Phosphorylation Modulates Clearance of Alpha-Synuclein Inclusions in a Yeast Model of Parkinson’s Disease

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Abstract

Alpha-synuclein (αSyn) is the main component of proteinaceous inclusions known as Lewy bodies (LBs), the typical pathological hallmark of Parkinson’s disease (PD) and other synucleinopathies. Although αSyn is phosphorylated at low levels under physiological conditions, it is estimated that ~90% of αSyn in LBs is phosphorylated at S129 (pS129). Nevertheless, the significance of pS129 in the biology of αSyn and in PD pathogenesis is still controversial. Here, we harnessed the power of budding yeast in order to assess the implications of phosphorylation on αSyn cytotoxicity, aggregation and sub-cellular distribution. We found that αSyn is phosphorylated on S129 by endogenous kinases. Interestingly, phosphorylation reduced αSyn toxicity and the percentage of cells with cytosolic inclusions, in comparison to cells expressing mutant forms of αSyn (S129A or S129G) that mimic the unphosphorylated form of αSyn. Using high-resolution 4D imaging and fluorescence recovery after photobleaching (FRAP) in live cells, we compared the dynamics of WT and S129A mutant αSyn. While WT αSyn inclusions were very homogeneous, inclusions formed by S129A αSyn were larger and showed FRAP heterogeneity. Upon blockade of αSyn expression, cells were able to clear the inclusions formed by WT αSyn. However, this process was much slower for the inclusions formed by S129A αSyn. Interestingly, whereas the accumulation of WT αSyn led to a marked induction of autophagy, cells expressing the S129A mutant failed to activate this protein quality control pathway. The finding that the phosphorylation state of αSyn on S129 can alter the ability of cells to clear αSyn inclusions provides important insight into the role that this posttranslational modification may have in the pathogenesis of PD and other synucleinopathies, opening novel avenues for investigating the molecular basis of these disorders and for the development of therapeutic strategies.

Introduction

Protein misfolding and aggregation is an unavoidable and widespread problem in biology. Cells evolved a series of quality control mechanisms to ensure overall proteostasis and, in some cases, to exploit the plasticity of diverse conformational states, including those concealed in protein aggregates, as in the case of certain types of prions. In other instances, protein aggregates can be detrimental [1,2]. Protein inclusions made of alpha-Synuclein (αSyn), known as Lewy bodies (LBs) are the pathological hallmark of Parkinson’s disease (PD) and other disorders known as synucleinopathies [3,4]. The normal function of αSyn is still unclear, but it is thought to be involved in the regulation of dopamine neurotransmission, vesicular trafficking and in synaptic function and plasticity [5]. Although αSyn is phosphorylated at low levels under physiological conditions, a striking 90% of αSyn is phosphorylated at S129 (pS129) in LBs [6]. However, the significance of pS129 in the pathogenesis of synucleinopathies is unresolved. While studies in Drosophila melanogaster [7] and transgenic mouse models of PD [8] showed that pS129 αSyn...
Protein aggregation is a common hallmark in neurodegenerative disorders, but is also associated with phenotypic plasticity in a variety of organisms, including yeasts. Alpha-synuclein (αSyn) forms aggregates that are typical of synucleinopathies, and is phosphorylated at S129, but the significance of phosphorylation in the biology and pathophysiology of the protein is still controversial. Exploring the power of budding yeast, we found phosphorylation reduced αSyn toxicity and inclusion formation. While inclusions formed by WT αSyn were homogeneous, those formed by S129A αSyn were larger and heterogeneous. Interestingly, clearance of αSyn inclusions was reduced in cells expressing S129A αSyn, correlating with deficient autophagy activation. The finding that phosphorylation alters the ability of cells to clear αSyn inclusions provides novel insight into the role phosphorylation may have in synucleinopathies, and suggests posttranslational modifications might constitute switches that control the aggregation and clearance of key proteins, opening novel avenues for the development of therapeutic strategies for these devastating disorders.

However, recent observations suggest the UPS may be less relevant in mediating αSyn clearance [25].

Here, we explored the power of yeast genetics in order to gain mechanistic insights into the role of S129 phosphorylation on αSyn biology. Using mutants of αSyn that attempt to mimic either phosphorylated (S129E) or unphosphorylated (S129A or S129G) states αSyn, we found that blocking phosphorylation increases αSyn toxicity and promotes the formation of cytosolic inclusions. Our data are consistent with the involvement of phosphorylation in the clearance of αSyn via autophagy.

Allotrogenic, our study provides insight into the role of S129 αSyn phosphorylation and opens novel avenues for additional studies in higher model systems.

Results

Blockade of αSyn S129 phosphorylation promotes toxicity and inclusion formation

To investigate the effect of αSyn phosphorylation in yeast, we used strains carrying two copies of human SNCA cDNA integrated in the genome, encoding either wild-type (WT), S129A, S129G, or S129E mutant αSyn fused to GFP (αSyn-GFP), in order to block (S129A or S129G) or mimic (S129E) phosphorylation, under the regulation of a galactose inducible promoter (GAL1) (Table 1). These strains were previously described and characterized [42] and display a moderate level of αSyn toxicity compared to strains described in other studies [16,27,28]. Growth and viability (colony-forming units, CFUs) of the different strains were assessed upon induction of αSyn expression (Figure 1A and S1A). We observed an initial lag phase of ~2 hours in cells expressing either WT or S129E αSyn-GFP, compatible with the carbon source switch (Figure S1A). Subsequently, these strains resumed growth and behaved similarly, growing slightly slower that the control cells (Figure 1A).

In contrast, cells expressing the S129A mutant exhibited a longer lag phase of ~6 hours (Figure S1A). During this period, cells expressing S129A αSyn-GFP lost viability, as indicated by propidium iodide (PI) staining of cells where membrane integrity was compromised (Figure 1B). This was confirmed by spotting assays (Figure S1B). In fact, 6 hours after induction of αSyn expression, 13.6 ± 0.2% of the yeast cells expressing S129A αSyn-GFP were PI positive, compared to only 2.1 ± 0.2% or 2.6 ± 0.5% of cells expressing WT or S129E αSyn-GFP, respectively (Figure 1B). Nonetheless, cells expressing S129A αSyn-GFP were able to adapt and to recover growth ~7.5 hours after expression induction, with a similar growth rate to those expressing WT or S129E αSyn-GFP (Figure 1A and S1A). These results suggest that yeast cells have mechanisms that allow them to cope with and to recover from toxicity induced by S129A αSyn. Still, cells expressing the mutant S129A αSyn-GFP failed to reach a final OD equivalent to that observed for cells expressing WT or S129E αSyn-GFP (Figure 1A). Altogether, these results demonstrate that expression of S129A αSyn is more toxic for yeast cells than expression of WT or S129E αSyn.

We then assessed the correlation between cytotoxicity and the subcellular distribution of αSyn-GFP. It was previously described that, initially, αSyn associates with the plasma membrane [16]. Upon increased accumulation of the protein, fluorescent foci appear adjacent to the plasma membrane and, finally, become cytoplasmic inclusions [16,27,41]. We imaged cells expressing the WT, S129A or S129E forms of αSyn-GFP by fluorescence microscopy and counted the percentage of cells displaying inclusions at different times after αSyn expression induction (1.5, 5 and 6 hours) (Figure 2A). Interestingly, in addition to being the...
### Table 1. Strains used in this study.

