Modeling Disease-Related Proteins in *Saccharomyces cerevisiae*: Insights into Alpha-Synuclein and TorsinA Biology

by

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ABSTRACT

The yeast *Saccharomyces cerevisiae* has long been used to model complex cellular processes. As a eukaryote, much of its fundamental biology is conserved with higher organisms. As a single-celled, genetically tractable organism, it can easily be utilized for both high-throughput screening and hypothesis-driven analysis. Therefore, many groups use yeast to model disease-related proteins.

One such model utilizes heterologous expression of α-synuclein (α-syn), a protein implicated in the progression of Parkinson's disease and other synucleinopathies. α-Syn expression in yeast is associated with many phenotypes that are recapitulated in higher organisms. Here, I used yeast to characterize two naturally occurring splice isoforms of α-syn, α-synΔ4 and α-synΔ6. Levels of these isoforms vary between synucleinopathies but little is known about their biology. I found that these splice isoforms display different localization patterns than full-length α-syn (α-synFL) and are less toxic in yeast. However, when expressed at a high level, both splice isoforms can exert toxicity and affect similar processes to α-synFL. Interestingly, the splice isoforms show differential responses to perturbations in sterol homeostasis. Studies concerning the relationship between sterol levels and synucleinopathy progression have been contradictory. Our findings reveal that α-synΔ4 is less sensitive to changes in sterol levels than α-synFL and α-synΔ6, suggesting that change in α-syn splice isoforms levels is a potential mechanism for these conflicting results.

I also describe an attempt to model torsinA pathobiology in yeast. Mutations in torsinA cause early onset torsion dystonia, a devastating motor disorder. This protein has been described to function in regulating endoplasmic reticulum (ER) stress through the unfolded protein response (UPR). While I was unable to recapitulate a role for torsinA in the UPR in yeast, this model can serve as a platform for discovery of torsinA cofactors that enable it to act in this capacity, especially as more is uncovered concerning torsinA's role in the UPR.

This thesis highlights both the strengths and limitations of modeling disease proteins in yeast. More specifically, my success with α-syn splice isoforms may provide insight into synucleinopathy etiology, while my inability to model torsinA-induced toxicity can inform subsequent attempts to study disease related proteins in yeast.

Thesis supervisor: Susan Lindquist
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For my parents, whose endless support has enabled all I have achieved

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I am also grateful to my thesis committee. Angelika Amon and Troy Littleton served on my committee from the beginning and provided much guidance concerning the direction of my projects, as well as my career. I would also like to thank David Housman and Mel Feany for joining my thesis defense committee – I look forward to hearing their comments on this work.

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Brooke Bevis, who is the lab manager of the Lindquist lab, has been my bay-mate for the entirety of my graduate career. She has guided me every step of graduate school and served not just a bay-mate, but also as a sounding board for all types of advice, both scientific and otherwise. Thank you, Brooke, for always being there for me and being such a great friend.

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Beyond being a member of the Lindquist lab, for the past five and a half years, I have been fortunate to work in the Whitehead Institute. The combination of being surrounded by exciting science and wonderful people has made me look forward to coming into lab every day. I am extremely grateful to all of the support staff at the Whitehead – from the facilities department to procurement services, for their hard work and friendly attitude. I need to thank the sixth floor glass wash and media prep teams for making the lab work we do so seamless. I especially need to thank the head of our media prep team, Dubi Azubuine, for the countless plates and bottles of yeast media he has made for me.

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I am fortunate to have both been born into and married into a wonderful family, all of whom I must thank for their support and encouragement. I especially want to thank both of my grandmothers, for constantly showing their love and pride, and my grandfathers, who have taught me the importance of always being interested in learning something new. It is from their examples that I decided to pursue such an advanced degree. Furthermore, from the time I met my husband, his family has welcomed me with open arms and been so encouraging to my career - I am so lucky to have gained them as my second family.

None of this would have been at all possible without the hard work of my parents, who always provided me with the best possible opportunities while I was growing up and always figured out what was best for me (even if it was science camp instead of basketball camp). Mom and Dad, thank you for everything – without you I would not be where I am today.

While my daughter Eva did not directly contribute to the progress of this work, there is no stronger motivation to get in to lab and work hard than knowing she is waiting for me to come home and play with her. Seeing what she could learn in her first year of life has also really put some pressure on me to keep up the pace. For that, I must thank her.

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Publications

Valastyan JS and Lindquist S. The Role of Protein Misfolding in Disease at a Glance. *Disease Models & Mechanisms*. *In preparation*.

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Poster and Oral Presentations

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Valastyan JS, Termine D, and Lindquist S. Expression of α-Synuclein Isoforms in *Saccharomyces cerevisiae* Elicits Differential Sterol Sensitivities.

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Valastyan J and Lindquist S. Utilizing *Saccharomyces cerevisiae* to understand the relationship between torsinA, protein trafficking and folding defects and alpha-synuclein.

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Chapter One:

Introduction

Parts of this chapter are in preparation for a review in *Disease Models and Mechanisms.*

Introduction

Proteins are one of the main cellular workhorses. According to the central dogma, the production of proteins involves the single step of translation. However, the unfolded amino acid chain that results from translation is far from biologically active. To function, a protein must first fold into its proper conformation. Although many aspects of folding are intrinsic to the biophysical properties of the protein itself, this process is still quite complex and susceptible to errors (Dill and MacCallum, 2012). Proteins consist of an elaborate arrangement of interior folds that collapse into a final structure, a process which is complicated by modest free energy gain associated with the correct folding of a protein as compared to its numerous misfolded states. Many misfolded proteins involved in disease contain one or more mutations that stabilize misfolded conformations. Furthermore, *in vivo*, protein folding is made even more difficult by the crowded environment of the cell, where proteins must assume their correct conformation while under constant barrage of neighboring molecules. These complications make it no surprise that many proteins assume the wrong conformation, often resulting in disease.

The cell has multiple methods for combating toxic misfolded proteins. First, chaperone pathways exist that either prevent proteins from misfolding in the first place or aid a misfolded protein in regaining its correct conformation (Hartl et al., 2011). Secondly, when it becomes clear that a misfolded protein cannot be properly refolded, systems exist to degrade these misfolded proteins (Nedelsky et al., 2008; Smith et al., 2011; Varshavsky, 2012). However, dysfunction of these pathways is also associated with number of diseases.

I will start this chapter with some specific examples of how protein misfolding can lead to disease, including both diseases caused by the loss of function (due to protein mislocalization or degradation), as well as gain of function mechanisms (such as mutations that cause a toxic novel

function, dominant negative mutations, and amyloid accumulation). I will then focus on neurodegenerative diseases, specifically synucleinopathies, a class of disorders characterized by the accumulation of the protein alpha synuclein (α-syn). I will review how the yeast *Saccharomyces cerevisiae* has contributed to our understanding of the function and dysfunction of α-syn. This chapter serves as an introduction to understand the framework underlying my thesis studies using *S. cerevisiae* to study splice isoforms of α-syn, as well as attempts to model the pathobiology of torsinA, a protein implicated in a separate neurological disorder, early onset torsion dystonia.

How protein folding problems can cause disease

Improper Localization

Because many proteins that localize to specific organelles must fold correctly in order to be trafficked properly, mutations that destabilize the correct fold can lead to improper subcellular localization of otherwise functional proteins. α-1-antitrypsin is a secreted protease inhibitor that, when mutated, leads to emphysema in a recessive, loss of function manner (Perlmutter, 2011). This occurs because mutant forms of this protein misfold and are retained in the endoplasmic reticulum (ER). If this protein is not secreted by hepatocytes into the circulation, it is unable to inhibit neutrophil elastase in the lung, which then causes extensive damage to this organ's connective tissue. However, the consequences of this mutated protein are more complicated; it is also incorrectly localized at an organ level. Due to impared secretion, the misfolded protein accumulates in the ER of hepatocytes, its site of synthesis, and leads to liver damage in a dominant gain of function manner (Hidvegi et al., 2005; Lomas et al., 1992). While damage to the lungs can be controlled with enzyme replacement therapy (Mohanka et al., 2012), liver accumulation has proven a greater challenge but some progress is being

made. Because aggregates in the liver are degraded by macroautophagy, drugs that enhance autophagy, including rapamycin and carbamazepine, have been shown to alleviate α-1-antitrypsin-induced hepatic toxicity (Hidvegi et al., 2010). Other therapy has focused on directly blocking the aggregation of mutant α-1-antitrypsin (Skinner et al., 1998).

Improper degradation

While degradation systems are essential for preventing the accumulation of non-functional misfolded proteins, they sometimes destroy proteins that, while mutant, could still function. For example, cystic fibrosis is caused by mutations in the plasma membrane chloride channel CFTR. The most common mutation of CFTR that cause cystic fibrosis is deletion of F508. This mutation destabilizes the protein causing it to misfold and be targeted for degradation (Qu et al., 1997); however, conditions that allow CFTRΔF508 to be folded properly and trafficked, such as lower growth temperature, show that the mutant protein can partially function (Denning et al., 1992). This finding prompted the discovery of a chemical chaperone of CFTRΔF508, or a small molecule that can allow a mutant, misfolded protein to regain its properly folded form (Brown et al., 1996). More recent work has uncovered the role of a multiprotein complex containing HSP90 and its co-chaperone AHA1 in CFTR degradation (Wang et al., 2006). Remarkably, upon knockdown of AHA1, CFTR ΔF508 becomes not only more stable, but its function can also be partially restored, suggesting that inhibition of chaperone systems may be therapeutically beneficial to patients with this mutation (Wang et al., 2006).

A second example is provided by Gaucher's disease, the most common lysosomal storage disease (Cox and Cachon-Gonzalez, 2011; Futerman and van Meer, 2004). It is caused by a variety of mutations in beta-glucosidase, leading to the buildup of the lipid, glucosylceramide.

The symptoms of Gaucher's disease vary widely (Grabowski, 2008) and, while this variability is not fully understood, it is thought to be related to extent of degradation of beta-glucosidase in the ER (Ron and Horowitz, 2005). Depending on the extent of ER processing, some fraction of the protein can be correctly processed, trafficked to the lysosome, and function, even in individuals carrying the disease-associated mutations. Currently, the most developed treatment for Gaucher's disease is enzyme replacement therapy, which is expensive and not curative (Grabowski, 2008). Therefore, there is great hope that a useful intervention for Gaucher's disease could be upregulation of chaperones that assist in the correct folding of beta-glucosidase (Sawkar et al., 2006). Indeed, both drugs that activate the unfolded protein response and chemcial chaperones that directly interact with and stabilize mutant beta-glucosidase have demonstrated efficacy in patient-derived cells (Sawkar et al., 2005; Sawkar et al., 2002).

Dominant negative mutations

A third way by which protein misfolding can cause disease is through a dominant negative mechanism. This occurs when a mutant protein antagonizes the function of the wild type (WT) and a phenotype results due to loss of protein activity even in a heterozygote. One example of this mechanism related to protein misfolding is epidermolysis bullosa simplex, a skin disorder characterized by severe blistering in response to injury. It is associated with mutations in the keratin genes *KRT5* or *KRT14*. Keratin forms long intermediate filaments that provide structure to the epidermis of the skin (Chamcheu et al., 2011). Disease-associated mutations in keratin cause the protein to misfold and aggregate, particularly in response to mechanical stress (Coulombe et al., 2009; Russell et al., 2004; Werner et al., 2004). Because a filament is

constructed of multiple keratin molecules, each filament tends to contain both WT and mutant protein in a heterozygote individual. The dominant nature of the disease is therefore explained by the fact that the mutant protein present in these filaments does not function properly, thus compromising the function of the entire filament. Like several other diseases discussed here, recent research has found chemical chaperones that may prevent the aggregation of mutant keratin and alleviate symptoms of the disease (Chamcheu et al., 2012).

A second example of dominant negative mutations that involve protein misfolding and predispose to disease is mutations in the homotetrameric transcription factor, p53. These mutations are one of the most common genetic alterations seen in cancer (Friedman et al., 1993). This is because p53 is responsible for regulating a host of pathways involved in maintaining genome integrity, including apoptosis, DNA damage repair, cell cycle regulation, and metabolism (Freed-Pastor and Prives, 2012). In the absence of stress, p53 is rapidly degraded by the proteosome in a process dependent on the ubiquitin ligase MDM2 (Kubbutat et al., 1997). In response to stresses like DNA damage, p53 is stabilized and able to stimulate transcription of its target genes. Many of the most common oncogenic mutations in p53 disrupt the core domain of the protein, preventing it from assuming its correctly folded conformation. These mutations have two effects. First, mutant p53 is still able to associate with other p53 monomers; however, the resulting tetramer does not function correctly, regardless of whether or not a WT copy of p53 is also present (Milner and Medcalf, 1991; Milner et al., 1991). Because of this, mutant p53 acts in a dominant negative manner; even in a heterozygote, most tetramers are dysfunctional. Secondly, mutant p53 is unable to interact with MDM2, and thus is more stable. This inappropriate accumulation of the mutant form of the protein makes it even less likely that a tetramer comprised solely of WT p53 will form. One family of small molecules

currently undergoing clinical trial for cancers dependent on p53 dysfunction are Nutlins. These compounds prevent MDM2 from interacting with and promoting degradation of WT p53, increasing the probability of formation of WT tetramers (Vassilev et al., 2004). Recently, small molecules that directly bind and restore WT function of mutant p53 have also been found (Gavrin et al., 2012).

Toxic novel function

Another way protein misfolding can cause a dominant phenotype is by allowing a protein to acquire a conformation that exerts a toxic novel function. One example is apolipoprotein E (APOE), a lipid transport molecule. One particular allele of *APOE*, *APOE4*, is found in at least one copy in 65-80% of patients with the disease (Farrer et al., 1997). The mutation in *APOE4* stabilizes a misfolded conformation of the protein; the WT protein has an extended domain that is compromised by an extra salt bridge in APOE4 (Dong and Weisgraber, 1996; Dong et al., 1994). This interaction changes the lipid affinity of APOE4 (Dong and Weisgraber, 1996; Dong et al., 1994), leads to disruption of mitochondrial function (Chen et al., 2011) and impairs neurite outgrowth (Nathan et al., 1994). It is also associated with increased levels of Aβ, the peptide that aggregates in brains of patients with Alzheimer's disease (Ma et al., 1994). This strongly implicates misfolded APOE in the pathogenesis of Alzheimer's disease. Due to the specific change in APOE4 structure, small molecules that prevent formation of the extra salt bridge may provide a therapeutic strategy for correcting the dysfunction of this protein. A recent study utilized a FRET-based assay to identify structure correctors that prevented APOE4 from forming the aberrant salt bridge that stabilizes its misfolded form (Brodbeck et al., 2011).

Compounds that corrected APOE4 misfolding also rescued APOE4-associated mitochondrial dysfunction and inhibition of neurite outgrowth.

A second example of mutation related to protein misfolding that leads to a toxic novel function for a protein is seen in *v-SRC*. This mutant form of this nonreceptor tyrosine kinase lacks an inhibitory phosphorylation site. While oncogenic *SRC* mutants have only been demonstrated in chicken fibrosarcoma, the lessons learned apply to other members of the SRC kinase family that are relevant to human cancers. Although v-SRC is constitutively active, it is also inherently less stable and requires the chaperone HSP90 for its maturation and proper localization; in contrast, WT SRC is much less HSP90-dependent (Bijlmakers and Marsh, 2000; Whitesell et al., 1994; Xu and Lindquist, 1993; Xu et al., 1999). Importantly, the activity of many oncogenic kinases, including other SRC family kinases, BCR-ABL, and BRAF, all display an increased requirement for assistance from the HSP90-based chaperone machinery [for recent review, see (Trepel et al., 2010)]. These finding have led to extensive efforts to understand and target chaperone function in the treatment of cancers (Mendillo et al., 2012; Whitesell et al., 2012)

Amyloid

No review of misfolded proteins and disease would be complete without discussion of the ability of stable amyloid fibers to accumulate and contribute to a variety of diseases. Amyloid pathogenesis differs from the mechanisms described earlier in that there are a wide range of diseases associated with different proteins, all of which accumulate as very stable and insoluble beta sheet fibers called amyloid. These diseases range from neurodegenerative disorders (including Alzheimer's disease, Parkinson's disease, and Huntington's disease) to amyloidoses

(such as familial amyloid polyneuropathy and primary systemic amyloidosis) (Caughey and Lansbury, 2003; Chiti and Dobson, 2006). In some cases, such as cataracts, disease is directly caused by amyloid fibril accumulation. In other cases, especially the neurodegenerative diseases, this is less clear. Recent evidence favors the theory that it is lower order oligomers that are responsible for disrupting cellular function and the amyloid deposits may be a protective mechanism that the cell uses to sequester these toxic species. Several of these proteins are also capable of forming pore-like structures that are hypothesized to disrupt membrane integrity, which is an additional potential toxicity mechanism (Lashuel et al., 2002). As previously discussed for α-1-antitrypsin deficiency, when pathogenesis is caused by the accumulation of protein aggregates, an attractive therapeutic strategy is to limit disease by blocking aggregate formation. Due to the role of amyloid accumulation in such a wide range of diseases and the shared pathway to fibril formation, much work has gone into creating therapeutics that target this amyloid fold, as opposed to targeting specific proteins. Indeed, antibodies have now been developed that recognize both amyloid fibrils and oligomers; an oligomer-specific antibody is able to block the toxicity of multiple types of oligomers in vitro. (Glabe, 2004; Kayed et al., 2003). Recent work has turned to developing antibodies that recognize both conformation and sequence, possibly allowing for more targeted therapeutics (Perchiacca et al., 2012). Small molecules that prevent aggregate formation (Ehrnhoefer et al., 2008) or enhance their degradation (Rubinsztein et al., 2007) have also been discovered.

The increasing prevalence of neurodegenerative disease

As mentioned above, neurodegenerative diseases are a major class of protein folding diseases and will be a major emphasis of the remainder of this thesis. With the rise of average lifespan of

individuals in developed countries, these diseases, associated with advancing age, are becoming increasingly prevalent. As of 2012, 12.5% of the American population over 65 is living with AD and another 2% are living with PD (www.alz.org). Even more staggering is the financial cost associated with these diseases. In the United States alone, the combined annual direct and indirect costs associated with AD are $183 billion (www.alz.org) and with PD are nearly $25 billion (www.pdf.org). Furthermore, as the baby boomer population continues to age, the costs associated with these late-onset diseases are expected to further skyrocket. For example, it is estimated that the cost of AD alone will be $1.1 trillion by 2050, not even accounting for inflation (www.alz.org). The need to find more effective and cheaper means of treating these diseases is desperate, and to accomplish this, we must gain a stronger understanding of their underlying causes.

Parkinson's disease

The second most prevalent neurodegenerative disease is PD, a major topic covered in this thesis. PD is the main cause of parkinsonism, a syndrome that presents with four main symptoms: resting tremor, rigidity of movement, bradykinesia/akinesia, and postural instability (Dauer and Przedborski, 2003). The majority of these symptoms are due to degeneration of dopaminergic neurons in the substantia nigra pars compacta, a region of the brain essential for motor control; however, other regions of the brain and autonomic nervous system degenerate as well, which account for the non-motor symptoms, including constipation, sleep disorders, anosmia, and depression (www.pdf.org). Unfortunately, by the time most symptoms present, there is already significant degeneration of various areas of the brain and the autonomic nervous system, making treatment difficult since there is no way to regain lost neurons. Beside

loss of specific brain regions, a striking feature of post-mortem brains of individuals with PD is the intracellular aggregates of proteins termed Lewy bodies (LB) and Lewy neurites. These accumulate in the remaining neurons of the affected brain regions and are named for Frederic Lewy, who first described the aggregates in 1912 (Rodrigues e Silva et al., 2010; Spillantini et al., 1997).

The majority of cases of PD and other parkinsonisms are sporadic, having no defined environmental or genetic cause. However, the small numbers of environmentally implicated or inherited cases have provided significant insight into the mechanisms of parkinsonism progression (Table 1). For example, exposure to the herbicide paraquat increases an individual's risk of developing PD, with an odds ratio confidence interval of 1.4-4.7 (Liou et al., 1997; Tanner et al., 2011). Paraquat acts as an oxidizing agent, which has led to examination of the role of superoxide radicals in PD progression (Bus et al., 1974). Environmental toxins have also drawn attention to mitochondrial complex I, as the street drug contaminant MPTP and the insecticide rotenone [odds ratio confidence interval 1.3-4.7 (Tanner et al., 2011)] both have been linked to environmentally related cases of parkinsonism and have been shown to inhibit this enzyme complex (Davey and Clark, 1996; Nicklas et al., 1985). MPTP was synthesized in the As and tested in primates as a treatment for a number of diseases; however, these tests were halted when the primates developed parkinsonisms. Interest in MPTP arose again from studies of drug users who developed parkinsonism symptoms upon exposure to contaminated drugs (Langston et al., 1983) and is now often used to model parkinsonism *in vivo*, while rotenone has mostly been used to create cell culture models (Betarbet et al., 2000).

PD is part of a spectrum of diseases termed parkinsonian disorders, all of which result in some loss of movement control, which can be due to degeneration of multiple brain regions and

can be combined with a wide variety of other symptoms, including dementia (Bohlhalter and Kaegi, 2011). Sixteen different PARK alleles have been associated with the familial cases of these disorders. Studies of these genes have provided insight into mechanisms driving the progression of these diseases. For example, four genes thought to contain causative alleles for genetically-linked cases of parkinsonism, *parkin* (PARK2), *PINK1* (PARK6)*, DJ-1* (PARK7), and *HTRA2/Omi* (PARK13), have been linked to regulating levels of oxidative stress and regulating of mitochondrial function (Bonifati et al., 2003; Darios et al., 2003; Palacino et al., 2004; Strauss et al., 2005; Valente et al., 2004a). Furthermore, eleven other PARK loci have been identified as genes important in familial PD and related disorders and we are learning more and more about the genes important for these heritable forms of the disease (Table 1.1) (Corti et al., 2011). For example, mutations in the metal transporter *ATP13A2* are associated with an autosomal recessively inherited form of parkinsonism with dementia (Gitler et al., 2009; Ramirez et al., 2006; Rentschler et al., 2012). Sharing ties to metal homeostasis, miners and welders who are exposed to heavy metals have an increased risk of parkinsonism (Racette et al., 2001; Sanchez-Ramos et al., 2011), tying cation exposure, especially manganese (Perl and Olanow, 2007), to development of parkinsonism. Additionally, *parkin* and *FBXO7*, the latter of which was found in a study looking for genes associated with Parkinsonian-pyramidal syndrome as opposed to canonical PD, have both been implicated in ubiqitination, which fits with the stereotypic role of protein accumulation in PD (Shimura et al., 2000; Shojaee et al., 2008). However, the most extensively studied gene implicated in PD progression is α-syn.

