

# Evaluation of $\alpha$ -Synuclein Degradation Pathways in a Budding Yeast Model

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## Neurodegenerative diseases

The nervous system controls and finely tunes most of the behaviors exhibited by animals. This system is made up of two parts: the central nervous system, consisting of the brain and spinal cord and the peripheral nervous system, which coordinates sensory input to the brain and motor output from the brain. The brain, in particular, contains approximately a hundred billion neurons that make several trillion connections to perform daily activities as diverse as sensation, perception, action, thought, motivation, emotions, memory, and homeostasis. However, what happens when these complex connections become dysfunctional or damaged? Unlike most other cells in our bodies, the brain and spinal cord neurons have severely limited abilities to recover from damage and disease, leading to permanent damage.

Disorders that lead to irreversible and progressive deterioration of the nervous system are categorized as neurodegenerative diseases or NDDs (Ross & Poirier, 2004). The most well known of these diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), multiple sclerosis (MS), amyotrophic lateral sclerosis or Lou Gehrig's disease (ALS) and prion diseases. NDDs are an enormous economic burden to society. For instance, the annual cost for AD NDD in the United States (National Institute of Neurological Disorders and Stroke, 2004). Nearly one million people in the United States, and seven to ten million worldwide are afflicted with PD (Parkinson's Disease Foundation, 2012). The estimated health care cost for PD patients in the United States alone is \$25 billion per year (Parkinson's Disease Foundation, 2012).

The common symptoms of PD include bradykinesia, increased muscle tone, resting tremor and abnormal postural righting reflexes (Olanow & Tatton, 1999; Galvin & Trokanowski, 2001). PD is devastating because these symptoms only start to appear when 60%-80% of these dopamine-producing cells are damaged (National Institute of Neurological Disorders and Stroke, 2004). If diagnosed early enough, symptoms can be treated with drugs for eight to fifteen years (The National Institute of Neurological Disorders and Stroke, 2004). However, a permanent cure is yet to be found.

### PD Causes

Both environmental and genetic factors contribute to PD resulting in sporadic (85-90%) and familial (10-15%) forms of PD (Fahn, 2008). The importance of understanding environmental causes of PD first surfaced when young drug addicts developed parkinsonian symptoms shortly after ingesting of MPTP (1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine), a by-product of synthetic heroin (Langston *et al.*, 1983). Furthermore, exposure to rotenone, a chemical used in organic farming as a pesticide (Betarbet *et al.*, 2000), *Helicobacter pylori* infection (Altschuler, 1996), exposure to heavy metals (Calne *et al.*, 1994), and exposure to free radicals (Maguire-Zeiss *et al.*, 2005) have also been linked with sporadic PD. How such

## Introduction

patients alone is 183 billion US dollars (Alzheimer's Disease Facts and Figures Report, 2011). Despite years of extensive research, cures for these neurodegenerative diseases remain elusive yet imperative for overcoming the human loss and economic burden.

Regardless of the diversity of symptoms that NDDs exhibit as a group, they have several important features in common. Firstly, in each NDD, a distinct group of cells die in an age-progressive manner responsible for that specifically cause the unique symptoms for that disease. Secondly, in those dying neurons, one or more proteins misfold and form fibrous aggregates that accumulate inside or outside the cells. This unifying pathology has laid the foundation for the current molecular understanding of NDDs, which are now also called proteinopathies or protein-misfolding diseases (Taylor *et al.*, 2002). Due to such shared pathology, understanding the mechanisms of protein misfolding-linked toxicity in one disease may unlock mysteries for the other NDDs. *My thesis focuses on understanding the molecular mechanisms of protein misfolding-linked toxicity of one such major NDD: Parkinson's disease (PD).*

### Parkinson's Disease

In 1817, in his short essay entitled, "An Essay on Shaking Palsy," James Parkinson first described the major symptoms of PD (Parkinson, 1817). Today, PD is the most common movement disorder and the second most common diverse factors actually cause PD pathology remains poorly understood.

Even though they are rare, genetic cases provide insight into PD, by allowing us to identify the underlying cellular mechanisms of PD. An increasing number of gene mutations are associated with PD. The most common genes include *SNCA* (more commonly known as  $\alpha$ -synuclein; Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998; Zarranz *et al.*, 2004), *Parkin* (Kitada *et al.*, 1998), *UCH-L1* (Liu *et al.*, 2002), *DJ-1* (Bonifati *et al.*, 2003), *PINK1* (Valente *et al.*, 2004) and *LRRK2/PARK8* (Funayama *et al.*, 2002; Paisan-Ruiz *et al.*, 2004). When mutated in patients, some genes cause autosomal-dominant PD (*UCHL-1*, *SNCA*, *LRRK2*), while other genes cause autosomal-recessive PD (*parkin*, *PINK1* and *DJ-1*),

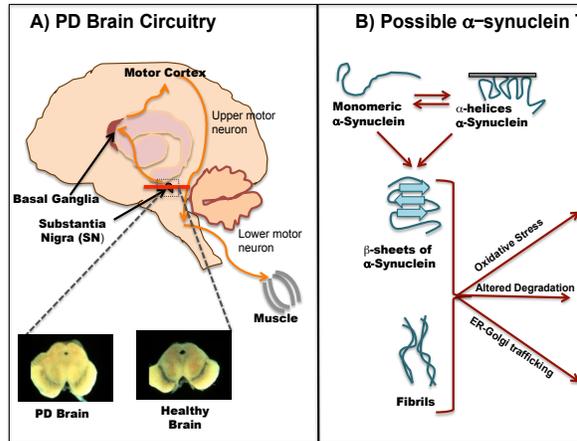
### PD Pathology

The death of dopamine producing cells in a region of the midbrain called the substantia nigra (SN) leads to PD. In a horizontal cross-section of a healthy brain, the SN appears as a dark band due to high levels of neuromelanin found in these dopamine neurons. However, in a PD brain, the dark band disappears due to progressive death of these cells in the SN (Figure 1A). In healthy individuals, controlled dopamine release from the SN results in inhibition of basal ganglia output, which leads to controlled excitation of upper motor neurons, allowing us to coordinate fine voluntary movements. The dysfunction of these inhibitory and excitatory mechanisms creates poorly coordinated slow movements, classifying PD as a hypokinetic movement disorder (Figure 1B; Purves *et al.*, 2008).

Most genetic and sporadic PD cases have protein aggregates within degenerating neurons, which Frederick Lewy first named as Lewy bodies in 1912.

The composition of Lewy bodies remained a mystery until Polymeropoulos and colleagues (1997) found that a mutation in the  $\alpha$ -synuclein gene of human chromosome 4 (A53T) caused autosomal-dominant PD

(Polymeropoulos *et al.*, 1997). This study and several others identified misfolded and aggregated  $\alpha$ -synuclein as the major component that makes up Lewy



**Figure 1: Parkinson's Disease Brain A.** PD brain circuitry: In a healthy brain, controlled dopaminergic effects from the SN inhibit basal ganglia output leading to activation of upper motor neurons that coordinate voluntary movements. In a PD brain, when dopamine neurons in the SN are deteriorated, upper motor neurons are less activated, leading to motor impairment symptoms in PD. **B.** Possible  $\alpha$ -synuclein Toxicity:  $\alpha$ -synuclein exists either in its unfolded monomeric form or as  $\alpha$ -helices bound to lipid membranes. The toxicity is a possible result of  $\alpha$ -helices undergoing conformational changes to form  $\beta$ -sheets and fibrils. Cell death might result from altered cellular mechanisms such as mitochondrial stress, altered vesicular transport and damaged degradation mechanisms.

The image of substantia nigra was retrieved via URL [http://img.medscape.com/fullsize/701/816/58977\\_substantia\\_nigra2.jpg](http://img.medscape.com/fullsize/701/816/58977_substantia_nigra2.jpg)

bodies (Polymeropoulos *et al.*, 1997; Spillantini *et al.*, 1998; Kruger *et al.*, 1998).  $\alpha$ -Synuclein was further identified as a major component of Lewy bodies linked to several other related NDDs, including dementia with Lewy bodies (DLB) and multiple-system atrophy (MSA), all of which are called  $\alpha$ -synucleinopathies (Galvin & Trojanowski, 2001).  $\alpha$ -Synuclein is clearly a central factor that contributes to all forms of PD pathogenesis.

### **$\alpha$ -Synuclein**

$\alpha$ -Synuclein is a small protein (140 amino acids) that belongs to the synuclein protein family, which also includes two other members:  $\beta$ -synuclein and  $\gamma$ -synuclein (George, 2002). Both  $\alpha$ - and  $\beta$ -synuclein are found primarily in brain cells, while  $\gamma$ -synuclein is found in the peripheral nervous system and retina. However,  $\alpha$ -synuclein is the only one of the three found in Lewy bodies (George, 2002).

Since 1997, intense research on the role of  $\alpha$ -synuclein in the nervous system indicates that its main functions include vesicle trafficking in the secretory pathway (Cooper *et al.*, 2006), neurotransmitter release at synaptic terminals (Jensen *et al.*, 1998; Cabin *et al.*, 2002), and maintaining synaptic plasticity by regulating actin dynamics (Bellani *et al.*, 2010).  $\alpha$ -Synuclein's diverse localization patterns in neurons correlate with its many functions. Mainly, this protein is localized in pre-synaptic terminals of neurons (Cabin *et al.*, 2002). It is also found in the cytoplasm (Clayton and George, 1998) and bound to endo-membranes (Davidson *et al.*, 1998) and the plasma membrane (Kahle, 2000; Eliezer *et al.*, 2001). Dixon *et al.* (2005) showed that  $\alpha$ -synuclein associates with the trans-Golgi network (TGN) as well. Unexpectedly,  $\alpha$ -synuclein has most recently been found outside of neurons in human body fluids, such as blood plasma and cerebrospinal fluid (Lee *et al.*, 2008).

The mechanism by which  $\alpha$ -synuclein aggregation is toxic to human neurons is one of the most debated questions in the field. The most well known hypothesis is that, monomeric  $\alpha$ -synuclein aggregates into small oligomers that are stabilized by forming  $\beta$ -sheets (Conway *et al.*, 1998; Spillantini *et al.*, 1998; El-Agnaf *et al.*, 1998; Hashimoto *et al.*, 1999; Narhi *et al.*, 1999). Further, these  $\beta$ -sheets undergo conformational changes to form fibrils (Caughey & Lansbury, 2003; Figure 1B). Deposition of these  $\beta$ -sheets and fibrils might occur later in the development of PD. However, a recent study has demonstrated that  $\alpha$ -synuclein forms helically-folded tetramers, which when destabilized, leads to misfolding and aggregation in PD (Bartels *et al.*, 2011). If this study is further substantiated, our perspective on  $\alpha$ -synuclein will change in the near future.

All three  $\alpha$ -synuclein familial mutations (A30P, A53T, E46K) enhance fibril formation, but not in the same ways. The A30P mutant specifically increases formation of protofibrils, but does not as readily assemble into larger aggregates as WT or A53T mutants, suggesting that the smaller protofibrils may be more toxic (Conway *et al.*, 1998). Many post-translational modifications on  $\alpha$ -synuclein, such as ubiquitination (Shimura *et al.*, 2001), phosphorylation (Okochi *et al.*, 2000; Fujiwara *et al.*, 2002; Fiske *et al.*, 2011) and nitration (Hodara *et al.*, 2004; Solvang

thesis, 2011) are thought to contribute to the pathologic state as well.

$\alpha$ -Synuclein concentration itself is key to PD pathogenesis. Overexpression of  $\alpha$ -synuclein increases cell death and toxicity in mammalian cells (Zhou *et al.*, 2000; Zach *et al.*, 2007; Zhou *et al.*, 2002), yeasts (Outeiro & Lindquist, 2003; Willingham *et al.*, 2003) and nematodes (Lakso *et al.*, 2003; van Ham *et al.*, 2008; Kuwahara *et al.*, 2006). Overexpression of  $\alpha$ -synuclein in mice results in accumulation and motor symptoms, without significant cell death, suggesting that disease does not require cell death (Kahle *et al.*, 2000; Giasson *et al.*, 2002). Most importantly, when  $\alpha$ -synuclein is directly expressed in the SN using viral vectors in mice (St Martin *et al.*, 2007), rats (Lauwers *et al.*, 2003; Yamada *et al.*, 2004) and primates (Kirik *et al.*, 2003) significant cell loss and aggregation occur, as the disease progresses.

The above studies suggest that  $\alpha$ -synuclein concentration leads to toxicity in midbrain neurons. However, disagreements still exist about the extent to which aggregation contributes to toxicity. Many new pathological links between  $\alpha$ -synuclein misfolding and other cellular mechanisms suggest that impairments in these connections, such as the induction of oxidative stress (Olanow *et al.*, 1999; Hsu *et al.*, 2000), altered ER-Golgi trafficking and synaptic vesicle systems (Cooper *et al.*, 2006; Withers *et al.*, 1997), and compromised proteasome and lysosome degradation (Tofaris *et al.*, 2001; Tanaka *et al.*, 2002; Vogiatzi *et al.*, 2008; Kuwahara *et al.*, 2008; Lee *et al.*, 2008; Figure 1B) may also contribute to neurotoxicity.

Firstly,  $\alpha$ -synuclein overexpression and misfolding directly induces mitochondrial deficits leading to oxidative stress (Hsu *et al.*, 2000). The oxidative metabolism of dopamine is a potential source of reactive oxygen species, (Olanow *et al.*, 1999) possibly making the SN vulnerable to damage. Moreover, mitochondrial dysfunction is detected in a proportion of PD patients (Schapira *et al.*, 1989; Mizuno *et al.*, 1989). Additionally, mitochondria are more sensitive to electron transport chain inhibitors like rotenone in the presence of  $\alpha$ -synuclein (Orth *et al.*, 2003). Overall, oxidative stress induced by  $\alpha$ -synuclein may lead to accumulating oxidants that damage many proteins and DNA and eventually trigger cell death (Figure 1B).

Secondly,  $\alpha$ -synuclein binds to synaptic vesicles (Withers *et al.*, 1997; Davidson *et al.*, 1998) and interrupts vesicle transportation. For example, A30P  $\alpha$ -synuclein alters exocytosis of catecholamine containing vesicles in PC12 cells and in chromaffin cells (Larsen *et al.*, 2006).  $\alpha$ -Synuclein overexpression causes a specific physiological impairment of neurotransmitter release in the absence of toxicity (Nemani *et al.*, 2010). In both yeast and mammalian cells,  $\alpha$ -synuclein overexpression alters ER-Golgi trafficking (Cooper *et al.*, 2006; Gosavi *et al.*, 2002; Smith *et al.*, 2005). Therefore,  $\alpha$ -synuclein may result in toxicity by altering the secretory pathway and vesicle transportation.

Finally, many studies have shown that  $\alpha$ -synuclein alters proteasomal-based protein degradation (Tanaka *et al.*, 2002; Snyder *et al.*, 2003; Lindersson *et al.*, 2004; Figure 1B). In addition,  $\alpha$ -synuclein also binds to lysosomal membranes, thus

preventing the functions of lysosome and chaperone-mediated autophagy (CMA; Cuervo *et al.*, 2004). Therefore, high  $\alpha$ -synuclein concentrations may lead to cellular toxicity due to disrupted degradation systems.

