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Dissecting the Molecular Pathway Involved in PLK2 Kinase-mediated α-Synuclein-selective Autophagic Degradation*

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Increasing lines of evidence support the causal link between α-synuclein (α-syn) accumulation in the brain and Parkinson's disease (PD) pathogenesis. Therefore, lowering α-syn protein levels may represent a viable therapeutic strategy for the treatment of PD and related disorders. We recently described a novel selective α-syn degradation pathway, catalyzed by the activity of the Polo-like kinase 2 (PLK2), capable of reducing α-syn protein expression and suppressing its toxicity *in vivo*. However, the exact molecular mechanisms underlying this degradation route remain elusive. In the present study we report that among PLK family members, PLK3 is also able to catalyze α-syn phosphorylation and degradation in living cells. Using pharmacological and genetic approaches, we confirmed the implication of the macroautophagy on PLK2-mediated α-syn turnover, and our observations suggest a concomitant co-degradation of these two proteins. Moreover, we showed that the N-terminal region of α-syn is important for PLK2-mediated α-syn phosphorylation and degradation and is implicated in the physical interaction between the two proteins. We also demonstrated that PLK2 polyubiquitination is important for PLK2·α-syn protein complex degradation, and we hypothesize that this post-translational modification may act as a signal for the selective recognition by the macroautophagy machinery. Finally, we observed that the PD-linked mutation E46K enhances PLK2-mediated α-syn degradation, suggesting that this mutated form is a *bona fide* substrate of this degradation pathway. In conclusion, our study provides a detailed description of the new degradation route of α-syn and offers new opportunities for the development of therapeutic strategies aiming to reduce α-syn protein accumulation and toxicity.

Parkinson's disease (PD)[2] is a neurodegenerative disorder characterized by the progressive loss of vulnerable neuronal populations in the brain and the accumulation of proteinaceous intraneuronal inclusions called Lewy bodies (1, 2). These inclusions mainly consist of a presynaptic protein, α-synuclein (α-syn) (3–5). Converging lines of evidence from neuropathological studies and experimental models support the central role of α-syn aggregation and toxicity in the pathogenesis of PD (3, 6). This α-syn abnormal accumulation is in part triggered by its gene duplications/triplications or by the impairment of its degradation (3, 6). Therefore, enhancing α-syn elimination may represent a viable therapeutic strategy for the treatment of PD and related disorders.

However, the question on how α-syn is eliminated *in vivo* has yielded controversial results (7). Although some studies reported specific elimination of the monomeric and fibrillar α-syn forms by the ubiquitin-proteasome system (8–10), others reported the degradation of this protein via the autophagy-lysosomal pathway, notably the chaperone-mediated autophagy (10–12). This controversy and the lack of known selective routes for α-syn elimination precluded the development of effective pharmacological approaches, aiming to enhance its turnover and to reduce its toxicity.

In this context we recently described a new selective route for α-syn degradation (13). This route implicates the autophagy degradation pathway and is dependent on PLK2 kinase activity, α-syn phosphorylation at Ser-129 and α-syn/PLK2 protein-protein interaction (13). Interestingly, activation of this degradation pathway by overexpressing PLK2 using adeno-associated viral-based approach significantly reduced α-syn protein levels and mitigated its toxicity in a rat model of PD (13). These results demonstrate that PLK2-mediated α-syn turnover may offer a unique opportunity to modulate, in a specific and selective manner, α-syn turnover and to reduce its toxicity *in vivo*. Nevertheless, the molecular mechanisms of this degradation route remain unexplored. The decortication of this cellular pathway will help to identify new pharmacological targets for the development of therapeutic strategies aiming to reduce the total α-syn protein burden and to suppress its toxicity. In the present study we sought to decipher the molecular machinery implicated in PLK2-mediated α-syn turnover *in vivo* using cell-based assays and a battery of pharmacological, genetic, and biochemical approaches.

Results

PLK2 and PLK3 Regulate α-Syn Protein Levels in a Ser-129 Phosphorylation-dependent Manner—Previous work by our group and others reported that α-syn is a *bona fide* substrate of the PLK family members *in vitro* (PLK1, PLK2, and PLK3) and

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[2] The abbreviations used are: PD, Parkinson's disease; α-syn, α-synuclein; 3MA, 3-methyladenine; PLK2, Polo-like kinase 2; ATG5 and ATG7, autophagy-related genes 5 and 7, respectively; SPAR, spine-associated RapGAP; PYR-41, [4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester; KDM, kinase dead mutant; Ub, ubiquitin.

