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Supplementary Materials for

A cullin-RING ubiquitin ligase targets exogenous α-synuclein and inhibits Lewy body–like pathology

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SUPPLEMENTARY MATERIALS

Supplementary materials and methods

Plasmids and cloning

Plasmid construction and DNA manipulations were performed following standard protocols. The DNA *sequences* of *all constructs* were verified by *sequencing prior to use.* The Flag-GFP-αSyn- and Flag-GFP-encoding plasmids (pcDNA5-Flag-GFP-αSyn and pcDNA5-Flag-GFP, respectively) were obtained by cloning the cDNA coding region of human αSyn (kindly provided by Dr. Say Yee How (*1*)) downstream the Flag tag and EGFP into the destination vector pcDNA5-Dest/FRT/TO derived from pcDNA5/FRT/TO (Invitrogen) by two-step PCR. The following primers were used to create pcDNA5-Flag-GFP-αSyn: EGFP forward, GGGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGACTACAAGGACGACGACGACAAGGTGAGCAAGGGCGAGGAGCTGTTCACC; EGFP reverse, GCTCTGGAAGTACAGGTTCTCCTTGTACAGCTCGTCCATGCCGAGAGTGATCC; αSyn forward, GAGAACCTGTACTTCCAGAGCGATGTATTCATGAAAGGACTTTCAAAGGC; αSyn reverse, GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTAGGCTTCAGGTTCGTAGTCTTGATACCCTTCCTCAGAAGGCATTTCATAAGCC. The following primers were used to construct pcDNA5-Flag-GFP: EGFP forward, GGGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGACTACAAGGACGACGACGACAAGGTGAGCAAGGGCGAGGAGCTGTTCACC; EGFP reverse, GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTATTACTTGTACAGCTCGTCCATGCCG. DN-Cul1, DN-Cul2, DN-Cul3 and DN-Cul4A expression vectors were obtained from Prof. Wade Harper through Addgene (www.addgene.org; plasmids 15818, 15819, 15820, and 15821, respectively). To generate the SKP1-V5 expression vector, bacterial clones transformed with SKP1 cDNA were obtained from hORFeome V5.1 (http://horfdb.dfci.harvard.edu/) and sub-cloned into pcDNA-DEST40 (Invitrogen). FBXL5-Flag and FBXL5-Δbox-Flag expression vectors were kindly provided by Prof. Richard Bruick (*2*).

Cell culture

SH-SY5Y, HEK293 and Cos7 cells were purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM, pH 7.3) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Only undifferentiated SH-SY5Y cells were used in this study. SH-SY5Y cell clones with inducible expression of Flag-GFP or Flag-GFP-αSyn were generated using the Flp-In/T-REx inducible expression system (Invitrogen). First, SH-SY5Y Flp-In/T-Rex cell clones were generated by stably transfecting SH-SY5Y T-Rex cell clones (kindly provided by Cristina Bellarosa and Claudio Tiribelli (*3*)) with the pFRT/*lacZeo* plasmid. Clones with the highest LacZ-Zeocin expression levels were co-transfected with the pcDNA5-Flag-GFP or pcDNA5-Flag-GFP-αSyn and pOG44 plasmids. Selection was carried out using 100 µg/ml zeocin and 100 µg/ml hygromycin B. The medium was changed every two days until individual cell clones were established. DN-Cul1 HeLa cell clones were generated using the same inducible

expression system; HeLa Flp-In/T-Rex cell clones (Invitrogen) were co-transfected with pcDNA5-DN-Cul1-Flag and pOG44 plasmids and selected with 50 µg/ml hygromycin B. To induce the expression of GFP, GFP-αSyn and DN-Cul1, cells were treated at the indicated times with 10 ng/ml doxycycline. Bafilomicyn-A was used at final concentration of 50 nM. The final concentrations of (FAC) and deferoxamine (DFO) were 100 µM and 50 µM, respectively. The MTT assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega) was performed according to the manufacturer's instructions.

Transfections

Transfection of plasmids (2 µg of total DNA) or siRNAs (20 pmol) was carried out with Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) transfection reagent, respectively, according to manufacturer's instructions. Unless otherwise indicated, cells were treated 48 h post-transfection in DMEM supplemented with 2% fetal calf serum and then extensively washed before harvest. siRNA target sequences were: Cul1, AATAGACATTGGGTTCGCCGT; SKP1, GAAAGGAAATCCGGAAATA; FBXL5, GAATCATGCTGAAGAGCGA and CAGCTATCTTAATCCTCAA; GFP, GCAAGCTGACCCTGAAGTTC; Luc, CTTCGAAATGTCCGTTCGGTT. siRNAs targeting genes encoding each of the 32 human FBX proteins (two sequences per gene) were purchased from Ambion (siRNA Silencer Select).

Co-immunoprecipitation and affinity purification

Cells were washed with ice cold PBS, lysed on ice with modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate, protease inhibitor cocktail), and immunoprecipitated with the indicated antibodies. Beads (protein-A sepharose, Sigma) containing precipitated proteins were collected by centrifugation, washed three times with modified RIPA buffer, and boiled at 95 °C for 5 min in 2X Laemmli sample buffer prior to electrophoresis. For metal-affinity purification of internalized His-iF-αSyn, cells were harvested in buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8). Lysates were incubated for 1 h at room temperature, then subjected to 10 cycles of strong sonication and centrifuged for 5 min at 2,000 g. The supernatants were incubated with pre-equilibrated Ni-NTA superflow resin (Qiagen) for 2 h. The resin was then sequentially washed with buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6) and buffer D (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). His-iF-αSyn was eluted with buffer E (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 3) and the pH of the eluent was neutralized with NaOH.

Antibodies

The following antibodies were used: Mouse monoclonal anti-αSyn (clone LB509) and rabbit polyclonal anti-V5 were purchased from Abcam. Rabbit polyclonal anti-αSyn and anti-LC3B were from Cell Signaling. Mouse monoclonal anti-Cul1 antibody (clone D5), anti-GFP (clone sc-9996), and anti-ubiquitin (clone sc-8017) were from Santa Cruz Biotechnology. Rabbit polyclonal anti-amyloid oligomer

A11 and anti-amyloid fibril OC were from Millipore. Mouse monoclonal anti-SKP1 (clone 1C10F4) and anti-p27 (clone pT187) were purchased from Invitrogen. Mouse monoclonal anti-Flag (clone M2) was from Sigma, and mouse affinity-purified anti-FBXL5 was from Neoclone.

ThT assay

The ThT binding assay was performed as described previously (*4*) using a 25 µM ThT solution in 25 mM sodium phosphate, pH 6.0. Aliquots (30 µl) of the protein samples containing amyloid aggregates were diluted into the ThT buffer, and fluorescence emission was measured at 25 °C with an excitation wavelength of 440 nm. Fluorescence emission was recorded at 484 nm on a Cary Eclipse Fluorescence Spectrophotometer (Agilent).

Circular dichroism and Fourier transform infrared spectroscopy

Circular dichroism spectra were recorded on a J-710 spectropolarimeter (Jasco). Far-UV CD spectra were recorded using a 1-mm path-length quartz cell at a protein concentration of 0.1 mg/ml. The mean residue ellipticity [θ] ($deg \cdot cm^2 \cdot dmol^{-1}$) was calculated from the formula $[\theta] = (\theta_{obs}/10) \cdot (MRW/lc)$, where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue molecular weight of the protein, *l* is the optical path length in cm, and *c* is the protein concentration in g/mL. The spectra were recorded in PBS buffer, pH 7.4.

