

Quantitative characterization of PP2A-derived peptides modulating the LRRK2:PP2A protein-protein interaction

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Abstract

Parkinson's disease (PD) is the most prevalent motor neurodegenerative disorder with currently no disease-modifying treatment. LRRK2 is a major PD genetic determinant, showing dephosphorylation of its heterologous phosphorylation sites (S910/S935) in disease. We previously proposed preventing LRRK2 S910/S935 dephosphorylation as a PD therapeutic approach attenuating LRRK2 hyperactivation. As PP2A phosphatase subunits are key regulators of this dephosphorylation, we targeted the LRRK2:PP2A protein-protein interaction (PPI) as strategy to prevent LRRK2 dephosphorylation. Using Microscale Thermophoresis, we measured the binding affinities of two PPP2CA-derived peptides, HT16 and QA16, for LRRK2, with dissociation constants ranging from 0.3 to 5 μ M. Strikingly, co-incubation of both peptides showed a sevenfold improved binding affinity, suggesting two distinct but allosterically coupled binding sites on LRRK2. Co-immunoprecipitation experiments in HEK293T cells further demonstrated that these peptides compete with PPP2CA for binding to LRRK2. AlphaFold2 structural modelling identified putative PPP2CA residues at the LRRK2 interaction interface. Alanine scanning of these residues clarified their contribution to the interaction pointing to Y130, D223 and H230 of PPP2CA as potential LRRK2 binding hotspots. These results provide the first quantitative characterization of the LRRK2:PPP2CA interaction interface and establish peptide molecular tools to develop selective PPI modulators blocking LRRK2 S910/S935 dephosphorylation as PD therapeutic approach.

1. Introduction

Parkinson's disease (PD) is the most prevalent motor neurodegenerative disorder, affecting over 7 million people worldwide, with no disease-modifying treatment currently available. Among the genetic determinants of PD, *LRRK2* (*Leucine-Rich Repeat Kinase 2*) stands as one of the most frequently mutated genes in familial forms of PD, with mutations such as G2019S accounting for a significant proportion of both familial and sporadic cases across diverse populations^{1,2}.

LRRK2 is a large multidomain protein harboring both GTPase and kinase activities, and its phosphorylation status is tightly correlated with disease progression. Two distinct phosphorylation clusters have been described: autophosphorylation sites concentrated within or proximal to the ROC domain, and heterologous sites located in an extended loop between leucine-rich repeats of the LRR domain (S860, S910, S935, S955, S973, S976). The phosphorylation state of these heterologous residues is governed by the balance between upstream kinases (CK1 α , PKA, IKK, I κ B)^{3–8}, and protein phosphatases, including PP1 and PP2A^{9,10}. An siRNA screen of the human phosphatome conducted by our group identified PP1 and PP2A isoforms as the primary phosphatases regulating LRRK2 dephosphorylation at residues S910/S935, with PP2A holoenzymes containing the phosphoprotein phosphatase 2A regulatory subunits 2A/B/C/D (PPP2R2A/B/C/D) playing a particularly prominent role⁹. We have also previously shown that regulation between LRRK2 and PP2A is bidirectional, and that LRRK2:PP2A complex formation is regulated by PAK6^{11,12}. PP2A dephosphorylates LRRK2 at S910/S935 heterologous residues and T1503 autophosphorylation residue, and in turn LRRK2 phosphorylates

PPP2CA at residue T304 altering both holoenzyme formation and catalytic activity^{9,11}. Therefore, LRRK2 and PP2A display intimate functional links.

The phosphorylation status at residues S910/S935 is important for 14-3-3 binding, which regulate cytosolic localization of LRRK2. Studies have shown that dephosphorylated LRRK2 (pathogenic mutants or kinase inhibited LRRK2) relocates subcellularly by templating onto microtubules^{13–17}. This accumulation is associated with the loss of 14-3-3 chaperone binding at the heterologous sites. Interestingly, both PP1 and PP2A subunits are found to bind to LRRK2^{10,18} and loss of 14-3-3 binding correlates with enhanced binding of PP1 and PP2A holoenzymes^{9,10}. Critically, dephosphorylation of these heterologous sites has been observed in several pathogenic LRRK2 mutants, in PD patient biofluids such as urine, in *post-mortem* brain tissue from PD patients, and is associated with increased alpha-synuclein release^{14,19–22}. Recent study from our laboratory have demonstrated that dephosphorylation at S910/S935 cluster renders LRRK2 sensible to hyperactivation by RAB29^{21,23}. Therefore, blocking LRRK2 dephosphorylation to restore and maintain basal phosphorylation at these sites represents a promising therapeutic strategy.

PP1 and PP2A are responsible for up to 90% of the phosphatase activity in eukaryotic cells, and are holoenzymes structurally composed of a catalytic subunit and a regulatory subunit (plus an additional scaffolding subunit for PP2A). Direct inhibition of PP1 or PP2A would carry major off-target consequences given their central roles in cellular signaling, and, importantly, PP2A might play a beneficial role in several neurodegenerative diseases as it has been shown to dephosphorylate α -syn and Tau^{24,25}. Therefore, a more selective strategy could consist in disrupting the LRRK2-phosphatase protein-protein interaction (PPI) specifically, without impairing other phosphatase substrates or the broader phosphatase interactome.

In the absence of structural data on the LRRK2:PP2A complex, a pepscan approach was recently used to map putative interaction interfaces between LRRK2 and PP2A catalytic subunit (PPP2CA). By screening overlapping peptide fragments of PPP2CA against full-length LRRK2, and reciprocally, Rebollo *et al.* (2026) identified two PPP2CA-derived peptides (M1 and M2) and three LRRK2-derived peptides (P1, P2, P3) capable of contributing to the complex interface, with M1 and P3 showing the ability to compete with the LRRK2:PP2A interaction in an *in vitro* co-immunoprecipitation assay²⁶. However, the binding affinities of these peptides for their respective partner remain uncharacterized.

Here, we report the quantitative biophysical characterization of PPP2CA-derived peptides using Microscale Thermophoresis (MST) and show a cooperative interaction between 2 peptides. We further demonstrate by co-immunoprecipitation that these peptides compete with PPP2CA for binding to LRRK2 in a cellular context. In addition, in the absence of structural data on the complex LRRK2:PP2A, we used an unbiased structural modelling AlphaFold approach to identify putative key interaction residues of PPP2CA and evaluated its contribution to the interaction by a mutational and alanine scanning analysis. Together, these results provide the first quantitative characterization of the PPP2CA:LRRK2 interaction

interface and establish these peptides as molecular tools for further development of selective PPI modulators in Parkinson's disease.

2. Results

2.1 Biophysical characterization of PP2A peptide binding to LRRK2 by Microscale Thermophoresis

To quantitatively characterize the interaction between the two PPP2CA-derived peptides identified by pepscan²⁶ and full-length LRRK2, we employed Microscale Thermophoresis (MST). As described in materials and methods, full-length recombinant LRRK2 was fluorescently labeled using a RED-NHS second-generation kit, and labeling quality was validated by size-exclusion chromatography prior to all experiments. Labeled LRRK2 was used at a fixed concentration of approximately 10 nM, and each peptide was titrated across a concentration range up to 500 μ M. All experiments were performed in triplicate at three laser power settings (Low, Medium, and High) to ensure robustness of the thermophoretic signal.

