Global Identification of Human Modifier Genes of Alpha-synuclein Toxicity

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GLOBAL IDENTIFICATION OF HUMAN MODIFIER GENES OF ALPHA-SYNUCLEIN TOXICITY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

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**ABSTRACT**


Alpha-synuclein is a small lipid binding protein abundantly expressed in the brain. Lewy body or Lewy-like pathology, primarily composed of misfolded alpha-synuclein, is a pathological feature shared by several neurodegenerative disorders, including Parkinson's disease (PD). Both missense mutations and increased copy numbers of the SNCA gene, encoding the alpha-synuclein protein, have been genetically linked to autosomal dominant PD. Other genetic variations affecting the expression of the SNCA gene have been associated with sporadic PD. Although the physiological function of alpha-synuclein is not well understood, its localization to plasma and vesicular membranes at the presynaptic terminals suggests a role in neurotransmission. How alpha-synuclein misfolds and deposits into the Lewy bodies or causes cytotoxicity to neurons remains unclear. Both membrane association and dosage-dependent toxicity of alpha-synuclein can be recapitulated in the budding yeast, *Saccharomyces cerevisiae*. Using yeast genetic screens, hundreds of suppressor and enhancer genes have been discovered, revealing complex cellular processes underlying the toxicity of alpha-synuclein. Mammalian homologs of several yeast suppressor genes exhibit similar protective effect in neurons, supporting the existence of conserved mechanisms pertinent to alpha-synuclein toxicity in yeast. We reasoned that screening yeast models expressing toxic high levels of alpha-synuclein may not uncover genes that promote aggregation and toxicity of the protein expressed at a low dosage. Furthermore, human genes without yeast homologs cannot be
identified in genetic screens using only yeast genes. To address these limitations, we expressed SNCA at a range of levels in yeast and found distinct membrane localizations of alpha-synuclein corresponding to various degrees of toxicity. Next, we constructed an overexpression library containing ~15,000 human-gene clones. Using this library, we identified new genes that enhance or suppress the toxicity of alpha-synuclein. Characterization of some modifier genes suggest that alpha-synuclein targets conserved cellular machineries in yeast, impairing endocytosis, actin cytoskeleton and cell polarity. Future validation of these modifier genes in neurons and animal models may provide new insights in alpha-synuclein pathology and the genetic basis of neurodegenerative disease.
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DEDICATION

This thesis is dedicated to the loving memory of my Father.
CHAPTER 1. INTRODUCTION

A. Alpha-synuclein

Alpha-synuclein is a lipid-binding protein encoded by the gene SNCA. It was originally identified in the electric organ of the Torpedo fish in 1980. Because this protein was discovered using an antibody to purify cholinergic vesicles in the electric organ, the protein is suggested to have a presynaptic role (1). This protein was later identified in the rat brain, and it was given the name “synuclein” (2,3). In humans, it was initially identified as an unrecognized component of amyloid structures in Alzheimer's disease patients' brain tissue in 1993. It was originally called NACP or the non-amyloid component of plaque (4). Later, two abundantly expressed human proteins were purified and sequenced from human brain. These two proteins of 140 and 134 amino acids were named alpha-synuclein and beta-synuclein respectively (5). Alpha-synuclein primarily shows a presynaptic localization (6), suggesting its role in the pre-synapse. In addition to its localization at the synapse, alpha-synuclein has also been found in the nucleus (7). However, since alpha-synuclein is a small protein of 140 amino acid residues, there is a possibility that the endogenous synuclein could enter the nucleus via simple diffusion.

The protein structure of alpha-synuclein has three distinct regions: N-terminal domain, NAC domain, and C-terminal domain (Figure 1.1). The protein contains a characteristic 11-residue sequence (consensus XKTKEGVXXXX), which forms an amphipathic alpha-helix in N-terminal domain (8). These repeats are highly conserved,
both across species and among the three different isoforms. This conservation suggests that this domain is important for its physiological function. These different domains have different functions in the protein. The amphipathic region of alpha-synuclein is responsible for its membrane binding character. The NAC domain is extremely hydrophobic, which is why it is thought to play a role in the aggregation of alpha-synuclein. The C-terminal tail is highly acidic, and is thought to be involved in its binding to other proteins (8,9). Remarkably, all of the Parkinson’s Disease associated mutations of the SNCA gene cluster within the N-terminal domain. Point mutations on the N-terminus of the protein include A53T, A30P, E46K, G51D, and H50Q (10–15). Patients with the A53T mutation in SNCA usually develop a severe form of PD that is accompanied by dementia. Whereas patients with the A30P mutation develop late-onset PD and usually show only mild dementia (16). In an in vitro study it was shown that A53T mutant alpha-synuclein aggregates more rapidly than wild type alpha-synuclein. But the A30P mutant alpha-synuclein forms aggregates at a much slower rate (17).

B. Alpha-synuclein expression in the brain

Because alpha-synuclein is highly expressed in the human brain tissue, this protein is suggested to play an important role in brain function. Indeed, previous studies have shown that alpha-synuclein regulates the distal pool of presynaptic vesicles (18,19) and dopamine release (20). Some studies also suggest its role as a chaperone (21). However, the complete functions and mechanisms of this protein are still unclear. Despite the known functions, there are potentially other functions remaining to be uncovered that
relate to alpha-synuclein’s ability in binding membranes, vesicles, or vesicle-associated membrane proteins.

C. Membrane-associated function of alpha-synuclein

The NAC region of alpha-synuclein is relatively hydrophobic and aggregation prone (8,9). Depending on membrane curvature, alpha-synuclein has been reported to adopt elongated or broken alpha-helical structures. Membranes containing lower curvature or larger diameter induce an elongated alpha-helix structure (22). On the other hand, membranes of high curvature, like small vesicles, induce a broken alpha-helix conformation (23,24). Alpha-synuclein preferentially binds to these smaller vesicles and is abundantly found in the small synaptic vesicles in the brain (25). Studies have shown that binding to membranes helps alpha-synuclein to oligomerize into multimers (26,27). On the other hand, the lipid binding capability of alpha-synuclein has been shown to induce membrane curvature. Alpha-synuclein can convert large vesicles into highly curved membrane tubules and vesicles (28).

In yeast, alpha-synuclein has been found to interact with acidic phospholipids, which is thought to promote formation of vesicle aggregations. In addition, alpha-synuclein aggregation is enhanced in yeast mutants that are capable of producing high levels of acidic phospholipids (29). Consistent with that, it has been shown that alpha-synuclein colocalizes with yeast membranes that are enriched with phosphatidylic acid (29). This protein can also interact with lipid rafts in both mammalian (30) and yeast models (31). Moreover, it has been reported that alpha-synuclein not only binds to the
lipid packs but it can also modulate lipid-packing (32). Studies have also shown that inhibition of sterol synthesis can play a role in the plasma membrane association of alpha-synuclein. This result suggests that a higher concentration of membrane sterols may be protective of synucleinopathy progression (33).

Previous studies have reported alpha-synuclein as a specific inhibitor of phospholipases D1 and D2, both in vitro and in vivo (34,35). This suggests that alpha-synuclein might be also involved in membrane biogenesis. Alpha-synuclein was purified as an inhibitor of phospholipase D2 (PLD2), suggesting its involvement in membrane trafficking (36,37). However, the physical interaction between alpha-synuclein and PLD2 has not been documented. Although genetic studies in yeast supported the role of alpha-synuclein in PLD inhibition (38), evidence from later studies have disproved this finding (39). Thus, the biological relevance of this finding is still uncertain. These studies suggest that alpha-synuclein is probably sensitive to both the type of lipids and the arrangement of lipids in the bilayer (40,41). However, the specific lipid environment and composition preferred by alpha-synuclein remains to be clarified.

D. Alpha-synuclein and vesicle trafficking

Chronic overexpression of alpha-synuclein leads to impairment of synaptic vesicle trafficking by inhibiting vesicle fusion (42). Similar vesicular trafficking impairment associated with Golgi fragmentation was observed as a result of alpha-synuclein aggregation in the mammalian cell line (43). Some studies using the neuroblastoma SH-SY5Y cell line showed that alpha-synuclein is secreted by the exosome (44). In addition,
overexpression of alpha-synuclein has been found to impair the ER-to-Golgi transport in non-neuronal cells. This blocking of vesicle trafficking is thought to be caused by inhibiting ER/Golgi SNARE protein functions (45). Interestingly, overexpression of alpha-synuclein in mammalian neurons leads to the formation of abnormally large vesicular structures (46), which may be attributed to the impairment of endocytosis (47).

In yeast, defective endocytosis has been suggested to promote alpha-synuclein inclusions and toxicity. Studies show that alpha-synuclein can induce aggregation of yeast Rab GTPase proteins (29). Rab GTPase regulates multiple steps of membrane trafficking, including vesicle budding and movement (48). It has been shown that a dysfunctional Ypt6 protein, which acts in retrograde endosome–Golgi transport, can exacerbate the vesicle aggregation (29). Another study has identified many yeast mutants that enhance alpha-synuclein aggregation and toxicity. These mutants were linked with endocytosis and vacuolar degradation (31). Together, these data suggest that alpha-synuclein toxicity in yeast is associated with endocytosis of the protein. It is suggested that vesicular recycling of the vesicles from the plasma membrane and vesicle fusion defects lead to the blocking of different vesicular trafficking path. This traffic block leads to the formation of alpha-synuclein inclusions.

Alpha-synuclein interacts with the vesicle-associated membrane proteins, such as VAMP2 or synaptobrevin2 with its C-terminal domain (50). This secretory protein is involved in neurotransmitter release. In yeast alpha-synuclein is localized to plasma membrane under low expression (38). It has been demonstrated that alpha-synuclein
targets the plasma membrane via the secretory pathway. Alpha-synuclein initially localizes to the plasma membrane before dispersing within the cytoplasm (51).

Alpha-synuclein is suggested to play role in synaptic homeostasis. The membrane curvature of a vesicle can be stabilized by the alpha-synuclein. The alpha-synuclein use its amphipathic helix to fold and fit into those lipid packaging spaces. This way they are able to sense and stabilize membrane curvature. The stable curvature of synaptic vesicles reduces the ability of vesicles completely fuse with the plasma membrane. These affects negatively to both endocytosis and exocytosis. On the other hand, alpha-synuclein via its protein interacting domain can act as a positive modulator of SNARE complex assembly. SNARE complex assembly which are involved in membrane fusion. So, alpha-synuclein is helping with the synaptic vesicle recycling by speeding up the membrane fusion and protein retrieval after exocytosis. Growing evidence suggests that alpha-synuclein can play a role in both endocytosis and exocytosis (52).

E. Molecular chaperone activity of alpha-synuclein

Alpha-synuclein is predicted to function as a molecular chaperone. Its biochemical structure suggests its capability to bind to other intracellular proteins. Alpha-synuclein shares homology both structurally and functionally with the 14-3-3 molecular chaperone proteins family (53). Consistent with a function similar to chaperone proteins, alpha-synuclein was found to suppress the aggregation of thermally denatured proteins via its C-terminal domain (54) and modulate the assembly of synaptic SNARE complexes (55).
F. Lewy body and synucleinopathies

Alpha-synuclein is famously linked to Parkinson’s disease (PD). Point mutations in SNCA were found to cause an autosomal dominant form of Parkinson’s disease (12). PD is an incurable neurodegenerative disorder, affecting six million people worldwide (56). The clinical phenotypes of PD include typical tremor, rigidity, and bradykinesia. The neuropathological phenotype of PD includes nerve cell loss in the substantia nigra region and the presence of Lewy bodies or protein inclusions (57). Studies have shown that alpha-synuclein is the main component of these Lewy body structures (58). Interestingly, while mutations in alpha-synuclein are seen only in a small fraction of the PD population, the presence of Lewy bodies were observed in more than 90 percent of PD cases (59,60). This genetic and neuropathological evidence of alpha-synuclein in PD indicates that this protein has a central role in PD pathogenesis. Since 90% of the PD cases are sporadic with similar pathology, understanding the molecular mechanism of SNCA is very important. Moreover, Lewy body or Lewy-like pathology containing alpha-synuclein aggregation is found in several neurodegenerative disorders in addition to PD (e.g. multiple system atrophy and dementia with Lewy bodies). Together, these disorders are known as synucleinopathies (16). Despite this prevalent phenotype of Lewy body, the aggregation mechanism of alpha-synuclein is still unclear.

G. Environmental factors of PD

In addition to genetic factors, it is evident that PD has a strong environmental influence. Multiple studies have confirmed that there is a higher risk of developing PD in
populations exposed to heavy metals and pesticides (61). Rotenone and paraquat are two pesticides that disrupt the respiratory chain in the mitochondria and cause oxidative stress. One study showed that the application of these pesticides could possibly influence PD progression (62). In addition, head injury and some antipsychotics has been linked to an enhanced risk of PD (63). Finally, PD is more prevalent in the aging population. The age dependence of the disease is a strong indication that environmental factors play a part in the PD progression.

H. Splice variants of alpha-synuclein

Alternative splicing in SNCA leads to the formation of various isoforms. The expression and abundance of these isoforms vary with the disease. Two of the most abundant isoforms include a deletion of exon four (alpha-synΔ4) or exon six (alpha-synΔ6). Alpha-SynΔ4 isoform is missing a part of the lipid-binding domain, leading to reduced toxicity and reduced membrane binding. Alpha-SynΔ6 isoform is missing a part of the protein-protein interaction domain. While this isoform also showed reduced toxicity, it did not show any reduction in membrane binding capability. These splice isoforms of SNCA display different levels of toxicity and localization. Also, these are found to block vesicular trafficking. Interestingly, these isoforms show differential response to OSH3 (sterol-binding protein) overexpression. (33).
I. Post translational modification of alpha-synuclein

Post translational modifications may be involved in the pathogenesis of PD. Over 90% of aggregated alpha-synuclein derived from PD patients’ brains are found phosphorylated at the serine 129 position, while only about 4% of alpha-synuclein derived from normal brain is phosphorylated at the same site (64). It remains unclear whether phosphorylation of alpha-synuclein impacts the fibrillation or aggregation process (65) or promote neuron degeneration (66).

J. The pathological form of alpha-synuclein

Alpha-synuclein aggregation is the pathological hallmark of PD, but whether it is the cause of the disease is a largely debated issue. An increasing amount of evidence from animal models, as well as data from genetic and biochemical studies, supports the idea that the processes involved in the alpha-synuclein oligomerization have central roles in the pathogenesis of PD (67). Despite being such a small protein, alpha-synuclein is highly dynamic. Because of its lipid-binding capability, it can exist in multiple different forms, which are responsible for the dynamic characteristics of this protein. The native state of alpha-synuclein is extensively debated. While some reported that alpha-synuclein purified from human cells form a helically folded tetramer (68), others found that alpha-synuclein predominantly exists as an unfolded monomer (69). Taken together, these studies suggest that alpha-synuclein exists under various conformational shapes and oligomeric states in a dynamic equilibrium, modulated by factors either accelerating or inhibiting its misfolding or aggregation.
K. The prion hypothesis

The prion hypothesis helps us understand the underlying mechanism of the aggregation formation of alpha-synuclein. According to this idea, in a rare and energetically unfavorable event, the pathological seed of alpha-synuclein is formed. This seed formation requires a high alpha-synuclein concentration. Once the pathological seeds are formed, physiological forms of protein can also change shapes and join the growing aggregates. Fragmentation of aggregates generates new seeds, accelerating the formation of new aggregates (69). Thus, if there is an abundance of alpha-synuclein in cells, it is likely that some may be misfolded forming aggregation. Clinical findings where patients with triplication in the SNCA gene suffer more severe disease progression seems to support this hypothesis. Because those patients show a higher expression of alpha-synuclein protein, the disease progression is higher in severity compared to patients with fewer copies of the SNCA gene (70).

L. Parkinson’s disease models

Several models based on overexpression of alpha-synuclein protein have been developed to study alpha-synuclein toxicity, from unicellular genetic models to nonhuman primates. In transgenic mice, the development of abundant alpha-synuclein filaments was observed upon overexpression of the human wild-type or mutant alpha-synuclein. These mice also exhibited some movement disorders (71,72). Nerve cell degeneration and severe paralysis were observed in a mouse line that expressed the PD-linked A53T mutation of the SNCA
gene (73). Furthermore, Lewy body-like inclusions and degeneration of nerve cells in the rodent substantia nigra region were also observed (74). In Drosophila, human alpha-synuclein expression led to the formation of Lewy body-like inclusions and locomotor impairment. In addition to the movement disorder, the flies also showed some age-dependent loss of some dopaminergic neurons (75). Similarly, in a Caenorhabditis elegans model, overexpression of the human SNCA gene resulted in dopaminergic nerve cell loss and motor deficits (76).

M. Yeast model of Parkinson’s disease

Despite the significant evolutionary distance, basic biological processes are highly conserved from yeast to humans. Vesicle trafficking pathways, protein folding, mitochondria, and lipid biology are similar in yeast and humans (77) Although the budding yeast Saccharomyces cerevisiae does not have any alpha-synuclein ortholog, several studies have validated the use of this organism as a powerful model to study alpha-synuclein modifiers. The alpha-synuclein yeast model is a well-established model. Researchers introduced single or multiple copies of PD-associated human SNCA gene to develop a model for PD. Several alpha-synuclein yeast models that have been developed using both wild-type (WT) and mutant SNCA genes (38).

Toxicity of alpha-synuclein is dosage-dependent in yeast as it is in higher organisms. Increasing the dosage of alpha-synuclein in terms of copy number leads to various changes in the yeast cell, including protein aggregation in the cytoplasm, cellular toxicity, defective vesicular trafficking, mitochondrial pathology, and reduced
proteasome degradation. In yeast, alpha-synuclein has been shown to be delivered to the plasma membrane through the secretory pathway (51). The alpha-synuclein yeast models could be used to perform an unbiased screening to identify the genetic modifiers of alpha-synuclein; these would include genes that alleviate yeast growth toxicity (suppressor) or worsen yeast growth (enhancer). Given the availability of genetic and cell biological tools, budding yeast could be considered as a useful model to perform genetic screens.

N. Overexpression screening in SNCA yeast model

Deletion and overexpression screenings are genetic tools that are generally used to understand biological pathways. Deletion leads to loss of function mutation and possible perturbation of cellular pathways. Alternatively, overexpression amplifies the function of the gene and, thus, introduces a mutant phenotype (78). This different approach may allow for overexpression to produce unique phenotypes which are often not possible to visualize by deletion. Overexpression genetic screening in the alpha-synuclein intermediate toxicity model (IntTox) have identified 77 modifiers, which are prominently associated with vesicle trafficking, metal ion transport, osmolyte synthesis, protein phosphorylation, and trehalose metabolism (79,80).

Ypt1, the yeast homolog of mammalian RAB1, is a GTPase that promotes the movement of vesicles from the ER to the Golgi complex. The YPT1 gene was identified as a suppressor in an overexpression screen of alpha-synuclein toxicity. The study suggested that alpha-synuclein is likely to inhibit the docking or fusion of vesicles to the Golgi complex, thereby inducing toxicity. This observation was further validated by
ultrastructural studies that showed that large cytoplasmic alpha-synuclein inclusions are accumulations of undocked vesicles associated with alpha-synuclein (81). It has also been shown that alpha-synuclein expression induces ER stress by blocking the degradation of misfolded ER proteins. These misfolded proteins require transport from the ER to the Golgi complex before degradation. Heat-shock and heat-shock response genes were also found to be potent suppressors of SNCA toxicity (82). Overexpression screening with PD-linked mutant A30P SNCA identified Ypp1 as a suppressor. Ypp1 antagonizes reactive oxygen species accumulation and toxicity. It binds to A30P mutant form of alpha-synuclein, and direct it towards degradation in the vacuole (83).

O. Enhancer screening in SNCA yeast model

Most of the genetic modifier screenings are focused on the identification of the suppressors that suppress alpha-synuclein toxicity. One of the first alpha-synuclein enhancer screens in yeast was performed using a strain with an intermediate level of alpha-synuclein expression and toxicity (79). In this study, they simultaneously screened for both enhancers and suppressors using the yeast genome library. They identified 20 yeast genes that could enhance the alpha-synuclein toxicity when overexpressed. Another enhancer screen was performed using the toxic alpha-synuclein model and the yeast deletion library. The study focused on the identification of the synthetic lethality of alpha-synuclein and the yeast genes. They identified the yeast genes that can induce alpha-synuclein toxicity when deleted. From the screen, 32% of the identified hits were related to lipid metabolism and vesicular transport (84).
P. Screening yeast models using a human gene library

The overexpression screening in yeast is generally performed using a plasmid library of ~5,500 protein-coding yeast ORFs cloned into yeast expression vectors. This library is very useful because yeast shares a large number of conserved cellular pathways, including autophagy, apoptosis, oxidative stress, etc. Usually, the next step after the identification of a yeast modifier gene is to test its human homologs. Following identification, the human homologs must be confirmed to modify the toxicity. Because many of the human genes do not have yeast homologs, however, those genes are not identified in the traditional screening. To address this limitation, our lab has developed a novel way to directly screen the human genes in yeast (85). We have generated a plasmid library of ~15,000 human genes, in which the collection of human gene ORFs were individually cloned into yeast expression vectors. This library enables us to directly screen for human genetic modifiers of alpha-synuclein toxicity in yeast.

Although Lewy bodies are observed in more than 90% of PD patients, the protein sequence or expression level of alpha-synuclein is not necessarily altered. We therefore hypothesize that there are genetic factors that increase the propensity of alpha-synuclein to misfold and enhance its toxicity. To test this hypothesis, we wanted to carry out a genome-wide screen of human genes in a yeast model that does not lead to alpha-synuclein aggregation or toxicity. The current genetic approaches for identifying genes enhancing the toxicity of alpha-synuclein in yeast have two limitations. First, yeast models express toxic levels of alpha-synuclein which might not be effective in
identifying the factors that trigger the initial molecular events promoting alpha-synuclein inclusions and toxicity. Second, genetic screens using only yeast genes cannot identify the human genes involved in the toxicity. To address this, we developed a new model, 1xSNCA, which express alpha-synuclein at a much lower level and does not lead to inclusions or toxicity in yeast. We also constructed an overexpression library containing ~15,000 human-gene clones. Using this library and SNCA yeast model, we identified enhancer genes that initiate the alpha-synuclein toxicity. This screen can provide us valuable information to uncover genetic buffering mechanisms that initiate alpha-synuclein proteinopathy.
Figure 1.1: The protein domain structure of alpha-synuclein.

