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## **Cover Page Footnote**

I would like to thank Dr. Shubhik DebBurman for the tremendous opportunity he has provided me in allowing me to conduct research in his laboratory, as well as his guidance and support throughout my research. I would also like to thank Michael White '07 and Michael Zorniak'07 for their aiding me throughout my research and for their advice. I would also like to thank Michael White'07, Michael Zorniak'07 and Sara Herrera'05, as their senior theses served as a basis for my senior thesis. Thank you to my lab peers, Alexandra Ayala'09, Michael Fiske'10, Lokesh Kukreja'08 for their support and company throughout my thesis project. Special thanks to my parents and family for their continued support and encouragement throughout my research. I would like to express gratitude to my senior thesis committee, Dr. Pliny Smith, Dr. Matthew Kelley and Dr. William Frost, as well as the National Institutes of Health for funding our laboratory.

# Insight into Familial and Sporadic Parkinson's Disease: $\alpha$ -Synuclein Mutant Analysis in a Fission Yeast Model

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## Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting six million people worldwide. It results from the specific loss of substantia nigra dopaminergic neurons, which accumulate large filamentous structures called Lewy bodies composed mostly of one misfolded and aggregated protein called  $\alpha$ -synuclein. The aggregation and membrane phospholipid binding ability of  $\alpha$ -synuclein are both linked to cellular toxicity in familial and sporadic forms of PD, but their relative contributions are not resolved. This thesis utilized a *Schizosaccharomyces pombe* (fission yeast) model to get further insight into the nature of  $\alpha$ -synuclein toxicity in these two forms of PD and the data was comparatively evaluated with previous studies done in budding yeast. Three studies were conducted. First, for insight into familial PD, we tested the hypothesis that the newest mutant E46K  $\alpha$ -synuclein is toxic in fission yeast, but instead found it to be surprisingly slightly toxic. This lack of major toxicity correlated with extensive  $\alpha$ -synuclein aggregation and the lack of plasma membrane localization. In contrast, in budding yeast, E46K is known to be toxic, membrane localized, and not aggregated. Next, for insight into sporadic Parkinson's disease, we first tested the hypothesis that alanine-76 in  $\alpha$ -synuclein as a major contributor to  $\alpha$ -synuclein's aggregation and found that an A76E mutant was indeed less aggregated in fission yeast. This finding supports past budding yeast work where A76E  $\alpha$ -synuclein is less plasma membrane localized. Lastly, we tested the hypothesis that post-translational modifications in  $\alpha$ -synuclein contribute to its aggregation and to its known higher migration when run on protein gels. Surprisingly, phosphorylation-deficient (S87A and S129A) and nitrosylation-deficient mutants (Y39F and Y125F) of  $\alpha$ -synuclein remained aggregated and their migration size on gels was unchanged. In contrast, these mutants are significantly toxic to budding yeast and maintain membrane localization. We suggest that fission yeast is more resistant than budding yeast to  $\alpha$ -synuclein toxicity possibly because membrane localization contributes toxicity, while aggregation protects. It is also possible that fission yeast more efficiently suppresses  $\alpha$ -synuclein toxicity by thus far unknown mechanisms. Importantly, the mutants studied in both models demonstrate that  $\alpha$ -synuclein intrinsically regulates its ability to aggregate and bind membrane phospholipids, providing new insight into the toxicity seen in both sporadic and familial PD.

## Introduction

### Neurodegenerative Diseases

Neurodegenerative diseases affect millions worldwide. These late onset progressive diseases, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease vary widely in symptoms. Despite symptom diversity, all neurodegenerative disease patient brains' contain characteristic proteinaceous deposits. The location of the deposits, the morphology of the deposits, and the specific neuronal cell type affected, however, differ by disease (Caughey and Lansbury, 2003). The specific mechanisms of protein aggregation and their link to neuronal cell death in each disease are not yet conclusive. Our interest lies in the molecular basis of Parkinson's disease (PD).

### Parkinson's disease

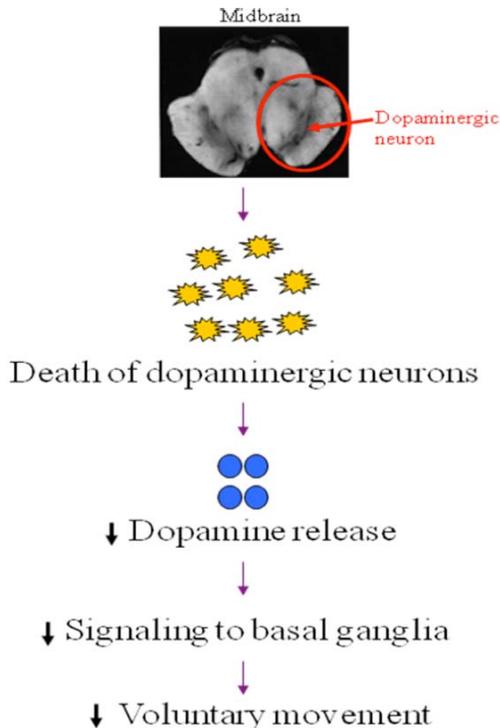
Parkinson's disease, first described by James Parkinson, is the second most common neurodegenerative disorder, affecting millions worldwide (Jain *et al.*, 2005). This common and fatal neurodegenerative disease, characterized by postural instability, resting and bradykinesia, affects 1 in 50 individuals over the age of 60 (Moghal *et al.*, 1994). Sporadic and familial are the two forms of Parkinson's disease. The sporadic form of PD is the most common, making up 90-95% of PD cases. The remaining 5-10% of PD cases are familial and caused by a mutation in one of six genes (Dauer *et al.*, 2003). Both forms of PD are linked to the death of midbrain dopaminergic substantia nigra neurons, which accumulate a misfolded and aggregated protein  $\alpha$ -synuclein (Spillantini *et al.*, 1997). As this specific cell death occurs, the release of the neurotransmitter dopamine decreases. The diminished amount of dopamine renders patients with a decreased ability to control and initiate movement, resulting in the clinical manifestations of PD (Figure 1; Dauer and Przedborski *et al.*, 2003). *My thesis will investigate the basis of cellular toxicity observed in both familial and sporadic PD.*

### Basis of Familial PD

Major breakthroughs occurred when the genes underlying familial PD were identified, as for first time a definitive cause of PD was revealed (Jain *et al.*, 2005). Two autosomal-dominant genes, ( $\alpha$ -synuclein and LRRK2) and three autosomal-recessive genes (*parkin*, DJ-1, and PINK1) have been associated with familial Parkinson's disease (Jain *et al.*, 2005).

Mutations in the *parkin* gene are associated with autosomal recessive, early-onset Parkinson's disease (Kitada *et al.* 1998). This gene is a ubiquitin protein ligase (Shimura *et al.* 2000). The gene DJ-1, for which there are few known mutations, is the first gene discovery which did not lead to a direct implication of an abnormality of the proteasome-lysosome system. While the exact function of the DJ-1 protein is unknown, it may serve a role in protecting neurons against oxidative stress and mitochondrial damage (Bonifati *et al.*, 2003). The UCHL-1 gene may have a biological role in PD due to its ubiquitin ligase activity and may possibly act in the same pathway as *parkin* (Liu *et al.*, 2002). PINK I, the third autosomal-recessive PD gene discovered, functions as a serine/threonine kinase of the Ca<sup>2+</sup>/calmodulin family and contains a mitochondrial targeting motif in the N-terminus. PINK I is thought to protect cells against apoptosis by maintaining mitochondrial membrane potential during exposure to proteasome inhibitors (Valente, E.M. *et al.* 2004). The last two of the aforementioned genes, LRRK2 and UCHL-1, both lead to the

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



**Figure 1. Pathology of Parkinson's disease:** Both forms of Parkinson's disease are characterized by very specific cell death. Upon autopsy, a severe loss of substantia nigra dopaminergic neurons in PD patients is observed. This neuronal loss results in a diminished release of the neurotransmitter dopamine, to the putamen and caudate nucleus, which in turn regulates the parts of the brain that control movement. The clinical manifestations of PD are a result of this decrease in dopamine.

autosomal-dependent form of PD. LRRK2 is part of a newly identified protein family referred to as ROCO. While the exact function of this protein is unknown, all other ROCO family proteins are involved in cytoskeletal rearrangement and may be implicated in apoptosis (Cohen *et al.*, 1997; Aysahl *et al.*, 2003).

My study focuses on the first gene discovered to cause familial PD,  $\alpha$ -synuclein, because not only is it linked to both familial and sporadic PD, but all familial mutant genes described above eventually lead to  $\alpha$ -synuclein misfolding and aggregation. As described below, three point mutations in this gene result in single amino acid substitutions in the  $\alpha$ -synuclein protein: A30P, A53T, and E46K (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997; Zarranz *et al.*, 2004, respectively). The examination of both wild-type and mutant versions of  $\alpha$ -synuclein is critical to the understanding of PD pathogenesis.

#### Wild-type $\alpha$ -synuclein is an aggregation prone protein

This natively unfolded protein of 140 amino acids has three major regions (Kaplan *et al.*, 2003). The first major region of this protein is an N-terminal amphipathic region consisting of amino acids 1-61. The central region consists of amino acids 61-95 (Weinreb *et al.*, 1996, Uversky *et al.*, 2002). Deletion of the central region prevents  $\alpha$ -synuclein aggregation *in vivo* and *in vitro*, suggesting that this region is essential for aggregation of the protein (Giasson *et al.*, 2000). Finally, the third region, a highly acidic C-terminal region consists of amino acids 95-140 (Uversky *et al.*, 2002). This C-terminal region may have an inhibitory role in the aggregation of  $\alpha$ -synuclein, as C-terminally truncated forms of  $\alpha$ -synuclein are

found to aggregate into filaments more readily than full-length wild-type  $\alpha$ -synuclein (Baba *et al.*, 1998). About 15% of Lewy bodies contain C-terminally truncated  $\alpha$ -synuclein (Baba *et al.*, 1998). This protein of unknown function is located throughout the central nervous system and is abundant in neurons, especially in pre-synaptic terminals (Jakes, R. *et al.*, 1994)  $\alpha$ -synuclein may be involved in maintenance of vesicular pools (Jenson *et al.*, 1998; Murphey *et al.*, 2000), synaptic plasticity (George *et al.*, 1995, Abeliovich *et al.*, 2000), phospholipid binding and lipid metabolism (Davidson *et al.*, 1998; Eliezer *et al.*, 2001; Sharon *et al.*, 2001; Outiero and Lindquist, 2003), microtubule binding (Alim *et al.*, 2004), and chaperone-like activity (Jenco *et al.*, 1998, Engelender *et al.*, 1999; Osterova *et al.*, 1999). The aggregation and misfolding of this protein contributes to the formation of Lewy bodies, which are hallmarks of the disease.

Many characteristics of A30P and A53T are now known and well-studied (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998). *In vitro*, self-aggregation occurs in wild-type, A30 and A53T (Bisaglia *et al.*, 2004; Bussell *et al.* 2005; Davidson *et al.*, 1997; Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998). Interestingly, the familial mutants vary in their abilities to bind lipids. Monomeric  $\alpha$ -synuclein binds to negatively charged phospholipid membranes and micelles *in vitro*, while A30P interferes with the  $\alpha$ -helical formation of the protein, rendering it unable to bind membranes (Bisaglia *et al.*, 2004; Bussell *et al.* 2005; Davidson *et al.*, 1997). While both mutants undergo nucleated polymerization, A53T has a greater propensity to form large polymers, while A30P has a lesser propensity to form large polymers compared to wild-type (Bussell *et al.* 2004; Jo *et al.*, 2000). Moreover, the A53T mutation results in the accelerated formation of filaments compare to both wild-type and an A30P  $\alpha$ -synuclein.

