Supporting Information

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SI Materials and Methods

Phosphopeptide Enrichment and MS. Phosphopeptide enrichment and MS analysis were performed as described previously (1). Briefly, after proteolytic cleavage of the samples with either trypsin or chymotrypsin, phosphopeptides were enriched with titanium dioxide. MS analysis of the samples was performed on an LTQ Orbitrap Velos mass spectrometer (Thermo-Fisher) coupled with an UltiMate 3000 RSLCnano-HPLC (Dionex). For MS/MS, peptides were fragmented by collision-induced dissociation and higher-energy collision-induced dissociation. A search was performed against the human subset of the UniProt database (2010–2012; 20,259 entries) using the Mascot search engine (version 2.3; Matrix Science) with the following parameters: 10 ppm as mass range for parent ions and 1 Da for fragment ions; carbamidomethyl as a fixed modification and methionine oxidation and serine/threonine, as well as tyrosine phosphorylation, as variable modifications. Depending on the enzyme used, the setting for the used proteolytic enzyme was set to trypsin or, in the case of the chymotrypsin-treated samples, the searches were performed with no enzyme specified. Scaffold 3_00_8 (Proteome Software) was used for downstream data analysis.

Peptides. Peptide synthesis was carried out in microwell plates (0.8-μmol synthesis scale) with an AutoSpot robot (Intavis) according to standard solid-phase Fmoc chemistry. For dye-labeled peptides, 2 μL 5.6-carboxyfluorescein [0.5 M in N,N-dimethylformamide (DMF)] was activated with 3.7 μL HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate 0.55 M in DMF) and 1 μL base mix (3 M 2.6-lutidine and 2 M diisopropylethylamine in DMF) and then coupled to the free amino group of the N-terminus of the respective peptide. After cleavage from the resin, peptides were purified by RP-HPLC on an Agilent 1260 Infinity system (Agilent Technologies). Quality control was performed by MALDI-TOF MS (Ultraflex; Bruker) and analytical RP-HPLC. Concentrations of fluorescein-labeled peptides were calculated by measurement of UV absorption at 492 nm in 50 mM potassium phosphate buffer (pH 9) with an extinction coefficient of 87,000 $L{\cdot}cm^{-1}{\cdot}M^{-1}$ being used.