Environmental	
Toxin	**Insight into PD**
MPTP	Mitochondrial Involvement
Rotenone	
Paraquat	
Heavy Metal Exposure	Role of Heavy Metals

Genetic		
PARK Locus	**Gene**	**Cellular Role**
PARK1/PARK4	*SNCA* (α-syn)	Synaptic function
PARK2	*Parkin*	Ubiquitin ligase and mitochondrial function
PARK3	*SPR*	Dopamine synthesis
PARK5	*UCHL1*	Deubiquitinating enzyme
PARK6	*PINK1*	Mitochondrial function and oxidative stress
PARK7	*DJ-1*	Chaperone and oxidative stress
PARK8	*LRRK2*	Kinase
PARK9	*ATP13A2*	Metal homeostasis and lysosome function
PARK10	Unknown	Unknown
PARK11	*GIGYF2*	Vesicular transport
PARK12	Unknown	Unknown
PARK13	*HTRA2/Omi*	Mitochondrial function
PARK14*	*PLA2G6*	Lipid Metabolism
PARK15*	*FBXO7*	Ubiquitin pathway
PARK16	Unknown	Unknown

Table 1.1. Summary of environmental toxins and genetic loci associated with PD and the insight they provide into PD progression. Underlined genes and/or cellular roles are not completely defined. *PARK14 and PARK15 were found based on roles in adult-onset levodopa-responsive dystonia-parkinsonism and Parkinsonian-pyramidal syndrome, respectively. (Ash et al., 2010; Corti et al., 2011; Dauer and Przedborski, 2003; Paisan-Ruiz et al., 2009; Sanchez-Ramos et al., 2011; Shojaee et al., 2008).

Alpha synuclein

α-Syn is prominently linked to PD progression, along with a number of other neurodegenerative diseases collectively termed synupcleinopathies. This association has drawn on two grounds. First, α-syn is a major component of LB (Spillantini et al., 1997). Second, as shown in Table 1, genetic variants at the α-syn locus are associated with familial forms of synucleinopathies. Two point mutants, A53T and A30P, are associated with familial PD, while a third point mutant, E46K, is linked with dementia with Lewy bodies (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Duplications and triplications of α-syn were shown to cause synucleinopathy and lower its average age of onset (Chartier-Harlin et al., 2004; Singleton et al., 2003). Furthermore, multiple SNPs associated with increased risk for sporadic PD have been linked to the α-syn locus (Mizuta et al., 2006).

Both *in vitro* and *in vivo* studies have shed light on structural properties of α-syn, as well as its endogenous function. It is a small, 14 kDa protein that is natively unfolded in an aqueous environment. In the presence of lipids, the N-terminus of α-syn forms amphipathic alpha helices, which associate with lipids (Davidson et al., 1998). This property is due to the presence of approximately six imperfect sequence repeats (KTKEGV) that, when oriented as an alpha helix, create an amphipathic distribution of the amino acids (Auluck et al., 2010). However, even in a lipid environment, the C-terminus of the protein remains unfolded (Eliezer et al., 2001). The C-terminus is thought to be important for protein-protein interactions of α-syn. Between the N- and C-termini is a region termed the NAC domain. This region was originally identified as the Non-Amyloid Component of amyloid deposits purified from brains of patients with AD (Ueda et al., 1993). Part of this region, especially amino acids 71-82, is important for the α-syn self-

interactions and toxicity (Giasson et al., 2001). Removal of this region, which makes α-syn more similar to its paralog β-synuclein (β-syn), abolishes its fibrilization ability.

As suggested by its name, α-syn localizes to the synaptic region of a neuron, as well as the nucleus (Maroteaux et al., 1988). While its nuclear role is not well understood, we are beginning to understand that α-syn plays a role in regulating vesicle fusion at the synapse. As will be discussed below, work in a number of model organisms, including the yeast, *Saccharomyces cerevisiae*, were important for elucidating this function of α-syn.

Saccharomyces cerevisiae as a model organism for neurodegenerative diseases

While it may initially seem like an unconventional choice for modeling neurodegenerative disease due to its lack of a nervous system, we have learned a great deal about the proteins involved in these disorders through studies in the single cell yeast, *S. cerevisiae*. There are many benefits to using *S. cerevisiae* as a model organism for studying cellular toxicity (Khurana and Lindquist, 2010). *S. cerevisiae* share many of the traits of other single-celled organisms, such as rapid generation time and inexpensive reagents for growth and manipulation. Furthermore, they have a compact genome and can exist as either a diploid or a haploid. This makes *S. cerevisiae* genetically tractable, with simple methods for expressing exogenous genes and creating deletion mutants for hypothesis-driven research, as well as many libraries available for high-throughput chemical and genetic analyses. This has allowed extensive characterization of the protein interactome of *S. cerevisiae*, which has been an invaluable tool for systems biology and allows for detailed dissection of pathways involved in a number of cellular processes (Petranovic and Nielsen, 2008). However, *S. cerevisiae* differ from bacteria in that they are eukaryotes, so they share similar subcellular compartments with higher organisms and display

significant conservation of fundamental cellular processes such as secretion, protein homeostasis, and mitochondrial function.

There has been a long history of learning about disease progression and complex processes through studies in *S. cerevisiae*, such as the function of the cancer-related protein v-src (Brugge et al., 1987) and the ability of HSP90 to modulate its toxicity (Xu and Lindquist, 1993), and the role of Bcl and Bax in apoptosis (Khoury and Greenwood, 2008). Furthermore, the scope of knowledge uncovered by studying proteins involved in many of the major neurodegenerative diseases in *S. cerevisiae* is remarkable.

One of the first yeast models of neurodegenerative disorders was the use of *S. cerevisiae* to examine proteins involved in a family of diseases termed poly(Q) expansion disorders. The most common member of this family is Huntington's disease, in which an extension of glutamine repeats in the protein Htt is directly associated with the severity of the disorder (The Huntington's Disease Collaborative Research Group, 1993). Similar to α-syn in synucleinopathies, Htt forms intracellular aggregates in brains of patients with Huntington's disease (DiFiglia et al., 1997). In a *S. cerevisiae* model, the first exon of Htt was expressed with polyQ tracts of varying lengths. Similar to the correlation between repeat length and disease severity, the more CAG repeats added to the protein, the more toxic expression of the protein was to *S. cerevisiae* (Giorgini et al., 2005). Furthermore, as the length of the CAG repeats was increased, the protein formed intracellular aggregates, reminiscent of those seen in the neurons of patients with Huntington's disease (Krobitsch and Lindquist, 2000). Once this model was established, it was used in a high-throughput screen to find small molecules capable of reversing the toxicity (Ehrnhoefer et al., 2006; Zhang et al., 2005) and genetic screens to identity genes that, when deleted, suppress (Giorgini et al., 2005) or enhance (Willingham et al., 2003)

Htt-induced toxicity. A variety of findings uncovered in *S. cerevisiae* studies have been validated in higher organism models (Khurana and Lindquist, 2010). Since using *S. cerevisiae* to study polyQ-induced toxicity, this concept has been expanded to employ yeast to study TDP-43 (implicated in amyotrophic lateral sclerosis) (Johnson et al., 2008), Aβ (implicated in AD) (Treusch et al., 2011), and α-syn.

Using yeast to model synucleinopathy

In 2003, two papers introduced a yeast model of α-syn-induced toxicity (Outeiro and Lindquist, 2003; Willingham et al., 2003). In this model, α-syn is expressed under the control of a galactose-inducible promoter. When expressed at a low level, α-syn was nontoxic and associated with the plasma membrane (Outeiro and Lindquist, 2003) (Figure 1.1). This localization fits with the lipophilic nature of the amphipathic helices of the N-terminus of α-syn. As α-syn was expressed at higher levels, the protein elicited cytotoxicity and began to localize to intracellular foci, reminiscent of LB (Figure 1.1). Immuno-electron microscopy (immunoEM) discerned that these foci were comprised of clumps of secretory vesicles decorated with α-syn (Gitler et al., 2008; Soper et al., 2008).

Figure 1.1 provides a summary of the phenotypes associated with α-syn expression in the three main strains created in our lab – NoTox, IntermediateTox (IntTox), and HighTox (HiTox), which express increasing levels of α-syn and, as implied by the names, show increasing levels of α-syn-induced toxicity. Throughout this work, these names will be used to describe strains showing the corresponding level of toxicity.

Results from a high-throughput screen shed light on the probable mechanisms involved in the accumulation of secretory vesicles upon expression of α-syn (Cooper et al., 2006). Almost all

genes in the yeast genome were tested to find genes that, when overexpressed, either enhanced or suppressed the toxicity elicited by α-syn expression. The largest class of modifiers uncovered from this screen was genes involved in endoplasmic reticulum (ER)-to-Golgi trafficking. Taken with the fact that α-syn reaches the plasma membrane by traveling on secretory vesicles originating from the ER (Dixon et al., 2005), it was hypothesized that α-syn is causing a block in ER-to-Golgi trafficking, thereby leading to the accumulation of vesicles and accounting for at least part of the toxicity mediated by α-syn. Indeed, expression of α-syn blocks trafficking of the endogenous yeast protein CPY, which normally traffics from the ER to the Golgi to the vacuole, acquiring experimentally discernible modifications in the process.

Importantly, this finding echoes those obtained from higher organisms. First, homologs of the hits from the overexpression screen, including those involved in ER-to-Golgi trafficking, were able to modify α-syn-induced toxicity in higher organism models of synucleinopathy. Furthermore, the fact that α-syn blocks ER-to-Golgi trafficking is consistent with the phenotype that results from α-syn deletion and overexpression in higher organisms. While deletion of α-syn in mice does not elicit any major phenotypes, electrophysiology studies uncovered that inappropriately large amounts of neurotransmitter are released in response to various stimuli, suggesting that the absence of α-syn allowed more synaptic vesicles to fuse with the presynaptic membrane. This implicates α-syn in stalling these vesicles (Abeliovich et al., 2000). Overexpression of α-syn in PC12 cells elicits the opposite response – lower quantities of dopamine are released from cells overexpressing α-syn than the wild type counterpart (Larsen et al., 2006).

Other phenotypes associated with α-syn overexpression can also be compared to features of synucleinopathies. Higher expression of α-syn in yeast elicited mitochondrial dysfunction

(Buttner et al., 2008; Flower et al., 2005; Su et al., 2010), as observed by higher levels of ROS production and downregulation of mitochondrially related genes as revealed by microarray analysis. This is reminiscent of the fact that other PD-related genes, such as *parkin* and *PINK1*, have been ascribed mitochondrial functions (Clark et al., 2006; Kitada et al., 1998; Valente et al., 2004b). Furthermore, a small molecule screen uncovered a compound that rescued yeast from α-syn-induced toxicity and reversed the mitochondrial signature (Su et al., 2010). This compound could rescue not only animal models of α-syn-induced toxicity, but also toxicity induced by treating rat midbrain neuronal culture with rotenone. This work helped to both prove the relevance of the model and show connections between α-syn and mitochondrially related genes and toxins implicated in PD progression, providing evidence that these were interrelated processes and not entirely separate means to induce PD.

The yeast model also has been helpful in showing ties between α-syn and another PD-related gene, *PARK9* (Gitler et al., 2009; Ramirez et al., 2006), which encodes the metal transporter ATP13A2. The yeast homolog of this gene, later named *YPK9*, was discovered in the previously described overexpression screen as a suppressor of α-syn-induced toxicity. More detailed analysis revealed that *PARK9* homologs could rescue higher organism models of α-syn-induced toxicity and uncovered that *YPK9* plays a role in manganese homeostasis. Intriguingly, while manganese exposure and mutations in *PARK9* had been tied to parkinsonism, this form of the disease shows no Lewy bodies and affects a different brain region than classical PD (Perl and Olanow, 2007; Smyth et al., 1973). There were no previously established ties between metal levels and α-syn, and therefore, the yeast model was able to provide a novel link between α-syn-induced toxicity, *PARK9*, and manganese induced parkinsonism.

Given the physical association of α-syn with lipids, it is not surprising that α-syn induces lipid-related phenotypes in yeast. The earliest description of α-syn expression in yeast reported an increase in lipid droplet formation in yeast expressing a toxic level of α-syn (Outeiro and Lindquist, 2003). Furthermore, the toxicity induced by α-syn expression is sensitive to the lipid environment in yeast, as the toxicity of α-syn-expressing cells is enhanced by inhibition of elongases required for the generation of very long chain fatty acids (Lee et al., 2011). Finally, the local lipid environment may be important for the formation of α-syn foci – one group showed an increased concentration of phosphatidic acid and that increases in acidic phospholipids increased the ability of α-syn to form foci (Soper et al., 2011), while another report found that α-syn associated with lipid rafts (Zabrocki et al., 2008).

Lipid rafts are highly enriched in sterols. Higher organisms, including humans, utilize cholesterol as their main sterol; however, in yeast, this role is fulfilled by ergosterol. Thorough analysis of two high-throughput analyses also revealed the importance of ergosterol in α-syn-induced toxicity. A flow algorithm, named ResponseNet, analyzes connections between differentially expressed genes found in microarray analyses and modifiers in an overexpression screen resulting from the same cellular perturbation (Yeger-Lotem et al., 2009). This is especially important because, in most cases, the hits from these two analyses are mutually exclusive, as hits from genetic screens tend to be biased toward transcriptional and regulatory proteins that can affect entire pathways, while microarray analyses tend to favor metabolic proteins involved in the response of a perturbation. ResponseNet was able to highlight genetic pathways affected by α-syn expression. One path highlighted by this algorithm was genes involved in ergosterol biosynthesis. Indeed, α-syn-expressing cells were found to be

hypersensitive to treatment with lovastatin, an inhibitor of HMG-CoA reductase, which an upstream biosynthetic gene a gene upstream in ergosterol synthesis.

There is already some evidence from human studies to suggest that this impact involving sterols is medically relevant for humans; however, the current state of the field is complicated by contradicting information. One study associated occurrences of PD with reduced levels of low-density lipoprotein (LDL) cholesterol (Huang et al., 2008). A case study also reported on a patient who developed reversible PD-related symptoms upon treatment with a statin (Muller et al., 1995). Conversely, another study found slower disease progression in PD patients on statins (Wolozin et al., 2007). However, this latter study may be confounded by the fact that individuals on statins have high levels of cholesterol to begin with or that statins affect a number of cholesterol-independent pathways (Goldstein and Brown, 1990) that may confound these studies. Nevertheless, this suggests an interesting relationship between cholesterol and PD progression.

(a)

α-Syn Expression

	NoTox	IntTox	HiTox
Foci Formation	Absent	Moderate	High
Growth Defect	Absent	Moderate	High
Vesicle Accumulation	Mild	Moderate	High
Mitochondrial Defect	Absent	Mild	Present
Lipid Droplet Accumulation	Absent	Mild	Present

(b)

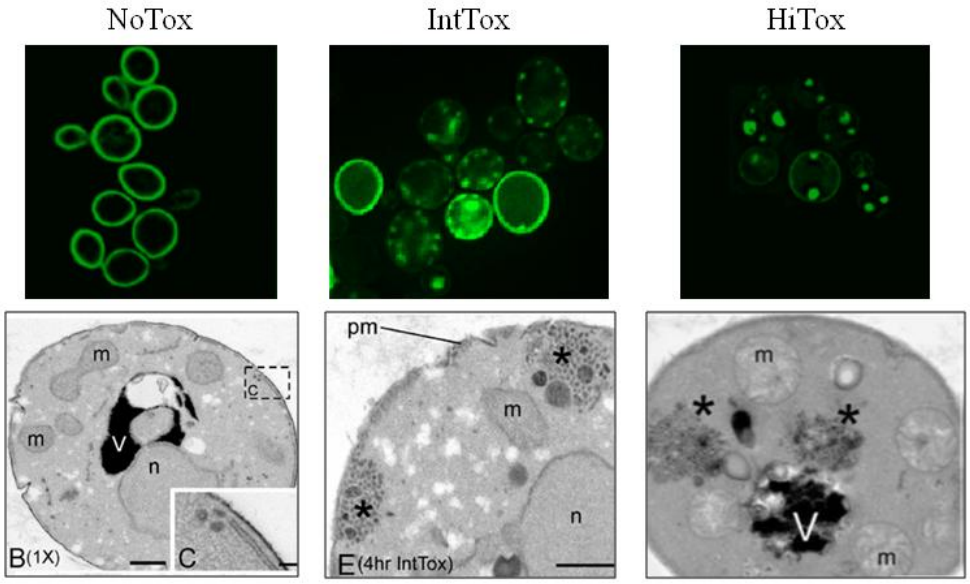

Figure 1.1. Summary of phenotypes associated with α-syn expression in *S. cerevisiae*. (a). Table describing the cellular phenotypes associated with three main strains of *S. cerevisiae* with increasing levels of α-syn expression. (b) Fluorescent and electron microscopy of the strains. m=mitochondria, n=nucleus, v=vacuole, pm=plasma membrane, *=accumulation of vesicles. Adapted from (Cooper et al., 2006; Gitler et al., 2008; Outeiro and Lindquist, 2003; Su et al., 2010)

α-Synuclein splice isoforms

The second chapter of this thesis highlights work studying the properties of two splice isoforms of α-syn in yeast. α-Syn is comprised of seven exons (Beyer and Ariza, 2012; Xia et al., 2001). The first two exons exists entirely in the 5' untranslated region of the protein, and thus was not used in any *S. cerevisiae*-based α-syn studies as endogenous *S. cerevisiae* regulatory sequences were used instead. Exons three and four form the majority of the N-terminus of the protein. All three disease-related point mutations of α-syn are found within these two exons, with A30P located in the third exon and E46K and A53T in the fourth. The remainder of the N-terminus and the NAC domain are encoded by exon five. Exon six and exon seven form the C-terminus and 3' untranslated region (Beyer, 2006). Four isoforms of α-syn have been identified in humans, α-syn140 (full length), α-syn126 (lacking exon four), α-syn112 (lacking exon six), and α-syn98 (lacking exons four and six) (Beyer et al., 2008a; Campion et al., 1995; Ueda et al., 1994).

Recent work has highlighted the pathophysiological relevance of these splice isoforms. Multiple studies have shown variation in levels of these splice isoforms in patients with synucleinopathies (Beyer et al., 2008a; Beyer et al., 2008b; Beyer et al., 2006; Beyer et al., 2004). Furthermore, these variations differ between distinct synucleinopathies. For example, in patients with dementia with Lewy bodies, levels of α-syn140 (full-length) and α-syn126 significantly decreased, while levels of α-syn112 and α-syn98 significantly increased (Beyer et al., 2008a; McLean et al., 2011). In PD, levels of all forms of α-syn increased, although to different extents (Beyer et al., 2008a; McLean et al., 2011). Although all of these studies are constrained by low sample size, the repeated reports of variation in α-syn isoform levels between patients and controls suggest there is some correlation between splicing and disease onset.

Furthermore, variability in the 3' untranslated region of α-syn, including risk alleles for sporadic PD, is associated with increased levels of α-syn splicing, further suggesting that variability in splice isoform level may be associated with PD (McCarthy et al., 2010).

There are also ties between α-syn splicing and toxin-driven models of PD. Treatment of SH-SY5Y dopaminergic cells with either MPP+ (the toxic byproduct of MPTP) or rotenone, two of the environmental toxins used to model PD, leads to an increase in exon five splicing (Kalivendi et al., 2009). Also, expression of α-syn98 renders PC12 cells more sensitive to rotenone and generates higher levels of reactive oxygen species than expression of α-syn140 (Ma et al., 2011).

Previous work on α-syn sequence truncations and mutants in *S. cerevisiae* allowed us to generate hypotheses on the properties of the various α-syn splice isoforms. One study examined a panel of α-syn point mutants for their toxicity and ability to bind to the plasma membrane in *S. cerevisiae* (Volles and Lansbury, 2007). They found a direct relationship between the mutant's ability to bind to the plasma membrane and its ability to induce toxicity, suggesting that lipid binding is important for the mechanism by which α-syn impedes cellular growth. Removal of a portion of the NAC region (AA 71-82) from α-syn (or expression of β-syn) destroyed the protein's ability to form foci and induce toxicity, but did not affect membrane binding (Soper et al., 2008). This work suggests that the biophysical properties of α-syn could be studied in *S. cerevisiae* and that the different isoforms may elicit differential localization and potential to induce toxicity.

S. cerevisiae rarely utilize splicing as a mechanism of genetic regulation (Dujon, 1996). Therefore, I generated expression constructs containing the alternatively spliced versions of α-syn by overlap extension PCR. These constructs were then integrated into *S. cerevisiae* genome to study the biophysical properties of the proteins and the mechanism by which each isoform of

α-syn induces toxicity. This study uncovered an interesting phenotype concerning differential sensitivity to lipid homeostasis, as will be discussed in chapter two.

Modeling early onset torsion dystonia

Early Onset Torsion Dystonia (EOTD) is a movement disorder characterized by involuntary muscle contractions that begins in childhood (Fahn, 1988). There is currently no cure for the disease, leaving the young individuals who are diagnosed with it to face a lifetime of medications and surgical treatments that sometimes can only partially alleviate the symptoms. It is caused by a single glutamic acid deletion in the gene torsinA (torsinAΔE), which is inherited in an autosomal dominant manner with reduced penetrance (Bressman et al., 1989; Ozelius et al., 1997).

TorsinA is a AAA+ ATPase that resides in the ER and nuclear membrane (Hewett et al., 2003; Kustedjo et al., 2000). This family of ATPases plays many roles in the cell (Hanson and Whiteheart, 2005), including protein remodeling, like the family member HSP104 (Parsell et al., 1994). This, combined with the fact that torsinA is often found in LB (Shashidharan et al., 2000a; Shashidharan et al., 2000b), led to the hypothesis that torsinA may also play a role in protein remodeling. Indeed, torsinA can untangle proteins *in vitro* (Burdette et al., 2010) and reduce aggregation of α-syn in H4 neuroglioma cells (McLean et al., 2002). TorsinA has also been implicated in relieving ER stress (Burdette et al., 2010; Chen et al., 2010) and affects trafficking (Hewett et al., 2007; Torres et al., 2004), which all may be linked to its role in protein remodeling. Many of these studies have also shown that the EOTD-related glutamic acid deletion blocks the function of torsinA.

In chapter three, I describe a model of torsinA function and/or toxicity in *S. cerevisiae*. Unfortunately, these goals were not met, as every assay I attempted failed to demonstrate a phenotype of torsinA expression. I hope that as more becomes known about torsinA function, especially its post-translational processing, which may require enzymes not conserved in *S. cerevisiae*, that my work can serve as the basis for improving the model and allow *S. cerevisiae* to be utilized to understand torsinA function in a manner similar to its use in studying α-syn.

Concluding remarks

It is striking to look back on the success of studying disease-related proteins in *S. cerevisiae*; however, upon closer examination of yeast as a model organism, it should not be surprising. In terms of cellular biology, *S. cerevisiae* is at the pinnacle of combining both ease of genetic analysis with conservation to higher eukaryotes at an intracellular level. While this single-celled organism may not be able to answer questions about how disease-related proteins affect organisms at a level of cell-cell communication or organ function, as it becomes clear that understanding the cellular defects associated with disorders helps us decipher the disease mechanism, we begin to appreciate the utility of such an elegant model.

