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Additional feedforward mechanism of Parkin activation via binding of phospho-UBL and RING0 in trans

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Abstract

Loss of function Parkin mutations lead to early-onset of Parkinson's disease. Parkin is an auto-inhibited ubiquitin E3 ligase activated by dual phosphorylation of its ubiquitin-like (Ubl) domain and ubiquitin by the PINK1 kinase. Herein, we demonstrate a competitive binding of the phospho-Ubl and RING2 domains towards the RING0 domain, which regulates Parkin activity. We show that phosphorylated Parkin can complex with native Parkin, leading to the activation of autoinhibited native Parkin in trans. Furthermore, we show that the activator element (ACT) of Parkin is required to maintain the enzyme kinetics, and the removal of ACT slows the enzyme catalysis. We also demonstrate that ACT can activate Parkin in trans but less efficiently than when present in the cis molecule. Furthermore, the crystal structure reveals a donor ubiquitin binding pocket in the linker connecting REP and RING2, which plays a crucial role in Parkin activity.

eLife assessment

This is a **useful** manuscript describing the competitive binding between Parkin domains to define the importance of dimerization in the mechanism of Parkin regulation and catalytic activity. The evidence supporting the importance of Parkin dimerization for an 'in trans' model of Parkin activity described in this manuscript is **solid**, but lacks more stringent and biochemical characterization of competitive binding that could provide more direct evidence to support the author's conclusions. This work will be of interest to those focused on defining the molecular mechanisms involved in ubiquitin ligase interactions, PINK-Parkin-mediated mitophagy, and mitochondrial organellar quality control.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra leading to motor defects. PD is primarily sporadic, occurring mainly in older people. Mutations in several genes, such as *PARK2* (Parkin) and *PARK6* (PINK1, PTEN-induced kinase 1), cause early-onset autosomal recessive juvenile parkinsonism (ARJP). Parkin and PINK1 function together in a common mitochondrial homeostasis pathway in which damaged mitochondria are cleared by autophagy (mitophagy)[1,2,3–7].

Parkin is an autoinhibited RBR family E3 ubiquitin ligase [8] consisting of an N-terminal ubiquitin-like (Ubl) domain followed by four Zn^{2+} binding domains RING0, RING1, inbetweenRING (IBR), and RING2 [9]. Parkin is a cytosolic protein activated following mitochondrial stress, mediated by PINK1 phosphorylation of Serine 65 (S65) on ubiquitin. Phosphorylation of ubiquitin enhances binding with Parkin and leads to the recruitment of Parkin to sites of damaged mitochondria [10–12]. On mitochondria, S65 of the Ubl domain of Parkin is phosphorylated by PINK1 [11,13–15], resulting in a fully active Parkin conformation. Fully active Parkin attaches new ubiquitin molecules on mitochondrial proteins, which are phosphorylated by PINK1 to recruit more cytoplasmic Parkin to the mitochondria, thus resulting in a positive feedforward amplification cycle [16]. Ubiquitination of mitochondrial proteins by Parkin also leads to the recruitment of autophagy receptors required for mitophagy [17,18].

Like other RBR-family E3 ligases, Parkin binds to an E2, and ubiquitin is transferred from E2 onto the catalytic C431 residue (on RING2) of Parkin before ubiquitination of lysines on target substrates [19,20]. On Parkin, several elements are present that maintain autoinhibited conformation of Parkin. The E2 binding site on RING1 is blocked by the Ubl domain and the short repressor (REP) element. Furthermore, C431 on RING2 is occluded by the RING0 domain of Parkin, which inhibits Parkin activity [8–21–23]. The phospho-Ubl domain binds within a basic patch (comprising K161, R163, and K211) on RING0 and displaces RING2 to expose C431 to activate Parkin [24]. In the structure of phospho-Parkin with RING2 removed, an activating element (ACT, 101–109), which is present in the linker region (77–140) between Ubl and RING0 domains, binds on the RING0 interface [24]. Mutations in the ACT are shown to affect Parkin activity negatively [24], suggesting their importance in Parkin regulation. Phospho-ubiquitin (pUb) binds in a pocket between RINGO and RING1, and activates Parkin allosterically [25–27]. pUb binding results in the displacement of the IBR domain, and the straightening of helix-1 of the RING1 domain [28]. Massive domain rearrangements have been proposed in the active state to allow the transfer of donor ubiquitin (bound between helix-1 and IBR) from E2 (on RING1) to C431 (on RING2) of Parkin [24,28,29,30].

