Alpha-Synuclein Toxicity is Caused by Mitochondrial Dysfunction

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Abstract

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, affecting roughly 1% of the population over the age of sixty years. Alpha-synuclein (aSyn) is a protein implicated in both familial and idiopathic forms of PD, yet despite the wealth of data implicating aSyn as a causative agent in PD, the mechanisms underlying its toxicity remain mostly unknown. Mitochondrial dysfunction is a major hallmark of PD, yet there is only limited evidence linking aSyn toxicity to mitochondrial dysfunction. My study establishes a novel aSyn model in respiring yeast cells, which allows me to explore how aSyn affects mitochondrial homeostasis and function. My data shows that mitochondrial fission and fusion, ER-mitochondria communication, and sphingolipid metabolism, interact genetically with aSyn toxicity. My work, therefore, indicates that aSyn impairs mitochondrial homeostasis, which might be a key contributor to neurodegeneration in PD.

Key Words

Parkinson’s disease, alpha-synuclein, neurodegenerative disorders, mitochondria, oxidative stress, mitophagy, mitochondrial fission/fusion, aggregation, oxidative phosphorylation, yeast model.
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List of Abbreviations

2xYT — 2x yeast extract tryptone
72Q — polyQ-expanded huntingtin protein
AD — Alzheimer’s disease
Amp — Ampicillin
BCA assay — Bicinchoninic acid assay
BSA — Bovine serum albumin
DMSO — Dimethyl sulfoxide
DNA — Deoxyribonucleic acid
ER — Endoplasmic reticulum
Gal — Galactose
GFP — Green fluorescent protein
H2O2 — hydrogen peroxide
HD — Huntington’s disease
Kan — Kanamycin
LB — Lysogeny Broth
Li — Lithium
MAT — Mating type
MPP+ — 1-methyl-4-phenylpyridinium
mRNA — Messenger RNA
NEM — N-Ethylmaleimide
O2^- — superoxide
OH^- — hydroxide radicals
OD600 — Optical Density at 600 nm
PBST — Phosphate buffered saline with tween
PCR — Polymerase chain reaction
PD — Parkinson’s disease
PEG — Polyethylene glycol
PGK-1 — Phosphoglycerate Kinase 1
PMSF — Phenylmethylsulfonyl fluoride
RNA — Ribonucleic acid
ROS — Reactive oxygen species
SD — Selective dextrose
SDD-AGE — Semi-Denaturing Detergent Agarose Gel Electrophoresis.
SDS — Sodium dodecyl sulfate
SDS-PAGE — Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGal — Selective galactose
SOD2 — Superoxide dismutase 2
TE — Tris-EDTA
UPS — Ubiquitin proteasome system
WT — Wild type
YNB — Yeast nitrogen base
YPD — Yeast extract peptone dextrose
Chapter 1

1 Introduction

1.1 Parkinson’s Disease

Parkinson’s Disease (PD) is the most common motor disorder and the second most common neurodegenerative disorder, with approximately 1% of the population over the age of 60 suffering from PD (1). Males are more likely to be affected by PD than females, at a ratio of 3:2, and the average life expectancy following diagnosis is 7-14 years (2). Symptoms of PD can be divided into motor and non-motor symptoms. Non-motor symptoms, while not present in all cases, include general mood disorders, depression and anxiety, dementia, sleep disorders as well as cognitive symptoms (3). Generally, cognitive symptoms entail difficulties with executive functioning, such as planning, controlling attention, and increased impulsivity. It is generally accepted that most non-motor symptoms present in the later stages of the disease, yet some evidence suggests certain cognitive disturbances might actually be the earliest sign of PD (4). Motor symptoms comprise what is termed “parkinsonism”, and include bradykinesia (slowed movements), resting tremor, forward gait, and decreased facial expressions (5).

The cause of these motor symptoms can be explained by examining the areas of the brain affected in PD, most notably the pars compacta of the substantia nigra in the midbrain, which is enriched in dopaminergic neurons. Approximately 80% of neurons in the pars compacta have already died by the time of diagnosis in most patients (6). The substantia nigra comprises part of a network of neurons termed the basal ganglia. The basal ganglia is generally involved in motor control and coordination, motivation, decision making, and control of eye movement. The pars compacta, more specifically, appears to be linked to motor control, albeit indirectly (7). For example, electrical stimulation of the pars compacta does not result in movement (as it would from stimulating the primary motor cortex), yet lesions in the pars compacta lead to Parkinson’s-like symptoms (8). As PD progresses, other parts of the brain begin to be affected by neurodegeneration, possibly accounting for the appearance of non-motor symptoms in later stages of the disease (9).
The most prominent pathological hallmark of PD is the appearance of Lewy bodies, which are large protein aggregates composed primarily of the protein alpha-synuclein (aSyn) (10). Initially, Lewy bodies were thought to cause neurodegeneration, yet recent evidence indicates a protective function (11).

Figure 1: A PET scan displaying dopamine storage capacity in basal ganglia of an unaffected (left) and PD affected brains (right) (132) B Cartoon depicting regions of the brain affected in PD (134). C Immunohistochemistry staining of a Lewy body (133).
The exact mechanisms underlying the cause of PD remain unknown, however, many causative agents have been identified (13). While the majority of cases of PD are idiopathic, i.e. without a known cause, a subset of approximately 5-15% of cases arise through inherited genetic mutations (14). These cases are termed familial PD (fPD) and have contributed much to our understanding of the causes of PD, generally. For instance, we now know that aSyn has a causative role in both idiopathic and fPD, and that mitochondrial dysfunction is intimately linked to PD, as mutations in three separate genes (PINK1, PARK2, PARK7) involved in mitochondrial homeostasis result in fPD (15).

There is presently no cure for PD nor are there therapies that halt or delay neurodegeneration. There are, however, treatment options, which provide relief from PD symptoms (16). Most common is treatment with the drug L-DOPA. L-DOPA is a molecular precursor of the neurotransmitter Dopamine (DA), which is converted into DA by neurons (17). As the neurons affected by PD are predominantly dopaminergic, the goal with L-DOPA therapy is to increase the amount of DA in the remaining neurons and compensate for the lack of synaptic activity resulting from the neurodegeneration of dopaminergic (DAergic) neurons (17). However, L-DOPA loses effectiveness after approximately five years and has undesired side effects, such as dyskinesia (18). The reason for the loss in efficacy is not completely understood, yet it is believed to be caused by the continual neurodegeneration of the remaining DAergic neurons, which necessitates treatment with higher L-DOPA doses, resulting in increased side effects (18). As a means of postponing the loss in efficacy, other drugs such as MAOIs (monoamine oxidase inhibitors) are often used in advance or in unison with L-DOPA. MAOIs work by inhibiting MAO, an enzyme which breaks down DA in the synaptic cleft (19). With the inhibition of MAO, DA can remain in the synaptic cleft longer and stimulate the post-synaptic neuron for an extended period of time. Studies seeking to determine whether treatment with MAOIs alone delays progression of PD symptoms compared to treatment with L-DOPA have remained inconclusive (20).

Other more invasive methods of treatment, such as deep brain stimulation, are applied regularly, yet the efficacy of such treatments remains debated (21). For this reason, it is paramount that the underlying causes of PD are uncovered so that targets for improved therapies can be identified.
1.2 Alpha-Synuclein

Alpha-Synuclein (aSyn) is an intrinsically disordered protein composed of 140 amino acids encoded by the SNCA gene (22). The N-terminal region of aSyn contains an amphipathic 60 residue sequence, while the central NAC (non-amyloid-β component) region (residues 61-95) is highly hydrophobic and involved in aggregation. The remaining residues 96-140 compose an unstructured highly acidic region (23).

aSyn can adopt an extended helical structure when bound to phospholipid bilayer membranes, yet it can also exist in many other forms when bound to other membranes, individual lipids, or other proteins (23). aSyn can exist both as a monomer, oligomer, protofibril, fibril, Lewy neurite, and finally as the main component of a Lewy body (24). Many of the conformational changes in aSyn are due to posttranslational modifications (25). Phosphorylation, nitration and modifications by DA and DA derivatives increase aSyn oligomerization (26). DA-aSyn modifications reduce degradation by chaperone-mediated autophagy of aSyn, while phosphorylation at Ser-129 also reduces aSyn clearance and degradation (27). Samples from PD unaffected brains show only 4% of Ser-129 phosphorylated aSyn, whereas PD patient samples reveal approximately 90% of aSyn in Lewy bodies is phosphorylated at Ser-129.

While the exact function of aSyn under non-pathological conditions remains mostly unknown, much has recently been uncovered regarding its’ biochemical and cellular properties both in vitro and in vivo. aSyn is believed to be involved in synaptic vesicle regulation during neurotransmitter release (28). Concordantly, aSyn has a high affinity for rounded lipid membranes and binding to these membranes can result in a conformational change of aSyn and also the bound membrane (29). Also, aSyn interferes with presynaptic terminal function, leading to a retraction of post-
synaptic dendrites, which is, at least according to some studies, the major cause of PD symptoms (30).

aSyn is also known to display great seeding ability, i.e. when injected into healthy brains, aggregated forms of aSyn can induce aSyn aggregation and neurodegeneration throughout many brain regions (31). Lewy bodies are the major pathological hallmark of PD, and it is through the study of Lewy bodies that aSyn was first identified as a potential causative agent in PD. Indeed, aSyn has a causal role in familial PD, as duplications or triplications of the SNCA gene leads to familial PD, as do several point mutations in SNCA (Figure 2) (32). Moreover, the evidence is overwhelming that aSyn also plays a causative role in idiopathic PD, as the vast majority of idiopathic cases show Lewy bodies (31, 30, 23). Due to the virtual omnipresence of Lewy bodies in PD, they were initially believed to be the most toxic species of aSyn and thus cause neurodegeneration, yet this idea has recently been challenged, with many studies indicating a protective role for Lewy bodies (24). For example, the number of Lewy bodies does not correlate with the severity of PD symptoms and approximately 10% of neurologically healthy individuals over the age of 60 have been found to contain Lewy bodies. Furthermore, certain forms of familial PD (i.e. those caused by PARK7 mutations) do not present with Lewy bodies at all (33). Thus, it is reasonable to assume that Lewy bodies can, in fact, be protective, acting as a compactor to sequester the more toxic forms of aSyn (34). In line with this idea, much evidence in the past decade indicates that aSyn oligomers are the most toxic form of aSyn (35).

Oligomers are aggregated forms of aSyn that are not yet fibrillar in nature. They can vary in molecular weight, with smaller unstable oligomers show weak seeding ability, while larger oligomers are more stable and have a high propensity for seeding (36). Evidence for the toxicity of oligomers come from both in vivo and in vitro studies (35, 37). Oligomers have been consistently identified in the areas of the brain most affected by neurodegeneration in PD patients (38). Oligomers also result in cell-death in cell models that develop aSyn-oligomers either through overexpression of aSyn or ectopic exposure to oligomers formed in vitro (24). Furthermore, expression of aSyn mutants that are more prone to oligomerization results in increased toxicity in both C. elegans and Drosophila models (39). Yet oligomers are unlikely the only toxic species of aSyn, as some evidence suggests Lewy neurites to contribute to PD, while other evidence suggests smaller, soluble forms of aSyn might be the most toxic (40).
In any case, aSyn has been shown to interfere with a variety of cellular functions, which can lead to PD-associated neurodegeneration. While the earliest and most dominant pathways leading to aSyn-induced toxicity remain unknown, a few main players have been identified (41). For instance, aSyn interferes with lysosomal function and autophagy (42). In mammalian cell models, bafilomycin, an inhibitor of autophagy, leads to an increase in aSyn aggregation and cell death. Conversely, treatment with rapamycin, a drug, which induces increased autophagy, reduced aSyn toxicity (43). There is also evidence that aSyn, more specifically aSyn oligomers, impair protein degradation by the 26S proteasome (44). The aSyn-yeast model, which will be discussed in detail below, highlights ER-Golgi trafficking and lipid metabolism as the main pathways through which aSyn elicits cellular toxicity (45).

My work presented here focuses on mitochondrial homeostasis as a major cellular pathway associated with aSyn toxicity. aSyn inhibits mitochondrial fusion in mammalian cell models, resulting in rounded, sometimes fragmented mitochondria (46). aSyn can also be imported into both the mitochondrial matrix and inner membrane space, where it inhibits complex I of the electron transport chain (47). Furthermore, mammalian cell models show that aSyn inhibits calcium import to the mitochondria from the ER by binding to the ER-mitochondria tethering protein VAPB (48). Despite the wealth of data regarding aSyn-mediated cellular dysfunctions, the “smoking gun” has still not been found and it remains mostly unclear to what degree each of the proposed mechanisms induces aSyn toxicity and contribute to PD. It is possible that one pathway leads to toxicity much earlier than others, or that they all equally and simultaneously contribute to aSyn toxicity. Much more work is therefore needed to better understand how aSyn contributes to PD.
1.3 Alpha-Synuclein Models

**Figure 3: Model systems to study aSyn.**
A Microscopic image of differentiated SH-SY5Y cells (49).
B Microscopic image of C. elegans (133).
C Drosophila melanogaster or fruit fly (134).
D Mouse (135)

To better understand the normal and pathological roles of aSyn in PD, various model systems and organisms have been utilized. These model systems range from tissue culture cells to unicellular organisms to non-mammalian and mammalian animal models.

One well-established aSyn mammalian cell model is the SH-SY5Y neuroblastoma cell line (Figure 3A). This cell line is advantageous as it is easy to grow, has dopaminergic characteristics, and can be differentiated into neuron-like cells. For instance, SH-SY5Y cells have led to discoveries regarding the nature of aSyn degradation, aSyn’s role in calcium homeostasis, and mitochondrial dysfunction (50). The disadvantages of this model are that SH-SY5Y cells are not truly
differentiated neuronal cells in the brain and that aSyn toxicity and aggregation arise only after extremely high aSyn expression or addition of other cellular stressors (48). Furthermore, these cells are generally cultured with glucose as a carbon source, causing approximately 90% of ATP to be generated through glycolysis (47). As neurons generate approximately 90% of their ATP through oxidative phosphorylation, this model does not provide an accurate representation of the metabolic activity that occurs in the neurons affected in PD, such as many processes related to mitochondria (51).

Animal models, which are most commonly used to model aSyn-toxicity, include the nematode C. elegans, the fruit fly Drosophila melanogaster, and mice or rat models (Figure 3B, C and D respectively). C. elegans are extremely useful to aSyn research since their entire connectome (nervous system) contains only eight DAergic neurons, which makes dopamine-related neurodegeneration quite easy to follow (52). The Drosophila model is advantageous as aSyn overexpression leads to a high level of degeneration of dopaminergic neurons and motor dysfunction (53). Finally, the rodent models are interesting models as they are phylogenetically much closer to humans and thus have a nervous system, which is quite similar to humans (54).