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Chapter Two:

Splice Isoform Studies Reveal that Sterol Depletion Relocalizes and Enhances Toxicity of α-Synuclein

This chapter are in preparation for submission to the *Journal of Cell Biology.*

Abstract

Synucleinopathies are a group of neurodegenerative diseases associated with the toxicity of alpha synuclein (α-syn). α-Syn splice isoform expression varies with disease, but it is not known how these isoforms affect the protein's function. Using yeast, we investigated two of the most abundant isoforms, which result in deletion of exon four (α-synΔ4) or exon six (α-synΔ6). α-SynΔ4, missing part of the lipid binding domain, had reduced toxicity and membrane binding. α-SynΔ6, missing part of the protein-protein interaction domain, also had reduced toxicity but no reduction in membrane binding. When expressed at high enough levels to cause toxicity, the splice isoforms generally duplicated the biological activities of wild-type α-syn, but responded differently to expression sterol binding proteins and to chemical inhibitors of sterol synthesis. Our findings suggest that the splice variants may be part of a protective response to α-syn toxicity. Given the prevalence of cholesterol-reducing drugs in modern medicine, the role of these isoforms in diverse synucleinopathies warrant further investigation.

Introduction

Alpha synuclein (α-syn) is a small, 14kDa protein associated with progression of synucleinopathies, a family of neurodegenerative diseases. These include Parkinson's disease (PD), multiple system atrophy, and dementia with Lewy bodies (Lee and Trojanowski, 2006), which are characterized by the degeneration of different neuronal cell types. The accumulation of misfolded α-syn is a hallmark of all of these diseases. Moreover, increased expression (through α-syn by copy number variation or the effects of regulatory SNPs) can drive these diseases (Kruger et al., 1998; Mizuta et al., 2006; Polymeropoulos et al., 1997; Zarranz et al., 2004). Indeed, many laboratories now model synucleinopathies by overexpressing α-syn (Feany and Bender, 2000; Lakso et al., 2003; Masliah et al., 2000; Outeiro and Lindquist, 2003).

The structural properties of α-syn provide insight into its biology. Its N-terminus is comprised of seven imperfect repeats that fold into amphipathic helices in the presence of lipids, allowing α-syn to interact with membranes (Davidson et al., 1998). α-Syn can also form toxic multimers, a property dependent on amino acids 71-82 (Giasson et al., 2001). Finally, the protein contains an unstructured C-terminal tail important for various protein-protein interactions (Eliezer et al., 2001). These basic biophysical properties generally affect α-syn functionality, allowing much simpler cell types, such as yeast, to be used in modeling α-syn-induced cellular pathologies.

Studying the cell biology of α-syn in *Saccharomyces cerevisiae* affords multiple advantages (Khurana and Lindquist, 2010). Yeast share fundamental eukaryotic cell biology with higher organisms, including vesicular trafficking, protein homeostasis, mitochondria, peroxisomes, autophagy and other functions of direct relevance to disease.

Moreover, yeast provide an unparalleled genetic toolbox, enabling both detailed hypothesis-driven analyses and unbiased screen-based discovery.

When α-syn is expressed at low levels in yeast, it localizes to the plasma membrane, highlighting its lipophilic nature. In both yeast and neurons, α-syn associates with lipid rafts, suggesting that α-syn displays similar lipid binding preferences in both cell types (Fortin et al., 2004; Zabrocki et al., 2008). At higher expression levels, α-syn foci form (Outeiro and Lindquist, 2003). Higher expression also elicits a number of cellular defects, including slowed growth, disrupted vesicle trafficking, lipid droplet accumulation, and mitochondrial dysfunction (Cooper et al., 2006; Outeiro and Lindquist, 2003). Importantly, these phenotypes have been verified in higher organism models of synucleinopathies. Studies in yeast also established a previously unknown link between α-syn and a second parkinsonism-associated protein, ATP13A2, and connected both proteins to manganese homeostasis (Gitler et al., 2009). Further, high-throughput screens identified compounds that rescue yeast and neuronal cells not only from α-syn-induced toxicity but also from mitochondrial poisons associated with PD (Su et al., 2010). Clearly, yeast models can provide insights on α-syn function that are directly relevant to neuropathology.

Here we use a yeast model to investigate naturally-occurring splice isoforms of α-syn. Full length α-syn (α-synFL) contains seven exons (Beyer and Ariza, 2012; Xia et al., 2001). (Note that exon numbering has recently been adjusted, due to discovery of a new exon in the 5'-untranslated region; Beyer and Ariza, 2012.). Exons one and two, as well as part of exon three, constitute the 5'-untranslated region of the mRNA. The remainder of exon three, as well as exon four and part of exon five comprise the N-terminal region of the protein, while the rest of exon five, as well as exons six and seven, create the C-terminal tail

of the protein. Both exon four and exon six are subject to alternative splicing, creating α-synΔ4 and α-synΔ6, respectively. These variants are differentially expressed in various synucleinopathies (Beyer et al., 2008; Campion et al., 1995; McLean et al.; Ueda et al., 1994) and recent work tied PD risk factors to increased splicing of exon six (Kalivendi et al., 2009; McCarthy et al., 2010). Whether this change in splicing contributes to pathology, is part of a protective mechanism, or is functionally inconsequential is unknown.

Here, we compared the biological effects of α-synΔ4 and α-synΔ6 with α-synFL. We characterized differences in the localization of each isoform and their relative toxicities. Testing the effects of 75 genes that modify the toxicity of α-synFL against the splice isoforms, we uncovered a differential response to the oxysterol-binding proteins Osh2p and Osh3p. This led us to study the sensitivity of α-synFL and the splice isoforms to perturbations in sterol synthesis.

Results and Discussion

Splice isoforms of α-syn display different toxicity

To investigate how variations in splicing affect the toxicity of α-syn we created strains that expressed equal copy numbers of α-synFL, α-synΔ4, or α-synΔ6 (Figure 2.1a). Genes directly encoding these variants were integrated into the *HIS3*, *LEU2*, *TRP1*, and *URA3* loci. Such transformants can contain multiple tandem integrants, which would confound this analysis. Therefore, we designed a PCR-based method to detect single integrants at each locus (Figure 2.S1) and generated strains containing single integrations of all isoform at each (Figure 2.1b). This allowed a precisely controlled comparison of isoform toxicities. All of the genes were driven by the same galactose-inducible promoter so that expression

could be induced by shifting cells to galactose containing media. Increasing copy numbers increased RNA expression. (Note that due to limiting concentrations of critical regulatory factors, mRNA expression does not increase commensurately with gene copy number; Figure 2.1c.)

Each protein variant accumulated to approximately the same level after galactose induction, indicating that the proteins have similar turnover rates (Figure 2.1d). As expected, strains carrying four copies (4x) of α-synFL displayed strong toxicity. In contrast, 4x α-synΔ6 strains showed only marginal toxicity and 4x α-synΔ4 strains grew as well as the GFP control strain (Figure 2.2a). Thus, when expressed on their own, the proteins have very different inherent capacities to derange cellular homeostasis.

The splice isoforms are not expressed in the absence of α-synFL in the brain. To assess the ability of these isoforms to contribute to the toxicity of α-synFL, we constructed strains with two copies (2x) of each splice isoform and two copies of α-synFL. Strains expressing only 2x α-synFL showed no growth defect, in keeping with the extreme dosage sensitivity previously reported for this protein in yeast and in humans (Outeiro and Lindquist, 2003). With this level of α-synFL expression however, the addition of two copies of either splice isoform was toxic. α-SynΔ4 enhanced toxicity to a lesser degree than α-synΔ6 (Figure 2.2b). Therefore, while both splice isoforms are intrinsically less toxic, they can nevertheless contribute to the toxicity of the full-length protein.

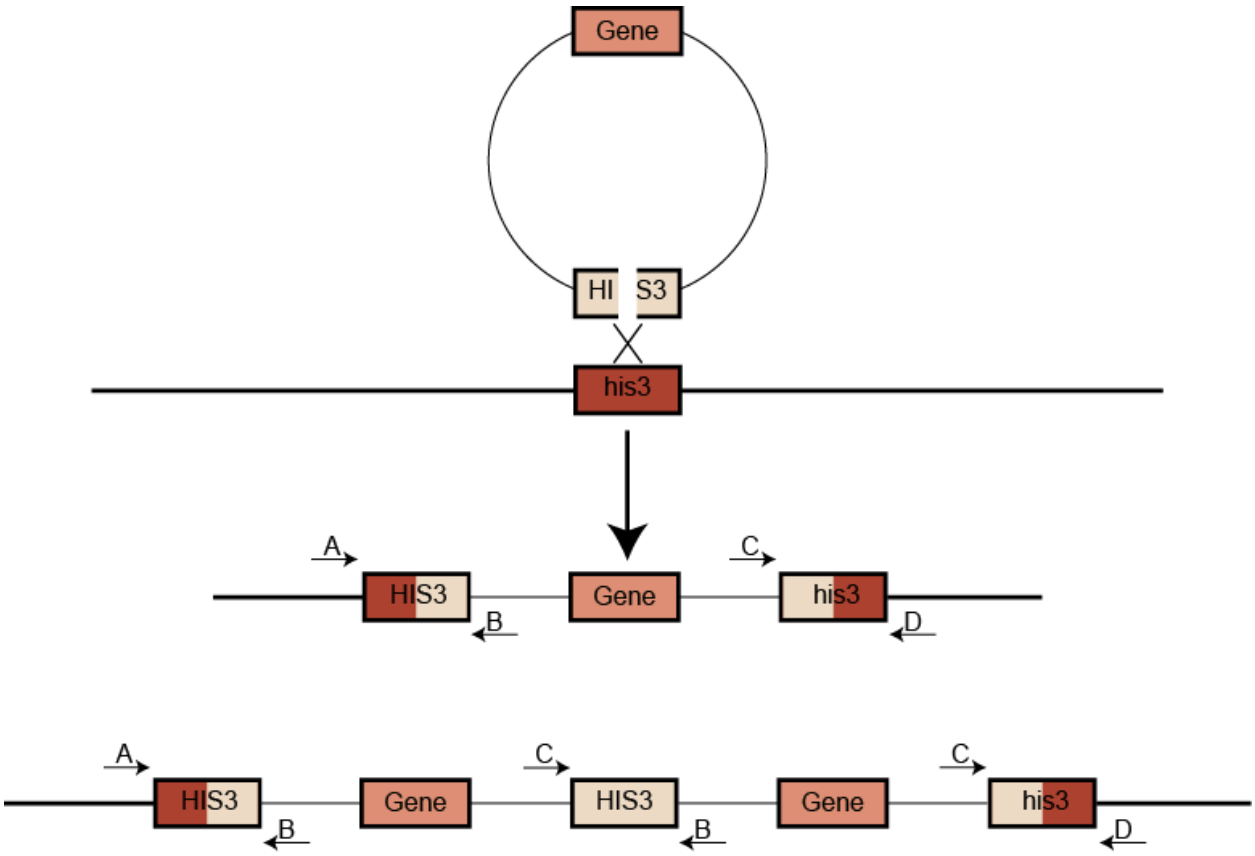

Figure 2.S1. Diagram showing the primer placement for testing for single integration of pRS plasmids.
Primers were designed to be used regardless of the identity of the inserted gene. Colony PCR was used with primer sets A+B, C+D, A+D, and B+C. A+B and C+D would result in a band if there was a correct insertion; A+D would result in a band if there was no correct insertion, and B+C would result in a band if there were tandem insertions. After this screening, long extension PCR was used with primers A+D to confirm the results.

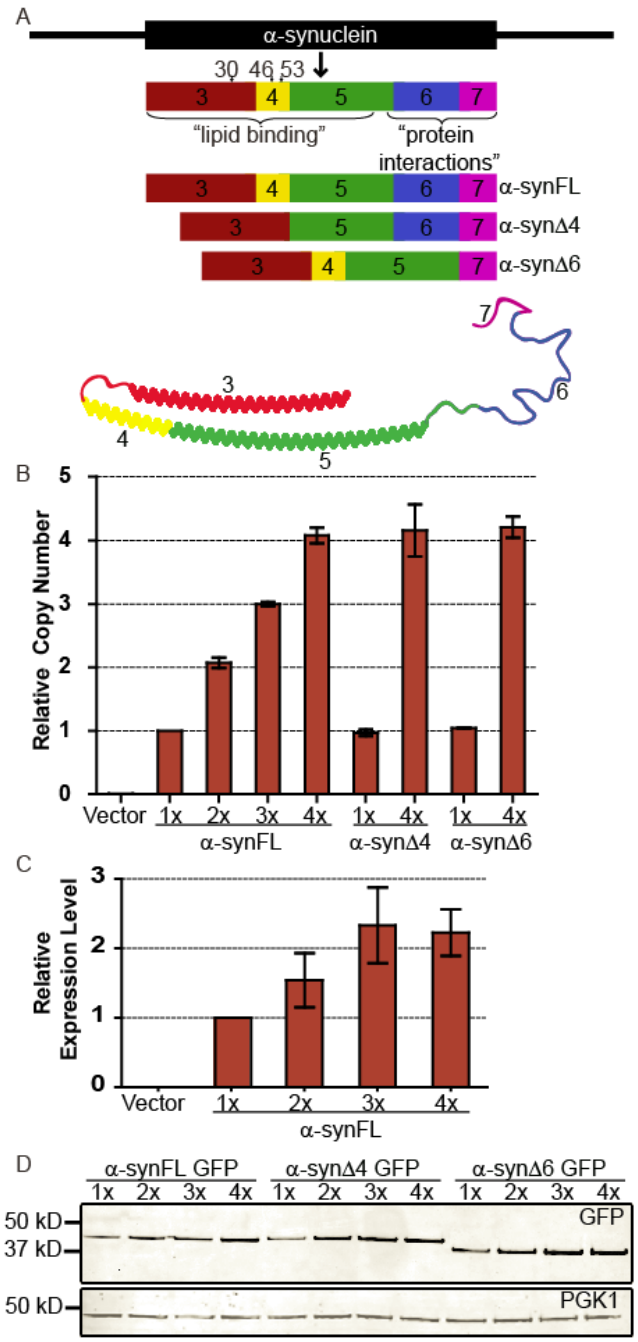

Figure 2.1. Creation of strains with equal copy numbers of the α-syn splice isoforms.

(a) Summary of isoforms tested.

(b) Real time PCR analysis of DNA levels of splice isoform strains, compared to one copy of α-synFL. 1x-4x represents α-syn copy number. n=2, error bars represent standard deviation.

(c) Real time PCR analysis of RNA levels from induction of α-synFL strains. 1x-4x represents α-syn copy number. Levels were normalized to PGK1, and compared to one copy of α-synFL. n=3, error bars represent standard deviation.

(d) Western blot analysis of strains with 1x-4x copies of splice isoforms. PGK1 = loading control.

Splice isoforms of α-syn display different localization

As previously reported, when first induced, α-synFL localizes to the plasma membrane. As it accumulates to toxic levels, it begins to form foci, first around the periphery of the cell and then extending into the interior (Gitler et al., 2008). In the 4x strains, after six hours of induction, α-synFL was observed at both locations (Figure 2.2c). α-SynΔ4 lacks a portion of the N-terminal amphipathic helix required for lipid binding and localized less efficiently to the plasma membrane. α-SynΔ6, which has the lipid binding domain intact, was primarily localized to the plasma membrane (Figure 2.2c). At this level of expression, α-synΔ4 and α-synΔ6 rarely formed foci. These results confirm the previously reported role of the N-terminal lipid binding-domain of α-syn in foci formation (Volles and Lansbury, 2007) and implicate the C-terminal region of α-syn as well. α-SynFL foci are the sites of accumulation of blocked vesicles (Gitler et al., 2008). Thus, this result suggest the C-terminal protein::protein interaction domain (Auluck et al., 2010), plays an important role in blocking vesicular trafficking.

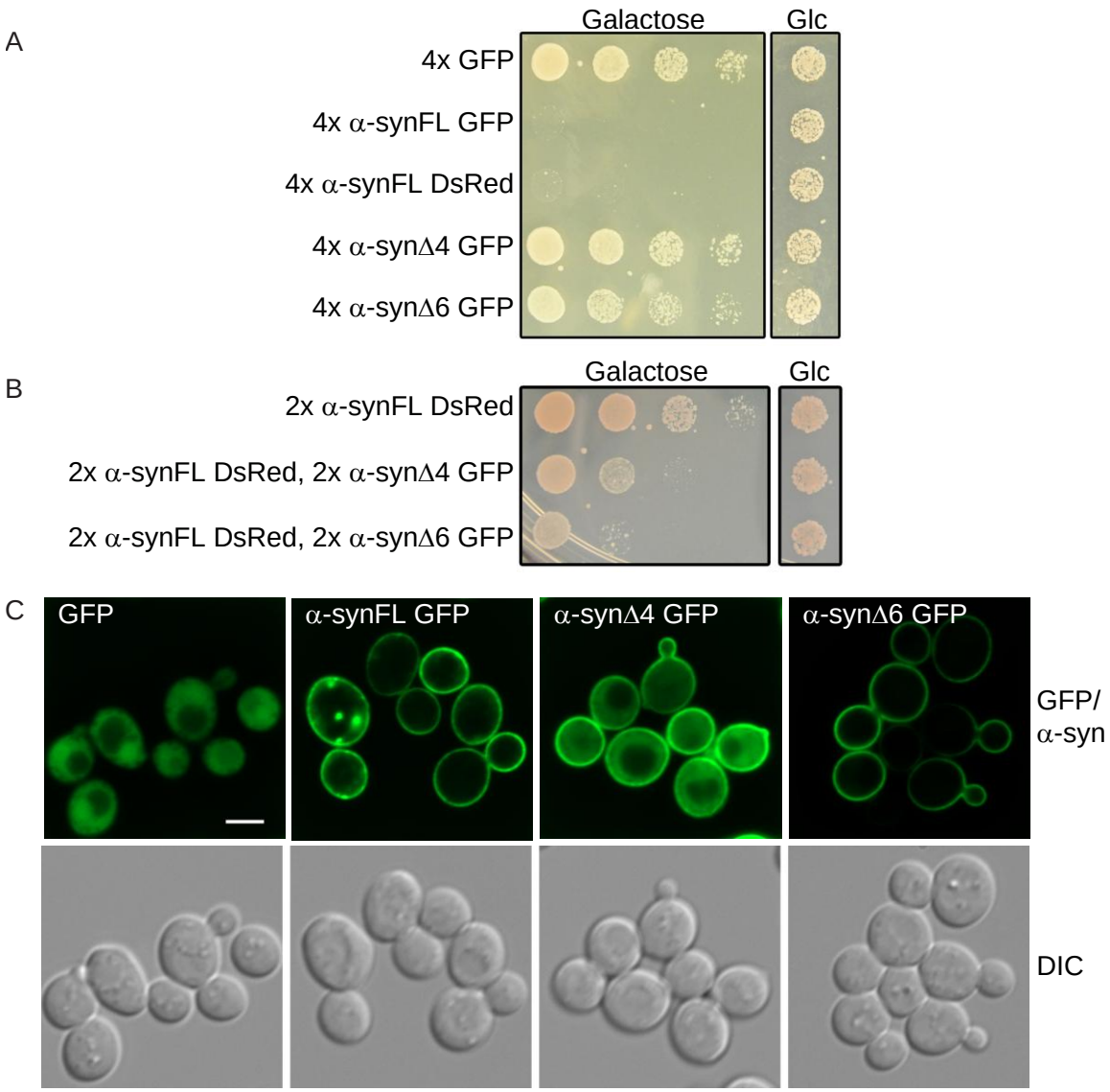

Figure 2.2. α-Syn splice isoforms elicit different toxicities and localization when expressed at the same level in *Saccharomyces cerevisiae*.

(a) Spot assay of α-syn-expressing strains with four copies of the indicated isoforms. Glc = glucose.

(b) Spot assay of strains co-expressing α-synFL and the splice isoforms. Glc = glucose.

(c) Microscopy reveals differential localization of splice isoforms. Fluorescent microscopy of 4x splice isoform strains. Scale bar = 5 μM.

Tandem plasmid integration results in strains with similar levels of toxicity

We have previously described several cellular processes that are affected by α-syn in yeast, and have determined the effect of most genes in the genome on α-syn toxicity (Cooper et al., 2006; Outeiro and Lindquist, 2003). Taking full advantage of yeast genetics to investigate functional differences between the splice isoforms required strains with similar levels of toxicity (Figure 2.3a). To create such strains, we isolated transformants with different numbers of tandem plasmid integrations, selecting those with similar levels of toxicity (Figure 2.3 b and c).

In these strains, α-synFL showed the expected localization pattern, forming small foci around the perimeter of the cells and larger foci in the cytoplasm (Figure 2.3d). α-SynΔ4 and α-synΔ6 also formed foci. However, α-synΔ4 still showed high levels of cytoplasmic localization.

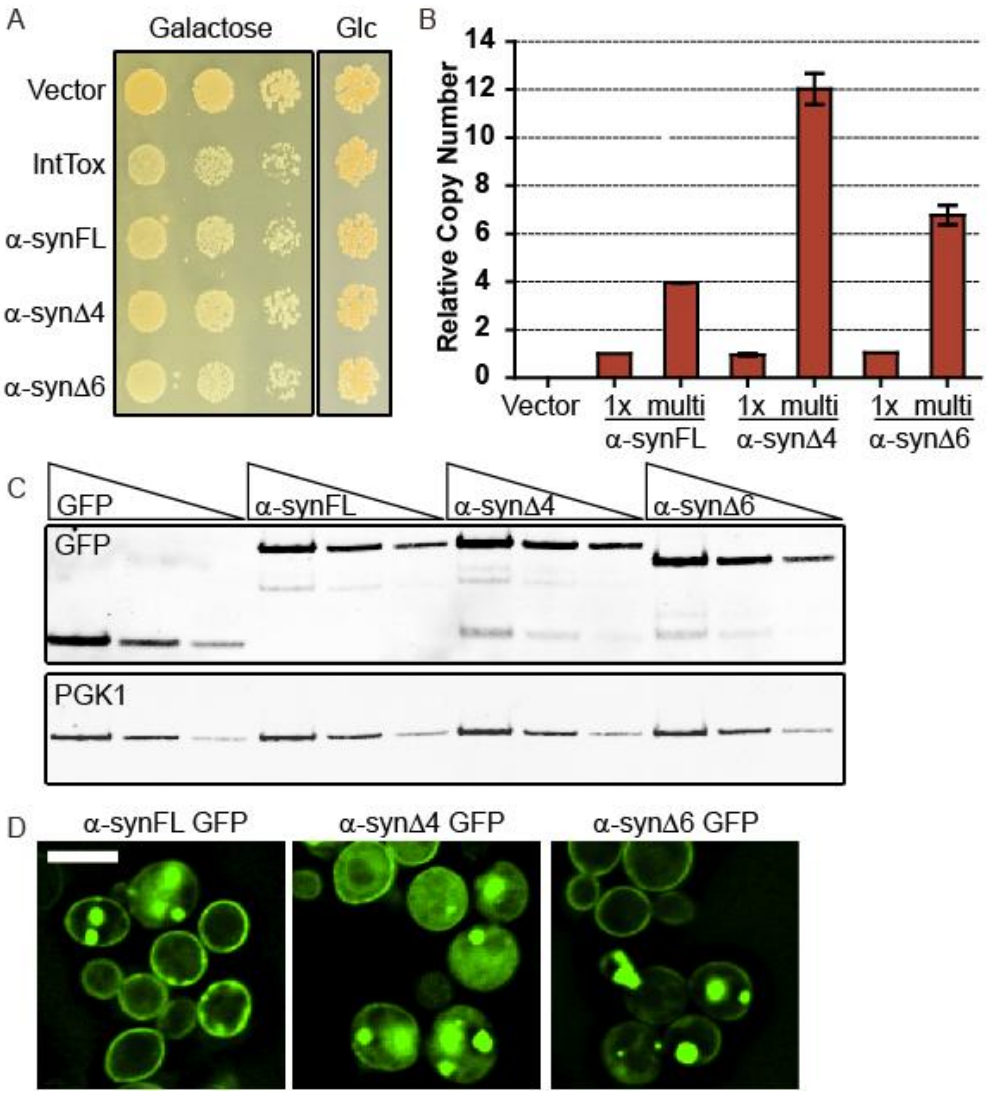

Figure 2.3. Higher levels of α-syn splice isoform expression leads to higher toxicity and foci formation.

