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online data

SUPPLEMENTARY ONLINE DATA

Inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser^{910}/Ser^{935}, disruption of 14-3-3 binding and altered cytoplasmic localization

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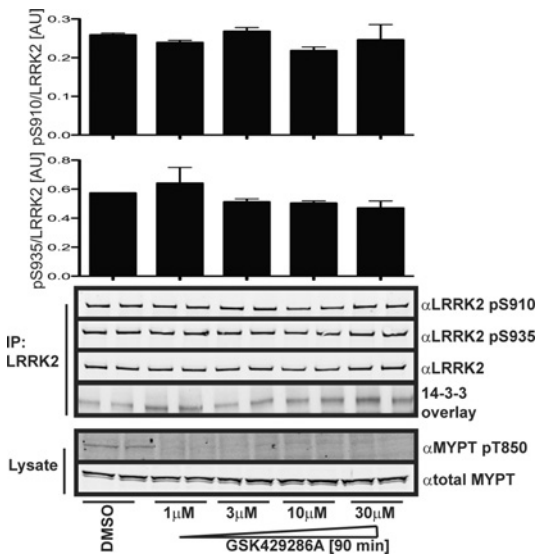

Figure S1 Phosphorylation of LRRK2 at Ser^{910} and Ser^{935} is not regulated by ROCK

Endogenous LRRK2 was immunoprecipitated (IP) with anti-LRRK2 100–500 (S348C) antibody from Swiss 3T3 cells treated with DMSO vehicle control or the indicated concentrations of the potent ROCK inhibitor GSK429286A for 90 min. Immunoprecipitates were subjected to immunoblot analysis with the indicated antibody as well as 14-3-3 overlay far-Western analysis. The immunoblot analysis was quantified by Odyssey® LI-COR analysis and the amount of LRRK2 phosphorylation is presented as the ratio of phosphospecific antibody to total LI-COR (phospho-Ser^{910}/LRRK2) in absorbance units (AU).

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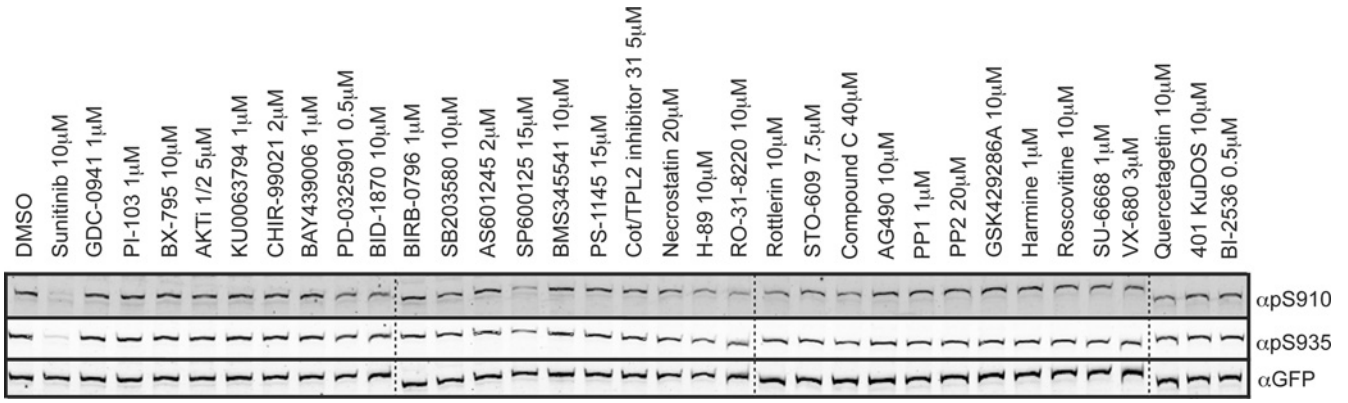

Figure S2 Effect of 33 signal transduction inhibitors on LRRK2 Ser^{910} and Ser^{935} phosphorylation

HEK-293 cells stably expressing GFP–LRRK2 were treated with DMSO, or the following inhibitors dissolved in DMSO, at the indicated concentration for 90 min: sunitinib (LRRK2 inhibitor [1]); GDC-0941 (PI3K inhibitor [2]); PI-103 (dual mTOR/PI3K inhibitor [3]); BX-795 {dual MARK/PDK1 (phosphoinositide-dependent kinase 1) inhibitor [4]}; AKTi1/2 {PKB (protein kinase B) inhibitor [5]}; KU0063794 (mTOR inhibitor [6]); CHIR-99021 {GSK3 (glycogen synthase kinase 3) inhibitor [7]}; BAY439006 (Raf inhibitor [8]); PD-0325901 (MEK1 inhibitor [9]); BID-1870 (RSK inhibitor [10]); BIRB-0796 (p38 MAPK inhibitor [11]); SB203580 (p38 MAPK inhibitor [12]); AS601245 (JNK inhibitor [13]); SP600125 (JNK inhibitor [14]); BMS345541 {IKK (inhibitor of nuclear factor κB kinase) inhibitor [15]}; PS-1145 (IKK inhibitor [16]); TPL2 inhibitor 31 {Cot/TPL2 (tumour progression locus 2) inhibitor [17]}; Necrostatin {RIPK (receptor-interacting serine/threonine protein kinase) inhibitor [18]}; H-89 {dual PKA (protein kinase A) /ROCK inhibitor [19]}; RO-31-8220 {PKC (protein kinase A) inhibitor [20]}; Rottlerin (PKC inhibitor [21]); STO-609 {CaMKK (calcium/calmodulin-dependent protein kinase kinase) inhibitor [22]}; Compound C {AMPK (AMP-activated protein kinase) inhibitor [23]}; AG490 {JAK (Janus kinase) inhibitor [24]}; PP1 (Src inhibitor [25]); PP2 (Src inhibitor [26]); GSK429286A (ROCK inhibitor [1]); Harmine (dual CDK (cyclin-dependent kinase)/DYRK (dual-specificity tyrosine-phosphorylated and -regulated kinase) inhibitor [27]); Roscovitine (CDK inhibitor [28]); SU-6668 (dual Src/Aurora kinase inhibitor [29]); VX-680 (Aurora kinase inhibitor [30]); Quercetagetin {PIMK (Pim kinase) inhibitor [31]}; 401 KuDOS {DNAPK (DNA-dependent protein kinase) inhibitor [32]}; and BI-2536 {PLK1 (Polo-like kinase 1) inhibitor [33]}. Following lysis, 30 μg of lysate was resolved by SDS/PAGE and immunoblotted for LRRK2 phosphorylation at Ser^{910} and Ser^{935}. Total LRRK2 was assessed by anti-GFP antibody immunoblotting. The immunoblots shown are representative of two independent experiments.