Transmission electron microscopy

Aliquots of the protein aggregation mixtures were examined by transmission electron microscopy with negative staining. A droplet of the sample was placed on a Butvar-coated copper grid (400-square mesh, TAAB Laboratory Equipment) or a carbon-coated copper grid (400-square mesh, Quantifoil Micro Tools), dried, and negatively stained with a droplet of uranyl acetate solution (1%, w/v). TEM pictures were taken on a Tecnai G^2 12 Twin (FEI Company) or on a Philips CM12 microscope operating at an excitation voltage of 100 kV.

Sequential extraction with detergents

Preparations of F-αSyn and iF-αSyn were spiked into Cos7 cell extracts and then sequentially extracted as follows: the extracts were homogenized in 100 µL of TBS+ buffer (50 mM Tris-HCl pH 7.4, 175 mM NaCl, 5 mM EDTA, and a protease inhibitor cocktail (Calbiochem, CA)) and centrifuged for 30 min at 120,000 × g. Pellets were sequentially extracted in: 1. TBS+ containing 1% of Triton X-100; 2. TBS+ containing 1% of Sarkosyl, and 3. 8 M urea containing 5% SDS. Each extraction step was followed by centrifugation for 20 min at 120,000 × g.

In vitro protein cross-linking

Each αSyn sample was diluted 1:1 in PBS to a final concentration of 125 µM and incubated with 1 mM homobifunctional dithiobis(succinimidyl) propionate crosslinker DSP (Thermo Scientific) for 15 min at room temperature. The mixtures

were then quenched with Tris-HCl (pH 7.5) to a final αSyn concentration of 50 mM and incubated for 30 min. The apparent molecular weight of the crosslinked αSyn species was then determined by denaturing SDS-PAGE.

Tryptic digestion

Cells were harvested in a buffer containing 8 M urea and 0.1 M NH_4HCO_3, and sonicated three times for 10 s with an ultrasonic probe device. Cell lysates (100 μg) were reduced with 12 mM dithiothreitol for 30 min at 32 °C and alkylated with 40 mM iodoacetamide for 45 min at 25 °C in the dark. Samples were diluted with 0.1 M NH_4HCO_3 to a final concentration of 2 M urea, and sequencing-grade porcine trypsin (Promega) was added to a final enzyme:substrate ratio of 1:100. Tryptic digestion was conducted at 32 °C for at least 16 h in the dark. The digestion was stopped by acidification to pH 3 with formic acid. The peptide mixtures were loaded onto Sep-Pak tC18 cartridges (Waters), desalted and eluted with 80% acetonitrile. All peptide samples were evaporated on a vacuum centrifuge to dryness, resolubilized in 0.1% formic acid, and immediately analysed by mass spectrometry.

Gene ontology, protein-protein interaction network analysis, and data visualization

Gene ontology (GO) enrichment analysis was performed using the functional annotation clustering tool Panther, using the highest classification stringency; 155 DAVID IDs were identified and clustered. The KEGG and protein-protein interaction (PPI) networks were obtained using the functional association network resource STRING (*5*) (http://string-db.org/; version 9.1). The STRING network was created with the following parameters: H. sapiens database, confidence score (0.7); active prediction methods all enabled; no more than 10 interactions; addition of white nodes: 5. Cytoscape (version 3.0.1) was used for network visualization.

Subcellular fractionation by sequential centrifugation

Subcellular fractionation of cultured cells was carried out following an established protocol with minor modifications (*6*). Three p15 cm dishes of 90 % confluent SH-SY5Y cells were washed twice with PBS and harvested by scrapping in 2 ml of ice-cold fractionation buffer (10 mM Tris/acetic acid pH 7.0, 250 mM sucrose). The cell suspensions were first homogenized using twenty repetitive strokes in a 5 ml Dounce homogenizer, and once they became homogeneously turbid, they were passed through a 25 gauge needle attached to a 2 ml syringe four times. The obtained cell lysates were cleared by a centrifugation step of 2,000 × g for 3 min at 4 °C to remove cell debris, and the resulting supernatant was then transferred to a new pre-chilled tube and centrifuged at 4,000 × g for 2 min to pellet the plasma membrane and nuclei. The supernatant was next ultracentrifuged at 100,000 × g for 2 min at 4 °C to pellet the mitochondria, endosomes, and lysosomes, while cytoplasmic proteins remained in the supernatant. Lysosomes were next isolated from these pellets by resuspending them in five volumes of pre-chilled miliQ distilled water. Resuspension was carried out gently by pipetting for 10 min. A final centrifugation step of 100,000 × g for 2 min at 4 °C was carried out to isolate lysosomes in the supernatant while mitochondria and endosomes were in the pellet.

In vitro ubiquitination assays

Human ubiquitin and Ube1 were purchased from Boston Biochem. Neddylated Cul1/Rbx1, Cdc34, and UbcH5 were prepared as described previously (*7, 8*). Ubiquitination assays were performed at 37 °C with 400 nM UbE1, 2 μM Cdc34 or UbcH5, 0.5 μM Cul1-N8/Rbx1, 50 μM ubiquitin and 1 μM αSyn fibrils in 40 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, and 1 mM dithiothreitol. SKP1 and FBXL5 were purified from two 10 cm plates of Cos7 cells transiently transfected with SKP1-V5 and FBXL5-Flag expression vectors. SKP1/FBXL5 complexes were immunoprecipitated with anti-Flag antibodies (Sigma) and eluted from the beads using 3X Flag peptide (Sigma). The reaction mix was quenched in 8 M guanidinium chloride sample buffer, and the samples were incubated for 16 h at room temperature. Finally, the samples were diluted 1:1 in Laemmli buffer and boiled for 2 min at 95 °C, and subsequently analyzed by SDS-PAGE and immunoblotting.

Immunofluorescence analyses

For immunofluorescence analysis of cultured cells, cells were seeded onto a glass coverslip and, after transfection and/or treatment, were fixed in 4% paraformaldehyde. A short incubation with phosphate buffer containing 0.001-0.005% of Triton X-100 was included before fixation in experiments with the OC antibody. For quantitative analyses, studies were performed blindly and automated whenever possible using the ImageJ cell counting tool, and at least two non-interacting investigators confirmed quantification results. Statistical analyses (one-way ANOVA with Dunnett's *post hoc* test) were performed with the software GraphPad Prism and STATISTICA (StatSoft, v7.0). Super-resolution images were acquired on an OMX V4 microscope using a low laser intensity (< 33%) and short exposure time (from 200 ms to 5 ms). The bleaching was measured on the complete stack, and the exposure time was adjusted to minimize bleaching. Stacks were analysed using Imaris (Bitplane); fibril volume (channel 1) detection was performed for all the samples with the same settings using the "Surface" function with 80-nm precision. The sum intensity on the channel 2 was collected in the "statistic" section of the αSyn particle volume. The values were fitted and plotted using IGOR (Wavemetrics). The background intensity resulting from nonspecific signal was determined by performing an immunostaining without the primary antibody but using the secondary antibody. To evaluate the nonspecific signal of the second channel, the linear correlation between fibril volume and sum intensity of channel 2 (referred to as background) was plotted.

Immunohistochemistry

For immunohistochemistry analyses of human tissues, 5-μm sections of shock-frozen brainstem or neocortex brain tissues were cut using a cryostat, fixed with 4% paraformaldehyde in PBS, and stored in 96% ethanol at 4 °C until use. Brain slices were washed with Tris-buffered saline (TBS) buffer, blocked and then incubated at 4 °C for 18 h with antibodies against αSyn, Cul1, and/or SKP1. The samples were visualized after washing and incubation for 1 h at room temperature with appropriate Alexa-488- or Alexa-594-conjugated secondary antibodies. Images were acquired with an Olympus FluoView1000 confocal microscope using a 10X lens

at a resolution of 1024 x 1024. Six fields were analyzed in each slice. For immunohistochemistry analyses of mouse brains, formalin-fixed tissues were cut into 5 μm sections that were deparaffinized through graded alcohols, immunostained with the indicated antibodies and finally visualized using DAB (Sigma-Aldrich, St. Louis, MO, USA). Hematoxylin counterstain was subsequently applied.

