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SUPPLEMENTARY ONLINE DATA

Identification of protein phosphatase 1 as a regulator of the LRRK2 phosphorylation cycle

Evy LOBBESTAEL*[1], Jing ZHAO†[1], Iakov N. RUDENKO‡, Aleksandra BEYLINA‡, Fangye GAO*, Justin WETTER§, Monique BEULLENS‖, Mathieu BOLLEN‖, Mark R. COOKSON‡, Veerle BAEKELANDT*, R. Jeremy NICHOLS†[1,2,3] and Jean-Marc TAYMANS*[1,2]

*Laboratory for Neurobiology and Gene Therapy, Department of Neurosciences, KU Leuven, Kapucijnenvoer 33, 3000 Leuven, Belgium, †The Parkinson's Institute, 675 Almanor Ave, Sunnyvale, CA 94085, U.S.A., ‡Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, 35 Convent Drive, Bethesda, MD 20892, U.S.A., §Primary and Stem Cell Systems, Life Technologies Corporation, 501 Charmany Drive, Madison, WI 53719, U.S.A., and ‖Laboratory for Biosignaling and Therapeutics, Department of Cellular and Molecular Medicine, KU Leuven, O&N I Herestraat 49 - box 901, 3000 Leuven, Belgium

MATERIALS AND METHODS

Constructs, antibodies and reagents

PP1α and PP1γ were cloned from PlasmidID clones HsCD00004106 and HsCD00005169. PP1β was cloned from PlasmidID clone HsCD00000467, the sequence has an L51P mutation, and was corrected to WT using the GeneArt Site-Directed Mutagenesis System (Invitrogen). PP2Aα was cloned from Addgene plasmid 10689 [1]. All of the constructs were inserted into the pcDNA5-FRT/TO backbone with an N-terminal FLAG tag. Constructs of truncated NIPP1 ($NIPP1_{143–224}$, residues 143–224) and I2 with N-terminal tags of eGFP and CFP respectively are described in [2]. 3× FLAG–LRRK2 constructs are described by Daniëls et al. [3]. eGFP-tagged constructs of LRRK2 were generated by cleaving out the 3× FLAG tag and ligating an eGFP sequence derived from the pEGFP-C1 plasmid. N-terminal eGFP-tagged LRRK2 used for the T-Rex cell line was cloned into the pcDNA5-FRT/TO backbone for generation of T-REx cell lines. All constructs were confirmed by restriction digest analysis and sequencing. Recombinant protein phosphatases were purchased from Calbiochem (EMD Millipore Chemicals). LRRK2 was detected with the anti-LRRK2 antibody N138/6 (Neuromab) or monoclonal rabbit anti-LRRK2 antibody MJFF-2 (clone C41-2, Epitomics). The immunoprecipitation of endogenous LRRK2 was performed with a monoclonal rabbit antibody directed against LRRK2 residues 100–500 (5097-5091; Epitomics). Sheep anti-LRRK2 $pSer^{910}$ (S357C) was used as described in [4] and monoclonal rabbit anti-$pSer^{910}$ and $pSer^{935}$ co-developed by the Michael J. Fox Foundation, Epitomics and University of Dundee were described in [5], polyclonal rabbit anti-LRRK2 $pSer^{935}$, $pSer^{955}$ and $pSer^{973}$ are described in [6]. FLAG M2 antibody was purchased from Sigma–Aldrich, eGFP antibody was produced in-house [7], from Roche (clones 7.1 and 13.1) or rabbit monoclonal anti-GFP from LifeTechnologies was used for PLAs. Mouse monoclonal anti-pan-PP1 antibody was described previously in [8] and anti-PP1α antibody was from Santa Cruz Biotechnology, Life Technologies (clone 10C6-10C3) or Cell Signaling Technologies. Anti-β-tubulin antibody was purchased from Sigma. Rat anti-pan-tubulin antibody was purchased from Santa Cruz Biotechnology. LiCOR DyeLight labelled 14-13-3 protein (recombinant His-tagged BMH1&2, a gift from Professor Dario Alessi, MRC Protein Phosphorylation and Ubiquitylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 5EH, U.K.) was prepared according to the manufacturer's instructions and used to detect 14-3-3 binding as described previously [6,9], calyculin A was obtained from Enzo Life Sciences, okadaic acid was purchased from Merck and LRRK2-IN1 was used as described in [10]. GFP-Trap Agarose was purchased from Chromotek.