#### Newly Discovered Mutant E46K

In 2004, Zarranz *et al.* discovered a novel  $\alpha$ -synuclein mutation, E46K, which is linked to familial PD. Upon autopsy of the Spanish patients in which the mutation was found, severe atrophy of the substantia nigra was observed. Severe neuronal loss resulted, as well as the presence of numerous Lewy bodies that were immunoreactive to  $\alpha$ -synuclein. Several *in vitro* studies have examined the effects of this mutation on phospholipid binding and aggregative properties of the protein. Interestingly some evidence suggested that E46K increased the rate of fibrillization compared to A53T, while similar studies proposed that the rate of fibrillization for both mutants was similar (Greenbaum *et al.*, 2005; Pandey *et al.*, 2005; Choi *et al.*, 2004). Interestingly, two types of aggregates have been found for E46K; These aggregates were larger than those found as a result of other mutations, such as A30P and A53T (Pandey *et al.*, 2005). Furthermore, E46K fibrillar aggregates have a smaller diameter than that of the WT protein (Raaij *et al.*, 2006). *Little is known about how the previously described in vitro evidence may translate in living cells, tissues and organisms and what factors contribute to E46K dependent toxicity.*

#### Basis of Sporadic PD

While Sporadic PD makes up for 90-95% of all PD, the exact causes are still unknown. Several hypotheses include oxidative stress, pesticides, proteasomal dysfunction, and mitochondrial dysfunction (Figure 2; Dauer and Przedborski, 2003). In PD, Lewy body's contain oxidatively modified  $\alpha$ -synuclein, which results in an enhanced ability for  $\alpha$ -synuclein to misfold and aggregate (Gaijsson *et al.*, 2000). The relationship between  $\alpha$ -synuclein misfolding and oxidative stress is still unclear, however, oxidative stress may contribute to sporadic PD, as the presence of oxidative stress results in certain proteins forming a complex with

aggregated  $\alpha$ -synuclein (Zhou *et al.*, 2004). Herbicides and pesticide, such as rotenone, maneb, and paraquat may also play a role in sporadic PD as well. These agricultural agents have been shown to reproduce specific features of PD when administered to mice (Uversky *et al.*, 2004). Interestingly, occupational exposure to organic solvents may also correlate with induction of fibrillation prone, partially folded  $\alpha$ -synuclein (Munishkina *et al.*, 2003). Moreover, mitochondrial dysfunction may contribute to sporadic PD as inhibition of mitochondrial complex I by the environmental toxins paraquat, rotenone, and MPTP, result in cell damage (Abou-Sleiman *et al.*, 2006) and an increased death of dopaminergic neurons (Mizuno *et al.*, 1989; Kweon *et al.*, 2004). Noteworthy is the invariable presence of  $\alpha$ -synuclein in Lewy bodies.

#### Is Alanine-76 Relevant?

Several *in vitro* studies have examined the contribution of alanine-76 to the aggregation of  $\alpha$ -synuclein. The deletion of a central stretch of  $\alpha$ -synuclein containing amino acids 61-95 resulted in the prevention of  $\alpha$ -synuclein aggregation in these *in vitro* systems, suggesting that this region is crucial for the protein's aggregation (Bodles *et al.*, 2001). Interestingly, a hydrophobic, 12 amino acid stretch of  $\alpha$ -synuclein, containing amino acids 70 to 82, was necessary and sufficient for filament assembly. The necessity of this specific region was determined through the deletion of the stretch, which results in the reduction of aggregation (Giasson *et al.*, 2001). Notably, the insertion of a single charge in this stretch, resulting in the mutation A76E, leads to the decrease in aggregation of  $\alpha$ -synuclein (Gaijsson *et al.*, 2001), suggesting that this alanine-76 is itself crucial to the aggregation of the protein. A mathematical model examining the effects of the substitution of a hydrophobic amino acid alanine to the hydrophilic amino acid glutamic acids also suggested the possibility of reduced aggregation (Chiti *et al.*, 2000). *Whether A76E results in the inhibition of aggregation, changes toxicity or alters  $\alpha$ -synuclein's membrane*

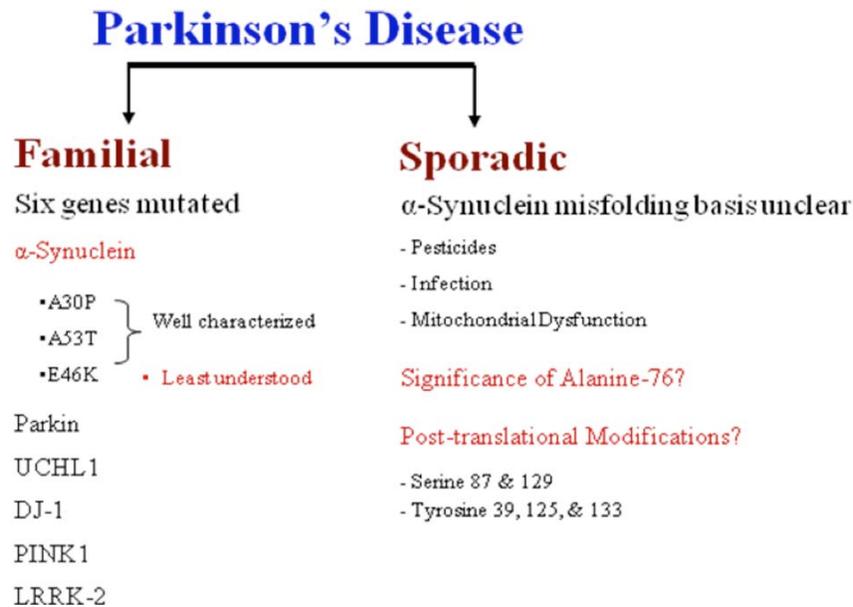
*phospholipid affinity in living cells and changes toxicity yet to be determined.*

#### Post-translational Modifications

While the molecular mechanism of  $\alpha$ -synuclein aggregation remains unknown, post-translational modifications may contribute to  $\alpha$ -synuclein misfolding and aggregation. Several post-translational modifications can occur on  $\alpha$ -synuclein, such as ubiquitination (Shimura *et al.*, 2001), glycosylation (Shimura *et al.*, 2001), nitrosylation (Takahashi *et al.*, 2002) and phosphorylation (Okoshi *et al.*, 2000; Fujiwara *et al.*, 2002). For this thesis, only two of these, phosphorylation and nitrosylation, are the subject of my investigation.

#### The Link between $\alpha$ -synuclein Phosphorylation and PD

As previously mentioned, Lewy bodies are the hallmarks of PD. Interestingly, in Lewy Bodies,  $\alpha$ -synuclein is selectively and extensively phosphorylated at serine-129 (Fujiwara *et al.*, 2002). *In vitro* evidence suggests that additional phosphorylation of  $\alpha$ -synuclein occurs at serine-87, however, phosphorylation at this site occurs less efficiently than that of serine-129 (Okochi *et al.* 2000). Evidence is lacking as to the kinase responsible for the phosphorylation of serine-87, however, several kinases are implicated in phosphorylation of  $\alpha$ -synuclein at serine-129. *In vivo* evidence suggests that Gprk2 (Chen and Feany, 2005) and casein kinase 1 and 2 (Okochi *et al.*, 2000) phosphorylate serine-129. There is conflicting evidence as to the relationship between phosphorylation, aggregation, and toxicity. One *in vivo* study suggests that phosphorylation of serine-129 enhances  $\alpha$ -synuclein toxicity and blocking of this phosphorylation site increases aggregate formation (Chen and Feany, 2005). Interestingly, a rat model suggests that the blocking of phosphorylation of serine-129 not only leads to a decrease in aggregate formation, but also an increase in intracellular toxicity and an exacerbation of  $\alpha$ -synuclein induced nigral pathology (Gorbatyuk *et al.*, 2008)



**Figure 2. Forms of Parkinson's Disease:** There are two forms of PD, sporadic and familial. Six genes are mutated in cases of PD. We focus on  $\alpha$ -synuclein, to which three missense mutations can occur. Two of these mutations, A30P and A53T, are well characterized. Less is known about the novel mutation, E46K. There are several hypotheses as to the causes of sporadic PD, including pesticides, infections, and mitochondrial dysfunction. Two factors may contribute to sporadic PD, including Alanine-76 and post-translational mutations.

### *The Link Between $\alpha$ -Synuclein Nitrosylation and PD Pathogenesis*

A second post-translational modification that can occur to  $\alpha$ -synuclein is nitrosylation. Nitrosylation is of interest as  $\alpha$ -synuclein inclusions in PD brains are strongly labeled for antibodies that specifically detect nitrated synuclein in autopsied PD brains (Trojanowski *et al.*, 1998; Duda *et al.*, 2000).  $\alpha$ -Synuclein is nitrated at several residues, including tyrosine-39, as well as tyrosine-125, 133, and 136 (Giasson *et al.*, 2000) by compounds such as peroxynitrate (Duda *et al.*, 2000). As a result of nitrosylation, several modifications can occur, including the change in conformation of the protein, which leads to increased protein aggregation. Most importantly, the nitration of  $\alpha$ -synuclein results in enhanced formation of SDS-insoluble, heat-stable high molecular mass aggregates *in vitro* (Souza *et al.*, 2000), the diminished binding of  $\alpha$ -synuclein to lipid vesicles, and the increase in fibril formation (Hodara *et al.*, 2004). *Whether post-translational mutations alter rate of  $\alpha$ -synuclein aggregation in living cells, and what consequences that has to cellular toxicity, is still not resolved.*

### *Yeast Models of PD*

Several models have been used in order to study of PD and more specifically,  $\alpha$ -synuclein. One of these models has been the mouse model (Caughey and Lansbury, 2003; Dauer and Przedborski, 2003). Transgenic mouse models reproduce many features of the disease. Worms (Lasko *et al.*, 2003), flies (Feany and Bender, 2000) and yeast (Outiero and Lindquist, 2003) are also effective models for PD.

*Budding Yeast Model: Saccharomyces cerevisiae* serves as an effective model for the examination of neurodegenerative diseases, including PD, prion diseases (Ma and Lindquist, 1999), Huntington's disease (Krobitsch and Lindquist, 2000; Muchowski *et al.* 2002), and amyotrophic lateral sclerosis (Kunst *et al.* 1997). Importantly, budding yeast shares with humans a high conservation of protein quality control pathways and of protein folding. Several investigations have concentrated on the study of familial mutants in yeast. In fact, four budding yeast models for studying  $\alpha$ -synuclein properties found that wild-type and A53T mutant  $\alpha$ -synuclein are plasma membrane associated proteins that can aggregate within cells, whereas the A30P mutant neither localizes to the plasma membrane nor aggregates (Outiero and Lindquist, 2003; Zabrocki *et al.*, 2005; Dixon *et al.*, 2005). However, the support for  $\alpha$ -synuclein toxicity to budding yeast is varied: two models demonstrate  $\alpha$ -synuclein aggregation itself is toxic to yeast (Outiero and Lindquist, 2003; Dixon *et al.*, 2005), while two others suggest that  $\alpha$ -synuclein-dependent toxicity requires an additional chemical or genetic insult, such as proteasomal inhibition and oxidative stress (Zabrocki *et al.*, 2005). Our lab has also developed a budding yeast model for the examination of  $\alpha$ -synuclein misfolding and aggregation. In our budding yeast model, wild-type and A53T display plasma membrane localization, supporting the protein's known *in vitro* binding abilities (Sharma *et al.*, 2006). In contrast, A30P localizes primarily throughout the cytoplasm, whereas the A30P/A53T mutant displays both phenotypes. In this model, toxicity is enhanced in cells exposed to an increased amount of oxidative stress or in cells with proteasomal impairment, supporting several *in vitro* studies (Sharma *et al.*, 2006).

