

# Mammalian ribosomal and chaperone protein RPS3A counteracts $\alpha$ -synuclein aggregation and toxicity in a yeast model system

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Accumulation of aggregated forms of  $\alpha$ Syn ( $\alpha$ -synuclein) into Lewy bodies is a known hallmark associated with neuronal cell death in Parkinson's disease. When expressed in the yeast *Saccharomyces cerevisiae*,  $\alpha$ Syn interacts with the plasma membrane, forms inclusions and causes a concentration-dependent growth defect. We have used a yeast mutant, *cog6* $\Delta$ , which is particularly sensitive to moderate  $\alpha$ Syn expression, for screening a mouse brain-specific cDNA library in order to identify mammalian proteins that counteract  $\alpha$ Syn toxicity. The mouse ribosomal and chaperone protein RPS3A was identified as a suppressor of  $\alpha$ Syn [WT (wild-type) and A53T] toxicity in yeast. We demonstrated that the 50 N-terminal amino acids are essential for this function. The yeast homologues of RPS3A were not effective in suppressing the  $\alpha$ Syn-induced growth defect,

illustrating the potential of our screening system to identify modifiers that would be missed using yeast gene overexpression as the first screening step. Co-expression of mouse RPS3A delayed the formation of  $\alpha$ Syn–GFP inclusions in the yeast cells. The results of the present study suggest that the recently identified extraribosomal chaperonin function of RPS3A also acts on the neurodegeneration-related protein  $\alpha$ Syn and reveal a new avenue for identifying promising candidate mammalian proteins involved in  $\alpha$ Syn functioning.

**Key words:** component of oligomeric Golgi complex 6 (COG6), Parkinson's disease, ribosomal and chaperone protein S3A (RPS3A),  $\alpha$ -synuclein, yeast screening.

## INTRODUCTION

Since the discovery that a point mutation (A53T) in *SNCA*, the gene encoding  $\alpha$ Syn ( $\alpha$ -synuclein), causes an autosomal dominant form of PD (Parkinson's disease) [1], there has been a strong interest in the physiological function of this protein and its connection with PD.  $\alpha$ Syn is a highly conserved, vertebrate-specific, 140-amino-acid protein that consists of an amphipathic N-terminal domain, an internal hydrophobic region and an acidic C-terminal tail. Misfolded  $\alpha$ Syn is the major component of the fibrillar cellular inclusions called LBs (Lewy bodies), the pathological hallmark of PD [2]. The abnormal accumulation of  $\alpha$ Syn in cellular inclusions has been associated with an entire spectrum of neurodegenerative diseases, collectively termed synucleinopathies [3]. Additional point mutations (A30P and E46K) in  $\alpha$ Syn and mutations that lead to increased  $\alpha$ Syn expression have been linked to autosomal dominant PD (as reviewed in [4]). Although familial PD caused by  $\alpha$ Syn mutations is very rare, PD characterized by  $\alpha$ Syn-containing LBs accounts for most of the sporadic cases of PD. Membrane-associated intermediates of fibrillization, or protofibrils, have been found to permeabilize presynaptic vesicles [5], interfere with vesicle trafficking [6], delay ER (endoplasmic reticulum)-to-Golgi transport by antagonizing ER/Golgi SNAREs (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptors) [7] and inhibit lysosomal function [8] and chaperone-mediated autophagy [9]. Cytosolic protofibrils are thought to

inhibit proteasomal protein degradation [10]. In dopamine-producing neurons these events lead to cell death through pathways that involve oxidative stress, mitochondrial dysfunction, ER stress and accumulation of misfolded proteins (as reviewed in [11]).

In yeast cells, human WT (wild-type) and A53T  $\alpha$ Syn associate with the plasma membrane after which they form cytoplasmic inclusions [12]. Many characteristics of  $\alpha$ Syn observed in other models have been described in yeast (as reviewed in [13,14]). Lindquist and colleagues have identified several multi-copy suppressors of  $\alpha$ Syn toxicity, including *YPT1* and *YPK9*, the yeast homologues of human *RAB1* and *PARK9* respectively [15,16]. Liang et al. [17] have identified several other yeast genes that, upon overexpression, counteract  $\alpha$ Syn-induced toxicity. The gene products played roles in ubiquitin-dependent protein catabolism, protein biosynthesis, vesicle trafficking and the response to stress.

We have taken the use of yeast as a tool for  $\alpha$ Syn studies a step further. We have used the *cog6* $\Delta$  (COG6 is component of oligomeric Golgi complex 6) yeast deletion strain, which is highly sensitive to moderate levels of  $\alpha$ Syn expression, to screen a brain-specific cDNA library for mammalian cDNAs that counteract  $\alpha$ Syn toxicity in yeast. We have isolated a cDNA clone encoding ribosomal and chaperone protein RPS3A (ribosomal and chaperone protein S3A) that suppresses yeast  $\alpha$ Syn toxicity, and it does this also independently from the *cog6* $\Delta$  deletion. The *Saccharomyces cerevisiae* homologues of RPS3A, Rps1A and Rps1B, were not effective in suppressing  $\alpha$ Syn toxicity and

Abbreviations used: COG6, component of oligomeric Golgi complex 6; ER, endoplasmic reticulum; 5-FOA, 5-fluoro-orotic acid; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HBx, hepatitis B protein X; HRP, horseradish peroxidase; LB, Lewy body; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NA, numerical aperture; PD, Parkinson's disease; Ppk1, phosphoglycerate kinase 1; PSMA2, proteasome subunit  $\alpha$  type 2; Spir1CT, C-terminus of Spire homologue 1; RPS3A, ribosomal protein S3A; SC, synthetic complete; SD, synthetic dextrose; SNARE, soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor;  $\alpha$ Syn,  $\alpha$ -synuclein; WT, wild-type; yeGFP, yeast-enhanced GFP; YPD, yeast extract/peptone/dextrose.

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**Table 1 Plasmids used in the present study***H.s.*, *Homo sapiens*; *M.m.*, *Mus musculus*; *S.c.*, *Saccharomyces cerevisiae*.

Plasmid name	Vector	Type	Selection marker	Coding sequence*
pESC/ $\alpha$ Syn	pESC-His	Multi-copy	<i>HIS3</i>	<i>H.s.</i> $\alpha$ Syn cDNA (WT)
pESC/ $\alpha$ SynA53T	pESC-His	Multicopy	<i>HIS3</i>	<i>H.s.</i> $\alpha$ Syn cDNA (A53T)
pESC/ $\alpha$ SynA30P	pESC-His	Multicopy	<i>HIS3</i>	<i>H.s.</i> $\alpha$ Syn cDNA (A30P)
pESC/ $\alpha$ Syn-GFP	pESC-His	Multi-copy	<i>HIS3</i>	<i>H.s.</i> $\alpha$ Syn cDNA (WT)-yeGFP C-terminal fusion
pRS403/ $\alpha$ Syn	pRS403	Integrative	<i>HIS3</i>	<i>H.s.</i> $\alpha$ Syn cDNA (WT)
pRS403/ $\alpha$ Syn-GFP	pRS403	Integrative	<i>HIS3</i>	<i>H.s.</i> $\alpha$ Syn cDNA (WT)-yeGFP C-terminal fusion
pRS405/ $\alpha$ Syn	pRS405	Integrative	<i>LEU2</i>	<i>H.s.</i> $\alpha$ Syn cDNA (WT)
pRS405/ $\alpha$ Syn-GFP	pRS405	Integrative	<i>LEU2</i>	<i>H.s.</i> $\alpha$ Syn cDNA (WT)-yeGFP C-terminal fusion
Empty vector	pVV214	Multi-copy	<i>URA3</i>	
pPSMA2	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> PSMA2 cDNA †
pRPS3A	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> RPS3A cDNA †
pSpir1CT	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> Spire homologue 1 cDNA amino acids 266–258*
pRPS1A	pVV214	Multi-copy	<i>URA3</i>	<i>S.c.</i> ORF <i>YLR441C</i> †
pRPS1B	pVV214	Multi-copy	<i>URA3</i>	<i>S.c.</i> ORF <i>YML063W</i> †
pRPS3A $\Delta$ 2-50	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> RPS3A cDNA M1, amino acids 51–264
pRPS3A $\Delta$ 164-264	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> RPS3A cDNA amino acids 1–163
pRPS3A $\Delta$ 114-264	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> RPS3A cDNA amino acids 1–113
pRPS3A $\Delta$ 51-264	pVV214	Multi-copy	<i>URA3</i>	<i>M.m.</i> RPS3A cDNA amino acids 1–50

\*Isolated from a mouse brain cDNA library in the screening (see the text for details).

†From the FLEXGene yeast protein-coding clone collection [41], Gateway® cloned.