However, animal models are not as ideal to study molecular and cellular mechanisms as cell models because of technical limitations. Furthermore, while the rodent nervous system is more similar to the human than that of C. elegans and of the fruit fly, rodent models still have severe limitations for PD research. For example, overexpression of aSyn does not lead to a pattern of neurodegeneration or behavioural symptomology that mimic PD (55). In fact, aSyn overexpression in mice does not lead to degeneration of the substantia nigra, the area most affected by neurodegeneration in PD. As an attempt to circumvent this problem, transgenic rodent models have been used in conjunction with small molecules, such as rotenone or MPTP, which result in neurodegeneration of DAergic neurons (55). The mechanisms and onset of drug-induced neurodegeneration, however, is not representative of the slowly progressing neurodegeneration that occurs in idiopathic PD. Collectively, while each aSyn model has its benefits, no model is perfect and thus different model systems must be utilized in accordance with the specific goals of a study.
1.4 Alpha-Synuclein Yeast Model

**Figure 4: Saccharomyces cerevisiae aSyn Model.** A Spotting assay (left) and fluorescence microscopy (right) of GFP-tagged aSyn under low expression. B Spotting assay (left) and fluorescence microscopy (right) of GFP-tagged aSyn under high expression.

The aSyn yeast model was originally developed in 2003 by Outeiro and Lindquist (44). Their model utilized the yeast *Saccharomyces cerevisiae*, or budding yeast, with either high or low expression of WT aSyn or the PD-associated aSyn mutants A53T and A30P fused C-terminally to GFP (green fluorescent protein). As aSyn expression was regulated by a galactose-inducible promoter, most experiments were completed with galactose as the carbon source. Outeiro and Lindquist reported that aSyn aggregation and toxicity followed a strictly dose-dependent profile, with low expression resulting in no aggregation and no toxicity and high expression resulting in high levels of aSyn aggregation and toxicity (Figure 4). Toxicity was inferred by performing spotting assays and then measuring the change in colony forming ability and growth of these colonies of the aSyn-expressing yeast strain compared to the empty vector control. While cell-death is not explicitly measured, spotting assays are a well-established assay in yeast to assess the toxicity associated with misfolded proteins, including alpha-synuclein (44, 57, 55).
Many studies have since explored the genetic interactions of aSyn in this yeast model using high-throughput screens to uncover genetic enhancers and suppressors of aSyn toxicity. These screens have revealed that at high levels of aSyn expression, gene deletions related to lipid metabolism and ER-Golgi vesicle trafficking (56) strongly enhance aSyn toxicity. Conversely, genetic screens searching for genetic suppressors of aSyn-toxicity found that genes involved in ER-Golgi vesicle trafficking were the most robust suppressors of aSyn toxicity when overexpressed together with high levels of aSyn expression (57). The most successful genetic suppressor identified in these screens was YPT1, the yeast orthologue to the mammalian RAB1. YPT1 encodes Rab GTPase, an enzyme involved in ER-Golgi trafficking. Overexpression of the Ypt1 protein leads to increased ER-Golgi vesicle trafficking. In fruit fly and nematode aSyn-models, RAB1 overexpression also significantly reduced neurodegeneration of dopaminergic neurons caused by aSyn expression (55). Thus, findings from the yeast model are replicated in other model systems.

However, one problem with this aSyn-yeast model is that genes related to mitochondrial homeostasis have not been identified as major genetic modifiers of aSyn toxicity, which is much in contrast to studies in virtually all other models. As mitochondrial dysfunction is intimately linked both to PD and aSyn-related dysfunctions, this lack of aSyn-mitochondrial interaction appears to be a shortcoming of the aSyn yeast model.

One possible explanation for the lack of genetic interactions pointing to mitochondrial dysfunction could be the use of the carbon sources used in the majority of previous aSyn yeast studies. The overwhelming majority of yeast work relies on aSyn expression using a galactose-inducible promoter, thus galactose is used as the sole carbon source to induce the expression of aSyn (58). In yeast, galactose is first converted into glucose-6-phosphate and then metabolized by glycolysis, which is called fermentation in microbes (59). In fact, yeast cells that are provided with ample fermentable carbon sources (e.g. glucose or galactose) will preferentially utilize glycolysis/fermentation to produce ATP before switching to primarily oxidative phosphorylation once the fermentable carbon sources are depleted. Furthermore, yeast deletion strains, which are completely deficient in oxidative phosphorylation, are still able to grow in glucose and galactose as a sole carbon source (60).

Of note, in neurons approximately 85% of ATP is generated through oxidative phosphorylation, thus their metabolic profile is substantially different from fermenting yeast (45).
Therefore, we argue that aSyn does, in fact, interfere with yeast mitochondria, yet due to the use of galactose as a carbon source, these pathways could not be discovered in previous studies as the mitochondria were not required in these yeast models. To address this problem, we used non-fermentable carbons sources, such as glycerol, which forces the cells to produce ATP via oxidative phosphorylation combined with low expression of aSyn in our revised yeast model. Our aSyn yeast model creates a metabolic profile that more accurately represents the metabolic profile of neurons affected by PD (45). Also, as yeast grown with glycerol divide very slowly, they are more similar to neurons, which typically do not divide after maturation (61). Finally, as glycerol requires yeast to utilize their mitochondria to produce ATP, mitochondrial homeostasis will be crucial for cell growth, thus any disturbances to the mitochondria caused by aSyn will be obvious.

1.5 Autophagy in PD

Autophagocytosis, or more simply autophagy, is the process by which a cell breaks down or degrades various components of the cytoplasm (62). Autophagy can be divided into two distinct classes, macroautophagy and microautophagy (60). Both macroautophagy and microautophagy can be selective or non-selective processes. Macroautophagy begins by the formation of a phagophore, which forms around the cargo until the cargo is fully engulfed. Once the cargo is sequestered, the enclosed capsule is considered an autophagosome. The autophagosome will then fuse with the vacuole (yeast and plants) or lysosome (animals) where its content is degraded (63). As opposed to macroautophagy, in microautophagy cargo is not transported to the vacuole/lysosome by autophagosomes but is sequestered directly by invagination of the vacuolar/lysosomal membrane. Microautophagy encompasses the degradation of mitochondria, peroxisomes, and components on the nucleus (61).

Autophagy serves many cellular functions, such as a response to nutrient depletion, infection, and organelle quality control (60). Caloric restriction increases chronological lifespan in a variety of unicellular and multicellular organisms, yet this extension in lifespan is prevented by blocking autophagy (64). Furthermore, inhibition of autophagy produces elevated levels of oxidative stress (65). In fact, drugs, such as rapamycin, which increase autophagy have also been shown to increase chronological lifespan in both cellular and animal models (66). Increased autophagy also protects cells from oxidative stress and toxicity caused by protein misfolding (63). Conversely bafilomycin,
a drug that inhibits autophagy, exacerbates the negative consequences of oxidative stress and protein misfolding (67). For example, bafilomycin increases aSyn-toxicity in both cellular and animal models overexpressing aSyn (68).

Dysfunctional autophagy is implicated in many diseases, including neurodegenerative diseases. In Alzheimer’s disease, PD, and Huntington’s disease, it is believed that inadequate autophagy at least partially causes the accumulation of toxic protein aggregates (69). Thus, autophagy is a crucial process which is indispensable for cellular homeostasis and may play an important role in neurodegeneration.

1.6 Oxidative Stress in PD

Oxidative stress refers to the imbalance between the concentration of reactive oxygen species (ROS) and the cell’s ability to detoxify these ROS and counteract the damages they cause. Common ROS include superoxide, hydrogen peroxide and the hydroxyl radical (70). ROS cause damage via the redox reactions they form with various cellular components. ROS oxidize lipids, proteins and DNA, resulting in a variety of negative consequences. Lipids that are oxidized by ROS can form unstable lipid radicals, which can then trigger a chain reaction known as lipid peroxidation (71). Lipid peroxidation results in damage to cell membranes and the production of carcinogens or mutagens such as malondialdehyde (72). While all amino acid residues are susceptible to oxidation, cysteines are highly susceptible to oxidation due to their nucleophilic thiol containing side chain (68). Oxidation of proteins may result in a change of conformation, solubility, loss of function or gain of toxic function (69). DNA oxidation most often occurs at the guanine bases, which have the highest propensity for oxidation relative to adenine, cytosine and thymine (73). DNA oxidation can lead to increased mutations of the genome (71).

Under physiological conditions, it is still unknown what processes are most responsible for ROS generation. Generally, it is believed that the majority of ROS are created as a byproduct of oxidative phosphorylation (68, 69). However, studies have shown that the rate of ROS generation is unaffected in cells lacking the ability to perform oxidative phosphorylation (74). Nonetheless, oxidative stress and increased ROS production is certainly implicated in a variety of diseases and disorders. For instance, increased oxidative stress is a hallmark of ageing, cancer, Alzheimer’s disease, bipolar disorder, schizophrenia and PD (68, 69, 75). Yet whether oxidative stress is causative in these diseases remains unknown.
1.7 Mitochondrial Dysfunction in PD

Mitochondria, often touted the powerhouses of the cell, are involved in many cellular processes, including energy production and specific cell-signaling pathways (76), such as those regulating apoptosis. The main components of the mitochondria are the outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), mitochondrial matrix, mitochondrial cristae, and the intermembrane space (74). The mitochondrial matrix is enclosed by the IMM and harbours all enzymes of the Krebs cycle, mitochondrial DNA (mtDNA), which encodes 13 mitochondrial proteins in humans, and nine in yeast (77). The OMM is involved in many signaling pathways and the initiation of fission and fusion events (78). The IMM harbours the proteins that build the electron transport chain (ETC) and forms the mitochondrial cristae.

The ETC is composed of complexes I-V, with Complex V being the site of ATP synthesis within oxidative phosphorylation where ADP is phosphorylated to generate ATP (75). Mitochondria generate approximately 30 molecules of ATP per molecule of glucose, compared to only two molecules of ATP per glucose produced by glycolysis or fermentation (74). However, the ETC regularly releases reactive oxygen species (ROS), such as superoxide, as a byproduct. Increased levels of ROS (oxidative stress) damages nucleic acids, proteins, and lipids, which can lead to cellular dysfunction and death (79). To counteract the production of ROS that accompanies oxidative phosphorylation, the cell employs various antioxidants, such as SOD2, which localizes to the mitochondria and converts superoxide to hydrogen peroxide and diatomic oxygen (80). Increased ROS production and decreased antioxidant function are associated with the negative effects of ageing as well as many neurodegenerative diseases (78).

While ATP production through the ETC is the one major function of mitochondria, they are also involved in many other cellular processes. For example, mitochondria have been implicated in cell-cycle regulation, pro-and anti-apoptotic signaling pathways, and calcium homeostasis (81). Therefore, mitochondrial dysfunction can lead to a suite of distinct but connected issues. Indeed, mitochondrial dysfunction is implicated in numerous highly diverse diseases. These diseases can either be primarily caused by mitochondrial dysfunction or can be characterized by secondary mitochondrial dysfunction (82). Primary mitochondrial diseases include mitochondrial myopathies, specific forms of diabetes mellitus, and deafness and myoneurogenic gastrointestinal
encephalopathy (80). Diseases with secondary mitochondrial dysfunction include many neurodegenerative diseases, such as Huntington’s disease, Amyotrophic Lateral Sclerosis (ALS), Alzheimer’s disease and PD (83). Mitochondrial dysfunction in PD is a relatively recent finding, yet there is now abundant evidence suggesting a causal role in PD pathology.

First, there is strong evidence for high levels of oxidative stress in the neurons affected by PD. These neurons show heightened levels of oxidized lipids, DNA, and carbonylated proteins (73). As the main producer of ROS in neurons are mitochondria, this suggests mitochondrial dysfunction. Much evidence has also linked mitochondrial dysfunction and oxidative stress to PD. For example, MPTP is a toxin, which after conversion to MPP+, inhibits complex I of the ETC in DAergic neurons and causes Parkinsonism. Further, patient samples from people suffering from PD show decreased complex I activity. Interestingly, this mitochondrial defect has been found in skeletal muscle and platelets, suggesting global mitochondrial dysfunction beyond neurons is concordant with PD (84). Moreover, complex I dysfunction can cause both increased ROS and apoptosis (85).

Other evidence for mitochondrial dysfunction in PD comes from both patient samples and PD models, which often show mitochondrial fragmentation, complex I deficiency and mtDNA mutations (86). Mitochondrial fragmentation is generally associated with pro-apoptotic signaling, yet it can also be a result of general cell stress or defects in the process of mitochondrial fusion (87).

Genetic cases of PD have also implicated mitochondrial dysfunction in PD pathogenesis, as mutations in the genes encoding parkin, PINK1, and DJ-1, all of which are associated with mitochondrial homeostasis, cause familial PD. PINK1 and parkin are proteins, which regulate mitophagy, i.e. the degradation of mitochondria via autophagy, while DJ-1 is a mitochondrial molecular chaperone, which protects the cell against oxidative stress (32). Animal models have shown that overexpressing parkin or PINK1 can be protective from MPTP toxicity (88). Furthermore, cell models have shown that overexpressing parkin or PINK1 can reverse the fragmented mitochondria phenotype caused by aSyn overexpression. However, while aSyn has been shown to interfere with mitochondrial homeostasis, the evidence linking this mitochondrial dysfunction and the resulting toxicity remains incomplete and more conclusive evidence is needed before aSyn toxicity can be directly linked to mitochondrial dysfunction.
1.8 Mitochondrial Homeostasis

Mitochondrial homeostasis describes all cellular processes involved in the proper biogenesis, maintenance, and degradation of mitochondria, such as mitochondrial fusion and fission, de novo mitochondrial synthesis, degradation of mitochondria via autophagy (i.e. mitophagy), and the communication between mitochondria and other cellular organelles, such as peroxisomes and the endoplasmic reticulum (ER).

1.8.1 Mitochondrial Fission and Fusion

Figure 5: Schematic displaying some of the key proteins involved in mitochondrial fission.
Figure 6: Schematic displaying some of the key proteins involved in mitochondrial fusion.

Mitochondria are constantly undergoing two opposing mechanisms, mitochondrial fission and mitochondrial fusion. Mitochondrial fission is the process by which mitochondria divide or fragment, leading to an increased number of smaller mitochondria. Mitochondrial fusion, on the other hand, is the process by which mitochondria fuse together to create a larger mitochondrial network (89). Both mitochondrial fission and fusion are highly conserved processes in all eukaryotes, including yeast and human cells.
The protein machinery used to facilitate mitochondrial fission in mammalian cells contains the proteins Drp1, Fis1, Mff, MiD49 and MiD51. In budding yeast, the key players are Dnm1, the yeast homolog to Drp1, Fis1 and Mdv1 (87). Dnm1, a dynamin-related GTPase, begins the fission process by clustering on mitochondrial microtubules and initiating membrane constriction and excision (90). Microscopy shows Dnm1 is recruited to and clusters around select areas of mitochondria before fission occurs (91). Fis1 binds to the outer mitochondrial membrane and then, facilitated by Mdv1, binds to Dnm1, which together initiate mitochondrial membrane constriction and fission (89).