The alpha-synuclein contains three distinct regions: N-terminal amphipathic region, hydrophobic NAC domain, and highly acidic C-terminal domain. All the PD-linked mutations are located in the N-terminal amphipathic region. The protein domain graphic was generated using Autodesk Sketchbook (www.sketchbook.com) and Microsoft PowerPoint.
CHAPTER 2: MATERIALS AND METHODS

Gateway Cloning

To transfer the gateway entry clone genes into a yeast vector, gateway cloning or LR reaction was used (86). In a microcentrifuge or PCR tube, each LR reaction consisted of 100 ng of entry clone plasmid DNA, ~150 ng of destination vector plasmid DNA, 1X LR clonase enzyme mix, and TE buffer (pH 8.0) to make a final reaction volume of 10 µl. After proper mixing of the reaction by pipetting, it was incubated overnight at 25°C. 5 µl of the reaction was transformed into competent bacteria DH5α cells following a standard bacterial transformation method (87). After successful transformation, two colonies were picked for each reaction, followed by plasmid isolation. The cloned plasmids were confirmed by restriction enzyme digestion.

Yeast Strains, Media, and Plasmids

Yeasts were grown in Yeast Extract–Peptone–Dextrose (YPD) media during mating. YPD media was also used for yeast strains with integrated plasmids. Yeast strains carrying non-integrating or centromeric plasmids were grown in synthetic dropout media. Synthetic media consists of all amino acids except for those used for selection of the plasmid. Each yeast culture media contained either 2% dextrose, galactose or raffinose as the carbon source. Yeast cultures were grown at 30°C in a shaking incubator at 200 RPM. Yeast strains, plasmids and PCR primers used in this study are listed in Tables 1, 2, and 3 respectively.
Serial Dilution / Spotting Assays

Yeast serial dilution assays were used to assess the growth and growth of a yeast strain. The yeast strains were pre-cultured in selective media with 2% raffinose or 2% glucose overnight. Cultures were normalized to an OD$_{600}$ = 1.0, serially diluted five-fold, and then 3.5 ul of culture were spotted to their respective synthetic agar plates containing 2% glucose, 2% galactose, or 3% glycerol with 1% ethanol. Agar plates were incubated at 30°C for 3-5 days, and pictures of the plates were taken every 24 hours.

One-step yeast transformation

We used standard PEG/lithium acetate method or One-step yeast transformation protocol to transfer yeast expression constructs (88). Briefly, 1ml of overnight grown yeast culture or freshly grown yeast cells from the agar plate were mixed in the transformation buffer: 80 µl 50% PEG3350, 10 µl 1M DTT, and 10 µl 1M LiAC. Next, 100 ng of plasmid DNA was added to the mixture and gently vortexed to mix. The sample was incubated in a 42°C water bath for one hour. The transformation mixture was vortexed every 15 minutes. Immediately after incubation, cells were spread using sterile glass beads onto their respective synthetic amino acid dropout or antibiotic-containing agar plates for selection. The plates were incubated in a 30°C incubator for 3-4 days. A negative control (without any plasmid DNA) was always included. The absence of colonies from the negative control ensures the efficiency of transformation.
Generating integrated single-copy SNCA yeast model

To identify the enhancers of alpha-synuclein toxicity in yeast we developed a yeast model expressing alpha-synuclein at a level that does not lead to aggregation or toxicity. We cloned the SNCA gene in both pAG303GAL-ccdB and pAG303GAL-ccdB-EYFP vectors. The cloned pRS303-SNCA and pRS303-SNCA-YFP were confirmed by sequencing and were transformed into W303 MATa yeast using a high throughput yeast transformation protocol (89) to generate yeast strains expressing 1xSNCA and 1xSNCA-YFP.

Fluorescence Microscopy

For all the fluorescence microscopy experiments in this study, we followed the same protocol. The yeast strains were pre-cultured in selective media with 2% raffinose or 2% glucose (for strains containing the constitutively active Gal3A368V allele) overnight until the culture reaches the mid to late log phase. The pre-culture was centrifuged at 1717 RCF for five minutes, washed twice with sterile ddH2O, and then resuspended in media containing 2% galactose to induce gene expression from the GAL1 promoter. The cultures were normalized to OD_{600}=1.0 and incubated for six hours in a 30°C shaking incubator. Right before microscopic analysis, one ml of the culture of each sample was taken into a small centrifuge tube and spinned down at 4218 RCF for one minute in a microcentrifuge to pellet cells. Cell pellets were resuspended in 200 µl of sterile ddH2O.
To prepare samples for microscopic analysis, 5 µl of the cell suspension was placed on a clean microscope slide and covered with a coverslip carefully to avoid bubbles. Images were obtained with an Olympus IX83 inverted fluorescent microscope and Olympus DP74 digital camera. Images were taken at 40X or 100x magnification using GFP (39002-AT-EGFP/FITC/Cy2/AlexaFluor 488), YFP (49003-ET-EYFP), CFP (49001-ET-ECFP), TxAED (39010-AT-Texas Red/mCherry/AlexaFluor 594), DsRed (49004-ET-CY3/TRITC), and TxAED-LongPass (19006-AT-Texas Red Longpass) filter cubes (CHROMA).

**Alpha-synuclein aggregation counting**

Fluorescence microscopy was done following the above-mentioned method. All samples were processed and imaged identically, using the same exposure time in the YFP channel. The ImageJ cell counter plugin was used to count cells and alpha-synuclein aggregates. First, without seeing the YFP signal, the total number of cells in a field of view were counted in the brightfield channel. Next, the number of cells in the field with visible alpha-synuclein aggregates were counted by analyzing the YFP channel. Using the following formula, the % cells with alpha-synuclein aggregates was calculated:

\[
\text{% Cells with SNCA aggregates} = \frac{\text{Cells with SNCA aggregates}}{\text{Total number of cells}}
\]
**Human gene plasmid library**

The human gene plasmid library is generated by Shuzen Chen, it is a resource of Quan Zhong and Shulin Ju lab. The library contains ~15,000 full-length human gene ORFs cloned into yeast expression vector pAG416Gal-ccdB. The whole human gene plasmid library was individually aliquoted into wells of costar plates and stored at -80°C.

**Library screen by transformation**

The human gene library was transformed into the yeast strain W303 MATa IntTox SNCA using a high throughput transformation protocol. Transformed yeasts were spotted on glucose and galactose agar plates to score yeast growth. Before spotting the transformed yeast were also saved as glycerol stocks in 96 well plates and stored at -80°C.

**Library screen by mating**

The human gene library was transformed into the yeast strain W303 MATα using a high throughput yeast transformation protocol. Transformed yeasts were saved as glycerol stocks in 96 well plates and stored at -80°C. To do the library screen by mating, this library was grown in YPD media together with the W303 MATα 1xSNCA yeast model. This culture was incubated overnight at 30°C. The culture was inoculated in double dropout selection media selecting for diploid yeast containing both plasmids. After
overnight incubation at 30°C, the culture was spotted on glucose and galactose agar plates to score yeast growth.

**Western Blot Analysis**

Post-alkaline extraction method (90) was used to extract yeast crude protein. Protein was separated using SDS/PAGE for 1.5 hours at 100 V. The gel was transferred to a PVDF membrane (Millipore) at 50V for two hours. After transfer, the membrane was rinsed with water and blocked with 5% nonfat dry milk in TBST for one hour at room temperature with gentle agitation. Next, the membrane was incubated with the primary antibody overnight at 4°C with agitation. The anti-GFP (Abcam), anti-PGK1 (Invitrogen) primary antibodies were used at a dilution of 1:10,000. After incubation, the membrane was washed three times, 10 minutes each in TBST and then incubated with a secondary antibody for one hour at room temperature with gentle agitation. AP-conjugated anti-rabbit and anti-mouse secondary antibodies were used at a dilution of 1:10,000 to target the primary antibodies of anti-GFP and anti-PGK1, respectively. Again, the membrane was washed three times in TBST. Next, one-step NBT/BCIP solution (Thermo Scientific) was used to develop the membrane. The membrane was repeatedly washed with distilled water to stop developing. The membrane was air-dried after developing and image was taken at various time points. Image quantification was performed using ImageJ software (6). Briefly, the background was subtracted from the protein bands and PGK1 bands. Then the ratios of the proteins against the corresponding PGK1 control were determined.
**FM4-64 staining**

The yeast strains were pre-cultured in selective media with 2% raffinose overnight until the culture reaches the mid to late log phase. The pre-culture was centrifuged at 1717 RCF for five minutes, washed twice with sterile ddH₂O, normalized to OD₆₀₀=1.0, and then resuspended in media containing 2% galactose to induce gene expression from the \textit{GALI} promoter. The cultures were incubated for six hours in a 30°C shaking incubator. We used Invitrogen FM4-64 dye to stain the endocytotic vesicles. After six hours of induction, the cells were harvested at 1717 RCF and resuspended in five ml YPD media. 200 µl of samples were separated in a microcentrifuge and kept on ice for the zero-minute control staining. 5 µl of FM4-64 dye (1.5 mM) was added to the rest of the YPD culture and incubated in dark in a 30°C shaking incubator for seven minutes. Then the culture was harvested and again suspended in YPD media for the “chase” experiment and incubated in dark in a 30°C shaking incubator. After 10, 20, 30, 45 and 90-minutes time points 200 µl of samples were taken out of the tube in a microcentrifuge and cells were harvested to observe by microscopy at 100x magnification. For the zero-minute staining cooled cells were stained by 0.2 µl of FM4-64 dye (1.5 mM) and quickly harvested using a 4°C microcentrifuge and washed with pre-cold YPD media and observed by microscopy. Images were taken at 100X magnification using 39010-AT-Texas Red/mCherry/AlexaFluor 594 or 49004-ET-CY3/TRITC filter cubes (CHROMA).
Actin staining

The yeast strains were pre-cultured in selective media with 2% raffinose overnight until the culture reaches the mid to late log phase. The pre-culture was centrifuged at 1717 RCF for five minutes, washed twice with sterile ddH$_2$O, normalized to OD$_{600}$=1.0, and then resuspended in media containing 2% galactose to induce gene expression from the \textit{GAL1} promoter. The cultures were incubated for six hours in a 30°C shaking incubator. The cells were fixed by adding formaldehyde directly to the culture medium to a final concentration of 3.7% and incubated for 10 minutes at room temperature with gentle agitation. Cells were harvested by spinning at 1717 RCF for five minutes and then resuspended in 1000 ml PBS solution containing 3.7% formaldehyde and incubated for one hour at room temperature with gentle agitation. Next, the cells were washed three times with PBS and were sonicated briefly (setting-3, microprobe, 30 seconds) using Fisher Scientific Sonic Dismembrator (Model 100) to disperse clumps. To stain the actin, we used Invitrogen Rhodamine Phalloidin dye. For each 200 µl of samples, 50 µl of dye (0.165 µM) was added and incubated for one hour in dark at room temperature with gentle agitation. Next, the cells were harvested and washed three times with PBS solution. 5 µl of the cell suspension was taken on a slide and a coverslip was added to visualize the cells by microscopy. We used Olympus IX83 inverted fluorescent microscope and Olympus DP74 digital camera. Images were taken at 100X magnification using 39010-AT-Texas Red/ mCherry/ AlexaFluor 594 or 49004-ET-CY3/TRITC filter cubes (CHROMA).
### TABLE 1: List of yeast strains used in this study

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<td>IntTox</td>
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<tr>
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<td>Deletion of △end3 in BY4741</td>
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The plasmids Gic2-PBD-RFP were gifts from Dr. Hay-Oak Park. The NoTox-WT, NoTox-A53T, NoTox-A30P, W303-SL-Control, and IntTox yeast strains were gifts from the laboratory of Dr. Susan Lindquist. The pAG416-GAL1-\textit{YPT1} plasmid construct was generated by Md Moydul Islam.
### TABLE 3: List of primers used in this study

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</table>

Alpha-synuclein, encoded by the SNCA gene, is a small lipid-binding protein primarily expressed in the brain. It is associated with multiple neurodegenerative diseases, including Multiple System atrophy, Parkinson’s Disease, and Dementia with Lewy bodies (16). The SNCA gene was identified as the first major causative gene associated with the early onset of familial PD (70). About ninety percent of PD cases are sporadic, and only ten percent are familial with known genetic causes (16). The pathological hallmark of PD is the presence of Lewy bodies, which are abnormal aggregations of alpha-synuclein. Lewy body pathology is observed in almost all familial and sporadic PD cases (67). Point mutations, duplication or triplication of SNCA gene, and genetic variations that elevate the expression of alpha-synuclein have been linked to familial and sporadic PD (94). Because of such prominent genetic and pathological connections of alpha-synuclein with neurodegenerative diseases, the SNCA gene has been studied extensively since 1993. Although years of research have yielded many important findings about alpha-synuclein, the cellular processes that initiate alpha-synuclein aggregation and toxicity have yet to be clarified.

To understand the factors that trigger alpha-synuclein toxicity, several genetic approaches have been applied. Including yeast, different model organisms have been used to study alpha-synuclein toxicity. The advantage of using a yeast model is the ease in performing genome-wide genetic screens (85).Hundreds of yeast genes have been
identified to modulate alpha-synuclein toxicity when deleted or overexpressed (79,80,83). These modifier genes led to the identification of conserved pathways involving the human homologs of yeast modifier genes.

One of the limitations of this genetic screen is that not all human genes necessarily have yeast homologs. The modifier effect of non-conserved human genes cannot be inferred from yeast modifier genes. Thus, the search for genetic modifiers is incomplete by considering only human genes that have yeast homologs. To address this limitation, we constructed an overexpression library containing approximately 15,000 human cDNA clones, each of which were individually cloned and expressed in yeast. Using this library, we tested whether yeast could help us identify human genetic modifiers of alpha-synuclein toxicity.

Most of the alpha-synuclein models rely on high-dosage overexpression of alpha-synuclein, which induces toxicity (79). This is consistent with PD cases where both high copy-number of SNCA gene and genetic variations increase expression of alpha-synuclein. This model, known as the IntTox strain, is useful in characterizing cellular defects induced by alpha-synuclein and in identifying genetic modifiers that can suppress toxicity. Because of the toxic level of alpha-synuclein expression, this high-dosage model may not be effective in uncovering enhancer genes that are involved in the early stages of initiating alpha-synuclein toxicity.

Instead, a non-toxic, low-dosage model of alpha-synuclein could be used to identify enhancer genes that are either involved in the initiation of alpha-synuclein toxicity or acting upstream of the toxicity. Thus, I generated an alpha-synuclein model
(1xSNCA) that expresses the protein at less than fifty percent of the established alpha-synuclein models. The new 1xSNCA is not toxic by itself, and, interestingly, reveals a polarized localization of alpha-synuclein. Using this new 1xSNCA model, we carried out a pilot genetic screening and identified one strong enhancer gene, intersectin 2 (ITSN2). The enhancer gene ITSN2 does not induce cytoplasmic inclusions of alpha-synuclein, but it increases cell size, suggesting distinct enhancer mechanisms. We next carried out a growth-based genome-wide genetic screening of human enhancer genes using our 1xSNCA model and identified five enhancer genes, including ITSN2. The enhancers did not change the alpha-synuclein expression level and did not necessarily induce the alpha-synuclein inclusions. In summary, we developed a low-dosage alpha-synuclein model, performed a genome-wide genetic screen, and identified human gene enhancers that induce alpha-synuclein toxicity.

**Aim 1A: Development of a new SNCA yeast model to identify human modifier genes that enhance alpha-synuclein toxicity.**

**Aim overview:** To identify enhancer genes that induce toxicity of alpha-synuclein expressed at a low dosage, I generated a yeast model carrying a single integrated copy of the SNCA gene (1xSNCA). This 1xSNCA model does not show any growth defects or exhibit any cytoplasmic inclusions of alpha-synuclein, regardless of whether the protein is tagged. Instead, alpha-synuclein appears to localize to polarized growth sites. I determined the expression level of alpha-synuclein in the 1xSNCA model, which is much lower than those in previously constructed SNCA yeast models. Using this low
expression 1xSNCA model, a pilot screen was carried out to identify human genes that enhance the toxicity of alpha-synuclein. A human enhancer gene that induced alpha-synuclein toxicity in the 1xSNCA yeast model was intersectin 2 (ITSN2). Expression of ITSN2 in 1xSNCA model led to enlarged cells, which was a phenotype not seen in the toxic SNCA model, or the IntTox strain. Furthermore, ITSN2 did not change the localization of alpha-synuclein in the 1xSNCA or IntTox strains. To determine the cellular localization of I TSN2, I tagged the ITSN2 protein with an N- or C-terminal fluorescence tag. The N-terminal tagged I TSN2 localizes to the plasma membrane, while the C-terminal tagged I TSN2 forms cytoplasmic foci. Unfortunately, neither N- nor C-terminal-tagged I TSN2 was able to induce toxicity in the 1xSNCA model, suggesting both N- and C-termini of I TSN2 are required for it to act as an enhancer. In contrast, only the C-terminal tagged I TSN2 enhances alpha-synuclein toxicity in the IntTox model. Despite inducing toxicity, the C-terminal tagged I TSN2 does not co-localize with alpha-synuclein inclusions.

**Results:** To identify more potent enhancer genes of alpha-synuclein, I generated a new SNCA yeast model with a single integrated copy of the SNCA gene. To generate this strain, a plasmid containing ORF of the SNCA gene controlled by a galactose inducible promoter was integrated at the *HIS3* locus in the yeast strain W303 *MATa*. The successful cloning was confirmed by sequencing. At the same time, W303 IH01 was used as a control strain where an empty vector plasmid pAG303GAL1-ccdB was integrated into the W303 *MATa* strain. To test the toxicity level of this new yeast strain, we performed a spotting assay. For spotting assay, all the precultured yeast strains were normalized to
make sure there were approximately similar number of cells for each of the testing strains. Next, yeast cells were serially diluted by five-fold and spotted onto agar plates of \( \text{HIS} \)- media containing galactose or glucose to turn gene expression on or off, respectively. Alpha-synuclein was not toxic in the new model of single copy SNCA integration compared to the IntTox model where 4-5 copies of SNCA gene were integrated (Figure 3.1A). To observe the localization of alpha-synuclein in the new model, another yeast strain was developed, named 1xSNCA-YFP. This strain carries a C-terminal EYFP-tagged single copy SNCA. Previous studies have shown that a C-terminal tagged SNCA yeast model shows similar cellular toxicity and growth defects as the untagged protein model \((79,38)\). This suggests that C-terminal tagged alpha-synuclein has similar biochemical activity as the non-tagged protein. We wanted to compare the localization of alpha-synuclein in the 1xSNCA-YFP to the IntTox model. To test this, the yeast strains were precultured overnight, shifted to media containing galactose, and grown for six hours to induce SNCA expression. Consistent with previous findings, alpha-synuclein in the IntTox model frequently induces cytoplasmic inclusions containing the protein, although there is some plasma membrane localization in some cells (Figure 3.1B). Interestingly, alpha-synuclein in the 1xSNCA-YFP model localizes to the plasma membrane and the polarized growth sites (Figure 3.1B).

The polarized localization of alpha-synuclein was not reported in previous studies of alpha-synuclein using yeast models. This unique cellular localization of alpha-synuclein might be due to the difference in the expression level. To test this, we wanted to confirm the expression level of alpha-synuclein in the 1xSNCA-YFP model. We carried out a western blot assay using antibodies against GFP and PGK1. PGK1 is an
essential gene that encodes a protein acting as a key enzyme in glycolysis and gluconeogenesis (95). The expression level of this protein should remain consistent between samples, so it was used as a loading control. The proteins for this experiment were extracted from IntTox, NoTox, 1xSNCA-YFP, and control strains after six hours of growth in galactose media to induce the gene expression. Although the SNCA in NoTox model carries a GFP tag, the IntTox and 1xSNCA-YFP both have EYFP tags. Since the GFP antibody can recognize both GFP and YFP, we used this antibody to perform the assay. Quantification of the western blot images showed that the expression of alpha-synuclein is 5.7-fold higher in the IntTox and 3.6-fold higher in the NoTox (Figure 3.2) models, compared to that in the 1xSNCA-YFP model.

Because this low-expression 1xSNCA model does not show growth defects or cytoplasmic inclusion of alpha-synuclein, we wanted to use this new model to identify human enhancer genes. I started with a pilot test using a collection of human gene enhancers identified using the IntTox model in our lab. I successfully transformed 349 screening hits of IntTox enhancer genes into the 1xSNCA yeast strain. This collection of yeast strains with the modifier genes were then precultured and spotted onto agar plates containing glucose or galactose. Potential enhancer genes or “hits” were identified as the human genes that showed a growth defect in yeast when co-expressed with SNCA. All the “hits” were then individually verified using serially diluted spotting assays (Figure 3.3). After verification, the human genes that induced toxicity only when co-expressed with SNCA, and not when it was expressed alone, were designated as human enhancer genes of the 1xSNCA model. Only one gene, ITSN2, was verified to show a strong enhancer phenotype in the low-expression 1xSNCA model.
ITSN2 encodes one of two intersectin proteins in humans. Intersectins are highly conserved scaffold protein in animals (96). Interestingly, in a Genome-Wide Association Study (GWAS), the presence of Single Nucleotide Polymorphisms (SNPs) in the ITSN1 gene, the paralog of ITSN2, was found to be associated with an increased risk of Parkinson’s Disease (97). To confirm the ITSN2-induced toxicity in all yeast SNCA models, we transformed ITSN2 in 1xSNCA, 1xSNCA-YFP, and IntTox yeast strains along with isogenic control strain. These yeast strains were serially diluted and spotted onto agar plates containing galactose or glucose to score the toxicity (Figure 3.4). The spotting assay shows that expression of ITSN2 alone is not toxic to yeast. When co-expressed with SNCA, however, yeast exhibit severe growth defects. ITSN2 induces toxicity in the IntTox, 1xSNCA, and 1xSNCA-YFP models. Moreover, the toxicity is more pronounced in the IntTox and 1xSNCA models than in the YFP-tagged 1xSNCA model.

ITSN2 induces toxicity in both the high and low expression models of alpha-synuclein, suggesting that ITSN2 is a potent enhancer of alpha-synuclein toxicity, regardless of SNCA dosage. To test whether ITSN2-induced toxicity leads to any change in the yeast morphology, we examined the toxic strain along with the control strains by microscopy. The 1xSNCA model and the isogenic control strain carrying either a vector control plasmid or ITSN2 expression plasmid were used in this experiment. These strains were cultured in galactose media for six hours to induce the co-expression of SNCA and ITSN2 before examination by microscopy. Strikingly, yeast cells show an enlarged cell size only when ITSN2 is co-expressed with SNCA (Figure 3.5). Such cell size
enlargement was not visible in yeast cells expressing either 1xSNCA or ITSN2 alone, suggesting the cell size enlargement is related to the toxicity.

