ABSTRACT

Title of Dissertation: THE RELATIONSHIP BETWEEN AUTOPHAGY, CELL SURVIVAL AND CELL DEATH IN A MODEL OF NEURODEGENERATION AND DEVELOPMENT.

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The catabolic degradation of proteins is vital for the proper function and homeostasis of all cells. Autophagy is one of the major catabolic systems, and it is involved in processes that are as diverse as cell survival, cell death, immune reaction, cancer and neurodegeneration. Neurodegenerative diseases often have the pathology of protein accumulation in inclusions, but it is unclear whether these inclusions cause cell toxicity. Here I show that autophagy has protective functions in a model of a polyglutamine neurodegenerative disease in *Drosophila*. Inhibition of autophagy in this model enhances polyglutmine-induced degeneration, while activation of autophagy suppresses degeneration. Moreover, I observed similar protein aggregates in the larval salivary glands of a *Drosophila* dynein light chain mutant. This dynein

light chain mutant is defective in autophagy, and their salivary glands fail to execute developmentally regulated programmed cell death. Ectopic activation of autophagy is sufficient to suppress the protein accumulation in dynein light chain mutant salivary glands. Both neurons and salivary glands are long-lived post-mitotic cells, and these cells are likely to have unique catabolic needs. Our data indicate that defects in catabolism are responsible for the neurodegenerative and salivary gland cell death defects that I observed, and could explain the association of autophagy with neurodegenerative diseases.

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By

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Chapter 1

Introduction

Neurodegenerative diseases

Neurodegenerative disease refers to a pathological condition that primarily affects neurons and causes neuronal dysfunction and neuronal death. These disorders most commonly arise in the elderly for no apparent reason, and progress in a relentless manner. Diseases of the nervous system combined account for more suffering and care than all other diseases (Cowan and Kandel 2001). In addition, as the prevalence of elderly people increases in society, the frequency and burden of these diseases increases.

Classification and diagnosis of the neurodegenerative diseases have been difficult because of overlapping symptoms. In practice, many diseases are classified by referencing their founder's name, such as Alzheimer's disease. Pathologically, the affected areas of the central nervous system have been used (Przedborski, Vila et al. 2003). For example, neurodegenerative diseases are classified to affect the cerebral cortex, the basal ganglia, the brain stem, cerebellum and the spinal cord, such as Spinobulbar Muscular Atrophy. Diseases of the cerebral cortex, such as in Alzheimer's disease, result in symptoms including memory loss and confusion (i.e. dementia) (Martin 1999). Diseases that predominately affect basal ganglia as in Parkinson's disease are characterized by involuntary movements (Nussbaum and Ellis 2003). Diseases of the brain stem, cerebellum and the spinal cord, such as

amyotrophic lateral sclerosis (ALS), commonly cause muscular atrophy and inability to move (Table 1). In recent years, elucidation of molecular basis of neurodegenerative diseases led to new classification systems. For instance, the terms "tri-nucleotide repeat disorders" and "polyglutamine expansion diseases" were introduced with the discovery of mutations that are caused by expansion of repeat regions in causative genes (Orr and Zoghbi 2007).

The factors that cause neurodegenerative diseases are not completely clear. Relatively small numbers of diseases, like the polyglutamine expansion diseases, have purely genetic causes; some are purely sporadic and others seem to be caused by both genetic and environmental factors. Certain types of neurodegenerative diseases are observed in particular geographical locations. For example, a type of ALS and Parkinson's disease (PD) combination (Guam-ALS) is observed in specific islands of Guam, and is associated with consumption of an indigenous plant species (Spencer, Nunn et al. 1987). In addition, exposure to a neurotoxin that is a side product of the analgesic drug meperidine synthesis is known to cause symptoms identical to Parkinson's disease (Bove, Prou et al. 2005). These examples suggest that neurodegenerative diseases can be caused by purely environmental factors. Investigation of genetic causes of these and other diseases is underway. The most common form of motor neuron degeneration, ALS, is caused by a dominant mutation in the superoxide dismutase (SOD) gene, but this only occurs in 2% of the reported cases (Rosen, Siddique et al. 1993). The causes of the rest of the cases are not known, but mutations in the dynein heavy chain in mouse models have been reported to cause ALS symptoms as well (Hafezparast, Klocke et al. 2003; Chen, Levedakou

et al. 2007). The most common neurodegenerative disorder, Alzheimer's disease, is caused by an autosomal dominant mutation in the amyloid precursor protein in 3-5% of reported cases. Although other mutations have been reported (Nussbaum and Ellis 2003), the cause of the majority of the cases is still unknown. The prevalence of Alzheimer's disease among those 65 to 69 years of age is 1%, and increases to 40-50% among people 95 years of age or older (Hy and Keller 2000). This implies that environmental or intrinsic causes that accumulate with age may increase predisposition to disease. Similarly, only a small fraction of all Parkinson's disease cases are linked to genetic mutations (Nussbaum and Ellis 2003).

In spite of their differences, most of the neurodegenerative diseases are characterized by protein inclusion formation in affected areas of the nervous system. This common observation led to the hypothesis that neurodegenerative diseases might share a common mechanism of pathology. Alzheimer's disease has been associated with two major kinds of inclusions. Extracellular inclusions are derived from the amyloid precursor protein (APP), and form β-sheet structures called "amyloids". Intracellular inclusions are formed by the microtubule-associated protein Tau (Hardy and Selkoe 2002). Parkinson's disease pathology is characterized by the formation of the Lewy bodies, which are protein inclusions with α-synuclein being the major constituent (Gai, Blessing et al. 1995). In ALS, surviving motor neurons develop protein inclusions called Bonina bodies that stain positive for SOD1 (Bruijn, Houseweart et al. 1998; Hafezparast, Klocke et al. 2003). Moreover, polyglutamine expansion diseases, such as Huntington's disease, are all associated with protein

inclusion formation (Zoghbi and Orr 2000). Studies of this common pathology may prove beneficial in understanding neurodegenerative diseases.

Polyglutamine expansion diseases

Polyglutamine expansion diseases are a group of progressive genetic diseases that are caused by increase in the number of naturally occurring CAG triplet (encoding glutamine) repeats in certain genes (Table 1). The number of CAG repeats is normal below approximately 40 repeats, but above this threshold neurodegenerative disease ensues. These diseases are inherited in a dominant fashion, which implies a gain of function effect of CAG expansion (La Spada and Taylor 2003). The significance of having CAG repeats in some genes is not known. The number of CAG repeats in normal alleles of causative genes are highly variable, and they can be as little as 4 CAG repeats in Huntingtin (Htt) in some species (Harjes and Wanker 2003). Therefore, having CAG repeat expansion may have little functional relevance to the protein. Whether expansion of CAG repeats alone causes neurodegeneration is a subject of debate (Orr and Zoghbi 2007). When only the first exon of the *htt* gene, which contains the polyglutamine stretch, is expressed in mice it causes neurodegeneration that is similar to Huntington disease (HD) (Mangiarini, Sathasivam et al. 1996). The CAG expansion in the androgen receptor (AR) that results in spinobulbar muscular atrophy (SBMA) does not cause loss of function of AR, since patients do not show many symptoms of testicular feminization (Doyu, Sobue et al. 1992). Moreover, deletion of AR in humans does not cause any symptoms of neurodegeneration (Brown, Lubahn et al. 1988; Brinkmann 2001). These examples support a model in which expansion of CAG itself causes the disease

and not changes in the function of the protein. Although its function is unknown, Htt has been suggested to be involved in vesicular transport along the axon tracts (Gunawardena, Her et al. 2003). This could fit a model where changes in the protein function lead to neurological dysfunction (discussed further below).

Models of polyglutamine expansion diseases

Polyglutamine expansion diseases are particularly well suited to model in animals due to their characterized mutations and dominant genetic nature. Models have been created in worms, flies, mice and non-human primates. Creation of these models has utilized either ectopic expression of expanded form of the causative protein by providing it in trans or by stable insertion of the gene into the genome. The HD models in *C. elegans* have been created by ectopic expression of expanded form of the first exon of Htt. Expression of 88, 128 or 150 glutamine repeats in the first exon resulted in impaired neuronal function in a length-dependent fashion (Faber, Alter et al. 1999; Parker, Connolly et al. 2001). In addition, ectopic expression of a polyglutamine stretch fused with GFP caused neurodegeneration with visible GFP punctate inclusions (Satyal, Schmidt et al. 2000). This shows that expression of expanded glutamine alone can recapitulate disease phenotype in *C. elegans*. Moreover, the convenience of RNAi screening methods and advancements in aging research in *C. elegans* have facilitated investigation of genetic modifiers of neurodegeneration in a model that expressed glutamine stretches alone (Brignull, Morley et al. 2006).

Drosophila is convenient for recapitulating polyglutamine expansion diseases due to its tissue specific expression tool, the UAS/GAL4 system (Brand and Perrimon

1993), and availability of various behavioral tests, including robust assays to measure motor activity. Expanded polyglutamine-encoding genes can be cloned and expressed in either subsets of neurons, the entire nervous system or in the eye. The fly eye has beneficial attributes for studying neurodegeneration, as it is not an essential organ and degeneration of cells can easily be evaluated based on the external structure of the eye. *Drosophila* was the first organism in which a polyglutamine expansion disease spinocerebellar ataxia 3 (SCA3) was modeled (Warrick, Paulson et al. 1998). Later HD, spinocerebellar ataxia 1 (SCA1) and spinobulbar muscular atrophy (SBMA) were also modeled in *Drosophila* (Jackson, Salecker et al. 1998; Fernandez-Funez, Nino-Rosales et al. 2000; Takeyama, Ito et al. 2002). The strength of using *Drosophila* as a model organism was recently demonstrated by a model of SCA3, which led to a novel discovery in a genetic screen for modifiers of disease symptoms. In this screen, the authors reported that, in addition to protein, ataxin-3 mRNA itself caused some aspects of neurodegeneration (Li, Yu et al. 2008). This finding implied that mRNAs may contribute to symptoms seen in the polyglutamine expansion diseases.

All of the polyglutamine diseases have been modeled in mice (Marsh, Lukacsovich et al. 2008). The first HD model in mice that recapitulated the disease symptoms was made by ectopic expression of an expanded glutamine first exon of Htt. In addition, knock-in models of HD were made in which glutamine repeat region of endogenous mouse *htt* was expanded by homologous recombination (Levine, Klapstein et al. 1999; Lin, Tallaksen-Greene et al. 2001). Symptoms of neurodegeneration in these models were milder than ectopic expression models. In

addition to HD models, SBMA mouse models were made by ectopic expression of polyglutamine-expanded full length or truncated AR (Bingham, Scott et al. 1995; Abel, Walcott et al. 2001). Neurodegeneration was seen only in males, and castration reversed disease progression indicating the need for the ligand of androgen receptor (AR), testosterone, for disease onset. Moreover, in a mouse model of SBMA hormone therapy with Leuprorelin that decreased release of testosterone, reversed SBMA symptoms (Katsuno, Adachi et al. 2003). This example shows that mouse models can successfully be used to assess the outcomes of therapeutic agents.

Recently a rhesus macaque model of HD expressing the first exon of Htt with various repeats of glutamine was developed using lentivirus as a gene delivery tool. These animals showed symptoms of HD including involuntary movements, swallowing and respiratory difficulty with pathology of inclusion formation in their brains (Yang, Cheng et al. 2008). In the future, non-human primate models could be used to better assess the outcomes of therapeutic chemicals that are being considered for use in humans.

Molecular Pathogenesis of polyglutamine expansion diseases

Transcription factor deactivation

Transcription factors are commonly found as part of the protein inclusions in degenerating cells and tissues in neurodegenerative diseases. This led to the hypothesis that changes in gene expression due to sequestration of transcription factors could lead to the symptoms of neurodegeneration (Okazawa 2003). CREB-binding protein (CBP) has been reported in protein inclusions caused by numerous

mutant proteins, including Htt and AR (McCampbell, Taylor et al. 2000; Nucifora, Sasaki et al. 2001). Although no direct evidence support this model, these results led to speculation that cell toxicity was caused either by titration or mislocalization of CBP. Similarly, the transcription factor Sp1 unnaturally interacts with polyglutamine-expanded Htt in its soluble form, and Sp1's transcriptional activity is repressed in early phases of HD, suggesting that repression of Sp1 could lead to some of the neurodegenerative symptoms (Dunah, Jeong et al. 2002). Furthermore, it was suggested that polyglutamine-expanded Htt leads to repression of brain derived neurotrophic factor because Htt's natural interaction with a transcription factor that activates neurotrophic factors is reduced (Zuccato, Tartari et al. 2003). Since neurotrophic factors are necessary for neuronal survival this could explain why neurons would be exclusively affected (Zweifel, Kuruvilla et al. 2005).

Loss of mutant protein function

Even though polyglutamine expansion diseases follow a dominant pattern of inheritance, it is possible that expansion of polyglutamine causes loss of natural protein function. If this is the case, deletion of causative genes should lead to neurodegeneration. Homozygous deletion of *htt* in mice is lethal (Duyao, Auerbach et al. 1995), and conditional *htt* knock-out caused progressive neurodegeneration in one report (Dragatsis, Levine et al. 2000). Moreover, ectopic expression of wild type Htt rescues neurodegenerative symptoms in a HD model (Leavitt, Guttman et al. 2001). These examples demonstrate that loss of Htt function can lead to some of the HD phenotypes, perhaps due to its regulation of brain derived neurotrophic factor. Moreover, in a mouse model of SCA1, which is caused by mutation of Ataxin-1,

neurodegenerative symptoms were decreased by incorporation of an extra copy of wild type *ataxin-1* into the genome (Bowman, Lam et al. 2007). By contrast to these examples, other mouse models with deletion in *htt* or *ataxin-1* did not result in neurodegenerative phenotypes (Duyao, Auerbach et al. 1995; Matilla, Roberson et al. 1998). In addition, homozygous HD patients have similar severity and progression of symptoms as heterozygous HD patients (Wexler, Young et al. 1987). Collectively, it is not clear whether loss of mutant gene function is a factor in polyglutamine expansion diseases.

Protein misfolding and protein stress

Expansion of polyglutamine beyond a threshold is likely to cause changes in protein structure. Proteins with expanded polyglutamines were found to assume novel confirmations in cell lines (Welch and Diamond 2001). Furthermore, in vitro experiments showed that proteins with polyglutamine expansions, unlike normal proteins, form a β-sheet dominated structure that results in toxicity once microinjected into cell lines (Nagai, Inui et al. 2007). These data imply that expansion of polyglutamine renders the proteins susceptible to misfolding. This could be one of the earlier events that eventually lead to protein inclusion formation and neurodegeneration. Overexpression of heat shock chaperones Hsp40 and Hsp70 was shown to ameliorate toxicity caused by expanded AR expression in cell lines (Kobayashi, Kume et al. 2000). Therefore, it is likely that protein stress ensues in cells due to misfolded proteins. Moreover, since, ubiquitin is very frequently found in protein inclusions of neurodegenerative disease samples, the ubiquitin-proteasome system could be involved in degradation of these proteins (Ross and Pickart 2004).

Proteasome inhibition and inclusion formation

Proteins with expanded polyglutamine stretches cause neurodegenerative diseases with the pathology of inclusion formation (Ross and Poirier 2004). The common observation that inclusions contain truncated forms of the polyglutamine expanded protein (Sieradzan, Mechan et al. 1999) led to the hypothesis that these proteins should initially be cleaved. Caspase-3 and caspase-6 have been implicated in the initial cleavage of Htt (Graham, Deng et al. 2006). Over-expression of a polyglutamine expanded Htt that is resistant to caspase-6 cleavage did not show any form of neurodegeneration, but this was not the case for a caspase-3 resistant form of Htt. It has been hypothesized that these cleavage products would first form soluble oligomers and later aggregate in protein inclusions together with other proteins (Kawaguchi, Kovacs et al. 2003). However, it is not clear how these inclusions cause toxicity. It is likely that these oligomers like other misfolded proteins are directed to the ubiquitin-proteasome system for degradation. However, the proteasome has been shown to be dysfunctional in many models of polyglutamine expansion diseases (Bence, Sampat et al. 2001; de Pril, Fischer et al. 2004; Ross and Poirier 2004; Pandey, Nie et al. 2007). The mechanism of proteasome inhibition is not known, but the reason is likely to be related to either aberrant oligomers or misfolded polyglutamine proteins.

The association of protein inclusion formation and neurodegeneration raises the question whether inclusions are toxic. Several lines of evidence in recent years suggested that inclusions are by products of protein stress and are not exclusively toxic to the cell. In a population of cells expressing polyglutamine-expanded Htt,

cells with inclusions were shown to survive significantly longer than cells without inclusions (Arrasate, Mitra et al. 2004). Cells without inclusions have more soluble oligomers of Htt with polyglutamine expansion, which positively correlated with shortened life span. Moreover, in a mouse model of SBMA soluble oligomers were detected several weeks before the onset of symptoms, and symptoms would start before inclusion formation was observed (Li, Chevalier-Larsen et al. 2007). Significantly, soluble oligomers cleared upon castration. These studies and others (Taylor, Tanaka et al. 2003; Slow, Graham et al. 2005) suggest that soluble oligomers are the toxic species, that toxicity may be achieved by inhibiting the proteasome, and that protein inclusion formation is an end point that stabilizes these oligomers. This speculation is challenged by the transcription factor deactivation hypothesis discussed above. Since aberrant oligomers and inclusions can sequester transcription factors, inclusions formed inside the nucleus could modify gene expression. It is likely that pathogenesis of polyglutamine expansion diseases is a result of combination of several factors (Shao and Diamond 2007).

Cell response to protein stress: the ubiquitin-proteasome system and autophagy

The ubiquitin-proteasome system

The proteasome system and autophagy are the two major catabolic systems in the cell. The proteasome system is the best characterized catabolic system (Etlinger and Goldberg 1977), and it is essential for the degradation of short lived proteins that function in critical signaling pathways. Among the pathways that the proteasome regulates are cell cycle progression, differentiation and development, modulation of

cell surface receptors, DNA repair, and the immune system (Ciechanover, Orian et al. 2000). Degradation of proteins by the proteasome system involves poly-ubiquitination of a target protein by the collaborative action of three enzymes followed by degradation by the 26S proteasome. Each ubiquitin moiety is activated by a ubiquitin activating enzyme (E1) by converting C-terminal Gly of ubiquitin to a high energy thiol ester by a Cys residue. Then a ubiquitin conjugating enzyme (E2) transfers this activated ubiquitin to the target protein that has been bound to a ubiquitin-protein ligase (E3). The target protein is poly-ubiquitinylated by successive repetitions of this reaction (Glickman and Ciechanover 2002). This central mechanism in the cytosol holds true for all the proteins that are targeted to the proteasome. Endoplasmic reticulum (ER) and nuclear proteins that are targeted for degradation are thought to be first transported out of their organelles.

During ER-associated degradation (ERAD), proteins or subunits in the ER that are misfolded or unassembled are targeted to the proteasome. These proteins are transported into the cytoplasm by a channel that contains Sec61 as a structural element, and then ubiquitinylated and degraded in the cytoplasm (Scott and Schekman 2008). Failure of ERAD leads to protein stress in the ER that can lead to species of protein oligomers that are often associated with inhibition of the proteasome and formation of protein inclusions in the cytoplasm (Bence, Sampat et al. 2001). The collective defensive response that the cell launches in an ER protein stress is referred to as unfolded protein response (UPR). It refers to a variety of adaptations that increase molecular chaperone expression, decrease overall translation rate and increase degradation of misfolded proteins (Schroder and Kaufman 2005).

In addition, prolonged UPR may activate apoptotic pathways (Hetz, Bernasconi et al. 2006). Several disorders have been associated with altered UPR, including cystic fibrosis (Johnston, Ward et al. 1998).

Autophagy

Three forms of autophagy have been described, including macro-autophagy, micro-autophagy and chaperone-mediated autophagy (Reggiori and Klionsky 2002). Macro-autophagy (hereafter referred to as autophagy), literally "self-eating", is classically regarded as a bulk degradation system of long-lived proteins and organelles, and is the best studied form of this process. Autophagy is used to deliver cargo, including proteins and organelles, to the lysosome for degradation (Klionsky and Emr 2000). Autophagy is activated in response to starvation conditions, and provides metabolic substrates that promote cell survival (Takeshige, Baba et al. 1992; Thumm, Egner et al. 1994). Recent evidence suggests that autophagy is not just a bulk, non-selective degradation system, but has more specific functions that entail intricate regulation. These include elimination of bacteria, antigen presentation and programmed cell death (Deretic 2005; Mizushima 2005). Autophagy commences with formation of a double membrane isolation vesicle in the nucleation site, which is called the pre-autophagosomal structure (PAS). The isolation membrane encapsulates cytoplasmic components to form the "autophagosome" which fuses with the lysosome where substrates are degraded by hydrolases. The origins of the PAS and isolation membrane are not clear. However, recent evidence suggests that ER membrane can serve as the source for the isolation membrane (Axe, Walker et al. 2008), but this remains controversial and it is possible that other membranes may be

used to initiate autophagy. Completion of the isolation membrane requires the activity of two conjugation systems (Ohsumi 2001). The Atg12 conjugation system results in the formation of the Atg12, Atg5 and Atg16 complex, while the Atg8 system results in conjugation of phosphatidylethanolamine (PE) to Atg8 (LC3 in mammals), which serves as a structural element of isolation membranes and autophagosomes (Kabeya, Mizushima et al. 2000; Xie, Nair et al. 2008). This has led to utilization of fluorescent protein tagged Atg8/LC3 as a marker of autophagosomes. Moreover, there is evidence suggesting that the Atg12-Atg5 complex promotes lipidation of Atg8 (Hanada, Noda et al. 2007). How the autophagosome meets with the lysosome is not clear, but once it docks and fuses to a lysosome, the inner autophagosome membrane with its contents enter the lysosome. In addition, autophagic and endocytic pathways can converge to form "amphisomes", which later are degraded by the lysosome (Berg, Fengsrud et al. 1998). This suggests an intimate interaction between vesicular trafficking pathways in the cell, and the regulation of both autophagy and endosome maturation by the Vps34, Vps15, and Atg6 (Beclin1 in mammals) complex supports this conclusion (Juhasz, Hill et al. 2008).

Autophagy in neurons and neurodegeneration

Studying autophagy in the nervous system has been challenging because of the difficulty to observe autophagic structures under physiological conditions. An investigation in the nervous system of mice carrying an autophagosome reporter transgene did not lead to any observation of autophagosomes under either physiological or under starvation conditions (Mizushima, Yamamoto et al. 2004). Indeed, for a long time autophagic structures had only been seen in the nervous

system upon severe stress, and during re-growth and regeneration upon injury (Rubinsztein, DiFiglia et al. 2005). Several studies have reported autophagic structures in the neurons of neurodegenerative disease models (Kegel, Kim et al. 2000; Nixon, Wegiel et al. 2005; Boland, Kumar et al. 2008). Most of these studies assumed that autophagy was involved in the turnover of organelles and cytoplasm during repair.

Landmark studies of *atg5* and *atg7* conditional knockout mice in the nervous system significantly advanced our understanding of the importance of autophagy in neurons (Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). These knockout mice had reduced longevity, severe motor deficiency at birth, and histological analyses of their nervous system revealed significant neuronal damage associated with DNA fragmentation. Histological and ultrastructural analyses revealed that mice lacking *atg5* and *atg7* had ubiquitinylated fibrillar inclusions in their nervous system that is reminiscent of neurodegenerative disease. Importantly, *atg7* mice had a normally functioning proteasome system. These results showed that autophagy was essential for nervous system function, and that basal autophagy in neurons is critical for the health and homeostasis of the brain. The inability to observe autophagic structures in the nervous system under physiological conditions has been attributed to a very efficient rate of autophagy such that autophagic structures could not be detected (Cuervo 2006; Boland, Kumar et al. 2008).

