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Insight Into Parkinson's Disease Using Yeast as a Model to Evaluate the Role of Autophagy Genes in α-Synuclein Toxicity

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Abstract

The misfolding and aggregation of the protein α-synuclein is the likely cause of Parkinson's disease (PD), a common incurable human neurodegenerative illness. A popular hypothesis is that increasing the degradation of α-synuclein may protect cells from its aggregation and toxicity. While the cellular organelle, lysosome, is pharmacologically implicated in degrading α-synuclein, no genetic evidence currently exists. We hypothesized that α-synuclein uses autophagy, an ancient route by which damaged proteins enter the lysosome. This hypothesis was tested in a budding yeast model wherein autophagy was compromised with individual autophagy gene knockouts required for the nucleation and expansion steps of autophagy. Three α-synuclein properties were assessed for potential increases: aggregation, accumulation, and toxicity. Surprisingly, with six genes examined thus far, we only observed subtle autophagic regulation of α-synuclein, unlike our prediction. To more completely understand autophagy-mediated α-synuclein degradation, the remaining proteins that comprise the nucleation, expansion, and fusion steps need similar examination.

Introduction

As we enter the 21st century, the world must confront a wide range of devastating and lethal epidemics such as cancer and HIV. However, due to the population's increasing longevity, society is confronting a new class of illness: neurodegenerative diseases. These ailments, characterized by the selective death of neurons in the Central Nervous System (CNS), are some of the most complex biological disorders. These illnesses are marked by neurological impediments such as motor deficits and memory loss. Some well-studied disorders include the prion disease, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), and Multiple Sclerosis (MS). These diseases can be further classified into two groups. The first are infectious – the disease causing agent is transmitted between people. Prion diseases are part of this category. The second are non-infectious and non-transmissible (AD, PD, HD, and MS), and arise due to genetic mutations (HD, PD, AD), sporadically (AD and PD), or as an auto-immune disorder (MS; Prusiner, 1982; Muchowski, 2002; Hauser et al. 2006; Rojo et al., 2008). All these diseases, however, are hallmarked by selective neuronal death and are believed to arise due to a protein that misfolds and accumulates in the neurons as inclusion bodies (IB). While the protective or toxic effects of this agent are currently under debate, research in this field has provided us with a better understanding of neurodegenerative diseases such as PD (Ross et al., 2005).

*This author wrote the paper as a senior thesis under the direction of Dr. Shubhik DebBurman.

Parkinson's disease

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease after Alzheimer's disease in the population over the age of 65 (de Silva et al. 2002). Currently, 4 million cases of PD are reported around the world, with 1.5 million people afflicted in the US. Sadly, the number of people with PD is expected to double within the next 30 years – ever-increasing the need for a treatment (Dorsey et al., 2007). Pathologically, PD is distinguished from other neurodegenerative diseases by the loss of select dopaminergic neurons in the substantia nigra pars compacta (SNpc; Barbosa et al., 1997). This degeneration, which results in the impairments of the motor cortex pathway, causes the clinical PD symptoms of bradykinesia, rigidity, and resting tremors (Schapira, 1999). In 1912, Friederich Lewy identified the characteristic trait of PD patients – the Lewy body (LB; Forster & Lewy, 1912).

Upon post-mortem analysis of PD patient brains, the dying and dead dopaminergic neurons of the SNpc are found to contain multiple and abnormal Lewy Bodies (Muchowski, 2002; Eriksen et al., 2005; Robinson, 2008). While many proteins are found in LBs, the major component of LBs are misfolded, aggregated, and ubiquinated α-synuclein (Dixon et al., 2005; Zabrocki et al., 2005). As such, α-synuclein is hypothesized to be the cause of PD (Outeiro & Lindquist, 2003; Cooper et al., 2006). Although familial inheritance of PD is known (15% of total PD cases), most causes of PD are sporadic (85% of total PD cases; Thomas & Beal, 2007; Figure 1).

Interestingly, most of our understanding about PD comes from the rarer genetic cases, as an imbalance in PD research exists (more is known about the genetic than sporadic causes of PD). At present, researchers believe that sporadic PD is linked to oxidative stress, mitochondrial and proteasomal dysfunction, environmental toxins, and the impairment of the Ubiquitin-Proteasome System – the major route for α-synuclein degradation (Muchowski et al., 2002; Dawson & Dawson, 2003; Moore et al., 2005; Cooper et al., 2006; Thomas & Beal, 2007; Fitzgerald & Plun-Favreau, 2008). In familial PD, substantial genetic evidence implicates mutations in the genes of the PARK locus to PD pathogenesis. These mutations have been linked to autosomal-dominant or autosomal-recessive inheritance (Gasser, 2009). Mutations in the UCHL1, LRRK2, and α-synuclein genes are linked to autosomal-dominant PD, while mutations in the Parkin, PINK1, and DJ-1 genes are found in autsomal-recessive PD (Leroy et al., 1998; Zimprich et al., 2004; Polymeropoulos et al., 1997; Kitada et al., 1998; Valente et al., 2004; Bonifati et al., 2003). Of these genes, α-synuclein is the most studied and linked protein to causing PD (Polymeropoulos et al., 1997; Spillantini et al., 1997; Chung et al., 2001). Upon further analysis of the LBs, α-synuclein is commonly found to be misfolded and accumulated in both idiopathic and genetic cases of PD (Giasson & Lee, 2003) . Currently, three familial α-synuclein mutations are linked to autosomal-dominant PD. The first is A53T, where the amino acid (protein building block) alanine is replaced by theronine (Polymeropoulos et al., 1997). The second is A30P, where an alanine is replaced with a proline (Krüger et al., 1998). The last familial mutant is the E46K mutation, where glutamic acid is replaced with lysine

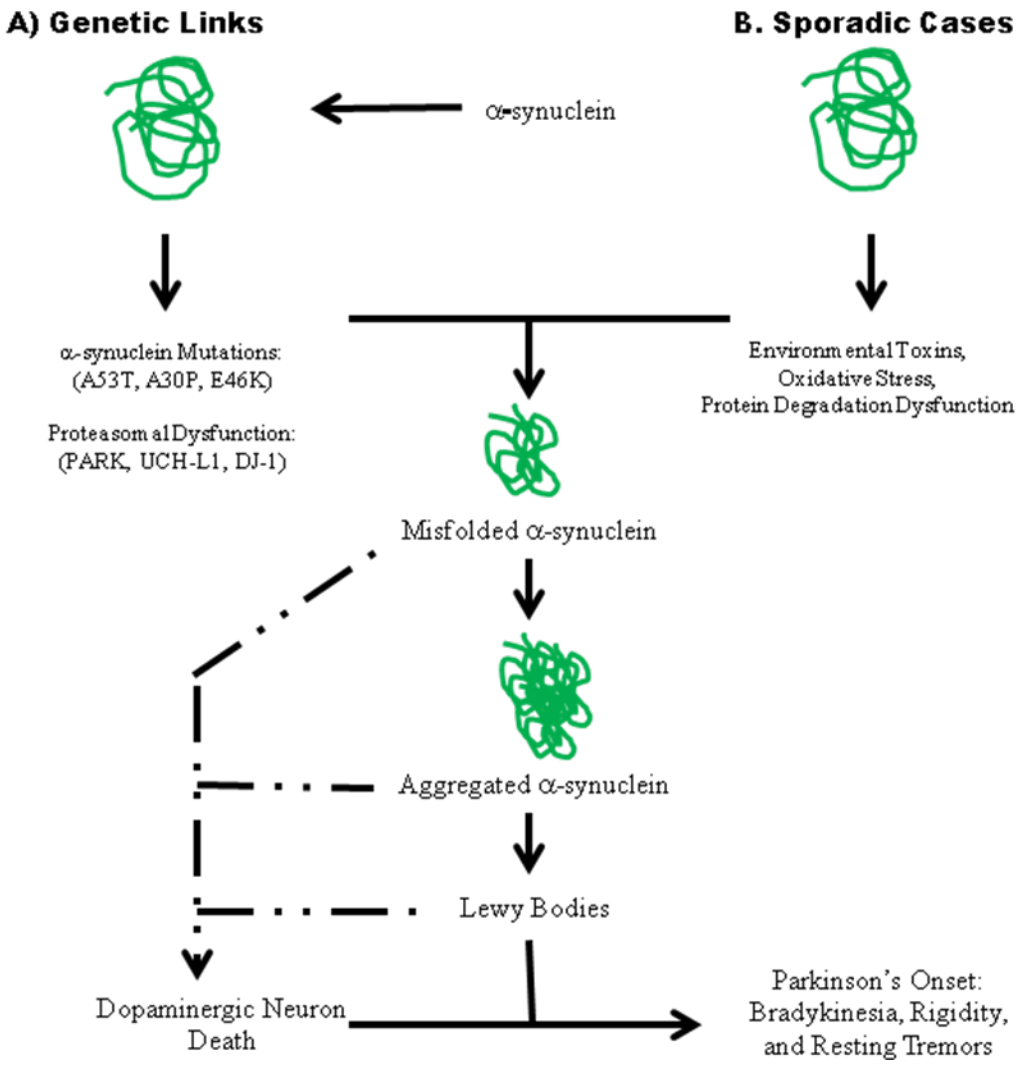

Figure 1: Molecular Basis of Parkinson's disease. (A) The three familial mutations of α-synuclein and mutations of the proteasomal degradation pathway are linked to genetic Parkinson's disease. Genetic mutations represent 5 % of all Parkinson's cases. **(B)** Idiopathic changes in α-synuclein, a protein believed to be responsible for Parkinson's, represent 95 % of all cases. Possible mutagens of α-synuclein could be oxidative stress, environmental toxins such as pesticides or other harmful chemicals, and or a dysfunctional protein degradation pathway. In both familial and sporadic Parkinson's α-synuclein aggregates form, this leads to the formation of Lewy Bodies, a hallmark in Parkinson's. It is then believed that these inclusions lead to cell death and ultimately to the onset of Parkinson's and its symptoms.

