

**DEFECTIVE AUTOPHAGY IN NEURODEGENERATION:
NOVEL ROLES FOR HUNTINGTIN AND OPTINEURIN IN REGULATING
AUTOPHAGOSOME DYNAMICS**

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A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

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Yvette C. Wong

DEDICATION

This thesis is dedicated to my parents
who have always shared with me their love and excitement for science
and never failed to encourage me to ask questions and make new discoveries in life.

ACKNOWLEDGMENTS

This work would not have been possible without the help and support of past and present Holzbaur lab members who have helped generate ideas, teach me new techniques and thoughtfully discussed my data. I am particularly grateful for Mariko Tokito who helped make numerous constructs for my studies and for Karen Wallace for helping me with mouse neuronal cultures. Without their help and patience, the studies presented in this thesis would not have been possible. I am also thankful for Meng-meng Fu and Sandra Maday who mentored me when I joined the Holzbaur lab and provided helpful experimental advice, and for Swathi Ayloo for her help in autophagosome purification for the huntingtin study. I would especially like to thank Eva Klinman for her help and support during my time in the lab, as well as Andrew Moore for following up on these studies. Most importantly, I would like to thank Dr. Erika Holzbaur for being a wonderful mentor and friend during my graduate school career. Her enthusiasm for science is always contagious and I have learned so much from her about conducting excellent science and being a great scientist. Erika's support and encouragement as well as her scientific insights have been invaluable and have made my graduate school experience one that I will always cherish.

I would also like to thank my thesis committee Dr. Robert Kalb, Dr. Alice Chen-Plotkin, Dr. Virginia Lee, Dr. Harry Ischiropoulos and Dr. Nancy Bonini for their guidance and support during my time in graduate school. I am also thankful for Dr. Michael Nusbaum, Dr. Joshua Gold and Jane Hoshi for their help with the Neuroscience Graduate Group, and my undergraduate research advisor Dr. Ron Harris-Warrick at Cornell University who inspired in me a love for neuroscience. I would also like to acknowledge my funding sources: Hearst Foundation Fellowship, 2011 (Y.C.W.) and National Institute of Health Grant R01 NS060698 (E.L.F.H.)

In addition, I would like to thank Danielle Mor and Madeleine Phillips for walking alongside me during graduate school, and Andrew Wang for his dedication and support of me. Finally, I am incredibly thankful to my parents for always encouraging me to succeed both in life and in graduate school.

ABSTRACT

DEFECTIVE AUTOPHAGY IN NEURODEGENERATION: NOVEL ROLES FOR HUNTINGTIN AND OPTINEURIN IN REGULATING AUTOPHAGOSOME DYNAMICS

Yvette C. Wong

Erika Holzbaur

Autophagy is an essential cellular degradative process that has been implicated in the pathogenesis of several neurodegenerative diseases including Huntington's disease and Amyotrophic Lateral Sclerosis (ALS). During autophagy, autophagosomes form around cargo such as mitochondria, and subsequently fuse with lysosomes to acidify and acquire enzymes to degrade internalized cargos. In neurons, constitutive autophagosome biogenesis preferentially occurs at the axon tip, followed by the robust retrograde axonal transport of autophagosomes back to the cell body. The mechanisms regulating both the axonal transport of autophagosomes and the selective degradation of damaged mitochondria have not yet been determined. Here, I report novel roles for huntingtin and optineurin in regulating these dynamics and show that this regulation is disrupted in models of neurodegenerative disease. Using live cell imaging of primary neurons, I demonstrate that huntingtin regulates autophagosome retrograde axonal transport via its interactions with dynein and the motor adaptor protein HAP1 (huntingtin-associated protein 1). Loss of either huntingtin or HAP1 disrupts autophagosome transport. We also find that expression of the polyglutamine expansion in huntingtin (polyQ-htt) which leads to Huntington's disease disrupts autophagosome transport, resulting in reduced autophagosome motility and inefficient cargo degradation. These observations support a model in which robust autophagosome transport is required for efficient lysosomal encounters along the axon; inhibition of this transport prevents efficient degradation of internalized cargos. To further explore the

mechanism regulating autophagy, I also examined the dynamics of selective mitochondrial degradation during PINK1 (PTEN-induced putative kinase 1)/parkin-dependent mitophagy. These studies identified optineurin as a novel autophagy receptor for damaged mitochondria. Optineurin is recruited to the outer mitochondrial membrane (OMM) following parkin-mediated ubiquitination of OMM proteins. Optineurin binds to ubiquitinated proteins via its UBAN domain, and subsequently recruits the autophagosome protein LC3 via its LC3 interacting region (LIR). This pathway is disrupted by either loss of optineurin or an ALS-associated E478G mutation in optineurin's ubiquitin binding domain, leading to inefficient mitochondrial degradation. Together, these studies provide new insights into the mechanisms driving autophagy and mitophagy, and further demonstrate that defects in autophagy may contribute to pathogenesis in both Huntington's disease and familial ALS.

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1 INTRODUCTION

1.1 Autophagy

Autophagy is a cellular degradative process in which cytosolic cargo are degraded by lysosomes (Mizushima et al., 2011). Autophagy can be divided into three subtypes: microautophagy, chaperone-mediated autophagy and macroautophagy. In microautophagy, cytosolic cargo are directly engulfed by the lysosome through invagination of the lysosomal membrane (Li et al., 2012). In chaperone-mediated autophagy (CMA), cargo are selectively recognized by the cytosolic chaperone hsc70 (heat shock-cognate protein of 70 KDa) via a pentapeptide amino acid motif such as KFERQ in the CMA cargo substrate (Cuervo and Wong, 2014). Upon substrate binding to hsc70, the substrate is brought to the surface of the lysosome where it binds to the cytosolic tail of LAMP-2A (lysosome-associated membrane protein type 2A), a single-span membrane protein, which induces LAMP-2A oligomerization into a translocation complex. LAMP-2A subsequently aids in the translocation of the CMA substrate across the lysosomal membrane into the lysosome where it is degraded (Cuervo and Wong, 2014).

In contrast, macroautophagy (hereafter referred to as autophagy) involves the formation of an autophagosome, a double-membrane organelle, which forms around cytosolic cargo and sequesters them within the autophagosome. The autophagosome subsequently fuses with lysosomes, which contain acidic hydrolases such as cathepsin proteases, to form an autolysosome. The autophagic cargo and the autophagosome inner membrane are subsequently degraded by the acidic hydrolases within the autolysosome, allowing for efficient recycling of macromolecules within the cell (Yang and Klionsky, 2010) (Figure 1.1).

The mammalian genes involved in autophagy were first identified in 1998, when Atg5 and Atg12 were found to regulate autophagosome biogenesis via a ubiquitin-like protein conjugation system (Mizushima et al., 1998). Since then, more than 30 autophagy genes have been identified which regulate autophagosome formation (Mizushima et al., 2011). In addition, while autophagy was previously thought to be solely a non-selective cargo degradation process which is upregulated during cellular stress such as starvation, autophagosomes have been found to selectively degrade cargo such as protein aggregates (aggrephagy) and mitochondria

(mitophagy) (Rogov et al., 2014). Selective autophagy is further mediated by autophagy receptors which preferentially bind ubiquitinated organelles or other cargos, and subsequently recruit the autophagosome protein light chain 3 (LC3) via their LC3-interaction region (LIR) motif (Birgisdottir et al., 2013; Stolz et al., 2014; Wild et al., 2014).

Recent work demonstrates that constitutive basal autophagy also occurs in neurons. These live cell imaging studies reveal a preferential formation of autophagosomes at the axon tip and their subsequent retrograde axonal transport towards the cell body (Lee et al., 2011; Maday et al., 2012). As there is increasing evidence implicating defective autophagy in neurodegeneration (Harris and Rubinsztein, 2012; Nixon, 2013; Wong and Cuervo, 2010), these studies provide a new model for studying neuronal autophagy and its potential defects during neurodegeneration.

1.1.1 Mechanisms of autophagosome biogenesis

Autophagosome biogenesis requires a cascade of different autophagy genes (Atgs) to initiate the formation, elongation and closure of the double-membrane autophagosome around its cargo. In non-selective autophagy, the protein complexes and the recruitment order of these different autophagy genes have been studied in great detail. However, it is important to note that these steps may be different during selective autophagy, such as in the degradation of ubiquitinated *Salmonella* (Fujita et al., 2013) or mitochondria (Itakura et al., 2012) (mitophagy; see Section 1.4) in which the omegasome/isolation membrane must selectively form around and engulf the ubiquitinated cargo, and thus the recruitment order of autophagy genes to selective autophagic cargo still remains to be examined.

Autophagosome biogenesis begins with the initiation, nucleation, and expansion of the autophagosome (Lamb et al., 2013) (Figure 1.1). During non-selective autophagy in mammalian cells, autophagosome biogenesis is initiated upon activation of the kinase ULK1 (Atg1 in yeast) and an ULK1/Atg13/FIP200 complex is recruited to the isolation membrane, the autophagosome precursor, and serves as a scaffold for the recruitment of downstream Atg proteins

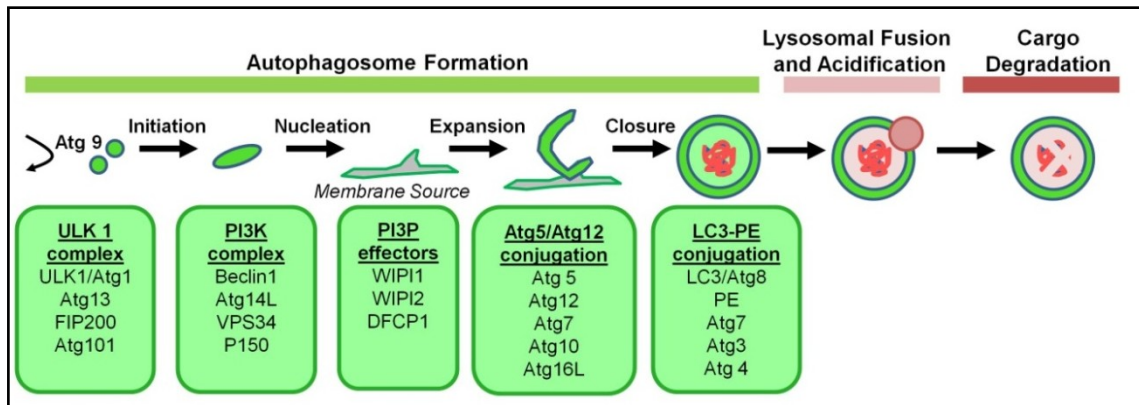


Figure 1.1: The autophagy pathway.

Autophagy begins with the formation of a double-membrane organelle (the autophagosome) around autophagic cargo. The autophagosome then fuses with lysosomes to acquire hydrolytic enzymes and acidification machinery to activate these enzymes, leading to autophagic cargo degradation. Autophagosome formation involves various autophagy protein complexes and two conjugation steps that can be divided into four parts: initiation, nucleation, expansion and closure.

(Shibutani and Yoshimori, 2014). Under nutrient rich conditions, ULK1 is negatively regulated by binding to the mammalian target of rapamycin complex 1 (mTORC1) complex, and is positively regulated by AMP-activated protein kinase (AMPK). However, upon amino acid starvation, mTORC1 is inactivated and dissociates from the ULK1 complex, allowing ULK1 to increase its kinase activity and facilitate autophagy initiation (Lamb et al., 2013).

In the subsequent nucleation step, the ULK1/Atg13/FIP200 complex recruits a class III PI3K (phosphatidylinositol 3-kinase) complex consisting of Beclin1, Atg14L, p150 and VPS34, which is responsible for generating the lipid PI3P (phosphatidylinositol 3-phosphate) by phosphorylating PI (phosphatidylinositol) (Lamb et al., 2013). ULK1 phosphorylates Beclin-1 on Ser14 to help activate this class III PI3K complex during autophagy (Russell et al., 2013)

PI3P generation is crucial for autophagosome formation and is localized to the membranes of autophagosomes. PI3P is able to recruit PI3P-binding proteins WIP11 and WIP12 (WD repeat domain phosphoinositide-interacting proteins) and DFCP1 (double FYVE-domain containing protein 1) to the forming isolation membrane (Axe et al., 2008). While it is still unclear what role DFCP1 plays (Axe et al., 2008), WIPs are considered to be essential PI3P effectors during autophagy which bridge PI3P generation with LC3 recruitment (Proikas-Cezanne et al.,

2015), as WIPI1 is essential for LC3 lipidation (Mauthe et al., 2011) and WIPI2 can bind Atg16L (Dooley et al., 2014).

Following the nucleation step, expansion of the autophagosome occurs via two ubiquitin-like conjugation steps. In the first step, Atg7 and Atg 10 act as E1-like and E2-like enzymes respectively, conjugating Atg12 with Atg 5. The Atg12/Atg5 complex then binds Atg16L via a direct interaction between Atg5 and Atg 16L (Shibutani and Yoshimori, 2014). The Atg12/Atg5/Atg16L complex is then recruited to the isolation membrane, potentially via Atg16L's interactions with FIP200 (Fujita et al., 2013; Gammoh et al., 2013; Nishimura et al., 2013) or WIPI2 (Dooley et al., 2014), but leaves the membrane upon completion of autophagosome formation (Shibutani and Yoshimori, 2014).

In the second step, Atg7 and Atg 3 act as E1-like and E2-like enzymes respectively, while Atg12/Atg5/Atg16L acts as an E3-like enzyme, to conjugate LC3 to the lipid PE (phosphatidylethanolamine) to form lipidated LC3 (also called LC3-II). This conjugation step occurs on the isolation membrane, generating lipidated LC3 on both the inner and outer surface of the autophagosome membrane during autophagy (Shibutani and Yoshimori, 2014). As lipidated LC3 stably associates with the autophagosome membrane, it has been used as a well-established marker for autophagosomes (Klionsky, DJ, 2012), and lipidated LC3 on the inner membrane is later degraded along with the internalized autophagic cargo. LC3 is thought to be crucial for isolation membrane expansion and closure of the autophagosome (Lamb et al., 2013). The LC3 family consists of LC3A, LC3B, LC3C, and its homologs GABARAP, GABARAPL1 and GABARAPL2. Of these, LC3B is the most prevalent and has been used most widely as an autophagosome marker.

The only transmembrane protein involved in autophagy is Atg9 which partially localizes to the trans-Golgi network and endosomes, and transiently colocalizes with LC3 in starvation-induced autophagy (Shibutani and Yoshimori, 2014). As Atg9 also localizes to small vesicles, these Atg9-positive vesicles have been proposed to supply the initial membrane source which later becomes the isolation membrane.

Several other membrane sources have also been proposed to contribute to the formation of the autophagosome membrane. These include the ER (endoplasmic reticulum) which forms DFCP1-positive omega-shaped cradles termed omegasomes as isolation membrane precursors of the autophagosome (Axe et al., 2008; Hayashi-Nishino et al., 2009). Mitochondria have also been suggested to contribute to the autophagosome membrane (Hailey et al., 2010), supporting evidence showing that autophagosomes form at ER-mitochondria contact sites (Hamasaki et al., 2013). In addition, other compartments such as ER exit sites where COPII vesicle formation occurs (Graef et al., 2013), the ER-Golgi intermediate compartment (Ge et al., 2013), the plasma membrane (Ravikumar et al., 2010) and recycling endosomes (Longatti et al., 2012; Puri et al., 2013) have also been found to contribute to autophagosome biogenesis (Shibutani and Yoshimori, 2014). Thus, the exact autophagosome membrane source still remains to be understood, as well as whether this membrane source might vary depending on the cell type, the method in which autophagy is induced, and the autophagic cargo being degraded. In neurons, autophagosomes forming at the axon terminal during basal constitutive autophagy were found to incorporate an ER membrane source rather than plasma membrane or mitochondria (Maday and Holzbaur, 2014).

Autophagosomes subsequently fuse with multiple lysosomes (Eskelinen, 2008; Yu et al., 2010) to acquire the necessary degradative machinery including hydrolytic enzymes such as proteases, lipases and nucleases, as well as the vacuolar ATPase (vATPase) proton pump which is needed to acidify the autolysosomal lumen for activation of the hydrolytic enzymes (Nixon, 2013). Following degradation of the internal autophagic contents, mTOR signaling is reactivated during prolonged starvation and is responsible for attenuating autophagy and generating proto-lysosomal tubules and vesicles that extrude from autolysosomes and ultimately mature into functional lysosomes (Yu et al., 2010). Thus, both the autophagic cargo and the lysosomal machinery is recycled by the cell to maintain cellular homeostasis in autophagy.

1.1.2 Selective autophagy via autophagy receptors

Multiple forms of selective autophagy have now been identified, including the selective degradation of protein aggregates (aggrephagy), mitochondria (mitophagy), ER (reticulophagy), ribosomes (ribophagy), peroxisomes (pexophagy), bacteria (xenophagy) and lipid droplets (lipophagy) (Rogov et al., 2014). However, identifying the mechanisms by which these selective cargo are targeted for autophagic degradation still remains to be examined.

Autophagy receptors that contain an LIR (LC3-interacting region) motif and also bind selective cargo either directly or indirectly by binding ubiquitin on the cargo have been found to regulate selective autophagic degradation. The currently identified autophagy receptors which directly bind both ubiquitin (via a ubiquitin binding domain) and LC3 (via a LIR domain) are SQSTM1/p62, neighbor of BRCA1 gene 1 (NBR1), optineurin, nuclear dot protein 52 kDa (NDP52), TAX1BP1/TRAFF6-binding protein (T6BP) and Tollip (Kirkin et al., 2009; Lu et al., 2014a; Pankiv et al., 2007; Thurston et al., 2009; Tumbarello et al., 2012; Wild et al., 2011). The activities of these receptors can be further regulated by different kinases. Phosphorylation of p62 by casein-kinase 2 (CK2) at S403 increases its affinity for polyubiquitin chains (Matsumoto et al., 2011), while phosphorylation of optineurin at S177 by TANK-binding kinase 1 (TBK1) increases its affinity for LC3 (Wild et al., 2011).

NBR1, p62 and optineurin have been found to regulate autophagic recognition of protein aggregates while NDP52, p62 and optineurin regulate bacterial clearance during xenophagy (Rogov et al., 2014). More recently, autophagic degradation of stress granules was identified (Buchan et al., 2013) and found to be regulated by NDP52 and p62 (Guo et al., 2014). However, the autophagy receptors for other selective autophagy pathways such as reticulophagy or lipophagy have not yet been identified.

In the case of mitophagy, the autophagy receptors that mediate the selective recognition of mitochondria by autophagosomes still remain to be established. In special cases of hypoxia-induced mitophagy or erythrocyte maturation, several mitophagy receptors have been identified including Nix (BNIP3L), BNIP3 and FUNDC1 (Liu et al., 2012; Novak et al., 2010; Zhang et al.,

2012). However, in other pathways of mitophagy such as PINK1 (PTEN induced putative kinase 1)/parkin-dependent mitophagy, it has been controversial which autophagy receptors are involved in regulating LC3 recruitment and autophagosome assembly around damaged mitochondria. Of the known autophagy receptors, only p62's role in parkin-mediated mitophagy has been previously investigated (Ding et al., 2010; Geisler et al., 2010; Narendra et al., 2010a; Okatsu et al., 2010), but this work has suggested conflicting roles for p62 as either an autophagy receptor (Ding et al., 2010; Geisler et al., 2010) or a regulator of perinuclear clustering of depolarized mitochondria (Narendra et al., 2010a; Okatsu et al., 2010). Thus, identifying the autophagy receptor responsible for recruiting autophagic machinery during mitophagy remains an important question in the field of mitochondrial degradation.

1.1.3 Neuronal Autophagy

Autophagy is particularly critical in neurons for the degradation of protein aggregates and damaged organelles such as mitochondria, as neurons are post-mitotic cells that cannot dispose of damaged cytosolic cargo through cell division. As neurons are highly polarized with both dendritic and axonal compartments, they must maintain active electrochemical signaling across these elaborate morphologies. In addition, neurons require recycling of proteins and organelles both at the synapse which are often long distances from the soma, as well as throughout the axon, dendrites and cell soma, thus making efficient autophagic degradation crucial for neurons.

The importance of autophagy in neurons was first demonstrated in 2006 when mice with neuronal-specific knockout of essential autophagy genes, Atg5 or Atg7, were found to exhibit neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). Atg5^{flox/flox}, nestin-Cre mice exhibited decreased motor coordination, balance and grip strength, and developed neuronal inclusion bodies which began as diffuse intracellular proteins that accumulated into cytoplasmic aggregates. In addition, these mice demonstrated neuronal loss which was most prominent in cerebellar Purkinje cells (Hara et al., 2006). Atg7^{flox/flox}, nestin-Cre mice also showed behavioral deficits including decreased motor coordination and limb-clasping reflexes, and inclusion body

formation which increased in number and size with age. These mice also demonstrated neuronal loss of cerebral and cerebellar cortices (Komatsu et al., 2006).

Autophagy is required for neuronal development and the maintenance of axonal homeostasis (Fimia et al., 2007; Komatsu et al., 2007; Wang et al., 2006). In particular, loss of Ambra1, a positive regulator of the Beclin1-dependent autophagy, resulted in severe neural tube defects in mouse embryos, associated with autophagy impairment and the accumulation of ubiquitinated proteins (Fimia et al., 2007). In addition, loss of Atg7 in Purkinje cells caused initial degeneration of axon terminals and progressive axonal swellings (Komatsu et al., 2007). Interestingly, dendritic or spine atrophy was not observed in conjunction with axonal dystrophy, suggesting that axon terminals may be more vulnerable to autophagy defects than dendrites.

Recent live cell imaging studies of basal autophagy in neurons using the well-established autophagosome marker GFP-LC3 (Klionsky, DJ, 2012) demonstrate that autophagosomes preferentially form at the axon tip (Maday et al., 2012)(Lee et al., 2011; Maday et al., 2012)(Lee et al., 2011; Maday et al., 2012)(Lee et al., 2011; Maday et al., 2012)(Lee et al., 2011; Maday et al., 2012)(Lee et al., 2011; Maday et al., 2012)(Lee et al., 2011; Maday et al., 2012) (Figure 1.2). In contrast to non-polarized cells in which autophagosome formation occurs in the cell body, autophagosomes in dorsal root ganglion, hippocampal and cortical neurons *in vitro* preferentially form at the axon tip at higher rates than along the axon or in the cell body (Maday and Holzbaur, 2014; Maday et al., 2012). These findings suggest that autophagy may be preferentially required in the distal axon to maintain synaptic function or mediate protein turnover.

During autophagosome formation in neurons, Atg13 and Atg5 are dynamically recruited to the axon tip (Maday and Holzbaur, 2014), followed by LC3 recruitment to the Atg13/Atg5 site. LC3 is first recruited as a puncta which grows into an autophagosome sphere with a diameter of ~800nm over 4-6 minutes (Maday et al., 2012), consistent with rates of autophagosome formation observed in non-neuronal cells (Koyama-Honda et al., 2013).

In neurons, neither plasma nor mitochondrial membrane are incorporated into nascent autophagosomes. Instead, neuronal autophagosomes form on or near Sec61 β -labeled

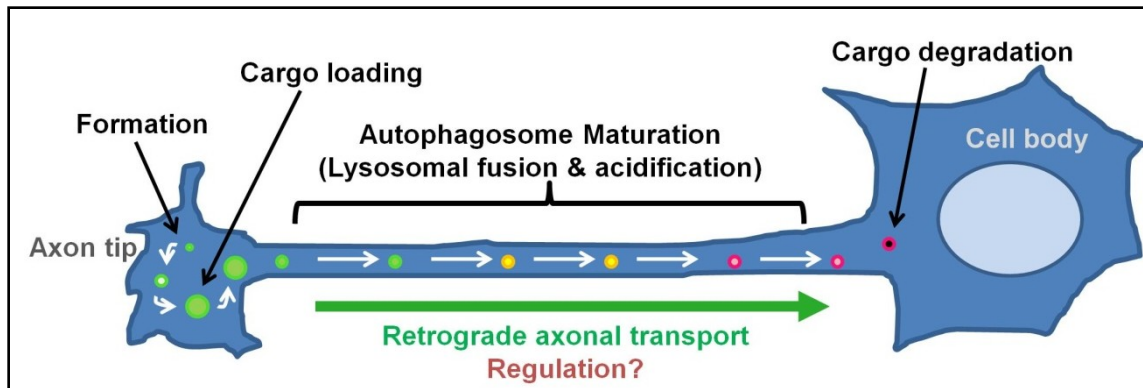


Figure 1.2: Autophagy in neurons.

Autophagosomes in neurons preferentially form at the axon tip. They subsequently undergo retrograde axonal transport back to the cell body, during which time they mature via fusion with lysosomes and acidify. However, the proteins that regulate and help scaffold motor proteins during retrograde axonal transport of autophagosome are not known.

ER in the distal axon, but not at ER exit sites (ERES) marked with Sec16L (Maday and Holzbaur, 2014). Similar to non-neuronal cells, autophagosomes form at the omegasome, a PI3P-enriched platform on the ER which recruits the protein DFCP1 (Axe et al., 2008; Koyama-Honda et al., 2013; Maday and Holzbaur, 2014).

Autophagosomes forming under basal autophagy in neurons are capable of engulfing mitochondrial fragments, as well as aggregate-prone proteins such as mutant SOD1^{G93A} (Maday et al., 2012). As autophagosomes leave the axon tip, they undergo dynein-driven retrograde transport (Lee et al., 2011; Maday et al., 2012) (see Section 1.2.3) and mature via fusion with LAMP1/Rab7-positive lysosomes that contain cathepsin proteases, leading to the formation of autolysosomes along the axon (Lee et al., 2011; Maday et al., 2012). Consistent with this model, autophagosomes acidify as they undergo axonal transport (Maday et al., 2012), observed using the dual color reporter mCherry-EGFP-LC3 in which the GFP signal is preferentially quenched as the compartment matures and acidifies (Kimura et al., 2007; Pankiv et al., 2007). This gradual acidification is likely due to lysosomal fusion and acquisition of the proton pump v-ATPase, which is necessary for cathepsin activation (Lee et al., 2011).

Thus, basal autophagy in neurons may act to efficiently recycle protein aggregates and damaged organelles from the axon terminal back to the cell body where amino acids and lipids

from the autophagic cargo can be reused. Further studying the regulation of this pathway in neurons will help expand our understanding of neuronal homeostasis.

1.1.4 Autophagy in neurodegenerative diseases

Neurodegenerative diseases such as ALS (Amyotrophic Lateral Sclerosis), Alzheimer's, Parkinson's and Huntington's disease are characterized by the progressive loss of neurons, the formation of protein inclusions, and the adult onset of clinical symptoms in the majority of patients. As autophagy is crucial for maintaining neuronal homeostasis and as defects in autophagy are sufficient to lead to neurodegeneration (Hara et al., 2006; Komatsu et al., 2006), it is not surprising that defective autophagy has been implicated in multiple neurodegenerative diseases (Harris and Rubinsztein, 2012; Nixon, 2013; Wong and Cuervo, 2010). Interestingly, while autophagy is efficient in younger neurons (Boland et al., 2008), autophagy proteins such as Beclin-1, Atg5 and Atg7 decline with age (Lipinski et al., 2010; Shibata et al., 2006), potentially leading to a decreased efficiency in autophagic induction and contributing to the late onset of many neurodegenerative diseases (Rubinsztein et al., 2011).

Autophagy is a critical pathway for degrading aggregated misfolded proteins, including mutant α -synuclein found in Parkinson's disease (Webb et al., 2003), mutant superoxide dismutase 1 (SOD1) found in ALS (Kabuta et al., 2006) and polyglutamine expansions in huntingtin (polyQ-htt) found in Huntington's disease (Ravikumar et al., 2002, 2004). Thus, defective autophagy results in the accumulation of mutant proteins, which may further exacerbate neuronal dysfunction during disease and contribute to neuronal loss. In addition, autophagy has also been found to regulate the turnover of proteins which are found in inclusions in various neurodegenerative diseases, including amyloid- β in Alzheimer's disease (Parr et al., 2012), wildtype α -synuclein in Parkinson's disease (Webb et al., 2003) and TDP-43 (transactive response DNA binding protein 43 kDa) in ALS (Scotter et al., 2014).

The autophagy pathway can be disrupted at multiple different stages in neurodegenerative disease, including defects in autophagosome biogenesis, cargo recognition,

autophagosome transport, autophagosome maturation, and lysosomal fusion (see Review in Chapter 4). Autophagosome biogenesis is upregulated in Alzheimer's disease (Lipinski et al., 2010) and ALS (Sasaki, 2011), while Parkinson's disease-associated mutants such as VPS35 downregulate autophagosome biogenesis by disrupting autophagosome formation (Schapansky et al., 2014; Winslow et al., 2010; Zavodszky et al., 2014). Autophagic cargo recognition has been proposed to be defective in Huntington's disease (Martinez-Vicente et al., 2010), and defective autolysosomal transport induced by inhibition of lysosomal proteolysis leads to dystrophic swellings that are immunopositive for APP (amyloid precursor protein) and characteristic of Alzheimer's disease (Lee et al., 2011). The step of autophagosome maturation and lysosomal fusion is also disrupted in multiple neurodegenerative diseases. In Alzheimer's disease patient brains, immature autophagosomes accumulate in dystrophic neurites (Nixon et al., 2005), and in Parkinson's disease, wildtype α -synuclein aggregates impair autophagy by delaying autophagosome maturation (Tanik et al., 2013). In ALS, ALS2/alsin regulates endolysosomal trafficking potentially by mediating endosomal fusion with autophagosomes (Hadano et al., 2010), and ALS-associated mutations in CHMP2B ((charged multivesicular body protein 2B) disrupt autophagosome maturation by inhibiting multivesicular body fusion with autophagosomes (Cox et al., 2010; Filimonenko et al., 2007; Lee et al., 2007).

In addition, mutations in the autophagy receptors p62 and optineurin are linked to ALS (Fecto et al., 2011; Maruyama et al., 2010), and the kinase TBK1 which phosphorylates both p62 and optineurin was also recently identified as a novel ALS gene (Cirulli et al., 2015; Freischmidt et al., 2015). Mutations in the kinase PINK1 and the E3 ubiquitin ligase parkin (PARK 2) which are responsible for regulating PINK1/parkin-dependent mitophagy are linked to Parkinson's disease (Kitada et al., 1998; Valente et al., 2004). Finally, several neurodegenerative disease-associated proteins including presenilin 1, ATP13A2 (PARK 9) and LRRK2 (Leucine-rich repeat kinase 2) have been found to regulate lysosomal acidification and fusion with autophagosomes, a critical step in autophagic degradation (Dehay et al., 2012; Hadano et al., 2010; Lee et al., 2010a; Usenovic et al., 2012). Thus, identifying the precise steps at which autophagy is

misregulated in different diseases will contribute to understanding the role of autophagy in the progression of neurodegenerative diseases (Harris and Rubinsztein, 2012).

1.2 Axonal transport in neurons

Axonal transport along microtubules is critical in neurons for maintaining neuronal function and homeostasis (Maday et al., 2014). Not surprisingly, defective axonal transport has been observed in many neurodegenerative disease models (Perlson et al., 2010), while mutations in motor proteins can directly cause neurodegeneration (Farrer et al., 2009; Puls et al., 2003; Zhao et al., 2001). Anterograde transport brings cargo from the cell body to the distal axon, allowing for newly synthesized organelles and synaptic proteins to be replenished at the axon terminal. In contrast, retrograde transport brings cargo from the distal axon back to the cell body, resulting in efficient clearance and recycling of misfolded proteins and damaged organelles from the axon terminal.

Different cargos exhibit different motility along the axon. Mitochondria, late endosomes and lysosomes, and mRNA protein granules exhibit bidirectional transport along the axon, while synaptic vesicle precursors, dense core vesicles, BDNF (brain derived neurotrophic factor) and APP are predominantly anterograde cargo. In contrast, autophagosomes, signaling endosomes such as Trk receptors, and injury signals such as STAT3 show predominantly retrograde motility (Maday et al., 2014).

In addition to the fast axonal transport characteristic of the cargos described above, there are also newly synthesized cargos which travel at much slower velocities referred to as slow axonal transport. These cargo can be divided into two different categories by their speeds: slow component a (such as tubulin and neurofilaments) which travel at 0.2-1mm/day, and slow component b (such as cytosolic proteins) which travel at 1-10mm/day (Roy, 2014).

Axon lengths are highly variable between neuronal populations in humans, ranging from 1 mm (for inhibitory interneurons) to 5 cm (retinal ganglion cell axons in the optic nerve), and can be as long as 1m (for motor neuron axons) (Twelvetrees et al., 2012). To ensure that transport is

efficiently directed across such long distances, the motor proteins that drive the axonal transport of cargos along microtubules must be properly regulated by adaptors or scaffolding proteins that regulate motor protein activity and cargo interactions.

1.2.1 Motor Proteins: kinesins and dynein/dynactin

Anterograde transport is driven by members of the kinesin superfamily, which are divided into 14 subfamilies. Of these, motor proteins in the kinesin-1, kinesin-2 and kinesin-3 subfamilies regulate axonal transport (Verhey and Hammond, 2009). Kinesin-1 members drive anterograde transport of many organelles and proteins at speeds of 0.5-1mm/sec and are highly processive motors which take 8 nm steps along a single protofilament towards the plus end of the microtubule. Kinesin-2 motors form homodimers or heterotrimers and drive the anterograde transport of N-cadherin and β -catenin (Teng et al., 2005) and are also associated with Rab7-positive late endosome/lysosomes (Castle et al., 2014; Hendricks et al., 2010). While both kinesin-1 and kinesin-2 motors have similar stall forces of 5 pN, kinesin-2 motors exhibit force-dependent detachment from microtubules (Schroeder et al., 2012). Kinesin-3 motors form highly processive motors upon cargo-mediated dimerization (Soppina et al., 2014) and drive transport of synaptic vesicle precursors and dense core vesicles (Hall and Hedgecock, 1991; Lo et al., 2011; Okada et al., 1995).

In contrast to the extended kinesin superfamily that drives anterograde transport, retrograde transport is exclusively driven by cytoplasmic dynein. The motor domain of dynein is encoded by a single gene (dynein heavy chain). In addition to dynein heavy chain, which dimerizes via its N-terminal tail domains, the dynein complex consists of five other subunits: dynein intermediate chain, dynein light-intermediate chain, and three classes of light chains (TCTEX, LC8 and Roadblock) (Roberts et al., 2013).

Dynein is activated by dynactin, a multiprotein complex which consists of the subunits p150^{Glued}, p62, p50 (dynactin), capping proteins (α and β), Arp1, p27, p25 and p22. The p150^{Glued} subunit forms a dimer within dynactin that binds directly to dynein intermediate chain,

and also binds microtubules via a cytoskeletal associated protein-glycine-rich (CAP-Gly) domain (Maday et al., 2014; Roberts et al., 2013). In vitro studies show that the microtubule-binding domains in the p150^{Glued} subunit of dynactin help increase dynein/dynactin motor processivity via increased association with microtubules (Ayloo et al., 2014; King and Schroer, 2000; Ross et al., 2006; Tripathy et al., 2014). In addition, the CAP-Gly domain of p150^{Glued} regulates the initiation of retrograde transport from the distal axon (Lloyd et al., 2012; Moughamian and Holzbaur, 2012), and is disrupted by mutations in the CAP-Gly domain which are associated with the neurodegenerative diseases Perry syndrome and distal hereditary motor neuropathy 7B (HMN7B) (Moughamian and Holzbaur, 2012). Mammalian cytoplasmic dynein transports cargos at speeds of 0.5 to 1mm/sec and exhibits a unitary stall force of 1pN (Mallik et al., 2004; Schroeder et al., 2010). Unlike kinesin motors, mammalian dynein does not exhibit robustly processive unidirectional transport on its own, but the processivity of the motor is significantly increased when functioning in a cohort with other dynein motors (Mallik et al., 2005) or by activators such as BicD2 (McKenney et al., 2014; Schlager et al., 2014).

1.2.2 Motor scaffolding proteins

Scaffolding proteins such as huntingtin, JIP1 (JNK-interacting protein 1), Milton/TRAKs, and Hook-1 are crucial for regulating motor proteins during organelle transport via their interactions with membrane-associated cargo receptors, components of kinesin and dynein motor complexes, or with signaling proteins including GTPases and kinases (Fu and Holzbaur, 2014). These different scaffolding proteins regulate the transport of different types of cargos such as lysosomes or mitochondria, allowing for precise regulation of organelle transport direction, speed and flux of different cargos.

Early endosome motility in fungi is regulated by Hok1/HookA, which recruits dynein and dynactin to endosomes and counteracts the activity of kinesin-3 motors (Bielska et al., 2014; Zhang et al., 2014b). The motility of late endosomes and lysosomes is regulated by the scaffold protein RILP (Rab-interacting lysosomal protein), which is recruited by the activated GTP-bound

form of Rab7 (Ras-related protein), and can subsequently recruit dynactin by binding the C-terminus of the p150^{Glued} subunit (Jordens et al., 2001). In addition, the bidirectional motility of RNA granules is regulated by the RNA-binding protein La which interacts with dynein rather than kinesin upon sumoylation (van Niekerk et al., 2007).

Mitochondria motility in *Drosophila* is regulated by the Milton/Mito complex in which Miro localizes to the outer mitochondrial membrane and subsequently recruits Milton (known as TRAK1 and TRAK2 in mammals), which binds directly to kinesin heavy chain (KHC) (Glater et al., 2006). Miro is capable of disrupting kinesin's association with mitochondria and stalling mitochondria at areas of high calcium via its two calcium-binding EF hand domains, either by inducing binding of KHC to Miro and inhibiting kinesin motor processivity (Wang and Schwarz, 2009), or by releasing KHC from the Milton/Miro complex on mitochondria (MacAskill et al., 2009).

The scaffolding protein JIP1 mediates the transport of several organelles including mitochondria, synaptic vesicles (Horiuchi et al., 2005), and APP-positive vesicles (Fu and Holzbaur, 2013; Muresan and Muresan, 2005). JIP1 can also bind dynactin and kinesin-1 in a phosphorylation-dependent manner (Fu and Holzbaur, 2013). Other members of the JIP family (JIPs 1-4) include JIP3 which also associates with dynein/dynactin and regulates the transport of axonal injury signals (Cavalli et al., 2005), and JIP4 which interacts with kinesin-1 and dynactin to regulate recycling endosome trafficking during cytokinesis (Montagnac et al., 2009).

Another scaffolding protein, huntingtin directly binds dynein intermediate chain (Caviston et al., 2007) and also interacts with dynactin (p150^{Glued} subunit) and kinesin via huntingtin-associated protein 1 (HAP-1), (Engelender et al., 1997; Li et al., 1998, 1995; McGuire et al., 2006; Twelvetrees et al., 2010). Huntingtin plays an important role in regulating the transport of many cargos including BDNF-positive vesicles, APP, Rab-11 positive recycling endosomes and GABA receptors (Gauthier et al., 2004; Her and Goldstein, 2008; Power et al., 2012; Twelvetrees et al., 2010). Axonal transport of these organelles is disrupted by expanded polyQ-htt in models

of Huntington's disease, suggesting that defective organelle transport may contribute to the pathogenesis of this neurodegenerative disease (see Section 1.3).

1.2.3 Autophagosome axonal transport

Recent live cell imaging studies in cultured neurons reveal robust retrograde transport of autophagosomes from the axon tip towards the cell body (Lee et al., 2011; Maday and Holzbaur, 2014; Maday et al., 2012). Autophagosomes exhibit bidirectional motility shortly after they are formed at the axon tip, which is likely driven by the alternating activities of kinesin-1 and dynein (Maday et al., 2012). However, they soon undergo robust retrograde axonal transport at speeds of ~0.5µm/sec in neurons from both the central nervous system (hippocampal neurons) and the peripheral nervous system (dorsal root ganglion neurons) (Maday and Holzbaur, 2014; Maday et al., 2012). Although some autophagosomes demonstrate bidirectional or anterograde motility along the axon, >80% of autophagosomes move in the retrograde direction with a flux of 1.7-1.9 autophagosomes travelling across 100 µm over 1 min (Maday and Holzbaur, 2014; Maday et al., 2012). As autophagosomes fuse with LAMP1-positive lysosomes and gradually acidify, as measured by GFP quenching with the mCherry-GFP-LC3 tandem construct, their motility becomes more bidirectional along the axon (Maday et al., 2012).

Autophagosome transport in neurons is driven by the retrograde motor dynein (Katsumata and Nishiyama, 2010; Lee et al., 2011; Maday et al., 2012) and its activator dynactin (Ikenaka et al., 2013). In addition, expression of CC1 which blocks dynein-dynactin interaction also inhibits autophagosome transport in dorsal root ganglion neurons cultured from adult GFP-LC3 transgenic mice (Maday et al., 2012). In a *C. elegans* knockdown of dynactin-1 in motor neurons, both motor defects and axonal degeneration were observed, along with impaired autophagosome motility in degenerating neurons (Ikenaka et al., 2013).

Dynein intermediate chain (DIC) comigrates with autophagosomes along the axon in dorsal root ganglion neurons, and both dynein and dynactin are present in autophagosome-enriched fractions isolated from mouse brain (Maday et al., 2012). Immunostaining of

endogenous DIC along the axon of mouse cortical neurons also shows colocalization with LC3-labeled autophagosomes (Lee et al., 2011). Not surprisingly, dynein mutations in both fly and mouse models lead to impaired autophagic clearance of polyQ-htt, a well-established substrate of autophagy (Ravikumar et al., 2002, 2005). Interestingly, both kinesin-1 and kinesin-2 motors also comigrate with autophagosomes along the axon and are found in autophagosome-enriched fractions (Maday et al., 2012). These observations suggest that there may be scaffolding proteins such as huntingtin or JIP1 which help inactivate kinesin and promote dynein activity on autophagosomes, allowing for the robust retrograde axonal transport of autophagosomes. Thus, further study of the regulation of autophagosome axonal transport in neurons will be important in advancing our understanding of both neuronal autophagy and motor protein activity regulation.

Although lysosomes are present at the axon tip as well as throughout the axon (Maday et al., 2012), autophagosomes preferentially fuse with lysosomes as they undergo retrograde transport towards the cell body (Lee et al., 2011). Disrupting lysosomal proteolysis by inhibiting cathepsins or suppressing lysosomal acidification results in decreased autolysosome and lysosome transport velocities, and leads to dystrophic axonal swellings (Lee et al., 2011). Thus, efficient lysosomal proteolysis and enzymatic function are required for autophagosome maturation as well as regulation of autolysosome and lysosome transport. However, it is still unclear whether the converse also occurs: whether autophagosome transport in neurons regulates autophagosome maturation and cargo degradation. In non-neuronal cells, it has been shown that inhibition of autophagosome transport leads to reduced lysosomal fusion events, which may lead to insufficient accumulation of degradative enzymes within the autophagosome (Jahreiss et al., 2008; Kimura et al., 2008; Ravikumar et al., 2005). Thus, it will be interesting to examine whether autophagosome transport is critical for autophagosome maturation and lysosomal fusion in neurons, and whether defects in autophagosome axonal transport ultimately lead to inefficient autophagic cargo degradation in neurons.

1.3 Huntington's disease

The autosomal dominant neurodegenerative disorder Huntington's disease is caused by polyglutamine (polyQ) expansions in exon 1 of the ubiquitously-expressed protein huntingtin (htt) (Macdonald et al., 1993) (Figure 1.3). Individuals with 35 glutamine repeats are at risk for developing the disease, while patients with 40 or more repeats invariably develop the disease. While patients with Huntington's disease typically have adult onset of clinical symptoms (30-50 years old), anticipation is present in the disease, such that the number of polyQ repeats increases in successive generations, resulting in earlier age of onset and increased disease severity (Imarisio et al., 2008).

Huntington's disease is characterized by both cognitive and motor dysfunction including depression, dementia, and episodes of chorea (Imarisio et al., 2008). The primary pathological hallmark of the disease is the loss of medium spiny neurons in the striatum expressing γ -aminobutyric acid (GABA). However, other neurons also degenerate at later stages of the disease including those in the cortex, globus pallidus and thalamus (Margulis and Finkbeiner, 2014). Similar to other neurodegenerative diseases such as Alzheimer's and Parkinson's disease, patients with Huntington's disease develop inclusion bodies, which in the case of Huntington's disease contain the mutant protein polyQ-htt. PolyQ-htt neuronal inclusion bodies are found in the cytoplasm and in the nucleus, and polyQ repeat length correlates with the number of inclusion bodies observed in patient brains (Margulis and Finkbeiner, 2014). Inclusion bodies form in response to polyQ-htt accumulation and may be protective by reducing levels of soluble polyQ-htt, rather than contributing to neuronal death (Arrasate et al., 2004). The accumulation of polyQ-htt in protein aggregates and inclusion bodies suggests that polyQ-htt is not efficiently degraded in neurons. Although the ubiquitin-proteasome (UPS) system may contribute to polyQ-htt degradation, autophagy is the main pathway by which both soluble and aggregated polyQ-htt are cleared (Qin et al., 2003; Ravikumar et al., 2002).

Huntingtin is a multi-domain protein consisting of 3144 aa, whose proposed functions include anti-apoptotic activity, transcriptional regulation and synaptic function (Gauthier et al.,

2004; Rigamonti et al., 2000; Saudou et al., 1998; Twelvetrees et al., 2010; Zuccato et al., 2003). Huntingtin has also been found to regulate organelle axonal transport by interacting with both retrograde and anterograde motor proteins (Caviston and Holzbaur, 2009). Expression of polyQ-htt is sufficient to disrupt axonal transport which is critical for maintaining neuronal homeostasis (Caviston and Holzbaur, 2009). However, the precise mechanisms which initially trigger neurodegeneration in Huntington's disease, and the reason for the late onset of clinical symptoms in patients still remain unclear.

In addition, as huntingtin is ubiquitously expressed throughout the body, it is still unknown why medium spiny neurons are preferentially affected in the disease (Gusella and MacDonald, 2006). The specific morphological, biochemical, and functional characteristics of these neurons have been proposed to contribute to their selective neurodegeneration (Han et al., 2010). In addition, huntingtin's interactions with neuronal-specific proteins such as the nucleotide-binding protein Rhes which sumolyates polyQ-htt and selectively localizes to the striatum has also been proposed to mediate neuronal selectivity in this disease (Subramaniam et al., 2009). Thus, the processes leading to neurodegeneration and how they contribute to selective neuronal vulnerability in Huntington's disease remain to be further examined.

1.3.1 Autophagy in Huntington's disease

Both soluble and aggregated polyQ-htt are cleared by autophagy (Qin et al., 2003; Ravikumar et al., 2002), and indeed, polyQ-htt is often used as a well-established substrate for measuring autophagic degradation. In Huntington's disease models, disruption of autophagy accelerates the formation of toxic polyQ-htt oligomers and ultimately results in cell death (Ravikumar et al., 2004; Sarkar et al., 2007). In addition, fly and mouse models with mutations in the retrograde motor dynein which is essential for autophagosome transport demonstrate impaired autophagic clearance of polyQ-htt (Ravikumar et al., 2005).

As polyQ-htt is robustly cleared by autophagy, upregulation of autophagy has been proposed as a therapeutic treatment for Huntington's disease. Upregulating autophagy via

inhibition of mTOR with the drug rapamycin in both fly and mouse models of Huntington's disease was initially found to decrease neurodegeneration and improved behavior and motor performance (Ravikumar et al., 2004). However, as mTOR-dependent molecules have been shown to cause negative side effects due to mTOR's multiple target pathways, mTOR-independent molecules such as lithium, trehalose, and rilmenidine, which also upregulate autophagy, have also been tested and found to clear polyQ-htt in different models, but still remain to be proven in human clinical trials (Martin et al., 2014a).