Mitochondrial fission is involved in processes, such as mitochondrial transport, mitophagy, and programmed cell death. Rodent knockout models have shown that fission is necessary for proper development, as Drp1 knockout studies in mice show prenatal lethality (92). In yeast, deletion of genes responsible for fission, such as DNM1 or FIS1 results in increased resistance to cell death, while cells also show defects in oxidative phosphorylation and mitophagy (87, 88).

The protein machinery necessary for mitochondrial fusion in mammalian cells contains the proteins Opa1 and the mitofusins Mfn1 and Mfn2. In yeast, the homolog for Opa1 is Mgm1, while Fzo1 is a conserved mitofusin. Two further crucial fusion machinery proteins are Ugo1 and Mdm30 (93). Studies suggest that Fzo1 is necessary to initiate the initial docking stage between two OMMs. Mgm1 mediates fusion of the IMM, which occurs separately, yet in temporal coordination with the fusion of the OMM (91). Ugo1 links Mgm1 and Fzo1 together to coordinate both IMM and OMM fusion. Mdm30 is required for Fzo1 ubiquitination, which initiates mitochondrial fusion (88, 91).

While our understanding of the cellular functions of mitochondrial fission and fusion remains incomplete, some progress can be reported. Both processes are crucial to mitochondrial homeostasis and function and, by extension, to general cell homeostasis (87). Furthermore, both processes seem to occur continuously, and often antagonistically. For example, deleting the gene FZO1 in yeast inhibits mitochondrial fusion and results in smaller, fragmented mitochondria, which is due to continued fission even in the absence of fusion. In contrast, deleting DNM1 produces the opposite phenotype, with elongated, sometimes globular mitochondrial networks. Interestingly, double deletion of both DNM1 and FZO1 results in normal mitochondrial morphology, indicative of the antagonistic nature of fission and fusion (87). Mitochondrial fusion
is necessary for both oxidative phosphorylation and mitochondrial biogenesis (94). Concordantly, cells that are deficient in mitochondrial fusion are unable to respire, have increased mtDNA mutations, decreased total mtDNA, and increased heterogeneity of mitochondrial content (87, 91). Mitochondrial content mixing was shown to be crucial as mitochondria that are unable to fuse their outer membranes have severe defects. Similar defects are observed for mitochondria that are able to fuse only their outer membranes and but not the inner membranes (95).

To conclude, both mitochondrial fission and fusion are opposing, necessary processes that work in concert to maintain mitochondrial homeostasis and general cellular health.

### 1.8.2 Mitochondrial Biogenesis and Mitophagy

![Figure 7: Simplified Depiction of Mitophagy](image)

Similar to mitochondrial fusion and fission, two other processes oppose each other in similar ways and are also critical to mitochondrial homeostasis. These two processes are mitophagy, and
mitochondrial biogenesis, or mitobiogenesis (96). Mitophagy is the selective degradation of mitochondria by autophagy, which can occur independently of general autophagy (94). Although the exact mechanisms and pathways of mitophagy are not completely understood, much progress has been made using both yeast and mammalian cell models (94).

Mitophagy occurs in three stages, starting with the initial fission of the mitochondrion to be degraded, followed by the engulfment into an autophagosome, and finally inclusion into the lysosome or vacuole (97). In yeast, the receptor Atg32 is required for the initiation of mitophagy. Mitophagy is initiated after Atg32 is phosphorylated and subsequently binds to the mitochondrial outer membrane (95). Atg32 then recruits various fission proteins to induce mitochondrial fission, and autophagy-related (ATG) proteins, such as Atg8, which facilitate the binding to isolation membranes. These isolation membranes fuse around the mitochondrion, resulting in an autophagosome that engulfs the mitochondrion targeted for degradation. The autophagosome then fuses with the lysosome or vacuole where the mitochondria are degraded (94, 95).

Mitophagy in mammalian cells occurs through a similar pathway, yet there are additional modes of mitophagy, which appear to be particular to mammalian cells. For example, there is no known parallel to PINK1/parkin-mediated mitophagy in yeast (98). In mammalian cells, loss of mitochondrial membrane potential causes the accumulation of the protein PINK1 at the OMM. PINK1 then recruits the protein parkin, an E3-like ubiquitin ligase, to the mitochondria where it is proposed to facilitate ubiquitination of the proteins in the OMM. Parkin is also believed to recruit various other downstream actors in mitophagy, such as LC3, the homolog of yeast Atg8, which is necessary for lysosome formation (99).

Mitophagy has been proposed to protect the cell against ROS and pro-apoptotic factors that mitochondria increasingly release during the course of ageing (100). In line with this theory, yeast cells deficient in mitophagy that are grown in non-fermentable carbon sources have a decreased chronological lifespan, increased ROS accumulation, and increased mtDNA mutations (101).

Mitobiogenesis describes the de novo synthesis of mitochondria. Mitobiogenesis involves the coordination of both the nuclear and mitochondrial genome to express mitochondrial proteins (102). In mammalian cells, there are two closely related master regulators of mitobiogenesis, PGC1-α and PGC1-β. PGC1-α facilitates mitobiogenesis by first activating the nuclear respiratory factor NRF-1, which in turn activates NRF-2. NRF-1 and NRF-2 work together with Sp1 (specific
protein 1) to begin expressing proteins necessary for mitochondrial biogenesis. Paramount to this process is the protein mTFA (mitochondrial transcription factor A), which is imported into the mitochondria where it activates expression of the proteins encoded by mtDNA (100). Mitochondrial biogenesis can be activated by many factors, such as cytosolic Ca\textsuperscript{2+} concentration, nitric oxide (NO), AMPK, SIRT1, and mitophagy. Generally, mitobiogenesis is upregulated in response to greater energetic demands (103). This can be a result of increased exercise in animals or decreased mitochondrial content on a cellular level (104).

While mitophagy relies solely on mitochondrial fission, mitobiogenesis relies on a concert of both fusion and fission (100, 101, 102). Mitofusins, for example, are downstream effectors of mitobiogenesis, while fission-related proteins, such as Drp1, are upregulated during mitobiogenesis (105). Mitochondrial fission may provide an increase in the number of individual mitochondria, which can be used as templates for mitobiogenesis (103). Mitochondrial fusion, however, appears necessary for the fusing of newly synthesized mitochondrial membranes (100). Indeed, mitophagy and mitobiogenesis appear to complement each other, with high levels of mitophagy often leading to an increase in mitobiogenesis (106).

Dysfunctions in mitobiogenesis have been implicated in numerous diseases and cell-death pathways (107). Overactivation of PGC-1\textalpha has been postulated as a cause of frataxin deficiency in Friedreich’s Ataxia (108). Yet many disorders relating to mitobiogenesis appear to be decreased and not increased PGC1-\textalpha activity. Insufficient mitobiogenesis, for example, has been implicated in Huntington’s disease, cardiovascular disease, ageing, diabetes, and renal diseases (100). In summary, both mitophagy and mitobiogenesis must be working in synchrony to maintain general cell health, as disruptions in either process can lead to cell dysfunction and death.
1.8.3 Prohibitins

![Diagram of Prohibitin Proteins]

**Figure 8: Simplified Representation of Prohibitin Proteins.** IMM= inter mitochondrial membrane and IMS= inter-membrane space.

Prohibitins are two highly conserved proteins, phb1 and phb2, encoded by the genes PHB1 and PHB2, respectively. They were originally named according to their role as negative regulators of cell proliferation, hence the name prohibitins. However, further analysis revealed the anti-proliferative activity of prohibitins is due to the untranslated 3’ region of the gene and not to the proteins themselves (109). The Phb1 and Phb2 are quite homologous, with approximately 50% identical amino acids. Each Phb has a hydrophobic region at the N-terminus, which anchors them to membranes, while the C-terminus flanks the cytosolic-facing coiled coil domain (110) (Figure 8). Together, Phb1 and Phb2 form large membrane-bound protein complexes approximately 1 MDa in size. Electron microscopy using prohibitins isolated from yeast revealed a ring-like structure, leading to the currently accepted model of the prohibitin membrane complex (107, 108).
Deletion of either PHB1 or PHB2 inhibit prohibitin complex assembly and the deletion of one prohibit encoding gene results in the reduction of proteins levels of the other prohibitin. This appears to be due to increased degradation of the remaining prohibitin protein as it can no longer form the prohibitin complex (107). Prohibitins have been purported to participate in a wide variety of functions, such as cell cycle regulation, transcriptional regulation, and apoptosis (111). However, prohibitins are best known for their role in mitochondrial homeostasis.

Prohibitins are involved in mitochondrial biogenesis, in establishing mitochondrial cristae morphology, and chaperone activity for mitochondrial proteins (109). As the exact molecular mechanisms by which prohibitins exert their functions remain somewhat unclear, most information relating to their function comes from knock-down, knockout, or gene deletion studies. For example, knock-down studies in mice and C. elegans reveal that prohibitins are necessary for embryonic development (112). In HeLa cells, knockdown of prohibitins results in fragmented mitochondria (109). Mouse embryonic fibroblasts revealed that a knockdown of either prohibitin resulted in abnormal mitochondrial cristae morphology (109).

Perhaps the most relevant finding to PD research is that Phb2 is a necessary receptor in parkin-mediated mitophagy (108). Phb2 binds to LC3 (ATG8 in yeast) following proteasomal-dependent OMM rupture. As Phb2 binds to LC3, then phagophores form around the mitochondrion eventually leading to mitophagy.

**1.9 Sphingolipids**

Sphingolipids are lipids that regulate many different cellular processes. Sphingolipids include ganglioside, cerebrosides, sphingomyelin, ceramide, and sphingosine. In terms of cellular structures, sphingolipids form lipid-rafts as parts of the cellular membranes (113). Sphingolipids are implicated in cell-cycle proliferation and regulation, ageing, endocytosis, stress responses, pro-survival and apoptotic pathway (114).

Most relevant to our work is the role sphingolipids play in mitochondrial-mediated apoptosis. Mitochondrial-mediated apoptosis occurs when certain stressors cause mitochondrial outer membrane permeabilization (MOMP), leading to the release of pro-apoptotic proteins, such as cytochrome c and Apaf1. These proteins will then initiate the caspase signaling pathway, an irreversible chain reaction culminating in cell death (112). How exactly sphingolipids induce these
events is not completely clear. One hypothesis is that aberrant sphingolipid levels physically change the permeability of the OMM leading to MOMP (115). Other studies have suggested that aberrant sphingolipid levels cause morphological errors in the ER as well as disrupt Ca^{2+} homeostasis, which then leads to mitochondrial-mediated apoptosis (116). Sphingolipids’ prosurvival and pro-apoptotic functions depend on their relative cellular levels, yet the regulation of sphingolipid metabolism is not fully understood. For example, in yeast, overexpression of the ceramidase-encoding gene YDC1 leads to decreased chronological lifespan and increased apoptosis (113). Mitochondrial fragmentation and dysfunction is also symptomatic of YDC1 overexpression. Conversely, reduced lifespan can be rescued by exogenous addition of ceramide, suggesting low ceramide levels as the cause for increased cell death (113).

Recent data from PD patient samples shows abnormal sphingolipid and sphingolipid enzyme levels in neurons. More specifically, Murphy et al (117) found that glucocerebrosidase levels and its enzymatic activity are reduced in brain regions with aSyn accumulation in early stages of PD. The enzyme glucocerebrosidase, which cleaves the beta-glucoside linkage of glucocerebroside (or glucosylceramide) is encoded by the gene GBA1. Mutations in the GBA1 gene result in the most common genetic risk factor for PD. Moreover, decreased lysosomal glucocerebrosidase levels correlated with increased aSyn accumulation and decreased ceramide levels.

![Diagram of aSyn accumulation, mitochondrial fragmentation, Apoptosis, Decreased lifespan, Membrane stability, Membrane fusion, aSyn clearance](image.png)

**Figure 9: Schematic showing effects of decreased (left) and increased (right) ceramide levels.**
1.10 Rationale, Hypothesis and Significance

1.10.1 Rationale

Mitochondrial dysfunction is clearly a major hallmark of PD and a major phenotype in many PD models, and aSyn is known as a key disease protein in PD. However, model systems have not yet shown conclusive evidence that causally links aSyn-toxicity to mitochondrial dysfunction. Furthermore, previous yeast-aSyn models have not implicated genes related to mitochondrial dysfunction as key players in aSyn-toxicity. We argue that this shortcoming is due to the metabolic profile of the yeast models and other experimental models used for aSyn research. Most of these cell models produce the majority of their ATP through either glycolysis or fermentation, while neurons produce the majority of their ATP through oxidative phosphorylation. Thus, we postulate that if the metabolic profile of the PD models were more similar to that of the neurons affected by PD, i.e. high levels of oxidative phosphorylation, mitochondrial dysfunction would be unmasked as a key pathologically relevant route of aSyn toxicity. This will allow us to assess the link between aSyn toxicity and mitochondrial function and mitochondrial homeostasis, by fission, fusion, ER-mitochondria communications, mitophagy, prohibitin function, and ceramide levels.

1.10.2 Hypothesis

We hypothesize that aSyn induces cellular toxicity by disrupting mitochondrial homeostasis. In order to test our hypothesis, I have pursued three specific aims.

1) To establish an aSyn-yeast model with low aSyn expression and high levels of oxidative phosphorylation.

2) To determine how mitochondria are affected by aSyn expression under growth conditions that elicit high oxidative phosphorylation activity.

3) To explore genetic interactions associated with mitochondrial homeostasis that enhance or suppress aSyn toxicity in our novel aSyn-yeast model.
1.10.3 Significance

aSyn is the most extensively studied protein in PD, yet it remains inconclusive how exactly aSyn causes neurotoxicity. Yeast studies have implicated disruptions in ER-Golgi transport as a possible cause of aSyn toxicity, yet PD patient samples show mitochondrial dysfunction to be a major hallmark of PD. Furthermore, mammalian cell models have shown mitochondrial dysfunction to occur with high levels of aSyn expression, yet often without any resultant toxicity. Our work will bridge the current gap between the mechanistic findings from aSyn cell models, and the pathological symptoms that are observed in PD patient samples by deciphering the genetic interaction between aSyn toxicity and mitochondrial function and homeostasis. This research also has great potential to uncover new therapeutic targets aimed at augmenting mitochondrial homeostasis for the treatment of PD.
Chapter 2

2 Materials and Methods

2.1 Materials

2.1.1 Yeast strains and media

Yeast strain BY 4742 (MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was used in this study. Yeast deletion strains were obtained from the Saccharomyces Genome Deletion Library, which was in turn purchased through ThermoFisher Scientific. The Genome Deletion Library is a collection of single yeast gene deletion strains which collectively, correspond to every non-essential gene deleted. Essential genes cannot be deleted as their deletion is nonviable.

