# Supporting Information

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## SI Materials and Methods

**Cell Culture and Transfections.** Cells were grown in DMEM containing 10% FBS in a 5% $CO_2$ atmosphere. HEK293 and SH-SY5Y cells were transiently transfected with N-terminal–tagged pRK5 plasmids using Lipofectamine 2000 (Invitrogen). Unless specified, 0.5 μg of each cDNA was used for the different transfections carried out in this study. For experiments using small interfering RNAs, cells were transfected with Lipofectamine 2000 as previously described (18) using siPIAS2 [5′-CCCACGAGUUUAGUUCAAtt-3′ (s17287), or scrambled siRNA control (Ambion)].

**Western Blot Analysis.** Samples were homogenized as previously described (8) and 5% was loaded per lane for steady-state experiments. Actin levels were used as loading control for intracellular protein analysis. For measurements of α-synuclein release to extracellular medium, 5% of medium was loaded per lane without any further purification. Total levels of loaded proteins from crude extracellular medium was determined by Ponceau S staining.

Blots were probed with the following antibodies: mouse anti-HA (Covance); rabbit anti-myc, rabbit anti-HA, mouse anti-actin, rabbit anti-LDH, rabbit anti–α-synuclein, mouse and goat anti-PIAS2 (sc-166494; sc-30879), mouse and rabbit anti-PIAS1 (sc-365127; sc-14016), and rabbit anti–SUMO-1 (sc-9060) obtained from Santa Cruz; mouse anti-myc and rabbit anti-Flag (Sigma); mouse anti–α-synuclein (BD Biosciences); and anti-SUMO1 (Abcam). For anti-SIAH and anti–synphilin-1 antibodies, the antibodies were generated and purified as previously described (8, 45). Quantification of enhanced chemiluminescence was carried out according to ImageMaster analysis.

**Human Tissues.** Postmortem human cerebral cortices from five idiopathic PD cases with dementia and age-matched controls, as well as substantia nigra from six idiopathic PD cases and age-matched controls were obtained from the Queen Square Brain Bank archive and were used for conventional steady-state analysis. Four substantia nigra from idiopathic PD cases and age-matched controls were obtained from Parkinson's UK Brain Bank and were used for proteinase K and formic acid assays.

For immunoblots, brains were processed as previously described (18). Briefly, brains were homogenized in buffer containing 50 mM Tris·HCl, 30 mM NaCl, 1% Triton, 0.1% SDS, 30 μM MG132, and protease inhibitor mixture. The extracts were subsequently clarified by centrifugation at 13,000 × *g* for 5 min. The samples (50 μg per lane) were resolved on SDS/PAGE, and levels of PIAS2 and loading controls were determined by Western blot analysis.

For the formic acid assays, brain samples were incubated in 100% formic acid for 1 h at 37 °C and sonicated. Samples were then diluted and neutralized with 1 M Tris base and resolved on SDS/PAGE. Total levels of SUMOylated proteins were determined by Western blot analysis.

**Coimmunoprecipitation Assays.** For coimmunoprecipitation experiments, transfected cells were lysed in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 30 μM MG132, and protease inhibitor mixture (MiniComplete; Roche). Cell extracts were clarified by centrifugation and incubated for 4 h with anti-HA or anti-Flag coupled to protein G beads (Sigma). Immunoprecipitates were washed with lysis buffer containing 500 mM NaCl, and coimmunoprecipitation was detected by Western blot.

For endogenous coimmunoprecipitation assays, rat brains were homogenized in coimmunoprecipitation buffer containing 1% Triton X-100 and 0.1% SDS. Brain homogenates were clarified by centrifugation at 13,000 × *g* for 5 min. The antibody to PIAS2 or PIAS1 was coupled to protein G beads (8) and incubated for 4 h with brain homogenate (2 mg/mL). Immunoprecipitates were washed with lysis buffer and detected by Western blot using an anti–α-synuclein antibody.