Several crystal structures of Parkin were solved in the last decade using various truncations in Parkin, which revealed new insights into the conformational changes during the intricate activation process of Parkin (**Extended Data Fig. 1A**). A few years ago, using the structure of truncated phospho-Parkin (RING2 removed) (**Extended Data Fig. 1A**), a model of phospho-Parkin was proposed wherein RING2 would be displaced from RING0 to occupy a pocket near the IBR domain (**Extended Data Fig. 1B**) [24,29]. However, the extent of conformational changes and domain rearrangements due to different regulatory elements of Parkin in the active state remains elusive. For example, it is not clear how and by what mechanism the displaced pUbl from RING1 would be recognized on RING0 in the cis molecule (as per the proposed model in **Extended Data Fig. 1B**) and not in the trans molecule, especially considering the likelihood of an encounter with a trans molecule in the crowded molecular environment. Previous cellular data coexpressing WT-Parkin and mutant Parkin constructs suggested the self-association of Parkin molecules after PINK1 activation at sites of damaged mitochondria [31]. However, a role for

phospho-ubiquitin-mediated recruitment of mutant Parkin, induced by co-expressed wild-type Parkin, could not be excluded. Furthermore, structural studies to understand the Parkin activation mechanism in the last decade have not captured any dimerization of Parkin *in vitro*[19–30].

Herein, using x-ray crystal structures, biophysical methods, and *in vitro* assays, we demonstrate the trans conformational changes in Parkin during the activation process, revealing novel insights into the Parkin activation mechanism. Our data suggest that the phospho-Ubl (pUbl) domain transiently binds to the basic patch on RING0 and competes with the RING2 domain. In addition to the previous observation that pUbl binding results in RING2 displacement, our new data show that the presence of RING2 restricts the binding of pUbl with the Parkin core, which establishes the competitive mode of interaction between RING2 and pUbl. The crystal structure of pUbl-linker (1–140) depleted Parkin (141–465)-pUb complex and supporting data show that RING2 is displaced transiently during the activation process and returns to its closed state after the removal of the pUbl domain from phospho-Parkin, suggesting dynamic nature of conformational changes during Parkin activation. Furthermore, we report Parkin dimerization, mediated by interactions between pUbl and the basic patch on the RING0 domain in trans. We also demonstrate that phospho-Parkin activates autoinhibited Parkin in trans, suggesting an additional feedforward mechanism of Parkin activation. Our data also reveals new insights into the regulation mediated by the ACT of Parkin, wherein the ACT is required for maintaining the enzyme kinetics. We show that similar to phospho-Ubl, ACT can also work in trans, though ACT is more efficient in cis. Furthermore, using x-ray crystallography and supporting experiments, we have characterized a new donor ubiquitin binding site in the linker region (408–415) between the REP element and RING2, which plays a crucial role in Parkin activity.

Results

Incorporation of molecular scissors to capture intricate dynamic conformations on Parkin

Previous studies using various biophysical methods showed that upon phosphorylation of the Ubl domain of Parkin, phospho-Ubl (pUbl) does not interact with the core of Parkin, lacking the Ubl domain [25–27]. However, the crystal structure of phospho-Parkin missing the RING2 (1–382) showed pUbl domain bound to the basic patch (K161, R163, K211) on the RING0 domain [24, 29]. RING2 shared a large surface with RING0, and the superimposition of phospho-Parkin (1–382, PDB 6GLC) and WT-Parkin (PDB 5C1Z) structures showed steric clashes between RING2, ACT, and pUbl (**Fig. 1A**). Therefore, we hypothesized whether the RING2 domain competes with the pUbl domain and thus blocks the interaction of pUbl with RING0. The latter hypothesis would also explain why previous attempts to study pUbl interactions show weak or no interactions between pUbl and Parkin in trans.

To capture crystal structures of protein-protein complexes, researchers use fusion constructs to allow the expression of two proteins in a single polypeptide chain. The fusion method increases the effective net concentration of two proteins in solution compared to mixing two proteins separately, thus stabilizing the interactions between two proteins. Earlier binding assays on Parkin failed to capture interactions in trans, and we speculated that this might be due to the lower net concentration of the domain in trans compared to the high net concentration of the fused domain. We hypothesized that untethering (cleavage of peptide bond) upon protease treatment would solve the above problem and enable us to capture the binding in trans using biophysical methods. To understand the above intricate mechanism, we introduced molecular scissors (human rhinovirus type 3C (HRV 3C) protease and tobacco etch virus (TEV) protease on Parkin constructs (**Fig. 1B**) to analyze the Ubl and RING2 domain rearrangements under native or phosphorylated

