Supporting Information

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SI Text

Molecular Determinants Controlling PLK2-Mediated α-Syn Phosphorylation. Several studies have reported that α-synuclein (α-syn) is a substrate of polo-like kinase 2 (PLK2) in vitro (1–3), in cell culture (1, 2), and in vivo (1). However, the molecular determinants controlling PLK2-mediated α-syn phosphorylation remain unexplored. Previous studies showed that amino acid substitutions K111→R or N210→A, mutating key residues in the PLK2 kinase domain (Fig. S1*A*), are sufficient to diminish PLK2 kinase activity (4–6). We generated both mutants and evaluated their ability to phosphorylate α-syn in HEK293T cells. As shown in Fig. S1*B*, each of these single mutations moderately decreased pS129 levels but failed to abolish α-syn phosphorylation by PLK2 (Fig. S1*B*, lanes 3 and 4). Nevertheless, the combination of these two mutations almost completely blocked PLK2 kinase activity on human α-syn, as shown by the drastic reduction in pS129 levels (Fig. S1*B*, lane 5). This form of PLK2 will be later referred to as the kinase dead mutant (PLK2 KDM). Next, we sought to determine whether other molecular determinants could control PLK2-mediated α-syn phosphorylation. As shown in Fig. S1*A*, members of the PLK family contain a conserved C-terminal polo box domain (PBD) that controls substrate recognition and interaction, subcellular localization, and kinase activity (7). Interestingly, PBD mutations (PLK2 pbm: W503F, H629A, K631M) (6) or its complete deletion (kinase domain alone, residues 1–340) did not affect α-syn phosphorylation in HEK cells (Fig. S1*B*, lanes 6 and 9). These data demonstrate that PLK2 PBD is not required for α-syn phosphorylation and suggest that other domains in PLK2 structure could be implicated in its interaction with α-syn.

Moreover, it has been reported that PLKs are activated by the phosphorylation of a critical threonine residue, at position T210 in the PLK1 structure, called the activation loop (8), and T239 in PLK2 (9) (Fig. S1*A*). The substitution of T239→V, to block PLK2 phosphorylation and autoactivation, did not affect PLK2's ability to phosphorylate α-syn, demonstrating that PLK2 activation is not required for α-syn phosphorylation.

Collectively, these data describe that, unlike other substrates, α-syn phosphorylation does not require PLK2 autoactivation or the implication of the PBD and suggest that other PLK2 domains are implicated in the PLK2/α-syn interaction.

Overexpression of α-Syn, but Not PLK2 WT or PLK2 KDM, Induces Dopaminergic Neuron Loss and Hemiparkinsonian Motor Deficits. To evaluate the toxicity of α-syn and PLK2 (WT or KDM), we overexpressed separately each transgene in the rat midbrain via AAV-mediated gene delivery. Four months postinjection, we revealed α-syn expression in the injected substantia nigra pars compacta (SNc) using a human-specific antibody against α-syn (Sc-211) (Fig. S4*A*). No signal was detected in PLK2-injected midbrain or in the noninjected side. In PLK2-injected midbrain only, we could detect overexpressed human PLK2 (Fig. S4*A*). It is worth noting that, despite the fact that the PLK2 antibody recognizes the rat form of the protein, we did not detect endogenous PLK2, suggesting that the level of PLK2 in rat dopaminergic neurons was below the threshold of detection of our antibody.

Quantitative analysis of dopaminergic toxicity using unbiased stereology revealed an extensive loss of tyrosine hydroxylase (TH)-positive neurons in the SNc, four months after injection of the AAV vector for α-syn overexpression ($36.2\% \pm 2.7\%$, versus the noninjected hemisphere; $n = 8$) (Fig. S4*B*). In contrast, neither the vector overexpressing PLK2 WT, nor the KDM, caused any comparable neuron loss, with an average degree of lesion that remained $<15\%$ ($13.3\% \pm 2.7\%, n = 8$ and $10.3\% \pm 1.9\%, n = 8$, respectively) (Fig. S4*B*). In addition, we found no difference between the active and kinase-dead forms, indicating that kinase activity had no significant role in the observed minor neuron loss. In the striatum, densitometry of TH immunolabeling showed a similar pattern of dopaminergic fiber loss, which was significant only for the α-syn–expressing vector, reaching $24.5\% \pm 4.4\%$ of fiber loss in the ipsilateral striatum (Fig. S4*C*). In PLK2-injected animals, striatal TH density remained comparable between both hemispheres ($<3\%$ loss) (Fig. 2*C*).