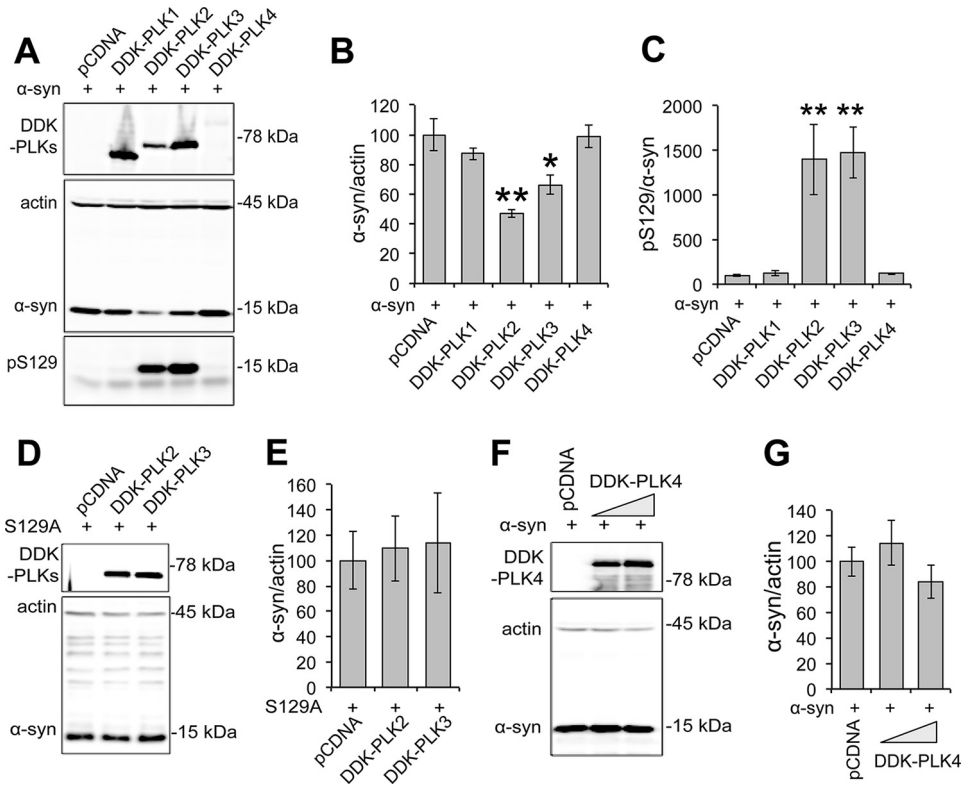

FIGURE 1. **PLK2 and PLK3 overexpression induced α-syn phosphorylation and degradation.** *A*, Western blot analysis illustrating the detection of DDK-PLKs (PLK 1–4), total α-syn, and phosphorylated α-syn at Ser-129 (*pS129*), 24 h post transfection in HEK-239T cells. Cells were transiently transfected with 1 μg of human α-syn plasmid and 0.5 μg of DDK-PLKs plasmids. For DNA titrations total DNA per transfection was equalized by the addition of pCDNA-empty plasmid. Total protein fraction was extracted in 1× Laemmli buffer. *B*, histograms representing the levels of total α-syn expressed as % of the control group and normalized against actin ($n = 3$). Quantification shows that only PLK2 and PLK3 induce a significant reduction of α-syn protein levels, with a more pronounced effect observed after PLK2 overexpression. *C*, histograms representing the levels of Ser(P)-129 signal were expressed as % of control group and normalized against total α-syn protein levels ($n = 3$). Quantification showed that only PLK2 and PLK3 induced significant accumulation of phosphorylated α-syn compared with the control group (14-fold increase). Western blot (*D*) and quantification (*E*) showing that PLK2 or PLK3 overexpression had no effect on the expression of the non-phosphorylatable α-syn mutant, S129A ($n = 4$). Western blot (*F*) and quantification (*G*) showing that overexpression of increasing concentration of PLK4 (1 μg and 2 μg) had no effect on α-syn protein levels, confirming that this kinase does not control α-syn expression *in vivo* ($n = 5$). *, $p < 0.05$ and **, $p < 0.01$ compared with α-syn+pCDNA condition, Tukey's multiple comparisons test.

in vivo (PLK2 and PLK3) (14, 15). Moreover, we recently showed that PLK2 overexpression catalyzes α-syn phosphorylation at Ser-129 and enhances its autophagic degradation *in vivo* in a PLK2 kinase activity-dependent manner (13). However, the question of whether other members of the PLK family are also able to regulate α-syn protein levels *in vivo* remains unexplored.

To address this question, we overexpressed human α-syn with PLK1, PLK2, PLK3, or PLK4 fused to a DDK tag in HEK-293T cells. 24 h post-transfection, the total protein fraction was extracted and analyzed by Western blot. First, we confirmed PLK overexpression using an anti-DDK antibody and detected their corresponding bands at the expected sizes (Fig. 1*A*). Interestingly, evaluation of α-syn protein expression revealed that only overexpression of PLK2 and PLK3, but not PLK1 and PLK4, was associated with a significant reduction of α-syn protein levels (−42%, $p < 0.01$, and −27%, $p < 0.05$%, respectively, compared with α-syn+pCDNA) (Fig. 1, *A* and *B*). These results confirm the role of PLK2 in the regulation of α-syn expression in mammalian cell lines and report for the first time a similar function of PLK3 *in vivo*. It is worth noting that PLK2 overexpression is associated with an enhanced α-syn degradation (−21%) compared with α-syn+PLK3 (Fig. 1, *A* and *B*). This observation highlights the superiority of PLK2 in the control of α-syn turnover and suggests that this kinase is the major enzyme regulating α-syn protein levels *in vivo*.

As we previously reported, we observed a dramatic accumulation of phosphorylated α-syn at Ser-129 (Ser(P)-129) after PLK2 and PLK3 overexpression, compared with the control group (~14-fold increase) (Fig. 1, *A* and *C*) (14). This observation suggests that PLK2 and PLK3-mediated reduction of α-syn protein levels might be associated with their capacity to efficiently phosphorylate α-syn at the residue Ser-129. To confirm the implication of Ser-129 phosphorylation on PLK2/3-mediated α-syn degradation, we co-overexpressed the non-phosphorylatable α-syn mutant form, S129A, with PLK2 or PLK3 in HEK-239T cells. Our results revealed that neither PLK2 nor PLK3 overexpression significantly affected α-syn S129A protein levels (Fig. 1, *D* and *E*), thus validating the importance of phosphorylation at Ser-129 residue on PLKs-mediated reduction of α-syn protein levels in living cells.

It is important to note that, in comparison to the other PLKs, PLK4 expression levels were very low and hardly detected by Western blot in our experimental conditions (Fig. 1*A*), which precludes conclusive statements regarding the effect of this kinase on α-syn turnover. To further investigate this question,