Interestingly, huntingtin itself has been localized to both the inner and outer membranes of liver-isolated autophagosomes (Atwal et al., 2007; Martinez-Vicente et al., 2010) and has been proposed to activate the endosomal-lysosomal system (Kegel et al., 2000). More recently, autophagic vacuoles from Huntington's disease models demonstrate defective recognition of cytosolic cargo such as mitochondria and lipids. Autophagosomes form at normal or enhanced rates, and successfully fused with lysosomes, but have decreased autophagic cargo content as observed by electron microscopy and immunoblot in liver-isolated autophagosomes (Martinez-Vicente et al., 2010). However, the exact mechanism by which huntingtin might regulate autophagic cargo recognition is still unclear, although polyQ-htt was found to have decreased interactions with the autophagy receptor p62 as compared to wildtype huntingtin.

In addition, huntingtin directly binds the autophagy receptor optineurin (Faber et al., 1998; Hattula and Peränen, 2000) and polyQ-htt delocalizes an optineurin/Rab8 complex from the Golgi, resulting in impaired post-Golgi trafficking to lysosomes (del Toro et al., 2009). Thus, further studying the role of huntingtin in regulating autophagy and how it is disrupted by polyQ-htt will be interesting, particularly as polyQ-htt itself is degraded by autophagy. Consequently, defective autophagy caused by polyQ-htt may be further exacerbated by the accumulation of inefficiently degraded polyQ-htt upon autophagic disruption.

1.3.2 Huntingtin as a motor scaffold

Huntingtin regulates the axonal transport dynamics of various organelles including BDNF-positive vesicles, APP, Rab-11 positive recycling endosomes, GABA receptors and isolated vesicles *in vitro* (Caviston et al., 2007; Gauthier et al., 2004; Gunawardena et al., 2003; Her and Goldstein, 2008; Power et al., 2012; Twelvetrees et al., 2010). Htt directly binds the dynein intermediate chain subunit of the retrograde motor dynein complex (Caviston et al., 2007), and interacts with the dynein-activator p150^{Glued} subunit of dynactin and the anterograde motor kinesin-1 via its direct binding to the adaptor protein HAP1 (huntingtin-associated protein-1) (Engelender et al., 1997; Li et al., 1998, 1995; McGuire et al., 2006; Twelvetrees et al., 2010) (Figure 1.3). HAP1 has been previously shown to regulate kinesin-dependent APP transport (McGuire et al., 2006; Yang et al., 2012) and anterograde GABA receptor trafficking (Twelvetrees et al., 2010). In addition, the activity of this huntingtin/HAP1 scaffolding complex may be carefully regulated *in vivo*, by post-translational modifications such as phosphorylation of Ser421 by the serine/threonine kinase Akt which recruits kinesin-1 to the dynactin complex, thus promoting anterograde transport (Colin et al., 2008).

Multiple studies have shown that polyQ-htt disrupts the axonal transport of various organelles, including the anterograde transport of APP and the bidirectional transport of BDNF and mitochondria (Chang et al., 2006; Gauthier et al., 2004; Gunawardena et al., 2003; Her and Goldstein, 2008; Lee et al., 2004; Orr et al., 2008; Song et al., 2011; Szebenyi et al., 2003; Trushina et al., 2004; Zala et al., 2008). Interestingly, transport of APP and BDNF is disrupted in striatal and hippocampal neurons but not in cortical neurons from Huntington's disease model mice (Her and Goldstein, 2008), consistent with the observation that striatal neurons preferentially degenerate in Huntington's patients. However, expression of wild type huntingtin was able to increase APP transport in all three neuron types (Her and Goldstein, 2008), suggesting that striatal and hippocampal neurons may be more susceptible to axonal transport defects caused by polyQ-htt. Interestingly, Ser421 phosphorylation of polyQ-huntingtin has been found to restore the anterograde transport of BDNF vesicles to wildtype levels, and was also able to increase the

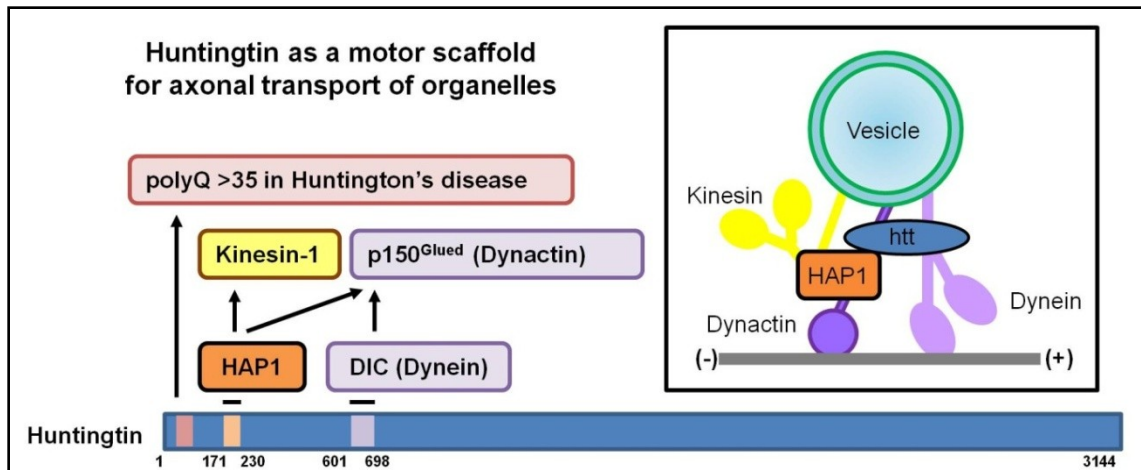


Figure 1.3: Huntingtin as a motor scaffold.

Huntingtin directly binds the retrograde motor DIC (dynein intermediate chain) and the adaptor protein HAP1 (huntingtin-associated protein 1). HAP1 in turn binds dynein-activator p150^{Glued} subunit of dynactin and the anterograde motor kinesin-1. Thus, the huntingtin/HAP1 complex may act to regulate the activity of microtubular motors during axonal transport of various organelles in both the retrograde (towards the cell body) and the anterograde (away from the cell body) direction.

interaction between huntingtin and dynactin (Zala et al., 2008). As axonal transport of organelles such as mitochondria and neurotrophic factors is critical for neuronal survival, polyQ-htt may contribute to Huntington's disease by disrupting axonal transport. In particular, the neurotrophic factor BDNF is critical for the survival of striatal medium spiny neurons, the neuronal type most vulnerable in Huntington's disease, and defective transport of BDNF may further contribute to the selective neuronal vulnerability observed in this disease (Gauthier et al., 2004).

Various explanations have been proposed for the defective axonal transport caused by polyQ-htt, including the decreased association of motor proteins with microtubules in cells expressing polyQ-htt (Gauthier et al., 2004), as well as the sequestration of motors into inclusion bodies in Huntington's disease brains (Trushina et al., 2004). JNK activity is also upregulated in Huntington's disease and is known to phosphorylate kinesin-1 resulting in reduced kinesin binding to microtubules which may disrupt anterograde cargo transport (Morfini et al., 2009). Interestingly, polyQ-htt has increased affinity for HAP1 compared to wildtype huntingtin (Li et al., 1995), suggesting that this enhanced interaction by polyQ-htt may disrupt a huntingtin/HAP1 motor scaffold complex formed on cargos during axonal transport. Thus, it will be important to

examine whether the transport of other organelles such as autophagosomes are also regulated by huntingtin/HAP1 and to understand the mechanisms by which polyQ-htt disrupts organelle transport. In addition, it will also be essential to further study the role of defective axonal transport in contributing to the pathogenesis of Huntington's disease and whether upregulating axonal transport might be a viable therapeutic target.

1.4 Mitophagy

Mitophagy is the homeostatic process of autophagic degradation of damaged mitochondria within the cell (Lemasters, 2005). As mitochondria generate toxic reactive oxygen species (ROS) and contain pro-apoptotic proteins, mitophagy ensures that damaged, depolarized and aged mitochondria do not release these harmful reagents into the cytoplasm and also prevents futile hydrolysis of ATP (Youle and Narendra, 2011). In addition, mitophagy helps degrade mitochondria during cytoplasmic remodeling and ensures the efficient elimination of damaged and mutated mtDNA that can disrupt mitochondrial function and lead to disease (Rodriguez-Enriquez et al., 2009; Suen et al., 2010).

Autophagic engulfment of mitochondria was first observed in early studies in hepatocytes, in association with activation of the mitochondrial permeability transition pore (mPTP) and loss of mitochondrial membrane potential ($\Delta\Psi_m$) (Tolkovsky, 2009). Since then, our understanding of mitophagy in yeast and mammalian cells has greatly advanced (Liu et al., 2014). In particular, the identification of two key players in mammalian mitophagy, PINK1 and parkin, has helped elucidate a PINK1/parkin pathway of mitophagy in mammalian cells (Matsuda et al., 2010; Narendra et al., 2008, 2010b; Vives-Bauza et al., 2010) (see Section 1.4.3). However, the exact mechanisms involved in recognizing damaged mitochondria and subsequently recruiting the autophagic machinery necessary to form the isolation membrane around damaged mitochondria still remain to be identified (Youle and Narendra, 2011). In addition, PINK1/parkin-independent pathways for mitophagy have also been identified,

suggesting that multiple types of mitophagy may occur in different cell types, which may be finely tuned both temporally and spatially to maintain mitochondrial homeostasis (Lemasters, 2014).

In yeast, screens for mutants defective in mitophagy initially identified Atg11 as an essential gene for selective mitophagy (Kanki and Klionsky, 2008). Atg11 is capable of recruiting the mitochondrial fission complex via an interaction with the fission GTPase protein Dnm1 (Mao et al., 2013) and disruption of this interaction blocks mitophagy, suggesting that mitochondrial fission machinery is crucial for progression of mitophagy in yeast. Subsequently, Atg32 was found to mediate Atg11's role in mitophagy (Kanki et al., 2009a; Okamoto et al., 2009). Atg32 is a single-pass membrane protein that localizes to mitochondria with its N terminus in the cytoplasm and C terminus in the mitochondrial intermembrane space. Atg32 binds Atg11 and also binds Atg8/LC3 proteins via its LIR/AIM (Atg8 interacting motif) (Kanki et al., 2009a; Okamoto et al., 2009). Processing of Atg32 may regulate mitophagy, as Ser114 is phosphorylated on Atg32 upon mitophagy induction and mediates Atg32's interaction with Atg11 (Aoki et al., 2011). In addition, cleavage of the C terminus of Atg32 by the mitochondrial i-AAA protease Yme1 is also required for mitophagy (Wang et al., 2013). Interestingly, Atg32 recruits Atg8 and Atg11 as an initial event in yeast mitophagy and occurs prior to autophagosome formation (Kondo-Okamoto et al., 2012). Another yeast protein Atg33 which localizes to mitochondria has also been identified in a screen for partial inhibition of mitophagy during starvation, although its precise role in mitophagy is still unclear (Kanki et al., 2009b). However, mitophagy in mammalian cells does not appear to follow a pathway similar to that identified in yeast.

1.4.1 Mitophagy pathways

In addition to PINK1/parkin-mediated mitophagy (see Section 1.4.3), several other mitophagy pathways have been identified in mammalian cells which are PINK1/parkin independent. Mitophagy induced by iron chelators does not require PINK1 stabilization or parkin activation, and can still occur in primary human fibroblasts from Parkinson's patients with parkin mutations (Allen et al., 2013). Expression of the E3 ubiquitin ligase Gp78 also regulates

mitophagy and induces mitochondrial fragmentation and degradation of mitofusins Mfn1 and Mfn2. Upon mitochondrial depolarization via carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), expression of Gp78 is sufficient to recruit LC3 to mitochondria-associated ER in cells lacking parkin (Fu et al., 2013). In addition, loss of GCN5L1, a component of the mitochondrial acetyltransferase machinery, induced mitophagy and increased mitochondrial turnover in a parkin-independent manner, suggesting that deacetylation of mitochondrial proteins may also initiate mitophagy (Webster et al., 2013).

Another pathway for mitochondrial degradation via lysosomes has also recently been observed, in which vesicles budding from the mitochondria carrying selected mitochondrial protein cargo, termed mitochondria-derived vesicles (MDVs), subsequently fuse with lysosomes (Soubannier et al., 2012). MDVs form in response to oxidative stress and do not require autophagosome proteins Atg5 or LC3 for fusion with lysosomes (Soubannier et al., 2012), suggesting that they provide an additional mechanism by which pieces of mitochondria may be degraded, rather than an entire mitochondria as in the case of classical mitophagy. Surprisingly, both parkin and PINK1 are required for the biogenesis of MDVs, and this vesicle formation is disrupted by a Parkinson's disease-associated mutation in parkin (McLelland et al., 2014).

Mitophagy is also upregulated in specialized cases during erythrocyte maturation, hypoxia and embryogenesis. During erythrocyte maturation, reticulocytes develop into mature erythrocytes over 2-3 days during which all their internal organelles including mitochondria are degraded. This form of mitophagy requires the mitophagy receptor Nix (also known as BNIP3L), a Bcl2-related protein which is located on the outer mitochondrial membrane (Sandoval et al., 2008; Schweers et al., 2007). Nix knockout mice develop anemia, while their erythrocytes retain mitochondria and demonstrate reduced survival (Sandoval et al., 2008). Ribosomal clearance is not disrupted in Nix knockout mice, demonstrating that Nix preferentially regulates mitochondrial clearance (Sandoval et al., 2008). Loss of Nix also does not disrupt autophagosome formation, but prevents the incorporation of mitochondria into autophagosomes (Schweers et al., 2007),

which is normally mediated by Nix's interaction with the autophagosome protein LC3/GABARAP via its N-terminal LIR domain (Novak et al., 2010).

In hypoxia-induced mitophagy, mitochondrial clearance is critical for downregulating oxidative phosphorylation to help prevent ROS accumulation. Upon hypoxia-inducible factor-1 (HIF-1) activation, mitophagy is initiated and requires constitutive expression of Beclin-1 and Atg5, and both Bnip3 and Nix expression (Bellot et al., 2009; Zhang et al., 2008). High levels of Bnip3 and Nix disrupt Bcl2 and Beclin1 interaction (Zhang et al., 2008), which allows for free Beclin1 to initiate Atg5-dependent autophagosome biogenesis. Selective degradation of mitochondria during hypoxia is regulated by the outer mitochondrial membrane protein FUNDC1 whose interactions with LC3 via its LIR motif are increased during hypoxic conditions (Liu et al., 2012).

Finally, during embryogenesis, sperm-derived paternal mitochondria are degraded upon fertilization, leaving only maternal mitochondria and mtDNA in the maturing oocyte. In *C. elegans*, paternal mitochondria are selectively targeted for LC3 autophagic degradation via sperm-derived components which trigger localized autophagosome biogenesis around paternal mitochondria (Al Rawi et al., 2011; Sato and Sato, 2011). In zygotes defective for autophagy, paternal mitochondria remain into the first larval stage (Sato and Sato, 2011).

1.4.2 Mitochondrial dynamics in mitophagy

In addition to maintaining mitochondrial homeostasis via autophagic degradation, mitochondria can undergo dynamic fission and fusion to help segregate their mtDNA content, transfer mitochondrial proteins, and regulate mitochondrial size (Burté et al., 2015). Importantly, defects in mitochondrial fission and fusion machinery also lead to human diseases including neurodegenerative diseases such as autosomal dominant optic atrophy (ADOA) and Charcot–Marie–Tooth neuropathy (CMT) (Itoh et al., 2013). In yeast, mitochondrial fission is regulated by the GTPase Dnm1 which is recruited to mitochondria by the mitochondria-localized proteins Fis1,

while outer and inner mitochondria membrane fusion is regulated by Fzo1 and Mgm1 respectively (Itoh et al., 2013).

In mammalian cells, Drp1 (dynamin-related protein 1) regulates fission and is recruited to mitochondria via Mff (mitochondrial fission factor) (Otera et al., 2010), as well as MiD49 and MiD51 (Palmer et al., 2011), but not Fis1 (Otera et al., 2010). However, there is evidence that MiD49 and MiD51 may be more important for regulating Drp1 recruitment (Losón et al., 2013; Palmer et al., 2013), while Mff and Fis1 may regulate the size and number of Drp1 puncta on mitochondria (Losón et al., 2013). Mammalian fusion is regulated by the mitofusins Mfn1 and Mfn2 which regulate outer mitochondrial membrane fusion (Chen et al., 2003; Koshiba et al., 2004) and Opa1 which regulates inner mitochondrial membrane fusion (Cipolat et al., 2004; Olichon et al., 2003; Song et al., 2009). More recently, mitochondrial fission was found to occur at sites where ER tubules contact mitochondria (Friedman et al., 2011).

The role of mitochondrial fission/fusion dynamics in relation to mitophagy is still unclear. In mammalian cells, elongated mitochondria in tubular networks are spared from autophagic degradation during nutrient starvation (Rambold et al., 2011). Consistent with this observation, inhibition of the fission machinery (Drp1 or Fis1) or overexpression of fusion machinery (Opa1) leading to elongated mitochondria downregulates mitophagy (Macvicar and Lane, 2014; Twig et al., 2008). In addition, parkin is known to ubiquitinate Mfn1 and Mfn2 leading to their proteasomal degradation (Tanaka et al., 2010). However, in yeast, fission-related genes have been reported to either regulate (Kanki et al., 2009b) or not regulate mitophagy (Mendl et al., 2011; Okamoto et al., 2009).

Efficient mitochondrial transport is also critical for proper distribution of mitochondria in cells, and is tightly coupled to autophagic degradation. Mitochondrial transport in neurons is regulated by the Milton/Miro complex which scaffolds the anterograde motor kinesin to mitochondria (Glater et al., 2006) and drives mitochondria axonal transport towards areas with high calcium and energy requirements (MacAskill et al., 2009; Wang and Schwarz, 2009) (see Section 1.2.2). Damaged mitochondria are stalled in axons for mitophagic degradation via release

of the Milton/kinesin motor protein complex from mitochondria via PINK1/parkin-dependent degradation of the adaptor Miro prior to mitophagy (Wang et al., 2011). Interestingly, there are more aged mitochondria in neurites than in the cell body, and the number of aged mitochondria can be increased by inhibiting autophagy (Ferree et al., 2013), further demonstrating that autophagy of aged and damaged mitochondria can be highly spatially regulated.

While cells must maintain efficient protein homeostasis in the cytoplasm, mitochondria must also undergo similar protein homeostasis within their matrix by activating the mtUPR (mitochondrial unfolded protein response) (Haynes and Ron, 2010). Mitochondrial chaperones such as 60kDa chaperone heat shock protein HSPD1 and its cofactor HSPE1, the 70kDa chaperone HSPA9 (also known as mortalin/Grp75/mtHsp70), and the co-chaperones DnaJA3, DnaJC19 and 20 are recruited to help unfold misfolded mitochondrial matrix proteins (Voos, 2013). In addition, mitochondrial proteases such as ClpP (caseinolytic mitochondrial matrix peptidase) and Lon are also involved in degrading misfolded proteins (Voos, 2013). Importantly, expression of misfolded proteins in the mitochondrial matrix or knockdown of the LON protease is sufficient to initiate PINK1/parkin mitophagy (Jin and Youle, 2013), showing that overload of the mtUPR is also tightly coupled to initiation of mitophagic degradation. Cells with deleterious mtDNA mutations have also been found to selectively clear mitochondria with mtDNA mutations via PINK1/parkin mitophagy (Suen et al., 2010).

1.4.3 PINK1/parkin pathway of mitophagy

Parkinson's disease is an adult onset neurodegenerative disease characterized by the formation of cytoplasmic Lewy bodies containing α -synuclein (Spillantini et al., 1997) and the degeneration of dopaminergic neurons in the substantia nigra, although other brain regions are also affected including the limbic and motor system (Braak and Braak, 2000). Both multiplications of the gene encoding α -synuclein (SNCA) (Chartier-Harlin et al., 2004; Singleton et al., 2003) and mutations in SNCA (Polymeropoulos, 1997) cause autosomal dominant forms of Parkinson's disease. Parkinson's disease patients exhibit tremors, bradykinesia and rigidity, and while

existing treatments such as Levodopa target these dopaminergic-related symptoms of the disease, other disabilities associated with Parkinson's disease such as dementia, freezing and falling are still not adequately treated (Schapira et al., 2014).

In addition to developing more targeted therapeutics, identifying biomarkers for Parkinson's disease will also help in creating more precise diagnostics and earlier recognition of Parkinson's disease in patients (Chen-Plotkin, 2014). Recently, plasma epidermal growth factor was identified as a biomarker for cognitive decline in Parkinson's disease (Chen-Plotkin et al., 2011), and the high density lipoprotein component Apolipoprotein A1 (ApoA1) was identified as a biomarker for Parkinson's disease age of onset, with lower levels of plasma ApoA1 corresponding to earlier age of onset (Qiang et al., 2013). Thus, further development of therapeutics, identification of biomarkers, and a better understanding of the cellular mechanism and the pathways that are disrupted in Parkinson's disease will contribute to advancing Parkinson's disease treatment.

The PINK1/parkin-mediated pathway of mitophagy is regulated by two proteins whose mutations cause autosomal recessive familial forms of Parkinson's disease: PINK1 (Valente et al., 2004) and parkin (Kitada et al., 1998) (Figure 1.4). Parkin was soon identified as an E3 ubiquitin ligase (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000) which ubiquitinates α -synuclein (Shimura et al., 2001) and the α -synuclein interacting protein synphilin-1 (Chung et al., 2001). Upon the discovery of mutations in the kinase PINK1 in Parkinson's disease, several groups demonstrated that both PINK1 and parkin were in the same molecular pathway using *Drosophila* models, with PINK1 acting upstream of parkin (Clark et al., 2006; Park et al., 2006b; Yang et al., 2006).

The role of parkin in mitophagy was first identified when HeLa cells expressing exogenous parkin showed preferential recruitment of parkin to depolarized mitochondria, and complete removal of mitochondria after 24h of mitochondrial depolarization by CCCP in the presence of parkin (Narendra et al., 2008). Subsequently, PINK1 was found to be necessary for

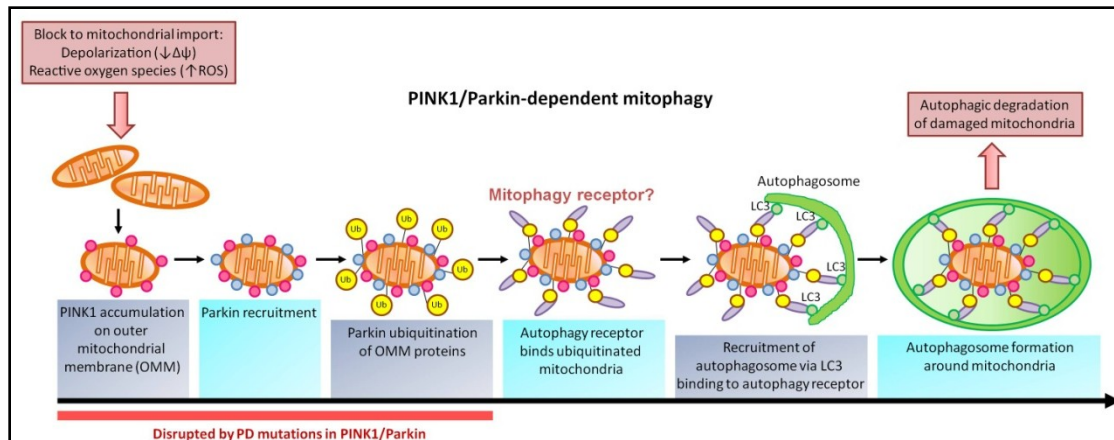


Figure 1.4: PINK1/parkin-dependent mitophagy.

Damage to mitochondria that blocks mitochondrial import leads to the accumulation of the kinase PINK1 on the outer mitochondrial membrane (OMM). PINK1 phosphorylates ubiquitin and the E3 ubiquitin ligase parkin, leading to parkin recruitment and activation. Parkin ubiquitinates OMM proteins, followed by the formation of the autophagosome around ubiquitinated mitochondria and subsequent mitochondrial degradation via autophagy (mitophagy). However, the autophagy receptors which bind ubiquitinated mitochondria and subsequently recruit LC3 on the autophagosomal membrane have not been identified.

parkin recruitment to mitochondria (Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010), demonstrating that both PINK1 and parkin act in a pathway regulating mitochondrial degradation. Importantly, Parkinson's disease-associated mutations in PINK1 and parkin disrupt their respective kinase and ubiquitinating activities (Lee et al., 2010b; Song et al., 2013; Sriram et al., 2005), leading to defective mitochondrial degradation.

Under normal conditions, PINK1 is imported through the TOM complex on the outer mitochondrial membrane and into the TIM complex of the inner mitochondrial membrane. It is then cleaved by the mitochondrial processing peptidase (MPP) (Greene et al., 2012), followed by cleavage by the rhomboid protease PARL (presenilin-associated rhomboid-like protein) (Jin et al., 2010; Meissner et al., 2011) between Ala103 and Phe104 resulting in a 52 kDa N-terminal-deleted fragment of PINK1 (Deas et al., 2011). This fragment is subsequently released and degraded in the cytosol by the proteasome by N-degron type 2 E3 ubiquitin ligases (Yamano and Youle, 2013), resulting in low levels of mitochondrial PINK1 under normal conditions.

However, upon disruption of mitochondrial function by depolarization (Narendra et al., 2008), increased ROS production (Yang and Yang, 2013), activation of the mtUPR (Jin and

Youle, 2013) or expression of the short mitochondrial isoform of ARF (smARF) (Grenier et al., 2014), PINK1 import into the inner membrane is blocked and is thus unable to be cleaved by PARL and MPP. Consequently, uncleaved PINK1 accumulates on the outer mitochondrial membrane bound to the TOM complex (Lazarou et al., 2012; Okatsu et al., 2013). PINK1 mitochondrial accumulation results in the recruitment of the E3 ubiquitin ligase parkin which ubiquitinates outer mitochondrial membrane proteins (Matsuda et al., 2010; Narendra et al., 2010b; Sarraf et al., 2013; Vives-Bauza et al., 2010), including mitochondrial fusion proteins Mfn1 and Mfn2 (Deng et al., 2008; Gegg et al., 2010; Poole et al., 2008; Tanaka et al., 2010; Ziviani et al., 2010), resulting in decreased fusion.

Following parkin recruitment, which occurs <30 minutes after mitochondrial damage (Narendra et al., 2008; Yang and Yang, 2013), autophagosome biogenesis occurs around the mitochondria. The transmembrane protein Atg9A and the ULK1/FIP200 complex are first independently recruited to mitochondria by an unknown mechanism (Itakura et al., 2012). However, as nonselective autophagosome biogenesis occurs at mitochondria-ER junctions (Hamasaki et al., 2013), and as autophagosomes form in response to mitochondrial depolarization even in the absence of parkin (Ding et al., 2010), it is possible that the recruitment of these initial autophagosome proteins to mitochondria does not require parkin. Alternatively, ubiquitin has been found to be crucial in recruiting Atg proteins including ULK1, Atg9, Atg14L and WIPI1 in the selective autophagy of ubiquitinated *Salmonella* (Fujita et al., 2013), suggesting that perhaps parkin ubiquitination is necessary for recruitment of upstream autophagy proteins to mitochondria.

Recruitment of both the Atg9A and the ULK1/FIP200 complex to mitochondria is responsible for recruitment of the Atg14 complex which generates PI3P on the omegasome, to which WIPI1/2 and DFCP1 bind (Itakura et al., 2012). In non-selective autophagy, recruitment of WIPI1/2 and DFCP1 is followed by the Atg12/Atg5/Atg16L complex which conjugates LC3 to PE (Lamb et al., 2013). However, in mitophagy, only Atg16L (and not Atg12 or Atg5) is recruited to mitochondria (Itakura et al., 2012), potentially since Atg16L can directly bind WIPI2 (Dooley et al.,

2014), ubiquitin (Fujita et al., 2013) and FIP200 (Fujita et al., 2013; Gammoh et al., 2013; Nishimura et al., 2013). Recruitment of DFCP1 is accompanied by recruitment of the autophagosome protein LC3 to the omegasome membrane generated around mitochondria (Yang and Yang, 2013), and ultimately leads to the formation of an autophagosome around the damaged mitochondria which is subsequently degraded over 24 hours via fusion with lysosomes (Narendra et al., 2008). Lipidated LC3 is required for closure of the autophagosome around mitochondria (Itakura et al., 2012). When mitophagy is initiated in the absence of lipidated LC3 in Atg3 KO MEFs, autophagosomes form which are not properly closed and do not contain mitochondrial cargo (Itakura et al., 2012), demonstrating that LC3 is essential for autophagic recognition of mitochondria.

Understanding how autophagosomes selectively engulf mitochondrial cargo during mitophagy remains an important unanswered question in the field. As mentioned above (see Section 1.1.2), autophagy receptors which contain an LIR (LC3-interacting region) motif and bind cargo either directly or indirectly by binding ubiquitin on the cargo may regulate selective recognition of mitochondria during PINK1/parkin mitophagy. As parkin ubiquitinates multiple proteins on the outer mitochondrial membrane (Sarraf et al., 2013), it has been proposed that classical autophagy receptors which bind ubiquitin via their ubiquitin binding domain might bind parkin-ubiquitinated mitochondrial proteins, and subsequently mediate LC3 recruitment and autophagosome assembly around damaged mitochondria. In particular, of the autophagy receptors identified to date (Rogov et al., 2014), only p62's role in parkin-mediated mitophagy has been examined, generating conflicting reports on whether p62 functions as either an autophagy receptor (Ding et al., 2010; Geisler et al., 2010) or a regulator of perinuclear clustering of depolarized mitochondria (Narendra et al., 2010a; Okatsu et al., 2010). Thus, identifying the autophagy receptor in PINK1/parkin mitophagy will be crucial for answering this question and further advancing our understanding of selective autophagosome biogenesis during mitophagy.

1.4.4 Mitophagy in neurons

As the majority of studies on PINK1/parkin mitophagy have been performed in non-neuronal cells, it is still unclear whether PINK1/parkin mitophagy normally occurs in neurons and at what rates (Amadoro et al., 2014; Grenier et al., 2013). In the initial study identifying parkin's role in mitophagy, there was a small increase in endogenous parkin observed in the mitochondrial fraction after CCCP treatment in cortical neurons, suggesting that endogenous parkin is recruited to depolarized mitochondria in neurons (Narendra et al., 2008). In neurons expressing exogenous parkin, parkin has been reported to be recruited to mitochondria upon CCCP treatment (Cai et al., 2012; Joselin et al., 2012; Koyano et al., 2013; McCoy et al., 2014) and valinomycin treatment (Seibler et al., 2011).

However, it has also been reported that CCCP treatment does not upregulate exogenous parkin recruitment to mitochondria in neurons since neurons depend on oxidative phosphorylation for survival (van Laar et al., 2011). Thus, unlike mitotic cells, which can switch to glycolysis and do not require mitochondria for survival, neurons may not have a mechanism for efficiently degrading all their mitochondria. Further, in contrast to the observation that parkin recruitment occurs within 30 minutes in immortalized cell lines, parkin recruitment in neurons has reported to occur 4 to 24 hours after mitochondrial depolarization (Cai et al., 2012; Joselin et al., 2012; van Laar et al., 2011; Seibler et al., 2011), and only occurs in 25-70% of neurons (Cai et al., 2012; Joselin et al., 2012; Koyano et al., 2013; McCoy et al., 2014; Seibler et al., 2011).

Recently, locally-induced damage to mitochondria in hippocampal axons was found to initiate mitophagy dependent on PINK1 and parkin. Exogenous fluorescently-tagged parkin, LC3-positive autophagosomes, and LAMP1-positive lysosomes were observed to rapidly translocate to damaged mitochondria within 20-40 minutes, and this was not observed in neurons from either PINK1 or parkin knockout mice (Ashrafi et al., 2014). These results further suggest that local mitophagy can occur in the axon and does not require damaged mitochondria to be retrogradely transported to the cell soma before they can be engulfed by autophagosomes, consistent with the

findings that damaged mitochondria are stalled in axons for mitophagic degradation in a PINK1/parkin-dependent manner (Wang et al., 2011).

In addition to damage-induced mitophagy, robust basal mitophagy has also been observed to occur at the axon tip, resulting in autophagosomes already containing mitochondrial fragments leaving the axon tip (Maday et al., 2012). Interestingly, these autophagosomes exhibit robust retrograde transport back towards the cell body (Maday et al., 2012), suggesting that at basal levels, the mitochondrial lipids and proteins may be efficiently recycled in the cell body and may be used for future mitochondrial biogenesis. Thus, further understanding whether PINK1 and parkin regulate basal mitophagy at the axon tip, as well as how often mitophagy is normally initiated in areas outside the axon tip will be important in further identifying the role that mutant PINK1 and parkin play in contributing to neurodegeneration in Parkinson's disease.

1.5 Optineurin

Optineurin (*optic neuropathy inducing*) was first named when mutations in optineurin were found to lead to primary open angle glaucoma (Rezaie et al., 2002). Subsequently, mutations in optineurin were also linked with ALS (van Blitterswijk et al., 2012; Cirulli et al., 2015; Iida et al., 2012; Maruyama et al., 2010; Millecamps et al., 2011), and optineurin has also been identified in a genome wide association study as a genetic risk factor for Paget's disease of bone (Albagha et al., 2010). Optineurin was recently found to be an autophagy receptor, binding ubiquitin via its UBA1 (Ubiquitin binding in ABIN and NEMO) domain (Zhu et al., 2007) and the autophagosome-associated protein LC3 via its LIR domain (LC3 interacting region) (Wild et al., 2011). However, in addition to a role in autophagy, optineurin also binds several other proteins and is involved in multiple cellular processes including vesicle trafficking, NF- κ B signaling, and the regulation of mitosis (Kachaner et al., 2012a).

Optineurin regulates vesicle trafficking through its interactions with multiple proteins including Rab8, huntingtin, myosin VI and transferrin receptor (TfR) (Kachaner et al., 2012a) (Figure 1.5). Optineurin was originally identified as a huntingtin-interacting protein (Faber et al.,

1998) and can recruit huntingtin to Rab8 vesicles, as optineurin is preferentially recruited to activated GTP-bound Rab8 via its N-terminus (Hattula and Peränen, 2000). As optineurin binds the actin motor myosin VI and as huntingtin interacts with microtubular motors dynein/dynactin and kinesin, optineurin and huntingtin have been proposed to regulate Rab8 vesicle motility on actin and microtubules (Sahlender et al., 2005) and the optineurin/huntingtin complex may regulate cargo transport switches between actin and microtubule tracks (Caviston and Holzbaur, 2009). In addition, optineurin's interaction with myosin VI also helps maintain Golgi ribbon structure (Sahlender et al., 2005), regulates polarized delivery of the epidermal growth factor receptor (EGFR) to the leading edge in migratory cells (Chibalina et al., 2010) and controls the basolateral delivery of membrane proteins sorted by the clathrin adaptor protein complex AP-1B (Au et al., 2007). Optineurin also regulates the trafficking of TfR (Nagabhushana et al., 2010; Park et al., 2010) through optineurin's negative feedback interaction with the GTPase-activating protein (GAP) TBC1D17 which it recruits to Rab8, leading to inactivation of Rab8 (Vaibhava et al., 2012).

Optineurin also regulates NF- κ B signaling and is 53% similar to NEMO (NF- κ B essential modulator), one of the proteins involved in NF- κ B signaling (Schwamborn et al., 2000). During classical NF- κ B signaling, cytokines such as TNF α bind to the surface receptor TNFR1 (TNF α receptor 1), leading to the formation of a cytosolic signaling complex involving TRADD (TNFR1-associated death domain protein), TRAF2 (TNF receptor associated factor 2) and RIP (receptor interacting protein). This signaling complex activates the kinase complex IKK (I κ B kinase), which consists of the catalytic subunits IKK α and β and the regulatory subunit NEMO/IKK- γ . This activated kinase complex then phosphorylates the inhibitory proteins I κ B α and I κ B β leading to their proteasomal degradation. NF- κ B, which is normally bound to I κ B α and I κ B β , is then free to enter the nucleus and induce transcription of genes regulating cell survival, inflammation and immunity (Kachaner et al., 2012a). Optineurin negatively regulates NF- κ B signaling by competing with NEMO for binding of polyubiquitinated RIP (Zhu et al., 2007). Optineurin further negatively regulates NF- κ B signaling by facilitating deubiquitination of

polyubiquitinated RIP by interacting with the deubiquitinase CYLD (Nagabhushana et al., 2011). Interestingly, NF- κ B binds to optineurin's promoter region and enhances optineurin expression (Sudhakar et al., 2009), allowing for a negative feedback loop on NF- κ B activation via optineurin.

During mitosis, the kinase Plk1 (Polo-like kinase) involved in regulating cell division cycle events phosphorylates optineurin at Ser177, leading to optineurin's localization to the nucleus where it enhances phosphorylation of its binding partner MYPT1 (myosin phosphatase target subunit 1) by Cdk1 (cyclin dependent kinase 1) (Kachaner et al., 2012b). Phosphorylated MYPT1 can then bind and inactivate Plk1 (Kachaner et al., 2012b), demonstrating another negative feedback loop involving optineurin, which in this case regulates Plk1 activity. Optineurin also negatively regulates type I interferon IFN β production during viral infection, by interacting with TBK1, a kinase which activates IRF3/7 (interferon regulatory factor 3/7) transcription factors (Mankouri et al., 2010). Optineurin has been proposed to both inhibit TBK1 phosphorylation of IRF3 (Sakaguchi et al., 2011), as well as activate TBK1 activity in response to lipopolysaccharide via optineurin binding to polyubiquitinated species (Gleason et al., 2011). Interestingly, optineurin is also a substrate of TBK1 kinase activity, and TBK1 phosphorylation of optineurin regulates autophagy (Wild et al., 2011).

1.5.1 Optineurin in glaucoma and ALS

Mutations in optineurin lead to normal tension glaucoma (Rezaie et al., 2002) and ALS (Maruyama et al., 2010) (Figure 1.5). Glaucoma is an optic neuropathy involving the progressive degeneration of retinal ganglion cells leading to peripheral vision loss, and is further exacerbated by damage to the optic nerve composed of retinal ganglion axons and glial cells (Weinreb et al., 2014). Glaucomas can be divided into two types: angle closure glaucoma (ACG) (in which the iridio-corneal angle is closed, blocking the drainage of aqueous humor, resulting in elevated intraocular pressure (IOP)) or primary open angle glaucoma (POAG) (in which the iridio-corneal angle remains 'open' and not anatomically hindered). In POAG, inefficient drainage of the aqueous humor still occurs, resulting in increased IOP, although there is a subset of primary open

angle glaucoma in which patients develop glaucoma without elevated IOP, known as normal tension glaucoma (NTG) (Weinreb et al., 2014). Thus, elevated IOP is an important risk factor for retinal degeneration in glaucoma, but is not necessary for retinal cell death as in the case of NTG.

Mutations in Myocilin/TIGR (trabecular meshwork inducible glucocorticoid response) (Stone, 1997), WDR36 (WD repeat 36) (Monemi et al., 2005), neurotrophin-4 (Pasutto et al., 2009) and CYP1B1 (Melki et al., 2004) have been linked to POAG, while mutations in optineurin lead to the POAG subset NTG (Rezaie et al., 2002). Optineurin mutations are associated with ~17% of families with NTG, of which the autosomal dominant E50K optineurin mutation is the most common, accounting for 13% of NTG cases (Rezaie et al., 2002). However, the mechanism by which optineurin mutations lead to glaucoma still remains to be understood.

A transgenic mouse model of E50K leads to progressive retinal degeneration (Chi et al., 2010) and expression of E50K optineurin, but not other glaucoma-related optineurin mutations (H26D, H486R, R545Q), leads to retinal ganglion cell death in cell culture (Chalasan et al., 2007). E50K optineurin also causes Golgi fragmentation (Park et al., 2006a), formation of larger autophagosomes (Shen et al., 2011), and has decreased binding to Rab8 compared to wildtype optineurin (Chi et al., 2010) but whether these observations are related to retinal ganglion cell degeneration is unclear. Another glaucoma-associated optineurin mutation H486R exhibits decreased interactions with CYLD and polyubiquitinated RIP, and is unable to inhibit TNF α -induced NF- κ B activation (Nagabhushana et al., 2011).

Mutations in optineurin also lead to familial ALS (Maruyama et al., 2010). ALS is a rapidly progressing adult-onset neurodegenerative disease characterized by degeneration of both lower and upper motor neurons, leading to progressive paralysis and death from respiratory failure 2-3 years after symptom onset (Renton et al., 2014). Approximately 10% of ALS cases are familial, and have been linked to mutations in multiple proteins including SOD1 (superoxide dismutase 1) (Rosen et al., 1993), the actin-binding protein profilin 1 (Wu et al., 2012), the RNA-binding proteins TDP-43 (Sreedharan et al., 2008) and FUS (fused in sarcoma) (Kwiatkowski et al., 2009; Vance et al., 2009), and the ubiquitin binding proteins VCP (valosin containing protein)

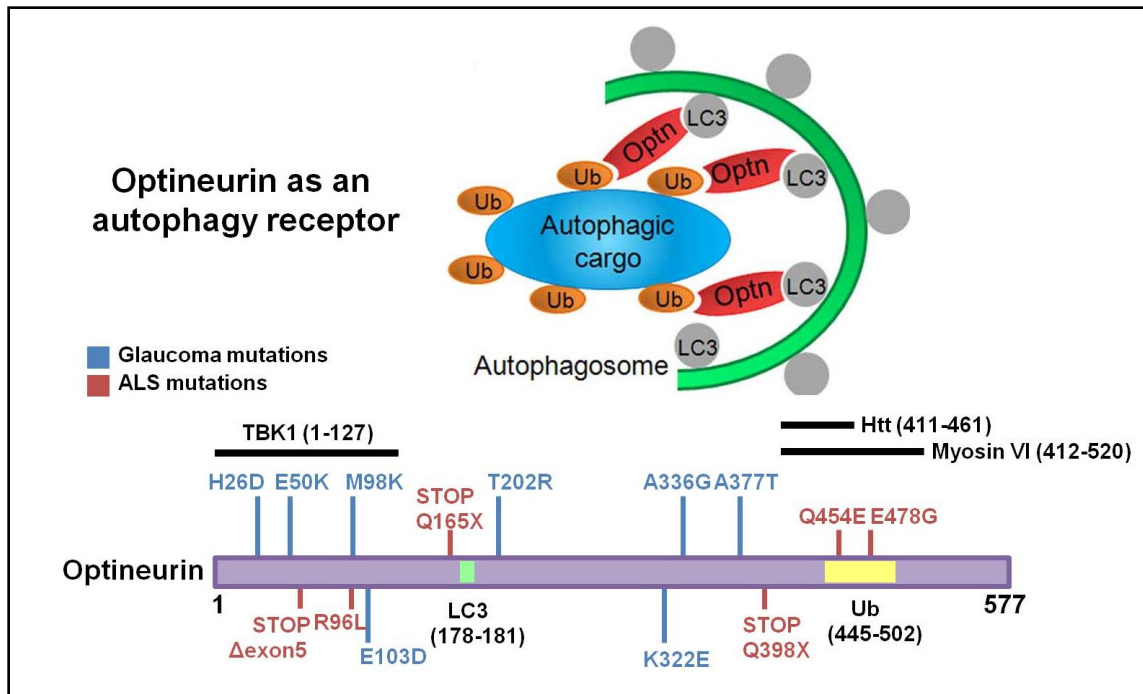


Figure 1.5: Optineurin as an autophagy receptor.

Optineurin interacts with many proteins including TBK1 (TANK-binding kinase 1), htt (huntingtin) and myosin VI. Mutations in optineurin have been linked to both glaucoma (blue) and ALS (red). Recently, optineurin was identified as an autophagy receptor which binds both the autophagosome protein LC3 and ubiquitin, allowing it to recruit autophagosome formation around autophagic cargo.

(Johnson et al., 2010), p62 (Fecto et al., 2011), ubiquitin 2 (Deng et al., 2011) and optineurin (Maruyama et al., 2010). More recently, hexanucleotide expansions in intron 1 of C9ORF72 have accounted for 40% of familial ALS cases (DeJesus-Hernandez et al., 2011; Renton et al., 2011). As these mutant proteins have very different cellular functions, it is still unclear what mechanisms lead to motor neuron death, and whether these different mutants converge into a single pathway which triggers neurodegeneration, or whether they are involved in various cellular mechanisms which are all capable of causing motor neuron death. Interestingly, TDP-43 was found to be a major component of the ubiquitin-positive neuronal inclusions found in both ALS and another neurodegenerative disease FTLD-TDP43 (frontotemporal lobar degeneration) (Neumann et al., 2006), demonstrating that these two different clinical conditions exist on the same spectrum and further posing new questions about the pathogenesis of ALS.

Several different mutations in optineurin including the E478G mutation in the UBAN domain have been identified in both familial and sporadic ALS patients (van Blitterswijk et al., 2012; Cirulli et al., 2015; Iida et al., 2012; Maruyama et al., 2010; Millecamps et al., 2011), but how these mutations cause motor neuron degeneration remain unclear. ALS-associated E478G and Q398X optineurin mutations have defective negative regulation of IRF3 activation during viral infection (Sakaguchi et al., 2011), while E478G optineurin is unable to inhibit TNF α -induced NF- κ B activation (Maruyama et al., 2010). In addition, the E478G optineurin mutation disrupts optineurin binding to ubiquitin, which is essential for its role as an autophagy receptor (Wild et al., 2011). Thus, it will be important to further understand the roles of optineurin and how specific mutations in optineurin lead to ALS. In addition, why different mutations in optineurin lead to two distinct diseases, glaucoma and ALS, is still unknown, although it is interesting to note that motor neurons have the longest axons across neuronal populations, followed by retinal ganglion cells.

1.5.2 Optineurin as an autophagy receptor

Optineurin is an autophagy receptor that preferentially binds K63-linked and linear ubiquitin chains via its UBAN domain (ubiquitin binding in ABIN and NEMO) (Zhu et al., 2007) and the autophagosome-associated protein LC3 via its LIR domain (LC3 interacting region) (Wild et al., 2011) (Figure 1.5). Optineurin was first identified as an autophagy receptor for ubiquitinated *Salmonella*, and is further regulated by TBK1-mediated Ser177 phosphorylation, which enhances the optineurin/LC3 binding affinity and upregulates autophagic degradation of *Salmonella* (Wild et al., 2011). Introducing a mutation in either the ubiquitin-binding domain of optineurin (DF474, 475NA) or in the LIR region (F178A) results in inefficient *Salmonella* degradation. Interestingly, optineurin targets the same bacteria as the autophagy receptors NDP52 and p62. However, while optineurin and NDP52 colocalize to the same subdomains on individual bacteria, these are different from the subdomains to which p62 colocalizes (Wild et al., 2011).

Optineurin was later found to recognize protein aggregates via its C-terminal coiled-coil domain in a ubiquitin-independent manner and regulate their autophagic degradation (Korac et

al., 2013). Interestingly, morpholino-silencing of optineurin in zebrafish results in a motor axonopathy phenotype (Korac et al., 2013), suggesting that optineurin may cause ALS via a loss of function mechanism. More recently, optineurin has been found to regulate autophagosome maturation during endosomal fusion (Tumbarello et al., 2012). The autophagy receptors NDP52, optineurin, T6BP are all capable of binding myosin VI, which can subsequently recruit Tom1, a myosin VI binding partner on endosomes. The myosin VI/Tom1 interaction has been proposed to mediate delivery of endosomal membranes to autophagosomes and promote autophagosome maturation and lysosomal fusion (Tumbarello et al., 2012). Thus, whether optineurin is capable of regulating the selective autophagic degradation of other types of autophagic cargo such as mitochondria during mitophagy, and whether optineurin might play additional roles during autophagosome formation and/or maturation still remain to be investigated.

