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Research Article

RESEARCH ARTICLE

Identification of novel protein phosphatases as modifiers of alpha-synuclein aggregation in yeast

Inês Caldeira Brás[1], Sandra Tenreiro[2], Andreia M. Silva[2] and Tiago F. Outeiro[1,2,3,4,*]

[1]Department of Experimental Neurodegeneration, Center for Nanoscale Microscopy and Molecular Physiology of the Brain, Center for Biostructural Imaging of Neurodegeneration, University Medical Center Goettingen, Walweg 33, 37073 Goettingen, Germany, [2]CEDOC – Chronic Diseases Research Center, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Rua Câmara Pestana n° 6, 6-A Edifício CEDOC II 1150-082 Lisboa, Portugal, [3]Max Planck Institute for Experimental Medicine, Hermann-Rein-Straße 3, 37075 Goettingen, Germany and [4]Institute of Neuroscience, The Medical School, Newcastle University, F ramlington Place, Newcastle Upon Tyne, NE2 4HH, UK

*Corresponding author: Department of Experimental Neurodegeneration University Medical Center Goettingen Waldweg 33, 37073 Goettingen, Germany. Tel: +49 (0) 5513913544; E-mail: tiago.outeiro@med.uni-goettingen.de

One sentence summary: In this article, we describe the use of yeast as a platform for the identification of phosphatases associated with synucleinopathies.

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ABSTRACT

Alpha-synuclein (aSyn) is a key player in a group of neurodegenerative diseases commonly known as synucleinopathies. Recent findings indicate phosphorylation in several aSyn residues can modulate its aggregation and subcellular localization, thereby affecting pathological processes. However, the precise molecular mechanisms governing aSyn phosphorylation are still unclear. Recent studies investigated the role of various families of protein kinases, such as the polo-like kinases, G protein-coupled receptor kinases or casein kinases. In contrast, our understanding of the phosphatases involved in the dephosphorylation of aSyn is rather limited. Here, we exploited the unique toolbox of the yeast *Saccharomyces cerevisiae* in order to identify novel phosphatases capable of modulating aSyn phosphorylation, inclusion formation and toxicity of human aSyn. In summary, given the association between aSyn phosphorylation and pathology in Parkinson's disease and other synucleinopathies, modulation of this post-translational modification may constitute an attractive target for therapeutic intervention.

Keywords: Parkinson's disease; alpha-synuclein; phosphorylation; phosphatases; neurodegeneration

INTRODUCTION

The recent worldwide increase in life expectancy resulted in a significant increase in the prevalence of several age-associated neurodegenerative diseases including Alzheimer's and Parkinson's disease (PD). PD belongs to a diverse group of neurodegenerative proteinopathies known as synucleinopathies. The progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* and the formation of Lewy bodies (LBs) composed of phosphorylated alpha-synuclein (aSyn) are the pathological hallmarks in this disorder (Spillantini *et al.* 1997; Spillantini *et al.* 1998; Goedert 2001; Wong and Krainc 2017). aSyn has been strongly linked to the pathogenesis of both familial

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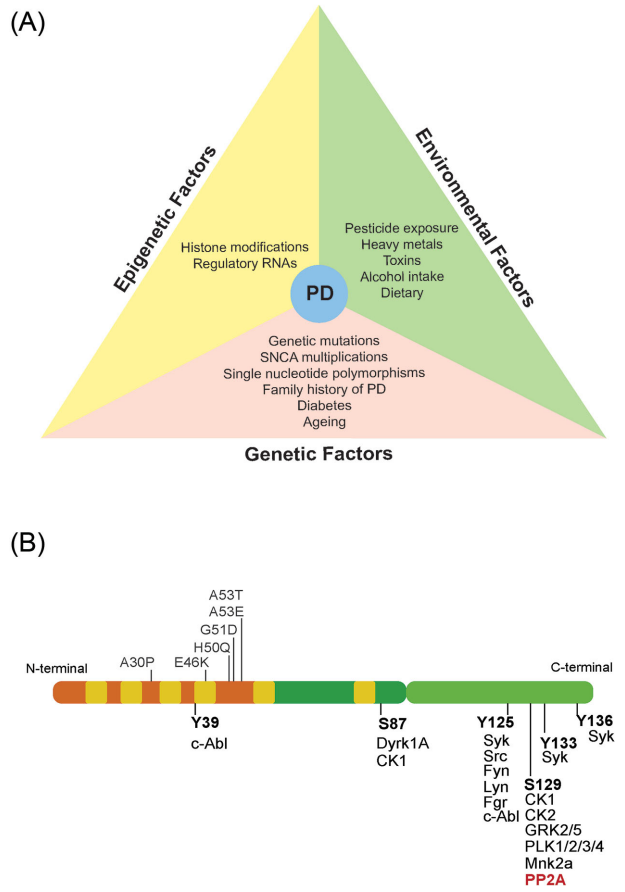

Figure 1. Risk factors associated with PD. (**A**) The etiology of PD is still unclear, but several risk factors have been described. These include environmental factors, genetic factors and epigenetics factors. (**B**) Schematic depicting the three main regions of aSyn. On top, PD-associated mutations are listed. Below, phosphorylation sites, and the known kinases (in black) and phosphatases (in red) involved in aSyn phosphorylation/dephosphorylation. In orange, the amphipathic N-terminal of the protein with the six mutations associated with familial PD (in gray); the hydrophobic central region that contains the nonamyloid-beta component (NAC) domain is represented in dark green and, in last, the acidic C-terminal is represented in light green. The protein contains imperfect KTKEGV repeats, which are represented in the figure as yellow squares.

and sporadic forms of PD (Fig. 1) (Polymeropoulos *et al.* 1997; Spillantini *et al.* 1997; Kruger *et al.* 1998; Zarranz *et al.* 2004; Appel-Cresswell *et al.* 2013; Lesage *et al.* 2013; Pasanen *et al.* 2014).