The peptides yielded well-defined binding isotherms with dissociation constants (K_d) in the sub-micromolar to low-micromolar range (Fig. 1). The two peptides derived from the catalytic subunit PPP2CA, HT16 and QA16 (M1 and M2²⁶), displayed affinities of $0.30 \pm 0.06 \mu$ M and $5 \pm 2 \mu$ M, respectively.

To confirm the specificity of the observed interactions, binding assays were repeated using heat-denatured LRRK2 (95°C, 5 min, in the presence of DTT and SDS). Under denaturing conditions, no binding signal was detected for HT16 or QA16, confirming that their interaction with LRRK2 is conformation-dependent and specific (Fig. 1).

Given that HT16 and QA16 both originate from alpha-helical regions of the PPP2CA catalytic subunit and are spatially proximal in the three-dimensional structure of PP2A (PDB: 3DW8)(Fig. 5), we investigated whether their simultaneous presence could influence their respective binding affinities for LRRK2.

Co-incubation of HT16 and QA16 prior to MST measurement resulted in a striking sevenfold improvement in binding affinity for both peptides (Fig. 1): the apparent K_d of HT16 shifted from $0.30 \pm 0.06 \mu$ M to $0.04 \pm 0.02 \mu$ M, and that of QA16 from $5 \pm 2 \mu$ M to $0.74 \pm 0.31 \mu$ M. This cooperative effect suggests that HT16 and QA16 engage two distinct but allosterically coupled binding sites on LRRK2, and that occupancy of one site enhances the affinity of the adjacent site. Competition assays at fixed concentrations will be required to formally establish the nature of this cooperativity.

2.2 PPP2CA-derived peptides modulate the LRRK2:PP2A complex *in vitro*

To assess whether the PPP2CA-derived peptides identified by pepscan were capable of interfering with the LRRK2:PP2A interaction in a cellular context, co-immunoprecipitation (Co-IP) experiments were performed in HEK293T cells co-transfected with eGFP-tagged LRRK2 and Flag-tagged PPP2CA. Immunocaptured complexes were treated with 1 mM of each peptide individually or in co-incubation, and the extent of complex disruption was quantified by western blot. Co-IP of the LRRK2:PPP2CA complex was clearly detected under DMSO control conditions. Treatment with the PPP2CA-derived peptides HT16 and QA16 yielded divergent results: QA16 displaced PPP2CA from LRRK2 by approximately 45%, while HT16 showed no detectable modulation of the LRRK2:PPP2CA interaction (Fig. 2). Co-incubation of HT16 and QA16 together did not result in a substantially greater displacement than either peptide alone.

2.3 PPP2CA-derived peptides binding and effect on LRRK2 *in cellulo*

To determine the effect of PPP2CA-derived peptides in a cellular context, we generated lentiviral eucaryotic expression plasmids encoding for mCherry-tagged HT16 and QA16 peptides. First, we determine whether the genetically encoded peptides could bind to LRRK2 *in cellulo*. We co-transfected HEK293T cells with eGFP-tagged LRRK2 and mCherry-tagged peptides and performed co-immunoprecipitation using GFP-Trap magnetic agarose beads. We observed that both genetically encoded peptides were co-immunoprecipitating with LRRK2, but not with eGFP negative control, suggesting that the co-immunoprecipitated peptides bind effectively to LRRK2 protein. Regarding both peptides, we observed that QA16 peptide binds stronger to LRRK2 than HT16 peptide (Fig. 3).

We next wonder whether these genetically encoded peptides could prevent dephosphorylation of LRRK2. We co-transfected HEK293T cells with eGFP-tagged LRRK2 and mCherry-tagged peptides, and we induced dephosphorylation by inhibition of LRRK2 with MLI-2, a potent and selective type I LRRK2 kinase inhibitor that induces dephosphorylation of S935 LRRK2 residue via recruitment of phosphatases. Treatment with 10 nM MLI-2 drastically reduced phosphorylation of S935 LRRK2. Co-expression of mCherry-peptides did not reach significance in blocking dephosphorylation in the presence of MLI-2 ($p = 0.17$ and 0.11), however peptide HT16 significantly enhanced LRRK2 pS935 rates in basal conditions (Fig. 4).

2.4 Mutational analysis of full-length PPP2CA

A. AlphaFold2 modelling and interface analysis of LRRK2:PPP2CA complex

The position of HT16 (HDLMELEFRIGGKSPDT, PPP2CA₆₃₋₇₈) and QA16 (QVYGFYDECLRKYGNA, PPP2CA₁₂₅₋₁₄₀)²⁶ were analyzed within the 3D structure of PPP2CA using UCSF ChimeraX^{27,28} and the available structure (PDB:3DW8). Both peptides form α -helix located at the surface of PPP2CA and are exposed to the solvent, consistent with their potential involvement in protein-protein interaction (Fig. 5).

We next asked whether these α -helices are facing the interaction interface with LRRK2. We used AlphaFold2 (AF2) to model the LRRK2:PPP2CA complex and we analyzed it using UCSF ChimeraX. Analysis of HT16 peptide region in the AF2 model did not render any potential interacting residue whilst analysis of QA16 peptide region identified Y127 and Y130 as potential contact residues with LRRK2. In the model, Y127 was positioned close to R1514 in the ROC domain (green) of LRRK2, while Y130 showed potential contact with K1314 and C1313 (Fig. 6).

We included additional residues based on their physiochemical properties and their potential contribution to protein-protein interaction in general, identifying the residues H63, R70 and K74 for HT16 peptide region, and the residues Y127, Y130, R135 and Y137 for QA16 peptide region. To determine the involvement of these residues in the interaction of LRRK2:PPP2CA we mutated these residues into alanine (alanine scanning) in full-length PPP2CA and we analyzed the implication of these residues by co-immunoprecipitation of LRRK2 with the different PPP2CA mutants. We observed that the mutants of HT16 did not change the interaction between LRRK2 and PPP2CA, whilst mutant Y130A of QA16 region caused a close to significant ($p = 0,0651$) decrease of interaction between LRRK2 and PPP2CA (Fig. 7).

The AF2 model also identified potential contacts with D223, E226 and H230 located outside the HT16 and QA16 regions (brown, Fig. 6) within PPP2CA. These residues showed a direct contact with S1889, H1911 and D1887, respectively, of KIN domain (blue) of LRRK2. Using the same approach, we generated mutants of these residues of PPP2CA and analyzed their effect by co-immunoprecipitation. In this case, we observed that when we replaced D223 and H230 by an alanine, the interaction was significantly reduced suggesting that these residues might also be involved in the interaction between LRRK2 and PPP2CA.

3. Discussion

The present study provides the first quantitative biophysical and biochemical characterization of the interaction between PP2A catalytic subunit-derived peptides and full-length LRRK2, and further supports their functional relevance in a cellular context through co-immunoprecipitation assays.

Using MST, two peptides of PPP2CA, HT16 and QA16, were found to bind to LRRK2 with dissociation constants ranging from 0.3 to 5 μ M. These affinities are in a concentration range consistent with peptide-based modulators of protein-protein interactions, and confirm that the interfaces identified by pepscan²⁶ correspond to measurable binding events rather than non-specific contacts. The loss of binding under denaturing conditions further supports the specificity and conformational dependence of these interactions.

