Molecular Determinant of α-Synuclein Pathotoxicity in Yeast Models

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Abstract

Parkinson disease (PD) is an incurable neurodegenerative disorder linked to the misfolding and aggregation of α-synuclein protein in dying neurons. Several molecular features of α-synuclein that appear to contribute to its properties are the familial mutant E46K, serine phosphorylation, and hydrophobic residues, but their exact role is unclear. I used two yeast models to examine how the E46K mutation, phosphorylation, and alanine-76 impact the properties of α-synuclein. My thesis research revealed three important findings: 1) The E46K familial mutation bound membranes in budding yeast, aggregated in fission yeast, and induced toxicity in a strain-specific manner. 2) Phosphorylation of α-synuclein influenced the protein's ability to aggregate, but the phosphorylation state did not determine toxicity in yeast. 3) Alanine-76 decreased membrane binding in budding yeast and aggregation in fission yeast. This thesis provided multiple lines of insight into the molecular determinants of α-synuclein toxicity. An increased understanding of α-synuclein's pathotoxic properties could lead to more effective therapeutic strategies.

Introduction

Neurodegenerative Diseases

At only three pounds, the human brain is by far the most important organ in the human body. The brain is our central processing unit; it is involved in every action we carry out, sensation we perceive, or thought we contemplate. From the feel of the sun's warmth on our skin to the complex muscle coordination necessary to walk up a flight of stairs, the brain plays a pivotal role in every action taken throughout life. This universal involvement in our lives requires a highly organized and overwhelmingly complex connection of neurons, the signaling cells of the nervous system, in the brain. In fact, the neuronal circuits of the brain are so complex that disruption of these specific connections results in a plethora of distinct neurological disorders, including schizophrenia, depression, autism, and epilepsy. In addition, a family of tragic, incurable disorders termed neurodegenerative diseases can afflict the brain as well. Neurodegenerative diseases increase in prevalence with age and have, unfortunately, become increasingly common in society as medical advances lead to increased longevity of the general population.

The unique environment of our brain contributes to its vulnerability to neurodegenerative disease in two important ways. Firstly, the adult brain is characterized by very select neurogenesis (birth of new neurons) (Purves et al., 2008). As such, neurons are not replaced in the same way that the skin or intestines replace lost cells. Secondly, cellular regeneration in the central nervous system (CNS) is constrained by a number of mechanisms (Grandpre et al., 2000). Neural circuits are precisely wired pathways of communication between cells that form when we are children. These regenerative barriers were favored by evolution to prevent incorrect wiring of neurons after damage occurs, as incorrect rewiring could lead to further harm (Purves et al., 2008). Once damage occurs in the CNS, it cannot be fixed, a property that makes studying neurodegeneration all the more important.

Each neurodegenerative disease is characterized by progressive death of neurons in a specific area of the brain or spinal cord. Several of the most well known neurodegenerative diseases are Alzheimer's disease (AD), multiple sclerosis, Huntington's disease (HD), prion diseases such as Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (mad cow disease), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD). The specificity of degeneration in each disease is quite astounding. For example, neurons in the cortex and hippocampus (involved in memory) degenerate in AD, resulting in the characteristic memory impairment seen in patients (Waldemar et al., 2007). In ALS, upper and lower motor neurons (cells essential for muscle movements) deteriorate, leading to the inability of sufferers to initiate voluntary movement (Shaw et al., 2001). Although neurodegenerative diseases are distinct in that they affect different regions of the brain, they are unified by the presence of protein deposits (often called aggregates) in or around the affected neurons (Taylor et al., 2002). These deposits form when the involved proteins come out of solution, similar to how cheese or yogurt comes out milk. In each neurodegenerative disease, one or two specific culprit proteins thought to be intimately involved in pathogenesis (disease development) compose a large portion of the aggregates. However, the role that these protein aggregates play in cell death in neurodegenerative diseases is not fully understood.

Some neurodegenerative diseases can be grouped together. One group of neurodegenerative diseases is termed the synucleinopathies, and this family includes dementia with lewy bodies (DLB), multiple system atrophy (MSA), lewy body dysphagia (LBD), and PD (Figure 1A and 1B). DLB primarily afflicts the cerebral cortex, and it results in dementia reminiscent of AD. MSA stems from loss of neurons in the putamen, globus pallidus, and caudate nucleus, and this disease leads to degeneration of movement and the body's autonomic functions. Lewy body dysphagia results from the degeneration of the vagus nerve, a nerve essential for muscle movements in the esophagus, and a hallmark symptom is difficulty swallowing. While these diseases afflict several regions of the brain, each one is characterized by the accumulation of misfolded and aggregated α-synuclein in the dying neurons (Spillantini et al., 1998; Wakabayashi, 1999; Burn et al., 2001; Heidebrink et al., 2001). Interestingly, DLB, PD, and MSA all involve the substantia nigra. My thesis research centers on increasing understanding of the molecular basis behind one specific synucleiopathy, PD, but my findings will help understand all synucleinopathies.

Introduction to Parkinson's Disease

PD is the most common movement-based disorder in the elderly and the second most common neurodegenerative disease after AD (Serulle et al., 2006; de Silviera et al., 2002). Over 4 million people suffer from the disorder worldwide, and this number is expected to double by 2030 (Dorsey et al., 2007; Jain et al., 2005). In PD patients, selective death of midbrain dopaminergic neurons in the

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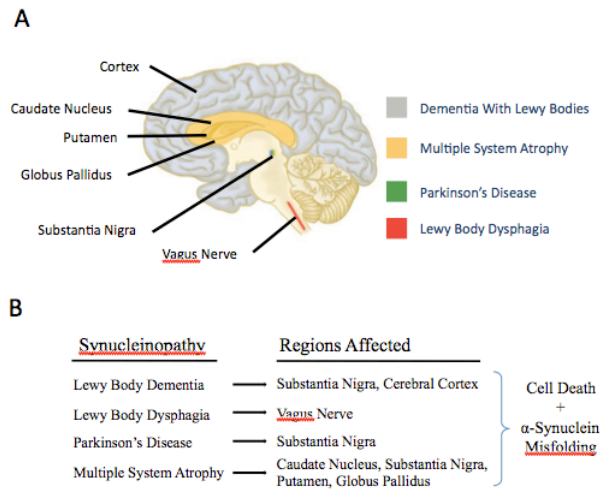

Figure 1: Synucleinopathies Impact Numerous Brain Regions
(A) Schematic of the brain & the structures involved in four synucleinopathies. Distinct regions of the brain are affected by individual synucleionpathic diseases. Blue: Lewy Body Dimentia. Orange: Multiple System Atrophy. Green: Parkinson's Disease. Red: Lewy Body Dysphagia (B) List of the regions afflicted by synucleionpathies. While each individual synucleinopathy afflicts a different region of the brain, they are all united in the presence of misfolded, aggregated α-synuclein within the dying neurons.

Brain image from: http://www.paulnussbaum.com/

substantia nigra pars compacta (SNpc) occurs (Barbosa et al., 1997). These neurons, localized to a 1-2mm wide strip and darkened by the pigment neuromelanin, are part of the basal ganglia, a neural circuit situated at the base of the forebrain responsible for the initiation of voluntary movement (Figure 2A; Olanow and Tatton, 1999). SNpc neurons project to the striatum, a structure that influences timing and coordination of muscle movements. Additionally, SNpc neurons synthesize the neurotransmitter dopamine, an important signaling molecule in this area of the brain. Diminished dopamine levels resulting from cell death further disrupt neuronal signaling. The loss of SNpc neurons in PD patients results in gaps in the neural circuit and the onset of several movement based symptoms, such as rigidity, resting tremors, and slow movement (Goedert et al., 2001). The exact cause of cell death in the SNpc is unknown, but many researchers believe the protein α-synuclein is the culprit (Outeiro and Lindquist, 2003; Cooper et al., 2006).

Upon examination under a light microscope, large protein accumulations are visible inside the dying neurons of PD patients (Muchowski, 2002; Eriksen et al., 2005; Robinson, 2008). These protein aggregates, as with all neurodegenerative diseases, are the hallmark symptom of PD. They were first identified by Friederich Lewy in 1912 and have since been termed Lewy Bodies (LBs; Forster and Lewy, 1912). Although LBs contain several different proteins, they are chiefly comprised of misfolded α-synuclein, a protein expressed throughout the brain (Spillantini et al., 1998). Interestingly, LBs are present in both of the major forms of PD: sporadic and familial.

Variety of Parkinson's Disease: Sporadic and Familial
Many diseases have both environmental or genetic causes. Diseases that arise without a known genetic cause are defined as sporadic. Conversely, diseases caused by heritable genetic mutations are termed familial. Over 90% of PD cases arise sporadically, and possible initiators are pesticide exposure (Ascherio et al., 2006), infection (Altschuler, 2007) and contact with heavy metals (Calne et al., 1994). Mitochondrial dysfunction (Langston et al., 1983; Langston et al., 1984) and free radical damage (Jenner and Olanow, 1996; Maguire-Zeiss et al., 2005) have also been implicated in disease onset. Additionally, sporadic PD is closely linked to the misfolding and aggregation of α-synuclein, but the exact cause remains unidentified (Dawson and Dawson, 2003; Greenamyre and Hastings, 2004).

A smaller percentage (5-10%) of PD is genetic in origin, and disease onset is linked to genetic mutations. Familial PD arises from autosomal recessive mutations in *Parkin, PINK1*, and *DJ-1* or autosomal dominant mutations in *UCHL1, LRRK2,* and *SNCA* (α-synuclein) (Leroy et al., 1998; Zimprich et al., 2004; Polymeropoulos et al., 1997; Kitada et al., 1998; Valente et al., 2004; Bonifati et al., 2003). These genetic mutations, specifically the mutants in the α-synuclein gene, have provided the field with unique insight into the mechanism behind PD because mutations known to cause disease allow scientists to directly study a direct link to disease.

To date, three point mutations in the α-synuclein gene are known to cause familial PD. Point mutations affect amino acids, the building blocks of proteins. Amino acids are analogous to beads on a piece of string. The sequence of beads determines whether or not the protein will acquire a correct shape (Alberts et al., 2009). Each bead has unique properties, and some of these beads are more important to the shape of a protein than others. In 1997, the first familial mutation in α-synuclein to cause PD was discovered in a family of Greek origin. The mutation results in an alanine to threonine (A53T) substitution in α-synuclein at the 53rd amino acid (Polymeropoulous et al., 1997). A year later, a second α-synuclein missense mutation was traced to a family in Germany. In this instance, proline replaces an alanine at the 30th amino acid (A30P; Krueger et al., 1998). The most recent α-synuclein mutant, discovered in 2004, results from a glutamic acid to lysine swap at the 46th amino acid (E46K; Zarranz et al, 2004). All three of the familial mutations lead to an increase in α-synuclein misfolding, providing support for the link between misfolded protein and cellular toxicity (Figure 2B; Conway et al., 1998; Giasson et al., 1999; Conway., et al 2000). In addition, familial PD is also caused by duplication or triplication of the α-synuclein gene, suggesting that one mechanism for the disease is over-expression of α-synuclein (Singleton et al., 2003; Chartier-Harlin et al., 2004). These familial mutations and the presence of α-synuclein in LBs of sporadic PD patients strongly implicate α-synuclein in the development of PD. My thesis sought to further understand α-synuclein's pathotoxic properties in both familial and sporadic PD.

A Closer Look at the Culprit: The α-Synuclein Protein
α-Synuclein belongs to the synuclein family of proteins. This small group of proteins contains two other members: β-synuclein, which is present in AD neurofibrillary lesions, and γ-synuclein, a protein closely linked to breast carcinoma progression (George, 2002; Bruening et al., 2000). Both β-synuclein and γ-synuclein differ from α-synuclein in that they do not cause PD (Uverskey et al., 2008). Interestingly, expression of β-synuclein in a mouse model actually protects from α-Synuclein induced toxicity (Fan et al., 2006). The exact functions of the synuclein proteins are not currently known (Uverskey et al., 2006).

α-Synuclein is a short, highly flexible protein found throughout the brain (Uéda et al., 1993; Jakes et al., 1994 ;Weinreb et al., 1996). The protein was initially described in relation to AD, as the non amyloid component (NAC) peptide of α-synuclein consistently localized to AD plaques. Interestingly, α-synuclein is also found associated with

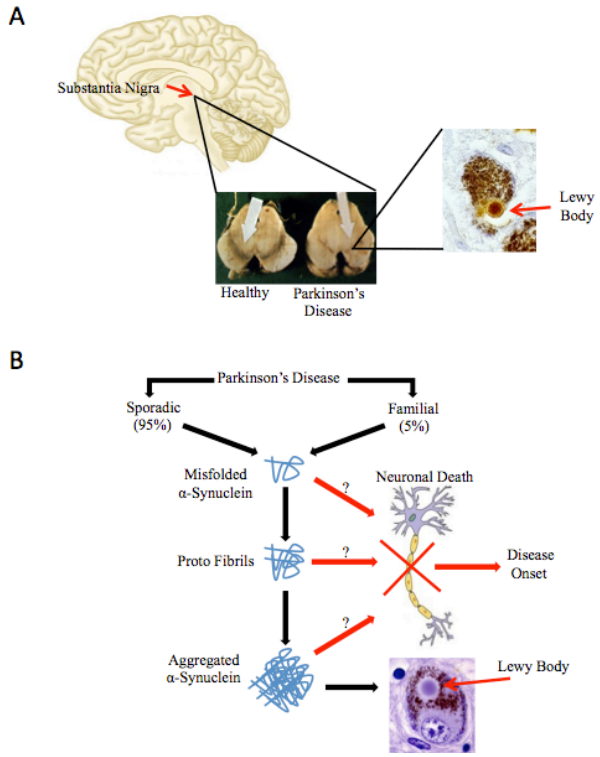

Figure 2: α-Synuclein and Cell Death (A) Diagram demonstrating where cell death occurs in Parkinson's disease (PD). The substantia nigra is located in the midbrain. A horizontal cut through this structure reveals the pigmented cells of the substantia nigra. Parkinson's diseased brains are characterized by a loss of pigmented neurons in the substantia nigra. In addition, accumulations of α-synuclein, termed Lewy Bodies, are visible in the dying cells when examined under a light microscope. (B) Flow chart demonstrating the similarities and differences of sporadic and familial forms of PD. In sporadic PD, wild-type α-synuclein misfolds and aggregates. Genetic mutations are responsible for the onset of familial PD. In either case, it is unclear whether the misfolded α-synuclein or a toxic intermediate is responsible for disease onset.

Brain image from http://www.paulnussbaum.com/.
Substantia nigra image from http://www.urmc.rochester.edu/neuroslides/slides/slide199.jpg.
Lewy body image from http://www.saigata-n.go.jp/saigata/rinken/neuropat/library/SN295LEWYSYNUCLX100.JPG
Lewy body image from http://neuropathology.neoucom.edu/chapter9/images9/9-lb2.jpg

tubulin, a similarity it shares with tau protein (Alim et al., 2004). α-Synuclein primarily localizes to pre-synaptic terminals of dopaminergic neurons (neurons that release dopamine). Dopamine is released from these neuron in vesicles, structures that can be thought of as transport containers, and α-synuclein is thought to be involved in vesicle trafficking and dopamine release (Maroteaux and Scheller, 1991; Cabin et al., 2002). α-Synuclein also appears to be involved in endocytosis, a mechanism that moves vesicles throughout cells, as expression of either WT or A53T disrupts endoplasmic reticulum (ER) to Golgi transport and enhances ER stress in a yeast model (Cooper et al., 2006).

While α-synuclein is normally a cytoplasmic protein, it also binds to phospholipid membranes (Clayton and George, 1998). This interaction is mediated by several KTKEGV motifs in α-synuclein amphipathic N-terminal domain (Soper et al., 2008). Additionally, α-synuclein has a natural tendency to aggregate due to its flexible structure. α-Synuclein aggregation occurs in a stepwise manner (Caughey et al., 2003). α-Synuclein monomers (single molecules) misfold and begin to coalesce into spherical protofibrils (short chains of α-synuclein). These protofibrils link together into longer chains, forming α-synuclein oligomers (Giasson et al., 1999). Subsequently, rapid fibril (or aggregate) formation occurs after the oligomers appear, resulting in the LBs visible in dying SNpc cells (Caughey et al., 2003). Both the domains of α-synuclein and key amino acids have been implicated in the protein's propensity to misfold and aggregate, but their exact contribution is still being evaluated (Baba et al., 1998; Soper et al., 2008). Exactly how α-synuclein contributes to toxicity and whether LBs are the toxic mechanism in PD is unclear.

Several hypotheses exist in the field as to how these LBs relate to neurotoxicity (Caughey et al., 2003). Over the past decade, support has increased for the idea that intermediate protofibrils, which precede LB formation, are responsible for toxicity in PD. While all three α-synuclein familial mutations enhance fibril formation, the A30P familial mutation specifically increases formation of protofibrils (Conway et al., 2000; Li et al., 2001). Electron microscopy revealed that α-synuclein protofibrils form pore-like structures in cell membranes, and these protofibrils can permeabilize synthetic membranes (Lashuel et al., 2000; Volles and Lansbury, 2002). Furthermore, α-synuclein's affinity for phospholipids is enhanced by protofibrils but not by fibrils (Volles and Lansbury, 2002; Ding et al., 2002). Thus, LB formation might be a protective response by the cells since fibril formation reduces the concentration of protofibril intermediates (Caughey et al., 2003). The properties of A53T and A30P familial mutants are well established, but the relationship between E46K and the impact it has on α-synuclein's aggregation is still being examined. α-Synuclein is also heavily modified by covalent bonds on specific amino acids in LBs. These modifications include ubiquitination (Shimura et al., 2001), glycosylation (Shimura et al., 2001), nitration (Hodara et al., 2004), and phosphorylation (Okochi et al., 2000; Fujiwara et al., 2002), but the role these modifications play in cellular death and aggregation is not fully understood and are all key research areas in PD (Sharon et al., 2001).

