Genetic Evidence in Yeast Model Implicates Autophagy in the Degradation of Parkinson's Disease Protein alpha-Synuclein

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Genetic Evidence in Yeast Model Implicates Autophagy in the Degradation of Parkinson’s Disease Protein α-Synuclein

Kayla Ahlstrand and Peter Sullivan*
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Lake Forest College
Lake Forest, Illinois 60045

Abstract

Parkinson’s disease (PD) is an incurable neurodegenerative disease resulting from dopaminergic neuronal death in the substantia nigra, likely due to the misfolding and aggregation of the protein α-synuclein. A major hypothesis is that increasing the degradation of α-synuclein reduces toxicity and aggregation. The lysosome is pharmacologically implicated in the degradation of α-synuclein. Macroautophagy (autophagy), a highly conserved catabolic process in eukaryotes, is a major route to the lysosome. The genetic link between PD and autophagy is currently tenuous and a subject of intense research in our lab. In this study, we hypothesized that basal autophagy would protect cells from α-synuclein toxicity, and tested this by genetically inhibiting the nucleation and fusion steps of autophagy in a budding yeast model. We assessed α-synuclein’s pathotoxicity linked properties (aggregation, accumulation and toxicity) in yeast autophagy gene (ATG) knockout strains needed for nucleation (ATG11 and ATG13) and two needed for fusion (VAM3 and VAM7). We are among the first to report that autophagy fusion step genes can regulate several of α-synuclein’s PD-related properties. Also, we found that the absence of any of these four genes altered α-synuclein localization, while three affected its accumulation, and none induced cellular toxicity. These new results support accumulating data from our lab for the role for autophagy in α-synuclein regulation, but will be further aided by completion of analysis of autophagy genes yet to be studied.

Introduction

Synucleinopathies lie in the broad realm of neurodegenerative disorders and are characterized by the aggregation of insoluble α-synuclein protein in various regions of the brain (Galvin et al., 2001). The inclusions created by α-synuclein play a major role in the onset of Parkinson’s Disease (PD), Lewy body variant of Alzheimer’s disease, dementia with Lewy bodies, neurodegeneration with brain iron accumulation type-1, and multiple system atrophy (Baba et al., 1998; Duda et al., 2000; Galvin et al., 2008; Spillantini et al., 1997, 1998; Tu et al., 1998). PD is the second most common neurodegenerative disease and results in resting tremors, bradykinesia, a shuffling gate, and postural instability (Olanow and Tatton 1999). In both sporadic and familial PD, dopaminergic neuronal death is found in the substantia nigra region of the midbrain, in which the misfolded and aggregated α-synuclein accumulates in intra-cellular inclusions called Lewy bodies (Spillantini et al., 1998). The relationship between α-synuclein and neuronal cell death in this devastating disease is still uncertain.

α-Synuclein is found abundantly near presynaptic terminals of neurons (Clayton and George 1998; Davidson et al., 1998; Kahl et al., 2000) and plays a role in regulating neurotransmitter vesicle pools (Jensen et al., 1998; Murphy et al., 2000), synaptic plasticity (George et al., 1995; Abellovich et al. 2000), phospholipid binding and transportation, and lipid metabolism (Davidson et al. 1998; Sharon et al., 2001). The fundamental properties of α-synuclein leading to PD include misfolding (Winreb et al., 1996; Uversky 2003), aggregating (Spillantini et al., 1998), and binding to cellular membranes (Sharma et al., 2005). α-Synuclein shows a gain in toxic function and expresses PD symptoms and pathology when over-expressed as wild type (WT) or one of its familial mutant forms (A30P or A53T) in mice (Masliah et al., 2000; Giasson et al., 2003), flies (Feany and Bender 2000; Auluck et al., 2002), and worms (Lasko et al., 2003). WT α-synuclein can polymerize in vitro; however, mutants A30P (Giasson et al., 1998) and E46K (Greenbaum et al., 2005) aggregate more quickly. The extent of aggregation is likely affected by α-synuclein accumulation and vice-versa. Thus, the mechanisms that control and, most importantly, decrease α-synuclein levels are important to understand. Varying degrees of evidence exist for whether the proteasome or the lysosome degrades α-synuclein and the relative contributions of these two pathways need further resolution.