(a) Spot assay of α-syn-expressing strains with different numbers of integrated copies but demonstrating equal levels of toxicity. IntTox is a previously created strain expressing α-synFL-YFP that was used in a high-throughput overexpression screen. Glc = glucose.

(b) Quantification of the number of α-syn copies inserted into the multi-copy strains by real time PCR in comparison to the 1x α-synFL strain. n=2, error bars represent standard deviation.

(c) Western blot analysis of α-syn splice isoforms in the same multi-copy strains shown in (a). PGK1 = loading control.

(d) Fluorescence microscopy of the same strains as shown in (a) expressing higher levels of α-syn. Scalebar = 5 μM.

Isoforms of α-syn block secretion

One of the earliest toxicities associated with α-syn expression is a block in secretory vesicle trafficking (Cooper et al., 2006). To assess if all splice isoforms could inhibit secretion, we used a simple halo assay for mating factor secretion. Cells of one mating type are spotted onto a lawn of the other, and the halos of growth arrest provide a qualitative measure of mating factor secretion. When tested on glucose plates, no α-syn was expressed and halos were roughly equal size. In contrast, when spotted on inducing galactose plates, strains expressing similarly toxic levels of all three α-syn splice isoforms showed similarly reduced zones of inhibition. This suggested that all three variants are capable of inhibiting secretion (Figure 2.4a). Importantly, this phenotype was not a simple generic affect of expressing a toxic protein; toxic levels of the Alzheimer's disease-related peptide Aβ (Treusch et al., 2011) did not significantly affect trafficking.

We next asked if the foci formed by the splice isoforms represent α-syn-blocked clumps of secretory vesicles, as is the case for α-synFL (Gitler et al., 2008). This phenotype can be probed by assaying colocalization of α-syn and secretory pathway proteins, such as Vps21p (Gitler et al., 2008). Indeed, colocalization was observed between Vps21p and α-synFL, as well as α-synΔ4 and α-synΔ6 (Figure 2.4b). In contrast, clatherin heavy chain protein Chc1p which is associated with endocytic vesicles did not show colocalization with any of the α-syn isoforms (Figure 2.S2a). This suggested that all three isoforms of α-syn result in accumulation of secretory vesicle clusters.

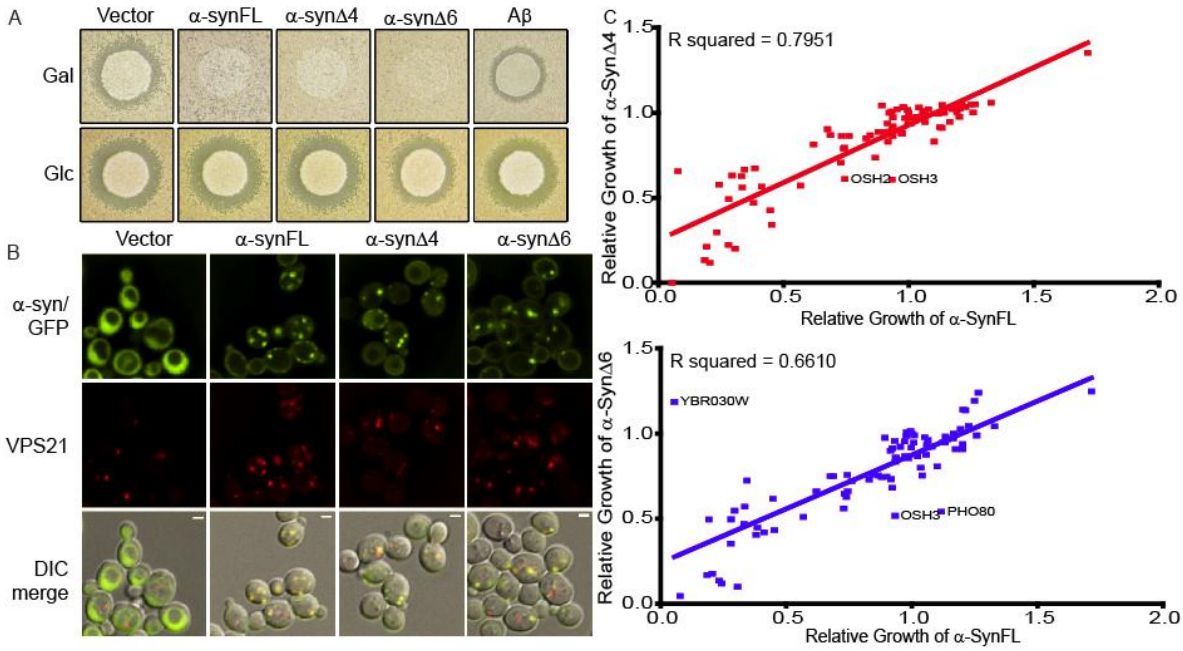

Figure 2.4. Splice isoforms elicit toxicity by similar, but not identical, mechanisms.

(a) Halo assays to test the secretion capacities of α-syn-expressing cells. Mat alpha cells were spotted on a lawn of *bar1* mat a cells and allowed to grow for two days. The zone of clearance surrounding the spot represents growth inhibition by secretion of alpha factor. Yeast expressing Aβ serve as a control. Gal = galactose, Glc = glucose.

(b) Fluorescent microscopy of cells expressing α-syn isoforms, tagged with GFP, and mKate-Vps21p. Scalebar = 2 μM.

(c) Relative growth of α-synFL-expressing strains transformed with genetic modifiers of toxicity compared to α-synΔ4- (red) or α-synΔ6- (blue) expressing strains. Each point represents the relative growth of the strain transformed with one genetic modifier. As is common in high throughput screens, some hits do not repeat upon respotting. All hits shown here repeated with the exception of YBR030W.

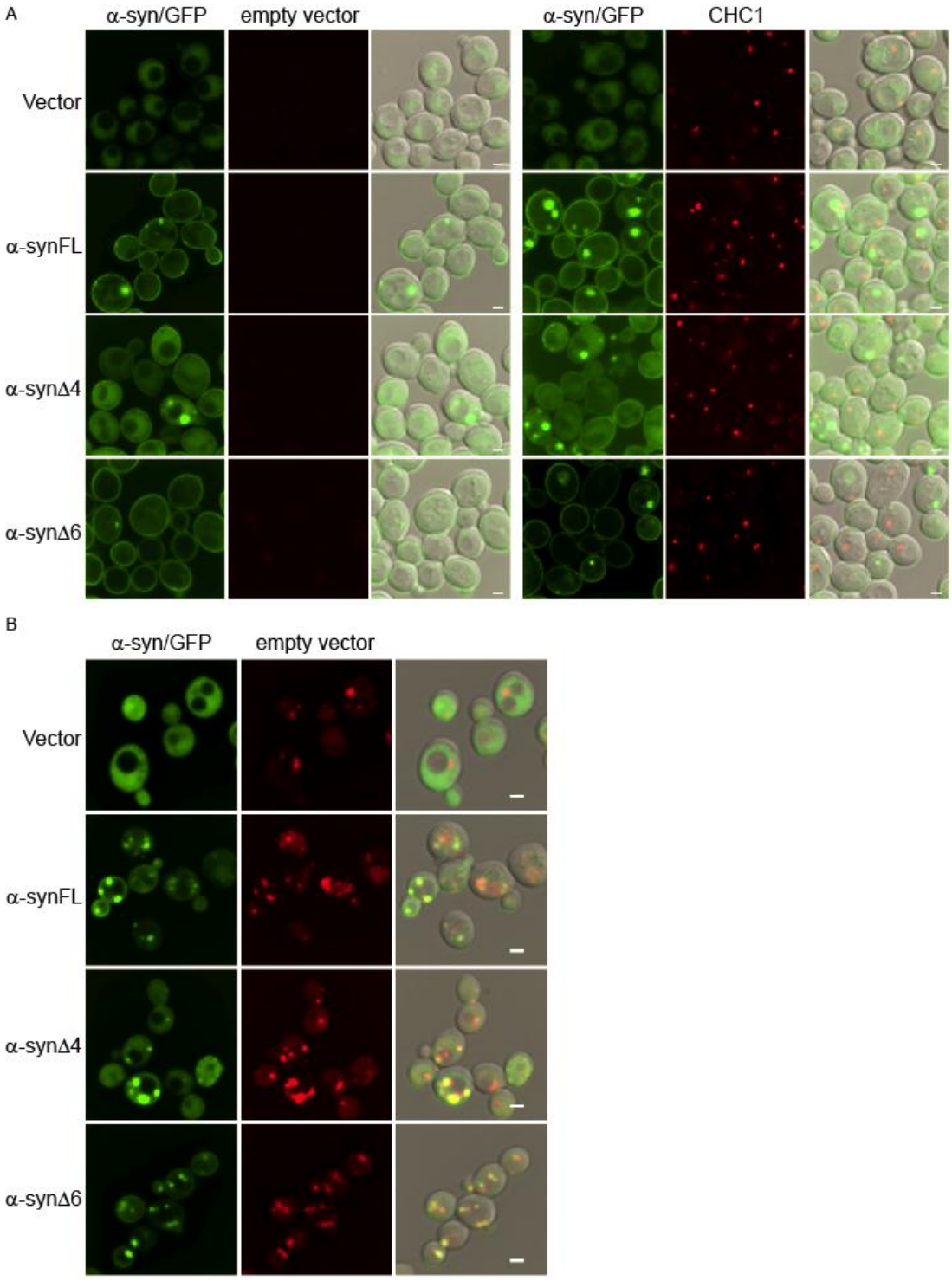

Figure 2.S2. Fluorescent microscopy of α-syn colocalization.

(a) Fluorescent microscopy of α-syn-GFP-expressing cells in addition to an empty vector or mKate-Chc1p. Scale bar = 2 μM.

(b) Fluorescent microscopy showing colocalization of α-syn-GFP and mKate-Vps21p. Scale bar = 2 μM.

α-Syn splice isoforms show differential response to OSH2 and OSH3 overexpression

To more thoroughly investigate the mechanisms by which the splice isoforms induce toxicity, we tested all 75 of the genes that were identified in an earlier screen as suppressors or enhancers of α-synFL-induced toxicity (Cooper et al., 2006). Each gene was tested in each strain at least three times to ensure the results were reproducible (Figure 2.S3).

Strikingly, most of the genes tested affected each of the splice isoforms in the same direction and to the same extent as α-synFL (Figure 2.4c). In congruence with the above results, all of the trafficking-related modifiers similarly affected α-synFL, α-synΔ4, and α-synΔ6. However, a small number of genes modified the toxicity of the two splice variants in a manner distinct from α-synFL. These effects were confirmed in individual spotting assays. Pho80p, a cyclin that monitors stress due to nutrient starvation, enhances α-synΔ6 toxicity while suppressing α-synFL and α-synΔ4 toxicity. Furthermore, Osh2p and Osh3p suppressed toxicity of the α-synFL-expressing strain but enhanced toxicity of α-synΔ4 and α-synΔ6.

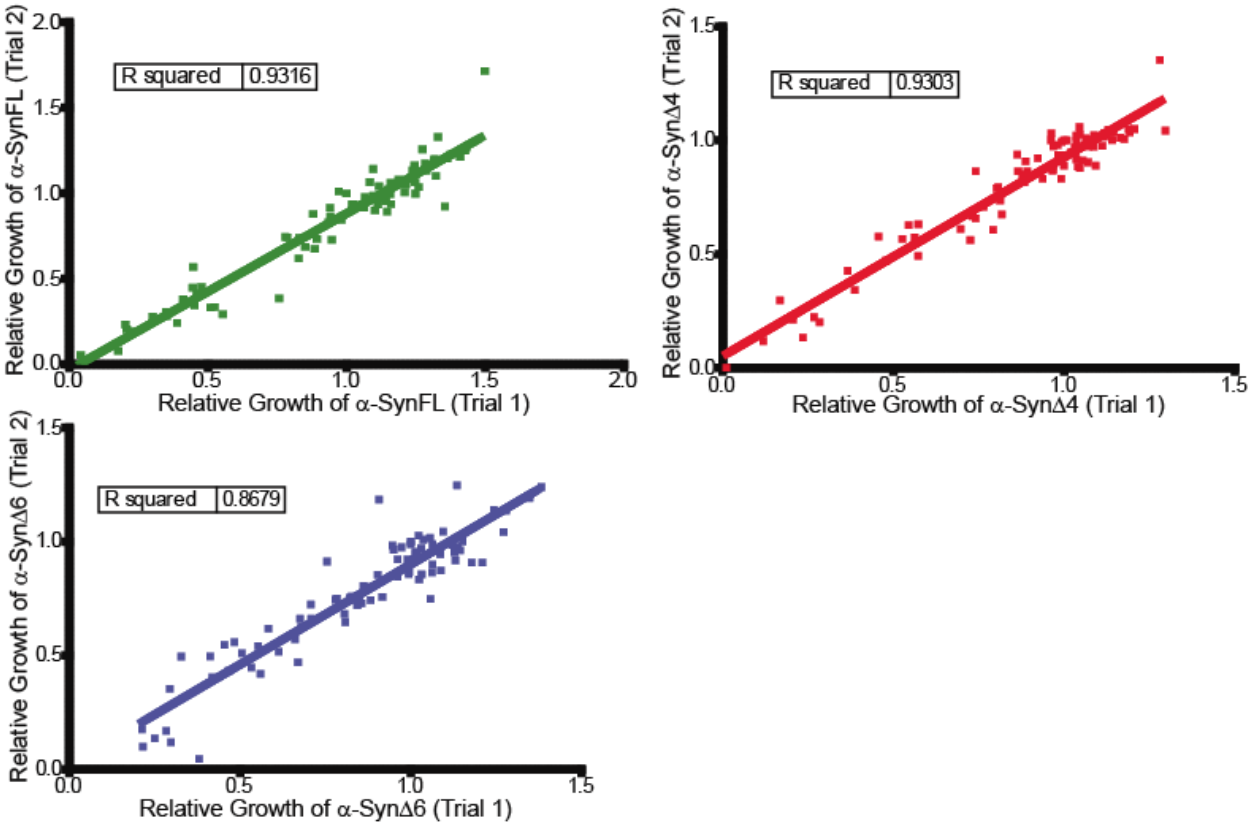

Figure 2.S3. High-throughput transformation of splice isoforms with genetic modifiers of α-syn-induced toxicity produced reproducible results.
Each spot on each graph represents the rate of growth of one splice isoform of α-syn strain transformed with one genetic modifier on two separate trials.

α-SynFL and α-synΔ6, but not α-synΔ4, elicit sensitivity to simvastatin and fluconazole

Given the role of Osh2p and Osh3p in mediating cellular sterol localization (Schulz and Prinz, 2007) and recent, somewhat perplexing connections between PD and cholesterol (Huang et al., 2008; Pahan et al., 2009; Wolozin et al., 2007) we focused on the relationship between sterols and α-syn toxicity. *S. cerevisiae* cells utilize ergosterol, rather than cholesterol as their principle sterol, but the two molecules are very similar and serve the same function in maintaining the correct fluidity of the plasma membrane. α-SynFL-expressing yeast were sensitive to treatment with statins, which inhibit of the enzyme HMG-CoA reductase, a rate-limiting step in the production of sterols in both yeast and humans (Yeger-Lotem et al., 2009). Therefore, we asked if cells expressing the splice isoforms of α-syn were similarly sensitive to treatment with simvastatin, a commonly prescribed statin in man.

α-synFL and α-synΔ6 expression caused sensitivity to doses of simvastatin that were not toxic to control cells; α-synΔ4 did not (Figure 2.5a). Statins inhibit a very early step in sterol production and disrupt multiple pathways related to α-syn-induced phenotypes, such as the production of quinones, dolichol formation, and prenylation. Therefore, to focus more precisely on sterol metabolism, we took advantage of a more specific inhibitor. Fluconazole inhibits 14α-demethylase, an enzyme specific to a late step in ergosterol synthesis. Yeast treated with fluconazole accumulate a modified sterol, 14α-methyl-3,6-diol, which increases membrane fluidity (Abe et al., 2009). We treated α-syn splice isoform-expressing yeast with concentrations of fluconazole that were not toxic to control cells. Again, α-synFL and α-synΔ6 were sensitive to fluconazole treatment, while α-synΔ4-expressing cells were not (Figure 2.5a). Therefore, a shift

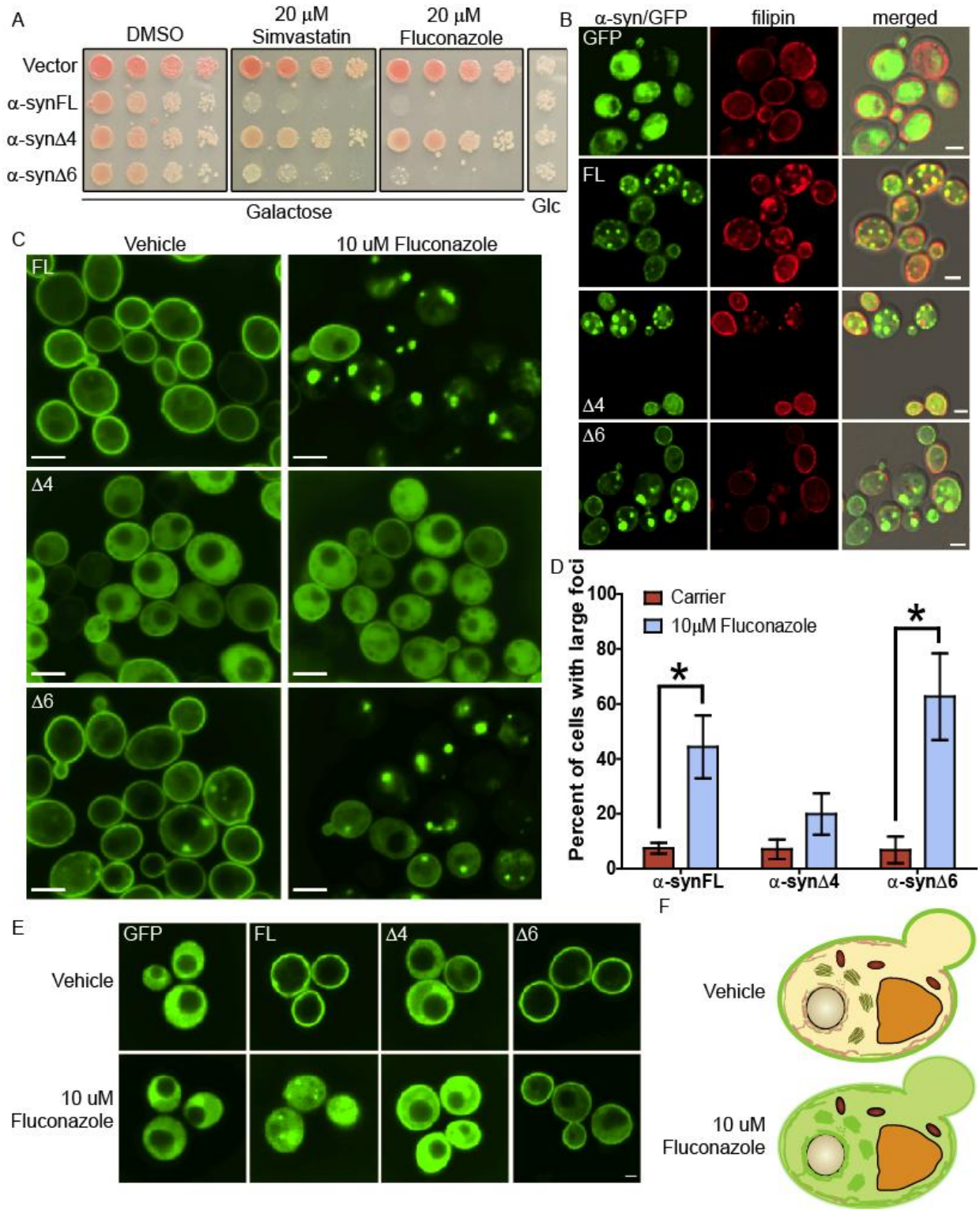
A
DMSO
20 μM Simvastatin
20 μM Fluconazole
Vector
α-synFL
α-synΔ4
α-synΔ6
Galactose
Glc
B
α-syn/GFP
filipin
merged
GFP
FL
Δ4
Δ6
C
Vehicle
10 uM Fluconazole
FL
Δ4
Δ6
D
Percent of cells with large foci
Carrier
10μM Fluconazole
α-synFL
α-synΔ4
α-synΔ6
E
GFP
FL
Δ4
Δ6
Vehicle
10 uM Fluconazole
F
Vehicle
10 uM Fluconazole

Figure 5. α-Syn splice isoforms show differential sensitivity to changes in ergosterol levels.

(a) Spot assay comparing the sensitivities of strains expressing α-syn splice isoforms to simvastatin and fluconazole. Glc = glucose.
(b) Fluorescent microscopy showing partial colocalization of α-syn-GFP and ergosterol (false-colored red). FL = α-synFL, Δ4 = α-synΔ4, Δ6 = α-synΔ6. Scalebar = 2 μM.
(c) Fluorescence microscopy showing the impact of fluconazole treatment on α-syn foci formation. Scalebar = 5 μM.
(d) Percent of cells with large α-syn foci after four hours of induction. n=3, error bars represent standard deviation, * = $p<0.05$ in a paired t-test.
(e) Fluorescence microscopy showing the impact of fluconazole treatment on α-syn localization. Fields lacking cells with foci were chosen to highlight membrane binding. Scalebar = 2 μM.
(f) Model suggesting the mechanism by which fluconazole enhances α-synFL and α-synΔ6 toxicity.

in sterol balance, and an increase in membrane fluidity, sensitizes cells to α-syn, but the spliced isoform α-synΔ4 resists this effect.

α-Syn-induced sensitivity to fluconazole can be attributed to an increased trafficking block

Since α-synFL foci represented lipid-rich, vesicular clusters blocked in trafficking by α-synFL protein, we asked if these clusters were enriched in sterols by staining with filipin, a fluorescent probe that binds sterols. In vector control cells, filipin displayed canonical staining of the plasma membrane, which has the highest sterol content in the cell (Zinser et al., 1991) (Figure 2.5b). In strains expressing all three isoforms of α-syn, filipin colocalized with to both the plasma membrane and to large intracellular α-syn foci (Figure 2.5b). Intriguingly, in many cases some foci stained with filipin and others did not. This might simply be a technical artifact, but it suggests heterogeneity in the membrane sterol content of vesicles trapped at these foci. In cells that had higher concentrations of filipin localized to α-syn foci, we saw less filipin staining at the plasma membrane. Therefore, α-syn causes a redistribution of endogenous sterols. One mechanism by which sterols arrives at the plasma membrane is by transport through the secretory pathway (Schulz and Prinz, 2007). Since α-syn expression blocks vesicular trafficking, sterol transport is also blocked by α-syn expression.