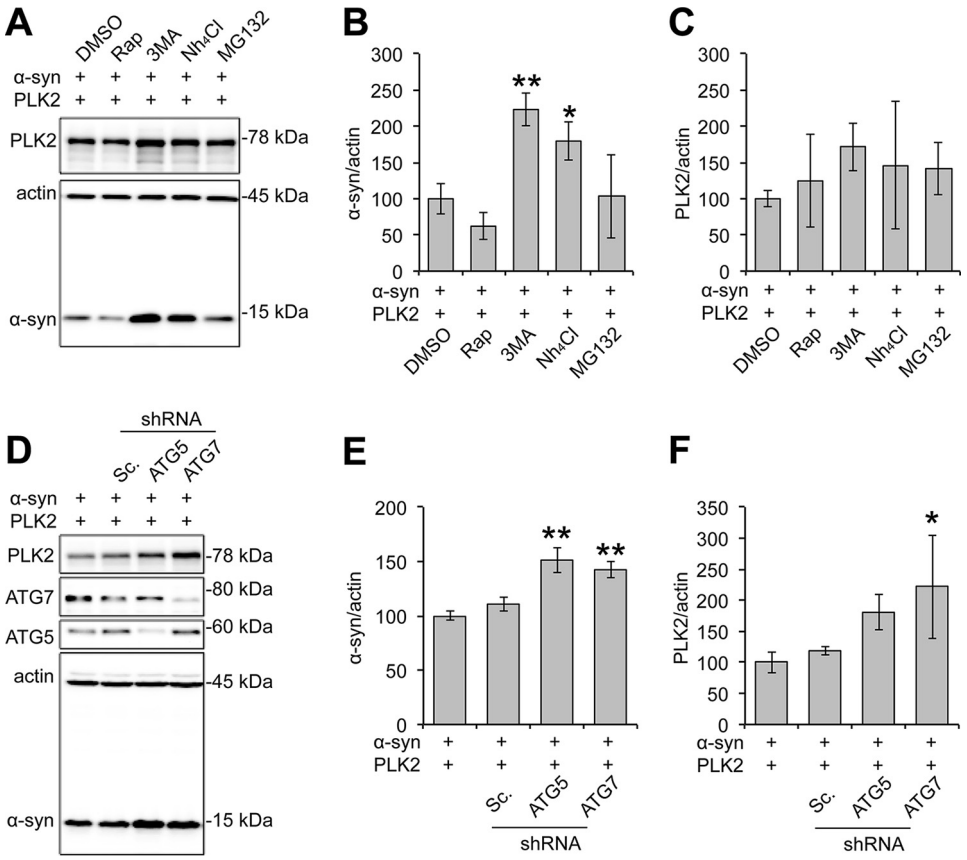

FIGURE 2. **PLK2 and α-syn were co-degraded via the macroautophagy pathway.** *A*, Western blot analysis of the effect of treatment with small molecules modulator of the protein degradation pathways on α-syn and PLK2 expression levels. HEK-239T cells were transiently transfected with 1 μg of α-syn and 0.5 μg of PLK2. 8 h post-transfection, cells were incubated for 12 h with the macroautophagy activator, rapamycin (*Rap*) (20 nM), the autophagy inhibitors 3MA (10 mM), and ammonium chloride (NH_4Cl; 30 mM), and finally with the proteasome inhibitor MG132 (10 μM). *B*, histograms representing the quantification of α-syn total expression levels as % for the control group and normalized against actin after treatment with small molecules ($n = 3$). Results show that treatment with Rap induced a decrease of α-syn protein levels, whereas treatment with autophagy inhibitors was associated with a significant accumulation of this protein. Inhibition of the proteasome degradation pathway using MG132 had no effect on α-syn protein levels. *C*, histograms representing the impact of treatment with degradation inhibitors on PLK2 protein levels ($n = 3$). Results demonstrate a slight accumulation of PLK2 after treatment with 3MA and NH_4Cl without reaching a significant level. *D*, Western blot analysis representing the effect of ATG5 and ATG7 knock-out on α-syn and PLK2 expression levels. Results show the dramatic decrease of ATG5 and ATG7 in the respective stable cell lines compared with the control and scrambled shRNA (*Sc.*) conditions, and an accumulation of α-syn and PLK2 in ATG-depleted cells. *E*, histograms presenting the quantification of α-syn total expression levels, as % for the control group and normalized against actin and demonstrating the significant accumulation of α-syn in ATG5 and ATG7-depleted cells ($n = 3$). *F*, histograms representing the impact of ATG5 and ATG7 knockdown on PLK2 protein levels; the results demonstrated a significant accumulation of this protein in ATG7-depleted cells ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$ compared α-syn+pCDNA condition; Tukey's multiple comparisons test.

we overexpressed α-syn in the presence of increasing concentrations of PLK4. Our results allowed better detection of PLK4 by Western blot (Fig. 1*F*) and confirmed the absence of the effect of the overexpression of this kinase on α-syn protein levels (Fig. 1, *F* and *G*) or on its phosphorylation at Ser-129 (data not shown).

PLK2 Enhances α-Syn Degradation via the Macroautophagy Pathway—To assess the relative contribution of the different protein degradation routes on α-syn clearance, we treated HEK-239T cells overexpressing α-syn and PLK2 with small molecule modulators of the lysosome autophagy or the proteasome degradation pathways. Our results revealed that activation of macroautophagy using the mTOR inhibitor rapamycin (20 nM) induced a further decrease of α-syn protein levels (-39%), although this effect did not reach statistical significance (Fig. 2, *A* and *B*). Moreover, treatments with the autophagy inhibitors 3-methyladenine (3MA; 10 mM) or ammonium chloride (NH_4Cl; 30 mM) suppressed PLK2-mediated α-syn turnover (Fig. 2, *A* and *B*). However, treatment with MG132 (10 μM), a potent inhibitor of the proteasome degradation pathway, had no effect on α-syn protein levels (Fig. 2, *A* and *B*). As we previously reported (13), these results confirm the key role of macroautophagy in PLK2-mediated α-syn turnover.

To further confirm the role of macroautophagy in PLK2-mediated α-syn clearance, we used HEK-239T cells stably expressing shRNA to deplete two key macroautophagy proteins, autophagy-related gene 5 (ATG5) and autophagy-related gene 7 (ATG7) (16), as confirmed by Western blot (Fig. 2*D*). As the control, we used cells overexpressing nonspecific scrambled shRNA. Our results showed a significant accumulation of α-syn in ATG5- and ATG7-depleted cells (+51%, $p < 0.01$; +42%, $p < 0.01$%, respectively, compared with control condition) (Fig. 2, *D* and *E*), thus confirming the implication of the macroautophagy on PLK2-mediated α-syn clearance.

Importantly, pharmacological and genetic inhibition of the macroautophagy resulted also in an accumulation of PLK2, which reached significance only after ATG7 knockdown (Fig. 2, *C* and *F*). Knowing that this protein is exclusively eliminated via

