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UNIVERSITY OF CALIFORNIA SAN DIEGO

Regulation of Parkinson's Disease-linked LRRK2

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biochemistry and Molecular Biophysics

by Amalia Villagran

Suarez

Committee in charge:

Professor Andres Leschziner, Chair
Professor Elizabeth Komives, Co-Chair
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The Dissertation of Amalia Villagran Suarez is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2026

DEDICATION

I want to dedicate this work to my husband, Charbel Velez. Thank you for being a pillar of support throughout these five years, during which we both faced many challenges and accomplished many things, even with our boys Marco and Leonardo. I will always remember the nights you waited patiently for me after a long purification day so we could eat dinner together, the days you took care of Marco when I was working in the lab, and the mornings I had to leave early and you were very tired but still stepped up to be the best dad and husband. I am grateful for your love and support, and I will show you the best in our new journey, which will be your medical school.

To my sister, Lourdes Villagran, I cannot believe we made it. Two immigrant girls trying to adapt to a new country while pursuing a better life. You are my sister, but also my mother, best friend, and mentor when I needed love, support, and guidance throughout my life. I appreciate your effort, selflessness, and empathy during the happiest and saddest times in our lives.

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To my mentor, Andres Leschziner, and Professor Samara Reck-Peterson, I am grateful for your support and encouragement throughout these five years of my PhD. Like I always said, I am very lucky to have worked with you and lucky that I got to explore and advance my scientific knowledge under your guidance. Also, I am thankful for your patience and understanding while I balanced both my PhD and family life, as I know it is not very common, but people like you and Samara make science a better place, and it reflects on every member of the lab and their future.

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PUBLICATIONS

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- Sanz Murillo, M., **Villagran Suarez, A.**, Dederer, V., Chatterjee, D., Alegrio Louro, J., Knapp, S., Mathea, S., and Leschziner, A.E. (2023). Inhibition of Parkinson's disease-related LRRK2 by type I and type II kinase inhibitors: Activity and structures. *Sci. Adv.* 9, eadk6191.
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ABSTRACT OF THE DISSERTATION

Regulation of Parkinson's Disease-linked LRRK2

by

Amalia Villagran Suarez

Doctor of Philosophy in Biochemistry and Molecular Biophysics

University of California San Diego, 2026

Professor Andres Leschziner, Chair
Professor Elizabeth Komives, Co-Chair

Mutations in the Leucine Rich Repeat Kinase 2 (LRRK2) are the most common cause of familial and sporadic Parkinson's Disease. LRRK2 is a 286kDa multidomain protein, containing two catalytic domains: a Ras-like GTPase (ROC) and a Kinase domain. LRRK2 has been reported to localize to the cytosol in its inactive form and co-localize with membranes, where it becomes activated and carries out its function of phosphorylating its Rab substrates. For LRRK2 to perform this function, it must undergo conformational changes where LRRK2 is converted from its inactive form, in which the LRR is docked on top of the kinase domain blocking its access to the activation

site, to an undocked LRR configuration where the active site is available for a Rab substrate to bind and undergo catalysis. This regulation of LRRK2 has been suggested to be the result from the intercommunication of the two catalytic domains, in which the ROC domain must exchange GDP for GTP to undock the LRR from the kinase domain, allowing the kinase domain to be accessible and activate subsequently.

This thesis reports high-resolution CryoEM structures, in combination with cell-based and in vitro assays, to explore the roles of the Kinase and ROC domain in the activation of LRRK2. Chapter 2 focuses on the structural determination of full-length LRRK2 and the C-terminal half, known as LRRK2^{RCKW} (ROC, COR, kinase and WD40 domains), using type I and II kinase inhibitors. The results reveal differences in active site of the most common familial mutation G2019S compared to wild-type LRRK2, structurally demonstrating its resistant and providing the basis for drug targeting of the mutant. We also show that LRRK2 N-terminal domain must fully undock from the kinase domain for it to close and adopt the active conformation, as even with the presence of a type I kinase inhibitor, full-length LRRK2 cannot fully close its kinase domain compared to LRRK2^{RCKW}. Chapter 3 investigates the role of the ROC domain in modulating LRRK2's activation via ROC nucleotide exchange, resulting in the first high resolution structure of active LRRK2 bound to a GTP analog, alongside multiple high-resolution structures of active and autoinhibited states.

We also characterized LRRK2 bound to different Designed Ankyrin Repeat Proteins (DARPs) and obtained cellular and biochemical assay data showing that different DARPs can shift the equilibrium of LRRK2 from its closed, autoinhibited state to its open, active state. Our results revealed how LRRK2 activation is dependent on the nucleotide exchange of its ROC domain, providing the first active structure of full-length LRRK2. Cell and biochemical assays

showed that LRRK2 mutants hyperactivation occurs through different parts of the activation pathway, where ROC-COR mutants lead to undocking of the N-terminus domain, and the kinase mutant G2019S acts directly on the kinase domain to increase its activity. Together, the work presented in this thesis provides new insights into the mechanism of LRRK2 activation and provides a framework for therapeutic development.

Chapter 1 : Introduction to Parkinson's Diseases-linked LRRK2

Mutations on the Leucine Rich Repeats Kinase 2 (LRRK2) are a genetic risk factor for familial Parkinson's Diseases (PD)¹. Mutations are known to increase LRRK2 kinase activity, and this increase in activity is suggested to be linked to the diseases²⁻⁵. Idiopathic PD carrying a wildtype-LRRK2 also show increase kinase activity postmortem⁶, suggesting that LRRK2 can be used to treat different types of PD, as it plays a major role on the disease. In cells, LRRK2 can be found in the cytosol, where it keeps and binds to the family of protein known as 14-3-3^{6,7-10} (Figure 1.1A), co-localize in the microtubule, where it forms filaments in high expressing LRRK2 cells¹²⁻¹⁵, and on membranes, where it can activate and interact with its substrate (Figure 1.1B). LRRK2 causes defects in the trafficking of different membrane-bound organelles: endosomes, lysosomes, autophagosomes and mitochondria^{16,17} (Figure 1.1C). This has made LRRK2 an important target for PD therapeutics with clinical trials ongoing.

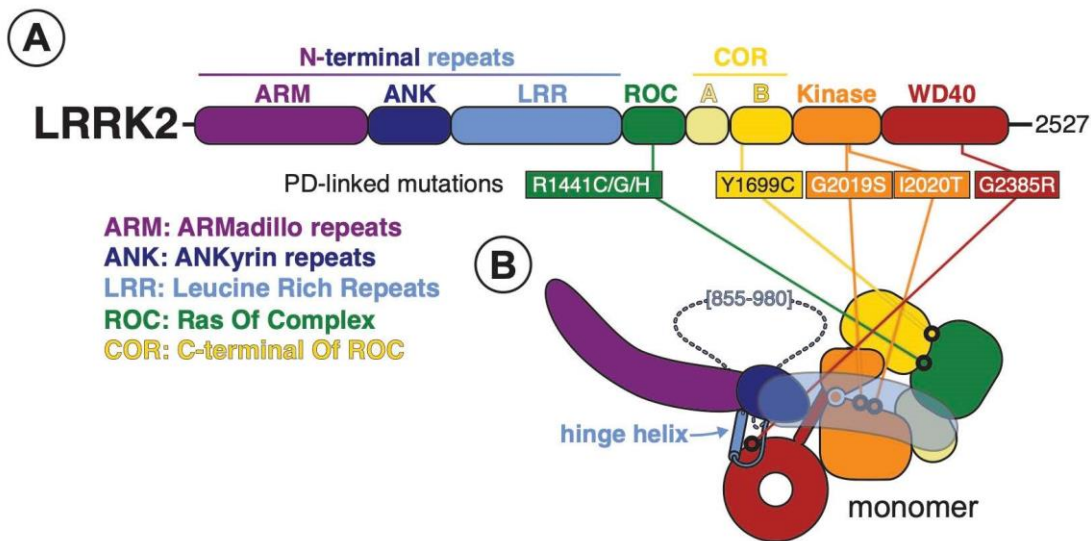


Figure 1.1. LRRK2 cellular localization.

Schematic representation of the cellular localization of LRRK2. **(A)** Cytosolic LRRK2 bound to the 14-3-3 dimer, maintaining autoinhibition. **(B)** Membrane LRRK2 interacting with Rabs and active. **(C)** Microtubule bound LRRK2 forming filaments.

LRRK2 is a large multi-domain 286kDa protein, it has an N-terminal half consisting of Armadillo (ARM), Ankyrin (ANK), and a Leucine Rich Repeats (LRR) domains, and a C-terminal half with two catalytic domains: Ras-family GTPases (Ras Of Complex, Roc) and a Kinase, alongside C-terminal OF ROC (COR) and WD40 domain¹⁸ (Figure 1.2). Previous structural work shows that LRRK2 is regulated by autoinhibition, the LRR which connects to the ROC GTPases domain, covers the kinase's active site, prompting it open and blocking access to its Rab substrates¹⁸. While LRRK2^{RCKW} show access to the active site of the Kinase domain and movement on the ROC-COR domains¹². Despite recent study proposed structures of LRRK2^{RCKW} bound to inhibitors generated using molecular dynamics, our findings show the first published experimental structures of inhibitor-bound LRRK2, an important gap in our understanding of this protein as a therapeutic target.

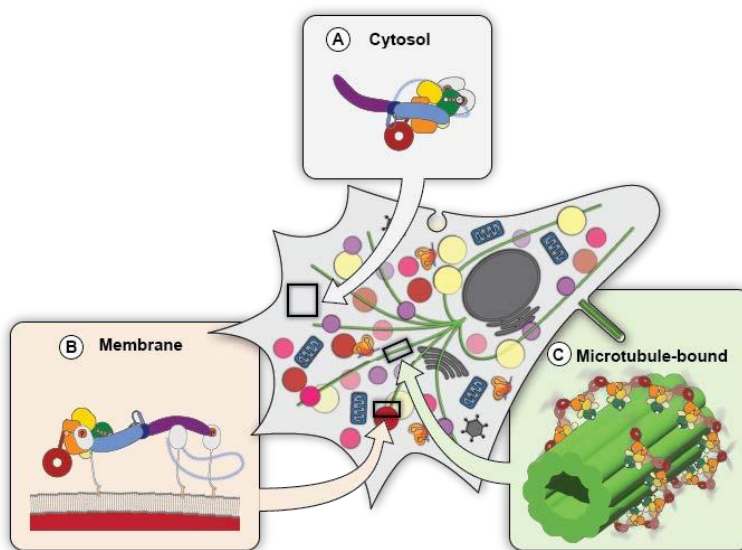


Figure 1.2 Domain architecture of LRRK2.

(A,B) Cartoon representation of LRRK2 domain architecture with the most common PD mutation located in the C-terminal half **(A)**, and structure of the LRRK2 monomer**(B)**.

Chapter 2 : Inhibition of Parkinson's disease-related LRRK2 by type I and type II kinase inhibitors: Activity and Structures

2.1 LRRK2 kinase inhibitors

Small molecules of diverse chemotypes have been developed as type-I kinase inhibitors for LRRK2 (Figure S1A). The first inhibitor to be introduced with an acceptable selectivity profile was LRRK2-IN-1¹⁹. However, its use for *in vivo* applications has been limited because of its low brain penetrance. HG-10-102-01, in contrast, was developed as a brain penetrant inhibitor²⁰, and its amino-pyrimidine based scaffold recently experienced a renaissance as a PROTAC warhead²¹. MLi-2, an inhibitor with high affinity and a superb selectivity profile has been important for the field²². Ever since its introduction in 2015 it has been regarded as the gold standard LRRK2 inhibitor and this chemical probe has been used in numerous studies, including clinical trials. Another powerful but less prominent inhibitor is PFE-360²³. Similar to MLi-2, its affinity for LRRK2 and its selectivity profile are both excellent. The last entry in our incomplete list is the type-I inhibitor DNL201 (developed as GNE0877)²⁴. This compound has been shown to efficiently inhibit LRRK2 in PD patients²⁵, and the related molecule DNL151 (renamed BIIB122) has currently entered phase 2b clinical testing (clinicaltrials.gov).

Less effort has been undertaken to develop type-II inhibitors for the LRRK2 kinase domain. To date, no selective type-II inhibitors have been published²⁶. However, several type-II inhibitors with a broad kinase target spectrum have been shown to bind to LRRK2 kinase with high affinity, namely ponatinib, GZD-824²⁷ and rebastinib²⁸. However, their numerous kinase off-targets prevent their application as chemical tools or for the treatment of PD.

The need for LRRK2 selective type-II inhibitors is not limited to their therapeutic potential.

The recent structural studies of LRRK2 listed above ^{27,29–32} have made it clear that conformational changes in LRRK2 driven by its kinase are likely to play central roles in its activation and subcellular localization. Having reagents that stabilize the LRRK2 kinase domain in either a closed, active-like conformation (type-I inhibitors), or in its open, inactive conformation (type-II inhibitors) would have a major impact on our ability to dissect its catalytic and scaffolding functions in cells and *in vivo*.

Here, we set out to bridge the gap in our understanding of the structural biology of LRRK2 inhibition. We present cryo-EM structures of LRRK2^{RCKW}, WT and carrying the PD-linked mutations G2019S and I2020T, bound to the LRRK2-specific type-I inhibitor MLI-2 or the broadspectrum type-II inhibitor GZD-824. We also present structures of full-length LRRK2 bound to these inhibitors to understand how the presence of the N-terminal repeats in LRRK2 affect inhibitor-induced conformational changes in the context of the full-length protein. The work presented here should help in the design of the next generation of LRRK2-specific inhibitors.

2.2 Results

Inhibitor binding stabilizes the LRRK2 kinase domain

The tight binding of small molecules to a protein usually stabilizes the protein and increases the temperature at which it denatures. We characterized the binding of diverse type-I and type-II inhibitors (Figure S2.1A, Table 2.1) by measuring the thermostability increase of a truncated LRRK2 protein consisting of its kinase and WD40 domains (“LRRK2^{KW}”) (Figure 2.1A) in the presence of inhibitors using differential scanning fluorimetry (DSF). Interestingly, the melting profile showed two clearly separated transitions most likely corresponding to the two domains, the kinase and the WD domain. In agreement with this hypothesis, only the melting curve of one of the phase transitions shifted in the presence of the diverse inhibitors tested suggesting that the transition at lower melting temperature corresponds to the kinase domain. However, in the absence of

inhibitors, LRRK2^{KW} was relatively unstable compared to other kinases (melting temperature (T_M) of 39°C)³³. Both type-I and type-II inhibitors stabilized LRRK2^{KW} substantially, with T_M shifts of up to 20°C, indicating strong binding to the kinase domain, while the melting curve of the WD domain remained unaffected (Figure S2.1B, D).

A mass spectrometry-based assay to monitor LRRK2 activity and inhibition

Increased kinase catalytic activity or impaired autoinhibition are features of LRRK2 PD variants^{34–37}. To compare the activity of the selected LRRK2 inhibitors, we developed a mass spectrometry (MS)-based kinase activity assay using an established endogenous substrate. We subjected the Rab8A GTPase domain (residues 6 to 176) to LRRK2^{RCKW} phosphorylation in the presence of varying concentrations of a given inhibitor and directly quantified the amount of phosphorylated Rab8A (pRab8A) by ESI-MS (Figure S2.1C). Most type-I and type-II inhibitors inhibited the phosphorylation reaction with IC₅₀ values between 7 and 50 nM. Surprisingly, the inhibitors HG-10-102-01 and LRRK2-IN-1 stood out with IC₅₀ values in the high nanomolar range. Altogether, there was a good correlation between the thermal shift data and the IC₅₀ values (Figure S2.1D).

Cryo-EM structures of LRRK2^{RCKW}(G2019S) bound to type-I and type-II inhibitors

To understand how inhibitors of the two main canonical types interact with the catalytic domain of LRRK2, we began by determining cryo-EM structures of LRRK2^{RCKW}, a well characterized construct consisting of the C-terminal half of LRRK2 containing all catalytic domains (Figure 2.1A)^{27,28,32,38}, bound to the LRRK2-specific inhibitor MLI-2 (type I) or the broad-spectrum GZD-824 (type II), in the presence of GDP as a ligand for the GTPase (ROC) domain. We used a variant of LRRK2^{RCKW} carrying the G2019S mutation, the most common mutation

associated with PD³⁹ and one of two, along with I2020T, located in the kinase domain (Figure 2.1, Figures S2.3, S2.4, and S2.5, Table S2.1).

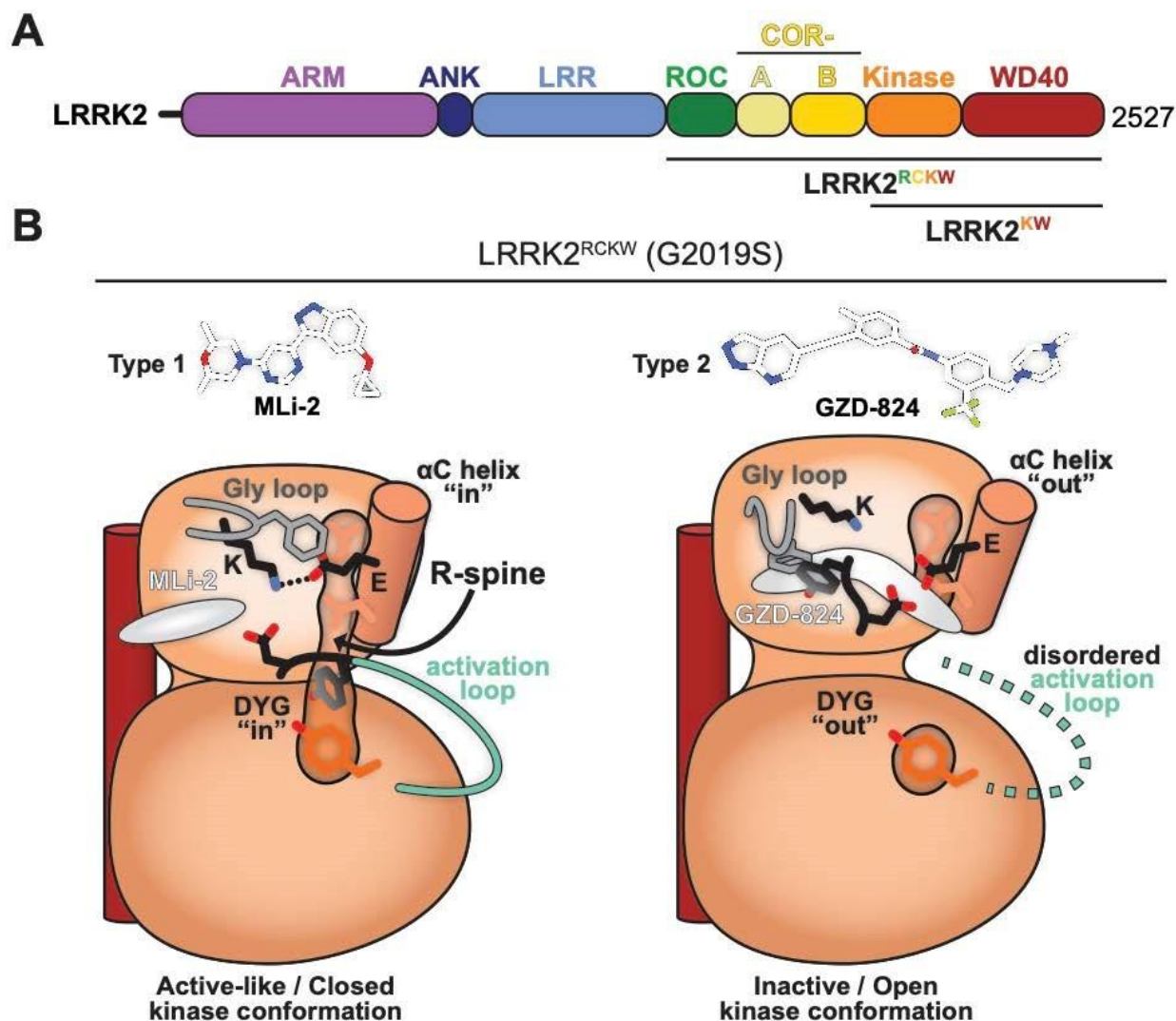


Figure 2.1 LRRK2^{RCKW}(G2019S) bound to MLI-2 and GZD-824.

(A) Domain architecture of LRRK2. The color coding of domains is used throughout this work. The three constructs used in this study—LRRK2, LRRK2^{RCKW}, and LRRK2^{KW}—are indicated. (B) Cartoon summary of the main features of the structures presented here. The inhibitors used, the type-I MLI-2 and type-II GZD-824, are shown in stick representation.

We solved these structures, as well as all additional cryo-EM structures reported here, bound to a LRRK2-specific Designed Ankyrin Repeat Protein (DARPin)⁴⁰ that we refer to as “E11”. This

DARPin was developed screening LRRK2^{RCKW}, and it tightly bound to its WD40 domain ⁴¹. We have found that adding the E11 DARPin to our cryo-EM samples consistently leads to structures with better resolution, most likely due to a reduction in the preferred orientation that LRRK2 and LRRK2^{RCKW} tend to adopt on cryo-EM grids, although we cannot rule out a contribution from their additional mass ⁴¹. For simplicity, we will omit “E11” from the names of the complexes discussed in this work. The resolutions of our maps were sufficient to reveal the molecular details of the kinase active site. The MLi-2 map had an overall resolution of 2.74Å and local resolutions as high as 2.4Å around the kinase’s active site (Figure S2.4 and Table S2.1). The GZD-824 map had an overall resolution of 3.11Å and local resolutions as high as 2.7Å around the kinase’s active site after focused refinement (Figure S2.5 and Table S2.1).

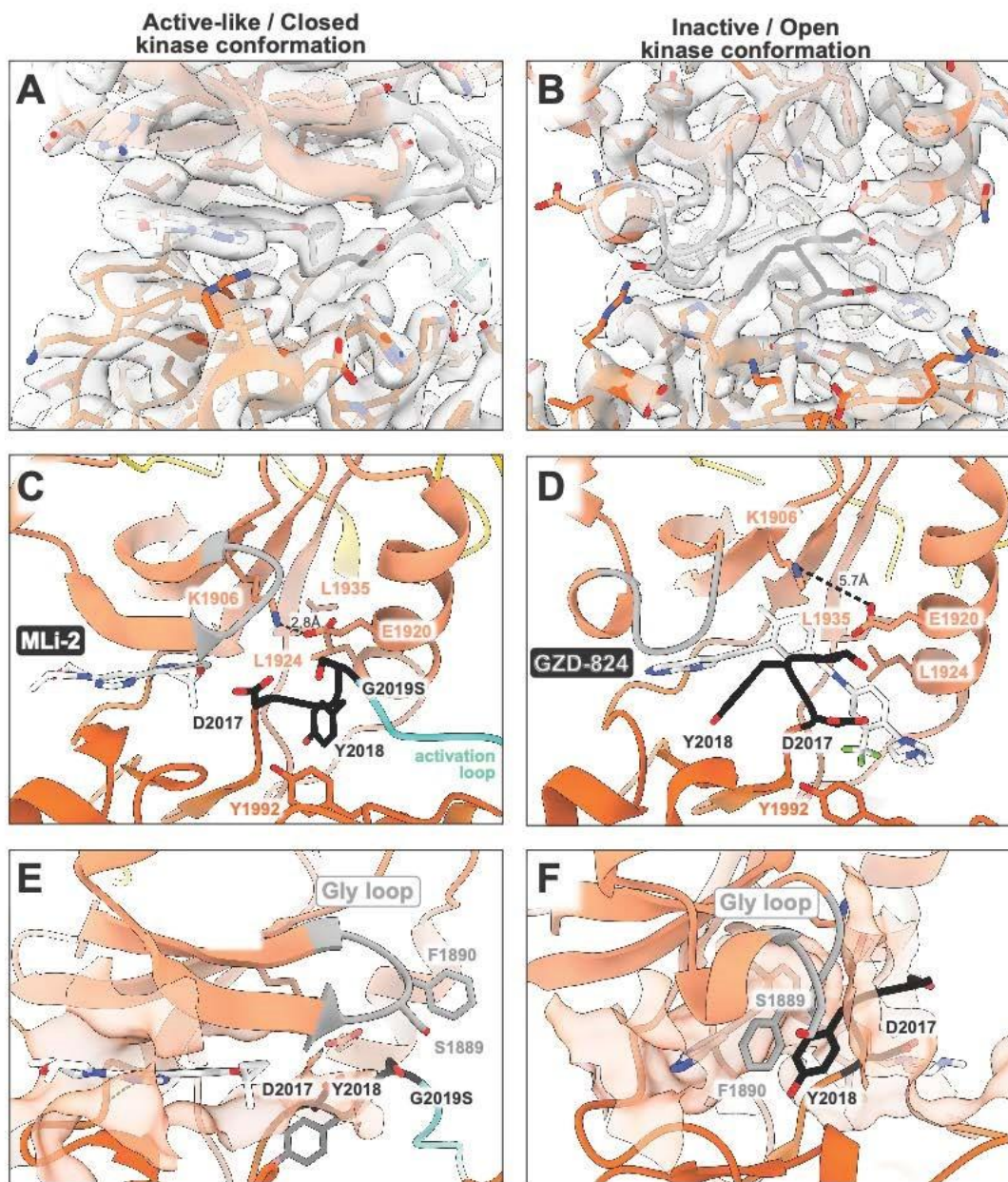


Figure 2.2 CryoEM of LRRK2^{RCKW}(G2019S) bound to MLI-2 and GZD-824.

(A,B) Cryo-EM maps and models for the inhibitor binding site and surrounding regions for MLI-2 (C) and GZD-824 (D) bound to LRRK2^{RCKW}(G2019S). (C,D) Close-up of the inhibitor binding site and kinase active site for the same structures shown in (A,B). Major features (G-loop, Lys1906Glu1920 interaction, DYG motif, R-spine, and activation loop) are indicated. (E,F) Inhibitor binding pocket. The semitransparent orange surface indicates the solvent accessible surface for those residues in contact with the inhibitor. Some of the features highlighted in (C,D) are shown here as well.

Figure 2.1B summarize the main features of the two structures. MLi-2 bound as expected in the ATP-binding pocket of LRRK2's kinase, with its indazole group making hydrogen bonds with the backbones of E1948 and A1950 in the kinase hinge region, as recently proposed based on molecular dynamics simulations³⁸. In contrast to those simulations, however, the glycine rich (G) loop was extended in our structure, as expected for an active kinase domain and F1890, located at the tip of the G-loop, was not involved in coordinating MLi-2. Also as expected, MLi-2 binding stabilized the DYG "in" and α C "in" conformation, a fully formed R-Spine, and an ordered activation loop, (Figure 2.1B, 2.2C,E). (Note: even though we will continue using the "DYG" nomenclature throughout the paper, DYG is DYS in the G2019S mutant.) We observed an inactive conformation with DYG "out", α C "out", a broken R-Spine, and a disordered activation loop in the presence of GZD-824 (Figure 2.1B,2.2D,F). Binding of GZD-824 to the ATP-binding pocket was shifted away from the hinge region relative to MLi-2; its pyrazolopyridine group partially overlapped with the location of the pyrimidine and indazole groups of MLi-2, with the rest of the molecule extending towards and past the α C helix. As expected from a larger inhibitor, the binding pocket for GZD-824 was enlarged by the DYG-out movement and opening of the allosteric back pocket, involving all the main components of the kinase's active site (Figure 2.2F). A major difference between the two structures was the conformation of their G-loops (Figure 2.2E,F). While the G-loop was in an extended conformation in LRRK2^{RCKW}(G2019S):MLi-2 (Figure 2.2E), it was sharply bent in LRRK2^{RCKW}(G2019S):GZD-824, with F1890 interacting with the pyrazolopyridine group in GZD-824 (Figure 2.2F). In turn, Y2018 from the DYG motif interacted with F1890 (Figure 2.2F).

Structural basis for decreased affinity of MLi-2 for LRRK2(G2019S)

G2019S is the most frequently identified LRRK2 mutation in PD patients³⁹. Although the

G2019S mutation increases LRRK2 kinase activity, as do the other most common mutations ³⁴⁻³⁷, LRRK2(G2019S) is unusual: it is the only variant that does not increase microtubule association in cells ^{42,43}, it shows the highest levels of autophosphorylation at S1292 ⁴⁴, and this mutation has been reported both to provide resistance to inhibition by MLi-2 ⁴⁵, and to be more sensitive to this inhibitor ²⁶. We therefore aimed to visualize, structurally, any differences that may exist in how inhibitors interact with LRRK2 carrying different PD-linked mutations. For this, we solved cryoEM structures of LRRK2^{RCKW} wild-type (WT), and I2020T, bound to MLi-2 (Figure 2.3, Figures S2.5, S2.6, and S2.7, Table S2.1) and compared them to the structure of LRRK2^{RCKW}(G2019S):MLi-2 presented above. We chose the I2020T mutation because of the different properties exhibited by G2019S and I2020T despite affecting neighboring residues. As it was the case with the structure of G2019S bound to MLi-2, these maps were of high enough resolution to reveal the details of the kinase's active site. The WT map had an overall resolution of 3.05Å and local resolutions as high as 2.8Å around the kinase's active site (Figure S2.6 and Table S2.1), while the I2020T map had an overall resolution of 2.74Å and local resolutions as high as 2.5Å around the kinase's active site (Figure S2.7 and Table S2.1).

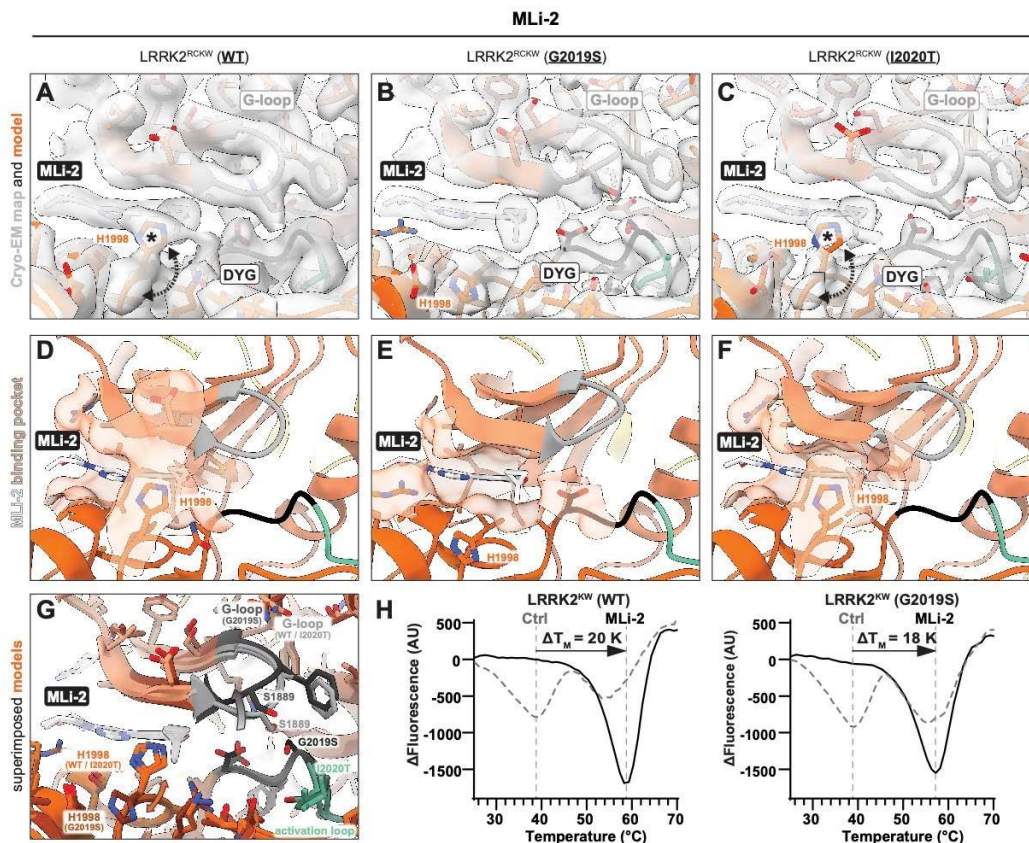


Figure 2.3 Binding of MLI-2 to LRRK2^{RCKW} wild-type (WT), G2019S, and I2020T.

(A-C) Cryo-EM maps and models for the MLI-2 binding site and surrounding regions for LRRK2^{RCKW} WT (A), G2019S (B), and I2020T (C). The asterisks in (A) and (C) highlight densities present in the WT and I2020T maps but absent from the G2019S one. The dashed arrows in (A) and (C) indicate that two different rotamers of H1998 can be accommodated by the maps of WT and I2020T. The rotamer pointing “up” (towards the kinase N-lobe) is shown in these panels. Note that only density accounting for the “down” rotamer is seen in G2019S (B). The Gloop and DYG motif are indicated in all three panels. (D-F) Inhibitor binding pocket. The semitransparent orange surface indicates the solvent accessible surface for those residues in contact with MLI-2. For LRRK2^{RCKW} WT and I2020T, these surfaces were generated using the “up” rotamer of H1998. The “up” rotamer of H1998 leads to a closing of the binding pocket in WT and I2020T (D, F) that is absent in G2019S (E). (G) Superposition of the models for MLI-2 bound to LRRK2^{RCKW} WT, G2019S, and I2020T. G2019S is shown in darker shades. The grey gradient-colored arrow in the G-loop indicates the movement of the loop in G2019S relative to WT and I2020T. The two-headed orange arrow indicates that two rotamers of H1998 in LRRK2^{RCKW} WT and I2020T can account for the density in our cryo-EM maps. (H) Differential Scanning Fluorimetry data measured for LRRK2^{RCKW} (WT) and LRRK2^{RCKW} (G2019S) in the presence and absence of MLI-2. In the absence of inhibitor, both proteins showed identical melting points suggesting that the mutant did not affect stability of the recombinant protein. Binding of the inhibitor resulted in a lower ΔT_M for LRRK2^{RCKW} (G2019S), suggesting weaker binding affinity of MLI-2.

Although the three structures—WT, G2019S, and I2020T—were very similar (Figure 2.3), two features were unique to LRRK2^{RCKW}(G2019S):MLi-2. First, the presence of a Ser instead of a Gly at position 2019 introduced a clash with the G-loop that pushed S1889 away from S2019 (Figure 2.3A-C,G). The second was an unexplained density we observed in the LRRK2^{RCKW}(WT):MLi-2 and LRRK2^{RCKW}(I2020T):MLi-2 cryo-EM maps, but not in that of LRRK2^{RCKW}(G2019S):MLi-2 (Figure 2.3A-C). This additional density in WT and I2020T could accommodate an alternative rotamer for H1998 (Figure 2.3A,B,G), which closes the binding site around MLi-2 (Figure 2.3D-F). Given the absence of density for this rotamer in G2019S, we predicted weaker binding of MLi-2 to LRRK2's kinase carrying the G2019S mutation. Although the accurate determination of sub-nanomolar inhibitor affinities for complex proteins is challenging, our DSF assay showed that the addition of MLi-2 to LRRK2^{KW}(G2019S) resulted in less thermal stabilization than that observed with LRRK2^{KW}(WT) (Figure 2.3H).

Structures of PD variants of LRRK2^{RCKW} bound to the type-II inhibitor GZD-824

Next, we solved cryo-EM structures of LRRK2^{RCKW} WT and I2020T bound to the broadspectrum type-II inhibitor GZD-824 (Figure 2.4, Figures S2.5, S2.8, and S2.9, Table S2.1) and compared them with the structure of the G2019S bound to GZD-824 discussed above. As with the structure of G2019S bound to GZD-824, the resolution of the maps allowed us to visualize the details of the kinase's active site. The WT map had an overall resolution of 2.9Å and local resolutions as high as 2.8Å around the kinase's active site (Figure S2.8 and Table S2.1), while the I2020T map had an overall resolution of 3.22Å and local resolutions as high as 2.8Å around the kinase's active site (Figure S2.9 and Table S2.1).

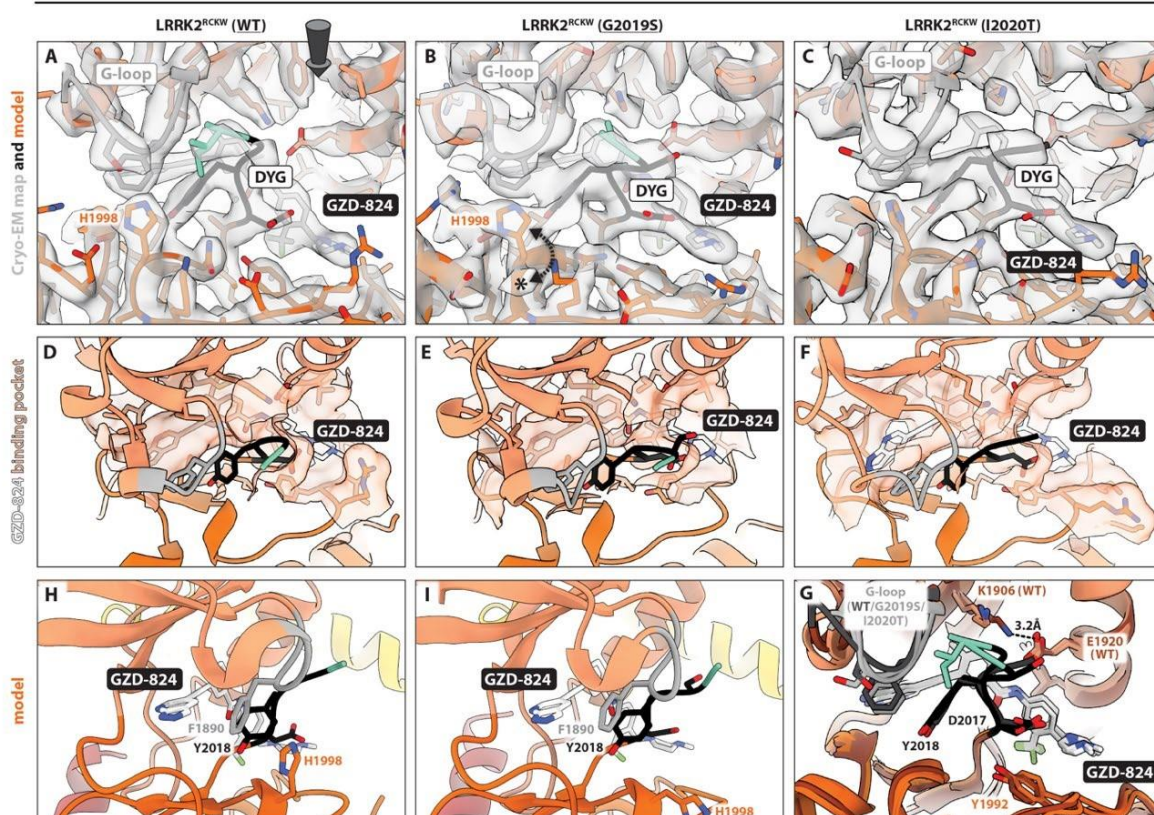


Figure 2.4 Binding of GZD-824 to LRRK2^{RCKW} wild-type (WT), G2019S, and I2020T.

(A,C) Cryo-EM maps and models for the GZD-824 binding site and surrounding regions for LRRK2^{RCKW}(WT):GZD-824 (A), LRRK2^{RCKW}(G2019S):GZD-824 (B), and LRRK2^{RCKW}(I2020T):GZD-824 (C). The asterisks in (B) highlights a density present in G2019S but absent in WT. The dashed arrow in (B) indicate that two different rotamers of H1998 can be accommodated by the map of G2019S. The rotamer that points “up” (towards the N-lobe of the kinase) is shown in panel (B). Note that only density accounting for the “up” rotamer is seen in WT (A). The G-loop and DYG motif are indicated in the panels. The arrow in panel (A) indicates the viewing direction for panels (D-F). (D-F) Inhibitor binding pocket. The semitransparent orange surface indicates the solvent accessible surface for those residues in contact with GZD-824. (G) Superposition of the models for GZD-824 bound to LRRK2^{RCKW} WT, G2019S, and I2020T. WT is shown in darker shades. (H,I) Pi-pi interactions in the GZD-824 binding pocket. The GZD-824 binding pocket is viewed from the direction of the kinase’s hinge motif for WT (H) and G2019S (I). The G-loop’s F1890 faces the pyrazolopyridine ring of the inhibitor and, in turn, interacts with the Y2018 of the DYG motif. H1998 is parallel to F1890 in the WT structure, where it adopts the “up” rotamer (H). G2019S can accommodate two rotamers for H1998 (B), and the model in (I) shows H1998 in its “down” rotamer, which places it away from the inhibitor’s binding site.