Several lines of genetic and chemical evidence support α-synuclein degradation via the proteasome. Mutations in the Parkin and C-terminal hydrolase-L1 proteins, both necessary for the proteasome pathway, are linked to proteasome dysfunction and PD pathogenesis (Schulz 2008; Leroy et al., 1998). Similarly, chemical inhibition of the proteasome results in PD pathogenesis in mice models (Petrucelli et al., 2002; McNaught et al., 2003; McNaught & Olanon 2006). Models over-expressing α-synuclein show that aggregated α-synuclein can lead to inhibition of the proteasome, further suggesting the relationship between PD and this pathway (Snyder et al., 2003). Also, α-synuclein aggregates have an abundance of ubiquitin molecules (Ciechanover and Brundin 2003), which tag proteins degraded by the proteasome (Hochstrasser 1996). These relationships indicate that α-synuclein directly interacts with the proteasome.

More recent evidence increasingly points to the lysosome also as a pathway for α-synuclein degradation (Lee et al., 2003; Webb et al., 2003; Cuervo et al., 2004; Vogiatzis et al., 2008). Increased accumulation of misfolded and aggregated α-synuclein results from the chemical inhibition of the lysosome (Lee et al., 2004). Additionally, mice with or without mutations for genes required for normal lysosomal function are shown to have increased neuronal death, neurodegenerative symptoms, and higher risks for the earlier onset of Alzheimer’s Disease, which is pathologically similar to PD (Butler et al., 2005). The lysosome is complex, however, in that there are multiple routes to this structure, including endocytosis and macroautophagy (autophagy) (Luzio et al., 2007; Klionsky). Evidence exists for α-synuclein degradation via endocytosis (Willingham et al., 2003; Alex Ayala, Lake Forest College senior thesis, 2009; Jaime Perez, Lake Forest College senior thesis, 2010). Autophagy is a cellular mechanism involved in the formation of double membrane structures called autophagosomes, which fuse with the lysosome to degrade its contents with enzymes (Klionsky and Ohsumi 1999). Autophagy is comprised of three steps (Figure 1). Using a mouse model for Huntington’s disease, a neurodegenerative disease
Figure 1: Steps of autophagy and associated genes. Shown are the three steps of autophagy accompanied by the genes associated with each step. Genes listed in green have already been assessed by Ray Choi, genes listed in red will be the target of assessment in this paper and genes listed in black remain to be assessed in the future.

Similar to PD, induction of autophagy protects against disease symptoms, while inhibition of autophagy has opposite effects (Ravikumar et al., 2004). Lately, Saccharomyces cerevisiae (budding yeast) has become a powerful model for exploring α-synuclein pathology (Outeiro and Lindquist 2003; Dixon et al., 2005; Flower et al., 2007; Sharma et al., 2006; Willingham et al., 2003; Zabrocki et al., 2005).

Recently, the DebBurman lab began evaluating autophagy using the budding yeast model. For his 2009 thesis, lab colleague Ray Choi analyzed a total of six knockout strains, two nucleation genes (ATG17 and VPS15), and four expansion genes (ATG1, ATG2, ATG8, and ATG18). His results predominately showed alterations in α-synuclein localization and no changes in toxicity or accumulation of α-synuclein (Ray Choi, Lake Forest College senior thesis, 2009). However, several genes still remain to be evaluated for nucleation (ATG6, ATG9, ATG11, ATG13, ATG14, and VPS34), expansion (ATG3, ATG4, ATG5, and ATG10), and all fusion-related genes (VAM3, VAM7, VIT1, and YKT6). We aimed to continue and extend Choi’s work by evaluating two gene knockouts for nucleation (atg11Δ, atg13Δ) and two gene knockouts for fusion (vam3Δ, and vam7Δ) in yeast cells. ATG11 functions in cytoplasmic to vacuole targeting (CVT) and brings receptor-bound cargo to the phagophore assembly site (PAS) (Yorimitsu and Klionsky 2005; Cheong et al., 2008), ATG13 is used in vesicle formation and in the CVT pathway (Funakoshi et al., 1997; Scott et al., 2000; Reggiori et al., 2004; Kabeya et al., 2005). VAM3 is required for vacuolar assembly and functions with VAM7 in vacuolar protein trafficking (Wada et al., 1992; Srivastava and Jones 1998; Sato et al., 1998). VAM7 also functions in the fusion of the lysosome and vacuole (Unger and Wickner 1998).