Yeast PD Models: A Research Alternative

Numerous models exist to study PD, including cell culture systems, *C. elegans, drosophila,* rat, and mice (Nass and Prezdborski, 2008). Neuronal culture systems provide direct evidence from living neurons, but they are fragile systems and cell culture reprograms their apoptotic and senescence pathways (Nass and Prezdborski, 2008). Both *C. elegans* and *Drosophila* models provide easily manipulable genetic systems, but genetic screens in these organisms are impractical due to the wide array of assays necessary (Nass and Prezdborski, 2008). While mammalian models provide terrific insight into the disease process in a species closely related to humans, genetic experiments are costly and difficult to perform (Nass and Prezdborski, 2008). Although they do not model the exact environmental conditions of a neuron, yeast provides an attractive alternative for PD research (Outiero and Lindquist, 2003; Brandis et al., 2006; Sharma et al., 2006).

For my thesis, I utilized two yeast PD models: budding yeast (Saccharomyces cerevisiae) and fission yeast (Schizosaccharomyces pombe). Both budding and fission yeast genomes share high homology with many human genes. In addition, these eukaryotic fungi synthesize, fold, and degrade proteins similar to humans, providing human relevance for discoveries using yeast (Nass and

Prezdborski, 2008). Practically, they are cheap, reproduce rapidly, and their entire genome is sequenced. Genetic knockouts of any non-essential genes are available in budding yeast, and a similar database currently in development for fission yeast (Nass and Prezdborski, 2008).

Budding yeast has a rich history of modeling human diseases, including cancer, mitochondrial disorders, and neurological diseases (Nass and Prezdborski, 2008). More relevant to my research, budding yeast serve as effective models for several neurodegenerative diseases, including prion diseases (Ma and Lindquist, 2002), AD (Komano et al., 1998), HD (Meriin et al., 2002), and ALS (Corson et al., 1998). In the PD field, a number of successful budding yeast model systems have been established since 2004 (Outeiro and Lindquist, 2003; Willingham et al., 2003; Dixon et al., 2005; Zabrocki et al., 2005; Sharma et al., 2006). Yeast do not normally make α-synuclein, so researchers use standard recombinant DNA technology to synthesize the protein in yeast. Susan Lindquist's lab developed the first budding yeast model in 2003. Their landmark paper clearly demonstrated a dose-dependent toxicity when yeast expressed α-synuclein. A single copy of the α-synuclein gene resulted in no toxicity and α-synuclein plasma membrane localization while two copies imparted significant toxicity and caused aggregation (Outiero and Lindquist, 2003). While the aggregates appeared reminiscent of classical LBs, future studies that visualized these aggregates in yeast suggested that at least some of the LBs are actually accumulations of α-synuclein associated with vesicles (Soper et al., 2008). α-Synuclein also disrupts ER-to-Golgi trafficking in yeast, a defect that is rescued by Rab1, providing additional evidence that the aggregates are vesicular in nature (Cooper et al., 2006). Thus, aggregates in yeast might be vesicles rather than true aggregates.

Our lab induces human α-synuclein in yeast using using methods similar to the previously mentioned studies. In budding yeast, WT and A53T α-synuclein localize primarily to the plasma membrane (Figure 3; Sharma et al., 2006). In contrast, A30P α-synuclein is typically diffuse throughout the cytoplasm, correlating well with previous research suggesting the proline substitution alters the interaction of the N-terminus with phospholipids (Sharma et al., 2006; Jensen et al., 1998). However, unlike the Lindquist lab, neither WT α-synuclein nor the familial mutants induce toxicity in budding yeast, which is most likely a result of a moderate expression levels (Sharma et al., 2006; Outiero and Lindquist, 2003). Interestingly, yeast with a genetically compromised proteasome (the cell's protein recycling center), exhibit significant toxicity when they express α-synuclein (Sharma et al., 2006). However, deletion of the numerous E1, E2, and E3 ubiquitin ligase genes (genes that mark proteins for degradation) does not affect toxicity (Herrera Thesis, 2005). However, deletion of manganese superoxide dismutase (SOD2; responsible for destroying damaging free radicals) and expression of α-synuclein coupled with a hydrogen peroxide challenge also proves lethal to yeast. Interestingly, when several other oxidative stress pathway genes are deleted, no toxicity is seen (Brandis Thesis, 2005; Sharma et al., 2006). Because of their ability to recapitulate several important PD related properties, our lab has also used budding yeast to research the role of endocytosis (Ayala Thesis, 2009; Perez Thesis, 2010), autophagy (Choi Thesis, 2009) and lipid synthesis (Kukreja Thesis, 2007), in α-synuclein biology.

In 2006, our lab also pioneered a fission yeast model to study α-synuclein to determine if α-synuclein behaves similar in another yeast species (Figure 3). Using different strength promoters, our lab demonstrated that α-synuclein aggregates in a concentration-dependent manner

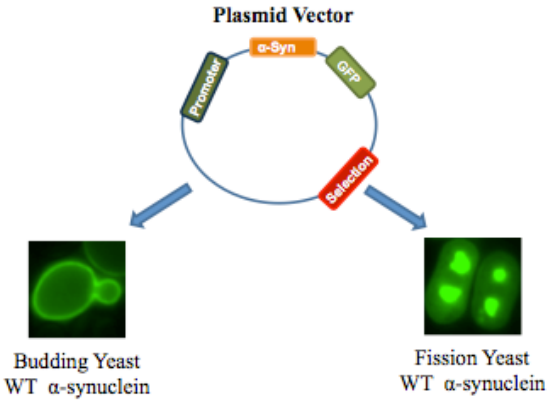

Figure 3: The DebBurman Lab Yeast Models (A) Diagram explaining budding and fission yeast models used by the DebBurman lab. Our expression system centers on a plasmid vector containing a human α-synuclein gene fused to green fluorescent protein (GFP). In budding yeast, expression is controlled by a galactose inducible promoter, and selection takes place using media lacking uracil. In fission yeast, expression is controlled by a thiamine repressible promoter, and selection takes place using media lacking leucine. WT α-synuclein associates with the plasma membrane in budding yeast. In fission yeast, WT α-synuclein aggregates in the cytoplasm.

in fission yeast (Brandis et al., 2006). The familial mutant A53T aggregates more aggressively than WT, but neither WT nor A53T localize to the plasma membrane (Brandis et al., 2006). In addition, despite extensive aggregation, no toxicity was seen with any α-synuclein variants (Brandis et al., 2006). This finding might suggest a protective role of aggregation, but it is not yet clear if the aggregates observed are true aggregates or accumulations of vesicular structures associated with α-synuclein.

My Focus

Several key questions in the PD field remain unsolved. 1) How is α-synuclein involved in the oxidative damage present in afflicted neurons? 2) By what route is α-synuclein degraded? 3) What role does α-synuclein have in neuronal death outside of the SNpc? 4) What are the properties of

the E46K familial mutant in living systems? 5) What role does covalent modification of α-synuclein have in disease pathogenesis? 6) Which structural aspects of α-synuclein contribute to its aggregation and membrane binding properties? My thesis will focus on the last three questions, and I will now provide more background for each question I address.

The Lonely Mutant: E46K

Despite its discovery nearly six years ago, surprisingly little is known about the E46K familial α-synuclein mutant. Discovered in a Spanish family in 2004, E46K is an autosomal dominant mutation (one mutated copy will cause PD) in the α-synuclein gene (Zarranz et al., 2004). Following its discovery, a group from Cambridge examined the E46K mutant *in vitro* and compared its properties to the A30P and A53T familial mutants. Two important observations were made. First, E46K enhanced α-synuclein binding to liposomes in a lipid binding assay by a factor of two compared to WT, A30P, and A53T. Second, E46K increased the formation of fibrils at a rate similar to A53T in a concentration-dependent manner. Interestingly, the E46K

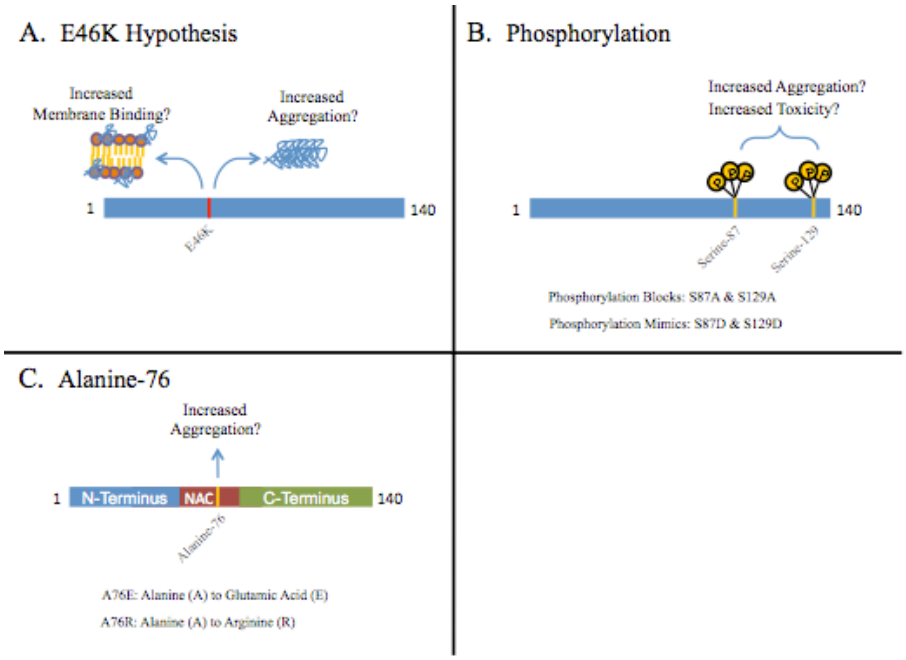

Figure 4: Properties of α-Synuclein (A) Diagram of α-synuclein demonstrating the location of serine-129 and serine-87, two primary sites of phosphorylation in Lewy Bodies. In vitro experiments demonstrated that serine phosphorylation enhances aggregation of α-synuclein. However, there is conflicting evidence as to the toxic nature of serine phosphorylation. (B) Diagram of α-synuclein demonstrating location of the E46K familial mutation. The mutation occurs when a glutamic acid (E) is mutated to lysine (K) due to a genetic mutation in the α-synuclein gene. The E46K mutant was shown to enhance membrane interaction and aggregation of α-synuclein in vitro. (C) Diagram of α-synuclein demonstrating the location of alanine-76 within the NAC domain of the protein. Alanine-76 was predicted to govern the hydrophobic properties of the protein, and the A76E and A76R mutations decreased aggregation of α-synuclein in vitro.

fibrils were more tightly twisted than A53T (Choi et al., 2004; Greenbaum et al., 2005).

In 2006, the E46K mutation was analyzed in a cell culture system. Researchers expressed WT and E46K in human neuroblastoma cells and assessed aggregation and the characteristics of the aggregates. The E46K mutant increased aggregation in cells, potentially due to an increase in surface charge. As previously stated, one hypothesis for α-synuclein induced toxicity involves interaction with phospholipids. The researchers hypothesized that this change in net charge might increase interactions with lipids and lead to an earlier onset of PD as compared to the sporadic form (Pandey et al., 2006).

Most recently, researchers investigated how the E46K mutation alters the conformation (shape) of α-synuclein. α-Synuclein is composed of three domains (regions): the N (aa1-57), NAC (aa61-95), and C (aa96-140) domains (Soper et al., 2008; Giasson et al., 2001). Previous studies demonstrate that A53T and A30P decrease the interaction between the N- and C-terminal regions of the protein (Bertonici et al., 2005). Disrupting the interaction between those two domains was suggested to contribute to the aggregation of these mutants *in vitro*. In contrast to A30P and A53T, the E46K mutant enhanced contact between the N- and C-terminus, suggesting that the interaction with the C-terminus does not play a role in the propensity of the protein to aggregate since A53T aggregates similarly to WT without enhancing this interaction (Rospigliosi et al., 2009). The properties of the E46K mutant still require further elucidation (Figure 4A). *The first goal of my thesis was to describe the properties of the E46K mutation in our budding and fission yeast models.*

The Mystery of Serine Phosphorylation

Phosphorylation involves the covalent addition of a phosphate group to a protein. This modification typically alters a protein's conformation, which is useful in regulating the on or off state of an enzyme (Alberts et al., 2008). The α-synuclein located in LBs is heavily phosphorylated at two serine residues: serine-129 and, to a lesser extent, serine-87 (Fujiwara et al., 2002; Palelogou et al., 2010). Several enzymes that phosphorylate proteins (called kinases) demonstrate the ability to phosphorylate α-synuclein in cell culture, including Lrrk2 (Chen and Feany, 2005), Gprk2 (Okochi et al., 2000), casein kinase 1 and 2 (Kim et al., 2005), and Dyrk1A (Sakamoto et al., 2009), but the kinase responsible for phosphorylation of α-synuclein in the LBs of PD patients has yet to be identified. *In vitro* fibrillization assays revealed that phosphorylation of α-synuclein enhances fibril formation (Fujiwara et al., 2002). α-Synuclein in LBs is also phosphorylated at several tyrosine residues, and research suggests tyrosine phosphorylation appears influential in preventing aggregation (Nakamura et al., 2001; Ellis et al., 2001; Chen et al., 2009).

Conflicting evidence exists in the field as to what role serine phosphorylation has in dopaminergic cell death. In 2005, the Feany lab demonstrated that an α-synuclein mutant that blocks phosphorylation at serine-129 through mutation to an alanine (S129A) attenuates toxicity in a *Drosophila* model (Chen and Feany, 2005). An α-synuclein phosphorylation-mimic mutant (S129D) enhanced toxicity in their fly model. The S129A mutation also increased inclusion formation, suggesting that LBs might be neuroprotective (Chen and Feany, 2005). However, a lab using a rat model to study phosphorylation found S129A to be extremely toxic as compared to wild-type (WT) and S129D synuclein. The S129D mutant increased inclusion formation in SNpc neurons (Gorbatyuk et al., 2008). A second paper published in 2009 also saw increased toxicity caused by S129A expression in a rat model (da Silveira et

al., 2009). Further complicating the issue is a recent study by the same authors of the *Drosophila* study. They found no difference in toxicity between rats expressing WT, S129A, and S129D α-synuclein (McFarland et al., 2009). Thus, the role that phosphorylation plays in toxicity is still unclear and may be organism and cell-dependent (Figure 4B). Describing the properties in yeast provides an additional organism to compare results in the field with. *The second goal of my thesis is to provide insight further into the role that α-synuclein serine phosphorylation has in PD pathogenesis by describing the properties of several phosphorylation mutants in our budding and fission yeast models.*

The Role of α-Synuclein's Hydrophobic Amino Acids

The N-terminus has been implicated in α-synuclein's ability to bind membranes due to its flexible nature and the presence of several imperfect KTKEGV amino acid repeats (Davidson et al., 1998; Perrin et al., 2000; Kim et al., 2006). Upon binding to membranes, the N-terminus shifts from an α-helical to a β-sheet conformation (Kim et al., 2006.) The A30P familial mutation, which occurs in repeat two, was shown to disrupt membrane binding *in vitro,* demonstrating how essential these repeats in the N-domain are for lipid interaction (Jensen et al., 1998). More recently, a 2008 study evaluated an α-synuclein N-terminal truncation mutant in a yeast model and found that the mutant failed to bind to plasma membranes, a well documented property of α-synuclein in budding yeast (Soper et al., 2008; Sharma et al., 2006; Outeiro and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005). These findings suggest that the proteins domains contribute significantly to α-synuclein's properties.

The NAC domain, also called the non-β-amyloid component, is a hydrophobic domain essential for aggregation and fibrillization both *in vitro* and *in vivo* (Giasson et al., 2001; Periquet et al., 2007). Within the NAC domain is a region of hydrophobic (water hating) residues from aa71-82 that was shown to be crucial for α-synuclein to aggregate (Giasson et al., 2001). On its own, the NAC domain is highly amyloidogenic and induces toxicity when expressed in PC12 (rat adrenal) and SHSY-5Y cells (neuroblastoma cancer cells) (Han et al., 1995; El-Agnaf et al., 1998a; El-Agnaf et al., 1998b; Bodles et al., 2001). In addition, β-synuclein, which does not aggregate as α-synuclein does, lacks the NAC domain (Biere et al., 2000). Addition of the NAC domain to β-synuclein is sufficient to induce aggregation of the protein (Biere et al., 2000). Soper et al. (2008) also demonstrated that, in yeast expressing a NAC truncation mutant, cytoplasmic aggregates failed to form.

