



## Phosphorylation, lipid raft interaction and traffic of $\alpha$ -synuclein in a yeast model for Parkinson

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### ABSTRACT

Parkinson's disease is a neurodegenerative disorder characterized by the formation of Lewy bodies containing aggregated  $\alpha$ -synuclein. We used a yeast model to screen for deletion mutants with mislocalization and enhanced inclusion formation of  $\alpha$ -synuclein. Many of the mutants were affected in functions related to vesicular traffic but especially mutants in endocytosis and vacuolar degradation combined inclusion formation with enhanced  $\alpha$ -synuclein-mediated toxicity. The screening also allowed for identification of casein kinases responsible for  $\alpha$ -synuclein phosphorylation at the plasma membrane as well as transacetylases that modulate the  $\alpha$ -synuclein membrane interaction. In addition,  $\alpha$ -synuclein was found to associate with lipid rafts, a phenomenon dependent on the ergosterol content. Together, our data suggest that toxicity of  $\alpha$ -synuclein in yeast is at least in part associated with endocytosis of the protein, vesicular recycling back to the plasma membrane and vacuolar fusion defects, each contributing to the obstruction of different vesicular trafficking routes.

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### 1. Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder characterized by the selective loss of dopaminergic neurons in the *substantia nigra pars compacta* and the formation of proteinaceous cytoplasmic inclusions in the affected neurons. These inclusions are known as Lewy bodies and are mainly composed of fibrillar  $\alpha$ -synuclein ( $\alpha$ -Syn) [1]. Familial forms of PD are associated with missense mutations (A30P, A53T and E46K) in the  $\alpha$ -Syn gene, as well as duplication or triplication of the  $\alpha$ -Syn locus, i.e. genetic alterations that accelerate  $\alpha$ -Syn misfolding and aggregation [2]. The exact molecular mechanisms that induce misfolding and toxicity of  $\alpha$ -Syn, especially in the more common sporadic forms of PD, are still elusive though several observations point to modifications such as C-terminal truncation, oxidation and phosphorylation or aberrant interactions with normal binding partners as possible causes [1,2]. Also, the function of  $\alpha$ -Syn is far from clear due to its apparent involvement in many cellular processes. However, converging observations suggest a role as regulator of dopamine neurotransmission and synaptic vesicular recycling [3]. A recent study with transgenic mice deficient for the cysteine-string protein- $\alpha$  demonstrated that

human  $\alpha$ -Syn can ameliorate complex assembly between plasma membrane and vesicular SNARE proteins. Interestingly, this activity depended on the capacity of  $\alpha$ -Syn to interact with phospholipids and hence was observed with wild type  $\alpha$ -Syn, but not with the A30P mutant [4]. Moreover, a study performed in PC12 cells led to the conclusion that  $\alpha$ -Syn has a role in a vesicle “priming” step, after secretory vesicle trafficking to “docking” sites, but before calcium-dependent vesicle membrane fusion [5].

Previously, we and others validated the yeast *Saccharomyces cerevisiae* as model system to study the biochemistry and toxicity of  $\alpha$ -Syn. Reminiscent to data produced by other models, the yeast system showed that  $\alpha$ -Syn localized to the plasma membrane, formed intracellular inclusions, and inhibited phospholipase D [6–8]. We extended these data and demonstrated that the formation of inclusions by wild type  $\alpha$ -synuclein (WT-Syn) and the clinical mutant A53T is a nucleation–elongation process initiated at the plasma membrane. Moreover, the failure of mutant A30P to form such inclusions could be attributed to its lower affinity for phospholipids since also this mutant formed aggregates when co-expressed with WT-Syn, which apparently formed the membrane-localized seeds necessary to induce the aggregation process [7]. Most interestingly, Cooper et al. [9] identified vesicular trafficking between the endoplasmic reticulum (ER) and Golgi as an early defect observed in yeast cells following expression of WT-Syn. Encouraged by these data

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and the observation of synthetic lethality induced by  $\alpha$ -Syn in some yeast mutants affected in protein sorting [8,10], we performed a genome-wide screening to isolate yeast deletion mutants that are characterized by mislocalization and/or enhanced inclusion formation of WT-Syn. This study led us to identify of several yeast orthologs and homologs of human proteins involved in vesicular transport and to demonstrate  $\alpha$ -Syn phosphorylation, N-terminal acetylation and lipid raft interaction as well as parameters determining  $\alpha$ -Syn toxicity in yeast.

## 2. Materials and methods

### 2.1. Yeast strains, plasmids and media

In this study we used the wild type BY4741 (*MATa his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0*) strain and its congenic deletion mutants [11]. The pUG23-syn plasmids for expression of  $\alpha$ -Syn from the methionine-repressible *MET25* promoter as well as the YEp181-syn plasmids for constitutive expression of  $\alpha$ -Syn were described previously [7]. The pUG35-syn plasmids were obtained by ligation of an *Xba*I and *Sall* fragment corresponding to the cDNA's of wild type  $\alpha$ -Syn or the mutants A53T or A30P from the pUG23-syn plasmids into pUG35 cut with the same restriction enzymes. Transformation procedures followed the standard lithium/polyethylene glycol method [12]. Transformants were grown at 30 °C in selective minimal glucose-containing medium (SD – Synthetic Dextrose) [12] supplemented with 1 mM methionine to suppress expression of  $\alpha$ -Syn, 20  $\mu$ M methionine to obtain moderate expression of  $\alpha$ -Syn, or without methionine to obtain overexpression of  $\alpha$ -Syn.

### 2.2. Determination $\alpha$ -Syn-mediated growth retardation

In order to test growth retardation caused by native  $\alpha$ -Syn, the growth profiles of the strains transformed with an empty vector or a YEp181 plasmid allowing for constitutive expression of WT-Syn were compared. Overnight precultures of three independent transformants were used to inoculate new cultures in glucose-containing synthetic medium at a starting OD<sub>600</sub> of 0.05. The growth profiles were then established by measuring OD<sub>600</sub> until the stationary phase was reached. Based on the log-transformed growth profiles, we calculated the half-times for growth of each mutant, i.e. the time necessary to reach half-maximal OD<sub>600</sub>, and took the difference in time ( $\Delta T$ ) when the strain was expressing native WT-Syn or transformed with the control plasmid.

### 2.3. Genome-wide screening of *S. cerevisiae* to identify genes important for $\alpha$ -Syn-EGFP fusion localization in the cell

The *S. cerevisiae* deletion strain collection, constructed in the BY4741 background (*MATa his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0*), was obtained from Euroscarf (Frankfurt, Germany). Each deletion strain (of a total of 4,857 viable strains available in the library) carries a replacement of a characterized or putative ORF by a *kanMX4* marker construct. Double mutants were obtained by crossing and subsequent tetrad analysis. The method for transformation of pUG35WT-syn plasmid into the yeast knockout collection and further selection was described previously [13].

To identify genes whose products are involved in localization of WT-Syn-EGFP fusion, the transformed collection was replica plated on SD plates containing 0.3 mM methionine. Each colony was then transferred to liquid medium without methionine and cells were grown till exponential phase. Each culture was subsequently inspected by fluorescent microscopy to observe localization of WT-Syn-EGFP. This screening was performed in duplicate. Mislocalization of WT-Syn-EGFP was further confirmed in the BY4741 strain transformed with pUG35WT-syn or pUG23WT-syn and

grown in SD medium containing 20  $\mu$ M methionine as described previously [7].

### 2.4. Antibodies and immunoblot analysis

$\alpha$ -Synuclein was detected with a rabbit polyclonal antibody directed against the C-terminus (Sigma) or an antibody specific for phosphorylated ser-129 (Wako, Japan).

Samples for Western blot analysis were prepared according to Zabrocki and coauthors [7]. Proteins were then separated by SDS-PAGE. To equilibrate the amount of protein, we compared the intensities obtained from Ponceau S staining of Immobilon-P (Millipore) after transfer of the proteins from gel to the filter, as well as by detection of *ADH2* expression (anti-*ADH2* antibody, Chemicon/Millipore, USA) that served as internal control of protein content. The antibody directed against Pma1 was a gift from B. André (ULB, Belgium).

Immunoreactions were quantified using TINA 2.08c program (Raytest, Belmont, USA). The immunoreactivity of phosphorylated  $\alpha$ -Syn was normalized against the signal obtained for the total amount of  $\alpha$ -Syn using the polyclonal anti-synuclein antibody. Experiments were done at least in duplicate with different transformants.

### 2.5. Microscopy

Fluorescence microscopy was performed with the OptiPhot (Nikon, Japan) microscope. Transformants with the *MET25*-controlled expression cassettes for EGFP-fused  $\alpha$ -Syn were cultured overnight in selective medium containing 1 mM methionine. From this preculture, cells were taken to inoculate a second culture (starting OD<sub>600</sub>=0.1) in selective medium containing 20  $\mu$ M methionine and incubated for 16–18 h at 30 °C (unless otherwise indicated) as described previously [7]. Cells were observed with the M-Plan 100 $\times$ /oil objective lens and the filter set with excitation filter 470 nm and 520LP emission filter (Nikon, Japan). The proportion of cells with the  $\alpha$ -Syn mislocalization within the population was then determined by inspection of at least 200 cells per culture.

For endocytosis of FM4-64 and visualization of vacuoles, yeast strains were grown in 50 ml of SD with 20  $\mu$ M methionine till OD<sub>600</sub>=1. Cells were harvested, washed in fresh medium and stained with 18  $\mu$ M FM4-64 dye (Molecular Probes) as it was described previously [7]. Vacuoles were visualized using 525/590LP filter set (Nikon, Japan) and counted manually. Cells with small clustered vacuoles were considered to have a vacuolar fusion defect.

### 2.6. Isolation of detergent-resistant lipid raft fractions

An amount equivalent to OD<sub>600</sub> 20 was taken of exponentially grown yeast cultures to perform the isolation of lipid raft fractions. The exponential cells were washed once with chilled water and lysed using a standard protocol with glass beads. Lysis was performed in TNE buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl and 5 mM EDTA) with addition of proteases inhibitors (benzamidine, PMSF and a cocktail of protease inhibitors (Roche)) [14]. Lysates were centrifuged at 500 g for 5 min at 4 °C, split in two parts. Triton-X-100 was added to 0.2% (or 1%, data not shown) to one part while TNE was added to the second part. Lysates were incubated on ice for 30 min, mixed with OptiPrep (Sigma) solution to reach 35% concentration and then overlaid with 1.2 ml of 30% OptiPrep solution in TXNE (TNE containing 0.1% Triton-X-100) and subsequently with 200  $\mu$ l of TXNE. The samples were centrifuged at 259 000 g for 8 h at 4 °C in SW55Ti rotor (Beckman, USA). Eleven equal fractions were taken and every fraction was precipitated by adding 50% trichloroacetic acid. Samples were centrifuged and protein pellets resuspended in 2 $\times$ SDS-PAGE buffer and loaded on SDS-PAGE gel. Equal volumes of

particular fractions were analyzed by SDS-PAGE and Western blotting.

