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PLK2 Modulates α-Synuclein Aggregation in Yeast and Mammalian Cells

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Abstract Phosphorylation of α-synuclein (aSyn) on serine 129 is one of the major post-translation modifications found in Lewy bodies, the typical pathological hallmark of Parkinson's disease. Here, we found that both PLK2 and PLK3 phosphorylate aSyn on serine 129 in yeast. However, only PLK2 increased aSyn cytotoxicity and the percentage of cells presenting cytoplasmic foci. Consistently, in mammalian cells, PLK2 induced aSyn phosphorylation on serine 129 and induced an increase in the size of the inclusions. Our study supports a role for PLK2 in the generation of aSyn inclusions by a mechanism that does not depend directly on serine 129 phosphorylation.

Keywords α-Synuclein · Parkinson's disease · Polo-like kinases · Aggregation · Serine 129 · Phosphorylation

Elisa Basso and Pedro Antas have equal contribution.

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Introduction

Pathological hallmarks of Parkinson's disease (PD) are loss of dopaminergic neurons in substantia nigra pars compacta and deposition of proteinaceous inclusions named Lewy bodies (LBs) [1]. The most abundant protein found in LBs is α-synuclein (aSyn), a small acidic protein involved in both sporadic and familial cases of PD [2, 3]. Oligomerized, truncated, oxidized, ubiquitinated, and phosphorylated aSyn species have been found in LBs, with ~90 % of aSyn being phosphorylated at serine 129 (Ser-129) [4–6]. The role of aSyn Ser-129 phosphorylation on PD pathogenesis is not yet fully resolved. In vitro studies demonstrate that phosphorylation of aSyn on Ser-129 inhibits fibrilization of the protein [7]. In a *Drosophila* model, substitution of the serine in position 129 with an alanine (S129A), to mimic the unphosphorylated form of the protein, resulted in suppression of dopaminergic cell loss and increase in aggregate formation [8]. In transgenic mouse models of PD, aSyn phosphorylation caused accelerated neuronal loss, suggesting a toxic effect of Ser-129 phosphorylation [9]. Furthermore, in rat models of PD using mutants that mimic the phosphorylated (S129D) or unphosphorylated state of the protein (S129A), two studies demonstrated that the S129A mutant was more toxic [10, 11]. Nevertheless, a separate study reported no differences in toxicity and aggregation propensity between the S129A and S129D aSyn mutants [12]. Several kinase families were shown to be able to phosphorylate aSyn at serine and tyrosine residues. The G-protein-coupled receptor kinases (GRK1, GRK2, GRK5, and GRK6) [13], the casein kinases (CKI and CKII) [4], the calmodulin-dependent kinase II [14], and the polo-like kinases (PLK1, PLK2, and PLK3) are reported to phosphorylate aSyn on Ser-129 [15–17]. Notably, PLK2 and PLK3, two members of PLK family, can both

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phosphorylate aSyn in vitro more efficiently than the other PLKs and are able to phosphorylate aSyn in monomeric and fibrillar states [17]. Here, we took advantage of the yeast system [18–20] to study the molecular effects of Ser-129 phosphorylation by PLKs on aSyn toxicity and inclusion formation. We then verified our findings using established mammalian cell models of aSyn oligomerization and inclusion formation. Our study demonstrates that aSyn inclusion formation is potentiated by PLK2 in a process that is conserved between yeast and mammalian systems.

Results

PLK2, But Not PLK3, Modulates aSyn Toxicity and aSyn Inclusion Formation in Yeast Cells

To better understand the function of aSyn Ser-129 phosphorylation, human aSyn was coexpressed with human PLKs in *S. cerevisiae* and the levels of Ser-129 phosphorylation were determined by immunoblotting. As expected, coexpression of aSyn with PLK2 and PLK3 resulted in a pronounced increase in the levels of aSyn Ser-129 phosphorylation, while PLK4 had no significant effect (Fig. 1a). The levels of PLK1 were much lower compared to the other kinases (Fig. 1a), precluding further studies on the effect of this kinase on the phosphorylation of aSyn. Thus, we focused on PLK2 and PLK3.

Initially, we investigated whether PLK2 or PLK3 altered the cytotoxicity induced by aSyn in yeast. For this purpose, strains expressing a moderately toxic version of aSyn-GFP were used [19]. The effect of PLK2 and PLK3 on cell growth was investigated by spotting assays. We found that coexpression of PLK2 with aSyn reduced cell growth, while PLK3 had no effect (Fig. 1b).

Next, we investigated whether the PLK2-induced toxicity was associated with the formation of intracellular inclusions. In 50 % of the cells, aSyn formed fluorescent foci (Fig. 1c). Interestingly, expression of PLK2 resulted in a 10 % increase of foci-positive cells, while no increase was observed with PLK3 (Fig. 1c).