1.6 Summary

In this chapter, I have introduced the autophagy pathway and highlighted the importance of both efficient axonal transport in neurons and properly regulated mitophagy (mitochondrial degradation via autophagy). In addition, I have introduced huntingtin (in which polyglutamine expansions lead to Huntington's disease) as a motor scaffold for organelle transport in neurons, and optineurin (in which mutations lead to glaucoma and familial ALS) as a recently identified autophagy receptor.

In the next few chapters, I will present the work I have done identifying novel roles for huntingtin and optineurin in the regulation of autophagosome dynamics and how these dynamics are disrupted in neurodegenerative disease models. In Chapter 2, I present our study demonstrating that huntingtin regulates the axonal transport of autophagosome in primary neurons, and show that this transport is disrupted by polyQ-htt in Huntington's disease models leading to defective cargo degradation (published in *Journal of Neuroscience*) (Wong and Holzbaur, 2014a). In Chapter 3, I present our work identifying optineurin as an autophagy receptor for damaged mitochondria in PINK1/parkin-dependent mitophagy, and show that an

ALS-associated mutation in optineurin disrupts efficient mitochondrial degradation (published in *Proceedings of the National Academy of Sciences*) (Wong and Holzbaur, 2014b). In Chapter 4, I review the work that has been done in the field of autophagy and neurodegeneration, and highlight defects in autophagy dynamics that have been found in Alzheimer's disease, Parkinson's disease, ALS and Huntington's disease (published in *Journal of Cell Science*) (Wong and Holzbaur, 2015).

Finally, in Chapter 5, I discuss the implications of our studies on the regulation of autophagosome transport by huntingtin and optineurin as a mitophagy receptor. I then describe future areas of study including studying the role of optineurin in neuronal mitophagy, as well as potential novel roles for actin in regulating mitochondrial quality control during mitochondrial fission and mitophagy.

2 THE REGULATION OF AUTOPHAGOSOME DYNAMICS BY HUNTINGTIN AND HAP1 IS DISRUPTED BY EXPRESSION OF MUTANT HUNTINGTIN LEADING TO DEFECTIVE CARGO DEGRADATION

All the work presented in chapter two was completed by Yvette Wong.

This chapter was written by Yvette Wong and Erika Holzbaur and was recently published in the
Journal of Neuroscience (Wong and Holzbaur, 2014a).

2.1 Introduction

Macroautophagy (referred to here as autophagy) is a critical cellular degradation pathway mediated by the autophagosome, a double-membrane vesicle that engulfs organelles and protein cargo (Xie and Klionsky, 2007). Defective autophagy leads to neurodegeneration (Hara et al., 2006; Komatsu et al., 2006) and is implicated in neurodegenerative diseases including Huntington's disease (HD) (Martinez-Vicente et al., 2010; Wong and Cuervo, 2010). In neurons, autophagosome formation is a polarized process (Maday et al., 2012). Nascent autophagosomes form and engulf cargo at the axon tip and subsequently undergo robust retrograde axonal transport towards the soma (Lee et al., 2011; Maday et al., 2012). Despite the pronounced unidirectional motility observed during autophagosome movement along the axon, both retrograde and anterograde motor proteins remain robustly associated with autophagosomes (Maday et al., 2012), suggesting that regulatory proteins such as scaffolding proteins may be necessary to promote efficient transport by controlling motor activity.

Huntingtin (htt) regulates the transport dynamics of various organelles (Caviston et al., 2007; Gauthier et al., 2004; Gunawardena et al., 2003; Her and Goldstein, 2008; Power et al., 2012). Htt directly binds the retrograde motor dynein (Caviston et al., 2007), and interacts with the dynein-activator dynactin and the anterograde motor kinesin-1 via the adaptor protein HAP1 (huntingtin-associated protein-1) (Engelender et al., 1997; Li et al., 1998, 1995; McGuire et al., 2006; Twelvetrees et al., 2010). Thus, the formation of a htt-HAP1-motor complex may regulate autophagosome dynamics along the axon.

Polyglutamine expansions in htt (polyQ-htt) cause HD, and both soluble and aggregated polyQ-htt are cleared by autophagy (Qin et al., 2003; Ravikumar et al., 2002). Disruption of autophagy accelerates polyQ-htt aggregate formation and cell death (Ravikumar et al., 2004; Sarkar et al., 2007), and defects in autophagic cargo loading (Martinez-Vicente et al., 2010) have been observed in HD models, further suggesting a link between defects in autophagy and HD.

Here, we find a role for htt and HAP1 as regulators of autophagosome transport. Using live cell imaging in primary neurons, we show that the htt/HAP1 complex enhances retrograde

motility, promoting efficient autophagosome transport towards the soma. Autophagosome transport is disrupted in primary neurons expressing pathogenic polyQ-htt and also in striatal cells from HD knock-in mice. Using RNAi to deplete endogenous htt, we find that htt is not required for the initial steps of autophagosome formation or cargo loading. Rather, the defective autophagosome transport observed in both htt-depleted neurons and polyQ-htt expressing neurons results in the aberrant accumulation of autophagosomes with engulfed mitochondrial fragments, suggesting that cargo degradation is impaired. Thus, defects in autophagosome transport and cargo degradation may contribute to the inefficient clearance of dysfunctional mitochondria and the accumulation of polyQ-htt observed in the neurons of patients with HD.

2.2 Results

2.2.1 Htt regulates autophagosome transport in neurons

Htt regulates the transport of organelles including BDNF (brain derived neurotrophic factor)-containing vesicles and recycling endosomes (Caviston et al., 2007; Gauthier et al., 2004; Gunawardena et al., 2003; Her and Goldstein, 2008; Power et al., 2012). Here we examined whether htt and its adaptor protein HAP1 regulate autophagosome dynamics. Htt has previously been localized to the outer membrane of liver-isolated autophagosomes (Atwal et al., 2007; Martinez-Vicente et al., 2010). To test whether htt and HAP1 are associated with neuronal autophagosomes, we isolated autophagosomes from mouse brain (Morvan et al., 2009; Strømhaug et al., 1998), enriching for LC3-II, the membrane-associated lipidated form of the autophagosome marker LC3 (Bampton et al., 2005; Kabeya, 2000; Klionsky, DJ, 2012). Htt and HAP1 isoforms HAP1a and HAP1b copurified in LC3-II-enriched fractions (Figure 2.1A), along with dynein, dynactin, and kinesin-1 as shown previously (Maday et al., 2012). Immunofluorescent staining of LC3 in axons from primary neurons showed ~65% of LC3 puncta colocalized with endogenous htt (Figure 2.1B,C). Endogenous HAP1 also showed >50% colocalization with LC3 puncta in axons of primary neurons (Figure 2.1D).

To study the regulation of autophagosome dynamics in primary neurons, we isolated dorsal root ganglion (DRG) neurons from GFP-LC3 transgenic mice (Mizushima et al., 2004) and analyzed GFP-LC3 labeled motility along neurites using live cell imaging. DRG neurites extend up to 1000 μ m from the soma (Perlson et al., 2009); microtubules in these neurites are uniformly polarized (Maday et al., 2012). Using this model system, we have found that autophagosomes form constitutively at neurite tips and undergo robust unidirectional transport towards the soma (Maday et al., 2012) driven by dynein and dynactin (Jahreiss et al., 2008; Kimura et al., 2008; Lee et al., 2011; Maday et al., 2012; Ravikumar et al., 2005).

We depleted htt from DRGs using fluorescently-labeled siRNA; ~80% of neurons were positive for the htt-siRNA, leading to reduced htt levels on immunoblot (Figure 2.2B). Htt depletion did not significantly decrease neurite length under our conditions (mock, $507 \pm 19.1 \mu$ m vs htt KD,

475 ± 15 µm). Autophagosomes in control neurons showed robust motility along the neurite, with the majority (70 ± 4.8%) moving in the retrograde direction (≥10 µm/3 min) while few (28 ± 4.5%) were stationary (<10 µm/3 min) as described (Maday et al., 2012). In contrast, htt-depleted neurons had a significantly decreased percentage of retrograde autophagosomes (45 ± 6.9%; $p < 0.01$) and a significant increase in bidirectional or stationary autophagosomes (52 ± 6.6%; $p < 0.01$) (Figure 2.2A,C). Further, autophagosomes in htt-depleted neurons exhibited reduced run lengths and run speeds; both net runs and individual runs were decreased (Figure 2.2D,E). To confirm specificity of knockdown, we rescued neurons depleted of endogenous htt with a full-length human htt-Q23 construct (WT-htt) resistant to the oligos used for siRNA. Rescue with WT-htt significantly increased the percentage of retrograde autophagosomes to levels similar to control neurons (Figure 2.2F,G) and significantly increased autophagosome net run lengths (htt KD, 17.2 ± 2.03 µm vs htt KD + WT-htt, 25.2 ± 2.41 µm; $p < 0.05$) and net run speeds (htt KD, 0.10 ± 0.012 µm/sec vs htt KD + WT-htt, 0.15 ± 0.015 µm/sec; $p < 0.05$). Importantly, these effects were

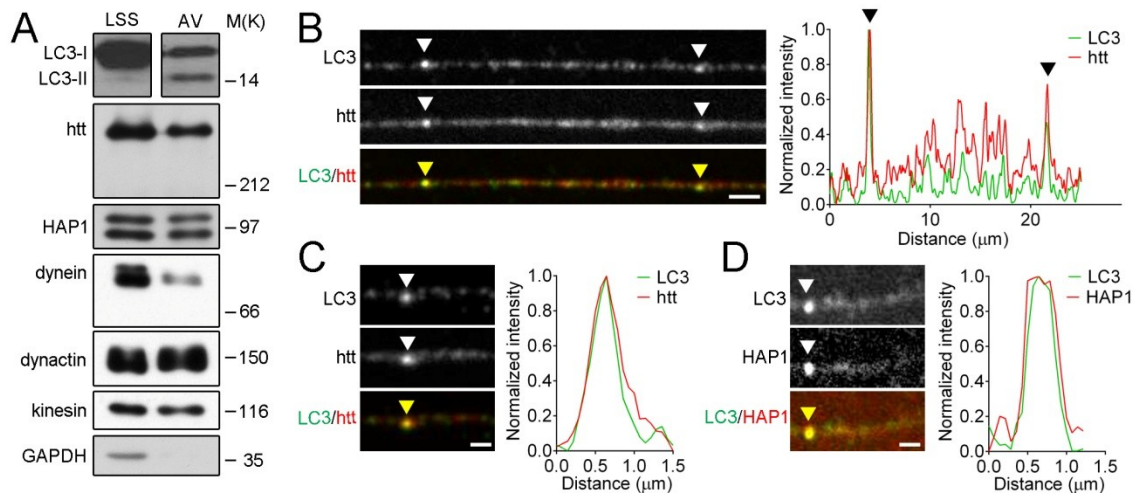


Figure 2.1: Htt associates with autophagosomes in neurons

(A) Autophagosome-enriched fraction (AV) prepared from mouse brain containing LC3-II, the lipidated form of LC3, is positive for htt, HAP1, the retrograde motor complex proteins dynein and dynactin, and the anterograde motor protein kinesin, but not for the cytoplasmic protein GAPDH. Equal total protein was loaded from the low speed supernatant fraction (LSS) obtained before purification for comparison by immunoblot. (B-D) Representative images with corresponding line scans of immunostaining of endogenous LC3 with htt (B, C) and HAP1 (D) in axons from primary DRG neurons demonstrate colocalization of htt and HAP1 with autophagosomes. Arrowheads highlight areas of colocalization. (Scale bars: B-D, 1 µm).

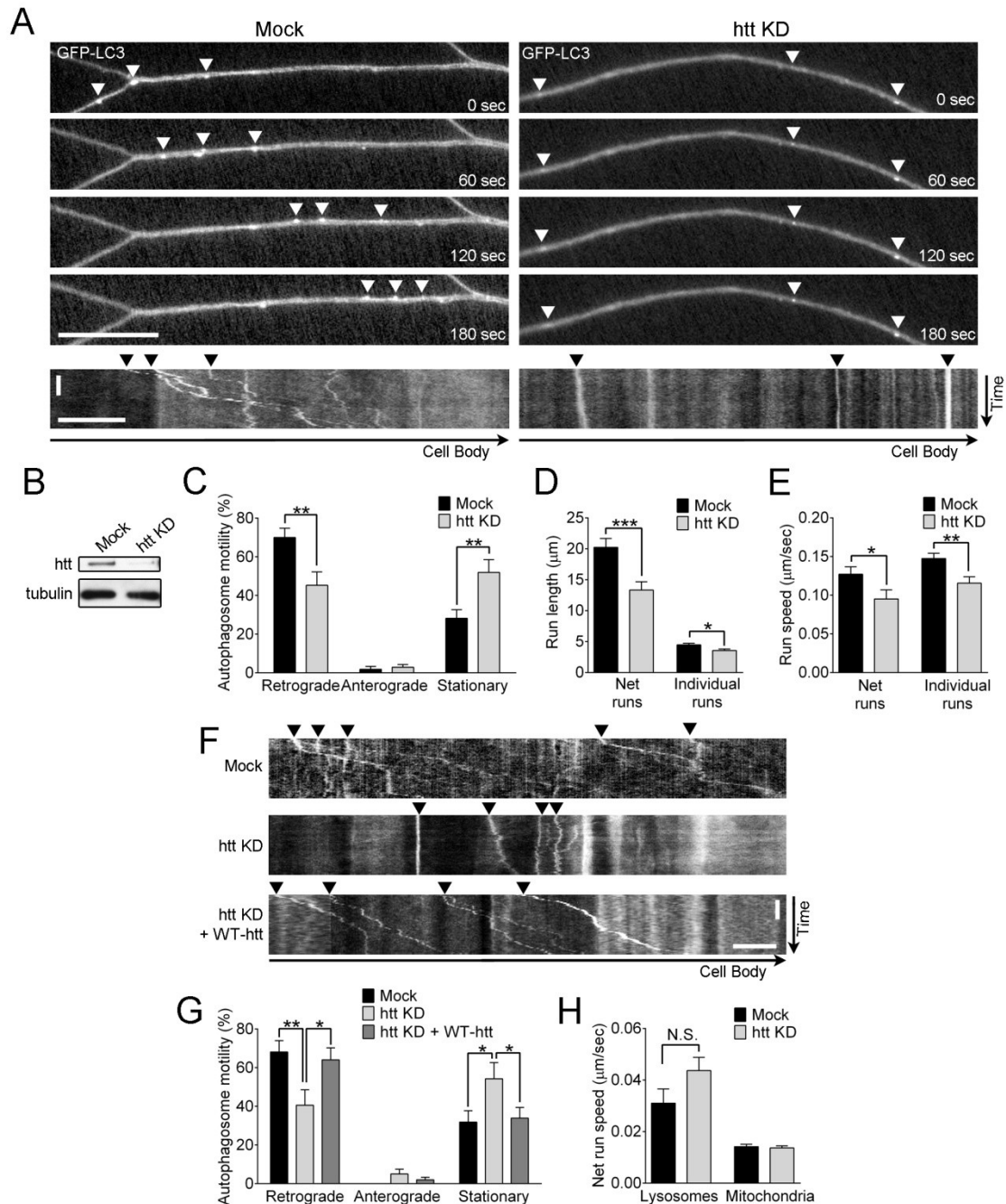


Figure 2.2: Htt regulates autophagosome transport in neurons.

(A) Representative time series and corresponding kymographs of GFP-LC3 autophagosome transport in axons of control (mock) and htt siRNA-depleted (htt KD) primary DRG neurons from GFP-LC3 transgenic mice. Autophagosomes in control neurons demonstrate robust retrograde transport (arrowheads) whereas autophagosomes in htt-depleted neurons show disrupted motility (arrowheads). (B) Htt protein levels are efficiently depleted by htt siRNA in immunoblot. (C) Depleting htt decreases the percentage of retrograde autophagosomes (moving $\geq 10 \mu\text{m}/3\text{min}$ in the retrograde direction) and increases the percentage of stationary autophagosomes (moving

<10 $\mu\text{m}/3\text{min}$) in neurons (mock, $n = 23$; htt KD, $n = 23$). The percentage of anterograde autophagosomes (moving $\geq 10 \mu\text{m}/3\text{min}$ in the anterograde direction) was not altered. (D, E) Run lengths (D) and run speeds (E) from autophagosome net runs (total distance traveled over 3 minutes) and individual runs (distance traveled before changing direction or speed) are reduced by htt depletion in primary neurons (net runs: mock, $n = 143$; htt KD, $n = 162$) (individual runs: mock, $n = 667$; htt KD, $n = 608$). (F) Representative kymographs of GFP-LC3 autophagosome transport demonstrate rescue of retrograde autophagosome transport (arrowheads) in htt-siRNA neurons expressing siRNA-resistant wildtype htt Q23 (htt KD + WT-htt) compared to htt siRNA-depleted (htt KD) primary DRG neurons. Rescued neurons have similar autophagosome transport to control neurons (mock). (G) Expression of wildtype htt rescues the percentage of retrograde autophagosomes in htt-siRNA neurons (mock, $n = 17$; htt KD, $n = 15$; htt KD + WT-htt, $n = 12$). (H) Htt depletion does not disrupt net run speeds of lysosomes or mitochondria in primary DRG neurons (lysosomes: mock, $n = 126$; htt KD, $n = 149$) (mitochondria: mock, $n = 122$; htt KD, $n = 177$). (Horizontal scale bars: A, F, 10 μm . Vertical scale bars, 1 min). Values represent means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

specific to autophagosomes, as neither lysosomal nor mitochondrial velocities were reduced by htt depletion (Figure 2.2H).

Htt directly binds dynein, and indirectly binds both dynactin and kinesin-1 via the adaptor HAP1 (Caviston et al., 2007; Engelender et al., 1997; Li et al., 1998, 1995; McGuire et al., 2006; Twelvetrees et al., 2010). Thus, htt might regulate autophagosome transport either by interacting with bound motor proteins via a scaffolding activity, or alternatively, by recruiting motor proteins to autophagosomes. To test the first hypothesis that htt acts as a scaffold to regulate efficient motor function, we depleted endogenous htt from GFP-LC3 primary DRG neurons and rescued with an siRNA-resistant wildtype htt construct (WT-htt) or dominant negative htt constructs unable to bind to either dynein (htt- Δdyn) or HAP1 (htt- ΔHAP1) (Pardo et al., 2010) (Figure 2.3B). While WT-htt could fully rescue defects in retrograde motility induced by htt depletion, autophagosomes in neurons expressing either htt- Δdyn or htt- ΔHAP1 demonstrated significantly disrupted autophagosome transport (Figure 2.3A,C). Moreover, both htt- Δdyn and htt- ΔHAP1 were unable to rescue autophagosome net run length or speed (Figure 2.3D,E). These results are consistent with the hypothesis that the interactions of htt and HAP1 with microtubule motors regulate their activity to promote efficient unidirectional transport.

We next tested the hypothesis that htt recruits motor proteins to autophagosomes. We depleted htt in DRG neurons and used live cell imaging to determine that both dynein (Figure

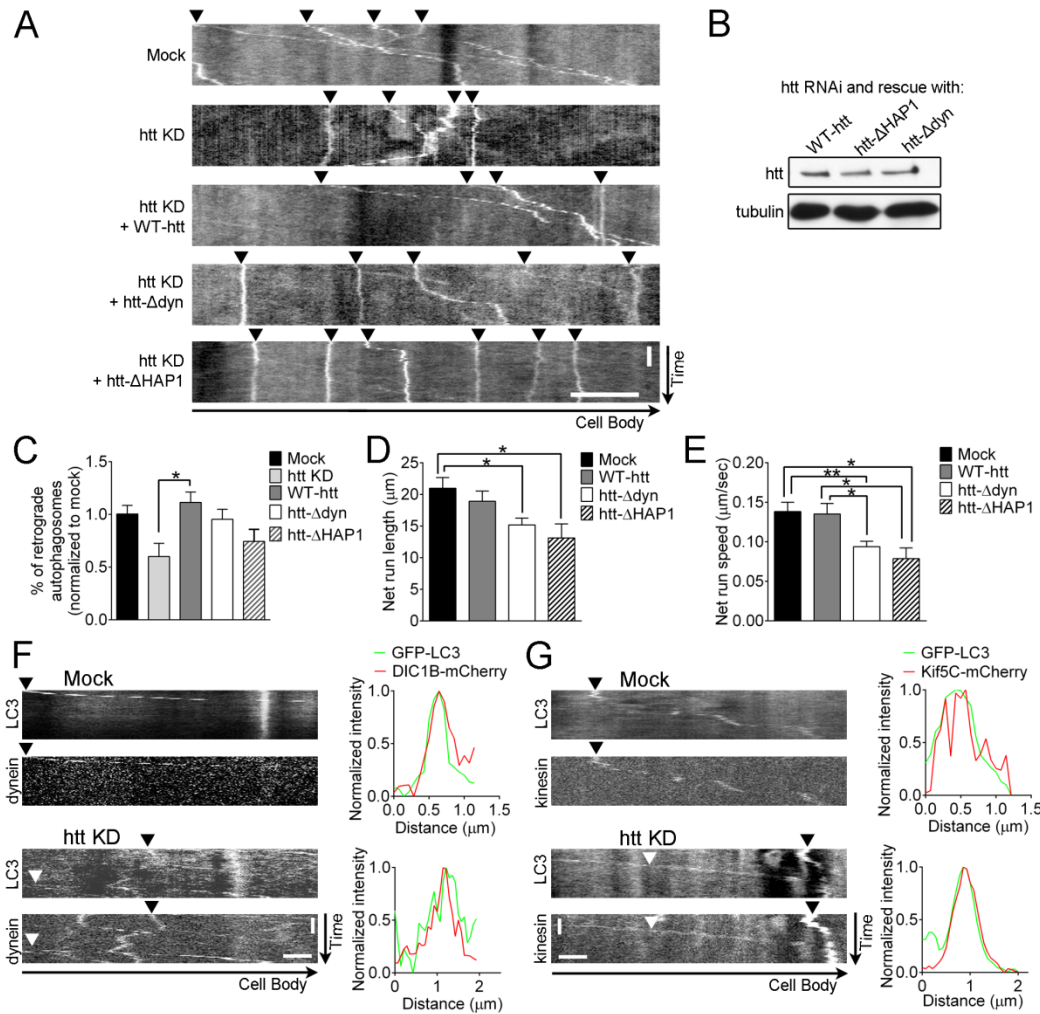


Figure 2.3: Htt regulates autophagosome transport by binding to dynein and HAP1.

(A) Representative kymographs of GPF-LC3 autophagosome transport demonstrate that siRNA-resistant dominant negative htt constructs which cannot bind dynein (htt KD + htt-Δdyn) or HAP1 (htt KD + htt-ΔHAP1) are unable to rescue autophagosome motility (arrowheads) in htt siRNA-depleted primary DRG neurons, as compared to normal full-length htt (htt KD + WT-htt). (B) WT-htt, htt-Δdyn and htt-ΔHAP1 are all efficiently expressed in neurons by immunoblot. (C) WT-htt rescues the percentage of retrograde autophagosomes to control levels while htt-Δdyn and htt-ΔHAP1 are unable to do so (mock, n = 41; htt KD, n = 14; WT-htt, n = 37; htt-Δdyn, n = 25; htt-ΔHAP1, n = 23). (D,E) Run lengths (D) and run speeds (E) from autophagosome net runs (total distance traveled over 3 minutes) are not rescued by htt-Δdyn or htt-ΔHAP1 compared to WT-htt (mock, n = 184; WT-htt, n = 135; htt-Δdyn, n = 207; htt-ΔHAP1, n = 67). (F) Representative kymographs and corresponding line scans show colocalization and co-transport (arrowheads) of a neuronal-specific isoform of the retrograde motor dynein (DIC1B-mCherry) with GFP-LC3 autophagosomes both in control neurons (mock) and htt-depleted (htt KD) primary DRG neurons. (G) Representative kymographs and corresponding line scans show colocalization and co-transport (arrowheads) of the anterograde motor kinesin (Kif5C-mCherry) with GFP-LC3 autophagosomes both in control neurons (mock) and htt-depleted neurons (htt KD). Line scan intensities are normalized per marker and per condition. (Horizontal scale bars: A, 10 μm; F, G, 2 μm. Vertical scale bars, 1 min). Values represent means ± SEM. *p < 0.05; **p < 0.01.

2.3F) and kinesin-1 (Figure 2.3G) were still co-transported with GFP-LC3-labeled autophagosomes as shown in representative images and corresponding line scans from single neurons. This suggests that motor proteins are still recruited to autophagosomes in the absence of htt, potentially by proteins such as FYCO1 (Pankiv et al., 2010). However, as we could not quantitatively assess the number of bound motors under each condition, we cannot exclude the possibility that htt depletion may decrease the number of motors recruited to autophagosomes.

2.2.2 Loss of HAP1 disrupts autophagosome transport

To further investigate the role of htt and HAP1 in regulating autophagosome dynamics, we depleted HAP1 in GFP-LC3-expressing DRG neurons using a fluorescently-labeled siRNA. HAP1-depleted neurons demonstrated significant disruption of autophagosome transport (Figure 2.4A), with an increased percentage of stationary autophagosomes (mock, $45 \pm 6.7\%$ vs HAP1 KD, $64 \pm 9.5\%$; $p < 0.001$) and significantly fewer autophagosomes moving in the retrograde direction (mock, $74 \pm 3.9\%$ vs HAP1 KD, $34 \pm 9.2\%$; $p < 0.001$) (Figure 2.4B). To confirm specificity of knockdown, we expressed an siRNA-resistant human HAP1 (hHAP1) construct, which rescued autophagosome motility to levels seen in control neurons (Figure 2.4A,B). Net run speeds and net run lengths of autophagosomes were also significantly decreased by HAP1 depletion and were rescued by hHAP1 expression (Figure 2.4C,D). HAP1 was previously shown to regulate neurite length in PC12 cells (Li et al., 2000; Rong et al., 2006), which have much shorter neurites than the DRGs studied here. However, we found that neurite length in primary DRG neurons 2 DIV was not affected by HAP1 depletion (mock, $424 \pm 12.3 \mu\text{m}$ vs HAP1 KD, $466 \pm 23.2 \mu\text{m}$) suggesting that the disruption of autophagosome transport induced by HAP1 depletion is not the result of reduced neurite outgrowth. Since HAP1 binds both dynactin and kinesin, we next investigated whether depleting HAP1 affected either retrograde or anterograde motor protein activity on autophagosomes.

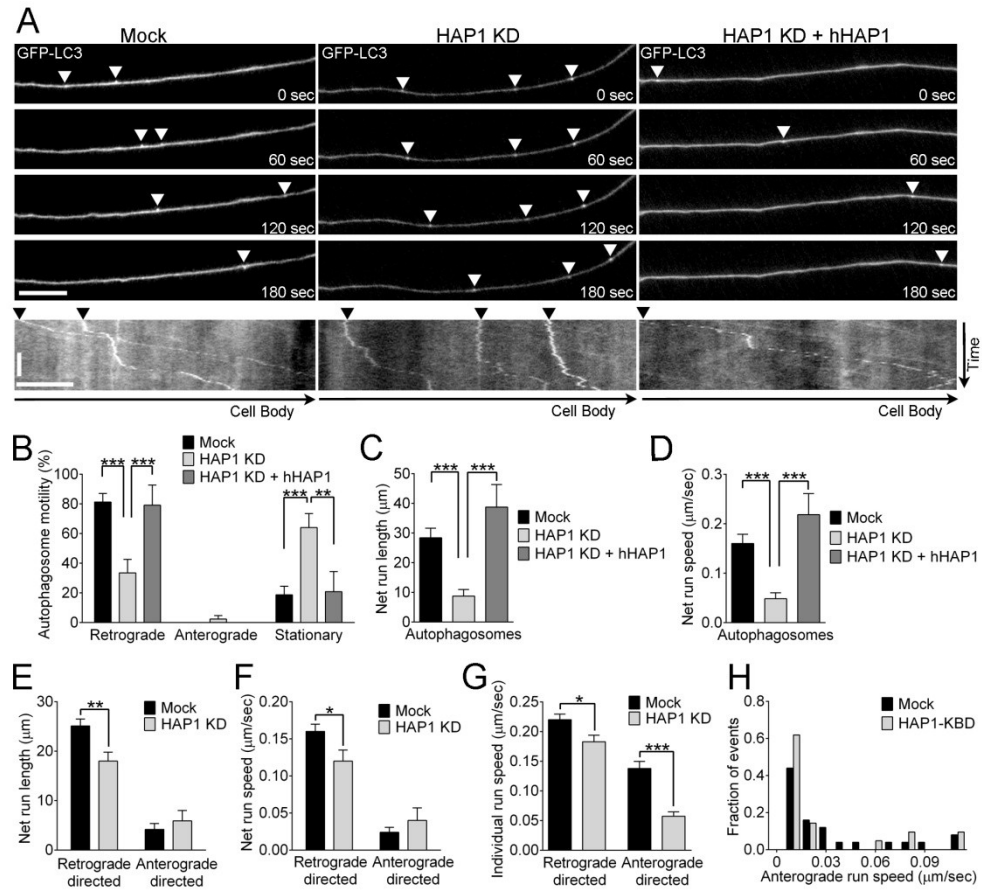


Figure 2.4: HAP1 depletion inhibits autophagosome transport in neurons.

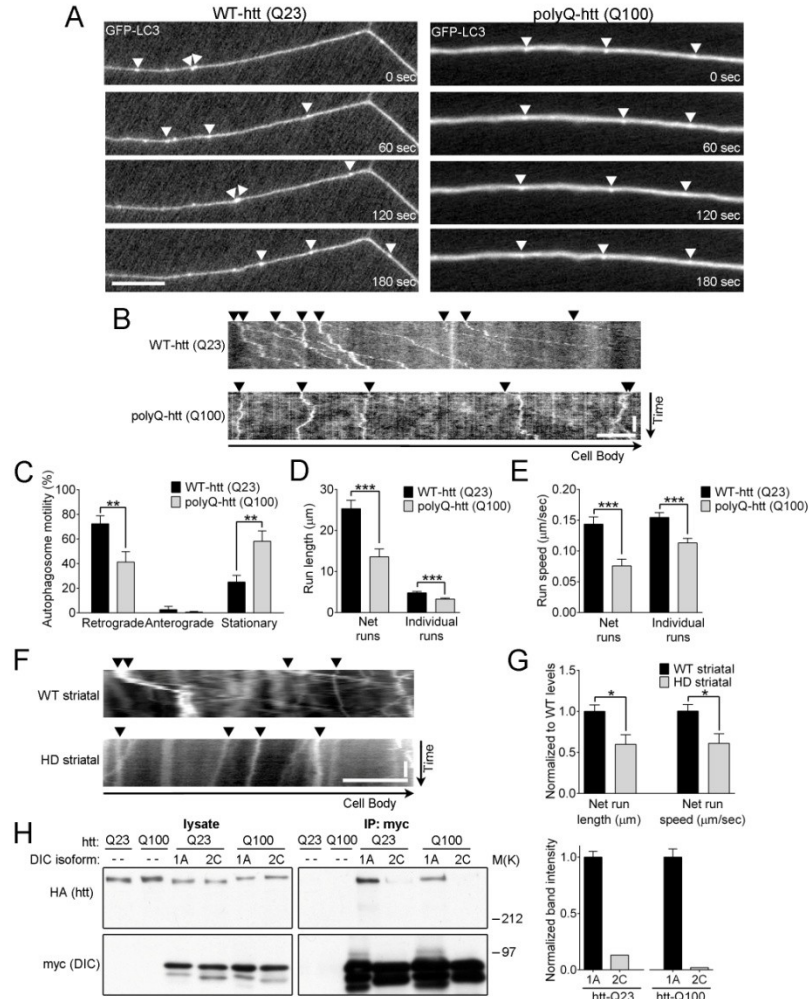
(A) Representative time series and corresponding kymographs of GFP-LC3 autophagosome transport in axons of primary DRG neurons demonstrate that autophagosome transport is disrupted in HAP1 siRNA-depleted neurons (HAP1 KD) compared to control neurons (mock) and is rescued by expression of siRNA-resistant human HAP1 (HAP1 KD + hHAP1) (arrowheads). Two autophagosomes are shown moving in control neurons (Mock) with one leaving the frame of view by 180 seconds (see corresponding kymograph). (B) Depleting HAP1 decreases the percentage of retrograde autophagosomes and increases the percentage of stationary autophagosomes in neurons which are rescued by expression of siRNA-resistant human HAP1 (HAP1 KD + hHAP1) (mock, n = 10; HAP1 KD, n = 14; HAP1 KD + hHAP1, n = 6). (C, D) HAP1 depletion reduces net run lengths (C) and net run speeds (D) of autophagosomes which are rescued by hHAP1 expression (mock, n = 52; HAP1 KD, n = 39; HAP1 KD + hHAP1, n = 17). (E, F) HAP1 depletion reduces the run lengths (E) and run speeds (F) of autophagosome net runs (total distance traveled over 3 minutes) in the retrograde direction but not in the anterograde direction (retrograde-directed: mock, n = 180; HAP1 KD, n = 107) (anterograde-directed: mock, n = 30; HAP1 KD, n = 30). (G) HAP1 depletion reduces individual run speeds in both retrograde and anterograde directions (retrograde-directed: mock, n = 709; HAP1 KD, n = 336) (anterograde-directed: mock, n = 269; HAP1 KD, n = 175). Retrograde-directed refers to runs of any length in the retrograde direction; anterograde-directed refers to runs of any length in the anterograde direction. (H) Expression of HAP1 lacking the kinesin binding domain (HAP1-KBD) results in an increase in the fraction of autophagosomes expressing slower anterograde run speeds (mock, n = 25; HAP1-KBD, n = 21). (Horizontal scale bar, 10 μ m. Vertical scale bar, 1 min). Values represent means $\pm$ SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

We found that depleting HAP1 specifically decreased the net run lengths and run speeds of retrograde-directed autophagosomes (Figure 2.4E,F), suggesting that the predominantly retrograde net runs of an autophagosome are regulated by HAP1. When we examined the speeds of individual runs, we found that HAP1 depletion decreased individual run speeds in both directions (Figure 2.4G), suggesting that HAP1 interacts with motor proteins to enhance motor activity for both retrograde and anterograde motors. This was further supported by expressing a dominant negative HAP1 inhibitor that blocks the HAP1-kinesin interaction (HAP1-KBD) (Twelvetrees et al., 2010). HAP1-KBD expression increased the percentage of slow-moving ($<0.01 \mu\text{m}/\text{sec}$) anterograde runs (Figure 2.4H), while depleting HAP1 increased the length of individual anterograde runs (mock, $1.16 \pm 0.089 \mu\text{m}$ vs HAP1 KD, $1.90 \pm 0.383 \mu\text{m}$; $p < 0.05$). Thus, disruption of HAP1 function in primary neurons induces prolonged anterograde runs but at slower speeds. Taken together, these results support a role for HAP1 in regulating autophagosome motility by promoting efficient retrograde-directed transport.

2.2.3 Pathogenic polyQ-htt disrupts autophagosome dynamics

PolyQ expansions in htt cause HD, an autosomal dominant neurodegenerative disorder, in which autophagy has been found to be critical in clearing both soluble and aggregated forms of pathogenic polyQ-htt (Qin et al., 2003; Ravikumar et al., 2002). To examine whether autophagosome transport is affected by polyQ-htt, we expressed siRNA-resistant wildtype htt (Q23) and polyQ-htt (Q100) in primary neurons depleted of endogenous htt and found that autophagosome motility was inhibited by polyQ-htt expression (Figure 2.5A,B). Neurons expressing polyQ-htt had a significant increase in the percentage of stationary autophagosomes ($58 \pm 8.5\%$) as compared to neurons expressing wildtype htt ($25 \pm 5.4\%$; $p < 0.01$), and exhibited decreased run lengths and speeds (Figure 2.5C-E).

Striatal neurons are among the cells selectively affected in HD, so we compared autophagosome motility in striatal cells from wildtype mice (HdhQ7/Q7) and from HD knock-in



mice (HdhQ111/Q111) (Trettel et al., 2000) using live cell imaging of mCherry-LC3. Striatal cells from HD mice demonstrated significant disruption of autophagosome dynamics with decreased net run lengths ($60 \pm 11.6\%$ of wildtype levels; $p < 0.05$) as well as decreased net run speeds ($61 \pm 11.7\%$ of wildtype levels; $p < 0.05$) (Figure 2.5F,G). These results further support the hypothesis that expression of polyQ-htt leads to misregulated autophagosome transport in neurons.

HD causes selective neurodegeneration although htt is ubiquitously expressed throughout the body (Gusella and MacDonald, 2006). The cell-type specificity observed in HD has been proposed to be mediated by htt's interactions with neuronal-specific proteins (Subramaniam et al., 2009). Thus, we examined the ability of htt to bind to differentially expressed isoforms of dynein intermediate chain (DIC), the major cargo-binding subunit of the dynein motor (Kuta et al., 2010). The DIC1 gene (DYNC1I1) is expressed in a neuron-specific manner to produce isoforms DIC1A, 1B and 1C, while the DIC2C isoform is a product of the DIC2 gene (DYNC1I2) and is expressed ubiquitously (Kuta et al., 2010; Myers et al., 2007; Pfister et al., 1996a, 1996b; Zhang et al., 2013). Here, we examined the ability of wildtype htt and polyQ-htt to bind to the neuronal-specific isoform DIC1A compared to the ubiquitously expressed isoform DIC2C. We co-expressed myc-tagged DIC isoforms with HA-tagged htt in COS7 cells, and immunoprecipitated using anti-myc antibody. Both wildtype htt and polyQ-htt bound strongly to neuronal-specific DIC1A, but much less binding was observed to the ubiquitously expressed DIC2C isoform (Figure 2.5H). This preferential association of both wild type and pathogenic htt with the neuronal-specific dynein isoform predicts that dynein-based transport of autophagosomes may be selectively impaired in neurons; this selectivity may contribute to the neuronal-selective degeneration seen in HD.

2.2.4 Htt is not required for autophagosome formation or cargo loading in neurons

To determine whether htt regulates other aspects of autophagy in neurons, we examined three initial steps that occur prior to autophagosome transport down the axon: autophagosome formation, cargo loading and initial fusion with late endosomes. In htt-depleted primary neurons,

we observed that autophagosomes continue to form, developing from small puncta into ring-like structures at the axon tip (Figure 2.6A). Depleting htt did not affect autophagosome density at the axon tip, the site of constitutive autophagosome formation, nor did htt depletion affect autophagosome density along the axon (Figure 2.6A).

Next, we examined cargo loading, focusing on ubiquitinated proteins and mitochondria. Autophagosomes in htt-depleted neurons still engulfed ubiquitinated proteins labeled by UbRFP (Figure 2.6B), with all autophagosomes positive for UbRFP in both mock and htt-depleted neurons (mock, $n = 41/41$ autophagosomes vs htt-depleted, $n = 30/30$ autophagosomes). Autophagosomes also effectively engulfed DsRed2-mito-labeled mitochondrial fragments (Maday et al., 2012) in both mock and htt-depleted neurons (Figure 2.6C).

Next, we investigated the fusion of autophagosomes with late endosomes, an initial step in compartment maturation (Eskelinen, 2008). Following formation at the axon tip, autophagosomes acquire the late endosomal marker LAMP1 as they initiate their transit along the axon (Maday et al., 2012). We examined the colocalization of GFP-LC3 and LAMP1-RFP at both the axon tip and along the axon in control and htt-depleted neurons. We saw no difference between control and htt-depleted neurons (Figure 2.6D); in both cases, few autophagosomes were LAMP1-positive at the axon tip, while the majority of autophagosomes along the axon were LAMP1-positive, consistent with previous observations (Lee et al., 2011; Maday et al., 2012).

We next examined whether autophagosomes in htt-depleted neurons are able to mature into autolysosomes. We used a dual-color mCherry-EGFP-LC3 reporter; the GFP moiety is preferentially quenched as the compartment matures and acidifies (Kimura et al., 2007; Pankiv et al., 2007), leading to an increase in red-only autophagosomes more proximal to the soma (Maday et al., 2012). Depleting htt did not significantly alter the percentage of red-only autophagosomes as compared to control neurons (mock, $62 \pm 3.8\%$ vs htt KD, $62 \pm 4.4\%$) (Figure 2.6E). In addition, htt depletion did not affect late endosome/lysosome motility (Figure 2.2H) or density along the axon (mock, $6.4 \pm 1.3/100 \mu\text{m}$ vs htt KD, $6.7 \pm 0.9/100 \mu\text{m}$).

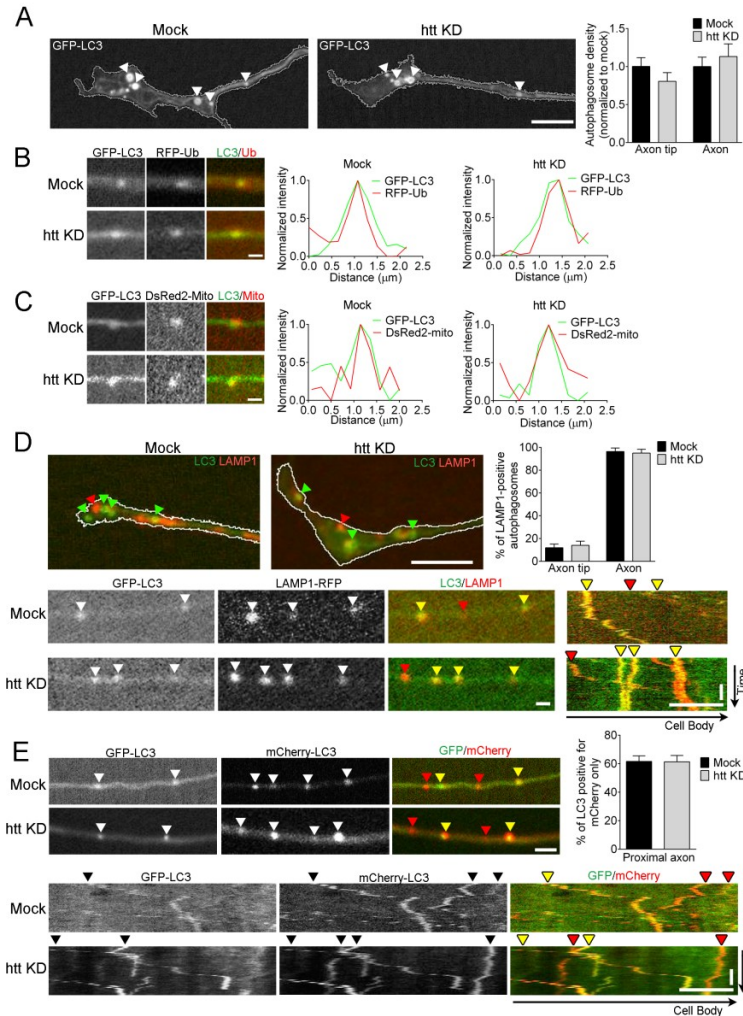


Figure 2.6: Htt is not required for initial steps of autophagosome formation, cargo loading and initial maturation.

(A) Representative confocal live-cell images of GFP-LC3 autophagosomes demonstrate autophagosomes forming (arrowheads) in the distal tip of the neurite from puncta into ring-like structures in both control (mock) and htt-depleted (htt KD) primary DRG neurons. Autophagosome density at the axon tip and along the axon is not disrupted by htt-depletion (axon tip: mock, $n = 10$; htt KD, $n = 13$) (axon: mock, $n = 23$; htt KD, $n = 23$). (B, C) Representative images and corresponding line scans of GFP-LC3 autophagosomes colocalized with RFP-Ub ubiquitinated protein cargo (B) and DsRed2-mito mitochondrial fragments (C) in axons of both control (mock) and htt-depleted (htt KD) neurons. Line scan intensities are normalized per marker and per condition. (D) Representative images of GFP-LC3 and LAMP1-RFP at the axon tip (top) and representative images and corresponding kymograph of GFP-LC3 autophagosomes positive for LAMP1-RFP (yellow arrowheads) along the axon (bottom) in both control (mock) and htt-depleted (htt KD) neurons (axon tip: mock, $n = 19$; htt KD, $n = 22$) (axon: mock, $n = 22$; htt KD, $n = 20$). (E) Representative images (top) and kymographs (bottom) of acidification of autophagosomes (red arrowheads) in neurons expressing mCherry-EGFP-LC3 in both control (mock) and htt-depleted (htt KD) primary DRG neurons. In acidic environments, the GFP moiety is preferentially quenched with persistent mCherry fluorescence (mock, $n = 16$; htt KD, $n = 13$). (Horizontal scale bars: A, D-E (kymographs), $10 \mu\text{m}$; B-E (images), $1 \mu\text{m}$). Vertical scale bars, 1 min .

2.2.5 Disruption of autophagosome transport by htt depletion leads to accumulation of autophagosomes with undegraded mitochondrial cargo

Previous studies have found that autophagosomes undergo multiple lysosomal fusion events (Eskelinen, 2008; Yu et al., 2010) and that defective autophagosome transport in non-neuronal cells leads to inefficient autophagosome fusion with lysosomes and decreased autophagic cargo degradation (Jahreiss et al., 2008; Kimura et al., 2008; Ravikumar et al., 2005). To examine whether autophagic cargo are inefficiently cleared when autophagosome transport is defective in neurons, we examined DsRed2-mito-labeled mitochondrial degradation in htt-depleted neurons. First, we examined whether mitochondrial morphology was disrupted by htt depletion but found that mitochondria had similar lengths and densities in both control and htt-depleted neurons from either wildtype mice or GFP-LC3 transgenic mice (Figure 2.7A,D,E). We also found that the percentage of fragmented mitochondria along the axon, a commonly used indicator of mitochondrial health (Song et al., 2011), was not increased in htt-depleted neurons (mock, $22 \pm 5.4\%$ vs htt-depleted, $26 \pm 4.7\%$) (Figure 2.7F).

To observe mitochondrial cargo fragments co-transporting with autophagosomes, we imaged mitochondria at increased exposure times. We found that at the axon tip, where autophagosomes first form and engulf their cargo, htt-depletion had no effect on the flux of mitochondrial engulfment, as measured by autophagosomes positive for mitochondrial fragments leaving the axon tip per μm per minute (mock, $0.47 \pm 0.19/100 \mu\text{m} \cdot \text{min}$ vs htt KD, $0.35 \pm 0.16/100 \mu\text{m} \cdot \text{min}$; $p = 0.63$), suggesting that mitochondria are engulfed in autophagosomes at equal rates in both mock and htt-depleted neurons. However, when we examined autophagosomes in the mid-axon, we found that autophagosomes in htt-depleted neurons contained a significantly higher percentage of mitochondrial fragments ($21 \pm 5.5\%$), compared to autophagosomes in mock neurons ($7 \pm 2.7\%$; $p < 0.05$) (Figure 2.7B,C,G), suggesting that mitochondria are not efficiently degraded within autophagosomes in htt-depleted neurons.