1. West AB, et al. (2005) Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci USA* 102(46):16842–16847.

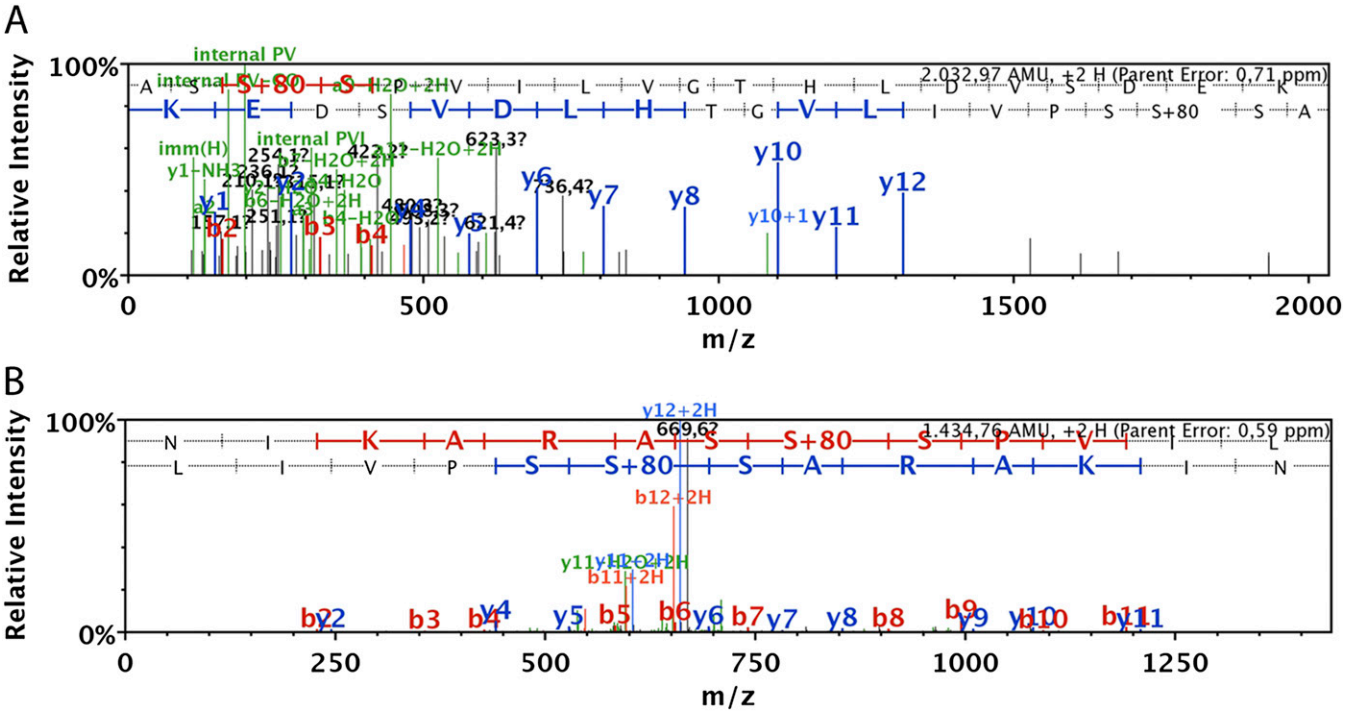

Fig. S1. Systematic phosphosite mapping by MS reveals unique PKA target sites within LRRK2. In vitro kinase assays were performed using a purified kinase-dead (K1906M) full-length or kinase-dead N-terminal deletion (Δ967) of LRRK2 as a substrate for purified catalytic subunit of PKA (60 min at 30 °C). Reaction products were precipitated with chloroform–methanol and digested with (*A*) trypsin or (*B*) chymotrypsin. Fragment spectra of the peptide bearing the unique PKA-phosphorylated serine residue 1444 are shown.

