Evaluating the Genetic Link between Autophagy and Parkinson's Disease in Budding Yeast Model

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Abstract

Parkinson's disease (PD) is an incurable neurodegenerative disease characterized by the death of midbrain dopaminergic neurons. The suspected cause of PD is the misfolding and aggregation of a brain protein, α-synuclein. A popular hypothesis is that increasing degradation of α-synuclein may protect the cell from aggregation and toxicity. While strong pharmacological evidence indicates that autophagy is involved in the degradation of α-synuclein in PD, the genetic link remains weak. For my senior thesis, I evaluated the hypothesis that the lysosome is the route for α-synuclein degradation by the autophagy pathway. Specifically, I studied the genetic link between autophagy and α-synuclein using a budding yeast model. My results demonstrate four significant findings. First, through genetic evidence, my results support the hypothesis that α-synuclein is regulated by the autophagy pathway. Of the six autophagy genes that I evaluated, deletion of each altered one or more α-synuclein PD-related properties. Second, these deletion effects on α-synuclein were gene-specific, indicating substrate-specificity. Third, α-synuclein localization exhibited a range of cellular distributions depending on the deleted gene. Finally, I did not observe α-synuclein dependent toxicity. Our lab's cumulative data further supports the hypothesis that the lysosome is the route for α-synuclein degradation by the autophagy pathway.

Introduction

Autophagy

Self-cannibalism elicits alarming and horrifying images. Yet, believe it or not, self-cannibalism is a normal process that our bodies use daily, such as between meal times. Autophagy is the cell biologist's term for such "self-eating." This degradation mechanism is an evolutionarily conserved degradation process designed to help cells and organisms survive conditions of stress, starvation, and infection or illness (Shintani & Klionsky, 2004). Its primary role is to break down old or damaged proteins, protein complexes, and organelles, both to protect cells from toxicity and as a way to obtain energy by recycling cellular components.

The scientific field has come a long way to understanding the molecular mechanisms of autophagy. New research suggests that a balance in autophagy is key to systemic health and an imbalance can result in diverse human diseases (Fig 1A). These diseases include diabetes, Crohn's disease, heart disease, aging, cancer and neurodegeneration. For example, Crohn's disease, an inflammatory disease of the gastrointestinal tract, is due to deficient autophagy. When autophagy is deficient, the organism responds by over-activating the inflammatory response leading to the symptoms of the disease (Cadwell et al., 2008). On the contrary, too much autophagy can also result in diseases such as Pompe disease, where the outcome is the disruption of the contractile apparatus of the muscle fibers (Shea & Raben, 2009). This disease is more difficult to treat because autophagy also interferes with the enzyme replacement therapy used to treat the disease (Shea & Raben, 2009). Overall, the wide range of diseases where autophagy plays a role demonstrates how integral this process is to the normal function of the cell. Thus, a balanced autophagy is critical for maintaining a healthy organism. My thesis explores the link between neurodegenerative diseases and autophagy.

Autophagy and Neurodegenerative Diseases

While autophagy is involved with many diseases, the research towards uncovering the role of autophagy in neurodegenerative diseases is still in its infancy. Neurodegenerative diseases are a class of disorders characterized by selective death of neurons in the central nervous system (Ross & Poirier, 2004). This death is believed to be due to the misfolding and aggregation of a protein particular to each disease (Fig 1B). The death of neurons leads to the symptoms seen in each of the diseases such as motor deficits or memory loss. Research shows that mice deficient for an important autophagy gene, Atg5, develop abnormal intracellular protein accumulations, which then develop into aggregates and inclusions (Hara et al., 2006). This suggests that autophagy is necessary for continued clearance of cytosolic proteins that otherwise have the potential of accumulating and interfering with proper neuronal function that can lead to neurodegeneration. Additional findings in mice show that the loss of Atg7, another essential autophagy gene, leads to behavioral defects, massive neuronal loss in the cerebral and cerebellar cortices, and accumulation of polyubiquitinated proteins (Komatsu et al., 2006). Furthermore, Atg7 is vital for proper membrane trafficking and turnover in the axons (Komatsu et al., 2007). When Atg7 is deleted in the cerebellar Purkinje cells, axonal dystrophy and degeneration occur (Komatsu et al., 2007). Thus, this data suggests the importance of autophagy in axonal homeostasis.

Research demonstrates that suppression of autophagy in neurons leads to neurodegenerative diseases (Komatsu et al., 2007). Conversely, induction of autophagy protects cells from toxicity in such diseases as Huntington’s (Ravikumar et al., 2004). For instance, Ravikumar et al. (2004) showed that induction of autophagy leads to protection against toxicity in both fly and mouse models. This neurodegenerative disease is characterized by a misfolding of a protein, huntingtin (Ross & Poirier, 2004). Similarly, in Parkinson’s disease, the culprit of the disease is a misfolded protein. Thus, what is the connection between autophagy and Parkinson’s disease?

Parkinson’s Disease

Parkinson’s disease (PD) is the second most common incurable progressive neurodegenerative disease after Alzheimer’s, affecting one million people in the United States and five million worldwide (Olanow et al., 2009). Unfortunately, the number of affected individuals is expected to double by 2030 (Olanow et al., 2009). The consequences of this will be a greater burden on the health care system and will increase the need for effective treatments (Dorsey et al., 2007). PD can develop at any age, though it is most common in older adults, with the peak of onset at age sixty (Fahn, 2008). With increased age, the prevalence and

*This author wrote the paper as a senior thesis in biology under the direction of Dr. Shubhi DebBurman.
incidence rise, with a lifetime risk of approximately 2% (Fahn, 2008).

PD can be characterized by both motor and nonmotor deficits (Fig 2A). Motor deficits include tremor-at-rest, bradykinesia, rigidity, loss of postural reflexes, flexed posture, and the freezing phenomenon (Fahn, 2008). Nonmotor functions affected include cardiovascular and gastrointestinal abnormalities, cognitive dysfunction, and depression (Fahn, 2008). Patients with PD usually live for twenty or more years post diagnosis and death is due to concurrent unrelated illness (Fahn, 2008).

Two forms of PD exist: sporadic and familial. The most common form of PD is sporadic, which accounts for 85-90% of PD cases (Fahn, 2008). Current research on sporadic PD demonstrates the links between PD and oxidative stress, mitochondrial and proteasomal dysfunction, and environmental toxins (Fahn, 2008). Familial PD accounts for the remaining 10-15% of all cases (Fahn, 2008). To date, the field has identified the following mutations in these seven genes: Parkin (Matsumine et al., 1997), UCH-L1 (Leroy et al., 1998), PINK1 (Valente et al., 2001), DJ-1 (Bonifati et al., 2002), LRRK2 (Funayama et al., 2002), ATP13A2 (Najim al-Din et al., 1994; Hampshire et al., 2001), and α-synuclein (Polymeropoulos et al., 1996). Out of all these familial genes, α-synuclein has been studied the most. The α-synuclein gene codes for a protein called α-synuclein, which has been linked to PD. The first α-synuclein missense mutation identified was of alanine to threonine at amino acid 53 (A53T) in an Italian family (Polymeropoulos et al., 1997). The second mutation discovered was A30P (alanine to proline on amino acid 30) (Kruger et al., 1998). Finally, the most recent mutation is E46K (glutamic acid to lysine on the amino acid 46) (Zarranz et al., 2004). This small change in the primary sequence of the α-synuclein has a crucial effect, resulting in an α-synuclein mutant protein that has a higher rate of misfolding when compared to normal α-synuclein (Polymeropoulos et al., 1997; George, 2002). If α-synuclein misfolds, it can no longer sustain its function in a cell, which can ultimately lead to the death of the cell.

The Function and Properties of α-Synuclein
α-Synuclein is a 140 amino-acid cytosolic protein that was initially identified as a precursor protein for the non-β amyloid component of the amyloid plaques of Alzheimer’s disease (Ueda et al., 1993). The DNA sequence of α-synuclein is homologous to two other proteins: β-synuclein and γ-synuclein (Gliasson et al., 2001). In vitro studies indicate that when either mutated α-synuclein or wild-type (WT) α-synuclein are expressed, aggregates of insoluble protofibrils form (Conway et al., 1998; Spillantini et al., 1998; El-Agnaf et al., 1998; Giasson et al., 1999), which are composed of β-sheets and amyloid-like filaments (El-Agnaf et al., 1998; Hashimoto et al., 1999; Narhi et al., 1999). Furthermore, mutated α-synuclein (A53T or A30P) has a faster rate fibrillation at higher concentrations than WT α-synuclein (Conway et al., 1998), with the A53T mutant being the fastest.