As with MLI-2, the three structures—WT, G2019S, and I2020T—were very similar (Figure 2.4G). Although all three structures assumed a DYG “out”, α C “out” conformation with a broken R-Spine and a disordered activation loop, the WT cryo-EM map showed a closer distance between K1906 and E1920 (Figure 2.4A,G). In all three cases, the G-loop was folded over the inhibitor to interact with its pyrazolopyridine and Y2018 from the DYG motif, as described above (Figure 2.4A-C,G).

We noticed differences in the rotamers adopted by H1998 in these structures, as had been the case with the MLI-2 ones. Here, however, the cryo-EM map for LRRK2^{RCKW}(G2019S):GZD824 could accommodate two rotamers for H1998 (Figure 2.4B), while WT (Figure 2.4A) showed density for a single rotamer. Although LRRK2^{RCKW}(I2020T):GZD-824 appeared to also show a single rotamer (Figure 2.4C), this cryo-EM map was of lower resolution and more anisotropic, making it difficult to establish this side chain conformation unambiguously. A potential role for H1998 in the GZD-824-bound structures is in the stabilization of F1890 in the G-loop, which packed against the pyrazolopyridine ring of the inhibitor (Figure 2.4H). The presence of an alternative rotamer in G2019S (Figure 2.4I) could result in weaker binding of GZD-824 to this mutant. This prediction is consistent with a study showing that GZD-824 inhibits LRRK2(WT) in an *in vitro* phosphorylation assay with an IC₅₀ ~4-fold lower than that for LRRK2(G2019S) ²⁶.

Structures of full-length LRRK2 bound to MLI-2 and GZD-824

On cryo-EM grids, LRRK2 monomers were shown to adopt an autoinhibited conformation where the N-terminal repeats (ARM-ANK-LRR) sterically block access to the kinase’s active site ²⁹. The repeats, which connect to the ROC domain, are held in place by interactions between the LRR domain and the kinase, and the ANK and WD40 domains ²⁹. This anchoring of the repeats restricts conformational changes within LRRK2. To determine whether the presence of the repeats would prevent some of the conformational changes we observed in the structures we obtained with

LRRK2^{RCKW}, we solved structures of full-length LRRK2(I2020T) bound to either MLi-2 or GZD824 (Figure 2.5, Figures S2.10 and S2.11, Table S2.1). As it was the case for all LRRK2^{RCKW} structures reported here, we solved the full-length ones in the presence of the E11 DARPin and GDP. The maps of full-length LRRK2 were of lower resolution than those for LRRK2^{RCKW}, but still sufficient to determine the main features of the kinase's active site. The map of full-length LRRK2 bound to MLi-2 had an overall resolution of 3.9Å, with a slightly higher local resolution of 3.8Å around the kinase's active site (Figure S2.10 and Table S2.1). The map of full-length LRRK2 bound to GZD-824 had an overall resolution of 3.4Å, with a slightly higher local resolution of 3.3Å around the kinase's active site (Figure S2.11 and Table S2.1).

As expected from the anchoring effect of the repeats, both LRRK2(I2020T):MLi-2 and LRRK2(I2020T):GZD-824 had their ROC-COR domains in a substantially more open (i.e. further away from the kinase) conformation than that seen in the equivalent structures with LRRK2^{RCKW} (Figure 2.5A,B). Next, we compared the kinase domains to see whether this difference in global conformation reflected differences in the states of the kinases themselves between LRRK2 and LRRK2^{RCKW} (Figure 2.5C-F). This was the case for MLi-2: while our structure of LRRK2^{RCKW}(I2020T):MLi2 showed an active-like, DYG “in”, α C “in” conformation with an ordered activation loop, the kinase in LRRK2(I2020T):MLi-2 had its α C in an “out” conformation and a disordered activation loop. This difference, in contrast, was absent from the GZD-824 structures; the kinases in LRRK2(I2020T):GZD-824 and LRRK2^{RCKW}(I2020T):GZD-824 were almost identical, the only difference being the formation of the salt bridge between K1906 and E1920 in LRRK2(I2020T):GZD-824 (Figure 2.5E,F).

To better understand this difference between MLi-2 and GZD-824, we compared the four structures carrying the I2020T mutation—LRRK2(I2020T):MLi-2, LRRK2^{RCKW}(I2020T):MLi2, LRRK2(I2020T):GZD-824, and LRRK2^{RCKW}(I2020T):GZD-824—by aligning them using the C-

lobe of their kinases (Figure 2.5G,H). We used vectors connecting equivalent α carbons to illustrate the conformational changes, and we quantified differences between structures by measuring the distance between the α carbons of M1335, a residue located at the beginning of the ROC domain (Figure 2.5G,H). This analysis suggested that the repeats may be preventing the kinase in LRRK2(I2020T):MLi-2 from reaching its fully closed / active-like conformation. While both MLi2 and GZD-824 led to relatively similar conformational differences between LRRK2 and LRRK2^{RCKW}, MLi-2 did seem to result in a slightly more closed conformation in LRRK2^{RCKW} (0.9Å between the α carbons of M1335) (Figure 2.5G), although we cannot determine whether this difference is significant at this point. The differences between the two types of inhibitors increased when we compared LRRK2(I2020T):MLi-2 to LRRK2(I2020T):GZD-824, and LRRK2^{RCKW}(I2020T):MLi-2 to LRRK2^{RCKW}(I2020T):GZD-824 (Figure 2.5H). As expected, the type-I inhibitor MLi-2 led to a more closed kinase conformation in LRRK2 than the type-II GZD824 (Figure 2.5H, left); the ROC domain in the MLi-2 structure is shifted towards the kinase by 3.4Å as measured by the distance between the M1335 residues. This difference increased to 5.8Å in LRRK2^{RCKW}, where the N-terminal repeats are absent(Figure 2.5H, right). Taken together, these observations suggested that the inability of MLi-2 to induce a fully closed / active-like state in the kinase of LRRK2(I2020T):MLi-2 was a result of the conformational constraining brought about by the anchored N-terminal repeats. Although we do not yet understand, mechanistically, what physiological processes lead to the release of the N-terminal repeats of LRRK2, and thus to full activation of the kinase, our work shows that MLi-2 was able to interact with the kinase both in the active-like state (LRRK2^{RCKW}) and in an intermediate activation state (LRRK2) (Figure 2.5C,D).

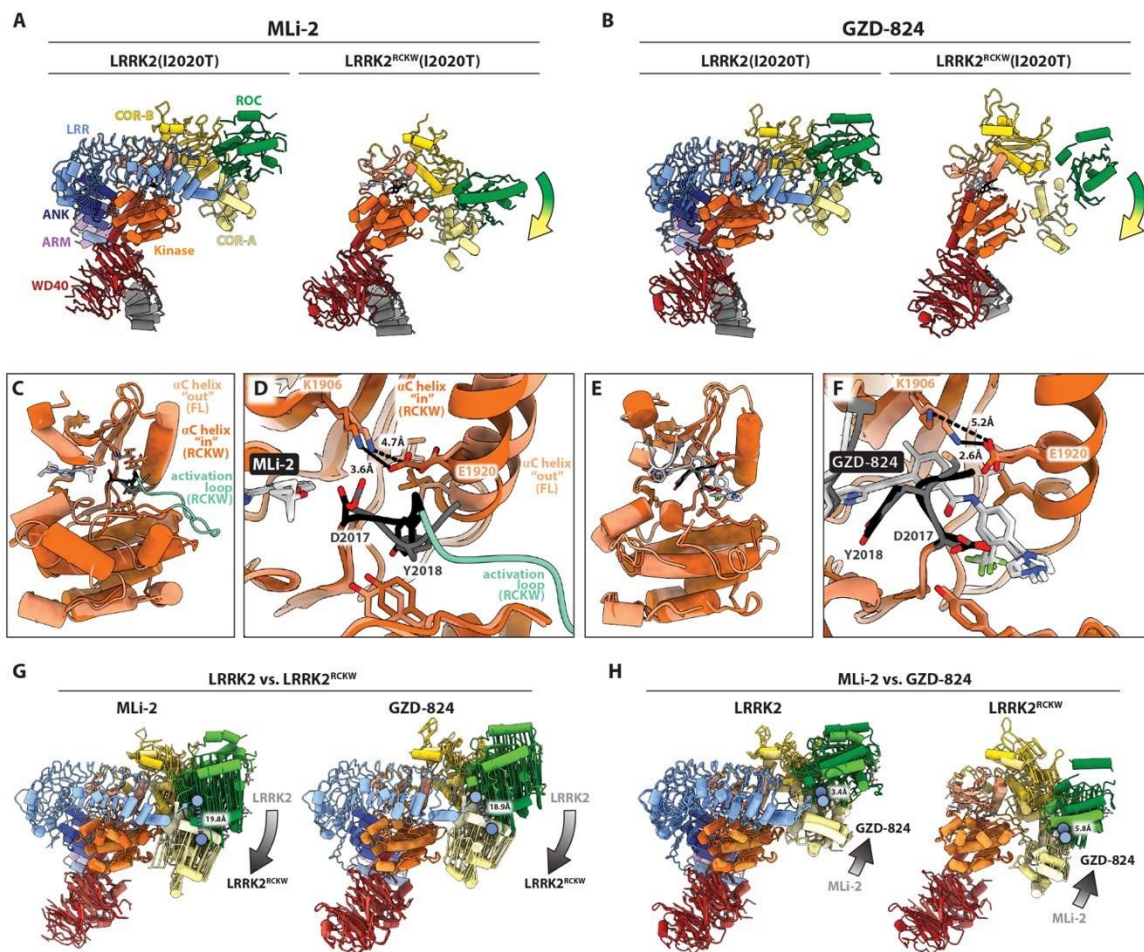


Figure 2.5. Structures of MLI-2 and GZD-824 bound to full-length LRRK2(I2020T)

(A) Structures of LRRK2(I2020T) (left) and LRRK2^{RCKW}(I2020T) (right) bound to MLI-2. (The structure of LRRK2^{RCKW}(I2020T):MLi-2 is the same one shown in **Figure 2**.) The colored arrow indicates the movement of the ROC-COR domains in LRRK2^{RCKW} relative to full-length LRRK2. (B) Same as in (A), for GZD-824. (The structure of LRRK2^{RCKW}(I2020T):GZD-824 is the same one shown in **Figure 3**.) (C) Overlay of the kinase domains from LRRK2(I2020T) and LRRK2^{RCKW}(I2020T) bound to MLI-2, highlighting the “out” conformation of the α C helix. In panels (C) to (F), lighter shades represent LRRK2 and darker shades represent LRRK2^{RCKW}. (D) Close-up of the kinase active sites in (C), with major features labeled. (E) Overlay of the kinase domains from LRRK2(I2020T) and LRRK2^{RCKW}(I2020T) bound to GZD-824, highlighting their similarity. (F) Close-up of the kinase active sites in (C), with major features labeled. (G) Conformational differences between LRRK2 and LRRK2^{RCKW} bound to either MLI-2 (left) or GZD-824 (right). Structures were aligned using the C-lobes of their kinase domains. Colored vectors connect the same α carbons between the two structures. The gradient arrows highlight the direction of the overall relative movement of the ROC-COR moiety. The blue circles mark M1335 at the beginning of the ROC domain; the distance separating the α carbons of M1335 in the two structures being compared is indicated. (H) Differences in the conformations adopted by LRRK2 (left) or LRRK2^{RCKW} (right) in the presence of either MLI-2 or GZD-824. All labels and indicators are the same as those in (G).

2.3 Discussion

Here, we have presented cryo-EM structures of LRRK2 and LRRK2^{RCKW} bound to the LRRK2-specific type-I inhibitor MLi-2, and the broad-spectrum type-II inhibitor GZD-824. These structures revealed the active-like state of LRRK2's kinase (in the case of MLi-2), and the structural differences involved in engaging the two main types of inhibitors.

By comparing LRRK2^{RCKW}(WT) with variants carrying the PD-linked mutations G2019S and I2020T, both of which are found within the kinase catalytic domain, we have begun to understand how some of these mutants might affect inhibitor binding. Similarly, comparing structures of inhibitor-bound LRRK2 and LRRK2^{RCKW} provided insights into how the N-terminal repeats of LRRK2 impact the interactions with inhibitors by constraining its conformational flexibility. These points are discussed further below.

The role of the G2019S mutation in LRRK2 inhibition

A comparison of our structures of LRRK2^{RCKW}(WT, G2019S, and I2020T) bound to MLi2 showed a difference in the conformations that H1998, located in the C-lobe, could adopt (Figure 2.3). The cryo-EM maps of WT and I2020T showed density that could be accounted for by two different rotamers of H1998, while G2019S could only accommodate one. Importantly, one of the rotamers in WT and I2020T, the one absent in G2019S, contributed to a larger MLi-2-binding interface that closed around the inhibitor. The absence of this additional interaction in G2019S suggested that MLi-2 might bind to G2019S with lower affinity, a prediction that was consistent with our DSF measurements of LRRK2^{KW} upon addition of MLi-2 (Figure S2.1).

A paper from the West group reported that the G2019S mutation made LRRK2 resistant to inhibition by MLi-2 *in vivo*. Although this would appear to agree with our structural and functional data, this study relied on measuring the loss of LRRK2-S935 phosphorylation as a proxy for

inhibition⁴⁵. While this is a frequently used biomarker for LRRK2 inhibition, it is a correlative one that is not directly dependent on LRRK2's kinase activity. A direct measurement of LRRK2's kinase activity, by quantifying the levels of Rab10 phosphorylation, was not possible in that study⁴⁵. On the other hand, data published by the Alessi group using purified LRRK2 and an *in vitro* assay with a peptide kinase substrate came to the opposite conclusion: that MLI-2 inhibited LRRK2(G2019S) ~2-fold better than LRRK2(WT)²⁶. As we discuss below, we hypothesize that these discrepancies may reflect the type of construct used (full-length LRRK2 in the Alessi study, LRRK2^{RCKW} in ours), and the potential roles played by the N-terminal repeats of LRRK2 in conformational changes and inhibitor binding. A better understanding of the potential effect of H1998 on the inhibition of different PD variants of LRRK2 will require combining *in vitro* assays with H1998 mutants in the context of an activated LRRK2, either by the removal of its N-terminal repeats or by more physiological means yet to be determined, with structural characterization of changes in the active site brought about by the combination of the PD and H1998 mutations.

We also saw differences in the rotamers that H1998 could adopt in our structures of LRRK2^{RCKW} bound to GZD-824 (Figure 2.4). In this case, the cryo-EM map of LRRK2^{RCKW}(G2019S):GZD824 showed density indicative of two rotamers for H1998, only one of which would contribute to inhibitor binding, while we only saw the latter in the cryo-EM map of the WT complex. The prediction, based on this structural difference, that LRRK2(WT) might bind more tightly than G2019S to GZD-824 was consistent with *in vitro* measurements of the IC₅₀ values for GZD-824 for these two variants²⁶. The G-loop adopted slightly different conformations in our LRRK2^{RCKW}(WT):GZD-824 and LRRK2^{RCKW}(G2019S):GZD-824 structures, and this could in principle have an effect on H1998, which is close to the G-loop in its “up” rotamer (Figure 2.4A,H). However, our model of LRRK2^{RCKW}(I2020T):GZD-824 was very similar to the one of G2019S (Figure 2.4G), yet the corresponding cryo-EM map appeared to show density for a single

H1998 rotamer, equivalent to that in WT. The caveat, which we noted earlier, is that the cryo-EM map for I2020T was of lower resolution and more anisotropic than those for WT and G2019S, so it was not possible at this point to rule out the possibility that the conformation of the G-loop was responsible for the difference between WT and G2019S mutant.

The role of the N-terminal repeats in LRRK2 inhibition

The N-terminal repeats of LRRK2 appear to play a double role in autoinhibition. On the one hand, the LRR domain physically blocks access to the kinase's active site²⁹. On the other, the LRR restricts the conformational flexibility of the C-terminal half of LRRK2 (LRRK2^{RCKW}), to which it is connected at the ROC domain, by interacting with the both the kinase (via the LRR domain) and the C-terminal helix (via the ANK domain)²⁹. Our analysis of the conformational differences between LRRK2 and LRRK2^{RCKW} bound to MLi-2 and GZD-824 (Figure 2.5) suggested that the constraints imposed by the N-terminal repeats had a bigger effect on type-I inhibitors. While our structures of LRRK2^{RCKW}:MLi-2 (Figures 2.1-3) showed the kinase in a fully closed / active-like state, as would be expected from a type-I inhibitor, the kinase in LRRK2(I2020T):MLi-2 was only partially closed, with its α C “out” and a disordered activation loop. We attribute this intermediate state to the tug-of-war between the MLi-2-induced closing of the kinase, and the constraining effect of the N-terminal repeats. We did not see this difference between LRRK2(I2020T):GZD-824 and LRRK2^{RCKW}(I2020T):GZD-824; the kinases in the two structures, in an inactive / open conformation, were almost identical (Figure 2.4).

Taken together, these comparisons suggest that the N-terminal repeats of LRRK2 prevented the kinase from reaching the fully closed / active-like state. At the same time, our structures showed that MLi-2 can bind to LRRK2 regardless of whether the kinase is fully closed

(LRRK2^{RCKW}) or in an intermediate activation state (LRRK2). Our results raise the possibility that IC₅₀'s measured using full-length LRRK2 (*in vitro*, in cells or *in vivo*) may be a combination of IC₅₀'s for at least two different populations: one population corresponds to the autoinhibited LRRK2, where the kinase cannot reach its fully active-like state, represented by our LRRK2(I2020T):MLi-2 structure while the other population corresponds to activated LRRK2, where the repeats have undocked and the kinase can reach its active-like state, represented by our LRRK2^{RCKW}(G2019S):MLi-2 structure.

Given the importance played by the N-terminal repeats in physiological LRRK2 activation, and likely in therapeutic LRRK2 inhibition, an important focus of future studies will be the role played by LRRK2's GTPase (the ROC domain) in its regulation. Since the ROC domain is where the N-terminal repeats join the C-terminal half of LRRK2 (LRRK2^{RCKW}), small conformational changes in the ROC domain could have an important effect on the position of the lever-like Nterminal repeats. A better understanding of this potential regulatory role for the ROC domain will require combining functional assays and structural studies where the nucleotide state of the GTPase can be precisely controlled.

Another element in LRRK2 likely to play a role in regulating the N-terminal repeats, and thus LRRK2's activation, is a loop in the LRR domain containing three phosphorylation sites: S910, S935, and S955. Phosphorylation of S910 and S935 is involved in recruiting 14-3-3 proteins, which are involved in keeping LRRK2 in the soluble cytosolic pool ^{46,47}. An important question is whether phosphorylation of these sites is dependent on the conformation of the N-terminal repeats. Recent work has shown that while treatment with type-I inhibitors leads to dephosphorylation of S935 in cells, treatment with type-II inhibitors does not ²⁶, suggesting that this effect may be dependent on the conformation of the N-terminal repeats.. So far, the loop containing these residues has been disordered in both the previously published structures of full-length LRRK2 ²⁹ and those

reported here. Since all these structures have represented the autoinhibited form of LRRK2, it is not clear at this point whether the inhibitor type-specific responses reflect different accessibility to the phosphorylation sites in the active and inactive forms of LRRK2, or to changes in the conformation of the loop itself between the two forms of LRRK2.

The structures presented here provide a blueprint for medicinal chemistry efforts to design new LRRK2-specific inhibitors, in particular type-II inhibitors for which we have no example of LRRK2 selective compounds. As mentioned in the introduction, selective type-II inhibitors are needed not only to expand the potential therapeutic arsenal to treat PD, but also to create a toolkit that allows inhibition LRRK2 in conformation-specific states to better understand the function of this protein in cells and *in vivo*.

2.4 Methods

Cloning, plasmid construction, and mutagenesis

Briefly, the DNA coding for LRRK2-WT residues 1327 to 2527 (taken from Mammalian Gene Collection) was PCR-amplified using the forward primer TACTTCCAATCCATGAAAAAGGCTGTGCCTTATAACCGA and the reverse primer TATCCACCTTTACTGTCACTCAACAGATGTTTCGTCTCATTTTTTCA. The amplicon was then inserted into the expression vector pFB-6HZB by ligation-independent cloning. This plasmid was used as a template for G2019S and I2020T site-directed mutagenesis (Q5 Site-Directed Mutagenesis Kit, NEB). According to Bac-to-Bac expression system protocols (Invitrogen), these plasmids were used for the generation of recombinant Baculoviruses.

Inhibitors

Stocks of the kinase inhibitors MLi-2 (10 mM; Tocris) and GZD-824 (10 mM; Cayman Chemical), were stored in DMSO at -20 C.

Dynamic scanning fluorimetry (DSF) assay

The assay was performed according to a previously established protocol ⁴⁸. Briefly, a solution of 4 μ M LRRK2^{KW} protein in assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 20 μ M GDP, 2.5 mM MgCl₂, 0.5% glycerol) was mixed 1:1000 with SYPRO Orange (Sigma). The inhibitors to be tested were added to a final concentration of 10 μ M. 20 μ L of each sample were placed in a 96-well plate and heated gradually from 25°C to 95°C. Fluorescence was monitored using an Mx3005P real-time PCR instrument (Stratagene) with excitation and emission filters set to 465 and 590 nm, respectively. Data was analysed with the MxPro software.

Table 2.1 Kinase inhibitors tested in this study.

Inhibitors are listed along with their SMILES formulae, vendor, and stock numbers.

Inhibitor	SMILES	Vendor	Order No
MLi-2	<chem>C[C@@H]1CN(C[C@@H](O1)C)C2=NC=N</chem> <chem>C(=C2)C3=NNC4=C3C=C(C=C4)OC5(CC5)</chem> <chem>C</chem>	Tocris Bioscience	1627091-47-7/ 5756
PF-360	<chem>N#CC1=CC(C2=CNC3=NC=NC(N4CCOCC</chem> <chem>4)=C32)=CN1C</chem>	MedChem Express	1527475-61-1/ HY-120085
HG-10-102-01	<chem>COC1=CC(C(N2CCOCC2)=O)=CC=C1NC3</chem> <chem>=NC(NC)=C(Cl)C=N3</chem>	MedChem Express	1351758-81-0/ HY-13488
LRRK2-IN-1	<chem>O=C(C1=CC(OC)=C(NC2=NC=C3C(N(C4=</chem> <chem>C(C(N3C)=O)C=CC=C4)C)=N2)C=C1)N5C</chem> <chem>CC(CC5)N6CCN(CC6)C</chem>	Merck Millipore	1234480-84-2/ 438193
DNL201	<chem>CC1=NN(C=C1NC2=NC=C(C(=N2)NC)C(F)</chem> <chem>(F)F)C(C)(C)C#N</chem>	MedChem Express	1374828-69-9
Rebastinib	<chem>CC(C)(C)C1=NN(C(NC(NC2=C(F)C=C(OC3</chem> <chem>=CC(C(NC)=O)=NC=C3)C=C2)=O)=C1)C4=</chem> <chem>CC=C5C(C=CC=N5)=C4</chem>	MedChem Express	1020172-07-9/ HY-13024
Ponatinib	<chem>CN(CC1)CCN1CC2=C(C(F)(F)F)C=C(NC(C</chem> <chem>3=CC=C(C)C(C#CC4=CN=C5N4N=CC=C5)</chem> <chem>=C3)=O)C=C2</chem>	MedChem Express	943319-70-8/ HY-12047
GZD-824	<chem>O=C(NC1=CC=C(CN2CCN(C)CC2)C(C(F)(</chem> <chem>F)F)=C1)C3=CC=C(C)C(C#CC4=CN=C(NN</chem> <chem>=C5)C5=C4)=C3</chem>	MedChem Express	1257628-77-5/ HY- 15666

Mass spectroscopy (MS)-based activity assay

To determine the IC₅₀ values for a set of LRRK2 inhibitors, the phosphorylation of the LRRK2 substrate Rab8A was assessed applying an MS-based activity assay. 50 nM LRRK2^{RCKW} protein was mixed with 5 μM substrate and varying concentrations of the inhibitors in assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 20 μM GDP, 2.5 mM MgCl₂, 0.5% glycerol). The reaction was started by adding 1 mM ATP and incubated at room temperature. The reaction was stopped by adding MS buffer (0.1 % formic acid in water) and subjected to an Agilent 6230 Electrospray Ionization Time-of-Flight mass spectrometer coupled with the liquid chromatography unit 1260 Infinity for analysis. 5 μL of the reaction mix was injected onto a C3 column and eluted at 0.4 mL/min flow rate using a solvent gradient of water to acetonitrile with 0.1% formic acid. Data was acquired using the MassHunter LC/MS Data Acquisition software and analyzed using the BioConfirm vB.08.00 tool (both Agilent Technology). The peak intensities of the unphosphorylated and phosphorylated Rab8A species were quantified and the relative kinase activity calculated. To determine the IC₅₀ values, a non-linear regression with variable slope was fitted to the datapoints with GraphPad Prism.

Protein purification: LRRK2^{RCKW} and LRRK2

For both LRRK2^{RCKW} and LRRK2 purifications, the pelleted Sf9 cells were washed with PBS, resuspended in lysis buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 20 mM imidazole, 0.5 mM TCEP, 5% glycerol, 5 mM MgCl₂, 20 μM GDP) and lysed by homogenization. The supernatant was cleared by centrifugation and loaded onto a Ni-NTA (Qiagen) column. After rinsing with lysis buffer, the His₆-Z-tagged protein was eluted in lysis buffer containing 300 mM imidazole. The eluate was then diluted to 250 mM NaCl mM with dilution buffer (50 mM HEPES pH 7.4, 0.5mM TCEP, 5% glycerol, 5mM MgCl₂, 20μM GDP) and loaded onto an SP sepharose column. His₆-Z-TEV-LRRK2^{RCKW}/His₆-Z-TEV-LRRK2 were eluted with a gradient of 250 mM to 2.5 M NaCl in

dilution buffer and then treated with TEV protease overnight to cleave the His₆Z. Contaminating proteins, the cleaved tag, uncleaved protein and TEV protease were removed by another combined SP sepharose Ni-NTA step. Finally, LRRK2^{RCKW} was concentrated and subjected to gel filtration in 20 mM HEPES pH 7.4, 700 mM NaCl, 0.5 mM TCEP, 5% glycerol, 2.5 mM MgCl₂, 20 μM GDP, while LRRK2 was concentrated and subjected to gel filtration in 20 mM HEPES pH 7.4, 200 mM NaCl, 0.5 mM TCEP, 5% glycerol, 2.5 mM MgCl₂, 20 μM GDP using an AKTA Xpress system with an S200 gel filtration column. Final yields, as calculated from UV absorbance, are typically 1.2-3.3 mg of LRRK2^{RCKW} and 0.9-2.2 mg of LRRK2 per liter of insect cell medium.

Cryo-EM sample preparation for LRRK2^{RCKW}:MLi2/GZD-824:E11 DARPin complexes

Purified LRRK2^{RCKW} (WT, G2019S or I2020T) was exchanged into 20 mM HEPES pH 7.4, 150 mM NaCl, 0.5mM TCEP, 5% glycerol, 2.5mM MgCl₂ and 20μM GDP. LRRK2^{RCKW} was incubated with E11-DARPin in a 1:1.25 molar ratio and either MLi-2 (20 μM) or GZD-824 (40 μM) for 10 minutes at RT and 15 minutes at 4°C. Complexes were diluted to a final concentration range of 4 to 6 μM in the same buffer before plunge freezing. 3 or 3.5 μl of LRRK2^{RCKW}:inhibitor:E11 DARPin complex were applied to a glow-discharged UltraAuFoil Holey Gold 200 mesh R2/2 grid (Quantifoil) and incubated in a FEI Vitrobot IV chamber at 4°C and 95% humidity for 20 sec. The excess liquid was blotted for 4 sec using filter paper 595 at blot force 3, and vitrified by plunging into liquid ethane cooled down to liquid nitrogen temperature.

Cryo-EM sample preparation for full length LRRK2(I2020T):MLi-2/GZD-824-E11 DARPin complex

Purified full-length LRRK2(I2020T) was incubated with E11 DARPin at a molar ratio of

1:1.25 and either MLi-2 (20 μ M) or GZD-824 (40 μ M) for 10 minutes at RT and 5-10 minutes at 4°C. Complexes were diluted to a 5 μ M final concentration in the same buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 5% glycerol, 2.5 mM MgCl₂) with the addition of a final 20 μ M GDP for LRRK2(I2020T):MLi-2:E11, or 100 μ M GMP-PNP for LRRK2(I2020T):GZD824:E11. 3 to 3.5 μ l of complex was applied to a glow-discharged UltrAuFoil Holey Gold 200 mesh R2/2 grid (Quantifoil) and incubated in a FEI Vitrobot IV chamber at 4°C and 95% humidity for 20 sec. The excess liquid was blotted for 4 sec using filter paper 595 at blot force 4, vitrified by plunging into liquid ethane cooled down to liquid nitrogen temperature.

Cryo-EM data collection

Cryo-EM data for LRRK2^{RCKW}(G2019S and I2020T):MLi-2:E11 DARPin, LRRK2^{RCKW}(WT, G2019S and I2020T):GZD-824:E11 DARPin and LRRK2(I2020T):MLi-2:E11 DARPin were collected on a Titan Krios G3 (Thermo Fisher Scientific) operated at 300 keV, equipped with a Falcon 4 direct electron detector (Thermo Fisher Scientific) and a Gatan BioContinuum energy filter. Images were collected at a nominal magnification of 130,000x in EFTEM mode (0.935 Å calibrated pixel size) using a 20-eV slit width in the energy filter and a cumulative electron exposure of ~55 electrons/Å². Data for LRRK2(I2020T)-E11:GZD-824 was collected on a Talos Arctica (FEI) operated at 200 keV, equipped with a Falcon 4i (Thermo Fisher Scientific) with a cumulative electron exposure of ~55 electrons/Å². Data were collected automatically using EPU software (Thermo Fisher Scientific). Data for LRRK2^{RCKW}:MLi-2:E11 DARPin were collected on a Titan Krios G3 (Thermo Fisher Scientific) operated at 300 keV, equipped with a K3 Summit direct electron detector (Gatan) and a Gatan BioContinuum energy filter. Images were collected at a nominal magnification of 105000x in EF-TEM mode (0.822 Å

calibrated pixel size) using a 20-eV slit width in the energy filter and a cumulative electron exposure of approximately 57 electrons/Å².

Data processing for LRRK2^{RCKW}:MLi-2:E11 DARPIn complexes

11,181, 8,402 and 10,488 movies were collected for LRRK2^{RCKW}-WT:MLi-2:E11 DARPIn, LRRK2^{RCKW}(G2019S):MLi-2:E11 DARPIn and LRRK2^{RCKW}(I2020T):MLi-2:E11 DARPIn complexes, respectively. They were aligned using MotionCor2 dose-weighted alignment option and CTF parameters were estimated on dose-weighted images using CTFFIND4 ⁴⁹. Micrographs with a CTF fit worse than 3.5-4Å (as determined by CTFFIND4, and varying among datasets) were discarded from further processing. Particles were picked using a Topaz model previously trained for each dataset. Several rounds of reference-free 2D classification yielded a stack of 574,664 (WT), 578,873 (G2019S) and 694,336 particles (I2020T), each containing both monomers and tetramers. All particles were extracted with a 400-pixel box using CryoSPARC ⁵⁰ and were separated based on oligomerization state (Figure S2.3, S2.6 and S2.7). Subsequently, abinitio jobs were run to further remove bad particles. The best ab-initio volume for the tetramer was used as an input for NU-Refinement (D2 symmetry). Then, particles were expanded based on the volume symmetry and used in subsequent jobs. After 3D Variability and C1-symmetry local refinement, we obtained maps at 3.05, 2.74 and 2.8Å, respectively. The best ab-initio map for the monomer, along with monomer particles (Figure S2.3, S2.6 and S2.7) were used as an input for a NU-Refinement. In all maps, FSC and local resolution estimations were performed using the routines implemented in CryoSPARC ⁵⁰.

Data processing for LRRK2(I2020T):MLi-2:E11 DARPIn complex

10,407 movies were collected for LRRK2(I2020T):MLi-2:E11 DARPIn and preprocessed as previously described. Micrographs with a CTF fit worse than 4Å (as determined by CTFFIND4)

were discarded from further processing. Particle picking was done following the same protocol for the LRRK2^{RCKW} datasets. Several rounds of 2D classification yielded 162,409 particles. After Abinitio job and a NU-Refinement, a final map at a resolution of 3.9Å was obtained (Figure S2.10).

Data processing for LRRK2^{RCKW}:GZD-824:E11 DARPin complexes

5,565, 7,988 and 8,386 movies were collected for LRRK2^{RCKW}WT:GZD-824:E11 DARPin, LRRK2^{RCKW}(G2019S):GZD-824:E11 DARPin and LRRK2^{RCKW}(I2020T):GZD-824:E11 DARPin complexes, respectively. They were aligned using MotionCor2 dose-weighted alignment option and CTF parameters were estimated on dose-weighted images using CTFFIND4⁴⁹. Micrographs with a CTF fit worse than 3.5-4.5 Å (as determined by CTFFIND4, and varying among datasets) were discarded from further processing. Particles were picked using a Topaz model previously trained for each dataset. Several rounds of reference-free 2D classification yielded a stack of 249,808, 759,293 and 735,633 monomer particles respectively; additionally, LRRK2^{RCKW}WT:GZD-824:E11 dataset also yielded 58,678 trimer particles. Particles were extracted with a 320-pixel box for monomers, and 400-pixel box for trimers using CryoSPARC⁵⁰ (Figure S2.4, S2.8 and S2.9). For all LRRK2^{RCKW} monomer particles, ab-initio jobs followed by heterogeneous refinement were run to sort particles. The best refined class was subject to a NUrefinement (C1 symmetry), followed by a local refinement with a mask surrounding the kinaseWD40-DARPin, yielding maps at resolutions of 3.10Å, 2.99Å, and 3.06Å, respectively. To account for the heterogeneity of the ROC-COR domain a 3D Variability job was done to further split the particles, followed by a local refinement with a mask surrounding the ROC-COR domain; this yielded maps at resolutions of 3.80Å, 3.50Å, and 3.63Å, respectively. For LRRK2^{RCKW} (trimer particles) an ab-initio job was run followed by a NU-refinement (C3 symmetry). Particles were expanded based on the volume symmetry and used in a C1-symmetry local refinement, yielding a map at 2.90Å resolution.

Data processing for LRRK2(I2020T):GZD-824:E11 DARPin complexes

4,102 movies were collected for LRRK2(I2020T):GZD-824:E11 and pre-processed as previously described. Micrographs with a CTF fit worse than 5.3Å (as determined by CTFFIND4) were discarded from further processing. Particle picking was done in the same manner as for the LRRK2^{RCKW} datasets, followed by several rounds of 2D classification, resulting in 1,029,496 particles. Particles were moved to Relion using pyem/csparc2star (<https://zenodo.org/doi/10.5281/zenodo.3576629>) and extracted to 3.8Å/pix. Particles were subject to 3D classification (5 classes) with alignment, 224,119 particles yielded the best 3D class of monomeric LRRK2(I2020T). Consensus 3D refinement using the best 3D class was done, followed by two rounds of 3D classification without alignment resulting in the best 3D class containing 60,465 particles. Particles were moved back to CryoSPARC³⁴, followed by a NUrefinement, yielding a map at 3.40Å resolution (Figure S2.11).

Model building and refinement of LRRK2^{RCKW}:MLi-2/GZD-824:E11 DARPin and LRRK2(I2020T):MLi-2/GZD-824:E11 DARPin

LRRK2^{RCKW} and LRRK2 models were built using the highest-resolution maps obtained for each complex. These maps corresponded to tetramers for LRRK2^{RCKW}(WT):MLi-2:E11, LRRK2^{RCKW}(G2019S):MLi-2:E11, and LRRK2^{RCKW}(I2020T):MLi-2:E11, a trimer for LRRK2^{RCKW}(WT):GZD-824:E11 and a monomer for FL(I2020T):MLi-2/GZD-824:E11 DARPin (see Figures S2.2, S2.4, S2.6-S2.11 for details on the cryo-EM workflow for each specific sample). To rule out structural differences in the kinase induced by oligomerization, we tested the fit of the models built using the higher-resolution oligomers into cryo-EM maps of the monomeric form of the same construct (Figure S2.5).

Available PDBs 6VP7, 7LHW and A1N for LRRK2^{RCKW}, LRRK2 and MLi-2, respectively, were used as starting points. Protein models were split into domains, docked into the corresponding cryo-EM maps, and merged. To obtain a GZD-824 model, electronic Ligand Building and Optimization Workbench (elBOW) software available in Phenix ⁵¹ was run using Simplified Molecular Input Line Entry System (SMILES) notation of molecule as an input. Inhibitor models (MLi-2 or GZD-824) were fitted in the map density and incorporated in the model. A combination of manual inspection of amino acids in Coot ^{52,53} and refinement of model into their maps in Phenix ⁵¹ was used to generate the final models.

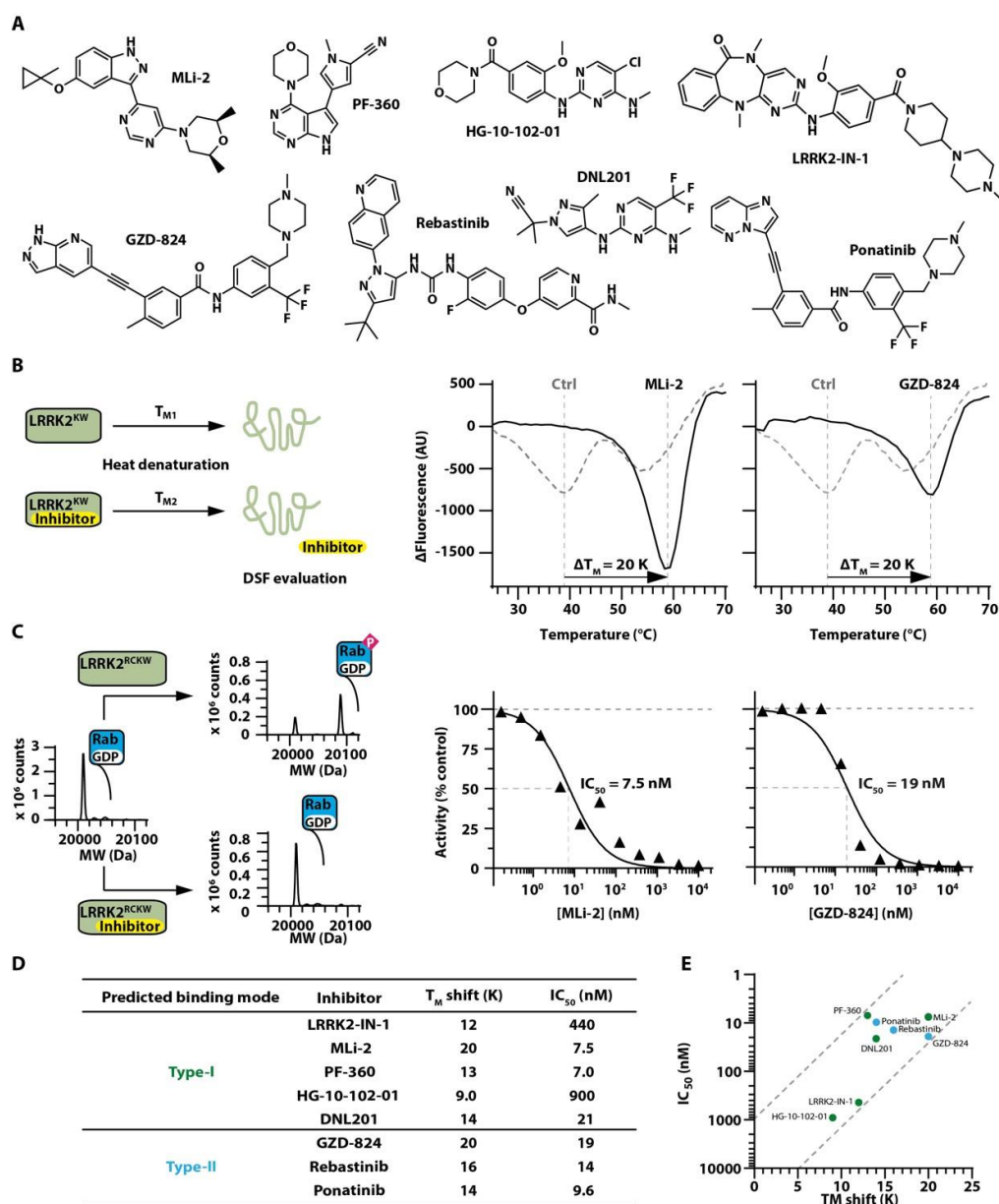
Analysis of conformational changes in LRRK2^{RCKW} and LRRK2

The segments linking equivalent α carbons in LRRK2^{RCKW} and LRRK2 were generated by aligning models by their C-lobes (residues 1949-2139) using UCSF ChimeraX ⁵⁴. Amino acids not present in the two models being compared were removed and primary sequences were aligned using ClustalW (EMBL-EBI)⁵⁵. Aligned sequences were used as an input for the public script PDBarrows.ipynb on Jupyter notebook, generating output arrows, which were colored to match the domains being linked. (Figure 2.5G,H).