To examine whether htt depletion affected the total level of mitochondrial degradation in neurons, we blotted for the mitochondrial marker Hsp60 in lysates from DRG neurons and saw a

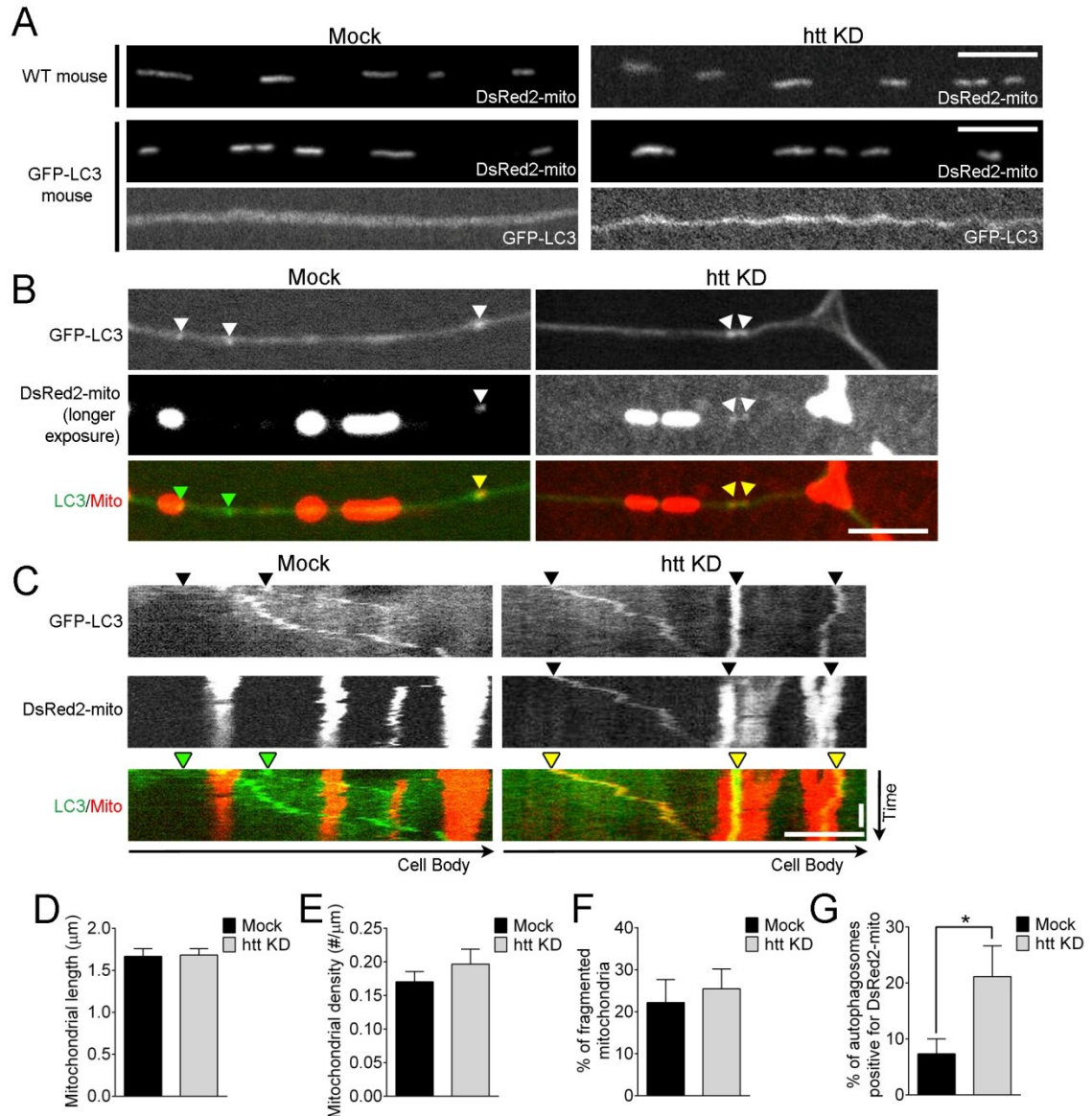


Figure 2.7: Depletion of htt leads to inefficient mitochondrial cargo degradation in autophagosomes.

(A) Htt depletion does not disrupt mitochondria morphology in either wildtype mice (WT) or GFP-LC3 transgenic mice. (B, C) Representative images (B) and kymographs (C) of increased DsRed2-mito mitochondrial fragments colocalized and co-transporting with GFP-LC3 autophagosomes (yellow arrowheads) in axons of htt-depleted neurons. Mitochondria were imaged at longer exposures to allow for visible co-transport of mitochondrial fragments with autophagosomes. Images and kymographs are taken from different neurons. (D-F) Htt depletion did not disrupt mitochondrial length (D) (mock, $n = 122$; htt KD, $n = 177$), density along the axon (E), or percentage of fragmented mitochondria (length $< 1 \mu\text{m}$) (F) in primary DRG neurons (mock, $n = 9$; htt KD, $n = 12$). (G) Htt depletion increased the percentage of GFP-LC3 autophagosomes containing DsRed2-mito mitochondria per neurite (mock, $n = 32$; htt KD, $n = 38$). (Horizontal scale bars, $10 \mu\text{m}$. Vertical scale bar, 1 min). Values represent means $\pm$ SEM. * $p < 0.05$.

small net increase in mitochondria protein levels in htt-depleted neurons (1.4 ± 0.3 ; mean $\pm$ SEM, $n = 6$), normalized to lysates from control neurons. This relatively limited increase is consistent with our observations from live imaging that total mitochondrial density along the axon is not altered, although there is an accumulation of undigested mitochondrial fragments in autophagosomes in transit along the axon. Together, these results suggest that disruption of autophagosome transport induced by htt depletion leads to inefficient degradation of mitochondrial fragments internalized into autophagosomes along the axon. This inefficient degradation does not result from a defect in initial acidification of the autophagosome or from significant alterations in lysosomal localization. Rather, defective autophagosome transport in htt-depleted neurons may lead to decreased encounters and thus decreased fusion with lysosomes along the axon resulting in inefficient cargo degradation (Kimura et al., 2008).

2.2.6 Defective autophagosome transport induced by polyQ-htt leads to inefficient degradation of engulfed mitochondria and accumulation of polyQ-htt aggregates

We next examined whether cargo degradation was similarly affected by expression of polyQ-htt. We expressed siRNA-resistant wildtype htt (Q23) and polyQ-htt (Q100) in primary neurons depleted of endogenous htt, and found that autophagosomes were still able to form at the axon tip (Figure 2.8A). Expression of polyQ-htt also did not affect autophagosome density at the axon tip or autophagosome density along the axon in our cultures (Figure 2.8A). To examine the effect of autophagosome transport on cargo degradation in polyQ-htt neurons, we again examined the clearance of DsRed2-mito-labeled mitochondrial fragments by autophagosomes, as defects in mitochondrial bioenergetics and dynamics have previously been observed in HD patients and animal models (Costa and Scorrano, 2012). In our neuronal cultures, mitochondria had similar lengths and densities in both WT-htt and polyQ-htt neurons (mitochondrial length: WT-htt, $1.44 \pm 0.05 \mu\text{m}$ vs polyQ-htt, $1.48 \pm 0.05 \mu\text{m}$) (mitochondrial density: WT-htt, $0.18 \pm 0.02/\mu\text{m}$ vs polyQ-htt, $0.20 \pm 0.01/\mu\text{m}$). The percentage of fragmented mitochondria along the axon also was not altered in polyQ-htt neurons (Figure 2.8D).

We also noted that polyQ-htt expression had no effect on the flux of mitochondrial engulfment, as measured by autophagosomes positive for mitochondrial fragments leaving the axon tip per μm per minute (WT-htt, $0.41 \pm 0.20/100 \mu\text{m} \cdot \text{min}$ vs polyQ-htt, $0.40 \pm 0.17/100 \mu\text{m} \cdot \text{min}$; $p = 0.94$), indicating that mitochondria are engulfed by autophagosomes at the axon tip at equal rates in neurons expressing either wildtype htt or polyQ-htt. However, when we examined autophagosomes along the axon, we found that expression of polyQ-htt caused a dramatic and significant increase in the percentage of mitochondrial fragments accumulated in autophagosomes traveling along the axon ($60 \pm 10.7\%$) compared to WT-htt neurons ($13 \pm 7.8\%$; $p < 0.05$) (Figure 2.8B,C,E). These results further support the hypothesis that defective autophagosome transport leads to reduced lysosomal fusion events along the axon and thus, autophagosomes are unable to maintain an appropriate luminal environment necessary for efficient cargo degradation.

To test this hypothesis, we examined the acidification state of GFP-LC3-labeled autophagosomes in neurons expressing polyQ-htt. For this experiment, we used the pH-sensitive probe LysoTracker Red because we found that this dye labels a broader fraction of autophagosomes in the proximal axon of wild type neurons ($95.1 \pm 2.7\%$) compared to the $62.0 \pm 3.8\%$ of autophagosomes in the proximal axon observed to exhibit red-only fluorescence from the tandem mCherry-EGFP-LC3 construct described above. Thus we reasoned that loss of LysoTracker Red staining would be a more comprehensive measure of changes in the acidity of this compartment. We examined autophagosomes at three distinct regions along the axon: the axon tip, the mid-axon, and the proximal axon ($<100\mu\text{m}$ from the cell body). We found that in both WT-htt and polyQ-htt neurons, few autophagosomes were acidified at the axon tip ($\sim 30\%$) while the majority of autophagosomes along the mid-axon were LysoTracker-positive ($\sim 90\%$) (Figure 2.8G), consistent with previous observations in wild type neurons (Maday et al., 2012). However, when we examined autophagosomes that had reached the proximal axon (closest to the cell body), we found a significant decrease in the percentage of autophagosomes that were LysoTracker-positive in polyQ-htt neurons as compared to WT-htt neurons ($p < 0.01$) (Figure

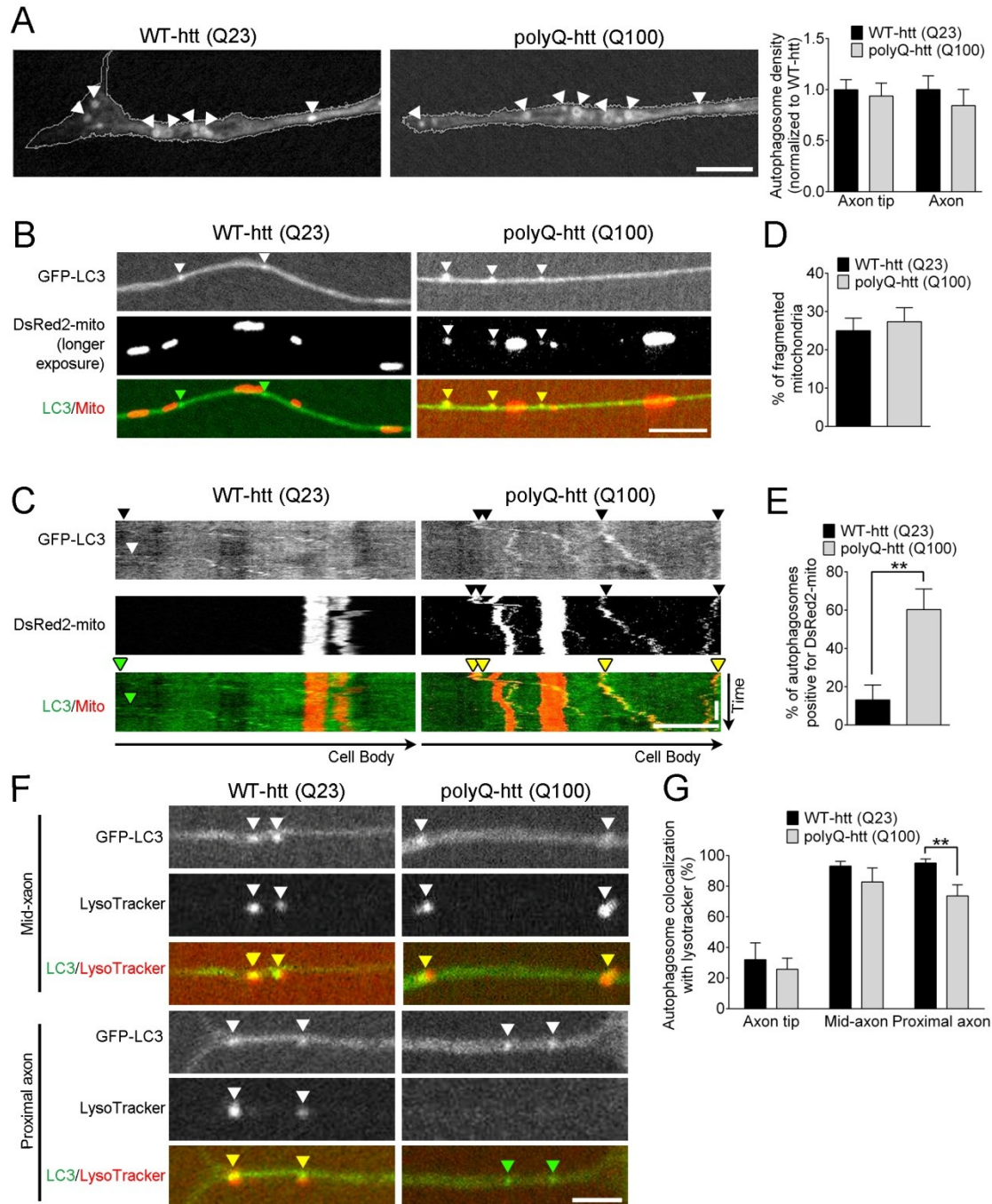


Figure 2.8: Pathogenic polyQ-htt causes inefficient mitochondrial cargo degradation in neurons.

(A) Confocal live-cell images showing GFP-LC3 autophagosomes forming (arrowheads) in the distal tip of the neurite from puncta into ring-like structures in htt siRNA-depleted primary DRG neurons from GFP-LC3 transgenic mice expressing either siRNA-resistant wildtype htt (Q23) or polyQ-htt (Q100). Autophagosome density at the axon tip and along the axon is not altered by expression of polyQ-htt (Q100) in primary DRG neurons (axon tip: WT-htt, n = 12; polyQ-htt, n = 11) (axon: WT-htt, n = 14; polyQ-htt, n = 17). (B,C) Representative images (B) and kymographs (C) of increased DsRed2-mito mitochondrial fragments colocalized and co-transporting with GFP-LC3.

LC3 autophagosomes (yellow arrowheads) in axons of polyQ-htt (Q100) expressing neurons. Mitochondria were imaged at longer exposures to allow for visible co-transport of mitochondrial fragments with autophagosomes. (D) PolyQ-htt expression did not disrupt percentage of fragmented mitochondria (length < 1 μ m) in primary DRG neurons (WT-htt, n = 12; polyQ-htt, n = 17). (E) PolyQ-htt expression increased the percentage of GFP-LC3 autophagosomes containing DsRed2-mito mitochondria per neurite (WT-htt, n = 12; polyQ-htt, n = 17). (F, G) Representative images (F) and quantification (G) showing similar levels of LysoTracker Red-positive autophagosomes at the axon tip and mid-axon, but reduced LysoTracker Red-positive autophagosomes in the proximal axon of neurons expressing polyQ-htt (Q100) compared to neurons expressing WT-htt (Q23) (axon tip: WT-htt, n = 20; polyQ-htt, n = 18) (mid-axon: WT-htt, n = 16; polyQ-htt, n = 15) (proximal-axon: WT-htt, n = 14; polyQ-htt, n = 14). (Horizontal scale bars: A-C, 10 μ m; F, 5 μ m. Vertical scale bar, 1 min). Values represent means $\pm$ SEM. ** p <0.01.

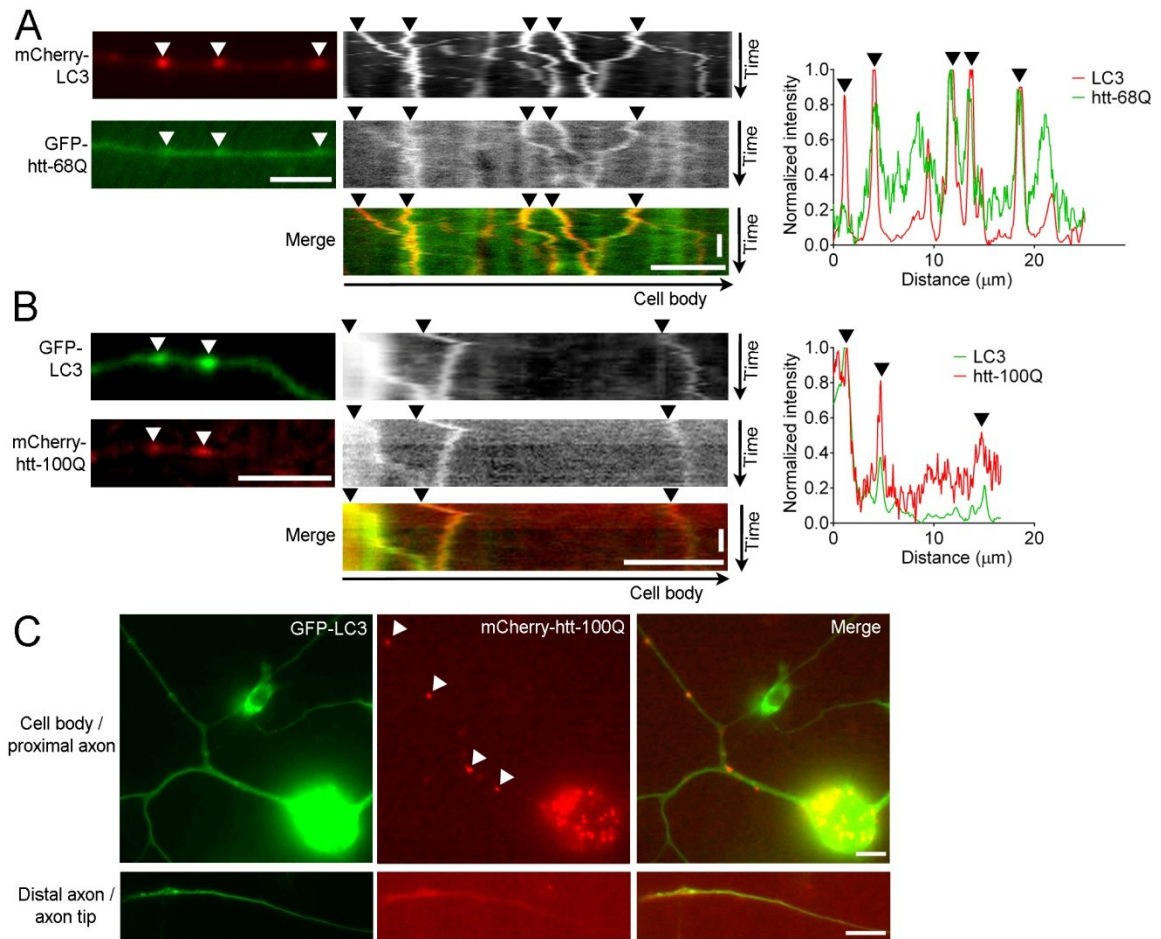


Figure 2.9: Disrupted autophagosome dynamics lead to inefficient clearance of pathogenic polyQ-htt from the distal axon.

(A, B) Representative images and kymographs and corresponding linescans show disease-associated cleaved N-terminal fragment of GFP-polyQ-htt (Q68) (A) and full-length mCherry-polyQ-htt (Q100) (B) colocalized and co-transporting as autophagic cargo with retrogradely moving autophagosomes (arrowheads) in axons of primary neurons. (C) Representative image of ineffective clearance and aggregate formation of mCherry-polyQ-htt (Q100) in the cell body, axon (arrowheads) and distal axon tip of primary neurons. (Horizontal scale bars, 10 μ m. Vertical scale bars, 1 min).

2.8F,G). These results are consistent with the hypothesis that autophagosomes undergoing disrupted transport in polyQ-htt neurons are unable to maintain an appropriate level of acidification necessary for efficient cargo degradation, possibly due to reduced lysosomal fusion events along the axon.

In HD, both soluble and aggregated pathogenic polyQ-htt are predominantly cleared by autophagy (Qin et al., 2003; Ravikumar et al., 2002). We used live cell imaging of primary neurons to further examine the role of autophagosomes in clearing polyQ-htt from the distal axon. Both the disease-associated N-terminal fragment of polyQ-htt (GFP-htt-68Q) and full-length polyQ-htt (mCherry-htt-Q100) were observed to co-transport with autophagosomes from the axon tip back to the cell body (Figure 2.9A,B). We also saw visible aggregates forming along the cell body and axon, and accumulating at the axon tip (Figure 2.9C), suggesting that early disruption of autophagosome dynamics may contribute to ineffective autophagic clearance of polyQ-htt in HD neurons, particularly from the distal axon and axon tip.

2.3 Discussion

Autophagosomes form constitutively at the axon tip in neurons and undergo robust retrograde transport towards the soma (Lee et al., 2011; Maday et al., 2012). Here we show that a htt/HAP1 complex drives retrograde-directed autophagosome transport by regulating motor protein activity, likely through a scaffolding mechanism. Htt is not required for autophagosome formation or cargo loading in neurons. Instead, defects in htt-mediated autophagosome transport lead to inefficient cargo degradation along the axon. Further, polyQ-htt-expressing neurons demonstrate defective autophagosome transport and inhibited degradative function, potentially contributing to inefficient autophagic polyQ-htt clearance in HD.

How might htt and HAP1 regulate autophagosome transport dynamics? Htt localizes to the outer membrane of autophagosomes (Atwal et al., 2007; Martinez-Vicente et al., 2010). Both htt and HAP1 are present in autophagosome-enriched brain fractions along with microtubule motors and colocalize with LC3-positive autophagosomes along axons of primary neurons. Autophagosome motility is driven by the retrograde dynein-dynactin complex (Jahreiss et al., 2008; Kimura et al., 2008; Lee et al., 2011; Maday et al., 2012) and the anterograde motor kinesin (Maday et al., 2012). We show that htt is likely not required for motor recruitment to autophagosomes, but rather acts to regulate motor activity. Htt binds directly to dynein (Caviston et al., 2007); here we found that abolishing the htt/dynein interaction affects the processivity of dynein, resulting in reduced autophagosome run lengths and speeds.

Abolishing the htt/HAP1 interaction also disrupted autophagosome transport resulting in more stationary autophagosomes. Since HAP1 binds to both dynactin, a required activator for retrograde transport, and kinesin-1, a major motor for anterograde transport (Engelender et al., 1997; Li et al., 1998; McGuire et al., 2006; Twelvetrees et al., 2010), HAP1 may play a key role in coordinating bidirectional motor activity. HAP1 has been previously shown to regulate kinesin-dependent APP (amyloid precursor protein) transport (McGuire et al., 2006; Yang et al., 2012) and anterograde GABA receptor trafficking (Twelvetrees et al., 2010). Here, we show that depleting HAP1 affected both retrograde and anterograde motility of autophagosomes, resulting

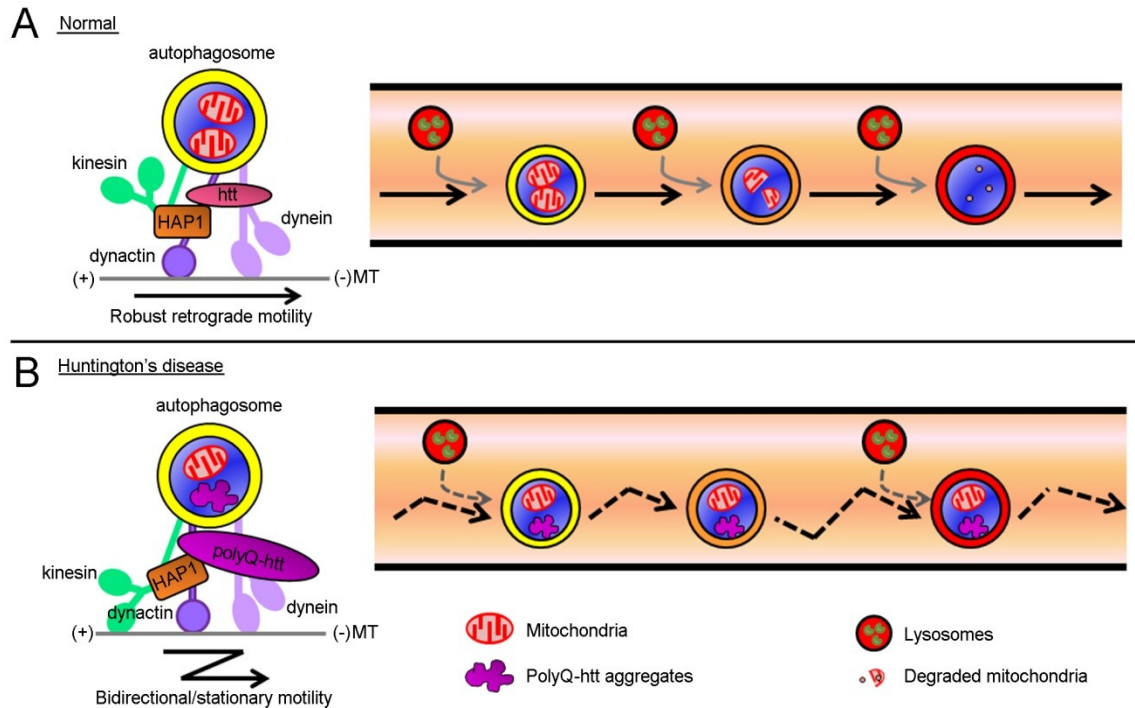


Figure 2.10: Model of htt/HAP1's regulation of autophagosome dynamics in neurons.

(A) Htt and HAP1 regulate the motor activity and processivity of microtubule motors dynein, dynactin and kinesin on autophagosomes via interactions among htt, HAP1, and neuronal-specific dynein isoforms to drive robust retrograde transport of autophagosomes back to the cell body in neurons along microtubules (MT). Retrograde autophagosome transport is necessary for efficient fusion with lysosomes for degradation of autophagic cargo such as mitochondria. (B) In HD, pathogenic polyQ-htt disrupts the htt/HAP1 motor protein complex on autophagosomes via altered polyQ-htt/HAP1 association. This misregulation of motors leads to bidirectional/stationary autophagosome dynamics in HD neurons, thereby disrupting the retrograde transport of autophagosomes necessary for efficient degradation of dysfunctional mitochondria and polyQ-htt.

in decreased velocities in both directions. In the retrograde direction, the htt/HAP1 complex may favor dynein-mediated motility by enhancing the affinity of the dynein-dynactin interaction through the formation of a quaternary (dynein-dynactin-HAP1-htt) complex. As depleting HAP1 also prolonged anterograde runs, but at slower speeds, HAP1 may also promote efficient autophagosome transport by limiting the frequency or length of anterograde runs. Since similar changes in autophagosome transport were observed upon depletion of HAP1 and expression of htt-ΔHAP1, htt and HAP1 are likely acting via the same pathway, consistent with previous studies (Gauthier et al., 2004). Taken together, these results suggest that the htt/HAP1 scaffolding complex promotes efficient retrograde motility by enhancing dynein-dynactin-driven movement

and by limiting kinesin-driven movement, leading to robust unidirectional autophagosome transport towards the soma (Figure 2.10A). The activity of this scaffolding complex may be carefully regulated *in vivo*, by post-translational modifications by the serine/threonine kinase Akt (Colin et al., 2008), or JNK (cJun N-terminal kinase) activity, which is upregulated in HD (Morfini et al., 2009). Since htt and HAP1 colocalize with a subpopulation of LC3-positive autophagosomes, it is also possible that other scaffolding proteins may regulate the transport of distinct or overlapping subpopulations of autophagosomes; work from our lab also implicates the JNK-binding scaffolding protein JIP1 in the regulation of autophagosome dynamics (Fu et al., 2014).

Interactions of htt with neuronal-specific proteins have been proposed to contribute to selective cell death in HD (Subramaniam et al., 2009). Our observations suggest that both normal and polyQ-htt preferentially interact with the neuronal-specific dynein isoform DIC1A as compared to the more ubiquitously expressed dynein isoform DIC2C. Thus, dynein-driven organelle transport may be selectively impaired in neurons and may contribute to HD neurodegeneration. Previous studies have shown that polyQ-htt expression disrupts the anterograde transport of APP-carrier vesicles and the bidirectional transport of BDNF and mitochondria (Chang et al., 2006; Gauthier et al., 2004; Gunawardena et al., 2003; Her and Goldstein, 2008; Lee et al., 2004; Orr et al., 2008; Song et al., 2011; Szebenyi et al., 2003; Trushina et al., 2004; Zala et al., 2008). Here, we show that polyQ-htt disrupts the predominantly retrograde transport of autophagosomes. Since both wild type htt and polyQ-htt both bind to dynein, autophagosome transport may be disrupted by the increased affinity of polyQ-htt for HAP1 described previously (Li et al., 1995). An alteration in the polyQ-htt/HAP1 motor protein complex may affect either dynein or kinesin motor activity, or a combination of both; specifically, a loss of motor coordination may be the basis for the decreased autophagosome motility we have observed in HD neurons (Figure 2.10B).

In htt-depleted neurons, we find that autophagosomes still form, and still internalize cargo including ubiquitinated proteins and mitochondria. Autophagosomes also undergo an initial fusion

with endosomes, becoming LAMP1-positive upon exit from the axon tip, and acidified, as measured by GFP-quenching. However, htt-depletion caused a significant increase in the fraction of autophagosomes containing undigested mitochondrial fragments. We asked whether this increase reflected an overall increase in mitophagy, possibly up-regulated in response to impaired mitochondrial health. However, we found that mitochondria health, as measured by density, motility and percent fragmentation, was not affected in htt-depleted neurons. Moreover, the rate of mitochondrial engulfment into autophagosomes at the axon tip was not altered by htt depletion, demonstrating that loss of htt does not increase mitophagic flux. In neurons expressing pathogenic polyQ-htt, we noted an even more pronounced accumulation of undigested mitochondrial fragments within autophagosomes along the axon, again in the absence of any evidence indicating an increase in mitophagic flux. Together, these observations support the hypothesis that defects in autophagosome transport lead to inefficient cargo degradation (Figure 2.10B).

We propose that the underlying mechanism linking these two observations is that transport inhibition leads to reduced lysosomal fusion events. This in turn may lead to insufficient accumulation of degradative enzymes within this compartment (Jahreiss et al., 2008; Kimura et al., 2008; Ravikumar et al., 2005) and a failure to maintain the acidified environment required for efficient degradation of engulfed proteins and organelles. In support of this interpretation, defects in autophagosome transport have previously been associated with impaired autophagic clearance and neurodegeneration (Ikenaka et al., 2013; Ravikumar et al., 2005). We found that autophagosomes in htt-depleted neurons still acquire the late endosomal marker LAMP1 and become acidified, suggesting that autophagosome-lysosome fusion is not fully impaired (Jahreiss et al., 2008). Htt depletion also did not affect lysosomal motility or density in neurons, as compared to observation in non-neuronal cells (Caviston et al., 2011), suggesting that inefficient cargo degradation was not caused by disrupted lysosomal localization. Taken together, our results indicate that defective autophagosome transport, rather than defects in initial acidification or altered lysosomal localization, lead to reduced lysosomal fusion events and inefficient cargo

degradation along the axon. In further support of this interpretation, we found that both WT-htt and polyQ-htt expressing neurons had similar levels of LysoTracker-positive autophagosomes in the mid-axon. However, at the proximal axon, polyQ-htt expressing neurons had lower levels of LysoTracker-positive autophagosomes, suggesting that polyQ-htt both disrupts autophagosome transport and causes a failure to maintain the acidified environment of autophagosomes as they move towards the proximal axon/cell body. The autophagy receptor optineurin (Wild et al., 2011), which binds htt, was recently found to mediate autophagosome maturation via the actin-based motor myosin VI (Tumbarello et al., 2012). Here we show that htt mediates efficient autophagosome maturation and cargo degradation by regulating microtubule-based motors to promote efficient autophagosome transport and this role is disrupted by expression of expanded polyQ-htt.

In HD models, inefficient clearance of polyQ-htt via autophagy (Qin et al., 2003; Ravikumar et al., 2002) accelerates the formation of toxic polyQ-htt oligomers and ultimately results in cell death (Ravikumar et al., 2004; Sarkar et al., 2007). Since disease-associated fragments and full-length polyQ-htt are cleared from the distal axon by retrograde autophagosome transport in neurons, early defects in transport may lead to inefficient polyQ-htt clearance which may further clog autophagosomes and inhibit the degradative machinery. In addition, inefficient clearance of mitochondrial fragments may contribute to defective mitochondrial bioenergetics in HD (Costa and Scorrano, 2012). In summary, we have identified htt and HAP1 as regulators of autophagosome dynamics and function in neurons. PolyQ-htt disrupts this complex, causing disrupted autophagosome transport and inefficient clearance of mitochondrial fragments and pathogenic polyQ-htt, potentially contributing to neuronal death in HD patients.

2.4 Materials and Methods

Reagents. GFP-LC3 transgenic mice, strain name B6.Cg-Tg(CAG-EGFP/LC3)53Nmi/NmiRbrc, were developed by N. Mizushima (Tokyo Medical and Dental University, Tokyo, Japan; (Mizushima et al., 2004)) and deposited into the RIKEN BioResource Center (Japan). Immortalized STHdhQ striatal cell lines from control HdhQ23/Q23 and HD knock-in Hdh Q111/Q111 mice were developed by M. MacDonald (Richard B. Simches Research Center, Boston, MA; (Trettel et al., 2000)) and deposited into the Coriell Cell Repositories (Camden, NJ). siRNA to htt (5'-GCAGCUUGUCCAGGUUUUUUU-3') and HAP1 (5'-GAAGUAUGUCCUCCAGCAAUU-3') were obtained from Dharmacon (Thermo Scientific). Constructs used were DsRed2-mito (gift from T. Schwarz, Harvard Medical School, Boston, MA), monomeric RFP-Ub (Addgene), LAMP1-RFP (Addgene), hHAP1a (gift from X. J. Li, Emory University, Atlanta, GA) and mCherry-EGFP-LC3 (gift from T. Johansen, University of Tromsø, Tromsø, Norway; (Pankiv et al., 2007)). Htt-Q23, htt-Q23-Δdyn, htt-Q23-ΔHAP1, htt-Q100, htt-68Q and HAP1-KBD were gifts from F. Saudou (Institut Curie, Orsay, France; (Pardo et al., 2010)) and J. Kittler (University College London, London, UK). pEGFP-LC3 (gift from T. Yoshimori, Osaka University, Osaka, Japan; (Kimura et al., 2007)), DIC1B (gift from K. Pfister, University of Virginia, Charlottesville, VA) and Kif5C tail (gift from M. Setou, Hamamatsu University School of Medicine, Shizuoka, Japan) were recloned into pmCherry (Takara Bio Inc.). DIC isoforms 1A and 2C (gifts from K. Pfister, University of Virginia, Charlottesville, VA) were recloned into pcDNA3.1mychisA (Invitrogen). Antibodies used were a polyclonal antibody against LC3B (Abcam) and monoclonal antibodies against htt (MAB2166; Chemicon), HAP1 (611302; BD Biosciences), dynein intermediate chain (clone 74.1; Millipore), dynactin p150^{Glued} (610474; BD Biosciences), kinesin-1 heavy chain (clone H2; Millipore), GAPDH (ab9484; Abcam), α-tubulin (DM1A; Sigma), HA (MMS-101P; Covance), myc (R950; Invitrogen) and Hsp60 (SPA-806; Enzo Life Sciences).

Cell culture and transfection. Dorsal root ganglia (DRGs) were dissected from spinal columns of adult mice of either sex under 1 year old. To isolate neurons, DRGs were treated with

20 U/ml papain (Worthington), followed by 2 mg/ml collagenase II (Gibco) and 2.4 mg/ml dispase II (Roche). Neurons were dissociated in HBSS (Gibco) supplemented with 5 mM HEPES and 10 mM D-glucose (pH 7.35) and purified using a 20% Percoll gradient (Sigma) for 8 min at $1,000 \times g$. DRG neurons were transfected with either 0.5-2 μ g plasmid DNA, 12-30 pmol siRNA or both using the Amaxa Basic Neuron SCN Nucleofector kit (Lonza) and then plated onto coverslips or glass-bottom dishes (FluoroDish, World Precision Instruments) which were coated with 0.01% poly-L-lysine and 20 μ g/ml laminin. Neurons were maintained for 2 DIV in F-12 media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin at 37°C in a 5% CO₂ incubator. Striatal cells were cultured and differentiated as previously described (Trettel et al., 2000) and imaged 2 DIV after differentiation. Both striatal cells and COS-7 cells were transfected using FuGENE 6 (Promega). All live-cell imaging was performed in low fluorescence nutrient medium (Hibernate A; BrainBits) supplemented with 2% B27 supplement (Invitrogen) and 2 mM GlutaMax (GIBCO). DRGs were labeled with 100 nM LysoTracker Red (Invitrogen) diluted in F-12 media for 30 min at 37°C in a 5% CO₂ incubator, followed by two washes with Hibernate A medium before imaging. All experiments involving animals were approved by the IACUC at the University of Pennsylvania.

Biochemistry. Autophagosome-enriched fractions were prepared from GFP-LC3 transgenic mice brains using a protocol adapted from Morvan et al. (2009) and Strømhaug et al. (1998) as described (Maday et al., 2012). Brains were homogenized in 10 ml of 250 mM sucrose in 10 mM Hepes-KOH (pH 7.4) using a 30-ml homogenizer with a round-bottom Teflon pestle. The homogenate was separated using a Nycondenz gradient and volumes of the gradient steps were then scaled proportionately for a rotor (SW41; Beckman Coulter). Equal total protein of low speed supernatant (LSS) and the autophagosome-enriched fraction (AV) were separated by SDS-PAGE and analyzed by western blot. For protein analysis of neuronal lysates, DRG neurons were cultured for 2 DIV, washed with Dulbecco's Phosphate-Buffered Saline (Gibco; pH 7.4) at room temperature and scraped into ice-cold lysis buffer (0.5mM DTT, 1mM Leupeptin, 1mM

Pepstatin-A and 1 mM TAME). Neuronal lysates were analyzed by SDS-PAGE and western blot according to standard protocols.

Immunofluorescence. For immunofluorescence analysis, DRG neurons were plated on coverslips and cultured for 2 DIV. Cells were washed once in PBS (150 mM NaCl in 50 mM NaPO₄, pH 7.4) and fixed in 4% paraformaldehyde with 4% sucrose in PBS for 5 min at room temperature. Cells were then washed twice in PBS and blocked and permeabilized in 2% (wt/vol) BSA and 0.1% (wt/vol) saponin in PBS for 1 hr at room temperature. Fixed cells were incubated in primary antibody for 1 hr, washed 3 × 5 min, incubated in secondary antibody for 1 hr, washed 3 × 5 min and mounted on glass slides with ProLong gold (Invitrogen).

Immunoprecipitation. To examine coimmunoprecipitation of htt with dynein isoforms, COS-7 cells were cotransfected for 24 hr with myc-tagged DIC isoforms and HA-tagged wildtype htt or polyQ-htt using FuGENE 6 (Promega). Cells were lysed in 80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, 50 mM NaCl, 0.5 mM DTT, 0.5% Triton X-100, and protease inhibitors (1 mM PMSF, 1 mM Leupeptin and 1 mM TAME). Lysates were immunoprecipitated using Protein G coupled Dynabeads (Invitrogen) incubated in myc antibody, and were subsequently washed in the above buffer without detergent. Equal total protein levels of lysates and eluates were analyzed by SDS-PAGE and western blot according to standard protocols. Measured band intensities of htt coimmunoprecipitating with DIC were normalized to account for differential DIC isoform expression and immunoprecipitation and are expressed as the ratio of DIC2C: DIC1A interaction for each htt construct.

Microscopy. Images of GFP-LC3 motility (Figure 2.2, 2.3A, 2.4, 2.5), mCherry-EGFP-LC3 motility (Figure 2.6E) and polyQ-htt (Figure 2.9) were acquired on an inverted epifluorescence microscope (DMI6000B; Leica) with an Apochromat 63× 1.4 NA oil immersion objective (Leica) in a temperature-controlled chamber (37°C). Digital images were acquired with a charge-coupled device camera (ORCA-R²; Hamamatsu Photonics) using LAS-AF software (Leica) at 1 frame every 3 sec for a 3 min duration. Dual-color videos were acquired as consecutive green-red images for a 3 min duration.

Images of lysosome and mitochondrial motility (Figure 2.2H), co-transport of GFP-LC3 with motors (Figure 2.3F,G), autophagosome biogenesis, GFP-LC3 co-transport with cargo, and maturation and fusion with lysosomes (Figure 2.6A-D, 7, 8) were acquired on a spinning-disk confocal (UltraVIEW VoX; PerkinElmer) on a Nikon Eclipse Ti microscope using an Apochromat 100× 1.49 NA oil immersion objective (Nikon) in a temperature-controlled chamber (37°C). Digital images were acquired with an EM charge-coupled device camera (C9100; Hamamatsu Photonics) using Volocity software (PerkinElmer) at 1 frame every 3-5 sec for a 3-10 min duration. Dual-color videos were acquired as consecutive green-red images for colocalization/co-transport experiments. Videos of mitochondria co-transported with autophagosomes were acquired using longer exposure times to allow visualization of mitochondrial fragments within autophagosomes. Neurons fixed for immunofluorescence experiments (Figure 2.1B-D) were also imaged using the confocal microscope described above. All images were assembled using ImageJ (National Institutes of Health) and Photoshop (Adobe).

Image Analysis. Kymographs were created using MetaMorph (Molecular Devices) for time-lapse epifluorescent images and the Multiple Kymograph plugin (submitted by J. Rietdorf and A. Seitz, European Molecular Biology Laboratory, Heidelberg, Germany) in ImageJ (NIH) for time-lapse confocal images. From these kymographs, GFP-LC3 puncta were classified as retrograde (moved $\geq 10 \mu\text{m}$ in the retrograde direction), anterograde (moved $\geq 10 \mu\text{m}$ in the anterograde direction) or stationary (moved less than $10 \mu\text{m}$ during the duration of the 3 min video). The percent motility of autophagosomes along the axon (either retrograde, anterograde or stationary) was calculated as a percentage of the total number of autophagosomes imaged per neuron. Run lengths and run speeds of organelles were manually measured for net runs (the total distance a puncta traveled during the 3 min video) and individual runs (the distance a puncta traveled before switching direction or speed) by drawing a slope from the beginning to the end of the run on the kymograph. Neurons from htt-depleted or htt rescue cultures were analyzed for the percentage of retrograde autophagosomes, and then normalized to the percentage of retrograde autophagosomes from control neurons of corresponding experiments, performed in parallel

(Figure 2.3C). For HAP1 depletion experiments, net runs and individual runs were further classified as retrograde-directed (moved any distance in the retrograde direction) or anterograde-directed (moved any distance in the anterograde direction).

To assess colocalization of LC3 with motors or cargo, line scans were generated using ImageJ (NIH) and normalized per protein and per condition. Autophagosome density at the axon tip was calculated using the total number of autophagosomes observed at the beginning of a video in a given axon tip (total area < ~50 μm from the end of a neurite). Autophagosome density along the axon was calculated from kymographs using the total number of autophagosomes observed divided by the length of the kymograph. The percentage of LAMP-1-positive GFP-LC3 puncta was calculated using the total number of autophagosomes observed at the axon tip (total area < ~50 μm from the end of a neurite) or along the axon (>100 μm from the end of a neurite) at a given time point. The percentage of mCherry-EGFP-LC3 puncta which had mCherry or GFP fluorescence was calculated as the total number of LC3-fluorescent puncta at the proximal axon (<100 μm from the cell body). The percentage of DsRed2-mito positive GFP-LC3 puncta was calculated using the total number of autophagosomes observed in a kymograph along the axon (>100 μm from the end of a neurite). Mitophagic flux was determined at the axon tip, as the percentage of DsRed2-mito positive GFP-LC3 puncta per μm per min observed at the distal axon tip (45-60 μm from the end of the neurite). The percentage of LysoTracker Red-positive GFP-LC3 puncta was calculated at the axon tip (< ~50 μm from the end of a neurite), the mid-axon (>100 μm from the end of a neurite) or the proximal axon (<100 μm from the cell body) at a given time point. Axon tips were outlined using threshold analysis in Image J (NIH).

Statistical Analysis. Statistics and graphing were performed using Prism (GraphPad) software. Comparisons of two data sets were performed using unpaired two-tailed Student's t test. Comparisons of % motility of autophagosomes for multiple data sets were performed using two-way ANOVA with Tukey's post-hoc test. All other comparisons of multiple data sets were performed using one-way ANOVA with Tukey's post-hoc test.

3 OPTINEURIN IS AN AUTOPHAGY RECEPTOR FOR DAMAGED MITOCHONDRIA IN PARKIN-MEDIATED MITOPHAGY THAT IS DISRUPTED BY AN ALS- LINKED MUTATION

All the work presented in chapter three was completed by Yvette Wong.

This chapter was written by Yvette Wong and Erika Holzbaur and was recently published in the
Proceedings of the National Academy of Sciences (Wong and Holzbaur, 2014b).

3.1 Introduction

Damaged mitochondria are selectively turned over in eukaryotic cells via mitophagy, a process in which double-membraned autophagosomes sequester and ultimately degrade mitochondria via lysosomal fusion (Wang and Klionsky, 2011; Youle and Narendra, 2011). This process is regulated by PINK1 (phosphatase and tensin homologue-induced putative kinase protein 1) and parkin, two proteins linked to hereditary forms of Parkinson's disease (Kitada et al., 1998; Valente et al., 2004). PINK1 is stabilized on the outer membrane of damaged mitochondria and recruits the E3 ubiquitin ligase parkin which ubiquitinates proteins on the outer mitochondrial membrane (OMM) (Gegg et al., 2010; Matsuda et al., 2010; Narendra et al., 2008, 2010b; Poole et al., 2010; Sarraf et al., 2013; Tanaka et al., 2010; Vives-Bauza et al., 2010; Ziviani et al., 2010). Parkin-mediated ubiquitination of damaged mitochondria is followed by autophagosome formation and engulfment of mitochondria (Wang and Klionsky, 2011; Youle and Narendra, 2011). However, the proteins involved in dynamically recruiting autophagic machinery to ubiquitinated damaged mitochondria still remain elusive.

Optineurin is an autophagy receptor, characterized by its ability to bind ubiquitin via its UBAN domain (Ubiquitin binding in ABIN and NEMO) (Zhu et al., 2007) and the autophagosome-associated protein LC3 via its LIR domain (LC3 interacting region) (Wild et al., 2011). Optineurin regulates autophagosome maturation (Tumbarello et al., 2012) and autophagic degradation of *Salmonella* and protein aggregates (Korac et al., 2013; Wild et al., 2011). However, optineurin's role in mitophagy has not been previously studied. Mutations in optineurin lead to primary open angle glaucoma (Rezaie et al., 2002) and Amyotrophic Lateral Sclerosis (ALS) (Maruyama et al., 2010), two neurodegenerative diseases in which mitochondrial defects have been observed (Cozzolino et al., 2013; Osborne and Del Olmo-Aguado, 2013). Thus, optineurin may play a role in regulating the autophagic turnover of damaged mitochondria during mitophagy.

Here, we use confocal live cell imaging to show that parkin is both necessary and sufficient to stabilize optineurin on the surface of damaged mitochondria. In the absence of parkin, optineurin puncta transiently localize to damaged mitochondria but do not remain stably

associated. In cells expressing parkin, optineurin is recruited to mitochondria following parkin recruitment and this recruitment is stabilized via the UBAN domain. Following optineurin recruitment, DFCP1 (double FYVE containing protein 1) puncta transiently localize to parkin/optineurin-labeled damaged mitochondria to mark the initial site of autophagosome formation (Axe et al., 2008). This is followed by LC3 recruitment and subsequent autophagosome formation around optineurin-labeled damaged mitochondria. Importantly, we find that depletion of optineurin inhibits autophagosome recruitment to damaged mitochondria, leading to increased levels of the mitochondrial matrix protein Hsp60 and mtDNA content within cells. This defect in mitochondrial degradation is rescued by wildtype optineurin, but not by the E478G UBAN mutant in optineurin causative for ALS (Maruyama et al., 2010), or by an optineurin LIR mutant unable to bind LC3 (Wild et al., 2011). Optineurin and p62, previously implicated in mitophagy (Ding et al., 2010; Geisler et al., 2010; Narendra et al., 2010a; Okatsu et al., 2010), are independently recruited to distinct domains on damaged mitochondria. In contrast to our observations with optineurin, depletion of p62 did not inhibit LC3 recruitment or efficient degradation of damaged mitochondria. Thus, our study shows an important role for the autophagy receptor optineurin in parkin-mediated mitophagy and provides support for the hypothesis that defective mitochondrial quality control may contribute to ALS pathogenesis.