| Description | Reference |
|-------------|-----------|
| W303-1A     | MATa; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1; ade2-1 | 95 |
| W303-1B     | MATa; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1; ade2-1 | 95 |
| VSY71       | W303-1A trp1-1::pRS304 TRP1++; ura3-1::pRS306 URA3+ | 96 |
| VSY72       | W303-1A trp1-1::pRS304 GAL1pr-SNCA(WT)-GFP TRP1++; ura3-1::pRS306 GAL1pr-SNCA(WT)-GFP::URA3+ | 96 |
| VSY73       | W303-1A trp1-1::pRS304 GAL1pr-SNCA(S129A)-GFP TRP1++; ura3-1::pRS306 GAL1pr-SNCA(S129A)-GFP::URA3+ | 96 |
| VSY74       | W303-1A trp1-1::pRS304 GAL1pr-SNCA(S129E)-GFP TRP1++; ura3-1::pRS306 GAL1pr-SNCA(S129E)-GFP::URA3+ | 96 |
| ΔSNCA S129G | W303 trp1-1::pRS304 GAL1pr-SNCA(S129G)-GFP TRP1++; ura3-1::pRS306 GAL1pr-SNCA(S129G)-GFP::URA3+ | This study |
| VSY71 pdr5  | VSY71 pdr5::KanMX4 | This study |
| VSY72 pdr5  | VSY72 pdr5::KanMX4 | This study |
| VSY73 pdr5  | VSY73 pdr5::KanMX4 | This study |
| VSY71.Δtag1 | VSY71.Δtag1::KanMX4 | This study |
| VSY72.Δtag1 | VSY72.Δtag1::KanMX4 | This study |
| VSY73.Δtag1 | VSY73.Δtag1::KanMX4 | This study |
| VSY71.Δtag7 | VSY71.Δtag7::KanMX4 | This study |
| VSY72.Δtag7 | VSY72.Δtag7::KanMX4 | This study |
| VSY73.Δtag7 | VSY73.Δtag7::KanMX4 | This study |
| VSY71 mCherry-Δtag8 | VSY71 leu2-3::p305 pATG8 2XmCherry-ATG8 LEU2+ | This study |
| VSY72 mCherry-Δtag8 | VSY72 leu2-3::p305 pATG8 2XmCherry-ATG8 LEU2+ | This study |
| VSY73 mCherry-Δtag8 | VSY73 leu2-3::p305 pATG8 2XmCherry-ATG8 LEU2+ | This study |
| VSY71 Pup1-RFP | VSY71 Pup1::p305PUP1-RFP LEU2+ | This study |
| VSY72 Pup1-RFP | VSY72 Pup1::p305PUP1-RFP LEU2+ | This study |
| VSY73 Pup1-RFP | VSY73 Pup1::p305PUP1-RFP LEU2+ | This study |
| VSY71 Bre5  | VSY71 pAG303GPD BRES HIS3+ | This study |
| VSY72 Bre5  | VSY72 pAG303GPD BRES HIS3+ | This study |
| VSY73 Bre5  | VSY73 pAG303GPD BRES HIS3+ | This study |
| VSY71 Ypt1  | VSY71 pAG303GPD YPT1 LEU2+ | This study |
| VSY72 Ypt1  | VSY72 pAG303GPD YPT1 LEU2+ | This study |
| VSY73 Ypt1  | VSY73 pAG303GPD YPT1 LEU2+ | This study |
| VSY71 Ykt6  | VSY71 pAG303GPD YKT6 LEU2+ | This study |
| VSY72 Ykt6  | VSY72 pAG303GPD YKT6 LEU2+ | This study |
| VSY73 Ykt6  | VSY73 pAG303GPD YKT6 LEU2+ | This study |
| VSY71 Ubp3  | VSY71 pAG303GPD UBP3 LEU2+ | This study |
| VSY72 Ubp3  | VSY72 pAG303GPD UBP3 LEU2+ | This study |
| VSY73 Ubp3  | VSY73 pAG303GPD UBP3 LEU2+ | This study |
| VSY71 Gyp8  | VSY71 pAG303GPD GYP8 LEU2+ | This study |
| VSY72 Gyp8  | VSY72 pAG303GPD GYP8 LEU2+ | This study |
| VSY73 Gyp8  | VSY73 pAG303GPD GYP8 LEU2+ | This study |
| VSY71 Pmr1  | VSY71 pAG303GPD PMR1 LEU2+ | This study |
| VSY72 Pmr1  | VSY72 pAG303GPD PMR1 LEU2+ | This study |
| VSY73 Pmr1  | VSY73 pAG303GPD PMR1 LEU2+ | This study |
| VSY71 Dcp1-DsRed | VSY71 pAG303GPD DCP1-DsRed LEU2+ | This study |
| VSY72 Dcp1-DsRed | VSY72 pAG303GPD DCP1-DsRed LEU2+ | This study |
| VSY73 Dcp1-DsRed | VSY73 pAG303GPD DCP1-DsRed LEU2+ | This study |
| VSY71 Cerulean-Ypt1 | VSY71 pAG303GPD Cerulean-YPT1 LEU2+ | This study |
| VSY72 Cerulean-Ypt1 | VSY72 pAG303GPD Cerulean-YPT1 LEU2+ | This study |
| VSY73 Cerulean-Ypt1 | VSY73 pAG303GPD Cerulean-YPT1 LEU2+ | This study |
| VSY71 Cerulean-Ypt31 | VSY71 pAG303GPD Cerulean-YPT31 LEU2+ | This study |
| VSY72 Cerulean-Ypt31 | VSY72 pAG303GPD Cerulean-YPT31 LEU2+ | This study |
| VSY73 Cerulean-Ypt31 | VSY73 pAG303GPD Cerulean-YPT31 LEU2+ | This study |
| VSY71 Cerulean-Sec4 | VSY71 pAG303GPD Cerulean-SEC4 LEU2+ | This study |
most toxic, the S129A mutant accelerated inclusion formation. At 3 hours post induction, the percentage of cells displaying inclusions was 3-fold higher for cells expressing the S129A mutant when compared to cells expressing WT protein (63.5 ± 1.8% compared to 23.7 ± 3.8%) (Figure 2A). After 6 hours of expression induction almost all cells expressing S129A displayed inclusions (98 ± 0.8%). In turn, the S129E mutation did not significantly affect the formation of aSyn-GFP inclusions (Figure 2A).

To confirm the effects observed with the S129A mutant were due to the inability of the protein for being phosphorylated, and not due to structural differences induced by the alanine residue, we also tested an aSyn mutant carrying an S129G substitution. Importantly, the results were identical to those observed with S129A aSyn-GFP (Figure S2). Thus, for subsequent experiments, we continued using the S129A aSyn-GFP phospho-resistant mutant.

We next assessed whether the differences in toxicity and inclusion formation were due to different expression levels of the different variants of aSyn-GFP tested. Using western blot analyses, we found all variants were expressed at similar levels after 6 hours of expression induction (Figure 2B). By using an antibody that specifically recognizes pS129-aSyn, we observed that human aSyn was phosphorylated on S129 residue by endogenous kinases (Figure 2B). Thus, together with the fact expression of both S129E and WT aSyn-GFP resulted in similar yeast growth phenotypes and percentage of cells with aSyn-GFP inclusions, we concluded that, in our yeast model, the S129E mutation mimics the phosphorylated state of aSyn at S129 (Figure 1 and 2A). For this reason, we continued our study using just WT and S129A aSyn-GFP. We detected species in fractions corresponding to molecular weights of 440 kDa (Figure 2D and S3). Interestingly, for S129A aSyn-GFP, we detected species in fractions corresponding to even higher molecular weights, since these eluted in the void volume of the column (Figure 2D and S3). The distribution of pS129 aSyn-GFP was also analyzed and showed a similar distribution to that of WT aSyn-GFP (Figure 2D). This result convincingly demonstrates, for the first time, that in the yeast model considerable large oligomeric species of aSyn-GFP are formed, an issue that had not been previously resolved in the field.

### Table 1. Cont.

| Description                           | Reference |
|---------------------------------------|-----------|
| VSY72 Cerulean- Sec4 pAG305GPD Cerulean-SEC4 LEU2+ | This study |
| VSY73 Cerulean- Sec4 pAG305GPD Cerulean-SEC4 LEU2+ | This study |
| VSY71 Cerulean-Ypt6 pAG305GPD Cerulean-YPT6 LEU2+ | This study |
| VSY72 Cerulean-Ypt6 pAG305GPD Cerulean-YPT6 LEU2+ | This study |
| VSY73 Cerulean-Ypt6 pAG305GPD Cerulean-YPT6 LEU2+ | This study |
| VSY71 Cerulean-Vps21 pAG305GPD Cerulean- VPS21 LEU2+ | This study |
| VSY72 Cerulean-Vps21 pAG305GPD Cerulean- VPS21 LEU2+ | This study |
| VSY73 Cerulean-Vps21 pAG305GPD Cerulean- VPS21 LEU2+ | This study |
| VSY71 Cerulean-Ypt52 pAG305GPD Cerulean-YPT52 LEU2+ | This study |
| VSY72 Cerulean-Ypt52 pAG305GPD Cerulean-YPT52 LEU2+ | This study |
| VSY73 Cerulean-Ypt52 pAG305GPD Cerulean-YPT52 LEU2+ | This study |
| VSY71 Cerulean-Ypt7 pAG305GPD Cerulean-YPT7 LEU2+ | This study |
| VSY72 Cerulean-Ypt7 pAG305GPD Cerulean-YPT7 LEU2+ | This study |
| VSY73 Cerulean-Ypt7 pAG305GPD Cerulean-YPT7 LEU2+ | This study |
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Modulation of aSyn S129A toxicity by modifiers of ER-to-Golgi trafficking defects

Several studies demonstrated that aSyn may disrupt multiple intracellular trafficking pathways in yeast [16,17,20,26-28,42,43]. However, the endoplasmic reticulum (ER)-to-Golgi vesicular trafficking impairment is one of the first defects following induction of aSyn expression [28]. This defect can be rescued by genes promoting ER-to-Golgi trafficking. In addition, genes negatively regulating ER-to-Golgi trafficking enhance aSyn toxicity [28]. To determine if the increased toxicity observed by the blockade of aSyn phosphorylation was also associated with ER-to-Golgi trafficking defects, we tested the effects of previously described suppressors (Ypt1, Ykt6, Ubp3, and Bre5) and enhancers (Gyp8 and Pmr1) of aSyn toxicity in yeast. These modifiers of aSyn toxicity were co-expressed with either WT or S129A aSyn-GFP and the effect on toxicity and inclusion formation was evaluated 6 hours after induction (Figures 3 and 4). As in our yeast model WT aSyn-GFP is moderately toxic, the suppression of toxicity was not remarkable in the spotting assay but became evident when cells were assessed for PI-staining, a more refined method to determine cell viability (Figure 3). Our findings were consistent with what was previously described [28]. Among the suppressors tested, only Bre5 was not able to rescue S129A aSyn-GFP toxicity in both assays (Figure 3). Expression of Ypt1, Ykt6 and Ubp3 significantly reduced the formation of both WT and S129A aSyn-GFP inclusions (Figure 4A) while the overexpression of Bre5 only decreased the formation of WT aSyn-GFP inclusions, and had no effect on S129A inclusion formation (Figure 4A).

