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ORIGINAL ARTICLE

α-Synuclein Budding Yeast Model

Toxicity Enhanced by Impaired Proteasome and Oxidative Stress

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder that results from the selective loss of midbrain dopaminergic neurons. Misfolding and aggregation of the protein α-synuclein, oxidative damage, and proteasomal impairment are all hypotheses for the molecular cause of this selective neurotoxicity. Here, we describe a *Saccharomyces cerevisiae* model to evaluate the misfolding, aggregation, and toxicity-inducing ability of wild-type α-synuclein and three mutants (A30P, A53T, and A30P/A53T), and we compare regulation of these properties by dysfunctional proteasomes and by oxidative stress. We found prominent localization of wild-type and A53T α-synuclein near the plasma membrane, supporting known in vitro lipid-binding ability. In contrast, A30P was mostly cytoplasmic, whereas A30P/A53T displayed both types of fluorescence. Surprisingly, α-synuclein was not toxic to several yeast strains tested. When yeast mutants for the proteasomal barrel (*doa3-1*) were evaluated, delayed α-synuclein synthesis and membrane association were observed; yeast mutant for the proteasomal cap (*sen3-1*) exhibited increased accumulation and aggregation of α-synuclein. Both *sen3*-1and *doa3-1* mutants exhibited synthetic lethality with α-synuclein. When yeasts were challenged with an oxidant (hydrogen peroxide), α-synuclein was extremely lethal to cells that lacked manganese superoxide dismutase Mn-SOD (*sod2*Δ) but not to cells that lacked copper, zinc superoxide dismutase Cu,Zn-SOD (*sod1*Δ). Despite the toxicity, *sod2*Δ cells never displayed intracellular aggregates of α-synuclein. We suggest that the toxic α-synuclein species in yeast are smaller than the visible aggregates, and toxicity might involve α-synuclein membrane association. Thus, yeasts have emerged effective organisms for characterizing factors and mechanisms that regulate α-synuclein toxicity.
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Index Entries: α-Synuclein; Parkinson's disease; neurodegeneration; *Saccharomyces cerevisiae*; protein misfolding; lipid binding; proteasome; aggregation; superoxide dismutase; oxidative stress.

Introduction

Parkinson's disease (PD) is a fatal and incurable neurodegenerative disorder characterized by resting tremors, postural instability, and bradykinesia (Olanow and Tatton, 1999). Although the molecular bases of sporadic PD are still unclear, so far mutations in six genes have been identified that cause familial PD: *α-synuclein* (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004), *parkin* (Kitada et al., 1998), *UCH-L1* (Funayama et al., 2002), *DJ-1* (Bonifati et al., 2003), *PINK1* (Valente et al., 2004), and *LRRK2* (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Both familial and sporadic forms of PD are linked to the death of midbrain substantia nigra neurons, which are found to accumulate misfolded and aggregated α-synuclein in cytoplasmic inclusions

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called Lewy bodies (Spillantini et al., 1998), and accumulate high levels of oxidative free radicals (Olanow and Tatton, 1999; Dawson and Dawson, 2003). It is still unclear how impaired protein degradation pathways and oxidative stress regulate α-synuclein misfolding and aggregation, and if such regulation causes cellular toxicity.

α-Synuclein is an abundant neuronal protein primarily localized in presynaptic terminals (Davidson et al., 1998; Clayton and George, 1998; Kahle et al., 2000). Although its neuronal function is still unknown, several properties might be relevant: maintenance of neurotransmitter vesicular pools (Jensen et al., 1998; Murphy et al., 2000), synaptic plasticity (George et al., 1995; Abeliovich et al., 2000), phospholipid binding and lipid metabolism (Davidson et al., 1998; Eliezer et al., 2001; Sharon et al., 2001; Outerio and Lindquist, 2003), microtubule binding (Alim et al., 2004), and chaperone-like ability (Jenco et al., 1998; Engelender et al., 1999; Ostrerova et al., 1999). In support of a gain-of-toxic function hypothesis for α-synuclein in PD, α-synuclein overexpression models for wild-type and its familial mutant forms (A30P and A53T) in mice (Masliah et al., 2000; Giasson et al., 2002), flies (Feany and Bender, 2000; Auluck et al., 2002), and worms (Lasko et al., 2003) display PD-like symptoms and pathology. Furthermore, purified wild-type, A30P, and A53T α-synuclein can all self-polymerize in vitro, but mutants oligomerize and/or aggregate more quickly (Conway et al., 1998, 2000; Giasson et al., 1999).

Several lines of evidence with PD point to ubiquitin-proteasome pathway (UPP) dysfunction. Intracellular α-synuclein aggregates are rich in ubiquitin molecules (Ciechanover and Brundin, 2003), and parkin and UCH-L1 mutations directly link UPP to α-synuclein misfolding (Kitada et al., 1998; Leroy et al., 1998). Parkin, a ubiquitin protein ligase, ubiquitinates an *O*-glycosylated form of α-synuclein (Shimura et al., 2001). Primary cell cultures that overexpress α-synuclein, when exposed to proteasome inhibitors, display decreased viability, but overexpression of parkin reduces this toxicity (Petrucelli et al., 2002). UCH-L1 possesses both ubiquitin ligase and hydrolase activities, but an increase in its ligase activity inhibits proteasomal degradation of α-synuclein (Leroy et al., 1998; Liu et al., 2002). In sporadic PD, proteasomal function is weakened in the substantia nigra neurons of PD patients, and reduced levels of proteasome activators are observed (McNaught and Jenner, 2001; McNaught et al., 2003). Direct inhibition of the proteasome in rat ventral mesencephalic cultures also results in dopaminergic neuronal death and formation of Lewy body-like inclusions (McNaught et al., 2002b). Rats treated with proteasome inhibitors in substantia nigra neurons develop PD-like symptoms (McNaught et al., 2002a). These findings support the notion that a compromised proteasome is unable to remove misfolded and aggregated proteins in both sporadic and familial cases of PD, but further in vivo genetic support is needed.

Oxidative damage and mitochondrial dysfunction have long been implicated as major factors in PD (Jenner and Olanow, 1996; Maguire-Zeiss et al., 2005). In sporadic PD, the death of dopaminergic neurons increases when complex I of the mitochondria is deficient (Mizuno et al., 1989; Kweon et al., 2004). Moreover, the inhibition of complex I leads to more reactive oxygen species (ROS), which can damage the electron-transport chain and lead to further production of ROS (Dauer and Przedborski, 2003). Autosomal recessive PD caused by DJ-1 mutations is linked to oxidative stress. DJ-1 is a redox-sensitive molecular chaperone expressed in oxidative cellular environments (Shendelman et al., 2004). Although the connection between α-synuclein misfolding and oxidative stress is still unclear, recently, certain proteins have been shown to form a complex with aggregated α-synuclein in vivo in the presence of oxidative stress and the PD-inducing agent, rotenone (Zhou et al., 2004).

Saccharomyces cerevisiae (budding yeast) is a powerful genetic system to model protein misfolding processes underlying human diseases (Outeiro and Muchowski, 2004), including prion disease (Ma and Lindquist, 1999), Huntington's disease (Krobitsch and Lindquist, 2000; Muchowski et al., 2000), and amyotrophic lateral sclerosis (Kunst et al., 1997). While this paper was being prepared, three *S. cerevisiae* models for α-synuclein have shed new insight into α-synuclein's pathology and biology (Outeiro and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005). Additionally, a yeast genetic screen revealed 86 genes that were synthetically lethal with overexpressed α-synuclein (Willingham et al., 2003).

Here, we describe our *S. cerevisiae* model to study α-synuclein's misfolding, aggregation, toxicity, and biological function and provide new data that support and significantly extend the previous findings. First, we determined expression, cellular localization, and in vivo stability of wild-type and mutant (A30P, A53T, and A30P/A53T) α-synuclein and the toxicity of each type toward yeast. Second, we assessed if genetically compromised proteasomes, using *sen3-1*

mutant (Sen3p is a 19S cap protein subunit [DeMarini et al., 1995]) or *doa3-1* mutant (Doa3p is a 20S barrel protein subunit [Arendt and Hochstrasser, 1999]), affected α-synuclein localization, aggregation, and toxicity. Finally, we analyzed if oxidants affected α-synuclein localization and toxicity in strains that lacked superoxide dismutase activity: either Cu,Zn-SOD/Sod1 (the major cytosolic enzyme) or Mn-SOD/Sod2 (the major mitochondrial enzyme). We found that α-synuclein localizes prominently near the plasma membrane, forms only minimal intracellular aggregates, and is not toxic to yeast. Proteasomal dysfunction is synthetically lethal with α-synuclein, but lethality does not require α-synuclein aggregation. Finally, Mn-SOD deletion selectively enhances α-synuclein's toxicity to yeast, without increasing visible intracellular aggregation.