### 3. Results

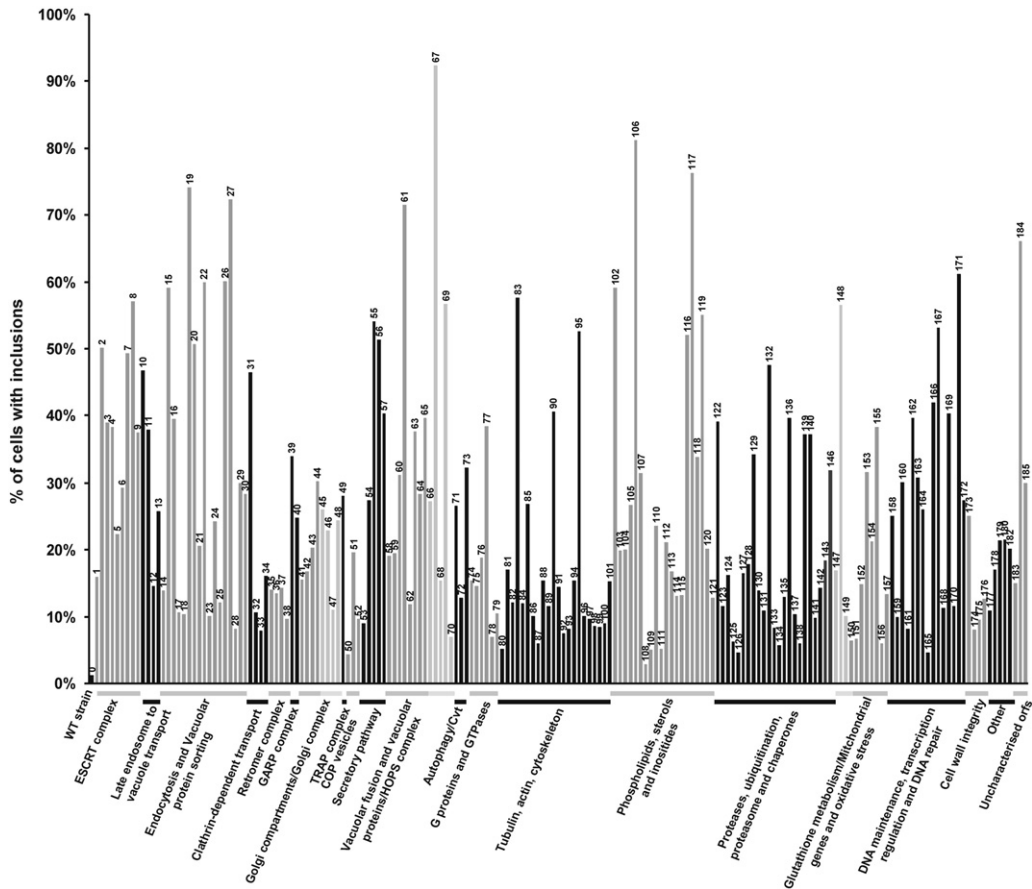
#### 3.1. Mislocalization of $\alpha$ -Syn in yeast mutants

It was previously demonstrated that expression of a WT-Syn-EGFP C-terminal fusion protein in yeast cells leads to the formation of amyloid inclusions, similarly to expression of native  $\alpha$ -Syn [6–8]. We took advantage of this ability to screen the genome-wide collection of yeast deletion strains with fluorescence microscopy to identify mutants that display mislocalization and/or enhanced inclusion formation as compared to the congenic wild type BY4741 yeast strain, when tested under conditions where the latter shows plasma membrane localization of the WT-Syn-EGFP fusion. As listed in Table S1 (Supplemental material) and quantified in Fig. 1, the screening retrieved 185 mutants that showed mislocalization of the fusion protein often together with more cells forming  $\alpha$ -Syn inclusion. Consistent with previous observations, these inclusions were either dense or more diffuse and in some mutants clearly vesicular membrane associated [7,15]. It should be noted that the amount of cells that formed  $\alpha$ -Syn inclusions varied considerably between mutants, ranging up to 95% in a mutant like *vps33 $\Delta$*  as compared to about 2% in the congenic wild type.

Dependent on the role of the gene deleted, the mutants were classified into functional groups (Fig. 1; Table S1–Supplemental material). This revealed that several mutants appeared to be affected in protein degradation or were lacking chaperones or proteins

involved in the ubiquitin–proteasome system. Other mutants were missing proteins involved in oxidative stress responses, including mitochondrial functions or proteins involved in glutathione metabolism. As such, our screening selected relevant mutants as indeed several recent studies performed in other model systems as well as on Parkinson patients implicated failure to properly degrade misfolded proteins, mitochondrial dysfunction and oxidative stress as important factors in the etiology of PD [16]. Previous studies in yeast also pointed to reciprocal effects between these processes and  $\alpha$ -Syn [7,10,17–21], providing further confidence that relevant mutants were selected. Nonetheless, the majority of mutants selected by our screening were affected in processes connected to vesicular traffic and protein sorting. This included not only strains lacking proteins involved in endocytosis, the secretory pathway or the vacuolar protein sorting (VPS) pathway but also strains defective in vacuolar biogenesis, cytosol-to-vacuole transport (*cvt*), actin organization as well as the metabolism and signaling of phospholipids, sterols and inositides. Interestingly, seven of these mutants were previously identified based on a genome-wide screening for yeast mutants that are synthetic sick or lethal upon expression of  $\alpha$ -Syn [10]. This included *arl3 $\Delta$* , *cog6 $\Delta$* , *sac2*, *vps24 $\Delta$* , *vps28 $\Delta$*  and *vps60 $\Delta$* , which all lack functions directly involved in vesicular traffic, and *opi3 $\Delta$* , which is affected in phospholipid metabolism. Also proteins that potentially modulate  $\alpha$ -Syn-induced dysfunction of ERAD (ER-Associated Degradation) and ER-stress [9] were recovered by our screening, including *Erv29*, an ER cargo receptor, and *Gyp1*, a GAP protein known to regulate the Rab GTPase *Ypt1*.

Since recent studies indicated that  $\alpha$ -Syn has roles in lipid metabolism and vesicle dynamics [3–5], we mainly focused on related



**Fig. 1.** Strains retrieved from the genome-wide screening for mutants with mislocalization and enhanced inclusion formation of  $\alpha$ -Syn. Shown is a bar diagram representing the relative number of cells with WT-Syn-EGFP inclusions (% of the total number of cells in the culture). The numbering of the different mutants corresponds to those given in Table S1 (Supplemental material). Also indicated is the process in which each mutant is affected.

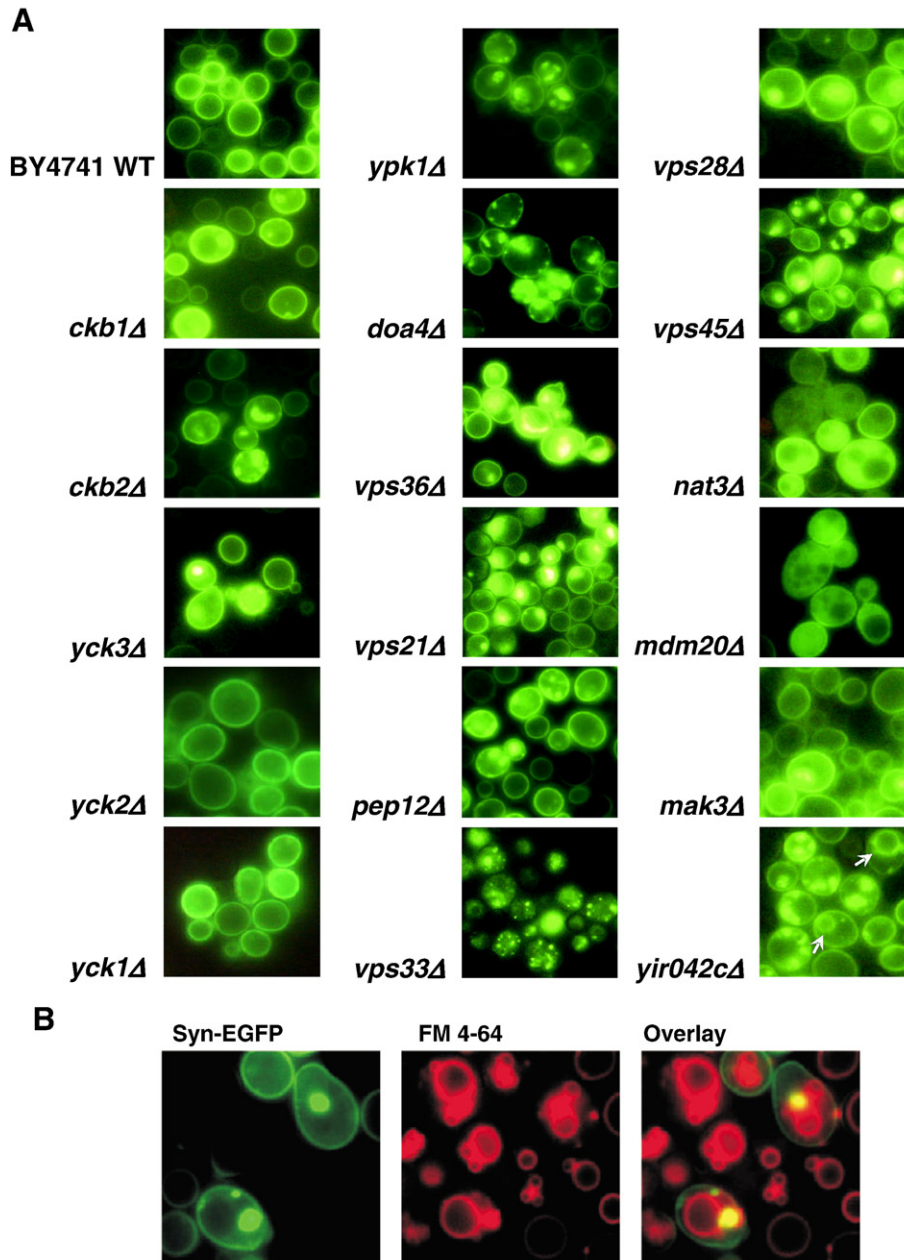
functions in yeast. Pictures displaying the intracellular distribution of the WT-Syn-EGFP fusion in mutants discussed in more detail in this paper are given in Fig. 2.