Specifically, in this study, we tested whether the three α-synuclein properties (aggregation, accumulation, and toxicity) would change in the four knockout strains assessed. We found increased α-synuclein aggregation in atg11Δ and vam7Δ, increased accumulation in atg11Δ, and no α-synuclein-dependent toxicity in yeast cells. We are also among the first to report that fusion related genes affect some of α-synuclein’s PD-related properties.

Materials and Methods

The following methods are described in detail in Sharma et al. (2006) and are briefly summarized below:

α-Synuclein Constructs

Human wild-type (WT) mutant α-synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and E46K were created from WT and α-synuclein and confirmed by sequencing (University of Chicago). WT and mutant α-synuclein were subcloned into pYES2.1 vectors (Invitrogen) after C-terminus GFP tagging as described in Sharma et al (2006).

Yeast Strains

BY4741, and all autophagy gene deletion strains derived from BY4741 parent strain, were purchased from Open Biosystems.

Yeast Expression

Budding yeast α-synuclein expression plasmid vectors (pYES2.1) were transformed into budding yeast strains as described in Burke et al. (2000). For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-
A. Microscopy

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>A30P</th>
<th>E46K</th>
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B. Expression

![Image](image7)

C. Serial Dilution Spotting

<table>
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<th>Galactose</th>
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</thead>
<tbody>
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</tr>
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<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
</tr>
<tr>
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<td><img src="image13" alt="Image" /></td>
</tr>
<tr>
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<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
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<tr>
<td>E46K</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
</tr>
</tbody>
</table>

D. Growth Curve

![Image](image18)

**Figure 2: α-Synuclein Properties in BY4741 parent strain.**

A. Microscopy: Live cell GFP imaging of α-synuclein distribution at 24 and 48 hrs. B. Expression: Western blot of α-synuclein and GFP alone expression (anti-V5) at 24 hours in BY4741 lysates. PGK serves as loading control. C. Serial Dilution Spotting: Five-fold serially diluted spotting-cells spotted on to glucose (non-induced) and galactose (induced) plates and grown for two days. D. Growth Curve: OD600 readings over 30 hr for BY4741 cells induced for α-synuclein, GFP, or vector alone.

URA). Presence of α-synuclein constructs was confirmed by polymerase-chain reaction. To regulate the expression of α-synuclein, the pYES2.1 vector contained a galactose inducible promoter (GAL1) allowing α-synuclein expression only when grown on in the presence of galactose.

**GFP Microscopy**

Yeast cells were grown in 5 mL SC-URA glucose overnight at 30°C and 200 rpm. After sufficient growth, cells were washed twice in 5 mL H2O by centrifuging at 1500 g for 5 minutes at 4°C. Cells were resuspended in 10 mL SC-URA galactose and incubated at 30°C and 200 rpm. At 24 and 48 hours, 1 mL of cell culture was centrifuged and 4 µL of cells were pipetted onto a slide. Cells were visualized under a Nikon TE2000-U fluorescent microscope and images were acquired using Metamorph 4.0 imaging software.

**Western Analysis**

Yeast cells were grown in glucose, washed, and resuspended in 10 mL galactose. At 24 and 48 hours, cell lysates were made from 2.5 x 10^7 cells/mL yeast cells. Cells were washed in 100 mM NaNO, and solubilized in Electrophoresis Sample Buffer (ESB) [2% sodium dodecyl sulfate (SDS), 80 mM Tris (pH 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/mL bromophenol blue, and a cocktail of protease inhibitors] and solubilizing agents (1% Triton-X 100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.7 µg/mL pepstatin A, 0.5 µg/mL leupeptin, 10 µg/mL E64, 2µg/mL aprotinin, and 2 µg/mL chymostatin). Samples were run on pre-cast 10-20% Tris-Glycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes and Western blot was performed with anti-V5 (Invitrogen) to visualize α-synuclein and anti-phosphoglycerokinase (PGK; Molecular Probes) as a loading control.