Materials and Methods

α-Synuclein Constructs

Human wild-type and A53T mutant α-synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and A30P/A53T mutant α-synuclein were created from wild-type and A53T mutant α-synuclein, respectively, using site-directed mutagenesis (Invitrogen). Wild-type and mutant α-synuclein cDNAs were subcloned into the pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). All α-synuclein forms were also tagged with green fluorescent protein (GFP) using a two-step cloning strategy, with GFP either tagged at the amino or carboxyl terminus. α-Synuclein cDNAs were first subcloned into mammalian expression vectors, pcDNA3.1/C-terminal GFP and pcDNA3.1/N-terminal GFP (Invitrogen) to be fused with GFP either at the amino or carboxyl terminus. Both forms of GFP-tagged α-synuclein genes were then PCR-amplified and subcloned into pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). Chemically competent *Escherichia coli* cells were transformed with α-synuclein, α-synuclein-GFP, and GFP-α-synuclein pYES2.1/V5-His-TOPO vectors. All constructs were verified by DNA sequencing (University of Chicago). The parent pYES2 vector (Invitrogen) and GFP in pYES2.1/V5-His-TOPO vector were used as controls.

Yeast Strains

For the majority of experiments, TSY623 (*MATα ade2-101 his3-Δ200 leu2-3,112 ura3-52*) was used (gift of Cold Spring Harbor Laboratory Yeast Genetics course, summer 2000). For the proteasome experiments, the following strains were gifted by Mark Hochstrasser (Yale, New Haven, CT): MHY803 (*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-*), MHY792 (parent strain, MHY803; *doa3* mutant), MHY810 (*MATa his3-Δ200 leu2-Δ1 lys1-1 met14 trp1-Δ1 ura3-Δ1::TRP1*), MHY898 (parent strain, MHY810; *sen3-1* mutant). For experiments in Fig. 4 (below), BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4743 (*MATa his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were purchased from Open Biosystems. For oxidative stress experiments, the parent strain was BY4741 for the Mn-SOD (YHR008C) and Cu,Zn-SOD (YJR104C) knockout strains (Open Biosystems).

Yeast Expression

α-Synuclein expression plasmid vectors were transformed into yeast strains, as described (Burke et al., 2000). 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%) or SC-URA raffinose (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.

Western Analyses

Yeast cells (2.5×10^7 cells/mL) were washed in 50 m*M* Tris (pH 7.5) and 10 m*M* NaN_3 and solubilized in electrophoresis sample buffer ([ESB] Burke et al., 2000) containing 2% sodium dodecyl sulfate (SDS), 80 m*M* 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 m*M* phenylmethylsulfonyl fluoride, 1 m*M* benzamidine, 1 m*M* 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% Tris-glycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes, and Western blot was performed with different monoclonal antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 (Invitrogen) for most blots, LB509

anti-α-synuclein (Santa Cruz, Biotech) in Fig. 1B,C (below), and anti-phosphoglycerokinase ([PGK] Molecular Probes) for most expression experiments, as a measure of loading control in duplicate blots. Chris Rochet (Purdue University, West Lafayette, IN) generously gifted purified recombinant α-synuclein made in *E. coli.*

Growth Curve Analysis

Cells were grown in 10 mL SC-URA glucose overnight at 30°C (200 rpm). Cells were harvested at 1500*g* for 5 min at 4°C and were washed twice in 5 mL H_2O. Cells were resuspended in 5 mL H_2O and counted. Flasks with 25 mL SC-URA galactose were each inoculated to 2.0×10^6 cells/mL density. At 0, 3, 6, 12, 18, 24, and 36 h and in duplicate measurements, 1 mL of cell culture was removed and placed in a cuvette to measure absorbance using a Hitachi U-2000 spectrophotometer. Averaged absorbance readings were plotted against time points to produce a growth curve.

Spotting Analysis

For spotting, cells were grown to mid-log phase in SC-URA raffinose (2%), normalized to equal densities (2×10^7 cells/mL), serially diluted (5-fold) into 96-well microwell plates, and spotted on SC-URA glucose (2%) or galactose (2%) plates using a 48-prong frogger (Dan-Kar) or manual pipetting. Photographs were scanned after 2 d of growth.

GFP Microscopy and Quantification

Cells were grown overnight in 10 mL SC-URA glucose at 30°C (200 rpm). Protein expression was induced with SC-URA galactose media, as described previously. After a desired amount of induction, cells were harvested at 1500*g* (4°C) for 5 min and were washed twice with 5 mL H_2O. Cells were resuspended in 100–1000 µL SC-URA glucose, and 10 µL cell suspension was pipeted onto a slide. Cells were visualized under either a Zeiss Axiovert-100 or a Nikon TE2000-U fluorescent microscope, and images were acquired and deconvoluted using Metamorph 4.0 imaging software. For quantification of various patterns of cellular distribution, typically cells were counted in several separate fields of the slide (by moving the platform in both axes at regular intervals) and until at least ~750 cells were counted. The ratio of cells displaying specific fluorescence patterns was calculated: general fluorescence (diffuse cytoplasmic localization), bright peripheral halos (plasma membrane localization), intracellular foci (possible cytoplasmic aggregation), and perivacuolar fluorescence.

Oxidative Stress Analyses

H_2O_2 was added to liquid expression media to a final concentration of 2 m*M*. Cells were grown in 2 m*M* H_2O_2 and galactose over a time course during which OD_{600} measurements were made to examine growth effects. Western blots were prepared to examine α-synuclein expression, and microscopy to examine cellular α-synuclein localization, all using the protocols described above.

Results

α*-Synuclein is Expressed in Yeast*

As the first step in developing our *S. cerevisiae* model, wild-type, A30P, A53T, and A30P/A53T mutant α-synuclein was expressed for 18–24 h in the strain TSY623. All forms of α-synuclein migrated at ~26–28 kDa, about 6–8 kDa higher than predicted (predicted size, 20 kDa: 14-kDa α-synuclein + 5- to 7-kDa V5-His6X tag [Fig. 1A]). At times, higher running α-synuclein bands (~30–34 kDa) comigrated (Fig. 1A), but, dimers, trimers, or higher order oligomers or SDS-insoluble aggregates were never observed. Although an anti-V5 antibody was used for protein detection in this and most other Western blots in this study, identical α-synuclein bands were seen with LB509 anti-α-synuclein antibody (Fig. 1B). This higher migration of α-synuclein is attributable to α-synuclein's intrinsic properties rather than the V5/His6X-tag or the protein gel, as non-V5/His-tagged α-synuclein made and purified from *E. coli* and probed with LB509 anti-α-synuclein antibody also ran higher than predicted at ~19 kDa (Fig. 1C [note that this protein does form a dimer marked with an asterisk {*}]), whereas V5/His6X-tagged GFP ran just as expected at ~34 kDa (Fig. 1D). Equal protein loading in this blot (and all subsequent blots) in this study was confirmed with either Coomassie stain and/or equal presence of PGK (data not shown).

Next, we tagged wild-type and the mutant α-synuclein with GFP, either at the amino terminus (subsequently termed GFP-α-synuclein) or the carboxyl terminus of α-synuclein (subsequently termed α-synuclein-GFP), because we did not know which placement would better maintain α-synuclein properties. On denaturing gels, both tagged versions migrated at ~58–60 kDa (again ~6–8 kDa above the ~52-kDa predicted size [Fig. 1D,E]). However, with