Interestingly, when HT16 and QA16 were incubated together, their apparent affinities increased approximately sevenfold, suggesting an “additive” effect between the two peptides. This observation may indicate that HT16 and QA16 engage two distinct but functionally coupled sites on LRRK2. One possible explanation is that binding of one peptide induces or stabilizes a conformational state of LRRK2

that facilitates binding of the second peptide. The proximity of the HT16 and QA16 helices in the three-dimensional structure of PPP2CA (code PDB 3DW8) also raises the possibility that their binding sites on LRRK2 could be spatially close. This type of cooperative binding effect could be of interest for the development of more potent inhibitors. In particular, bifunctional or bivalent molecules combining pharmacophoric elements of both peptides could potentially take advantage of this cooperativity to achieve higher binding affinities. However, in co-immunoprecipitation, only QA16 efficiently disrupted the LRRK2:PPP2CA complex by approximately 45%, suggesting that QA16 may represent a more functionally relevant interaction element. In addition, experiments using mCherry-tagged HT16 and QA16, genetically encoded peptides, showed that QA16 may associate to LRRK2 *in cellulo*, supporting its ability to interact with LRRK2 in a cellular context. Although both peptides bind to LRRK2 in MST experiments, these results suggest that they may have different roles in the interaction. QA16 appears to contribute more strongly to the LRRK2:PPP2CA interaction, while the role of HT16 may depend on the cellular or structural context. These observations led us to provide additional structural information on the localization, accessibility and spatial organization of these peptides within PPP2CA, allowing us to better understand their different contributions to the LRRK2:PPP2CA interface.

To better characterize the interface, the highest-ranked AF2 model was used to select residues for mutagenesis. The predictive complex model was used as a guide rather than as a definitive representation of the interaction. Mutagenesis identified Y130, within QA16 and D223, H230 within another helix of PPP2CA, as key virtual residues for complex formation. The effects observed for Y130A, D223A and H230A were consistent with the contacts proposed by AF2. Interestingly, Y127A had no detectable effect despite a predicted interaction with R1514 in the ROC domain. These results show that a contact predicted by AF2 does not necessarily indicate an important contribution to the interface, as these contacts remains predictions, and highlight the importance of experimental mutational analysis.

One limitation of our structural analysis is that the AF2 prediction only considers the catalytic subunits of PP2A. However, PP2A forms a heterotrimeric complex and is active in association with regulatory subunits^{29–31}. These subunits could modify the accessibility of some regions of PPP2CA by masking residues or interactions sites that are exposed when the catalytic subunit is considered alone, or by inducing conformational changes. In addition, in a cellular context, additional protein partners may interact with PPP2CA or LRRK2 and influence complex formation or accessibility of specific regions. This cellular environment may partly explain the difference observed between AF2 prediction, MST and Co-IP experiments. Studying the interaction with the full-length PP2A may provide a more physiological context and help to better understand the role of HT16 and QA16.

Interestingly, our results are also consistent with the observations of Athanasopoulos *et al.* (2026)¹¹, who identified the ROC domain as a region involved in the interaction with PPP2CA. This provides further supports for the involvement of ROC in the LRRK2:PPP2CA interaction. In addition, the effects observed with the D223A and H230A mutations point to the KIN domain as another potential interaction region, suggesting that different regions of LRRK2 may contribute to the interaction with PP2A. An important next step would be to mutate the corresponding residues on LRRK2 (KIN and ROC domain) that are

predicted to interact with the key PPP2CA residues identified in this study: D223, H230, Y130. Comparing the effects of mutations on both sides of the interface would provide more information on the relevance of the contacts predicted by the AF2 model and help us to define more precisely the LRRK2:PPP2CA interaction interface.

Finally, a higher peptide concentration was required to observe competition in Co-IP experiments compared with the K_d values measured by MST. This difference is not unexpected given the fundamentally different conditions and may be related to the complexity of the cell lysate, the presence of other partners, peptide stability, or the accessibility of the binding site, all of which may influence peptide binding and competition with full-length proteins. Future studies could focus on improving peptide stability and affinity, for example through stapling, cyclization or the use of non-natural amino acids.

Comparison with the recently published work of Rebollo *et al.* (2026)²⁶, who identify both PPP2CA and LRRK2-derived interfering peptides, is informative. While that study demonstrated competition of the LRRK2:PP2A complex by peptides P3 and M1 in Co-IP assays, no binding affinities were reported. Our quantitative MST data, the possible cooperative binding between QA16 and HT16, Co-IP experiments, and site-directed mutagenesis extend these observations by providing additional information on the molecular basis of the LRRK2:PPP2CA interaction and provide a basis for the structure-guided optimization of these peptides as selective PPI modulators.

Several limitations should nevertheless be considered. While the increased apparent affinity observed when HT16 and QA16 were combined is consistent with a cooperative binding effect, further experiments will be required to fully characterize the molecular basis of this observation. In addition, the structural model proposed remains predictive and should therefore be considered as a framework for guiding further experimental investigation of the interaction interface.

A crucial goal in developing protein-protein interaction inhibitors targeting the interaction between LRRK2 and its regulatory phosphatases is not only to block binding of phosphatases to LRRK2 but also to prevent dephosphorylation. Indeed, LRRK2 is dephosphorylated in disease, and while the phosphorylation state of LRRK2 does not correlate with LRRK2 hyperactivation in basal conditions³², we recently showed that dephosphorylated LRRK2 is exquisitely sensitized to hyperactivation by upstream activators such as Rab29²³. Therefore, phosphatase-binding blockers should be tested for their ability to prevent LRRK2 dephosphorylation. In our experimental conditions, the two mCherry-peptides HT16 and QA16 were tested for their ability to prevent LRRK2 dephosphorylation at the site of interest (S935) in the presence of MLi-2. A slight but non-significant trend toward modulation was observed in dephosphorylation conditions, while HT16 enhanced LRRK2 S935 phosphorylation in basal condition (Fig. 4), indicating a potential for PP2A blocking peptides to preserve LRRK2 phosphorylation. Further experiments will be required to confirm this effect. Other peptides sequences, such as the helix containing residues D223 and H230, could be also evaluated in this assay. For example, their ability to

modulate the phosphorylation state of purified LRRK2 could be investigated using an enzymatic assay in the presence of active recombinant PPP2CA.

4. Conclusion

In summary, we have provided the first quantitative characterization of the binding affinities of PPP2CA catalytic subunit-derived peptides for full-length LRRK2 and identified a possible cooperative binding behavior between two catalytic subunit-derived peptides. We demonstrated the ability of QA16 to selectively modulate the LRRK2:PPP2CA complex in a cellular context. Experiments with genetically encoded peptides support its ability to interact with LRRK2 *in cellulo*. In addition, site-directed mutagenesis identified key residues involved in complex formation and supported the implication of ROC domain, while identifying the KIN domain as another potential interaction region. Together these results provide further insight into the LRRK2:PPP2CA interaction interface and provide rational basis for the development of selective protein-protein interaction modulators targeting this complex with the aim of favoring LRRK2 phosphorylation at S910/S935 as a therapeutic approach for Parkinson's disease.