**Table 2 Yeast strains used in the present study**

Strain name	Genotype	Reference
BY4742	<i>MAT<math>\alpha</math> his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	[42]
<i>cog6</i> $\Delta$	BY4742 <i>cog6</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>vps52</i> $\Delta$	BY4742 <i>vps52</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>dpp1</i> $\Delta$	BY4742 <i>dpp1</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>opi3</i> $\Delta$	BY4742 <i>opi3</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>sod2</i> $\Delta$	BY4742 <i>sod2</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>vps24</i> $\Delta$	BY4742 <i>vps24</i> $\Delta$ :: <i>KANMX4</i>	[43]
BY2E	BY4742 <i>his3</i> $\Delta$ 1::pRS403 <i>leu2</i> $\Delta$ 0::pRS405	The present study
BY2 $\alpha$ Syn	BY4742 <i>his3</i> $\Delta$ 1::pRS403/ $\alpha$ Syn <i>leu2</i> $\Delta$ 0::pRS405/ $\alpha$ Syn	The present study
<i>cog6</i> $\Delta$ 2E	BY4742 <i>cog6</i> $\Delta$ :: <i>KANMX4</i> <i>his3</i> $\Delta$ 1::pRS403 <i>leu2</i> $\Delta$ 0::pRS405	The present study
<i>cog6</i> $\Delta$ 2 $\alpha$ Syn	BY4742 <i>cog6</i> $\Delta$ :: <i>KANMX4</i> <i>his3</i> $\Delta$ 1::pRS403/ $\alpha$ Syn <i>leu2</i> $\Delta$ 0::pRS405/ $\alpha$ Syn	The present study
<i>cog6</i> $\Delta$ 2 $\alpha$ Syn-GFP	BY4742 <i>cog6</i> $\Delta$ :: <i>KANMX4</i> <i>his3</i> $\Delta$ 1::pRS403/ $\alpha$ Syn-GFP <i>leu2</i> $\Delta$ 0::pRS405/ $\alpha$ Syn-GFP	The present study
<i>rps1a</i> $\Delta$	BY4742 <i>rps1a</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>rps1b</i> $\Delta$	<i>MAT<math>\alpha</math> his3</i> $\Delta$ 1 <i>leu2</i> $\Delta$ 0 <i>met15</i> $\Delta$ 0 <i>ura3</i> $\Delta$ 0 <i>rps1b</i> $\Delta$ :: <i>KANMX4</i>	[43]
<i>rps1a</i> $\Delta$ / <i>rps1b</i> $\Delta$	BY <i>rps1a</i> $\Delta$ $\alpha$ /BY <i>rps1b</i> $\Delta$ $\alpha$	The present study

thus could not have been identified in a yeast overexpression screen, which illustrates the power of our mouse brain cDNA screening strategy. Because RPS3A also strongly reduced  $\alpha$ Syn-GFP inclusion formation, we suggest that its recently described chaperonin function [18] is involved in preventing  $\alpha$ Syn from folding into a harmful conformation, possibly by stabilizing the recently described helically folded form [19].

## EXPERIMENTAL

### Strains, plasmids and media

The yeast strains and plasmids used are shown in Tables 1 and 2 respectively. The cDNA library was constructed by Invitrogen, using mRNA isolated from mouse brain. Double-stranded cDNA was cloned in a Gateway Topo cloning vector and the library was swapped into the pVV214 yeast expression vector [20].  $\alpha$ Syn is expressed from the *GALI* promoter, either on the multi-copy

plasmid pESC-HIS (Agilent Technologies) or integrated in two copies in the genome, via integrative vectors pRS403 and pRS405 (Stratagene).

Cells were pre-grown in non-inducing raffinose (2% w/v) selective medium (SC-His, SC-Ura, SC-Leu-His, SC-Ura-Leu-His, where SC is synthetic complete lacking histidine, uracil, leucine and histidine, and uracil, leucine and histidine respectively; BIO101) and then shifted to galactose (2% w/v) selective medium for induction of  $\alpha$ Syn expression. For microscopy, cells were grown in raffinose (2% w/v) selective medium, after which they were transferred to galactose (2% w/v) selective medium for 15 h. For sporulation, sodium acetate (0.5%) agar plates were used. For loss of pVV214-derived plasmids, agar plates (SC, 2% glucose) with 0.1% 5-FOA (5-fluoro-orotic acid) were used. To test for the presence of the KanMX gene-disruption cassette, cells were streaked on to YPD (yeast extract/peptone/dextrose: 1% yeast extract, 2% peptone, 2% glucose) agar plates containing 300 mg/l geneticin (G418, Invitrogen).

## Growth assays

Yeast cells were grown in raffinose minimal medium to mid-exponential phase at 30°C. A dilution series ( $D_{600} = 1, 0.1, 0.01$  and 0.001) was made with sugar-free minimal medium. Then, 5  $\mu$ l of each dilution was spotted on to agarose plates with galactose or glucose minimal medium, which were incubated at 30°C. The assays were evaluated at 1, 2 and 3 days, but the results were qualitatively similar and quantitatively clearer at 3 days. Hence the latter are shown in the Figures. For all direct comparisons between strains or plasmids, the growth assays were done on one and the same agarose plate. For growth assays in liquid media, cells were also cultured in raffinose minimal medium to mid-exponential phase at 30°C and growth curves were determined in liquid minimal galactose medium, inoculated at  $D_{600} = 0.05$  and incubated at 30°C.  $D_{600}$  was measured every 1 h for 3 days in a Bioscreen C System.  $\alpha$ Syn toxicity was enhanced in WT cells by the addition of 9% (v/v) DMSO.

## Screening of the cDNA library

The *cog6 $\Delta$ 2 $\alpha$ Syn* strain was transformed with the cDNA library after which the cells were plated out on galactose. From the transformants obtained, those that could not grow on plates containing galactose (for induction of  $\alpha$ Syn expression) and 5-FOA (for loss of the cDNA plasmid) were selected for an  $\alpha$ Syn toxicity test. From the colonies that showed good growth, the cDNA plasmids were isolated and retransformed in the *cog6 $\Delta$ 2 $\alpha$ Syn* strain. With these transformants the  $\alpha$ Syn toxicity test was repeated to confirm the suppression of  $\alpha$ Syn toxicity by the cDNA plasmids isolated.

## Western blot analysis

Cells were grown at 30°C in selective raffinose medium until they reached exponential phase. They were then transferred for 15 h to galactose medium to induce  $\alpha$ Syn expression. Protein extracts were obtained as described in [21]. Mouse brain extract in SDS/PAGE loading buffer was obtained from Sigma-Aldrich. SDS/PAGE was performed using Invitrogen NuPAGE Novex 4–12% Bis-Tris Gels.  $\alpha$ Syn and  $\alpha$ Syn-GFP protein levels were determined by Western blot analysis using a rabbit anti- $\alpha$ Syn antibody (Cell Signaling Technology), diluted 1:500, and compared with Pgk1 (phosphoglycerate kinase 1) levels determined by Western blotting using a mouse anti-Pgk1 antibody (Molecular Probes), diluted 1:500, or to Gapdh (glyceraldehyde-3-phosphate dehydrogenase) using a mouse anti-Gapdh antibody (Millipore), diluted 1:1000. Secondary antibodies used were HRP (horseradish peroxidase)-conjugated donkey anti-(rabbit IgG) ECL antibody and HRP-conjugated sheep anti-(mouse IgG) ECL antibody (GE Healthcare), both diluted 1:5000. RPS3A protein levels were determined by Western blot analysis using a custom-made affinity-purified chicken IgY polyclonal antibody (Genway), diluted 1:1000. As the secondary antibody HRP-conjugated goat anti-(chicken IgY) Fc fragment (Genway) was used, diluted 1:5000.

## Complementation analysis

The heterozygous diploid strain *rps1a $\Delta$ /RPS1A rps1b $\Delta$ /RPS1B* (obtained by mating of *rps1a $\Delta$*  with *rps1b $\Delta$* ) was transformed with a plasmid, after which transformants were sporulated (as described previously [22]) and growth of the segregants was assessed on SD (synthetic dextrose) lacking uracil (SD-Ura),

SD plates containing 5-FOA, a medium where loss of the *URA3*-encoding plasmids is induced, and on YPD plates with geneticin, where only the segregants that contain at least one KanMX-disrupted gene can grow. The genotypes of the segregants were confirmed by PCR on the genomic DNA.

## Fluorescence microscopy

Laser-scanning microscopy was performed with an LSM510 system (Carl Zeiss), using Koehler illumination and a high-NA (numerical aperture) objective (C-Apochromat 40 $\times$ /NA1.2/W). Yeast cell cultures were placed between two 100- $\mu$ m-thick cover slips. The 488-nm line of an argon-ion laser (acousto-optical tunable filter 15%) was used to excite yeGFP (yeast-enhanced GFP). Fluorescence of yeGFP was captured through a BP505-530 filter.

