Insight Into Parkinson’s Disease: Does α-Synuclein Use the MVB/Endocytosis Pathway as a Route for Degradation in the Lysosome?

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Abstract

Parkinson’s disease is a fatal and incurable human neurodegenerative disorder that destroys midbrain neurons. The misfolding, accumulation, and aggregation of the protein α-synuclein is thought to kill these cells. Enhancing α-synuclein degradation may help prevent its accumulation and aggregation, while protecting cells against toxicity. For this thesis, we used the model organism budding yeast to evaluate the hypothesis that α-synuclein is degraded by the cellular organelle lysosome via a specific route: the MVB/endocytosis pathway. Specifically, we evaluated whether three disease-related properties of α-synuclein (aggregation, accumulation, and toxicity) worsened in yeast strains that were individually deleted for genes coding for proteins required for the MVB/endocytosis pathway. In support of our hypothesis, each gene deletion altered one or more α-synuclein properties. While our data indicates that the MVB pathway is a route for α-synuclein degradation, the specificity and extent of α-synuclein involvement with proteins within the ESCRT complexes appears unexpectedly complex.

Introduction

Neurodegenerative Diseases

The human brain is considered the most extraordinarily complex organ to have evolved in all living organisms. It is thought to control all behaviors, from simple movements and sensations to attributes as complex as emotions, language, culture, and personality. Due to the brain’s complexity, it is logical that the malfunction of its neurons leads to tragic illnesses, including addiction, depression, and schizophrenia. Among such brain disorders, neurodegenerative diseases are a specific family of devastating and fatal ailments that result from the progressive death of specific neurons. There is no cure for these diseases, and they affect millions of people worldwide. Sadly, the longer we live the more these diseases become a burden on society’s healthcare and economy.

The major neurodegenerative diseases are Alzheimer’s disease (AD), Huntington disease (HD), prion diseases, Lou Gehrig’s disease (ALS), and Parkinson’s disease (PD). In each disease, a different set of neurons dies interrupting highly specialized networks in the brain controlling unique functions like learning and memory (in AD), initiation and termination of movement (in PD or HD) or muscle control (in ALS). In addition to cell death, the pathogenesis of these neurodegenerative diseases is linked to another common signature: the misfolding of a specific protein unique to each disease (Figure 1A). Such abnormal protein deposits may result in amyloid plaques outside dying cells (AD and prion diseases) or cytoplasmic inclusions inside dying cells (HD and PD; Taylor et al., 2002). Despite substantial progress during the past decade, many biological mysteries linking protein misfolding and cellular death remain unsolved. My thesis focuses on better understanding the molecular basis of one neurodegenerative disease: PD.

Insight into Parkinson’s Disease

Although all neurodegenerative diseases are fatal, Parkinson’s disease is among the most devastating due to its high occurrence in the adult population. In fact, PD is the second most common neurodegenerative disease, affecting over one million people in North America (Greenamyre and Hastings, 2004). PD patients suffer from the selective loss of dopaminergic neurons in a region of the brain known as the substantia nigra. Loss of these specific cells contributes to the onset of PD symptoms, such as resting tremors, slowness in movement, and muscular rigidity (Forno, 1996; Olanow and Tatton, 1999). These neurons form part of the nigrostriatal pathway in the basal ganglia and project from the substantia nigra to the striatum, a structure that controls timing and coordination of movement. Additionally, neurons in this region synthesize the neurotransmitter dopamine, which controls signaling in the basal ganglia. Thus, loss of dopamine production results in motor deficiency (Figure 1B).

As a member of the neurodegenerative disease family, PD is characterized not only by cell death, but also by the presence of protein inclusions in the dying neurons that control movement. Post mortem examination of PD brains reveals the presence of aggregates composed mainly of two proteins, α-synuclein and ubiquitin. These deposits, termed Lewy bodies, are found in both sporadic and familial cases of PD (Lücking and Brice, 2000; Spillantini et al., 1998). Over ninety percent of PD cases are sporadic. This form of PD is commonly linked to the misfolding of normal α-synuclein, however, its cause is not well understood. Furthermore, some sporadic cases are caused by environmental toxins, such as MPTP and pesticides (Langston et al., 1983; Dawson and Dawson, 2003; Greenamyre and Hastings, 2004).

Alternatively, a second form of PD known as familial PD, is inherited due to its genetic component. Unlike sporadic cases, aggregates in familial PD contain one of three mutant α-synuclein proteins caused by point mutations in the α-synuclein gene (A30P, A53T, or E46K; Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004). Furthermore, familial cases of PD have also been linked to mutations in five other genes. These mutations have served as genetic tools that provide evidence that oxidative stress and proteasomal dysfunction are linked to PD pathogenesis. The role of mitochondrial oxidative stress is supported by mutations in the DJ-1 protein, and the mitochondrial protein and associated protein PINK1 and LRRK2 (Bonifati et al., 2003; Paisan-Ruiz et al., 2004; Valente et al., 2004; Zimprich et al., 2004). Studies concerning mutations in ubiquitin proteasome system (UPS) proteins UCH-L1 and parkin support the proteasomal dysfunction hypothesis (Oda et al., 1998; Funayama et al., 2002). The study of these five mutations provides insight into the possible mechanisms underlying sporadic PD. More specifically, scientists believe that oxidative stress and/or an impaired ubiquitin-proteasome system could also contribute to sporadic forms of PD. However, given the invariant

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A. Neurodegenerative diseases are characterized by cell death; however, they are also characterized by a common problem: protein misfolding. Each disease has a culprit protein that misfolds and accumulates. Parkinson’s Disease Pathway. The motor cortex controls movement of our muscles, and the basal ganglia controls timing and coordination of that movement. In PD, dopaminergic neurons in the substantia nigra (SNpc) die (X). Death on these neurons leads to dopamine deficiency. A decline in dopamine levels interrupts the nigrostriatal pathway causing less voluntary movement. What is α-Synuclein? The culprit protein in all forms of PD is α-synuclein. This protein, which is found to aggregate in all PD patients, is 140 amino acids in length and is commonly found in presynaptic terminals of substantia nigra neurons (Maroteaux et al., 1988; Kaplan et al., 2003). Although its function is unknown, α-synuclein colocalizes with synaptic vesicles and is thought to regulate vesicular transport (Cabin et al., 2002; Outeria and Lindquist, 2003). As previously mentioned, α-synuclein misfolding can result from genetic mutations or a random event in the normal α-synuclein protein leading to the formation of Lewy bodies. In vitro studies show that both
wild-type and mutant α-synuclein are capable of self-fibrillation. However, mutant forms of this protein are more likely to aggregate (Conway et al., 1998; Glasson et al., 1999; Conway et al., 2000). Fibers form much more quickly if preformed proteins fibers are present. Furthermore, this model accounts for the protein misfolding observed in other neurodegenerative diseases, such as α-synuclein in Alzheimer’s disease, huntingtin in Huntington’s disease, and prion proteins in Creutzfeldt-Jacob disease (Caughey and Lansbury, 2003; Perutz and Windle, 2001). We are thus left with the question, does protein misfolding play a role in disease pathogenesis?

