# Evaluating Factors That Affect α-Synuclein Toxicity in Yeast Models: Protein Concentration, Phospholipids, & Oxidants

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Abstract

**Parkinson's disease (PD) is a progressive neurodegenerative disease caused by the death of midbrain dopaminergic neurons. The misfolding and aggregation of a membrane phospholipid binding protein, α-synuclein, and the accumulation of oxygen radicals play a ruinous role in this selective cell death. How the protein's aggregation and phospholipid binding properties contribute to its toxic nature is still unclear. To better understand α-synuclein's toxic mechanism, our lab previously developed comparative models in fission yeast and budding yeast. Using the model organisms, my thesis explored three related questions. First, I tested the hypothesis that α-synuclein aggregation follows the nucleation polymerization theory, which requires that aggregates increase with concentration and over time. I analyzed α-synuclein aggregation properties when it is expressed at moderate levels and compared my data with published work with high and low levels of α-synuclein expression (Brandis *et al.* 2006). I found that moderately expressed α-synuclein formed fewer and later aggregates compared with highly expressed α-synuclein, indicating a threshold concentration of α-synuclein was critical for aggregation process. Together, both the published (Brandis et al., 2006) and the current thesis studies provide strong live cell support for the nucleation polymerization model. In my second study, I tested the hypothesis that increasing phospholipid association of α-synuclein would enhance cellular toxicity of the protein. Using a chemical approach, I induced phospholipid synthesis of both fission yeast and budding yeast with dimethyl sulfoxide (DMSO), a known inducer (Murata et al., 2003). Instead of demonstrating α-synuclein-dependent toxicity, DMSO exerted an unexpected α-synuclein-independent toxicity in both yeasts, in addition to inducing a lethal morphology defect in budding yeast. Moreover, instead of inducing plasma membrane localization of α-synuclein in either yeast, DMSO altered α-synuclein localization in both yeasts into as-yet unidentified cytoplasmic structures. We speculate that some of these structures may be internal membrane bound organelles. To test for membrane phospholipid binding specificity, α-synuclein localization was analyzed in a phosphatidylserine-deficient budding yeast strain. We observed no loss of plasma membrane localization suggesting that other phospholipids may regulate such specificity to α-synuclein. Lastly, I tested the hypothesis that α-synuclein toxicity will enhance in a combinatorial manner with oxidative stress in fission yeast, which has been an organism resistant to α-synuclein's toxicity. In my preliminary results, I have instead found an unexpected α-synuclein-independent toxicity to the oxidant, hydrogen peroxide. These related studies, together, illustrate the usefulness of yeasts in evaluating genetic and environmental factors that regulate α-synuclein toxicity linked to PD.**

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

## Introduction

Neurodegenerative diseases are characterized by the progressive loss of function and death of neurons located in the central nervous system. These illnesses most of the time strike humans as they age. Some examples of neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS; also known as Lou Gehrig's disease), Huntington's disease, and Multiple Sclerosis (MS). These disorders have genetic, sporadic (most common) and infectious (less common) causes. Pathologically, these diseases have some commonalities. When a neurodegenerative disease afflicts a human being, it leads to death of specific set of neurons within the brain. For example in Huntington's disease, the degeneration of neuronal cells occurs particularly in the caudate nucleus and putamen of the basal ganglia responsible for motor function, creating symptoms of jerky uncontrolled movement (Brown et al., 1999).

Another common pathological feature that has been observed in these neurodegenerative diseases is the accumulation of abnormally folding protein into insoluble clumps. The protein aggregates either inside cytoplasm or extracellularly of dying neurons. The mechanism of protein misfolding and aggregating in relation to neuronal death is not clearly known (Ross and Poirier, 2004). The misfolding protein can arise from a mutation in the gene that transcribes it, or sporadic events with no known causes.

### *Parkinson's Disease*

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder. Classical symptoms include tremors, rigidity, slowness in movement, and poor balance and coordination. Most cases of PD are idiopathic, with unknown causes, and occur during late ages (http://www.nlm.nih.gov/medlineplus/parkinsonsdisease.html ). Approximately 10% of PD cases are known to be genetically linked (Eriksen et al., 2003). The disease is fatal with no cure in sight, but over the last thirty years, varieties of drugs have been developed to cope with the symptoms.

The manifestation of Parkinson's disease occurs due to the death of dopaminergic neurons in the substantia nigra pars compacta (Figure 1). The pathogenic mechanisms for sporadic cases of PD are thought to involve mitochondrial impairment and oxidative stress (Chua and Tang, 2006) which can arise from exposure to pesticides, rural living, farming, and drinking well water (Jenner P, 2001; Sherer et al., 2002). Moreover, when post mortem PD brains are examined, cytosolic inclusions called Lewy bodies appear as the prominent feature of this disease (Lücking, and Brice, 2000).

Presently, key findings in PD research point to the hypothesis that the misfolded protein α-synuclein plays a critical role in the pathogenesis. First, the Lewy bodies in the dying neurons contain a fibrillar form of misfolded α-synuclein (Spillantini et al., 1997). Also, three missense mutations in the α-synuclein gene were discovered as a cause of autosomal dominantly inherited PD. Alanine at 53$^{rd}$ codon is replaced with threonine (A53T) in an Italian-American Greek family (Polymeropoulos et al., 1997). Also,

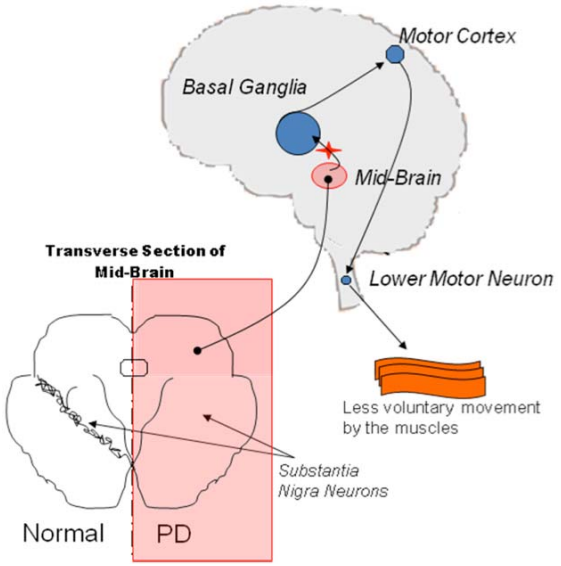



**Figure 1. Manifestation of Parkinson's disease from its pathology to its symptoms.** The symptoms of Parkinson's disease result from the loss of dopaminergic cells in the ventral region of the substantia nigra. Because these neurons project to the striatum, their loss means changes in the activity of the neural circuits within the basal ganglia which regulate movement. In essence, there is an increase in inhibitory signal out of substantia nigra (denoted by red star), enventually leading to excitatory signal to the motor cortex and lower motor neurons which cause a hypokinetic movement condition in PD.

alanine at 30$^{th}$ condon is replaced with proline in a German family (Kruger et al., 1998). Lastly, glutamic acid at 46$^{th}$ codon is replaced with lysine in a Spanish family (Zarranz et al., 2004).

The normal function of α-synuclein is still clearly not understood. α-Synuclein is a natively unfolded protein composed of 140 amino acid residues (Kaplan et al., 2003). It is localized mainly at the presynaptic terminals shown by immunocytochemical and subcellular fractionation studies (Maroteaux et al., 1988). The expression of this protein is high with ongoing synaptic activity in the brain regions such as olfactory bulb, cerebral cortex, hippocampus and substantia nigra (Kaplan et al., 2003). Because α-synuclein is present in the presynaptic neurons, many believe it is important in neuronal plasticity (Clayton and George, 1999), regulating neurotransmitter release, and neuronal differentiation (Lücking, and Brice, 2000).

Using model organisms, transgenic mice (Masliah et al., 2000), and flies (Feany and Bender, 2000) carrying the human form α-synuclein have been shown to generate Parkinsonian phenotypes; specific dopaminergic neurons degenerate, the α-synuclein forms clumps, and the motor deficits are exhibited (Rochet et al., 2004). Besides the mutations in α-synuclein, there are five other genes known to cause familial PD: *parkin* (Kitada et al., 1998), *DJ-1* (Bonifati et al., 2003), ubiquitin carboxyl-terminal esterase L1 (*UCHL1*—Funayama et al., 2002), phosphatase and tensin homologue (PTEN)-induced kinase 1 (*PINK1*—Valente et al., 2004), and leucine-rich repeat kinase 2 (*LRRK2*—Paisan-Ruiz et al., 2004). Interestingly, while mutations in other genes result in functionally impaired or inactive protein products, studies of α-synuclein show mutant α-synuclein expression leads to a gain in function of the protein (Eriksen et al., 2003). The substantia nigral dopaminergic neurons of adult rat are selectively vulnerable to high levels of either wild-type or A53T mutated human α-synuclein (Kaplan et al., 2003). In contrast, the loss of α-synuclein appears to have minimal effects. When α-synuclein was knocked out in mice, the organism developed normally similar to wild-type (Abeliovich et al., 2000). Besides the use of animal models for research on PD, budding yeast has also been used to study the pathological properties of α-synuclein (Outeiro and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005).

My thesis is divided into three studies in an attempt to understand three questions. What is the mechanism of α-synuclein aggregation *in vivo*? How α-synuclein's interactions with lipids influence α-synuclein aggregation formation and cellular toxicity *in vivo*? How does oxidative stress contribute to α-synuclein aggregate formation and toxicity? *The following sections will provide the basis of my thesis studies, my aims and hypotheses, and explanation for the model organisms I use to carry out my research.*

*Model organisms to study PD*

*Saccharomyces cerevisiae* (budding yeast) has emerged as a powerful genetic system to model protein misfolding-linked neurodegenerative diseases (Outeiro and Muchowski, 2004), including 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). Using budding yeast models, properties of α-synuclein protein in live cells are being uncovered. Wild-type and A53T mutant α-synuclein were found to associate with plasma membrane and aggregate within cells; whereas the A30P mutant neither localizes to the plasma membrane nor aggregates (Outeiro and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005; Sharma et al., 2006). However, α-synuclein toxicity is not present independently in all of these budding yeast models. In fact, two models demonstrate that α-synuclein aggregation itself causes toxicity (Outeiro and Lindquist, 2003; Dixon et al., 2005). The other two studies find that α-synuclein-dependent toxicity requires an additional chemical or genetic insult, such as proteasomal inhibition and oxidative stress (Zabrocki et al., 2005; Sharma et al., 2006) or coexpression of the microtubule-associated protein tau (Zabrocki et al., 2005). Overexpression of α-synuclein in yeast can lead to abnormal lipid accumulation (Outeiro and Lindquist, 2003) and disrupt vesicle trafficking (Outeiro and Lindquist, 2003; Zabrocki et al., 2005). Further dissecting the unknown molecular pathway of pathogenic misfolded α-synuclein, inhibition of phospholipid metabolism (Outeiro and Lindquist, 2003) and secretory pathway dysfunction (Dixon et al., 2005) can also increase α-synuclein-dependent toxicity. In budding yeast, so far, there are 86 identified genes when absent with overexpressed α-synuclein are synthetically lethal.

I will also employ another family of yeast, *Schizosaccharomyces pombe* (fission yeast). The organism's genome is sequenced which provides us an opportunity to do molecular biology work with it. In the past, fission yeast has provided insight into the mechanism of the cell cycle (Fantes and Beggs, 2000), DNA repair and recombination (Davis and Smith, 2001), and checkpoint controls needed for stability (Humphrey, 2000). Needless to say, fission yeast use as a model organism for protein misfolding neurodegenerative diseases, including PD, has been sparse. Similar to budding yeast, fission yeast share with humans a high conservation of protein folding, stress response, and protein quality control pathways (Wood *et al.* 2002) making the organism a potential good model for PD neuron.

*Mechanism of α-synuclein misfolding and aggregation*

The process of Lewy body formation from misfolded α-synuclein was first tested in *in vitro* experiments. Studies suggest that α-synuclein aggregation follows a hypothetical model called nucleation polymerization (Conway et al., 2000). Previously, misfolded protein aggregation in many neurodegenerative diseases, for instance, β-amyloids in Alzheimer's Disease, huntingtin in Huntington's Disease, and prion proteins in Transmissible Spongiform Encephalopathies were predicted by this model (Caughey and Lansbury, 2003; Perutz and Windle, 2001). In essence, the model states that soluble protein monomers aggregate into polymers over time. The process is facilitated by the formation of a small oligomer that seeds the polymerization process (Figure 2A). Increasing the oligomerization of the protein (seeds) by increasing protein concentration facilitates faster rate of polymerization. Many *in vitro* studies observed the aggregation of wild type and familial mutant (A30P and A53T) forms of purified recombinant α-synuclein follow this model (Conway et al., 1998, 2000; Giasson et al., 1999; Nahri et al., 1999; Wood et al., 1999; Li et al., 2001; Shtilerman et al., 2002). Though α-synuclein fibril formation proposed by nucleation polymerization model has been extensively studied *in vitro*, evidence to support this model *in vivo* are still being conducted.

The nucleation polymerization model proposes an explanation for how misfolded α-synuclein aggregates into the amyloid fibrils similar to Lewy bodies in dying neurons of PD patients (Caughey and Lansbury, 2003). Only recently, fission yeast (*Schizosaccharomyces pombe*) was developed as a PD model organism to study α-synuclein's misfolding, aggregation, and cytotoxic properties (Brandis et al., 2006). In this *in vivo* study, cellular toxicity was absent, but α-synuclein formed aggregates extensively over time and concentration (see Appendix Figuire A.1.). This new study provides evidence for the *in vivo* support of nucleation polymerization model for α-synuclein aggregation. Two concentration levels (high and low) of the protein were expressed in the fission yeast (Figure 2B), but analysis with moderate expression of α-synuclein was not conducted. Thereby, the results provided us with an incomplete picture for the support of the nucleation polymerization model. *For my first thesis goal, I evaluated α-synuclein misfolding, aggregation and toxic properties in fission yeast when the protein was expressed at moderate concentrations.*

*α-Synuclein interactions with lipid membranes*

Many studies have shown α-synuclein to bind to phospholipids (Davidson et al., 1998; Jo et al. 2000; Eliezer et al. 2001; Dixon, et al. 2005; Kim et al. 2006; Rochet et al., 2004) and, this interaction proves to be important in physiological functions such as neuronal plasticity (Lücking, and Brice, 2000; Clayton, and George 1999), neuronal differentiation (Lücking, and Brice, 2000; Kaplan et al., 2003) and secretion of neurotransmitters (Lücking, and Brice, 2000; Fortin et al., 2005; Kaplan et al., 2003; Maroteaux et al., 1988). Though the biology of α-synuclein interaction with lipids has been extensively elucidated with *in vitro* experiments, the *in vivo* consequences of such interaction in PD pathogenesis remain unclear. Close examination of α-synuclein membrane binding *in vivo* will help us elucidate the pathology of the protein in humans.