SUPPLEMENTARY FIGURES

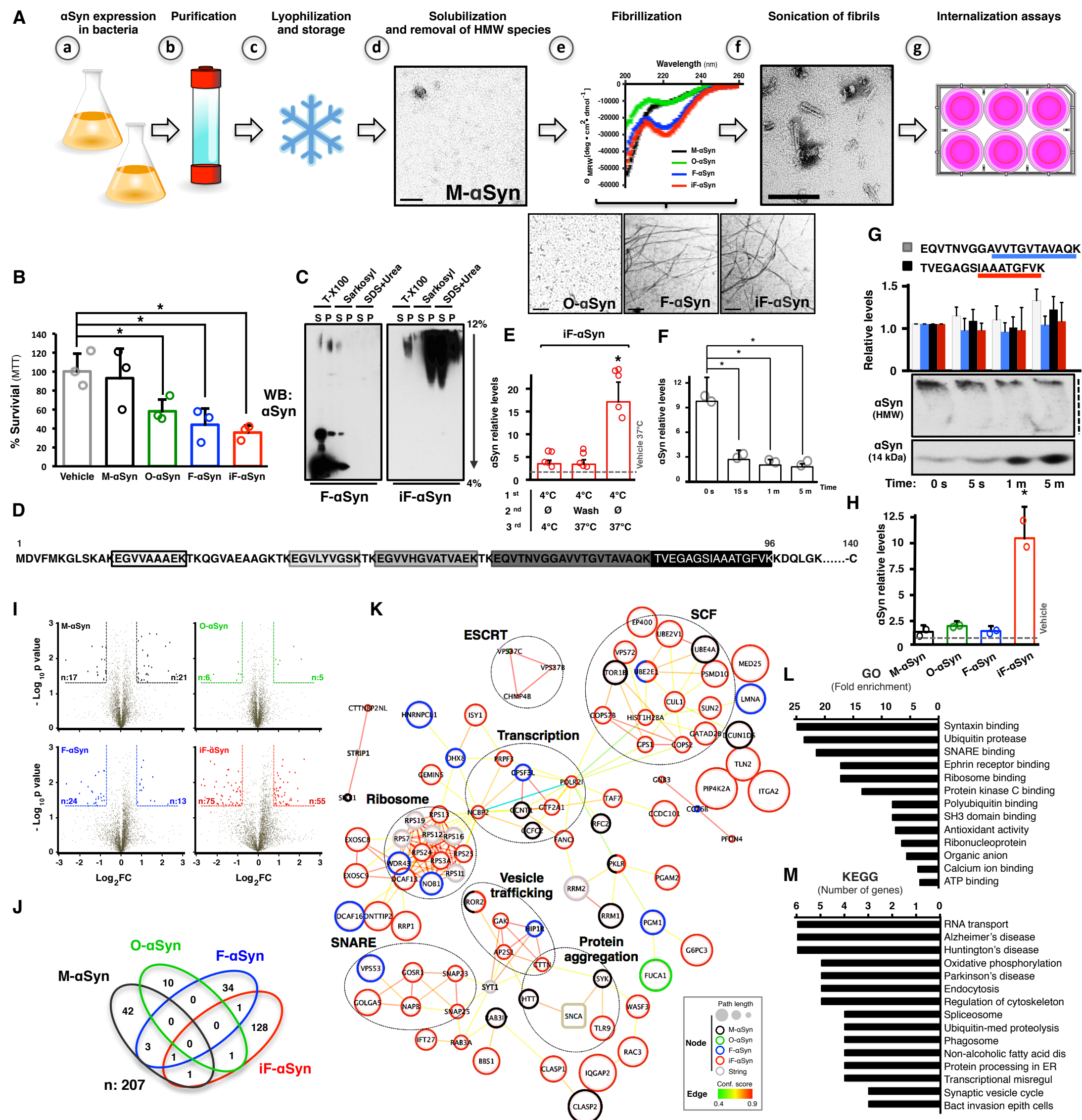
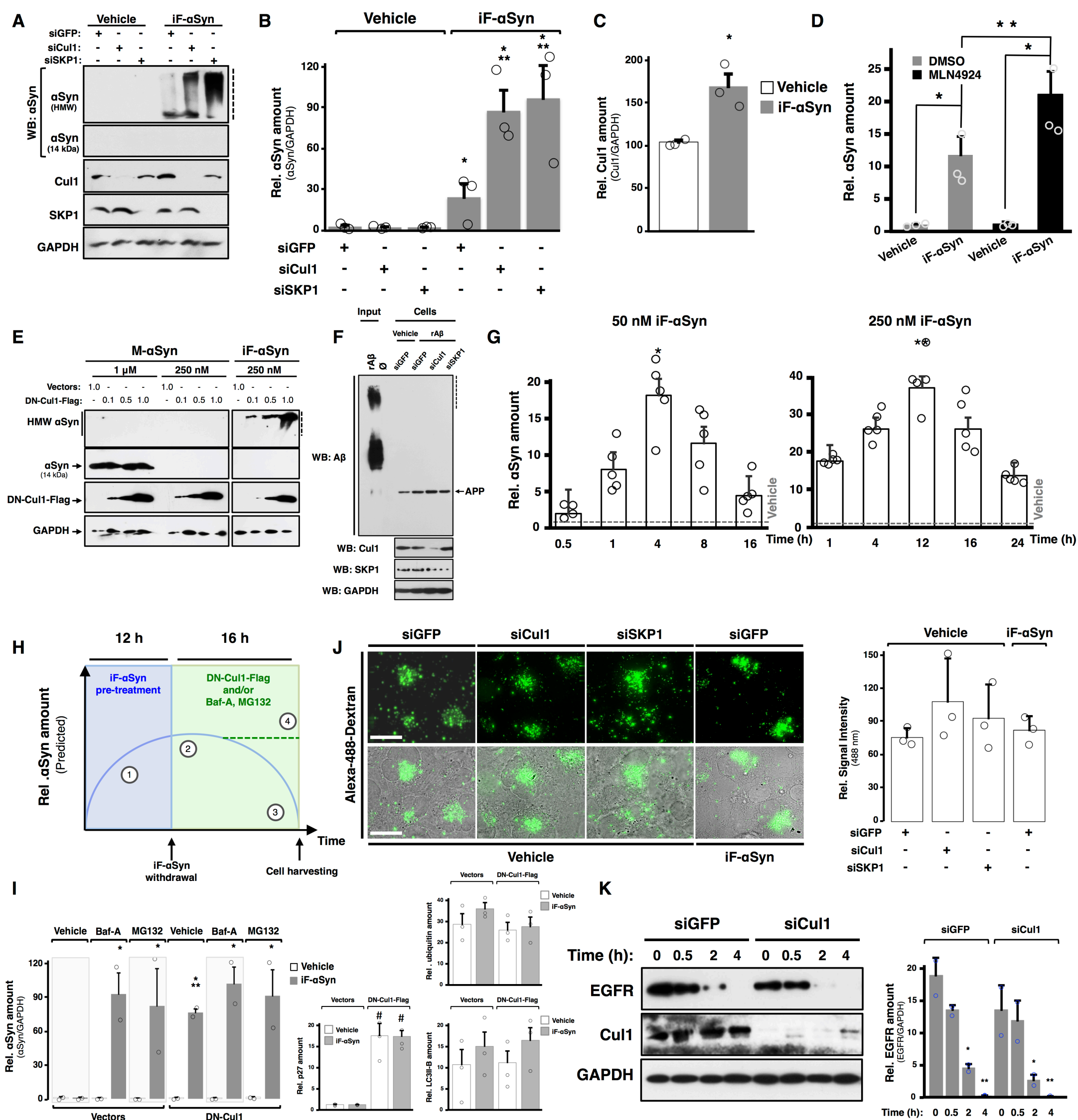