In light of the controversy regarding the potentially toxic role of protein aggregation in PD, understanding how α-synuclein acquires its shape is an important question. In 2003, researchers modeled α-synuclein aggregation in relation to hydrophobicity, charge, and tendency to convert from α-helix to β-sheet (Chiti et al., 2003). They predicted that alanine-76 within the NAC domain would be a key contributor to α-synuclein's hydrophobic properties because the A76R and A76E mutants would significantly alter the hydrophobicity of the polypeptide chain (Chiti et al., 2003). In addition, *in vitro* analysis of A76E and A76R indicated that these two mutants aggregate more slowly than WT α-synuclein (Giasson et al., 2001). Thus, alanine-76 appears potentially relevant because it affects a pathology-linked property: protein aggregation (Figure 4C). However, no PD patients actually have an A76E or A76R mutation. *A third goal of my thesis is to assess the relevance of alanine-76 using our budding and fission yeast models.*

Hypothesis and Aims

The overall goal of my study was to better understand the intrinsic determinants and chemical modifications that generate toxicity and alter the properties of α-synuclein. Thus, I investigated three specific areas of PD research: 1) the E46K familial mutant 2) serine phosphorylation 3) individual amino acid alanine-76. Each chapter of my thesis addresses one of these research areas.

Study One: E46K Familial Mutant Analysis

Hypothesis and Aims: The E46K familial mutant enhances α-synuclein's association with phospholipids and propensity to aggregate. To test this hypothesis, I assessed E46K localization, accumulation, and toxicity in budding yeast and fission yeast.

Main Findings: E46K associated with plasma membranes in budding yeast and aggregated in fission yeast. Expression of the E46K mutant did not enhance toxicity in budding yeast, but it did prove toxic to one fission yeast strain.

Study Two: α-Synuclein Phosphorylation Mutant Analysis

Hypothesis and Aims: Phosphorylation at serine-87 and -129 contributes to aggregation and the toxic properties of α-synuclein. To test this hypothesis, I assessed localization, accumulation, and toxicity of several α-synuclein phosphorylation mutants in budding yeast and fission yeast.

Main Findings: Mutants that block phosphorylation at serine-87 and serine-129 altered aggregation in fission yeast. Mutations that mimic phosphorylation also significantly altered localization in fission yeast. Neither the phosphorylation deficient nor mimic mutants significantly altered localization in budding yeast nor enhanced toxicity in either yeast.

Study Three: Alanine-76 Analysis

Hypothesis and Aims: Alanine-76 contributes to α-synuclein aggregation and membrane association in yeast. To test this hypothesis, I assessed localization, accumulation, and toxicity of the A76E and A76R α-synuclein mutants in budding and fission yeast.

Main Findings: Both the A76E and A76R decreased membrane association in budding yeast and reduced aggregation in fission yeast, although to differing extents in each organism. However, neither mutant was more toxic to yeast than wild-type α-synuclein.

Materials and Methods

Experimental Design

My study began by creating several α-synuclein point mutations. For study 1, I generated the A53T, A30P, & E46K α-synuclein familial mutants. For study 2, I generated the α-synuclein phosphorylation deficient mutants S87A & S129A. Structurally, alanine (A) lacks the hydroxyl group where a phosphate group is added on a serine (S). An alanine substitution should effectively block phosphorylation at these two serine residues. I also generated α-synuclein phosphorylation mimic mutants S87D & S129D. An aspartic acid (D) mimics the protein conformation of phosphorylated α-synuclein. These mutants will model a constitutively phosphorylated state. For study 3, I generated the A76E & A76R alanine-76 α-synuclein mutants. Alanine (A) is a nonpolar hydrophobic residue. Glutamic acid (E) is a polar, negatively charged, hydrophilic residue. Arginine (R) is a polar, positively charged, hydrophilic residue. As per the mathematical prediction, replacing a hydrophobic residue with a hydrophilic residue should influence the protein's ability to aggregate and alter solubility. These mutations were confirmed by sequencing at the University of Chicago.

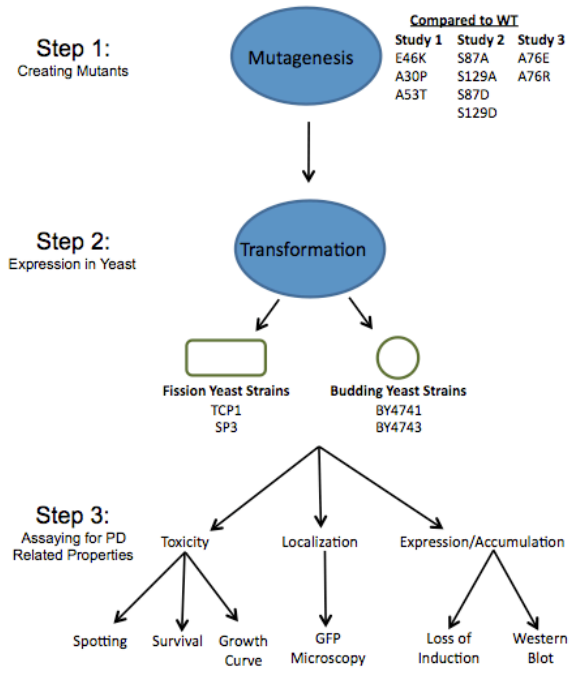

Figure 1: Experimental Design. Desired mutants were created using Invitrogen site directed mutagenesis kit. Mutants created for study 1: A53T, A30P, and E46K. Mutants created for study 2: S87A, S129A, S87D, and S129D. Mutants created for study 3: A76E and A76R The mutants were then transformed into either budding yeast or fission yeast. Upon transformation, mutants were characterized using several techniques.

After sequencing confirmation, the mutants were transformed into either budding yeast strains BY4741 and BY4743 or fission yeast strains TCP1 & SP3. After successful transformation, I began conducting experiments on the mutants (Figure 5A). See below for more detailed experimental methods.

In all three studies, all mutants were compared to three controls: Wildtype α-synuclein (WT), parent plasmid (PP), and green fluorescent protein (GFP). The WT control allows comparison of yeast expressing the point mutants to yeast expressing normal, WT α-synuclein. The PP control demonstrates that the vector alone has no effect on yeast, and the GFP control confirms that the GFP protein is not toxic to yeast. In all cases, α-synuclein's properties were assessed using four well-established yeast assays. Live cell GFP microscopy allowed for visualization of where the α-synuclein mutants localized. OD600 growth curves and serial dilution spotting assays measured toxicity in the yeast resulting from α-synuclein expression. Western blots were used to assess α-synuclein expression and accumulation. I also utilized our lab's newly developed survival assay to determine the nature of alpha-synuclein toxicity and loss of induction assay to determine protein accumulation and turnover.

All methods were adapted from Sharma et al. 2006 or Brandis et al. 2006 except for loss of induction and survival assay.

α-Synuclein Constructs

cDNAs of wildtype (WT) and A53T α-synuclein were given to us by Christopher Ross at Johns Hopkins University. The A30P and E46K mutants were created using site-directed mutagenesis on WT α-synuclein (Invitrogen). The mutations were sequenced at University of Chicago to confirm successful amino acid substitution. The α-synuclein cDNAs were first subcloned into the mammalian expression vector pcDNA3.1/C-terminal GFP to fuse α-synuclein with GFP at the C-terminus. α-Synuclein cDNAs were then PCR-amplified and subcloned into the pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). I transformed the WT and mutant α-synuclein-GFP fusions into chemically competent E. coli cells for replication and storage. The empty pYES2.1 vector and GFP in the pYES2.1/V5-His-TOPO vector served as controls. The above strategy was also used to generate the fission yeast α-synuclein constructs in the pNMT1/V5-His-TOPO vector. The S87A, S129A, S87A/S129A, and A76E mutations were created using site-directed mutagenesis. The E46K mutant was previously created by Michael White'07.

Yeast Strains

Parent budding yeast strains BY4741 (mat a) and BY4743 (diploid), and fission yeast parent strains TCP1 and SP3 were purchased from Open Biosystems.

Yeast Expression

Both pYES2.1 and pNMT1 expression vectors were transformed into budding and fission yeast strains as described in Burke et al 2000. For budding yeast, cells were grown and stored in synthetic complete media that lacked the uracil nucleotide (SC-Ura). Expression was regulated by using SC-Ura+glucose (repressor of GAL promoter) or SC-Ura+galactose (inducer of GAL promoter). For fission yeast, cells were grown and stored in media lacking the essential amino acid leucine (PDM-Leu). Expression was regulated using Edinburg Minimal Media (EMM) lacking thiamine (repressor of promoter) or with 10mM thiamine added (no repressor present). Yeast cells were grown overnight in either 2% SC-Ura+glucose (budding yeast) or EMM+T (fission yeast) at 30°C, washed with water twice, and diluted to desired concentration in 2% SC-Ura+galactose (budding yeast) or EMM-T (fission yeast) to induce expression.

Mutant Creation

The Invitrogen site-directed mutagenesis kit was used to create the E46K, S87A, S129A, S87D and S129D, A76E, and A76R mutants. The pYES2.1 or pNMT1 WT α-synuclein vector was used as a template for mutagenesis for the S87A, S129A, and A76E mutants. The S87A/S129A was created by mutating the S87A plasmid with the S129A primers. The following primers were used:

E46K FP: 5' ATGTAGGCTCCAAAAACAAGAAGGGAGTGGTGC 3'
E46K RP: 5' CTTGGTTTTGGAGCCTACATAGAGAACACC 3'

S87A FP: 5' GTGGAGGGAGCAGGGGCCATTGCAGCAGCC 3'
S87A RP: 5' CCCTGCTCCCTCCACTGTCTTCTGGGCTAC 3'

S129A FP: 5' GCTTATGAAATGCCTGACGCCGGAAGGGTATC 3'
S129A RP: 5' AGGCATTTCATAAGCCTCATTGTCAGGATC 3'

S87D FP: 5' GTGGAGGGAGCAGGGGACATTGCAGCAGCC 3'
S87D RP: 5'CCCTGCTCCCTCCACTGTCTTCTGGGCTAC 3'

S129D FP: 5' GCTTATGAAATGCCTGACGAGGAAGGGTATC 3'
S129D RP: 5' AGGCATTTCATAAGCCTCATTGTCAGGATC 3'

A76E FP: 5'-CAGTGGTGACGGGTGTGACAGGAGTAGCCCAGA-3'
A76E RP: 5'- CAACCTCCTCGTCACCACTGCCCACACTGT-3'

A76R FP: 5'- CAGTG GTGACG GGT GTGACACGAGTAGCCCAG A-3'
A76R RP: 5'- CAACCTCCTCGTCACCACTGCCCACACTGT-3'

The mutagenesis product was transformed into One Shot Max Efficiency Dh5α-T1 competent cells, grown in LB+Ampicilin media, and mini-prepped (Qiagen Mini-prep Kit) to isolate plasmid DNA. The purified plasmid was sequenced at the University of Chicago DNA Sequencing Facility.

Western Analysis
Budding or fission yeast cells at 2. 5x10 7 cells/mL concentration were washed with 100mM NaN3 and solubilized in electrophoresis sample buffer (ESB; Burke, 2000). The ESB contained 2% sodium dodecyl sulfate (SDS), 80 mM Tris (Ph 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/ml bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton-X 100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 10 mg/ml E64, 2mg/ml aprotinin, and 2 mg/ml chymostatin). Lysates were electrophoresed at 130 volts on a 10-20% Tris-Glycine gel (Invitrogen) with 1X SDS running buffer. SeeBlue (Invitrogen) molecular ladder was used as a standard. Gels were transferred to PVDF membranes using a semi-dry transfer method and probed using the desired antibodies. To detect α-synuclein, either a mouse monoclonal anti-V5 or anti-α-synuclein (Invitrogen) was used followed by an anti-mouse secondary antibody. Anti-phosphoglycerokinase (PGK; Molecular probes) was used as a loading control for budding yeast and Anti-beta-actin (Abcam) was used as a loading control for fission yeast. The protein was visualized by detecting for alkaline-phosphatase activity.

OD600 Growth Curve Analysis
Yeast cells were grown in either 10mL SC-Ura+glucose (budding yeast) or EMM+T (fission yeast) overnight at 30°C and 200 rpm. To collect cells, yeast were pelleted at 1500 x g for 5 minutes at 4°C. They were washed twice with 5 mL H2O, re-suspended in 10mL H2O, and counted using a hemocytometer to determine cell density. Flasks containing 25mL SC-Ura+galactose (budding yeast) or EMM-T (fission yeast) were inoculated to a density of 2.0x106 cells/mL. Duplicate spectrophotometer 600nm absorbance measurements of 1mL of cells in a plastic cuvette were taken at 0, 3, 6, 12, 18, 24, 36, and 48 hours post-induction. The spectrophotometer model was a Hitachi U-2000 Spectrophotometer. A growth curve was generated by plotting the average absorbance readings of three separate experiments vs. time in Microsoft Excel. A student's T-test was used to determine significance.

Serial Dilution Spotting
Yeast cells were grown in either 10mL SC-Ura+glucose (budding yeast) or EMM+T (fission yeast) overnight at 30°C and 200 rpm. To collect cells, yeast were pelleted at 1500 x g for 5 minutes at 4°C. They were washed twice with 5 mL H2O, re-suspended in 10mL H2O, and counted using a hemocytometer to determine cell density. 2.0x106 cells were removed and pelleted. The supernatant was removed, and cells were resuspended in 1mL H2O. Cells were diluted 5 fold in a 96 well microtitier plate and spotted onto SC-Ura+glucose and Sc-Ura+galactose (budding yeast) or EMM+T and EMM-T (fission yeast) growth plates. Cells were grown for 24 hours, and pictures were taken using an HP Canoscan scanner. Images were imported into Adobe Photoshop CS2.

GFP Microscopy
Yeast cells were grown in either 10mL SC-Ura+glucose (budding yeast) or EMM+T (fission yeast) overnight at 30°C and 200 rpm. To collect cells, yeast were pelleted at 1500 x g for 5 minutes at 4°C. They were washed twice with 5 mL H2O, re-suspended in 10mL H2O, and counted using a hemocytometer to determine cell density. Flasks containing 25mL SC-Ura+galactose (budding yeast) or EMM-T (fission yeast) were inoculated to a density of 2.0x107 cells/mL. 1mL of cell culture was pelleted at 5000 rpm for 1 minute. 900uL of supernatant was removed. The remaining 100uL of cell culture was vortexed, and 5-10uL of sample was pipette onto a glass slide. Cells were visualized using a Nikon TE2000-U fluorescent microscope, and images were collected and quantified using Metamorph 4.0 software.

Survival Assay
Yeast cells were grown in either 10mL SC-Ura+glucose (budding yeast) or EMM+T (fission yeast) overnight at 30°C and 200 rpm. To collect cells, yeast were pelleted at 1500 x g for 5 minutes at 4°C. They were washed three times with 5 mL H2O, re-suspended in 10mL H2O, and counted using a hemocytometer to determine cell density. Flasks containing 25 mL SC-Ura+galactose (budding yeast) or EMM-T (fission yeast) were inoculated to a density of 2.0 x 10^7 cells. At 8 and 18 hours post induction, cell density was determined. 1.8 x 10^6 cells were removed and washed once with 1 mL of H2O. The cells were resuspended in 1 mL of water, and diluted 1:1000. 300mL of 1:1000 diluted cells (1000 cells) were spread on large plates containing SC-Ura+Galactose and SC-Ura+Glucose (budding yeast) or EMM-T and EMM+T (fission yeast).

Loss of Induction
Yeast cells were grown in either 10mL SC-Ura+glucose (budding yeast) or EMM+T (fission yeast) overnight at 30°C and 200 rpm. To collect cells, yeast were pelleted at 1500 x g for 5 minutes at 4°C. They were washed three times with 5 mL H2O, re-suspended in 10mL H2O, and counted using a hemocytometer to determine cell density. Flasks containing 25 mL SC-Ura+galactose (budding yeast) or EMM-T (fission yeast) were inoculated to a density of 2.0 x 10^7 cells and grown for 24 hours. After 24 hours, lysates were prepared as described in the western blot section. The yeast cells were then pelleted at 1500 x g for 5 minutes at 4°C. They were washed three times with mL H2O, and flasks containing 25 mL of Sc-Ura+glucose (budding yeast) or EMM+T (fission yeast) were inoculated at 2.0 x 10^7 density. Lysates were prepared at 0, 6, 12, and 24 hours after inoculation in repressing media. Western blot was used to detect the protein.