*In vitro evidence:* In test tube studies, α-synuclein is shown to exist in at least two structural forms. The free α-synuclein is largely unfolded in solution or cytosol, but goes through helical conformations upon binding to lipids (Eleizer et al., 2001). The binding is spontaneous driven by the charged lipid head groups (Kim et al., 2006). The cause of α-synuclein toxicity outside the living system has been investigated. Volles et al. (2001) considered protofibrils, an α-synuclein form between monomer and fibril to be neurotoxic. They bind more tightly to lipid membranes than the monomeric and fibrillar form of α-synuclein (Volles et al., 2001). If protofibrils were present in the cells, the cellular environment probably would try to cope with protofibril toxicity by driving the protein to fibril form. Indeed, evidence of α-synuclein fibril formation through protective cellular mechanism has been seen. In model systems of PD, α-synuclein fibrils form without associated toxicity (Rochet et al., 2000). But the protofibril membrane permeabilization activity could crucially account for the toxicity of α-synuclein in PD (Scherzer and Feany, 2004; Conway et al., 2001).

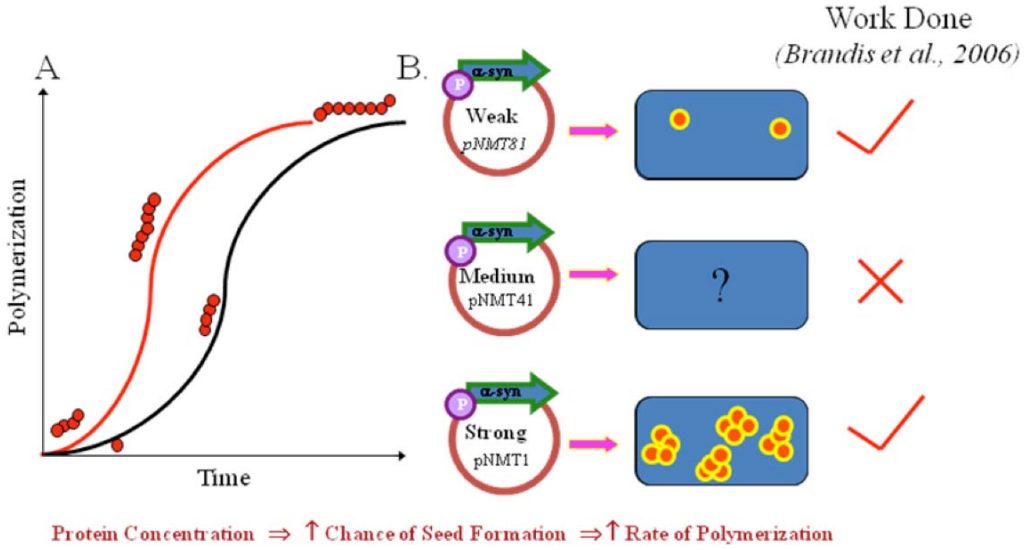



**Figure 2. Hypothesis for misfolded a-synuclein aggregation in fission yeast.** A. Illustration of the nucleation polymerization model. The process of polymerization for the protein starts as soluble monomer, changing to oligomer (seed) and then finally precipitating out of the solution as polymer. This process is time and concentration dependent (initial concentration on the red curve is higher in comparison to the black curve). B. Brandis et. al (2006) employ vectors to express a-synuclein in vivo at various concentration levels. Accordant with the nucleation polymerization hypothesis, a-synuclein aggregates increased as the protein concentration increased. No analysis was done where a-synuclein is moderately expressed.

*In vivo evidence:* A correlation of α-synuclein membrane binding with cellular toxicity exists (Dixon et al., 2005; Chua and Tang, 2006; Cooper et al., 2006). Similar to binding of α-synuclein to artificial phospholipid vesicles, α-synuclein's lipid binding in the cells is mediated by α-helix formation in the N-terminal repeat region (Kim et al., 2006). However, PD familial A30P mutation, but not A53T, reduces membrane binding. In contrast to *in vitro* study with artificial membranes, α-synuclein interaction with biological membranes is rapidly reversible and is not driven by electrostatic attraction. Rather, α-synuclein's lipid binding demonstrates to be complex in live cells as it is not determined solely by intrinsic properties of the protein or the membranes (Kim et al., 2006).

Using cell cultures and animal models of PD, possible pathological interaction between α-synuclein and lipids has been linked more closely. Transgenic mouse model for PD shows that the α-synuclein primarily localizes to highly lipid neuronal region of synaptosomes (Khale et al., 2003). In the yeast *Saccharomyces cerevisiae* PD model, both the WT and the A53T isoforms of α-synuclein localize to the plasma membrane (Dixon et al., 2005; Outeiro and Lindquist, 2003). They have been delivered to the membrane via the secretory pathway. In contrast, the A30P mutant protein disperses within the cytoplasm and has no binding with the plasma membrane. When their expression is elevated, WT and A53T, but not A30P, are toxic to cells. In this study, a direct relationship is present between plasma membrane localization of α-synuclein and its toxicity in yeast (Dixon et al., 2005).

Yeast continues to serve as a good model system for PD. A screen of several thousand library clones in yeast identified 25 non-toxic α-synuclein sequence variants (Volles et al., 2007). Interestingly, these variants had a mutation to either proline or glutamic acid, which cause a defect in membrane binding. Based on these findings, it is possible yeast toxicity arise by α-synuclein binding directly to membranes at levels sufficient to disrupt cell's homeostasis (Volles et al., 2007).

In our lab, we work with two yeast model organisms that express α-synuclein. However, only budding yeast show α-synuclein dependent toxicity (Sharma et al., 2006). These cells also have localization of α-synuclein to the plasma membrane. We speculate that the lack of membrane association in fission yeast may be due to changes in plasma membrane phospholipid composition. Budding and fission yeasts have slight different phospholipid composition, particularly phosphatidylethanolamine (Fernandez et al., 1986). In *in vitro* study, evidence of α-synuclein interaction with phospholipids is strong. The protein is known to interact with the phosphatidylinositol signaling pathway (Narayanan et al., 2005) and preferentially binds to acidic phospholipids such phosphatidylethanolamine (PE) and phosphatidylserine (PS); (Jo et al., 2000; Perrin et al., 2000).

Both *in vitro and in vivo* evidence give us clues about how might α-synuclein, in lipid binding state, become toxic given that the pathological conditions exist (e.g. oxidative stress); (Figure 3). In living cells, α-synuclein reversible lipid binding property can be altered to one which is irreversible when there is cellular oxidative stress. Dopaminergic neurons are possibly vulnerable to oxidative stress because dopamine quinones from the oxidation of dopamine stabilize lipid bound α-synuclein species (Conway et al., 2001).

We would like to determine whether the induction of phospholipids production directly correlates with the increase of α-synuclein's interaction with phospholipids and cellular toxicity. Secondly, we would like to determine which specific phospholipid(s) are possibly involved in α-synuclein binding to the plasma membrane. If those phospholipid(s) are lost, would we observe reduction in α-synuclein toxicity? *Thus, in my second thesis goal, I investigated these questions in budding and fission yeast by genetically and chemically regulating the cellular phospholipid content.*

*Oxidative Stress and PD*

Oxidative stress occurs largely due to mitochondrial dysfunction where stress is put on the cell by the production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and the hydroxyl radical (Abou-Sleiman et al., 2006). ROS within the cells can damage cellular structures which comprise of lipid membranes via lipid peroxidation (Giorgio Lenaz, 1998). ROS can also modulate important metabolic and other cellular activities by oxidizing critical proteins, and causing mutations in the mitochondrial DNA (mtDNA) (Alam et al, 1997). The inhibition of complex I of the mitochondria has been shown to generate ROS, which can damage the electron-transport chain and lead to the further production of ROS (Dauer, and Przedborski, 2003). In PD human brains and model organisms, several studies have confirmed the deficiency of complex I of the mitochondria (Parker 1989; Schapira, 1998; Simon et al., 2000; Valente et al., 2004; Devi et al., 2008). Interestingly, the death of dopaminergic neurons has been shown to increase when complex I of the mitochondria is deficient (Kweon et al., 2004).

Several PD inducing molecular species have been discovered which induce oxidative stress and lead to death of dopaminergic neurons. For example, the drug 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) can cause extensive death of dopaminergic neuronal in the substantia nigra. This drug has been used to model PD in non-human primates (Burns et al., 1986), mice (Ogawa et al., 1985), rats (Saghal et al., 1984) and zebrafish (Bretaud et al., 2004) resulting in an acute and irreversible parkinsonian syndrome. When examined closely, the dose of MPTP inhibited function of complex I of the mitochondrial electron transport chain. Mitochondrial dysfunction was also exhibited in rat model for PD where the organism was exposed to rotenone, a specific mitochondrial complex I inhibitor. The exposure to rotenone brought about key features of PD, particularly the cytoplasmic, α-synuclein fibrils (Dawson and Dawson, 2003).

The role of oxidative stress in PD using yeast models appears to be promising. Recently, α-synuclein's toxicity can be enhanced with oxidative stress in budding yeast (Sharma et al., 2006). On the contrary, fission yeast model for PD can be robust when α-is overexpressed (Brandis et al., 2006). Perhaps, additional stress on fission yeast is required for occurrence of α-synuclein cellular toxicity. The path to cellular toxicity might involve that both α-synuclein expression and oxidant accumulation are present (Figure 4). *Thus for my third thesis goal, I evaluate the toxicity of α-synuclein in fission yeast when the organism exhibits oxidative stress.*

*Hypotheses and Aims*

*Study 1: Hypothesis: α-Synuclein misfolding and aggregation in live cells will follow the nucleation-polymerization model.* The aim of the first study was to moderately express α-synuclein in fission yeast and compare α-synuclein aggregation and toxicity with previously seen. I predicted that α-synuclein would aggregate at an intermediate level and aggregates would appear later in time course than at higher concentration. Plus, these cells would not be toxic.

*Study 2: Hypothesis: Increasing phospholipid association of α-synuclein will enhance cellular toxicity of the protein.* The aim was to increase (via DMSO treatment) or decrease (via deletion of a gene) cellular phospholipids and

observe any difference in α-synuclein's localization and cellular toxic properties. I predicted that α-synuclein in the budding yeast would localize more to the plasma membrane in the presence of DMSO compared to the control condition. In the fission yeast, I predicted that the chemical would decrease the formation of cytoplasmic aggregates and increase α-synuclein's plasma membrane localization. Also, both yeasts would incur cellular toxicity.

*Study 3: Hypothesis: α-Synuclein dependent toxicity will enhance in the presence of oxidative stress.* The aim of this study was to evaluate the effects of α-synuclein dependent toxicity in the presence of oxidative stress using fission yeast as a model organism. We added exogenous oxidant (hydrogen peroxide), and I predicted that α-synuclein toxicity will enhance in a combinatorial manner with oxidative stress in fission yeast. Lastly, I predicted that α-synuclein aggregates would be altered under oxidative stress.

The next three sections will detail the materials and methods, results and discussion of the three studies—Chapter 1: Live cell evidence of nucleation polymerization model of α-synuclein misfolding and aggregation: Concentration Is Key; Chapter 2: Does cellular toxicity require α-synuclein association with lipids?; Chapter 3: Does oxidative stress trigger cellular toxicity in α-synuclein expressing fission yeast?

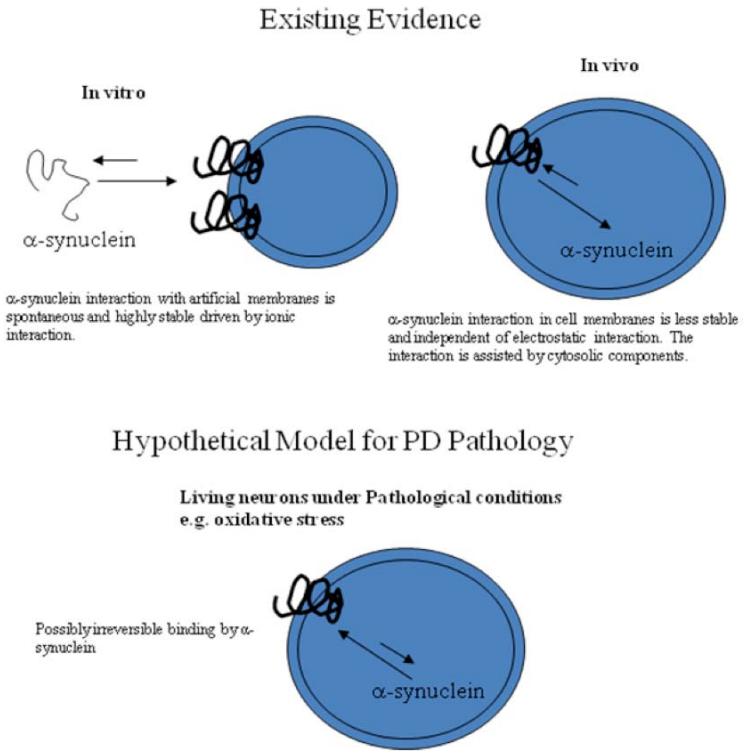



**Figure 3. a-Synuclein's lipid binding properties.** Could the way a-synuclein bind in living system under pathological condition like oxidative stress may provide clue to mechanism of a-synuclein dependent toxicity? Such cellular condition would contain free oxygen radicals. α-Synuclein rapid dissociation from lipid membrane may be impacted. Though otherwise regulated step by the cellular components, the tight irregular lipid-binding of α-synuclein is hypothesized to cause damage to cells. Figure adapted from Kim, Y. S. et al (2006).

