

Characterization of α -synuclein aggregation and synergistic toxicity with protein tau in yeast

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A yeast model was generated to study the mechanisms and phenotypical repercussions of expression of α -synuclein as well as the coexpression of protein tau. The data show that aggregation of α -synuclein is a nucleation–elongation process initiated at the plasma membrane. Aggregation is consistently enhanced by dimethyl sulfoxide, which is known to increase the level of phospholipids and membranes in yeast cells. Aggregation of α -synuclein was also triggered by treatment of the yeast cells with ferrous ions, which are known to increase oxidative stress. In addition, data are presented in support of the hypothesis that degradation of α -synuclein occurs via autophagy and proteasomes and that aggregation of α -synuclein disturbs endocytosis. Reminiscent of observations in double-transgenic mice, coexpression of α -synuclein and protein tau in yeast cells is synergistically toxic, as exemplified by inhibition of proliferation. Taken together, the data show that these yeast models recapitulate major aspects of α -synuclein aggregation and cytotoxicity, and offer great potential for defining the underlying mechanisms of toxicity and synergistic actions of α -synuclein and protein tau.

Aberrant aggregation of specific proteins is a common pathological hallmark of several neurodegenerative disorders. The neuropathology of Parkinson's disease (PD) is marked by fibrillary cytoplasmic inclusions in degenerating dopaminergic neurons. These inclusions contain mainly ubiquitin and α -synuclein and are known as Lewy bodies. α -Synuclein is an abundant, presynaptic protein of 140 residues containing seven imperfect N-terminal repeats, presumed to function in vesicle binding. The middle portion of α -synuclein is a hydrophobic domain, termed non-amyloid component domain, and important in the aggregation of α -synuclein [1]. Three mutations in α -synuclein, A30P, E46K and A53T, are associated with early-onset familial forms of PD, but the mutant proteins show differences in neurotoxicity and physical properties [2–5].

Although the etiology of the common, sporadic form of PD remains unknown, some studies highlight the importance of phosphorylation of α -synuclein at Ser129 and Tyr125 to promote fibril formation [1,6,7].

Alzheimer's disease (AD) is defined by extraneuronal plaques composed of aggregated amyloid ($A\beta$) peptides and by intracellular paired helical filaments and neurofibrillary tangles. In the absence of β -amyloid, paired helical filaments and neurofibrillary tangles are also evident in many other tauopathies, including frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) and Pick's disease [8]. Protein tau is a microtubule-associated protein expressed as six isoforms by differential mRNA splicing, and contains zero or two N-terminal inserts of unknown function and three or four microtubule-binding domains. The

Abbreviations

AD, Alzheimer's disease; EGFP, enhanced green fluorescent protein; FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; GFP, green fluorescent protein; PD, Parkinson's disease; SD, synthetic dextrose; YPD, yeast peptone dextrose.

identification of many exonic and intronic mutations in the tau gene in patients with FTDP-17 established that mutant and even wild-type protein tau is sufficient to cause neurodegeneration and dementia [9,10]. Binding of tau to microtubuli is dynamically controlled by differential expression of isoforms and by reversible phosphorylation of many sites by many different kinases, including GSK-3 β and cdk5 [9,11,12]. Phosphorylation of tau appears to affect its aggregation, as tau is invariably hyperphosphorylated in neurofibrillary tangles isolated from brain of patients with AD or other tauopathies [13]. It is still a matter of debate whether hyperphosphorylation is a cause or a consequence of tangle formation. Moreover, conformational changes in protein tau appear to be essential in the development of tau pathology [14–16].

Considerable overlap in the pathology of AD and PD has been reported. For instance, tau and α -synuclein pathologies were observed in familial AD, in Down's syndrome, in the Lewy body variant of AD (LBVAD), in the parkinsonism–dementia complex of Guam, and in members of the Contursi kindred [17,18]. In transgenic mice, expression of the pathogenic human α -synuclein A53T mutant induced severe motor impairment due to the formation of abundant α -synuclein and tau inclusions in neurons. Furthermore, mice expressing solely human wild-type α -synuclein or human mutant protein tau-P301L did not form inclusions, but only by combined expression in double-transgenic mice, α -synuclein-positive and tau-positive inclusions developed in oligodendrocytes. This suggests that α -synuclein and protein tau directly or indirectly interact with each other [19]. *In vitro*, α -synuclein appeared to interact directly with tau and to stimulate protein kinase A-dependent phosphorylation of tau [20], and both proteins were found to promote mutual fibrilization [21,22].

Different model systems have been developed to study the pathophysiology of neurodegenerative diseases, although no model displays all the hallmarks associated with AD or PD. Apart from transgenic rodents with stable or transient expression of particular proteins engaged in neurodegenerative diseases [23–26], less complex systems and organisms are in use, e.g. mammalian cell lines [27], *Drosophila melanogaster* and *Caenorhabditis elegans* [10,28,29]. Most recently, humanized yeast cells were shown to recapitulate several fundamental aspects related to PD and the pathogenicity of α -synuclein [30] as well as to AD and processing of amyloid precursor protein [31].

We studied the effects of expression of α -synuclein as well as coexpression of protein tau and confirm the effects reported for expression of α -synuclein in yeast

[30]. In addition, we present data supporting the hypothesis that aggregation of α -synuclein is initiated by nucleation at the plasma membrane followed by elongation. Moreover, aggregation is enhanced by treatment with Me₂SO, which in yeast is known to increase levels of phospholipids to form new membranes. Finally, treatment of yeast cells with ferrous ions triggered not only the formation of reactive oxygen species but also increased aggregation of α -synuclein. Further data support the hypothesis that degradation of α -synuclein occurs by autophagy and proteasomes and that aggregation of α -synuclein interferes with endocytosis. As in double-transgenic mice, the coexpression of α -synuclein and protein tau was synergistically toxic in yeast cells. The combined data underline the potential offered by these yeast models for defining the underlying mechanisms of toxicity and synergistic actions of α -synuclein and protein tau.

Results and Discussion

α -Synuclein creates amyloid-like aggregates in yeast

To study the pathology induced by α -synuclein in a well-defined cellular model system, we expressed the wild-type α -synuclein (wt-synuclein) and the clinical mutant proteins A30P and A53T in the W303-1A wild-type yeast strain and its isogenic *pho85 Δ* mutant. *PHO85* encodes the orthologue of human cdk5, a cyclin-dependent kinase known to play a central role in neurodegeneration [32,33] that is found in Lewy bodies [34,35]. We initially used high-copy-number plasmids whereby the expression of α -synuclein was under control of the strong constitutive *TPII* promoter. Consistent with a previous report [30], this approach yielded moderate and comparable expression levels of wt-synuclein and the A53T mutant and much higher levels of the A30P mutant in both wild-type and the *pho85 Δ* mutant cells (Fig. 1A). Lower expression of wt-synuclein and the A53T mutant could result from selective pressure to reduce average plasmid copy numbers to avoid a possible toxic effect [30]. However, we did not observe significant differences in growth between wild-type and *pho85 Δ* cells overexpressing α -synuclein, not even under more demanding growth conditions (Fig. 1B). To circumvent the possibility of counter-selection, we constructed centromeric plasmids that express α -synuclein or C-terminal α -synuclein-enhanced green fluorescent protein (EGFP) fusion proteins under the control of the inducible *MET25* promoter, which is induced by depletion of methionine from the medium and is

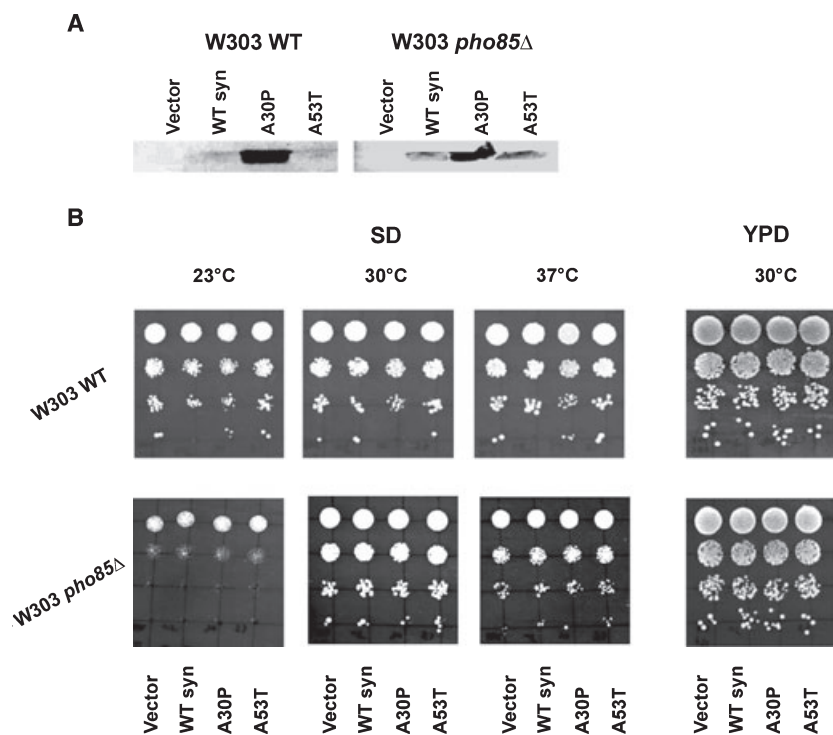


Fig. 1. Expression of human α -synuclein in *Saccharomyces cerevisiae*. (A) Western blot analysis and (B) growth of wild-type or isogenic *pho85* Δ cells overexpressing native wild-type (WT syn) or mutant (A30P, A53T) α -synuclein from the constitutive *TPI1* promoter. The strains were grown on SD medium until early exponential phase. Equal amounts of cells were sampled for immunodetection or for spot assays to monitor growth on selective (SD) or rich (YPD) medium at 23 °C, 30 °C or 37 °C as indicated.

repressed by methionine concentrations exceeding 0.3 mM. Even when the transformants were selected in media containing 1 mM methionine, the concentrations of wt-synuclein and the mutant proteins after derepression in methionine-free medium were similar for native and EGFP-fusion proteins and comparable to those obtained with the *TPI*-controlled expression system (Fig. 2A, and data not shown). Furthermore, all transformants remained viable after derepression and displayed similar growth curves to strains transformed with empty vectors (data not shown). Hence, we concluded that expression of α -synuclein did not induce toxicity in our strains.