ITSN2 was initially identified as an enhancer for the IntTox model, which I again confirmed (Figure 3.4). Given the cell enlargement phenotype induced by the ITSN2 in 1xSNCA model, we wanted to test whether this phenotype is also present when ITSN2 is expressed in the IntTox model. This was tested in the IntTox model and its isogenic control strain. Both strains were transformed with a vector control plasmid and ITSN2. These four strains were cultured in galactose media for six hours to induce the expression of SNCA and ITSN2 before microscopy. The observation of live yeast cells showed that no cell size enlargement was visible in any of the yeast cells, regardless of whether SNCA and ITSN2 were expressed alone or together (Figure 3.6). This suggests that the enhancer mechanism of ITSN2 might be different between the low-dosage and high-dosage SNCA models.

Next, we wanted to see if ITSN2 induces any changes in the subcellular localization of alpha-synuclein in 1xSNCA-YFP and IntTox models. We transformed the 1xSNCA-YFP and IntTox yeast strains with either a vector control plasmid or ITSN2 expression plasmid. These yeast strains were then precultured and grown in galactose media for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed by microscopy using the YFP filter to visualize alpha-synuclein (Figure 3.7). We observed that ITSN2 does not change the localization of alpha-synuclein in the 1xSNCA-YFP or IntTox yeast models. Alpha-synuclein is localized to the plasma membrane in 1xSNCA-YFP regardless of ITSN2 expression. We did not see a
pronounced cell size enlargement in the 1xSNCA-YFP model. This is consistent with my observation that ITSN2-induced toxicity of this 1xSNCA-YFP model was moderate as seen in Figure 3.4. The IntTox model had a mixed population of yeast cells, where some showed plasma membrane localization of alpha-synuclein, and others showed cytoplasmic inclusions regardless of ITSN2 expression. The plasma membrane-localized alpha-synuclein observed in the 1xSNCA model with ITSN2 expression suggests that cytoplasmic inclusion of alpha-synuclein is not a requirement for cellular toxicity.

ITSN2 induces toxicity in SNCA models but does not change the localization of alpha-synuclein. Thus, we wanted to determine the localization of ITSN2 in yeast. To visualize ITSN2 in yeast, we generated two clones of ITSN2 tagged with ECFP. The tag on the N-terminus for one and on the C-terminus for the other. Next, we transformed the control yeast strain W303 IH01 with these two tagged versions of ITSN2. The yeast strains were cultured in galactose media for six hours to induce the expression of ITSN2. Next, the live yeast cells were observed by microscopy using CFP filters (Figure 3.8). Interestingly, the cellular localization of ITSN2 in yeast is altered by the N- or C-terminal fluorescent tags. The N-terminal ECFP-tagged ITSN2 localized to the plasma membrane, whereas the C-terminal ECFP-tagged ITSN2 localized to the cytoplasm, showing a punctate structure. The effect of the fluorescent tag in changing cellular localization of ITSN2 suggests that altering localization of ITSN2 might also alter its function or effect on toxicity.

The change in the cellular localization of ITSN2 by N- or C-terminal fluorescent tags led us to test whether the tagged ITSN2 has the same function in yeast as the non-
tagged ITSN2. To test if the tagged ITSN2 can also induce the alpha-synuclein toxicity in the SNCA models, I introduced the N-terminal ECFP-tagged ITSN2 into the IntTox, 1xSNCA-YFP, and 1xSNCA strains. These yeast strains were also transformed with an ECFP-tagged empty vector plasmid. Next, these yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose. The growth of the yeast cells after three days showed that ECFP-ITSN2 does not induce toxicity of alpha-synuclein in the IntTox, 1xSNCA-YFP, or 1xSNCA models (Figure 3.9). This suggests that the N-terminal part of the ITSN2 protein is required for inducing alpha-synuclein toxicity.

The N-terminal ECFP-tagged ITSN2 localizes to the plasma membrane in the control strain but does not induce alpha-synuclein toxicity in any of the SNCA models. Thus, we wanted to check if the N-terminal ECFP-tagged ITSN2 also shows a plasma membrane localization in the 1xSNCA model and whether it co-localizes with alpha-synuclein. 1xSNCA-YFP strain transformed with ECFP-ITSN2 were grown in galactose media for six hours to induce the expression of SNCA and ITSN2. The live yeast cells were observed in the CFP or YFP filters to visualize ITSN2 and alpha-synuclein, respectively (Figure 3.10). We observed that ECFP-ITSN2 localized to the plasma membrane and co-localized with alpha-synuclein in the 1xSNCA-YFP model. Here, the cell size of the 1xSNCA-YFP model was not enlarged, which is consistent with the finding that ECFP-ITSN2 does not induce toxicity in SNCA models. Next, we questioned whether the C-terminal ECFP-tagged ITSN2 is able to induce toxicity of alpha-synuclein in SNCA models. To test this, we transformed the 1x
SNCA, 1xSNCA-YFP, IntTox, and isogenic control yeast strains with a C-terminal ECFP-tagged ITSN2 and a control vector plasmid. These yeast strains were precultured in raffinose media before serially diluted spotting onto agar plates containing galactose or glucose (Figure 3.11). ITSN2-ECFP induces the toxicity of alpha-synuclein in the IntTox model but not in the 1xSNCA models. This suggests that the C-terminal of ITSN2 is important for inducing alpha-synuclein toxicity in the 1xSNCA model but not in the IntTox model. Overall, these findings suggest distinct mechanisms underlying the toxicity of alpha-synuclein in these two models.

The C-terminal ECFP-tagged ITSN2 shows a cytoplasmic localization with small punctate structures in the control strain. It does not induce alpha-synuclein toxicity in 1xSNCA models but induces toxicity in the IntTox model. So, we wanted to determine the localization of ITSN2-ECFP in the 1xSNCA-YFP and IntTox models and whether it co-localizes with alpha-synuclein inclusions. 1xSNCA-YFP and IntTox strain transformed with ECFP-ITSN2 were precultured and shifted to galactose media for six hours to induce the expression of SNCA and ITSN2. Next, live yeast cells were observed by microscopy in the CFP or YFP filters to visualize ITSN2 and alpha-synuclein, respectively (Figure 3.12). ITSN2-ECFP is localized throughout the cytoplasm as small punctate structures. The ITSN2-ECFP does not co-localize with the plasma membrane localized alpha-synuclein of the 1xSNCA-YFP model (Figure 3.12). Interestingly, ITSN2-ECFP also does not co-localize with the cytoplasmic inclusions of alpha-synuclein in the IntTox model either. This data suggests that ITSN2-ECFP does not induce toxicity via direct interaction with alpha-synuclein in the IntTox model.
**Aim1B: Human gene enhancers of alpha-synuclein toxicity.**

**Aim overview:** The successful identification of a human gene enhancer using 1xSNCA model indicates that it is possible to identify other potent human enhancers. To this end, we carried out a growth-based genome-scale genetic screening of human genes enhancing toxicity of alpha-synuclein. To perform this screening, we used our overexpression library containing approximately 15,000 human cDNA clones. The library screen was based on a mating strategy where genetic material of a yeast from the library was transferred to the 1xSNCA model by mating. After screening the library, we identified and verified five human genes that induce alpha-synuclein toxicity in the 1xSNCA model. These enhancers do not necessarily induce the formation of alpha-synuclein inclusions in the 1xSNCA-YFP model. To understand the enhancer mechanism, I carried out a western blot assay and found that the enhancer genes do not increase the expression levels of alpha-synuclein. This suggests the enhancers do not induce toxicity in the 1xSNCA model by altering the alpha-synuclein protein levels.

**Results:** ITSN2 is the only enhancer gene that was identified from my pilot screen of human genetic enhancers. ITSN2 enhances alpha-synuclein toxicity in the 1xSNCA, 1xSNCA-YFP, and IntTox models. However, the ITSN2-induced changes in cell morphology appear to be different between the IntTox and 1xSNCA models. These findings support our hypothesis that the low-expression 1xSNCA model is useful in identifying unique enhancers with distinct enhancer mechanism. These findings
motivated us to carry out a genome-scale genetic screen using the 1xSNCA model to identify more potent enhancers.

To do a genome-wide genetic screen, we used our plasmid library containing 14,827 human ORFs cloned into the pAG416GAL1-ccdB vector. Instead of transforming all the clones one by one, we introduced the human genes into our 1xSNCA model by yeast mating. Here, we took advantage of the collection of haploid MATα control strains already transformed with the human-genome library. This collection of yeast strains was mated with the MATa haploid 1xSNCA yeast strain (Figure 3.13). Next, this diploid collection of yeast strains was precultured in media containing glucose and then spotted onto agar plates containing glucose or galactose using a robotic spotting machine. The growth of these yeast strains was followed to identify those human gene clones that enhance the toxicity of alpha-synuclein in the 1xSNCA yeast strain. Images of the agar plates were taken on days 2 and 3 of spotting using the “Phenobooth” colony counter (98). Next, we analyzed the photos using the Phenobooth analyzer to identify the colonies that were showing a growth defect compared to the yeast strain expressing SNCA alone with an empty vector plasmid. The strongest primary enhancer “hits” were then individually verified using serial-diluted spotting assays.

After completion of the growth-based genome-scale genetic screening using our 1xSNCA model, we analyzed the growth of the colonies. After analysis of the growth of the yeast cells six of the “hits” were scored as strong enhancers. I wanted to verify these “hits” to confirm their enhancer phenotype in the 1xSNCA model. I first revived the glycerol stocks of the haploid MATα control strains transformed with those particular
human-genes and a vector control plasmid. Next, the haploid $MATa$ 1x SNCA yeast strains were mated with those yeast strains. The diploid yeast cells were selected using drop-out media, precultured in glucose media, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 3.14). The USO1, BORG4, CEGT, PI51A, and ITSN2 genes were identified as enhancers, but the CCNE1 gene was not able to enhance the toxicity in the 1xSNCA model. To confirm that these human genes are not toxic by themselves in yeast, I also mated the haploid $MATa$ control strains harboring the human genes with an isogenic control strain W303 IH01. Independent identification of ITSN2 as an enhancer again strengthens our previous finding. ITSN2 does not induce a similar level of toxicity in this diploid 1xSNCA model compared to the haploid model (Figure 3.4), suggesting that the induced toxicity of alpha-synuclein might be affected by ploidy in yeast.

The four other genes identified as the enhancer of the 1xSNCA model share some interesting features. The USO1 gene, also known as “General vesicular transport factor p115” is a transport protein. This protein recycles between the cytosol and the Golgi apparatus. It interacts with a set of COPII vesicle-associated SNAREs to form a cis-SNARE complex (99). USO1 is also associated with a neurological disease called “Autosomal Dominant Non-Syndromic Intellectual Disability 6” (100). BORG4 is also known as “CDC42 Effector Protein 4”. The BORG4 protein interacts with Rho family GTPases and is shown to bind both CDC42 and TC10 GTPases. It is suggested that it has a role in actin filament assembly and cell shape control (101). The enhancer gene CEGT is Ceramide Glucosyltransferase. This is an enzyme that plays an important role in the biosynthesis of glycosphingolipids. Glycosphingolipids are part of the cell membrane.
containing lipid and sugar. These are essential components of membrane microdomains that are responsible for membrane trafficking and signal transduction (102). CEGT is also associated with two neurological diseases “Gaucher Disease Type I” and “Sandhoff Disease” (103). The PI51A or Phosphatidylinositol-4-Phosphate 5-Kinase Type 1 Alpha gene is involved in a variety of cellular processes, including actin cytoskeleton organization, cell adhesion, cell migration, and phagocytosis (104).

The five verified enhancer genes of the 1xSNCA model were also verified in the 1xSNCA-YFP model. In the 1xSNCA-YFP model, the enhancer genes showed a similar pattern of toxicity. Previously we have tested that ITSN2 induces cell size enlargement and does not induce alpha-synuclein inclusions in the 1xSNCA-YFP model. We wanted to find out if the four other enhancers show similar alpha-synuclein localization or cell size enlargement. To test this, we performed an overnight culture of diploid 1xSNCA-YFP yeast carrying either a vector control plasmid or human enhancer genes. Then we shifted the yeast cultures to galactose media and cultured for six hours to induce the expression of SNCA. We examined live yeast cells by microscopy in the YFP filter to visualize alpha-synuclein. I found that there were two categories of enhancers showing two different localizations of alpha-synuclein (Figure 3.15). The ITSN2, BORG4, and CEGT enhancers induced toxicity without affecting the plasma membrane localization of alpha-synuclein. But the USO1 and PI51A enhancers induced the formation of cytoplasmic inclusions of alpha-synuclein in some cells. This observation suggests that there are distinct enhancer mechanisms underlying the induced toxicity of alpha-synuclein. It further supports that alpha-synuclein inclusion formation is not necessary to
induce toxicity, and these enhancers likely act on the early event of alpha-synuclein toxicity.

The enhancers identified by the genome-wide screen had various levels of toxicity. When these enhancers were expressed in the 1xSNCA-YFP model, two of them led to cytoplasmic inclusions of alpha-synuclein while three of them did not change alpha-synuclein localization. To determine whether the change in localization of alpha-synuclein was due to a difference in expression level, I performed a western blot analysis on alpha-synuclein expression in the enhancer-induced toxic strains. I tested the diploid 1xSNCA-YFP strains harboring a vector control plasmid, USO1 expression plasmid, or ITSN2 expression plasmid. I picked two enhancers of two different categories. ITSN2 does not change the plasma membrane localization of alpha-synuclein. USO1 induces cytoplasmic inclusions of alpha-synuclein: USO1. Protein was extracted from the yeast strains precultured overnight and shifted to media containing galactose and grown for six hours. The western blot was performed using an antibody against GFP and the loading control protein PGK1 (Figure 3.16). The signal intensity of the alpha-synuclein protein bands was normalized to the intensity of the PGK1 loading control. There is no increase of the alpha-synuclein protein in the 1xSNCA-YFP model when co-expressing ITSN2 and USO1. This result suggests that their enhancer effect does not involve changes in alpha-synuclein expression. In addition, the difference in their effects on alpha-synuclein cellular localization is independent from expression levels. Instead, different enhancer mechanism may be involved in the difference in toxic effects.
Figure 3.1. The yeast models with a single integrated copy of the SNCA gene (1xSNCA) do not show growth defects or contain cytoplasmic inclusions of alpha-synuclein. **A)** The haploid 1x SNCA, 1xSNCA-YFP, and IntTox yeast strains along with their isogenic control strain (W303 IH1), precultured in raffinose media, were serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C (top panel). **B)** The haploid 1xSNCA-YFP and IntTox yeast strains were precultured in raffinose media for overnight and shifted to media containing 2% galactose at OD_{600} 1.0 and grown for six hours to induce the expression of SNCA-EYFP. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein (bottom panel). Scale Bar = 10 μm.
Figure 3.2. Expression of alpha-synuclein in the 1xSNCA model is lower than that in previously constructed SNCA yeast models. Alpha-synuclein expression in the haploid IntTox, NoTox, and 1xSNCA-YFP was analyzed by western blot. Protein was extracted from the yeast strains precultured in raffinose media for overnight and shifted to media containing 2% galactose at OD_{600} 1.0 and grown for six hours. Western blot analysis was performed using antibodies against GFP to target SNCA-YFP and against the loading control protein, PGK1. Quantification was performed using ImageJ software. The signal intensity of the alpha-synuclein protein bands was normalized to the intensity of PGK1 bands. The values are normalized to the ratio of SNCA/PGK1 in 1xSNCA yeast.
Figure 3.3. A pilot screen of human genes that enhance toxicity of alpha-synuclein in the 1xSNCA model. The 585 enhancer and suppressor human gene clones identified in the IntTox yeast model were transformed into the MAT a haploid 1xSNCA yeast strain (STEP 1). This collection of yeast strains was precultured in media containing raffinose and then spotted onto agar plates containing glucose (gene off) or galactose (gene on). The growth of the yeast strains was followed to identify those human gene clones that enhance the toxicity of alpha-synuclein in the haploid 1xSNCA yeast strain. The red squares indicate examples of a candidate enhancer (STEP 2). All enhancer “hits” were then individually verified using the serial dilution and spotting assay (STEP 3).
Figure 3.4. ITSN2 induces toxicity of alpha-synuclein in yeast. The haploid 1x SNCA, 1xSNCA-YFP, and IntTox yeast strains along with their isogenic control strain (W303 IH1) were transformed with the pAG416GAL1-ITSN2 expression plasmid and the control vector plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after three days of growth at 30°C.
Figure 3.5. Co-expression of ITSN2 and alpha-synuclein in the 1xSNCA model leads to enlarged cells. The haploid 1x SNCA yeast and isogenic control strains bearing either the vector control plasmid pAG416GAL1-ccdB or the pAG416GAL1-ITSN2 expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in brightfield. Scale Bar = 10 μm.
Figure 3.6. Expression of ITSN2 in the IntTox model does not alter cell shape. The haploid IntTox yeast bearing either the vector control plasmid pAG416GAL1-ccdB or the pAG416GAL1-ITSN2 expression plasmid were precultured in raffinose media. Following overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the bright field. Scale Bar = 10 μm.
Figure 3.7. ITSN2 does not change the localization of alpha-synuclein in the 1xSNCA-YFP or IntTox yeast models. The haploid 1xSNCA-YFP and IntTox yeast bearing either the vector control plasmid pAG416GAL1-ccdB or the pAG416GAL1-ITSN2 expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD_{600} 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 μm.
Figure 3.8. Cellular localization of ITSN2 is altered by N- or C-terminal fluorescent tag. Control strain (W303 IH1) transformed with pAG416GAL1-ITSN2-ECFP and pAG416GAL1-ECFP-ITSN2 expression plasmids were precultured in raffinose media. After overnight growth, they were shifted to media containing 2\% galactose at OD\textsubscript{600} 1.0 and grown for six hours to induce the expression of ITSN2. Live yeast cells were observed at 100X magnification in the bright field and CFP filter to visualize ITSN2. Scale Bar = 10 \mu m.
Figure 3.9. N-terminal ECFP-tagged ITSN2 does not induce toxicity of alpha-synuclein. The haploid 1x SNCA, 1xSNCA-YFP, and IntTox yeast strains along with isogenic control strain (W303 IH1) were transformed with the pAG416GAL1-ECFP-ITSN2 expression plasmid and the control vector plasmid pAG416GAL1-ECFP-ccdB. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after three days of growth at 30°C.
Figure 3.10. N-terminal ECFP-tagged ITSN2 co-localizes with alpha-synuclein in the **1xSNCA-YFP model**. The haploid 1xSNCA-YFP yeast strain transformed with pAG416GAL1/ECFP-ITSN2 expression plasmid was precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the bright field, and CFP or YFP filters to visualize ITSN2 and alpha-synuclein respectively. Scale Bar = 10 µm.
Figure 3.11. C-terminal ECFP-tagged ITSN2 induces toxicity of alpha-synuclein in the IntTox model but not in the 1xSNCA models. The haploid 1x SNCA, 1xSNCA-YFP, and IntTox yeast strains along with their isogenic control strain (W303 IH1) were transformed with the pAG416\textit{GAL1-ITSN2-ECFP} expression plasmid and the control vector plasmid pAG414\textit{GAL1-ccdB-ECFP}. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after three days of growth at 30°C.
Figure 3.12. C-terminal tagged ITSN2 does not co-localize with alpha-synuclein. The haploid 1xSNCA-YFP and IntTox yeast strains transformed with the pAG416GAL1-ITSN2-ECFP expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the brightfield. CFP and YFP filters were used to visualize ITSN2 and alpha-synuclein, respectively. For better contrast, the CFP and YFP signals were shown as red and gray, respectively, in the merged image using ImageJ. Scale Bar = 10 μm.
Figure 3.13. Fitness based genome-scale genetic screening of human genes

enhancing toxicity of alpha-synuclein. The plasmid library containing 14,827 human ORFs cloned in the pAG416GALI-ccdB vector transformed into the haploid MATα control strains were mated with the MATa haploid 1xSNCA yeast strain (STEP 1). This collection of yeast strains was precultured in media containing glucose and then spotted onto agar plates containing glucose (gene off) or galactose (gene on). The growth of the yeast strains was followed to identify those human gene clones that enhance the toxicity of alpha-synuclein in the 1xSNCA yeast strain. The red square indicates examples of candidate enhancer (STEP 2). All enhancer “hits” were then individually verified using the serial dilution and spotting assay (STEP 3).
**Figure 3.14. Human genes induce toxicity of alpha-synuclein in the 1xSNCA model.**

The haploid 1x SNCA yeast strain was mated with its isogenic control strain (W303 IH1) bearing the pAG416GAL1-ccdB control vector. Diploid yeast cells were precultured in selective drop-out glucose media before serially diluted spotting onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C. (*not verified as an enhancer*)
Figure 3.15. Enhancers do not necessarily induce the formation of alpha-synuclein inclusions in the 1xSNCA-YFP model. Diploid 1xSNCA-YFP yeast bearing either a vector control plasmid or human enhancer genes under the pAG416GAL1-ccdB vector were precultured in glucose media for overnight and shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 µm.
Figure 3.16. Enhancer genes do not increase the expression levels of alpha-synuclein in the 1xSNCA model. Alpha-synuclein expression was analyzed by western blot. The strains tested were in the diploid 1xSNCA-YFP yeast. These strains included yeast with only a vector control plasmid, pAG416GAL1-USO1, or pAG416GAL1-ITSN2. Protein was extracted from the yeast strains precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at an OD$_{600}$ of 1.0 and grown for six hours. A western blot was performed using antibodies against GFP to target SNCA-YFP and against the loading control protein, PGK1. Quantification was performed using ImageJ software. Quantification shown below the membrane is the signal intensity of the alpha-synuclein protein bands normalized to the intensity of PGK1 bands. The values are normalized to the ratio of SNCA/PKG1 in yeast without enhancer.
Aim 2: Characterization of ITSN2 induced alpha-synuclein toxicity.

We developed a low-dosage alpha-synuclein yeast model and did a genome-wide genetic screening using human genes. We identified five enhancer genes that can induce alpha-synuclein toxicity in the 1xSNCA model. ITSN2 is one of the human genes that was identified in the pilot screen and in the complete library screen. It was verified as a strong enhancer of the 1xSNCA model. ITSN2 induces alpha-synuclein toxicity across different SNCA yeast models. These models include the 1xSNCA, NoTox, and IntTox yeast models, which express low, intermediate, and high levels of alpha-synuclein, respectively. Our goal was to understand the mechanism of ITSN2 induced alpha-synuclein toxicity in SNCA models.

