Evaluating α-Synuclein's Interaction with Cellular Phospholipids and Potential Toxicity in Yeast Models for Parkinson's Disease

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ABSTRACT

Parkinson's disease is a progressive neurodegenerative disease caused by the death of midbrain dopaminergic neurons. The misfolding and aggregation of α-synuclein plays a ruinous role in this disease, but how the protein becomes toxic is unclear. Using yeasts as model organisms for studying α-synuclein properties, our study explores the hypothesis that α-synuclein toxicity depends on plasma membrane phospholipid binding. First, using a chemical approach, we induced phospholipid synthesis in both fission and budding yeast with dimethyl sulfoxide (DMSO), a known inducer [1]. Instead of regulating α-synuclein-dependent toxicity, DMSO unexpectedly exerted its own toxicity in both yeasts, in addition to inducing a lethal morphology defect in budding yeast. Moreover, instead of inducing plasma membrane localization of α-synuclein in either yeast, DMSO altered α-synuclein localization in both yeasts into as-yet unidentified cytoplasmic structures. We speculate that some of these structures may be internal, membrane bound organelles. To test for membrane phospholipid binding specifically, α-synuclein localization was analyzed in a phosphatidylserine-deficient budding yeast strain. We observed no loss of plasma membrane localization, suggesting that other phospholipids may regulate such specificity to α-synuclein. Together, these related studies illustrate the usefulness of yeasts in evaluating genetic and environmental factors that regulate α-synuclein toxicity linked to Parkinson's disease.

I.   INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder in which patients suffer from resting tremors, postural rigidity, bradykinesia, and poor balance. These symptoms are the result of the specific death of dopaminergic neurons in the substantia nigra *pars compacta* [2]. Most cases of PD are idiopathic and occur during late age. Approximately 10% of PD cases are genetically linked [3]. PD is fatal and incurable, but over the last thirty years a variety of drugs have been developed to treat the symptoms.

Key findings in PD research point to the hypothesis that the protein α-synuclein plays a critical role in pathogenesis. PD is characterized by cytoplasmic accumulation of misfolded α-synuclein, Lewy body, within the affected dying dopaminergic neurons [4]. There are three mutations in the α-synuclein gene that cause dominant and familial PD; A53T [5], A30P [6], and E46K [7]. Transgenic mice [8] and flies [9] expressing the human form of α-synuclein are well established models for replicating the PD phenotypes [10].

In recent years, the role of lipids has become significant in PD pathology. The interaction of α-synuclein with lipids and vesicular membranes are evident in Lewy bodies [11-12]. α-Synuclein is found in lipid-rich cytosolic fractions where the structure of amino and carboxyl termini of this protein share homology with fatty acid-binding proteins [13]. In the transgenic mouse model for PD, α-synuclein localizes primarily to the lipid-rich neuronal region of synaptosomes

[14]. In yeast, *Saccharomyces cerevisiae,* models, both WT and A53T α-synuclein localize to the plasma membrane [15-16]. When α-synuclein expression is elevated in the yeast, WT and A53T, but not A30P, are toxic to cells [15]. Yeast continues to serve as a good model system for PD. A screen of several thousand library clones in yeast identified 25 non-toxic α-synuclein sequence variants [17]. Interestingly, most of these variants contain a mutation in the N-terminal region to either proline or glutamic acid, which cause a defect in membrane binding. Hence, it is possible that toxicity arose by α-synuclein binding directly to phospholipid membranes [17]. Moreover, the over-expression of α-synuclein in yeast can lead to abnormal lipid accumulation [16], disrupt endocytosis [16, 18] and impair membrane trafficking in endoplasmic reticulum [19]. A genetic screen in budding yeast identified 86 genes that were lethal when absent with over-expressed α-synuclein [20]. Surprisingly, the two largest groups of these genes were involved in lipid metabolism and vesicle-mediated transport [20]. Previously, our lab has worked with both budding and fission yeast to model PD [21-22]. We also find that α-synuclein localizes to the plasma membrane and exhibits cellular toxicity in budding yeast [21]. However, in fission yeast, α-synuclein neither goes to the plasma membrane nor is it toxic [22].

Here, we further investigated the link between α-synuclein's interaction with phospholipids and cellular toxicity using budding and fission yeast models. We developed two approaches; 1) chemical, using DMSO to up-regulate phospholipid synthesis, and 2) genetic, using a gene-knockout budding yeast strain to down-regulate phospholipid synthesis. We hypothesized that increasing phospholipid association of α-synuclein would enhance cellular toxicity of the protein. We predicted that α-synuclein in budding yeast would localize more to the plasma membrane in the presence of DMSO compared to cells left untreated. In fission yeast, we predicted that DMSO would decrease the formation of cytoplasmic aggregates and increase α-synuclein's plasma membrane localization. Additionally, both yeasts would incur α-synuclein dependent cellular toxicity in the presence of DMSO. In the knockout strain, we predicted a loss of α-synuclein membrane localization with reduction in cellular toxicity.

II.   MATERIALS AND METHODS

a.   *S. Cerevisiae* Expression Vectors

The pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen) was used for α-synuclein expression in budding yeast. The following two vector constructs were previously created for each experiment: green fluorescent protein (GFP), and wild-type α-synuclein. α-synuclein was tagged at the C-terminus with GFP. The pYES2.1/V5-His-TOPO vector and the GFP tagged vector were used as expression controls.

b.   *S. Pombe* Expression Vectors

Polymerase chain reaction (PCR) was used to amplify C-terminal green fluorescence protein (GFP)-tagged wild-type α-synuclein fusion cDNA from the α-synuclein-GFP containing pYES2/TOPO *S. cerevisiae* vectors constructed by Sharma et al [21]:

forward primer, 5'-GGGGCCAAGCTTGCCATGGATGTATTCATGAAAGGA-3';

reverse primer, 5'-TTTGTAGAGCTCATACATGCCATG-3'.

Similarly, PCR was used to amplify GFP cDNA from GFP-pYES/TOPO *S. cerevisiae* vectors constructed by Sharma et al. [21]:

forward primer, 5'CCCGGGACCATGGCCAGCAAAGGAGAAG-3';

reverse primer, 5'-TTTGTAGAGCTCATACATGCCATG-3'.