The precise molecular determinants of aSyn misfolding and aggregation in synucleinopathies are still unclear, but imbalances in phosphorylation and in other post-translational modifications (PTMs) have been described to play important roles in aSyn biology and pathobiology. aSyn is ubiquitylated (Spillantini *et al.* 1998; Shimura *et al.* 2001) and phosphorylated (Fujiwara *et al.* 2002) in LBs. Furthermore, aSyn can also undergo other PTMs, such as sumoylation (Krumova *et al.* 2011), acetylation (Bartels *et al.* 2014; de Oliveira *et al.* 2017) or glycation (Vicente Miranda *et al.* 2017), and these PTMs can modulate its aggregation and toxicity in different model systems.

The levels of aSyn phosphorylation are regulated by a balance between phosphorylation by protein kinases, and dephosphorylation by protein phosphatases. aSyn can be phosphorylated in several serine, tyrosine and threonine residues (Tenreiro, Eckermann and Outeiro 2014) (Fig. 1B). Most studies have focused on the phosphorylation on serine 129 (S129) (Tenreiro, Eckermann and Outeiro 2014). This PTM is consistently observed in LBs in PD, as well as in dementia with Lewy bodies (DLB) and in multiple system atrophy (MSA) (Fujiwara *et al.* 2002; Saito *et al.* 2003; Anderson *et al.* 2006). While only 4% of soluble, monomeric aSyn appears phosphorylated under physiological conditions, approximately 90% is phosphorylated in LBs of patients with PD (Fujiwara *et al.* 2002; Anderson *et al.* 2006; Zhou *et al.* 2011, Lue *et al.* 2012; Walker *et al.* 2013).

Despite previous efforts, there is still no consensus on whether phosphorylation promotes or inhibits aSyn aggregation and neurotoxicity (Saito *et al.* 2003; Oueslati, Fournier and Lashuel 2010; Basso *et al.* 2013; Tenreiro, Eckermann and Outeiro 2014; Oueslati 2016; Samuel *et al.* 2016). Despite this controversy, aSyn S129 phosphorylation is routinely used as a marker of aSyn aggregation (Kragh *et al.* 2009; Kuwahara *et al.* 2012; Febbraro *et al.* 2013; Tenreiro, Eckermann and Outeiro 2014).

Assuming that phosphorylation of aSyn affects its biology/pathobiology, one might assume that pharmacological modulation of phosphorylation may be used as a therapeutic target for synucleinopathies. In fact, inhibition of relevant aSyn kinases was attempted based on both the druggability and past success of kinase inhibitors for other disorders (Zhang, Yang and Gray 2009). Several tyrosine kinases phosphorylate aSyn, including c-Abl (Mahul-Mellier *et al.* 2014; Brahmachari *et al.* 2016), Syk (Negro *et al.* 2002), and the Src-family kinases—Src (Ellis *et al.* 2001), Fyn (Nakamura *et al.* 2001), Lyn and c-Frg (Negro *et al.* 2002). Likewise, phosphatases that reduce phosphorylation are equally important in maintaining protein phosphorylation status, but these have received little attention as drug targets, as phosphatases seem to be even less specific (Braithwaite *et al.* 2012).

Protein phosphatases fall into four major families established on different structures, specificity and catalytic mechanisms. These include serine/threonine-specific phosphoprotein phosphatases (PPP), magnesium-dependent protein phosphatases (PPM/PP2C), aspartate-based protein phosphatases and phosphotyrosine phosphatases (PTP) (Lillo *et al.* 2014). The PPP family is among the most highly conserved family in eukaryotes, and can be further divided into different subgroups: PP1, PP2/PP2A and PP3/PP2B (Table S1, Supporting Information) (Sancenon *et al.* 2012). Phosphatases are difficult to study since many of them are only functional as part of complexes with one or more regulatory subunits. However, phosphatases can rival the kinases in terms of substrate specificity, depending on the different regulatory subunits involved in the protein phosphatase complexes.

The *Saccharomyces cerevisiae* toolbox is still unrivaled among eukaryotes, enabling powerful large-scale screens. This model system has been used to investigate the biology and pathobiology of aSyn (Outeiro and Lindquist 2003), and several molecular aspects of PD have been recapitulated in yeast (Tenreiro *et al.* 2017). Over the years, several genetic and pharmacological screens have been performed to identify modifiers of aSyn toxicity and aggregation (Willingham *et al.* 2003; Cooper *et al.* 2006; Flower *et al.* 2007; Liang *et al.* 2008; Zabrocki *et al.* 2008; Su *et al.* 2010; Tardiff and Lindquist 2013, Dhungel *et al.* 2015). The molecular impact of phosphorylation in aSyn has been investigated in yeast, using heterologous expression of human kinases or phosphatases together with human aSyn (Zabrocki *et al.* 2008; Gitler *et al.* 2009).