Consistent with previously reported data [30], the EGFP-fusion proteins of both wt-synuclein and the A53T mutant were localized at the membrane during the first hours after induction. Interestingly, the proteins started to aggregate upon prolonged induction, and small inclusions became visible, located close to the membrane (Fig. 2B). The inclusions often transformed into larger cytoplasmic aggregates in up to 5% of wild-type cells and about 10% of *pho85* Δ mutant cells when the cultures reached the late exponential phase. The A30P mutant, on the other hand, was exclusively located in the cytoplasm and did not give rise to any inclusions (Fig. 2C), which is consistent with the observation of reduced vesicle binding of the A30P mutant in other models [36]. It is evidently also

in line with the reduced affinity of the A30P mutant for membranes and lipid surfaces [2,37,38]. Interestingly, coexpression of native wt-synuclein with the A30P-EGFP fusion protein resulted in the formation of inclusions containing A30P-synuclein (Fig. 2C). This indicates that the A30P mutant can form inclusions on the nuclei provided by wt-synuclein, demonstrating that the A30P mutant is mainly defective in nucleation. Furthermore, we noted that cells expressing wt-synuclein contained less, but larger inclusions per cell than the A53T mutant (Fig. 2C), suggesting that wt-synuclein nucleates less efficiently than the A53T mutant and that elongation is primarily determined by the availability and distribution of the remaining protein over the preformed nuclei. Therefore, the data provide additional evidence for the nucleation-elongation hypothesis, which was formulated on the basis of *in vitro* aggregation studies with cell-free extracts as well as purified recombinant α -synuclein, demonstrating that membrane-bound α -synuclein has a higher tendency to aggregate than the free cytosolic form and that nuclei act as seeds [3,4,39].

To rule out the possibility that aggregation was influenced by the presence of the EGFP tags and to demonstrate the presence of β -sheeted aggregates, we performed thioflavin-S staining and analysed in parallel *pho85* Δ cells expressing tagged or untagged native synuclein. Staining was carried out on sphero-

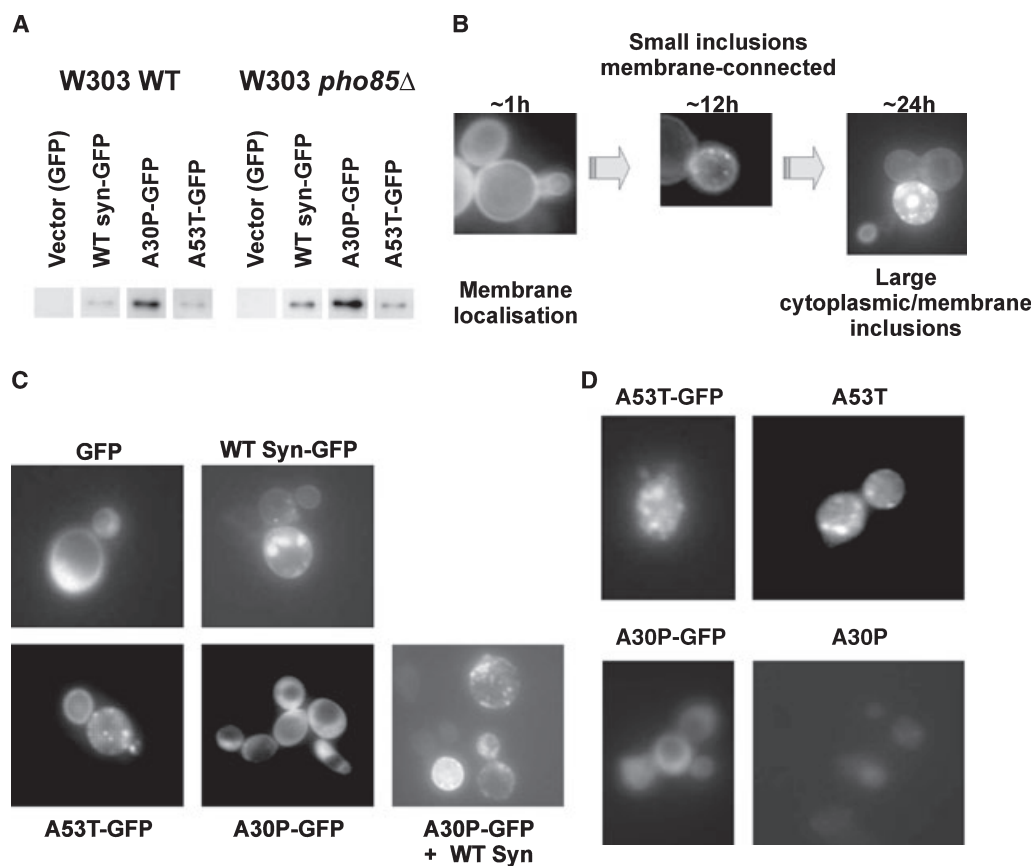


Fig. 2. Expression of α -synuclein–EGFP fusion proteins and the formation of inclusions. (A) Western blot analysis of wild-type or isogenic *pho85Δ* cells expressing C-terminal EGFP fusions of wild-type (WT syn) or mutant (A30P, A53T) α -synuclein under the control of the inducible *MET25* promoter. (B) Time-dependent and expression-dependent redistribution of wt-synuclein–EGFP fusion proteins in *pho85Δ* cells after derepression in methionine-free SD medium for 1 h, 12 h or 24 h, monitored by fluorescence microscopy. As indicated, the fusion protein was initially (1 h) localized at the plasma membrane, but, upon prolonged derepression (12 h), small membrane-connected inclusions became visible, which further (24 h) often converted into larger cytoplasmic inclusions. (C) Cellular localization of EGFP and C-terminal EGFP fusions of wt-synuclein, A53T and A30P after 24 h of derepression. (D) Thioflavin-S staining visualized amyloid-like aggregates in *pho85Δ* cells overexpressing either native or EGFP-fused mutant A53T synuclein in contrast with cells overexpressing native or EGFP-fused mutant A30P synuclein.

plasts, as intact yeast cells are not permeable to thioflavin-S. Spheroplasting of cells expressing α -synuclein–EGFP fusion proteins showed, as expected, a considerable decrease in the amount of cells with inclusions, and those cells contained only a few large membrane-disconnected aggregates. Nevertheless, these cytoplasmic inclusions reacted with thioflavin-S. Similarly, large cytoplasmic inclusions were also visible to a comparable extent in spheroplasts created from cells expressing native wt-synuclein (data not shown) or the A53T mutant (Fig. 2D), indicating that these proteins formed amyloid-like aggregates. In contrast, no thioflavin-S staining was found in spheroplasts from cells expressing the A30P mutant either as native or as EGFP-fusion protein (Fig. 2D).

Aggregation of α -synuclein is proportional to its expression level and the lipid content of yeast cells

The results described above confirmed the importance of α -synuclein–membrane interaction in the formation of aggregates. Moreover, recent genome-wide screens performed in yeast linked lipid metabolism to α -synuclein-induced cellular toxicity [40,41]. Therefore, we examined whether enhanced lipid biosynthesis would be sufficient to increase α -synuclein aggregation. This was accomplished by treatment of the yeast cells with Me_2SO , which is known to stimulate lipid biosynthesis and increase phospholipids in their membranes [42].

The addition of Me₂SO to the culture medium up to 10% was not toxic, although growth of all the yeast strains was slower. Staining with DiOC₆ [42] confirmed enhanced plasma and intracellular membrane formation in yeast cells grown in 10% (v/v) Me₂SO for 18 h (Fig. 3A). Moreover, in the strains expressing synuclein, a dramatic enhancement in the number of cells with inclusions was evident (Fig. 3B). Most interestingly, the effect of Me₂SO was not restricted to wt-synuclein and the A53T mutant, as the A30P mutant also formed aggregates when grown in the presence of 10% (v/v) Me₂SO. In all cases, the number of cells containing aggregates was about, or even above, 80% on treatment with 10% (v/v) Me₂SO. Note that treatment for 18 h with 4% Me₂SO also increased the number of cells with inclusions formed by wt-synuclein and the A53T mutant in the wild-type strain, but not for the A30P mutant. Remarkably, the effects triggered by 4% (v/v) Me₂SO were more pronounced in the *pho85Δ* deletion strain, and even the A30P mutant still formed aggregates under these conditions (Fig. 3B). Besides the very pronounced effect on the number of cells with inclusions, Me₂SO significantly increased the size of the inclusions formed by wt-synuclein and by both mutants (data not shown). In addition, Me₂SO increased up to sixfold the amount of α -synuclein protein detected by western blotting in wild-type cells and up to threefold in *pho85Δ* cells (Fig. 3C). This can be ascribed to enhanced synthesis of α -synuclein as Me₂SO stimulates derepression of the *MET25* promoter [42], although the Me₂SO-induced aggregation may also reduce the turnover of α -synuclein by preventing its degradation.