Structure depictions

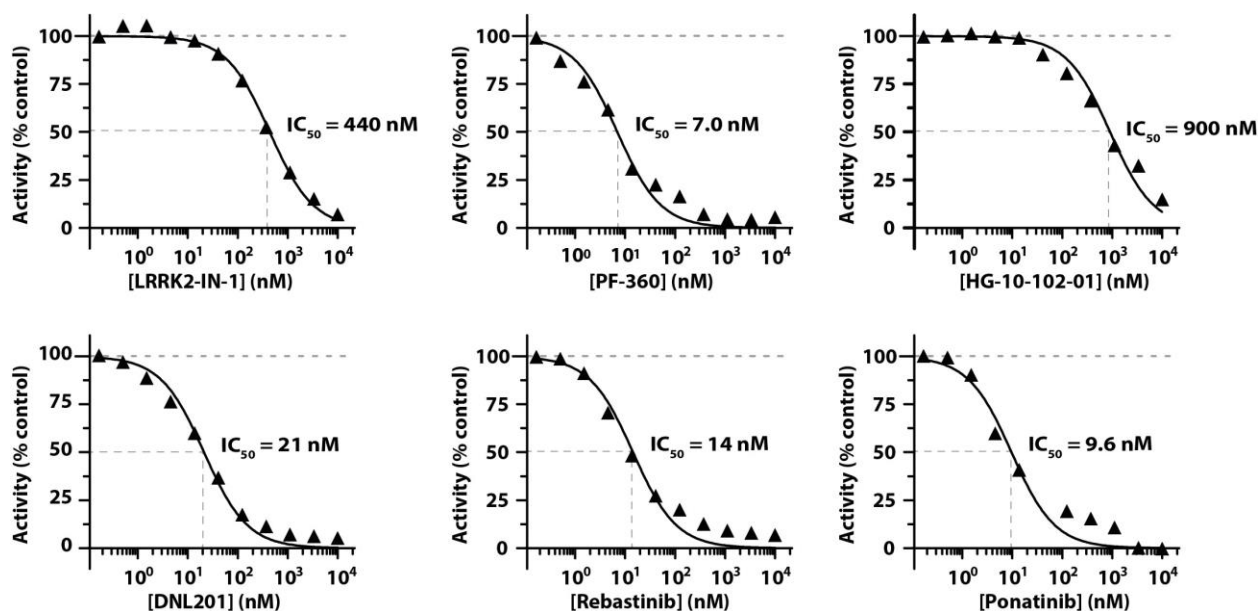
All structure-related figures were prepared using ChimeraX ⁵⁴.

2.5 Supplementary Data and Figures



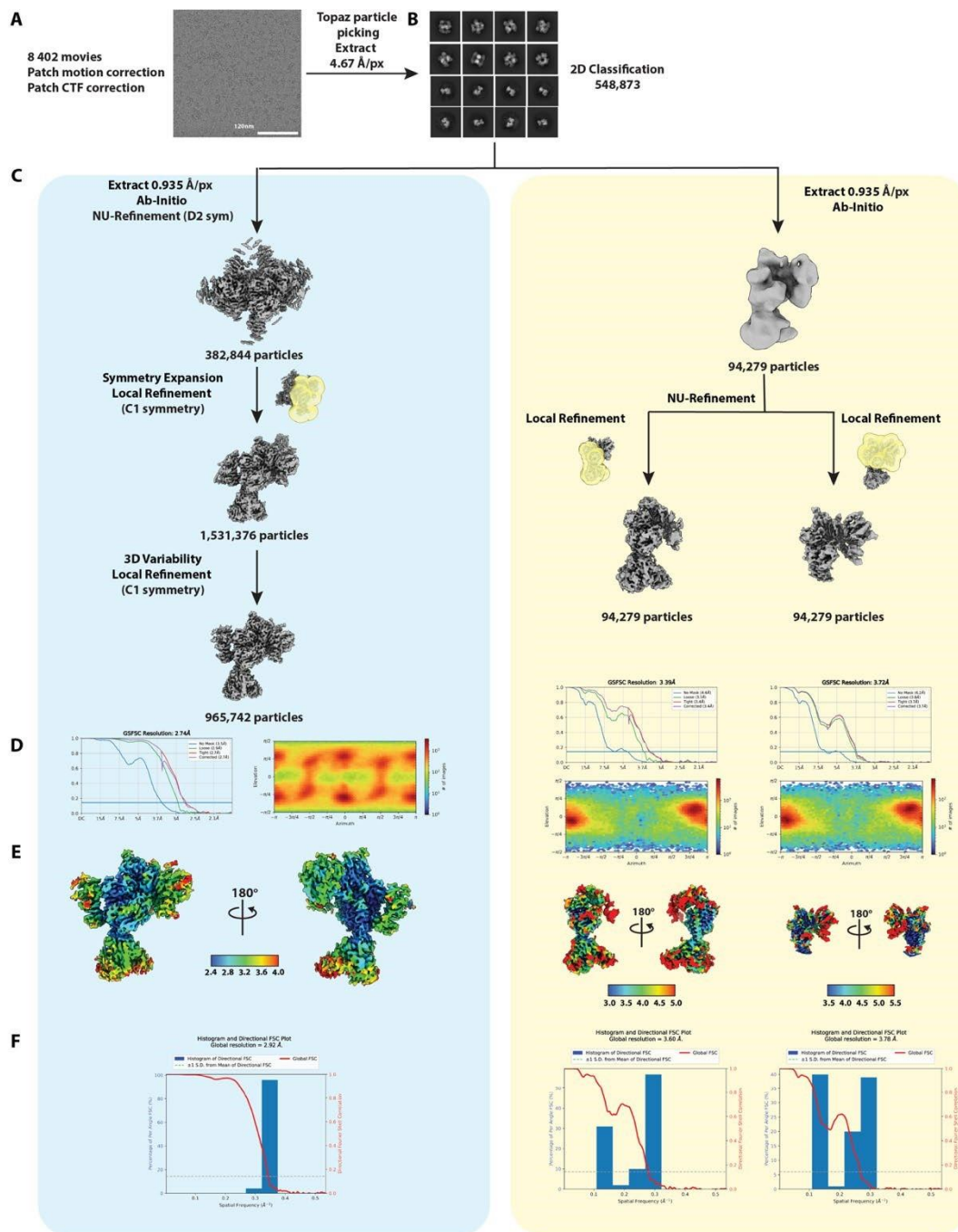
Supplementary Figure 2.1 Stabilization and inhibition of LRRK2's kinase by type-I and type-II inhibitors.

(A) Prominent inhibitors of the LRRK2 kinase. (B) The binding of kinase inhibitors stabilized LRRK2^{KW} as determined in a DSF assay. Shown are the first derivatives of the melting curves. The biphasic melting of LRRK2^{KW} indicated that the kinase and WD40 domains melt independently of each other. (C) The impact of type-I and type-II inhibitors on LRRK2^{RCKW} kinase activity was assessed with an MS-based activity assay. The inhibitors prevented LRRK2^{RCKW} from phosphorylating Rab8A with nanomolar IC₅₀ values. (D) T_M shifts (for LRRK2^{KW}) and IC₅₀s (for LRRK2^{RCKW}) obtained for the type-I and type-II inhibitors shown in (A). (E) Plot of IC₅₀s as a function of T_M shifts for the inhibitors in (E). The Pearson correlation coefficient between the two measurements is -0.67



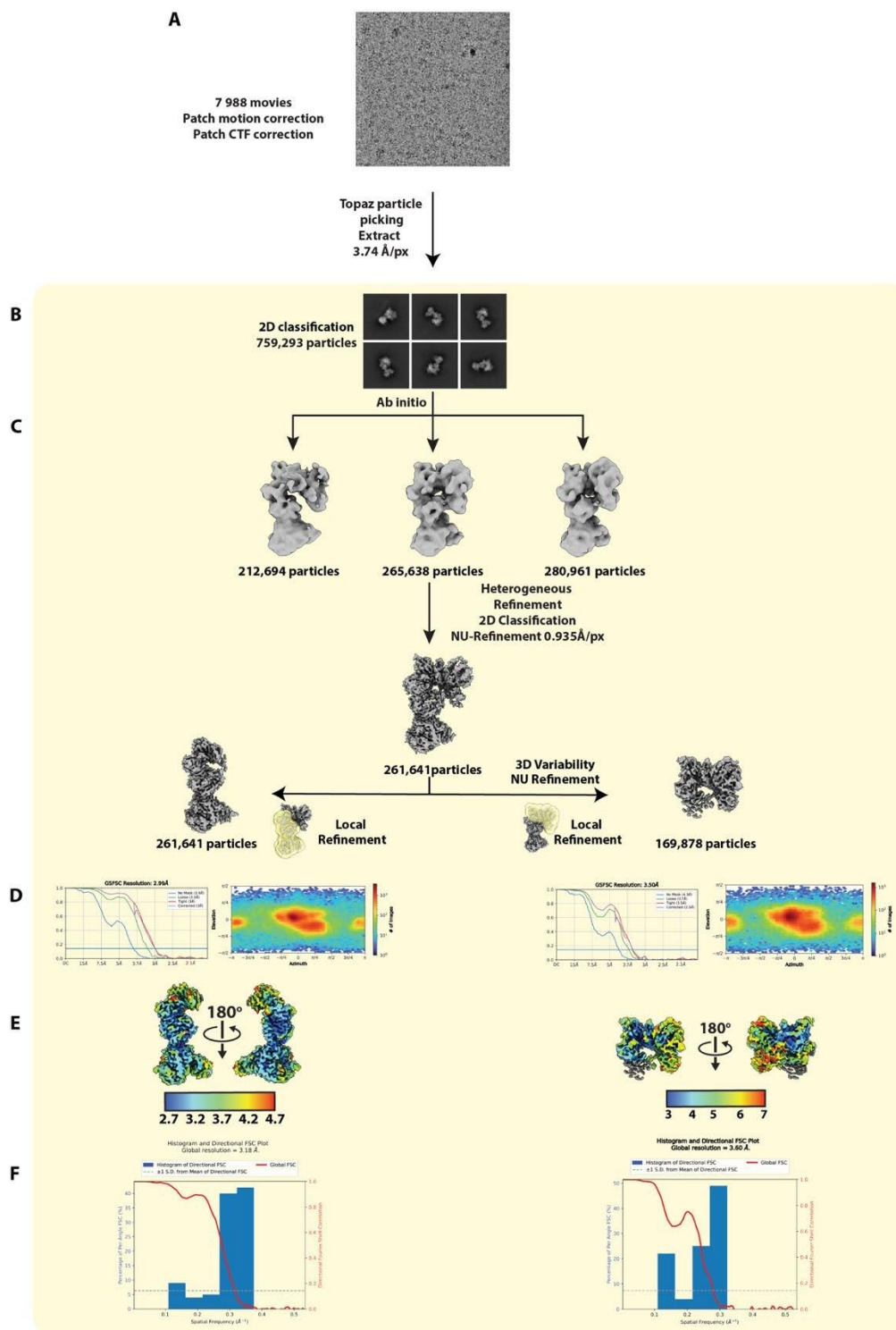
Supplementary Figure 2.2 LRRK2 inhibition curves obtained from the MS-based activity assay.

The physiological LRRK2 substrate Rab8A was subjected to phosphorylation by LRRK2^{RCKW}. Varying concentrations of the indicated inhibitors were present in the reaction mixtures allowing for the determination of IC_{50} values.



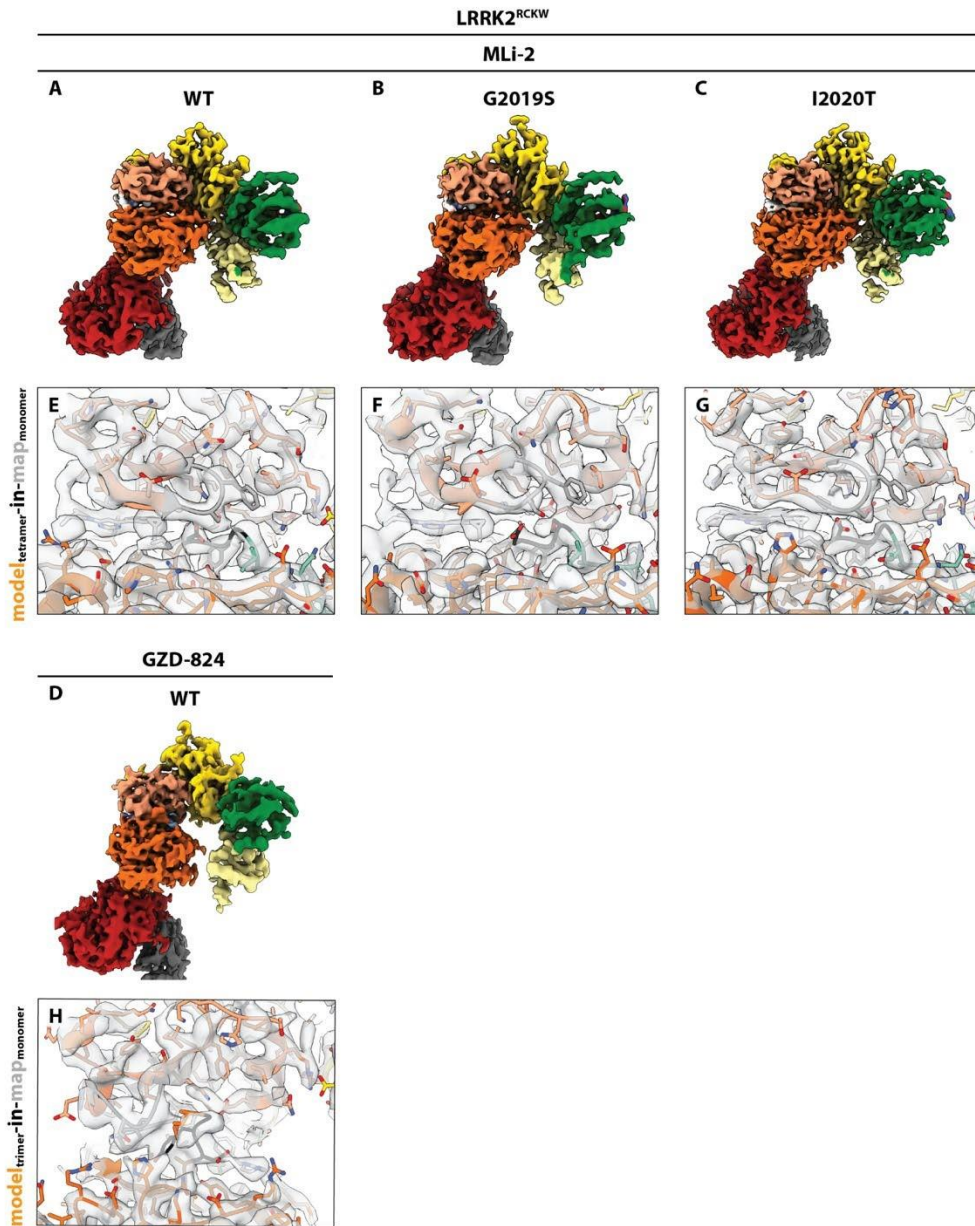
Supplementary Figure 2.3 Cryo-EM workflow for LRRK2^{RCKW}(G2019S):MLi-2.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots and Euler angle distributions (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2^{RCKW}(G2019S):MLi-2 tetramer (blue panel) and monomer (yellow panel).



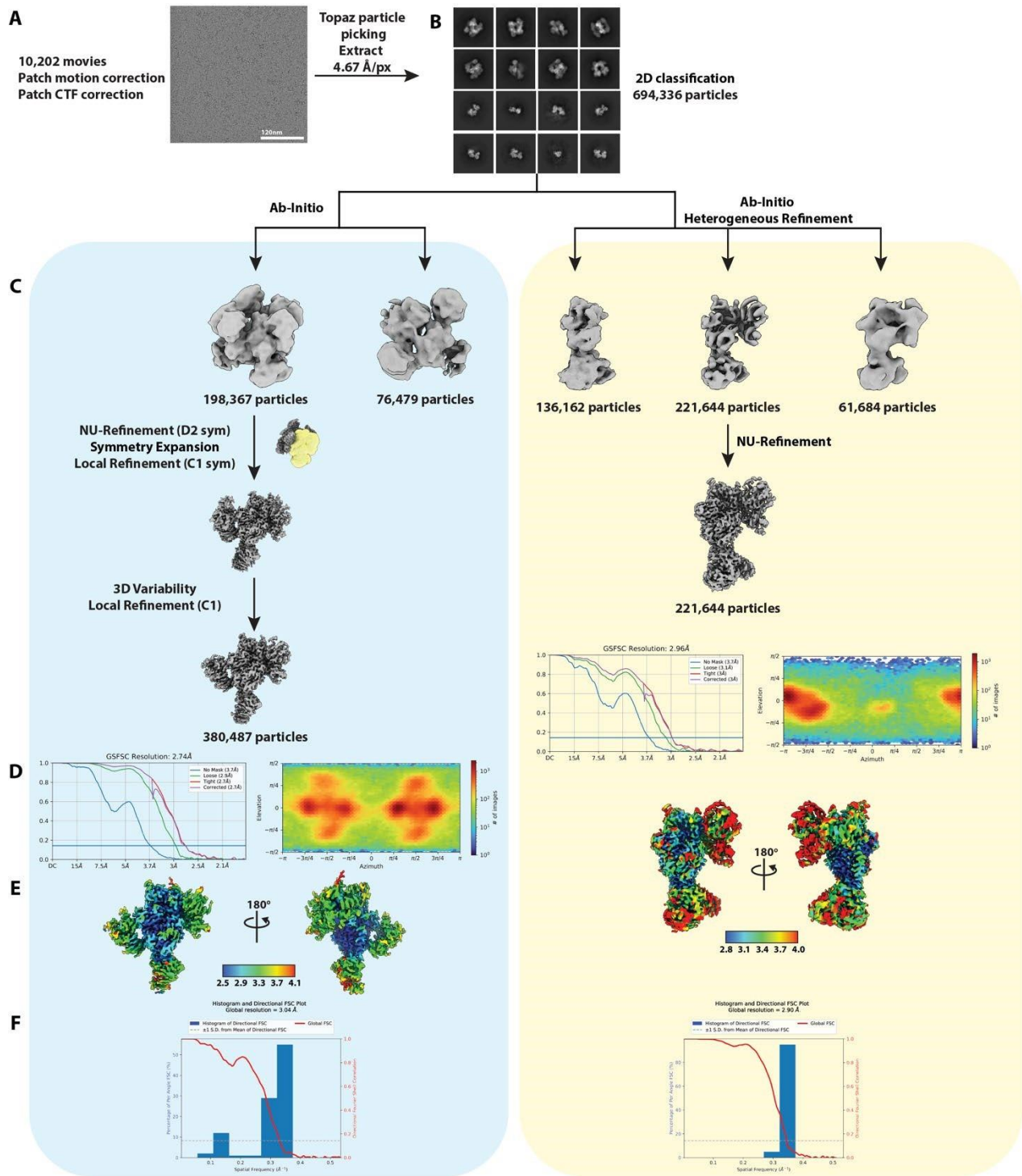
Supplementary Figure 2.4 Cryo-EM workflow for LRRK2^{RCKW}(G2019S):GZD-824.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots and Euler angle distributions (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2^{RCKW}(G2019S):GZD-824 monomer.



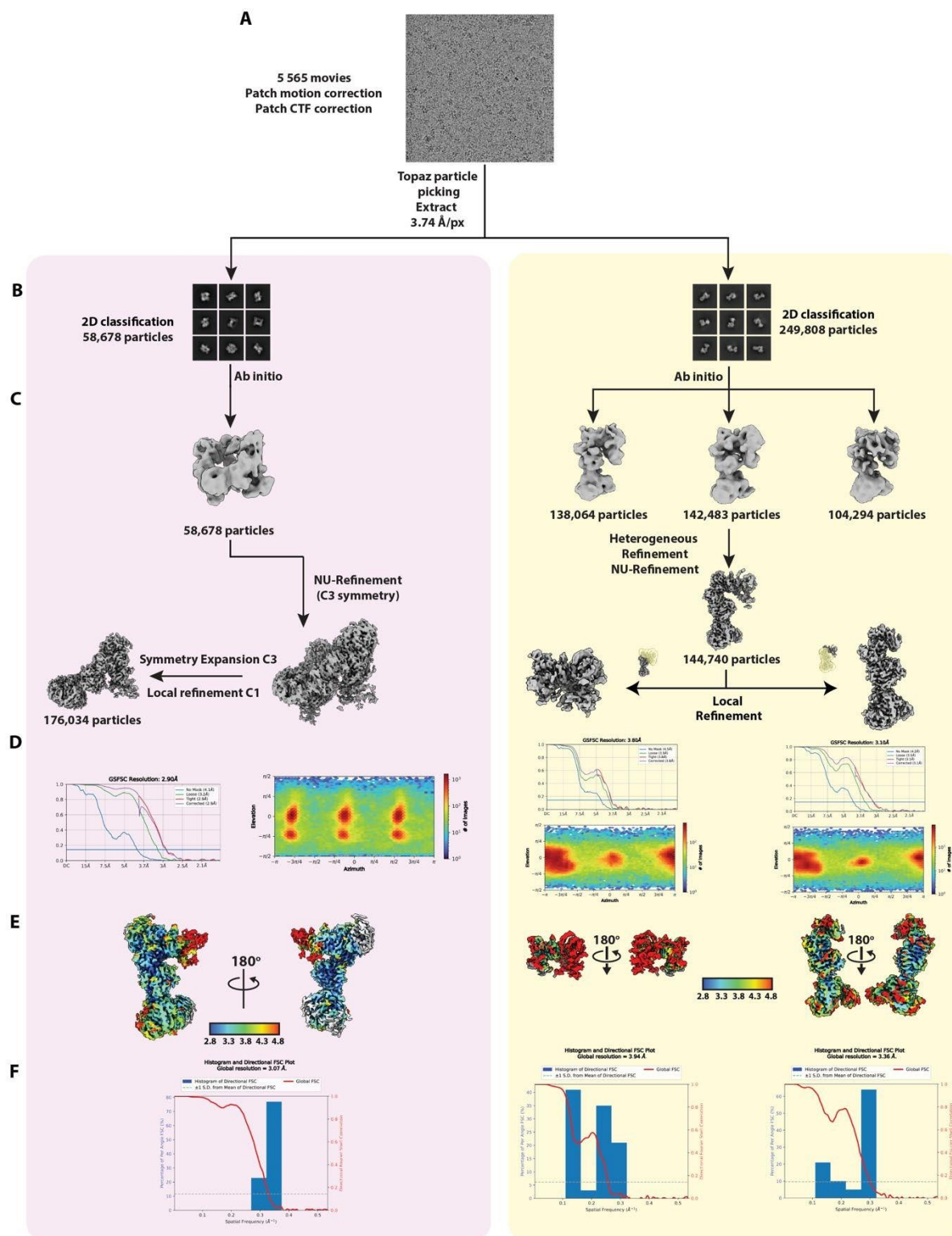
Supplementary Figure 2.5 Cryo-EM maps of monomeric LRRK2^{RCKW}'s account for models built into higher-resolution trimeric and tetrameric maps.

(A-D) Cryo-EM maps of the monomeric form of LRRK2^{RCKW}(WT):MLi-2 (A), LRRK2^{RCKW}(G2019S):MLi-2 (B), LRRK2^{RCKW}(I2020T):MLi-2(C), and LRRK2^{RCKW}(WT):GZD824 (D). Maps are colored according to the domain color scheme shown in Figure S1A. The DARPin E11 is shown in dark grey. (E-H) The models built using the higher-resolution tetrameric (E-G) and trimeric (H) cryo-EM maps are fitted into the monomeric maps (see Methods for details on the cryo-EM data processing) to highlight that the kinase features discussed in the text (G-loop, DYG motif, α C helix, K1906-E1920 pair, and activation loop) are not dependent on the oligomeric state of LRRK2^{RCKW}. The panels show the fit in and around the kinase's active site.



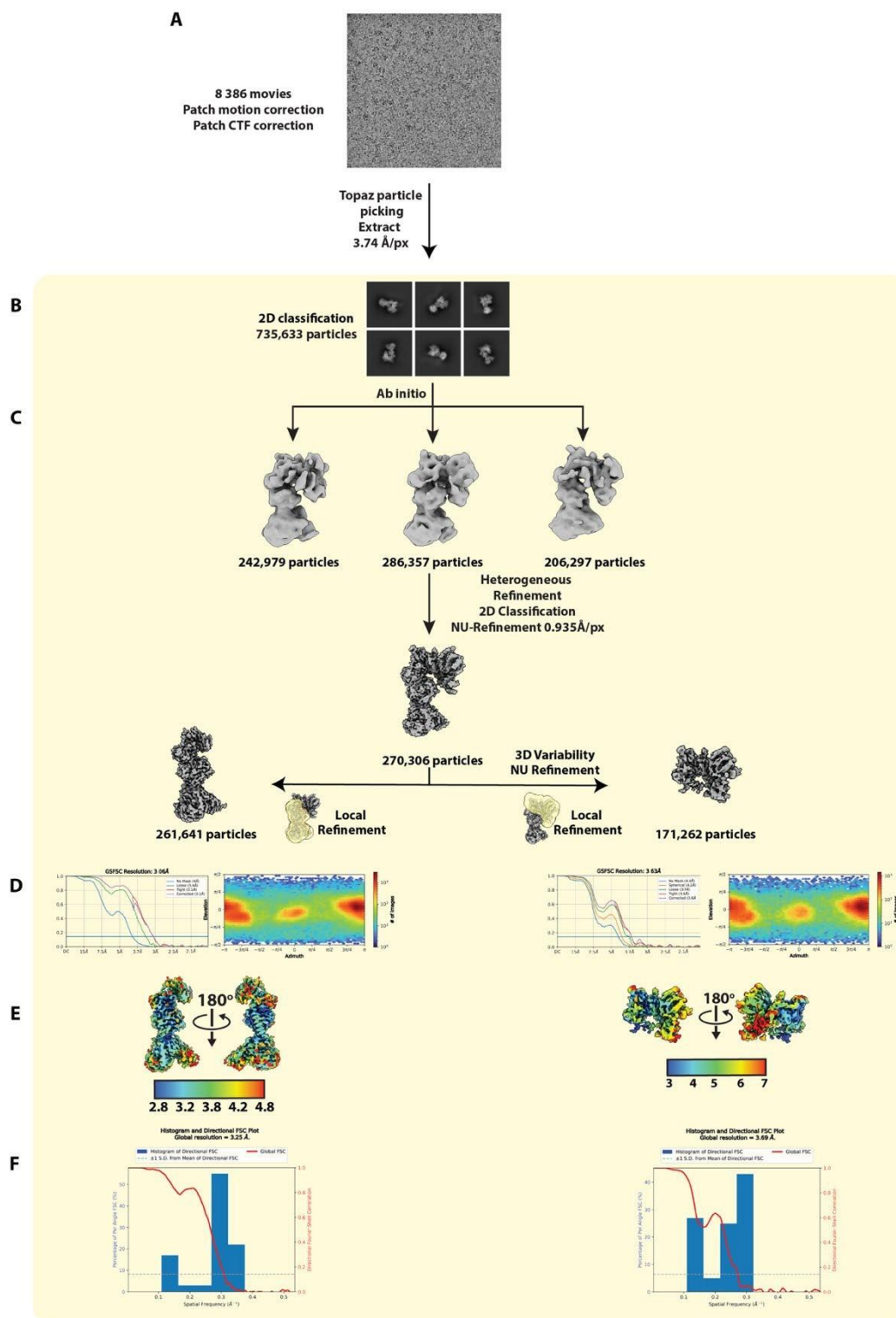
Supplementary Figure 2.7 Cryo-EM workflow for LRRK2^{RCKW}(I2020T):MLi-2.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots and Euler angle distributions (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2^{RCKW}(I2020T):MLi-2 tetramer (blue panel) and monomer (yellow panel).



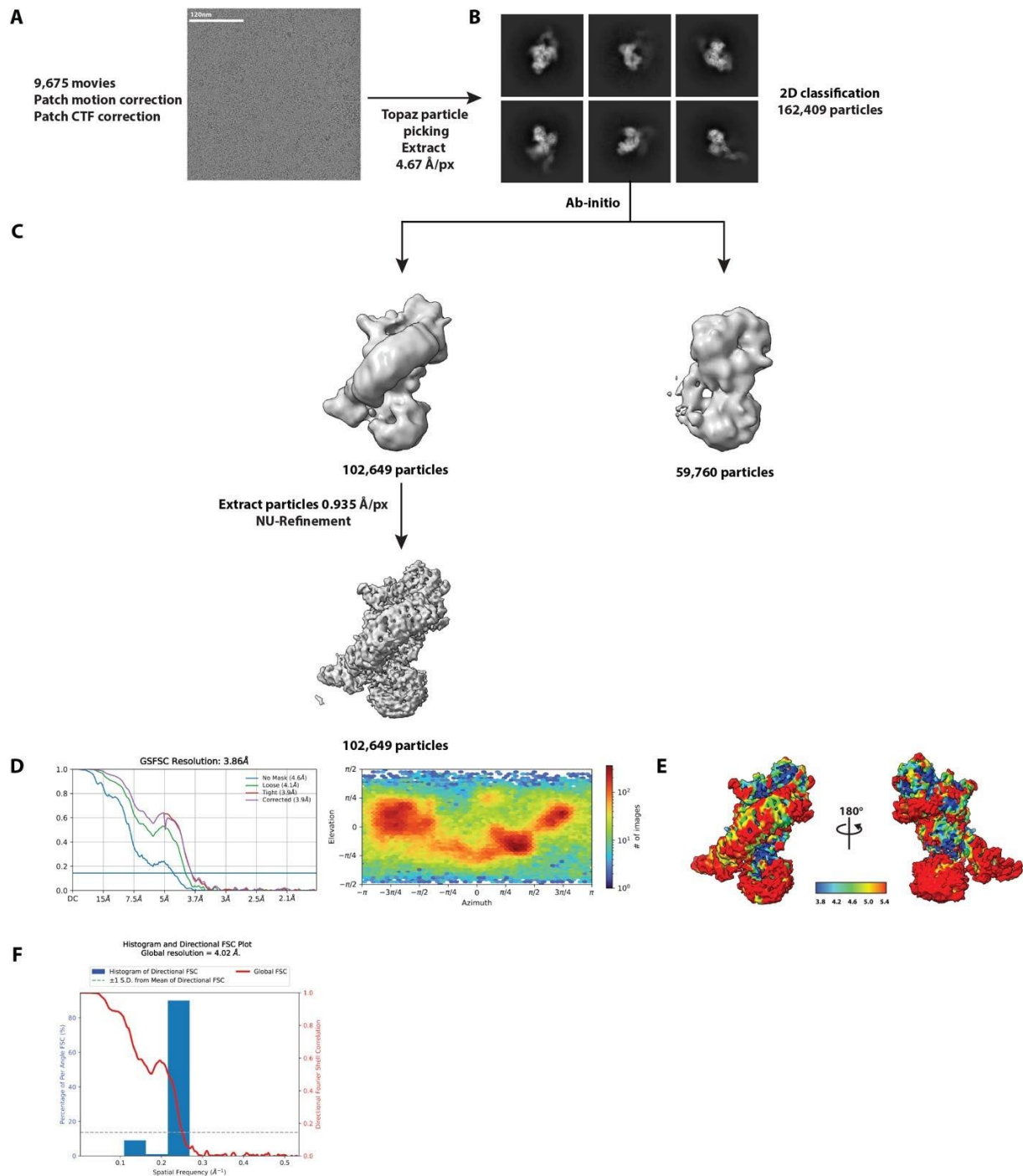
Supplementary Figure 2.8 Cryo-EM workflow for LRRK2^{RCKW}(WT):GZD-824.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots and Euler angle distributions (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2^{RCKW}(WT):GZD-824 trimer (pink panel) and monomer (yellow panel).



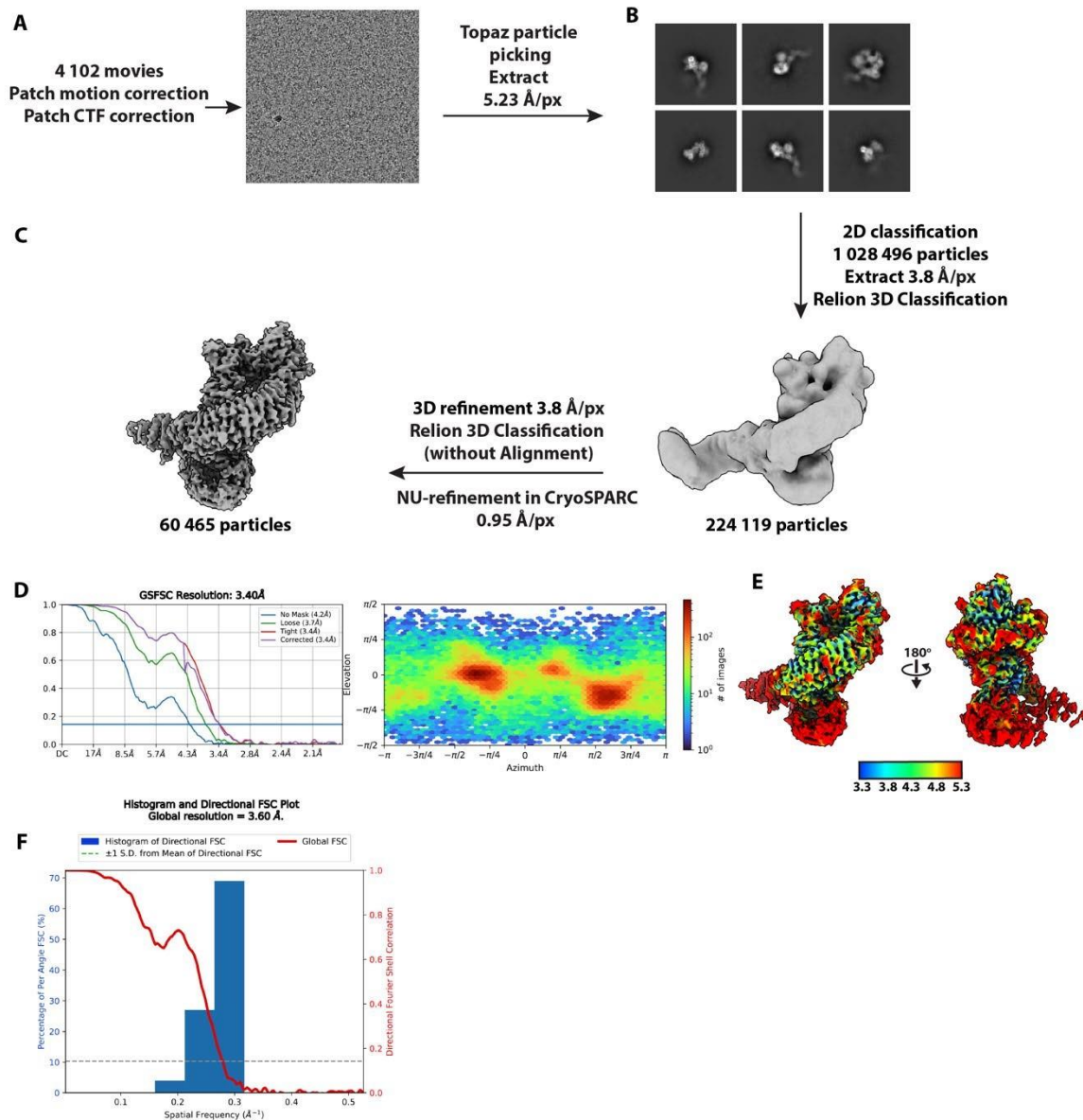
Supplementary Figure 2.9 Cryo-EM workflow for LRRK2^{RCKW}(I2020T):GZD-824.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots and Euler angle distributions (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2^{RCKW}(I2020T):GZD-824 monomer.



Supplementary Figure 2.10 Cryo-EM workflow for LRRK2(I2020T):MLi-2.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots and Euler angle distributions (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2(I2020T):MLi-2.



Supplementary Figure 2.11 Cryo-EM workflow for LRRK2(I2020T):GZD-824.

Representative micrograph (A), 2D class averages (B), data processing strategy (C), FSC plots (D), local resolution maps (E), and 3D FSC plot (F) for LRRK2(I2020T):GZD-824.