These PCR products were subcloned, according to the manufacturer's protocol (Invitrogen), into fission yeast pNMT-1 TOPO-TA expression vector. The following two vector constructs were previously created for each experiment: GFP, and wild-type α-synuclein. α-synuclein was tagged at the C-terminus with GFP. The pNMT-1 vector and GFP in pNMT-1 vector served as expression controls.

c.   Yeast Strains

The TCP1 strain (h-*leu1-32*; Invitrogen) of fission yeast was kindly

provided by Judy Potashkin, Rosalind Franklin University of Medicine and Science. BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and *cho1Δ* knockout strain were purchased from Open Biosystems.

d. Yeast Transformation

S. Pombe strains were transformed with pNMT TOPO-TA vectors [23] and *S. Cerevisiae* were transformed with pYES2.1/V5-His-TOPO vectors (see table 1.) using the lithium-acetate transformation method [24].

e. *S. Pombe* Expression

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

f. *S. Cerevisiae* Expression

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

g. Fluorescence Microscopy

Yeast cells were first grown overnight at 30°C in EMM containing thiamine (10uM) if working with *S. Pombe* or in SC-Ura glucose if working with *S. Cerevisiae*. After 24hr, cells were pelleted at 1500g for 5 minutes, washed twice in 10ml dH$_2$O, resuspended in 10 ml EMM without thiamine/SC-Ura galactose, of which 125 mL cells were used to inoculate 25 mL EMM without thiamine/SC-Ura galactose (to express α-synuclein). At desired expression time points for microscopy, cells were harvested at 1500g (4°C) for 5 min and were washed in 5 mL water. Then cells were resuspended in 100-1000 uL EMM+T/SC-Ura glucose, of which 10 uL was pipetted onto a slide. Slide of cell culture was viewed using Nikon TE-2000U fluorescence microscope at 1000X magnification. Images were deconvoluted using MetaMorph software version 4.2. Cells were first viewed under differential interference contrast (DIC) microscopy, and then viewed for GFP fluorescence. For analysis, the number of cells in the field containing α-synuclein aggregates, intracellular structure localization or plasma membrane local-ization. The field was then moved three turns on the field control knob, and the process was repeated in a new field. At least 750 cells were evaluated for each sample. α-Synuclein phenotypes were scored as percent of total cells observed.

h. Growth Curve

Cells were grown in 5 ml PDM-Leu/SC-Ura glucose overnight at 30°C in the incubator which rotates at 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice in 5 ml H$_2$O. Cells were re-suspended in 5 ml H$_2$O and were counted. Flasks with 25 ml EMM/SC-Ura galactose were each inoculated with 2.0×10^6 cells/ml density. At 0, 6, 12, 18, 24, 36 and 48 hours, and in duplicate measurements, 1 ml of cell culture was removed and placed in a cuvet to measure absorbance using a Hitachi U-2000 Spectrophotometer. Averaged absorbance readings were plotted against time points to produce a growth curve.

i. Spotting Analysis

For spotting, cells were grown to mid-log phase in PDM-Leu/SC-Ura glucose normalized to equal densities (2×107 cells/mL), serially diluted (5-fold) into 96-well microwell plates. Using a multi-channel pipettor, culture is spotted on EMM +
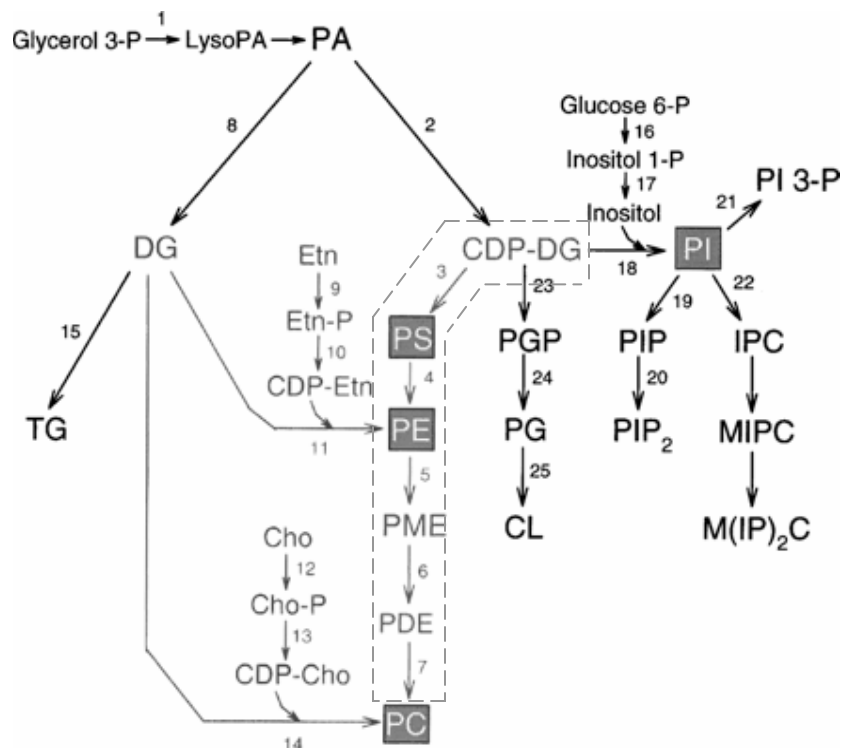
Figure 1. Phospholipid biosynthetic pathways in *Saccharomyces cerevisiae*. Reaction 3, in our study, is terminated due to the absence of PS synthase. The four major phospholipids (PC, PE, PI, and PS) are indicated by boxes [25].

thiamine or EMM – thiamine plates (*S. Pombe)* and SC-Ura glucose or galactose plates (*S. Cerevisiae)*. Photographs of the plates were scanned after 2 days of growth.

j.   Western Analyses

Yeast cells (2.5×10^7 cells/mL) were washed in 50 mM Tris (pH 7.5) and 10 mM NaN$_3$ and solubilized in electrophoresis sample buffer ([ESB] Burke et al [24]) containing 2% sodium dodecyl sulfate (SDS), 80 mM Tris (pH 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/mL bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.7 µg/mL pepstatin A, 0.5 µg/mL leupeptin, 10 µg/mL E64, 2 µg/mL aprotinin, and 2 µg/mL chymostatin). Samples were run on precast 10–20% Trisglycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to

PVDF membranes, and Western blot was performed with different monoclonal antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 (Invitrogen) for most blots to probe for α-synuclein, and anti-phospho-glycerokinase ([PGK] Molecular Probes) and Anti β-Actin (Abcam) for most expression experiments, as a measure of loading control in duplicate blots.