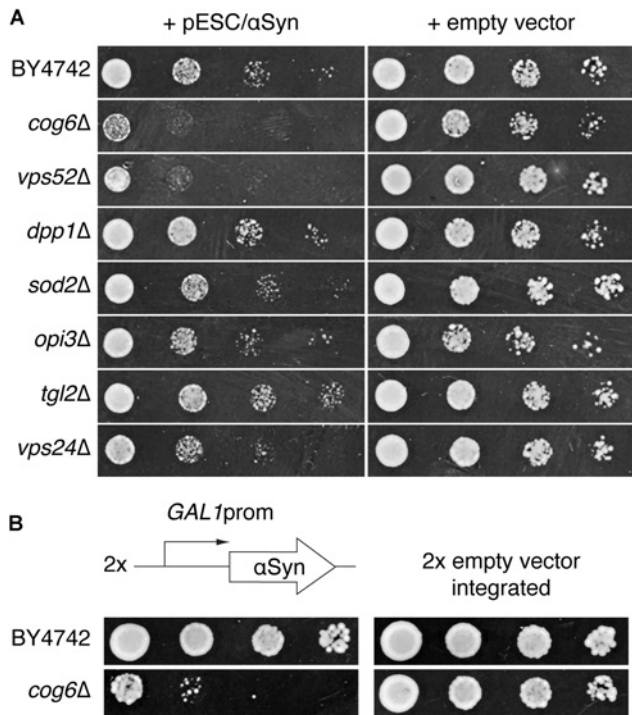
## RESULTS

### Establishment of a yeast screening system for isolation of suppressors of $\alpha$ Syn toxicity

We have tested yeast deletion strains reported to be sensitive to  $\alpha$ Syn expression [12,23] for those that showed the highest sensitivity, in order to minimize possible artefacts due to high overexpression of  $\alpha$ Syn in the yeast model system. For that purpose, we transformed the strains with a multi-copy vector containing human WT  $\alpha$ Syn (NCBI accession number AAI08276) under the control of a galactose-inducible promoter and tested the transformants for residual growth on selective galactose medium. Under the conditions used in the present study  $\alpha$ Syn expression caused, at most, a slight growth inhibition of the *dpp1 $\Delta$* , *opi3 $\Delta$* , *sod2 $\Delta$* , *tgl2 $\Delta$*  and *vps24 $\Delta$*  strains and isogenic WT strain BY4742. A strong growth defect was observed in the *vps52 $\Delta$*  strain, but the *cog6 $\Delta$*  strain was most sensitive to galactose-induced expression of  $\alpha$ Syn (Figure 1A). To obtain a stable cell line for screening purposes we have integrated two galactose-inducible copies of the  $\alpha$ Syn ORF into the genome of the *cog6 $\Delta$*  strain. The resulting transformants (*cog6 $\Delta$ 2 $\alpha$ Syn*) showed a strong growth defect on galactose (Figure 1B). On glucose all strains grew well and also cells transformed with empty vectors showed the same growth as untransformed cells (results not shown). Hence we used the *cog6 $\Delta$ 2 $\alpha$ Syn* strain in the subsequent screening for mammalian suppressor clones. In contrast with the observations of Outeiro and Lindquist [24], we did not observe an  $\alpha$ Syn-induced growth defect in our WT strain with two galactose-inducible copies of  $\alpha$ Syn integrated in the genome.

### Screening for suppressors of $\alpha$ Syn toxicity with a mouse brain cDNA library

Total mRNA was isolated from mouse brain and used for construction of a cDNA library, custom-made by Invitrogen. The cDNA library was subcloned into the yeast expression vector pVV214 [20] and the resulting library was transformed into the *cog6 $\Delta$ 2 $\alpha$ Syn* strain. We obtained approximately 36000 independent transformants of which approximately 500 transformants were able to grow on the galactose-containing plates. Only the colonies that did not grow on galactose after loss of the cDNA (*URA3*) plasmid on medium with 5-FOA, a substance converted into the toxic fluorouracil by orotidine 5'-monophosphate decarboxylase, the *URA3* gene product [25], were retained for plasmid isolation. After retransformation of the



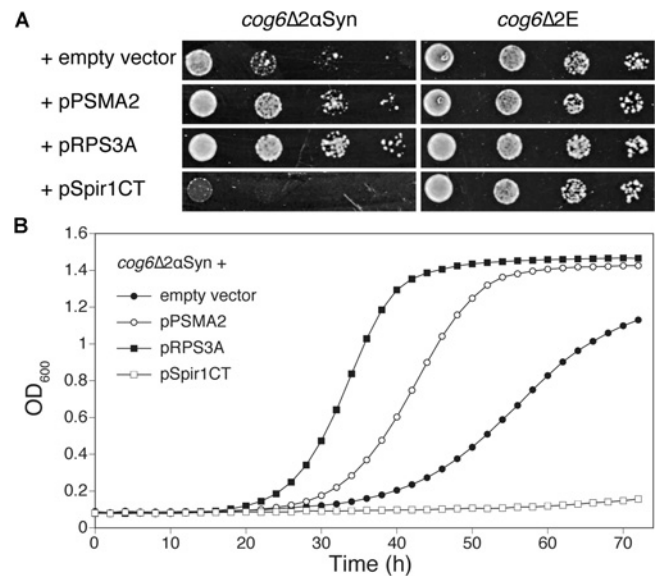
**Figure 1** The *cog6Δ* strain is highly sensitive to  $\alpha$ Syn expression

(A) Yeast strains BY4742, *cog6Δ*, *vps52Δ*, *dpp1Δ*, *sod2Δ*, *opi3Δ*, *tgl2Δ* and *vps24Δ* were transformed with the pESC/ $\alpha$ Syn plasmid for galactose-induced expression of  $\alpha$ Syn or empty vector pESC-His and a growth assay on SC-His plates containing galactose was performed as described in the Experimental section. (B) Two copies of human WT  $\alpha$ Syn, under the control of the *GAL1* promoter, or empty vectors pRS403 and pRS405 were integrated into the genome of the BY4742 and *cog6Δ* strains. A growth assay on SC-Leu-His plates containing galactose was performed as described in the Experimental section.

isolated plasmids in the *cog6Δ2αSyn* strain, we found two clones that strongly reduced the growth defect of *cog6Δ2αSyn* cells on  $\alpha$ Syn-inducing medium: they encoded the mouse ribosomal protein S3A (pRPS3A, NCBI accession number NP\_058655) and the proteasome subunit  $\alpha$  type 2 (pPSMA2, NCBI accession number NP\_032970). One of the isolated cDNA plasmids surprisingly enhanced cell death upon retransformation in the *cog6Δ2αSyn* strain (Figure 2A). This cDNA plasmid had no growth-inhibitory effect in the *cog6Δ* strain without  $\alpha$ Syn, indicating that the cDNA product apparently enhances  $\alpha$ Syn toxicity. This plasmid encodes a fragment of the actin organizer protein Spire homologue 1 (NCBI accession number NP\_919336) containing the 263 C-terminal amino acids (termed pSpir1CT). The three plasmids had a corresponding effect on the growth rate of *cog6Δ2αSyn* cells in liquid galactose medium (Figure 2B). As a Bioscreen C device was used for the attenuation readings, the measurements are saturated at a  $D_{600}$  of approximately 1.4. Hence this does not indicate that the cells have reached stationary phase.

#### RPS3A suppresses $\alpha$ Syn toxicity in other backgrounds than the *cog6Δ* strain, but does not influence $\alpha$ Syn protein levels

A drawback of doing the screening in a sensitive mutant strain is that suppressors isolated could be acting on the sensitivity of that strain, rather than reducing the toxicity of  $\alpha$ Syn. To address this issue, we investigated whether the observed effects were also