Using the budding yeast model, our lab has recently shown that E46K confers toxicity to budding yeast (Sara Herrera Thesis, 2005; Michael White Thesis, 2007). These studies demonstrate that E46K toxicity is ploidy-specific and strain-specific. Furthermore, E46K  $\alpha$ -synuclein expressing cells have extensive plasma membrane localization and show reduced protein expression when compared to wild-type  $\alpha$ -synuclein. Like E46K, A76E and

four post-translational modification mutants (S129A, S87A, Y39A, Y125F) have been examined and result in the toxicity of budding yeast cells (Michael Zorniak Thesis, 2007; Sara Herrera Thesis, 2005). Interestingly, plasma membrane localization decreases for A76E suggesting that alanine-76 facilitates membrane phospholipids association (Michael Zorniak Thesis, 2007). All four post-translational modification mutants, however, maintain primarily membrane localization. Thus, our budding yeast model points to membrane localization as a link to  $\alpha$ -synuclein toxicity.

*Fission Yeast Model:* Our lab has also developed and published a fission yeast model for PD that supports the nucleation polymerization theory for  $\alpha$ -synuclein degradation (Brandis *et al.*, 2006). *Schizosaccharomyces pombe* (fission yeast), like budding yeast, also serves as a powerful model organism in cell biology, as it provides insight into eukaryotic cell cycle (Fantes and Beggs, 2000), DNA repair and recombination (Davis and Smith, 2001), and checkpoint controls needed for stability (Humphrey, 2000). Like budding yeast, fission yeast shares with humans a high conservation of protein folding and protein quality control pathways (Wood *et al.*, 2002). In fission yeast,  $\alpha$ -synuclein-A30P and combination mutant A30P/A53T are cytoplasmically diffuse, while wild-type  $\alpha$ -synuclein and A53T form prominent inclusions. This inclusion formation occurs in a time and concentration-dependent manner (Brandis *et al.*, 2006). A53T forms aggregates at a faster rate than WT- $\alpha$ -synuclein. Most interesting, however, are the lack of  $\alpha$ -synuclein plasma membrane localization and the lack of  $\alpha$ -synuclein-dependent toxicity.

Both fission and budding yeast serve as effective models for examining  $\alpha$ -synuclein properties. First, yeast is inexpensive. Secondly, they grow quickly, which allows for faster experimentation than other models. Importantly, their entire genome has been sequenced, which allows us to make genetic mutations and examine the molecular basis of the disease. Lastly, yeast make, fold and degrade proteins similarly to humans. Since this disease is one involving protein misfolding, this property is important.

While a majority of work in our lab has previously focused on the characterization of  $\alpha$ -synuclein in a budding yeast model, the goal of my thesis was to further examine how E46K, A76E, and the four post-translational modification mutants (S129A, S87A, Y39F, Y125F) affect the intrinsic aggregation and phospholipid binding properties of  $\alpha$ -synuclein in a fission yeast model and compare my data with those obtained from budding yeast.

### *Hypotheses and Aims*

I conducted three related studies to test the following hypotheses. For each hypothesis, I evaluated  $\alpha$ -synuclein mutant expression through Western blot analysis, toxicity through OD600 growth curves and spotting assays, and localization/aggregation through live cell GFP microscopy in fission yeast.

Hypothesis I: To gain insight into familial PD, I tested the hypothesis that *E46K mutant  $\alpha$ -synuclein is toxic to live fission yeast cells and displays both plasma membrane binding and aggregation.* I predicted that *E46K would aggregate more than wild-type  $\alpha$ -synuclein.* My findings are detailed in Chapter I.

Hypothesis II: To gain insight into sporadic PD, I hypothesized that *alanine-76 contributes to  $\alpha$ -synuclein aggregation.* I predicted that *the A76E mutation would decrease aggregate formation due to its decreased hydrophobicity.* My findings are detailed in Chapter II. \*Note: The following three figures (Figures 3-5) summarize our lab's budding yeast findings and illustrate the properties that are yet to be examined in fission yeast.

A-synuclein Properties in Fission Yeast

	WT	A30P	A53T	E46K
Diffuse	-	+	-	?
Aggregate	+	-	+	?
Lipid Binding	-	-	-	?
Toxicity	-	-	-	?

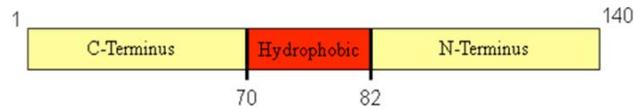
B. E46K Properties in Budding Yeast

E46K	Budding Yeast
Diffuse	-
Aggregate	-
Lipid Binding	+
Toxicity	+

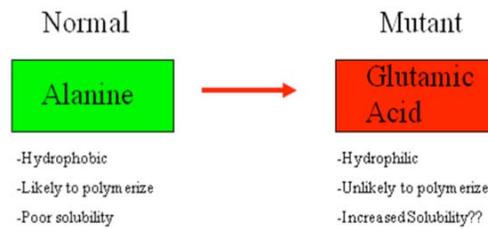
**Figure 3. Known E46K Properties in Yeast Models.**

A. Characterization of several  $\alpha$ -synuclein mutants in a fission yeast model showed that A30P displayed diffuse localization, whereas aggregation of  $\alpha$ -synuclein was observed in A53T, similarly to WT  $\alpha$ -synuclein. E46K properties in fission yeast remain to be examined.  
 B. In budding yeast, E46K mutant  $\alpha$ -synuclein confers toxicity, display membrane localization.

A.  $\alpha$ -Synuclein Domains



B. Alanine-76



C. A76E Properties in Fission Yeast

A76E	Budding Yeast
Toxicity	Slight
Aggregation	None
Membrane Localization	Less

**Figure 4. Mode of Alanine-76 Action.**

A.  $\alpha$ -Synuclein is a protein of 140 amino acids with three major regions. The central hydrophobic region of the protein is thought to be critical to the aggregation of the protein.  
 B. Alanine-76 may contribute to the aggregation of  $\alpha$ -synuclein, as it is a hydrophobic amino acid, which is likely to polymerize. The A76E mutation may increase the solubility of the protein due to the hydrophilic nature of glutamic acid.  
 C. In budding yeast, A76E confers slight toxicity, results in a lack of aggregation and reduction in membrane localization.

### A. Phosphorylation and Nitrosylation on $\alpha$ -Synuclein

Site	Relevance to PD	Citation
S87	S87 is phosphorylated <i>in vitro</i> . Evidence on effect of ser-87 phosphorylation on fibril formation is lacking.	Okochi <i>et al.</i> , 2000
S129	Phosphorylation of ser-129 increases fibril formation <i>in vitro</i> .	Fujiwara <i>et al.</i> , 2002
Y39	Nitration of Y39 results in a diminished binding to lipid vesicles and in the increase of fibril formation	Hodara <i>et al.</i> , 2004
Y125	Lack of nitration at this site results in the reduction of dimerization of $\alpha$ -synuclein	Takahashi <i>et al.</i> , 2002

### B. Post-Translational Effects on $\alpha$ -Synuclein in Budding Yeast

Mutation	Toxicity	Lipid Binding	Aggregation
S87A	+	+	-
S129A	+	+	-
Y39F	+	+	-
Y125F	+	+	-

**Figure 5. Effects of Post-translational Modification Mutants on  $\alpha$ -Synuclein.**

A. Several studies have examined the phosphorylation and nitrosylation of  $\alpha$ -synuclein. The effect of phosphorylation have been examined *in vitro*. *In vivo* examination of the post-translational modifications' is lacking and the effects on these mutations on toxicity and aggregation of  $\alpha$ -synuclein are yet to be determined.

B. Post-translational mutations were examined in a budding yeast model. All four modification mutants showed toxicity as well as localization to the plasma membrane. Post-translationally modified  $\alpha$ -synuclein displayed lack of aggregation.

### Methods

**Study Site:** DebBurman Laboratory, Lake Forest College, Lake Forest, Illinois.

**Vectors:** All forms of  $\alpha$ -synuclein and GFP were expressed using the *pNMT1-TOPO* DNA vector (Invitrogen).

**$\alpha$ -Synuclein:** C-terminally GFP tagged human wild-type, A30P, A53T, E46K, A76E, A30P/E46K, A53T/E46K, A30P/A53T/E46K, S89A, S129A, Y39F, and Y125F were used.

**Expression of  $\alpha$ -synuclein:** Fission yeast cells were grown in PDM-leucine media. This media allows for the growth of the fission yeast without allowing for the expression of  $\alpha$ -synuclein and GFP. A thiamine repressible within our vector was used in order to manipulate expression of the proteins  $\alpha$ -synuclein and GFP. Cells are first grown overnight. These are then centrifuged at 2500 rpm at 4°C. They are subsequently washed with water twice, after which they are inoculated in EMM-thiamine liquid media. This media allows for the growth of fission yeast cells as well as the expression  $\alpha$ -synuclein and GFP.

**Site-Directed Mutagenesis:** The A30P/E46K, A53T/E46K and A30P/A53T.E46K combination mutants were made using Gene Taylor Site-Directed Mutagenesis Systems and Taq High Fidelity polymerase (Invitrogen; Figure 3). C-terminally GFP-tagged A30P, A53T, and A30P/A53T mutant  $\alpha$ -synuclein constructs were used for this site-directed mutagenesis. The DNA mutagenesis product was transformed into One Shot Max Efficiency Dh5 $\alpha$ -T1 Competent Cells. Yeast colonies were then mini-prepped (Qiagen Mini-prep Kit) in order to isolate the mutated plasmid. The mutation of  $\alpha$ -synuclein was confirmed by

sending DNA to the University of Chicago Cancer Research Center DNA Sequencing Facility.

**Transformation of Yeast Strains:** WT-GFP and A76E TCP1 fission yeast cells were previously transformed by Michael Zorniak 07'. E46K fission yeast and all post-translation mutants were transformed by Isaac Holmes (Table 1).

**Bacterial Whole Cell Polymerase Chain Reaction (PCR):** Amplification of bacterial DNA was achieved using Bacterial Whole Cell PCR. Each reaction is set up in a 0.2 ml microcentrifuge tube. The following was added to each reaction: 7  $\mu$ L H<sub>2</sub>O, 2.5  $\mu$ l Forward primer, 2.5  $\mu$ l reverse primer, 12.5  $\mu$ l Master mix (MgCl<sub>2</sub>, Buffer, Taq DNA polymerase, dNTP) and 0.5  $\mu$ l of bacterial cells (Qiagen mini-prep product). A similar reaction is set up as a positive control with control plasmid. The PCR program was as follows: 95°C 30 sec., 55°C 30 sec., 72°C 30 sec. (this cycle is repeated 29 times), 72 °C 30 min., 4°C indefinitely.