**Purification of Recombinant Proteins.** His–α-synuclein was expressed in BL-21 bacterial cells as previously described (17). After 16 h of induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), bacterial cells were lysed and cleared from debris, and His–α-synuclein was purified using TALON beads according to the manufacturer's instructions (BD Biosciences). All variants of His–α-synuclein were verified for their purity and aggregation state by SDS/PAGE and Western blots analysis before in vitro experiments.

GST-PIAS2 and GST-PIAS1 were expressed in BL-21 bacterial cells, induced by the addition of IPTG and purified as previously described (8). The purified RanBP2 catalytic region, GST-RanBP2ΔFG, was purchased from Enzo. Unless stated otherwise, both proteins were used at a concentration of 200 ng per reaction.

**In Vitro Binding Assays.** GST proteins (GST-PIAS2 and GST-PIAS1 full length and control GST-Parkin amino acids 77–237) (0.2 μg) were incubated with 5 μg/mL His–α-synuclein coupled to TALON beads for 1 h at 4 °C in buffer containing 50 mM Tris·HCl (pH 7.4), 140 mM NaCl, 1% Triton X-100, 30 μM MG132, and protease inhibitors (Complete; Roche). Beads were washed in the same buffer with 500 mM NaCl. Bound proteins were detected by Western blot.

**In Vitro Ubiquitination Assays.** His–α-synuclein was purified from bacteria using TALON beads according to the manufacturer's instructions (BD Biosciences). Recombinant His–α-synuclein was incubated in a reaction medium containing 40 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM DTT, 1 mM ATP-γ–S, 7.5 μg of ubiquitin, 1 μM ubiquitin aldehyde, 100 ng of E1, and 200 ng of UbcH5b, in the presence or absence of 500 ng of SIAH-2. Ubiquitination reactions with Nedd4 were carried out with concentrations equimolar to SIAH-2. Reactions were incubated at 37 °C for 1 h, terminated by the addition of SDS sample buffer, and resolved on SDS/PAGE. Ubiquitinated α-synuclein was determined by Western blot using anti–α-synuclein antibody.

For the in vitro SUMOylation/ubiquitination coupled assays, samples were incubated at 37 °C for 1 h with purified components of the in vitro SUMOylation assays as above, in the presence or absence of 200 ng of PIAS2. Samples were then incubated for an additional hour at 37 °C with purified components of the in vitro ubiquitination assays, in the presence or absence of SIAH-2. Reactions were terminated by the addition of SDS sample buffer and resolved on SDS/PAGE. SUMOylated/ubiquitinated α-synuclein was determined by Western blot.

**In Vivo SUMOylation and Ubiquitination Assays.** Transfected HEK293 cells were treated with proteasome, autophagy, and lysosomal inhibitors (10 μM lactacystin, 10 mM 3-methyladenine, 25 mM ammonium chloride) for 16 h, and harvested cells were lysed by hot lysis as described (17) to prevent further addition or removal

of posttranslational modifications during the immunoprecipitation process. Briefly, cells were lysed in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, and 4% SDS and boiled at 100 °C for 5 min. Lysates were sonicated and diluted 20-fold with lysis buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 30 μM MG132, and protease inhibitor mixture (Mini Complete; Roche). Samples were centrifuged at 13,000 × *g* for 5 min, and the supernatant was incubated with anti-Flag (Flag-SUMO1 or Flag-ubiquitin) or anti-HA (HA–α-synuclein) antibodies coupled to beads (Sigma) for 4 h at 4 °C. After incubation, beads were extensively washed with final lysis buffer containing 500 mM NaCl. Controls with recombinant purified deSUMOylase Ulp1 were carried out by adding the deSUMOylase (2 μg) to the washed immunoprecipitates for 1 h. Immunoprecipitates were loaded on a SDS/PAGE gel for Western blot analysis. The specificity of SUMO–α-synuclein signal was determined by the disappearance of the SUMO–α-synuclein signal in Western blots in samples treated with Ulp1.