To clarify the role of PLK2-mediated phosphorylation on aSyn toxicity and inclusion formation, experiments were carried out in the presence of a kinase dead mutant (DM) of PLK2, carrying a K111M substitution [21]. As expected, the PLK2 kinase dead mutant (PLK2DM) prevented the phosphorylation on Ser-129 without affecting the levels of aSyn (Fig. 1d). The effect of PLK2DM on the cytotoxicity of aSyn was also tested on spotting assays, and a decrease in cell growth was detected, as observed for the WT PLK2 protein. We next asked whether PLK2DM affected the distribution of aSyn in yeast cells. Surprisingly, no difference was observed when compared to wild type PLK2 (Fig. 1c).

Further data on the coexpression of aSyn with PLK4, a member of the PLK family that was not able to increase aSyn phosphorylation levels on Ser-129 (Fig. 1a), did not reveal any increase in aSyn foci formation or toxicity (Suppl. fig. S1). These results confirmed that the phenotype observed is not correlated with aSyn phosphorylation on Ser-129 but is specific for PLK2.

Altogether, these findings suggest that the role of PLK2 on aSyn inclusion formation and toxicity might results from additional effects of the protein that are not solely dependent on its kinase activity and, consequently, on the direct phosphorylation of aSyn on Ser-129.

Next, in order to assess the biochemical state of aSyn-GFP in the fluorescent foci, we performed ultracentrifugation analysis in sucrose gradients. We observed an increase in the levels of aSyn species with higher molecular weight in cells coexpressing PLK2, PLK2DM, and PLK3 (Fig. 1e). PLK2DM promoted the accumulation of similar types of aSyn species to those formed in the presence of PLK2 (Fig. 1e), confirming that the effect of PLK2 on aSyn inclusion formation is independent from its ability to phosphorylate aSyn on Ser-129.

PLK2 Promotes aSyn Inclusion Formation in Human Cells

In order to validate the results obtained in yeast, we investigated the effect of PLK2 on aSyn oligomerization in a human cell line (H4 cells). First, we assessed the effect of PLK2 on aSyn oligomerization using bimolecular fluorescence complementation (BiFC) (Fig. 2a) [22]. Cells stably expressing the aSyn BiFC constructs were transfected with PLK2 and analyzed by fluorescence microscopy (Fig. 2b). The overall fluorescence of the cells was not changed in the presence of PLK2. However, in 10 % of the cells coexpressing PLK2, we observed the presence of punctate foci, suggesting oligomers were recruited into larger inclusions (Fig. 2b). The role of PLK2 on inclusion formation was then investigated using the BI2536 PLK2 inhibitor [15]. This treatment resulted in the elimination of aSyn inclusions (Fig. 2b). In parallel, the levels of aSyn Ser-129 phosphorylation were evaluated by immunoblotting analysis. As expected, we detected a significant increase in aSyn Ser-129 phosphorylation in the presence of PLK2, while treatment with BI2536 reduced phosphorylation (Fig. 2c), indicating that the BI2536 PLK2 inhibitor was effective.

PLK2 Induces aSyn Aggregation in CAD Cells

To further confirm the effect of PLK2 on aSyn aggregation propensity, we used another mammalian cell system. PLK2 was coexpressed with aSyn and synphilin-1, an established paradigm of aSyn aggregation that results in the formation of LB-like inclusions [23]. Synphilin-1 is a known aSyn