How might fluconazole treatment affect α-syn localization? Incubation with fluconazole elicited a striking relocalization of α-synFL and α-synΔ6 but not α-synΔ4 (Figure 2.5c). α-SynFL- and α-synΔ6-expressing cells treated with fluconazole were much more likely to form foci, even at a time point in which untreated cells show few foci (Figure 2.5d). These foci colocalize with Vps21p, a second secretory vesicle marker, suggesting that these foci are all α-

syn decorating clumps of secretory vesicles (Gitler et al., 2008) (Figure 2.S2b). Focusing on cells without significant foci, it is also clear that, for α-synFL and α-synΔ6, some of the protein redistributed to the cytoplasm (Figure 2.5e). This suggests that reductions in the plasma membrane sterol content disrupt plasma membrane binding of α-synFL and α-synΔ6, but not α-synΔ4. This might be due to a specific failure of α-syn to bind 14α-methyl-3,6-diol or, more broadly, to the impact of fluconazole on membrane fluidity (Abe et al., 2009). α-SynΔ4 binds membranes more weakly and does not respond to fluconazole treatment, suggesting a role for exon four in sterol-mediated membrane binding.

This data also suggests one mechanism by which changes in membrane sterol content affect α-syn toxicity. In yeast, the plasma membrane has the highest ratio of ergosterol:phospholipids (Zinser et al., 1991) and, therefore, simvastatin and fluconazole treatments most strongly affect the dynamics of α-syn interactions with the plasma membrane. α-Syn induces toxicity not at the plasma membrane, but instead in the secretory pathway (Cooper et al., 2006). Consequently, when α-syn is expressed at nontoxic levels, the plasma membrane may serve as a reservoir for the protein, reducing α-synFL and α-synΔ6 interaction with secretory vesicles. (α-SynΔ4, which has a lower affinity for membranes overall is less affected.) Depletion of sterols from the plasma membrane would liberate α-syn (either due to reduced sterol binding or changes in membrane fluidity). This would afford a greater opportunity for α-syn to interact in a toxic manner with vesicles in the secretory pathway (Figure 2.5f).

Both α-synΔ4 and α-synΔ6 respond differently to OSH2 and OSH3 overexpression than α-synFL, yet a differential response to fluconazole is only seen by α-synΔ4. Therefore, the mechanism for these phenotypes must be disparate. At present the reason for this disparity

remains unclear. OSH2 and OSH3 belong to a large family of conserved oxysterol-binding proteins with overlapping functions in membrane sterol relocalization that remain to be deciphered, both yeast and mammalian cells. Recent work implicates Osh3p in regulating lipid concentration at ER/plasma membrane contact sites (Stefan et al., 2011). It is tempting to speculate that α-syn is blocking transport between these two organelles and that reorganization of the membranes by OSH proteins impacts the binding or functionality of α-syn. This change in membrane architecture may allow the splice isoforms to differentially interact with the membrane and impart toxicity. The mechanisms remain opaque at present but α-syn may provide a useful to in disentangling OSH protein functions.

Here we have shown that the splice isoforms of α-syn exhibit different toxicities and membrane binding properties *in vivo*. Furthermore, these toxicities change in response to alterations in intracellular sterol levels. Our work benefited greatly from the genetic toolbox available for yeast. Such investigations are far more difficult in neurons but, given the current state of confusion about the ties between cholesterol and synucleinopathies (Huang et al., 2008; Pahan et al., 2009; Wolozin et al., 2007), the effort seems warranted. Since statins are one of the most heavily prescribed drugs in the developed world, determining their effects on α-syn dynamics, and the role of α-syn splice isoforms on those dynamics, would seem an imperative for development of the best possible therapeutic strategies for treating patients with these diseases.

Materials and Methods

Materials

Simvastatin was obtained from Sigma-Aldrich. Fluconazole was obtained from VWR.

Yeast strains and growth conditions

All experiments were performed in the W303 yeast background, unless otherwise noted. The secretion assays utilized a *bar1* mutant strain, *MATa bar1 leu2 ura3 trp1 his2 ade1*, which was a kind gift from the Fink lab. The standard lithium acetate transformation protocol was used for all yeast transformations (Gietz et al., 1992; Gietz et al., 1995). To select for single integrants, about 50 ng of cut plasmid DNA was used in the transformation. This resulted in a mix of single and tandem integrants. To select for tandem integrants, about 500 ng of cut plasmid DNA was used in the transformation.

Synthetic media included 0.67% yeast nitrogen base without amino acids (Fischer Scientific), supplemented with amino acids as needed (MP Biomedicals) and 2% sugar. YP media included 1% yeast extract, 2% peptone, 2% glucose adjusted to pH 7.0, and 2% sugar. Plates included 2% agar.

For galactose induction, unless otherwise noted, cells were grown to log phase for 6-8 hours in synthetic media containing glucose before being diluted into synthetic media containing raffinose for overnight growth. Log phase cells were then diluted into synthetic media containing galactose for induction. Unless otherwise specified, cells were induced for six hours.

Plasmid construction

α-Syn splice isoforms were generated using overlap extension PCR with Pfu Turbo (Agilent Technologies). A seventh exon of α-syn was only recently found. Therefore, in many previous works, α-synΔ4 is refered to as α-syn lacking exon 3 or α-syn126 while α-synΔ6 is referred to as α-syn lacking exon 5 or α-syn112 (Beyer and Ariza, 2012). The second round of PCR also added

the sequences required for subcloning via BP reaction into pDONR221 (Invitrogen) (Hartley et al., 2000; Walhout et al., 2000). The resulting entry clone was then utilized in an LR reaction to move the insert to a variety of necessary entry vectors, all from the pAG series (Alberti et al., 2007).

For colocalization studies, PCR with Pfu Turbo was used to add XhoI and PstI sites to α-synFL. This was digested and subcloned into the Gateway® TagRFP-AS-N entry clone (Evrogen), which was then used in LR reactions to move the insert into one of the pAG series vectors.

For screening previously established genetic modifiers of α-syn-induced toxicity, the hits were cherry-picked from the Yeast FLEXGene collection (Hu et al., 2007). Any gene used in low-throughput was cherry-picked from the original library and sequence-verified. If required in a different vector backbone, the gene was moved to pDONR221 by BP reaction, then to a vector of the pAG series by LR reaction. mKate-CHC1 and mKate-VPS21 were constructed with the same protocol and transformed into pRS-GPD-mKate-ccdB.

Single insertion PCR analysis

Colony PCR was used to screen all created strains for those with a single insertion in the correct locus (see Figure S1 A). Primers used for this analysis were:
HIS3-TRP1-URA3-C (GGCTTAACTATGCGGCATCAGAGC), HIS3-A (CGACGCTTTGTCTTCATTCA), HIS3-B (CGCATATGATCCGTCGAGT), HIS3-D (ACCACTTGCCACCTATCACC), TRP1-A (GCTGACAGGGAAATGGTCAG), TRP1-B (CGATTTCGGCCTATTGGTTA), TRP1-D (CCCCCTGCGATGTATATTTT), URA3-A (AATGTGGCTGTGGTTTCAGG), URA3-B (CGTTGGAGTCCACGTTCTTT), URA3-D (GGCGAGGTATTGGATAGTTCC), LEU2-A (GGCACAAAGGCAATGAGACT), LEU2-B (CGGCATCAGAGCAGATTGTA), LEU2-C

(TCTGTGCGGTATTTCACACC), LEU2-D (AACGGATCTCCAGATCATCG). Correct strains were those that PCR-amplified bands with primers A+B and C+D, but not A+D and B+C. Those that were correct by this first screening were analyzed by long extension PCR (Roche).

Real time PCR

Genomic DNA was extracted using the YeaStar Genomic DNA Kit (Zymoresearch) or RNA was extracted using an RNeasy Mini Kit (Qiagen) and converted to cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). Levels of α-syn and PGK1 (control) were then measured using a QuantiFast SYBR Green PCR Kit (Qiagen) in triplicate. Primer pairs used for this analysis were: α-syn (TGGCTGAGAAGACCAAAGAGC and CTTGCCCAACTGGTCCTTTTTG), PGK1 (AATCGGTGACTCCATCTTCG and GTGTTGGCATCAGCAGAGAA).

Fluorescent microscopy

Cells were induced following standard galactose induction before visualization by fluorescent microscopy. Cells were spun down, washed, and resuspended in 1x PBS before being viewed with a Plan Apochromat 100x/1.40 NA oil objective lens on a Nikon Eclipse Ti microscopy at room temperature. Images were taken with a CoolSNAP HQ camera (Photometrics). Z stacks were taken above and below the plane of focus, which were then deconvoluted by using the 3D deconvolution algorithm in the NIS-Elements HR software.

For localization studies in the presence of fluconazole, cells were induced as outlined above. To examine localization changes in the presence of 10 μM fluconazole, the drug was added during growth in raffinose and cells were treated overnight before being induced with galactose for four hours. DMSO was used as the vehicle. After four hours, cells were imaged as above, and

foci were counted from GFP pictures taken of fields chosen in the DIC channel to allow for unbiased field selection.

For filipin staining, cells were induced as outlined above. Following wash, they were resuspended in 4% formaldehyde, diluted from 16% formaldehyde (Ted Pella) in PBS, for 15 minutes. Following two washes in PBS, cells were stained with 500 μg/mL filipin (Cayman Chemical) for 10 minutes in the dark before visualization. Similar results were obtained from unfixed cells.

Spotting assays

Cells were grown overnight to saturation in synthetic media containing raffinose. Every strain was diluted to a starting OD600=1.0 and five-fold serial dilutions were made before spotting on inducing (galactose) or non-inducing (glucose) plates.

Western blots

Following induction, cells were lysed by bead beating in lysis buffer (10mM HEPES pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.5 mM PMSF, and 1x protease inhibitor (Roche)). The lysates were then incubated with 0.5% Tween before spinning to remove beads and debris. Protein concentration was quantified using the BCA assay (Pierce Protein Research) then normalized. Samples were run on a 10% Bis-Tris Gel (Invitrogen). This was transferred to PDVF membrane and blocked with 5% milk in PBS. For protein visualization, either anti-GFP (Roche) or anti-PGK1 (Molecular Probes) was used as a primary antibody and a dye-conjugated anti-mouse IgG (LICOR Biosciences) was used as a secondary antibody. The blot was visualized using Odyssey Infrared Imaging System (LICOR Biosciences).

Secretion assay

Strains were struck onto a YP-glucose plate and grown at 30 degrees overnight. The next day, *bar1* cells were grown in YP-glucose for 4 hours before diluting to plate a lawn on both YP-glucose and YP-galactose plates. After drying, the test strains were resuspended in sterile water. For uninduced controls, 10 μL of culture diluted to an OD600=1.0 was spotted in the center of the YPD plate. For the induced plates on YP-galactose, an OD600=1.0 was used for toxic disease model strains, while an OD600=0.04 was used for the vector control to account for their different rates of growth. Pictures were taken after two days of growth at 30 degrees.

Screening α-syn enhancers and suppressors

A standard lithium acetate transformation protocol was adapted for use with 96-well plates (Cooper et al., 2006; Gietz et al., 1992; Gietz et al., 1995). Following transformation, cells were grown to saturation in synthetic media with glucose lacking uracil to select for cells that took up the plasmid. Once at saturation, they were spotted onto synthetic media plates with either glucose or galactose. Following two days of growth, galactose plates were scanned and the density of the spot was analyzed using ImageQuant TL. This was repeated for each strain at least three times.

Statistics

All statistical analysis was performed using the Student's t-test, with the exception of goodness of fit analysis where a linear regression was used to determine R squared.

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Chapter Three:

TorsinA and the TorsinA-Interacting Protein Printor Have no Impact on Endoplasmic Reticulum Stress or Protein Trafficking in Yeast

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Abstract

Early-onset torsion dystonia is a severe, life-long disease that leads to loss of motor control and involuntary muscle contractions. While the molecular etiology of the disease is not fully understood, a mutation in an AAA+ ATPase, torsinA, has been linked to disease onset. Previous work on torsinA has shown that it localizes to the endoplasmic reticulum, where there is evidence that it plays roles in protein trafficking, and potentially also protein folding. Given the high level of evolutionary conservation among proteins involved in these processes, as well as the previous successes achieved in examining other proteins involved in complex human diseases in yeast, we hypothesized that *Saccharomyces cerevisiae* might represent a useful model system for studying torsinA function and the effects of its mutants. Since torsinA is proposed to function in protein homeostasis, we tested cells for their ability to respond to various stressors, using a fluorescent reporter to measure the unfolded protein response, as well as their rate of protein secretion. TorsinA did not impact these processes, even after co-expression of its recently identified interacting partner, printor. In light of these findings, we propose that yeast may lack an additional cofactor necessary for torsinA function or proteins required for essential post-translational modifications of torsinA. Alternatively, torsinA may not function in endoplasmic reticulum protein homeostasis. The strains and assays we describe may provide useful tools for identifying and investigating these possibilities and are freely available.

Introduction

A glutamic acid deletion (ΔE) in torsinA causes early-onset torsion dystonia, a devastating autosomal dominant neurofunctional disease that impacts patients as children or young adults and has no cure (Bressman et al., 1994). The torsinA protein carries a signal sequence in its N-terminus that localizes the protein to the contiguous lumen of the endoplasmic reticulum (ER) and nuclear membrane (Giles et al., 2009; Hewett et al., 2003; Kustedjo et al., 2000). C-terminal to this is a hydrophobic region that allows torsinA to interact with membranes (Liu et al., 2003). The remainder of the protein encompasses an AAA+ ATPase domain, which includes the site of the dystonia-associated mutation (Ozelius et al., 1997).

While AAA+ ATPase domains have been ascribed diverse functions, many are involved in protein remodeling (Hanson and Whiteheart, 2005). Yeast have AAA+ ATPases that participate in protein folding, such as HSP104 (Parsell et al., 1994), but none are homologs of torsinA. However, there is precedent for successfully studying AAA+ ATPases in heterologous environments, as HSP104 has been shown to function in neurons, which lack an HSP104 homolog (Perrin et al., 2007).

TorsinA acts in protein trafficking and the dystonia-associated mutation in torsinA disrupts this process (Hewett et al., 2007; Torres et al., 2004). Due to the homology between torsinA and the Hsp100 family, it was hypothesized that torsinA may function as a chaperone. Indeed, recent work has shown that torsinA can untangle protein aggregates *in vitro* and rescue both nematodes and mouse embryonic fibroblasts from ER stress (Burdette et al., 2010; Chen et al., 2010). However, work in PC6.3 cells showed that while the ER stressor dithiothreitol (DTT) induces post-translational modification of torsinA in rat PC6.3 cells, torsinA cannot rescue ER stress or protein aggregation of polyglutamine (Gordon et al., 2011). This discrepancy may arise

from differences between cell lines. The latter paper was the only one to use a neuronal cell line, which may be the most representative of the cell type impacted by dystonia. TorsinA is present in Lewy bodies (LB), the proteinaceous aggregates that are a hallmark of Parkinson's disease (PD) (Shashidharan et al., 2000a; Shashidharan et al., 2000b). TorsinA is able to reduce the aggregation of a principal component of LB, alpha-synuclein (α-syn), in H4 neuroglioma cells, and lessen the neuronal death caused by α-syn expression in nematodes (Cao et al., 2005; McLean et al., 2002). This aspect of torsinA biology is not completely understood since torsinA is localized to the ER (Hewett et al., 2003) and LB and α-syn exist in the cytoplasm (McLean et al., 2002; Shashidharan et al., 2000a), but could potentially be explained by ER associated protein degradation (ERAD) (Giles et al., 2008); although more work is needed on this topic. Hence, while data suggest certain cellular functions for torsinA, the details of this role in protein homeostasis is far from understood and it is unclear how the disease-associated mutation of torsinA interferes with its proposed chaperone function.

Many studies have revealed the utility of modeling complex biology in the yeast *Saccharomyces cerevisiae*. Yeast have an unrivaled genetic toolbox for both hypothesis-based and unbiased high-throughput analyses (Khurana and Lindquist). Furthermore, yeast have been vital in understanding processes involved in protein homeostasis, including the unfolded protein response (UPR) and the role of chaperones (Lindquist and Craig, 1988; Mori, 2009). More recently, yeast have been utilized to model the cell biology of neurodegenerative diseases, including polyglutamine (polyQ) diseases (e.g., Huntington's disease) (Krobitsch and Lindquist, 2000), synucleinopathies (e.g., PD) (Outeiro and Lindquist, 2003), and Alzheimer's disease (Bharadwaj et al., 2008; Caine et al., 2007; Middendorp et al., 2004).

Studies of this nature concerning the cell-biological role of α-syn have been particularly

successful. α-Syn elicits toxicity when expressed in yeast, as well as cellular phenotypes reminiscent of those observed in PD patients (Outeiro and Lindquist, 2003). From high-throughput screens, our lab and others began to elucidate how α-syn expression alters normal cellular functions. One such screen assayed the consequences of overexpressing individual yeast genes on α-syn-induced toxicity; this revealed conserved cellular processes perturbed by α-syn expression in yeast and higher eukaryotes, including ER-to-Golgi trafficking, and established a link between α-syn and *PARK9*, a gene previously associated with PD by genetic analyses, but not known to be functionally tied to α-syn (Cooper et al., 2006; Gitler et al., 2009; Yeger-Lotem et al., 2009). A second yeast-based screen discovered chemical compounds capable of rescuing not only α-syn-induced toxicity in neurons, but also toxicity triggered by the drug rotenone, which represents an independent model for PD (Su et al., 2010).

Motivated by the success of these prior yeast models, here we attempt to investigate the cellular effects of torsinA expression in *Saccharomyces cerevisiae*. Upon expression of torsinA in yeast, we find that torsinA does not detectably impact protein folding and secretion under the many conditions that we have tested. Although we would not normally report negative results, we do so for several reasons. First, due to the past success of modeling complex diseases in yeast, we foresee others attempting similar studies and hope that this work will serve as a starting point for their analyses. Secondly, it is possible that our failure to detect significant changes upon torsinA expression may be due to missing cofactors, improper protein processing, or subcellular environmental differences between yeast and higher eukaryotes. As these topics are understood in greater detail, this model may come to serve as a useful tool for the discovery of torsinA function and torsinA-interacting partners. For these reasons, all plasmids created in this study will be made freely available from Addgene.

Results

Expression of torsinA in the yeast ER requires a yeast ER localization signal

TorsinA is a resident ER protein (Hewett et al., 2003; Kustedjo et al., 2000). However, expression of full-length torsinA in yeast, driven by a high-expression galactose-inducible promoter, resulted in the formation of large cytoplasmic aggregates (data not shown). Since we were interested in studying the ability of torsinA to act specifically in the ER, we wished to localize the protein to this cellular compartment. To accomplish this, we replaced the endogenous ER localization sequence of torsinA (Giles et al., 2009) with the localization sequence of the yeast ER-resident protein, Kar2p (Figure 3.1a) (Tokunaga et al., 1992). Importantly, in this construct, only the portion of torsinA that has been shown previously to be cleaved following ER localization was replaced (Hewett et al., 2003; Liu et al., 2003), leaving all functional domains of the final protein intact. This caused torsinA to localize to the ER, as visualized by C-terminal green fluorescent protein (GFP) tagging of torsinA (Figure 3.1B). Even in the ER, the protein still aggregated significantly when expressed from the galactose-inducible promoter (data not shown). Therefore, the expression level of torsinA was decreased by using the MET25 promoter. For all experiments other than microscopy, the GFP tag was replaced with an HDEL (ER retention) motif to ensure that any torsinA mistakenly trafficked to the Golgi apparatus would be returned to the ER (Pelham, 1989).

While the data presented below exclusively utilize this form of torsinA, in which the endogenous localization sequence was replaced with that from KAR2, pilot studies were performed using two other fusion constructs. The first attached the KAR2 localization sequence to full-length torsinA and the second utilized torsinA lacking both the endogenous signal sequence and hydrophobic domain. Both of these forms of the protein elicited similar results to

those outlined below (data not shown).

Multiple isoforms of torsinA were used in these analyses (Figure 3.1a). TorsinAΔE, the mutation associated with early-onset torsion dystonia, lacks a glutamic acid at position 302 or 303 (Ozelius et al., 1997). It was hypothesized that this form of the protein may lose torsinA-associated activity, as it has previously been shown that torsinAΔE acts by a dominant-negative mechanism (Torres et al., 2004). Furthermore, the ATPase activity of torsinA is required for its chaperone function (Chen et al., 2010). Therefore, control constructs were created by inserting two previously characterized mutations in the AAA+ domain into both torsinA and torsinAΔE - K108A or E171Q, which inactivate ATP binding and hydrolysis, respectively (Naismith et al., 2004).

KAR2-tagged torsinA displayed predominantly ER-localized, non-aggregated expression, suggesting the signal sequence was sufficient for relocalization (Figure 3.1b) (Ronicke et al., 1997). Some cytoplasmic foci were still observed and low-level GFP was also seen in the vacuole (arrowhead points to one example), suggesting that some of the protein was being degraded (Figure 3.1b). We performed a spotting assay to assess the impact of torsinA expression on the yeast growth rate. None of the forms of torsinA examined impaired or accelerated the rate of growth, suggesting that torsinA expression did not significantly perturb any yeast cellular processes essential for growth (Figure 3.1c). Importantly, torsinAΔE did not diminish the growth rate of yeast, suggesting that the mutation does not act by a dominant, gain of function mechanism to impede cell viability in our model.