Table 1: List of α-Synuclein Constructs

α-Synuclein Construct	Vector	Yeast Strains	Chapter
PP	pYES2.1, pREP1	BY4741, BY4743, TCP1, SP3	1, 2, 3
GFP	pYES2.1, pNMT1	BY4741, BY4743, TCP1, SP3	1, 2, 3
WT-GFP	pYES2.1, pNMT1	BY4741, BY4743, TCP1, SP3	1, 2, 3
E46K-GFP	pYES2.1, pNMT1	BY4741, BY4743, TCP1, SP3	1
A53T-GFP	pYES2.1, pNMT1	BY4741, TCP1	1
A30P-GFP	pYES2.1, pNMT1	BY4741, TCP1	1
S87A-GFP	pYES2.1, pNMT1	BY4741, TCP1	2
S129A-GFP	pYES2.1, pNMT1	BY4741, TCP1	2
S87D-GFP	pYES2.1, pNMT1	BY4741, TCP1	2
S129D-GFP	pYES2.1, pNMT1	BY4741, TCP1	2
A76E-GFP	pYES2.1, pNMT1	BY4741, TCP1	3
A76R-GFP	pYES2.1, pNMT1	BY4741, TCP1	3

Chapter 1: The Familial Mutant E46K Enhances Membrane Association, Aggregation, and Toxicity

Results

Familial Mutant E46K Associates with Membranes in Budding Yeast Strain BY4741

The first α-synuclein property I evaluated was localization. I began my experiments in the widely used budding yeast strain BY4741. To visualize α-synuclein localization, I used live cell-green fluorescent protein (GFP) microscopy. All α-synuclein and mutants are tagged to GFP, a protein that fluoresces blue when exposed to blue light. This unique property of GFP allows us to visualize where α-synuclein localizes. Previous work in our lab showed that WT α-synuclein associates with the plasma membrane in this strain (Sharma et al., 2006). I saw that A53T α-synuclein associates with membranes and occasionally aggregates at 24 and 48 hours (Figure 1A and 1B). In contrast, the A30P mutation sporadically associates with plasma membranes and consistently localizes throughout the cytoplasm at 24 and 48 hours (Figure 1A and 1B). In support of my hypothesis, GFP microscopy revealed that E46K α-synuclein also associated with the plasma membrane of BY4741 at 24 and 48 hours, just like WT and A53T (Figure 1A and 1B). No intracellular aggregates were visible, a marked difference from the A53T mutation (Figure 1A and 1B; Sharma et al., 2006).

I then examined a second α-synuclein property: toxicity. To assess toxicity of E46K in budding yeast, I utilized an OD600 growth curve and serial dilution spotting, two well established yeast assays (Outiero and Lindquist, 2005; Soper et al., 2008). The OD600 growth curve uncovered no defect in growth between BY4741 cells not expressing α-synuclein (PP and GFP) and cells that expressed either WT or E46K α-synuclein (Figure 1C). This lack of toxicity was confirmed by serial dilution spotting (Figure 1D). BY4741 cells expressing WT or E46K α-synuclein grew equally well as cells expressing the empty vector or GFP on the spotting plates (Figure 1D). As a control, all cells grew equally well on repressing media (Figure 1D).

Lastly, I evaluated the final α-synuclein property I set out to examine: expression. To do so, I used a standard Western blot. Western blot failed to detect a change in expression between BY4741 cells expressing WT or E46K at 24 or 48 hours.

E46K Also Associates with Membranes in Diploid Budding Yeast Strain BY4743

Yeasts exist in different strains, a trait that is similar to how humans have different races and ethnicities. To ascertain if diploidy or strain background influences the properties of E46K α-synuclein, I also evaluated E46K in the diploid budding yeast strain BY4743. Once again in support of my hypothesis, E46K associated with the plasma membrane of this strain at 24 and 48 hours, similar to BY4741 (Figure 2A and 2B). Additionally, little to no aggregation was apparent in these cells (Figure 2B). α-Synuclein and the E46K familial mutant were non-toxic to BY4743 yeast cells, as cells expressing either α-synuclein variants grew similar to cells expressing the empty vector or the GFP protein in OD600 growth curves and serial dilution spotting (Figure 2C and 2D). The only difference between the haploid and diploid strains was that E46K expression decreased in BY4743 (compare Figure 1E to 2E).

Familial Mutant E46K Aggregates in Fission Yeast and Exhibits Strain Specific Toxicity

I next investigated α-synuclein's properties in the second yeast model organism: fission yeast. I began my analysis of E46K in fission yeast by examining the localization of the protein at 24 and 48 hours post induction. Previous work in our lab illustrated that WT α-synuclein forms aggregates in the cytoplasm of fission yeast (Brandis et al., 2006). In support of my hypothesis, E46K formed clearly visible aggregates in the TCP1 yeast strain (Figure 3A). The E46K phenotype was once again similar to that of the A53T mutant, which also formed aggregates in the yeast (Brandis et al., 2006). However, it differed from the cytoplasmically diffuse phenotype characteristic of A30P (Figure 3A and 3B). The E46K mutation also enhanced α-synuclein's association with an unidentified organelle that appears to be the yeast nucleus. This association was most prominent at 24 hours but decreased at 48 hours (Figure 3B). Interestingly, unlike in budding yeast, E46K does not associate with the plasma membrane (compare Figure 1A to 3A).

I next examined how E46K impacted the growth of fission yeast. Unlike in budding yeast, the OD600 growth curve uncovered a slight α-synuclein-dependent delay in fission yeast growth where even WT was toxic (WT: 18hrs $p=.0021$; E46K: 18hrs $p= 0.0036$; WT: 24hrs $p= 0.0096$; E46K 24hrs $p= 0.00079$; Figure 3C). However, contrary to my hypothesis, the E46K mutation did not further enhance this α-synuclein-dependent toxicity relative to WT (Figure 3C). Serial dilution spotting confirmed both the slight α-synuclein-dependent toxicity and that E46K did not further alter it (Figure 3D). No delay in growth was apparent on repressing (+thiamine) plates (Figure 3C).

Lastly, I assessed expression levels in TCP1 fission yeast cells expressing E46K α-synuclein. Western blot demonstrated no change in steady state expression levels between fission yeast cells expressing WT or E46K α-synuclein at 24 or 48 hours (Figure 3E). I also examined accumulation and protein turnover of E46K in TCP1 cells using a loss of induction assay where cells were first grown in inducing media (-thiamine) for 24 hours and then transferred to repressing media (+thiamine). Protein levels were assessed over 24 additional hours. Western blot of TCP1 yeast cell lysates prepared at several time points post α-synuclein production indicated that degradation of both WT and E46K α-synuclein occurred at similar rates in TCP1 cells (Figure 1E).

E46K is Selectively Toxic to SP3

I also analyzed the E46K mutation in a second fission yeast strain, SP3, to determine if E46K exhibited strain specific properties. GFP microscopy revealed that WT α-synuclein associated with endomembrane structures and aggregated at 24 hours post induction (Figure 4A and 4B). By 48 hours, both aggregation and endomembrane association occurred simultaneously in most cells (Figure 4A and 4B). In contrast to WT, the E46K mutation aggregated less and more strongly associated with numerous internal membranous structures in the yeast at both 24 and 48 hours (Figure 4A and 4B). These findings did not support the hypothesis that E46K would increase aggregation, but it did support my prediction that E46K would increase association with lipids. Interestingly, despite the strong association with internal membranes, E46K did not enhance association with the plasma membranes of the yeast.

I next assessed α-synuclein-dependent toxicity in SP3 yeast cells to determine how this interesting localization phenotype altered cell growth. Similar to TCP1, I saw a clear α-synuclein-dependent toxicity in the growth curves (WT: 18 hrs $p=0.0016$; E46K: 18 hrs $p= 0.000026$; WT: 24 hrs $p= 0.0000027$; E46K 24 hrs $p= 0.00000084$; Figure 4C) and serial dilution spotting (Figure 4D). Additionally, the E46K mutant was selectively toxic to SP3 fission yeast

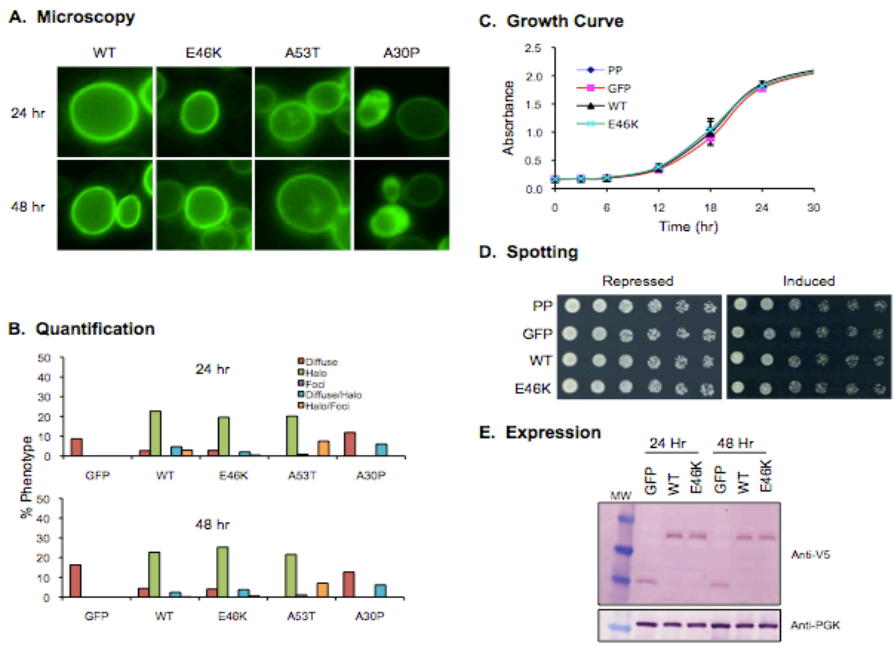

Figure 1: Characterization of E46K in BY4741 Budding Yeast
A. Microscopy: WT & E46K α-synuclein localization at 24 & 48 hours post induction. n=2
B. Quantification: Quantification of 750 WT & E46K expressing cells scored for several phenotypes. n=2
C. Growth Curve: OD600 readings recorded over 30 hours for BY4741 cells induced for PP, GFP, WT & E46K α-synuclein. n=3
D. Spotting: Yeast expressing PP, GFP, WT, & E46K α-synuclein spotted onto glucose (repressed) & galactose (induced) plates after five-fold serial dilutions. n=3
E. Accumulation & Expression: Western blot at 24 & 48 hours of GFP, WT, & E46K α-synuclein expression (anti-V5) with galactose media. PGK: loading control. n=2

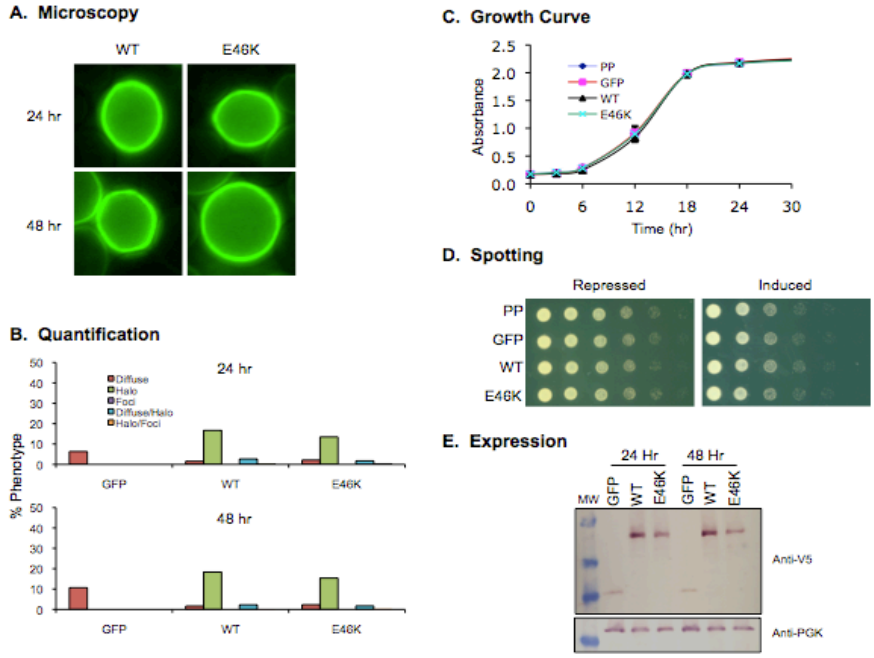

Figure 2: Characterization of E46K in BY4743 Budding Yeast
A. Microscopy: WT & E46K α-synuclein localization at 24 & 48 hours post induction. n=2
B. Quantification: Quantification of 750 WT & E46K expressing cells scored for several phenotypes. n=2
C. Growth Curve: OD600 readings recorded over 30 hours for BY4743 cells induced for PP, GFP, WT & E46K α-synuclein. n=3
D. Spotting: Yeast expressing PP, GFP, WT, & E46K α-synuclein spotted onto glucose (repressed) & galactose (induced) plates after five-fold serial dilutions. n=3
E. Accumulation & Expression: Western blot at 24 & 48 hours of GFP, WT, & E46K α-synuclein expression (anti-V5) with galactose media. PGK: loading control. n=2

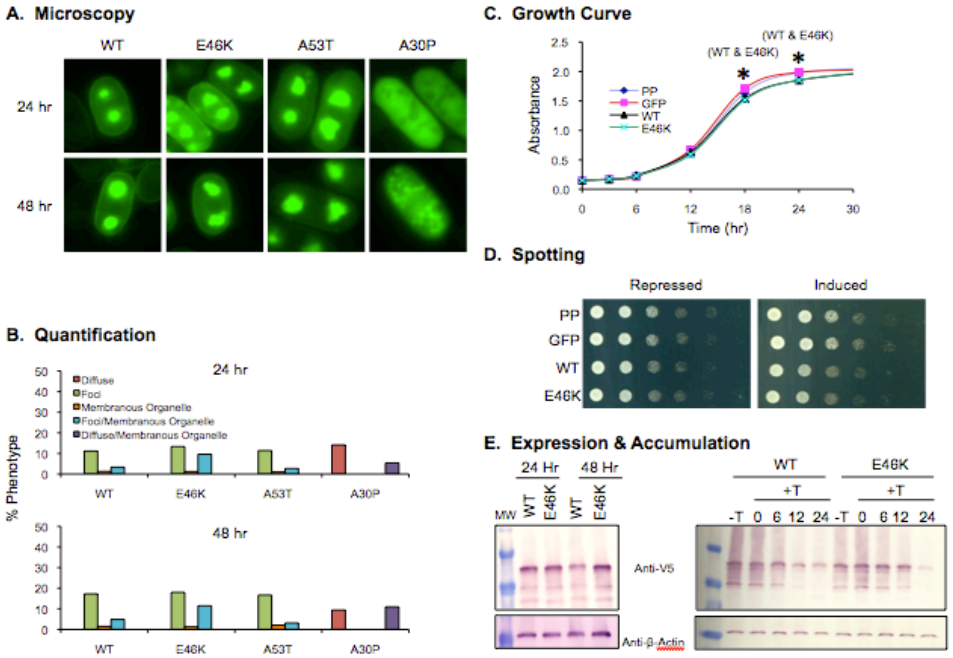

Figure 3: Characterization of E46K in TCP1 Fission Yeast

A. Microscopy: WT & E46K α-synuclein localization at 24 & 48 hours post induction. n=2.

B. Quantification: Quantification of 750 WT & E46K expressing cells scored for several phenotypes. n=2.

C. Growth Curve: OD600 readings recorded over 30 hours for TCP1 cells induced for PP, GFP, WT, & E46K α-synuclein. Asterisk (*) indicates p <.05 compared to PP (WT: 18hrs p=.0021; E46K: 18hrs p= 0.0036; WT: 24hrs p= 0.0096; E46K 24hrs p= 0.00079) n=3.

D. Spotting: Yeast expressing PP, GFP, WT, & E46K α-synuclein spotted onto +thiamine (repressed) & -thiamine (induced) plates after five-fold serial dilutions. n=3

E. Expression & Accumulation: LEFT- Western blot at 24 & 48 hours of WT & E46K α-synuclein expression (anti-V5) with -thiamine media. n=2. RIGHT- Loss of induction Western blot of lysates prepared at 24 hrs –T, 0 hrs +T, 6 hrs +t, 12 hrs +T & 24 hrs +T. n=2. β-Actin : loading control.