The observation of autophagic vacuoles in neurodegenerative models, and the presence of protein inclusions in autophagy mutant mice, led to extensive investigation of the role of autophagy in neurodegenerative diseases. Autophagy

could either degrade cells or be activated to cope with the protein stress that leads to inclusion formation during neurodegeneration (Levine and Kroemer 2008). In support of the latter possibility, activation of autophagy reduced the toxicity of Htt polyglutamine expansion in fly eyes and motor activity of mice respectively (Ravikumar, Vacher et al. 2004). In this study, we investigated the role of autophagy in a fly model of SBMA (Chapter 2).

Autophagy and the proteasome system

The observation of ubiquitin-positive protein inclusions in *atg* gene knockout mice suggests that the function of the proteasome system and autophagy are related. This may imply that proteasome substrates can be degraded by autophagy. In a cell line model of Parkinson's disease the mutant form of α-synuclein, which causes the disease, was found to be degraded by both the proteasome system and autophagy (Webb, Ravikumar et al. 2003; Cuervo, Stefanis et al. 2004). Therefore, the proteasome system and autophagy are capable of degrading the same substrate.

While studies on the relationship of the proteasome system and autophagy are in their infancy, several candidate proteins emerged that may be involved in this relationship. HDAC6 is a cytoplasmic histone deacetylase that belongs to a different class than nuclear histone deacetylases (Grozinger, Hassig et al. 1999). It is primarily found in the cytoplasm associated with microtubules. It has a ubiquitinylated protein binding domain, and it was shown to bind to dyneins (Hubbert, Guardiola et al. 2002). It was suggested that HDAC6 is directly involved in the formation of specialized protein inclusions that are surrounded by membranes called "aggresomes" (Kawaguchi, Kovacs et al. 2003; Boyault, Gilquin et al. 2006).

Another protein that may link the ubiquitin-proteasome system and autophagy is p62. It has an ubiquitinylated protein binding domain, a PB1 domain that facilitates polymerization, and an LC3/Atg8 binding domain (Ichimura, Kumanomidou et al. 2008). The involvement of p62 in inclusion formation was confirmed in hepatocytes derived from autophagy deficient mice. While autophagy mutants developed ubiquitin positive inclusions, autophagy and p62 double mutants failed to form inclusions (Komatsu, Waguri et al. 2007). p62 and ubiquitin-positive protein inclusions were formed in a cell line model of Huntington's disease that resulted in proteasome inhibition, and the p62 in inclusions were physically bound to and co-localized with Atg8, suggesting that inclusions were recognized and degraded by autophagic machinery (Bjorkoy, Lamark et al. 2005). Moreover, a recent study characterized the crystal structure of Atg8 and p62 binding. In this study, point mutations in the Atg8 binding domain resulted in ubiquitin positive inclusion formation. These results suggest that p62 provides a direct link between ubiquitinylated proteins and autophagy (Ichimura, Kumanomidou et al. 2008).

Finally, the autophagy linked FYVE protein (Alfy) was suggested to be involved in autophagic clearance of ubiquitinylated proteins. Alfy has a FYVE domain that binds to phosphatidylinositol-3-phosphate, which is necessary for induction of autophagy (Simonsen, Wurmser et al. 2001). In starved HeLa cells, Alfy co-localizes with Atg8 showing its association with autophagic machinery. In addition, during proteasome inhibition of HeLa cells Alfy localized to ubiquitin positive protein inclusions (Simonsen, Birkeland et al. 2004). In *Drosophila*, mutations in the Alfy's homolog *blue cheese* cause progressive neurodegeneration with amyloid like protein

inclusion pathology (Finley, Edeen et al. 2003). These findings imply that Alfy may function in catabolic degradation of proteins.

Dyneins and neurodegeneration

Dynein: a unique molecular motor protein

Molecular motors are specialized protein complexes that provide directed movement in cells. They are composed of three main families: actin based myosins, microtubule based kinesins and dyneins (Schliwa and Woehlke 2003). Myosins and kinesins have been extensively classified into at least 17 and 14 classes respectively, since they are composed of components with high sequence variability. By contrast, dyneins have been classified into only 2 classes (axonemal and cytoplasmic) due to their high sequence conservation across species and cellular compartments (Hook and Vallee 2006). Myosins are classically localized to cell cortex and are involved in maintaining cell shape. However, a number of so-called unconventional myosins have been found to be involved in many key cellular events including organelle transport and endocytosis (Wu, Jung et al. 2000). Kinesins are involved in movement of cargo towards plus end of microtubules (towards the cortex), while dyneins towards minus end (towards the nucleus). Even though they both function on microtubules, kinesins and dyneins are structurally and functionally distinct. Nevertheless, they are interdependent, since inhibiting either dyneins or kinesins disrupts movement on microtubules in both directions (Martin, Iyadurai et al. 1999). This interdependence could be due to the necessity of dyneins to be carried by kinesins to the distal ends of the cell whence they can start their retrograde movement (Ligon, Tokito et al. 2004).

Dyneins are members of the ATPase superfamily with a large (~3000 amino acid) modular motor region that is composed of 6 ATPase modules, which collectively make up the dynein heavy chain (DHC, ~500 kDa) (Shpetner, Paschal et al. 1988). The dynein heavy chain binds to a variety of smaller subunits: intermediate chains (ICs, ~60-140 kDa), light intermediate chains (LICs, 30-60 kDa) and light chains (LCs, 8-30 kDa) (Vallee, Williams et al. 2004). The classic view of a dynein motor is that each dynein complex is composed of two DHCs, two ICs, two LICs and a variable number of LCs (Vallee, Wall et al. 1988). However, this view has been challenged in more recent years (Wickstead and Gull 2007). Moreover, ICs bind to an important binding partner, $p150^{Glued}$ that is a structural element of dynactin, which increases efficiency of dynein movement (Karki and Holzbaur 1995; Vaughan and Vallee 1995).

LCs, which bind to the dynein complex through the ICs, are primarily responsible for cargo recognition. Three classes of LCs have been identified that are specific for the cytoplasmic dynein: Tctex, LC8 and LC7. Tctex-1 has been found to interact with rhodopsin, Trk receptor and others (Tai, Chuang et al. 1999; Yano, Lee et al. 2001). LC7 has been found to interact with TGFβ receptor (Tang, Staub et al. 2002). LC8 is the most conserved of the light chains and has been found to interact with numerous cargos (Jaffrey and Snyder 1996; Puthalakath, Huang et al. 1999; Lo, Kan et al. 2005). Interestingly, LC8 was shown to function in the regulation of binding partners without the involvement of motor activity or association with other dyneins (Puthalakath, Huang et al. 1999). Moreover, while higher plant genomes contain LC8 homologous sequences, they do not contain sequences of any of the other dynein

components (Lawrence, Morris et al. 2001; Wickstead and Gull 2007). This implies that LC8 could have functions that are different than motor activity in plants, even though, it cannot be ruled out that these findings could be due to unusual dynein sequence variability in plants.

Dyneins in the mechanism of autophagic movement

The need for autophagosomes to dock and fuse with lysosomes implies that there is a mechanism to move either of these organelles to meet with each other in the cytoplasm. There are several lines of evidence indicating that once formed, autophagosomes move in a dynein-dependent fashion towards the centrosome, where lysosomes are thought to be concentrated. First, microtubule disruption in hepatocytes significantly reduces autophagosome-lysosome fusion events (Kochl, Hu et al. 2006). Second, microtubule destabilizing agents such as nocodazole inhibit the formation and regular movement of autophagosomes in cell lines (Fass, Shvets et al. 2006; Kimura, Noda et al. 2008). Third, inhibition of dynein components, the dynein intermediate chain or the $p150^{Glued}$ disrupts regular movement of autophagosomes (Kimura, Noda et al. 2008). Since microtubule disruption inhibits autophagosome formation, dyneins may also be involved in bringing cytoplasmic components destined for autophagic degradation to the pre-autophagosomal structure, although there is no direct evidence for this possibility.

Dynein mediated transport in neurons

Endocytic vesicles that are transported by dyneins from axon ends to the cell body in neurons are also called “signaling endosomes” due to their cargo that contains signaling molecules. The most studied example is transport of the family of

neuronal growth factors (NGFs) collectively known as neurotrophins. The NGFs that are released from the axon bind to tyrosine kinases (Trks) or to p75 receptors and this is followed by the formation of endocytic vesicles (Grimes, Beattie et al. 1997; Bronfman, Tcherpakov et al. 2003). Following endocytosis, NGFs influence axon and dendrite growth, and neuronal differentiation (Zweifel, Kuruvilla et al. 2005). For the signal to take any effect, the endocytic vesicle has to be transported through the axon to the cell body (Ye, Kuruvilla et al. 2003). Retrograde transport of NGFs is essential for neuronal survival since removal of NGFs from axon ends but not from the cell body leads to cell death (Campenot 1977). This retrograde transport is dynein dependent, since disruption of dynein activity impairs the transport of NGFs (Heerssen, Pazyra et al. 2004). In addition to signaling endosomes, dyneins also carry proteins with nuclear localization signal (NLS), such as transcription factors, to the nucleus. Following neuronal injury, retrograde transport of NGFs decrease significantly, while transport of NLS containing proteins increases (Raivich, Hellweg et al. 1991). The interaction of dyneins and NLS containing proteins are maintained by “importins”, which also mediate nuclear import of these signals through the nuclear core complex (Hanz and Fainzilber 2006). Downregulation of this signaling by ectopic expression of dominant negative NLS proteins impairs regeneration of neurons (Hanz, Perlson et al. 2003). Intriguingly, in injured neurons the dynein-importin complex can transport some signaling proteins without an NLS through interactions with vimentin or dynactin (Perlson, Hanz et al. 2005).

Dynein mutations that result in neurodegeneration

The importance of retrograde transport and therefore of dyneins for neuronal survival led to investigations of the outcomes of dynein mutations in the nervous system. Since peripheral neurons have long axons spanning throughout the body, the effects of dynein loss is likely to be considerable in these neurons. Retrograde transport was defective in a mouse model of the motor neuron disease ALS which contained a dominant negative form of SOD1 (*sod1*^{G93A}) (Murakami, Nagano et al. 2001). However, only a minority of ALS cases is due to mutations in *sod1* (Cleveland and Rothstein 2001). Other mutations that caused ALS in mice were identified in the dynein heavy chain (Hafezparast, Klocke et al. 2003): heterozygous *loa*/+ and *cra*/+ mice had significant muscle and motor neuron loss with a pathology of protein inclusion formation that are positive for neurofilaments and SOD1. Another mouse mutation in the dynein heavy chain called *sprawling* (*swl*) had a phenotype similar to *loa* (Chen, Levedakou et al. 2007). Interestingly, ALS phenotype of the *sod1*^{G93A} mice is partially rescued in the double mutant mice *sod1*^{G93A}*/loa* (Kieran, Hafezparast et al. 2005) and in *sod1*^{G93A}*/cra* but not in *sod1*^{G93A}*/swl* (Teuchert, Fischer et al. 2006). This partial rescue could be due to inability to transport the dominant negative SOD1^{G93A} within the motor neurons of *loa* and *cra* heterozygotes. This hypothesis is supported by the detection of SOD1 in protein inclusions that appear in the motor neurons of the *loa*/+ and *cra*/+ mice. The inability of *swl* to ameliorate *sod1*^{G93A} phenotype could be due to *swl* being a weaker allele however, this subject is under debate (Strom, Gal et al. 2008). The aforementioned mutants are all in dynein heavy chain. This raises the question of whether mutations in other subunits of dynein could cause neuron disease. A

mutation was found in the structural element of dynactin, p150^{Glued}, in a family with progressive motor neuron disease (Puls, Jonnakuty et al. 2003). Later a mouse line carrying the mutant form of the p150^{Glued} was generated (Lai, Lin et al. 2007). These mice had slowly progressing motor neuron disease with the pathology of motor neuron loss and decrease in locomotor functions. Interestingly, another mouse line of the same p150^{Glued} mutation was described to result in a weak form of the polyglutamine expansion disease spinobulbar muscular atrophy (SBMA) with a pathology of distal motor neuron degeneration (Chevalier-Larsen, Wallace et al. 2008). In this mouse line, however, retrograde transport was normal, perhaps due to weaker expression of the mutant form of p150^{Glued}. Furthermore, transcription of dynactin and the rate of retrograde transport were found to be significantly reduced in a mouse model of SBMA expressing mutant human androgen receptor (Katsuno, Adachi et al. 2006). This mouse model displays protein inclusions and loss of motor neurons. Upon castration these symptoms disappear together with the restoration of dynactin transcript and of the rate of retrograde transport.

In addition to mutations in dynein, a mutation in a kinesin was found to cause motor neuron disease in humans and a mouse model (Zhao, Takita et al. 2001). Taken together these studies demonstrated the importance of microtubule based transport and of dyneins in neuronal function.

Cell death

The death of neurons is a common pathogenic observation in neurodegenerative diseases. Although dysfunctional neurons that have not yet died may contribute to

neurodegenerative symptoms, studies of neuronal death will help to complete our understanding of the disease process.

Three morphological forms of cell death have been observed during development, including apoptotic, autophagic and non-lysosomal (necrotic) cell death (Schweichel and Merker 1973; Clarke 1990). Apoptotic cell death is the most studied and is characterized by DNA fragmentation, cell condensation, and membrane blebbing (Kerr, Wyllie et al. 1972). Apoptotic machinery is triggered by cytochrome c release from the mitochondria followed by the assembly of the apoptosome that includes the apoptotic protease activating factor (Apaf-1) and caspase 9 (Liu, Kim et al. 1996; Zou, Henzel et al. 1997). Even though this process is generally well conserved, it's been suggested that the apoptosome assembly does not require cytochrome c release in *Drosophila* (Dorstyn, Read et al. 2002). Apoptosome assembly is followed by downstream activation of effector caspases such as caspase-3, which degrades many cell substrates thereby triggering cell death (Timmer and Salvesen 2007). Last stage of apoptosis is removal by a phagocyte following the recognition of the "eat me" signal on the plasma membrane of the apoptotic cell (Savill and Fadok 2000). The best characterized of these signals is the phospholipid phosphatidylserine (Fadok, Voelker et al. 1992). The contribution of apoptosis to neurodegenerative diseases is not clear. Several studies reported activation of caspases in neurodegenerative disease models, which may imply cell death by apoptosis (Wootz, Hansson et al. 2004; Yamada, Iwatsubo et al. 2004). In addition, loss of function of the Apaf-1 homolog in *Drosophila* reduced neurodegeneration in a model of HD, supporting the role of apoptosis in neurodegeneration. By contrast, neurodegenerative symptoms

were dependent on caspase-6 mediated cleavage of Htt in another model of HD suggesting that caspase activation may serve to initiate the disease process rather than killing the cell (Graham, Deng et al. 2006).

Autophagic cell death is characterized by the presence of numerous autophagosomes and autolysosomes containing cytoplasmic components in dying cell (Baehrecke 2005). Unlike apoptosis, cells dying with an autophagic morphology are not cleared by phagocytosis, and instead they degrade using their own lysosomal enzymes. Caspase activation has also been implicated in autophagic cell death. *Drosophila* larval salivary glands die as part of the developmental program in a caspase and autophagy dependent manner, without phagocytosis (Martin and Baehrecke 2004; Berry and Baehrecke 2007). In addition, it has been reported that brain damage due to constriction of blood supply (cerebral ischemia) involves cell death by caspase activation with autophagic morphology (Rami and Kogel 2008). By contrast, autophagic cell death was observed in cell lines that were defective in activating caspases (Shimizu, Kanaseki et al. 2004). Moreover, it is well established that ectopic activation of autophagy is sufficient to kill cells (Scott, Juhasz et al. 2007). Although autophagy has been associated with many examples of dying cells in diverse taxa, the precise role of autophagy during cell death requires further investigation.

Necrotic cell death is due to short, detrimental insults that result in swelling of organelles and loss of cellular integrity (Schweichel and Merker 1973). Necrotic cell death might be an alternative to other forms of cell death and it does not utilize the caspase or the autophagy machinery (Oppenheim, Flavell et al. 2001).

Concluding remarks

Disruption of the fine balance between anabolism and catabolism is likely to have important consequences to the cell. Dysfunctional catabolism and protein stress has been linked to toxicity in neurodegenerative diseases. Here, I first investigate the relationship between the proteasome system and autophagy in a *Drosophila* model of spinobulbar muscular atrophy (SBMA). Next, I investigate the consequences of a mutation in dynein light chain that causes defects in protein clearance, larval salivary gland cell death, and locomotor activity in *Drosophila*.

Table 1 : Common Neurodegenerative Diseases

Name	Cause	Symptoms	Pathology
Non-Polyglutamine Expansion Diseases			
Alzheimer's disease (adult onset [7th to 9th decades], progressive)	Autosomal dominant (3 to 5 % of cases), sporadic. Mutations in amyloid precursor protein (APP), Presenilins.	Memory loss, confusion.	Irreversible dementia, neuronal and synaptic loss, protein inclusions.
Amyotrophic Lateral Sclerosis (adult onset [5th to 6th decades], progressive)	Mutation of superoxide dismutase 1 (SOD1, less than 20% of cases), sporadic.	Muscle weakness, lack of coordination of movements.	Degeneration of motor neurons, protein inclusions and axonal swelling. Death within 3 to 5 years of diagnosis.
Parkinson's disease (adult [6th to 7th decades], progressive)	Autosomal recessive, autosomal dominant, multiple loci, α-synuclein, sporadic.	Tremor, rigidity, repetitive movements, postural instability.	Loss of dopaminergic neurons in substantia nigra, protein inclusions called Lewy bodies.
Polyglutamine Expansion Diseases			
Dentatorubral-pallidoluysian atrophy (DRPLA) (adult or juvenile onset, progressive)	Autosomal dominant, CAG expansion of Atrophin-1.	Uncontrollable movements (chorea), ataxia, lack of coordination, dementia.	Central nervous system atrophy, protein inclusions.
Huntington's disease (adult onset, progressive)	Autosomal dominant, CAG expansion of Huntingtin (HTT).	Uncontrollable movements (chorea), cognitive impairment, depression, compulsive behavior.	Neuronal loss of striatum, frontal and temporal cortex. Protein inclusions in the brain.
Kennedy's disease (Spinal bulbar muscular atrophy). Seen in adult males, progressive.	Autosomal dominant, CAG expansion of androgen receptor (AR). Requires testosterone.	Tremor of the hands, muscular atrophy, impairment of motor functions.	Loss of motor neurons, protein inclusion formation.
Spinocerbellar ataxias (Types 1, 2, 3, 6, 7, 17, adult onset, progressive)	Autosomal dominant, CAG expansion of ataxin (ATXN).	Lack of coordination of movements.	Neuronal loss, protein inclusions, blindness, early fatality.

Sources: Martin 1999; Orr and Zoghbi 2007, OMIM

Chapter 2

The proteasome and autophagy are cytoprotective in a *Drosophila* model of Spinal Bulbar Muscular Atrophy

This study is part of a published collaborative work referenced below:

Pandey, U. B., Z. Nie, Y. Batlevi, Y. *et al*. (2007). "HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS." Nature 447(7146): 859-63.

Abstract

The neurons of most neurodegenerative disorder patients contain protein inclusions, which suggest that insufficient protein degradation might be a factor in disease. The ubiquitin proteasome system and the autophagy are the major contributors of catabolic degradation. We observed that when the proteasome is inhibited by expression of a dominant negative subunit, autophagy is induced. We generated a *Drosophila* model of spinal bulbar muscular atrophy (SBMA) that recapitulates the characteristics of this disease in the eye, including ligand- and length-dependent degeneration. We show that the proteasome system is impaired in this SBMA model, and autophagy is induced. Moreover, further induction of autophagy by ectopic expression of PTEN is cytoprotective, and impairment of autophagy enhances degenerative phenotypes. We suggest that proteasome inhibition contributes to neurodegenerative symptoms, and that autophagy acts to compensate for proteasome impairment in neurodegenerative diseases.

Introduction

Protein inclusion formation is a pathological hallmark of neurodegenerative diseases, but the significance of inclusion formation is not known. Protein inclusions frequently contain the fragments of the mutant protein, such as either Htt or androgen receptor in the contexts of either Huntington's disease or SBMA, in addition to other proteins, including transcription factors, heat shock proteins and ubiquitin (McCampbell, Taylor et al. 2000; de Pril, Fischer et al. 2004). Polyglutamine expansion diseases are caused by gain of function mutations, and are transmitted in a dominant fashion (Gurney, Pu et al. 1994; Orr and Zoghbi 2007). Importantly, complete deletion of the genes responsible for some of these diseases does not cause neurodegeneration (Brown, Lubahn et al. 1988; Brinkmann 2001). How the expansion of polyglutamine leads to toxicity is not fully understood. It has been reported that proteins with large numbers of glutamines tend to misfold (Welch and Diamond 2001). This observation led to the hypothesis that neurodegenerative diseases could be protein confirmation diseases in which the capacity of misfolded protein load is increased beyond the ability of the cell to degrade these proteins (Nedelsky, Todd et al. 2008). There is increasing evidence that inclusions are not toxic but the smaller soluble oligomers are, and inclusions are formed as an end result (Taylor, Tanaka et al. 2003; Arrasate, Mitra et al. 2004; Li, Chevalier-Larsen et al. 2007).

The hypothesis of toxic misfolded protein load led to investigations of the degradation pathways in the context of neurodegenerative diseases. If a newly synthesized protein cannot be refolded by molecular chaperones it is ubiquitinylated

and targeted to the 26-S ubiquitin proteasome for degradation (Ciechanover, Orian et al. 2000). The presence of ubiquitin in protein inclusions of neurodegenerative disease patients may suggest proteasome's involvement in the effort to degrade aberrant proteins before they accumulate in inclusions. The proteasome was found to be inhibited in neurodegenerative diseases (Bence, Sampat et al. 2001; Ross and Pickart 2004). In addition, there is evidence that proteasome inhibition occurs before protein inclusion formation (Bennett, Bence et al. 2005). Therefore, it has been suggested that protein inclusions form as a result of protein stress and inability of the proteasome to degrade misfolded proteins (Johnston, Ward et al. 1998). Protein inclusions could serve as an intermediary step to degrade aberrant proteins by other catabolic processes in the cell (Taylor, Tanaka et al. 2003).

The other major degradation mechanism in the cell is autophagy, which utilizes lysosomes to degrade cellular components that are sequestered in double-membrane vesicles. Autophagy is used for the turnover of long-lived proteins and organelles (Klionsky and Emr 2000; Mizushima 2005). Autophagic structures were found in multiple neurodegenerative disease models (Kegel, Kim et al. 2000; Nixon, Wegiel et al. 2005; Boland, Kumar et al. 2008). Pharmacological induction of autophagy in cell lines of neurodegenerative disease models can degrade aberrant proteins, including polyglutamine expanded Huntingtin and proteins with polyalanine expansion (Ravikumar, Duden et al. 2002; Ravikumar, Vacher et al. 2004).

The polyglutamine expansion diseases are caused by the expansion of the naturally occurring glutamine repeat regions in some proteins (Zoghbi and Orr 2000). These diseases include the Huntington's disease, spinal bulbar muscular atrophy

(SBMA), spinocerebellar ataxias (SCAs) and the dentatorubral pallidoluysian atrophy (DRPLA) (Table 1). SBMA was the first disease to be associated with glutamine repeat expansion. It is seen in people with polyglutamine expanded androgen receptor (AR) and it is transmitted in a dominant fashion (Brown, Goss et al. 1989; La Spada, Wilson et al. 1991). High concentrations of AR's ligand testosterone is required for disease to occur, hence it is a male-specific disease. In mouse models of SBMA, female littermates do not show the disease and castration of males stops the disease progression (Li, Chevalier-Larsen et al. 2007). As in other polyglutamine expansion diseases, symptoms start in adults and progress over time. Most people without the disease have approximately 20 glutamine repeats in their AR, and 40 repeats of glutamine is the threshold for symptoms to occur. Patients who have AR with higher numbers of polyglutamine repeats start seeing symptoms earlier in life and disease symptoms progress more rapidly (Doyu, Sobue et al. 1992). Major symptoms are muscular cramps, atrophy, tremor of the hands and androgen insensitivity.