Taken together, increased  $\alpha$ -synuclein concentration interrupts many cellular mechanisms, making further investigations of each of these mechanisms critical for better understanding of the molecular basis of PD pathology. *For my thesis, I focused on understanding the connections between  $\alpha$ -synuclein toxicity based on altered cellular protein degradation systems.*

#### **$\alpha$ -Synuclein Degradation**

When proteins are dysfunctional or misfolded beyond repair, cells act efficiently to get rid of them. In eukaryotes, the proteasome and lysosome are the main protein degradation sites. The extent to which  $\alpha$ -synuclein accumulates and forms aggregates is determined by a dynamic equilibrium between its production and its degradation. Therefore, it is important to know how  $\alpha$ -synuclein is degraded.

##### *Proteasomal Degradation of $\alpha$ -synuclein*

The ubiquitin proteasome system (UPS) typically degrades short-lived, misfolded and damaged proteins that function in the cytoplasm and nucleus (Sherman *et al.*, 2001). First, multiple ubiquitin molecules are attached to proteins as markers through a series of reactions mediated by enzymes called ubiquitin ligases (E1, E2 and E3). Next, these marked proteins are degraded at the proteasome (Figure 2). Finally, all the degradation products such as peptide fragments and amino acids are recycled to produce new proteins (Pickart *et al.*, 2000). UPS is the most studied  $\alpha$ -synuclein degradation pathway.

Many lines of evidence indicate that  $\alpha$ -synuclein is degraded at the proteasome.  $\alpha$ -Synuclein directly

binds with the proteasome cap (Bennett *et al.* 1999; Tofaris *et al.*, 2001); proteasome inhibition results in loss of SN neurons (McNaught *et al.*, 2004) and mice with genetically impaired UPS recapitulate many neuropathological and behavioral features of PD (Xie *et al.*, 2010). Direct genetic evidence tied to familial PD is even more compelling. Leroy *et al.* (1998) found a missense mutation in the UCHL1 gene (Ubiquitin carboxy-terminal hydrolase1) to cause familial PD. UCHL1 is a de-ubiquitinating protein, which hydrolyses bonds between ubiquitin and adducts, an important step for recycling of mono-ubiquitin (McNaught *et al.*, 2002). In its absence, labeling of proteins with ubiquitin for degradation is reduced (McNaught *et al.*, 2002). Furthermore, another familial PD mutant gene the parkin was identified. Parkin is a ubiquitin ligase (E3) that ubiquitinates misfolded  $\alpha$ -synuclein (Kitada *et al.*, 1998). Non-ubiquitinated form of  $\alpha$ -synuclein accumulates in dopamine neurons, in the absence of parkin (Shimura *et al.*, 2001; Zhang *et al.*, 2000). Two other familial PD-causing mutants, Pink1 and DJ-1, form a complex with parkin to alter parkin-dependent ubiquitination and degradation of substrates (Xiong *et al.*, 2009; Tang *et al.*, 2006; Moore *et al.*, 2005). Due to strong evidence, proteasome was thought as the major and only  $\alpha$ -synuclein degradation site, for a long time. However, evidence for lysosomal degradation of  $\alpha$ -synuclein is now more evident.

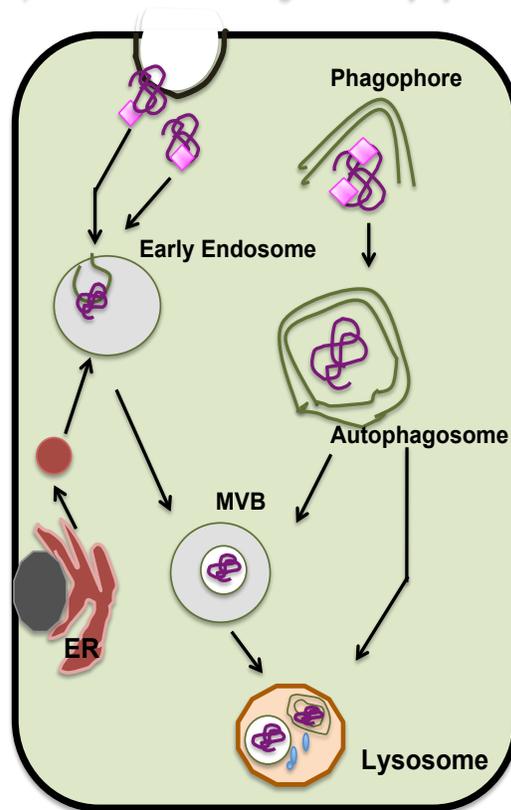
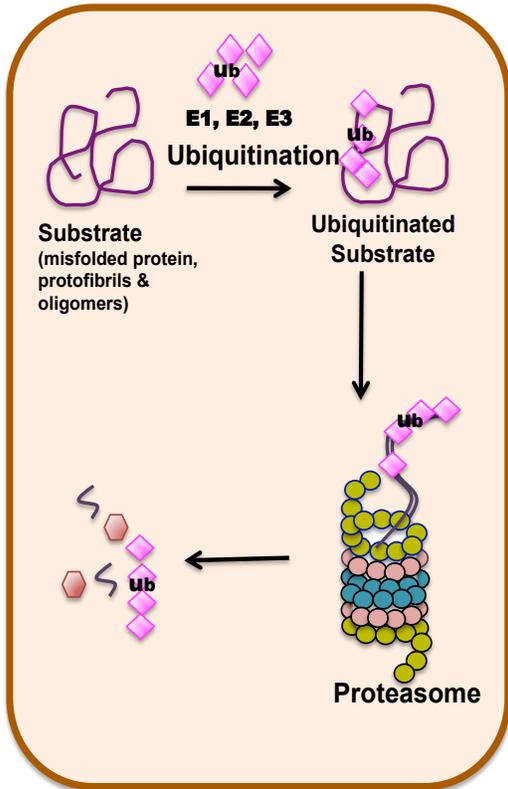
##### *Lysosomal degradation of $\alpha$ -synuclein*

The other main degradation organelle of eukaryotic cells is the lysosome, which contains acidic hydrolases that break down all forms of cellular macromolecules and pathogens (Rubinsztein, 2006). Three main routes direct cargo to the lysosome for degradation: phagocytosis, endocytosis and autophagy (Aderem & Underhill, 1999; Mellman, 1996; Yormitsu & Klionsky, 2005).

Autophagy is the “self-cannibalism” mechanism that recycles long-lived proteins in a cell (Yormitsu & Klionsky, 2005). Several lines of pharmacological and genetic evidence provide support for use of this route for a-synuclein degradation. Inhibition of autophagy

**Figure 2: Cellular degradation mechanisms:** A. Misfolded protein degradation by proteasome: misfolded proteins are polyubiquitinated through a

A) Misfolded Protein Degradation by Proteasome      B) Misfolded Protein Degradation by Lysosome



series of reactions mediated by ubiquitin ligase enzymes E1, E2, and E3 and are directed to the proteasome for degradation **B.** Misfolded protein degradation by the lysosome: misfolded or non-functional proteins are directed to the lysosome either by autophagy, where a membrane is formed around a targeted region of the cell, separating the contents from the rest of the cytoplasm, which eventually fuses with the lysosome for degradation or by endocytosis, where the lipid membrane forms a pocket, which then pinches off into the cell to form a vesicle inside the endosome that fuses with the lysosome for degradation.

leads to accumulation of both wild-type and familial mutants of a-synuclein (Vogiatzi *et al.*, 2008, Anglade *et al.*, 1997; Stefanis *et al.*, 2001). In PC12 cells, autophagy activation reduces a-synuclein aggregates (Webb *et al.*, 2003). Autophagy routes a-synuclein to the lysosome and the A53T mutant blocks this pathway (Cuervo *et al.*, 2004; Vogiatzi *et al.*, 2008). Finally, previous DebBurman lab members have shown that autophagy genes regulate a-synuclein in yeast models for PD (Choi thesis, 2009; Konnikova thesis, 2011). Due to autophagy's involvement in many neurodegenerative diseases, it has been explored extensively as an a-synuclein degradation route.

**Endocytosis**

Endocytosis is also known as the MVB (multi vesicular body) or ESCRT (Endosomal Sorting Complex Required for Transport) pathway. This pathway maintains cell equilibrium by internalizing, transporting, sorting, and degrading macromolecules with the help of protein complexes called ESCRTs (Mellman, 1996). Proteins from the plasma membrane, trans-Golgi network (TGN) and cytoplasm are selectively taken into the endosomal compartments. These endosomes eventually fuse with the lysosome

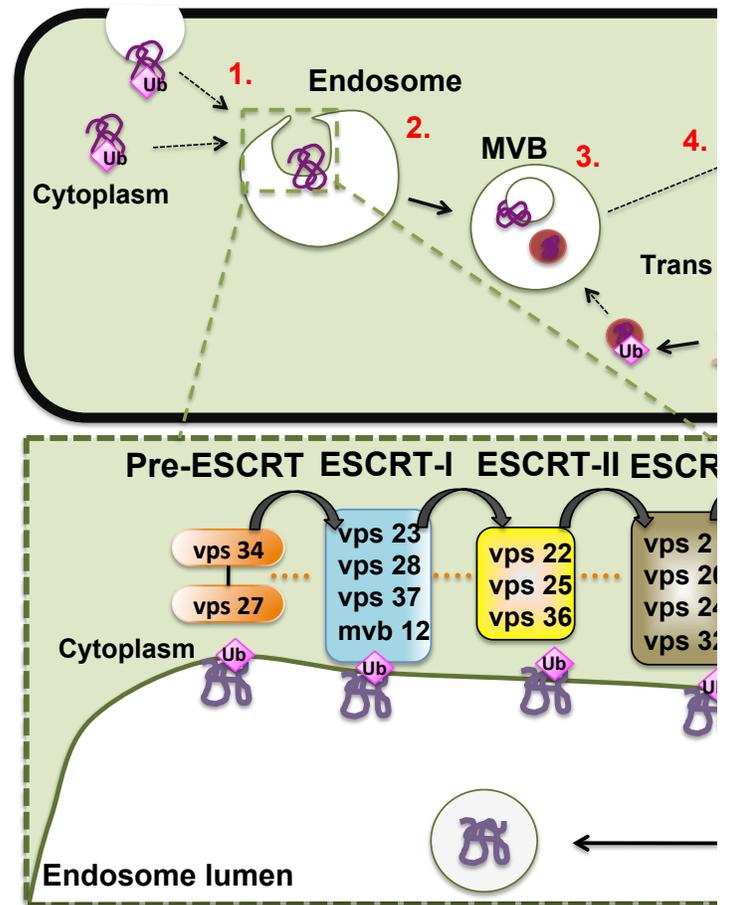
Since phagocytosis is the process of internalizing and directing pathogens (such as bacteria and viruses) to the lysosome for degradation (Aderem & Underhill, 1999), it is unlikely to function as an a-synuclein degradation route and will be excluded from this discussion. Evidence for both autophagy and endocytosis will be discussed below.

**Autophagy**

for degradation (Mellman, 1996; Figure 3). In this highly selective process, K63-linked ubiquitin tag substrates and directs them to the lysosome for degradation (Raiborg *et al.*, 2003; Katzmann *et al.*, 2001). Endocytosis is one of the most conserved pathways from yeast to humans.

In yeast and humans, the endocytosis pathway comprises of class E Vps (vacuolar protein sorting) proteins. At least seventeen of these proteins function together as complexes (pre-ESCRT, ESCRT-I, ESCRT-II, ESCRT-III and post-ESCRT; Katzman *et al.*, 2001; Babst *et al.*, 2002a; Babst *et al.*, 2002b). These ESCRT complexes recognize ubiquitinated cargo and deform the endosome membrane, allowing the cargo to be internalized within vesicles (MVBs) that form within the endosome lumen. The ESCRT-III complex recruits post-ESCRT proteins, Vps4 and Doa4 to disassemble and release the membrane, forming these MVBs (Figure 3; Katzmann *et al.*, 2001; Babst *et al.*, 2002). The most well known functions of endocytosis are internalization of food and maintaining plasma membrane composition (Mellman, 1996). However, endocytosis is also involved in many other functions.

The ESCRTs protect against disorders such as cancer, bacterial infections and neurodegeneration. For instance, endocytosis controls Epidermal Growth Factor Receptor (EGFR) concentration, which is key to controlled cell growth and proliferation. Defective endocytosis of this receptor leads to excessive signaling by EGFR, resulting in



**Figure 3: The endocytosis pathway** TOP: First, the substrate proteins for the endocytosis route are recruited from the plasma membrane, trans ER-Golgi network, and the cytoplasm. Second, ubiquitin is removed and cargo is taken into the endosome. Third, vesicles are formed inside the endosomes, which eventually matures into MVBs. Finally, these MVBs are fused with the lysosome for degradation. BOTTOM: Ubiquitinated cargo is recognized with the help of the protein complexes (the pre-ESCRT, ESCRT-I, ESCRT-II, ESCRT-III and the post-ESCRT) that comprise the endocytosis pathway.

cancer (Fedler *et al.*, 1990). ESCRT proteins also control Notch signaling involved in animal growth patterning (Radtko & Raj, 2003). In the absence of ESCRT protein Vps25, Notch receptors accumulate and induce cell proliferation (Chao *et al.*, 2004; Reynolds-Kenneally & Mlodzik, 2005). Mycobacteria that cause tuberculosis evade phagocytosis in order to replicate (Philips *et al.*, 2008). However, ESCRTs are capable of controlling mycobacteria replication, either

by supporting autophagy or by mediating the fusion of phagosomes, containing mycobacteria with the lysosome, further suggesting the involvement of ESCRT mediated degradation (Phillips *et al.*, 2008).

Importantly, various NDDs are linked to compromised endocytosis. Endosomal abnormalities precede the established pathological markers of b-amyloid plaques and neuro fibrillary tangles (NFTs) in AD (Keating *et al.*, 2006). Missense mutations in the ESCRT-III protein Vps2 develop amyotrophic lateral sclerosis (ALS; Parkinson *et al.*, 2006) and frontotemporal dementia (FTD; Skibinski *et al.*, 2005). Finally, functional Vps24 is required for autophagic clearance of the HD-linked misfolded Huntington protein (Filimonenko *et al.*, 2007). Given these links, the role of endocytosis in PD, and especially its involvement in a-synuclein degradation is worth further examination.

### Endocytosis and PD

Several recent studies have strengthened the link between PD and endocytosis. Deletion of three MVB pathway genes, *vps28*, *vps60* and *vps24* cause a-synuclein dependent toxicity in a yeast model (Willingham *et al.*, 2003). Flower *et al.* (2007) showed a gene, which they named *YPP1*, routes A30P a-synuclein to the yeast lysosome by interacting with many endocytosis proteins. Moreover, in a worm model of PD, four endocytic gene deletions worsen motor abnormalities and cause accumulation of a-synuclein (Kuwahara *et al.*, 2008). Endocytosis also directs extracellular a-synuclein that is present in cerebrospinal fluid and blood plasma to the lysosome for degradation (Lee *et al.*, 2008). These studies provided early evidence for a link between endocytosis and a-synuclein regulation.

However, a comprehensive analysis of endocytosis sorting proteins in regulating a-synuclein was still lacking when Jessica Price '06, Mithaq Vahedi '08, Alex Ayala '09 and Jaime Perez '10 initiated a long-term molecular genetic study of endocytosis in our lab. They analyzed how three a-synuclein pathological properties (accumulation, localization, and cellular toxicity) were affected in the absence of individual genes that make the proteins of ESCRT complexes Pre-ESCRT, ESCRT-I, ESCRT-II, and ESCRT-III. They analyzed effects of thirteen individual ESCRT gene deletions and found nine of them to effect a-synuclein localization, eight to effect accumulation and one to show consistent toxicity (Table 1). However, they had not analyzed the genes that controlled the final post-ESCRT step. As *Chapter One of my thesis*, I filled this gap in knowledge and analyzed the post-ESCRT step in regulating pathological properties of a-synuclein.