An insight into monomeric α-synuclein demonstrates that it does not contain any stable structure and has become known as a “natively unfolded” protein (Uversky, 2003). This implies that, due to its inherent plasticity, monomeric α-synuclein can adopt a series of conformations depending on its environment. However, in cases of increased concentration, α-synuclein undergoes self-assembly into dimers and small oligomers, which allows for its stabilization (Uversky, 2003). Interestingly, in Lewy bodies, α-synuclein is found in the form of protofibrils and small oligomers (Spillantini et al., 1997). This suggests that the formation of protofibrils is linked to cell death (Goldberg & Lansbury, 2000; Kaplan et al., 2003; Volles & Lansbury, 2003). Furthermore, both in vitro studies and in vivo studies show that α-synuclein is found aggregated in neurons (Baba et al., 1998; Trojanowski et al., 1998). This aggregation, through an unknown mechanism, ultimately leads to neurotoxicity (Ding et al., 2002; Sharon et al., 2003; Periquet

<table>
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<th>Human Diseases</th>
<th>Role of Autophagy</th>
<th>Protective or Harmful?</th>
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</tr>
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<td>Atherosclerosis</td>
<td>Degradation of apolipoprotein B</td>
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<td>Crohn's Disease</td>
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</tr>
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<td>Maintenance of β-cells</td>
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</tr>
<tr>
<td>Diabetes</td>
<td>Removes damaged organelles</td>
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<tr>
<td>Pompe's Disease</td>
<td>Destroys contractile apparatus</td>
<td>Harmful</td>
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<th>Culprit Protein</th>
<th>Degradation Problems?</th>
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<td>Alzheimer’s Disease</td>
<td>Amyloid-β peptide</td>
<td>Yes</td>
</tr>
<tr>
<td>Huntington’s Disease</td>
<td>Huntingtin</td>
<td>Yes</td>
</tr>
<tr>
<td>Prion Disease</td>
<td>Prion Protein</td>
<td>Yes</td>
</tr>
<tr>
<td>ALS</td>
<td>SOD1</td>
<td>Yes</td>
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Figure 1: Human diseases and autophagy. A. The figure demonstrates the various diseases that are linked to autophagy. The role of autophagy is suggested in each of the diseases and whether autophagy serves a protective or harmful role in the disease. References: 1) Mathew et al., 2009; 2) Ohsaki et al., 2006; 3) Cadwell et al., 2008; 4) Hubbard et al., 2010; 5) Jung et al., 2010; 6) Shea & Raben, 2009. B. The figure demonstrates neurodegenerative diseases and the culprit protein linked to each of the diseases. Furthermore, whether the disease is due to a degradation problem is assessed. References: 1) Hara et al., 2006, Komatsu et al., 2006; 2) Yang et al., 2011; 3) Ravikumar et al., 2004; 4) Zhang et al., 2011; 5) Hiseke et al., 2010.
Additionally, accumulation of α-synuclein has been seen in Caenorhabditis elegans (Lakso et al., 2003). Movement (Feany & Bender, 2000). Similar results have been seen in mice, with loss and impaired climbing abilities, indicating difficulty in movement (Masliah et al., 2000). Overexpression of WT α-synuclein results in severe motor defects that lead to paralysis, death, and appearance of insoluble α-synuclein inclusions located in the dopaminergic neurons, neuronal dysfunction, death, and appearance of insoluble α-synuclein inclusions (Devilal et al., 2008) and to the plasma membrane of vesicles (Kamp & Beyer, 2006). Lastly, studies demonstrate that overexpression of α-synuclein leads to mitochondrial dysfunction (Martin et al., 2006; Stichel et al., 2007; Su et al., 2010). In C. elegans, α-synuclein binds to mitochondria through an unknown mechanism, resulting in mitochondrial fragmentation. In this study, mitochondrial fragmentation could be rescued by coexpression of Parkin, DJ-1, or Pink1, each of which are the genes that, when mutated, are associated with familial PD (Kamp et al., 2010).

When WT α-synuclein is found highly modified through phosphorylation and nitration, and these modifications have been linked to toxicity (Shimura et al., 2001; Hodara et al., 2004; Fujiwara et al., 2002; Okochi et al., 2000). My colleague, Keith Solvang ‘11, investigated the properties of modified α-synuclein in his senior thesis.

Parkinson’s disease is a gain-of-function disease, meaning that in PD, α-synuclein gains novel properties. When WT α-synuclein or mutant α-synuclein (A53T or A30P) is overexpressed, it is toxic to neuroblastoma cells and results in cell death (Osterova et al., 1999). Moreover, overexpression of WT α-synuclein in some transgenic mouse lines results in PD-like pathogenesis: development of cytoplasmic α-synuclein inclusions, loss of dopaminergic synapses, and motor impairment (Masliah et al., 2000). Furthermore, overexpression of A53T mutant α-synuclein in mouse lines results in severe motor defects that lead to paralysis, death, and appearance of insoluble α-synuclein inclusions and Lewy body-like inclusions (Gliason et al., 2002). In Drosophila, expression of human wild-type α-synuclein or mutant α-synuclein results in the formation of Lewy body-like inclusions located in the dopaminergic neurons, neuronal loss, and impaired climbing abilities, indicating difficulty in movement (Feany & Bender, 2000). Similar results have been seen in Caenorhabditis elegans (Lakso et al., 2003). Additionally, accumulation of α-synuclein is seen in the brain of PD patients (Xu et al., 2002; Devilal et al., 2008; Shehadeh et al., 2009).

Another important property of α-synuclein is that it binds the plasma membrane and endomembrane within the cell. In yeast studies, WT and E46K α-synuclein is found to localize to the plasma membrane (Outeiro & Lindquist, 2003; Dixon et al., 2005; Sharma et al., 2006; Cooper et al., 2006). Additionally, studies have demonstrated that α-synuclein binds to the inner membrane of mitochondria (Devilal et al., 2008) and to the plasma membrane of vesicles (Kamp & Beyer, 2006). Lastly, studies demonstrate that overexpression of α-synuclein leads to mitochondrial dysfunction (Martin et al., 2006; Stichel et al., 2007; Su et al., 2010). In C. elegans, α-synuclein binds to mitochondria through an unknown mechanism, resulting in mitochondrial fragmentation. In this study, mitochondrial fragmentation could be rescued by coexpression of Parkin, DJ-1, or Pink1, each of which are the genes that, when mutated, are associated with familial PD (Kamp et al., 2010).

Out of these three genes, Parkin codes for an E3 ubiquitin ligase involved in proteasomal degradation. Therefore, mitochondrial dysfunction, due to α-synuclein, can be rescued by proteasomal degradation. This suggests that studying α-synuclein degradation could uncover possibilities for cell survival. α-Synuclein degradation is the primary focus of my thesis.

**Degradation of α-Synuclein**

Proteins are one of the building blocks of life; they are able to perform diverse cellular functions that are driven by their specific amino acid sequences and resultant structures. Like all "machinery," protein molecules can acquire wear and tear and become damaged as a natural consequence of cellular stress and age (Rubinsztein, 2006). To handle recycling of such damaged proteins, cells have evolved two degradation machines: the proteasome and the lysosome (Rubinsztein, 2006). The proteasome degrades short-lived proteins that are found in the nucleus or cytoplasm, while the lysosome degrades longer-lived proteins that are found in the plasma membrane and endomembrane within the cell. In yeast studies, WT and E46K α-synuclein is found to localize to the plasma membrane (Outeiro & Lindquist, 2003; Dixon et al., 2005; Sharma et al., 2006; Cooper et al., 2006). Additionally, studies have demonstrated that α-synuclein binds to the inner membrane of mitochondria (Devilal et al., 2008) and to the plasma membrane of vesicles (Kamp & Beyer, 2006). Lastly, studies demonstrate that overexpression of α-synuclein leads to mitochondrial dysfunction (Martin et al., 2006; Stichel et al., 2007; Su et al., 2010). In C. elegans, α-synuclein binds to mitochondria through an unknown mechanism, resulting in mitochondrial fragmentation. In this study, mitochondrial fragmentation could be rescued by coexpression of Parkin, DJ-1, or Pink1, each of which are the genes that, when mutated, are associated with familial PD (Kamp et al., 2010).

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degrades proteins that are found bound to the plasma membrane. The question remains how α-synuclein is degraded. It is localized to different parts of the cell, both the proteasome and the lysosome are likely involved in its degradation (Fig 3). I will first focus on the proteasome and then on the lysosome.