5. Materials and Methods

5.1 Peptide synthesis and solubilization

Two PPP2CA-derived peptides were chemically synthesized by GL biochem (Shanghai, China) based on sequences identified by pepscan analysis²⁶: two from the catalytic subunit PPP2CA (HT16: HDLMELFRIGGKSPDT; QA16: QVYGFYDECLRKYGNA). Peptide identity and purity were confirmed prior to use.

For solubilization, approximately 2 mg of each peptide were accurately weighted and dissolved at 10 mM in 100% DMSO. Stock solutions were subsequently diluted to 1 mM in PBS-T (PBS 1X, 0.05% v/v Tween-20) to reach a final DMSO concentration of 10%.

5.2 Fluorescent labeling of LRRK2

Full-length recombinant LRRK2 (ref: A15198, 746 nM) was fluorescently labeled using the Monolith RED-NHS 2nd generation labeling kit (NanoTemper Technologies), following the manufacturer's instructions with modification due to sub-optimal protein concentration relative to kit recommendations.

Briefly, the protein was first transferred into PBS-T by buffer exchange. The NHS-ester dye was dissolved in 30 μ L DMSO and brought to a concentration 2–3-fold above the protein molar concentration. Dye and protein solutions were mixed at a 1:1 ratio (200 μ L final volume) and incubated in the dark for 30 minutes at room temperature. Labeled protein was then purified by size-exclusion chromatography on a pre-equilibrated purification column provided with the kit, and eluted as six fractions of 100 μ L each. The fluorescence intensity of each fraction was measured on the Monolith NT.115 instrument to identify protein-containing fractions. Fractions exhibiting fluorescence intensity between 400 and 800

fluorescence units (FU) were selected, aliquoted, and stored at -80°C for further use. The labeled protein was estimated to be at approximately 10 nM based on fluorescence intensity at 100% LED power.

5.3 Microscale Thermophoresis binding assays

All MST experiments were performed on a Monolith NT.115 instrument (NanoTemper Technologies) using the red detection channel at 100% LED power. Measurements were conducted at three laser power settings (Low, Medium, and High) to ensure consistency of the thermophoretic signal across conditions.

Binding assays with HT16 and QA16 were performed in PBS-T supplemented with 10% DMSO (PBS-TD) to a 5% DMSO in the final experiment. For each peptide, a serial 1:1 dilution series was prepared in PBS-TD starting from a stock solution of 1 mM to final concentration 500 μM after adding LRRK2 labeled, yielding 16-point titration curves. Labeled LRRK2 at approximately 20 nM was mixed in equal volumes with each dilution point immediately prior to loading into standard or premium capillaries (NanoTemper Technologies). Each experiment was performed in triplicate ($n = 3$), and dissociation constants (K_d) were determined by fitting the normalized fluorescence signal (F_{norm}) as a function of ligand concentration using the MO.Affinity Analysis software (NanoTemper Technologies).

To assess the cooperative effect of HT16 and QA16, reciprocal co-incubation experiments were performed. A serial dilution of one peptide was prepared in the presence of the other peptide at a constant concentration. The second peptide was included in the dilution buffer at 2 mM, resulting in a concentration of 1 mM in dilution series. Samples were then mixed with an equal volume of labeled protein, resulting in a final concentration of 500 μM for the peptide maintained constant and a maximum concentration of 500 μM for the serially diluted peptide.

5.4 Denaturation control assay

To confirm the specificity of all observed binding events, denaturation controls were performed for each peptide. Labeled LRRK2 was denatured by incubation at 95°C for 5 minutes in the presence of 40 mM DTT and 4% (v/v) SDS. MST measurements were then repeated under identical conditions using denatured LRRK2 as the fluorescent species. Peptides showing no binding signal with denatured LRRK2 were considered to interact specifically with the native conformation of the protein. Peptides retaining a binding signal under denaturing conditions were classified as non-specific binders and excluded from further analysis.

5.5 Cell culture and transfection

HEK293T cells (ATCC ref CRL-3296) were grown as described above^{21,23}. Cells were cultured in Dulbecco modified Eagle's medium containing high glucose (Sigma-Aldrich, D6429), supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific 15595309), non-essential amino acids (Fisher Scientific, 12084947) and 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Fisher Scientific, 15140122).

HEK293T cells were seeded onto 100 mm plates and co-transfected at 70–80% confluence with 12 μg of eGFP-LRRK2, 2 μg of GFP or 2 μg of eGFP-PPP2R2A and 2 μg of Flag-PPP2CA constructs for

LRRK2:PPP2CA co-immunoprecipitation, or with 12 µg of eGFP-LRRK2 or 2 µg of GFP and 5 µg of mCherry-Peptide constructs for LRRK2:peptide co-immunoprecipitation using polyethylenimine (PEI, branched) (Sigma-Aldrich, 408727). To evaluate the effect of mCherry-Peptides on LRRK2 phosphorylation, cells were seeded onto 12-well plates and co-transfected at 70–80% confluence with 1 µg of 3xFlag-LRRK2 and 0.5 µg of mCherry or mCherry-Peptide constructs using polyethylenimine (PEI, branched) (Sigma-Aldrich, 408727). After 48 h, the cells were lysed in Lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.2% Triton-X100, 5 mM MgCl₂, 10% glycerol) containing EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail.

DNA constructs and site-directed mutagenesis

pLV CSJ empty vector (CMV), pLV CSJ eGFP-LRRK2, pLV CSJ 3xFlag-LRRK2, pcDNA5D FRT/TO Flag-PPP2CA and vLenti eGFP-PPP2R2A were previously described⁹. Constructs expressing mCherry-Peptides (HT16 or QA16) were designed into a lentiviral backbone and obtained from VectorBuilder. pLV mCherry construct was kindly provided by Dr. Pantelis Tsoulfas (Addgene plasmid 36084). peGFP-C1 was obtained from Clontech. PPP2CA mutants were generated by QuikChange II site-directed mutagenesis kit (Agilent Technologies). All plasmids were obtained from overnight bacterial cultures grown at 37 °C using PureYield™ Plasmid Midiprep System (Promega, A2495) according to manufacturer's instructions. Plasmids identity and mutagenesis were verified by sequencing of the entire coding region (Eurofins Genomics GmbH, Germany).

5.6 Co-immunoprecipitation

Co-immunoprecipitation experiments were performed as previously described with minor modifications²³. Briefly, transfected HEK293T cells were lysed in ice-cold lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Lysates were cleared by centrifugation at 13,200 rpm for 10 minutes at 4°C. Immunoprecipitation was performed using anti-GFP magnetic beads (Proteintech, gtma) to pull down eGFP-LRRK2 and its associated partners. After one wash with lysis buffer, for peptide competition assays, captured complexes were incubated with either DMSO, 1 mM of each peptide individually or in co-incubation for 30 minutes at 4°C. Following two additional washes with lysis buffer, bound proteins were eluted by boiling in a solution of 2X NuPAGE™ LDS Sample Buffer and Bolt™ Sample Reducing Agent and resolved by SDS-PAGE on 4–12% Bis-Tris gels (NuPAGE, Invitrogen). Proteins were transferred to nitrocellulose membranes and immunodetected using anti-Flag antibody to detect PPP2CA, anti-GFP antibody to detect LRRK2 and anti-α-tubulin as loading control. Chemiluminescent signals were acquired with Amersham ImageQuant™ 800 western blot imaging system, and quantified by densitometric analysis using Image Studio software. Results are expressed as percentage of the DMSO-treated control condition.