For immunoprecipitation of SUMOylated proteins in human brain tissues, frontal cortices were homogenized by hot lysis as described above for transfected cells. Lysates were incubated with goat anti-SUMO1 antibody (sc-6375; Santa Cruz) coupled to protein G beads (Thermo) for 4 h at 4 °C. After incubation, beads were extensively washed with final lysis buffer containing 500 mM NaCl. As for the SUMOylation from cells, the specificity of SUMO–α-synuclein signal in brain tissues was determined by incubation of the immunoprecipitates with 2 μg of deSUMOylase (Ulp1) for 1 h before the SDS/PAGE. Immunoprecipitates were loaded on an SDS/PAGE gel for Western blot analysis.

**Cycloheximide Chase Experiments.** Transfected cells were incubated with 50 μM cycloheximide for the indicated times. Cells were harvested for the time specified above and were lysed in buffer containing 50 mM Tris·HCl (pH 7.4), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 30 μM MG132, and protease inhibitor mixture (MiniComplete; Roche). Cell extracts were centrifuged for 5 min at 13,000 × *g*. The supernatants were then analyzed by SDS/PAGE and analyzed by Western blot.

**Proteinase K Assays.** Transfected HEK293 cells as well as human brain tissues (substantia nigra) were processed in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, and 1% Triton X-100. Lysates were sonicated for 10 s and incubated with 1 μg/mL proteinase K for the indicated times, as previously described (34). Following digestion, lysates were boiled for 5 min, run on SDS/PAGE, and analyzed by Western blot.

**Immunocytochemistry.** Transfected SH-SY5Y cells were incubated with 2 μg/mL proteinase K for 10 min at 37 °C, fixed in 4% paraformaldehyde for 15 min, and blocked in PBS containing 0.2% Triton X-100 and 5% normal goat serum. The cells were labeled with anti-HA or anti-myc antibodies, as previously described (8). Immunolabeling was detected using FITC- and $Cy^3$-labeled secondary antibodies (Jackson Laboratories). α-Synuclein aggregates were determined by processing the cells 36 h after transfection and analyzed by confocal microscopy analysis using a Zeiss LSM 700 confocal microscope (Zeiss). Optical sections were obtained under a 63× immersion objective at a definition of 1,024 × 1,024 pixels with the pinhole diameter adjusted to 1 μm. All sections were acquired using the same laser parameters and image magnification. Ten different fields were examined for each condition in every experiment, and data are representative of at least three independent experiments.

**Primary Neuronal Cultures.** Primary cortical cultures were prepared from Sprague-Dawley rats at embryonic day 18, as described (46). After decapitation, the embryos were harvested and the cortices were dissected and maintained in Hank's balanced salt solution. Samples were digested by incubation for 10 min with trypsin (Beit-Haemek Biological Industries) at 37 °C. After trituration with glass Pasteur pipettes and removal of clumps, the suspension was filtered through a 0.7-μm filter (BD Biosciences). The cells were collected by centrifugation and resuspended in Neurobasal medium supplemented with 5% B27 (Invitrogen) and 0.5 mM L-glutamine (Beit-Haemek Biological Industries). Neurons were plated in six-well plates coated with poly-D-lysine (Sigma) at a density of $2 \times 10^6$ cells per well.

**Immunohistochemistry of Human Tissues.** Paraffin-embedded sections (8 μm) of midbrain from three idiopathic PD cases were deparaffinized in xylene followed by graded rehydration. Endogenous peroxidase activity was blocked with methanol/0.3% $H_2O_2$. Sections were pretreated by pressure cooking in citrate buffer at pH 6.0 for 10 min, and nonspecific protein binding was blocked in 10% normal goat serum in PBS for 30 min at room temperature. Primary antibody was incubated at 4 °C for 16 h, immunostained by the avidin–biotin peroxidase complex method (Dako), and developed with 3.3-diaminobenzidine (Vector Laboratories). Anti-SUMO1 (Abcam), anti-PIAS2 (Santa Cruz), and anti–α-synuclein (BD Biosciences) antibodies were used in the experiments. Sections were counterstained with Mayer's hematoxylin.