**Western Analysis:** Cells were grown in PDM-leucine at 30°C over night. These cells were centrifuged for 5 minutes at 4°C and subsequently washed twice with water. The washed pellet was then inoculated in EMM-thiamine media, which allows for the expression of  $\alpha$ -synuclein. Cells were counted and a cell density of 2.5X 10<sup>7</sup> was isolated 24 hours post-induction. The isolation process required the centrifugation of the cells in a countertop centrifuge at (34,000) rpm. Cells were lysed using electrophoresis solubilizing buffer (ESB) and small glass beads. 10  $\mu$ L of each lysate and See Blue mass ladder (Invitrogen) were loaded into Tris-glycine gels (Invitrogen). Chambers of the gel box were filled with 1X running buffer (29.0 g Tris-base, 144.0 g Glycine, 500 mL, SDS, 1L H<sub>2</sub>O at pH 8.7). The lysates were electrophoresed at ~130 V. Protein transfer to PVDF membranes was performed using Semi-Dry Transfer Apparatus (Biorad), methanol, water, and 1X transfer buffer (18.3g Tris-base,

Construct	Expression Vector	Strain
WT $\alpha$ -synuclein-GFP	pNMT1	TCP1
Parent Plasmid $\alpha$ -synuclein-GFP	pNMT1	TCP1
A30P $\alpha$ -synuclein-GFP	pNMT1	TCP1
A53T $\alpha$ -synuclein-GFP	pNMT1	TCP1
E46K $\alpha$ -synuclein-GFP	pNMT1	TCP1
A30P/E46K $\alpha$ -synuclein-GFP	pNMT1	TCP1
A53T/E46K $\alpha$ -synuclein-GFP	pNMT1	TCP1
A30P/A53T/E46K $\alpha$ -synuclein-GFP	pNMT1	TCP1
A76E $\alpha$ -synuclein-GFP	pNMT1	TCP1
S89A $\alpha$ -synuclein-GFP	pNMT1	TCP1
S129A $\alpha$ -synuclein-GFP	pNMT1	TCP1
Y39F $\alpha$ -synuclein-GFP	pNMT1	TCP1
Y125F $\alpha$ -synuclein-GFP	pNMT1	TCP1
Parent plasmid- $\alpha$ -synuclein-GFP	pNMT1	Sp
WT $\alpha$ -synuclein-GFP	pNMT1	Sp
E46K $\alpha$ -synuclein-GFP	pNMT1	Sp

**Table 1. Transformed *Schizosaccharomyces pombe* Strains:** All  $\alpha$ -synuclein constructs were previously transformed into *S. pombe*. Newly synthesized A30P/E46K, A53T/E46K, and A30P/A53T/E46K combination mutants were transformed into *S. pombe*. The DNA construct is presented in the left column, the expression vector is presented in the center column and the DNA construct is presented in the right column.

90.0 g Glycine, 500 mL H<sub>2</sub>O, at pH 8.3). Blocking was extended to three hours and blotting was performed using Anti-V5/Alkaline phosphatase,  $\beta$ -actin mouse monoclonal antibody and Anti-mouse secondary antibody (Invitrogen). All washes with antibody wash performed during the blotting process were extended from 5 minutes to 10 minutes. Membrane allowed to air dry and then photographed

**Growth Curve Analysis:** Cell density was assessed via an optical density assay at a 600nm wavelength. Cells were grown overnight in 10 mL of PDM-leucine media at 30°C. The 10 ML cell culture is transferred to a centrifuge tube and centrifuged at 1500 xg for 5 minutes at 4°C and subsequently washed with water. Cells were re-suspended in 10 mL of DI H<sub>2</sub>O and counted using a hemocytometer to determine cell density. A cell culture of 2.0x 10<sup>7</sup> was inoculated in 35 mL of EMM-leucine and EMM+leucine media and grown in a shaken incubator at 30°C for 48 hours. Two 1 mL cultures were removed from each flask at 0, 3, 6, 18, 24, 36, and 48 hours and placed in two cuvettes for duplicate readings. Readings were made using the Hitachi U-2000 Spectrometer at a 600nm wavelength.

**Cell Spotting/ Growth Rate Analysis:** Cells were grown in 10 mL of PDM-Leucine liquid media overnight at 30°C. Each 10 ml sample was centrifuged at 1500xg and subsequently washed with 5 mL of sterile H<sub>2</sub>O. The resulting pellet was re-suspended in 10 mL of DI H<sub>2</sub>O and counted. A 2.7x10<sup>7</sup> cell/mL cell culture was removed from the 10 mL culture, centrifuged and water was removed. The resulting pellet was re-suspended in 1 mL of H<sub>2</sub>O. A 100  $\mu$ l sample of the 2.7x10<sup>7</sup> cells/mL were pipetted into the first well of a 96-well microliter plate. The cultures were 5-fold serially diluted across the plate. 2  $\mu$ l of each dilution was plated onto EMM+thiamine and EMM-thiamine plate. Once all the samples were plated, the plates were stored in a 30°C incubator for ~48 hours or until colonies were visible. Plates were then photograph and stored at 4°C.

**Time-Course Microscopy:** Time-course microscopy was used in order to analyze the localization of wild-type and mutant  $\alpha$ -synuclein during expression in fission yeast cells. Images were taken using a Nikon TE-2000-U fluorescent microscope and Metamorph® 6.0 software. One milliliter of each sample (WT-GFP, A76E-GFP, E46K-GFP, S89A-GFP, S129-GFP, Y39F-GFP, and Y125F-GFP) was removed from

an inoculated culture in EMM-thiamine media. Each sample was centrifuged in a tabletop centrifuge at (34000 rpm) for 1 minute after which about ~80% of the media was poured off. Ten  $\mu$ l of each culture was pipetted onto a glass slide and covered with a cover slip. Two images were obtained for each culture, one being a differential interference contrast (DIC) and the second being a fluorescence image. At each time point, 15 DIC and 15 fluorescence images were taken to ensure that at least 750 cells were imaged.

## Results

### *E46K Is Slightly to Fission Yeast Strain TCP1*

Our first goal was to characterize toxicity familial mutant E46K-GFP  $\alpha$ -synuclein in fission yeast. First, optical density analysis at 600 nm (OD600) was performed in order to analyze E46K effects on cell growth. As was previously reported (Brandis *et al.* 2006), parent vector, GFP alone, and wildtype  $\alpha$ -synuclein expressing cells were not toxic to fission yeast (Figure 6A). Despite significant differences ( $p = .013251$ ), we consider E46K to be minimally toxic (Figure 6A). Serial spotting was used as a second method of examining cellular toxicity. No difference in colony survival was observed between wild-type and E46K- $\alpha$ -synuclein, and this survival was similar to that of cells containing parent vector expressing GFP alone (Figure 6B). Therefore, together both assays revealed that E46K is minimally toxic to fission yeast cells.

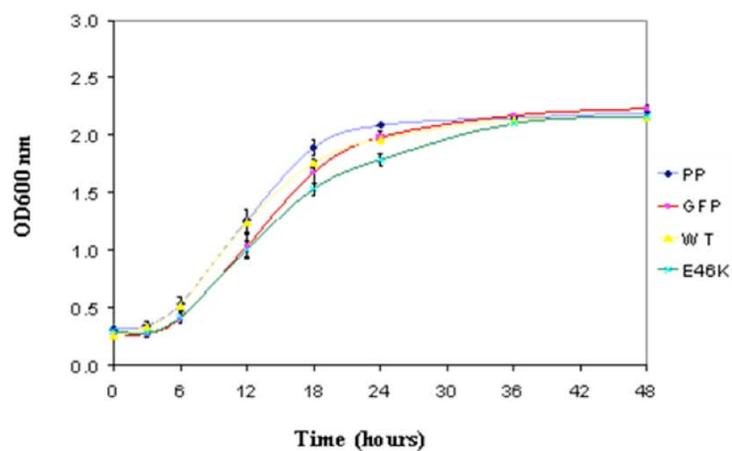
### *Similar Expression of E46K and Wildtype $\alpha$ -Synuclein in TCP1*

To determine if E46K resulted in  $\alpha$ -synuclein accumulation or in the presence of higher order aggregated protein, Western Blot analysis of E46K at 24 hours post-induction of  $\alpha$ -synuclein in TCP1 showed no difference in expression between wild-type and E46K expressing cells in TCP1. Furthermore, we see the presence of higher molecular weight aggregates (Figure 6C).

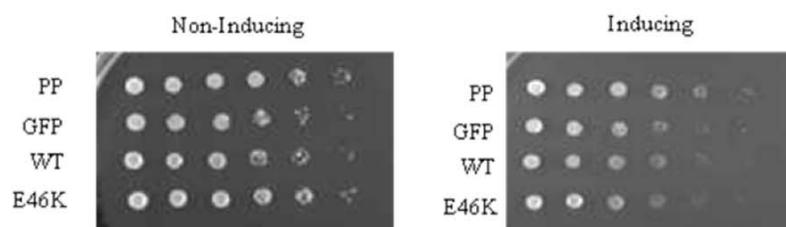
### *E46K $\alpha$ -Synuclein Aggregates Similarly to Wild-Type in TCP1*

To evaluate if E46K aggregates and/or localizes to the plasma membrane, live cell GFP microscopy was conducted. As previously reported (Brandis *et al.*, 2006), wildtype and A53T formed distinct cytoplasmic aggregates,