In addition to forming aggregates, α-synuclein tends to interact with phospholipid membranes and, like other phospholipid-binding proteins, it contains a cytosolic fatty acid binding signature (Sharon et al., 2001). Upon interaction with lipids, α-synuclein which is normally unfolded in cells, adopts a helical conformation (Eleizer et al., 2001). Both interactions, transmembrane, in vitro studies reveal α-synuclein’s phospholipid and vesicle binding characteristics (Davidson et al., 1998; Jo et al., 2000; Eleizer et al., 2001). Furthermore, membrane-localized α-synuclein is observed in rat and budding yeast models (Maroteaux and Scheller, 1991; Outorio and Lindquist, 2003; Dixon et al., 2005; Sharma et al., 2006). In the past, studies have shown that both α-synuclein aggregation and membrane-binding properties, and their role in PD pathogenesis, remains an unsolved mystery.

Throughout the past decade extensive work focused on characterizing the mutant forms of α-synuclein. Similarly to wild-type (WT) α-synuclein, familial mutants A53T and E46K localize to the plasma membrane in yeast (Outorio and Linquist, 2003; Dixon et al., 2005; Sharma et al., 2006; White thesis, 2007). In contrast, A30P has reduced membrane localization and is found diffuse throughout the cytoplasm (Dixon et al., 2005; Sharma et al., 2006). Overexpression of WT and A30T α-synuclein is toxic to yeast cells. However, whereas A53T α-synuclein transgenic mice form neuronal inclusions leading to the paralysis and death, WT α-synuclein transgenic mice remain unaffected (Outorio and Linquist, 2003; Dixon et al., 2005; Glasson et al., 2003). The last familial mutant, A30P, generates only subtle toxicity in yeast (Outorio and Linquist, 2003; Dixon et al., 2005).

The Five Conundrums

Although the past decade celebrated many advances in the field, five key questions concerning the molecular basis of PD remain unanswered. Firstly, it is still unclear whether α-synuclein is implicated in PD pathogenesis. Secondly, the mechanism regulating α-synuclein misfolding and aggregation is not well understood. Furthermore, if α-synuclein is the causative agent in PD, then its toxic form, whether membrane-bound or aggregated, remains unclear. Thirdly, we are unaware of the specific relationship between the ubiquitin-proteasome (UPS) degradation pathway and the α-synuclein. It is unknown whether UPS deficiency leads to α-synuclein aggregation or if α-synuclein impedes UPS function in PD patients. Fourthly, further research is needed to elucidate the role of oxidative stress in PD pathogenesis. Finally, all routes for α-synuclein degradation have not yet been established. Since α-synuclein is present in all forms of PD, understanding its degradation pathway could shed light on how to enhance its removal, which may be a possible target for therapy. This last question will be the focus of my thesis. More specifically, I investigated the MVB/endocytosis pathway as an alternative route for α-synuclein degradation.

Cells must remove damaged or aged proteins to maintain proper function. In cells, degradation in the endocytotic route leads to the removal of endocytosed proteins, which may be a possible target for therapy. This last question will be the focus of my thesis. More specifically, I investigated the MVB/endocytosis pathway as an alternative route for α-synuclein degradation.

Proteasome: A Known Route for α-Synuclein Degradation

For the past fifteen years, studies suggested that α-synuclein was degraded strictly by the proteasome. This assumption was supported primarily by mutations in two genes encoding proteins involved in the ubiquitin-proteasome system: UCHL1 and parkin. Mutations in either of these genes are known to cause familial PD. UCHL1 is a proteolytic enzyme, and its activity inhibits α-synuclein degradation (Funayama et al., 2002; Liu et al., 2002). Parkin is an E3 ubiquitin ligase essential for the attachment of ubiquitin to damaged proteins, signaling their degradation (Kitada et al., 1996). More importantly, mutations in the parkin gene are toxic to neurons in the substantia nigra. Additionally, the neurons expressing mutant forms of parkin accumulate non-ubiquinated α-synuclein (Giasson and Goedert, 2001). These observations suggest that parkin is needed to add ubiquitin molecules to α-synuclein in order for α-synuclein’s degradation to occur.

Another line of evidence for α-synuclein degradation via the proteasome lies in in vitro and in vivo studies, which demonstrate that pharmacological inhibition of the proteasome leads to the selective loss of dopaminergic neurons in mice and the death of rat adrenal medulla cells (Nair et al., 2006; Rideout et al., 2002). Furthermore, inhibition of proteolytic activity in 26/20S proteasomes and UCHL1 induces α-synuclein aggregation and toxicity (Mytilineou et al., 2004). Proteasome inhibition in mice increases cytoplasmic p53, a protein involved in apoptotic signaling. This suggests that the p53 pathway may be relevant to the neurodegeneration seen in PD patients (Nair et al., 2006). Also, rat adrenal medulla cells expressing A53T α-synuclein show diminished proteasomal activity, and symptomatic α-synuclein transgenic mice express similar deficiencies in the UPS as patients with sporadic forms of PD (Goedert, 2001; Stefani et al., 2001; Chen et al., 2006). Proteasomal defects in A53T-expressing cells are possibly explained by the early appearance of mutant α-synuclein inclusions.

Lysosome: An Alternative Route for α-Synuclein Degradation

Until recently, the proteasome was thought to be the only site for α-synuclein turnover. However, mounting evidence suggests that the lysosome is also responsible for α-synuclein degradation. Damaged proteins take one of two routes when targeted for lysosomal degradation: autophagy or endocytosis. Furthermore, a biosynthetic route known as the cytoplasm-to-lysosome pathway is also capable of transporting cytosolic proteins, such as Aminopeptidase I (API), to the lysosome for degradation (Scott and Klionsky, 1995; Scott et al., 1996; Figure 3A).