α -Synuclein is eliminated by proteasomal degradation and by rapamycin-induced autophagy

The finding that some forms of inherited PD are caused by mutations in E3 ubiquitin ligase (Parkin) and ubiquitin carboxyl-terminal hydrolase L1 indicates that proteasomal dysfunction contributes to the pathogenesis of PD [1]. A number of studies investigated the effect of proteasomal inhibition on α -synuclein degradation with conflicting results, i.e. α -synuclein appeared not to be subject to proteasomal degradation [43], whereas others reported that proteasomal inhibition triggered accumulation and aggregation of α -synuclein [44] and even that α -synuclein inhibited the proteasome [45].

We tested the effect of the proteasome inhibitor, lactacystin, on the formation of wt-synuclein inclusions in *pho85Δ* cells. As lactacystin cannot penetrate

intact yeast cells, the experiments were performed on spheroplasts. Incubation of spheroplasts for 4 h with 50 μ M lactacystin increased the number of cells with inclusions from about 10% to more than 40% (Fig. 4A), showing that the proteasome is also actively involved in synuclein turn-over in yeast cells.

Another potential pathway for clearance of aggregate-prone proteins involves degradation via autophagy, a process involving the formation of autophagosomes and their subsequent delivery to the vacuole in yeast [46] or the lysosome in mammals [47]. A role for autophagy has been well documented for Huntington disease [48], but some observations suggested that it also has a role in PD [49]. As in mammalian cells, autophagy in yeast is induced by rapamycin-dependent inhibition of the Tor kinases [46].

Incubation of wild-type cells or *pho85Δ* cells over-expressing α -synuclein-EGFP with 50 nM rapamycin for 30 min almost completely inhibited the formation of inclusions (Fig. 4B). Moreover, rapamycin almost completely annihilated the strong inducing effect of Me₂SO on the formation of α -synuclein inclusions in a concentration-dependent manner, i.e. treatment of wild-type cells with 10% (v/v) Me₂SO together with 50 nM rapamycin reduced the number of cells with inclusions from 80% to 20% (Fig. 4C). Analysis by western blot consistently revealed a dramatic decrease in α -synuclein concentrations in rapamycin-treated cells (Fig. 4D). Remarkably, *pho85Δ* cells appeared to be less sensitive to rapamycin treatment than wild-type yeast cells as at least 10-fold higher concentrations of rapamycin were required to reduce the number of cells with inclusions after Me₂SO treatment to less than 50%. In addition, wild-type cells expressing either wt-synuclein or mutant α -synuclein responded similarly and with comparable sensitivities, whereas *pho85Δ* cells expressing wt-synuclein responded more markedly than *pho85Δ* cells expressing the A53T mutant or the A30P mutant (Fig. 4C). For the latter, we repeatedly observed an increase in the number of cells with inclusions after treatment with low concentrations of rapamycin. Most likely, this relates to the lower affinity of A30P for lipids and membranous compounds as described above. It should be noted that the differences observed between the two strains were not reflected in or proportional to the expression levels of α -synuclein as determined by Western blot analysis (Fig. 4D).

In conclusion, the data described above demonstrate that the levels of expression and aggregation of α -synuclein in yeast cells are controlled by the proteasome as well as by the autophagocytic pathway.

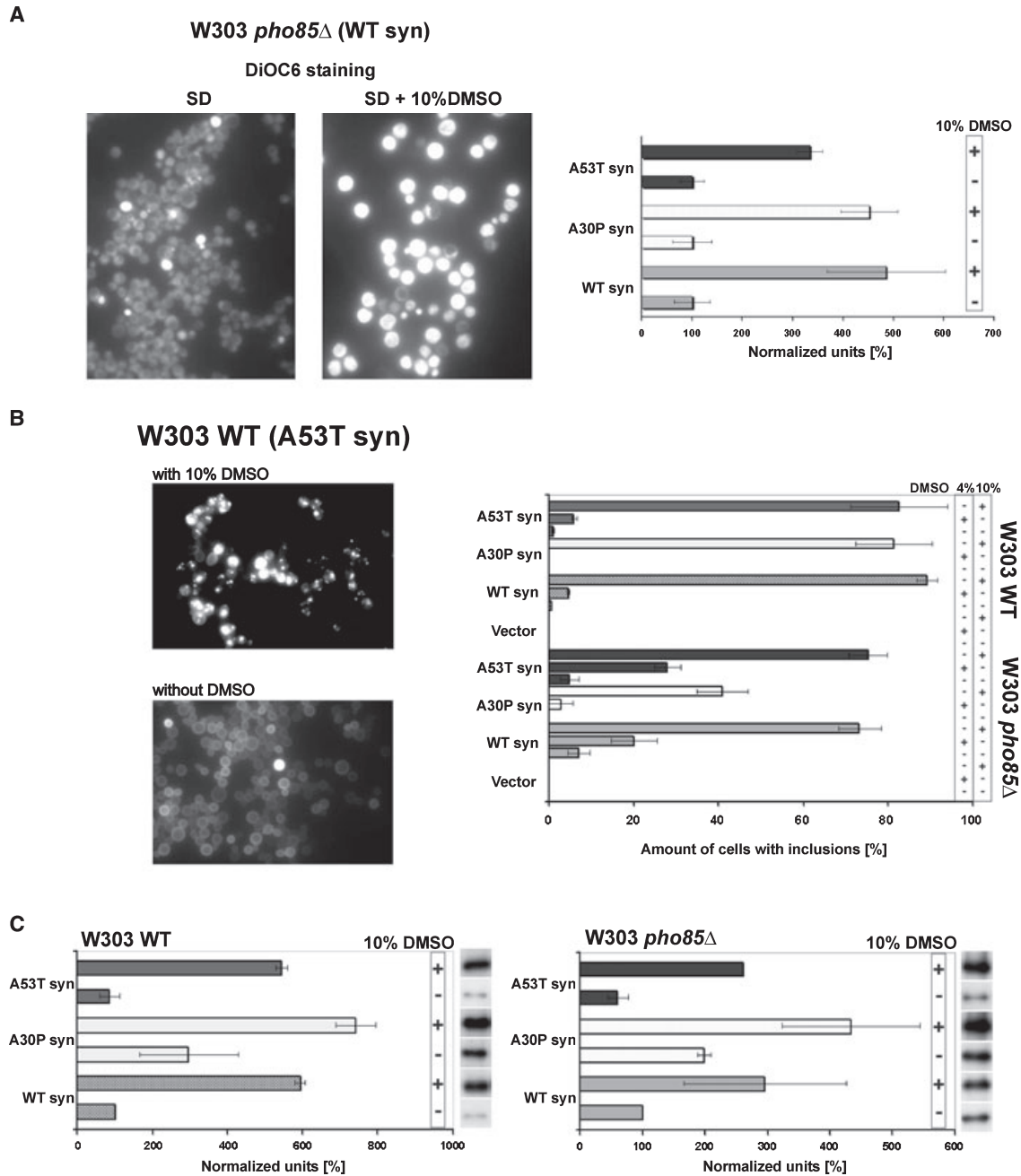


Fig. 3. Multiple effects triggered by Me₂SO treatment of cells overexpressing α -synucleins. (A) *Pho85* Δ cells overexpressing native wt-synuclein, A30P and A53T were grown for 18 h on methionine-free SD medium with or without 10% (v/v) Me₂SO and then stained with the lipophilic dye DiOC₆. The left panel shows typical images of the *pho85* Δ cells overexpressing native wt-synuclein when grown on medium with or without 10% Me₂SO. The right panel shows the quantification where the amount of fluorescence from DiOC₆ obtained with α -synuclein expressing cells grown on medium with 10% (v/v) Me₂SO was normalized to the amount of fluorescence of the same cells grown on medium without Me₂SO. (B) Wild-type and *pho85* Δ cells overexpressing α -synuclein-EGFP fusion proteins were grown on methionine-free SD medium with or without addition of 10% (v/v) Me₂SO for 18 h. Typical images of wild-type cells overexpressing EGFP-fused A53T synuclein are shown in the left panel. The right panel displays the percentage of cells forming inclusions of EGFP-fused wt-synuclein, A30P or A53T in cultures grown in the absence or presence of 4% or 10% Me₂SO. (C) Equal amounts of cells (A₆₀₀) from (B) were sampled for immunodetection with α -synuclein antibodies. Graphs show the proportion of α -synuclein-EGFP fusion normalized to the amount of wt-synuclein expression found in wild-type cells (left panel) or *pho85* Δ cells (right panel) when grown on minimal medium without Me₂SO.