Intersectins (ITSN) are a family of highly conserved scaffold protein in animals (96). In mammals, there are two types of intersectins known as ITSN1 and ITSN2. Both of these proteins are structurally very similar as both share similar domains and are found in two major isoforms - long and short. Interestingly, ITSN1 long isoform is highly expressed in the nervous system while the short isoform is expressed ubiquitously (105). They are involved in the Clathrin-mediated endocytosis process. It is thought to regulate the formation of clathrin-coated vesicles (106). Overexpression of intersectins leads to endocytic defects in multiple cell types (107). Interestingly, in a Genome-Wide Association Study (GWAS), the presence of SNPs in the ITSN1 gene was found to be associated with increased risk of Parkinson’s Disease (97).
Structurally, human intersectins have two Eps15-homology (EH) domains, an extended KLERQ coiled-coil domain, five Src-homology 3 (SH3) domains, and C-terminal DH domains. The DH domain of ITSN contains a Guanine nucleotide exchange factor (GEF) present in the long isoform of ITSN2. This domain is responsible for the activation of Cdc42, which is a small GTPase that regulates several pathways including endocytosis and actin polymerization (106). The short isoform of ITSN2 is truncated in the C-terminus and does not have the DH domain (108). The ITSN2 gene has three homologs in yeast named Sla1, Ede1, and Pan1. All three of the homologous proteins localize in the polarized growth site of the plasma membrane (109), and are involved in clathrin-mediated endocytosis process (110).

The C terminal domain of the ITSN2 activates Cdc42. Activated Cdc42 and ITSN2 interact with WASL and other WASP proteins and regulates the actin-related protein-2/3 (ARP2/3) complex, resulting in actin filament nucleation and initiation of actin polymerization (108). WASL or WASP Like Actin Nucleation Promoting Factor is a protein highly expressed in neural tissues. It is a member of the Wiskott-Aldrich syndrome (WAS) protein family (107).

We noticed that co-expression of ITSN2 and alpha-synuclein in the nontoxic SNCA model induces enlarged cell size. Enlarged cell size could be induced by a defect in vesicle turnover or disruption of the actin cytoskeleton (111). The role of ITSN2 in endocytosis and as a GEF factor of Cdc42 lead us to examine endocytosis and actin network in the SNCA models expressing ITSN2.
To understand the function of ITSN2 in endocytosis, I used a classic FM4-64 assay to monitor endocytosis in the SNCA models showing ITSN2 induced toxicity. To examine the actin cytoskeleton structure, I used Rhodamine Phalloidin dye. I used two mutant SNCA yeast strains, showing two different localizations of alpha-synuclein to test if membrane association of alpha-synuclein is required for ITSN2 to induce toxicity. By overexpressing and deleting yeast homologs of ITSN2, I examined whether ITSN2 played the similar role of its homolog in yeast. In addition, I generated an ITSN2 isoform lacking the C-terminus (ITSN2\textsuperscript{short}) to test the GEF factor-mediated function of ITSN2.

We found that the enhancer effect of ITSN2 requires membrane-localized alpha-synuclein; it can only induce toxicity in the membrane-bound SNCA yeast strains. It was evident by the FM4-64 assays that the expression of ITSN2 together with alpha-synuclein drastically delayed the endocytosis process in the 1xSNCA and NoTox models. Consistent with endocytosis delay effects, we also found abnormal actin structures in the 1xSNCA model expressing ITSN2. The frequently observed actin patches observed in this strain are the characteristic feature of immobile endocytosis sites. This abundance of depolarized actin patches is an indicator of a delay of endocytosis at an early stage. The deletion of \textit{Sla1} or the yeast homolog of ITSN2, further enhanced the toxicity of alpha-synuclein induced by ITSN2. This indicates that the effect of ITSN2 potentially is interfering with normal yeast endocytosis function, and this effect must be in collaboration with alpha-synuclein. Also, the expression of the short isoform of ITSN2 (ITSN2\textsuperscript{short}), which lacks its C-terminal domains required for activating Cdc42, failed to induce toxicity in 1xSNCA and NoTox models. Consistent with the lack of an enhancer phenotype, ITSN2\textsuperscript{short} does not lead to defective endocytosis in the 1xSNCA model.
Furthermore, the C-terminal of ITSN2 is also required to disrupt the polarized localization of activated Cdc42. Mis-regulation of the Cdc42 protein may contribute to the growth defect observed in the ITSN2 induced toxicity.

The enhancer effect of ITSN2 is different in the IntTox model as compared to that in the 1xSNCA or NoTox model. The expression of ITSN2\textsuperscript{short} or ITSN2-ECFP could enhance alpha-synuclein toxicity in the IntTox model. Interestingly, ITSN2-ECFP expression did not exacerbate the endocytosis defect of alpha-synuclein, suggesting that the enhancer effect of ITSN2 might involve different mechanisms in the non-toxic and toxic SNCA models.

**Aim 2A: Co-expression of ITSN2 and SNCA delays endocytosis in yeast**

**Aim overview:** In this sub-aim, we focus on examining the effect of ITSN2 and SNCA on endocytosis. Consistent with our previous findings, ITSN2 induces toxicity of alpha-synuclein in the 1x-SNCA model, co-expression of ITSN2 and SNCA in the NoTox model leads to enlarged cells and growth defects. Similarly, it does not induce alpha-synuclein inclusions in the NoTox model, as seen in the 1xSNCA model. Using two PD-associated mutant forms of the SNCA gene, we discovered that ITSN2 can only induce toxicity when alpha-synuclein is membrane-localized and not cytosolic. I monitored the endocytosis process using the FM4-64 method and found that the expression of ITSN2 leads to defective endocytosis in the 1xSNCA and NoTox yeast models. To better understand the function of ITSN2 in yeast, I tested whether ITSN2 can complement the function of its homolog, Sla1. Surprisingly, ITSN2 does not complement the growth
defect of the $\Delta sla1$ mutant. Instead, ITSN2 enhances the growth defects of the $\Delta sla1$ mutant presumably by enhancing its endocytosis defects. Furthermore, deletion of $SLA1$ enhances the toxicity of alpha-synuclein induced by ITSN2. In contrast, deletion of $END3$, which encodes a protein functioning together with Sla1, does not induce toxicity of alpha-synuclein, suggesting a specific functional relationship between alpha-synuclein with ITSN2 or Sla1. Furthermore, overexpression of $EDE1$, another homolog of ITSN2 involved in regulating the initiation of endocytosis in yeast, did not alter ITSN2-induced toxicity of alpha-synuclein. This suggests that the enhancer effect of ITSN2 likely does not involve defects in the initiation step of endocytosis. Consistent with this finding, we observed internalization of FM4-64 and the formation of actin patches corresponding to yeast endocytic sites in the 1xSNCA and NoTox models expressing ITSN2. Interestingly, ITSN2 induces depolarized actin patches in the 1xSNCA model, consistent with perturbation of polarized endocytosis in yeast.

**Results:** The enhancer gene ITSN2 induces toxicity of alpha-synuclein in 1x SNCA, 1xSNCA-YFP, and IntTox yeast models. The 1xSNCA strains are low expression models, and IntTox is a high expression model, as mentioned previously. We wanted to check if ITSN2 could induce toxicity in a nontoxic but intermediate expression model, such as the NoTox yeast strain. The NoTox model does not show a toxic phenotype, but the alpha-synuclein expression is higher than the 1xSNCA model (Figure 3.2B). To test if ITSN2 could induce toxicity in NoTox model, I carried out a spotting assay. The haploid NoTox yeast strain along with its isogenic control strain (W303 SL-Control) were
transformed with an ITSN2 expression plasmid and a control vector plasmid. The transformed yeasts were precultured then serially diluted and spotted onto agar plates containing galactose or glucose. The results show that ITSN2 can induce alpha-synuclein toxicity in the NoTox model (Figure 4.1). This suggests that ITSN2 can induce alpha-synuclein toxicity regardless of alpha-synuclein expression level.

We found that ITSN2-induced toxicity in 1xSNCA model was accompanied with cell size enlargement (Figure 3.5). But ITSN2-induced toxicity does not increase the cell size in the high expression IntTox model (Figure 3.6). So, we wanted to test whether ITSN2 induced toxicity can alter the cell size in the NoTox model. To test this, I precultured the NoTox yeast strain along with its isogenic control strain (W303 SL-Control) transformed with ITSN2 and a control vector plasmid. These yeast strains were shifted to galactose media and grown for six hours to induce the expression of SNCA and ITSN2. Then live yeast cells were observed by microscopy. I found that the expression of ITSN2 in the NoTox model leads to enlarged cells (Figure 4.2). This cell size enlargement is not observed when SNCA and ITSN2 are expressed separately. This suggests that the enhancer mechanism of ITSN2 for the NoTox and the 1xSNCA might be similar.

Next, I wanted to find out if the expression of ITSN2 in the NoTox model could change the plasma membrane localization of alpha-synuclein. The NoTox model carries C-terminal GFP-tagged alpha-synuclein, which was visualized by microscopy. I precultured the NoTox yeast strain and the isogenic control strain (W303 SL-Control) transformed with ITSN2 and a control vector plasmid. These yeast strains were shifted to
galactose media and grown for six hours to induce the expression of SNCA and ITSN2. Next, live yeast cells were observed by microscopy using the GFP filter to visualize alpha-synuclein. Similar as observed in the 1xSNCA model, expression of ITSN2 does not induce alpha-synuclein inclusions in the NoTox model either (Figure 4.3). This finding also suggests that the enhancer mechanism of ITSN2 for the NoTox and the 1xSNCA might be similar.

As seen in Figure 4.3, alpha-synuclein exclusively localizes to the plasma membrane. We also have two other NoTox models that carry two different PD-linked mutant forms of the SNCA gene: SNCA-A53T and SNCA-A30P (38). These two models exhibit distinct localizations of alpha-synuclein in yeast. The A53T mutation in SNCA leads to the plasma-membrane localization of alpha-synuclein, similar to the wild-type. However, the SNCA with A30P mutation is completely cytosolic and does not localize to the plasma membrane. We thought to take advantage of these two PD-linked mutations to ask the question of whether the membrane localization of alpha-synuclein is required to induce toxicity by ITSN2. To test this, the NoTox yeast models containing SNCA-WT, SNCA-A53T, and SNCA-A30P along with the isogenic control strain (W303 SL-Control), were transformed with ITSN2 expression plasmid and a control vector plasmid. Next, these yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose. ITSN2 induces alpha-synuclein toxicity in strains expressing the wild-type or the A53T mutant, but does not induce any growth defect in the SNCA-A30P strain (Figure 4.4). This result indicates that ITSN2 requires membrane-localized alpha-synuclein to induce toxicity.
Next, we wanted to determine the localization of alpha-synuclein in the SNCA-A30P strain with or without ITSN2 expression. We tested the NoTox SNCA-WT, and SNCA-A30P strains bearing either a vector control plasmid or ITSN2 expression plasmids mentioned in Figure 4.4. These strains were precultured overnight and shifted to galactose for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed by microscopy. We found that the ITSN2-induced toxic strain NoTox SNCA-WT shows plasma membrane localization of alpha-synuclein. However, the NoTox SNCA-A30P strain, which does not induce ITSN2-mediated toxicity, shows cytosolic localization of alpha-synuclein (Figure 4.5). This suggests that ITSN2 requires membrane-localized alpha-synuclein to induce toxicity, and it cannot induce alpha-synuclein toxicity when it is localized to the cytoplasm.

These findings are consistent with known functions of ITSN2. ITSN2 is involved in the clathrin-mediated endocytosis process. It is thought to regulate the formation of clathrin-coated vesicles (110). It has also been reported that overexpression of ITSN2 leads to endocytic defects in multiple cell types (107). So, we wanted to check if overexpression of ITSN2 would lead to defective endocytosis in the yeast model or not. We transformed the haploid 1xSNCA yeast strain and the control strain (W303 IH1) with ITSN2 expression plasmid and a vector control plasmid. We carried out an FM4-64 assay to assess the defects in endocytosis in these strains. FM4-64 is a lipophilic dye that is widely used to visualize the dynamics of endocytosis pathways. To carry out this assay, the yeast cells were precultured and shifted to galactose media for six hours to induce the expression of SNCA and ITSN2. Next, the live yeast cells were incubated with FM4-64 dye to monitor endocytosis. Following the chasing experiment as described in the
methods section, we viewed the cells by microscopy in the dsRed filter (Figure 4.6). There is, indeed, a defect in endocytosis only when ITSN2 is co-expressed with SNCA. Specifically, the internalization of the dye within the endocytosis vesicle is hampered, and small “dots” of dye appear close to the plasma membrane (as shown by the arrow in Figure 4.6 at 45 minutes). When SNCA is expressed alone, however, there is no defect; the internalization of the vesicle is similar to that of the control strain W303 IH1, where the dye is fully internalized in most cells and reaches the vacuolar membrane within the 20 to 45 minutes time point. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes were counted from randomly selected fields of view for all four strains. We found that the fractions of cells that show fully internalized FM4-64 is significantly lower in the yeast strains co-expressing ITSN2 and SNCA (Table 4). However, ITSN2 when expressed alone also show endocytosis defect to some extent. This result suggests that ITSN2 might induces alpha-synuclein toxicity in 1xSNCA strain via the endocytosis pathway.

This led to the question of whether the NoTox model also exhibits endocytosis defects when ITSN2 is co-expressed. To test this question, I transformed the haploid NoTox yeast strain with ITSN2 expression plasmid. The control (W303 SL-Control) strain transformed with a vector control plasmid was used as a control. We carried out a similar FM4-64 assay to assess the defects in endocytosis in these strains. These yeast cells were precultured and shifted to galactose media for six hours to induce the expression of SNCA and ITSN2. Next, the live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis. The cells were viewed by microscopy in the TxRed filter (for FM4-64) and GFP (for SNCA). A drastic delay of endocytosis was observed
when ITSN2 is co-expressed with SNCA as compared to the control strain (Figure 4.7). Here cells from randomly selected fields showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes were counted. The fraction of cells that show fully internalized FM4-64 is lower in the yeast strain co-expressing ITSN2 and SNCA (Table 5). This finding suggests that ITSN2 induces alpha-synuclein toxicity in NoTox strain via the endocytosis pathway. Thus, the enhancer mechanisms of ITSN2 may be similar in the NoTox and the 1xSNCA models.

The ITSN2 gene has three homologs in yeast named SLA1, EDE1, and PAN1. These homologs localize in the polarized budding site of the plasma membrane (109). All three of the homologs are involved in clathrin-mediated endocytosis (110). END3 is another yeast gene that is also involved in endocytosis. The End3 protein forms a complex with two of the ITSN2 homolog proteins Sla1p and Pan1p (112). Deletion of the homologs of ITSN2 induces endocytosis defects and also shows a growth defect in the yeast (113). To understand whether ITSN2 can complement the function of its homologs in yeast, I performed a spotting assay. I acquired the yeast deletion strains of \( \Delta sla1 \), \( \Delta end3 \), and \( \Delta ede1 \). PAN1 is an essential gene in yeast and we do not have the \( \Delta pan1 \) deletion strain. Since the deletion collection of yeast is in the BY4741 background, I used a wild type (WT) BY4741 strain as a control for this experiment. We wanted to test whether ITSN2 can rescue \( \Delta sla1 \) and \( \Delta ede1 \) strains from the deletion-induced toxicity. To test that, \( \Delta sla1 \), \( \Delta end3 \), \( \Delta ede1 \), and WT yeast strains were transformed with ITSN2 expression plasmid and a vector control plasmid. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.8). ITSN2 does not rescue growth defects of the \( \Delta sla1 \) and \( \Delta ede1 \) mutants. Interestingly,
ITSN2 enhances the growth defect of the $\Delta sla1$ mutant but not the $\Delta end3$ or $\Delta ede1$ mutants. This result indicates that overexpression of ITSN2 may disrupt Sla1-related endocytic steps in yeast.

Next, I tested whether ITSN2 enhances endocytosis defects in the $\Delta sla1$ mutant. I carried out an FM4-64 assay to assess the defects in endocytosis in the haploid $\Delta sla1$ yeast strain transformed with the ITSN2 expression plasmid and a vector control. The yeast cells were precultured and shifted to galactose media for six hours to induce the expression of SNCA and ITSN2. Next, the live yeast cells were incubated with lipophilic dye FM4-64 to monitor the endocytosis. The cells were viewed by microscopy in the TxRed filter. ITSN2 expression exacerbates endocytosis defects observed in the $\Delta sla1$ mutant (Figure 4.9). Cells from randomly selected fields showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes were counted. I found that the fractions of cells that show fully internalized FM4-64 is lower in the $\Delta sla1$ mutant yeast strain expressing ITSN2 (Table 6). This suggests that ITSN2 enhances the cellular defects of the $\Delta sla1$ mutant by enhancing the endocytosis defects.

Considering that overexpression of ITSN2 may disrupt Sla1-related endocytic step, we tested whether the deletion of $SLA1$ would exacerbate the toxicity of alpha-synuclein. To test this, I integrated single copy SNCA into the $\Delta sla1$ and WT yeast strains. Next, I transformed these strains with ITSN2 expression plasmid and a vector control plasmid. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.10). Interestingly, deletion of $SLA1$
enhances the toxicity of alpha-synuclein by itself and in combination with ITSN2 (last row of Figure 4.10).

In contrast, deletion of END3, which encodes a protein that directly interact with Pan1 and Sla1, does not alter toxicity of alpha-synuclein. I integrated a single copy SNCA gene into the haploid Δend3 yeast strain and WT yeast strains. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.11). Deletion of END3 does not induce toxicity of alpha-synuclein. This suggests that alpha-synuclein toxicity is specific to ITSN2 or SLA1.

The two deletion mutants, Δsla1 and Δede1, disrupt two distinct steps of endocytosis, the late-coat and early-coat assembly, respectively. ITSN2 enhances the growth defect of the Δsla1 deletion mutant but not the Δede1 mutant (Figure 4.8). We next tested if overexpression of EDE1 affects ITSN2 induced toxicity of alpha-synuclein. I transformed the haploid 1xSNCA yeast strain and the control strain (W303 IH1) already bearing ITSN2 expression plasmid and a vector control plasmid with EDE1 expression plasmid and a vector control plasmid. Then these yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.12). Overexpression of EDE1 does not alter ITSN2 induced toxicity of alpha-synuclein. It is worth mentioning that I failed to generate the SNCA integrated Δede1 strain to test if deletion of EDE1 induces toxic phenotype. Overall, our findings support a model in which endocytic defects induced by deletion of SLA1 or overexpression of ITSN2 enhances toxicity of alpha-synuclein. Such defects likely do not occur at the initiation step, involving Ede2.
Using the FM4-64 assay, we observed that co-expression of ITSN2 and SNCA delays endocytosis in yeast. Because regulated assembly of actin networks is a critical part of endocytosis, we tested if co-expression of ITSN2 and SNCA in yeast could lead to abnormal actin assembly. I transformed the haploid 1xSNCA yeast strain and control strain (W303 IH1) with ITSN2 expression plasmid or a vector control plasmid. The yeast cells were precultured and shifted to galactose media for six hours to induce the expression of SNCA and ITSN2. Next, the cells were fixed with formaldehyde and stained with Rhodamine Phalloidine dye. Rhodamine Phalloidine is a fluorescent dye that is used to stain the actin filaments and patches. To visualize the actin structures, the stained cells were mounted on a slide and observed by microscopy in the dsRed filter (Figure 4.13). We found that only the yeast strain expressing both ITSN2 and SNCA together were showing depolarized actin patches all over the cell membrane. In contrast, the yeast strains expressing either SNCA or ITSN2 show normal polarized localization of the actin patches similar to the control W303 IH1 strain. This result shows that ITSN2 can induce depolarized actin structures in the 1xSNCA model. Depolarized actin patches are consistent with mis-regulation of endocytosis, further supporting a model in which ITSN2 induces toxicity in the 1xSNCA model by disrupting normal endocytosis.

Aim 2B: The enhancer effect of ITSN2 in the 1xSNCA model depends on its C-terminal domains.

Aim overview: In this sub-aim, we focus on studying the role of the Cdc42 GEF domains in the C-terminal of ITSN2 in its enhancer effect. In Aim1 (Chapter 3), we found that the
C-terminal domain of ITSN2 is likely important for its enhancer effect, as the C-terminal tagged ITSN2 fails to induce alpha-synuclein toxicity in the 1xSNCA model. Consistent with this finding, we observed that ITSN2\textsuperscript{short} failed to induce toxicity of alpha-synuclein in the 1xSNCA or NoTox models. Furthermore, the expression of ITSN2 did not lead to defective endocytosis in the 1xSNCA model. Suggesting the important role of the C-terminal domain of ITSN2 in the induction of alpha-synuclein toxicity. Interestingly, the expression of ITSN2\textsuperscript{short} induces abnormal actin clumps. It suggests that regions other than the C-terminal can also affect actin in an adverse way. Given the distinct actin abnormality, ITSN2\textsuperscript{short} is more likely interfering with the directionality of actin polymerization instead of a hold up of endocytosis at the plasma membrane. One of the key regulators of actin assembly and cell polarity, Cdc42 was found to be affected in the ITSN2 induced toxicity of alpha-synuclein. The activated Cdc42 is depolarized in yeast co-expressing SNCA and ITSN2. This could explain the growth defect observed in the ITSN2 induced toxicity of the 1xSNCA model. However, ITSN1 expression does not lead to any growth defect or depolarized actin structures in the 1xSNCA yeast model. It also showed polarized activated Cdc42, suggesting that the growth defect, depolarized actin, and depolarized Cdc42 are all related to the ITSN2-induced alpha-synuclein toxicity. And this toxicity is exerted through the C-terminal domain of ITSN2 by mis-regulation of Cdc42. We also found that expression of another human gene from the same pathway as ITSN2, WASL, further enhance ITSN2-induced toxicity in the 1xSNCA yeast model. These findings suggest that WASL and ITSN2 act in the same pathway to induce alpha-synuclein toxicity. Contrary to the absence of enhancer effect in the 1xSNCA models, the expression of both ITSN1 and ITSN2\textsuperscript{short} leads to growth
defects in the IntTox yeast model. Furthermore, expression of ITSN1 or ITSN2 (both long and short forms) does not change the cell size in this toxic model. These findings resemble our previous observation on ITSN2-ECFP, which induces toxicity only in the IntTox model but not in the 1xSNCA models. Finally, I found that endocytosis in the IntTox model was not exacerbated by ITSN2-ECFP expression. This suggests that the enhancer mechanisms are different in the IntTox model as compared to the 1xSNCA models.