Table 1: Yeast Deletion Strains Used in this Study

<table>
<thead>
<tr>
<th>Gene Deleted</th>
<th>Transcribed Protein’s Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG32/ YIL146C</td>
<td>Required for mitophagy.</td>
</tr>
<tr>
<td>FIS1/ YIL065C</td>
<td>Required for mitochondrial fission.</td>
</tr>
<tr>
<td>DNM1/ YLL001W</td>
<td>Required for mitochondrial fission.</td>
</tr>
<tr>
<td>MDV1/ YJL112W</td>
<td>Required for mitochondrial fission.</td>
</tr>
<tr>
<td>MDM30/ YLR368W</td>
<td>Required for mitochondrial fusion.</td>
</tr>
<tr>
<td>MMM1/ YLL006W</td>
<td>Required for ER-mitochondria communication.</td>
</tr>
<tr>
<td>MDM10/ YAL010C</td>
<td>Required for ER-mitochondria communication.</td>
</tr>
<tr>
<td>UGO1/ YDR470C</td>
<td>Required for mitochondrial fusion.</td>
</tr>
<tr>
<td>FZO1</td>
<td>Required for mitochondrial fusion.</td>
</tr>
<tr>
<td>YDC1/ YPL087W</td>
<td>Required for ceramide metabolism.</td>
</tr>
<tr>
<td>IRE1/ YHR079C</td>
<td>Mediates unfolded protein response.</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>HAC1/YFL031W</td>
<td>Mediates unfolded protein response.</td>
</tr>
<tr>
<td>SAC2/ YDR484W</td>
<td>Component of golgi-associated retrograde protein complex.</td>
</tr>
<tr>
<td>PEX2/ YJL210W</td>
<td>Functions in peroxisomal matrix protein import.</td>
</tr>
<tr>
<td>OPI3/ YJR073C</td>
<td>Enzyme used in phosphatidylcholine biosynthesis.</td>
</tr>
<tr>
<td>ARL3/YPL051W</td>
<td>GTPase that regulates membrane traffic to Golgi apparatus.</td>
</tr>
<tr>
<td>VPS28/ YPL065W</td>
<td>Involved in protein sorting into endosomes.</td>
</tr>
<tr>
<td>COG6/ YNL041C</td>
<td>Involved in fusion of transport vesicles to Golgi membrane.</td>
</tr>
<tr>
<td>MIP1/ YOR330C</td>
<td>Mitochondrial DNA polymerase.</td>
</tr>
<tr>
<td>PHB1/ YGR132C</td>
<td>Forms prohibitin complex in inner-mitochondrial membrane</td>
</tr>
<tr>
<td>PHB2/ YGR231C</td>
<td>Forms prohibitin complex in inner-mitochondrial membrane</td>
</tr>
<tr>
<td>SOD2/ YHR008C</td>
<td>Localizes to mitochondria and protects against oxidative stress.</td>
</tr>
</tbody>
</table>

All descriptions are modified versions of the descriptions available on the SGD website. A more detailed description is found in the introduction.

### 2.1.2 Yeast Media

Yeast-peptone-dextrose (YPD) rich media (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose) and selective dextrose (SD) media 2% glucose, 1X yeast nitrogen base (YNB), 6 g/L l-isoleucine, 2 g/L L-arginine, 4g/L L-lysine HCl, 6 g/L L-phenylalanine, 1 g/L L-threonine, and 1g/L L-methionine) in either liquid media or agar plates (20g/L) were used to grow yeast cells. SD media was supplemented with 4 different amino acids (4g/L L-tryptophan, 6g/L L-leucine, 2 g/L
L-histidine-monohydrate) depending on the selectivity maker of the plasmid. Yeast media was also made with the same formula above yet using different carbon sources. These alternate carbon sources are listed below:

Glycerol Media: 2% glycerol

Oleic Acid Media: 0.1% oleic acid + 0.05% Tween 40

Myristic Acid Media: 0.1% oleic acid + 0.05% Tween 40.

Oleic and myristic acid media was made according to the formula described by Ralf Erdmann et al, 1989.

2.1.3 E. coli strains and media

Escherichia coli strain DH5α. Genotype F−F80lacZΔM15 Δ(lacZYA-argF) 169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1 was used in this study. Subcloning Efficiency DH5α Competent Cells (Invitrogen, Cat. No. 18265-017) were used for the cloning work in this study. E. coli cultures were grown in Lysogeny Broth (LB) media (10 g/L NaCl, 10g/L tryptone, and 5 g/L yeast extract) with antibiotic resistance (100 µg/mL ampicillin or kanamycin, depending on selectivity markers) for transformations and cloning. 2x Yeast-tryptone (2xYT) media (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl) was used for recovery of E. coli cells after transformation.

2.1.4 DNA Plasmids

pYX142-mtGFP was obtained from Addgene (plasmid #45050) and originally developed in the Westermann lab (118). The pDONR201 plasmid containing the ORF for human wild-type alpha-synuclein plasmid was provided by the Duennwald lab. Using the standard Gateway Cloning Protocol (Invitrogen Protocol, (119) the alpha-synuclein encoding ORF was recombined into pAG413GPD-ccdB and pAG416-ccdB-EGFP destination vector (120). The plasmids were transformed and purified using the Qiagen Plasmid Miniprep Kit. Low copy yeast expression plasmids are yeast episomal centromere plasmids (YCP) that produce 1-2 copies of the plasmid per cell, whereas high copy yeast expression plasmids (2 micron) are yeast episomal plasmids (YEP) that produce about 100 copies per cell (121).
Table 2: Plasmids created using Gateway Cloning in this study

<table>
<thead>
<tr>
<th>Template</th>
<th>Destination Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-Synuclein</td>
<td>pAG413GPD-ccdB</td>
</tr>
<tr>
<td>Alpha-Synuclein</td>
<td>pAG416GPD-ccdB-EGFP</td>
</tr>
<tr>
<td>Alpha-Synuclein</td>
<td>pAG304Gal-ccdB-EGFP</td>
</tr>
<tr>
<td>Alpha-Synuclein</td>
<td>pAG304303Gal-ccdB-EGFP</td>
</tr>
</tbody>
</table>
2.1.5 Antibodies
The antibodies used in this study are shown in Table 3.

Table 3: Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Supplier</th>
<th>Use</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Sigma</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
<tr>
<td>Alpha-Synuclein</td>
<td>Abcam</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
<tr>
<td>RFP</td>
<td>Thermo Fisher</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
<tr>
<td>Histone</td>
<td>Abcam</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
<tr>
<td>PGK-1</td>
<td>Antibodies-online</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
<tr>
<td>Rabbit (Alexa 680)</td>
<td>Life Technologies</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
<tr>
<td>Mouse (Alexa 680)</td>
<td>Life Technologies</td>
<td>Western Blot</td>
<td>1:2500</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 High-Efficiency Yeast Transformation
Yeast transformations were performed according to standard PEG/lithium acetate method. A single colony of yeast cells are inoculated into 3 mL of YPD liquid or SD media and incubated at 30°C with shaking overnight. The liquid culture is then combined with 27 mL of YPD liquid to make a 30 mL liquid culture and incubated at 30°C shaking till the cells have reached log phase (an OD$_{600}$ of 0.4 to 0.5). The culture is then centrifuged at 2000 x g for 5 minutes. The supernatant is aspirated off and the pellet is washed with 3 mL of sterile water. The cells are centrifuged again at the same speed and time. The pellet is resuspended in 2 mL of 100 mM Li-Acetate in TE buffer after the wash step and incubated at 30°C shaking for 10 minutes. The culture is centrifuged again after incubation and the pellet is resuspended in 100 µl of Li-Acetate per transformation.
Each transformation is composed of 100 µL cell suspension, 250 µl transformation (1 X TE, 40% PEG, and 100mM Li-Acetate), 12µl salmon sperm DNA, 1µl (0.3~0.5 µg) plasmid DNA, and 25µl DMSO and in the order listed and vortexed thoroughly. The cells were then allowed to recover at 30°C shaking for 30 minutes, following a 20-minute heat shock at 42°C shaking. After recovery, the cells are centrifuged for 1 minute at 2000 xg, the supernatant aspirated, and the pellet resuspended in 100 µl TE buffer. The cells are then plated onto selective agar plates.

2.2.2 E. Coli Transformation

We perform E. coli transformations to replicate and amplify plasmid DNA, resulting in abundant amounts of DNA. 100 µl aliquots of transformation competent DH5α cells are thawed on ice from storage at -80°C; 1-5 µl (0.1~0.5 µg) of plasmid DNA is added to the cells and mixed thorough by gently flicking the tubes (the competent cells should not be vortexed). The cells are allowed to recover on ice for about 30 mins and heat shocked at 42°C for 45 seconds. The cells rest on ice for 2 mins before 1 mL of 2xYT is added and the cells allowed to recover at 37°C in a shaking incubator for at least 1 hour. Following recovery, the cells are centrifuged at 10,000 xg for one minute, the supernatant aspirated off, and resuspend in 100 µl of 2xYT. The suspension is plated on LB agar plates with selective antibiotic depending on the antibiotic resistance of the vector.

2.2.3 Gateway Cloning

The alpha-synuclein constructs were created through the standard procedures of Gateway cloning and consists of three steps, Polymerase Chain Reaction (PCR) amplification, BP recombination, and LR recombination following the Gateway cloning protocol developed by Invitrogen (117).

2.2.3.1 PCR

In brief, PCR was performed to obtain a template for the BP recombination reaction. We used an adapted version of the touchdown PCR program in order to avoid non-specific proliferation as a side-product of the reaction. The reaction cycle for PCR program protocol includes two cycles. The first cycle is repeated 10 times with an annealing temperature of 60°C; the second cycle is repeated 20 times with an annealing temperature of 57°C. The reaction mixture for PCR consists
of 2 µl of template DNA (~200 ng), 2µl of 100 mM forward primer, 2µl of 100 mM reverse primer, 2 µl of 100 mM dNTPs, 1µl of Q5 polymerase (New England BioLabs), and 20µl of 5x Q5 buffer (New England BioLabs). The forward and reverse primers are created using the Custom Primers - OligoPerfect™ Designer (Thermo Fisher).

**Agarose Gel Electrophoresis of PCR Products**

PCR products were then loaded into a 1% agarose gel and run in TAE buffer for approximately 30 min at 120 V. The PCR products were then cut out and purified using the Bioneer Pacific Acuprep Gel Purification Kit according to the provided protocol.

### 2.2.3 BP Reaction

The BP recombination reaction was performed by combining 2 µl of destination vector plasmid DNA (about 100-200 ng of DNA) or PCR product, 1 µl of pDONR vector (150 ng/µl), and 2 µl of 5X BP Clonase (Invitrogen). The mixture is vortexed and centrifuged twice to ensure thorough mixing and then allowed to incubate overnight at 37°C. 1 µl of Proteinase K (Invitrogen) solution is added to the reaction following incubation and left to react at 37°C for 10 minutes. 2 µl of this reaction mixture is then used to transform into Subcloning Efficiency DH5α Competent Cells (Invitrogen, Cat. No. 18265-017) following the protocol provided by the manufacturer. The cells are plated in LB kanamycin (kan) resistant agar plates and incubated overnight at 37°C. Colonies are picked from the plates and inoculated into LB kan liquid overnight at 37°C. The plasmids are then extracted from the E. coli cells by using the High-Speed Plasmid Mini Kit (Qiagen); the resulting DNA is in pDONR vector backbone.

### 2.2.3.3 LR Reaction

The LR recombination reaction uses 1 µl of the product (100-300 ng) from the BP reaction, i.e. the pDONR vector, in combination with 2 µl of destination vector (150 ng/µl), 13 µl of TE buffer, and 4 µl of LR Clonase (Invitrogen). The destination vectors used are listed in section 2.1.4. The mixture is vortexed and centrifuged twice and allowed to incubate overnight at 37°C. The resulting procedure is the same as described for the BP recombination reaction—Proteinase K is added to
the mixture, incubated, and transformed into competent cells. The destination vectors are ampicillin (amp) resistant and therefore must be plated on LB amp agar plates and then inoculated in LB amp liquid media.

DNA Analysis

Once Gateway Cloning was completed, we would verify successful cloning with a diagnostic restriction digest at regions flanking the gene. If necessary, we would also send constructs to the Robarts Research Institute at the University of Western Ontario for DNA sequencing to verify its identity using pDONR and specifically designed sequencing primers.

2.2.4 Yeast Spotting Assays

Spotting assays are performed by first inoculating yeast cells in 3 mL in SD media and incubated overnight in a shaking incubator at 30°C shaking at 220 rpm. 100 µl of the cells are then taken in an Eppendorf tube to be diluted 1:10 in water to measure the OD$_{600}$, which indicates cell density. In a 96-well plate, we dilute our cell cultures to a cell density normalized to OD$_{600}$=1 in the first row of wells, followed by five serial dilutions of 1:5 in the subsequent 5 wells. We then use a 48-prong Frogger (V&P Scientific) to spot the samples on YPD, SD, and SGal, Raf, glycerol, oleic acid, and myristic acid plates lacking selective amino acid markers. The YPD plates are used as growth and spotting controls, whereas the SD, SGal, Raf, glycerol, oleic acid, and myristic acid plates reflect the toxicity (e.g. of aSyn) of the induced yeast cells. The incubation period varies with the type of plate; YPD plates are incubated 2 days; SD plates 2 days; glycerol, oleic acid, and myristic acid plates 3-5 days. The plates are all incubated at 30°C. The plates are documented during the entire test period to monitor the growth of the yeast colonies by taking photographs using a digital camera. Images of the spotting assays grown on YPD plates are available in the supplementary figures.

2.2.4.1 Spotting Assay Quantification

Once spotting assays were photographed, they were quantified. The image was first modified on Adobe Photoshop such that the area of the plate with no yeast growing was completely black, while yeast growth was completely white. The image was then opened in ImageJ and a circular
selection that covered an area slightly smaller than the average single spot was used to gather measurements. For SD plates, the third dilution was used for quantification after 2 days of growth. The mean gray value was used to represent densitometry, where a value of 0 was indicative of the background, while a value of 255 was representative of complete growth. For SGal and Raf plates, the same protocol was used but quantifications were taken at day 3. For glycerol, oleic acid, and myristic acid plates, quantifications were taken on day 4, yet they included both dilution 3 and 4, as this results in consistent maximal densitometry values. Vector controls were then normalized to produce a value of 1, and the alpha-synuclein expressing strain then normalized accordingly.

### 2.2.5 Fluorescent microscopy

Microscopy imaging of GFP or RFP tagged constructs was performed by first inoculating yeast cells in SD media at 30°C overnight. The cells are then washed twice with sterile water, resuspended in SD media, and separately in other growth media of interest (i.e. glycerol or oleic acid) and incubated at 30°C. After incubating for time frames ranging from 6-24 hours, small samples of the culture are placed on a microscope slide. The cells are imaged on Olympus BX-51 Bright Field/Fluorescence Microscope and images were captured using an equipped CCD camera (Spot Pursuit). Exposure and magnification settings were kept consistent for each microscopy session unless specified otherwise.

