

Suppression of α -synuclein toxicity and vesicle trafficking defects by phosphorylation at S129 in yeast depends on genetic context

Vicente Sancenon¹, Sue-Ann Lee^{1,2}, Christina Patrick⁵, Janice Griffith⁶, Amy Paulino⁵, Tiago F. Outeiro^{7,8}, Fulvio Reggiori⁶, Eliezer Masliah⁵ and Paul J. Muchowski^{1,2,3,4,*}

¹Gladstone Institute of Neurological Disease, 1650 Owens Street, San Francisco, CA 94158, USA, ²Biomedical Sciences Graduate Program, ³Department of Biochemistry and Biophysics and ⁴Department of Neurology, University of California, San Francisco, USA, ⁵Department of Pathology and Medicine, University of California, San Diego, La Jolla, CA 92093, USA, ⁶Department of Cell Biology, University Medical Center Utrecht, Utrecht, CX 3584, The Netherlands, ⁷Cellular and Molecular Neuroscience Unit and ⁸Instituto de Medicina Molecular, Instituto de Fisiologia, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

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The aggregation of α -synuclein (α Syn) is a neuropathologic hallmark of Parkinson's disease and other synucleinopathies. In Lewy bodies, α Syn is extensively phosphorylated, predominantly at serine 129 (S129). Recent studies in yeast have shown that, at toxic levels, α Syn disrupts Rab homeostasis, causing an initial endoplasmic reticulum-to-Golgi block that precedes a generalized trafficking collapse. However, whether α Syn phosphorylation modulates trafficking defects has not been evaluated. Here, we show that constitutive expression of α Syn in yeast impairs late-exocytic, early-endocytic and/or recycling trafficking. Although members of the casein kinase I (CKI) family phosphorylate α Syn at S129, they attenuate α Syn toxicity and trafficking defects by an S129 phosphorylation-independent mechanism. Surprisingly, phosphorylation of S129 modulates α Syn toxicity and trafficking defects in a manner strictly determined by genetic background. Abnormal endosome morphology, increased levels of the endosome marker Rab5 and co-localization of mammalian CKI with α Syn aggregates are observed in brain sections from α Syn-overexpressing mice and human synucleinopathies. Our results contribute to evidence that suggests α Syn-induced defects in endocytosis, exocytosis and/or recycling of vesicles involved in these cellular processes might contribute to the pathogenesis of synucleinopathies.

INTRODUCTION

Synucleinopathies comprise a subset of neurodegenerative disorders characterized by the accumulation of cytoplasmic inclusions, or Lewy bodies (LBs), that contain the protein α -synuclein (α Syn) in selected populations of neurons [Parkinson's disease (PD) and dementia with Lewy bodies (DLB)] or glia [multiple system atrophy (MSA)]. Although the etiology of these disorders is unknown, the discovery of mutations in the α Syn gene (*SNCA*) that cause PD implicates α Syn in the pathogenesis of synucleinopathies (1).

The precise cellular function of α Syn is unclear. α Syn is a pre-synaptic protein that stimulates the formation of synaptic

vesicles and neuronal transmission *in vitro* and *in vivo* (2–5). Importantly, the discovery that multiplications of the α Syn locus cause PD suggests that neurotoxicity is a quantitative trait of α Syn (6). Therefore, overexpression of α Syn has been widely used to study the molecular mechanisms of disease pathogenesis in a variety of model systems. In addition to other phenotypes, overexpression of α Syn appears to disrupt vesicular transport in cell-based and *in vitro* models, and in patients with PD (7–11). Yeast has proven useful as model to reconstitute α Syn dose-dependent cellular toxicity and vesicular transport defects. α Syn was shown to block ER-to-Golgi transport (12) and other intracellular trafficking pathways (13,14) at toxic concentrations. These trafficking

*To whom correspondence should be addressed. Tel: +1 4157342515; Fax: +1 4153550824; Email: pmuchowski@gladstone.ucsf.edu

failures correlate with an accumulation of intracellular vesicles (13,15). Interestingly, α Syn toxicity in yeast and other model organisms can be modulated by manipulating the expression of genes involved in vesicular trafficking (12,16–20).

Posttranslational modifications of α Syn *in vivo* may play an important role in the pathogenesis of PD and other synucleinopathies. The most abundant modification of α Syn in LBs is the phosphorylation of serine 129 (S129) (21,22). This residue is located within a casein kinase (CK) consensus recognition site and is phosphorylated by yeast and mammalian CKs (14,22–24) and other kinases (25–28) in cellular and animal models. However, the relevance of S129 phosphorylation for pathogenesis remains controversial. Discordant studies in rats and *Drosophila* argue for protective, innocuous and detrimental effects of phosphorylation on neurodegeneration (29–32). Moreover, whether phosphorylation influences α Syn-induced intracellular trafficking defects has not been evaluated.

In this study, we show that late-exocytic, early-endocytic and/or recycling transport of plasma membrane (PM) proteins is disrupted by constitutive expression of α Syn in yeast. Yeast casein kinase 1 (Yck1) attenuates this defect by a phosphorylation-independent mechanism. However, blocking α Syn phosphorylation dramatically enhances α Syn toxicity and trafficking defects in a strain-specific manner in yeast, suggesting that genetic context determines the sensitivity to changes in the phosphorylation state of α Syn. We also report early endosome (EE) alterations and co-localization of mammalian CKI δ with α Syn-positive inclusions in mouse models and human synucleinopathy brains, providing evidence that endosome anomalies and CKI δ sequestration may contribute to the pathogenesis of synucleinopathies.

RESULTS

Overexpression of α Syn causes vesicles to accumulate in yeast

Wild-type (WT) α Syn-GFP ectopically expressed in yeast from the galactose-inducible promoter of the *GALI* gene accumulates in intracellular deposits that were initially described as inclusions (33). The earliest inclusions form at 3.5 h of induction in the cell periphery and subsequently spread toward the cell interior (13). Immuno-electron microscopy (IEM) studies revealed that the inclusions observed by fluorescence microscopy are composed of α Syn-positive clusters of vesicles (13,15). To further investigate the composition of these clusters, we examined the ultrastructure and the subcellular localization of α Syn by IEM over time in yeast expressing α Syn-GFP by the *GALI* promoter. Within the first 4 h of induction, α Syn-GFP immunoreactivity was almost exclusively observed at the PM (data not shown). At 6 h, we observed small clusters of vesicles in the vicinity of the PM (Supplementary Material, Fig. S1A and B). The vesicles were homogeneous in size (~20–40 nm in diameter), morphology and electron density, suggesting a common origin. At 12 h, the clusters were enlarged, and their vesicular content became heterogeneous in morphology and size (up to ~100 nm in diameter), consistent with multiple origins of vesicles due to a widespread trafficking defect as reported (13) (Supplementary

Material, Fig. S1C and D). Notably, α Syn-GFP immunoreactivity was detected on the surface of the vesicles at 6 and 12 h, in agreement with previous reports showing that the α Syn inclusions observed by fluorescence microscopy correspond to these clusters of α Syn-positive vesicles.

Constitutive α Syn expression disrupts late-exocytic, early-endocytic and/or recycling trafficking in yeast

Although the Lindquist group initially showed that α Syn inclusions co-localize with Ypt1, an endoplasmic reticulum (ER)-to-Golgi trafficking marker (12), a follow-up study by the same group showed co-localization with diverse trafficking 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), suggesting that α Syn may disrupt multiple intracellular trafficking routes in yeast (13). To investigate the precise origin of vesicles that accumulate after α Syn expression in yeast, we evaluated the effect of constitutively expressing untagged α Syn from a glycerol-3-phosphate dehydrogenase (*GPD1*) promoter in a 2 μ m plasmid on the steady-state localization of a series of GFP-tagged protein markers for different intracellular trafficking pathways (34–36) (Fig. 1A). GFP-Snc1, a transmembrane exocytic SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor), is targeted to the PM through the secretory pathway and subsequently recycled to the PM via EEs and the Golgi. Ste2-GFP, the transmembrane α -factor pheromone receptor, and GFP-Snc1-Sso1, an engineered variant of GFP-Snc1 containing a transmembrane domain of the SNARE Sso1, are targeted to the PM through the secretory pathway and, subsequently, after endocytosis, to the vacuolar lumen via EE and LEs. The SNARE GFP-Pep12 is targeted to the membrane of the prevacuolar complex (PVC) via the carboxypeptidase Y (CPY) biosynthetic pathway (ER to Golgi to PVC/LE to vacuole). The transmembrane proteins GFP-Phm5 and Sna3-GFP are targeted to the vacuolar lumen via the CPY pathway by ubiquitin-dependent and -independent mechanisms, respectively. GFP-Nyv1-Snc1, an engineered variant of the SNARE Nyv1 containing the transmembrane domain of Snc1, is targeted to the vacuole membrane via the alkaline phosphatase (ALP) pathway (ER to Golgi to vacuole).

To quantitatively assess the effect of α Syn on trafficking, we counted the percentage of cells that exhibit a mislocalization phenotype, considered as any anomaly in the localization pattern of a trafficking marker that differs from the pattern displayed by the majority of cells that do not express α Syn. Constitutive expression of untagged α Syn from the *GPD1* promoter selectively prevented the proper targeting of GFP-Snc1 to the PM and of GFP-Snc1-Sso1 and Ste2-GFP to the vacuole lumen in ~50–85% of cells, but not of GFP-Pep12 and GFP-Nyv1-Snc1 to the vacuole membrane or GFP-Phm5 and Sna3-GFP to the vacuole lumen (Fig. 1B and C). The trafficking routes that are unique to the protein markers perturbed by untagged α Syn include Golgi-to-PM, PM-to-endosome and endosome-to-Golgi. These observations suggest that, at the steady-state expression levels achieved by the *GPD1* promoter, untagged α Syn impairs the delivery of proteins to the PM from the Golgi and/or their subsequent

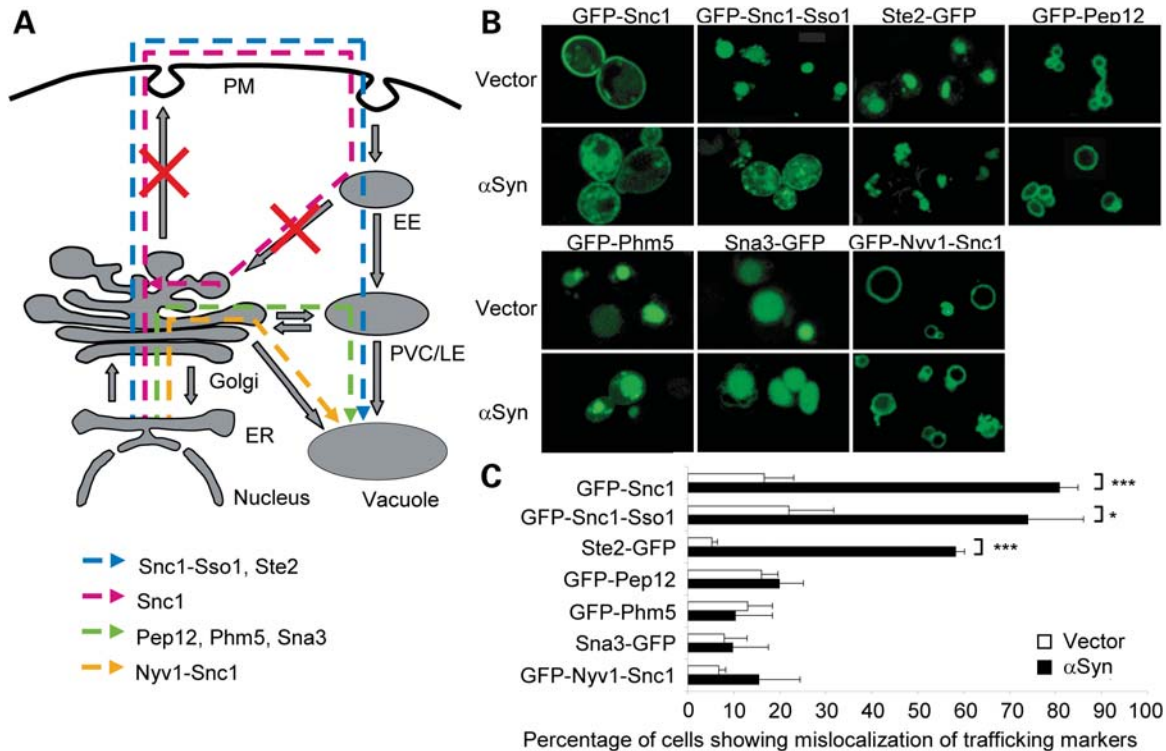


Figure 1. Untagged α Syn overexpression causes the mislocalization of late-exocytic and early-endocytic protein markers. (A) Schematic representation of the trafficking routes of the indicated protein markers in yeast. Red crosses indicate putative trafficking steps blocked by constitutive α Syn expression. EE, early endosome; ER, endoplasmic reticulum; PM, plasma membrane; PVC/LE, prevacuolar complex/late endosome. (B) Effect of untagged α Syn on the localization of the indicated protein trafficking markers. BY4741 strain co-transformed with plasmids for the expression of the indicated protein markers, and untagged WT α Syn under the control of a *GPD1* constitutive promoter or the corresponding empty vector was imaged in the logarithmic phase. (C) Quantification of the percentage of cells from (B) exhibiting mislocalization of the indicated trafficking markers. Error bars represent the standard deviation of three experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's *t*-test.