5.7 AlphaFold modelling

DeepMind's machine learning model, AlphaFold2, was used to predict the structure of the complex between human LRRK2 (UniProt reference ID Q5S007) and PPP2CA (UniProt reference ID P67775). The

AlphaFold2 code was downloaded from DeepMind's official GitHub repository (<https://github.com/deepmind/alphafold>). The computations were performed on a workstation equipped with an NVIDIA RTX A5000 GPU (24 GB). The system was running Linux (Ubuntu 20.04), CUDA 11, Python 3.8, and TensorFlow 2.3.1.

Sequence alignments were performed using the standard UniRef90 databases. Five complex models were generated using AlphaFold2 with the recommended configurations provided by DeepMind and with structural templates disabled to obtain *de novo* models. The resulting models were evaluated based on the confidence metrics provided by AlphaFold2, and the highest-ranked model was selected for further structural analysis. This model was subsequently subjected to a final energy minimization step prior to analysis of the predicted LRRK2:PPP2CA interaction interface.

5.8 Structural analysis and visualization

Three-dimensional structures of PP2A subunits were retrieved from the Protein Data Bank (PDB entry: 3DW8) and visualized using ChimeraX (UCSF)^{27,28}. Peptide sequences identified by pepscan and MST were mapped onto the corresponding structural coordinates to illustrate their localization within the catalytic subunit. Predictive structural models of the LRRK2:PPP2CA interaction were generated using AlphaFold2 multimer and are presented as illustrative models to support the interpretation of experimental data using ChimeraX (UCSF).

Declarations

Conflicts of Interest:

The authors declare no conflict of interest.

Author Contribution

AJLO, MS, ML performed the experiments, data analysis, and statistical analysis; LMG, ADS, SY and PM assisted with experimental methods development; MS, AJLO, NL, ML and JMT contributed to data interpretation; JMT, NL and ML proposed, conceived, designed the study and acquired fundings; MS, AJLO, JMT and ML wrote the manuscript; all contributors reviewed and approved the final version of the manuscript.

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Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request. Supplemental raw data pertaining to the western blot figures has been supplied as supplemental data.

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Figures

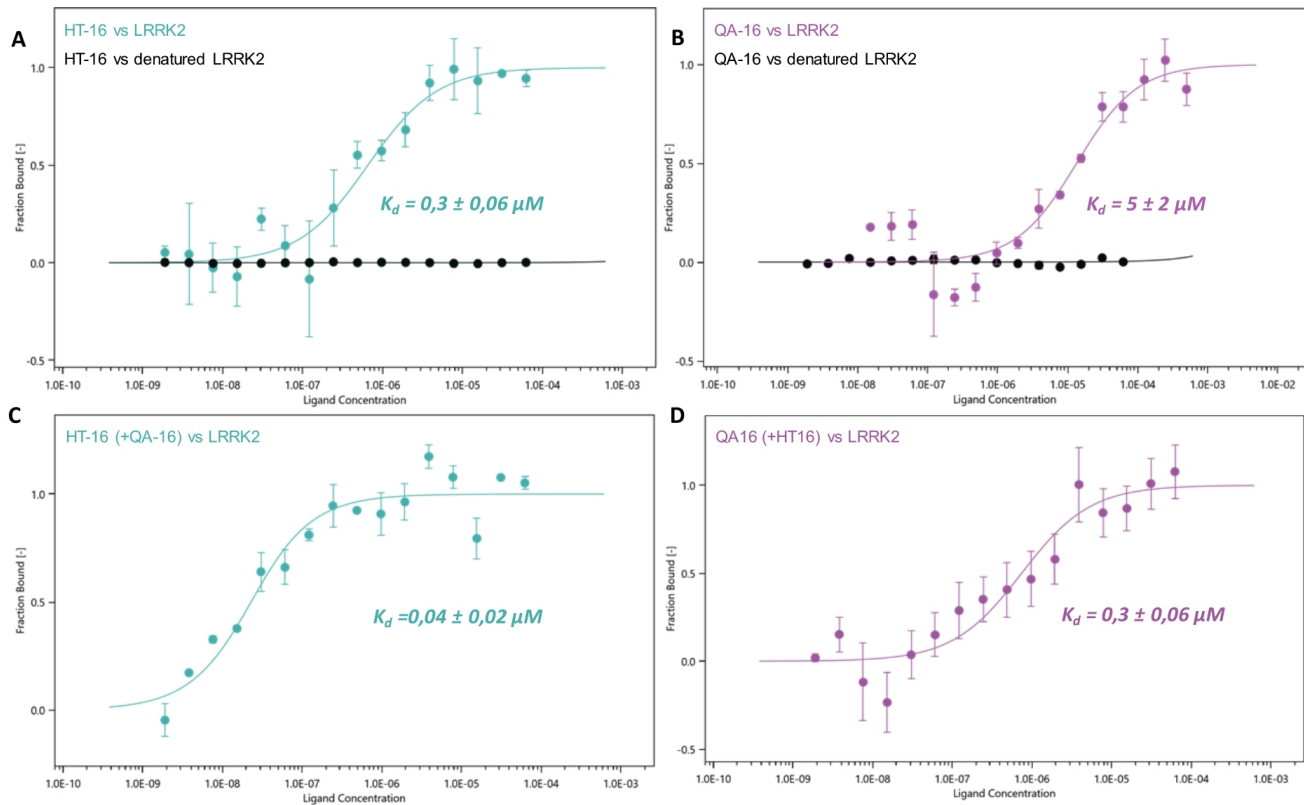


Figure 1

Biophysical characterization of PPP2CA-derived peptides binding to full-length LRRK2 by Microscale Thermophoresis (MST). Binding isotherms of selected PPP2CA-derived peptides titrated against RED-NHS-labeled recombinant LRRK2 (final concentration: ~10 nM). **(A)** Titration of HT16 vs LRRK2 ($K_d = 0.30 \pm 0.06 \mu M$, in turquoise) compared to HT16 vs LRRK2 under denaturing conditions (in black). **(B)** Titration of QA16 vs LRRK2 ($K_d = 5 \pm 2 \mu M$, in purple) compared to QA16 vs LRRK2 under denaturing conditions (in black). **(C)** Titration of HT16 (constant concentration of QA16, 500 μM) vs LRRK2 ($K_d = 0.04 \pm 0.02 \mu M$, in turquoise). **(D)** QA16 (constant concentration of HT16, 500 μM) vs LRRK2 ($K_d = 0,74 \pm 0,31 \mu M$, in purple).