2.6 Tables

Supplementary Table 2.1 Cryo-EM data collection, refinement, and validation statistics

	LRRK2 ^{RCKW} (WT): MLi-2:E11 DARPin (tetramer) (EMDB-41709) (PDB 8TXZ)	LRRK2 ^{RCKW} (G2019S): MLi-2:E11 DARPin (tetramer) (EMDB-41754) (PDB 8TZC)	LRRK2 ^{RCKW} (I2020T): MLi-2:E11 DARPin (tetramer) (EMDB-41758) (PDB 8TZG)
Data collection and processing			
Magnification	105000	130000	130000
Voltage (kV)	300	300	300
Electron exposure (e-/Å ²)	57	55	55
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.822	0.935	0.935
Symmetry imposed	D2	D2	D2
Initial particle images (no.)	574 664	548 873	694 336
Final particle images (no.)	584 196	965 742	380 487
Map resolution (Å)	3.05	2.74	2.74
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	2.8 – 3.6	2.4 - 4.0	2.5-4.1
Refinement			
Initial model used (PDB code)	6VP7	6VP7	6VP7
Model resolution (Å)	3.05	2.74	2.74
FSC threshold	0.143	0.143	0.143
Model composition			
Non-hydrogen atoms	6261	7623	7095
Protein residues	814	998	907
Nucleotide	1	1	1
Ligand	1 1	1 1	1 1
Water			
<i>B</i> factors (Å ²)			
Protein	42.37	93.28	47.54
Nucleotide	212.72	132.81	121.15
Ligand	23.59	78.41	34.58
Water	12.48	70.18	25.11
R.m.s. deviations			
Bond lengths (Å)	0.019	0.008	0.003
Bond angles (°)	1.312	1.034	0.701
Validation			
MolProbity score	2.30	2.21	1.83
Clashscore	8.85	7.97	8.06
Ramachandran plot			
Favored (%)	93.92	93.74	94.31
Allowed (%)	5.96	6.26	5.58
Disallowed (%)	0.12	0.00	0.12

Supplementary Table 2.1 (continued). Cryo-EM data collection, refinement, and validation statistics

	LRRK2 ^{RCKW} (G2019S):GZD-824:E11 DARPin ^a (monomer) Composite map (EMDB-41728) (PDB 8TYQ)	LRRK2 ^{RCKW} (G2019S):GZD-824:E11 DARPin ^a (monomer) Consensus refinement (EMDB-41798)	LRRK2 ^{RCKW} (G2019S):GZD-824:E11 DARPin ^a (monomer) Focused refinement Kinase-WD40 (EMDB-41799)	LRRK2 ^{RCKW} (G2019S):GZD-824:E11 DARPin ^a (monomer) Focused refinement ROC-COR (EMDB-41797)
Data collection and processing				
Magnification	130000	130000	130000	130000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	55	55	55	55
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	759,293	759,293	759,293	759,293
Final particle images (no.)	261,641/169,878	261,641	261,641	169,878
Map resolution (Å)	--	3.10	2.99	3.50
FSC threshold	--	0.143	0.143	0.143
Map resolution range (Å)	2.7.-7	2.7-6	2.7-4.2	3-7
Refinement				
Initial model used (PDB code)	6VP7	--	--	--
Model resolution (Å)	--	--	--	--
FSC threshold	--	--	--	--
Model composition				
Non-hydrogen atoms	8523	--	--	--
Protein residues	1068	--	--	--
Nucleotide	0	--	--	--
Ligands	1	--	--	--
<i>B</i> factors (Å ²)				
Protein	171.59	--	--	--
Nucleotide	--	-- --	-- --	-- --
Ligand	48.03	--	--	--
R.m.s. deviations				
Bond lengths (Å)	0.007	-- --	-- --	-- --
Bond angles (°)	0.904	--	--	--
Validation				
MolProbity score	1.72	-- --	-- --	-- --
Clashscore	10.14	--	--	--

Supplementary Table 2.1 (continued). Cryo-EM data collection, refinement, and validation statistics

Ramachandran plot				
Favored (%)	96.86	--	--	--
Allowed (%)	3.16	-- --	-- --	-- --
Disallowed (%)	0.00			
^a composite map				
	LRRK2 ^{RCKW} (I2020T): GZD-824:E11 DARPin (monomer) Composite map (EMDB- 41753) (PDB 8TZB)	LRRK2 ^{RCKW} (I2020T): GZD-824:E11 DARPin ^a (monomer) Consensus refinement (EMDB- 41802)	LRRK2 ^{RCKW} (I2020T): GZD-824:E11 DARPin ^a (monomer) Focused refinement Kinase-WD40 (EMDB-41794)	LRRK2 ^{RCKW} (I2020T): GZD-824:E11 DARPin ^a (monomer) Focused refinement ROC-COR (EMDB-41795)
Data collection and processing				
Magnification	130000	130000	130000	130000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	55	55	55	55
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	735,633	735,633	735,633	735,633
Final particle images (no.)	261,641/171,262	270,306	261,641	171,262
Map resolution (Å)	--	3.22	3.06	3.63
FSC threshold	--	0.143	0.143	0.143
Map resolution range (Å)	--	2.8-7	2.8-4.4	3-7
Refinement				
Initial model used (PDB code)	6VP7	--	--	--
Model resolution (Å)	--	--	--	--
FSC threshold	--	--	--	--
Model composition				
Non-hydrogen atoms	7499	--	--	--
Protein residues	1012	--	--	--
Nucleotide	0 1	--	--	--
Ligands				

Supplementary Table 2.1 (continued). Cryo-EM data collection, refinement, and validation statistics

<i>B</i> factors (Å ²)				
Protein	145.4	--	--	--
Nucleotide	--	---	---	---
Ligand	33.66			
R.m.s. deviations				
Bond lengths (Å)	0.004	---	---	---
Bond angles (°)	0.594			
Validation				
MolProbity score	1.94	---	---	---
Clashscore	10.46			
Ramachandran plot				
Favored (%)	93.94	--	--	--
Allowed (%)	5.96	---	---	---
Disallowed (%)	0.11			
^a composite map				
	FL(I2020T): MLi-2:E11 DARPin (monomer) (EMDB-41759) (PDB 8TZH)	FL(I2020T): GZD-824: E11 DARPin (monomer) (EMDB-41757) (PDB 8TZF)	LRRK2 ^{RCKW} (WT): GZD-824:E11 DARPin (trimer) (EMDB-41756) (PDB 8TZE)	
Data collection and processing				
Magnification	130000	150000	130000	
Voltage (kV)	300	200	300	
Electron exposure (e-/Å ²)	50	55	55	
Defocus range (µm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	
Pixel size (Å)	0.935	0.95	0.935	
Symmetry imposed	C1	C1	C3	
Initial particle images (no.)	162,409	224,119	58,678	
Final particle images (no.)	102,649	60,465	176,034	
Map resolution (Å)	3.9	3.4	2.90	
FSC threshold	0.143	0.143	0.143	
Map resolution range (Å)	3.8 -5.0	3.3-5.3	2.8-4.5	
Refinement				
Initial model used (PDB code)	7LHW	7LHW	6VP7	
Model resolution (Å)	3.9	3.4	2.90	
FSC threshold	0.143	0.143	0.143	
Model composition				
Non-hydrogen atoms	11,593	12,156	6251	
Protein residues	1,606	1,671	787	
Nucleotide	1 1	1 1	0 1	
Ligands				
<i>B</i> factors (Å ²)				
Protein	96.31	174.38	121.98	
Nucleotide	102.80	87.23	--	

Supplementary Table 2.1 (continued). Cryo-EM data collection, refinement, and validation statistics

Ligand	82.68	109	45.07
R.m.s. deviations			
Bond lengths (Å)	0.003	0.003	0.007
Bond angles (°)	0.791	0.557	1.096
Validation			
MolProbity score	2.18	1.99	2.42
Clashscore	15.15	9.96	9.49
Ramachandran plot			
Favored (%)	91.79	92.4	93.54
Allowed (%)	8.21	7.60	6.32
Disallowed (%)	0.00	0.00	0.13

2.7 Contributions and Acknowledgments

Author Contributions

All authors were involved in experimental design. Villagran Suarez, A.; Sanz-Murillo, M.; Alegrio Louro, J.; performed the cryo-EM work (data collection and analysis). Dederer, V.; Chatterjee, D.; Mathea, S.; performed assays and mass spectrometry. Villagran Suarez, A.; SanzMurillo, M.; Mathea, S; Knapp, S; Reck-Peterson, S.; Leschziner, A.E.; wrote the manuscript edited it. The dissertation author was the first author and primary contributor to this work.

Acknowledgments

Chapter 2, in full, is a reprint of the material as it appears in *Science Advances*: Inhibition of Parkinson's Disease-related LRRK2 by type-I and type-II kinase inhibitors: activity and structures. Sanz-Murillo, M.; Villagran Suarez, A.; Dederer, V.; Chatterjee, D.; Knapp, S.; Mathea, S.; Reck-Peterson, S.L.; Leschziner, A.E.

We would like to thank Samara Reck-Peterson for feedback on the manuscript. We also thank the UC San Diego Cryo-EM Facility, and the Physics Computing Facility at UC San Diego for IT support. This work was supported by Aligning Science Across Parkinson's grant ASAP000519 (SK, AEL). AVS was supported by the Molecular Biophysics Training Grant (NIH Grant T32 GM139795) and an NSF Postgraduate Fellowship.

Chapter 3 The structural basis for LRRK2's activation and autoinhibition

3.1 LRRK2 GTPases Role

Several studies have examined the role of the ROC GTPase domain in regulating LRRK2 activity, but the results are not consistent. Earlier work showed that addition of GTP or non-hydrolyzable GTP analogs to cell lysates correlates with increased LRRK2 kinase activity^{56,57}, and that pathogenic mutations in the ROC–COR region can enhance GTP binding^{56,58} and affect GTP hydrolysis^{59,60}. However, while nucleotide-dependent effects were frequently observed in lysate-based assays, they were reduced or absent when nucleotides were added to immunoprecipitated or purified LRRK2, indicating a strong dependence on experimental and cellular context^{56,57}. Structurally, the direct connection between the LRR, which blocks access to the kinase active site, and the ROC domain makes its nucleotide state a prime candidate to regulate LRRK2. In support of this role, the interface between ROC and the adjacent COR-B domain is a hotspot for PD-linked mutations (Figure 3.1A,B), and some of these mutations have been shown to alter GTP binding^{56,58} or hydrolysis. However, no structural studies have systematically examined the effect of the ROC nucleotide state on LRRK2 activation and autoinhibition. All structures of LRRK2 reported to date where the nucleotide state of the ROC domain could be determined, including those capturing an active conformation, were bound to GDP. . All structures of LRRK2 reported to date where the nucleotide state of the ROC domain could be determined^{61–66}, including those capturing an active conformation, were bound to GDP.

Here, we set out to determine how the nucleotide state of the ROC GTPase regulates LRRK2 activation and autoinhibition, structurally and mechanistically. Understanding this regulation could open new therapeutic avenues to allosterically inhibit LRRK2's kinase activity.

The accumulated evidence has shown that different types of kinase inhibitors stabilize different LRRK2 conformations^{62–64}, and that these conformations have different cellular localizations and/or interactomes^{35,43,46,47,62}. Although the clinical relevance of these differences is not yet clear, they suggest that developing multiple approaches for targeting LRRK2 is desirable. In parallel, there is growing evidence that LRRK2 plays an important role in the lung and that loss of function is linked to both pulmonary fibrosis and tumorigenesis. Thus, allosteric activators of LRRK2 may be useful therapeutics as well.

The foundation of this study is an unbiased structural approach to understand if and how the nucleotide state of LRRK2's ROC domain correlates with LRRK2's active and autoinhibited conformations. Throughout this work, we use 'activated' to refer to any LRRK2 conformation where its N-terminal repeats are undocked, and 'autoinhibited' to refer to those conformations where the N-terminal repeats are docked and thus blocking access to the kinase active site. Note that, in this context, activated refers to accessibility to the kinase active site and not to kinase activity itself.

3.2 Results

Cryo-EM reveals that GDP loss from the GTPase domain leads to LRRK2 activation

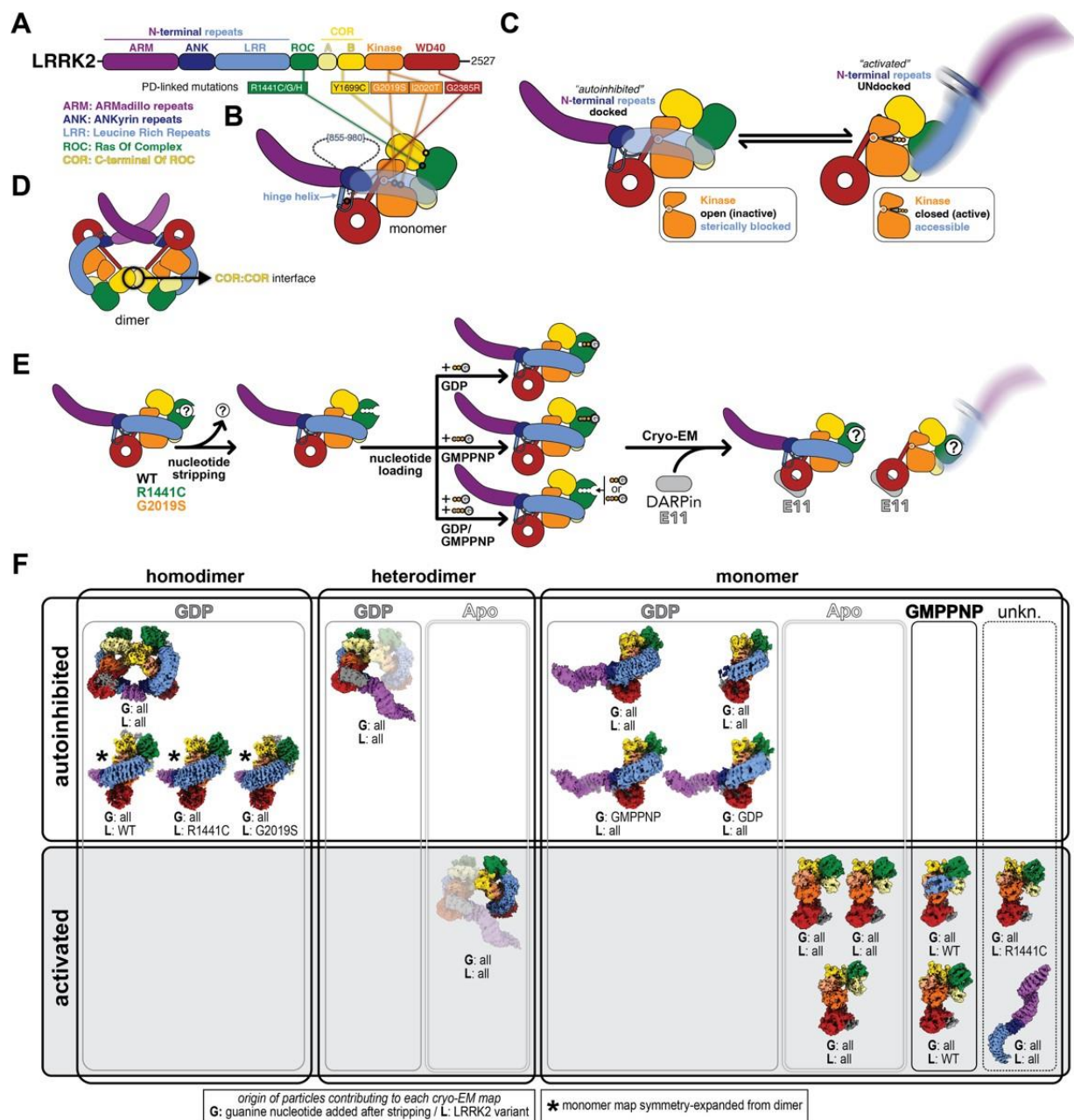
To determine structures of LRRK2 in different nucleotide states, we used a nucleotide exchange protocol to strip off the GDP that is typically bound to purified LRRK2 (see Methods). We then incubated LRRK2 with one of the following: (1) GDP; (2) the non-hydrolyzable GTP analog GMPPNP; or (3) a mixture of GDP and GMPPNP (Figure 3.1E). We used three different LRRK2 variants: wild-type and the PD-linked mutants G2019S and R1441C (Figure 3.1A, 3.1B). G2019S, found in the kinase's active site, is the most common PD-linked mutation in LRRK2. R1441C is found in the ROC domain at the interface between ROC and COR-B (Figure 3.1A,

3.1B). We collected data from the resulting samples and combined all the data sets into one for the initial data processing. Later, we split some of the data according to the LRRK2 variant or the nucleotide used in the samples (see Methods and Figures S3.1-S3.7). All samples were incubated with the Designed Ankyrin Repeat Protein (DARPin) E11, which we previously showed binds to the WD40 domain and does not affect kinase activity *in vitro*⁷⁰. The reason for including DARPin E11 in our samples is that its presence increases the range of orientations LRRK2 adopts on cryoEM grids, which leads to higher resolution structures⁷⁰.

Data processing revealed a significant level of compositional and conformational heterogeneity in our samples, as expected, including multiple cryo-EM maps representing activated LRRK2 (Figure 3.1F, and Figure S3.1). We were able to build models for 16 distinct cryo-EM maps (Figure 3.1F, and Figures S3.1-S3.7). Nine of these maps corresponded to autoinhibited LRRK2 and could be classified into three states (Figure 3.1F): a LRRK2 monomer with the N-terminal repeats docked; the dimeric form of this species; and a mixed dimer where one LRRK2 protomer had its repeats docked (autoinhibited) while the other had them undocked (activated). The eight maps representing activated LRRK2 could be classified into three structures (Figure 3.1F): the same mixed dimer (which is counted twice as it contains both autoinhibited and activated LRRK2); structures showing only the C-terminal half of LRRK2 containing the ROC, COR, Kinase, and WD40 domains; and structures showing only the N-terminal repeats.

Figure 3.1 Cryo-EM reveals that GDP loss from the GTPase domain leads to LRRK2 activation.

(A,B) Cartoon representations of the domain architecture (A) and structure of the LRRK2 monomer (B). These cartoons represent the autoinhibited conformation of LRRK2. Common PDlinked mutations are indicated in (A) and (B). We show the LRR as semi-transparent to make the active site of the kinase visible and use the same depiction in (C). **(C)** Cartoon representation of the autoinhibited and activated forms of LRRK2; this nomenclature is used throughout this work. Note: Although the cartoon for activated LRRK2 here shows the kinase in its closed or active conformation, we use ‘activated’ in the text to indicate only the conformation of the N-terminal repeats (i.e. undocked) regardless of the conformation of the kinase. **(D)** Cartoon representation of the LRRK2 dimer, mediated by COR:COR interactions. **(E)** Schematic representation of the strategy we used for our cryo-EM analysis: LRRK2 [WT, R1441C, or G2019S] was stripped of its nucleotide and then incubated with GDP, GMPPNP, or a mixture of both. Before preparing cryoEM grids, samples were incubated with DARPin E11, which binds to the WD40 domain and reduces the preferred orientation of LRRK2 on cryo-EM grids²³. Note: E11 is omitted from all cartoons of LRRK2 going forward for simplicity, although it is present in the cryo-EM maps. Data was collected from individual samples, but all data sets were merged and processed together initially, with sorting done after initial structures had been obtained and refined (see Methods and Figures S3.1-S3.7 for the data processing pipeline). **(F)** Table of all cryo-EM structures of LRRK2 obtained in this study for which models were built, as well as a map for the N-terminal repeats, for which no model was built. Structures are sorted according to whether they represent an autoinhibited or activated form of LRRK2, by the nucleotide state we determined for their GTPase domains (Apo, GDP, GMPPNP, or unknown), and whether they are dimeric or monomeric. For the heterodimers, the highlighted monomer is the one to which the descriptors apply. We indicate, below each map, what particles contributed to it based on the guanine nucleotide added to the sample (‘G’) and the LRRK2 variant (‘L’) used.



Autoinhibited LRRK2 is bound to GDP

Next, we determined the nucleotide state of ROC for the highest resolution map representing the monomeric form of autoinhibited LRRK2 (Figure 3.2A, and Figures S3.2, S3.3). In agreement with previous structures^{61,63,64}, this map fit GDP better than GMPPNP (Figure 3.2B, 3.2C). We then asked which data sets had contributed to this structure. Surprisingly, we found that particles coming from samples treated with any of the three nucleotide conditions contributed to the map, with a majority coming from samples treated with GMPPNP. We split the data by nucleotide added, and generated new maps comprised of particles from samples that had been treated either with GDP (Figure 3.2D) or GMPPNP (Figure 3.2E). This revealed that the map arising from samples treated with GMPPNP also accommodated GDP better than GMPPNP (Figure 3.2F, 3.2G). We interpreted this as incomplete nucleotide stripping, with some LRRK2 molecules maintaining their originally bound GDP throughout the process.

To test this, we prepared a sample where we extended the nucleotide stripping step from 1h to 24h; while samples stripped for 1h showed 23-35% of their particles in the autoinhibited conformation, the sample stripped for 24h had 100% of the particles in the active state (Figure 3.2H and Figure S3.8). Taken together, our data show that autoinhibited LRRK2 is in a GDP bound state.

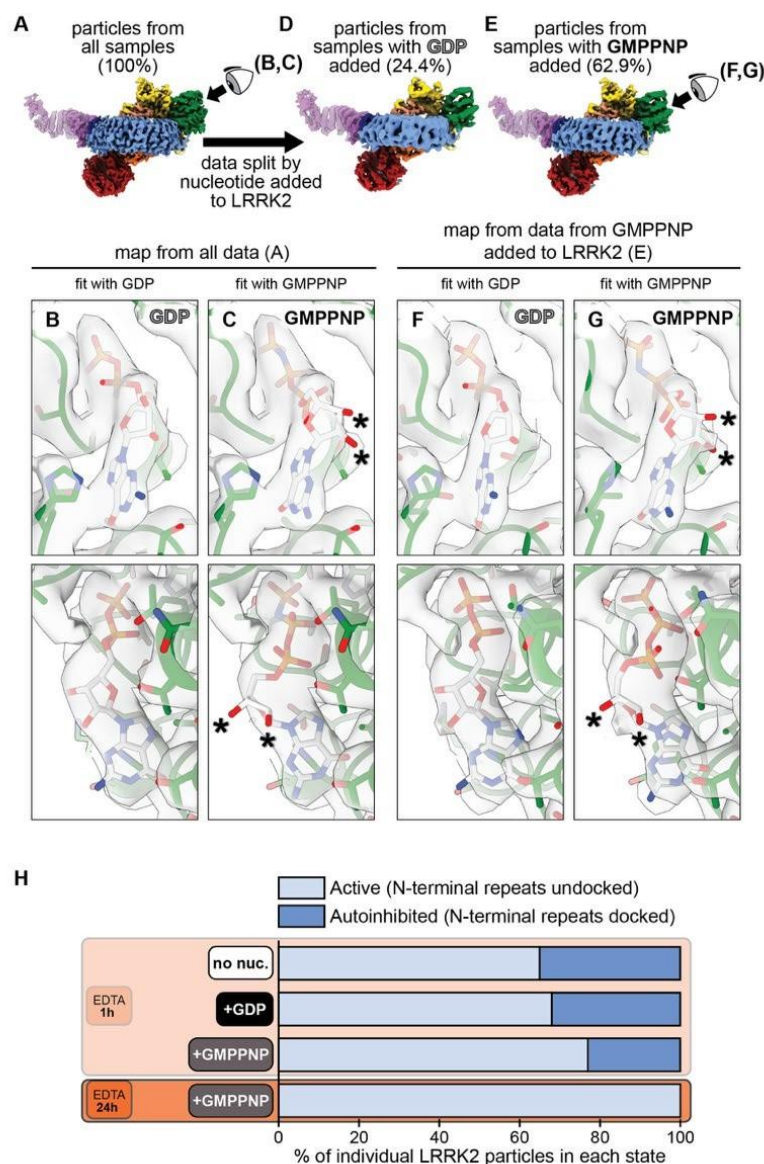


Figure 3.2 Autoinhibited LRRK2 is bound to GDP.

(A) Cryo-EM map of autoinhibited LRRK2 comprised of particles coming from all samples (i.e. not sorted based on the nature of the samples). **(B,C)** Close-ups of the nucleotide-binding pocket for the map in (A) with the model built and refined containing either GDP (B), or GMPPNP (C). The asterisks mark the 2'-OH and 3'-OH of the ribose in GMPPNP, as these are the features whose fit in the map differs most between the two nucleotides. Once we determined that the map in (A) was comprised of particles coming from samples treated with GDP (24.4%), GMPPNP (62.9%), and GDP + GMPPNP (12.7%), we reconstructed maps containing particles arising from samples treated only with GDP **(D)** or GMPPNP **(E)**. **(F, G)** Close-ups of the nucleotide-binding pocket for the map in (E) with the model built and refined containing either GDP (F), or GMPPNP (G). As in (B,C), asterisks mark the 2'-OH and 3'-OH of the ribose in GMPPNP. **(H)** Distribution of LRRK2 particles (cryo-EM data) into active and autoinhibited conformations as a function of the length of the nucleotide stripping treatment (EDTA) and nucleotide added after it. (See Figure S3.8 for data processing pipeline.)

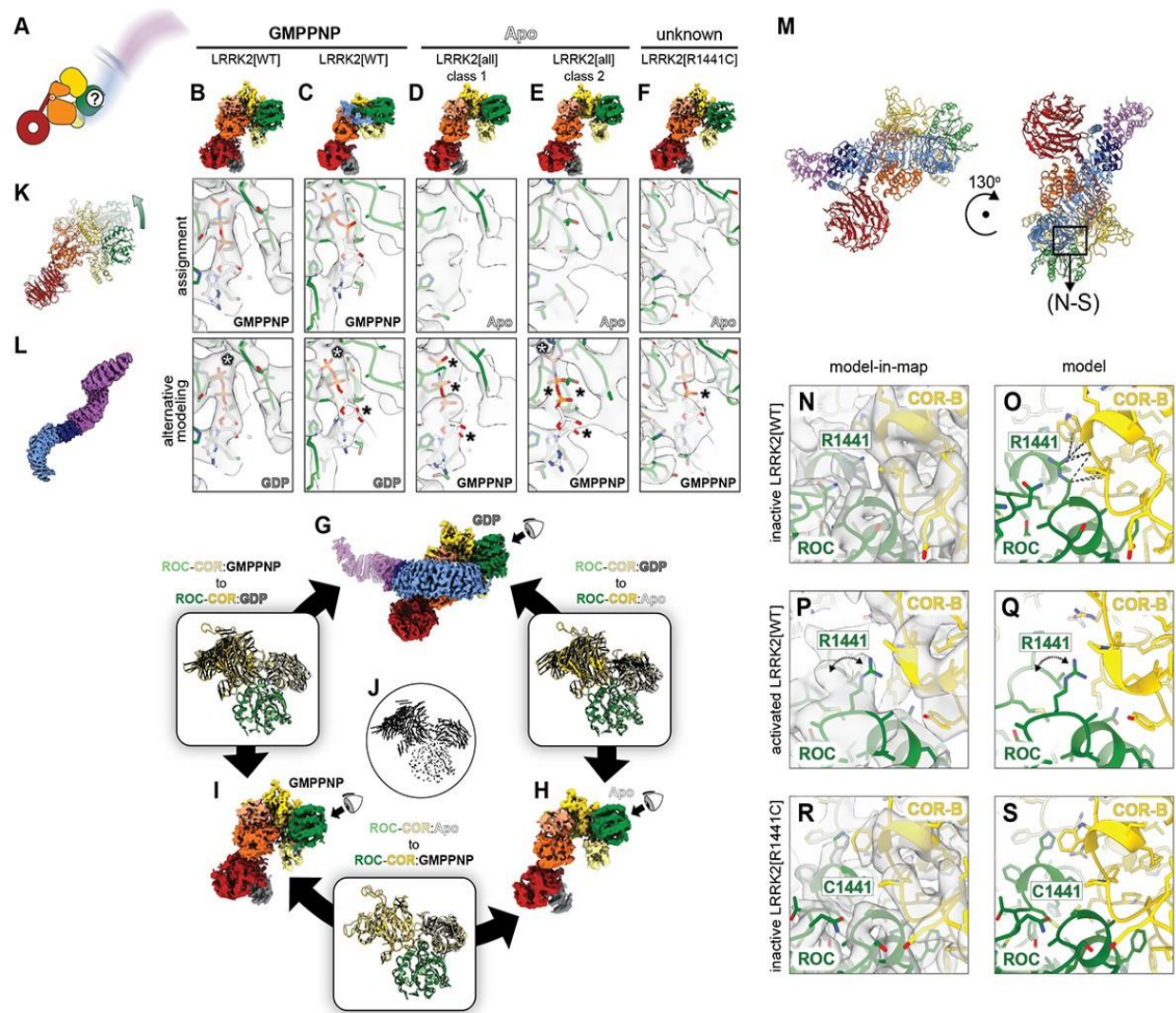
Active LRRK2 is either bound to GMPPNP or lacks nucleotide

We next focused on the structures of activated LRRK2, where the N-terminal repeats are undocked and thus not seen in our cryo-EM maps because the repeats are flexible relative to the C-terminal half of LRRK2. (Figure 3.3A-3.3F, and Figures S3.4, S3.6). The activated structures of LRRK2 also differed from the GDP-bound, autoinhibited form of LRRK2 in the conformational heterogeneity of their ROC and COR-A/B domains, which show flexibility relative to the kinase and to each other (Figure 3.3G-3.3J, Figure S3.9).

We obtained five volumes representing activated LRRK2 for which we were able to build models (Figure 3.3B-3.3F, and Figures S3.4-S3.6) and could determine the nucleotide state of the ROC domain for four of them; two volumes contained GMPPNP (Figure 3.3B, 3.3C) while two lacked nucleotide (Figure 3.3D, 3.3E). These structures suggest that LRRK2 activation requires the loss of GDP, which our data show correlates with the autoinhibited form, and that the activated state was maintained either in the absence of nucleotide or when GTP (GMPPNP here) was bound. One GMPPNP-bound structure of activated LRRK2 was unique because we observed density for the C-terminal portion of the LRR, which connects to the GTPase (Figure 3.3C), possibly representing an intermediate of activation or autoinhibition (Figure 3.3K, and Figure S3.9B, S3.9C).

Figure 3.3 Activated LRRK2 is bound to GMPPNP or lacks nucleotide.

(A) Schematic representation of activated LRRK2, with its repeats undocked. **(B-F)** Guanine nucleotide states of the ROC domains of activated LRRK2. Maps are shown for 5 different forms of activated LRRK2. Below the maps, we show close-ups of the nucleotide binding pocket of the ROC domain. The top row shows the nucleotide assignments we made based on the analysis of the structures. The bottom row shows an alternative modeling to highlight the reasons why it was rejected. The asterisks highlight problems with the fitting: black asterisks mark portions of the nucleotide that do not fit inside the density while white asterisks mark parts of the map that are not accounted for by the nucleotide or come too close to it. The models on the top row were refined in the presence of GMPPNP (B, C) or absence of nucleotide (D-F). The panels on the bottom row show the same maps and protein models as in the top row, with the alternative nucleotide modeled in by aligning the structure that contains it to the one shown in the panel and then displaying only the nucleotide. For panels labeled ‘GDP’, we aligned the ROC domain from LRRK2:G10:GDP (Figure 3.5E). For those labeled ‘GMPPNP’, we aligned the structure of LRRK2[WT]:GMPPNP (this figure, panel (B)). **(G-J)**, Conformational changes in ROC-COR between different nucleotide states of LRRK2. We aligned the ROC-COR modules of autoinhibited LRRK2:GDP (G), activated LRRK2:Apo (H), and activated LRRK2:GMPPNP (I) by their GTPase domains. The panels between the structures show the ROC-COR modules from the two structures along with vectors, in black, connecting equivalent alpha carbons between the structures (shown by themselves as an example in (J)). **(K)** Superposition of the model for activated LRRK2 with partial LRR density (C), and the C-terminal half from autoinhibited LRRK2 (LRRK2:G10:GDP, Figure 3.5E) to highlight that the former is in the activated conformation. The autoinhibited structure is shown in dim colors, and the green arrow shows the different positions of the ROC-COR module in the two models. **(L)** Cryo-EM map, colored by domain, of the undocked N-terminal repeats of LRRK2. The images contributing to this map originated from the same particles that gave rise to the maps of the C-terminal half of LRRK2 shown in this figure. **(M-S)** R1441 is involved in stabilizing the ROC:COR-B interface (the area within the box in (M)). **(N, O)** Model-in-map (N) and model alone (O) for close-ups of the ROC:COR-B interface in autoinhibited LRRK2 (the LRRK2:G10:GDP structure) showing interactions between R1441 and COR-B. **(P, Q)** The same interface in the activated form of LRRK2[WT]:GMPPNP. Because the conformational changes in ROC-COR in activated LRRK2 involve a movement of COR-B away from ROC, R1441 no longer interacts with COR-B. The cryo-EM map of activated LRRK2[WT]:GMPPNP (P) shows density for two conformations of R1441, neither of which interacts with COR-B. The dashed arrow indicates the two conformations. **(R, S)** Model-in-map and model alone for activated LRRK2[R1441C] showing the R1441C mutation. A comparison with panels (N, O) shows how the stabilizing interaction between ROC and COR-B in autoinhibited LRRK2 cannot happen in the R1441C mutant.



We wanted to test the idea that removal of GDP was sufficient to activate LRRK2. We first tested this structurally: we applied our nucleotide-stripping protocol to LRRK2 without subsequently adding back a nucleotide. Data processing of images collected from this sample. The 24h incubation in the ‘mock’ nucleotide stripping (i.e. without EDTA) leads to some loss of LRRK2 and an associated reduction in activity relative to an untreated sample (Figure 4B and Figure S3.10D). Incubation with EDTA, however, leads to increased phosphorylation of Rab8a relative to both untreated and ‘mock’ treated LRRK2 (Figure 3.4B and Figure S3.10D).

One molecular species of LRRK2 that was conspicuously absent from our data sets was a dimer of activated LRRK2s (Figure 3.1F). We observed dimers of the autoinhibited form, and mixed dimers with one autoinhibited and one activated protomer. However, none of our data sets contained a dimer where both LRRK2 protomers had their repeats undocked. We wondered if activation of LRRK2 might destabilize the dimer. This was indeed the case: activating LRRK2 with the nucleotide-stripping protocol resulted in a shift from dimers to monomers, as measured by mass photometry (Figure 3.4C and 3.4D and Figure S3.18A).

We also solved a structure of the undocked N-terminal repeats (Figure 3.3L, and Figure S3.7), which contained a significant fraction of particles coming from monomeric activated LRRK2. Our ability to obtain this structure shows that the repeats remain structured when undocked from the C-terminal half of LRRK2. Importantly, modeling suggested that the Nterminal repeats could not redock when LRRK2 is in its activated conformation (Figure S3.11). Without LRRK2 going back to the conformation seen in its GDP-bound state, docking would require flexibility in the repeats that we have not seen in our data (Figure S3.11). Thus, the combination of conformational changes at the ROC-COR interface and the rigidity of the Nterminal repeats appear to be key elements in LRRK2’s regulation.

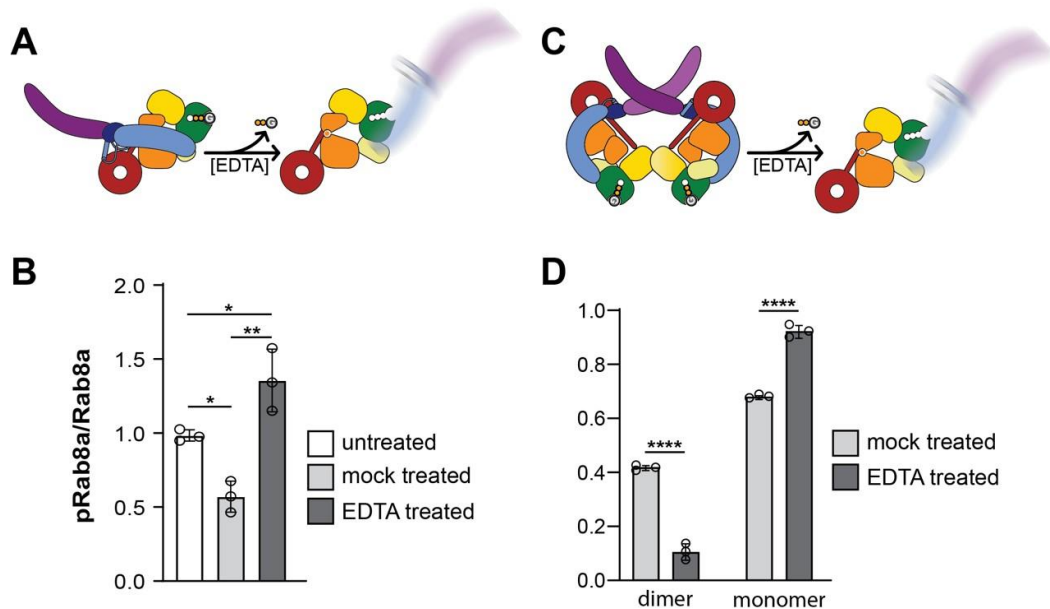


Figure 3.4 Removal of GDP activates LRRK2 and disrupts the dimer form.

(A) Schematic representation of the hypothesis being tested: that removal of GDP by exposing LRRK2 to the nucleotide stripping protocol leads to its activation. (B) LRRK2[WT] was incubated with ('EDTA treated') or without EDTA ('mock treated') for 24 hours followed by addition of 20mM MgCl₂ and further incubation for 24 hours. Untreated LRRK2 ('untreated') was used immediately upon thawing from frozen aliquots. All LRRK2 samples were incubated with Rab8a in the presence of ATP, with one negative control lacking ATP. Background-corrected pRab8a was normalized to the average pRab8a in the untreated LRRK2 + ATP condition from [n=3] independent Western blots. Images were processed in ImageJ. All data were analyzed using a oneway ANOVA with Tukey's multiple comparison correction. *p = 0.0246 for untreated vs 'mock'; *p = 0.0378 for untreated vs 'EDTA'; **p = 0.0011 for 'mock' vs 'EDTA'. (See Figure S3.15 for representative immunoblot.) (C) Schematic representation of the hypothesis being tested: that removal of GDP by exposing LRRK2 to the nucleotide stripping protocol destabilizes the dimeric form. (D) Fraction of LRRK2 monomers and dimers relative to (monomer + dimer) for LRRK2 exposed to the nucleotide stripping protocol or mocked-stripped (see Methods). Total particle counts corresponding to the molecular weights of either monomer or dimer were determined from mass photometry traces. The fraction of dimers contributing to the total (monomer + dimer) number of LRRK2 landing events was analyzed with one-way analysis of variance (ANOVA) and corrected for multiple comparisons using Tukey's test. Individual data points represent 3 technical repeats. ****p < 0.0001. See Figure S3.18A for mass photometry traces and particle counts. showed that a significant fraction of the LRRK2 molecules were in their active conformation (i.e. the N-terminal repeats are not seen) (Figure S3.10A-S3.10C). Next, we tested this functionally by measuring the effect of GDP removal on LRRK2 phosphorylation of Rab8a in vitro (Figure 3.4A).

The 24h incubation in the ‘mock’ nucleotide stripping (i.e. without EDTA) leads to some loss of LRRK2 and an associated reduction in activity relative to an untreated sample (Figure 4B and Figure S3.10D). Incubation with EDTA, however, leads to increased phosphorylation of Rab8a relative to both untreated and ‘mock’ treated LRRK2 (Figure 3.4B and Figure S3.10D).

One molecular species of LRRK2 that was conspicuously absent from our data sets was a dimer of activated LRRK2s (Figure 3.1F). We observed dimers of the autoinhibited form, and mixed dimers with one autoinhibited and one activated protomer. However, none of our data sets contained a dimer where both LRRK2 protomers had their repeats undocked. We wondered if activation of LRRK2 might destabilize the dimer. This was indeed the case: activating LRRK2 with the nucleotide-stripping protocol resulted in a shift from dimers to monomers, as measured by mass photometry (Figure 3.4C and 3.4D and Figure S3.18A).

We also solved a structure of the undocked N-terminal repeats (Figure 3.3L, and Figure S3.7), which contained a significant fraction of particles coming from monomeric activated LRRK2. Our ability to obtain this structure shows that the repeats remain structured when undocked from the C-terminal half of LRRK2. Importantly, modeling suggested that the Nterminal repeats could not redock when LRRK2 is in its activated conformation (Figure S3.11). Without LRRK2 going back to the conformation seen in its GDP-bound state, docking would require flexibility in the repeats that we have not seen in our data (Figure S3.11). Thus, the combination of conformational changes at the ROC-COR interface and the rigidity of the Nterminal repeats appear to be key elements in LRRK2’s regulation.