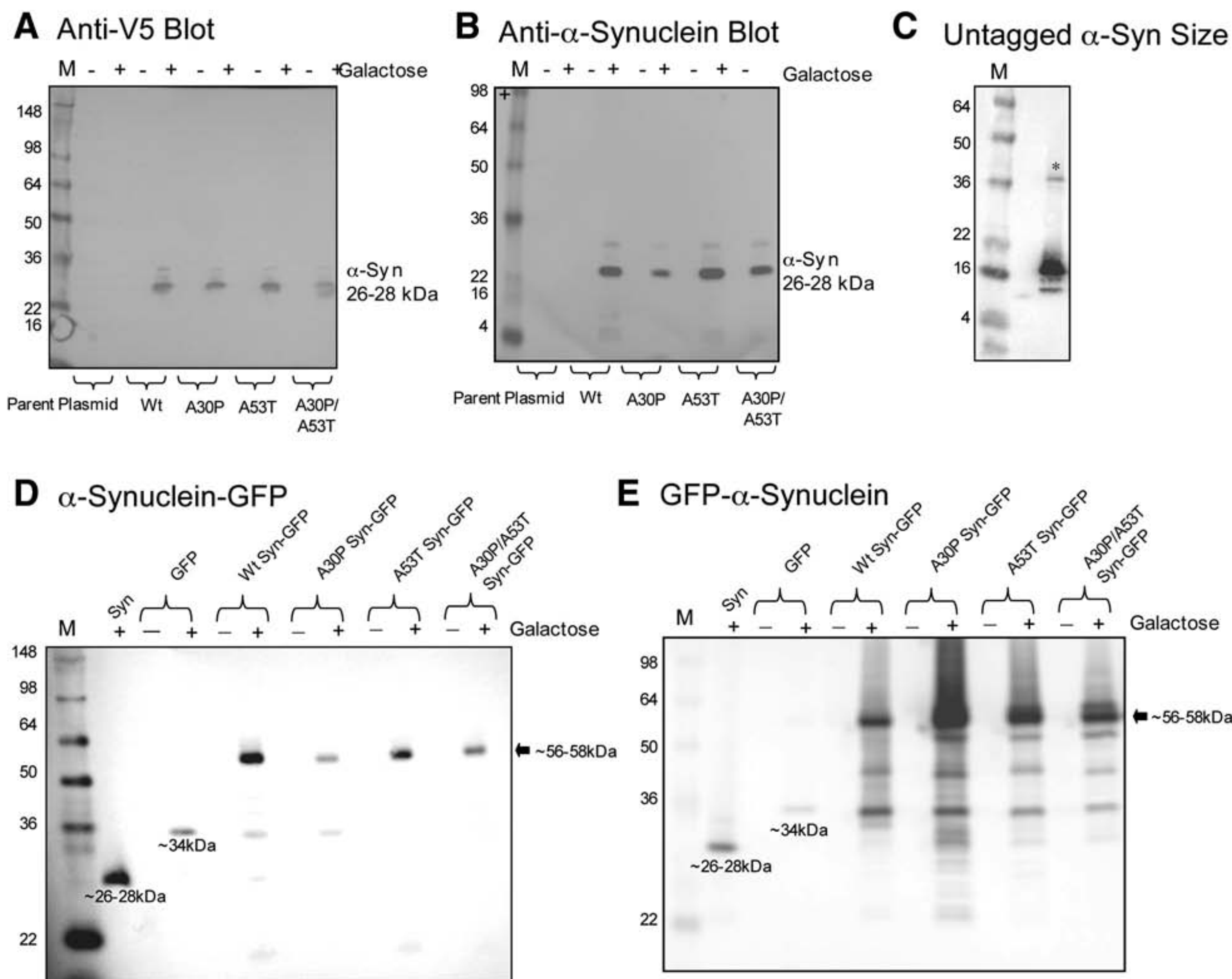


Fig. 1. α-Synuclein expression in yeast. **(A)** Western blotting of TSY623 yeast lysates demonstrate that wild-type (Wt), A30P, A53T, and A30P/A53T α-synuclein were expressed to similar levels and migrated at ~26–28 kDa on denaturing gels (probe: anti-V5 antibody). **(B)** Wt, A30P, A53T, and A30P/A53T α-synuclein were also probed with LB509 anti-α-synuclein antibody, and identical bands at ~26–28kDa were detected. **(C)** Expression of bacterially made recombinant α-synuclein lacking the V5/His6X tag migrated higher than expected (19 kDa instead of 14 kDa [probe: LB509]). **(D)** α-Synuclein-GFP (GFP tagged at carboxyl terminus) expression: α-Synuclein alone migrated at ~26–28kDa, GFP at ~34 kDa (expected size), and the major band for the α-synuclein-GFP was observed at ~58–60 kDa (probe: anti-V5). **(E)** GFP-α-synuclein (GFP tagged at amino terminus) expression: The major band for GFP-α-synuclein was observed at ~58–60 kDa, but the fusion protein displayed many degradation products (probe: anti-V5). M = molecular weight marker; + = presence of galactose.

GFP-α-synuclein we observed multiple lower migrating bands below the prominent band at ~58–60 kDa (Fig. 1E), which could be indicative of degradation either during cell lysis or in vivo. We reasoned that α-synuclein-GFP was relatively more stable and used it for most subsequent experiments.

α-Synuclein is Not Toxic to Yeast

To test the hypothesis that α-synuclein is toxic to yeast, OD_{600} and spotting analyses were done with cells expressing untagged or GFP-tagged α-synuclein. We observed little to no differences between the growth of cells carrying the parent pYES2 vector and cells expressing wild-type and mutant α-synuclein, irrespective of GFP tag (Fig. 2A). Serial spotting of cells expressing C-terminally tagged α-synuclein-GFP on glucose and galactose plates confirmed the absence of toxicity (Fig. 2B [similar data obtained with untagged α-synuclein and GFP-α-synuclein is not shown]).

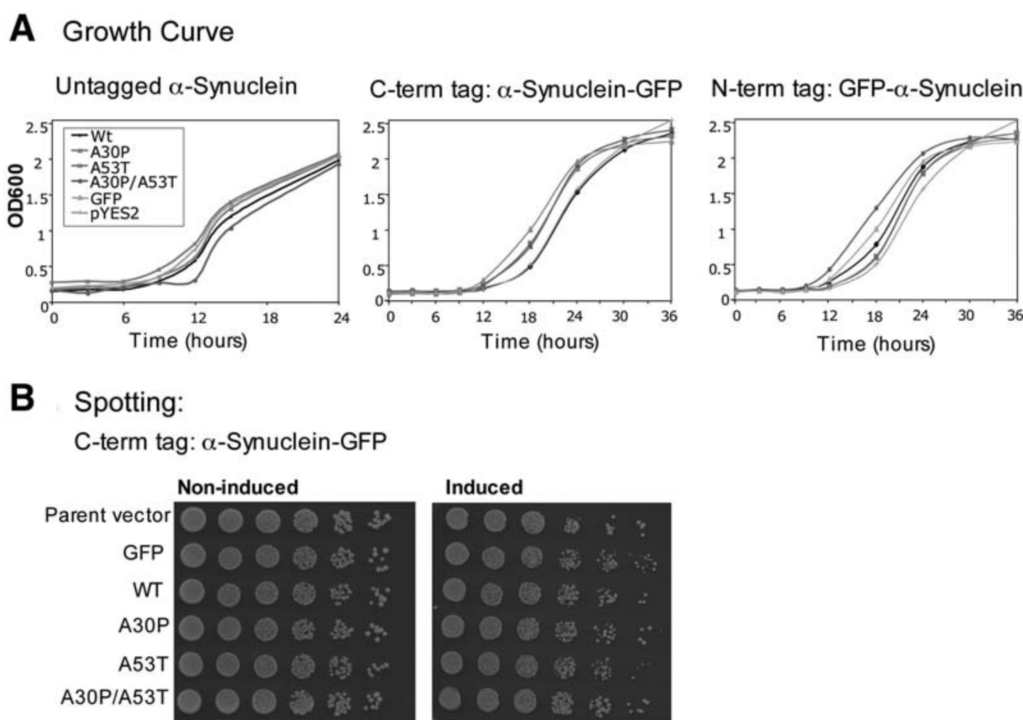


Fig. 2. α-Synuclein toxicity analysis in yeast. **(A)** Growth curve: OD_{600} readings were taken at several time points for yeast cells induced for α-synuclein expression by growth in galactose: (Left) Untagged α-synuclein; (middle) α-synuclein-GFP; (right) GFP-α-synuclein. **(B)** Serial spotting: Growth was also analyzed by spotting fivefold serially diluted yeast cells containing α-synuclein-GFP on inducing (galactose) or noninducing plates (glucose). In both assays, compared with cells with the parent vector or GFP alone, cells with α-synuclein did not grow significantly slower.