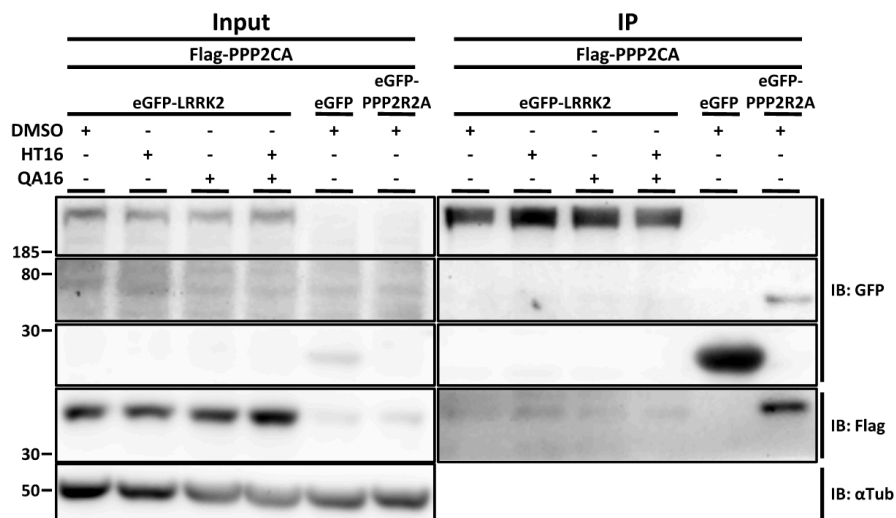
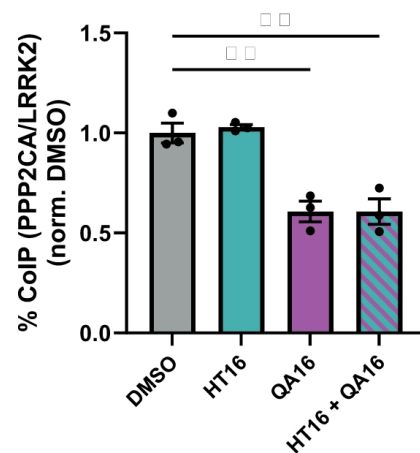


Figure 2

Modulation of the LRRK2:PPP2CA complex by PPP2CA-derived peptides. Co-IP performed in HEK293T cells co-transfected with eGFP-LRRK2 and Flag-PPP2CA, treated with 1 mM of each peptide individually or in co-incubation. (Left) Representative western blot showing immunoprecipitated complexes and corresponding inputs, revealed with antibodies against Flag (PPP2CA), GFP (LRRK2) and α -tubulin as loading control. (Right) Densitometric quantification of PPP2CA signal normalized to the DMSO control condition (set to 1). Data are expressed as mean $\pm$ s.e.m. ($n=3$ independent experiments); $**p < 0.01$.

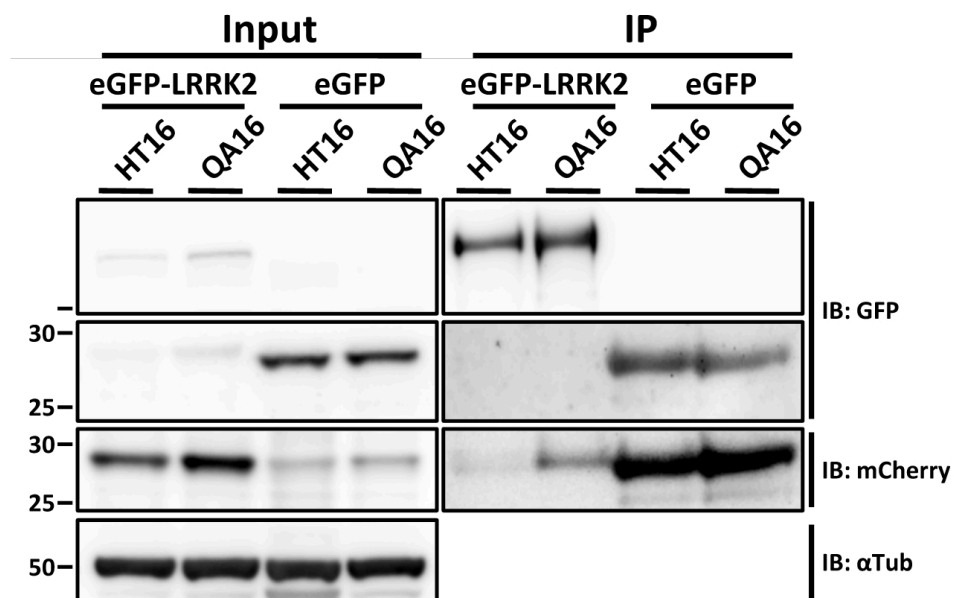
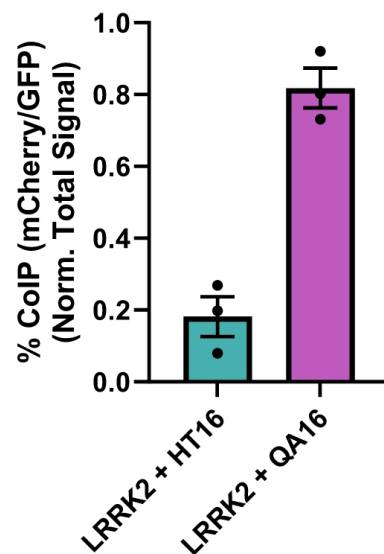


Figure 3

Genetically encoded peptides bind LRRK2. Co-IP performed in HEK293T cells co-transfected with eGFP-LRRK2 and mCherry-peptides. (Left) Representative western blot showing immunoprecipitated complexes and corresponding inputs, revealed with antibodies against GFP (LRRK2), mCherry (peptides) and α -tubulin as loading control. (Right) Densitometric quantification of LRRK2:peptides binding normalized to total signal. Data are expressed as mean $\pm$ s.e.m. (n=3 independent experiments).

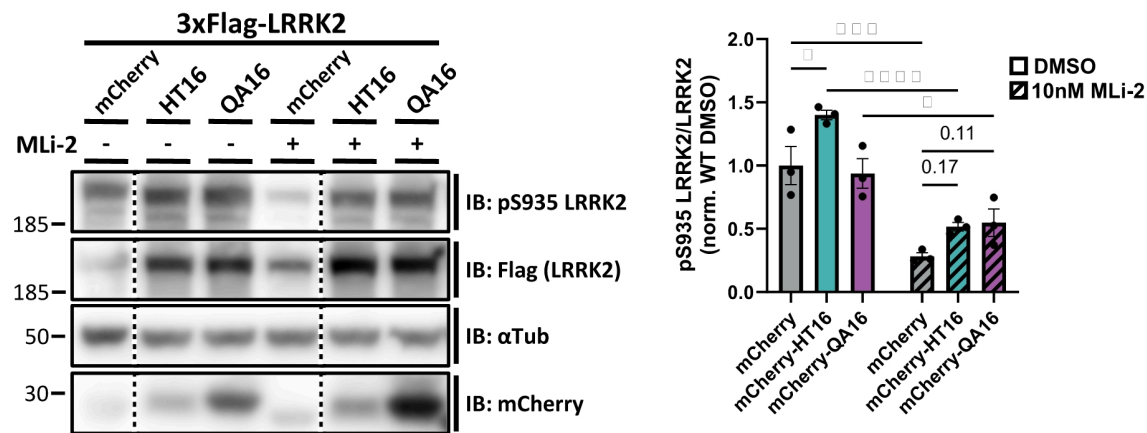


Figure 4

Effect of genetically encoded peptides on pS935 LRRK2 upon inhibition. (A) HEK293T cells were co-transfected with 3xFlag-tagged LRRK2 and mCherry or mCherry-peptides, and treated with DMSO or 10 nM MLI-2 where indicated. Representative western blot revealed with antibodies against phosphorylated S935 LRRK2 (pS935 LRRK2), flag (total LRRK2), mCherry (peptides) and α -tubulin as loading control. (B) Quantification of ratio of pS935 LRRK2 vs LRRK2 of western depicted in (A). Bars represent mean $\pm$ s.e.m. (n=3 independent experiments); *p < 0.05, *** p < 0.001, **** p < 0.0001.