endocytic and recycling trafficking. In contrast, other markers that use the ER-to-Golgi pathway but bypass the PM (GFP-Pep12, GFP-Phm5, Sna3-GFP and GFP-Nyv1-Snc1) do not exhibit localization defects in α Syn-expressing cells, suggesting that ER-to-Golgi trafficking is not impaired.

We next analyzed the subcellular distribution of α Syn-GFP and the exocytic SNARE Snc1 by co-IEM (Fig. 2). Consistent with the fluorescence microscopy studies, Snc1 immunoreactivity was detected in the α Syn-GFP-positive vesicular clusters, confirming anomalies in PM delivery, endocytic and/or recycling trafficking of Snc1. As α Syn has been reported to block the delivery of the dye FM 4-64 to the vacuole, but not its uptake (33), we propose that α Syn impairs post-endocytic and/or recycling trafficking that follows vesicle budding from the PM (Fig. 1A), at least at early stages after expression.

To gain further insights in the trafficking steps disrupted by α Syn, we investigated the subcellular distribution of GFP-Snc1-Sso1 and the dye FM 4-64 in two subsets of yeast trafficking mutants (Supplementary Material, Fig. S2A): first, in loss-of-function deletion mutants defective in intra-Golgi (*ypt31 Δ*), endocytic (*end3 Δ* , *ypt51 Δ*), recycling (*ypt31 Δ*), endosome-to-Golgi (*vps35 Δ*) and endosome-to-vacuole (*vps23 Δ* , *did3 Δ*) transport, endosome and vacuole homotypic fusion (*ypt7 Δ*) and actin remodeling (*sac6 Δ*); second, in temperature-sensitive secretory mutants defective in

ER-to-Golgi (*sec7-4*, *sec18-1*), intra-Golgi (*sec7-4*) and Golgi-to-PM (*sec1-1*) transport at permissive [room temperature (RT)] and non-permissive (37°C) temperatures. Among all of the mutants studied, *did3 Δ* and *vps23 Δ* appear to most closely resemble the mislocalization pattern of GFP-Snc1-Sso1 caused by α Syn, suggesting a late-endocytic defect (Supplementary Material, Fig. S2B and C). However, the mislocalization phenotype in α Syn-expressing cells seems to rather be unique, suggesting that multiple trafficking steps are affected, in agreement with a previous study (13).

Yck1 attenuates α Syn-induced toxicity and trafficking defects through an S129 phosphorylation-independent mechanism

There is increasing evidence that α Syn disrupts endocytic trafficking. For example, in yeast α Syn overexpression perturbs the subcellular distribution of the endocytic tracker FM 4-64 (33) and diverse endocytic protein markers (13). The endocytic pathway modulates α Syn toxicity in *Caenorhabditis elegans* (17). Therefore, we reasoned that genes that regulate endocytosis might also modify α Syn-induced growth defects and vesicle accumulation in yeast. To test this hypothesis, we assessed whether Yck1 and Yck2, two functionally redundant PM-associated members of the CKI protein family that promote the endocytosis of PM proteins (37,38), modulate

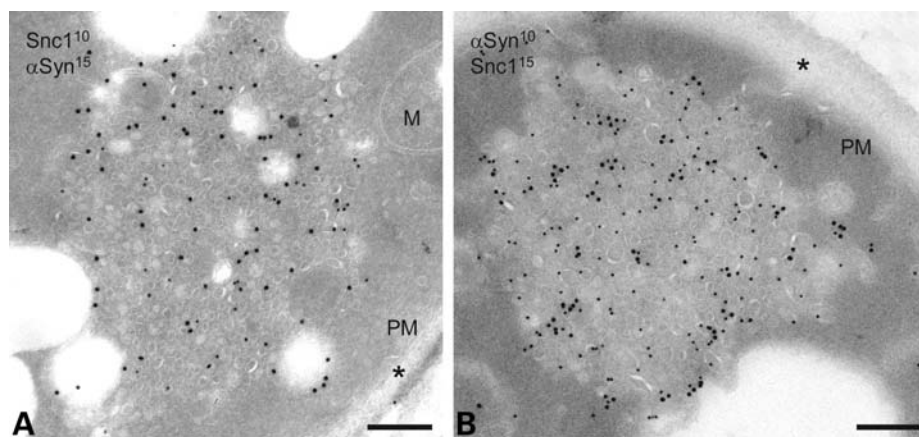


Figure 2. α Syn-induced vesicular clusters contain the exocytic SNARE Snc1. Strain FRY346 carrying an integrated δ xMYC-SNC1 fusion and transformed with a plasmid for the expression of α Syn-GFP under the control of a constitutive *GPD1* promoter was grown to the logarithmic phase, and cells were processed for IEM. In the left panel (A), cryosections were first incubated with anti-myc antibodies and then with anti GFP antibodies (α Syn¹⁵, 15 nm gold particles; Snc1¹⁰, 10 nm gold particles), whereas in the right panel (B) the sequence of the antibodies was inverted (α Syn¹⁰, 10 nm gold; Snc1¹⁵, 15 nm gold particles). PM, plasma membrane; M, mitochondria. The asterisk denotes the cell wall. Bar, 200 nm.

α Syn toxicity. For these studies, we generated a yeast strain carrying two stably integrated copies of the human α Syn gene fused to GFP under the control of the *GAL1* inducible promoter (which allows to control for gene-specific effects in non-inducing conditions) in the BY4741 strain (Table 1). As positive and negative controls, we deleted the genes *TLG2*, a known α Syn toxicity modifier encoding a SNARE required for the targeting of Yck2 to the PM (20,39), and *SPO14*, a gene encoding a yeast phospholipase D that does not modify α Syn toxicity (40), respectively. In contrast to a previous study (14), we found that deletion of *YCK1* and *YCK2* genes significantly increased the growth defect caused by α Syn-GFP (Fig. 3A and Supplementary Material, Fig. S3).

To confirm these genetic interactions, we reasoned that pharmacologic inhibition of CKI activity in yeast expressing α Syn should phenocopy the *yck1* Δ or *yck2* Δ alleles, and this effect should be more dramatic in yeast lacking either one of the two functionally redundant enzymes. Therefore, we tested the effect of the CKI-specific inhibitor D4476 on the viability of cells expressing α Syn-GFP from two genomic loci. To make cells more sensitive to the compound, we knocked out the multidrug resistance gene *PDR1* in all the strains tested. Although *YCK1* or *YCK2* are not individually essential for cell growth, deletion of both genes results in growth arrest. As expected, the CKI-specific inhibitor D4476 decreased yeast growth in a concentration-dependent manner (Supplementary Material, Fig. S4). Notably, this effect was more pronounced in cells expressing α Syn-GFP, and was significantly increased in cells lacking one of the two redundant enzymes (Supplementary Material, Fig. S4), suggesting that CKI activity counteracts the detrimental effects of α Syn overload.

To determine whether the increase in growth inhibition caused by the loss of CKI function correlates with increased trafficking defects, we studied the localization of mCherry-tagged Snc1-Sso1 in WT and *yck1* Δ cells carrying two or zero integrated copies of the galactose-inducible α Syn-GFP gene. In cells not expressing α Syn-GFP, mCherry-Snc1-Sso1

is correctly delivered to the vacuolar lumen (Fig. 3B). In contrast, expression of α Syn-GFP prevented the proper targeting of mCherry-Snc1-Sso1 in \sim 30% of WT cells (Fig. 3B and C). Note that the defect in these cells is less marked than in cells constitutively expressing untagged α Syn from a 2 μ m plasmid, where \sim 75% of cells are affected (Fig. 1C). However, deletion of *YCK1* did not aggravate this phenotype (Fig. 3B and C), indicating that the enhancement of growth defects by the *yck1* Δ mutation is not associated with an enhancement of endocytic trafficking defects.

Next, we investigated the ability of *YCK1* to reverse α Syn-induced toxicity and trafficking defects (Fig. 3D and E). We observed that *YCK1* overexpression attenuates the growth defect caused by two copies of the α Syn-GFP gene in WT and *yck1* Δ cells. In addition, in contrast to the absence of trafficking defect enhancement upon deletion of *YCK1*, overexpression of *YCK1* significantly reduced the percentage of cells showing abnormal localization of GFP-Snc1-Sso1 in cells constitutively expressing untagged α Syn (Fig. 3E). Interestingly, Ypt1, a Rab GTPase that also suppresses untagged α Syn toxicity (12), decreased mislocalization of GFP-Snc1-Sso1 to the same extent. These results suggest that Yck1 and Ypt1 may alleviate α Syn toxicity, at least in part, by directly promoting PM endocytosis.