Results: As mentioned earlier, there are two major isoforms of ITSN2—long and short. The DH domain of ITSN2 that contains the GEF is found in the long isoform of ITSN2. This domain is responsible for the activation of Cdc42. The short isoform of ITSN2 is truncated in the C-terminus and does not have the DH domain (108). To test whether the endocytosis defect induced by ITSN2 in the 1xSNCA model is via its activation of Cdc42, we generated the short isoform of ITSN2, ITSN2\textsuperscript{short}. The ITSN2\textsuperscript{short} was generated using specific primers designed to amplify the short form of the ITSN2 using the long-form ITSN2 as a template. Next, I transformed the 1xSNCA yeast strain and the control strain (W303 IH1) with ITSN2\textsuperscript{short} and a vector control plasmid. Next, these yeast strains along with the previously transformed 1xSNCA and control strains with full-length ITSN2 were cultured to perform a spotting assay. The yeast strains were serially diluted and spotted onto agar plates containing galactose or glucose. ITSN2\textsuperscript{short} does not enhance alpha-synuclein toxicity in the 1xSNCA model (Figure 4.14). This finding suggests that the C terminal of ITSN2 is critical to induce alpha-synuclein toxicity.
Next, we wanted to find out if the short form ITSN2 loses its capability to induce alpha-synuclein toxicity in the NoTox model. I transformed the NoTox yeast strain and the control strain (W303 SL-Control) with ITSN2\textsuperscript{short} in an expression plasmid and a vector control plasmid. These yeast strains, along with the previously transformed NoTox and control strains with full-length ITSN2, were cultured to perform a spotting assay. The yeast strains were serially diluted and spotted onto agar plates containing galactose or glucose. ITSN2\textsuperscript{short} does not enhance alpha-synuclein toxicity in the NoTox model (Figure 4.15). This result again suggests that the C terminal of ITSN2 is critical to induce alpha-synuclein toxicity.

These findings made us question whether the short ITSN2 can induce endocytosis defects in SNCA models. To test this, we carried out an FM4-64 assay to examine endocytosis in these strains. I transformed the haploid 1xSNCA yeast strain and the control strain (W303 IH1) with ITSN2\textsuperscript{short} to perform this assay. Here, the control strain (W303 IH1) transformed with an empty vector plasmid was used as control. These yeast cells were precultured and shifted to galactose media for six hours to induce the expression of SNCA and ITSN2\textsuperscript{short}. Next, live yeast cells were incubated with the dye FM4-64 to monitor the endocytosis. Following the chasing experiment as mentioned in the methods section, I viewed the cells by microscopy in the dsRed filter. Expression of ITSN2\textsuperscript{short} does not lead to defective endocytosis in the 1xSNCA model (Figure 4.16). However, the ITSN2\textsuperscript{short} expression by itself led to a slight defect in endocytosis in the yeast. Cells from randomly selected fields showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes were counted. The expression of ITSN2\textsuperscript{short} does not lead to any significant defect in endocytosis in the 1xSNCA model.
(Table 7). However, like ITSN2, ITSN2\textsuperscript{short} also by itself delay endocytosis to some extent. This suggests ITSN2 and its short form both, by themselves, delay endocytosis but, in combination with SNCA, ITSN2 but not ITSN2\textsuperscript{short} further exacerbate the defects in endocytosis.

Next, we wanted to confirm if short ITSN2 leads to similar depolarized actin patches in the 1xSNCA model as seen in ITSN2 induced toxicity in Figure 4.13. To test this, I carried out an actin staining assay using the Rhodamine Phalloidine. I used the same strains mentioned in Figure 4.16 to perform this assay. The yeast cells were precultured, shifted to galactose media, and grown for six hours to induce the expression of SNCA and ITSN2\textsuperscript{short}. Then the cells were fixed with formaldehyde and stained with Rhodamine Phalloidine dye. The stained cells were mounted on a slide and observed by microscopy in the dsRED filter (Figure 4.17). Expression of ITSN2\textsuperscript{short} in 1xSNCA strain as well as in the control strain does not lead to depolarized actin patches; the cells are polarized and patches are located to the bud site. However, the ITSN2\textsuperscript{short} expression does induce some abnormal actin clumps in 1xSNCA strain as well as in the control strain. This suggests that although ITSN2\textsuperscript{short} can cause actin dysregulation in yeast although it does not induce toxicity in 1xSNCA or in the control.

ITSN2\textsuperscript{short} does induce alpha-synuclein toxicity, endocytosis defect, and depolarization of actin in the 1xSNCA model. This suggests that the C-terminus of ITSN2 is responsible for inducing alpha-synuclein toxicity since the DH domain of ITSN2 is located in the C terminal which carries GEF that regulates the activation of Cdc42. We wish to test if ITSN and ITSN2\textsuperscript{short} affect activation of Cdc42 or localization
of the activated form Cdc42 at the polarized growth sites. To test this, I integrated Gic2-PBD-RFP to the haploid 1xSNCA and control (W303 IH1) strains. Gic2-PBD-RFP plasmid allows us to visualize the activated form of the Cdc42 protein. The integrated yeast strains were again transformed with ITSN2 and ITSN2<sup>short</sup> expression plasmid or a vector control plasmid. These strains were precultured overnight and shifted to galactose for six hours to induce the expression of SNCA and ITSN2. The live yeast cells were viewed by microscopy in the dsRED filter to visualize activated Cdc42 (Figure 4.18). We observed that the activated Cdc42 is depolarized in yeast expressing SNCA and ITSN2. However, this depolarization of activated Cdc42 was not observed in yeast cells expressing SNCA and ITSN2<sup>short</sup>, nor in strains expressing ITSN2 alone. In fact, the 1xSNCA strain was exhibiting more polarized Cdc42 compared to the control (Figure 4.18B). This result suggests that the C terminal of ITSN2 is responsible for the depolarization of activated Cdc42 and thus inducing alpha-synuclein toxicity in the 1xSNCA model.

In mammals, there are two types of intersectins known as ITSN1 and ITSN2. Both of these proteins are structurally very similar as both share similar domains and are found in two major isoforms - long and short. Interestingly, ITSN1 long isoform is highly expressed in the nervous system and ITSN1 short isoform is expressed ubiquitously. Both ITSN1 and 2 play a role in endocytosis, and overexpression of ITSN1 or 2 leads to endocytic defects. To understand if these two proteins function similarly in yeast, we wanted to see if ITSN1 can induce alpha-synuclein toxicity in the 1xSNCA model. To test this, I transformed the haploid control strain (W303 IH1) and 1xSNCA yeast strain with ITSN2 and ITSN1 expression plasmid and vector control plasmid. Next, these yeast
strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.19). The results of the spotting assay show that ITSN1 expression does not lead to any growth defect in the 1xSNCA yeast model. This result suggests that ITSN1 might function differently than ITSN2 in yeast.

Next, we wanted to ask if the expression of ITSN1 could induce depolarized actin structures in the 1xSNCA model. To test this, I carried out an actin staining experiment using the same yeast strains mentioned in Figure 4.19. The yeast strains were precultured, shifted to galactose media, and grown for six hours to induce the expression of SNCA and ITSN1. Next, the cells were fixed with formaldehyde and stained with Rhodamine Phalloidine dye. The stained cells were mounted on a slide to observe by microscopy in the dsRED filter (Figure 4.20). Expression of ITSN1 in 1xSNCA strain as well as in the control strain does not lead to any significant depolarized actin structures; the cells are polarized and patches are located to the bud site. However, some cells were larger in size compared to the control yeast strain. This result suggests that ITSN1 might function differently than ITSN2 because it does not induce depolarized actin structures in yeast.

Activated Cdc42 is depolarized in yeast exhibiting ITSN2-induced toxicity of alpha-synuclein. Thus, we hypothesized that activated Cdc42 should be polarized in the 1xSNCA model expressing ITSN1 that does not induce alpha-synuclein toxicity. To test this, I used the same 1xSNCA strain integrated with Gic2-PBD-RFP from Figure 4.18. Next, the yeast strain was transformed with ITSN1 and ITSN2 expression plasmid. These strains were precultured overnight, shifted to galactose, and grown for six hours to induce the expression of SNCA and ITSN1 and ITSN2. The live yeast cells were viewed by
microscopy in the dsRED filter to visualize activated Cdc42 (Figure 4.21). We observed
that the activated Cdc42 is polarized in yeast expressing SNCA and ITSN1, whereas cells
expressing SNCA and ITSN2 show completely depolarized Cdc42. This result is
consistent with the idea that ITSN1 does not induce alpha-synuclein toxicity due to its
inability to depolarize Cdc42. This further support our speculation that the alpha-
synuclein toxicity induced by ITSN2 is through depolarization of Cdc42, which is
mediated by its C terminal domain.

We know that the C terminal domain of the ITSN2 activates the Cdc42. Activated
Cdc42 and ITSN2 interact with WASL and other WASP proteins; they also regulate the
actin-related protein-2/3 (ARP2/3) complex, resulting in the actin filament nucleation and
initiation of actin polymerization (108). WASL, or WASP-Like Actin Nucleation
Promoting Factor, is a protein highly expressed in neural tissues. It is a member of the
Wiskott-Aldrich syndrome (WAS) protein family (107). It is evident that WASL acts
downstream of ITSN2 in the cytoskeletal organization. So, we wanted to check if WASL
can also induce alpha-synuclein toxicity in the SNCA model. I individually crossed the
haploid control (W303 IH1 MATa) and 1xSNCA-YFP yeast strains with the W303 MATα
strain transformed with the pAG416GAL1-WASL expression plasmid or the
pAG416GAL1-ccdB vector control plasmid. I carried out a spotting assay to assess the
growth. Yeast strains were precultured, serially diluted, and spotted onto agar plates
containing galactose or glucose (Figure 4.22). WASL does not induce toxicity of alpha-
synuclein in the diploid 1xSNCA models.
Next, we wanted to determine whether WASL expression can induce alpha-synuclein inclusions or change cell shape. To test this, I cultured the same strains mentioned in Figure 4.22 to observe them by microscopy. These strains were precultured overnight, shifted to galactose, and grown for six hours to induce the expression of SNCA and WASL. The cells were observed in the YFP filter to visualize alpha-synuclein (Figure 4.23). WASL expression does not change the cell shape in the diploid 1xSNCA model. Also, no alpha-synuclein inclusions were observed in the 1xSNCA model when WASL was expressed.

Next, we wanted to ask if WASL expression together with ITSN2 will be able to induce toxicity in the yeast. To test this, I used the haploid W303 IH1 and 1xSNCA yeast strains transformed with ITSN2 expression plasmid or vector control plasmid. These strains were then transformed with WASL expression plasmid or vector control plasmid. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.24). Co-expression of WASL and ITSN2 does not induce toxicity in the control strain. However, WASL further enhances the ITSN2 induced alpha-synuclein toxicity in the 1xSNCA yeast model.

We hypothesize that the ITSN2 enhancer mechanism in the IntTox model is different in the 1xSNCA and NoTox models. ITSN2 induces alpha-synuclein toxicity in the 1xSNCA and NoTox models. Both of these models show enlarged cell shape and defect in endocytosis upon ITSN2 expression. Neither of these models shows any growth defect when short ITSN2 is expressed. Therefore, ITSN2-induced alpha-synuclein toxicity acts on a similar pathway in the nontoxic SNCA models. ITSN2 also induces
toxicity of alpha-synuclein in the high expression IntTox model. But a different enhancer mechanism may be involved, because no changes in cell size was observed, when ITSN2 is expressed in the IntTox strain. Furthermore, CFP- tagged ITSN2 failed to induce toxicity in the 1xSNCA model and NoTox models, but it clearly has a strong enhancer phenotype in the IntTox model. Finally, ITSN1 failed to induce alpha-synuclein toxicity or actin depolarization in the 1xSNCA model. It is possible that ITSN1 and ITSN2 function differently in yeast. But ITSN1 might still act as an enhancer in the IntTox model. To test these, I carried out a spotting assay. I transformed the haploid control strain (W303 IH1) and IntTox yeast strain with ITSN2, ITSN2\textsuperscript{short}, and ITSN1 expression plasmid and a vector control plasmid. Next, these yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 4.25). ITSN1 and ITSN2\textsuperscript{short}, which failed to induce toxicity in the nontoxic SNCA models, induce growth defects in the IntTox yeast model. These results suggest that ITSN2 enhancer mechanism is different in toxic and nontoxic SNCA models.

Next, we wanted to test if the ITSN1 and ITSN2\textsuperscript{short}-induced alpha-synuclein toxicity could change the cell shape in the IntTox yeast model. I used the IntTox strain transformed with ITSN2\textsuperscript{short}, ITSN1, and a vector control plasmid (as described for Figure 4.25) to carry out a microscopy experiment. These yeast strains were precultured, shifted to galactose media, and grown for six hours to induce the expression of SNCA and both intersectins. Live yeast cells were observed by microscopy in the YFP filter to visualize alpha-synuclein (Figure 4.26A). The images show that the expression of ITSN1 or ITSN2\textsuperscript{short} does not change cell shape in the IntTox model. In addition, when the cells with alpha-synuclein inclusions were counted, we found that there was no significant
difference in the percent of cells with alpha-synuclein inclusions among these three strains (Figure 4.26B). Therefore, ITSN1 or ITSN2\textsuperscript{short} -induced alpha-synuclein toxicity in the IntTox model likely involves different mechanisms.

It has been previously reported that overexpression of alpha-synuclein in yeast leads to an endocytosis defect in the IntTox model. To confirm that result, I carried out an FM4-64 assay with the haploid IntTox yeast strain and control (W303 IH1) strains to assess the defect in the endocytosis process. These yeast cells were precultured, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Next, the live yeast cells were incubated with FM4-64 dye to monitor the endocytosis. Following the chasing experiment, I viewed the cells by microscopy in the TxRED filter. Alpha-synuclein expression leads to defective endocytosis in the IntTox model (Figure 4.27). We found that the endocytosis vesicles failed to reach the vacuole. The vesicles mislocalize and are sequestered into the cytoplasmic inclusions of alpha-synuclein, whereas the control yeast strains show normal internalization of the endocytosis vesicles within 20 minutes of chasing. Cells from randomly selected fields showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes were counted. I found that the expression of alpha-synuclein in the IntTox model leads to a defect in endocytosis (Table 8). This suggests that the IntTox model is more prone to toxicity because of its underlying endocytosis defects.

We reported in Aim 1 (Chapter 3) that ITSN2-ECFP failed to induce growth defects in the 1xSNCA and NoTox models. Interestingly, ITSN2-ECFP enhances the growth defect in the IntTox model since the IntTox model by itself shows a defect in
endocytosis. We thought to find out whether ITSN2-ECFP expression would exacerbate the endocytosis defect in the IntTox yeast model and thus enhance the toxicity. To test this, I transformed the haploid IntTox yeast strain with ITSN2-ECFP or vector control plasmid and carried out an FM4-64 assay. These yeast cells were precultured, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Next, the live yeast cells were incubated with FM4-64 dye to monitor the endocytosis. Following the chasing experiment, I viewed the cells by microscopy in the TxRED filter. ITSN2-ECFP expression does not exacerbate the defective endocytosis in the IntTox yeast model (Figure 4.28). The cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar were also counted from randomly selected fields. I found that the expression of ITSN2-ECFP in the IntTox model does not exacerbate the underlying endocytosis defects (Table 9). This suggests that the ITSN2 enhancer mechanism is different in the IntTox model compared to the 1xSNCA model.

To find out whether the toxicity is due to an increase in alpha-synuclein inclusions, I analyzed the images from Figure 4.28. The number of cells carrying alpha-synuclein inclusion were counted at the zero-minute time point to get the fraction of cells with alpha-synuclein inclusions (Figure 4.29). ITSN2-ECFP expression does not lead to a significant change in alpha-synuclein inclusions. This suggests that ITSN2 can induce alpha-synuclein toxicity in the IntTox model without altering alpha-synuclein inclusions.
Figure 4.1. ITSN2 induces the toxicity of alpha-synuclein in the NoTox model. The haploid NoTox yeast strain along with its isogenic control strain (W303 SL-Control, Table 01) were transformed with the pAG414GAL1-ITSN2 expression plasmid and the pAG414GAL1-ccdB control vector plasmid. These yeast strains were precultured in raffinose media, and then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after three days of growth at 30°C.
Figure 4.2. Co-expression of ITSN2 and SNCA in the NoTox model leads to enlarged cells. The haploid NoTox yeast and isogenic control yeast strain (W303 SL-Control) bearing either a vector control plasmid pAG414GAL1-ccdB or pAG414GAL1-ITSN2 expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the bright field. Scale Bar = 10 µm.
Figure 4.3. Expression of ITSN2 in the NoTox model does not induce alpha-synuclein inclusions. The haploid NoTox yeast bearing either a vector control plasmid pAG414GAL1-ccdB or pAG414GAL1-ITSN2 expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the bright field and GFP filter to visualize alpha-synuclein. Scale Bar = 10 μm.
Figure 4.4. ITSN2 requires membrane-localized alpha-synuclein to induce toxicity.

The control haploid strain (W303 SL-Control, Table 01) and haploid NoTox yeast models, which contain SNCA-WT, SNCA-A53T and SNCA-A30P, were transformed with the pAG414GAL1-ITSN2 expression plasmid and the pAG414GAL1-ccdB vector control plasmid. These yeast strains were precultured in raffinose media, and then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.5. The enhancer effect of ITSN2 is specific to membrane-associated alpha-synuclein. The haploid NoTox yeast models, SNCA-WT and SNCA-A30P bearing either a vector control plasmid pAG414GAL1-ccdB or pAG414GAL1-ITSN2 expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and ITSN2. Live yeast cells were observed at 100X magnification in the bright field. Scale Bar = 10 µm.
Figure 4.6. Expression of ITSN2 leads to defective endocytosis in the 1xSNCA yeast model. The haploid 1xSNCA yeast strain and the control strain (W303 IH1) were transformed with the pAG416GALI-ITSN2 expression plasmid and the pAG416GALI-ccdB control plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and/or ITSN2. Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and they were viewed at the indicated time points at 100x magnification in the dsRED (for FM4-64) filter. Endocytosis vesicles stranded at the plasma membrane are marked with white arrows on the image. Scale Bar = 10 μm.
Table 4. Expression of ITSN2 leads to defective endocytosis in the 1xSNCA yeast model

The haploid 1xSNCA yeast strain and its isogenic control strain (W303 IH1), transformed with p416Gal-ITSN2 or the p416Gal-ccdB vector were stained with FM 4-64 and chased at 30 °C. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes after 45 minutes of chase were counted from randomly selected field of views of a single experiment (n=1). The fractions of cells that show fully internalized FM4-64 dye and their corresponding calculated 95% Confidence Interval for proportion are shown.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of cells counted</th>
<th>Fraction of cells with fully internalized FM4-64 to vacuolar membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (W303 IH1)</td>
<td>160</td>
<td>0.45±0.08</td>
</tr>
<tr>
<td>1xSNCA</td>
<td>97</td>
<td>0.51±0.10</td>
</tr>
<tr>
<td>ITSN2</td>
<td>77</td>
<td>0.28±0.10</td>
</tr>
<tr>
<td>1xSNCA+ITSN2</td>
<td>184</td>
<td>0.04±0.03</td>
</tr>
</tbody>
</table>
Figure 4.7. Expression of ITSN2 leads to defective endocytosis in the NoTox model.

The haploid NoTox yeast strain and the control strain (W303 SL-Control) were transformed with the pAG414GAL1-ITSN2 expression plasmid and the pAG414GAL1-
ccdB control plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and/or ITSN2. Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and viewed at the indicated time points at 100x magnification in the TxRED (for FM4-64) and GFP (for SNCA) filters. Endocytosis vesicles stranded at the plasma membrane is marked with white arrows on the image. Scale Bar = 10 μm.
Table 5. Expression of ITSN2 leads to defective endocytosis in the NoTox model.

The haploid NoTox yeast strain and its isogenic control strain (W303 SL-Control), transformed with p414Gal-ITSN2 and p414Gal-ccdB vector respectively were stained with FM 4-64 and chased at 30 °C. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes after 45 minutes of chase were counted from randomly selected field of views of a single experiment (n=1). The fractions of cells that show fully internalized internalized FM4-64 dye and their corresponding calculated 95% Confidence Interval for proportion are shown.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of cells counted</th>
<th>Fraction of cells with fully internalized FM4-64 to vacuolar membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sl-Control</td>
<td>162</td>
<td>0.85±0.05</td>
</tr>
<tr>
<td>NoTox SNCA+ITSN2</td>
<td>132</td>
<td>0.05±0.04</td>
</tr>
</tbody>
</table>
Figure 4.8. ITSN2 enhances the growth defect of the Δsla1 mutant. The Δsla1, Δend3, Δede1 and WT yeast strains (BY4741) were transformed with the pAG416GAL1-ITSN2 expression plasmid and the pAG416GAL1-ccdB vector control plasmid. These yeast strains were precultured in glucose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.9. ITSN2 enhances endocytosis defect in the Δsla1 mutant. The haploid Δsla1 yeast strain was transformed with the pAG416GAL1-ITSN2 expression plasmid and the pAG416GAL1-ccdB vector control. These yeast cells were precultured in glucose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of ITSN2. Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and viewed at the indicated time points at 100x magnification in the TxRED (for FM4-64) filters. Scale Bar = 10 μm.
Table 6. ITSN2 enhances endocytosis defect in the \( \Delta sla1 \) mutant.

The haploid \( \Delta sla1 \) yeast strain, transformed with p416Gal-ITSN2 or the p416Gal-ccdB vector were stained with FM 4-64 and chased at 30 °C. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes after 45 minutes of chase were counted from randomly selected field of views of a single experiment (n=1). The fractions of cells that show fully internalized FM4-64 dye and their corresponding calculated 95% Confidence Interval for proportion are shown.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of cells counted</th>
<th>Fraction of cells with fully internalized FM4-64 to vacuolar membranes</th>
</tr>
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<tbody>
<tr>
<td>( \Delta sla1 )</td>
<td>112</td>
<td>0.75±0.08</td>
</tr>
<tr>
<td>( \Delta sla1 )+ITSN2</td>
<td>120</td>
<td>0.47±0.09</td>
</tr>
</tbody>
</table>
Figure 4.10. Deletion of SLA1 further enhances the toxicity of alpha-synuclein induced by ITSN2. The pAG416GAL1-ITSN2 expression plasmid and the pAG416GAL1-ccdB vector control plasmid were transferred into haploid Δsla1 and WT yeast strains (BY4741) already transformed with the pAG303GAL1-1xSNCA expression plasmid and the pAG303GAL1-ccdB vector plasmid. These yeast strains were precultured in glucose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.11. Deletion of END3 does not induce toxicity of alpha-synuclein. The haploid ∆end3 yeast strain and WT yeast strains (BY4741) were transformed with the pAG303GAL1-1xSNCA plasmid. These yeast strains were precultured in glucose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.12. Overexpression of *EDE1* does not alter *ITSN2* induced toxicity of *alpha-synuclein*. The haploid 1xSNCA yeast strain and the control strain (W303 IH1) transformed with the pAG414*GAL1*-*ITSN2* expression plasmid or the pAG414*GAL1*-ccdB control plasmid were transformed with the pAG416*GAL1*-*EDE1* or the p416*GAL1*-ccdB vector control plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.13. ITSN2 induces depolarized actin structures in the 1xSNCA model. The haploid control strain (W303 IH1) and 1xSNCA yeast strains were transformed with either the pAG416GAL1-ITSN2 expression plasmid or a vector control. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Then cells were fixed with 3.7% formaldehyde and stained with Rhodamine Phalloidine to visualize actin in the dsRED filter at 100x magnification. Scale Bar = 10 μm.
Figure 4.14. **ITSN2<sub>short</sub> does not induce the toxicity of alpha-synuclein in the 1xSNCA model.** The haploid control strain (W303 IH1) and 1xSNCA yeast strain were transformed with the pAG416GALI-ITSN2, pAG416GALI-ITSN2<sub>short</sub> (short isoform of ITSN2) expression plasmid and the pAG416GALI-ccdB vector control plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
<table>
<thead>
<tr>
<th>NoTox</th>
<th>Gene ON Galactose</th>
<th>Gene OFF Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>ITSN2</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Vector</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>ITSN2</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>ITSN2&lt;sup&gt;short&lt;/sup&gt;</td>
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<tr>
<td>+</td>
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</tr>
<tr>
<td>-</td>
<td>ITSN2&lt;sup&gt;short&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.15. *ITSN2<sup>short</sup> does not induce the toxicity of alpha-synuclein in the NoTox model.* The haploid NoTox yeast strain and the control strain (W303 SL-Control) were transformed with the pAG414GAL1-ITSN2, pAG414GAL1-ITSN2<sup>short</sup> (short isoform of ITSN2) expression plasmid and the pAG414GAL1-ccdB vector control plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.16. Expression of ITSN2\textsuperscript{short} does not lead to defective endocytosis in the 1xSNCA model. The haploid 1xSNCA yeast strain and the control strain (W303 IH1) were transformed with the pAG416GAL1-ITSN2\textsuperscript{short} expression plasmid and the
p416GALI-ccdB control plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD\(_{600}\) 1.0 and grown for six hours to induce the expression of SNCA and/or ITSN2. Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and viewed at the indicated time points at 100x magnification in the dsRED (for FM4-64) filter. Scale Bar = 10 μm.
Table 7. Expression of ITSN2\textsuperscript{short} does not lead to defective endocytosis in the 1xSNCA model.

