Does α-Synuclein use Endocytosis as a Route for Degradation by the Lysosome?

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Abstract

Parkinson's disease (PD) is an incurable fatal brain disorder linked to three disease-related properties that result from α-synuclein accumulation: its misfolding, aggregation, and cellular toxicity. Accelerating α-synuclein degradation might provide therapy by reducing its accumulation. We tested if the lysosome degrades α-synuclein by a specific route, endocytosis, in a budding yeast model for PD. Specifically, we evaluated if α-synuclein accumulation, aggregation, and toxicity worsened in seven yeast strains that had individual gene deletions, which control specific steps within endocytosis. We report three significant findings. Firstly, all seven genes affected at least one α-synuclein PD-related property, thus providing accumulating genetic evidence for the endosome pathway as a regulator of α-synuclein degradation. Secondly, each gene affected α-synuclein properties to different extents, suggesting substrate specificity for endocytosis steps. Lastly, none of the genes contributed additional α-synuclein-dependent toxicity. Together, our data suggests that α-synuclein is degraded by the lysosome using the endocytosis route.
Introduction

Introduction

Neurodegenerative Diseases
Most of the cells in our body have regenerative power. Unfortunately, regeneration in the central nervous system is extremely restricted. This lack of regeneration can be particularly devastating, because if these cells are destroyed or damaged, permanent disability ensues.

Our society is afflicted by a variety of human brain disorders. Amongst them are a group of disorders classified as the neurodegenerative diseases. The word neurodegeneration gives us a hint of what these disorders involve: the deterioration or death of neurons in the brain specific to the disease. Neurodegenerative diseases affect millions of people worldwide, and by 2040, they will surpass cancer as the leading cause of death. Unfortunately, no cure currently exists. Understanding the molecular and cellular mechanisms by which these diseases operate might eventually lead to more effective treatments and ultimately a cure (Lozano et al., 2005).

The best-studied neurodegenerative diseases include Alzheimer's disease, Lou Gehrig's disease, Creutzfeldt-Jacob disease, Huntington disease, Parkinson's disease (PD), and prion diseases. They vary in symptoms and onset, but one common characteristic is the presence of an abnormal protein unique to each disease. Each particular protein misfolds, aggregates, usually leading to accumulation as intracellular or extracellular inclusions in the brain of patients (Taylor et al., 2002). In most cases, these proteins are thought to gain a toxic new function leading to pathogenesis, but data is not yet conclusive. My thesis focused on the molecular basis of one such disease, PD.

Parkinson's Disease
PD afflicts around four million people worldwide, making it amongst the most prevalent neurodegenerative disease (Lozano et al., 2005). First characterized in the 1800s by physician James Parkinson, classic symptoms include tremors, muscular rigidity, slowness of movement, and impaired balance and coordination. Half of the patients develop symptoms after age 60, suggesting that the disease not only affects the elderly since the other half develop symptoms before then. Currently, no cure for PD exists, but treatment for the symptoms is available. Unfortunately, most patients eventually become resistant to the medication (Lozano et al., 2005).

Similar to other neurodegenerative diseases, PD is linked to the misfolding and accumulation of a particular protein, α-synuclein (Spillantini, et al., 1998). A hallmark symptom of PD is the accumulation and aggregation of α-synuclein into structures called lewy bodies found in the substantia nigra region of the midbrain. These individuals experience loss of dopaminergic neurons in this area of the brain (Giasson et al., 1999; Spillantini et al., 1998, Abeliovich et al., 2000; Lozano et al., 2000). The basal ganglia consists of a group of nuclei that organize motor behavior and the substantia nigra is one component of this pathway. Therefore, the substantia nigra is essential for voluntary movement. Due to this fact, substantia nigra cell death leads to movement disorders since the death of these neurons interferes with the normal signaling to the basal ganglia (Figure 1A; Schmidt and Oertel, 2006). The brain has two pathways through the basal ganglia which function to either initiate volitional movement (direct pathway) or to suppress inappropriate movements (indirect pathway) (Purves, 2008). PD affects the direct pathway to the basal ganglia because it results from the inability to initiate movement, therefore making it a hypokinetic disease (Figure 1B; Purves, 2008).

PD occurs in two forms: sporadic or familial. An estimated 95 percent of PD cases are sporadic (Schmidt and Oertel, 2006). The misfolding and accumulation of normal wild-type (WT) α-synuclein is linked to the sporadic case of PD. α-Synuclein aggregates and accumulates because of decreasing mitochondrial complex 1 activity, oxidative stress, and environmental factors such as chemicals or toxins (Dawson and Dawson, 2003). For example, the uptake of 1-Methyl-4-phenyl-4-propionoxy-piperidine (MPPP), a byproduct of synthetic heroin, caused PD-like symptoms in patients (Langston et al., 1983). The cause for the misfolding and accumulation of WT α-synuclein is not well understood. Because of the importance of these characteristics in PD pathogenesis, they are essential to examine further.

In contrast, familial PD is caused by genetic factors. Studies of families demonstrate that point mutations in the α-synuclein gene cause PD. These mutations include the A30P (Polymeropoulos et al., 1997), A53T (Kruger et al., 1998) or E46K (Zarranz et al., 2004) changes. Six other genes have been identified in the onset of familial PD: *parkin* (Kitada et al., 1998), UCH-L1 (Liu et al., 2002), DJ-1 (Bonifati et al., 2003), PINK1 (Valente et al., 2004), LRRK2 (Funayama et al., 2002; Paisan-Ruiz et al., 2004), and PARK9 (Ramirez et al., 2006). The *parkin* E3 ubiquitin ligase and UCH-L1 ubiquitin hydrolase gene mutations are involved

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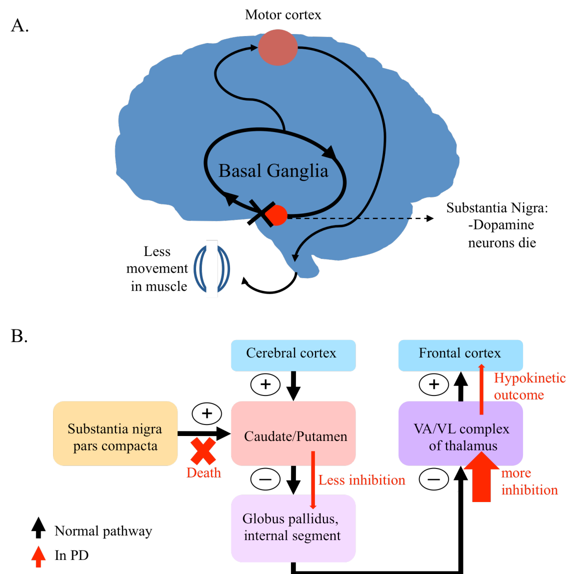


Figure 1: Parkinson's disease Pathway. A. The motor cortex controls movement of our muscles, while the basal ganglia controls timing and coordination of that movement. The substantia nigra is part of the basal ganglia. This pathway is disrupted in PD by the loss of the dopaminergic neurons in the substantia nigra. This leads to less/shaky voluntary movement because the basal ganglia cannot send signals to the motor cortex, which would send instructions for movement. B. Direct pathway through the basal ganglia. In PD (red), the inputs by the substantia nigra are diminished, making it more difficult for a transient inhibition of the caudaute and putamen. This results in less inhibition of the globus pallidus and in turn more inhibition of the VA/VL complex of the thalamus, which results in less thalamic excitation of the motor cortex (Adapted from Purves, 2008).

in interfering with the ubiquitin proteasome system (Kitada et al., 1998; Liu et al., 2002). DJ-1 gene mutations are implicated in the pathogenesis of PD because of its possible role in response to oxidative stress (Bonifati et al., 2003). PINK1 is involved in the phosphorylation of mitochondrial proteins, as a response mechanism for mitochondrial dysfunction, and its mutation has been linked to PD progression (Valente et al., 2004). A mutation in protein LRRK2 has similarly been linked to pathogenesis due to possible phosphorylation of α-synuclein protein or causing accumulation due to kinase activity (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Studies demonstrate that the LRRK2 mutation causes a significant increase in kinase activity, suggesting a gain in PD pathology (Gloeckner et al., 2006). The most recently identified gene in familial PD has been PARK9, since mutations in this gene cause loss of function. Loss of PARK9 might be involved in the accumulation of proteins because of degradation dysfunction through either the proteasome or lysosome (Ramirez et al., 2006). Most importantly PARK9 protects cells from manganese toxicity, since manganese is an environmental factor in PD (Gitler et al., 2009). The mechanisms involved in progression to familial PD are a large area of study that still needs to be fully uncovered. Studying α-synuclein properties is important in order to understand PD pathogenesis, since α-synuclein misfolds in both sporadic and familial forms of the disease.

α-Synuclein
α-Synuclein has been implicated in a variety of other neurodegenerative diseases including AD (Lucking and Brice, 2000). As mentioned previously, the misfolding and aggregation of α-synuclein leads to Lewy body formation in the brains of patients. α-Synuclein is part of a larger family of proteins including α-, β-, and γ-synuclein (George, 2009). The protein has a molecular mass of 19 kDA, is 140 amino acids long (Jakes et al., 1994), and is composed of the N-terminal amphiphathic domain, the hydrophobic middle domain, and the acidic C-terminal domain (Lucking and Brice, 2000). α-Synuclein's flexible nature is due to its unfolded C-terminal region (Eliezer et al., 2001).