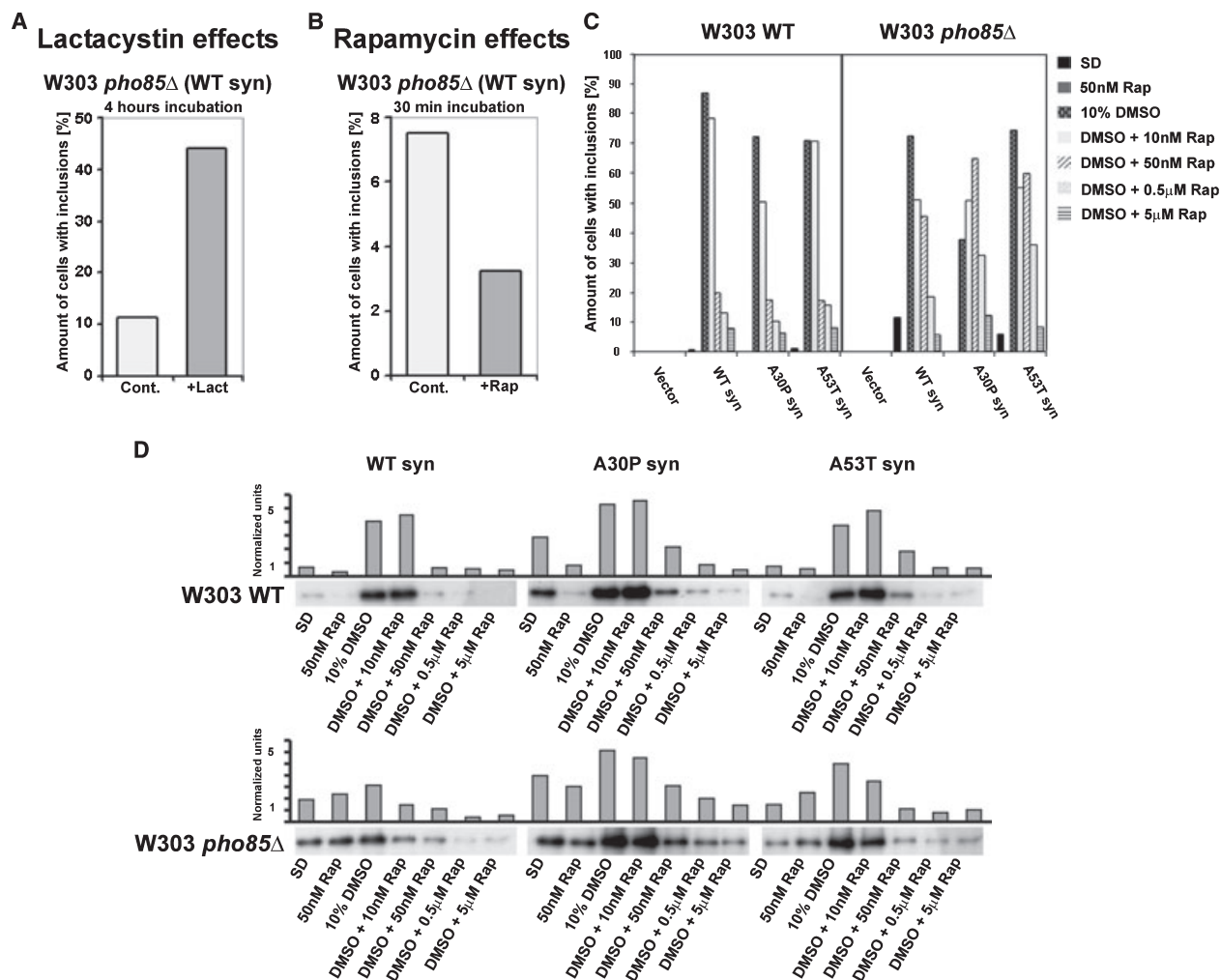


Fig. 4. Inhibition of the proteasome and induction of autophagy influences the expression and aggregation of α -synuclein in yeast. (A) Percentage of spheroplasts with inclusions of EGFP-fused wt-synuclein in spheroplast preparations treated for 4 h with 50 μ M lactacystin (+ lac) or with 1% (v/v) Me₂SO as control (cont.) (B) Yeast strains overexpressing EGFP fusions of wt-synuclein were treated with 50 nM rapamycin (+ Rap) or with 1% (v/v) Me₂SO as control (cont.) for 30 min. The percentage of cells with inclusions of EGFP-fused wt-synuclein was determined by visual inspection of at least 400 cells. (C) Yeast cells expressing EGFP fusions of wt-synuclein, A30P or A53T were grown on methionine-free SD medium with or without 10% Me₂SO and treated with the indicated concentrations of rapamycin. The graph shows the percentage of cells with inclusions based on results of three experiments with independent cultures. (D) Equal amounts of cells from the cultures described in (C) were used to prepare total protein extracts for immunodetection of α -synuclein. All samples were quantified and represented in the bar diagrams showing expression as normalized units relative to the expression of wt-synuclein in untreated wild-type cells, which was set as 1 unit.

α -Synuclein expression in yeast interferes with endocytosis

The observation that *pho85* Δ cells were less responsive to rapamycin was surprising as it has been reported that the *pho85* Δ strain is more sensitive to rapamycin-induced growth inhibition [50] and that Pho85 acts as a negative regulator of autophagy [51]. However, it has also been documented that in *pho85* Δ cells the vacuole, which in yeast

functions analogously to the mammalian lysosome, is enlarged, almost completely transparent, and internally disorganized, and studies on endocytosis of fluorescent dyes such as FM4-64 and LY showed that *pho85* Δ cells are defective in endosomal transport to, and fusion with, the vacuole [50]. In addition, it was reported that overexpression of green fluorescent protein (GFP)-fused α -synuclein caused aberrant accumulation of the dye FM4-64 in yeast cells [30].

We confirmed these phenotypes in our strains, as overexpression of wt-synuclein or A53T mutant led to the accumulation of FM4-64 in many intermediates and retarded transport to the vacuole, a phenomenon

that seemed more pronounced in *pho85 Δ* cells than wild-type cells and that was further aggravated on treatment with 10% (v/v) Me₂SO (Fig. 5A,B). Note that the inclusions formed by wt-synuclein or the