3.2 Results

3.2.1 Optineurin transiently associates with damaged mitochondria in the absence of parkin.

In untreated HeLa cells, GFP-labeled optineurin (GFP-Optn) is predominantly cytosolic (Ying and Yue, 2012) with few puncta per cell that rarely localize to mitochondria (top panel, Figure 3.1A and Figure 3.S1B). We induced mitochondrial damage with the mitochondrial uncoupler carbonyl-cyanide m-chlorophenylhydrazone (CCCP) which depolarizes mitochondria. Mitochondria became more fragmented (bottom panel, Figure 3.1A and Figure 3.S1A) but remained localized throughout the cell after 1 h CCCP treatment. Following CCCP treatment, ~25% of optineurin puncta were observed to transiently associate with damaged mitochondria (bottom panel, Figure 3.1 A-B and Figure 3.S1C). Following dissociation from mitochondria, optineurin puncta either rebind to a neighboring mitochondria or remained cytosolic (Figure 3.1C).

Optineurin puncta preferentially localized to a single tip of an elongated mitochondria rather than along the length of the organelle (Figure 3.1D-F, Figure 3.S1D). After 2 h of CCCP treatment, mitochondria became fragmented and punctate, while 6 h after treatment, the majority of mitochondria were circular rather than elongated (Figure 3.S2A-B). Fewer optineurin puncta localized to mitochondrial tips following prolonged mitochondrial damage (Figure 3.1B). However, even at 6 h CCCP, we observed optineurin puncta that transiently localized to a single point on damaged circular mitochondria or colocalized with extremely fragmented punctate mitochondria (Figure 3.S2B). Importantly, immunostaining of endogenous optineurin in CCCP-treated HeLa cells confirmed the specific localization of optineurin to the tips of damaged mitochondria (Figure 3.S2C-D). Thus, in HeLa cells, which express a low level of endogenous parkin (Denison et al., 2003; Pawlyk et al., 2003), optineurin specifically but transiently localizes to the tips of damaged mitochondria.

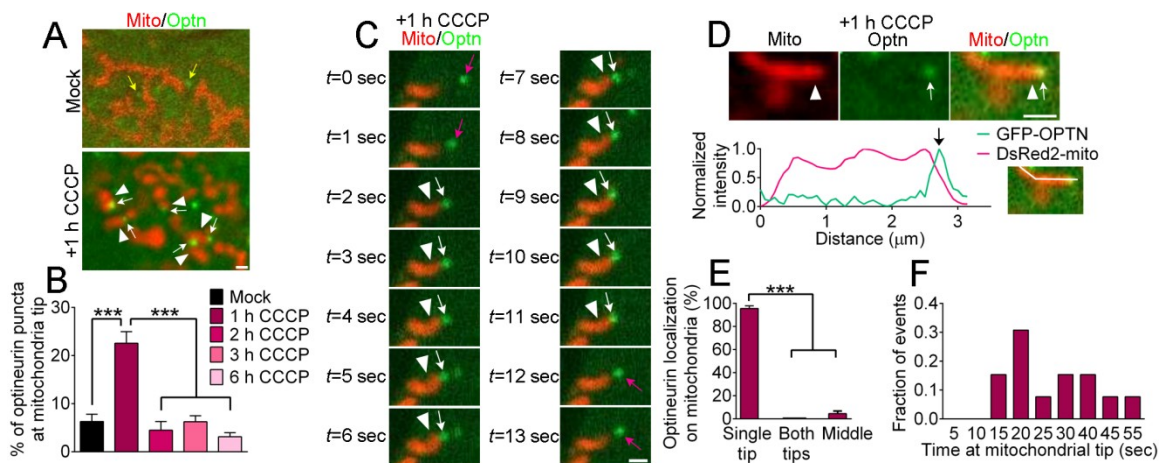


Figure 3.1: Optineurin transiently associates with damaged mitochondria in the absence of parkin.

(A) 1 h CCCP (20 μ M) treatment of HeLa cells causes mitochondrial fragmentation. Optineurin puncta (GFP-Optn) in control cells do not associate with mitochondria (DsRed2-mito) (yellow arrows). In HeLa cells in the absence of parkin, CCCP-induced mitochondrial damage causes optineurin puncta to transiently associate (white arrows) with the tips of fragmented mitochondria (arrowheads). (B) Optineurin preferentially localizes to damaged mitochondria immediately following CCCP treatment. (C) Time series of optineurin recruitment from the cytosol (pink arrows) to a mitochondrial tip (white arrows, optineurin puncta; arrowheads, mitochondria). (D) Example of optineurin (arrow) localized to a mitochondrial tip (arrowhead). (E, F) After 1 h CCCP treatment of HeLa cells, optineurin preferentially localizes to the mitochondrial tip for ~30 sec. (Scale bars: A, C, and D, 1 μ m.) Values represent means $\pm$ SEM. ***P < 0.001.

3.2.2 Parkin ubiquitination stabilizes optineurin recruitment to the outer membrane of damaged mitochondria.

Next, we used live cell microscopy to image the dynamics of parkin during mitophagy in CCCP-treated HeLa cells. The E3 ubiquitin ligase parkin is recruited from the cytosol to the OMM of damaged mitochondria via a PINK1-dependent pathway (Matsuda et al., 2010; Narendra et al., 2008, 2010b; Vives-Bauza et al., 2010), where it ubiquitinates OMM proteins including mitochondrial fusion proteins Mfn1 and Mfn2 (Gegg et al., 2010; Poole et al., 2010; Sarraf et al., 2013; Tanaka et al., 2010; Ziviani et al., 2010). We expressed YFP-Parkin in HeLa cells treated with CCCP for 1 h and showed that parkin was gradually recruited to the surface of mitochondria (DsRed2-mito), as previously noted (Narendra et al., 2008) (Figure 3.S3A). Under confocal microscopy, we could visualize parkin rings forming around spherical mitochondrial fragments within a single z plane, indicative of parkin recruitment to the mitochondrial surface (Figure 3.2A).

About 50% of mitochondria were parkin-labeled by 30 min, and 95% of mitochondria were parkin-labeled by 1 h CCCP treatment (Figure 3.S3B-D). Prolonged mitochondrial damage (2 h CCCP) resulted in the aggregation of fragmented mitochondria, with parkin recruited to the surface of each fragment (Figure 3.2B-C), rather than to the surface of the entire mitochondrial aggregate. Further mitochondrial damage (6 h CCCP) resulted in the perinuclear localization of mitochondrial aggregates with parkin still stably localized to the surface of individual fragments (Figure 3.S3E).

Next, we examined the dynamics of GFP-Optn in CCCP-treated HeLa cells expressing parkin. In contrast to the transient localization of optineurin puncta to mitochondrial tips observed in the absence of parkin, parkin expression induced the stable recruitment of optineurin all around the surface of damaged mitochondria (Figure 3.2D). Using confocal microscopy, we imaged a single z plane and observed optineurin rings surrounding spherical fragments of mitochondria (Figure 3.2E). Using time-lapse video microscopy, we found that optineurin was gradually recruited from the cytosol to the surface of damaged mitochondria (Figure 3.S4A). Few mitochondria were labeled with optineurin after 30 min of CCCP treatment, but 60% of mitochondria were labeled with optineurin by 1 h CCCP treatment (Figure 3.2F) with optineurin rings forming around mitochondrial fragments throughout the cell (Figure 3.S4B). After 2 h CCCP treatment, the majority of mitochondria were surrounded by optineurin (Figure 3.2G); this extensive colocalization remained after 6 h CCCP treatment (Figure 3.S5A). Importantly, immunostaining of endogenous optineurin also showed a parkin-dependent stabilization of recruitment to the surface of damaged mitochondria, confirming our observations with the live cell GFP-Optn probe (Figure 3.2H).

Next, we compared the dynamics of parkin and optineurin recruitment to damaged mitochondria, by co-expressing fluorescently-tagged optineurin and parkin with sBFP-mito. We found that after 30 min – 1.5 h CCCP treatment, some mitochondria were labeled with parkin only (Figure 3.2I), but all optineurin-labeled mitochondria were parkin-positive (Figure 3.2 J-K). For a given mitochondria, parkin was recruited first, followed by optineurin (Figure 3.2I). At 1 h CCCP,

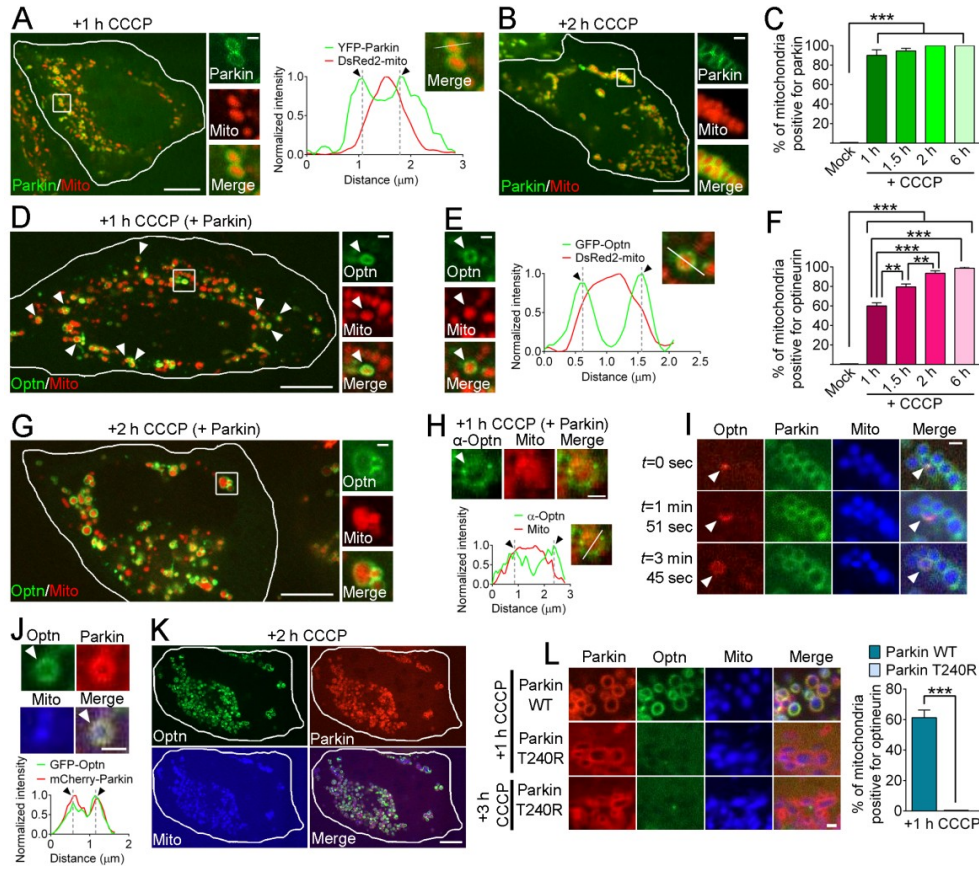


Figure 3.2: Parkin ubiquitination stabilizes optineurin recruitment to the outer membrane of damaged mitochondria.

(A) Confocal image of HeLa cell expressing DsRed2-mito and YFP-parkin after 1 h CCCP treatment. Parkin is recruited to damaged mitochondria, seen as parkin rings around spherical mitochondrial fragments within a single z plane, with corresponding linescan (right). (B) 2 h CCCP treatment causes aggregation of parkin-labeled mitochondria. (C) The majority of mitochondria are parkin-positive after 1 h CCCP treatment. (D) Confocal image of HeLa cell expressing parkin, GFP-Optn and DsRed2-mito treated with CCCP for 1 h. Optineurin is stably recruited to the surface of damaged mitochondria in the presence of parkin. (E) Optineurin localization on the outer membrane of damaged mitochondria with corresponding linescan (right). (F) Optineurin recruitment lags parkin recruitment. (G) Optineurin is recruited to the majority of mitochondria following 2 h CCCP treatment. (H) Immunostaining of endogenous optineurin in 1 h CCCP-treated HeLa cells expressing parkin and sBFP-mito (pseudo-colored red) with corresponding linescan (lower). Optineurin is recruited to the outer membrane of damaged mitochondria. (I) Confocal time series of a CCCP-treated HeLa cell expressing mCherry-Optn, YFP-parkin and sBFP-mito showing optineurin recruitment only occurs following parkin recruitment to damaged mitochondria. An optineurin puncta is first localized to the surface of a parkin-labeled mitochondria, and gradually grows into a ring. (J) CCCP-treated HeLa cells expressing GFP-Optn, mCherry-parkin and sBFP-mito. Confocal image and corresponding linescan (bottom) of damaged mitochondria with both optineurin and parkin localized to its outer membrane. (K) Both optineurin and parkin localize to mitochondria following 2 h CCCP treatment. (L) A catalytically-inactive Parkinson's disease-associated parkin mutant T240R is unable to recruit optineurin after either 1 or 3 h CCCP. (Scale bars: A, B, D, G, and K, 10 μ m; Insets in A, B, D and G, 1 μ m; E, H, I, J and L, 1 μ m.) Values represent means $\pm$ SEM. **P < 0.01; ***P < 0.001.

67% of parkin-positive mitochondria were also positive for optineurin, and by 1.5 h CCCP, 84% of parkin-positive mitochondria were also positive for optineurin. By 2 h CCCP treatment, most mitochondria were positive for both parkin and optineurin (Figure 3.S5B-D), and remained so after 6 h of CCCP treatment (Figure 3.S5E-H). Expression of mCherry-Optn (rather than GFP-Optn), YFP-parkin and sBFP-mito in HeLa cells provided similar results, with stepwise recruitment of parkin and then optineurin to damaged mitochondria (Figure 3.S5I-J).

To test whether parkin's E3 ubiquitin ligase activity is necessary for optineurin recruitment, we compared the effect of wildtype parkin with a catalytically inactive Parkinson's disease-associated T240R mutant in parkin's RING1 domain (Sriram et al., 2005). After 1 h CCCP, wildtype parkin was robustly recruited to damaged mitochondria (n=87/87 cells), while parkin T240R showed low levels of recruitment to damaged mitochondria (n=112/112 cells) (Figure 3.2L). Optineurin was recruited to mitochondria in cells expressing wildtype parkin (61.3 ± 5.0 % of mitochondria after 1 h CCCP), but was not recruited to any mitochondria in T240R parkin-expressing cells (0% of mitochondria after 1 h CCCP) (Figure 3.2L). After 3 h CCCP, parkin T240R showed increased recruitment to damaged mitochondria, suggesting that T240R parkin can be recruited to damaged mitochondria but perhaps at a slower rate compared to wildtype parkin. However, even at this time point optineurin was not recruited to damaged mitochondria in T240R parkin-expressing cells (n=54/54 cells) (Figure 3.2L), demonstrating that optineurin's recruitment to damaged mitochondria is dependent on parkin's E3 ubiquitin ligase activity.

3.2.3 Optineurin is dynamically recruited to damaged mitochondria.

To spatially regulate the induction of mitochondrial damage, we expressed Mito-KillerRed in HeLa cells expressing parkin and GFP-Optn. Illumination of Mito-KillerRed (Mito-KR) with 561-nm light induces generation of reactive oxygen species (ROS) within the mitochondrial matrix

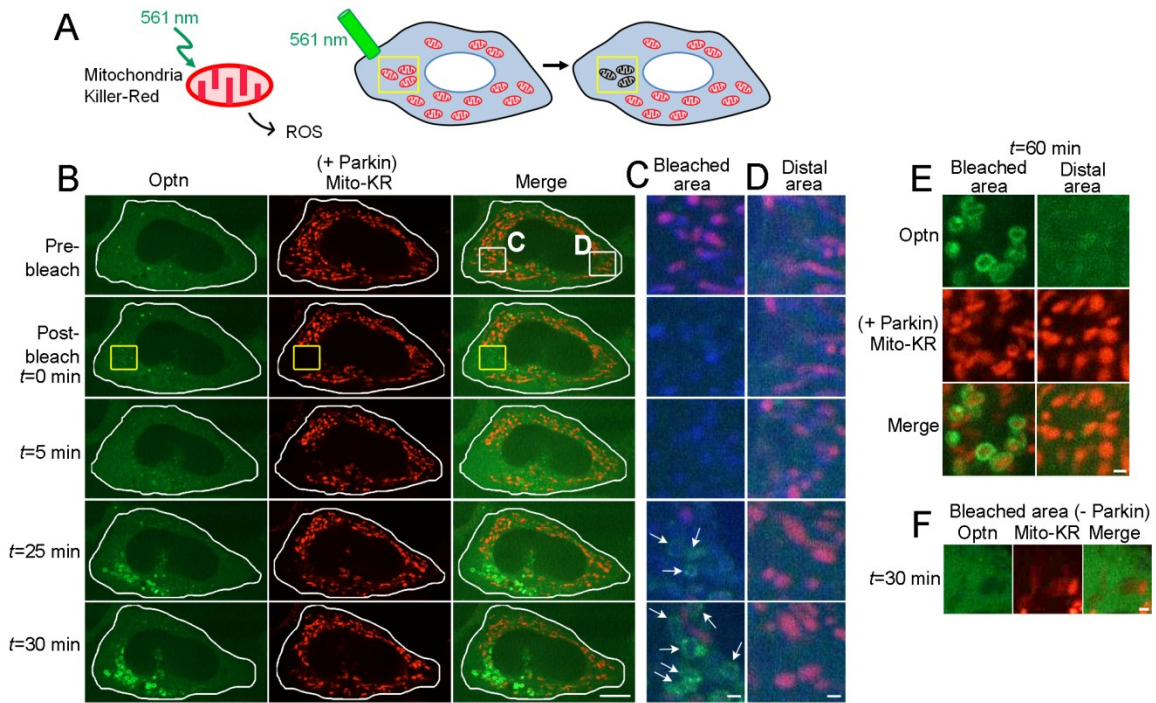


Figure 3.3: Optineurin is dynamically recruited to damaged mitochondria via parkin.

(A) Schematic: illumination of mitochondrial-targeted Killer-Red (mito-KR) by 561nm light leads to the generation of reactive oxygen species (ROS) within the mitochondrial matrix. Illumination of a defined region within the cell (yellow box) allows for spatiotemporally-controlled damage of a specific mitochondrial population (black mitochondria). (B) Confocal image of HeLa cell expressing parkin, GFP-Optn, mito-KR and sBFP-mito. A defined region was illuminated (yellow box) leading to Mito-KR bleaching and induced mitochondrial damage. Optineurin is recruited to the surface of damaged mitochondria beginning ~25 min after bleaching. (C,D) Optineurin recruitment is spatially restricted to mitochondria near the bleached region rather than to a distal unbleached region of the cell. (E) Optineurin remains localized to mitochondria in the bleached area 1 h after bleaching. (F) Optineurin is not recruited to damaged mitochondria in a HeLa cell not expressing exogenous parkin, demonstrating that optineurin recruitment to spatiotemporally-restricted damaged mitochondria also requires parkin. (Scale bars: B, 10 μ m; C–F, 1 μ m.)

(Bulina et al., 2006), resulting in damage to a spatially-localized population of mitochondria within the cell (Figure 3.3A) and parkin recruitment (Yang and Yang, 2013). Optineurin localization was predominantly cytosolic prior to bleaching (top panel; Figure 3.3B). Bleaching of the Mito-KillerRed signal within the illuminated region (yellow box; Figure 3.3B) led to the striking recruitment of optineurin to the surface of damaged mitochondria within 25 min, seen as the formation of optineurin rings around mitochondria labeled with sBFP-mito (Figure 3.3B-C). After 30 min, optineurin was dynamically recruited to additional mitochondria surrounding the bleached region (Figure 3.3B-C). In contrast, optineurin was not recruited to mitochondria in a region of the

cell distal to the point of focally-induced mitochondrial damage (Figure 3.3D). Optineurin remained localized to the surface of damaged mitochondria 1 h post-bleaching proximal to the illuminated region, but was not recruited to mitochondria in the distal region (Figure 3.3E). Importantly, in HeLa cells that did not express exogenous parkin, we did not observe recruitment of optineurin to damaged mitochondria even 30 min after bleaching (Figure 3.3F). Thus, expression of parkin leads to the spatiotemporally-restricted recruitment and stabilization of optineurin on damaged mitochondria.

3.2.4 Optineurin stabilization on damaged mitochondria via its UBAN domain is disrupted by an ALS-linked mutation.

We hypothesize that the parkin-mediated ubiquitination of proteins on the OMM (Gegg et al., 2010; Poole et al., 2010; Sarraf et al., 2013; Tanaka et al., 2010; Ziviani et al., 2010) stabilizes the association of optineurin with damaged mitochondria, since optineurin can bind ubiquitin via its UBAN domain (Zhu et al., 2007). To test this hypothesis, we expressed the ALS-associated E478G ubiquitin-binding deficient mutant (optn-E478G), which has a point mutation in the UBAN domain (Maruyama et al., 2010; Wild et al., 2011). In HeLa cells expressing parkin and treated with CCCP, we found that optn-E478G was not recruited to the surface of damaged mitochondria, but instead remained predominantly cytosolic (Figure 3.4A). Almost none of the mitochondria (1%) were surrounded by optn-E478G, compared to the 60% of mitochondria surrounded by wildtype optineurin following 1 h CCCP treatment (Figure 3.4A). Next, we performed confocal time-lapse video imaging and found that after >45 min of CCCP treatment, the majority of damaged mitochondria were parkin-positive but negative for GFP-Optn-E478G (Figure 3.S6A). We did observe transient interactions of GFP-Optn-E478G puncta with damaged mitochondrial fragments (white arrows, Figure 3.4B and Figure 3.S6A). However, unlike wildtype optineurin, optn-E478G did not remain stably associated with damaged mitochondria, dissociating after ~ 6 min (yellow arrows, Figure 3.4B). Strikingly, even after prolonged mitochondrial damage,

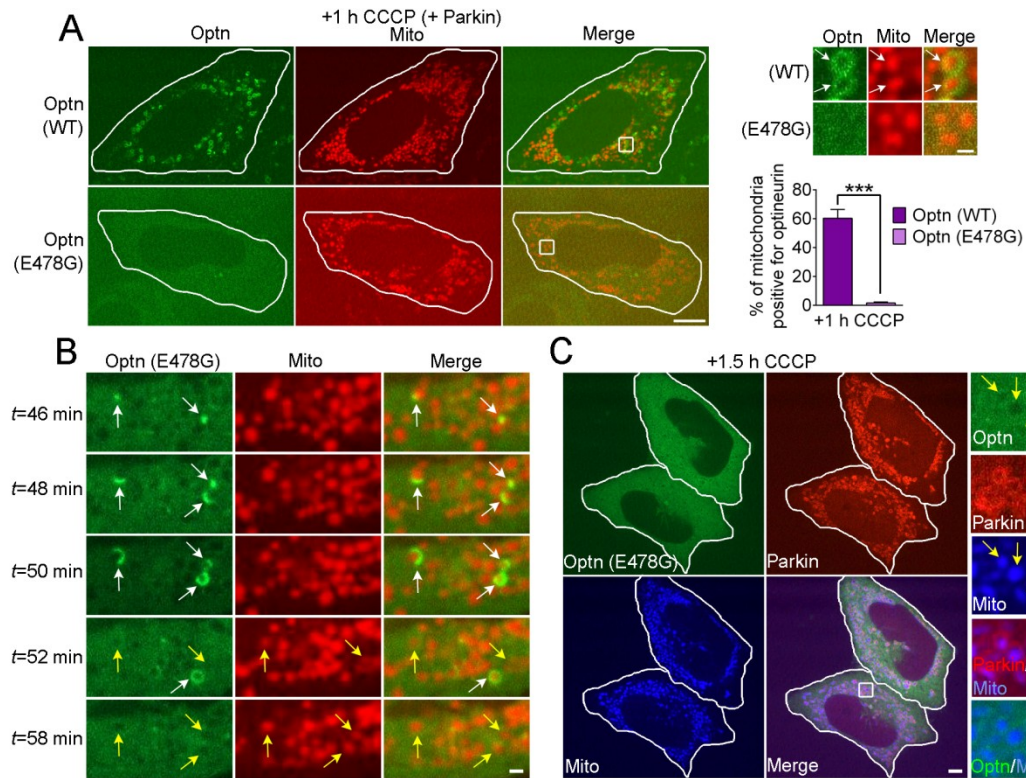


Figure 3. 4: Optineurin stably associates with damaged ubiquitinated mitochondria via its UBAN domain and is disrupted by an ALS-linked mutation.

(A) Confocal image of CCCP-treated HeLa cell expressing parkin, sBFP-mito (pseudo-colored red) and either wildtype mCherry-Optn or an ALS-associated UBAN mutant mCherry-Optn-E478G which has deficient ubiquitin binding (optn-E478G) (pseudo-colored green). Wildtype optn shows clear recruitment to the surface of damaged mitochondria (white arrows on magnified image) after 1 h CCCP, while optn-E478G does not stably associate with damaged mitochondria. Quantification of optineurin recruitment to damaged mitochondria showing that wildtype optineurin is preferentially recruited and stabilized on mitochondria compared to the E478G mutant. (B) Time lapse showing optn-E478G puncta are transiently recruited to the outer surface of damaged mitochondria (white arrows). However, optn-E478G does not remain stably associated, resulting in mitochondria not labeled with optn-E478G (yellow arrows). (C) Optn-E478G remains cytosolic even after 1.5 h CCCP treatment and does not localize to parkin-positive mitochondria (yellow arrows). (Scale bars: A, 10 μ m; C, 5 μ m; Insets in A and C, 1 μ m; B, 1 μ m.) Values represent means $\pm$ SEM. ***P < 0.001.

optn-E478G remained cytosolic (yellow arrows, Figure 3.4C), while wildtype optineurin had formed rings around parkin-positive mitochondria. These results indicate that ubiquitin binding is essential to stabilize the association of optineurin with damaged mitochondria, and further support the hypothesis that optineurin binds parkin-ubiquitinated OMM proteins via its UBAN domain.

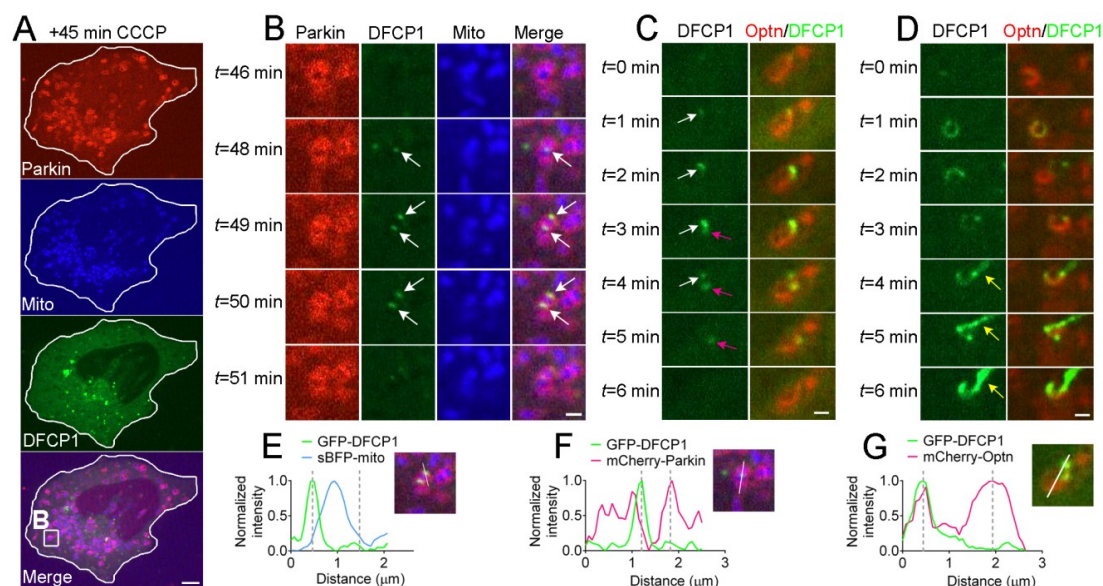


Figure 3.5: DFCP1 omegasome formation occurs after parkin/optineurin recruitment to damaged mitochondria.

(A) HeLa cell expressing sBFP-mito, mCherry-parkin and the omegasome marker GFP-DFCP1 treated with CCCP for 45 min. DFCP1 puncta are transiently recruited to parkin-positive mitochondria. (B) Time lapse images of boxed area in (A) following CCCP treatment showing DFCP1 puncta recruited to parkin-labeled mitochondria for ~3 min before leaving (arrows). (C) Two examples of a DFCP1 puncta gradually forming on an optineurin-labeled mitochondria over ~3 min and then disappearing (white arrows, pink arrows). (D) Example of DFCP1 recruitment to the surface of an optineurin-labeled mitochondria and later forming DFCP1 tubules (yellow arrows) which extend outward. (E-G) Linescans showing DFCP1 localization with parkin and optineurin on the outer membrane of damaged mitochondria, from HeLa cells expressing GFP-DFCP1 with sBFP-mito, mCherry-parkin or mCherry-Optn. (Scale bars: A, 5 μ m; B-D, 1 μ m.)

3.2.5 DFCP1 omegasomes form on parkin/optineurin-labeled damaged mitochondria.

Autophagy receptors such as optineurin have been proposed to recruit autophagic machinery to cargo (Wild et al., 2014). To test this hypothesis, we examined the omegasome-localized protein DFCP1, which marks the initial site of autophagosome formation (Axe et al., 2008; Hayashi-Nishino et al., 2009) and localizes to damaged mitochondria (Itakura et al., 2012; Yang and Yang, 2013). We expressed GFP-DFCP1 in CCCP-treated HeLa cells expressing parkin and using time lapse confocal imaging, found that DFCP1 puncta appeared dynamically on mitochondria at various time points from 45 min to 1.5 h of CCCP treatment. DFCP1 puncta transiently localized to parkin-positive mitochondria for ~3-5 min (Figure 3.5A-B,E-F).

To examine whether optineurin recruitment occurs prior to omegasome formation, we co-expressed DFPC1 and optineurin in HeLa cells also expressing parkin. We observed optineurin recruitment to mitochondria after 1 h CCCP treatment (Figure 3.S7A). DFPC1 puncta transiently (Axe et al., 2008; Hayashi-Nishino et al., 2009) and localizes to damaged mitochondria (Itakura et al., 2012; Yang and Yang, 2013). We expressed GFP-DFPC1 in CCCP-treated HeLa cells expressing parkin and using time lapse confocal imaging, found that DFPC1 puncta appeared dynamically on mitochondria at various time points from 45 min to 1.5 h of CCCP treatment. DFPC1 puncta transiently localized to parkin-positive mitochondria for ~3-5 min (Figure 3.5A-B,E-F).

To examine whether optineurin recruitment occurs prior to omegasome formation, we co-expressed DFPC1 and optineurin in HeLa cells also expressing parkin. We observed optineurin localized to optineurin-labeled mitochondria (Figure 3.5C-D,G) and remained on the surface of mitochondria for 3-5 min (see two examples - white and pink arrows, Figure 3.5C). We also observed DFPC1 puncta that began as ring-like structures around optineurin-labeled mitochondria and formed extensions outwards from the optineurin ring (Figure 3.5D). We found that DFPC1 localized to damaged mitochondria already positive for optineurin (n=54/54 mitochondria), indicating that omegasome formation occurs downstream of optineurin recruitment. To test if optineurin is required for DFPC1 recruitment, we depleted optineurin using siRNA and found that DFPC1 could still localize to damaged mitochondria (Figure 3.S7B). In addition, the number of DFPC1 puncta on mitochondria per cell after 1 h CCCP was not affected by optineurin depletion (mock, $12.1 \pm 1.1\%$; optn KD, $9.1 \pm 0.7\%$, $p = 0.08$). Thus, DFPC1 omegasome formation occurs subsequent to optineurin recruitment but is localized to damaged mitochondria in an optineurin-independent pathway.

3.2.6 Optineurin recruits autophagosomes to damaged mitochondria via its LIR domain.

We next examined autophagosome formation using the well-established marker GFP-LC3 (Kabeya, 2000). Autophagosomes form from omegasomes on damaged mitochondria (Yang

and Yang, 2013), and we asked if this occurs downstream of optineurin recruitment to damaged mitochondria. We imaged GFP-LC3 and optineurin dynamics in HeLa cells expressing parkin. After 1 h CCCP treatment, we saw the formation of LC3-positive autophagosomes around mitochondria surrounded by optineurin (Figure 3.6A). As expected, we only observed autophagosome formation around mitochondria that had already recruited optineurin (n=185/185 mitochondria). At this timepoint, not all optineurin-labeled mitochondria were engulfed by autophagosomes (yellow arrow, Figure 3.6B), indicating that autophagosome engulfment of damaged mitochondria does not occur simultaneously throughout the cell, but rather, is a gradual, dynamic process which may depend on the depolarized state of each individual mitochondria. To observe the timeframe of autophagosome formation, we examined HeLa cells treated after 1.5 h CCCP and found that by this time, ~70% of optineurin-labeled mitochondria were engulfed by LC3-positive autophagosomes (Figure 3.6C and Figure 3.S7C).

Using time-lapse video microscopy, we saw that autophagosome formation around optineurin-labeled mitochondria began as a small LC3 puncta on the surface of the mitochondria and grew into a sphere which enveloped the entire mitochondria, seen as an LC3 ring in a single z-plane under confocal microscopy (white arrow, Figure 3.S7D). This process of LC3 puncta to ring formation lasted ~5 min, similar to the kinetics of basal autophagy observed in primary neurons (Maday et al., 2012).

Next, to test whether optineurin is an autophagy receptor for damaged mitochondria, we siRNA-depleted optineurin in parkin-expressing HeLa cells and examined whether GFP-LC3 autophagosomes could still be recruited to damaged mitochondria. siRNA depletion of optineurin lead to >90% knockdown of endogenous optineurin (Figure 3.S8A). In control cells, we observed autophagosome formation around mitochondria after 1 h CCCP treatment (Figure 3.6D), in ~80% of cells. However, in optineurin-depleted cells, almost none of the mitochondria were engulfed by autophagosomes (Figure 3.6E); the percentage of total mitochondria that were engulfed by LC3 was significantly decreased compared to control cells (Figure 3.6F). Indeed, the percentage of cells that demonstrated any autophagic engulfment of mitochondria after 1 h CCCP treatment

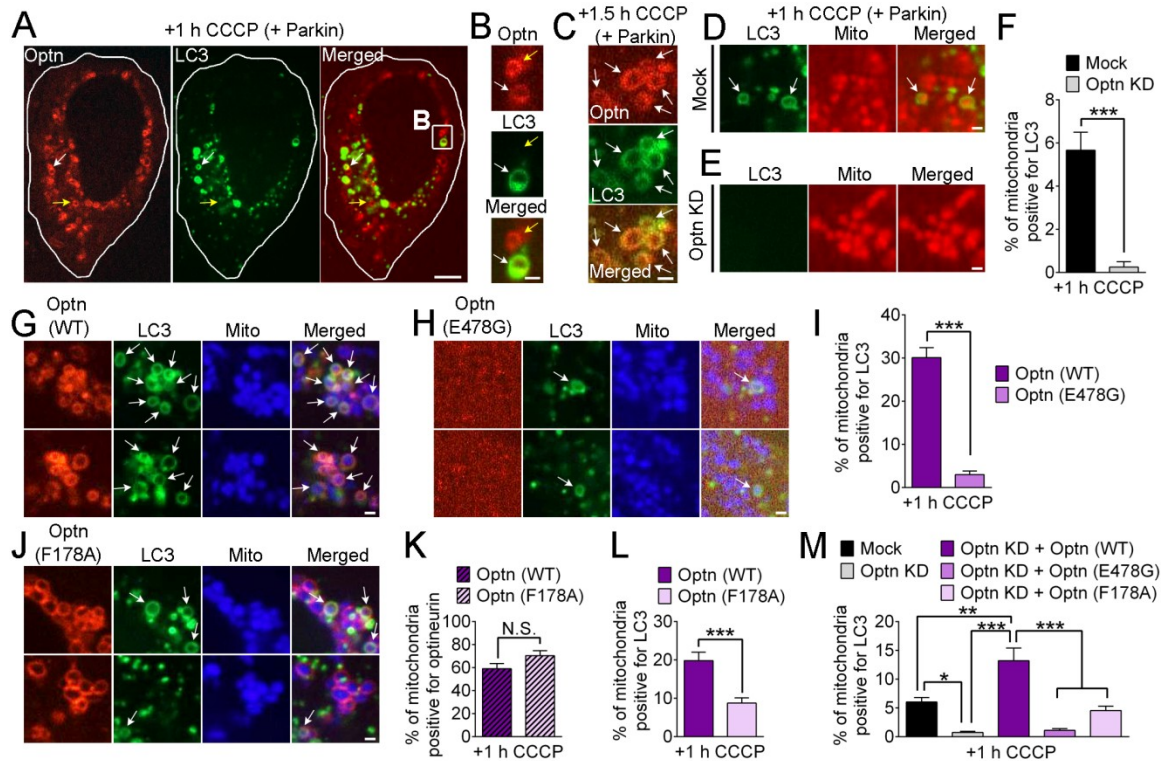


Figure 3.6: Optineurin recruits LC3 autophagosomes to damaged mitochondria via its LIR domain.

(A,B) Confocal images of a HeLa cell expressing parkin, mCherry-Optn and the autophagosome marker GFP-LC3 treated with CCCP for 1 h. Optineurin is recruited to the surface of damaged mitochondria via parkin expression. LC3 autophagosomes dynamically form around optineurin-labeled damaged mitochondria (white arrows). Not all optineurin-labeled mitochondria are LC3-positive at this time point (yellow arrows). (C) Increased recruitment of LC3 to optineurin-labeled mitochondria after 1.5 h CCCP treatment (white arrows). (D-F) HeLa cells expressing parkin, GFP-LC3 and sBFP-mito (pseudo-colored red). Formation of LC3 autophagosomes around damaged mitochondria (white arrows) in control cells with endogenous levels of optineurin (Mock) after 1 h CCCP treatment is inhibited by siRNA knockdown of endogenous optineurin (Optn KD). (G) HeLa cell expressing parkin, mCherry-Optn, GFP-LC3 and sBFP-mito after 1 h CCCP treatment showing accelerated LC3 autophagosome engulfment of optineurin-labeled mitochondria in cells with increased optineurin expression (white arrows). (H,I) Expression of ALS-associated ubiquitin-binding-deficient mCherry-Optn-E478G inhibits LC3 recruitment to mitochondria (white arrows). (J-L) Expression of LC3-binding-deficient mCherry-Optn-F178A which localizes to damaged mitochondria inhibits LC3 recruitment to mitochondria (white arrows). (M) Optineurin depletion disrupts LC3 autophagosome formation around mitochondria, and is rescued by siRNA-resistant wildtype optineurin, but not siRNA-resistant optineurin E478G or F178A. (Scale bars: A, 5 μ m; B- E, G-H and J, 1 μ m.) Values represent means $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001. N.S. (not significant).

was dramatically decreased by optineurin depletion (mock, $78.5 \pm 6.8\%$; optn KD, $11.8 \pm 4.3\%$, $p = 0.001$).

After 2 h CCCP treatment, optineurin-depleted cells still demonstrated a substantial lag in their ability to recruit LC3 to mitochondria, with less than 30% of cells containing any LC3-engulfed mitochondria. Even after 24 h CCCP treatment, recruitment of LC3 autophagosomes to damaged mitochondria in optineurin-depleted cells was still strikingly inhibited. These results demonstrate that optineurin plays a crucial role as an autophagy receptor in parkin-dependent mitophagy by recruiting LC3 autophagosomes to damaged mitochondria.

We next examined whether increased levels of optineurin could enhance recruitment of autophagosomes to mitochondria. We overexpressed wildtype optineurin in parkin-expressing HeLa cells and found that this induced a striking increase in the percentage of mitochondria engulfed by autophagosomes (Figure 3.6G). In contrast, expression of the ALS-associated optn-E478G mutant resulted in significantly slower rates of autophagosome formation, with only 3% of mitochondria engulfed after 1 h CCCP treatment (Figure 3.6H), as compared to ~30% of mitochondria in cells expressing wildtype optineurin (Figure 3.6I).

To test if optineurin recruited LC3 to damaged mitochondria via its LIR domain, we expressed an optineurin F178A LIR mutant unable to bind LC3 (Wild et al., 2011). Optineurin F178A was robustly recruited to damaged mitochondria after 1 h CCCP similar to wildtype optineurin (Figure 3.6J-K), but caused a defect in LC3 recruitment to mitochondria (Figure 3.6J, L), demonstrating that optineurin induces LC3 recruitment and autophagosome formation around mitochondria via its LIR domain.

Finally, we depleted optineurin using siRNA and tested whether we could rescue the defect of LC3 recruitment to damaged mitochondria by expressing siRNA-resistant optineurin in parkin-expressing HeLa cells. After 1 h CCCP, optineurin depletion decreased LC3 recruitment to damaged mitochondria; this defect was rescued by expression of the siRNA-resistant wildtype optineurin. In contrast, expression of either an siRNA-resistant optineurin E478G or F178A mutant were unable to rescue the percentage of LC3-engulfed mitochondria (Figure 3.6M). Taken

together, these results demonstrate that optineurin is an autophagy receptor for damaged mitochondria which is capable of recruiting autophagosomes to mitochondria.

3.2.7 Optineurin and p62 are independently recruited to different domains on damaged mitochondria and have distinct roles in mitophagy.

There are currently five known autophagy receptors which bind both ubiquitin and LC3: p62/SQSTM1, NBR1, NDP52, T6BP and optineurin (Kirkin et al., 2009; Pankiv et al., 2007; Thurston et al., 2009; Tumbarello et al., 2012; Wild et al., 2011). Among these proteins, only p62's role in parkin-mediated mitophagy has been investigated (Ding et al., 2010; Geisler et al., 2010; Narendra et al., 2010a; Okatsu et al., 2010), but this work has suggested conflicting roles for p62 as either an autophagy receptor (Ding et al., 2010; Geisler et al., 2010) or a regulator of perinuclear clustering of depolarized mitochondria (Narendra et al., 2010a; Okatsu et al., 2010). Thus, we asked whether optineurin and p62 act in the same pathway during parkin-dependent mitophagy.

In parkin-expressing cells, we found that optineurin was recruited to the entire surface of damaged mitochondria (Figure 3.7A), while p62 preferentially localized to domains between adjacent mitochondria (Figure 3.7B-C) as previously observed (Narendra et al., 2010a; Okatsu et al., 2010). Expression of p62 also accelerated the formation of mitochondrial aggregates after 1 h CCCP (Figure 3.7B). These results further support a role for p62 as a regulator of mitochondrial aggregation and clustering during parkin-dependent mitophagy (Narendra et al., 2010a; Okatsu et al., 2010).

Next, we examined whether p62 regulates optineurin recruitment to damaged mitochondria by using a p62 siRNA, which lead to >90% knockdown of endogenous p62 (Figure 3.S8B). We found that p62 depletion had no effect on optineurin recruitment to mitochondria damaged with 1 h CCCP (Figure 3.7D-E). We also found that p62 depletion by siRNA did not disrupt LC3 recruitment to damaged mitochondria after 1 h CCCP (Figure 3.7D,F), further

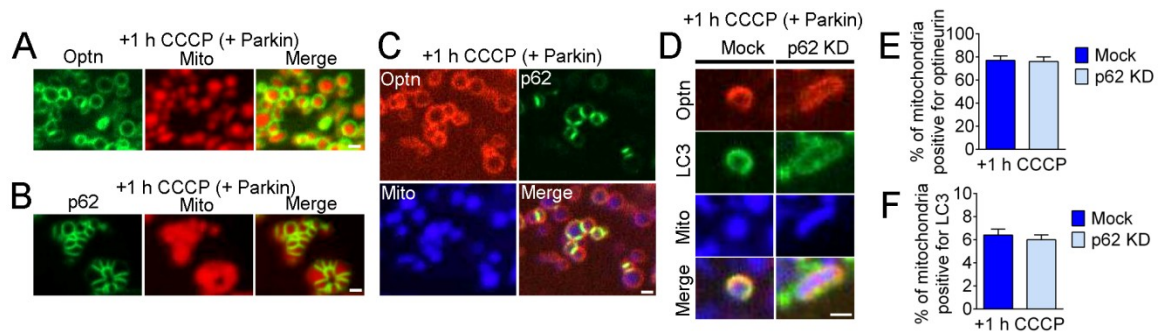


Figure 3.7: Optineurin and p62 localize to different domains on damaged mitochondria and have distinct roles in mitophagy.

(A) GFP-Optn is recruited to the entire surface of damaged mitochondria after 1 h CCCP in parkin-expressing HeLa cells. (B) GFP-p62 preferentially localizes to the domains between adjacent mitochondria and p62 expression accelerates mitochondrial aggregation after 1 h CCCP. (C) mCherry-Optn and GFP-p62 localize to different domains on damaged mitochondria. (D-F) p62 depletion by siRNA results in elongated mitochondria but does not inhibit optineurin or LC3 recruitment to mitochondria after 1 h CCCP (right panel, D). (Scale bars: A-D, 1 μ m.) Values represent means $\pm$ SEM.

suggesting that p62 acts as a regulator of mitochondrial aggregation rather than as an autophagy receptor during parkin-dependent mitophagy. Interestingly, although depletion of p62 resulted in more tubular mitochondria after 1 h CCCP (right panel, Figure 3.7D), both optineurin and LC3 were still recruited to these elongated mitochondria. We also examined whether optineurin might regulate p62 recruitment to damaged mitochondria. In cells depleted of optineurin, p62 was still recruited to damaged mitochondria (Figure 3.S8C), and still preferentially localized between adjacent mitochondria (Figure 3.S8D-E). Thus, our results suggest that while optineurin is an autophagy receptor in mitophagy recruiting LC3 to damaged mitochondria, p62 regulates mitochondrial aggregation following mitochondrial damage. Moreover, optineurin and p62 are recruited independently to separate domains on damaged mitochondria, potentially facilitating their distinct roles during parkin-dependent mitophagy.

3.2.8 The autophagy receptor optineurin is necessary for efficient mitochondrial degradation in parkin-mediated mitophagy.

To examine whether autophagosome recruitment and engulfment of damaged mitochondria leads to mitochondrial degradation, we analyzed levels of the mitochondrial matrix

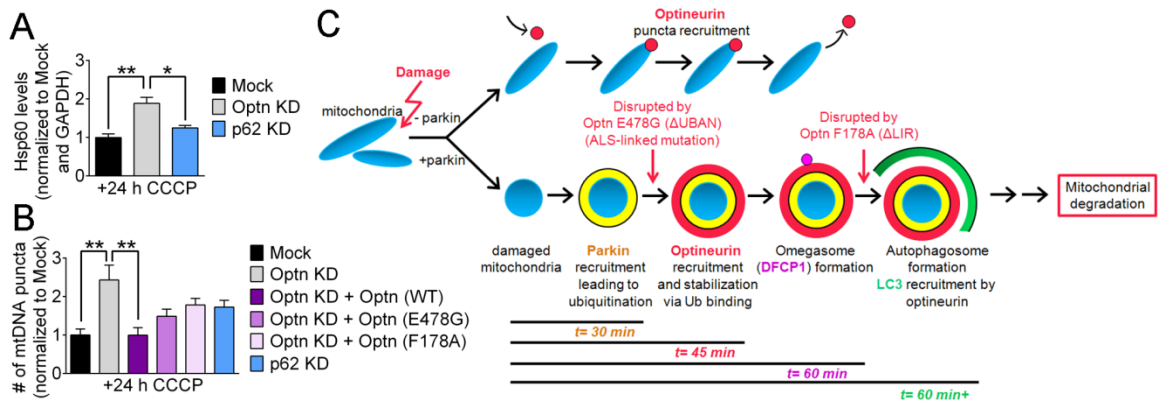


Figure 3.8: Optineurin is an autophagy receptor for damaged mitochondria and regulates mitochondrial degradation.