Figure S1. ***Internalization of insoluble fibrils leads to αSyn accumulation and proteome alterations.*** (**A**) Schematic representation of the workflow of the αSyn internalization experiments that includes experimental electron microscopy (d-f) and circular dichroism (e) data. (a) Recombinant αSyn is expressed in bacteria and (b) purified as previously described (see materials and methods). Pure (> 95%) αSyn was then lyophilized and stored at -20 °C (c). Monomeric αSyn (d) was obtained by dissolving the lyophilized protein in buffer following a removal of HMW pre-existing species by ultracentrifugation and filtration. αSyn oligomers and fibrils (e) were obtained by incubating 250 µM of monomeric protein in orbital agitation at 37 °C for the indicated times. With exception of fibrils, which were fragmented by sonication prior use (f), all samples were used in uptake experiments (g) immediately after the incubation. Scale bar: 100 nm. (**B**) Plot showing the cell viability of SH-SY5Y cells treated with the indicated αSyn species for 72 h. Data show the mean of three biological replicates + SD. * $p < 0.05$ compared to vehicle (one-way ANOVA followed by the *post hoc* Dunnett's test for multiple comparisons). (**C**) F-αSyn and iF-

αSyn were subjected to sequential extraction with Triton-X100, sarkosyl, and 8 M urea plus 4% SDS, and supernatants (S) and pellets (P) were analyzed by WB. (**D**) Amino acid sequence of human αSyn. The tryptic peptides quantified by SRM are contained within boxes. (**E**) Bar graph showing the amount of αSyn in cells treated with iF-αSyn and subjected to a washing step to remove non-internalized αSyn (included in all uptake experiments). In this experiment, SH-SY5Y cells were incubated at 4 °C and treated with iF-αSyn for 6 h (1st step). Non-internalized αSyn was removed from the cell cultures by this washing step (2nd step; Ø: no wash). During the third step, cells were kept at 4 °C or incubated at 37 °C for 16 h to promote αSyn internalization. Cells were then harvested and αSyn was quantified by SRM. * $p < 0.05$ indicate significant differences of 4°C / Ø / 37°C compared to the rest of the treatments. (one-way ANOVA followed by the *post hoc* Fisher's test for multiple comparisons). Red dashed line, αSyn levels in vehicle-treated cells incubated at 37 °C. (**F and G**) iF-αSyn was solubilized by a strong pulse of sonication of 5 s or 1 or 5 min, and then used to treat SH-SY5Y cells for 16 h. (**F**) Quantification of αSyn quantities by SRM. Data show the mean + SD. * $p < 0.05$ non-sonicated fibrils ($t = 0$ s) compared to the rest of the treatments (one-way ANOVA with Tukey's *post hoc* test). (**G**) Sonicated iF-αSyn was subjected to SRM analyses to quantify two tryptic peptides (black and grey), and two peptides to monitor αSyn spontaneous degradation (red and blue). WB of sonicated iF-αSyn is shown at the bottom. Dashed line, stacking gel. Note that the acute strong sonication pulse used in this experiment is > 50 times stronger than the soft prolonged sonication step applied to F- and iF-αSyn prior to cell treatment in all internalization experiments (see Fig. S1A and materials and methods). (**H**) Quantification of αSyn quantities by shotgun proteomics in cells treated with M-, O-, F- and iF-αSyn. Data show the mean + SD. * $p < 0.001$ iF-αSyn compared to the rest of the treatments (one-way ANOVA according to the Progenesis software). (**I**) The experiment of figs. 1E, F and G (proteome-wide quantification of proteins from αSyn-treated cells) was replicated in an independent experiment that also shows a more pronounced proteomic response triggered by iF-αSyn (DEPs for M-, O-, F- are 38, 11 and 37, respectively while 130 DEPs were found for iF-αSyn). The results are expressed in volcano plots using the same criteria that fig. 1E. (**J**) Venn diagram of the overlap of 207 DEPs obtained in the second independent experiment. (**K**) Protein-protein interaction network analysis of DEPs from αSyn-treated cells obtained in the second experiment. Clusters of functionally related DEPs are indicated by dashed ovals. (**L**) Gene ontology (GO) and (**M**) KEGG pathway analyses based on the STRING database. Only processes with enrichment fold > 2 and represented genes > 3 were considered significantly enriched for GO and KEGG analyses, respectively.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siGFP
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αSyn (HMW)
αSyn (14 kDa)
Cul1
SKP1
GAPDH
Rel. αSyn amount (αSyn/GAPDH)
Rel. Cul1 amount (Cul1/GAPDH)
Rel. αSyn amount
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MLN4924
M-αSyn
1 μM
250 nM
Vectors:
DN-Cul1-Flag:
HMW αSyn
DN-Cul1-Flag
Input
Cells
rAβ
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APP
WB: Cul1
WB: SKP1
WB: GAPDH
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250 nM iF-αSyn
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16 h
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Time
iF-αSyn withdrawal
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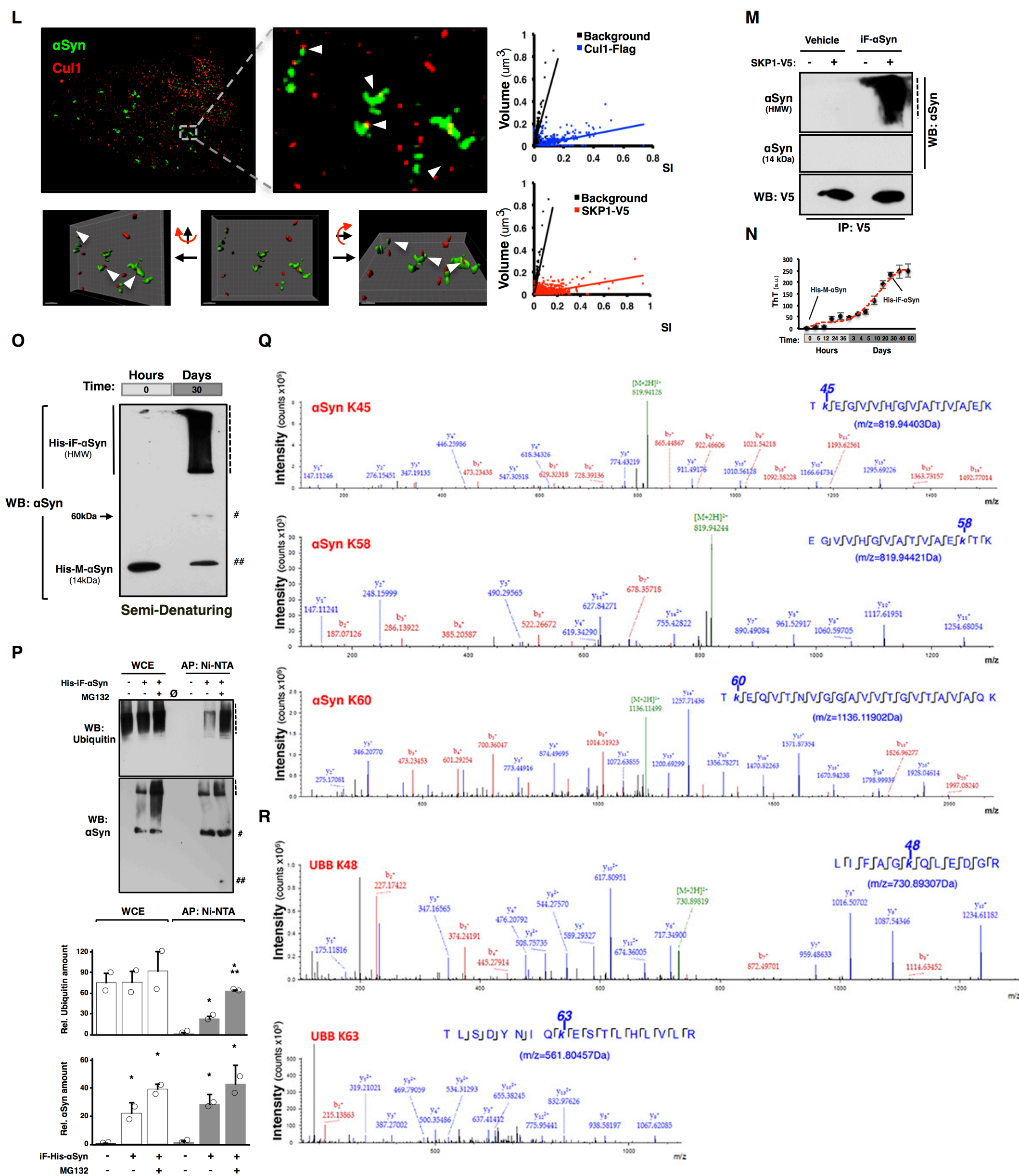
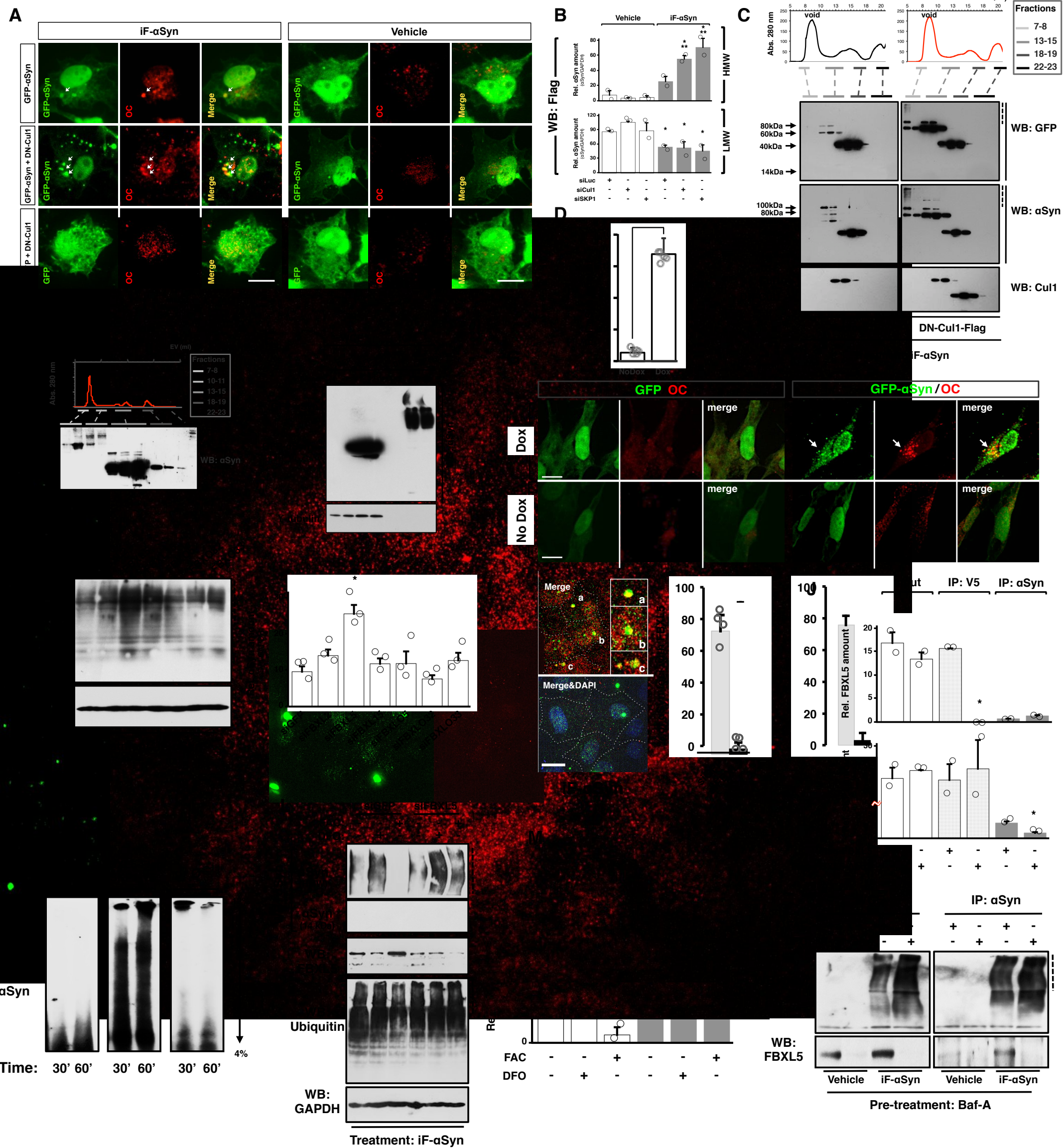