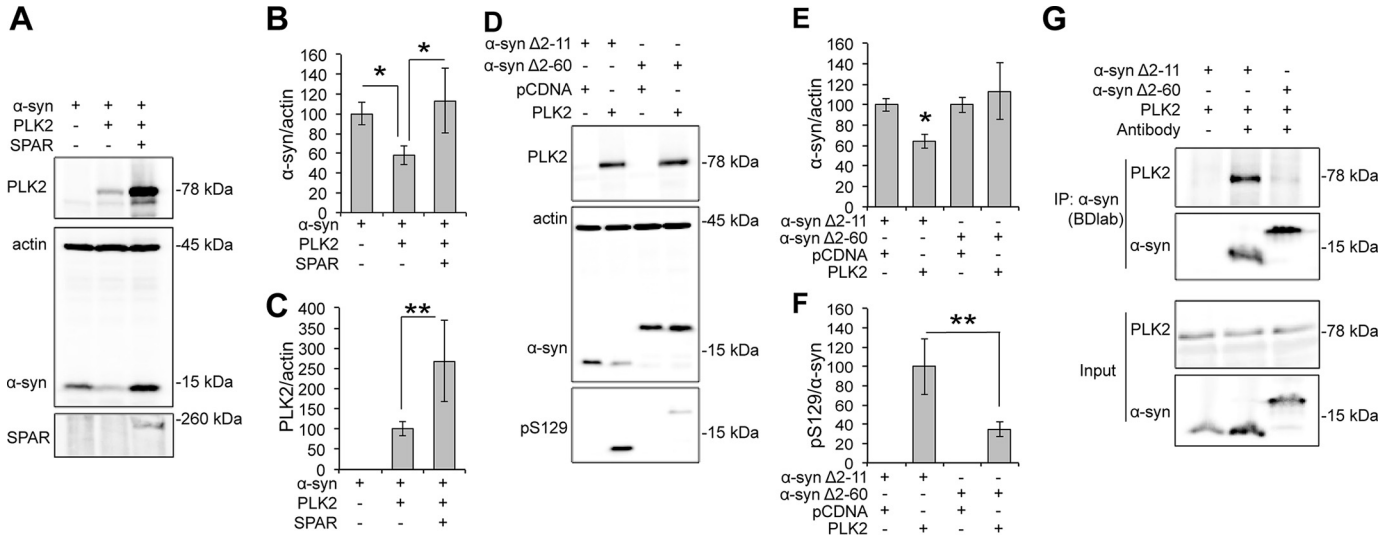

FIGURE 3. **α-Syn N-terminal region was required for its PLK2-mediated phosphorylation and degradation.** *A*, Western blot illustrating the expression levels of α-syn and PLK2 in the presence of SPAR, which disrupts the formation of α-syn/PLK2 protein complex. *B*, quantification of α-syn protein levels showing that SPAR overexpression suppresses PLK2-mediated α-syn elimination ($n = 3$). *C*, quantification of PLK2 protein levels showing that SPAR overexpression induced a significant accumulation of PLK2 ($n = 3$). *D*, Western blot analysis of the total α-syn and Ser(P)-129 protein levels 24 h post transfection in HEK-239T cells. Cells were transiently transfected with 0.5 μg of α-syn Δ2–11 or 1 μg of α-syn Δ2–60 and 0.5 μg of PLK2 plasmids, and the total protein fraction was collected directly in 1X Laemmli buffer. *E*, histograms representing the quantification of α-syn protein levels, normalized against the actin expression, and showing that the N-terminal truncation Δ2–60, but not Δ2–11, affects α-syn degradation ($n = 3$). *F*, histograms representing the quantification of Ser(P)-129 levels after PLK2 overexpression, normalized against total α-syn protein expression ($n = 3$). The results show that Δ2–60 truncation induced a significant reduction of α-syn phosphorylation levels compared with α-syn Δ2–11. *G*, co-immunoprecipitation (*IP*) of PLK2 with N-terminal truncated α-syn (Δ2–11 or Δ2–60) showing that the deletion of the entire N-terminal regions (Δ2–60) is sufficient to block PLK2 and α-syn protein-protein transfection. *, $p < 0.05$ compared α-syn+pCDNA condition; Tukey's multiple comparisons test. For the comparison of PLK2 protein levels, **, $p < 0.01$ compared with α-syn+PLK2 condition using Student's *t* test.

the proteasome degradation pathway (data not shown) (17–19), our data suggest that PLK2 is concomitantly co-degraded with α-syn via the macroautophagy pathway.

α-Syn N Terminus Is Required for Its Interaction with PLK2, Phosphorylation, and Degradation—We recently showed that α-syn/PLK2 protein-protein interaction is required for PLK2-mediated α-syn turnover (13). Moreover, the co-accumulation of these two proteins after inhibition of the macroautophagy pathway suggests that they might be co-degraded (Fig. 2). To verify this hypothesis, we exposed the α-syn·PLK2 protein complex to a competitive interaction by overexpressing another PLK2 substrate, spine-associated RapGAP(SPAR), which precludes PLK2·α-syn complex formation (18, 20). As we previously reported, SPAR overexpression reversed PLK2-mediated α-syn degradation and restored α-syn expression levels (+51%, $p < 0.05$, compared with α-syn+PLK2) (Fig. 3, *A* and *B*), confirming the importance of α-syn·PLK2 physical interaction in PLK2-mediated α-syn clearance. More importantly, the evaluation of PLK2 protein levels revealed a significant accumulation of this protein after SPAR overexpression (2.5-fold increase, $p < 0.01$, compared with α-syn+PLK2) (Fig. 3, *A* and *C*). This observation endorses the hypothesis that PLK2 and α-syn interact together and then the protein complex is degraded by selective autophagy.

Next, we sought to map α-syn and PLK2 interaction. A recent work by Wang et *al.* 21 reported that the first 11 amino acids (2–11) of α-syn structure are required for its interaction and phosphorylation by CDC5, the yeast ortholog of human PLK2, emphasizing the role of α-syn N-terminal segment in the interaction between the two proteins. To assess the importance of this region on α-syn interaction with PLK2, phosphorylation, and degradation in mammalian cell lines, we overexpressed PLK2 either with the truncated form of α-syn missing the first 11 amino acids (α-syn Δ2–11) or with the construct missing the entire amphipathic N-terminal region (α-syn Δ2–60). Evaluation of α-syn total protein levels revealed that Δ2–11 truncation did not affect PLK2-induced α-syn degradation (−46%, $p < 0.05$ compared with Δ2–11+pCDNA), whereas the deletion of the entire N-terminal segment completely suppressed α-syn clearance (Fig. 3, *D* and *E*). Moreover, the evaluation of Ser(P)-129 levels revealed that Δ2–60 truncation significantly reduced α-syn phosphorylation by PLK2 at Ser-129 compared with α-syn Δ2–11 (−60%, $p < 0.01$) (Fig. 3, *D* and *F*). Together, our data demonstrate that the entire N-terminal region is implicated in PLK2-mediated α-syn phosphorylation at Ser-129 and degradation. It is important to note that Δ2–60 truncation appears to affect the protein structure and disrupt its migration in SDS-PAGE, as reflected by the detection of its corresponding band at ~25 kDa (Fig. 3*D*).

To further confirm the role of α-syn N terminus in its physical interaction with PLK2 in mammalian cell line, we performed a coimmunoprecipitation assay of PLK2 and N-terminal truncated α-syn forms. Our results showed that α-syn Δ2–11 immunoprecipitation was associated with the pulldown of PLK2, whereas α-syn Δ2–60 immunoprecipitation resulted in a significant reduction in PLK2 levels (Fig. 3*G*), suggesting that Δ2–60 truncation compromised the interaction between the two proteins. This observation confirms the importance of the α-syn N-terminal region (2–60) on α-syn·PLK2 interaction and complex formation.

Collectively, our results demonstrate that α-syn N terminus region is important for its phosphorylation and degradation

mediated by PLK2 and confirm that α-syn·PLK2 protein complex formation is essential for the two proteins co-turnover.

Ubiquitin System Is Required for PLK2 and α-Syn-selective Macroautophagic Co-degradation—For several years, the macroautophagy degradation pathway has been considered as a non-selective cellular process (22). However, in the last few years, converging evidence demonstrated that this degradation route is also selective, and emphasis was made on the role of the ubiquitin system in this selectivity (23–26). To verify whether ubiquitination is required for PLK2-mediated α-syn autophagic turnover and may confer selectivity to this process, we treated HEK-239T cells overexpressing α-syn and PLK2 with [4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester (PYR-41) (12.5 μM), a potent and irreversible inhibitor of E1 ubiquitin ligase (27–30). Evaluation of α-syn expression levels showed that PYR-41 treatment suppressed PLK2-mediated α-syn degradation and restored α-syn protein levels to normal levels (Fig. 4, *A* and *B*). This observation highlights the potential role of the ubiquitin system in α-syn clearance in living cells. In parallel, PYR-41 treatment also induced a significant accumulation of PLK2 (2.5-fold increase, $p < 0.01$ compared with the non-treated condition) (Fig. 4, *A* and *C*), supporting the hypothesis that PLK2 and α-syn are co-degraded via the common autophagic degradation pathway.