There is high conservation, from yeast to humans, in the three groups of serine/threonine protein phosphatases, PP2A, PP4 and PP6, and their interactors (Fig. 2; Table S1, Supporting Information). Thus, based on the sequence similarities of

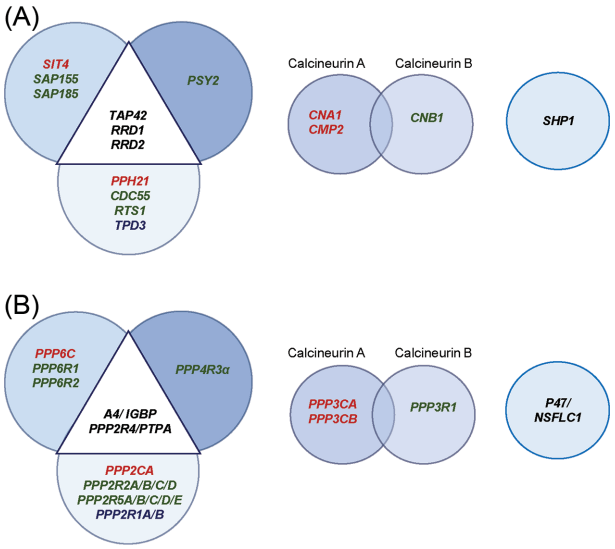

Figure 2. Genes selected for the focused screen in yeast. (**A**) Saccharomyces *cerevisiae* genes and (**B**) *Homo sapiens* homologues. Boxes with similar shapes and colors represent different families. (Left) The three closely related groups of serine/threonine protein phosphatases (PP). PP2A, PP4 and PP6 are conserved throughout eukaryotes. Genes encoding catalytic subunits are shown in red, canonical scaffolding subunits in blue and regulatory subunits are in green; putative common interactors for the subunits are shown in black in the central triangle. (Middle) Calcineurin consists in a catalytic subunit calcineurin A and a tightly associated calcium-binding regulatory subunit, calcineurin B. (Right upper) Shp1, p47/NSFLC1 *Homo sapiens* homolog, promotes cell cycle progression by positive regulation of PP1.

protein phosphatases in yeast and mammals, major findings can be expected from the use of yeast as a toolbox. For example, understanding PP2A activities in PD has been difficult since this phosphatase can be assembled with different subunits. In yeast, each PP2A subunit can be individually expressed, simplifying the study of the relevance of each subunit on the function of the complete PP2A.

Here, we performed a focused screen to identify phosphatases able to modulate aSyn-associated phenotypes in yeast (Fig. 2A; Table S1, Supporting Information). Among 15 proteins tested, Shp1, Cna1, Cnb1, Rrd2, Sap155 and Sit4 reduced the percentage of cells with aSyn intracellular inclusions. Interestingly, these proteins did not modulate aSyn phosphorylation on S129 (aSyn pS129), indicating the importance of studying the effect on other aSyn residues. In total, our study suggests that modulation of the activity of protein phosphatases may prove a useful approach for treating PD and other synucleinopathies in ways that do not depend only on the phosphorylation of aSyn on S129.

RESULTS

In order to identify phosphatases that can modulate aSyn-associated phenotypes in yeast, we performed a focused screen of serine/threonine protein phosphatases with human homologs (Fig. 2). We assessed the effects of the selected phosphatases on aSyn cytotoxicity, and determined their effect on aSyn pS129 and on inclusion formation in yeast. In particular, we focused on three closely related groups of serine/threonine protein phosphatases, PP2A, PP4 and PP6 and known interactors (Fig. 2; Table S1, Supporting Information).

We co-expressed aSyn fused to GFP (aSyn-GFP) using a galactose-inducible promoter (*GAL1*) along with 15 proteins