α Syn contains a consensus CKI phosphorylation site on S129 that is phosphorylated by yeast and mammalian CKI *in vitro* and *in vivo* (14,24). To assess whether α Syn is phosphorylated at S129 in yeast, we monitored the levels of total and phosphorylated α Syn-GFP (pS129) over time in WT cells carrying two copies of the α Syn-GFP gene under the control of the *GAL1* promoter. Both α Syn and its phosphorylated form were detected after 1 h of inducing α Syn-GFP expression, indicating that α Syn is rapidly phosphorylated in yeast (Fig. 3F). To test whether CKI modulates directly or indirectly the phosphorylation of α Syn at S129 in our model, we compared the relative levels of pS129 in the WT, *yck1* Δ and *yck2* Δ strains after 1 h of induction. The *spo14* Δ strain was included as negative control. As shown previously (14), we

Table 1. Strains used in this study

Strain	MT ^a	Genotype	Source
BY4741	<i>MATa</i>	<i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(75)
FRY346	<i>MATa</i>	BY4741 <i>TPI1pr-8xMYC-SNC1::URA3</i>	This study
Y5563	<i>MATα</i>	<i>can1Δ::MFA1pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LYS2+</i>	(76)
VSY1	<i>MATa</i>	BY4741 <i>ade2Δ0::SNCA(WT)-GFP NatR</i>	This study
VSY2	<i>MATα</i>	Y5563 <i>trp1Δ0::SNCA(WT)-GFP URA3+</i>	This study
VSY4	<i>MATα</i>	VSY2 <i>ade2Δ0::SNCA(WT)-GFP NatR</i>	This study
VSY17	<i>MATa</i>	VSY4 <i>yck1Δ0::KanR</i>	This study
VSY58	<i>MATa</i>	VSY4 <i>yck2Δ0::KanR</i>	This study
VSY59	<i>MATa</i>	VSY4 <i>tlg2Δ0::KanR</i>	This study
VSY60	<i>MATa</i>	VSY4 <i>spo14Δ0::KanR</i>	This study
<i>yck1Δ</i>	<i>MATa</i>	BY4741 <i>yck1Δ0::KanR</i>	(87)
<i>yck2Δ</i>	<i>MATa</i>	BY4741 <i>yck2Δ0::KanR</i>	(87)
<i>spo14Δ</i>	<i>MATa</i>	BY4741 <i>spo14Δ0::KanR</i>	(87)
<i>tlg2Δ</i>	<i>MATa</i>	BY4741 <i>tlg2Δ0::KanR</i>	(87)
<i>pdr1Δ</i>	<i>MATa</i>	BY4741 <i>pdr1Δ0::KanR</i>	(87)
<i>did3Δ</i>	<i>MATa</i>	BY4741 <i>did3Δ0::KanR</i>	(87)
<i>end3Δ</i>	<i>MATa</i>	BY4741 <i>end3Δ0::KanR</i>	(87)
<i>rcy1Δ</i>	<i>MATa</i>	BY4741 <i>rcy1Δ0::KanR</i>	(87)
<i>sac6Δ</i>	<i>MATa</i>	BY4741 <i>sac6Δ0::KanR</i>	(87)
<i>vps23Δ</i>	<i>MATa</i>	BY4741 <i>vps23Δ0::KanR</i>	(87)
<i>vps35Δ</i>	<i>MATa</i>	BY4741 <i>vps35Δ0::KanR</i>	(87)
<i>ypt7Δ</i>	<i>MATa</i>	BY4741 <i>ypt7Δ0::KanR</i>	(87)
<i>ypt31Δ</i>	<i>MATa</i>	BY4741 <i>ypt31Δ0::KanR</i>	(87)
<i>ypt51Δ</i>	<i>MATa</i>	BY4741 <i>ypt51Δ0::KanR</i>	(87)
RSY255	<i>MATα</i>	<i>ura3-52 leu2-3,-112</i>	(88)
RY782	<i>MATα</i>	<i>his4-619 ura3-52 sec1-1</i>	(88)
RY112	<i>MATa</i>	<i>his4-619 ura3-52 leu2-3,-112 trp1-289 sec 7-4</i>	(88)
RY271	<i>MATα</i>	<i>his4-619 ura3-5 sec18-1</i>	(88)
VSY5	<i>MATα</i>	VSY4 <i>pdr1Δ0::KanR</i>	This study
VSY24	<i>MATa</i>	VSY17 <i>yck1Δ0::kanRΔ0::LEU2</i>	This study
VSY25	<i>MATa</i>	VSY24 <i>pdr1Δ0::KanR</i>	This study
W303-1A	<i>MATa</i>	<i>can1-100 his3-11 15 leu2-3 112 trp1-1 ura3-1 ade2-1</i>	(89)
VSY67	<i>MATa</i>	W303-1A <i>ura3-1::pRS306 URA3+</i>	This study
VSY68	<i>MATa</i>	W303-1A <i>ura3-1::GAL1pr-SNCA(WT)-GFP URA3+</i>	This study
VSY69	<i>MATa</i>	W303-1A <i>ura3-1::GAL1pr-SNCA(S129A)-GFP URA3+</i>	This study
VSY70	<i>MATa</i>	W303-1A <i>ura3-1::GAL1pr-SNCA(S129E)-GFP URA3+</i>	This study
VSY71	<i>MATa</i>	VSY67 <i>trp1-1::pRS304 TRP1+</i>	This study
VSY72	<i>MATa</i>	VSY68 <i>trp1-1::GAL1pr-SNCA(WT)-GFP TRP1+</i>	This study
VSY73	<i>MATa</i>	VSY69 <i>trp1-1::GAL1pr-SNCA(S129A)-GFP TRP1+</i>	This study
VSY74	<i>MATa</i>	VSY70 <i>trp1-1::GAL1pr-SNCA(S129E)-GFP TRP1+</i>	This study
VSY75	<i>MATa</i>	VSY71 <i>yck1Δ0::KanR</i>	This study
VSY76	<i>MATa</i>	VSY72 <i>yck1Δ0::KanR</i>	This study
VSY77	<i>MATa</i>	VSY73 <i>yck1Δ0::KanR</i>	This study
VSY78	<i>MATa</i>	VSY74 <i>yck1Δ0::KanR</i>	This study
VSY79	<i>MATα</i>	Y5563 <i>trp1Δ0::pRS405 LEU2+</i>	This study
VSY80	<i>MATα</i>	Y5563 <i>trp1Δ0::SNCA(WT)-GFP LEU2+</i>	This study
VSY81	<i>MATα</i>	Y5563 <i>trp1Δ0::SNCA(S129A)-GFP LEU2+</i>	This study
VSY82	<i>MATα</i>	Y5563 <i>trp1Δ0::SNCA(S129E)-GFP LEU2+</i>	This study
VSY83	<i>MATα</i>	VSY79 <i>pdr1Δ0::pRS465 URA3+</i>	This study
VSY84	<i>MATα</i>	VSY80 <i>pdr1Δ0::SNCA(WT)-GFP URA3+</i>	This study
VSY85	<i>MATα</i>	VSY81 <i>pdr1Δ0::SNCA(S129A)-GFP URA3+</i>	This study
VSY86	<i>MATα</i>	VSY82 <i>pdr1Δ0::SNCA(S129E)-GFP URA3+</i>	This study

^aMating type.

observed a modest (~30%) decrease in the relative levels of pS129 in the *yck1Δ* and *yck2Δ* mutants compared with the WT strain (Fig. 3G and H), confirming that CKI contributes partially to the phosphorylation of S129.

To determine whether the attenuation of growth and trafficking defects by Yck1 is mediated by direct phosphorylation of αSyn, we measured the levels of pS129 in WT and *yck1Δ* cells transformed with a plasmid for the overexpression of

YCK1 or the corresponding empty vector at 5.5 and 11 h of induction (Fig. 3I). As control, we treated cells with a combination of the phosphatase inhibitors (PI) okadaic acid and activated Na₃VO₄. *YCK1* overexpression did not increase αSyn phosphorylation in either WT or *yck1Δ* cells, suggesting that the attenuation of toxicity and trafficking defects by *YCK1* is not mediated by direct phosphorylation of αSyn at S129, but rather through the phosphorylation of other targets.