The haploid 1xSNCA yeast strain and its isogenic control strain (W303 IH1), transformed with p416Gal-ITSN2\textsuperscript{short} or the p416Gal-ccdB vector were stained with FM 4-64 and chased at 30 °C. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes after 45 minutes of chase were counted from randomly selected field of views of a single experiment (n=1). The fractions of cells that show fully internalized FM4-64 dye and their corresponding calculated 95% Confidence Interval for proportion are shown.

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</tr>
</thead>
<tbody>
<tr>
<td>Control (W303 IH1)</td>
<td>114</td>
<td>0.75±0.08</td>
</tr>
<tr>
<td>ITSN2\textsuperscript{short}</td>
<td>275</td>
<td>0.41±0.06</td>
</tr>
<tr>
<td>1xSNCA+ ITSN2\textsuperscript{short}</td>
<td>102</td>
<td>0.47±0.10</td>
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</tbody>
</table>
Figure 4.17. Expression of ITSN2\textsuperscript{short} induces abnormal actin clumps. Actin structures of the isogenic haploid W303 IH1 and 1xSNCA yeast strains transformed with the p416GAL1-ITSN2\textsuperscript{short} expression plasmid and the pAG416GAL1-ccdB vector control plasmid were analyzed using fluorescent microscopy. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD\textsubscript{600} 1.0 and grown for six hours to induce the expression of SNCA. Then cells were fixed with 3.7% formaldehyde and stained with Rhodamine Phalloidin to visualize actin in the dsRED filter at 100x magnification. Abnormal actin structure is marked with a white arrow on the image. Scale Bar = 10 \( \mu \text{m} \).
Figure 4.18. Activated Cdc42 is depolarized in yeast exhibiting ITSN2 induced toxicity of alpha-synuclein. Isogenic haploid 1xSNCA and control (W303 IH1) strains with integrated Gic2-PBD-RFP transformed with the pAG416GAL1-ITSN2, and the p416GAL1-ITSN2<sub>short</sub> expression plasmid or the pAG416GAL1-ccdB vector control plasmid were analyzed by fluorescent microscopy to visualize activated Cdc42 protein. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD<sub>600</sub> 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were viewed under microscope at 100x magnification in the dsRED (for Cdc2) filter. Scale Bar = 10 µm
Figure 4.19. ITSN1 expression does not cause growth defect in the 1xSNCA model.

The haploid control strain (W303 IH1) and 1xSNCA yeast strain were transformed with the pAG416\textit{GAL1-ITSN2}, pAG416\textit{GAL1-ITSN1} expression plasmids and a vector control plasmid pAG416\textit{GAL1-ccdB}. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.20. ITSN1 does not induce depolarized actin structures in the 1xSNCA model. The haploid control strain (W303 IH1) and 1xSNCA yeast strains were transformed with either pAG416GAL1-ITSN1 expression plasmid or a vector control plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Next, cells were fixed with 3.7% formaldehyde and stained with Rhodamine Phalloidine to visualize actin in the dsRED filter at 100x magnification. Scale Bar = 10 μm.
Figure 4.21. Activated Cdc42 exhibits polarized localization in yeast expressing ITSN1 in the 1xSNCA model. Isogenic haploid 1xSNCA strain with integrated Gic2-PBD-RFP transformed with the pAG416GALI-ITSN2, and p416GALI-ITSN1L expression plasmids were analyzed by fluorescent microscopy to visualize activated Cdc42 protein. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were viewed under microscope at 100x magnification in the dsRED (for Cdc42) filter. Scale Bar = 10 μm.
Figure 4.22. Expression of WASL alone does not induce the toxicity of alpha-synuclein in diploid 1xSNCA models. The haploid control (W303 IH1 MATa) and 1xSNCA-YFP yeast strains were individually crossed with W303 MATα transformed with the pAG416GAL1-WASL expression plasmid or the pAG416GAL1-ccdB vector control plasmid. The resulting diploid yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
**Figure 4.23. WASL expression does not change cell shape or induce alpha-synuclein inclusions in the diploid 1xSNCA model.** The haploid control (W303 IH1 $MAT_a$) and 1xSNCA-YFP yeast strains were individually crossed with W303 $MAT_\alpha$ transformed with the pAG416GAL1-WASL expression plasmid or the pAG416GAL1-ccdB vector control plasmid. The resulting diploid yeast strains were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at $OD_{600}$ 1.0 and grown for six hours to induce the expression of SNCA and WASL. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 $\mu$m.
Figure 4.24. WASL further enhances ITSN2 induced Toxicity in 1xSNCA yeast model. The haploid W303 IH1 and 1xSNCA yeast strains transformed with the p414GAL1-ITSN2 expression plasmid or the pAG414GAL1-ccdB vector control plasmid were transformed with the pAG416GAL1-WASL or the pAG416GAL1-ccdB vector control plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.25. ITSN1 and ITSN2\textsuperscript{short} expression leads to growth defects in the IntTox yeast model. The haploid control strain (W303 IH1) and IntTox yeast strain were transformed with the pAG416\textit{GAL1}-ITSN2, pAG416\textit{GAL1}-ITSN2\textsuperscript{short}, and pAG416\textit{GAL1}-ITSN1 expression plasmids and the pAG416\textit{GAL1}-ccdB vector control plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 4.26. ITSN1 or ITSN2\textsuperscript{short} expressed in the IntTox model does not change cell shape or alpha-synuclein inclusions. A) The IntTox yeast strain transformed with the pAG416\textit{GAL1-ITSN2}\textsuperscript{short}, pAG416\textit{GAL1-ITSN1} expression plasmids and the pAG416\textit{GAL1-ccdB} vector control plasmid precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD\textsubscript{600} 1.0 and grown for six hours to induce the expression of SNCA, ITSN1, and ITSN2\textsuperscript{short}. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 \textmu m. B) The percentage of yeast cells with alpha-synuclein inclusions were calculated for each yeast strain. Detailed methods of the calculation of percentage of cells with alpha-synuclein inclusions can be found in the methods. Shown is the percentage from single experiment with estimated 95% confidence interval of the fraction.
Figure 4.2. Defective endocytosis in the IntTox model. The haploid control strain (W303 IH1) and IntTox yeast strains were precultured in raffinose media. After overnight
growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and viewed at the indicated time points at 100x magnification in the dsRED (for FM4-64), YFP (for SNCA) filters. Scale Bar = 10 μm.
Table 8. Defective endocytosis in the IntTox model.

The haploid IntTox yeast strain and its isogenic control strain (W303 IH1) were stained with FM 4-64 and chased at 30 °C. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes after 45 minutes of chase were counted from randomly selected field of views of a single experiment (n=1). The fractions of cells that show fully internalized FM4-64 dye and their corresponding calculated 95% Confidence Interval for proportion are shown.

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<th>Strain</th>
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<th>Fraction of cells with fully internalized FM4-64 to vacuolar membranes</th>
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<td>Control (W303 IH1)</td>
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<td>IntTox</td>
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Figure 4.28. ITSN2-ECFP expression does not exacerbate endocytosis defect in the IntTox yeast model. The haploid IntTox yeast strain transformed with the pAG416GAL1-ITSN2-ECFP or the pAG416GAL1-ccdb-ECFP expression plasmids were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD600 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and they were viewed at the indicated time points at 100x magnification in the dsRED (for FM4-64), YFP (for SNCA), and CFP (for ITSN2) filters. Scale Bar = 10 μm.
Table 9. ITSN2 expression does not exacerbate the defective endocytosis in IntTox yeast model.

The haploid IntTox yeast strain and its isogenic control strain (W303 IH1), transformed with pAG416Gal-ITSN2-ECFP or pAG416Gal-ccdb-ECFP vector were stained with FM 4-64 and chased at 30 °C. Cells showing either fully or incompletely internalized FM4-64 dye to their vacuolar membranes after 45 minutes of chase were counted from randomly selected field of views of a single experiment (n=1). The fractions of cells that show fully internalized FM4-64 dye and their corresponding calculated 95% Confidence Interval for proportion are shown.

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<th>Strain</th>
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<td>IntTox</td>
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<tr>
<td>IntTox+ITSN2-ECFP</td>
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Figure 4.29. ITSN2-ECFP expression does not lead to a significant change in alpha-synuclein inclusions. The haploid IntTox yeast strain transformed with the pAG416GAL1-ITSN2-ECFP or the pAG416GAL1-ccdb-ECFP expression plasmids were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were viewed at 100x magnification in the YFP (for SNCA) filters. The percentage of yeast cells with alpha-synuclein inclusions were calculated for each yeast strain. Detailed methods of the calculation of percentage of cells with alpha-synuclein inclusions can be found in the methods. Shown is the average and SEM from two separate experiments.
Aim 3: Characterization of human suppressor genes of alpha-synuclein toxicity.

Aim Overview: The high expression yeast model of alpha-synuclein exhibits growth defects. This phenotype could be utilized to identify suppressor genes that alleviate the toxicity of alpha-synuclein. The advantage of using yeast as a model is to carry out genome-wide genetic screens. Hundreds of yeast genes have been found that modulate alpha-synuclein toxicity. Based on their homology and several other properties, scientists have constructed a “humanized” modifier network of alpha-synuclein (114). However, many human genes do not have yeast homologs, and thus they cannot be readily identified from yeast modifier genes by homology. To identify human genes involved in the regulation of alpha-synuclein toxicity, we used our yeast expression human gene library to identify human suppressor genes of alpha-synuclein toxicity using the toxic IntTox model. Our screen involved introducing each of the ~15,000 human genes into the IntTox model, followed by identification of hits based on colony sizes and final verification of the phenotypes to confirm the rescue of toxicity. We reasoned that human genes, even without yeast homologs, may directly act on alpha-synuclein and alleviate its toxicity in yeast. Alternatively, human genes may affect conserved cellular processes in yeast and indirectly prevent the toxicity. We identified 141 human suppressor genes that can rescue the IntTox model from alpha-synuclein toxicity. Among those suppressors, two functional groups are enriched: Homeobox proteins and genes involved in ribosome biogenesis.
We took those two groups and individually verified their suppressor phenotype. To understand the suppressor mechanism, I carried out western blot assays to examine expression of alpha-synuclein in the presence of the suppressor genes. I showed that expression of these genes did not alter the levels of alpha-synuclein protein. For at least two of the suppressor genes, I observed a significant reduction in cytoplasmic inclusions of alpha-synuclein. Interestingly, I also discovered that alpha-synuclein inclusions colocalize with the master regulator protein of cell polarization, Cdc42. The sequestering of Cdc42 to alpha-synuclein inclusions could be one of the potential mechanisms underlying cellular toxicity of alpha-synuclein. Interestingly, I found that the expression of the yeast homeobox protein, HMLalpha2, suppresses the toxicity of alpha-synuclein in the IntTox model without changing the protein levels of alpha-synuclein. Similarly, it also did not reduce the number of alpha-synuclein inclusions. Yeast homeobox and ribosome proteins are reported to increase bipolar budding of yeast. A similar bipolar budding pattern is also observed in diploid yeast whereas haploid yeast shows an axial budding pattern. Interestingly, diploid IntTox model does not show any growth defect with similar expression levels of alpha-synuclein. Also, the diploid IntTox model exhibited significantly fewer alpha-synuclein inclusions. Almost complete plasma membrane localization of alpha-synuclein was observed. To understand this mechanism of axial and bipolar budding, I introduced the yeast AXL1 gene into the IntTox model. The AXL1 gene has been reported to increase the axial budding. Consistent with our hypothesis, expression of AXL1 enhances alpha-synuclein toxicity in the IntTox model. This toxic phenotype was not accompanied by any change in the protein levels or the alpha-synuclein inclusions in the IntTox model.
Results: Using our yeast expression human gene library, we aimed to identify the human genetic modifiers that can rescue the IntTox yeast strain from alpha-synuclein induced toxicity. To accomplish this genome-wide genetic screen, we transformed the plasmid library into the haploid IntTox yeast strain. The plasmid library contains 14,827 human ORFs cloned into the galactose inducible vector. Next, this collection of yeast transformants were precultured in media and spotted onto agar plates containing glucose or galactose. The candidate suppressors were identified by following the growth of the yeast strains containing human gene clones that suppressed the toxicity of alpha-synuclein. All candidate suppressors or “hits” were then individually verified using the serial dilution and spotting essay (Figure 5.1).

After verification, we identified 141 human suppressor genes that can suppress alpha-synuclein toxicity in the IntTox model. Interestingly 16% (23/141) of the suppressor genes are proteins involved in Ribosomal biogenesis, another 26% (22/141) of the suppressor genes are homeobox proteins (Figure 5.2).

To verify the suppressor phenotype of the IntTox model expressing homeobox and ribosome proteins, we carried out a serial dilution spotting assay. The haploid IntTox yeast strains were transformed with the homeobox and ribosome protein suppressor genes, as well as a vector control plasmid. The control strain (W303 IH01) transformed with a vector control plasmid was used as a control. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 5.3).
Images of the agar plates show that the IntTox strains carrying the homeobox or ribosome proteins can rescue the yeast from alpha-synuclein toxicity.

To determine whether the suppressor-mediated rescue of toxicity in the IntTox model is due to a change in the expression of alpha-synuclein, we checked the protein levels of alpha-synuclein. We used the strains mentioned in Figure 5.3 to perform a western blot assay. Protein was extracted yeast cells precultured overnight, shifted to galactose media, and grown for six hours. The signal intensity of the alpha-synuclein protein bands was normalized to the intensity of control PGK1 bands. Then, the values were normalized to the ratio of SNCA/PGK1 in yeast without the suppressors. From three independent repeats, we found that there was no significant change in the protein levels of alpha-synuclein when suppressor genes were co-expressed (Table 10). Figure 5.4 is a representative image of the western blot assay showing alpha-synuclein protein levels in the IntTox model carrying either a homeobox suppressor (DLX5), ribosome protein suppressor (RPS24) or a vector control plasmid. This suggests that human gene suppressors do not suppress the alpha-synuclein toxicity by changing the protein levels of alpha-synuclein.

Since the human gene suppressors do not change the protein levels of alpha-synuclein, we wanted to understand whether the suppressors would lead to a decreased cytoplasmic inclusion of alpha-synuclein. To test this, we used the IntTox yeast strains mentioned in Figure 5.4, IntTox model carrying either a homeobox suppressor (DLX5), ribosome protein suppressor (RPS24) or a vector control plasmid. These yeast strains were precultured overnight, shifted to galactose media, and grown for six hours to induce
the expression of SNCA. Next, live yeast cells were observed by microscopy in the YFP filter to visualize alpha-synuclein (Figure 5.5A). We counted the cells containing cytoplasmic inclusions or showing plasma-membrane localized alpha-synuclein. We calculated the fraction of cells containing cytoplasmic inclusions and carried out three independent repeats of the experiment. The homeobox protein (DLX5) and the ribosome protein (RPS24) significantly reduce alpha-synuclein inclusions in the IntTox model (Figure 5.5B). This result suggests that the homeobox and ribosome protein suppressors may rescue the IntTox model from alpha-synuclein toxicity by reducing the extent to which alpha-synuclein forms cytoplasmic inclusions in the cell.

We have found that enhancer gene ITSN2-induced toxicity in the 1xSNCA model could induce depolarized Cdc42. Cdc42 is an important cell cycle mediator and also involved in various cellular processes, including actin organization and cell polarity. We wanted to check the localization of Cdc42 in the IntTox model. Unfortunately, we were unable to test the activated Cdc42 experiment in the IntTox model because the signal of the YFP tagged alpha-synuclein of IntTox is too strong and bleed through the DsRed channel, which we use to monitor activated from Cdc42. We therefore transformed the haploid control strain (W303 IH1) and IntTox yeast strains with an expression plasmid bearing ECFP-\textit{CDC42} under the constitutive GPD promoter. It was previously shown that N-terminal tagged Cdc42 is able to retain its function. These yeast strains were precultured overnight, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Live yeast cells were observed by microscopy in the YFP and CFP filter to visualize alpha-synuclein and Cdc42, respectively (Figure 5.6). Cdc42 is mislocalized and sequestered to alpha-synuclein inclusions in the IntTox model. This
suggests that mislocalization of Cdc42 may contribute to the observed dosage-dependent toxicity of alpha-synuclein in the IntTox model.

Next, we wanted to understand if the suppressor can rescue the IntTox model by altering the mislocalization of Cdc42. To test this, we used the IntTox strain mentioned in Figure 5.6 carrying ECFP-\textit{CDC42}. I transformed this yeast strain with DLX5, RPS24, \textit{YPTI}, and vector control plasmids. \textit{YPTI} is a yeast suppressor gene of the IntTox model, and it is involved with vesicle trafficking (79). The transformed yeast strains were precultured overnight, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Next, live yeast cells were observed by microscopy in the YFP and CFP filter to visualize alpha-synuclein and Cdc42 respectively (Figure 5.7). We found that the yeast strains carrying the suppressor genes still show mislocalization of Cdc42 to alpha-synuclein inclusions.

Next, we counted the cells carrying cytoplasmic inclusions and the cells showing a plasma membrane localization of alpha-synuclein to calculate the fraction of cells showing cytoplasmic inclusions. The homeobox protein (DLX5), the ribosome protein suppressor (RPS24), and \textit{YPTI} all lead to reduced alpha-synuclein inclusions in the IntTox model (Figure 5.8). These findings are consistent with the possibility that the suppressor rescues the toxicity of the IntTox model by reducing cytoplasmic inclusions of alpha-synuclein, indirectly alleviating defects in Cdc42 mislocalization.

Next, we wanted to test whether expression of Cdc42 will lead to any changes in the growth of IntTox model or any changes in the suppressor phenotype. To this end, I used the set of yeast strains used in the microscopy analysis in Figure 5.7 to check the
toxicity level of IntTox model in the presence of Cdc42 expression. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 5.9). Images of the agar plates show that extra plasmid copy of CDC42 does not alter IntTox toxicity. In addition, expression of Cdc42 does not change the effect of the suppressor genes on alpha-synuclein toxicity in the IntTox model, either. This suggests that the findings of Figures 5.7 and 5.8 were not influenced by CDC42 overexpression.

The homeobox domains are highly conserved and are often involved in transcription regulation (115). Since these human homeobox proteins rescue yeast from alpha-synuclein induced cellular toxicity, we wanted to test whether the yeast homeobox genes can do so as well. To test that, I carried out a growth assay using a yeast homolog of a homeobox gene, HMLALPHA2. I transformed the control haploid strain (W303 IH1) and haploid IntTox yeast strains with HMLALPHA2 expression plasmid and a vector control plasmid. These yeast strains were precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 5.10). Images show that the expression of yeast HMLALPHA2 gene can suppress the toxicity of alpha-synuclein in the IntTox model. This suggests that the human homeobox protein is functioning in a similar pathway to suppress alpha-synuclein toxicity in yeast.

Next, we wanted to find out if the HMLALPHA2 expression could change the protein levels of alpha-synuclein. To test that, we checked the protein levels of alpha-synuclein in the IntTox strains mentioned in Figure 5.10 by a western blot assay. Protein was extracted from the yeast cells precultured overnight, shifted to galactose media, and
grown for six hours. The signal intensity of the alpha-synuclein protein bands was normalized to the intensity of Pgk1 bands. Then, the values were normalized to the ratio of SNCA/PGK1 in yeast without the suppressor. We found that there is no significant change in the protein levels of alpha-synuclein when HMLALPHA2 is co-expressed (Figure 5.11). Although HMLALPHA2 can rescue yeast from alpha-synuclein toxicity, the protein levels of alpha-synuclein remains unchanged. This result is similar to that of human homeobox suppressor genes.

Since overexpression of HMLALPHA2 does not change the protein levels of alpha-synuclein, we wanted to test whether HMLALPHA2 expression would lead to a decreased cytoplasmic inclusion of alpha-synuclein, similar to the human suppressors. To test this, I used the IntTox yeast strains mentioned in figure 5.10 carrying the HMLALPHA2 gene and vector control plasmid. These yeast strains were precultured overnight, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Live yeast cells were observed by microscopy in the YFP filter to visualize alpha-synuclein (Figure 5.12A). We, then, counted the cells carrying cytoplasmic inclusions and the cells showing a plasma membrane localization of alpha-synuclein to get the fraction of cells showing cytoplasmic inclusions. We carried out three independent repeats of the experiment. The yeast homeobox suppressor HMLALPHA2 reduces formation of cytoplasmic inclusions, although the difference is not statistically significant (Figure 5.12B). It is possible that HMLALPHA2 mediates the rescue of alpha-synuclein toxicity in the IntTox model in a similar way as the human suppressors.
We wondered if similar suppressor mechanisms could be involved in the toxicity rescue by homeobox and ribosomal proteins in the IntTox yeast model. Interestingly, overexpression of the yeast homeobox *HMLALPHA2* and ribosome protein *RPS7A* both increase the bipolar budding pattern in yeast (116). Yeast has two different budding patterns depending on its polarity: axial budding pattern and bipolar budding pattern. This budding pattern of yeast depends on its ploidy. The haploid yeast shows an axial budding pattern, and diploid yeast shows bipolar budding pattern (118). This information leads to a hypothesis that reduction of axial budding or an increase of bipolar budding will be able to rescue yeast from alpha-synuclein toxicity. To test this hypothesis, we generated a diploid IntTox model by crossing the haploid MATα IntTox model with its isogenic haploid MATα wild-type strain carrying a vector control plasmid. The haploid MATα IntTox model was also transformed with the same vector control plasmid. Both haploid and diploid IntTox yeast strains were then precultured, serially diluted, and spotted onto agar plates containing galactose or glucose (Figure 5.13). The spotting results show that the diploid IntTox model does not show any growth defect. This supports our hypothesis that a non-axial budding helps the yeast to recover from alpha-synuclein toxicity.