Next, we assessed the effect of human α-syn or PLK2 on rat motor performance, using the cylinder test to measure spontaneous forelimb activity. Before injection, animals equally used both forelimbs, with ~50% of contacts with the cylinder made by each forepaw. However, four months postinjection, animals overexpressing human α-syn displayed a motor asymmetry ($9.2\% \pm 2\%$), reflecting the reduced use of the forepaw contralateral to the injected hemisphere (Fig. S4*D*). Altogether, these data confirm that human α-syn overexpression is toxic to DA neurons, in contrast to the AAV-mediated overexpression of PLK2 WT or the KDM, which do not cause any major DA neuron loss in the current experimental conditions. Therefore, we next combined the two vectors to determine whether PLK2 activity could modify α-syn toxicity in vivo.

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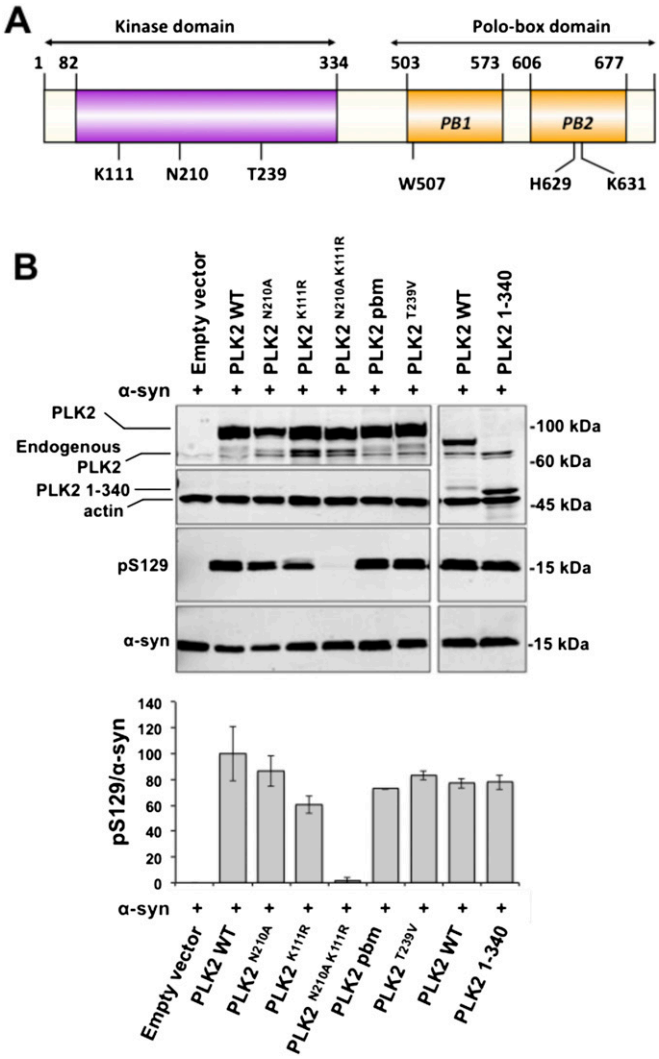

Fig. S1. (*A*) Schematic representation of PLK2 showing the kinase domain and polo-box domain and highlighting the key residues involved in regulating PLK2 kinase activity and substrate interactions. (*B*) Western blot and optical-density quantification from HEK cells transfected with α-syn (1 μg) and GFP-tagged (lanes 1–7) or Flag-tagged PLK2 (lanes 8 and 9). The results show that mutation of the key residues of polo-box domain (PLK2 pbm: W503F, H629A, K631M) that were shown to inhibit substrate recognition and interaction, subcellular localization, and kinase activity or inhibit PLK2 phosphorylation and activation (T239V) did not affect PLK2-induced α-syn phosphorylation in HEK cells.

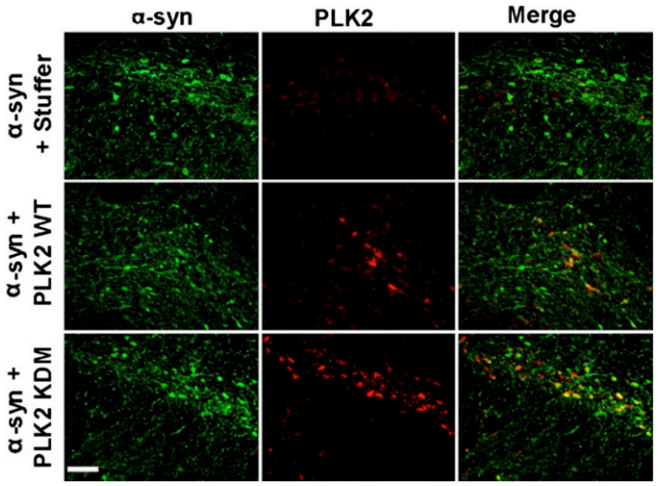

Fig. S2. α-Syn and PLK2 colocalize in the majority of the dopaminergic neurons. Photomicrographs illustrating the expression of α-syn and PLK2 in the injected brains. The merge showed that the majority of PLK2-positive cells also express α-syn. (Scale bar: 100 μm.)