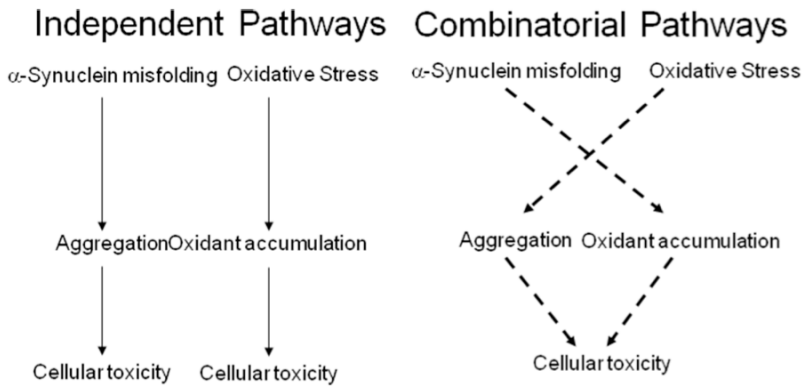



**Figure 4. Pathways for oxidative stress and a-synuclein cellular toxicity.** The independent pathways state that a-synuclein aggregation and oxidant accumulation are toxic to the cell via separate pathways. The combinatorial pathways proposes that a-synuclein misfolding may lead to oxidant accumulation, and oxidative stress may influence a-synuclein aggregation. Both outcomes perhaps together contribute to cellular toxicity.

**Materials and Methods**

*S. Cerevisiae Expression Vectors*
The pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen) was used for all α-synuclein expression in budding yeast. The following three vector constructs were previously created for each experiment: green fluorescent protein (GFP), and wild-type α-synuclein. α-Synuclein was tagged at the C-terminus with GFP. For all experiments, the pYES2.1/V5-His-TOPO vector and the GFP tagged vector were used as expression controls (See Table 1 for List of Constructs)

*S. Pombe Expression Vectors*
Polymerase chain reaction (PCR) was used to amplify C-terminal green fluorescence protein (GFP)-tagged α-synuclein (wild-type, A30P, A53T, A30P/A53T) fusion cDNA from the α-sunclein-GFP containing pYES2/TOPO *S. cerevisiae* vectors constructed by Sharma et al.(2006): forward primer, 5'-GGGGCCAAGCTTGCCATGGATGTATTCATGAAAGGA-3'; reverse primer, 5'-TTTGTAGAGCTCATACATGCCATG-3'. Similarly, PCR was used to amplify GFP cDNA from GFP-pYES/TOPO *S. cerevisiae* vectors constructed by Sharma et al. (2006): forward primer, 5'CCCGGGACCATGGCCAGCAAAGGAGAAG-3'; reverse primer, 5'-TTTGTAGAGCTCATACATGCCATG-3'.

These PCR products were subcloned, according to the manufacturer's protocol (Invitrogen), into each of these two fission yeast pNMT TOPO-TA expression vectors: pNMT1 (for high expression), and pNMT41 (for intermediate expression) vectors. The following three vector constructs were previously created for each experiment: green fluorescent protein (GFP), and wild-type α-synuclein. α-Synuclein was tagged at the C-terminus with GFP. The pNMT-1/41 vectors and GFP in pNMT-1/41 vector served as expression controls (see Table 1 for List of Constructs).

*Yeast Strains*
The TCP1 strain (h-*leu1-32*; Invitrogen) of fission yeast was kindly provided by Judy Potashkin, Rosalind Franklin University of Medicine and Science. BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and CHO1Δ knockout strain were purchased from Open Biosystems.

*Yeast Transformation*
*S. pombe* strains were transformed with pNMT TOPO-TA vectors (Alfa et al., 1993) and *S. Cerevisiae* were transformed with pYES2.1/V5-His-TOPO vectors (see table 1.) using the lithium-acetate transformation method (Burke et al., 2000).

*S. Pombe Expression*
For selection, yeast cells were grown on synthetic-complete media lacking leucine (PDM-leu). Presence of α-synuclein constructs was confirmed by polymerase chain reaction (PCR). The pREP vector, containing a thiamine repressible promoter, allowed for regulated α-synuclein expression. Yeast cells were first grown overnight in PDM-leucine (10uM thiamine) media at 30°C. Cells were washed with water and diluted to log-phase (5 × 10^6 cells/mL) in EMM lacking thiamine media to induce expression and grown to the time points desired for various measurements.

*S. Cerevisiae Expression*
For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-URA). Presence of α-synuclein constructs was confirmed by polymerase chain reaction (PCR). The pYES2.1 vector, containing a galactose-inducible promoter (GAL1), allowed for regulated α-synuclein expression. Yeast cells were first grown overnight in SC-URA glucose (2%) media at 30°C. Cells were washed with water and diluted to log-phase (5 × 10^6 cells/mL) in SC-URA galactose (2%) media to induce expression and grown to the time points desired for various measurements.

*Fluorescence Microscopy*
Yeast cells were first grown overnight at 30$^{o}$C in Edinburgh minimal medium (EMM) containing thiamine (10uM) if working with *S. Pombe* or in SC-URA glucose if working with *S. Cerevisiae*. After 24h, cells were pelleted at 1500g for 5 minutes, washed twice in 10ml $dH_2O$, resuspended in 10 ml EMM without thiamine/SC-URA galactose, of which 125 mL cells were used to inoculate 25 mL EMM without thiamine/SC-URA galactose (to express α-synuclein). At desired expression time points for microscopy, cells were harvested at 1500g (4$^{o}$C) for 5 min and were washed in 5 mL water. Then cells were resuspended in 100-1000 uL EMM+T/SC-URA glucose, of which 10 uL was pipetted onto a slide. Slide of cell culture was viewed using Nikon TE-2000U fluorescence microscope at 1000X magnification. Images were deconvoluted using MetaMorph software version 4.2. In order to quantify α-synuclein aggregates, cells were first viewed under differential interference contrast (DIC) microscopy, and total cell count in the field was determined and viewed for GFP fluorescence. For analysis on *S. Pombe,* the number of cells in the field containing 1, 2, and 3+ aggregates or intracellular structure localization was determined. In case of analysis on *S. Cerevisiae,* the number of cells in the field which display halo, or aggregates (foci) was determined. The field was then moved three turns on the field control knob, and the process was repeated in a new field. At least 750 cells were evaluated for each treatment. Aggregates were scored as percent of total cells in the field that expressed 1, 2, and 3+ aggregates.

*Western Analyses*
Yeast cells (2.5x10$^{7}$ cells/ml) were washed in 50 mM Tris (pH 7.5), 10 mM $NaN_3$ and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000) containing 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). Samples were run on pre-cast 10-20% Tris-Glycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes and Western blot was performed. For analysis on *S. Cerevisiae*, anti-V5 AP monoclonal antibody and anti-PGK (Molecular Probes) for loading control was used. For *S. Pombe*, anti-V5 AP monoclonal antibody and anti-beta actin (Molecular Probes) for loading control was employed. The immunodetection protocol from Invitrogen Western Breeze kit was used to detect the alkaline phosphatase activity.

*Growth Curve*
Cells were grown in 5 ml PDM-Leu/SC-URA glucose overnight at 30$^{o}$C in the incubator which rotates at 200 rpm. Cells were harvested at 1500 x g for 5 min at 4¢XC, and were washed twice in 5 ml $H_20$. Cells were re-suspended in 5 ml $H_20$ and were counted. Flasks with 25 ml EMM/SC-URA galactose were each inoculated with 2.0x10$^{6}$cells/ml density. At 0, 6, 12, 18, 24, 36 and 48 hours, and in duplicate measurements, 1 ml of cell culture was removed and placed in a cuvet to measure absorbance using a Hitachi U-2000

Spectrophotometer. Averaged absorbance readings were plotted against time points to produce a growth curve.

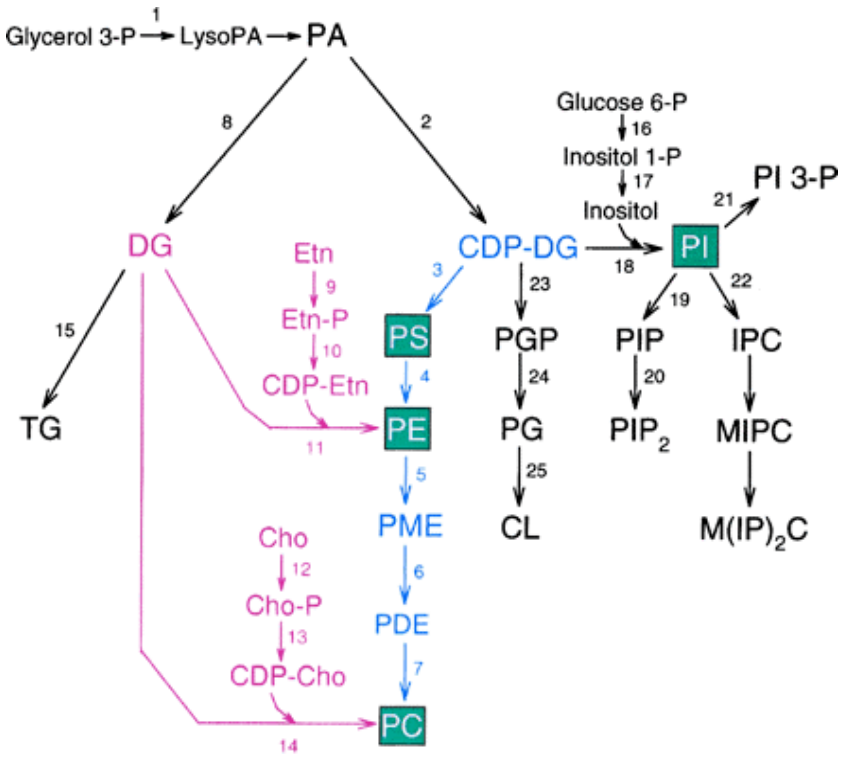
*Spotting Analysis*
For spotting, cells were grown to mid-log phase in PDM-leu (*S. Pombe*) or in SC-URA glucose (2%) (*S. Cerevisiae*), normalized to equal densities (2 × 107 cells/mL), serially diluted (5-fold) into 96-well microwell plates. Using a multi-channel pipettor, culture is spotted on EMM + thiamine or EMM – thiamine plates in the case when working with *S. Pombe* and SC-URA glucose (2%) or galactose (2%) plates when working with *S. Cerevisiae*. Photographs were scanned after 2 d of growth.

*DMSO Treatment*
Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Cells were grown in 10 ml PDM-leu (*S. Pombe*) or in SC-URA glucose (2%) (*S. Cerevisiae*) overnight at 30°C. Steps of cell harvesting and calculating cell density for inoculation was done according to the appropriate experiment. The cells which expressed α-synuclein cloned in pNMT-1 vector were used when treating *S. Pombe* (TCP1) with DMSO. When treating *S. Cerevisiae* (BY4741) with DMSO, cell which expressed α-synuclein in pYES2 vector were used. Before inoculation, DMSO was added in concentrations ranging from 0 to 10% in EMM media (*S. Pombe*) or in SC-URA galactose media (*S. Cerevisiae*). The concentration of DMSO exposure did not exceed 10%. A higher dose than 10% of DMSO would do harm to the cell (Zabrocki et al., 2005). After the DMSO mixed well in the inducing media, yeast cells were inoculated into it. Then the cells were observed at the desired time points for the following examinations: Western Analysis, Growth Curves, Spotting and Fluorescence Microscopy.

*Analysis on cho1Δ strain*
The deficiency in phospholipid content was produced using a knocked out yeast strain that lacks the gene CHO1 which encodes for phosphatidylserine synthase. The phospholipid biosynthetic pathway (see below; adapted Carman and Zeimetz, 1996) in yeast is a reference for understanding how specific phospholipid-deficient state is established. For this thesis, the main pathway of import is in blue and is termed the de novo pathway for synthesis of PS, PE and phosphotidycholine (PC). The purple pathways, called the Kennedy pathway are for using exogenous choline or ethanolamine to produce those phospholipids. The parent strain is BY4741. To stop PS production (it would still have all the rest of the phospholipids if ethanolamine present), we grew cho1Δ yeast strain in 1 mM ethanolamine in every selected media. Protocols written above for *S. cerevisiae* experiments such as growth curves, and live cell microscopy were followed.

*$H_2O_2$ treatment in S. Pombe*
Protocols written above for *S. Pombe* experiments such as growth curves, spotting and live cell microscopy were followed. $H_2O_2$ was added to the inducing liquid media EMM without thiamine or 25ml EMM agar plates (spotting analysis) to a final concentration of 0.4mM or 2mM. The control culture was cells untreated with $H_2O_2$.

*Statistical Analyses*
Statistical significance was established using a T-Test comparing the means of control cells containing no α-synuclein at 18 and 24 hours in treated versus untreated conditions.