### 2.2.6 Western Blot

#### 2.2.6.1 Protein Extraction

A 4 mL yeast culture is first inoculated in SD media overnight. The culture is then spun down at 2000 xg, washed twice, and resuspended in 5 mL of SD or glycerol overnight. The culture is then spun down and washed once with water. The supernatant is discarded and the pellet is resuspended in 200 µl of lysis buffer (50 mM Hepes pH 7.5, 5mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, 50 mM NEM, 2mM PMSF, and 1X Sigma protease inhibitor tablet) and transferred into an Eppendorf tube. We then add 100 µl of glass beads (ca. 500 µm in diameter) to physically disrupt the cell walls by vortexing them 6 times for 30 sec intervals and cooling on ice for 30 secs between these intervals. We then spin the culture at 5000 xg for 10 mins and collect the supernatant in a fresh Eppendorf tube.
2.2.6.2 Normalization of Protein Concentrations
We perform a BCA Protein Assay to determine the concentration of protein in the sample. The assay was performed according to the Thermo Scientific Pierce BCA Protein Assay Kit Instructions. After obtaining the concentration and normalizing the total protein amount in each sample per blot, we dilute the samples with 4x reducing SDS buffer (0.25M Trisma Base pH 6.8, 8.0% SDS, 40% sterile glycerol, 10% β-mercaptoethanol, 0.04% bromophenol blue).

2.2.6.3 SDS-PAGE
We run SDS-PAGE with the samples on an 8-16% gradient gel (Bio-Rad Criterion TGX Stain-Free Precast Gels) or 12% acrylamide gels at 220 V for about 50 mins. The gel is then transferred onto a Nitrocellulose or PVDF membranes (BioRad) using the Bio-Rad Trans-Blot Turbo machine following the manufacturer’s protocol.

2.2.6.4 Immunoblotting and Analysis
Following the gel transfer, we block the membrane using 5% skim milk powder (Carnation) in Phosphate Buffered Saline with 0.01 % (v/v) Tween (PBST) and incubate for 1 hr on a shaker. The membrane is then incubated in primary antibody overnight on a shaker at 4° C. Following incubation with the primary antibody, we wash the membrane with 50 ml aliquots of PBST at 10 min intervals for an hour on a shaker and then incubate in the secondary antibody for 1 h on a shaker. The membrane is washed again with 50 ml aliquots of PBST in 10 min intervals on a shaker for an hour. The membrane is then documented using the ChemiDoc MP System (Bio-Rad) and analyzed using Image Lab (Bio-Rad) and Prism 6 (Graph Pad).

2.2.7 Sedimentation Assay
2.2.7.1 Protein Extraction
The sedimentation assay was adapted from Theodoraki et al. (2012) and Shiber et al. (2013). A 4 mL yeast culture is first inoculated in SD media overnight. The culture is then spun down at 2000 xg, washed twice, and resuspended in 5 mL SD or glycerol media overnight. We use OD_{600}=1 amount of cells from the 5 mL culture. The 1 OD_{600} culture is then further spun down and washed
once with water. The supernatant is discarded and the pellet is resuspended in 200 µl of lysis (100 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1mM DTT, 5% glycerol, 0.5% TritonX-100, 50 mM NEM, 2mM PMSF, and 1X Sigma protease inhibitor tablet) and transferred into an Eppendorf tube. Acid-washed glass beads (425-600 µm, Sigma) are then added to physically lyse the cells by vortexing them 6 times for 30 secs intervals and cooling it on ice for 30 secs between the intervals. The tubes were pierced with a 16-gauge needle and the lysates (both pellet and supernatant) are collected in a fresh Eppendorf tube by centrifugation in pulses to separate lysates and glass beads.

Normalization of Protein Concentrations - See Section 2.2.6.2

Protein Extraction Continued

50 µl of the lysate was taken out and mixed with equal volume of SUMEB Buffer (8M Urea, 1% SDS, 10 mM MOPS, 10 mM EDTA, and 0.01% bromopheno blue) in a new tube, this aliquot represents total lysates. The rest of the lysate was spun down at 500 xg for 15 min at 4°C. 100 µl of the supernatant was transferred into a new tube and mixed with 100 µl of SUMEB buffer, this represents the supernatant portion of the lysate. The remaining supernatant from the lysate was aspirated off. The pellet was resuspended with 100 µl of the lysis buffer (without protease inhibitors) and 100 µl of SUMEB buffer, this represents the pellet portion of the lysate. The samples were boiled at 80°C for 5 min and 25 µl of the samples were loaded onto a 12% acrylamide gel. The gel is then run according to the SDS-PAGE and immunoblotting procedures described in sections 2.2.6.3 and 2.2.6.4

2.2.8 Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE)

2.2.8.1 Protein Extraction

A 4 mL yeast culture is first inoculated in SD media overnight. The culture is then spun down at 2000 xg, washed twice, and resuspended in 5 mL of SD or glycerol overnight. The culture is then spun down and washed once with water. The supernatant is discarded and the pellet is resuspended in 300 µl of lysis buffer (100 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 8 mM PMSF, and protease inhibitor cocktail) and transferred into an Eppendorf tube. We then add 100 µl of glass beads (ca. 500 µm in diameter) to physically disrupt the cell walls by vortexing them 6 times for 30 sec intervals and cooling on ice for 30 secs between these intervals.
We then use a 20G syringe to puncture the bottom of the tube and spin the culture at 5000 xg for 1 min and collect the lysate in a fresh tube below.

Normalization of Protein Concentrations - See Section 2.2.6.2

2.2.8.2 Agarose Gel Electrophoresis

We use 1.8% agarose gels with 0.1% SDS running at 80 V for about 2 hours in TAE buffer with 1% SDS. The gel is then transferred onto a Nitrocellulose or PVDF membranes (BioRad) according to the Whatman: Turboblotter Rapid Downward Transfer Systems protocol overnight.

Immunoblotting and Analysis - Following transfer, we follow the same protocols for immunoblotting and analysis as described in section 2.2.6.4.

2.2.9 Reactive Oxygen Species Assay

The reactive oxygen species (ROS) assay was carried out following the protocol described by Joel James et al, 2015. A 4 mL yeast culture is first inoculated in SD media overnight. The culture is then spun down at 2000 xg, washed twice, and resuspended in 5 mL of SD or glycerol media, treated or untreated with 100 µM H2O2 overnight. The OD of the cells is then measured (as described in section 2.2.4) and the samples are accordingly normalized to have equal amounts of cells. H2DCF-DA was then added to the cultures at a concentration of 10 µM and the cells were incubated in the dark for 30 min. Cells were then pelleted and resuspended in 2M Lithium acetate for 2 minutes with light agitation. Cells were again pelleted and resuspended in 0.1% SDS with a drop of chloroform and shaken vigorously for 2 minutes. Cells were then pelleted, and the supernatant was loaded into a 96 well plate and the fluorescence was measured using the Cytation 5 Cell Imaging Multi-Mode Reader fluorescence protocol.

2.2.10 Creation of Petite Yeast Strains

The creation of rho\(^0\) yeast, or petite yeast strains was achieved by following the protocol described by Thomas D. Fox et al, 1991 (122). Yeast were grown to saturation overnight in SD media with only 0.67% YNB and 25 µg/ml ethidium bromide. 10 µl was then taken from the culture grown to
saturation and inoculated in fresh ethidium bromide-containing media as described above and
grown overnight. We then performed spotting assays on selective media (as described in section
2.2.4) to allow for isolation of single colonies. Single colonies were then restreaked on a new plate
of selective media and replicative plating was used to confirm the yeast were petite and respiratory
deficient.

2.2.11 Replica Plating

Replica plating was achieved by first streaking out the yeast strains of interest onto a new plate of
selective media. This plate was then incubated at 30°C for two days. We then transferred the yeast
onto a sterile velvet cloth draped over a sterile transfer block. Next, we pressed our new plates of
interest onto the velvet cloth to allow for transfer of the yeast. These plates were then incubated at
30°C for 2-4 days, depending on the carbon source used.

2.2.12 Statistical Analysis

Statistical analysis of the viability assays, western blots, and aggregation quantifications, were
completed using the GraphPad Prism 6 software. Statistical significance was obtained by
performing unpaired t-tests to compare the means and standard deviations between the control data
set and the experiment data set (each at a minimum of three biological replicas). Significance levels
are indicated using asterisks, where * is p<0.05.
3 Results

3.1 Expansion of the Alpha-Synuclein (aSyn) Yeast Model – Aggregation and Toxicity in Respiring Cells

The previously established alpha-synuclein (aSyn) yeast model focused on growth in fermentable carbon sources, such as glucose and galactose. In yeast, these sugars are mostly metabolized through glycolysis and cells are rapidly dividing until they reach stationary phase. In contrast, neurons, including those affected in Parkinson’s disease (PD), mostly metabolize sugar trough oxidative phosphorylation and mature neurons usually do not divide. To mimic this metabolic activity and lack of cell division in yeast, we determined aSyn toxicity and aggregation in non-fermentable carbon sources, such as glycerol and potassium acetate.

When grown on the fermentable carbon source glucose, spotting assays reveal only a mild toxicity in yeast cells expressing moderate levels of aSyn, with the aSyn-expressing yeast cells showing 12% reduced growth compared to the vector control (Figure 10A, B). However, when the carbon source is switched to glycerol, toxicity increases and the aSyn-expressing shows 40% reduced growth compared to the vector control (Figure 10A, B).

Western blots probing for aSyn reveal no significant change in aSyn expression when cells are grown with either glucose or glycerol as a carbon source (Figure 10C). This shows that the increase in aSyn toxicity is not merely due to increased expression or stability of aSyn in glycerol, as changing the carbon source did not change aSyn protein levels.

We then performed a sedimentation assay to test whether aSyn aggregation is increased in cells grown in glycerol. The sedimentation assay was performed in parallel with cells grown in glucose or glycerol as a carbon source. We find that growth in glycerol results in a 46% increase of insoluble aSyn compared to growth in glucose (Figure 10C).

In conclusion, growing cells with glycerol as opposed to glucose results in a significant increase in both aSyn toxicity and aggregation.
In order to visualize aSyn conformational changes, we analyzed aSyn carboxy-terminally fused to the green fluorescent protein (aSyn-GFP) in wild-type (WT) yeast cells using fluorescent microscopy with cells grown with either glucose or glycerol as a carbon source. Cells grown in glucose did not contain any aSyn aggregation at moderate expression levels, yet approximately 20% of cells grown in glycerol did contain aggregates (Figure 11A), which confirms the sedimentation analysis of aSyn aggregation.

We next explored how aSyn affects mitochondrial morphology during both growth in glucose and glycerol. We therefore transformed the reporter protein mtGFP (mitochondria targeted GFP) into WT aSyn expressing an empty vector control yeast cells. Microscopy revealed no noticeable difference in mitochondrial morphology between the vector and aSyn-expressing strain when grown in glucose. However, the mtGFP signal is much less intense in the aSyn-expressing strain grown in glycerol compared to the vector control grown in glycerol (Figure 11B). Western blots probing for mtGFP confirmed the microscopy results as mtGFP levels are reduced by 36% in the aSyn-expressing strain grown in glycerol compared to the vector control (Figure 11C).

Changing the carbon source from glucose to glycerol in yeast creates a metabolic profile that is similar to neurons, with high levels of respiration, or oxidative phosphorylation (123) and low cell division activity. Our moderate aSyn-expression yeast model reveals that this switch to oxidative phosphorylation due to growth in glycerol produces an increase in both aSyn aggregation and toxicity. Furthermore, microscopy and biochemistry reveal that aSyn causes a defect in mitochondrial homeostasis that is noticeable only when cells are respiring due to growth in glycerol. These findings establish a new yeast model using respiring cells and point to mitochondrial dysfunction as a contributor to aSyn toxicity.
A  
Vector  
aSyn  

Glucose
Glycerol

1-5 Dilution

B  

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aSyn Growth/Vector Growth

C  

Glucose  
Glycerol

aSyn
PGK1

14 kDa
45 kDa

D  

Glucose  
Glycerol

aSyn

Insoluble/Soluble

Glucose  
Glycerol

*
Figure 10: Characterization of aSyn-yeast glycerol model. A Spotting assays of wild type yeast cells expressing a vector control or aSyn, grown in either glucose or glycerol. B Quantification of the spotting assays. Graphed data represents the growth value of the vector control divided by the aSyn-expressing strain. C Western blots probed for α-aSyn and loading control (α-PGK1) prepared with protein lysates from yeast cells grown in glucose or glycerol. Quantification of three independent experiments is shown in the right part of the panel. D Sedimentation assay probed for α-aSyn prepared with protein lysates from yeast cells grown in either glucose or glycerol. Quantification of three independent experiments are shown in the right part of the panel.
Figure 11: Fluorescence microscopy of GFP-tagged aSyn and cells expressing mtGFP in the absence and presence of aSyn. A. Microscopy of GFP-tagged aSyn in WT yeast cells grown in either glucose or glycerol. Quantification of cells containing aggregates is displayed on the right from three independent experiments. B Fluorescence microscopy of WT yeast cells expressing mtGFP in the absence or presence of aSyn grown in either glucose or glycerol. C Western blots probing for mtGFP in WT yeast in the absence or presence of aSyn grown in either glucose or glycerol probed for GFP and Histone H3 as a loading control. Quantification of three independent experiments is presented on the right.
3.2 aSyn toxicity and aggregation depend on metabolic activity

While most of our work below was completed using either glucose or glycerol as a carbon source, we also characterized aSyn aggregation and toxicity under conditions with diverse metabolic profiles. To this end, we performed both spotting assays and microscopy using seven different carbon sources: glucose, raffinose and galactose are fermentable carbon sources, whereas potassium acetate, oleic acid and myristic acid are non-fermentable. Potassium acetate is metabolized primarily through oxidative phosphorylation, yet oleic acid and myristic acid undergo beta-oxidation prior to oxidative phosphorylation.