In this study we describe an *in vivo* model of SBMA that recapitulates the disease in the fly eye. We show that expression of the expanded AR in the eye causes degeneration in a polyglutamine length-dependent fashion. The proteasome is inhibited in this model, and autophagy is induced. Genetic activation of autophagy ameliorates this degeneration. Moreover, proteasome impairment alone causes eye degeneration and autophagy induction, implying that the disease symptoms are due to misfolded protein stress caused by proteasome inhibition.

Materials and Methods

Fly Strains:

Drosophila stocks were maintained on standard fly food either at 25^{O}C or at room temperature. Dihydrotestosterone (DHT) was dissolved in absolute ethanol and added to liquid fly food to a final concentration of 1 mM. The GMR-GAL4 strain was obtained from Bloomington Stock Center (Indiana University, Bloomington, IN). uas-DTS7 strain was provided by Dr. Eric Fortier (Syracuse University, Syracuse, NY). uas-CL1-GFP strain and the human AR with 12, 20, 45, 52, 121 CAG repeats containing strains were provided by Dr. J. Paul Taylor (St. Jude Children's Medical Center, Memphis, TN). uas-Atg6 RNAi and uas-Atg12 RNAi (Pandey, Nie et al. 2007) strains were used to down-regulate autophagy. uas-PTEN (Huang, Potter et al. 1999) strain was used to activate autophagy.

Electron Microscopy:

Scanning electron microscopy (SEM) samples were collected and fixed in 2.5% gluteraldehyde (EMS, Fort Washington, PA) in PBS, and post-fixed for 15-30 minutes in 1.5% osmium tetroxide (Stevens Metallurgical, NY) in PBS. Samples were then dehydrated in ethanol, immersed in hexamethyldisilazane (Polysciences Inc. Warrington, PA) and dried in a dessicator for three days. Specimens were then coated with gold:palladium using a Denton DV-503 vacuum evaporator, and analyzed using an AMRAY 1820D scanning electron microscope.

For TEM evaluation, fly heads were fixed with 2.5% gluteraldehyde/2% formaldehyde with 0.1 M sodium cacodylate and stored at 4°C until embedding.

Heads were post-fixed with 2% osmium tetroxide followed by an increasing gradient dehydration step using ethanol and propylene oxide. Heads were then embedded in LX-112 medium (Ladd) and sections were cut ultrathin (90 nm), placed on uncoated copper grids, and stained with 0.2% lead citrate and 1% uranyl acetate. Images were examined with a JEOL-1010 electron microscope (JEOL) at 80 kV.

Immunohistochemistry:

To image imaginal eye discs, wandering third instar larvae were dissected in standard *Drosophila* saline containing 128mM NaCl, 4mM MgCl2, 2mM KCl, 0.4mM CaCl2, 70mM sucrose and 5mM HEPES29. After dissection, whole larval brains containing attached eye imaginal discs were fixed in 4% paraformaldehyde for 30 minutes on ice and washed with standard saline three times. Eye imaginal discs were mounted in Glycergel Mounting Medium (DAKO) and viewed on a Bio-Rad MRC 1024 ES confocal laser scanning module on a Nikon Eclipse E600 and examined by using LaserSharp software.

Results:

Drosophila model of spinal bulbar muscular atrophy

In order to develop a *Drosophila* model of SBMA, transgenic flies containing full length human AR with 12, 20, 45, 52, and 121 glutamine stretches were expressed in the eye by GMR-GAL4 using the UAS/GAL4 system (Brand and Perrimon 1993). Expression of AR with different glutamine stretches did not cause a considerable defect in the eye morphology (Figure 2-1, top row). By contrast, expression of AR with different glutamine stretches in flies reared on AR's natural ligand

dihydrotestosterone (DHT) containing food resulted in considerable degeneration of the eye in a polyglutamine (polyQ) stretch length dependent manner (Figure 2-1, bottom row). This degeneration is most severe in the posterior margin and entails a disorganization of the ommatidial array, fusion of ommatidia, and disorganization of ommatidial bristles. The polyQ stretch length dependency mirrors the disease in humans, since the disease symptoms are more severe in patients that have AR with higher numbers of glutamine repeats.

To investigate the relationship between polyglutamine length and age of disease onset, we took advantage of the ligand-dependency of this model. Flies expressing AR containing 12, 52 and 121 glutamine repeats were reared on standard fly food until eclosion, and then adults were transferred to DHT-containing food. These adults were regularly transferred to fresh food that contained DHT, and they were observed daily for changes in the eye morphology. Flies expressing AR with 121 glutamine repeats exhibited a polyQ- length dependent degeneration earlier than flies expressing AR with 52 glutamine repeats (Figure 2-2, bottom and middle rows). The average number of days to the onset of the degenerative phenotype in flies expressing AR with 52 glutamine repeats was 23.7 days. This average was 18.9 days in flies expressing AR with 121 glutamine repeats. Flies expressing AR with 12 glutamine repeats did not show a significant degeneration by day 60 after when the experiment was stopped (Figure 2-2, top row). Earlier onset of degeneration with increasing polyQ length mirrors the disease in humans, since the disease symptoms start earlier in life in patients that have AR with higher numbers of glutamine repeats. Taken together, expression of human AR with different polyQ stretches in the *Drosophila*

eye provides a model with the characteristics of the age of onset and severity of the SBMA in human patients.

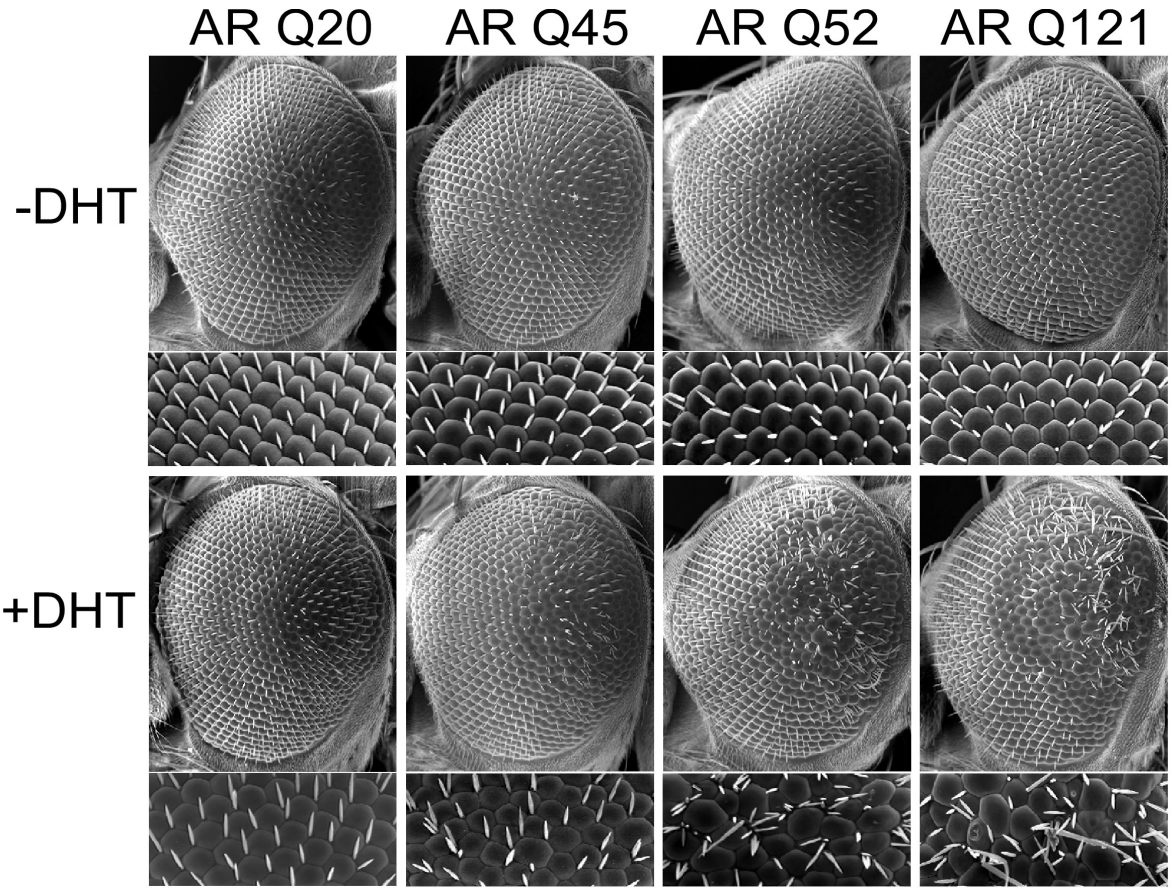

Figure 2-1: Eye degeneration is enhanced by increased polyglutamine stretch in the AR.

SEM pictures of the *Drosophila* eye of 1 day old flies showing the SBMA model generated by expressing human androgen receptor (AR) with various lengths of polyglutamine stretches. Expression of human AR in the eye causes degeneration as long as they are raised on DHT containing food and degeneration is enhanced with increasing polyglutamine stretch (bottom row). There is no significant effect on the eye when flies are raised on food lacking DHT (top row). Detailed genotypes in order: *+/+; gmr gal4/+; uas-AR Q20, +/+; gmr gal4/+; uas-AR Q45, +/+; gmr gal4/+; uas-AR Q52, +/+; gmr gal4/+; uas-AR Q121.*

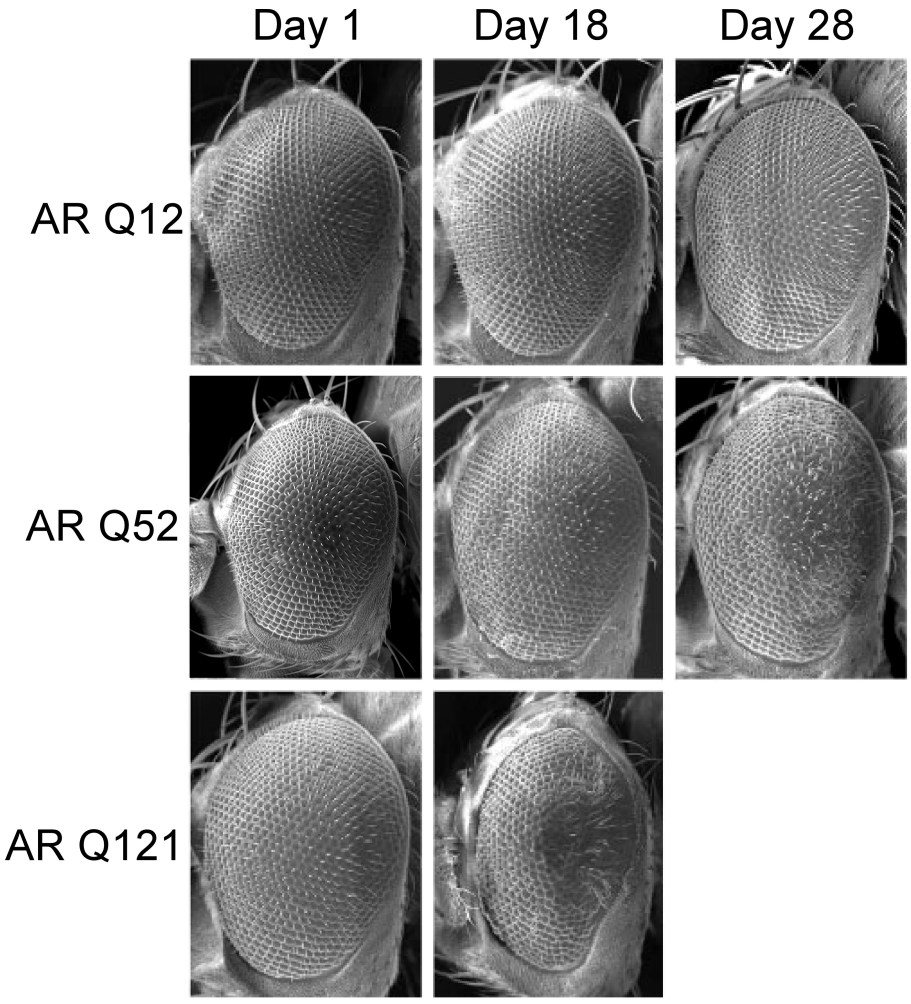

Figure 2-2: Increase in polyglutamine stretch causes earlier onset of degeneration in the eye.

SEM images of AR Q12, AR Q52 and AR Q121 expressing flies that were raised on regular food and aged on DHT containing food starting first day of adult life. Fly heads were dissected and fixed on indicated ages. First appearance of degeneration in AR Q121 expressing flies is by day 18 (bottom row), and of AR Q52 expressing flies is by day 28 (middle row), while no degeneration is observed in AR Q12 expressing flies (Genotype: *+/+; gmr gal4/+; uas-AR Q12)* by day 60, indicating that length of polyglutamine stretch determines age of onset of degeneration (Quantification of phenotypes done by Udai Pandey).

Genetic impairment of the proteasome results in activation of autophagy

Previous studies have suggested that the proteasome is inhibited in neurodegeneration models (Lindsten, de Vrij et al. 2002). Therefore, we hypothesized that proteasome impairment alone may lead to activation of autophagy since maintenance of catabolism is essential for cellular homeostasis. DTS7 is temperature sensitive, dominant negative form of the β2 subunit of the proteasome (Belote and Fortier 2002). When it is expressed in the eyes of the flies reared at 22^{O}C, eye morphology is normal (Figure 2-3A). By contrast, when flies expressing DTS7 are reared at 28^{O}C, there is a substantial degeneration in the eye (Figure 2-3B). When examined by TEM, autophagic vacuoles were rare or absent in the eyes of the flies reared at 22^{O}C (data not shown). However, there were numerous autophagic vacuoles in the retina of the flies expressing DTS7 reared at 28^{O}C (Figure 2-3C). Among these were vacuoles that contained multi-vesicular bodies (Figure 2-3D), and organelles (Figure 2-3E), which both typically appear during autophagy. This indicates that autophagy is activated beyond normal levels when the proteasome is inhibited.

A genetic approach was used to test whether autophagy was cytotoxic or cytoprotective upon proteasome inhibition. Downregulation of autophagy with *atg6* or *atg12* RNAi in the eyes of the flies reared at 22^{O}C did not result in a considerable change in the eye compared to DTS7 expression alone (Figure 2-4, top row). However, downregulation of autophagy with the same RNAi constructs in the eyes of the flies reared at 28^{O}C, enhanced the degeneration seen with DTS7 expression alone (Figure 2-4, bottom row). These results imply that upon proteasome inhibition,

activation of autophagy has a protective role, since impairing activation of autophagy results in enhancement of the proteasome inhibition phenotype.

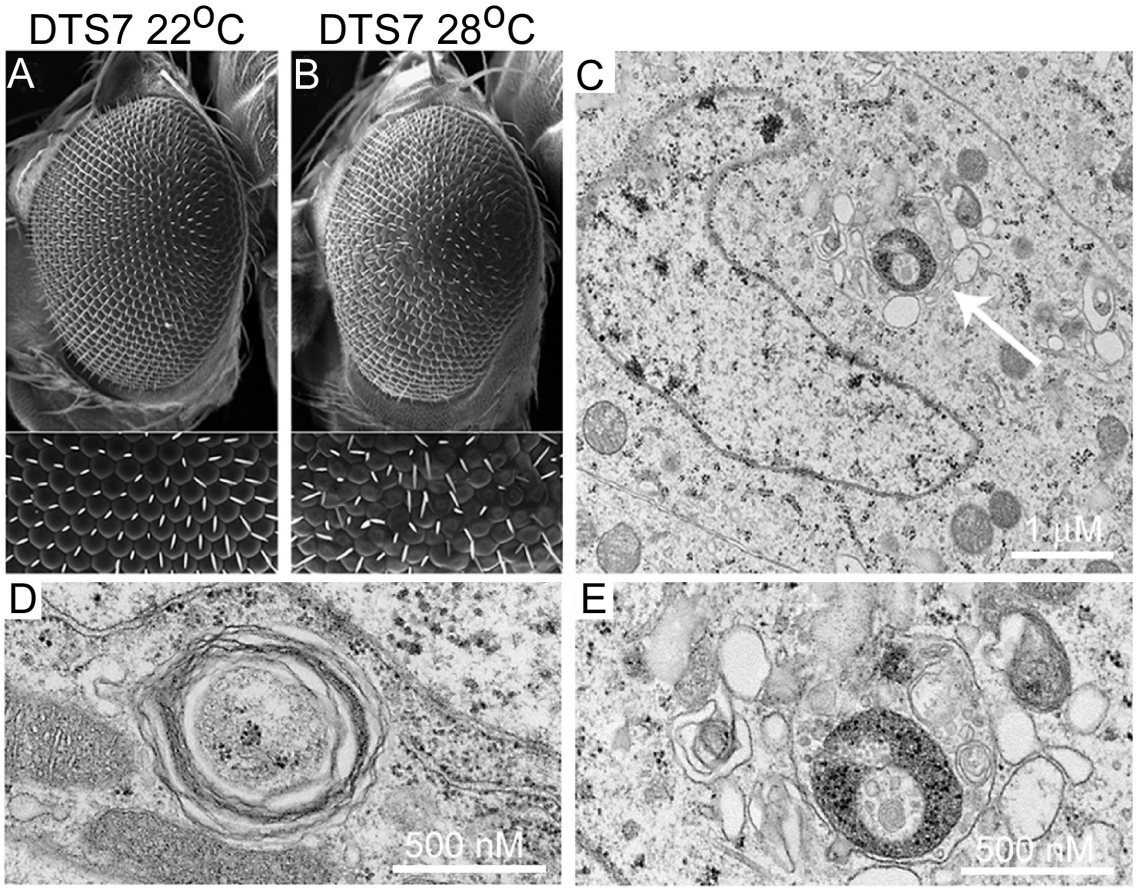

Figure 2-3: Inhibition of the proteasome activates autophagy.

Effect of expressing the temperature sensitive, dominant negative proteasome subunit in the eye (A, B). uas-DTS7 expressing fly eyes are normal when they are reared at 22°C (A), while they have rough eye phenotype when they are reared at 28°C (B), genotype: *w; gmr-GAL4/+; uas-DTS7/+*. TEM images of retinal sections of DTS7 expressing flies raised at 28°C show the appearance of autophagic vacuoles and multi-vesicular bodies (C and D respectively). (E) shows high magnification of the area indicated by the arrow in (C) (TEM analyses were done by J. Paul Taylor).

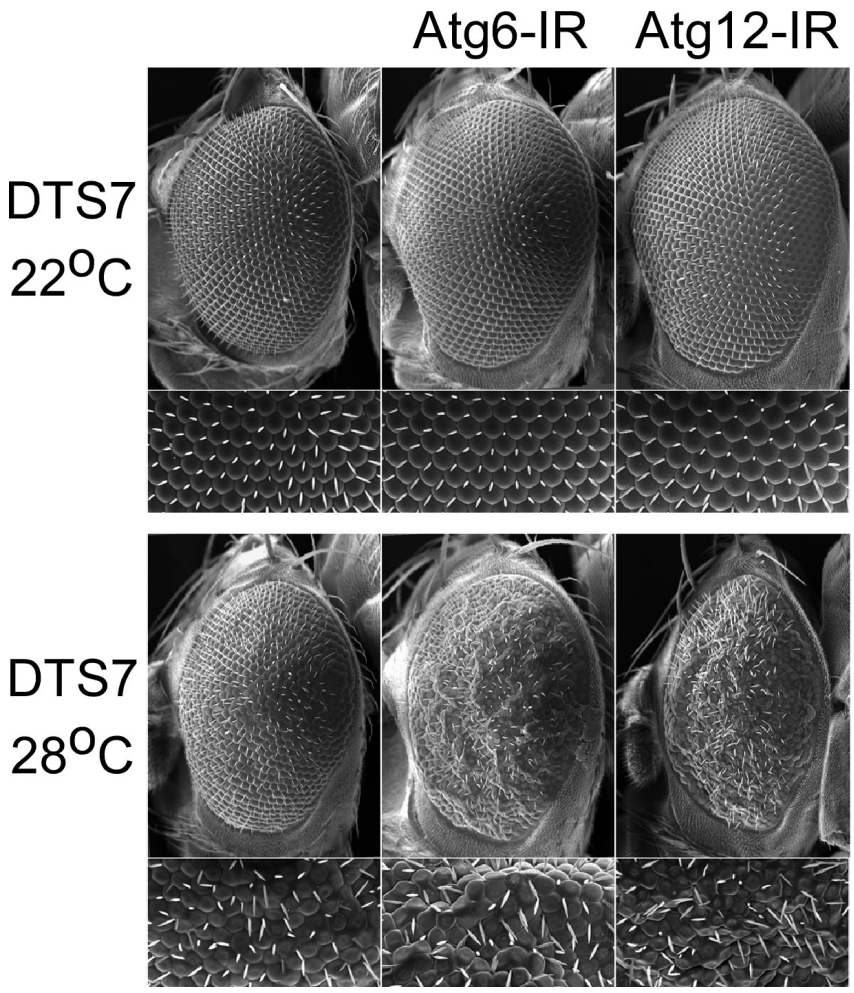

Figure 2-4: Inhibition of autophagy enhances degeneration caused by proteasome impairment.

(Top row) Downregulation of essential autophagy genes *atg6* and *atg12* by RNAi in DTS7 expressing animals does not have an effect on eye morphology at the permissive temperature 22°C. (Bottom row) Eye degeneration seen in DTS7 is enhanced when autophagy is downregulated by *atg6* and *atg12* RNAi at restrictive temperature 28°C, indicating that inhibition of both the proteasome and autophagy cause enhanced toxicity than inhibiting only the proteasome or autophagy alone. Genotypes in order: *w; gmr-GAL4/+; uas-DTS7/+*, *w; gmr-GAL4/uas-Atg6-IR; uas-DTS7/+*, *uas-Atg12-IR/+; gmr-GAL4/+; uas-DTS7/+*.

The Proteasome is inhibited in the SMBA model

Inhibition of the proteasome has been implicated in a wide variety of neurodegenerative diseases (Bence, Sampat et al. 2001). In order to study the proteasome in the fly SBMA model, we utilized transgenic flies expressing a fluorescent reporter of proteasome function. CL1-GFP is a fusion between GFP and a short polypeptide that renders GFP a target for the proteasome (Dantuma, Lindsten et al. 2000). Since this fusion protein is a target for the proteasome, it is degraded under normal conditions (Figure 2-5B). However, when proteasome is impaired CL1-GFP is retained in the cell and is detectable by fluorescence microscopy. We tested this in the eye imaginal discs of the third instar larvae by utilizing the temperature sensitive nature of the DTS7 transgene. No CL1-GFP was detected in the eye imaginal discs of flies expressing DTS7 at 22^{O}C. By contrast, CL1-GFP is detected in flies expressing DTS7 at 28^{O}C (Figure 2-5C, D). This showed that the CL1-GFP fusion reporter can be reliably used to detect proteasome impairment. We used this system to test whether the proteasome was inhibited in the SBMA model. In the flies expressing AR with 121 glutamine repeats in the eye in the absence of DHT, CL1-GFP was not detected. By contrast, when the same flies were reared on food containing DHT, CL1-GFP was detected (Figure 2-5E, F). This shows that in the fly SBMA model the proteasome is inhibited in a ligand-dependent manner.