**Quantification and Statistics.** Quantification of specific bands obtained by enhanced chemiluminescence reactions was carried out using the ImageMaster analysis (GE Healthcare Life Sciences). Statistical analysis was performed by means of repeated-measures one-way ANOVA with Bonferroni's multiple-comparison test or by two-tailed Student's *t* test using GraphPad Prism software, version 6.03 (GraphPad).

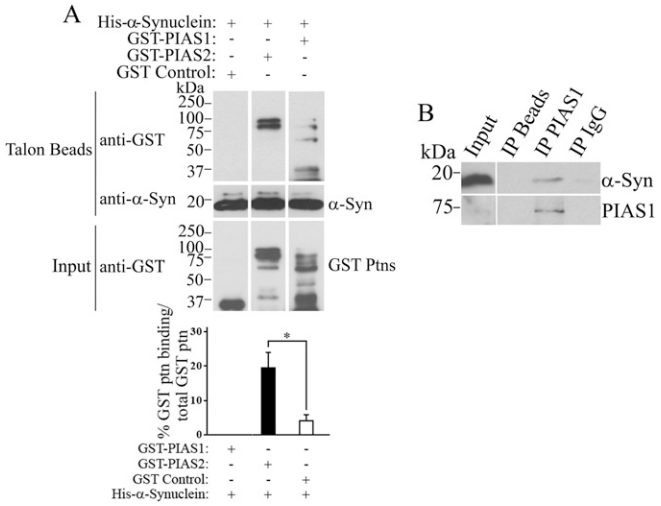


**Fig. S1.** PIAS2 and PIAS1 interact with α-synuclein. (*A*) In vitro binding of PIAS2 and PIAS1 to α-synuclein. Recombinant purified His–α-synuclein was incubated with GST-PIAS2, GST-PIAS1, or GST-control protein (GST-parkin amino acids 77–237). Binding of GST proteins with His–α-synuclein was detected with anti-GST antibody. Levels of His–α-synuclein bound to TALON resin and GST protein loading controls are shown in the *Middle* and *Lower*, respectively. Graph depicts the percentage of GST-PIAS2 and GST-PIAS1 bound to His–α-synuclein relative to the total amount of each GST protein added per incubation. Values represent the average ± SEM of three experiments. *Different from control at $P < 0.05$ (Student's *t* test). (*B*) Endogenous PIAS1 was immunoprecipitated from rat brain lysate using anti-PIAS1 antibody (*Lower*), and coimmunoprecipitation was visualized with anti–α-synuclein antibody (*Upper*).