Regarding the two enhancers of aSyn toxicity, Gyp8 and Pmr1, expression of an extra copy of these genes exacerbated WT aSyn toxicity as expected [28], and had a similar effect on S129A aSyn.
Gyp8 and Pmr1 also increased the formation of aSyn inclusions in WT aSyn-GFP expressing cells without affecting the percentage of cells with S129A aSyn-GFP inclusions, which is already high and therefore precludes the ability to detect an additional increase (Figure 4A). To determine if the phenotypes observed resulted from an effect of the modifiers on the levels of WT and S129A aSyn-GFP, we preformed western blot analyses. We found that total aSyn and pS129 levels were not affected by the co-expression of the modifiers (Figure 4B).

Altogether, these results indicate that ER-to-Golgi vesicle trafficking defects associated with S129A aSyn toxicity can also be rescued by suppressors of WT aSyn toxicity.

Blockade of aSyn phosphorylation compromises its degradation

We postulated that the different biochemical properties might arise from differences in protein clearance of the S129A aSyn-GFP. To test this, aSyn expression was stopped after 6 hours by replacing galactose by glucose (to repress the GAL1 promoter) and aSyn-GFP clearance was followed for 6 hours (Figure 5A). We found 49.6 ± 0.4% of cells expressing S129A aSyn-GFP contained inclusions, while only 8.6 ± 1.2% of the ones expressing the WT form presented aSyn-GFP inclusions, after the clearance period (Figure 5B and C). This corresponds to a reduction of about 50% and 70% when compared to the initial amount of cells with inclusions, respectively (Figure 5C). At 0 hours of clearance (6 hours after aSyn expression induction), S129A expressing cells presented fewer inclusions per cell (Figure 5D). These inclusions were larger and more heterogeneous in size than those formed by WT aSyn-GFP (Figure 5E). After 6 hours of clearance, no significant differences in the size of the inclusions formed by WT or S129A aSyn-GFP were detected (Figure 5E), due to an increase in size of the inclusions formed by WT aSyn-GFP compared to zero of clearance (Figure 5E).

To further investigate how phosphorylation affects aSyn inclusion formation and characteristics, cells were analysed by flow cytometry (Figure 5F). At 0 hours of clearance, both strains presented a homogeneous distribution of the GFP fluorescence showing a single population of cells (Figure 5F). However, 6 hours after clearance, two populations were visible for both WT and S129A aSyn-GFP, one with weaker (sub-population a) and one with stronger GFP (sub-population b) signal (Figure 5F). We found that the strain expressing S129A aSyn-GFP accumulated more cells in the population displaying stronger GFP signal (11 ± 3.5% versus 1.4 ± 1.0% for WT aSyn-GFP). Together with the results from the fluorescent microscopy, these findings are consistent with cells accumulating inclusions of different sizes, with the larger inclusions displaying stronger GFP signal (Figure 5F).

Next, we evaluated the protein levels of S129A and WT aSyn-GFP at 0 and 6 hours of clearance by western blot analyses. As expected from the results in Figure 2C, no differences were observed at 0 hours of clearance (Figure 6A). In addition, at 0 hours of clearance, both WT and S129A aSyn-expressing cells presented similar levels of Triton-X-Insoluble protein (TI), 55.37 ± 2.23% and 50.69 ± 9.47%, respectively (Figure 6B). However, 6 hours after clearance, the levels of WT aSyn-GFP (41.93 ± 6.04%) were around 32% below those of S129A aSyn-GFP (73.01 ± 19.45%) (Figure 6B), and this was accompanied by a significant increase of the S129A aSyn-GFP in the TI fraction (69.98 ± 6.11%) (Figure 6B). Importantly, during the clearance period, the levels of pS129 aSyn did not change significantly for the WT aSyn-expressing strain (Figure S4).
Based on the morphological and biochemical differences observed for inclusions formed by WT or S129A aSyn-GFP, we hypothesized that the dynamics of aSyn in the inclusions might differ. To test this, we first used high-resolution 4D imaging in WT and S129A aSyn-expressing cells. Images were acquired every 10 min for 18 hours after induction of aSyn expression (Figure 7 and Supplementary Movie S1 and S2). Initially, WT aSyn-GFP distributed preferentially along the plasma membrane. In contrast, S129A aSyn-GFP formed inclusions sooner than the WT aSyn-GFP (Figure 7). The percentage of cells with inclusions in the population of cells expressing WT or S129A aSyn-GFP was...
determined and the results confirm that S129A mutation in aSyn promotes inclusions formation in yeast (Figure 7B).

To further compare the protein dynamics in the inclusions formed by WT or S129A aSyn-GFP, we next used Fluorescence Recovery After Photobleaching (FRAP) and calculated kinetic parameters based on exponential fitting to the FRAP plots (Figure 8). Under the assumption that aSyn-GFP diffusion is very fast compared both to binding and to the timescale of the FRAP...
experiment, i.e., that binding dominates and diffusion is not detected in the FRAP recovery, an exponential fit to each FRAP recovery curve enables the determination of the aSyn-GFP immobile fraction (IF) and mean residence time (T) (Figure S5A). After 6 hours of aSyn expression induction, the inclusions formed by the WT aSyn-GFP were homogeneous with respect to the relative fluorescence recovery profile (Figure 8A). The residence time for these inclusions was 39.3 ± 0.7 seconds and the immobile fraction, which corresponds to static or long-term bound aSyn-GFP, was 19.3 ± 7.9% (Figure S5A). In contrast, the inclusions formed in cells expressing S129A aSyn-GFP were heterogeneous, and could be distinguished in three main groups, based on their FRAP recovery profiles. In group I the immobile fraction was 55.7 ± 7.5% and the mean residence time was 17.2 ± 0.8 seconds. In group II, the inclusions behaved as the ones formed in cells expressing WT aSyn-GFP, with a mean residence time of 35.2 ± 11.9 seconds and an immobile fraction of 24.1 ± 12.4%. Finally, in group III, inclusions presented a lower
Phosphorylation Modulates aSyn Clearance in Yeast

A

Gal

aSyn expression
ON

0 h

Glu

aSyn expression
OFF

6 h

B

WT

S129A

0 h clearance

6 h clearance

C

y = -8.5135x + 97.534

y = -11.988x + 76.649

% of cells with inclusions

Clearance Time (h)

D

WT

S129A

0 h clearance

6 h clearance

E

Inclusion size (μm²)

0 h clearance

6 h clearance

F

Empty

WT

S129A

0h clearance

6h clearance

Sub-population b

Frequency of cells

a

b

5 μm

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mean residence time of 14.9±3.1 seconds and an immobile fraction of 12.7±7.0%. We next determined the mean area and fluorescence intensity of the inclusions analyzed in the FRAP experiments before photobleaching. Inclusions formed by S129A aSyn-GFP were in general larger and presented stronger fluorescence than those formed by WT aSyn-GFP, suggesting the existence of heterogeneity in the inclusions (Figure 8B and C). To test whether inclusion heterogeneity was associated with reduced viability of cells expressing non-phosphorylatable S129A aSyn, we performed FRAP only in PI positive cells containing inclusions. In those cells, the initial fluorescence of the inclusions was not recovered at all and the fluorescence signal was unstable (Figure S6), in contrast to the patterns observed for the WT and S129A inclusions presented in Figure 8. Next, we compared protein dynamics of S129A aSyn-GFP inclusions 6 hours after blocking aSyn expression with that of inclusions at 0 hours of clearance. Again, we distinguished three types of inclusions based on the protein dynamics profiles (Figure 8A). However, after 6 hours of