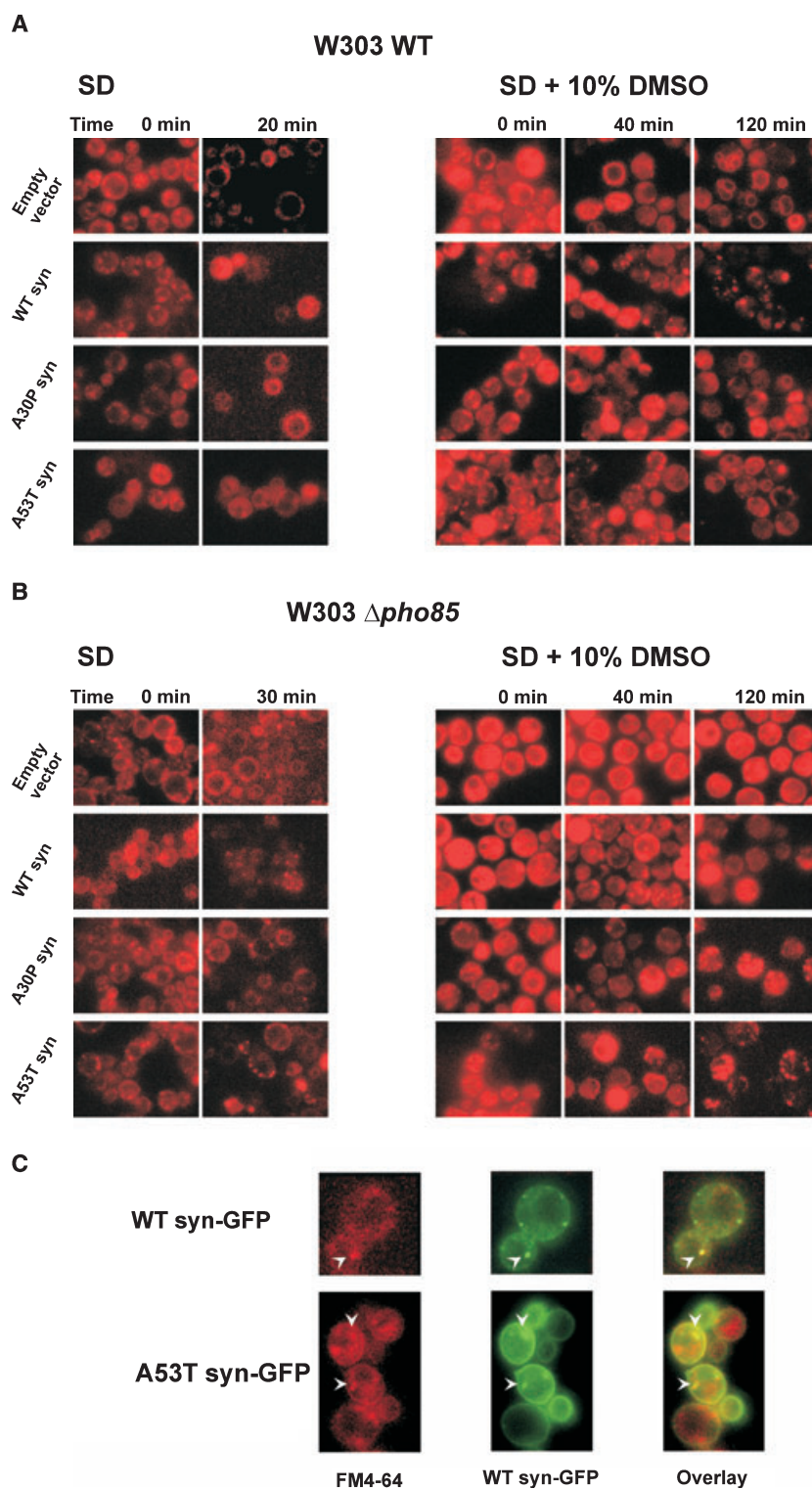


Fig. 5. Aggregation of α -synuclein retards endocytosis. Wild-type (A) or *pho85 Δ* (B) cells overexpressing wt-synuclein-EGFP, A53T-EGFP or A30P-EGFP as well as EGFP alone were grown for 18 h on methionine-free SD medium without or with 10% (v/v) Me₂SO, and stained with FM4-64. Endocytosis of FM4-64 was followed until the control strains, expressing only EGFP, and at least one of the synuclein expression strains showed staining of the vacuolar membrane, except for the *pho85 Δ* cells treated with 10% Me₂SO, which apparently could not reach this stage. (C) Fluorescence of FM4-64 and α -synuclein-EGFP was examined on the same cells using Texas Red and GFP mode settings on a fluorescence microscope. Overlaying of the images was performed with Adobe Photoshop 5.0 software. As illustrated for wild-type cells grown in the absence of Me₂SO, inclusions of wt-synuclein-EGFP and A53T-EGFP colocalized with FM4-64-stained endosomal intermediates (white arrows).

A53T mutant often colocalized with the FM4-64-stained vesicles in both wild-type (Fig. 5C) and *pho85Δ* cells (data not shown). Overexpression of the A30P mutant, which remains cytoplasmic and does not form aggregates, did not affect endocytosis of FM4-64. However, this mutant also caused retardation of endosomal transport when its aggregation was induced by treatment with 10% (v/v) Me₂SO. This correlation suggested that it is actually the aggregation of α -synuclein that disturbs the endocytic pathway. Together, the data also point to the interesting feature that aggregation of α -synuclein is not restricted to the plasma membrane and may also occur on intracellular membranes or, alternatively, that the cells attempt to remove the aggregates formed at, and bound to, the plasma membrane via transport to the vacuole by the endocytic pathway. As the α -synuclein aggregates are first observed at the plasma membrane (Fig. 2B) before becoming localized in the cytoplasm, we favor the last hypothesis.

Iron ions increase oxidative stress and promote α -synuclein aggregation

Recent studies indicated that iron and other metal ions may contribute to the pathology of PD [52,53]. Iron ions are not only identified in Lewy bodies [54], but

in vitro Fe²⁺ ions promote the formation of filamentous aggregates of α -synuclein [55]. More recently, the promoting effect of iron ions on α -synuclein aggregation was documented in SH-SY5Y neuroblastoma cells which overexpress α -synuclein [56,57]. The mechanism of iron-enhanced formation of fibrils remains to be elucidated and may be due to direct alterations in the secondary structure of α -synuclein [55] or to damage caused by oxidative stress through hydroxyl radicals generated via a Fenton-type reaction [57].

We added Fe²⁺ ions to yeast cells to stimulate free radical production [58,59] and monitored the effect on aggregation of α -synuclein. Detection of reactive oxygen species with DHR123 [60] confirmed the increase in free radical production caused by Fe²⁺ ions (Fig. 6A). Simultaneously, a sixfold increase in the number of cells with inclusions of wt-synuclein was observed, despite a slight decrease in the expression level of α -synuclein on western blot (Fig. 6B). In contrast, Fe²⁺ ions did not induce aggregation of the A30P mutant.

We further analysed growth in the presence or absence of Fe²⁺ ions and found that they caused general but not α -synuclein-dependent growth retardation, as cells either expressing α -synuclein or transformed with an empty plasmid displayed similar growth curves (data not shown). The data show that, although Fe²⁺

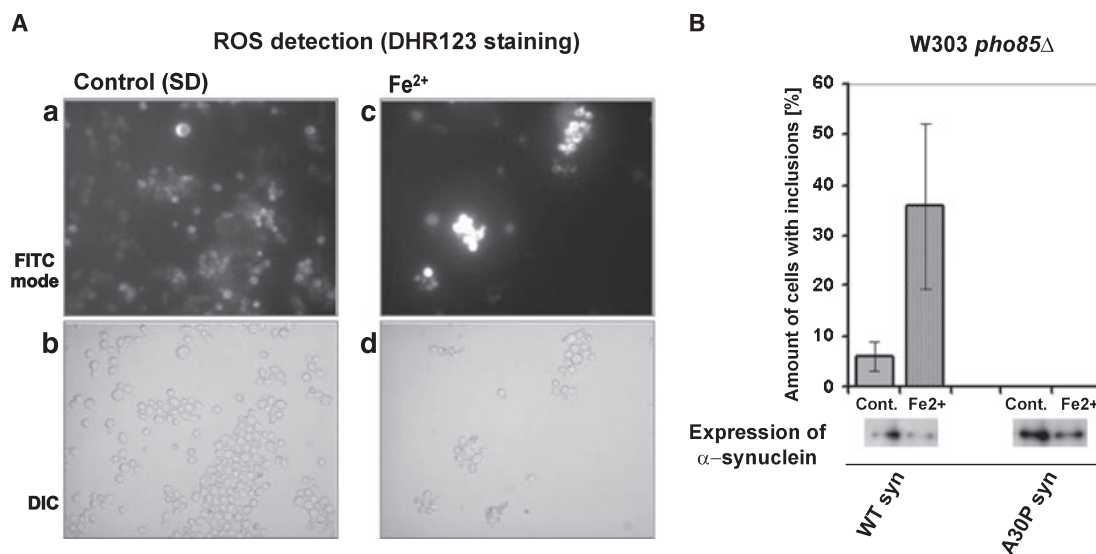


Fig. 6. Ferrous ions increase the formation of inclusions of α -synuclein-EGFP. (A) *Pho85Δ* transformants were grown on methionine-free SD medium for 24 h to induce expression of native wt-synuclein. Subsequently, the culture was split and one half was treated for 30 min with 20 mM FeSO₄, while the other half was treated with 20 mM (NH₄)₂SO₄ as control. The pictures show reactive oxygen species detection in *pho85Δ* cells expressing native wt-synuclein treated with 20 mM (NH₄)₂SO₄ (A,B) or 20 mM FeSO₄ (C,D) as visualized with DHR123 staining and fluorescence microscopy (A,C) and the corresponding digital input computer (DIC) images (B,D). (B) Bar diagram showing the percentages of *pho85Δ* cells expressing wt-synuclein-EGFP (left) or A30P-EGFP (right) that contain inclusions when treated with or without FeSO₄. Equal amounts of cells were also sampled to prepare total extracts for immunodetection of α -synuclein as illustrated below the diagram.

ions induced a surplus of aggregated α -synuclein, this is not toxic to the yeast strains used in this study.

Coexpression of protein tau with α -synuclein increased the α -synuclein toxicity

The absence of any significant effects of α -synuclein expression or aggregation on growth of transformed yeast cells directed us to examine whether coexpression with protein tau would enhance toxicity, as described for double-transgenic mice [19]. Therefore, wild-type α -synuclein or either clinical mutant were coexpressed with the wild-type human tau-2N/4R isoform (wt-tau)

or with tau-P301L mutant (P301L-tau) associated with FDTP-17 [10]. Wild-type yeast cells that combine the expression of wt-tau and α -synuclein displayed a marked reduction in growth in comparison with cells expressing only one of the two proteins (Fig. 7A). In contrast, coexpression of wt-tau with either α -synuclein mutant did not cause a significant growth reduction, although some delay in growth was observed for the A53T mutant. Interestingly, however, the A53T mutant yielded a similar growth-retarded phenotype to wt-synuclein when coexpressed with tau-P301L. The same trend, but more pronounced, was also observed in *pho85 Δ* cells, when growth was monitored on