Figure S2. ***An SCF E3 ubiquitin ligase targets internalized αSyn for ubiquitination and degradation.*** (**A**) WB of Cos7 cells transfected with siRNAs specific for GFP (siGFP), Cul1 (siCul1) or SKP1 (siSKP1) and treated with iF-αSyn. (**B**) Bar graph showing the quantities of αSyn determined by WB in cells transfected with siGFP, siCul1 or siSKP1 and treated with iF-αSyn. Data show the mean + SEM. * and ** $p < 0.05$ compared to vehicle and siGFP, respectively (one-way ANOVA with Tukey's *post hoc* test). (**C**) Bar graph showing the quantities of Cul1 determined by WB in iF-αSyn-treated cells. Data show the mean + SEM. * $p < 0.05$ compared to vehicle (unpaired, two tails distribution Student's *t* test). (**D**) Bar graph showing the quantities of αSyn determined by SRM in cells treated with MLN4924 and/or iF-αSyn. Data show the mean + SD. * and ** $p < 0.05$ compared to vehicle and DMSO, respectively (one-way ANOVA followed by the *post hoc* Tukey's test). (**F**) WB of Cos7 cells transfected with empty vectors or with different amounts of the DN-Cul1-Flag expression vector. After transfection the cells were treated with 1 μM or 250 nM of M-αSyn (left) or 250 nM of iF-αSyn (right). Note that accumulation of αSyn was