k.   Analysis on cho1Δ strain

The deficiency in phospholipid content was produced using a knocked out yeast strain that lacks the gene CHO1 which encodes for phosphatidylserine synthase. The phospholipid biosynthetic pathway (see Figure 1; adapted [25]) in yeast is a reference for understanding how specific phospholipid-deficient state is established. For this study, the main pathway of import is surrounded with dashed boxes and is termed the de novo pathway for synthesis of PS, PE and phosphotidycholine (PC). The
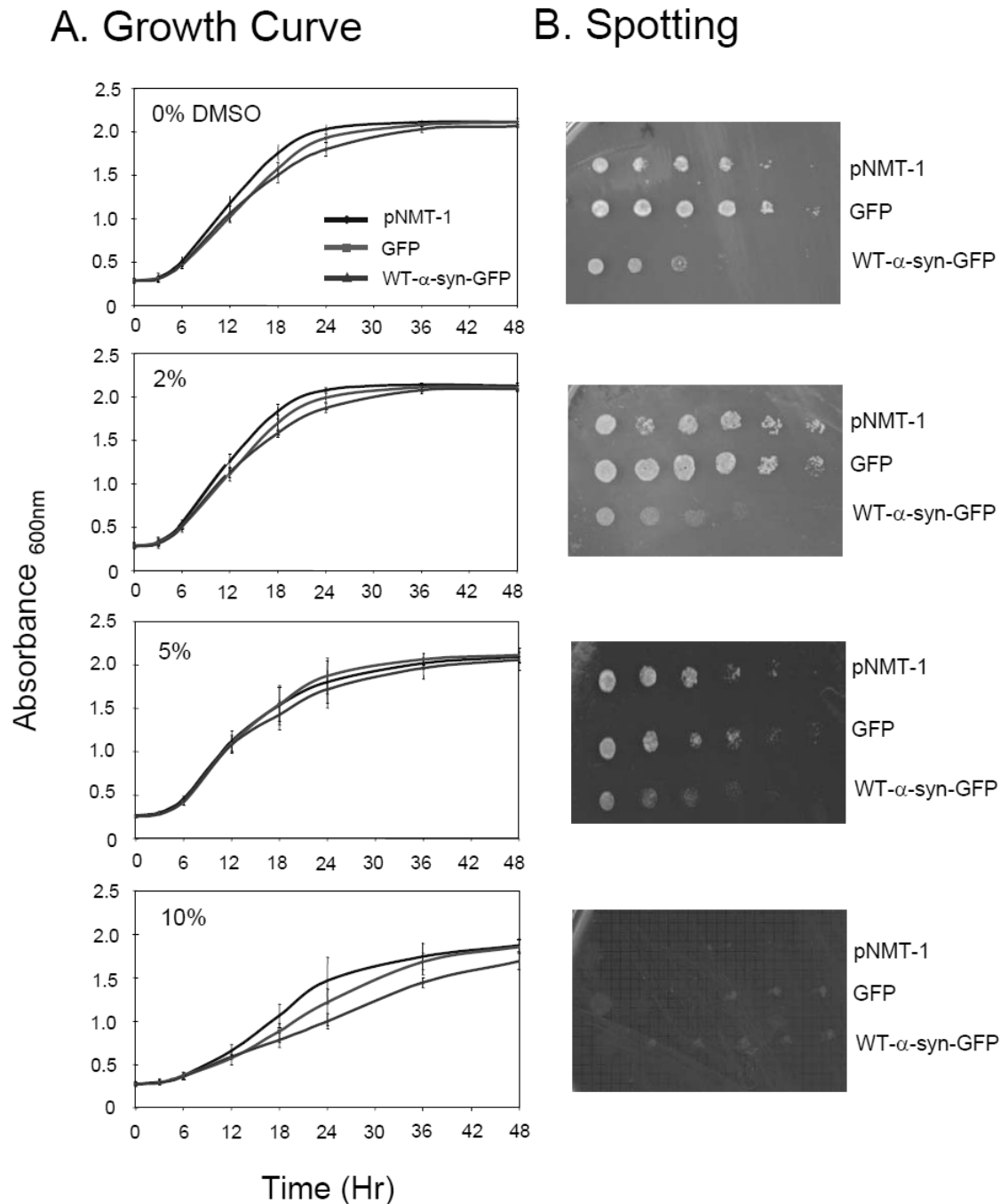
A. Growth Curve

B. Spotting

Figure 2. Fission yeast treated with DMSO. **A.** *Growth Curve*: Fission yeast were grown in EMM-T for 48 hours expressing WT (wild-type) α-synuclein along with pNMT-1 parent plasmid and GFP as controls. Over this time course, cells were treated with 2%, 5% or 10% DMSO and compared to the cells that were not treated. Each of the points on the curves represents the mean from three trials on measurement of absorbance. In addition, the standard error of the mean for a 95% confidence interval is represented by the y-bar on each of the points on the growth curves. Significant growth difference exists between pNMT-1 in 0% DMSO (n=3, p18hr = 0.007 and p24hr= 0.098) and pNMT-1 in 10% DMSO. **B.** *Spotting*: Serially diluted cells expressing parent pNMT-1 plasmid, GFP, or WT α-synuclein were spotted onto EMM-T (inducing) plates. This experiment was repeated two separate times.