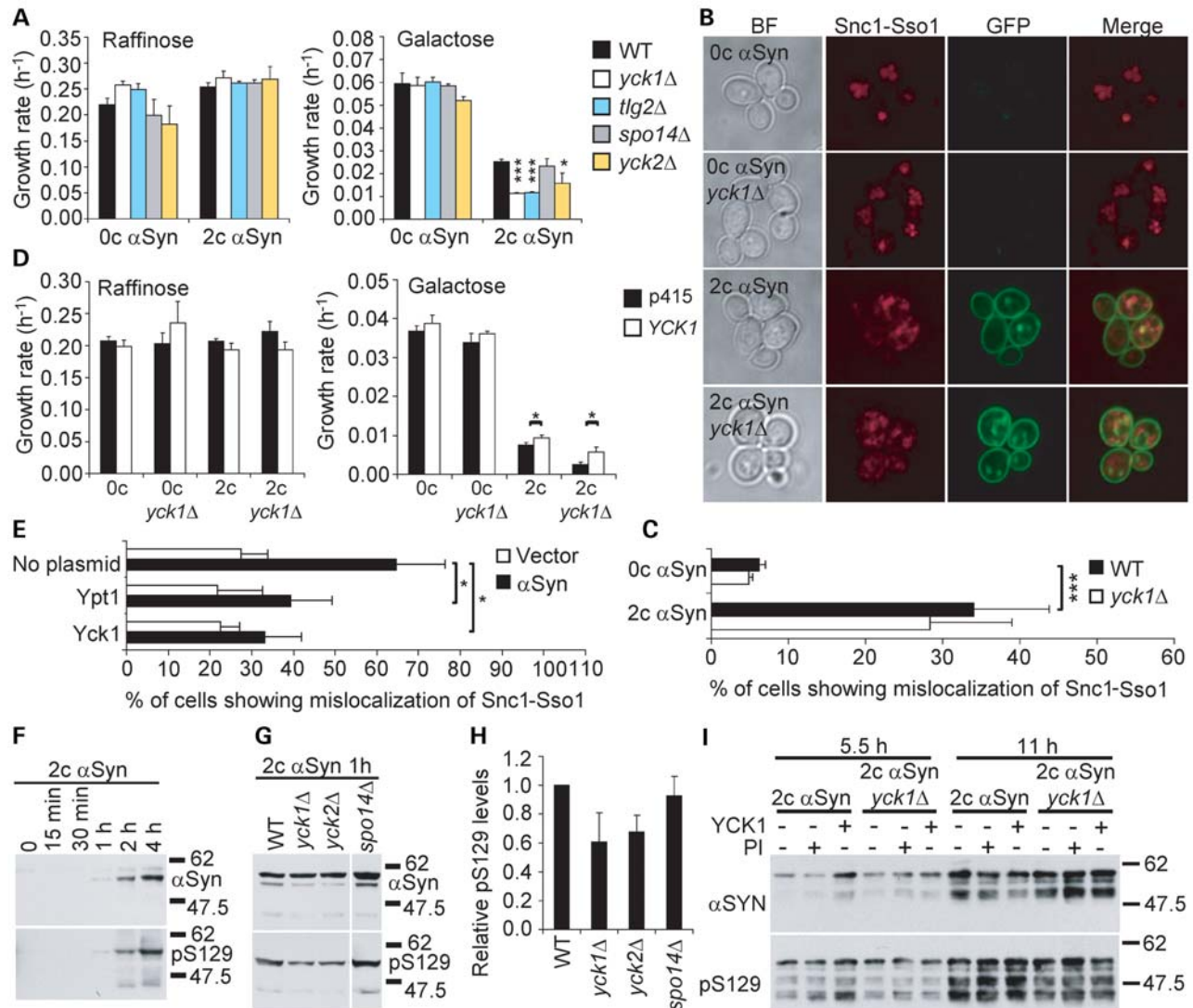


Figure 3. Yck1 attenuates α Syn-induced toxicity and trafficking defects through an S129 phosphorylation-independent mechanism. (A) Deletion of CKI genes (*yck1* Δ , *yck2* Δ) enhances the growth defect caused by α Syn overexpression in the BY4741 genetic background. Growth rates of WT cells or the indicated mutant strains containing either two copies (2c) or no copies (0c) of the α Syn-GFP gene in the BY4741 genetic background in conditions that do (galactose) or do not (raffinose) induce the expression of α Syn-GFP. The *tlg2* Δ and *spo14* Δ mutants are included as positive and negative controls, respectively. Growth rates were determined as the slope of the growth curves shown in Supplementary Material, Figure S3, during the logarithmic phase. (B) Deletion of *YCK1* does not enhance the defects in the trafficking of the marker Snc1-Sso1 caused by α Syn-GFP. WT or *yck1* Δ cells containing two copies (2c) or no copies (0c) of the α Syn-GFP gene in the BY4741 genetic background were transformed with a plasmid for the expression of trafficking marker mCherry-Snc1-Sso1, induced overnight in galactose-containing media and imaged in the logarithmic phase. (C) Quantification of the percentage of cells from (B) showing mislocalization of mCherry-Snc1-Sso1. (D) Overexpression of *YCK1* partially attenuates α Syn-induced growth defects. Growth rates of WT or *yck1* Δ cells containing two copies (2c) or no copies (0c) of the α Syn-GFP gene in the BY4741 genetic background and transformed with a plasmid for the overexpression of *YCK1* or the corresponding empty plasmid (p415). (E) Yck1 and Ypt1 partially restore targeting of GFP-Snc1-Sso1 to the vacuolar lumen. BY4741 cells expressing GFP-Snc1-Sso1 were transformed with either untagged WT α Syn under the control of a constitutive *GPD1* promoter or the corresponding empty vector and the indicated plasmids for the overexpression of *YPT1* or *YCK1*. After transfer to a galactose-containing medium, cells were imaged in the logarithmic phase. The percentage of cells exhibiting mislocalization of GFP-Snc1-Sso1 is shown. (F) Kinetics of α Syn induction and phosphorylation in WT cells containing two copies of the α Syn-GFP gene in the BY4741 genetic background. Cells were grown to the logarithmic phase ($OD_{600} \approx 0.8$) in raffinose-containing media and expression of α Syn was induced with galactose. Aliquots were collected at the indicated times and levels of phosphorylated (pS129) and total α Syn were analyzed by western blot. The soluble fraction is shown. The earliest α Syn was detected after 1 h of induction. (G) Deletion of *YCK1* or *YCK2* reduces α Syn phosphorylation modestly. The indicated strains were induced for 1 h and analyzed by western blot as described in (F). (H) Quantification of the S129 phosphorylation levels was estimated as the ratio of the band densities of phosphorylated relative to total α Syn from (G). (I) Overexpression of *YCK1* does not increase α Syn phosphorylation at S129. WT or *yck1* Δ cells containing two copies (2c) or no copies (0c) of the α Syn-GFP gene in the BY4741 genetic background and transformed with a plasmid for the overexpression of *YCK1* or the corresponding empty vector were grown to the logarithmic phase in raffinose-containing medium and induced with galactose. Aliquots were collected at the indicated times for western blot analysis. The indicated cultures were treated with the PI okadaic acid and activated Na_2VO_4 for 15 min before being collected. Error bars represent standard deviations from three experiments in (A), (C), (D) and (E) and two experiments in (H). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; (A, C, D and E) Student's *t*-test.

Phosphorylation regulates the toxicity and trafficking defects caused by α Syn in a genetic context-dependent manner

The role of S129 phosphorylation in disease pathogenesis is unclear, as contradictory results have been published in different models (29–32,41,42). To investigate the effect of S129 phosphorylation on α Syn-induced toxicity and trafficking defects in yeast, we generated strains that stably express WT α Syn-GFP or the phosphorylation mutants S129A-GFP or S129E-GFP from one or two genomic loci in the BY4741 strain background (Table 1). As expected, α Syn-GFP reduced cell growth in a dose-dependent manner (Fig. 4A). However, replacement of S129 by A or E did not alter α Syn-GFP toxicity or expression levels (Fig. 4A and B). These results suggest that phosphorylation at S129 does not modulate α Syn toxicity in the BY4741 strain background (although phosphorylation of other targets by Yck1 and Yck2 modulates α Syn toxicity as shown before). However, since the S129 phosphorylation status appears to govern α Syn neurotoxicity in fly and rat models (31,43), but not in two other models (29,32), we reasoned that variations in the genetic background of the models might account for the differential sensitivity of cells to the α Syn phosphorylation status.

To test this hypothesis, we generated strains that stably express WT α Syn-GFP or the phosphorylation mutants S129A-GFP or S129E-GFP from one or two genomic loci in the W303-1A genetic background (Table 1). This strain carries a mutation in the *YBP1* gene that increases its sensitivity to oxidative stress (44), a known mechanism of α Syn toxicity (45). Whereas one copy of any of the three α Syn-GFP alleles had no impact on yeast growth, two copies were detrimental in an allele-specific manner (Fig. 4C). Although the S129A mutation, which prevents phosphorylation, caused a dramatic increase in the growth defect caused by WT α Syn-GFP, the S129E-GFP mutation, which mimics phosphorylation, had no effect, suggesting that preventing phosphorylation enhances α Syn toxicity in the W303-1A strain background. Interestingly, the S129A-GFP mutation caused a significant ~6-fold increase in the percentage of cells with α Syn-GFP inclusions in comparison with WT α Syn-GFP without altering expression levels (Fig. 4D–F), suggesting that blocking phosphorylation of S129 enhances trafficking defects caused by α Syn-GFP on the W303-1A genetic background.

To confirm this hypothesis, we studied the trafficking of the dye FM 4-64 in the strains with two copies of the WT α Syn-GFP gene, or the S129A-GFP and S129E-GFP mutations in the W303-1A background. As reported previously (33), WT α Syn-GFP impaired the delivery of the dye to the vacuolar membrane (Fig. 4G and H). The S129A-GFP mutation significantly enhanced this defect, indicating that the enhancement of toxicity correlates with increased trafficking defects. Importantly, these defects were typically observed only in cells with α Syn inclusions (Fig. 4I), regardless of the α Syn variant expressed, confirming that the formation of inclusions is an indication of underlying trafficking defects and suggesting a molecular link between phosphorylation and trafficking.

To verify whether the modulation of α Syn toxicity by Yck1 observed in the BY4741-derived strain is mediated by S129 phosphorylation or an independent mechanism, we deleted or overexpressed *YCK1* in strains expressing two copies of the WT α Syn-GFP gene, or the S129A-GFP and S129E-GFP mutations in the W303-1A background. Unexpectedly, either deletion or overexpression of *YCK1* did not alter the growth defect of these strains, whether expressing WT α Syn-GFP or the S129-GFP mutations (Fig. 4J). These observations demonstrate that the genetic modification of a toxic phenotype is profoundly influenced by the genetic background and may help explain paradoxical observations reported in different models of α Syn toxicity.

Evidence of endosome anomalies and CK1 δ mislocalization in α Syn transgenic mice and in synucleinopathy brains

To determine whether defects in early-endocytic trafficking are a conserved pathologic feature of synucleinopathies, we studied the subcellular localization of the EE protein marker Rab5 in brain tissues from a transgenic (tg) mouse model of synucleinopathy (46) and human DLB/PD. In tg mice, overexpression of human α Syn results in the formation of α Syn-positive inclusion-like structures in the neocortex, hippocampus and substantia nigra (SN) by 2 months of age (46). In non-tg mice, Rab5 labeled discrete punctuate endosomes in the cytosol of cortical neurons at 6 months of age, whereas α Syn stained discrete puncta in the cell periphery. However, in tg animals, Rab5 labeled abnormally swollen endosomes in neurons that contained α Syn-positive intracellular inclusions (Fig. 5). Interestingly, the swollen endosomes co-localized with small granular aggregates of α Syn, but were excluded from large Lewy-body-like inclusions. Similarly, in cortical sections of human control subjects, Rab5 and α Syn exhibited a punctuate pattern that rarely co-localized. However, in human DLB/PD cases, Rab5 stained abnormal endosomal compartments that co-localized with α Syn granular aggregates but not with LBs. Although the nature of the enlarged Rab5-positive endosomes is unknown and they may not be equivalent to the accumulation of vesicles observed in yeast-overexpressing α Syn, the presence of these abnormal endosomes suggests that dysfunction of the endocytic pathway may occur in α Syn tg mice.

To examine the early-endocytic trafficking machinery in pathologic states *in vivo*, we analyzed by western blot the levels of Rab5 and two other EE markers, Rab4 and EEA1, in the α Syn tg mice and in a tg mouse model of Alzheimer's disease (AD), a non-strict form of synucleinopathy in which a processed fragment of α Syn deposits in extracellular amyloid plaques. In this model, expression of mutant human amyloid precursor protein (APP) leads to the formation of plaques in the frontal cortex by 4 months of age (47). At 6 months of age, both models exhibited elevated levels of monomeric and high molecular weight (HMW) species of α Syn in detergent-insoluble brain fractions (Fig. 6A and B). This change correlated with an apparent accumulation of Rab4 and HMW forms of Rab5, but no alterations in the mobility or levels of EEA1.

To validate these observations, we analyzed Rab5, Rab4 and EEA1 levels in two subgroups of human amyloidopathies,