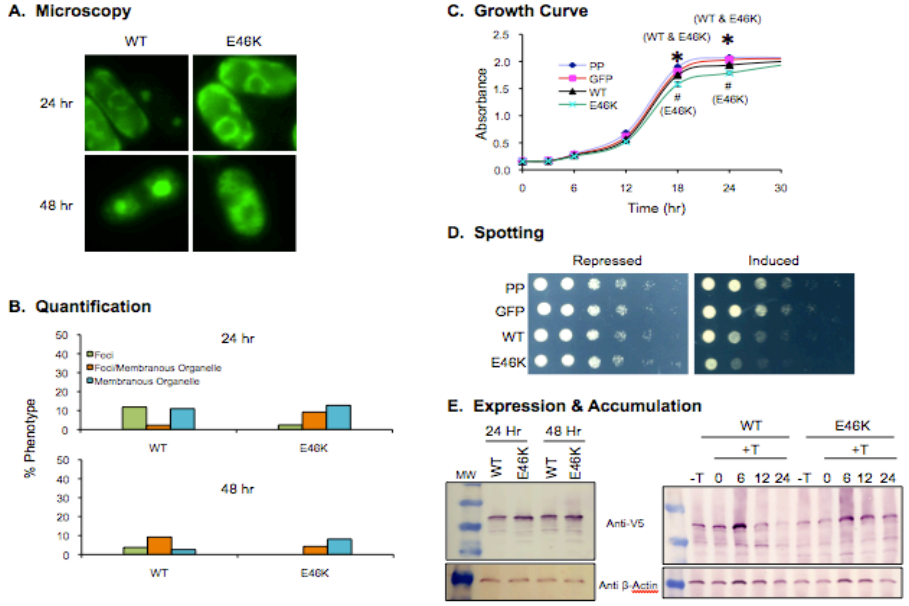

Figure 4: Characterization of E46K in SP3 Fission Yeast

A. Microscopy: WT & E46K α-synuclein localization at 24 & 48 hours post induction. n=2

B. Quantification: Quantification of 750 WT & E46K expressing cells scored for several phenotypes. n=2

C. Growth Curve: OD600 readings recorded over 30 hours for cells induced for PP, GFP, WT, & E46K α-synuclein. Asterisk (*) indicates significant difference compared to PP. (WT: 18 hrs p=0.0016; E46K: 18 hrs p= 0.000026; WT: 24 hrs p= 0.0000027; E46K 24 hrs p= 0.00000084). Pound (#) indicates significance compared to WT. (E46K: 18 hrs p= 0.0065; E46K: 24 hrs p= 0.00056). n=3

D. Spotting: Yeast expressing PP, GFP, WT, & E46K α-synuclein spotted onto +thiamine (repressed) &-thiamine (induced) plates after five-fold serial dilutions. n=3

E. Expression & Accumulation: LEFT- Western blot at 24 & 48 hours of WT & E46K α-synuclein expression (anti-V5) with -thiamine media. n=2 RIGHT- Loss of induction Western blot of lysates prepared at 24 hrs –T, 0 hrs +T, 6 hrs +t, 12 hrs +T & 24 hrs +T. β-Actin: loading control. n=1

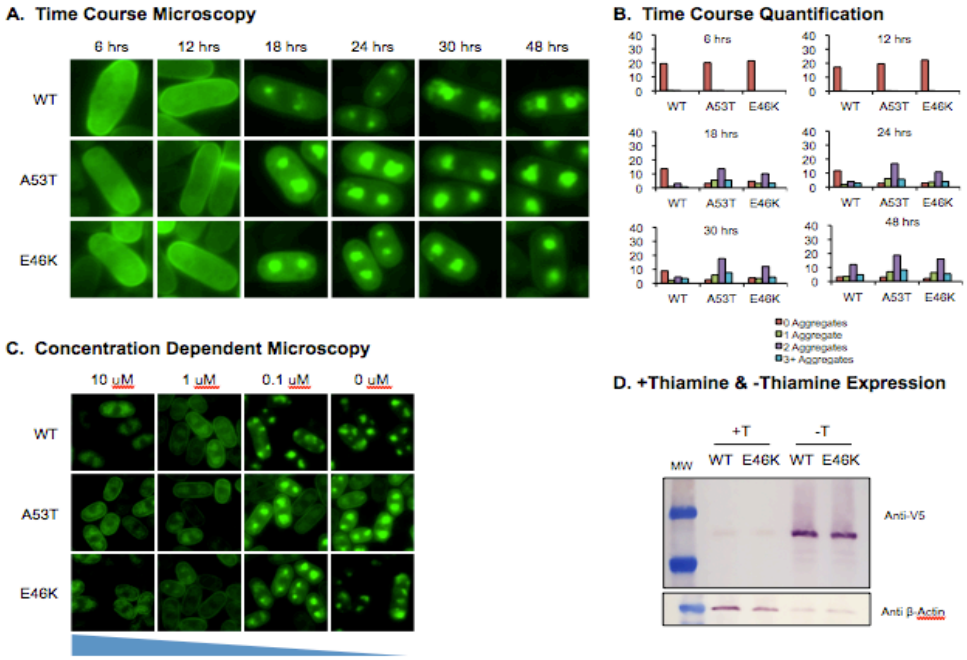

Figure 5: Characterization E46K Aggregation Rate in TCP1 Fission Yeast
A. Time Course Microscopy: TCP1 yeast cells expressing WT, A53T, or E46K α-synuclein at 6, 12, 18, 24, 30, & 48 hours post induction with -thiamine media. n=2
B. Time Course Quantification: Quantification of 750 cells for number of aggregates present. n=2
C. Concentration Dependent Microscopy: TCP1 yeast cells expressing WT, A53T, or E46K α-synuclein at 24 hours post induction with 10 uM, 1 uM, 0.1 uM, or 0 u M +thiamine media. n=2
D. -Thiamine & +Thiamine Expression: Western blot of TCP1 cells expressing WT or E46K (anti- V5) at 24 hours after inoculation in -thiamine or +thiamine media. β-actin: loading control. n=2

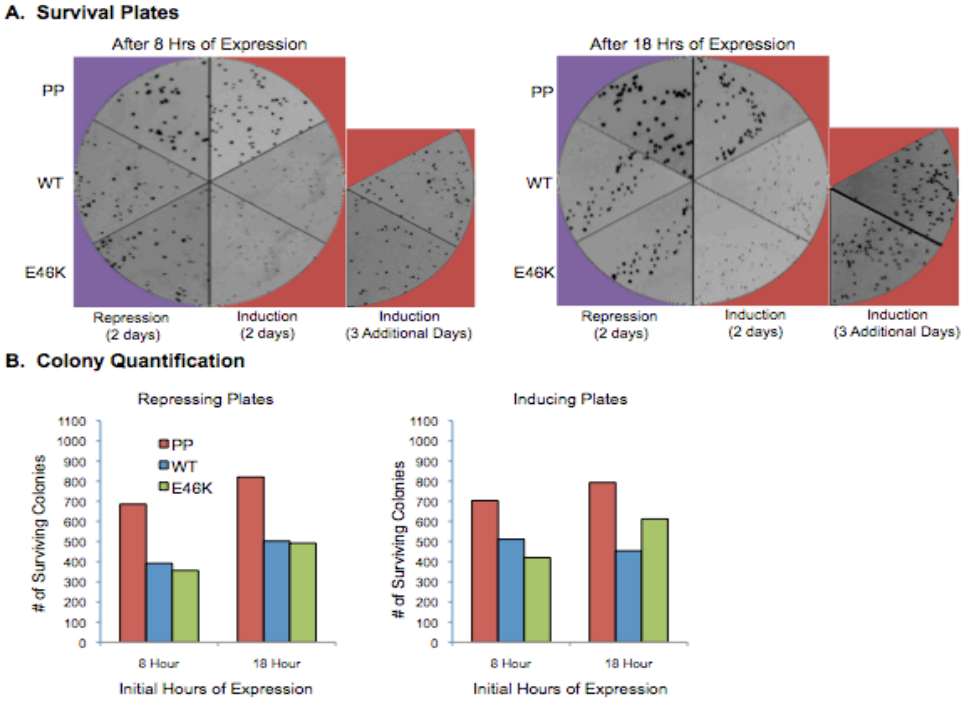

Figure 6: Characterization of E46K Survival in TCP1 Fission Yeast
A. Survival Plates: LEFT- Combined pictures of TCP1 cells plated on +thiamine (purple) or -thiamine (red) after expressing PP, WT, or E46K α-synuclein for 8 hrs. RIGHT- Combined pictures of TCP1 cells plated on +thiamine (purple) or -thiamine (red) after expressing PP, WT, or E46K α-synuclein for 18 hrs. n=2
B. Survival Quantification: The number of surviving colonies on +thiamine (left) or -thiamine (right) plates was counted & plotted for both time points. n=2

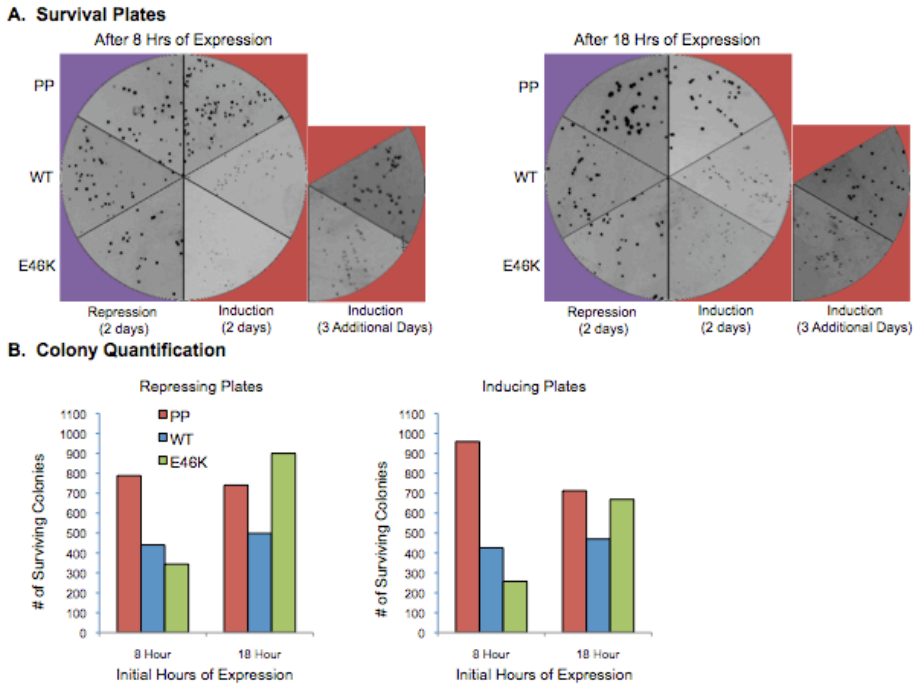

Figure 7: Characterization of E46K Survival in SP3 Fission Yeast
A. Survival Plates: LEFT- Combined pictures of SP3 cells plated on +thiamine (purple) or -thiamine (red) after expressing PP, WT, or E46K α-synuclein for 8 hrs. RIGHT- Combined pictures of SP3 cells plated on +thiamine (purple) or -thiamine (red) after expressing PP, WT, or E46K α-synuclein for 18 hrs. n=2
B. Survival Quantification: The number of surviving colonies on +thiamine (left) or -thiamine (right) plates was counted & plotted for both time points. n=2

compared to WT. Yeast cells expressing E46K grew more slowly than cells expressing WT α-synuclein in both OD600 growth curves (E46K: 18 hrs p= 0.0065; E46K: 24 hrs p= 0.00056) and serial dilution spotting (Figure 4D). No difference in growth was seen on the control plates (Figure 4C).

Expression levels were also assessed in SP3 cells expressing E46K to determine if the toxicity was due to α-synuclein accumulation. No change in steady state expression levels between cells expressing WT or the E46K familial mutant was seen (Figure 4E). Interestingly, the loss of induction blot suggested that a slight increase in E46K α-synuclein remained at 12 and 24 hours post inoculation in +thiamine media compared to WT (Figure 4E). However, the loading of the blot is not equal, and I am currently repeating the experiment (Figure 4E).

E46K α-Synuclein Aggregates Faster than WT

According to the nucleation polymerization hypothesis, amyloid proteins aggregate into polymers in a time and concentration-dependent manner (Caughey et al., 2003). *In vitro* data suggests that E46K aggregates faster than WT α-synuclein (Choi et al., 2004). I first addressed whether or not time-dependence held true in living cells by visualizing cells expressing WT, E46K, or A53T α-synuclein at 6, 12, 18, 24, 30, and 48 hours post induction with inducing media. My data shows that WT, A53T, and E46K localize to the cytoplasm of TCP1 fission yeast cells at 6 and 12 hours post induction. However, at 18 hours E46K and A53T began to form distinct aggregates in the cytoplasm of the yeast cells (Figure 5A). WT α-synuclein began to aggregate as well, but the inclusions are not as intense as the two familial mutants, and it appeared fewer cells with aggregates were present (Figure 5A). Based on these qualitative observations, I quantified the number of cells with aggregates and the number of aggregates at all time points (Figure 5B). Eventually, WT α-synuclein aggregated at a rate similar to E46K and A53T, but not until 48 hours post induction (Figure 5B). These findings are similar to those from Brandis et al. (2006), although A53T aggregated more aggressively than seen in this study.

I next investigated if E46K aggregation was concentration-dependent. Fission yeast cells expressing WT, A53T, or E46K were grown in media containing 0 uM, 0.1 uM, 1.0 uM, or 10 uM thiamine. None of the α-synuclein variants aggregated at 10 uM or 0.1 uM concentration and instead were weakly expressed in the cytoplasm (Figure 5C). However, all α-synuclein variants began to aggregate at .1 uM concentration (Figure 5C). Interestingly, the A53T mutant formed larger aggregates inside of TCP1 cells (Figure 5C). The WT and E46K mutants aggregated as well, but α-synuclein appeared to be associating with endomembrane organelles in a small but significant portion of cells, just like in SP3 (Figure 5C). Qualitatively, E46K α-synuclein most resembled WT at all concentrations (Figure 5C).

Given the weak fluorescence of cells under repressing conditions, I also examined α-synuclein expression in repressing and inducing media. In fact, WT and E46K expression was detected faintly at 24 hours even under repressing conditions (Figure 5D). Expression was significantly enhanced when optimally induced (Figure 5D).

Therefore, E46K follows the prediction of the nucleation polymerization model on its path to intracellular aggregation.

E46K impairs Growth of TCP1 and SP3 Fission Yeast Cells

To determine the nature of α-synuclein-dependent toxicity in fission yeast, I utilized a newly developed survival assay. In this experiment, 1000 fission yeast that produced α-synuclein for 8 or 18 hours were plated onto inducing plates (continue to produce α-synuclein) or repressing plates (halts α-synuclein production). The number of surviving colonies were counted and graphed. Smaller colony size would indicate a cell division/growth defect while reduction in colony number would indicate cell death. Qualitative observation of growth plates indicated that the colony size of TCP1 and SP3 cells that continued to express either WT or E46K α-synuclein were smaller than PP containing cells or WT or E46K expressing yeasts plated on repressing media (Figure 6A and 7A). It took an additional 3 days of growth for the colonies to reach a similar size, indicating a severe growth defect (Figure 6A and 7A). Interestingly in SP3, where E46K α-synuclein was more toxic than WT, E46K expressing cells plated on inducing plates formed even smaller colonies than WT expressing cells plated on the same media, and they never reached the same colony size after 3 additional days of growth (Figure 6A and 7A). Preliminary quantification of the colony numbers on each plate indicated a general decrease for cells expressing WT or E46K α-synuclein, although more trials are needed to establish whether cell death is occurring (Figure 6B and 7B).

Discussion

While over 90% of PD cases are sporadic in origin, 10% arise from known mutations in several PD associated genes. Three point mutations in the α-synuclein gene account for part of the 10% of familial PD cases: A30P, A53T, and E46K, the most recently discovered of the three mutants. Since E46K's discovery in 2004, only a handful of studies have evaluated the mutant's properties. Insight into how these familial mutants cause familial PD could provide important clues to what contributes to α-synuclein toxicity in PD. The first goal of my thesis was to characterize the properties of the E46K α-synuclein mutation in two budding yeast and two fission yeast strains. My three main findings were 1) E46K α-synuclein associated with the plasma membrane in budding yeast 2) E46K aggregated in fission yeast 3) E46K exhibits strain specific toxicity in fission yeast.

E46K Associates with Plasma Membrane Phospholipids in Budding Yeast

In support of my hypothesis, live cell GFP microscopy illustrated that E46K associated with the plasma membrane of budding yeast. This study is the first to demonstrate E46K association with membranes in living cells. E46K α-synuclein's affinity for lipids is well documented. *In vitro* lipid binding assays demonstrate that E46K α-synuclein binds to liposomes two times faster than either WT or the familial mutants A30P and A53T (Choi et al., 2004). Additionally, NMR solution spectroscopy confirmed that E46K binds more readily to lipids than WT, A53T or A30P (Choi et al., 2004). Recent solution NMR work also suggested that E46K α-synuclein adopts a structure that favors lipid binding more rapidly than WT or other α-synuclein mutants (Bodner et al., 2010). Interestingly, a recent study described conformational changes induced by the E46K mutation. These researchers also used NMR spectroscopy, but they discovered that the E46K mutation enhances N to C-terminal contact compared to WT and the other two familial α-synuclein mutants (Rospigliosi et al., 2009). Their finding was surprising, as the E46K mutation occurs in one of the KTKEGV amino acid repeats that are essential for membrane association. The N-terminus of α-synuclein is important in mediating membrane association (Soper et al., 2008), and the E46K mutation's impact on the N to C-terminal contact might result in a conformation that more easily adopts a helical structure, the structural form required for α-synuclein to interact with lipids (Soper et al., 2008).

Surprisingly, no plasma membrane phospholipid association was apparent in fission yeast despite extensive plasma membrane association in budding yeast. However, fission yeast and budding yeast are significantly divergent on the evolutionary time scale. As the two yeast species are separated by almost 400 million years of evolution, inevitable differences in plasma membrane composition are likely (Roux et al., 2010). α-Synuclein might have an increased affinity because of differences in the plasma membrane composition of budding yeast in comparison to fission yeast, providing a logical explanation for this difference.

Previous work in our lab is consistent with my findings. Michael White's thesis in 2007 evaluated the E46K mutant in several budding yeast strains. His results suggested that E46K associates with plasma membranes in BY4741 and BY4743 budding yeast strains (White Thesis, 2007). He also conducted work in several other budding yeast strains, including BY4742, TSY623, and W303. E46K initially localized to the membrane in all yeast strains, but later localized to the vacuole after 24 hours in the TSY623 strain. Interestingly, vacuolar localization of E46K in TSY623 was slower compared to WT, supporting the hypothesis that E46K α-synuclein has an intrinsic affinity for membranes (White Thesis, 2007). However, unbeknownst to him, his protein variants had a mutation at the 140th amino acid that was accidentally inserted during PCR based subcloning of the α-synuclein gene. Overall, my findings detailing E46K membrane association in BY4741 and BY4743 agree with Mike White's thesis work.