Figure 5. S129A aSyn inclusions clearance is compromised. (A) Schematic representation of the treatments performed: aSyn expression was induced for 6 hours in galactose medium (Gal) and then yeast cells were transferred to glucose medium (Glu) to repress aSyn expression for 6 hours. (B) Intracellular localization of WT or S129A aSyn-GFP and (C) percentage of yeast cells containing aSyn-GFP inclusions, assessed by fluorescence microscopy at the indicated time points of aSyn clearance (**p<0.001, *p<0.01; two-tailed unpaired t-test with Welch’s correction). (D) Percentage of cells presenting 3 or less inclusions, or 4 or more inclusions per cell (**p<0.01, *p<0.05; Unpaired two-tailed t-test), and (E) inclusions size (µm²) at 0 or 6 hours of aSyn clearance. (F) SSC (side scatter) and aSyn-GFP fluorescence in the indicated yeast cells, assessed by flow cytometry, at 0 and 6 hours after aSyn clearance. A sub-population of yeast cells presenting higher levels of GFP fluorescence is distinguishable after 6 hours of WT or S129A aSyn clearance (left panel). The frequency of cells of this sub-population is represented in the right upper panel. In the right lower panel a fluorescence microscopy picture exemplifies the different GFP intensities observed in aSyn yeast cells after 6 hours of clearance (a) indicates cells with lower levels of GFP fluorescence and (b) sub-population of yeast cells presenting higher levels of GFP fluorescence). A representative result is shown from at least three independent experiments. Values represent the mean ± SD.
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Figure 6. The percentage of Triton-X insoluble S129A aSyn increases during clearance. (A) WT and S129A aSyn-GFP protein levels in yeast cells assessed by western blot analysis of total protein extracts at the indicated time points of aSyn-GFP clearance (left panel). Densitometric analysis of the immunodetection of aSyn relative to the intensity obtained for GAPDH, used as loading control, presented in arbitrary units (a.u.) (right panel) (**p<0.001, *p<0.05; one way ANOVA with Bonferroni’s multiple comparison test). (B) Triton-X soluble (TS) and Triton-X insoluble (TI) fractions of aSyn WT or S129A assessed by western blot analysis (upper panel) and determination by densitometric analysis of the percentage of TI aSyn, at the indicated time points of aSyn clearance (lower panel) (*p<0.05; Mann-Whitney test). GAPDH was used as an internal control for the experiment. A representative result is shown from at least four independent experiments. Values represent the mean ± SD.
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clearance, the immobile fraction as well as the mean residence time increased in the S129A aSyn-GFP inclusions when compared to 0 hours (Figure S5B). The presence of inclusions with different aSyn-GFP dynamics in the cell led us to hypothesize that the protein was partitioning between distinct subcellular protein quality control compartments. In particular, we hypothesized these compartments might be either the “juxtanuclear quality control” compartment (JUNQ), that is in close proximity to the nucleus and colocalizes with the proteasome, the “insoluble protein deposit” (IPOD), that colocalizes with the autophagic marker Atg8 [44], or P-bodies, cytoplasmic RNA-protein (RNP) granules that contain non-translating mRNAs as a cellular response to stress [45]. To verify these hypotheses, we performed fluorescence microscopy using established sub-cellular markers, namely Atg8 (IPOD), Pup1 (the beta 2 subunit of the 20S proteasome, JUNQ) and Dcp1 (P-Bodies). However, we observed no colocalization between WT or S129A aSyn-GFP with any of these markers (Figure S7).

aSyn inclusions in yeast cells colocalize with diverse trafficking markers including Ypt1 (ER-to-Golgi), Ypt31 (late Golgi), Sec4 (secretory vesicles-to-PM), Ypt6 (endosome-to-Golgi), Vps21 and Ypt52 (early-to-late endosome) and Ypt7 (LE-to-vacuole) [27]. Considering that we observed that S129A aSyn also relates to defects in ER-to-Golgi trafficking, we asked whether blocking pS129 could alter the normal distribution of aSyn inclusions. Thus, we compared the colocalization of WT and S129A aSyn-GFP inclusions using the trafficking markers indicated above. We found that blocking pS129 did not significantly alter the localization of aSyn inclusions (Figure S8).

aSyn has previously been shown to interact with membranes and, at low levels of WT aSyn expression, it localizes mostly to the plasma membrane [16]. Since aSyn also begins to aggregate at the membrane in small vesicles, we sought to investigate its association with the endocytic machinery. In order to visualize plasma membrane to vacuole endocytic trafficking, we used the monocarboxylate-proton symporter Jen1, which undergoes internalization through the endocytic pathway with subsequent vacuolar degradation [46,47]. At the initial stages of aSyn inclusion formation we observed almost exclusive colocalization of WT and S129A aSyn-GFP inclusions with Jen1, from the plasma membrane, in early endocytic vesicles, to the vacuole (Figure 9 and Supplementary Movie S3 and S4). As aSyn inclusion formation becomes more severe, the vesicles no longer reach the vacuole and accumulate in larger Jen1-positive inclusions, which are likely to be late endosomes (Figure 9). WT aSyn inclusions are present either as single vesicle or smaller clusters, while S129A aSyn inclusions at late stages of aggregation represent large clusters of vesicles (Figure 9).

Unphosphorylatable aSyn impairs induction of autophagy

Next, we evaluated whether phosphorylation altered the clearance of aSyn in the cell. First, we tested protein degradation by the ubiquitin-proteasome system (UPS). For this, we deleted the PDR5 gene in the strains expressing aSyn-GFP to ensure chemical inhibition of the proteasome by MG132 [48]. The expression levels of aSyn-GFP in the Apd3Δ3 mutants and in the original strains were similar (Figure S9A). Western blot analysis revealed a marked increase in the levels of ubiquitinated proteins in MG132 treated cells, confirming pharmacological proteasome inhibition was achieved (Figure S9C). However, we found that proteasome inhibition did not alter the levels of either WT or S129A aSyn-GFP, nor the percentage of cells displaying aSyn inclusions (Figure S9B). We observed a striking increment in ubiquitinated proteins relative to 3 hours of aSyn clearance (Figure S9B), suggesting the aSyn-induced proteasome inhibition was at least partially reversible.

Afterwards, we analyzed the contribution of autophagy to the clearance of WT and S129A aSyn-GFP by comparing ATG8 induction and autophagic flux. We used the mCherry-Atg8

Figure 7. Blocking aSyn phosphorylation reduces membrane interactions and accelerates inclusion formation. (A) Time lapse confocal imaging comparing the distribution of WT or S129A aSyn-GFP at the indicated time points after induction of aSyn expression. Nuclei were visualized by co-expressing NLS-TagRFP657. (B) Percentage of WT and S129A aSyn-GFP expressing cells presenting inclusions over time. \( n_{\text{wt}} = 48 \) cells, \( n_{\text{S129A}} = 55 \) cells. Inclusions were recognized by measuring GFP intensity. doi:10.1371/journal.pgen.1004302.g007
processing assay [49,50], and inserted the reporter under the control of the endogenous ATG8 promoter in the genome of the strains expressing WT or S129A aSyn-GFP (Table 1). ATG8 induction was measured by quantifying the increase of total mCherry signal (mCherry-Atg8 and free mCherry signal) normalized to the loading control (GAPDH) (Figure 10A), reflecting autophagy induction [49,50]. On the other hand, autophagic flux was quantified by measuring the vacuolar degradation of the Atg8 domain of the reporter (ratio of free mCherry to total mCherry signal) by western blot analysis [49,50], reflecting the vacuolar transfer and degradation of autophagosomes (Figure 10A). Interestingly, WT aSyn-GFP induced a 2-fold increase in the levels of Atg8 which decreased gradually during the clearance period (Figure 10A). In contrast, the levels of Atg8 remained unaltered throughout induction and clearance in cells expressing S129A aSyn-GFP (Figure 10A). No alterations in autophagic flux were observed with either WT or S129A aSyn-GFP.

Using flow cytometry, we confirmed these results and established a correlation between autophagy induction (measured by mCherry fluorescence intensity) and aSyn-GFP signal (Figure 10B). At 0 hours of clearance the mCherry-Atg8 median fluorescence intensity (MFI) in cells expressing S129A aSyn-GFP were considerably higher than those in cells expressing WT aSyn, and decreased gradually during the clearance period to near basal levels (Figure 10B). These results are consistent with the western blot analysis described above (Figure 10A). As expected, it was visible that, during clearance, the fluorescence of either WT or S129A aSyn-GFP decreases (Figure 10B). However, in both cases a sub-population of cells with larger and brighter inclusions maintained stronger GFP fluorescence after 6 hours of clearance. In this sub-population, higher levels of mCherry-Atg8 were observed both in the cells expressing WT or S129A aSyn-GFP, indicating that autophagy induction is more pronounced in cells with bigger and brighter aSyn inclusions (Figure 10B). However, in this sub-population, the cells expressing S129A...
aSyn-GFP displayed lower levels of mCherry-Atg8 than cells expressing WT aSyn-GFP both at 0 and 6 hours of clearance (Figure 10B).

A second line of evidence for the effect of pS129 on aSyn clearance by autophagy was obtained by genetically modulating this pathway. For these studies, we deleted \textit{ATG1} and \textit{ATG7} genes in the WT and S129A aSyn-expressing strains. Atg1 is a kinase playing an important role in autophagy initiation [51] and its mutant is defective in autophagy [52] while Atg7 is an activator of Atg6 and is required for the formation of autophagic bodies [53]. Deletion of \textit{ATG1} and \textit{ATG7} did not significantly affect WT or S129A aSyn-GFP expression levels after 6 hours of induction (Figure 11A). However, when aSyn expression was turned off and clearance was followed during 18 hours, \textit{D}atg7 resulted in higher levels of WT and S129A aSyn-GFP (Figure 11A). Deletion of \textit{ATG1} and \textit{ATG7} significantly increased WT aSyn pS129 levels after 0 hours of clearance (6 hours after aSyn expression induction), an effect that was not observed after 6 hours of clearance (Figure S10).

Deletion of \textit{ATG1} and \textit{ATG7} also significantly increased the percentage of cells displaying aSyn inclusions after 6 hours of clearance (Figure 11B). Flow cytometry experiments confirmed the fluorescence microscopy results, showing a significant increase in the population of cells displaying stronger GFP signal when autophagy was impaired due to \textit{ATG1} or \textit{ATG7} deletion (Figure 11C). Moreover, \textit{D}atg7 and \textit{D}atg7 S129A aSyn-GFP expressing cells also exhibited a higher percentage of PI positive cells, indicating that impairment of autophagy increased S129A aSyn-GFP toxicity, an effect that was not observed for WT aSyn-GFP expressing cells (Figure 11D).