Isolation and culture of primary neurons

Primary cortical neurons were prepared from newborn (P_0; post-natal day zero) pups (C57BL/6J). Neuronal cells were dissociated by papain (Worthington), plated on to 6-well plates precoated with poly-D-lysine (BD Biosciences) at a density of 2.5×10^6 cells/well in Basal Medium Eagle (Sigma–Aldrich) supplemented with B27, N2, glutaMAX-I, penicillin/streptomycin (Invitrogen) and 0.45% glucose (Sigma–Aldrich). The medium was replaced by half every 2 days. Cytosine arabinoside (3 μM; Sigma–Aldrich) was added for the first 3 days to inhibit glial cell proliferation. At 14 DIV (days in vitro), neurons were treated for 30 min with 20 or 100 nM calyculin A, 100 nM okadaic acid, 1 μM LRRK2-IN1, 20 nM calyculin A and 1 μM LRRK2-IN1 or DMSO alone. Cells were collected and spun down at 16000 g for 10 min. The pellet was lysed in 30 μl of buffer containing 10 mM Tris/HCl, 2% SDS, protease inhibitors cocktail (Roche), and HALT phosphatase inhibitor cocktail (Thermo Scientific), sonicated and subjected to immunoblot analysis. All mouse work followed the guidelines approved by the Institutional Animal Care and Use Committees of the National Institute of Child Health and Human Development.

In vitro dephosphorylation

For the dephosphorylation of radioactively labelled LRRK2, HEK-293T cells expressing 3× FLAG–LRRK2 were grown to confluence in 10-cm-diameter petri dishes then metabolically labelled as described above. Following labelling, cells were lysed and 3× FLAG–LRRK2 was purified as described above. FLAG-M2 beads holding the purified 3× FLAG–LRRK2 were rinsed in dephosphorylation buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT and 0.02% Triton) and protein was eluted using 3× FLAG peptide (0.1 mg/ml, Sigma). Protein

[1] These authors contributed equally to this study.
[2] Correspondence may be addressed to either of these authors (email jnichols@parkinsonsinstitute.org or jean-marc.taymans@med.kuleuven.be).
[3] R. Jeremy Nichols is a consultant for LifeTechnologies Corporation.

(approximately 10 μg/ml) was incubated with a panel of recombinant phosphatase catalytic subunits (PP1α, $PP2A_1$, $PP2A_2$, PP2B, PP2Cα and PPλ, all from Calbiochem, Merck Millipore) at the limiting concentration of 12.5 units per ml, as well as an alkaline phosphatase control (250 units/ml) (Fermentas, Thermo Scientific) for 30 min at 30 °C. Reactions were stopped by the addition of SDS loading dye. Samples were resolved on Nupage 3–8 % Tris-acetate gels. Incorporated ^{32}P was detected by autoradiography using a Storm 840 phosphorescence scanner (GE Healthcare). The same membranes were stained with Ponceau S (Sigma) to correct for protein loading and probed with the monoclonal rabbit anti-LRRK2 antibody MJFF-2 to confirm the presence of LRRK2.

Co-immunoprecipitation (co-IP)

Per 10-cm-diameter dish of T-REx HEK-293 cells overexpressing GFP–LRRK2, cells were washed once with PBS and lysed in situ with 500 μl of lysis buffer (50 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.27 M sucrose, 1 mM benzamidine and 1 mM PMSF and was supplemented with 1 % Nonidet P40) on ice, then centrifuged at 15000 *g* at 4 °C for 15 min. Protein concentrations were determined using the Bradford method with BSA as the standard. Cell lysates were subjected to immunoprecipitation with GFP-Trap agarose beads at 5 μl beads per 500 μl lysate for 1 h at 4 °C. Beads were washed twice with lysis buffer supplemented with 300 mM NaCl, then twice with buffer A (50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.1 mM EGTA and 0.27 M sucrose). Immune complexes were incubated in NuPAGE LDS (lithium dodecyl sulphate) sample buffer at 70 °C for 10 min, then boiled for 5 min.