Some of the first studies that contribute to the idea that the lysosome is a site for α-synuclein degradation were conducted by Cuervo et al. (2004). Cuervo and his
DNA molecules code for specific amino acid sequences that determine the shape of all proteins found in our cells. Usually proteins fold into their correct structure and help maintain order in cells. However, sometimes proteins misfold and are a threat to the cell. Although normal proteins can misfold, this is more common when a mutation is present. Misfolded proteins can be degraded by the lysosome or the proteasome. Damaged proteins found in the cytoplasm or the nucleus are degraded by the proteasome, whereas extracellular or membrane proteins are degraded by the lysosome. Unfortunately, when misfolded proteins are unable to be degraded they may aggregate and accumulate and in many cases cause cell death. This can also occur if the proteasome and/or lysosome are compromised.

The aforementioned studies reinforce the notion that the lysosome is a site for α-synuclein degradation. However, evidence was primarily focused on chaperone-mediated autophagy; in fact, research in the field neglected the possibility that the second route, the endocytosis/lysosome pathway, could also mediate α-synuclein degradation. The endocytosis pathway is relevant because a genetic screen in yeast revealed that specific knockouts of essential endocytotic proteins increase α-synuclein toxicity (Willingham et al., 2003). Three out of the 86 genes found to be toxic encoded vesicular transporting proteins (vps) vps24, vps26, and vps28. Similarly, recent studies provide evidence, which suggests that deficient endocytosis is linked to PD pathogenesis (Kuwahara et al., 2008). They found that the loss of four endocytosis-related genes (pa-2, aps-2, eps-8, rab-7) caused neurons to accumulate α-synuclein in C. elegans. Furthermore, these neurons had impaired neuromuscular transmission causing the worms to exhibit motor impairments.

**Multivesicular Body/Endosome Pathway**

Proteins transport within the endocytosis pathway depends on the formation of multivesicular bodies (MVB). Multivesicular bodies are essential compartmentalizing vesicles that have multiple functions within a cell. Studies in Drosophila and C. elegans demonstrate the role of MVB in regulating development and neuronal differentiation (Artavanis-Tsakonas et al., 1999; Berset et al., 2001). The study of the MVB pathway is also important for understanding other related mechanisms, such as budding of viruses. This is of importance because HIV-1, for example, follows a process of the MVB pathway (Strack et al., 2000).

Endocytosis is a route taken by transmembrane proteins to reach the lysosome for degradation. Many proteins have cytoplasmic domains that mediate their ability to be removed by endocytosis. For example, epidermal growth factor receptor (EGFR), a well-characterized substrate for this pathway, contains such a domain. However, a mutation in the domain inhibits proper endocytosis and ultimately, degradation of the protein (Felder et al., 1990). In addition, yeast proteins Ste3, Gap1, Tat2t and mammalian proteins GHR, MHCI, E-Cadherin are tagged with ubiquitin and transported to the lysosome by the MVB sorting pathway (Kutzmann et al., 2002). Lysosomal degradation of Ste2p, also a cell surface protein, in
Figure 3: MVB/Endocytosis pathway

A. Two major and one minor routes for lysosome degradation:
(I) Endocytosis: Plasma membranes invaginate to isolate damaged or old membrane proteins in a vesicle called an endosome. Proteins are then sorted into compartments within the endosome to create a multivesicular body. Fusion between multivesicular bodies and the lysosome is the last step to transport the protein into the lysosome for its degradation. (II) Autophagy: Damaged proteins in the cytoplasm are engulfed by an autophagosome, which is then transported to the lysosome. Fusion between the two allows the damaged protein to be transported into the lysosome. (III) Cytoplasm-to-vacuole (Ctv): This is a biosynthetic pathway similar to autophagy. Damaged enzymes in the cytoplasm are engulfed by a membrane creating a vesicle to transport the enzyme to the lysosome for degradation.

B. Early endosomes recruit ESCRT-I (vps28, vps23, vps32, mvb12), ESCRT-II (vps25, vps36, vps22), and ESCRT-III (vps24, vps20, vps2, vps32) complexes to their membrane. The damaged protein (cargo) binds to ESCRT-I and is transported from one complex to the next (following the arrows) until it is ready to be engulfed by the endosome membrane. Once in a separate compartment within endosome the structure is a multivesicular body (MVB). MVBs then fuse with the lysosome.

S. cerevisiae was the first to be studied closely to monitor the role of ubiquitin in this pathway. In order for proteins to be correctly sorted into the lysosome, they must first be tagged with ubiquitin. Endocytosis initiates after the cytoplasmic domain of transmembrane proteins undergo monoubiquitylation (Hicke, 2001; Hicke and Dunn, 2003; Strous et al., 1996). Membranes containing ubiquitin-tagged proteins will create early endosomes by invagination. However, if all Ste2 lysine residues are mutated, ubiquitin cannot bind to it, and the protein is not degraded (Terrell et al., 1998).

Sorting of proteins into multivesicular bodies (MVB) is a complex process facilitated by the interactions between many endosomal sorting complexes required for transport (ESCRT). These sorting complexes are found on the MVB membrane and consist of vacuolar protein sorting (vps) proteins, which help transport the damaged cargo across the MVB membrane. Each complex consists of specialized class E vacuolar (vps) proteins. ESCRT-I is made up of vps37, vps28, vps23 and mvb12 (Alam and Sundquist, 2007). Of these four proteins vps28 physically interacts with ESCRT-II, which consists of vps22, vps36 and two vps25 subunits (Babst et al., 2002; Hierro et al., 2004). Finally, ESCRT-III is made up of vps2, vps20, vps24 and vps32. ESCRT-II and ESCRT-III physically interact via the binding between vps25 and vps20 (Babst et al 2002; Hurley, 2006). Although, studies were performed in a simple, unicellular fungus, other research suggests that the pathway is highly conserved between yeast and humans (Katzmann et al., 2001).