Altogether these results indicate that blocking aSyn phosphorylation impacts on autophagy induction, suggesting cells process phosphorylated aSyn in a distinct way.

Figure 9. Plasma membrane to vacuole endocytic trafficking of WT and S129A aSyn. (A) aSyn inclusions localize to membrane and endosomal compartments. Cells coexpressing Jen1-mCherry and WT or S129A aSyn-GFP were grown at 30°C in glucose and shifted to galactose for 6 hours. Jen1-mCherry is constitutively synthesized, trafficked to the membrane, and re-absorbed through the endocytic pathway, eventually ending up in the vacuole. Since mCherry is pH stable it is visible in the vacuole. aSyn-GFP inclusions colocalize with Jen1-mCherry in endosomal compartments throughout the entire endocytic pathway. (B) aSyn foci cluster with vesicles. Images shown are still frames from a time-lapse series showing internalization of aSyn through the endocytic pathway. In some cases of low level of aSyn aggregation enters the vacuole. As the aggregation level increases, however, aSyn-containing vesicles are trapped, likely in late endosomes. (C) aSyn inclusions are present as vesicles or vesicle clusters (arrows). Expression of S129A aSyn-GFP was induced for 8 hours. 3D reconstruction and single z sections (2D) are shown. Each z series was acquired with 0.4 micron step size and 21 total steps.

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Figure 10. S129A αSyn impairs ATG8 induction. (A) Autophagy evaluated by mCherry-Atg8 processing assay in yeast cells expressing mCherry-Atg8 under the regulation of the endogenous promoter alone (control) or together with either WT or S129A αSyn, at the indicated time points, assessed by western blot (upper panel) (h: hours of induction; hc: hours of clearance). ATG8 induction quantified by the total mCherry signal (mCherry-Atg8 and free mCherry signal, detected with anti-mCherry) (lower left panel); autophagic flux quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free mCherry to total mCherry signal) (lower right panel). (**p-value< 0.001; one way ANOVA with Bonferroni’s multiple comparison test). Results shown are from one representative experiment from a total of five independent experiments. (B) αSyn-GFP and mCherry-Atg8 fluorescence intensity in yeast cells expressing mCherry-Atg8 alone (control) or together with either WT or S129A αSyn, at the indicated time points, assessed by flow cytometry (upper panel). mCherry-Atg8 MFI from all cells (lower left panel) and from the indicated subpopulation (lower right panel) (**p<0.001; one way ANOVA, with Bonferroni’s multiple comparison test). Yeast cells not expressing αSyn-GFP or mCherry-Atg8 were also used as an additional control (empty). A representative result is shown from at least four independent experiments. Values represent the mean ± SD.

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Discussion

Here, we found that WT αSyn-GFP is strongly phosphorylated at S129 by endogenous yeast kinases and S129E mutant αSyn-GFP mimics the behavior of WT protein, in particular with respect to cytotoxicity and inclusion formation. This contrasts with other reports that showed the S129E αSyn-GFP mutant fails to mimic the effect of αSyn S129 phosphorylation [54,55]. It is possible that the discrepancy is due to differences in the systems used but, importantly, it suggests S129E αSyn mutant might constitute a valid approach for the assessment of the cellular responses involved in the accumulation of phosphorylated αSyn. The S129A mutation was found to promote αSyn fibrillization [10,56]. However, this was not consensual in all cell and animal models where S129A was expressed. In SH-SY5Y cells, a neuroblastoma cell line with dopaminergic characteristics, S129A αSyn expression reduces inclusion formation [12], while in a Drosophila model, S129A αSyn expression results in the accumulation of increased levels of αSyn oligomers, but not of mature fibrils [7]. In our yeast model, expression of the phosphorylation-deficient S129A αSyn-GFP resulted in an exacerbation of αSyn toxicity concomitantly with a reduction in cellular viability and an increase in αSyn inclusion formation. This phenotype does not appear to be related to specific structural consequences of this mutation on αSyn, but rather to the blockade of S129 phosphorylation, as similar results were obtained when S129G αSyn-GFP, a different αSyn phospho-resistant mutant, was expressed.

Despite the accumulating evidence favoring the hypothesis that soluble αSyn oligomers, rather than insoluble protein aggregates, are the cytotoxic species in PD, the question is still unresolved [57,38]. In yeast, αSyn inclusions were described as clusters of vesicles [26,27,42], raising questions about whether αSyn accumulations actually displayed biochemical properties compatible with the formation of protein aggregates. However, at least some of these accumulations are indeed amyloid-like and β-sheeted aggregates, as they react with thioflavin S [41] or thioflavin T [59]. Here, we biochemically characterized the αSyn-GFP species that are formed in yeast cells using two complementary approaches (sucrose gradients and size exclusion chromatography) and clearly demonstrate the formation of large oligomeric species. Moreover, we established a correlation between increased inclusions formation and exacerbation of cytotoxicity and the formation of oligomeric species with higher molecular weight for the S129A αSyn-GFP mutant. Interestingly, we did not observe differences in the TX-insoluble fractions of cells expressing either WT or S129A mutant αSyn. These results are consistent with other reports where large αSyn soluble oligomers are also considered to constitute the toxic species [60].

Among the various cellular defects that have been implicated in the etiology of synucleinopathies, vesicular trafficking impairment has emerged as a major component of αSyn-dependent toxicity in yeast and in other model organisms [16,17,20,26–28,42,43]. Both genetic and chemical modulation of vesicular trafficking module αSyn toxicity [17,20,28]. In this study we show that pS129 blockade exacerbates vesicular trafficking defects that can be relieved by overexpression of YPT1, YKT6 and UBP3, genes that increase forward transport between ER and Golgi. Ypt1, the Rab guanosine triphosphatase whose mammalian ortholog Rab1 is able to prevent dopaminergic neuron loss [28], plays an essential role in the tethering and docking of the transport vesicle with the Golgi [61]. Ykt6, the soluble NSF (N-ethylmaleimide–sensitive factor) attachment protein receptor protein (SNARE), increases forward transport by increasing the likelihood of membrane vesicles from the ER tethering to Golgi target membranes [62]. In turn, the ubiquitin protease Ubp3, together with its cofactor Brc5, function to deubiquitinate the COPII coat protein Sec23p, and likewise promote vesicle exit from the ER [63]. Interestingly, the Brc5 cofactor, which also suppresses αSyn toxicity [28], was not able to restore S129A αSyn-GFP induced trafficking defect. This suggests that αSyn phosphorylation may modulate the way the protein interacts with components of the trafficking pathway, as one might expect.

Moreover, GP8 and PAR1 whose overproduction negatively regulates ER-Golgi trafficking, exacerbate S129A αSyn-GFP toxicity. Gyp8, is a negative regulator of Ypt1, that therefore inhibits ER-to-Golgi trafficking [64], while Pmr1 is the major Golgi membrane P-type ATPase ion pump responsible for transporting Ca²⁺ and Mn²⁺ ions into the Golgi apparatus, both of which are important for proper processing and trafficking of proteins through the secretory pathway [65].

Proteostasis is a central concept in the context of several disorders [66]. An imbalance between the rates of protein synthesis, clearance, and aggregation, caused by proteostasis dysfunction, could favor accumulation and/or formation of protein oligomers and inclusions that contribute to cytotoxicity [67]. We found that blocking αSyn phosphorylation impaired the turnover of αSyn. During clearance of S129A αSyn, we observed a significant increase of the TX-insoluble fraction, concomitantly with the attenuation αSyn-induced cytotoxicity. Our observations are consistent with those in a study in Drosophila where reduced αSyn toxicity was correlated with an increase in detergent-insoluble αSyn [68] and suggests this may constitute a defense mechanism.

Using high-resolution 4D imaging we found that WT αSyn has a lag phase during which it preferentially associates with the plasma membrane, whereas the S129A forms inclusions almost immediately. Furthermore, blocking αSyn phosphorylation alters the dynamics of αSyn in inclusions suggesting there are distinct populations of αSyn accumulating with different kinetics in inclusions. Based on this, we defined three groups of inclusions based on the recovery after photobleaching.

The presence of an immobile fraction of αSyn is common to all FRAP recovery curves, albeit at different proportions for each cell type.
of inclusions. However, blocking aSyn phosphorylation enables the protein to establish transient interactions with inclusions, which occur significantly faster than with WT aSyn.