**Phospholipid biosynthetic pathways in *Saccharomyces cerevisiae*.** Reaction 3 in our study is terminated due to absence of PS synthase. The four major phospholipids (PC, PE, PI, and PS) are indicated by green boxes (Carman and Zeimetz, 1996).

| Construct | Expression Vector | Strain |
|---|---|---|
| ***Used in Chapter 1 Study*** | | |
| No cDNA (parent plasmid) | pNMT41 | TCP1 |
| GFP | pNMT41 | TCP1 |
| A30P *α*-Synuclein-GFP | pNMT41 | TCP1 |
| A53T *α*-Synuclein-GFP | pNMT41 | TCP1 |
| A30P/A53T *α*-Synuclein-GFP | pNMT41 | TCP1 |
| ***Used in Chapter 2 and 3 Studies*** | | |
| No cDNA (parent plasmid) | pNMT1 | TCP1 |
| GFP | pNMT1 | TCP1 |
| WT *α*-Synuclein-GFP | pNMT1 | TCP1 |
| No cDNA (parent plasmid) | pYES2.1 | BY4741 |
| GFP | pYES2.1 | BY4741 |
| WT *α*-Synuclein-GFP | pYES2.1 | BY4741 |
| No cDNA (parent plasmid) | pYES2.1 | *cho1Δ* |
| GFP | pYES2.1 | *cho1Δ* |
| WT *α*-Synuclein-GFP | pYES2.1 | *cho1Δ* |

**Table 1. GFP-tagged α-synuclein constructs created for thesis experiments.**

**Chapter 1: Live cell evidence of nucleation polymerization model of α-synuclein misfolding and aggregation: Concentration Is Key**

**Results**

*Moderately expressed α-synuclein aggregates in live cells*

Our first goal was to moderately express α-synuclein in fission yeast. Therefore, we used the PNMT-41 vector for α-synuclein expression, which was previously shown to achieve moderate expression by Western blot analysis (Brandis et al., 2006; see Appendix Figure A.1.). For this study, we used two familial mutants (A30P and A53T) because they localize differently under high expression in fission yeast: A30P remains cytoplasmically and A53T aggregates extensively (Brandis et al., 2006; see Appendix Figure A.1.). Using C-terminal GFP tagged α-synuclein constructs, we then examined α-synuclein localization in live cells by GFP microscopy. Time course microscopy revealed that A53T α-synuclein formed aggregates as early as 18 hour (Figure 5A). Quantification of aggregates confirmed this qualitative finding (Figure 5B). In contrast, A30P did not form aggregates and remained cytoplasmically throughout the time course. Interestingly, the double mutant A30P/A53T behaved similar to A30P indicating the dominance of A30P over A53T (Figure 5A). Therefore, under moderate expression, these three mutants achieve the same localization patterns, seen under high expression of α-synuclein (Brandis et al., 2006; Appendix Figure A.1).

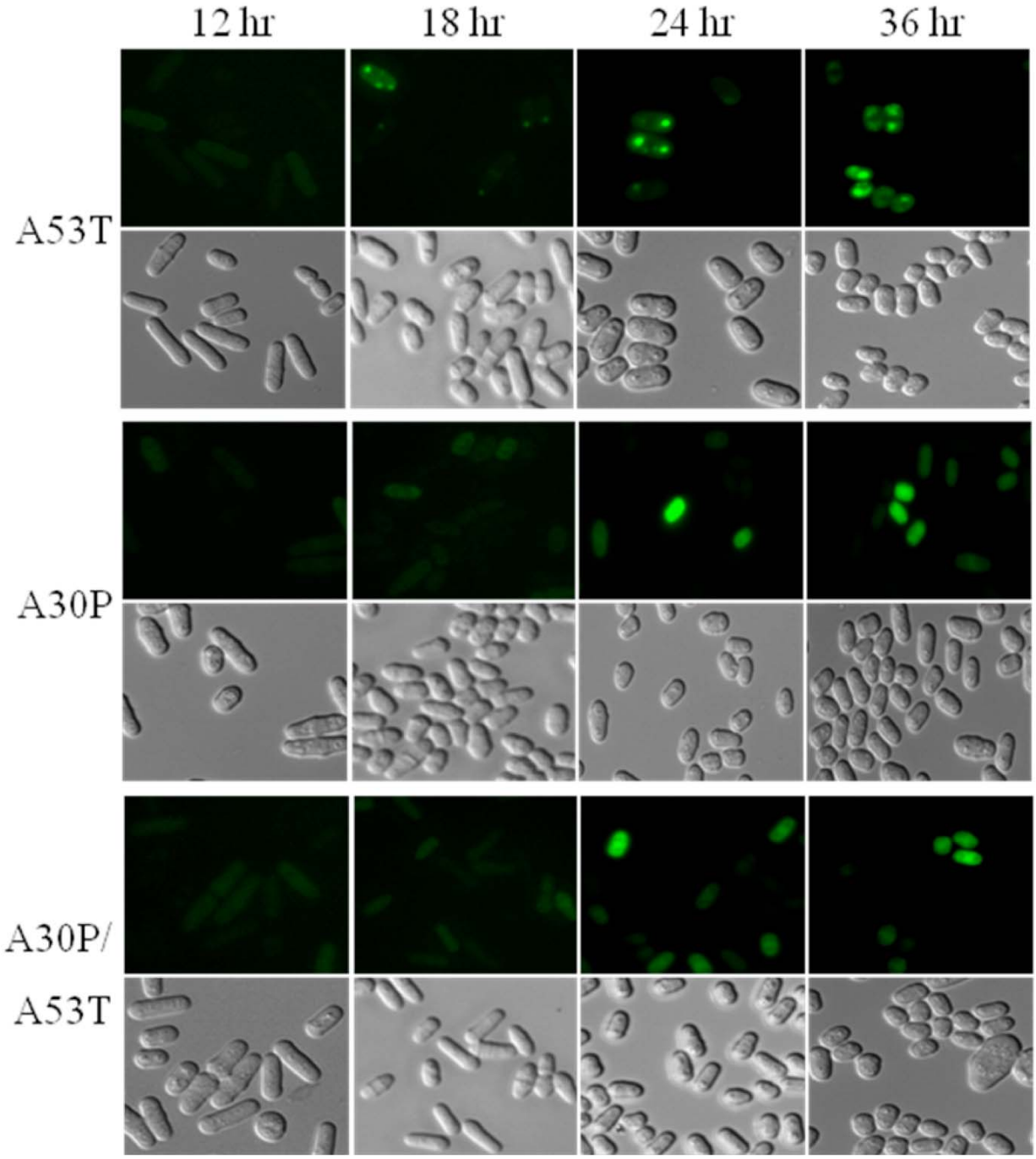
*Moderately expressed α-synuclein aggregates lesser and later*

Next, we compared the aggregation of A53T at high and moderate concentrations over a time course. Firstly, highly expressed α-synuclein aggregates earlier than moderately expressed α-synuclein (Figure 5C). Secondly at every point of the time course, highly expressed a-synuclein formed greater number of aggregates than moderately expressed synuclein (Figure 5C). This intermediate aggregates formation with moderately expressed α-synuclein supports our initial hypothesis.

*Moderately expressed α-synuclein is not toxic*

Our last goal was to examine toxicity of cells moderately expressing α-synuclein. As expected, control cells containing just pNMT-1 grew healthy (Figure 5D). Cells expressing either A30P, A53T or A30P/A53T grew just as well as the control cells, demonstrating a lack of toxicity (Figure 5D). This lack of toxicity supports our initial hypothesis.

pNMT41 Medium Expression



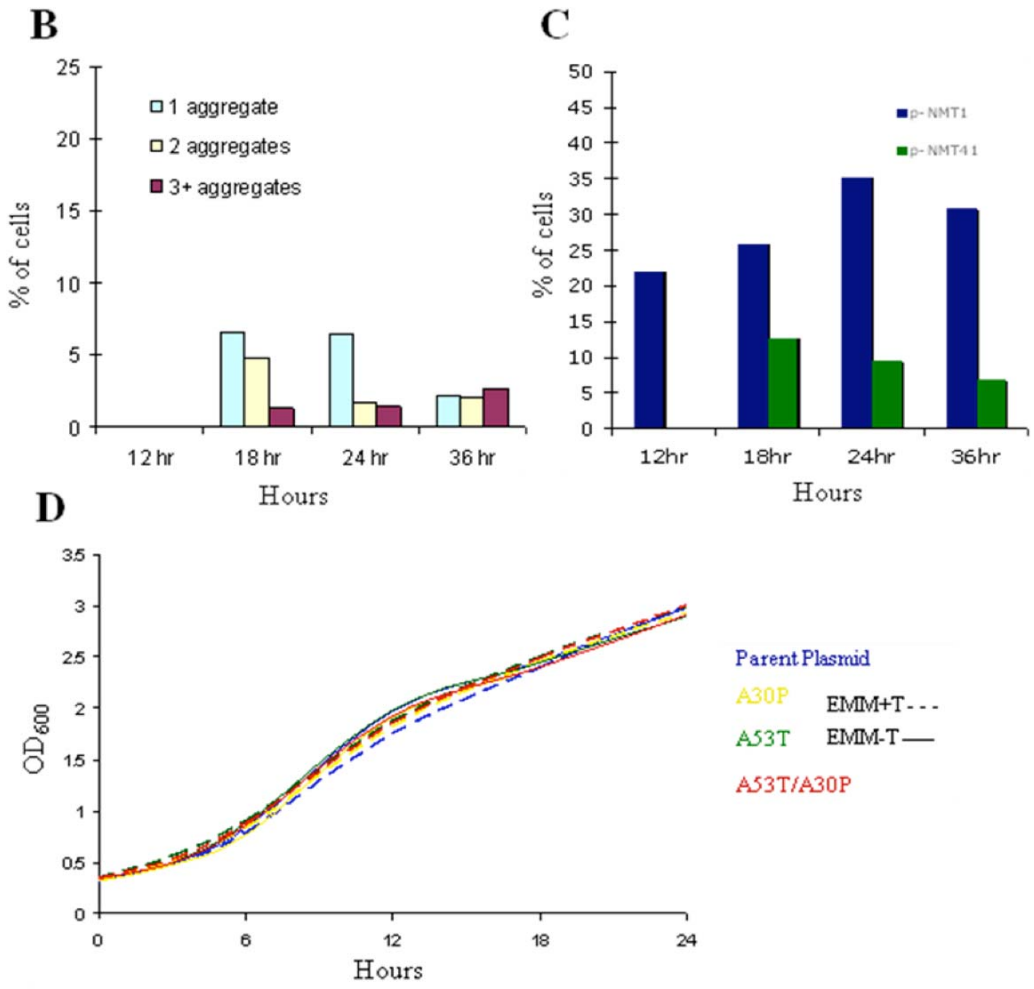



**Figure 5. Analysis of moderately expressed a-synuclein.** A. α-Synuclein was expressed in moderate concentration by pNMT41 promoter vector. These yeast were grown in EMM without thiamine. Images were captured at the indicated times over 36 hours for cells expressing A53T, A30P and A30P/A53T a-synuclein. B. Time course of quantified A53T α-synuclein aggregation. Cells, cultured in EMM media without thiamine, expressed α-synuclein in moderate concentrations. The number of aggregates formed was was quantized in cells expressing A53T α-synuclein over a 36-hr time course. Cells were scored in terms of containing 1 aggregate (blue bar), 2 aggregates (yellow bar), or 3 or more aggregates (red bar). Bars represent percentage of total cells counted in each sample that exhibited the designated number of aggregates per cell. C. Comparison of moderate to high expression of quantified A53T α-synuclein aggregation. Comparison of quantified data on A53T α-synuclein aggregates with high expression (blue bar) (Quantification data provided by Brandis et al. 2006) compared to moderate expression (green bar) over the 36-hr period. Bars represent percentage of total cells that had 1 or more aggregates per cell. D. Growth curve of cells moderately expressing a-synuclein. OD600 measurements were obtained over 48 hours for cells containing A30P, A53T, and A30/A53T forms of α-synuclein in pNMT41 vector three separate times and averaged (n=3). Cells grown in thiamine (dashed lines) served as control.

## Discussion

With the past study by Brandis et al. (2006) and this current study, we have now completed the successful development of a fission yeast as a model organism to study the misfolding, aggregation, and cytotoxic properties of α-synuclein linked to Parkinson's disease. Specifically, the work with fission yeast sheds provocative insight into the ideas that concentration of α-synuclein is important for the protein's aggregation and α-synuclein aggregation may protect again cellular toxicity.

*α-Synuclein aggregation: concentration is key*

Our first finding is that the protein's polymerization activity is concentration dependent. At high concentrations, many α-synuclein aggregates are observed, while at low concentrations α-synuclein aggregates are absent (Brandis et al, 2006). In the current study, at moderate concentration, α-synuclein forms an intermediate number of aggregates. According to data by Brandis et al. (2006), the protein expression of moderate concentration was only slightly higher than the protein expression of the low concentration. The low concentration of α-synuclein was not enough to lead to the formation of insoluble aggregates. Therefore, the evidence indicates that slightly higher (moderate) concentration of α-synuclein is required to form aggregates. We consider the moderate concentration or a concentration near to it as the threshold point of α-synuclein protein concentration that must be present to turn the soluble protein into insoluble aggregates. As protein concentration increases, the oligomers polymerize to form greater numbers of aggregates. Moreover, α-synuclein aggregation activity is time-dependent. Overall, Brandis et al. (2006) and this study display both concentration and time-dependent α-synuclein

aggregation properties which support the grounds for the nucleation polymerization model *in vivo*.

*Toxicity: is membrane localization key?*
Our second finding is that fission yeast presents itself as a robust organism despite the extensive formation of α-synuclein aggregates. To this date, in a fission yeast model, membrane localization of α-synuclein has not been observed with cytotoxicity. In the budding yeast model, there is evidence of membrane localization with toxicity (Sharma et al., 2006). This leads to the hypothesis that membrane localization might be key to cytotoxicity. We did not observe α-synuclein dependent toxicity in fission yeast, but we still suspect that toxicity requires α-synuclein membrane localization. In a previous study by Rochet et al. (2004), membrane localization of α-synuclein was shown to be essential in creating toxic protofibrils. Under a different study, the destruction of vesicular membranes by protofibrillar α-synuclein was directly observed by atomic force microscopy (Volles et al., 2001). In our lab, the toxicity of budding yeast was observed when α-synuclein localized at the cell periphery. In budding yeast, the localization of α-synuclein to the plasma membrane happened prior to the formation of α-synuclein aggregates. With the exception of A30P, wild-type and A53T α-synuclein localized to the plasma membrane before forming inclusions (Sharma et al., 2006). On the other hand, in our fission yeast model, neither toxicity nor membrane association was observed. Overall, a connection between α-synuclein-dependent toxicity and a α-synuclein's association with the plasma membrane is strongly implicated.