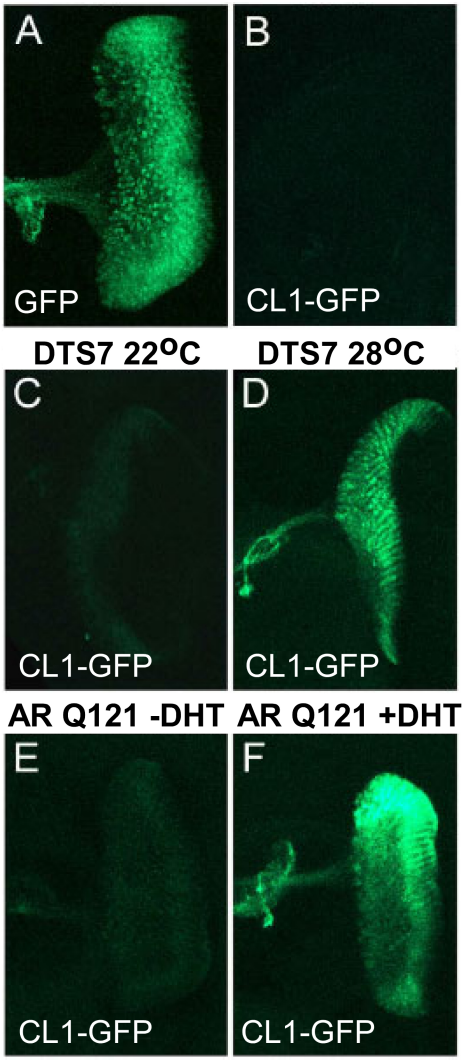

Figure 2-5: The proteasome is inhibited in flies expressing AR Q121 on DHT food.

Confocal microscopy images of the proteasome reporter expressed in the imaginal eye discs from 3rd instar larvae. (A) GFP expression alone showing that GFP alone is not degraded. (B) CL1-GFP expression alone, indicating that it is degraded in controls. (C-F) CL1-GFP expression with indicated genotypes. (C,D) CL1-GFP accumulation, indicating proteasome inhibition, is observed only at restrictive temperature when the dominant negative proteasome subunit (DTS7) is expressed. (E,F) CL1-GFP is accumulated only when AR Q121 expressing flies were raised on

DHT food, indicating that the proteasome is inhibited in the SBMA model. Genotypes: (A) *w; gmr-GAL4/+; uas-GFP/+*. (B) *w; gmr-GAL4/+; uas-CL1-GFP/+*. (C) *w; gmr-GAL4/+; uas-CL1-GFP/uas-DTS7*. (D) *w; gmr-GAL4/+; uas-CL1-GFP/uas-DTS7*. (E) *w; gmr-GAL4/+; uas-CL1-GFP/ uas-AR Q121*. (F) *w; gmr-GAL4/+; uas-CL1-GFP/ uas-AR Q121* (Work performed by Zhiping Nie).

Role of autophagy in the SBMA model

To determine the role of autophagy in the SBMA model, we tested the effect of autophagy downregulation. Downregulation of autophagy by *atg6* or *atg12* RNAi in the eyes of flies expressing AR with 52 glutamine repeats did not result in a considerable change in the eye compared to expression of AR with 52 glutamine repeats alone (Figure 2-6, top row). However, down-regulation of either *atg6* or *atg12* in the eyes of flies reared on DHT food enhanced eye degeneration in flies expressing AR with 52 glutamine repeats (Figure 2-6, bottom row). These data indicate that in the fly SBMA model, autophagy protects the cell from polyglutamine-expanded protein toxicity. Furthermore, our data suggest that polyglutamine expression induces proteasome inhibition (Figure 2-5: The proteasome is inhibited in flies expressing AR Q121 on DHT food. , and that autophagy may be induced to compensate for altered proteasome function. If this is true, ectopic induction of autophagy under the conditions of proteasome inhibition should suppress degeneration.

To investigate this possibility, we used the class I PI3 kinase pathway inhibitor, PTEN. The class I PI3 kinase pathway positively regulates growth, and negatively regulates autophagy (Baehrecke 2005). In the presence of DHT, co-expression of AR with 52 glutamine repeats and PTEN, suppressed the rough eye phenotype compared to the expression of AR with 52 repeats alone (Figure 2-7A,B and C). This is due to activation of autophagy by PTEN, since, downregulation of autophagy in the same background by *atg12* or *atg6* RNAi restores the degenerative phenotype (Figure 2-7D and E). In addition, the degenerative phenotype of AR with 52 glutamine repeats

expression was significantly decreased when flies were reared on food containing rapamycin (data not shown). Rapamycin activates autophagy by inhibiting TOR, which is a downstream component of the PI3 kinase pathway (Scott, Schuldiner et al. 2004). These data imply that autophagy is activated in the SBMA model in order to cope with the protein stress that is caused by proteasome inhibition.

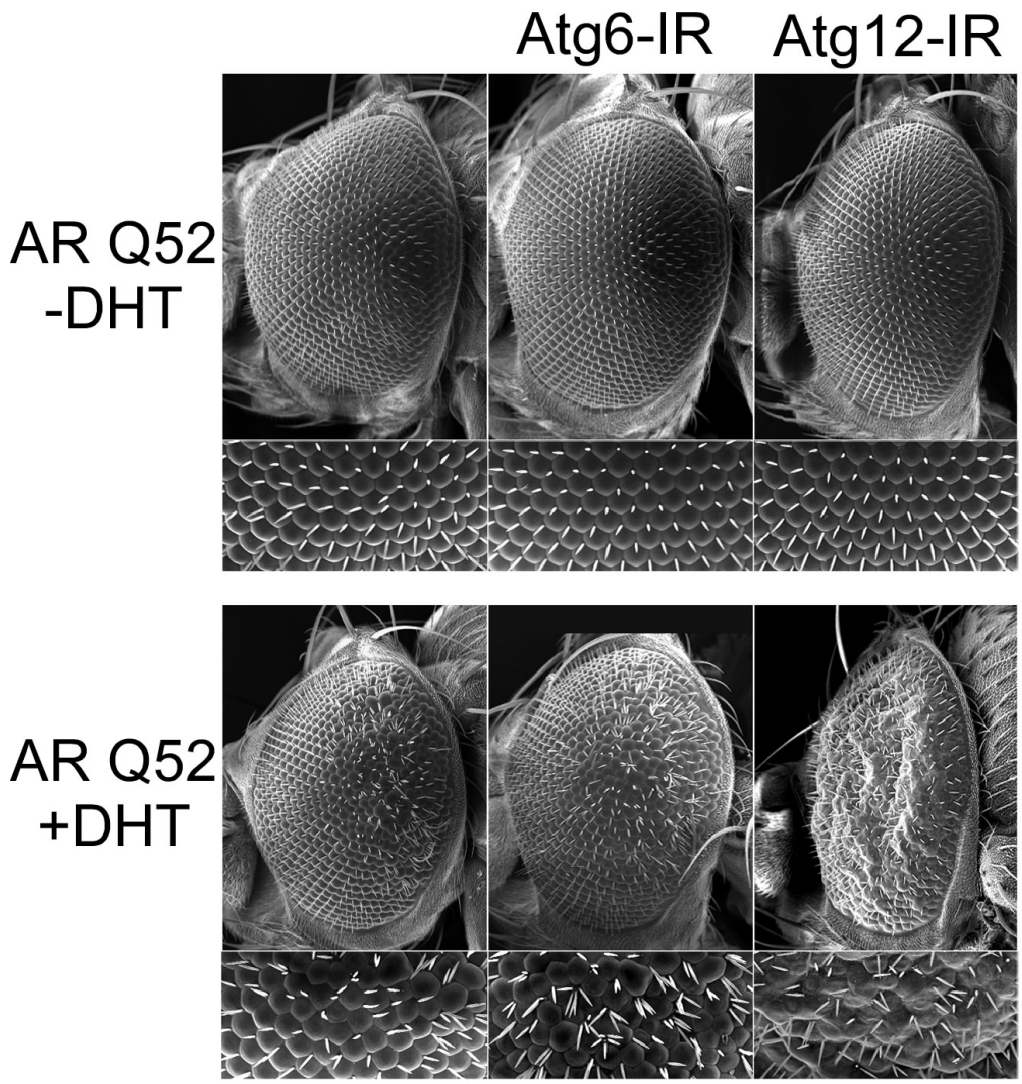

Figure 2-6: Inhibition of autophagy enhances degeneration of the eye in the SBMA model.

(Top row) Downregulation of essential autophagy genes *atg6* and *atg12* by RNAi has no effect on eye phenotype of 1 day old flies expressing AR Q52 reared on regular food. (Bottom row) Eye degeneration seen in AR Q52 expressing 1 day old flies on DHT containing food is enhanced when autophagy is downregulated, suggesting that autophagy is protective in the SBMA model. Genotypes in order: *w; gmr-GAL4/+; uas-AR Q52/+*, *w; gmr-GAL4/uas-Atg6-IR*; *uas-AR Q52/+*, *uas-Atg12-IR/Y; gmr-GAL4/+; uas-AR Q52/+*.

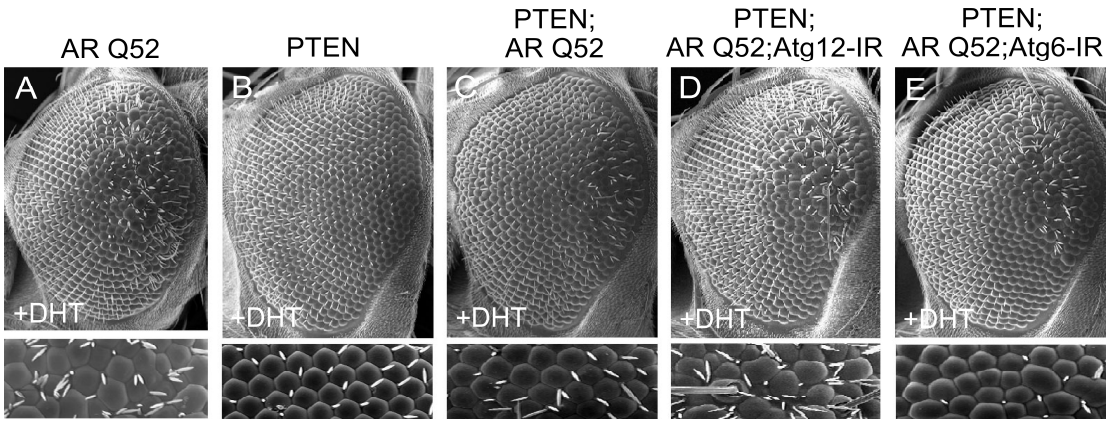

Figure 2-7: Activation of autophagy reduces degeneration in the eye expressing AR Q52.

(A) AR Q52 expressing 1 day old flies reared on DHT food show eye degeneration. (B) PTEN expression alone in 1 day old flies reared on DHT food show no degeneration. (C) PTEN and AR Q52 expressing 1 day old flies reared on DHT food indicates decreased eye degeneration compared to AR Q52 expression alone (A). This decrease is autophagy dependent, since down-regulation of autophagy genes by *atg12* (D) and by *atg6* RNAi (E) reverses the effect of ectopic PTEN expression on eye degeneration, seen when AR Q52 is expressed alone (A). Genotypes in detail: (A) *w; gmr-GAL4/+; uas-AR Q52/+*, (B) *w; gmr-GAL4/+; uas-PTEN/+*, (C) *w; gmr-GAL4/+; uas-AR Q52/uas-PTEN*, (D) *uas-Atg12-IR/Y; gmr-GAL4/+; uas-AR Q52/uas-PTEN*, (E) *w; gmr-GAL4/uas-Atg6-IR; uas-AR Q52/uas-PTEN* .

Discussion

We tested the hypothesis that proteasome inhibition contributes to polyglutamine-induced neurodegeneration. The ubiquitin-proteasome system is the first line of defense against the accumulation of misfolded proteins. It is one of the major catabolic processes that is involved in essential cellular functions as diverse as the cell cycle progression, modulation of cell surface receptors, and the immune system (Ciechanover, Orian et al. 2000). In neurons, the proteasome contributes to specialized functions including axon pruning, and regulating the protein content of synapses (Patrick 2006). The proteasome's role to degrade misfolded proteins could be critical for neurons since neurons are long-lived post mitotic cells that are likely to be more sensitive to accumulating cellular stress. This could also explain why familial neurodegenerative diseases affect the nervous system exclusively, even though all tissues should carry the same mutation. In this study, we have shown that proteasome inhibition by ectopic expression of DTS7 causes eye degeneration (Figure 2-3). Similarly, in an SBMA model created by ectopic expression of glutamine-expanded AR, we observed eye degeneration (Figure 2-1). Importantly, the proteasome is also impaired in the SBMA model (Figure 2-5). The observation of eye degeneration by either inhibition of the proteasome alone or by the ectopic expression of mutant AR implies that proteasome impairment could contribute to the degeneration associated with the SBMA model. How do polyglutamine expanded proteins inhibit the proteasome? Since the protein inclusions stain positive for the mutant protein and ubiquitin, the proteasome may attempt to degrade polyglutamine expanded proteins at first. It's been reported that the proteasome cannot degrade proteins with long glutamine stretches (Venkatraman, Wetzel et al. 2004). Therefore,

mutant proteins may occupy the proteasome and this may lead to impairment. However, this subject is under debate (Michalik and Van Broeckhoven 2004).

Autophagic structures have been associated with the nervous tissue in neurodegenerative diseases, while they are not easily detected in the nervous system under physiological conditions (Mizushima, Yamamoto et al. 2004). Autophagy is one of the major catabolic systems in the eukaryotic cell. Therefore, we can hypothesize that autophagy is induced in cells under protein stress to help degrade the misfolded proteins. In this study we show that autophagy is induced when the proteasome is impaired by DTS7 expression alone (Figure 2-3), and the eye degeneration phenotype of DTS7 is enhanced when autophagy is impaired (Figure 2-4). In addition, the eye degeneration phenotype of DTS7 expression is rescued with induction of autophagy by rapamycin treatment (Pandey, Nie et al. 2007). These data imply that induction of autophagy is sufficient to alleviate the toxic effects of proteasome impairment, probably by reducing protein stress. This is important since it points to a possible relationship between the two catabolic pathways, and that autophagy could at least partially compensate for the ubiquitin proteasome system. Similar to proteasome inhibition alone, in the SBMA model the eye degeneration phenotype is enhanced when autophagy is impaired (Figure 2-6) and the degeneration phenotype is reduced when autophagy is activated by PTEN expression (Figure 2-7) or by rapamycin treatment (Pandey, Nie et al. 2007). Collectively this data implies that the degenerative phenotype that is observed in the SBMA model is partially, if not entirely, due to proteasome impairment (Figure 2-5), and induction of autophagy helps clear the protein stress that ensues as a result of proteasome impairment (Figure

2-7). Once induced, autophagy could be degrading either the small soluble protein oligomers, the contents of the protein inclusions, or both. It has been suggested that the soluble oligomers are the toxic species perhaps due to their potential to impair the proteasome (Abel, Walcott et al. 2001). It has also been suggested that inclusion formation may help degrade the toxic species in a neurodegenerative disease model (Taylor, Tanaka et al. 2003). Moreover, inclusion formation was found to be mediated by proteins like p62 (Bjorkoy, Lamark et al. 2005; Komatsu, Waguri et al. 2007) and HDAC6 (Iwata, Riley et al. 2005), which are both associated with autophagy. Finally, overexpression of HDAC6 ameliorates the degenerative phenotype of SBMA model that is shown here in an autophagy dependent manner (Pandey, Nie et al. 2007). Collectively, these findings fit into a model in which misfolded proteins are first accumulated into inclusions with the activities of proteins like p62 and HDAC6, later these proteins help move the proteins in inclusions into the autophagic machinery for degradation. HDAC6 fits this model since it is a cytoplasmic, microtubule associated protein that could be a part of a molecular motor involved in carrying cargo associated with this process (Kawaguchi, Kovacs et al. 2003).

It is not clear how proteasome impairment leads to toxicity in the context of neurodegenerative diseases. We can speculate that an overwhelmed proteasome could lead to failure in many regulatory pathways that the proteasome controls, and this defect eventually results in cell death. In the case of neurons, the dysfunctional cells cannot be replaced. Therefore, it is likely that a dysfunctional neuron lives until it dies with cellular catastrophe. The accumulation of numerous dysfunctional or

dying cells could eventually lead to symptoms observed in these diseases. This might also contribute to the late onset nature of neurodegenerative diseases. Our study implies that therapeutic treatments that increase levels of autophagy, like rapamycin treatment, could delay the disease progression. However, it should be noted that autophagy has been implicated in cell death (Lee and Baehrecke 2001; Berry and Baehrecke 2007). While low levels of autophagy are essential for physiological functions and cell survival, high levels of autophagy are detrimental to the cell (Baehrecke 2005; Berry and Baehrecke 2007; Scott, Juhasz et al. 2007). A complete understanding of polyglutamine protein toxicity and development of therapeutic strategies will require deeper understanding of this disease and its relationship to autophagy.

Chapter 3

Dynein light chain 1 is required for autophagy, protein clearance and cell death in *Drosophila*

Abstract

Autophagy is a conserved catabolic pathway that is important for turnover of long-lived proteins and organelles, and has been implicated in cell survival, tumor progression, protection from infection, neurodegenerative disease and cell death. Autophagy and caspases are required for type II autophagic cell death of *Drosophila* larval salivary glands during development, but the mechanisms that regulate these degradation pathways are not understood. We conducted a forward genetic screen for genes that are required for salivary gland cell death, and here we describe the identification of *Drosophila* dynein light chain 1 (*ddlc1*) as a gene that is required for type II cell death with autophagy. Autophagy is attenuated in *ddlc1* mutants, but caspases are active in these cells. *ddlc1* mutant salivary glands develop large fibrillar protein inclusions that stain positive for amyloid-specific dyes and ubiquitin. Ectopic expression of Atg1 is sufficient to induce autophagy, clear protein inclusions, and rescue degradation of *ddlc1* mutant salivary glands. Furthermore, *ddlc1* mutant larvae have decreased motility, and mutations in *ddlc1* enhance the impairment of motility that is observed in a *Drosophila* model of polyglutamine disease. Significantly, this decrease in larval motility is associated with the accumulation of p62 in muscles. These results indicate that Ddlc1 is required for protein clearance by autophagy that is associated with autophagic cell death and neurodegeneration.

Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved catabolic pathway (Mizushima, Noda et al. 1998; Xie and Klionsky 2007). Pioneering studies of yeast identified genes that are required for autophagy (*atg* genes) during starvation (Takeshige, Baba et al. 1992; Thumm, Egner et al. 1994; Klionsky and Emr 2000). Autophagy is also activated during starvation in animals (Mizushima, Yamamoto et al. 2004), but the complexity of higher animals, and the association of autophagy with neurodegenerative diseases, bacterial clearance, antigen presentation, tumor suppression/survival and cell death (Mizushima 2005), raises questions about how autophagy is regulated and how cargo is recruited and delivered to lysosomes in multicellular organisms.

Autophagy involves the formation of an isolation membrane around cytoplasmic components to form the autophagosome, and content of this structure is degraded by fusion with lysosomes (Klionsky and Emr 2000). The formation of the isolation membrane requires the action of the Atg8 and the Atg12 conjugation pathways. Atg12 is activated by Atg7 in an ATP dependent manner, and becomes conjugated with Atg5 and Atg16 through an Atg10 intermediate. Atg8 (LC3 in mammals) is cleaved by Atg4, and activated by Atg7 before it is conjugated to phosphatidylethanolamine (PE) through an Atg3 intermediate (Ohsumi 2001). Atg8-PE is a structural element of the autophagosomal membrane. While much is known about the formation of autophagosomes, several important questions about the regulation of autophagy remain unanswered. For example, it is not known whether cytoplasmic components are directed to the site of autophagosome formation, and it is

not completely clear how autophagosomes and lysosomes find each other to complete degradation. Nevertheless, there is some evidence that autophagosome formation and proper movement and fusion with lysosomes depends on intact microtubules (Fass, Shvets et al. 2006; Kochl, Hu et al. 2006). Moreover, autophagosome movement was shown to be dependent on dynein motor function since down regulation of the dynein intermediate chain or p150 disrupts autophagosome movement (Kimura, Noda et al. 2008).

The role of autophagy in programmed cell death has been a subject of debate (Baehrecke 2005; Levine and Yuan 2005). Autophagy has a well established role in survival, and decreased autophagy gene function can promote cell death (Boya, Gonzalez-Polo et al. 2005). The association of autophagy with neurodegenerative diseases (Nixon, Wegiel et al. 2005) resulted in interest about the role of this catabolic process in cell survival and death. Autophagy plays a protective role against neurodegeneration in disease models (Bjorkoy, Lamark et al. 2005; Pandey, Nie et al. 2007; Boland, Kumar et al. 2008), as well as under physiological conditions (Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). The role of autophagy in cell survival has also been demonstrated in cancer (Qu, Yu et al. 2003; Yue, Jin et al. 2003). In this case, rapidly growing tumors utilize autophagy degradation products as nutrients. Alternatively, if degradation of cellular components reaches higher levels this may lead to cell death of cancer cells (Mathew, Karantza-Wadsworth et al. 2007). Autophagosomes have been associated with dying cells during development (Schweichel and Merker 1973; Clarke 1990), and recent studies indicate that autophagy can promote the degradation and clearance of cells during cell death

(Berry and Baehrecke 2007; Qu, Zou et al. 2007). This apparent paradox raises questions about how autophagy is regulated in the context of cell death.

Drosophila larval salivary glands possess type II cell death morphology during development, and contain numerous autophagosomes (Lee and Baehrecke 2001; Martin and Baehrecke 2004; Berry and Baehrecke 2008). A rise in the steroid 20-hydroxyecdysone 12 hours after puparium formation triggers cell death, and glands are completely destroyed within 4 hours (Jiang, Baehrecke et al. 1997; Lee and Baehrecke 2001). Salivary glands fail to completely degrade in *atg* mutants, and induction of autophagy by expression of Atg1 is sufficient to induce caspase-independent cell death (Berry and Baehrecke 2008). Caspases are also required for salivary gland degradation, and function in the fragmentation of DNA, cleavage of nuclear Lamins, and likely many other caspase substrates in these cells (Martin and Baehrecke 2004). Inhibition of both caspases and autophagy has a stronger persistence phenotype than inhibiting either of these pathways alone, indicating that caspases and autophagy work in an additive manner to degrade salivary glands (Berry and Baehrecke 2008). Therefore, larval salivary glands in *Drosophila* are degraded with a unique form of cell death that requires both caspases and autophagy.

Dyneins are conserved microtubule dependent molecular motors involved in retrograde transport of cargo in the cell. They are composed of heavy, intermediate, light intermediate and light chains. Heavy chains regulate movement on microtubules in an ATP dependent fashion, while light chains are involved in cargo recognition (Vallee, Williams et al. 2004). The smallest dynein light chain (LC8/PIN in vertebrates and Ddlc1/Cutup in *Drosophila*) encodes a 8kD protein that is highly

conserved from *Chlamydomonas* to humans (King, Barbarese et al. 1996). LC8 binds to a variety of cargo as a dimer via a conserved motif (Lo, Naisbitt et al. 2001; Rodriguez-Crespo, Yelamos et al. 2001), including neuronal nitric oxide synthase (nNOS), inhibitor of NF-κB (IκBα), nuclear respiratory factor-1 (NRF-1), p53-binding protein 1 (53BP1) and the estrogen receptor (ER) (Jaffrey and Snyder 1996; Crepieux, Kwon et al. 1997; Herzig, Andersson et al. 2000; Lo, Kan et al. 2005; Rayala, den Hollander et al. 2005). In *Drosophila*, Ddlc1 is cytoplasmic and ubiquitously expressed. Complete loss of function of *ddlc1* causes embryonic lethality and ectopic apoptosis (Dick, Ray et al. 1996). Ectopic activation of apoptosis could be due to Ddlc1's inhibitory effect on proapoptotic Bcl-2 family members, such as Bim as has been demonstrated in mammalian cells (Puthalakath, Huang et al. 1999). Partial loss of function mutations in *Drosophila ddlc1* cause female sterility and alter proper sensory neuron development with defects in axon guidance (Phillis, Statton et al. 1996). Ddlc1's crucial role in oogenesis has been demonstrated since it is required for proper localization of the swallow and gurken RNAs in the oocyte (Schnorrer, Bohmann et al. 2000; Wang, Hare et al. 2004; Rom, Faicevici et al. 2007).