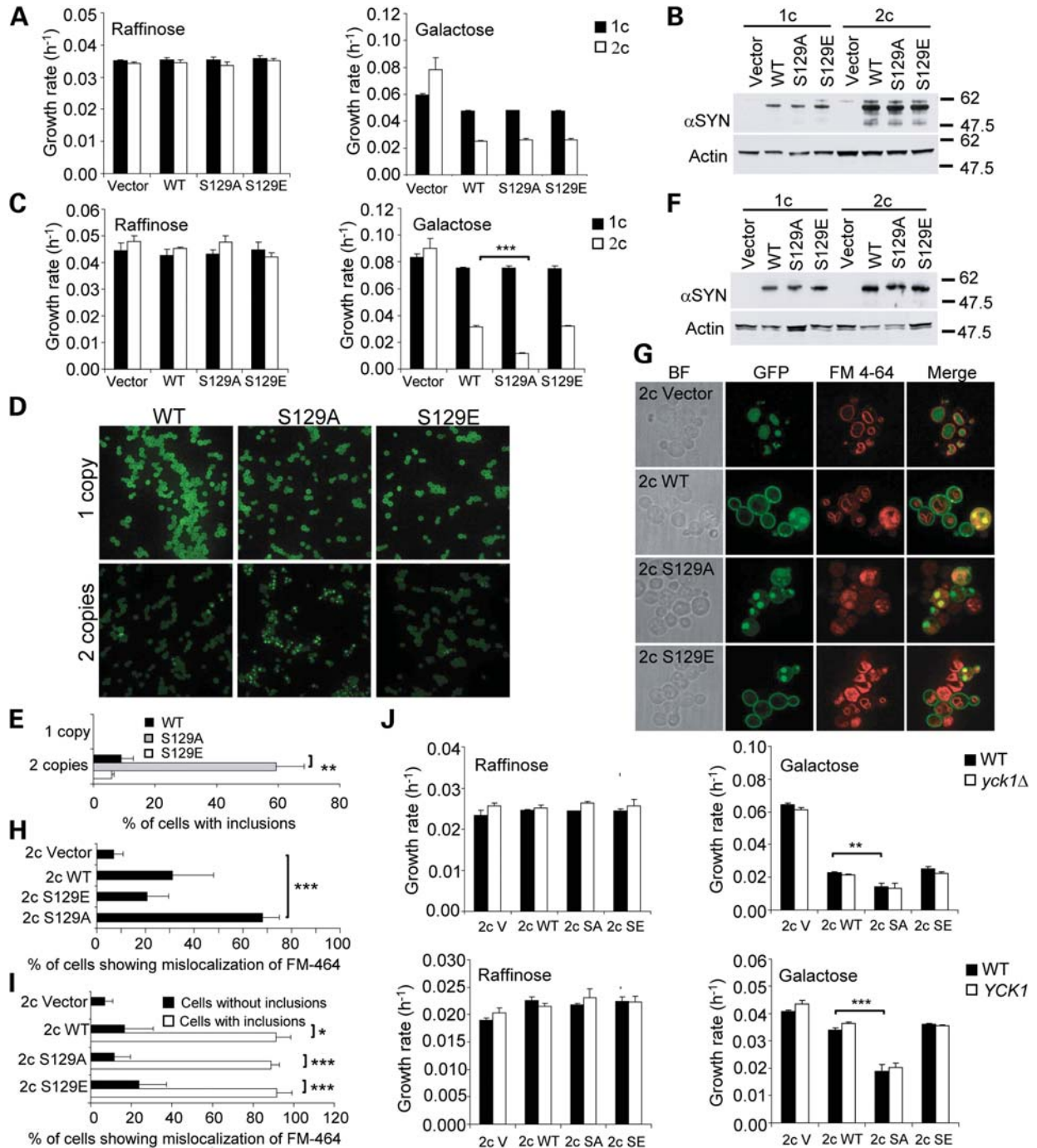


Figure 4. Preventing phosphorylation of S129 increases α Syn-induced toxicity and trafficking defects in a genetic context-dependent manner. (A) Growth rates of strains containing one (1c) or two (2c) copies of either the wild type (WT) α Syn-GFP gene or the phosphorylation mutants (S129A-GFP, S129E-GFP) or the corresponding empty vector in the BY4741 genetic background in conditions that do (galactose) or do not (raffinose) induce the expression of α Syn. (B) S129-GFP mutations do not alter the levels of α Syn in the BY4741 background. Western blot of strains from (A) grown for 8 h in galactose-containing medium. (C) Growth rates of strains containing one (1c) or two (2c) copies of either the WT α Syn-GFP gene or the phosphorylation mutants (S129A-GFP, S129E-GFP), or the corresponding empty vector in the W303-1A genetic background in conditions that do (galactose) or do not (raffinose) induce the expression of α Syn. (D) Localization of α Syn-GFP in the strains from (C) imaged in the logarithmic phase. (E) Quantification of the percentage of cells from (D) exhibiting α Syn inclusions. (F) S129 mutations do not alter the levels of α Syn-GFP in the W303-1A background. Western blot of strains from (C) grown for 8 h in galactose-containing medium. (G) Preventing S129 phosphorylation enhances mislocalization of FM 4-64 caused by α Syn-GFP in the W303-1A background. Strains from (C) were co-stained with the dye FM 4-64 and imaged in the logarithmic phase. (H) Quantification of the percentage of total cells from (G) showing mislocalization of the dye FM 4-64. (I) Quantification of the percentage of cells with and without inclusion from (G) showing mislocalization of the dye FM 4-64. A high percentage of cells with inclusions display anomalies in the localization pattern of FM 4-64. (J) Deletion or overexpression of *YCK1* in the W303-1A background does not modify α Syn-induced growth defects. Growth rates of strains from (A) in which *YCK1* was either deleted (*yck1 Δ , upper panels) or overexpressed (*YCK1*, lower panels) in conditions that do (galactose) or do not (raffinose) induce the expression of α Syn-GFP. SA, S129A; SE, S129E. (C, E, H and I) Error bars represent standard deviations from three experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; (C, E, H, I and J) Student's *t*-test.*

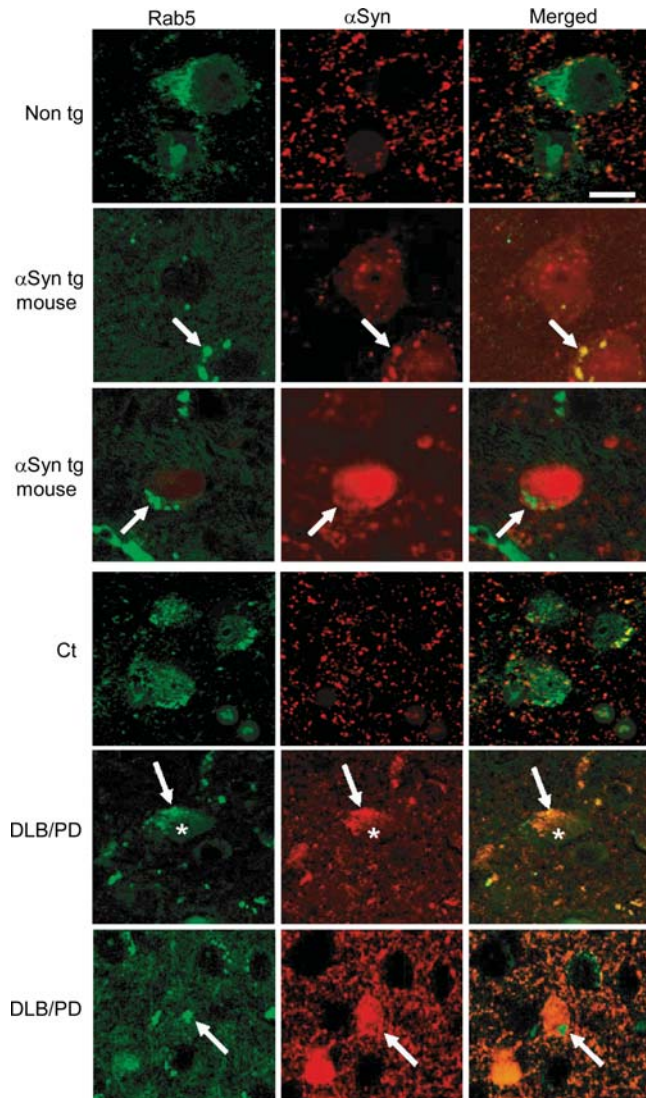


Figure 5. Abnormal endosome morphology in α Syn tg mice and in synucleinopathy brains. Abnormally enlarged Rab5-positive endosomes co-localize with granular inclusions of α Syn and accumulate in the vicinity of α Syn inclusions in α Syn tg mice and human DLB/PD. Cortical sections from α Syn tg mice or DLB/PD cases were double-labeled with antibodies against α Syn and Rab5 and detected with Tyramide Red or FITC-conjugated secondary antibodies, respectively. Images of non-tg animals and control (Ct) subjects are included for reference. Arrows indicate the Rab5-positive compartments. Scale bar, 10 μ m.

including DLB/PD and AD. Consistent with the mouse studies, detergent-insoluble HMW species of α Syn accumulated in strict synucleinopathies and AD cases compared with age-matched control subjects (Fig. 6C and D). In addition, levels of Rab5, but not Rab4 or EEA1, were markedly elevated in DLB/PD and AD relative to controls. As in the mouse models, Rab5 exhibited a mobility shift to HMW forms in all the amyloidopathy cases studied, whereas the mobility of Rab4 or EEA1 was unchanged. Although the functional significance of the Rab5 mobility shift is unknown, the co-localization of α Syn granular aggregates with enlarged endosomes and the correlation between accumulation of α Syn

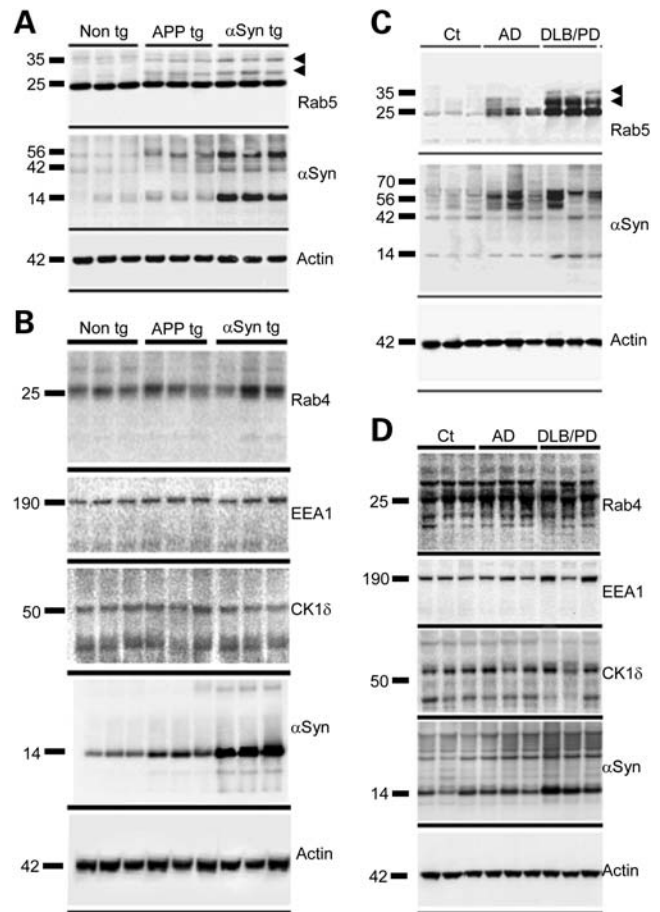


Figure 6. Analysis of levels of the EE markers Rab5, Rab4 and EEA1, and of mammalian CK1 δ in mice models and human synucleinopathies. Brain particulate fractions from α Syn tg, APP tg or non-tg mice (A and B) or from human DLB/PD or AD patients or control (Ct) subjects (C and D) were analyzed by western blot with the indicated antibodies. Arrowheads indicate HMW forms of Rab5.

HMW species and Rab5 suggest a causative role for α Syn in EE dysfunction. Consistent with this hypothesis, using small hairpin RNA-mediated gene knock down and overexpression studies, we recently found that *stx7*, *Vps24*, *Vps28*, *Vps34*, *Vps45* and *Vps52*, proteins involved in endosomal transport, modulate α Syn toxicity in a dopaminergic SH-SY5Y neuroblastoma cell line and in primary neurons (Lee *et al.*, manuscript in review).

To investigate a possible involvement of mammalian CKI proteins in the pathogenesis of synucleinopathies *in vivo*, we studied the subcellular localization of CKI δ , involved in vesicle transport (48,49), in brain sections from α Syn tg mice and human DLB/PD (Fig. 7). As expected, CKI δ localized predominantly to the cell periphery in cortical neurons from non-tg animals. In contrast, CKI δ co-localized with α Syn inclusions in tg animals. Importantly, this association was also found in neuronal inclusions in human DLB/PD, consistent with the notion that CKI δ may be sequestered by/recruited to α Syn inclusions in synucleinopathies.

Elevated levels of CKI δ mRNA have been detected in AD brains (50), suggesting that upregulation of CKI δ may be a

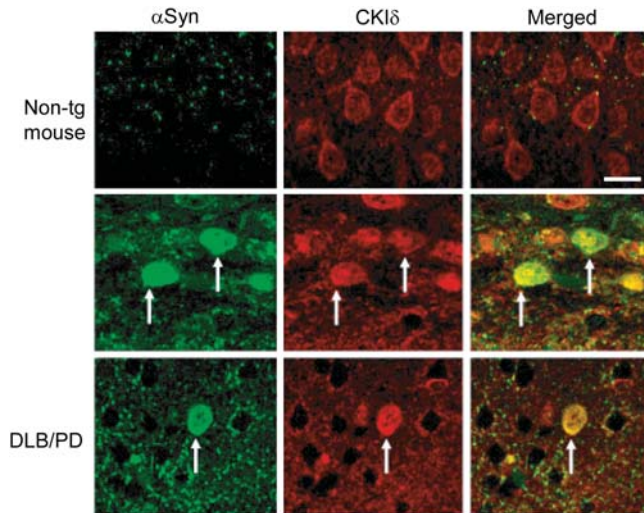


Figure 7. CK1 δ co-localizes with α Syn inclusions in α Syn tg mice and human DLB/PD. Cortical sections from non-tg mice, α Syn tg mice or human DLB/PD cases were double-labeled with antibodies against α Syn and CK1 δ and detected with FITC and Tyramid Red-conjugated secondary antibodies, respectively. Arrows indicate the α Syn-positive inclusions. Scale bar, 10 μ m.

compensatory response in AD. To investigate whether CK1 δ is upregulated in synucleinopathies, we analyzed the levels of CK1 δ by western blot in brains from α Syn and APP tg mice and human DLB/PD and AD cases (Fig. 6B and D). We did not detect any significant changes in the levels of CK1 δ in mice models or human synucleinopathies, indicating that CK1 δ is not upregulated in response to α Syn accumulation. CK1 δ levels were also apparently unchanged in AD cases, suggesting that the observed mRNA upregulation in AD may not lead to increased CK1 δ synthesis, or that the upregulation is tissue-specific and undetectable in whole-brain homogenates.