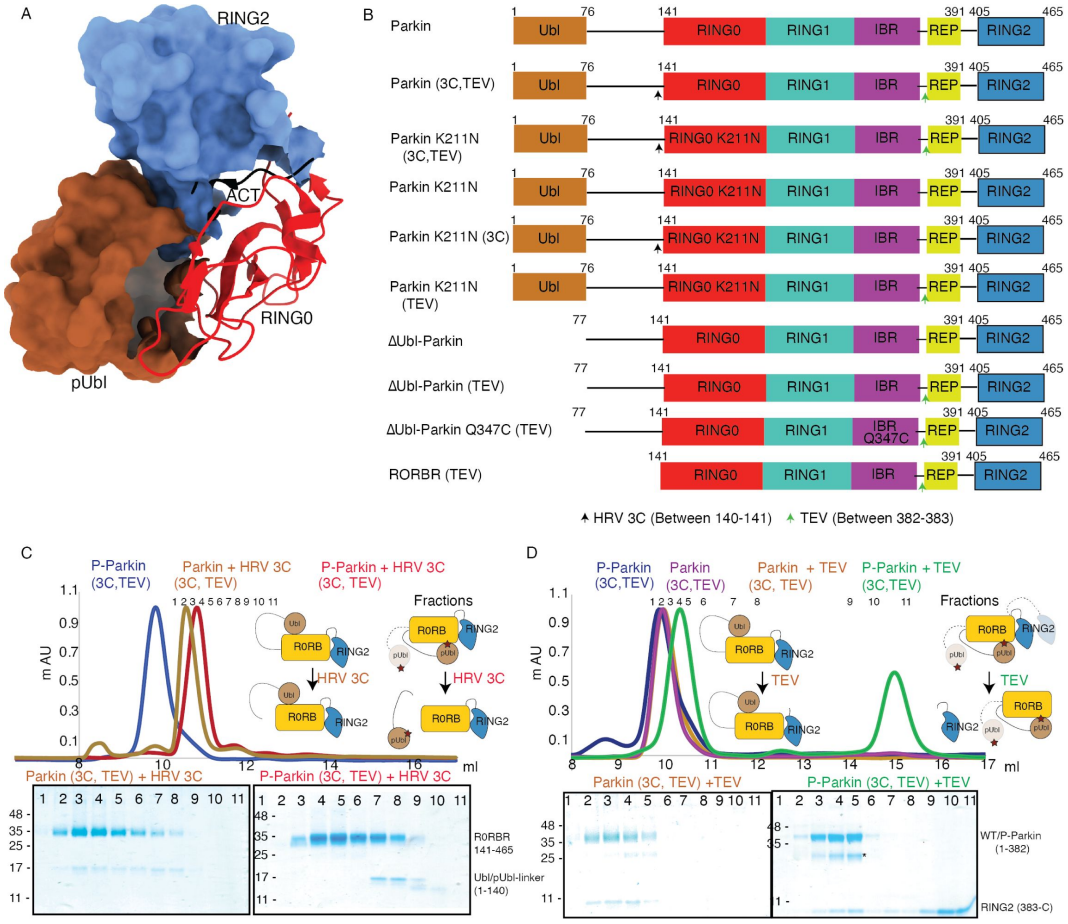

Figure 1

Incorporation of molecular scissors to study dynamic conformation upon Parkin phosphorylation

A. Superimposition of WT-Parkin (PDB 5C1Z) and phospho-Parkin (PDB 6GLC) structures. RING2 (blue), pUbl (brown), RING0 (red), and ACT (black) are shown. For clarity, other Parkin domains are not included.
B. Schematic representation of Parkin domains and various constructs used in this study. HRV 3C and TEV sites incorporated in the Parkin construct are marked with black and green arrows, respectively.
C. Size-exclusion chromatography (SEC) assay shows the binding/displacement of Ubl-linker (1–140) under native or phosphorylated conditions. A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown in the lower panel. A schematic representation is used to explain SEC data.
D. Size-exclusion chromatography (SEC) assay shows binding/displacement of RING2 (383–465) under native or phosphorylated conditions. Coomassie-stained gels of indicated peaks are shown in the lower panel. TEV as contamination is indicated (*).

states. We introduced HRV 3C (between 140th -141st residue) or TEV (382nd -383rd) sites in the loop regions of Parkin (**Fig. 1B**) to avoid any artifacts due to perturbations in native interactions on protein.

First, we tested the ubiquitination activity of Parkin (3C, TEV)to ensure that the inclusion of protease sites did not affect the protein folding or function, which is confirmed by the similar activity of Parkin (3C, TEV) as of the native Parkin construct (**Extended Data Fig. 2**). Furthermore, we noticed that Ubl-linker (1140) co-elute with R0RBR (141–465) in native Parkin (3C, TEV) after treatment with 3C protease, suggesting a stronger interaction between Ubl and the Parkin core (**Fig. 1C**). However, in phosphorylated Parkin (3C, TEV) treated with 3C, pUbl-linker (1–140) did not form a complex with R0RBR (141–465), suggesting a poor/no interaction between phospho-Ubl with the core of Parkin (**Fig. 1C**). Furthermore, in native Parkin (3C, TEV) treated with TEV, RING2 (383–465) co-eluted with Parkin (1–382), suggesting a stronger interaction between RING2 and the Parkin core in the native Parkin (**Fig. 1D**). However, in phospho-Parkin (3C, TEV) treated with TEV, RING2 (383–465) eluted separately from the Parkin (1–382), suggesting that phosphorylation of the Ubl domain results in the displacement of the RING2 domain (**Fig. 1D**). All the above data confirmed that inclusion of molecular scissors on Parkin constructs do not affect Parkin folding. Previous observations that phosphorylation of Ubl weakens Ubl and Parkin interaction, and displacement of RING2 in phospho-Parkin, were validated using our assay. Also, respective proteases only cleaved (untethered) the peptide bond without affecting the native interactions between Parkin domains.