These vps proteins have numerous interactions with each other. Studies using budding yeast as a model show that vps27 is recruited by vps24 on the endosome membrane where it binds to the ubiquitinated cargo. Simultaneously, vps27 recruits ESCRT-I (Piper et al., 1995; Katzmann et al., 2001).
Katzmann et al., 2003). Interaction between monoubiquitinated cargo and ESCRT-I will recruit ESCRT-II and ESCRT-III to the membrane, recruiting all three ESCRT complexes enables the monoubiquitinated cargo to be transported across the membrane so that it can then invaginate and deposit the cargo into vesicles within the endosome’s lumen (Babts et al., 2002). Damaged cargo is sorted into compartments within the endosome, which give rise to the multivesicular body. This MVB then fuses with the lysosome and deposits its contents allowing for cargo proteolysis. All substrates of the MVB/endoctosis pathway take the same route. However, our question is specific to one possible substrate: α-synuclein.

Gap in Knowledge: What We Still Do Not Know
Although past evidence for lysosomal degradation of α-synuclein exists, research has been limited to only pharmacological studies (Oestreich et al., 2007; Lee et al., 2004). Furthermore, proteosomal degradation of α-synuclein, a well studied route, is supported by pharmacological, genetic, and biochemical evidence. Thus, in order to recognize the lysosome as a site for α-synuclein degradation genetic and biochemical studies are also needed. Until now, only one study showed α-synuclein toxicity in endocytosis-deficient strains (Willingham et al., 2003). Further, direct confirmation of the toxicity observed is still needed. Furthermore, not all PD-linked α-synuclein properties, such as localization and accumulation, were previously evaluated. In addition, whether MVB/endoctosis protein complexes are utilized similarly by all protein substrates is poorly analyzed. Lastly, if α-synuclein uses this pathway, it is unknown whether all protein or protein complexes will be critical. My hypothesis is that the MVB/endoctosis pathway is a route for α-synuclein degradation by the lysosome. To test this hypothesis, I selected two genes from each ESCRT complex. For ESCRT-I, I chose to evaluate the role of vps28 because its elimination was previously shown to be toxic (Willingham et al., 2003) and mvb12 because it was recently discovered at the time I began my research (Oestreich et al., 2007). For ESCRT-II, vps25 and vps36 were chosen randomly. Lastly, for ESCRT-III I chose to evaluate vps24 because a deletion was shown to be toxic (Willingham et al., 2003) and vps20 was chosen randomly. I predicted that deletion of any one of these genes would increase α-synuclein toxicity and change its localization (Figure 4). To evaluate the role of all six proteins I performed the following aims:

Aim 1: To compromise ESCRT-I genes (vps28 and mvb12) in budding yeast and determine whether α-synuclein accumulated, changed localization, and/or induced toxicity.

Aim 2: To compromise ESCRT-II genes (vps25 and vps36) in budding yeast and determine whether α-synuclein accumulated, changed localization, and/or induced toxicity.

Aim 3: To compromise ESCRT-III genes (vps24 and vps20) in budding yeast, and determine whether α-synuclein accumulated, changed localization, and/or induced toxicity.

Aim 4: This aim evaluated whether the correct amino acid glycine would alter α-synuclein properties in parent or MVB pathway compromised strains (vps28Δ, vps25Δ, vps24Δ). The following sections include methods and materials, results, discussion, and conclusions of the studies that address the above aims.

Results
Experimental set up: The role of the MVB/endoctosis pathway in α-synuclein regulation was examined in budding yeast strains lacking specific genes that encode protein complexes ESCRT-I, II, or III. Specifically, for each ESCRT complex, two knockout strains were analyzed and compared to the isogenic parent strain BY4741, vps28Δ and mvb12Δ for ESCRT-I, vps25Δ and vps36Δ for ESCRT-II, and vps20Δ and vps24Δ for ESCRT-III. These seven yeast strains were transformed with five plasmid expression vectors. Two served as controls (pYES2 parent vector and GFP vector) used to corroborate that the vector alone and the addition of the GFP protein was not detrimental to the yeast strains. The three remaining vectors consisted of WT α-synuclein and α-synuclein familial mutants A30P and E46K. All α-synuclein variants were tagged with GFP at the C-terminus. Four assays were used to assess PD-related α-synuclein properties. Toxicity and growth patterns were assessed by optical density growth curves and serial dilution spotting, localization was visualized by live cell fluorescence microscopy, and aggregation and accumulation was observed by Western blotting.
Figure 4: Hypothesis for altered α-synuclein properties in yeast with compromised MVB. A. Yeast with intact MVB/endocytosis sorting pathways will be healthy and α-synuclein bind the membrane. B. Yeast with compromised MVB/endocytosis pathways (vps knockouts) will form aggregates and bind less to the membrane. Additionally α-synuclein will be toxic to cells. C. Table showing the genes that make ESCRT-I, II and III. The genes examined in this study have a checkmark.

α-Synuclein is Membrane-Bound and Non-Toxic in Parent Strain BY4741
First, α-synuclein was examined in parent strain BY4741, which served as a comparative control for all subsequent strains. The following PD-related α-synuclein properties noted confirm the published findings in the field and in our lab (Outerio and Lindquist, 2003; Sharma et al., 2007; M. White thesis 2007): 1) neither WT nor familial mutants A30P and E46K were toxic (Figure 5A & B). 2) WT and E46K α-synuclein are primarily plasma membrane bound, while A30P was predominantly diffuse throughout the cytoplasm (Figure 5D & E). 3) Protein expression was similar for all α-synuclein expressing cells (Figure C).

α-Synuclein is Toxic to ESCRT-I Deficient Strain vps28Δ
Next, we evaluated the effects of α-synuclein in ESCRT-I compromised yeast vps28Δ. Compared to pYES2 and GFP growth curves, WT, A30P, and E46K cells showed delayed growth in vps28Δ cells (Figure 6A). This α-synuclein-dependent toxicity was confirmed by spotting analysis (Figure 6B). Importantly, α-synuclein-expressing vps28Δ cells exhibited compromised growth compared to parent strain BY4741 (compare Figure 5A to Figure 6A). These findings are in support of our hypothesis. Although WT and E46K α-synuclein localization in vps28Δ cells did not shift dramatically compared to BY4741, weakened membrane affinity and increased cytoplasmic localization was observed.
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Table 1. List of Transformed Budding Yeast Strains
during early expression. Over time, however, all α-synuclein localization patterns resembled those of parent strain BY4741 (compare Figure 5D & E to Figure 6D & E). Finally, contrary to what we hypothesized, no increase in the α-synuclein expression in vps28Δ was seen compared to parent strain BY4741 levels (compare Figure 5C to Figure 6C). Therefore, the vps28Δ toxicity was best correlated with changes in α-synuclein localization.