DISCUSSION

A number of recent studies have shown that α Syn overexpression causes vesicle trafficking defects in a wide variety of model systems (7–10,12,13,15,17) by perturbing SNARE function (11,51,52). In yeast, an early effect of expressing α Syn by a strong galactose-inducible system is an ER-to-Golgi block that precedes a global trafficking failure (12,13). This trafficking collapse is accompanied by the formation of α Syn-positive vesicular clusters that co-localize with protein markers of several trafficking routes, including ER-to-Golgi, intra-Golgi, Golgi-to-PM, EE-to-LE, and LE-to-vacuole (13,15). The presence of endosome-to-Golgi markers in the clusters is controversial since Gitler *et al.* (13) detected Ypt6, but Soper *et al.* (15) failed to detect Vps17 or Vps29. Importantly, the Rab GTPase Ypt1 and the SNARE Ykt6, involved in ER-to-Golgi vesicle-mediated transport, suppress α Syn-induced trafficking defects (11–13).

In this study, we present evidence of the steady-state impairment of late-exocytic, early-endocytic and/or recycling trafficking by constitutive expression of α Syn in yeast from the *GPD1* promoter. In agreement with previous studies, we

observed that trafficking defects coincide with an accumulation of α Syn-positive vesicles that originate in the vicinity of the cell surface and progressively expand toward the cell interior. The vesicles co-label with Snc1, an exocytic SNARE that is targeted to the PM and subsequently internalized and recycled for re-use via EE and the Golgi, implying that at least some of the vesicles originate in the Golgi or the PM. We propose that, in our system, α Syn blocks a trafficking step that follows vesicle budding from the Golgi or the PM and precedes fusion to target membranes. However, we cannot exclude the possibility that the observed vesicles constitute a cellular response to cope with the α Syn overload by compartmentalizing the toxic protein.

In our studies, α Syn did not impair the targeting of a number of protein trafficking markers traversing the ER and the Golgi toward the vacuole, indicating that ER-to-Golgi transport is unaffected in our model. The discrepancy between our findings and previous studies may be reconciled by differences in the expression levels, toxicity and duration of the insult attained by distinct expression systems (53,54). Whereas prior studies used a galactose-inducible promoter, our studies used a constitutive promoter to assess the effects of α Syn expression on protein trafficking. Interestingly, in the current study, we show that Ypt1 rescues the mislocalization of GFP-Snc1-Sso1 caused by constitutive α Syn expression. Although it is well established that Ypt1 functions in ER-to-Golgi trafficking, this protein also facilitates the recycling of internalized PM proteins (55). Therefore, we propose that Ypt1 rescues α Syn toxicity by promoting, at least in part, the recycling of PM proteins. Consistent with this interpretation, Ypt6, which is involved in endosome-to-Golgi and intra-Golgi retrograde transport (56), was also shown to partially suppress α Syn toxicity (12,13). Thus, although it is conceivable that the trafficking defects that we observed might be secondary to the sustained imposition of the primary ER-to-Golgi block, in our system, α Syn appears to directly impair late-exocytic, early-endocytic and/or recycling trafficking without affecting other pathways.

Our observations contribute to accumulating evidence that trafficking defects are a conserved mechanism of pathogenesis in human synucleinopathies. In yeast, Rab GTPases governing multiple trafficking steps are sequestered by α Syn-induced vesicle clusters (13). Similarly, in humans, members of the Rab family implicated in exocytosis (Rab3a), endocytosis (Rab5) and polarized traffic (Rab8a) interact aberrantly with α Syn in DLB and co-localize with α Syn glial inclusions in MSA (57–59). Conversely, Rab1 (involved in ER-to-Golgi transport), Rab3a and Rab8a are neuroprotective in cellular and animal models in which α Syn is overexpressed (12,13). In catecholaminergic cells, α Syn impairs exocytosis, leading to an accumulation of docked vesicles (9). Finally, clusters of dense core vesicles have been observed in the perimeter of LBs (15,60,61).

Our study provides evidence of anomalies in endosome morphology and the endocytic Rab5 in pathologic states *in vivo*. In mouse models and human synucleinopathies, EE are abnormally enlarged in cortical neurons and Rab5 co-localizes with α Syn granular inclusions and accumulates abnormally in detergent-insoluble fractions from brain lysates. Similarly, we observed an increase in the levels of Rab4 in α Syn tg mice, but

not in human DLB/PD cases, whereas the levels of EEA1 were unchanged in mice and humans. Both Rab4 and Rab5 are GTPases involved in early endocytic trafficking, although they differ in their functional specialization. Whereas Rab5 regulates the fusion between endocytic vesicles and EE, as well as the homotypic fusion between EE (62), Rab4 controls the function or formation of endosomes involved in endocytic recycling (63). In contrast, EEA1 is a Rab5 effector (64). Therefore, α Syn appears to alter the function of endocytic Rab GTPases without altering the levels of downstream effectors. In agreement with our observations, inhibition of Rab5 GTPase activity results in the formation of unusually large early endocytic structures (65), a phenotype mimicked by the overexpression of α Syn. These alterations suggest that endocytic trafficking defects might also occur and contribute to neuronal dysfunction in synucleinopathies. In striking similarity to our yeast studies, an RNAi screen in the nematode *C. elegans* showed that endocytosis-defective mutants potently exacerbate α Syn neurotoxicity (17). Worms that overexpressed α Syn displayed decreased neurotransmitter release, similar to endocytosis-defective mutants. These authors also reported that the knock down of a CK1 gene enhances α Syn toxicity, and showed that α Syn phosphorylated at S129 accumulated in mutants defective in endocytosis (17).

In agreement with this model, we found that deletion of *YCK1* or *YCK2*, two redundant kinases of the CKI family that promote the endocytosis and delivery of PM proteins to the vacuole, led to an increase in α Syn toxicity. Interestingly, *yck1* Δ cells accumulate cargo normally destined for the vacuole in an endocytic compartment, but do not affect trafficking through the CPY and ALP pathways to the vacuole (37). These results are further supported by our previous studies in which the yeast SNARE Tlg2 was identified as a loss-of-function enhancer of α Syn toxicity (20). Tlg2 participates in endosome-to-Golgi recycling and is required for targeting Yck2 to the PM (39), suggesting that exacerbation of α Syn toxicity in *tlg2* Δ cells might be due, at least in part, to decreased CKI activity at the PM. Although we did not detect any significant increase in trafficking defects upon deletion of *YCK1*, we observed a reduction in α Syn-induced growth and trafficking defects upon overexpression of *YCK1*. Although Yck1 contributes modestly to the phosphorylation of α Syn at S129, our results indicate that the attenuation of the toxicity and trafficking defects is not mediated by increased phosphorylation of α Syn. Thus, it is conceivable that CKI activity protects against α Syn toxicity by directly promoting PM endocytosis. This hypothesis is consistent with the observation that knocking down the *C. elegans* *YCK1* ortholog *csnk-1* by RNAi causes synaptic deficits selectively in α Syn tg worms (17).

The function of S129 phosphorylation in physiologic and pathologic conditions is unclear. Although only a small fraction of α Syn is phosphorylated in the healthy brain, α Syn is hyperphosphorylated at S129 in pathologic lesions (21,22,66). However, the relevance of S129 phosphorylation to disease pathogenesis is unknown since conflicting observations have been reported. Mimicking phosphorylation has been shown to be neuroprotective (31) or innocuous (29,32) in rats, but detrimental in *Drosophila* (30), and SH-SY5Y and oligodendroglial cells (41,42). We showed that, in yeast, the

effect of S129 phosphorylation on α Syn toxicity is exquisitely dependent on genetic context; although blocking S129 phosphorylation is innocuous in BY4741-derived yeast strains, this markedly increased the toxicity and trafficking defects caused by α Syn in W303-1A-derived strains, supporting a protective role for phosphorylation in specific genetic contexts. Interestingly, W303-1A carries a mutation in the *YBP1* gene that decreases oxidative stress responses and increases the sensitivity to oxidative stress. This genetic variability could contribute to the differential sensitivity between the two strain backgrounds to S129A α Syn. Consistent with this hypothesis, α Syn causes oxidative stress and ROS accumulation and increases the vulnerability of yeast to hydrogen peroxide (16,67). Conversely, antioxidants and genes involved in the stress response suppress α Syn toxicity in yeast (18,68).

Taken together, our studies indicate that α Syn toxicity is linked to trafficking defects and that this phenotype is modulated by phosphorylation-dependent and -independent pathways. For example, the attenuation of toxicity and trafficking defects by *YCK1* appears to be uncoupled from S129 phosphorylation. However, the relative contribution of each pathway to α Syn toxicity appears to be dependent on the genetic landscape of the cell.

A previous study by Zabrocki *et al.* (14) showed that Yck1, Yck2, Yck3 and CKII phosphorylate α Syn at S129. However, although deletion of *YCK1* and *YCK2* modestly alleviated an α Syn-induced growth defect and stabilized α Syn at the PM, deletion of *YCK3* and the four subunits of CKII (*CKA1*, *CKA2*, *CKB1* and *CKB2*) exacerbated the growth defect and resulted in α Syn accumulation in intracellular compartments. The latter observation is consistent with the genetic context-dependent enhancement of α Syn toxicity and inclusion formation by the S129A allele we report in this study. Therefore, despite some discrepancies regarding the effects of CKs on α Syn toxicity, the general conclusion arising from both studies is that impairment of endocytic trafficking can at least partially account for increased α Syn toxicity.

There is substantial evidence that mammalian CKs phosphorylate α Syn at S129 in cultured cells and *in vivo* (22–24). In neurons, a number of mammalian CKI isoforms associate with synaptic vesicles, and the phosphorylation of CKI substrates is thought to regulate synaptic vesicle trafficking and neurotransmission. Mammalian CK substrates include proteins implicated in synaptic vesicle formation (AP-3 adaptor complex) (69), docking and fusion (p65) (70) and exocytosis (synaptotagmin I) (71), and in the storage of neurotransmitters (VMAT2) (72). Interestingly, some CKI mRNA isoforms (α , δ and ϵ , but especially δ) are dramatically upregulated in the hippocampus and associated with tau-containing neurofibrillary tangles in AD and other dementias (50). Here, we describe for the first time the co-localization of CKI δ with LBs in DLB/PD, suggesting that CKI δ may be sequestered in a manner that might prevent proper phosphorylation of its canonical substrates in synucleinopathies.