Phospho-Ubl domain and RING2 domain have a competitive mode of binding on RING0 domain

Previous models of Parkin activation suggested permanent displacement of RING2 after Ubl phosphorylation (**Extended Data Fig. 1B**). We wanted to test whether RING2 and pUbl affect the binding of each other on Parkin, which would suggest a competitive binding mode between pUbl and RING2 on the RING0 domain, and a dynamic displaced or bound states of pUbl and RING2. To test the competitive mode of binding between pUbl and RING2 on RING0, and thus affecting the binding of each other, we performed the SEC assay after sequential treatment with HRV 3C and TEV on Parkin (3C, TEV). Interestingly, pUbl-linker (1–140) co-eluted with Parkin core (141–382) upon 3C treatment on fractions that were collected after TEV treatment on phospho-Parkin (3C, TEV) which led to displacement of RING2 (383–465) (**Fig. 2A**). Similarly, RING2 (383–465) co-eluted with Parkin core (141–382) upon TEV treatment on fractions that were collected after 3C treatment on phospho-Parkin (3C, TEV) which led to displacement of pUbl-linker (1–140) (**Fig. 2B**). This data confirmed that pUbl and RING2 competitively bind on RING0. The binding of one negatively affected the binding of the other, unlike previous observations, which only showed phosphorylation of Ubl leading to RING2 displacement.

Our data in **Fig. 2B** suggested dynamic displacement of RING2 as untethering of RING2 after pUbl wash-off resulted in stabilization of interactions between RING2 and Parkin core. To further confirm, we crystallized the phospho-Parkin (3C, TEV)-pUb complex after treatment with 3C protease. Treatment with 3C led to displacement of the pUbl-linker (1–140) from the Parkin core (141–465). The overall structure of pUbl-linker (1–140) depleted Parkin (141–465)-pUb complex was determined at 3.3 Å (**Extended Data Table 1**), and showed similar conformation as seen in previously solved structures of Parkin in autoinhibited state (**Fig. 2C**). The crystal structure showed RING2 bound to RING0, which confirmed that RING2 was only transiently displaced from the RING0 domain in phospho-Parkin and returned to its original position after removal of pUbl-linker (**Fig. 2C**), further confirming our SEC data (**Fig. 2B**). The crystal structure also revealed that the REP element is bound to the RING1, similar to the autoinhibited state of Parkin (**Fig. 2C**). Phospho-ubiquitin was bound to the basic patch between RING0 and RING1, and resulted in similar conformational changes in IBR and helix (connecting RING1-IBR domains) (**Fig. 2C**). In the asymmetric unit, two molecules of Parkin bound to pUb were seen; however, in one of the