### Combined inhibition of endocytosis and autophagy

To date, several labs, including our own, have shown that both the proteasome and lysosome can regulate a-synuclein (McNaught *et al.*, 2004; Xie *et al.*, 2010; Lee *et al.*, 2008; Cuervo *et al.*, 2004; Sharma *et al.*, 2006; Perez thesis, 2010; Konnikova thesis, 2011). Emerging evidence suggests that the proteasomal and lysosomal pathways can regulate and even compensate for one another. Holen *et al.* (1995) showed that chemical inhibition of both autophagy and endocytosis in rat hepatocytes result in accumulation of

endosomes and reduction of degradation of specific substrates. Some studies have found a close relationship between autophagy and endocytosis pathways (Berg *et al.*, 1998; Eskelinen, 2005). The number of autolysosomes decreases, leading to decreased degradation via lysosome in the absence of ESCRT proteins in mouse cortical neurons (Lee *et al.*, 2007) and *Drosophila* larvae (Rusten *et al.*, 2007). Furthermore, ESCRTs are now known to be involved in autophagosome fusion (Rusten & Stenmark, 2009). Therefore, a logical prediction is that inhibition of more than one lysosomal route should further elevate a-synuclein pathological properties, but no existing studies have examined this possibility. *For Chapter Two of my thesis*, I addressed this gap in knowledge and assessed a-synuclein pathological properties under deficient endocytosis and autophagy conditions in yeast.

### Evaluation of compensatory responses

A cell responds to stress induced by misfolded or aggregated proteins in two major ways. It either tries to fold proteins back into their original shape or get rid of the irreparable or excessive proteins (Muchowski & Wacker, 2005). Therefore, the induction of ubiquitin-tagging and heat shock responses may represent the activation of cellular defenses to protect against protein misfolding induced toxicity in cells. In yeast models of PD, it is unclear if cells activate such systems when a-synuclein misfolding induces toxicity. *In chapter three of my thesis*, I addressed this gap in knowledge by examining heat shock and ubiquitin responses in a-synuclein expressing cells compromised for autophagy and endocytosis.

### Ubiquitination response

One of the major pathological features of PD is the presence of ubiquitinated a-synuclein in Lewy bodies in neurons. As discussed earlier, ubiquitination directs cargo to both the lysosome and the proteasome (Pickart *et al.*, 2000; Raiborg *et al.*, 2003; Katzmann *et al.*, 2001). However, the exact type of ubiquitin modification recognized by each pathway is different (Korolchuk *et al.*, 2010). For instance, UPS recognizes K48-linked ubiquitin chains (Long *et al.*, 2008), while lysosomal degradation pathways recognize K63-linked chains (Lauwers *et al.*, 2009). Given that a-synuclein uses both degradation pathways, inhibition of one pathway may activate the other as a compensatory mechanism. The literature indicates that such compensations do occur. Impairment of the UPS causes upregulation of autophagy, as a compensation mechanism in *Drosophila* (Pandey *et al.*, 2007) and in human colon tumor cell lines (Ding *et al.*, 2007). Activation of autophagy protect against cell death caused by proteasome inhibition in both cell and mouse models (Pan *et al.*, 2008). Impaired autophagy also results in impaired degradation of specific UPS substrates (Korolchuk *et al.*, 2009; Qiao *et al.*, 2009). However, whether such compensation occurs in PD models is not well examined. *The first goal of Chapter Three of my thesis was to assess the activation of ubiquitination responses in a-synuclein expressing cells that were impaired for lysosomal degradation.*

### Heat Shock Response

Under a-synuclein-induced stress coupled with

deficient degradation mechanisms, cells might also initiate a heat shock protein response. Heat shock proteins (Hsps) are found in all organisms and are among the most highly conserved family of proteins between bacteria and humans (Lindquist, 1986). When protein homeostasis is challenged, these proteins are activated and engage mainly in folding proteins back into their correct shape and preventing their aggregation. This large family of proteins is classified based on molecular weight, and its major members include Hsp27, Hsp40, Hsp70, Hsp90 and Hsp104 (Muchowski & Wacker, 2005).

Overexpression studies on Hsp70, Hsp40, and Hsp27 have demonstrated protective effects of Hsps in animal models of neurodegenerative diseases (Muchowski & Wacker, 2005). Hsp70 overexpression protects neurons from  $\beta$ -amyloid-mediated toxicity in models of AD (Margrane *et al.*, 2004; Smith *et al.*, 2005). In addition, Hsp70 overexpression significantly reduces the toxicity mediated by  $\alpha$ -synuclein in PD mice (Klucken *et al.*, 2004). Furthermore, Hsp90 co-localizes with  $\alpha$ -synuclein filaments of Lewy bodies and its expression is increased in the PD brain in correlation with the change of insoluble  $\alpha$ -synuclein (Uryu *et al.*, 2006). Despite several established yeast models for PD, whether or not  $\alpha$ -synuclein expressing yeasts cells induce the heat shock response is not known. *Therefore, the second goal of Chapter Three of my thesis was to assess heat shock protein response in lysosomal degradation altered  $\alpha$ -synuclein expressing yeast strains.*

#### **Model Organism: Budding Yeast, *Saccharomyces cerevisiae***

I have chosen budding yeast as my model organism because it provides fundamental insights into basic eukaryotic biology and many human diseases, including cancer (Nass and Prezdborski, 2008). Importantly, budding yeast has been an exceptional models that provide new insights into several major NDDs, including PD (Outeiro and Lindquist, 2003), polyglutamine disorders such as HD (Meriin *et al.*, 2002), AD (Treich *et al.*, 2011), and TDP43-linked ALS (Johnson *et al.*, 2008). Most relevant to my thesis, the genes for autophagy and endocytosis were not only discovered in budding yeast, but also are highly conserved from yeast to humans (Katzman *et al.*, 2001; Klionsky & Emr, 2000). Practically, budding yeast is inexpensive, easy to manipulate genetically and fast to reproduce.

Our lab uses budding yeast as a model to study  $\alpha$ -synuclein biology and PD-linked pathology (Sharma *et al.*, 2006; Fiske *et al.*, 2011a; Fiske *et al.*, 2011b). We express  $\alpha$ -synuclein at moderate levels in budding yeast, which is non-toxic to budding yeast cells, and it localizes predominantly to the plasma membrane (Sharma *et al.*, 2006; Fiske *et al.*, 2011; Figure 6). We have previously utilized single gene deletion strains of budding yeast to study both endocytosis and autophagy (Ayala Thesis, 2009; Choi thesis; 2009; Perez thesis, 2010; Konnikova thesis, 2011).

#### **Study one: Regulation of $\alpha$ -synuclein by Post-ESCRT proteins**

*Hypothesis & Aims:* I hypothesized that post-ESCRT

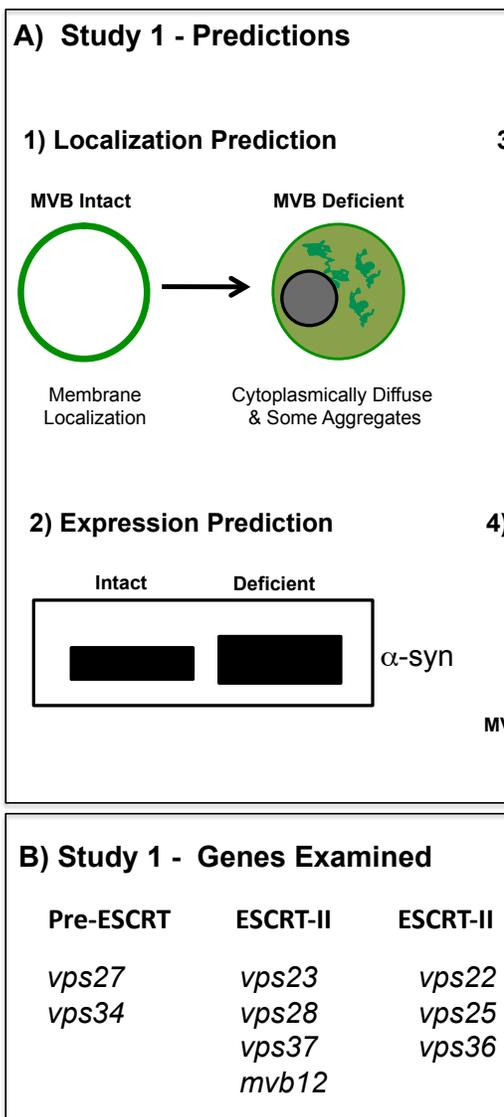
genes regulate  $\alpha$ -synuclein localization, accumulation, and toxicity. My aim was to assess how individual deletion of post-ESCRT genes (*vps4 $\Delta$* , *vps60 $\Delta$* , *doa4 $\Delta$* , *vta1 $\Delta$* ) affects these three pathological properties in budding yeast (Figure 4).

*Predictions and main findings:* I predicted that deletion of each of the above genes would result in one or more of the following changes: change in localization, increased accumulation, or induced toxicity (Figure 4). I report three main findings: First, all four-gene deletions affected at least two pathological properties of  $\alpha$ -synuclein. Second, not all gene deletions affected each property to the same extent, suggesting substrate specificity in binding. Third, I did not observe  $\alpha$ -synuclein dependent toxicity in any of the post-ESCRT gene deletion strains.

#### **Study two: Combined inhibition of endocytosis and autophagy**

*Hypothesis and Aims:* I hypothesized that the double inhibition of autophagy and endocytosis would further elevate  $\alpha$ -synuclein accumulation, change  $\alpha$ -synuclein localization and enhance toxicity. My aim was to assess these properties in chemically (and temporarily) inhibited autophagy in five different endocytosis deletion strains (*vps34 $\Delta$* , *vps28 $\Delta$* , *vps37 $\Delta$* , *vps2 $\Delta$* , and *vps4 $\Delta$* ).

*Predictions and main findings:* I expected at least one elevated  $\alpha$ -synuclein pathological property in the doubly inhibited yeast strains (Figure 5). I report increased toxicity and  $\alpha$ -synuclein accumulation in one endocytosis deletion strain (*vps28 $\Delta$* ) under impaired lysosomal degradation.



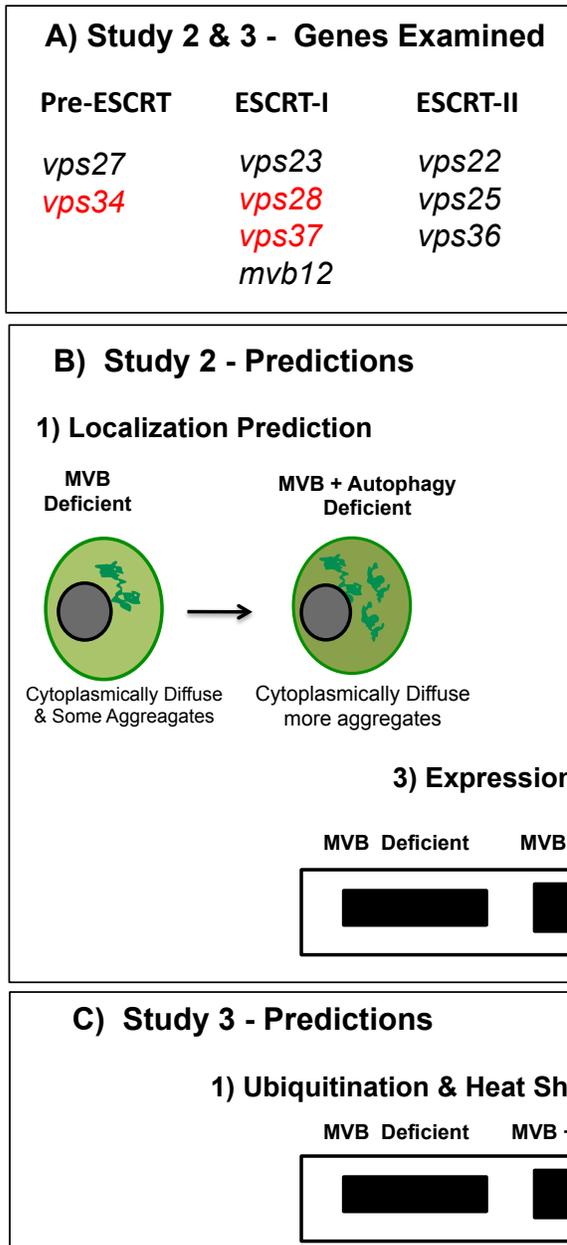
**Hypothesis and Aims:** I hypothesized that yeast would compensate for deficient lysosomal regulation of  $\alpha$ -synuclein by activating proteasomal degradation and activating heat shock response. My aim was to measure ubiquitination levels (as a molecular marker for selective degradation) and activation of those Hsps most closely linked to NDDs (Hsp 70 and Hsp40) using ubiquitin- and Hsp-specific antibodies in strains (*vps34 $\Delta$* , *vps28 $\Delta$* , *vps37 $\Delta$* , *vps2 $\Delta$* , and *vps4 $\Delta$* ) where both autophagy and endocytosis are inhibited.

**Predictions and Main Findings:** I predicted increased ubiquitination and Hsp70/40 levels in the doubly inhibited strains (Figure 5). I do not yet have consistent findings to report as most of my time was spent optimizing protocols for the various antibodies.

**Figure 4: Study One: Predictions and genes examined.** A. Predictions for endocytic regulation of  $\alpha$ -synuclein: I induced  $\alpha$ -synuclein expression in both the endocytosis intact and the endocytosis deficient yeast. The endocytosis intact strain should degrade  $\alpha$ -synuclein via the endosome-lysosome route, leading to healthy cells. On the other hand, we predict some, but not all endocytosis deficient yeast will show: 1. Change in localization 2.  $\alpha$ -synuclein dependent toxicity 3. Increased  $\alpha$ -synuclein accumulation, and 4. Decreased degradation.

B. A table showing the genes that make ESCRT-I, II and III and the post-ESCRT complexes. The genes examined in this study are shown in red.

**Study three: Evaluation of compensatory responses**



**Figure 5: Study Two & Three: Predictions and genes examined.** **A.** A table showing the genes that make ESCRT-I, II and III and the post-ESCRT complexes. The genes examined in Chapter Two and Three are shown in red. **B.** I induced  $\alpha$ -synuclein expression in endocytosis deficient strains and in both endocytosis and autophagy deficient yeast strains. I predict some, but not all, lysosomal degradation deficient yeast will show increased 1) change in localization 2) toxicity and 3) expression. **C.** Furthermore, increased ubiquitination and heat shock response will be seen in lysosomal degradation deficient strains.

**Table 1: Summary of  $\alpha$ -synuclein changes in endocytosis deficient strains.** Adapted from Thesis of Jaime Perez'10.

Strain	Localization	Toxicity	Expression
<b>Pre-ESCRT</b>			
<i>vps34</i> $\Delta$	Yes	None	Yes
<i>vps27</i> $\Delta$	Yes	None	None
<b>ESCRT-I</b>			
<i>vps23</i> $\Delta$	No	None	None
<i>vps28</i> $\Delta$	Yes	Toxic	Yes
<i>vps37</i> $\Delta$	Yes	None	Yes
<i>mvb12</i> $\Delta$	No	None	Yes
<b>ESCRT-II</b>			
<i>vps22</i> $\Delta$	Yes	None	None
<i>vps25</i> $\Delta$	Yes	None	Yes
<i>vps36</i> $\Delta$	Yes	None	Yes
<b>ESCRT-III</b>			
<i>vps20</i> $\Delta$	No	None	Yes
<i>vps24</i> $\Delta$	Yes	None	None
<i>vps32</i> $\Delta$	No	None	None
<i>vps2</i> $\Delta$	Yes	None	Yes

## Materials and Methods

All of the following methods were adapted from Sharma et al (2006) and Fiske et al (2011). However, I have described the methods briefly below.