**Chapter 2: Does α-synuclein binding to phospholipids regulate its toxicity?**

**Results**

*DMSO induces α-synuclein independent toxicity in fission yeast*
Our first goal was to evaluate if a global induction of phospholipid synthesis would increase α-synuclein-dependent toxicity in fission yeast. We used a known phospholipid inducer in yeasts, the organic solvent DMSO (Murata et al., 2003). Toxicity was assessed in two ways. First, OD600 growth curves were performed on cells with or without a-synuclein and treated them with 0, 2, 5, and 10% DMSO. As previously reported (Brandis et al. 2006), wildtype α-synuclein-GFP expressing cells that lacked DMSO grew well (Figure 6A). However, as DMSO concentration increased, and particularly at 10%, the growth curve shifted significantly to the right, indicating a toxic effect (Figure 6B-C). Surprisingly, this toxicity was not α-synuclein dependent, as it was seen even with cells that expressed GFP alone or only contained parent vector (Figure 6A-C). α-Synuclein, at best, enhanced this DMSO toxicity by a very small margin (Figure 6C). Secondly, we evaluated colony survival on plates by serial dilution. Cell cultures either containing parent vector, GFP or, wild-type α-synuclein serially diluted 5 times, were spotted onto sets of repressing (+thiamine) and inducing (-thiamine) plates, that contained 0, 2, 5, and 10% DMSO. Similar to growth curve analysis, we observed a striking dose-dependent toxicity with DMSO that was not dependent of the presence of a-synuclein (Figure 7).

Together, both toxicity assays indicate that DMSO exerted an unexpected toxicity in fission yeast independent of α-synuclein presence.

*DMSO alters α-synuclein localization to internal structures in fission yeast*
Next, we evaluated if DMSO would induce any plasma membrane localization of α-synuclein in fission yeast. α-synuclein localization was assessed by live cell GFP imaging. As previously reported (Brandis et al. 2006), wildtype α-synuclein-GFP cells not treated with DMSO showed distinct cytoplasmic aggregates of α-synuclein (Figure 8). DMSO treatment, at 5 and 10%, induced α-synuclein to alter its localization to internal cellular structures (Figure 8). Upon quantification, we determined that with increasing DMSO concentration, while α-synuclein aggregates were still present, an increasing percent of cells began to display internal cellular structures (Figure 9).

*DMSO also induces α-synuclein independent toxicity in budding yeast*
We conducted parallel studies in budding yeast using the strain BY4741 and asked if DMSO would increase α-synuclein-dependent toxicity. Again, we assessed toxicity in two ways. First, OD600 growth curves were performed on cells with or without α-synuclein expression and treated them with 0, 2, 5, and 10% DMSO. Wildtype α-synuclein-GFP expressing cells, independent of DMSO treatment, exhibited slight toxicity (Figure 10A). However, as DMSO concentration increased, and particularly at 10%, the growth curve shifted significantly to the right, indicating a toxic effect (Figure 10B-C). However, like seen with fission yeast, the toxicity was not specific to α-synuclein. Cells that expressed GFP alone or only contained parent vector also exhibited similar magnitude of growth inhibition (Figure 10A-C). Secondly, we evaluated toxicity by spotting analysis. Cell cultures either containing parent vector, GFP or, wild-type α-synuclein were spotted onto sets of repressing (glucose) and inducing (galactose) plates, that contained 0, 2, 5, and 10% DMSO. Because this 10% DMSO toxicity was unexpected and contrary to published findings (Murata et al. 2003), we wondered if this DMSO toxicity was the result of the carbon source, galactose. But we found that 10% DMSO exhibited the same toxicity in cells grown on glucose (Figure 11A.) Lastly, we even considered that the toxic effect of DMSO possibly was peculiar to the BY4741 strain we had used. However, 10% DMSO demonstrated the same toxicity in a second yeast strain, TSY623 (Figure 11B).

Similar to growth curve analysis, we observed a striking dose-dependent toxicity with DMSO that was not dependent of the presence of α-synuclein (Figure 12). Together, both toxicity assays indicate that DMSO exerted an unexpected toxicity in budding yeast independent of α-synuclein presence.

*DMSO induces α-synuclein aggregates and aberrant morphology*
Next, we evaluated if DMSO would induce additional plasma membrane localization of α-synuclein in budding yeast. As previously reported (Sharma et al. 2006), untreated wildtype α-synuclein-GFP cells showed α-synuclein localizing to the plasma membrane. After these cells were treated with DMSO for 18 hour and 24 hour, GFP images were captured. At 5% DMSO, two major changes occurred: 1) Cells adopted aberrant morphology and they appeared elongated and sickle-shaped rather than as normal round shape; and 2) α-synuclein gained some intracellular localization that appeared like aggregates (Figure 13). Quantification of cellular morphology indicated that the aberrant cellular morphology was highest at 5% DMSO treatment (Figure 14). Localization patterns indicated that in the normal shaped cells α-synuclein primarily localized at the plasma membrane, while in the abnormal shaped cells, α-synuclein significantly aggregated in the cytoplasm (Figure 15).

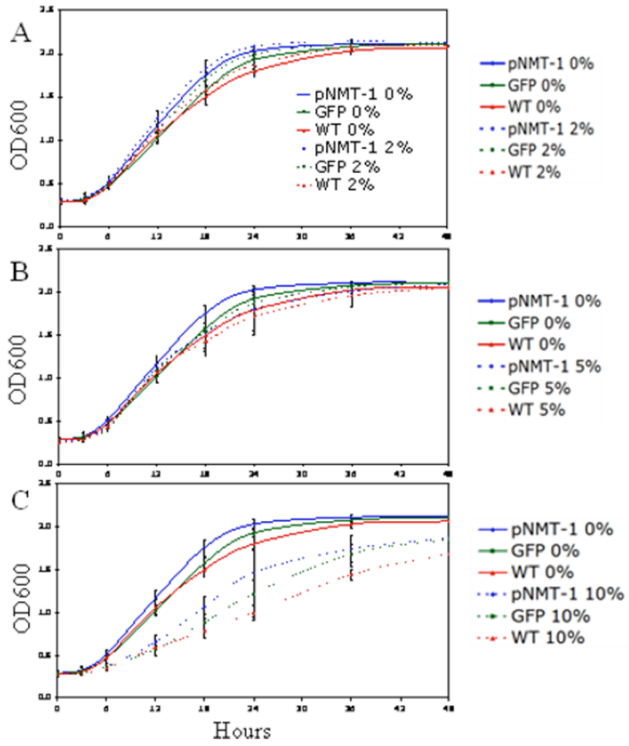



**Figure 6. Growth curves of fission yeast cells treated with DMSO.** Fission yeast cells were grown in EMM-T for 48 hours (x-axis) to induce parent pNMT-1 plasmid, GFP, and WT (wild-type) a-synuclein. Over this time course, the cells were treated with 2% (A), 5% (B) or 10% (C) DMSO (dashed lines) and compared to the cells that were not treated with DMSO (solid lines). Each of the points on the curves represents the mean from three trials on measurement of absorbance (y-axis). In addition, the standard error of the mean for a 95% confidence interval are represented by the y-bar on each of the points on the growth curves. Significant growth difference exists between pNMT-1 in 0% DMSO (n=3, P18hour= 0.007764 and P24hour= 0.098409) and pNMT-1 in 10% DMSO.

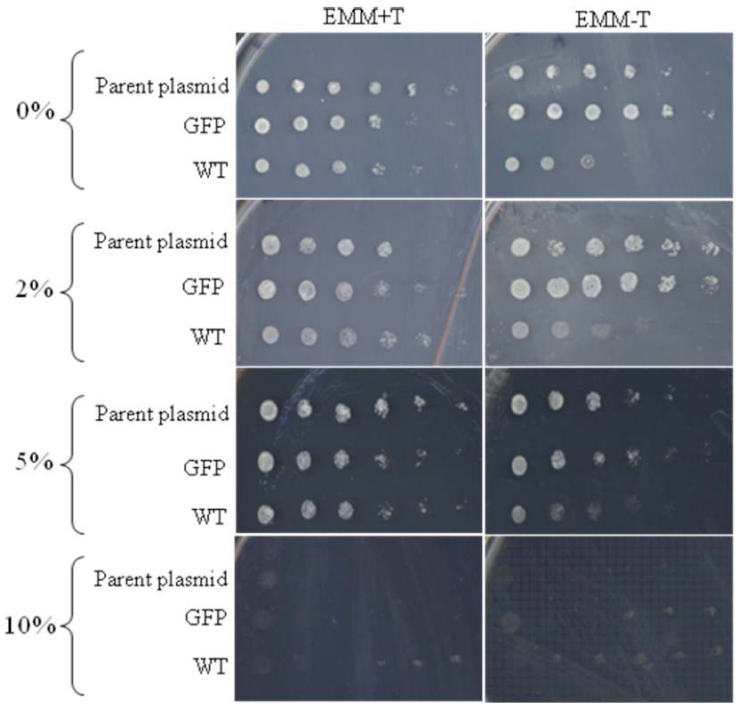



**Figure 7. Serial Spotting of fission yeast cells treated with DMSO.** Serially diluted cells expressing parent pNMT-1 plasmid, GFP, or WT a-synuclein were spotted onto EMM+T (repressing) and EMM-T (inducing) plates. This experiment was repeated two separate times.

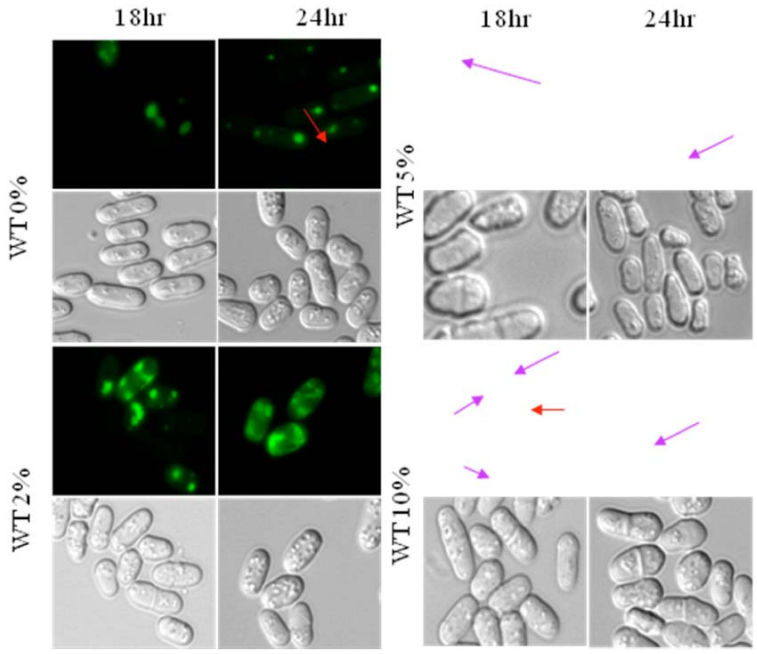



**Figure 8. Microscopy of fission yeast cells treated with DMSO.** Images were captured at 18 hour and 24 hour of induction in EMM-T. Aggregates (red arrow) in these cells started to form at 18 hour. With increasing concentrations of DMSO, a-synuclein began to localize to intracellular structures (purple arrow).

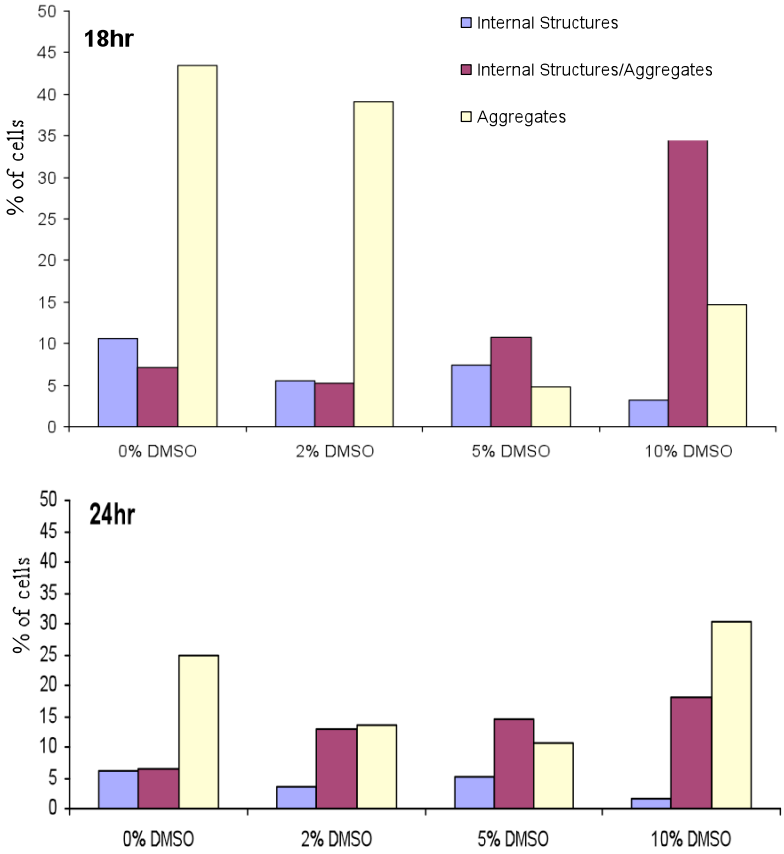



**Figure 9. Quantification of a-synuclein phenotypes in fission yeast cells.** Cells expressing WT a-synuclein were counted and characterized by the following phenotypes: internal structure localization (blue bar), internal structure localization and aggregates (purple bar), or aggregates (yellow bar). Bars represent percent of 600-800 total cells which display those various a-synuclein phenotypes at different concentration of DMSO.