The following key α-synuclein findings may help us better understand PD: 1) The protein's normal function is not well understood, but data indicates it is highly expressed in the brain, particularly in presynaptic nerve terminals (Kaplan et al., 2003; Jakes et al., 1994). 2) α-Synuclein deletion mice exhibit a reduction in striatal dopamine neurons, supporting the finding that the protein is an essential presynaptic regulator of dopamine neurotransmission (Abeliovich et al., 2000). 3) Lewy bodies have an increased accumulation of fibrillar α-synuclein, which point to their role in disease pathogenesis (Goldberg and Lansbury, 2000; Kaplan et al., 2003). 4) The presence of a weak transient or residual secondary structure in the protein is what may be playing a role in amyloid fibril formation, therefore resulting in aggregation (Eliezer et al., 2001). 5) Cytoplasmic concentrations of dopamine have been demonstrated to promote and stabilize protofibrillar intermediates, linking the dopaminergic selectivity of α-synuclein (Conway et al., 2001). 6) Data on the cytotoxicity of α-synuclein protofibrils has been inconclusive, but research demonstrates that α-synuclein protofibrils are the ones that tightly bind to vesicles and cause membrane permeabilization and destruction, a toxic effect (Volles et al., 2001). 7) To further support α-synuclein's gain of function characteristics in PD, experiments in animal models show PD-like symptoms with α-synuclein over-expression. This was demonstrated with wild-type α-synuclein and familial mutants A30P and A53T in mice (Masilah et al., 2000), flies (Feany and Bender, 2000), and worms (Lakso et al., 2003). 8) In yeast models, WT and E46K α-synuclein enhanced cell toxicity (Dixon et al., 2005). WT and the other familial mutant, E46K localize to the plasma membrane, confirming α-synuclein's membrane binding affinity. The familial mutant A30P localizes to the cytoplasm (Ouitero and Lindquist, 2003; Dixon et al., 2006; Sharma et al., 2006). However, the role of α-synuclein membrane binding in PD pathogenesis remains unknown. 9) Since it is found associated in diverse cellular locations, α-synuclein degradation is likely complex.

Major α-Synuclein Questions
The past decade provided tremendous insight into the PD field. Questions regarding the molecular basis of PD still remain unanswered. Is the accumulation and inclusion formation of α-synuclein causing the progression of PD? If this is true, what is the causative agent? From the studies with the familial causing PD genes, the role of oxidative stress in PD pathogenesis needs to be fully understood. Studying the effect of mitochondrial dysfunction's role in the disease is a priority. A major gap in the field is whether α-synuclein accumulation causes PD. If so, a better understanding of the different degradation routes α-synuclein takes is essential for developing treatments. My thesis specifically focused on this last question.

The Degradation Problem
α-Synuclein is found in many cell locations (Figure 2A). α-Synuclein is mostly found in the cytoplasm, while studies demonstrate an affinity to phospholipids along with vesicle binding (Kahle, 2000; Davidson et al., 1998; Eliezer et al., 2001). Recently, data indicate that α-synuclein is delivered to the plasma membrane through its interactions with the ER-Golgi secretory pathway (Dixon et al., 2005). Lastly, small

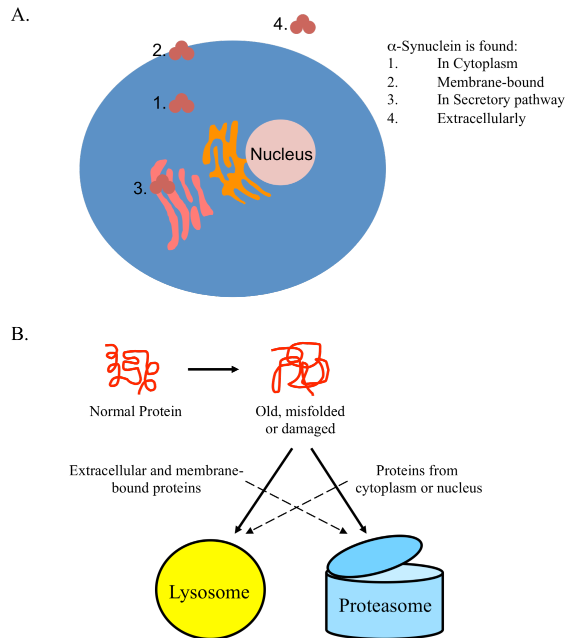


Figure 2: α-Synuclein location and possible degradation routes. A. α-Synuclein is found in several places throughout a cell: 1. In cytoplasm, 2. Membrane-bound, 3. In secretory pathway, and 4. Extracellularly. B. Old, misfolded or damaged proteins need to be degraded. Extracellular and membrane-bound proteins use the lysosome as a route, while nuclear cytoplasmic proteins use the proteasome.

amounts of α-synuclein are secreted from cells and are present in human body fluids, such as the blood plasma and cerebrospinal fluid (Lee et al., 2008).

α-Synuclein degradation is likely complex due to its localization (Figure 2A). The cell has mechanisms to degrade old, damaged, or misfolded proteins, as eliminating such proteins is critical for the health of an organism. These mechanisms break proteins down to their constituent amino acids through one of two different degradation routes: the ubiquitin proteasome system (UPS) or the lysosome (Figure 2B). Given α-synuclein's localization, its degradation is likely complex. Data reveals that prion proteins, another set of proteins linked to neurodegeneration, can use both pathways. In mice brains, the prion protein is deposited in the lysosome (Laszlo et al., 1992), while in yeast it uses the proteasome for degradation (Ma and Lindquist, 2001). Depending on where α-synuclein is localized, it might use one or both routes. I first examine the evidence for the proteasome as a site for α-synuclein degradation, followed by the lysosome.

Route One: The Proteasome

The ubiquitin proteasome system (UPS) is one of two recycling mechanisms in the cell. The proteasome usually degrades proteins from the cytoplasm or the nucleus. Exceptions to this rule exist, however (Figure 2B; Alberts, 2004). The UPS uses ubiquitin, a small protein that is attached to molecules that need to be degraded, as a marker for the molecules' destruction. The enzymes that add the ubiquitin protein recognize signals for misfolding or chemical damage in the proteins.

Until 2004, the PD field believed α-synuclein was degraded solely by the proteasome, because α-synuclein is found in the cytoplasm. Several lines of genetic evidence support the proteasome as an α-synuclein degradation route. Patients with two mutant genes involved in the ubiquitin-proteasome system caused familial PD (UCH-L1 and *parkin*). UCH-L1 is a neuronal enzyme that hydrolyses ubiquitin, and studies show that α-synuclein aggregates when mutated UCH-L1's concentration exceeds a certain threshold (Liu et al., 2002). *Parkin* is an E3 ligase that ubiquitylates α-synuclein among other proteins and tags them for degradation (Liu et al., 2002; Kitada et al., 1998). Several lines of genetic and pharmacological evidence further provide a link to the importance of the proteasome in α-synuclein degradation. A 26S proteasome subunit mouse knockout model resulted in mice with intra-neuronal Lewy-like inclusions and neurodegeneration in the nigrostriatal pathway (Bedfort et al., 2008). Pharmacological studies have shown that proteasome inhibition, by lactacystin, for example, leads to α-synuclein inclusion formation (Rideout et al., 2002; Sawada et al., 2004). Proteasome inhibition in mice increases cell death through activation of cytoplasmic p53. Findings suggest that p53 abnormalities may play a role in dopaminergic cell death (Nair et al., 2006). These results highlight the proteasome as a key organelle in α-synuclein degradation.

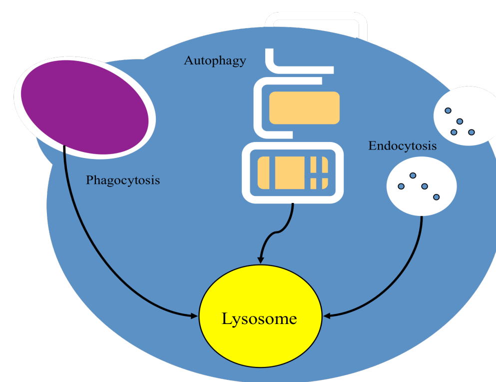


Figure 3: Three routes to the lysosome. There are three major routes to the lysosome: phagocytosis, autophagy, and endocytosis. In phagocytosis, the cell engulfs bacteria or viruses for degradation. In autophagy, a membrane forms and eventually surrounds the organelle, then transporting it to the lysosome. Lastly, during endocytosis, the plasma membrane invaginates and material from outside of the cell or from the plasma membrane is tagged to the lysosome.

Route Two: The Lysosome

The lysosomal system is the second recycling mechanism in the cell which degrades proteins from the plasma membrane or from outside of the cell. The cell has three pathways to the lysosome: phagocytosis, autophagy and endocytosis (Figure 3). Extracellular particles, such as bacteria, are taken up through phagocytosis. Once the particle is bound to the cell, special receptors trigger invagination of the membrane. The vesicle then pinches off and this vacuole is now a phagosome. Then enzymes begin breaking the bacteria down and transport it to the lysosome; once it fuses with the lysosome, it becomes a phagolysosome (Alberts, 2004). Since α-synuclein is not a bacterium, phagocytosis is not studied as a degradation route.