A. Microscopy

B. Quantification
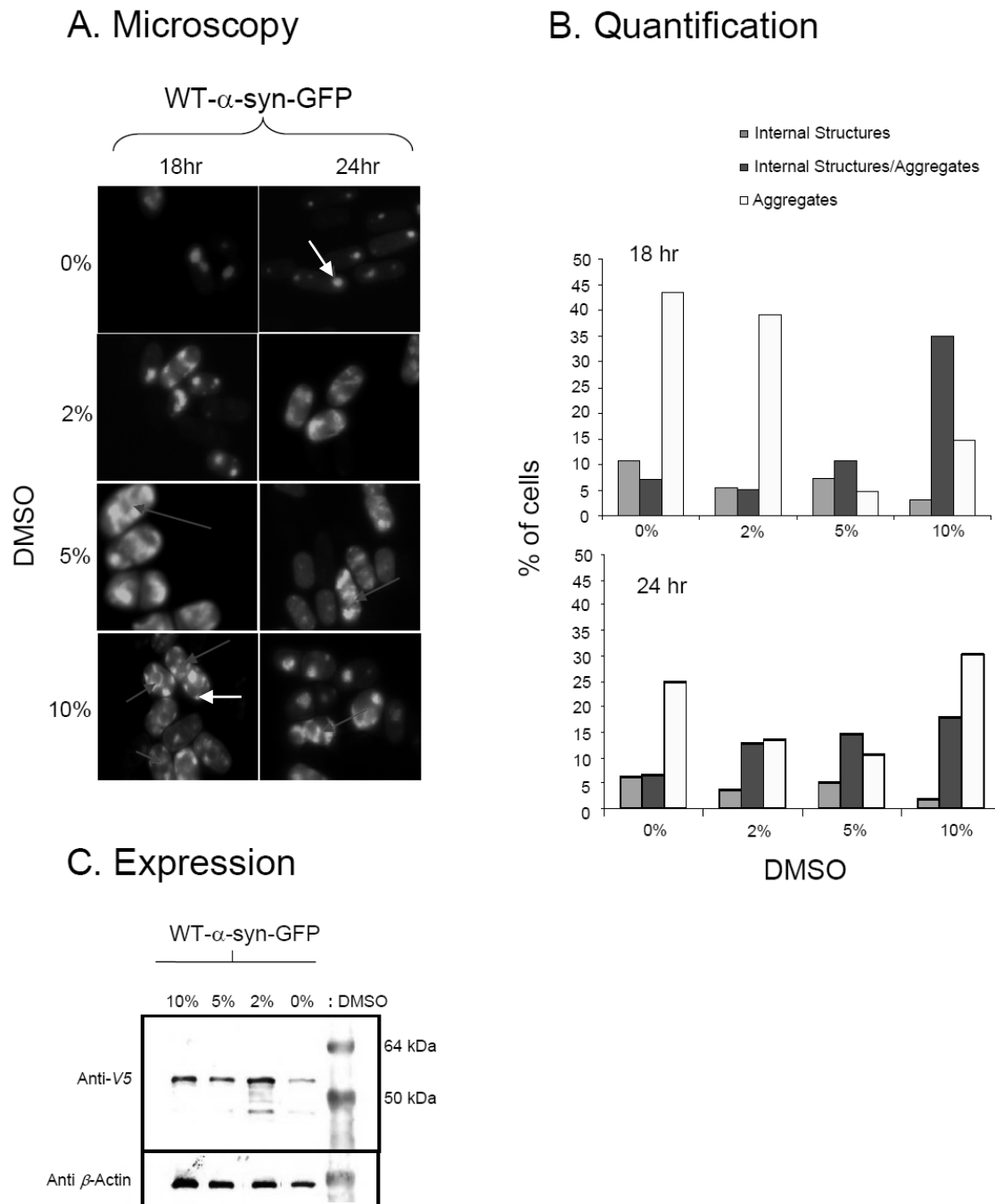
C. Expression

Figure 3. GFP Microscopy and α-synuclein expression in fission yeast cells treated with DMSO. *A. Microscopy:* Images were captured at 18 and 24 hours after induction in EMM-T. Aggregates (white arrow) in these cells started to form at 18 hours. With increasing concentrations of DMSO, α-synuclein began to localize to intracellular structures (gray arrow). *B. Quantification:* Cells expressing WT-α-synuclein were counted and characterized by the following phenotypes: internal structure localization (light gray bar), internal structure localization and aggregates (dark gray bar), or aggregates (white bar). Bars represent the percent of 600-800 total cells which display those various α-synuclein phenotypes at different concentration of DMSO. *C. Expression:* For all the samples, α-synuclein expression was induced for 24 hr in EMM-T media and detected using anti-V5. Anti β-Actin antibody showed equal loading of protein.

gray pathways, called the Kennedy pathway, are for using exogenous choline or ethanolamine to produce those phospholipids. The parent strain is BY4741. To stop PS production (it would still have all the rest of the phospholipids if ethanolamine present), we grew cho1Δ yeast strain in 1 mM ethanolamine in every selected media. Protocols written above for *S. cerevisiae* experiments such as growth curve, and fluorescence microscopy were followed.

l.   Statistical Analyses

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

m. DMSO Treatment

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Cells were grown in 10 ml PDM-leu or in SC-Ura glucose overnight at 30°C. Procedure for cell harvesting and calculating cell density for inoculation was done according to the appropriate experiment discussed above. DMSO was added to the selected media at concentrations of 0%, 2%, 5% and 10%. Afterwards, yeast cells were inoculated into the media. Then the cells were observed at the desired time points for the following experiments: Western Analysis, Growth Curve, Spotting Analysis and Fluorescence Microscopy.

III.   RESULTS

a. DMSO induces α-synuclein independent toxicity in fission yeast

Our first goal was to evaluate if a global induction of phospholipid synthesis would increase α-synuclein-dependent toxicity in fission yeast. We used a known phospholipid inducer in yeast, the organic solvent DMSO [1]. Toxicity was assessed in two ways. First, OD600 growth curves were performed on cells with or without α-synuclein and with DMSO treatment. As previously reported [22], without DMSO α-synuclein expressing cells grew well (Figure

2A). However, with DMSO cells were toxic in a concentration dependent manner (Figure 2A). Surprisingly, this toxicity was not α-synuclein dependent, cells that expressed GFP alone or contained the parent vector were also growth impaired (Figure 2A). α-Synuclein, at best, enhanced DMSO toxicity minimally (Figure 2A). Secondly, we evaluated colony survival on plates by serial dilution. Similar to growth curve analysis, we observed a striking dose-dependent toxicity with DMSO that was not dependent on the presence of α-synuclein (Figure 2B). Together, both toxicity assays indicate that DMSO exerted an unexpected toxicity in fission yeast independent of α-synuclein presence.

b. DMSO alters α-synuclein localization to internal structures in fission yeast

Next, we evaluated if DMSO would induce plasma membrane localization of α-synuclein in fission yeast. Localization was assessed by live cell GFP imaging. As previously reported [22], wild-type α-synuclein in fission yeast showed cytoplasmic aggregates (Figure 3A). DMSO induced α-synuclein to alter its localization to internal cellular structures (Figure 3A). Upon quantification, we determined that with increasing DMSO concentration, a greater percent of cells began to display α-synuclein localization to internal cellular structures (Figure 3B). However, the expression of α-synuclein remained the same with DMSO treatment (Figure 3C).