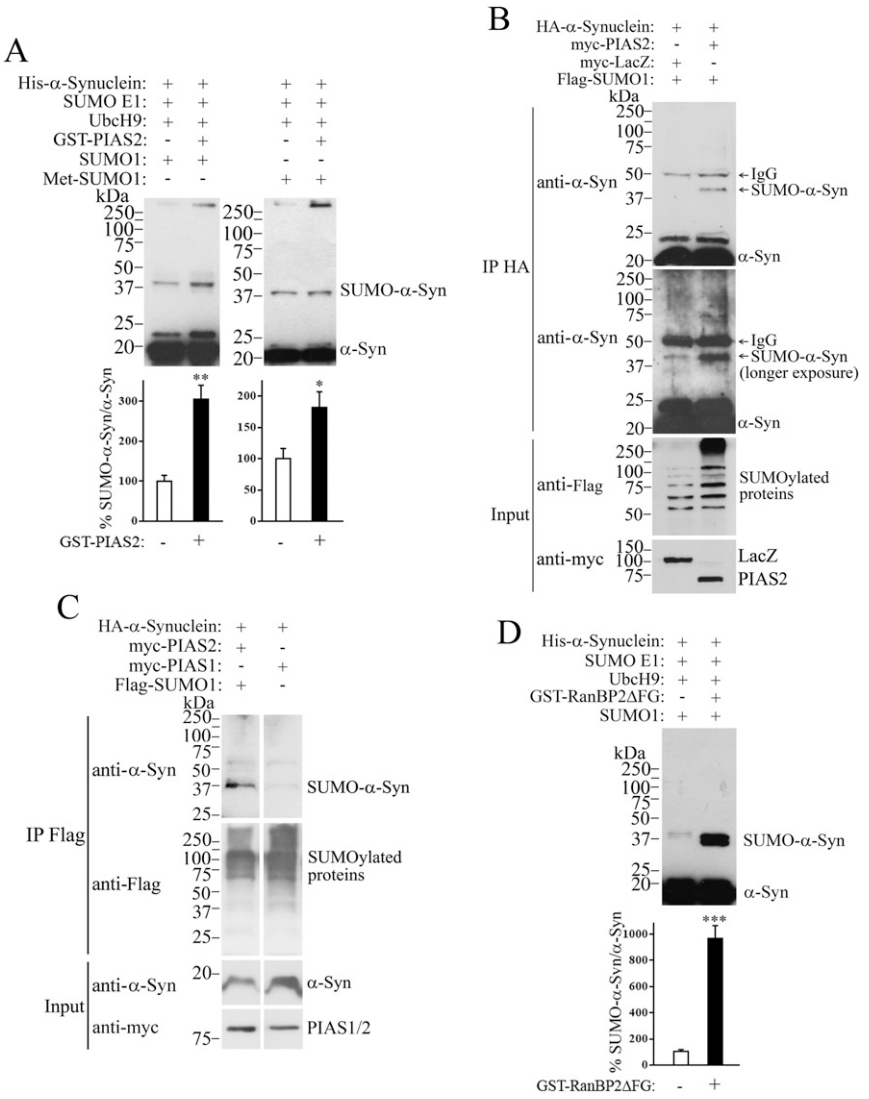


**Fig. S2.** Different E3 SUMO ligases SUMOylate α-synuclein. (*A*) In vitro SUMOylation by PIAS2 was carried out by incubating His–α-synuclein with SUMOylation components and 0.2 μg of PIAS2, either with SUMO1 (*Left*) or methylated SUMO1 (Met-SUMO1) (*Right*). Levels of SUMOylated α-synuclein were quantified with anti–α-synuclein antibody. Graphs represent the percentage of in vitro SUMOylated α-synuclein relative to total α-synuclein, in the presence of SUMO1 (left graph) and Met-SUMO1 (right graph). (*B*) HA–α-synuclein from transfected HEK293 cells was immunoprecipitated with an anti-HA antibody, and SUMOylated α-synuclein was detected with anti–α-synuclein (*Upper*). Longer exposure with anti–α-synuclein antibody reveals the basal levels of α-synuclein SUMOylation in the absence of ectopic PIAS2 expression (second panel). (*C*) HEK293 cells were cotransfected with myc-PIAS1 or myc-PIAS2. SUMOylated proteins were immunoprecipitated with anti-Flag antibody, and SUMOylated α-synuclein was detected with anti–α-synuclein antibody (first panel). Immunoprecipitated SUMOylated proteins were detected with anti-Flag antibody (second panel). (*D*) In vitro SUMOylation by RanBP2 was carried out by incubating His–α-synuclein with SUMOylation components, in the absence and in the presence of the catalytic region of RanBP2 (GST-RanBP2ΔFG). Levels of SUMOylated α-synuclein were quantified with anti–α-synuclein antibody. Graph represents the percentage of in vitro SUMOylated α-synuclein relative to total α-synuclein. Values represent the average ± SEM of three experiments. Different from control at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (Student's *t* test).

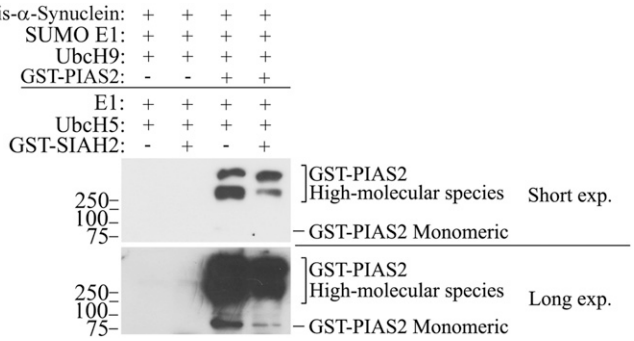


**Fig. S3.** Formation of high–molecular-weight species of PIAS2 upon SUMOylation and ubiquitination reactions. Different exposure times of GST-PIAS2 blot like that shown in Fig. 2*A*. After SUMOylation and ubiquitination reactions, high–molecular-weight PIAS2 species appear on short exposures (*Upper*). Monomeric GST-PIAS2 protein is detectable after longer exposure of the blot (*Lower*).