(A) Quantification of Hsp60 by immunoblot after 24 h CCCP in parkin-expressing HeLa cells normalized to control cells and loading control GAPDH. Optineurin siRNA knockdown, but not p62 siRNA knockdown, leads to increased Hsp60 mitochondrial matrix protein content, indicating inefficient mitochondrial degradation. (B) Quantification of mtDNA by immunofluorescence after 24 h CCCP in parkin-expressing HeLa cells. Optineurin siRNA knockdown, but not p62 siRNA knockdown, results in the accumulation of inefficiently degraded mitochondria. siRNA-resistant wildtype optineurin, but not siRNA-resistant optineurin E478G or F178A, rescues efficient clearance of damaged mitochondria. (C) Model: In the absence of parkin, optineurin puncta transiently localize to the tips of damaged mitochondria but do not remain stably associated. In the presence of parkin, parkin is first recruited to the outer membrane of damaged mitochondria, followed by optineurin recruitment via its UBAN domain to parkin-ubiquitinated mitochondria. Next, the omegasome protein DFCP1 is transiently recruited to optineurin-labeled mitochondria, marking the initial site of autophagosome formation. Optineurin then recruits LC3 to mitochondria via its LIR domain leading to autophagosome engulfment and mitochondrial degradation. A mutation in the UBAN domain (ALS-associated E478G) disrupts optineurin recruitment while a mutation in the LIR domain (F178A) disrupts LC3 recruitment. Time line (bottom) indicates approximate half-time for each step of the parkin-optineurin-DFCP1-LC3 pathway after induced mitochondrial damage. Values represent means $\pm$ SEM. *P < 0.05; **P < 0.01.

protein Hsp60 by immunoblot as a marker of mitochondrial protein content. We found that in parkin-expressing cells treated with 24 h CCCP, siRNA depletion of optineurin induced a significant increase in Hsp60 levels (normalized to loading control) compared to control cells (Figure 3.8A and Figure 3.S9A). In contrast, siRNA depletion of p62 did not induce a significant increase in Hsp60 levels relative to control cells. We also observed a striking increase in the levels of the outer mitochondrial membrane protein Tom20 upon optineurin depletion, but not upon p62 depletion (Figure 3.S9A). Next, we immunostained for mitochondrial DNA (mtDNA) as another measure of mitochondrial degradation. After 24 h CCCP, the majority of mitochondria in parkin-expressing cells were degraded, with only a few mitochondria remaining in each cell

(Figure 3.S9B). Again, siRNA depletion of optineurin led to a striking increase in the number of mitochondria remaining, indicating defective mitochondrial degradation (optn KD, 2.4 ± 0.4 fold of mitochondria in control cells, $p < 0.01$) (Figure 3.8B and Figure 3.S9C). This defect in mitochondrial degradation was rescued by expressing an siRNA-resistant wildtype optineurin (Figure 3.8B and Figure 3.S9D). However, efficient mitochondrial degradation was not rescued by either an siRNA-resistant optineurin E478G ALS-linked UBAN mutant, or an F178A LIR mutant (Figure 3.8B and Figure 3.S9E-F). In addition, p62 siRNA-depletion did not significantly increase the number of mitochondria remaining (Figure 3.8B and Figure 3.S9G). Together, these data further suggest that p62 is not a required receptor for the mitophagy of damaged mitochondria. In contrast, optineurin functions as an autophagy receptor for damaged mitochondria by binding to both ubiquitin and LC3, and this is critical for efficient mitochondrial clearance via mitophagy.

3.3 Discussion

In this study, we used live cell microscopy to image the dynamics of the parkin-optn-DFCP1-LC3 pathway for mitophagy. We found that optineurin recruitment to damaged mitochondria occurs subsequent to parkin recruitment but prior to autophagosome formation. The precise timing of optineurin's recruitment to mitochondria suggests a key role in linking parkin activity to the induction of autophagosome formation during mitophagy. Optineurin is recruited to parkin-ubiquitinated mitochondria and is stabilized on damaged mitochondria by its UBA domain. Optineurin subsequently recruits LC3 via its LIR domain, resulting in autophagosome formation around mitochondria (see model, Figure 3.8C). Disruption of either of these steps leads to inefficient autophagic engulfment of mitochondria and the accumulation of damaged mitochondria within the cell.

Previous studies have shown that early autophagic machinery, including the omegasome marker DFCP1 (Axe et al., 2008; Hayashi-Nishino et al., 2009), is recruited independently and prior to LC3 during mitophagy (Itakura et al., 2012). We found that although DFCP1 was recruited to damaged mitochondria independent of optineurin, DFCP1 recruitment temporally follows optineurin recruitment. Thus, optineurin might also play additional roles in regulating proteins involved in autophagosome formation prior to LC3 assembly. Interestingly, optineurin binds both huntingtin and myosin VI (Faber et al., 1998; Sahlender et al., 2005), two proteins recently shown to regulate autophagosome motility (Wong and Holzbaur, 2014a) and maturation (Tumbarello et al., 2012), further suggesting a scaffolding role for optineurin in autophagosome formation and dynamics.

There are currently five known autophagy receptors which bind both ubiquitin and LC3: p62/SQSTM1, NBR1, NDP52, T6BP and optineurin (Kirkin et al., 2009; Pankiv et al., 2007; Thurston et al., 2009; Tumbarello et al., 2012; Wild et al., 2011). Here, we examined the interaction between two of these receptors: p62 and optineurin. We found that p62 and optineurin are independently recruited to different domains on damaged mitochondria, thereby allowing them to perform distinct functions during mitophagy. p62 preferentially localized to domains

between adjacent mitochondria and accelerated mitochondrial aggregation, but did not regulate LC3 recruitment to mitochondria. These results are consistent with previous reports showing that p62 is not an autophagy receptor for damaged mitochondria but rather, clusters damaged mitochondria via its PB1 oligomerization domain (Narendra et al., 2010a; Okatsu et al., 2010). In contrast, we found that optineurin uniformly localized across the surface of damaged mitochondria and serves as a robust autophagy receptor for damaged mitochondria. Optineurin expression accelerated LC3 recruitment and facilitated autophagic engulfment and degradation of damaged mitochondria. Interestingly, p62 and optineurin have also been observed to localize to separate subdomains on ubiquitinated *Salmonella* (Wild et al., 2011), suggesting that p62 and optineurin may also play distinct roles in other types of selective autophagy. Since optineurin and NDP52 were found to localize to common subdomains on ubiquitinated *Salmonella* (Wild et al., 2011), it will be interesting to examine whether these other autophagy receptors have similar roles to optineurin in recruiting autophagic machinery to mitochondria during parkin-mediated mitophagy, which would provide further insight as to whether there are overlapping roles for autophagy receptors during selective autophagy.

Efficient turnover of damaged mitochondria is essential for maintaining cellular homeostasis, particularly in neurons, which have high energy demands (Itoh et al., 2013). PINK1 and parkin, proteins linked with neurodegeneration in Parkinson's disease (Kitada et al., 1998; Valente et al., 2004), are key players in regulating mitochondrial degradation via mitophagy (Wang and Klionsky, 2011; Youle and Narendra, 2011). Here, we show that optineurin, mutations in which are causative for diseases including glaucoma and ALS (Albagha et al., 2010; Maruyama et al., 2010; Rezaie et al., 2002), is recruited to damaged mitochondria downstream of PINK1 and parkin, but upstream of autophagosome formation and engulfment. Importantly, we find that an ALS-associated optineurin UBAN mutant is unable to stably associate with damaged mitochondria, leading to a reduced rate of autophagic engulfment and degradation of damaged mitochondria. Interestingly, ALS-associated valosin-containing protein (VCP) was also recently

implicated in PINK/parkin mitophagy (Kim et al., 2013). Thus, our results further highlight a potential role for mitophagy defects in ALS that may contribute to motor neuron death.

3.4 Materials and Methods

Reagents. Constructs used include: DsRed2-mito (gift from T. Schwarz, Harvard Medical School, Boston, MA) recloned into pSBFP2-C1 (Addgene), pEGFP-OPTN and HA-OPTN E478G (gifts from I. Dikic, Goethe University, Frankfurt, Germany) recloned into pmCherry (Takara Bio Inc.) and pEGFP, YFP-parkin and mCherry-parkin (gifts from R. Youle, National Institute of Health, Bethesda, MD) recloned into pSBFP2-C1 (Addgene) and an untagged parkin construct, DFCP1 (Addgene) recloned into pEGFP, pEGFP-LC3 (gift from T. Yoshimori, Osaka University, Osaka, Japan), GFP-p62 (Addgene) and pKillerRed-dMito (Evrogen). We generated mutant mCherry-parkin T240R and mCherry-OPTN F178A constructs by site-directed mutagenesis. We also generated siRNA-resistant mCherry-OPTN wildtype, E478G and F178A constructs against the Optn siRNA described below. Antibodies used were against optineurin (ab23666, Abcam), tubulin (T9026, Sigma), β -catenin (610154, BD Biosciences), p62 (ab56416, Abcam), Hsp60 (SPA-806, Enzo Life Sciences), GAPDH (ab9484, Abcam), Tom20 (sc-11415, Santa Cruz), (DNA (61014, Progen) and an Alexa fluorophore-conjugated secondary antibody from Molecular Probes (Invitrogen). The siRNA to optineurin (5'-CCACCAGCTGAAAGAAGCC-3') (Sahlender et al., 2005) and to p62 (5'-GCATTGAAGTTGATATCGAT-3') (Pankiv et al., 2007) were obtained from Dharmacon (Thermo Scientific).

Cell Culture and Transfections. HeLa cells were cultured in DMEM medium (Corning) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C in a 5% CO₂ incubator. Cells were transfected using FuGENE 6 (Promega). CCCP (Sigma-Aldrich) was given at 20 μ M. Knockdown experiments were performed with 60nM siRNA using Lipofectamine RNAiMAX (Invitrogen). For knockdown and rescue experiments, cells were transfected with siRNA-resistant constructs 1 day after siRNA expression. For immunofluorescence analysis, cells were plated on coverslips and fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Fixed cells were incubated in primary antibody for 1 hr, washed 3 $\times$ 5 min, incubated in secondary antibody for 1 hr, washed 3 $\times$ 5 min and mounted on glass slides with ProLong gold (Invitrogen). For immunoblot analysis, cells were lysed and

analyzed by SDS-PAGE and Western blot according to standard protocols. Tubulin, β -catenin and GAPDH were used as loading controls for their varying molecular weights.

Live Cell Imaging and Analysis. All images were acquired on a spinning-disk confocal (UltraVIEW VoX; PerkinElmer) on a Nikon Eclipse Ti microscope using an Apochromat 100 $\times$ 1.49 NA oil immersion objective (Nikon) in a temperature-controlled chamber (37°C). Digital images were acquired with an EM charge-coupled device camera (C9100; Hamamatsu Photonics) using Volocity software (PerkinElmer) at 1 frame every 1-30 sec or 1 frame/min. Dual-color videos were acquired as consecutive green-red images, and tri-color videos were acquired as consecutive green-red-blue images. To induce spatiotemporally-regulated mitophagy, we photobleached a region of a HeLa cell expressing pKillerRed-dMito with a 561-nm laser at 100% for 100 iterations. HeLa cells were transferred into phenol-red-free medium (Invitrogen; supplemented with fetal bovine serum and penicillin/streptomycin) for live cell imaging. Line scans were generated using ImageJ (NIH) and normalized per protein and per condition. In linescans, dashed lines and arrows highlight peaks of protein fluorescent intensity. Cell and nucleus outlines were drawn using Photoshop (Adobe). Immunoblots were quantified using ImageJ (NIH). Anti-DNA preferentially stained mitochondrial DNA in HeLa cells as previously noted (Legros et al., 2004) and mtDNA nucleoids were counted per cell from a single confocal image. For each condition, >800 mtDNA puncta were counted from $n \geq 9$ cells (N=3 experiments). All images were assembled using ImageJ (NIH) and Photoshop (Adobe). Statistics and graphing were performed using Prism (GraphPad) software. Data were analyzed using unpaired two-tailed Student's t test (two data sets) or one-way ANOVA with Tukey's post-hoc test (multiple data sets).

3.5 Supplemental Information

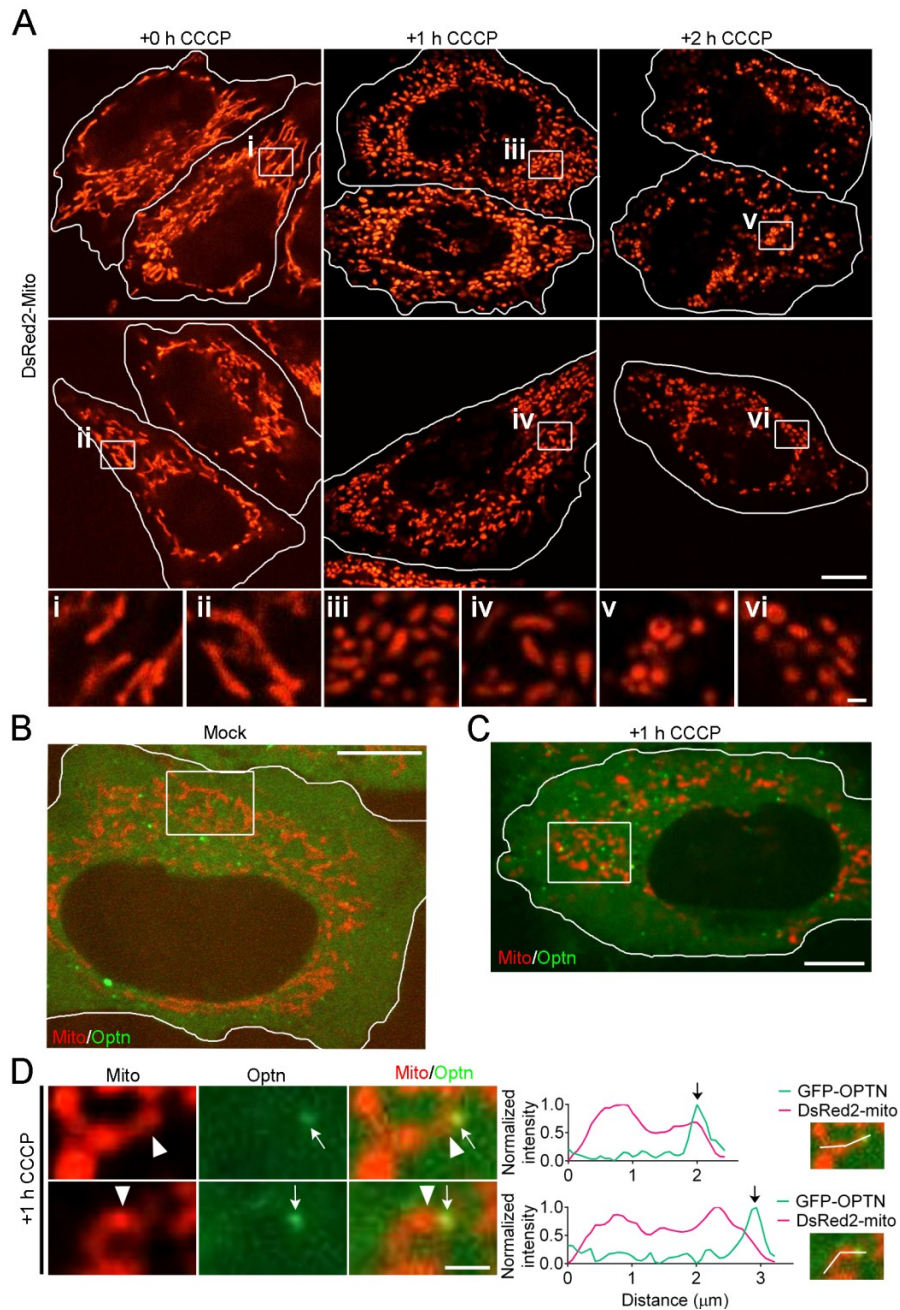


Figure 3.S 1: Optineurin puncta localize to tips of mitochondrial fragments in the absence of parkin.

(A) CCCP treatment of HeLa cells expressing DsRed2-mito results in mitochondrial fragmentation. (B) Non-CCCP treated HeLa cells (Mock) expressing DsRed2-mito and GFP-optn. Optineurin puncta do not associate with mitochondria. Boxed area corresponds to Figure 3.1A (top). (C) In CCCP-treated cells (20 μM), optineurin puncta are transiently recruited to tips of fragmented mitochondria. Boxed area corresponds to Figure 3.1A (bottom). (D) Examples of optineurin puncta (white arrows) localized to the tip of fragmented mitochondria (arrowheads). Corresponding line scans show optineurin's localization to mitochondrial tip. (Scale bars: A-C, 10 μm ; Inset in A, D, 1 μm .)

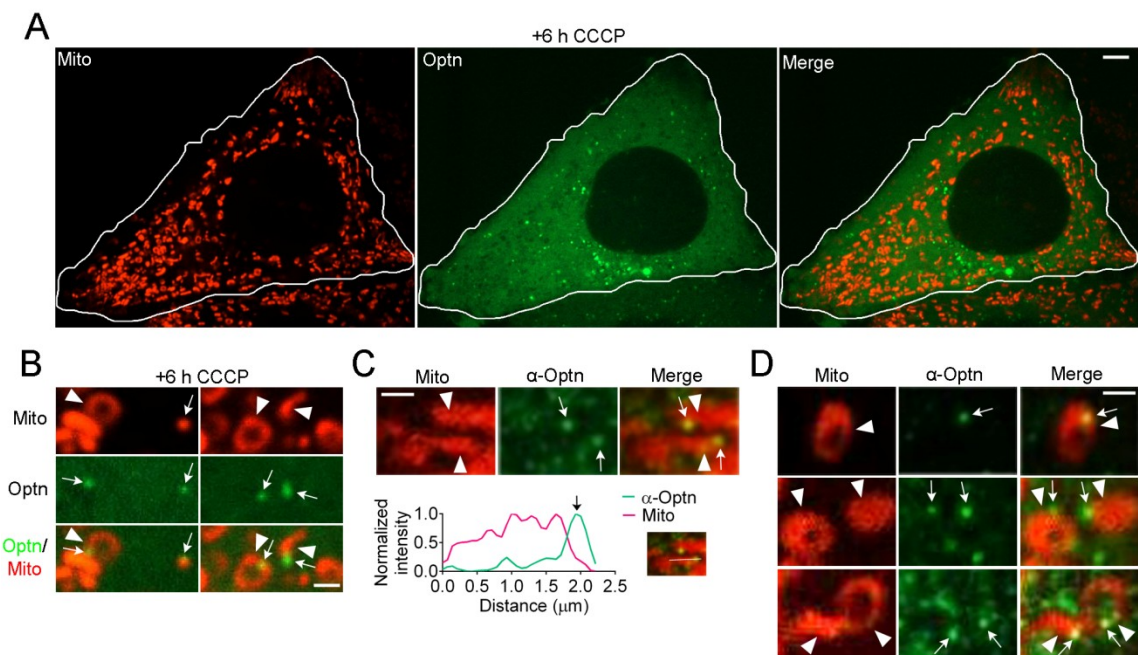


Figure 3.S 2: Optineurin localization to mitochondria after prolonged damage in the absence of parkin.

(A) HeLa cell expressing DsRed2-mito and GFP-Optn treated with CCCP for 6 h, causing severe mitochondrial damage resulting in fragmented and circular mitochondria. (B) Examples of optineurin puncta (white arrows) localized to a singular point on circular mitochondria (arrowheads) and to the tips of fragmented mitochondrial puncta (white arrows) after 6 h CCCP treatment. (C) Immunostaining of endogenous optineurin in HeLa cells treated with 1 h CCCP showing optineurin puncta localization (white arrows) to the tip of damaged mitochondria (arrowheads). Corresponding linescan shows optineurin's localization to mitochondrial tip. (D) Examples of immunostaining of endogenous optineurin in CCCP-treated HeLa cells showing optineurin puncta (white arrows) localized to a singular point on circular damaged mitochondria (arrowheads). (Scale bars: A, 5 μ m; B-D, 1 μ m.)

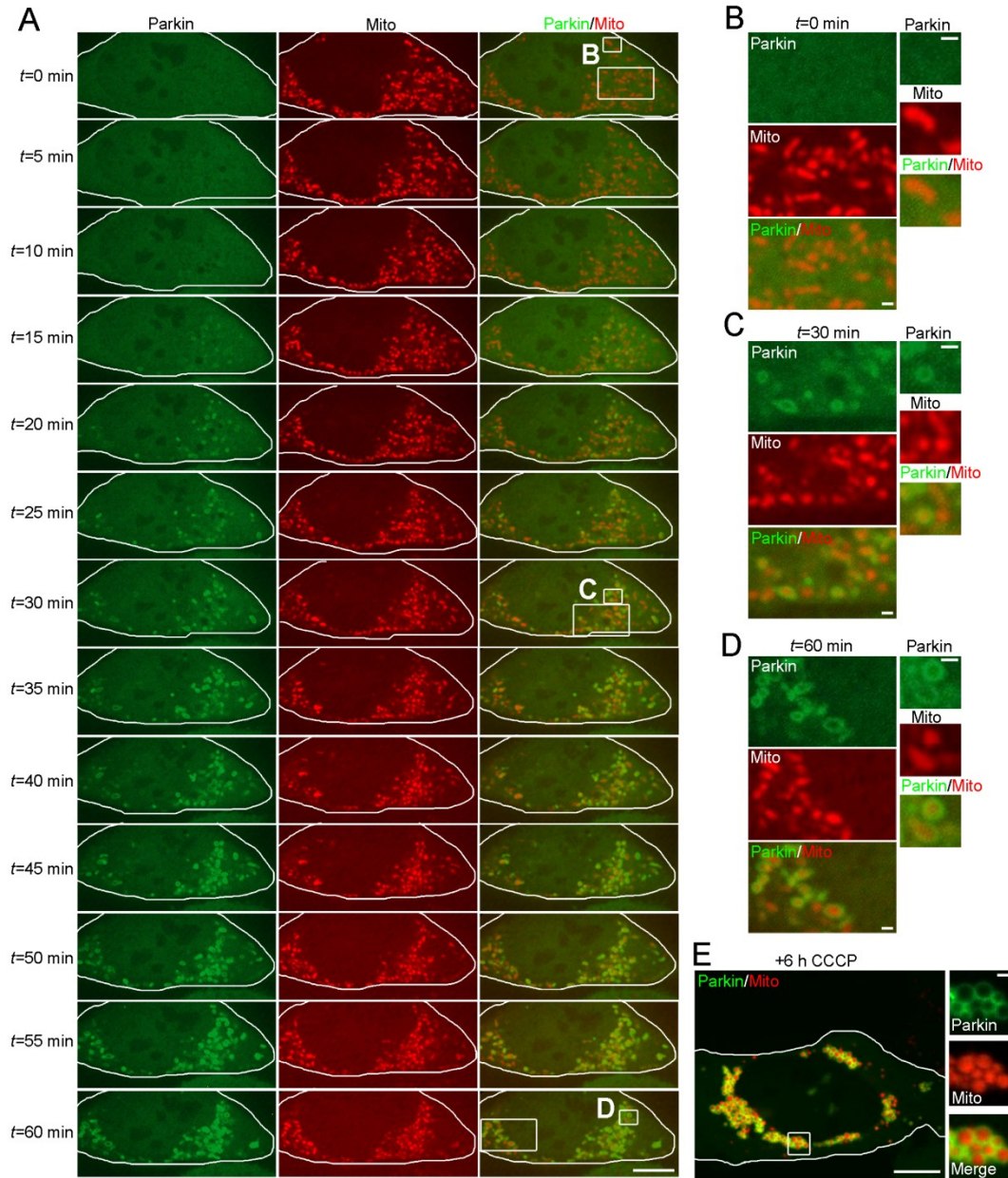


Figure 3.S 3: Dynamics of parkin recruitment to damaged mitochondria.

(A) Confocal time series of a HeLa cell expressing DsRed2-mito and YFP-parkin treated with CCCP over 1 h, showing gradual parkin recruitment to the surface of damaged mitochondria. (B) Magnified view of boxed areas in (A) ($t=0$ min). Parkin is cytosolic and not recruited to mitochondria which have not yet been damaged. (C) Magnified view of boxed areas in (A) ($t=30$ min). Parkin is gradually recruited to the outer membrane of damaged mitochondria, visualized as parkin rings forming around spherical mitochondrial fragments within a single z plane using confocal microscopy. (D) Magnified view of boxed areas in (A) ($t=60$ min). Parkin is recruited to the majority of damaged mitochondria. (E) 6 h CCCP treatment causes perinuclear localization and complete aggregation of all mitochondria while parkin remains stably associated. Images (right) show higher magnification of boxed area. (Scale bars: A and E, 10 μm ; B-D and Inset in E, 1 μm .)

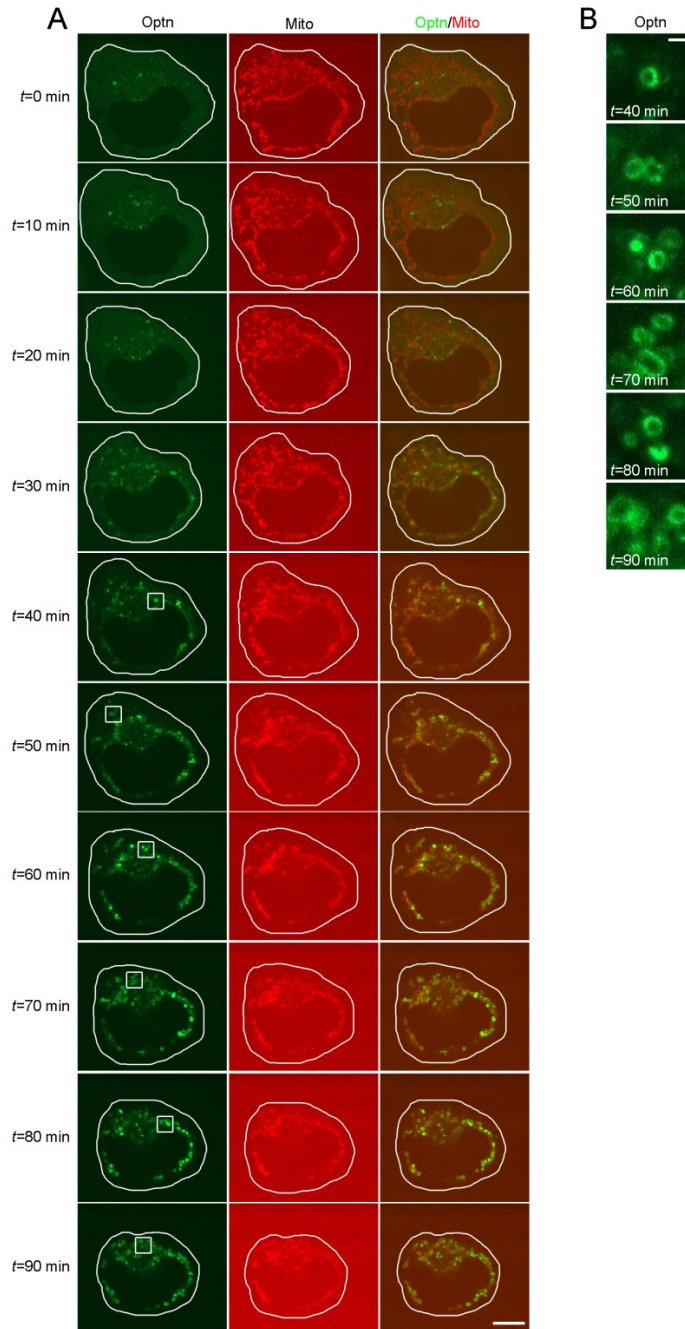


Figure 3.S 4: Dynamics of parkin-dependent optineurin recruitment and stabilization on the surface of damaged mitochondria.

(A) Confocal time series of a HeLa cell expressing parkin and sBFP-mito (pseudocolored red) treated with CCCP over 1.5 h, showing gradual optineurin recruitment to the surface of damaged mitochondria. (B) Magnified view of boxed areas in (A). Optineurin is gradually recruited to the outer membrane of damaged mitochondria, resulting in optineurin rings forming around mitochondrial fragments within a single z plane using confocal microscopy. (Scale bars: A, 10 μm ; B, 1 μm .)

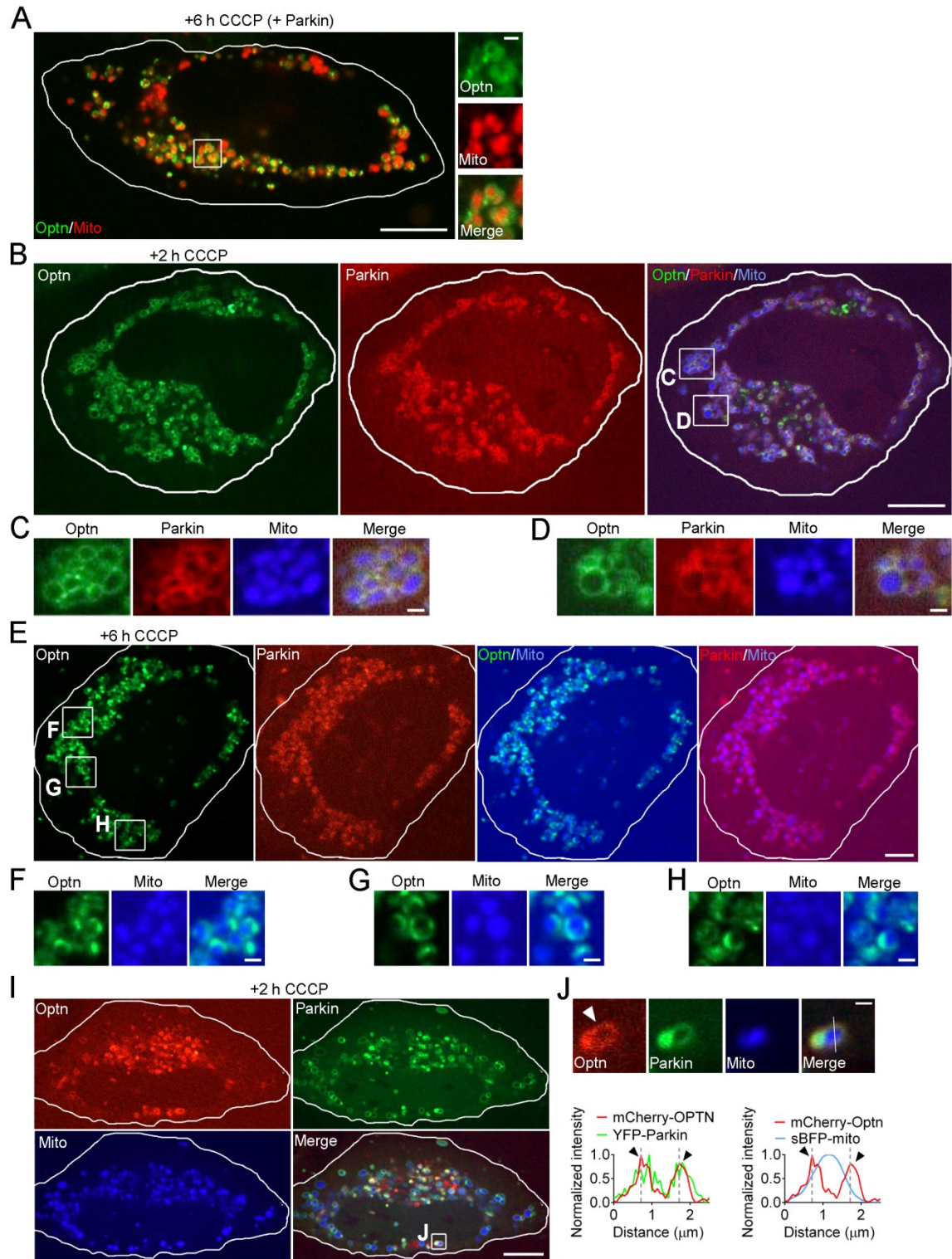


Figure 3.S 5: Optineurin localization on the outer membrane of parkin-labeled damaged mitochondria.

(A) Optineurin is preferentially localized to distinct patches on the surface of damaged mitochondria following 6 h CCCP treatment. (B) Confocal image of a HeLa cell expressing GFP-Optn, mCherry-parkin and sBFP-mito. Both parkin and optineurin are recruited to the majority of mitochondria following 2 h CCCP treatment. (C,D) Magnified images of boxed areas in (B). (E) Confocal image of a HeLa cell expressing GFP-Optn, mCherry-parkin and sBFP-mito showing that both parkin and optineurin remain localized to the mitochondrial surface even after 6 h CCCP treatment. (F-H) Magnified images of boxed areas in (E). Optineurin is preferentially localized to distinct patches on the surface of damaged mitochondria following 6 h CCCP treatment. (I) Confocal image of a HeLa cell expressing mCherry-Optn, YFP-parkin and sBFP-mito after 2 h CCCP treatment, showing that optineurin and parkin are localized to the mitochondrial surface (irrespective of their fluorescent tag). (J) Magnified image of boxed area in (I). Corresponding linescans show that both optineurin and parkin localize on the outer surface of damaged mitochondria. (Scale bars: A, 10 μ m; B, E, and I, 5 μ m; Inset in A, C, D, F-H, and J, 1 μ m.)

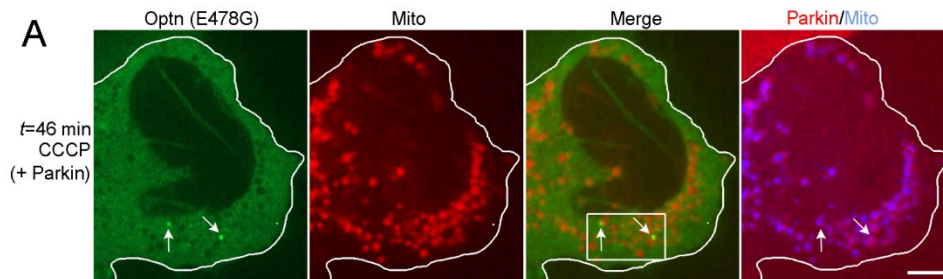


Figure 3.S 6: ALS-linked optineurin E478G does not stably associate with parkin-labeled damaged mitochondria.

(A) Confocal image of HeLa cells expressing sBFP-mito, mCherry-parkin and GFP-Optn-E478G treated with CCCP. Optn-E478G remains cytosolic, with a few puncta localized to damaged mitochondria (pseudo-colored red) (white arrows). Parkin is still recruited to damaged mitochondria (right panel). Boxed area corresponds to Figure 3.4B time lapse image. (Scale bar: A, 5 μ m).

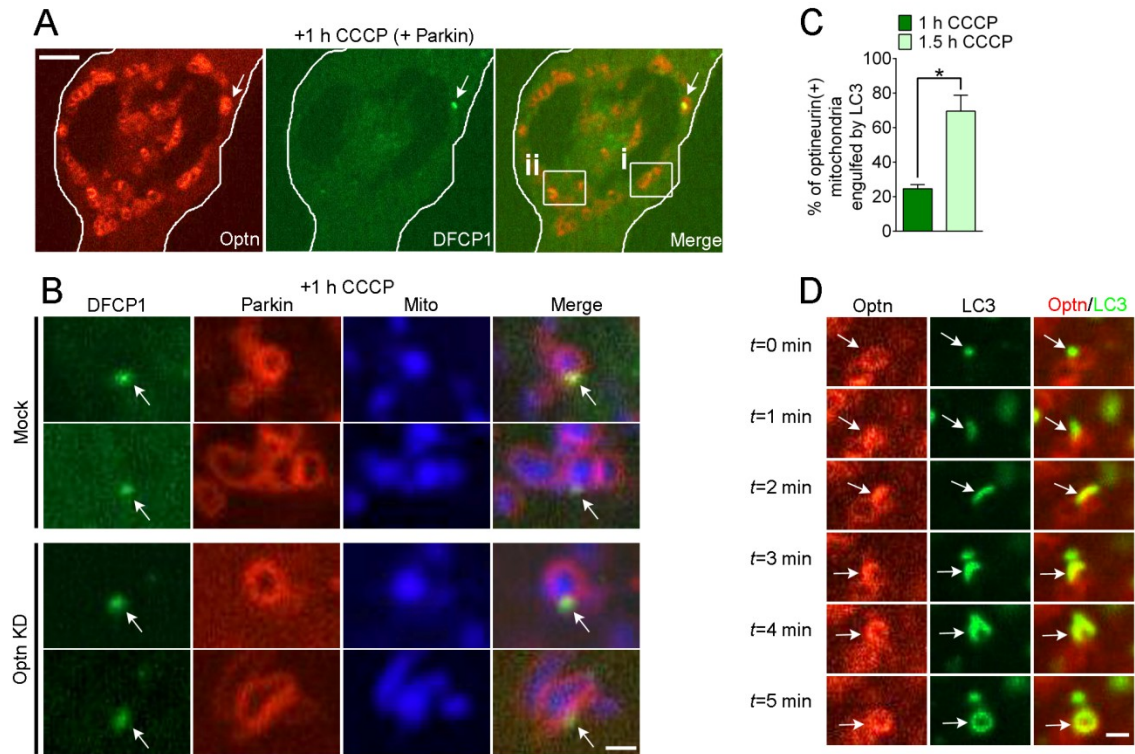


Figure 3.S 7: Optineurin-independent recruitment of DFCP1 and subsequent autophagosome formation around optineurin-labeled damaged mitochondria.

(A) Confocal image of HeLa cell expressing parkin, mCherry-Optn and the omegasome marker GFP-DFCP1 treated with CCCP for 1 h. Optineurin is recruited to the surface of damaged mitochondria, seen as optineurin rings within a single z plane. DFCP1 puncta are dynamically recruited to optineurin-labeled mitochondria (white arrow). Boxed area i corresponds to Figure 3.5C. Boxed area ii corresponds to Figure 3.5D. (B) GFP-DFCP1 (white arrows) is transiently recruited to parkin-labeled mitochondria after 1 h CCCP even after optineurin siRNA depletion. (C) Quantification of LC3 recruitment to optineurin-labeled mitochondria. (D) Confocal time series images from a HeLa cell expressing parkin, mCherry-Optn and the autophagosome marker GFP-LC3 treated with CCCP for 1 h, showing example of autophagosome formation. An LC3 puncta localizes to the side of an optineurin-labeled mitochondria and gradually forms a ring that surrounds an optineurin-labeled mitochondria (white arrows). (Scale bars: A, 5 μ m; B, D, 1 μ m). Values represent means $\pm$ SEM. *P < 0.05.

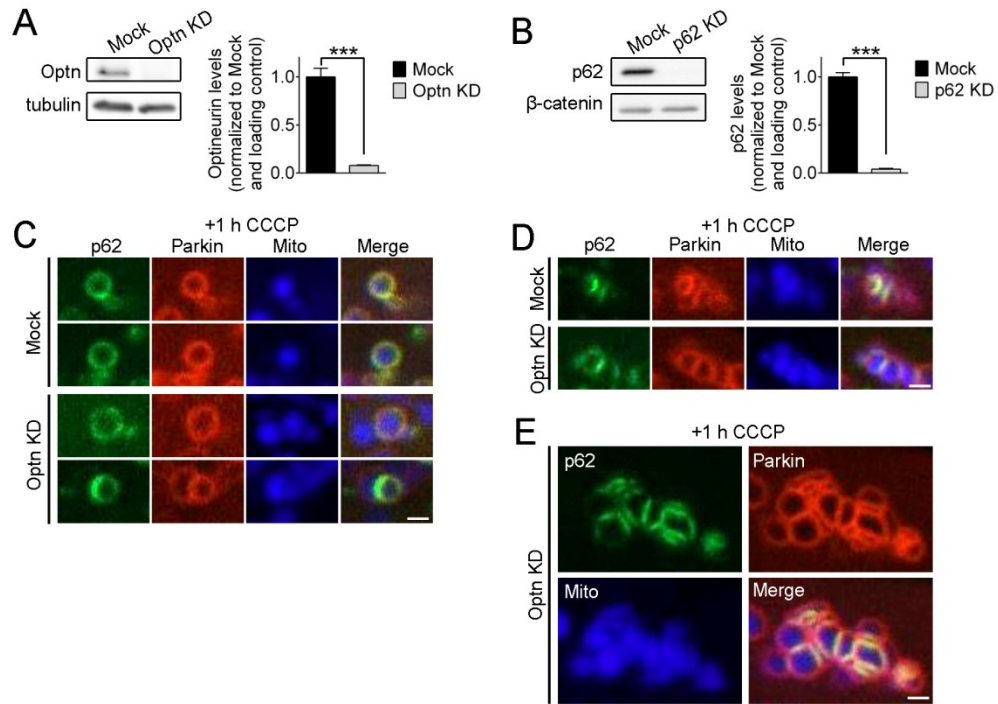


Figure 3.S 8: Optineurin does not regulate p62 recruitment to damaged mitochondria.

(A) Optineurin knockdown by siRNA (Optn KD) leads to efficient depletion of endogenous optineurin in HeLa cells as seen by immunoblot (Quantification on right). (B) p62 knockdown by siRNA (p62 KD) leads to efficient depletion of endogenous p62 in HeLa cells as seen by immunoblot (Quantification on right). (C) GFP-p62 is recruited to parkin-labeled damaged mitochondria after 1 h CCCP even after optineurin siRNA depletion. (D-E) GFP-p62 preferentially localizes to the domain between adjacent aggregated mitochondria after 1 h CCCP even after optineurin siRNA depletion. (Scale bar: C-E, 1 μ m.) Values represent means $\pm$ SEM. *** $P < 0.001$.

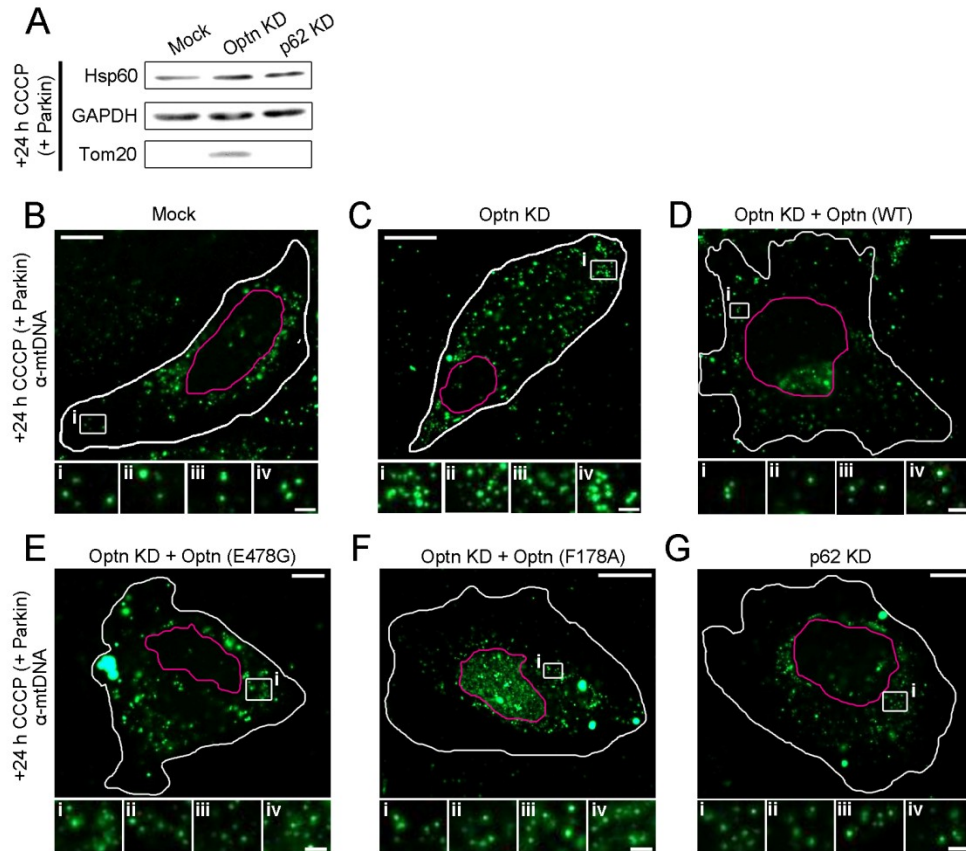


Figure 3.S 9: Optineurin regulates efficient mitochondrial degradation via its UBAN and LIR domains.

(A) Immunoblot of mitochondrial matrix protein Hsp60, mitochondrial outer membrane protein Tom20 and cytosolic protein loading control GAPDH after 24 h CCCP in parkin-expressing HeLa cells. Optineurin siRNA depletion leads to a significant increase in levels of Hsp60 and Tom20 relative to loading control, while p62 siRNA depletion does not induce a significant increase in levels of Hsp60 or Tom20 relative to loading control. (B-G) Confocal images of immunofluorescence staining of mitochondrial DNA in parkin-expressing HeLa cells after 24 h CCCP. Mitochondria are not efficiently degraded upon optineurin siRNA depletion after 24 h CCCP (C). In cells depleted of endogenous optineurin by siRNA, expression of siRNA-resistant wildtype optineurin is able to efficiently clear damaged mitochondria after 24 h CCCP (D). Expression of siRNA-resistant optineurin E478G UBAN mutant (E) or F178A LIR mutant (F) does not result in efficient clearance of damaged mitochondria after 24 h CCCP. p62 depletion by siRNA does not significantly inhibit mitochondrial degradation. Bottom: High-magnification of mitochondrial DNA puncta from different cells (i corresponds to inset from low-magnification cell image). Nuclei are outlined in pink. (Scale bars: B-G, 5 μ m; Insets in B-G, 1 μ m.)

4 AUTOPHAGOSOME DYNAMICS IN NEURODEGENERATION

This chapter was written by Yvette Wong and Erika Holzbaur and was published as a review in the *Journal of Cell Science* (Wong and Holzbaur, 2015).

4.1 Introduction

Autophagy is a cellular degradation process in which cytosolic cargo are degraded by lysosomes, and can be divided into three subtypes: microautophagy, chaperone-mediated autophagy and macroautophagy. In microautophagy, cytosolic cargo are directly engulfed by the lysosome through invagination of the lysosomal membrane (Li et al., 2012), while in chaperone-mediated autophagy, cargo are selectively recognized by cytosolic chaperones which deliver them to a translocation complex on the lysosomal membrane (Cuervo and Wong, 2014).

In contrast, macroautophagy (hereafter referred to as autophagy) involves the formation of the autophagosome, a double-membrane organelle, which forms around cytosolic cargo and subsequently degrades its cargo by lysosomal fusion (Yang and Klionsky, 2010). In addition to non-selective cargo degradation during cellular stress, autophagosomes can selectively degrade protein aggregates, mitochondria (mitophagy), endoplasmic reticulum (ER-phagy) and peroxisomes (pexophagy) (Rogov et al., 2014). Selective autophagy is mediated by autophagy receptors which preferentially bind ubiquitinated organelles or other cargos, and subsequently recruit the autophagosome protein light chain 3 (LC3) via their LC3-interaction region (LIR) motif (Stolz et al., 2014; Wild et al., 2014).

There is increasing evidence implicating defective autophagy in neurodegeneration (Harris and Rubinsztein, 2012; Nixon, 2013; Wong and Cuervo, 2010). However, the unique dynamics of autophagosomes in neurons have only recently been studied using live cell imaging, revealing a preferential formation of autophagosomes at the axon tip and their subsequent retrograde axonal transport towards the cell body (Lee et al., 2011; Maday et al., 2012). These studies provide a new model for studying neuronal autophagy and its potential defects during neurodegeneration.