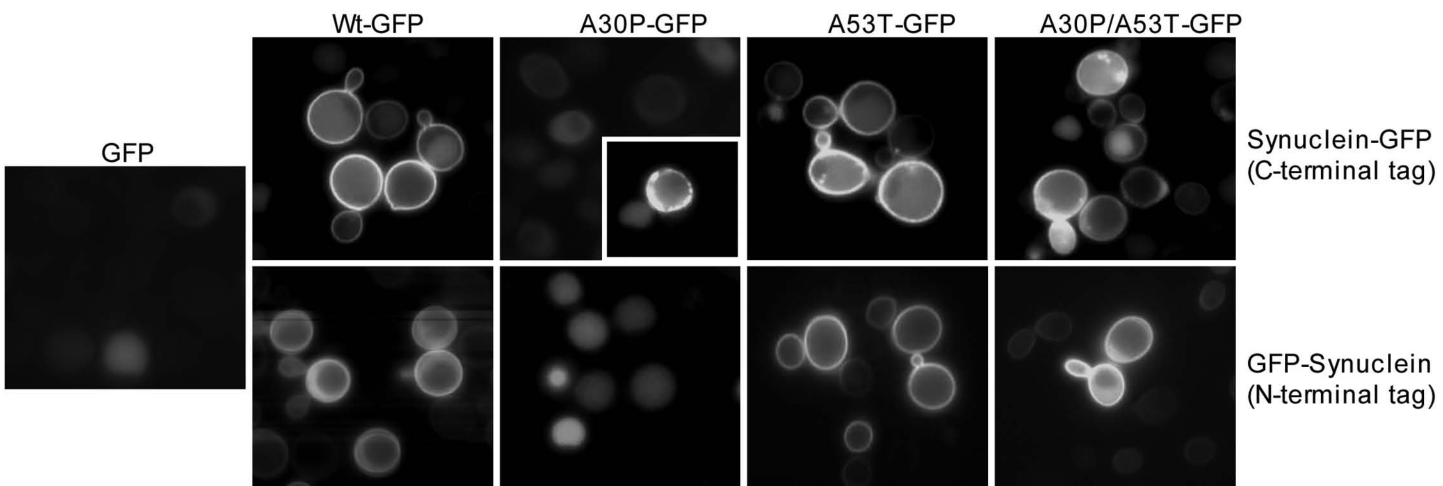
α-Synuclein Localizes Near Yeast Plasma Membranes

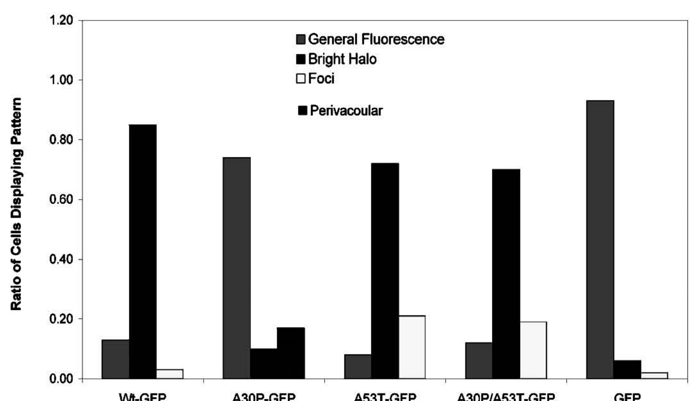
α-Synuclein-GFP revealed surprising and intense peripheral localization within 18–24 h of induction in TSY623 yeast. Both wild-type and A53T mutants distinctly localized near cell membranes, and only in some cells did the A53T mutant additionally form small cytoplasmic foci (which we considered as aggregates [Fig. 3A, top]). In contrast, in cells expressing the A30P mutant, α-synuclein did not localize to the cell periphery. Instead, A30P was diffusely distributed throughout the cytoplasm (Fig. 3A), and some cells exhibited intense perivacuolar fluorescence (inset, Fig. 3A). The A30P/A53T mutant displayed an intermediate and variable phenotype between A30P and A53T: Many cells displayed plasma membrane fluorescence like A53T, almost all had a more intense cytoplasmically diffuse background as in A30P, and only some cells displayed aggregates. Control cells expressing GFP alone localized diffusely in the cytoplasm but exhibited much lower fluorescence intensity compared with those expressing A30P α-synuclein (Fig. 3A). The location of the GFP tag did not significantly influence α-synuclein localization (Fig. 3A, cf. top and bottom panels). We used a 2 μ plasmid to express α-synuclein, which presumably led to variable expression in the same population of transformed cells and gave us the advantage of visualizing a range of fluorescence patterns, as is evident in most images.

Finally, we quantified the fluorescent phenotypes of cells displaying halos (plasma membrane localization), foci (aggregation), and general fluorescence (cytoplasmic localization [Fig. 3B,C]). Irrespective of GFP tag placement, between 60% and 80% of wild-type, A53T, and A30P/A53T cells that fluoresced showed membrane localization, whereas A30P only exhibited perivacuolar and cytoplasmic fluorescence. Only with C-terminal GFP-tagged α-synuclein did the wild-type, A53T, and A30P/A53T forms display aggregates (up to 20% of cells).

α-Synuclein Localization and Toxicity Assessment in Different Strains

Because we did not find α-synuclein to be strongly toxic to the TSY623 strain, we expressed wild-type α-synuclein localization and toxicity in other strains





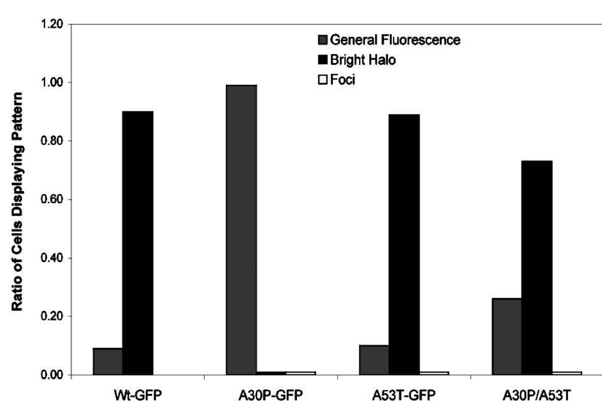


Fig. 3. α-Synuclein localization in yeast. **(A)** Live cell GFP fluorescence images of GFP-tagged α-synuclein at 24 h, with α-synuclein-GFP in the top row and GFP α-synuclein in the bottom row. GFP alone is on the extreme left. For GFP-α-synuclein, wild-type (Wt), A53T, and A30P/A53T displayed plasma membrane localization near yeast plasma membranes. A30P was cytoplasmic. A53T and A30P/A53T displayed aggregates. N-terminal tagged GFP-α-synuclein displayed similar patterns, except that no foci were observed for any form of α-synuclein. GFP alone showed diffuse fluorescence. **(B,C)** GFP-fluorescing cells were quantified for both α-synuclein-GFP and **(B)** GFP-α-synuclein **(C)**. Cells (750) of each transformed strain were counted and scored for four types of fluorescence patterns: general fluorescence (diffuse cytoplasmic localization), bright peripheral halos (plasma membrane localization), intracellular foci (possible cytoplasmic aggregation), and perivacuolar fluorescence (vacuoles were confirmed by either DIC or phase-contrast microscopy).