To investigate if polyubiquitination is important for PLK2-mediated α-syn degradation, we overexpressed α-syn and PLK2 with the lysine-less ubiquitin mutant (Ub-K0). Mutation of the lysine residues precludes the formation of polyubiquitin chains, and this mutant is theoretically only capable of mediating mono- or multiubiquitination (31). We then evaluated α-syn expression levels and observed that Ub-K0 overexpression inhibited PLK2-mediated α-syn degradation (Fig. 4, *D* and *E*). This result demonstrates that the formation of polyubiquitin chains is required for PLK2-mediated α-syn turnover. Importantly, we also observed that Ub-K0 overexpression induced a significant accumulation of PLK2 (Fig. 4, *D* and *F*), thus further confirming PLK2 and α-syn concomitant degradation.

Next, we sought to determine which protein in α-syn·PLK2 complex is polyubiquitinated. To address this question, we overexpressed α-syn and PLK2 in the presence of HA-ubiquitin and treated the cells with NH_4Cl to inhibit α-syn·PLK2 co-degradation followed by immunoprecipitation using the anti-HA antibody. Using an anti-PLK2 antibody, we detected a smear of proteins corresponding to PLK2 protein with HA immunoreactivity, indicating that PLK2 was indeed polyubiquitinated (Fig. 4*G*). As controls, we overexpressed PLK2 kinase dead mutant (KDM) and α-syn S129A mutant, which preclude PLK2 and α-syn interaction and suppress α-syn degradation (13). Our results revealed the absence of polyubiquitinated PLK2 in these conditions, highlighting the importance of PLK2 kinase activity, α-syn phosphorylation, and the interaction between the two proteins in PLK2 polyubiquitination (Fig. 4*G*). Moreover, using anti-α-syn antibody, we could not detect any α-syn signal after HA pulldown (data not shown). Collectively, our data demonstrate that PLK2 polyubiquitination is essential for α-syn·PLK2 complex degradation process.

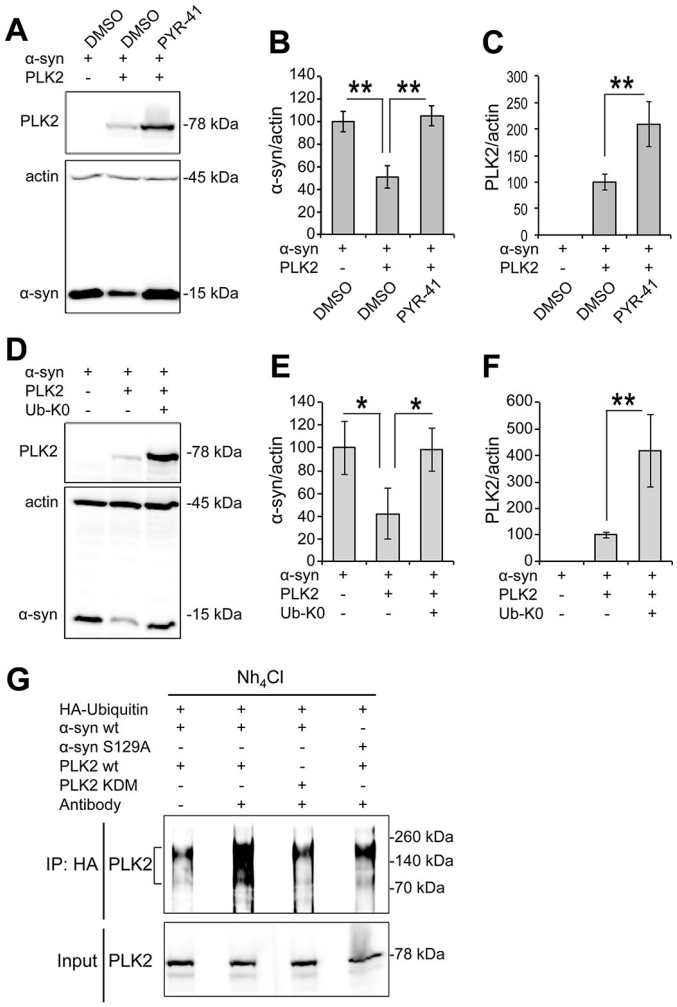

FIGURE 4. **Polyubiquitination was implicated in PLK2-mediated α-syn degradation.** *A*, Western blot illustrating the effect of ubiquitin system inhibition using PYR-41 on α-syn and PLK2 protein levels. HEK-239T cells were transfected with 1 μg of α-syn and 0.5 μg of PLK2 plasmids. 24 h post-transfection cells were treated with PYR-41 (12.5 μM) for 12 h. *B*, quantification of α-syn protein levels, expressed as % of the control and normalized against actin, demonstrates that treatment with PYR-41 suppresses PLK2-mediated α-syn degradation ($n = 3$). *C*, quantification of PLK2 protein levels, expressed as % of the control (α-syn+PLK2+DMSO) and normalized against actin, shows that PYR-41 induce a significant accumulation of PLK2 ($n = 3$). *D*, Western blot illustrating the effect of ubiquitin mutant missing the lysine residues (*Ub-K0*) on α-syn and PLK2 protein levels. HEK-239T cells were transfected with 1 μg of α-syn, 0.5 μg of PLK2, and 2 μg of Ub-K0 plasmids. *E*, quantification of α-syn protein levels, expressed as % of the control and normalized against actin, demonstrates that Ub-K0 overexpression restored α-syn expression to normal levels ($n = 3$). *F*, quantification of PLK2 protein levels, expressed as % of the control (α-syn+PLK2+DMSO) and normalized against actin, shows that Ub-K0 overexpression induces a significant accumulation of PLK2 ($n = 3$). *G*, HEK-239T cells were transfected with HA-tagged ubiquitin (HA-Ub) and either PLK2 (wt or KDM) or with α-syn (WT or S129A) and treated with NH_4Cl for 12 h. Cell Lysates were immunoprecipitated (*IP*) with anti-HA antibody, and Western blots were immunoblotted using PLK2 antibody. *Brackets* indicate expected a high molecular weight smear of ubiquitinated PLK2. **, $p < 0.01$ and *, $p < 0.05$ compared with α-syn+pCDNA+DMSO condition; Tukey's multiple comparisons test. For the comparison of PLK2 protein levels, **, $p < 0.01$ compared with α-syn+PLK2 condition using Student's *t* test.