This interesting phenotype of diploid IntTox model leads us to the question of whether the absence of growth defect is due to a change in the protein levels of alpha-synuclein. To test this, we carried out a western blot assay using the haploid and diploid IntTox yeast strains mentioned in Figure 5.13. An isogenic haploid control strain was used here as a control. Protein was extracted from the yeast cells precultured overnight, shifted to galactose media, and grown for six hours. The signal intensity of the alpha-
synuclein protein bands was normalized to the intensity of PGK1 bands. There is no significant change in the protein levels of alpha-synuclein in the haploid and diploid IntTox yeast strains (Figure 5.14). This suggests that the change in the toxicity phenotype in the diploid IntTox model is not due to a change in protein levels of alpha-synuclein.

To understand the nontoxic phenotype of the diploid IntTox model, we wanted to look into the localization of alpha-synuclein. To find this, we cultured the haploid and diploid IntTox strains to observe them under microscope. These yeast strains were precultured overnight, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Live yeast cells were observed by microscopy in the YFP filter to visualize alpha-synuclein (Figure 5.15A). We counted the cells carrying cytoplasmic inclusions and the cells showing a plasma membrane localization of alpha-synuclein to get the fraction of cells showing cytoplasmic inclusions. The diploid IntTox model exhibits much less alpha-synuclein inclusions compared to the haploid IntTox model (Figure 5.15B). This suggests that the diploid yeast model has the capability to avoid cytoplasmic inclusions and thus rescuing the yeast from alpha-synuclein toxicity.

If axial budding promotes toxicity of alpha-synuclein, genetic changes that induces axial budding may act as an enhancer in the IntTox model. To test this, we picked up a mating-related gene AXL1. This gene, when overexpressed, significantly increases axial budding in yeast (117). Following this published data, we cloned the AXL1 gene under a high copy number plasmid, pAG426GAL1-ccdB, and transformed it into the IntTox model as well as in the control strain. A similar vector control plasmid was also transformed into those strains. These yeast strains were then precultured, serially...
diluted, and spotted onto agar plates containing galactose or glucose (Figure 5.16). The spotting results show that the expression of yeast AXL1 gene enhances the toxicity of alpha-synuclein in the IntTox model. The overexpression of AXL1 alone does not induce toxicity in the control strain, suggesting that it specifically enhances alpha-synuclein toxicity. However, in the spotting image, the toxic phenotype of the IntTox model by itself does not exhibit any growth defects. We reason that this is due to the presence of high-copy plasmid pAG426GAL1-ccdB. Similar suppression of toxicity has also been observed when other toxic yeast models (e.g. FUS and TDP-43, unpublished data) were transformed with this expression plasmid. The findings of this spotting experiment suggest that yeast genes increasing the axial budding pattern can enhance alpha-synuclein toxicity in the IntTox model.

Next, we wanted to understand if the Axl1 mediated increase of alpha-synuclein toxicity in the IntTox model is due to a change in the protein levels of alpha-synuclein. To test that, we carried out a western blot assay using the IntTox models mentioned in Figure 5.16. Protein was extracted from the yeast cells precultured overnight, shifted to galactose media, and grown for six hours. The signal intensity of the alpha-synuclein protein bands was normalized to the intensity of PGK1 bands (Figure 5.17). The values were normalized to the ratio of SNCA/PGK1 in haploid IntTox strain carrying vector control plasmid. We found that the expression of AXL1 does not change the protein levels of alpha-synuclein. This result suggests that Axl1 does not induce alpha-synuclein toxicity by enhancing protein expression.
Overexpression of \textit{AXL1} enhances alpha-synuclein toxicity but does not increase the protein levels of alpha-synuclein. Next, we wanted to ask if this enhancer mechanism involves increased cytoplasmic inclusions of alpha-synuclein. To test this, we examined the localization of alpha-synuclein in the IntTox strain carrying \textit{AXL1} or the vector control plasmid. These yeast strains were precultured overnight, shifted to galactose media, and grown for six hours to induce the expression of SNCA. Next, live yeast cells were observed by microscopy in the YFP filter to visualize alpha-synuclein (Figure 5.18A). We, then, counted the cells carrying cytoplasmic inclusions and the cells showing a plasma membrane localization of alpha-synuclein to get the fraction of cells showing cytoplasmic inclusions. We carried out three independent repeats of the experiment. The results show that the expression of \textit{AXL1} does not alter the formation of alpha-synuclein inclusions in the IntTox model (Figure 5.18A). This result suggests that Axl1 induced alpha-synuclein toxicity of the IntTox model is not through a change in the number of inclusions; thus, other mechanisms may be involved.
Figure 5.1. Genome-scale genetic screening of human suppressor genes. The plasmid library containing 14,827 human ORFs cloned in the pAG416GAL1-ccdB vector was transformed into the haploid IntTox yeast strain using a high-efficiency transformation protocol (STEP 1). This collection of yeast strains was precultured in media containing raffinose and then spotted onto agar plates containing glucose (gene off) or galactose (gene on). The growth of the yeast strains was followed to identify those human gene clones that suppressed the toxicity of alpha-synuclein in the haploid IntTox yeast strain. The green squares indicate examples of candidate suppressors (STEP 2). All suppressor “hits” were then individually verified using the serial dilution and spotting essay (STEP 3).
Figure 5.2. Two main groups of human suppressor genes of alpha-synuclein toxicity.

Nineteen percent (27/141) of genes that suppress alpha-synuclein toxicity in IntTox yeast model belong to the ribosomal protein group and fifteen percent (21/141) of suppressor genes belong to the homeobox protein group.
Figure 5.3. Homeobox and ribosome proteins suppressing the toxicity of alpha-synuclein in the InTox model. The haploid IntTox yeast strains were transformed with the human suppressor genes under the pAG416GAL1-ccdB vector. These yeast strains were then precultured in raffinose media, serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the galactose plates are shown here. The pictures were taken after two days of growth at 30°C.
Table 10. The human gene suppressors do not change protein levels of alpha-synuclein. The haploid IntTox yeast strains transformed with the human suppressor genes in the pAG416GAL1-ccdB vector were analyzed by western blot. Protein was extracted from the yeast cells precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD_{600} 1.0 and grown for six hours. A western blot was performed using an antibody against GFP and the loading control protein PGK1. Quantification was performed using ImageJ software. The signal intensity of the alpha-synuclein protein bands normalized to the intensity of PGK1 bands. The values were normalized to the ratio of SNCA/PGK1 in yeast without suppressor. Shown is the average and standard deviation (STD) from three separate experiments.
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**Figure 5.4. The human gene suppressors do not change protein levels of alpha-synuclein.** Haploid IntTox yeast strains transformed with either a control vector plasmid or the pAG416GALI-DLX5 and the pAG416GALI-RPS24 expression plasmids were analyzed by western blot. Protein was extracted from the yeast cells precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours. A western blot was performed using an antibody against GFP and the loading control protein PGK1. Quantification was performed using ImageJ software. Quantification shown below the membrane is the signal intensity of the alpha-synuclein protein bands normalized to the intensity of PGK1 bands. The values are normalized to the ratio of SNCA/PGK1 in yeast without suppressor.
Figure 5.5. The suppressors lead to a significant reduction in alpha-synuclein inclusions. A) Haploid IntTox yeast strains transformed with either a vector plasmid or the pAG416GAL1-DLX5 and the pAG416GAL1-RPS24 expression plasmid were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD600 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 μm. B) The percentage of yeast cells with alpha-synuclein inclusions were calculated for each yeast strain. Detailed methods of the calculation of percentage of cells with alpha-synuclein inclusions can be found in the methods. Shown is the average and SEM from three separate experiments.
Figure 5.6. Cdc42 is mislocalized and sequestered to alpha-synuclein inclusions in the IntTox model. The haploid control strain (W303 IH1) and IntTox yeast strains were transformed with the pAG415GPD-ECFP-CDC42 expression plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD\textsubscript{600} 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were viewed by microscopy at 100x magnification in the CFP (for Cdc42), YFP (for SNCA) filters. Scale Bar = 10 μm.
Figure 5.7. The suppressors do not lead to any significant change in the mislocalization of Cdc42. The IntTox yeast strain harboring the pAG415GPD-ECFP-CDC42 plasmid were transformed with the pAG416GAL1-DLX5, pAG416GAL1-RPS24, and the pAG416GAL1-YPT1 expression plasmids. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD_{600} 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were viewed under microscope at 100x magnification in the CFP (for Cdc42), YFP (for SNCA) filters. The blue Cdc42 were converted into red and merged with yellow alpha-synuclein using ImageJ software. Scale Bar = 10 μm.
Figure 5.8. Expression of suppressors reduce alpha-synuclein inclusions in the IntTox model. The IntTox yeast strain harboring the pAG415GPD-ECFP-CDC42 plasmid were transformed with the pAG416GAL1-DLX5, pAG416GAL1-RPS24, and the pAG416GAL1-YPT1 expression plasmids. These yeast cells were precultured in raffinose media for overnight growth. Next, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were viewed by microscopy at 100x magnification in the YFP filters to visualize alpha-synuclein. The percentage of yeast cells with alpha-synuclein inclusions were calculated for each yeast strain. Detailed methods of the calculation of percentage of cells with alpha-synuclein inclusions can be found in the methods. Shown is the percentage from single experiment with estimated 95% confidence interval of the fraction.
Figure 5.9. Expression of *CDC42* does not alter the effect of the suppressor genes on alpha-synuclein toxicity in the IntTox model. The IntTox yeast strain harboring the pAG415GPD-ECFP-*CDC42* plasmid were transformed with the pAG416*GAL1*-DLX5, pAG416*GAL1*-RPS24, and the pAG416*GAL1*-*YPT1* expression plasmids. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 5.10. Expression of yeast *HMLALPHA2* gene suppresses the toxicity of alpha-synuclein in the IntTox model. The control haploid strain (W303 IH1) and haploid IntTox yeast strains were transformed with the pAG416*GAL1-HMLALPHA2* expression plasmid and a control vector plasmid. These yeast strains were precultured in raffinose media, serially diluted, and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after three days of growth at 30°C.
Figure 5.11. Expression of HMLALPHA2 does not change the protein levels of alpha-synuclein. Haploid IntTox yeast strains transformed with either a control vector plasmid or the pAG416GAL1-HMLALPHA2 expression plasmid were used for western blot. Protein was extracted from the yeast strains precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours. Western blot was performed using an antibody against GFP and the loading control protein PGK1. Quantification was performed using ImageJ software. Quantification shown below the membrane is the signal intensity of the alpha-synuclein protein bands normalized to the intensity of PGK1 bands. The values are normalized to the ratio of SNCA/PGK1 in yeast without HMLalpha2.
Figure 5.12. Expression of *HMLALPHA2* does not reduce alpha-synuclein inclusions in the IntTox model. A) The haploid IntTox yeast strains were transformed with the pAG416*GAL1-HMLALPHA2* expression plasmid and a vector control plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 µm. B) The percentage of yeast cells with alpha-synuclein inclusions were calculated for each yeast strain. Detailed methods of the calculation of percentage of cells with alpha-synuclein inclusions can be found in the methods. Shown is the average and SEM from three separate experiments.
Figure 5.13. The diploid IntTox model does not show growth defects. A) Haploid IntTox yeast strain \((\text{MAT} a)\) were transformed with a vector plasmid or mated with control yeast strain of the opposite mating type \((\text{w}303 \text{MAT}\alpha)\) transformed with the same vector plasmid to generate a diploid IntTox yeast strain. These yeast strains along with a haploid and diploid control strains were precultured in raffinose media, serially diluted, and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after two days of growth at 30°C.
Figure 5.14. Diploid IntTox model does not alter the protein levels of alpha-synuclein. Haploid IntTox yeast strain (MATa) were transformed with a vector plasmid or mated with control yeast strain of the opposite mating type (w303 MATα) transformed with the same vector plasmid to generate a diploid IntTox yeast strain. These yeast strains, along with an isogenic haploid control strain, were used for western blot. Protein was extracted from the yeast strains precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD_{600} 1.0 and grown for six hours. Western blot was performed using an antibody against GFP and the loading control protein PGK1. Quantification was performed using ImageJ software. Quantification shown below the membrane is the signal intensity of the alpha-synuclein protein bands normalized to the intensity of PGK1 bands.
Figure 5.15. Diploid IntTox model exhibits less alpha-synuclein inclusions. A)

Diploid IntTox generated by mating with control yeast strain of the opposite mating type (w303 \textit{MAT}^\alpha\) and Haploid IntTox yeast strain (\textit{MAT}^a\) were precultured in raffinose media. After overnight growth, they were shifted to media containing 2\% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 \textmu m. B) The percentage of yeast cells with alpha-synuclein inclusions were calculated for each yeast strain. Detailed methods of the calculation of percentage of cells with alpha-synuclein inclusions can be found in the methods. Shown is the average and SEM from three separate experiments.
Figure 5.16. Expression of yeast *AXL1* gene enhances the toxicity of alpha-synuclein in the IntTox model. The control haploid strain (W303 IH1) and haploid IntTox yeast strains were transformed with the pAG426GAL1-AXL1 expression plasmid and a control vector plasmid. These yeast strains were precultured in raffinose media then serially diluted and spotted onto agar plates containing galactose (gene on) or glucose (gene off). The pictures of the plates were taken after three days of growth at 30°C.
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![Western Blot](image)

**Figure 5.17. Expression of AXL1 does not change the protein levels of alpha-synuclein.** Haploid IntTox yeast strains transformed with either a control vector plasmid or the pAG426GAL1-AXL1 expression plasmid were used for western blot. Protein was extracted from the yeast strains precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD\textsubscript{600} 1.0 and grown for six hours. A western blot was performed using an antibody against GFP and the loading control protein PGK1. Quantification was performed using ImageJ software. Quantification shown below the membrane is the signal intensity of the alpha-synuclein protein bands normalized to the intensity of PGK1 bands. The values are normalized to the ratio of SNCA/PGK1 in yeast with vector control.
Figure 5.18. Expression of *AXL1* does not alter the formation of alpha-synuclein inclusions in the IntTox model. The haploid IntTox yeast strains were transformed with the pAG426GAL1-AXL1 expression plasmid and a vector control plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. Live yeast cells were observed at 100X magnification in the bright field and YFP filter to visualize alpha-synuclein. Scale Bar = 10 μm.
CHAPTER 6. DISCUSSION

Development of a new SNCA yeast model

Alpha-synuclein is a small lipid binding protein primarily expressed in the neurons. A pathological hallmark of Parkinson's disease (PD) is the formation of Lewy body, primarily composed of the abnormal aggregation of alpha-synuclein (57). Lewy body and Lewy related pathology are frequently observed in several other neurodegenerative disease, including Alzheimer’s disease, Dementia with Lewy body and Multiple Systems Atrophy (16). Both missense mutations and increased copy numbers of the SNCA gene encoding alpha-synuclein lead to PD. Despite decades of research, the precise function of alpha-synuclein and the mechanisms underlying its toxicity to neurons remain unclear.

Membrane-associated localization, dosage-dependent formation of cytoplasmic inclusions and cellular toxicity of alpha-synuclein can be faithfully recapitulated in a simple eukaryote, the budding yeast, *Saccharomyces cerevisiae*. Genetic screens of yeast cells expressing toxicity level of alpha-synuclein have identified hundreds of suppressor and enhancer genes, revealing complex cellular processes underlying alpha-synuclein toxicity. Mammalian homologs of several yeast modifier genes were found to have a similar effect in neurons, supporting the existence of conserved mechanisms pertinent to alpha-synuclein toxicity in yeast (114).

We reasoned that current genetic approaches studying toxicity of alpha-synuclein in yeast have limitations. Yeast models containing toxic levels of alpha-synuclein might
not be effective in uncovering factors that trigger the initial molecular events promoting alpha-synuclein inclusions and toxicity. To address this, we developed a new SNCA model, 1xSNCA, expressing alpha-synuclein at a much lower level that does not lead to inclusions or toxicity in yeast (Figure 3.1). In this new model, alpha-synuclein localizes to the polarized growth sites. This finding is consistent with it being targeted to the plasma membrane via polarized exocytosis in yeast. Indeed, localization of alpha-synuclein to the plasma membrane appears to depend on functional secretory pathway in yeast (51). Alpha-synuclein has an N-terminal vesicle binding domain that binds to the lipids and a C-terminal protein-binding domain by which it interacts with VAMP proteins. VAMP is Vesicle associated membrane proteins that are involved in vesicle fusion (50). The polarized localization of alpha-synuclein protein in yeast suggests that it targets the polarized budding zone either by binding to a lipid moiety with its N-terminal region or by binding to other proteins that are targeted the polarized growth site.

Interestingly, the yeast homolog of VAMP protein SNC1 also shows similar polarized localization (29,119) as alpha-synuclein. This finding suggests the 1xSNCA model is recapitulating the function of alpha-synuclein in yeast.

**Identification of human modifier genes that enhance alpha-synuclein toxicity in the 1xSNCA model**

Different approach has been taken to understands the factors that trigger alpha-synuclein toxicity. Using yeast model several genetic screens has been performed. Many of those enhancer genes showed conserved mechanisms of to alpha-synuclein toxicity in yeast.
One of the limitations of yeast genetic screens is that one can only identify the human genes that has conserved yeast homolog. Since most human genes do not have functionally conserved yeast homologs, the search for human gene modifiers is incomplete. To address this limitation, we constructed an overexpression library containing ~15,000 human-gene clones. Using this library, I carried out genome-wide genetic screening and identified five human genes that can enhance the toxicity of the 1xSNCA model (Figure 3.14). None of the five human genes by themselves is toxic to yeast. This suggest that these human genes may share common cellular targets or directly interact with alpha-synuclein to induce its toxicity.

Enhancer genes share membrane-associated functions

All five human enhancer genes that we identified share membrane-associated functions, including vesicle-mediated cellular trafficking, cytoskeleton or cell polarity regulation, and lipid biosynthesis. Interestingly, two of the enhancer genes induced alpha-synuclein inclusions in the 1xSNCA model (Figure 3.15). Whereas the other three enhancer genes did not induce alpha-synuclein inclusions but still induced growth defects. So, the 1xSNCA model was able to identify two different classes of enhancers. One group of enhancers induced alpha-synuclein inclusions and another group did not induce inclusions. The different localization of the alpha-synuclein upon human enhancer gene expression suggest different enhancer mechanisms may be involved.

One of the human gene enhancers that induce alpha-synuclein inclusions in 1xSNCA model is the USO1. The USO1 gene encodes the “General vesicular transport
factor p115”, a protein that recycl[es between the cytosol and the Golgi apparatus (99). USO1 is highly conserved gene from yeast to human. The yeast homolog of human USO1 is essential for vesicle transport from ER to Golgi (120). Yeast USO1 has not been identified as a modifier gene that affects the toxicity of alpha-synuclein. Overexpression of alpha-synuclein has been shown to block ER-Golgi traffic in yeast (79). It is possible that overexpression of human USO1 interferes with yeast Uso1 function, thus compromising exocytosis process and leading to growth defects in yeast cells. It has been reported that alpha-synuclein accumulates in the cytoplasm in secretory mutants (51). So, a compromised exocytosis could be one explanation why alpha-synuclein inclusions were observed upon USO1 expression in yeast.

To understand how USO1 compromise with exocytosis, I need to first determine cellular localization of human USO1. I can tag the USO1 protein with a fluorescence tag and check its localization by comparing to other marker proteins of ER or Golgi in yeast. I can also measure the ER to Golgi transport using CPY transport analysis. In yeast, secretory transport can be monitored by analyzing the maturation of carboxypeptidase Y (CPY). CPY is a highly studied protein which is synthesized as a proenzyme then modified into different forms in the ER (p1), Golgi complex (p2) and the mature form (m) after delivery to the vacuole (121). To test the ER to Golgi transport the 1xSNCA yeast strain expressing USO1 can be subjected to pulse–chase analysis. CPY can be recovered by immunoprecipitation. The SDS-PAGE of the CPY will reveal the protein size which will also indicate the modification of the protein. If we observe that all the proteins are in their p1 state that will indicate there is a defect in the ER to Golgi transport.
PI51A is another human enhancer gene that was identified from our screen. PI51A is a kinase that catalyzes the phosphorylation of phosphatidylinositol 4-phosphate (PI4P) to form phosphatidylinositol 4,5-bisphosphate (PI4,5P2) (104). This human gene also has a yeast homolog, MSS4 (122). It is possible that overexpression of PI51A in yeast perturbs the synthesis, localization and homeostasis of phosphoinositides, which critically regulate membrane trafficking. Both exocytosis and endocytosis have been previously implicated as cellular processes that modulate alpha-synuclein toxicity. Furthermore, cytoplasmic inclusions of alpha-synuclein contain clustering vesicles (29). Given the observed inclusions of alpha-synuclein induced by PI51A, it would be interesting to determine the effect of PI51A overexpression on cellular distribution of PI4P and PI4,5P and vesicle-mediated membrane trafficking in both the wild-type yeast and 1xSNCA strains.

The human enhancer gene, BORG4 induces alpha-synuclein toxicity in the 1xSNCA model without the formation of any inclusions. The specific function of this protein is still largely unknown. This protein has been reported to bind to CDC42 in a GTP-dependent manner. The protein was suggested to have a role in inducing actin filament assembly and cell shape control (101). Thus, the enhancer effect of this protein may involve yeast Cdc42. To test this, I can monitor Cdc42 localization and activation in yeast strains expressing BORG4. I can integrate the Gic2-PBD-RFP to the haploid 1xSNCA and control (W303 IH1) strains carrying BORG4. The Gic2-PBD-RFP or Gic2–p21 binding domain (PBD)–RFP is a reporter for GTP-Cdc42. So, Gic2-PBD-RFP plasmid allows us to visualize the activated form of the Cdc42 protein. In WT cells Gic2-PBD-RFP is localized to the bud site of growing yeast (93). I would expect to see a
change in Cdc42 localization if the enhancer mechanism involves interfering with Cdc42 function. Similar to what we have observed in ITSN2 induced toxicity in 1xSNCA model. Where Cdc42 showed a completely depolarized localization.