Second ESCRT-I Strain mvb12Δ Enhances α-Synuclein Accumulation

A second ESCRT-I knockout strain, mvb12Δ, was evaluated to further elucidate what was seen in vps28Δ and the role of this first complex in the regulation of α-synuclein. Surprisingly, while cells expressing α-synuclein grew equal to pYES2-GFP expressing cells (compare Figure 5A & B to Figure 7A & B), fluorescence microscopy revealed subtle shifts in WT expression in mvb12Δ compared to parent strain BY4741 (compare Figure 5C to Figure 7C). Therefore, while both ESCRT-I proteins affected α-synuclein, they affected different properties.

ESCRT-II vps25Δ Strain has Subtle Effects on α-Synuclein Localization

We then evaluated the role of ESCRT-II in the regulation α-synuclein. Cells expressing α-synuclein in vps25Δ grew similarly to pYES2 and GFP cells and to parent strain BY4741 (compare Figure 5A & B to Figure 8A & B). Fluorescence microscopy revealed subtle shifts in WT and E46K α-synuclein localization from primarily membrane-bound in parent strain BY4741 to weak membrane-bound and diffuse in vps25Δ cells. A30P localization remained unchanged (compare Figure 5D & E to Figure 8D & E). Lastly, α-synuclein expression was similar in vps25Δ and parent strain BY4741 (compare Figure 5C to Figure 8C). Therefore, only one α-synuclein property was subtly affected by vps25Δ.

α-Synuclein Shifts Localization and Accumulates in ESCRT-II vps36Δ

The second ESCRT-II gene examined was vps36Δ. All vps36Δ cells, including α-synuclein expressing and non-expressing cells, had similar growth patterns (Figure 9A & B). Not surprisingly, all vps36Δ cells had delayed growth compared to parent strain BY4741 due to their decrease utilization of a carbon source. What was surprising, however, was that irrespective of α-synuclein expression the delay in growth was equal for all vps36Δ cells (compare Figure 5A & B to Figure 9A & B). Compared to BY4741, early WT and E46K expression shifted from their predominantly plasma membrane-bound localization to a variety of different patterns in vps36Δ cells. Phenotypes observed included membrane-bound with aggregates, punctate foci, and intracellular foci surrounding the vacuole or diffuse throughout the cytoplasm. Over time, however, WT and E46K became predominantly membrane-bound. Although A30P was predominantly diffuse throughout the cytoplasm, in later expressing cells A30P appeared more heterogeneous: as membrane bound with diffuse α-synuclein or punctate foci (compare Figure 5D & E to Figure 9D & E). Additionally, all α-synuclein variants accumulated in vps36Δ (compare Figure 5C to Figure 9C). Therefore, ESCRT-II proteins impacted α-synuclein differently.

ESCRT-III vps20Δ Behaves Similarly to ESCRT-II vps36Δ

Our final goal was to evaluate the role of ESCRT-III proteins in mediating α-synuclein degradation. vps20Δ cells displayed a non-α-synuclein-dependent delay in growth compared to parent strain BY4741, much like ESCRT-II vps36Δ cells (compare Figure 5A & B to Figure 10A & B). This strain also has decreased carbonsource utilization (Cheng et al., 2007). Additionally, WT and E46K localization was predominantly membrane-bound; however, other expression patterns such as membrane-bound with intracellular aggregates surrounding the vacuole and membrane-bound with aggregates were also present during early expression. A30P localization patterns remained unchanged compared to those of parent strain BY4741 (compare Figure 5D & E to Figure 10D & E). As seen in previous strains (mvb12Δ and vps36Δ), α-synuclein also accumulated in vps20Δ (compare Figure 5C to Figure 10C).

Second ESCRT-III Strain vps24Δ is Toxic to A30P and Alters Localization

Unexpectedly, the second ESCRT-III strain vps24Δ did not have similar effects on α-synuclein as the first evaluated strain vps20Δ. Toxicity in this strain was specific to A30P-expressing cells, which had delayed growth compared to parent strain BY4741. WT and E46K-expressing cells grew similarly to pYES2, GFP, and parent strain BY4741 (compare Figure 5A & B to Figure 11A & B). Furthermore, WT and E46K shifted from their membrane-bound localization to a strong expression within the cytoplasm. A30P localization patterns remained unchanged (compare Figure 5D & E to Figure 11D & E). Finally, no accumulation was seen compared to parent strain BY4741 (compare Figure 5C to Figure 11C). Therefore, both ESCRT-III proteins play a role in all three PD-related α-synuclein properties.

The Role of α-Synuclein’s Last Amino Acid

Last summer, we realized that our budding yeast were transformed with α-synuclein’s expressing vector containing a point mutation in its last amino acid. This unexpected error was due to PCR-based subcloning that utilized a faulty primer sequence. The mutation consisted of an alanine to glycine switch in the 140th amino acid. After this discovery, we immediately corrected the amino acid sequence and re-transformed all the budding yeast strains with the corrected sequence. Prior to realizing this error, however, many ESCRT knockout strains had been evaluated: vps28Δ and mvb12Δ for ESCRT-I, vps25Δ for ESCRT-II, and vps24Δ for ESCRT-III. This unexpected error, however, provided us with a tool to examine the importance of the 140th amino acid in α-synuclein properties and PD.

All three strains between α-synuclein-Glycine140 and α-synuclein-Alanine140 revealed no changes between the vast majority of yeast strains examined. A small set of strains did, however, exhibit subtle differences between the two α-synuclein sequences. The observed findings are summarized below:

1. Growth curves were similar for α-synuclein-Alanine140 and α-synuclein-Glycine140 mvb12Δ and vps25Δ. A difference was observed in vps28Δ cells where α-synuclein-Alanine140 was toxic, whereas α-synuclein-Glycine140 was not. The only change in vps24Δ cells was the observation of A30P-dependent toxicity in the cells expressing α-synuclein-Alanine140.

2. Localization patterns were mostly consistent between α-synuclein-Glycine140 and α-synuclein-Alanine140.
expressing cells. Differences observed were contained to vps28Δ and vps24Δ cells (compare Figures 2 and 4).

3. Accumulation patterns were also consistent between α-synuclein-Alanine140 and α-synuclein-Glycine140 expressing cells with the exception of parent strain BY4741. In this strain, E46K α-synuclein-Glycine140 expression was never detected, however E46K α-synuclein-Alanine140 was present in all strains (compare Figures 5 to Appendix Figure 1).