**Spotting Analysis**

Yeast cells were grown in 5 mL SC-URA glucose overnight at 30°C (200 rpm), centrifuged at 1500 g for 5 min at 4°C, washed twice in 5 mL H2O, and resuspended in 10 mL H2O. Cells were counted and diluted to 2.0 x 10^7 cells/mL then serially diluted (5-fold) into 96-well microwell plates. Pipetting was used to spot cells onto SC-URA glucose (2%) and galactose (2%) plates. Plates were incubated at 30°C until colonies could be seen up to the fifth dilution.

**Growth Curve Analysis**

Yeast cells were grown in 5 mL SC-URA glucose overnight at 30°C and 200 rpm. Cells were centrifuged at 1500 g for 5 min at 4°C, washed twice in 5 mL H2O and resuspended in 10 mL H2O. Cells were counted and diluted to 2.0 x 10^6 cell/
In our budding yeast model of α-synuclein, we analyzed how α-synuclein PD-related properties were affected when two steps of autophagy were compromised: nucleation and fusion. In nucleation, the genes ATG11 and ATG13 were deleted, while in fusion, VAM3 and VAM7 were deleted. Deletion strains were compared with the isogenic parent strain, BY4741. The experiments were set up with two controls (vector and GFP). The vector and GFP controls ensured that any resulting changes in α-synuclein characteristics were not due to the vector or GFP. Three GFP-tagged α-synuclein forms were studied: WT α-synuclein and two familial mutants (A30P and E46K). Three PD-related characteristics were assessed: aggregation/localization by live cell GFP microscopy, accumulation by Western blotting, and toxicity by optical density 600nm growth curves and serially diluted spotting.

BY4741 Parent Strain Localizes to Plasma Membrane Without Toxicity
The results from BY4741 were used as a control to compare the effects of α-synuclein observed in the gene deletion strains. As with previous data, BY4741 showed plasma membrane localization in WT (Sharma et al., 2006) and E46K (Michael White, Lake Forest College senior thesis, 2007; Michael Fiske, Lake Forest College senior thesis, 2010) and cytoplasmic diffuseness in A30P (Fig. 2A). No accumulation of α-synuclein was seen in any of the α-synuclein expressing cells of BY4741 (Fig. 2B). Finally, we looked at toxicity in BY4741 α-synuclein expressing cells. No toxicity was seen in any of the α-synuclein expressing cells, as each strain grew similar to one another in both the serial dilution spotting and the growth curves (Fig. 2C and D). These results were used as a control to compare effects of α-synuclein observed in the gene deletion strains.

Absence of ATG11 Increases Aggregation and Induces α-Synuclein-Independent Toxicity
Next, we assessed the changes in α-synuclein’s three PD-related properties in atg11Δ cells, the first of two nucleation-compromised strains we studied. When compared to the parent strain, atg11Δ induced a strong change in localization in WT and E46K α-synuclein, which, rather than being associated with the plasma membrane, was more aggregated in the cytoplasm over a 48-hour time course (Fig. 3A). Interestingly, this aggregation correlated well with
Absence of VAM3 Alters Localization and Increases 3D). confirmed by the assessment of was also seen in cells (Fig 3C). The surprising lack of toxicity in galactose time constraints, this experiment has not yet been repeated. control for the Wester did not appear to have any accumulation (Fig. 3B). The aggregation (Fig. 3A). We found a possible increase in localization or aggregation when compared to BY4741 (Fig. 3A). A30P and E46K did not show any changes in localization of α-synuclein in WT and both familial mutants. Each showed a mild increase in cytoplasmic diffuseness of α-synuclein (compare Fig. 4A to 2A). Similar to atg11Δ, we saw an increase in accumulation of α-synuclein in E46K but not in WT or A30P (Fig. 4B). We then assessed toxicity with serial dilution spotting and saw no α-synuclein toxicity in galactose-induced cells compared to non-induced cells (Fig. 4C). Growth curves of vam3Δ confirmed the lack of α-synuclein toxicity (Fig. 4D).