### *a-Synuclein vectors*

The vectors for this study pYES2.1, green fluorescent protein (GFP), Wild-type  $\alpha$ -synuclein (WT) and E46K mutant  $\alpha$ -synuclein have been created earlier as described in Fiske et al (2011).

### *Yeast Strains*

The budding yeast parent strain BY4741 (mat a) and MVB deletion strains *vps34 $\Delta$* , *vps28 $\Delta$* , *vps37 $\Delta$* , *vps2 $\Delta$* , *vps4 $\Delta$* , *vps60 $\Delta$* , *doa4 $\Delta$* , *vta1 $\Delta$*  were purchased from yeast ORF collection at open biosystems. Each Post-ESCRT vps deletion strain was transformed with four plasmid vectors (Table 2). The rest of the strains had been transformed by previous lab members (Ayala thesis, 2009; Perez thesis, 2010).

### *Yeast Transformation*

$\alpha$ -Synuclein expression plasmid vectors were transformed into yeast strains, as described in Burke et al (2000). Yeast cells were selected by growing them on synthetic-complete media that lacks uracil (SC-URA). Polymerase chain reaction (PCR) confirmed the presence of  $\alpha$ -synuclein vector.

### *Yeast a-Synuclein Expression*

The galactose-inducible promoter (GAL1) of the pYES2.1 vector, allowed for regulated  $\alpha$ -synuclein expression. For all the experiments, yeast cells were first grown overnight in SC-URA glucose (2%) at 30°C and at 200 rpm. Cells were washed twice with water and diluted to a concentration of  $5 \times 10^6$  cells/ml in SC-URA galactose (2%) media to induce  $\alpha$ -synuclein expression and grown to the desired time points.

**Experimental Controls:** The pYES2.1 alone, GFP in pYES2.1, wild-type in pYES2.1, and E46K in pYES2.1 inserted into the MVB pathway intact parent isogenic BY4741 strain served as positive controls. Parent plasmid (pYES2.1/PP) was used to determine whether the vector alone had an effect on the strain. GFP in the pYES2.1 was used to show that expression of GFP to visualize was not harmful to the cells.

I used four yeast assays, Western analysis, GFP live-cell microscopy, Serial dilution spotting, and Survival assay to assess the  $\alpha$ -synuclein properties in budding yeast. GFP live cell microscopy was used to visualize the localization of  $\alpha$ -synuclein. Western analysis was used either to assess expression and accumulation or loss of accumulation over time of  $\alpha$ -synuclein.  $\alpha$ -Synuclein dependent toxicity was analyzed using either Serial dilution spotting assay or Survival assay. Each one of these assays is described in brief below.

### *Western Analysis*

Budding yeast cells were harvested from SC-Ura+galactose media at  $2.5 \times 10^7$  cells/ml concentration at each desired time point. Cell pellets were washed

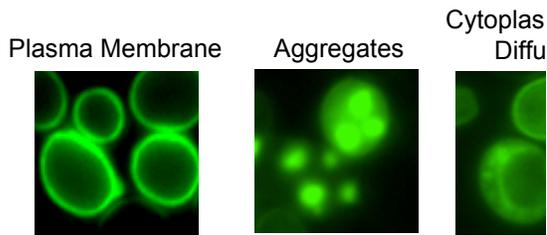
with 100 mM Na<sub>3</sub> and solubilized in electrophoresis sample buffer (ESB; Burke et al., 2000). ESB was prepared using 2% sodium dodecyl sulfate (SDS), 80 mM Tris (pH 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/ml bromophenol blue, and a mixture of protease inhibitors and solubilizing agents, which included 1% Triton-X 100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 1 mM sodium orthovanadate, 0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 10 mg/ml E64, 2mg/ml aprotinin, and 2 mg/ml chymostatin. Samples were then electrophoresed at 130 volts on a 10-20% Tris-Glycine gel (Invitrogen) with 1X SDS running buffer. SeeBlue (Invitrogen) served as the standard molecular ladder.

After running the SDS gels, they were equilibrated in 1x SDS transfer buffer and transferred to Polyvinylidene fluoride (PVDF) membranes using the semi-dry transfer method.  $\alpha$ -Synuclein was detected using a mouse monoclonal anti-V5 (Invitrogen) followed by an anti-mouse secondary antibody. To detect ubiquitination, K48 and K63 linkage specific monoclonal antibodies (Cell Signaling) were used followed by an anti-rabbit secondary antibody. Heat shock protein activation was detected using Hsp70 and Hsp40 monoclonal antibodies provided by Elizabeth Craig at the University of Wisconsin-Madison. Anti-phosphoglycerokinase (PGK; Molecular probes) was used as a loading control. The visualizing solution contained NBT (nitro-blue tetrazolium chloride), BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) and alkaline phosphatase buffer. At least two thirds of the trials, with similar PGK levels, had to be consistent for acceptance as valid data.

### *GFP Microscopy*

A density of  $2.0 \times 10^7$  cells/ml was transferred to 25 ml of sc-Ura+galactose. At each specific time point, 1 ml of cell culture was removed and pelleted at 5000 rpm for one minute. 900  $\mu$ l of supernatant was removed. A sample of 4  $\mu$ l was taken from the remaining 100  $\mu$ l and observed under the Nikon TE2000-U fluorescent microscope. Metamorph 4.0 software was used to collect and quantify ~750 cell images. Fluorescence images were assessed for phenotype (see below). Next, the percentages of cells expressed each phenotype was calculated (N=2). Given its localization to the plasma membrane in both wild-type and E46K, phenotypes other than the plasma membrane localization indicated a pathological property of change in  $\alpha$ -synuclein localization. Depending on the percentage of cells with the plasma membrane localization, the alteration was categorized as a strong change (if cells retain  $\leq 40\%$  plasma membrane localization), a moderate change (if cells retain  $\geq 40\%$  but  $\leq 80\%$  plasma membrane localization) or no change (if cells retain  $\geq 80\%$  of plasma membrane localization).

I used a slightly different procedure to visualize  $\alpha$ -synuclein localization in both autophagy and endocytosis compromised strains. After 18 hours of  $\alpha$ -synuclein expression, autophagy was chemically inhibited with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) treatment and images were collected and quantified 6 hours after the treatment using the same procedure as above (N=2).



#### Spotting Assay

Yeast cells were cultured in 10 ml SC-Ura+glucose at 30°C and 200 rpm. To collect cells, yeast were pelleted at 1500xg for 5 minutes at 4°C. Cells were washed twice with 5 ml of H<sub>2</sub>O, re-suspended in 10 ml H<sub>2</sub>O, and counted using a hemocytometer to determine cell density. 2.0x10<sup>6</sup> cells/ml were removed and pelleted. The supernatant was removed, and cells were resuspended in 1 ml of H<sub>2</sub>O. Cells were diluted 10-fold (5X) in a 96 well microtiter plate and spotted onto SC-Ura+glucose and Sc-Ura+galactose growth plates. Pictures of the ScUra+glucose and Sc-Ura+galactose plates were taken at 24 hours and 48 hours respectively using an HP Canoscan scanner. Images were imported into Adobe Photoshop CS3 (N=3).

#### Survival Assay

I utilized the survival assay to determine both growth (colony size) and toxicity (colony number) in autophagy and endocytosis compromised strains. The cultured yeast cells were counted using a hemocytometer and a density of 2.0x10<sup>7</sup> cells/ml was transferred to 25 ml of sc-Ura+galactose. 12 hours after a-synuclein induction, the cells were treated with 0.1 mM PMSF (autophagy inhibitor). 18 hours after induction, cell density was determined. 1.8x10<sup>7</sup> cells/ml were removed and washed once with 1 ml of H<sub>2</sub>O. The cells were re-suspended in 1ml of H<sub>2</sub>O, and diluted 1:1000. 55.5 ul of diluted cells (500 cells) were spread on large plates containing SC-Ura+Galactose and SC-Ura+Glucose (N=3). Independent samples t-test was used to run statistics on colony number (SPSS software).

#### Loss-of-Induction

After growing cells overnight, the cells were counted using a hemocytometer to determine a cell density of 2.0x10<sup>7</sup> cells/ml and these cells were inoculated and cultured in flasks containing 25 ml SC-Ura+galactose. After 24 hours, lysates were prepared as described in the Western Analysis. The yeast cells were then pelleted at 1500xg for 5 minutes at 4°C. They were washed three times with 5 ml H<sub>2</sub>O, and a cell density of 2.0x10<sup>7</sup>/ml was inoculated in flasks containing 25 ml of Sc-Ura+glucose. Lysates were prepared at 0, 6, 12, and 24 hours after inoculation in Sc-Ura+glucose (a-synuclein repressing media). The

Western analysis was carried out to detect the protein. (N=2)

#### Statistical Analysis

Western blotting was conducted for consisted results of three times with consistent PGK levels on each blot. GFP microscopy was conducted at least two times with consistent results. If the two trials looked different, I performed a third trial and then performed quantification of all of the trials as described earlier. Using Metamorph 4.0, 700 DIC pictures were counted and corresponding fluorescence images were assessed for phenotype and percentages of cells expressing that phenotype were determined. After quantification of each trial, an average was taken. More trials are needed to perform statistical significance tests. However, percentage change in localization was taken into consideration to interpret data. Spotting assay was repeated for consistent results of three times (N=3). For the survival assay, the difference between colony numbers was analyzed using independent samples t-test using SPSS software.

**Table 2. List of transformed budding yeast strains used in my thesis.** The other strains used in my study were transformed by previous lab members (Ayala thesis, 2009; Perez thesis, 2010)

No cDNA (Parent Plasmid)	pYES2.1	BY4741 <i>vps4Δ</i> <i>vps60Δ</i> <i>doa4Δ</i> <i>vta1Δ</i>
GFP	pYES2.1	BY4741 <i>vps4Δ</i> <i>vps60Δ</i> <i>doa4Δ</i> <i>vta1Δ</i>
Wild-type- a-synuclein-GFP	pYES2.1	BY4741 <i>vps4Δ</i> <i>vps60Δ</i> <i>doa4Δ</i> <i>vta1Δ</i>
E46K- a-synuclein-GFP	pYES2.1	BY4741 <i>vps4Δ</i> <i>vps60Δ</i> <i>doa4Δ</i> <i>vta1Δ</i>

**Chapter 1: Post-ESCRT Genes of Endocytosis  
Regulate  $\alpha$ -Synuclein Pathology**

## Results

**Experimental set-up:** The role of post-ESCRT genes in regulating a-synuclein properties was assessed in strains with individual gene deletions that code for the post-ESCRT proteins (*vps4Δ*, *vps60Δ*, *doa1Δ* and *vta1Δ*). Each of these strains and the endocytosis-intact strain (BY4741) were transformed with four plasmid expression vectors: vector alone (pYES2), vector with green fluorescent protein (GFP), vector with wild-type a-synuclein attached to GFP, vector with E46K a-synuclein attached to GFP (Table 1). Cells containing pYES2 and GFP vectors served as negative controls as they did not express a-synuclein. I examined three PD-related properties in a-synuclein expressing cells. First, a-synuclein localization was determined using live-cell GFP Fluorescence Microscopy. Second, cellular a-synuclein was determined using Western blotting, which detects specific cellular protein levels. Third, a-synuclein-dependent toxicity was assessed using a ten-fold serial dilution assay (Spotting). Results for each of these assays will be discussed below.

### *a-Synuclein properties in endocytosis-intact strain*

First, I analyzed a-synuclein properties (localization, accumulation and toxicity) in the endocytosis-intact BY4741 strain (Figure 6). In subsequent experiments, the extent to which each of these properties in individual gene deletion strains differed from that of the control strain indicated involvement of post-ESCRT proteins in regulating a-synuclein (Figure 6). Both wild-type and E46K a-synuclein localize to the plasma membrane in more than 90% of the yeast cells over 48 hours of expression (Figure 6A). Western blotting indicated both wild-type and E46K a-synuclein were expressed at similar levels over this time period (Figure 6B). Finally, spotting assays showed that individual expression of either wild-type or E46K a-synuclein alone was not toxic to yeast cells (Figure 6C). These data recapitulated published findings from our lab (Sharma *et al.*, 2006).

### *a-Synuclein localization altered in all post-ESCRT deletion strains*

I first analyzed the localization patterns of wild-type (Figure 7) and E46K a-synuclein in live cells (Figure 8) for each post-ESCRT gene deletion strain over a 48-hours. My analysis was both qualitative and quantitative (Figures 7 and 8). In support of my hypothesis, all four genes regulated a-synuclein localization, as less a-synuclein was at the plasma membrane and more shifted to the cytoplasm in each gene deletion strain, occasionally forming intracellular aggregates at the cytoplasm (Figures 7 and 8). Representative live-cell GFP images are shown for both wild-type (Figure 7A) and E46K a-synuclein (Figure 8A). Both wild-type and E46K a-synuclein showed similar patterns of localization over 48 hours in all of the deletion strains as discussed below.

The deletion of *Vps4* produced the strongest change in wild-type a-synuclein localization, where only 37% and 20% of cells retained plasma membrane localization by 24 hours (Figure 7B) and 48 hours (Figure 7C), respectively. This pattern was also seen in the *doa4Δ* and the *vps60Δ* strains, with only 30% and 32% cells respectively, retaining plasma membrane

localization after 48 hours (Figures 7B and 7C). In the *vta1Δ* strain, however, wild-type a-synuclein took longer to shift to the cytoplasm, with 68% of cells still at the plasma membrane after 48 hours (Figures 7B and 7C). Similar to wild-type a-synuclein, the E46K mutant showed the most altered localization in the *vps4Δ* strain, followed by the *doa4Δ* and the *vps60Δ* strains, with the least change in the *vta1Δ* strain.

### *a-synuclein accumulates in all individual post-ESCRT deletion strains*

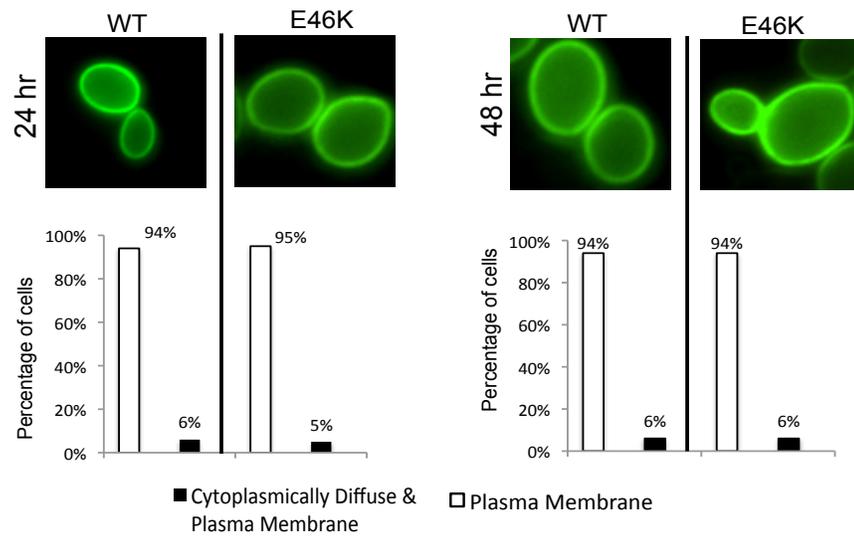
Next, I compared a-synuclein concentration in individual post-ESCRT deletion strains to that of the control strain (Figure 9A). At 24 hours, wild-type expression was higher only in the *vps4Δ* strain. After 48 hours, a-synuclein expression increased in all deletion strains. However, each strain was affected to a different extent (Figure 9A top panel). In contrast, E46K a-synuclein expression was initially increased in all deletion strains, but this increase disappeared in the *vta1Δ* strain after 48 hours (Figure 9A bottom panel). In summary, all deletion strains showed increase in a-synuclein expression within the 48-hour time period.