observed when exogenous M-αSyn was used at 1μM but not 250 nM replicating data of fig. 1. (**G**) Bar graph showing αSyn quantities determined by SRM in HeLa cells treated with vehicle (horizontal dashed line) or 50 nM or 250 nM of iF-αSyn for the indicated times. Data show the mean + SD. * and ** $p < 0.001$ compared to the rest of the treatments (one-way ANOVA followed by the *post hoc* Dunnett's test). (**H**) Schematic representation of the experiment of fig. 2C. In this cellular model it is expected that 1) a 12 h pre-treatment with iF-αSyn allows αSyn to be internalized and to accumulate within cells; 2) after removal of extracellular αSyn, no significant αSyn uptake is expected, and therefore αSyn intracellular levels are mainly determined by the degradation rate; DN-Cul1 expression is induced and/or lysosomal degradation is inhibited at this stage; 3) internalized αSyn is degraded over 16 h; 4) if lysosome function is inhibited or SCF components are depleted, degradation of internalized αSyn is prevented leading to its accumulation (dashed line). (**I**) Bar graph of the relative amounts of αSyn (left), p27 (middle), ubiquitin and LC3II-B (right) determined by WB of the experiment of fig. 2C. Data show the mean + SEM. * and ** $p < 0.05$ compared to vehicle or vectors and DN-Cul1, respectively (unpaired Student's *t* test). (**J**) Microscopy images of Cos7 cells transfected with siGFP, siCul1, or siSKP1 and treated with extracellular AlexaFluor-488-labeled dextran. Lower panels show the merged fluorescence and bright fields. On the right is a bar graph showing the signal intensity at 488 nm. Data show the mean + SEM. No significant differences were observed according to one-way ANOVA followed by the *post hoc* Tukey's test. (**K**) WB of Cos7 cells transfected with siGFP or siCul1 and treated with EGF for the indicated time points. On the right is shown the bar graph of the relative amounts of EGFR determined by WB. Data show the mean + SEM. * and ** $p < 0.05$ and $p < 0.01$ compared to siGFP (unpaired Student's *t* test). (**L**) Immunofluorescence analyses using super-resolution microscopy of Cos7 cells expressing Cul1-Flag (in red) and treated with iF-αSyn. 3D reconstruction analysis is shown in the bottom. Arrows indicate co-localization of αSyn and Cul1. On the left is plotted the correlation of the volume of internalized αSyn particles (y-axis) and the sum of the intensities (SI, x-axis) of the background (black) or Cul1-Flag (blue). Data from a similar experiment with cells expressing V5-SKP1 (red) is also shown. Scale bar: 10 nm. (**M**) WB of immunoprecipitates from Cos7 cells transfected with an empty vector (-) or an SKP1-V5 expression vector (+) and treated iF-αSyn. Immunoprecipitation was carried out with anti-V5 antibodies. (**N and O**) Recombinant 6XHis-tagged αSyn was incubated for the indicated times and analyzed by thioflavin-T (**N**) and WB (**O**). (**P**) WB of WCE and affinity purified αSyn obtained from Cos7 cells treated with His-iF-αSyn. Quantification of the blots is shown on the right. Data show the mean + SEM. * and ** $p < 0.05$ compared to vehicle and MG132, respectively (one-way ANOVA followed by the *post hoc* Tukey's test). (**Q**) MS/MS spectra of αSyn di-glycine (GG)-modified tryptic peptides embedding K45/K58/K60 are shown. The sequence of each tryptic precursor peptide, the modified lysine, and the precursor m/z value are indicated. (**R**) MS/MS spectra of ubiquitin di-glycine (GG)-modified tryptic peptides embedding K48 and K63 are shown. The sequence of each tryptic precursor peptide, the modified lysine, and the precursor m/z value are indicated. Dashed lines, stacking gels. # and ## indicate αSyn-immunoreactive bands of 15 and 60 kDa respectively.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Vehicle
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Vehicle
iF-αSyn
WB: Flag
HMW
LMW
siLuc
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siSKP1
C
Fractions
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13-15
18-19
22-23
void
EV (ml)
WB: GFP
WB: αSyn
WB: Cul1
DN-Cul1-Flag
80kDa
60kDa
40kDa
14kDa
100kDa
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merge
Dox
No Dox
Merge
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IP: αSyn
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Pre-treatment: Baf-A

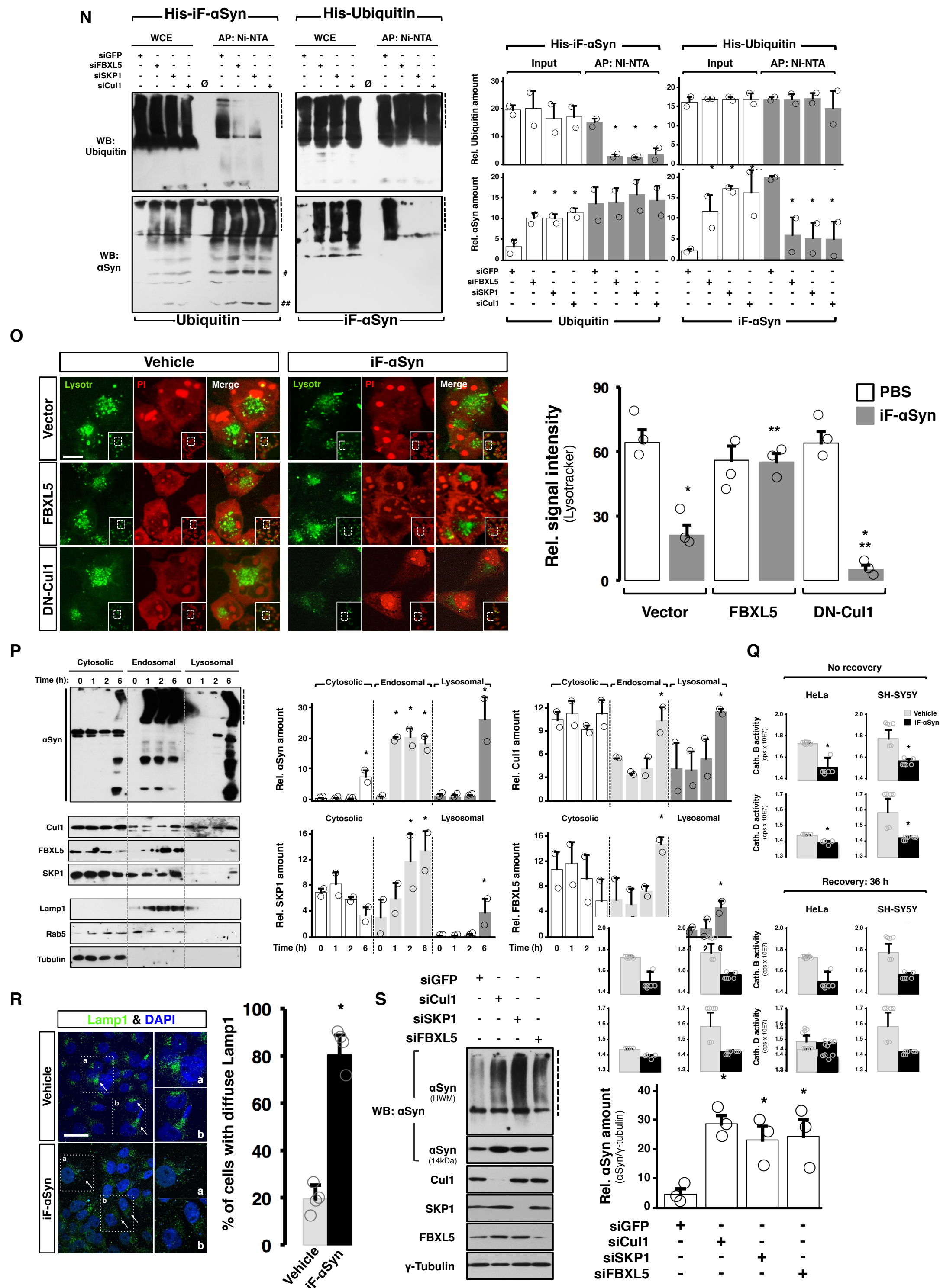

Figure S3. ***An SCF inhibits the prion-like properties of extracellular αSyn*****.** (**A**) Immunofluorescence and confocal microscopy analyses of Cos7 cells transfected with GFP or GFP-αSyn and/or DN-Cul1 and treated with iF-αSyn. Scale bar 10 µm. (**B**) Bar graph showing the quantities of Flag-tagged GFP-αSyn determined by WB in cells transfected with siLuc, siCul1 or siSKP1 and treated with iF-αSyn. HMW and LMW, upper and lower part of the gel, respectively. Data show the mean + SEM. * and ** $p < 0.05$ compared to vehicle and siLuc, respectively (one-way ANOVA