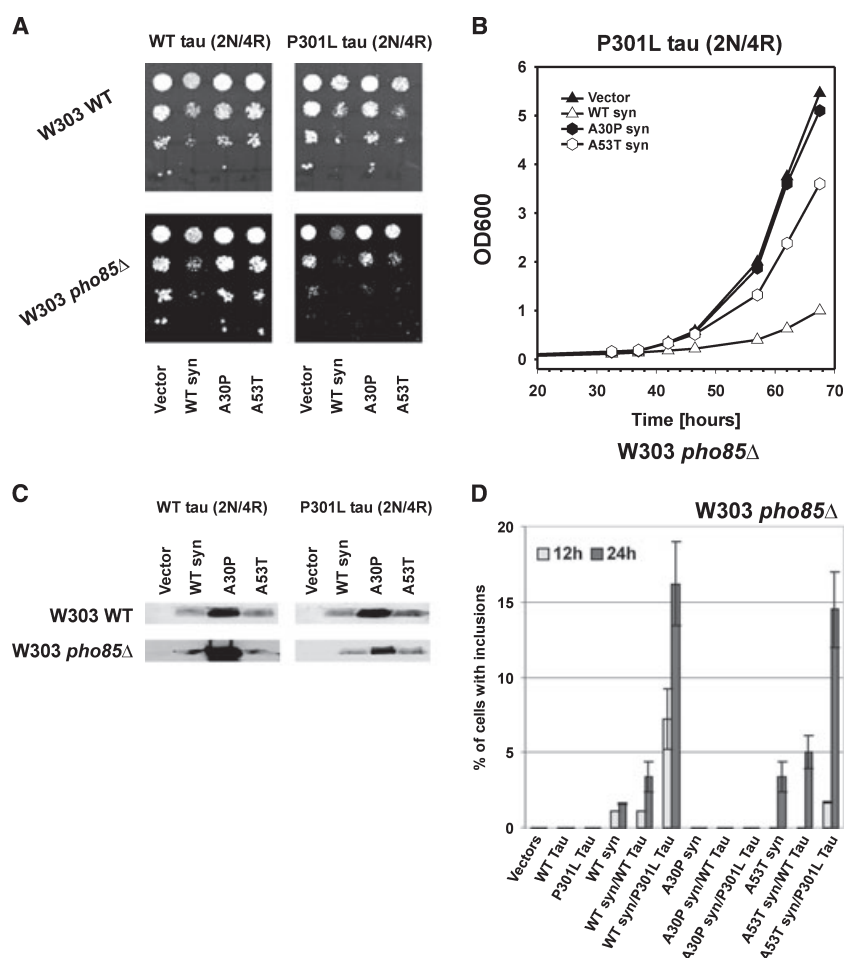


Fig. 7. Coexpression of α -synuclein and human protein tau induces toxicity in yeast. (A) Equal amounts of wild-type cells (upper panels) and *pho85 Δ* (lower panels) cells expressing wt-tau (left) or tau-P301L (right) alone or in combination with wt-synuclein, A30P or A53T were spotted in serial dilutions on SD medium and allowed to grow for 3 days. (B) Growth on liquid SD medium of *pho85 Δ* cells expressing tau-P301L alone (\blacktriangle) or in combination with wt-synuclein (\triangle), A30P (\bullet) or A53T (\circ). (C) Western blot analysis of wild type cells (upper panels) and *pho85 Δ* (lower panels) cells expressing wt-tau (left) or tau-P301L (right) alone or in combination with wt-synuclein, A30P or A53T. (D) Strains expressing various combinations of wild-type or mutant protein tau with wild-type or mutant α -synuclein-EGFP were grown on methionine-free SD medium until early (12 h; light grey bars) and late (24 h; dark grey bars) exponential growth phase, and the percentages of cells with α -synuclein inclusions were determined. The graph presents data from three independent experiments.

synthetic medium (Fig. 7A,B). On rich yeast peptone dextrose (YPD) medium, the *pho85 Δ* cells grew slower at increased temperatures when coexpressing wild-type or A53T α -synuclein with tau-P301L, while the corresponding wild-type cells were growing normally (data not shown).

The cellular levels of α -synuclein were similar in strains overexpressing α -synuclein alone or in combination with wt-tau or tau-P301L, ruling out the possibility that tau protein caused toxicity by increasing α -synuclein expression (Fig. 7C). On the contrary, consistent with the data presented above (Fig. 1), expression of the A30P α -synuclein mutant was always the highest, although no growth retardation was ever noted.

Next, we investigated whether the observed toxicity was related to the increased aggregation of α -synuclein. Therefore, the various strains with combinations of α -synuclein and tau were tested using *MET25* plasmids to express EGFP-tagged versions of α -synuclein. A significant increase in the number of cells that formed aggregates of wt-synuclein or the A53T mutant was noted when coexpressed with wt-tau and even more so when coexpressed with mutant tau-P301L (Fig. 7D). This confirms most recent data obtained *in vitro* as well as in double-transgenic mice *in vivo* [19,21,22], demonstrating that tau promotes the fibrilization of α -synuclein. It should be noted that the number of cells containing α -synuclein aggregates by coexpression of human protein tau was lower than that obtained by treatment with Fe^{2+} ions, for which, however, no growth inhibitory effects were observed. Hence, the toxicity caused by coexpression of α -synuclein and protein tau has to include interference of tau with processes other than those inducing α -synuclein aggregation. In this context, the observation of a more pronounced toxicity in a *pho85 Δ* background is of particular interest given that *PHO85* encodes the yeast orthologue of human *cdk5* [32]. *Cdk5* does not appear to affect phosphorylation of synuclein [61], but this kinase has a well-established role in phosphorylation and paired helical filament formation of protein tau in human brain [8,12,13]. Therefore, it is tempting to speculate that, in yeast also, the toxicity of human tau may depend on its phosphorylation status, which involves *Pho85*.

In conclusion, we have studied the expression of α -synuclein in yeast cells, modulation by inhibition of the proteasome, induction of autophagy, effects induced by Me_2SO and Fe^{2+} ions, and the coexpression of protein tau. The data confirm and extend considerably a recent report on the expression of α -synuclein in yeast [30] and demonstrate that

aggregation of α -synuclein in yeast cells is initiated by nucleation at the plasma membrane. Subsequent further aggregation is enhanced by Me_2SO , which is known to increase the levels of phospholipids and new membranes in yeast [42]. Fe^{2+} ions increased the aggregation of α -synuclein, probably indirectly by increasing oxidative stress, although without marked negative effects on growth or survival of the cells. Importantly, further data demonstrate that degradation of α -synuclein occurs by autophagy and by proteasomes and that the aggregation of α -synuclein interferes with endocytosis. Coexpression of human protein tau with α -synuclein synergistically increased the toxicity for the yeast cells, although the number of cells containing synuclein aggregates was less than obtained with Fe^{2+} ions. The combined data strongly indicate that it is not the aggregation of synuclein *per se* that is responsible for growth retardation or toxicity, and point to interference of synuclein with membranes and endocytic pathways as the major culprit. In fact, the formation of aggregates would be protective to the cells by decreasing the concentrations of active synuclein, as recently claimed for huntingtin [62]. The data underline the potential offered by the yeast models for defining the underlying fundamental mechanisms involved in the normal function, as well as the pathology and synergistic action of α -synuclein and protein tau. Although these mechanisms will ultimately have to be tested in neuronal and animal models, the yeast-based models have the advantage that they are less complex and better genetically defined. An additional benefit is the ease and rapidity with which yeast cells can be manipulated and grown, making them also ideal tools for large-scale screening to identify various factors, including cDNA and peptide libraries or chemical compounds, which may interfere with the pathological action of α -synuclein or protein tau.

Experimental procedures

Yeast strains and media

In this study we used the W303-1A (Mat a *can1-100 ade2-1 his3-11 trp1-1 ura3-1 leu2-3, 112*) wild-type strain and its isogenic *pho85 Δ* mutant. Deletion of *PHO85* was achieved by one-step replacement of the gene with the appropriate PCR deletion cassette containing a *HIS3* or *TRP1* auxotrophic marker as described previously [63].

Transformation of yeast was performed using a standard lithium/poly(ethylene glycol) method [64]. Yeast cells were grown in rich medium (YPD) or selective minimal glucose-containing medium (synthetic dextrose; SD) deficient in the

required amino acids [65]. Transformants with the *MET25*-controlled expression cassettes for native or EGFP-fused α -synuclein were cultured overnight in selective medium with 1 mM methionine. From this preculture, cells were taken to inoculate a second culture (starting $A_{600} = 0.1$) on selective medium without methionine which was then grown for 18 h at 30 °C unless otherwise indicated.

Plasmids

Oligonucleotide primers were used to amplify a full-length α -synuclein sequence from pYX212- α -syn plasmids containing cDNA for wild-type α -synuclein or the mutants A30P and A53T (gift from n.v. ReMYND; <http://www.remynd.com>). The 0.4-kb products were digested with *XbaI* and *SalI* restriction enzymes and ligated in correct orientation into pUG23 plasmid to obtain controlled expression via the *MET25* promoter of the native protein or C-terminally fused α -synuclein-EGFP protein (kindly provided by J. H. Hegemann, Heinrich-Heine-Universität, Düsseldorf, Germany). The multicopy (2 μ) plasmids YEp181- α -syn (WT, A30P and A53T) were generated by ligation of fragments obtained after digestion of the pYX212- α -syn plasmids with *NcoI* and *XhoI* into YEp181 containing a *TP11* promoter. Human tau isoforms [2N/4R tau (wt) and 2N/4R P301L tau] were inserted into the episomal pYX212 plasmid (R&D Systems, Minneapolis, MN) under control of constitutive *TP11* promoter.

Spot assay

Growth was monitored on solid medium by spot assay by making 10-fold serial dilutions of exponentially growing cultures, starting from $A_{600} = 1.0$. Samples of each dilution were spotted on to YPD and SD (without appropriate amino acid for selection) media. Plates were incubated at 23, 30, or 37 °C for 2–5 days as indicated.

