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**Yeast Cells Provide Insight into
Alpha-Synuclein Biology and Pathobiology**

Supporting Online Material, *Science Online*

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Materials and Methods

Plasmid constructions

Alpha-synuclein (α Syn) WT, A53T or A30P sequences (a kind gift from Dr. Peter Lansbury, Harvard University) were cloned into p426GPD (*S1*) as *SpeI-HindIII*-digested products of PCR amplification. GFP, CFP and YFP fusions were constructed in the same vector series (*S1*) by inserting the XFP (X = G, C or Y) coding sequence as a *ClaI-XhoI*-digested PCR product in frame with α Syn. Constructs were verified by DNA sequencing. α Syn alone or fused to XFP was subcloned into p416GPD, p423GPD, p425GPD, p426GAL (*S2*) or the integrative vectors from the pRS series (pRS306 and pRS304), (*S3*) as *SacI-KpnI* fragments.

Yeast Strains and Genetic Procedures

Genotypes of yeast strains are listed in Table SI. Yeast manipulations were performed and media were prepared using standard procedures (S4). Transformations of yeast were carried out using a standard lithium acetate procedure (S5). Yeast strains carrying the galactose-inducible α Syn constructs were pre-grown in raffinose medium (no repression of the galactose-inducible promoter) prior to galactose medium, to allow rapid, synchronous induction of expression.

Spotting Experiments

Cells carrying either one or two copies of the α Syn-GFP fusions were grown overnight at 30 °C in liquid media containing raffinose until they reached log or mid-log phase.

Cultures were then normalized for OD₆₀₀, serially diluted and spotted onto solid media containing either glucose or galactose, after which they were grown at 30 °C for 2-3 days.

Cells carrying the 2 μ plasmids were grown in synthetic medium lacking uracil, for plasmid selection, serially diluted and spotted onto rich medium (YPD) or synthetic medium lacking uracil.

Plasmid Maintenance Studies

Cells carrying 2 μ plasmids were grown in non-selective medium (YPD) until they reached log or mid-log phase and then plated onto YPD and synthetic drop-out solid media. Plasmid loss was assessed by comparing the number of cells growing on drop-out

versus rich medium. Cells carrying A30P mutant α Syn maintained the plasmid at higher copy-number, hence the higher levels of expression shown in Fig. 1A.

SDS-PAGE and Immunoblottings

Yeast cells expressing α Syn were grown to mid-log phase, harvested, washed with water and lysed in ethanol, with glass beads. The precipitated proteins were spun down at 13,000 rpm for 15 min., at 4 °C, and the pellets resuspended in standard SDS-sample buffer. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotting was performed following standard procedures. In Fig. 1A results with the Syn-1 antibody (Transduction Laboratories) are shown. Immunoblotting was also performed with antibodies LB509 (Zymed Laboratories) and anti-GFP (Roche). Each stained the same single band.

Microscopy

For analysis of α Syn-GFP distribution, strains carrying either one or two copies of α Syn-GFP were grown overnight in raffinose medium. After normalizing the culture density, expression was induced in galactose for 8 hours, at which time the levels and distribution of α Syn had reached steady state. Cells were then imaged with a Zeiss Axioplan II microscope equipped with MetaMorph (UIC) for acquisition. For analysis of α Syn distribution 2D and/or 3D deconvolution was applied (Huygens Essentials, SVI). Membrane localization precedes the formation of inclusions, neither of which is observed with A30P, supporting earlier suggestions that lipids promote α Syn oligomerization (S6), a process associated with neurodegeneration (S7).

Co-expression of α Syn with other aggregation-prone proteins, such as transthyretin (TTR) or the prion protein (PrP), did not result in co-aggregation, demonstrating that coalescence of α Syn is a nucleated, highly selective process.

Immunofluorescence (IF) was performed according to standard procedures (*S4*). In short, cell walls were fixed with formaldehyde, digested with Zymolyase 100T (Seikagaku Corporation, Tokyo, Japan), and processed for IF. Syn-1 antibody was from Transduction Laboratories. LB509 was from Zymed Laboratories. The anti-ubiquitin antibody was from Dako. Alexa-fluor antibodies were from Molecular Probes.

To label lipids, cells grown to mid-log phase were stained with the lipophylic dye Nile Red (final concentration of 0.5 μ g/ml) for 5 min (*S8*), washed with PBS and imaged.

Images shown in Fig. 3A were acquired using the same exposure time.