The three fermentable carbon sources (galactose, raffinose, glucose) I tested did not show different aSyn toxicity (Figure 12B). Also, fluorescent microscopy did not reveal any changes in aSyn localization or aggregation (Figure 13). In contrast, growth with Potassium acetate resulted in an increase in aSyn toxicity similar to that observed for glycerol (Figure 12A). Microscopy also revealed increased aSyn aggregation (Figure 13). We found that growth in oleic acid did not result in a significant increase in toxicity compared to glucose, despite the apparent change in aSyn localization (Figure 12A, B, Figure 13). Myristic acid, however, resulted in increased aSyn toxicity (60% reduced growth) (Figure 12A). Yeast cells grown in myristic acid also contained large aSyn aggregates (Figure 13).
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**B**

![Images showing different conditions and substrates](image)

**C**

![Bar graph showing aSyn Growth/Vector Growth](image)
Figure 12: aSyn toxicity in yeast cells grown in different carbon sources: A Different carbon sources and the predominant type of metabolism used by WT yeast cells to generate ATP. B Spotting assays of wild type yeast cells expressing a vector control or aSyn, grown in different carbon sources. C Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
Figure 13: Fluorescence microscopy of aSyn-GFP. A Microscopy of aSyn-GFP expressed in WT yeast cells grown in fermentable carbon sources. B Microscopy of aSyn-GFP expressed in WT yeast cells grown in non-fermentable carbon sources.
3.3 Previously established enhancers of aSyn toxicity do not alter toxicity in our model

As previously mentioned, the aSyn-yeast model using high expression has been extensively characterized. The genetic interactions identified using this model did not indicate any connection between aSyn and mitochondrial homeostasis and function (44, 54, 56). We explored whether these previously established genetic enhancers reproduced in our low expression model grown in glucose and in glycerol. Of the six deletion strains we chose to represent the previously established enhancers of aSyn toxicity, we found no increase in toxicity using our low-expression model grown in glucose and only the deletion of VPS28 cause showed increased aSyn toxicity in glycerol (Figure 14 A, B). These data indicate that growth conditions and expression levels of aSyn are key to specific genetic interactions.
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B

aSyn Growth/Vector Growth

![Graph](image17.png)

Growth

WT  Δopi3  Δpex2  Δarl3  Δvps28  Δcog6  Δsac2

Glucose
Figure 14: Toxicity of previously established enhancers. A Spotting assays of WT yeast and previously established enhancers expressing a vector control or aSyn, grown with glucose or glycerol. B Quantification of the spotting assays grown with glucose. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain.
3.4 Petite-cells show no difference in aSyn toxicity and aggregation

Petite-cells yeast cells have their mtDNA deleted or mutated to the extent that they can no longer express genes required to perform oxidative phosphorylation. These petite cells are therefore respiratory deficient and provide a reliable control for disruptions in mitochondrial homeostasis independent of oxidative phosphorylation. Petite cells were created using the mutagen ethidium bromide or as a result of the deletion of the MIP1 gene. We transformed GFP-tagged and WT aSyn into a petite (rho) and Δmip1 yeast strain. Spotting assays revealed no increase in aSyn toxicity in the petite or Δmip1 yeast strain when compared to WT yeast (figure 15A). Furthermore, microscopy revealed no difference in aSyn aggregation between the WT yeast and petite strain suggesting that aSyn aggregation and toxicity is not different in respiratory deficient yeast cells than WT yeast cells.
Figure 15: Spotting assays of WT and petite yeast cells expressing aSyn-GFP.  A Spotting assays of WT, Δ mip1 and rho° yeast cells expressing a vector control or aSyn, grown in glucose.  B Quantification of the spotting assays grown in glucose. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.  C Microscopy of aSyn-GFP expressed in WT and petite yeast grown in glucose.
3.5 Oxidative stress does not exacerbate aSyn toxicity and aggregation

Oxidative stress is a hallmark of PD, yet whether it is a result or cause of PD remains unknown (63). Here, we tested the connection between oxidative stress and aSyn toxicity by expressing aSyn in yeast cells deleted for SOD2 and by exposing WT yeast cells expressing aSyn to H\textsubscript{2}O\textsubscript{2} treatment. SOD2 encodes the gene for mitochondrial superoxide dismutase, which acts as an antioxidant that converts superoxide, a byproduct of oxidative phosphorylation, to H\textsubscript{2}O\textsubscript{2} and diatomic oxygen. Yeast cells lacking Sod2 activity are unable to grow on non-fermentable carbon sources (124). H\textsubscript{2}O\textsubscript{2} treatment is a well-established method of exposing yeast cells to oxidative stress (125). Spotting assays revealed no change in aSyn toxicity in WT yeast treated with 100\textmu M H\textsubscript{2}O\textsubscript{2}, and in the Δsod2 strain (Figure 16A, B) grown in glucose. Furthermore, aSyn aggregation was unchanged after 6 hours of 100\textmu M H\textsubscript{2}O\textsubscript{2} treatment and in the Δsod2 strain grown in glucose (Figure 16C). These results indicate that oxidative stress alone that not increase aSyn toxicity and aggregation. Also, when we combine H\textsubscript{2}O\textsubscript{2} treatment with growth on glycerol plates, we detect no increased aSyn toxicity to vector control yeast cells.
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H2O2 100 µM

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aSyn GFP

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Figure 16: Oxidative stress and aSyn toxicity: A Spotting assays of WT yeast in the absence or presence of 100µM H₂O₂ and Δsod2 yeast expressing a vector control or aSyn, grown in glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain using three biological replicas. C Microscopy of aSyn-GFP expressed in WT yeast +/- 100µM H₂O₂ and in Δsod2 yeast cells when grown in glucose.
3.6 Bafilomycin exacerbates aSyn toxicity only under respiring conditions

To investigate how autophagy interacts with aSyn toxicity in our model, we performed spotting assays on plates treated with either 10µg/ml rapamycin or 300nM bafilomycin (126), with either glucose or glycerol as a carbon source. Rapamycin increases autophagy through inhibition of the TOR pathway, while bafilomycin blocks autophagy through its inhibition of the vacuolar H^+ ATPase (124). Rapamycin reduced aSyn toxicity on glucose, as indicated by a 7% increase in growth for the aSyn expressing strain. Surprisingly, the aSyn-expressing yeast cells grew 20% better than the vector control when treated with bafilomycin (Figure 17A, B). These effects were reversed when glycerol was used as a carbon source. The growth of the aSyn-expressing strain was reduced to 7% that of the vector control when treated with bafilomycin, while rapamycin resulted in no significant change in toxicity. Therefore, our results indicate that rapamycin treatment produces no phenotype while bafilomycin strongly exacerbates aSyn toxicity only in cells grown under respiring conditions.
Figure 17: aSyn toxicity in the presence of rapamycin and bafilomycin. A Spotting assays of WT yeast in the absence or presence of 100µg/ml rapamycin or +/- 300nM bafilomycin expressing a vector control or aSyn, grown in glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
3.7 Defects in ERMES exacerbates aSyn toxicity and increases aggregation

To test whether the endoplasmic reticulum (ER) is a target of aSyn’s disruption in mitochondrial function, we utilized a yeast strain with the MMM1 gene deleted (Δmmm1) in our aSyn-yeast model. MMM1 is required for the formation of the Endoplasmic Reticulum-Mitochondrial-Encounter Structure (ERMES) complex in yeast. The ERMES complex is key for the contact between the ER and mitochondria and exchange of important molecules between the two organelles, such as calcium ions and lipids. As this deletion strain is respiratory deficient and cannot grow in non-fermentable carbon sources, all work had to be completed with glucose media. Spotting assays reveal that the Δmmm1 strain resulted in a 53% decrease in growth of the aSyn expressing strain when compared to the vector control (Figure 18A, B). In comparison, aSyn expressed in WT yeast grows only 12% less than the vector control. To determine how aSyn aggregation is affected by the loss of the ERMES complex, we expressed aSyn-GFP into Δmmm1 cells. Fluorescence microscopy showed that the number of cells appearing with aggregates when grown in glucose rose from 0% in WT yeast cells to approximately 35% in the Δmmm1 cells (Figure 18C). Therefore, aSyn toxicity and aggregation is exacerbated by disruption of the ERMES.
Figure 18: aSyn toxicity and aggregation in ERMES deficient cells. A Spotting assays of WT and Δmmm1 yeast expressing a vector control or aSyn, grown in glucose. B Quantification of the spotting assays grown in glucose. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas. C Microscopy of aSyn-GFP expressed in WT and Δmmm1 yeast grown in glucose. D Quantification of cells containing aSyn aggregates in WT and Δmmm1 yeast cells grown in glucose.
3.8 Prohibitin deficiency exacerbate aSyn toxicity

Prohibitins are highly conserved proteins encoded by the genes PHB1 and PHB2 in yeast. To investigate how aSyn interacts genetically with prohibitins, we tested PHB1 or PHB2 deleted cells in our aSyn-yeast model. Spotting assays revealed only a moderate increase in toxicity in the aSyn-expressing Δphb1 strain when grown in glucose, yet no increase in toxicity when grown with glycerol (Figure 19A, B). A large increase in toxicity was observed in the aSyn-expressing Δphb2 strain when grown on glucose and on glycerol. The aSyn-expressing Δphb2 strain grew 90% less than the vector control in glucose and 93% less than the vector control in glycerol (Figure 19A, B).

We next determined how aSyn affects mitochondrial morphology in Δphb2 cells grown in glucose and glycerol. We therefore transformed mtGFP (mitochondria-targeted GFP) into Δphb2 aSyn and an empty vector expressing yeast strains. We reported no mitochondrial morphological abnormalities in the vector control Δphb2 strain in either glucose or glycerol when compared to WT (as previously reported) (107). However, we observed a notable morphological change, and an overall reduction in mtGFP signal in the Δphb2 aSyn-expressing strain when grown with glycerol (Figure 20A). Western blots probing for mtGFP in the Δphb2 deletion strains showed a significant decrease (35%) in mtGFP signal in the aSyn-expressing strain when grown in glucose compared to the vector control. When the cells were grown with glycerol, however, the variability of mtGFP signal intensities was so high that a significant difference could not be assessed.
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1:5 Dilution

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Vector

+ aSyn
**Figure 19: aSyn toxicity in yeast cells deleted for PBH1 or PBH2.** A Spotting assays of WT, Δphb1 and Δphb2 yeast cells expressing a vector control or aSyn, grown in either glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
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- **Glucose**
  - Vector
  - Δphb2: mtGFP 27 kDa, Histone 17 kDa
  - +aSyn
  - Δphb2: mtGFP 27 kDa, Histone 17 kDa

- **Glycerol**
  - Vector
  - Δphb2: mtGFP 27 kDa, Histone 17 kDa
  - +aSyn
  - Δphb2: mtGFP 27 kDa, Histone 17 kDa

Bar graph showing normalized signal intensity for mtGFP under different conditions.
Figure 20: Fluorescence microscopy of WT and Δphb2 yeast cells expressing mtGFP and aSyn. A Fluorescence microscopy of WT yeast (top) and Δphb2 yeast (bottom) expressing mtGFP +/- aSyn grown in either glucose or glycerol as a carbon source. B Western blots probing for mtGFP in Δphb2 yeast in the absence or presence of aSyn grown in either glucose or glycerol as a carbon source. Probed with α-GFP and α-Histone H3 as a loading control. Quantification of three biological replicas is presented on the right.
3.9 Mitochondrial Homeostasis – Fission and Fusion

3.9.1 Deletion of mitochondrial fission genes reduces aSyn toxicity and aSyn aggregation

Previous studies have shown that aSyn inhibits mitochondrial fusion and increases mitochondrial fragmentation (127). We thus tested if aSyn toxicity is reduced in yeast strains deficient in mitochondrial fission. Mitochondrial fission in yeast is carried out by multi-protein complex and cannot occur without the proteins encoded by the genes DNM1, FIS1 or MDV1 (87). To determine how aSyn interacts with mitochondrial fission, we transformed aSyn into three different yeast strains which each had a gene deleted required for mitochondrial fission, namely Δdnm1, Δfis1, and Δmdv1. Spotting assays revealed no significant change in aSyn-toxicity in these deletion strains when grown in glucose (Figure 21A). However, both Δdnm1 and Δfis1 rescued aSyn-toxicity when grown in glycerol, where the Δdnm1 aSyn-expressing strain grew 19% better than the vector control, and the Δfis1 aSyn-expressing strain grew 11% better than the vector control. With the vector controls of both Δdnm1 and Δfis1 growing worse in glycerol than WT yeast cells, we observe that aSyn toxicity is indeed reduced in Δdnm1 and Δfis1 yeast cells grown in glycerol (Figure 21A, B).

We next determined how aSyn affects mitochondrial morphology during both growth in glucose and glycerol in the Δfis1 strain, as a representative of the fission deficient yeast. We therefore transformed mtGFP (mitochondria-targeted GFP) into Δfis1 aSyn and an empty vector expressing yeast strains. When grown in glucose, we noticed long tubular mitochondrial networks in the vector control, indicative of fission-deficient mitochondria (90) (Figure 22 A). The aSyn-expressing strain displayed no noticeable difference in mitochondrial morphology in comparison to the vector control when grown with glucose. When cells were grown in glycerol, the aSyn-expressing yeast cells showed reduced mtGFP signal and altered mitochondrial morphology. Indeed, western blots probing for mtGFP confirmed that the mtGFP protein levels in the aSyn-expressing Δfis1 strain was 22% lower than in the vector control when grown in glycerol. (Figure 23B).
As aSyn-toxicity was reduced by in the Δfis1 strain, we sought to determine how aSyn aggregation is affected. To this end, we transformed aSyn-GFP into Δfis1 yeast cells. Fluorescence microscopy revealed a ~50% reduction in aSyn aggregation when grown in glycerol, where 7% of Δfis1 cells showed aggregates, compared to 20% in WT (Figure 22B).
Figure 21: Genetic interactions of aSyn and mitochondrial fission genes. A Spotting assays of WT, Δdnm1, Δfis1 and Δmdv1 yeast expressing a vector control or aSyn, grown in either glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
A

Vector WT +aSyn WT

Glucose mtGFP Brightfield mtGFP Brightfield

Glycerol mtGFP Brightfield mtGFP Brightfield

Vector Δfis1 + aSyn Δfis1

Glucose mtGFP Brightfield mtGFP Brightfield

Glycerol mtGFP Brightfield mtGFP Brightfield

B

Glucose

Vector Δfis1 +aSyn Δfis1

mtGFP 27 kDa
Histone 17 kDa

Glycerol

Vector Δfis1 +aSyn Δfis1

mtGFP 27 kDa
Histone 17 kDa

mtGFP

Normalized Signal Intensity

Vector Δfis1 Δfis1 Glucose

Vector Δfis1 Δfis1 Glycerol

Vector aSyn aSyn Glucose

Vector aSyn aSyn Glycerol
Figure 22: Fluorescence microscopy of WT and Δfis1 yeast cells expressing mtGFP in the absence or presence of aSyn. A Fluorescence microscopy of WT yeast (top) or Δfis1 yeast (bottom) cells expressing mtGFP in the absence of presence of aSyn grown in either glucose or glycerol as a carbon source. B Western blots probing for mtGFP in Δfis1 yeast cells in the absence or presence of aSyn grown in either glucose or glycerol as a carbon source. Western blots were probed with α-GFP and α-Histone H3 as a loading control. Quantification of three biological replicas is presented on the right.
Figure 23: aSyn aggregation in fission deficient yeast cells. A Fluorescence microscopy of aSyn-GFP in WT and Δfis1 yeast cells, grown in glucose or glycerol as a carbon source. B Quantification (three biological replicas) of cells containing aSyn aggregates in WT and Δfis1 yeast cells grown in glucose or glycerol.
3.9.2 Deletion of mitochondrial fusion genes exacerbates aSyn toxicity and disrupts mitochondrial homeostasis

As previous studies have shown aSyn inhibits mitochondrial fusion and increases the number of fragmented mitochondria (122), we hypothesized that aSyn toxicity would be increased in yeast strains deficient in mitochondrial fusion. Mitochondrial fusion in yeast is carried out by many different proteins, yet it cannot occur without the proteins encoded by UGO1, FZO1 or MDM30 (88). To determine how aSyn genetically interacts with mitochondrial fusion, we transformed aSyn into three different yeast strains, each of which bearing a deletion of a gene required for mitochondrial fusion: Δfzo1, Δugo1 and Δmdm30. As these three deletion strains are respiratory deficient, spotting assays and western blots could only be completed using glucose as a carbon source. Spotting assays revealed no increase in toxicity between the vector control and aSyn-expressing Δfzo1 strain (Figure 24A). However, the aSyn-expressing Δugo1 strain grew 30% less than the vector control, while the aSyn-expressing Δmdm30 strain grew 90% less than the vector control. Therefore, the inhibition of mitochondrial fusion greatly exacerbates aSyn toxicity.