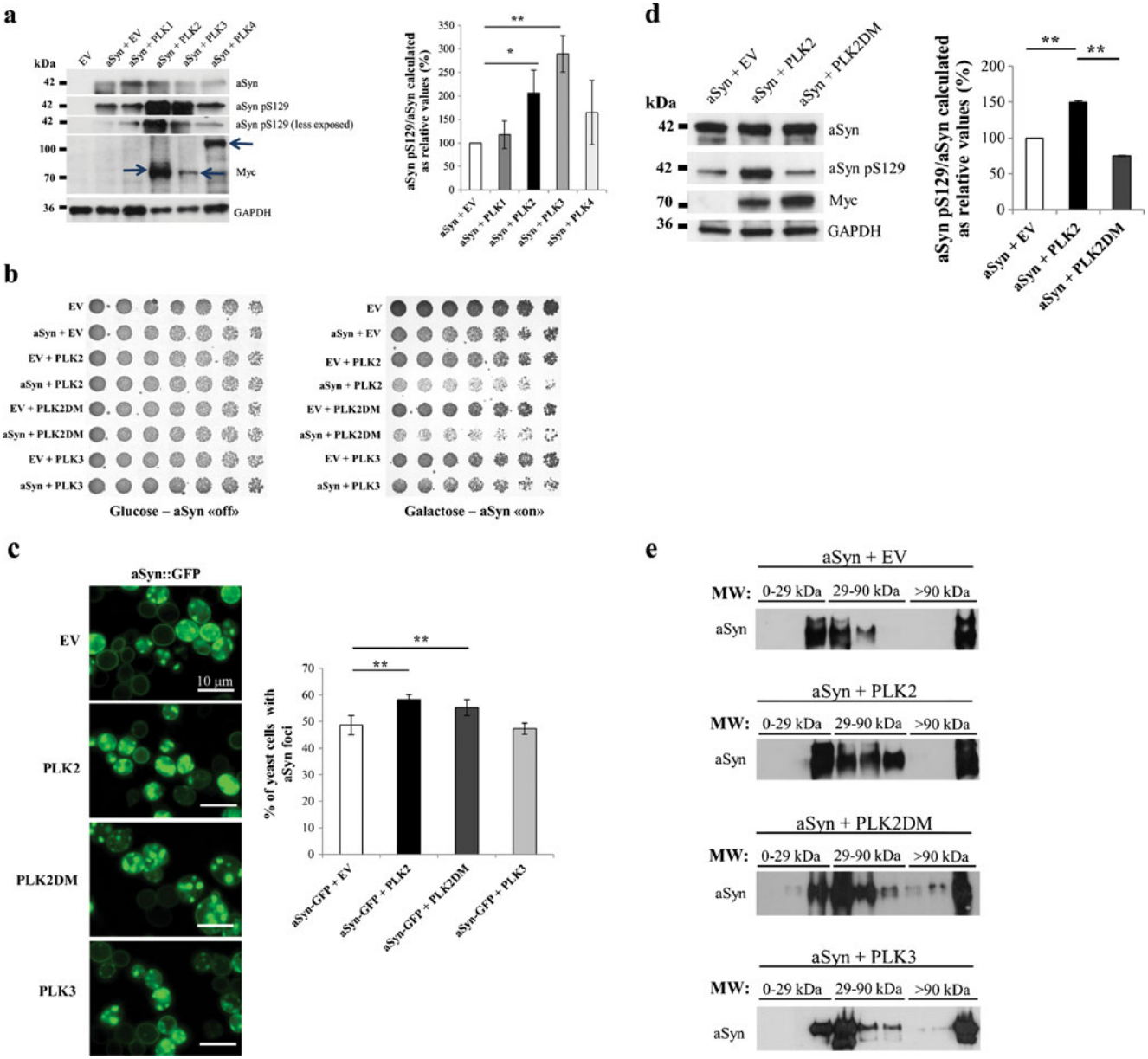

Fig. 1 PLK2 increases aSyn Ser-129 phosphorylation, cytotoxicity, and foci formation in yeast cells. **a** Phosphorylation levels of aSyn on Ser-129 when expressed either alone (aSyn + empty vector (EV)) or with PLK1 (68 kDa), PLK2 (78 kDa), PLK3 (71 kDa), and PLK4 (104 kDa) determined by immunoblotting (*left panel*). pSER129 overexposed blot showing phosphorylation levels in all the conditions examined. Densitometric analysis of the immunodetection of aSyn Ser-129 phosphorylation levels was normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the aSyn + EV condition (*upper panel*). **b** Spotting assay of yeast cultures coexpressing aSyn (aSyn + EV) or empty vector (EV) with PLK2 or PLK3 or PLK2DM (dead kinase mutant version of PLK2). The cell suspensions were adjusted to $OD_{600\ nm}$=0.05±0.005 and used to prepare 1/2 serial dilutions that were applied as spots (4 µl) onto the surface of the solid medium either with glucose (control) or galactose (induced of aSyn and PLKs expression) as carbon source. **c** Fluorescence microscopy and quantification of the number of cells presenting aSyn foci in cells expressing aSyn-GFP fusion protein alone (aSyn-GFP + EV) or together with PLK2 or PLK3 or PLK2DM. **d** Phosphorylation levels of aSyn on Ser-129 when expressed either alone (aSyn + EV) or together with either PLK2 or PLK2DM determined by immunoblotting (*upper panel*). Densitometric analysis of the Ser-129 phosphorylation levels normalized for the total amount of aSyn (mean ± s.e.m.) (*upper right panel*). **e** The oligomeric species formed in yeast cells expressing aSyn alone (aSyn + EV) or with PLK2, or PLK2DM or PLK3 were resolved using sucrose gradients. The resulting fractions were applied to a SDS-PAGE gel followed by immunoblotting with an antibody anti-aSyn (*lower panel*). All the data shown are representative of at least three independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (*=p<0.05, **=p<0.005)

interacting protein [24], present in the core of LBs [25] and may be involved in aSyn recruitment to inclusion bodies [25, 26]. A cathecolaminergic mouse cell line (CAD) was cotransfected with aSyn and synphilin-1 together with each