This chapter will summarize recent work on the autophagy pathway in neurons and the defects observed at each step of the process in ALS, Alzheimer's, Parkinson's and Huntington's disease. These steps include autophagosome formation at the axon tip, cargo engulfment of

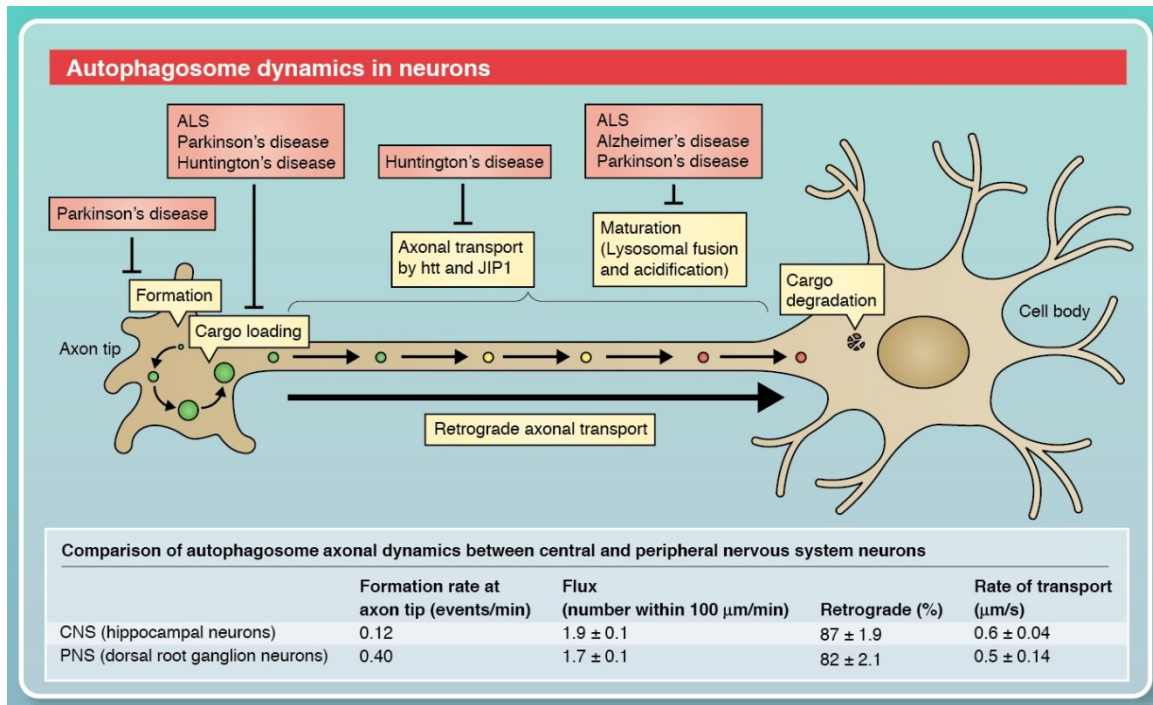


Figure 4.1: Defective autophagosome dynamics in neurodegenerative disease.

protein aggregates and damaged mitochondria (mitophagy), axonal transport of autophagosomes, lysosomal fusion and cargo degradation (Figure 4.1).

4.2 Autophagy is an essential homeostatic pathway in neurons

Like other cell types, neurons accumulate protein aggregates and damaged organelles such as mitochondria that must be degraded via autophagy to maintain cellular homeostasis. However, neurons are post-mitotic cells that are highly polarized with both dendritic and axonal compartments, which can extend over distances many times greater than their cell soma. Consequently, neurons require motor proteins to actively supply proteins and organelles to these distal processes, and to drive the transport of signaling molecules and degradative compartments back to the cell body. Thus, due to the maintenance of active electrochemical signaling and elaborate morphologies, neurons have high energetic demands. They require efficient recycling of proteins and organelles both at the synapse, as well as throughout the axon, dendrites and cell

soma, making autophagic degradation particularly crucial in neurons. Autophagy is also required for neuronal development and the maintenance of axonal homeostasis (Fimia et al., 2007; Komatsu et al., 2007; Wang et al., 2006).

Not surprisingly, defective autophagy induces protein aggregation and neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). While autophagy is efficient in younger neurons (Boland et al., 2008), autophagy proteins such as Beclin-1, Atg5 and Atg7 decline with age (Lipinski et al., 2010; Shibata et al., 2006), potentially contributing to the late onset of many neurodegenerative diseases (Rubinsztein et al., 2011).

4.3 Autophagosome biogenesis in neurons

Neurons exhibit robust constitutive autophagy, with autophagosomes forming preferentially at the axon tip (Maday et al., 2012). This observation suggests that autophagy is required for protein and organelle turnover distally, perhaps to balance the net flux of proteins and organelles arriving via anterograde transport (Maday et al., 2014). Distal biogenesis may also be coupled to synaptic function. There is also evidence that autophagosome formation can be induced along the axon in order to clear damaged mitochondria (Ashrafi et al., 2014).

Autophagosome biogenesis at the axon tip begins with the dynamic recruitment of Atg13 and Atg5 to double-FYVE containing protein 1 (DFCP-1) omegasomes, a phosphatidylinositol 3-phosphate (PI3P)-enriched omega-shaped ER structure that serves as a platform for autophagosome biogenesis (Maday and Holzbaur, 2014). This recruitment is followed by the incorporation of lipidated LC3 into the developing autophagosome over a period of four to six minutes (Maday et al., 2012) (Figure 4.2).

Autophagosome formation does not appear to be impaired in the majority of neurodegenerative diseases, and may even be upregulated. Autophagy is transcriptionally upregulated in Alzheimer's disease patient brains, potentially via an amyloid- β -mediated increase in reactive oxygen species (ROS) production and phosphoinositide 3 kinase (PI3K) activity (Lipinski et al., 2010). ALS patients also exhibit increased levels of the autophagy initiation

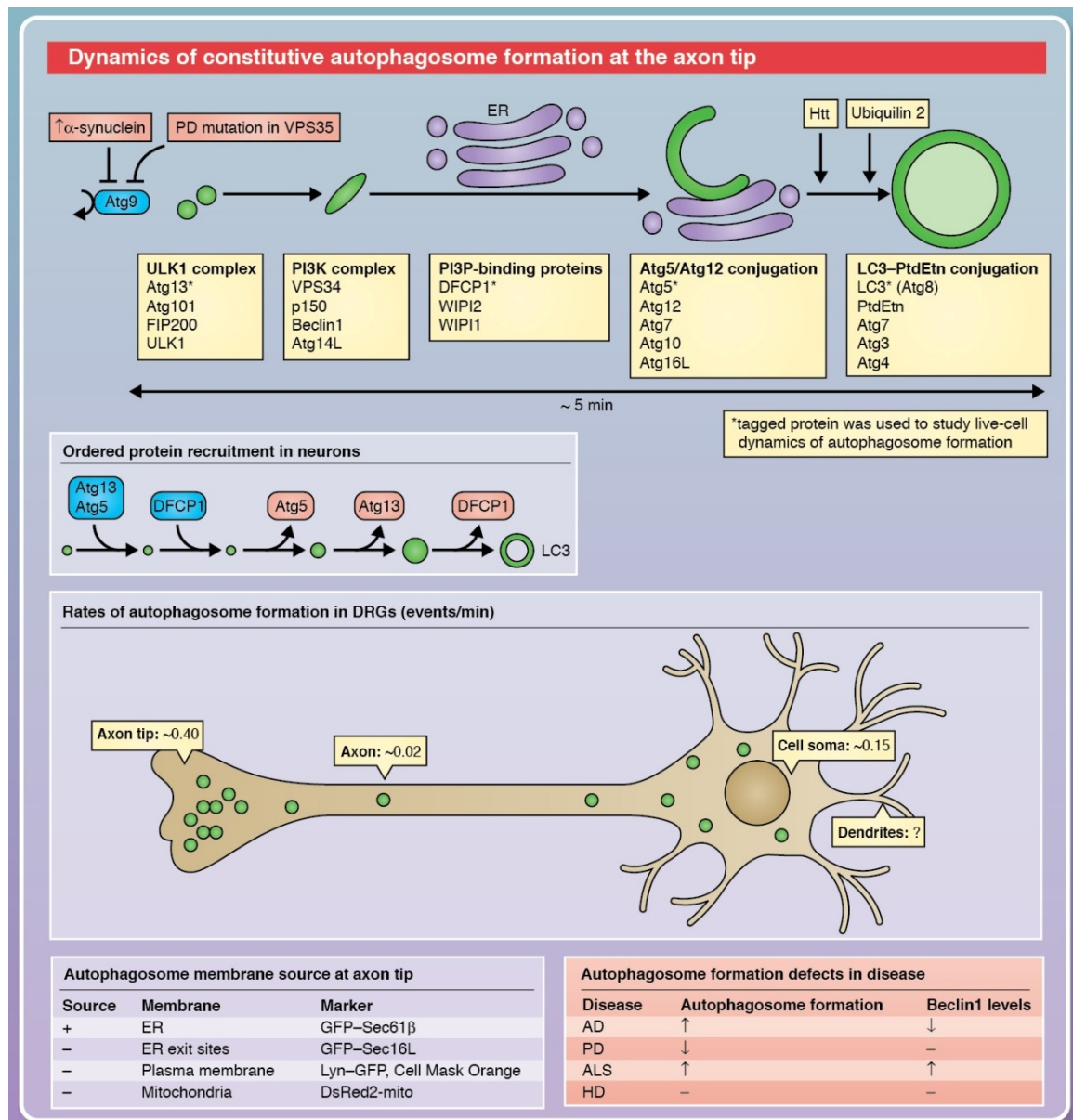


Figure 4.2: Dynamics of autophagosome formation at the axon tip.

proteins Beclin-1 and Atg5/Atg12 (Hetz et al., 2009) and an overall increase in autophagosomes (Sasaki, 2011). In Huntington's disease models, autophagosome formation at the axon tip and density along the axon are not altered (Baldo et al., 2013; Martinez-Vicente et al., 2010; Wong and Holzbaur, 2014a).

Conversely, in Parkinson's disease, α -synuclein overexpression inhibits autophagy by inhibition of Rab1a, leading to Atg9 mislocalization and defective omegasome and autophagosome formation (Winslow et al., 2010). A Parkinson's disease-associated mutation in VPS35, a member of the retromer complex, also disrupts autophagosome formation due to abnormal Atg9 trafficking (Zavodszky et al., 2014). Another Parkinson's disease-associated protein, *leucine-rich repeat kinase 2 (LRRK2)*, also localizes to autophagosome membranes and loss of LRRK2 disrupts autophagic induction (Schapansky et al., 2014).

Certain disease-associated proteins directly regulate autophagic formation. Myristoylation of a caspase-3-cleaved fragment of huntingtin promotes membrane curvature during autophagosome formation (Martin et al., 2014b), while ubiquilin 2, an ALS-associated protein which binds to LC3, promotes autophagosome formation (Rothenberg et al., 2010). With recent advances in our understanding of neuronal autophagy, it will be critical to examine the role of localized autophagosome formation in neurodegeneration.

4.4 Cargo loading of disease-associated proteins

Autophagy is the predominant degradation pathway for protein aggregates, including mutant α -synuclein (Webb et al., 2003), mutant superoxide dismutase 1 (SOD1) (Kabuta et al., 2006) and polyglutamine expansions in huntingtin (polyQ-htt) (Ravikumar et al., 2002, 2004). Autophagy can also regulate the turnover of other disease-associated proteins including amyloid- β (Parr et al., 2012), wildtype α -synuclein (Webb et al., 2003) and transactive response DNA binding protein 43 kDa (TDP-43) (Scotter et al., 2014).

Protein aggregates can be degraded by selective autophagy, which involves the recruitment of autophagy receptor proteins, including SQSTM1/p62, neighbor of BRCA1 gene 1 (NBR1), optineurin, nuclear dot *protein* 52 kDa (NDP52), and TAX1BP1/TRAFF6-binding protein (T6BP), to ubiquitinated proteins (Birgisdottir et al., 2013; Rogov et al., 2014; Stolz et al., 2014) (Figure 4.3). Interestingly, mutations in both p62 and optineurin are causative for rare forms of familial ALS (Fecto et al., 2011; Maruyama et al., 2010). The activities of these receptors are regulated by

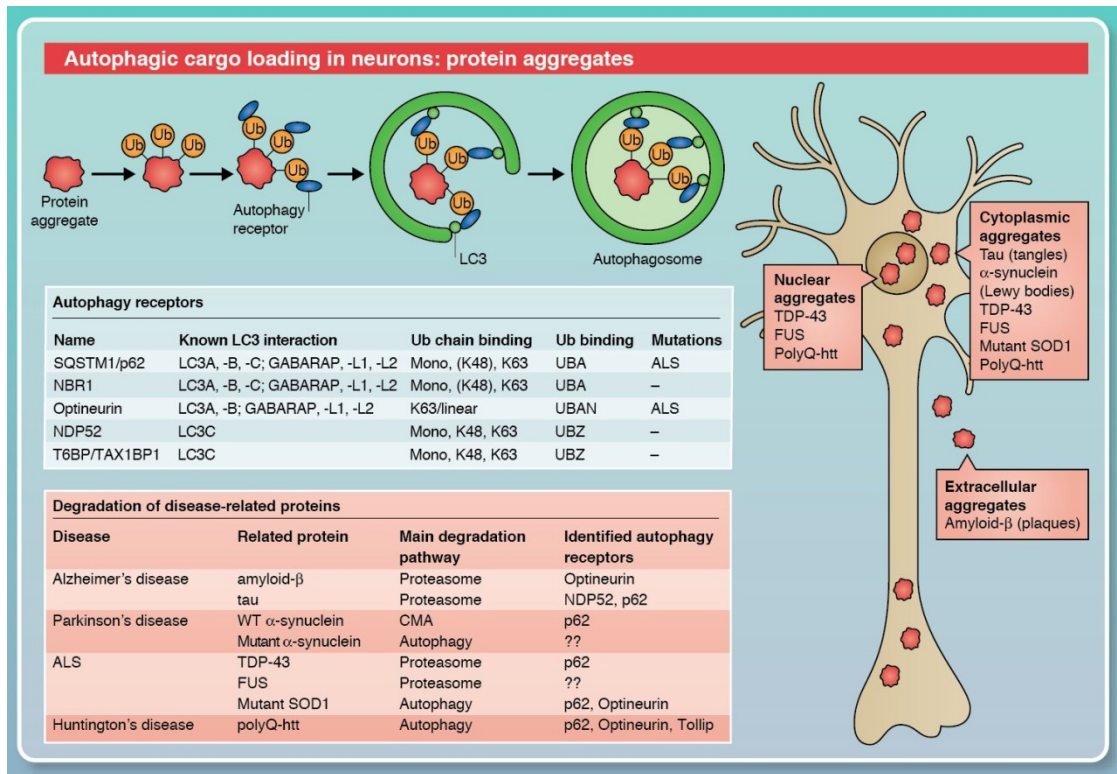


Figure 4.3: Autophagic degradation of disease-relevant protein aggregates.

different kinases. Phosphorylation of p62 by casein-kinase 2 (CK2) at S403 increases its affinity for polyubiquitin chains (Matsumoto et al., 2011), while phosphorylation of optineurin at S177 by TANK-binding kinase 1 (TBK1) increases its affinity for LC3 (Wild et al., 2011). Although recent work has begun to identify the autophagy receptors responsible for selective autophagy of various disease-associated proteins, the dynamics of their recruitment and whether different receptors may have redundant roles remain unclear.

Alzheimer's disease pathology is characterized by the formation of hyperphosphorylated tau tangles and amyloid-β plaques. Autophagic degradation of tau is regulated by nuclear factor erythroid-2-related factor 2 (Nrf2)-mediated induction of the autophagy receptor NDP52 (Jo et al., 2014) and the Alzheimer's disease-associated locus phosphatidylinositol binding clathrin assembly protein (PICALM/CALM), which controls the endocytosis of soluble NSF attachment protein receptors (SNAREs) that are involved in the autophagy pathway (Moreau et al., 2014).

Autophagy also regulates amyloid- β production (Yu et al., 2005), and defective autophagy leads to the accumulation of intraneuronal amyloid precursor protein (APP) (Nilsson et al., 2013). In microglia, amyloid- β is degraded by autophagy via the autophagy receptor optineurin (Cho et al., 2014) (Figure 4.3).

In Parkinson's disease, α -synuclein accumulates and forms Lewy body inclusions. Mutant forms of α -synuclein (A53T and A30P) are degraded predominantly by autophagy, rather than by chaperone-mediated autophagy (Cuervo et al., 2004; Webb et al., 2003). In contrast, chaperone-mediated autophagy degrades both wildtype α -synuclein and LRRK2, but becomes impaired by Parkinson's disease-associated mutations in either protein (Cuervo et al., 2004; Orenstein et al., 2013).

In ALS, autophagy induction enhances TDP-43 turnover and increases survival of ALS neuronal models (Barmada et al., 2014; Caccamo et al., 2009). Cytoplasmic TDP-43 and fused in sarcoma (FUS) localize to stress granules, which are degraded by selective autophagy in a process termed granulophagy (Buchan et al., 2013; Ryu et al., 2014). While soluble TDP-43 is primarily degraded by the proteasome, aggregated TDP-43 is cleared by autophagy (Scotter et al., 2014). ALS-associated mutant SOD1 is predominantly cleared by autophagy (Kabuta et al., 2006) that involves the autophagy receptor optineurin (Korac et al., 2013).

Huntington's disease is caused by polyQ-htt. Autophagy is critical for degrading polyQ-htt (Qin et al., 2003; Ravikumar et al., 2002) and, accordingly, upregulation of autophagy increases survival in Huntington's disease models (Ravikumar et al., 2004). The autophagy receptor p62 is recruited to the vicinity of aggregated polyQ-htt, and reduced p62 levels lead to increased polyQ-htt-mediated cell death (Björkøy et al., 2005). Other autophagy receptors for polyQ-htt include optineurin (Korac et al., 2013) and Tollip, a member of CUET ubiquitin-binding adaptor proteins (Lu et al., 2014a). PolyQ-htt may be targeted to autophagosomes by several mechanisms, including its acetylation at K444 by CREB-binding protein (CBP) (Jeong et al., 2009) and activation of the insulin receptor substrate-2 (Yamamoto et al., 2006).

Using live cell imaging in cultured neurons, recent studies have found that autophagosomes that undergo axonal transport engulf both mutant SOD1 (Maday et al., 2012) and polyQ-htt (Wong and Holzbaur, 2014a), further demonstrating that autophagy contributes to protein turnover in the axon. Thus, autophagy may play a crucial role in regulating aggregate formation both at synapses and along the axon.

4.5 Mitophagy in neurodegenerative diseases

Mitochondrial degradation through parkin-mediated mitophagy has drawn much attention, with many key discoveries made within the past year. Parkin-mediated mitophagy has been primarily implicated in Parkinson's disease, as this pathway involves PTEN induced putative kinase 1 (PINK1; also known as PARK6) and parkin (PARK 2), two genes linked to familial Parkinson's disease (Kitada et al., 1998; Valente et al., 2004).

PINK1 is normally cleaved by the protease presenilin-associated rhomboid-like protein (PARL) on the inner mitochondrial membrane, leading to its degradation (Deas et al., 2011; Greene et al., 2012; Jin et al., 2010; Meissner et al., 2011; Whitworth et al., 2008). Upon mitochondrial damage, such as depolarization (Narendra et al., 2008), increased ROS production (Yang and Yang, 2013), activation of the mitochondrial unfolded protein response (mtUPR) (Jin and Youle, 2013) or expression of the short mitochondrial isoform of ARF (smARF) (Grenier et al., 2014), PINK1 accumulates on the outer mitochondrial membrane and subsequently recruits the E3 ubiquitin ligase parkin to ubiquitinate outer mitochondrial membrane proteins (Matsuda et al., 2010; Narendra et al., 2010b; Sarraf et al., 2013; Vives-Bauza et al., 2010) (Figure 4.4). Parkinson's disease-associated mutations in PINK1 and parkin disrupt their respective kinase and ubiquitinating activities (Lee et al., 2010b; Song et al., 2013; Sriram et al., 2005), leading to defective mitochondrial degradation. Depletion of DJ-1, another Parkinson's disease-associated gene, disrupts both mitochondrial dynamics and morphology (Hao et al., 2010; Irrcher et al., 2010; Thomas et al., 2011). In addition, two other Parkinson's disease associated proteins, F-box domain-containing protein (Fbxo7; also known as PARK15) and *sterol regulatory element binding*

transcription factor (SREBF1) are also implicated in mitophagy (Burchell et al., 2013; Ivatt et al., 2014).

Recent progress now implicates parkin-mediated mitochondrial degradation in ALS. We recently found that ALS-associated protein optineurin is a novel autophagy receptor for damaged mitochondria that undergo parkin-mediated mitophagy (Wong and Holzbaur, 2014b). Optineurin is dynamically recruited to ubiquitinated mitochondria via its ubiquitin-binding domain (UBAN) downstream of parkin recruitment. Recruitment of optineurin leads to autophagosome formation, mediated by the interaction between its LIR motif (Wild et al., 2011) and LC3, resulting in the engulfment and degradation of damaged mitochondria. An ALS-associated E478G mutation in optineurin's UBAN (Maruyama et al., 2010) inhibits optineurin recruitment to damaged mitochondria, resulting in inefficient mitochondrial degradation (Wong and Holzbaur, 2014b). Another ALS-associated protein, valosin containing protein (VCP/p97) (Johnson et al., 2010), also translocates to damaged mitochondria downstream of parkin, and aids in the proteasomal degradation of ubiquitinated mitofusins Mfn1 and Mfn2 (Kim et al., 2013; Tanaka et al., 2010). Disease-associated VCP mutants are inefficiently recruited to mitochondria and disrupt mitochondrial clearance (Kim et al., 2013; Kimura et al., 2013). In addition, p62 is linked to ALS (Fecto et al., 2011) and regulates mitochondrial clustering through its *Phox and Bem1p (PB1)* oligomerization domain (Narendra et al., 2010a; Okatsu et al., 2010). Together, these studies demonstrate a role for ALS-associated proteins optineurin, VCP and p62 in mitophagy, and further implicate mitochondrial damage in ALS pathogenesis. Of note, loss of either TDP-43 or FUS, two RNA binding proteins involved in ALS, leads to decreased levels of parkin, which might also disrupt mitophagy initiation (Lagier-Tourenne et al., 2012; Polymenidou et al., 2011). Defective mitochondrial dynamics have also been implicated in Huntington's disease (Costa and Scorrano, 2012). These defects may be exacerbated by inefficient mitophagy due to defective autophagic recognition of mitochondrial cargo (Martinez-Vicente et al., 2010), or disrupted autophagosome transport along the axon leading to inhibition of cargo degradation (Wong and Holzbaur, 2014a). Thus, defective mitophagy, once thought to be a hallmark of familial

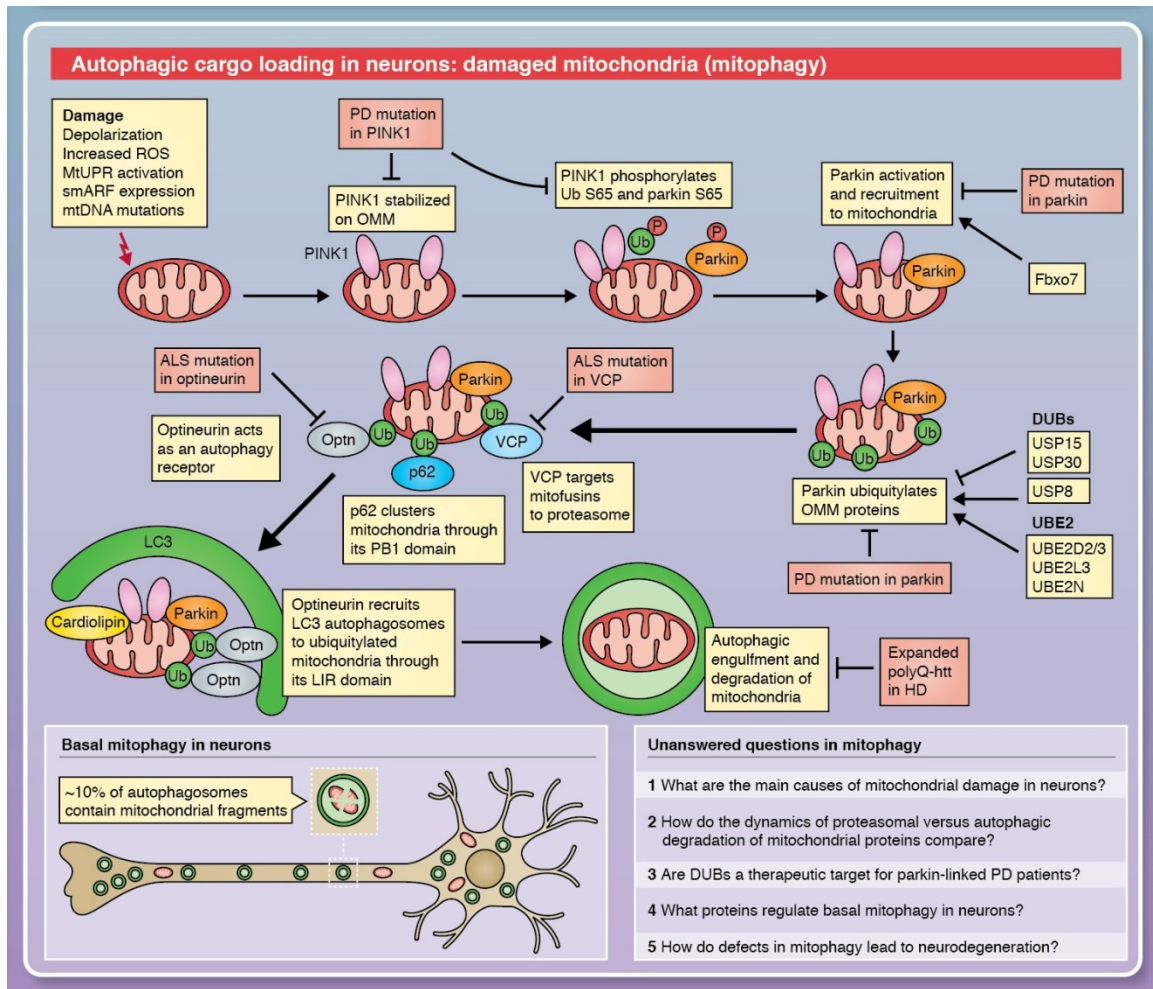


Figure 4.4: Defective mitophagy in neurodegenerative diseases.

Parkinson's disease, may be a common characteristic of multiple neurodegenerative diseases, and in fact represent a point of vulnerability in the neuron.

Live cell imaging studies of mitophagy in neurons initially found that upon mitochondrial depolarization of the whole cell, autophagosome engulfment preferentially occurred around mitochondria in the cell soma (Cai et al., 2012). However, localized damaged to mitochondria in neuronal axons was also found to induce autophagosome formation locally (Ashrafi et al., 2014). As healthy mitochondria normally undergo bidirectional transport in axons, damaged mitochondria may be stalled in axons due to release of the Milton/kinesin motor protein complex from mitochondria via PINK1/parkin-dependent degradation of the adaptor Miro (Wang et al.,

2011). In addition, neurons demonstrate a robust pathway of constitutive basal mitophagy at the axon tip (Maday et al., 2012); this pathway is impaired by expression of polyQ-htt (Wong and Holzbaur, 2014a).

Recent work on mitophagy has filled important gaps in our understanding of the PINK1/parkin pathway. PINK1 binds to phosphoglycerate mutase family member 5 (PGAM5) in the inner mitochondrial membrane (Lu et al., 2014b), but upon cleavage, translocates to the cytosol for proteasomal degradation (Fedorowicz et al., 2014; Yamano and Youle, 2013). Upon mitochondrial damage, parkin is recruited to damaged mitochondria downstream of PINK1, which phosphorylates Ser 65 on the ubiquitin-like (UBL) domain of parkin (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012) and Ser 65 on ubiquitin, leading to parkin activation (Kane et al., 2014; Kazlauskaitė et al., 2014; Koyano et al., 2014; Ordureau et al., 2014; Zhang et al., 2014a).

Several E2 ubiquitin-conjugating enzymes (UBE2) were recently identified for parkin, including UBE2D2/3 and UBE2L3, which charge parkin with ubiquitin, and UBE2N, which regulates mitochondrial clustering (Fiesel et al., 2014; Geisler et al., 2014). Three different deubiquitinating enzymes (DUBs) were also recently found to regulate mitophagy, with USP30 and USP15 opposing mitophagy by deubiquitinating parkin substrates (Bingol et al., 2014; Cornelissen et al., 2014), and USP8 promoting mitophagy by deubiquitinating parkin itself (Durcan et al., 2014). Another E3 ubiquitin ligase, Gp78, also ubiquitinates mitofusin1 and mitofusin2 to induce mitophagy (Fu et al., 2013).

Downstream of mitochondrial ubiquitination, our recent work identifies optineurin as an autophagy receptor, which recruits autophagosomes to ubiquitinated mitochondria, leading to mitochondrial degradation (Wong and Holzbaur, 2014b). Cardiolipin, an inner mitochondrial membrane phospholipid, also binds the autophagosome protein LC3 (Chu et al., 2013), suggesting that there may be alternative mechanisms of autophagosome recruitment. As many of these studies were performed in non-neuronal cells, it will be important to establish which of these steps also occur in neurons and may be defective during neurodegeneration. Robust basal mitophagy occurs in neurons (Maday et al., 2012), but it is unclear whether this constitutive

process occurs via PINK1/parkin-dependent or independent pathways (Allen et al., 2013; Bingol et al., 2014; Grenier et al., 2013; Strappazzon et al., 2015; Webster et al., 2013). In contrast, locally-induced mitophagy in the axon has been shown to be dependent on both PINK1 and parkin (Ashrafi et al., 2014).

4.6 Regulation of autophagosome transport in neurodegeneration

Vesicles and organelles undergo transport along the axon on microtubule tracks, moving in either the retrograde (towards the soma) or anterograde (away from the soma) directions. Retrograde movement is driven by the motor dynein and its adaptor dynactin, while anterograde transport is driven by kinesins. Scaffold proteins such as JIP1, JIP3, huntingtin, Milton/TRAKs, and Hook-1 bind motor proteins and regulate their activity, and thus are crucial for regulating organelle transport (Fu and Holzbaur, 2014). Cargos move differentially along the axon; while mitochondria and lysosomes move bidirectionally, APP and dense core vesicles move predominantly in the anterograde direction, and signaling endosome transport is primarily retrograde. Efficient axonal transport is critical to supply the distal synapse with newly synthesized material, and to clear damaged proteins and organelles.

Huntingtin is a scaffolding protein which binds dynein directly (Caviston et al., 2007) and also interacts with dynactin (p150^{Glued} subunit) and kinesin through Huntingtin-associated protein 1 (HAP-1), (Engelender et al., 1997; Li et al., 1998, 1995; McGuire et al., 2006; Twelvetrees et al., 2010). Huntingtin localizes to the outer membrane of autophagosomes (Atwal et al., 2007; Martinez-Vicente et al., 2010) and regulates autophagosome transport through its interactions with dynein and HAP-1 (Wong and Holzbaur, 2014a). Autophagosome axonal transport is also regulated by JIP1, which binds dynactin and kinesin-1 in a phosphorylation-dependent manner (Fu and Holzbaur, 2013), and also binds LC3 through its LIR domain. JIP1 binding to LC3 competitively disrupts JIP1-mediated activation of kinesin, inhibiting anterograde motility and

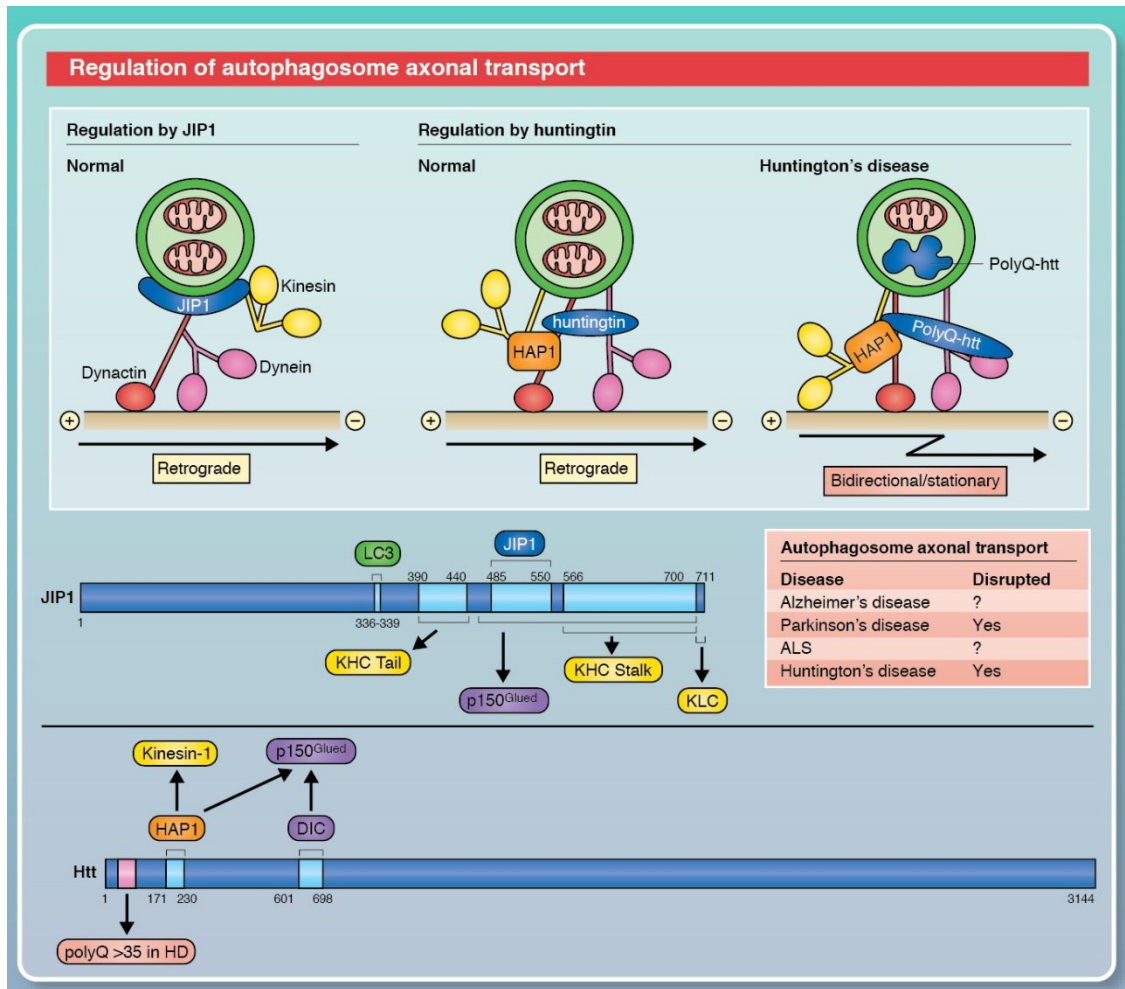


Figure 4.5: Regulation of autophagosome axonal transport.

favoring retrograde transport of autophagosomes (Fu et al., 2014). Thus, similar to other organelles, autophagosome axonal transport is tightly regulated by scaffolding proteins to sustain efficient transport, which is critical for maintaining neuronal homeostasis.

Defective lysosomal and autolysosomal transport have been observed upon inhibition of lysosomal proteolysis, which lead to dystrophic swellings that are immunopositive for APP and characteristic of Alzheimer's disease (Lee et al., 2011). In neurons harboring α -synuclein aggregates induced by the addition of preformed α -synuclein fibrils, autophagosomes demonstrate decreased motility (Volpicelli-Daley et al., 2014). In Huntington's disease models, polyQ-htt disrupts autophagosome axonal transport, resulting in defects in compartment

acidification and defective degradation of engulfed mitochondrial fragments (Wong and Holzbaur, 2014a).

In contrast, autophagosome transport was not disrupted in sensory neurons from early or late stage ALS model SOD1^{G93A} mice despite the formation of SOD1^{G93A} aggregates along the axon (Maday et al., 2012). Thus, defects in autophagosome transport may be specific to certain neurodegenerative diseases. Alternatively, autophagosome transport may be disrupted in disease-specific neuronal populations (ex. motor neurons in ALS). As defective transport leads to disrupted autophagosome maturation (Fu et al., 2014; Wong and Holzbaur, 2014a), transport defects may contribute to neuronal accumulation of autophagic cargo, such as damaged mitochondria or protein aggregates.

4.7 Autophagosome maturation and lysosomal fusion in disease

Autophagosome maturation involves fusion of the autophagosome with LAMP1/Rab7-positive lysosomes that contain cathepsin proteases, leading to the formation of autolysosomes (Lee et al., 2011; Maday et al., 2012). Autophagosomes also gradually acidify as they undergo axonal transport, likely due to lysosomal fusion and acquisition of the proton pump v-ATPase; the resulting acidification is necessary for cathepsin activation (Lee et al., 2011) (Figure 4.6).

In Alzheimer's disease patient brains, immature autophagosomes accumulate in dystrophic neurites (Nixon et al., 2005), potentially caused by defective cathepsin-mediated proteolysis or impaired autophagosome transport (Boland et al., 2008; Lee et al., 2011). Presenilin 1 mutations, the most common cause for familial Alzheimer's disease (Sherrington et al., 1995), disrupt the targeting of the v-ATPase V0a1 subunit from the ER to lysosomes (Lee et al., 2010a). As the v-ATPase is necessary for efficient acidification of both lysosomes and autophagosomes, this further implicates defects in the cellular degradation machinery in the pathogenesis of familial Alzheimer's disease.

In Parkinson's disease, wildtype α -synuclein aggregates impair autophagy by delaying autophagosome maturation (Tanik et al., 2013) and mutant α -synuclein A53T causes

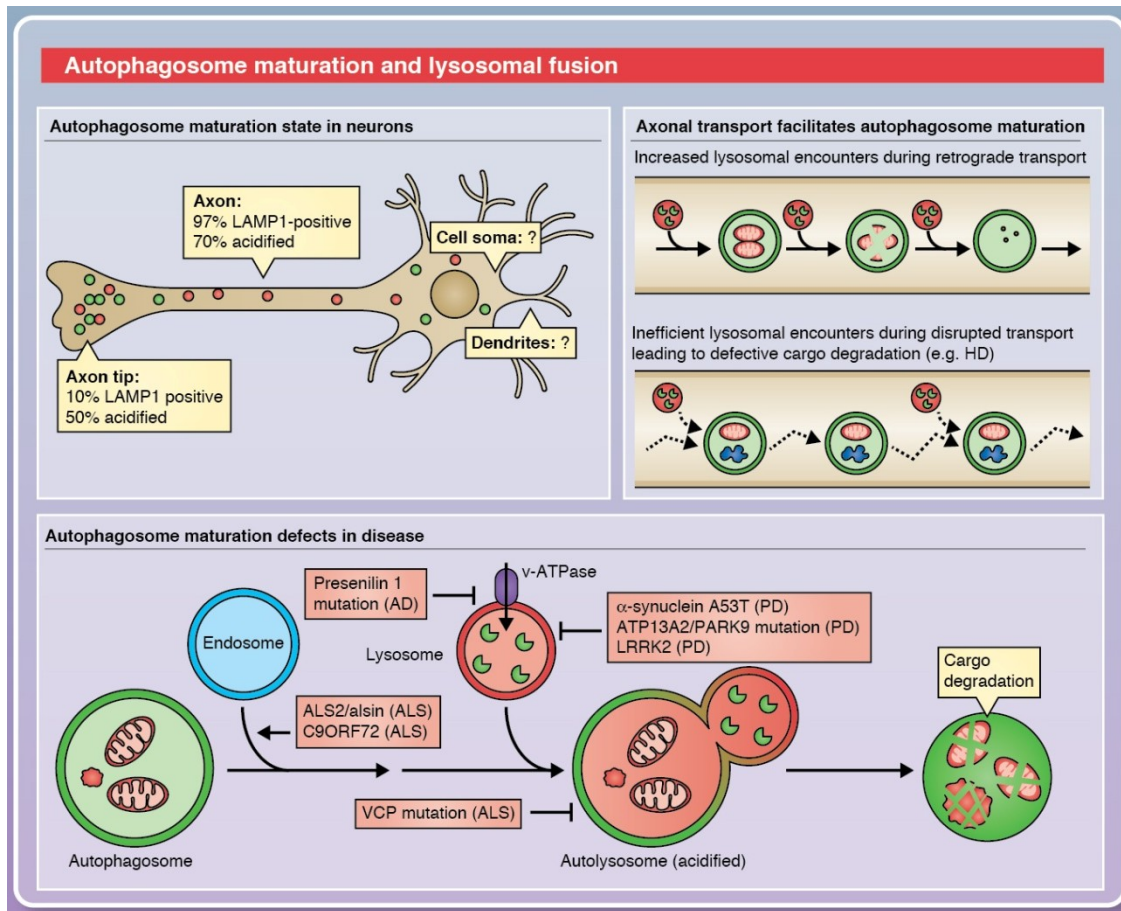


Figure 4.6: Regulation of autophagosome maturation.

accumulation of autophagic-vesicular structures and impaired lysosomal hydrolysis (Stefanis et al., 2001). Parkinson's disease-linked mutations in another gene, ATP13A2 (PARK9) that encode a lysosomal P-type ATPase, also lead to defects in lysosomal acidification, the processing of lysosomal enzymes and the clearance of autophagic substrates (Dehay et al., 2012; Usenovic et al., 2012). Finally, the Parkinson's disease-associated protein LRRK2, which has been implicated in endosomal-lysosomal trafficking, localizes to autophagosomes and activates a calcium-dependent pathway that leads to increased autophagosome formation and a decreased number of acidic lysosomes (Alegre-Abarrategui et al., 2009; Gómez-Suaga et al., 2012; MacLeod et al., 2013).

In contrast, the ALS-associated protein ALS2/alsin regulates endolysosomal trafficking potentially by mediating the fusion between endosomes and autophagosomes (Hadano et al., 2010), and another ALS-associated protein, VCP, also regulates autophagosome maturation (Ju et al., 2009; Tresse et al., 2010). Further, ALS-associated mutations in the protein CHMP2B disrupt autophagosome maturation by inhibiting the fusion of autophagosomes with multivesicular bodies (Cox et al., 2010; Filimonenko et al., 2007; Lee et al., 2007). More recently, the ALS and frontotemporal dementia-associated protein C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011) has been implicated in endosomal trafficking (Farg et al., 2014).

As efficient lysosomal fusion and protease activity are crucial for autophagy, defects in this final step of autophagy can disrupt cargo degradation, even if previous steps in the autophagy pathway are functional. Thus, it will be important to further study the dynamics of autophagosome maturation and lysosomal fusion in neurons and whether these dynamics are impaired during neurodegeneration.

4.8 Conclusion

Recent studies demonstrate that constitutive autophagosome formation in neurons follows an ordered and spatially regulated pathway, with preferential formation at the axon tip. As cellular architectures differ among neuronal populations, the spatially-restricted formation of autophagosomes may contribute to selective neuronal vulnerability during neurodegeneration. Autophagosomes subsequently mature by fusion with axonal lysosomes as they undergo retrograde axonal transport towards the cell body. This new model of neuronal autophagy provides a novel framework for understanding how defects in this pathway may contribute to neurodegeneration. As there is evidence for localized mitophagy along the axon and in the soma, it will also be interesting to further examine the regulation of locally induced autophagosome biogenesis in neurons.

Several key questions in the field of neuronal autophagy and degeneration still remain unresolved. Does the autophagosome initiation pathway that has been established in non-

neuronal cells follow the same pathway in neurons? Does selective autophagy of lipids or ER occur in neurons? How might synaptic activity regulate the location and rate of autophagosome formation? Is autophagy differentially regulated in axons and dendrites? How effectively can autophagy be up-regulated in response to protein aggregation or mitochondrial dysfunction? How is mitophagy regulated in neurons? What factors mediate autophagosome/lysosomal fusion along the axon, and are these steps disrupted in disease? It will also be important to further study neuronal autophagy *in vivo* to better understand whether misregulated autophagy is upstream or downstream of the initial neurodegenerative insult, and ultimately to determine whether modulation of autophagy is a viable therapeutic target for the treatment of neurodegenerative diseases.

5 CONCLUSIONS AND FUTURE DIRECTIONS

Autophagy is critical for degrading damaged cytoplasmic organelles and proteins to maintain cellular homeostasis. Thus, studying the live cell dynamics of this pathway is essential for understanding the regulation of basal cellular homeostasis. In addition, examining the defects that may arise in this pathway in Huntington's and ALS models enables us to further understand the importance of autophagy in neuronal survival, and identify potential mechanisms that may contribute to neurodegeneration in these diseases. The work described in the previous chapters expands our understanding of autophagosome dynamics in living cells and identifies novel proteins that are involved in the regulation of these dynamics.

In Chapter 2, we identified huntingtin as the first known motor scaffolding protein to regulate autophagosome transport. By studying basal autophagy in neurons, we found that huntingtin regulates autophagosome transport along axons via its interactions with the retrograde motor protein dynein and the motor adaptor HAP1. Both huntingtin and HAP1 are required for the efficient retrograde axonal transport of autophagosomes, and polyQ-htt disrupts this transport resulting in more stationary autophagosomes throughout the axon. Importantly, defects in autophagosome transport lead to the accumulation of undegraded mitochondria in autophagosomes transported down the axon. Thus, the defects observed in the presence of polyQ-htt suggest that defective autophagosome transport may contribute to neurodegeneration in Huntington's disease.

In Chapter 3, we examined autophagosome dynamics in the selective pathway of mitochondrial degradation during PINK1/parkin-dependent mitophagy. Using live cell imaging, we identified optineurin as a novel autophagy receptor which mediates autophagosome formation around damaged mitochondria during mitophagy. We found that optineurin is recruited to damaged mitochondria downstream of parkin by binding to parkin-ubiquitinated mitochondria. Optineurin can subsequently recruit the autophagosome protein LC3, which is required for efficient autophagic engulfment of damaged mitochondria. Interestingly, we found that an ALS-associated E478G mutation in optineurin's ubiquitin binding domain is unable to be efficiently recruited to damaged mitochondria, leading to inefficient mitochondrial degradation and the

accumulation of damaged mitochondria. Thus, defective mitophagy may contribute to neurodegeneration in patients with optineurin-associated ALS.

These studies further demonstrate the importance of well-regulated autophagosome dynamics for maintaining neuronal homeostasis and extends our knowledge of autophagy defects in neurodegenerative diseases (as reviewed in Chapter 4). Below, I first summarize the implications and questions raised based on our two studies and propose a model for huntingtin and optineurin's roles in autophagy, and secondly discuss future directions investigating novel roles for optineurin and actin in regulating mitochondrial degradation and dynamics in neurons.

5.1 Regulation of autophagosome transport by huntingtin

Huntingtin has previously been shown to regulate the transport of bidirectional cargo such as recycling endosomes, and anterograde-directed cargo such as BDNF, APP and GABA receptors (Gauthier et al., 2004; Her and Goldstein, 2008; Power et al., 2012; Twelvetrees et al., 2010). However, our study described in Chapter 2 is the first to demonstrate huntingtin's regulation of the axonal transport of a retrograde-directed organelle - the autophagosome. This regulation is mediated by huntingtin's interaction with dynein intermediate chain (Caviston et al., 2007), a member of the retrograde dynein motor complex. Both huntingtin and dynein have been previously localized to vesicles purified from mouse brain (Caviston et al., 2007). We found that huntingtin colocalizes with dynein-positive autophagosomes in neuronal axons and is also present in autophagosome-enriched fractions isolated from mouse brain, along with dynein intermediate chain and the p150^{Glued} subunit of dynactin. Interestingly, depletion of huntingtin did not disrupt the recruitment of dynein or kinesin to autophagosomes. This suggests that other adaptors such as FYCO1 may recruit microtubular motor proteins to autophagosome (Pankiv et al., 2010), while huntingtin acts to scaffold and regulate their motor activity once they have been recruited. Huntingtin was also recently found to regulate the retrograde-directed transport of the BDNF receptor TrkB in dendrites via TrkB's interactions with huntingtin and dynein (Liot et al., 2013). Thus, huntingtin is capable of regulating dynein-based retrograde cargo transport in both

axons and dendrites, demonstrating that huntingtin is a crucial motor scaffold for organelle transport across neurites.