We determined how aSyn affects mitochondrial morphology during both growth in glucose and glycerol in the Δmdm30 strain, which we chose as a representative of the fusion deficient yeast strains. We therefore transformed mtGFP (mitochondria-targeted GFP) into Δmdm30 cells expressing aSyn or an empty vector control. When grown in glucose, we noticed fragmented mitochondrial networks in the vector control, indicative of fusion-deficient mitochondria (88) (Figure 25A). This phenotype appeared to be exacerbated by growth with glycerol. We also noticed a large decrease in mtGFP signal in the aSyn-expressing strain when grown both in glucose and glycerol, in comparison to their respective vector controls. Furthermore, the aSyn-expressing strain contained severely deformed mitochondria when grown in glycerol. Western blots probing for mtGFP confirmed the microscopy results as the mtGFP signal in the aSyn-expressing Δmdm30 strain was 30% lower than the vector control. (Figure 25B).

As aSyn-toxicity was exacerbated in the Δmdm30 strain, we sought to determine how aSyn aggregation might be affected. To this end, we transformed aSyn-GFP into Δmdm30 yeast cells.
Fluorescence microscopy revealed no significant change in aSyn aggregation compared to the WT yeast cells when grown in glucose or glycerol. (Figure 26B).
Figure 24: Genetic interactions of aSyn and mitochondrial fusion gene deletions. A Spotting assays of WT, Δfzo1, Δugo1 and Δmdm30 yeast expressing a vector control or aSyn, grown in either glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
Figure 25: Fluorescence microscopy of WT and Δmdm30 yeast cells expressing mtGFP in the absence or presence of aSyn. A Fluorescence microscopy of WT yeast (top) or Δmdm30 yeast (bottom) expressing mtGFP in the absence or presence of aSyn grown in either glucose or glycerol as a carbon source. B Western blots probing for mtGFP in Δmdm30 yeast in the absence or presence of aSyn grown in glucose as a carbon source. Probed with α-GFP and α-Histone H3 as a loading control. Quantification of three biological replicas is presented on the right.
**Figure 26: aSyn aggregation in fusion deficient yeast.** A Fluorescence microscopy of aSyn-GFP in WT and Δmdm30 yeast grown in glucose or glycerol as a carbon source. B Quantification of cells containing aSyn aggregates in WT and Δmdm30 yeast grown in glucose or glycerol from three biological replicas.
3.9.3 Mitophagy inhibition prevents aSyn-associated reduction in mtGFP signal

The interplay between aSyn and mitophagy remains unresolved, with some studies suggesting that mitophagy is protective, while others suggest that it is required for aSyn-induced toxicity (128). We thus tested if defects in mitophagy altered aSyn toxicity in our resiping yeast model. In yeast, mitophagy, yet not general autophagy, can be inhibited by deletion of the gene ATG32. We therefore transformed aSyn constructs and vector controls into the Δatg32 strain. After performing spotting assays on media containing glucose and glycerol, we found no significant change in toxicity when aSyn was expressed in the Δatg32 strain (Figure 27A).

We also tested if aSyn changed mitochondrial morphology in mitophagy-deficient yeast cells. To this end, we transformed mtGFP into Δatg32 yeast cells expressing aSyn or an empty vector. We observed no noticeable difference between the WT vector control and Δatg32 vector control strains. Furthermore, we did not notice any differences in mitochondrial morphology or mtGFP signal intensity between the vector control and aSyn-expressing Δatg32 strains (Figure 28A). This was the case for cells grown in glucose and glycerol. Western blots probing for mtGFP also confirmed our microscopy results, as there was no significant change in mtGFP signal between the vector and aSyn-expressing Δatg32 strains when grown in glucose and glycerol (Figure 28A, B). Therefore, aSyn does not cause a reduction in mtGFP signal in mitophagy deficient cells.

To determine if aSyn aggregation is affected by mitophagy, we transformed aSyn-GFP into the Δatg32 yeast strain. Fluorescence microscopy revealed a ~50% reduction in aSyn aggregation in the Δatg32 yeast strain compared to WT yeast cells when grown in glycerol, with 12% of the Δatg32 cells containing aggregates compared to 20% in WT yeast (Figure 29).
Figure 27: Genetic interactions of aSyn and a mitophagy gene deletion. A Spotting assays of WT and ∆atg32 yeast expressing a vector control or aSyn, grown in either glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
Figure 28: Fluorescence microscopy of WT and Δatg32 yeast cells expressing mtGFP in the absence or presence of aSyn. A Fluorescence microscopy of WT yeast (top) or Δatg32 yeast (bottom) expressing mtGFP in the absence or presence of aSyn grown in either glucose or glycerol as a carbon source. B Western blots probing for mtGFP in Δatg32 yeast in the absence or presence of aSyn grown in glucose or glycerol as a carbon source probed with an anti-GFP antibody and an anti-Histone H3 antibody as a loading control. Quantification of three biological replicas is presented on the right.
Figure 29: aSyn aggregation in mitophagy deficient yeast cells. A Fluorescence microscopy of aSyn-GFP in WT and Δatg32 yeast, grown in glucose or glycerol as a carbon source. B Quantification of cells containing aSyn aggregates in WT and Δatg32 yeast grown in glucose or glycerol.
3.10 Deletion of YDC1 rescues aSyn toxicity

Sphingolipids are a class of lipids involved in pro- and antiapoptotic signalling, specifically through mitochondrial-mediated apoptosis (113). We explored a possible interaction between sphingolipid metabolism and aSyn activity. To this end, we utilized the Δydc1 strain and incorporated it into our aSyn-yeast model. YDC1 encodes an enzyme involved in ceramide degradation, thus the Δydc1 deletion strain will have elevated levels of ceramide compared to WT. We performed spotting assays in both glucose and glycerol as carbon sources. Remarkably, the Δydc1 deletion completely rescued aSyn toxicity when grown on both glucose and glycerol (Figure 30A), providing evidence of a genetic interaction between aSyn and sphingolipid metabolism.
Figure 30: aSyn toxicity and ceramide metabolism. A Spotting assays of WT and Δydc1 yeast expressing a vector control or aSyn, grown with either glucose or glycerol. B Quantification of the spotting assays. Graphed data represents the growth value of the vector control divided by the aSyn-expressing strain from three biological replicas.
3.8 Toxic pathways of Huntington fragment different from aSyn

Mitochondrial homeostasis is intimately linked to general cell homeostasis, thus, it is possible that disruptions in mitochondrial homeostasis observed in this study may predispose cells to increased vulnerability or even cell death due to the expression of many different misfolded proteins (85). To determine whether our genetic enhancers and suppressors of aSyn toxicity are specific to aSyn and not toxic proteins in general, we transformed 72Q, a Huntington fragment into deletion strains that showed strong genetic interactions with aSyn and performed spotting assays. We found that Δfis1, a suppressor of aSyn toxicity, was an enhancer of 72Q toxicity. While toxicity was also increased by 72Q in some Δfis1 strains when grown on glycerol, variability in toxicity was so high that a significant difference was not assessed. This high variability in 72Q toxicity has been previously reported and might be indicative of a Rnq-prion forming interaction. Furthermore, Δmdm30, one of the strongest enhancers of aSyn toxicity did not interact genetically at all with 72Q (Figure 31A, B), i.e. did not exacerbate 72Q toxicity. Therefore, we conclude that the genetic pathways that we identified as genetic modifiers of aSyn toxicity and aggregation are specific to aSyn independent of their growth conditions (glucose and glycerol).
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Graph A: Growth Measurements for WT, Δdnm1, Δfis1, Δugo1, and Δmdm30 in glucose and glycerol media.

Graph B: aSyn Growth/Vector Growth for WT, Δdnm1, Δfis1, Δugo1, and Δmdm30 in glucose and glycerol media.
Figure 31: Toxic polyQ expansion protein and mitochondrial homeostasis. A Spotting assays of WT, Δdnm1, Δfis1, Δugo1, Δmdm30 yeast expressing a vector control or 72Q, grown in either glucose or glycerol. B Quantification of the spotting assays. Graphed data is the growth value of the vector control divided by the 72Q-expressing strain from three biological replicas.
3.9 Summary of findings

- Increasing oxidative phosphorylation increases aSyn toxicity and aggregation in WT yeast.
- aSyn causes abnormal mitochondrial morphology in respiring yeast cells.
- aSyn toxicity and aggregation is increased by defects in ERMES.
- aSyn toxicity is exacerbated by deletion of PHB2 gene and mitochondrial morphology is severely affected.
- Deletion of YDC1 rescues aSyn toxicity.
- Inhibition of mitochondrial fusion exacerbates aSyn toxicity.
- Inhibition of mitochondrial fission rescues aSyn toxicity.
Chapter 4 Discussion

Section 4.1: A novel alpha-Synuclein model in respiring yeast

One of the most profound findings of my work is that yeast cells that use oxidative phosphorylation for energy production show increased aSyn toxicity and aggregation (Figure 9, 10). This increased toxicity and aggregation is not caused merely by higher levels of aSyn protein levels, as western blots have confirmed that our aSyn expression levels were equal when yeast cells were grown in fermentable and non-fermentable carbon sources (Figure 9). Furthermore, by performing spotting assays with a diverse array of carbon sources, which cause different rates of cell divisions, we have excluded the possibility increased aSyn toxicity and aggregation is solely based by slow cell division rates. We found that none of the fermentable carbon sources tested here resulted in an increase in aSyn toxicity or aggregation, while all non-fermentable carbon sources resulted in the appearance of aSyn aggregates (Figure 11, 12). The rate of cell division is an important variable for accurate yeast models because mature neurons typically do not divide at all. Also, rapidly dividing cells, such as yeast cells grown in glucose, distribute misfolded proteins between mother and daughter cell as an important mechanism of cellular protein quality control. In essence, our novel aSyn yeast model uses both a respiratory metabolism and a low rate of cell division to mimic the neurons affected by aSyn toxicity more closely.

Fermentable carbon sources allow yeast to create the majority of their ATP through either glycolysis or fermentation, processes that occur independently of mitochondria (121). However, non-fermentable carbon sources such as oleic acid, myristic acid, potassium acetate and glycerol must be metabolised through oxidative phosphorylation, thus utilizing mitochondria (121). Our results demonstrate that aSyn is toxic in cells with mitochondria that perform oxidative phosphorylation and many other metabolic and cellular functions.

As increasing oxidative phosphorylation increased both aSyn aggregation and toxicity, we explored whether aSyn also causes damage to mitochondria, as represented by altered morphology and protein import. Changes in mitochondrial morphology are difficult to objectively quantify, as there are no standard methods for differentiating fragmented from tubular mitochondria. Therefore, we performed western blots probing for mtGFP, which was used as an indicator of total mitochondrial protein content and protein import. There was no significant difference in mtGFP
levels between the vector control and aSyn-expressing yeast cells when grown in glucose, yet there was a 36% reduction in mtGFP signal in the aSyn-expressing strain compared to the vector control when grown with glycerol (Figure 10). This indicates that aSyn causes disruptions in mitochondrial homeostasis leading that might contribute to aSyn toxicity.

While the genetic pathways in yeast that interact with aSyn have already been explored in various studies, most studies utilized a high expression level of aSyn in cells grown in fermentable carbon sources. To determine whether these genetic interactions also exist in our low expression model, we incorporated six deletion strains, which have previously been characterized as enhancers of aSyn toxicity using the high expression model (44, 55). None of the selected gene deletions resulted in increased toxicity when yeast cells were grown in glucose with low expression of aSyn (Figure 13). In fact, in our model aSyn produced more toxicity in WT yeast grown with glycerol than the genetic enhancers grown on glucose. Also, only the deletion of VSP28 showed increased aSyn toxicity when yeast cells were grown in media inducing oxidative phosphorylation, whereas the other five deletions did not alter aSyn toxicity. These results suggest that the genetic interactions that determine aSyn toxicity in our low expression respiring yeast model are profoundly different from the one identified in the previously describes models. Our novel yeast model thus has great potential to unravel genetic networks and cellular mechanisms of aSyn toxicity in future high through-put screens that have been unexplored before.

**Section 4.2: Oxidative Stress and aSyn Toxicity**

Oxidative stress is one of the major hallmarks of PD. Neurons affected by PD show damage to proteins, lipids and nucleic acids caused by oxidative stress (73). However, whether oxidative stress is a cause or early even in PD, or merely a downstream effect remains unclear (81). When we induced oxidative stress either by adding hydrogen peroxide or by the deletion of the antioxidant gene SOD2 in our novel aSyn model we found that neither aSyn toxicity nor aggregation was increased. Thus, our data does not support the notion that oxidative stress causes aSyn aggregation and toxicity. These findings extend even to increasing the levels of oxidative stress caused by oxidative phosphorylation in our model.
Section 4.3: Autophagy and Mitophagy Interact Genetically with aSyn Toxicity

**aSyn and Autophagy**

Previous studies have shown that inhibiting autophagy e.g. by bafilomycin increases both aSyn toxicity and aggregation, whereas increasing autophagy by rapamycin treatment increases aSyn clearance and reduces its toxicity (42). Here we tested how general autophagy influenced aSyn toxicity in our novel yeast model. We found that rapamycin slightly reduced aSyn toxicity when grown in glucose yet produced no reduction in toxicity when grown in glycerol (Figure 16). Bafilomycin did not increase aSyn toxicity when grown with glucose, yet it strongly increased aSyn toxicity in glycerol. It is plausible that clearance of toxic aSyn by autophagy is crucial to cellular fitness when the mitochondria are producing the majority of ATP via oxidative phosphorylation. In any case, our data document that aSyn-toxicity is highly dependent on cellular metabolic activity even when autophagy is impaired.

**aSyn and Mitophagy:**

In addition to general autophagy, we tested how aSyn toxicity is modulated by mitophagy. Mitophagy, i.e. the degradation of mitochondria via autophagy, is a crucial process in mitochondrial homeostasis and impaired mitophagy has been implicated in PD (96), specifically in cases of Parkinsonism caused by mutations in the genes encoding the genes for parkin and PINK1.