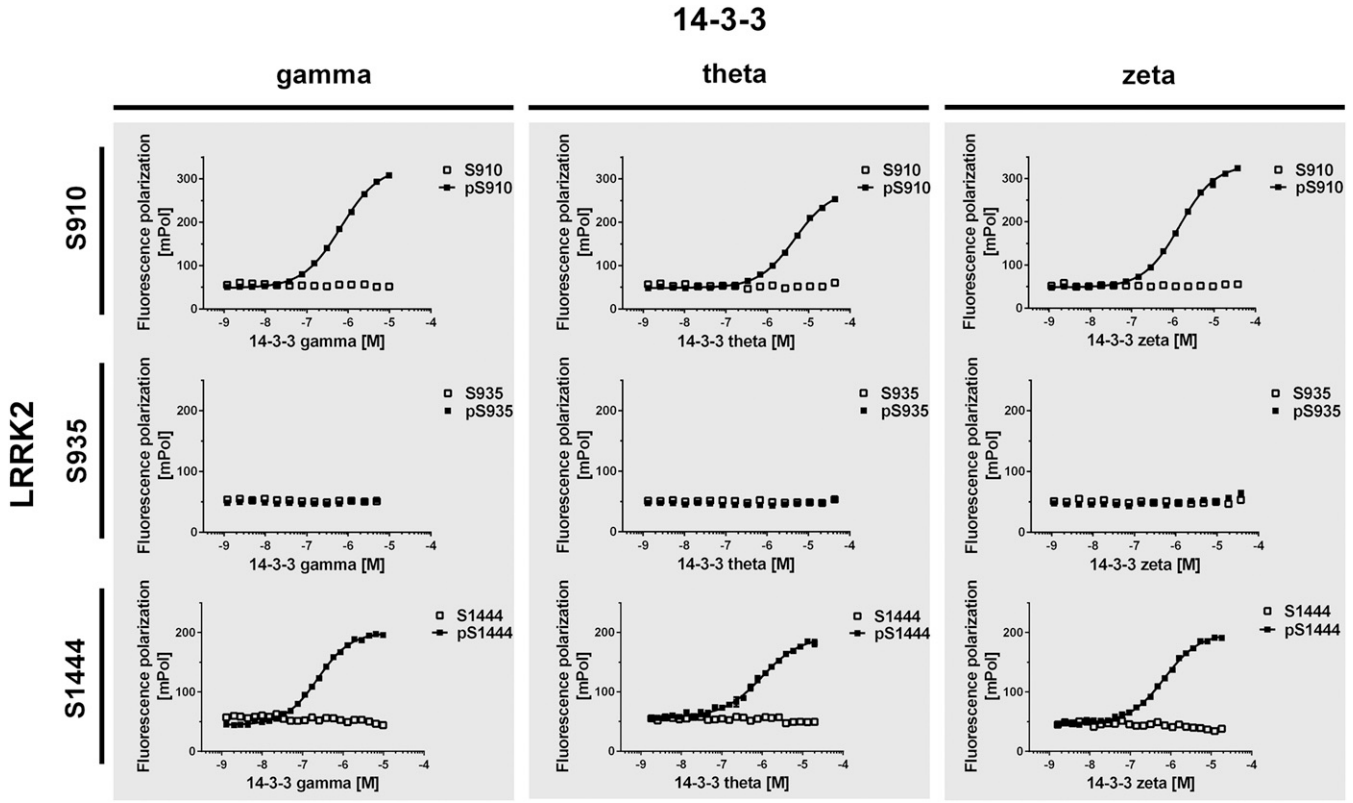

Fig. S2. Binding of GST–14-3-3 to synthetic peptides corresponds to the newly identified 14-3-3 binding region of LRRK2. K_d determination based on fluorescence polarization for 14-3-3 proteins with phosphorylated (solid symbols) or nonphosphorylated (open symbols) peptides covering S910, S935, or S1444. Various concentrations of GST–14-3-3 protein (14-3-3 gamma, theta, or zeta) were incubated with 10 nM of the corresponding synthetic peptide. Each data point represents the mean ± SEM from triplicate measurements. K_d values are shown in Table 1.

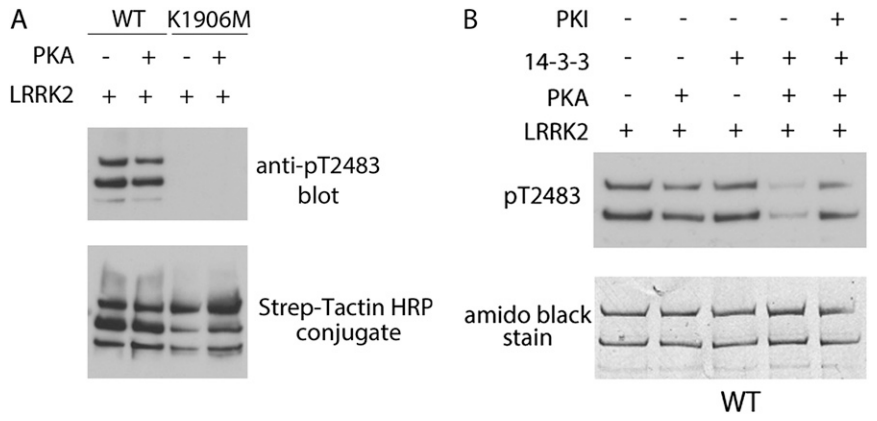

Fig. S3. LRRK2 kinase activity is modulated by phosphorylation of S1444 and 14-3-3 binding. (*A*) Kinase activities of LRRK2 WT or K1905M (1 μg each) were measured by monitoring LRRK2 autophosphorylation in the presence or absence of PKA (9 U) in vitro. Samples were separated on SDS/PAGE and then immunoblotted with anti-pT2483 antibody (*Upper*). Strep-Tactin HRP antibody confirmed equal protein loading (*Lower*). (*B*) Kinase activity of LRRK2 WT (1 μg) was measured by monitoring LRRK2 autophosphorylation in the presence of PKA (9 U) and/or GST–14-3-3 gamma (10 μg) as well as PKI (10 μM) in vitro. Samples then were separated via SDS/PAGE and immunoblotted with anti-pT2483 antibody. Amido Black staining confirmed equal protein loading.