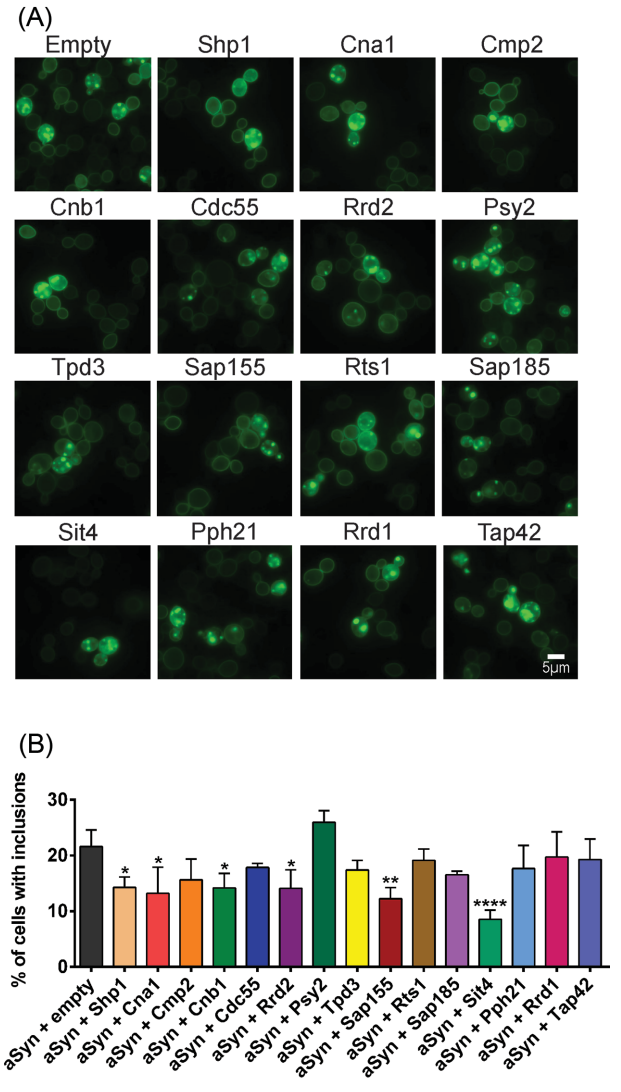

Figure 3. Effect of the indicated phosphatases on aSyn inclusion formation in yeast. (**A**) Fluorescence microscopy images of cells expressing aSyn-GFP alone [aSyn + empty vector (empty)] or together with the selected phosphatases (Shp1, Cna1, Cmp2, Cnb1, Cdc55, Rrd2, Psy2, Tpd3, Sap155, Rts1, Sap185, Sit4, Pph21, Rrd1 and Tap42). Images were obtained 8 h after aSyn-GFP expression induction in galactose-containing medium. A representative picture is shown from three independent experiments. (**B**) Quantification of the percentage of cells presenting aSyn inclusions (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$). Values represent the mean ± SD from three independent experiments.

from the Open Biosystems yeast open reading frame (ORF) collection (Gelperin *et al.* 2005), to assess the effect of each ORF on aSyn inclusion formation in yeast. Expression of human aSyn-GFP in the W303.1A yeast strain results in a moderate level of aSyn toxicity. Co-expression of aSyn-GFP with Shp1, Cna1, Cnb1, Rrd2, Sap155 and Sit4 resulted in a significant reduction in the percentage of cells with aSyn inclusions (Fig. 3). Sit4 exerted the strongest reduction, and the other phosphatases did not affect the formation of aSyn inclusions (Fig. 3B). Interestingly, none of the selected phosphatases significantly affected the total levels of aSyn or of S129 phosphorylation (Fig. 4; Table S2, Supporting Information). We further investigated the effect of the phosphatases on aSyn-induced cytotoxicity by spotting assays (Fig. 5A; Table S3, Supporting Information). The expression of the phosphatases alone (except Tpd3 and Rts1) resulted in a