For FM4-64 staining, cells were grown in raffinose medium overnight, densities of cultures were then normalized and the cultures split in two. One half of the culture was grown in galactose medium while the other was grown in raffinose medium. After 8 hours cells were harvested, washed and stained with FM4-64 dye as previously described (*S9*). In this experiment we employed a toxic Htt derivative described previously (*S10*), but used a single integrated copy of the gene, which is as toxic as the α Syn gene. In contrast with the high copy 2 μ plasmid, previously reported to affect FM4-64 distribution (*S11*), a single integrated copy of this Htt gene did not affect FM4-64 distribution.

Electron microscopy of yeast cells expressing α Syn was performed as described (S12), after cell wall digestion with zymolyase 100T (Seikagaku Corporation, Tokyo, Japan).

Supporting Figures

Fig. S1

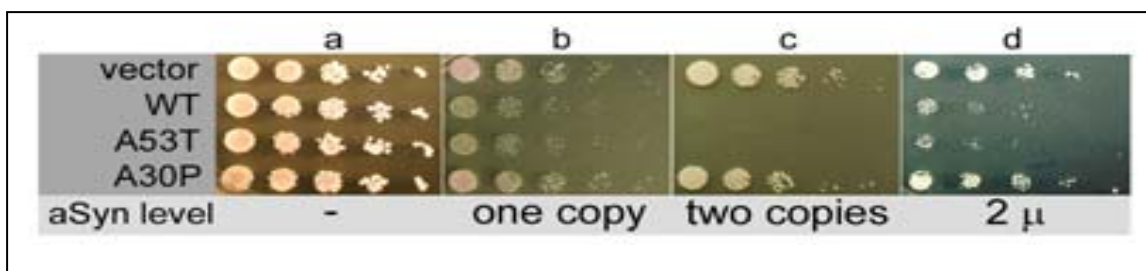


Fig. S1 In liquid cultures, which formed the basis for all the experiments reported in the paper, one copy of α Syn had little effect on growth. On plates it slightly inhibits growth. One copy (b) of α Syn WT or A53T had a mild effect on growth whereas two copies (c) completely inhibited it. 2 μ plasmid expression of WT and A53T α Syn caused mild inhibition of cell growth (d). The A30P mutant had no detectable effect on growth on this assay. Cells of all strains grew equally well when α Syn expression was not induced (a). Slight differences in toxicity in liquid and on plates are very useful in genetic screens.

Fig. S2

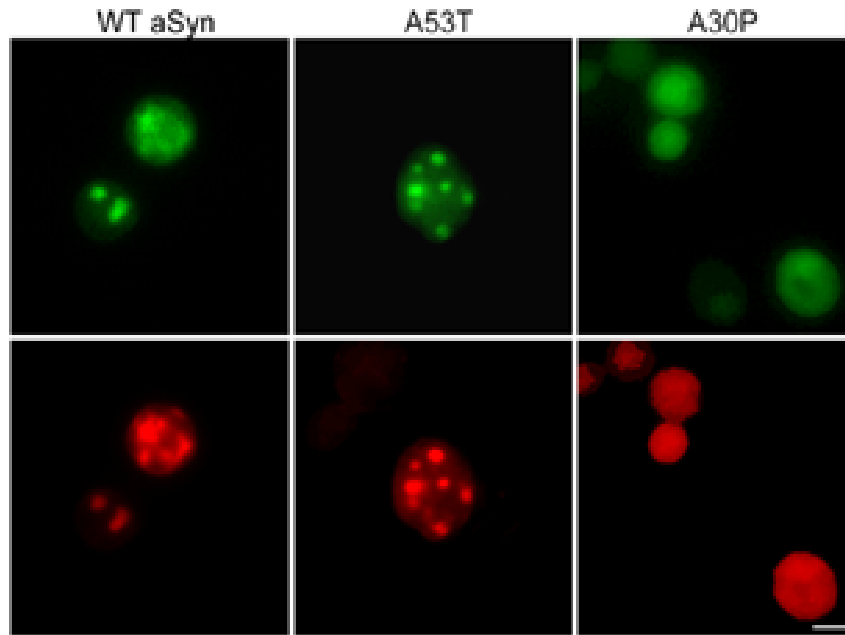


Fig. S2. α Syn-GFP produces the same pattern of localization when detected by GFP fluorescence or by immunofluorescence (IF) localization with the anti- α Syn antibody Syn-1, confirming that GFP localizations are not an artifact of proteolysis. Cells expressing WT α Syn-GFP, A53T or A30P from a 2 μ plasmid were processed for IF using the Syn-1 antibody followed by an Alexa Fluor 594-conjugated anti-mouse secondary antibody. GFP-positive inclusions (top) overlap with inclusions stained with the Syn-1 antibody (bottom), indicating the presence of full-length α Syn-GFP in the inclusions. The fixation procedure for IF eliminated the ability to assess the highly specific nature of α Syn at the plasma membrane. Scale bar, 1 μ .