When the synthesis of aggregation-prone proteins surpasses the degradation capacity of the cell, different quality-control mechanisms that are conserved from yeast to mammalian cells actively sequester aggregated proteins as a protective cellular response [58]. Misfolded aggregated proteins partition between two cellular compartments: the JUNQ and the IPOD compartment, which may serve a protective function and facilitate aggregate clearance. We postulated that the differences in sizes, number and dynamics of the inclusion formed by WT and S129A aSyn might reflect the distribution of aSyn between these compartments where misfolded proteins display distinct relative exchange rates with the soluble cytosolic pool [44,69,70]. Non-phosphorylated S129A aSyn accumulating in a less mobile fraction could indicate the protein was localizing to the IPOD, the preferred destination of protein aggregates. Likewise, the S129A aSyn in group III indicated the exchange of soluble aSyn with the cytosolic pool, as described for the JUNQ compartment. Unexpectedly, we found no colocalization of WT or S129A aSyn with neither IPOD nor JUNQ, suggesting that aSyn is not actively sorted to these cellular compartments in yeast. Moreover, we also did not observe colocalization of WT or S129A aSyn-GFP with P-bodies. Alternatively, we did detect colocalization of S129A aSyn-GFP with vesicular markers including Ypt31 (late Golgi), Sec4 (secretory vesicles-to-PM), Ypt6 (endosome-to-Golgi), Vps21 and Ypt52 [EE-to-late endosome (LE)] and Ypt7 (LE-to-vacuole).

We observed that initial stages of aSyn inclusion formation occur in conjunction with receptor-mediated endocytosis of the plasma membrane synporter Jen1. The alteration in the course of endocytosis caused by the presence of aSyn in endosomal compartments affects trafficking, causes accumulation of vesicles and eventually leads to cell death. The deficiency in the delivery of late endosomes carrying aSyn to the vacuole is more pronounced in the strain expressing S129A aSyn. This prompted us to evaluate the effect of this mutant on endocytosis. The clustering of the endosomal compartment caused by the presence of aSyn on the membrane, slows down endocytic trafficking and eventually blocks internalization of vesicles. While small inclusions of WT and S129A aSyn are cleared, large clusters of S129A aSyn accumulate proximally to the vacuole and, with new cycles of endocytosis, the size of the clusters becomes unbearable for the cell.

Given that early endosome functions are essential for autophagy and for endocytosis-UPS, deregulation of endocytosis by the presence of aSyn in vesicles disrupts not only vesicular trafficking but also major degradation pathways. Vesicles and membranous structures were also observed to accumulate at the periphery of Lys in PD brains [26], reinforcing the relevance of this mechanism in the context of aSyn pathobiology.

Posttranslational modifications modulate the degradation of aggregate-prone proteins by the UPS and/or autophagy. Ubiquitination generally determines whether a protein is degraded via the UPS or autophagy [71]. Interestingly, phosphorylation of mutant huntingtin (Htt), the protein associated with Huntington’s disease, was found to precede and regulate additional posttranslational modifications, including ubiquitination, SUMOylation, and acetylation, enhancing its normal clearance by the proteasome and lysosome [72]. In particular, acetylation of mutant huntingtin promotes its targeting to autophagosomes, facilitating its specific degradation by the autophagy/lysosomal pathway [73].

The impact of aSyn S129 phosphorylation on its clearance only started to be investigated recently. In neuronal cell lines, it was observed that proteasome inhibition results in increased levels of pS129 aSyn as an outcome of either increased activity of the kinase(s) involved or decreased phosphatase activity, together with decreased degradation of pS129 aSyn by the proteasome in an ubiquitin-independent manner [74,75].

Recently, overexpression of GRK6, one of the kinases capable of phosphorylating aSyn at S129 [76], was found to moderately increase aSyn toxicity in a rat model of familial PD [77]. In contrast, another recent study showed that the overexpression of another kinase, the Polo-like kinase 2 (PLK2) [78], is protective by mediating selective autophagy clearance of pS129 aSyn [79]. This apparent discrepancy could be the reflex of the different efficiencies of these kinases to phosphorylate aSyn at S129. In our yeast model, we completely abolished aSyn phosphorylation by replacing S129 with neutral and phosphorylation-resistant amino acids (alanine or glycine). In fact, our results are consistent with those recently reported [79], since we observed that blocking aSyn phosphorylation compromises its degradation. The clearance of the inclusions formed by S129A aSyn was slower than that of inclusions formed by WT aSyn. Moreover, our findings suggest that autophagy is the main mechanism involved in aSyn clearance in our yeast PD model. Whereas the accumulation of WT aSyn led to a marked induction of autophagy, cells expressing the S129A mutant failed to activate this pathway. Thus, we postulate that S129 phosphorylation might constitute a switch to sense and induce the autophagocytic pathway, and that blocking phosphorylation impairs autophagic induction, albeit without altering autophagic flux. Genetic impairment of yeast autophagy by deletion of ATG1 and ATG7 did not significantly affect the levels of WT or S129A aSyn after 6 hours of induction suggesting that, at this time point, clearance by autophagy is not superimposed to protein synthesis or that cells could compensate autophagy impairment through other clearance pathways, as suggested by studies performed in other cellular models [38,39,80]. However, ATG7 deletion increases the defect on the clearance of S129A aSyn-GFP but it also has a significant effect on WT aSyn-GFP clearance. This effect could be due to the accumulation of different aSyn species formed by S129A or WT aSyn, as we clearly demonstrated in this study. While soluble and smaller oligomeric species of aSyn could be more easily cleared by the proteasome and CMA, as reported in other models [32,33,81,82], the larger oligomeric species formed by S129A aSyn-GFP could specifically require autophagy function for clearance. Our observation that aSyn accumulation leads to impairment of proteasome function, as
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Materials and Methods

Plasmids and yeast strains

The yeast strains used in this work are described in Table 1. VSY71 to VSY74 contain double genome insertions of GAL1-KanMX4/WT, S129A or S129E-GFP or of the empty vectors and were previously described [42].

Plasmids p304 GAL1pr-SYC4/S129G-GFP and p306 GAL1pr-SYC4/S129G-GFP were generated by site directed mutagenesis of the corresponding plasmids carrying the WT SYC4 gene. These plasmids were linearized with EcoRV for integration into W303-1A and W303-1B strains, respectively. The correct insertion of the integrative vectors was verified by PCR and the haploid strains were used to generate a diploid strain by mating. Diploids were selected in minimal medium by URA and TRP prototrophy. The 1A and W303-1B strains, respectively. The correct insertion of the plasmids were linearized with EcoRV for integration into W303-1B strains, respectively. The correct insertion of the integrative vectors was verified by PCR and the haploid strains were used to generate a diploid strain by mating. Diploids were selected in minimal medium by URA and TRP prototrophy.

The diploids M5 and M6 used for the remainder of this work were generated in a similar way to diploids M1 and M2 described in [42]. Briefly, diploids M1 and M2 were crossed to the diploids containing the empty vectors pRS316 and pRS305 to generate diploids M5 and M6 containing the empty vectors pRS305 and pRS306, respectively.

Materials and methods

Yeast strains were obtained from the Yeast Genetic Stock Centre (Yeast Genetic Stock Centre, 1000 Mapleton Drive, Bloomington, IN, USA) or from Dr. Aaron Gitler, Stanford University, cloned in Gateway entry clones [87]. These plasmids were integrated in the VSY71, VSY72 and VSY73 genome to generate new strains (Table 1).

The vesicles markers Ypt1, Ypt31, Sec4, Ypt6, Vps21, Ypt52 and Ypt7 used on fluorescence microscopy experiments were constructed by Gitler and co-workers [27] and were obtained from Addgene (pAG416GPD-Cerulean-YPT1, 18848; pAG416GPD-Cerulean-YPT31, 18849; pAG416GPD-Cerulean-SEC4, 18844; pAG416GPD-Cerulean-YPT6, 18845; pAG416GPD-Cerulean-YPS21, 18842; pAG416GPD-Cerulean-YPT52, 18843; pAG416GPD-Cerulean-YPT7, 18847). These clones were used to generate entry clones by recombination cloning into a Gateway pDONR221 vector (Invitrogen). These clones were then used to generate new integrative vectors in the pAG305 GPD-Cerulean-ccdB vector which were verified by DNA sequencing. These plasmids were integrated in the VSY71, VSY72 and VSY73 genome to generate new strains (Table 1).

The P-bodies marker encoding the gene DCP1 cloned in pBG1005-DCP1 was obtained from the Open Biosystems Yeast ORF Collection and used to generate an entry clone into Gateway pDONR221 vector (Invitrogen). This clone was then used to generate a new integrative vector in the pAG305 GPD-ccdB-DrRed vector which was verified by DNA sequencing. This plasmid was integrated in the VSY71, VSY72 and VSY73 genome to generate new strains (Table 1). Construction of the pmESC, mCherry plasmid was performed by ligating BamHI-JEV1-Xhol fragment generated by PCR amplification of chromosome DNA with oligonucleotides containing flanking corresponding restriction enzyme sites into BamHI-pESC-mCherry plasmid. mCherry was subcloned into pESC (LEU) plasmid following Xhol/NdeI restriction sites.

Yeast transformations were carried out using a standard lithium acetate procedure and all the genome insertions were confirmed by two independent PCRs following standard procedures [80].

Yeast culture conditions and CFUs determination

For aSyn expression induction experiments, yeast cells were pre-grown in YEP-Raffinose (peptone 2%, yeast extract 1%, raffinose 1%) liquid media at 30°C, with orbital agitation (200 rpm) for 24 hours (doubling time: ~3 hours). After 24 hours, optical density at 600 nm (OD600 nm) was measured and yeast cells were diluted to a standardized OD600 nm = 3×10^-3 (~2.5×10^5 cells/mL) in YEP-Raffinose liquid media and grown at 30°C, with orbital agitation (200 rpm). After 24 hours, OD600 nm was measured. The volume of yeast culture needed to inoculate a new culture with an initial standardized OD600 nm = 0.2 (~7×10^6 cells/mL) was centrifuged (3000 rpm, at 30°C for 4 min). Cells were then resuspended in YEP-Galactose (peptone 2%, yeast extract 1%, galactose 1%) liquid media and incubated at 30°C, with orbital agitation (200 rpm), for 6 hours. The cell viability was assessed by counting CFUs after incubation of culture aliquots for 2 days at 30°C on YEP-glucose agar plates.