c. DMSO also induces α-synuclein–independent toxicity in budding yeast

We conducted parallel studies in budding yeast using the strain BY4741, and asked if DMSO would increase α-synuclein-dependent toxicity. First, OD600 growth curves were performed on cells with or without α-synuclein expression and treated them with DMSO. α-Synuclein expressing cells, without DMSO, exhibited slight toxicity (Figure 4A). However, as DMSO concentration increased, especially at 10%, the growth curve shifted significantly to the right, indicating a toxic effect (Figure 4A). Toxicity was not specific to α-synuclein; cells
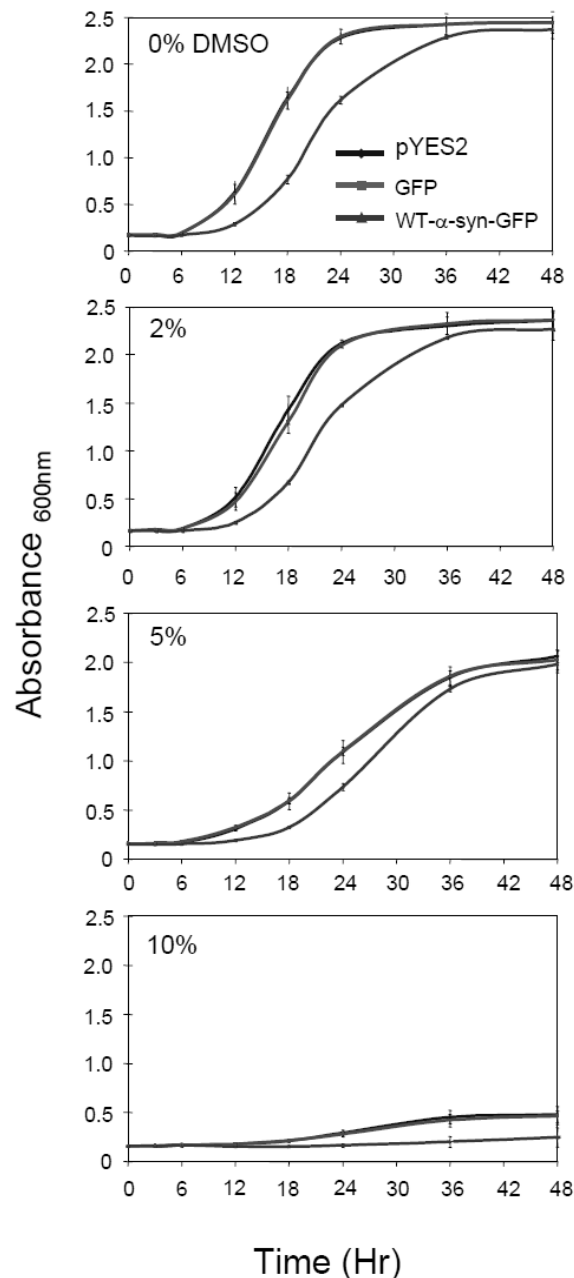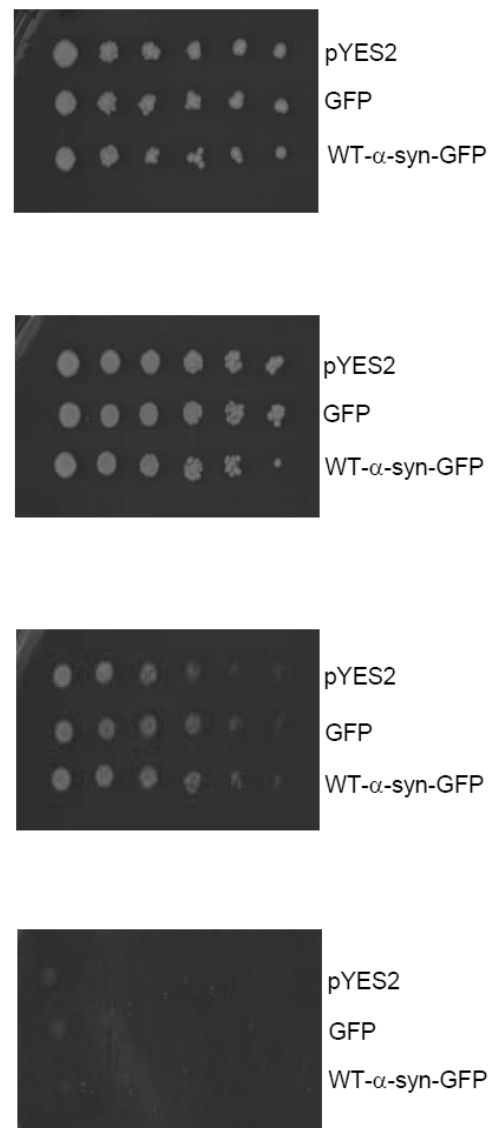
Figure 4. Budding yeast BY4741 treated with DMSO. *A. Growth Curve:* BY4741 cells were grown in Sc-Ura galactose for 48 hours to induce parent pYES2 plasmid, GFP, and WT a-synuclein. Over this time-course, cells were treated with 2% (A), 5% (B) or 10% (C) DMSO (dashed lines) and compared to the cells that went without DMSO treatment (solid lines). Each of the points on the curves represents the mean from three trials on measurement of absorbance. In addition, the standard error of the mean for a 95% confidence interval is represented by the y-bar on each of the points on the growth curves. Significant growth difference exists between pYES2 in 0% DMSO (n=3, p18hr= 0.004 and p24hr= 0.0005) and pYES2 in 10% DMSO. *B. Spotting:* Serially diluted cells expressing parent pYES2 plasmid, GFP, or WT α-synuclein were spotted onto SC-Ura galactose (inducing) plates. This experiment was repeated two separate times.

A. Evaluating Sugar Dependence
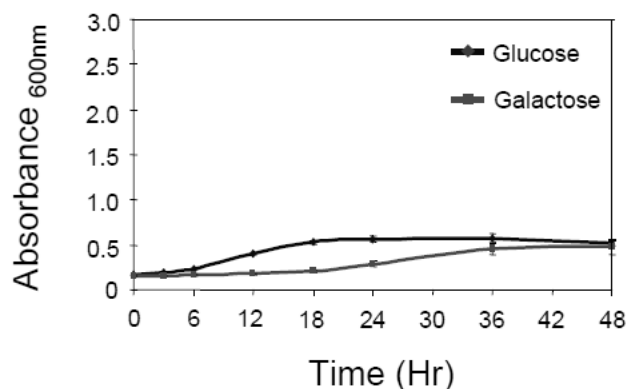
B. Evaluating Strain Dependence
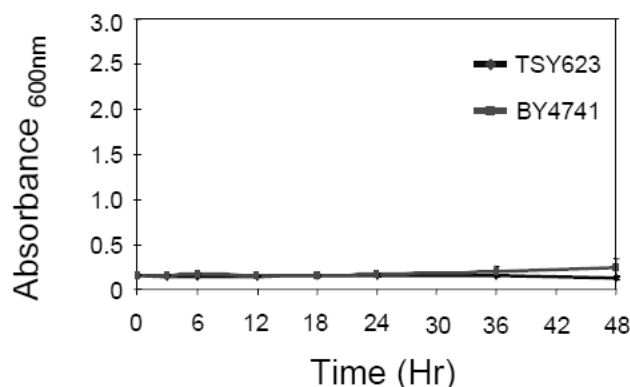
Figure 5. Budding yeast toxicity analysis with DMSO. **A.** *Evaluating Sugar Dependence:* BY4741 budding yeast cells with parent plasmid were grown in SC-Ura galactose and glucose media to test if slow growth in 10% DMSO is partially due to change of sugar in the media. **B.** *Evaluating Strain Dependence:* We tested another a-synuclein expressing budding yeast strain, TSY623, for its growth in 10% DMSO condition. Each of the points on the growth curves represents the mean from three trials. In addition, the standard error of the mean for a 95% confidence interval is represented by the y-bar on each of the points on the growth curves.

that expressed GFP alone or contained parent vector were also growth impaired (Figure 4A). Secondly, we evaluated toxicity by serial dilution spotting. Similar to growth curve analysis, we observed a striking dose-dependent toxicity with DMSO that was not dependent on the presence of α-synuclein (Figure 4B). Because this 10% DMSO toxicity was unexpected and contrary to published findings [1], we inquired if the toxicity was a result of the sugar source, galactose. However, we found that 10% DMSO exhibited the same toxicity in cells grown on glucose (Figure 5A.) Lastly, we even considered that the toxic effect of DMSO may be specific to the BY4741 strain. Yet, 10% DMSO demonstrated the same toxicity in another yeast strain, TSY623 (Figure 5B).