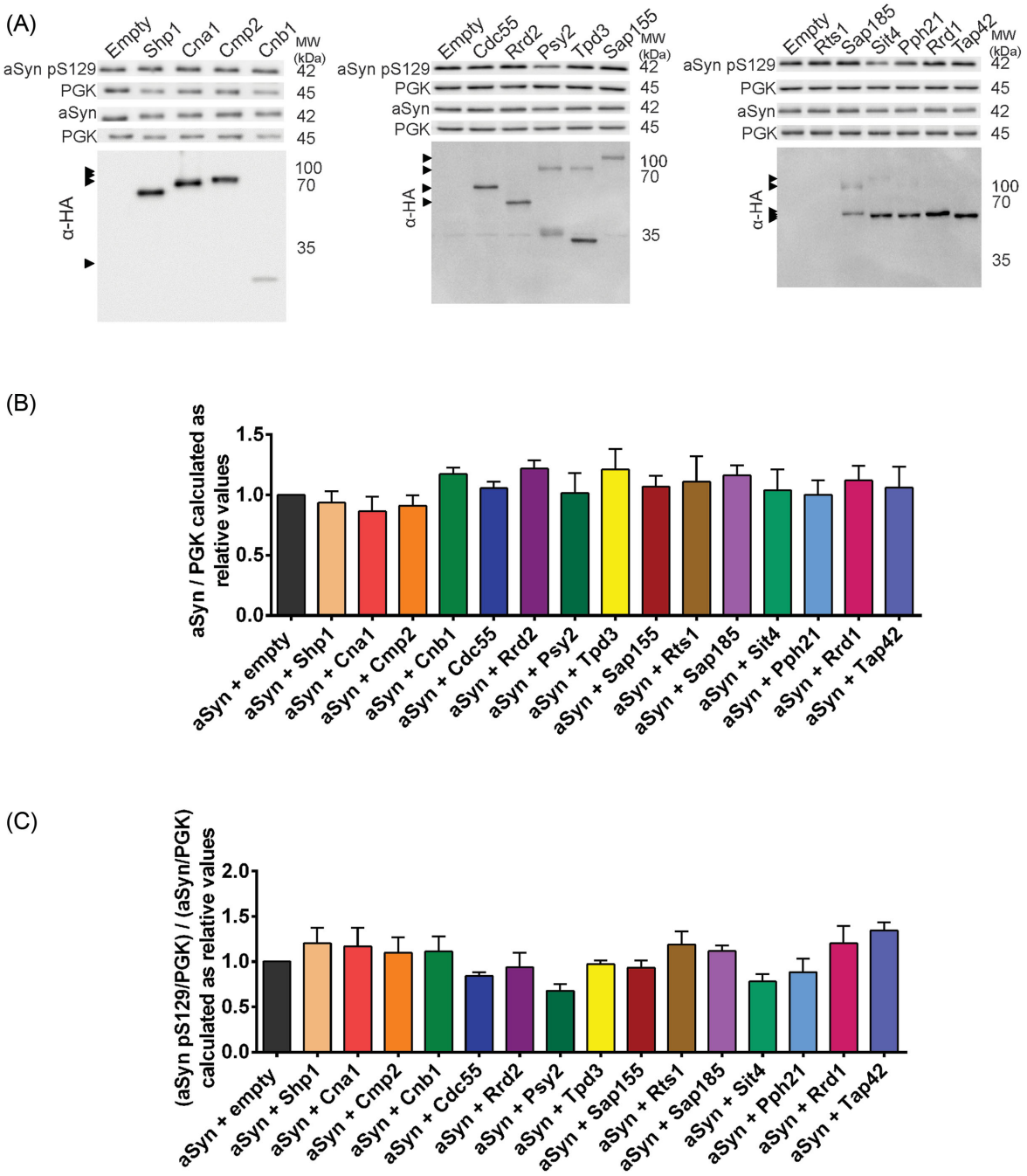

Figure 4. Effect of the indicated phosphatases on the levels of total aSyn and of pS129 in yeast. (**A**) aSyn protein and aSyn phosphorylated on serine 129 (aSyn pS129), when expressed aSyn alone [aSyn + empty vector (empty)] or with phosphatases (Shp1, Cna1, Cmp2, Cnb1, Cdc55, Rrd2, Psy2, Tpd3, Sap155, Rts1, Sap185, Sit4, Pph21, Rrd1 and Tap42), assessed by immunoblotting, 8 h after aSyn-GFP and phosphatases expression induction. Phosphatases expression was detected by western blot using an antibody against HA flag. Correspondent molecular weight (kDa) is indicated by arrowheads. (**B**) Densitometry analysis of the immunodetection of aSyn-GFP normalized to PGK, used as loading control, presented in arbitrary units (a.u.). (**C**) Densitometry analysis of the immunodetection of pS129 aSyn-GFP relative to the intensity obtained for total aSyn and normalized by PGK, used as loading control, presented in arbitrary units (a.u.). A representative result is shown from three independent experiments. Values represent the mean ± SD from three independent experiments.

reduction of cell growth, suggesting that the overexpression of these genes is toxic to yeast cells (Fig. 5A; Table S3, Supporting Information). Expression of Shp1, Cdc55, Sap155 and Sap185 resulted in higher toxicity when co-expressed with aSyn-GFP, in contrast to Cmp2 and Pph21 that enhanced cell growth (Fig. 5A; Table S3, Supporting Information). No effects were observed when the other proteins were co-expressed together with aSyn-GFP.

To further investigate the effect of the selected phosphatases (Sap155, Rrd2 and Sit4) on aSyn-mediated toxicity, we performed flow cytometry measurements using Propidium Iodide (PI) as a marker of toxicity by measuring plasma membrane