**Proteasomal Degradation**

The proteasome is a large, barrel-shaped catalytic complex that is part of the ubiquitin-proteasome system (UPS; Fig 3). This mechanism degrades intracellular soluble proteins that are found in the cytoplasm, nucleus, and endoplasmic reticulum and are mutated, misfolded, denatured, misplaced, or damaged (Davies, 2001; Goldberg, 2003). Over the years, compelling evidence suggests that UPS dysfunction is linked to Parkinson’s disease. In familial PD, patients with either of two mutated genes, Parkin (Matsumine et al. 1997) or UCH-L1 (Leroy et al., 1998), exhibit PD-like symptoms, and postmortem analyses demonstrate formation of Lewy bodies. In sporadic PD, proteasome structure and function are altered in the substantia nigra (McNaught et al., 2003). In vitro studies demonstrate that inhibition of the proteasome results in a selective degeneration of dopamine neurons paired with formation of inclusions that are stained positively for both α-synuclein and ubiquitin (McNaught et al., 2002; Rideout et al., 2005). Furthermore, injections into the substantia nigra or striatum of proteasomal inhibitors induce dopamine neuron degeneration with inclusions in rats (McNaught et al., 2002; Fornai et al., 2003). In rats, systematic administration of proteasome inhibitors leads to the development of PD-like symptoms with PET scans demonstrating progressive loss of dopaminergic nerve terminals in the striatum. Postmortem analyses of these animals display striatal dopamine depletion and formation of inclusions in the substantia nigra (McNaught et al., 2004). Numerous studies have demonstrated that α-synuclein is degraded by the proteasome (Bennett et al., 1999; Tofaris et al., 2001). Specifically, wild-type α-synuclein has been shown to be a substrate for both the 20S and 26S proteasomes and degraded mostly in an ubiquitin-independent manner. However, the story differs for the A53T α-synuclein mutant. Unlike WT, both in vivo and in vitro studies established that the A53T α-synuclein mutant protein does not undergo proteasomal degradation (Stefanis et al., 2001; Tanaka et al., 2001). These studies serve as compelling evidence that α-synuclein is degraded by the proteasome, but also suggest the occurrence of other types of α-synuclein degradation, particularly for the mutant protein.

**Lysosomal Degradation**

The journey of α-synuclein degradation does not end with the proteasome. In 2003, Webb et al. hinted that the lysosome is also involved. Three pathways to the lysosome exist: phagocytosis, endocytosis, and autophagy (Fig 3). Viruses and bacteria use the phagocytosis pathway; this pathway is not used by α-synuclein. Substantial evidence in the field suggests that endocytosis is the route for α-synuclein degradation (Willingham et al., 2003; Kuwahara et al., 2008; Perez Thesis, 2010; Ayala Thesis, 2009). However, autophagy also appears to have a role. When Webb et al. (2003) specifically inhibited autophagy, α-synuclein accumulated in the cell. Chemical induction of autophagy with rapamycin leads to decreased α-synuclein levels, as compared to the control. Three types of autophagy exist: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. In macroautophagy, the whole organelle or portion of the cytosol is sequestered directly by the lysosome for degradation. CMA involves the direct translocation of cytosolic proteins that contain a particular pentapeptide motif to the lysosome (Rubinsztein, 2006). Substantial evidence in the field exists that CMA is one of the pathways used by α-synuclein (Cuervo et al., 2004; Vogiatzi et al., 2008). When the lamp2a gene, which codes for a lysosomal receptor for CMA, is deleted, α-synuclein accumulates (Vogiatzi et al., 2008). Macroautophagy degrades the bulk of cytoplasmic proteins and organelles found in the cytoplasm (Rubinsztein et al., 2005; Fig 4). When macroautophagy is pharmacologically inhibited, α-synuclein accumulates (Vogiatzi et al., 2008), which suggests that this mechanism (hereafter referred to as autophagy) also degrades α-synuclein. Furthermore, expression of mutant α-synuclein in cells results in the accumulation of autophagic-vesicular structures, suggesting that autophagy is involved in the degradation of mutant α-synuclein (Angiade et al., 1997; Stefanis et al., 2001). Moreover, Prigione et al. (2010) demonstrated an induced autophagy response in peripheral blood mononuclear cells of PD patients while α-synuclein in these cells was in a soluble form. They showed that the lack of aggregated α-synuclein can not be due to the UPS activity, since increased activation of the system was not detected. Thus, these cumulative studies further support the hypothesis that the autophagy route degrades α-synuclein.

**Figure 4: Autophagy pathway**

The figure demonstrates the three steps of the autophagy pathway: nucleation, expansion, and fusion. In the nucleation step, the membrane begins to form around the material that needs to be degraded. In the expansion step, the autophagosome forms. In the fusion step, the autophagosome fuses with the lysosome. In each step, there are numerous proteins involved that are organized into complexes. Complex I and II operate in the nucleation step. Complex I regulates induction of autophagy, followed by vesicle nucleation (as demonstrated by the red arrow) regulated by complex II. Complexes III, IV, and V operate in the expansion step. Complex II and complex III work together to expand the vesicle by adding more plasma membrane. Complex V retrieves Atg9 from the plasma membrane, preparing the autophagosome for the fusion step. The fusion step is regulated by a different set of proteins. For my thesis, I studied genes from complexes I, II, III, IV, and V. The figure is adapted from Nakatowa et al. (2009).
A.

1) Toxicity Prediction

![Toxicity Prediction Diagram]

2) Localization Prediction

![Localization Prediction Diagram]

3) Expression Prediction

<table>
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<tr>
<th>Genes Examined</th>
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<th>Complex II</th>
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Figure 5: Predictions and genes evaluated. A. I evaluated three properties of α-synuclein in autophagy deficient strains compared to the autophagy intact strains: toxicity, localization, and expression. 1) For the toxicity analysis, I predicted that I would see more cell death in the autophagy deficient strains since I will see fewer cells (black circles) on the plate as compared to when autophagy is intact. 2) For the localization analysis, I predicted that WT and E46K α-synuclein (represented by green) will change localization. In autophagy intact cells, WT and E46K α-synuclein localizes to the plasma membrane. In autophagy deficient cells, I predicted that WT and E46K α-synuclein would become cytoplasmically diffuse and form some aggregates. A30P α-synuclein would stay cytoplasmically diffuse and form many aggregates. In WT, E46K, and A30P, I predicted the vacuole (represented by V) will stay dimly lit. 3) For expression analysis, I believed α-synuclein would accumulate in the autophagy deficient strains as compared to the autophagy intact strain (as demonstrated by a cartoon of a Western blot). B. The genes in red are the novel genes that I examined. The genes in black are those that have already been evaluated by our lab but were re-assessed by me once again. The genes in blue were not studied.

Autophagy proceeds in three steps: nucleation, expansion, and fusion with the lysosome (Fig 4). The nucleation step is composed of the membrane starting to form around the items that need to be degraded. During expansion, an autophagosomal forms. Finally, during the fusion step, the autophagosomal fuses with the lysosome for the degradation to occur. The conventional method of studying autophagy has been through the discovery of autophagy genes (Shintani & Klionsky, 2004; Suzuki & Ohsumi, 2007). A new, elaborate way of thinking about autophagy genes is by grouping them into complexes (Fig 4). In particular, five main complexes are involved in the process. When autophagy is induced through either starvation or rapamycin, the following complexes form: Atg1 kinase complex (Complex I), Ptdlns 3-kinase complex (Complex II), Atg8 conjugated system complex (Complex III), Atg12 conjugated system complex (Complex IV), and Atg2-Atg18 complex and Atg9 (Complex V). Complex I and Complex II are involved with the initial step of nucleation. Once autophagy is activated, the proteins involved in Complex I bind to the lipid membrane and work with Complex II to attract other autophagy proteins to the preautophagosomal structure (PAS). The function of Complex III and Complex IV is to continue building the plasma membrane until it forms the autophagosome. Once the autophagosome is complete, Complex IV removes Atg9 from the membrane, and the autophagosome is ready for fusion with the lysosome.

For my thesis, I evaluated complexes I, II, III, IV, and V. In particular, I evaluated Atg29 and Atg31 in complex I, Atg6 and Atg14 in complex II, Atg7 in complex III (which is also found in complex IV), and Atg9 in complex V. Atg29 and Atg31 cooperate with Atg17 for the recruitment of other Atg proteins to PAS. Atg6, also known as Vps30, has an unknown function but is known to be one of the substrates of the complex. Atg14 is responsible for the recruitment of Ptdlns 3-kinase complex to the PAS and is required for localizing additional Atg proteins to the PAS. Atg7 belongs to the E1 family of enzymes, which are ubiquitin-activating enzymes. Finally, Atg9 is a transmembrane protein that is involved in autophagic vesicle formation. While the exact function is unknown, Atg9 may be involved in membrane delivery to the PAS.