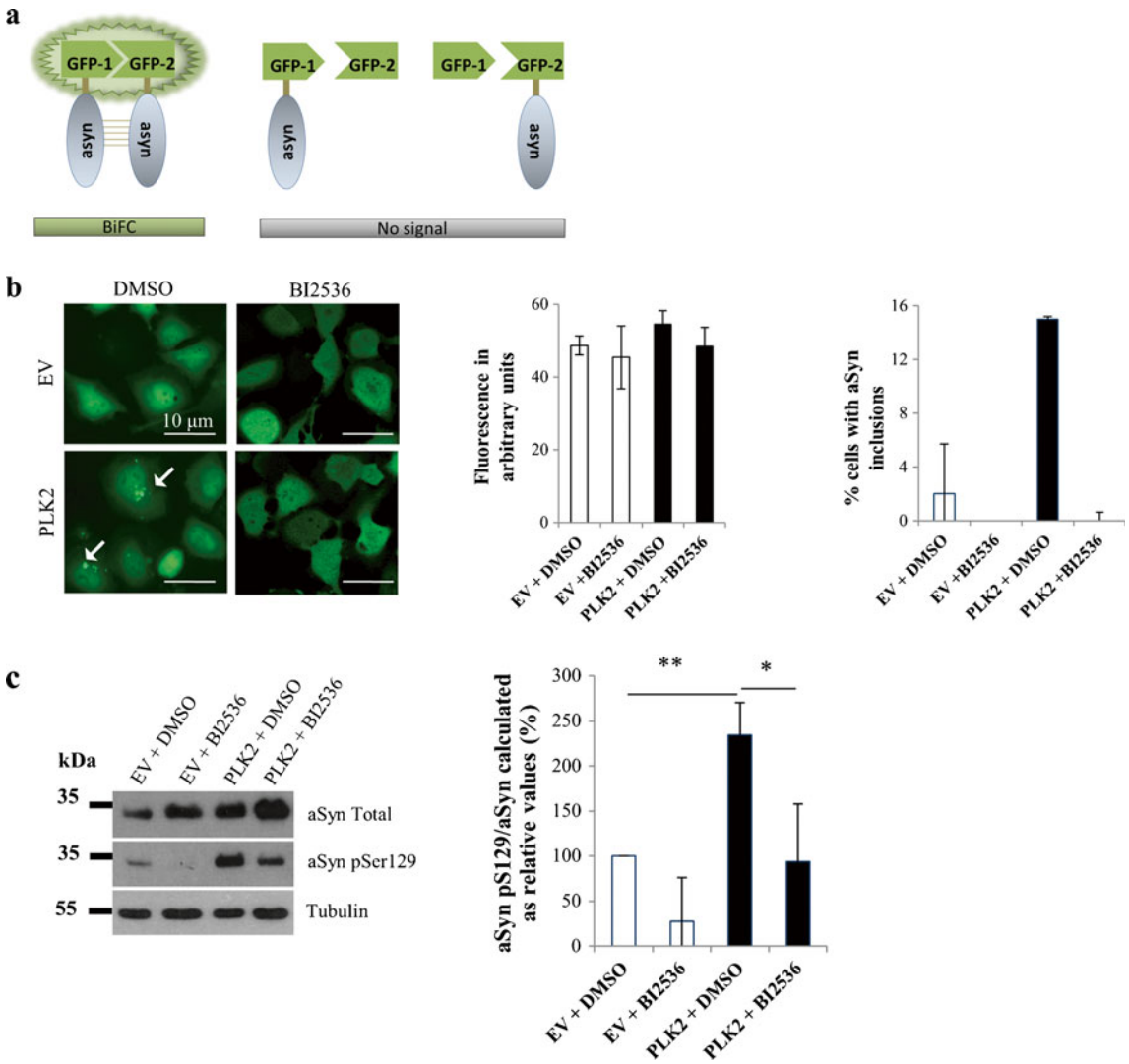

Fig. 2 PLK2 does not interfere with aSyn oligomerization. **a** The BiFC assay. Two aSyn molecules are fused to two nonfluorescent halves of a fluorescent reporter, in this case, GFP. If the proteins interact, they bring together the halves of the reporter protein and reconstitute the functional fluorophore. Protein complementation occurs only when aSyn is fused to fragments of GFP and not observed when GFP fragments alone are expressed. **b** Microscopy analysis of H4 cells stably transfected with GN-link-aSyn + aSyn-GC and transiently cotransfected either with PLK2 or an empty vector (EV) in the presence (BI2536) or absence (DMSO) of a kinase inhibitor. aSyn fluorescence intensity is quantified in arbitrary units. The percentage of cells with aSyn inclusions is shown. **c** H4 cells stably transfected with GN-link-aSyn + aSyn-GC were immunoblotted 48 h post-transient cotransfection either with PLK2 or empty control (EV) in the presence (BI2536) or absence (DMSO) of a kinase inhibitor using antibodies against aSyn phosphorylated on Ser-129 and total aSyn. Ser-129 phosphorylation levels were normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the EV + DMSO condition. **a** All data presented are representative of three independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (*=$p<0.05$), (**=$p<0.005$)