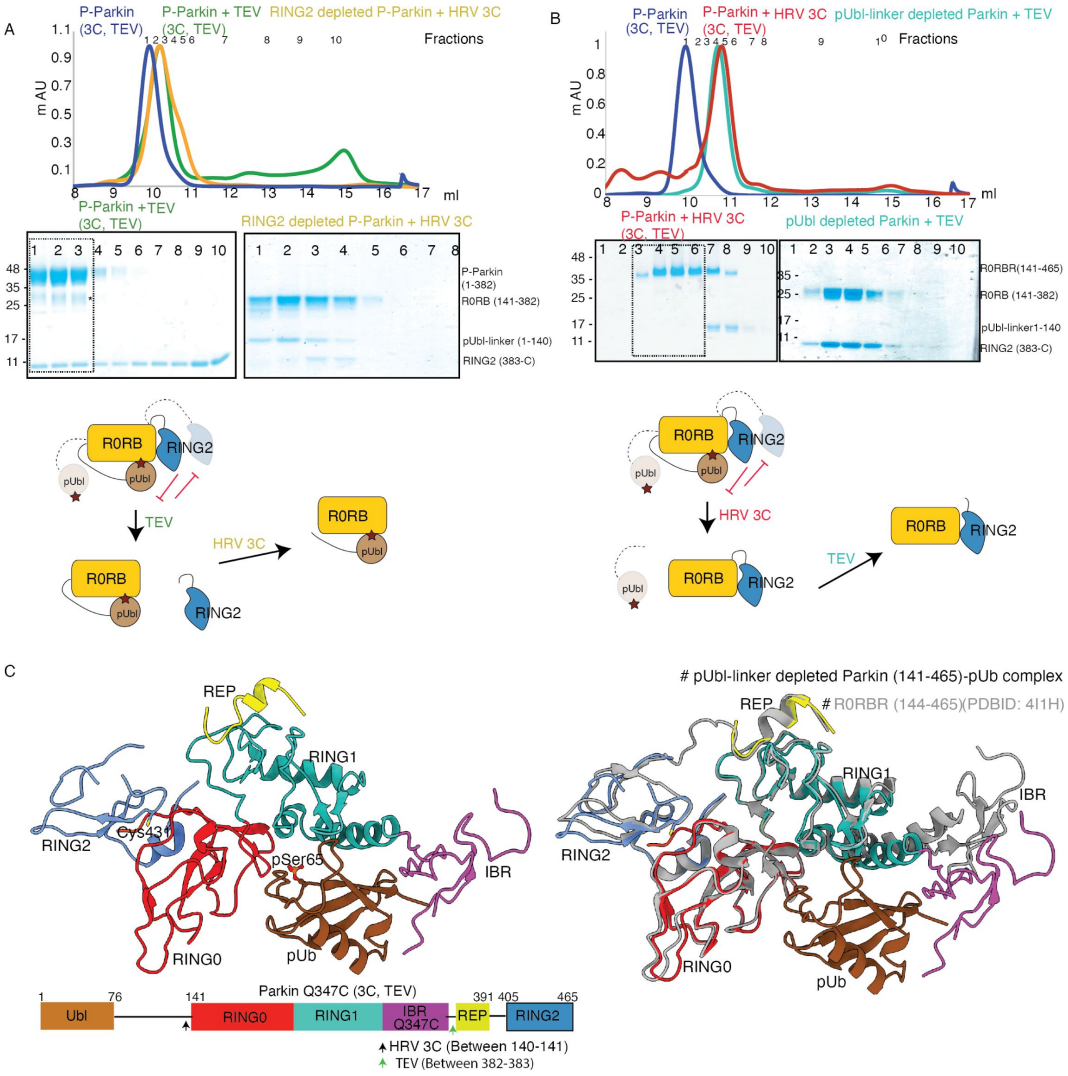

Figure 2

Characterization of competing mode of binding between pUbl and RING2

A. SEC assay shows depletion of RING2 (383–465) from phospho-Parkin stabilize pUbl-linker (1–140) binding with Parkin (141–382) after treatment with 3C protease. Fractions that were pooled for subsequent proteolysis are highlighted in the box.
B. SEC assay shows depletion of pUbl-linker (1–140) from phospho-Parkin stabilize RING2 (383–465) binding with Parkin (R0RB, 141–382) after treatment with TEV protease. Fractions that were pooled for subsequent proteolysis are highlighted in the box.
C. Crystal structure of pUbl-linker (1–140) depleted Parkin (141–465) complex with pUb (brown). Different domains of Parkin are colored, as shown in the left panel. Catalytic C431 is highlighted. Structure of pUbl-linker (1–140) depleted Parkin (141–465)-pUb complex (colored as in the left panel) is superimposed with R0RBR structure (PDB 4I1H, grey) in the right panel. A schematic representation of the Parkin Q347C (3C, TEV) construct used for crystallization is shown at the bottom.

Parkin molecules, no density was observed in the IBR region (**Extended Data Fig. 3**). Overall, this data suggested that pUbl and RING2 exist in a dynamic state in phospho-Parkin (pUbl binding<->RING2 open<->pUbl displaced<->RING2 closed), and compete for binding on RING0.

K211N mutation on Parkin perturbs RING2 displacement, not pUbl displacement

As phosphorylation of Ubl resulted in displacement of pUbl from Parkin core (**Fig. 1C**), we wondered whether interactions between pUbl and the basic patch (comprising K161, R163, and K211) on RING0 played a key role in pUbl displacement from RING1. Interestingly, similar to phospho-Parkin (3C, TEV) (**Fig. 2C**), pUbl-linker (1–140) remained flexible in phospho-Parkin K211N (3C, TEV) and eluted separately from Parkin core (141–465) on SEC (**Fig. 3A**). This data suggests that binding of pUbl with the basic patch on RING0 domain may not be the driving force for pUbl displacement. Further, to confirm that displacement of the RING2 domain is mediated by pUbl binding in the basic patch (K161, R163, and K211) on the RING0 domain, we tested the RING2 displacement using phospho-Parkin K211N (3C, TEV). N211 resulted in stabilization of the RING2 (383–465) domain on phospho-Parkin K211N (1–382) upon TEV treatment, and the two fragments co-eluted on SEC (**Fig. 3A**). Although pUbl was displaced in phospho-Parkin K211N, Parkin activity was drastically reduced (**Fig. 3B**), suggesting RING2 displacement, not Ubl displacement, is a major cause of Parkin activation. We also noticed a basal level of Parkin activity in the lanes without any activator (pUb), which was reduced in the Parkin K211N mutant (**Fig. 3B**). To understand the conformational changes upon mutation in the basic patch on RING0, we also crystallized phospho-Parkin R163D/K211N/Q347C (3C)-pUb complex after treatment with 3C protease, which washed off pUbl-linker (1–140) from Parkin core (141–465). This complex resulted in better crystals diffracting up to 2.35 Å. The overall structure of the pUbl-linker (1–140) depleted Parkin R163D/K211N/Q347C (141–465)-pUb complex (hereafter R0RBR R163D/K211N-pUb complex) was similar to the autoinhibited structure wherein RING2 was bound on RING0 and REP element was bound on RING1 (**Fig. 3C**).