### 3.2. Phosphorylation of $\alpha$ -Syn in yeast is mediated by plasma membrane casein kinases and correlates with its inclusion formation and toxicity

Among the genes involved in lipid metabolism and signaling our screening identified Ckb1 and Ckb2, the regulatory subunits that control substrate specificity of the yeast casein kinase II (CK-II). The catalytic subunits of CK-II are redundantly encoded by *CKA1* and *CKA2* [22]. CK-II kinases are essential for growth and besides their role in transducing survival signals, they appear to have a function in

phospholipid and ceramide synthesis [23–25]. As shown in Fig. 2, the deletion of each of the regulatory subunits caused a similar phenotype with WT-Syn-EGFP not only being present at the plasma membrane but also in foci that may correspond to the ER and Golgi complex [23]. Mutants for the catalytic CK-II subunits were not retrieved by our screening and a more thorough analysis confirmed that these strains did not give rise to a clear mislocalization phenotype as WT-Syn-EGFP remained predominantly at the plasma membrane, similar as in the congenic wild type strain (data not shown).

Also the casein kinase I (CK-I) Yck3 was retrieved by our screen. Yck3 is known to regulate the Vps/HOPS (Homotypic Fusion and Vacuole Protein Sorting) complex required for homotypic vacuolar fusion and fusion of transport vesicles to the vacuole [26–28].



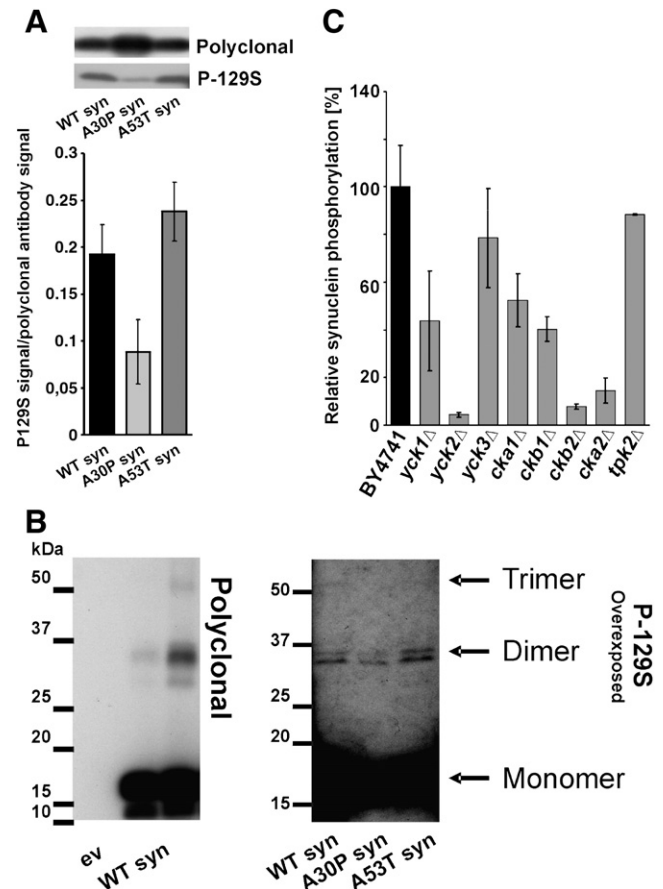
**Fig. 2.** Mislocalization and inclusion formation of  $\alpha$ -Syn in specified yeast mutants. (A) Fluorescence microscopic visualization and intracellular localization of the WT-Syn-EGFP fusion protein in the BY4741 wild type yeast strain (BY4741WT) and the congenic deletion mutants that are discussed in more detail in this paper. The mutants were ordered according to the functions in which they are affected. Categories include mutants lacking protein kinases (*ckb1Δ*, *ckb2Δ*, *yck3Δ*, *yck2Δ*, *yck1Δ* and *ypk1Δ*), vesicular trafficking mutants (*doa4Δ*, *vps36Δ*, *vps21Δ*, *pep12Δ*, *vps33Δ*, *vps28Δ* and *vps45Δ*) and mutants affected in N-terminal acetyltransferase activities (*nat3Δ*, *mdm20Δ*, *mak3Δ* and *yir042cΔ*). For the *yir042cΔ* mutant the arrows indicate association of the WT-Syn-EGFP fusion protein with the vacuolar membrane. (B) Overlay of WT-Syn-EGFP localization and endocytosed FM 4-64 in the *yck3Δ* mutant showing accumulation of  $\alpha$ -Syn inclusions at the periphery of the vacuolar membrane.

Consistently, its deletion caused enhanced  $\alpha$ -Syn accumulation in vesicles at the periphery of vacuoles as demonstrated by localization of WT-Syn-EGFP in cells stained with the endocytosed dye FM4-64 (Fig. 2A and B). In addition to Yck3, the yeast genome encodes two other CK-I kinases, i.e. Yck1 and Yck2, both localized at the plasma membrane where they phosphorylate and mark proteins for subsequent ubiquitination and endocytosis [29]. In strains carrying a deletion of YCK1 or YCK2, WT-Syn-EGFP remained at the plasma membrane, similarly as in the wild type strain (data not shown).

Since CK-I and CK-II were reported to phosphorylate  $\alpha$ -Syn at ser-129 *in vitro* and *in vivo* in mammalian cells [30] and transgenic mice [31], we examined whether casein kinases phosphorylated  $\alpha$ -Syn in yeast as well. First, we expressed WT-Syn or the clinical mutants A30P or A53T as EGFP fusion or native protein in the BY4741 wild type strain and analyzed the phosphorylation status of these proteins. Consistent with previously reported data [6,7], the EGFP-tagged A53T was plasma membrane-localized, similar to WT-Syn-EGFP, while the EGFP-tagged A30P mutant was predominantly cytoplasmic with only a minor fraction at the plasma membrane (data not shown). Also consistent was that WT-Syn and the A53T mutant are expressed at lower levels when compared to the A30P mutant, as shown by Western blot analysis of the native proteins obtained from exponentially growing wild type cells (Fig. 3A). Subsequently, we analyzed ser-129 phosphorylation of  $\alpha$ -Syn. This revealed that all  $\alpha$ -Syn isoforms were phosphorylated and that the level of phosphorylation of the A30P mutant was significantly lower than that of WT-Syn or the A53T mutant (Fig. 3A). These data suggest an apparent positive correlation between  $\alpha$ -Syn phosphorylation, as reported here, and the ability to bind the plasma membrane, form inclusions and to display toxicity in yeast, as previously demonstrated [6,7]. Interestingly, overexposure of the Western blots demonstrated that a fraction of the phosphorylated  $\alpha$ -Syn is also present as dimers in yeast, again with the lowest levels obtained for the A30P mutant (Fig. 3B). Two independent studies demonstrated that these  $\alpha$ -Syn dimers preferentially bind lipid membranes and vesicles *in vitro* and, consistent with our data, both studies demonstrated reduced membrane binding of A30P as compared to WT-Syn or A53T [32,33]. Note that these *in vitro* studies were performed with recombinant non-phosphorylated  $\alpha$ -Syn obtained from bacteria, indicating that phosphorylation of  $\alpha$ -Syn is not a prerequisite for membrane binding.

Next, we analyzed the involvement of CK-I and CK-II for phosphorylation of  $\alpha$ -Syn. Therefore, we constitutively expressed native WT-Syn in the corresponding yeast deletion mutants and monitored again its phosphorylation during exponential growth. Although the level of ser-129 phosphorylation was affected in each of these mutants as compared to the congenic wild type strain, the most dramatic decreases were observed in the strains missing Yck2, Cka2 or Ckb2 (Fig. 3C). As control, a *tpk2* $\Delta$  mutant was included, which lacks one of the catalytic PKA subunit. This mutant displayed ser-129 phosphorylation levels similar to that of the wild type strain. We also analyzed whether decreased  $\alpha$ -Syn phosphorylation, by the lack of Yck2, would affect dimer-formation but could not observe any significant difference in the amount of  $\alpha$ -Syn dimers formed in wild type and *yck2* $\Delta$  cells (data not shown).

Given that phosphorylation of ser-129 defines  $\alpha$ -Syn neurotoxicity and inclusion formation in a *Drosophila* model and that it is specifically associated to  $\alpha$ -Syn aggregation in patients with Dementia with Lewy Bodies (DLB) [34,35], we wondered whether lowering ser-129 phosphorylation would ameliorate the  $\alpha$ -Syn-induced toxicity in our yeast model. To this end, we compared growth for the wild type strain and the casein kinase mutants without or with expression of native WT-Syn. To get a quantitative measurement that takes into account possible growth differences specific for the yeast mutants, we calculated the  $\alpha$ -Syn-induced reduction in half-time for growth ( $\Delta T$ ) for each strain; i.e. the difference in time necessary to reach the half-maximal OD<sub>600</sub> for a strain when constitutively expressing native WT-



**Fig. 3.** Phosphorylation of  $\alpha$ -Syn and the involvement of casein kinases. (A) Phosphorylation of native WT-Syn and the clinical mutants A30P and A53T in the wild type strain BY4741 as detected by immunodecoration using a phospho-ser129 specific monoclonal antibody and quantified relative to intensity obtained for immunodecoration with a polyclonal  $\alpha$ -Syn antibody. (B) (Left) Detection of oligomeric forms produced by native WT-Syn in the BY4741 wild type strain by Western blot analysis of total protein extracts loaded at a final amount of 5  $\mu$ g and 10  $\mu$ g using the polyclonal  $\alpha$ -Syn antibody. (Right) Detection of oligomeric forms produced by native WT-Syn and the mutants A30P and A53T in BY4741 cells in total protein extracts loaded at a final amount of 10  $\mu$ g using the monoclonal phospho-ser129 specific antibody. (C) Quantification of the relative amount of phosphorylation of native WT-syn in BY4741, a control strain lacking the PKA subunit Tpk2 and mutants lacking a particular casein kinase as indicated. The level of phosphorylation of WT-Syn in BY4741 was set at 100%.

Syn or when transformed with the control plasmid (Table 1). This revealed that particularly the deletion of YCK1 and YCK2 reduced the  $\Delta T$  values. All other casein kinase mutants displayed higher  $\Delta T$  values as compared to the wild type strain. Thus, although both CK-I and CK-II kinases affect ser-129 phosphorylation of WT-Syn in yeast, only deletion of the plasma membrane-localized CK-I kinase ameliorated growth of yeast cells expressing native WT-Syn.