Confluent dishes of A549 cells were treated with 2 μM LRRK2-IN1 for 90 min, or 20 nM calyculin A for 30 min. Then cell lysates were prepared and subjected to immunoprecipitation as described above for GFP-Trap with 6 μg/10-cm-diameter dish of anti-LRRK2 100–500 (Epitomics) or rabbit IgG (Bethyl) for 2 h at 4 °C.

Gel electrophoresis and immunoblotting

The protein content of cell lysates was determined using the BCA protein determination assay (Pierce Biotechnology) or the Bradford method. Cell lysates were boiled and resolved by electrophoresis on a NuPage 3–8 % tris-acetate gradient gel, 4–12 % Bis-Tris gradient gel or 12.5 % SDS gel. Separated proteins were transferred on to a PVDF membrane (Bio-Rad Laboratories) or Protran Nitrocellulose membrane (Whatman, GE Healthcare) and non-specific binding sites were blocked for 30 min in PBST (PBS supplemented with 0.1 % Triton X-100) or TBST (Tris-buffered saline with 0.1 % Tween 20) and 5 % (w/v) non-fat dried skimmed milk powder or 5 % BSA. After overnight incubation at 4 °C with the appropriate antibodies, blots were washed three times with PBST. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako) for 1 h, blots were washed again as mentioned before. Bands were visualized using ECL (GE Healthcare) or LI-COR dual probes (Lincoln). To normalize the signal of phospho-specific antibodies to LRRK2 expression levels, blots were stripped after detection of the phospho-specific signal and reprobed with anti-LRRK2 antibody. To strip antibodies from a blot, the blot was incubated with methanol for 30 s, followed by two wash steps of 5 min with distilled water and two wash steps with PBST. Next, the blot was incubated with stripping buffer (62.5 mM Tris/HCl, pH 6.8, 2 % SDS and 100 mM 2-mercaptoethanol) for 30 min at 70 °C, followed by two 10 min wash steps with PBST. Immunodetection on stripped blots was performed as described above. Densitometric analysis of the bands on the blot autoradiograms and immunoreactivity were performed using Aida Analyzer v1.0 (Raytest) or Odyssey infrared imaging system (Lincoln).

TR-FRET LanthaScreen Cellular assay for LRRK2 $pSer^{935}$

LanthaScreen $pSer^{935}$ assays were performed essentially as described previously [11]. In brief, U2OS cells were transduced with 20 % BacMam LRRK2–GFP virus overnight. Cells were replated at 10000 cells per well in 384 well plates. The cells were either left untreated or treated with 0.093, 1 or 10 μM LRRK2-IN1 inhibitor or in the presence of the indicated dilution series of calyculin A for 30 min. Cells were directly lysed in the presence of terbium-labelled $pSer^{935}$ antibody and, after 3 h incubation, TR-FRET ratios for terbium and GFP were read on a PerkinElmer EnVision Plate Reader.

Imunocytochemistry and proximity ligation assay

For immunocytochemistry, cells were grown on coverslips and washed with PBS and fixed with 4 % paraformaldehyde for 15 min, followed by two wash steps with PBS. After permeabilization for 5 min with PBST, a 30 min blocking step with 10 % goat serum (Dako cytomation) in PBS was performed. This was followed by a 2 h incubation with FLAG M2 and in-house anti-eGFP antibody in PBS. After three washes with PBS for 5 min, the cells were incubated for 1 h with secondary antibody (Alexa Fluor®-conjugated antibody, 1:500 dilution, Molecular Probes) and washed three times for 5 min with PBS. The coverslips were mounted on a microscope slide with Mowiol (Sigma) supplemented with DAPI (Roche). Visualization was performed by confocal laser-scanning microscopy (Fluoview 1000, Olympus) using a 100 × lens.