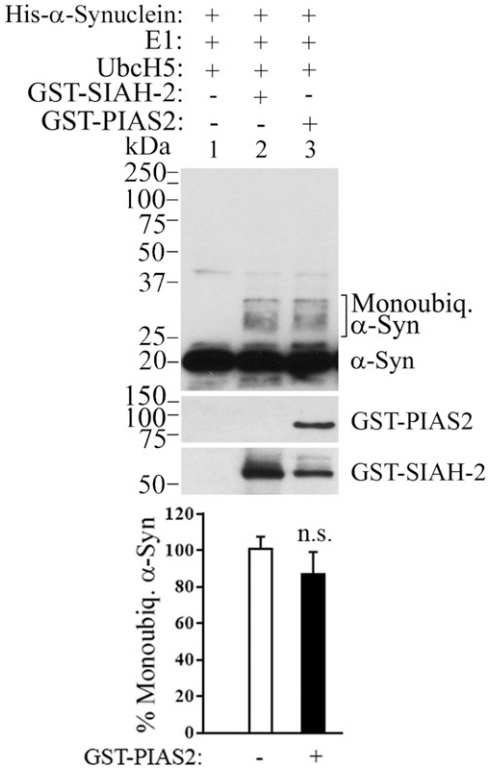


**Fig. S4.** PIAS2 does not inhibit α-synuclein monoubiquitination in the absence of SUMOylation components. In vitro ubiquitination reactions were carried out by incubating His–α-synuclein with purified ubiquitination components, in the absence and in the presence of purified GST-SIAH-2. Addition of GST-PIAS2 without SUMOylation components (lane 3) had no effect on α-synuclein monoubiquitination (graph). Levels of His–α-synuclein monoubiquitination were determined with anti–α-synuclein antibody. Values represent the average ± SEM of three experiments. n.s., not a significant difference (Student's *t* test).

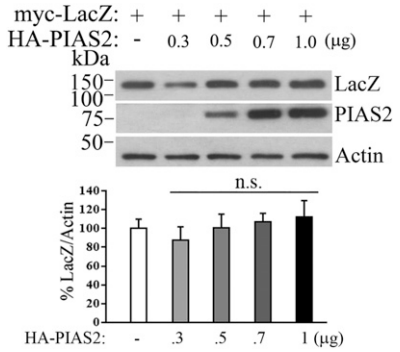


**Fig. S5.** PIAS2 does not promote the accumulation of LacZ control protein. Steady-state levels of myc-LacZ and HA-PIAS2 from transfected HEK293 cells were monitored with anti-myc and anti-HA antibodies, respectively. Graph represents the percentage of myc-LacZ (relative to β-actin) with increasing amounts of HA-PIAS2. Values represent the average ± SEM of three independent experiments. n.s., not significantly different from control (repeated-measures one-way ANOVA with Bonferroni post hoc test).