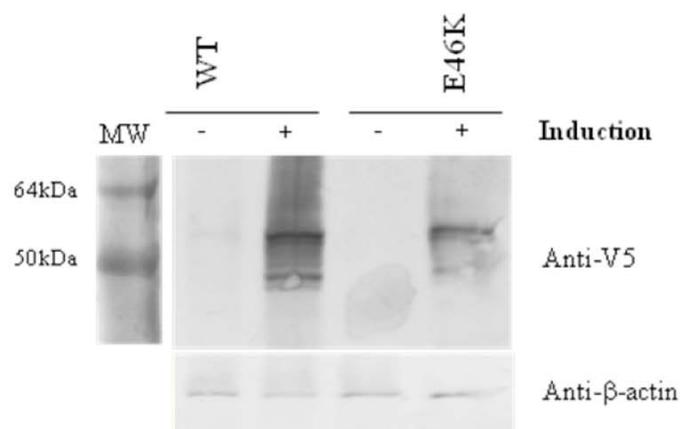
## A. Growth Curve



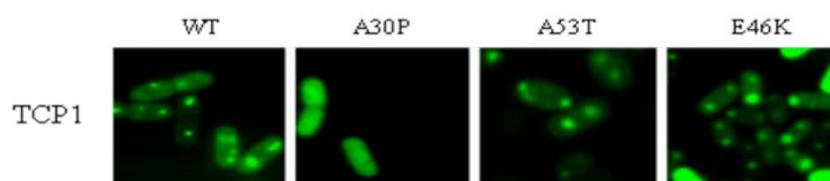
## B. Cell Spotting



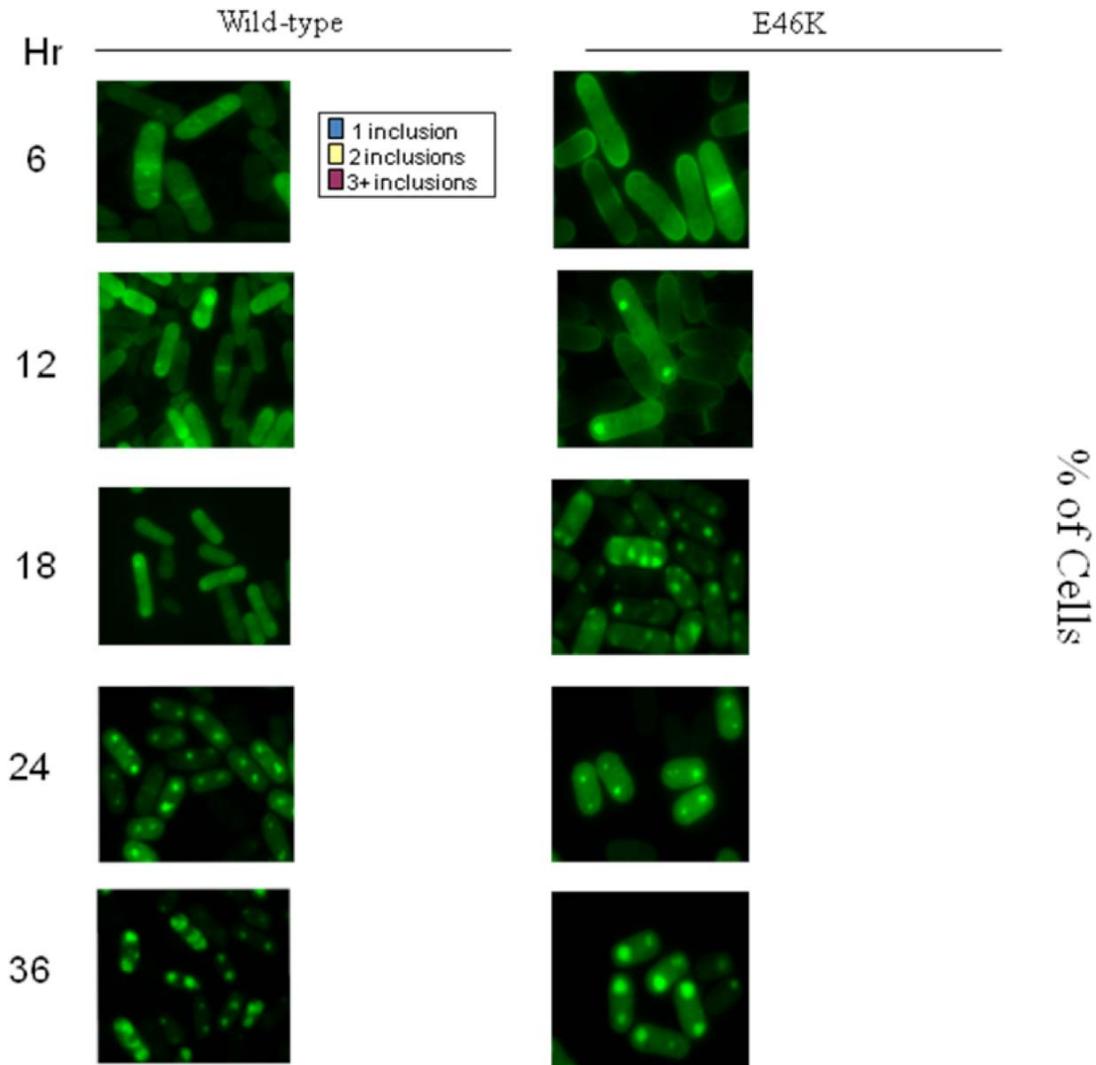
## C. Protein Expression



## D. Live Cell Microscopy

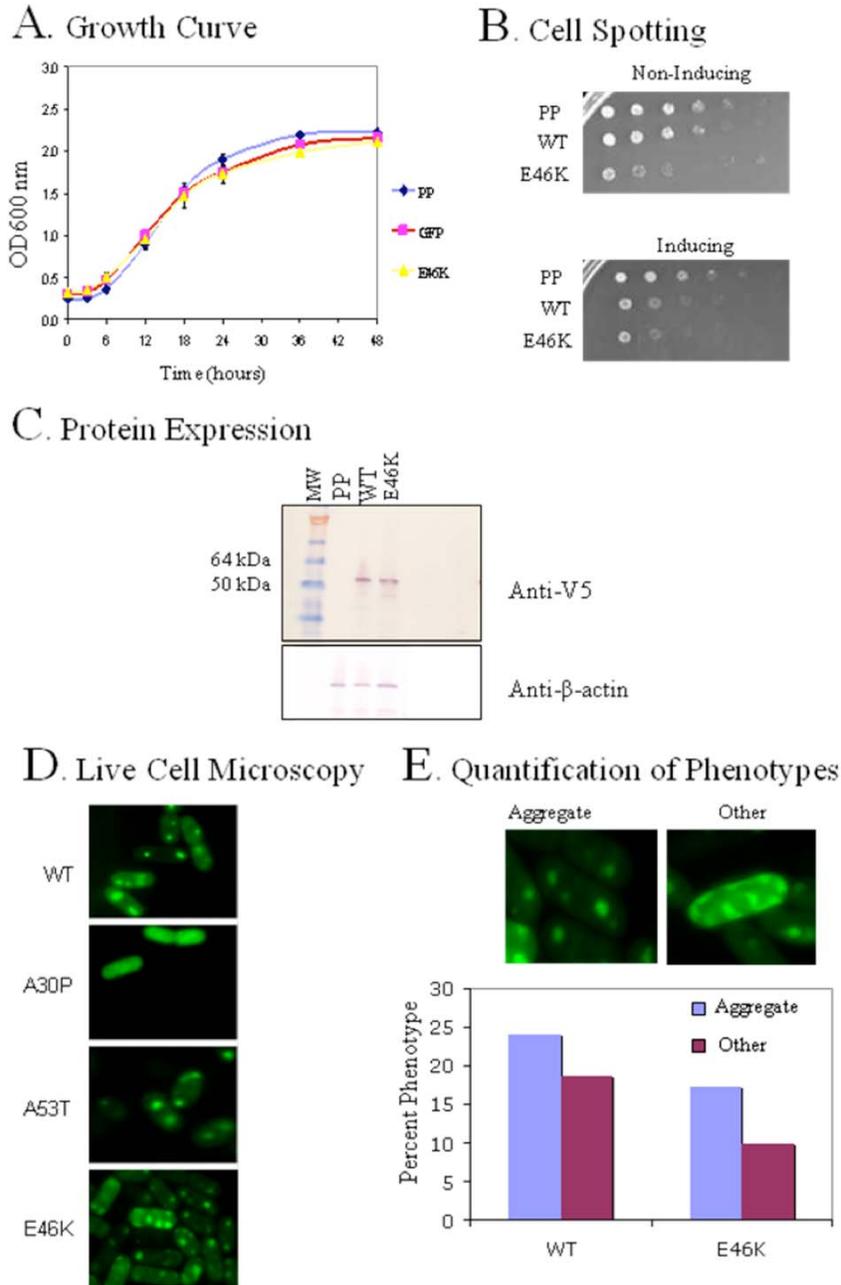


## E. Live Cell Microscopy and Quantification of Phenotypes



**Figure 6. Characterization of E46K  $\alpha$ -synuclein in Fission Yeast Strain TCP1**

- Growth Curve Analysis: Growth Curve during 48 hours of expression for Parent plasmid (blue line), GFP (red line), WT-GFP (yellow line) and E46K (green line). Culture density indicated by absorbance (nm) on the y-axis and time (hrs) after  $\alpha$ -synuclein induction. Data points representative of mean of three trials. Standard Error of the Mean (S.E.M.) bars given for each for each data point. Slight difference in growth observed between WT-GFP and E46K in fission yeast in  $\alpha$ -synuclein inducing media. (n=3, p=0.13251)
- Cell Spotting: TCP1 cells were serially diluted and spotted on  $\alpha$ -synuclein inducing (EMM+thiamine) and non-inducing (EMM+thiamine) media to evaluate effect of  $\alpha$ -synuclein expression on cell growth. Media plate with  $\alpha$ -synuclein repressing solid media EMM+thiamine to the left and media plate with  $\alpha$ -synuclein inducing solid media EMM+thiamine to the right. No difference in growth observed between WT-GFP and E46K on  $\alpha$ -synuclein inducing or non-inducing media (n=3).
- Western Analysis: Cells were grown in  $\alpha$ -synuclein inducing (EMM+thiamine) and non-inducing (EMM+thiamine) media for 24 hours. At 24 hours, western analysis was performed. Blots were probed with an anti-V5 antibody, which binds exclusively to the V5 tag on  $\alpha$ -synuclein.  $\alpha$ -synuclein bands are visible at ~54 kDa. No difference in expression observed between E46K and WT.
- Live Cell Microscopy: Wild-type (Brandis *et al.*, 2006) and E46K fission yeast cell constructs were C-terminally tagged with GFP. Fluorescent images of wild-type E46K  $\alpha$ -synuclein fission yeast taken over 36-hour time course at 1000X magnification. Images taken at 6, 12, 18, 24 and 36 hours post-induction.
- Quantification of Phenotypes: A total of 750 cells were imaged for quantification. At each time point, cells were quantified as having one (blue), two (yellow) or three plus (maroon) inclusions. Percentage of cells exhibiting each phenotype plotted. Percentage of aggregates increases in a time-dependent manner.



**Figure 7. Characterization of E46K  $\alpha$ -synuclein in Fission Yeast Strain Sph-**

- A. Growth Curve Analysis: Growth Curve during 48 hours of expression for Parent plasmid (blue line), WT-GFP (pink line), and E46K (yellow line). Culture density indicated by absorbance (nm) on the y-axis and time (hrs) after  $\alpha$ -synuclein induction. Data points represent the mean of three trials. Standard Error of the Mean (S.E.M.) bars given for each for each data point. No difference in growth observed between WT-GFP and E46K in fission yeast strain Sp in  $\alpha$ -synuclein inducing media. (n=3; p=.36058)
- B. Cell Spotting: PP, GFP, WT and E46K Sph- cells were serially diluted and spotted on  $\alpha$ -synuclein inducing (EMM-thiamine) and non-inducing (EMM+thiamine) media to evaluate effect of  $\alpha$ -synuclein expression on cell growth. Media plate with  $\alpha$ -synuclein repressing solid media EMM+thiamine to the left and media plate with  $\alpha$ -synuclein inducing solid media EMM-thiamine to the right. No difference in growth observed between WT-GFP and E46K on  $\alpha$ -synuclein inducing or non-inducing media (n=3).
- C. Western Analysis: Cells were grown in  $\alpha$ -synuclein inducing (EMM-thiamine) media for 24 hours. At 24 hours, Western analysis was performed. Blots were probed with an anti-V5 antibody, which binds exclusively to the V5 tag on  $\alpha$ -synuclein.  $\alpha$ -synuclein bands are visible at ~54 kDa.
- D. Live Cell Microscopy: E46K fission yeast cell constructs C-terminally tagged with GFP. Fluorescent images of E46K  $\alpha$ -synuclein fission yeast taken over 36 hour time course at 1000X magnification. Images taken at 18 hours post-induction.
- E. Quantification of Phenotypes: A total of 750 cells were imaged for quantification. At each time point, cells were quantified as having either one (blue), two (yellow), or three plus (maroon) inclusions. Percentage of cells exhibiting each phenotype are plotted. Percentage of aggregates increases in a time-dependent manner.

while A30P is cytoplasmically diffuse (Figure 6D). Similar to wild-type and A53T, E46K also formed distinct aggregates in TCP1, ranging from 1-5 per cell. Because A53T aggregates faster and earlier than the wildtype form in fission yeast (Brandis *et al.* 2006), we asked if E46K did the same. Over a 36-hour time course, we observed no difference in the timing or extent of  $\alpha$ -synuclein aggregation between wild-type and E46K (Figure 6E). Notably, at no time during this time course, does E46K localize to the plasma membrane (Figure 6E), again behaving similarly to wild-type and A53T, which do not localize to fission yeast plasma membrane either (Figure 6D; and Brandis *et al.* 2006).