The second pathway to the lysosome is autophagy and a method that the cell uses to get rid of unwanted or damaged organelles and proteins found in the cytoplasm. Autophagy is a stimulus-induced (self-cannibalism)

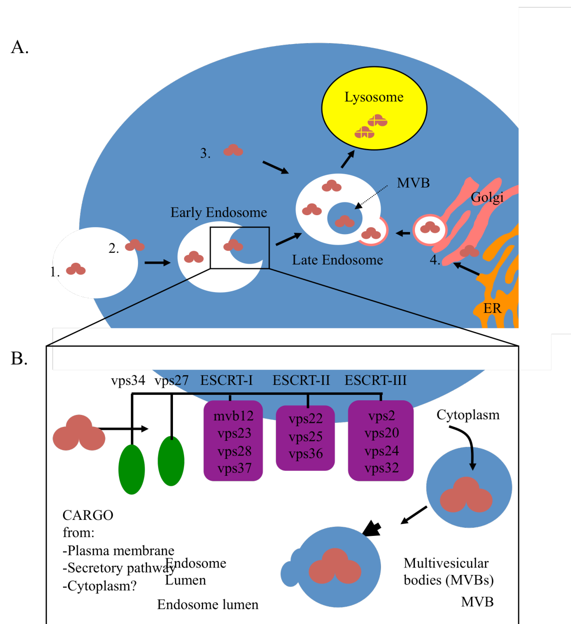


Figure 4: MVB/Endocytosis Pathway. A. The endocytic pathway brings in proteins from outside or from the plasma membrane into the cell and to the lysosome. It also brings in proteins that the lysosome needs to break down substances. These proteins are made in the ER. It does this using multivesicular bodies (MVBs), which are vesicles that form inside the late endosome. B. MVBs are formed when the endosome membrane invaginates into the lumen. The invagination is made possible by the endosomal-sorting complex required for transport (ESCRT). The ESCRT complex helps form MVBs which then make it possible for those membrane-bound proteins to be degraded once the endosome and lysosome fuse.

mechanism: for example, if the cell is experiencing amino acid starvation the cell will degrade nonessential cellular components. The process begins with the formation of a membrane around an organelle, a process called nucleation. The membrane expands until the organelle is completely enclosed by a double membrane, creating an autophagosome (expansion). Lastly, this vesicle is then transported to the lysosome for degradation and the union of an autophagosome and a lysosome is called fusion (Alberts, 2004).

In endocytosis, the third route to the lysosome, fluid or small molecules are taken from the outside of the cell, from the plasma membrane, or from the ER-Golgi secretory pathway to the lysosome. This happens when the plasma membrane buds in and then pinches off to form an endosome. This material is eventually taken to the lysosome for digestion (Alberts, 2004).

Several lines of pharmacological evidence now point to the lysosome as another α-synuclein degradation route: 1) Evidence demonstrates no changes in levels of α-synuclein with proteasome inhibition (Rideout et al., 2004; Ancolio et al., 2000). 2) Studies performed with human neuroblastoma and rat embryonic cortical neuron cell cultures with inhibited lysosome show rapid accumulation of oligomeric α-synuclein (Lee et al., 2004). Cuervo et al. (2004) conducted experiments demonstrating WT α-synuclein is internalized and degraded by the lysosome through chaperone-mediated autophagy (CMA). The A30P and A53T mutants, on the other hand, were not degraded using this mechanism. 3) Results were confirmed in similar experiments with inhibited CMA and macroautophagy, resulting in WT α-synuclein accumulation (Vogliatzi et al., 2008). 4) Other experiments with autophagy found evidence of the presence of α-synuclein in autophagy-like vesicles. Data also showed that autophagy inducers, such as rapamycin, increased the clearance of α-synuclein in PC12 cell lines (Webb et al., 2003).

The studies above focus mostly on autophagy and less on endocytosis; almost all evidence is pharmacological. My thesis, therefore, focused on the endocytosis route.

Multivesicular body/Endosome Pathway

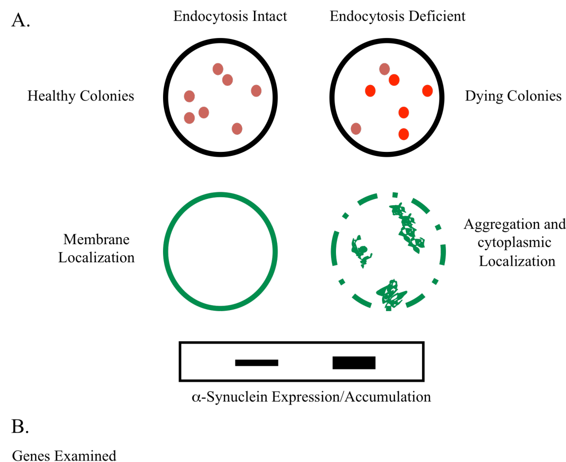
The main function of the endocytosis pathway is to import proteins located extracellularly or proteins on the membrane to the lysosome. Endocytosis also brings in proteins from the ER that the lysosome needs to break down substances (Figure 4A).

Transmembrane proteins are delivered to the endosome lumen in multivesicular bodies (MVBs), which are vesicles that form inside the late endosome. The sorting of proteins into the MVB pathway is a complex, multistep process. Monoubiquitination of these transmembrane proteins signals their sorting into an MVB (Katzmann et al., 2001). A common substrate that uses the MVB/endosome pathway is the epidermal growth factor receptor (EGF-R). The signal for this protein to become degraded by MVBs is tyrosine phosphorylation, and mutations impair its degradation (Fedler et al., 1990). Experiments with growth hormone receptor (GHR) demonstrate that ubiquitin needs to be present for GHR degradation by endocytosis (Strous et al., 1996). In accordance, other proteins such as Ste3, Gap1, Tat2t in yeast and GHR, MHCII, E-Cadherin in mammals also uses ubiquitin as a signal for degradation by the MVB/endocytosis pathway (Katzmann et al., 2002).

The invagination of the endosome membrane is made possible by subcomplexes, the endosomal-sorting complex required for transport (pre-ESCRT, ESCRT I, II, III and post-ESCRT). The ESCRT complexes helps form MVBs which then make it possible for those membrane-bound proteins to be degraded once the endosome and lysosome fuse (Figure 4B; Katzmann et al., 2001; Katzman et al., 2002).

The ESCRT complexes are composed of less than 20 proteins, known as class E Vacuolar Protein Sorting (*vps*) proteins, since the vacuole is the yeast counterpart of the lysosome (Katzmann et al., 2002). Mutations in these genes cause defective sorting of transmembrane proteins from the plasma membrane to the vacuolar lumen by means of the MVB pathway (Hierro et al., 2004). *vps34*, a protein kinase, is involved at the pre-ESCRT step with initiation and cargo recognition. *vps34* is also responsible for the synthesis of a specific phospholipid, phosphatidylinositol 3-phosphate, which then forms a complex at the membrane with *vps15* to regulate protein sorting (Herman & Erm, 1990; Stack et al.,1993). Moreover, pre-ESCRT protein *vps27* is recruited to the early endosome by its interaction with the ubiquitinated cargo. *vps27* then recruits ESCRT-I complex (composed of *mvb12, vps23, vps28,* and *vps37*) into the membrane, these proteins are responsible for the cargo sorting. ESCRT-I recruits ESCRT-II (composed *of vps22, vps25,* and *vps36*), and ESCRT-II recruits ESCRT-III (composed of *vps2, vps20, vps24,* and *vps32*) into the membrane (Babst et al., 2002). ESCRT-III is responsible for cargo sequestration and finally MVB vesicle formation. Post-ESCRT steps include *vps4*, vps60 and vta1. These are involved in disassembly and membrane release (Lee and Gao, 2008).

A few studies have focused on the endocytosis/MVB pathway as a mechanism for α-synuclein degradation. First, genome-wide yeast deletions identified two endocytosis genes, vps24 and vps28, that demonstrated α-synuclein dependent toxicity (Willingham et al., 2003).



Pre-ESCRT	ESCRT-I	ESCRT-II	ESCRT-III
vps34 ✓	mvb12	vps22 ✓	vps2 ✓
vps27 ✓	vps23 ✓	vps25	vps20
	vps28	vps36	vps24
	vps37 ✓		vps32 ✓

Figure 5: Predictions and genes examined. A. Predictions for endocytotic regulation of α-synuclein. We induced α-synuclein expression in both the endocytosis intact and the endocytosis deficient yeast. The endocytosis intact is expected to degrade α-synuclein via the endosome-lysosome pathway, leading to healthy cells. On the other hand, we predict some, but not all endocytosis deficient yeast will increase α-synuclein accumulation, toxicity and change localization. B. Table showing the genes that make ESCRT-I, II and III. The genes examined in this study have a checkmark.

Also, phosphorylated α-synuclein accumulated in C. elegans with endocytosis gene knockouts (Kuwahara et al., 2008). Even though α-synuclein is mostly a cytoplasmic protein, small amounts of its aggregated form are found outside the cells in PD patients, in particular in the blood plasma and cerebrospinal fluid. Lee et al., (2008) showed that accumulated extra cellular α-synuclein was internalized using the endocytosis pathway and eventually degraded by the lysosome (Lee et al., 2008). Rab5, a GTPase, seems to be an important factor in neuronal endocytosis. One study showed that Rab5 is critical for the degradation of exogenous α-synuclein in cells (Sung et al., 2001). In a yeast model, the familial mutant A30P binds to endocytic protein YPP1 at the plasma membrane, causing the budding of endocytic vesicles in receptor mediated endocytosis, and eventually targeted to the vacuole for degradation, and thus again linking endocytosis in α-synuclein degradation by the lysosome (Flower et al., 2007). Therefore, if α-synuclein uses this pathway, it should interact with all or some of the other ESCRT pathway proteins involved.