(Zarranz et al., 2004). These mutations permit α-synuclein to misfold at a faster rate compared to normal α-synuclein (Polymeropoulos et al. 1997; George, 2002). Thus, conformational changes in α-synuclein could not only lead to loss-of-normal-function, but to the increased susceptibility of the accumulation and aggregation of α-synuclein (Spillantini et al., 1997; Lashuel et al., 2002; Dev et al., 2003). Nevertheless, the role α-synuclein has in PD pathogenesis is still unknown (Figure 1; Polymeropoulos et al. 1997).

The Folding & Function of the Protein α-Synuclein

α-Synuclein, a 140-amino acid protein, is an interesting protein as it has a natural tendency to misfold and is naturally found unfolded in the cell (Weinreb et al., 1996; Uversky, 2003). It is expressed throughout the brain, but is predominantly found in the dopaminergic, cortical, and noradrenergic neurons. Furthermore, it localizes in the pre-synaptic terminals of neurons (Spillantini et al., 1997; Chiba-Falek et al., 2007). Within the neurons, α-synuclein is cytoplasmically located, but has an especially high binding affinity for phospholipids (Kahle et al., 2000). While the current function of α-synuclein is unknown, it is implicated in dopamine transmission, synaptic vesicle dynamics, and in learning and adaptation (Outeiro & Lindquist, 2003).

While substantial evidence is found in the PD field, much more insight is needed to understand PD pathogenesis. Major questions that are currently being researched in the field are seeking what role free radicals have in oxidative stress and mitochondrial dysfunction and how they are linked to PD? Are wild-type (WT) and mutant α-synuclein toxic to organisms? Is α-synuclein toxic in its soluble or insoluble form in *vivo*? Do Lewy bodies play a protective role in neurons or are they harmful? Lastly, what cellular mechanism is responsible for α-synuclein degradation?

My thesis will focus on this latter topic and examine α-synuclein digestion via autophagy. However, before I discuss protein degradation, I will review protein synthesis.

Normal Protein Synthesis

α-Synuclein, like all proteins, is initially transcribed in the nucleus from DNA into an mRNA molecule. After which, the mRNA is exported into the cytoplasm where it is translated by ribosomes into an amino acid chain – protein in its primary structure. With the help from protein chaperones, proteins fold into its secondary (α-helix and β-sheets) and tertiary structures. If multiple tertiary proteins interact, the resulting protein is in a quaternary state (Englander et al., 2007). Currently, two mechanistic theories of protein folding exist. The first is pre-determined by the amino acid sequence of the protein, which depends on cooperative structural characteristics. The second model, known as the independent unrelated pathway, states that proteins folds through intermediate structures, until the final conformation is reached (Englander et al., 2007; Krishna et al., 2007). After proteins are in its final structure, they are transported within the cell, to neighboring cells, or to the extracellular matrix – where they will perform their cellular function(s). Once their function is fulfilled or if they are no longer needed, cells will eventually degrade these proteins.

Protein Degradation Pathways

Proteins are degraded for multiple reasons; some proteins are short-lived and are quickly degraded to prevent further function, while other proteins that have mutated or improperly folded (initially or due to other factors such as: denaturation, environment, or age) are also degraded (Englander et al., 2007). If these unnecessary and damaged proteins are left in the cell, they may prove toxic and subsequently fatal to the cell due to their aberrant functions. Therefore, proteins are degraded to maintain and protect normal cellular homeostasis (Bader et al., 2007). The recycled amino acids are then used in future protein synthesis.

Cells have evolved two pathways for protein degradation: the proteasome and lysosome (Figure 2). Proteins found cytoplasmically are degraded by the proteasome via the ubiquitin-proteasome pathway (UPP), while proteins found in the plasma membrane or extracellularly are degraded via the lysosome (some cross-talk occurs). The α-synuclein protein is unique since it is found in the cytoplasm and attached to the plasma membrane (Kahle et al., 2000). Due to its dual localization, α-synuclein may be potentially degraded by the proteasome or by the lysosome. In fact, research shows that the co-chaperone CHIP (carboxy-terminus of Hsp70 interacting protein) may act as a molecular determinant of where α-synuclein will be degraded; the protein's TPR domain sends α-synuclein to the proteasome, while the protein's U-box domain sends α-synuclein to the lysosome (Shin et al., 2005; Engelender, 2008).

The Proteasome & α-Synuclein

The proteasome is a multi-unit organelle located in the cytoplasm and nucleus of the cell that degrades proteins (Figure 2; Coux et al., 1996; Bader et al., 2007). Before proteins can be degraded by the proteasome, they must be tagged by ubiquitin – a signal for degradation – multiple times via the ubiquitin-proteasome pathway (Bader et al., 2007). This process, known as ubiquitination, adds ubiquitin with the following enzymes. E1 activates ubiquitin; E2 conjugates ubiquitin; and E3 ligases, or attaches ubiquitin and the protein (Hedge and Upadhya, 2007; van Tijn et al., 2008). The proteasome, which recognizes this chain of ubiquitin, internalizes the protein, but prevents ubiquitin from entering and recycles it back into the cell (van Tijn et al., 2008). Once inside the proteasome, the protein is degraded by a large enzyme complex (26s) into reusable amino acids (Bader et al., 2007; van Tijn et al., 2008).

In several major neurodegenerative disorders, defects in the ubiquitin-proteasome pathway, due to genetic or environmental factors, may be responsible for IB formation and disease pathogenesis. Aberrant mutations in ataxin-1 are linked to spinocerebellar ataxia type 1 (SCA1). When proteasome is inhibited in mice in the presence of mutant ataxin-1, not only did neurodegeneration occur, but the mutant proteins formed IBs (Cummings et al., 1999). In AD, ubiquitin not only associates with non-degraded tau tangles, but is found mutated in AD patients (Morishima-Kawashima et al., 1993; van Leeuwen et al., 1998). Furthermore, the extended polyQ repeats of the huntingtin protein prevents proteasomal function (mechanism unknown), allowing for the aggregation of huntingtin (Impairment of the ubiquitin–proteasome system by protein aggregation).

Similarly, mutations in genes required for the ubiquitin-proteasome pathway are linked to PD pathogenesis. In healthy neuronal cells, the Parkin protein, an E3 enzyme, labels proteins, such as modified α-synuclein, for degradation (Kitada et al., 1998; Shimura et al., 2001; Schulz, 2008). Additionally, mutations in the ubiquitin C-terminal hydrolase-L1 (UCH-L1) protein, a de-ubiquitinating protein, are also linked to PD pathogenesis (Leroy et al., 1998; Liu et al., 2002). Thus, direct genetic evidence implicates and supports the hypothesis that proteasome dysfunction is a cause of PD. Furthermore, α-synuclein found in LBs reveal non-degraded and multi-ubiquinated α-synuclein – reinforcing the proteasome in PD (Ghee et al., 2000; Tanaka et al., 2001; Snyder et al., 2003). These findings were further strengthened when researchers discovered that Parkin deficient *Drosophila* had PD pathology similar to humans (Greene et al., 2003). Lastly, chemical inhibition of the proteasome by epoxomicins or by MB132 results in reduced cell viability and in the formation of PD-like LBs in murine models (Petrucelli et al., 2002; McNaught et al., 2004; McNaught & Olanow, 2006). Thus, supported by genetic and chemical studies, proteasomal dysfunction appears a likely cause of PD pathogenesis.

The Lysosome & α-Synuclein

Unlike the substantial genetic evidence that implicates the proteasome and PD, the role of the lysosome in α-synuclein degradation is not supported by the literature. Lysosomes are enclosed organelles that contain acid hydrolase enzymes and proteases, which degrade proteins and other large molecules, such as aged organelles, bacteria, and nutrients (de Duve et al., 1955). These materials are transported to the lysosome in vesicular bodies via three independent pathways. Due to its importance in protein degradation, researchers hypothesize that the lysosome has a role in degrading α-synuclein.