E46K Mutant Is a Bona Fide Substrate for PLK2-mediated α-Syn Degradation—Previous work reported that α-syn PD-linked mutations (A30P, E46K, and A53T) affect α-syn degradation via the chaperone-mediated autophagy pathway (32, 33) and the proteasome-ubiquitin system (33, 34). To assess

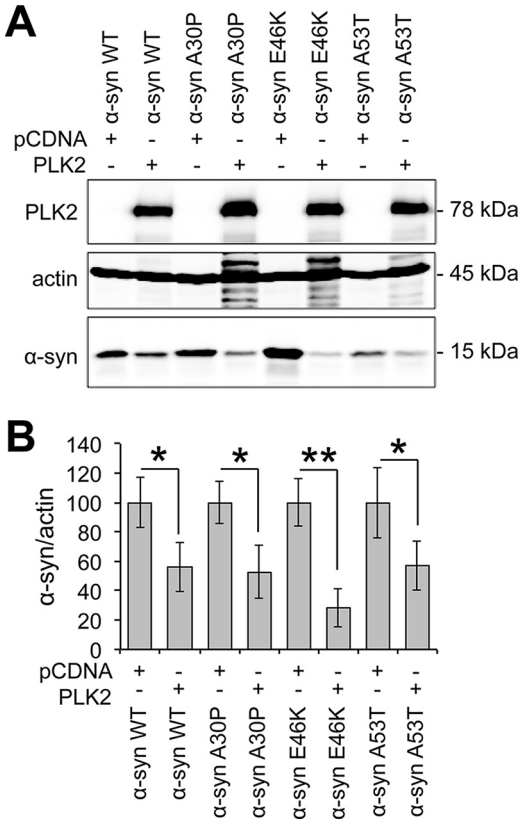

FIGURE 5. **E46K mutant was a bona fide substrate for PLK2-mediated α-syn degradation.** *A*, Western blot illustrating the expression levels of PLK2 and total α-syn 24 h post transfection in HEK-239T cells. Cells were transiently transfected with 1 μg of WT or PD-linked mutant forms of α-syn and 0.5 μg of PLK2 plasmids, and the total protein fraction was collected directly in 1× Laemmli buffer. *B*, histograms representing α-syn total protein levels in the presence or absence of PLK2 ($n = 4$). The results show that the expression levels of the PD mutant forms are reduced in presence of PLK2, confirming that all the PD mutants are eliminated through the PLK2-mediated degradation pathway. Interestingly, E46K mutant exhibited more pronounced reduction of its expression levels after PLK2 overexpression. **, $p < 0.01$ and *, $p < 0.05$, compared α-syn+pCDNA condition; Tukey's multiple comparisons test.

whether PD-linked mutations could also affect PLK2-mediated α-syn degradation, we overexpressed PLK2 with α-syn WT, A30P, E46K, and A53T in HEK-239T cells. Western blot analysis showed that protein levels of α-syn mutants are reduced after PLK2 overexpression, indicating that the protein harboring the PD-linked mutations is also eliminated via the PLK2-mediated degradation pathway (Fig. 5, *A* and *B*). Interestingly, although PLK2 overexpression induced a 40–50% decrease of A30P and A53T α-syn levels, E46K mutant exhibited a more marked reduction (−70%) of the total protein levels (Fig. 5*B*), suggesting that this mutant could represent a *bona fide* substrate of PLK2-induced degradation route. Together, our results demonstrate that the PD-linked mutations do not affect PLK2-mediated autophagic degradation of α-syn and reveal that the E46K mutation may enhance α-syn elimination via the PLK2-mediated degradation pathway.

Discussion

In the present study we describe for the first time detailed structural and molecular mechanisms underlying PLK2-mediated α-syn turnover. We show that PLK2 interacts with and phosphorylates α-syn at the residue Ser-129. This post-translational modification seems to play an important role in PLK2·α-syn complex formation. Subsequently, the complex is polyubiquitinated, specifically the PLK2, which might facilitate its recognition and elimination by the macroautophagy machinery (Fig. 6).

PLK2 and PLK3 Enhance α-Syn Turnover in Living Cells—In a previous work we reported that among the kinases catalyzing α-syn phosphorylation at Ser-129 *in vivo* (*i.e.* the G protein-coupled receptor kinases (GRKs) 3, 5, and 6), only PLK2 induces α-syn selective autophagic degradation (13). In the present study, we report that another PLK family member, namely the PLK3, is also able to enhance α-syn clearance in mammalian cell culture. This observation suggests that these two kinases may play a common physiological role by controlling α-syn protein levels *in vivo*. Importantly, PLK2 and PLK3 exhibit different subcellular localization in mammalian cell lines (cytosolic localization for PLK2 and membrane-associated and nuclear localization for PLK3) (14, 35), suggesting that these kinases may independently regulate α-syn expression at different cell compartments.

Since our first report of the PLK2-mediated α-syn degradation (13), findings from independent groups supported our observation. In 2014 Tenreiro *et al.* (36) confirmed the role of Ser-129 phosphorylation in α-syn-selective degradation and showed that Ser-129 → Ala substitution affects α-syn autophagic elimination in a yeast model of PD . More recently, a study by Wang *et al.* (37) reported the implication of PLK3 in the selective degradation of the prion protein. Interestingly, similar to PLK2-mediated α-syn degradation, prion protein turnover is governed by PLK3 kinase activity and PLK3/prion protein-protein interaction (37). These observations suggest that PLKs-mediated elimination of α-syn and prion protein may implicate common cellular pathways and suggest a possible role of these kinases in the selective elimination of "toxic" and misfolded proteins *in vivo*.

The exact physiological role of PLK2/PLK3-mediated α-syn degradation remains elusive. However, the implication of PLKs and α-syn in common cellular pathways suggests a synergistic role of these partners in different physiological cell processes. For instance, given the role α-syn in the regulation of neurotransmitter release and synaptic vesicle trafficking (3, 38), one can speculate that PLK2 and PLK3 may modulate the presynaptic activity through the regulation of α-syn phosphorylation and expression levels. In addition to their role in the regulation of the post-synaptic density activity and architecture (18, 20, 39), our data suggest that PLK2 and PLK3 may play a more global role in the regulation of synaptic homeostasis in the brain.

Importance of Ser-129 Phosphorylation on PLK2/PLK3-induced α-syn Turnover—Converging lines of evidence support the important role of α-syn Ser-129 phosphorylation in its degradation mediated by PLK2: 1) we report that only PLK2 and PLK3, the two PLK members capable to catalyze α-syn phosphorylation at Ser-129, induce α-syn clearance; 2) Ser-129 → Ala substitution to block phosphorylation precludes PLK2-mediated α-syn degradation; 3) PLK2 kinase activity is required to enhance α-syn elimination (13). The importance of this post-translational modification could be associated to its key role in the modulation of α-syn/PLK2 protein-protein