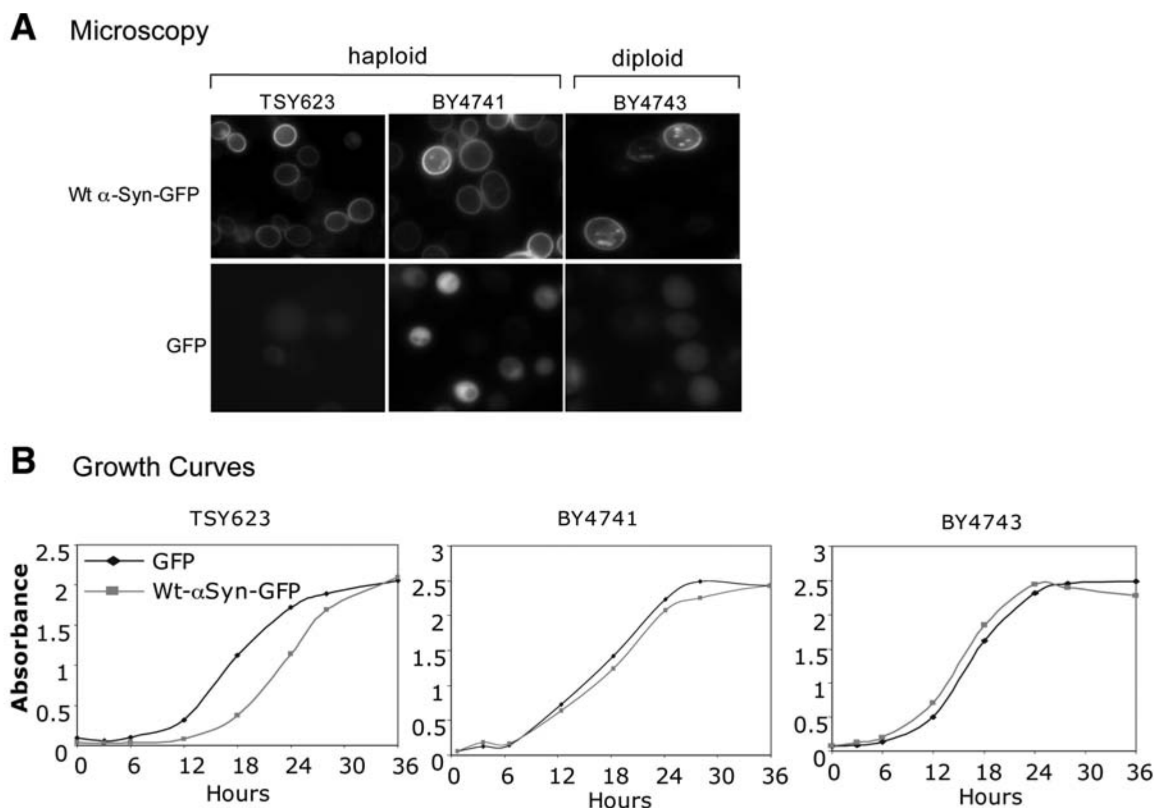


Fig. 4. α-Synuclein localization and toxicity in different strain backgrounds. Wild-type (Wt) α-synuclein-GFP and GFP alone were evaluated in three strains (haploid: TSY623, BY4741; diploid: BY4743). **(A)** Microscopy: Wt α-synuclein localized near plasma membranes in all three strains but showed variable amounts of aggregates between the strains. GFP displayed similar diffuse fluorescence in all strains. **(B)** Growth curves: OD_{600} was assessed over a 36-h time course for GFP and Wt α-synuclein in all three strains. Compared with GFP alone, cells expressing Wt α-synuclein did not show a significant growth change in any strain.

to evaluate strain dependence (TSY623, BY4741) and ploidy (BY4743 is diploid, whereas TSY623 and BY4741 are haploid). GFP-tagged fluorescence of wild-type α-synuclein demonstrated dominant plasma membrane association in all strains (Fig. 4A). Some BY4741 and BY4743 cells displayed multiple aggregates of wild-type α-synuclein, whereas the TSY623 strain had none (Fig. 4A); but these aggregates did not impart toxicity, as measured by growth curves (Fig. 4B [similar lack of toxicity with serial spotting is not shown]). Lack of toxicity was also seen in the W303 strain (not shown). For analysis in two other strains (MHY803 and MHY810), *see* Proteasomal Cap Impairment (*sen3-1*) Enhances α-Synuclein Accumulation and Aggregation.

Proteasomal Cap Impairment (sen3-1) *Enhances α-Synuclein Accumulation and Aggregation*

To genetically assess the relationship between α-synuclein and the UPP pathway, we transformed wild-type, A30P, A53T, and A30P/A53T α-synuclein into two proteasomal mutant strains and corresponding isogenic parents. *sen3-1* compromises cap function (Sen3p is a subunit of the 19S′ regulatory cap that activates the 20S′ proteasome barrel [DeMarini et al., 1995]). *doa3-1* compromises proteasomal barrel function (Doa3p is a proteolytic catalytic subunit of the 20S′ proteasomal barrel [Arendt and Hochstrasser, 1999]). We predicted an increase in α-synuclein accumulation and toxicity in both mutants.

Consistent with this hypothesis, wild-type and A30P α-synuclein expression was higher in *sen3-1* compared with its isogenic parent (Fig. 5A [A53T and A30P/A53T data exhibited similar increases]; data not shown). Two higher running α-synuclein bands (~30–34 kDa) also became more prominent in *sen3-1*, but dimers, trimers, or higher order α-synuclein oligomers were not detected. α-Synuclein-GFP localization was altered in *sen3-1*, with cells displaying

α-synuclein aggregates, even with A30P (Fig. 5B). A53T cells with aggregates increased by 50%, wild-type and A30P cells increased by ~10%, whereas A30P/A53T cells were modest at ~5% (Fig. 5C). Interestingly, all α-synuclein forms, except A30P, continued to display membrane localization (Fig. 5B).

Proteasomal Barrel Impairment (doa3-1) *Delays α-Synuclein Synthesis and Membrane Localization*

In contrast to what we discovered in *sen3-1*, all four forms of α-synuclein were expressed to lower levels in *doa3-1* compared with its isogenic parent (Fig. 5A; data not shown). But when examined over a longer time course, although A53T remained poorly expressed, wild-type, A30P, and A30P/A53T eventually regained expression to levels seen in isogenic parent (Fig. 5D). Surprisingly, in *doa3-1*, α-synuclein initially did not localize to the plasma membrane and instead aggregate-like fluorescence near the periphery was observed for all four α-synuclein forms (Fig. 5B). We hypothesized that these small aggregates would accumulate into larger cytoplasmic inclusions over time because of the proteasome barrel's compromised ability to degrade α-synuclein. Instead, α-synuclein distributed at the plasma membrane over time (Fig. 5E). Presumably, the small peripheral aggregates at 18–24 h were initial spots of membrane interaction that eventually spread across the entire membrane.

Synthetic Lethality of α-Synuclein With Both Proteasomal Mutants

Finally, we assessed α-synuclein-dependent toxicity when the proteasome was impaired. Wild-type, A30P, and A53T were strongly toxic in *sen3-1* compared with growth of the parent (Fig. 6A, left). Similarly, strong synthetic lethality was observed in *doa3-1* strains expressing α-synuclein (Fig. 6A, right). This striking toxicity was reiterated through serially spotting analysis (Fig. 6B), although both *sen3-1* and *doa3-1* expressed mild growth defects, even when not expressing α-synuclein (glucose plates).

Lack of Mn-SOD is Toxic to Yeasts That Express α-Synuclein

We next examined the combined effect of oxidative stress and α-synuclein expression in yeast, as oxidative stress has been increasingly linked to the misfolding and aggregation of α-synuclein (Jenner and Olanow, 1996; Maguire-Zeiss et al., 2005). First, we asked if α-synuclein would be more toxic to yeast that lacked one of the two major superoxide dismutases in yeast, Cu,Zn-SOD (*sod1*Δ) or Mn-SOD (*sod2*Δ). Then we asked if toxicity would be enhanced if these cells were exposed to oxidants. Without α-synuclein, yeast strains lacking either SOD were not toxic, irrespective of oxidant treatment (Fig. 7A, bottom). However, wild-type α-synuclein was selectively toxic to *sod2*Δ, but not *sod1*Δ (top). A30P (middle) and A53T (data not shown) cells exhibited similar Mn-SOD-dependent toxicity. Hydrogen peroxide strikingly exacerbated *sod2*Δ toxicity (top and middle), whereas *sod1*Δ viability was preserved. Wild-type and A30P α-synuclein retained their respective cellular localization patterns in cells that lacked either SOD (Fig. 7B). Even under toxic conditions (*sod2*Δ), visible α-synuclein aggregates were absent (Fig. 7B). Interestingly, adding oxidant reduced wild-type α-synuclein expression in Mn-SOD-lacking cells (top) but maintained A30P distribution (bottom).

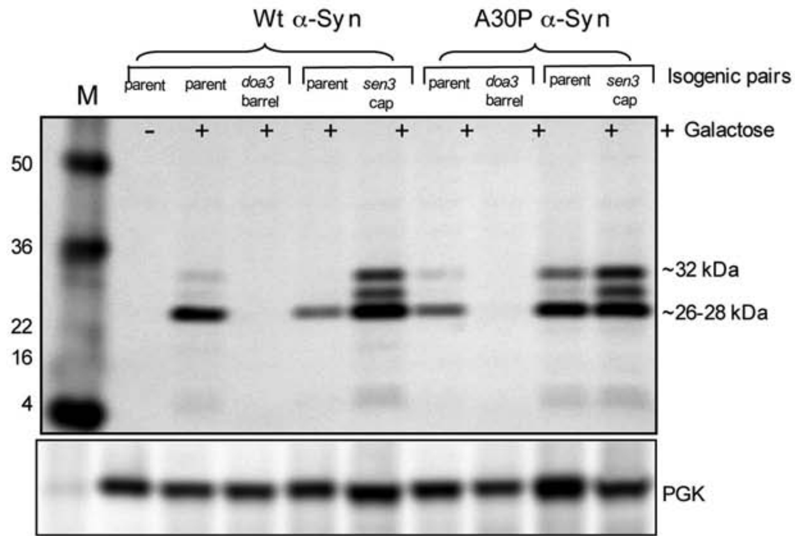
Discussion

Our budding yeast model demonstrates the benefits of using *S. cerevisiae* to dissect protein misfolding, aggregation, degradation, and toxicity mechanisms linked to neurodegeneration. As elaborated below, proteasomal dysfunction and oxidative stress diversely impact α-synuclein localization and aggregation, and both impairments induce cellular toxicity.