Gap in Knowledge: What we still do not know

Proteasome degradation of α-synuclein is supported by pharmacological, genetic and biochemical evidence. In order for the lysosome to receive a similarly wide level of support, additional genetic and biochemical studies need to be done. Past evidence for α-synuclein degradation by the lysosome mostly focused on pharmacological studies (Cuervo et al., 2004; Lee et al., 2003). Alex Ayala '09 undertook the first study of the MVB/endocytosis protein complexes, and it was one of the few times where several PD-linked α-synuclein properties, accumulation, localization, and toxicity, were studied. She found that all six strains examined altered at least one of the three α-synuclein PD-related properties (Table 1; Ayala Thesis, 2009). She reported three significant findings: 1) The MVB/endocytosis pathway is implicated in α-synuclein degradation, 2) Some ESCRT genes subtly regulate α-synuclein properties, and 3) An overall absence of toxicity and how we can understand those differences. The rest of the MVB/endocytosis genes still need to be analyzed (Figure 5B). My thesis filled this gap in knowledge using the same budding yeast model.

Table 1: Past evaluation of α-synuclein degradation by endocytosis. Ayala Thesis (2009) evaluated α-Synuclein degradation toxicity, localization, and accumulation for six total ESCRT protein knockout strains. The changes were assessed as a strong, weak or none when compared to BY4741.

Strain:	Localization	Toxicity	Accumulation
ESCRT-I: *mvb12Δ* *vps28Δ*	None Weak	Non Toxic Weak	Weak Weak
ESCRT-II: *vps25Δ* *vps36Δ*	Weak Weak	Non Toxic Non Toxic	Weak Strong
ESCRT-III: *vps20Δ* *vps24Δ*	Weak Strong	Non Toxic Toxic	Strong None

Budding Yeast

S. cerevisiae, budding yeast, was used as a model organism to evaluate the role of the endocytosis/MVB pathway in α-synuclein degradation. The MVB/endosome pathway is best studied in budding yeast and because of its importance, the MVB pathway to the lysosome has been conserved from yeasts to humans (Katzman et al., 2001). Budding yeast are extremely powerful molecular and biological tools because their genome is known and the entire gene deletion library is available (OpenBiosystems). This eukaryotic model system is effective for the study of different neurodegenerative diseases. Budding yeast are used in the study of Huntington's disease and PD among others (Willingham et al., 2003; Outeiro and Lindqust, 2004, Dixon et al., 2005, Sharma et al., 2006).

Hypothesis and Aims

My hypothesis was that the MVB/endosome pathway is a route by which α-synuclein is targeted to the lysosome.

To test this hypothesis, I examined pre-ESCRT (*vps34Δ* and *vps27Δ*), ESCRT-I (*vps37Δ* and *vps23Δ*), ESCRT-II (*vps22Δ*) and ESCRT-III (*vps2Δ* and *vps32Δ*) genes. To evaluate the role of all seven proteins I evaluated the following aim:

Aim: To compromise individual ESCRT genes in budding yeast and determine whether α-synuclein accumulated, changed localization, and/or induced toxicity.

I predicted that the deletion of each of these genes would change one or more of the following: increase cytoplasmic localization of α-synuclein, increase α-synuclein accumulation and induce toxicity (Figure 5A).

We report three significant findings. Firstly, all seven genes affected at least one α-synuclein PD-related property and thus providing accumulating genetic evidence for the MVB/endosome pathway as a regulator of α-

synuclein degradation. Secondly, each gene affected α-synuclein properties to different extents, suggesting substrate specificity for endocytosis steps. Lastly, none of the genes contributed additional α-synuclein-dependent toxicity. Together, our data suggests that α-synuclein is degraded by the lysosome using the endocytosis route.

Methods and Materials

Except for the Loss of Induction assay the following methods have all been adapted from Sharma et al., (2006) and described briefly again.

α-Synuclein Constructs
The vectors used in this study (pYES2.1, GFP, WT α-synuclein, and the A30P and E46K familial mutants) were created as previously described in Sharma et al., (2006).

Yeast Strains
Parent strain BY4741 (mat a) and MVB deletion strains *vps27Δ*, *vps34Δ, vps23Δ, vps37Δ, vps22Δ, vps2Δ,* and *vps32Δ* were purchased from Open Biosystems.

Yeast Expression
α-Synuclein expression plasmid vectors were transformed into above budding yeast strains as described in Burke et al., (2000). Yeast cells were grown on media lacking uracil (SC-URA) for selection. Using polymerase-chain reaction (PCR), the presence of α-synuclein constructs was confirmed. The expression of α-synuclein was regulated using a galactose inducible promoter (GAL1) in the pYES2.1 vector. Yeast cells were grown overnight in SC-URA glucose (2%) media at 30ºC. Cells were washed with water and diluted to 5 x 10^6 cells/ml in SC-URA galactose (2%) media to induce expression and grown to the time points desired for various experiments mentioned below.

Western Blot Analysis
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were centrifuged at 1500xg for 5 minutes at 4°C, and washed three times with 5ml water. Cells were then transferred to 25ml of SC-URA galactose at 30°C at 200 rpm. At 24 hours, 2.5x107 cell density was calculated to create cell lysates. Cells were washed with 100 mM NaN3, solubilized in ESB (electrophoresis sample buffer), and small beads were added to break up contents. Samples were run on Tris-Glycine gels using SDS containing running bugger. SeeBlue was used as the molecular weight standard. Gels were transferred to a PVDF membrane. Western blot was created by washing membranes with different antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 to code for α-synuclein, and anti-PGK (phosphoglycerolkinase) as a loading control.

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 as previously described. After 24 and 48 hours, 1ml of culture was pipetted into a 1.5 ml microcentrifuge tube and 10 ml cell suspension was pipetted onto a slide. Cells were visualized under a Nikon TE2000-U fluorescent microscope and images were acquired and analyzed using Metamorph 4.0 imaging software.

Growth Curve Analysis
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were centrifuged at 1500xg for 5 minutes at 4°C, and washed three times with 5ml water. Cells were resuspended in 10ml water and counted. 2.0x106 cell density was inoculated into 25ml galactose and grown in a shaking incubator at 30°C, 200 rpm for 48 hours. At specific time points (0, 3, 6, 12, 18. 24, 36 and 48 hours) 1 ml of culture was removed from each culture and pipetted into two cuvetes for duplicate readings. Readings were taken using a Hitachi U-2000 Spectrophotometer set at 600 nm.

Spotting
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were centrifuged at 1500xg for 5 minutes at 4°C, and washed three times with 5ml water. Cells were resuspended in 10ml water and counted. 2.0x107 cell density were placed into a 1.5 ml centrifuge tube and centrifuged at 2500 rpm for 5 minutes. Water was removed and cells were resuspended in 1ml of sterile DI water. Cells were 5-fold serially diluted into 96-microwell plates and spotted on SC-URA glucose and galactose plates using a multi-channel pipette. Photographs were taken after 24 hours of growth for glucose plates and 48 hours for galactose plates.

Loss of Induction
Cells were grown overnight in 5 ml SC-URA glucose at 30°C, 200 rpm. They were washed twice in 5 ml water. Cells were re-suspended in 10 ml water and 4 ml were transferred into 46 ml of SC-URA galactose and grown for 24 hours. At 24 hours, cells were counted and lysates were prepared. The culture was then transferred to a 50 ml centrifuge tube, washed 3 times with 10 ml water and re-suspended in 15 ml. 5 ml of culture was transferred to 45 ml of SC-URA glucose. Lysates were prepared for galactose 24 hrs, glucose 0 hrs, glucose 6 hrs, glucose 12 hrs, and glucose 24 hrs, and Western Blot was performed as described above.

Statistics
Western blots, loss of induction blots, and microscopy pictures at each time point were repeated twice. Growth curves and 3 sets of glucose/galactose spotting were repeated three times. Growth curves were placed into a cumulative graph. Two-tailed distribution, two sample equal variable t-tests were performed on all cumulative growth curves at 18 and 24 hours. Each α-synuclein plasmid vector was compared to the parent plasmid. Microsoft excel was used to perform the t-test on the growth curves. For microscopy, Metamorph 4.0 imaging software was used to count 750 DIC cells, and the corresponding fluorescent pictures of cells were scored for phenotype and percent was calculated.

Results

Experimental Set-Up:
Localization was analyzed through fluorescent microscopy, toxicity through growth curves and five-fold dilution spotting on plates, and accumulation through Western blots and loss of induction assays. Each strain was compared to the isogenic parent strain, BY4741, and transformed with five plasmid vectors (Table 2). The pYES2.1 parent plasmid and GFP plasmid served as negative controls. The three synuclein vectors contained wild-type (WT) or two familial mutants (A30P and E46K).

BY4741: α-Synuclein is membrane bound in parent strain
First, we analyzed α-synuclein properties in BY4741 strain because it displays normal endocytosis and does not contain any endocytosis gene deletions. The findings in this strain served as controls for the rest of the gene deletion strains we then studied. As previously shown (Sharma et al., 2006), we also found that both WT and E46K α-synuclein localized

Table 2. List of Transformed Budding Yeast Strains

No cDNA	pYES2.1	BY4741
(parent plasmid)		*vps37Δ*
		vps27Δ
		vps37Δ
		vps23Δ
		vps22Δ
		vps2Δ
		vps32Δ
Alanine140-GFP	pYES2.1	BY4741
		vps37Δ
		vps27Δ
		vps37Δ
		vps23Δ
		vps22Δ
		vps2Δ
		vps32Δ
WT-α-Synuclein-Alanine140-GFP	pYES2.1	BY4741
		vps37Δ
		vps27Δ
		vps37Δ
		vps23Δ
		vps22Δ
		vps2Δ
		vps32Δ
A30P-α-Synuclein-Alanine140-GFP	pYES2.1	BY4741
		vps37Δ
		vps27Δ
		vps37Δ
		vps23Δ
		vps22Δ
		vps2Δ
		vps32Δ
E46K-α-Synuclein-Alanine140-GFP	pYES2.1	BY4741
		vps37Δ
		vps27Δ
		vps37Δ
		vps23Δ
		vps22Δ
		vps2Δ
		vps32Δ

to the plasma membrane but A30P was diffuse within the cytoplasm (Figure 6A, left). This qualitative observation was quantifiably confirmed (Figure 6A, right). WT and E46K α-synuclein were equally expressed in these cells, while A30P expression was reduced (Figure 6B). Lastly, α-synuclein was not toxic to yeasts as assessed by both five-fold serial dilution plating (Figure 6C) and growth curves (Figure 6D).