For spot assays cell suspensions were adjusted to OD600nm = 0.05±0.005 and used to prepare 1/3 serial dilutions that were applied as spots (4 μl) onto the surface of theYPD rich medium used as control or YEP-Galactose medium and incubated at 30°C for 2–3 days.
For aSyn clearance experiments, OD$_{600}$ of the 6 hours induced cultures was measured and the volume of yeast culture needed to inoculate a new culture with an initial standardized OD$_{600}$ = 0.2 (~7×10$^6$ cells/ml) was centrifuged (3000 rpm, at 30°C for 4 min). Cells were washed in PBS and resuspended in YEP-Glucose (peptone 2%, yeast extract 1%, glucose 2%) liquid media and incubated at 30°C, with orbital agitation (200 rpm), for 6 hours.

For fluorescence microscopy or flow cytometry analysis, adenine was added to the growth media at a final concentration of 0.16 mg/mL to avoid background interactions by the red pigment production due to ade2 auxotrophic marker of the used yeast strain. Adenine supplementation did not alter growth phenotypes of the tested yeast strains.

**Flow cytometry**

Yeast cell membrane integrity was evaluated with PI staining using a BD LSR Fortessa. Yeast cells were incubated with PI 5 µg/mL for 15 min. As a positive control, cells boiled for 10 min were used (data not shown).

Autophagy induction was determined measuring fluorescence intensity of mCherry-Atg8 under the regulation of the natural promoter [85] in cells co-expressing WT or S129A aSyn-GFP, using a BD FACSAria III equipped with a 561 nm laser for excitation and a 600 LP mirror in conjunction with a 610/20 BP filter for detection (BD Biosciences, San Jose, CA). Fluorescence intensity of WT or S129A aSyn-GFP was measured in simultaneous using a 488 nm laser for excitation and a 502 LP mirror in conjunction with a 530/30 BP filter for detection (BD Biosciences, San Jose, CA).

A minimum of 10,000 events were collected for each experiment. Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Results were expressed as median fluorescence intensity (MFI) of a molecule.

**Protein extraction and western blot analysis**

For total protein extraction, yeast cells were lysed in Tris-HCl buffer pH 7.6 supplemented with protease and phosphatases inhibitor cocktail (Roche, Mannheim, Germany), with glass beads (3 cycles of 30 seconds in the beadbeater and 1 min on ice). Cell debris was removed with a smooth centrifugation (700 g, 3 min, 4°C) and the supernatant was collected. The supernatant was sonicated (10 seconds at 10 mA, Soniprep 150 from Sanyo). Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific Inc, Illinois, USA). The same amount of total protein was loaded in the SDS-PAGE for the detection of mCherry-Atg8 levels. As WT and mutant aSyn expression cells exhibit slightly distinct growth rates, equal volumes of total protein, corresponding to the same number of cells (normalized based on OD$_{600}$) were applied to the SDS-PAGE for aSyn quantification in induction, clearance and proteasome pharmacological inhibition experiments, in order to avoid bias in the protein measurement levels due to a cell dilution effect.

Protein sample buffer (200 mM Tris-HCl pH 6.8, 6% 2-mercaptoethanol, 8% SDS, 40% glycerol, 0.4% bromophenol blue) was added to each protein sample and heated for 10 min at 100°C before acrylamide gel loading. Protein samples were run in SDS-PAGE and transferred to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad), as specified by the manufacturer. Immunoblotting was performed following standard procedures with the listed antibodies: aSyn (BD Transduction Laboratories, San Jose, CA, USA), pS129-aSyn (Wako Chemicals USA, Inc., Richmond VA, USA) and DsRed (Clontech Laboratories, Inc. USA). GAPDH (Ambion, Cambridgeshire, UK) or PGK (Life Technologies, Grand Island, NY, USA) were used as loading control.

The band intensity of the different immunoblots signals was estimated using ImageJ software (NIH, Bethesda, MD) and normalized against the corresponding GAPDH or PGK signal. In particular, aSyn levels were determined by calculating the ratio between aSyn/GAPDH and normalized to the control (mean ± SD); pS129-aSyn levels were determined by doing the ratio between both values: (pS129/GAPDH)/(aSyn/GAPDH) and normalized to the control (mean ± SD). Autophagy induction was quantified by the determination of the fold increase of total mCherry signal (mCherry-Atg8 and free mCherry signal, detected with anti-DsRed) normalized to GAPDH [89]; autophagic flux was quantified by measuring the vacular degradation of the Atg8 domain reporter (ratio of free mCherry to total mCherry signal) [50].

**Triton soluble and insoluble fractions**

Total protein was extracted and quantified with the BCA protein assay kit (Thermo Fisher Scientific Inc, Illinois, USA). 200 µg of total protein was incubated with 1% Triton X-100 on ice, for 30 min. Protein fractions were separated by centrifugation at 15000 g for 60 min at 4°C. The top soluble protein fraction (Triton-soluble, TS) was collected and the insoluble protein fraction (Triton-insoluble, TI) pellet was resuspended in 40 µl of 2% SDS Trit-HCl buffer pH 7.4 by pipetting and subsequent sonication (10 seconds). Equal volumes of TS and TI were loaded and resolved by SDS-PAGE.

**Sucrose gradient and size exclusion chromatography**

Total protein from cells expressing WT or S129A aSyn was obtained and applied on a 5 to 30% sucrose gradient as described before [90,91]. Fractions were collected, precipitated for 4 hours at 4°C in trichloroacetic acid, washed in acetone three times and suspended in protein sample buffer (0.5 M Tris-HCl, pH 6.8, Glycerol, 10% SDS, 0.1% Bromophenol Blue). Proteins were resolved by SDS-PAGE and estimation of the molecular sizes for each fraction was previously described [92].

Size exclusion-fast protein liquid chromatography (SEC-FPLC) was performed with total protein lysates from cells expressing WT or S129A aSyn-GFP extracted as described for sucrose gradient [90,91], centrifuged at 16000 g for 4 min and filter with 0.45 µM PVDF (Whatman) to remove any insoluble particles. Samples (3 mg of protein in final volume of 500 µL) were analyzed on a Superose 6 10/300 GL column (GE Healthcare, Uppsala, Sweden) using a FPLC system with UV-M II detector (GE Healthcare, Uppsala, Sweden). The samples were eluted with 50 mM ammonium acetate, pH 7.4 at a flow rate of 500 µL/min and the UV absorbance was monitored at 280 nm. To estimate the molecular weight of the protein samples, High Molecular Weight and Low Molecular Weight gel filtration calibration kits were used (GE Healthcare, Uppsala, Sweden). Fractions of 500 µL were collected, precipitated overnight at 4°C in trichloroacetic acid, washed in acetone three times and resuspended in protein sample buffer (0.5 M Tris-HCl, pH 6.8, Glycerol, 10% SDS, 0.1% Bromophenol Blue), and were resolved by SDS-PAGE.

**Fluorescence 3D time-lapse (4D imaging)**

For time-lapse imaging, VSY yeast cells transformed with pESC-Leu G4LTPr NLS-TagRFP657 were pre-grown overnight in Synthetic complete (SC) medium without leucine (SC-Leu) raffinose liquid media at 30°C, with orbital agitation (200 rpm). OD$_{600}$ was measured and yeast cells were diluted to a standardized OD$_{600}$ = 0.8 (~2.4×10$^7$ cells/ml) in SC-Leu

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raffinose liquid media and grown at 30°C, with orbital agitation (200 rpm). After 6 hours, cells were seeded on concanavalin A-coated 4-well microscope plates (Greiner Bio-One GmbH) for about 10 min. Then all media was replaced by SC-Leu Galactose for aSyn and NLS-TagRFP657 expression induction. Confocal 3D movies were acquired using a dual point-scanning Nikon AIR-si microscope equipped with a Planapo Peizo stage (MCL), using a 60x PlanApO IR water objective NA 1.27 and PlanApO VC oil objective 960x (NA 1.40, 0.35 micron slices, and 0.5% laser power (from 5 mW 488 laser and 40 mW 561 laser). Prior to imaging the point-spread function was visualized with 100 nm fluorescence beads in order to adjust the correction ring of the objective to the coverslip thickness. Movies were acquired in resonant-scanning mode. Z-stacks were acquired every 10 min for 18 hours with some exceptions. Each z-series was acquired with 0.35 micron step size and 30 total steps, images - in galvano scanning mode (0.4 micron slices). Image processing was performed using NIS-Elements software.