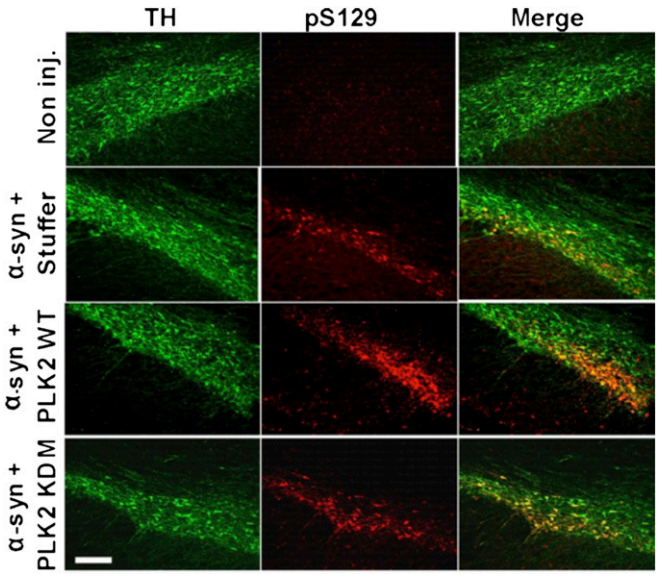

Fig. S3. Localization of pS129 expression in the dopaminergic neurons. Photomicrographs showing that detection of phosphorylated α-syn at S129 is in the majority of the TH-positive neurons. In the condition where α-syn is overexpressed with PLK2 WT, the levels of pS129 are dramatically increased compared with the conditions α-syn + Stuffer or α-syn + PLK2 KDM. (Scale bar: 100 μm.)