Based on our observations and previous studies, we have generated a model to describe the cascade of pathogenic events that lead to neuronal dysfunction and death in synucleinopathies (Fig. 8). As α Syn is normally a synaptic vesicle-associated protein, we hypothesize that, under physiologic conditions, CKI δ regulates neurotransmission by

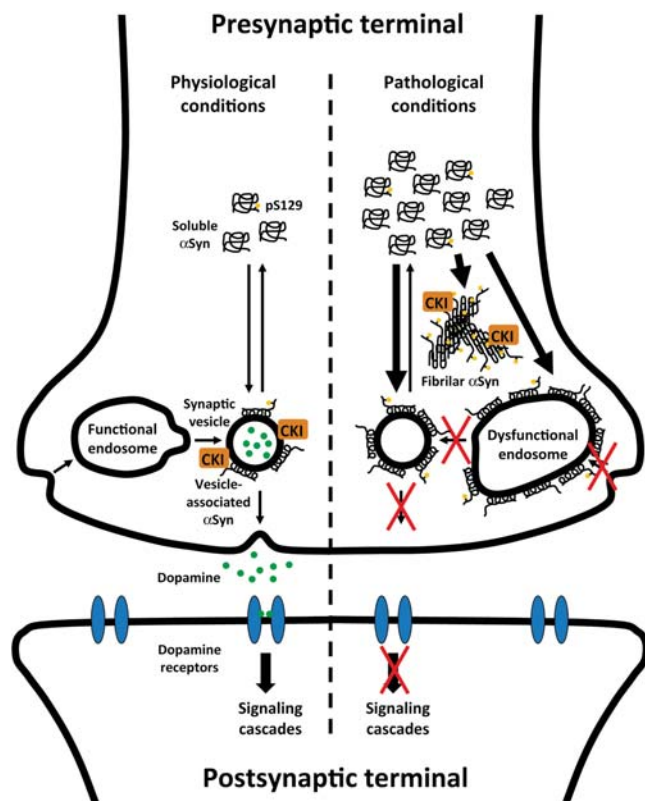


Figure 8. A model depicting how α Syn accumulation may cause neurotransmission defects in PD. We hypothesize that, under normal conditions, CKI regulates α Syn function and, possibly, association with synaptic vesicles and neurotransmitter release by phosphorylating synaptic proteins, including α Syn at S129. Under pathologic conditions in which α Syn accumulates and synaptic trafficking and neurotransmission are compromised, CKI may play a protective role in attenuating vesicular trafficking defects and restoring synaptic transmission. However, when all binding sites in synaptic vesicles are saturated, excess α Syn may associate with other compartments, such as endosomes, leading to abnormally enlarged endosomes and defects in synaptic vesicle homeostasis and neurotransmission. In addition, excess α Syn may form insoluble fibrillar deposits that sequester CKI, depleting CKI activity and enhancing synaptic defects. Red crosses indicate possible trafficking blocked by α Syn.

phosphorylating synaptic proteins, potentially including α Syn. Under pathologic conditions, the progressive accumulation of α Syn may lead to sequestration of Rab GTPases and deficits in vesicular endocytic/exocytic/recycling trafficking that ultimately impair neurotransmitter release. We speculate that when all binding sites in synaptic vesicles are saturated, excess α Syn may associate with other compartments, including endosomes, leading to sustained defects in synaptic vesicle homeostasis and neurotransmission. CKI δ -dependent phosphorylation of vesicular substrates, including α Syn, may play a protective role by attenuating trafficking defects and stabilizing synaptic transmission. In addition, excess α Syn deposited in LBs may irreversibly sequester CKI δ and other vesicle-associated proteins, resulting in the loss of CKI δ activity and reduced protection against these defects. In summary, our study provides additional evidence that vesicular trafficking defects involving endocytosis and exocytosis and CKI δ dysfunction may be relevant for the pathogenesis of synucleinopathies.

MATERIALS AND METHODS

Plasmids

Plasmids p426GPD α Syn(WT)GFP, p426GAL α Syn(WT)GFP, pGS416 (*GFP-SNC1*), pGSSO416 (*GFP-SNC1-SSO1*), pGNS416 (*GFP-NYVI-SSO1*), pPEP416 (*GFP-PEP12*), pPHM5 (*GFP-PHM5*) and pSNA3416 (*SNA3-GFP*) have been described (33–36).

Plasmid pSTE2416 was created by replacing the *SNA3* gene from plasmid pSNA3416 (36) with the *STE2* gene as a *HindIII*–*AgeI*-digested product of PCR amplification.

Plasmid p8xmycSNC1416 (8xMYC-SNC1) was created by replacing the *GFP* gene from plasmid pGS416 with the 8xMYC sequence as a *HindIII*–*EcoRI*-digested product of PCR amplification. Plasmid p8xmycSNC1406 was then created by subcloning the *TP11pr-8xMYC-SNC1* fusion from plasmid p8xmycSNC1416 into the *XhoI* and *BamHI* sites of the pRS406 integrating vector (73). p8xmycSNC1406 was linearized with *EcoRV* for integration.

Plasmid pmCheSSO416 (*mCherry-SNC1-SSO1*) was created by replacing the *GFP* tag from plasmid pGSSO416 with the *mCherry* gene from plasmid pmCheV5ATG8406 (74) as an *XhoI*–*EcoRI* fragment. Plasmid pmCheSSO415 was created by subcloning the *TP11pr-mCherry-SNC1-SSO1* fusion from plasmid pmCheSSO416 into the *XhoI* and *SacI* sites of the plasmid p415TEF (54).

Plasmid p423GPD α SYN(WT)GFP was created by subcloning the *SNCA*(WT)-*GFP* fusion from plasmid p426GAL α SYN(WT)GFP into the *SpeI* and *XhoI* sites of plasmid p423GPD (54).

Plasmid p423GPD α SYN(WT) was created by subcloning *SNCA*(WT) from plasmid p426GPD α SYN(WT)GFP into the *SacI* and *XhoI* sites of p423GPD (54).

Plasmids p426GAL α SYN(S129A)GFP and p426GAL α SYN(S129E)GFP were created by recombining PCR-amplified *SNCA*(S129A) and *SNCA*(S129E) (kindly provided by Dr Robert Edwards, UCSF) into *BamHI*-linearized p426GAL α SYN(WT)GFP. Plasmids pRS304 α SYN(WT)GFP, pRS304 α SYN(S129A)GFP, pRS304 α SYN(S129E)GFP, pRS306 α SYN(WT)GFP, pRS306 α SYN(S129A)GFP, pRS306 α SYN(S129E)GFP were then created by subcloning the *GAL1pr-SNCA*(WT, S129A and S129E)-*GFP* fusions from the p426GAL-derived plasmids into the *SacI* and *KpnI* sites of the integrating vectors pRS304 and pRS306 (73). Plasmids pRS304(MCS-) and pRS306(MCS-), lacking the multiple cloning site, were created by digesting pRS304 and pRS306 with *SacI* and *KpnI*, blunting the ends with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs) and re-circularizing the plasmids. pRS304- and pRS306-derived plasmids were linearized with *EcoRV* for integration into the W303-1A strain.

Plasmids pRS405TRP1 α SYN(WT)GFP, pRS405TRP1 α SYN(S129A)GFP and pRS405TRP1 α SYN(S129E)GFP were generated by sequential insertion of the *GAL1pr-SNCA*(WT, S129A and S129E)-*GFP* fusions from the p426GAL-derived plasmids into the *SacI* and *KpnI* sites of the integrating vector pRS405, followed by insertion of the first and the last 300 bp of the *TRP1* ORF in inverted order (3'5') separated by an *XmaI* site into the *SacI* site of plasmid pRS405 for gamma integration into the *TRP1* locus. Plasmid

pRS405TRP13'5' was generated by removing the *GAL1pr-SNCA*(WT, S129A and S129E)-*GFP* insert within the *SpeI* and *XhoI* sites, blunting the ends with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs) and re-circularizing the plasmids.

Plasmids pRS406PDR1 α SYN(WT)GFP, pRS406PDR1 α SYN(S129A)GFP and pRS406PDR1 α SYN(S129E)GFP were generated by sequential insertion of the *GAL1pr-SNCA*(WT, S129A and S129E)-*GFP* fusions from the p426GAL-derived plasmids into the *SacI* and *KpnI* sites of the integrating vector pRS406, followed by insertion of the first and the last 300 bp of the *PDR1* ORF in inverted order (3'5') separated by an *MfeI* site into the *SacI* site of plasmid pRS406 for gamma integration into the *PDR1* locus. Plasmid pRS406PDR13'5' was generated by removing the *GAL1pr-SNCA*(WT, S129A and S129E)-*GFP* insert within the *AgeI* and *XhoI* sites, blunting the ends with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs) and re-circularizing the plasmids.

pRS405- and pRS406-derived plasmids were linearized with *XmaI* and *MfeI*, respectively, for integration into the Y5563 strain.

Plasmids p425GALYPT1 and p425GALYCK1 were created by subcloning *YPT1* and *YCK1* from the Yeast ORF collection (Open Biosystems) into p425GAL (53) (Addgene), using Gateway Technology (Invitrogen).

Plasmid p415YCK1 was created by cloning *YCK1* with its endogenous promoter into plasmid p415TEF (54) as a *SacI*-*BamHI*-digested product of PCR amplification from yeast genomic DNA.

Yeast strains and manipulation

The strains used in this study are summarized in Table 1.

To generate strain VSY1, the *GAL1pr-SNCA*(WT)-*GFP* fusion was PCR-amplified from plasmid p426GAL α SYN(WT)GFP, and the *MX4-NatR* cassette was PCR-amplified from plasmid p4339 (kindly provided by Dr Charles Boone, University of Toronto). Primer sequences were designed to enable the merging of both fragments in a third PCR reaction and the site-directed integration of the resulting *GAL1pr-SNCA*(WT)-*GFP-NatR* fusion at the *ADE2* locus by homologous recombination in the BY4741 strain (75).

To generate strain VSY2, the *GAL1pr-SNCA*(WT)-*GFP* fusion was PCR-amplified from plasmid p426GAL α SYN(WT)GFP, and the *URA3-MX6* cassette was PCR-amplified from plasmid p4348 (kindly provided by Dr Charles Boone, University of Toronto). Primer sequences were designed to enable the merging of both fragments in a third PCR reaction and the site-directed integration of the resulting *GAL1pr-SNCA*(WT)-*GFP-URA3* fusion at the *TRP1* locus by homologous recombination in the Y5563 strain (76).

To generate strain VSY4, strains VSY1 and VSY2 were crossed. Diploid cells were selected on SD-Ura + G418 (Invitrogen)/ClonNAT (Werner BioAgents) plates and sporulated. Spores were germinated and haploid cells of both mating types were selected in SD-Arg/Lys/Ura + canavanine (Sigma-Aldrich)/thialysine (Sigma-Aldrich)/G418/clonNAT

plates. *MAT α* cells were selected by their inability to grow on SD-His plates, and the mating type was subsequently confirmed by mating test.

Strains VSY53-61 were obtained from a cross between strain VSY4 and the corresponding deletion strains.

Strains *pdr1 Δ* , *yck1 Δ* , *yck2 Δ* , *tlg2 Δ* and *spo14 Δ* were retrieved from the Yeast *MAT α* Genome Deletion Collection (Open Biosystems).

Strain VSY5 was generated by disruption of the *PDR1* gene from strain VSY4 with the *kanMX4* cassette obtained by PCR amplification of genomic DNA from strain *pdr1 Δ* .

Strain VSY24 was generated by disruption of the *kanMX4* cassette from strain VSY17 with the *LEU2* cassette obtained by PCR amplification from plasmid pRS415.

Strain VSY25 was generated by disruption of the *PDR1* gene from strain VSY24 with the *kanMX4* cassette obtained by PCR amplification of genomic DNA from strain *pdr1 Δ* .

Strain FRY346 was generated by integration of *EcoRV*-linearized p8xmycSNC1406 plasmid in the BY4741 strain.

Strains VSY67 to VSY74 were generated by consecutive integration of *EcoRV*-linearized pRS306- and pRS304-derived plasmids in the W303-1A strain.

Strains VSY75-78 were generated by disruption of the *YCK1* gene from strains VSY71-78 with the *kanMX4* cassette obtained by PCR amplification of genomic DNA from strain *yck1 Δ* .

Strains VSY79-86 were generated by consecutive integration of *XmaI*-linearized pRS405-derived plasmids, followed by integration of *MfeI*-linearized, pRS406-derived plasmids in the Y5563 strain.

Strains BY4741 and FRY346 were transformed with the indicated plasmids, using the one-step protocol (43) and cultured in synthetic complete medium without the corresponding nutrients for auxotrophic selection and with the indicated carbon sources.

The integrated strains were transformed using the standard protocol (77) and cultured in rich (YEP) medium with the indicated carbon sources.

Yeast growth curves

The indicated strains were inoculated in triplicate in 100 μ l of raffinose-containing medium in 96-well plates and grown for 48 h to the stationary phase. Cultures were then diluted 100-fold in raffinose- and galactose-containing media and incubated at 30°C. The OD₆₀₀ was recorded at the indicated times. Growth rates were determined as the slope of the growth curves during the logarithmic phase.