E46K is more Aggregation Prone Than WT α-Synuclein

My work also suggests that E46K aggregates more rapidly than WT α-synuclein in fission yeast, again supporting my hypothesis. In fact, E46K aggregation rates more closely matched those of the A53T α-synuclein familial mutant. Both *in vitro* and *in vivo* evidence exists supporting my findings. Previously, E46K was shown to promote aggregation at a rate similar to A53T in *in vitro* systems (Choi et al., 2004; Greenbaum et al., 2005). Additionally, the KTKEGV repeats in α-synuclein's N-terminus appear to influence aggregation rates (Koo et al., 2008). A recent study created glutamic acid to lysine point mutants within several of these repeats. Synthetic E(XX)K mutations at these points decreased fibrillization lag time *in vitro* (Harada et al., 2009). Subsequent work detailed E46K's properties in cell culture systems, where the familial mutant readily formed intracellular aggregates (Pandey et al., 2006). My work adds to knowledge of E46K properties in living cells. Overall, the evidence suggests that the E46K mutation enhances α-synuclein aggregation due to the mutation's location in the N-terminus of α-synuclein. As mentioned before, NMR spectroscopy suggests that the E46K mutant enhances N to C-terminal contact. Koo et al. (2008) also demonstrated that the E46K mutant resulted in N to NAC region contact. The NAC region is crucial for aggregate formation (Soper et al., 2008). E46K mutations also result in an overall decrease in negative charge of the entire α-synuclein protein (Koo et al., 2008). This interaction with the NAC domain, compounded by an overall decrease in negative charge, is hypothesized to increase aggregation rates, providing an explanation for E46K's impact on aggregation.

In stark contrast to my findings in fission yeast, little to no aggregation was apparent in BY4741 or BY4743 budding yeast cells expressing E46K α-synuclein. The most likely explanation has to do with the strength of the promoter in each of the vectors. Aggregation of α-synuclein increases as α-synuclein concentration increases (Outiero and

Lindquist, 2003). The galactose inducible promoter found in the pYES2 budding yeast vector results in moderate α-synuclein expression. However, the fission yeast cells are under the control of the pNMT1 vector, which uses a thiamine repressible promoter. The pNMT1 promoter is a stronger promoter than the pYES2 promoter, resulting in a significantly increased expression. Therefore, this increased α-synuclein expression might result in aggregation in fission yeast (Brandis et al., 2006) and plasma membrane association in budding yeast (Sharma et al., 2006). Other labs that utilize a stronger expression system see aggregation in budding yeast (Outeiro and Lindquist, 2005), but when they use a moderate expression vector, they also see membrane association (Dixon et al., 2005; Zabrocki et al., 2005).

Stephanie Valtierra '08 assessed E46K's properties in fission yeast cells for her senior thesis. However, her work was also conducted with an uncorrected α-synuclein variant containing a mutation at α-synuclein's final amino acid. Despite the mutation at this location, my findings on E46K's impact on aggregation agree. Her time course microscopy with WT, A30P, A53T, and E46K expressing cells demonstrated that E46K α-synuclein aggregated more rapidly than WT. Similar to my findings, E46K most closely resembled A53T in its aggregation tendency.

E46K is Selectively Toxic to SP3 Fission Yeast

In support of my hypothesis, serial dilution spotting and OD600 growth curve analysis revealed E46K dependent toxicity in SP3 fission yeast cells. However, contrary to my hypothesis, E46K did not enhance toxicity in TCP1 fission yeast or two budding yeast strains, as demonstrated by the same growth assays. One of the most important research avenues in PD biology is the identification of the toxic α-synuclein species. In the field, both membrane association (Volles et al., 2003) and aggregation (Outiero and Lindquist, 2003; Caughey et al., 2003) are correlated with toxicity. *In vitro*, α-synuclein has the ability to permeabilize synthetic vesicles through the formation of protofibrils, the intermediates formed before mature aggregates (Volles et al., 2003). These protofibrils form pore-like structures on the membrane, ultimately destabilizing them. The A30P familial mutation enhances the formation of these protofibrils, suggesting that protofibril association with the membrane is the toxic mechanism. However, the A53T mutant, which also causes familial PD, enhances fibrillization and aggregation of α-synuclein. This finding, in addition to the correlation between aggregation and pathology, suggests that aggregation might be the pathotoxic feature of α-synuclein (Choi et al., 2004).

However, in SP3 yeast expressing E46K, neither plasma membrane association nor aggregation was the dominant phenotype. Instead, α-synuclein appeared to be interacting with the endomembrane system of the fission yeast. In 2008, researchers demonstrated that over-expression of α-synuclein in budding yeast cells disrupts ER to Golgi trafficking (Cooper et al., 2006; Gitler et al., 2008). Additionally, genetic suppression of ER trafficking rescues some yeast strains from α-synuclein-dependent toxicity (Su et al., 2010). It is possible that E46K might be interacting with the ER of the fission yeast cells, as accumulations of α-synuclein were visible outside of an organelle reminiscent of the nucleus. The fission yeast's ER surrounds the nucleus, and thus α-synuclein might be accumulating here. This accumulation might be disrupting ER to Golgi activity, resulting in toxicity. It is interesting to note that E46K also enhances association with endomembrane systems in TCP1; however no added toxicity is apparent. The endomembrane association in TCP1 fission yeast is slightly different from that in SP3. In TCP1, endomembrane association usually involves only what appears to be the ER. In SP3, E46K α-synuclein also enhances association with vesicles inside of the cells. Thus it might be the disruption of vesicle integrity that mediates toxicity rather than accumulation on or around the ER. As well, the SP3 might be more sensitive to ER disruption than TCP1, which would account for the strain specific difference in toxicity. Another possibility is that the lipid composition of SP3 yeast differs from that of TCP1 in a way that enhances α-synuclein association with intracellular organelles. Finally, the endomembrane localization in TCP1 might be a minor phenotype. A majority of cells might display α-synuclein aggregates, which is not toxic. Since only few cells exhibit a potentially toxic endomembrane phenotype, an insignificant number of cells die and this toxicity is not detected by the growth assays.

Additionally, the toxicity I see might not be a result of cell death. The OD600 growth curve and spotting assay illustrate some sort of toxicity, but they do not provide evidence as to whether or not the cells actually die or simply grow more slowly. However, the survival assay results suggest that, as a result of the toxic effects of WT and E46K, cells are dividing less rapidly when they express α-synuclein. Quantification of these results revealed no clear indication that the WT and E46K cells actually survive less well. More repeats will be necessary to clarify a relation between colony survival and toxicity. Cell stains, such as FM464 or propidium iodide, could also be used to identify activation of apoptosis pathways or the onset of necrosis.

Stephanie Valtierra also saw α-synuclein endomembrane association and E46K dependent toxicity in SP3 fission yeast (Valtierra Thesis, 2008). However, she did not see the vesicular association that is evident in my microscopy data. E46K α-synuclein was still toxic to these cells, and this suggests that ER accumulation might be the key to toxicity in this strain. A related question is whether or not A53T or A30P will be toxic to SP3 yeasts and if so, what phenotype they will display.

Table 2: Summary of Familial Mutant Properties in Both Yeast Models

	Budding Yeast		Fission Yeast	
	Localization?	Toxicity?	Localization?	Toxicity?
A53T	Mostly Plasma Membrane Some Aggregation	None	Aggregates	No
A30P	Some Plasma Membrane Mostly Cytoplasm	None	Cytoplasm	No
E46K	Mostly Plasma Membrane	None	Aggregates	Strain Specific

Chapter 2: Serine Phosphorylation Alters α-Synuclein Aggregation Without Affecting Toxicity

Results

Phosphorylation Mutants Slightly Alter α-Synuclein Membrane Binding in BY4741

Similar to my approach from the previous chapter, my first goal was to assess the properties of the phosphorylation deficient (S87A and S129A) and phosphorylation mimic (S87D and S129D) mutants in BY4741 budding yeast. I began by determining if they altered α-synuclein localization in our budding yeast model. S87A α-synuclein localized primarily to the plasma membrane of budding yeast at 24 and 48 hours (Figure 3A). The second phosphorylation deficient mutant, S129A, also localized to the plasma membrane at 24 and 48 hours post induction (Figure 3A and 3B). The S87D and S129D phosphorylation mimic α-synuclein mutants localized to both the cytoplasm of the

budding yeast at 24 hours, although a majority of cells still retained membrane association as well. By 48 hours, S87D and S129D α-synuclein localized to the plasma membrane, similar to WT and the phosphorylation deficient mutants (Figure 3A and Figure 3B).

I next determined what effect the phosphorylation deficient and mimic mutants had on α-synuclein toxicity in budding yeast. The OD600 growth curve revealed no delay in growth of budding yeast cells expressing any of the α-synuclein phosphorylation mutants in comparison to the three controls (Figure 3C). Serial dilution spotting on solid plate media confirmed this result (Figure 3D).

Lastly, I assessed expression levels in cells expressing S87A, S129A, S87D, or S129D α-synuclein in budding yeast. Expression was similar among the phosphorylation mutants and WT, as illustrated by Western blot (Figure 3E).

Phosphorylation Mutants Alter α-Synuclein Localization in TCP1 in Complex Ways

My second goal was to assess the properties of the α-synuclein phosphorylation mutants in our fission yeast model. In support of my hypothesis, S87A and S129A α-synuclein decreased aggregation in the fission yeast model at 24 and 48 hours (Figure 4A and 4B). Additionally, both S87A and S129A α-synuclein enhanced association with what might be the yeast nucleus, endoplasmic reticulum, or other to-be-identified endomembrane structures (Figure 4A). Surprisingly, the phosphorylation mimics also altered α-synuclein localization. S87D α-synuclein localized to the cytoplasm and aggregated in fission yeast at 24 hours. S129D α-synuclein also increased cytoplasmic localization at 24 hours, but in a more complex way than S87D (Figure 4A and 4B). Unexpectedly, S129D associated with an intracellular structure, similar to the phosphorylation deficient mutants (Figure 4A).

I next examined what effect these phosphorylation mutants had on toxicity in the fission yeast model. Firstly, I assessed growth of fission yeast cells expressing either WT α-synuclein or one of the phosphorylation mutants using an OD600 growth curve. Neither the phosphorylation mimic nor phosphorylation deficient mutants altered toxicity according to the growth curve. However, similar to my TCP1 E46K spotting results, I noted all α-synuclein expressing yeast grew slower on inducing plates than yeast containing the empty vector or expressing GFP, but none of the phosphorylation mutants enhanced or abated this toxicity (Ch 2, Figure 2D and Ch 1, Figure 2D).

Finally, I assessed expression of the phosphorylation mutants in the fission yeast model. The S87A, S129A, and S87D mutants all appear to be expressed at a slightly higher level than WT (Figure 4E). Interestingly, these are all the phosphorylation mutants that have a phenotype least similar to WT α-synuclein.

Discussion

Covalent modifications of proteins are implicated in both normal and disease states. Both tau and α-synuclein are proteins that undergo phosphorylation and are linked to neurodegeneration. Thus, understanding how phosphorylation relates to disease onset is a significant step in developing treatment for these incurable, fatal diseases. The second goal of my thesis was to determine how serine phosphorylation of α-synuclein influences PD pathogenesis. I reported three key findings that I will discuss further. 1) Blocking serine phosphorylation decreases aggregation. 2) Blocking serine phosphorylation did not alter α-synuclein's phospholipid interactions. 3) Neither blocking serine phosphorylation nor mimicking a phosphorylated state influences the toxic properties of α-synuclein.

Does Phosphorylation Impact Aggregation?

Normally, α-synuclein forms intracellular aggregates in our fission yeast model (Brandis et al., 2006). In support of my hypothesis, GFP live cell microscopy revealed that the phosphorylation mimics decreased aggregation in fission yeast. The S87A and S129A mutants resulted in a complex phenotype that included less aggregation and increased cytoplasmic localization. Several studies have investigated serine phosphorylation's role in aggregation, but the findings do not always agree. An *in vivo* fly model showed that over-expression of the S129D mutant or the expression of WT and GprK2, a kinase that phosphorylates α-synuclein, did not enhance aggregation (Chen and Feany, 2005). Furthermore, no increase or decrease in inclusion formation was seen in a rat model expressing WT or S129A mutants (McFarland et al., 2009). However, substantial evidence exists to support the notion that serine phosphorylation promotes aggregation. In 2008, a study by Fujiwara et al. illustrated that serine-129 phosphorylation prompts the formation of α-synuclein fibrils more readily than WT *in vitro*. Additionally, an S129A phosphorylation deficient mutant failed to aggregate in human neuroblastoma cells, also suggesting that phosphorylation enhances aggregation (Smith et al., 2005). An *in vivo* rat model also found increased aggregation in SNpc cells expressing an S129D mutant (Gorbatyuk et al., 2008). My findings in fission yeast provide further evidence that serine phosphorylation promotes aggregation of α-synuclein, although the exact role of serine phosphorylation is still controversial.

Most recently, my findings in fission yeast partially contradict a second study by Palelogou et al (2008). They reported that phosphorylation of α-synuclein inhibits aggregation *in vitro,* and the S129A mutant formed significantly more fibrils compared to WT *in vitro.* The most plausible explanation for my conflicting findings has to do with my model systems. They used an *in vitro* model while I study the protein in yeast. The environment of a living organism is extremely complex, and an *in vitro* system cannot recapitulate the exact conditions of a living cell. As mentioned previously, *in vitro* and *in vivo* systems do not always agree. Another possibility to explain the conflicting findings has to do with when phosphorylation of α-synuclein actually occurs. It is possible that phosphorylation of α-synuclein takes place after aggregation, and phosphorylation might not actually increase aggregation *in vivo.* It might be that aggregation results in a structure more conducive to phosphorylation. Thus, identifying which kinases phosphorylate α-synuclein in LB's is essential to understanding the role that phosphorylation plays in aggregation.

Previous work by Stephanie Valtierra in fission yeast demonstrated that both phosphorylation-deficient mutants aggregated more rapidly than WT. However, increasing evidence suggests that what appear to be aggregates in our fission yeast model might actually be collections of α-synuclein associated with vesicles. This correlates well with the recent finding that suggests the aggregates in LBs are actually accumulations of α-synuclein in vesicles (Soper et al., 2008). Thus, blocking phosphorylation might be enhancing α-synuclein's association with the endomembrane system.

Aggregation of α-synuclein has been linked to cellular toxicity throughout the history of PD research (Conway et al., 1998; Baba et al., 1998). Any research that provides insight as to what contributes to α-synuclein aggregation is valuable. Here, I discovered that blocking phosphorylation decreases aggregation in a fission yeast

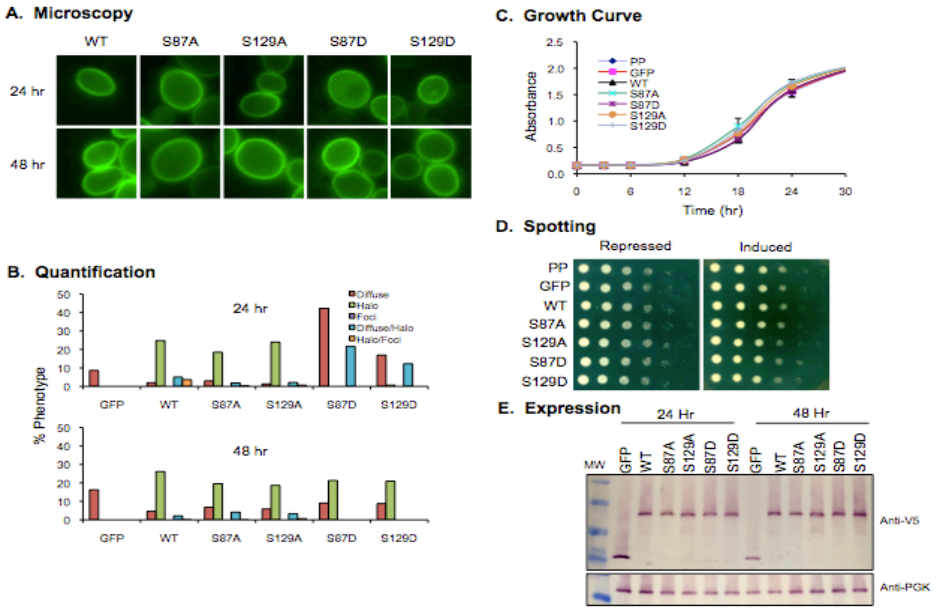

Figure 1: Characterization of Phosphorylation Mutants in Budding Yeast
A. Microscopy: WT, S87A, S129A, S87D, & S129D α-synuclein localization at 24 & 48 hours post induction. n=2
B. Quantification: Quantification of 750 GFP, WT, S87A, S129A, S87D, & S129D expressing cells scored for several phenotypes. n=2
C. Growth Curve: OD600 readings recorded over 30 hours for BY4741 cells induced for WT, S87A, S129A, S87D, & S129D α-synuclein. n=3
D. Spotting: Yeast expressing PP, GFP, WT, S87A, S129A, S87D, or S129D α-synuclein spotted onto glucose (repressed) & galactose (induced) plates after five-fold serial dilutions. n=3
E. Expression: Western blot at 24 & 48 hours of WT, S87A, S129A, S87D, & S129D α-synuclein expression (anti-V5) with galactose media. PGK: loading control. n=2