The PD-linked R1441C/G/H mutations likely promote undocking of the N-terminal repeats

R1441 is located at the interface between the ROC GTPase and the COR-B domain (Figure

3.1A, 3.1B), a region where several PD-linked mutations cluster. To understand how the R1441C/G/H mutations increase Rab phosphorylation by LRRK2⁷¹, we first compared structures of LRRK2[WT] in its autoinhibited and activated conformations (Figure 3.3M-3.3S). In GDPbound, autoinhibited LRRK2[WT], R1441 packs against the COR-B domain, likely contributing to the stabilization of this interface, and thus to autoinhibition (Figure 3.3N, 3.3O). In contrast, the structure of GMPPNP-bound, activated LRRK2[WT] revealed density that can accommodate R1441 in two different rotamers; one pointing towards COR-B and the other away from it (Figure 3.3P, 3.3Q); importantly, neither rotamer interacts with COR-B in the activated state, where CORB has moved away from ROC. We hypothesize that R1441, which is far away from the nucleotide binding pocket and thus unlikely to directly affect binding or hydrolysis, acts as a latch that helps stabilize the ROC-COR interactions as COR-B approaches ROC when LRRK2 returns to its autoinhibited conformation. This interaction between R1441 and COR-B is absent in the PD-linked R1441C mutant (Figure 3.3R, 3.3S), which would therefore favor the activated state of LRRK2. A comparison of cryo-EM data obtained from untreated LRRK2 (i.e. not exposed to the nucleotide-stripping protocol) either WT or carrying the R1441C mutation showed a moderate increase in the fraction of particles with their N-terminal repeats undocked in LRRK2[R1441C] (Figure S3.12). Thus, our data suggest that the increased Rab phosphorylation seen in LRRK2[R1441C/G/H] is a result of a shift in the LRRK2 population towards its activated conformation rather than a direct effect on the catalytic activity of the kinase. A test of this hypothesis is discussed below.

Development of DARPins to control LRRK2's activation

The data presented above show that autoinhibited LRRK2 is bound to GDP and that its loss leads to LRRK2 activation. Our data also suggest that the coupling between nucleotide and activation state is mediated by the conformation of the ROC-COR module, whose flexibility is constrained when the N-terminal repeats are docked. Given this, we wondered if the conformation (docked or undocked) of the N-terminal repeats would in turn regulate nucleotide binding and hydrolysis in the ROC domain. To test this, we needed a way to promote or prevent the undocking of the N-terminal repeats that did not require directly exchanging the nucleotide of the GTPase. We had previously identified Designed Ankyrin Repeat Proteins (DARPins) that bound to LRRK2⁷⁰. The first DARPin we characterized⁷⁰, E11, bound to the WD40 domain in an area where it does not interact or interfere with any other part of LRRK2, and did not affect LRRK2 kinase activity in vitro⁷⁰ (Figure 3.5K). Here, we screened for other DARPins that might alter LRRK2's activation state and identified two additional DARPins that bind to the WD40 domain: one that activates LRRK2 (C12), and one that stabilizes its autoinhibited conformation (G10) (Figure 3.5). DARPin C12 binds at a location on the WD40 domain that is sterically incompatible with the docked conformation of the N-terminal repeats (Figure 3.5A-3.4D, Figure S13). DARPin G10 binds both to the WD40 domain and to the N-terminal repeats, bridging them (Figure 3.5E-3.4H, Figure S3.14). As predicted from the structures (Figure 3.5I, 3.4J), DARPin C12 increased LRRK2s kinase activity towards Rab8a in vitro, whereas DARPin G10 decreased it (Figure 5K, Figure S15).

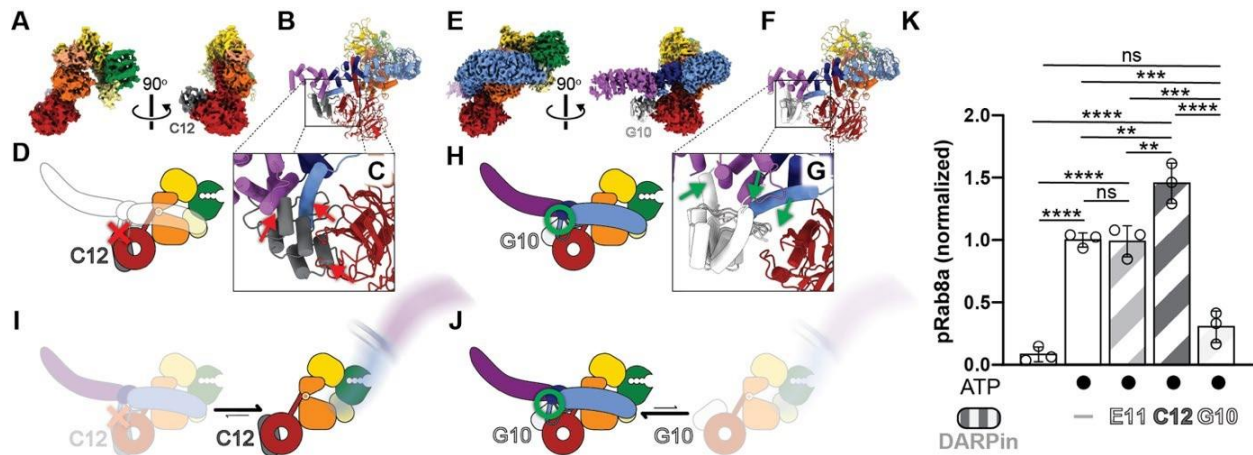


Figure 3.5 Cryo-EM structures of LRRK2 and LRRK2^{RCKW} bound to DARPins C12 and G10.

(A) Cryo-EM map of LRRK2^{RCKW} with DARPin C12 bound to its WD40 domain. (B, C) Model of full-length LRRK2 bound to C12 showing the clashes that would result between the N-terminal repeats and C12 (B), and close-up of those clashes (C). (D) Cartoon illustration of the clashes. (E) Cryo-EM map of LRRK2 with DARPin G10 bound to the WD40 domain. (F, G) Structure of LRRK2 bound to G10 showing that G10 interacts both with the WD40 domain and the N-terminal repeats (F), and close-up of the interactions (G). (H) Cartoon illustration of the interactions. (I) DARPin C12 is predicted to activate LRRK2 by promoting the conformation with the N-terminal repeats undocked. (J) DARPin G10 is predicted to inhibit LRRK2 by stabilizing the conformation with the N-terminal repeats docked. (K) In vitro Rab8a phosphorylation assays. LRRK2[WT] was incubated with Rab8a in the presence of ATP (with a no ATP control) plus one of the following: no DARPin, E11, C12, or G10. Background-corrected pRab8a was normalized to the average of pRab8a in the LRRK2 + ATP condition from [n=3] independent Western blots. Images were processed in ImageJ; all data were analyzed using a one-way ANOVA with Tukey's multiple comparison correction. p = non-significant for No ATP vs LRRK2 + G10, all other comparisons with No ATP ****p<0.0001. LRRK2 vs LRRK2+E11 were non-significant; **p=0.0042 for LRRK2 vs LRRK2+C12; ***p=0.0001 for LRRK2 vs LRRK2+G10; **p=0.0036 for LRRK2+E11 vs LRRK2+C12; ***p=0.0002 for LRRK2+E11 vs LRRK2+G10; and ****p<0.0001 for LRRK2+C12 vs LRRK2+G10

G2019S and R1441H increase Rab phosphorylation by LRRK2 via different mechanisms

Our structural analysis suggested that R1441C/G/H mutations activate LRRK2 by favoring the undocked conformation of the N-terminal repeats (Figure 3.3M-3.3S). G2019S, on the other hand, is in the catalytic DYG triad in the kinase's active site. Thus, we hypothesized that these two

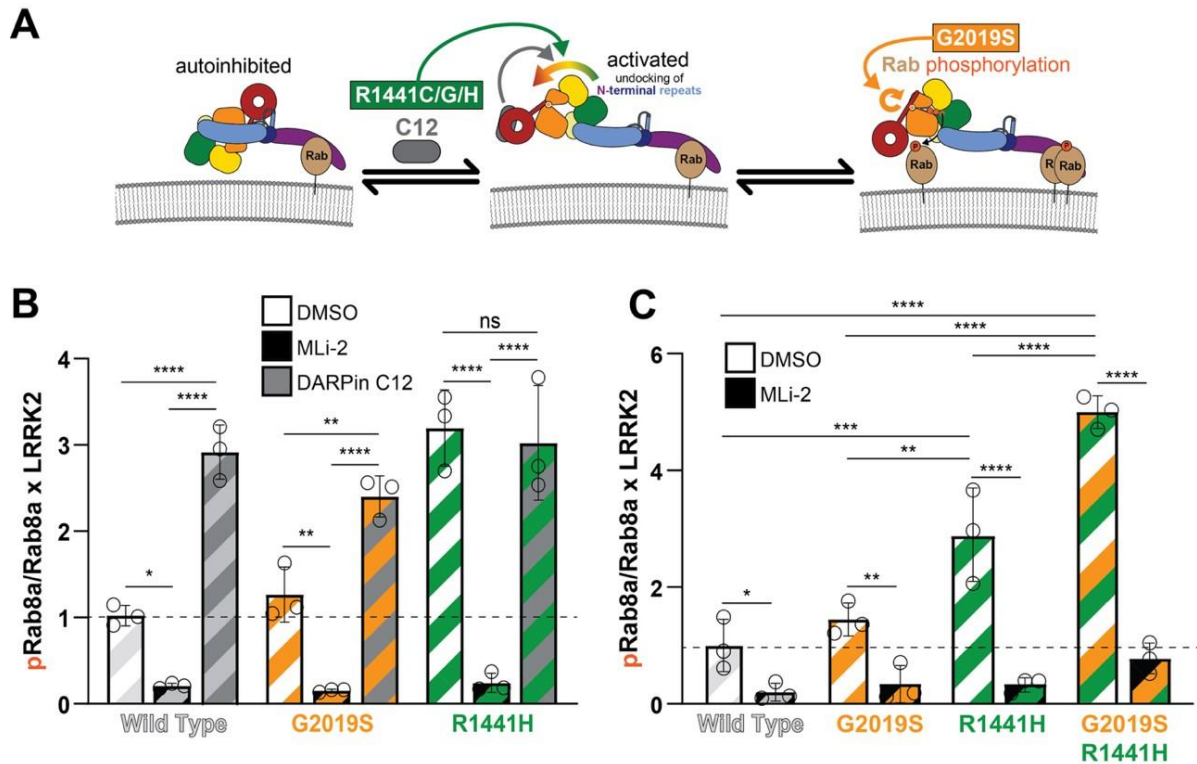


Figure 3.6 The PD mutants R1441C/G/H and G2019S increase Rab phosphorylation by different mechanisms.

(A) Proposed mechanism of activation of LRRK2 by the PD-linked mutations R1441C/G/H and G2019S. R1441C/G/H act by promoting the release of autoinhibition, while G2019S increases the activity of the kinase itself. Like R1441C/G/H, DARPin C12 also promotes the release of autoinhibition. **(B)** Quantification of pRab8a (pT72) / Rab8a x LRRK2, normalized to the mean of LRRK2[WT] + DMSO condition from [n=3] independent Western blots. HEK 293T coexpressing GFP-11-tagged LRRK2[WT], LRRK2[G2019S], or LRRK2[R1441H] and GFP-Rab8a with or without Flag-tagged DARPin C12 were treated with either DMSO, MLi-2, or nothing in the C12 condition. Samples were immunoblotted for LRRK2, phospho-Rab8a (pT72), GFP, and Flag. Images were processed on ImageJ, and all data were analyzed using a two-way ANOVA with Tukey's multiple comparison correction. **** $p < 0.0001$ for WT+C12 vs DMSO and MLi-2 and * $p = 0.0161$ for WT+DMSO vs MLi-2; ** $p = 0.0011$ and **** $p < 0.0001$ G2019S+C12 vs DMSO and MLi-2; ** $p = 0.0013$ G2019S+DMSO vs MLi-2. **** $p < 0.0001$ R1441H+MLi-2 vs C12 and DMSO; non-significant R1441H DMSO vs C12. Error bars $\pm$ SD. **(C)** Quantification of pRab8a (pT72) / Rab8a x LRRK2 normalized to the mean of LRRK2[WT] + DMSO condition. HEK 293T co-expressing LRRK2[WT], LRRK2[G2019S], LRRK2[R1441H] or the double mutant LRRK2[G2019S/R1441H] and GFP-Rab8a were treated, processed and analyzed as before (B). DMSO vs MLi-2: * $p = 0.0229$ WT, ** $p = 0.0031$ G2019S, and *** $p < 0.0001$ R1441H and DM; non-significant G2019S+DMSO vs WT+DMSO; ** $p = 0.0016$ G2019S+DMSO vs R1441H+DMSO; *** $p = 0.0001$ R1441H+DMSO vs WT+DMSO; **** $p < 0.0001$ all comparisons with the G2019S/R1441H double mutant. Error bars $\pm$ SD.

PD-linked mutations lead to increased Rab phosphorylation in cells by acting in different steps of

the LRRK2 activation/phosphorylation pathway (Figure 3.6A): R1441C/G/H mutations enrich the population of activated LRRK2, while G2019S activates the kinase itself. We tested this in two different ways.

First, we used the C12 DARPin, which activates LRRK2 by favoring the undocking of the N-terminal repeats. Mirroring our results in vitro (Figure 3.5K), expressing DARPin C12 in cells led to a significant increase in the phosphorylation of Rab8a by LRRK2[WT] (Figure 3.6B). This effect is not exclusive to Rab8a; we observed a similar increase in the phosphorylation of Rab10 (Figure S3.16). If R1441C/G/H mutations increase activity by favoring undocking of the repeats, as we hypothesized, the presence of C12 should not lead to a significant additional increase in Rab phosphorylation. LRRK2 G2019S, on the other hand, acts on the kinase itself and should be further activated by C12. This was indeed the case when we measured Rab8a phosphorylation in cells coexpressing C12 and either LRRK2[G2019S] or LRRK2[R1441H] (Figure 3.6B and Figure S3.17A).

A second prediction from our hypothesis (Figure 3.6A) is that the activating effects of the R1441C/G/H and G2019S mutations should be additive to some extent, as they act in series, with R1441C/G/H mutations increasing the pool of activated LRRK2, and G2019S increasing the kinase activity of that pool. Indeed, the increase in Rab8a phosphorylation in cells by the double mutant LRRK2[R1441H/G2019S] was close to the sum of the effects seen with the single mutants (Figure 3.6C and Figure S3.17B).

Taken together, these data support the hypothesis that even though R1441C/G/H and G2019S both lead to an increase in Rab phosphorylation in cells, they do so by acting on different steps in the LRRK2 pathway.

Nucleotide exchange and hydrolysis by ROC are regulated both by the N-terminal repeats and the Rab substrate

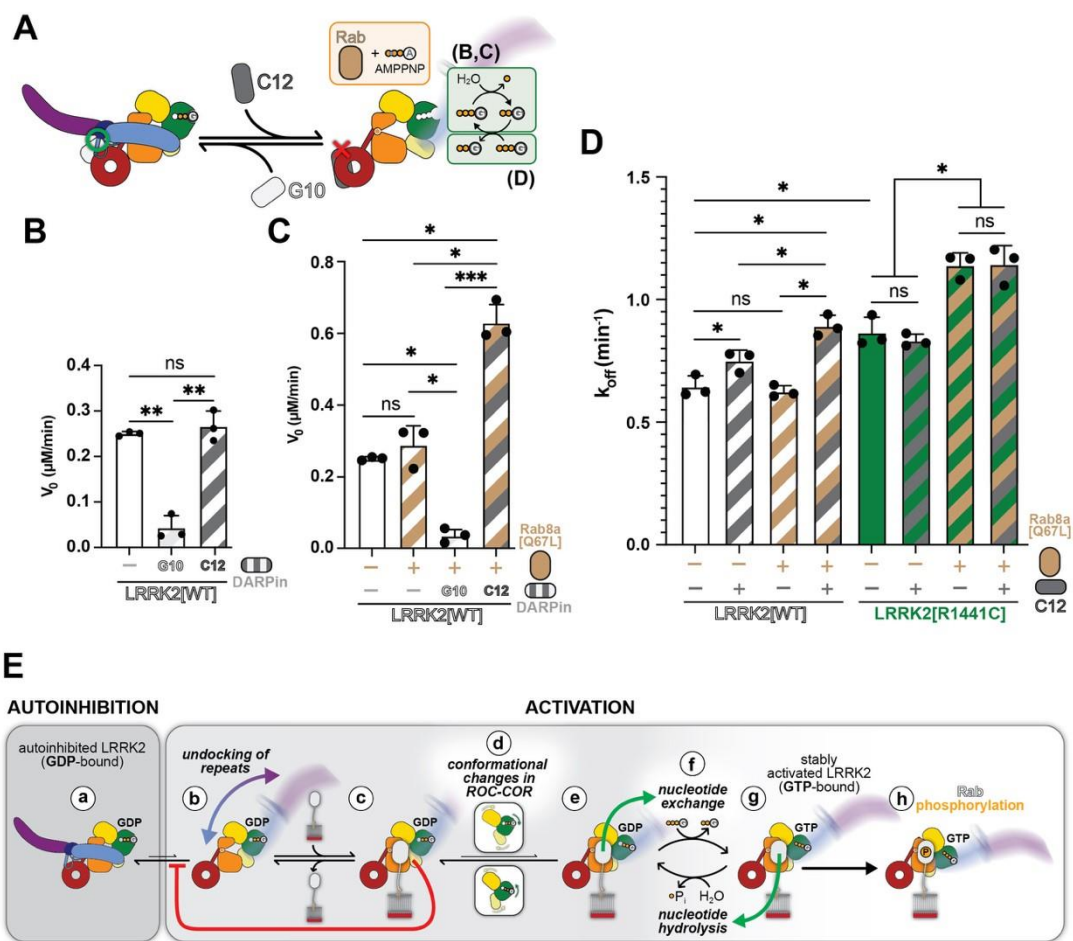
We next tested the effect of DARPins C12 and G10 on the GTPase activity of LRRK2. Our hypothesis (Figure 3.7A) was that G10 would stabilize the autoinhibited conformation of LRRK2 and thus prevent cycles of nucleotide exchange and hydrolysis, while C12 would stimulate them by promoting the undocking of the N-terminal repeats. Our initial data agreed with our prediction that G10 would inhibit the GTPase activity of LRRK2; however, C12 had no effect on it (Figure 3.7B). We wondered whether undocking of the N-terminal repeats was not sufficient to stimulate GTP hydrolysis, and if adding a Rab substrate might. Indeed, the combination of C12 and Rab8a (the Q67L variant that is locked in its GTP-bound state) led to a significant activation of the GTPase (Figure 3.7C). Importantly, Rab8a[Q67L] on its own had no effect on the baseline GTPase activity of LRRK2 (Figure 3.7C), suggesting that undocking of the N-terminal repeats by C12 is required for Rab8a to exert its effect on hydrolysis.

The effect of Rab8a on the rate of GTP hydrolysis could be the result of an increase in hydrolysis itself, an increase in the rate of GDP release, or a combination of both. To address this, we measured the rate of GDP release from LRRK2 pre-loaded with fluorescently labeled nucleotide in a single-turnover assay (see Methods). Consistent with our model where undocking of the N-terminal repeats lowers the affinity for GDP, the rate of GDP release from LRRK2[WT] was increased by the addition of DARPin C12 (Figure 3.7D). Addition of Rab8a on its own had no effect on GDP release, but the combination of C12 and Rab8a led to a small but significant increase relative to C12 alone, indicating that Rab8a is promoting GDP release (Figure 3.7D).

Lastly, we wanted to confirm that the effects we were seeing with C12 were due to its promoting the undocked conformation of LRRK2. For this, we turned to LRRK2[R1441C], which our data showed favors the undocked conformation of LRRK2 (Figure 3.6). Consistent with those results, GDP release from LRRK2[R1441C] was faster than from LRRK2[WT] and was in turn insensitive to the addition of C12 (Figure 3.7D). Finally, addition of Rab8a to LRRK2[R1441C] significantly increased GDP release, and was again insensitive to C12, supporting the idea that Rab8a is acting downstream of the undocking of the N-terminal repeats. Taken together, our data showed that Rab8a stimulates both GDP release and GTP hydrolysis in activated LRRK2.

Figure 3.7 The PD mutants R1441C/G/H and G2019S increase Rab phosphorylation by different mechanisms.

(A) Proposed mechanism of activation of LRRK2 by the PD-linked mutations R1441C/G/H and G2019S. R1441C/G/H act by promoting the release of autoinhibition, while G2019S increases the activity of the kinase itself. Like R1441C/G/H, DARPin C12 also promotes the release of autoinhibition. **(B)** Quantification of pRab8a (pT72) / Rab8a x LRRK2, normalized to the mean of LRRK2[WT] + DMSO condition from [n=3] independent Western blots. HEK 293T coexpressing GFP-11-tagged LRRK2[WT], LRRK2[G2019S], or LRRK2[R1441H] and GFP-Rab8a with or without Flag-tagged DARPin C12 were treated with either DMSO, MLi-2, or nothing in the C12 condition. Samples were immunoblotted for LRRK2, phospho-Rab8a (pT72), GFP, and Flag. Images were processed on ImageJ, and all data were analyzed using a two-way ANOVA with Tukey's multiple comparison correction. ****p<0.0001 for WT+C12 vs DMSO and MLi-2 and *p=0.0161 for WT+DMSO vs MLi-2; **p=0.0011 and ****p<0.0001 G2019S+C12 vs DMSO and MLi-2; **p=0.0013 G2019S+DMSO vs MLi-2. ****p<0.0001 R1441H+MLi-2 vs C12 and DMSO; non-significant R1441H DMSO vs C12. Error bars $\pm$ SD. **(C)** Quantification of pRab8a (pT72) / Rab8a x LRRK2 normalized to the mean of LRRK2[WT] + DMSO condition. HEK 293T co-expressing LRRK2[WT], LRRK2[G2019S], LRRK2[R1441H] or the double mutant LRRK2[G2019S/R1441H] and GFP-Rab8a were treated, processed and analyzed as before (B). DMSO vs MLi-2: *p=0.0229 WT, **p=0.0031 G2019S, and ***p<0.0001 R1441H and DM; non-significant G2019S+DMSO vs WT+DMSO; **p=0.0016 G2019S+DMSO vs R1441H+DMSO; ***p=0.0001 R1441H+DMSO vs WT+DMSO; ****p<0.0001 all comparisons with the G2019S/R1441H double mutant. Error bars $\pm$ SD.



3.3 Discussion

The nucleotide state of the ROC GTPase controls LRRK2 activation and autoinhibition

The work presented here, together with contributions from several other groups in the field, has led us to propose a model for how the nucleotide state of the ROC domain regulates the activation and autoinhibition of LRRK2 (Figure 3.7E). Our data also showed that the common PDlinked mutations R1441C/G/H and G2019S act on different steps of the activation mechanism and provided an explanation for how PD-associated mutations found on either side of this ROC-COR interface (e.g. N1437H, Y1699C) lead to increased Rab phosphorylation. Our structural data showed that the autoinhibited conformation of LRRK2 was always accompanied by a ROC domain bound to GDP (Figure 3.2), in agreement with all structures of this species published to date where the nucleotide state could be determined^{61,63,64}. This suggests that the docked conformation of the N-terminal repeats stabilizes a conformation of ROC with high affinity for GDP (Figure 3.7E(a)). This is consistent with our observation that complete stripping of GDP from autoinhibited LRRK2 requires long treatment with EDTA (Figure 3.2H and Figure S3.8).

Our model proposes that undocking of the N-terminal repeats and the resulting increased conformational flexibility in the ROC-COR module leads to a decrease in the ROC domain's affinity for GDP (Figure 7E(b-e)). This is supported by the increase in the rate of GDP release from LRRK2[R1441C], a mutant we have shown favors the undocked conformation of the Nterminal repeats (Figure 3.3N-3.3S and Figure 3.6). It is likely that any protein that interacts with LRRK2 and stabilizes its N-terminal domains in the undocked conformation will act as an activator by promoting nucleotide exchange, leading to stably active GTP-bound LRRK2. While a few activators have been identified—including Rab12^{72,73}, Rab32^{74,75}, and GABARAP^{76,77}—we do not yet understand whether they exert their effects by recruiting LRRK2 to membranes or additionally by somehow stabilizing the undocked conformation once LRRK2 is on the membrane.

Our data also revealed that the Rab substrate plays a role in stimulating the GTPase cycle in the ROC domain, accelerating both GDP release and GTP hydrolysis (Figure 3.7C and 3.7D, Figure 3.7E(e-g)). This mechanism allows the N-terminal repeats to act as a substrate sensor; promoting GTP hydrolysis to return LRRK2 to its GDP-bound state puts LRRK2 in a state compatible with a return to autoinhibition, a process that is prevented if Rab substrate is available to sterically block redocking of the N-terminal repeats (Figure 3.7E(c)). If re-docking is prevented, ROC-COR remains in the conformation with low affinity for GDP (Figure 3.7E(e)), leading to GDP release, GTP binding, and a return of LRRK2 to its stably active conformation (Figure 3.7E(g-h)).

Several studies over the years have addressed the role of the GTPase in regulating LRRK2 activity. While some are consistent with the work we presented here, others are not. In general, work done in cells or with lysates agrees with our observations: addition of GTP or non-hydrolyzable GTP analogs to cell lysates correlates with increased LRRK2 kinase activity^{78,79}. Similarly, LRRK2 localization and association with microtubules in cells correlate more closely with the ability to bind GTP than with kinase activity⁸⁰, and we now understand that binding to microtubules requires LRRK2 to be in its active conformation^{62,81,82}. Also in agreement with our data are observations that pathogenic mutations in the ROC–COR region can enhance GTP binding^{78,80} and affect GTP hydrolysis^{83,84}, and that disruption of guanine nucleotide binding by mutations in the P-loop of the GTPase domain (e.g., K1347A or T1348N) generally impaired kinase activity and showed cellular phenotypes across multiple systems^{79,85,86}. However, while nucleotide dependent effects were frequently observed in lysate-based assays, they were reduced or absent when nucleotides were added to immunoprecipitated or purified LRRK2, indicating a strong

dependence on experimental and cellular context^{78,79}. In addition, measurements of GTPase activity have yielded inconsistent results: while some studies reported low intrinsic hydrolysis and predominantly GTP-bound LRRK2 in cells⁸⁵, others showed that mutations that either increase or decrease GTP hydrolysis reduce kinase activity⁸⁶, suggesting that nucleotide binding is more important than hydrolysis in regulating kinase activity. Finally, there is evidence pointing to a role for autophosphorylation of the ROC domain in regulating GTP hydrolysis⁸⁴. It is difficult to determine where discrepancies arise from as the studies are not directly comparable. They differ in whether work was done in cells, with lysates, or, when using purified protein, on whether LRRK2 was immunoprecipitated or recombinantly expressed. They also report on a variety of assays (nucleotide binding, hydrolysis, Michaelis-Menten kinetics) and both auto- and substrate phosphorylation. Our work has shown that the activation state of LRRK2 is sensitive to how the protein is treated in guanine nucleotide exchange protocols. Given this, it is likely that some of the disagreements in the literature reflect differences in the distribution of active and autoinhibited populations of LRRK2 in cells and lysates vs. purified protein, or because of differences in the nucleotide exchange protocols used.

The active form of LRRK2 is likely a monomer

Several lines of evidence lead us to propose that the active form of LRRK2 is a monomer. Previous work has shown that disruption of dimerization is activating^{82,87}. Stripping LRRK2 of its bound GDP, a process that is activating and leads both to undocking of the N-terminal repeats (Figure S10) and an increase in kinase activity in vitro (Figure 3.4A and 3.4B, and Figure S3.11), correlates with a reduction in the dimeric form of LRRK2 (Figure 3.4C and 3.4D, and Figure S3.18A). Finally, in agreement with our mass photometry data, modeling a dimer in which both LRRK2s are activated showed that the conformational changes in the COR-A and COR-B domains

involved in activation lead to significant changes and clashes at the dimer interface (Figure S18BS3.18H).

In contrast to our observations and proposal, it has been suggested that LRRK2 is activated in the context of a tetramer⁶⁵. This model was based on a cryo-EM structure where LRRK2 formed a dimer of dimers, with each dimer containing one protomer of autoinhibited LRRK2 and one protomer of activated LRRK2, where its repeats were undocked. LRRK2 is known to dimerize, and structural work suggests that the highest affinity dimerization interface is the one mediated by interactions between its C-terminal Of ROC (COR) domains (COR-A and COR-B)^{61,62} (Figure 3.1D); this was the interface mediating the dimers that formed the tetramer. Given this, the model predicts that disrupting dimerization at the COR interface, which in turn would disrupt the tetramer, should result in a decrease in LRRK2 activity. However, the opposite is true: disrupting the dimer interface increases Rab phosphorylation, both in vitro⁸⁷ and in cells^{82,87}. While the physiological relevance of the reported tetramer remains to be determined, it is possible that its appearance was a consequence of the high concentrations of LRRK2 used for cryo-EM (2128 μ M)⁶⁵.

We hypothesize that one role of dimerization in LRRK2 may be, as we discuss further below, to stabilize the autoinhibited state by preventing the conformational changes in the ROCCOR module linked to activation.

The ROC-COR module as a regulatory nexus

Our work points to the ROC-COR module as a regulatory nexus in LRRK2, with the conformation of ROC-COR being coupled to the nucleotide state of the ROC GTPase. A recent cryo-EM structure of a bacterial Roco protein (which contain the LRR-ROC-COR module but lacks a kinase) showed undocking of its LRR domain when its GTPase was in a GTP-like state⁸⁸.

We propose that stabilizing the ROC-COR module in a specific conformation, as we suggest LRRK2 dimerization does for the autoinhibited form, may be a strategy that other regulatory factors use. Identifying such factors may lead to novel targets for PD therapeutics.

The best-established regulators of LRRK2 are 14-3-3 proteins⁸⁹⁻⁹². These proteins, which function as dimers, bind to phosphorylated residues (pS910 and pS935) in a flexible loop in the LRR domain, and are understood to keep LRRK2 in its autoinhibited form in the cytosol⁴⁷. The pS910 and pS935 marks are used as biomarkers in the diagnosis of LRRK2-linked PD, as the majority of pathogenic mutations that increase LRRK2 activity suppress biomarker site phosphorylation⁴⁶.

A structure of LRRK2 bound to 14-3-3 was recently reported⁶⁶. In this structure, a 14-3-3 dimer was bound simultaneously to the COR-A and COR-B domains of LRRK2 and densities for two phosphoserines were observed interacting with 14-3-3. The authors proposed that 14-3-3 maintains LRRK2 in an autoinhibited state by restricting the movement of the N-terminal repeats, as these are tethered to 14-3-3 via the loop containing pS910 and pS935. The authors also noted that 14-3-3 could only fully engage with autoinhibited LRRK2; the conformational changes in COR-A and COR-B during activation would prevent 14-3-3 from binding to both in the active form of LRRK2⁶⁶.

Based on the data presented here, we propose that the role of the pS910/pS935 residues in the loop is not to restrict the conformation of the N-terminal repeats, but rather to recruit 14-3-3 to LRRK2, as the loop is long enough to accommodate undocking even when bound to 14-3-3. In this model, 14-3-3's main function would be to clamp together the COR-A and COR-B domains to prevent the conformational changes that lead to GDP release and LRRK2 activation.

Interestingly, binding of 14-3-3 to LRRK2 prevents dimerization by blocking the CORCOR interface⁶⁶. Since LRRK2 dimerization and 14-3-3 binding both engage COR-A and CORB in their autoinhibited conformation, they could be alternative mechanisms to prevent the conformational changes required for LRRK2 activation. Understanding the interplay among these different regulatory mechanisms could also lead to novel therapeutic targets.

Nucleotide control of the docking and undocking of the N-terminal repeats

Our structural data suggest a mechanistic model for how the nucleotide state of ROC might control the docking and undocking of the N-terminal repeats (Figure S3.19). The LRR are connected to the ROC-COR module in the autoinhibited state of LRRK2 via a short two-stranded β -sheet (Figure S3.19A, S3.19B). At one end, both β -strands connect to the ROC domain. At the other end, they connect to different domains: one goes from the LRR to ROC, and the other from ROC to COR-A. This arrangement means that changes in the relative orientations of ROC and COR-A, as happens during activation of LRRK2, would destabilize this β -sheet. In fact, the β sheet is absent from all our structures of activated LRRK2. We only saw it in the structure of GMPPNP-bound activated LRRK2 where partial density for the LRR is present (Figure S3.19C-S3.19F). The potential importance of this β -sheet is highlighted by the high conservation of the sequences that encompass it (Figure S3.19G, S3.19H). A closer look at changes in the ROC domain that correlate with its nucleotide state suggests that a conformational switch may be controlling the presence or absence of the β -sheet, with GDP stabilizing it and GMPPNP leading to its disruption (Figure S3.19I-S3.19L).

Roadmap for therapeutics

The conformation of the ROC-COR module changes when the N-terminal repeats undock and no longer constrain it (Figure 3.7E(c-e)). Additional changes in the interfaces between the ROC

and COR domains occur in response to the nucleotide bound to ROC (Figure 3.3G-3.3J). These changes provide a roadmap for identifying small molecules that stabilize either the GDP-bound autoinhibited LRRK2 or the GTP-bound or Apo activated LRRK2. The importance of the interface between ROC and COR-B for PD has been appreciated for a long time as it is a hot spot for disease-linked mutations (Figure 3.1A, 3.1B). Molecules that stabilize the autoinhibited LRRK2 would represent a novel category of allosteric LRRK2 kinase inhibitors for the treatment of PD, distinct from the Type-1 kinase inhibitors that are and were previously in clinical trials (clinicaltrials.gov). Molecules that activate LRRK2 could also be interesting therapeutically, as loss of LRRK2 activity has been linked to both lung fibrosis and lung tumorigenesis in rodents⁶⁷⁻⁶⁹.

Limitations of the study

Our model for how the nucleotide state of the ROC domain controls the activation and autoinhibition of LRRK2 is largely based on the correlation between nucleotide state and conformation in our cryo-EM data. Direct evidence for the interplay between the conformation of LRRK2 and nucleotide binding and/or hydrolysis at the ROC domain would require single-molecule FRET experiments that simultaneously measure conformational changes and nucleotide binding and release. Additionally, our inferences about the relative affinities for the different nucleotides—high affinity for GDP in autoinhibited LRRK2, and lower affinity for GDP and preference for GTP in the activated state—are based on the nucleotide states seen in cryo-EM data and how these nucleotide states vary in response to the nucleotide stripping and exchange protocol. Because our data suggest that nucleotide affinity is conformation dependent, obtaining dissociation constants would be extremely challenging experimentally. Finally, although we proposed a mechanism for how the nucleotide state of ROC might control docking and undocking of the

Nterminal repeats, this model remains hypothetical. Additional structures and structure-guided functional assays will be required to test it.

3.4 Methods

Data availability

All cryo-EM maps and models have been deposited in the EM Data Bank and Protein Data Bank, respectively. All accession numbers are listed in Table S3.1.

Expression and purification of LRRK2

For LRRK2 expression, DNA coding for full-length LRRK2-WT (residues 1-2527) or LRRK2^{RCKW} (residues 1327-2527) (from the Mammalian Gene Collection) was polymerase chain reaction (PCR)-amplified, and the amplicon was inserted into the expression vector pKL: (RRID:Addgene_110741). The plasmid was used as template for G2019S and R1441C sitedirected mutagenesis (Q5 Site-Directed Mutagenesis Kit, NEB). The resulting plasmids were used to generate recombinant baculovirus, according to Bac-to-Bac expression system protocols (Invitrogen). The virus was used to infect SF9 insect cells (RRID:CVCL_0549) with a density of 2×10^6 cells/ml. Infected cells were cultured in serum-free Insect-XPRESS Medium (Lonza) at 27°C for 3 days post infection. For LRRK2 purification, pelleted Sf9 cells were washed with phosphate-buffered saline (PBS), resuspended in lysis buffer [50mM Hepes (pH 7.4), 500mM NaCl, 20mM imidazole, 0.5mM TCEP, 5% glycerol, 5mM MgCl₂, and 20μM GDP] and lysed by homogenization. The supernatant was cleared by centrifugation and loaded onto a Ni-NTA (Qiagen) column. After rinsing with lysis buffer, the His₆-Z-tagged protein was eluted in lysis buffer containing 300mM imidazole. The eluate was then diluted to 250mM NaCl with dilution buffer [50mM Hepes (pH 7.4), 0.5mM TCEP, 5% glycerol, 5mM MgCl₂, and 20μM GDP] and loaded onto an SP Sepharose column (Cytiva). His₆-Z-TEV-LRRK2^{RCKW}/His₆-Z-TEV-LRRK2

were eluted with a gradient of 250mM to 2.5 M NaCl in dilution buffer and then treated with recombinantly expressed Tobacco Etch Virus (TEV) protease overnight to cleave the His₆-Z. Contaminating proteins, the cleaved tag, uncleaved protein, and TEV protease were removed by another combined SP sepharose Ni-NTA (Cytiva) step. Lastly, both LRRK2 and LRRK2^{RCKW} were concentrated and subjected to gel filtration in 20mM Hepes (pH 7.4), 700mM NaCl, 0.5mM TCEP, 5% glycerol, 2.5mM MgCl₂, and 20μM GDP, using an ÄKTAexpress system with an S190 16/200 gel filtration column (GE Healthcare). Final yields, as calculated from ultraviolet absorbance, were in the range of 0.9 to 2.2 mg for LRRK2 and LRRK2^{RCKW} respectively per liter of insect cell medium.

Expression and purification of DARPins C12 and G10

DARPin E11, C12 and G10 were cloned into a pQE30 vector (Qiagen) containing an Nterminal 8× His tag and a C-terminal 3x-FLAG tag, as previously described⁷⁰. Plasmids were transformed into *E. coli* Rosetta cells. Cells were grown at 37 °C with shaking until the *A*₆₀₀ reached 1.0. Then, the temperature was reduced to 18 °C and protein expression was induced by adding 0.5mM IPTG. After 18 (H) the cells were harvested by centrifugation and resuspended in lysis buffer (50mM Hepes pH 7.4, 500mM NaCl, 20mM imidazole, 0.5mM TCEP, and 5% glycerol) prior to lysis by sonication. The lysate was cleared by centrifugation and loaded onto preequilibrated Ni-NTA Sepharose beads (GE HealthCare). The beads were washed with 50 column volumes of lysis buffer and protein was eluted in lysis buffer supplemented with 300mM imidazole. The eluate was concentrated to 5 ml and subjected to SEC using an S190 16/200 column (GE HealthCare) in SEC buffer (20mM Hepes pH 7.4, 150mM NaCl, 0.5mM TCEP, and 5% glycerol). Final concentrations for DARPin E11, C12 and G10 were: 6.3mg/mL, 3.6mg/mL and 3mg/mL respectively.

Kinase activity assays with DARPins

The impact of DARPin binding on kinase activity was assessed by measuring the phosphorylation of Rab8a. The reaction was set up using 100 nM LRRK2 and incubated for 40 minutes at room temperature with 1.5 μ M His-Rab8a T22N and with or without 1mM ATP, 10 μ M DARPin (E11, C12, or G10), or 2.5 μ M Mli-2 in reaction buffer (50mM HEPES pH 7.4, 150mM NaCl, 0.5mM TCEP, 5mM MgCl₂, 5% glycerol, 20 μ M GTP, 2 μ M GDP). Reactions were quenched using SDS-PAGE sample buffer, run on an SDS-PAGE gel, and then transferred via Western blot for immunoblot analysis to visualize LRRK2 (1:1000, MJFF2 (c41–2); Abcam ab133474, Lot # GR3445288–3; RRID:[AB_2713963](#)), His-Rab8a (1:2000, 6*His Monoclonal antibody; Proteintech 66005, Lot # 10027680; RRID: [AB_3086567](#)), and phospho-Rab8a (pT72) (1:500, Abcam 230260, Lot #GR3216587–1;RRID:[AB_2814988](#)). All membranes were imaged using the Biorad Chemidoc MP Imaging system. Band intensities were quantified using ImageJ (version 2.14.0). One-way ANOVA with Tukey’s multiple comparison correction was performed using Graphpad Prism (version 10.5.0, RRID: [SCR_002798](#)) on the ratio of background corrected phospho-Rab in each condition to the average of phospho-Rab in the LRRK2 alone condition (N=3).