Untethering of the linker connecting IBR and RING2 allows pUbl binding in trans

We next investigated whether the competitive binding between pUbl and RING2 to the RING0 could explain previous reports [19–30] observing the lack of interaction between pUbl and Parkin (lacking Ubl domain) in trans. To test this, we used phospho-Parkin K211N, which would not allow the binding of pUbl in the RING0 pocket of the same molecule, and tested its interaction with AUbl-Parkin. However, no complex formation between phospho-Parkin K211N and AUbl-Parkin was seen on SEC (**Fig. 4A**). We next validated this finding using isothermal titration calorimetry (ITC), which did not show any detectable interaction between phospho-Parkin K211N and AUbl-Parkin (**Fig. 4A**), consistent with previously published reports.

As our data suggested that the fused domain outcompetes the untethered domain (**Fig 2**), we wondered whether this may explain the lack of detectable binding in trans. To test this, we used AUbl-Parkin (TEV) treated with TEV as acceptor Parkin, which overcomes the problem of higher net concentration of the fused competing RING2 domain. Acceptor AUbl-Parkin (TEV) was treated with TEV, and TEV was removed using an affinity column followed by SEC. SEC showed co-elution of ΔUbl-Parkin (77–382) and RING2 (383–465), confirming that TEV cleaved (untethered) the peptide bond (connecting IBR and REPRING2) without affecting the native interactions between ΔUbl-Parkin (77–382) and RING2 (383–465) (**Fig 4B**). Incubation of phospho-Parkin K211N with untethered ΔUbl-Parkin (TEV) led to the displacement of RING2 (383–465) from ΔUbl-Parkin (77–382), and a stable trans complex between phospho-Parkin K211N and ΔUbl-Parkin (77–382) by SEC analysis (**Fig. 4B**). The ITC showed a strong affinity (K_d = 1.1 ± 0.3 μM) between phospho-Parkin K211N and untethered ΔUbl-Parkin (TEV) (**Fig. 4B**), which further supported the SEC data.