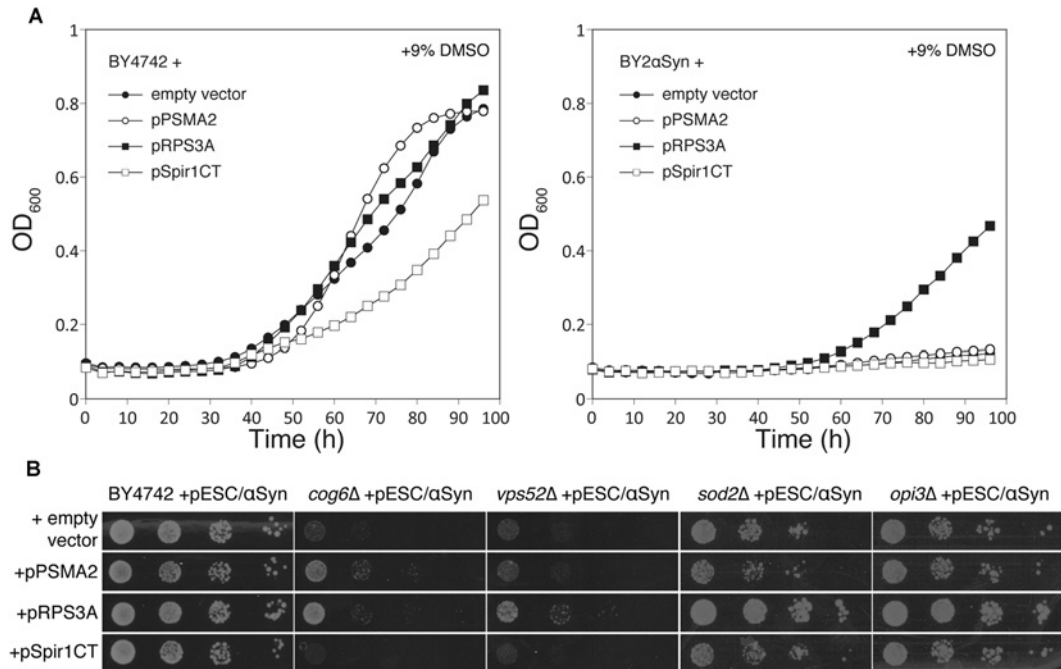


**Figure 2** Two mammalian suppressors and one enhancer of  $\alpha$ Syn toxicity have been identified

(A) Strain *cog6Δ2αSyn* and the control strain not expressing  $\alpha$ Syn were transformed with empty vector pV214 or with the mouse cDNA plasmids isolated in the screening, as indicated on the Figure. A growth assay was performed on SC-Ura-Leu-His galactose plates, as described in the Experimental section. (B) Growth curves were determined in SC-Ura-Leu-His galactose (2% w/v) medium, using a Bioscreen C device as described in the Experimental section. Strains: *cog6Δ2αSyn* + empty vector (●), pPSMA2 (○), pRPS3A (■), pSpir1CT (□).

present in other backgrounds. Because no clear effects of  $\alpha$ Syn or the other brain proteins could be observed in WT BY4742 cells grown in normal inducing medium, we performed growth experiments in the presence of DMSO. Addition of DMSO was reported to enhance the amount of  $\alpha$ Syn-induced inclusions, leading to growth reduction [26]. Addition of 9% (v/v) DMSO caused a much stronger growth inhibition of cells expressing  $\alpha$ Syn than in cells without  $\alpha$ Syn, but only RPS3A counteracted the DMSO-induced growth inhibition of the BY2 $\alpha$ Syn strain, and it had no effect in the absence of  $\alpha$ Syn. PSMA2 did not affect the growth of either strain with or without  $\alpha$ Syn or DMSO, whereas Spir1CT enhanced the DMSO-induced growth defect, independent of  $\alpha$ Syn expression (Figure 3A). RPS3A also improved the growth of BY2 $\alpha$ Syn in the presence of 9 mM  $ZnSO_4$ , which was demonstrated to increase  $\alpha$ Syn toxicity [27] (results not shown). When we tested the suppressors and enhancer in other  $\alpha$ Syn-sensitive strains (Figure 3B), we observed that PSMA2 only suppresses the  $\alpha$ Syn-sensitivity of the *cog6Δ* strain, whereas RPS3A also counteracted the  $\alpha$ Syn-induced growth defect in the strains *vps52Δ*, *sod2Δ* and *opi3Δ*. For Spir1CT, we did not observe an increased growth defect in the strains with weak  $\alpha$ Syn-sensitivity: BY4742, *sod2Δ* and *opi3Δ*. We conclude that only RPS3A is directly affecting  $\alpha$ Syn toxicity, whereas PSMA2 is a suppressor of the  $\alpha$ Syn-sensitivity in the *cog6Δ* background and the C-terminus of Spire homologue 1 enhances lethality in cells that are challenged with various stresses, but not specifically  $\alpha$ Syn-induced lethality.

None of the isolated mouse cDNAs, nor the deletion of *COG6*, significantly influenced  $\alpha$ Syn protein levels, as judged by Western blotting experiments (Figure 4A). Mouse  $\alpha$ Syn from a commercially available mouse brain extract (Sigma) migrated slightly higher than the yeast-expressed human  $\alpha$ Syn,



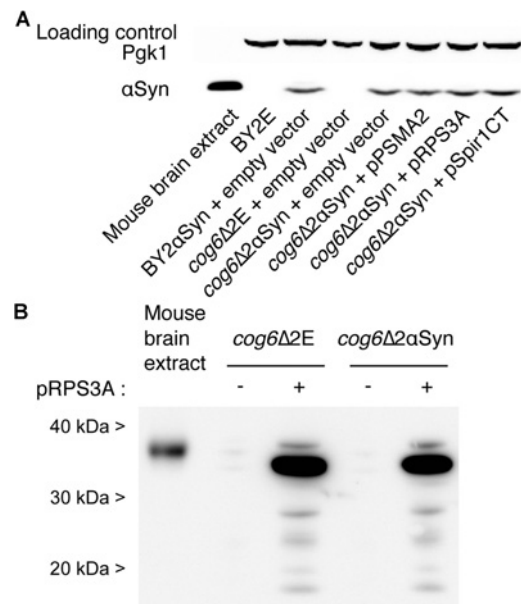
**Figure 3** PS3A counteracts the  $\alpha$ Syn-induced growth defect irrespective of the genetic background

(A) RPS3A counteracts DMSO-enhanced toxicity of  $\alpha$ Syn and has no effect on DMSO toxicity in a strain not expressing  $\alpha$ Syn, PSMA2 does not influence the DMSO-enhanced  $\alpha$ Syn toxicity and Spir1CT enhances the DMSO-induced growth defect independent of  $\alpha$ Syn expression. Left-hand panel: BY4742 without  $\alpha$ Syn; right-hand panel: BY2 $\alpha$ Syn. Plasmids: empty vector (●), pPSMA2 (○), pRPS3A (■), pSpir1CT (□). Growth curves in SC–Ura galactose medium with 9% DMSO were obtained as described in the Experimental section. (B) RPS3A suppresses  $\alpha$ Syn toxicity in  $\alpha$ Syn-sensitive strains *cog6* $\Delta$ , *vps52* $\Delta$ , *sod2* $\Delta$  and *opi3* $\Delta$ . PSMA2 only affects the growth of  $\alpha$ Syn-expressing *cog6* $\Delta$  cells. Spir1CT does not enhance the weak  $\alpha$ Syn-sensitivity of strains BY4742, *sod2* $\Delta$  or *opi3* $\Delta$ . Transformants, as indicated on the Figure, were spotted on to SC–Ura–His galactose plates as described in the Experimental section.

possibly due to the seven-amino-acid differences between the two proteins. A custom-made antibody against RPS3A (from Cell Signaling Technology) readily detected the yeast-expressed RPS3A (Figure 4B). Both in the yeast extracts and in the mouse brain extract from Sigma, mouse RPS3A migrated approximately 6 kDa higher than its predicted molecular mass of 29.89 kDa. Several lower-molecular-mass bands were present in the yeast extracts from yeast cells with pRPS3A, and the strongest band was the second largest form, approximately 2 kDa smaller than the form present in the mouse brain extract from Sigma. Very weak bands of similar sizes can be seen in the extracts from cells carrying the empty vector instead of pRPS3A, probably due to cross-reactivity of the antibody with the yeast homologues of RPS3A. There are no indications that the presence of  $\alpha$ Syn influences the protein levels or the post-translational processing of RPS3A. Despite several attempts using both the specific antibodies and an anti-c-Myc antibody against a Myc-tagged version of  $\alpha$ Syn, we were unable to reproducibly demonstrate co-immunoprecipitation of RPS3A with  $\alpha$ Syn, or vice versa.

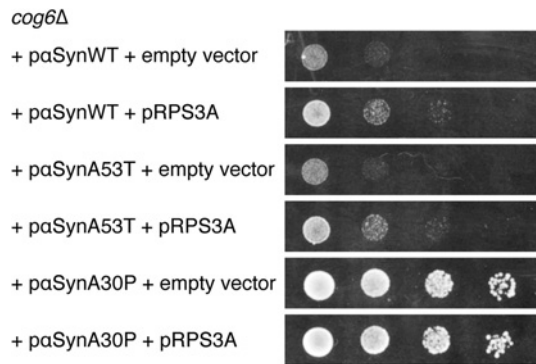
#### Mouse RPS3A suppresses $\alpha$ Syn A53T toxicity in yeast

It was shown previously that, when expressed in yeast,  $\alpha$ Syn A53T induces a growth defect similar to  $\alpha$ Syn WT. In contrast with WT and A53T,  $\alpha$ Syn A30P does not localize to the plasma membrane, but remains dispersed throughout the cytoplasm and does not induce a growth defect in yeast [24]. When expressed in yeast strain *cog6* $\Delta$ , we observed that  $\alpha$ Syn A53T causes a strong growth defect that is suppressed by co-expression of RPS3A. The expression of  $\alpha$ Syn A30P does not result in a growth defect in the *cog6* $\Delta$  background (Figure 5).



**Figure 4** Western blot experiments of mammalian proteins expressed in yeast

(A) Deletion of *COG6* and co-expression of brain proteins do not significantly change expression levels of  $\alpha$ Syn. Western blot analysis 15 h after induction on galactose (2% w/v). Primary antibodies: (top) anti-yeast Pgk1 (produced in mouse) as a loading control, (bottom) anti-human  $\alpha$ Syn (produced in rabbit). (B)  $\alpha$ Syn expression does not change the appearance of RPS3A in SDS/PAGE. Western blot analysis 15 h after induction on galactose (2% w/v). Primary antibody: anti-mouse RPS3A (produced in chicken). The molecular mass in kDa is indicated on the left-hand side.