followed by the *post hoc* Fisher's test). (C) Flag-GFP-αSyn-expressing HeLa cells were transfected with an empty vector (Vectors) or a DN-Cul1-Flag expression vector. After transfection the cells were treated with iF-αSyn for 16 h and whole cell lysates were fractionated by size exclusion chromatography (SEC). Fractions containing soluble monomers and small oligomers (fractions 17-19), partially insoluble HMW oligomers (fractions 13-15), and insoluble HMW αSyn (fractions 7 and 8) were analyzed by WB. EV, elution volume. (**D to G**) Characterization of SH-SY5Y cell clones stably expressing Flag-GFP or Flag-GFP-αSyn. (**D**) Bar graph showing the amount of αSyn determined by SRM in non-induced (NoDox) and induced (Dox) cells. The results are expressed as mean + SD. * $p < 0.01$ (unpaired Student's *t* test). (**E**) SEC profile of the GFP-Flag-αSyn expressed in SH-SY5Y cell clones. The indicated fractions were analyzed by WB. (**F**) WB of whole cell extracts (WCE) and conditioned media (CM) from cell clones stably expressing GFP or GFP-αSyn. The cytoplasmic protein gamma-tubulin was analyzed as control of CM. (**G**) Representative confocal microscopy analysis of GFP- or GFP-αSyn-expressing SH-SY5Y cell clones. Immunofluorescence was carried out with the conformational antibody OC. Scale bar: 10 μm. Arrow: foci containing GFP-αSyn. (**H**) WB of Cos7 cells transfected with the indicated siRNAs and treated with iF-αSyn. Quantification of the blots is shown on the right. Data show the mean + SEM. * $p < 0.001$ compared to siGFP (one-way ANOVA followed by the *post hoc* Dunnett's test). (**I**) Immunofluorescence and confocal microscopy analyses of control and FBXL5-depleted Cos7 cells treated with iF-αSyn. Number of foci containing both αSyn and FBXL5 in control siGFP- and siFBXL5-treated cells is shown on the right. Data show the mean + SD. * $p < 0.01$ compared to siGFP (unpaired Student's *t* test). Scale bar: 10 μm. (**J**) In vitro ubiquitination assays using immobilized Flag-FBXL5/SKP1-V5 complexes, obtained from transiently-transfected Cos7 cells treated with vehicle (see Fig. 3F). Reaction mixtures contained sonicated iF-αSyn as substrate and recombinant ubiquitin, E1, E2 and neddylated Cul1 as enzymatic source. Samples were analyzed by WB using anti-αSyn antibodies. Sonicated iF-αSyn and a reaction mix in which Flag-FBXL5/SKP1-V5 complexes were omitted (Ø) are also shown. (**K**) WB of immunoprecipitates from SH-SY5Y cells pre-treated with Baf-A and either FAC or DFO and subsequently treated with iF-αSyn. Immunoprecipitation was carried out using anti-αSyn antibodies. (**L**) WB of control or FBXL5-depleted Cos7 cells pre-treated with FAC or DFO and subsequently exposed to iF-αSyn. Quantification of the blots is shown on the right. Data show the mean + SEM. * and ** $p < 0.05$ compared to untreated cells and cells transfected with siGFP, respectively (one-way ANOVA followed by the *post hoc* Fisher's test). (**M**) Quantification of the blots of fig. 3E. Data show the mean + SEM. * $p < 0.01$ compared to Flag-FBXL5 (unpaired Student's *t* test). (**N**) WB of either 6XHis-tagged αSyn or 6XHis-tagged ubiquitin purified under denaturing conditions from cells treated with His-iF-αSyn (left) or iF-αSyn and expressing His-ubiquitin (right). # and ##, αSyn immunoreactive bands of 15 and 60 kDa, respectively. Quantification of the blots is shown on the right. Data show the mean + SEM. * $p < 0.05$ compared to siGFP (one-way ANOVA followed by the *post hoc* Fisher's test). (**O**) Confocal microscopy analyses of Cos7 cells expressing FBXL5 or DN-Cul1 and treated iF-αSyn. Cells were stained with Lysotracker to visualize acidic vesicles whereas propidium iodide (PI) was used to visualize cell morphology. Quantification of the signal intensity at 488 nm is shown on the right. Data show the mean + SEM. * and ** $p < 0.05$ compared to PBS and vector (one-way ANOVA followed by the *post hoc* Fisher's test). (**P**) WB of cytoplasm-, endosome-, and lysosome-enriched fractions obtained from SH-SY5Y cells treated with iF-αSyn

for the indicated times. Quantification of the blots is shown on the right. Data show the mean + SEM. * $p < 0.05$ compared to time = 0 (one-way ANOVA followed by the *post hoc* Fisher's test). (**Q**) HeLa and SH-SY5Y cells were treated with iF-αSyn for 12 h and then harvested (No recovery) or incubated in media devoid of αSyn for 36 h and then harvested (Recovery 36 h). Activity levels of cathepsin B (upper panel) and cathepsin D (lower panel) were determined in whole cell lysates immediately after harvesting. Data show the mean + SD. * $p < 0.05$ compared to vehicle (unpaired Student's *t* test). Cps, counts per seconds. (**R**) Confocal microscopy of BV-2 cells treated with vehicle or iF-αSyn and immunostained for Lamp1. DAPI was used to stain nuclei. Data show the mean + SD. * $p < 0.05$ compared to vehicle (unpaired Student's *t* test). (**S**) WB analyses of SH-SY5Y cells overexpressing human αSyn and depleted of Cul1, SKP1 or FBXL5. Quantification of the blots is shown on the right. Data show the mean + SEM. * $p < 0.05$ compared to vehicle (one-way ANOVA followed by the *post hoc* Tukey's test). Dashed lines, stacking gels.