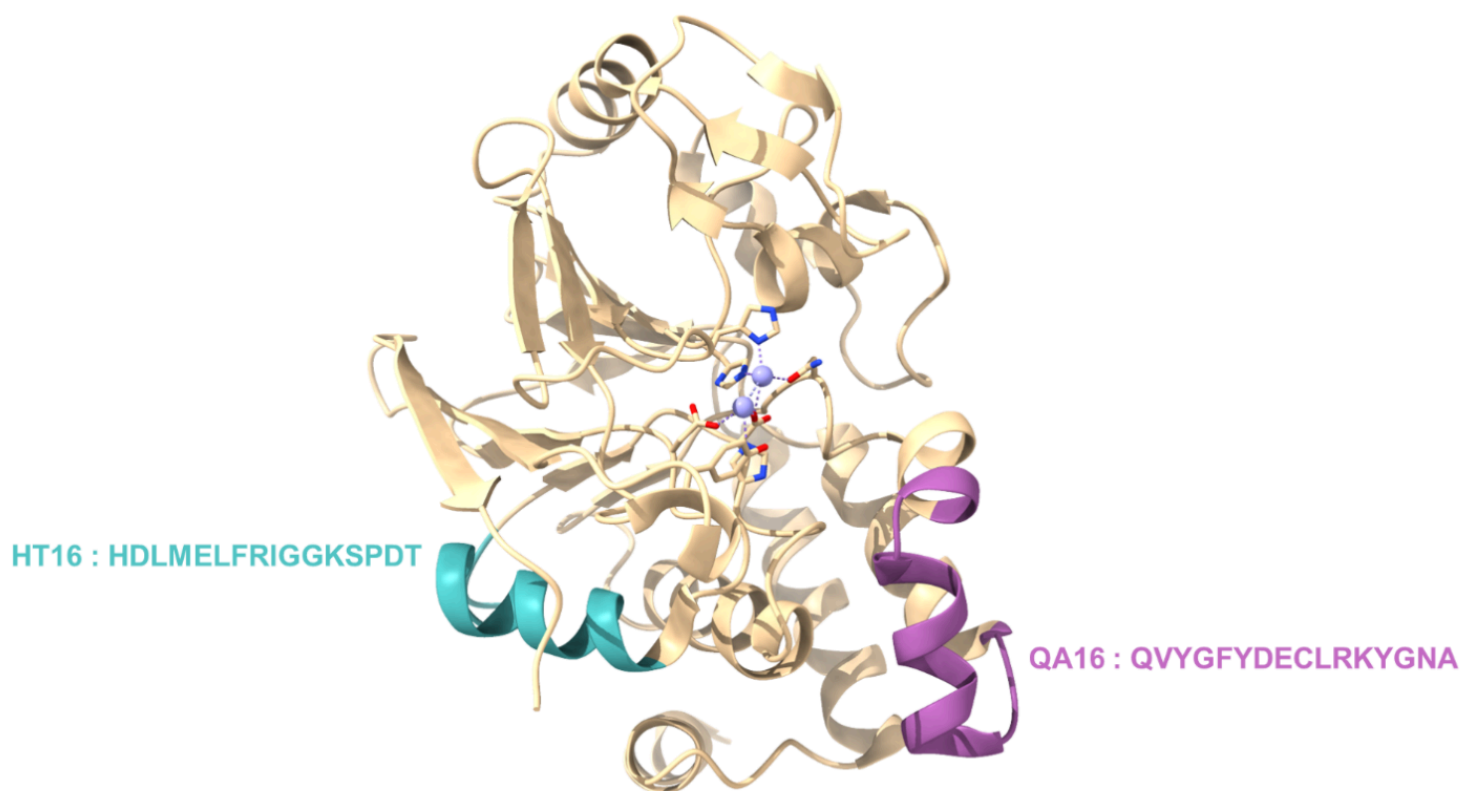


Figure 5

3D structure of the PP2A catalytic subunit (PPP2CA). Localization within the 3D structure of the helical peptides (α helices), HT16 (in turquoise) and QA16 (in purple) identified using the pepscan strategy. The 3D structure was visualized using Chimera X software based on structure available in the Protein data bank (PDB code: 3DW8)