**Figure 5** RPS3A counteracts  $\alpha$ Syn A53T toxicity and  $\alpha$ Syn A30P is not toxic in *cog6* $\Delta$  yeast cells

Strain *cog6* $\Delta$  was transformed with the plasmids indicated. A spot dilution growth assay was performed on SC-Ura-His galactose plates, as described in the Experimental section.

### Mouse RPS3A complements the deletion of its yeast homologues RPS1A and RPS1B, but the yeast homologues do not suppress the *cog6* $\Delta$ 2 $\alpha$ Syn growth defect

Mammalian RPS3A has two homologous proteins in yeast, Rps1A and Rps1B. We found that double deletion of the corresponding genes is lethal and that pRPS3A can overcome the lethality of the *rps1a* $\Delta$  *rps1b* $\Delta$  strain (Figure 6A). This indicates that mouse RPS3A probably contributes to the ribosome activity of the yeast. We have cloned the yeast homologues into the pVV214 vector (pRPS1A and pRPS1B, NCBI accession numbers NP\_013546 and NP\_013648), and evaluated the capacity of the resulting plasmids to complement the lethality of the *rps1a* $\Delta$  *rps1b* $\Delta$  deletion strain. Complementation was observed and *rps1a* $\Delta$  *rps1b* $\Delta$  + pRPS1A or pRPS1B even grew slightly better than *rps1a* $\Delta$  *rps1b* $\Delta$  + pRPS3A (Figure 6B), indicating that all three plasmids allow the cells to produce functional ribosomal subunits. When we transformed plasmids pRPS1A and pRPS1B into the *cog6* $\Delta$ 2 $\alpha$ Syn strain and assessed growth on galactose, they did not suppress  $\alpha$ Syn-induced lethality, neither on agar plates (Figure 6C) nor in liquid medium (Figure 6D). pRPS1A even seemed to slightly enhance the  $\alpha$ Syn-induced growth defect. Again, the plateau reached in the growth curves is due to saturation of the Bioscreen C reading at this  $D_{600}$  value, which should not be interpreted as the cells entering stationary phase. We can conclude that the suppression of  $\alpha$ Syn toxicity by RPS3A is a specific function of the mammalian protein, which is absent from its yeast counterparts.

### Identification of the domain of RPS3A necessary for suppression of $\alpha$ Syn toxicity in yeast

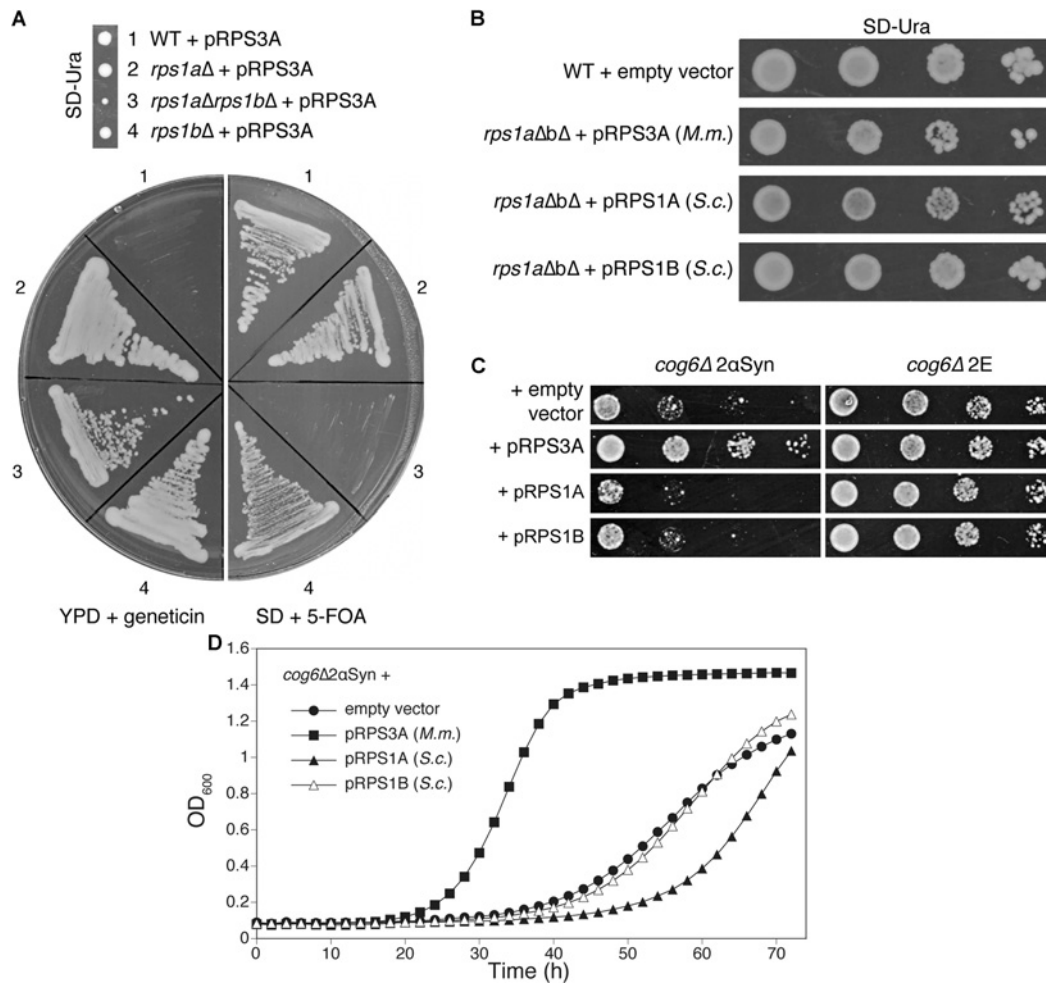
In the study by Lim et al. [18] it was shown that 50 N-terminal amino acids of RPS3A are essential to interact with and to increase the solubility of the HBx (hepatitis B protein X) in hepatocellular carcinoma cells, whereas from the C-terminus up to 101 amino acids could be deleted without affecting this chaperone function. We have created a yeast expression clone in which the coding sequence for the 50 N-terminal amino acids except the start codon was removed (pRPS3A $\Delta$ 2-50) as well as clones for expression of C-terminal deletions of 101 amino acids (pRPS3A $\Delta$ 164-264), 151 amino acids (pRPS3A $\Delta$ 114-264) and 214 amino acids (pRPS3A $\Delta$ 51-264), as represented in

Figure 7(A). By expressing these plasmids in our yeast strain, we have evaluated which domain is essential and which is sufficient for suppression of  $\alpha$ Syn toxicity in yeast. From the growth assay (Figure 7B), we can conclude that the 50 N-terminal amino acids are indeed essential for this function of RPS3A, as pRPS3A $\Delta$ 2-50 has lost the ability to suppress  $\alpha$ Syn toxicity. However, they are not sufficient because pRPS3A $\Delta$ 51-264 also does not result in restored growth of *cog6* $\Delta$ 2 $\alpha$ Syn on galactose. Because plasmid pRPS3A $\Delta$ 164-264 still improves growth on galactose (albeit to a lesser extent than full-length RPS3A) but pRPS3A $\Delta$ 114-264 does not, we conclude that amino acids 51–164 are also essential for the suppression of  $\alpha$ Syn toxicity.

To evaluate whether the truncations affect the ribosomal function of RPS3A, we have transformed the heterozygous diploid strain *RPS1A/rps1a* $\Delta$  *RPS1B/rps1b* $\Delta$  with the different plasmids and sporulated the transformants to evaluate which of the plasmids could complement the synthetic lethality of the *rps1a* $\Delta$  *rps1b* $\Delta$  strain. Only when the plasmid expressing full-length RPS3A was transformed, were four growing spores obtained (Figure 7C), indicating that none of the truncation mutants had retained the ribosomal function of RPS3A.