PLAs were performed on eight-well CC2 chamber slides. Cells were fixed and permeabilized as in the immunocytochemistry experiments, then blocked with Duolink Blocking Solution for 30 min at 37 °C. Immediately after blocking, the cells were incubated with the indicated primary antibodies in the Duolink Antibody Diluent for 1 h at room temperature (20–24 °C). The cells were washed twice with Duolink Wash Buffer A for 5 min before the incubation of the PLUS and MINUS PLA probes. The cells were incubated with PLA probes for 1 h at 37 °C, followed by two washes with Wash Buffer A for 5 min. The cells were then incubated with pre-mixed Ligation-Ligase solution at 37 °C for 30 min. After two washes with Wash Buffer A for 2 min, the cells were incubated with pre-mixed Amplification-Polymerase solution for 60–90 min at 37 °C. Finally, the cells were washed twice with Duolink Wash Buffer B for 10 min, followed by 0.01 × Wash Buffer B for 1min. The slides were dried in the dark and mounted with Duolink II Mounting Medium. Images were quantified with the Duolink Image Tool Software (Version 1.0.1.2).

Quantification of skein-like structures

HEK-293FT cells (Invitrogen) were seeded on coverslips and transfected with 3 × FLAG–LRRK2 plasmids for WT and series of mutants: S910A/S935A, R1441C, R1441G, Y1699C, K1906M, G2019S and I2020T. At 24 h after transfection cells were treated with DMSO, 1 μM LRRK2-IN1, 5 nM calyculin A, or 1 μM LRRK2-IN1 and 5 nM calyculin A for 30 min at

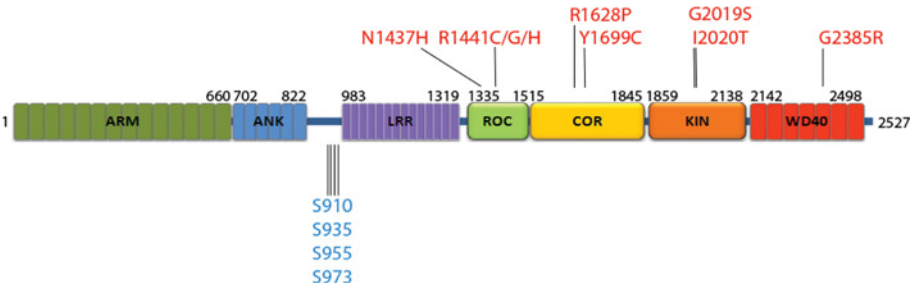

Figure S1 Schematic representation of the LRRK2 domain structure

Cellular phosphorylation sites reported by at least two independent studies (reviewed in [12]) and investigated in the present study are depicted in blue, pathogenic LRRK2 mutations or risk factors are shown in red.

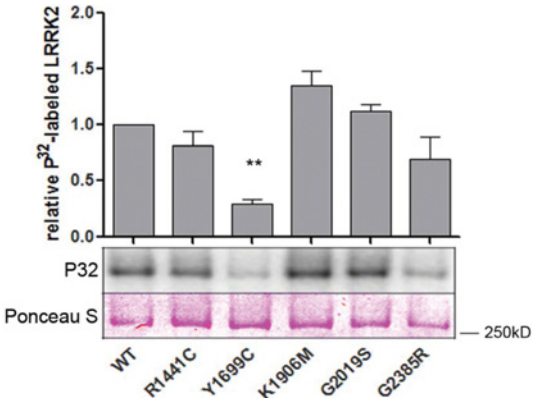

Figure S2 LRRK2 mutants show decreased metabolic labelling

Metabolic labelling of WT LRRK2 and mutations R1441C, Y1699C, K1906M, G2019S and G2385R ^{32}P values were controlled for expression level and normalized to WT LRRK2 values. Results are consistent with reduced phosphorylation levels at Ser^{910} and Ser^{935} as previously reported (see text for more details). Representative blots are shown (with molecular mass indicated in kDa) and the histogram shows quantifications of four independent experiments, results are means ± S.E.M. $^{**}P < 0.01$.