*Aggregation and lack of E46K Toxicity is Not Strain Specific*  
Finally, to assess whether the lack of major E46K toxicity was specific to TCP1 or if this property was universal to multiple fission yeast strains, we repeated the TCP1 assays in a second fission yeast strain, Sp. Both the growth curve and serial spotting on plates revealed that E46K was not toxic in Sp (Figures 7A and B;  $p=.36058$ ). Furthermore, no difference in expression was observed between wild-type and E46K expressing cells in Sp. Also absent was the presence of higher molecular weight aggregates. Similar to observations in TCP1, E46K formed distinct cytoplasmic aggregates (Figure 7D), resembling wildtype and A53T aggregation cells previously reported for Sp1 (Brandis *et al.*, 2006). Interestingly, live cell microscopy revealed a second intracellular phenotype for  $\alpha$ -synuclein, common to both wildtype and E46K forms; because we have not been able to confirm the identity of these internal structures yet, we have for now scored it as "other" (Figure 7E).

## Discussion

This study represents the first assessment of E46K  $\alpha$ -synuclein in a fission yeast model and is one of the first studies of E46K in any living cell. Very little is still known about E46K properties in cells and tissues and nearly all published work describes its properties in *in vitro* systems. Here, we report that E46K aggregates extensively in live cells without plasma membrane association and does not confer major toxicity to fission yeast. As will be discussed below, these fission yeast findings significantly differ from E46K  $\alpha$ -synuclein characteristics in budding yeast. Therefore, in addition to giving new insight into the nature of  $\alpha$ -synuclein toxicity, our study stresses the importance of using both fission and budding yeast models when evaluating protein misfolding and toxicity in yeast systems.

### *E46K Promotes Extensive Aggregation in Fission Yeast*

As hypothesized, E46K aggregates extensively in our fission yeast model. This aggregation is reminiscent of the characteristic aggregation seen in Lewy bodies in human PD brains (Choi *et al.*, 2004). Therefore, fission yeast serves as a good model for examining E46K. Furthermore, this live cell aggregation supports *in vitro* data about E46K, which demonstrate that E46K increases the rate of fibrillization of  $\alpha$ -synuclein (Greenbaum *et al.*, 2005; Pandey *et al.*, 2005; Choi *et al.*, 2004).

In fission yeast, E46K aggregation resembles that of A53T and WT in its propensity to aggregate and this aggregation is notably different from A30P's cytoplasmic localization (Dixon *et al.* 2005; Outiero and Lindquist, 2003; Zabrocki *et al.* 2005). Our fission yeast model findings that A53T and WT have a high propensity to aggregate are comparable to those found in *in vitro* studies. Furthermore, our findings that familial mutant A53T aggregates at a faster rate than WT are also comparable to *in vitro* studies (Bussell *et al.* 2004; Jo *et al.*, 2000). Surprisingly, E46K does not aggregate faster than wild-type. While WT and A53T

mutations promote the accumulation of intermediate aggregate, or protofibrils, E46K does not, suggesting that the extent of aggregation may be critical for disease and toxicity linked to disease (Greenbaum *et al.*, 2005).

### *E46K Surprisingly Results in Lack of Membrane Association*

The phospholipid binding abilities of E46K have been thoroughly examined *in vitro* and suggest that E46K increases  $\alpha$ -synuclein's phospholipid binding abilities (Choi *et al.*, 2004). This observation may explain E46K mutant  $\alpha$ -synuclein's membrane binding affinity and the modest intracellular aggregation in budding yeast. (White, 2007; See Appendix A, pg 60). While we had hypothesized that E46K would result in membrane binding, E46K in fission yeast lacks membrane binding. Differences in the membrane binding properties between fission and budding yeast may be attributable to differences within the phospholipid membranes of the yeast themselves. It is a possibility that there may be reduced or altered plasma membrane phospholipid composition, or differences in membrane proteins with which  $\alpha$ -synuclein interacts, leading to the lack of membrane association (Brandis *et al.*, 2006).

### *E46K Confers Slight Toxicity in One Strain of Fission yeast*

The most notable finding is the unexpected lack of major E46K dependant toxicity. A major hypothesis in the field of neurodegenerative diseases had been that aggregation was key to cellular toxicity (Geodert *et al.*, 2001). In recent times, this position has changed as aggregation is now thought to be protective or minimally harmless. One possibility to explain the major E46K dependent toxicity is the extent of aggregation in E46K  $\alpha$ -synuclein. Perhaps, aggregation happens to fast in fission yeast, and less intermediates form. Possibly, intermediate aggregates are the causative factor of toxicity, as also suggested by the extensive aggregation and lack of toxicity seen in WT and A53T (Brandis *et al.*, 2006). In our yeast models, most cells displaying predominant membrane localization also exhibit  $\alpha$ -synuclein-dependent toxicity (Brandis *et al.*, 2006; Sharma *et al.*, 2006), therefore, a second reason that E46K may not result in major toxicity may be the lack of membrane association seen in E46K  $\alpha$ -synuclein. Lastly, the lack of major toxicity in fission yeast may be due to the difference in the genomes of the yeast themselves. The fission yeast genome may provide a protective response that is not observed in budding yeast. While these possible protective mechanisms are not known, uncovering these mechanisms may help us better understand the disease.

## Chapter 2: Characterization of A76E $\alpha$ -synuclein in Fission Yeast

### Results

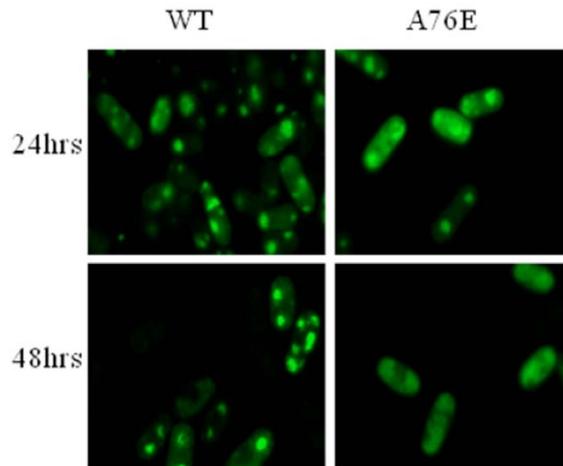
#### *A76E is Less Aggregated*

First, we evaluated whether the mutation A76E reduced  $\alpha$ -synuclein aggregation in fission yeast, live cell microscopy was conducted. As we had hypothesized, A76E reduced  $\alpha$ -synuclein aggregation, compared to wild-type  $\alpha$ -synuclein (Figure 8A). Quantification of this microscopy confirmed that wild-type  $\alpha$ -synuclein formed distinct intracellular aggregates in most cells, as previously reported in Brandis *et al.* 2006, while A76E expressing cells demonstrated a significant shift of  $\alpha$ -synuclein localization to the cytoplasm, with many cells now showing a combined cytoplasmically diffuse/aggregation phenotype (Figure 8B). Interestingly, no  $\alpha$ -synuclein membrane localization was observed with A76E, as would be predicted for this more hydrophilic mutant (Figure 8A,B).

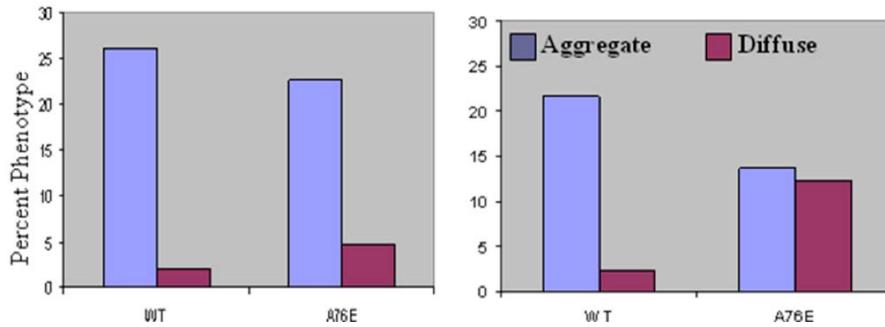
*Expression of  $\alpha$ -Synuclein Unchanged by A76E*  
 Next, to evaluate if A76E affected the expression of  $\alpha$ -synuclein, Western blot analysis of A76E cells was

performed at 24 hours post-induction of  $\alpha$ -synuclein. A76E cells and wild-type expressing cells showed no difference in expression, despite the reduction in aggregation (Figure 8C).

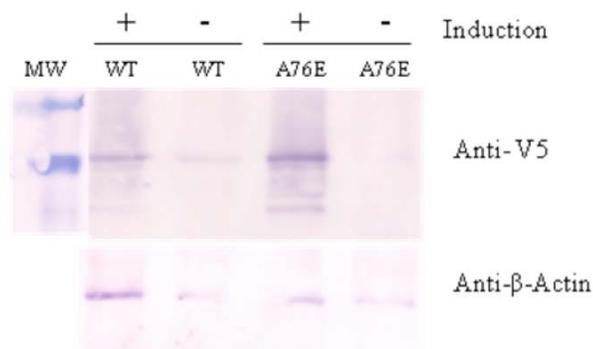
### A. Live Cell Microscopy



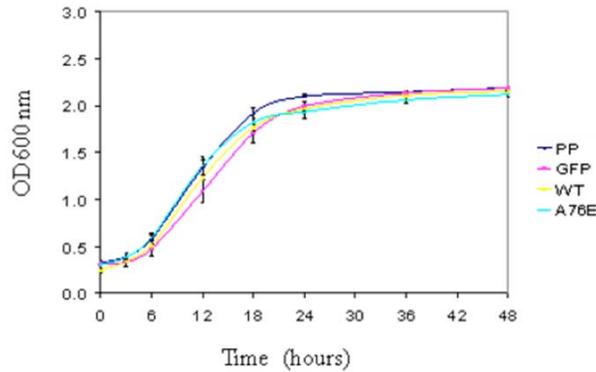
### B. Quantification of Phenotypes



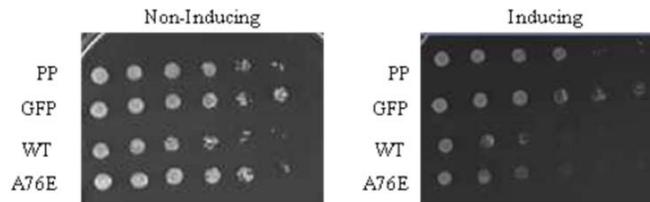
### C. Protein Analysis



## D. Growth Curve



## E. Cell Spotting



### Figure 8. Characterization of A76E α-synuclein in Fission Yeast

A. Live Cell Microscopy: A76E fission yeast cell constructs C-terminally tagged with GFP. Fluorescent images of A76E α-synuclein fission yeast taken over 36 hour time course at 1000X magnification. Images taken at 12 and 24 hours post-induction.

B. Quantification of Phenotypes: A total of 750 cells were imaged for quantification. At each time point, cells were quantified as having either diffuse fluorescence (maroon), or aggregate (blue). Percentage of cells exhibiting each phenotype at 24 and 48 hours plotted. A76E reduces aggregation of α-synuclein in fission yeast.

C. Western Analysis: Cells were grown in α-synuclein inducing (EMM-thiamine) and non-inducing (EMM+thiamine) media for 24 hours. At 24 hours, western analysis was performed. Blots were probed with an anti-V5 antibody, which binds exclusively to the V5 tag on α-synuclein. α-synuclein bands are visible at ~52 kDa. There is no difference in expression between A76E and WT.