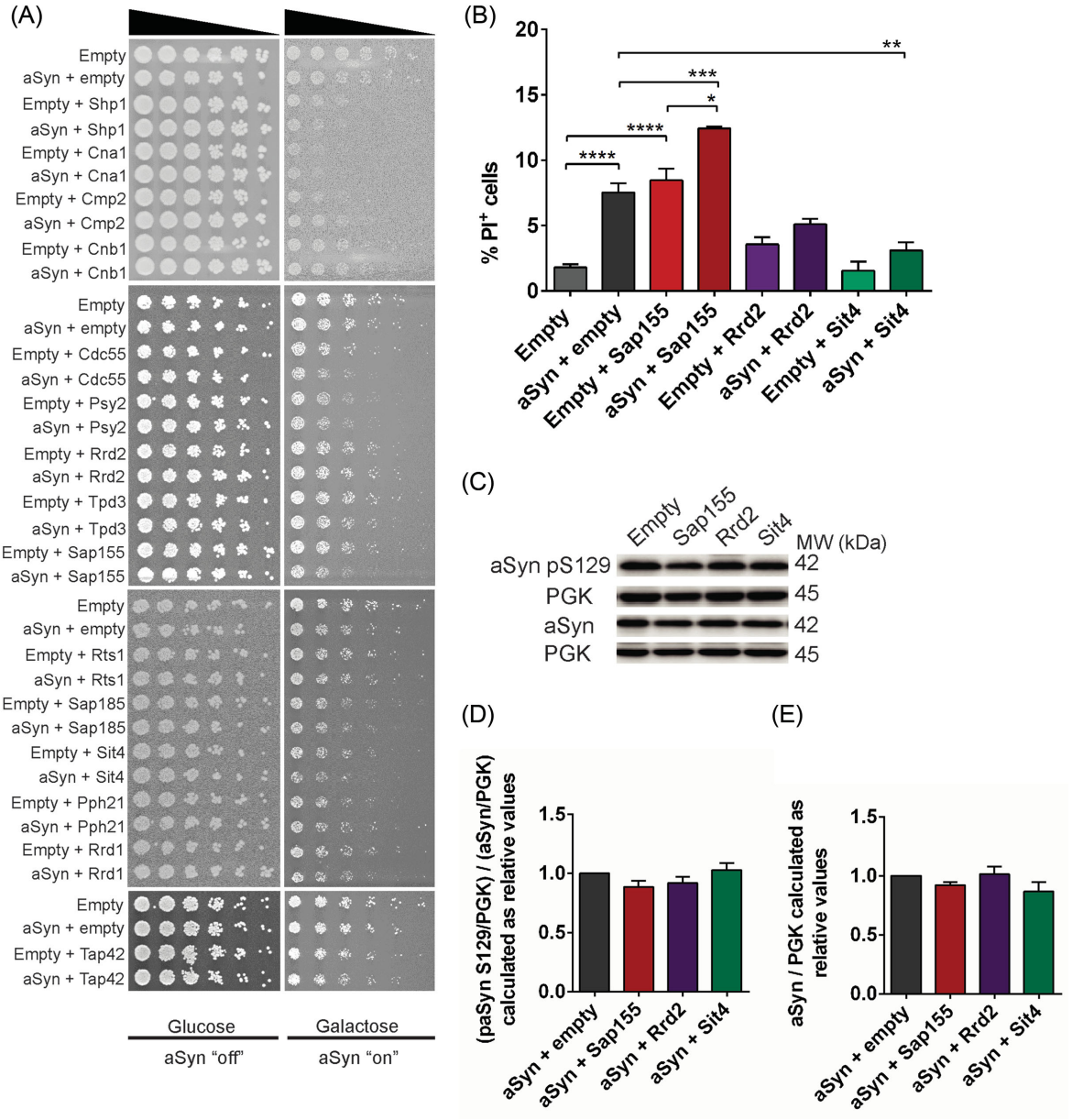

Figure 5. Effect of the phosphatases on aSyn toxicity in yeast. (**A**) Spotting assays of yeast cells co-expressing aSyn alone [aSyn + empty vector (empty)] or with phosphatases (Shp1, Cna1, Cmp2, Cnb1, Cdc55, Rrd2, Psy2, Tpd3, Sap155, Rts1, Sap185, Sit4, Pph21, Rrd1 and Tap42). Cell suspensions were adjusted to the same OD_{600nm}, serially diluted and spotted onto the surface of the solid medium containing either glucose (control) or galactose at the indicated proportions as carbon sources. (**B**) aSyn-GFP versus PI fluorescence in yeast cells co-expressing aSyn alone [aSyn + empty vector (empty)] or together with phosphatases, assessed by flow cytometry in the GFP (530/30 BP) channel, 16 h after induction of expression of aSyn-GFP and phosphatases. (**C**) aSyn protein and aSyn pS129 when expressed aSyn alone [aSyn + empty vector (empty)] or with phosphatases, assessed by immunoblotting, 16 h after induction of expression of aSyn-GFP and phosphatases. (**D**) Densitometry analysis of the immunodetection of aSyn-GFP normalized by PGK, used as loading control, presented in arbitrary units (a.u.). (**E**) Densitometry analysis of the immunodetection of pS129 aSyn-GFP relative to the intensity obtained for total aSyn and normalized by PGK, used as loading control, presented in arbitrary units (a.u.). A representative result is shown from three independent experiments. Values represent the mean ± SD from three independent experiments (****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

integrity 16 h after induction of protein expression. As expected, expression of aSyn alone increased the percentage of PI-positive (PI^+) cells when compared to control cells (Fig. 5B). Consistently, with the results obtained in the spotting assays (Fig. 5A; Table S3, Supporting Information), expression of Sap155 increased toxicity, and this was further increased upon co-expression with aSyn (Fig. 5B). In contrast, co-expression of Sit4 with aSyn was protective when compared with the expression of aSyn alone (Fig. 5A and B). Expression of Rrd2 had no significant effect (Fig. 5B), in agreement with the results observed in the spotting assay (Fig. 5A; Table S3, Supporting Information).

Interestingly, no significant alterations in the total levels of aSyn or of pS129 aSyn were observed after 16 h of protein induction in cells co-expressing aSyn with each of the phosphatases (Fig. 5C–E).

The effect of a subset of phosphatases (Tap42, Cmp2 and Rrd1) was also evaluated in a yeast strain stably expressing aSyn,