Pre-ESCRT: Both Proteins Regulate α-synuclein Localization

Our next goal was to examine α-synuclein property changes in the first of two pre-ESCRT compromised strains: *vps34Δ*. In support of our hypothesis, both WT and E46K α-synuclein were quantifiably less localized at the plasma membrane at the end of 48 hours of expression (Figure 7A). Interestingly, A30P did not alter its localization pattern and, as expected, GFP remained unchanged (Figure 7A). Moreover, WT and E46K α-synuclein both also increased accumulation in *vps34Δ* cells, as shown in Western blots at 24 hours of expression (Figure 7B, top). To confirm this accumulation, we assessed protein turnover of both WT and E46K α-synuclein by the loss of induction assay, which consequently supported the accumulation despite unequal loading (Figure 7B, bottom). However, the α-synuclein localization change and accumulation did not correlate with an increase in cellular toxicity as shown by both toxicity assays (Figure 7C & D).

Secondly, we examined *vps27Δ* cells to assess if pre-ESCRT proteins regulate α-synuclein in similar or different ways. Similar to *vps34Δ,* WT and E46K α-synuclein were diffuse through cytoplasm and localized at the plasma membrane through 48 hours of expression, while A30P again did not alter its localization pattern (Figure 8A). Surprisingly, all three forms of α-synuclein compared equally to parent strain in the Western blot (Figure 8B, top). To confirm this lack of accumulation, we assessed protein turnover of WT α-synuclein using loss of induction and consequently saw no change between *vps27Δ* and parent strain (Figure 8B, bottom). Finally, α-synuclein was not toxic as shown through spotting (Figure 8C) and growth curve assays (Figure 8D).

ESCRT I: Lack of vps37 Accumulates α-synuclein

We then examined the two genes encoding the ESCRT I complex, starting with *vps37Δ*. Supporting our predictions, α-synuclein demonstrated a minor change in localization in WT and E46K through 48 hours with less localization at the plasma membrane, while A30P remained the same (Figure 9A). Furthermore, WT α-synuclein showed increased accumulation in *vps37Δ*, as seen through Western blots and loss of induction blots (Figure 9B). Although Western blot did not show accumulation for E46K, loss of induction analysis demonstrated accumulation (Figure 9B, bottom right). Finally, α-synuclein was not toxic as shown through both toxicity assays (Figure 9C & D).

The remaining ESCRT I gene deletion was *vps23Δ*. Unlike *vps37Δ* cells, *vps23Δ* cells had a very subtle change in localization when compared to parent strain, only a minority of cells expressed WT and E46K α-synuclein through the cytoplasm at 48 hours (Figure 10A). Moreover, α-synuclein expression was similar in both *vps23Δ* and parent strain despite unequal loading (Figure 10B). Just as in *vps37Δ*, the expression of α-synuclein did not cause toxicity to the cells (Figure 10C & D).

ESCRT II: vps22 does not affect α-synuclein

Our next goal was to examine the remaining ESCRT II strain: *vps22Δ*. Refuting our hypothesis, WT and A30P did not alter their localization pattern dramatically over 48 hours when compared to parent strain. Some E46K α-synuclein is diffuse within cytoplasm at 48 hours (Figure 11A). α-Synuclein compared equally to parent strain in the Western blot and loss of induction (Figure 11B). Lastly, α-synuclein was not toxic to the cells (Figure 11C & D).

ESCRT III: vps2 Alters α-synuclein Localization and Accumulation

Our last goal was to examine one of two remaining ESCRT III compromised strains, *vps2Δ*, for its three PD-related α-synuclein characteristics. Supporting our belief, both WT and E46K α-synuclein were quantifiably less localized at the plasma membrane at the end of 48 hours of expression, while A30P remained the same (Figure 12A). Moreover, WT

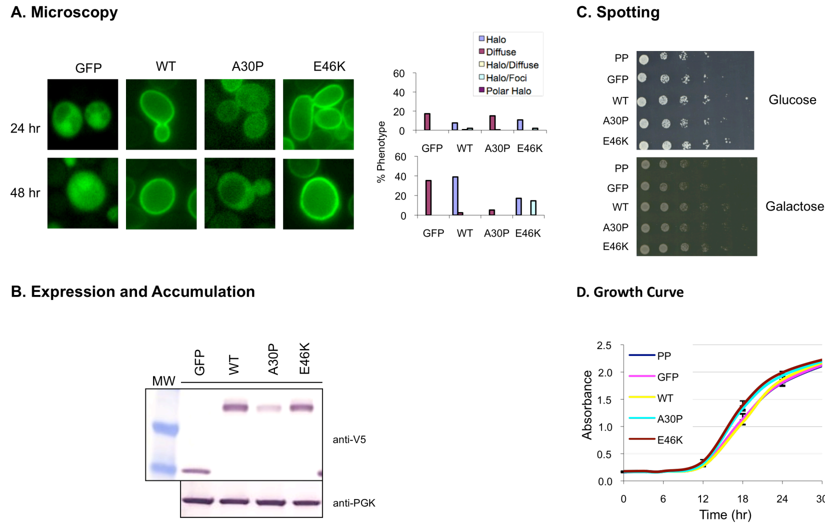


Figure 6: α-Synuclein is membrane bound in parent strain

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. WT and E46K α-synuclein localizes to the membrane. Instead, A30P α-synuclein was diffuse throughout the cytoplasm and shows no membrane localization (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in BY4741 were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting was used to assess the amount of WT and familial mutant α-synuclein present in BY4741 yeast at 24 hrs. WT and E46K expression were similar while A30P expression was reduced (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in BY4741 budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.992, WT, p=0.593, A30P, p=0.085, E46K, p=0.0604, at 24 hours: GFP, p=0.913, WT, p=0.498, A30P, p=0.060, and E46K growing better, p=0.015).

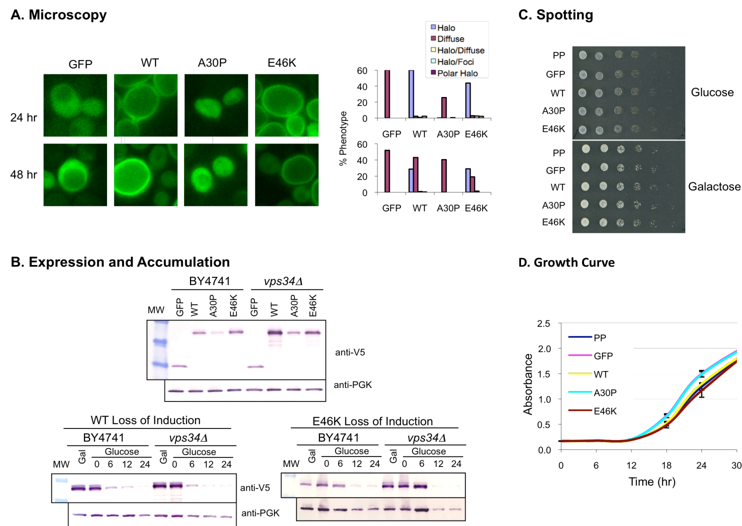


Figure 7: a-Synuclein accumulation and localization changes in *vps34Δ*

A. LEFT α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. WT and E46K α-synuclein were less localized at the plasma membrane at the end of 48 hours of expression. A30P did not alter its localization pattern, and as expected GFP remained unchanged (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps34Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps34Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. WT and E46K α-synuclein both showed increased accumulation in vps34Δ cells, as shown in both Western blots at 24 hours of expression and by loss of α-synuclein induction over the next 24 hours (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps34Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP growing better, p=0.020, WT, p=0.833, A30P growing better, p=0.018, E46K, p=0.699, at 24 hours: GFP growing better, p=0.022, WT, p=0.460, A30P growing better, p=0.018, and E46K, p=0.617

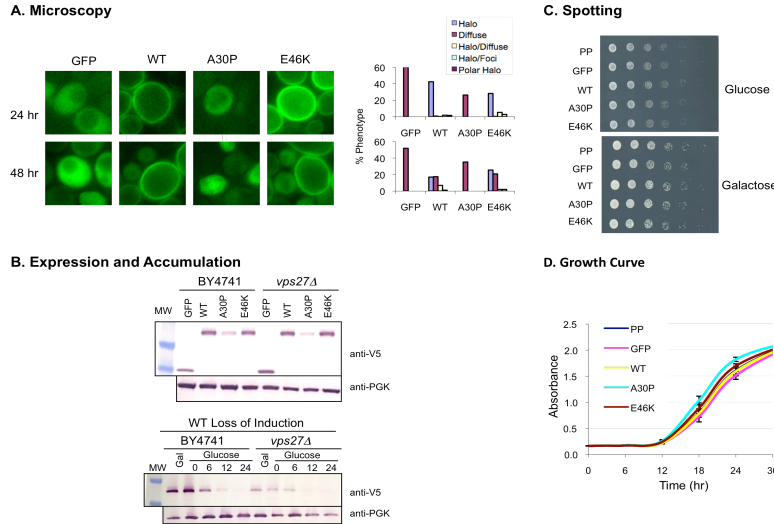


Figure 8: Changes in α-synuclein localization in second pre-ESCRT *vps27Δ*

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. WT and E46K α-synuclein were less localized at the plasma membrane at the end of 48 hours of expression. A30P did not alter its localization pattern (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps27Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps27Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. Surprisingly, all three forms of α-synuclein compared equally to parent strain in the Western blot and WT results were confirmed in loss of induction blot (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps27Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.617, WT, p=0.936, A30P, p=0.064, E46K, p=0.494, at 24 hours: GFP, p=0.432, WT, p=0.897, A30P growing better, p=0.050, and E46K, p=0.384).