Here we characterize a *ddlc1* mutant that was isolated in a forward genetic screen for genes that are required for salivary gland cell death in *Drosophila*. We show that salivary glands of *ddlc1* animals are defective in autophagy and fail to die normally. The salivary gland cells that fail to die develop large amyloid-like protein inclusions that can be degraded by ectopic expression of Atg1, a regulator of autophagy. Furthermore, we show that *ddlc1* enhances motility defects in a model of

the polyglutamine neurodegenerative disease spinobulbar muscular atrophy, and suggest that this is because of the accumulation of protein aggregates in muscles.

Materials and Methods

Drosophila strains

A collection of 1,475 single P element-*lacZ* insertion mutations were screened to identify genes that were expressed 13 hours after pupariation. From this screen *l(1)G0371* was found as a P element-induced mutation of *ddlc1* (also known as *cutup, ctp*). The *ddlc1* precise excision line was generated by crossing the *l(1)G0371* line to Δ 2-3 transposase as described (Robertson, Preston et al. 1988) and precise excision was confirmed by DNA sequencing. The uas-Ddlc1 strain was created as described below. uas-p35 (Hay, Wolff et al. 1994) was used to inhibit effector caspases. uas-Atg1^{GS10797} and uas-Atg1^{6B} (Scott, Juhasz et al. 2007) were used for ectopic induction of autophagy. uas-Sgs3-GFP (Biyasheva, Do et al. 2001) was used to track the Glue protein in salivary glands. Fkh-Gal4 (Berry and Baehrecke 2007) was used to drive salivary gland specific expression. The uas-AR Q20 and uas-AR Q52 lines were used for the Spinal Bulbar Muscular Atrophy model (Pandey, Nie et al. 2007). uas-GFP-LC3 (Rusten, Lindmo et al. 2004) was used as a marker of autophagosomes, and *tubulin*-GFP-LAMP1 (courtesy of H. Krämer) was used as a marker of lysosomes. Wild-type Canton-S or the precise excision of the *l(1)G0371* P-element were used as controls.

uas-Ddlc1 transgenic flies

To create the uas-Ddlc1 rescue construct, the *ddlc1* open reading frame (ORF) with flanking EcoRI and XbaI flanking restriction sites was amplified from the Berkeley Drosophila Genome Project cDNA library and cloned using the TA cloning kit (Invitrogen). The *ddlc1* ORF was sequenced, isolated from the TA vector by restriction digestion with EcoRI and XbaI, and inserted in the pUAST *Drosophila* transformation vector. The uas-Ddlc1 construct was then used to generate transgenic *Drosophila* (Best Gene Inc. Chino Hills, CA).

Salivary gland histology

Animals of indicated genotypes were staged as new white prepupae and aged at 25^{o}C until either 2 hours or 12 hours after head eversion, fixed in FAAG overnight, dehydrated, embedded in paraffin, sectioned and stained with Weigert's hematoxylin and Pollack's Trichrome as previously described (Muro, Berry et al. 2006). DNA fragmentation was detected by TUNEL assay using the Apoptotag kit (Chemicon) according to manufacturer's protocols. To detect amyloidosis with Thioflavin-S, paraffin sections were rehydrated, stained with Mayer's Hematoxylin (Fluka Chemie, Switzerland) for 1 minute, stained with 1% Thioflavin-S (Sigma) for 5 minutes, followed by dehydration and mounting in Vectashield with DAPI (Vector Laboratories). For Congo red staining, paraffin sections were rehydrated, stained with Mayer's Hematoxylin for 1 minute, equilibrated in NaCl saturated 80% ethanol for 20 minutes, stained with 0.2% Congo Red (Sigma) in saturated NaCl solution for 1 hour, and dehydrated. Sections were examined with a Zeiss Axio Imager.Z1 microscope.

Caspase substrate assays

The EnzChek Caspase-3 Assay kit (Molecular Probes) was used according to manufacturer's protocols. Either Canton-S control, precise excision of the *l(1)G0371* P-element control or *ddlc1 l(1)G0371* P-element mutant pupae were staged as new white prepupae, aged to 4 hours after puparium formation, whole animal lysates were isolated and reaction buffer containing the Z-DEVD-AMC was added followed by incubation at 37^0C for 30 minutes. Fluorescence was detected by using a spectrometer. To confirm the specificity of the assay for measuring caspase-3-like activity, Ac-DEVD-CHO was added to Canton-S lysate as a competitive inhibitor. In addition, the pan-caspase inhibitor p35 was ubiquitously expressed by using the *daughterless*-GAL4 driver. Each experiment was repeated three times.

Fluorescence microscopy

For autophagosome and lysosome detection using uas-GFP-LC3 and *tub*-GFP-LAMP1, pupae were staged as new white prepupae, aged to either 6 hours after puparium formation, 2 hours or 12 hours after head eversion. Salivary glands were dissected in PBS, stained with Hoechst 33342 to detect DNA, mounted in PBS, and immediately imaged with Zeiss Axio Imager.Z1 with Apotome. The number of GFP-positive puncta was quantified using Ziess automatic measurement software. The value for each gland was calculated by the mean number of punctae from three fields of vision.

Immunohistochemistry

For immunohistochemistry, salivary glands were dissected from pupae 2 hours after head eversion, fixed in 4% paraformaldehyde and processed as previously

described (Martin and Baehrecke 2004). Rabbit anti-Ref(2)P was used at 1:1000, mouse anti- mono- and poly-ubiquitinylated proteins (clone FK2, Biomol) was used at 1:500 concentrations. For neuromuscular junction staining, third instar larvae were dipped in calcium free saline solution (128 mM NaCl, 2mM KCl, 4mM $MgCl_2$-$6H_2O$, 35.5 mM Sucrose, 5 mM HEPES, 1mM EGTA), filleted open and cleared of non-neuromuscular organs in a dissection dish and fixed in 4% paraformaldehyde for 10 minutes. The preparations were then washed with phosphate buffered triton-x100 (0.2 %) solution and stained with anti-Ref(2)P as above. Preparations were stained with secondary antibody and/or with anti-HRP conjugated antibody used at 1:200, and mounted in Vectashield medium with DAPI (Vector Laboratories, CA).

Electron microscopy

Freshly dissected salivary glands were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH7.4, post-fixed in buffered 1% osmium tetroxide, dehydrated through a graded ethanol series and propylene oxide and infiltrated for embedding in SPI-pon/Araldite. Ultrathin sections (80nm) were stained with uranyl acetate and lead citrate before examination in a Philips CM12 transmission electron microscope.

Protein extracts and western blotting

Salivary glands were dissected 1.5 hours after head eversion, placed in Laemmli buffer (0.1% glycerol, 2% SDS, 0.125 M Tris [pH 6.8], 0.05% β-mercaptoethanol, and 0.05% bromophenol blue) and boiled for 5 minutes. Equal amounts of total protein were separated on 12% SDS-polyacrylamide gels. Separated proteins were transferred to 0.45 µm Immobilon-P membranes (Millipore). Membranes were stripped with 25 mM glycine-HCL, pH 2, 15% SDS buffer between antibodies as

described by the manufacturer. Anti- Ref(2)P was used at 1: 5000, mouse anti- mono- and poly-ubiquitinylated proteins (clone FK2, Biomol) were used at 1:10,000 and anti-β-tubulin was used at 1:50 concentration.

Larval motility assay

15 wandering third instar larvae were collected from the indicated genotypes, washed in PBS and individually placed on a 1% agarose gel cast in an 80mm petri dish. The petri dish was placed on gridlines spaced by 5 mm. The larvae were allowed to acclimate for 1 minute, and then the number of grid lines that the posterior end of the larvae passed in 2 minutes was recorded for each larva. The average number of grid lines passed was calculated for each experiment. Each experiment was repeated three times. The average of three experiments is reported for each genotype.

Results

Dynein light chain 1 is required for salivary gland degradation

Several genes, including steroid-regulated transcription factors, caspases, autophagy genes and regulators of growth, have been identified that are required for proper destruction of larval salivary glands (Baehrecke 2000; Neufeld and Baehrecke 2008). In addition, hundreds of genes are induced within 4 hours of salivary gland degradation (Lee, Clough et al. 2003), suggesting that many additional genes may function in the death of these cells. To identify additional genes that function in salivary gland programmed cell death, we screened a collection of 1,475 stocks with a single lethal P-element-*LacZ* enhancer trap reporter for expression 13 hours after

puparium formation, and failure of salivary gland degradation at 24 hours after puparium formation. From this screen, we identified the P-element line *l(1)G0371* inserted into the dynein light chain 1 gene *ddlc1*, also known as *cutup* (*ctp*). The location of the P-element insertion was mapped to 124 bp downstream of the first exon. This *ddlc1* allele is pupal lethal and their head eversion occurs at 15 hours after puparium formation.

Histological examination of *ddlc1* mutant animals 12 hours after head eversion revealed a significant defect in salivary gland cell death. All *ddlc1* pupae failed to complete salivary gland degradation, with animals having either intact or partially fragmented glands (Figure 3-1B, C, F). By contrast, most control animals lack salivary glands (Figure 3-1A, F). Significantly, precise excision of the *l(1)G0371* P-element reverted the defect in salivary gland degradation (data not shown). We confirmed the salivary gland cell death defect of *ddlc1* by using a different allele (*ddlc1*^{e73}), which was previously reported to have axon guidance defects (Phillis, Statton et al. 1996). 100% of *ddlc1*^{e73} animals failed to destroy their salivary glands by 12 hours after head eversion (data not shown). To further confirm that the failure in salivary gland degradation phenotype was caused by loss of *ddlc1* function, we performed a transgenic rescue experiment (Brand and Perrimon 1993). We generated transgenic flies and expressed Ddlc1 in the salivary glands of *ddlc1* mutant flies. Histological examination indicated that expression of Ddlc1 in salivary glands largely rescued the *ddlc1* mutant defect in degradation of this tissue 24 hours after puparium formation (Figure 3-1D, E, F). These data indicate that *ddlc1* is necessary for complete salivary gland degradation.

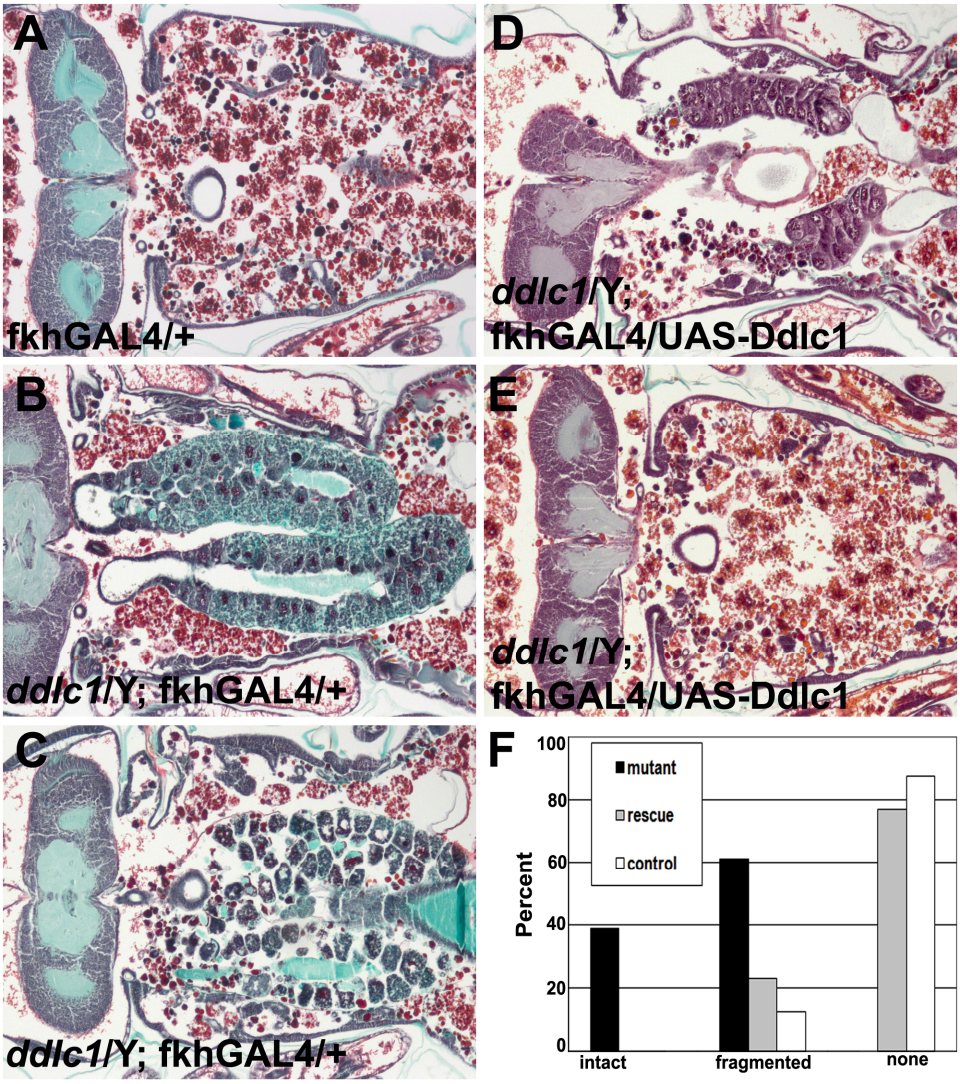

Figure 3-1: Dynein light chain 1 is required for salivary gland degradation.

(A-E) Representative paraffin sections of pupae 12 hours after head eversion. (A) Almost all control animals lack salivary glands (n=16). (B,C) All *ddlc1* mutant animals fail to degrade their salivary glands with some being intact (B) and others being fragmented (C) (n=28). (D, E) Expression of Ddlc1 in salivary glands largely rescues the degradation defect phenotype of *ddlc1* mutants with a small number of animals having fragmented salivary glands (D) and most having none (E) (n=22).

(F) Percentage of animals with intact, fragmented and no salivary glands. *ddlc1* mutant pupae in black, rescue in gray, and control in white bars. Detailed genotypes: (A) *fkhGAL4/+*, (B,C) *ddlc1/Y; +/+ ; fkhGAL4/+* (D,E) *ddlc1/Y ; uas-Ddlc1/+ ; fkhGAL4/ uas-Ddlc1*.

ddlc1 mutants have altered caspase levels

Ectopic caspase activity was previously shown to be present in *Drosophila ddlc1* mutants (Dick, Ray et al. 1996) and Ddlc1 inhibits the pro-apoptotic activity of Bim in mammalian cells (Puthalakath, Huang et al. 1999). Since caspases are required to complete salivary gland cell degradation (Lee and Baehrecke 2001; Martin and Baehrecke 2004; Berry and Baehrecke 2008), we hypothesized that *ddlc1* may regulate caspases in salivary glands. Caspase-dependent DNA fragmentation was detected in both the control and the *ddlc1* mutant salivary glands by the TUNEL assay 2 hours after head eversion (Figure 3-2A, B). Even though *ddlc1* mutant salivary glands fail to be degraded, they had premature DNA fragmentation before the rise in steroid that triggers DNA fragmentation and cell death in wild-type salivary glands (data not shown). Caspase activity levels were quantified using the fluoregenic caspase substrate DEVD-AMC. Both wild-type and control animals lacking the P-element following precise excision had comparable levels of caspase activity, and this activity could be inhibited by either a chemical inhibitor (Z-VAD-FMK) or genetically by expression of the pan-caspase inhibitor p35 (Figure 3-2C) (Hay, Wolff et al. 1994; Lee and Baehrecke 2001; Martin and Baehrecke 2004; Berry and Baehrecke 2008). Although *ddlc1* mutant animals have reduced caspase activity (Figure 3-2C), the presence of caspase activity and fragmented DNA suggests that residual cysteine protease activity may be the reason that salivary gland degradation is not completely inhibited in *ddlc1* mutants.

To test if caspases contribute to *ddlc1* mutant salivary gland degradation, we expressed p35 in *ddlc1* mutant salivary glands and examined them using paraffin

histology. As previously shown (Lee and Baehrecke 2001; Berry and Baehrecke 2007), all control animals that express p35 in salivary glands possess partly degraded condensed salivary gland cell fragments 24 hours after puparium formation (Figure 3-2D). In control *ddlc1* mutants, 39% of animals have intact salivary glands, while 61% are partly degraded (Figure 3-2D). By contrast, p35 expression in the salivary glands of *ddlc1* mutant animals results in nearly complete inhibition of degradation, with 96% of these animals having intact salivary glands. These data indicate that caspases are active and contribute to the partial salivary gland degradation phenotype in *ddlc1* mutant animals, and suggest that Ddlc1 influences non-caspase factor(s) that participate in the death of this tissue.

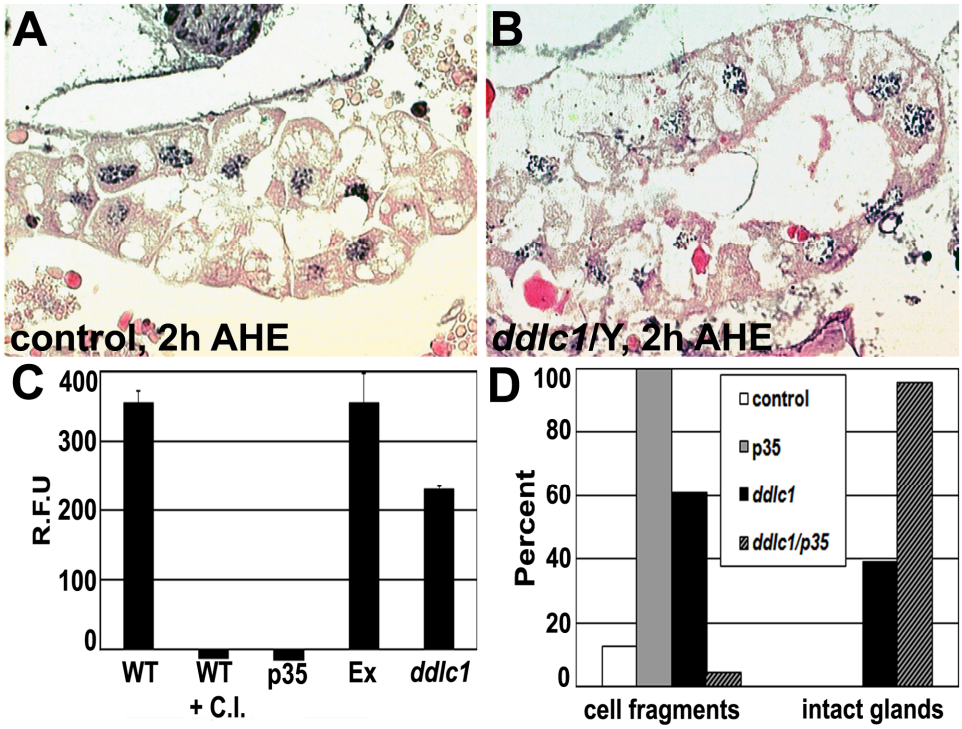

Figure 3-2: *ddlc1* mutants have altered caspase levels.

(A, B) TUNEL assay to detect DNA fragmentation in control (A) and *ddlc1* mutant (B) salivary glands 2 hours after head eversion (AHE) (n=5/genotype). (C) Caspase activity of pupal lysates collected at 4 hours after puparium formation (n=3) measured by cleavage of fluorogenic caspase-3 substrate Z-DEVD-AMC. The strains used for caspase activity measurements include WT: wild-type Canton S, WT + C.I.: wild-type Canton S + caspase inhibitor DEVD-CHO, p35: *daGAL4 x uas-p35*, Ex: precise excision of the P element, *ddlc1*: *ddlc1/Y*. (D) Percentage of animals of indicated genotypes with salivary gland cell fragments and intact salivary glands. Graph represents quantification of persistent salivary glands in paraffin sections of pupae 12 hours after head eversion. Detailed genotypes: Control (*fkhGAL4/+)* white bar (n=16), p35 expression (*fkhGAL4/uas-p35*) gray bar (n=13), *ddlc1* mutant (*ddlc1/Y;*

+/+; fkhGAL4/+) black bars (n=28), and p35 expression in *ddlc1* mutant animals (*ddlc1/Y; fkhGAL4/uas-p35),* striped bars (n=24). Error bars in (C) represent standard error. R.F.U: Relative Fluorescence Units.

ddlc1 mutant glands have impaired autophagy

Autophagosome formation and *atg* genes are induced just prior to salivary gland cell death, and autophagy is required for complete degradation of this tissue (Lee and Baehrecke 2001; Lee, Clough et al. 2003; Berry and Baehrecke 2007; Berry and Baehrecke 2008). We tested whether autophagy is defective in *ddlc1* mutant salivary glands by using the autophagosome marker GFP-LC3 (Rusten, Lindmo et al. 2004). As expected, the number of GFP-LC3 puncta increased in control salivary glands following the rise in steroid that triggers salivary gland cell death (Figure 3-3A, B, D). By contrast, the number of GFP-LC3 puncta was significantly lower in *ddlc1* mutant salivary glands at the same stage (Figure 3-3C, D). In addition, the number of GFP-LC3 puncta did not increase in *ddlc1* mutant salivary glands 10 hours later (Figure 3-3D). These data indicate that *ddlc1* mutant salivary glands are impaired in autophagosome formation.

Since lysosomes are required for autophagy, we tested if lysosome numbers are altered in *ddlc1* mutant salivary glands by expressing a GFP-Lysosome associated membrane protein 1 (LAMP1) reporter transgene (Rusten, Lindmo et al. 2004). In controls, the number of GFP-LAMP1 puncta increases following the rise in steroid that triggers salivary gland cell death (Figure 3-3E, F, H). Although the number of GFP-LAMP1 puncta was lower in *ddlc1* mutant salivary glands at the same stage (Figure 3-3G, H), the number of GFP-LAMP1 puncta increases 10h later (Figure 3-3H). These data indicate that although lysosome numbers are reduced in *ddlc1* mutant salivary glands, lysosome biogenesis appears to occur in this tissue. Consistent with this conclusion, the endosome-associated marker GFP-Rab5 is

identical in *ddlc1* mutant and wild-type salivary glands following the rise in steroid that triggers salivary gland cell death (data not shown). Taken together, these data indicate that *ddlc1* mutant salivary glands are defective in autophagosome biogenesis, while they can assemble new lysosomes.