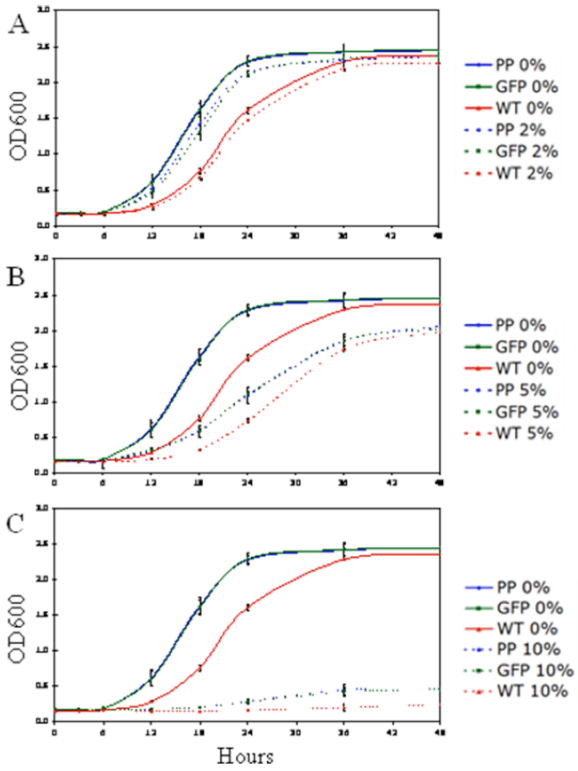



**Figure 10. Growth curves of BY4741 treated with DMSO.** BY4741 budding yeast cells were grown in Sc-ura galactose for 48 hours (x-axis) to induce parent pYES2 plasmid, GFP, and WT a-synuclein. Over this time course, the cells were treated with 2% (A), 5% (B) or 10% (C) DMSO (dashed lines) and compared to the cells that went without DMSO treatment (solid lines). Each of the points on the curves represents the mean from three trials on measurement of absorbances (y-axis). In addition, the standard error of the mean for a 95% confidence interval are represented by the y-bar on each of the points on the growth curves. Significant growth difference exists between pYES2 in 0% DMSO (n=3, P18hour= 0.00374 and P24hour= 0.000497) and pYES2 in 10% DMSO.

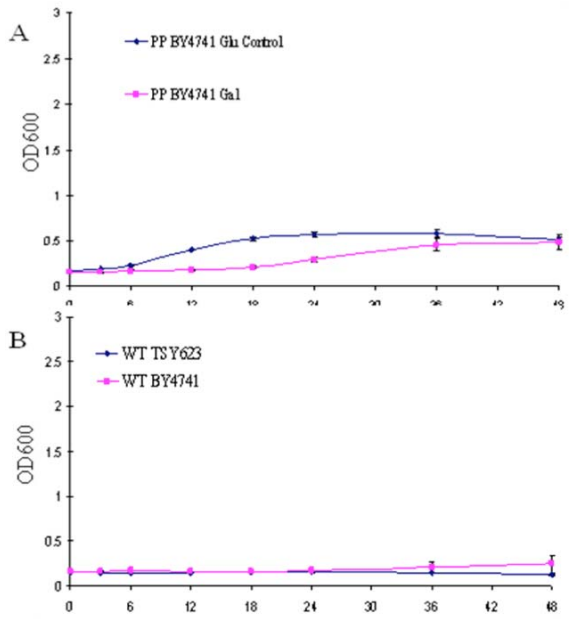



**Figure 11. Growth curves in 10% DMSO of BY4741 (glucose control) and TSY623.** A. BY4741 budding yeast cells with parent plasmid were grown in Sc-ura galactose and glucose media to test if slow growth in 10% DMSO is partially due to change of sugar in the media. B. We tested another a-synuclein expressing budding yeast strain, TSY623, for its growth in 10% DMSO condition. Each of the points on the growth curves represents the mean from three trials. In addition, the standard error of the mean for a 95% confidence interval are represented by the y-bar on each of the points on the growth curves.

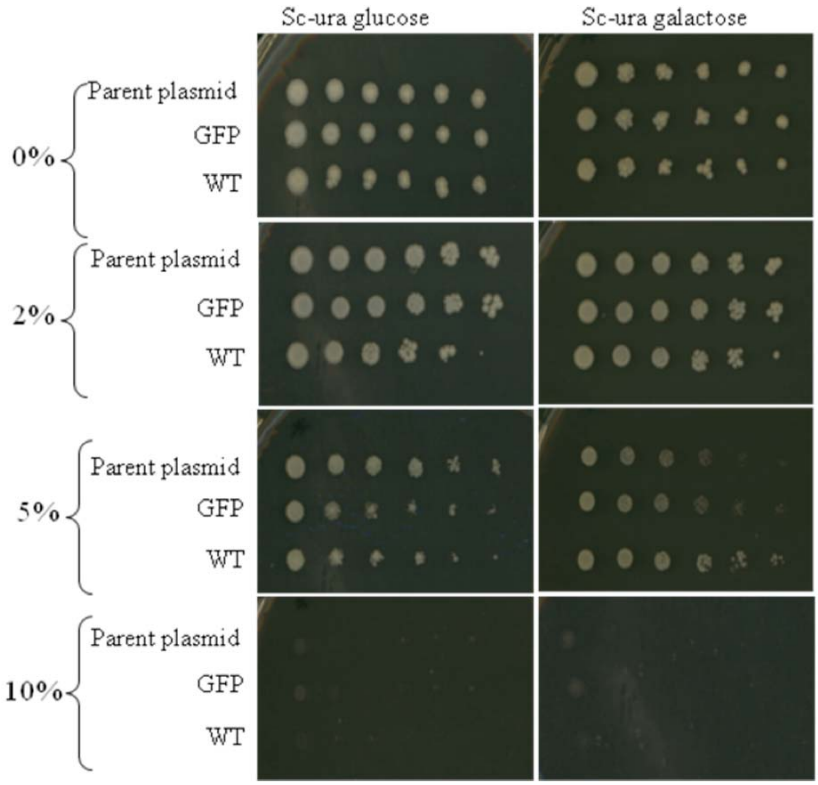



**Figure 12. Serial Spotting of BY4741 treated with DMSO.** Serially diluted cells expressing parent pYES2 plasmid, GFP, or WT a-synuclein were spotted onto Sc-ura glucose (repressing) and Sc-ura galactose (inducing) plates. This experiment was repeated two separate times.

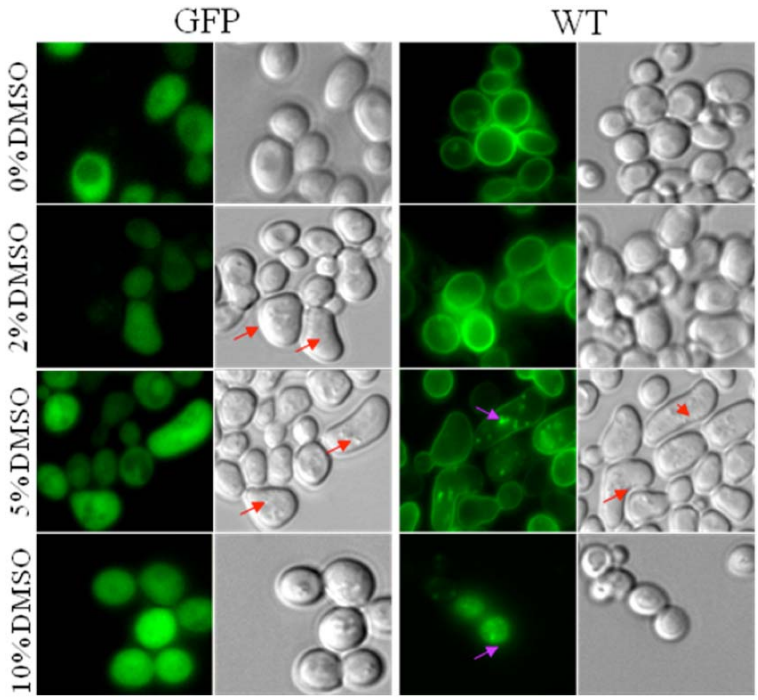



**Figure 13. Microscopy of BY4741 treated with DMSO.**Images were captured at 18 hour of induction in Sc-ura galactose. Without DMSO treatment, most of the cells showed WT a-synuclein localized to the plasma membrane (halo) and GFP diffused in the cytoplasm. With increasing concentrations of DMSO, both GFP and WT cells showed aberrant morphology (red arrow). The aberrant morphological cells expressing wildtype a-synuclein showed increasing number of cytoplasmic a-synuclein aggregates (purple arrow).

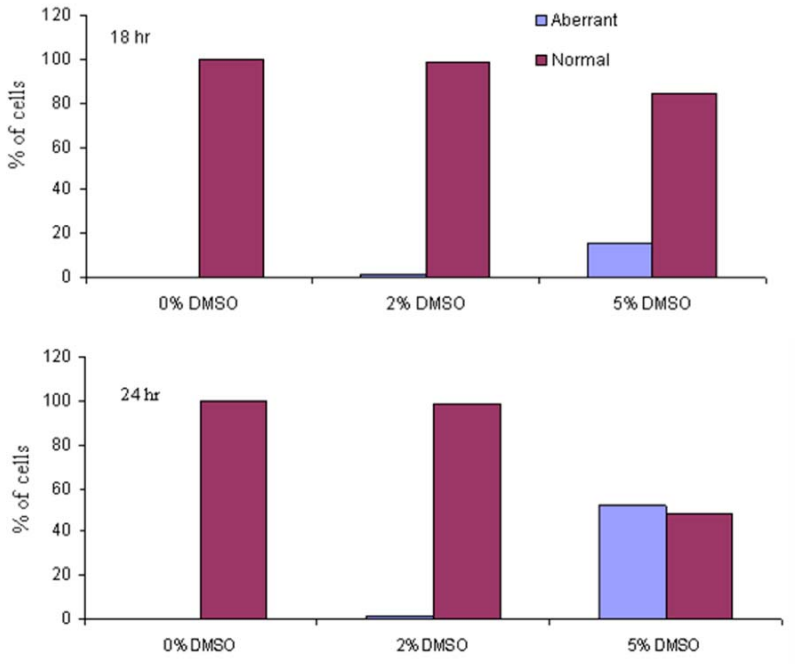



**Figure 14. Quantification of cell morphology with DMSO treatment.** DIC images of cells expressing WT a-synuclein were counted and characterized by those showing aberrant morphology (blue bar) or normal oval shape (purple bar). Bars represent percent of 600-800 total cells which display those two different DIC images of the cells at various concentrations of DMSO. The appearance of aberrant morphology increased with higher concentration of DMSO and over time when quantifying cells at 18 hour versus 24 hour.

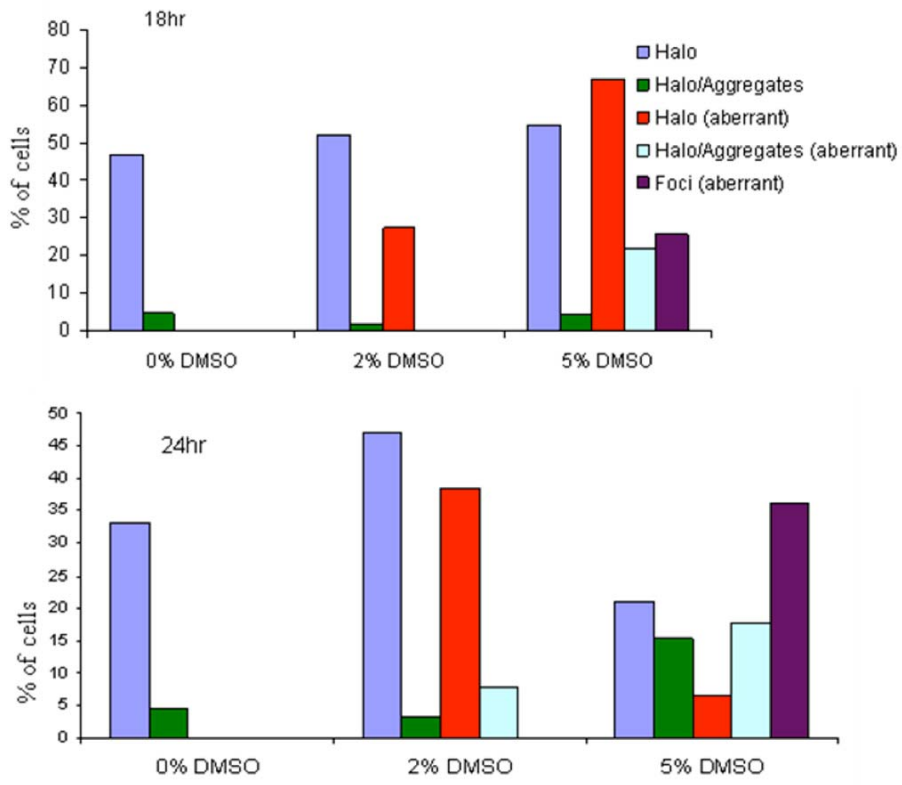



**Figure 15. Quantification of a-synuclein localization with DMSO treatment.** Cells expressing WT a-synuclein were counted and quantified by those showing halo (blue bar), halo/aggregates (green bar), halo in aberrant yeast (red bar), halo/aggregates in aberrant yeast (sky blue bar) or foci in aberrant yeast (red bar). Bars represent percent of 600-800 total cells which display those various a-synuclein phenotypes at concentrations of 0%, 2%, and 5% DMSO.