Discussion

How α-synuclein is degraded by cells is an unresolved, therapeutically relevant issue for the future treatment of PD. Until recently, most of the studies in the field suggested that α-synuclein was degraded by the proteasome (Rideout et al., 2001; Chen et al., 2006). In recent years, however, pharmacological evidence suggests that α-synuclein can also be degraded by the lysosome (Lee et al., 2004; Cuervo et al., 2004; Willingham et al., 2003; Kuwahara et al., 2008). The goal of my thesis was to provide genetic evidence for the MVB/endocytosis pathway as a regulator of α-synuclein degradation. We previously hypothesized that some budding yeast strains lacking specific ESCRT genes regulated all α-synuclein accumulation and/or shifts in its localization patterns while not all knockout strains had the same effects on α-synuclein accumulation in these strains compared to the overexpression of Ypk9, an uncharacterized yeast gene, a single property while leaving the rest unaffected. In yeast, some ESCRT II is not necessary for the degradation of MVB/endocytosis pathway substrates. Furthermore, ESCRT-II consists of vps22, vps36, and two vps25 proteins; however, interactions are strongest between vps22 and vps36 (Slater et al., 2006). Therefore, ESCRT-II, specifically the vps25, protein is most dispensable in the regulation of mammalian MVB/endocytosis substrates. In other substrates, it is well established that depletion of one protein in the MVB/endocytosis pathway may not be enough to inhibit the degradation of a specific substrate, a phenomenon that may be occurring in our yeast models lacking vps25 and mvb12.

MVB/endocytosis Pathway is Implicated in α-Synuclein Degradation

Our first major finding is that we provide genetic evidence that the genes controlling the MVB/endocytosis pathway regulate PD-related α-synuclein properties. These data provide new and needed evidence to strengthen the notion that the lysosome is a site for α-synuclein degradation. For example, while not all knockout strains had the same effects on α-synuclein, five out of the six strains evaluated caused α-synuclein accumulation and/or shifts in its localization patterns (vps28Δ, mvb12Δ, vps36Δ, vps25Δ, and vps24Δ). On the other hand, only two strains were toxic (vps28Δ and vps24Δ). These findings are in agreement with Willingham et al. (2003; Table 2).

We were somewhat surprised that none of the evaluated ESCRT genes regulated all α-synuclein properties examined (toxicity, accumulation, and localization). However, this tendency to not affect all pathologically-relevant properties has been previously observed. For example, deletion of the heat shock protein Ssa3 is toxic to yeast; this deletion, however, has no effect on α-synuclein accumulation (Flower et al., 2005). Furthermore, overexpression strains also show alterations in a single property while leaving the rest unaffected. In yeast, overexpression of Ypk9, an uncharacterized yeast gene, suppresses α-synuclein-induced toxicity; however, accumulation in these strains is unchanged compared to the control (Gitter et al., 2009). In our lab, compromised oxidative stress response strain sso2Δ shows consistent toxic effects, but no change in localization patterns (Brandis thesis, 2006).

Furthermore, most other labs focus primarily on one α-synuclein property while neglecting to report others (Table 3). For example, Willingham et al. (2003) identified 86 genes that enhanced α-synuclein toxicity when deleted. Toxicity was evaluated exclusively by serial spotting analysis while α-synuclein accumulation and localization were not addressed. Therefore, alteration of one or two properties is not unexpected and is generally found to be sufficient evidence to suggest the role of ESCRT proteins in α-synuclein regulation. Nevertheless, as an experimental standard, our lab investigates all three.

Some ESCRT Genes Subtly Regulate α-Synuclein Properties

Our second notable finding is that not all ESCRT genes appear to regulate α-synuclein to the same extent. ESCRT-II vps25Δ strain had minimal effect on α-synuclein localization, whereas mvb12Δ had a major effect on α-synuclein accumulation compared to parent strain BY4741. In these strains, all other α-synuclein properties remained unchanged. For reasons explained below, we were not surprised by these results.

As shown by Bowers et al. (2005), ESCRT-II-deficient mammalian cells are capable of properly degrading a model substrate protein, EGFR. Specifically, the depletion of vps25 and vps22 has no effect on EGFR down-regulation. Additionally, they found that ESCRT-II is not necessary for the degradation of MVβ/endocytosis pathway substrates. Furthermore, ESCRT-II consists of vps22, vps36, and two vps25 proteins; however, interactions are strongest between vps22 and vps36 (Slater et al., 2006). Therefore, ESCRT-II, specifically the vps25, protein is most dispensable in the regulation of mammalian MVB/endocytosis substrates. In other substrates, it is well established that depletion of one protein in the MVB/endocytosis pathway may not be enough to inhibit the degradation of a specific substrate, a phenomenon that may be occurring in our yeast models lacking vps25 and mvb12.

Understanding Toxic Differences

The third notable finding is that, unexpectedly, most ESCRT knockout strains did not exhibit toxic effects on α-synuclein. In fact, four out of the six strains evaluated did not alter growth patterns compared to parent strain BY4741 (mvb12Δ, vps25Δ, vps36Δ, and vps24Δ). While vps28Δ cells exhibited α-synuclein-dependent toxicity for all α-synuclein variants, vps24Δ was only toxic to A30P expressing cells. After evaluating a list of previously examined knockout yeast strains by our lab, we realized that α-synuclein-dependent toxicity is rarely observed (Table 4A). Furthermore, a genetic screen revealed that only 86 out of approximately 4,000 knockout strains induced α-synuclein toxicity in yeast (Table 4B).

We chose to evaluate A30P toxicity separately because, unlike WT and E46K, this familial mutant has unique properties and is predominantly localized to the cytoplasm (Sharma et al., 2007; Outerio and Lindquist, 2003). Additionally, while overexpression of WT and E46K is toxic to yeast, overexpression of A30P is not (Dixon et al., 2005; Outerio and Lindquist, 2003). Interestingly, Flower et al. (2009) found that YPF1, an essential gene in yeast of unknown function, exclusively suppresses A30P toxicity and facilitates its transport to the vacuole for degradation. This gene had no effect on WT α-synuclein or other familial mutants. Much like WT and E46K, A30P was toxic in vps28Δ; however, it was also toxic in vps24Δ. Therefore, A30P toxicity is dependent upon the manipulation of specific genes. In our study, deletion on vps28 and vps24 was enough to induce A30P toxicity.