In fact, genetic and pharmacological evidence implicates lysosomal dysfunction in other neurodegenerative disorders. Mice that lack and have mutations in Cathepsin D and Cytacin C, genes required for normal lysosome function, are shown to have increased neuronal death, neurodegenerative symptoms, and increased risks for the earlier onset of AD (Koike et al., 2000; Nixon, 2004; Shacka et al., 2007). Furthermore, mice deficient in the beclin-1, an autophagy gene, display motor defects and form Lewy Bodies (Mizushima et al., 2008). Interestingly, when lysosomal activity is stimulated by the chemical Z-Phe-Ala-diazomethylketone (PADK), the cell increased the clearance of toxic proteins linked to AD (Butler et al., 2005).

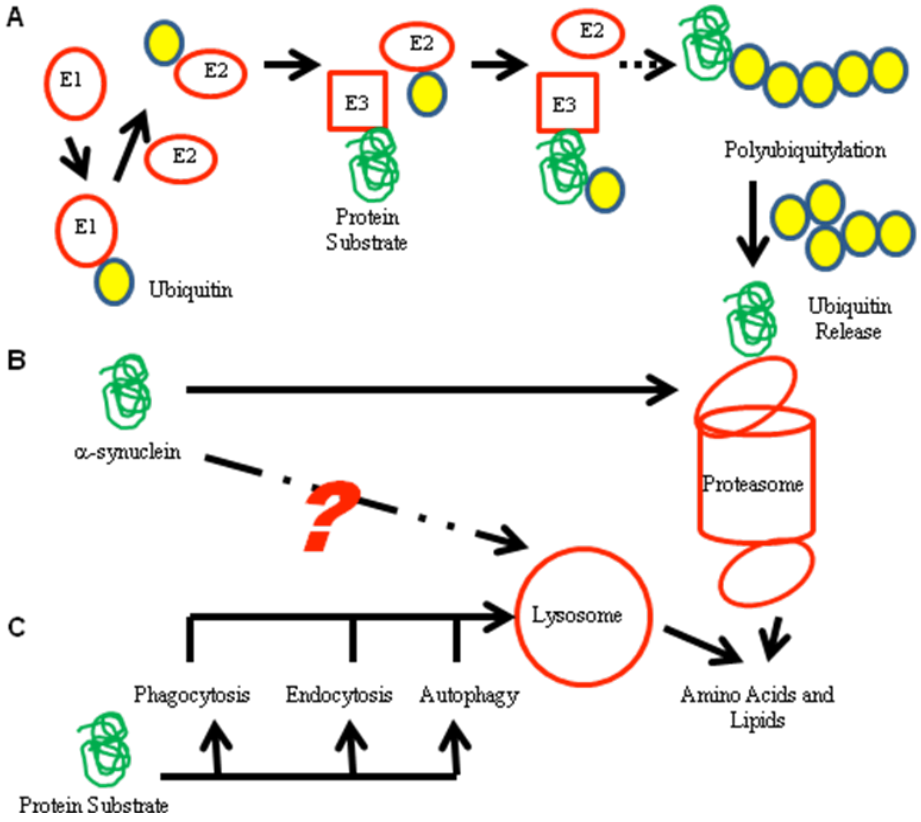

Figure 2: Cellular Degradation Pathways: Eukaryotic cells have evolved two independent pathways for the degradation of unnecessary cellular waste, which helps maintain cellular homeostasis. The first is the Ubiquiting-Proteasome Pathway (UPP). In this trafficking system, only proteins found in the cytoplasm and nucleus are degraded. The cells, however, must be poly-ubiquinated before the proteasome will recognize the protein to be recycled. Another organelle used to digest waste is the lysosome. Protein embedded in the plasma membrane or extracellularly is degraded by this pathway. There are three paths to the membrane. The first is phagocytosis, used for digestion of foreign particles. The second is endocytosis, used for the uptake of small solutes and proteins. The third is autophagy. In autophagy the cell eats its old and damaged organelles and protein. The end-product of both systems releases amino acids and lipids used for future synthesis. α-synuclein, which is found both in the cytoplasm and attached to the plasma membrane, is believed to be degraded by both the proteasome and lysosome.

In PD pathology, Lee et al (2004) found that the lysosome inhibitors rotenone, Baf, or Cathespin I reduced lysosomal degradation and caused the accumulation and aggregation of α-synuclein. Furthermore, Cathepsin D, a protease found in the lysosome, is also linked to the degradation of α-synuclein (Qiao et al., 2008). When Cathepsin D levels are reduced, the levels of α-synuclein dramatically increase (Sevlever et al., 2008). The possible role of lysosomal degradation of α-synuclein came in 2004 where chemical inhibition of the lysosome by ammonium chloride increased the levels of α-synuclein, which led to eventual cell death (Cuervo et al.). Nevertheless, the role of the lysosome in PD pathogenesis is still not well understood.

Pathways to the Lysosome

Proteins and other large molecules destined for lysosomal digestion must first bypass the plasma membrane and then be transported to the lysosome. This transportation of particles is achieved by three independent pathways (De Duve & Wattiaux, 1966; Luzio et al., 2007).

The first trafficking mechanism is phagocytosis. In this process, the cell engulfs foreign pathogens and apoptotic cells (De Duve & Wattiaux, 1966; Luzio et al., 2007). Once these targets have been identified, a signaling cascade is initiated. This pathway allows for the plasma membrane to reorganize and encircle the pathogens in vesicles known as phagosomes (Luzio et al., 2007). However, no evidence supports α-synuclein degradation through the pagocytic-lysosomal pathway as this path degrades bacterial agents.

The second lysosomal pathway is endocytosis. In this route, cells engulf material by invagination of the plasma membrane. The formed vesicle then migrates to the lysosome. However, during this path, the vesicular body matures from an early endosome to a late endosome; the latter containing more multivesicular bodies (Luzio et al., 2007). Current evidence implicates the onset of neurodegeneration due to mutations in the endosomal sorting complex required for transport (ESCRT). Patients with mutations in the vesicular protein sorting2B (VPS) develop amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (Raiborg & Stenmark, 2009). Interestingly, in 2007 Hamamichi et al. found VPS 41 neuroprotective from α-synuclein-induced degeneration in the dopaminergic neurons of *Caenorhabditis elegans* (C. *elegans*). As such, dysfunctions in ESCRT may be responsible for α-synuclein aggregation (Willingham et al., 2003; Alex Ayala's thesis).

The last trafficking system responsible for transport to the lysosome is called autophagy.

The Role & Mechanism of Autophagy in the Cell

Autophagy was discovered in the 1950s by Christian de Duve, the same man who discovered the lysosome (De Duve & Wattiaux, 1966). However, autophagy remained an obscure process until recently, where it is now at the forefront of scientific research (Klionsky, 2007; Levine & Koremer, 2008). Literally translated from Greek, Autophagy means self-eating. In autophagy, the cell sequesters cytoplasmic cargo (old, damaged, and or aggregated proteins, old and damaged organelles, and invading microorganisms) in a double-membrane lipid vesicle known as an autophagosome. This vesicle is then transported to the lysosome where the material will be degraded. Three classifications of autophagy exist: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Figure 3; hereafter referred to as autophagy; Klionsky, 2007; Levine & Koremer, 2008). Most of our understanding and knowledge about autophagy has come from macroautophagy, as it has been studied the most compared to the other forms of autophagy. My thesis will focus on this type of autophagy (Mariño & López-Otín, 2004; Bandhyopadhyay & Cuervo, 2007; Suzuki & Ohsumi, 2007; Klionsky, 2007; Levine & Koremer, 2008).

Generally, autophagy is viewed as a bulk-degradation apparatus that recycles old and damaged proteins and organelles. Furthermore, the universal belief on autophagy is that its levels are upregulated in cells facing conditions of stress and or starvation (De Duve & Wattiaux, 1966; Mizushima et al., 2004; Yorimitsu & Klionsky, 2005). During these challenging states, the cells consume themselves to acquire the necessary nutrients to survive and maintain basic functions. During these harsh conditions, autophagy non-selectively degrades proteins and organelles to acquire these nutrients (Nair & Klionsky, 2005). However, autophagy does not only fight stressful states, but is maintained at basal in the cell during nutrient rich and stress free times (Mizushima et al., 2001; Mariño & López-Otín, 2004; Nixon et al., 2005). Under these conditions, autophagy selectively degrades unnecessary organelles to control biogenesis (Nair & Klionsky, 2005).

Interestingly, if autophagy is over active, this process may actually kill the cells (Shintani & Klionsky, 2004). This process is similar to apoptosis or cell suicide. As such, these two mechanisms are categorized as follows: apoptosis is called programmed cell death (PCD) I, while autophagy is called PCD II (Cardenas-Aguayo Mdel et al., 2003; Shintani & Klionsky, 2004). Autophagy is viewed as a safety net in the cell, preventing unnecessary or improper cell death (Shintani & Klionsky, 2004). Therefore, autophagy maintains cellular homeostasis and survival during healthy and sickly times.