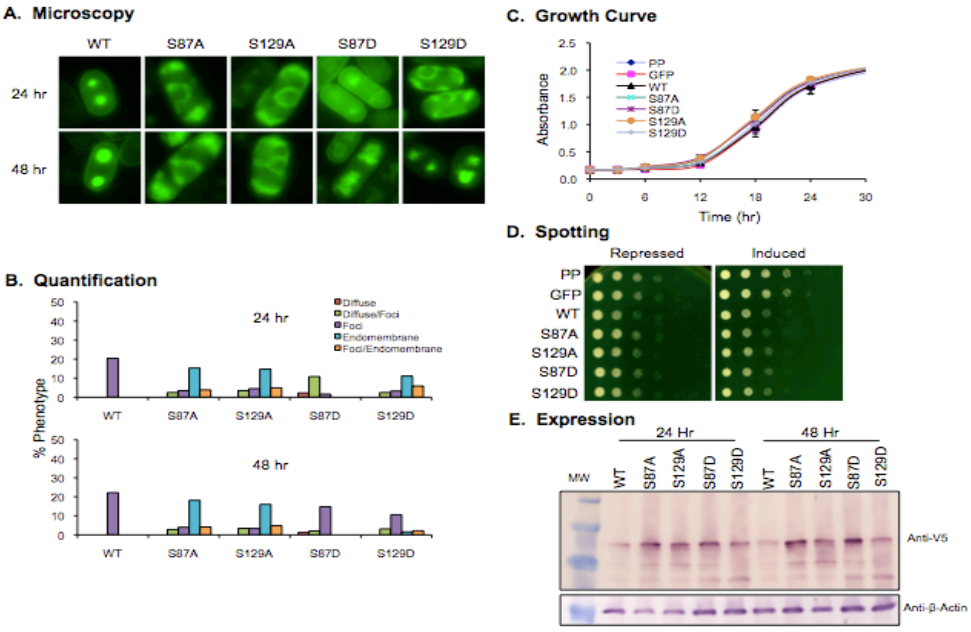

Figure 2: Characterization of Phosphorylation Mutants in Fission Yeast
A. Microscopy: WT, S87A, S129A, S87D, & S129D α-synuclein localization at 24 & 48 hours post induction. n=2
B. Quantification: Quantification of 750 WT, S87A, S129A, S87D, & S129D expressing cells scored for several phenotypes. n=2
C. Growth Curve: OD600 readings recorded over 30 hours for TCP1 cells induced for WT, S87A, S129A, S87D, & S129D α-synuclein. n=3
D. Spotting: Yeast expressing PP, GFP, WT, S87A, S129A, S87D, & S129D α-synuclein spotted onto +thiamine (repressed) & -thiamine (induced) plates after five-fold serial dilutions. n=3
E. Expression: Western blot at 24 & 48 hours of WT, S87A, S129A, S87D, & S129D α-synuclein expression (anti-V5) with -thiamine media. β-Actin: loading control. n=2

model, suggesting that phosphorylation of α-synuclein promotes the aggregation of the protein. My findings contribute to the growing link between phosphorylation and aggregation.

What Role Does Phosphorylation Have in Membrane Interactions?

α-Synuclein's affinity for membranes is a well-documented phenomenon. In the budding yeast model, WT α-synuclein is routinely localized to the plasma membrane (Sharma et al., 2006). Interestingly, mimicking serine phosphorylation eliminated this interaction, but only at 24 hours of induction. This finding suggests that phosphorylation decreases α-synuclein's interaction with membranes. My findings complement a recently published article that suggests phosphorylated α-synuclein decreases interactions with phospholipids (Palelogou et al., 2010). This study saw increased membrane association with the S87A mutant *in vitro.* α-Synuclein requires an alpha helical structure to interact with membranes, and Palelogou et al. (2010) suggest phosphorylation at serine-87 disrupts the required shape necessary for the protein-membrane interaction. Interestingly, Palelogou et al. (2010) did not see decreased membrane association with an S87E mutant, a mutant similar to the S87D variants I used, and they suggest that serine to glutamic acid mimic mutants might not fully recapitulate phosphorylation. However, I did see a decrease in membrane affinity with an S87D mutant. Two possible explanations exist. Although similar, glutamic acid (E) and aspartic acid (D) are not identical in structure and might mimic phosphorylation to different degrees. Additionally, since membrane association returned by 48 hours, aspartic acid might only partially recapitulate serine phosphorylation. Regardless, my study provides the first evidence that serine-87 phosphorylation influences membrane association in a living organism.

Previous work in our lab by Sara Herrera and Stephanie Valtierra characterized phosphorylation mutants S87A and S129A amino acid in budding and fission yeast, respectively (Hererra Thesis, 2005 and Valtierra Thesis, 2008). Unknowingly, however, the variants that they worked with had a single amino acid mutation at the 140th amino acid. Sara Herrera and I both saw similar membrane association in budding yeast with the phosphorylation deficient mutants (Herrera Thesis, 2005) while Stephanie Valtierra and I both saw interaction of α-synuclein with unidentified intracellular organelle (Valtierra Thesis, 2008).

Membrane association is one of the properties of α-synuclein most closely linked to toxicity (Maroteaux and Scheller, 2005; Dixon et al., 2005). As such, furthering our understanding of the molecular determinants of α-synuclein's membrane association is crucial to understanding disease pathogenesis. My results suggest that serine-87 phosphorylation influences membrane interactions in budding and fission yeast and that serine-129 phosphorylation alters membrane association in fission yeast. However, further research is necessary to understand how phosphorylation influences membrane interactions.

Is Phosphorylation Related to Toxicity?

None of my findings in our two yeast models suggest that phosphorylation affects cellular toxicity, as neither the phosphorylation-blocking nor mimic mutants enhanced or diminished toxicity. My findings contradict work done on phosphorylation mutants by Sara Hererra. She showed that phosphorylation blocking mutants were significantly toxic to budding yeast (Herrera Thesis, 2005). However, my findings do match Stephanie Valtierra's work in fission yeast. Her results suggested that the S87A and S129A mutants were non-toxic to fission yeast cells (Valtierra Thesis, 2008). Sara Hererra's work was on the uncorrected α-synuclein variants, which might explain the differences seen in toxicity. Similarly, although Stephanie Valtierra and I both failed to see a relationship between phosphorylation and toxicity, she did not see the α-synuclein-dependent toxicity apparent in my spotting. Again, her work was on the uncorrected variants, and the 140th amino acid mutation might have prevented α-synuclein from adopting the shape necessary to induce toxicity.

In the field, the role of phosphorylation in toxicity is a complicated topic. Conflicting evidence exists as to whether or not phosphorylation enhances or protects from toxicity. Some researchers link toxicity to phosphorylation (Chen and Feany, 2005) while others find that lack of phosphorylation promotes toxicity (Gorbatyuk et al., 2008; de Silveira et al., 2009). Still others find no difference between phosphorylation states and toxicity (McFarland et al., 2009). My findings support the notion that phosphorylation of α-synuclein does not influence toxicity in our yeast models. It is interesting to note that, despite the variety of phenotypes observed (membrane associated, cytoplasmically diffuse, endomembrane associated, aggregated), no increase in toxicity was induced by either phosphorylation-mimic or -deficient mutants. It is possible that the kinase necessary for phosphorylation α-synuclein at the precise time to form the toxic species does not exist in yeast, yet our lab does have unpublished data that suggests α-synuclein is phosphorylated at serine-129 in budding yeast. Recent evidence has suggested that phosphorylation might promote the formation of protective, mature fibrils rather than toxic intermediates (Paleologou et al., 2010). Additionally, recent findings have suggested that tyrosine phosphorylation might have opposing influences on α-synuclein-dependent toxicity, and this tyrosine phosphorylation might mask toxicity resulting from serine phosphorylation (Chen et al., 2009). Combining serine and tyrosine mutations might provide insight into the mechanism by which serine phosphorylation impacts toxicity.

Clarification of the role that phosphorylation plays in PD is critical for the development of PD treatments. If phosphorylation actually leads to toxicity, development of drugs that inhibit the responsible kinases could provide an effective therapy for the disease. Likewise, if phosphorylation is protective, enhancing phosphorylation might ward off PD. Either way, more research is needed to identify the specific role of phosphorylation in α-synuclein's properties and disease onset.

Table 3: Summary of Phosphorylation Findings

Study	Model	Aggregation Conclusion	Toxicity Conclusion
Fujiwara et al., 2002	*In vitro*	Phosphorylation Enhances Aggregation	N/A
Smith et al., 2005	Cell Culture	Phosphorylation Enhances Aggregation	N/A
Chen and Feany, 2005	*Drosophila*	Phosphorylation Prevents Aggregation	Phosphorylation Promotes Toxicity
Gorbatyuk et al., 2008	Rat	Phosphorylation Enhances Aggregation	Phosphorylation Prevents Toxicity
McFarland et al., 2009	Rat	No effect	No effect
Palelogou et al., 2009	*In vitro*	Phosphorylation Prevents Aggregation	N/A
DeSilvia et al., 2009	Mice	Phosphorylation Prevents Aggregation	Phosphorylation Prevents Toxicity
This Thesis	Yeast	Phosphorylation Prevents Aggregation	No effect

Chapter 3: Alanine-76 Influences Membrane Association and Aggregation in Yeast Models

Results

Alanine-76 Mutants Alter Localization in Budding Yeast Without Inducing Toxicity

Again, my first goal was to characterize the A76E and A76R mutants in budding yeast. I first assessed the localization of the A76E and A76R α-synuclein mutants in budding yeast strain BY4741. As previously demonstrated by our lab, WT α-synuclein associates with the plasma membrane of budding yeast at 24 and 48 hours post induction (Sharma et al., 2006). In support of my hypothesis, A76E α-synuclein localized to the cytoplasm of the yeast at 24 hours after induction and remained in the cytoplasm at 48 hours (Figure 1A and Figure 1B). Similar to A76E, A76R localized to the cytoplasm at 24 hours, although some plasma membrane association was still present. However, unlike A76E, the A76R α-synuclein localized back to the plasma membrane by 48 hours (Figure 1A and 1B).

I next evaluated how the alanine-76 mutants altered the toxic properties of α-synuclein in budding yeast. My OD600 growth curve indicated that budding yeast cells expressing either A76E or A76R experienced no delay in growth in comparison to cells expressing WT, PP, or GFP (Figure 1C and 1D). Serial dilution spotting confirmed this result. The cells expressing either the A76E or A76R mutant grew equally well on solid inducing media (Figure 1D).

I finally determined how α-synuclein expression levels changed in the budding yeast as a result of the A76E or A76R mutations. Western blot illustrated no evident difference in expression levels at 24 or 48 hours between cells expressing WT, A76E or A76R α-synuclein (Figure 1E).

A76E and A76R α-Synuclein Differentially Alter Localization in Fission Yeast

My second goal was to evaluate the A76E and A76R mutants in fission yeast. I first assessed localization of A76E and A76R in fission yeast strain TCP1. In support of my hypothesis, A76E α-synuclein strongly localized throughout the cytoplasm of the fission yeast cells with only minor aggregation (Figure 2A and 2B). The A76E α-synuclein remained in the cytoplasm after 48 hours, although more significant aggregates formed by this time. A76R α-synuclein localized to the cytoplasm as well at 24 hours, but more prominent aggregates were visible at this time point compared to A76R (Figure 2A and 2B). By 48 hours, A76R more resembled wildtype α-synuclein than A76E. Additionally, some membrane association was apparent at 24 and 48 hours in fission yeast cells expressing A76R α-synuclein (Figure 2A and 2B). These results indicate A76R appears to confer membrane affinity in budding yeast and fission yeast.

Next, I determined how the A76E and A76R mutants altered α-synuclein toxicity in fission yeast. My OD600 growth curve revealed no change in growth between cells expressing PP, GFP, WT, or the two alanine-76 mutants (Figure 2C). As well, serial dilution spotting demonstrated that the A76E and A76R α-synuclein mutants grew equally well compared to WT on inducing plate media (Figure 2D). As previously seen, an α-synuclein-dependent toxicity was observed in the serial dilution spotting plates. Cells expressing WT, A76E or A76R α-synuclein grew less well compared to cells expressing PP or GFP (Figure 2D).

I finally assessed α-synuclein expression levels in cells expressing WT, A76E, or A76R α-synuclein. No significant difference was observed in cells expressing WT, A76E, or A76R α-synuclein at 24 or 48 hours (Figure 2E).

Discussion

A protein's properties result from its amino acid sequence. Each amino acid has specific characteristics which contribute to the overall properties of a protein. Research suggests that α-synuclein's propensity to aggregate, a property closely linked to toxicity, is mediated by a stretch of amino acids from aa71-82 (Chiti et al., 2003; Giasson et al., 2001). As such, understanding how these amino acids contribute to aggregation is an important avenue of research. The final goal of my thesis was to determine the contribution of alanine-76, an amino acid within this stretch, to the hydrophobic properties of α-synuclein: membrane association and aggregation. My research revealed three key findings: 1) The A76E and A76R α-synuclein mutants decreased membrane association in budding yeast and aggregation in fission yeast. 2) A76E more dramatically relocalized α-synuclein than A76R. 3) Neither A76E nor A76R influenced α-synuclein toxicity.

How Relevant Is Alanine-76?

In support of my hypothesis, GFP live cell microscopy of the A76E and A76R α-synuclein mutants revealed a decrease in membrane association in budding yeast. Although both mutants altered α-synuclein's localization, A76E did so more drastically. Previous work has demonstrated that deletion of several hydrophobic residues from the hydrophobic middle domain is sufficient to prevent aggregation formation (Giasson et al., 2001; Periquet et al., 2007). My findings suggest that a single amino acid mutation can have a significant impact on the shape and properties of a protein. A single hydrophobic to hydrophilic mutation resulted in a significant increase in the solubility of the protein. Recent work has demonstrated that deletions of only two of these hydrophobic residues decreased mature of α-synuclein fibril formation and increased oligomerization (Waxman et al., 2009). Interestingly, the two amino acids that decreased aggregations the most when deleted together were aa76 and aa77. This deletion evidence and my point mutation findings suggest that small numbers of amino acids can significantly impact the properties of α-synuclein. Additionally, alanine-76 appears quite relevant to α-synuclein aggregation.

Previous work in our lab assessed an A76E mutant in budding and fission yeast containing an incorrect 140th amino acid (Zorniak Thesis, 2007). The uncorrected A76E mutant resulted in a similar increase in cytoplasmic localization as seen in my study (Zorniak Thesis, 2007).

The Importance of Shape

My GFP live cell microscopy images demonstrated that the A76E mutation resulted in a more prominent redistribution of α-synuclein than A76R. Chiti et al. (2003) hypothesized that the A76E mutation would decrease the hydrophobicity of α-synuclein less than A76R. More specifically, their statistics suggested A76E would increase solubility by a factor of three while A76R would result in a fourfold increase in solubility. Thus, A76R should have resulted in a more significant decrease in aggregation and membrane binding than A76E. However, this prediction was the opposite of what my results demonstrated. Not only did A76R aggregate more than A76E, A76R also associated with plasma membranes in fission yeast, a phenotype our lab rarely sees. However, an *in vitro* study by Giasson et al. (2001) demonstrated that A76E slowed fibril formation more than A76R. The environment of a living cell is infinitely complex, and it is hard to predict with perfect accuracy how a protein will behave in a cell. The previous *in vitro* data and my thesis results clearly demonstrate this.