D. Growth Curve Analysis: Growth Curve during 48 hours of expression for parent plasmid (dark blue), GFP (pink), WT-GFP (yellow line) and A76E (light blue line). Culture density indicated by absorbance (nm) on the y-axis and time (hrs) after α-synuclein induction. Data points representative of mean of three trials. Standard Error of the Mean (S.E.M.) bars given for each data point. No difference in growth observed between WT-GFP and A76E in fission yeast in α-synuclein inducing media. (n=3; p=241208).

E. Cell Spotting: PP, GFP, WT-GFP and A76E α-synuclein expressing cells were serially diluted and spotted on α-synuclein inducing (EMM-thiamine) and suppressing (EMM+thiamine) media to evaluate effect of α-synuclein expression on cell growth. Media plate with α-synuclein repressing solid media EMM+thiamine to the left and media plate with α-synuclein inducing solid media EMM-thiamine to the right. No difference in growth observed between WT-GFP and A76E on α-synuclein inducing or suppressing media.

### Lack of Toxicity Observed for A76E Expressing Cells

Lastly, we asked whether the decreased aggregation would alter α-synuclein toxicity in fission yeast. OD600 growth curves demonstrated that A76E cells grew just as well as those containing parent vector, or expressing GFP alone or wildtype α-synuclein (Figure 8D; p=.241208), indicating that decreased aggregation did not increase or decrease toxicity. Serial spotting confirmed a lack of A76E induced toxicity, as no difference in colony survival was observed between wild-type and A76E-α-synuclein, and this level of colony survival was similar to that of cells containing parent vector or expressing GFP alone (Figure 8E). Collectively, both assays indicated that A76E does not induce toxicity in fission yeast cells.

## Discussion

In most biological systems, despite the significant expenditure of energy to avoid the natural tendency of some proteins to aggregate and form harmful deposits, some proteins nonetheless misfold and aggregate. The misfolding,

aggregation, and membrane phospholipid binding of α-synuclein is linked to both familial and sporadic forms of PD. This study provides strong relevance of alanine-76 to PD, by reporting that the substitution mutant A76E significantly reduces α-synuclein aggregation in live cells, without increasing plasma membrane association, or conferring toxicity to fission yeast.

### The Contribution of Alanine-76 to Aggregation of α-Synuclein

Our first notable finding is that A76E reduces α-synuclein aggregation in fission yeast, providing critical live cell support for the hypothesis that alanine-76 is an important regulator of α-synuclein aggregation. Previously, *in vitro* studies had implicated alanine-76 within α-synuclein as an intrinsic contributor to its aggregation and pathogenesis (Chiti *et al.*, 2000; Gaijsson *et al.*, 2001). Through deletion analysis, Giasson *et al.* (2001) identified a hydrophobic stretch of twelve amino acids (70-82) thought to be responsible for α-synuclein aggregation. Both Giasson *et al.* (2000) and Chiti *et al.* (2001) pointed to alanine-76 as being the key amino acid within this stretch that contributed the

hydrophobicity necessary for aggregation. Our finding provides new evidence to extend these studies, and indicates that in sporadic and familial PD, alanine-76 may be critically involved in misfolding and aggregation of  $\alpha$ -synuclein.

Because A76E replaces hydrophobicity with a negative charge, it should also reduce the ability of  $\alpha$ -synuclein to associate with the plasma membrane. In fact, we do not see a redistribution of  $\alpha$ -synuclein to the plasma membrane in fission yeast. Instead, it redistributes in the cytoplasm. Further evidence for reduced plasma membrane localization comes from our budding yeast model, where A76E reduces membrane localization, without any increase in aggregation (Michael Zorniak Thesis 2007; See appendix B, pg 62). In this model, not surprisingly, an increase in the number of live cells displaying a diffuse/halo phenotype correlated with the loss of membrane localization, indicative of a more hydrophilic  $\alpha$ -synuclein. Therefore, in both fission and budding yeast models, we observed a reduction in the amount of aggregation and membrane localization, indicative of the contribution of alanine-76 to the hydrophobic properties of  $\alpha$ -synuclein.

#### Lack of A76E Induced Toxicity

The second notable finding was that while A76E resulted in reduced  $\alpha$ -synuclein aggregation, it did not affect toxicity in fission yeast. Although reduced, aggregation is still observed in live cells, therefore, its' possible that these remaining aggregates are protective rather than toxic (Rochet *et al.*, 2004), and thus prevents toxicity. Additionally, the lack of  $\alpha$ -synuclein membrane localization may contribute to the absence of toxicity in A76E fission yeast cells. In support of this notion, our A76E budding yeast model demonstrates a reduced  $\alpha$ -synuclein membrane localization and yet is toxic (Michael Zorniak Thesis, 2007; See appendix, pg 63). We

believe that this budding yeast toxicity is due to absence of visible aggregation and yet enough retention of membrane localization to be toxic to yeast. Thus, our budding and fission yeast data for A76E are consistent with E46K results, and provides general support for an emerging hypothesis in the PD field for  $\alpha$ -synuclein toxicity being linked to its association with plasma membranes.

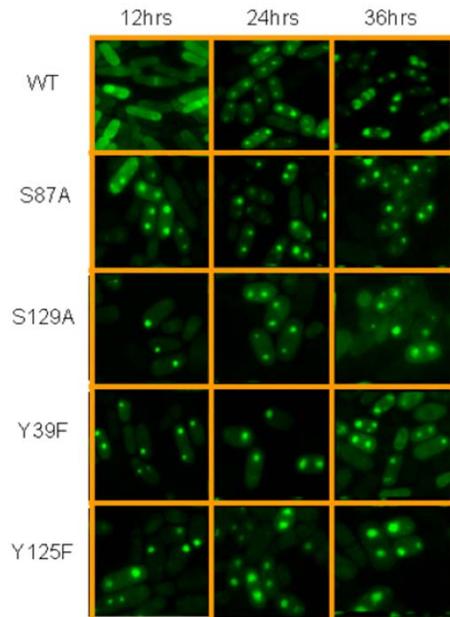
### Chapter 3: Characterization of Post-translational Modification Mutations in Fission Yeast

#### Results

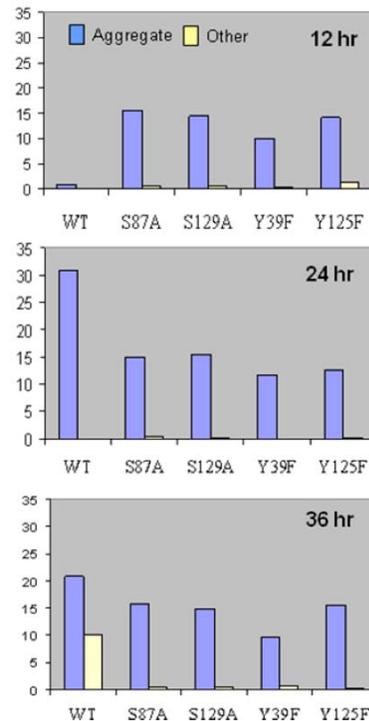
##### Post-translational Modification Mutants Maintain Aggregation

Our first goal was to test the prediction that one or more of the nitrosylation-deficient (Y39A and Y125F) and phosphorylation-deficient (S87A and S129A) mutants would reduce  $\alpha$ -synuclein aggregation in fission yeast cells. To this end, we evaluated the effect of post-translational mutations on  $\alpha$ -synuclein localization in fission yeast cells through live cell GFP microscopy (Figure 9A). As expected, over a time course, wild-type  $\alpha$ -synuclein aggregated by 24 hours and remained aggregated (Figure 9A-B). At 12 hours, however, the amount of aggregation of all four post-translational modification mutants was higher than wild-type  $\alpha$ -synuclein, contrary to our hypothesis (Figure 9A-B). But by 36 hours, aggregation levels between post-translational mutants and wild-type  $\alpha$ -synuclein were similar (Figure 9A-B). Interestingly, we observed a second intracellular localization pattern ("other") for wild-type and all four mutant forms of  $\alpha$ -synuclein; but the number of cells that exhibited this phenotype were fewer. This "other" phenotype was similar to the type we had observed in wild-type and E46K  $\alpha$ -synuclein in Chapter 1.

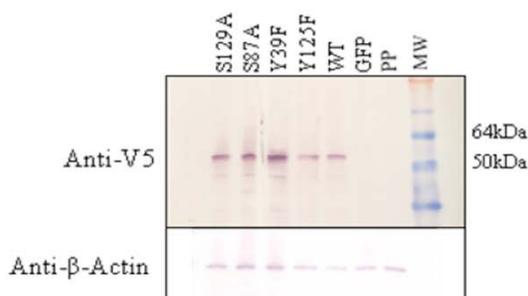
#### A. Live Cell Microscopy



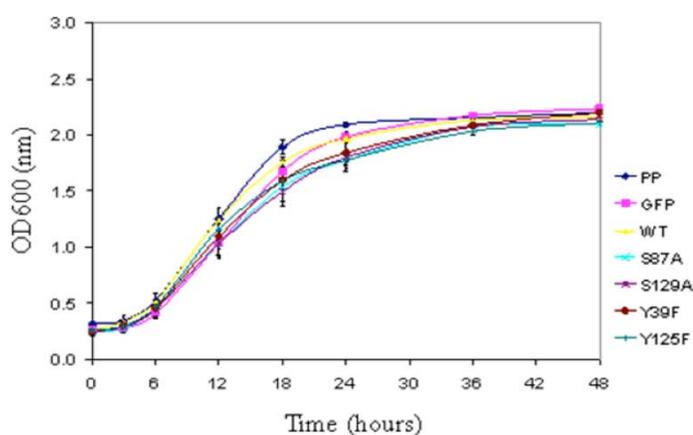
#### B. Quantification of Phenotypes



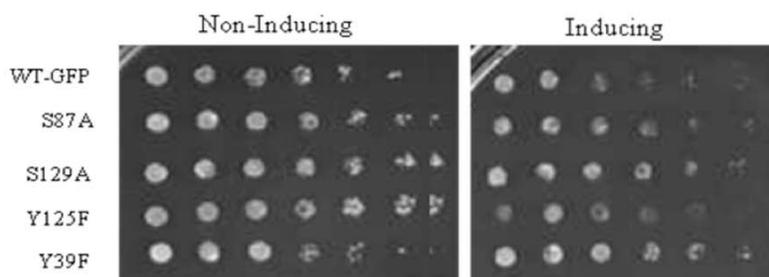
## C. Protein Expression



## D. Growth Curve



## E. Cell Spotting



**Figure 9. Characterization of Post-translationally Mutant  $\alpha$ -synuclein in Fission Yeast**

A. Live Cell Microscopy: All fission yeast cell constructs C-terminally tagged with GFP. Fluorescent images of WT-GFP, S87A, S129A, Y39F, and Y125F  $\alpha$ -synuclein fission yeast taken over 36 hour time course at 1000X magnification. Images taken at 12, 24 and 36 hours post-induction.

B. Quantification of Phenotypes: A total of 750 cells were imaged for quantification. At each time point, cells were quantified as having either aggregate fluorescence (dark blue), aggregate/ diffuse fluorescence (maroon), other (yellow). Percentage of cells exhibiting each phenotype at 12, 24, and 36 hours plotted.