Microscopy

Fluorescence microscopy was performed with the Axio-plane2 (Zeiss, Oberkochen, Germany) microscope. Cells grown to mid-exponential or late exponential phase in SD with methionine were washed in medium depleted for methionine, resuspended in the same medium and incubated for 3–24 h at 30 °C to induce production of α -synuclein-EGFP fusion proteins before observation. Cells were placed on to microscopy slides pretreated with 0.1% (w/v) poly(L-lysine). The proportion of cells containing fluorescent inclusions within the population was then determined by inspection of at least 400 cells per culture. Cells were observed with a 100/1.3 (Zeiss Plan-NeoFLUOR oil) objective, and the filter mode with excitation filter BP470/20 and LP515 long pass emission filter (Zeiss) was used.

Thioflavin-S staining and lactacystin-mediated proteasome inhibition

Thioflavin-S staining (Thio S; Sigma) was performed using a modified method of Kimura and coworkers [66]. To prepare spheroplasts, cells from mid-exponential, late exponential, or stationary phase were centrifuged at 420 *g* for 2 min, washed twice with sodium phosphate buffer, pH 6.5, before fixation with 4% formaldehyde for 15 min. Then the cells were washed with 0.1 M sodium phosphate buffer, pH 6.5, supplemented with 1.2 M sorbitol. Cells were resuspended in the same buffer supplemented with 1.2 M sorbitol and 0.002% (v/v) 2-mercaptoethanol, and 200 μ g Zymolyase 100T (ICN) was added. Cells were incubated for 30–60 min at 30 °C, washed three times with 0.1 M sodium phosphate buffer containing 1.2 M sorbitol, and, after addition of 10% (w/v) SDS (20 μ L per 1 mL cells), the spheroplasts were used for staining. Thioflavin-S was added to 0.001% concentration (fresh 1% stock in water) and, after incubation for 10–20 min, the spheroplasts were washed three times with buffer containing 1% gelatin, 0.12 M NaCl and 0.1% (v/v) Tween 20. They were then resuspended in mounting medium [90% (v/v) glycerol with 1 mg·mL⁻¹ *p*-phenylenediamine (pH 9.0)], and placed on to microscopy slides pretreated with 0.1% poly(L-lysine) for observation using the fluorescence microscope.

To determine the effect of proteasome inhibition, cells overexpressing EGFP fusions of wt-synuclein were used for spheroplasting as described above. The spheroplasts were treated with 50 μ M lactacystin or with 1% (v/v) Me₂SO as control for 4 h, and the percentage of spheroplasts with inclusions of EGFP-fused wt-synuclein was determined by inspection of at least 400 spheroplasts under the fluorescence microscope.

DiOC₆, FM4-64 and DHR123 staining

DiOC₆ (3,3'-dihexyloxycarbocyanine iodide; Acros Organics, Geel, Belgium) staining was carried out as described by Block-Alper and coworkers [67] with minor changes. Strains overexpressing native α -synuclein were grown on methionine-containing SD medium for 24 h. Then new cultures were started in selective medium lacking methionine with or without addition of Me₂SO to final concentrations of 4% or 10% (v/v). After growth for 18 h, cells ($A_{600} = 1$) were harvested and washed with 1 mL sterile Tris/EDTA buffer. After centrifugation at low speed, the cell pellet was resuspended in 1 mL Tris/EDTA buffer, and 1 μ L DiOC₆ solution (stock solution 1 mg·mL⁻¹ in ethanol) was added. Cells were mixed and analyzed immediately.

For FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)hexatrienyl]pyridinium dibromide; Molecular Probes) staining, strains overexpressing EGFP-fused synuclein as well as control strains expressing solely EGFP were

grown until late exponential growth phase and examined for inclusion formation under the fluorescence microscope with fluorescein isothiocyanate filter set. Next, the cells were stained with FM4-64 dye for 20 min at 0 °C, washed, and incubated at 15 °C to induce transport of FM4-64 dye to endocytic intermediates. At this point, fluorescence of FM4-64 dye and EGFP was examined under the fluorescence microscope with Texas Red and GFP modes. Overlaying of images was performed with Adobe PHOTOSHOP 5.0 software.

DHR123 staining was as described previously [60].

Antibodies and immunoblot analysis

Western blot analyses were performed as previously described [68] making use of Immobilon-P (Millipore, Billerica, MA) membranes. α -Synuclein was detected with an anti- α -syn rabbit polyclonal antibodies directed against the C-terminus (Sigma). Alkaline phosphatase-conjugated (Santa-Cruz Biotech, Santa Cruz, CA) or horseradish peroxidase-conjugated (Bio-Rad, Hercules, CA) rabbit antibodies were used as secondary antibodies. For detection, the NBT/BCIP or ECL methods (CL-Xposure Film; Pierce Perbio, Bezons, France) were used.

Samples for western blot analysis were performed as follows. Equal amounts of cells (A_{600}) were pelleted by centrifugation, resuspended in 4 vol. SDS/PAGE buffer, and boiled for 5 min at 95 °C. Proteins were then separated by SDS/PAGE. To equilibrate the amount of protein, we compared the intensities obtained from Coomassie blue staining of SDS/polyacrylamide gels or Ponceau S staining of poly(vinylidene difluoride) membranes (Millipore) after transfer of the proteins from the gel.

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References

- 1 Recchia A, Debetto P, Negro A, Guidolin D, Skaper SD & Giusti P (2004) α -Synuclein and Parkinson's disease. *FASEB J* **18**, 617–626.
- 2 Jo E, Fuller N, Rand RP, St George-Hyslop P & Fraser PE (2002) Defective membrane interactions of familial Parkinson's disease mutant A30P alpha-synuclein. *J Mol Biol* **315**, 799–807.
- 3 Lee HJ, Choi C & Lee SJ (2002) Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form. *J Biol Chem* **277**, 671–678.
- 4 Lee HJ & Lee SJ (2002) Characterization of cytoplasmic alpha-synuclein aggregates. Fibril formation is tightly linked to the inclusion-forming process in cells. *J Biol Chem* **277**, 48976–48983.
- 5 Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B *et al.* (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol* **55**, 164–173.
- 6 Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Tokio K & Iwatsubo T (2002) Alpha-synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* **4**, 160–164.
- 7 Ellis CE, Schwartzberg PL, Grider TL, Fink DW & Nussbaum RL (2001) Alpha-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. *J Biol Chem* **276**, 3879–3884.
- 8 Lee VM, Goedert M & Trojanowski JQ (2001) Neurodegenerative tauopathies. *Annu Rev Neurosci* **24**, 1121–1159.
- 9 Spillantini MG & Goedert M (1998) Tau protein pathology in neurodegenerative diseases. *Trends Neurosci* **21**, 428–433.
- 10 Goedert M (2004) Tau protein and neurodegeneration. *Semin Cell Dev Biol* **15**, 45–49.
- 11 Mandelkow E-M & Mandelkow E (1998) Tau in Alzheimer's disease. *Trends Cell Biol* **8**, 425–427.
- 12 Noble W, Olm V, Takata K, Casey E, Mary O, Meyerson J, Gaynor K, LaFrancois J, Wang L, Kondo T *et al.* (2003) Cdk5 is a key factor in tau aggregation and tangle formation *in vivo*. *Neuron* **38**, 555–565.
- 13 Buee L, Bussiere T, Buee-Scherrer V, Delacourte A & Hof PR (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev* **33**, 95–130.
- 14 Garcia-Sierra F, Ghoshal N, Quinn B, Berry RW & Binder LI (2003) Conformational changes and truncation of tau protein during tangle evolution in Alzheimer's disease. *J Alzheimers Dis* **5**, 65–77.
- 15 Weaver CL, Espinoza M, Kress Y & Davies P (2000) Conformational change as one of the earliest alterations of tau in Alzheimer's disease. *Neurobiol Aging* **21**, 719–727.
- 16 Terwel D, Lasrado R, Snauwaert J, Vandeweert E, Van Haesendonck C, Borghgraef P & Van Leuven F (2005) Changed conformation of mutant tau-P301L underlies the moribund tauopathy, absent in progressive, non-lethal axonopathy of tau-4R/2N transgenic mice. *J Biol Chem* **280**, 3963–3973.