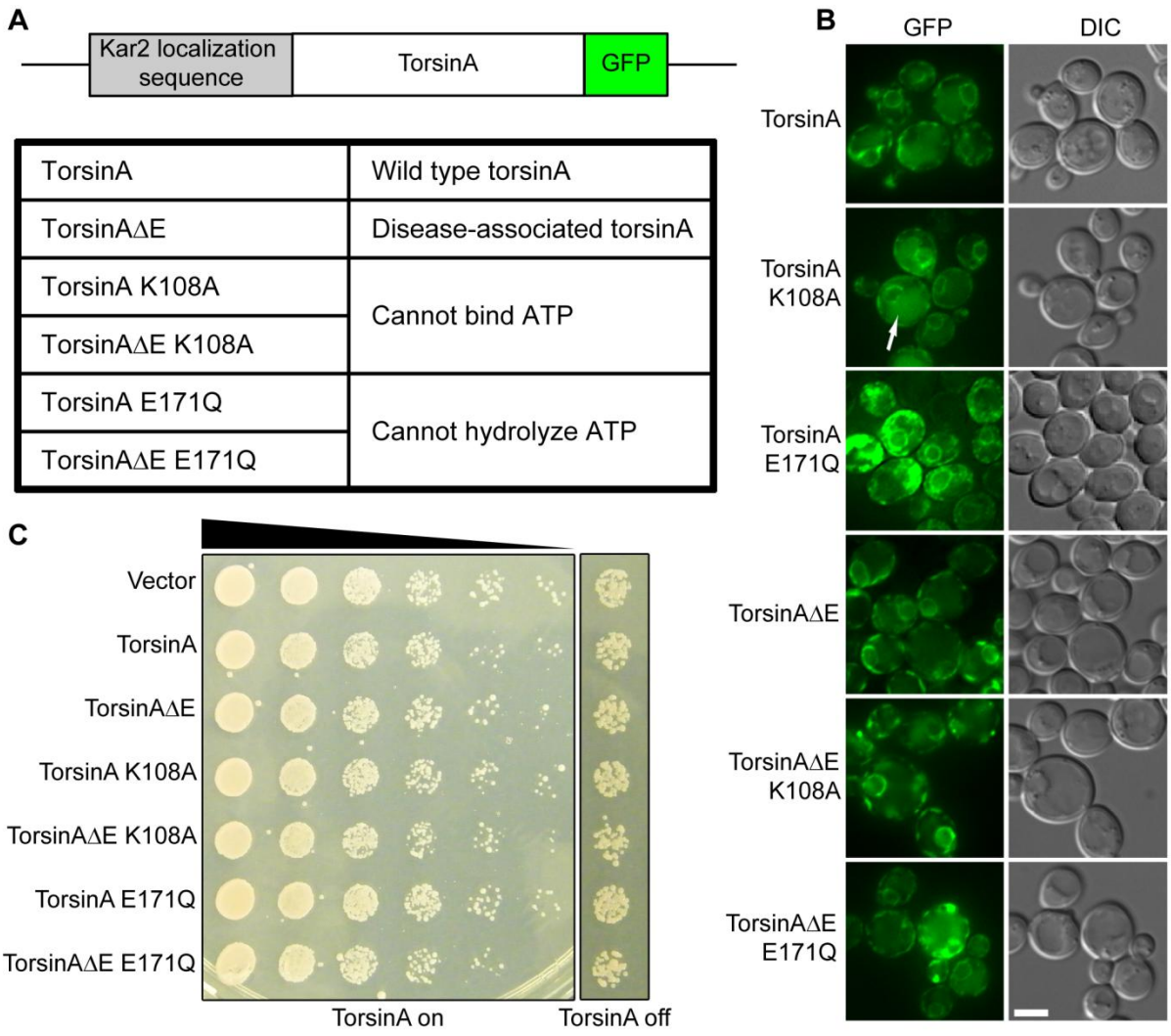

Figure 3.1. TorsinA can be expressed in the endoplasmic reticulum of yeast.
(a) Diagram of constructs created for this work (upper panel) and summary of the different forms of torsinA used in these experiments (lower panel). TorsinA was localized to the endoplasmic reticulum (ER) using the signal sequence of the endogenous yeast protein KAR2 and an HDEL sequence.
(b) Microscopy of yeast strains used. TorsinA was localized to the contiguous lumen of the nuclear envelope and ER. Some signal was also seen in the vacuole (arrow head), suggesting a portion of the protein was degraded. A representative frame is shown for each strain. Scale bar = 2 μM.
(c) Growth of torsinA-expressing yeast on plates. Each row is a 5-fold dilution of the previous row. Expression of wild type (WT) or mutant torsinA did not impact the growth rate. Uninduced plates included 1 mM methionine and induced plates lacked methionine.

Expression of torsinA in yeast does not allow for recapitulation of its roles in protein homeostasis and trafficking

To test if torsinA could function as a chaperone, yeast strains were assayed for ability to overcome protein folding perturbations in the presence of wild type (WT) and mutant torsinA. To monitor the onset of the UPR, we utilized a previously described reporter that consists of GFP driven by four copies of the promoter from a UPR-responsive gene (UPRE), thus allowing dose-dependent monitoring of an upregulation in the UPR (Cox et al., 1993; Pollard et al., 1998). Each torsinA construct elicited a slight, yet significant, basal increase in the UPR in yeast, as compared to an empty vector (Figure 3.2a), likely due to the expression of extra protein molecules in the ER. This was not torsinA-specific, since the reporter gene luciferase, when expressed in this manner, elicited the same response (data not shown). For this reason, we assayed the ability of WT torsinA to modulate the UPR in comparison to mutant forms of the protein, hypothesizing that any biologically meaningful effects of torsinA expression should be attenuated by the disease-associated mutation and/or the mutations that inactivate ATP binding. None of the torsinA isoforms elicited an UPR that was significantly different from the others, suggesting that the rise in UPR was not due to mutant torsinA dysfunction (Figure 3.2a).

This assay was used to study the ability of torsinA to impinge upon the UPR induced by oxidative protein damage in yeast by treating torsinA-expressing cells with DTT. DTT causes oxidative ER stress by inhibiting disulfide bond formation, thus eliciting the UPR (Pollard et al., 1998). Recent work has suggested that a *C. elegans* homolog of torsinA, *ooc-5*, can act as a sensor of intracellular oxidation, as it contains a disulfide bond important for ATP/ADP binding (Zhu et al., 2008). DTT triggered the UPR in our strains; however, none of the torsinA isoforms tested lowered the levels of DTT-induced UPR (Figure 3.2a). To ensure that our reporter system was

sensitive to the functions of proteins known to reduce oxidative protein damage, we expressed the yeast protein, ero1p. This protein is an oxidase that is necessary for oxidative protein folding and can reduce levels of UPR upon stress (Frand and Kaiser, 1998; Pollard et al., 1998). Indeed, we observed that it significantly reduced the level of DTT-induced UPR (Figure 3.2a).

As a second assay to gauge the ability of torsinA to reduce cellular stress associated with oxidative damage in yeast, torsinA was expressed in a strain containing the *ero1-1* mutation, resulting in a temperature-sensitive ero1p that limits growth at 37°C (Frand and Kaiser, 1998). Neither torsinA nor torsinAΔE impacted growth of *ero1-1* at this restrictive temperature (Figure 3.2b).

Next, we asked if torsinA could reduce the levels of the UPR induced by sources other than oxidative stress in yeast. A constitutively unfolded mutant form of carboxypeptidase Y (CPY*) is an ER-associated degradation (ERAD) substrate that can elicit the UPR (Travers et al., 2000). Coexpression of any form of torsinA with CPY* had no significant effect on the level of the UPR observed (Figure 3.2a). Additionally, pilot studies were performed to test the ability of torsinA to reduce the UPR elicited by tunicamycin, a glycoprotein synthesis inhibitor; however, no significant differences were observed (data not shown).

Multiple studies have documented that torsinA plays a role in vesicular trafficking (Granata et al., 2008; Hewett et al., 2007; Torres et al., 2004). Similar to the chaperone system that oversees protein folding, secretion is a highly conserved pathway with many proteins displaying extensive homology between yeast and higher organisms (Bennett and Scheller, 1993). Therefore, we investigated whether torsinA expression altered protein trafficking in yeast. To do so, we utilized a previously established assay for secretion that monitors trafficking of the endogenous yeast protein invertase to the plasma membrane, where it cleaves sucrose,

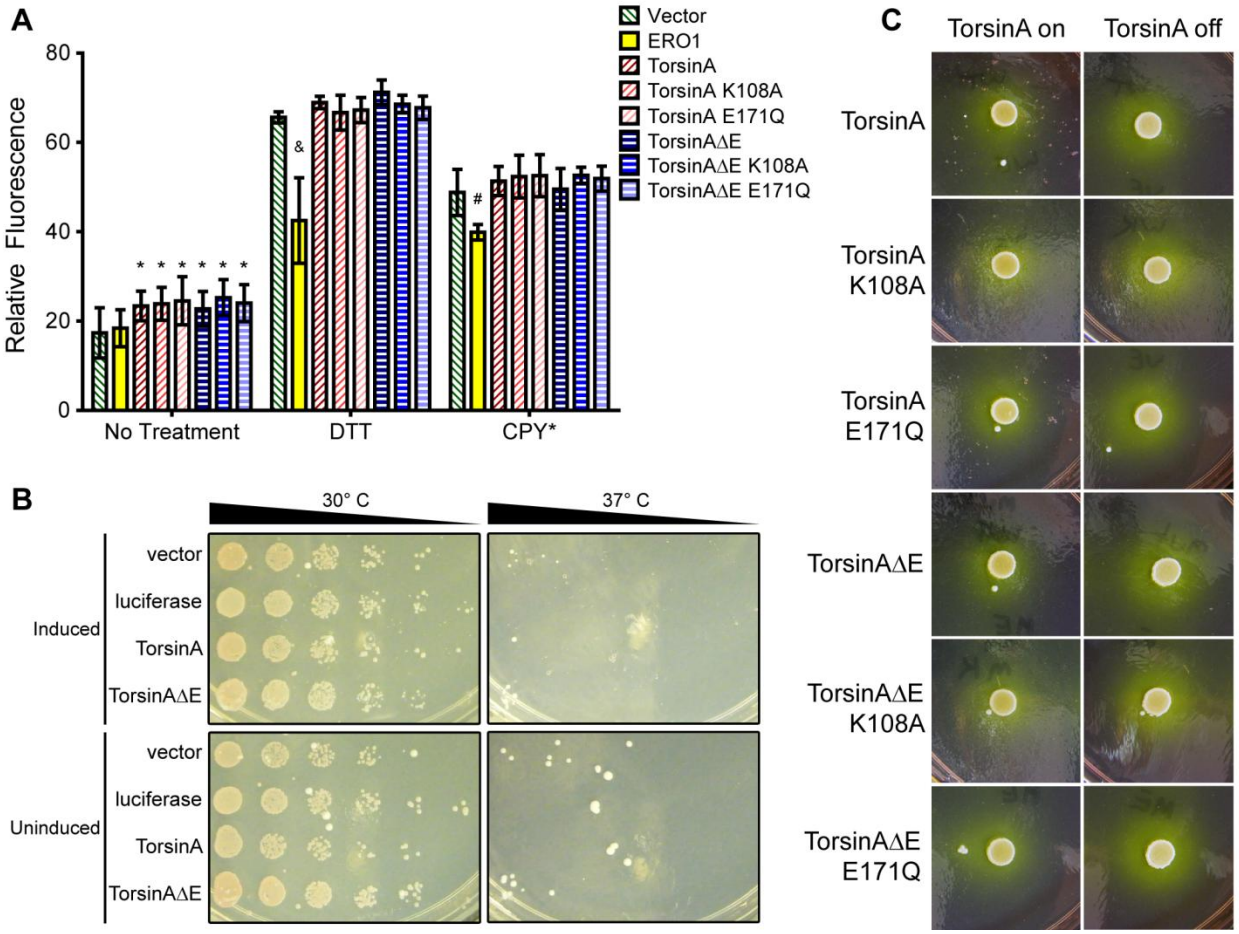

Figure 3.2. TorsinA does not impact the unfolded protein response or trafficking in yeast.
(a) A construct containing GFP driven by a unfolded protein response (UPR) sensitive promoter (UPRE-GFP) was used to monitor levels of the unfolded protein response (UPR) upon stress with 1.5 mM dithiothreitol (DTT) or mutant carboxypeptidase Y (CPY*). ERO1 served as a positive control. TorsinA is not able to reduce UPR levels caused by either stressor. Statistical analysis was conducted in comparison to the vector control strain with a 1-tailed Student's t-test. * = $p<0.05$, # = $p<0.005$, & = $p<0.001$. N=6 independent trials per sample.
(b) Growth of ero1-1 in the presence and absence of torsinA at 37°C. Each row is a 5-fold dilution of the previous row. TorsinA is not able to rescue the growth defect by the *ero1-1* mutation. Uninduced plates included 1 mM methionine and induced plates lacked methionine.
(c) Trafficking of invertase, as monitored by halos produced by growth of torsinA-expressing strains on plates containing bromocresol purple (BCP). TorsinA does not impact the rate of secretion.

resulting in a local pH change (Nishikawa and Nakano, 1993). This change can then be monitored using the pH-sensitive dye bromocresol purple (BCP). Upon spotting torsinA-expressing strains onto BCP/sucrose plates, no change was observed in the diameter of the halo that resulted from this pH change, suggesting that secretion was not impacted by any of the torsinA forms (Figure 3.2c).

It has also been suggested that torsinA can prevent the progression of aggregate-associated neurodegeneration in models of synucleinopathies and polyQ disorders (Cao et al., 2005; McLean et al., 2002). Using the previously described yeast model of α-syn-induced toxicity (Outeiro and Lindquist, 2003), we asked whether concomitant expression of torsinA and α-syn could rescue this phenotype; however, torsinA had no significant impact on the rate of growth in conjunction with α-syn expression (Figure 3.3).

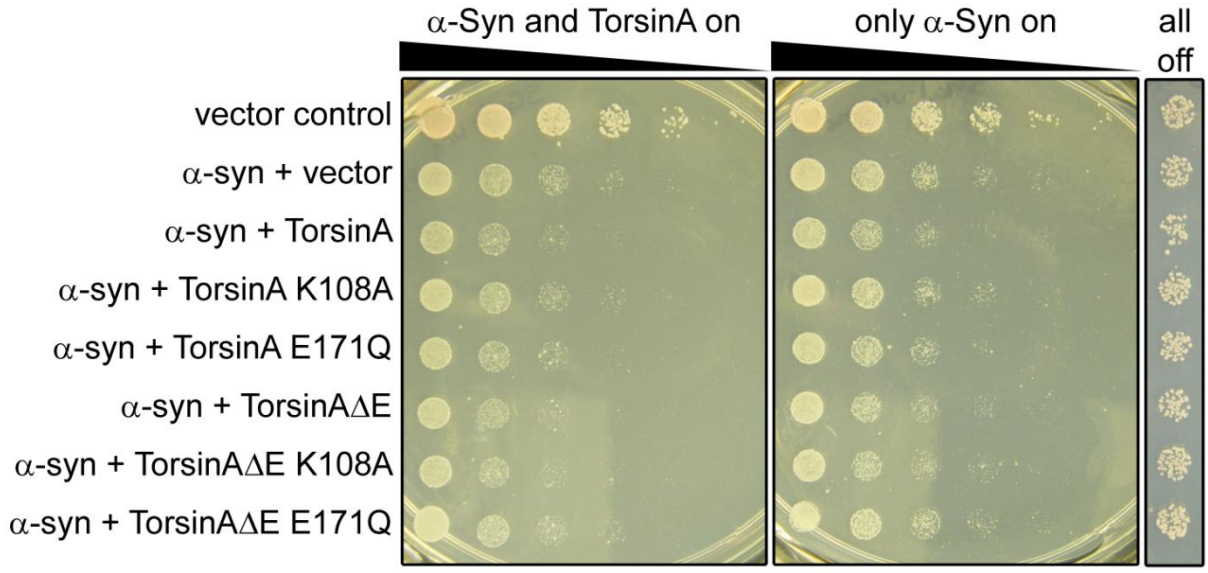

Figure 3.3. TorsinA cannot rescue α-synuclein-induced toxicity in yeast.
Assay showing the ability of yeast to grow in the presence or absence of α-synuclein (α-syn) with and without torsinA. Each row is a 5-fold dilution of the previous row. TorsinA cannot rescue α-syn-induced toxicity.

Addition of the torsinA-interacting protein printor is not sufficient to uncover the role of torsinA

The above-cited data suggested that torsinA expression in yeast failed to affect any of the cellular processes in which this protein has been shown to act in higher eukaryotic cells. We hypothesized that this might be attributable to the fact that yeast lack certain essential cofactor(s) that are required for torsinA function. Therefore, we reassessed the ability of torsinA to function in the above-stated pathways in the presence of the newly-discovered torsinA-interacting protein, printor (Giles et al., 2009). The association between torsinA and printor was discovered through a yeast two-hybrid screen, suggesting that these two proteins could, at a minimum, physically interact within a yeast cell. Furthermore, this prior work demonstrated that printor selectively binds torsinA lacking ATP, suggesting printor could act as a cofactor of torsinA. TorsinA and printor specifically interact in the ER, and not in the nuclear envelope. For this reason, printor was localized to the ER using the Kar2 and HDEL localization sequences, as described above for torsinA. Co-expression of WT torsinA and printor in yeast failed to elicit differential cellular phenotypes upon examination of CPY*- or DTT-induced UPR or invertase trafficking (Figure 3.4a & b, respectively). TorsinAΔE and printor elicited a small reduction of UPR induction upon co-expression of CPY* (Figure 3.4a). While this reduction was statistically significant, it is unlikely to be biologically relevant since torsinAΔE was previously show to be inactive.

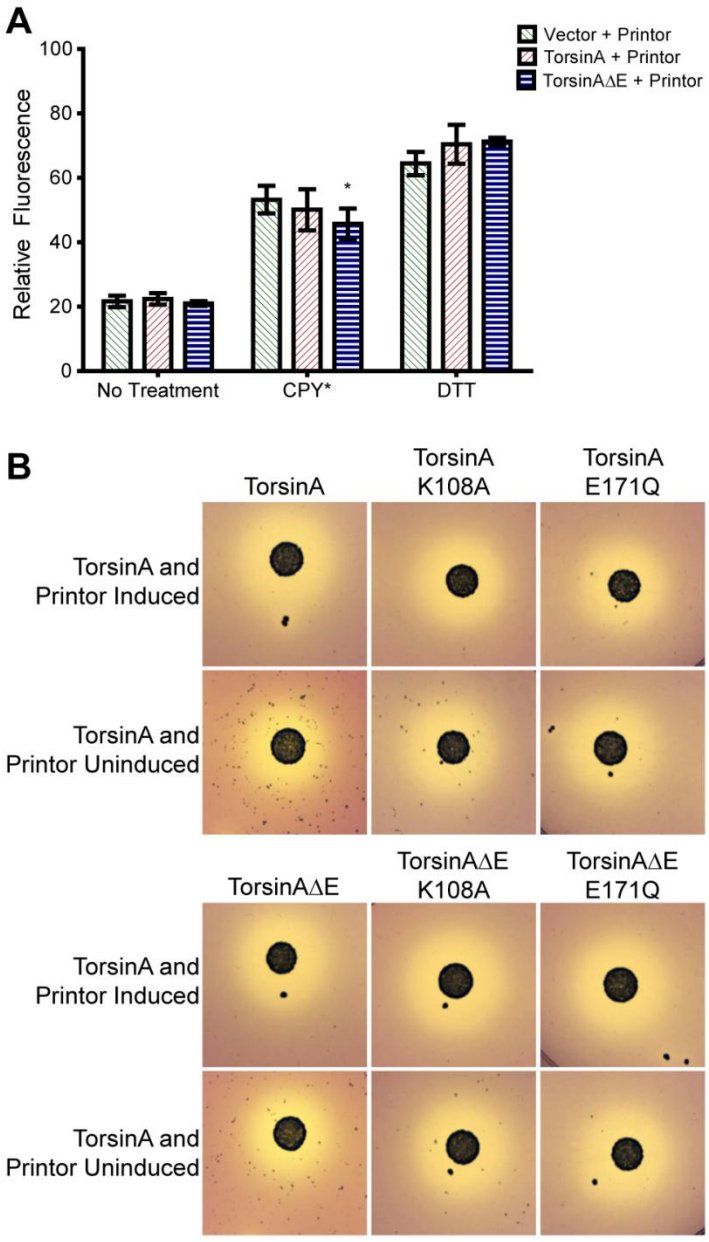

Figure 3.4. Coexpression of torsinA and printor does not uncover a phenotype stemming from torsinA expression.

(a) The UPR of the indicated yeast strains was monitored by flow cytometry to detect expression from the UPRE-GFP construct upon stress with CPY* or 1.5 mM DTT. Coexpression of printor does not allow WT torsinA to reduce UPR-related stress. A 1-tailed Student's t-test was used to compare relative fluorescence of torsinA strains to the vector control. *=$p<0.01$. N=6 independent trials per each sample.

(b) The impact of torsinA with and without printor on trafficking of invertase. Cells were spotted on plates containing the pH sensitive dye, BCP. Simultaneous expression of torsinA and printor does not impact the rate of secretion.

Discussion

TorsinA has been proposed to function in two highly conserved cellular processes: protein folding and protein trafficking. As such, we hypothesized that torsinA expression in yeast would impact these pathways. However, we failed to uncover a role for torsinA-mediated control of protein folding and secretion in *S. cerevisiae*. This observation was surprising because other protein folding and remodeling factors, such as HSP104, are readily analyzed when heterologously expressed in evolutionarily divergent organisms. Moreover torsinA was shown to reduce protein aggregation *in vitro*, and we hypothesized that these effects would also extend to our yeast system (Burdette et al., 2010).

However, our present findings can be easily reconciled with this previous work. First, the above-cited *in vitro* study utilized torsinA lacking both its signal sequence and its hydrophobic domain. Secondly, the *in vitro* work, in contrast to previous *in vivo* work in worms with the full-length torsinA protein(Chen et al., 2010), was unable to uncover a functional difference between torsinA and torsinAΔE (Burdette et al., 2010). It is possible that the same cofactors or post-translational modifications that are necessary to explain the difference between the *in vitro* and *in vivo* experiments also account for the inability of torsinA to be biologically active in yeast. It is also possible that torsinA must be expressed in subcellular locations other than the ER to mediate its function, which would not occur in our model due to the presence of the exogenous HDEL tag, which was added to increase the level of torsinA retained in the ER. Alternatively, there may be some difference in the ER environment of the yeast cells as compared to higher eukaryotic cell, which might prohibit or is redundant with torsinA activity.

There are a number of other possible explanations for these disparities. While the majority of AAA+ ATPases act as protein remoldeing factors, many have quite specific substrates (Hanson

and Whiteheart, 2005), and it is possible that yeast lack torsinA's substrate(s). Furthermore, the UPR may serve a broader range of functions in vertebrates than in yeast (Rutkowski and Hegde, 2010) and torsinA may function in an aspect of UPR signaling not conserved in yeast. Finally, as mentioned above, recent evidence suggests that torsinA may not act in general protein homeostasis. Work in neuronal lines has failed to recapitulate the role of torsinA in protein folding, which, noteably, was only seen upon overexpression of torsinA in other models (Gordon et al., 2011). Furthermore, torsinA has been knocked out in a variety of model organisms, but none of these studies have reported a protein homeostasis-related phenotype. This suggests that torsinA may serve a different principal function, such as its role in maintaining nuclear envelope structure, which has previously been shown to partially explain the neuron-specific sensitivity of torsinA mutation (Goodchild et al., 2005).

As more is discovered about torsinA function and its interacting proteins through neuronal culture or transgenic animals, the work presented here may provide a platform for further investigation using yeast. Furthermore, this yeast model might prove helpful in discovering such cofactors, as it could be screened to identify human genes that interact genetically with torsinA. For this reason, all of the plasmids created for this project will be made freely available through Addgene for distribution to others who are interested in testing these hypotheses. While we failed to uncover a phenotype for torsinA expression in yeast, we are optimistic that future work can improve this model and make useful steps toward dissecting the function of torsinA and the dysfunction associated with its mutant form.

Materials and Methods

Materials

Dithiothreitol, tunicamycin, methionine, and bromocresol purple (BCP) were purchased from Sigma.

Construction of plasmids

Except where noted, standard Gateway cloning methods were used for cloning (Hartley et al., 2000; Walhout et al., 2000). pDONR221 (Invitrogen) was used as the donor plasmid and the pAG series of plasmids (Alberti et al., 2007) were used as destination vectors.