of the four PLKs. The levels of aSyn and Ser-129 phosphorylation were evaluated by immunoblotting analysis. Both PLK2 and PLK3 induced aSyn Ser-129 phosphorylation, while PLK1 had no effect (Fig. 3a). The lower expression levels of PLK4 precluded further conclusions on the effects of this kinase (Fig. 3a).

Interestingly, coexpression of aSyn with PLK3, but not PLK2, resulted in a significant reduction in the levels of aSyn (Fig. 3a), in agreement with previous studies [17]. Thus, it was not possible to further evaluate the role of PLK3 on aSyn phosphorylation or aggregation using this cell model.

Next, the effect of PLK2 on aSyn aggregation was investigated by immunocytochemistry. We found that PLK2 slightly altered the percentage of cells displaying aSyn inclusions, although this did not reach statistical significance (Fig. 3b). Nevertheless, the accumulation of larger aSyn inclusions was observed in the presence of PLK2, confirming an effect on aSyn aggregation propensity (Fig. 3b).

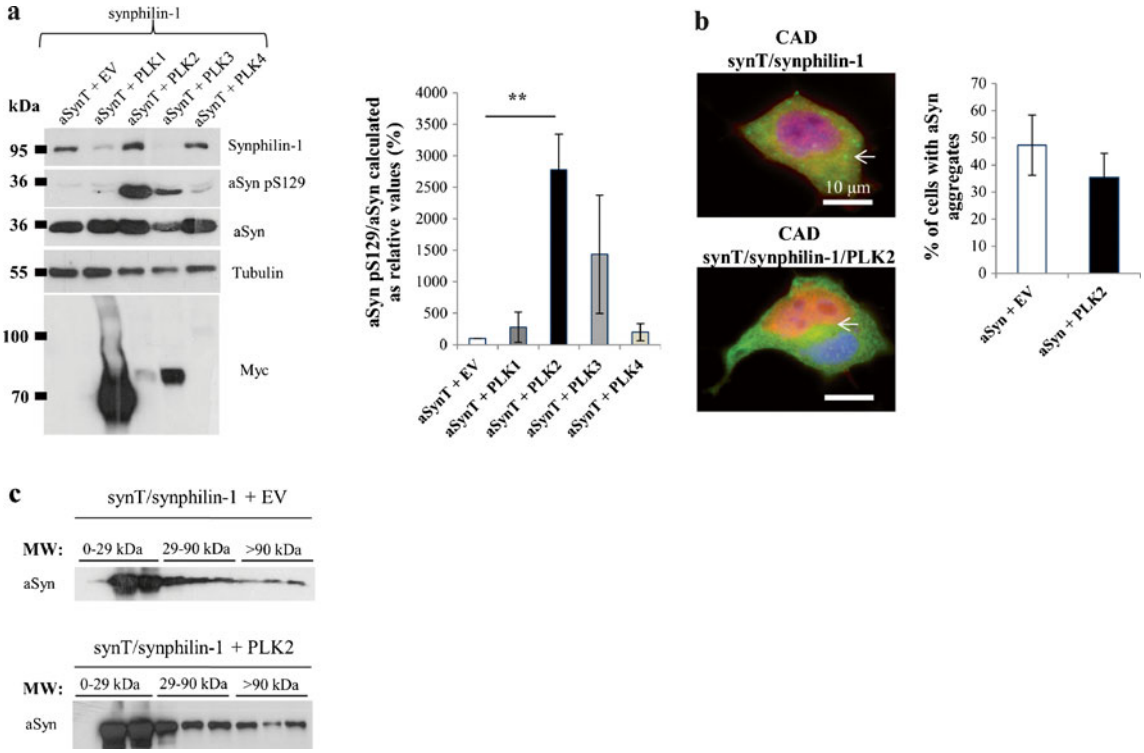

Fig. 3 PLK2 induces aSyn phosphorylation and increases the size of aSyn aggregates in CAD cells. **a** CAD cells were transiently transfected with aSynEGFPΔ155 (aSynT + EV), synphilin-1, and PLK1, PLK2, PLK3, and PLK4. Cells were lysed and immunoblotted for phosphorylated aSyn, total aSyn, synphilin-1, and PLKs (myc-tag). Ser-129 phosphorylation levels were normalized for the total amount of aSyn (mean ± s.e.m.) and relative to the aSynT + EV. **b** aSynT-(aSynEGFPΔ155), synphilin-1-, and PLK2-expressing cells were immunostained for aSyn and synphilin-1. The number of cells containing aSyn aggregates is plotted as a percentage of total transfected cells and relative to the aSynT + EV condition. **c** Lysates of CAD cells expressing aSynT (aSynEGFPΔ155) alone or with PLK2 were ultracentrifuged in sucrose gradient and analyzed by immunoblotting using antibodies against aSyn phosphorylated on Ser-129 and total aSyn. All data presented are representative of three independent experiments. Statistical analysis was performed using two-tailed Student's *t* test for unpaired data (**=$p<0.005$)

To assess the biochemical nature of the aSyn aggregates formed in the presence of PLK2, ultracentrifugation in sucrose gradients was performed. In agreement with the data obtained in yeast, we observed an increase of aSyn higher order oligomeric species (Fig. 3c).

Discussion

Here, we explored established yeast and mammalian cell systems to investigate the molecular role of Ser-129 phosphorylation in the formation of aSyn inclusions. We found that PLK2 and PLK3 phosphorylate aSyn in yeast. Interestingly, only PLK2 increased aSyn cytotoxicity and the formation of intracellular inclusions in this model. Likewise, we found that a kinase dead PLK2 (PLK2DM), which was unable to increase aSyn phosphorylation levels on Ser-129, increased the number of cells presenting aSyn foci and decreased cell growth, similar to the active form of the kinase. Additionally, although PLK3 leads to higher levels of Ser-129 phosphorylation than PLK2, it did not increase aSyn cytotoxicity or inclusion formation in yeast, while PLK4 did not increase aSyn phosphorylation on Ser-129 or foci formation. Altogether, these data suggest that PLK2 increases aSyn foci formation independently of the levels of phosphorylation of aSyn on Ser-129 and that the action of PLK2 on aSyn might involve additional effects on targets other than just aSyn, or on other phosphorylation sites in aSyn.