Due to similar WT and E46K α-synuclein localization properties, toxicity was evaluated jointly. WT and E46K α- synuclein-expressing cells were toxic in vps28Δ. In addition to
Table 2: Summary of α-synuclein properties in endocytosis-deficient strains. α-Synuclein toxicity, localization, and accumulation were evaluated for all six ESCRT protein knockout strains. Changes were assessed as a strong, weak or none.

<table>
<thead>
<tr>
<th>Knockout Strain</th>
<th>Toxicity</th>
<th>Localization</th>
<th>Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESCRT-I: vps2Δ</td>
<td>Weak</td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>ESCRT-I: mve12Δ</td>
<td>None</td>
<td>None</td>
<td>Weak</td>
</tr>
<tr>
<td>ESCRT-II: vps2Δ</td>
<td>None</td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>ESCRT-II: vps3Δ</td>
<td>None</td>
<td>Weak</td>
<td>Strong</td>
</tr>
<tr>
<td>ESCRT-III: vps2Δ</td>
<td>None</td>
<td>Weak</td>
<td>Strong</td>
</tr>
<tr>
<td>ESCRT-III: vps24Δ</td>
<td>Strong (A30P)</td>
<td>\Strong</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 3: Summary of α-synuclein properties assessed in published yeast models. This table indicates whether one, two, or all three α-synuclein properties were assessed by other studies. Properties evaluated are indicated by a yes, while properties not evaluated are indicated by a no.

<table>
<thead>
<tr>
<th>Yeast models of α-synuclein</th>
<th>Toxicity</th>
<th>Localization</th>
<th>Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outerio and Lindquist (2003)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Willingham et al. (2003)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Liang et al. (2008)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Gitler et al. (2007)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Volles and Lansbury (2007)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sharma et al. (2006) &amp; all DebBurman lab theses</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 4: Summary of α-synuclein toxicity in yeast deletion strains. A. Number yeast deletion strains that induced α-synuclein-dependent toxicity in 14 different studies in the DebBurman lab. B. Number of yeast deletion strains that induced α-synuclein-dependent toxicity from a genetic screen of ~4000 strains.

<table>
<thead>
<tr>
<th>Specific Study in DebBurman Lab</th>
<th>No. of Strains evaluated</th>
<th>Toxicity</th>
<th>Specific Study in DebBurman Lab</th>
<th>No. of Strains evaluated</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. Sharma, 2004</td>
<td>2</td>
<td>2</td>
<td>S. Herrera, 2005</td>
<td>8</td>
<td>Only 1</td>
</tr>
<tr>
<td>J. Price, 2004</td>
<td>1</td>
<td>1</td>
<td>S. Vahedi, 2005</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>A. Paul, 2004</td>
<td>1</td>
<td>No</td>
<td>M. Vahedi, 2006</td>
<td>6</td>
<td>None</td>
</tr>
<tr>
<td>K. Brandis, 2005</td>
<td>4</td>
<td>Only 1</td>
<td>J. Wang, 2006</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>I. Vaidya, 2005</td>
<td>3</td>
<td>None</td>
<td>M. Zomel, 2006</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>R. Shrestha, 2005</td>
<td>3</td>
<td>None</td>
<td>L. Kukreja, 2006</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>M. Stevenson, 2005</td>
<td>1</td>
<td>No</td>
<td>R. Choi, 2009</td>
<td>6</td>
<td>Only 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Knockout Study</th>
<th>No. of Knockout Strains</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willingham et al., 2003</td>
<td>~4000</td>
<td>86</td>
</tr>
</tbody>
</table>

Cytoplasmic localization there was still membrane-bound α-synuclein whenever toxicity was present. Contrary to this, in vps24Δ, WT and E46K shifted to a strong cytoplasmic localization and did not show toxicity. Correlations between membrane affinity and toxicity have previously been documented. For example, mutant α-synuclein, causing a decrease in membrane affinity, promotes survival in yeast (Volles and Lansbury, 2007). Similarly, membrane-binding WT and A53T familial mutants are toxic to yeast when overexpressed (Dixon et al., 2005). When overexpressed, A30P remains in the cytoplasm and is not toxic. We believe that WT and E46K toxicity is dependent on membrane localization. However, membrane binding alone is not sufficient for toxicity. Many of the strains I evaluated retained membrane affinity but did not show toxicity (mvb12Δ, vps20Δ, vps36Δ, and vps25Δ). Thus, the necessary amount of membrane binding needed for cells to reach their toxicity threshold remains unclear.

Furthermore, we believe that the proteasome is compensating for the compromised MVB/endocytosis pathway in the strains exhibiting no growth impairment and a shift in α-synuclein’s localization to the cytoplasm (vps25Δ, vps36Δ, vps24Δ and vps20Δ). Many studies show compensatory effects between the proteasome and lysosome when needed. For example, Laszlo et al. (1992) found that mammalian prion PrP is deposited in the lysosome of mice brains; however, in yeast, PrP also accumulates in the cytoplasm when the proteasome is inhibited (Ma and Lindquist, 2001). In addition, molecules usually degraded by the lysosome can enter the proteasome pathway during incomplete autophagy (Settembre et al., 2007). Thus, when needed the two degradation pathways may be interchangeable. Additionally, α-synuclein degradation in the proteasome is well documented, and the shift to the cytoplasm places α-synuclein in an ideal site for proteasome degradation. Finally, our vps24Δ was not toxic to WT α-synuclein as observed Willingham et al. (2003). This discrepancy may be due to their higher α-synuclein expression in yeast.

The Role of the 140th Amino Acid
Our fourth finding is that the 140th amino acid is negligible for PD-related α-synuclein properties. These findings were expected since the switch from the alanine to glycine occurred at the last amino acid which rarely contributes to the structure of a protein. The difference between α-synuclein-Glycine 140 and α-synuclein-Alanine is a methyl group, which is a neutral group that is of relative small size compared to the α-synuclein protein. Furthermore, unlike other amino acids in the α-synuclein protein, the 140th amino acid is not known to undergo post-translational modifications (Anderson et al., 2006).

Rarely, subtle differences were observed between α-synuclein-Glycine and α-synuclein-Alanine140. The differences
observed in parent strain BY4741. E46K expression, vps24Δ toxicity, and vps26Δ localization and toxicity. Therefore, the research conducted prior to the discovery of the error is relevant and both α-synuclein sequences behave similarly.