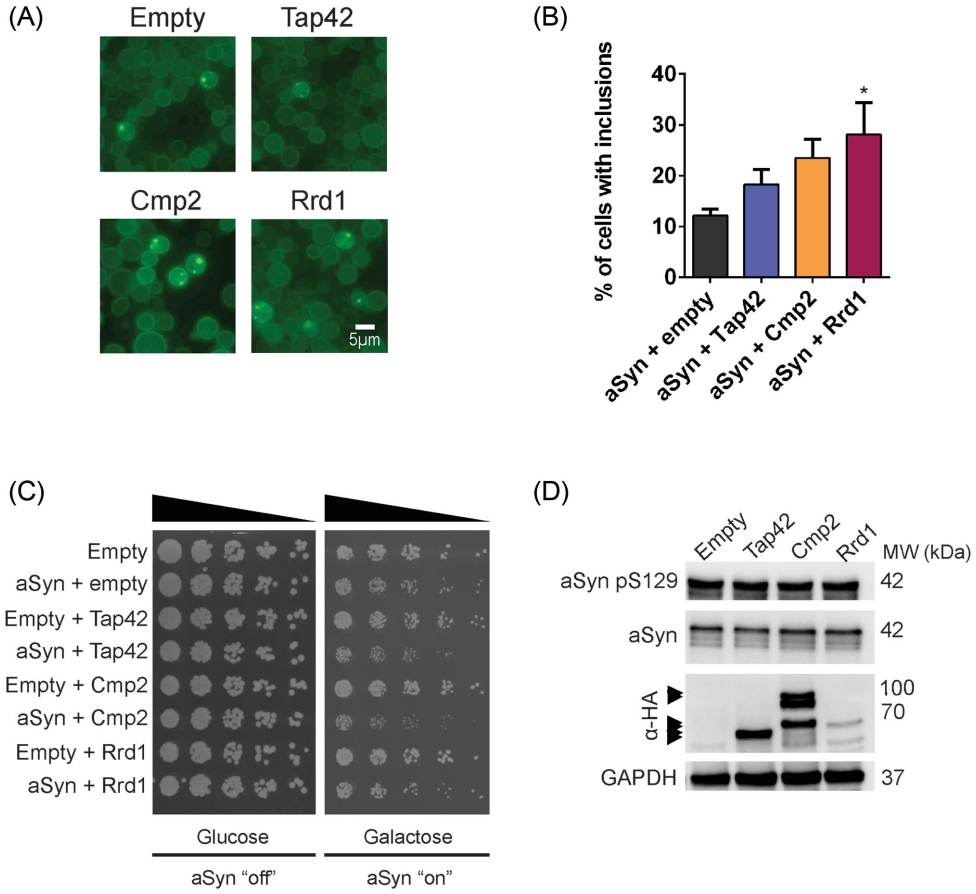

Figure 6. Effect of the phosphatases on aSyn toxicity using a yeast strain stably expressing aSyn. (**A**) Fluorescence microscopy images of cells expressing aSyn-GFP alone [aSyn + empty vector (empty)] or together with the selected phosphatases (Tap42, Cmp2 and Rrd1). Images were obtained 6 h after aSyn-GFP expression induction in galactose-containing medium. A representative picture is shown from three independent experiments. (**B**) Quantification of the percentage of cells presenting aSyn inclusions ($^*p < 0.05$). Values represent the mean ± SD from three independent experiments. (**C**) Spotting assays of yeast cells co-expressing aSyn alone [aSyn + empty vector (empty)] or with phosphatases (Tap42, Cmp2 and Rrd1). Cell suspensions were adjusted to the same OD_{600nm}, serially diluted and spotted onto the surface of the solid medium containing either glucose (control) or galactose. A representative image is shown from three independent experiments. (**D**) aSyn protein and aSyn pS129, when expressed aSyn alone [aSyn + empty vector (empty)] or with phosphatases (Tap42, Cmp2 and Rrd1), assessed by immunoblotting, 6 h after aSyn-GFP and phosphatases expression induction. Phosphatases expression was detected by immunoblot using an antibody against HA-flag. Correspondent molecular weight (kDa) is indicated by arrowheads.

which results in higher levels of toxicity (Fig. 6). In this model, two insertions of the cDNA encoding aSyn are inserted in the yeast genome (Outeiro and Lindquist 2003). We found that Rrd1 slightly increased the percentage of cells with aSyn inclusions (Fig. 6A and B) but induced no significant differences either in aSyn toxicity (Fig. 6C) or in the levels of aSyn or aSyn p129 protein (Fig. 6D).

DISCUSSION

Here, we report that overexpression of Shp1, Cna1, Cnb1, Rrd2, Sap155 and Sit4 together with aSyn-GFP resulted in a significant reduction in the percentage of cells with aSyn inclusions. Interestingly, overexpression of protein phosphatases alone resulted in different degrees of cytotoxicity. Co-expression of Cmp2 and Pph21 with aSyn-GFP improved cell growth when compared with the respective controls, in opposite to Shp1, Cdc55, Sap155 and Sap185 that exacerbated aSyn toxicity. Surprisingly, none of the phosphatases tested modulated the levels of aSyn pS129. Nevertheless, it is possible that the phosphorylation levels in other aSyn residues might be affected, and this will require additional investigation.

Our study also found no strict correlation between aSyn aggregation and toxicity, in line with other reports. It is conceivable that the expression of phosphatases may result in an indirect role of these proteins in cell viability by interfering with other pathways, and not by directly affecting aSyn phosphorylation.

Dysregulation of calcium levels is one of the early pathological hallmarks in neurodegenerative diseases, which lead to the loss of synaptic integrity and axonal neurodegeneration (Shah *et al.* 2017). Calcineurin is widely distributed in mammalian tissues, and is most abundant in the brain. As a serine/threonine protein phosphatase, calcineurin participates in a number of cellular processes and calcium-dependent signal transduction pathways, in addition to regulating transcription, dephosphorylating calcium channels, GTPases and microtubule binding proteins (Aramburu *et al.* 2000). In yeast, Cna1 is one of the isoforms of the catalytic subunit A, and Cnb1 is the regulatory subunit B of calcineurin. Our data indicate that overexpression of Cna1 or Cnb1 reduce the percentage of cells with aSyn inclusions but without changes in cell toxicity. This suggests a possible link between alterations in calcium levels and the formation of aSyn inclusions.

Two PTPA orthologues, Rrd1 and Rrd2, exist in *S. cerevisiae*. These proteins are identical to the human PTPA, a protein that stimulates the tyrosine phosphatase activity of PP2A *in vitro* (Van Hoof *et al*. 1994, 1998). In the case of Rrd2, we observed a reduction in the percentage of cells with aSyn inclusions. Interestingly, we found that Rrd1 slightly increased the percentage of cells with aSyn inclusions in a yeast strain stably expressing aSyn, which results in higher levels of toxicity. Previous studies indicate that the Rrd proteins may be involved in regulating the equilibrium between phosphorylation and dephosphorylation activities in various regulatory kinase pathways that govern a broad range of cellular processes. Our findings suggest a mild protective effect of Rrd1 phosphatase on aSyn toxicity.

The yeast Sit4 protein is the catalytic subunit of the CAPP, a heterotrimeric complex that also includes Tpd3 and Cdc55 proteins as regulatory subunits (Nickels and Broach 1996). This is a serine-threonine protein phosphatase related to PP2A family, and it has homology with human PP6 that are involved in cell cycle regulation (Bastians and Ponstingl 1996). As expected for a protein phosphatase, Sit4 regulates a wide range of biological processes, including control lifespan, mitochondrial function and cell cycle progression (Sutton, Immanuel and Arndt 1991; Barbosa *et al*. 2016), the UPS (Singer *et al*. 2003) and trafficking from the ER to the Golgi complex (Bhandari *et al*. 2013). Co-expression of Sit4 with aSyn resulted in a strong reduction of the percentage of cells with aSyn inclusions and no differences in the cell growth in the spotting assays. However, after 16 h of induction of expression, we found that Sit4 was protective when compared with the expression of aSyn alone. This suggests a possible protective effect of this phosphatase that is lost at later time points, when other cellular pathways may already be affected.