α-Synuclein's Prevalent Membrane Association and Modest In Vivo Aggregation

Wild-type and A53T α-synuclein's affinity for yeast plasma membranes is consistent with several recent lines of prior evidence indicating α-synuclein's interaction with lipids. α-Synuclein binds to fatty acids and phospholipids in vitro (Davidson et al., 1998), and its aggregation is regulated by fatty acids, with polyunsaturated fatty acids promoting aggregation and saturated fatty acids inhibiting it (Sharon et al., 2001). Physiologically, α-synuclein is hypothesized to interact with dopamine transporters on the neuronal membrane and thus regulate the amount of dopamine that enters the cell (Wersinger et al., 2003). All recent yeast models for α-synuclein have also reported its membrane localization (Outeiro and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005). Additionally, Willingham et al. (2003) conducted a yeast genetic screen and found that several membrane transport and lipid metabolism proteins regulated α-synuclein's toxicity.

A Expression: *doa3* Barrel vs. *sen3* Cap Mutant



B Microscopy: *doa3* Barrel vs. *sen3* Cap Mutant

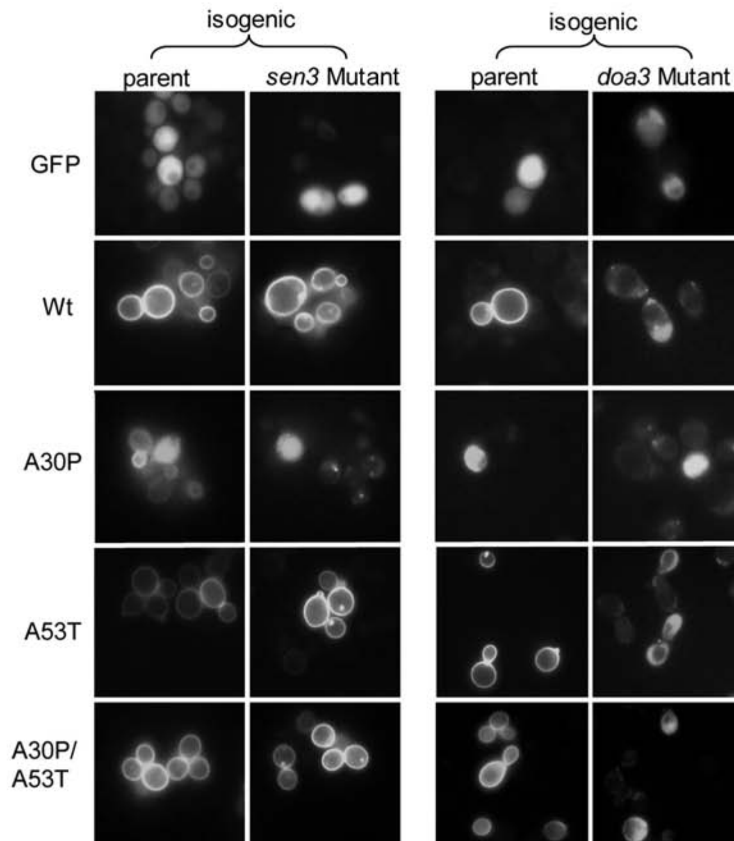


Fig. 5. (*Continued*)

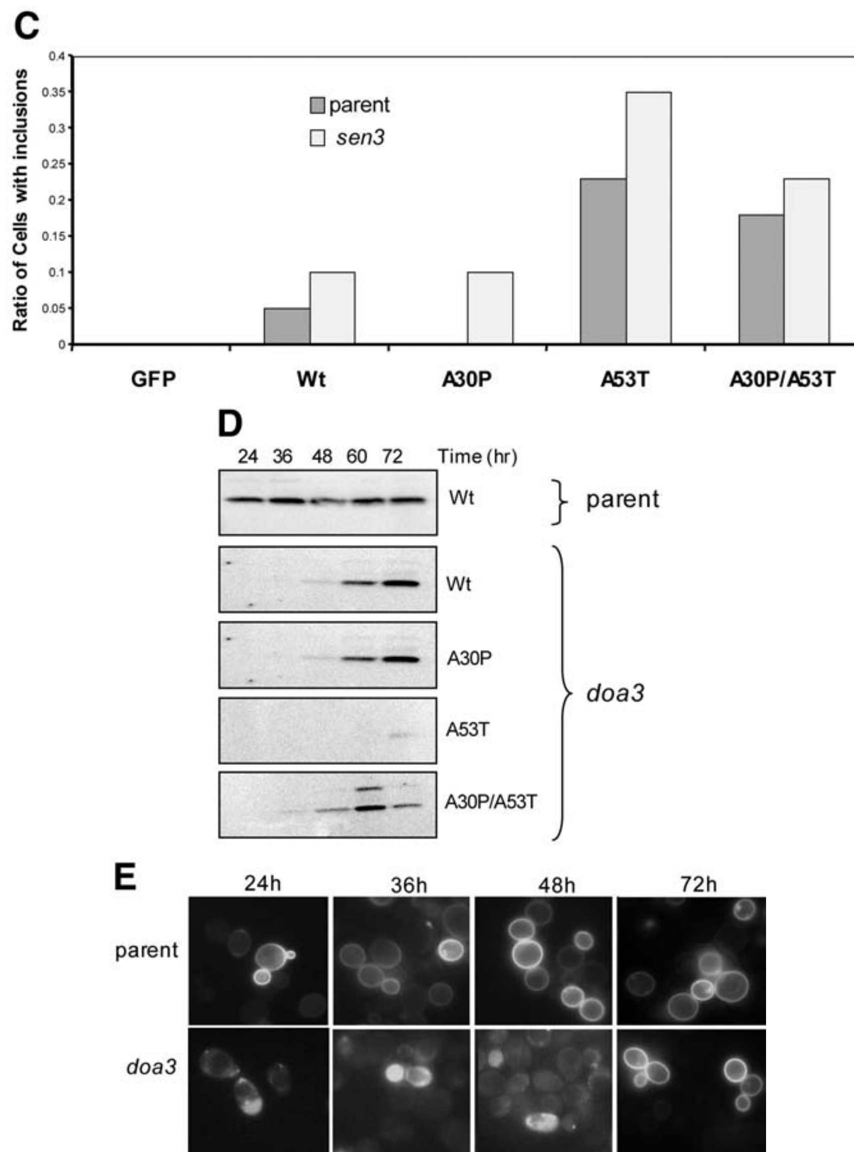
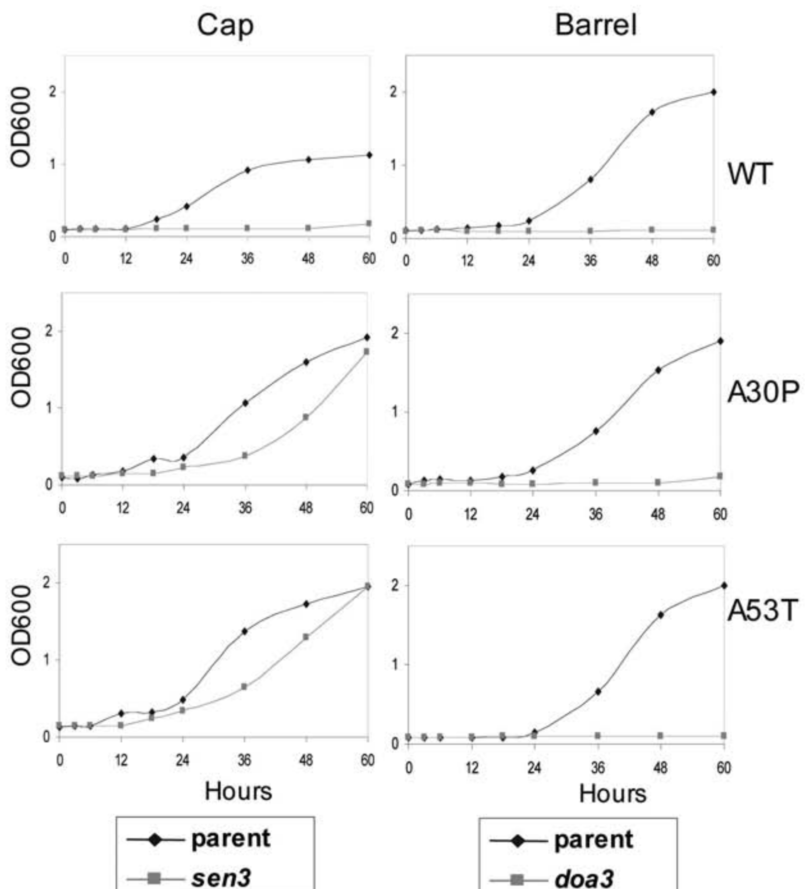