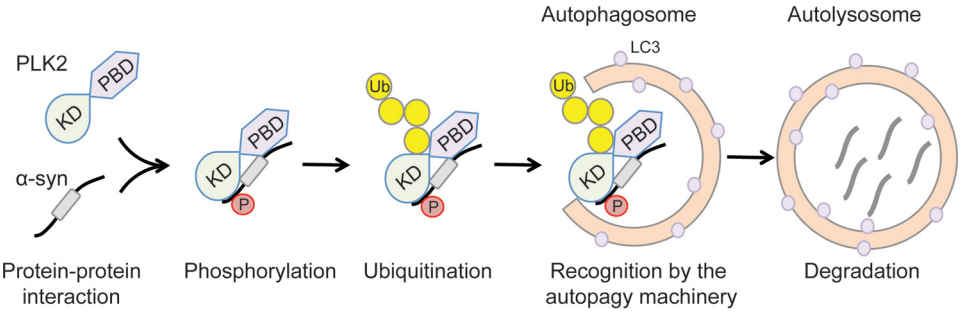

FIGURE 6. **Schematic representation of the molecular events underlying PLK2-mediated α-syn degradation.** PLK2 interacts with and phosphorylated α-syn at the residue Ser-129. Then the complex is recognized by the ubiquitin system, and a polyubiquitin chain is added to PLK2, which may facilitate its recognition by the macroautophagy machinery. *KD*, kinase domain; *PBD*, polo box domain; *P*, Ser-129 phosphorylation.

interaction. Indeed, we previously showed that mutations blocking phosphorylation of α-syn at Ser-129 (S129A) or PLK2 kinase activity (K111R, N210A) hinder α-syn and PLK2 protein-protein interaction and suppress α-syn turnover (13). Moreover, the impact of Ser-129 on α-syn protein structure (40) suggests that Ser(P)-129-related α-syn conformation changes may facilitate the interaction with PLK2 and stabilize the protein complex during the degradation process, giving rise to the concept of phosphorylation-dependent PLK2·α-syn complex formation. This concept of phosphorylation-dependent protein interaction has been previously described for several protein partners. For instance, phosphorylation of Ataxin-1 at the residue Ser-776 serves as a binding platform for RNA binding motif protein 17 (RBM17) and facilitates their interaction in spinocerebellar ataxia type1, a polyglutamine expansion neurodegenerative disease (41). Other studies reported that phosphorylation is also able to regulate the formation of large protein heteromultimers, *i.e.* tyrosine hydroxylase (TH) and 14-3-3 proteins, resulting in a tight regulation of the TH enzymatic activity (42).

Protein-Protein Interaction Is Required for PLK2·α-Syn Co-degradation—Pharmacological and genetic manipulation of the macroautophagy pathway resulted in α-syn and PLK2 co-accumulation, indicating that these two proteins might be co-eliminated. This observation supports our hypothesis suggesting that PLK2 and α-syn interact together and form a stable protein complex that is recognized and eliminated by the degradation machinery (Fig. 6). Given the importance of the protein-protein interaction in this cell process, we sought to map α-syn and PLK2 interaction, and our results revealed the implication of the entire N-terminal region of α-syn (amino acids 1–60), as shown by pulldown assay. Moreover, the deletion of this region (Δ2–60) significantly affected α-syn phosphorylation and degradation induced by PLK2, thus confirming the importance of this region in PLK2-mediated α-syn phosphorylation and degradation.

Previous work by Wang *et al.* (21) studied α-syn and PLK2 interaction in yeast cells. Contrary to our observation, their data showed that deletion of the 11 first amino acids of α-syn is sufficient to alter the interaction and the phosphorylation of α-syn by CDC5, the yeast ortholog of human PLK2 (21). This apparent discrepancy could be due to the fact that although orthologs, the protein structures of CDC5 and PLK2 are different (only 30% homology; UniProt), which may implicate distinct protein regions and conformations for the interaction with α-syn. Furthermore, this observation suggests that α-syn interaction with PLKs is differently regulated in mammalian and in yeast cells, which may reflect distinct physiological roles in different living cells.

Polyubiquitination Is Required for α-Syn and PLK2 Co-degradation—In the present study we report that inhibition of the ubiquitin system, more specifically the polyubiquitination, blocked PLK2-mediated α-syn degradation, revealing that this post-translational modification plays a key role in this cell process. Moreover, we observed that PLK2 is subjected to polyubiquitination, suggesting that the interaction with α-syn may facilitate PLK2 recognition by the ubiquitin ligases and enhance its ubiquitination.

The role of polyubiquitination on PLK2-mediated α-syn elimination remains unknown. However, recent findings suggest that this post-translational modification plays an important role in the selective autophagic degradation of several proteins and serves as a recognition signal for cargo recruitment (23–26). At the molecular level, the polyubiquitin moieties are recognized by the proteins adaptors (*i.e.* p62/SQSTM1 and NRB1), which contain a ubiquitin-associated domain (43), and then they facilitate their recruitment by the autophagy machinery. Therefore, our observations suggest that PLK2 ubiquitination may confer specificity and selectivity to the newly described α-syn elimination route.

PD-linked α-Syn E46K Mutant Is a Bona Fide Substrate for PLK2-mediated Protein Degradation—Previous studies reported that PD-linked mutations (A30P, E46K, A53T) affect α-syn elimination by the proteasome or the chaperone-mediated autophagy degradation pathways (32–34). However, in our model, these mutations had no effect on the PLK2-mediated α-syn turnover. This observation suggests that the new α-syn elimination route may be differently regulated due to the fact that α-syn is eliminated in a context of a protein complex.

Furthermore, our results showed that elimination of E46K α-syn form is enhanced compared with the other mutants, suggesting that this form may represent a good substrate of PLK2-mediated elimination route. Interestingly, in a previous work we observed that, among the PD mutants, E46K enhances α-syn phosphorylation at Ser-129 *in vivo* (44). Given the important role of Ser-129 phosphorylation in the regulation of PLK2 and α-syn protein-protein interaction, one can speculate that the E46K-associated increase of α-syn phosphorylation may

facilitate its interaction with PLK2 and consequently boost its elimination. However, this observation goes against the pathogenic nature of the E46K mutation. This disagreement suggests that elimination of this α-syn mutant could be enhanced in normal physiological conditions and it might be affected in the context of aging or PD pathogenesis. This hypothesis is supported by several observations reporting a dramatic dysfunction of protein degradation pathway, notably the autophagy pathway, during aging (45, 46) and in PD-diseased brains (47, 48), which lead to protein accumulation and neuronal loss.

Moreover, neuropathological and experimental studies reported that E46K mutation promotes α-syn aggregation (49, 50) and enhances its accumulation within the ER/microsomes in α-syn transgenic mouse brains and PD patient post-mortem tissues (51). These observations suggest that increased α-syn E46K aggregation or its sequestration in ER/microsomes in diseased brains may alter its subcellular localization and consequently affect its elimination by the PLK2-mediated degradation pathway. Further analyses are required to validate these hypotheses, and the use of α-syn E46K-based genetic mouse model of PD can help address this question *in vivo* (44).