Overexpression of Sap155 alone was toxic to yeast cells, and this was further increased upon co-expression with aSyn-GFP. Also, we found a reduction in the percentage of cells with aSyn inclusions. Sap 155 is a protein phosphatase that interacts with Sit4 (Luke *et al*. 1996) and also is a negative modulator of potassium efflux by inhibiting Sit4 (Manlandro *et al*. 2005). Thus, the toxic effect of Sap155 overexpression might be related with changes in potassium homeostasis, and due to interactions with Sit4.

PP1 plays a role in several aspects of eukaryotic cell physiology. In the budding yeast, the essential gene GLC7 encodes for the catalytic subunit of PP1, which regulates glycogen metabolism, actin cytoskeleton and gene expression (Cannon 2010). Shp1 was originally isolated as a suppressor of the toxic effect of Glc7 overexpression (Zhang *et al*. 1995). In addition, Shp1 serves as a cofactor for Cdc48 in ubiquitin-dependent protein degradation (Hartmann-Petersen *et al*. 2004), and interacts with the ubiquitin fold autophagy protein Atg8 during autophagosome biogenesis (Krick *et al*. 2010). Accumulating evidence suggest that Shp1 targets Cdc48 for its diverse functions, such as in endoplasmic reticulum-associated degradation (ERAD), in ER/Golgi membrane dynamics and in DNA replication (Tran, Tomsic and Brodsky 2011). Overexpression of this phosphatase resulted in cytotoxicity, and this was further exacerbated upon co-expression with aSyn, demonstrating a detrimental effect caused by Shp1 expression. Interestingly, the co-expression of Shp1 with aSyn-GFP resulted in a decrease of the percentage of cells with aSyn inclusions.

CONCLUDING REMARKS AND OUTLOOK

A major unresolved issue in the field of PD and other synucleinopathies is whether aSyn accumulation in insoluble protein aggregates is a cause or a consequence of the pathological alterations that cause the clinical features of the different diseases. In this context, PTMs, chemical alterations that modulate protein folding, function and subcellular localization, emerge as important molecular events that can modulate aSyn biology and pathobiology. Phosphorylation is one of the most studied PTMs in aSyn, but it remains unclear what are the biological consequences of this PTM on aSyn. Thus, it is essential to understand which are the key enzymes involved in the phosphorylation and dephosphorylation of aSyn, and also the residues that can be phosphorylated.

Given the high degree of conservation between yeast and human phosphatases, we reasoned that the yeast toolbox would enable us to gain novel insight into the effect of these enzymes on aSyn aggregation and toxicity in yeast. Notably, our study identified novel genetic modifiers of aSyn aggregation and toxicity that are involved in a variety of cellular functions, demonstrating that focused genetic screens, such as the one we describe herein, can accelerate our understanding of both players and pathways associated with aSyn pathobiology. Our findings can now be further dissected in more complex model organisms, amenable to other types of functional studies.

Ultimately, the study of phosphatases capable of dephosphorylating aSyn and of modulating its aggregation and cytotoxicity may lead to the identification of novel targets for therapeutic intervention in PD and other synucleinopathies.

MATERIALS AND METHODS

Plasmids

The plasmids used in this work were p426GAL (empty vector as control) (Mumberg, Muller and Funk 1995) and p425GAL_aSyn-GFP (Outeiro and Lindquist 2003). The phosphatases were expressed in yeast cells using the Open Biosystems Yeast ORF collection (Gelperin *et al*. 2005). These are in the 2 micron URA3 BG1805 backbone (Gelperin *et al*. 2005).

The pAG415GAL_*TAP42*-HA, pAG415GAL_*CMP2*-HA and pAG415GAL_*RRD1*-HA were cloned using the gateway system using the BG1805 vectors from Open Biosystems Yeast ORF collection, as described by Alberti, Gitler and Lindquist 2007.

Yeast strains and procedures

The yeast strain W303.1A (*MAT*a, *leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) was used throughout the study.

The strains with genomic insertions used were W303-1A trp1-1::pRS304 TRP1+; ura3-1:: pRS306 URA3+ as control, and W303-1A trp1-1:: pRS304 GAL1pr-SNCA(WT)-GFP TRP1+; ura3-1:: pRS306 pRS306GAL1pr-SNCA(WT)-GFP::URA3+ expressing aSyn (Outeiro and Lindquist 2003).

Yeast transformations were performed using the Lithium Acetate method (Gietz *et al*. 1995). Yeast cells were cultured in SC minimal medium without the vector auxotrophies as described before (Macedo *et al*. 2015). Protein extraction, western blot analyzes, fluorescence microscopy and flow cytometry were performed as previously described (Tenreiro *et al*. 2014; Macedo *et al*. 2015).

Statistical analyses

GraphPad PRISM 6 software (GraphPad Sofware, Inc.) was used to perform the statistical evaluation of all data. Results are shown as the average ± standard deviation of at least three independent experiments. Results were analyzed by means of a

one-way ANOVA followed by a Dunnett's Multiple Comparison Test for comparison of averages between the various groups. Results were considered significant only when $P < 0.05$.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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