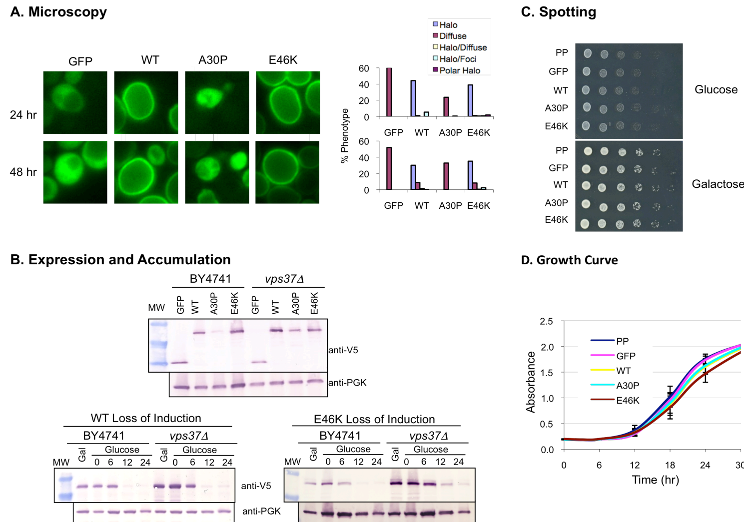


Figure 9: First ESCRT I strain vps37Δ exhibits accumulation

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. α-Synuclein had a minor change in localization in WT and E46K through 48 hours with less localization at the plasma membrane, while A30P remained the same (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps37Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps37Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. WT α-synuclein showed increased accumulation in *vps37Δ,* as seen through Western blots and loss of induction blots. Although Western blot did not show accumulation for E46K, loss of induction analysis demonstrated accumulation (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps37Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.784, WT, p=0.641, A30P, p=0.682, E46K, p=0.504, at 24 hours: GFP, p=0.852, WT, p=0.386, A30P, p=0.439, and E46K, p=0.163).

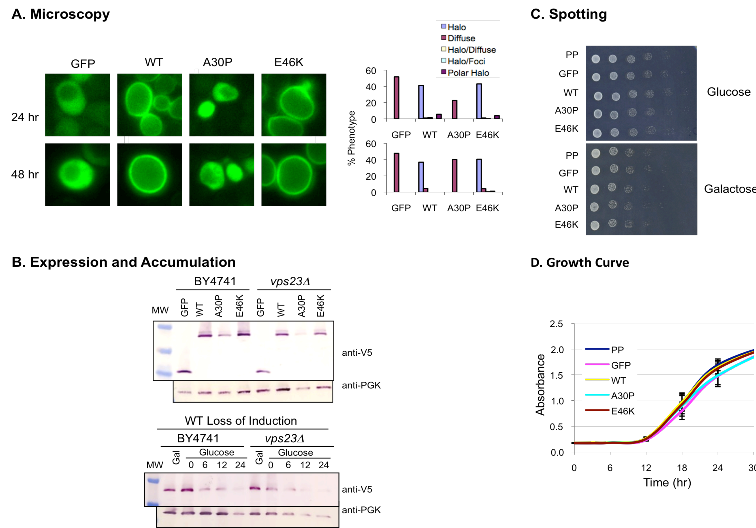


Figure 10: Subtle α-synuclein localization and accumulation in *vps23Δ*

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. *vps23Δ* cells had a very subtle change in localization when compared to parent strain, only a minority of cells expressed WT and E46K α-synuclein through the cytoplasm at 48 hours (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps23Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps23Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. α-Synuclein expression was similar in both *vps23Δ* and parent strain (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps23Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.495, WT, p=0.988, A30P, p=0.785, E46K, p=0.865, at 24 hours: GFP, p=0.303, WT, p=0.806, A30P, p=0.430, and E46K, p=0.691).

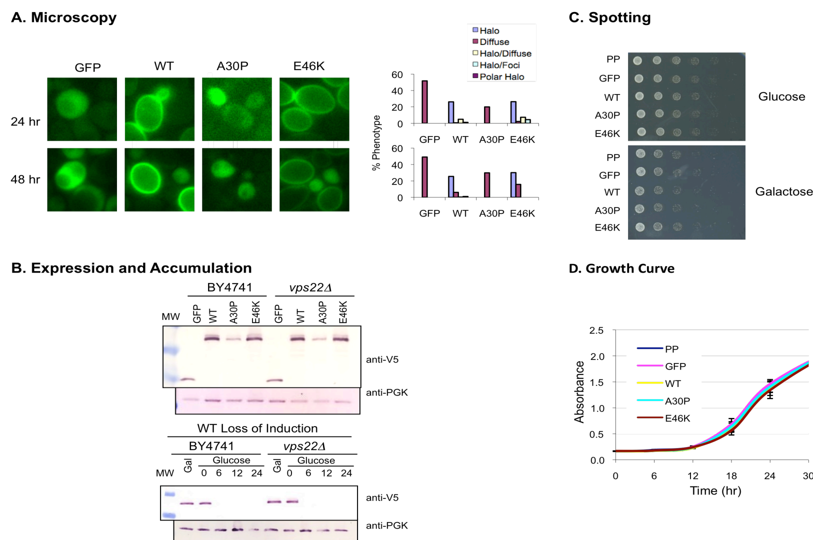


Figure 11: ESCRT II *vps22Δ* exhibit few changes on α-synuclein

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. WT and A30P did not alter their localization pattern dramatically over 48 hours. Some E46K α-synuclein is diffuse within cytoplasm at 48 hours (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps22Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps22Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. α-Synuclein compared equally to parent strain in the Western blot (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3)

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps22Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.486, WT, p=0.760, A30P, p=0.696, E46K, p=0.763, at 24 hours: GFP, p=0.570, WT, p=0.989, A30P, p=0.831, and E46K, p=0.894).

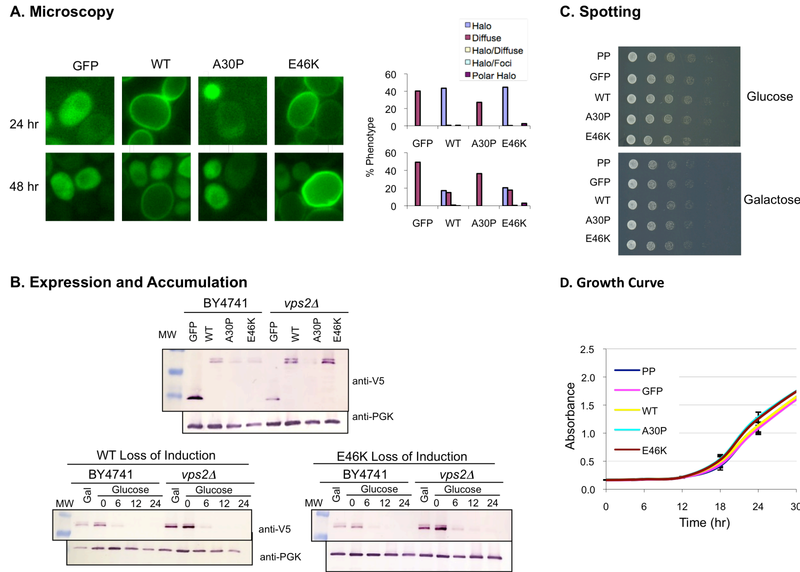


Figure 12: ESCRT III strain *vps2Δ* expresses high localization to cytoplasm

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. WT and E46K α-synuclein were quantifiably less localized at the plasma membrane at the end of 48 hours of expression while A30P remained the same (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps2Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps2Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. WT and E46K α-synuclein showed increased accumulation in *vps2Δ* as demonstrated by Western and loss of induction blots (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps2Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.836, WT, p=0.547, A30P, p=0.307, E46K, p=0.342, at 24 hours: GFP, p=0.896, WT, p=0.851, A30P, p=0.170, and E46K, p=0.316).