Assessment of the biochemical state of aSyn using ultracentrifugation in sucrose gradients revealed an increase in oligomeric species with higher molecular weight, with PLK2, PLK2DM, and PLK3. Nonetheless, PLK2 and PLK2DM induced a different pattern of distribution of aSyn species, confirming a specific role for PLK2 in aSyn foci formation and toxicity in yeast cells.

The results obtained in yeast were then validated in human cells. The BiFC assay enabled us to investigate the role of PLK2 in a model of aSyn dimerization/oligomerization because the reconstitution of the functional GFP protein reports the formation of aSyn oligomers (Fig. 2a) [22]. The formation of aSyn cytoplasmic inclusions was observed in cells

coexpressing PLK2. Using BI2536, a known inhibitor of PLK2 kinase activity, we further assessed the role of aSyn Ser-129 phosphorylation on the formation of aSyn inclusions. BI2526 reduced aSyn phosphorylation, as expected, and affected PLK2-mediated aSyn inclusion formation.

To further explore the effect of PLK2 on aSyn aggregation, we used the a model based on the coexpression of a C-terminally modified form of aSyn (SynT) and synphilin-1 [23] in CAD cells. The interaction of synphilin-1 with aSyn is known to be dependent on several factors; ubiquitylation is essential for its aggregation into LBs [27], while phosphorylation can modulate either inclusion formation, by altering its interaction with aSyn [28], or its ubiquitylation state [29]. Further studies using the aSyn S129A phosphorylation mutant showed decreased synphilin-1 ubiquitylation and formation of intracellular inclusions [30]. Our results demonstrated that PLK1 was not able to increase the levels of aSyn Ser-129 phosphorylation but affected the levels of synphilin-1. As described above, in our cell model, the levels of aSyn phosphorylation may have affected synphilin-1 protein levels and, consequently, the formation of aSyn larger inclusions.

On the other hand, we also found that PLK2 significantly increased aSyn Ser-129 phosphorylation and inclusion formation, in agreement with the results obtained in yeast. As PLK2 does not alter the levels of synphilin-1 and induces the formation of aSyn inclusions, these results further imply that the formation of aSyn inclusions requires specific factors, validating the effect of PLK2 in this cellular model of aSyn inclusion formation. Despite the ability of PLK3 to increase the phosphorylation levels of aSyn, we could not further investigate its role on aSyn aggregation, as it drastically reduced the levels of aSyn, suggesting it might promote its degradation. The biochemical nature of the aSyn inclusions formed in the presence of PLK2 was also assessed and, in agreement with the data obtained in yeast cells, PLK2 caused an overall increase in larger Syn species.