Table S1. PKA phosphorylation sites in LRRK2 K1906M and Δ967 identified by MS (PKA-treated LRRK2)

Residue	Peptide sequence*	Start	End	Enzyme	Peptides recovered	
					LRRK2	LRRK2 Δ967
T833	(R)KQ**T**NIASTLAR(M)	831	841	Tryp	3	—
	(L)RKQ**T**NIASTL(A)	830	839	Chym	1	—
S850	(R)YQMK**S**AVEEGTASGSDGNFSEDVLSK(F)	846	871	Tryp	1	—
S858	(K)SAVEEGTA**S**GSDGNFSEDVLSK(F)	850	871	Tryp	2	—
S860	(K)SAVEEGTASG**S**DGNFSEDVLSK(F)	850	871	Tryp	3	—
S908	(K)K**S**NSISVGEFYR(D)	907	918	Tryp	5	—
	(L)VKKK**S**NSISVGEF(Y)	904	916	Chym	2	—
S910	(K)KSN**S**ISVGEFYR(D)	907	918	Tryp	31	—
	(L)VKKKSN**S**ISVGEFY(R)	904	917	Chym	9	—
S933	(R)H**S**NSLGPIFDHEDLLK(R)	932	947	Tryp	10	—
S935	(R)HSN**S**LGPIFDHEDLLKR(K)	932	947	Tryp	6	—
S954	(K)IL**S**SDDSLR(S)	952	960	Tryp	5	—
S955	(K)ILS**S**DDSLR(S)	952	960	Tryp	3	—
	(L)S**S**DDSLRSSKL(Q)	954	964	Chym	1	—
S958	(L)SSDD**S**LRSSKL(Q)	954	964	Chym	1	—
S962	(K)ILSSDDSLRS**S**K(L)	952	963	Tryp	1	—
	(L)SSDDSLRS**S**KL(Q)	954	964	Chym	1	—
S971	(R)H**S**DSISSLASER(E)	970	981	Tryp	8	3
S973	(R)HSD**S**ISSLASER(E)	970	981	Tryp	9	10
	(L)QSHMRHSD**S**ISSL(A)	965	977	Chym	1	—
S976	(R)HSDSIS**S**LASER(E)	970	981	Tryp	1	1
S973/S976	(R)HSD**S**IS**S**LASER(E)	970	981	Tryp	—	1
S979	(R)HSDSISSLA**S**ER(E)	970	981	Tryp	—	1
S1443	(K)ARA**S**SPVILVGTHLDVSDEK(Q)	1440	1460	Tryp	4	3
	(F)NIKARA**S**SPVIL(V)	1437	1449	Chym	1	—
S1444	(R)AS**S**PVILVGTHLDVSDEK(Q)	1442	1460	Tryp	4	5
	(F)NIKARAS**S**PVIL(V)	1437	1449	Chym	3	—
T1849	(R)L**T**IPISQIAPDLILADLPR(N)	1848	1866	Tryp	—	1
S2166	(R)NA**S**IWLGCGHTDR(G)	2164	2176	Tryp	—	2

Chymotryptic (Chym) or tryptic (Tryp) phosphopeptides derived from PKA-treated LRRK2 K1906M or K1906M Δ967 overexpressed and purified from Sf9.
*Residues in boldface and underlined denote phosphorylated residues within the LRRK2 peptides.

Table S2. PKA phosphorylation sites in LRRK2 K1906M and Δ967 identified by MS (untreated—control)

Residue	Peptide sequence*	Start	End	Enzyme	Peptides recovered	
					LRRK2	LRRK2 Δ967
S910	(K)SN**S**ISVGEFYR(D)	908	918	Tryp	1	—
	(K)SN**S**ISVGEFYR(D)	908	918	Chym	1	—
S955	(K)ILS**S**DDSLR(S)	952	960	Tryp	1	—
	(K)ILS**S**DDSLR(S)	952	960	Chym	1	—
S971	(R)H**S**DSISSLASER(E)	970	981	Tyrp	—	2
S973	(R)HSD**S**ISSLASER(E)	970	981	Tyrp	4	8
	(R)HSD**S**ISSLASER(E)	970	981	Chym	4	—
S975	(R)HSDSI**S**SLASER(E)	970	981	Tyrp	1	1
S976	(R)HSDSIS**S**LASER(E)	970	981	Tyrp	—	1
S979	(R)HSDSISSLA**S**ER(E)	970	981	Tryp	—	1

Chymotryptic (Chym) or tryptic (Tyrp) phosphopeptides derived from untreated LRRK2 K1906M or K1906M Δ967 overexpressed and purified from Sf9.
*Residues in boldface and underlined denote phosphorylated residues within the LRRK2 peptides.