Gap in Knowledge

While pharmacological and genetic evidence exists in support of proteasomal degradation of α-synuclein, only pharmacological evidence exists in support of autophagy. In order to demonstrate that autophagy is involved, further genetic evidence needs to be obtained. Evaluation of all of the genes involved in the pathway is vital to understanding autophagy’s role because this approach would demonstrate if some genes are more important than others in the guidance of α-synuclein through this degradation pathway. Furthermore, evaluation of all of the autophagy genes will demonstrate whether a substrate dictates the specificity of autophagy: toxicity, altered localization, and accumulation. Ray Choi ’09 began the first genetic study of α-synuclein in PD pathol. Our lab is interested in substrate selective therapy, determining which genes are critical for α-synuclein regulation is important. Moreover, genetic screens need to be performed, but they need to evaluate all three properties seen in PD pathology: toxicity, altered localization, and accumulation. Our Lab’s Model Organism

Numerous animal models are used to study the role of α-synuclein in PD. These models include flies (Feany & Bender, 2000), mice (Giasson et al., 2002), worms (Lakso et al., 2003), yeast (Outeiro & Lindquist, 2003). Yeast is a single cell fungus that has a short generation time, fully sequenced genome, and well-established molecular genetics (Kaebelrlein et al., 2001). Yeast is a single cell fungus that has a short generation time, fully sequenced genome, and well-established molecular genetics (Kaebelrlein et al., 2001). Most of the pathways in yeast are evolutionarily conserved between higher eukaryotes such as humans (Kaebelrlein et al., 2001). Autophagy was first characterized in yeast, and to date, 33 Atg genes have been identified in budding yeast that are responsible for the three steps of autophagy (Klionsky et al., 2003; Suzuki et al., 2007; Ventritti & Cuervo, 2007). Also, yeast is a relatively inexpensive model organism and easy to work with in an undergraduate setting. Our lab uses budding yeast as a model to study α-synuclein (Sharma et al., 2006; Brandis et al., 2006). α-Synuclein is not toxic to budding yeast cells, and wild-type α-synuclein localizes predominantly...
through genetic evidence, my results support the hypothesis that α-synuclein is regulated by the autophagy pathway. Of the six autophagy genes that I evaluated, deletion of each altered one or more α-synuclein PD-related properties. Secondly, these deletion effects on α-synuclein were gene-specific, indicating substrate-specificity. Thirdly, α-synuclein localization exhibited a range of cellular distributions depending on the deleted gene. Finally, I did not observe α-synuclein dependent toxicity.

Methods
All of the methods described below were adopted from Sharma et al. (2006), and they are all described again in less detail.

α-Synuclein Constructs
The vectors for this study, pYES2.1, pNMT1, GFP, WT α-synuclein, A30P and E46K mutant α-synuclein, have been created earlier as described in Sharma et al. (2006). Table 2 shows the transformed yeast strains.

Yeast Strains
For budding yeast parent strain BY4741 (mat a) and ATG deletion strains atg29, atg31, atg6, atg14, atg7, atg9 were purchased from Open Biosystems (Table 2).

Yeast Expression
The pYES2.1 vectors were transformed into budding yeast following procedures described in Burke et al. (2000).

to the plasma membrane (Sharma et al., 2006). However, other labs have seen α-synuclein dependent toxicity in budding yeast (Outeiro & Lindquist, 2003). We use single gene deletion strains to study α-synuclein-dependent toxicity, localization, and expression. Recently, research was conducted on autophagy by the DebBurman lab, and the researchers saw that when autophagy is compromised, α-synuclein is not toxic to the cells (Choi Thesis, 2009; Table 1). In some particular deletion strains, a change in localization and increased expression of α-synuclein is seen (Choi Thesis, 2009). To date, no studies have evaluated autophagy in budding yeast.

Hypothesis and Aims
My hypothesis was that autophagy is a route by which α-synuclein is degraded by the lysosome. To test my hypothesis, I evaluated complex I (atg29Δ and atg31Δ), complex II (atg6Δ and atg14Δ), complex III and IV (atg7Δ), and complex V (atg9Δ; Fig 5B).

Aim: To evaluate the effects of compromised autophagy on α-synuclein toxicity, expression, and localization in budding yeast. 

Prediction: I predict that each of the deleted genes (atg6Δ, atg7Δ, atg14Δ, atg31Δ) will result in an alteration of α-synuclein localization, induce cellular toxicity, and increase α-synuclein expression (Fig 5A).

Results: My results demonstrate four distinct findings. First, through genetic evidence, my results support the hypothesis that α-synuclein is regulated by the autophagy pathway. Of the six autophagy genes that I evaluated, deletion of each altered one or more α-synuclein PD-related properties. Secondly, these deletion effects on α-synuclein were gene-specific, indicating substrate-specificity. Thirdly, α-synuclein localization exhibited a range of cellular distributions depending on the deleted gene. Finally, I did not observe α-synuclein dependent toxicity.

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Localization</th>
<th>Toxicity</th>
<th>Expression</th>
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<tbody>
<tr>
<td>Complex I</td>
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<td>None</td>
</tr>
<tr>
<td></td>
<td>atg13Δ</td>
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<tr>
<td></td>
<td>atg29Δ</td>
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</tr>
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</tr>
<tr>
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<td></td>
<td>atg14Δ</td>
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<td></td>
<td>vps15Δ</td>
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Table 1: Summary Chart of all Atg genes analyzed (pre thesis)
The summary chart summarizes all of the genes that have been evaluated thus far in our lab. The genes were originally evaluated by the following: Ray Choi '09: atg13Δ, atg27Δ, atg31Δ, atg29Δ, vps15Δ, Peter Sullivan '12: atg11Δ, atg18Δ; Kayla Ahlstrand '12: atg13Δ, vam4Δ; Peter Sullivan '12: atg11Δ, atg13Δ.
Budding yeast cells were grown in the media lacking SC-Ura. Expression of α-synuclein was regulated by a galactose-inducible promoter. α-Synuclein expression was repressed when cells were grown in SC-Ura+Glucose, and expression was turned on when cells were grown in SC-Ura+Galactose. Cells were grown overnight at 30 °C in 2% SC-Ura+Glucose. Then, the cells were washed twice, and the desired concentration was transferred to 2% SC-Ura+Galactose in order to induce expression of the protein.

Western Blot Analysis
Yeast cells were grown overnight in 5 mL of SC-Ura+Glucose media at 30 °C with shaking at 200 rpm. The cells were then washed two times and transferred to the α-synuclein expression media. At 24 and 48 hours lysates were prepared. 2.5x107 cells/mL were first washed twice with 100 mM Na3, then solubilized in ESB (electrophoresis sample buffer), and small beads were added to the contents to break down the cells. The lysates were run on 10-20% Tris-Glycine gels at 130 volts with a 1x buffer containing SDS. As a standard, SeeBlue molecular ladder was used. After electrophoresis of the lysates, the gel was transferred to PVDF membranes using a semi-dry transfer method. The Western blot was then probed with various antibodies for detection of α-synuclein and for phosphoglycerokinase (PGK), which was the loading control. To detect α-synuclein, a mouse monoclonal anti-V5 was used, and to detect PGK, anti-PGK antibody was used.

GFP Microscopy
Yeast cells were grown overnight in 5 mL of SC-Ura+Glucose media at 30 °C at 200 rpm. The cells were then washed two times and transferred to α-synuclein expression media. Cells were then inoculated to 2.6x107 cells/mL in flasks containing 25 mL of SC-Ura+Galactose. At specific time points (24 and 48 hours), 1 mL of cell culture was removed from the flasks, and 10 μL of sample was pipetted onto a glass slide for fluorescent microscopic examination. Using a Nikon TE2000U fluorescent microscope, cells were visualized and analyzed using Metamorph 4.0 software.

Using Metamorph 4.0, 750 DIC pictures were counted and corresponding fluorescence images were assessed for phenotype and percentages of cells expressing that phenotype were determined. Below are examples of phenotypes that I assessed:

![Example of phenotypes](image)

OD600 Growth Curve Analysis
Yeast cells were grown overnight in 5 mL of SC-Ura+Glucose media at 3 °C at 200 rpm. Cells were then centrifuged for 5 minutes at 4 °C, followed by two washes with 5 mL of sterile water. The cells were then re-suspended in 10 mL of sterile water and counted to determine cell density using a hemocytometer. Cells were then inoculated to 2.6x106 cells/mL in flasks containing 25 mL of SC-Ura+Galactose. At 0, 3, 6, 12, 18, 24, 36, and 48 hours, and in duplicate measurements, 1 mL of cell culture was removed and placed in a cuvette to measure absorbance at 600 nM using a Hitachi U-2000 Spectrophotometer.