In order to determine whether this increase in a-synuclein concentration was due to less degradation, I conducted loss-of-induction experiments in two deletion strains that showed the most accumulation (*vps4Δ* and *doa4Δ*). This assay measures how much a-synuclein remains in cells over time after its expression is turned off. I compared a-synuclein levels over 24 hours in these strains with BY4741 cells (Figure 9B). Both wild-type and E46K a-synuclein disappeared more slowly in the *vps4Δ*, indicating impaired degradation. In the *doa4Δ* strain, wild-type and E46K a-synuclein was additionally maintained at higher levels throughout the time course, also supporting impaired degradation.

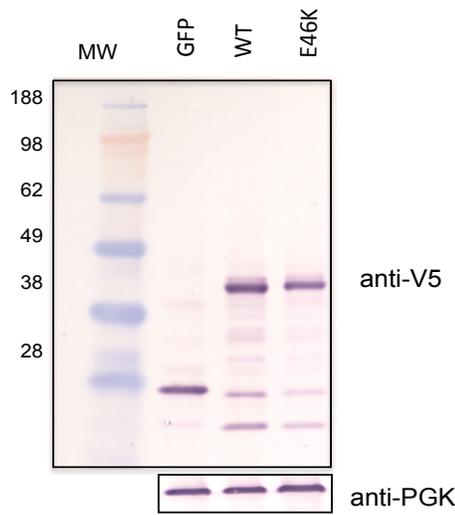
### *Absence of a-synuclein toxicity in Post-ESCRT deletion strains*

Even though the deletion of individual post-ESCRT proteins affected both localization and expression of a-synuclein, it did not result in a-synuclein-dependent toxicity in any of the deletion strains with wild-type or E46K a-synuclein (Figure 10). However, I observed a media-dependent decrease in growth in both the *vps4Δ* and the *doa4Δ* strains. This decrease indicates that, while these genes might not be essential for yeast survival, they impact growth variably in different carbon sources.

### A. Localization



### B. Expression



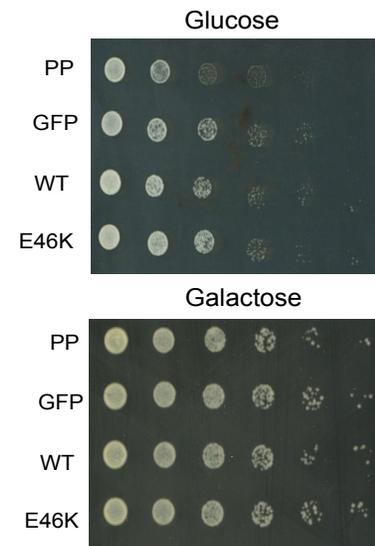
**Figure 6: Analysis of  $\alpha$ -synuclein properties in the BY4741 strain**

**A.** Localization: endocytosis intact strain was visualized using fluorescence microscopy at 24 and 48 hours in wild-type and E46K. Approximately 750 cells were quantified (N=2).

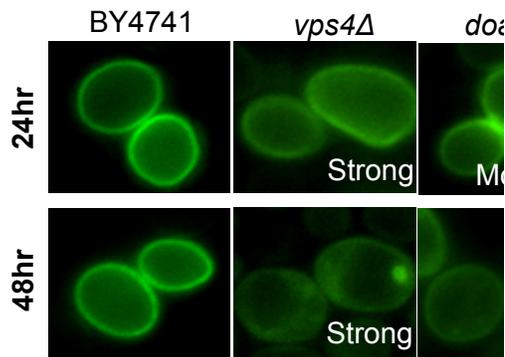
**B.** Accumulation: Western blotting was

used to evaluate the expression of  $\alpha$ -synuclein at 24 and 48 hours comparing the wild-type and E46K  $\alpha$ -synuclein constructs to GFP (N=2). **C.** Toxicity: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). The abbreviations are as follows: PP (parent vector); GFP (green fluorescent protein); wild-type and E46K (N=3).

### C. Toxicity

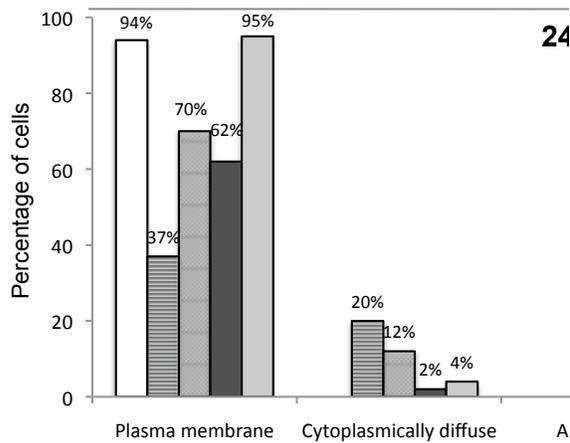


### A. Qualitative Representation

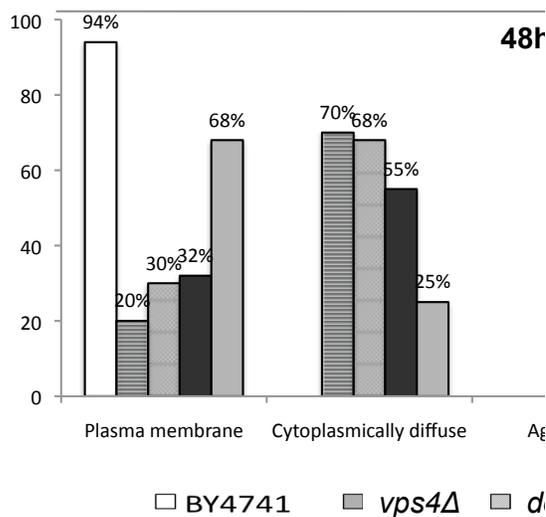


**Figure 7: Microscopy and quantification of wild-type  $\alpha$ -synuclein in the post-ESCRT deletion strains:** **A.** Microscopy images of  $\alpha$ -synuclein localization at 24 and 48 hours.  $\alpha$ -Synuclein tagged with GFP was visualized using fluorescence microscopy. **B.** Quantification of approximately 750 cells of each of the deletion strains at 24 hours (N=2). **C.** Quantification of approximately 750 cells of each of the deletion strains at 48 hours (N=2). Depending on the percentage of cells with the plasma membrane localization phenotype, the alteration was categorized as a strong change (if cells retain  $\leq 40\%$  plasma membrane localization), moderate change (if cells retain  $\geq 40\%$  but  $\leq 80\%$  plasma membrane localization) and no change (if cells retain  $\geq 80\%$  of plasma membrane localization).

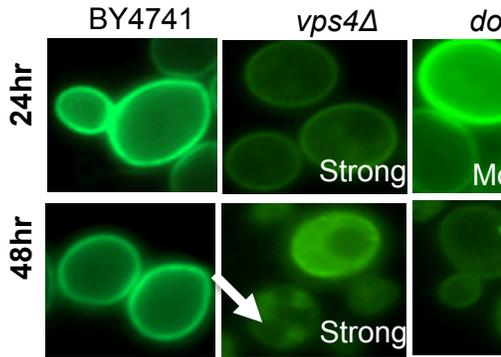
### B. Quantification of WT $\alpha$ -Syn



### C. Quantification of WT $\alpha$ -Syn

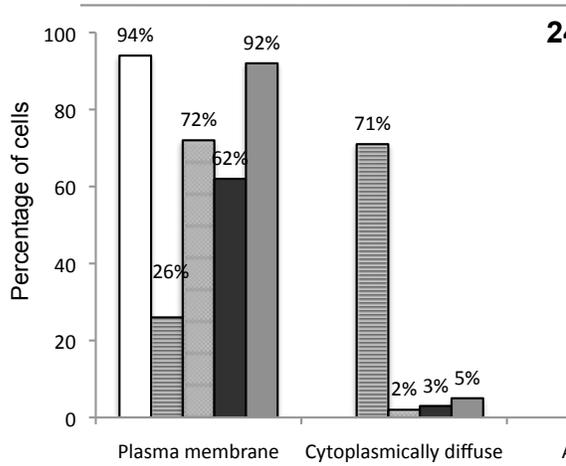


### A. Qualitative Representation

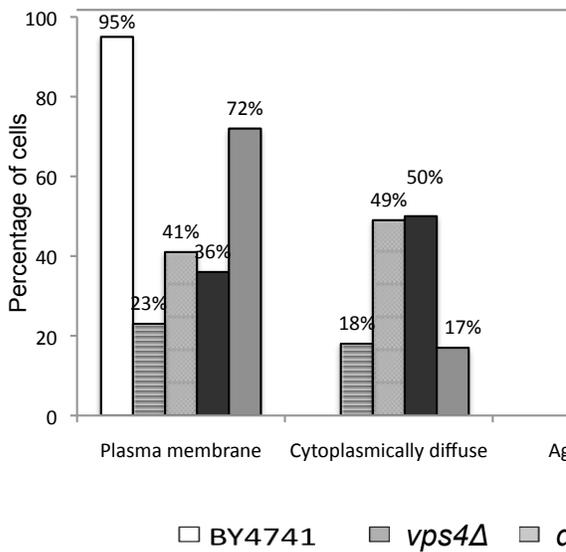


**Figure 8: Microscopy and quantification of E46K  $\alpha$ -Synuclein in the post-ESCRT deletion strains:** **A.** Microscopy images of  $\alpha$ -synuclein localization at 24 and 48 hours in deletion strains.  $\alpha$ -Synuclein tagged with GFP was visualized using fluorescence microscopy. **B.** Quantification of approximately 750 cells of each of the deletion strains at 24 hours (N=2). **C.** Quantification of approximately 750 cells of each of the deletion strains at 48 hours (N=2). Depending on the percentage of cells with the plasma membrane localization phenotype, the alteration was categorized as a strong change (if cells retain  $\leq 40\%$  plasma membrane localization), moderate change (if cells retain  $\geq 40\%$  but  $\leq 80\%$  plasma membrane localization) and no change (if cells retain  $\geq 80\%$  of plasma membrane localization).

### B. Quantification of E46K $\alpha$ -Syn

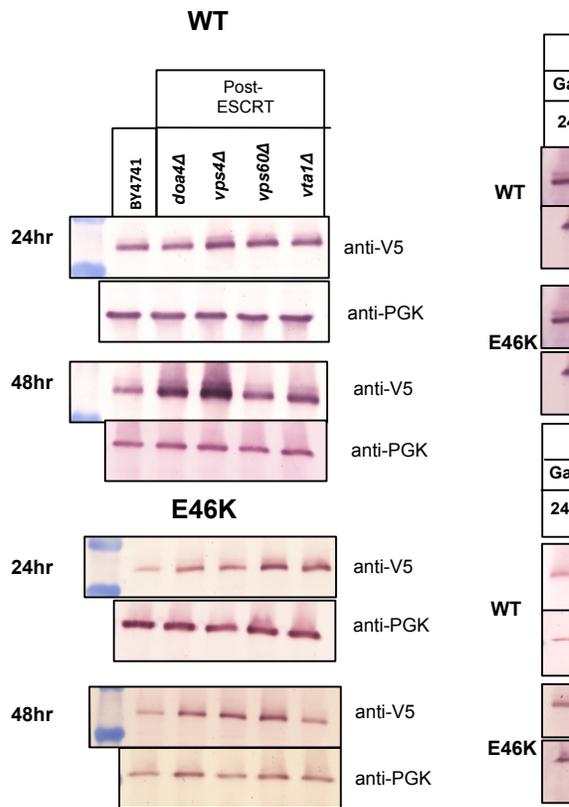


### C. Quantification of E46K $\alpha$ -Syn

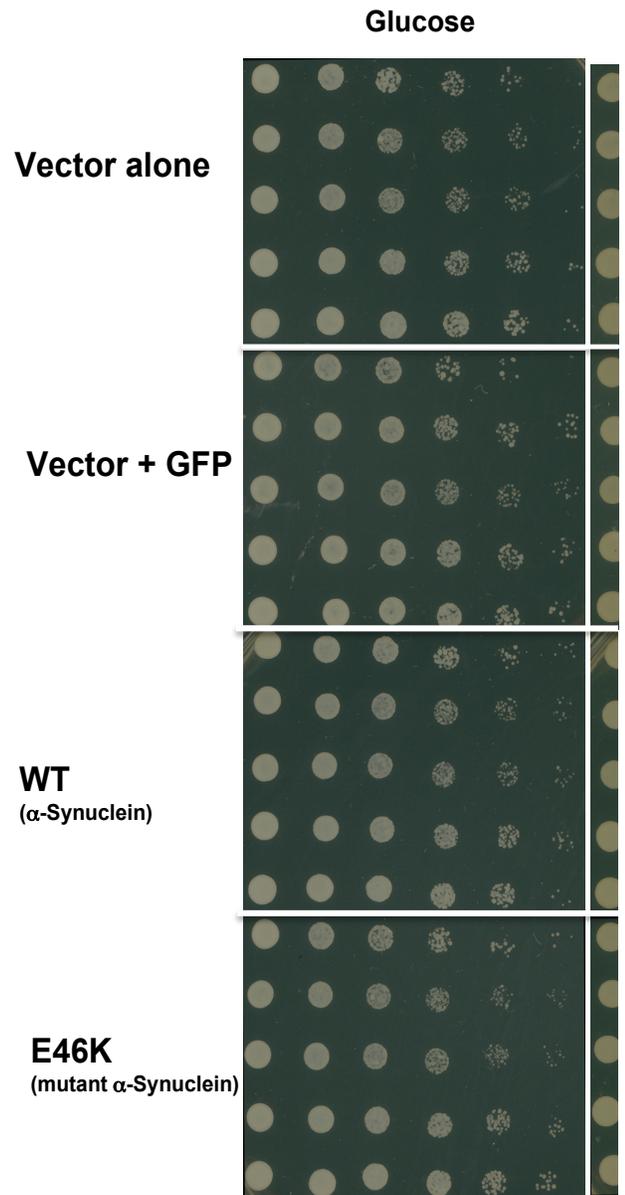


□ BY4741    ■ *vps4* $\Delta$     ■ do

## A. Accumulation



**Figure 9: Accumulation and loss of induction of the post-ESCRT deletion strains:** **A.** Accumulation: Western blotting was used to evaluate the expression of  $\alpha$ -synuclein at 24 and 48 hours by comparing the deletion strains to the endocytosis intact parent strain BY4741 (N=3) **B.** Loss of induction assays were carried out in strains (*vps4* $\Delta$  and *doa4* $\Delta$ ) that showed more accumulation compared to the BY4741 to assess the loss of accumulation of  $\alpha$ -synuclein over time (N=3).



**Figure 10: Toxicity analysis of the post-ESCRT deletion strains:** Spotting: Ten-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). The *vps4* $\Delta$  and the *doa4* $\Delta$  show media dependent and  $\alpha$ -synuclein independent slow growth. Plasmid vector and GFP vector served as controls (N=3).