d. DMSO induces α-synuclein aggregates and aberrant morphology

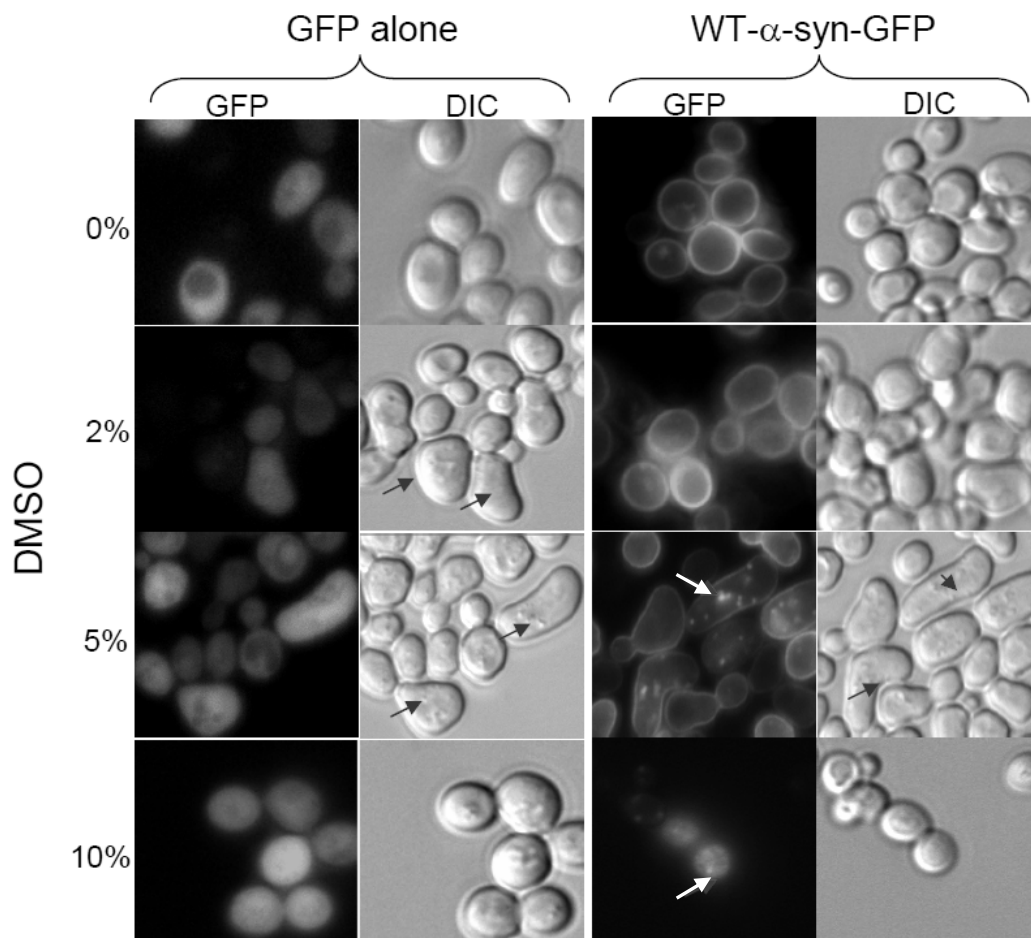Next, we evaluated if DMSO would induce greater plasma membrane localization of α-synuclein in budding yeast.

Figure 6. GFP Microscopy of BY4741 treated with DMSO. Images were captured at 18 hours after induction in SC-Ura galactose. Without DMSO treatment, most of the cells showed WT α-synuclein localized to the plasma membrane and GFP diffuse in the cytoplasm. With increasing concentrations of DMSO, both GFP and WT cells showed aberrant morphology (dark gray arrow). The aberrant morphological cells expressing WT α-synuclein showed increasing number of cytoplasmic α-synuclein aggregates (white arrow).

As previously reported [21], untreated α-synuclein-expressing cells showed α-synuclein localized to the plasma membrane. Images were captured at both 18 and 24 hours post-induction. At 5% DMSO, two major changes occurred: 1) Cells adopted an aberrant morphology and appeared elongated and sickle-shaped rather than the normal round shape and 2) α-synuclein gained intracellular localization to unknown compartments and was aggregated (Figure 6). Quantification indicated that the aberrant cellular morphology was highest at 5% DMSO treatment (Figure 7A). Localization patterns indicated that in the normal shaped cells α-synuclein primarily localized to the plasma membrane, while in the abnormal shaped cells it formed cytoplasmic aggregates (Figure 6 and 7B). The expression of α-synuclein remained the same with DMSO treatment (Figure 7C).

e. Lack of PS impairs yeast growth

Our third goal was to evaluate whether α-synuclein's association with the plasma membrane was specific to a major membrane phospholipid. Moreover, a loss of a major phospholipid, phosphatidylserine (PS), from cellular membranes may change α-synuclein toxicity in budding yeast. PS

deficiency was achieved by using a yeast strain knocked-out for the CHO1 gene. This knockout grew significantly slower than the parent strain, BY4741 (Figure 8B). When cells finally started to grow, *cho1Δ* strain expressing WT α-synuclein exhibited similar growth curves compared to the control cells (parent plasmid and GFP; Figure 8B).

f. Lack of PS preserves α-synuclein localization

Nevertheless, we still evaluated α-synuclein localization in *cho1Δ* cells by live-cell-GFP imaging to determine if the absence of PS altered α-synuclein's membrane localization. Both at 24 and 36 hours, WT α-synuclein localized to the plasma membrane in *cho1Δ* and the parent strain (Figure 8A), indicating that α-synuclein association with the plasma membrane did not solely require PS.

IV.   DISCUSSION

Here, we have examined α-synuclein's interaction with phospholipids as a potential basis for its cellular toxicity. Our first major finding was that DMSO exerted cellular toxicity independent of α-synuclein expression along with altering α-synuclein's localization and, in budding yeast, changing cellular morphology. Our second major finding was that we found PS to be less critical to α-synuclein's phospholipid membrane binding.