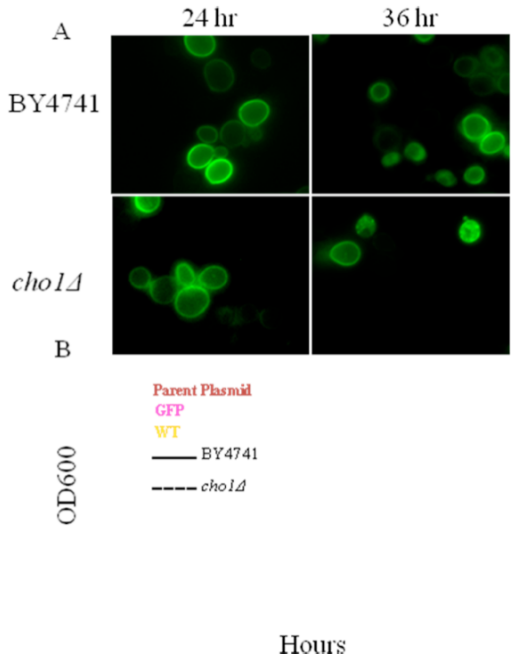



**Figure 16. Analysis of *cho1Δ* strain expressing a-synuclein.** *A. Microscopy of parent strain BY4741 and cho1Δ cells expressing WT a-synuclein.* Images were captured at 24 and 36 hour of induction in Sc-Ura Galactose. In CHO1 knockout strain, the WT a-synuclein plasma membrane localization is intact similar to WT a-synuclein in parent strain. *B. Growth curves of cho1Δ cells expressing isoforms of a-synuclein.* No observable differences in growth exist between a-synuclein expressing *cho1Δ* cells and the *cho1Δ* controls, but significant growth difference exists between BY4741 (n=3, P18hour= 0.003636217and P24hour= 0.000723) and pYES2 in *cho1Δ* cells. Each of the points on the curves represents the mean from three trials on measurement of absorbances (y-axis). In addition, the standard error of the mean for a 95% confidence interval are represented by the y-bar on each of the points on the growth curves.

*Lack of phosphatidylserine makes yeast toxic*

Our third goal was to evaluate whether α-synuclein association with plasma membrane was specific to a major membrane phospholipid. We began this analysis by asking whether a loss of a major phospholipid, phosphatidylserine (PS), from cellular membranes would change α-synuclein toxicity in budding yeast. PS deficiency was achieved by using a yeast strain knocked out for the CHO1 gene (which codes for the enzyme phosphatidylserine synthase that synthesizes PS in budding yeast). Unfortunately, this knockout strain grew significantly slower than the wild-type strain BY4741 indicating a general toxicity making it difficult to assess α-synuclein dependent effect (Figure 16B). When the cells finally started to grow, *Cho1Δ* strains expressing α-synuclein tagged GFP (wildtype, A30P, A53T, and E46K) exhibited similar growth curves compared to the controls cells (pYES2 and GFP; Figure 16A).

*Similar α-synuclein localization pattern in cho1Δ yeast and BY4741 cells*

Nevertheless, we still evaluated α-synuclein localization in surviving *Cho1Δ* cells by live cell GFP imaging to determine if the absence of PS altered membrane localization of α-synuclein. Both at 24 and 36 hours, Wild-type α-synuclein localized to the plasma membrane in *Cho1Δ* and the parent BY4741 strain (Figure 16B), indicating that in these cells, α-synuclein associated with the plasma membrane without needing PS.

**Discussion**

We inquired about whether α-synuclein's association with phospholipid plasma membrane was critical to cellular toxicity and whether α-synuclein's membrane interaction was specific to certain phospholipids. Our first major finding is that DMSO exerted cellular toxicity independent of α-synuclein expression along with changing α-synuclein's phenotype and, specifically in budding yeast, changing cellular morphology. Our second major finding is that we found PS to be less critical to α-synuclein's phospholipid membrane binding.

*DMSO affects viability and influence aberrant cellular morphology*

The slow growth observed in both fission yeast and budding yeast was due to DMSO. Supporting evidence come from the growth curves of control cells, which expressed no α-synuclein and exhibited slow growth. Organic solvents, like DMSO, have been known to negatively affect the metabolism of microorganisms (Zhang et al., 2003). Previous finding on DMSO treatment to yeast showed that 10% concentration in liquid media will reduce the density of the cell culture by half (Murata et al., 2003). In our study, during mid log phase (24hr), 10% DMSO media was shown to reduce the density of fission yeast cells by approximately half. Interestingly, same concentration of DMSO had a dramatic effect on the growth of the budding yeast cells which grew tenth of the density of cells untreated with DMSO. The chemical also induced budding yeast to have abnormal morphologies described in detail in the results section. These aberrant cell shapes were present starting at 2% DMSO, and then higher at 5% DMSO. I predict that these cells are having difficulty dividing properly. They may have duplicated their DNA since the cell volume enlarged and appeared to be twice the size of normal yeast. Similar results have been observed with another haploid yeast

*Torulaspora delbrueckii* that was induced into a diploid by treatment with DMSO (Sasaki and Ohshima, 1987). Indeed, DMSO affects the mammalian cell cycle too. The chemical inhibits the differentiation of myoblasts into muscle cells (Blau and Epstein, 1979) and the differentiation of the β-lymphocyte-derived cell line into IgM-producing cells. Besides the fact that DMSO is an inducer of cellular phospholipid, its influence may also be to disrupt cell cycle, as it may be exemplified in this study with yeast.

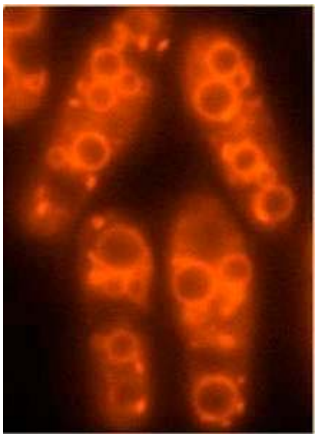
*DMSO increases α-synuclein's intracellular localization in fission yeast*

With increasing concentrations of DMSO, α-synuclein level of toxicity was not enhanced. However, the expression of α-synuclein inside the cell became different. α-Synuclein may have localized to the vacuolar membrane. FM4-64 dye, which is a vacuole stain, applied on fission yeast show vacuolar membrane structures (see image below). These intracellular structures resemble what α-synuclein localized to in large number when the yeast cells were treated with DMSO. Though FM4-64 stain was not used in this thesis study, another yeast study by Pellens et al. (2005) showed colocalization of FM4-64 stained vacuoles with wild-type α-synuclein. Hence, we speculate that α-synuclein possibly interactd ewith the vacuolar membranes in our fission yeast model.

DMSO globally increases the phospholipids production in the cell (Murata et al., 2003). Besides the phospholipids in the plasma membrane, DMSO perhaps increased the interaction of α-synuclein with phospholipids to other membrane bound organelles in the cell. In addition, the formation of α-synuclein aggregates was also increased around the intracellular membranes. The lipid membrane interaction here demonstrates to be important for α-synuclein aggregation, but it does not enhance α-synuclein cellular toxicity. Toxicity and α-synuclein's plasma membrane localization are conceivably correlated, but these two events are still missing in our fission yeast model for PD and, as-yet they can not be discredited.

*DMSO also induces α-synuclein localization in budding yeast*

α-Synuclein is slightly toxic to the BY4741 budding yeast strain. Increasing DMSO concentration did not cause further α-synuclein dependent toxicity. Moreover, the localization of the protein toward the plasma membrane was diminished with higher DMSO concentrations. We predicted that α-synuclein in budding yeast would localize more to the plasma membrane. Surprisingly, the protein formed non-toxic aggregates in the cytoplasm when treated with 5% and 10% DMSO. Our predictions are not well supported by these findings.



imbs.massey.ac.nz/Hyamslab/fission.htm

There are two views on how DMSO may have increased α-synuclein aggregation in the cytoplasm. First, the chemical has global effects on lipid biosynthesis, as half of the genes in the ergosterol, sphingoglycolipid and fatty acid biosynthetic pathways are down-regulated (Zhang et al., 2003). So, the down regulation may impact α-synuclein lipid interaction in yeast because the protein has been shown to bind to those fatty acids and phospholipids (Sharon et al., 2001; Kaplan et al., 2003; Salem et al., 2007; Davidson et al., 1998; Jo et al., 2000). Overall, we speculate that high concentration of DMSO (5-10%) reduces some of the phospholipids and fatty acids from the yeast plasma membrane and weaken α-synuclein interaction with the plasma membrane structure. In effect, the reduced membrane binding of the α-synuclein would result in a higher cytosolic concentration of α-synuclein, leading to α-synuclein aggregate formation.

The alternative view is that the DMSO up-regulates various groups of lipids particularly phospholipids. Genes such as INO1 and OPI3 in yeast, encoding key enzymes in phospholipid biosynthesis, are significantly elevated with DMSO treatment (Murata et. al., 2003). Because α-synuclein has shown to preferentially bind to other phospholipids such as PI and PE *in vitro* (Jo et al., 2000), α-synuclein's interaction with the plasma membrane maybe induced and in the process form lipid-bound aggregates (Lee et al., 2002; Sharma et al., 2006). However, under cellular context, α-synuclein membrane association is different from test tube condition. In cells, the lipid binding is rapidly reversible (Fortin et al., 2005). Therefore, it is possible that aggregates are formed with increase of α-synuclein interaction with membranes as the DMSO concentration increases in yeast (Pellens et al., 2005). Subsequently, α-synuclein's interaction with the plasma membrane weakens and results in higher cytoplasmic aggregates.

*Does α-synuclein bind to non-PS phospholipids?*

There exist four major cellular phospholipids: PC, PE, phosphatidylinositol (PI), and PS. We only analyzed one knockout strain that lacks phosphatidylserine synthase which makes PS. In the budding yeast model for PD, α-synuclein's localization to the plasma membrane is a prominent feature. In this current study, the localization to the plasma membrane was not lost as PS was lost from the cellular phospholipid membranes. We expected some if not all the reduction of α-synuclein's membrane localization. In *in vitro*, α-synuclein is found to bind weakly to PS. The protein's phospholipid affinity is higher with other phospholipids (Jo et al., 2000). This may explain why the protein remained localized to the plasma membrane in the yeast knockout strain because perhaps the protein's lipid binding is not specific to PS. On the other hand, it is also possible that the interaction of α-synuclein with PS is strong; no *in vivo* evidence is present to refute it otherwise. In this case, α-synuclein may have begun to interact with other phospholipids in the absence of PS.

Also, the growth of the knockout yeast in inducing galactose media was drastically slowed in comparison to BY4741 parent strain. Usually when yeast's sugar is changed from glucose to galactose, they grow slower, but the slow growth of knockout strain indicated to us that PS is an important major phospholipid in yeast. Evidently with these sick cells, it also meant that I could not get a dependable measurement on the level of toxicity of α-synuclein expression.

**Chapter 3: Does oxidative stress enhances α-synuclein's toxicity in fission yeast?**

**Results**

*$H_2O_2$ induces α-synuclein independent toxicity in fission yeast*

Our first goal was to evaluate how α-synuclein dependent toxicity can increase in fission yeast. α-Synuclein has minimal toxic effect in fission yeast. Though extensive α-synuclein aggregates formulated, the fission yeast remains robust (Brandis et al., 2006). $H_2O_2$ perhaps will function as an additional insult on the existing stress of α-synuclein expression (Manning-Bog et al., 2002; Abou-Sleiman et al., 2006). Eventually, the treatment could enhance α-synuclein dependent toxicity. We treated the cells with various concentrations of $H_2O_2$. However, as $H_2O_2$ concentration increased, and particularly at 2mM, the growth curve shifted to the right, indicating a slight toxic effect (Figure 17A-B). Surprisingly, this toxicity was not α-synuclein dependent, as it was seen even with cells that expressed GFP alone or only contained parent vector (Figure 17A-B). Secondly, we evaluated colony survival on plates by serial dilution. Cell cultures either containing parent vector, GFP or, wild-type α-synuclein serially diluted 5 times, were spotted onto sets of repressing (+thiamine) and inducing (-thiamine) plates, that contained 0, 0.4mM and 2mM $H_2O_2$. Similar to growth curve analysis, we observed a striking dose-dependent toxicity with $H_2O_2$ that was not dependent on the presence of α-synuclein (Figure 18).

Together, both toxicity assays indicate that at 2mM concentration $H_2O_2$ exerted an unexpected toxicity in fission yeast independent of α-synuclein presence.

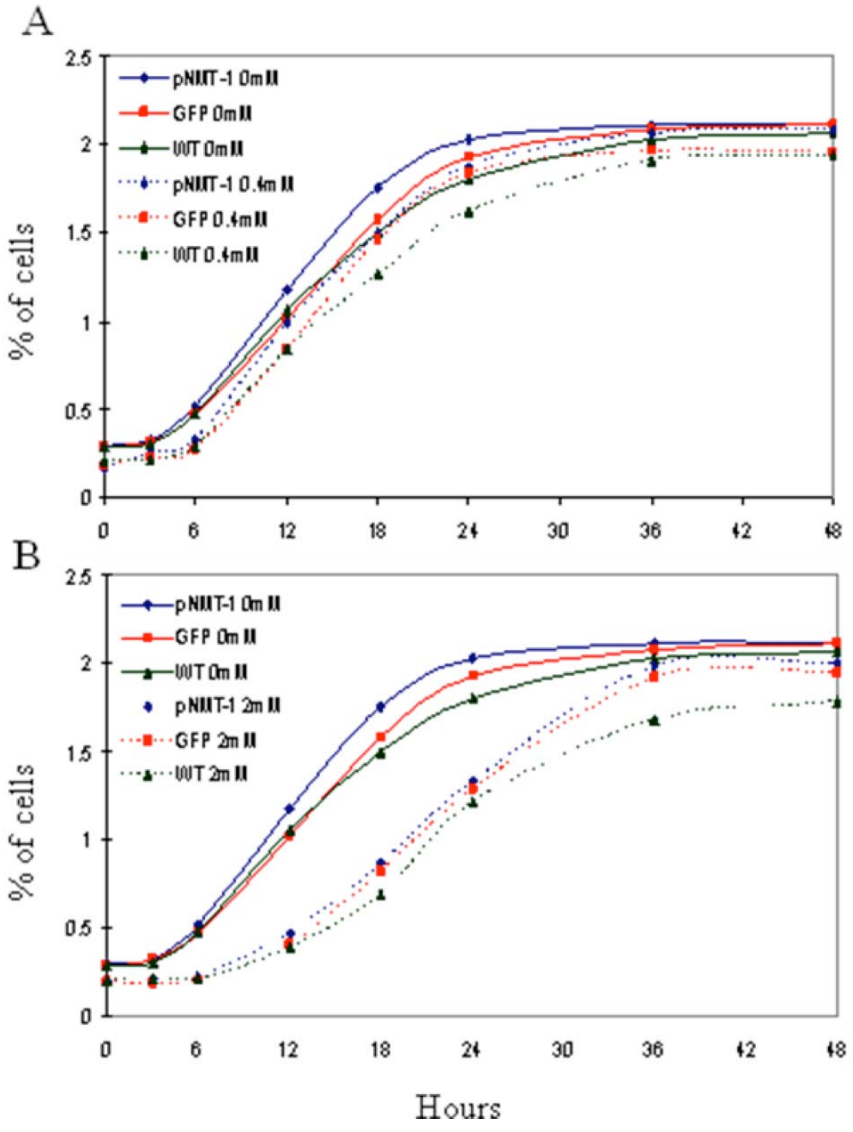



**Figure 17. Growth curves of fission yeast cells treated with $H_2O_2$.** Fission yeast cells were grown in EMM-T for 48 hours (x-axis) to induce parent pNMT-1 plasmid, GFP, and WT a-synuclein. Over this time course, the cells were treated with 0.4mM(A), or 2mM (B) $H_2O_2$ (dashed lines) and compared to the cells that were not treated with $H_2O_2$ (solid lines). Each of the points on the curves represents the mean from two trials on measurement of absorbances (y-axis); (n=2, P18hour=0.002008 and P24hour= 0.017602 between pNMT-1 in 0mM $H_2O_2$ and pNMT-1 in 2mM $H_2O_2$)