### Mouse RPS3A and RPS3A $\Delta$ 164-264 reduce $\alpha$ Syn-GFP inclusion formation in the *cog6* $\Delta$ 2 $\alpha$ Syn-GFP strain

A previous study showed that  $\alpha$ Syn-GFP constructs in yeast are not subject to proteolysis, as opposed to related fusions in mammalian cells [12], and that the fusion with GFP does not influence the localization of  $\alpha$ Syn. Hence we integrated two copies of a construct encoding galactose-inducible  $\alpha$ Syn-GFP into the genome of the *cog6* $\Delta$  strain, resulting in the strain *cog6* $\Delta$ 2 $\alpha$ Syn-GFP. The C-terminal fusion of GFP to  $\alpha$ Syn did not influence its toxicity in the *cog6* $\Delta$  background and RPS3A and RPS3A $\Delta$ 164-264 also suppressed  $\alpha$ Syn-GFP toxicity in the *cog6* $\Delta$ 2 $\alpha$ Syn-GFP strain, whereas the other truncations did not (results not shown). We studied the intracellular localization of  $\alpha$ Syn as a function of time after induction of  $\alpha$ Syn-GFP expression with galactose and observed three patterns of intracellular localization; at early time points  $\alpha$ Syn-GFP was located nearly exclusively at the plasma membrane, later on (starting 6 h after the induction of  $\alpha$ Syn expression) part of the cells showed both localization at the plasma membrane and in cytosolic inclusions and finally (starting 12 h after the induction of  $\alpha$ Syn expression) some cells showed  $\alpha$ Syn-GFP fluorescence exclusively in cytosolic inclusions (Figure 8A). Co-expression of RPS3A and RPS3A $\Delta$ 164-264 clearly delays the shift of  $\alpha$ Syn-GFP from the plasma membrane to the cytosolic inclusions, whereas cells transformed with the other truncation mutants behave much like empty-vector-transformed cells (Figure 8B). These effects were quantified by counting the cells with  $\alpha$ Syn-GFP localized in inclusions, 15 h after induction of  $\alpha$ Syn-GFP expression with galactose (Figure 8C). A Western blot was performed with cell extracts from these strains and showed that co-expression of RPS3A or its truncation forms does not influence  $\alpha$ Syn-GFP expression levels (Figure 8D). Although it has been shown previously that inclusion formation is not a prerequisite for  $\alpha$ Syn-induced toxicity in yeast [23,28,29], we observe here that in the *cog6* $\Delta$ 2 $\alpha$ Syn-GFP strain, toxicity is strongly correlated with the extent of cytosolic inclusion formation and that RPS3A counteracts this. As for the suppression of the  $\alpha$ Syn-induced growth defect, deletion of the 50 N-terminal amino acids abolishes the effect, whereas RPS3A with 101 C-terminal amino acids deleted retains much of the activity against inclusion formation.



**Figure 6** Plasmids pRPS3A (mouse) and pRPS1A and pRPS1B (yeast) complement synthetic lethality of *rps1aΔ rps1bΔ* double deletion, but only pRPS3A suppresses the  $\alpha$ Syn-induced growth defect

(A) Heterozygous diploid strain *rps1aΔ/RPS1A rps1bΔ/RPS1B* was obtained by crossing *rps1aΔ* with *rps1bΔ*. After transformation of this strain with pRPS3A, sporulation and tetrad dissection, the segregants were streaked on to YPD plates with geneticin and SD plates with 5-FOA. (B) *rps1aΔ rps1bΔ* cells carrying pRPS1A or pRPS1B were obtained by tetrad dissection of transformed heterozygous diploids. A growth assay on SD-Ura plates was performed with the indicated strains as described in the Experimental section. *M.m.*, *Mus musculus*; *S.c.*, *Saccharomyces cerevisiae*. (C) Strain *cog6Δ*2 $\alpha$ Syn and the control strain not expressing  $\alpha$ Syn were transformed with the indicated plasmids. A growth assay was performed on SC-Ura-Leu-His galactose plates, as described in the Experimental section. (D) Growth curves were determined in SC-Ura-Leu-His galactose (2% w/v) medium, using a Bioscreen C device as described in the Experimental section. Strains: *cog6Δ*2 $\alpha$ Syn + empty vector (●), pRPS3A (■), pRPS1A (▲), pRPS1B (△).

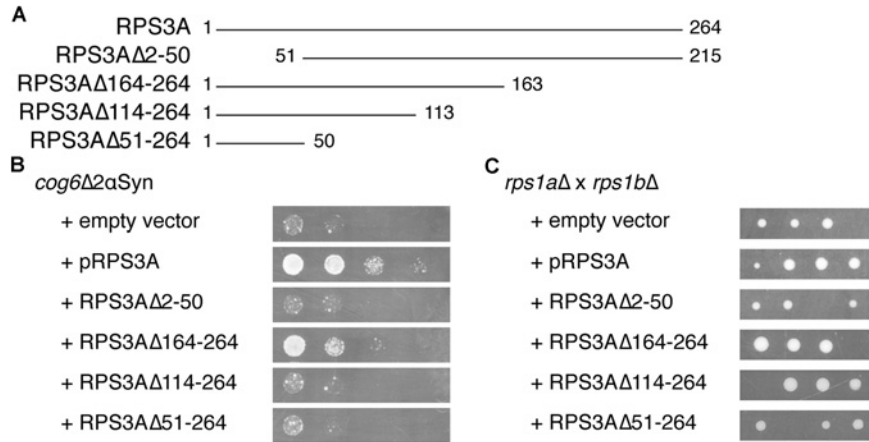
## DISCUSSION

### The *cog6Δ*2 $\alpha$ Syn screening system was used to identify mammalian brain proteins that suppress $\alpha$ Syn toxicity in yeast

Efficient screening of the mouse brain cDNA library in yeast depended on a strong  $\alpha$ Syn-induced toxicity phenotype. To avoid excessive overexpression of  $\alpha$ Syn, we opted for using a deletion mutant with increased sensitivity to  $\alpha$ Syn overexpression. In our hands, the strains *dpp1Δ*, *opi3Δ*, *sod2Δ*, *tgl2Δ* and *vps24Δ* were only slightly or not at all sensitive to  $\alpha$ Syn expressed from the *GAL1* promoter on a multi-copy plasmid, in contrast with what was observed in a previously reported screening of the yeast deletion collection [12]. The sensitivity of the strains *cog6Δ* and *vps52Δ* to  $\alpha$ Syn expression was much higher, and integration of two galactose-inducible copies of WT human  $\alpha$ Syn in the strain *cog6Δ* (*cog6Δ*2 $\alpha$ Syn) resulted in a sufficiently strong growth defect allowing efficient screening of the brain cDNA library for suppressors of  $\alpha$ Syn-induced toxicity. The advantage in using such a sensitive strain for the cDNA screening is that a toxicity

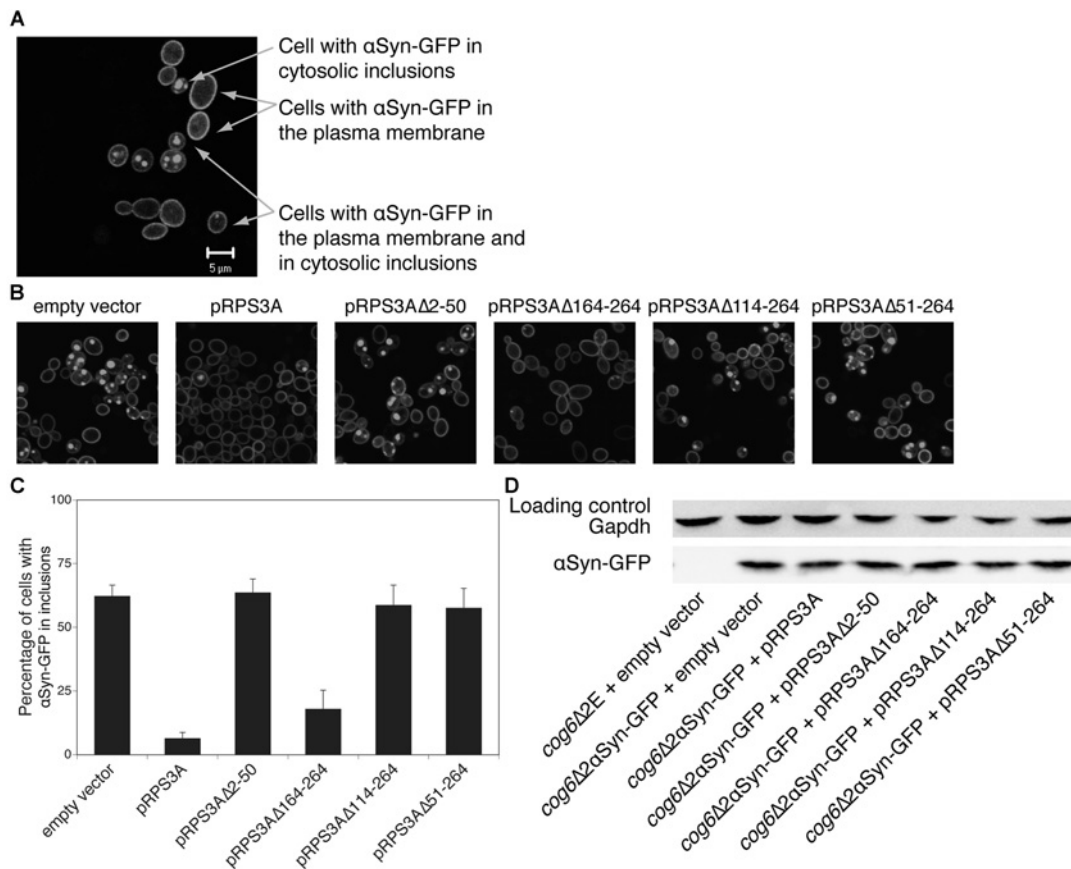
phenotype can be obtained with much lower  $\alpha$ Syn expression levels. In this way, we can avoid isolating suppressors that may just counteract toxicity caused by a high overload of foreign protein or due to irrelevant toxicity caused by very high  $\alpha$ Syn levels. Cog6 is a non-essential component of the COG complex, a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments [30]. Cog6 is proposed to mediate the interaction of the COG complex with t-SNARE (target SNARE) proteins [31,32]. Apparently, the Cog6 protein plays an essential role in the resistance of WT *S. cerevisiae* cells to  $\alpha$ Syn toxicity. The fact that we did not observe  $\alpha$ Syn-induced lethality in a WT yeast strain with two galactose-inducible copies of  $\alpha$ Syn integrated, whereas Outeiro and Lindquist [24] describe a strong growth defect, can be explained by differences in the  $\alpha$ Syn expression levels due to different chromosomal integration sites, or by differences in the genetic background of the strains (BY4742 instead of W303-1A).