Autophagosome transport in axons is further regulated by huntingtin's interaction with the motor adaptor HAP1. As HAP1 binds both dynactin, a required activator for retrograde transport, and kinesin-1, a major motor for anterograde transport (Engelender et al., 1997; Li et al., 1998; McGuire et al., 2006; Twelvetrees et al., 2010), HAP1 may play a key role in coordinating bidirectional motor activity. Our results suggest that a htt/HAP1 scaffolding complex promotes efficient retrograde motility of autophagosomes by enhancing dynein-dynactin-driven movement and by limiting kinesin-driven movement, leading to robust unidirectional autophagosome transport towards the soma. However, the activity of this scaffolding complex may be additionally regulated *in vivo*, by post-translational modifications by the serine/threonine kinase Akt (Colin et al., 2008) or by JNK (cJun N-terminal kinase) which is upregulated in Huntington's disease (Morfini et al., 2009). Thus, it will be interesting to examine how modification of either of these kinases regulates autophagosome transport both in wildtype and Huntington's disease models. Indeed, Akt-dependent phosphorylation of polyQ-htt rescues the axonal transport of BDNF vesicles to wild type levels potentially by restoring the interaction between polyQ-htt and motor proteins (Zala et al., 2008), suggesting that autophagosome transport defects in Huntington's disease may similarly be rescued by post-translational modifications.

Huntingtin has been previously localized to the outer membrane of autophagosomes (Atwal et al., 2007; Martinez-Vicente et al., 2010), and we found that huntingtin colocalizes with autophagosomes in neuronal axons to regulate autophagosome transport along axons (Wong and Holzbaur, 2014a). In addition, several recent studies have linked huntingtin to the regulation of autophagosome biogenesis, suggesting that huntingtin may regulate additional steps during autophagy. A new posttranslational modification of huntingtin involving myristoylation of a caspase-3-cleaved fragment of huntingtin was recently found to induce autophagosome formation resulting in abnormally large autolysosomal structures. As this fragment is highly similar to the BATS (Barkor/ATG14L autophagosome targeting sequence) domain which regulates membrane

curvature, it was proposed that a fragment of huntingtin may regulate membrane formation during autophagosome biogenesis (Martin et al., 2014b). In addition, the C-terminal fragment of huntingtin may also regulate autophagosome biogenesis via its interactions with ULK1, Beclin 1, Atg13, BNIP3/BNIP3L/NIX and the autophagy receptor p62. Moreover, this fragment also contains an LIR domain allowing it to directly interact with both LC3B and GABARAP1 (Ochaba et al., 2014).

In addition, huntingtin was found to promote the selective autophagy of protein aggregates, lipids and mitochondria but had no effect on autophagosomes biogenesis during constitutive or starvation-induced autophagy (Rui et al., 2015). The authors proposed that huntingtin initiates autophagosome formation around selective autophagic cargo by recruiting ULK1 from the negatively regulated ULK1/mTOR/Raptor complex to selective autophagic cargo, via huntingtin's interaction with p62. ULK1 could then initiate autophagosomes biogenesis on p62-labeled selective cargo. Additionally, huntingtin was found to increase p62's interaction with K63-branched ubiquitin chains. Thus, further examining the role of huntingtin in regulating selective autophagy in neurons will be important, as well as whether the induction of selective autophagy might be spatially regulated in different compartments of the neuron. In our study, we similarly found that depleting huntingtin in primary neurons did not disrupt the rate of constitutive autophagosome formation at the axon tip. However, as we saw a defect in degradation of mitochondria within autophagosomes leaving the axon terminal, it remains to be examined whether the mitophagy we observed at the axon terminal occurs an active process of classical selective autophagy (ubiquitination of damaged mitochondria and organized autophagosome biogenesis specifically around mitochondria) or whether these mitochondrial fragments are engulfed by autophagosomes in a non-specific process along with other cytosolic cargo in a constitutive manner. As we have not observed robust parkin or optineurin recruitment to mitochondria at the axon terminal in dorsal root ganglion neurons (unpublished data), it is likely that the mitophagy we observe occurs constitutively and would explain why depletion of

huntingtin did not affect cargo loading of mitochondrial fragments into autophagosomes at the axon terminal.

Huntingtin also directly binds optineurin (Faber et al., 1998; Hattula and Peränen, 2000), which binds LC3 and regulates selective autophagic degradation of *Salmonella*, protein aggregates and damaged mitochondria (Korac et al., 2013; Wild et al., 2011; Wong and Holzbaaur, 2014b). Thus, it is also possible that huntingtin might be recruited via optineurin to autophagosomes during selective autophagy. In addition, it remains to be examined whether optineurin interacts with huntingtin on autophagosomes to regulate huntingtin's motor scaffolding ability and interaction with HAP1.

Our lab also recently identified JIP1 as a second motor scaffolding protein which regulates autophagosome axonal transport in neurons (Fu et al., 2014). JIP1 interacts with kinesin heavy chain (KHC) to relieve KHC auto-inhibition, but also binds dynactin; binding to dynactin competitively inhibits kinesin-1 activation. This binding is further regulated by JNK-dependent phosphorylation of Ser421 (Fu and Holzbaaur, 2013), allowing JIP1 to act as a molecular switch between retrograde and anterograde-directed transport. JIP1 also contains a LIR domain allowing it to directly bind the autophagosome adaptor LC3 and regulate retrograde-directed autophagosome transport (Fu et al., 2014). Both JIP1 and huntingtin colocalize to the same autophagosomes in neuronal axons (unpublished data), suggesting that JIP1 and huntingtin might both be necessary to coordinate motor protein and act to regulate motor protein complex activity on the same autophagosomes. Alternatively, it is possible that JIP1 and huntingtin might be activated at different spatial and temporal points and thus alternately regulate motor proteins during autophagosome transport. In particular, as both huntingtin and JIP1 can be further phosphorylated by Akt and JNK respectively to regulate the direction and rate of organelle transport, it is possible that post-translational modification may help regulate the activity of these two scaffolding proteins. Thus, further study of the regulation of autophagosome transport via huntingtin and JIP1 will be important for understanding the roles of motor scaffolding proteins in regulating autophagy.

Previous studies have shown that polyQ-htt expression disrupts the axonal transport of multiple cargos including BDNF and mitochondria (Chang et al., 2006; Gauthier et al., 2004; Gunawardena et al., 2003; Her and Goldstein, 2008; Lee et al., 2004; Orr et al., 2008; Song et al., 2011; Szebenyi et al., 2003; Trushina et al., 2004; Zala et al., 2008). We found that polyQ-htt expression disrupts autophagosome axonal transport prior to the formation of polyQ-htt aggregates (Wong and Holzbaur, 2014a), suggesting that initial defects in autophagosome transport are not the result of physical blockade of protein aggregates present along the axon, or by the sequestration of motor proteins or adaptors into polyQ-htt aggregates. Rather, since huntingtin's interaction with HAP1 is crucial for regulating autophagosome transport, we propose that the polyQ-htt/HAP1 complex necessary for this regulation is disrupted by the increased affinity of polyQ-htt for HAP1 (Li et al., 1995). Interestingly, we found that both wild type htt and polyQ-htt were able to bind dynein intermediate chain. However, both normal and polyQ-htt preferentially interacted with the neuronal-specific dynein isoform DIC1A as compared to the more ubiquitously expressed dynein isoform DIC2C (Wong and Holzbaur, 2014a) suggesting that dynein-driven organelle transport may be selectively impaired in neurons and may contribute to neurodegeneration in Huntington's disease.

Importantly, we found that defects in autophagosome axonal transport lead to defective autophagosome acidification and cargo degradation in neurons (Fu et al., 2014; Wong and Holzbaur, 2014a). We propose that transport inhibition leads to reduced lysosomal fusion events which in turn leads to insufficient accumulation of degradative enzymes and a failure to maintain the acidified environment required for efficient degradation of engulfed proteins and organelles within the autophagosome (Jahreiss et al., 2008; Kimura et al., 2008; Ravikumar et al., 2005). Indeed, defects in autophagosome transport have previously been associated with impaired autophagic clearance and neurodegeneration (Ikenaka et al., 2013; Ravikumar et al., 2005). Interestingly, we found that autophagosomes in htt-depleted neurons still acquire the late endosomal marker LAMP1, suggesting that autophagosome-lysosome fusion is not fully impaired (Jahreiss et al., 2008) and lysosomal density was also not impaired in neurons (Wong and

Holzbaur, 2014a). However, at the proximal axon, polyQ-htt expressing neurons had lower levels of LysoTracker-positive autophagosomes, suggesting that polyQ-htt both disrupts autophagosome transport and causes a failure to maintain the acidified environment of autophagosomes as they move towards the proximal axon/cell body.

Thus, properly regulated autophagosome transport is crucial for efficient cargo degradation in neurons. This is particularly true in Huntington's disease in which autophagy is the predominant pathway for clearing polyQ-htt (Qin et al., 2003; Ravikumar et al., 2002). Indeed, inefficient autophagic degradation has been shown to accelerate the formation of toxic polyQ-htt oligomers and cause cell death (Ravikumar et al., 2004; Sarkar et al., 2007). As both disease-associated fragments and full-length polyQ-htt are cleared from the distal axon by retrograde autophagosome transport in neurons, early defects in autophagosome transport may lead to inefficient polyQ-htt clearance which may further disrupt autophagosome transport and inhibit autophagic degradation. In addition, inefficient autophagic clearance of mitochondrial fragments during mitophagy may contribute to defective mitochondrial bioenergetics in Huntington's disease (Costa and Scorrano, 2012). Thus, our study identifies a novel autophagy defect associated with polyQ-htt in Huntington's disease that may contribute to polyQ-htt accumulation as observed in patients. Recently, defective autophagosome transport was also observed in a model of Parkinson's disease in which neurons harboring α -synuclein aggregates induced by the addition of preformed α -synuclein fibrils also demonstrated decreased autophagosome motility (Volpicelli-Daley et al., 2014). Consequently, defective autophagosome transport may also be found in other neurodegenerative diseases and may be a crucial factor contributing to neurodegeneration that has not been previously studied.

5.2 Optineurin's role in PINK1/parkin-dependent mitophagy

Although autophagy receptors for specialized cases of mitophagy such as hypoxia-induced mitophagy or erythrocyte maturation have been identified, (Liu et al., 2012; Novak et al., 2010; Zhang et al., 2012), the autophagy receptors responsible for recognizing damaged

mitochondria and recruiting autophagosome assembly around mitochondria during PINK1/parkin-dependent mitophagy have not been previously identified. We identified the ALS-associated protein optineurin as a novel autophagy receptor for PINK1/parkin-dependent mitophagy as described in Chapter 3 which is crucial for recruiting the autophagosome protein LC3 to damaged mitochondria and facilitate autophagic degradation during mitophagy (Wong and Holzbaur, 2014b).

Using live cell microscopy, we found that optineurin is dynamically recruited to damaged mitochondria subsequent to parkin recruitment but prior to LC3-labeled autophagosome formation around mitochondria. While other studies have examined the dynamics of parkin recruitment and autophagosome formation around damaged mitochondria (Narendra et al., 2008; Yang and Yang, 2013), our study is the first to examine the precise recruitment dynamics of an autophagy receptor to damaged mitochondria. Importantly, our study establishes an ordered parkin-optineurin-DFCP1-LC3 pathway of dynamic protein recruitment during PINK1/parkin mitophagy. Interestingly, optineurin is recruited ~10-15 minutes following parkin recruitment rather than immediately after, suggesting that there is a time lag during which parkin is actively ubiquitinating outer mitochondrial membrane proteins, followed by the binding of cytosolic optineurin to ubiquitin via its UBA domain. Upon damage to a selective population of mitochondria (as compared to damage to the total mitochondrial pool), optineurin demonstrates slightly faster recruitment dynamics, potentially due to the fact that there are fewer damaged mitochondria which need to recruit optineurin from its cytosolic pool.

Following optineurin recruitment, there is at least another 10-15 minutes before LC3 is recruited to damaged mitochondria. During this time, upstream autophagic machinery including the ULK1 complex, PI3K complex, and PI3P binding proteins such as WIPI1, WIPI2 and DFCP1 might be recruited to damaged mitochondria. Indeed, we observe the transient recruitment of DFCP1 to optineurin-positive damaged mitochondria prior to LC3 recruitment, although DFCP1 is still present on the mitochondria when LC3 is initially recruited. Interestingly, while both the proteins parkin and optineurin are robustly recruited to all damaged mitochondria within a

relatively short time frame (i.e. all mitochondria become parkin-positive within ~20 minutes of each other), autophagosome formation around damaged mitochondria does not happen simultaneously. Indeed, although autophagosome formation around mitochondria begins around one hour after mitochondrial damage, there are mitochondria which have not been engulfed by autophagosomes even after 2 hours. This delay in autophagosome biogenesis may be due to the complex cascade of autophagy protein complexes and membrane source required to form an autophagosome, as compared to the more efficient recruitment of cytosolic proteins such as parkin or optineurin.

There are currently six identified autophagy receptors which bind both ubiquitin and LC3: optineurin, p62/SQSTM1, NBR1, NDP52, T6BP and Tollip (Kirkin et al., 2009; Lu et al., 2014a; Pankiv et al., 2007; Thurston et al., 2009; Tumbarello et al., 2012; Wild et al., 2011). Besides our work on optineurin, only the role of p62 in mitophagy has been studied, generating conflicting reports on its role as either an autophagy receptor (Ding et al., 2010; Geisler et al., 2010) or a regulator of perinuclear clustering of depolarized mitochondria (Narendra et al., 2010a; Okatsu et al., 2010). We found that p62 and optineurin were independently recruited to damaged mitochondria as expected since they both bind ubiquitin and are not known to interact with each other. However, we found that optineurin and p62 localized to different domains on damaged mitochondria similar to p62 and optineurin's localization on ubiquitinated *Salmonella* (Wild et al., 2011). p62 preferentially localized to domains between adjacent mitochondria and accelerated mitochondrial aggregation, but did not regulate LC3 recruitment to mitochondria. These results are consistent with previous reports showing that p62 is not an autophagy receptor for damaged mitochondria but rather clusters damaged mitochondria via its PB1 oligomerization domain (Narendra et al., 2010a; Okatsu et al., 2010). In contrast, we found that optineurin uniformly localized across the surface of damaged mitochondria and served as a robust autophagy receptor for damaged mitochondria.

As both optineurin and p62 bind ubiquitin and LC3 and are recruited to damaged mitochondria, it is interesting that optineurin preferentially recruits LC3 to mitochondria while p62

does not. One possibility is that since p62 can homodimerize via its PB1 domain or heterodimerize with another autophagy receptor NBR1 which also contains a PB1 domain (Birgisdottir et al., 2013), this dimerization might induce a conformational change preventing p62 from efficiently binding LC3 via its LIR domain, located between the PB1 and ubiquitin binding domains. In contrast, optineurin's coiled coiled regions which may facilitate in its oligomerization are located in the middle of the protein, with its LIR domain at the N terminus and the ubiquitin binding domain at the C terminus. Interestingly, the protein domain sequence of the autophagy receptor NDP52 and T6BP are more similar to that of optineurin than to p62 (Birgisdottir et al., 2013), and both optineurin and NDP52 localize to the same subdomains on ubiquitinated *Salmonella* (Wild et al., 2011). Thus, NDP52 and T6BP might also be recruited to damaged mitochondria during mitophagy and regulate autophagic engulfment along with optineurin.

A second possibility is that as p62 preferentially localizes to regions between adjacent damaged mitochondria, p62 may be unable to efficiently induce LC3 recruitment and autophagosome formation around an entire mitochondria, a step necessary for successful mitophagy. In contrast, as optineurin localizes to the entire surface of the mitochondria, it might be more able to efficiently recruit LC3 around the mitochondria. Alternatively, the differences in ubiquitin and LC3 binding domains between optineurin and p62 (Birgisdottir et al., 2013) might also explain why p62 is not a robust mitophagy receptor. Optineurin contains a UBA domain for binding ubiquitin which preferentially binds K63-linked and linear ubiquitin chains, while p62 contains a UBA domain which preferentially binds K63-linked and mono ubiquitin although its phosphorylation by CK2 increases its affinity for polyubiquitin chains (Matsumoto et al., 2011). In addition, optineurin's LIR domain does not bind LC3C, while p62's LIR domain binds LC3A,-B,-C and GABARAP, -L1, -L2. Thus, further understanding the differences between p62 and optineurin's interactions with ubiquitin, LC3 and potentially other autophagy related protein may help elucidate the different roles they play in mitophagy. Ultimately, studying the functions of the different autophagy receptors in mitophagy and whether they have overlapping roles will be crucial for understanding how PINK1/parkin mitophagy is regulated.

Our study fills in an important gap in the PINK1/parkin pathway by identifying the autophagy receptor optineurin which links parkin recruitment and ubiquitination of mitochondrial proteins to the autophagic recognition of damaged mitochondria via LC3 autophagosome recruitment. However, another key step in the pathway that is not yet understood has been how stabilization of the kinase PINK1 on the outer mitochondrial membrane leads to the recruitment of the E3 ubiquitin ligase parkin from the cytosol to mitochondria. Recently, several groups independently demonstrated that PINK1 phosphorylates Ser65 on ubiquitin, contributing to parkin activation (Kane et al., 2014; Kazlauskaitė et al., 2014; Koyano et al., 2014; Ordureau et al., 2014; Zhang et al., 2014a). PINK1 was previously also shown to phosphorylate Ser65 on the ubiquitin-like (UBL) domain of parkin (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012), but this phosphorylation is not sufficient to activate parkin. Although identifying this PINK1-mediated phosphorylation step of ubiquitin furthers our understanding of the pathway, it is still unclear how parkin is recruited and stabilized on mitochondria, and whether the recruitment of parkin occurs prior or subsequent to ubiquitin phosphorylation by PINK1.

We found that the ALS-associated E478G mutation in optineurin's ubiquitin binding domain prevents its recruitment to damaged mitochondria, leading to inefficient autophagosome engulfment and degradation of mitochondria. However, there are other ALS-linked mutations in optineurin which are located in other domains that are not known to disrupt optineurin's ubiquitin binding ability (Kachaner et al., 2012a). Thus, it will be important to examine whether these other ALS-associated mutations also prevent efficient mitophagy. As optineurin has been proposed to oligomerize into stable trimers via its coiled coiled domains and ubiquitin binding domain (Gao et al., 2014), it will be interesting to examine whether mutations in optineurin's coiled coiled domains disrupt its ability to oligomerize and whether this oligomerization is important for its rapid recruitment to damaged mitochondria.

Mutations in optineurin are linked to both glaucoma (Rezaie et al., 2002) and ALS (Maruyama et al., 2010), but how mutations in the same protein lead to these two very different neurodegenerative diseases remains unknown. The autosomal dominant E50K mutation in

optineurin accounts for ~13% of normal tension glaucoma cases (Rezaie et al., 2002), but the how this mutation disrupts mitophagy has never been studied. As mitochondria defects have been proposed to contribute to neurodegeneration of retinal ganglion cells in glaucoma (Osborne and Del Olmo-Aguado, 2013), it is highly possible that mitophagy defects may contribute to optineurin-associated cases of glaucoma. In addition, as glaucoma mutations such as M98K are located in close proximity to ALS mutations such as R96L (Kachaner et al., 2012a), it will also be interesting to see whether these neighboring mutations which lead to different diseases result in similar defects during mitophagy.

Defects in the mitophagy pathway have been predominantly associated with Parkinson's disease, as mutations in PINK1 and parkin are linked to familial Parkinson's disease and lead to defective mitochondrial degradation (Kitada et al., 1998; Valente et al., 2004). Our study demonstrates that an ALS mutation in optineurin disrupts its recruitment to mitochondria downstream of PINK1 and parkin, and also leads to defective mitochondrial degradation. Thus, defects in the same mitochondrial quality control pathway may contribute to multiple neurodegenerative diseases. However, as optineurin also regulates other cellular roles including vesicle trafficking, NF- κ B signaling and autophagic degradation of other substrates such as bacteria and protein aggregates, these additional roles may contribute to the clinical and pathological differences between Parkinson's diseases and optineurin-associated familial ALS.

Interestingly, another ALS-linked protein VCP (valosin-containing protein) has also been implicated in PINK/parkin mitophagy (Tanaka et al., 2010). VCP (also called p97, or Cdc48 in yeast) is a hexameric protein from the AAA family and contains two ATPase domains (Meyer et al., 2012). VCP is involved in multiple cellular functions including ERAD (endoplasmic reticulum-associated degradation), autophagosome maturation, endolysosomal sorting, and DNA repair. However, VCP is best characterized for its ability to bind ubiquitinated proteins via its N terminus, and extract them from membranes or protein complexes to help unfold them for proteasomal degradation (Meyer et al., 2012). Using *Drosophila* models, VCP was found to complement PINK1 deficiency but not parkin deficiency. VCP is recruited to parkin-mediated ubiquitinated

mitochondrial proteins downstream of parkin recruitment (Kim et al., 2013), and aids in the proteasomal degradation of mitofusin 1 and mitofusin 2 (Tanaka et al., 2010). VCP is also required for mitochondrial degradation via PINK1/parkin mitophagy, and pathogenic ALS mutations in VCP lead to inefficient mitochondrial degradation (Kim et al., 2013). Thus, mutations in two different ubiquitin-binding ALS-associated proteins, VCP and optineurin, lead to inefficient mitochondrial degradation and further highlight a potential role for mitophagy defects in ALS that may contribute to motor neuron death. Interestingly, defects in the selective autophagic degradation of stress granules during granulophagy were also observed upon expression of pathogenic mutations in VCP (Buchan et al., 2013). Thus, it will be interesting to see whether ALS-linked mutations in optineurin also disrupt granulophagy, as well as whether familial ALS might be characterized by defective autophagic degradation of selective autophagy substrates including mitochondria, stress granules, ER, ribosomes and protein aggregates.

As previously mentioned, the protein p62 is also recruited to damaged mitochondria during PINK1/parkin mitophagy, and we confirmed its role in clustering damaged ubiquitinated mitochondria. Mutations in p62 have also been linked to ALS (Fecto et al., 2011), although the role of these mutations in disrupting mitochondrial degradation have not been well characterized. Recently, the kinase TBK1 which phosphorylates both p62 and optineurin, and whose binding to and phosphorylation of optineurin enhances its interaction with LC3 (Wild et al., 2011), was also implicated in familial ALS via a loss of function mechanism (Cirulli et al., 2015; Freischmidt et al., 2015). Thus, further examining the role of TBK1 in regulating mitophagy will be interesting and whether enhancing TBK1 kinase activity can rescue defective mitophagy.

We found that optineurin and p62 were independently recruited to damaged mitochondria via their ubiquitin binding, suggesting that optineurin and VCP may also be independently recruited via ubiquitin. In addition, mutations in another ubiquitin binding protein, ubiquilin 2, have also been linked to ALS (Deng et al., 2011), although the role of ubiquilin 2 in mitophagy has not been studied. Ubiquilin 2 contains both a UBL (ubiquitin-like domain) and a UBA (ubiquitin binding domain) which interacts with both K48 and K63 chain ubiquitin. As parkin predominantly

adds K63-branched ubiquitin chains on outer mitochondrial membrane proteins (Sarraf et al., 2013), it is highly likely that ubiquilin 2 can also bind ubiquitinated mitochondria during mitophagy, downstream of parkin ubiquitinating activity. Interestingly, ubiquilin 4, another member of the ubiquilin family, has been identified as an autophagy receptor binding both ubiquitin and LC3 via a non-canonical LC3 interacting region, and is able to consequently recruit ubiquilin 1 to autophagosomes (Lee et al., 2013). Thus, it will be important to examine whether ubiquilin 2 localizes to autophagosomes and is recruited to damaged mitochondria during their selective autophagic degradation.

As mutations in various ubiquitin-binding proteins have been linked to ALS (Renton et al., 2014) and ubiquitin serves as a tag for both proteasomal and autophagic degradation, defects in overall neuronal proteostasis may contribute to ALS pathogenesis. We have found that an ALS-associated optineurin mutation leads to defective mitophagy. However, further work needs to be done to further examine the role of mitophagy in leading to motor neuron degeneration in cases of other ALS-linked mutations in optineurin and in other ubiquitin binding proteins including p62 and ubiquilin 2. Moreover, as other non-ubiquitin binding proteins such as TDP-43, FUS and profilin have also been linked to familial ALS (Renton et al., 2014), it will be crucial to examine whether there are mitophagy defects in these cases of ALS. Finally, it will be important to observe whether defective mitochondrial autophagic degradation is also observed in sporadic ALS cases by examining mitochondrial and autophagy markers in patient brain and motor neurons, as well as in cell culture models using iPSC (induced pluripotent stem cell)-derived neural stem cells from ALS patient fibroblasts.

5.3 Proposed models for huntingtin and optineurin's roles in autophagy

How does huntingtin regulate autophagosome transport in neurons?

Based on our studies, I propose a model in which huntingtin regulates the axonal transport of autophagosomes in neurons, and disruption of this transport contributes to defective

autophagy and polyQ-htt proteostasis in Huntington's disease. In our model, kinesin and dynein/dynactin motor proteins are first recruited to the autophagosome outer membrane via FYCO1 or other motor adaptors following autophagosome biogenesis at the axon terminal in neurons. Subsequently, huntingtin is recruited from the cytosol to the autophagosome at the axon terminal via its direct interaction with dynein intermediate chain in the dynein motor complex and may be further stabilized on the autophagosome by its interactions with LC3 on the autophagosome outer membrane via its LIR domain. Huntingtin is recruited to autophagosomes at the axon terminal in both selective and non-selective forms of autophagy, and during both starvation-induced and basal autophagy in neurons. HAP1 is also recruited by binding huntingtin, the p150^{Glued} subunit of dynactin and kinesin, resulting in a stable huntingtin/HAP1 motor adaptor complex on the autophagosome outer membrane. This complex then functions to promote dynein motor retrograde activity on the autophagosome, resulting in efficient retrograde transport of autophagosomes back towards the cell body.

In this model, JIP1 is also recruited to the same autophagosomes following autophagosome biogenesis via its interactions with kinesin heavy chain and dynactin, and is further stabilized on the autophagosome outer membrane by binding LC3 via its LIR domain. JIP1 binds motors in separate motor complexes on the autophagosome outer membrane, and its binding to dynactin inhibits kinesin-1 activation, thereby decreasing anterograde motility of autophagosomes and allowing for dynein/dynactin motors to drive autophagosomes in the retrograde direction. Thus, huntingtin/HAP1 and JIP1 motor complexes together act to promote efficient retrograde transport of autophagosomes by promoting dynein activity and inhibiting kinesin activity respectively. These two motor complexes on the autophagosome outer membrane are further regulated by Akt and JNK phosphorylation activity respectively, in which local phosphorylation along the axon further regulates autophagosome motility to allow for decreased motility in areas of increased lysosomal density to promote autolysosomal fusion. Efficient autophagosome axonal transport is required for the autophagosome to encounter lysosomes along the axon. As the autophagosome fuses with multiple lysosomes along the axon, the

resulting autolysosome gradually acidifies and accumulates the necessary enzymes for cargo degradation. Thus, the majority of autophagic cargo is degraded by the time the autophagosome has reached the cell body. At this point, the autophagosome membrane and its internal contents are recycled and huntingtin detaches from the autophagosome and returns to its cytosolic pool.

In Huntington's disease, polyQ-htt disrupts multiple cellular processes including the axonal transport of multiple organelles including autophagosomes, BDNF vesicles and recycling endosomes. In our model, axonal transport of organelles is disrupted by polyQ-htt which competes with wild type huntingtin for binding to HAP1 in the huntingtin/HAP1 motor scaffold complex, resulting in a tighter polyQ-htt/HAP1 motor scaffold complex which has defective binding and regulation of both dynein/dynactin and kinesin motors. This defective motor complex results in inefficient axonal transport of retrograde, anterograde and bidirectional organelles. In particular, autophagosome axonal transport is no longer robustly retrograde and becomes predominantly stationary or bidirectional with decreased net velocities, leading to decreased encounters with lysosomes and inefficient maturation and degradation of autophagic cargo along the axon.

Autophagy is the predominant mechanism for degrading both soluble and aggregated polyQ-htt. As such, efficient autophagosome transport and degradation are also critical for polyQ-htt turnover. In this model, disruption of autophagosome transport by polyQ-htt leads to decreased autophagic cargo degradation including that of polyQ-htt itself. This excess polyQ-htt is then able to further out compete wild type huntingtin for the regulation of motor proteins on autophagosomes, leading to an even greater disruption of autophagosome transport and further inefficiency in polyQ-htt degradation. As polyQ-htt accumulates within the cell, it further disrupts other cellular processes such as transcription, metabolic function and cellular proteostasis which contribute to neuronal dysfunction. In addition, excess polyQ-htt may form inclusion bodies which sequester motor proteins and motor adaptors such as HAP1 and JIP1, leading to further disruption of organelle transport. These inclusions may also sequester important protein

machinery involved in selective autophagy such as p62 and optineurin, leading to an additional defect in selective autophagic degradation in Huntington's disease.

Thus, in this 'run-away train' model, what begins as a defect in autophagosome axonal transport results in inefficient polyQ-htt turnover and accumulation which further exacerbates autophagosome transport and contributes to additional polyQ-htt-induced defects in other cellular processes leading to neurodegeneration. As this process of inefficient polyQ-htt autophagic degradation and polyQ-htt accumulation is gradual, this may contribute to the late onset of degeneration observed in most Huntington's disease patients. Thus, specifically increasing the rate of polyQ-htt degradation or preventing polyQ-htt production without disrupting autophagic degradation of other cargo may help to stop this 'run-away train' and prevent neurodegeneration in Huntington's disease.

How does optineurin regulate autophagic degradation of mitochondria?

I propose a model in which optineurin acts as a key autophagy receptor for damaged mitochondria, and ALS-associated optineurin mutations lead to a loss of function resulting in inefficient autophagic degradation of selective cargo and the accumulation of dysfunctional mitochondria. In PINK1/parkin-mediated mitophagy, mitochondria which lose their mitochondrial protein import ability accumulate PINK1 on their outer mitochondrial membrane. PINK1 subsequently phosphorylates ubiquitin and parkin in the cytosol, leading to gradual parkin recruitment and stabilization on the outer mitochondrial membrane. Parkin then ubiquitinates outer mitochondrial membrane proteins with K63-branched ubiquitin chains. Optineurin is then recruited from the cytosol to mitochondria by binding ubiquitin via its UBAN domain, and may oligomerize on the mitochondrial membrane into either trimers or hexamers as it is gradually recruited, which could in turn facilitate its ability to efficiently recruit LC3.

As optineurin is localized throughout the cytosol, optineurin can be efficiently recruited upon mitochondrial ubiquitination to damaged mitochondria which might be distributed throughout the neuron in the cell body, axon or dendrites. In addition, optineurin recruitment may not be restricted to parkin-ubiquitinated mitochondria, as any E3 ubiquitin ligase which is able to robustly

ubiquitinate mitochondrial proteins with K63-branched chains will be sufficient to induce optineurin recruitment. Thus, other mechanisms of mitochondrial damage which do not impair mitochondrial import and do not initiate PINK1/parkin-mediated mitophagy may also require optineurin provided that mitochondria are robustly ubiquitinated.

Following optineurin recruitment, the proteins involved in autophagosome biogenesis are recruited to mitochondria leading to isolation membrane and omegasome formation around the mitochondria. In the absence of optineurin, DFCP1 ER-based omegasomes are still able to form around damaged mitochondria, suggesting that there are other proteins which may be able to initiate autophagosome biogenesis around damaged mitochondria leading to omegasome formation. One possible protein is p62, which is also recruited to damaged mitochondria and could subsequently recruit huntingtin, which in turn might bind and scaffold initial autophagosome biogenesis proteins such as ULK1, Beclin 1 and Atg13. It is also possible that in addition to optineurin, other autophagy receptors such as NDP52 and T6BP localize to mitochondria and also bind and recruit huntingtin to initiate autophagosome biogenesis. Finally, there may be additional mechanisms independent of autophagy receptors and huntingtin which facilitate in recruiting early autophagic machinery to damaged mitochondria.

Optineurin subsequently plays a crucial role in the final step of autophagosome formation around mitochondria. In our model, optineurin localized on the outer mitochondrial membrane recruits LC3 via its LIR domain from the cytosol to the forming autophagosome. As LC3 is needed for isolation membrane expansion and final closure of the autophagosome around the mitochondria, this recruitment step by optineurin is critical for ensuring that the damaged mitochondria is fully enclosed within an autophagosome and thus efficiently degraded via autophagy.

As other autophagy receptors such as NDP52 and T6BP are also recruited to ubiquitinated mitochondria, these proteins could also facilitate recruitment of LC3 to damaged mitochondria in the final stages of autophagosome formation but to a lesser extent than optineurin, potentially due to their decreased binding affinity for LC3. In addition, other proteins

such as p62 and NBR1 which are also recruited to ubiquitinated mitochondria might help to cluster neighboring damaged mitochondria by forming both homo- and heterodimers via their PB1 domains to link adjacent mitochondria.

The ALS-linked E478G mutation in optineurin's UBAN domain is unable to be efficiently recruited to damaged mitochondria, leading to decreased autophagosome formation around mitochondria and inefficient mitochondrial degradation. This E478G mutation disrupts optineurin's ubiquitin binding but also has decreased oligomerization capability compared to wild type optineurin since it cannot stably bind to itself or wild type optineurin. Thus, although the E478G exists as a heterozygous mutation in patients, I predict that it leads to ALS via a loss of function mechanism. In particular, as optineurin functions optimally in autophagy as either a trimer or hexamer in our model, the presence of a heterozygote mutation dramatically decreases the probability of forming a functional trimer or hexamer comprised of only wild type optineurin (12.5% for trimers; 1.5625% for hexamers). As the majority of ALS-linked mutations in optineurin are either deletion mutations or mutations in coiled coiled regions which are crucial for its oligomerization, I predict that optineurin leads to familial ALS via a loss of function mechanism. In contrast, glaucoma-linked mutations in optineurin might play a more crucial role via gain of function mechanisms by disrupting additional cellular processes such as inducing Golgi fragmentation.

As healthy mitochondria are critical for neuronal metabolism and survival particularly in neurons with long axons such as motor neurons, defective mitophagy caused by optineurin loss of function in familial ALS plays a critical role in contributing to the neuronal death observed in ALS. However, optineurin also regulates the selective autophagy of pathogens and protein aggregates, and defects in these processes may further contribute to neurodegeneration in ALS. Interestingly, a large subset of ALS-linked proteins have now been linked to both autophagy and mitophagy, including optineurin, p62, VCP, ubiquilin 2 and TBK1 suggesting that the loss of function of these proteins leading to inefficient autophagy and accumulation of unhealthy mitochondria may be the initial trigger for neuronal dysfunction in this subset of familial ALS

cases. Other familial cases of ALS which have been linked to defective RNA biology such as TDP-43 and FUS mutations and C9ORF72 hexanucleotide expansions cases may also exhibit defective autophagy, such as impaired autophagic degradation of protein aggregates or RNA stress granules during granulophagy, which may further exacerbate defective neuronal homeostasis. Thus, defects in autophagy play a critical role in at least a subset of familial ALS cases and may also contribute to the pathogenesis in sporadic ALS cases. Understanding the autophagy defects in both selective and non-selective autophagy and identifying the precise steps which are disrupted in sporadic ALS cases will thus be essential for further elucidating the mechanisms which lead to neurodegeneration in ALS.

Consensus model for huntingtin and optineurin's roles in autophagy

In summary, I have presented two models for the roles of huntingtin and its binding partner optineurin in regulating autophagosome dynamics. In both selective and non-selective autophagy, huntingtin is recruited to the fully formed autophagosome at the axon terminal to regulate motor protein activity and promote efficient retrograde transport towards the cell body. In the selective autophagy of damaged mitochondria, optineurin is recruited to ubiquitinated mitochondria and plays a critical role in ensuring autophagosome closure around mitochondria by its recruitment of LC3. However, as huntingtin and optineurin interact with each other, optineurin may also play an additional role during selective autophagy by recruiting huntingtin to selective autophagic cargo.

In contrast to the first role of huntingtin on the outer autophagosome membrane in regulating the transport of both selective and non-selective autophagosomes formed at the axon tip, huntingtin may also be recruited during specific cases of selective autophagy throughout the neuron that are not restricted to the axon tip to facilitate autophagosome biogenesis. During selective autophagy, a pool of huntingtin may be recruited at an earlier step of autophagosome biogenesis to selective ubiquitinated autophagic cargo via huntingtin's interactions with optineurin and/or p62. This pool of huntingtin subsequently acts to scaffold proteins involved in initial autophagosome biogenesis events such as ULK1, Beclin 1 and Atg13 to facilitate

autophagosome formation on selective autophagic cargo. This interaction is upregulated during selectively-induced autophagy to promote selective autophagy of cargo such as mitochondria and lipids. Huntingtin either detaches from p62 and optineurin prior to the closure of the autophagosome, or may become incorporated inside the autophagosome if it remains attached to the autophagic cargo via its binding to optineurin and/or p62. Thus, this pool of huntingtin remains distinct from the second pool of huntingtin which is subsequently recruited to motor proteins on the outer autophagosome membrane to regulate autophagosome transport following the closure of a fully formed autophagosome.

The models I have proposed above highlight the essential roles for huntingtin and optineurin in regulating autophagosome biogenesis and dynamics in neurons. In addition, they demonstrate how mutations in these two proteins could lead to neurodegeneration in Huntington's disease and familial ALS, and evince the importance of further studying the regulation of autophagy in neurons.

5.4 Future Directions

5.4.1 Optineurin as a mitophagy receptor in neurons

As our study identifying the ALS-associated protein optineurin as an autophagy receptor in PINK1/parkin mitophagy was performed in non-neuronal immortalized HeLa cells, we plan to extend our studies into neurons as ALS primarily affects upper and lower motor neurons. Importantly, we hope to confirm that optineurin is also recruited to damaged mitochondria in neurons, and that the E478G ALS-associated mutation in optineurin which we found to cause defective mitophagy in HeLa cells also causes a similar defect in primary neurons. Optineurin has been shown to colocalize with mutant SOD1 protein aggregates in motor neurons in an ALS mouse model of human SOD1^{G93A}, and optineurin depletion in zebrafish overexpressing SOD1^{G93A} causes motor axonopathy (Korac et al., 2013), suggesting that optineurin is also able to regulate autophagic degradation in neurons.

To examine optineurin's role in mitophagy in neurons, it will be important to first establish a robust protocol for inducing PINK1/parkin-dependent mitophagy in neurons. Selectively inducing reactive oxygen species (ROS) production in the mitochondrial matrix by activating the mito-KillerRed (mitoKR) construct in a select mitochondrial population is sufficient to recruit both parkin and optineurin to damaged mitochondria in HeLa cells (Wong and Holzbaur, 2014b; Yang and Yang, 2013). In addition, activating mitoKR in the axons of neurons grown in antioxidant-free media is able to induce recruitment of parkin and LC3 to damaged mitochondria (Ashrafi et al., 2014). Mitochondria in neurons may also be damaged using other methods such as introducing mtDNA mutations or disrupting mtUPR (mitochondrial unfolded protein response) by depleting cells of mitochondrial chaperones such as HSP60 or mortalin/mtHSP70 to examine whether PINK1/parkin mitophagy is induced.

Following parkin recruitment to damaged mitochondria in neurons, it will be important to test whether optineurin is subsequently recruited, and whether the rate of optineurin recruitment in neurons is similar to that in HeLa cells. In addition, it will be crucial to examine whether the parkin/optineurin/DFCP1/LC3 pathway of ordered protein recruitment observed in HeLa cells is also seen in neurons, and whether optineurin regulates LC3 recruitment. It will also be interesting to examine whether optineurin expression is upregulated in response to prolonged mitochondrial damage, which might represent a feedback loop in which the neuron senses mitochondrial damage and upregulates the expression of its autophagic machinery including that of autophagy receptors. As other autophagy receptors such as NDP52 and T6BP might also be involved in mitophagy, observing the recruitment dynamics of these proteins to mitochondria in both HeLa cells and primary neurons will be essential for expanding our understanding of mitophagy regulation.

Studying whether motor neurons selectively degenerate in optineurin knockout mice will help us further understand the mechanisms behind the selective neuronal degeneration observed in ALS. In addition, it will be interesting to test whether depolarized mitochondria accumulate in different neuronal types (such as cortical, hippocampal or motor neurons) in optineurin knockout

mice using mitochondrial markers such as TMRE, as well as whether these neurons accumulate ROS, have Ca^{2+} overload, or have increased mtDNA mutations in their remaining mitochondria. Finally, it will be important to compare mouse models with either ALS or glaucoma mutations in optineurin to examine whether defects in mitophagy are observed in either or both mouse models.

Our lab previously showed that basal levels of mitophagy occur in the axon terminal of dorsal root ganglion neurons without induction of mitochondrial damage, suggesting that mitophagy is normally initiated in these neurons (Maday et al., 2012). However, neither parkin nor optineurin were found to demonstrate robust recruitment to mitochondria at the axon terminal of these neurons during this level of basal mitophagy (unpublished data), suggesting that autophagic engulfment of mitochondria at the axon terminal may be occurring in a non-selective manner or in a pathway independent of parkin and optineurin. Thus, further studying the regulation of basal mitophagy will also be crucial for identifying the rate at which PINK1/parkin mitophagy is initiated in healthy neurons and for understanding how often optineurin is recruited to damaged mitochondria. In particular, it may be possible that the PINK1/parkin pathway is only initiated in neurons upon certain types of mitochondrial damage which block mitochondrial import, a necessary step for stabilizing PINK1 on the outer mitochondrial membrane and initiating PINK1/parkin mitophagy (Youle and Narendra, 2011).

5.4.2 Novel roles for actin in mitophagy and mitochondrial fission

Optineurin binds the actin motor myosin VI and this interaction mediates autophagosomes fusion with endosomes positive for the myosin VI-binding partner Tom1 (Tumbarello et al., 2012). However, optineurin's association with an actin motor suggests that actin itself may be recruited during mitophagy and may play a role during autophagosome biogenesis. Indeed, actin was recently found to be recruited during starvation-induced non-selective autophagy (Aguilera et al., 2012), as well as during the autophagy of protein aggregates (Lee et al., 2010c).

Upon nutrient deprivation in HeLa cells, actin has been shown to colocalize with the autophagy proteins Atg14 and Beclin1 and PI3P-rich structures labeled with DFCP1. In addition, depolymerization of actin decreases the number of autophagic vesicles formed in response to starvation but does not disrupt autophagosome maturation or colocalization with ULK1, Atg5, or LC3 (Aguilera et al., 2012). In contrast, another report demonstrated that actin is recruited by HDAC6 (histone deacetylase-6) to ubiquitinated proteins in response to proteasomal inhibition with the drug MG-132 in mouse embryonic fibroblasts (MEFs) during autophagy (Lee et al., 2010c). HDAC6 was found to recruit actin in a cortactin-dependent manner to help stimulate autophagosome/lysosome fusion, and HDAC6 deficiency resulted in autophagosome maturation failure. In addition, actin was not found to be required for starvation-induced autophagy and actin was proposed to preferentially regulate selective autophagy (Lee et al., 2010c). Thus, both the precise role of actin in selective and non-selective autophagy, as well as the temporal dynamics of actin recruitment and polymerization during autophagosome biogenesis and maturation remain to be better characterized.

Our preliminary data demonstrate that actin filaments form around damaged mitochondria during PINK1/parkin mitophagy following parkin recruitment in HeLa cells. Thus, identifying the actin-binding proteins that facilitate actin polymerization on mitochondria will be important to understand how actin is recruited and the rate at which it is recruited to damaged mitochondria. In addition, examining the temporal dynamics of actin recruitment will contribute to our understanding of actin's role during autophagy. Actin may be recruited prior to optineurin recruitment, or alternatively, during the later step of autophagosome biogenesis during DFCP1 recruitment. Thus, identifying the precise time point of actin recruitment will provide insight into whether actin helps to scaffold autophagy receptors prior to autophagosome biogenesis, or whether it might play a more active role during autophagosome formation by modeling the autophagosome membrane or the mitochondria itself. Further, it will be interesting to examine whether actin subsequently recruits myosin motors to the mitochondria during mitophagy which may help facilitate autophagosome formation or fusion with endosomes/lysosomes.

To further study the role of actin in mitophagy, it will be useful to modulate actin dynamics, either by depolymerization actin using drugs such as cytochalasin D or latrunculin, or by expressing different actin binding proteins such as formins which help polymerize actin, or actin disassembly factors such as cofilin. Upon modulation of actin dynamics, it will be important to study whether the PINK1/parkin pathway is still initiated leading to parkin recruitment, as well as whether optineurin and other autophagic machinery including Atg proteins, DFCEP1 and LC3 are still recruited to damaged mitochondria. Thus, examining whether actin is required for efficient mitochondrial regulation would demonstrate an exciting novel role for actin in mitophagy.

Interestingly, mutations in the actin binding protein profilin have also been linked to ALS (Wu et al., 2012). Profilin is responsible for binding actin and facilitating actin polymerization and elongation of actin filaments. As we found that an ALS-associated mutation in optineurin disrupts mitochondrial degradation, it would be exciting to see if profilin also acts in the same mitophagy pathway by regulating actin filament elongation on damaged mitochondria and whether ALS-associated mutations in profilin disrupt actin polymerization leading to defective mitochondrial degradation.

Although the role of actin in mitophagy has never been examined, actin has previously been shown to regulate mitochondrial dynamics by facilitating mitochondrial fission. However, the mechanism by which actin regulates mitochondrial fission still remains unclear. Preventing actin polymerization with either cytochalasin D or latrunculin A leads to decreased mitochondrial fission (De Vos et al., 2005). In addition, loss of formin INF2 (inverted formin 2) or myosin II results in strikingly elongated mitochondria, implicating them as regulators of mitochondrial fission (Korobova et al., 2013, 2014). Recently, actin was found to transiently assemble on the outer mitochondrial membrane immediately following mitochondrial depolarization in HeLa cells and MEFs (Li et al., 2014). In addition, actin localizes to a subpopulation of mitochondria in HeLa cells in which mitochondrial damage has not been induced. To further examine the role of actin in regulating mitochondrial fission, we plan to investigate actin dynamics in healthy cells (without inducing mitochondrial damage) using live cell imaging and observe whether actin is dynamically

recruited to mitochondria and the time frame of this recruitment. Further, we plan to identify whether actin recruitment occurs subsequent to mitochondrial fission events, and whether actin is preferentially regulated to mitochondria which have recently undergone fission.

We also plan to study what role actin plays upon its recruitment to mitochondria. Actin may act to limit mitochondrial motility by anchoring it to the intracellular matrix, or alternatively, actin may help to prevent mitochondrial fusion by creating an actin fence around mitochondria and inhibiting the fusion of the outer and inner mitochondrial membrane which is required for mitochondrial fusion. Actin may also recruit actin-binding proteins to the mitochondria which might play an active role in remodeling mitochondria membrane morphology via force generation or regulate the activity of proteins on the outer mitochondrial membrane such as the GTPase mitochondrial fission protein (Drp1) and fusion proteins (mitofusins). Thus, further understanding the role of actin in regulating mitochondrial dynamics will be important for expanding our knowledge of mitochondrial fission dynamics, which is a critical process in maintaining mitochondrial homeostasis. Finally, examining whether actin is recruited during PINK1/parkin mitophagy in neurons, and whether actin is also transiently recruited to mitochondria to regulate mitochondrial fission/fusion dynamics will provide greater insight into neuronal homeostasis and the potential defects which might occur in neurodegenerative diseases.

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