TorsinA and torsinAΔE were obtained as a generous donation from Xandra Breakefield. Overlap extension PCR (Pogulis et al., 1996) was used to add the KAR2 and HDEL localization sequences. These constructs were expressed in pAG416Gal-ccdB or pAG416Gal-ccdB-GFP (Alberti et al., 2007). These were then cut with XbaI and XhoI and subcloned into the same sites of 416-Met25. Site-directed mutagenesis was used to create K108A and E171Q mutations with Pfu Turbo (Stratagene).

pRS313-CPY*-HA was a generous gift from Antony Cooper. After sequencing, mutagenic PCR was used to create the same mutation in CPY in pDONR221, which was then used in an LR reaction to move to pAG413-Gal-ccdB (Alberti et al., 2007).

pPW0533, the 304 4xUPRE-GFP reporter, was a generous donation from Peter Walter. Following digestion in within the TRP1 gene, it was transformed into W303 to make a stable strain with an integrated copy.

Printor cDNA was purchased from Kazusa DNA Research Institute (product ID ORK00224) (Nagase et al., 2000). Overlap extension PCR (Pogulis et al., 1996) was used to add the Kar2 and

HDEL localization sequences and subclone into pDONR221.

Table 3.1. Primers used.

#	Purpose	Sequence
112	Mutagenic PCR TorsinA E171Q	GGTCCATCTTCATATTTGATCAAATGGATAAGATGCATGAGGC
113	Mutagenic PCR TorsinA E171Q	GCCTGCATGCATCTTATCCATTTGATCAAATATGAAGATGGACC
114	Mutagenic PCR TorsinA K108A	GGTGGACAGGCACCGGCGCTAATTTCGTCAGCAAGATCATCG
115	Mutagenic PCR TorsinA K108A	CGATGATCTTGCTGACGAAATTAGCGCCGGTGCCTGTCCACC
121	Overlap extension PCR for KAR2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAAAATGTTTTTCA ACAGACTAAGCGC
124	Overlap extension PCR for torsinA	GGGGACCACTTTGTACAAGAAAGCTGGGTTATCATCGTAGTAATAA TCTAAC
126	Overlap extension PCR for torsinA	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACAATTCATCATGA TCATCGTAGTAATAATC
138	Overlap extension PCR for torsinA	CCCAGGCTGATGGGCTCCACACCTCTAACTAAAACATTGG
139	Overlap extension PCR for torsinA	CCAATGTTTTAGTTAGAGGTGTGGAGCCCATCAGCCTGGG
140	Mutagenic PCR CPY*	CAAGATTTCCACATCGCTAGGGAATCCTACGCCGGCC
141	Mutagenic PCR CPY*	GGCCGGCGTAGGATTCCCTAGCGATGTGGAAATCTTG
181	Overlap extension PCR for KLHL14	CCAATGTTTTAGTTAGAGGTATGTCCAGATCCGGGGACAGGACCTC CACC
182	Overlap extension PCR for KLHL14	GGGTTTTACAATTCATCATGTTTGTTGTATGGTACACAAGAGGGCA G
183	Overlap extension PCR for KLHL14	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACAATTCATCATGT TTG
184	Overlap extension PCR for KLHL14	GAAAGCTGGGTTTTTGTTGTATGGTACACAAGAGGGCAG
185	Overlap extension PCR for KLHL14	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTGTTG

Yeast strains and growth conditions

With the exception of CKY558 (*ero1-1* containing strain; generous gift from Chris Kaiser) (Frand and Kaiser, 1998), all experiments were done in the W303 genetic background. The standard lithium acetate transformation protocol was used for all yeast transformations (Gietz et al., 1992; Gietz et al., 1995). Minimal media included 0.67% yeast nitrogen base without amino acids (Fischer Scientific), supplemental amino acids (minus those needed as selectable markers) (MP Biomedicals), and 2% sugar, with 2% agar included in plates. All liquid growth was performed at 30°C with aeration. Except where noted, growth on plates was performed at

30°C. For galactose induction (CPY* and α-syn), strains were grown overnight in SRaff and diluted into SGal. For methionine induction (torsinA and printor), cells were grown overnight in 1 mM methionine overnight and diluted into media lacking methionine.

Spotting assays

Cells were grown overnight in SRaff plus 1mM methionine. Strains were diluted to OD_{600}=1.0 and serially diluted five-fold for six total repetitions before spotting on the appropriate agar plates.

UPRE induction assays

Cells were grown overnight in appropriate media. Strains were diluted to OD_{600}=0.1 in inducing media. DTT was used at the indicated concentrations in order to induce a UPR. After 6 hours of induction, cells were subjected to flow cytometry (Guava PCA-96, Millipore).

Microscopy

Cells were grown to log phase in non-inducing media, then moved to inducing media for 5 hours. Live cells were visualized by using a Zeiss Axiovert 200 microscope. Z-stacks were taken and deconvoluted using the nearest neighbor algorithm from the Axiovision software.

BCP trafficking assays

Cells were grown overnight in SD with 1 mM methionine media. They were diluted to an OD_{600} = 1.0 and spotted onto BCP plates containing (as measured in weight/volume) 0.67% yeast nitrogen base without amino acids (Fischer Scientific), supplemental amino acids (minus

those needed as selectable markers) (MP Biomedicals), 2% sugar, 2% agar, 0.0032% BCP, and 0.5x PBS. Plates were grown for 3 days at 30°C.

Statistics

All data are shown as mean ± standard deviation. A 1-tailed Student's T –test was performed for all comparative statistical analysis.

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Chapter Four:

Conclusion: Lessons from Disease Models in Yeast

Reasons to model human disease in *Saccharomyces cerevisiae*

There are many reasons why the yeast *Saccharomyces cerevisiae* has become such a popular model organism. Importantly, because yeast are eukaryotic, most of these pathways, including cell cycle regulation, DNA damage repair, vesicular trafficking, protein processing and secretion, mitochondrial function, and lipid biology, are well conserved with higher organisms. Its genome is fully sequenced and is 100 times smaller than that of humans. They lack many redundant genes found in higher organisms, making it easier to study a phenotype associated with deletion of a gene. Furthermore, most *S. cerevisiae* genes lack introns, making their approximately 6000 genes much easier to define and characterize. This has facilitated the creation of a vast array of yeast-based genetic libraries that can be utilized for high-throughput, unbiased screening, ranging from deletion and tagged libraries to collections of overexpression plasmids that encompass most of the 6000 genes.

Beyond impressive tools for unbiased analyses, *S. cerevisiae* is a tractable organism for biological research for other reasons. They grow quickly and require only inexpensive reagents. This allows for large quantities of yeast to be grown, which is especially important for protein-level analyses. Furthermore, due to the fact that they have robust homologous recombination pathways, *S. cerevisiae* can be easily transformed with plasmids that can either alter endogenous genes or introduce novel sequence elements. Finally, since they have been studied so extensively, powerful and intricate methods have been developed for studying many fundamental biological processes, allowing researchers to deeply probe the specifics of these pathways. These features allow studies in yeast to progress much faster than those in higher organisms; experiments that take weeks in yeast would take months to years in mice.

These traits have prompted scientists to turn to *S. cerevisiae* in order to obtain information on some of the most complex biological processes, including work that has important implications for human disease. In one study, approximately 25% of a group of human disease genes selected from a wide variety of pathways were found to have yeast homologs based on sequence alone (Foury, 1997). For genes with yeast homologs, understanding the function of the protein in yeast often provides great insight into its function in human cells. One topic that has been widely studied in this manner is cancer. Many of the genes that control the yeast cell cycle have human homologs relevant for the progression of cancer (Hartwell, 2002). An example of this is the role of aneuploidy in cancer (Pfau and Amon, 2012). Healthy cells maintain the correct complement of chromosomes through carefully orchestrated events during mitosis. In contrast, many cancer cells have some degree of aneuploidy, which can occasionally be quite dramatic. Work in yeast unveiled that this aneuploidy results in proteotoxic stress and increased sensitivity to inhibitors of protein degradation, which was later recapitulated in mammalian cells (Tang et al., 2011; Torres et al., 2007). This information can now be applied to developing therapeutic strategies for cancer (Pfau and Amon, 2012; Thompson et al., 2010)

Studies in yeast have aided in understanding ties between DNA repair and cancer progression as well. For example, it is known that simple sequence repeats are genetically unstable, especially in the background of other mutations often found in cancer cells; however, the underlying basis for this instability was unknown. Using yeast to discover genes involved in the process of DNA repair revealed *MSH2*, the human homolog of which was later found to be mutated in a hereditary form of colon cancer (Fishel et al., 1993; Strand et al., 1993).

Yeast cells have also been essential for studies of proteins involved in rarer genetic disorders. One example of this is Friedreich's ataxia, a neurological disorder caused by a mutation in the gene *FRDA1*. Deletion of the yeast homolog of *FRDA1*, *YFH1*, resulted in improper accumulation of iron in the mitochondria and disruption of respiration (Babcock et al., 1997; Wilson and Roof, 1997). This early insight into the function of FRDA1 has driven current research, including development of iron chelators as a therapeutic intervention for individuals with Friedreich's ataxia (Becker and Richardson, 2001).

Yeast are also useful for modeling diseases in which there either is not a yeast homlog or a yeast homolog has yet to be discovered. I have already discussed yeast models of the synucleinopathy-related protein α-syn, and yeast models have been developed for proteins involved in other neurodegenerative diseases, such as Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Johnson et al., 2009; Krobitsch and Lindquist, 2000; Treusch et al., 2011). Yeast have also been used to heterologously express and study cancer-related genes, including caspases and p53 (Pereira et al., 2012). Furthermore, a third class of genes that have been successfully studied in yeast are those involved in kidney disease (Kolb et al., 2011). This includes ion channels, plasma membrane transport proteins, aquaporins, and signaling proteins. Many of these proteins are complex, containing multiple membrane spanning domains or require interactions with other proteins for their correct folding and localization. The fact that these proteins function similarly in yeast as mammalian cells is remarkable and highlights the high level of cellular conservation between yeast and higher organisms.

Finally, one of the biggest advantages of the yeast system is the potential using screens to accumulate vast amounts of data including protein-protein interactions, gene expression

profiles, and effects of overexpressing or deleting genes. As modeling through systems biology becomes more advanced, algorithms are being used to uncover complex biological processes employing this data from yeast and discovering ties to human disease (Petranovic and Nielsen, 2008). These networks can highlight pathways perturbed by expressing heterologous disease-associated proteins in yeast, as elucidated through unbiased analyses (Yeger-Lotem et al., 2009).

Of course, *S. cerevisiae* do have some limitations in terms of their suitability for modeling complex disease. Processes involving protein interactions with partners that are not fully understood and lack yeast homologs are less likely to be easily studied. This may have contributed to problems I experienced with modeling dystonia, as discussed below. Other pathways, while conserved in yeast, are much simpler than in higher organisms, so studies in yeast miss details that may be essential for function. One example of this is the electron transport chain in the mitochondria: yeast replace the entirety of complex I (45 subunits in mammals) with a single enzyme, Ndi1p (Carroll et al., 2006; De Vries et al., 1992). Furthermore, and perhaps more importantly in the context of human disease, as single-celled organisms, yeast are not an ideal model for disease processes that rely on cellular contacts or creation of full tissues or organs. This becomes especially relevant when considering systems like immune function or neuronal communication.

However, while yeast sometimes lack processes identical to those involved in human disease progression, yeast may have a simpler system that can still provide important insight. α-Syn is an example of this. Yeast do not display the process of regulated secretion that α-syn is thought to play a role in governing: all secretion in yeast is constitutive. However, studying the

mechanism by which α-syn hinders constitutive secretion in yeast has provided insight into how α-syn affects specialized secretion in the nervous system.

In this thesis, I have described two projects, one successfully modeling the splice isoforms of α-syn in yeast and the other failing to find a phenotype associated with torsinA expression in yeast. Herein I will outline future directions for both of these projects and discuss what they tell us about the strengths and limitations of using yeast to model human disease.

Future directions: α-syn splice isoforms

Within this thesis, I used yeast to assess the biophysical properties of two splice isoforms of α-syn, one lacking exon four (α-synΔ4) and one lacking exon six (α-synΔ6). These splice isoforms are differentially expressed in synucleinopathies; however, little is known about their biology. My studies revealed differential toxicity and localization of the splice isoforms as compared full length α-syn (α-synFL). Using yeast as our model organism enabled such thorough investigation of the biological differences between these isoforms. We were able to generate strains that we knew expressed the same levels of each isoforms. Such matched strains would be much more difficult to construct in higher organisms, highlighting the power of using yeast to study heterologous genes.

We further utilized yeast in high-thoughput analyses to ask how the splice isoforms differentially affect cell biology. Again, these studies would have been technically much more challenging, or impossible, in higher organisms. We discovered that expression of the splice isoforms differentially sensitize yeast to changes in sterol biology. There is a complicated history of the role that sterols play in synucleinopathy progression. Some studies show high cholesterol levels to be associated with increased risk of synucleinopathy, while others suggest

the opposite (Huang et al., 2008; Muller et al., 1995; Wolozin et al., 2007). We found that cells expressing α-synFL and α-synΔ6 are also sensitive to drugs that block sterol synthesis, while control cells and cells expressing α-synΔ4 are resistant. Therefore, it is possible that there are ties between splice isoforms levels and differential impact of cholesterol on synucleinopathy progression, but significant future work is required to fully understand the biology of the splice isoforms in this and other contexts.

First, more work is necessary to understand the membrane binding properties of the splice isoforms. α-SynFL has been shown to bind sterols (Fantini et al., 2011; Fortin et al., 2004) and addition of cholesterol promotes α-synFL binding to a vesicle bilayer *in vitro* (Kamp and Beyer, 2006). This suggests that α-synΔ4 may be less able to interact with membrane sterols; consequently, it would be interesting to investigate the *in vitro* lipid binding properties of this splice isoform.

Some studies of this type are already underway. Single-molecule fluorescence resonance energy transfer (FRET) studies revealed that α-synFL folds differently in the presence of varying concentrations of the lipid mimic SDS, depending on whether SDS is in a flat membrane conformation versus a spherical micelle (Ferreon et al., 2009). Through collaboration, the Deniz lab is currently investigating the ability of the splice isoforms to assume these various conformations in similar ways and will hopefully be able to extend these studies to examining the affect of adding cholesterol to these membranes. This approach, combined with assays to investigate the ability of the splice isoforms to interact with different membranes would provide a more complete picture of the lipid binding properties of the α-syn splice isoforms.

While we highlight intracellular localization differences between the α-syn splice isoforms in yeast, this should be extended to tissue culture cells. There are two options for pursuing this.

First, it has been found that transgenic mice modestly overexpressing α-synFL-GFP show that it localizes to synaptic boutons in hippocampal neurons (Scott and Roy, 2012; Scott et al., 2010). While it is time consuming and expensive to make transgenic mice, mice expressing α-synΔ4-GFP and α-synΔ6-GFP would be very informative to examining the localization of the splice isoforms. A second option focuses more on the membrane binding properties of the splice isoforms, although it utilizes HeLa cells instead of neuronal cells. Previous work found that it is possible to visualize the membrane binding properties of α-syn by using digitonin to permeabilize HeLa cells transfected with α-syn (Fortin et al., 2004). This led to a loss of cytoplasmic α-syn and revealed that the remaining α-syn localized to lipid rafts, which are regions of the membrane concentrated in specific proteins and lipids such as cholesterol. This suggests that α-syn may associates more tightly with this area of the membrane. Membrane binding to lipid rafts was lost by the disease-associated variant α-synA30P, which has been extensively shown to be unable to bind lipids. It would be interesting to repeat this experiment with cells expressing α-synΔ4 and α-synΔ6, and upon altering cellular sterol content, in attempt to recapitulate my yeast results.

In this thesis, I also showed that α-synΔ4-expressing yeast cells are less sensitive to sterol inhibition than α-synFL- or α-synΔ6-expressing yeast. Extending these studies to neuronal culture has proven challenging. I attempted to investigate whether rat primary neuronal cells expressing α-syn splice isoforms also showed differential sensitivities to statins. This was difficult due to the extreme sensitivity of these cells to even small concentrations of the drugs. One option would be to work with other cell types that may be less sensitive to statins; however, using cells other than neurons may be less relevant to disease and α-syn overexpression is not toxic to all types of mammalian cells, complicating these analyses.

Another avenue for future research is the mechanism by which OSH proteins differentially regulate α-syn-induced toxicity. Here, I showed that OSH2 and OSH3 enhance α-synΔ4- and α-synΔ6-induced toxicity, while they suppress the toxicity elicited by α-synFL. Little is known regarding the mechanism of action of OSH2 and OSH3, but a recent paper demonstrated that OSH3 localizes to endoplasmic reticulum (ER)-plasma membrane contact sites and regulates local phosphatidylinositol 4-phosphate levels at these sites (Stefan et al., 2011). Given the localization of α-syn to the plasma membrane and its impact on secretory vesicles originating from the ER, it will be important to determine if α-syn localizes to these same contact sites. In work not reported here, I also found that the other OSH family members have no impact on toxicity induced by any of the α-syn isoforms. This potentially provides insight into important differences between the functionality of OSH family members.

Finally, there were other genes that, when overexpressed, differentially modified the splice isoforms as compared to α-synFL. Most only produced small differences, but the most notable unexplored difference was that PHO80 suppressed toxicity of α-synFL and α-synΔ4 and enhanced α-synΔ6 (data not shown). PHO80 is a cyclin that has been linked to nutrient sensing, as well as some pathways previously associated with α-syn-induced toxicity, such as calcium homeostasis and metal accumulation. This suggests that the α-synΔ6 may differentially affect these processes, which should be studied further. Also, given that α-synΔ6 lacks a portion of the C-terminal tail of the protein important for protein-protein interactions, it could serve as a useful tool for studying potential interacting partners of α-synΔ6 to α-synFL.

Future directions: torsinA

Here, I also outlined my attempts to use yeast to study the dystonia-related protein torsinA. It has previously been shown that torsinA regulates ER stress (Chen et al., 2010). Given the high level of conservation in ER stress pathways and genes between yeast and higher organisms, I hypothesized that I may be able to recapitulate torsinA's role regulating ER stress in yeast, even though yeast lack an endogenous torsinA homolog. Unfortunately, in contrast to the situation encountered upon heterologous expression of α-syn in yeast, I was unable to uncover any stress-related phenotypes associated with torsinA expression in yeast. However, this early work can serve as a platform for future torsinA studies. As cofactors of torsinA are discovered, our model can be utilized to test if these modifiers are necessary for torsinA to function in yeast. There is still many unknowns concerning the role of torsinA in ER stress and I am optimistic that future work will uncover details necessary for modeling torsinA biology in yeast.

In the time since I have ceased attempting to model the consequences of torsinA in yeast, some advances have been made in this field that may provide new insight into modeling this protein's cellular function. TorsinA was found to act in endoplasmic reticulum associated degradation (ERAD) (Nery et al., 2011). These authors also found that ERAD was inhibited by overexpression of the disease-associated mutant form of torsinA as well as downregulation of wild type torsinA. If there was a defect in ERAD upon overexpression of mutant torsinA in my studies, the unfolded protein response reporter I used in these studies would have detected it, but is relevant to consider in designing future studies.

Yeast two-hybrid analysis of a torsinA isoform lacking the first forty amino acids yielded an interaction partner, Printor, which was also shown to physically interact with torsinA in higher organisms (Giles et al., 2009). Notably, full-length torsinA failed to produce any positive hits in

this same screen. These first forty amino acids encode the signal sequence and a hydrophobic domain. The authors reasoned that removal of this domain might simply interfere with the ability of the protein to translocate into the nucleus, which would be essential for the assay. However, this modification could also be interfering with other critical aspects of torsinA function when expressed in yeast. I performed some preliminary studies with torsinA lacking the first 40 amino acids but all of my results still suggested that this form of torsinA was not active in my assays. Any future work on this topic should consider these results carefully. It is possible that modification or removal of other domains may allow more fruitful studies, although the biological significance of such modifications could temper enthusiasm of any positive results obtained.

Another possible problem with modeling torsinA in yeast stems from the fact that yeast lack some of the known binding partners of torsinA, such as LULL1 and LAP1 (Goodchild and Dauer, 2005). While these proteins are thought to be more important for the ability of torsinA to target the nuclear envelope than for its activity in the ER, it is possible that torsinA requires these interactions for its role in regulating ER stress. Ectopic coexpression of these proteins with torsinA in yeast may help to create a functional dystonia model. Future work in higher organisms, especially efforts to define other accessory proteins necessary for torsinA function, may provide insights that will allow successful creation of this yeast model.

I was fortunate to be able to publish our unsuccessful attempts to model TorsinA in yeast, due to the extremely rigorous way in which I attacked this problem. However, these types of publications are rarely seen in the literature. This is a shame. Examination of unsuccessful experiments can be extremely helpful. They provide important insights that allow scientists to more carefully craft future experimental efforts. Furthermore, they prevent other labs from

duplicating the same studies and wasting valuable time and financial resources. Gone are the days where one must scour paper journals in a library to find articles of interest. We live in an age where a simple internet search will quickly reveal any publication on any topic. With the rise of internet-based journals, which do not have the same limitations imposed by publishing costs and are able to focus on judging science more for its thoroughness and quality of investigation, there is an obvious forum for these types of results. Hopefully, this will prompt more scientists to craft these types of publications and expand the scientific dialogue to include both positive and negative results, when they are properly and rigorously controlled.

Future directions: modeling human disease proteins in yeast

Looking to the future, what can we learn from successes and failures of studying human disease proteins in yeast that will allow us to better select novel proteins to attempt to model? First, it is important to strongly consider what is already known about the protein of interest. The success of the yeast models expressing proteins involved in neurodegenerative diseases is tightly linked to their function. They are all small proteins or peptides that cause cellular dysfunction by interrupting highly conserved processes. In the case of α-syn, its primary binding partner and site of induced toxicity are cellular membranes, a component of every eukaryotic cell. Its primary impact is on vesicle secretion, an extremely conserved process. These factors contribute into the ability of α-syn dysfunction to be effectively modeled in yeast. Other neurodegenerative disease proteins that have been successfully modeled in yeast are similar. For example, Aβ, a peptide associated with Alzheimer's disease, induces toxicity upon expression in yeast and affects similar processes to those in higher organisms (Treusch et al., 2011). Again, Aβ is a small peptide that is inherently prone to aggregation, affecting highly

conserved processes like endocytosis. Importantly, however, these proteins are not simply causing toxicity by aggregating and blocking up protein turnover systems. Each protein triggers a unique signature of phenotypes in yeast, many of which have been found to be relevant to their corresponding disease.

While torsinA regulates conserved cellular processes, it also is a complex protein with multiple domains and many non-conserved binding partners. These factors probably limited my success in modeling torsinA biology in yeast. However, while these types of proteins may be more complicated to model in yeast, this should not be viewed as a complete deterrent. As noted above, there has been much success in modeling transporters involved in renal function in yeast. These proteins are complex transmembrane proteins that one might assume would be hard to express heterologously. The fact that yeast have proteins that function similarly, while not direct homologs, may provide one clue to the reason for the success of these studies. Therefore, even if there are doubts concerning the ability to create a yeast-based model for certain proteins, the results may be surprisingly fruitful, as long as experiments are designed rationally, keeping in mind the limitations of the yeast system.