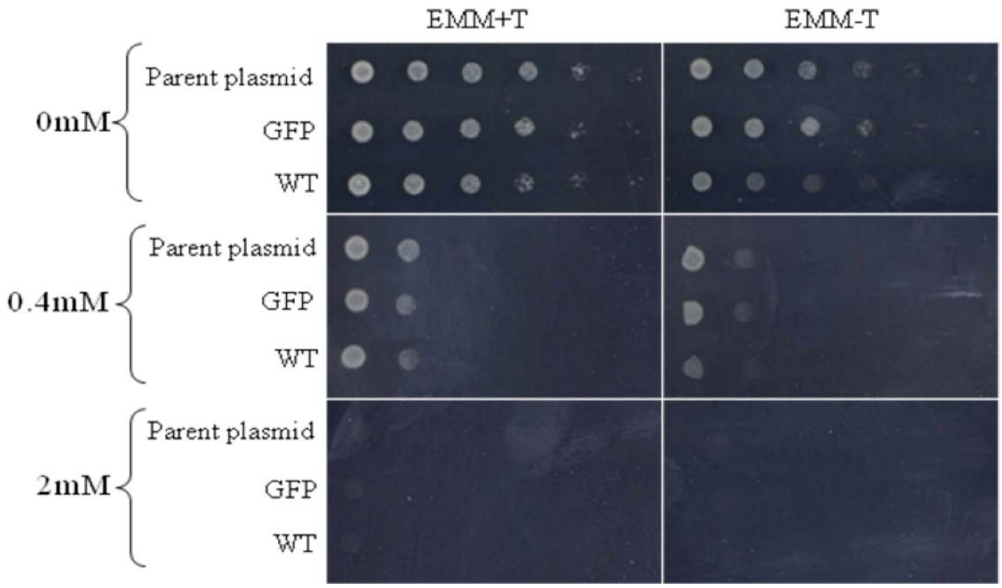



**Figure 18. Serial Spotting of fission yeast cells treated with $H_2O_2$.** Serially diluted cells expressing parent pYES2 plasmid, GFP, or WT a-synuclein were spotted onto EMM+T (repressing) and EMM-T (inducing) plates. This experiment was repeated two separate times.

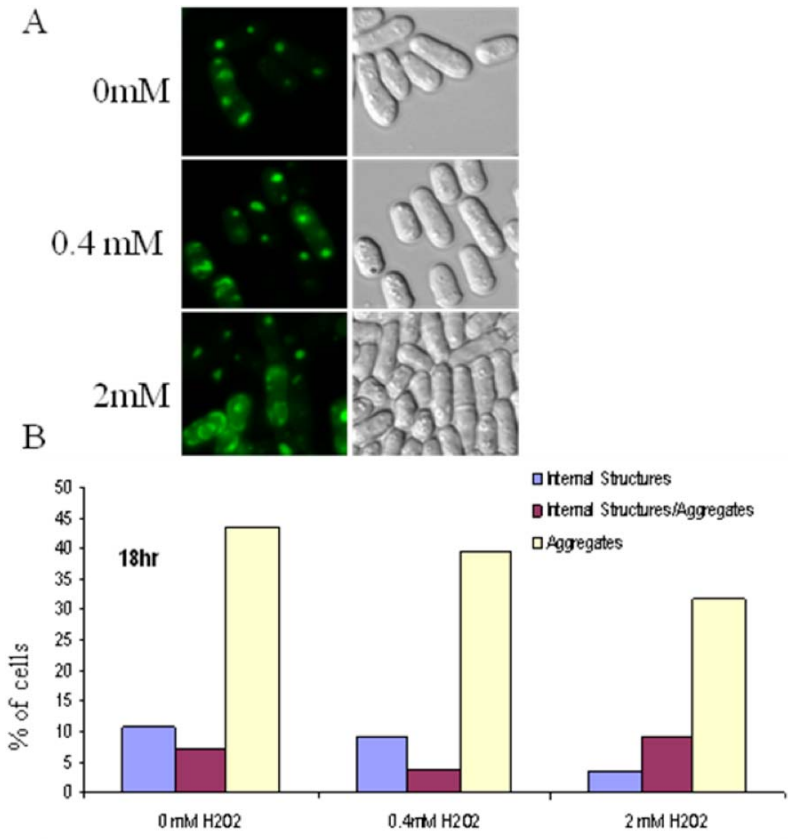



**Figure 19. Microscopy and quantification of a-synuclein phenotypes in fission treated with $H_2O_2$.** Images were captured at 18 hour of induction in EMM-T (A). Aggregates were noticeable with treatment of $H_2O_2$, along with small percent of cells showing a-synuclein localizing to intracellular structures. Aggregate formation and localization to intracellular structures did not increase with higher dose of H2O2 treatment (B). Cells expressing WT a-synuclein were counted and characterized by those showing intracellular structure localization (blue bar), intracellular structure localization and aggregates (purple bar), or aggregates (yellow bar). Bars represent percent of 600-800 total cells which display those various a-synuclein phenotypes at different concentration of $H_2O_2$.

*$H_2O_2$ induces alteration of α-synuclein localization to internal structures*

Next, we examined the phenotype of α-synuclein in the fission yeast to determine whether α-synuclein localization changed from aggregates. Images for cells expressing α-synuclein were taken at 18 hour post-induction. As the concentration of $H_2O_2$ was increased to 2mM, the predominant phenotype was aggregates. The lesser common phenotype was the localization of the protein into internal cellular structures. In fact, some cells had both of these α-synuclein localization patterns (Figure 19A). Quantification indicated that majority of cells had α-synuclein aggregates, meanwhile, greater percent of cells began to show both α-synuclein intracellular localization and aggregates with increasing $H_2O_2$ concentrations (Figure 19B).

**Discussion**

There is increasing evidence that oxidative stress is involved in Parkinson's disease. Yet, a connection between α-synuclein misfolding and oxidative stress is not fully understood. Examined initially by Brandis et al. (2006), our fission model organism does not exhibit toxicity when α-synuclein is overexpressed. We inquired whether additional stress (e.g. oxidative) on fission yeast is critical for toxicity to occur. Our major finding is that $H_2O_2$ exerted toxicity on fission yeast surprisingly at low concentration, independent of α-synuclein expression along with altering slightly α-synuclein's phenotype.

*No enhancement of α-synuclein toxicity with $H_2O_2$ treatment*

$H_2O_2$ is a powerful oxidizing agent, but its interaction with α-synuclein inside the cell may not be enough to cause α-synuclein dependent cell death. Additional cellular dysfunctions maybe required to occur before α-synuclein dependent toxicity elevates. We presume that there are protective measures in fission yeast to suppress α-synuclein toxicity. For example, anti-oxidant gene sod2 was shown to protect α-synuclein toxicity in yeast cells (Willingham et al., 2003). When the gene is deleted, α-synuclein toxicity was exhibited and further enhanced with $H_2O_2$ treatment (Brandis et al., 2006). Perhaps certain gene(s) are responsible for regulating α-synuclein toxicity in fission yeast, especially under oxidative stress.

*$H_2O_2$ toxicity in yeast*

The slow growth of fission yeast in media containing 2mM $H_2O_2$ was a surprising finding when we measured toxicity. On the contrary, the 2mM $H_2O_2$ does not impair growth of budding yeast (Sharma et al., 2006). In fact, the lethal dose is significantly higher and closer to 15-25mM in most budding yeast strains (McEwen et al., 1984). Though fission yeast must be producing antioxidants and enzymes to break down reactive oxygen species generated by $H_2O_2$, they might not be adequate to maintain cellular redox homeostasis as the concentration rises to 2mM (Chen et al., 2003). One study points out that a 2mM $H_2O_2$ concentration inhibits the fission yeast cell growth by 58% (Pekmez et al., 2008). Also, the increased $H_2O_2$ concentrations lower the Glutathione (GSH) levels; 2.0 mM $H_2O_2$ treatment showed significant decrease in GSH level in fission yeast (Pekmez et al., 2008). GSH is an abundant thiol which has been shown to protect against the deleterious effects of reactive oxygen species in budding yeast cells (Grant et al., 1998). GSH expression in budding yeast and fission yeast may perhaps be different hence leading to difference in tolerance for $H_2O_2$ treatment. Along with GSH, there might be group other regulatory genes in response to oxidative stress that may be responsible for why fission are sensitive to $H_2O_2$ treatment.

**Conclusion**

Using budding and fission yeast models of Parkinson's disease, we have evaluated factors, protein concentration, phospholipids, and oxidants that can potentially regulate α-synuclein's cellular toxicity. In the process, we have discovered that though these factors may not induce α-synuclein's cellular toxicity, they can alter α-synuclein's localization inside the cell. In addition, to our surprise, chemical such DMSO can alter the morphology of budding yeast cells.

Currently, our major conception from current, and including previous, findings *in vitro* and *in vivo* studies suggest that α-synuclein-dependent toxicity is better correlated with plasma membrane localization, rather than with intracellular aggregation. We are inching toward understanding α-synuclein's mode of toxicity. Willingham et al. (2003) conducted a yeast genetic screen and found that several membrane transport and lipid metabolism proteins regulated α-synuclein's toxicity.

We do not think that fission yeast is a robust organism as we once thought. The slight increase in $H_2O_2$ in media was toxic to these yeasts. Perhaps, α-synuclein is toxic to fission yeast, but in what form and with what molecules it associates that trigger its toxicity are yet to be resolved. Some α-synuclein expressing fission yeast might be so toxic that they die early in the time course. We only microscopically observe these cells 18-48 hour α-synuclein post-induction. Interestingly, during this time period, the cells appear to be non-toxic despite the extensive formation of aggregates. Similarly, PD patient's brain when examined during autopsy consists of α-synuclein aggregation within the substantia nigral neurons. These aggregates may perhaps be forming in a nucleation-polymerization dependent manner provided by the evidence we have contributed in this thesis. At the same time, there has been a significant decrease in the number of substantia nigral neurons. The neurons which persisted longer in this progressive neurodegenerative disease—available during autopsy—yield α-synuclein in Lewy Bodies (Spillantini et al., 1998). It is possible that α-synuclein aggregates are ultimately neuroprotective where the cells are trying to sequester the protein all together and make misfolded α-synuclein a better cellular target for degradation.

*Criticisms and Limitations*

DMSO was widely used in this study, but how the chemical induced change in cellular phospholipid biosynthesis in our yeasts is unclear. Up to date, we are the first to analyze the effects of DMSO on fission yeast. Plus, the effects of the chemical on budding yeast have varied, where one study analyzed it to be an up-regulator of phospholipids and fatty acids and another study finds it to be a down-regulator of phospholipids and fatty acids. $DiOC_6$ is a commercially available dye that we have not employed but can use to detect changes in cellular phospholipids. In our fission yeast cells treated with DMSO, we observed α-synuclein localizing to different places inside the cells, possibly membrane bound organelles like vacuoles. α-Synuclein vacuolar localization remains unconfirmed because we have not shown this specific α-synuclein localization with any fluorescent marker or stain.

Lastly, there are inconsistencies between the results from growth curves and spotting analyses in our second study and third study of this thesis. Wild-type α-synuclein expressing fission yeast grew better in liquid media than on spotting agar plates. In budding yeast, wild-type α-synuclein grew slower in liquid media than on spotting agar plates. Investigation in the third study reflected similar problems. The addition of moderate 0.4mM $H_2O_2$

concentration did not reduce cell growth in liquid media, but it did on agar plates irrespective of whether the cells expressed α-synuclein. Overall, we think that more reliable results come from the growth curve analyses because first, they have been repeated more times than spotting, and second, in growth curve analyses, the cell culture is suspended in liquid media that is considerably homogenous.

*Future Research*

In the immediate future, we plan to do staining with α-synuclein expressing yeast cells that go through DMSO treatment. With $DiOC_6$, we will be able to see possible changes in the cellular phospholipids. We will possibly use FM4-64 dye on DMSO treated fission yeast cells to stain vacuoles. The finding from the stain will be able to substantiate or refute our observation that DMSO induces α-synuclein to interact with vacoular membrane structures. In our third study, we discerned that fission yeast cells at 2mM $H_2O_2$ are dying in response to oxidative stress by the treatment of $H_2O_2$. Commercially available FUN stain will be a useful method to measure the rise of reactive oxygen species in the cell as the growth of fission yeast succumb to $H_2O_2$ treatment. For our further future investigations, we will explore some of the following questions: why α-synuclein does not go to the plasma membrane in fission yeast and be a non-toxic cytoplasmic aggregate; are other major membrane phospholipids such PE, PI, and PC critical to α-synuclein membrane localization and toxicity.

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