Paradoxically, previous studies in yeast have reported that functional mitophagy is a prerequisite for aSyn toxicity (126). Our findings, however, show that aSyn toxicity was not altered in a mitophagy deficient yeast strain (Δatg32) irrespective of their metabolic activity.

As previous tests with the mtGFP reporter showed a reduction in mtGFP, we aimed to uncover the cause of this aSyn-associated mtGFP reduction. To this end, we utilized the Δatg32 strain to determine if inhibiting mitophagy would prevent the aSyn-induced reduction in mtGFP signal. We found no reduction in mtGFP signal in the aSyn-expressing Δatg32 strain when grown in either glucose or glycerol, indicating increased mitophagy as a cause in the mtGFP reduction. In essence, our results do not confirm a connection between mitophagy and aSyn toxicity.
Section 4.4: Loss of ERMES Function Increases aSyn Toxicity and Aggregation

Much evidence links aSyn to mitochondrial dysfunction, yet the underlying genetic, cellular, and molecular mechanism remain unknown. Recent studies have shown that aSyn disrupts mitochondrial-ER contact sites, termed ERMES in yeast or MAM in mammalian cells (47). As ER-mitochondrial contact sites are important for Ca\(^{2+}\) homeostasis, lipid exchange and general mitochondrial homeostasis and previous studies have purported that aSyn toxicity arises from the disruption of these contact sites. Our data show that disruptions in the ERMES complex by deletion of the MMM1 gene results in both an increase in aSyn aggregation and toxicity (Figure 17) thus suggesting a strong genetic interaction between aSyn toxicity and ERMES a part of mitochondrial homeostasis.

Section 4.5: Impaired Prohibitin function Exacerbates aSyn Toxicity and Results in Abnormal Mitochondrial Morphology

Abnormal prohibitin levels have recently been documented in neurons of people affected by PD (129). Further, prohibitins play a major role in mitochondrial homeostasis. We accordingly reasoned that prohibit function is linked to aSyn toxicity. On glucose, aSyn toxicity was exacerbated by the deletion of both yeast prohibit encoding genes, \(\Delta\text{phb1}\) and \(\Delta\text{phb2}\) (Figure 19). When grown on glycerol, however, only \(\Delta\text{phb2}\) exacerbated aSyn toxicity. We also noted that approximately 1/4 of the aSyn-expressing \(\Delta\text{phb2}\) strains would develop spontaneous suppressors of aSyn toxicity. The greater toxic interaction between aSyn and the \(\Delta\text{phb2}\) strain was a surprising finding as both prohibitin proteins are equally required to form the functional prohibitin complex (107). Moreover, deletion of either prohibitin gene results in a reduction of protein levels of the remaining prohibitin protein (110). The two prohibitin proteins, however, are not identical and some recent findings regarding Phb2 might account for the difference in aSyn toxicity in cells grown in glycerol. For example, McFarland and colleagues found that aSyn interacts with Phb2 at OMM but not with Phb1 (130). While we have provided no physical evidence to this end, it is plausible that aSyn interferes with Phb2 function independent of Phb1 or the intact prohibitin complex.

Fluorescence microscopy and western blots monitoring mtGFP revealed a significant decrease in mtGFP signal in aSyn-expressing \(\Delta\text{phb2}\) yeast cells only when cells were grown with glucose
(Figure 20). This was puzzling as growth in glycerol tends to further decrease the mtGFP signal in the aSyn expressing strain. One possible explanation could be that the spontaneous suppressors we observed during our spotting assays also grew in the liquid cultures with glycerol used for the western blots and could alter the overall outcome of the microscopy and protein assessment of mtGFP. This can also explain the high variability in the mtGFP signal which made it impossible to obtain reliable, statistically significant results (Figure 20B). Nonetheless, our results clearly show that aSyn has a strong genetic interaction with both prohibitins, and that mitochondrial homeostasis is severely affected by aSyn in prohibitin deficient cells.

Section 4.6: Alkaline dihydroceramidase Deficiency Rescues aSyn Toxicity

Ceramides are a family of lipids which are involved in many cellular processes, including mitochondrial-mediated apoptosis. We found that the Δydc1 cells, i.e. yeast cells deleted for alkaline dihydroceramidase, which mostly mediates hydrolysis of dihydroceramide to a free fatty acid and dihydrosphingosine showed no aSyn toxicity when grown in both glucose and glycerol. Ceramide and sphingolipids, in general, are only recently beginning to come into the light as key players in PD.

Our data show that ceramide metabolism not only interacts with aSyn toxicity but also imply that increased ceramide levels rescue aSyn toxicity. The mechanism through which this occurs is unknown, yet there are a few possible explanations. First, ceramide levels act downstream of aSyn-induced mitochondrial dysfunction. Low ceramide levels are known to cause both mitochondrial fragmentation and induce apoptosis, while exogenous addition of ceramide rescues these defects (113). Thus, it is possible that as aSyn inhibits mitochondrial fusion and interferes with mitochondrial homeostasis, the increased ceramide levels caused by the deletion of Ydc1 protect against mitochondrial-mediated apoptosis. Second, ceramides might protect mitochondria from aSyn-induced damage. Ceramide depletion results in mitochondrial membrane instability, resulting in increased fission. In contrast, increased ceramide levels in membranes have been reported to both increase membrane stability and act as recruiting platforms for the mitochondrial membrane fusion machinery (131). Therefore, it is possible that increased ceramide levels do not greatly inhibit mitochondrial fission but do counteract the fusion-inhibiting nature of aSyn which otherwise would lead to cellular dysfunction and death. It also possible that both of these suggested mechanisms act in concert to reduce aSyn-induced cell death. Clearly, future mechanistic
experiments that directly assess the concentrations of ceramides and how they interact with aSyn in mitochondrial membranes are required to decipher the underlying mechanisms.

**Section 4.7: aSyn Interacts Genetically with Mitochondrial Fission and Fusion**

Fragmented mitochondria are one of the major cellular hallmarks of PD and many other neurodegenerative diseases (85). This aberrant increase in fragmented mitochondria can have several different causes, including mitochondrial stress, programmed cell death, increased mitochondrial fission, or decreased mitochondrial fusion (85). Here we determined how mitochondrial fission and fusion genetically interact with aSyn toxicity and aggregation.

We found that yeast cells deleted for genes required for mitochondrial fission decreased both aSyn aggregation and toxicity when cells were grown in glycerol. This indicates that mitochondrial fission is implicated in both aSyn aggregation and toxicity. It is plausible to deduce that aSyn induces fragmentation by inhibiting mitochondrial fusion, rather than by increasing mitochondrial fission thus disturbing the balance between fusion and fission, which is crucial for proper mitochondrial homeostasis. In line with this interpretation, microscopy of the mtGFP reporter showed a reduction in mtGFP signal in aSyn-expressing fission-deficient yeast cells when grown in either glucose or glycerol (Figure 22) indicating increased mitochondrial abnormalities.

We next tested if aSyn interacts genetically with mitochondrial fusion. Our results show that yeast cells bearing deletions of genes necessary for mitochondrial fusion show increased aSyn toxicity. Furthermore, our mitochondrial reporter mtGFP revealed severe morphological abnormalities from the aSyn-expressing fusion-deficient yeast cells, and western blots probing for mtGFP confirmed that mtGFP levels were significantly reduced.

To confirm that these genetic interactions between aSyn and mitochondrial fission and fusion were aSyn specific, we also transformed a slightly toxic Huntington fragment, 72Q, into four representative fission and fusion gene deletion strains. We found that 72Q toxicity was not enhanced by any of the aSyn enhancers and that 72Q toxicity was surprisingly enhanced by the Δfis1 strain, a suppressor of aSyn toxicity. Therefore, these findings highlight the specificity by which aSyn interferes with mitochondria and suggest that fragmented mitochondria sensitize cells to aSyn toxicity, whereas increased mitochondrial fusion protects cells from aSyn toxicity.
**4.8: Conclusion**

Our work has provided evidence that aSyn disrupts mitochondrial homeostasis resulting in toxicity. We have found that aSyn follows not only a dose-dependent toxicity, but also a metabolic-dependent toxicity. Switching the carbon source from glucose to glycerol, i.e. from fermentative to respiration, induces oxidative phosphorylation and increases both aSyn toxicity and aggregation. As neurons produce the majority of their ATP through oxidative phosphorylation, we conclude that our novel respiring aSyn yeast model more accurately represents the metabolic profile of neurons affected in PD. Furthermore, we have shown that halting mitochondrial fission was protective for aSyn toxicity while halting mitochondrial fusion enhanced aSyn toxicity. We thus deduce that aSyn toxicity arises through an inhibition of mitochondrial fusion, which then results in a cascade of effects such as decreased ATP production and apoptosis.

**4.9: Limitations**

Yeast is a powerful tool to study genetic interactions of misfolded proteins associated with neurodegeneration, such as ALS, HD, and PD. Yet, work in yeast models has some obvious limitations. Yeast is a unicellular organism, unlike neurons affected by PD, which precludes work in yeast models to study mechanisms of neurodegeneration based on cell-cell interactions. Furthermore, yeast do not have the main morphological features of neuron, nor can they be excited by synaptic activity. Most relevant to the work presented here, there are aspects of mitochondrial function and homeostasis that differ between yeast and mammalian cells. For example, inhibiting mitophagy appears to be quite lethal to neurons, yet the effects of inhibiting mitophagy in yeast is less severe. In the following chapter, provide solutions to these limitations which could be implemented in future experiments.

**4.10: Future Work**

First, we should validate our main results in neuronal cells, e.g. neuronal cell line, such as SH-SY5Y cells, which have been useful for PD studies before. An important experiment would be to show that increasing oxidative phosphorylation in a neuronal cell line increases aSyn toxicity and aggregation. This may be somewhat difficult to achieve as many cultured mammalian cells use glycolysis to generate the majority of their ATP. Yet, changing carbon sources, such as replacing...
glucose with pyruvate, can increase oxidative phosphorylation activity, where aSyn toxicity and aggregation, and mitochondrial function and homeostasis can be assessed.

Of note, neurons do not only produce approximately 90% of their ATP through oxidative phosphorylation, but the oxidative phosphorylation activity within each neuron changes dramatically based on increased or decreased synaptic activity. To reflect this activity-based alteration in oxidative phosphorylation and the ensuing requirement for proper mitochondrial function and homeostasis, we would likely need to utilize aSyn animal models, such as C. elegans, Drosophila melanogaster, and rodent models.

We have shown that the genetic enhancers and suppressors of aSyn toxicity we discovered in this study do not interact in the same way with a toxic Huntington fragment. In future experiments, it will be important to test how mitochondrial homeostasis generally, and fusion and fission specifically are modulated by other PD-related proteins. PINK1 and parkin are both involved in mitophagy and DJ-1 functions as an oxidative chaperone in mitochondrial homeostasis. It will thus be informative to analyze the effect of PINK1, parkin, and DJ-1 on mitochondrial fusion and fission alone and in connection with aSyn in SH-SY5Y cells and PD animal models.

The last piece of the puzzle we would like to solve is uncovering how exactly aSyn induces mitochondrial dysfunction. Finding proteins that physically interact with and are inhibited by aSyn seems an obvious approach, yet these experiments have already been previously conducted with limited success. Instead, focusing on aSyn’s interaction with lipids and membranes as the main cause of aSyn toxicity might be more promising. For instance, future experiments could seek to explore how aSyn affects lipid metabolism and membrane integrity and how it relates to aSyn toxicity and mitochondrial dysfunction.

**4.11: Significance**

aSyn is a key PD protein, yet how exactly it causes neurodegeneration remains unknown. Clearly, research on aSyn has been complicated by the wide range of cellular processes that interact with aSyn. These processes include ER-Golgi trafficking, ER-mitochondrial communication, autophagy, lipid metabolism and cellular protein homeostasis. Our study identifies mitochondrial homeostasis, in particular, mitochondrial fusion and fission, as an early and specific mechanism by which aSyn causes cellular toxicity and possibly as a promising therapeutic target to treat PD.
Figure 32: Simplified schematic depicting the lifecycle of mitochondria in a healthy cell (top) and a cell expressing aSyn (bottom).
Supplementary Figures

Figure 33: Spotting assays on YPD of WT yeast expressing empty vector or aSyn.

Figure 34: Spotting assays on YPD of WT yeast and mitochondrial fission gene deletions expressing empty vector or aSyn.

Figure 35: Spotting assays on YPD of WT yeast and mitochondrial fusion gene deletions expressing empty vector or aSyn.
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Michael G. Tauro

Education

2015: University of Toronto Bachelor of Science, Neuroscience, Graduated December 2015.

2017: Master of Science candidate at Western University, Anticipated graduation of January, 2019

Honours and Awards

2018-2019: Parkinson Society Southwestern Ontario: Graduate Student Research Program Award
Value: $15,000

Presentations and Abstracts


2015: “Experimental Evidence for the Utility of Nociception in Osteichthyes” Research proposal created by myself under the supervision of Dr. Carin Wittnich. Purpose was to devise a study that could test whether less complex nervous systems (such as that of the elasmobranchi and osteichthyes) are capable of experiencing pain in the same way as more complex mammalian nervous systems. Progress of study was monitored bi-weekly by Dr. Wittnich for a total of 100 hours.

2015: “Neuroanatomical Explanation for Differences in Aggressive Behaviour Between Sea Lions, Dolphins and Manatees” Manuscript completed by myself as a requirement for the physiology field course PSL379 held in Puerto Aventuras, Mexico and at the University of Toronto. Consisted of observing animal behaviour and then providing a neuroanatomical explanation for observed behaviour differences as well as an evolutionary rationale as to why said neuroanatomical differences developed.

2014: Completed my required placement for an Exercise and Mental Health course at U of T at the drug and addiction centre “Vita Novus”. Purpose was to gain knowledge on how exercise might help those in recovery from drug addiction. Achieved by asking clients how they find exercise benefits them during recovery while also exercising together. Also involved reading relevant studies on exercise and addiction. Wrote an exercise routine for the clients I was working with that they could follow after the completion of my placement.

Volunteer Experience

2018: Walk-it for Parkinson’s. Assisted in planning, set up, and registration of event. Also presented a poster geared towards a layman audience.


International Experience
2015: Completed a field course in Mexico (PSL379) studying aggressive behaviours in sea lions, dolphins and manatees and then provided a neuroanatomical explanation as to why there are differences in levels of aggression across species.

2011: Completed a semester abroad at the University of Siena, Italy. Helped improve my fluency in Italian while also providing an excellent international learning experience

**Work Experience**

2013-2015: Employed at Addiction Canada during the summer.

2012: Quality control at Vins Plastics.

**Sports Involvements**

2012-2015: Varsity Fencing Team

2013-2015: Varsity Rugby Team

**Languages:** Fluent and literate in English and Italian.