Spotting Analysis
Yeast cells were grown overnight in 5 mL of SC-Ura+Glucose media at 3 °C at 200 rpm. Cells were then centrifuged for 5 minutes at 4 °C, followed by two washes with 5 mL of sterile water. The cells were then re-suspended in 10 mL of sterile water and counted to determine cell density using a hemocytometer. A density of 2.6x106 cells/mL was removed and cells were resuspended in 1 mL of sterile water. Cells were diluted five-fold and plated onto SC-Ura+Glucose agar plates for loading control and onto SC-Ura+Galactose agar plates to assess growth with the gal-induced expression of α-synuclein. After 24 hours, digital photographs were taken using an HP Canoscan scanner, and images were imported using Adobe Photoshop CS2 program.

Statistics
Western blot analysis was conducted at least two times with consistent results during both of the trials. Meaning that for each Western blot, I needed to repeat the trials until I saw the same results twice. GFP microscopy was conducted at least two times with consistent results. If the two trials looked different, I performed a third trial and then proceeded with quantification of all of the trials. Using Metamorph 4.0, 750 DIC pictures were counted and corresponding fluorescence images were assessed for phenotype and percentages of cells expressing that phenotype were determined. After quantification of each trial, an average was taken. Both OD600 growth curves and spotting analysis were performed three times. For OD600, a cumulative graph was plotted with absorbance on the y-axis and time points on the x-axis. At 12, 18, and 24 hours, two-tailed distribution, two sample, equal variable t-tests were performed on cumulative graphs to check for statistical significance.

Results
Experimental Setup: The role of autophagy in the regulation of α-synuclein was evaluated using budding yeast strains that had single gene deletions for the proteins that belong to autophagy pathway complexes I (atg29Δ, atg31Δ), II (atg6Δ, atg14Δ), III (atg7Δ), IV (atg7Δ), and V (atg9Δ; Fig 5B). For each complex, single gene deletion strains were analyzed by comparisons to the autophagy intact parent strain. Ten deletion strains previously analyzed by our lab were re-quantified and re-grouped to fit with the modern classification of autophagy complexes (Choi Thesis, 2009; Danny Sanchez ’11; Kayla Ahlstrand ’12; Peter Sullivan ’12). The gene deletion strains and the parent strain were transformed with five plasmid expression vectors. pYES2 parent vector and Green Fluorescent Protein (GFP) vector served as negative controls, since neither of them express α-synuclein. The other three vectors expressed WT α-synuclein and two α-synuclein familial mutants, A30P and E46K. The α-synuclein vectors were all tagged with GFP on the C-terminus for live cell illumination of α-synuclein. PD-related α-synuclein properties were assessed using four assays. Toxicity was assessed by performing 1) growth curves and 2) five-fold serial dilution spotting. 3) Localization of α-synuclein was assessed using live fluorescence GFP microscopy. 4) Expression was analyzed with Western blotting.

In intact autophagy, α-synuclein localizes to the plasma membrane and is non-toxic
Before I began evaluation of autophagy (Atg) genes, I confirmed α-synuclein properties in the parent BY4741 strain in which autophagy is intact. These α-synuclein characteristics served as controls for all autophagy gene deletion strains. As previously shown (Sharma et al., 2006),
live cell GFP microscopy demonstrated WT and E46K α-synuclein localized to the plasma membrane, while A30P α-synuclein was cytoplasmically diffuse (Fig 6A). I saw similar expression of WT and E46K α-synuclein, using Western blot with V5 antibody against α-synuclein, with phosphoglycerate kinase (PGK) serving as protein loading control (Fig 6B). Finally, neither WT nor mutant α-synuclein was toxic to these yeast cells, assessed by OD-600 growth curves (Fig 6C) and serial dilution spotting on growth plates (Fig 6D).

Complex I: Increased α-synuclein expression and mislocalization without toxicity
The purpose of my first set of experiments was to examine five autophagy genes which encode proteins in complex I. I saw a strong change in localization in atg31Δ for both WT and E46K (Fig 7A). At 48 hours, WT α-synuclein was cytoplasmically diffused in approximately 59% of the cells. In atg13Δ and atg17Δ I observed a moderate change in α-synuclein localization in WT and E46K (Fig 7A). In atg29Δ, a moderate change was observed for E46K (Fig 7A). E46K was diffused in 20% of atg29Δ cells; however, the diffuse localization pattern differed from the parent strain. Interestingly, α-synuclein accumulated around a vacuole, and the vacuole was dimly lit compared to the cytoplasm. I observed no change in localization in atg1Δ when compared to the parent strain (7A). A30P α-synuclein demonstrated dominant cytoplasmic diffusion in the examined strains in complex I (Fig 7A).

Complex II: α-Synuclein mislocalization and increased expression remains the key
My next goal was to examine four autophagy genes that encode proteins in complex II. I observed a strong change in localization in Vps34Δ for WT α-synuclein with only 39% of the cells retaining plasma membrane localization at 48 hours (Fig 8A). E46K α-synuclein localized to the plasma membrane in 56% of the cells, indicating a moderate change
Figure 7: Analysis of Complex I

A. TOP: α-Synuclein localization in complex I: α-synuclein tagged with GFP was visualized using fluorescence microscopy at 24 and 48 hours. BOTTOM: Quantification of approximately 750 cells of each transformed construct of each of the deletion strains. Based on the quantification, the changes in the phenotype are characterized as strong, moderate or none. In the case of WT and E46K α-synuclein: Cells that retain ≥ 80% of plasma membrane localization are said to have no change. Cells that retain ≥ 40% but ≤ 80% plasma membrane localization are said to have a moderate change. Finally cells that retain ≤ 40% percent of plasma membrane localization are said to have a strong change. In the case of A30P α-synuclein, cells that retain ≥ 80% of cytoplasmic diffusion are said to have no change; cells that retain ≥ 40% but ≤ 80% of cytoplasmic diffusion are said to have moderate change; and cells that retain ≤ 40% percent of cytoplasmic diffusion are said to have strong change. (n=2)

B. Growth curves: One of the assays to evaluate toxicity was Optical Density at 600 nm to compare the growth of the negative controls (parent vector and GFP) to α-synuclein constructs (WT, A30P, and E46K) in each of the deletion strains. At 12, 18, and 24 hours, a two-tailed distribution t-test was performed. When I do not mark the time points with an asterisk, it means that the p-value is greater than 0.005. (n=3)

C. Spotting: Five-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). * indicates non-α-synuclein dependent toxicity. (n=3)

D. Western Blot: Western blotting was used to evaluate the expression of α-synuclein at 24 and 48 hours by comparing the deletion strains to the parent strain BY4741 where autophagy is intact. (n=2)

In localization compared to the parent strain, I saw moderate change in WT and E46K α-synuclein localization in atg6Δ (Fig 8A). Interestingly, in atg6Δ, while A30P α-synuclein remained cytoplasmically diffuse, the pattern of localization resembled atg29Δ: intense cytoplasmic fluorescence with a dimly lit vacuole (Fig 8A). Moderate change in localization was noted for WT α-synuclein in Vps15 and a strong change for E46K. At 48 hours, WT and E46K α-synuclein localized to the plasma membrane and formed small cytoplasmic aggregates (Fig 8A). A30P α-synuclein remained cytoplasmically diffuse in all of the deletion strains of complex II (Fig 8A). Once again, I saw no α-synuclein dependent toxicity in any deletion strains.

Instead, growth curve and spotting experiments demonstrated that vps15Δ exhibited non-α-synuclein dependent toxicity (Fig 8B & C; marked by red asterisk). Similarly, growth curves demonstrate slight growth impairment in vps34Δ (Fig 8B & C; marked by blue asterisk). Interestingly, atg6Δ and atg14Δ cells, based on growth curves, grew slightly better than the parent strain (Fig 8B; marked by black asterisk). WT and E46K α-synuclein expression increased in atg6Δ (Fig 8D 24 hr; second lane). E46K α-synuclein expression increased in vps34Δ over 48 hours (Fig 8D 24 hr & 48 hr; fifth lane). Also, I observed a weak increase in expression in atg14Δ as compared to the parent strain (Fig 8D 24 hr; third lane). In vps15Δ, I noted
Figure 8: Analysis of Complex II

A. TOP: α-Synuclein localization in BY4741, autophagy intact strain, and the complex II deletion strains: α-synuclein tagged with GFP was visualized using fluorescence microscopy at 24 and 48 hours. BOTTOM: Quantification of approximately 750 cells of each transformed construct of each of the deletion strains. Based on the quantification, the changes in the phenotype are characterized as strong, moderate or none. In the case of WT and E46K α-synuclein, cells that retain ≥80% of plasma membrane localization are said to have no change; cells that retain ≥40% but ≤80% of plasma membrane localization are said to have moderate change; and cells that retain ≤40% percent of plasma membrane localization are said to have strong change. In the case of A30P α-synuclein, cells that retain ≥80% of cytoplasmic diffusion are said to have no change; cells that retain ≥40% but ≤80% of cytoplasmic diffusion are said to have moderate change; and cells that retain ≤40% percent of cytoplasmic diffusion are said to have strong change.