Transforming the *cog6Δ*2 $\alpha$ Syn strain with the mouse-brain-specific cDNA library, elimination of false positives using



**Figure 7** The 50 N-terminal amino acids of RPS3A are essential for its suppression effect on  $\alpha$ Syn toxicity; truncation of 101 C-terminal amino acids results in residual activity

(A) Schematic representation of the truncated alleles of RPS3A that were cloned into vector pV214 for the present study. (B) Truncation of the 50 N-terminal amino acids of RPS3A abolishes its capacity to counteract  $\alpha$ Syn toxicity in strain *cog6Δ2αSyn*, whereas deletion of the 101 C-terminal amino acids results in residual suppression activity; deletion of larger C-terminal domains (151 or 214 amino acids) results in the absence of  $\alpha$ Syn toxicity suppression. Strain *cog6Δ2αSyn* was transformed with the indicated plasmids and a growth assay was performed on SC–Ura–Leu–His galactose plates as described in the Experimental section. (C) None of the truncated versions of RPS3A can complement synthetic lethality of the *rps1aΔ rps1bΔ* strain. Heterozygous diploid strain *rps1aΔ/RPS1A rps1bΔ/RPS1B* was transformed with the indicated plasmids, transformants were sporulated and tetrad dissection was performed on SD–Ura plates.



**Figure 8** RPS3A and RPS3AΔ164-264 counteract  $\alpha$ Syn–GFP inclusion formation in strain *cog6Δ2αSyn-GFP*

(A) Three  $\alpha$ Syn–GFP localization patterns are observed, i.e. cells with only plasma membrane localization, cells with only cytosolic inclusions of  $\alpha$ Syn–GFP and cells with both localizations. (B) Sample images of GFP localization in *cog6Δ2αSyn-GFP* cells transformed with the indicated plasmids, 15 h after induction on galactose (2% w/v). (C) Approximately 200 cells of each strain were counted and divided according to the presence of inclusions. The effect of the plasmids indicated on the localization pattern is shown in the histograms. Values are means  $\pm$  S.E.M. obtained by repeating the experiment with three independent transformants. (D) Co-expression of RPS3A or one of its truncation forms does not influence  $\alpha$ Syn–GFP protein levels. Western blot analysis, 15 h after induction with galactose (2% w/v). Primary antibodies: (top) anti-yeast Gapdh (produced in mouse) as a loading control; (bottom) anti-human  $\alpha$ Syn (produced in rabbit).



5-FOA and confirmation by retransformation and growth assays resulted in the identification of two suppressors of the  $\alpha$ Syn-induced growth defect: the mouse PSMA2 and RPS3A. One of the isolated cDNA plasmids surprisingly enhanced the growth defect upon retransformation in the *cog6* $\Delta$  $\alpha$ Syn strain, but it had no growth-inhibitory effect in the *cog6* $\Delta$  strain without  $\alpha$ Syn, indicating that the cDNA product apparently enhances  $\alpha$ Syn toxicity. This cDNA encodes a fragment of the Spire homologue 1 protein, containing the 263 C-terminal amino acids (Spir1CT).

A drawback of the use of the *cog6* $\Delta$  $\alpha$ Syn strain for the screening is that some of the hits may not represent suppressors of the  $\alpha$ Syn-induced toxicity, but rather those of the *cog6* $\Delta$ -induced sensitivity. This can be easily controlled by assessing the effects of the isolated suppressors on  $\alpha$ Syn-induced growth defects in yeast strains with a different background. As PSMA2 could only suppress the growth defect of *cog6* $\Delta$  cells, but not of *vps52* $\Delta$ , *sod2* $\Delta$ , *opi3* $\Delta$  or BY2 $\alpha$ Syn cells challenged with DMSO, we had to conclude that PSMA2 is a suppressor of the *cog6* $\Delta$ -induced sensitivity. Apart from the fact that PSMA2 does not alter the  $\alpha$ Syn protein levels in strain *cog6* $\Delta$  $\alpha$ Syn, we have no lead on the mechanism by which PSMA2 counteracts the sensitivity induced by the deletion of *COG6*. Similarly, the C-terminus of Spire homologue 1 could not enhance the mild  $\alpha$ Syn toxicity in BY4742, *sod2* $\Delta$  and *opi3* $\Delta$ , but did increase the growth defect of the BY4742 strain challenged with DMSO in the absence of  $\alpha$ Syn. Plasmid pSpir1CT is thus an enhancer of the lethality in yeast strains challenged with various stresses, but not specific for  $\alpha$ Syn. Kerkhoff et al. [33] describe how overexpression of an N-terminally truncated form of mouse Spire homologue 1, a protein only 37 amino acids shorter than the protein encoded by the cDNA we isolated, in a murine fibroblast 3T3 cell line strongly inhibited the transport of the VSV-G (vesicular stomatitis virus G) protein to the plasma membrane and how a transport step in the exocytosis pathway was blocked. Overexpression of the full-length Spire homologue 1 had no effect [33]. Similarly, a clone encoding the full-length cDNA of Spire homologue 1 did not enhance  $\alpha$ Syn-induced growth inhibition in the *cog6* $\Delta$  $\alpha$ Syn strain (results not shown).

### Mammalian RPS3A suppresses $\alpha$ Syn toxicity and counteracts $\alpha$ Syn-GFP inclusion formation in yeast cells

The ribosome has no well-studied link with  $\alpha$ Syn misfolding or aggregation. However, there are some studies that show a possible link of RPS3A with neurodegeneration. One group found that *Rps3a* gene expression is up-regulated in mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [34]. Furthermore, RPS3A protein levels are significantly higher in the striatum of MPTP- and methamphetamine-treated mice than in control mice [35]. One genetic linkage study claimed that a mutation in *RPS3A*, SNP *rs498055*, is linked to late-onset Alzheimer's disease [36]; however, that conclusion was later contradicted by three other studies [37–39].

In the present study we show that mouse RPS3A (which differs only in two amino acids from human RPS3A) can counteract toxicity caused by human WT  $\alpha$ Syn, in several  $\alpha$ Syn-sensitive yeast deletion mutants, as well as in WT cells treated with DMSO or ZnSO<sub>4</sub>, a strong indication that RPS3A directly affects  $\alpha$ Syn, rather than reducing the sensitivity of the yeast cells. RPS3A was also shown to suppress the yeast growth defect caused by  $\alpha$ Syn A53T. The suppression activity is not related to the synthesis or breakdown of  $\alpha$ Syn, as indicated by Western blot analysis of the  $\alpha$ Syn protein content. We observed that

the most prominent band for RPS3A in Western blot analysis of yeast extracts is approximately 2 kDa smaller than the band detected in mouse brain extracts, which could indicate some post-translational processing in yeast which does not occur in mouse brain cells, or vice versa.