Cells deprived of CK-I activity, such as *yck1* $\Delta$  *yck2*<sup>ts</sup> mutants at restrictive temperature, were reported to show stabilization of transporters and receptors at the plasma membrane, consistent with a role of these casein kinases in the initial steps of endocytosis [36–38]. Interestingly, CK-I deficient cells display synthetic lethality when combined with disruption of the clathrin heavy chain function, suggesting that the CK-I kinases operate in an endocytic pathway that is parallel to the clathrin-dependent pathway [36]. In line with this, we found that the deletion of *CHC1*, encoding the clathrin heavy chain, ameliorated the growth upon native WT-Syn expression to similar extend as the deletion of YCK1 (Table 1), though the level of  $\alpha$ -Syn phosphorylation at ser-129 remained comparable to that observed in wild type cells (Fig. 4). Reduced  $\alpha$ -Syn toxicity was also observed in cells lacking Rvs161, a member of the BAR-domain family

**Table 1**  
Comparison of difference in the growth of yeast strains with or without overexpression of WT-Syn

Strain	$\Delta T \pm \text{SEM}$ [h]
BY4741	5.74 ± 0.50
<i>yck1Δ</i>	3.54 ± 0.12
<i>yck2Δ</i>	4.37 ± 1.63
<i>yck3Δ</i>	6.96 ± 1.48
<i>tpk2Δ</i>	5.23 ± 0.97
<i>cka1Δ</i>	6.03 ± 2.16
<i>cka2Δ</i>	8.35 ± 2.17
<i>ckb1Δ</i>	7.39 ± 0.76
<i>ckb2Δ</i>	6.24 ± 0.68
<i>ypk1Δ</i>	11.69 ± 1.87
<i>ypk2Δ</i>	2.47 ± 0.67
<i>doa4Δ</i>	9.49 ± 1.3
<i>chc1Δ</i>	3.25 ± 2.33
<i>erg2Δ</i>	15.71 ± 1.18
<i>erg6Δ</i>	5.45 ± 0.95
<i>nat1Δ</i>	5.3 ± 1.67
<i>nat3Δ</i>	0.94 ± 1.68
<i>pep12Δ</i>	8.78 ± 1.42
<i>rsv161Δ</i>	2.95 ± 1.44
<i>vps21Δ</i>	9.67 ± 1.96
<i>vps28Δ</i>	7.13 ± 0.13
<i>vps33Δ</i>	4.40 ± 1.60
<i>vps45Δ</i>	6.44 ± 1.35
<i>YIR042cΔ</i>	6.66 ± 0.16
<i>vps21Δ vps33Δ</i>	4.33 ± 1.22
<i>vps28Δ vps33Δ</i>	4.75 ± 1.75
<i>vps21Δ yck1Δ</i>	8.80 ± 0.20
<i>vps28Δ yck1Δ</i>	12.83 ± 1.89

$\Delta T$  [h]: describes the difference in time necessary to reach half-maximal OD<sub>600</sub> when a strain was expressing native WT-Syn or transformed with the control plasmid.

SEM: standard error of the mean obtained from three independent growth curves.

of proteins that includes amphiphysin (Table 1). In mammalian brain, amphiphysins function in synaptic vesicle endocytosis where they appear to sense membrane curvature and are involved in the release of the forming vesicle [39,40]. In yeast, the Rvs proteins have been attributed an additional role in targeting secretory vesicles to the plasma membrane [41]. This may explain why the *rsv161Δ* displayed comparable low  $\alpha$ -Syn phosphorylation levels as the mutants lacking CK-I kinases (Fig. 4), since indeed, WT-Syn was reported to reach the plasma membrane via the secretory pathway [8].

Taken together, the picture emerging from the data described above is that yeast mutants affected in the initial steps of endocytosis, i.e. impaired CK-I-mediated phosphorylation events at the plasma membrane or impaired formation and internalization of endocytic vesicles, are characterized by a reduced  $\alpha$ -Syn-induced toxicity.

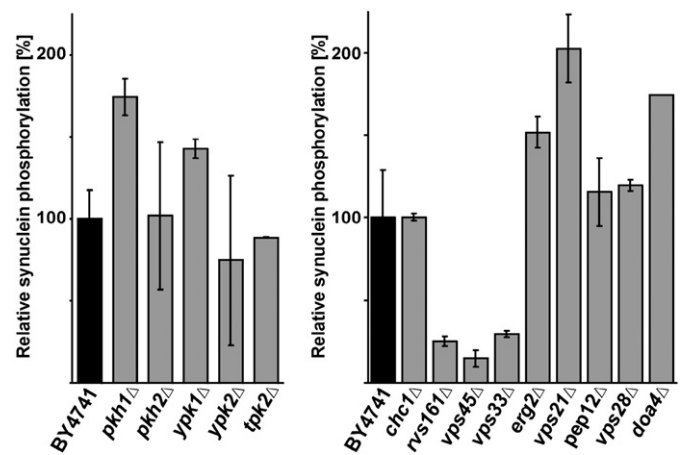
### 3.3. Hampered endosomal transport and increased recycling results in enhanced $\alpha$ -Syn phosphorylation and $\alpha$ -Syn toxicity

Recent studies demonstrated the serum- and glucocorticoid-regulated kinase *sgk1* to be associated with cell death in several animal PD models [42]. Our screening recovered the SGK homologue Ypk1 [43] as a protein kinase that upon deletion leads to accumulation of  $\alpha$ -Syn on intracellular and plasma membrane-localized vesicles (Fig. 2). Ypk1 and its closely related kinase Ypk2 function in sphingolipid signaling where they act downstream of the yeast PDK1 orthologues Pkh1 and Pkh2, respectively [44]. Analysis of  $\alpha$ -Syn phosphorylation revealed that deletion of Pkh1 or its substrate Ypk1 resulted in increased levels of ser-129 phosphorylation while deletion Pkh2 or Ypk2 yielded, respectively, similar and slightly reduced levels as those observed for the wild type strain or the *tpk2Δ* control strain (Fig. 4). This difference in phosphorylation was reflected in the growth properties of the mutants, as we found significantly increased  $\Delta T$  values and enhanced  $\alpha$ -Syn-mediated toxicity in the *ypk1Δ* mutant

strain in contrast to the *ypk2Δ* mutant that displayed lower  $\Delta T$  values in comparison to the wild type strain (Table 1).

Both Ypk1 and Ypk2 play a role in endocytosis but despite their close homology their functions appear not to be redundant. A recent study on rapid ligand-induced internalization of the pheromone receptor Ste2 established that Ypk2 is specifically required to phosphorylate Myo5, a myosin I protein [45]. Myosins bear an actin-activated ATPase and promote Arp2/3-dependent actin assembly facilitating the formation and further invagination of clathrin coated vesicle [46]. As such, Ypk2 appears to be involved in the initial steps of vesicle formation and therefore it was not surprising that the *ypk2Δ* mutant was characterized by a reduced  $\alpha$ -Syn toxicity, similar as the *chc1Δ* and *rsv161Δ* mutants described above (Table 1). The exact point for interception of Ypk1 with endocytosis remains to be determined. However, one study clearly demonstrated that it fulfils functions that cannot be replaced by Ypk2 and which are downstream of cargo phosphorylation and ubiquitination [47]. In addition, *ypk1Δ* mutants were reported to be cold-sensitive and hypersensitive to rapamycin, in contrast to *ypk2Δ* cells [44]. This link between Ypk1 and rapamycin was confirmed as the kinase associates with TORC1 and TORC2 in a novel type of detergent-resistant membrane domains (DRMs) distinct from plasma membrane rafts. Analysis of Ste3 internalization and liquid-phase endocytosis led these authors to conclude that TORC1 is likely to function in a post-vesicle-internalization step [48]. Based on this information, it is feasible that also Ypk1 operates after vesicle internalization. Therefore, we hypothesized that the observed increased  $\alpha$ -Syn toxicity that differentiates the *ypk1Δ* mutant from the *ypk2Δ* mutant (Table 1) relates to impaired traffic of the newly formed vesicle.

To reject or confirm this hypothesis, we analyzed the mutant that lacks the C-8 sterol isomerase Erg2. This mutant was also reported to have a post-vesicle-internalization defect [49,50]. Our analysis showed that the *erg2Δ* mutant combined increased  $\alpha$ -Syn phosphorylation with enhanced toxicity as well and thus had similar characteristics as those of *ypk1Δ* cells upon expression of WT-Syn (Table 1, Fig. 4). Other mutants affected in the downstream steps of endocytosis revealed an equivalent phenotype (Table 1, Fig. 4). This included strains lacking the Rab GTPase Vps21 or the t-SNARE Pep12/Vps6, both class D Vps proteins involved in traffic from early endosomes to the prevacuolar compartment [51,52], the ESCRT-I (Endosomal Sorting Complex Required for Transport) mutant lacking Vps28, required in cargo selection for the vacuolar degradation/MVB



**Fig. 4.** Phosphorylation  $\alpha$ -Syn in mutants affected in endocytosis or biosynthetic protein sorting routes. Quantification of the relative amount of phosphorylation of native WT-Syn in BY4741, a control strain lacking the PKA subunit Tpk2 and mutants lacking the PDK-orthologs Pkh1 or Pkh2 and the SGK-orthologs Ypk1 or Ypk2 as indicated (left) or mutants lacking proteins involved in different vesicular trafficking routes as specified (right). The level of phosphorylation of WT-Syn in BY4741 was set at 100%.

pathway [53] and the mutant missing Doa4, the deubiquitylating enzyme that recycles ubiquitin from endocytosed material [54]. Taken together, the data confirmed that endocytosis mutants, which are affected in post-vesicle-internalization steps and therefore fail to properly deliver endocytosed material to the vacuole, share a phenotype of enhanced  $\alpha$ -Syn phosphorylation and increased  $\alpha$ -Syn toxicity. Next, we introduced the deletion of *YCK1* in the *vps21 $\Delta$*  or *vps28 $\Delta$*  strains. As described above, the deletion of *YCK1* reduced  $\alpha$ -Syn toxicity presumably by lowering the endocytic rate. However, this deletion could not prevent the toxicity enhancement conferred by the lack of Vps21 or Vps28, indicative that post-vesicle-internalization defects have a major impact on  $\alpha$ -Syn toxicity (Table 1). The latter was further exemplified upon analysis of the *vps45 $\Delta$*  mutant. The Vps45 protein is not required for delivery of endocytosed material to the vacuole but is essential for fusion of Golgi-derived vesicles to the prevacuolar compartment and transport of Cvt vesicles from the cytoplasm to the vacuole [55,56]. In contrast to the endocytosis mutants, the *vps45 $\Delta$*  strain combined reduced  $\alpha$ -Syn phosphorylation with an  $\alpha$ -Syn toxicity that was only slightly higher than that observed in the wild type strain (Table 1, Fig. 4). This observations pointed us to another important aspect. Since the deletion of *VPS45* blocks biosynthetic trafficking, several of the soluble vacuolar proteases and vacuolar membrane proteins do not reach the degrading compartment [55]. Therefore, it appears that there is no apparent correlation between reduced vacuolar degrading activity and  $\alpha$ -Syn toxicity. In fact, careful re-examination of the wild type strain BY4741 and mutants retrieved from our screening failed to show that the WT-Syn-EGFP fusion protein entered into the vacuolar lumen, even not under conditions of sustained overexpression or conditions where EGFP fusions of yeast permeases, such as the ammonium permease Mep2, readily entered the vacuole (data not shown). Moreover, examination of a yeast *pep4 $\Delta$*  mutant that fails to degrade proteins in the vacuole because it lacks the Pep4 protease did not reveal accumulation or enhanced inclusion formation of WT-Syn-EGFP, but a similar expression level and plasma membrane localization of the fusion protein as in the wild type strain (data not shown).