Absence of VAM7 Changes Localization of α-Synuclein
To further understand the fusion step, we assessed a second gene, VAM7, in each of α-synuclein’s three PD-related properties. We compared vam3Δ results to both BY4741 and the other compromised fusion cells, vam3Δ. When compared to BY4741, vam7Δ showed significant changes in localization and aggregation of α-synuclein. WT and E46K both had stronger cytoplasmic diffusion of α-synuclein while A30P had increased aggregation (compare Fig. 4A to 2A). These changes in localization and aggregation were significantly different from the other compromised fusion gene, vam3Δ. There was no accumulation of α-synuclein in WT or either familial mutant. Again, this differs from vam3Δ, in which a strong accumulation of E46K was seen (Fig. 4B). Like each of the other knockout strains we assessed, serial dilution spotting of vam7Δ showed no toxicity in galactose-induced cells compared to non-induced cells (Fig. 4C). The lack of toxicity was confirmed by growth curve analysis of vam7Δ (Fig. 4D).

Discussion
Dysfunction in autophagy, a major protein degradation route to the lysosome in our cells, may be responsible for increased protein accumulation that contributes to the pathology of different neurological disorders (Rubinsztejn et al., 2006). The misfolding, aggregation, and eventual accumulation of α-synuclein are likely causes of dopaminergic cell death in PD patients (Lee et al., 2004). By genetically inhibiting the nucleation and fusion steps of autophagy, we hypothesized that autophagy protects cells

### Table 1: Summary of α-synuclein properties in autophagy gene deletion strains

<table>
<thead>
<tr>
<th>Deletion Strain</th>
<th>Increased Aggregation</th>
<th>Increased Accumulation</th>
<th>Increased Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>atg11Δ</td>
<td>strong</td>
<td>strong (E46K)</td>
<td>non-syn dependent</td>
</tr>
<tr>
<td>atg13Δ</td>
<td>weak</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>atg17Δ</td>
<td>none</td>
<td>weak</td>
<td>none</td>
</tr>
<tr>
<td>Vps15Δ</td>
<td>weak</td>
<td>none</td>
<td>non-syn dependent</td>
</tr>
<tr>
<td>atg1Δ</td>
<td>weak</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>atg2Δ</td>
<td>strong</td>
<td>strong (E46K)</td>
<td>none</td>
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<tr>
<td>atg3Δ</td>
<td>weak</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>atg4Δ</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>atg8Δ</td>
<td>weak</td>
<td>none</td>
<td>none</td>
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<tr>
<td>atg18Δ</td>
<td>weak</td>
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</tr>
<tr>
<td>vam3Δ</td>
<td>weak</td>
<td>strong (E46K)</td>
<td>none</td>
</tr>
<tr>
<td>vam7Δ</td>
<td>strong</td>
<td>weak</td>
<td>none</td>
</tr>
</tbody>
</table>

increased accumulation for E46K, but WT and A30P α-synuclein expression was similar to levels in parent cells (Fig. 3B). Serial dilution spotting of atg11Δ demonstrated strong cellular toxicity compared to the parent cells (compare Fig. 3C to Fig. 2C), but this toxicity was not α-synuclein dependent, since GFP and vector cells were also toxic even without galactose induction. This surprising α-synuclein-independent toxicity, in the absence of ATG11, was confirmed in growth curve assessments (Fig. 3D).

Absence of ATG13 Increases Cytoplasmic Diffusion of WT α-Synuclein
Our next step was to compromise another nucleation gene, ATG13, to understand how other nucleation genes contributed to each of α-synuclein’s three PD-related properties. When we compromised nucleation with atg13Δ cells, we saw striking differences from the other nucleation gene, atg11Δ. Compared to BY4741 parent cells, atg13Δ showed mild changes in localization and aggregation in WT α-synuclein. Instead of synuclein being cytoplasmically diffuse, we saw intracellular aggregates form over 48 hours (Fig. 3A). A30P and E46K did not show any changes in localization or aggregation when compared to BY4741 (compare Fig. 2A to 3A). Compared to atg11Δ, atg13Δ showed strikingly less changes in localization and aggregation (Fig. 3A). We found a possible increase in accumulation of α-synuclein in E46K, while WT and A30P did not appear to have any accumulation (Fig. 3B). The control for the Western analysis did not develop, and due to time constraints, this experiment has not yet been repeated. Serial dilution spotting of atg13Δ did not show α-synuclein toxicity in galactose-induced cells compared to non-induced cells (Fig 3C). The surprising lack of toxicity seen in atg11Δ was also seen in atg13Δ cells. This lack of toxicity was confirmed by the assessment of atg13Δ growth curves (Fig. 3D).