Critiques and Limitations
The findings in this thesis are limited by the absence of key experiments. We have not yet performed a vacuum stain in order to verify that α-synuclein is not transported to the vacuole. Furthermore, a vesicle stain is needed to evaluate the integrity of the MVB/endocytosis pathway. Finally, we have not completed immuno precipitation assays to assess interactions between MVB proteins and α-synuclein.

Future Directions
Future research will complete analysis on remaining ESCRT-I (vps23 and vps37), II (vps22) and III (vps2 and vps32) proteins and their ability to regulate α-synuclein toxicity, localization, misfolding, and accumulation. This will help us better understand the MVB/endocytosis pathway and its role in α-synuclein degradation. To further our research, we would like to develop vesicle and vacuolar stains in order to visualize α-synuclein and its route to the lysosome. Also, we would like to test synergistic effects by combining MVB dysfunction with either pharmacologically compromised proteasomes or induced oxidative stress. We suspect that the proteasome is compensating for lysosomal loss, thus we expect to see α-synuclein-specific toxicity in cells which have compromised proteasomes and lysosomes. Another possibility would be to verify whether proteasomal enzyme expression increases in the strains where WT and E46K shifted to the cytoplasm. Finally, we aim to evaluate induction of apoptosis, autophagy, and vacuolar integrity as cellular responses in MVB-deficient cells expressing α-synuclein.

Conclusion
The cause of α-synuclein degradation is a puzzling question that remains unanswered. A likely contributor to the onset of this neurodegenerative disease is the accumulation and aggregation of the α-synuclein protein. For many years now, scientists have sought to find mediators of α-synuclein in the hopes of understanding the pathogenesis behind PD. If the cause of PD is in fact α-synuclein and its aggregated form, then duty lies in understanding how this protein is removed from cells and why it is not properly degraded in PD patients. It was thus my goal to further understand the role of the MVB/endocytosis pathway in the degradation of α-synuclein. The use of a yeast model was ideal for this project because genes of the MVB/endocytosis pathway were first discovered in yeast. Additionally, yeast have shown to be a great model for studying this pathway and the substrates that use it.

After examining α-synuclein properties (toxicity, localization, and accumulation) in six ESCRT-deficient budding yeast strains, we are confident that the MVB/endocytosis pathway is a route for α-synuclein degradation. Even though alterations in all three properties evaluated where never observed at once, we were able to see changes in α-synuclein localization and/or expression in all knockout strains compared to those of parent strain BY4741. Furthermore, α-synuclein-dependent toxicity was observed in one ESCRT-deficient strain. Combining the outcomes of all the knockout strains provided a complex picture of the role of MVB/endocytosis pathway in regulating α-synuclein.

Methods and Materials
Methods from Sharma et al. 2006.

α-Synuclein Constructs
Human wild-type and A33T mutant α-synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and E46K were created from wild-type and α-synuclein using site-directed mutagenesis (Invitrogen) and mutations were confirmed by sequencing (University of Chicago). Wild-type and mutant α-synuclein cDNAs were subcloned into the pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). All α-synuclein forms were tagged with GFP using a two-step cloning strategy, with GFP either tagged at the N- or C-terminus. α-Synuclein cDNAs were first subcloned into mammalian expression vectors, pCDNA3.1 C-terminal GFP (Invitrogen) in order to be fused with GFP at the C-terminus. GFP tagged α-synuclein genes were then PCR-amplified and subcloned into pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). Chemically competent E. coli cells were transformed with the α-synuclein-cDNAs, and GFP-α-synuclein pYES2.1/V5-His-TOPO vectors. The parent pYES2.1 vector (Invitrogen) and GFP in pYES2.1/V5-His-TOPO vector were used as controls.

Correcting the 140th Amino Acid Mutant
All α-synuclein-Glycine140 was reconstructed to α-synuclein-Alanine140 using site-directed mutagenesis. The following primers were used:

Gal-1 forward primer 5' AATATACCTCTATATCTTAACGTTC-3'
V5C-term Reverse primer 5' ACGGAGAAGGTAGGATGAT-3'

All α-synuclein constructs were confirmed by sequencing at University of Chicago.

Yeast Strains
Parent strain BY4741 (mat a) and MVB knockout strains vps2Δ, vps12Δ, vps2δ, vps36Δ, vps20Δ and vps24Δ were purchased from Open Biosystems.

Yeast Expression
α-Synuclein expression plasmid vectors were transformed into the above budding yeast strains as described (Burke et al. 2000). For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-Ura). Table 1 summarizes all transformed stains used in this study. Presence of α-synuclein constructs was confirmed by PCR. The pYES2.1 vector, containing a galactose inducible promoter (GAL1), allowed for regulated α-synuclein expression. Yeast cells were first grown overnight in SC-Ura glucose (2%) or SC-Ura rafnose (2%) media at 30°C and then spun in a centrifuge at 200 rpm. Cells were washed with water and diluted to log-phase (5 x 10⁶ cells/ml) in SC-Ura galactose (2%) media to induce expression and grown to the time points desired for various measurements.

Western Analysis
Yeast cells (2.5x10⁷ cells/mL) were washed in 50 mM Tris (pH 7.5), 10 mM NaH3 and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000) containing 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 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 10 mg/ml Edta, 2mg/ml aprotinin, and 2 mg/ml chymostatin). Samples were run on a 10% Tris-Glycine gel (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 different monoclonal antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 (Invitrogen) and anti-phosphoglycerokinase (PGK; Molecular Probes) for most expression experiments, as a measure of loading control in duplicate blots.

Growth Curve Analysis
Cells were grown in 10 ml SC-Ura glucose overnight at 30°C, 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice in 5 ml H2O and were counted. Flasks with 25 ml SC-Ura galactose were each inoculated to 2.0x10⁵ cells/ml density. 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 cuvet to measure absorbance using a Hitachi U-2000 Spectrophotometer. Averaged absorbance readings were plotted against time to produce a growth curve.

GFP Microscopy
Cells were grown overnight in 10 ml SC-Ura glucose at 30°C at 200 rpm. Protein expression was induced with SC-Ura galactose media. After a time specific induction, cells were harvested at 1500 x g at 4°C for 5 minutes and were washed twice in 5 ml H2O. Cells were suspended in 100-1000 ml SC-Ura glucose, and 10 ml cell suspension was pipetted
onto a slide. Cells were visualized using a Nikon TE2000-U fluorescent microscope and images were acquired and analyzed using Metamorph 4.0 imaging software."

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