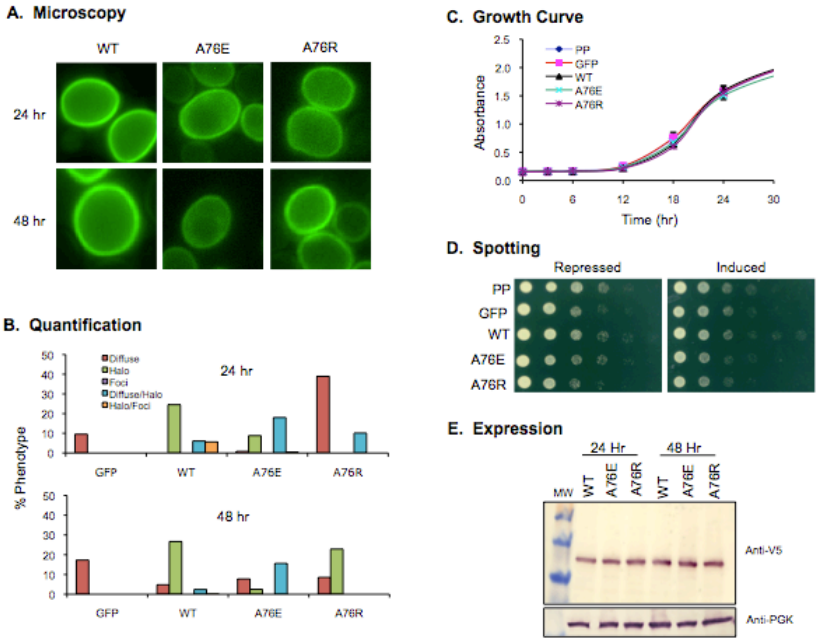

Figure 1: Characterization of Alanine-76 Mutants in Budding Yeast
A. Microscopy: WT, A76E, & A76R α-synuclein localization at 24 & 48 hours post induction. n=2
B. Quantification: Quantification of 750 GFP, WT, A76E, & A76R expressing cells scored for several phenotypes. n=2
C. Growth Curve: OD600 readings recorded over 30 hours for BY4741 cells induced for PP, GFP, WT, A76E, & A76R α-synuclein. n=3
D. Spotting: Yeast expressing PP, GFP, WT, A76E & A76R α-synuclein spotted onto glucose (repressed) and galactose (induced) plates after five-fold serial dilutions. n=3
E. Expression: Western blot at 24 & 48 hours of WT, A76E & A76R α-synuclein expression (anti-V5) with galactose media. PGK: loading control. n=2

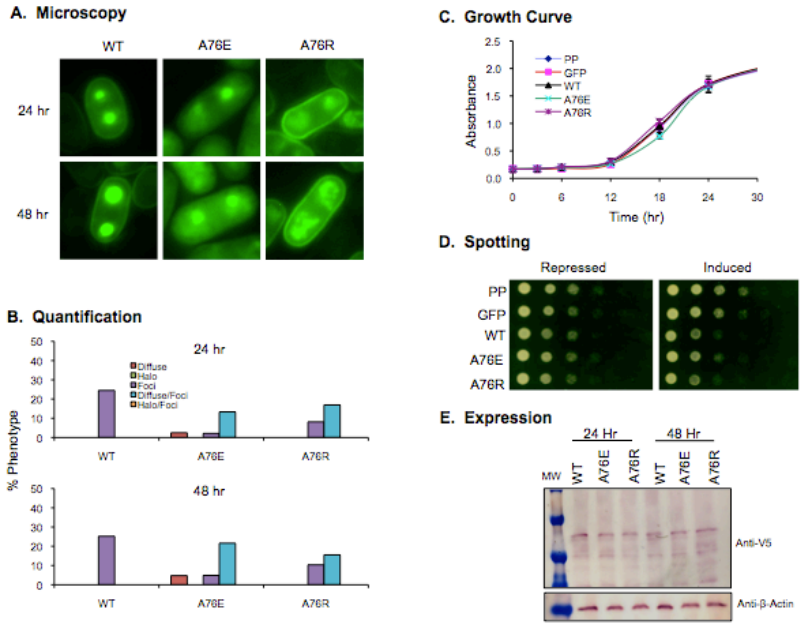

Figure 2: Characterization of Alanine-76 Mutants in Fission Yeast
A. Microscopy: WT, A76E, & A76R α-synuclein localization at 24 & 48 hours post induction. n=2
B. Quantification: Quantification of 750 WT, A76E, & A76R expressing cells scored for several phenotypes. n=2
C. Growth Curve: OD600 readings recorded over 30 hours for TCP1 cells induced for WT, A76E, & A76R α-synuclein. n=3
D. Serial Dilution Spotting: Yeast expressing PP, GFP, WT, A76E or A76R α-synuclein spotted onto +thiamine (repressed) and -thiamine (induced) plates after five-fold serial dilutions. n=3
E. Accumulation & Expression: Western blot at 24 & 48 hours of WT, A76E & A76R α-synuclein expression (anti-V5) with -thiamine media. β-Actin: loading control. n=2

Surprisingly, I saw A76R regularly associate with the plasma membrane of fission yeast. Glutamic acid is negatively charged while arginine is positively charged. One possible explanation is that a positive charge alters the shape in a manner that confers membrane affinity. Since only a single nucleotide change (A76E) dramatically affected aggregation, a different point mutation might be sufficient to induce a shape change that increases membrane affinity.

Are Aggregates Toxic?

Several lines of evidence support the notion that aggregation is a protective mechanism employed by cells to prevent α-synuclein toxicity. Firstly, the A30P familial mutant promotes the formation of protofibrils rather than mature fibrils (Lansbury 1999, Goldberg and Lansbury 2000). Secondly, the formation of protofibrils coincides with the fragmentation of the Golgi apparatus and loss of cell viability in a cell culture model (Gosavi et al., 2005). If aggregates are protective, then eliminating aggregation should increase α-synuclein-dependent toxicity. However, both the A76E and A76R mutations decreased aggregation in fission yeast without an increase in toxicity, suggesting that aggregation might not be protective. However, in addition to the increased cytoplasmic α-synuclein, yeast expressing either A76E and A76R α-synuclein still formed aggregates. Thus, even a small amount of aggregation might prove protective to cells. If this is the case, perhaps a certain threshold of aggregation is required to confer protection. Also, our budding yeast model might not express sufficient levels of α-synuclein to induce toxicity, as our expression system is only moderate. Other labs that significantly over express α-synuclein in budding yeast do see toxicity (Outiero and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005).

Emerging evidence suggests that α-synuclein's interaction with phospholipid membranes induces toxicity (Volles et al., 2001). During the aggregation process, protofibrils intermediates are generated (Caughey et al., 2003). Research suggests that these protofibrils might possibly induce cellular toxicity by generating pores in the plasma membrane (Volles and Lansbury, 2002). Additionally, α-synuclein protofibrils were shown to permeabilize membranes *in vitro* (Lashuel et al., 2000). In budding yeast, membrane association decreased with both the A76E and A76R mutations. However, the effect was more pronounced in A76E expressing cells. Regardless, neither mutation induced toxicity. If membrane associated α-synuclein decreased and aggregates were not formed, it is likely that protofibrils were produced. However, no toxicity resulted from this loss of membrane association, suggesting that protofibrils might not be the toxic species in PD.

My evidence is inconclusive as to whether aggregates or protofibrils generate toxicity in PD. A final possibility rests with the yeast organism itself. Yeasts are incredibly hardy organisms that have evolved to survive in extreme conditions over millions of years. As such, these cells might harbor an unknown mechanism that provides them protection against moderate levels of α-synuclein. Uncovering the mechanism behind this tolerance to α-synuclein toxicity could prove valuable in developing therapeutic treatments.

Table 4: Summary of Alanine-76 Predictions and Results

Mutant	Aggregation Prediction	Actual Result	Membrane Association Prediction	Actual Result
A76E	**Less** than WT	**Less** than WT	**Less** Than WT	**Less** than WT
A76R	**Less** than A76E	**Similar** to WT	**Less** than A76E	**Similar** to WT

Conclusion

My Findings

My thesis has provided insight into the molecular determinants of α-synuclein pathotoxicity using budding yeast and fission yeast as models. I successfully investigated the role of the newly discovered E46K familial mutant, serine phosphorylation, and alanine-76 in α-synuclein's pathotoxic properties: membrane association and aggregation. The E46K mutant associated with membranes in budding yeast and aggregated in fission yeast, both results that support my hypothesis. This familial mutant was also toxic to the SP3 fission yeast strain but not TCP1, a discovery that only partially supports my hypothesis. Serine phosphorylation appears to subtly regulate membrane association but significantly alter aggregation, the latter of which supports my hypothesis. Surprisingly, in contrast to my hypothesis, no toxicity was seen in either yeast model despite a multitude of phenotypes. Alanine-76 appears relevant to α-synuclein aggregation and membrane association since, in support of my hypothesis, the A76E and A76R mutants altered both properties in our yeast models. Unexpectedly, no change in toxicity was coupled with a change in phenotype. These studies provide insight into the complex nature of α-synuclein toxicity and the potential power of yeast models.

The Perplexing Nature of α-Synuclein Toxicity

An important question in the field revolves around the α-synuclein shape that causes cell death in PD. As previously discussed, α-synuclein has a natural tendency to aggregate (Spilantini et al., 1998) and associate with phospholipid membranes (Clayton and George, 1998). In yeast, toxicity is seen both when α-synuclein aggregates (Outiero and Lindquist, 2003) and when α-synuclein associates with plasma membranes (Dixon et al., 2005; Sharma et al., 2006). However, other yeast models do not see toxicity when α-synuclein associates with the plasma membrane (Zabrocki et al., 2005; Sharma et al., 2005). Additionally, α-synuclein is slightly toxic to our fission yeast model when it appears to aggregate and when it associates with intracellular organelles. The inconsistent findings regarding toxicity and the variety of phenotypes linked to toxicity suggest that some other shape altogether might be toxic.

A theory regarding α-synuclein toxicity that is gaining support in the field is an idea that an intermediate formed during the aggregation process causes toxicity in PD. α-Synuclein aggregation is a complex process that occurs in a stepwise manner. Aggregation begins when a single α-synuclein molecule misfolds (Caughey et al., 2003). This single molecule attracts other misfolded α-synuclein, forming small chains of α-synuclein that consist of 10-15 protein molecules linked together (Caughey et al., 2003; Lansbury et al., 2000). These shorter chains of α-synuclein, called protofibrils, are invisible to the eye because they are still soluble in the cell. Thousands of protofibrils begin to clump together, forming longer and longer chains until they reach the size of the classic LBs seen in PD patients.

Recently, scientists have begun to focus on these soluble protofibrils as the source of toxicity in PD as well as other neurodegenerative diseases involving protein misfolding, such as AD (amyloid-beta protein) & HD (huntingtin protein; Caughey et al., 2003). The data supporting this notion, which is mainly from test tube studies, is twofold. First, each of the α-synuclein familial mutants, which result in aggressive, early onset PD, enhance protofibril formation (Polymeropolous et al., 1997; Krueger et al., 1998; Lansbury et al., 2002; Zarranz et al., 2004). Second, even though protofibrils are soluble, atomic force microscopy has demonstrated that protofibrils form ring like

structures in synthetic membranes, suggesting they may decrease membrane stability and integrity (Ding et al., 2002). However, protofibrils have never been observed in living cells.

Although much evidence points to protofibril intermediates as the source of toxicity, the very nature of protofibrils limits our ability to identify them as they are soluble in the cellular environment. Beyond test tubes, there are no good assays that directly detect protofibrils in living cells. Atomic force microscopy is the only technique currently powerful enough to visualize protofibrils at the microscopic level. However, atomic force microscopy is only possible on purified protein, thus making identification of protofibrils in live cells problematic (van Heel et al., 2000). Cryo-electron microscopy, which is electron microscopy conducted at supercooled temperatures, can also visualize protofibrils. Although the cells would be killed prior to visualization, cryo-electron microscopy might be more suitable to detecting protofibrils in living cells since it preserves membrane characteristics (van Heel et al., 2000).

I think my data supports the protofibril hypothesis in an important way. It is convenient to say that aggregation is toxic because I see aggregation and toxicity in fission yeast compared to membrane association and no toxicity in budding yeast. However, I see a variety of α-synuclein localization patterns in fission at the same time that I see equal levels of toxicity (WT: aggregation, E46K & phosphorylation deficient mutants: endomembrane association, A76E: cytoplasmic localization). This suggests that none of the localization phenotypes are more toxic than the others. α-Synuclein aggregates in a concentration dependent manner (Caughey et al., 2003). Expression levels in our fission yeast model are significantly higher than in budding yeast. The increased α-synuclein concentration in fission yeast might facilitate the formation of protofibrils, which might account for the toxicity seen when fission yeast express α-synuclein. On a large scale, α-synuclein is localizing to a variety of areas in the cell, but on a smaller scale, invisible protofibrils are killing the cell, resulting in toxicity. I also saw enhanced toxicity in SP3 fission yeast. A future study to evaluate the A53T and A30P familial mutants in this fission yeast strain is warranted, as enhanced toxicity might provide further evidence for the protofibril hypothesis.

However, apart from protofibrils, it is possible that a general increase in α-synuclein levels causes toxicity. Both duplication and triplication of the α-synuclein gene result in PD (Singleton et al., 2003; Chartier-Harlin et al., 2004). Additionally, familial PD genes UCHL1 & Parkin that encode ubiquitin ligases that cause PD when mutant (Leroy et al., 1998; Kitada et al., 1998), suggesting that increased α-synuclein levels are toxic. I only see α-synuclein dependent toxicity in fission yeast, an organism in which we express much higher levels of α-synuclein compared to budding yeast.

Future Studies to Extend My Findings

My thesis raises several unanswered questions: 1) What organelle did the E46K mutation and phosphorylation mutants increase association with in fission yeast? 2) Does the E46K mutant kill cells or simply slow growth? 3) Does α-synuclein become phosphorylated in budding and fission yeast cells? 4) Are the α-synuclein aggregates I see in fission yeast true aggregates?

Both the E46K and phosphorylation deficient mutants localize to numerous intracellular organelles in fission yeast. Numerous chemicals can stain the nucleus of yeast cells, allowing us to determine the identity of this organelle. One well established nucleus stain that we have used before in our lab is DAPI. It is also possible that α-synuclein is associating with the ER of fission yeast. Numerous ER markers are commercially available and could provide insight as to whether or not α-synuclein is interacting with the endomembrane systems of the yeast. The FM464 stain could be used to identify vacuolar structures in the yeast that α-synuclein might associate with as well.

An α-synuclein-dependent toxicity was uncovered in fission yeast, and an E46K dependent toxicity was apparent in the SP3 yeast strain. However, whether or not these cells are dying by apoptosis or necrosis is unclear. Propidium iodide is a stain that differentiates between necrotic or apoptotic cells, as it is only permeable to cells whose membranes have been compromised. This stain could be used to differentiate necrotic and apoptotic yeast cells that express the α-synuclein mutants. Furthermore, perfecting the survival assay and repeating this experiment on TCP1 and SP3 cells expressing WT and E46K is essential to completing the toxicity puzzle, as one possible explanation for cell death is an accumulation of α-synuclein.

Whether or not α-synuclein is phosphorylated in yeast remains an open question. While the phosphorylation mutants clearly alter α-synuclein's properties, whether this is due to an altered phosphorylation state or a protein shape change brought about by the amino acid substitutions remains to be answered. Antibodies specific to phosphorylated α-synuclein are commercially available. My lab recently purchased these antibodies, and preliminary work was done using them by Keith Solvang '11. However, due to time constraints, this work was not completed in the spring. Western analysis using these antibodies is crucial to understand how phosphorylation relates to the protein's properties.

Microscopy results from this study suggest that the aggregates I see in fission yeast might not be true aggregates. Recent evidence from Virginia Lee's lab at the University of Pennsylvania demonstrated that the aggregates they see in budding yeast are actually accumulations of α-synuclein associated with vesicles. My microscopy results also suggest that these aggregates might be vesicular accumulations of α-synuclein. Vesicle markers are commercially available and could be used to determine the identity of these structures. Additionally, electron microscopy would provide an answer as to whether or not these are true aggregates.

The Power of Yeast Overshadows Limitations

While addressing my hypotheses, I simultaneously provided evidence for the power of yeast models to study a neurodegenerative disease. Numerous organisms exist to study neurodegenerative diseases, and each one has benefits and drawbacks. Cell culture models allow us to study a disease in the relevant cell type, but neuronal cultures are fragile and lab conditions alter their natural state. Fruit flies and *C. elegans* provide pliable genetic systems, but they are unsuitable for large scale screens. Mice allow us to study a disease in a whole vertebrate organism, but experiments take months to complete and they are not as powerful genetically as other models.

My yeast model does face several limitations. Firstly, yeast do not fully model the properties of a neuron. Neurons are highly specialized cells designed to conduct electrical signals over long distances to other neurons (Purves et al., 2008). In contrast, yeast are a unicellular fungus that does not send electrical signals. Additionally, the substantia nigra cells that first degenerate in PD are unique in that they synthesize dopamine (Purves et al., 2008). Previous research demonstrates that α-synuclein forms a covalent adduct with dopamine, and this interaction might have potential implications in neuronal degeneration (Conway et al., 2001). Yeast cells do not synthesize

dopamine, and therefore this potential property of α-synuclein is not recapitulated in yeast models.

However, yeast function as a powerful discovery platform. Due to their tractable genetics, it is possible to conduct large scale screens for modifiers of α-synuclein toxicity. Willingham et al. (2003) conducted a screen of over 4000 genes and identified 86 genes that modified the toxicity of α-synuclein. These genes included oxidative stress enzymes, lipid synthesis proteins, and endocytosis proteins, amongst others. Each of these areas is a heavily researched field in PD. Gitler et al. (2009) also used yeast to screen for modifiers of α-synuclein toxicity and began examining these genes in more complex animals, such as *Drosophila* and *C. Elegans*. This study is a perfect example of a discovery made in yeast that was being scaled up for research in more complex organisms in which such a screen would not have been possible. As such, my findings could potentially be examined in a more complex organism to gain further insight. One such opportunity yet to be seized by another lab is the study of E46K in mice.

Yeast continues to emerge as a powerful model for disease as evidenced by the growing number of diseases modeled by this single-celled fungi. They are cheap, grow rapidly, have well established genetics, and much of the protein folding machinery is conserved between yeast and humans. As such, yeast models for neurodegenerative diseases should not be underestimated.

The Importance of a Cure

PD is rapidly becoming an increasing public health concern as the human race lives longer. Without effective treatments, the number of PD sufferers is expected to double by 2030 (Dorsey et al., 2007). Although my thesis provided significant insight into three specific areas of PD research, numerous questions remain in the field. Understanding how familial PD mutants cause disease could provide enhanced treatment for suffers of genetic PD. Additionally, understanding how chemical modification relates to toxicity might allow development of drugs that modulate modification to treat PD. On the same idea, the structural components that contribute to α-synuclein's properties might also serve as potential drug targets. It is clear that α-synuclein is involved in PD onset, and understanding how this mysterious protein contributes to disease is essential to developing effective therapies for this debilitating neurodegenerative disease.

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