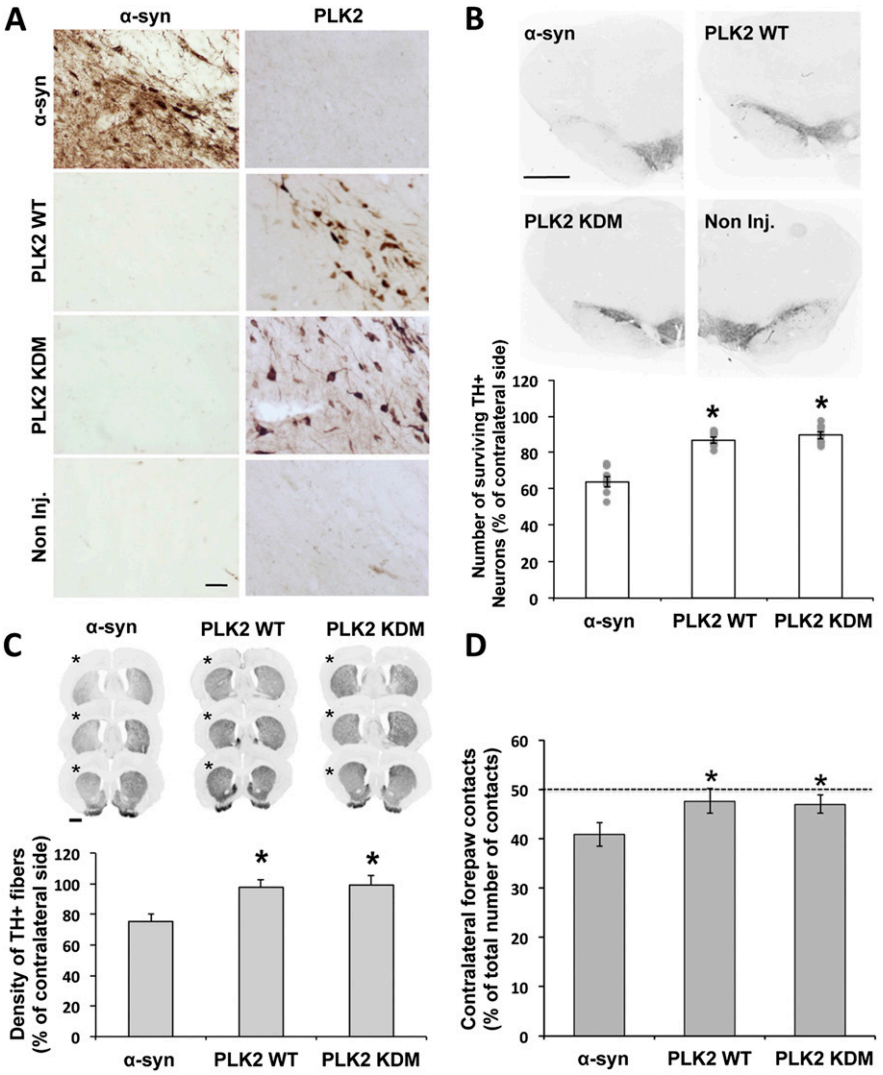

Fig. S4. α-Syn overexpression, but not PLK2 WT or PLK2 KDM, induces dopaminergic toxicity in vivo. (*A*) Immunohistochemistry illustrating the expression of human α-syn and PLK2 in the injected midbrains. The revelation showed α-syn signal in the injected SNc; however, no signal was detected in PLK2-injected midbrain or in the noninjected side. PLK2 immunoreactivity (PLK2 WT or KDM) was exclusively detected in PLK2-injected midbrains. (Scale bar: 50 μm.) (*B*) Photomicrographs illustrating the dopaminergic staining in the injected midbrains, and histograms showing the quantification of the dopaminergic loss in the injected SNc, compared with the contralateral side. The analysis revealed that only α-syn overexpression induced an extensive neuronal loss (~35%); however, PLK2 overexpression induced less than 10% of TH neuronal loss. (Scale bar: 1 mm.) (*C*) Illustration of TH level in the striatum. Optical-density quantification showed that only the overexpression of α-syn, but not PLK2 WT or KDM, induced a TH fiber loss in the ipsilateral striatum (*). (*D*) Histograms illustrating the induction of hemiparkinsonian motor impairment after the overexpression of human α-syn and PLK2. Four mos postinjection, only α-syn overexpression, but not PLK2, exhibited a large forelimb asymmetry. *$P < 0.05$ compared with α-syn-injected group, one-way ANOVA followed by Newman–Keuls post hoc test.

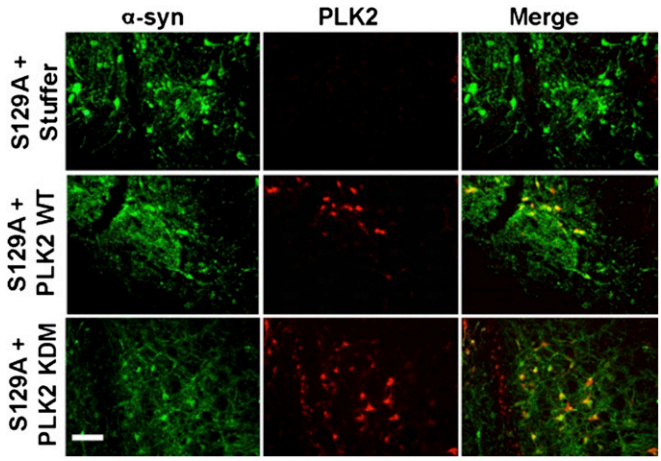

Fig. S5. Colocalization of S129A and PLK2 in the majority of the dopaminergic neurons. Photomicrographs illustrating the expression of S129A and PLK2 in the injected brains. The merge showed that the majority of PLK2-positive cells also express S129A. (Scale bar: 100 μm.)

Table S1. List of primers used to generate PLK2 and α-syn mutants

Constructs	Primer forward	Primer reverse
PLK2 K111R	5′CAAAGTCTACGCCGCACGAATTATTCCTCACAGCAGAG3′	5′CTCTGCTGTGAGGAATAATTCGTGCGGCGTAGACTTTG3′
PLK2 N210A	5′GAGATCTCAAACTAGGGGCCTTTTTT3′	5′AAAAAAGGCCCCTAGTTTGAGATCTC3′
PLK2 T239V	5′GGAACACAGAAGGAGAGTGATATGTGGTACCCC3′	5′GGGGTACCACATATCACTCTCCTTCTGTGTTCC3′
α-Syn S129A	5′CTTATGAAATGCCTGCTGAGGAAG3′	5′CTTCCTCAGCAGGCATTTCATAAG3′