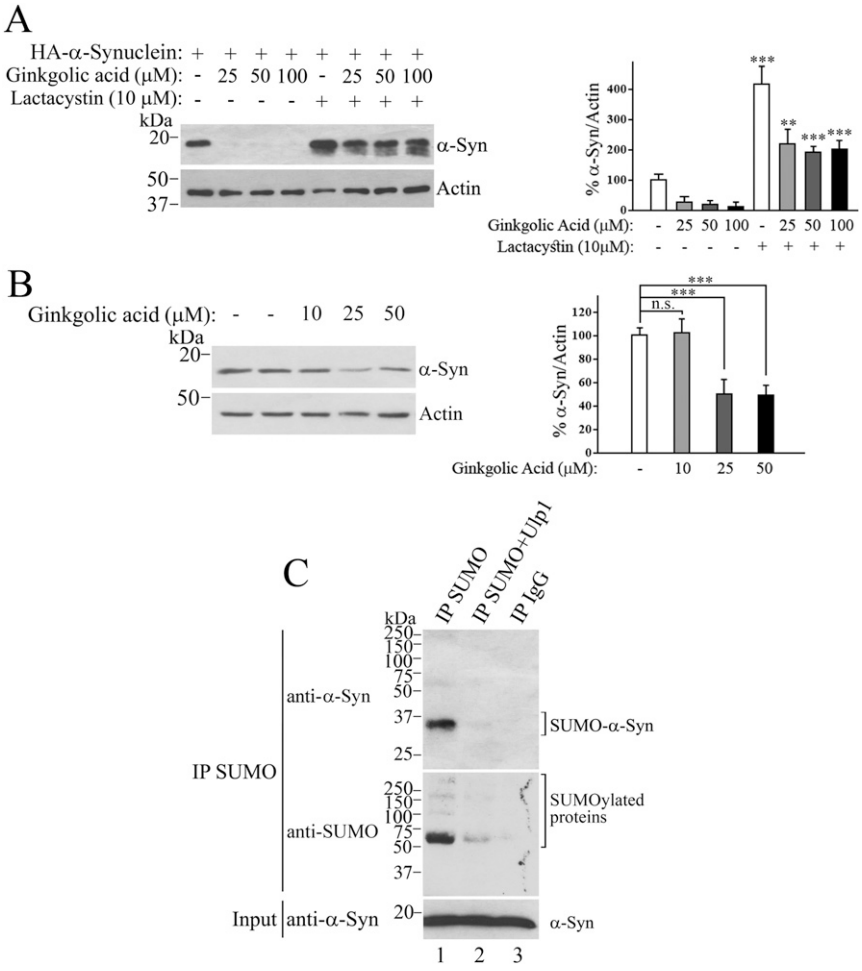


**Fig. S6.** Inhibition of SUMOylation promotes α-synuclein proteasomal degradation. (*A*) Transfected HEK293 cells were treated with increasing concentrations of the SUMOylation inhibitor ginkgolic acid for 16 h, in the presence or absence of the proteasome inhibitor lactacystin. Levels of α-synuclein were monitored with anti–α-synuclein antibody (*Upper*). Graph depicts the percentage of α-synuclein steady-state levels normalized to β-actin. (*B*) Neurons were treated with increasing concentrations of ginkgolic acid for 16 h. Levels of endogenous α-synuclein were monitored with anti–α-synuclein antibody (*Upper*). Graph depicts the percentage of endogenous α-synuclein steady-state levels normalized to β-actin. (*C*) Lysates from human frontal cortex were immunoprecipitated with anti-SUMO1 (lane 1) or anti-IgG antibodies (lane 3). As an additional control, SUMO1 immunoprecipitate was incubated with the purified deSUMOylase Ulp1 (lane 2). Levels of SUMOylated α-synuclein were determined using anti–α-synuclein antibody (*Upper*). Immunoprecipitated SUMOylated proteins are shown in the *Middle*. Figures are representative of three independent experiments. Values represent the average ± SEM of three experiments. Different from control at $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively. Student's *t* test comparing lactacystin and respective control without lactacystin (*A*) or repeated-measures one-way ANOVA with Bonferroni post hoc test (*B*). n.s., not significant.