Pharmacologic inhibition of CKI activity

For the CKI inhibitor studies, strains *pdr1 Δ* (0c α Syn in *pdr1 Δ*), VSY5 (2c α Syn in *pdr1 Δ*) and VSY24 (2c α Syn in *pdr1 Δ yck1 Δ*) were grown in raffinose-containing medium to the stationary phase, diluted to OD₆₀₀ = 0.1 in raffinose- and galactose-containing media and dispensed in 100 μ l of aliquots to 96-well plates. Aliquots were treated in triplicate with the indicated concentrations of D4476 (Calbiochem, CA, USA) or vehicle DMSO alone.

Table 2. Characteristics of human cases analyzed in this study

Group	Age (years)	Gender M/F ^a	Blessed score (range)	Braak stage (range)	Plaques per mm ²	Tangles per 0.1 mm ²	Lewy bodies	Brain weight (g)
Non-demented (<i>n</i> = 4)	83 ± 2	2/2	0–1	0–1	0.25	0	0	1150 ± 40
AD (<i>n</i> = 6)	81 ± 2	3/3	13–33	5–6	25 ± 3	5 ± 1	0	1070 ± 35
DLB/PD (<i>n</i> = 8)	83 ± 1	5/3	6–33	2–4	28 ± 3	2 ± 1	3 ± 1	1110 ± 60

^aMales/Females.

Phosphorylation assays

For the time-course experiment, strain VSY4 (2c α Syn in WT) was grown to the logarithmic phase ($OD_{600} \approx 0.8$) in raffinose-containing medium, and α Syn expression was induced by adding 0.2% galactose. At the indicated times, 8 ml of aliquots were collected. For protein extraction, cells were collected by centrifugation, washed with water and resuspended in 200 μ l of extraction buffer [200 mM Tris, pH 8.0, 150 mM ammonium sulfate, 10% glycerol, 1 mM EDTA, 1 μ M microcystin LR, 200 μ M activated Na_3VO_4 and 1 \times complete protease inhibitor cocktail (Roche)] and 100 μ l of acid-washed glass beads (425–600 μ m) (Sigma-Aldrich). Cells were lysed by vortexing two times for 5 min at 4°C. Supernatants were separated from cell debris and beads by centrifugation at 5000 r.p.m. for 5 min and then cleared by centrifugation at 13 000 r.p.m. for 30 min. The soluble fractions (supernatant) were separated by SDS–PAGE and proteins analyzed by immunoblot with mouse anti- α Syn (1:20 000) (BD Transduction Laboratories) and anti-S129 phospho-specific antibodies (1:20 000) (JH22.11A5, Elan Pharmaceuticals).

For comparison of α Syn phosphorylation levels in different deletion backgrounds, strains VSY4, VSY17, VSY58 and VSY60 were grown to the logarithmic phase ($OD_{600} \approx 0.8$) in raffinose-containing medium, and α Syn expression was induced by adding 0.2% galactose for 1 h. Samples were treated as described before, and soluble fractions were analyzed by western blot. Band densities were quantified with ImageQuant 5.2 (Molecular Dynamics).

To assess α Syn phosphorylation in *YCK1*-overexpressing cells, the indicated strains were grown for 12 h to the logarithmic phase in raffinose-containing medium and induced with 2% galactose. At 5.5 and 11 h, 10 ml of aliquots were collected. When indicated, cultures were treated with 500 nM microcystin and 200 μ M cell-permeable Na_3VO_4 for 15 min prior to cell harvesting. Samples were analyzed as described before.

Analysis of α Syn levels in yeast

Strains VSY67-74 and VSY79-86 were grown to the logarithmic phase in raffinose-containing medium and induced with 2% galactose for 8 h. Samples were processed and analyzed by western immunoblot as described before.

Analysis of α Syn, Rab5, Rab4, EEA1 and CK1 δ cellular levels

Brain homogenates were solubilized in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES, pH 7.4, 140 mM NaCl,

1 mM EDTA, 1 mM Na_3VO_4 , 20 mM β -glycerophosphate and proteinase inhibitor cocktails) and separated into cytosolic and particulate fractions by centrifugation. Twenty milligrams of the particulate fractions were resolved by SDS–PAGE and blotted onto membranes before being decorated with rabbit polyclonal anti- α Syn (Chemicon), mouse monoclonal anti-Rab5 (BD Transduction Laboratories), mouse anti-human EEA1 (BD Transduction Laboratories), mouse anti-human Rab4 (BD Transduction Laboratories), goat anti-CK1 δ (C-18) (Santa Cruz Biotechnology) and mouse monoclonal anti-actin (Chemicon).

Mouse models

For this study, 12 heterozygous tg, 6-month-old mice expressing human α Syn under the regulatory control of the platelet-derived growth factor- β promoter (Line D) (46) and 12 littermate non-tg, age-matched controls were used. These animals were selected because they display abnormal accumulation of detergent-insoluble α Syn, develop cytoplasmic α Syn-immunoreactive inclusion-like structures in the brain and display neurodegenerative and motor deficits that mimic certain aspects of DLB/PD (46,78–80). Comparisons of the patterns of α Syn and Rab5 distribution were performed with six tg 6-month-old mice that mimic AD-like pathology by expressing the human mutant APP (line 41) under the thyl promoter (47).

Human cases and neuropathologic evaluation

This study examined a total of 18 subjects (Table 2), including 8 cases of DLB/PD, 6 cases of Alzheimer's disease (AD) and 4 non-demented controls. Autopsy material was obtained from patients studied neurologically and psychometrically at the Alzheimer Disease Research Center/University of California, San Diego (ADRC/UCSD). For each case, paraffin sections from 10% buffered formalin-fixed neocortical, limbic system and sub-cortical material stained with hematoxylin and eosin (H&E) and thioflavin-S were used for routine neuropathologic analysis (81,82) that included the Braak stage (83). The diagnosis of DLB/PD was based on the clinical presentation of dementia, followed by parkinsonism and the pathologic findings of LBs in the locus coeruleus, SN or nucleus basalis of Meynert, as well as in cortical and subcortical regions. LBs were detected using H&E anti-ubiquitin and anti α Syn antibodies as recommended by the Consortium on DLB criteria for a pathologic diagnosis of DLB/PD (84). In addition to the presence of LBs, the great majority of these cases

display sufficient plaques and tangles to be classified as Braak stages III–IV. Specifically, DLB/PD cases had abundant plaques in the neocortex and limbic system but fewer tangles compared with AD cases.

Fluorescence microscopy of yeast

For the S129 mutagenesis study, strains VSY67-74 were grown to the stationary phase at 30°C in raffinose-containing medium and diluted 100-fold in galactose-containing medium. Cells were induced for 16 h, mounted and sealed as described.

For the trafficking studies, strain BY4741 co-transformed with plasmids p423GPD α SYN(WT) or p423GPF and pGS416 (*GFP-SNC1*), pGSSO416 (*GFP-SNC1-SSO1*), pGNS416 (*GFP-NYV1-SSO1*), pPEP416 (*GFP-PEP12*), pPHM5 (*GFP-PHM5*), pSNA3416 (*SNA3-GFP*) or pSTE2416 (*GFP-STE2*) was grown for 12 h to the logarithmic phase at 30°C in glucose-containing medium and mounted as described. Strains BY4741, *yck1 Δ* , VSY4 and VSY17 transformed with plasmid pmCheSSO416 (*mCherry-SNC1-SSO1*) were grown for 12 h to the logarithmic phase at 30°C in galactose-containing medium and mounted as described.

For FM 4-64 labeling, the indicated strains were grown for 12 h in medium containing glucose [deletion and temperature-sensitive mutant strains transformed with plasmid pGSSO416 (*GFP-SNC1-SSO1*)] or galactose (strains VSY71-74) at 30°C (deletion strains and VSY71-74) or RT (temperature-sensitive strains). A total of 0.16 culture ODs were centrifuged and resuspended in 40 μ l of medium containing 40 μ M FM 4-64 to a final OD₆₀₀ of 4.0. Cells were pulsed with the dye for 15 min, washed with 1 ml of H₂O, resuspended in 0.5 ml of unlabeled medium and incubated for 1 h to let the dye internalize. The deletion and VSY71-74 strains were labeled at 30°C, whereas the temperature-sensitive strains were pre-incubated at 37°C or RT for 30 min prior to the FM 4-64 pulse and kept at the same temperature throughout all the steps for imaging.

For the rescue studies, strain BY4741 co-transformed with plasmids p423GPD α SYN(WT) or p423GPF alone, and pGSSO416 (*GFP-SNC1-SSO1*), was transformed with plasmids p425GALYPT1, p425GALYCK1 or left untransformed (no plasmid). Cells were grown in glucose-containing medium at 30°C to the stationary phase and diluted down 20-fold in galactose-containing medium. Cultures were induced for 16 h and prepared for imaging as described.

For the mutagenesis, trafficking, rescue and FM 4-64 studies, cells were imaged with a Nikon Plan Apo VC 100 \times (N.A. 1.4) objective on a spinning disc confocal microscope (Nikon) and images were acquired with a Cascade II digital camera (Photometrics), using Micro-Manager 1.3 (University of California, San Francisco).

IEM of yeast

For single-labeling, strain BY4741 transformed with the plasmid p426GAL α Syn(WT)GFP was grown at 30°C in glucose-containing medium to the early logarithmic phase, washed with water, resuspended in galactose-containing medium and incubated for 6 or 12 h. For double-labeling,

strain FRY346 transformed with plasmid p423GPD α SYN(WT)GFP was grown for 12 h at 30°C in glucose-containing medium to the logarithmic phase. In both cases, cells were fixed with 2% glutaraldehyde–0.2% para-formaldehyde and prepared for the Tokuyasu cryosectioning procedure according to a protocol optimized for *Saccharomyces cerevisiae* (85). Ultrathin sections were incubated first with antibodies recognizing the tags and subsequently with protein A-gold conjugates (86). After standard staining with uranyl and embedding in methylcellulose, sections were visualized in a JEOL 1010 electron microscope, and images were recorded on Kodak 4489 sheet films.

For single-labeling, a polyclonal anti-GFP antiserum was used (Abcam). For double-labeling, a monoclonal anti-myc antibody (Santa Cruz Biotechnology) and the same anti-GFP antiserum were used. Untransformed cells were treated in the same way and used as background controls.

Immunofluorescence of mammalian cells

For the co-localization studies, sections from the temporal cortex of DLB/PD and control cases and from α Syn tg and non-tg mice were used. Free-floating 40 mm thick vibratome sections were washed with Tris-buffered saline (TBS, pH 7.4), pre-treated in 3% H₂O₂ and blocked with 10% serum (Vector), 3% bovine serum albumin (Sigma-Aldrich) and 0.2% gelatin in TBS-Tx.

Double-immunofluorescence analyses were performed utilizing the Tyramide Signal AmplificationTM-Direct (Red) system (NEN Life Sciences). Specificity of this system was tested by deleting each primary antibody. For the Rab5 studies, sections were double-labeled with monoclonal antibodies against α Syn (1:20 000) (Cell Signaling) and Rab5 (1:75) (Vector) and detected with Tyramide Red and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:75) (Vector), respectively. For the CK1 δ studies, sections were double-labeled with the polyclonal antibodies against α Syn (1:200) (Chemicon) and CK1 δ (C-18) (Santa Cruz Biotechnology) and detected with FITC and Tyramide Red-conjugated secondary antibodies (1:75) (Vector), respectively. Sections were imaged with a Zeiss 63 \times (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss) with an attached MRC1024 laser scanning confocal microscope system (BioRad). All sections were processed simultaneously under the same conditions and experiments were performed twice for reproducibility.

Statistical analyses

Statistical analysis was performed using Prism 5 (GraphPad Software). Student's *t*-test was run for pairwise comparisons between groups at a single condition, and ANOVA for repetitive measurements was run for pairwise comparisons between groups throughout multiple treatments. Significance *P*-values were **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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