Previous studies demonstrated the ability of PLK2 to phosphorylate aSyn in monomeric and fibrillar states [17]. Here, we provide evidence that PLK2 may be involved in the formation of aSyn aggregates in a way that is not solely dependent on its ability to phosphorylate aSyn on Ser-129. Recent studies showed that aSyn may directly impair PLK2 activity and, consequently, inhibit the signaling pathway regulated by PLK2, namely p38 MAPK [31]. Furthermore, PLK2 has been also involved in the inhibition of the mammalian target of rapamycin (mTOR) pathway, in a tumor growth context, through the interaction with tuberous sclerosis complex 1 (TSC1) [32]. Interestingly, this pathway is also a known regulator of the autophagy degradation system that, together with the ubiquitin–proteasome system, is known to be responsible for the clearance of aSyn [33].

Although further studies will be needed to elucidate which PLK2 signaling cascades interfere with aSyn inclusion formation, our results, obtained combining yeast and mammalian cell models, provide novel experimental support in favor of PLK2 as a target for therapeutic intervention in synucleinopathies.

Materials and Methods

Cloning of PLKs, aSyn, and Synphilin-1

The yeast expression vector p426GAL was used to express human aSyn under a galactose-inducible promoter and fused with GFP, as described before [19]. The PLK mammalian expression plasmid pCMV6 was a kind gift from Dr. Hilal Lashuel, École polytechnique fédérale de Lausanne, Switzerland. For the PLK expression in yeast cells, the PLK1 Myc-FLAG gene was excised from pCMV6 and cloned into the *Cla*I and *Eco*RI sites of pRS423GAL, while PLK2 Myc-FLAG, PLK3 Myc-FLAG, and PLK4 Myc-FLAG genes were excised from pCMV6 and cloned into the *Cla*I and *Bam*HI sites of p423GAL. The p423GAL PLK2 K111M mutant was generated by site-directed mutagenesis using primers: 5′ primer: CAAAGTCTACGCCGCAATGAT TATTCCTCACAGCAG and 3′ primer: CTGCTGTGA GGAATAATCATTGCGGCGTAGACTTTG (Stratagene: QuikChange™ Site-Directed Mutagenesis Kit). Other plasmids used in this study were previously described: SynT (aSynEGF deletion mutant (WTSynEGFPΔ155)) [23] and synphilin-1 [24].

Yeast Strains Cultures and Determination of Cell Survival

In this study, we used the wild type W303.1a *MAT*a strain (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*). Yeast cultures were transformed using standard lithium acetate protocol. All strains were grown in synthetic complete medium without histidine and uracil (SC-His-Ura) [6.7 g l^{-1} Yeast Nitrogen Base (BD Biosciences), appropriate amino acid dropout mix (Sunrise Science Products), 1 % (w/v) raffinose, or 1 % (w/v) galactose].

Yeast cells carrying the galactose-inducible aSyn-GFP construct alone or in combination with PLKs were pregrown in SC-His-Ura 1 % raffinose for 24 h and then diluted back in order to reach an optical density (OD_{600}) of 0.1 after an overnight growth. For growth assays on solid medium, a serial dilution of exponentially growing cells was made in SC-His-Ura 1 % raffinose medium and then spotted on SC-His-Ura 1 % galactose or SC-His-Ura 1 % glucose (control)

agar plates, which were then incubated at 30 °C for at least 48 h.

Cell Culture and Transfections

Mouse CAD cathecolaminergic cells (kind gift from C. Zurzolo, Instituto Pasteur, Paris, France) and human H4 neuroglioma cells (HTB-148; ATCC, Manassas, VA, USA) were maintained in Opti-MEM (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. CAD cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and H4 cells with FuGENE™ 6 (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Compound Treatment of Transfected H4 Cells

H4 cells were stably transfected with GN-link-aSyn + aSyn-GC as previously described [22]. The PLK2 inhibitor BI2536, APMU (*N*-[4-(4-aminothieno[2,3-d]pyrimidin-5-yl)phenyl]-*N*′-(3-methylphenyl)urea), was a kind gift from Prof. Poul Henning Jensen, Institut for Biomedicin–Medicinsk Biokemi, Aarhus Universitet. The inhibitor was diluted in DMSO and cells were treated for 2 h at a final concentration of 1 μM. Cells were washed and lysed as described below.

Western Blot Analysis

For quantitative analysis, equal amounts of yeast cells coexpressing aSyn and PLKs were taken 8 h postinduction of aSyn expression, harvested, and lysed in tris–HCl buffer pH 7.4 with glass beads in the presence of protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Cell debris were spun down at 2,600 rpm for 5 min and supernatants containing total lysate were collected and sonicated (10 s at 10 mA, Soniprep 150 for Sanyo). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotting was performed following standard procedures.