If not reduced vacuolar degradation, then what caused the combined phenotype of enhanced  $\alpha$ -Syn phosphorylation or  $\alpha$ -Syn toxicity? We reasoned that this could be due to enhanced recycling of  $\alpha$ -Syn from endosomes back to the plasma membrane. The rationale behind this is that several studies on endocytosis of yeast plasma membrane proteins indicated recycling to be very rapid in mutants that block traffic along the vacuolar degradation pathway, resulting in an apparent plasma membrane stabilization of permeases like Gap1 and Fur4 [57–59]. Studies on recycling identified the HOPS mutant *vps33 $\Delta$*  as a strain where endocytosed proteins, i.e. the v-SNARE and VAMP ortholog Snc1, remain trapped in a hypophosphorylated form in recycling-incompetent endosomes [58]. Hence, to substantiate that recycling may as well apply to  $\alpha$ -Syn, we analyzed the *vps33 $\Delta$*  mutant in more detail. As expected, WT-Syn-EGFP was mostly found in inclusions in this strain (Figs. 2 and 5), yielding more than 90% of the mutant cells with inclusions (Fig. 1 and Table S1—Supplemental

material). Analysis of mutant  $\alpha$ -Syn showed a similar phenotype for EGFP-tagged A53T, while for EGFP-tagged A30P fewer cells with clear inclusion were found (Fig. 5). Hence, inclusion formation of wild type and mutant  $\alpha$ -Syn in the *vps33 $\Delta$*  mutant appeared to correlate with the plasma membrane binding properties of the proteins.

Despite the high score of inclusion formation with WT-Syn, the *vps33 $\Delta$*  strain displayed a lower  $\Delta T$  value as the congenic wild type strain, indicative that there was decreased toxicity upon expression of native WT-Syn and underscoring the lack of correlation between  $\alpha$ -Syn toxicity and the number of cells with inclusion (Table 1). Furthermore, examination of the phosphorylation level of native WT-Syn confirmed that the protein accumulated in its hypophosphorylated form in this mutant (Fig. 4). Intriguing is that the *vps33 $\Delta$*  strain was reported not to have discernible vacuoles and to secrete vacuolar proteases [60,61]. Thereby, our analysis again confirmed the lack of correlation between reduced vacuolar degradation and elevated levels of  $\alpha$ -Syn phosphorylation or toxicity.

To substantiate that enhanced  $\alpha$ -Syn recycling does explain the increased  $\alpha$ -Syn toxicity characteristic for post-vesicle-internalization mutants, we deleted *VPS33* in the *vps21 $\Delta$*  and *vps28 $\Delta$*  mutants. As expected, this led to a significant reduction of the  $\alpha$ -Syn toxicity since the *vps21 $\Delta$ vps33 $\Delta$*  and *vps28 $\Delta$ vps33 $\Delta$*  double mutants displayed  $\Delta T$  values comparable to that of the single *vps33 $\Delta$*  deletion mutant (Table 1).

### 3.4. N-terminal acetyltransferases determine $\alpha$ -Syn membrane binding specificities

Apart from studies on ser-129 phosphorylation, Anderson et al. [35] reported the presence of N-terminal acetylated  $\alpha$ -Syn in brain cytosol and Lewy bodies. We retrieved the catalytic subunit Nat3 of the N-terminal acetyltransferase NatB complex from our screening. This protein shows most homology to human NAT5. In addition to NatB, yeast cells encode two other N-terminal acetyltransferases, i.e. NatA and NatC, which are characterized by the catalytic subunits Ard1 and Mak3 [62]. Although N-terminal acetylation is a common modification of eukaryotic proteins, the biological role is far from understood but may be subtle and not absolute for most proteins. To demonstrate the importance of N-terminal acetylation for  $\alpha$ -Syn localization, we compared the cellular distribution of the WT-Syn-EGFP fusion protein in several Nat mutants. As shown in Fig. 2 and quantified in Fig. 6A, disruption of NatB activity by deletion of either the catalytic subunit, Nat3, or the auxiliary subunit, Mdm20, led to a more cytoplasmic localization of WT-Syn-EGFP, demonstrating that NatB is indispensable for the proper plasma membrane targeting of  $\alpha$ -Syn. In contrast, disruption of NatA or NatC by deletion of Ard1, Nat1 or Mak3, or disruption of Nat4, known to be involved in histone acetylation [63], did not seem to have a major effect since the fusion protein remained predominantly localized at the plasma membrane, similar as in the wild type strain. These data are in line with the substrate specificities attributed to the different yeast N-terminal acetyltransferases. For NatB this comprises Met-Glu and Met-Asp

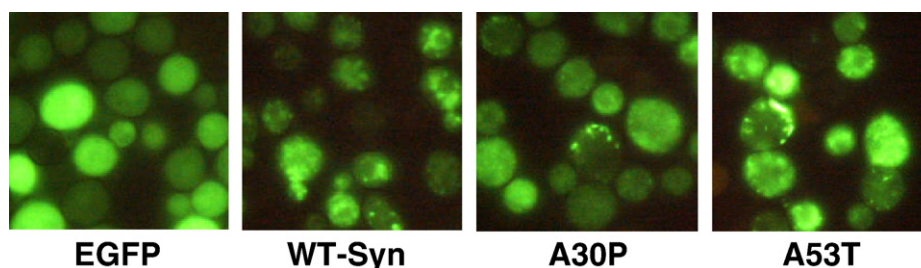
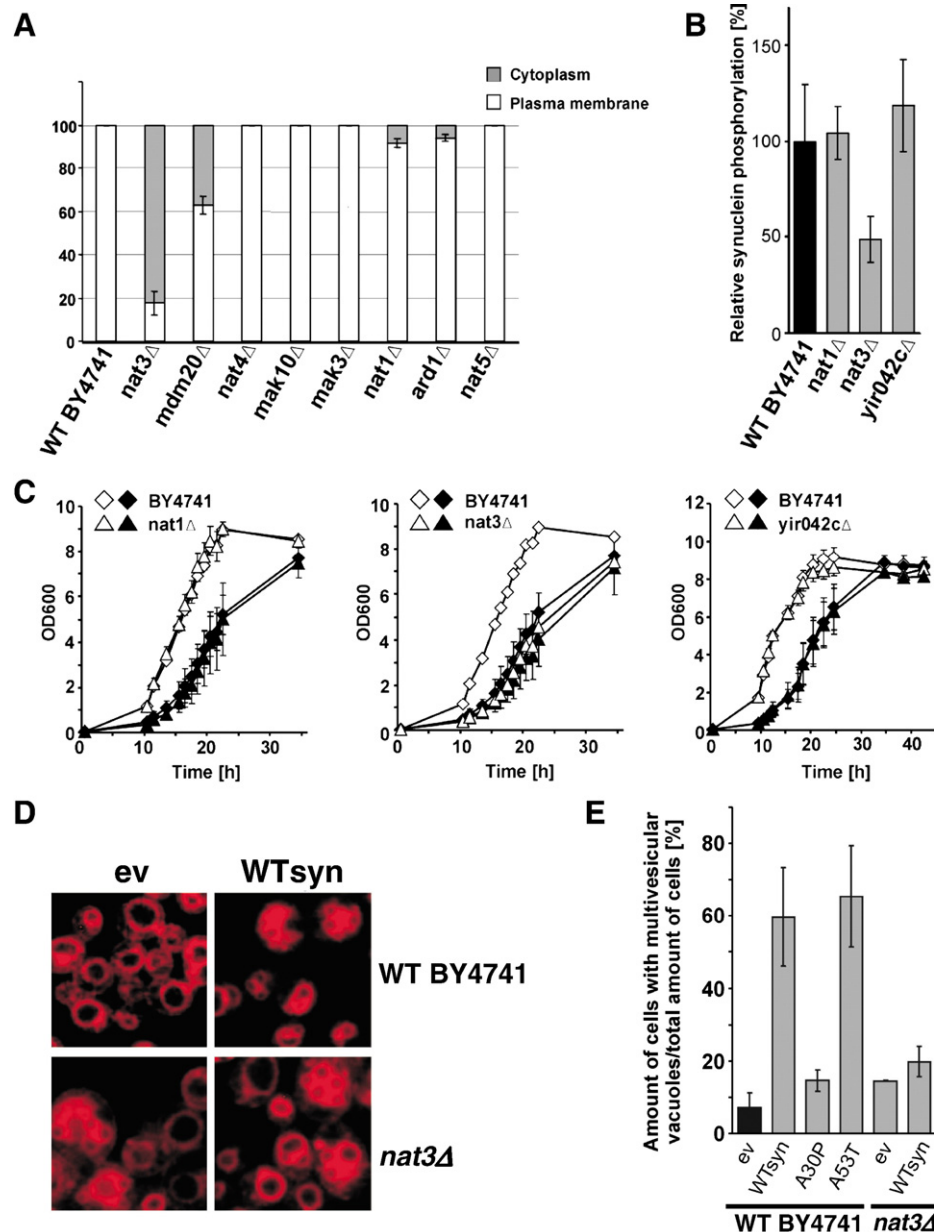


Fig. 5. Accumulation of  $\alpha$ -Syn in recycling-incompetent endosomes in the *vps33 $\Delta$*  mutant. Fluorescence microscopic visualization and intracellular localization of EGFP and EGFP-tagged WT-Syn, A30P or A53T in *vps33 $\Delta$*  mutant cells grown for 18 h in selective medium containing 20  $\mu$ M methionine.



**Fig. 6.** Influence of N-terminal acetylation on localization, phosphorylation and toxicity of  $\alpha$ -Syn. (A) Amount of cells with predominant plasma membrane localization or cytoplasmic localization of WT-Syn-EGFP in cultures from the wild type yeast BY4741 or congeneric mutants lacking catalytic or regulatory components of N-terminal acetyl transferases as indicated. (B) Quantification of the relative amount of ser-129 phosphorylation of native WT-Syn in BY4741 and congeneric *nat1 $\Delta$* , *nat3 $\Delta$*  or *yir042c $\Delta$*  mutants. (C) Growth profiles of the BY4741 wild type strain ( $\diamond$ ,  $\blacklozenge$ ) and congeneric mutants lacking Nat1, Nat3 or Yir042c ( $\Delta$ ,  $\blacktriangle$ ) transformed with an empty plasmid ( $\diamond$ ,  $\Delta$ ) or a construct allowing for expression of native WT-Syn ( $\blacklozenge$ ,  $\blacktriangle$ ). All data represent the mean of at least three independent experiments. (D) Endocytosis of FM4-64 in the BY4741 wild type strain and the *nat3 $\Delta$*  mutant each transformed with a control plasmid (ev) or expressing native WT-Syn. The dye was added to the cells at 0 °C. Cells were then shifted to room temperature to start endocytosis and pictures were taken after 45 min. (E) Quantification of cells with multi-vesicular vacuoles. Given are the percentages obtained for the BY4741 wild type strain or the *nat3 $\Delta$*  mutant transformed with a control plasmid (ev) or expressing native WT-Syn, A30P or A53T as indicated.

termini [62], the latter corresponding to the N-terminus of  $\alpha$ -Syn, making this protein indeed a potential substrate. Interestingly, only the deletion of Nat3 but not that of other transferases caused a significant reduction in the  $\alpha$ -Syn phosphorylation level (Fig. 6B), which is in agreement with the data described above suggesting that phosphorylation of  $\alpha$ -Syn occurs, at least in part, when the protein is plasma membrane associated.