## Discussion

NDDs are characterized by the accumulation of abnormal protein aggregates in affected brain regions and impairment of protein degradation processes that may contribute to toxic protein accumulation. Due to its complex localization, a-synuclein degradation is important to understand in PD pathogenesis, which will help in designing future PD therapies. More than four years ago, the DeBBurman lab initiated an analysis of endocytosis as a lysosomal route that regulates a-synuclein degradation. In this first chapter of my thesis, I completed this ongoing study by analyzing the involvement of the post-ESCRT genes in endocytosis in regulating several PD related properties of a-synuclein.

I share three notable findings that support my hypothesis: (1) All four post-ESCRT proteins regulate a-synuclein's PD related properties; (2) These post-ESCRT proteins affect each pathological property of a-synuclein to different extents, suggesting possible substrate specificity; (3) I find a surprising lack of a-synuclein-dependent toxicity. Together, past work from our lab and my work strengthens genetic support that endocytosis is a likely route for a-synuclein degradation at the lysosome. To date, my colleagues and I have examined seventeen endocytosis genes and found that thirteen effect a-synuclein localization, twelve regulate its accumulation, and one causes consistent a-synuclein dependent toxicity (Table 3). Each of these findings will be discussed in more detail below.

### *Post-ESCRT genes regulate a-synuclein pathological properties*

All four post-ESCRT gene deletion strains I examined, altered a-synuclein localization and increased its accumulation. However, none of them resulted in toxicity. Previously, our lab had shown similar lines of evidence for the pre-ESCRT, ESCRT-I, ESCRT-II and ESCRT-III steps (Table 3), where a-synuclein localization was the most widely affected change, followed by accumulation. The least affected property was toxicity. My study completes the first full examination of all major endocytosis genes in regulation of these three specific a-synuclein pathological properties.

I observed that a-synuclein significantly shifted from the plasma membrane to the cytoplasm in all post-ESCRT deletion strains. In most of the deletion strains, intracellular aggregates were not dominant, although present. This striking localization pattern suggests either that a-synuclein still forms aggregates in the cytoplasm that are too small for microscopic visualization or that most of this a-synuclein is soluble. The aggregate-like formations in some cells could either be *bona fide* misfolded aggregates or accumulating vesicles (containing or interacting with a-synuclein) that result from disrupted endocytosis. Previous studies in yeast (Babst *et al.*, 2002a; Katzmann *et al.*, 2001; Babst *et al.*, 2002b) and mammalian cells (Doyotte *et al.*, 2005; Malerod *et al.*, 2007; Razi *et al.*, 2006) have demonstrated that depletion of the individual ESCRT proteins alters endocytic vesicle distribution in addition to the inhibition of the endocytic trafficking. Future biochemical analysis

of a-synuclein aggregate-like inclusions with endocytosis pathway-specific stains will confirm this possibility.

Evidence for ESCRT pathway's involvement in a-synuclein degradation is still emerging. Willingham *et al.*'s (2003) study of yeast genetic screens clearly indicated toxicity mediated by a-synuclein in *vps28Δ*, *vps60Δ* and *vps24Δ*. Unlike studies in our lab, Willingham and colleagues did not study a-synuclein localization changes or accumulation. Here I provide results for these properties (Table 3). Another study showed that internalized a-synuclein move through the endosomal pathway and get degraded in the lysosome, which ultimately results in the degradation of the a-synuclein aggregates from the culture medium (Lee *et al.*, 2008). Tofaris *et al.* (2011) showed that overexpression of Nedd4, a ubiquitin ligase, increases down regulation of a-synuclein by the endosomal-sorting complex required for transport. Furthermore, they found that disruption of Rsp5p (Nedd4 ortholog) in yeast decreases a-synuclein degradation, while enhancing inclusion formation and toxicity (Tofaris *et al.*, 2011). Most importantly, a mutation in *vps35* causes late onset PD (Zimprich *et al.*, 2011; Vilariño-Güell *et al.*, 2011). Along with these findings, my results provide strong evidence for a-synuclein regulation by endocytosis.

Finally, our studies contribute to increasing support for endocytosis defects in a variety of NDDs. For example, CHMP2B (homolog of yeast Vps2, an ESCRT-III protein) positive inclusions form in AD patients, suggesting involvement of the endocytic pathway in other age-related diseases. Interestingly, the absence of *snf7* (*Vps32*; a ESCRT-III protein) in cortical neurons causes neuronal loss, suggesting neurodegeneration linked to the endosomal-lysosome pathway (Lee *et al.*, 2007).

### *Possible substrate specificity*

All four post-ESCRT proteins regulate a-synuclein pathological properties, but to differing extents. It is possible that Doa4 and Vps4 are more involved in recruitment of a-synuclein than the others. Sp8/UBPY, the mammalian ortholog of budding yeast Ubp4/Doa4, constitutively co-precipitates in a bivalent manner with the well-known endocytosis-pathway substrate EGFR, suggesting substrate specificity in degradation (Alwan & van Leeuwen, 2007). Furthermore, Bowers *et al.* (2006) demonstrated that EGFR degradation was not affected by deletions in the ESCRT-II genes, *vps25* and *vps22*, indicating that each ESCRT pathway protein is unlikely to interact directly with and critically regulate each protein substrate that uses this pathway to enter the endosome lumen. Thus, our results suggest post-ESCRT proteins possess substrate specificity for a-synuclein. In the future, providing evidence for direct interaction between these ESCRT proteins and a-synuclein will strengthen this idea.

### *Absence of toxicity*

Interestingly, none of the post-ESCRT gene deletions result a-synuclein dependent toxicity in yeast. However, out of seventeen genes examined in our lab (Table 3), only one (*vps28Δ*) resulted in consistent

a-synuclein-dependent toxicity. There are many possible reasons for this highly selective toxicity. First, as discussed earlier, not all proteins might be involved in regulating a-synuclein levels to the same extent, and perhaps Vps28 is the most important protein in this pathway for a-synuclein regulation. Furthermore, Willingham et al (2003) found five genes (*vps28*, *vps24*, *vps60*, *sac2* and *cog6*) involved in endosomal protein sorting and vesicular transport that cause toxicity in the absence of a-synuclein. Even though we examined three of them (*vps28*, *vps24*, *vps60*), we only found

*vps28Δ* to be consistently toxic. The difference between my study and Willingham et al (2003) can be attributed to different a-synuclein expression levels. Willingham et al (2003) expressed a-synuclein at higher levels than we did, and concentration is a critical contributor to toxicity (Outeiro and Lindquist, 2003). While lower a-synuclein expression levels reduce overall toxicity in our studies, it also allows us to uncover the most critical regulators of a-synuclein.

Finally, it is possible that several other ESCRT machinery proteins, other degradation pathways, or stress responses compensate for and protect against the toxicity that could otherwise be generated by accumulating cytoplasmic a-synuclein in endocytosis deletion strains. Several studies have shown cross-

regulation of degradation pathways achieve the degradation of essential proteins (Rideout *et al.*, 2004; Pandey *et al.*, 2007; Luo & Le, 2010). In the second and third chapters of my thesis, I address the interplay between different degradation mechanisms and other compensatory responses.

Strain	Localization	Toxicity	Expression
<b>Pre-ESCRT</b>			
<i>vps34Δ</i>	Yes	None	Yes
<i>vps27Δ</i>	Yes	None	None
<b>ESCRT-I</b>			
<i>vps23Δ</i>	No	None	None
<i>vps28Δ</i>	Yes	Toxic	Yes
<i>vps37Δ</i>	Yes	None	Yes
<i>mhb12Δ</i>	No	None	Yes
<b>ESCRT-II</b>			
<i>vps22Δ</i>	Yes	None	None
<i>vps25Δ</i>	Yes	None	Yes
<i>vps36Δ</i>	Yes	None	Yes
<b>ESCRT-III</b>			
<i>vps20Δ</i>	No	None	Yes
<i>vps24Δ</i>	Yes	None	None
<i>vps32Δ</i>	No	None	None
<i>vps2Δ</i>	Yes	None	Yes
<b>Post-ESCRT</b>			
<i>vps4Δ</i>	Yes	None	Yes
<i>vps60Δ</i>	Yes	None	Yes
<i>vta1Δ</i>	Yes	None	Yes
<i>doa4Δ</i>	Yes	None	Yes

**Table 3: Summary of findings in the endocytosis-deficient strains:** Compiled data indicating whether each of the three pathological properties were affected in either wild-type or E46K strains. Data for Pre-ESCRT, ESCRT-I, ESCRT-II, and ESCRT-III were obtained from previous theses (Ayala thesis, 2009; Perez thesis,

2010). All the previous findings are included in Appendix (Figures 1, 2, & 3)

## **a-Synuclein Regulation**

## Results

**Experimental set-up:** The role of both endocytosis and autophagy in regulating a-synuclein together was assessed by chemically inhibiting autophagy in five endocytosis deficient strains (*vps34Δ*, *vps28Δ*, *vps37Δ*, *vps2Δ* and *vps4Δ*). I chose these five strains because they are some of the strains that showed the most altered a-synuclein pathology related properties in previous studies (Appendix Figures 1, 2 & 3). Each of these strains and the endocytosis-intact strain (control) were transformed with wild-type a-synuclein attached to a GFP vector (Ayala thesis, 2009; Perez thesis, 2010; Table 1). Autophagy was temporarily inhibited using a well-known autophagy inhibitor, phenyl methyl sulfonyl fluoride (PMSF; Liang *et al.*, 1999; Takeshige *et al.*, 1992). As in study one, I assessed three PD-related a-synuclein properties. First, I used a survival assay to determine a-synuclein dependent growth and toxicity. Second, I determined a-synuclein expression using Western blotting, which detects specific protein levels. Third, I assessed a-synuclein localization using Fluorescence Microscopy.

### Growth and Toxicity

To measure cellular toxicity I used the survival assay (instead of the serial dilution spotting assay used in Chapter One) as it allows the assessment of both yeast colony size and its numbers. In this assay, after expression of a-synuclein in endocytosis-deficient strains for 12 hours, I treated cells with 0.1 mM PMSF (autophagy inhibitor) for another 6 hours. 500 yeast cells from either PMSF-treated or non-treated conditions were then grown in either a-synuclein expressing (galactose) or repressing (glucose) media for 3 or 4 additional days (Figure 11A).

First, I analyzed the appearance of colonies and colony size. In doubly inhibited cells, out of all five strains examined, only two strains (*vps28Δ* and *vps2Δ*) showed a-synuclein dependent delay in colony appearance and reduction in colony size (Figure 11B and 11C). In these two strains, yeast colonies took at least one additional day to appear on galactose plates (Figure 11B). Upon appearance, the PMSF treated cells were smaller than the non-PMSF treated cells in those plates (Figure 11B). The *vps28Δ* and the *vps2Δ* strains that contained only the pYES2 vector did not show any such toxicity, confirming that effects on colony size were due to expression of a-synuclein and not due to just the vector (Figure 11A). The decrease in colony appearance and size on glucose plates further established a-synuclein-dependent toxicity.

Next, I assessed the number of yeast colonies that survived on each plate, using independent samples t-tests for all of five strains and for the BY4741 strain (Figure 12). In cells that lacked Vps28, PMSF treatment indeed resulted in significant reduction of colony number when they expressed a-synuclein (N=3; d(f)=2, p=0.04). Furthermore, there were significantly fewer PMSF treated colonies compared to non-PMSF-treated colonies (N=3; d(f)=2, p= 0.001). Furthermore, the absence of Vps28 alone did not reduce colony number (N=3; d(f)=2, p= 0.67).

Although there were fewer colonies in a-synuclein expressing *vps2Δ*, this decrease was not significant compared to non-PMSF treated cells (N=3;

d(f)=2, p= 0.27) or cells that did not express a-synuclein (N=3; d(f)=2, p= 0.08).

In contrast, I did not see a-synuclein dependent toxicity in cells that lacked Vps34, Vps37 or Vps4 and were untreated with PMSF (*vps34Δ*: N=3; d(f)=2 p= 0.81, *vps37Δ*: N=3; d(f)=2, p= 0.87, and *vps4Δ*: N=3; d(f)=2, p=1.0). Adding PMSF did not reduce colony size either [*vps34Δ*: N=3; d(f)=2, p= 0.87), *vps37Δ*: N=3; d(f)=2, p= 0.79), and *vps4Δ*: N=3; d(f)=2, p= 0.67)]. As expected, a-synuclein expression in BY4741 cells treated with PMSF did not reduce colony size either (N=3; d(f)=2, p= 0.91). In summary, the double inhibition of autophagy and endocytosis caused a-synuclein-dependent toxicity only in cells that lacked Vps28.

### a-Synuclein Expression

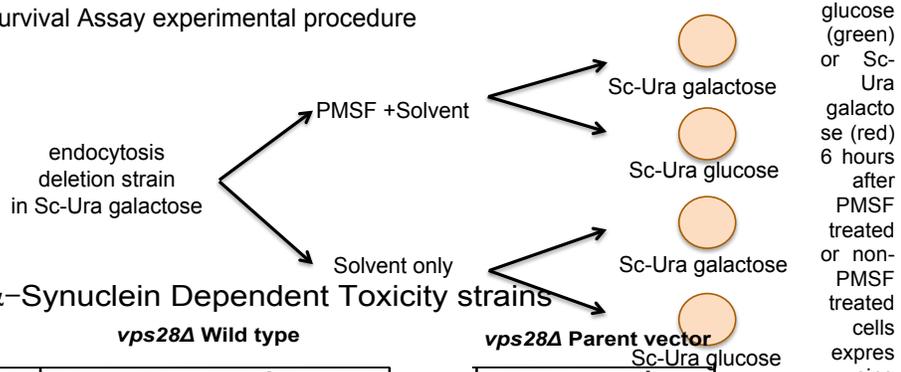
Next, to evaluate a-synuclein accumulation, I compared cellular a-synuclein levels using Western blotting in the five endocytic gene deletion strains and the BY4741 strain that were treated with or without PMSF (Figure 13). Interestingly, only *vps28Δ* showed PMSF dependent increase in a-synuclein levels (Figure 13: lane 5 and 6). In all the other strains (*vps34Δ*, *vps37Δ*, *vps2Δ*, *vps4Δ*, and BY4741), I did not see PMSF-dependent a-synuclein accumulation. PGK served as a loading control.