At the molecular level, autophagy occurs in three steps: nucleation, expansion, and fusion. The genes responsible for each step in *Saccharomyces cerevisiae* is found in Figure 3.To begin, a signal initiates autophagy by activating a pre—autophagosomal structure (PAS; Suzuki & Ohsumi, 2007; Levine & Koremer, 2008). This structure is located near the vacuole and contains the necessary materials (lipids and atg proteins) for forming the autophagosome (Suzuki & Ohsumi, 2007; Klionsky & Emr, 2000). After this signal, the PAS enters the nucleation step where it expands into a double-lipid membrane known as the phagophore or isolation membrane (Suzuki & Ohsumi, 2007; Levine & Koremer, 2008; Figure 3). As the IM grows autophagy enters the expansion step. In this stage, the IM recognizes and surrounds its cargo (cytoplasm and damaged proteins or organelles) and seals close; the final vesicle structure is called the autophagosome (Klionsky & Emr, 2000). However, before the material is degraded, the atg proteins that formed this structure are recycled in the cell for further use. Finally, in the last autophagy step, the vesicle fuses with the lysosome and the marked material is degraded (Suzuki & Ohsumi, 2007). The nutrients are then recycled back into the cell for further use.

But why should we look at autophagy in relation to neurodegenerative diseases? Specifically, what role does it have in α-synuclein degradation and in PD pathogenesis?

Linking Autophagy to Neurodegeneration

When mice are deficient in autophagy-related genes 5 or 7 (Atg 5 or Atg7), these animals begin to show signs of characteristic neurodegeneration with neuronal death and IB formation; Hara et al., 2006; Komatsu et al., 2006). Similarly, autophagy levels increase in mice during the early stages of AD, which are evidenced by the increase in autophagosomes containing amyloid β protein in AD neurons (Yang et al., 2009). Furthermore, when the mammalian target of rapamycin (mTOR, a gene required for autophagy) is pharmacologically inhibited, clearance of polyglutamine proteins in HD increases (Ravikumar, 2004). From these results, autophagy appears to serve as a neuroprotector and may be a potential pharmacological therapy in neurodegenerative diseases.

Having established a link between HD and autophagy, research turned to discover an autophagic link with PD. Currently, no genetic evidence supports autophagy's role in PD. Interestingly, histological evidence shows a substantial increase in the levels of autophagosomes in the dead and dying neurons of PD patients (Anglade et al., 1997 Williams et al., 2006). While these observations do not provide a genetic link to PD, the pathology is similar to what was seen in AD and HD models – suggesting a possible PD – autophagy link. Nevertheless, while no genetic evidence exists, some pharmacological experiments link autophagy and PD. When rampamycin is introduced to cell cultures – inhibiting mTOR – autophagy levels increase and help clear α-synuclein (Berger et al., 2006). On the other hand, when autophagy is inhibited in cell cultures with lithium, α-synuclein aggregates in the cytoplasm and in autophagosomes (Ferrucci et al. 2008). Current evidence shows that autophagy degrades α-synuclein and that α-synuclein may actually inhibit this process, hinting at a possible PD autophagy link (Gomez-Santos et al., 2007; Bandhyopadhyay & Cuervo, 2007; Sarkar et al., 2007; Martinez-Vicente et al., 2008; Wyttenbach et al., 2008; Vogiatzi et al., 2008; Kabuta & Wada, 2008; Xilouri et al., 2008; Wei et al., 2009).

The following question remains: what, if any, role does autophagy have in PD? Furthermore, as yeast have at least 30 atg genes, is a single atg gene responsible for the degradation of α-synuclein (Suzuki & Ohsumi, 2007)? Is a complex of genes required to degrade α-synuclein, knowing that these proteins interact with each other? Is one autophagy step more important than another? These questions will be best examined and answered in a budding yeast model (Figure 4).

Why Study Autophagy in Budding Yeast?

While yeast are commonly viewed in relation to the production of bread and beer, these organisms have become a useful tool in molecular biology. As the *Saccharomyces cerevisiae*, Baker's yeast, genome has been sequenced and is available online, we can easily manipulate yeast genetically. Additionally, a knockout library

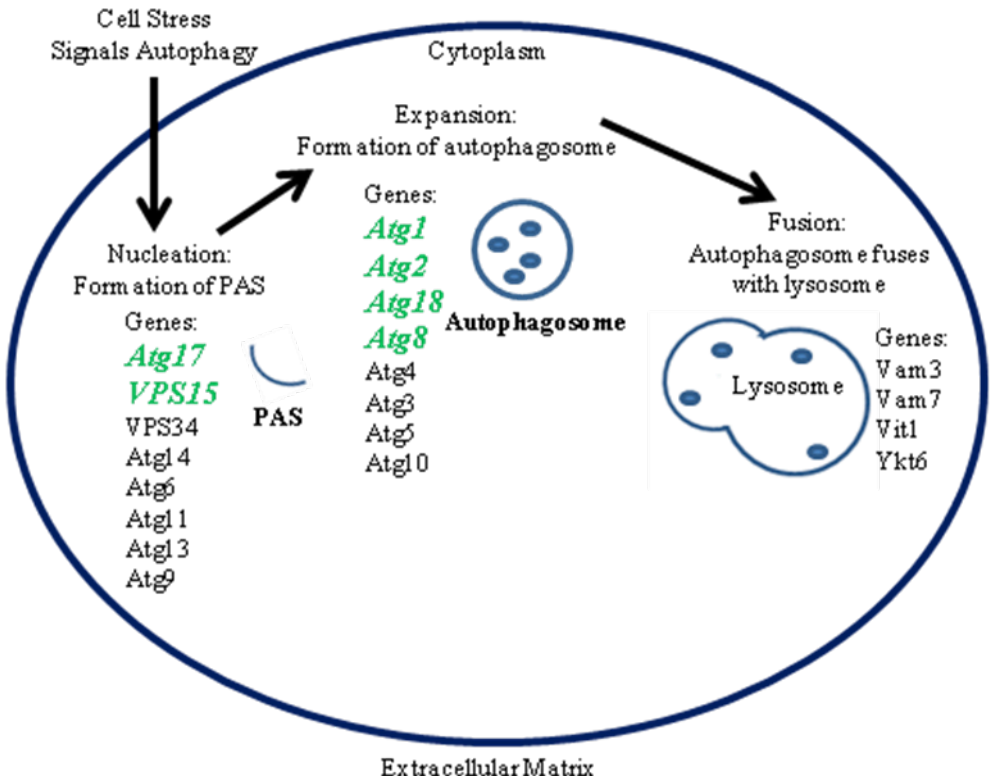

Figure 3: The steps of macroautophagy (autophagy) and their associated genes. Autophagy, in cells, is at a constant basal level to maintain homeostasis and a balance between synthesis and degradation of protein and organelles. However, cells can up-regulate autophagy in stressful and or nutrient-poor conditions to help it survive. The three major steps of autophagy are nucleation, expansion, and fusion. In the nucleation step, a pre-autophagosome structure forms around the target molecule. In the expansion step, the autophagosome is formed and the target completely encircled by the lipid bilayer. Also at this stage, autophagic bodies are formed in the autophagaosome. In the last step of autophagy, the autophagaosome binds to the lysosome or vacuole and releases its content for degradation. The lysosome will release the end-products of the digestion which are amino acids.

of all yeast genes is readily available, allowing for the study of individual genes. Furthermore, yeast and humans have high levels of evolutionary conservations in genes and in mechanisms such as cell cycle, intracellular transport, and protein synthesis, folding, and degradation (Miller-Flemming et al., 2008; Winderickx et al., 2008). Specifically, autophagy and its genes are highly conserved between yeast and humans (Klisonky & Emr, 2000). Furthermore, autophagy genes were fist discovered in and are best understood in yeast. Lastly, yeast are cheap, easy to maintain, and reproduce quickly, making it possible to perform acquire data promptly (Outerio & Lindquist, 2003; Miller-Flemming et al., 2008; Winderickx et al., 2008).

The versatility of budding yeast in molecular biology is evidenced by its use to study a wide range of protein linked neurodegenerative disorders such as prion diseases (Sondheimer & Lindquist, 2000), AD (Komano et al., 1998), HD (Meriin et al., 2002), ALS (Corson et al., 1998), fronto-temporal dementia with Parkinsonism linked to chromosome 17 (FTDP 17; Vandebroek et al., 2006), and PD (Outerio & Lindquist, 2003; Willingham et al., 2003).

The first α-synuclein yeast model papers were published in 2003 (Outerio & Lindquist, Willingham et al.). These labs found that wild-type (WT) and A53T α-synuclein bind the plasma membrane, form inclusions, alters vesicle transport, and induces toxicity in a concentration dependent manner (Outerio & Lindquist, 2003, Dixon et al., 2005). However, A30P α-synuclein is cytoplasmically diffuse and is not toxic to yeast (Outerio & Lindquist, 2003, Dixon et al., 2005; Soper et al., 2008). Similarly, the Muchowski lab preformed a genomic-wide screen and identified 86 knockout strains that were lethal when overexpressed with α-synuclein (Willingham et al. 2003). Nevertheless, their findings successfully reproduced the findings established by other labs (Willingham et al., 2003).These results then suggest that α-synuclein localization at the plasma membrane may be toxic to cells (Dixon et al., 2005).