a. DMSO affects viability and influences aberrant cellular morphology

Organic solvents, like DMSO, are known to negatively affect the metabolism of micro-organisms [26]. Previous findings on DMSO treatment to yeast showed that a 10% concentration in liquid media reduces the cell density by half [1]. In our study, during mid-log phase, 10% DMSO also reduced the density of fission yeast cells by approximately half. But, to our surprise, this concentration reduced growth by nearly 90% in two strains of budding yeast. Such a high level of toxicity has not been previously reported. Interestingly, in both types of yeasts, the DMSO toxicity was the highest when α-synuclein was also expressed in these cells, suggesting that α-synuclein

added to the toxicity, but only slightly. 5% DMSO induced budding yeast (but not fission yeast) to produce abnormal morphologies, reminiscent of cell cycle arrest. The cells are often 2-3 times the normal size suggesting the occurrence of incomplete cytokinesis. In support of this notion, DMSO can convert a haploid yeast strain of *Torulaspora delbrueckii* into a diploid [27]. Indeed, DMSO can also perturb the mammalian cell cycle, inhibiting the differentiation of myoblasts into muscle cells [28] and the differentiation of β-lymphocyte-derived cell line into IgM-producing cells [29]. The mechanism by which DMSO affects yeast cell cycle, cellular morphology, and eventually cellular death remains unknown.
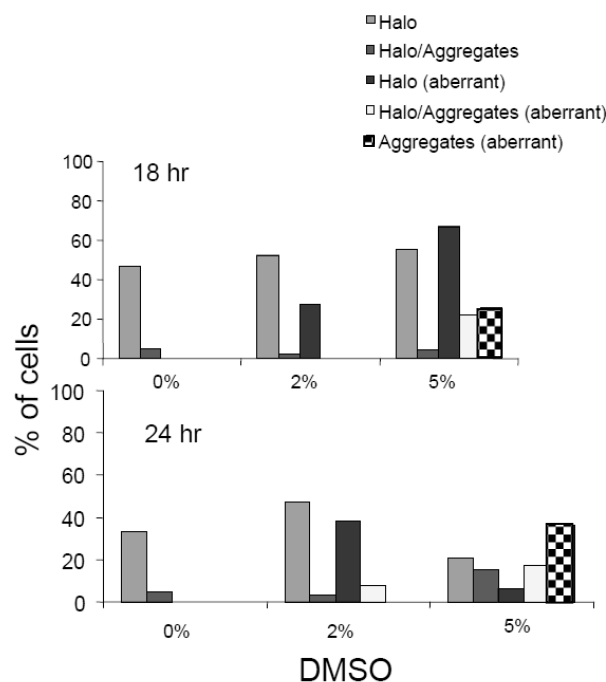
b. DMSO increases α-synuclein's intracellular localization in fission yeast

DMSO has a dramatic influence on α-synuclein cellular localization in both types of yeast. In DMSO treated fission yeast, α-synuclein surprisingly does not redistribute from intracellular aggregate to the plasma membrane, which is contrary to our hypothesis. Instead it is found in intracellular locales that are different from and in addition to the usual aggregates. We suggest that the new phospholipids that were induced by DMSO [1] in fission yeast are likely to be intracellular vesicle pools (rather than newly added plasma membrane components). These new phospholipid vesicles bind to α-synuclein, redistributing some of it from forming aggregates. It is also possible that some of these internal α-synuclein locales are yeast prevacuoles or the vacuole itself, since α-synuclein is known to target to the vacuole/lysosome for its degradation [30]. In support of the latter notion, FM4-64 dye, which is a vacuole stain, applied to fission yeast, fluoresces vacuolar membrane structures [31] in a pattern reminiscent of the α-synuclein localization we see in DMSO-treated fission yeast. Though FM4-64 stain was not used in this study, Pellens et al. [32] showed colocalization of FM4-64 stained vesicles with WT α-synuclein in yeasts. Lastly, it is also possible that the aggregated pool of α-synuclein in fission yeast (seen with and without DMSO) may actually be the result of similar intracellular phospholipid interactions. Our data suggests that such

A. Quantification [Morphology]
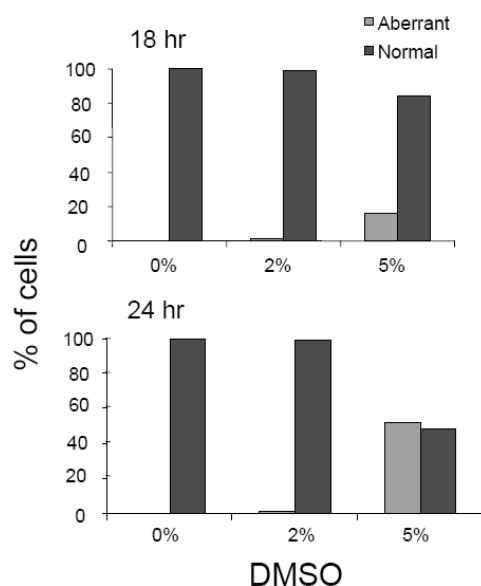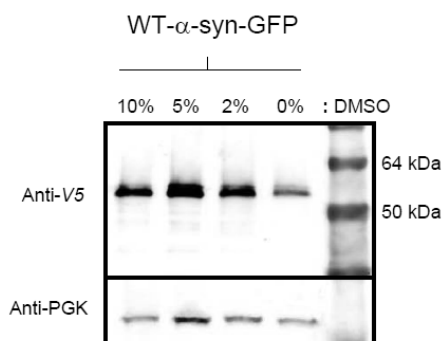
B. Quantification [Localization]

Figure 7. Quantification and α-synuclein expression in budding yeast cells treated with DMSO. **A.** *Quantification of Morphology:* DIC images of cells expressing WT α-synuclein were counted and characterized by those showing aberrant morphology (light gray bar) or normal oval shape (light gray bar). Bars represent the percent of 600-800 total cells which display those two different DIC images of the cells at various concentrations of DMSO. The appearance of aberrant morphology increased with a higher concentration of DMSO and over time. **B.** *Quantification of α-Synuclein Localization:* Cells expressing WT α-synuclein were counted and quantified by those showing halo (light gray bar), halo/aggregates (medium gray bar), halo in aberrant yeast (dark gray bar), halo/aggregates in aberrant yeast (checked bar) or aggregates in aberrant yeast (dark gray bar). Bars represent percent of 600-800 total cells which display those various α-synuclein phenotypes at concentrations of 0%, 2%, and 5% DMSO. **C.** *Expression:* For all samples, α-synuclein expression was induced for 24 hours in galactose media and detected using anti-V5. The lower blot was probed with anti-PGK as a loading control.
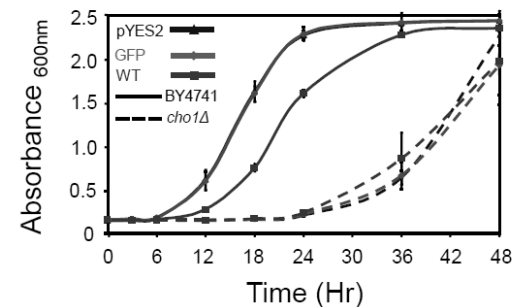
A. Microscopy
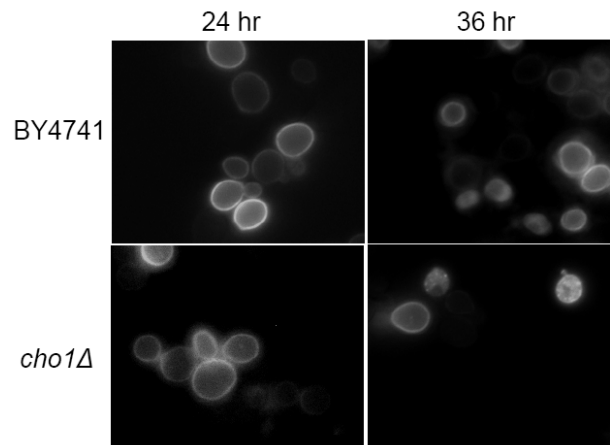
B. Growth Curve