B. Growth curves: One of the assays to evaluate toxicity was Optical Density at 600 nm to observe the growth of the negative controls (parent vector and GFP) α-synuclein constructs (WT, A30P, and E46K) in each of the deletion strains. At 12, 18, and 24 hours, a two-tailed distribution t-test was performed. When I do not mark the time points with an asterisk, it means that the p-value is greater than 0.005. * represents time points where non-α-synuclein dependent toxicity was observed for vps15Δ (for all the time points, p-value < 0.005). * represents time points where better growth is observed for atg6Δ and atg14Δ (for all the time points, the p-value < 0.005). (n=3)

C. Spotting: Five-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). * indicates non-α-synuclein dependent toxicity. (n=3)

D. Western Blot: Western blotting was used to evaluate the expression of α-synuclein at 24 and 48 hours by comparing the deletion strains to the parent strain BY4741 where autophagy is intact. (n=2)

decreased expression of E46K α-synuclein at 24 hours, with no change in expression in WT (Fig 8D 24hr; forth lane).

Complex III: Less altered α-synuclein localization and expression

My next step was to examine four genes that encode proteins in complex III. It is important to remember that Atg7 operates in complex III and complex IV. I saw a moderate change in localization in atg7Δ in WT α-synuclein (Fig 9A). At 24 and 48 hours, WT α-synuclein demonstrated cytoplasmic diffusion in 30% of the cells, while in the parent strain it was diffuse in 6% of the cells. Interestingly, I saw a change in A30P α-synuclein localization in atg8Δ compared to the parent strain (Fig 9A). In atg8Δ more than 30% of the cells exhibited cytoplasmic localization with aggregates. Surprisingly, I did not see alterations in localization of atg3Δ or atg4Δ; more than 80% of the cells retained patterns of localization seen in the parent strain (Fig 9A).

As seen with previous complexes, no deletion strains demonstrated α-synuclein dependent toxicity. Surprisingly, I did observe toxicity in atg7Δ, but the pattern was neither α-synuclein dependent nor α-synuclein independent (Fig 9B & C; marked by red asterisk). After evaluating the expression of these four genes, only E46K α-
Figure 9: Analysis of Complex III

A. TOP: α-Synuclein localization in BY4741, an autophagy intact strain, and the complex III deletion strains: α-synuclein tagged with GFP was visualized using fluorescence microscopy at 24 and 48 hours. BOTTOM: Quantification of approximately 750 cells of each transformed construct of each of the deletion strains. Based on the quantification, the changes in the phenotype are characterized as strong, moderate or none. In the case of WT and E46K α-synuclein, cells that retain ≥ 80% of plasma membrane localization are said to have no change; cells that retain ≥ 40% but ≤ 80% of plasma membrane localization are said to have moderate change; and cells that retain ≤ 40% percent of plasma membrane localization are said to have strong change. In the case of A30P α-synuclein, cells that retain ≥ 80% of cytoplasmic diffusion are said to have no change; cells that retain ≥ 40% but ≤ 80% of cytoplasmic diffusion are said to have moderate change; and cells that retain ≤ 40% percent of cytoplasmic diffusion are said to have a strong change. (n=2)

B. Growth curves: One of the assays to evaluate toxicity was Optical Density at 600 nm to observe the growth of the negative controls (parent vector and GFP) to α-synuclein constructs (WT, A30P, and E46K) in each of the deletion strains. At 12, 18, and 24 hours, a two-tailed distribution t-test was performed. When I do not mark the time points with an asterisk, it means that the p-value is greater than 0.005 * represents time points where neither non-α-synuclein dependent toxicity nor α-synuclein dependent toxicity was observed in atg7Δ (for all the time points, p-value < 0.005). (n=3)

C. Spacing: Five-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). * indicates non-α-synuclein dependent toxicity. (n=3)

D. Western Blot: Western blotting was used to evaluate the expression of α-synuclein at 24 and 48 hours by comparing the deletion strains to the parent strain BY4741 where autophagy is intact. (n=2)

Complex V: α-Synuclein mislocalizes in a variety of phenotypes

Lastly, I looked at three genes that encode proteins in complex V. All of the deletion strains demonstrated altered localization (Fig 10A). I observed a strong change in localization of both WT and E46K α-synuclein in atg2Δ. At 48 hours, approximately 26% of the WT α-synuclein cells retained the plasma membrane localization, and also 26% of cells had a cytoplasmic localization (Fig 10A). In the majority of the cells of atg2Δ, WT α-synuclein formed cytoplasmic aggregates and localized to the plasma membrane. I found E46K α-synuclein cytoplasmically diffused in 25% of the cells and the other 25% of the cells localized to the plasma membrane with the formation of aggregates (Fig 10A). In 32% of the cells, A30P α-synuclein exhibited intense membrane fluorescence with a cytoplasmically diffused background. I saw a moderate change in localization in WT α-synuclein and a strong change in E46K α-synuclein in atg9Δ. At 48 hours, E46K α-synuclein was cytoplasmically diffused in approximately 75% of the cells. A30P α-synuclein was diffused with the presence of aggregates in 28% of the cells at 48 hours in atg18Δ, while in the parent strain, A30P α-synuclein always localized cytoplasmically with no aggregates (Fig 10A). Once again, I saw no α-synuclein dependent toxicity in any of the autophagy
Figure 10: Analysis of Complex V
A. TOP: α-Synuclein localization in BY4741, an autophagy intact strain, and the complex V deletion strains: α-synuclein tagged with GFP was visualized using fluorescence microscopy at 24 and 48 hours. BOTTOM: Quantification of approximately 750 cells of each transformed construct of each of the deletion strains. (n=2) Quantification of BY4741 autophagy intact and deletion strains: Quantification of approximately 750 cells of each transformed construct of each of the deletion strains. Based on the quantification, the changes in the phenotype are characterized as strong, moderate or none. In the case of WT and E46K α-synuclein, cells that retain ≥ 80% of plasma membrane localization are said to have no change; cells that retain ≥ 40% but ≤ 80% of plasma membrane localization are said to have moderate change; and cells that retain ≤ 40% percent of plasma membrane localization are said to have strong change. In the case of A30P α-synuclein, cells that retain ≥ 80% of cytoplasmic diffusion are said to have no change; cells that retain ≥ 40% but ≤ 80% of cytoplasmic diffusion are said to have moderate change; and cells that retain ≤ 40% percent of cytoplasmic diffusion are said to have strong change. (n=2)
B. Growth curves: One of the assays to evaluate toxicity was Optical Density at 600 nm to observe the growth of the negative controls (parent vector and GFP) to α-synuclein constructs (WT, A30P, and E46K) in each of the deletion strains. At 12, 18, and 24 hours, a two-tailed distribution t-test was performed. When I do not mark the time points with an asterisk, it means that the p-value is greater than 0.005. (n=3)
C. Spotting: Five-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). (n=3)
D. Western Blot: Western blotting was used to evaluate the expression of α-synuclein at 24 and 48 hours by comparing the deletion strains to the parent strain BY4741 where autophagy is intact. (n=2)

Deletions. Spotting and growth curve assays demonstrated similar growth for all of the α-synuclein deletion strains compared to the parent strain (Fig 10B & 10C). Interestingly, I saw an increase in expression in both WT and E46K α-synuclein in atg9Δ and atg18Δ compared to the parent strain, with the loading control being equal (Fig 10D; WT 24 hr; E46K 24 hr & 48 hr; third & forth lanes). Surprisingly, no change in expression was observed for atg2Δ (Fig 10D; second lane).