In our lab, we have also reproduced the findings in the field. Budding yeast that expressed WT and A53T α-synuclein localized at the plasma membrane, while A30P α-synuclein was cytoplasmically diffuse (Sharma et al., 2004). However, we did not observe toxicity in any of our yeast expressing these synucleins (Sharm et al., 2004). We have also examined the third known α-synuclein mutant: E46K (Zarranz et al., 2004) E46K α-synuclein localized to the plasma membrane and was more toxic than the other familial mutants (Herrera Thesis, 2004; White Thesis, 2004). Furthermore, Michael Zorniak, who examined mitochondrial dysfunction, found decreased α-synuclein expression, no cellular toxicity, and for formation of intracellular aggregates in one of his two examined strains (2007). Lastly, we found that the formation of α-synuclein aggregates was dependent on α-synuclein expression levels (Kukreja, 2008). Additionally, when DMSO was applied to budding yeast, α-synuclein localized in unidentified cytoplasmic structures and had an α-synuclein independent toxicity (Kukreja, 2008).

While our lab has had similar results to other yeast labs, this summer (2008) we discovered an error in the 140th

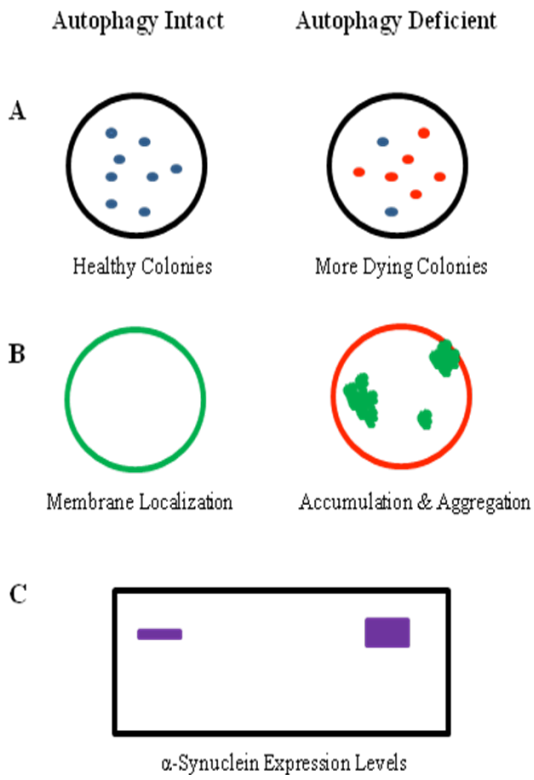

Figure 4: *Saccharomyces cerevisiae* autophagy knockout models. In WT and knockout yeast, we induced α-synuclein expression through our galactose promoter region. The normal cells should degrade α-synuclein via the autophagy-lysosome pathway and will be a healthy cell. However, in the Atg knockout yeast, we hypothesize that the increased α-synuclein expression will not be degraded by autophagy. Thus, α-synuclein will aggregate in our yeast model and prove toxic.

amino acid of our α-synuclein – where a glycine residue was mistaken for an alanine residue (Sharma, 2004). Upon discovery, we quickly corrected all our α-synuclein constructs to the correct α-synuclein – 140 Alanine. Since then, we have repeated major findings in our lab and found that most α-synuclein properties remained the same between the α-synuclein – 140 Alanine and the α-synuclein – 140 Glycine constructs. These results were expected as the difference between glycine and alanine is a methyl side chain – a conservative difference – should have no substantial impact on the folding of the protein. Furthermore, we did not find any suggestions that the 140th amino acid of α-synuclein undergoes modification in the literature. Thus, having established a viable model organism with evolutionary conservation with humans, our budding yeast will provide insight into autophagy's role in α-synuclein degradation and PD pathogenesis in humans (Miller-Flemming et al., 2008).

Hypothesis & Aims

My hypothesis was that autophagy is a route for α-synuclein degradation by the lysosome and I tested this hypothesis using a genetic approach in budding yeast. Of the 30 plus autophagy genes discovered and characterized in yeast, my thesis examined a total of six genes: four from the nucleation step and two from the expansion step.

Aim 1: To compromise nucleation step genes (Atg 17 and VPS15) by individual gene knockout strains in budding yeast and to examine if α-synuclein changed its cytoplasmic localization away from the plasma membrane, increased cellular accumulation, and made the cells more toxic

Aim 2: To compromise expansion step genes (Atg 1, Atg2, Atg8, and Atg 18) by individual gene knockout strains in budding yeast and to examine if α-synuclein changed its cytoplasmic localization away from the plasma membrane, increased cellular accumulation, and made the cells more toxic

Aim 3: To compare the pathological properties of α-synuclein – 140 Glycine to α-synuclein – 140 Alanine and to test the prediction that little to no change would be observed between them.

Results

The following results provide data for the two autophagy steps (nucleation and expansion) analyzed in their regulation of α-synuclein toxicity in budding yeast. A total of six knockout strains, two nucleation genes (Atg17 and VPS 15) and four expansion genes (Atg1, Atg2, Atg8, and Atg18), were compared with the isogenic parent BY4741 strain. Each strain was analyzed with two control vectors: pYES2 and GFP, and three α-synuclein-GFP vectors. The pYES2 construct verifies that introduction of the plasmid does not affect yeast; while the GFP construct ensures that the GFP protein is not toxic to the yeast. The three α-synuclein vectors examined were WT (linked with sporadic PD) and A30P and E46K (both familial PD mutants). We used four assays to examine α-synuclein's PD linked properties. Toxicity was measured with OD600 growth curves and spotting analysis. Expression and accumulation were measured with Western blot analysis. α-Synuclein localization was visualized with live cell GFP microscopy.

α-Synuclein Presents Well Known Characteristics in Parent BY4741 Strain

As previously published (Sharma et al., 2006), WT α-synuclein was predominantly at the plasma membrane and not toxic in BY4741 (Fig. 5). A30P α-synuclein localized diffusely throughout the cell and was not toxic (Sharma et al., 2006). Lastly, E46K α-synuclein localized to the plasma membrane and was not toxic (White et al, manuscript in prep). α-synuclein characteristics in this strain will serve as comparative controls for the autophagy compromised strains examined below because these knockout strains are derived from and are genetically isogenic to BY4741.

α-Synuclein Accumulation Alters in Nucleation Compromised Strain vps15Δ

Our first goal was to determine if α-synuclein expression would affect growth in nucleation compromised *vps15Δ* yeast. *vps15Δ* cells grew at an equal rate, irrespective of whether they expressed α-synuclein or not, but they exhibited a non- α-synuclein-dependent delayed growth when compared to BY4741 (Fig. 6). While the literature shows that *vps15Δ* yeast are viable, no evidence suggests a delay in growth compared to WT (Giaever et al., 2002). Serial spotting analysis confirmed growth curve observations (Fig. 6). Thus, we did not observe the toxicity in *vps15Δ* predicted by our hypothesis. Next, we measured α-synuclein accumulation in *vps15Δ* cells with Western Blot analysis and noted a marked decrease of α-synuclein expression in A30P cells, but not in WT, E46K, or GFP cells. Lastly, α-synuclein localization in WT and E46K cells was initially more heterogeneous (a combination of plasma membrane

localization and intracellular aggregates) in *vps15Δ* compared to predominantly plasma membrane localization in BY4741. A30P intensity was initially lower but reached BY4741 levels by 48 hours.

α-Synuclein Alters its Localization in Nucleation Comprised Strain atg17Δ

To evaluate specificity or universality of regulation by nucleation step genes, a second gene knockout strain, *atg17Δ*, was studied. Surprisingly, *atg17Δ* cells expressing α-synuclein grew better than cells that did not express synuclein (Fig. 7). However, serial spotting plates contradicted the growth curve data (Fig. 7), making it difficult to fully interpret the impact on toxicity. Western Blot analysis showed no change in synuclein expression in *atg17Δ* cells, but showed a marked decrease in GFP alone expression (Fig. 7). Lastly, *atg17Δ* cells decreased GFP fluorescence over time. In *atg17Δ* cells, WT and E46K α-synuclein localization shifted from polarized plasma membrane localization to full plasma membrane localization with some intracellular aggregates. A30P did not alter its localization. Collectively, these results suggest subtle regulation of the nucleation genes *vps15Δ* and *atg17Δ* in degrading α-synuclein as some, but not all, α-synuclein properties examined were altered.

α-Synuclein Alters its Localization in Expansion Comprised Strain atg18Δ

Next we examined the expansion step in autophagy, by first evaluating the atg18 gene. Again, contrary to our hypothesis, all *atg18Δ* cells, with or without synuclein, grew at a comparable rate to BY4741 cells (Fig. 8). Western blots revealed no change in *atg18Δ* α-synuclein expressing cells compared to BY4741, but we observed decreased GFP alone expression in cells (Fig. 8). Plasma membrane localization of E46K became more intense over time in *atg18Δ* when compared to BY4741 cells, but remained constant for WT or GFP alone. While A30P was initially diffuse, it formed more intracellular aggregates over time than it does in BY4741.