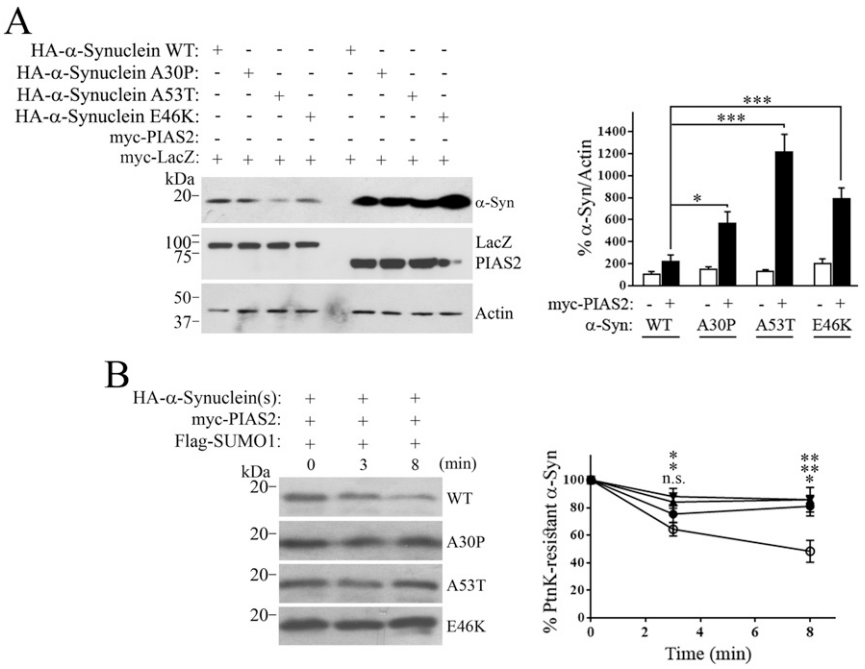


**Fig. S7.** SUMOylation increases the accumulation and aggregation of α-synuclein disease mutants. (*A*) Steady-state levels of WT and mutant α-synuclein species in transfected HEK293 cells, and in the presence of myc-PIAS2 or control myc-LacZ, were monitored with anti-HA antibody (*Upper*). Levels of myc proteins are determined with anti-myc antibody (*Middle*). Graph depicts the percentage of α-synuclein (WT, A30P, A53T, and E46K) steady-state levels normalized to β-actin. (*B*) HEK293 cells transfected with α-synuclein (WT, A30P, A53T, and E46K) and PIAS2 were digested with proteinase K for indicated times. The remaining proteinase-resistant α-synuclein was determined by Western blot analysis with anti-HA antibody. Graph depicts the percentage of proteinase K-resistant HA–α-synuclein (WT, ○; A30P, ▲; A53T, ●; and E46K, ▼) that remains over the indicated time points. Values represent the average ± SEM of three to four independent experiments. Different from WT at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Repeated-measures one-way ANOVA with Bonferroni post hoc test. n.s., not significant.

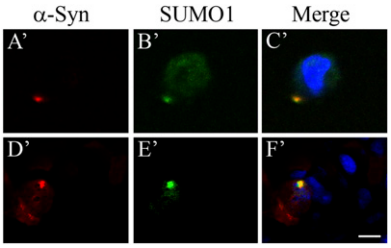


**Fig. S8.** SUMO1 colocalizes with α-synuclein aggregates in cells. SH-SY5Y cells were transfected with HA–α-synuclein, Flag-SUMO1, and myc-PIAS2. Cells were treated with proteinase K before fixation and processed for immunocytochemistry with anti-HA (red, *A*′ and *D*′) and anti-SUMO1 (green, *B*′ and *E*′) antibodies. Colocalization of SUMO1 with α-synuclein inclusions was determined by confocal microscopy (*C*′ and *F*′). (Scale bar, 25 μm.)

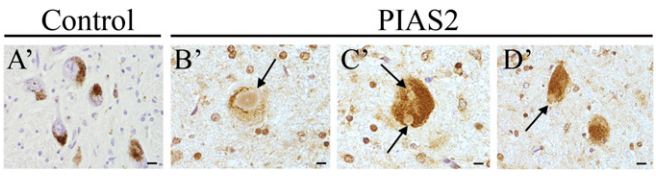


**Fig. S9.** PIAS2 is present in Lewy bodies. Immunohistochemistry of substantia nigra from PD patients using anti-PIAS2 antibody. Control was carried out in the absence of the primary antibody. Arrows point to PIAS2-positive Lewy bodies. Sections were counterstained with hematoxylin. [Scale bars, 10 μm (*A*′ and *D*′) and 5 μm (*B*′ and *C*′).]