Discussion

Determining the degradation mechanism of α-synuclein can uncover therapeutic possibilities for treating Parkinson’s disease, as α-synuclein accumulation is a key pathological hallmark. Recent evidence puts autophagy at the forefront of the pathways responsible for degradation of α-synuclein; however, the genetic evidence linking autophagy and α-synuclein is still weak. Four years ago, DebBurman lab began evaluating the hypothesis that autophagy regulates α-synuclein degradation by studying in a budding yeast model an initial set of single gene deletions of proteins that form the autophagy pathway (Choi Thesis, 2009 and other lab projects). The purpose of my thesis was to continue the evaluation of the remaining autophagy genes. I also assessed the previously studied genes by putting them and the novel genes that I studied into a modern classification of the autophagy pathway. The modern classification organizes the genes into five functional complexes (I, II, III, IV, and V) that are responsible for the formation of the autophagosome. For my thesis, I assessed genes from all of the five complexes. I elaborate on four significant findings below.
First, through genetic evidence, my results support the hypothesis that α-synuclein is regulated by the autophagy pathway. Out of the six autophagy genes that I evaluated, each altered one or more α-synuclein PD-related properties. Secondly, the effects on α-synuclein were gene-specific, indicating substrate-specificity. Thirdly, α-synuclein localized in a spectrum of patterns when autophagy was deficient in individual gene-deletion strains. Finally, I did not observe α-synuclein dependent toxicity.

**α-Synuclein is regulated by the autophagy pathway**

In support of my hypothesis, my results demonstrate α-synuclein degradation by the autophagy pathway. Of the six novel autophagy genes that I examined, each affects at least one or more PD-related property of α-synuclein (Table 3; bolded genes). Particularly, atg6Δ, atg7Δ, atg9Δ, atg29Δ, and atg31Δ alter α-synuclein localization, while α-synuclein expression increases in all strains except atg31Δ, where I observed a loss of expression. Also, I reevaluated and confirmed previous findings of our lab with ten autophagy gene-deletions by organizing them into functional complexes (I, II, III, and V). Out of the ten genes that I reevaluated and confirmed, eight of them have altered properties (Table 3; non-bolded genes). Specifically, three of them, vps34Δ, atg18Δ, and atg13Δ, have altered localization and increased expression. Similar to previous studies, I demonstrate that atg2Δ, atg8Δ, atg17Δ, and vps15Δ alter localization of α-synuclein and that atg1Δ had increased expression. Consequently, our combined work demonstrates that out of the sixteen genes evaluated, thirteen showed altered localization; eleven showed a change in expression. None of the genes showed α-synuclein dependent toxicity. Interestingly, atg7Δ, atg31Δ, and vps15Δ all display non-α-synuclein dependent toxicity, suggesting their importance to yeast survival. Our cumulative efforts demonstrate convincing genetic evidence that autophagy is the route for α-synuclein degradation.

For a scientific community to consider an autophagy gene a regulator of α-synuclein, do all PD-related α-synuclein properties need to be altered or just a few? Our lab focuses on performing a thorough evaluation of three properties of α-synuclein: toxicity, localization, and expression. Manipulation of these properties is central to the pathogenesis of PD. The majority of leading yeast labs that have studied α-synuclein (Table 4) focuses more on the evaluation of only one of these properties and are also looking at other pathways, such as secretory pathways, with electron microscopy. My lab consistently studies three important markers of α-synuclein linked to PD pathology. Therefore, we believe that if we demonstrate a change in one or two PD-related properties, we demonstrate the regulation of α-synuclein by the pathway being studied.

Over the years, DebBurman lab has studied diverse pathways including oxidative stress (Brandis Thesis, 2006; Kukreja Thesis, 2008), ubiquitin proteasome system (Herrera Thesis, 2005), chaperones (Shrestha, 2007; unpublished findings), mitochondrial function (Zorniak Thesis, 2007), and phospholipid synthesis (Kukreja Thesis, 2008) due to their ability to regulate α-synuclein pathogenesis. With the exception of endocytosis, which had the most striking effect on all α-synuclein properties that our lab examines (Perez Thesis, 2010 and Ayala Thesis, 2009), none of the other pathways strongly altered more than one property of α-synuclein. Even in endocytosis, only one gene out of the 17 demonstrates α-synuclein dependent toxicity (Ayala Thesis, 2009). After thorough examination of these pathways, we conclude that α-synuclein can be degraded by both endocytosis and autophagy.

Our results belong to the first study to address the question of α-synuclein degradation by autophagy in a yeast model, genetically. As described earlier, important pharmacological studies demonstrate that autophagy is involved in degradation for α-synuclein (Bugyi et al., 2004, Vogtzi et al., 2008). My hypothesis is further supported by mammalian genetic studies. Crews et al. (2010) evaluation of the pathology of the brain of patients with dementia with Lewy bodies revealed an inverse positive relationship between Atg7 and α-synuclein as Atg7 levels decrease. Furthermore, electron microscopy analysis demonstrates a presence of abnormal autophagosomes or α-synuclein positive neurons that are absent in the control cases. Similar results are seen in the transgenic mouse model of PD (Crews et al., 2010). This accumulation of α-synuclein and neuronal deficits in transgenic mice is rescued by the delivery of Atg7. Therefore, this study once again supports the hypothesis that decreased autophagy degradation of α-synuclein leads to its accumulation.

A study by Winslow et al. (2010) noted that overexpression of wild-type α-synuclein impairs macroautophagy in mammalian cells and in transgenic mice through inhibition of Rab1a. When Rab1a is overexpressed, the defect is rescued. Overexpression of α-synuclein or Rab1α knockout causes mislocalization of Atg9, which results in a decrease in omegasomal formation, a precursor to autophagosome formation. This study further demonstrates that α-synuclein is degraded by autophagy and can support my findings. I observed a strong increase in expression of WT and E46K α-synuclein and a shift in localization of α-synuclein from the plasma membrane to the cytoplasm when Atg9 is deleted (Table 3; bolded genes). Since numerous studies indicate that Atg1 is vital for autophagosome formation, I predict that impaired degradation of α-synuclein and its accumulation in the cytoplasm is due to this deletion. The studies described above demonstrate that α-synuclein does use the autophagy route. Our cumulative findings extend existing literature by adding genetic support in a more comprehensive way by evaluating more genes and properties.

**Substrate specificity implied in α-synuclein degradation**

After evaluating the majority of the autophagy genes, I saw that α-synuclein is regulated to a varying degree by each gene. Some of the genes have an effect on two properties of α-synuclein while others alter only one property. For example, atg6Δ and vps34Δ strongly alter localization, while atg11Δ, atg8Δ, and atg19Δ alter localization while having strongly increased expression. I saw a change in localization but no expression in atg17Δ, vps15Δ, and atg2Δ. Finally, I observe no change in any of the properties in atg3Δ and atg4Δ. This varying alteration in α-synuclein properties suggests the following question: Do all autophagy substrates interact with all autophagy proteins or does each substrate interact with a subset specific to the substrate? My results indirectly demonstrate that the interaction takes place with a subset of autophagy proteins.

Although the autophagy pathway is controlled by 33 genes, only 17 are recognized as needed for core machinery of autophagosomal formation, indicating that some genes are more important than others (Klionsky et al., 2003). As described earlier, mice with single gene deletions of Atg5 or Atg7 show progressive neurodegeneration and accumulation of ubiquitin-positive protein aggregates in the neural cells (Hara et al., 2006; Komatsu et al., 2006). This suggests that both Atg5 and Atg7 proteins regulate the Atg12 conjugation system. When deleted, the lack of these proteins causes damage to the cell. Furthermore, an interaction between Atg11 and Atg15 is vital for the induction of autophagy under starvation conditions. In both yeast and Drosophila, when Atg13 is deleted, autophagy induction fails (Chang & Neufeld, 2009). Atg1 is a serine/threonine kinase,
is vital for the formation of PAS under such conditions as starvation, and is needed for dissociation of Atg proteins from the ER during autophagy induction (Cheong & Klionsky, 2008). Additionally, proper cycling of Atg9 requires Atg1. Atg9 plays an important role in autophagosome formation because it cycles between the ER and mitochondria and supplies the lipids to create autophagosomes. In the absence of Atg9, Atg proteins fail to be recruited to the PAS thus preventing autophagosome formation (Reggiori et al., 2005). To recap, even though both Atg1 and Atg9 belong to a particular complex, they seem to carry out the primary role of that complex and trigger the subordinate role of the genes belonging to their complex.

Since not all of the Atg genes play a vital role in the autophagosome formation and propagation, perhaps not all genes regulate α-synuclein. In the field, the current evidence, while limited, suggests substrate specificity during the autophagy degradation process. Proteins like Atd6p and p62 selectively indicate degradation by autophagy (Onodera & Ohsumi, 2004; Pankiv et al., 2007). For example, p62, which is commonly found in inclusion bodies containing polyubiquitinated protein aggregates, is selectively recognized by light chain 3 (LC3), which has a mammalian homologue, Atg8 (Pankiv et al., 2007). Furthermore, some organelles as ribosomes and peroxisomes are degraded by selective autophagy (Kraft et al., 2008). Atg7α cells abolish degradation of the ribosomes but not atg19α cells, indicating substrate specificity (Kraft et al., 2008). Moreover, pathogens’ degradation is linked to selective autophagy. γ-Herpesviruses (γ-HVs), which includes pathogens like Epstein Barr virus (mononucleosis) and Kaposi’s sarcoma, associate HIV, code for antiprototic protein Bcl2s, which promotes viral replication and pathogenesis. Sinha et al. (2008) shows that a homolog M11 of Bcl2s regulates Beclin 1, Atg6 homolog in yeast, and results in inhibition of autophagy. Such examples illustrate selective autophagy. Therefore, not all autophagic proteins are equally required for degradation of specific substrates.