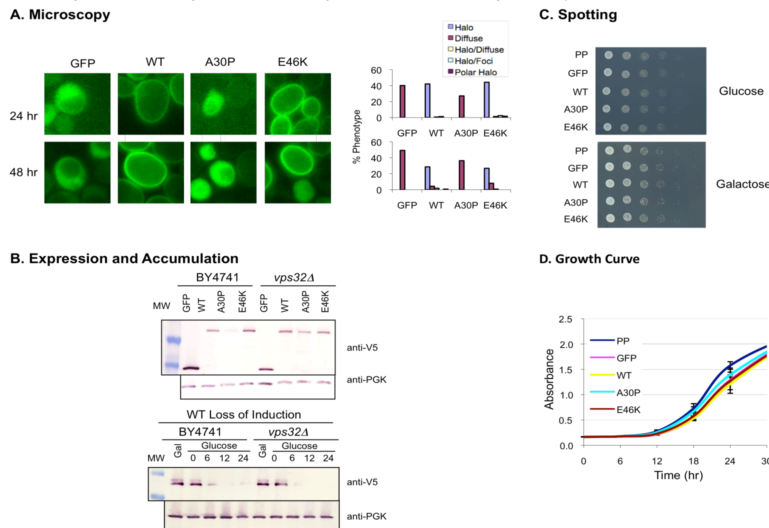


Figure 13: Subtle shift in localization in ESCRT III *vps32Δ* strain

A. LEFT- α-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged α-synuclein at 24 and 48 hrs. *vps32Δ* cells had a subtle change in localization (n=2). RIGHT- Localization quantification of 750 cells of each transformed α-synuclein construct in *vps32Δ* were counted and scored for five different florescence localization patterns: halo, diffuse, halo and diffuse, halo and foci, and polar halo (n=2).

B. Protein expression: Western blotting and loss of induction were used to assess the amount of WT and familial mutant α-synuclein present in *vps32Δ* yeast at 24 hrs. Protein expression was compared to BY4741 parent strain which served as a control. α-synuclein expression was similar in both *vps32Δ* and parent strain BY4741 (n=2).

C. Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, A30P, and E46K showed no impaired growth (n=3).

D. Growth curve: Optical density 600 nm was used to evaluate growth in in *vps32Δ* budding yeast expressing WT, A30P, and E46K α-synuclein (induced in galactose). All α-synuclein expressing cells grew similarly to PP control (n=3, at 18 hours: GFP, p=0.071, WT, p=0.075, A30P, p=0.642, E46K, p=0.250, at 24 hours: GFP, p=0.014, WT, p=0.094, A30P, p=0.261, and E46K, p=0.138).

Table 3: Summary of α-synuclein changes in endocytosis-deficient strains. α-Synuclein toxicity, localization, and accumulation have been evaluated for thirteen total ESCRT protein knockout strains. The strains done as part of this thesis are in bold. The changes were assessed as a strong, weak or none when compared to parent strain.

Strain:	Localization	Toxicity	Accumulation
Pre-ESCRT:			
vps34Δ	**Strong**	**Non Toxic**	**Strong**
vps27Δ	**Strong**	**Non Toxic**	**None**
ESCRT-I:			
mvb12Δ	None	Non Toxic	Weak
vps23Δ	**Weak**	**Non Toxic**	**None**
vps28Δ	Weak	Weak	Weak
vps37Δ	**Weak**	**Non Toxic**	**Strong**
ESCRT-II:			
vps22Δ	**Weak**	**Non Toxic**	**None**
vps25Δ	Weak	Non Toxic	Weak
vps36Δ	Weak	Non Toxic	Strong
ESCRT-III:			
vps2Δ	**Strong**	**Non Toxic**	**Strong**
vps20Δ	Weak	Non Toxic	Strong
vps24Δ	Strong	Toxic	None
vps32Δ	**Weak**	**Non Toxic**	**None**

and E46K α-synuclein showed increased accumulation in *vps2Δ* as demonstrated by Western blots and loss of induction blots (Figure 12B). As usual, the expression of α-synuclein did not cause toxicity to the cells as shown through spotting (Figure 12C) and growth curve assays (Figure 12D).

To finally complete our picture, we examined *vps32Δ,* since it was our remaining ESCRT III gene deletion. Refuting our hypothesis, *vps32Δ* cells had only a subtle change in localization unlike *vps2Δ* (Figure 13A). In accordance to past strains, α-synuclein expression was similar in both *vps32Δ* and parent strain BY4741 (Figure 13B). The expression of α-synuclein did not cause toxicity to the cells as demonstrated by both toxicity assays (Figure 13C & D).

Discussion

Understanding how α-synuclein is degraded is of significant therapeutic value if α-synuclein is the culprit protein in PD. To build on current pharmacological evidence, the field needs more genetic analysis on whether α-synuclein degradation is through the MVB/endosome pathway to the lysosome. The DebBurman lab began evaluating the ability of several ESCRT proteins within the endocytosis pathway to regulate α-synuclein two years ago (Ayala Thesis, 2009). The goal of my thesis was to complete assessment of the remaining ESCRT-I, -II, and -III proteins, as well as the two pre-ESCRT proteins. We report three findings of notable interest. Firstly, my thesis further contributes to the accumulating genetic evidence for the MVB/endosome pathway as a regulator of α-synuclein degradation. Secondly, each gene affected α-synuclein properties to different extents, suggesting substrate specificity for endocytosis steps. Lastly, none of the genes contributed additional α-synuclein-dependent toxicity. Table 3 is a summary of the work accomplished so far within the MVB/endosome pathway by my thesis and that by Alex Ayala. For the future, post-ESCRT proteins remain to be analyzed.

The MVB/endosome Pathway is Involved in Degrading α-synuclein

A first notable finding is that my data supports the hypothesis that α-synuclein is degraded through the MVB/endosome pathway to the lysosome. Of the seven genes I examined, all impacted one or more of the three α-synuclein-PD related properties (Table 3). Specifically, α-synuclein accumulation increased in three strains (*vps34Δ, vps37Δ,* and *vps2Δ*), while α-synuclein localization became more cytoplasmic to different extents in all seven strains. This data adds to previous findings in our lab (Table 3; Ayala Thesis, 2009). Previously, α-synuclein accumulation increased in five of the six strains examined (*mvb12Δ, vps28Δ, vps25Δ, vps36Δ,* and *vps20Δ*). Additionally, an overlapping set of five strains had increased cytoplasmic localization and some formed aggregates *(vps28Δ, vps25Δ, vps36Δ, vps20Δ,* and *vps24Δ).* Lastly, two strains exhibited α-synuclein-dependent toxicity (*vps24Δ* and *vps28Δ*; Table 3). Therefore, our combined work shows that eight of thirteen strains examined changed accumulation while twelve out of thirteen strains affected localization patterns. Our work provides strong genetic support for α-synuclein degradation by the MVB/endocytosis pathway.

The DebBurman lab utilizes a powerful budding yeast model for PD to assess three major PD-related α-synuclein properties: localization, accumulation and toxicity, while most other published models focus on fewer (Table 4). For example, Willingham et al. (2003), when using genome wide yeast screening to discoverer modifiers of α-synuclein properties, only focused on toxicity. Within that study, two MVB pathway genes, *vps24* and *vps28,* enhanced α-synuclein toxicity when deleted, confirmed by Ayala Thesis (2009). Through this work, suggested the MVB/endocytosis route as a possible pathway for α-synuclein degradation. Accumulation and localization were not examined. Our lab saw changes in at least one and sometimes two of the three α-synuclein-PD related properties. We believe our approach is more rigorous and therefore adds additional evidence establishing the ESCRT genes' involvement in α-synuclein degradation.

The fact that most ESCRT deletion genes affected several α-synuclein-PD related properties is unusual since evaluation of other pathways, such as oxidative stress (Brandis Thesis, 2006; Kukreja Thesis, 2008), ubiquitin proteasome system (Herrera Thesis, 2005), chaperones (Shrestha, 2007; unpublished findings), mitochondrial function (Zorniak Thesis, 2007), phospholipid synthesis (Kukreja Thesis, 2008), and autophagy (Choi Thesis, 2009) did not demonstrate such a high degree of regulation for multiple α-synuclein properties. For example, only four of six genes important for the nucleation and expansion steps of autophagy were found to subtly regulate α-synuclein properties when deleted (Choi Thesis, 2009). This suggests a subtler role for autophagy and point to the MVB/endocytosis route as a more likely route for α-synuclein degradation.

An important genetic study with yeast previously demonstrated that MVB proteins interact with YPP1 in targeting A30P α-synuclein to the vacuole (the yeast equivalent of the lysosome) for degradation (Flower et al., 2009). YPP1 is a protein that binds to A30P α-synuclein at the plasma membrane, directing it to the vacuole through endocytosis. YPP1 suppressed accumulation and toxicity of A30P α-synuclein, and the deletion caused accumulation and toxicity (Flower et al., 2009). The MVB genes examined

Table 4: 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.

Yeast models of a-synuclein	Toxicity	Localization	Accumulation
Outerio and Lindquist (2003)	Yes	Yes	No
Willingham et al. (2003)	Yes	No	No
Liang et al. (2008)	Yes	No	No
Gitler et al. (2007)	Yes	No	No
Volles and Lansbury (2007)	Yes	Yes	No
Sharma et al. (2006) & all DebBurman lab theses	Yes	Yes	Yes

in my thesis are essential components in another aspect of endocytosis: the formation of MVBs. Researchers demonstrated that α-synuclein binds to YPP1 endocytic protein for degradation through changes in accumulation and localization when YPP1 was deleted. When I evaluated endocytosis deletion strains, I also saw changes in accumulation and localization, providing further evidence for α-synuclein degradation through endocytosis. What is still puzzling is that in my study we see few A30P α-synuclein changes when the ESCRT machinery is compromised.