Fluorescence microscopy and FRAP experiments

The percentage of cells with aSyn inclusions, number of aSyn inclusions per cell and size of aSyn inclusions were determined by fluorescence microscopy using a Zeiss Axiosvert 200 M (Carl Zeiss) widefield fluorescence microscope equipped with a cooled CCD camera (Roper Scientific CoolSnap HQ) to acquire images containing at least 700 cells per strain, which were then manually counted using ImageJ. Colocalization studies were performed with a Zeiss Axiosvert 200 M (Carl Zeiss) widefield fluorescence microscope equipped with a cooled CCD camera (Roper Scientific CoolSnap HQ) or a Zeiss LSM 710 inverted laser scanning confocal microscope (Carl Zeiss) using a Plan-Neofluar 63x/1.4 oil immersion objective. EGFP fluorescence was detected using the 488 nm laser line of an Ar laser (25 mW nominal output) and a custom wavelength detection window set to 493–556 nm. mCherry and RFP fluorescence were detected using a 561 nm DPSS laser (15 mW) and a custom detection window set to 569–797 nm.

Yeast cells were grown as described above. At the indicated time points cells were collected by centrifugation and resuspended in PBS and 0.5% low melting agarose on a microscope slide.

FRAP experiments were performed using a Zeiss LSM 710 inverted laser scanning confocal microscope equipped with a large incubator (Pecon, Erbach, Germany) maintained at 30°C. Images were acquired using a PlanNeofluar 63x/1.4 objective. A series of 80 z-stacks consisting of 5 different focal planes spaced 0.7 μm apart (frame size 512×512, pixel width 91 nm and pixel time 4.44 μs) were acquired at intervals of 2 seconds with pinhole set to 1 Airy unit. In each FRAP experiment a single inclusion, focused at the central focal plane of the z-stack, was bleached using the 488 nm laser line of an Ar laser (25 mW nominal output) and a custom wavelength detection window set to 493–556 nm. mCherry and RFP fluorescence were detected using a 561 nm DPSS laser (15 mW) and a custom detection window set to 569–797 nm.

Intracellular localization of the WT, S129A or S129G aSyn-GFP were assessed by western blot analysis of total protein extracts 6 hours after aSyn-GFP expression induction. PGK was used as loading control. (C) Intracellular localization of the WT, S129A or S129G aSyn-GFP (left panel) and percentage of yeast cells containing aSyn inclusions (right panel), after 6 hours of aSyn expression induction, assessed by fluorescence microscopy (*p<0.01; one way ANOVA and post-hoc Tukey test). Results shown are from one representative experiment from at least three independent experiments. Values represent the mean ± SD. (TIF)

Supporting Information

Figure S1 S129A aSyn is more toxic than WT aSyn. (A) Cell viability determined by CFUs during the initial time points of the growth curve (shown in Figure 1A) of yeast cells expressing either WT (□), S129A (▲) or S129E (○) aSyn-GFP, compared to cells that are not expressing the human protein (△). Cells used as inoculum were exponential-phase cells cultivated in raffinose medium that at time zero were transferred to galactose medium to induce aSyn expression. Values are representative from three independent experiments. (B) Spotting assay of the indicated yeast cells. The cell suspensions with adjusted OD600nm were serially diluted and spotted onto the surface of solid medium containing either glucose (control) or galactose (induced aSyn expression) as carbon source. A representative result is shown from at least three independent experiments. (TIFF)

Figure S2 S129G aSyn behaves similarly to S129A aSyn. (A) Spotting assay of the indicated yeast cells. The cell suspensions with adjusted OD600nm were serially diluted and spotted onto the surface of solid medium containing either glucose (control) or galactose (induced aSyn expression) as carbon source. Results shown are from one representative experiment from at least three independent experiments. (B) WT, S129A or S129G aSyn-GFP expression and pS129 levels in yeast cells assessed by western blot analysis of total protein extracts 6 hours after aSyn-GFP expression induction. PGK was used as loading control. (C) Intracellular localization of the WT, S129A or S129G aSyn-GFP (left panel) and percentage of yeast cells containing aSyn inclusions (right panel), after 6 hours of aSyn expression induction, assessed by fluorescence microscopy (*p<0.01; one way ANOVA and post-hoc Tukey test). Results shown are from one representative experiment from at least three independent experiments. Values represent the mean ± SD. (TIF)

Figure S3 Elution profiles of SEC from total protein extracts of cells expressing WT or S129A aSyn-GFP. Equal amounts of total protein (~3 mg) from cells not expressing aSyn (empty) or expressing either WT or S129A aSyn-GFP were separated in a Superose 6 10/300 GL column. A calibration curve for SEC was
performed using protein molecular weight markers (ferritin, 440 kDa; aldolase, 150 kDa; conalbumin, 75 kDa; ovalbumin, 44 kDa, and aprotinin, 6.5 kDa). The elution time of the protein markers is indicated in the figure. Results shown are from one representative experiment from at least three independent experiments. Values represent the mean ± SD. (TIFF)

**Figure S4** S129 phosphorylation levels of WT aSyn are not altered during aSyn clearance. WT aSyn and pS129 levels at the indicated time points of aSyn clearance (left panel). Densitometric analysis of the pS129-aSyn levels by determining the ratio of the pS129 and the total levels of aSyn [pS129/PGK]/(aSyn/PGK) and normalized to the control. Results shown are from one representative experiment from at least four independent experiments. Values represent the mean ± SD. (TIFF)

**Figure S5** aSyn protein dynamics in inclusions. FRAP recovery curves of the aSyn WT and S129A inclusions shown in Figure 5 are well fit with a single exponential curve. FRAP experiments were performed at (A) 0 hours and (B) at 6 hours of clearance. The aSyn immobile fraction (I) and the aSyn mean residence time (T) values were calculated from the single exponential fit. Values represent the mean ± SD. (TIFF)

**Figure S6** aSyn inclusions from PI-positive cells expressing S129A aSyn do not recover after photobleaching. (A) Time lapse recording of the fluorescence recovery after photobleaching of aSyn inclusions in three representative PI positively marked cells expressing aSyn S129A after 6 hours of expression induction and the corresponding (B) FRAP recovery curve of cells shown in (A). (TIFF)

**Figure S7** Inclusions formed by WT or S129A aSyn do not colocalize with IPOD, JUNQ or P-bodies markers. Confocal microscopy images of cells co-expressing either WT or S129A aSyn-GFP and mCherry-Atg8, Pup8-RFP or Dcp1-DsRed after 6 hours of aSyn expression induction. Results shown are from one representative experiment from at least three independent experiments. (TIFF)

**Figure S8** Inclusions formed by WT or S129A aSyn colocalize with vesicular trafficking markers. Fluorescence microscopy images of cells co-expressing either WT or S129A aSyn-GFP, and the indicated vesicular trafficking markers with N-terminus fusions with cerulean, after 6 hours of aSyn expression induction. Results shown are from one representative experiment from at least three independent experiments. (TIFF)

**Figure S9** The proteasome is not involved in the clearance of S129A aSyn-GFP. (A) WT, S129A aSyn-GFP expression levels in WT or Adpδ5 yeast cells assessed by western blot analysis of total protein extracts 6 hours after aSyn-GFP expression induction. (B) Percentage of Adpδ5 cells with WT or S129A aSyn-GFP inclusions, before and after 3 hours of clearance in the presence of MG132 in DMSO or only DMSO. (C) WT and S129A aSyn-GFP expression levels at the same time points and subject to the same treatments as in (B), determined by western blot analysis of protein total extracts from Adpδ5 yeast cells. The total levels of ubiquitinated proteins were also evaluated by immunoblotting to confirm the effectiveness of the MG132 as proteasome activity inhibitor. Results shown are from one representative experiment from at least three independent experiments. Values represent the mean ± SD. (TIFF)

**Figure S10** Impairment of autophagy do not affect aSyn S129 phosphorylation. WT aSyn-GFP pS129 levels assessed by western blot analysis of total protein extracts at the indicated time points of aSyn-GFP clearance (left panel). Densitometric analysis of the pS129-aSyn levels by determining the ratio between pS129 and the total levels of aSyn [pS129/PGK]/(aSyn/PGK) and normalized to the control (right panel) (*p<0.05; one way ANOVA with Bonferroni’s multiple comparison test). A representative result is shown from at least three independent experiments. Values represent the mean ± SD. (TIFF)

**Movie S1** High-resolution 4D imaging of yeast cells expressing WT aSyn-GFP. Nuclei are marked with NLS-TagRFP657. Images were acquired every 10 min for 18 hours after induction of aSyn expression. (AVI)

**Movie S2** High-resolution 4D imaging of yeast cells expressing S129A aSyn-GFP. Nuclei are marked with NLS-TagRFP657. Images were acquired every 10 min for 18 hours after induction of aSyn expression. (AVI)

**Movie S3** High-resolution 4D imaging of yeast cells expressing WT aSyn-GFP and Jen1-mCherry. Images were acquired every 10 min after induction of aSyn expression. (AVI)

**Movie S4** High-resolution 4D imaging of yeast cells expressing WT aSyn-GFP and Jen1-mCherry. Images were acquired every 10 min after induction of aSyn expression. (AVI)

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**Author Contributions**

Conceived and designed the experiments: ST CNS DK TFO. Performed the experiments: ST DW MMRP PA DM RRR TA MW FM AG. Analyzed the data: ST MMRP JR MW DM. Wrote the paper: ST MMRP.

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