Several α-Synuclein Properties Affected in Expansion Comprised Strain atg2Δ

Next, to evaluate specificity or universality of regulation by expansion genes, we examined a second gene, *atg2*. Growth curves demonstrated that α-synuclein cells had a delayed growth compared to the other *atg2Δ* cells, suggesting some toxicity (Fig. 9). However, serial spotting plates did not capture this reduced WT growth (Fig. 9). Interestingly, Western analysis showed increased accumulation of WT synuclein and GFP alone, but not A30P or E46K, when compared to expression in BY4741 cells (Fig. 9). Importantly, all forms of α-synuclein (WT A30P, and E46K) localized as intracellular aggregates over time, losing their initial membrane localization (WT and E46K) of cytoplasmically diffuse status (A30P), showing significant α-synuclein dependent change (Fig. 9).

α-Synuclein Alters its Localization in Expansion Comprised Strain atg8Δ

Because the expansion step involves at least ten genes, we examined another gene knockout, *atg8Δ*. Irrespective of whether they expressed α-synuclein or not, *atg8Δ* cells grew at an equal rate to BY4741 (Fig. 10). Serial spotting analysis confirmed this observation (Fig. 10). Western blot analysis revealed no changed in accumulation when compared to BY4741 cells (Fig. 10). Lastly, live cell microscopy in *atg8Δ* revealed unexpected intracellular aggregate formation of not just α-synuclein variants but also of GFP alone, indicating a non-synuclein-dependent localization effect.

α-Synuclein Alters its Localization in Expansion Comprised Strain atg1Δ

Lastly, we examined another expansion gene atg1. Irrespective of whether they expressed α-synuclein or not, *atg1Δ* cells grew at an equal rate to BY4741 (Fig. 11). Serial spotting confirmed this analysis (Fig. 11). Western blot analysis revealed no change in accumulation when compared to BY4741 cells (Fig. 11). Lastly, live cell microscopy in *atg1Δ* cells revealed unexpected intracellular aggregate formation in GFP and A30P α-synuclein expressing cells over time, while WT and E46K α-synuclein remained unchanged when compared to BY4741 cells (Fig. 11).

Collectively, these results suggest subtle regulation of the expansion genes in degrading α-synuclein. In *atg2Δ* yeast, we observed changes in growth and localization. In *atg18Δ* yeast, E46K and A30P localization were α-synuclein, but the other properties were untouched. Interestingly, *atg8Δ* had little impact on α-synuclein specific properties, but instead, its effect on protein localization appeared more global. Lastly, *atg1Δ* did not appear to have significant effects on α-synuclein specific properties.

Does α-Synuclein's 140th Amino Acid Matter?

During the summer of 2008, our lab discovered a mutation in our α-synuclein construct where the terminal 140th amino acid was a mutant (glycine) instead of the correct amino acid (alanine). We, therefore, corrected all of our α-synuclein constructs and proceeded with experimentation (as seen with the above results). However, before this discovery, my autophagy research (and that done by my two colleagues, Alina Konnikova and Julian Mclain) had utilized the α-synuclein constructs that carried the glycine mutant in four knockout strains (*atg1Δ, atg2Δ, atg17Δ, and atg18Δ*). Here, I will provide a brief summary of results we obtained and compare it to the α-synuclein data derived from the corrected amino acid (See Appendix figures: 1 – 5)

1. Serial spotting analysis revealed no difference between α-synuclein – 140 Glycine and α-synuclein – 140 Alanine, in any of the four strains tested (compare B in Appendix figures 1 – 5).

2. Growth curves reveal no differences between α-synuclein – 140 Glycine and α-synuclein – 140 Alanine, as above. However, one notable change was that E46K growth was rescued in α-synuclein – 140 Alanine (compare A in Appendix figures 1 – 5).

3. Live cell microscopy of all vector constructs (α-synuclein – 140 Glycine and α-synuclein – 140 Alanine) in *atg1Δ, atg2Δ, atg17Δ,* and *atg18Δ* strains are mostly comparable to BY4741, with some subtle exceptions (compare D in Appendix figures 1 – 5).

4. Lastly, Western blot analysis revealed reduced accumulation of E46K α-synuclein – 140 Glycine in BY4741 compared to *atg1Δ* , *atg2Δ, atg17Δ,* and *atg18Δ*. However, E46K α-synuclein – 140 Alanine expression was comparable to *atg1Δ, atg2Δ, atg17Δ,* and *atg18Δ* (compare C in Appendix figures 1 – 5).

Together, these results reveal that most of the α-synuclein properties examined were similar, with some subtle changes between α-synuclein – 140 Glycine and α-synuclein – 140 Alanine.

Discussion

While substantial genetic evidence links α-synuclein degradation via the proteasome, increasing evidence implicates autophagy mediated lysosomal degradation (Webb et al., 2003; Gomez-Santos et al., 2007; Bandhyopadhyay & Cuervo, 2007). Nevertheless, this hypothesis in the field is still tentative as more research is required. As such, my thesis investigated autophagy's role in the clearance of α-synuclein in yeast cells. We inquired whether autophagy knockouts would be toxic in *Saccharomyces cerevisiae* expressing α-synuclein. We hypothesized an increase in α-synuclein related toxicity in our autophagy deficient strains, due to increased accumulation. Our results, however, lead to three notable conclusions as they unexpectedly did not show dramatic changes in the α-synuclein properties examined. First, we found subtle regulation of α-synuclein in our knockout strains. Secondly, different autophagy knockouts had different effects on the α-synuclein properties in BY4741. Lastly, none of the genes examined has an effect on α-synuclein toxicity. Nonetheless, while our results do provide some insight into autophagy's role in α-synuclein's PD pathogenesis, more research is needed for conclusive results.

Subtle Regulation of α-Synuclein in Autophagy Knockouts

Contrary to our hypothesis, the six-autophagy genes we examined did not have major effects on α-synuclein related PD pathogenesis. Instead, the knockouts we studied had subtle alterations in the three examined α-synuclein properties. Furthermore, not all properties investigated were affected in each knockout strain. For example, while growth in α-synuclein expressing *atg17Δ* yeast grew faster than BY4741, the other properties of this strain were mostly unaffected, but still comparable to BY4741. Similarly, *atg2Δ* mutants revealed a dramatic shift in α-synuclein localization (foci formation), but growth and accumulation were comparable to BY4741.

While our results predominantly show α-synuclein alterations in one of four assays (change in localization, but not in toxicity or accumulation), these results cannot exclude autophagy from having a role in α-synuclein PD pathogenesis. Past research with yeast gene knockout strains have demonstrated α-synuclein regulation without affecting all known PD-linked properties. Previous research of oxidative stress in our lab has shown extensive α-synuclein related toxicity in the *sod1Δ and sod2Δ* strains, but no change in α-synuclein localization (Sharma et al., 2006).

Research in the Gitler lab (2009) shows similar impairments to single assays of their PD yeast model. They found that the overexpression of Ypk9 rescued BY4741 yeast from α-synuclein induced toxicity, but observed no alteration in the other α-synuclein properties. Similarly, Flower et al. (2005) found increased toxicity in their *Sac3Δ*, a heat shock protein, FY23 yeast strain, but found no change in α-synuclein expression. Even though our current results mostly show subtle autophagic regulation of α-synuclein properties in one assay, we cannot decidedly determine autophagy's potential role in PD pathogenesis as single changes have significant consequences in our own and other labs' yeast models.

Thus, despite its subtle role, autophagy can still be implicated in our yeast model. As previously stated, autophagy is required for basic cellular homeostasis and maintenance. As seen in figure 3, autophagy is a complex, multi-step pathway composed of at least 30 genes (Suzuki & Ohsumi, 2007). Since each autophagy step contains at least seven genes, one or two of these genes of the overall process may play a critical role in PD pathogenesis. For example, two autophagy genes in mice had an important role in basal autophagy and in the onset of neurodegeneration: Atg5 and Atg7 (Komatsu et al., 2006; Hara et al., 2006). Mice deficient in either gene, in the absence of other mutations, displayed characteristic symptoms of neurodegeneration. Furthermore, when beclin 1 (yeast homologue: apg6p) expression is reduced in mice, they show earlier-than-expected AD pathogenesis (Pickford et al., 2008). To this extent, our research has only examined a fraction of the relevant autophagy genes of the nucleation and expansion steps in yeast. Therefore, we have yet to determine if a critical gene(s) exists in PD pathogenesis.