Mouse CAD cathecolaminergic cells and human H4 neuroglioma cells were lysed with NP-40 buffer in the presence of protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Lysates were cleared from debris by a 13,000 rpm centrifugation for 30 s at 4 °C and then subjected to SDS-PAGE. After blotting, the membranes were incubated using antibodies listed below, followed by incubation with HRP-labeled secondary antibodies. After washing three times in TBS with 0.1 % Tween (TBS-T, pH 7.4), the immunoblots were developed using ECL (Millipore, Billerica, MA, USA). The primary antibodies used were specific for Venus–synphilin-1 (Sigma-Aldrich, St. Louis, MO, USA), aSyn (BD Transduction Laboratories, San Jose, CA, USA), phospho-Ser-129 aSyn (Wako Chemicals USA, Inc., Richmond ,VA, USA), and Myc-PLKs (Cell Signaling Technology, Inc., Danvers, MA, USA). GAPDH (Ambion, Cambridgeshire, UK) and α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) antibodies served as loading control for yeast and cell lines, respectively.

Immunocytochemistry and Microscopy Analysis

Fluorescence microscopy of yeast cells was performed with a Zeiss Axiovert 200 M Widefield Fluorescence microscope equipped with a digital Axiocam from Zeiss (objective 100×, Plan-Apochromat, Oil, NA (1.40)). Transformants expressing aSyn alone or with PLK2 or PLK3 were pregrown on SC-His-Ura 1 % raffinose as described above and then on SC-His-Ura 1 % galactose medium for 8 h at 30 °C with shaking. Yeast cells were subsequently spin down and visualized under the microscope. The proportion of cells presenting aSyn inclusions was then determined by counting at least 1,000 cells per strain using ImageJ software. Significance was calculated using Student's two-tailed unpaired *t* test. A value of $p<0.05$ was considered to be statistically significant.

Transfected CAD cells were fixed with 4 % paraformaldehyde. Cells were permeabilized in PBS containing 0.1 % Triton X-100 and blocked in 1.5 % normal goat serum containing PBS for 1 h. Cells were incubated with primary antibody for 2 h at RT or overnight at 4 °C (mouse anti-aSyn; BD Transduction Laboratories, San Jose, CA, USA; rabbit anti-Venus–synphilin-1; Sigma-Aldrich, St. Louis, MO, USA) followed by secondary antibody incubation for 1 h (goat anti-mouse IgG-Alexa488, Invitrogen Corporation, Carlsbad, CA, USA and a donkey anti-rabbit IgG-Alexa568, Invitrogen Corporation, Carlsbad, CA, USA). Slides were subjected to fluorescence microscopy with a Zeiss Axiovert 200 M Widefield Fluorescence microscope equipped with a digital Axiocam from Zeiss (objective 40×, EC Plan-NeoFluar, Dry, NA (0.75)). The proportion of cells with aSyn inclusions within the population was then determined by counting at least 100 cells per condition using ImageJ software. For BiFC-GFP reconstitution assay, H4 stable cell line expressing aSyn dimers/oligomers was bioimaged as previously described [22]. All data were analyzed using Student's two-tailed unpaired *t* test. A value of $p<0.05$ was considered to be statistically significant.

Velocity Gradient

Yeast spheroplasts were prepared with the following procedure: yeast cells expressing aSyn alone or together with PLK2 or PLK3 were harvested by centrifugation, washed with sterile

water, suspended in spheroplasting solution (tris pH 7.5 20 mM, MgCl2 0.5 mM, BME 50 mM, sorbitol 1.2 M, and zymolyase 0.5 mg/ml), and incubated at 30 °C for 30 min. Samples were centrifuged at 800*g* for 5 min at room temperature and the supernatant was completely removed. Yeast spheroplasts and CAD cells were lysate in a solution containing (tris pH 7.4 1 M, NaCl 5 M, SDS 0.4 %, Triton X-100 0.2 %) with inhibitors for proteases/kinases/phosphatases and were placed 20 min on ice. Then, cells were mechanically disrupted by forcing the solution to pass through a 25 G syringe six times. One milligram of total protein was applied on a 5 to 30 % sucrose gradient. Centrifugation was conducted at 4 °C with a swinging bucket rotor (SW-55Ti rotor, Beckman Instruments, Inc., Palo Alto, CA, USA) in a Beckman XL-90 S/N ultracentrifuge at 450,000 rpm for 16 h. Nine fractions were collected and precipitated for 4 h at 4 °C in trichloroacetic acid. Proteins were then washed in acetone three times and resuspended in protein sample buffer (0.5 M tris–HCl, pH 6.8, glycerol, 10 % (w/v) SDS, 0.1 % (w/v) Bromophenol Blue), resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted against total aSyn following standard procedures. Velocity gradient procedure and estimation of the molecular sizes for each fraction were previously described [34].

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Conflict of interest None.

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