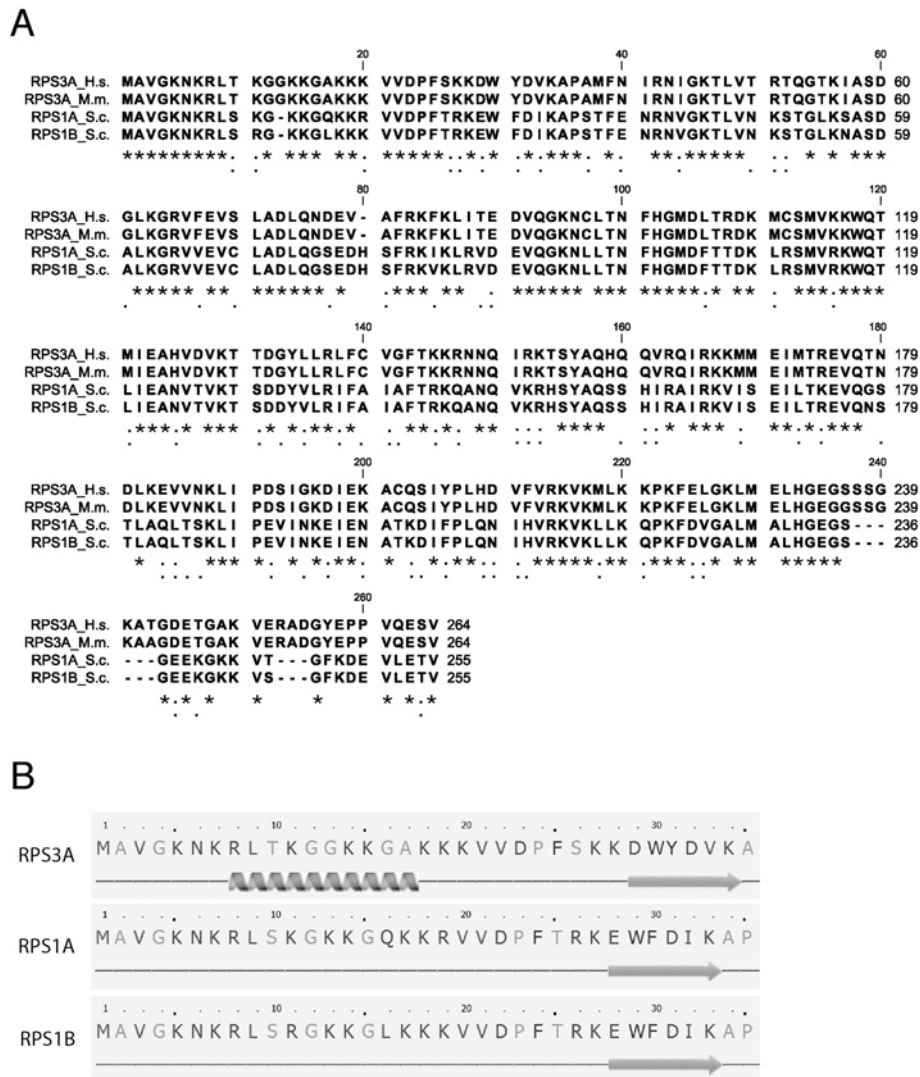
The fact that we failed to reproducibly demonstrate co-immunoprecipitation between RPS3A and  $\alpha$ Syn argues against a tight physical interaction, but it does not necessarily mean that the two proteins do not interact at all. The physical interaction could be too transient in nature to be detected in a co-immunoprecipitation experiment, but still able to induce a structural change in  $\alpha$ Syn.

### RPS3A probably counteracts $\alpha$ Syn aggregation and toxicity through its extraribosomal chaperone function

When overexpressed in hepatocellular carcinoma cells, RPS3A was recently found to exert an extraribosomal chaperoning activity on HBx, preventing its aggregation into inclusions [18]. As we observe that RPS3A strongly counteracts the formation of  $\alpha$ Syn-GFP inclusions in yeast cells, we suggest that the chaperoning function of RPS3A may also prevent the formation of toxic  $\alpha$ Syn species, possibly by stabilization of a much less toxic plasma-membrane-bound species.

Because RPS3A can only partially complement the synthetic lethality of the *rps1a* $\Delta$  *rps1b* $\Delta$  strain and overexpression of yeast Rps1a or Rps1b does not suppress  $\alpha$ Syn toxicity, it seems that the effect on  $\alpha$ Syn is not related to the essential ribosomal function that is also exerted by the yeast homologues, but rather to an additional function that is specific for the mammalian counterpart. Lim et al. [18] found that the 50 N-terminal amino acids of RPS3A are essential for the interaction with and chaperone function on the HBx protein. In our yeast system, we observe that deletion of the 50 N-terminal amino acids abolishes the ability of RPS3A to suppress the  $\alpha$ Syn-induced growth defect and inclusion formation, whereas the deletion of the 101 C-terminal amino acids did not affect suppression activity. When 151 or 214 amino acids are removed from the C-terminus, the suppression activity completely disappears. Hence, we have shown that the 50 N-terminal amino acids are essential for the chaperone function of RPS3A, but they are not sufficient. The fact that pRPS3A $\Delta$ 164-264 has reduced suppression activity compared with full-length pRPS3A could be an indication that the N-terminus of RPS3A does play a role in promoting chaperone activity, or in the stability of the protein in yeast cells. Further evidence uncoupling the chaperone function of RPS3A from its ribosomal function comes from the fact that pRPS3A $\Delta$ 164-263 does not support growth of *rps1a* $\Delta$  *rps1b* $\Delta$  cells, indicating that it cannot function as a ribosomal protein, whereas it does retain an effect on  $\alpha$ Syn aggregation and toxicity.

An alignment of mouse RPS3A with its yeast counterparts RPS1A and RPS1B shows 57% and 58% identities at the amino acid level respectively (Figure 9A). For completeness, the human RPS3A sequence (RPS3A\_H.s., NCBI accession number NP\_000997) was also included in the alignment. It only differs from its mouse homologue in two amino acid positions, both located in the C-terminal part of the protein. The strongest sequence differences between the mammalian and the yeast proteins are in the 24 C-terminal amino acids of RPS3A, which show a very poor alignment, with large gaps. When the amino acid sequences were submitted to the secondary structure prediction program Phyre2 [40], the most prominent difference was in an  $\alpha$ -helical region from Arg<sup>8</sup> to Ala<sup>17</sup> in mammalian RPS3A, which was not predicted in yeast RPS1A or RPS1B (Figure 9B). Since



**Figure 9** Differences in primary and predicted secondary structures of mouse or human RPS3A and yeast RPS1A or RPS1B

(A) Sequence alignment of human (RPS3A\_H.s.), mouse (RPS3A\_M.m.) RPS3A, and yeast RPS1A and RPS1B (RPS1A\_S.c. and RPS1B\_S.c.). (B) Screen shots (only the N-terminal parts) of the result of secondary structure prediction by the 'Protein Homology/AnalogY Recognition Engine' Phyre2 [40] of mouse RPS3A, and yeast RPS1A and RPS1B. A helix under the sequence represents a predicted  $\alpha$ -helix, an arrow represents a predicted  $\beta$ -sheet.

the N-terminal domain of RPS3A is essential for its chaperone function, we can speculate that this predicted  $\alpha$ -helix may play an important role in establishing the chaperone activity.

## Conclusions

The present study has shown that a yeast selection system, employing a specific deletion strain highly sensitive to  $\alpha$ Syn expression, can be used to directly isolate mammalian genes encoding proteins that affect  $\alpha$ Syn-induced toxicity in yeast. One isolated suppressor (PSMA2) and one enhancer (the C-terminus of Spire homologue 1) did not act on  $\alpha$ Syn directly, but mouse ribosomal subunit RPS3A clearly counteracted  $\alpha$ Syn toxicity in all yeast backgrounds tested, suggesting a direct effect on the  $\alpha$ Syn protein, possibly through its recently discovered extraribosomal chaperoning activity [18]. Overexpression of the yeast homologues of RPS3A did not result in suppression, suggesting a unique chaperone function for the mammalian

protein. This illustrates the power of our screening set-up in identifying mammalian-specific effectors of  $\alpha$ Syn phenotypes. The RPS3A clone strongly reduced the formation of  $\alpha$ Syn-GFP inclusions in the *cog6* $\Delta$  $\alpha$ Syn-GFP strain, indicating that it stabilizes an  $\alpha$ Syn species with reduced toxicity and reduced propensity to form inclusions. The 50 N-terminal amino acids of RPS3A are essential for the effects on  $\alpha$ Syn aggregation and toxicity, but this domain alone is not sufficient. Thus far, the functional interaction between RPS3A and  $\alpha$ Syn has only been demonstrated in yeast cells. The next logical step will be to examine whether RPS3A can also influence  $\alpha$ Syn-related phenotypes (such as inclusion formation and toxicity) in mammalian cells, in particular primary neuron cells.

Our discovery of a chaperone protein protecting against  $\alpha$ Syn-induced toxicity suggests the possibility that  $\alpha$ Syn is also toxic in normal human brain cells, but that its toxicity is suppressed by an active chaperone system, composed of RPS3A and possibly other chaperones. When neural cells age, this protective system may slowly degenerate, and/or under environmental stress, the

system may become overloaded, causing  $\alpha$ Syn to become toxic. Overexpression of  $\alpha$ Syn or expression of specific mutant forms of  $\alpha$ Syn, as in familial PD, may put a higher burden on the protective system so that the toxicity, and thus the onset of PD, would start at an earlier age.

## AUTHOR CONTRIBUTION

Stijn De Graeve, Sarah Marinelli, Frank Stolz and Jurgen Vandamme created the strains and plasmids used in the present study. Stijn De Graeve and Sarah Marinelli performed the cDNA library screening, the different yeast growth assays and the protein assays. Stijn De Graeve, Sarah Marinelli and Jurgen Vandamme performed the confocal microscopy experiments, and were advised herein by Jelle Hendrix and Yves Engelborghs. The paper was prepared by Stijn De Graeve, Sarah Marinelli and Johan Thevelein. The project was supervised by Frank Stolz, Patrick Van Dijk and Johan Thevelein.

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