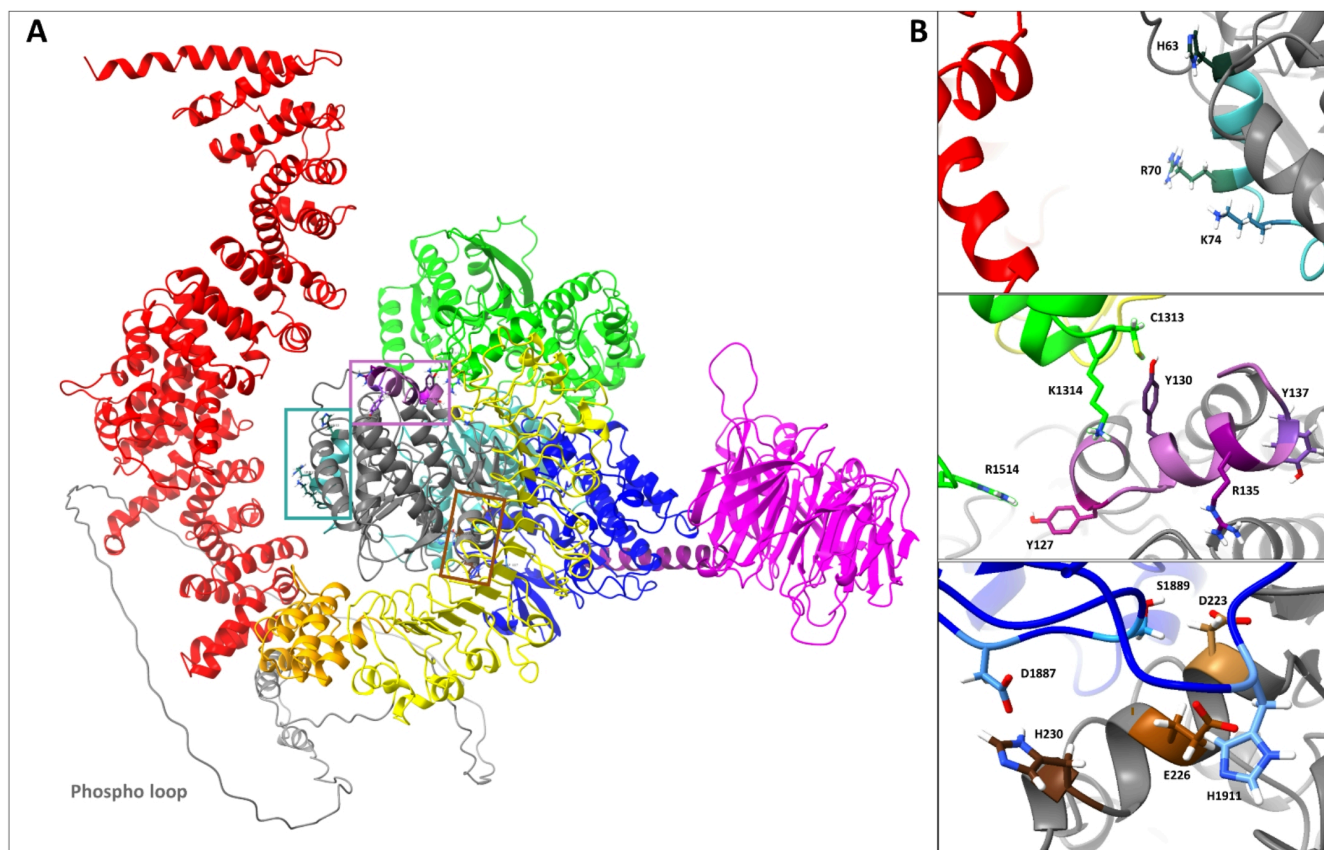


Figure 6

AlphaFold2-predicted complex and mapping of protein-protein interface residues. (A) The LRRK2:PPP2CA predicted by AlphaFold2 is shown with two interacting partners represented in distinct colors: PPP2CA in grey and LRRK2 in different colors. (B) Residues located in the HT16 (in turquoise, focus on region in turquoise box in panel A), QA16 (in purple, focus on region in purple box in panel A), and residues in other helix of PPP2CA (in different shades of brown, focus on region in brown box in panel A) at the interaction interface and involved in contacts between the two proteins are highlighted. Each structure was visualized using ChimeraX software.

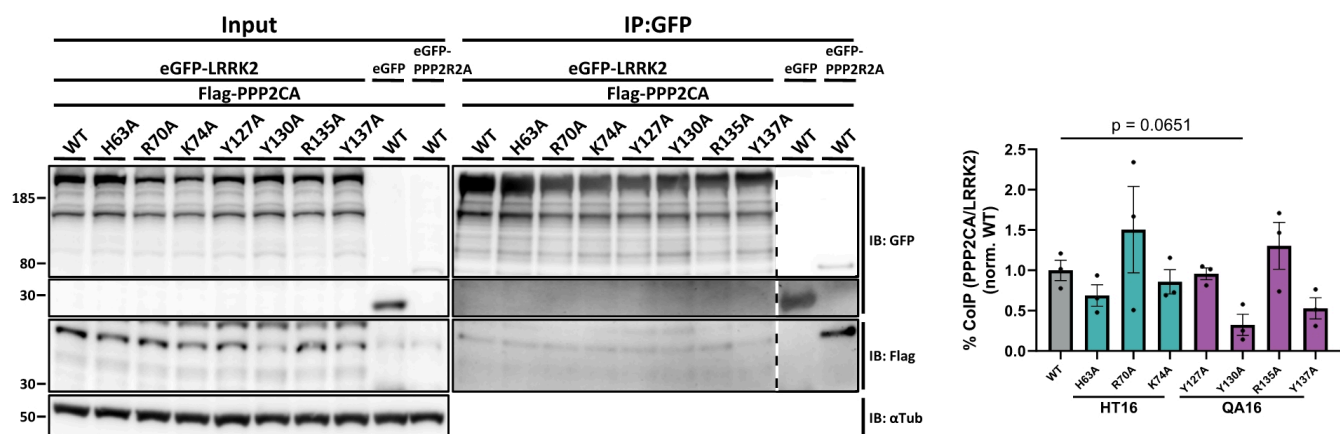


Figure 7

Co-immunoprecipitation of LRRK2 and PPP2CA peptide mutants. (A) HEK293T were co-transfected with eGFP-tagged LRRK2 and Flag-tagged PPP2CA containing alanine mutants at predicted hot spots for LRRK2:PPP2CA interaction within the HT16 and QA16 interfering peptide sequences and cell lysates subjected for immunoprecipitation with anti-GFP magnetic beads followed by western blot analysis with an anti-Flag or anti-GFP antibody. (B) Quantification of co-immunoprecipitation between LRRK2 and PPP2CA of blots depicted in (A). Bars represent mean $\pm$ s.e.m. (n=3 independent experiments).

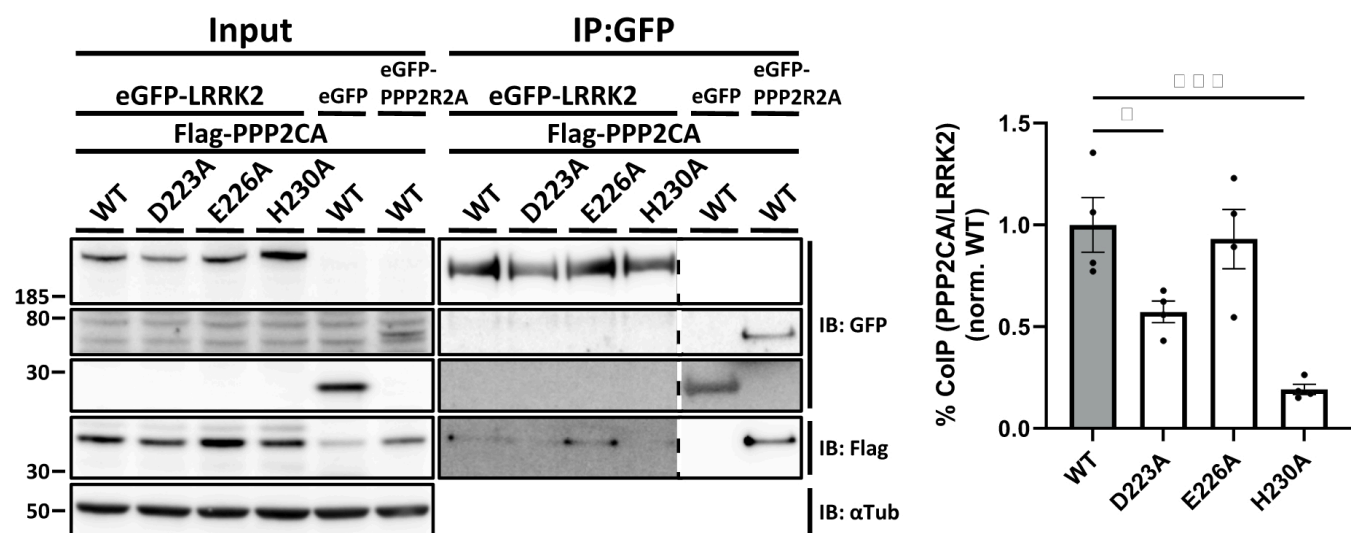


Figure 8

Co-immunoprecipitation of LRRK2 and PPP2CA mutants. (A) HEK293T were co-transfected with eGFP-tagged LRRK2 and Flag-tagged PPP2CA mutants and cell lysates subjected for immunoprecipitation

with anti-GFP magnetic beads followed by western blot analysis with an anti-Flag or anti-GFP antibody. (B) Quantification of co-immunoprecipitation between LRRK2 and PPP2CA of blots depicted in (A). Bars represent mean $\pm$ s.e.m. (n=4 independent experiments); *p < 0.05, ***p < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [LRRK2PPP2AmanuscriptSupplementalRawdata.pdf](#)