Concluding remarks

While yeast have been used as a model system for decades, there has been a recent explosion in using them to model disease via expression of heterologous proteins, especially proteins involved in neurodegenerative diseases. This is likely because yeast are a simple system with an already developed set of powerful assays, yet share significant cellular conservation with higher eukaryotes. While some disease-related proteins are more difficult to model in yeast than others, the scientific advances that can derive from an effective yeast model often outweigh

the risk that the protein may not function in yeast. Hopefully more labs will undertake this challenge, resulting in more yeast-based models of human disease-related proteins as well as the accompanying scientific advances that yeast models can yield, which can truly advance our treatment using human disease.

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Appendix One:

Using Split-GFP to Study the Self-Interaction of α-Synuclein

Abstract

Split-GFP has been widely used as a method to determine if two proteins are able to physically interact within the context of a living cell. Previous split-GFP studies have already shown that α-syn-α-syn self-interactions occur in tissue culture cells. We extended this study to examine these interactions in the yeast *Saccharomyces cerevisiae*, with the goal of enabling a screen for factors that either increase or decrease α-syn-α-syn self-interaction. While we were able to create a system that was bright enough for us to visualize these interactions using low-throughput fluorescent microscopy, the signal was too dim to be used in high-throughput applications. This limited the utility of our model for making broad observations about the localization of α-syn-α-syn interactions in the yeast cell.

Introduction

α-Synuclein (α-syn) is a small lipophilic protein associated with a number of neurodegenerative disorders, collectively termed synucleinopathies, the most prevalent of which is Parkinson's disease. Mutations in and genomic multiplications of the gene encoding α-syn are associated with early-onset forms of these diseases. Furthermore, the protein itself is a principle component of Lewy bodies, intracellular aggregates that form in the surviving neurons of patients with certain synucleinopathies (Dauer and Przedborski, 2003). While large protein aggregates are prominent in brain slices, many believe it is actually smaller-order oligomers that drive disease progression (Conway et al., 2000).

The earliest papers published on α-syn expression in yeast showed that the protein was able to localize to the plasma membrane, as well as both small peripheral and large intracellular foci (Outeiro and Lindquist, 2003). It was later found that these foci represent clumps of secretory vesicles, which are decorated with α-syn (Gitler et al., 2008). These vesicles accumulate because α-syn inhibits the ability of vesicles formed in the endoplasmic reticulum (ER) to fuse with the Golgi apparatus.

Previous efforts in our lab to uncover higher-order protein species of α-syn upon expression in yeast have had variable results (unpublished observations). Since α-syn expression in yeast does elicit toxicity, this toxicity in the absence of higher-order aggregates fits with the model that dynamic oligomers, which would be harder to extract, are the toxic species as opposed to stable amyloid aggregates. It is unclear if this is due to the technical constraints of studying protein-protein interactions following protein extraction or if the cellular environment of *S. cerevisiae* inhibits α-syn self-interactions. To answer this, we developed a method using split-GFP to look at α-syn-α-syn self-interactions *in vivo*.

The split-GFP method utilizes the fact that GFP can be split into two halves, each of which is non-fluorescent on its own. However, when brought physically close enough together, the two halves are able to assemble into a functional fluorescent molecule. When these two halves of GFP are used to tag proteins, it is assumed that complementation will only occur to a significant degree if the two tagged proteins directly interact (Hu et al., 2002). This was successfully used to study α-syn interactions in cultured mammalian cells (Outeiro et al., 2008); however, in this study, complemented split-GFP fluorescence could be seen throughout the cell with no specific intracellular localization discernible. Here, we utilized this same method to examine if α-syn self-interactions occur in *S. cerevisiae*.

Results

Expressing α-syn tagged with split-GFP

Throughout this study, "splitN" will be used to describe the N-terminal half of GFP and "splitC" will be used to describe the C-terminal half. Early attempts at creating constructs to express α-syn tagged with each side of split-GFP, as described in (Outeiro et al., 2008), failed due to low or no expression of α-syn-splitC. In these constructs, splitN contained amino acids 1-155 of GFP, while splitC contained amino acids 156-238 of GFP. Therefore, we turned to a split-GFP system developed for expression in yeast (Barnard et al., 2008; Barnard and Timson, 2010), in which splitN contained amino acids 1-157 of GFP and splitC contained amino acids 158-238 of GFP. While multiple combinations of these two sets were tried, we were surprised to find that the pair that showed the highest amount of fluorescence was splitN with amino acids 1-155 of GFP and splitC with amino acids 158-238 of GFP (Figure A1.1a). A construct was generated that

expressed both splitN-α-syn and α-syn-splitC within the same construct, which will be referred to as α-syn-splitTotal (Figure A1.1b).

Strains were created for α-syn-splitC alone and α-syn-splitTotal that displayed approximately the same toxicity. All strains made with splitN-α-syn alone showed no toxicity. A Western blot elucidated the reason for this. There was much lower expression of splitN-α-syn than α-syn-splitC, even in the α-syn-splitTotal strains that have equal numbers of integrated copies (Figure A1.2). This is likely due to instability of the splitN-α-syn, since moving splitN-α-syn to the site on the plasmid from which α-syn-splitC was being expressed did not increase expression of splitN-α-syn (data not shown).We can conclude that splitN-α-syn and α-syn-splitC stably interact as evidenced by the higher band that appears in the α-syn-splitTotal sample at a slightly higher molecular weight than α-syn-GFP.

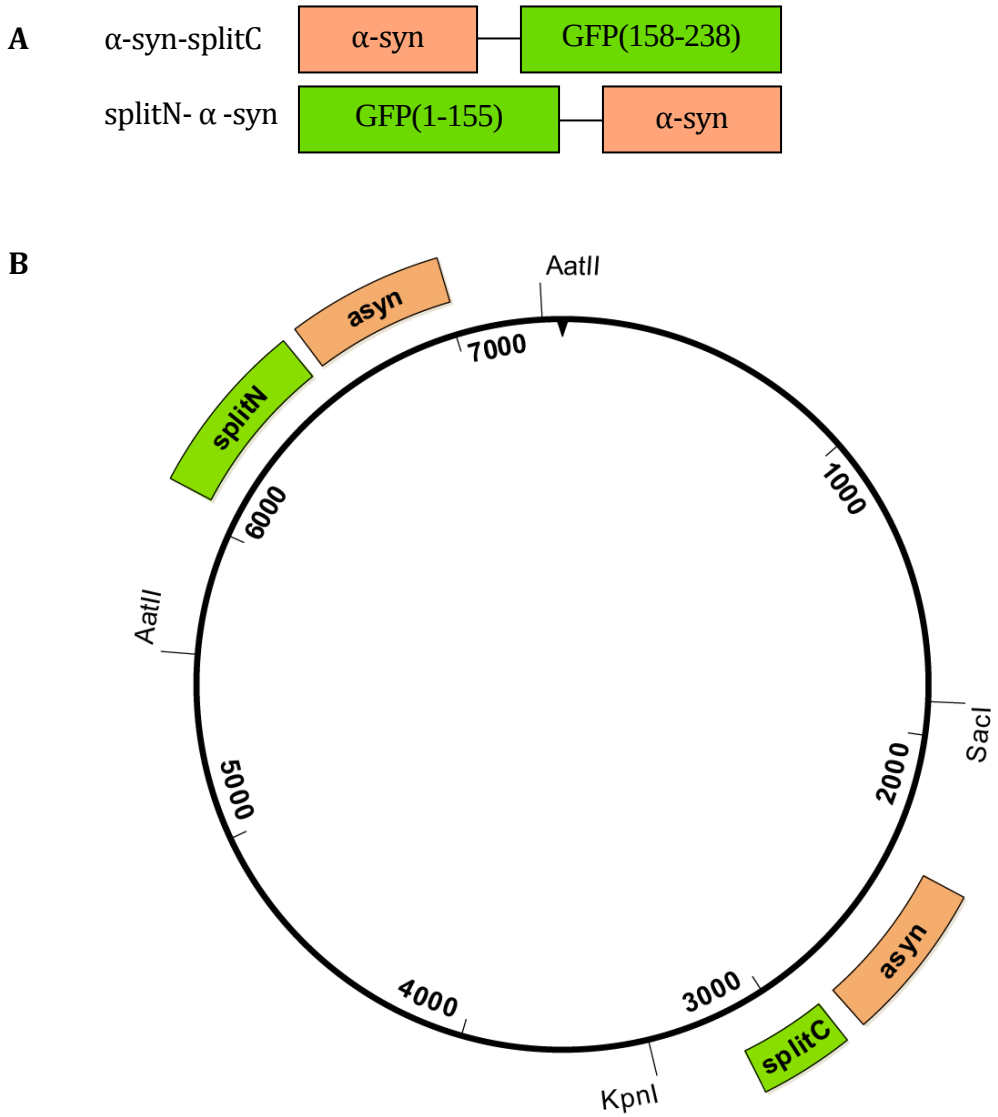

Figure A1.1. Summary of constructs used.

(a) Separate α-syn constructs were tagged either C-terminally with the final 81 amino acids of GFP or N-terminally with the first 155 amino acids of GFP. These constructs are referred to as splitC or splitN, respectively.

(b) In an attempt to express equal copy numbers of the α-syn-splitC and splitN-α-syn constructs, both were subcloned into the same yeast integrating vector. This ensured that, while multiple copies of the vector may be inserted, the copy number ratio of α-syn-splitC to splitN-α-syn should remain 1:1.

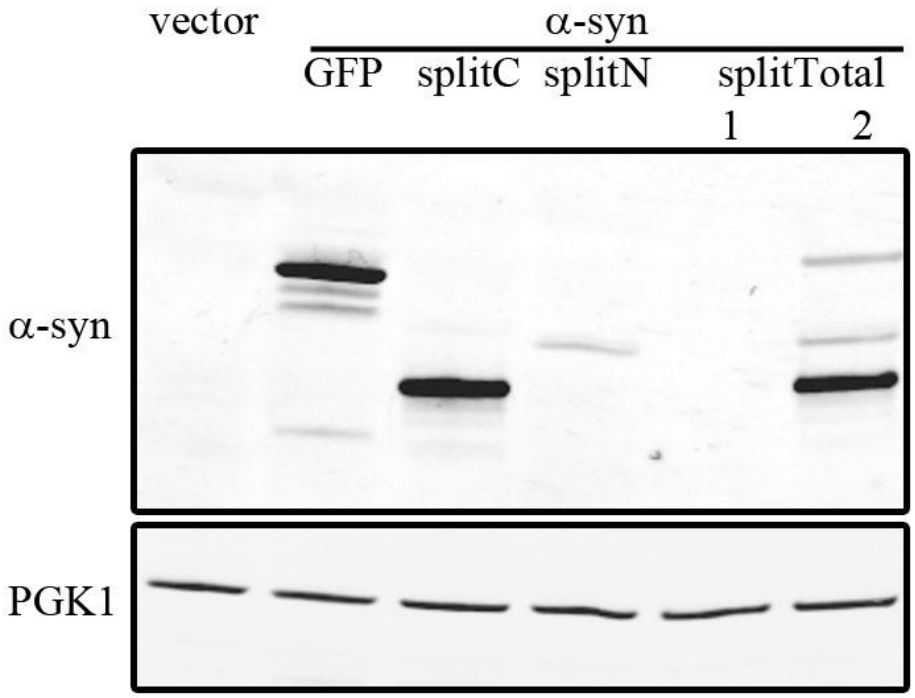

Figure A1.2. Expression of split-GFP constructs.
Proteins were extracted from cells expressing α-syn tagged with splitC and/or splitN. When α-syn-splitC and splitN-α-syn are expressed simultaneously, there are lower levels of splitN-α-syn, even though both constructs are expressed from the same plasmid. However, evidence that splitN and splitC can stably interact is provided by the highest band in the splitTotal 2 lane, which is stable even though the samples were boiled for five minutes and run through an SDS-PAGE gel.

Visualization of α-syn self-interaction

When viewed by fluorescent microscopy, α-syn-splitTotal displayed an interesting localization pattern. For comparison, a strain of α-syn-GFP that elicits the same level of toxicity as the α-syn-splitTotal strain was visualized. As previously shown, α-syn-GFP shows even distribution of α-syn around the plasma membrane, small foci at the periphery, and large intracellular foci. Although expressed at a high enough level to induce toxicity, the control strain containing α-syn-splitC showed no fluorescence (figure A1.3). The other control plasmid splitN-α-syn could not be expressed at a high enough level to induce toxicity; however, when expressed at a level similar to the amount of splitN-α-syn in the α-syn-splitTotal strain, splitN-α-syn showed no fluorescence. α-syn-splitTotal, however, was fluorescent in a non-contiguous pattern around the plasma membrane, suggesting that α-syn-α-syn interactions occur at specific locations. Notably, while α-syn-GFP showed fluorescent intracellular foci, α-syn-splitTotal did not show any foci.

Unfortunately, while specific fluorescent patterns were seen, the signal was very weak. This made further analysis of these cells difficult and precluded their use in high-throughput applications, as the signal could not be significantly detected from background in flow cytometry. Due to this low signal, this project was not continued.

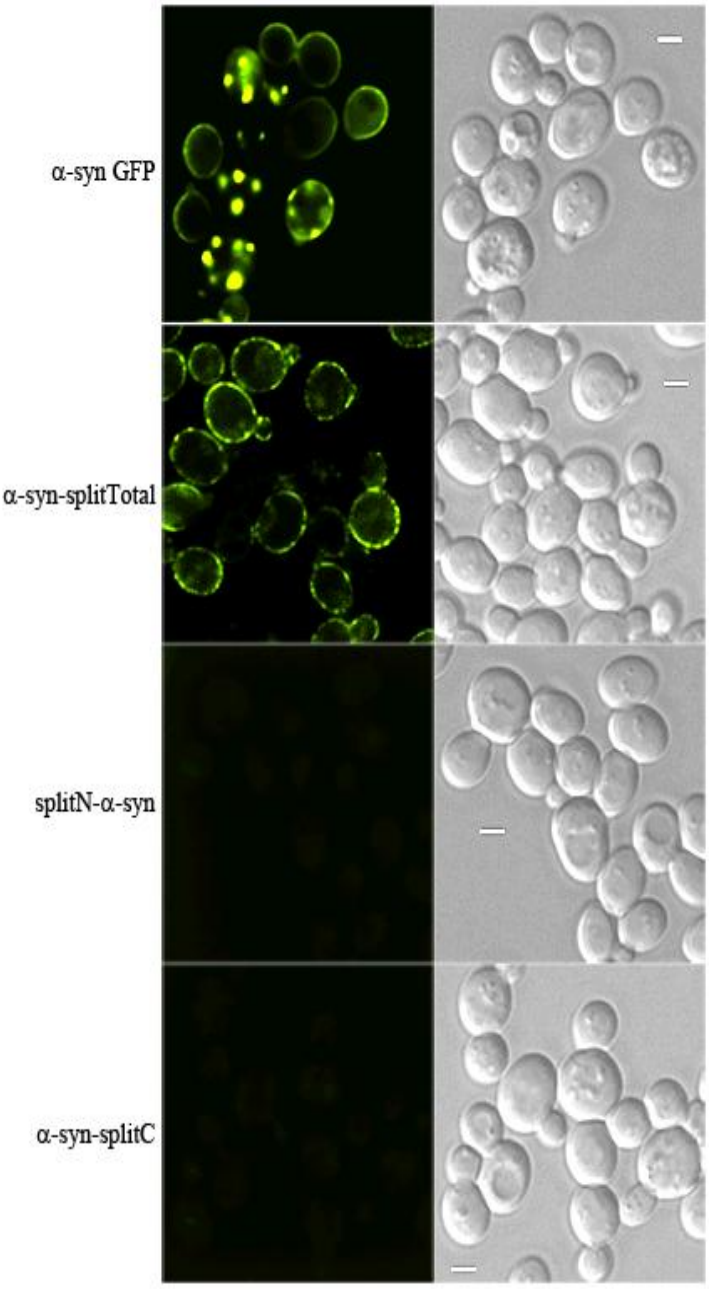

Figure A1.3. Visualization of α-syn-α-syn self-interactions.
Cells were grown in galactose to induce α-syn expression before visualization by fluorescent and DIC microscopy. α-Syn-GFP is seen uniformly at the plasma membrane and intracellular foci. α-Syn-splitTotal is seen at specific locations at the plasma membrane but not in intracellular foci, although α-Syn-splitTotal expressed at a level to induce the same toxicity as α-syn-GFP. Scale bar = 2μM.

Discussion

In this study, we examined the α-syn-α-syn self-interactions that occur in yeast using split-GFP. The N-terminal fragment of GFP was fused to the N-terminus of one α-syn construct while the C-terminal fragment of GFP was fused to the C-terminus of another α-syn construct. We found that the splitN-α-syn construct was expressed at a significantly lower level, even when the same number of genomic copies was integrated. Possibly due to this lower expression of splitN-α-syn, the signal achieved in α-syn-splitTotal was quite dim, preventing in-depth study of the localization pattern. However, we were still able to make some interesting initial observations.

One observation was that α-syn-splitTotal showed fluorescence only at specific peripheral locations and not in intracellular foci. This occurred even in strains with high levels of toxicity that would normally be associated with α-syn foci formation. We present two hypotheses that could explain this observation. First, it is possible that when α-syn localizes to foci, it assumes a confirmation that prevents α-syn-α-syn self-interactions, even though there is a high local concentration of α-syn. Secondly, it is possible that the stabilized α-syn-α-syn self-interaction of α-syn-splitTotal (as demonstrated by the fact that these complexes are resistant to denaturing by reducing agent and boiling) allows α-syn to be toxic when expressed at a lower level, such that these foci are not being formed. Previous studies have found that these foci represent an accumulation of secretory vesicles, studded with α-syn and various secretory proteins (Gitler et al., 2008). This question could be answered in a number of ways. One option is to perform immuno-electron microscopy, looking for the presence of accumulated vesicles with α-syn colocalization. Secondly, one could co-express α-syn-splitTotal with α-syn-RFP to look if there is accumulation of foci containing α-syn-RFP with or without green signal. However, this method

could be confounded by bleed through of the red signal since the signal from α-syn-splitTotal is so dim.

Furthermore, it was previously shown (Outeiro and Lindquist, 2003), and reproduced here, that α-syn-GFP shows uniform localization around the entirety of the plasma membrane, while α-syn-splitTotal showed signal at specific points along the membrane. There are many possible explanations for what these spots could be, since it is known that the cell periphery is not uniform, but instead contains a multitude of different local domains. We would like to offer two hypotheses. First, it is known that α-syn causes toxicity by blocking trafficking from the ER to the Golgi. Indeed, some of the early aggregates of α-syn that form are accumulations of secretory vesicles appearing around the cell surface. This localization pattern is somewhat reminiscent of the peripheral ER, and this might be the site of α-syn-α-syn self-interactions. Secondly, many studies have shown that α-syn prefers to interact in sterol-rich environments. It is possible that the sites of increased α-syn-α-syn self-interaction represent lipid rafts, which are enriched in the sterol ergosterol. This question could be answered by looking for colocalization with ER proteins or proteins that localize to lipid rafts. However, these colocalization studies would be complicated by the low signal level of α-syn-splitTotal.

It is likely that one of the reasons that the GFP signal level is so low is due to low levels of the splitN-α-syn protein. While it is not known if this low expression is due to instability of the RNA or protein, it is unlikely to be due to lower levels of transcription, as expression of the construct from the same site on the plasmid as α-syn-splitC did not significantly change expression levels. Stability of the RNA could be determined by using real time PCR to compare RNA levels of α-syn-splitC to splitN-α-syn from plasmids where they are inserted into the same site. This result

could be used to inform educated modifications to the splitN-α-syn construct in an attempt to increase the stability of the RNA or protein.

Materials and Methods

Construction of plasmids

GN-link-α-syn was a kind gift from Brad Hyman (Outeiro et al., 2008). For use in the *S. cerevisiae* system, overlap extension PCR was used to attach flanking AatII sites and the Gal promoter to GN-link-α-syn. This was then subcloned into the AatII site of pRS304 to make pRS304-splitN-α-syn.

SplitC (BYP6335) was a kind gift from David Timson (Barnard et al., 2008). Overlap extension PCR was used to attach this construct to the C-terminus of α-syn and add SpeI and XhoI sites. This was subcloned into 413-Gal (Ronicke et al., 1997). This was then cut with KpnI and SacI to isolate the construct from promoter to terminator and subcloned into pRS304 to make pRS304-α-syn-splitC or pRS304-splitN-α-syn to make pRS304-splitTotal-α-syn.

Table A1.1. Primers Used.

#	Purpose	Sequence
353	AatIIGal-f	GGGACGGACGTCACGGATTAGAAGCCGCCGAGCGGG
354	Gal-SplitN-f	CCCCGGATTCTAGAACTAGTATGGTGAGCAAGGGCGAGGAGCTGTTCACC
355	Gal-SplitN-r	GGTGAACAGCTCCTCGCCCTTGCTCACCATACTAGTTCTAGAATCCGGGG
356	AatII-α-syn-r	GGGACGGACGTCGTCGACTTAGGCTTCAGGTTCGTAGTCTTG
404	SpeI-α-syn-f	GGGGACACTAGTATGGATGTATTCATGAAAGG
405	α-syn-splitC-f	CAAGACTACGAACCTGAAGCCGGAGGATCTGGAGGG
406	α-syn-splitC-r	CCCTCCAGATCCTCCGGCTTCAGGTTCGTAGTCTTG
446	XhoI-splitC-r	GGGACGCTCGAGTTACTTGTACAGCTCG

Yeast strains and growth conditions

All experiments were performed in the JK9-3d yeast background. All yeast transformations

were completed using the standard lithium acetate transformation protocol (Gietz et al., 1992; Gietz et al., 1995). All growth was done at 30°C, with aeration for liquid growth.

To induce α-syn expression, cells were grown to log phase in glucose-supplemented minimal media [0.67% yeast nitrogen base without amino acids (Fischer Scientific), supplemental amino acids (minus those needed as selectable markers) (MP Biomedicals), and 2% sugar]. Cells were then diluted in minimal media with raffinose for overnight growth to log phase, at which time cells were diluted into minimal media with galactose to be induced for six hours.

Western blot

After 6 hours of induction, bead beating was used to lyse cells in lysis buffer (10mM HEPES pH7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.5 mM phenylmethanesulfonylfluoride (PMSF), and 1x protease inhibitor (Roche)). The product was incubated with 0.5% Tween and then separated from the beads and cell debris. A BCA assay (Pierce Protein Research) was used to quantify protein concentration. Normalized levels of protein were boiled for five minutes in NuPage LDS sample buffer (Invitrogen) with reducing agent and run on a 10% Bis-Tris SDS-PAGE Gel (Invitrogen) and transferred to PDVF membrane. After blocking with 5% milk in PBS, the membrane was probed with either mouse anti-α-syn (BD Biosciences) or rabbit anti-PGK1 (antibodies-online.com) and a dye-conjugated anti-mouse or anti-rabbit IgG secondary antibody (LICOR Biosciences). The blot was visualized using the Odyssey Infrared Imaging System (LICOR Biosciences).

Microscopy

Cells were induced by the method described above. After mounting on a slide, a Nikon Eclipse Ti was used to visualize cells by fluorescent microscopy. Z-stacks were taken. After normalizing signal over the Z-stack, the images were deconvoluted using the 3D deconvolution algorithm. The brightness of the images was adjusted to highlight the localization of the α-syn-split-GFP strain, which is significantly dimer than α-syn tagged with GFP.

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