C. Western blot Analysis: Cells were grown in  $\alpha$ -synuclein inducing (EMM-thiamine) media for 24 hours. At 24 hours, western analysis was performed. Blots were probed with an anti-V5 antibody, which binds exclusively to the V5 tag on  $\alpha$ -synuclein.  $\alpha$ -synuclein bands are visible at ~52 kDa. There is no difference in expression between post-translational modification mutants and WT.

D. Growth Curve Analysis: Growth Curve during 48 hours of expression for parent plasmid (blue), GFP (pink), WT-GFP (yellow line), S87A (light blue line), S129A (purple), Y39F (brown line), and Y125F (green line). Culture density indicated by absorbance (nm) on the y-axis and time (hrs) after  $\alpha$ -synuclein induction on x-axis. Data points representative of mean of three trials. Standard Error of the Mean (S.E.M.) bars given for each for each data point. No major difference in growth observed between WT-GFP, S87A, S129A, Y39F, and Y125F fission yeast in  $\alpha$ -synuclein inducing media. (n=3; S87A: p=.060802, S129A: p=.026591, Y39F: p=.040414, Y125F: p=.024439)

E. Serial Spotting: WT-GFP, S87A, S129A, Y39F, and Y125F  $\alpha$ -synuclein expressing cells were serially diluted and spotted on  $\alpha$ -synuclein inducing (EMM-thiamine) and suppressing (EMM+thiamine) media to evaluate effect of  $\alpha$ -synuclein expression on cell growth. Media plate with  $\alpha$ -synuclein repressing solid media EMM+thiamine to the left and media plate with  $\alpha$ -synuclein inducing solid media EMM-thiamine to the right. No difference in growth observed between WT-GFP, S87A, S129A, Y39F, and Y125F on  $\alpha$ -synuclein inducing or suppressing media.

### *Post-translational Modification Mutants Maintain Size on Gels*

We performed Western blot analysis at 24 hours post-induction in order to test the hypothesis that one of these four post-translational modifications was responsible for the abnormal migration (about 10 kDa higher) of  $\alpha$ -synuclein in yeast lysates. Interestingly, all four mutants maintained a migration size similar to wild-type  $\alpha$ -synuclein on SDS-PAGE gels (Figure 9C).

### *Post-translational Modification Mutants Do Not Result in Major Toxicity*

Lastly, we evaluated the effects of these four  $\alpha$ -synuclein mutants on cellular toxicity, using optical density assay and serial spotting analysis. In fact, we observed slight difference in growth curves between parent vector, GFP alone, wild-type  $\alpha$ -synuclein, and any of the four  $\alpha$ -synuclein mutants (Figure 9D). Despite significant toxicity, we do not consider this toxicity to be major (S87A:  $p=0.060802$ , S129A:  $p=0.026591$ , Y39F:  $p=0.040414$ , Y125F:  $p=0.024439$ ). Nor did we observe any difference within all these strains, in their colony formation on serially diluted spotted plates (Figure 9E). Collectively, both assays demonstrate that post-translational mutations do not regulate  $\alpha$ -synuclein toxicity in fission yeast.

## **Discussion**

Several factors may contribute to the misfolding and aggregation of  $\alpha$ -synuclein and aid in the progression of PD. Among those suggested in PD literature, are specific posttranslational modifications on  $\alpha$ -synuclein, phosphorylation on serine-87 and serine-129, and nitrosylation on tyrosine-39 and tyrosine-125. Despite some supporting *in vitro* evidence, whether these modifications actually aid in PD pathogenesis or are harmless or even beneficial has not been resolved. Our major finding here is that neither the phosphorylation-deficient mutants nor the nitrosylation-deficient mutants reduce  $\alpha$ -synuclein aggregation in fission yeast, nor do they affect toxicity. When combined with parallel studies done in a budding yeast model (Sara Herrera Thesis, 2005, See appendix C pg 65), our conclusion is that all of these post-translational modifications are, in fact, potentially beneficial to cells.

### *Why Don't Post-Translational Deficient Mutants Reduce Aggregation?*

The most notable finding is lack of reduction in aggregation of the four post-translational modification mutants. The simplest explanation could be that neither phosphorylation nor nitrosylation of  $\alpha$ -synuclein affect aggregate formation *in vivo*. What is the evidence that these modifications do enhance aggregation? In the case of phosphorylation, *in vitro* evidence suggests that (i)  $\alpha$ -synuclein is phosphorylated at serine-87 and serine-129 (Fujiwara *et al.*, 2002); (ii) this phosphorylation promotes the formation of fibrils *in vitro* (Fujiwara *et al.*, 2002); and (iii) mutating phosphorylation sites decreases inclusion formation (Smith *et al.*, 2005). However, other studies contest some of these findings. First, blocking phosphorylation of serine-129 increases aggregate formation (Chen and Feany, 2005). Secondly, Lee *et al.* (2004) find that S129A does not have any impact on inclusion formation.

In the case of nitrosylation, *in vitro* evidence suggest that (i)  $\alpha$ -synuclein is nitrosylated at tyrosine 39 and tyrosine-125 (Takahashi *et al.*, 2002); (ii) an increase in the ratio of nitrating agent to results in the augmentation of  $\alpha$ -synuclein aggregates (Paxinou *et al.*, 2001) and  $\alpha$ -synuclein oligomers (Souza *et al.*, 2000) and (iii) tyrosine-125 may play a critical role in  $\alpha$ -synuclein dimerization, as a lack of this

residue significantly decreases dimerization compared to  $\alpha$ -synuclein lacking tyrosine-39 (Takahashi *et al.*, 2002). Our *in vivo* fission yeast evidence, however, does not correlate with *in vitro* evidence, our nitration and phosphorylation deficient mutants do not alter the aggregation rates of the protein. Interestingly, in parallel studies in budding yeast (Sara Herrera Thesis, 2005; See appendix C, pg 64), none of these mutants affected aggregation. Instead, all of them maintain plasma membrane localization. A second explanation for lack of aggregations differences due to post-translation modification could be that such modifications do not happen in yeast. It is important to note that we have not tested for either  $\alpha$ -synuclein phosphorylation or nitrosylation in our yeast model. Furthermore, it is possible that more than one post-translational modification is necessary to alter rate of  $\alpha$ -synuclein aggregation.

### *Why Don't Post-Translational Modification-Deficient Mutants Reduce Toxicity?*

Our second notable finding is that all four mutants lack major toxicity in fission yeast. A simple reason could be that the post-translational modification mutants retained high levels of aggregation comparable to wild-type. As previously discussed in chapters I and II, these aggregates may be protective, so the presence of aggregation may lead to the lack of toxicity seen observed in these mutants, as well as wild-type, A53T, A76E, E46K. Secondly, these mutants do not localize to the plasma membrane in fission yeast, which may have been a key factor to generate toxicity. We know from the budding yeast model, where significant toxicity was observed with four all mutants, a significant amount of membrane localization existed (Sara Herrera, 2005; See Appendix C, pg 61). Most interestingly, a recent study using a rat model of PD demonstrates that S129A accelerates neurodegeneration, while constitutive phosphorylation of serine 129 protects against neurodegeneration (Gorbatyuk *et al.*, 2008).

Understanding a possible mechanism for neuron protection in mice may be important for the possibility of therapeutic treatment. Furthermore, understanding the protective mechanisms that fission yeast may have to protect it from the phosphorylation-deficiency dependent toxicity observed in other models may also help us understand the mechanism of neurodegeneration.

### *Why Don't Post-Translational Modification-Deficient Mutants Migrate More Quickly?*

We tested the hypothesis that these post-translational mutations were responsible for the aberrant size (~10 kDa higher) of  $\alpha$ -synuclein. Interestingly, fission yeast post-translational modification mutants, like budding yeast post-translational modification mutants (Sara Herrera thesis, 2005; See appendix C, pg 61), do not influence the size change of  $\alpha$ -synuclein. A possibility exists that these modifications, if these occur at all, are not responsible for the aberrant size of  $\alpha$ -synuclein. Furthermore, it is possible that other post-translational modifications such as glycosylation and ubiquitination (Shimura *et al.*, 2001), or a combination of modifications could result in the increased size of  $\alpha$ -synuclein. Lastly, the higher migration of  $\alpha$ -synuclein could be due to other factors, such as the possible change in conformation of the protein. Adopting a specific conformation could result in the decreased motility of the protein through the gel, however, little is known about the specific conformational changes that may result in the increased size of  $\alpha$ -synuclein.

## **Conclusion**

This thesis examined several hypotheses that focused primarily focused on the aggregation and phospholipid

binding properties of  $\alpha$ -synuclein and how one or both are linked to cellular toxicity. Our findings provide new and converging insights into the pathogenesis that underlies both forms of PD, sporadic and familial. In our first study, we found that E46K, the newest familial  $\alpha$ -synuclein mutant, aggregates extensively in fission yeast, as we had hypothesized. Contrary to our prediction, however, E46K  $\alpha$ -synuclein does not aggregate faster than wild-type  $\alpha$ -synuclein and lacks plasma membrane localization. In our second study, we found that the  $\alpha$ -synuclein mutant A76E reduces the rate of  $\alpha$ -synuclein aggregation, supporting our hypothesis that alanine-76 is a key contributor to  $\alpha$ -synuclein's aggregation propensity. In our final study, we found that phosphorylation and nitrosylation deficient  $\alpha$ -synuclein mutants maintain both aggregation and do not affect toxicity, contrary to our hypothesis that such post-translational modifications of  $\alpha$ -synuclein facilitate its aggregation and possibly confer toxicity. Interestingly, we also found that post-translational modification mutants did not change the size of  $\alpha$ -synuclein, implicating other factors in the aberrant migration of  $\alpha$ -synuclein in yeast models.

Together, these three studies point to key amino acid residues within  $\alpha$ -synuclein that control their aggregation status. This thesis also further confirms fission yeast as a model organism that is particularly resistant to  $\alpha$ -synuclein toxicity. For this reason, it is an attractive system in which to identify novel factors that provide such protection.

#### *Budding and Fission Models: Both Powerful Tools for $\alpha$ -synuclein Characterization*

While several differences have emerged in the characterization  $\alpha$ -synuclein properties in budding and fission yeast, these differences stress the usefulness of using both models in the examination of  $\alpha$ -synuclein misfolding and aggregation. Fission yeast is excellent for understanding the basis for aggregation of  $\alpha$ -synuclein, while budding yeast serves as an effective model  $\alpha$ -synuclein membrane phospholipid localization. Together, both yeast models provide a more complete assessment of factors necessary for cellular toxicity. The differences in  $\alpha$ -synuclein properties between the two yeasts may be due to differences in their genomes, or to differences in their expressed proteins.

#### *Limitations*

We do not know the nature of the aggregates that form in fission yeast and whether they are truly cytoplasmic or linked to specific cytoplasmic structures/organelles. Using organelle specific markers or dyes may provide clarity. A second major limitation is that the quantification examined using GFP microscopy is a non-blind process. A possibility exists that this process could result in slight variations of results if performed by different people. Lastly, while effective, our unicellular model does not allow us to examine the physical manifestations of the disease that are observed in other models.

#### *Future Studies*

Future studies will include the characterization of combination mutants of E46K and the two other familial mutants A30P and A53T to assess the contribution of one mutant over the other in controlling aggregation. Similarly, we will conduct experiments with combination mutants of more than post-translational modifications, since single mutants did influence toxicity or reduce  $\alpha$ -synuclein migration size. Fuller characterization of these  $\alpha$ -synuclein mutants may increase our understanding of not only  $\alpha$ -synuclein function, but also the mechanisms of its aggregation and cellular toxicity, which may potentially help us in developing treatment for PD.

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