Kinase activity assay with nucleotide-stripped LRRK2

LRRK2[WT] was incubated with (‘EDTA treated’) or without EDTA (‘mock treated’) for 24 hours followed by addition of 20mM MgCl₂ and further incubation for 24 hours. Untreated LRRK2 was used immediately upon thawing from frozen aliquots. The effect of nucleotide stripping on kinase activity was assayed by measuring the phosphorylation of Rab8a as described above. The reaction was set up using 100 nM LRRK2 and incubated for 40 minutes at room temperature with 1.5 μ M His-Rab8a T22N and with or without 1mM ATP, in reaction buffer (50mM HEPES pH 7.4, 150mM NaCl, 0.5mM TCEP, 5mM MgCl₂, 5% glycerol). All LRRK2 samples were incubated with Rab8a in the presence of ATP, with one negative control lacking ATP.

Background-corrected pRab8a was normalized to the average pRab8a in the untreated LRRK2 + ATP condition from [n=3] independent Western blots. Images were processed in ImageJ.

Guanine nucleotide exchange of LRRK2 samples for cryo-EM

We prepared a working stock of 0.1M EDTA (pH 7.5) by diluting 0.2M EDTA pH 7.5 in half with Exchange Buffer (20mM Hepes pH 7.4; 200mM NaCl and 20 μ M TCEP). Ten μ L aliquots of full-length LRRK2 at concentrations of 11–20 μ M were mixed with 0.5 μ L of 0.1M EDTA (pH 7.5) (for a final EDTA concentration of 5mM) and incubated for 30 min at 4 °C. After the incubation, a Zeba micro desalting column (Thermo Fisher Scientific) was equilibrated with Exchange Buffer according to the manufacturer's instructions. A total of 10.5 μ L of sample was applied to the spin column, followed by 3 μ L of Exchange Buffer, and centrifuged for 2 min at 1000 $\times$ g at 4 °C. After centrifugation, 1.3 μ L of 10mM nucleotide (GMP-PNP, Abcam AB146660, or GDP, Sigma-Aldrich G7127) was added followed by 1.3 μ L of 20mM MgCl₂ to the 13.5 μ L sample, for a total volume of 16.1 μ L and a final concentration of 0.8mM nucleotide and 1.6mM MgCl₂. For mixed-nucleotide samples, 1.3 μ L of 750 μ M GMP-PNP and 250 μ M GDP were added, followed by 1.3 μ L of 20mM MgCl₂ to the 13.5 μ L sample, for a total volume of 16.1 μ L and a final concentration of 0.8mM total nucleotide (200 μ M GDP and 600 μ M GMP-PNP) and 1.6mM MgCl₂. Samples were incubated for 30–60 min at 4 °C.

Some of our initial cryo-EM analysis revealed that the nucleotide stripping protocol described above was incomplete, and that some LRRK2 remained bound to GDP even in samples that had been stripped and incubated with GMPPNP (Figure 3.2). To address this issue, we tested a longer EDTA treatment. Ten μ L of purified full-length LRRK2 (15 μ M) was incubated with 5mM EDTA (pH 7.5) for 24 hr at 4 °C. After incubation, a Zeba micro desalting column was equilibrated with Exchange Buffer as described above. A total of 10.5 μ L of sample was applied to the spin

column, followed by 3 μL of Exchange Buffer, and centrifuged for 2 min at $1000 \times g$ at 4°C . After centrifugation, 1.3 μL of 10mM GMP-PNP was added, followed by 1.3 μL of 20mM MgCl_2 , and the sample was incubated for 24 hr at 4°C , for a final concentration of 0.8mM nucleotide and 1.6mM MgCl_2 .

Guanine nucleotide exchange of LRRK2 samples for kinase activity and fluorescence measurements

Full-length human LRRK2 at 20 μM was thawed on ice and diluted to 500nM in a nucleotide stripping buffer (50mM HEPES, 150mM NaCl, 1mM TCEP, 5mM EDTA, and 1mM GMPPNP or GDP). Because of the incomplete nucleotide exchange we detected in our cryo-EM samples that had been stripped of their nucleotide for 30-60min at 4°C , each sample was incubated at room temperature for 3 hours. We verified that LRRK2 was stable at room temperature for this long by measuring fluorescence emission spectra of LRRK2 incubated at room temperature for up to 3 hours (Figure S3.20). After the stripping, 10mM MgCl_2 was added to neutralize EDTA, then samples were incubated for another 30 minutes at room temperature and nucleotide (GMPPNP or GDP) was added to a final 100 μM . Each sample (LRRK2 with GMPPNP or LRRK2 with GDP) was transferred into mini dialysis devices (Thermo Scientific, 69572) and dialyzed against 300mL of nucleotide exchange buffer (50mM HEPES, 150mM NaCl, 5mM MgCl_2 , 1mM TCEP, and 0.1 μM GMPPNP or GDP) overnight in the cold room with gentle stirring. The next day samples were spun down on a tabletop centrifuge at maximum speed and directly used for the measurements.

Guanidine nucleotide exchange for mass photometry

12.7 μM of LRRK2[WT] in 5 μL of buffer (20mM Hepes pH 7.4, 150mM NaCl, 5% glycerol, 0.5mM TCEP, 20 μM GDP and 2.5mM MgCl_2) was mixed with one of the following: (1) 0.28 μL of 100mM EDTA pH 7.5 and 0.28 μL of 100 μM GDP ('EDTA treated'), or (2) 0.28 μL of

milliQ H₂O and 0.28 μ L of 100 μ M GDP ('mock treated'). Samples were incubated on ice for 2 hours, at which point 0.6 μ L of 20mM MgCl₂ were added, and samples were incubated on ice for another 24 hours. Samples were then diluted using with 20mM Hepes pH 7.4, 150mM NaCl, 5% glycerol, 0.5mM TCEP, and 1mM MgCl₂, to a final concentration of LRRK2[WT] of 100nM and incubated on ice for 30 minutes before mass photometry measurements.

Total particle counts corresponding to the molecular weights of either monomer or dimer were determined from mass photometry traces. The percentage of dimers contributing to total monomer/dimer LRRK2 landing events were analyzed with one-way analysis of variance (ANOVA) and corrected for multiple comparisons using Tukey's test.

Sample preparation for Cryo-EM: nucleotide-exchanged LRRK2

LRRK2, following nucleotide stripping and exchange, was incubated with MLi-2 (10mM; Tocris, RRID: Addgene_226784), and E11-DARPin, purified as previously described⁷⁰. The incubation was performed at a molar ratio of 1:1.5:1.25 (LRRK2:MLi-2:E11) for 10 min at room temperature, followed by 15 min at 4 °C. The resulting complex was diluted to a final concentration of 7 μ M LRRK2 in Exchange Buffer (20mM HEPES, pH 7.4, 200mM NaCl, 20 μ M TCEP) and supplemented with 21 μ M DDM (GOLDBIO: DDM5_69227). A volume of 2.8–3.5 μ L was applied to glow-discharged UltraAUfoil Holey Gold 300 mesh R1.2/1.3 grids (Quantifoil, Q350AR13A) and incubated in a FEI Vitrobot IV chamber at 4 °C and 95 % humidity for 5 s.

Excess liquid was blotted for 2 s using filter paper 595 at blot force 4, and the grids were vitrified by plunging into liquid ethane cooled to liquid-nitrogen temperature.

One data set for LRRK2^{R1441C} was acquired on a Talos Arctica (FEI) operated at 200 keV and equipped with a Falcon 4i detector (Thermo Fisher Scientific). Images were recorded at a nominal magnification of 150,000 $\times$ in EF-TEM mode (0.95 Å/pixel) with a cumulative electron

exposure of approximately $55 \text{ e}^-/\text{\AA}^2$. The remaining data sets for LRRK2^{WT}, LRRK2^{G2019S}, and LRRK2^{R1441C} were acquired on a Titan Krios G3 (Thermo Fisher Scientific) operated at 300 keV, equipped with a Falcon 4 direct electron detector and a Gatan BioContinuum energy filter. Images were collected at a nominal magnification of 130,000 $\times$ in EF-TEM mode (0.935 $\text{\AA}$ /pixel) using a 20 eV energy filter slit width, with a cumulative electron exposure of approximately $55 \text{ e}^-/\text{\AA}^2$.

Sample preparation for Cryo-EM: DARPin-bound LRRK2 and LRRK2^{RCKW}

For LRRK2 bound to G10-DARPin, a 1:5 molar ratio (LRRK2:G10) was incubated for 10 min at room temperature, followed by 15 min at 4 °C. The complex was diluted to a final concentration of 5 μ M LRRK2 in buffer containing 20mM HEPES (pH 7.4), 200mM NaCl, 20 μ M TCEP, 20 μ M GDP, 2.5mM MgCl₂, and 5 % glycerol, and supplemented with 21 μ M DDM (GOLDBIO: DDM5_69227). A 2.8 μ L volume was applied to glow-discharged UltraAUfoil Holey Gold 300 mesh R1.2/1.3 grids (Quantifoil, Q350AR13A) and incubated in a FEI Vitrobot IV chamber at 4 °C and 95 % humidity for 5 s. Excess liquid was blotted for 2 s using filter paper 595 at blot force 4, and the grids were vitrified by plunging into liquid ethane cooled to liquid nitrogen temperature. Data was collected under the same conditions described above on a Titan Krios G3.

As previously described⁷⁰ LRRK2^{RCKW}[R1441H]:C12-DARPin was prepared by desalting LRRK2 into a buffer containing 20mM HEPES (pH 7.4), 150mM NaCl, 20 μ M TCEP, 20 μ M GDP, 2.5mM MgCl₂, and 5 % glycerol using the Zeba micro desalting column (Thermo Fisher). LRRK2^{RCKW} was then incubated with C12-DARPin in a ratio of 1:1.25 (LRRK2:C12) for 10 min at room temperature, followed by 15 min at 4 °C. The sample was diluted to a final LRRK2^{RCKW} concentration of 6 μ M. A 4 μ L volume was applied to glow-discharged UltraAUfoil Holey Gold

300 mesh R1.2/1.3 grids (Quantifoil, Q350AR13A) and incubated in a FEI Vitrobot IV chamber at 4 °C and 95 % humidity for 20 s. Excess liquid was blotted for 4 s using filter paper 595 at blot force 4, and the grids were vitrified by plunging into liquid ethane cooled to liquid-nitrogen temperature. Images were collected at a nominal magnification of 36000x in EF-TEM mode (1.16 Å/pixel), with a cumulative electron exposure of approximately 51 e⁻/Å². Data was collected on a FEI Talos Arctica operated at 200 kV and equipped with a K2 summit direct electron detector (Gatan).

Cryo-EM data data processing and model building

Movies for all datasets were pre-processed using Patch MotionCor and Patch CTF estimation in CryoSPARC⁹³ (RRID:SCR_016501) to perform motion correction and estimate the contrast transfer function (CTF). Micrographs with a CTF fit worse than 5.5 Å were excluded from further processing. Particles were picked for all datasets using a Topaz⁹⁴ model (version 0.2.4, <https://cb.csail.mit.edu/topaz/>) previously trained on the dataset. For combined datasets, several additional rounds of Topaz training and two-dimensional (2D) classification were performed. Particle stacks were extracted at the original pixel size. Ab initio and heterogeneous refinement jobs were then used to discard poor-quality particles, followed by non-uniform refinement and local refinement (see Figures S1-S6, S9, S13, and S14). To build the initial LRRK2 model, the highest-resolution local maps obtained for the LRRK2:G10 complex were used (EMD-70981). The available structure of full-length LRRK2⁶¹ (PDB 7LHW) was used as the starting point, yielding the final model PDB 9OXH. This model was subsequently split into domains, docked into the corresponding cryo-EM maps using UCSF ChimeraX⁵⁴ (version 1.9, RRID:SCR_015872), and merged into one chain. For models containing density for MLI-2, we built the model for the part

of the map corresponding to the kinase and WD40 domains starting from our previous structure of MLI-2-bound LRRK2^{RCKW} (PDB 8TXZ)⁶³. For DARPIN C12 and G10, initial models were generated using AlphaFold 3⁹⁵. Models were fit into the density maps using Coot⁹⁶ (version 0.9.8, www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/, RRID:SCR_014222) and refined in Phenix to generate the final models. Maps for LRRK2^{WT}[GMPPNP] and LRRK2^{R1441C} were improved with Resolve in Phenix⁹⁷. Data collection and refinement statistics are summarized in Table S3.1.

Movies for LRRK2^{RCKW}:C12 DARPIN complex, were collected and pre-processed using Patch MotionCor and Patch CTF estimation in CryoSPARC⁹³ (RRID:SCR_016501). Micrographs with CTF fit worse than 4 Å were excluded from further processing. Particles obtained with blob picker in CryoSPARC were used to train a Topaz⁹⁴ model. After several rounds of 2D Classification, particles were extracted with a 288-pixel box and used for subsequent processing. Two final local refinements were performed using focused masks. The first refinement targeted the C-lobe of the kinase, the WD40 domain, and the C12 DARPIN, while the second focused on the ROC domain, COR-A, COR-B, and the N-lobe of the kinase. These refinements yielded maps at 3.48 Å and 3.6 Å resolution, respectively. Local refinement procedures were conducted to evaluate map quality and directional resolution anisotropy. The available structure of LRRK2^{RCKW} (PDB:6VP7) was fitted into the 3D maps using UCSF ChimeraX⁵⁴ and used as a starting point for model building, which was performed in Coot⁹⁶. The built structures were refined using real-space refinement as implemented in Phenix⁹⁷. Data collection and refinement statistics are summarized in Table S3.1.

Quantitation of the distribution of autoinhibited and activated LRRK2 in samples treated with EDTA for 1h vs. 24h

This analysis included particle counts from samples subjected to the nucleotide stripping protocol for 1h and then incubated with GDP and/or GMPPNP yielding active volumes (see Figures S3.4C–3.4D for monomers and Figure S3.2F for mixed dimers) or autoinhibited volumes (see Figures S3.2C–3.2D and Figure S3.2F for mixed dimers), excluding data for the mutants (LRRK2[G2019S] and LRRK2[R1441C]). The counts were then further separated according to the nucleotide added after stripping. We also used particle counts from LRRK2 subjected to 1h nucleotide stripping without subsequent addition of nucleotide (see Figure S3.8) and data from LRRK2 subjected to 24h nucleotide stripping followed by addition of GMPPNP (see Figures S3.5D–3.5F). The final relative abundance of particles corresponding to the active and autoinhibited states of LRRK2[WT] in the different samples is presented in Figure 3.2H and Figure S3.8D.

Cryo-EM data collection for untreated (no nucleotide stripping) LRRK2[WT] and LRRK2[R1441C]

Cryo-EM data were collected on a Talos Arctica transmission electron microscope operated at 200 kV equipped with a Gatan K3 direct electron detector. Images were acquired at a nominal magnification of 36,000 $\times$, corresponding to a calibrated super-resolution pixel size of 0.5505 Å/pixel (1.101 Å/pixel in counting mode). Movies were collected using Leginon v3.7 at a dose rate of 21.22 e⁻/Å²/s over 2.40 s (total dose 50.92 e⁻/Å²), fractionated into 40 frames. A total of 2,371 movies were collected over a defocus range of 1.7–2.6 μ m. Ice thickness was estimated as described previously.

On-the-fly processing was performed using MotionCor2 for motion correction and CTFFIND4 for CTF estimation within the Appion pipeline. Motion-corrected, dose-weighted micrographs (1.101 Å/pixel) were imported into cryoSPARC. Initial particle picking was

performed using Warp, and picks from each dataset (WT and R1441C) were used to generate templates for cryoSPARC-based template picking.

Cryo-EM data processing for untreated (no nucleotide stripping) LRRK2[WT] and LRRK2[R1441C]

Motion-corrected micrographs from WT and R1441C datasets were combined prior to downstream processing to ensure consistent classification and minimize bias. After curation based on CTF quality and ice thickness (2,257 micrographs), initial particle picking was performed in cryoSPARC using template-based methods, yielding 1,420,224 particles. Representative particles were selected and used for Topaz-based particle picking, resulting in a refined dataset of 424,769 particles. This was subjected to two rounds of 2D classification to remove junk particles, yielding a final cleaned subset of 92,497 particles for downstream analysis.

These particles were used for ab initio reconstruction and heterogeneous refinement to separate junk, flexible, and autoinhibited conformations. Particles corresponding to autoinhibited states were selected, re-centered, and re-extracted at 3.03 Å/pixel (32,759 particles). These particles were further classified by ab initio reconstruction and heterogeneous refinement to resolve autoinhibited monomeric and dimeric states. The autoinhibited dimer subset was subjected to 3D classification in cryoSPARC into two classes, yielding a mixed monomer/dimer class (11,468 particles) and a dimer class (9,033 particles).

Final particle distributions were determined by tracing particles back to their dataset of origin following joint classification. Particles were grouped into four structural categories:

Activated LRRK2 (“RCKW”), Autoinhibited Monomer, Autoinhibited Dimer, and Mixed Dimer.

The mixed monomer/dimer class was conservatively treated as contributing equally to active and inactive states for downstream quantification.

GTP turnover assay

GTP hydrolysis was monitored using a real-time inorganic phosphate detection assay (EnzCheck Phosphate Assay Kit; Thermo Fisher Scientific/Invitrogen, E6645). This assay relies on the enzymatic conversion of the chromogenic substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) by purine nucleoside phosphorylase (PNP) in the presence of inorganic phosphate. Phosphate released during GTP hydrolysis drives the MESG/PNP reaction, resulting in a spectral shift in absorbance from 330 nm to 360 nm, which was continuously recorded using a spectrophotometer (Agilent G6860AA). Reactions were carried out in 60 μ L volumes in quartz cuvettes and contained 1x reaction buffer (50mM Tris-HCl, 150mM KCl, 1mM MgCl₂, pH 7.5), MESG substrate (0.2mM final), and PNP (1 U/mL final). Absorbance at 360 nm was monitored in real time for 3 min at 22°C. Phosphate release was quantified from the change in absorbance using the extinction coefficient of 2-amino-6-mercapto-7-methylpurine ($\epsilon = 11,000 \text{ M}^{-1}\text{cm}^{-1}$). To minimize phosphate contamination, all solutions were prepared with P_i-free water, and cuvettes were thoroughly rinsed with deionized water between measurements. For DARPin assays, purified LRRK2 protein (1 μ M final) was incubated with or without individual DARPins (G10 or C12; 1.2 μ M final) for 30 min at room temperature prior to measurement. For Rab8a Q67L-dependent assays, LRRK2 (1 μ M) was pre-incubated with G10 or C12 (1.2 μ M) for 30 min, followed by the addition of Rab8a Q67L (20 μ M). After an additional 10 min incubation, GTP hydrolysis rates were measured as described above.

In vitro guanine nucleotide exchange assay

Nucleotide exchange on LRRK2 was measured using a fluorescence-based assay as described previously⁹⁸. Recombinant LRRK2 protein (1 μ M) was preloaded with 1mM BODIPYFL-GDP (G22360, Thermo Fisher Scientific) in exchange buffer containing 20mM HEPES (pH

7.2), 150mM KCl, 5% glycerol, and 1mM DTT. The loading reaction was performed at 4 °C for 5 h. Nucleotide loading was terminated by the addition of MgCl₂ to a final concentration of 10mM. To initiate nucleotide exchange, BODIPY-FL–GDP–loaded LRRK2 was rapidly mixed with C12 (1.2μM) and/or Rab8A Q67L (20μM) in exchange buffer supplemented with 5mM GTP. Reactions were carried out in black 384-well plates. Fluorescence signals were monitored in real time using a microplate reader (Cytation 5, Agilent BioTek) with excitation at 488 nm and emission at 535 nm. Measurements were recorded every 10 s for 6 min.

Analysis of phosphorylated Rab8a in cells

For in-cell LRRK2 activity assays, 1.2×10^6 293T cells were plated into each well in six well plates and incubated at 37 °C in 5% CO₂. After two days, cells were transfected with 500ng GFP-Rab8a (EGFP C1 vector backbone; Addgene; RRID: Addgene_49543) plus or minus 500ng 8xHis-DARPin C12-3xFlag (pcDNA3.1 vector backbone) and 1000ng of full-length LRRK2 wild type, G2019S, R1441H, or the double mutant G2019S+R1441H (all LRRK2 in the pCDNA5 vector backbone) using polyethylenimine (Polysciences, catalog # 23966-1). After 48 hours, control cells not transfected with DARPin C12 were treated with either DMSO or MLI-2 (Cayman Chemical Company, catalog #19305; 5μM) and incubated for an hour. Cells were washed three times in cold DPBS then lysed in 300uL cold RIPA buffer per well (50mM Tris pH8, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Sodium Deoxycholate, 1mM DTT in the presence of cOmplete mini EASYpack protease inhibitor cocktail and the PhosSTOP EASYpack phosphatase inhibitor cocktail (Sigma-Aldrich, catalog # 05056489001; Roche, 04906837001)). Cell lysates were vortexed twice for 30 seconds then rotated for 15 minutes at 4°C. Lysates were clarified by centrifugation at 17,000g for 15 minutes at 4°C, and the supernatant was moved to a new tube. SDS-PAGE loading buffer and reducing agent (1X, Invitrogen, catalog # NP0007 and NP0009) were added to the supernatant which was then boiled at 95°C for 10 minutes. Samples were spun

again at 13,000g for five minutes, and supernatant was moved to a new tube. Samples were either used immediately or stored at -20°C . For immunoblotting, samples were processed as described previously⁷⁰. In short, the membrane was cut at the 70kDa molecular weight marker and the top portion was immunoblotted for LRRK2 with a specific antibody (1:1000, MJFF2 (c41–2); Abcam catalog # ab133474, Lot # GR3445288–3; RRID:[AB_2713963](#)) in 1% BSA. The lower portion was immunoblotted using antibodies for phospho-Rab8a (pT72) (1:1000, Abcam catalog # 230260, Lot # GR3216587–1; RRID:[AB_2814988](#)), GFP (1:2500, Santa Cruz Biotechnology catalog # sc-9996, Lot # C2523; RRID:[AB_627695](#)), and GAPDH (1:3000, Cell Signaling Technology catalog # 2118, Lot # 16; RRID:[AB_561053](#)) in 1% milk. To visualize DARPin C12, cell lysate was reduced in Tricine SDS sample buffer (Invitrogen, catalog # LC167). Samples were run on Tricine 10%-20% gels (Invitrogen, catalog # EC66252BOX) and run in tricine running buffer (Invitrogen, catalog # LC1675) for 1 hour and 20 mins at 125V. Protein was then transferred onto polyvinylidene fluoride (PVDF) transfer membrane (0.2 μm pore size) for 75 min at 300 mA at 4°C . Samples were then blocked in 5% milk for an hour and incubated for 16 hours at 4°C in primary rabbit anti-FLAG polyclonal antibody (ptg labs, catalog # 20543-1-AP, Lot # 00106090; RRID:[AB_11232216](#)) in 1% milk. Samples were washed three times in Tris buffered saline buffer with 0.05% Tween-20 (TBS-T) for 10 minutes then incubated with goat anti-rabbit Licor680 antibody (LI-COR biosciences; catalog # 926-32211, Lot # D40109-05; RRID:[AB_621843](#)) in 4% milk for 1 hour at room temperature. All membranes were imaged using the Li-Cor imaging system. Intensities of phospho-Rab8a, GFP, and LRRK2 bands were measured using ImageJ (version 2.14.0). Two-way ANOVA were conducted on the ratios of phosphorylated Rab8a to total Rab8a and LRRK2, normalized to the mean of the wild type LRRK2 plus DMSO group using GraphPad Prism (version 10.5.0, RRID: ⁴³) with Tukey's multiple comparison correction.

RMSD analysis

The RMSD values reported in Figure S9 were obtained by doing pairwise comparisons between the ROC-COR modules of the structures shown in the figure. Specifically, we extracted the part of each model corresponding to residues 1332-1878 (ROC, COR-A, and COR-B). For each pairwise comparison, models were aligned to each other in ChimeraX⁵⁴ (“match”), using only their backbones. Once aligned, the RMSD for the entire ROC-COR module was calculated in ChimeraX⁵⁴ (“rmsd”), also using only the backbone atoms.

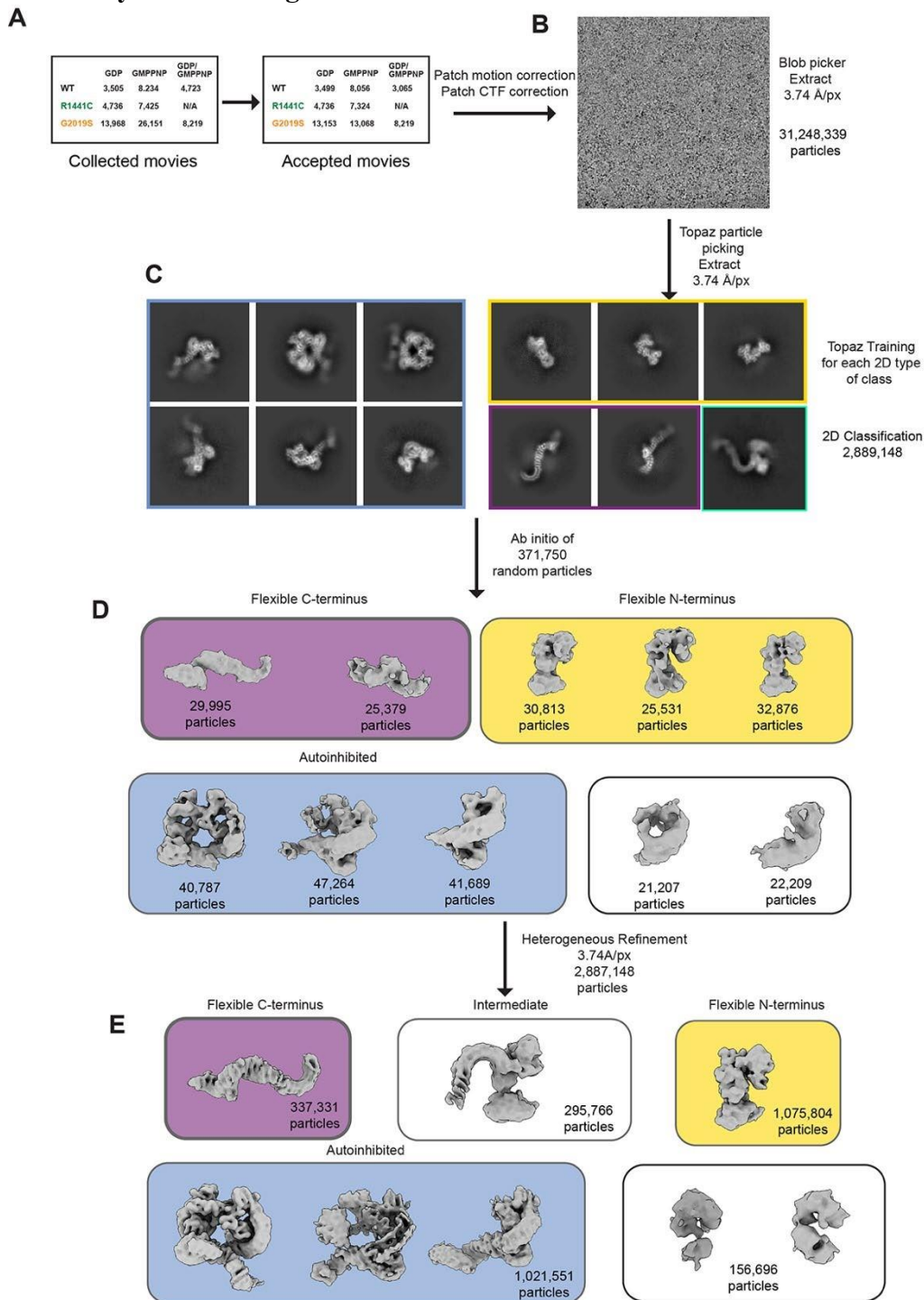
Figure preparation

All structural representations were prepared using ChimeraX⁵⁴.

Quantification and Statistical Analysis

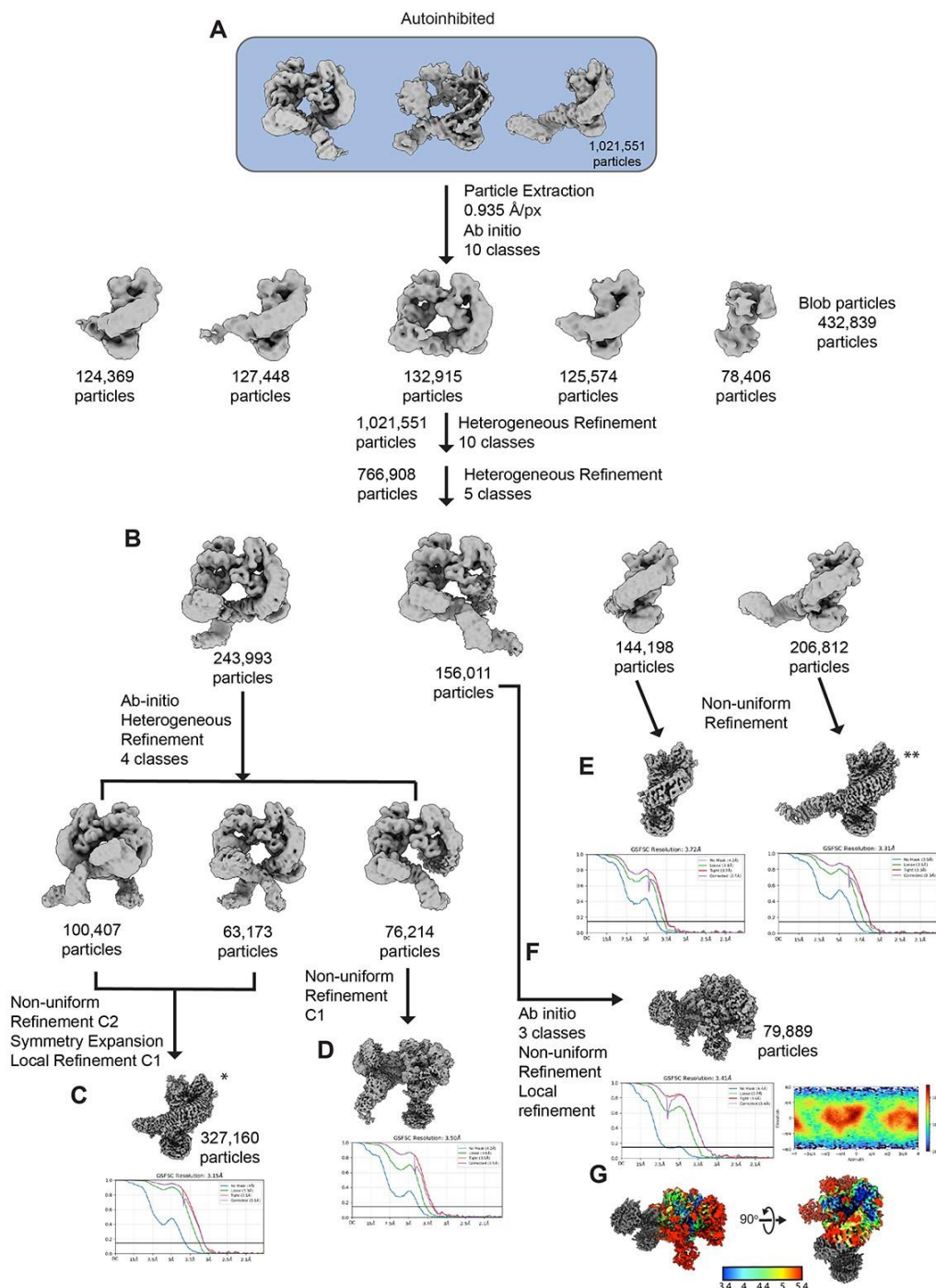
Statistical tests are noted in figure legends. All data are shown as means $\pm$ s.d., and all analyses were performed using Prism 10 software (Graphpad).

3.5 Supplementary Data and Figures



Supplementary Figure 3.1 Overall Cryo-EM processing pipeline for the combined data set.

(A) Number of movies that were collected and kept for further processing. (B, C) Representative micrograph (B) and class averages (C). (D) Data processing strategy. (E) Splitting of data into volumes representing different states/conformations of LRRK2.

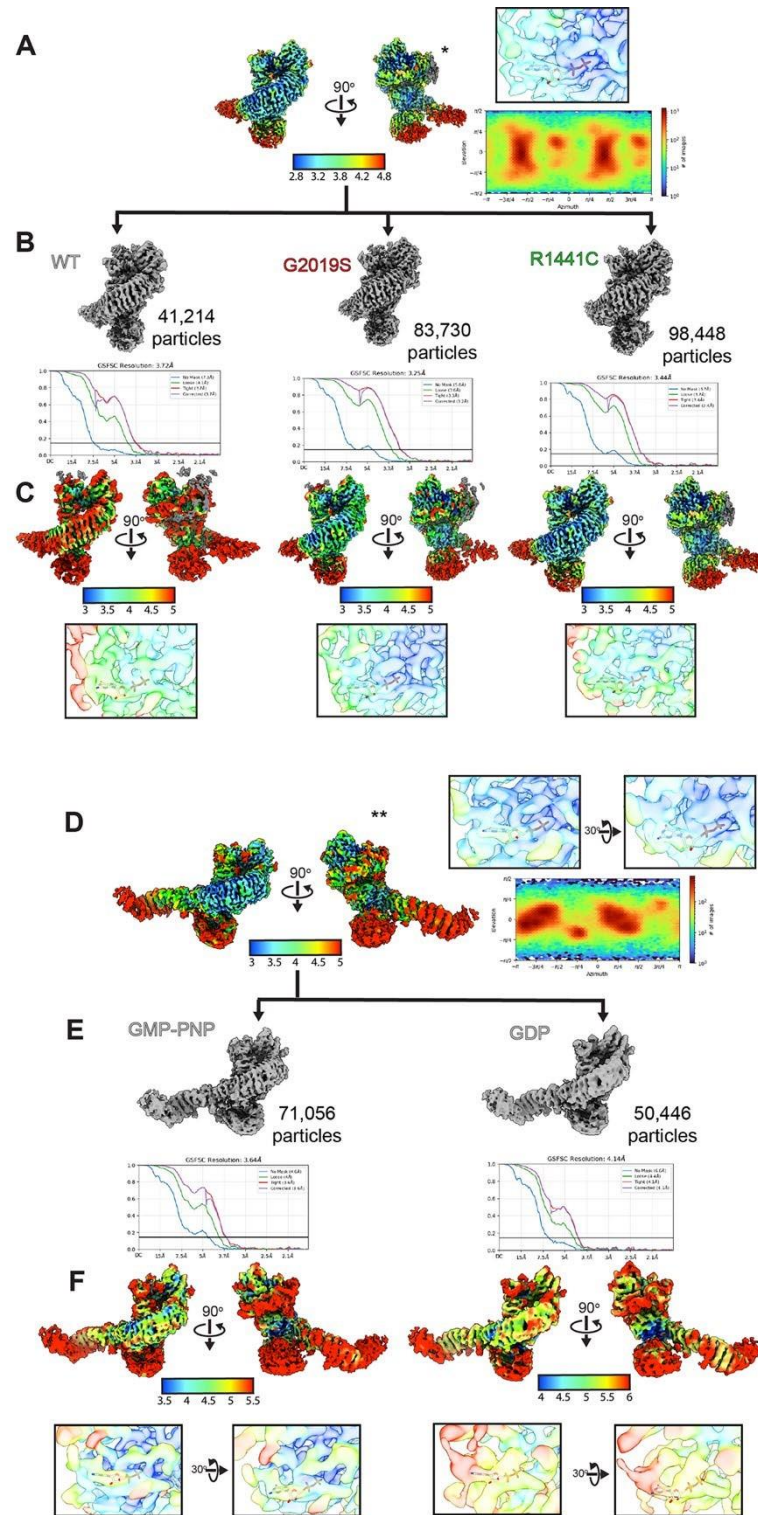


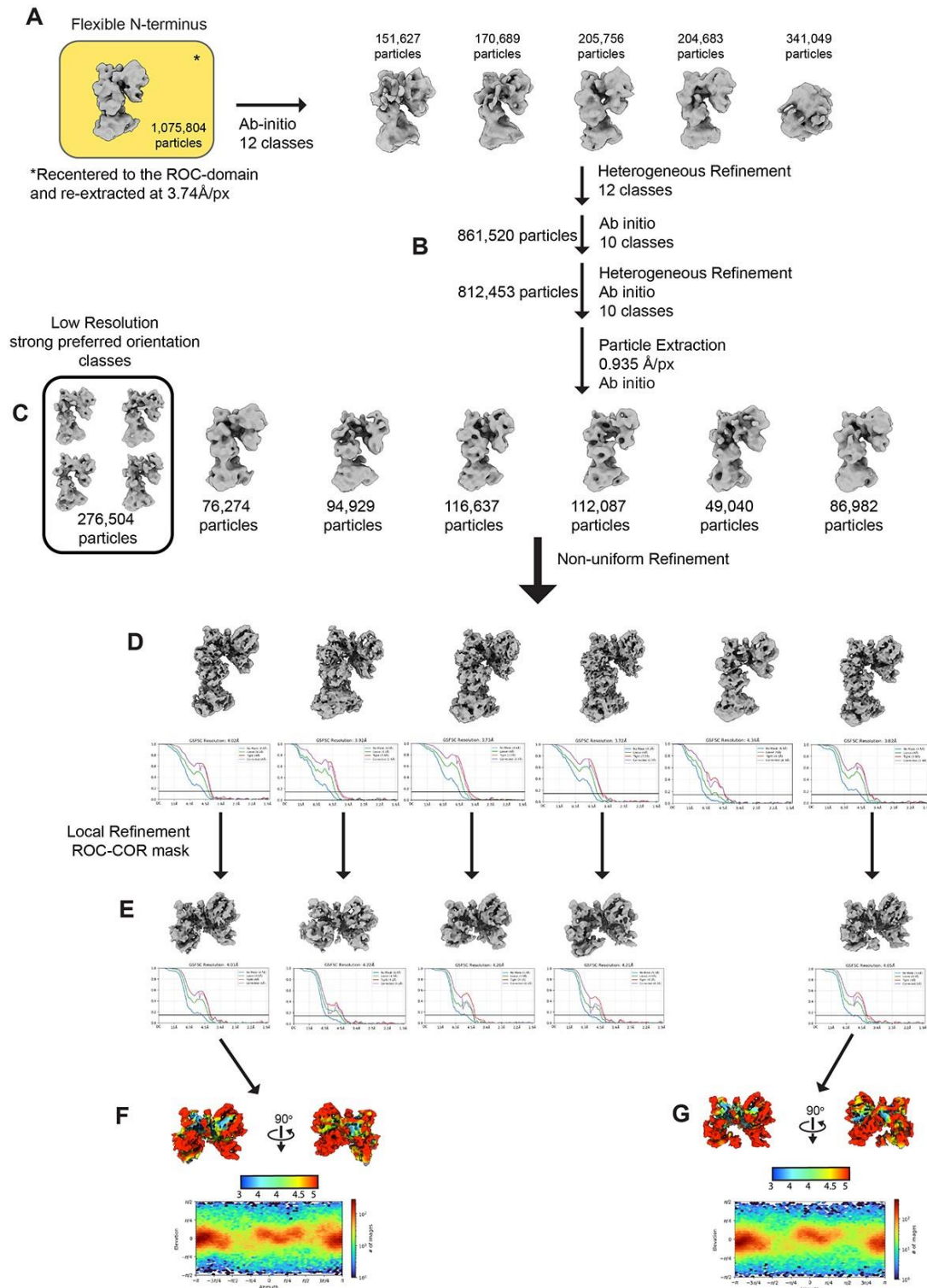
Supplementary Figure 3.2 Cryo-EM processing pipeline for the combined data set: autoinhibited LRRK2 and mixed dimers.