Fig. 5. α-Synuclein expression and localization in *sen3* (proteasomal cap mutant) and *doa3* (proteasomal barrel mutant). **(A)** Western blot: For all samples, expression was induced for 24 h and detected using anti-V5. +, induction by galactose; M, molecular-weight marker. Lower blot: identical blot probed with anti-PGK antibody. Wild-type (Wt) and A30P α-synuclein expression was higher in *sen3* cap mutant strain (MHY898) than in isogenic parent (MHY810). Wt and A30P α-synuclein expression in *doa3* barrel mutant strain (MHY792) was lower compared with the isogenic parent (MHY803). **(B)** Microscopy: Live cell imaging at 24 h of induction, evaluating Wt and mutant α-synuclein-GFP (A30P, A53T, A30P/A53T) in *sen3* cap and *doa3* barrel mutants, alongside their respectively matched isogenic parent strains. GFP alone demonstrated diffuse cytoplasmic fluorescence across all strains. Wt, A53T, and A30P/A53T α-synuclein all had reduced fluorescence and lack of membrane association in the barrel mutant strain and showed aggregate formation while retaining membrane association in the *sen3* mutant strain. A30P α-synuclein localized in the cytoplasm in all strains but showed some aggregate formation in the *sen3* mutant strain. **(C)** *sen3* analysis: Quantification of fluorescence patterns supported increased aggregation of α-synuclein in the 19S′ (cap) mutant strains, with the A53T mutant showing the highest increase. Cells (750) were counted for each sample type. **(D)** Western blot of α-synuclein time course in *doa3*: Expression of Wt and mutant α-synuclein in isogenic parent and *doa3* mutant strain was analyzed over a period of 24–72 h (probe: anti-V5). In *doa3*, Wt and mutant α-synuclein did not express significantly until 48–60 h after induction. **(E)** Microscopy of α-synuclein time course in *doa3*: Wt α-synuclein was analyzed in *doa3* and its isogenic parent strain 24–72 h after expression. In *doa3*, α-synuclein ultimately localized to the periphery by 72 h of induction, instead of coalescing into aggregates.



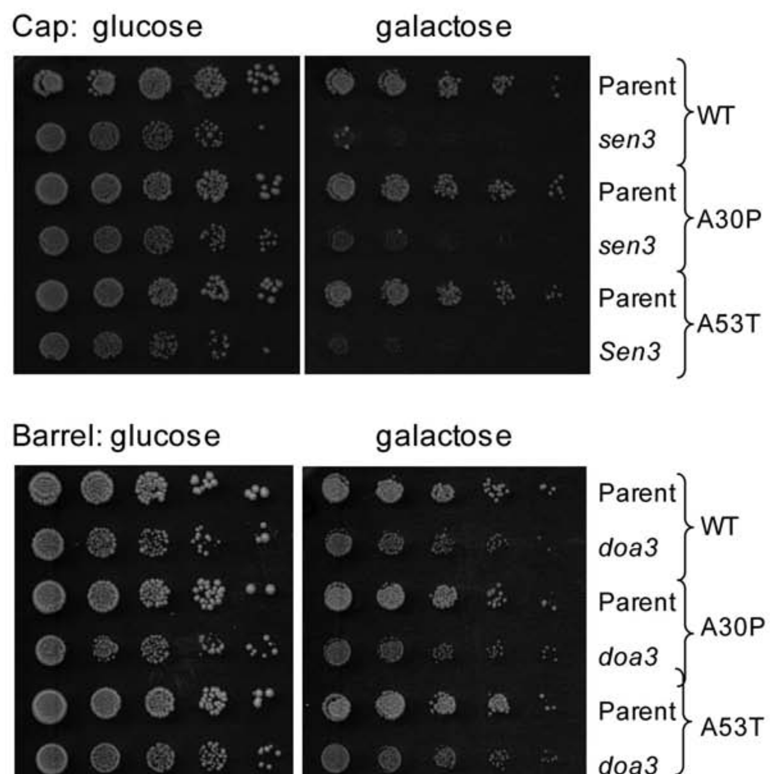


Fig. 6. Toxicity analysis of *sen3* and *doa3* strains expressing α-synuclein. **(A)** Growth curve: OD_{600} time course for cells expressing wild-type (WT), A30P, and A53T α-synuclein over 60 h shown in *sen3* mutant and isogenic parent (left) and *doa3* mutant and isogenic parent (right). **(B)** Serial spotting: Cells expressing WT, A30P, and A53T α-synuclein in *sen3* mutant and isogenic parent (top) and *doa3* mutant and isogenic parent (bottom) were spotted in galactose and glucose plates. In both assays, all three forms of α-synuclein were toxic to *sen3* and *doa3* compared with respective parent strains.