Apart from PI51A, CEGT is another enhancer gene that is involved in lipid biosynthesis. CEGT is a ceramide glucosyltransferase, an enzyme that plays an important role in the biosynthesis of glycosphingolipids. Recently it has been reported that heterozygous loss-of-function mutations in GBA, encoding glucocerebrosidase, drastically increase the risk of developing PD and other synucleinopathies (123). Glucocerebrosidase catalyzes the reverse enzymatic reaction of CEGT by converting glucosylceramide to ceramide. Interestingly, glucosylceramide was shown to be toxic to neurons (124). It would be interesting to test if overexpression of human CEGT in yeast may lead to the production of glucosylceramide and how glucosylceramide may contribute to the induced growth defects.

**ITSN2 induces toxicity of alpha-synuclein in the SNCA models**

The ITSN2 gene enhances alpha-synuclein toxicity in the 1xSNCA, 1xSNCA-YFP, and IntTox models. Intersectins (ITSN) are highly conserved scaffold protein in animals (96). In mammals, there are two types of intersectins known as ITSN1 and ITSN2. Both of these proteins are structurally very similar as both share similar domains and both are expressed as long and short forms, due to alternative splicing. Structurally, human intersectins have two Eps15-homology (EH) domains, an extended KLERQ coiled-coil domain, five Src-homology 3 (SH3) domains, and C-terminal DBL (or RhoGEF domain),
PH and C2 domains (Figure 6.1). The RhoGEF domain of ITSN only presents in the long isoform of intersectins. This domain is named as it acts as the Guanine nucleotide exchange factor (GEF) of Cdc42. CDC42 is a highly conserved small GTPase. Yeast Cdc42 regulates endocytosis and actin networks (93). The short isoform of ITSN is truncated in the C-terminus and does not have the RhoGEF domain (108).

ITSN1 long isoform is highly expressed in the nervous system and ITSN1 short isoform is expressed ubiquitously (108). Interestingly, in a Genome-Wide Association Study (GWAS), SNPs in ITSN1 gene was found to be associated with increased risk of Parkinson’s Disease (97). Intersectins are involved in the Clathrin-mediated endocytosis process. It is thought to regulate the formation of clathrin-coated vesicles (106). It has also been reported that overexpression of ITSN2 leads to endocytic defects in multiple cell types (107). ITSN1 and ITSN2 have three homologs in yeast named Sla1, Ede1, and Pan1. These homologs localize in the polarized growth site of the plasma membrane (109). All three of the homologs are involved in Clathrin-mediated endocytosis (110).

**ITSN2 requires membrane-localized alpha-synuclein to induce toxicity**

The low-copy number integration of the SNCA gene in yeast (NoTox model) has been applied to two mutated SNCA genes associated with PD. The A53T mutation in SNCA does not change membrane localization of alpha-synuclein, while the A30P mutation in SNCA disrupts its membrane localization giving rise to the production of alpha-synuclein in the cytosol (38). Interestingly, ITSN2 can only induced toxicity when expressed in the A53T SNCA mutant model. The co-expression of A30P SNCA and ITSN2 does not
induce any growth defect. This suggest that ITSN2 only induces the toxicity of membrane-localized alpha-synuclein. Most of the known function of ITSN2 is related to endocytosis. It is possible that ITSN2 induce toxicity in SNCA models by interfering with the endocytosis process, originated from the plasma membrane. To identify the localization of ITSN2 in yeast I tried to add a fluorescence tag at either the N- or C-terminal of the protein (Figure 3.8). Interestingly the C-terminal tagged and N-terminal tagged ITSN2 showed different localization of the protein (Figure 3.8). N-terminal tagged ITSN2 localizes to the plasma membrane, while C-terminal tagged ITNS2 forms cytoplasmic foci. These findings suggest that cellular localization of ITSN2 is regulated by its N- and C-terminal domains, which are known to bind membranes containing PIP2 and CDC42, respectively (108). Unfortunately, neither version of the tagged ITSN2 was able to induce toxicity in the 1xSNCA model (Figure 3.9 and Figure 3.11). This suggest that tagging the ITSN2 affect its enhancer ability. To understand the actual localization of the ITSN2 we can do immunostaining using ITSN2 specific antibody (125). This will allow us to visualize the location of ITSN2 within the yeast cell without tagging it.

**ITSN2 delays endocytosis and induce depolarized actin structures in SNCA models**

It was evident that the expression of ITSN2 together with alpha-synuclein drastically delayed the endocytosis process. We used a FM4-64 dye to monitor endocytosis in yeast. The progress of endocytosis in yeast are measured by the internalization of the FM4-64 dye. When SNCA and ITSN2 is co-expressed in yeast, we observed that the endocytosis vesicles failed to be targeted to the vacuoles and some appear to be trapped in the plasma
membrane even after 45 minutes of chasing (Figure 4.6 and 4.7). This is a huge delay compared to the wild type yeast which completes membrane invagination and vesicle scission within a few seconds (126). Consistent with a delay of endocytosis at the plasma membrane, we found actin patches, corresponding to membrane invagination sites of endocytosis, formed at the plasma membrane but appear to be depolarized when SNCA and ITSN2 are co-expressed (Figure 6.2). We suggest this delay of endocytosis might involve the yeast homologs of ITSN2, all of which regulate endocytosis in yeast and require membrane localization of alpha-synuclein.

**ITSN2 does not complement its homologs function in yeast and SLA1 enhances**

**ITSN2 mediated alpha-synuclein toxicity**

The ITSN2 gene has three homologs in yeast named Sla1, Ede1, and Pan1. The deletion of *SLA1* or the yeast homolog of ITSN2 cannot be complemented by ITSN2. Instead, ITSN2 enhances the growth defects of the △sla1 mutant (Figure 4.8) likely by further delaying the endocytosis defects (Figure 4.9). In addition, deletion of *SLA1* enhances the toxicity of alpha-synuclein either alone or together with ITSN2 expression (Figure 4.10). Sla1 is a late coat protein and shares homology with ITSN2 by its SH3 domains (112). Sla1 forms a complex with Ede1 and Pan1 resembling functional homolog of ITSN2. Sla1 has an intimate functional relation with Sla2. Sla2 is a middle coat protein which is essential for endocytosis. Sla2 interacts with both early and late cote proteins. Importantly, deletion of both *SLA1* and *SLA2* is lethal to yeast (112). We therefore speculate that ITSN2 might interact with Sla2 function thus enhancing growth defects of
the SLA1 deletion mutant (Figure 4.8). For example, ITSN2 might block other late coat proteins (including Sla1) to interact with Sla2. In this regard, a lack of any growth of the SLA1 deletion strain bearing SNCA and ITSN2 plasmids (Figure 4.10) suggests that SNCA and ITSN2 co-expression may disrupt the function of Sla2. So, an additional deletion of SLA1 is lethal to yeast. It is worth noting that deletion of SLA2 exhibits similarly depolarized actin (131) as observed in the 1xSNCA model with ITSN2 expression. We cannot exclude the possibility of yeast proteins other than Sla2 are involved in the mis-regulation of actin polymerization and Cdc42 polarization in the 1xSNCA model with ITSN2 expression. But the observation of stranded endocytosis vesicle in different assays (Figure 6.2) is consistent with the idea that the delay of endocytosis occurs primarily at the plasma membrane, likely during the middle and late coat assembly or vesicle scission, involving the function of Sla1 and Sla2.

The enhancer effect of ITSN2 in the 1xSNCA model depends on its C-terminal domains

The C terminal of ITSN2 is critical for Cdc42 regulation. Because the DH domain of ITSN2 contains a GEF which is responsible for the activation of Cdc42 (108). In yeast, activated Cdc2 is localized to the polarized growth sites. Depolarize Cdc42 resembles a dysregulation of the protein. That’s exactly what we saw in figure 4.18 when both ITSN2 and alpha-synuclein was co-expressed. This suggest that the enhancer effect of ITSN2 in the 1xSNCA model might depend on its C-terminal domains. Consistent with our finding where we showed that C-terminal tagged ITSN2 failed to induce alpha-synuclein toxicity
in 1xSNCA model (Figure 3.11). In addition, ITSN2\textsuperscript{short}, which does not have the C-terminal part, also failed to induce toxicity in 1xSNCA and NoTox models (Figure 4.14 and 4.15). Finally, ITSN2\textsuperscript{short} expression does not lead to severe endocytosis delay in the 1xSNCA model (Figure 4.16). These findings support our hypothesis that C-terminal DH domains is responsible for ITSN2 induced alpha-synuclein toxicity. To further confirm this hypothesis, we can generate a mutant ITSN2 lacking only the C-terminal DH domain and test if it is able to induce alpha-synuclein toxicity.

**Expression of ITSN2\textsuperscript{short} form induces abnormal actin clumps**

The endocytosis regulation is tightly regulated. The initiation phase takes relatively long time, during this time the plasma membrane is not bent. But once the marker proteins, coat proteins get assembled on to the plasma membrane the invagination starts. This step is driven by actin polymerization, it is very well orchestrated and happens within very defined time points (126). When ITSN2 and SNCA is co-expressed we observed completely depolarized actin patches (Figure 4.13). But when we co-expressed ITSN2\textsuperscript{short} and SNCA we observed some polarized along with some depolarized actin structures which might be due to some growth condition variation. But one striking observation was consistent in repeated experiment which is the presence of large clumps of actin (Figure 4.17). ITSN2\textsuperscript{short} could induce these abnormal clumps of actin in both 1xSNCA model and in WT yeast. This phenotype has been observed in some yeast mutants (127). It often happens when actin polymerization at the plasma membrane for endocytosis is misoriented. Instead of pulling the invagination of the plasma membrane for
polymerizing, it goes to the opposite direction. Our findings suggest that the direction of actin polymerization is perturbed in cells with ITSN2\textsuperscript{short} expression. The ITSN2\textsuperscript{short}, lacking a C-terminal domain to act on Cdc42, still contains the N-terminal that capable of binding to membrane lipids and the middle region with protein-binding ability. The N-terminal of ITSN2 interacts with crucial lipid regulator of endocytosis, PI4,5P. The middle SH3 domains may bind to endocytosis proteins (108). We speculate that ITSN2\textsuperscript{short} interacts with a network of lipids and proteins which impair proper actin polymerization, although such mis-regulation does not lead to severe endocytosis delay or growth defects in the 1xSNCA model.

\textbf{ITSN1 does not induce toxicity in the 1xSNCA model}

Although ITSN1 and ITSN2 is highly similar, we didn’t observe a similar enhancer effect for ITSN1 in non-toxic SNCA yeast models (Figure 4.19). One possibility could be that the two intersectins have different interacting partners in yeast. This could be due to the differences in their respective protein-protein domains of the two proteins. Indeed, it has been reported that ITSN1 and ITSN2 may interact with different proteins (128). Different interacting proteins of the two intersectins may explain their different effects on alpha-synuclein toxicity. It is worth mentioning that although ITSN1 fails to induce growth defect in the 1xSNCA, but it leads to enlarged cell size (Figure 4.20). However, this enlargement of cell size is less pronounced as compared to those in the 1xSNCA model expressing ITSN2. It is possible that cellular defects induced by ITSN1 is not enough to
affect growth. I can clone ITSN1 in a high-copy plasmid (e.g. pAG426GAL1-ITSN1) to expression ITSN1 at a higher level in the 1xSNCA model and test its enhancer effect.

The ITSN2 enhancer mechanism is different in the IntTox model

Interestingly, the ITSN enhancer effect was different in the IntTox model as compared to the 1xSNCA model. Notably, all three proteins, ITSN1, ITSN2, and ITSN2\textsuperscript{short}, induce toxicity in the IntTox model (Figure 4.26), yet neither ITSN1 nor ITSN2\textsuperscript{short} acts as an enhancer in the 1xSNCA model. Alpha-synuclein is almost exclusively localized to the plasma membrane In the 1xSNCA model, while in the IntTox model, alpha-synuclein forms cytoplasmic inclusions. Apart from the difference in alpha-synuclein localization, the IntTox model has defective ER to Golgi transport (45). Exocytosis is an essential cellular process for yeast to grow. Given that ITSN1, ITSN2, and ITSN2\textsuperscript{short} do not increase alpha-synuclein inclusions (Figure 4.27), nor do they increase the cell size or exacerbate endocytic defects (Figure 4.29) in the IntTox strain, we speculate that intersectins may enhance the toxicity of alpha-synuclein by further impairing exocytosis. To test this hypothesis, I can monitor secretory pathway or ER-to-Golgi transport in IntTox strains expressing ITSN1, ITSN2, and ITSN2\textsuperscript{short}.

Genome-scale genetic screening of human suppressor genes

The development of the IntTox model allows the initiation of new research to study the toxicity of alpha-synuclein using yeast as a model system. This model is particularly
useful to identify suppressor genes that can alleviate the toxicity of alpha-synuclein and unveil molecular mechanism of alpha-synuclein toxicity. Given that many human genes do not have yeast homologs, therefore they modifier effect cannot be readily inferred from yeast suppressor genes by homology. Towards an effort to identify human genes involved in the regulation of alpha-synuclein toxicity, we used a newly constructed yeast expression human gene library to identify human genes that suppress alpha-synuclein toxicity in the IntTox model. Among the identified 141 human suppressor genes, two functional groups were enriched, the Homeobox proteins and genes involved in ribosome biogenesis.

**Ribosome proteins suppressing the toxicity of alpha-synuclein in the IntTox**

In previously performed genetic screens in yeast, 13 yeast ribosome subunits and other proteins involved in translation regulation have been identified as suppressors of alpha-synuclein toxicity (129). The suppressor effect of the human ribosome-associated proteins is not very surprising considering that ribosome genes are highly conserved throughout eukaryotes. Ribosome proteins play a pivotal role in protein synthesis. Ribosome assembly and translation are essential cellular processes that require the coordination of many (>200) ribosome assembly proteins (130). This process is linked to diverse fundamental processes, including cellular growth and cell division. Identification of ribosome subunits as suppressors suggest possible translational defects induced by toxic levels alpha-synuclein.
Homeobox proteins suppressing the toxicity of alpha-synuclein in the InTox

The human homeobox suppression of alpha synuclein is surprising because many these genes are specialized in embryonic development, a process that is absent in the unicellular yeast. To understand how human homoeobox proteins might target exert suppressor effects, I tested the effect of a yeast homeobox protein on the toxicity of alpha-synuclein. Yeast genome has seven genes encoding five homeobox proteins. All of them are transcription factors. Four genes encode two transcription factors, A1 and Alpha 2, responsible for the regulation of the mating type specific genes in yeast. In haploid yeasts, several genes are turned on only in one but not the other mating type. In diploid cells, both A1 and Alpha 2 are expressed, which silence haploid specific genes.

We wondered if human homeobox proteins may suppress the toxicity of alpha-synuclein by silencing haploid-specific genes. Indeed, the yeast homeobox protein, alpha2 (encoded by the HMLALPHA2 gene, suppresses alpha-synuclein toxicity in the IntTox model (Figure 5.10). Expression of alpha2 in haploid IntTox model should silence the haploid specific genes. Notably, it has been reported that expression of HMLALPHA2 in haploid yeast induces bipolar budding, typical for diploid yeast (116). Consistent with the hypothesis that silencing haploid-specific genes rescues alpha-synuclein toxicity, diploid IntTox model does not show any growth defect and exhibits less alpha-synuclein inclusions (Figure 5.12). In contrast, expression of yeast AXL1 enhances the toxicity of alpha-synuclein in the IntTox model (Figure 5.16). AXIL is a yeast gene when overexpressed increase axial budding, typical for haploid cells (117). These findings support our hypothesis that haploid specific genes modulate alpha-synuclein toxicity.
**Cdc42 is mislocalized and sequestered to alpha-synuclein inclusions in the IntTox model**

Both ribosome and homeobox proteins change the budding pattern of yeast. Suggesting that polarized growth critically underlies alpha-synuclein toxicity. We have shown intriguing connections of ITSN2 and Cdc42 in the low expression alpha-synuclein models. Cdc42 is a master regulator of polarized cell growth in yeast. So, we tested whether Cdc42 could also be involved in the IntTox model toxicity. As expected, Cdc42 is mislocalized and sequestered to the alpha-synuclein inclusions in the IntTox model. This sequestration could compromise the normal function of Cdc42, we don’t know the exact mechanism how this sequestration is contributing to the SNCA toxicity. It would be interesting to determine cellular localization active form of Cdc42 in the IntTox model.

Unfortunately, we could not use the Gic2-PBD-RFP plasmid in the IntTox model as we did for the non-tagged 1xSNCA model monitor Cdc42 activation. The signal of the YFP-tagged alpha-synuclein in the IntTox model bleeds through the DsRed filter, which we use to capture Gic2-PBD-RFP that binds activated form of Cdc42. We can reengineer the Gic2-PBD-RFP plasmid to change the tag from RFP to mTFP1. This should allow us to visualize both alpha-synuclein and activated Cdc42 and confirm if there is a defect in Cdc42 polarization in the IntTox model.

Extra plasmid copy of Cdc42 does not suppress the toxicity of the IntTox model (Figure 5.9), arguing against a dosage-dependent effect (i.e. sequestration of Cdc42 to alpha-synuclein inclusions limit the availability of the protein). It is worth mentioning
that the promoter we used in these experiments is a constitutive promoter (the GPD promoter), which is less active on galactose. It is possible that more Cdc42 protein is needed to rescue toxic phenotype of the IntTox model. To test this, I can clone Cdc42 under the GAL1 promoter or expresses it from high-copy number plasmid.

**CONCLUSION**

Membrane-associated localization, dosage-dependent formation of cytoplasmic inclusions and cellular toxicity of alpha-synuclein can be faithfully recapitulated in a simple eukaryote, the budding yeast, *Saccharomyces cerevisiae*. Genetic screens of yeast cells expressing toxicity level of alpha-synuclein have identified hundreds of suppressor and enhancer genes, revealing complex cellular processes underlying alpha-synuclein toxicity (81). Mammalian homologs of several yeast modifier genes were found to have a similar effect in neurons, supporting the existence of conserved mechanisms pertinent to alpha-synuclein toxicity in yeast.

We reasoned that current genetic approaches studying toxicity of alpha-synuclein in yeast have two limitations. First, yeast models containing toxic levels of alpha-synuclein might not be effective in uncovering factors that trigger the initial molecular events promoting alpha-synuclein inclusions and toxicity. Second, human genes without yeast homologs cannot be identified in genetic screens using only yeast genes.

To address these limitations, we developed a new SNCA model, 1xSNCA, expressing alpha-synuclein at a much lower level that does not lead to inclusions or
toxicity in yeast. In this new model, alpha-synuclein localizes to the polarized growth sites, consistent with it being targeting to the plasma membrane via polarized exocytosis. Further, we constructed an overexpression library containing ~15,000 human-gene clones. Using this library, we identified human-gene enhancers and suppressors of alpha-synuclein toxicity. Five enhancer genes identified using the 1xSNCA model all have membrane-associated functions, including vesicle-mediated cellular trafficking, cytoskeleton or cell polarity regulation, and lipid biosynthesis. Over one third of the suppressor genes, identified in the toxic model, are homeobox proteins or proteins involved in ribosome biogenesis. Characterization of human enhancer and suppressor genes suggest that alpha-synuclein, when expressed in yeast, affects endocytosis, exocytosis, and Cdc42 regulated polarized growth.

Membrane trafficking in yeast, while it undergoes polarized growth, involves tightly coupled polarized exocytosis and endocytosis at an “active zone” of budding. Such membrane-associated cellular events resemble similar process in a presynaptic nerve terminal, where both exocytosis and endocytosis are under precise control and involve proteins highly conserved from yeast to human. It would be extremely exciting to validate these the enhancers and suppressors in neurons or animal models. Such efforts will provide new insights into the genetic buffering in neurodegeneration and expand our understanding of PD from a global perspective.
Figure 6.1. The protein domain structure of ITSN1 and ITSN2.

The human intersectins showing two Eps15-homology (EH) domains, an extended KLERQ coiled-coil domain, five Src-homology 3 (SH3) domains (A to E), and C-terminal DH domains with PH and C2. The length of long and short form is mentioned above the structure. The protein domain graphic was generated using Autodesk Sketchbook (www.sketchbook.com) and Microsoft PowerPoint.
Figure. 6.2. The ITSN2 induced toxicity of alpha-synuclein in the 1xSNCA model leads to stranded endocytosis vesicles and depolarized actin patches. The haploid 1xSNCA yeast strain was transformed with the pAG416GAL1-ITSN2 expression plasmid. These yeast cells were precultured in raffinose media. After overnight growth, they were shifted to media containing 2% galactose at OD$_{600}$ 1.0 and grown for six hours to induce the expression of SNCA. For **FM4-64 assay**: Live yeast cells were incubated with lipophilic dye FM4-64 to monitor endocytosis at 30°C, and viewed at 45 minutes time points at 100x magnification in the TxRED (for FM4-64) filter. For **actin staining**: Cells were fixed with 3.7% formaldehyde and stained with Rhodamine Phalloidine to visualize actin in the dsRED filter at 100x magnification. Scale Bar = 10 μm.
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Abbreviations

CPY (Carboxypeptidase Y)

DTT (Dithiothreitol)

ECFP (Enhanced Cyan Fluorescent Protein)

EGFP (Enhanced Green Fluorescent Protein)

EH (Eps15-homology)

ER (Endoplasmic Reticulum)

EYFP (Enhanced Yellow Fluorescent Protein)

FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl) pyridinium dibromide)

GWAS (Genome-Wide Association Study)

HIS (Histidine)

LEU (Leucine)

LiAc (Lithium acetate)

OD (Optical Density)

ORFs (Open Reading Frames)

PAGE (Polyacrylamide Gel Electrophoresis)

PCR (Polymerase Chain Reaction)

PD (Parkinson’s Disease)

PEG (Polyethylene Glycol)

PGK1 (phosphoglycerate kinase 1)
PLD (Phospholipase D)

PVDF (Polyvinylidene Difluoride)

RCF (Relative Centrifugal Force)

RPM (Revolutions Per Minute)

SDS (Sodium Dodecyl Sulfate)

SEM (Standard Error Of The Mean)

SH3 (Src-homology 3)

SNARE (Soluble NSF (N-Ethylmaleimide-Sensitive Factor) Attachment Protein Receptor)

TBST (Tris-Buffered Saline + Tween 20)

TRP (Tryptophan)

URA (Uracil)

VAMP (Vesicle associated membrane proteins)

WT (Wild Type)

YPD (Yeast, Peptone, Dextrose)