Analysis of the growth profiles demonstrated that expression of native WT-Syn did not significantly influence the growth of the *nat3 $\Delta$*  mutant, in contrast to the other *nat* mutants (Fig. 6C, Table 1). This suggested that NatB could be involved in the process leading to toxicity of  $\alpha$ -Syn. To elaborate on this, we took a closer look to several phenotypic parameters known to be dependent on NatB. Mutants

carrying a *NAT3* or *MDM20* deletion were reported to have a mitochondrial inheritance defect [64,65]. However, a comparative analysis of the wild type BY4741 strain with or without expression of native WT-Syn did not reveal differences in the distribution of mitochondria from the mother to daughter cells (data not shown), excluding that  $\alpha$ -Syn expression would primarily interfere with mitochondrial inheritance. Yeast *nat3 $\Delta$*  mutants also lack stable actin filaments due to the loss of acetylation of actin and tropomyosin-1 [65,66]. Though several genes involved in actin cytoskeleton organization were indeed recovered from our screen, including the tropomyosin1 gene *TPM1*, the corresponding mutants clearly show  $\alpha$ -Syn inclusions while cells deprived from NatB activity are characterized by a diffuse cytoplasmic localization of  $\alpha$ -Syn. This makes it

unlikely that NatB-dependent defects in the actin cytoskeleton would be a primary denominator for  $\alpha$ -Syn toxicity, which is in line with the conclusion drawn from an elaborate growth study stating that the phenotypes of the *nat3* $\Delta$  mutant are not predominantly caused by alterations in the actin cytoskeleton [67].

Cells deprived of Nat3 were additionally reported to have vacuolar fusion defects [26]. To address whether such a phenotype is obtained upon  $\alpha$ -Syn expression, we monitored endocytosis of the fluorescent dye FM4-64 until a steady state was obtained. In wild type cells transformed with the empty vector or expressing the A30P mutant, FM4-64 staining resulted in visualization of a single or a few large vacuoles within 20 min. In cells transformed with WT-Syn or A53T a steady state was reached only 45 min. after internalization of the fluorescent dye and many cells displayed staining of multiple clustered small vacuoles (Fig. 6D, E). In the *nat3* $\Delta$  mutant, the difference between the control or cells expressing WT-Syn was not that pronounced and although some vacuolar fusion defects were evident in these strains, the amount of cells with such a defect was comparable to the numbers obtained with A30P-expressing wild type cells (Fig. 6D, E). Thus, deletion of *NAT3* appears to prevent  $\alpha$ -Syn-induced vacuolar fusion defects. This would be consistent with a lower input into endocytosis as further suggested by the observation that WT-Syn is more cytoplasmic in the *nat3* $\Delta$  mutant.

Next to NatB, our screening identified an additional putative GCN5-related N-acetyltransferase (GNAT) encoded by *YIR042c*. Deletion of this ORF often resulted in  $\alpha$ -Syn-EGFP localization in vacuolar membranes (Fig. 2), providing an additional confirmation that  $\alpha$ -Syn is sorted along the vacuolar degradation pathway. In contrast to disruption of Nat3, deletion of *YIR042c* did not affect  $\alpha$ -Syn phosphorylation or the level of  $\alpha$ -Syn-mediated toxicity significantly when compared to the congenic wild type strain (Fig. 6B, C).

### 3.5. $\alpha$ -Syn binds to DRMs in yeast in an ergosterol-dependent manner

Recently, it was shown that  $\alpha$ -Syn can associate with lipid rafts and that this interaction is important to mediate the synaptic localization in mouse brain [68]. Lipid rafts are enriched in cholesterol and sphingolipids and are believed to correspond biochemically to so-called DRMs (detergent-resistant membrane fractions) because they are not readily solubilized in non-ionic detergents [69]. The identification in our screen of genes involved in synthesis of sphingolipid and ergosterol, the yeast counterpart for cholesterol, suggested that  $\alpha$ -Syn could interact with rafts also in yeast. To address this issue, we expressed  $\alpha$ -Syn as native or EGFP fusion in wild type cells, solubilized the membranes in 0.2% Triton-X-100 at 0 °C and separated the insoluble membranes by flotation on an Optiprep gradient. Western blotting of the different gradient fractions revealed that a minor portion of both native as well as EGFP-fused WT-Syn is recovered in the upper detergent-resistant fractions that also contained Pma1, a known DRM-associated protein that served as positive control (Fig. 7A). Immunodetection with the antibody specific for phosphorylated ser-129 confirmed the presence of minute amounts of phosphorylated  $\alpha$ -Syn in the same fractions. We then also fractionated membranes obtained from cells expressing the native A30P protein. In comparison to WT-Syn, the A30P mutation decreases DRM association in yeast, which is consistent to the observations made in cells of higher eukaryotes [68]. Finally, when membranes were isolated from cells expressing native EGFP, this soluble protein was solely present in fractions that separated more to the bottom of the gradient.

To assess directly the role of ergosterol in the interaction of  $\alpha$ -Syn with DRMs in yeast, we analyzed different mutants affected in ergosterol biosynthesis, i.e. *erg6* $\Delta$ , *erg28* $\Delta$  and *erg24* $\Delta$ . In contrast to the *erg24* $\Delta$  strain, the *erg6* $\Delta$  and *erg28* $\Delta$  mutants were both recovered from our screening as they combined plasma membrane localization with accumulation of inclusions of WT-Syn-EGFP (Table S1—Supple-

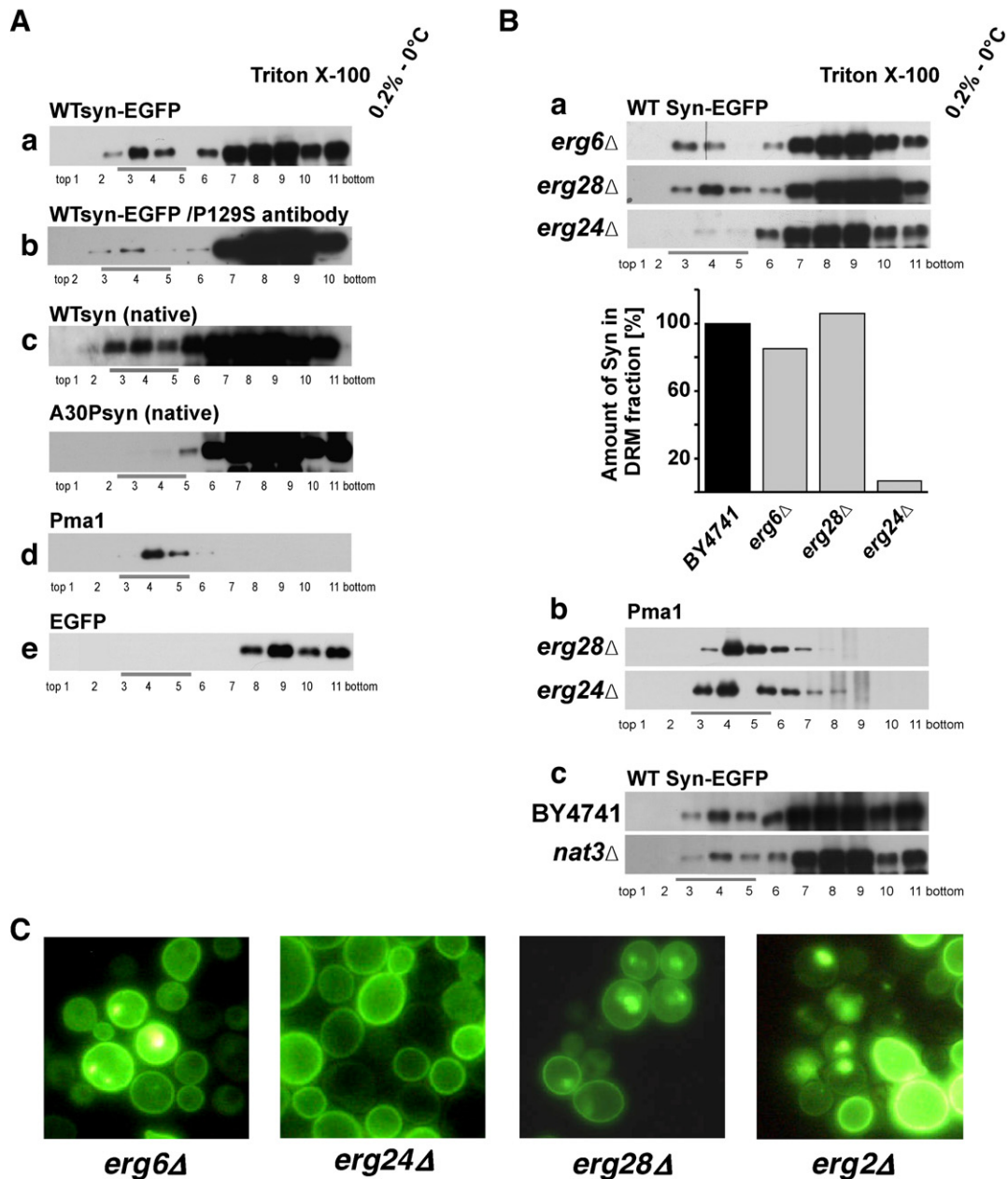
mental material). Indeed, *ERG6* encodes the ER-resident delta-24-sterol methyltransferase required for C-24 methylation of zymosterol, while *ERG28* encodes a tethering function required to form the biosynthetic complex at the ER. Both genes are not essential under standard growth conditions, indicative that yeast cells tolerate some degree of difference in ergosterol biosynthesis [70,71]. In contrast, *ERG24* encodes the sterol C-14 reductase, which is essential for aerobic growth on rich medium but not on synthetic medium, the growth condition used in this study [70]. Interestingly, deletion of *ERG6* or *ERG28* had only a moderate effect on the presence of WT-Syn-EGFP in the upper detergent-resistant fractions, while deletion of *ERG24* displaced WT-Syn-EGFP to the more dense gradient fractions and thus abrogated an interaction between  $\alpha$ -Syn and DRMs (Fig. 7B). This observation is in line with data obtained in neuronal cells and mice on the cholesterol requirement for the interaction of  $\alpha$ -Syn to membrane rafts [68,72]. As expected, the deletion of *ERG24* did not affect the association of Pma1 with DRMs (Fig. 7B), which is consistent with the finding that this association is largely independent of the ergosterol content in rafts [73]. Note, however, that  $\alpha$ -Syn still localized to the plasma membrane in the *erg24* $\Delta$  strain, indicative that besides lipid rafts the protein must also interact with other membranous components (Fig. 7C). Furthermore, the *erg6* $\Delta$  and *erg24* $\Delta$  mutants displayed similar  $\alpha$ -Syn phosphorylation levels (data not shown), suggesting that  $\alpha$ -Syn phosphorylation occurs before the interaction with DRMs or that the latter is not a prerequisite  $\alpha$ -Syn phosphorylation.