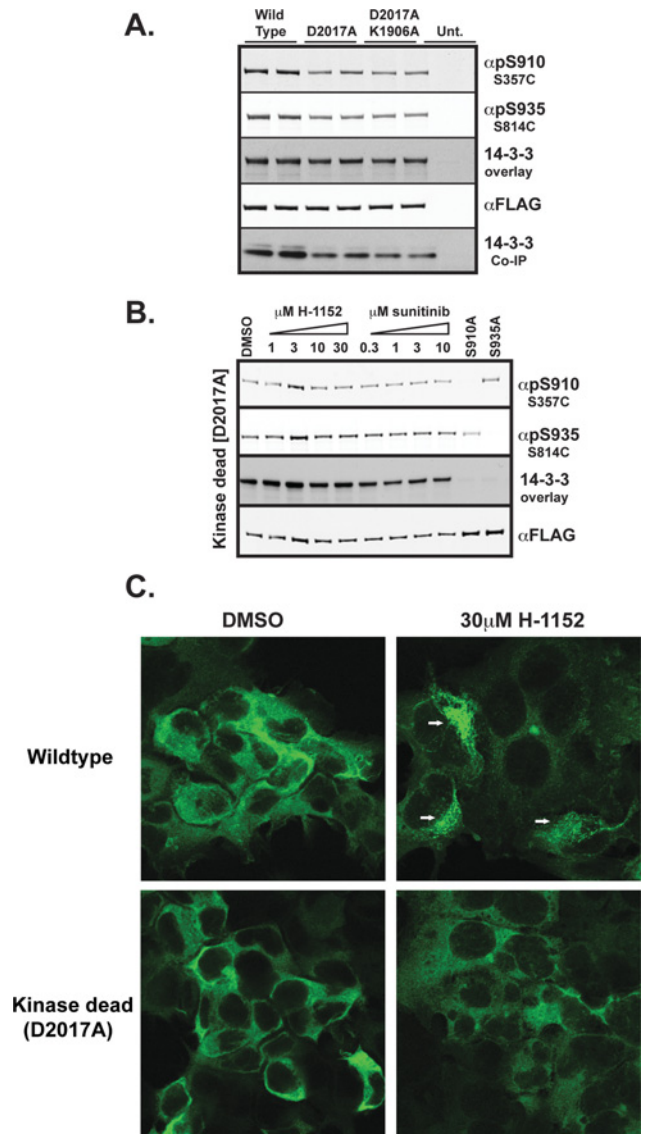

Figure S3 Analysis of Ser^{910} and Ser^{935} phosphorylation in kinase-inactive mutants of LRRK2

(**A**) FLAG-tagged wild-type LRRK2 and the indicated kinase-inactive versions, LRRK2(D2017A) and a double kinase-inactive LRRK2(K1906A/D2017A) mutant (that should be completely devoid of even trace levels of kinase activity), were transiently expressed in HEK-293 cells and immunoprecipitated with anti-FLAG beads. Immunoprecipitates were analysed by immunoblot analysis with anti-FLAG (for total LRRK2), anti-phospho-Ser^{910} (αpS910) and anti-phospho-Ser^{935} (αpS935) antibodies as well as a 14-3-3 overlay assay. Co-immunoprecipitated 14-3-3 was assessed by immunoblotting with the anti-pan-14-3-3 antibody. (**B**) HEK-293 cells transiently expressing FLAG–LRRK2(D2017A) were treated with DMSO vehicle control or the indicated concentrations of H-1152 or sunitinib for 90 min. Cells were lysed in lysis buffer supplemented with 0.5% NP-40 and 150 mM NaCl and subjected to anti-FLAG antibody immunoprecipitation. Immunoprecipitates were resolved by SDS/PAGE (4–12% Novex gels) and subjected to immunoblotting with anti-FLAG (for total LRRK2), anti-phospho-Ser^{910} (αpS910) and anti-phospho-Ser^{935} (αpS935) antibodies, as well as a 14-3-3 overlay assay. (**C**) Stable-inducible T-REx cells lines harbouring the indicated forms of LRRK2 were induced for 24 h with 0.1 μg/ml doxycycline to induce expression of GFP–LRRK2. The indicated cell lines were treated in the absence or presence of the indicated dose of H-1152 for 90 min prior to fixation. Representative fluorescent micrographs of GFP–LRRK2 localization are shown. Cytoplasmic aggregates resembling inclusion bodies of GFP–LRRK2 are indicated with white arrows.

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