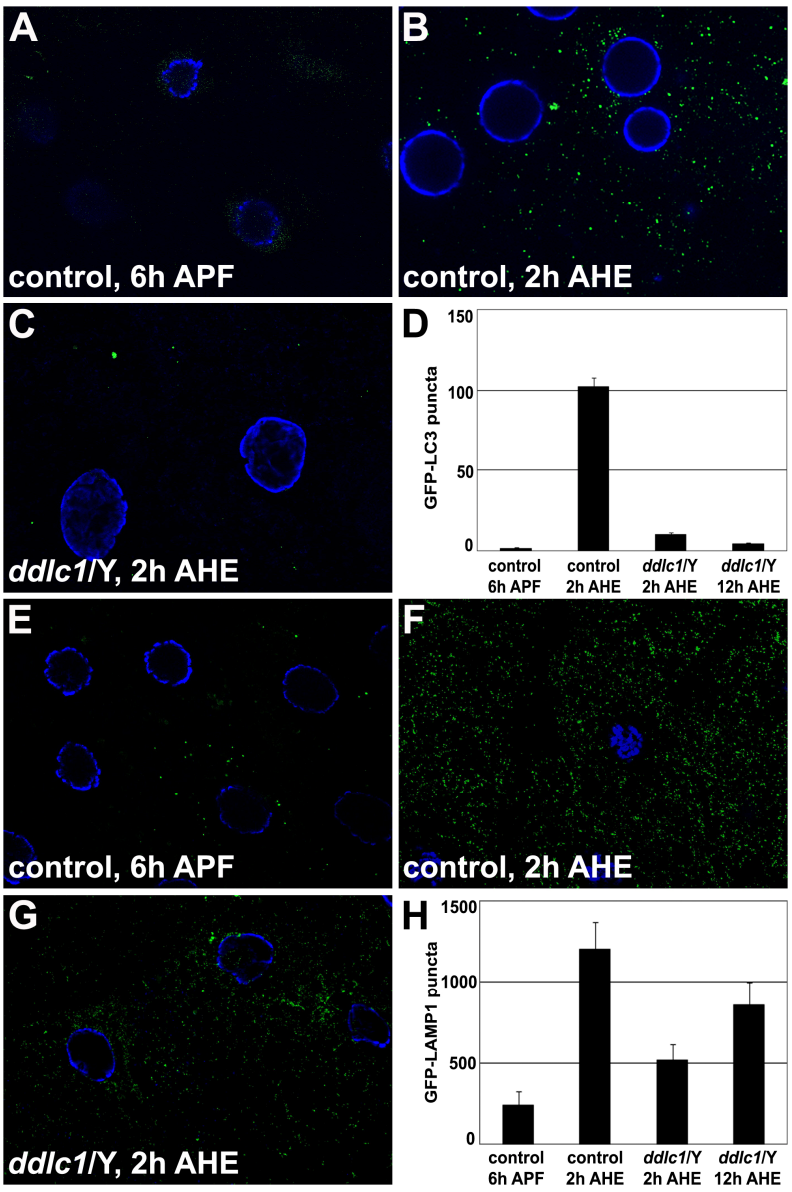

Figure 3-3: Autophagy is attenuated in *ddlc1* mutants.

(A-C) Representative fluorescence microscopy images of salivary glands expressing GFP-LC3 (green) and DNA (blue). (A) Control animal salivary glands have little to no puncta before cell death at 6 hours after puparium formation (APF) (n=16). (B) Control animal salivary glands have a large number of puncta 2 hours after head eversion (AHE) (n=20). (C) *ddlc1* mutant salivary glands have few puncta 2 hours

AHE (n=17). Detailed genotypes: (A, B) *ex; +/+; fkhGAL4/uas-GFP-LC3*, (C) *ddlc1*/Y; +/+; *fkhGAL4/uas-GFP-LC3*. (D) Average number of GFP-LC3 puncta in the genotypes shown in A-C, and in *ddlc1* mutant animals 12 hours AHE (n=23), detailed genotype: *ddlc1/Y; +/+; fkhGAL4/uas-GFP-LC3*. Error bars represent standard error. (E-G) Representative fluorescence microscopy images of salivary glands expressing GFP-LAMP1 (green) and DNA (blue). (E) Control animals have little LAMP1 puncta 6 hours APF (n=19). (F) Control animals have a large number of LAMP1 puncta 2 hours AHE (n=19). (G) *ddlc1* mutant glands have fewer LAMP1 puncta than controls 2 hours AHE (n=25). Detailed genotypes: (E, F) *ex; tub-GFP-LAMP1/+*, (G) *ddlc1/Y; tub-GFP-LAMP1/+*. (H) Average number of GFP-LAMP1 puncta in the genotypes shown in (E-G), and in *ddlc1* mutant animals 12 hours AHE (n=21), detailed genotype: *ddlc1/Y; tub-GFP-LAMP1/+*. Error bars represent standard error. ex: Line with the P element precisely excised.

ddlc1 mutants contain amyloid-like protein inclusions that can be degraded by induction of autophagy

The observation of fewer autophagosomes in *ddlc1* mutant salivary gland cells prompted us to investigate their morphology by transmission electron microscopy (TEM). Wild-type salivary gland cells contain autophagosomes and autolysosomes that are typically observed in these cells following the rise in steroid that triggers their death (Figure 3-4A). Although *ddlc1* mutant salivary glands contained some autophagic structures, numerous fibrillar inclusions were present. These inclusions appear to be dispersed throughout the cytoplasm and were not surrounded by membranes (Figure 3-4B). The Glue protein is produced and secreted by the salivary glands to help pupae attach to surfaces during pupariation. *ddlc1* mutant animals can secrete the Glue protein normally (Figure 3-6). Therefore, it is unlikely that these inclusions form due to aggregation of the Glue protein.

The accumulation of protein inclusions is the most common outcome of neurodegenerative diseases (Ross and Poirier 2004). Protein inclusion formation has been associated with proteasome inhibition (Bence, Sampat et al. 2001; Pandey, Nie et al. 2007). Therefore, we tested if the proteasome was inhibited in the *ddlc1* mutant salivary glands by expressing the GFP fused CL1 reporter (Dantuma, Lindsten et al. 2000). GFP-CL1 is normally degraded by the proteasome, and if the proteasome is inhibited it accumulates in the cell and can be detected as a GFP signal. Expression of GFP-CL1 alone and in the *ddlc1* mutant background did not result in GFP-CL1 accumulation in the gland (data not shown). By contrast, inhibition of the proteasome by expressing a temperature sensitive dominant negative subunit of the proteasome

(Belote and Fortier 2002) resulted in GFP-CL1 accumulation (data not shown). These results suggest that the proteasome system is not inhibited in the *ddlc1* mutant salivary glands. The fibrillar nature of the *ddlc1* mutant salivary gland inclusions are similar to β-sheet structures that are observed in amyloids associated with neurodegenerative diseases (Ross and Poirier 2004). Therefore, we tested if the protein inclusions in *ddlc1* mutant salivary glands are similar to protein inclusions seen in neurodegenerative diseases by performing histological tests that are typically used to detect amyloidosis in the clinic (Maezawa, Hong et al. 2008). Thioflavin-S stains of the *ddlc1* mutant salivary gland paraffin sections contained numerous brightly-stained foci that are indicative of amyloids (Figure 3-4D), while control glands did not possess these structures (Figure 3-4C). Similarly, congo-red staining of inclusions was only present in experimental *ddlc1* mutant salivary glands (data not shown).

Protein inclusions that are associated with neurodegeneration typically contain ubiquitin and p62, proteins that are associated with the ubiquitin proteasome system and autophagy (Ross and Pickart 2004; Bjorkoy, Lamark et al. 2005). Therefore, we tested whether the protein inclusions that are observed in *ddlc1* mutant salivary glands contain ubiquitin and *Drosophila* p62 ortholog Ref(2)P (Nezis, Simonsen et al. 2008). While both Ubiquitin and Ref(2)P were detected in wild-type salivary gland cells, they were not co-localized (Figure 3-4E). By contrast, Ubiquitin and Ref(2)P were co-localized in large puncta in *ddlc1* mutant salivary gland cells (Figure 3-4F). Furthermore, Ref(2)P levels were elevated and higher molecular weight

proteins were ubiquitinylated in *ddlc1* mutant salivary glands compared to wild-type salivary glands (Figure 3-4G).

Protein inclusions accumulate when catabolic pathways, including autophagy, are defective during neurodegeneration (Iwata, Riley et al. 2005; Pandey, Nie et al. 2007; Pan, Kondo et al. 2008). The reduced number of GFP-LC3 autophagosomes and accumulation of protein aggregates in *ddlc1* mutant salivary glands (Figures 3-3 and 3-4) prompted us to test if expression of the Atg1 kinase, a protein that is sufficient to induce autophagy (Berry and Baehrecke 2007; Scott, Juhasz et al. 2007), is sufficient to inhibit the accumulation of protein aggregates in these mutants. Significantly, the thioflavin-S positive inclusions in *ddlc1* mutant salivary gland cells were absent when we expressed a moderate strength Atg1 transgene in this tissue (Scott, Juhasz et al. 2007) (Figure 3-4D, I). In addition, TEM sections of these glands indicated that fibrillar inclusions were absent in glands with enhanced degradation (data not shown) or reduced in moderately degraded glands (Figure 3-4B, H). Therefore, since *ddlc1* glands are defective in autophagosome formation (Figure 3-3), we propose that the cell death and protein inclusion defects in *ddlc1* mutant salivary glands are due to impairment of an early step in autophagy.

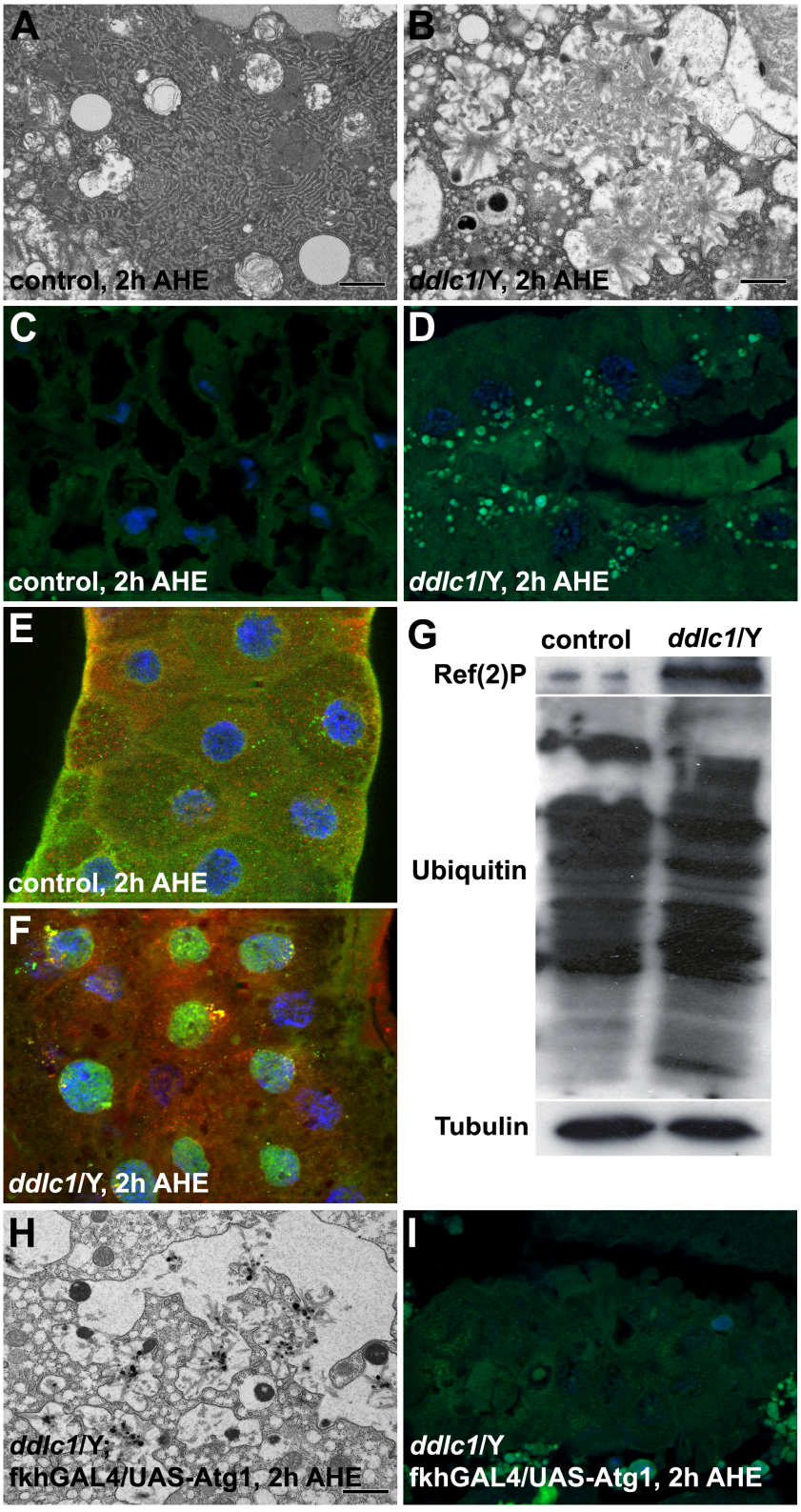

Figure 3-4: *ddlc1* mutants contain amyloid-like protein inclusions that are reduced when autophagy is induced.

(A,B) TEM images of salivary glands. Bars represent 1µm. (A) Wild-type Canton S control salivary glands 2 hours AHE. (B) *ddlc1* mutant salivary glands 2 hours AHE possess numerous large fibrillar inclusions. (C,D) Thioflavin-S-stained paraffin sections of pupae. (C) Wild-type Canton S control salivary glands 2 hours AHE possess no Thioflavin-S-stained foci (n=10 animals). (D) *ddlc1* mutant salivary glands 2 hours AHE possess numerous bright green foci indicative of Thioflavin-S-staining (n=10 animals). (E,F) Immunohistochemistry images of salivary glands 2 hours AHE, Ref(2)P (green), Ubiquitin (red), DNA (blue). (E) Wild type Canton S control salivary gland showing no co-localization of Ref(2)P and Ubiquitin 2 hours AHE. (F) *ddlc1* mutant salivary glands contain large puncta with co-localization of Ref(2)P and Ubiquitin 2 hours AHE. (G) Ref(2)P and Ubiquitin immuno-blot of salivary glands isolated from control and *ddlc1* mutant animals 2 hours AHE. Tubulin is a control for loading and transfer. (H, I) Expression of Atg1 in *ddlc1* mutant salivary glands 2 hours AHE. (H) TEM image of gland showing attenuation of fibrillar inclusions (compare with B), bar represents 1µm. (I) Thioflavin-S-stained paraffin sections contain little or no foci in salivary glands (compare with D) (n=10), detailed genotype*: ddlc1/Y; +/+; fkhGAL4/uas-Atg1*^{GS10797}.

ddlc1 enhances motility defects in a Spinal Bulbar Muscular Atrophy model

We have previously shown that protein clearance by autophagy plays an important role in an eye model of the neurodegenerative disorder spinal bulbar muscular atrophy (SBMA) (Pandey, Nie et al. 2007). SBMA is characterized by progressive muscular atrophy in males, and is caused by polyglutamine expansion in the androgen receptor (AR) in the presence of the AR ligand testosterone (Takeyama, Ito et al. 2002). Similar to our previous studies in a *Drosophila* eye model, we observed a polyglutamine length- and ligand-dependent decrease in motor activity when we expressed AR in motor neurons of third instar larvae (Figure 3-5A). In addition, *ddlc1* mutant larvae possess motility defects, and loss of *ddlc1* enhances the SBMA defect in motor activity when expanded polyglutamine-containing AR is expressed in motor neurons (Figure 3-5B).

Significantly, *ddlc1* mutant larval muscles have numerous p62 positive inclusions (Figure 3-5G), while wild type larval muscles have a diffuse staining of p62 (Figure 3-5F). In addition, these inclusions become larger when AR Q52 is expressed in the presence of DHT (Figure 3-5H). Moreover, *ddlc1* mutants and *ddlc1* mutants expressing AR Q52 with DHT occasionally have punctate Ref(2)P staining pattern in their motor neurons (Figure 3-5D, E), and wild type motor neurons do not (Figure 3-5C). Ref(2)P positive inclusions were previously observed in cases where catabolism was compromised as discussed above. Therefore, these data suggest that the motor activity defect of *ddlc1* larvae could be due to catabolic defects in the neuromuscular tissue. Enlargement of Ref(2)P positive inclusions (Figure 3-5H) and enhancement of

motility defect in *ddlc1* mutants (Figure 3-5B) indicates that expressing AR Q52 increases the catabolic defects and toxicity seen in *ddlc1* mutants alone.

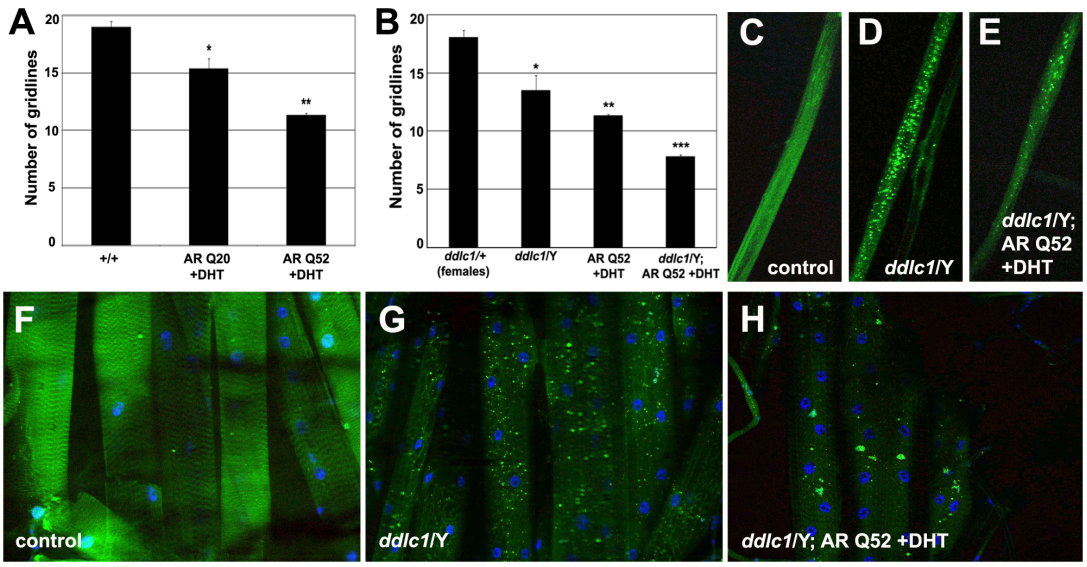

Figure 3-5: *ddlc1* enhances motility defects in a model of Spinal Bulbar Muscular Atrophy, and this phenotype is associated with Ref(2)P inclusions in axons and muscles.

(A,B) Average number of grid lines passed by the posterior end of larvae. (A) The motility of either wild-type Canton S larvae (+/+), or larvae expressing full-length human androgen receptor in motor neurons containing 20 glutamines (AR Q20) or 52 glutamines (AR Q52) and raised on food containing dihydrotestosterone (DHT) were analyzed (n=3 x15 larvae). (B) *ddlc1/Y* mutant larvae have motility defects compared to control female siblings (*ddlc1*/+), and the motility defect of AR 52Q expression is enhanced when it is expressed in *ddlc1* mutant larvae in the presence of DHT (n=3 x 15 larvae). "*" indicates a $p< 0.01$ significant difference, "**" indicates a $p< 0.001$ significant difference from wild-type (+/+ or *ddcl1*/+ controls). "***" indicates a $p< 0.001$ significant difference of AR Q52 expression alone from the AR Q52 expression in the *ddlc1* mutant larvae. Detailed genotypes in order within graphs: (A) *d42GAL4/+, w; uas-hAR- Q20/d42GAL4, w; uas-hAR Q52/d42GAL4.* (B) *ddlc1/+* ♀

, *ddlc1/Y, w; uas-hAR Q52/d42GAL4, ddlc1/Y; uas-hAR Q52/d42GAL4*. (C-E) Ref(2)P staining (green) in third instar larval motor neurons. (C) Wild-type Canton S control showing no punctate staining (n=6 animals). (D) *ddlc1* mutant neurons contain Ref(2)P positive punctate staining (n=4). (E) *ddlc1* mutant expressing hAR Q52 in the presence of DHT showing Ref(2)P positive punctate staining (n=4 animals). (F-H) Ref(2)P staining (green) of third instar larval muscles. (F) Wild-type Canton S control muscles contain little or no punctate Ref(2)P staining (n=6). (G) *ddlc1* mutant larval muscles contain Ref(2)P positive punctate staining (n=4). (H) *ddlc1* mutant larvae expressing hAR Q52 in the presence of DHT have large Ref(2)P positive punctate staining (n=4). DHT: dihydrotestosterone.

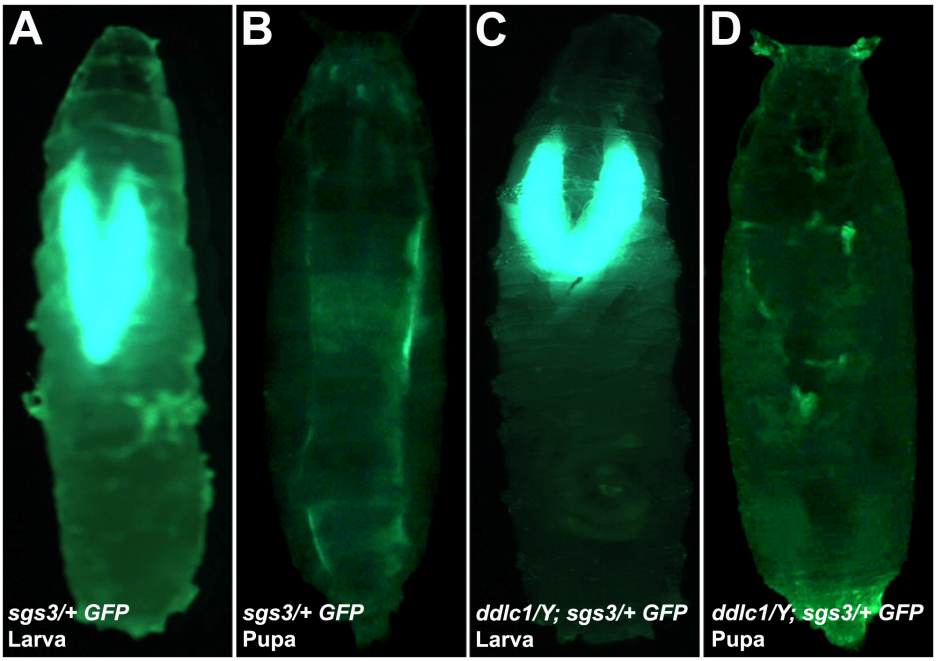

Figure 3-6: *ddlc1* salivary glands are not defective in secretion of the Glue protein

(A) Control larva with salivary glands filled with the GFP-tagged Glue protein. (B) Control white pre-pupa after secretion of the Glue protein. Some Glue protein is observed attached to the cuticle and none is in the salivary glands. (C) *ddlc1/Y* larva with salivary glands filled with the GFP-tagged Glue protein. (D) *ddlc1/Y* white pre-pupa after secretion of the Glue protein showing that the *ddlc1* mutation does not inhibit secretion of the glue protein.

Discussion

Drosophila ddlc1 was identified in a forward genetic screen for genes that are required for type II cell death with autophagy in salivary glands (Figure 3-1). Ddlc1 has been implicated in the regulation of cell death through physical interactions with Bim (Puthalakath, Huang et al. 1999) and p53 binding protein (Lo, Kan et al. 2005). Both of these cases imply that Ddlc1 functions in cell death by influencing the activity of caspase regulators and caspases. By contrast, we found that *ddlc1* mutant animals possess caspase activity that is only slightly lower than controls, and our genetic studies indicate that factors other than caspases are influenced by *ddlc1*, and required for salivary gland destruction (Figure 3-2). Significantly, *ddlc1* mutant salivary glands are defective in autophagosome formation even though they assemble new lysosomes (Figure 3-3). Thus, failure in salivary gland degradation is expected, since degradation of this tissue requires autophagy (Lee and Baehrecke 2001; Berry and Baehrecke 2007). Although autophagy is an important regulator of salivary gland cell death, we cannot exclude the possibility that *ddlc1* regulates other processes that are required for destruction of this tissue. In support of this possibility, *ddlc1* mutant salivary glands have a stronger defect in their degradation than autophagy *atg* gene mutants (Berry and Baehrecke 2007).