(A) Data processing strategy. (B) Processing of the autoinhibited dimer. (C) Volume and FSC curve for the dimer after symmetry expansion. (D) Volume and FSC curve for the asymmetric dimer (autoinhibited protomer). (E) Volumes and FSC curves for autoinhibited monomers. (F) Volume, FSC curve and Euler angle distribution for the asymmetric dimer (activated protomer). (G) Local resolution map. * and ** indicate volumes whose processing continues in Figure S3.3.

Supplementary Figure 3.3 Cryo-EM processing pipeline for autoinhibited LRRK2 and mixed dimers: splitting by nucleotide added or by LRRK2 variant.

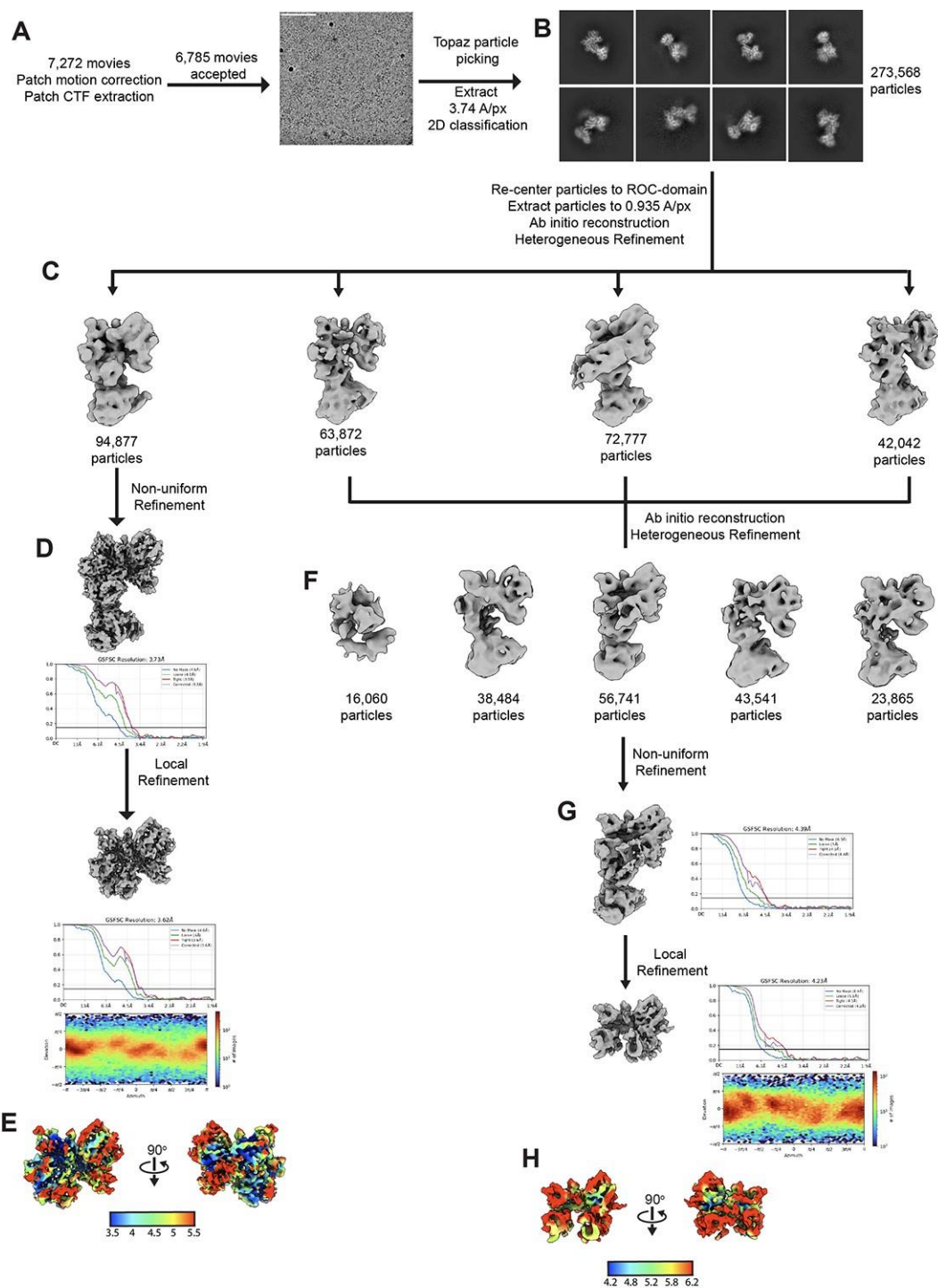
(A) Local resolution map and Euler angle distribution for the dimer after symmetry expansion. **(B)** Data splitting by LRRK2 variant; volumes and FSC curves. **(C)** Local resolution map in the vicinity of the nucleotide-binding pocket of the ROC domain. **(D)** Local resolution map and Euler angle distribution for the monomer. **(E)** Data splitting by the guanine nucleotide added to the sample; volumes and FSC curves. **(F)** Local resolution map in the vicinity of the nucleotidebinding pocket of the ROC domain.





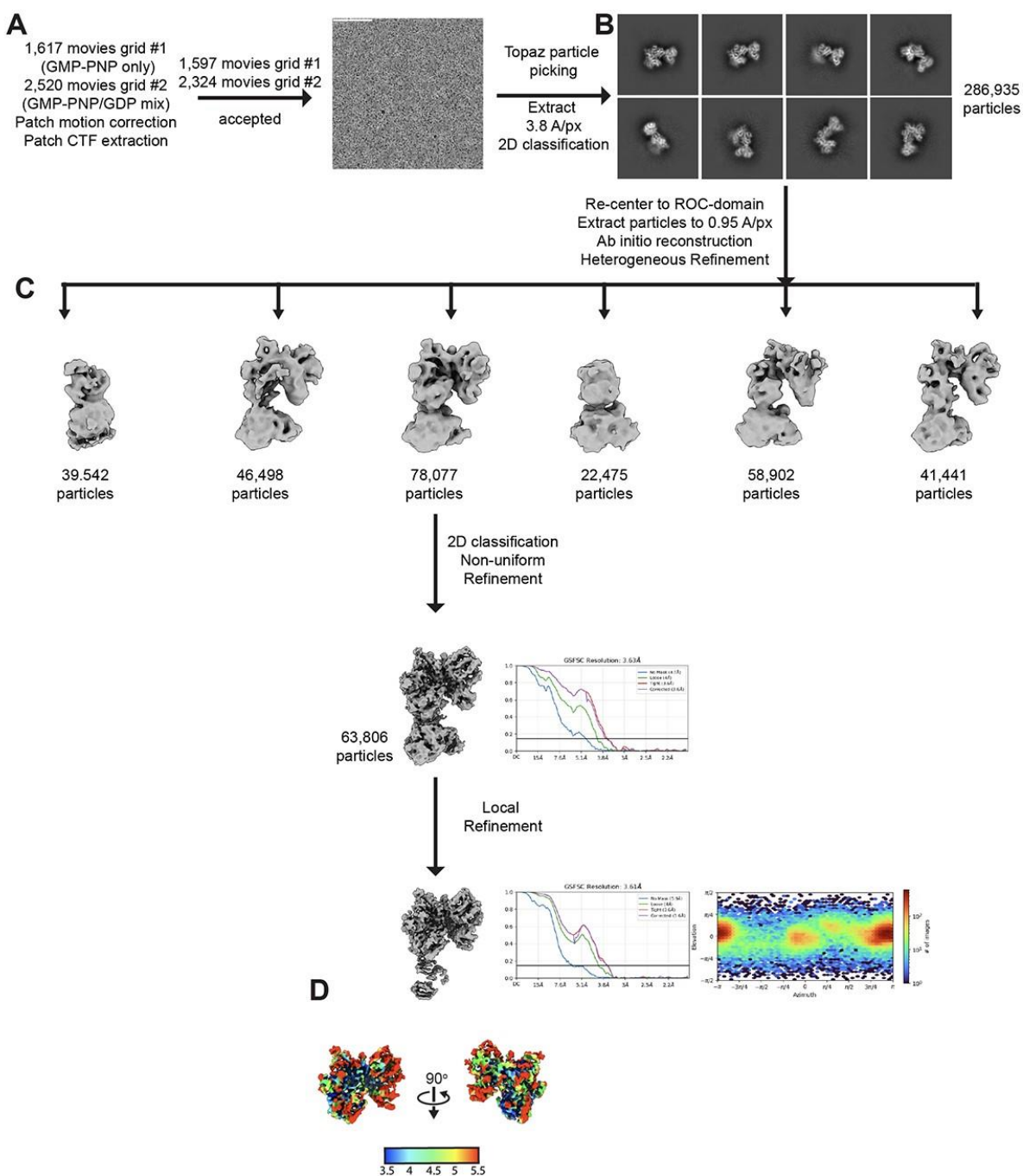
Supplementary Figure 3.4 Cryo-EM processing pipeline for the combined data set: activated LRRK2 (C-terminal half).

(A, B) Data processing strategy. (C) Splitting of activated volumes by conformation. (D) Volumes and FSC curves of activated LRRK2. (E) Local refinement volumes and FSC curves. (F, G) Local resolution maps and Euler angle distributions for activated LRRK2 (apo).



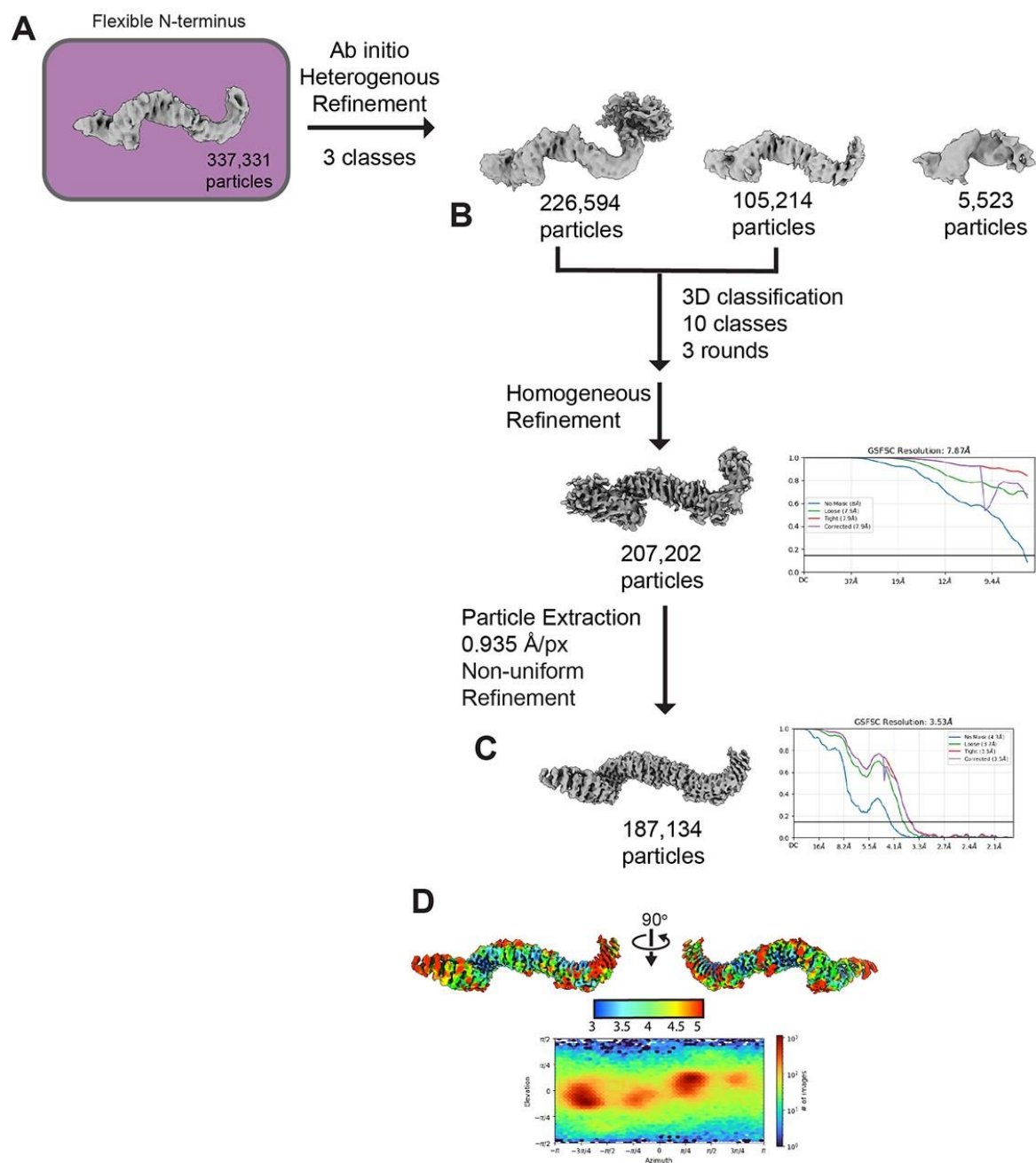
Supplementary Figure 3.5 Cryo-EM data processing pipeline for LRRK2 incubated with GMPPNP for 24h, active and semi-active conformations.

(A) Number of movies collected and kept for further processing, and representative micrograph. (B) 2D class averages. (C, F) Data processing strategy. (D) Volume, FSC curve and Euler angle distribution, and (E) local resolution map of the ROC-COR domain of activated LRRK2. (G) Volume, FSC curve and Euler angle distribution. (H) Local resolution map of the ROC-COR domain of 'semi-activated' LRRK2.



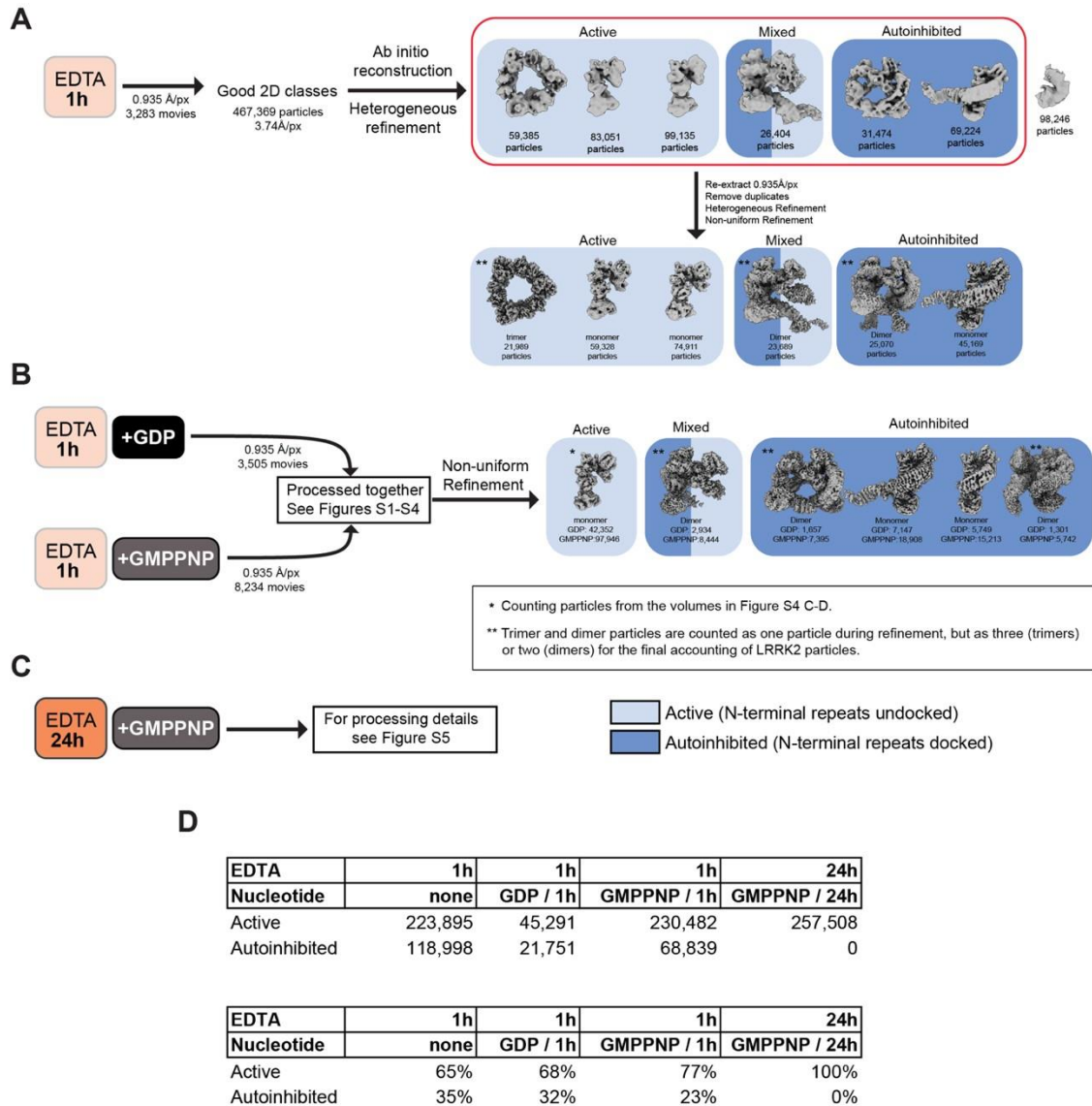
Supplementary Figure 3.6 Cryo-EM processing pipeline for activated LRRK2[R1441C].

(A) Number of movies collected and kept for further processing, and representative micrograph. (B) 2D class averages. (C) Data processing strategy. (D) Volume, FSC curve, Euler angle distribution, and local resolution map of the ROC–COR domain of activated LRRK2[R1441C].



Supplementary Figure 3.7 Cryo-EM data processing pipeline for the N-terminal repeats.

(A, B) Data processing strategy. (C) Volume and FSC curve. (D) Local resolution map and Euler angle distribution



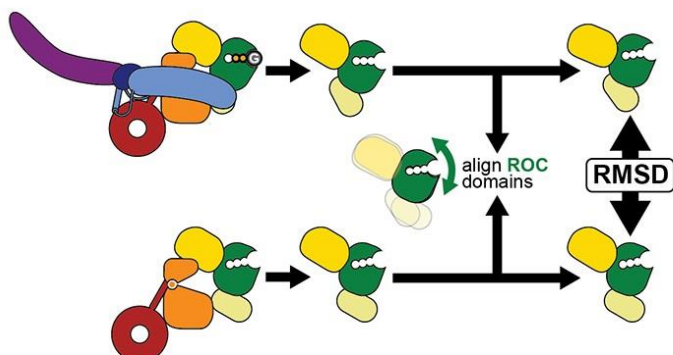
Supplementary Figure 3.8 Distribution of autoinhibited and activated LRRK2 in samples that were treated with EDTA for 1h vs. 24h.

(A) Cryo-EM data processing of nucleotide-stripped LRRK2 (EDTA treatment only), yielding active LRRK2 in monomeric, trimeric, and mixed dimer forms, as well as autoinhibited LRRK2 in monomeric, dimeric, and mixed dimer forms. (B) Data classification for LRRK2 after nucleotide stripping and 1h incubation with GDP or GMPPNP, yielding active LRRK2 monomers and mixed dimers, and autoinhibited monomers, dimers, and mixed dimers. (C) Data classification for LRRK2 after nucleotide stripping for 24h and incubation with GMPPNP for 24h, yielding exclusively active LRRK2 monomers (see Figure S5 for data processing details). (D) Number and relative abundance (%) of particles corresponding to the active and inactive conformations of LRRK2 derived from cryo-EM datasets of LRRK2 subjected to EDTA treatment for 1h or 24h and then incubated with one of the following: no nucleotide, GDP, GMPPNP. The graph in Figure 2H corresponds to the relative abundances shown in the table.

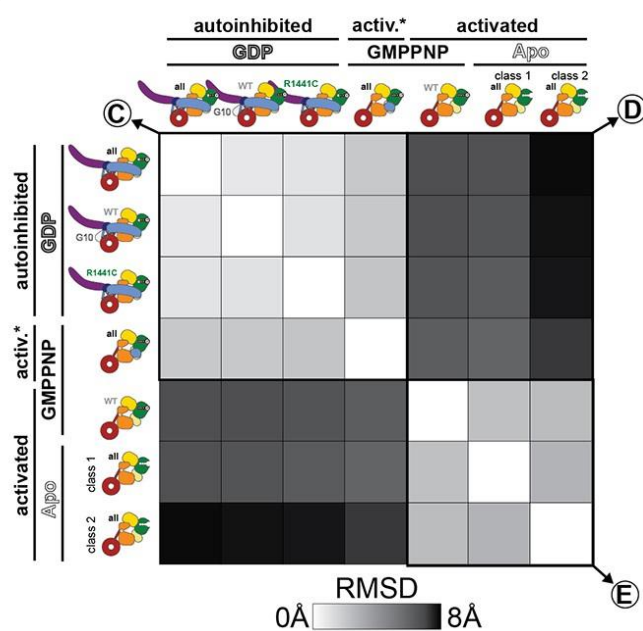
Supplementary Figure 3.9 Quantification of conformational differences among structures of autoinhibited and activated LRRK2.

(A) Schematic representation of the approach used here to quantify conformation differences between structures. We focused our analysis on the ROC-COR module, where the largest conformational changes take place in the C-terminal half of LRRK2 (LRRK2^{RCKW}). For any pair of structures—in this example an autoinhibited LRRK2:GDP and an activated LRRK2:Apo—we extracted their ROC-COR modules and aligned them to each other by their ROC domains. We then measured the Root Mean Squared Deviation (RMSD) between the two ROC-COR modules (see Methods). Those values are shown in the matrices in greyscale. (B) Matrix of pairwise comparisons (RMSDs) for 7 structures: 3 of autoinhibited LRRK2, 3 of activated LRRK2, and the one structure of activated LRRK2 where we saw the LRR partially docked (labeled ‘activated*’ here). For all structures, we indicated the LRRK2 variant and the nucleotide state of the ROC domain. The RMSD scale used in this matrix is shown at the bottom. Three quadrants, which are replotted separately with different RMSD scales, are highlighted with thicker lines and their panels indicated. (C-E), The 3 quadrants highlighted in (B) are shown with RMSD scales expanded to reveal differences among each subset of structures.

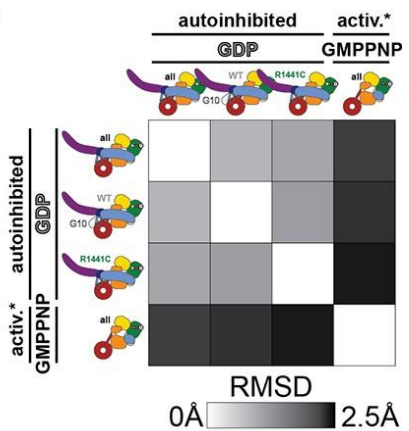
A



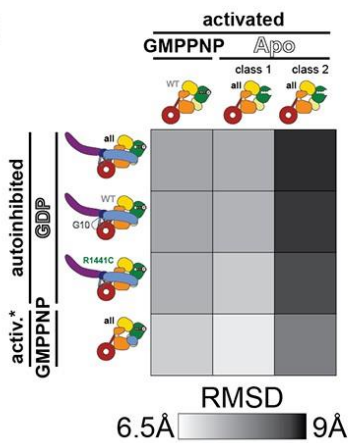
B



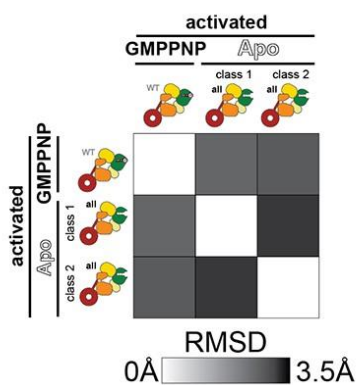
C

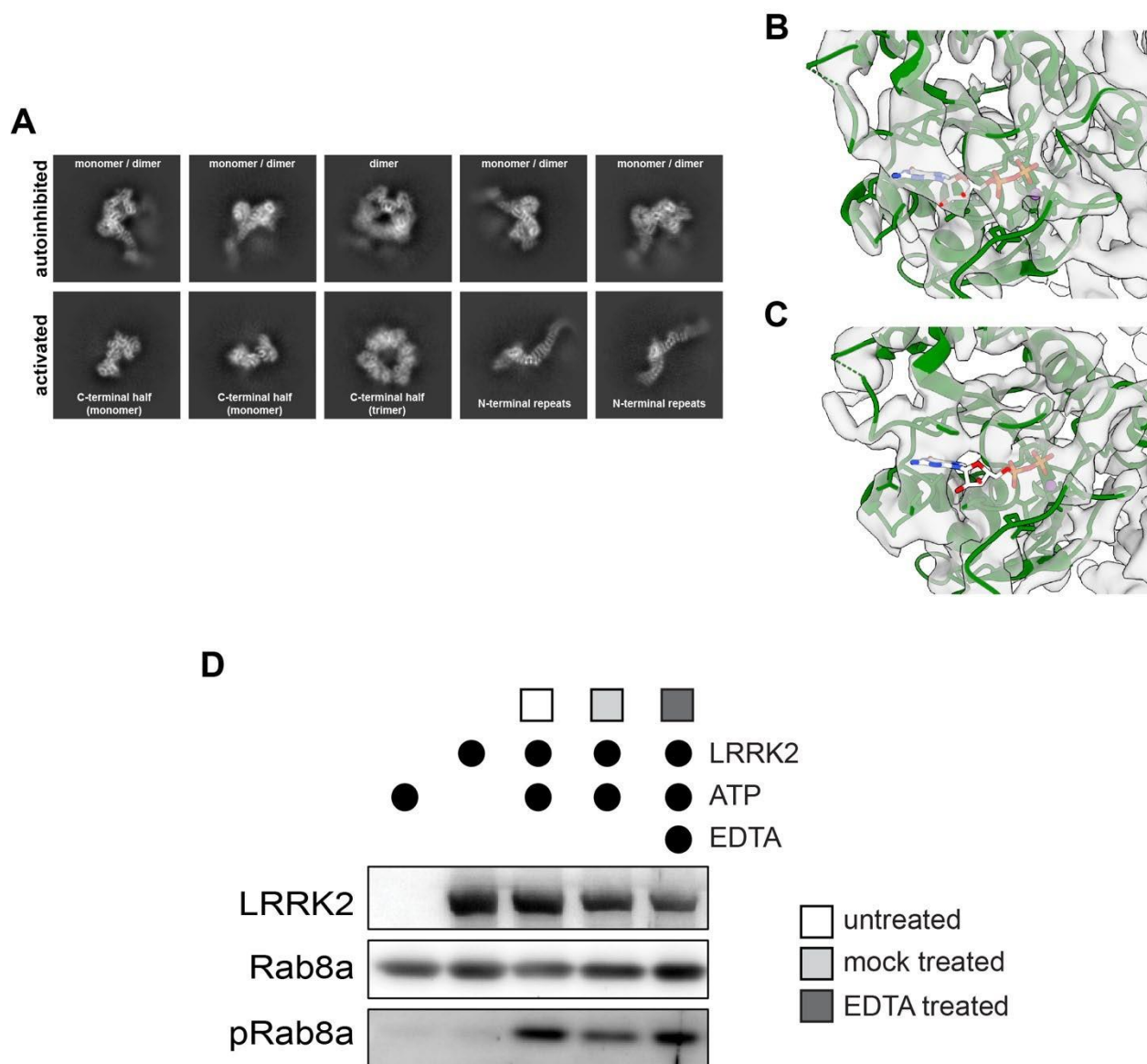


D



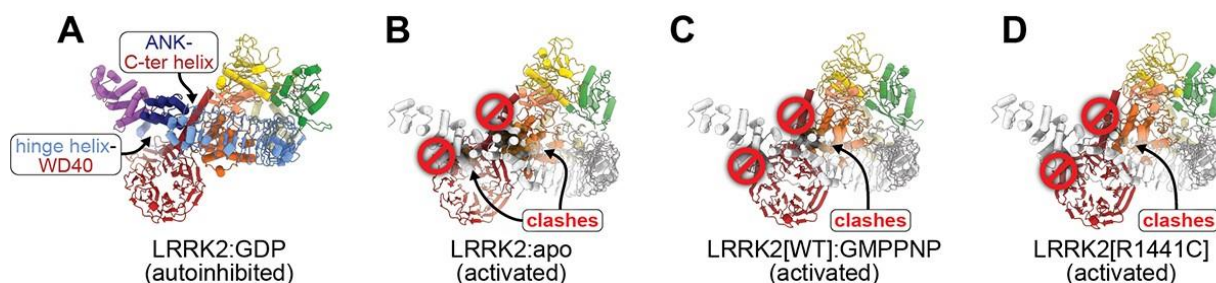
E





Supplementary Figure 3.10 The nucleotide stripping protocol activates LRRK2.

(A) 2D class averages for LRRK2 that was subjected to the nucleotide-stripping protocol but to which no additional nucleotide was added. The top and bottom rows show species of autoinhibited (top) and activated (bottom) LRRK2. (B, C) Close-ups of the ROC domain for reconstructions obtained from the data shown in (A) for autoinhibited (B) and activated (C) LRRK2. The density in autoinhibited LRRK2 (B) can accommodate a nucleotide (GDP was modeled here based on the data discussed in the manuscript) while activated LRRK2 (C) cannot (D) Representative immunoblot for in vitro Rab8a phosphorylation assays with or without treatment with EDTA (Figure 4B). ATP was omitted in one condition as a negative control.

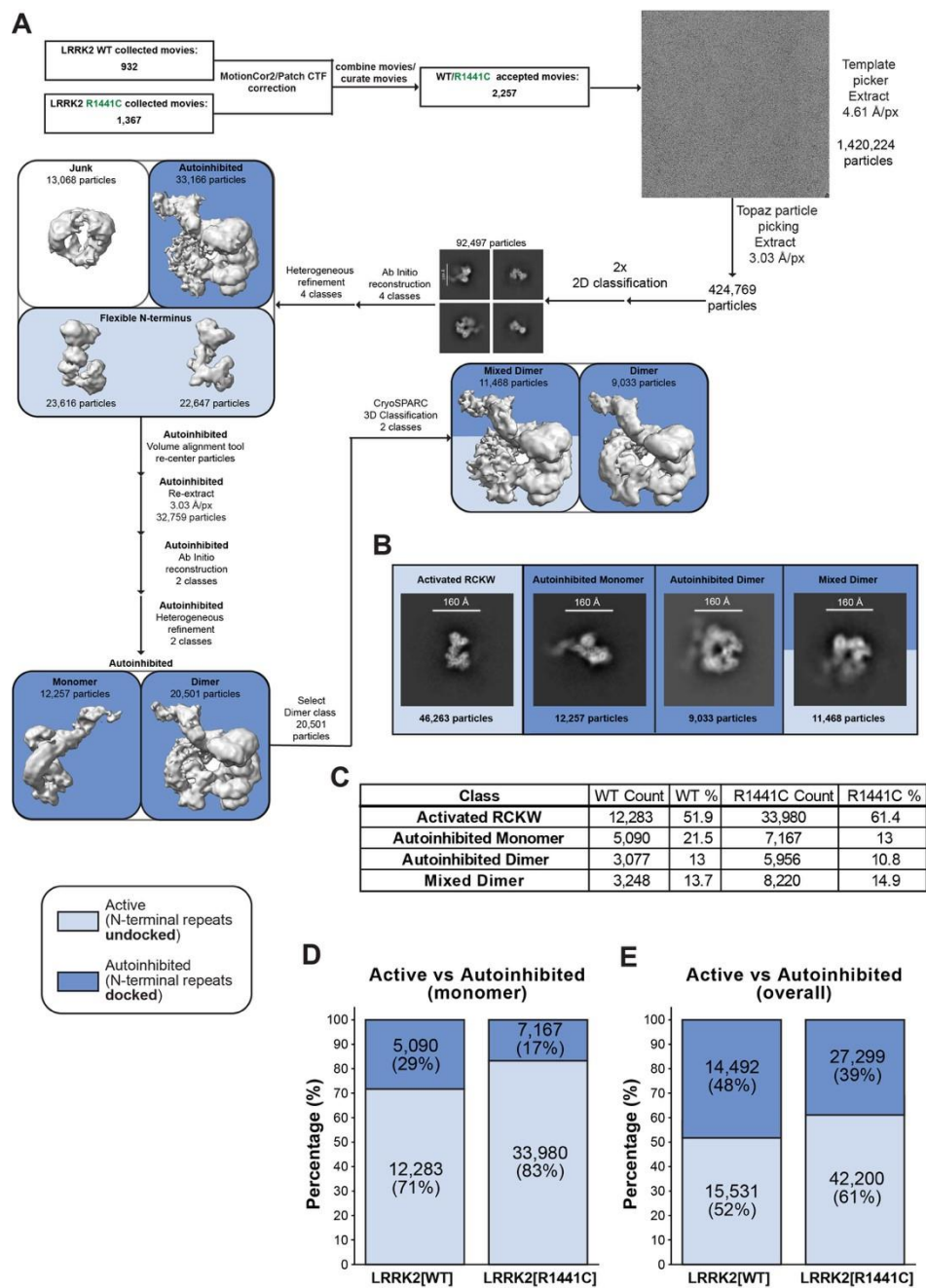


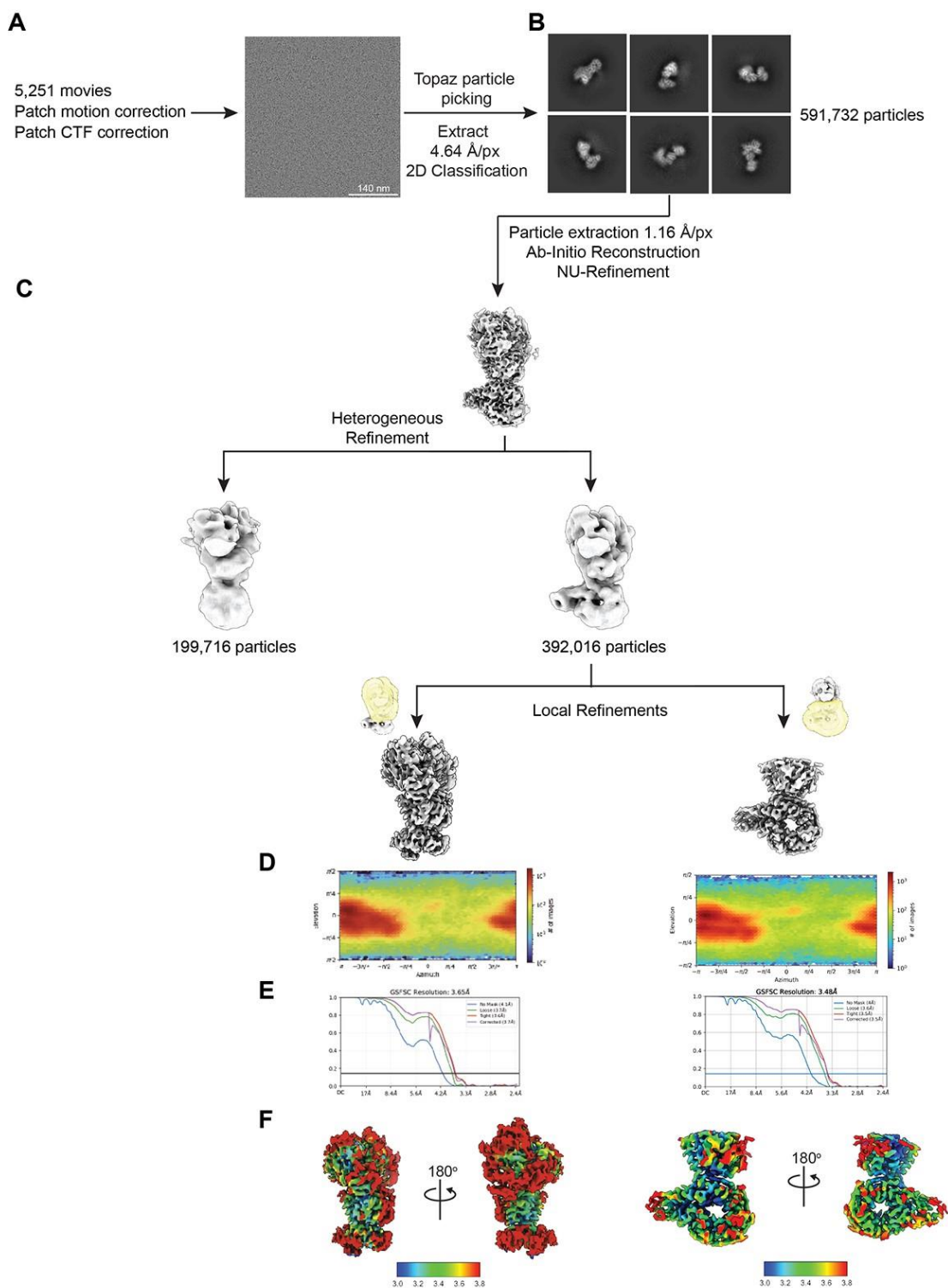
Supplementary Figure 3.11 Modeling the redocking of the N-terminal repeats in the context of activated LRRK2.

(A) Structure of GDP-bound, autoinhibited LRRK2 highlighting the two main interactions that stabilize the docked conformation: one between the hinge helix in the LRR and the WD40 domain, and the other between the ANK domain and the C-terminal helix the follows the WD40. **(B-D)**, We modeled the N-terminal repeats into three of our structures of activated LRRK2: LRRK2:Apo (class 2) (Figure 3E), LRRK2[WT]:GMPPNP (Figure 3B), and LRRK2[R1441C] (Figure 3F). In all cases, we took a fragment comprising the N-terminal repeats and the ROC domain from autoinhibited LRRK2 and aligned that to each of the structures of activated LRRK2 via their ROC domains. These panels show that the interactions shown in (A) cannot form when the C-terminal half of LRRK2 is in its active conformation without additional flexibility in the N-terminal repeats, which we did not observe in our data. Furthermore, there are clashes between the repeats and the C-terminal half in all three models.

Supplementary Figure 3.12 The mutation R1441C increases the fraction of LRRK2 in the active conformation (N-terminal repeats undocked).

(A) Data processing workflow for the combined LRRK2[WT] and LRRK2[R1441C] datasets. Light blue denotes active states and dark blue denotes autoinhibited states. (B) Representative 2D class averages for Activated LRRK2 (“RCKW”), Autoinhibited Monomer, Autoinhibited Dimer, and Mixed Dimer states derived from the combined dataset (79,021 total particles). (C) Particle counts and distributions across structural classes for LRRK2[WT] and LRRK2[R1441C] datasets. (D) Partition of active and autoinhibited states for monomeric LRRK2[WT] and LRRK2[R1441C]. (E) Partition of active and autoinhibited states for all species of LRRK2[WT] and LRRK2[R1441C]. Mixed dimers are counted twice, contributing one protomer to the active counts and one protomer to the autoinhibited counts.