Figure 8. Analysis of *cho1Δ* expressing α-synuclein. **A.** *Microscopy:* Images were captured at 24 and 36 hour post-induction in SC-Ura galactose. In CHO1 knockout strain, the WT α-synuclein plasma membrane localization is intact similar to WT α-synuclein in parent strain. **B.** *Growth curve:* No observable differences in growth exist between α-synuclein expressing *cho1Δ* cells and the *cho1Δ* controls, but significant growth difference exists between BY4741 (n=3, p_{18hr}= 0.004 and p_{24hr}= 0.0007) and pYES2 in *cho1Δ* cells. Each of the points on the curves represents the mean from three trials on measurement of absorbance (y-axis). In addition, the standard error of the mean for a 95% confidence interval is represented by the y-bar on each of the points on the growth curves.

membrane interaction appears more important for α-synuclein aggregation, rather than its cellular toxicity. Toxicity may indeed require α-synuclein's plasma membrane localization. Given the lack of α-synuclein's plasma membrane localization in fission yeast, it perhaps explains why the α-synuclein addition to DMSO toxicity is at best minimal.

c. DMSO also alters α-synuclein localization in budding yeast

Contrary to our prediction for budding yeast, the localization of α-synuclein at the plasma membrane diminished with increasing DMSO levels. Instead, increased pools of intracellular α-synuclein aggregates appeared at 2 and 5% DMSO treatment. We offer two suggestions for this unexpected redistribution. First, like we suggested for fission yeast, DMSO increases α-synuclein aggregation in the cytoplasm as a result of increased pool of newly made intracellular phospholipids that do not go to the plasma membrane. Secondly, DMSO is also known to regulate

half of the genes in the ergosterol, sphingoglycolipid and fatty acid biosynthetic pathways [26]. This down-regulation may impact α-synuclein:lipid interaction in yeast because the α-synuclein binds fatty acids and phospholipids [13, 33-36]. In effect, the reduced plasma membrane binding of α-synuclein would result in higher cytosolic concentration of α-synuclein, leading it to form non-lipid containing aggregates.

d. Does α-synuclein bind to non-PS phospholipids?

In the budding yeast model for PD, α-synuclein localization to the plasma membrane is a prominent feature [21]. In the current study, the localization to the plasma membrane is not lost when PS is eliminated out from the cell. We had expected some, if not all, the reduction of α-synuclein's plasma membrane localization, because *in vitro* α-synuclein binds to PS [36]. But the protein does bind to other phospholipids as well [36], which may explain why α-synuclein remained localized to the plasma membrane in the PS knockout strain. Perhaps, the

protein's cellular phospholipid binding does not involve PS, or if it does, it's not limited to it. PS is an important phospholipid in yeast growth evidenced by the poor growth of yeast that lack PS.   This poor growth prevented us from accurately assessing effects that PS may have had on α-synuclein toxicity.

V.   CONCLUSION

We demonstrate a surprising finding that an organic solvent, DMSO, exerts cellular toxicity and changes morphology in two yeast models we had established for studying Parkinson disease. Budding yeasts exhibit greater vulnerability to DMSO in comparison to fission yeasts. Secondly, DMSO altered α-synuclein's localization to yet-unidentified intracellular structures. Our lab speculates that these structures are vacuoles or other phospholipid containing structures. Our future investigation for the link between α-synuclein's interaction with phospholipids and cellular toxicity is leading us to explore these future questions: why α-synuclein forms non-toxic cytoplasmic aggregates? Why does it not localize to the plasma membrane in fission yeast? Are other major membrane phospholipids, such as phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI) critical to α-synuclein plasma membrane localization?
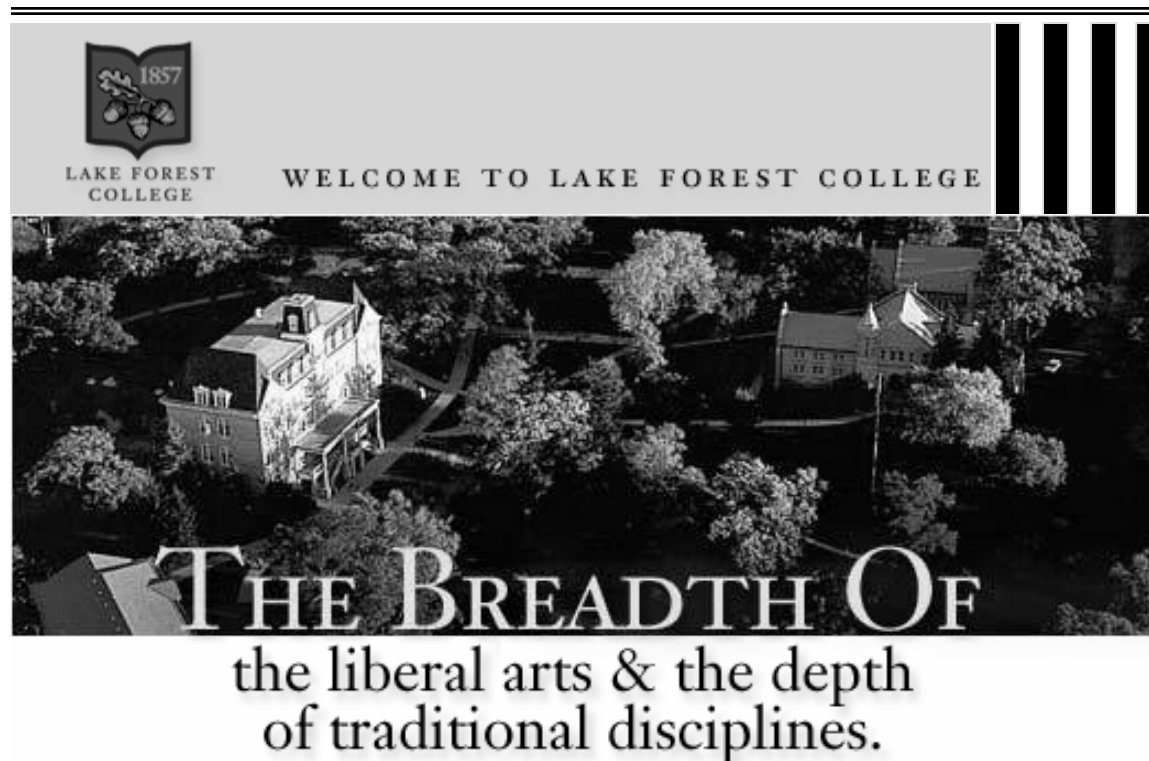
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