Ddlc1 is best known as a dynein motor component, but it also functions in motor-independent processes (Barbar 2008). For example, Ddlc1 and Bim influence caspase activity and cell death in a dynein motor-independent manner, by Ddlc1 binding to and restriction of Bim pro-apoptotic activity (Puthalakath, Huang et al. 1999). Alternatively, Ddlc1 is part of a dynein motor in its regulation of the p53

binding protein and DNA damage-induced p53 nuclear localization (Lo, Kan et al. 2005). It is not completely clear how Ddlc1 regulates autophagy and salivary gland destruction. An intact microtubule network is necessary for proper autophagy (Fass, Shvets et al. 2006; Kochl, Hu et al. 2006) and disruption of dynactin impairs autophagy (Kimura, Noda et al. 2008), so it is possible that Ddlc1 is part of a dynein motor that is required for autophagy in salivary glands. However, we did not observe any defect in salivary gland degradation in Dynein heavy chain mutants (data not shown), and this suggests that Ddlc1 may be regulating autophagy in a motor-independent manner. Expression of the autophagy regulator Atg1 was sufficient to rescue salivary gland degradation (Figure 3-4). This suggests that Ddlc1 may either influence signaling upstream of Atg1. Alternatively, Ddlc1 may facilitate the localization of either autophagosomal cargo or autophagy regulatory factors that are needed to form autophagosomes. Future work should resolve these possible models for Ddlc1 function in dying salivary glands.

The clearance of unwanted and damaged proteins is an essential component of cell homeostasis, and autophagy is one of the critical pathways for removal of such proteins (Klionsky and Emr 2000; Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). Significantly, defects in protein clearance have been associated with several neurodegenerative disorders (Bjorkoy, Lamark et al. 2005; Pandey, Nie et al. 2007; Boland, Kumar et al. 2008). To our surprise, protein inclusions accumulate in *ddlc1* mutant non-neuronal salivary glands (Figure 3-4). The fibrillar nature of these inclusions is extremely reminiscent of β-sheet structures that are observed in neuronal amyloids, and staining with amyloid specific thioflavin-s supports this observation

(Figure 3-4). We speculate that this protein inclusions form because of attenuated autophagy in *ddlc1* mutants and this conclusion is supported by the fact that activation of autophagy by expression of Atg1 reduces these inclusions (Figure 3-4). In addition, the levels of p62 increased and were localized with ubiquitin positive inclusions in *ddcl1* mutant salivary glands (Figure 3-4).

p62 and ubiquitin accumulate in aging brain of conditional autophagy deficient mice and in flies (Komatsu, Waguri et al. 2007; Nezis, Simonsen et al. 2008). In addition, p62-associated protein inclusions were degraded by autophagy in a model of Huntington's disease (Bjorkoy, Lamark et al. 2005), and disruption of the LC3/Atg8 binding domain in p62 resulted in the formation of ubiquitin-positive inclusions (Ichimura, Kumanomidou et al. 2008). These and other studies suggest similarities between the role of autophagy in neuronal protein aggregation disorders and in *ddlc1* mutant salivary glands, and this prompted us to study the influence of *ddlc1* mutations on the nervous system. We studied the influence of decreased Ddlc1 function, as well as a model of the polyglutamine disorder SBMA, on larval locomotor activity. Both *ddlc1* mutant larvae and the SBMA model larvae exhibited decreased motor activity (Figure 3-5). In addition, we observed that expression of AR Q52 in the motor neurons of *ddlc1* mutant larvae enhanced motility defect and the size of p62-positive inclusions in muscles. This observation suggests that the increased size of protein inclusions in *ddlc1* muscle is triggered in a cell non-autonomous manner by altered motor neuron function. This phenomenon can be explained by interdependency of muscles and axons. Axon growth and innervation is dependent on neurotrophic growth factors produced by muscles (Howe and Mobley

2005). Likewise, formation of proper muscle structure requires signaling input from the innerving neurons (Maggs, Huxley et al. 2008). Significantly, in a *C. elegans* model of polyglutamine disease, mutations in axonal GABAergic or cholinergic signaling disrupted protein homeostasis in the muscle causing enhanced polyglutamine inclusion formation and motility defects (Garcia, Casanueva et al. 2007). Moreover, these mutations caused temperature sensitive proteins to go into inclusions in the muscle at permissive temperatures. Therefore, we speculate that AR Q52 expression in the motor neurons might cause signaling imbalance in the neurons that further negatively impacts protein homeostasis in the muscle of *ddlc1* larvae. It should be noted that we did not observe p62 positive inclusions in the muscles of larvae expressing AR Q52 in the motor neurons alone (data not shown). These observations suggest that *ddlc1* mutation increases the propensity of protein stress and inclusion formation in the larval muscle perhaps due to Ddlc1's involvement in protein degradation. Accordingly, when AR Q52 is expressed in the motor neurons in *ddlc1* background there is a higher likelihood to form protein inclusions (Figure 3-5).

Autophagy is a catabolic process that clears material from the cytoplasm to promote physiological processes associated with organism, tissue and cell homeostasis. Here we identify *ddlc1* as a factor that is required for autophagy in dying cells, and show that loss of *ddlc1* leads to accumulation of p62-associated aggregates in the non-neuronal salivary gland and muscle cell types. These results raise the question of why these tissues exhibit *ddlc1* mutant defects. Larval salivary glands and muscles have several common features, including that they are made of

endo-replicating cells that do not divide, they have very large cells with extensive cytoplasm, and that they die after larval development with a type II autophagic cell death morphology. Therefore, one possibility is that these non-dividing cells rely more heavily on autophagy for clearance of either unwanted or damaged proteins than diploid cells, and that decreased autophagy leads to protein aggregates. Alternatively, these are relatively long-lived cells, and it is possible that at stages approaching death autophagy increasingly promotes clearance of cytoplasm. Therefore, impairment of autophagy results in protein accumulation at the time of cell death. Future studies should resolve how Ddlc1 functions in the regulation of protein clearance, autophagy and cell death.

Chapter 4

Summary and Future Directions

The relationship between the proteasome and autophagy in neurodegenerative disease

The balance between anabolic and catabolic processes is crucial to maintain cellular homeostasis. The ubiquitin-proteasome system and autophagy are the two main catabolic degradation systems. The proteasome system is well studied, and it is known to be a key regulator of many cellular activities (Ciechanover, Orian et al. 2000; Glickman and Ciechanover 2002). By contrast, autophagy is poorly understood. Autophagy has been implicated in roles as diverse as turnover of organelles and long lived proteins, cell survival, cell death, neurodegenerative diseases and cancer (Klionsky and Emr 2000; Mizushima 2005). It is possible that the proteasome system and autophagy communicate to maintain the appropriate quantity of catabolic degradation. The discovery of the HDAC6 and p62 proteins that are associated with polyubiquitinylated proteins and autophagy raised the question whether these proteins link the proteasome and autophagy (Bjorkoy, Lamark et al. 2005; Iwata, Riley et al. 2005). Our studies indicate that when the proteasome is inhibited *in-vivo,* autophagy is induced to compensate for lack of degradation and to maintain homeostasis (Chapter 2). Moreover in the SBMA neurodegenerative disease model used in this study, proteasome inhibition is compensated for by autophagy (Chapter 2). HDAC6 appears to play a role in the autophagic degradation of proteins that were targeted to the proteasome but could not be degraded by this mechanism. It has been suggested that HDAC6 could be part of a molecular motor

that moves cargo to inclusions, which are later degraded by autophagy (Kawaguchi, Kovacs et al. 2003). This hypothesis is supported by our studies which show that autophagic rescue of degeneration in the eye is dependent on HDAC6 (Pandey, Nie et al. 2007). However, the precise mechanism as to how the proteasome and the autophagy communicate, and HDAC6's role in this communication, is not entirely understood. It is of interest to investigate the binding partners of HDAC6 by yeast two hybrid screens, as well as by co-immunoprecipitation and proteomic analyses, in conditions of proteasome impairment. HDAC6 may be found to bind to molecular motors and to proteins that could be causing proteasome impairment, such as polyglutamine expanded proteins. It would also be interesting to investigate if proteasome inhibition is a prerequisite for the induction of autophagy during protein stress, or if accumulation of unwanted proteins alone induces autophagy. Monitoring autophagy and proteasome levels during protein mis-expression may help us understand these possibilities and the relationship between proteasome inhibition and autophagy.

Our results suggest that the induction of autophagy is protective against the degenerative effects of polyglutamine expanded AR expression (Chapter 2). It should be noted that we have not directly tested whether polyglutamine expanded AR causes protein stress, or alternatively if stress is caused by the accumulation of other proteins. However, we know that turnover of polyglutamine expanded AR is significantly reduced in degenerative conditions (Pandey, Nie et al. 2007). Either direct inhibition of the proteasome using a genetic approach, or indirect inhibition of the proteasome by the expression of polyglutamine expanded AR causes eye

degeneration (Chapter 2). Therefore, the basis of toxicity in the SBMA model is the proteasome impairment. This has important consequences for polyglutamine expansion diseases. In patients, progressive proteasome inhibition is likely to be an important contributor to toxicity, and the reason for protein inclusion formation. However, this alone cannot explain the mechanism of all polyglutamine expansion diseases since different diseases have different symptoms and they affect different neurons. This implies that either the natural function of the glutamine expanded protein or new functions it acquires might be involved in the outcomes of particular diseases. Investigating the changes in gene and protein expression profiles in the SBMA model in the absence and presence of DHT could provide directions for deciphering the disease mechanisms in addition to proteasome inhibition. Therapies that involve relieving the effects of proteasome inhibition, such as increasing the rate of autophagy, could delay the occurrence of symptoms. However, this is complicated by the side effect of activating autophagy in tissues that are not involved in neurodegenerative symptoms, and that ectopic autophagy could induce cell death (Baehrecke 2005; Scott, Juhasz et al. 2007). Another approach for therapy could be to screen known chemicals for their ability to upregulate HDAC6's activity, since we have shown that overexpression of HDAC6 alone is enough to reduce the eye degeneration phenotype (Pandey, Nie et al. 2007).

Autophagy in catabolism and cell death

Larval salivary glands die in an autophagy- and caspase-dependent manner during development of *Drosophila* (Lee and Baehrecke 2001; Berry and Baehrecke 2007). We identified Ddlc1 as one of the effectors of salivary gland cell death in a forward

genetic screen (Chapter 3). *ddlc1* mutants were previously reported to have ovarian development and axon path finding defects (Dick, Ray et al. 1996). *ddlc1* mutant salivary glands were defective in autophagy, and as a result they had a considerable cell death defect (Chapter 3). The complete mechanism of autophagy is not entirely understood (Klionsky 2005). For example, it is not known how autophagosomes form and are directed to lysosomes in the cytoplasm. Our data indicate that Ddlc1 is involved in autophagosome formation (Chapter 3). However, we could not identify the mechanism of Ddlc1's contribution to autophagosomes. Since ectopic expression of Atg1 could activate autophagy in *ddlc1* mutants, Ddlc1 is likely to be involved in an upstream, yet unidentified regulation of autophagy. We were limited in our ability to do immunohistochemical analyses of *ddlc1* mutant glands due to unavailability of a working antibody. In the future, either Ddlc1 antibodies or Ddlc1 fused to a fluorescent protein could be generated to follow this protein in the cell (as long as this fusion protein does not inhibit Ddlc1 function). If Ddlc1 is co-localized with LC3 or Atg1 and not with lysosomes, this would support Ddlc1's function in earlier steps of autophagosome formation. Ddlc1 could also be immunoprecipitated to identify binding partners. Dynein motor binding partners would confirm Ddlc1's involvement in a dynein motor. We speculated that Ddlc1 could be involved in bringing cargo to autophagosomes (Chapter 3). This could be tested in cell lines or yeast in which autophagosomal cargo can be traced (Xie, Nair et al. 2008). For example, this cargo may not be found in autophagosomes in *ddlc1* mutants.

We observed amyloid like inclusions in the *ddlc1* mutant salivary glands (Chapter 3). Our data suggest that this is due to defects in autophagic degradation, as the

proteasome was not inhibited (data not shown). Ref(2)P/p62, which functions in protein inclusion formation during protein stress (Bjorkoy, Lamark et al. 2005; Komatsu, Waguri et al. 2007; Nezis, Simonsen et al. 2008), was increased in *ddlc1* glands. It is of interest to investigate if *ddlc1* and *ref(2)p* double mutants could still form protein inclusions in the salivary gland. If inclusions cannot form, this will confirm Ref(2)P's function in inclusion formation in this tissue. Moreover, it is of interest to determine the protein species that accumulate in inclusions. Our data suggested that only a few ubiquitinylated species were upregulated in the *ddcl1* glands (Chapter 3). These or other proteins that are upregulated in coomassie stained protein gels could be isolated and identified by mass spectrometry. A considerable amount of glue protein is produced and secreted during pupariation to enable pupae to attach to surfaces (Biyasheva, Do et al. 2001). Our data suggest that *ddlc1* glands are not defective in secreting the glue protein (Chapter 3). Therefore, it is unlikely that it is the glue protein that aggregates in *ddlc1* glands.

To conclude, our studies illustrate the importance of compensatory autophagic degradation in neurodegenerative diseases. In addition, impairment of autophagy through a mutation in *ddlc1* caused inclusion formation that was reminiscent of neurodegenerative disease in non-neuronal salivary gland cells. These observations are in agreement with the protein stress hypothesis of neurodegenerative diseases, which proposes that impairment of catabolic degradation causes protein stress and inclusions. Emergence of autophagy as a protective mechanism in neurodegenerative diseases is likely to lead to new possibilities for therapy.

References

Abel, A., J. Walcott, et al. (2001). "Expression of expanded repeat androgen receptor produces neurologic disease in transgenic mice." Hum Mol Genet **10**(2): 107-16.

Arrasate, M., S. Mitra, et al. (2004). "Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death." Nature **431**(7010): 805-10.

Axe, E. L., S. A. Walker, et al. (2008). "Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum." J Cell Biol **182**(4): 685-701.

Baehrecke, E. H. (2000). "Steroid regulation of programmed cell death during Drosophila development." Cell Death Differ **7**(11): 1057-62.

Baehrecke, E. H. (2005). "Autophagy: dual roles in life and death?" Nat Rev Mol Cell Biol **6**(6): 505-10.

Barbar, E. (2008). "Dynein light chain LC8 is a dimerization hub essential in diverse protein networks." Biochemistry **47**(2): 503-8.

Belote, J. M. and E. Fortier (2002). "Targeted expression of dominant negative proteasome mutants in Drosophila melanogaster." Genesis **34**(1-2): 80-2.

Bence, N. F., R. M. Sampat, et al. (2001). "Impairment of the ubiquitin-proteasome system by protein aggregation." Science **292**(5521): 1552-5.

Bennett, E. J., N. F. Bence, et al. (2005). "Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation." Mol Cell **17**(3): 351-65.

Berg, T. O., M. Fengsrud, et al. (1998). "Isolation and characterization of rat liver amphisomes. Evidence for fusion of autophagosomes with both early and late endosomes." J Biol Chem **273**(34): 21883-92.

Berry, D. L. and E. H. Baehrecke (2007). "Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila." Cell **131**(6): 1137-48.

Berry, D. L. and E. H. Baehrecke (2008). "Autophagy functions in programmed cell death." Autophagy **4**(3): 359-60.

Bingham, P. M., M. O. Scott, et al. (1995). "Stability of an expanded trinucleotide repeat in the androgen receptor gene in transgenic mice." Nat Genet **9**(2): 191-6.

Biyasheva, A., T. V. Do, et al. (2001). "Glue secretion in the Drosophila salivary gland: a model for steroid-regulated exocytosis." Dev Biol **231**(1): 234-51.

Bjorkoy, G., T. Lamark, et al. (2005). "p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death." J Cell Biol **171**(4): 603-14.

Boland, B., A. Kumar, et al. (2008). "Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease." J Neurosci **28**(27): 6926-37.

Bove, J., D. Prou, et al. (2005). "Toxin-induced models of Parkinson's disease." NeuroRx **2**(3): 484-94.

Bowman, A. B., Y. C. Lam, et al. (2007). "Duplication of Atxn1l suppresses SCA1 neuropathology by decreasing incorporation of polyglutamine-expanded ataxin-1 into native complexes." Nat Genet **39**(3): 373-9.
Boya, P., R. A. Gonzalez-Polo, et al. (2005). "Inhibition of macroautophagy triggers apoptosis." Mol. Cell. Biol. **25**: 1025-1040.
Boyault, C., B. Gilquin, et al. (2006). "HDAC6-p97/VCP controlled polyubiquitin chain turnover." EMBO J **25**(14): 3357-66.
Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**(2): 401-15.
Brignull, H. R., J. F. Morley, et al. (2006). "Modeling polyglutamine pathogenesis in C. elegans." Methods Enzymol **412**: 256-82.
Brinkmann, A. O. (2001). "Molecular basis of androgen insensitivity." Mol Cell Endocrinol **179**(1-2): 105-9.
Bronfman, F. C., M. Tcherpakov, et al. (2003). "Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome." J Neurosci **23**(8): 3209-20.
Brown, C. J., S. J. Goss, et al. (1989). "Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism." Am J Hum Genet **44**(2): 264-9.
Brown, T. R., D. B. Lubahn, et al. (1988). "Deletion of the steroid-binding domain of the human androgen receptor gene in one family with complete androgen insensitivity syndrome: evidence for further genetic heterogeneity in this syndrome." Proc Natl Acad Sci U S A **85**(21): 8151-5.
Bruijn, L. I., M. K. Houseweart, et al. (1998). "Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1." Science **281**(5384): 1851-4.
Campenot, R. B. (1977). "Local control of neurite development by nerve growth factor." Proc Natl Acad Sci U S A **74**(10): 4516-9.
Chen, X. J., E. N. Levedakou, et al. (2007). "Proprioceptive sensory neuropathy in mice with a mutation in the cytoplasmic Dynein heavy chain 1 gene." J Neurosci **27**(52): 14515-24.
Chevalier-Larsen, E. S., K. E. Wallace, et al. (2008). "Lysosomal proliferation and distal degeneration in motor neurons expressing the G59S mutation in the p150Glued subunit of dynactin." Hum Mol Genet **17**(13): 1946-55.
Ciechanover, A., A. Orian, et al. (2000). "Ubiquitin-mediated proteolysis: biological regulation via destruction." Bioessays **22**(5): 442-51.
Clarke, P. G. (1990). "Developmental cell death: morphological diversity and multiple mechanisms." Anat Embryol (Berl) **181**(3): 195-213.
Clarke, P. G. H. (1990). "Developmental cell death: morphological diversity and multiple mechanisms." Anat. Embryol. **181**: 195-213.
Cleveland, D. W. and J. D. Rothstein (2001). "From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS." Nat Rev Neurosci **2**(11): 806-19.
Cowan, W. M. and E. R. Kandel (2001). "Prospects for neurology and psychiatry." JAMA **285**(5): 594-600.

Crepieux, P., H. Kwon, et al. (1997). "I kappaB alpha physically interacts with a cytoskeleton-associated protein through its signal response domain." Mol Cell Biol **17**(12): 7375-85.
Cuervo, A. M. (2006). "Autophagy in neurons: it is not all about food." Trends Mol Med **12**(10): 461-4.
Cuervo, A. M., L. Stefanis, et al. (2004). "Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy." Science **305**(5688): 1292-5.
Dantuma, N. P., K. Lindsten, et al. (2000). "Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells." Nat Biotechnol **18**(5): 538-43.
de Pril, R., D. F. Fischer, et al. (2004). "Accumulation of aberrant ubiquitin induces aggregate formation and cell death in polyglutamine diseases." Hum Mol Genet **13**(16): 1803-13.
Deretic, V. (2005). "Autophagy in innate and adaptive immunity." Trends Immunol **26**(10): 523-8.
Dick, T., K. Ray, et al. (1996). "Cytoplasmic dynein (ddlc1) mutations cause morphogenetic defects and apoptotic cell death in Drosophila melanogaster." Mol Cell Biol **16**(5): 1966-77.
Dorstyn, L., S. Read, et al. (2002). "The role of cytochrome c in caspase activation in Drosophila melanogaster cells." J. Cell Biol. **156**: 1089-1098.
Doyu, M., G. Sobue, et al. (1992). "Severity of X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG repeat in androgen receptor gene." Ann Neurol **32**(5): 707-10.
Dragatsis, I., M. S. Levine, et al. (2000). "Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice." Nat Genet **26**(3): 300-6.
Dunah, A. W., H. Jeong, et al. (2002). "Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease." Science **296**(5576): 2238-43.
Duyao, M. P., A. B. Auerbach, et al. (1995). "Inactivation of the mouse Huntington's disease gene homolog Hdh." Science **269**(5222): 407-10.
Etlinger, J. D. and A. L. Goldberg (1977). "A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes." Proc Natl Acad Sci U S A **74**(1): 54-8.
Faber, P. W., J. R. Alter, et al. (1999). "Polyglutamine-mediated dysfunction and apoptotic death of a Caenorhabditis elegans sensory neuron." Proc Natl Acad Sci U S A **96**(1): 179-84.
Fadok, V. A., D. R. Voelker, et al. (1992). "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages." J. Immunol. **148**(7): 2207-2216.
Fass, E., E. Shvets, et al. (2006). "Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes." J Biol Chem **281**(47): 36303-16.
Fernandez-Funez, P., M. L. Nino-Rosales, et al. (2000). "Identification of genes that modify ataxin-1-induced neurodegeneration." Nature **408**(6808): 101-6.

Finley, K. D., P. T. Edeen, et al. (2003). "blue cheese mutations define a novel, conserved gene involved in progressive neural degeneration." J Neurosci **23**(4): 1254-64.
Gai, W. P., W. W. Blessing, et al. (1995). "Ubiquitin-positive degenerating neurites in the brainstem in Parkinson's disease." Brain **118 (Pt 6)**: 1447-59.
Garcia, S. M., M. O. Casanueva, et al. (2007). "Neuronal signaling modulates protein homeostasis in Caenorhabditis elegans post-synaptic muscle cells." Genes Dev **21**(22): 3006-16.
Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol Rev **82**(2): 373-428.
Graham, R. K., Y. Deng, et al. (2006). "Cleavage at the caspase-6 site is required for neuronal dysfunction and degeneration due to mutant huntingtin." Cell **125**(6): 1179-91.
Grimes, M. L., E. Beattie, et al. (1997). "A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA." Proc Natl Acad Sci U S A **94**(18): 9909-14.
Grozinger, C. M., C. A. Hassig, et al. (1999). "Three proteins define a class of human histone deacetylases related to yeast Hda1p." Proc Natl Acad Sci U S A **96**(9): 4868-73.
Gunawardena, S., L. S. Her, et al. (2003). "Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in Drosophila." Neuron **40**(1): 25-40.
Gurney, M. E., H. Pu, et al. (1994). "Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation." Science **264**(5166): 1772-5.
Hafezparast, M., R. Klocke, et al. (2003). "Mutations in dynein link motor neuron degeneration to defects in retrograde transport." Science **300**(5620): 808-12.
Hanada, T., N. N. Noda, et al. (2007). "The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy." J Biol Chem **282**(52): 37298-302.
Hanz, S. and M. Fainzilber (2006). "Retrograde signaling in injured nerve--the axon reaction revisited." J Neurochem **99**(1): 13-9.
Hanz, S., E. Perlson, et al. (2003). "Axoplasmic importins enable retrograde injury signaling in lesioned nerve." Neuron **40**(6): 1095-104.
Hara, T., K. Nakamura, et al. (2006). "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice." Nature **441**(7095): 885-9.
Hardy, J. and D. J. Selkoe (2002). "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics." Science **297**(5580): 353-6.
Harjes, P. and E. E. Wanker (2003). "The hunt for huntingtin function: interaction partners tell many different stories." Trends Biochem Sci **28**(8): 425-33.
Hay, B. A., T. Wolff, et al. (1994). "Expression of baculovirus P35 prevents cell death in Drosophila." Development **120**(8): 2121-9.
Heerssen, H. M., M. F. Pazyra, et al. (2004). "Dynein motors transport activated Trks to promote survival of target-dependent neurons." Nat Neurosci **7**(6): 596-604.