Fig. S3

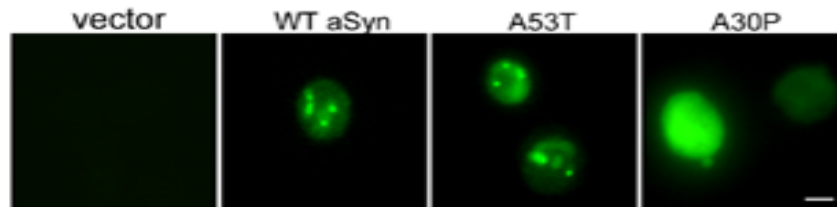


Fig. S3. α Syn aggregation is not an artifact of fusion to GFP. IF of cells expressing WT, A53T and A30P α Syn from 2 μ plasmids was performed with the Syn-1 antibody followed by an Alexa Fluor 488-conjugated anti-mouse secondary antibody. Inclusions were similar in number, size and distributions to those observed with the 2 μ plasmids of α Syn-GFP fusions, but membrane localization was reduced due to the fixation and permeabilization procedures required for IF. Scale bar, 1 μ .

Fig. S4

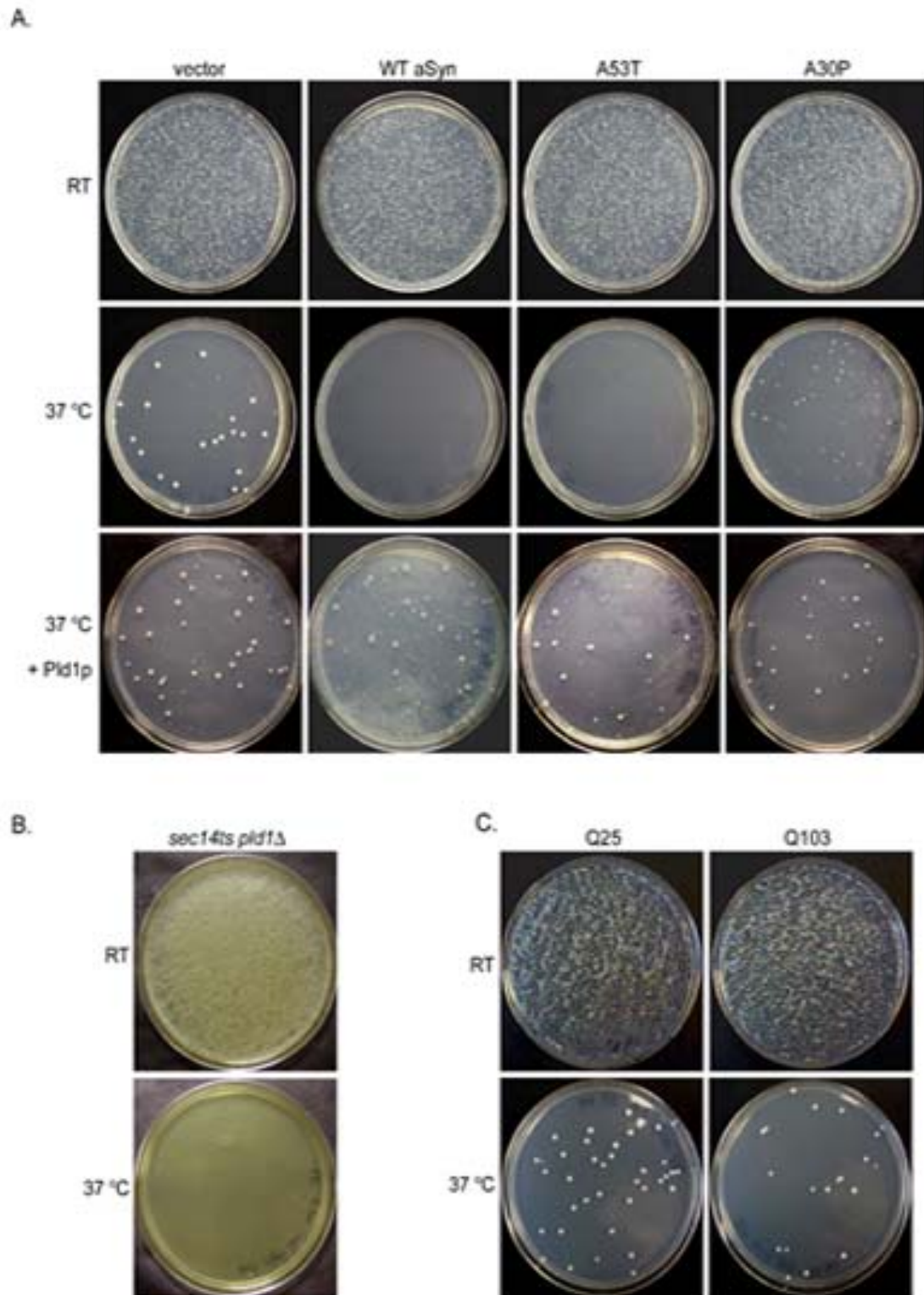


Fig. S4. α Syn inhibits PLD in *sec14ts* cells. Liquid cultures were grown overnight in synthetic selective medium for the plasmid and equal numbers of cells were plated onto solid selective medium and incubated at either the permissive (30 °C) or non-permissive temperature (37 °C). (A) *sec14ts* cells expressing α Syn WT, A53T and A30P from 2 μ plasmids grow normally at room temperature (RT), with those expressing WT or A53T α Syn growing slightly slower than those carrying an empty vector control or those expressing A30P (top and data not shown). When cells are incubated at 37 °C PLD activity is required for the accumulation of ‘bypass mutations’ for growth. Cells expressing WT and A53T α Syn, unlike those expressing A30P or carrying an empty vector, are not able to accumulate those mutations due to PLD inhibition (middle row). Upon introduction of yeast PLD1 from an extrachromosomal plasmid in the same cells, the ability to accumulate ‘bypass mutations’ was restored in cells expressing WT and A53T α Syn. (B) *sec14ts* cells in which the *PLD* gene is deleted are not able to acquire the ‘bypass mutations’ required for growth at 37 °C. (C) The same *sec14ts* cells expressing normal (Q25) or mutant (Q103) Htt exon-1 show normal growth at RT and acquire the ‘bypass mutations’ that enable them to grow at 37 °C at the same rate as vector controls. Thus, the effect of α Syn on PLD activity is specific.