37 °C. Immediately after treatment, cells were fixed with 4 % paraformaldehyde for 20 min, permeabilized with 0.1 % Triton X-100 for 10 min and stained with primary anti-FLAG M2 antibody (2 ng/μl final concentration) and secondary anti-mouse Alexa Fluor® 488-conjugated antibody (1:500 dilution, Invitrogen). Nuclei were stained with TO-PRO-3 iodide (Invitrogen). A total of 100–150 LRRK2-transfected cells per sample were counted over multiple fields on a 100 × objective for the presence of skein-like structures.

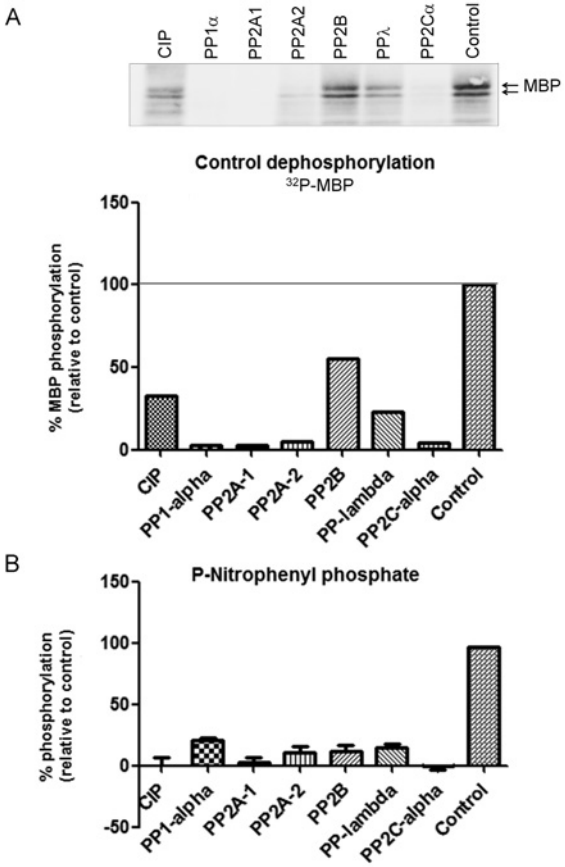

Figure S3 Control of *in vitro* phosphatase activity of recombinant phosphatases

Recombinant phosphatases were tested on generic substrates in the same conditions as those used to dephosphorylate LRRK2 *in vitro* (see Figure 1 of the main text). (A) The ability of different phosphatases to dephosphorylate PKA-phosphorylated MBP *in vitro* was tested. MBP was first labelled with radioactive phosphates by *in vitro* phosphorylation by PKA in the presence of [γ-^{32}P]ATP, then submitted to dephosphorylation (in the presence of 2 μM staurosporine to inhibit PKA) using the indicated panel of phosphatases as described in the Materials and methods section. Samples were analysed by SDS/PAGE and blotted to PVDF membranes as described in the Materials and methods section. Shown is the autoradiogram blot illustrating the incorporated ^{32}P levels. The graph in the lower panel of (A) shows the quantification of incorporated ^{32}P in MBP for each phosphatase relative to the control (non-dephosphorylated MBP). (B) The ability of different phosphatases to dephosphorylate pNPP (Sigma) *in vitro* was tested. Dephosphorylation was tested according to the manufacturer's instructions. Shown are quantifications of triplicate measures (means ± S.E.M.) relative to the maximum dephosphorylation given for alkaline phosphatase (for which values were set to zero,). CIP, calf intestinal alkaline phosphatase.

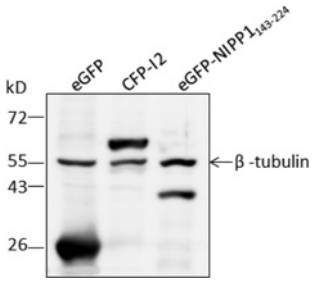

Figure S4 Overexpression of PP1 inhibitors

GFP immunoblot showing overexpression of eGFP, CFP–I2 and eGFP–NIPP1$_{143–224}$ in HEK-293T cells. β-Tubulin was used to control for equal loading.

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