### a-Synuclein Localization

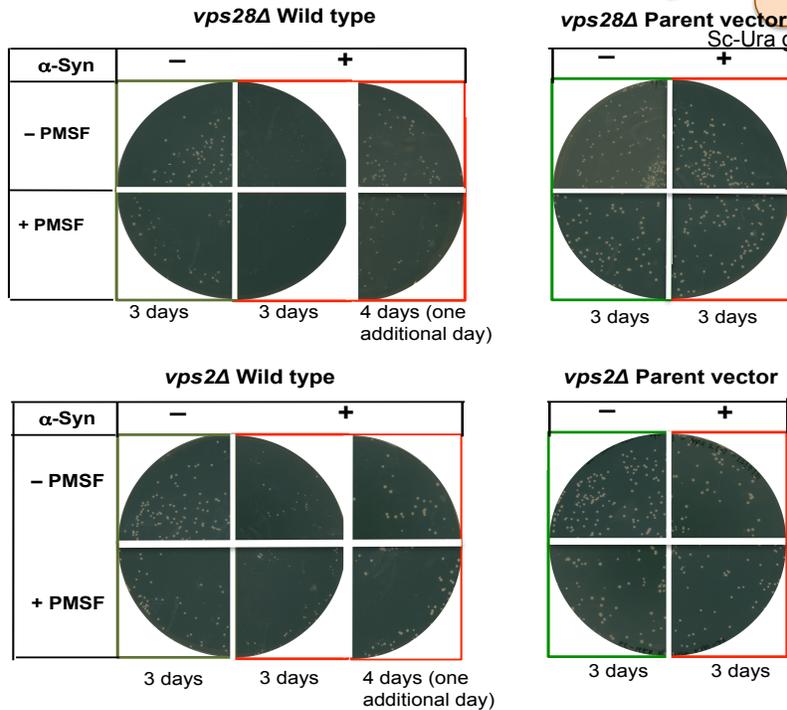
Finally, I determined if the enhanced toxicity and increased a-synuclein levels seen in PMSF-treated *vps28Δ* cells were correlated to increased a-synuclein aggregation. For this analysis, I evaluated a-synuclein localization with live cell GFP microscopy in all five endocytic gene-deletion strains; however, I show data here for only two strains that exhibited a-synuclein-dependent effects on survival (*vps28Δ* and *vps2Δ*). The data for other strains (*vps34Δ*, *vps37Δ*, and *vps4Δ*) are included in the appendix (Figure 4).

Surprisingly, I did not see an increase in a-synuclein aggregation in either of the two strains examined, when treated with PMSF (Figure 14A and B; Appendix Figure 4). All three treatments (*vps28Δ*, *vps28Δ*+PMSF and *vps28Δ*+solvent) showed similar localization profiles, indicating a shift of a-synuclein localization from the plasma membrane to the cytoplasm without significant aggregation (Figure 14A and B).

A. Survival Assay experimental procedure



B.  $\alpha$ -Synuclein Dependent Toxicity Strains



WT (wild-type) and Parent Vector are shown (N=3). These wild-type strains took one additional day to grow in galactose after treating with PMSF.

B.

Characterization of non-toxic strains: Combined pictures of cells plated on Sc-Ura glucose (green) or Sc-Ura galactose (red) 6 hours after PMSF treated or non-PMSF treated cells expressing wild-type (N=3).

C. Non-Toxic Strains

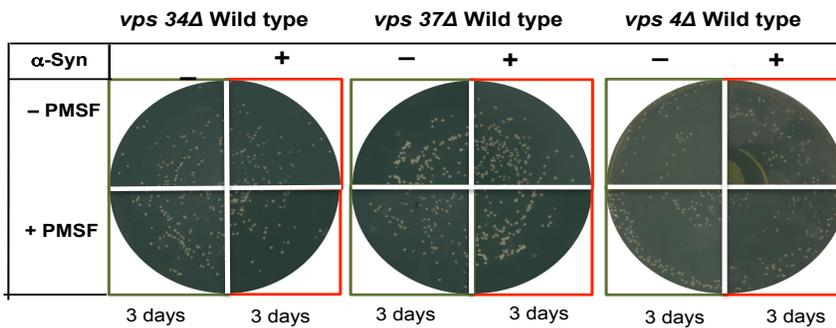
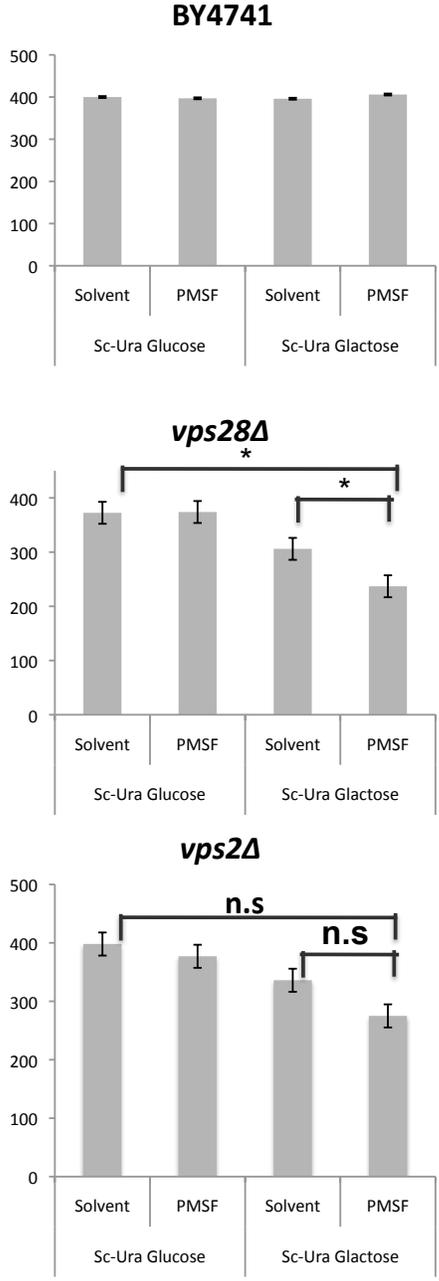


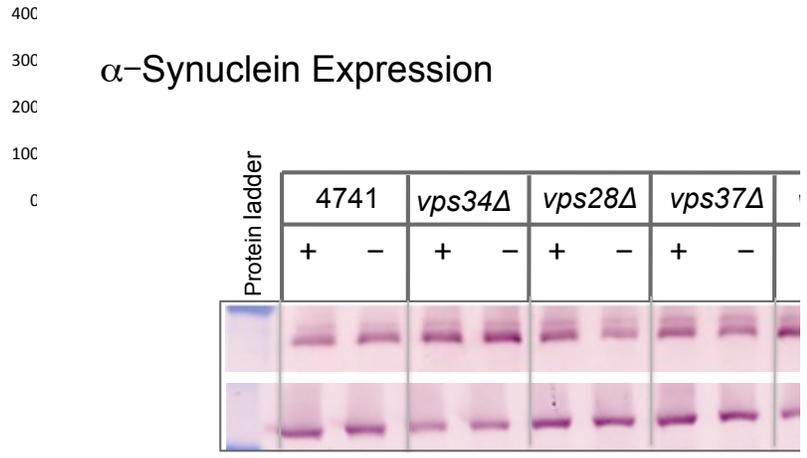
Figure 11: Survival assays of lysosomal degradation deficient strains. A. Experimental procedure for survival assays. Each endocytosis deletion strain

grown in  $\alpha$ -synuclein expressing media for 12 hours was treated with either PMSF or the solvent alone for another 6 hours. 500 of the cells were grown in either  $\alpha$ -synuclein expressing or repressing media plates. B. Survival plates of  $\alpha$ -synuclein dependent toxicity strains: Combined pictures of cells plated on Sc-Ura



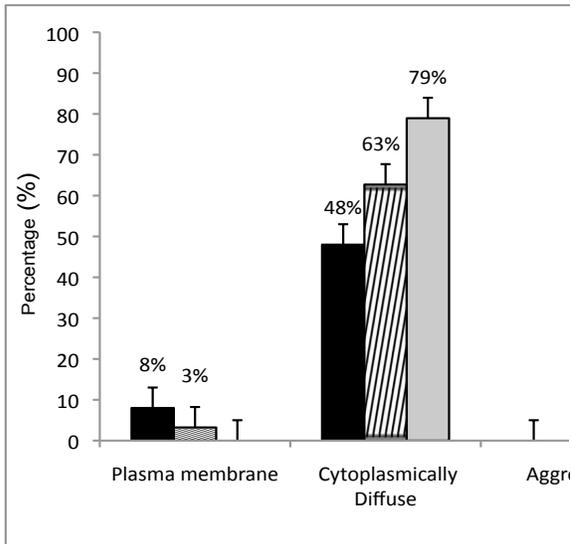
**Figure 12: Survival assay quantification of lysosomal degradation-deficient strains:** Number of colonies survived in each plate in all the strains (N=3). There was a significant decrease in colony number when both routes were impaired in *vps28Δ* (Unpaired t-test,  $p < 0.05$ ). In *vps28Δ*, there was also a significant difference between colony numbers in the endocytosis deficient strains and the lysosomal routes deficient strains (independent samples t-test,  $p < 0.05$ ). There was no significant PMSF dependent toxicity (independent samples t-test,  $p = 0.27$ ) or the lysosomal

deficient toxicity (independent samples t-test,  $p = 0.08$ ) in *vps2Δ*.

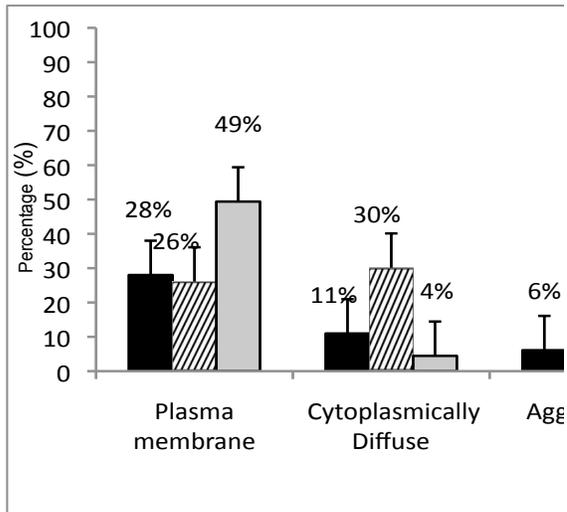


**Figure 13: α-Synuclein expression in lysosomal degradation-deficient strains:** Western blotting was used to evaluate the expression of α-synuclein in selected strains, 6 hours after PMSF treatment and 18 hours after a-synuclein induction. V5 identified a-synuclein levels. PGK levels served as the loading control. The *vps28Δ* strain showed PMSF dependent increase in a-synuclein expression. (N=2). The other strains did not show any PMSF dependent increase in expression.

### A) $\alpha$ -synuclein localization in *vps2*



### B) $\alpha$ -Synuclein Localization in *vp*



■ WT    ▨ WT (    ■ WT (

**Figure 14: Microscopy and quantification of wild-type  $\alpha$ -synuclein in *vps28Δ* and *vps2Δ*: A.  $\alpha$ -synuclein localization and quantification of the *vps28Δ*:  $\alpha$ -synuclein tagged with GFP was visualized using fluorescence microscopy 6 hours after PMSF treatment and 24 hours after  $\alpha$ -synuclein induction. Approximately 750 cells of each deletion strain treated with or without PMSF were quantified (N=2).**

## Discussion

Studies from our lab (Sharma *et al.*, 2006; Konnikova thesis, 2011; Perez thesis, 2010) and by others (Leroy *et al.*, 1998; Shimura *et al.*, 2001; Xie *et al.*, 2010; Tanaka *et al.*, 2001; Vogiatzi *et al.*, 2008; Kuwahara *et al.*, 2008; Lee *et al.*, 2008) have provided strong support that  $\alpha$ -synuclein is degraded at the lysosome (using both endocytosis and autophagy routes) and at the proteasome. It is possible that both pathways are impaired in PD, but whether such double impairment worsens  $\alpha$ -synuclein pathology simultaneously, is not clear. The second chapter of my thesis examined this question, by testing the hypothesis that autophagy and endocytosis routes regulate  $\alpha$ -synuclein interdependently. I expected to observe elevated  $\alpha$ -synuclein pathological properties when both routes were inhibited. Here, I share preliminary evidence for an increased cellular toxicity and  $\alpha$ -synuclein accumulation, when both endocytosis and autophagy are comprised. My data set suggests close interactions between autophagy and endocytosis in regulating  $\alpha$ -synuclein and implicates vps28 as an important molecule in this regulation.

As hypothesized, the co-inhibition of endocytosis and autophagy increased  $\alpha$ -synuclein-dependent toxicity and accumulation, but this effect was highly specific and depended on the exact gene (*vps28*) that was deleted to compromise endocytosis. Interestingly, this same strain (*vps28 $\Delta$* ) is the only one strain that produces  $\alpha$ -synuclein dependent toxicity even without autophagy inhibition (Chapter One). It is possible that toxicity would have achieved significant levels and  $\alpha$ -synuclein would have accumulated in *vps2 $\Delta$*  cells, if we had overexpressed  $\alpha$ -synuclein at higher levels. A few studies in animal models have examined how simultaneous inhibition of both routes regulates  $\alpha$ -synuclein aggregation, accumulation, or toxicity. However, my preliminary data supports a previous study that examined other lysosomal-bound protein substrates that use these routes. Holen *et al.* (1995) found that chemical inhibition of both autophagy and endocytosis in rat hepatocytes increase endosome formation and reduce specific substrate degradation.

Notably, autophagy inhibition alone did not cause significant toxicity in the BY4741 strain. This finding is not surprising and agrees with previous genetic studies. Individual deletion of autophagy genes does not result in  $\alpha$ -synuclein dependent toxicity in budding yeast (Choi thesis, 2009; Konnikova thesis, 2011). Although Willingham *et al.* (2003) uncovered 86 gene deletions that caused  $\alpha$ -synuclein toxicity in budding yeast, none of them were autophagy genes.

Current research suggests that endocytosis and autophagy routes to the lysosome are connected at the final steps in more complex ways than previously thought. It now appears that autophagosomes fuse with both endosomes and lysosomes during the last steps of autophagy (Rusten and Stenmark, 2009). Inhibition of this fusion step results in accumulation of autophagosomes and a consequent decrease in post-fusion structures. Specifically, efficient autophagic degradation requires functional ESCRTs (Eskelinen, 2005). The absence of ESCRT proteins results in

increased numbers of amphisomes and autolysosomes (Filimonenko *et al.*, 2007; Nara *et al.*, 2002). While molecular mechanisms behind these fusion steps will need much more clarification, my preliminary data strengthens the argument that ESCRT proteins (specifically Vps28) are involved in autophagic regulation.

Not only do different routes to the lysosome interact with and regulate each other, inhibiting these intersecting lysosomal routes likely activate the proteasome pathway as a compensatory mechanism. It is already established that when the proteasome is inhibited, autophagy gets activated (Pan *et al.*, 2008; Pandey *et al.*, 2007). In the final chapter of my thesis, I addressed the question of whether the proteasome becomes activated when the lysosomal pathways are compromised.

**Chapter 3: Evaluation of Compensatory Responses  
under Deficient**

**Lysosomal Degradation**

## Results

*Experimental set-up:* I analyzed two compensatory cellular responses that could have been triggered due to defective lysosomal degradation: proteasomal activation and heat shock protein response. These responses were measured in the same endocytic gene-deletion strains (*vps34Δ*, *vps28Δ*, *vps37Δ*, *vps2Δ* and *vps4Δ*) that were examined in Chapter Two. In these strains and the control BY4741 strain, autophagy was chemically inhibited with 0.1 mM PMSF (as done in Chapter Two). I used Western blotting to determine the levels of ubiquitin proteins and two major heat shock proteins, Hsp70 and Hsp40.

### *Ubiquitination response*

Covalent attachment of ubiquitin to short lived or damaged proteins serves as the signal that initiates their selective degradation (Hershko *et al.*, 2000; Hershko & Ciechanover, 1982). While K63-linked ubiquitin chains act as a signal for both UPS and lysosome degradation pathways (Lauwers *et al.*, 2009), K48-linked ubiquitin chains indicate selective proteasomal degradation (Long *et al.*, 2008). Therefore, I used antibodies for both of these linkages to determine overall stimulation of ubiquitination, when lysosomal degradation was damaged.