Absence of VAM3 Alters Localization and Increases Accumulation
We then looked into compromising the less-studied fusion step of autophagy. The first gene we assessed was VAM3. Compared to BY4741, vam3Δ had mild changes in the localization of α-synuclein in WT and both familial mutants. Each showed a mild increase in cytoplasmic diffuseness of α-synuclein (compare Fig. 4A to 2A). Similar to atg11Δ, we saw an increase in accumulation of α-synuclein in E46K but not in WT or A30P (Fig. 4B). We then assessed toxicity with serial dilution spotting and saw no α-synuclein toxicity in galactose-induced cells compared to non-induced cells (Fig. 4C). Growth curves of vam3Δ confirmed the lack of α-synuclein toxicity (Fig. 4D).

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<td>vam7Δ</td>
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against α-synuclein toxicity. Here we report four notable findings: First and foremost, we are among the first to report that the autophagy fusion step genes can regulate several of α-synuclein’s PD-related properties. Secondly, compromised autophagy consistently alters α-synuclein localization (sometimes by enhancing aggregation). Thirdly, compromised autophagy only modestly increases accumulation. Lastly, inhibiting autophagy leads to a surprising lack of toxicity. These new results support past data from our lab on the analysis of several other autophagy gene deletions (Ray Choi, Lake Forest College senior thesis, 2009; see Table 1). Together, they provide accumulating evidence that autophagy is a likely route for α-synuclein degradation.

Fusion Step in Autophagy Regulates α-Synuclein
In strong support of our hypothesis, our first notable finding was that fusion genes regulate α-synuclein, as vam3Δ and vam7Δ affected both α-synuclein localization and accumulation. Previously, only nucleation and expansion step genes were studied and shown to alter α-synuclein cellular localization (Ray Choi, Lake Forest College senior thesis, 2009). We do not know of a published report that has evaluated fusion genes in regulating α-synuclein in any model organism. This finding is important because it shows that all steps of autophagy are likely interacting with α-synuclein, making a strong case that it is a bona fide substrate for the autophagy pathway. Fusion step genes (and the formation of autophagosomes) have been implicated in other human diseases (Toddle et al., 2009) suggesting the general importance of this step in cellular function. One of the four fusion genes (YKT6 in yeast) serves in lysosome trafficking and neuronal function (Hasegawa et al., 2003). Therefore, it will be important to extend our analysis to the rest of the fusion genes.

Compromised Autophagy Subtly Alters α-Synuclein Mislocalization and Aggregation
Also in strong support of our hypothesis, our second notable finding was that α-synuclein localization changes (atg11Δ and vam7Δ) and intracellular aggregates form (atg11Δ) in cells with compromised autophagy. Similarly, Ray Choi found a variety of localization changes and a strong increase in aggregation in atg2Δ (see Table 1). Other lab colleagues studying endocytosis, Alex Ayala and Jaime Perez, found altered localization in a majority of the strains they analyzed, which makes both autophagy and endocytosis candidates for α-synuclein degradation.

Deleting atg11Δ previously resulted in a severe defect in Cvt vesicle formation (Shintanti et al., 2004). Since the Cvt complex is surrounded by the autophagosome, which ultimately fuses with the lysosome to degrade proteins, it is possible that any deficiency in the Cvt pathway could affect the ability to degrade α-synuclein (Baba et al., 1997). Another study showed the accumulation of polyQ40 aggregates, which are found in cells of patients with Huntington’s disease, in C. elegans muscle cells and the subsequent muscle dysfunction by inactivating autophagy genes (orthologs of yeast ATG6, ATG7 and ATG18) (Jia et al., 2007).