Conclusions and Impact for PD Therapeutic Strategies—In the last decade the question on how α-syn is degraded *in vivo* has yielded controversial results (7). Our previous (13) and present studies describe for the first time a new specific and selective degradation pathway of α-syn (Fig. 6). This specificity is in part governed by a synergistic role of two post-translational modifications: phosphorylation and polyubiquitination. Although α-syn phosphorylation at Ser-129 stabilizes its interaction with PLK2 and the formation of protein complex, polyubiquitination of PLK2 seems to play a role in the selective recognition, recruitment, and degradation by the autophagic machinery.

The decortication of PLK2-mediated α-syn degradation machinery offers a unique opportunity to understand this cellular process and to develop new pharmacological strategies to manipulate α-syn protein levels and to reduce its toxicity in a specific and selective manner.

Materials and Methods

Cell Culture—HEK-293T cells were maintained at 37 °C and 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Life Technologies) and 1% penicillin/streptomycin (Gibco, Life Technologies). Cell passage numbers did not exceed 20. HEK-239T stable cell lines overexpressing human ATG5 shRNA (Sigma, TRCN0000151474), human ATG7 shRNA (Sigma, TRCN0000007587), or nonspecific shRNA (Addgene Plasmid #1864) were kindly provided by Dr Stéphane Gobeil (Université Laval, Quebec, Canada).

Plasmids—The plasmids used in this study are listed below: pCDNA-human α-syn, pCDNA-human α-syn S129A, pCMV6-DDK-PLK1, -PLK2, -PLK3, and -PLK4, and pCMV6-PLK2 K111R N210A (PLK2 KDM) were kindly provided by Prof. Hilal Lashuel (Brain and Mind Institute, EPFL, Switzerland) (14). pAAV-human α-syn Δ2–11 was kindly provided by Dr. Bernard Schneider (Brain and Mind Institute, EPFL, Switzerland). pCDNA-human α-syn Δ2–60 and pCDNA human α-syn A30P, E46K, and A53T plasmids were kindly provided by Prof. Seung-Jae Lee (Neuroscience Research Institute, Seoul National University College of Medicine, Seoul, Korea). pCDNA-myc-SPAR plasmid was kindly provided by Prof. Christina Spilker (Leibniz Institute for Neurobiology, Magdeburg, Germany). pRK5-HA-Ubiquitin-K0 was a gift from Prof. Ted Dawson (Addgene plasmid # 17603) (52).

Plasmid Transfection and Treatment with Small Molecules—HEK-293T cells were grown in 6-well plates to 40–50% confluency and transfected with 1 μg of pCDNA-human α-syn and 0.5 μg of pCMV6-DDK-PLKs plasmids using calcium phosphate transient transfection protocol. For DNA titrations, total DNA per transfection was equalized by the addition of pCDNA-empty plasmid. To modulate the autophagy degradation pathway, 8 h post-transfection HEK-239T cells were incubated for 12 h with rapamycin (20 nM) (Sigma, R0395), 3MA (10 mM) (Sigma, M9281), or ammonium chloride (NH_4Cl; 30 mM) (Sigma, A9434). To inhibit the proteasome degradation pathway, cells were treated with MG132 (10 μM) (Sigma, M8699). To block the ubiquitination process, transfected cells were incubated for 12 h in the presence of PYR-41 (F5100, UBPBio), a potent inhibitor of ubiquitin-activating enzyme (E1).

Western Blot (SDS-PAGE)—24 h post-transfection (12 h post treatment with small molecules) cells were washed and collected in ice shield PBS-1X and pelleted by centrifugation (1000 × *g*) at 4 °C for 3 min. The total protein fraction was extracted by dissolving the cell pellet in 1× Laemmli buffer (63 mM Tris-HCl (pH 6.8), 10% glycerol, 0.1% 2-mercaptoethanol, 0.0005% bromphenol blue, 2% SDS) and incubated at 95 °C for 20 min for DNA denaturation. 5 μl of the total protein fraction (corresponding to 25–35 μg of proteins) was loaded per well in a 12% SDS-PAGE. Gels were run at 130 V for 75 min, and the proteins were transferred to nitrocellulose membranes (Bio-Rad) using a semidry transfer machine (Bio-Rad). The membranes were heated in PBS 1× in a microwave for 1 min then incubated in a blocking solution (3% BSA in PBS 1×) at room temperature for 2 h. After the blocking step, the membranes were incubated overnight at 4 °C with the primary antibodies in the blocking solution. The primary antibodies were: mouse anti-α-syn (1:1000, BD Transduction Laboratories), mouse anti-Ser(P)-129 (1:5000, pSyn#64, Wako Pure Chemical Industries), rabbit anti-PLK2 (1:500, sc-25421 Santa Cruz Biotechnology), mouse anti-β-actin (1:15000, Applied Biological Materials Inc.), mouse anti-DDK (1:1000, Origene). The day after, the membranes were washed 3 times with PBS-Tween 0.1% (PBST), then incubated with the appropriate secondary antibodies, goat anti-rabbit, or anti-mouse (1:25000; Jackson ImmunoResearch) followed by the addition of the chemiluminescence reagents (Luminata; Millipore). Band intensities were detected using myECL imager (Thermo Scientific) and quantified using Fiji software.

Co-immunoprecipitation—PLK2 and truncated α-syn co-immunoprecipitation assay has been performed as we previously described (13). Briefly, 24 h post-transfection, cells were lysed using a tissue homogenizer in immunoprecipitation buffer (10 mM Tris·HCl (pH 7.4), 5 mM EGTA, 2 mM DTT, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 2 mM ATP) complemented with protease inhibitor (1:200), PMSF (1:100), and phosphatase inhibitor mixture 2 and 3 (1:200). For the pulldown of ubiquitinated

proteins, cells where treated with NH_4Cl for 12 h, then the cells were lysed in immunoprecipitation buffer supplemented with 2 mM deubiquitinases inhibitor, *N*-ethylmaleimide (NEM, Sigma). After centrifugation for 20 min at 16,000 × *g*, cleared lysate was collected and then incubated overnight at 4 °C under rotation with the appropriate antibody (anti-α-syn, BD Transduction Laboratories, or anti-HA tag, AB9110, Abcam). The day after, Dynabeads (Invitrogen) were washed, equilibrated in lysis buffer, and then added to the cell lysate and antibody mixture and incubated overnight at 4 °C under rotation. After 3 washes in PBS-Tween 0.01%, beads were incubated with 1× loading buffer at 95 °C for 10 min, and the samples were electrophoresed on 12% SDS-PAGE.

Statistical Analysis—All assays were performed in at least three independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparisons test. The comparison of PLK2 protein expression levels was performed using Student's *t* test. $p < 0.05$ was required for rejection of the null hypothesis. All values were expressed as the means ± S.D., and the software used for the statistical analysis was Prism v. 6 (GraphPad, La Jolla, CA).

Author Contributions—A. O. conceived the study, performed the experiments, analyzed the results, and wrote the manuscript. M. D. and M. B. performed the experiments and analyzed the results.

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