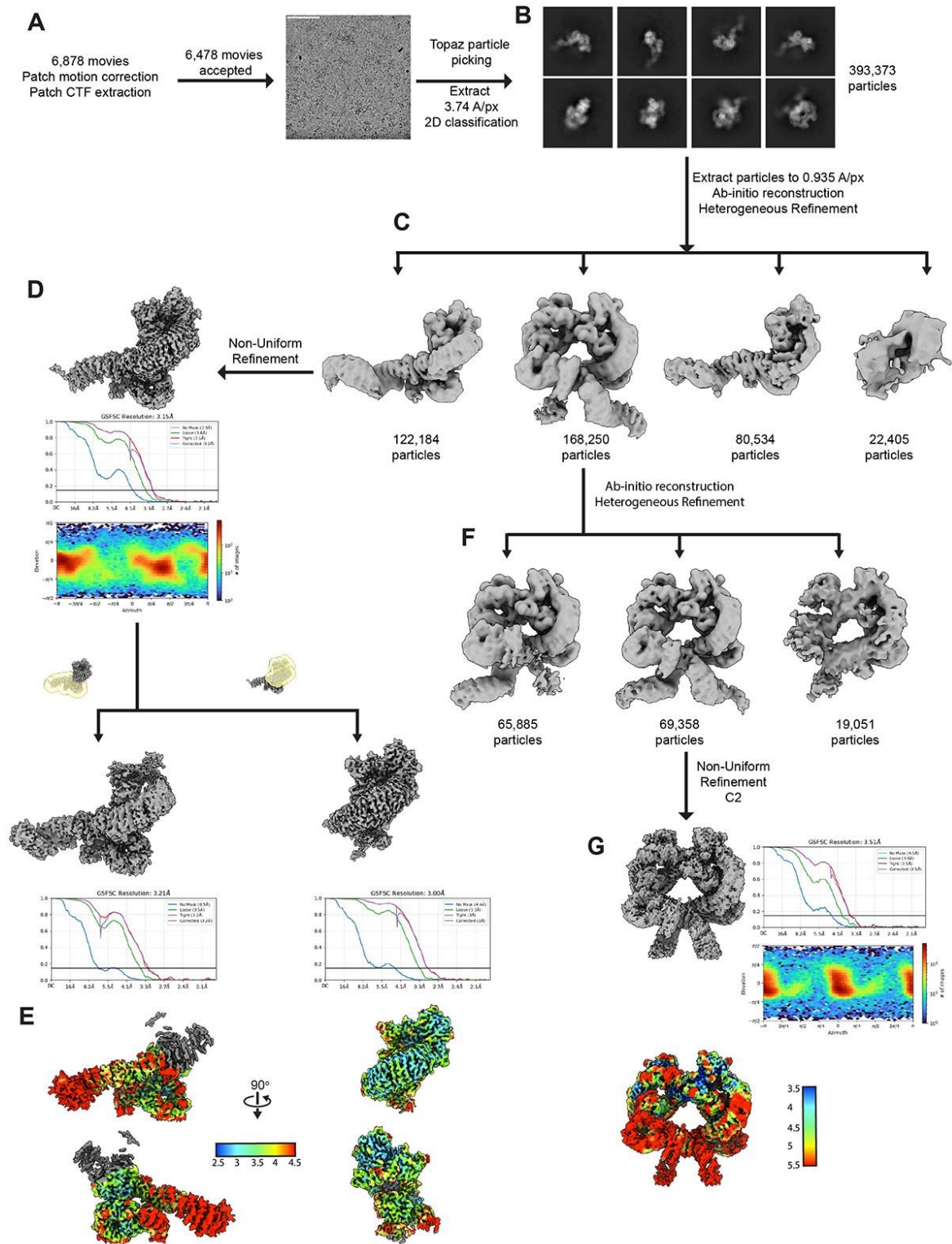


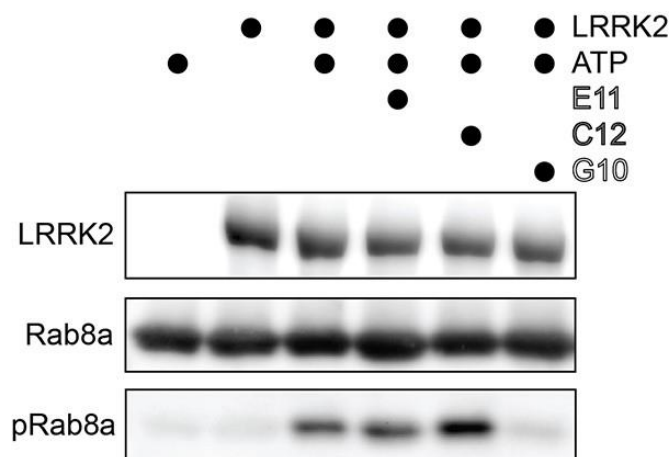
Supplementary Figure 3.13 Cryo-EM data processing pipeline for LRRK2^{RCKW} bound to DARPin C12.

(A) Number of movies collected and kept for further processing, and representative micrograph. (B) 2D class averages. (C) Data processing strategy. (D, E) Euler angle distribution (D) and FSC curve (E) for the volumes shown above. (F) Local resolution map.

Supplementary Figure 3.14 Cryo-EM data processing pipeline for LRRK2 bound to DARPin G10.

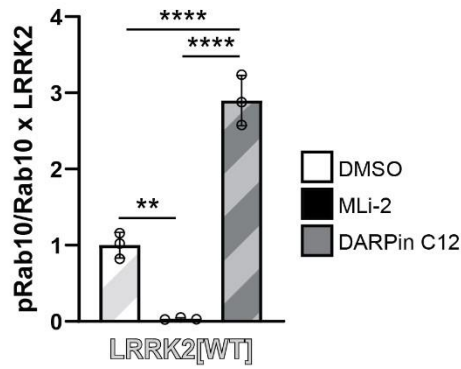
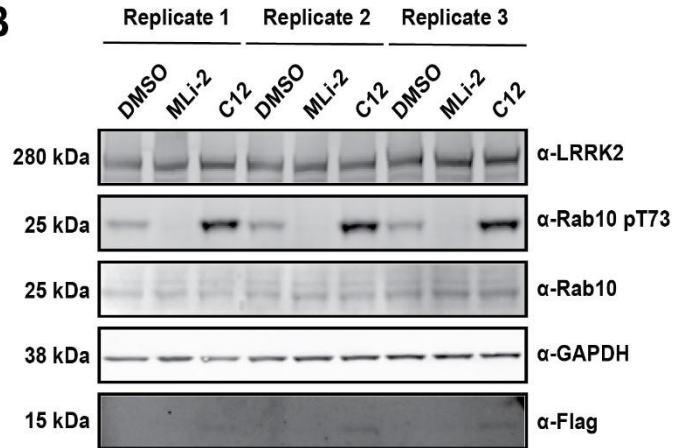
(A) Number of movies collected and kept for further processing, and representative micrograph. (B) 2D class averages. (C) Data processing strategy. (D) Processing of monomeric LRRK2:G10, volume, Euler angle distribution and FSC curve. (E) Local resolution map of monomeric LRRK2:G10. (F) Processing of dimeric LRRK2:G10. (G) Volume, FSC curve, Euler angle distribution and local resolution map of dimeric LRRK2:G10.





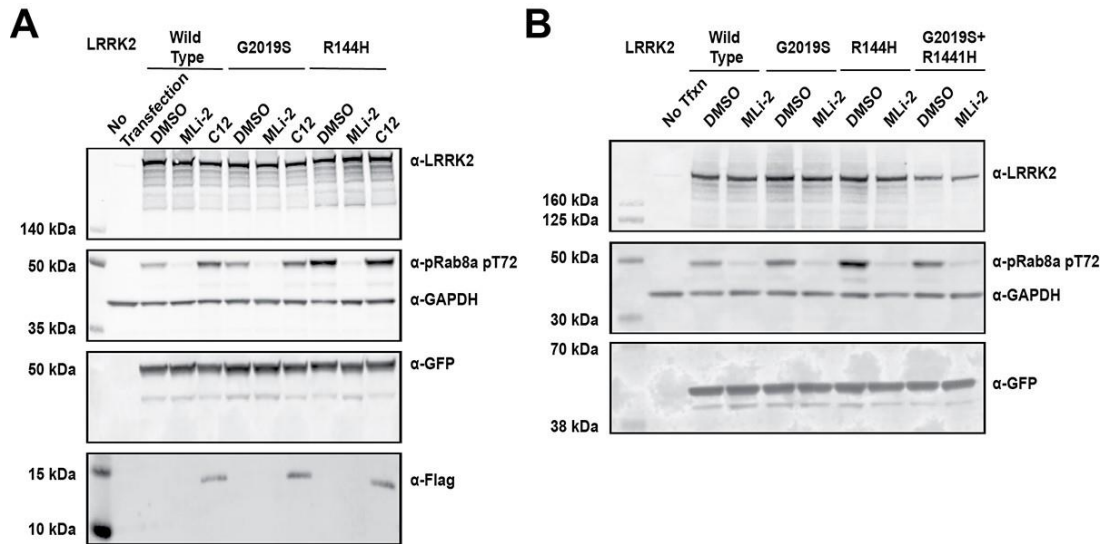
Supplementary Figure 3.15 Representative immunoblot for Figure 3.4K (in vitro Rab8a phosphorylation assays with DARPins E11, C12, and G10).

LRRK2[WT] was incubated with Rab8a in the presence of ATP and either no DARPIn or one of the following DARPins: E11, C12, or G10. ATP was omitted in one condition as a negative control.

A**B**

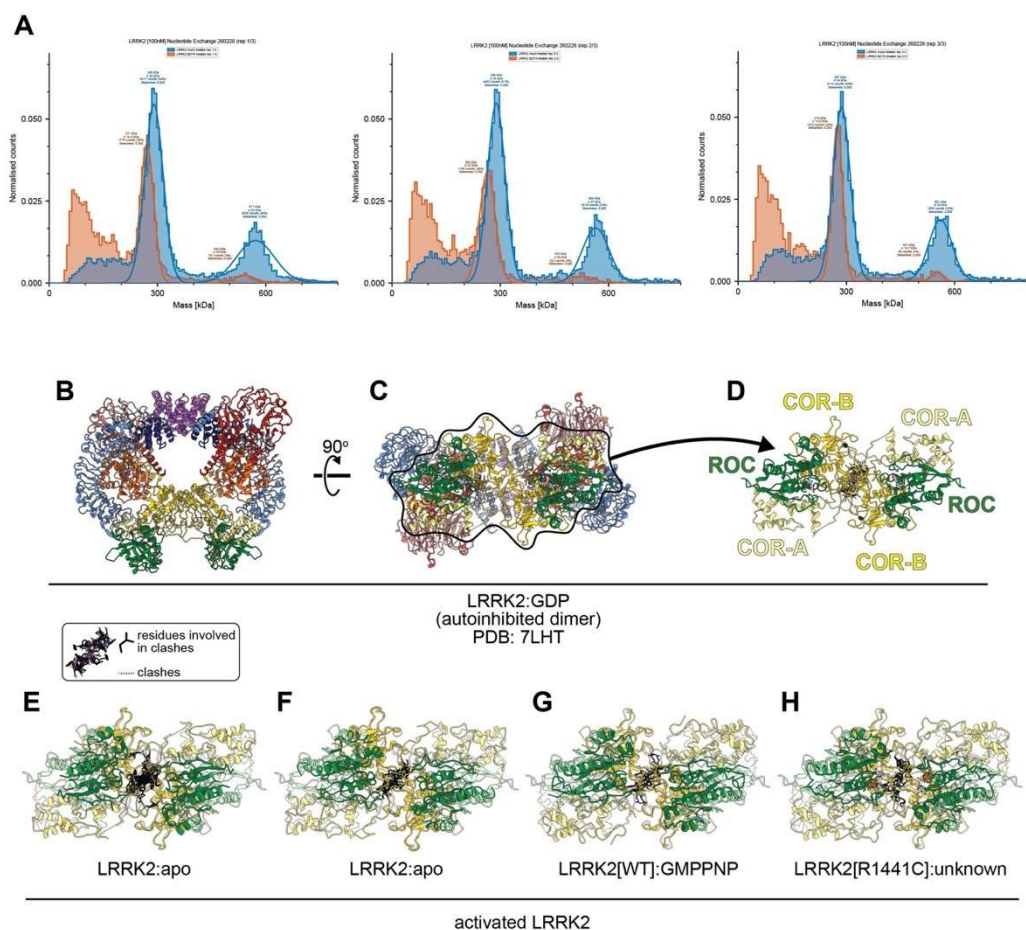
Supplementary Figure 3.16 (A) Quantification and (B) immunoblot of endogenous pRab10 (pT73) / Rab10 x LRRK2 normalized to the mean of LRRK2[WT] + DMSO condition.

HEK 293Ts co-expressing LRRK2[WT] were treated with either DMSO or MLi-2 (5μM) for 1 hour. Cells were lysed and immunoblotted for LRRK2, Rab10, phosphor-Rab10 (T73), GAPDH, and Flag-tagged DARPin C12. All data were analyzed using a two-way ANOVA with Tukey's multiple comparison correction: DMSO vs MLi-2: ** $p=0.0036$; MLi-2 vs DARPin C12: **** $p<0.0001$; DMSO vs DARPin C12: **** $p<0.0001$. Error bars $\pm$ SD.



Supplementary Figure 3.17 Representative immunoblots for Figure 6B (effect of DARPin C12 on Rab8a phosphorylation in cells by LRRK2[WT], LRRK2[G2019S], and LRRK2[R1441H] and Figure 6C (Rab8a phosphorylation in cells by LRRK2[G2019S], LRRK2[R1441H], and LRRK2[G2019S/R1441H]).

(A) Representative immunoblot from 293T cells transiently transfected with GFP 11-tagged LRRK2[WT], LRRK2[R1441H], or LRRK2[G2019S] and its substrate GFP-Rab8a plus or minus Flag-tagged DARPin C12. LRRK2 and its mutants with GFP-Rab8a alone were treated with either DMSO or MLi-2 (5 μ M) for 1 hour. Lysed cells were immunoblotted for LRRK2, GFP-Rab8a, phospho-Rab8a (pT72), and GAPDH. Samples were immunoblotted for Flag separately. (B) Representative immunoblot from 293T cells transiently transfected with GFP 11-tagged LRRK2[WT], LRRK2[R1441H], LRRK2[G2019S], or LRRK2[G2019S/R1441H] and its substrate GFP-Rab8a and treated with either DMSO or MLi-2 (5 μ M) for 1 hour. Lysed cells were immunoblotted for LRRK2, GFP-Rab8a, phospho-Rab8a (pT72), and GAPDH.



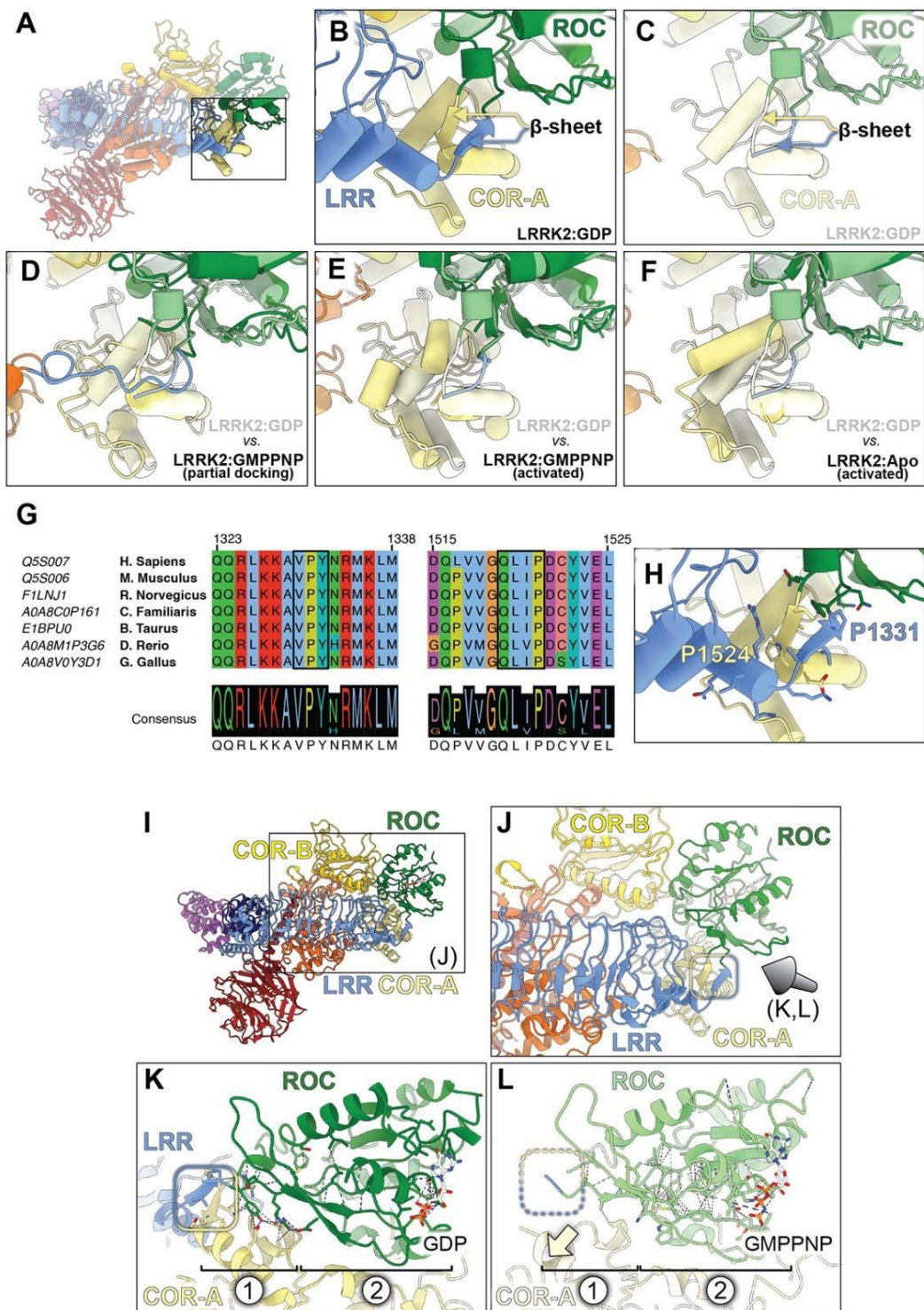
Supplementary Figure 3.18 The active state of LRRK2 is likely to be a monomer.

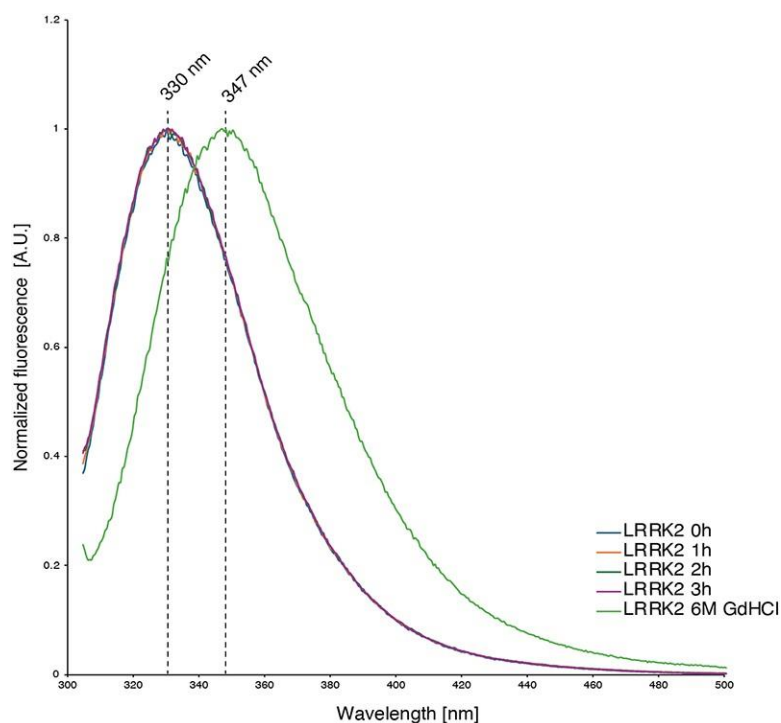
(A) Treatment of LRRK2 with EDTA shifts the population from the dimeric to the monomeric form. LRRK2 was either treated with EDTA for 2h (as in our nucleotide stripping protocol) or left

untreated (mock treatment) (see Methods). The resulting samples were subjected to mass photometry to measure the abundance of LRRK2 monomers and dimers. The three technical replicates of the mass photometry measurements quantified in Figure 4D are shown here. (B-H) Modeling of activated LRRK2 in the context of the autoinhibited dimer. (B,C) Two views of the dimeric form of autoinhibited LRRK2:GDP (PDB: 7LHT). The view in (C) is from the dimeric interface, which involves the COR-B and COR-A domains. (D) Structure of the ROC-COR moieties of the LRRK2 dimer, showing the contacts (dashed black lines) involved in formation of the dimer. (E-H) Modeling of dimer interfaces for activated LRRK2. We took the ROC-COR moieties from four structures of activated LRRK2—LRRK2:Apo (1) (E), LRRK2:Apo (2) (F), LRRK2[WT]:GMPPNP (G), and LRRK2[R1441C] (nucleotide undetermined) (H)—and aligned them to the two COR-B domains that form the dimer interface in autoinhibited LRRK2. Residues involved in clashes are shown in black, and the clashes between them, as identified in ChimeraX, as purple dashed lines. Overlaid on these models is a semi-transparent version of panel (D) to highlight the conformational changes between the dimer interface in autoinhibited LRRK2 and the models shown here.

Supplementary Figure 3.19 Model for nucleotide control of the docking and undocking of the N-terminal repeats.

(A, B) In the autoinhibited (GDP-bound) form of LRRK2, the N-terminal repeats of LRRK2 connect to the GTPase (ROC) (A), via one strand of a two-stranded β -sheet (B). The other β -strand connects ROC to the COR-A domain (B). (C) Same panel as in (B), but with the N-terminal repeats of LRRK2 removed and the structure shown in lighter shades. This structure will be used as reference in panels (d-f) to illustrate the conformational changes between autoinhibited LRRK2 (in lighter shades) and different structures of activated LRRK2 (in the regular darker shades). (D) Comparison between autoinhibited LRRK2 and the structure of GMPPNP-bound LRRK2 (Figure 3C) with partial redocking of the LRR. Formation of the β -sheet is seen in this structure. (E, F) Comparison between autoinhibited LRRK2 and GMPPNP-bound (E) or Apo (F) activated LRRK2. The β -sheet is not formed in these structures. (G) Sequence alignment of the regions that encompass the two β -strands. (H) Close-up of the β -sheet and neighboring region, with the residues included in the alignment shown. The two prolines that mark the ends of the β -strands (P1331 and P1524) are labeled. (I, J) Structure of autoinhibited LRRK2, bound to GDP (I), with the region inside the square enlarged in (J). The blue/yellow rounded square in (J) shows the β -sheet connecting the LRR to the C-terminal half of LRRK2. The arrow indicates the viewing direction in panels (K) and (L). (K, L) Close-ups of the ROC domain and the region where it connects to the LRR and COR-A domains in the autoinhibited (GDP-bound) (K) and activated (GMPPNP-bound) (L) forms of LRRK2. The blue/yellow rounded square in (K) shows the β -sheet while the dotted version in (L) indicates its absence in the activated form of LRRK2. In addition to the presence or absence of this β -sheet, the two states differ in a set of interactions seen adjacent to the β -sheet when it is formed (region (1) in panel (K)), or close to the nucleotide in the activated (GMPPNP) form of LRRK2 (region (2) in panel (L)). The autoinhibited state of LRRK2 (K) shows several interactions in (1) and few in (2), while the reverse is seen in the activated state (L). The yellow arrow in (L) highlights the movement of the COR-A domain away from ROC responsible for breaking the β -sheet in activated LRRK2.





Supplementary Figure 3.20 Fluorescence emission spectra for LRRK2 incubated at room temperature for different times.

To determine the stability of LRRK2 incubated at room temperature for a few hours (needed for the nucleotide-exchange protocol), we measured fluorescence spectra for LRRK2[G2019S] incubated at room temperature at one-hour intervals from 0h (control) to 3h. We also measured the fluorescence spectrum of LRRK2 in 6M guanidinium hydrochloride as an unfolded control. LRRK2 was diluted to 1.56 μ M (or 40 μ M tryptophan).

3.6 Tables

Supplementary Table 3.1 Cryo-EM data collection, refinement, and validation statistics

	LRRK2 autoinhibited bound to G10 DARPin (EMDB70981) (PDB 9OXH)	LRRK2 autoinhibited bound to G10 DARPin map focused (top) (EMDB- 73497)	LRRK2 autoinhibited bound to G10 DARPin map focused (WD40DARPin) (EMDB-73499)	LRRK2 autoinhibited (EMDB- 70604) (PDB 9OM2)
Data collection and processing				
Magnification	130000	13000	130000	130000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ / Å ²)	50	50	56	55
Defocus range (µm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	2889148	2889148	2889148	2889148
Final particle images (no.)	122184	122184	122184	206812
Map resolution (Å)	3.15	3.00	3.21	3.31
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.5-4.5	2.5-3.5	2.8-5	3-5
Refinement				
Initial model used (PDB code)	7LHW	---	---	9OXH
Model resolution (Å)	---	---	---	---
FSC threshold	---	---	---	---
Model resolution range (Å)	---	---	---	---
Map sharpening <i>B</i> factor (Å ²)	---	---	---	---
Model composition				
Non-hydrogen atoms	14799	---	---	13980
Protein residues	1912	---	---	1782
Ligands	1	-	-	0
<i>B</i> factors (Å²)				
Protein	49.32 41.31	---	---	80.09
Nucleotide	33.05	---	---	58.07
Ligand		-	-	---
R.m.s. deviations				
Bond lengths (Å)	0.004	---	---	0.003
Bond angles (°)	0.933	-	-	0.660

Validation					
MolProbity score	1.98 7.07	---	---		2.16
Clashscore	0.06	---	---		12.71
Poor rotamers (%)		-	-		0.64
Ramachandran plot					
Favored (%)	88.35	---	---		90.23
Allowed (%)	10.96	---	---		9.60
Disallowed (%)	0.69	-	-		0.17

Supplementary Table 3.2 (Continued) Cryo-EM data collection, refinement, and validation statistics

	LRRK2 GMP-PNP bound active state (EMDB-72756) (PDB 9Y7I)	LRRK2 GMP-PNP bound active state focused map ROC-COR (EMDB-73485)	LRRK2 GMP-PNP bound active state focused map ROC-COR resolveEM (EMDB-73485)
Data collection and processing			
Magnification	13000	13000	13000
Voltage (kV)	300	300	300
Electron exposure (e-/Å ²)	56	56	56
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1
Initial particle images (no.)	273568	273568	273568
Final particle images (no.)	94877	94877	94877
Map resolution (Å)	3.73	3.73	3.73
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	3-5	3-5	3-5
Refinement			
Initial model used (PDB code)	9OXH, 8TXZ	--	---
Model resolution (Å)	---	---	---
FSC threshold	---	---	---
Model resolution range (Å)	---	---	---
Map sharpening <i>B</i> factor (Å ²)	---	---	---
Model composition			
Non-hydrogen atoms	8192	---	---
Protein residues	1101	---	---
Ligands	2	-	-
<i>B</i> factors (Å ²)			
Protein	139.15	---	---
Nucleotide	---	---	---
Ligand	142.72	-	-
R.m.s. deviations			
Bond lengths (Å)	0.010	---	---
Bond angles (°)	1.187	-	-
Validation			
MolProbity score	2.16 7.93	---	---
Clashscore	0.38	---	---
Poor rotamers (%)		-	-
Ramachandran plot			
Favored (%)	90.63	---	---
Allowed (%)	9.09	---	---
Disallowed (%)	0.28	-	-

Supplementary Table 3.3 (Continued) Cryo-EM data collection, refinement, and validation statistics

	LRRK2 GMPPNP bound inactive state (EMDB-72649) (PDB 9Y7A)	LRRK2 GMPPNP bound inactive state Refinement (EMDB-73487)	LRRK2 GMPPNP bound inactive state focus map ROC-COR (EMDB-73491)	LRRK2 GMPPNP bound inactive state focus map ROC-COR resolveEM (EMDB-73492)
Data collection and processing				
Magnification	130000	130000	130000	130000
Voltage (kV)	300	300	300	300
Electron exposure (e-/Å ²)	56	56	56	56
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	273568	273568	273568	273568
Final particle images (no.)	56741	56741	56741	56741
Map resolution (Å)	4.40	4.40	4.40	4.40
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	4-7	4-7	3-7	3-7
Refinement				
Initial model used (PDB code)	9OXH	---	---	---
Model resolution (Å)	---	---	---	---
FSC threshold	---	---	---	---
Model resolution range (Å)	---	---	---	---
Map sharpening <i>B</i> factor (Å ²)	---	---	---	---
Model composition				
Non-hydrogen atoms	6384	---	---	---
Protein residues	1028	---	---	---
Ligands	1	-	-	-
<i>B</i> factors (Å²)				
Protein	184.06	---	---	---
Nucleotide	---	---	---	---
Ligand	170.47	-	-	-
R.m.s. deviations				
Bond lengths (Å)	0.004	---	---	---
Bond angles (°)	0.880	-	-	-

Validation				
MolProbity score	1.99	---	---	---
Clashscore	7.38	---	---	---
Poor rotamers (%)	0	-	-	-
Ramachandran plot				
Favored (%)	82.26	---	---	---
Allowed (%)	16.83	---	---	---
Disallowed (%)	0.91	-	-	-

Supplementary Table 3.4 (Continued) Cryo-EM data collection, refinement, and validation statistics

	LRRK2 active state (R1441C) (EMDB72768) (PDB 9YCD)	LRRK2 active state (R1441C) Focused map ROC-COR (EMDB-73051)	LRRK2 active state (R1441C) Focused map ROC-COR (EMDB-73051)	LRRK2 autoinhibited flex-ARM (EMDB-70982) (PDB 9OXI)
Data collection and processing				
Magnification	130000	130000	130000	130000
Voltage (kV)	300	300	300	300
Electron exposure (e-/Å ²)	50	50	50	55
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	286935	286935	286935	2889148
Final particle images (no.)	63806	63806	63806	144198
Map resolution (Å)	3.63	3.63	3.63	3.72
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	3-9	3-8	3-	3-6
Refinement				
Initial model used (PDB code)	9OXH, 8TXZ	---	---	9OXH
Model resolution (Å)	---	---	---	---
FSC threshold	---	---	---	---
Model resolution range (Å)	---	---	---	---
Map sharpening <i>B</i> factor (Å ²)	---	---	---	---
Model composition				
Non-hydrogen atoms	8602	---	---	12100
Protein residues	1117	---	---	1531
Ligands	1	-	-	0

<i>B</i> factors (Å ²)				
Protein	140.67	---	---	92.50
Nucleotide	---	---	---	47.60
Ligand	56.29	-	-	---
R.m.s. deviations				
Bond lengths (Å)	0.004	---	---	0.003
Bond angles (°)	0.978	-	-	0.812
Validation				
MolProbity score	2.14	---	---	1.92
Clashscore	10.52	---	---	7.09
Poor rotamers (%)	0.11	-	-	0
Ramachandran plot				
Favored (%)	88.41	---	---	90.74
Allowed (%)	11.22	---	---	8.99
Disallowed (%)	0.37	-	-	0.27

Supplementary Table 3.5 (Continued) Cryo-EM data collection, refinement, and validation statistics

	LRRK2 after symmetry expansion (G2019S) (EMDB-72556) (PDB 9Y68)	LRRK2 after symmetry expansion (R1441C) (EMDB-71012) (PDB 9OYA)	LRRK2 asymmetry dimer (EMDB-72751) (PDB 9YBL)	LRRK2 after symmetry expansion (EMDB-72555) (PDB 9Y67)
Data collection and processing				
Magnification	130000	130000	130000	13000
Voltage (kV)	300	300	300	300
Electron exposure (e-/Å ²)	55	55	55	55
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	2889148	2889148	2889148	2889148
Final particle images (no.)	83730	98448	79889	41214
Map resolution (Å)	3.25	3.44	3.44	3.72
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	3-5	3-5	3-6	3-6
Refinement				
Initial model used (PDB code)	9OXH	9OXH	9OXH	9OXH
Model resolution (Å)	---	---	---	---
FSC threshold	---	---	---	---
Model resolution range (Å)	---	---	---	---

Map sharpening <i>B</i> factor (Å ²)	---	---	---	---
Model composition				
Non-hydrogen atoms	13046	14270	15523	13173
Protein residues	1700	1801	2071	1713
Ligands	0	0	0	0
<i>B</i> factors (Å ²)				
Protein	109.89	102.36	117.93	126.71
Nucleotide	76.92	75.05	38.41	85.35
Ligand	---	---	--	---
R.m.s. deviations				
Bond lengths (Å)	0.003	0.004	0.004	0.004
Bond angles (°)	0.660	0.809	0.743	0.946
Validation				
MolProbity score	1.93	1.92	1.98 8.25	2.04 8.77
Clashscore	7.17	6.74	0.06	0.14
Poor rotamers (%)	0	0		
Ramachandran plot				
Favored (%)	90.56	90.15	88.25	89.07
Allowed (%)	9.38	9.79	11.35	10.51
Disallowed (%)	0.06	0.06	0.40	0.42

Supplementary Table 3.6 (Continued) Cryo-EM data collection, refinement, and validation statistics

	LRRK2 active state 1 (APO ROC) EMDB-72762) (PDB 9YC8)	LRRK2 active state 1 (APO ROC) focus map ROC-COR EMDB-73496)	LRRK2 active state 2 (APO ROC) EMDB-72756) (PDB 9YBP)	LRRK2 active state 2 (APO ROC) EMDB-72756) (PDB 9YBP)
Data collection and processing Magnification				
	130000	130000	13000	13000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	55	55	55	55
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	2889148	2889148	2889148	2889148
Final particle images (no.)	76274	76274	86982	86982
Map resolution (Å)	4.02	4.01	4	4.05
FSC threshold	0.143	0.143	0.143	0.143

Map resolution range (Å)	3.5-7.2	3.5-7.4	3.4-8	3-8
Refinement		---		---
Initial model used (PDB code)	9OXH		9OXH	
Model resolution (Å)	---	---	---	---
FSC threshold	---	---	---	---
Model resolution range (Å)	---	---	---	---
Map sharpening B factor (Å ²)	---	---	---	---
Model composition				
Non-hydrogen atoms	7822	---	6587	---
Protein residues	1051	---	990	---
Ligands	1	-	0	-
B factors (Å ²)				
Protein	127.40	---	119.59	---
Nucleotide	86.07	---	---	---
Ligand	---	-	-	-
R.m.s. deviations				
Bond lengths (Å)	0.003	---	0.002	---
Bond angles (°)	0.662	-	0.669	-
Validation				
MolProbity score	2.20	---	1.88	---
Clashscore	6.41	---	6.61	---
Poor rotamers (%)	0.26	-	0	-
Ramachandran plot				
Favored (%)	88.81	---	93.55	---
Allowed (%)	10.99	---	6.03	---
Disallowed (%)	0.20	-	0.42	-

Supplementary Table 3.7 (Continued) Cryo-EM data collection, refinement, and validation statistics

	LRRK2 autoinhibited (EMDB-72941) (PDB 9YGT)	LRRK2 autoinhibited (EMD-72978)	LRRK2 active state 3 (APO ROC) (EMD-72978)
Data collection and processing			
Magnification	13000	1300	1300
Voltage (kV)	300	300	300
Electron exposure (e-/Å ²)	55	55	55
Defocus range (µm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	0.935	0.935	0.935
Symmetry imposed	C1	C1	C1
Initial particle images (no.)	2889148	2889148	2889148
Final particle images (no.)	129152	50446	112087

Map resolution (Å)	3.42	4.14	3.72
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	3-5	4-6	3-6
Refinement		--	--
Initial model used (PDB code)	9OM2		
Model resolution (Å)	---	---	---
FSC threshold	---	-	-
Model resolution range (Å)	---	---	---
Map sharpening <i>B</i> factor (Å ²)	---	---	---
Model composition			
Non-hydrogen atoms	13934	---	---
Protein residues	1774	---	---
Ligands	0	---	---
<i>B</i> factors (Å ²)			
Protein	55.10	---	---
Nucleotide	48.81	---	---
Ligand	---	-	-
R.m.s. deviations			
Bond lengths (Å)	0.005	---	---
Bond angles (°)	0.939	-	-
Validation			
MolProbity score	2.04	---	---
Clashscore	8.79	---	---
Poor rotamers (%)	0	-	-
Ramachandran plot			
Favored (%)	89.49	---	---
Allowed (%)	10.29	---	---
Disallowed (%)	0.23	-	-

Supplementary Table 3.8 (Continued) Cryo-EM data collection, refinement, and validation statistics

LRRK2 ^{RCKW} bound to C12 DARPin (EMDB73338) (PDB-9YQK)	LRRK2 ^{RCKW} map focused on C-terminal kinase domain, WD40 and C12 DARPin (EMD-73280)	LRRK2 ^{RCKW} map focused on ROC, COR- A, CORB and Nterminal kinase domain (EMD-73281)	LRRK2 ^{RCKW} bound to DARPin C12 consensus map (EMD-73282)
--	---	--	--

Data collection and processing				
Magnification	36000	36000	36000	36000
Voltage (kV)	200	200	200	200
Electron exposure (e-/Å ²)	51	51	51	51
Defocus range (μm)	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0	-1.0 to -3.0
Pixel size (Å)	1.16	1.16	1.16	1.16
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	591732	591732	591732	591732
Final particle images (no.)	392016	392016	392016	392016
Map resolution (Å)		3.48	3.6	3.72
FSC threshold		0.143	0.143	0.143
Map resolution range (Å)	3-5	3-5	3-5	3-5
Refinement				
Initial model used (PDB code)	6VP7			
Model resolution (Å)	---	---	---	---
FSC threshold	---	-	-	-
	---	---	---	---
Model resolution range (Å)				
Map sharpening <i>B</i> factor (Å ²)	---	---	---	---
Model composition				
Non-hydrogen atoms	8925	---	---	---
Protein residues	1123	---	---	---
Ligands	0	---	---	---
<i>B</i> factors (Å ²)				
Protein	103.92	---	---	---
Nucleotide	---	---	---	---
Ligand	-	-	-	-
R.m.s. deviations				
Bond lengths (Å)	0.003	---	---	---
Bond angles (°)	0.659	-	-	-
Validation				
MolProbity score	2.48	---	---	---
Clashscore	11.95	---	---	---
Poor rotamers (%)	0	-	-	-
Ramachandran plot				
Favored (%)	92.43	---	---	---
Allowed (%)	7.48	---	---	---
Disallowed (%)	0.09	-	-	-

3.7 Contributions and Acknowledgments

Author Contributions

All authors were involved in experimental design. AVS, TB, MS-M, EM, KHVN, and DR performed the cryo-EM work (data collection and analysis). KSH and KJS performed the cellbased assays. WG performed the GTPase and GDP release assays. EM performed the mass photometry experiments. LZ and KMS developed the nucleotide-exchange assay. AVS, KSH, TB, MS-M, KS, EX, VD, SM, SK, SLR-P, and AEL were involved in the development and characterization of the DARPinS. AVS, KSH, TB, MS-M, EM, KHVN, WG, KS, RF, DR, SLRP, and AEL performed data analysis. JI made the model animation. AVS, SLR-P, and AEL wrote the manuscript, and AVS, KSH, TB, KJS, SK, KMS, SLR-P, and AEL edited it.

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Chapter 4: Conclusion and future directions

4.1 Conclusion

The work presented in this thesis provides a significant step forward in understanding how LRRK2 is autoregulated. In Chapter 2, we presented the first high-resolution structures of LRRK2 bound to kinase inhibitors, unveiling the first mechanistic insight into how activation of LRRK2 involves undocking of the repeats. Because the kinase does not fully close in the presence of type I inhibitors alone, the docking of the repeats preserves its autoinhibited state and prevents movement of the ROC-COR domain and, subsequently, the kinase domain. In Chapter 3, we obtained the first high-resolution structures of activated LRRK2 bound to a GTP analog and in the APO state (ROC domain), generating the first mechanistic insight into how nucleotide exchange in the ROC domain controls the equilibrium of its conformation. The structural change of the ROC-COR domains and undocking of the repeats start the GTPase cycle, which is essential for LRRK2 kinase activation. All the structures and findings in this thesis give the initial framework for understanding LRRK2 activation and guiding future therapeutic development.

4.2 Future directions

In summary, the results of this thesis offer a detailed mechanistic understanding of how LRRK2 is regulated. In the work of Chapter 3, we found that Rab8a enhances LRRK2 activation, suggesting that it works downstream in our proposed models. Recently, a preprint published structures of LRRK2 bound to different Rabs⁹⁹ and described how they potentially act in LRRK2 activation. While these structures show how different Rabs bind to autoinhibited LRRK2 and LRRK2^{RCKW} at different sites, they are positioned far from the kinase active site for phosphate transfer to occur. A structure of Rab bound to LRRK2's active site is a crucial element to understanding LRRK2's regulation by Rabs.

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