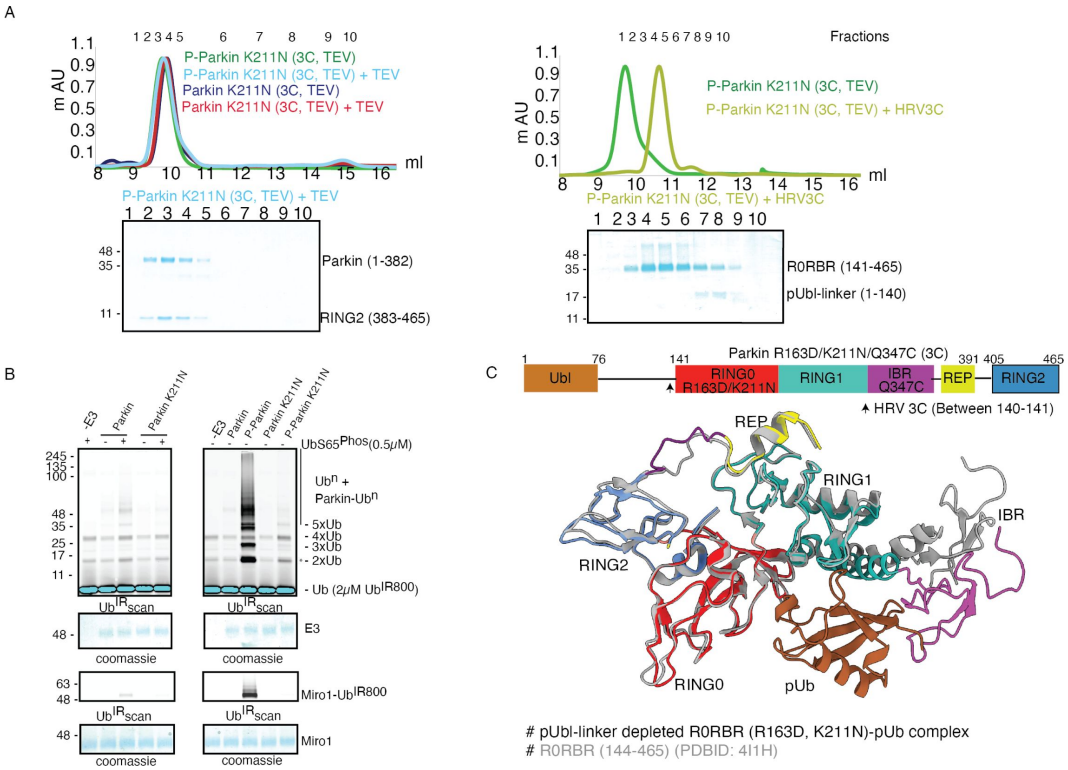

Figure 3

K211N mutation affects RING2 displacement, not pUbl

A. Size-exclusion chromatography (SEC) assay to test the displacement of RING2 (left panel) or pUbl-linker (right panel) after phosphorylation of Parkin K211N (3C, TEV).
B. Ubiquitination assay to test the activity of Parkin K211N in the presence of pUb or using phosphoParkin K211N. The middle panel shows a Coomassie-stained loading control. A non-specific, ATP-independent band is indicated (*). The lower panel shows Miro1 ubiquitination for the respective proteins in the upper lane. Coomassie-stained gel showing Miro1 is used as the loading control of substrate ubiquitination assay.
C. Crystal structure of pUbl-linker (1–140) depleted R0RBR (R163D/K211N)-pUb complex. The superimposed apo R0RBR structure (PDB 4I1H) is shown in grey. A schematic representation of the Parkin R163D/K211N/Q347C (3C) construct used for crystallization is shown at the top.