Supporting Tables

Table S1. Strains used in this study.

Yeast Strain	Genotype
W303-1A	<i>MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1</i>
sec14ts a	<i>MAT a his3-D200 lys2-801 sec14-1 ura3-52</i>
sec14ts α	<i>MAT α ade2-1D1 his3-D200 trp1-D sec14-1 ura3-52</i>
sec14ts spo14 Δ a	<i>MAT a his3-D200 lys2-801 sec14-1 ura3-52 spo14::KAN</i>
sec14ts spo14 Δ α	<i>MAT α ade2-1D1 his3-D200 trp1-D sec14-1 ura3-52 spo14::KAN</i>
cki1 Δ	<i>MAT a his3 ura3 cki1::HIS3</i>
sec14ts cki1 Δ	<i>MAT a his3 lys2 trp1 ura3 cki1::HIS3 sec14^{ts}-3</i>

Table S2. Plasmids used in this study.

Plasmids	Type of Plasmid	Source or Reference
p42X*GPD	2 μ	(S1)
p426Gal	2 μ	(S2)
p416/PQ25	CEN	(S13)
p416/PQ103	CEN	(S13)
pRS304	Integrative	(S3)
pRS306	Integrative	(S3)
p426GPD-GFP	2 μ	This study
p426GPD-CFP	2 μ	This study
p426GPD-YFP	2 μ	This study
p426GPD- α SynWT	2 μ	This study
p426GPD- α SynA53T	2 μ	This study
p426GPD- α SynA30P	2 μ	This study
p42XGPD- α SynWT-N \dagger FP	2 μ	This study
p42XGPD- α SynA53T-N \dagger FP	2 μ	This study
p42XGPD- α SynA30P-N \dagger FP	2 μ	This study
pRS304- α SynWT-GFP	Integrative	This study
pRS304- α SynA53T-GFP	Integrative	This study
pRS304- α SynA30P-GFP	Integrative	This study
pRS306- α SynWT-GFP	Integrative	This study
pRS306- α SynA53T-GFP	Integrative	This study
pRS306- α SynA30P-GFP	Integrative	This study
pYES2-FLAG103Q	2 μ	(S10)
pPLD1	CEN	(S14)

*X=3, 5 or 6; \dagger N=C, G or Y

Supporting References

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