While a potential autophagy gene may exist, our results may be due to redundancies that exist in the autophagic pathway – at least 30 genes are responsible for this complex pathway (Suzuki & Ohsumi, 2007). Even though we examined subtle changes of α-synuclein in our nucleation and expansion knockouts, another atg gene of the same step may compensate for this loss of function. Yang et al. (2006) found that atg22p, in yeast, has overlapping functions with avt3p and avt4p – vacuolar efflux proteins. Additionally, mammalian atg1 is known to have redundant functions (Lee et al., 2007). Therefore, nucleation and expansion genes may have possible complementary and or redundant roles in yeast, leading to the subtle changes observed. As with critical autophagy genes, this redundancy has yet to be established in yeast.

Proteins do not act independently in the cell, but function together as a large piece of machinery; autophagy proteins are no exception. Most, if not all, autophagy genes perform their functions as part of a large complex of proteins. Atg1 forms complexes with Atg13 and Atg17; it also forms a complex with atg11 (Kamada et al. 2000; Kabeya et al., 2005). Furthermore, the Atg12, Atg5, Atg7, Atg10, and Atg16 complex is required for autophagosome formation (Wang & Klionsky, 2003). In 2005, Cheong et al. found that their *atg17Δ* yeast had only minor autophagy defects. This finding suggests that even if we were to remove a protein required for autophagy complexes, we would not hinder this process, but rather make in inefficient. Thus, the alteration we observed in some α-synuclein properties in our six *atgΔ* strains could be explained by autophagy's reduced functionality.

Not All Autophagy Genes Affect α-Synuclein Similarly

Despite the subtle changes we observed in our autophagy knockout strains, each knockout strain had interesting and differing effects on the α-synuclein properties examined. Most of the strains studied had only one major change in our four evaluations, with some subtle, if any, changes in the other assays. In *atg17Δ*, α-synuclein appears not to be toxic as these constructs allow yeast to grow faster. In *atg8Δ* yeast, α-synuclein localization in A30P and E46K changed over time when compared to BY4741. However, we did notice two strains that did have changes in more than one of our assays. The first was *atg2Δ*; in this strain, α-synuclein localization dramatically changed over time and WT α-synuclein had a growth delayed compared to the four other constructs. The second strain was *vps15Δ* where growth, localization, and accumulation in one α-synuclein construct were altered.

Similar to the idea that one autophagy gene is important for α-synuclein degradation, not all autophagy genes have an equal role in this catabolic process (Suzuki & Ohsumi, 2007). In autophagy, of the 30 known genes thus far, 16 of these genes are part of the basic machinery needed to form the IM, while the 14 other genes work in concert with these basic proteins (Suzuki & Ohsumi, 2007; Mari & Reggioir, 2007). Of the six genes we examined, all genes, but atg17, are required for the core autophagy

machinery (Xie & Klionsky, 2007). Additionally, certain genes are known to be more functionally important than others in this process. One protein of great importance is atg9 – part of the core proteins. The function of atg9 is to sequester the lipid membranes needed for the formation of the PAS (Xie & Klionsky, 2007). When this gene is loss, autophagy is greatly hindered (Lang et al., 2000). Taken together, our knockouts may have less importance in the autophagic pathway than other genes. Furthermore, they may have a less specific role in degrading α-synuclein. However, due to the limited research on the six genes I studied, we cannot state their important role in autophagy or in the degradation of α-synuclein.

No Effects on α-Synuclein Related Toxicity in Autophagy Knockouts

While my data may implicate α-synuclein regulation by autophagy due to the change in its localization, these results are a stark contrast to our prediction on toxicity. As previously stated, the most surprising result we obtained was in our *atg17Δ* yeast where the three α-synuclein constructs grew at a faster rate than that of our controls. However, we did observe some toxicity as *vps15Δ* yeast grew at a slower rate than that of our control. Even WT α-synuclein showed some delayed growth in our *atg2Δ* yeast. Nevertheless, these strains did not reveal the toxicity we expected, nor did the three other examined strains.

As aforementioned, some compensation in the nucleation and expansion steps of autophagy may be due to possible redundancies in the pathway. However, this complementary effect is not limited to autophagy genes themselves, but can be expanded to three other degradation pathways in yeast: proteasomal degradation, apoptosis, and chaperone-mediated autophagy. As previously stated, α-synuclein can be degraded by both the proteasome and the lysosome. Therefore, α-synuclein in our autophagy knockout yeast may be increasingly degraded by the proteasome, or at least at sufficient levels to prevent noticeable toxicity in these yeasts. Webb et al. (2003) have shown that inhibition of either the proteasome or autophagy causes an upregulation in activity of the other respective pathway, suggesting some levels of compensation (Ding et al., 2007; Settembre et al., 2007; Raben et al., 2008; Venkatachalam et al., 2008; Zhang et al., 2009)

This similar effect is observed in another pathway to maintain the health of an organism. Apoptosis, like autophagy, is an example of programmed cell death (PCD). Due to their similarity in cellular function, these two pathways are tightly related and have some compensatory effects. Two proteins that regulate and differentiate between PCD I and PCD II are the DAPk and DRP-1 family of proteins (Shintani & Klionsky, 2004; Mizushima et al., 2008). Due to the tight regulation between the two PCDs, the results of these papers suggest that inhibition of autophagy may lead to increases in apoptotic activity and vice versa (Maiuri et al., 2009; Mizushima et al., 2008). In 2006, a paper showed that caplain cleavage of Atg5 induced apoptosis (Yousefi et al., 2006). Furthermore, loss of the bax protein in mice, upregulated autophagy, while loss of Atg 5 or Atg 7 increased apoptosis in neuronal cells (role of bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes; Hara et al., 2006; Komatsu et al., 2006). Therefore, yeast that were overcome by α-synuclein related toxicity increased their apoptotic response early in their growth to help maintain the populations survival (Chronological aging leads to apoptosis in yeast). Nevertheless, apoptosis in yeast, as well as the link between the two PCD mechanisms are not fully understood. Thus, more research is required before this hypothetical connection can be established.

A last compensatory mechanism for autophagy is CMA. Unlike autophagy, CMA undergoes a molecule by molecule degradation pathway. Specific proteins are sequestered by a chaperone protein that transports this complex to the lysosome. Nevertheless, while these two pathways are mostly independent, recent genetic evidence reveals cross-talk between autophagy and CMA as lowered CMA activity increased autophagy levels (Kaushink et al., 2008). The opposite effect is also observed (Massey et al., 2006; Kaushik et al., 2008). However, these results show only partial compensation due to the redundancies between the two autophagys. Taken together, one pathway is not enough to compensate for the other. Nevertheless, the partial CMA compensation may be enough to have prevented the toxicity we expected in our autophagy knockout strains (Kaushik et al., 2008).

A last explanation for this lack of observable toxicity may be linked to the physical traits of yeast – their stress response. For example, yeast used to brew beer must respond to different temperatures, oxidative conditions, and pH levels must adapt to these stressful conditions to survive (Gibson et al., 2007). Therefore, the yeast stress response is particularly adept to helping these organisms survive a variety of harsh and unstable environments. This same survival skill may be activated in my autophagy deficient yeast; in addition to the upregulation of the three other degradation pathways. With these results, we cannot exclude an autophagic regulation of α-synuclein related PD toxicity.

Only Subtle Differences Between α-Synuclein – 140 Glycine and α-Synuclein – 140 Alanine Constructs

While the data from this thesis may or may not implicate autophagy in α-synuclein degradation, our results, nevertheless, supported our third aim: there would be little or no change between the two α-synuclein constructs. One reason we did not expect any change is that the difference between α-synuclein – 140 Glycine and α-synuclein – 140 Alanine is due to the conservative difference between amino acids – a methyl group. To begin, the methyl group is a microscopic addition when compared to the entire protein. Second, this group does not change the polarity of the protein as hydrogen and methane do not carry an overall charge. These two factors should then not affect the tertiary structure of the protein significantly. Lastly, α-synuclein that is found in Lewy Bodies is known to undergo post-translational modification at the following sites: 12th, 21st, 23rd, 87th, 115th, 119th, 122nd, 129th, 133rd, and 135th (Fujiwara et al., 2002; Anderson et al., 2006), but no known modification is known to occur on α-synuclein at the 140th. Therefore, since the terminal amino acid does not appear to have great functional importance, there should be subtle changes, if any, between the α-synuclein constructs.

The most notable change between the two α-synuclein constructs came in BY4741 Western Blots. The E46K expression in the corrected α-synuclein increased compared to mutant α-synuclein (Fig x. appendix x). Secondly, corrected E46K was no longer toxic compared to the mutant α-synuclein. In our knockouts strains, the most prominent change between the two α-synuclein constructs was with the shift in localization. Even with these changes in corrected α-synuclein, the α-synuclein properties did not dramatically differ from the mutant α-synuclein. We can therefore conclude that the data from three years of research in our lab are still valid for reference and that the 140th amino acid has slight alterations on α-synuclein.

Criticisms and Limitations

While we believe that autophagy is dysfunctional in our knockout strains, we never measured autophagy. Therefore,

we cannot determine if autophagy has ceased. Instead of directly measuring autophagy, we measure its effects indirectly. Furthermore, once we ensure autophagy deficiency, FM4-64 staining will ensure that α-synuclein is sent to and degraded by the lysosome. Finally, evaluation of the other atg genes and inducing autophagy in our atg knockouts will help elucidate the possible genetic links between α-synuclein degradation and autophagy.