- 17 Kurosinski P, Guggisberg M & Gotz J (2002) Alzheimer's and Parkinson's disease: overlapping or synergistic pathologies? *Trends Mol Med* **8**, 3–5.
- 18 Lee VM, Giasson BI & Trojanowski JQ (2004) More than just two peas in a pod: common amyloidogenic properties of tau and alpha-synuclein in neurodegenerative diseases. *Trends Neurosci* **27**, 129–134.
- 19 Giasson BI, Forman MS, Higuchi M, Golbe LI, Graves CL, Kotzbauer PT, Trojanowski JQ & Lee VM (2003) Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science* **300**, 636–640.
- 20 Jensen PH, Hager H, Nielsen MS, Hojrup P, Gliemann J & Jakes R (1999) Alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. *J Biol Chem* **274**, 25481–25489.
- 21 Giasson BI, Lee VM & Trojanowski JQ (2003) Interactions of amyloidogenic proteins. *Neuromolecular Med* **4**, 49–58.
- 22 Kotzbauer PT, Giasson BI, Kravitz AV, Golbe LI, Mark MH, Trojanowski JQ & Lee VM (2004) Fibrillization of alpha-synuclein and tau in familial Parkinson's disease caused by the A53T alpha-synuclein mutation. *Exp Neurol* **187**, 279–288.
- 23 Frenagut PO & Chesselet MF (2004) Alpha-synuclein and transgenic mouse models. *Neurobiol Dis* **17**, 123–130.
- 24 Hutton M, Lewis J, Dickson D, Yen SH & McGowan E (2001) Analysis of tauopathies with transgenic mice. *Trends Mol Med* **7**, 467–470.
- 25 Shimohama S, Sawada H, Kitamura Y & Taniguchi T (2003) Disease model: Parkinson's disease. *Trends Mol Med* **9**, 360–365.
- 26 Van Leuven F (2000) Single and multiple transgenic mice as models for Alzheimer's disease. *Prog Neurobiol* **61**, 305–312.
- 27 Kahle PJ, Neumann M, Ozmen L & Haass C (2000) Physiology and pathophysiology of alpha-synuclein. Cell culture and transgenic animal models based on a Parkinson's disease-associated protein. *Ann NY Acad Sci* **920**, 33–41.
- 28 Bonini NM & Fortini ME (2003) Human neurodegenerative disease modeling using *Drosophila*. *Annu Rev Neurosci* **26**, 627–656.
- 29 Lakso M, Vartiainen S, Moilanen AM, Sirvio J, Thomas JH, Nass R, Blakely RD & Wong G (2003) Dopaminergic neuronal loss and motor deficits in *Caenorhabditis elegans* overexpressing human alpha-synuclein. *J Neurochem* **86**, 165–172.
- 30 Outeiro TF & Lindquist S (2003) Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science* **302**, 1772–1775.
- 31 Edbauer D, Winkler E, Regula JT, Peshold B, Steiner H & Haass C (2003) Reconstitution of gamma-secretase activity. *Nat Cell Biol* **5**, 486–488.
- 32 Huang D, Patrick G, Moffat J, Tsai LH & Andrews B (1999) Mammalian Cdk5 is a functional homologue of the budding yeast Pho85 cyclin-dependent protein kinase. *Proc Natl Acad Sci USA* **96**, 14445–14450.
- 33 Shelton SB & Johnson GV (2004) Cyclin-dependent kinase-5 in neurodegeneration. *J Neurochem* **88**, 1313–1326.
- 34 Nakamura S, Kawamoto Y, Nakano S, Akiguchi I & Kimura J (1997) p35^{nck5a} and cyclin-dependent kinase 5 colocalize in Lewy bodies of brains with Parkinson's disease. *Acta Neuropathol (Berl)* **94**, 153–157.
- 35 Takahashi M, Iseki E & Kosaka K (2000) Cyclin-dependent kinase 5 (Cdk5) associated with Lewy bodies in diffuse Lewy body disease. *Brain Res* **862**, 253–256.
- 36 Jensen PH, Nielsen MS, Jakes R, Dotti CG & Goedert M (1998) Binding of alpha-synuclein to brain vesicles is abolished by familial Parkinson's disease mutation. *J Biol Chem* **273**, 26292–26294.
- 37 Perrin RJ, Woods WS, Clayton DF & George JM (2000) Interaction of human alpha-Synuclein and Parkinson's disease variants with phospholipids. Structural analysis using site-directed mutagenesis. *J Biol Chem* **275**, 34393–34398.
- 38 Bussell R Jr & Eliezer D (2004) Effects of Parkinson's disease-linked mutations on the structure of lipid-associated alpha-synuclein. *Biochemistry* **43**, 4810–4818.
- 39 Wood SJ, Wypych J, Steavenson S, Louis JC, Citron M & Biere AL (1999) Alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. *J Biol Chem* **274**, 19509–19512.
- 40 Scherzer CR, Jensen RV, Gullans SR & Feany MB (2003) Gene expression changes presage neurodegeneration in a *Drosophila* model of Parkinson's disease. *Hum Mol Genet* **12**, 2457–2466.
- 41 Willingham S, Outeiro TF, DeVit MJ, Lindquist SL & Muchowski PJ (2003) Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. *Science* **302**, 1769–1772.
- 42 Murata Y, Watanabe T, Sato M, Momose Y, Nakahara T, Oka S & Iwahashi H (2003) Dimethyl sulfoxide exposure facilitates phospholipid biosynthesis and cellular membrane proliferation in yeast cells. *J Biol Chem* **278**, 33185–33193.
- 43 Ancolio K, Alves da Costa C, Ueda K & Checler F (2000) Alpha-synuclein and the Parkinson's disease-related mutant Ala53Thr-alpha-synuclein do not undergo proteasomal degradation in HEK293 and neuronal cells. *Neurosci Lett* **285**, 79–82.
- 44 McNaught KS, Bjorklund LM, Belizaire R, Isacson O, Jenner P & Olanow CW (2002) Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. *Neuroreport* **13**, 1437–1441.
- 45 Lindersson E, Beedholm R, Hojrup P, Moos T, Gai W, Hendil KB & Jensen PH (2004) Proteasomal inhibition

- by alpha-synuclein filaments and oligomers. *J Biol Chem* **279**, 12924–12934.
- 46 Noda T, Suzuki K & Ohsumi Y (2002) Yeast autophagosomes: *de novo* formation of a membrane structure. *Trends Cell Biol* **12**, 231–235.
- 47 Klionsky DJ & Emr SD (2000) Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717–1721.
- 48 Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O’Kane CJ & Rubinsztein DC (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* **36**, 585–595.
- 49 Webb JL, Ravikumar B, Atkins J, Skepper JN & Rubinsztein DC (2003) Alpha-synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* **278**, 25009–25013.
- 50 Huang D, Moffat J & Andrews B (2002) Dissection of a complex phenotype by functional genomics reveals roles for the yeast cyclin-dependent protein kinase Pho85 in stress adaptation and cell integrity. *Mol Cell Biol* **22**, 5076–5088.
- 51 Wang Z, Wilson WA, Fujino MA & Roach PJ (2001) Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of cAMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Mol Cell Biol* **21**, 5742–5752.
- 52 Gotz ME, Double K, Gerlach M, Youdim MB & Riederer P (2004) The relevance of iron in the pathogenesis of Parkinson’s disease. *Ann NY Acad Sci* **1012**, 193–208.
- 53 Kaur D & Andersen J (2004) Does cellular iron dysregulation play a causative role in Parkinson’s disease? *Ageing Res Rev* **3**, 327–343.
- 54 Castellani RJ, Siedlak SL, Perry G & Smith MA (2000) Sequestration of iron by Lewy bodies in Parkinson’s disease. *Acta Neuropathol (Berl)* **100**, 111–114.
- 55 Uversky VN, Li J & Fink AL (2001) Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson’s disease and heavy metal exposure. *J Biol Chem* **276**, 44284–44296.
- 56 Hasegawa T, Matsuzaki M, Takeda A, Kikuchi A, Akita H, Perry G, Smith MA & Itoyama Y (2004) Accelerated alpha-synuclein aggregation after differentiation of SH-SY5Y neuroblastoma cells. *Brain Res* **1013**, 51–59.
- 57 Matsuzaki M, Hasegawa T, Takeda A, Kikuchi A, Furukawa K, Kato Y & Itoyama Y (2004) Histochemical features of stress-induced aggregates in alpha-synuclein overexpressing cells. *Brain Res* **1004**, 83–90.
- 58 Avery SV (2001) Metal toxicity in yeast and the role of oxidative stress. *Adv Appl Microbiol* **49**, 111–142.
- 59 Stadler N, Hofer M & Sigler K (2001) Mechanisms of *Saccharomyces cerevisiae* PMA1H⁺-ATPase inactivation by Fe²⁺, H₂O₂ and Fenton reagents. *Free Radic Res* **35**, 643–653.
- 60 Wysocki R & Kron SJ (2004) Yeast cell death during DNA damage arrest is independent of caspase or reactive oxygen species. *J Cell Biol* **166**, 311–316.
- 61 Nakamura T, Yamashita H, Takahashi T & Nakamura S (2001) Activated Fyn phosphorylates alpha-synuclein at tyrosine residue 125. *Biochem Biophys Res Commun* **280**, 1085–1092.
- 62 Arrasate M, Mitra S, Schweitzer ES, Segal MR & Finkbeiner S (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature* **431**, 805–810.
- 63 Wach A, Brachat A, Pohlmann R & Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**, 1793–1808.
- 64 Gietz D, St Jean A, Woods RA & Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**, 1425.
- 65 Kaiser C, Michaelis S & Mitchell A (1994) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- 66 Kimura Y, Koitabashi S, Kakizuka A & Fujita T (2002) Circumvention of chaperone requirement for aggregate formation of a short polyglutamine tract by the co-expression of a long polyglutamine tract. *J Biol Chem* **277**, 37536–37541.
- 67 Block-Alper L, Webster P, Zhou X, Supekova L, Wong WH, Schultz PG & Meyer DI (2002) INO2, a positive regulator of lipid biosynthesis, is essential for the formation of inducible membranes in yeast. *Mol Biol Cell* **13**, 40–51.
- 68 Towbin H, Staehelin T & Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications 1979. *Proc Natl Acad Sci USA* **76**, 4350–4354.