Additionally, my results provide insight into the question of whether all complexes are equally important or not. I observe that the alteration of α-synuclein properties varies with the complex. For example in Complex I, the deletion of four out of the five genes shows altered properties. However, in Complex III, only two of the four genes studied showed altered properties of α-synuclein. Thus, the degree of the properties’ alteration in each of the complexes can suggest that some are more responsible than others in regulating α-synuclein.

α-Synuclein displays a surprisingly wide range of cellular localization

Surprisingly, in atg2Δ, atg13Δ, atg31Δ, and vps15Δ, I saw for both wild-type and E46K α-synuclein the following localization patterns: plasma membrane association, cytoplasmic diffusion, plasma membrane association with aggregates, and cytoplasmic diffusion with aggregates. This was a notable finding because my lab does not see such a range of patterns when studying other pathways. Furthermore, in A30P α-synuclein, I saw cytoplasmic localization with aggregates in atg8Δ, atg8Δ, atg13Δ, atg18Δ, and atg29. I found the presence of aggregates interesting because studies done by other labs suggest that the nature of these aggregates is cytoplasmic vesicles (Soper et al., 2008). Soper et al. (2008) reveals, through electron microscopy, that α-synuclein initially localizes to the plasma membrane and subsequently forms accumulations of vesicles (Reggiori et al., 2005). By performing EM, they confirm that α-synuclein is present in these vesicles and is not present in the form of amyloid fibrils, which suggests an alternate mechanism for α-synuclein cellular toxicity in PD (Soper et al., 2008). Pathological studies of the brain revealed that accumulations of vesicles are found surrounding Lewy bodies (Graham & Klionsky, 2008).

Furthermore, Soper et al. (2008) determined two types of vesicles: secretory vesicles and ER-Golgi transport vesicles. Interestingly, they did not find that A30P α-synuclein expressing cells have accumulations of vesicles, which is contrary to my results. Cooper et al. (2006) demonstrated that accumulation of α-synuclein inhibits ER-Golgi trafficking. If autophagy is the route for α-synuclein degradation, then compromising it will result in α-synuclein accumulation leading to ER-Golgi trafficking inhibition. I hypothesize that ER-Golgi trafficking inhibition leads to accumulation of the vesicles in these cells. The field speculates that one of the functions of α-synuclein is lipid metabolism. Perhaps, the accumulated α-synuclein causes an accumulation of lipid droplets that then interferes with vesicle trafficking as seen in Outeiro and Lindquist (2003). Soper et al. (2008) states that at least two types of vesicles are present. Therefore, a possibility of other types of vesicles or, perhaps, autophagosomes exists. In the future, it would be interesting to further investigate the nature of these vesicles.

No α-synuclein dependent toxicity was observed

To my surprise, while most of the gene deletions alter the properties of α-synuclein localization and expression, I did not observe toxicity. This aspect of my hypothesis was not supported since I expected cell death after the deletions. However, it is interesting to see that out of the 16 genes studied, 4 had non-autophagic dependent toxicity. This could mean that Atg genes are involved in other mechanisms that are vital for cell survival, which shows that the autophagy pathways are complex and still not well understood. The link between α-synuclein and toxicity has been one of the hardest to unravel. For example, a study done by Willingham et al. (2003) shows that out of 4850 genes examined, only 86 demonstrated α-synuclein dependent toxicity. Out of these 86 genes identified to cause α-synuclein dependent toxicity, none were Atg genes. Thus, my results support the findings of Willingham et al. (2003). Interestingly, some of the genes identified by Willingham et al. (2003), such as PARK9, can rescue α-synuclein toxicity when exposed to magnesium (Gitler et al., 2009). Based on my results and the literature, I can hypothesize that toxicity is not positively correlated with the alteration of α-synuclein localization and accumulation.

Low α-synuclein expression, activation of apoptosis, or yeast excretion of plasmids are some of the reasons for the lack of α-synuclein dependent toxicity. Perhaps, since I used a low to moderate α-synuclein expressing system, I did not express α-synuclein at a level that would be toxic to the cells. Other labs demonstrate that when α-synuclein is expressed at a higher level, it is toxic to the cells (Outeiro & Lindquist, 2003). If there was minimal toxicity, I might have not seen it because of the cross-talk between autophagy and apoptosis. Perhaps, apoptosis increases in cells experiencing α-synuclein dependent toxicity. Yang et al. (2009) demonstrated that in PC12 cell lines expressing α-synuclein, inhibition of autophagy results in a negative correlation between autophagy and apoptosis. Measurement of apoptosis markers can be one of my future studies when evaluating autophagy deletions. Another possible reason why I did not see toxicity could be because the yeast were excreting the plasmid and keeping just enough plasmid to survive, thus preventing noticeable toxicity (Outeiro & Lindquist, 2003). The excretion of plasmid can explain why I do not see any α-synuclein expression in the atg31Δ deletion.
Furthermore, in mammalian cells, evidence demonstrates that under autophagy inducing conditions, other degradation mechanisms can be activated in order to decrease accumulation of misfolded protein and possible toxicity (Wyttenbach et al., 2008). As mentioned earlier, two primary mechanisms exist in the cell for degradation of proteins: the proteasome and the lysosome. Evidence in the field demonstrates that when a proteasome is compromised, the activity of autophagy is up-regulated, which suggests compensatory pathways (Zhu et al., 2010; Pandey et al., 2007). Moreover, evidence shows compensating mechanisms taking place between the routes to the lysosome. Midorikawa et al. (2010) showed that degradation of rhodopsin is dependent on both the endosomal pathway and autophagy. When Atg7 or Atg8 is deleted, they see an increase in the amount of rhodopsin localization to Rab7-positive late endosomes, followed by retinal degeneration (Midorikawa et al., 2010). Rab7 overexpression suppresses rhodopsin accumulation and retinal degeneration, which accelerates the endosomal degradation pathway. Perhaps, α-synuclein is degraded in the same manner as rhodopsin. Additionally, other genes could have compensated for the single-gene deletions. Since yeast are very evolutionarily adapted organisms, deletion of proteins in the complex could have taken over the job of the protein that is not produced. Thus, such compensation is a limitation of a single-gene deletion study.

Criticisms, limitations, and future studies

One of the limitations of this study is using single-gene knockouts. I could not evaluate if the reason why I did not see toxicity was due to compensation by other genes. Furthermore, I never actually measured the level of autophagy, which can be easily done by looking at LC3-II levels by Western blotting. Additionally, my study suggests interaction between α-synuclein and the Atg proteins, or the lack of interaction. Both of these interactions need to be studied by our lab in the future. The biochemical interactions can be demonstrated using immunofluorescence or immunoprecipitation. Moreover, the nature and the type of aggregates I saw needs to be established.

I have completed the evaluation of four out of the five complexes involved in autophagosome formation. In order to complete the study, the last complex needs to be examined. Furthermore, the analysis of the fusion step of autophagosome needs to be completed. Once we have a clear understanding of which genes regulate α-synuclein, we can study multiple-gene deletions and their effect on the properties of α-synuclein. I believe that a compensatory mechanism takes place between the pathways to the lysosome and between the lysosome and the proteasome. It would be interesting to see what would happen if autophagy and endocytosis are compromised or autophagy and the proteasome. Moreover, once we confirm which genes are responsible, we can perform gene-rescue experiments.

Conclusion

PD is an incurable neurodegenerative disease that afflicts millions worldwide (Fahn, 2008). The goal of the field is to create therapeutic treatments that will alleviate the symptoms and, perhaps, cure the disease. Over the years, autophagy has become an important topic of discussion suggesting that studying autophagy might shed light on neurodegenerative diseases. The goal of this study was to demonstrate a genetic link between α-synuclein and autophagy. I was able to show that α-synuclein is regulated by autophagy but that not all of the autophagy genes are responsible for this regulation. Furthermore, each of the genes has its own profile of regulation. However, more research is needed to determine the exact mechanism of this regulation and what happens in PD. My lab offers the first study of α-synuclein regulation by autophagy in a yeast model. Future studies will further the understanding of this complex pathway and possibly offer treatments that patients have long been awaiting.

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