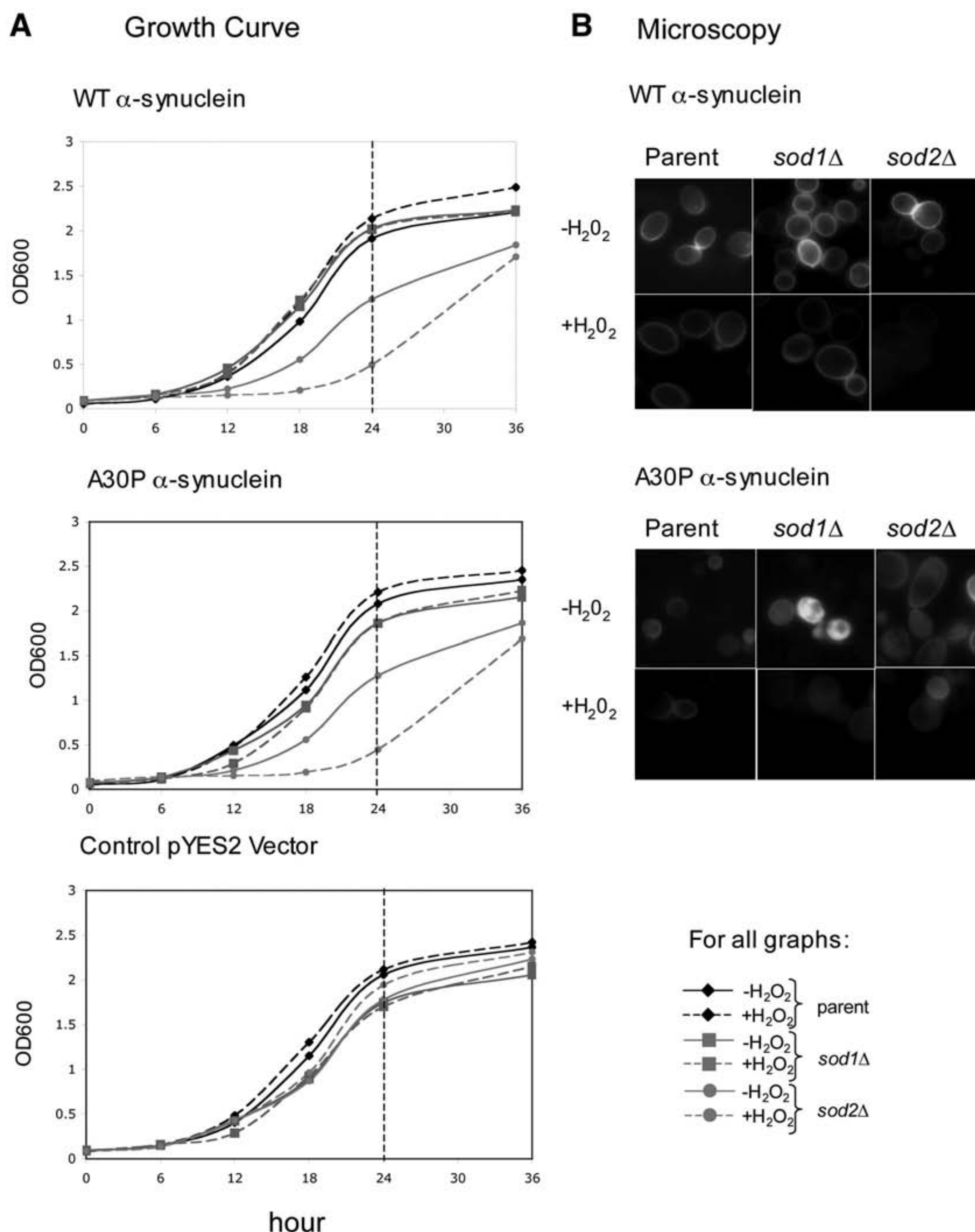


Fig. 7. Toxicity and localization analysis of α-synuclein in yeast lacking superoxide dismutases. **(A)** Growth curves: Cells lacking either Cu,Zn-SOD (*sod1*Δ) or Mn-SOD (*sod2*Δ) and control cells that expressed both enzymes were grown in galactose media that induced α-synuclein expression over 36 h, with and without 2 m*M* H_2O_2. Cells that contained parent pYES2 vector served as control. OD_{600} measurements were taken at several points to establish growth curves for various yeast strains (Top: Wild-type [WT] α-synuclein; middle: A30P α-synuclein; bottom: pYES2 vector). Cells transformed with control plasmid exhibited similar growth rates with and without H_2O_2 treatment. With WT and A30P α-synuclein-expressing cells, *sod2*Δ cells grew slower than *sod1*Δ and parent strains. *sod2*Δ cells grew even slower with H_2O_2 treatment but not *sod1*Δ strains. **(B)** Microscopy: Shown are GFP images of α-synuclein at 24 h of induction (corresponds to broken lines in **A**). Neither *sod1*Δ *n*or *sod2*Δ strain*s* altered WT (top) or A30P (bottom) α-synuclein membrane localization, nor did the addition of the oxidant, except that WT α-synuclein expression appears inhibited in surviving cells.

Compared with wild-type α-synuclein, A53T and A30P/A53T mutants are more prone to aggregation in budding yeast. This finding is consistent with published in vitro and in vivo studies, which show that α-synuclein aggregation is accelerated by the A53T mutation (Conway et al., 1998; Narhi et al., 1999; Giasson et al., 2002). In contrast, the A30P mutant does not localize to yeast plasma membranes. Although some studies reveal that the A30P mutation greatly reduces α-synuclein's ability to bind lipids (Jensen et al., 1998; Cole et al., 2002), others find little effect on lipid binding (Perrin et al., 2000). The other yeast models also found that A30P α-synuclein failed to localize to the periphery (Outeiro and Lindquist, 2003; Dixon et al., 2005; Zabrocki et al., 2005). We propose that the A30P mutant, as a result of lack of membrane association, remains mostly soluble in yeast, whereas wild-type and A53T α-synuclein, aided by membrane association, follow a misfolding pathway by which some aggregated α-synuclein accumulates into small inclusions.

Lack of Toxicity

Unlike the α-synuclein toxicity seen in other model organisms (including mice, flies, and worms), we find that α-synuclein expression by itself is not toxic to yeast. We suspect that we did not achieve high enough overexpression to induce sufficient misfolding of the protein needed for high levels of aggregation or toxicity to ensue. At low levels of expression, perhaps budding yeast provides sufficient protective mechanisms against α-synuclein's toxicity, including some of the 86 protective genes discovered recently (Willingham et al., 2003). That elevating α-synuclein expression can induce toxicity supports our reasoning (Outeiro and Lindquist, 2003; Dixon et al., 2005). However, it should be noted that Zabrocki et al. (2005) reported a lack of α-synuclein toxicity in yeast despite overexpression under stringent growth conditions.

Proteasomal Deficiency Leads to α-Synuclein-Dependent Toxicity

The synthetic lethality that ensues in yeast when proteasomal mutants are combined with α-synuclein expression underscores the contribution of the UPP pathway in α-synuclein misfolding and toxicity. Dixon et al. (2005) also identified the yeast proteasomal barrel mutants *pre1* and *pre2* (*PRE2* and *DOA3* are the same genes) as synthetically toxic in combination with α-synuclein. Several hypotheses have been proposed to explain UPP contribution in PD: (1) Protein aggregates result from a dysfunctional UPP; (2) aggregates first form and then inhibit the UPP and prevent degradation of their own and other cellular proteins; (3) both processes are at work. Our *sen3-1* data support the former case, as α-synuclein accumulates and aggregates in this cap mutant. Given that α-synuclein binds the proteasomal cap in a highly specific manner (Snyder et al., 2003) and *sen3-1* has severe defects in the degrading of many polyubiquitinated substrates (DeMarini et al., 1995), we hypothesize that the mutant cap reduces the efficiency of α-synuclein binding and entry into the proteasome barrel. Thus, α-synuclein accumulates in the cytoplasm and some of it aggregates into small inclusions.

In *doa3-1* (the barrel mutant), we propose that because this strain cannot clear many polyubiquitinated substrates as efficiently (Arendt and Hochstrasser, 1999), substrate accumulation increases the overall cellular stress, causing the yeast to reduce synthesis of selective proteins (such as α-synuclein) that are likely to cause additional stress. Apparently, small concentrations of α-synuclein are enough to induce toxicity in this extremely vulnerable strain. The surviving cells simply do not accumulate enough α-synuclein to form visible aggregates.

Mitochondrial SOD Protects Against the Combined Stress of Oxidant and α-Synuclein

The synthetic lethality of Mn-SOD absence with α-synuclein expression in yeast strongly supports prior evidence that oxidative damage and mitochondrial dysfunction contribute to PD (Jenner and Olanow, 1996; Hashimoto et al., 1999; Sherer et al., 2002; Dawson and Dawson, 2003; Song et al., 2004; Maguire-Zeiss et al.; 2005; Testa et al., 2005). Magnesium superoxide dismutase (Mn-SOD) is known to protect neurons against oxidative stress, and its absence can lead to neurodegeneration (Lebovitz et al., 1996; Andreassen et al., 2001; Hinerfeld et al., 2004; Liang and Patel, 2004; Lynn et al., 2005). We speculate that oxidant-induced mitochondrial oxidative damage is particularly enhanced in the presence of α-synuclein, leading to cytotoxicity. In support of this notion, mitochondrial-associated metabolic proteins have been found selectively oxidized in A30P α-synuclein transgenic mice (Poon et al., 2005).

That Cu,Zn-SOD absence does not induce α-synuclein toxicity in yeast is reminiscent of the Willingham et al. (2003) yeast genetic screen for enhancers of

α-synuclein toxicity that uncovered Mn-SOD as an enhancer, but not Cu,Zn-SOD. Interestingly, whereas Mn-SOD is cytoprotective against hydroperoxide-induced apoptosis in PC12 cells, Cu,Zn-SOD is not (Pias et al., 2003). Yet, Cu,Zn-SOD can induce α-synuclein aggregation in vitro (Kim et al., 2002; Kang and Kim, 2003) and protect against paraquat-induced PD phenotype (Thiruchelvam et al., 2005). Perhaps significant oxidative damage in the absence of Cu,Zn-SOD is averted by other cytoplasmic antioxidants, including catalases, which metabolize hydrogen peroxide, and glutathione, which scavenges free radicals. Therefore, it will be important to test not only if yeast catalases and glutathione-regulating enzymes regulate α-synuclein toxicity like Mn-SOD does but also if the absence of any these enzymes combined with lack of Cu,Zn-SOD results in α-synuclein toxicity.

α-Synuclein Toxicity Without Aggregation

Why do *sod2Δ* and *doa3-1* strains not exhibit visible aggregates of α-synuclein despite the toxicity? Possibly, α-synuclein aggregate-containing cells might be so toxic that they die early in the time course, when microscopy does not facilitate α-synuclein detection because of low expression. Alternately, visible aggregates are not essential to toxicity. The true toxic agent(s) in neurodegenerative diseases still remains unresolved (Sisodia, 1998; Taylor et al., 2002; Caughey and Lansbury, 2003). We do not rule out the possibility that visible α-synuclein aggregates might aid in toxicity in yeast, but our data points to nonvisible soluble oligomers (possibly protofibrils) as more likely candidates for toxicity.

α-Synuclein Membrane Localization and Toxicity

Recently, Rochet et al. (2004) proposed that membrane association of α-synuclein might be linked to its cytotoxicity. In budding yeast, in all instances of toxicity except A30P, we note that α-synuclein is associated with the plasma membrane; but in our recent fission yeast model for α-synuclein (Brandis et al., 2006), although α-synuclein aggregates in a strikingly nucleation-polymerization-dependent manner in live cells, it is neither toxic nor does it localize to the plasma membrane. Together, the two yeast models support that α-synuclein-dependent toxicity is better correlated with plasma membrane localization, rather than with intracellular aggregation. But, whether α-synuclein membrane association is ultimately protective, harmful, or neutral to the cells will need future molecular clarification.

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