Weak α-Synuclein Accumulation in Deficient Autophagy
Our third finding of weak α-synuclein accumulation supported our hypothesis to a lesser extent than mislocalization and aggregation. Only the E46K strains of atg11Δ, atg13Δ and vam3Δ showed increased accumulation, although the results of atg13Δ have not yet been repeated due to time constraints. Previous work by Ray Choi shows similar results with the six knockout strains he assessed. Of these six previously studied strains, only vps15Δ and E46K of atg2Δ showed any increase in α-synuclein accumulation (Table 1). These effects may have been milder than expected, because the specific gene knockouts may not be crucial to α-synuclein degradation. Not all genes are necessary for autophagy to function (Suzuki and Ohsumi, 2007). While ATG13, VAM3, and VAM7 are required for autophagy (Kabeya et al., 2005; Klionsky and Emr, 2000), ATG11 is not. It is, however, required for Cvt pathways (Stromhaug et al., 2004). This emphasizes that each gene has a different function, some of which may not be necessary for degrading α-synuclein, resulting in the mild effects that we saw in accumulation. However, in relation to Huntington’s disease, a previous study showed that mice with ATG5 and ATG7 knockouts have increased polyubiquitinated protein accumulation in neurons and developed neuronal degeneration (Hara et al., 2006).

Surprising Lack of Toxicity
We saw a surprising lack of toxicity in all the strains assessed. The only toxicity seen was in atg11Δ, yet this toxicity was not α-synuclein dependent, as we observed that both α-synuclein expressing and non-expressing cells showed similar toxicity. This was not in accordance with our hypothesis but was similar to the results previously found by Ray Choi, which also show α-synuclein dependent toxicity.

One explanation for the lack of toxicity is that another pathway is compensating for the inhibition of the autophagy pathway to the lysosome. Either the proteasome (Webb et al., 2003) or the CMA pathway (Kaushik et al., 2008) can be upregulated with the inhibition of autophagy. A recent study showed that the inhibition of autophagy produces an increase in ubiquitinated proteins suggesting that the proteasomal pathway is capable of compensating for impaired autophagy (Venkatachalam, K. et al., 2008). These compensatory routes may be enough to prevent toxicity from being expressed to the extent expected.

Other factors may compensate for compromised autophagy, including the physical traits of yeast and other genes. Yeast adapt to several stressful conditions, such as different temperatures, oxidative conditions, and pH levels when used to produce beer (Gibson et al., 2007). This survival skill may also be present in autophagy deficient yeast cells. Moreover, it is possible that another gene is compensating for the knocked out gene or that multiple genes must be knocked out to produce toxicity. In addition, our model has little to moderate levels of α-synuclein, which may explain the small amount of toxicity. In the Lindquist lab, however, a moderate to high level of α-synuclein produces cellular toxicity (Outeiro & Lindquist, 2003). Thus, we may have to increase α-synuclein expression levels to see any toxicity is our model.

Lastly, previous studies in our lab on oxidative stress and the proteasome have shown clear α-synuclein related toxicity in several strains (Sharma et al, 2006; Alex Ayala, Lake Forest College senior thesis, 2009; Jaime Perez, Lake Forest College senior thesis, 2010). Thus, we cannot completely disregard toxicity related to α-synuclein expression.

Future Studies
Our next goal is to assess all remaining nucleation, expansion, and fusion genes. We can also examine the induction of autophagy in which we would predict to see the opposite results as originally hypothesized for compromising autophagy. It is not until we complete these experiments that we can come to a complete conclusion of α-synuclein degradation via autophagy.
Conclusion

Our research aimed to uncover the possible link between autophagy and α-synuclein related PD pathogenesis. Most notably, we are among the first to analyze the fusion genes and report that these genes affect α-synuclein properties. In addition, we found a surprising lack of toxicity and subtle changes in α-synuclein localization and accumulation. With incomplete analyses and future experiments, however, we cannot establish whether or not autophagy plays a role in α-synuclein degradation.

Revealing a definite connection between autophagy and PD gives hope for PD treatment. Scientists hypothesize that increasing autophagy may decrease protein aggregates in Huntington's disease, which is pathologically similar to PD (Ravikumar et al., 2004; Sarkar et al., 2007). If similarly applied to PD patients, the potential to ameliorate the devastating effects of this disease may drastically increase.

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