Future Studies

The first studies to be preformed will be the complete analysis of all remaining nucleation, expansion, and fusion genes. We will also need to verify α-synuclein localization to the lysosome with FM4-64 staining. The next steps will be to analyze induced autophagy with different nutrient starvation conditions, chemical upregulation of autophagy, or other stress promoting environments. (As of yet, we have only studied basal autophagy). With these experiments, we will have a better understanding of autophagy's role in controlling α-synuclein related toxicity. Additionally, all these experiments will need to be repeated in fission yeast as a complete knockout library is now available. As a last study, we will need to examine if other degradation pathways are compensating for the loss of autophagy.

Conclusion

The goal of this research was to establish a connection between autophagy and α-synuclein related PD pathogenesis. While our results surprisingly did not reveal the toxicity we expected, autophagy did appear to subtly regulate the degradation of α-synuclein. The most significant change we observed was the mislocalization of α-synuclein from the parent BY4741 strain. Nonetheless, we cannot decidedly state if autophagy play or does not play a role in degrading α-synuclein as more research must be conducted.

However, if we are able to establish an autophagic – PD link, the potential for PD treatments greatly arise. In other neurodegenerative disease, scientists hypothesize that upregulation of autophagy may remove toxic protein aggregates in HD (Ravikumar et al., 2004; Sarkar et al., 2007). It may also be possible to use this increased activity to prevent the onset of disease; a similar treatment option may be available to PD patients (Pan et al., 2008). Taken together, establishing an autophagic genetic link with PD could potentially help improve the quality of life in PD patients.

Methods

Methods described in detail in Sharma et al 2006.

α-Synuclein Constructs

A30P and E46K α-synuclein were produced using site-directed mutagenesis (Invitrogen) from WT α-synuclein – sequencing was confirmed by the University of Chicago. A53T α-synuclein cDNAs and human WT α-synuclein were a gift from Christopher Ross (Johns Hopkins University). WT and mutant α-synuclein cDNAs were subcloned into the pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). All α-synuclein cDNAs were subcloned into the mammalian expression vector pcDNA3.1/C-terminal GFP (Invitrogen) where they were fused with GFP at the C-terminus. These α-synuclein constructs were PCR-amplified and subcloned into pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). Chemically competent E. coli cells were transformed with the α-synuclein, α-synuclein-GFP, and GFP-–α-synuclein pYES2.1/V5-His-TOPO vectors.

All α-synuclein – 140 Glycine constructs were reconstructed to α-synuclein – 140 Alanine using site-directed mutagenesis. The following primers were used:

Gal-1 forward primer
5'-AATATACCTCTATACTTTAACGTC-3'

V5C-term Reverse primer
5'-ACCGAGGAGAGGGTTAGGGAT-3

All α-synuclein – 140 Alanine constructs were confirmed by sequencing (University of Chicago).

α-synuclein Expression Vectors

The pYES2.1/V5-His-TOPO DNA vector (Invitrogen) was used for α-synuclein expression in budding yeast. Four constructs were previously created for each experiment: green fluorescent protein (GFP), wild-type α-synuclein, A30P α-synuclein, and E46K α-synuclein. The pYES2.1/V5-His-TOPO DNA vector and the GFP tagged vector were used as controls (See Table 1 for List of Constructs).

Yeast Strains: The BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and atgΔ knockout strains (atg1Δ, atg2Δ, atg8Δ, atg17Δ, atg18Δ, and vps15Δ) were purchased from Open Biosytsems.

Transformation of Yeast Strains: The GFP and α-synuclein WT, A30P, and E46K plasmids were transformed into BY4741, atg1Δ in 4741, atg2Δ in 4741, atg8Δ in 4741, atg17Δ in 4741, atg18Δ in 4741, and vps15Δ in 4741. All plasmids had C-terminus GFP tags in the pYES2.1/V5-His-TOPO vector. Transformations were confirmed by whole-cell yeast polymerase chain reaction (PCR).

α-Synuclein Expression: α-synuclein and GFP expression was regulated by a galactose inducible promoter (GAL1) within the pYES2.1/V5-His-TOPO. Yeast were grown in SC-Uracil glucose overnight for analysis. The glucose media suppressed α-synuclein expression. The yeast were centrifuged (2500 rpm at 4 °C for 5 min) and then washed. This process was repeated twice to insure glucose removal. The yeast were then inoculated in SC-Uracil galactose and incubated at30 °C and 200 rpm shaking to induce α-synuclein expression.

Growth Curve Analysis: Cells were grown overnight in 5 mL of SC-Uracil glucose at 30 °C and 200 rpm shaking. The cells were centrifuged (2500 10^7 cells were removed, pelleted, and re-suspended in 1 mL of water. Each culture had 100 μL transferred to a 96-well microtiter plate and were serially diluted, 5-fold, across the plate. The cultures were then plated onto three SC-Uracil galactose and three SC-Uracil glucose plates. Each plate was then incubated at 30 °C and photographed 2 days post-growth.

GFP – Live Cell Fluorescent Microscopy: 1ml of cells were taken from a parallel running Growth Curve and pelleted at 14,000 rpm 24 and 48 hours post-α-synuclein expression. The galactose media was poured off and the cells were re-suspended in the remaining media. 10 μL of each culture was pipetted onto a glass slide with a cover slip. All images were taken using a Nikon TE2000-U fluorescent microscope at 1000X magnification and Metamorph® 6.0 software. Cells were first viewed with differential interference contrast (DIC), and then the fluorescence images of the same cells were taken. A minimum of 750 cells for each culture were examined.

rpm for 5 min at 4 °C) and washed. This process was repeated twice. Cells were re-suspended in 10 mL of nanopure H_20 and counted using a hemocytometer. Flasks with 25ml SC-Uracil galactose were inoculated to a cell density of 2.0 x 10^6 cells/mL and incubated at 30 °C and 200 rpm shaking for 48 hours. At 0, 3, 6, 12, 18, 24, 36, and 48 hours post-galactose innoculation, cell density was measured by pipetting 1 mL from each culture, in duplicate, into a cuvet. The cuvets were analyzed using a Hitachi U-2000 spectrophotometer to determine culture density. Average absorbance versus time was plotted on a graph for growth curves.

Growth Analysis through Cell Spotting: Cells were grown overnight in 5 mL of SC-Uracil glucose at 30 °C and 200 rpm shaking. The cells were centrifuged (2500 rpm for 5 min at 4 °C) and washed. This process was repeated twice. Cells were re-suspended in 10 mL of nanopure H_20 and counted using a hemocytometer. A density of 2.0 x

Western Blotting: Cells were grown in SC-Uracil glucose overnight. They were harvested and centrifuged at 2500 rpm at 4 °C for 5 min then washed with water (repeated twice). The cells were then

transferred to SC-Uracil galactose. Cells were taken 24 and 48 hours post-α-synuclein expression and counted to a density of 2.5 x 10^7. The cells were centrifuged at 14,000 rpm in order to make cell lysates. Electrophoresis -solubilizing buffer (ESB) was added to each colony, heat shocked at 100°C, and lysed using small glass beads. The ESB contains numerous protease inhibitors and Sodium Dodecyl Sulfate (SDS) detergent. The lysates were stored at -20 °C. Tris-glycine gels (Invitrogen) were used to run the cell lysates. 10 μL of See Blue mass ladder (Invitrogen) was used to determine protein mass. 20 μL of each cell lysate was transferred into the gel and ran at 130 V with 1X running buffer (29.0 g Tris-Base, 144.0 g Glycine, 10.0 g SDS, 1 L H_2O, and pH 8.7). The protein in the gel was transferred to PVDF membranes using the Semi-Dry Transfer apparatus (Biorad). Anti-V5 monoclonal (Invitrogen) antibody was used to analyze α-synuclein expression, while anti-Phosphoglycerate Kinase (PGK, Invitrogen) was used for loading control. The immunodetection protocol from Invitrogen Western Breeze kit was used to detect the alkaline phsophatase activity. The gel was dried and photographed.

Table 1. List of Transformed Budding Yeast Strains

Construct	Expression Vector	Strain
No cDNA & GFP	pYES2.1	BY4741, *atg1Δ, atg2Δ, atg8Δ, atg17Δ, atg18Δ, vps15Δ*
WT-α-Synuclein- 140 Alanine-GFP	pYES2.1	" "
A30P-α-Synuclein-140 Alanine-GFP	pYES2.1	" "
E46K-α-Synuclein-140 Alanine-GFP	pYES2.1	" "
WT-α-Synuclein-140Glycine-GFP	pYES2.1	BY4741, *atg1Δ, atg2Δ, atg17Δ, atg18Δ*
A30P-α-Synuclein-140Glycine-GFP	pYES2.1	" "
E46K-α-Synuclein-140Glycine-GFP	pYES2.1	" "

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