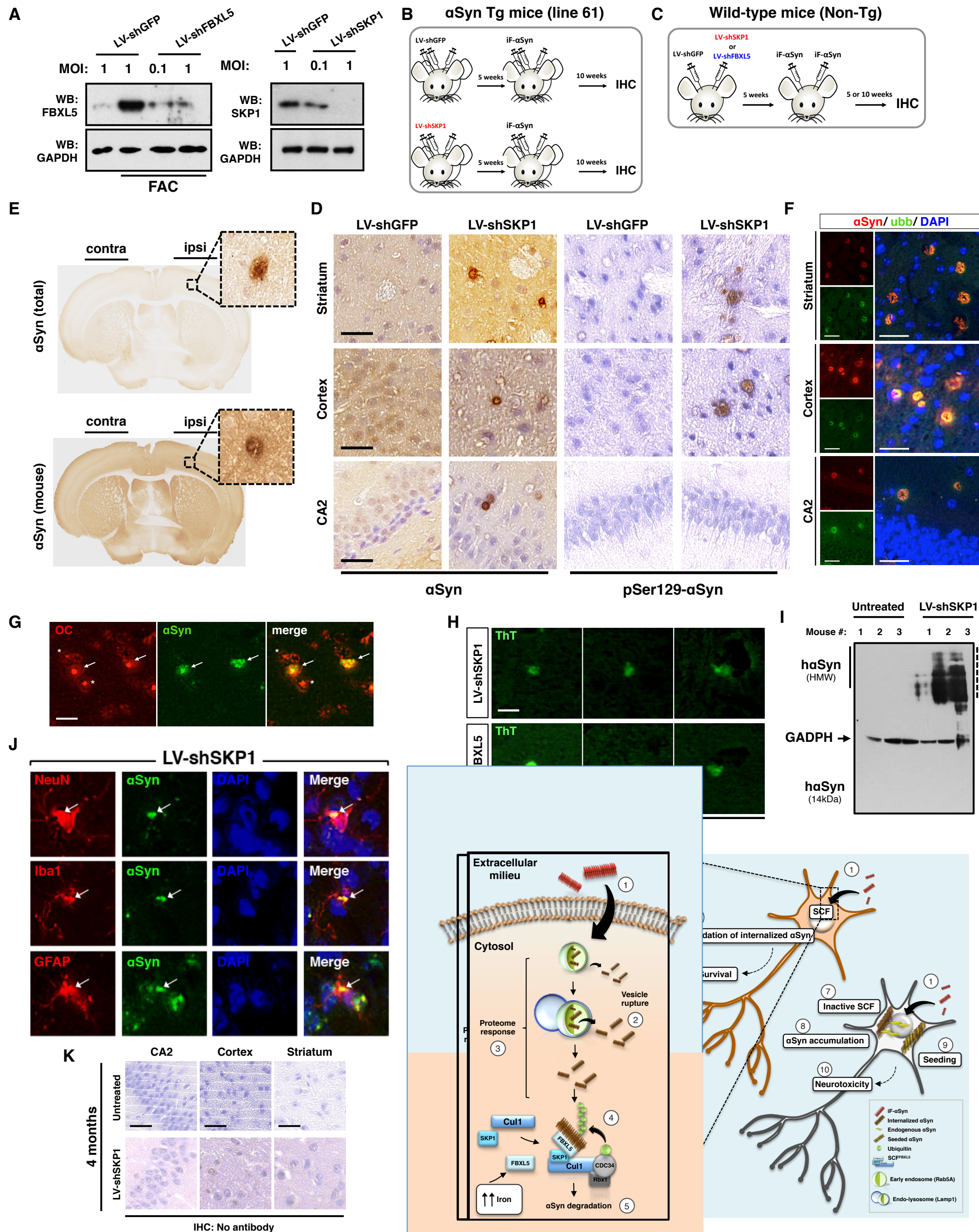

Figure S4. ***SCF^{FBXL5} inhibits L*** ***d by extracellular αSyn fibrils.*** (**A**) Mouse neuroblastoma ed with LV-shSKP1 and LV-shFBXL5 to test efficacy of sil was used as a negative control. MOI, multiplicity of infection. FAC, ferric ammonium citrate. (B and C) Schematic representation of the experimental design used to evaluate SCF^{FBXL5} effect on LB-like pathology induced by extracellular αSyn fibrils in (**B**) αSyn transgenic and (**C**) non-transgenic wild-type mice. (**D**) Representative coronal section of brains of wild-type mice injected contralaterally (contra) with LV-shGFP (*n=6*) and ipsilaterally (ipsi) with LV-shFBXL5 (*n=6*) followed by bilateral administration of iF-αSyn. Sections were stained with antibodies specific for total (top) or mouse-specific (bottom) αSyn. (**E**) Representative images of paraffin-embedded sections of striatum, cortex, and

CA2 of wild-type mice injected with LV-shGFP (*n=6*) and LV-shSKP1(*n=6*) (left and right hemisphere, respectively) and subsequently with iF-αSyn. Sections were subjected to immunohistochemistry analyses at 10 weeks using αSyn and pSer129-αSyn antibodies. Scale bar: 25 μm (**F and G**) Immunofluorescence analyses of brain sections of LV-shSKP1-injected mice using (**F**) human αSyn and (**F**) ubiquitin or (**G**) OC antibodies. (**H**) Brain slices of animals injected with LV-shSKP1 and LV-shFBXL5 were stained with thioflavin-T (ThT). Scale bar 10μm. (**I**) WB analyses of brain lysates of control (untreated) and LV-shSKP1-injected mice (*n=3*). (**J**) Immunofluorescence analyses of brain sections of LV-shSKP1-injected mice using human αSyn and NeuN (upper), Iba1 (middle) or GFAP (lower) antibodies. (**K**) Immunohistochemistry analyses of untreated and the same animal of Fig. S4D (injected with LV-shSKP1 and iF-αSyn injected) showing LB-like αSyn and αSyn ^{pSer129}-positive inclusions. In this experiment the primary antibody was omitted in order to confirm the specificity of the staining. (**L**) Hypothetical model of SCF^{FBXL5} action on internalized αSyn and LB pathogenesis and spread. *Left panel:* αSyn fibrils are taken up from the extracellular milieu by endocytosis (**1**). Upon vesicle rupture (**2**), αSyn is released to the cytoplasm (**3**), triggering an orchestrated proteome response involving SCF ubiquitin ligases, among others (**3**). SCF^{FBXL5}, which is stabilized by high cellular iron levels, mediates αSyn ubiquitination (**4**). Finally, ubiquitinated αSyn is degraded (**5**). *Right panel:* (**6**) In healthy neurons (orange), internalized αSyn is efficiently degraded by SCF^{FBXL5}, which plays a protective role against αSyn-mediated neurotoxicity. A pathological process might arise from impaired SCF^{FBXL5} activity (**7**), leading to αSyn accumulation (**8**), seeded aggregation of endogenous αSyn and its recruitment into newly formed LBs (**9**), and neurotoxicity (**10**).

Table S4. Gene ontology enrichment analysis according to the Panther database (GO molecular function, experimental). DEPs from SH-SY5Y cells treated with iF-αSyn were used for the analysis. Only biological processes with a p value < 0.05 were considered as significantly enriched.

Biological process terms	P value	Fold enrichment
Syntaxin binding	1.06E-06	26
Ubiquitin-specific protease binding	3.24E-03	25
SNARE binding	3.28E-06	23
Ephrin receptor binding	5.91E-03	18
Ribosome binding	5.91-03	18
Protein kinase C binding	8.38E-03	14
Polyubiquitin binding	2.33E-02	9
SH3 domain binding	2.33E-02	9
Antioxidant activity	2.62E-02	8
Ribonucleoprotein complex binding	3.55E-02	7
Organic anion transmembrane transporter activity	4.41E-02	6

Table S5. KEEG pathway enrichment analysis according to the STRING database. DEPs from SH-SY5Y cells treated with iF-αSyn were used as input. Only processes with a p value < 0.05 and number of genes > 3 were considered significantly enriched.

	Biological process terms	P value	Number of genes
1			
	GO:1100 – Metabolic pathways	1.82E-01	12
2			
	GO:3013 – RNA transport	1.11E-03	6
	GO:5010 – Alzheimer's disease	1.69E-03	6
	GO:5016 – Huntington's disease	2.77E-03	6
3			
	GO:190 – Oxidative phosphorylation	3.31E-03	5
	GO:5012 – Parkinson's disease	4.40E-03	5
	GO:4144 – Endocytosis	1.64E-02	5
	GO:4810 – Regulation of actin cytoskeleton	2.23E-02	5
4			
	GO:3040 – Spliceosome	1.54E-02	4
	GO:4120 – Ubiquitin-mediated proteolysis	1.99E-02	4
	GO:4145 – Phagosome	2.40E-02	4
	GO:4932 – Non-alcoholic fatty liver disease (NAFLD)	2.68E-02	4
	GO:4141 – Protein processing in the endoplasmic reticulum	3.64E-02	4
	GO:5203 – Transcriptional regulation in cancer	3.78E-02	4
5			
	GO:4721 – Synaptic vesicle cycle	1.10E-02	3
	GO:5100 – Bacterial invasion of epithelial cells	1.86E-02	3
	GO:3008 – Ribosome biogenesis in eukaryotes	1.86E-02	3
	GO:4260 – Cardiac muscle contraction	2.00E-02	3
	GO:4666 – FC gamma R-mediated phagocytosis	2.94E-02	3
	GO:240 – Pyrimidine metabolism	4.18E-02	3