Finally, to investigate whether or not the reduced plasma membrane binding of  $\alpha$ -Syn in NatB-deficient cells would be due to an altered lipid raft interaction, we also analyzed the *nat3* $\Delta$  mutant. As shown in Fig. 7B, WT-Syn-EGFP was still present in DRM fractions isolated from *nat3* $\Delta$  cells with levels comparable to those obtained for the congenic wild type strain. This suggests that N-terminal acetylation of  $\alpha$ -Syn is not essential for its interaction with DRMs but solely for the interaction with other membrane constituents.

## 4. Discussion

Although the precise function of  $\alpha$ -Syn is still not clear, studies in mammalian cell lines and transgenic mice suggested a role in synaptic vesicle dynamics. Also in yeast, several observations linked  $\alpha$ -Syn to vesicular traffic. Expression of WT-Syn or the A53T mutant were reported to retard endocytosis [6,7] and to block ER-to-Golgi transport thereby causing ER-stress [9]. In addition, genes with a function in vesicular traffic in yeast have been identified as modulators of  $\alpha$ -Syn-induced toxicity [9,10]. The latter demonstrated that interference with vesicular traffic could underlie the observed toxicity of  $\alpha$ -Syn in yeast cells. However, there are several closely connected vesicle trafficking routes and it remains to be elucidated how and where  $\alpha$ -Syn intercepts with vesicular transport and how this may lead to toxicity in yeast. This is further illustrated by the retrieval with our screen of mutants lacking components of the tethering and sorting complexes TRAPP (TRANSPORT Protein Particle), COG (Conserved Oligomeric Golgi), GARP/VFT (Golgi-Associated Retrograde Protein/Vps Fifty Three), ESCRT, HOPS and the Class C Vps complex, which define different trafficking routes in yeast [53] and which were all found to display mislocalization and enhanced inclusion formation of  $\alpha$ -Syn.

Toxicity in yeast is mainly observed with WT-Syn and the A53T mutant and to a much lesser extent with the A30P mutant [6,7]. This difference closely reflects the membrane binding capacity of the proteins, since WT-Syn and A53T were found to initially localize at the plasma membrane where they start to form inclusions, while the A30P mutant was shown to remain predominantly cytoplasmic without inclusion formation [6–8]. Dixon et al. showed that delivery of WT-Syn and A53T to the plasma membrane occurs through association with vesicular intermediates of the secretory pathway [8]. Sorting into secretory vesicles and correct targeting to the plasma membrane in



**Fig. 7.** Lipid raft interaction of  $\alpha$ -Syn. (A) Interaction to lipid rafts of native WT-Syn, the native A30P mutant or the wild type  $\alpha$ -Syn fused to EGFP (WT-syn-EGFP) in the BY4741 strain. Detection of  $\alpha$ -Syn in the different samples was done using the polyclonal  $\alpha$ -Syn antibody (a, c) or with the monoclonal phospho-ser129 antibody (b). Also shown are the positive (d) and negative (e) controls with the detection of Pma1 or EGFP, respectively. (B) Interaction to lipid rafts of WT-Syn (a) or Pma1 (b) in the specified mutants affected in ergosterol biosynthesis (a, b) or in the *nat3Δ* mutant (c). Extracts were prepared in the presence or absence of 0.2% Triton-X-100 at 0 °C as indicated. The quantification displays the amount of  $\alpha$ -Syn bound to DRMs (fractions 3, 4, and 5) versus the soluble fraction (fractions 7 to 11). The results are relative to the data obtained with the wild type strain where the amount of  $\alpha$ -Syn present in DRM fractions is set at 100%. (C) Fluorescence microscopic visualization and intracellular localization of EGFP-tagged WT-Syn in ergosterol biosynthesis mutants grown for 18 h in selective medium containing 20  $\mu$ M methionine.

yeast requires the incorporation of plasma membrane resident proteins into detergent-insoluble domains or lipid rafts, a process that occurs before exit of the Golgi apparatus [73,74]. In mouse brain,  $\alpha$ -Syn was indeed reported to be associated to lipid rafts [68] and we provide the first evidence that also in yeast a minor fraction of native and EGFP-fused  $\alpha$ -Syn co-purifies with the detergent-insoluble membrane domains. Moreover, the interaction between  $\alpha$ -Syn and lipid rafts in mammalian cells was shown to be abrogated by depletion of cholesterol or the introduction of the A30P mutation in  $\alpha$ -Syn [68] and the same parameters were found to determine the presence of  $\alpha$ -Syn in the detergent-resistant fractions of yeast membranes.

Recent studies described the blockage of ER-to-Golgi transport [9] and accumulation of ER-Golgi transport vesicles and secretory

vesicles [15] as early phenomena underlying the  $\alpha$ -Syn-mediated growth defect in yeast. Hence, it is tempting to speculate that these phenomena depend on the interaction of  $\alpha$ -Syn with lipid domains in the membranous compartments and vesicles. The observation that the  $\alpha$ -Syn mutant A30P combines a strongly reduced DRM interaction with failure to form inclusions, to accumulate vesicles or to induce a significant growth defect, supports such a dependency. For WT-Syn, however, our results were not that conclusive since comparable amounts of the protein appear to be present in DRM fractions isolated from wild type cells, where WT-Syn is mostly targeted to the plasma membrane, from the ergosterol biosynthesis mutants *erg6Δ* and *erg28Δ*, which are both characterized by enhanced  $\alpha$ -Syn inclusion formation, or from the *nat3Δ* mutant,

which displays a more cytosolic localization of WT-Syn. Nonetheless, there are differences between the strains for the distribution of WT-Syn over the various optiprep fractions, including those that contain DRMs, and we cannot rule out completely that these differences might be important. In addition, WT-Syn failed to properly interact with DRMs when expressed in the *erg24Δ* mutant and although we still observed plasma membrane localization of the WT-Syn-EGFP fusion protein, the latter failed to form inclusions under the conditions used for the mutant collection screening. It should also be noted that only a minimal portion of WT-Syn expressed in yeast co-purifies with DRMs and thus, that the majority of the protein is found in the detergent soluble fractions. This makes WT-Syn different from typical lipid raft associated proteins and it possibly indicates that the association of WT-Syn with DRMs could be indirect via an interaction with another protein. In mammalian cells,  $\alpha$ -Syn was recently shown to interact with the septin, Sept4, a presynaptic scaffold present in lipid rafts that also binds other proteins like the dopamine transporter, syntaxin and SNAP25. Interestingly, this study revealed Sept4 to function as a suppressor for  $\alpha$ -Syn phosphorylation, oligomerization and neurotoxicity [75] and it was suggested Sept4 plays an important role in vesicle assembly and release [76].

Our data established that  $\alpha$ -Syn is phosphorylated at ser-129 in yeast cells and that this is dependent on the CK-I and CK-II casein kinases. Again, this is in line with data obtained from studies in higher eukaryotic systems [30,31] but while these and other studies [34,35] indicated that  $\alpha$ -Syn phosphorylation is an early event in the pathology of Parkinson's disease, the yeast model only confirmed that reducing the ser-129 phosphorylation by deletion of the plasma membrane-localized CK-I kinase Yck1 or Yck2 coincided with a drop of  $\alpha$ -Syn-mediated growth retardation or toxicity. The depletion of the CK-II kinase activity did not ameliorate cell growth but this may not be surprising since CK-II is important for the transduction of survival signals, both in mammalian and yeast cells [25].

Interestingly, both Yck1 and Yck2 are known to phosphorylate and thereby stimulate the internalization of several yeast plasma membrane proteins, including the pheromone receptors Ste2 and Ste3 [29,36,77], the uracil permease Fur4 [37,38] and the maltose permease Mal61 [78]. In addition, Yck1 also associates to the plasma membrane glucose sensor Rtg2 to promote the phosphorylation and degradation of Mth1 and Std1, two proteins required for the regulation of glucose transporters in yeast [79]. This led us to assume that the reduced toxicity of  $\alpha$ -Syn in the *yck1Δ* and *yck2Δ* mutants could relate to a lower endocytic rate. This assumption was further supported by the observation that also mutants lacking other proteins involved in the formation and internalization endocytic vesicles, i.e. the clathrin heavy chain Chc1, the amphiphysin-homolog Rvs161 or the SGK2-homolog Ypk2, displayed reduced  $\alpha$ -Syn toxicity as well. In contrast, mutants with post-vesicle-internalization defects were characterized by enhanced  $\alpha$ -Syn toxicity. Together, these data suggest that in addition to obstruction of ER-to-Golgi transport and the secretory pathway,  $\alpha$ -Syn toxicity must also arise from interference with endocytic processes. As such,  $\alpha$ -Syn behaves similar to expanded polyQ Huntingtin fragments, which also gain toxicity in yeast by obstructing early steps of endocytosis [80,81].

The increased  $\alpha$ -Syn toxicity in mutants with post-vesicle-internalization defects appeared to be specific as it did not occur with the *vps45Δ* mutant, lacking a protein involved in traffic between Golgi and the prevacuolar compartment and transport of Cvt vesicles from the cytoplasm to the vacuole [55,56]. One could argue that a block in endocytosis prevents efficient vacuolar degradation of  $\alpha$ -Syn and other proteins and that this would be the cause of enhanced  $\alpha$ -Syn toxicity in the post-vesicle-internalization mutants. However, the toxicity of  $\alpha$ -Syn was similar in wild type cells and in the *vps45Δ* mutant, which fails to properly deliver proteases to the vacuole [55], and it was even reduced in the *vps33Δ* mutant, which does not have discernible vacuoles [60,61]. This makes it unlikely that the lack of

vacuolar degradation capacity would be the primary reason for the observed increased  $\alpha$ -Syn toxicity. Moreover, in none of the strains analyzed in our screening we observed WT-Syn-EGFP to enter the vacuolar lumen, an observation that is in line with data from a study recently performed by Witt and coworkers. They identified Ypp1, a protein involved in receptor-mediated endocytosis, as multicopy suppressor of the toxicity triggered by A30P when yeast cells are challenged with peroxide-induced oxidative stress. In unraveling the molecular mechanism underlying this suppression, they showed that Ypp1 binds to A30P and drives this protein to vacuolar degradation via the endocytic pathway. Interestingly, Ypp1 does not interact with WT-Syn and in consequence fails to induce its vacuolar degradation or to rescue yeast cells from the toxicity induced by WT-Syn [82].