Other studies continue to demonstrate and support my findings for α-synuclein degradation by the endocytic pathway. Sung et al., (2001) demonstrated that exogenous α-synuclein uses Rab5, a GTPase involved in the regulation of endocytosis, a to enter the cell, resulting in inclusion formation and cell death. Lee et al., (2007) further demonstrated that endocytosis degrades aggregated forms of extracellular α-synuclein. Moreover, in a genetic screen of over 1,500 genes in C. elegans, ten gene deletions caused α-synuclein toxicity. Out of those ten, four are involved in the endocytic machinery. The genes included apa-2 and aps-2 which are necessary in recruiting clathrin and cargo receptors to the plasma membrane for invagination into endosomes. Data demonstrate that defective synaptic vesicle endocytosis leads to α-synuclein toxicity and accumulation of phosphorylated α-synuclein, again providing a pathogenic link between α-synuclein and endocytosis (Kuwahara et al., 2008). Moreover, another study provided further evidence that aggregated α-synuclein interacts with clathrin during receptor-mediated endocytosis (Liu et al., 2007). The studies above further demonstrate α-synuclein internalization through endocytosis, however our data advances the field in providing specific evidence of α-synuclein interacting with the MVB proteins on the endosome membrane.

α-Synuclein is Regulated by a Subset of ESCRT Proteins

My second notable finding is that while all evaluated genes had an effect on at least one or more α-synuclein PD-related properties, each had a different profile of regulation. While all ESCRT proteins are involved in α-synuclein regulation, most consistent regulation appears to be in the pre-ESCRT component. Strong/weak subtle regulation in ESCRT-I and –III was apparent, and the least amount of regulation occurred in ESCRT-II (Table 3).

Since all the proteins examined are involved in sorting membrane proteins into MVBs, it is possible that some of them play a more significant role in directing α-synuclein into MVBs to be degraded. An important question is whether all substrates for the MVB pathway need to interact with each protein that comprises an ESCRT complex. Surprisingly, not much evidence yet exists. One of the best-known substrates for the MVB pathway, the EGF-R, interacts less with ESCRT-II. In mammalian cells, Bowers et al., (2006) demonstrated that EGF-R degradation was not affected by deletions in ESCRT-II proteins *vps25* and *vps22,* and therefore does not play as large of a role in endosomal transport as the other complexes. This question of α-synuclein specificity continues to be a mystery, since evidence with the human immunodeficiency virus (HIV) receptor Gab, another well-studied endocytosis substrate, comes from examining only a few key proteins in the ESCRT machinery. HIV interacts with membrane-bound Gag protein, which uses Tsg101 (*vps2 in* yeast) to gain access to the rest of the machinery involved in MVB vesicle budding. When Tsg101 was depleted, researchers demonstrated that there was a decrease in the viral proteins released from the cell. Therefore a functional MVB pathway is required for HIV release (Garrus et al., 2001). Additionally, a similar interaction with Tsg101 is seen with another virus, Ebola (Martin-Serrano et al., 2001). Since neither EGF-R nor HIV Gab receptor interact with all ESCRT complex proteins, α-synuclein might interact with certain ESCRT components more than others in its degradation.

Moreover, within one particular ESCRT component, we see varying degrees of changes between each protein in that complex (Table 3). The notion of compensation is important when discussing the specificity of α-synuclein degradation. Perhaps the removal of one specific protein in yeast will not be enough to alter α-synuclein degradation, or any other substrate using the MVB/endocytosis pathway. The remaining proteins in a particular ESCRT complex could compensate for the deletion of one protein. This makes the study of individual gene deletions difficult.

Furthermore, when looking at individual gene deletions, as shown by our data, not one individual gene deletion changed all three α-synuclein PD-related properties examined (Table 3). Each gene deletion had a different phenotype. This might demonstrate that not all pathologically significant properties have to be affected in order to determine the gene's importance in modulating α-synuclein. Past work in our lab parallels this notion. Brandis Thesis (2006) demonstrated that yeast deficient for sod2, a gene involved in oxidative stress response, have consistent toxicity without a shift in localization. Another example of this can be demonstrated in a study where Ypk9 was over-expressed in yeast, here Ypk9 altered localization and suppressed toxicity, however, immunobloting and fluorescence quantifications showed that the over-

expression of ypk9 did not affect α-synuclein levels (Gitler et al., 2009).

Consistent Absence of Toxicity

Our final notable finding is the surprising absence of α-synuclein-dependent toxicity in most strains evaluated (Table 3). We expected to see cells dying or growing slower due to α-synuclein expression because the literature demonstrates such toxicity (Ouitero and Lindqusist, 2004). The same strains that exhibited α-synuclein-dependent toxicity in our lab *(vps24* and *vps28*) were the same ones that Willingham et al., 2003 demonstrated in his genome wide screen to discover α-synuclein modifiers (Table 3; Ayala Thesis, 2009). Willingham et al. (2003) didn't demonstrate toxicity in the rest of the deletion strains we examined. Moreover, toxic deletion strains *vps24* and *vps28* did not exhibit the most dramatic shifts in the other α-synuclein-PD related properties. Other gene deletions expressed strong changes in both localization and accumulation. Therefore, toxicity is not entirely dependent on accumulation and localization changes.

Furthermore, in order to be a regulator of α-synuclein, gene deletions do not always need to alter toxicity. In Willingham et al., (2003), around 4000 genes were examined and only 86 exhibited α-synuclein-dependent toxicity (Table 5A). There are many other genes linked to PD that did not express toxicity in that study, such as PARK9 and YPP1. Therefore, the link between α-synuclein and toxicity is unknown.

The surprising level of toxicity in this study has been reproduced in past studies in our lab (Table 5B). This also demonstrated that our moderate α-synuclein expression model does exhibit α-synuclein-dependent toxicity in certain cases. Perhaps the advantage to our moderate expression model is that it allows us to uncover really strong α-synuclein modifiers.

One suggestion for the lack of toxicity could be attributed to compensation by the other degradation pathway, the proteasome, when the MVB/endosome pathway is compromised. Therefore, this compensation could help in clearing any toxic α-synuclein present. Research demonstrates that the opposite is true. When the proteasome pathway is impaired, autophagy acts as a compensatory system. This was demonstrated in studies with cultured neuronal cells (Rideout et al., 2004) and in flies (Pandey et al., 2007). This compensation could also work visa versa, and instead of compensating for autophagy, compensate for endocytosis. Thus, the two degradation pathways may be interchangeable when needed. Additionally, the shift of α-synuclein to the cytoplasm places the protein in an ideal site for proteasome degradation, and such degradation is well documented.

One last explanation for the absence of toxicity is since the localization of α-synuclein is changing from the plasma membrane, perhaps these cells need a minimum level of α-synuclein at the plasma membrane in order for it to be toxic. Studies show that α-synuclein disrupts normal membrane processes that eventually lead to toxicity (Volles and Lansbury, 2007).

Table 5: Summary of α-synuclein toxicity in yeast deletion strains. A. Number yeast deletion strains that induced α-synuclein-dependent toxicity in 15 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.

A.

Knockout Study	No. of Knockout Strains	Toxicity
Willingham et al., 2003	~4000	86

B.

Specific Study in DebBurman Lab	No. of Strains evaluated	Toxicity	Specific Study in DebBurma Lab	No. of Strains evaluated	Toxicity
N. Sharma, 2004	2	2	S. Herrera, 2005	8	Only 1
J. Price, 2004	1	1	S. Vahedi, 2005	2	None
A. Paul, 2004	1	None	M. Vahedi, 2006	6	None
K. Brandis, 2005	4	Only 1	J. Wang, 2006	2	None
T. Vaidya, 2005	3	None	M. Zorniak, 2007	2	None
R. Shrestha, 2005	3	None	L. Kukreja, 2008	1	None
M. Stevenson, 2005	1	None	R. Choi, 2009	6	Only 1
			A. Ayala, 2009	7	2

Critiques, Limitations, and Future Studies

The biggest limitation in our study is that genetic manipulations don't display what biochemical studies show, the actual interaction between a specific ESCRT protein and α-synuclein. Co-immunoprecipitation or co-immunofluorescence assays would be effective at demonstrating this interaction. Some experiments that could improve and confirm the findings conducted here include vacuolar and vesicle staining. A vacuolar stain could verify that α-synuclein is not transported to the vacuole. A vesicle stain would also be helpful in evaluating integrity of the MVB/endocytosis pathway. Future studies could also focus on actually measuring if proteasome degradation of α-synuclein is increasing during MVB dysfunction. Other studies could examine the combined effects of different ESCRT protein gene deletions with α-synuclein because the combined effects of two compromised genes could result in more drastic effects to cells. It might also be beneficial to study combined MVB dysfunction with compromised proteasomes. Moreover, studies that overexpress certain ESCRT genes could be effective at demonstrating their protective effect on the three α-synuclein PD-related properties. Lastly, future studies need to examine the post-ESCRT proteins and their ability to regulate α-synuclein degradation. Their role in the disassembly and final step of the protein sorting process is crucial.

Conclusion

Currently no treatment for the burden caused by neurodegenerative disorders exists. It is important to study amyloid proteins that form fibrils since current research is hinting that many proteins have the sequence capable of triggering amyloid formation (Schnabel, 2010). The research being done will help in understanding the mechanism by which α-synuclein is removed from PD patients and eventually lead to the development of information that will help in the cure of PD and similar diseases.

This study focused on one of the mechanisms by which α-synuclein is removed from cells. The three significant findings of this study together suggest that the pre-ESCRT and ESCRT I, II, and III steps in the MVB/endosome pathway are involved in degrading α-synuclein, although in an unexpectedly complex way. Results support our hypothesis that the MVB/endosome pathway is a route by which α-synuclein is targeted to the lysosome. While all four ESCRT complexes appear to be involved, not all of their protein components may be contributing to the regulation of α-synuclein degradation.

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