Herzig, R. P., U. Andersson, et al. (2000). "Dynein light chain interacts with NRF-1 and EWG, structurally and functionally related transcription factors from humans and drosophila." J Cell Sci **113 Pt 23**: 4263-73.
Hetz, C., P. Bernasconi, et al. (2006). "Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha." Science **312**(5773): 572-6.
Hook, P. and R. B. Vallee (2006). "The dynein family at a glance." J Cell Sci **119**(Pt 21): 4369-71.
Howe, C. L. and W. C. Mobley (2005). "Long-distance retrograde neurotrophic signaling." Curr Opin Neurobiol **15**(1): 40-8.
Huang, H., C. J. Potter, et al. (1999). "PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development." Development **126**(23): 5365-72.
Hubbert, C., A. Guardiola, et al. (2002). "HDAC6 is a microtubule-associated deacetylase." Nature **417**(6887): 455-8.
Hy, L. X. and D. M. Keller (2000). "Prevalence of AD among whites: a summary by levels of severity." Neurology **55**(2): 198-204.
Ichimura, Y., T. Kumanomidou, et al. (2008). "Structural basis for sorting mechanism of p62 in selective autophagy." J Biol Chem **283**(33): 22847-57.
Iwata, A., B. E. Riley, et al. (2005). "HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin." J Biol Chem.
Jackson, G. R., I. Salecker, et al. (1998). "Polyglutamine-expanded human huntingtin transgenes induce degeneration of Drosophila photoreceptor neurons." Neuron **21**(3): 633-42.
Jaffrey, S. R. and S. H. Snyder (1996). "PIN: an associated protein inhibitor of neuronal nitric oxide synthase." Science **274**(5288): 774-7.
Jiang, C., E. H. Baehrecke, et al. (1997). "Steroid regulated programmed cell death during *Drosophila* metamorphosis." Development **124**: 4673-4683.
Johnston, J. A., C. L. Ward, et al. (1998). "Aggresomes: a cellular response to misfolded proteins." J Cell Biol **143**(7): 1883-98.
Juhasz, G., J. H. Hill, et al. (2008). "The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila." J Cell Biol **181**(4): 655-66.
Kabeya, Y., N. Mizushima, et al. (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." Embo J **19**(21): 5720-8.
Karki, S. and E. L. Holzbaur (1995). "Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex." J Biol Chem **270**(48): 28806-11.
Katsuno, M., H. Adachi, et al. (2003). "Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy." Nat Med **9**(6): 768-73.
Katsuno, M., H. Adachi, et al. (2006). "Reversible disruption of dynactin 1-mediated retrograde axonal transport in polyglutamine-induced motor neuron degeneration." J Neurosci **26**(47): 12106-17.

Kawaguchi, Y., J. J. Kovacs, et al. (2003). "The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress." Cell **115**(6): 727-38.
Kegel, K. B., M. Kim, et al. (2000). "Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy." J Neurosci **20**(19): 7268-78.
Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-57.
Kieran, D., M. Hafezparast, et al. (2005). "A mutation in dynein rescues axonal transport defects and extends the life span of ALS mice." J Cell Biol **169**(4): 561-7.
Kimura, S., T. Noda, et al. (2008). "Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes." Cell Struct Funct **33**(1): 109-22.
King, S. M., E. Barbarese, et al. (1996). "Brain cytoplasmic and flagellar outer arm dyneins share a highly conserved Mr 8,000 light chain." J Biol Chem **271**(32): 19358-66.
Klionsky, D. J. (2005). "The molecular machinery of autophagy: unanswered questions." J Cell Sci **118**(Pt 1): 7-18.
Klionsky, D. J. and S. D. Emr (2000). "Autophagy as a regulated pathway of cellular degradation." Science **290**(5497): 1717-21.
Kobayashi, Y., A. Kume, et al. (2000). "Chaperones Hsp70 and Hsp40 suppress aggregate formation and apoptosis in cultured neuronal cells expressing truncated androgen receptor protein with expanded polyglutamine tract." J Biol Chem **275**(12): 8772-8.
Kochl, R., X. W. Hu, et al. (2006). "Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes." Traffic **7**(2): 129-45.
Komatsu, M., S. Waguri, et al. (2006). "Loss of autophagy in the central nervous system causes neurodegeneration in mice." Nature **441**(7095): 880-4.
Komatsu, M., S. Waguri, et al. (2007). "Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice." Cell **131**(6): 1149-63.
La Spada, A. R. and J. P. Taylor (2003). "Polyglutamines placed into context." Neuron **38**(5): 681-4.
La Spada, A. R., E. M. Wilson, et al. (1991). "Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy." Nature **352**(6330): 77-9.
Lai, C., X. Lin, et al. (2007). "The G59S mutation in p150(glued) causes dysfunction of dynactin in mice." J Neurosci **27**(51): 13982-90.
Lawrence, C. J., N. R. Morris, et al. (2001). "Dyneins have run their course in plant lineage." Traffic **2**(5): 362-3.
Leavitt, B. R., J. A. Guttman, et al. (2001). "Wild-type huntingtin reduces the cellular toxicity of mutant huntingtin in vivo." Am J Hum Genet **68**(2): 313-24.
Lee, C. Y. and E. H. Baehrecke (2001). "Steroid regulation of autophagic programmed cell death during development." Development **128**(8): 1443-55.

Lee, C. Y., E. A. Clough, et al. (2003). "Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila." Curr Biol **13**(4): 350-7.
Levine, B. and G. Kroemer (2008). "Autophagy in the pathogenesis of disease." Cell **132**(1): 27-42.
Levine, B. and J. Yuan (2005). "Autophagy in cell death: an innocent convict?" J. Clin. Invest. **115**: 2679-2688.
Levine, M. S., G. J. Klapstein, et al. (1999). "Enhanced sensitivity to N-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease." J Neurosci Res **58**(4): 515-32.
Li, L. B., Z. Yu, et al. (2008). "RNA toxicity is a component of ataxin-3 degeneration in Drosophila." Nature **453**(7198): 1107-11.
Li, M., E. S. Chevalier-Larsen, et al. (2007). "Soluble androgen receptor oligomers underlie pathology in a mouse model of spinobulbar muscular atrophy." J Biol Chem **282**(5): 3157-64.
Ligon, L. A., M. Tokito, et al. (2004). "A direct interaction between cytoplasmic dynein and kinesin I may coordinate motor activity." J Biol Chem **279**(18): 19201-8.
Lin, C. H., S. Tallaksen-Greene, et al. (2001). "Neurological abnormalities in a knock-in mouse model of Huntington's disease." Hum Mol Genet **10**(2): 137-44.
Lindsten, K., F. M. de Vrij, et al. (2002). "Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation." J Cell Biol **157**(3): 417-27.
Liu, X., C. N. Kim, et al. (1996). "Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c." Cell **86**(1): 147-57.
Lo, K. W., H. M. Kan, et al. (2005). "The 8-kDa dynein light chain binds to p53-binding protein 1 and mediates DNA damage-induced p53 nuclear accumulation." J Biol Chem **280**(9): 8172-9.
Lo, K. W., S. Naisbitt, et al. (2001). "The 8-kDa dynein light chain binds to its targets via a conserved (K/R)XTQT motif." J Biol Chem **276**(17): 14059-66.
Maezawa, I., H. S. Hong, et al. (2008). "Congo red and thioflavin-T analogs detect Abeta oligomers." J Neurochem **104**(2): 457-68.
Maggs, A. M., C. Huxley, et al. (2008). "Nerve-dependent changes in skeletal muscle myosin heavy chain after experimental denervation and cross-reinnervation and in a demyelinating mouse model of Charcot-Marie-Tooth disease type 1A." Muscle Nerve **38**(6): 1572-84.
Mangiarini, L., K. Sathasivam, et al. (1996). "Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice." Cell **87**(3): 493-506.
Marsh, J. L., T. Lukacsovich, et al. (2008). "Animal models of polyglutamine diseases and therapeutic approaches." J Biol Chem.
Martin, D. N. and E. H. Baehrecke (2004). "Caspases function in autophagic programmed cell death in Drosophila." Development **131**(2): 275-84.
Martin, J. B. (1999). "Molecular basis of the neurodegenerative disorders." N Engl J Med **340**(25): 1970-80.

Martin, M., S. J. Iyadurai, et al. (1999). "Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport." Mol Biol Cell **10**(11): 3717-28.
Mathew, R., V. Karantza-Wadsworth, et al. (2007). "Role of autophagy in cancer." Nat Rev Cancer **7**(12): 961-7.
Matilla, A., E. D. Roberson, et al. (1998). "Mice lacking ataxin-1 display learning deficits and decreased hippocampal paired-pulse facilitation." J Neurosci **18**(14): 5508-16.
McCampbell, A., J. P. Taylor, et al. (2000). "CREB-binding protein sequestration by expanded polyglutamine." Hum Mol Genet **9**(14): 2197-202.
Michalik, A. and C. Van Broeckhoven (2004). "Proteasome degrades soluble expanded polyglutamine completely and efficiently." Neurobiol Dis **16**(1): 202-11.
Mizushima, N. (2005). "The pleiotropic role of autophagy: from protein metabolism to bactericide." Cell Death Differ **12 Suppl 2**: 1535-41.
Mizushima, N., T. Noda, et al. (1998). "A protein conjugation system essential for autophagy." Nature **395**: 395-398.
Mizushima, N., A. Yamamoto, et al. (2004). "In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker." Mol Biol Cell **15**(3): 1101-11.
Murakami, T., I. Nagano, et al. (2001). "Impaired retrograde axonal transport of adenovirus-mediated E. coli LacZ gene in the mice carrying mutant SOD1 gene." Neurosci Lett **308**(3): 149-52.
Muro, I., D. L. Berry, et al. (2006). "The Drosophila caspase Ice is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process." Development **133**(17): 3305-15.
Nagai, Y., T. Inui, et al. (2007). "A toxic monomeric conformer of the polyglutamine protein." Nat Struct Mol Biol **14**(4): 332-40.
Nedelsky, N. B., P. K. Todd, et al. (2008). "Autophagy and the ubiquitin-proteasome system: Collaborators in neuroprotection." Biochim Biophys Acta.
Neufeld, T. P. and E. H. Baehrecke (2008). "Eating on the fly: function and regulation of autophagy during cell growth, survival and death in Drosophila." Autophagy **4**(5): 557-62.
Nezis, I. P., A. Simonsen, et al. (2008). "Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain." J Cell Biol **180**(6): 1065-71.
Nixon, R. A., J. Wegiel, et al. (2005). "Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study." J Neuropathol Exp Neurol **64**(2): 113-22.
Nucifora, F. C., Jr., M. Sasaki, et al. (2001). "Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity." Science **291**(5512): 2423-8.
Nussbaum, R. L. and C. E. Ellis (2003). "Alzheimer's disease and Parkinson's disease." N Engl J Med **348**(14): 1356-64.
Ohsumi, Y. (2001). "Molecular dissection of autophagy: two ubiquitin-like systems." Nat Rev Mol Cell Biol **2**(3): 211-6.

Okazawa, H. (2003). "Polyglutamine diseases: a transcription disorder?" Cell Mol Life Sci **60**(7): 1427-39.
Oppenheim, R. W., R. A. Flavell, et al. (2001). "Programmed cell death of developing mammalian neurons after genetic deletion of caspases." J Neurosci **21**(13): 4752-60.
Orr, H. T. and H. Y. Zoghbi (2007). "Trinucleotide repeat disorders." Annu Rev Neurosci **30**: 575-621.
Pan, T., S. Kondo, et al. (2008). "Neuroprotection of rapamycin in lactacystin-induced neurodegeneration via autophagy enhancement." Neurobiol Dis **32**(1): 16-25.
Pandey, U. B., Z. Nie, et al. (2007). "HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS." Nature **447**(7146): 859-63.
Parker, J. A., J. B. Connolly, et al. (2001). "Expanded polyglutamines in Caenorhabditis elegans cause axonal abnormalities and severe dysfunction of PLM mechanosensory neurons without cell death." Proc Natl Acad Sci U S A **98**(23): 13318-23.
Patrick, G. N. (2006). "Synapse formation and plasticity: recent insights from the perspective of the ubiquitin proteasome system." Curr Opin Neurobiol **16**(1): 90-4.
Perlson, E., S. Hanz, et al. (2005). "Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve." Neuron **45**(5): 715-26.
Phillis, R., D. Statton, et al. (1996). "Mutations in the 8 kDa dynein light chain gene disrupt sensory axon projections in the Drosophila imaginal CNS." Development **122**(10): 2955-63.
Przedborski, S., M. Vila, et al. (2003). "Neurodegeneration: what is it and where are we?" J Clin Invest **111**(1): 3-10.
Puls, I., C. Jonnakuty, et al. (2003). "Mutant dynactin in motor neuron disease." Nat Genet **33**(4): 455-6.
Puthalakath, H., D. C. Huang, et al. (1999). "The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex." Mol Cell **3**(3): 287-96.
Qu, X., J. Yu, et al. (2003). "Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene." J Clin Invest **112**(12): 1809-20.
Qu, X., Z. Zou, et al. (2007). "Autophagy gene-dependent clearance of apoptotic cells during embryonic development." Cell **128**: 931-946.
Raivich, G., R. Hellweg, et al. (1991). "NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration." Neuron **7**(1): 151-64.
Rami, A. and D. Kogel (2008). "Apoptosis meets autophagy-like cell death in the ischemic penumbra: Two sides of the same coin?" Autophagy **4**(4): 422-6.
Ravikumar, B., R. Duden, et al. (2002). "Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy." Hum Mol Genet **11**(9): 1107-17.

Ravikumar, B., C. Vacher, et al. (2004). "Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease." Nat Genet **36**(6): 585-95.
Rayala, S. K., P. den Hollander, et al. (2005). "Functional regulation of oestrogen receptor pathway by the dynein light chain 1." EMBO Rep **6**(6): 538-44.
Reggiori, F. and D. J. Klionsky (2002). "Autophagy in the eukaryotic cell." Eukaryot Cell **1**(1): 11-21.
Robertson, H. M., C. R. Preston, et al. (1988). "A stable genomic source of P element transposase in Drosophila melanogaster." Genetics **118**(3): 461-70.
Rodriguez-Crespo, I., B. Yelamos, et al. (2001). "Identification of novel cellular proteins that bind to the LC8 dynein light chain using a pepscan technique." FEBS Lett **503**(2-3): 135-41.
Rom, I., A. Faicevici, et al. (2007). "Drosophila Dynein light chain (DDLC1) binds to gurken mRNA and is required for its localization." Biochim Biophys Acta **1773**(10): 1526-33.
Rosen, D. R., T. Siddique, et al. (1993). "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis." Nature **362**(6415): 59-62.
Ross, C. A. and C. M. Pickart (2004). "The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases." Trends Cell Biol **14**(12): 703-11.
Ross, C. A. and M. A. Poirier (2004). "Protein aggregation and neurodegenerative disease." Nat Med **10 Suppl**: S10-7.
Rubinsztein, D. C., M. DiFiglia, et al. (2005). "Autophagy and its possible roles in nervous system diseases, damage and repair." Autophagy **1**(1): 11-22.
Rusten, T. E., K. Lindmo, et al. (2004). "Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway." Dev Cell **7**(2): 179-92.
Satyal, S. H., E. Schmidt, et al. (2000). "Polyglutamine aggregates alter protein folding homeostasis in Caenorhabditis elegans." Proc Natl Acad Sci U S A **97**(11): 5750-5.
Savill, J. and V. Fadok (2000). "Corpse clearance defines the meaning of cell death." Nature **407**(6805): 784-8.
Schliwa, M. and G. Woehlke (2003). "Molecular motors." Nature **422**(6933): 759-65.
Schnorrer, F., K. Bohmann, et al. (2000). "The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of Drosophila oocytes." Nat Cell Biol **2**(4): 185-90.
Schroder, M. and R. J. Kaufman (2005). "The mammalian unfolded protein response." Annu Rev Biochem **74**: 739-89.
Schweichel, J.-U. and H.-J. Merker (1973). "The morphology of various types of cell death in prenatal tissues." Teratology **7**: 253-266.
Schweichel, J. U. and H. J. Merker (1973). "The morphology of various types of cell death in prenatal tissues." Teratology **7**(3): 253-66.
Scott, D. C. and R. Schekman (2008). "Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins." J Cell Biol **181**(7): 1095-105.

Scott, R. C., G. Juhasz, et al. (2007). "Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death." Curr Biol **17**(1): 1-11.
Scott, R. C., O. Schuldiner, et al. (2004). "Role and regulation of starvation-induced autophagy in the Drosophila fat body." Dev Cell **7**(2): 167-78.
Shao, J. and M. I. Diamond (2007). "Polyglutamine diseases: emerging concepts in pathogenesis and therapy." Hum Mol Genet **16 Spec No. 2**: R115-23.
Shimizu, S., T. Kanaseki, et al. (2004). "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." Nat Cell Biol **6**(12): 1221-8.
Shpetner, H. S., B. M. Paschal, et al. (1988). "Characterization of the microtubule-activated ATPase of brain cytoplasmic dynein (MAP 1C)." J Cell Biol **107**(3): 1001-9.
Sieradzan, K. A., A. O. Mechan, et al. (1999). "Huntington's disease intranuclear inclusions contain truncated, ubiquitinated huntingtin protein." Exp Neurol **156**(1): 92-9.
Simonsen, A., H. C. Birkeland, et al. (2004). "Alfy, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes." J Cell Sci **117**(Pt 18): 4239-51.
Simonsen, A., A. E. Wurmser, et al. (2001). "The role of phosphoinositides in membrane transport." Curr Opin Cell Biol **13**(4): 485-92.
Slow, E. J., R. K. Graham, et al. (2005). "Absence of behavioral abnormalities and neurodegeneration in vivo despite widespread neuronal huntingtin inclusions." Proc Natl Acad Sci U S A **102**(32): 11402-7.
Spencer, P. S., P. B. Nunn, et al. (1987). "Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant excitant neurotoxin." Science **237**(4814): 517-22.
Strom, A. L., J. Gal, et al. (2008). "Retrograde axonal transport and motor neuron disease." J Neurochem **106**(2): 495-505.
Tai, A. W., J. Z. Chuang, et al. (1999). "Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1." Cell **97**(7): 877-87.
Takeshige, K., M. Baba, et al. (1992). "Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction." J Cell Biol **119**: 301-11.
Takeshige, K., M. Baba, et al. (1992). "Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction." J Cell Biol **119**(2): 301-11.
Takeyama, K., S. Ito, et al. (2002). "Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in Drosophila." Neuron **35**(5): 855-64.
Tang, Q., C. M. Staub, et al. (2002). "A novel transforming growth factor-beta receptor-interacting protein that is also a light chain of the motor protein dynein." Mol Biol Cell **13**(12): 4484-96.
Taylor, J. P., F. Tanaka, et al. (2003). "Aggresomes protect cells by enhancing the degradation of toxic polyglutamine-containing protein." Hum Mol Genet **12**(7): 749-57.

Teuchert, M., D. Fischer, et al. (2006). "A dynein mutation attenuates motor neuron degeneration in SOD1(G93A) mice." Exp Neurol **198**(1): 271-4.
Thumm, M., R. Egner, et al. (1994). "Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*." FEBS Lett. **349**: 275-280.
Thumm, M., R. Egner, et al. (1994). "Isolation of autophagocytosis mutants of Saccharomyces cerevisiae." FEBS Lett **349**(2): 275-80.
Timmer, J. C. and G. S. Salvesen (2007). "Caspase substrates." Cell Death Differ **14**(1): 66-72.
Vallee, R. B., J. S. Wall, et al. (1988). "Microtubule-associated protein 1C from brain is a two-headed cytosolic dynein." Nature **332**(6164): 561-3.
Vallee, R. B., J. C. Williams, et al. (2004). "Dynein: An ancient motor protein involved in multiple modes of transport." J Neurobiol **58**(2): 189-200.
Vaughan, K. T. and R. B. Vallee (1995). "Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150Glued." J Cell Biol **131**(6 Pt 1): 1507-16.
Venkatraman, P., R. Wetzel, et al. (2004). "Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins." Mol Cell **14**(1): 95-104.
Wang, L., M. Hare, et al. (2004). "Dynein light chain LC8 promotes assembly of the coiled-coil domain of swallow protein." Biochemistry **43**(15): 4611-20.
Warrick, J. M., H. L. Paulson, et al. (1998). "Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in Drosophila." Cell **93**(6): 939-49.
Webb, J. L., B. Ravikumar, et al. (2003). "Alpha-Synuclein is degraded by both autophagy and the proteasome." J Biol Chem **278**(27): 25009-13.
Welch, W. J. and M. I. Diamond (2001). "Glucocorticoid modulation of androgen receptor nuclear aggregation and cellular toxicity is associated with distinct forms of soluble expanded polyglutamine protein." Hum Mol Genet **10**(26): 3063-74.
Wexler, N. S., A. B. Young, et al. (1987). "Homozygotes for Huntington's disease." Nature **326**(6109): 194-7.
Wickstead, B. and K. Gull (2007). "Dyneins across eukaryotes: a comparative genomic analysis." Traffic **8**(12): 1708-21.
Wootz, H., I. Hansson, et al. (2004). "Caspase-12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS." Biochem Biophys Res Commun **322**(1): 281-6.
Wu, X., G. Jung, et al. (2000). "Functions of unconventional myosins." Curr Opin Cell Biol **12**(1): 42-51.
Xie, Z. and D. J. Klionsky (2007). "Autophagosome formation: core machinery and adaptations." Nat. Cell Biol. **9**: 1102-1109.
Xie, Z., U. Nair, et al. (2008). "Atg8 controls phagophore expansion during autophagosome formation." Mol Biol Cell **19**(8): 3290-8.
Yamada, M., T. Iwatsubo, et al. (2004). "Overexpression of alpha-synuclein in rat substantia nigra results in loss of dopaminergic neurons, phosphorylation of alpha-synuclein and activation of caspase-9: resemblance to pathogenetic changes in Parkinson's disease." J Neurochem **91**(2): 451-61.

Yang, S. H., P. H. Cheng, et al. (2008). "Towards a transgenic model of Huntington's disease in a non-human primate." Nature **453**(7197): 921-4.
Yano, H., F. S. Lee, et al. (2001). "Association of Trk neurotrophin receptors with components of the cytoplasmic dynein motor." J Neurosci **21**(3): RC125.
Ye, H., R. Kuruvilla, et al. (2003). "Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons." Neuron **39**(1): 57-68.
Yue, Z., S. Jin, et al. (2003). "Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor." Proc Natl Acad Sci U S A **100**(25): 15077-82.
Zhao, C., J. Takita, et al. (2001). "Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta." Cell **105**(5): 587-97.
Zoghbi, H. Y. and H. T. Orr (2000). "Glutamine repeats and neurodegeneration." Annu Rev Neurosci **23**: 217-47.
Zou, H., W. J. Henzel, et al. (1997). "Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3." Cell **90**(3): 405-413.
Zuccato, C., M. Tartari, et al. (2003). "Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes." Nat Genet **35**(1): 76-83.
Zweifel, L. S., R. Kuruvilla, et al. (2005). "Functions and mechanisms of retrograde neurotrophin signalling." Nat Rev Neurosci **6**(8): 615-25.