Studies on the yeast amino acid transporter Gap1 and the uracil permease Fur4, indicated that these proteins gradually dissociate from DRMs during trafficking along the endocytic pathway to recycle back to the plasma membrane [74,83]. Interestingly, yeast mutants with post-vesicle-internalization defects have higher rates of recycling and therefore display an apparent stabilization of transporters, permeases and receptors at the plasma membrane [57–59]. Our data with the *vps33Δ* mutant indicate that recycling also applies to  $\alpha$ -Syn and that it underlies, at least in part,  $\alpha$ -Syn toxicity in yeast cells. The *vps33Δ* mutant is known to have recycling deficiencies [58] and it was the only yeast strain with a post-vesicular-internalization defect that did not display enhanced  $\alpha$ -Syn toxicity levels and this despite the highest score for inclusion formation of all mutants tested. Furthermore, the additional deletion of *VPS33* in mutants with a post-vesicle-internalization defects counteracted the  $\alpha$ -Syn-induced growth defect.

When compared to the congenic wild type strain, mutants affected in endocytosis steps downstream of vesicle internalization were characterized by enhanced ser-129 phosphorylation of  $\alpha$ -Syn. In contrast, the *vps33Δ* mutant kept  $\alpha$ -Syn trapped as a hypophosphorylated protein, similar to what has been reported for the v-SNARE Snc1 [58]. This implies that recycling allows for accumulation of phosphorylated  $\alpha$ -Syn. Although this aspect was not studied in detail, it is tempting to speculate that this is due to phosphorylation of the accumulated  $\alpha$ -Syn at the plasma membrane by Yck1 and Yck2. Accumulation at the plasma membrane would also allow to reach the critical concentration of  $\alpha$ -Syn necessary for self-assembly and inclusion formation, a phenomenon we and others demonstrated to initiate at the plasma membrane [7,8]. Here we should mention that there is still a controversy whether or not  $\alpha$ -Syn fibrilization occurs in yeast cells. Indeed, while we previously reported that native and EGFP-tagged  $\alpha$ -Syn formed thioflavin-S positive and thus amyloid-like aggregates [7], it was more recently argued based on EM studies that the  $\alpha$ -Syn accumulations do not contain filamentous structures but solely clusters of membranous vesicles [15]. Whatever the nature of the inclusions, their formation depends on the binding of  $\alpha$ -Syn to membranes. In this context it is interesting to note that recent studies in yeast suggested recycling to go along with changes in the lipid content between the plasma membrane and endosomal membranes [74,83], similar to observations made in mammalian cells where sphingolipids are restricted to enter the lysosomal degradative pathway but recycle back from endosomes to the cell surface [84,85].

To date, different recycling pathways have been described in yeast and proteins can directly recycle from endosomes to the plasma membrane or first traverse through the Golgi system [57,58]. As such, recycling could feed in and accelerate the toxic effect of  $\alpha$ -Syn on ER–Golgi traffic previously described by Lindquist and coworkers [9].

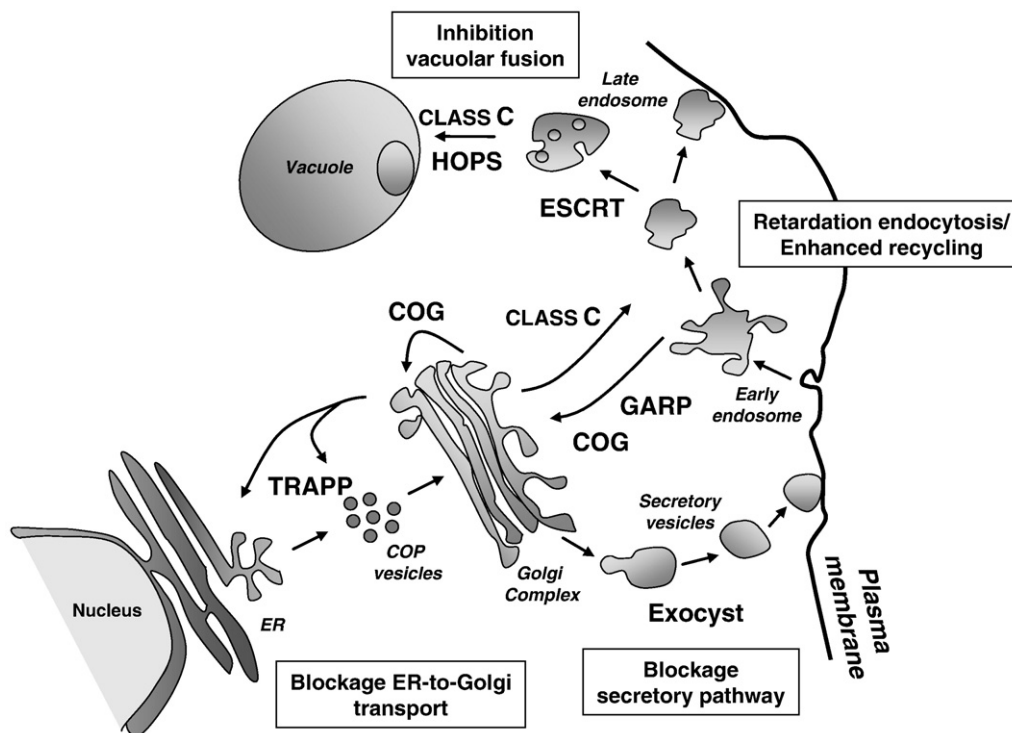
In wild type yeast cells, we found WT-Syn and A53T, but not A30P, to induce vacuolar fusion defects in wild type cells. Evidence that vacuolar fusion defects underlie  $\alpha$ -Syn-induced toxicity comes from a recent study performed by Cooper and coworkers. They identified several suppressors for the  $\alpha$ -Syn-induced toxicity in yeast and this included Ykt6, a v-SNARE protein that apart from a role in retrograde

transport to the *cis*-Golgi has also an essential function in vacuolar fusion [9,86–88]. Moreover, we showed that the disruption of the *N*-acetyltransferase NatB partially suppressed this  $\alpha$ -Syn-induced vacuolar fusion defect and that this coincided with failure of  $\alpha$ -Syn to trigger toxicity in the *nat3* $\Delta$  mutant. Although we did not measure acetylation of  $\alpha$ -Syn directly, there is a good chance that  $\alpha$ -Syn is directly subject to *N*-terminal acetylation by NatB since its *N*-terminus fits with the proposed consensus sequence of Nat B substrates. Moreover,  $\alpha$ -Syn is *N*-terminally acetylated in neurons [35]. Whether this means that *N*-terminal acetylation of  $\alpha$ -Syn is required to trigger the vacuolar fusion phenotype cannot be concluded as disruption of NatB itself already influenced the phenotype to some extent. Interestingly, disruption of NatB altered the intracellular localization of WT-Syn from the plasma membrane to a more cytoplasmic distribution and this went together with lowering ser-129 phosphorylation of WT-Syn. Again this corroborated a link between plasma membrane binding, phosphorylation and toxicity of  $\alpha$ -Syn. Taken together, it is feasible to state that WT-Syn in the NatB mutant behaves similarly as the clinical A30P mutant in wild type yeast cells. Hence, it would be appealing to investigate whether WT-Syn and the A30P mutant are differentially acetylated and whether possible differences relate directly to the above described YPP1-induced vacuolar degradation. Indeed, although the biological function of *N*-terminal acetylation is not well understood, it was previously suggested to play a crucial role in impeding protein turnover [89]. Note, however, that in human brain the *N*-terminally acetylated protein is present in Lewy bodies as well as brain cytosol [35], suggesting that the modification is not specific for the formation of insoluble  $\alpha$ -Syn aggregates. However, this does not exclude that *N*-terminal acetylation could be a trigger in the process leading to the precursors of aggregates, i.e. the soluble  $\alpha$ -Syn protofibrils. Also intriguing is the characterization of the putative GCN5-related acetyltransferase (GNAT), Yir042c, as a protein preventing vacuolar membrane targeting of  $\alpha$ -Syn. Several GNAT proteins have been identified to date and subsets of this protein family are known to have different substrate specificities [90]. So far, the role and

substrates of Yir042c are elusive but based on the observation that the *YIRO472c* deletion results in binding of  $\alpha$ -Syn to the vacuolar membrane, we speculate that either the protein has a role in lipid metabolism or that it acts as modulator of the lipid binding properties of  $\alpha$ -Syn.

Another important aspect disclosed by our analyses is that there is no strict correlation between the percentage of cells that formed  $\alpha$ -Syn inclusions and  $\alpha$ -Syn toxicity observed in the corresponding yeast culture. For instance, for the *ypk1* $\Delta$  and the *vps45* $\Delta$  mutants 13% and 60% of the cells formed inclusions, respectively, and both strains display enhanced  $\alpha$ -Syn toxicity as compared to the wild type strain, while conversely for the *vps33* $\Delta$  mutant we counted 93% inclusion-positive cells but observed a lower  $\alpha$ -Syn toxicity as in the wild type strain. This indicates that self-assembly or inclusion formation of  $\alpha$ -Syn is not toxic *per se* but that it is rather the disruption of normal membrane processes by  $\alpha$ -Syn that determines its toxicity. A similar conclusion was reached by Volles and Lansbury, who analyzed randomly generated  $\alpha$ -Syn mutants [91]. Based on their study, toxicity in yeast cells solely correlated to the membrane binding ability of  $\alpha$ -Syn and not with the fibrilization rate of the protein as measured *in vitro*. Moreover, when expressed in the fission yeast *Schizosaccharomyces pombe*, human  $\alpha$ -Syn does not target the plasma membrane and the protein is not toxic despite its extensive aggregation [92].

To summarize, our data suggest the model depicted in Fig. 8 where in addition to blockage of ER-to-Golgi transport and the secretory pathway as previously described [9,15],  $\alpha$ -Syn gains toxicity in yeast upon its association with forming vesicles at the plasma membrane, a process that is, at least in part, controlled by the plasma membrane resident CK-I kinases. Thereby  $\alpha$ -Syn enters the vacuolar degradation pathway but since it is excluded to enter the vacuole,  $\alpha$ -Syn recycles from endosomes back to the plasma membrane. This continuous cycle might eventually lead to saturation and blockage of endocytosis and vesicular trafficking routes that merge with the endocytic protein sorting machinery. Thus, it appears that the properties ascribed to  $\alpha$ -



**Fig. 8.** Interference of  $\alpha$ -Syn with vesicular trafficking in yeast. Shown are different vesicular trafficking routes in yeast and the tethering and sorting complexes involved (TRAPP, COG, GARP, ESCRT, HOPS, Exocyst and the Class C Vps complex). Expression of  $\alpha$ -Syn in yeast affects vesicular traffic at different stages as highlighted in the text boxes, thereby leading to a blockage of ER-to-Golgi transport and the secretory pathway, retardation of endocytosis, enhanced recycling and vacuolar fusion defects.

Syn for the control of vesicular dynamics and vesicular recycling in neurons [3–5] are maintained when the protein is expressed in yeast, making the latter a prime model to study the biology and pathobiology of  $\alpha$ -Syn.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.06.010.

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