As these were new antibodies to our lab, I had to develop conditions for optimal protein detection in yeast cell lysates. In my first several attempts, both K63 and K48 ubiquitination were not detectable under initial experimental conditions (Example blot: Figure 15A). In successive trials, I increased variable exposure times to both primary and secondary antibodies. Finally, I detected both forms of ubiquitination with overnight exposure to the primary antibodies and two-hour exposure to the secondary antibody (representative blot: Figure 15B). Currently, I am still optimizing conditions for protein detection. While these are still preliminary observations, I have found higher K48-linked ubiquitin levels in the *vps37Δ*, the *vps2Δ*, and the *vps4Δ* strains compared to the BY4741, the *vps28Δ*, and the *vps34Δ* strains. However, these changes were not PMSF-dependent (Figure 15B). K63-linked ubiquitin response was variable in most experiments including the blot shown. Therefore, conclusions cannot be derived (Figure 15B). In all of these trials, PGK served as a positive loading control.

### *Heat shock response*

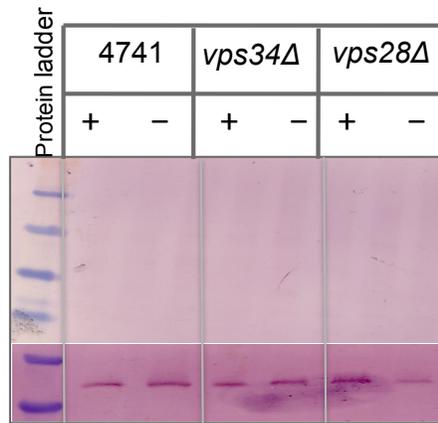
When protein homeostasis is challenged, different Hsps are activated and each Hsp engages to re-fold misfolded proteins back into their functional shape and prevent them from aggregating (Lindquist, 1986). The most common Hsps that are activated under diseased conditions are Hsp70, Hsp40 and Hsp90 (Morimoto & Santoro, 1998). Furthermore, Hsp70 assists in stabilizing and folding many proteins, and is found in many cellular locations (Lindquist, 1986). While all Hsps should ultimately be screened for activation, as a first step in this direction, I analyzed the activation of Hsp70 and Hsp40 using antibodies.

In my first several attempts at developing optimal blotting conditions with our yeast lysates, the Hsp70 and Hsp40 antibodies produced excessively high non-specific staining backgrounds (example blots:

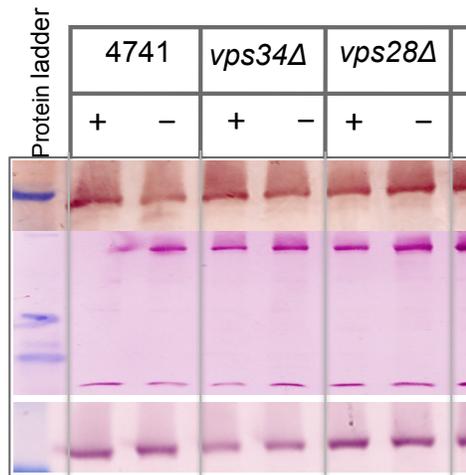
Figure 16A). In successive trials, I reduced both the concentration of primary antibodies and the exposure times to these antibodies, changing one variable at a time. Finally, for Hsp40 detection, when I reduced the dilution of the primary antibody from 1:1000 to 1:2000 and reduced exposure time from one hour to thirty minutes, the resulting blots produced fewer background signals (Example blot: Figure 16B). I now consistently observe PMSF-independent increases in Hsp40 levels in all five endocytic gene deletion strains compared to the BY4741 strain. For Hsp70 detection, I have reduced the antibody dilution from 1:600 to 1:12000 and the exposure time from one hour to thirty minutes. While I have reduced non-specific background staining for these blots, I have not yet strengthened the detection of Hsp70 itself (Figure 16B). Currently, I am still optimizing conditions for Hsp70 and can make no early conclusions. Here, PGK levels served as a positive loading control.

## Ubiquitination

A.

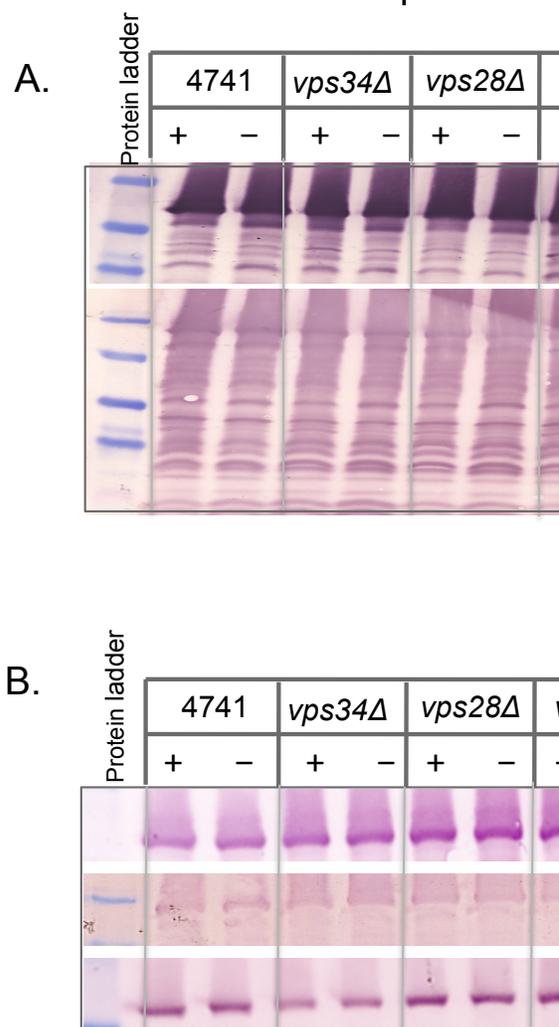


B.



**Figure 15: Ubiquitin response in lysosomal degradation deficient strains:** **A.** Undetectable ubiquitination response: K48-linked ubiquitin and K63-linked ubiquitin signals of BY4741 and deletions strains treated with or without PMSF. (N=3). **B.** K48-linked ubiquitin and K63-linked ubiquitin signals of BY4741 and deletions strains treated with or without PMSF. Ubiquitination response: higher K48-linked ubiquitination in *vps37Δ*, *vps28Δ* and *vps4Δ* independent of PMSF treatment. The exposure time to primary antibody was increased from 4 hours to overnight and secondary antibody was increased from 1 hour to 2 hours. (N=2). PGK served as the loading control.

## Heat Shock Protein Expression



**Figure 15: Heat shock protein response in lysosomal degradation deficient strains: A.** Hsp70 and Hsp40 protein levels of BY4741 and Deletions strains treated with or without PMSF with background signals (N=2) **B.** Heat Shock Protein response after changed conditions. Exposure time: 30 mins. Dilution: 1/ 2000. Overall increase in Hsp40 levels in deletion strains was seen. No PMSF dependent difference was observed. Hsp70 conditions still need to be optimized Exposure time: 30 mins Dilution: 1/12000 (N=2). PGK served as the loading control.

## Discussion

Eukaryotic cells respond to misfolded and accumulated proteins either by degrading or re-folding them into their correct shape to protect against potential toxicity (Muchowski & Wacker, 2005). In this final chapter of my thesis, I assessed whether yeast cells respond to  $\alpha$ -synuclein-induced stress under deficient lysosomal degradation by activating coping mechanisms to reduce overall toxicity. If similar mechanisms are activated in PD, they may offer additional therapeutic targets. I hypothesized that co-inhibition of endocytosis and autophagy in  $\alpha$ -synuclein expressing yeast cells would activate proteasome-based degradation and major heat shock proteins. I assessed K48 and K63-based ubiquitination and Hsp70/Hsp40 using Western blotting. My experimental protocols are still being optimized and my data set is still preliminary. While I am unable to make conclusions toward my hypothesis, I report two early qualitative observations: there is a general increase in (1) K48 ubiquitination and (2) Hsp40 levels in multiple endocytic gene deletion strains irrespective of autophagy inhibition.

### *Increased K48 poly-ubiquitination*

Even though I hypothesized that I would see more proteasomal activation when both lysosomal routes were co-inhibited, I did not see a PMSF-dependent difference in K48 poly-ubiquitination. However, the observation of PMSF-independent increase in poly-ubiquitination in three of the five strains suggests possible direction of proteins ( $\alpha$ -synuclein and other protein substrates) towards the proteasome. K48-linked ubiquitin chains indicate selective proteasomal degradation (Chau *et al.*, 1989; Deng *et al.*, 2000; Lim *et al.*, 2005; Lim *et al.*, 2006; Long *et al.*, 2008). According to some studies, a decrease in K48-ubiquitination and an increase in K63-ubiquitination indicate a dysfunctional proteasome (Lim *et al.*, 2005; Lim *et al.*, 2006; Avanson & Ellison, 1994). Therefore, the K48 ubiquitination increase that I observe might indeed reflect proteasomal activation.

In contrast, other studies show that inhibition of lysosomal degradation can also impair UPS and cause an increase in ubiquitinated protein inclusions in the cytoplasm (Korolchuk *et al.*, 2009; Qiao & Zhang, 2009). Qiao and Zhang (2009) further suggest that the heat shock response is activated preferentially over the proteasome, under altered lysosomal conditions. However, none of the above studies verified whether those protein inclusions were K48-linked or K63-linked. In general, the ubiquitination status of  $\alpha$ -synuclein needs more thorough examination in most animal PD models or in patients. I hope to continue optimizing the experimental conditions for K63 ubiquitination and replicate K48 ubiquitination studies in the future.

### *Heat Shock Protein Response*

I hypothesized that Hsp70 and Hsp40 expression would increase when endocytosis and autophagy were co-inhibited. In my preliminary data, I did not observe a PMSF-dependent difference in expression thus could not support this overall hypothesis. Nonetheless, I observed PMSF-independent increase in Hsp40 expression in all of the

endocytic gene deletion strains compared to the BY4741, indicating that comprising endocytosis could be enough to trigger this compensatory mechanism. This early observation agrees with previous studies on other neurodegenerative diseases in the field. Hsp70 and Hsp40 are two of the most common Hsps, involved in neurodegenerative diseases (Sherman *et al.*, 2001). Many studies have found protective functions of Hsps under protein misfolding and aggregating conditions (Auluck *et al.*, 2002; Mclean *et al.*, 2002; Magrane *et al.*, 2004). For instance, inclusion bodies formed in neurodegenerative diseases co-localize with various Hsps (Sherman *et al.*, 2001) suggesting of an unsuccessful attempt to re-fold or degrade those culprit aggregates. Additionally, Hsp70 rapidly associates and disassociates with mutant Huntington and regulate their formation (Kim *et al.*, 2002; Chai *et al.*, 2002). Finally, RNA-mediated interference (RNAi) of Hsp70 and Hsp90 in cell culture increases the accumulation of insoluble, aggregates of AD protein, tau (Dou *et al.*, 2003). In yeast, absence of *apj* gene (yeast ortholog of Hsp40) results in a-synuclein mediated toxicity (Willingham *et al.*, 2003). Even though, the observed trend with Hsp40 needs further substantiation, it suggests that protein-folding responses are activated in yeast cells under a-synuclein induced toxicity. In the future, in addition to repeating the Hsp40 experiments and optimizing Hsp70 antibody, I hope to analyze activation of other Hsps, including Hsp104 and Hsp90.

Heat shock proteins also rescues neurodegeneration in a variety of animal models for diverse NDDs (Auluck *et al.*, 2002; Mclean *et al.*, 2002; Magrane *et al.*, 2004). Over-expression of Hsp70 saves neurons from intracellular Ab-plaque mediated toxicity in cell culture (Magrane *et al.*, 2004). Co-expression of human Hsp70 prevents wild-type or mutant a-synuclein mediated toxicity in dopaminergic neurons of *Drosophila* (Auluck *et al.*, 2002). Over-expression of Hsp40 or Hsp70 in a a-synuclein cell model can decrease a-synuclein aggregation (McLean *et al.*, 2002). Finally, Hsp90 and Hsp40 are known suppressors of a-synuclein toxicity in yeast PD models (Flower *et al.*, 2005). Therefore, optimizing experimental conditions in our yeast PD models to obtain consistent results in the future will provide insight into compensation by Hsps. Understanding these protective mechanisms of Hsps may help us identify possible therapeutic targets in the future.

### Limitations, Criticisms and Future Studies

Each of my three studies had some limitations, which if overcome in the future will provide more insight into a-synuclein regulation. Most of them are due to limitations in advanced molecular techniques. I will discuss limitations of each of the studies below.

While my first study brought to an end a five-year long evaluation of endocytic genes in our budding yeast model for PD, several limitations can be addressed in the future. Firstly, I used single gene deletion strains to determine the involvement of ESCRT proteins. The absence of toxicity might be due to compensation by other endocytosis genes or by other degradation mechanisms, as shown in other literature. In the future, multiple gene deletions and confirmation of individual gene deletions by genetic rescue to recover the effects might provide strong evidence for endosomal regulation of a-synuclein. Secondly, my data set does not indicate direct physical interaction of ESCRT proteins with a-synuclein nor do we show evidence that a-synuclein is within the endosome or lysosome. Biochemical analysis such as co-immunoprecipitation, and co-immunofluorescence might help determine whether a-synuclein directly interact and co-localize with these endocytosis proteins. Thirdly, biochemical analysis of aggregate-like formations we observed in microscopy images needs to be carried out in the future to determine their composition. Furthermore, endosome and vacuole containing will confirm that these compartments contain a-synuclein under normal conditions or are devoid of it when degradation is impaired.

The second study required many trials of new methodologies leaving much room for improvement in the future. The first limitation is the assumption of autophagy inhibition by PMSF. Though PMSF prevents degradation of autophagic bodies, we should directly measure the robustness of the autophagy response in our yeast cells. This might also help rule out solvent effects. Different solvents or other chemical autophagic inhibitors such as 3-methyladenine should also be assessed (Kabeya *et al.*, 2003; Selgen and Gordon, 1982).

Finally, the third study requires optimizing antibody conditions for ubiquitin, Hsp70 and Hsp40. Furthermore, utilization of biochemical assays (Verma *et al.*, 2004) that can differentiate between proteins at proteolytic state or aggregates will provide evidence whether impairment of one pathway impairs or activates the other. Roles of other heat shock proteins such as Hsp90 and Hsp104 should be expanded on in the future.

## Conclusion

PD affects nearly one million people in the U.S. and seven to ten million worldwide (Parkinson's Disease Foundation, 2012). After decades of research on cellular mechanisms underlying Parkinson's disease, a cure remains unknown. One of the most intensely investigated areas in the field is how  $\alpha$ -synuclein (one of the main contributors to PD pathogenesis) gets degraded. Many degradation mechanisms control  $\alpha$ -synuclein pathology suggesting a wider range of protein quality control mechanisms than previously thought. Our lab's research, including my thesis work, has contributed to the field the knowledge that  $\alpha$ -synuclein is regulated by both the lysosome and the proteasome.

In my first study, I provided genetic evidence for involvement of endocytosis in  $\alpha$ -synuclein regulation by demonstrating that four post-ESCRT genes regulate  $\alpha$ -synuclein location and cellular concentration. In my second study, I provided evidence for simultaneous regulation of  $\alpha$ -synuclein by endocytosis and autophagy routes. In my final study, I report preliminary trends that still need additional experimental support that heat shock proteins and ubiquitin responses could get activated in response to altered regulation of  $\alpha$ -synuclein by the lysosome. These three studies suggest that  $\alpha$ -synuclein affects many cellular mechanisms. Yeasts are powerful model organisms through which we can gain insight into human disease pathology. Insight into  $\alpha$ -synuclein degradation and associated compensatory responses mechanisms may lead to possible therapeutics giving hope to millions of humans who are afflicted with PD.

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