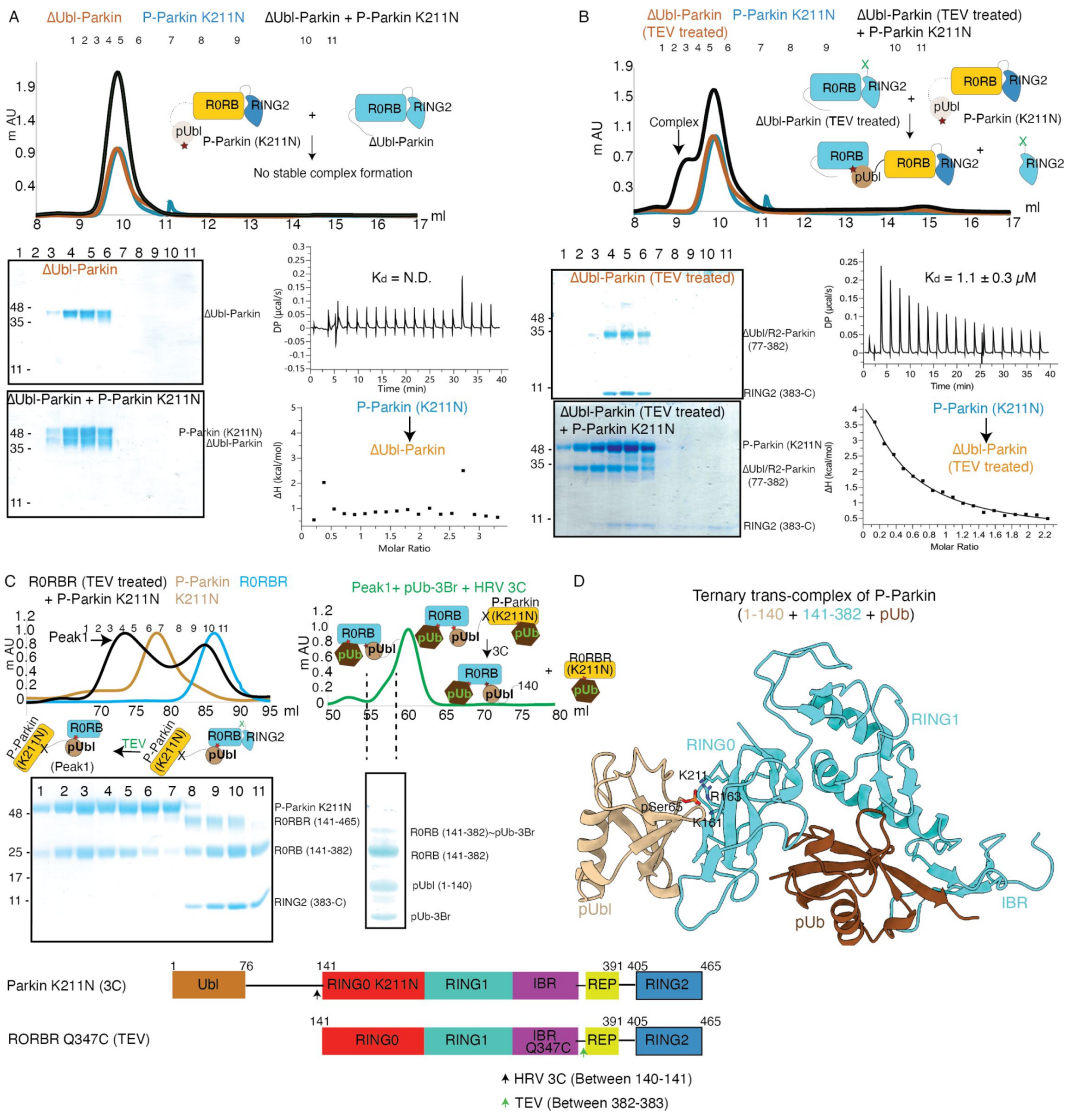

Figure 4

Untethering of the linker between IBR-RING2 allows Parkin and phospho-Ubl interaction in trans

A. Binding assay between phospho-Parkin K211N and AUbl-Parkin. A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown in the lower panel. A schematic representation is used to explain SEC data. Isothermal Titration Calorimetry assay between phosphoParkin K211N and AUbl-Parkin is shown in the lower panel. N.D. stands for not determined.

B. Binding assay between phospho-Parkin K211N and untethered AUbl-Parkin (TEV). A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown in the lower panel. A schematic representation is used to explain SEC data. Isothermal Titration Calorimetry assay between phospho-Parkin K211N and untethered ΔUbl-Parkin (TEV) is shown in the lower panel. The dissociation constant (K_d) is shown.

C. SEC assay to test binding between untethered R0RBR Q347C (TEV) and phospho-Parkin K211N (3C), and displacement of RING2 (383–465) from R0RBR, the left panel. The peak1 (black) containing R0RB (141–382) and phospho-Parkin K211N complex was incubated with pUb-3Br, followed by HRV 3C protease, to purify ternary trans-complex of phospho-Parkin (1–140 + 141–382 + pUb) on SEC, the right panel. The concentrated fractions from the shoulder (highlighted with a dashed line) of the peak in the right panel were loaded on SDS PAGE to confirm complex formation. A schematic representation of the Parkin constructs used for crystallization is shown at the bottom.

D. Crystal structure of the trans-complex of phospho-Parkin with pUb (brown) shows phospho-Ubl domain (wheat) bound to RING0 (cyan) domain of Parkin (cyan).

Further, to confirm that untethering does not affect the native interactions between RING2 and RING0 domains, we purified and determined the structure of untethered R0RBR (TEV) Parkin (**Extended Data Fig. 4A**). Co-elution of R0RB (141–382) and RING2 (383–465) fragments on SEC (**Extended Data Fig. 4B**) and crystal structure analysis showing intact native interactions between RING2 and RING0 (**Extended Data Fig. 4C**) excluded the possibility of artifact.

To understand the molecular details of the complex observed in **Figure 4B**, we used Parkin K211N (3C) as a donor of pUbl-linker (1–140) and R0RBR Q347C (TEV) Parkin as an acceptor of pUbl-linker (1–140). Phospho-Parkin K211N (3C) formed a stable complex with untethered R0RBR Q347C (TEV), and RING2 (383–465) was removed from R0RBR Q347C (TEV) (**Fig. 4C**). The fractions containing the complex of phospho-Parkin K211N and R0RB (141–382) upon treatment with 3C protease followed by incubation with pUb-3Br showed co-elution of components of the ternary trans-complex (R0RB (141–382), pUbl-linker (1–140), and pUb) on SEC (**Fig. 4C**). The crystal structure of the ternary trans-complex of phosphoParkin (pUbl-linker (1–140) + R0RB (141–382) + pUb) was solved at 1.92 Å (**Extended Data Table 1**) which further confirmed trans-complex formation between Parkin molecules (**Fig. 4D**, **Extended Data Fig. 5**). In the crystal structure, the pUbl domain from the donor molecule (phospho-Parkin K211N (3C)) was bound to the basic patch of RING0 on the acceptor molecule (untethered R0RBR (TEV)) (**Fig. 4D**) in trans. The conformation observed in the trans complex was similar to phospho-Parkin (1–382) structure with fused pUbl domain and untethered/truncated RING2 in a cis molecule [24,29]. Interestingly, the linker connecting pUbl and RING0 remained disordered in all the structures [24,29]. Therefore, it would be difficult to say whether, in the previous cis structures, the pUbl bound to RING0 was from the same molecule or different molecules. Moreover, the fusion of pUbl with RING0 and untethering/truncation of RING2, as in the earlier structures [24,29], could favor pUbl binding with RING0 in cis. Our data established that keeping pUbl and RING2 untethered from their binding partner RING0, thus reducing the artifact due to the higher net concentration of the fused domain with RING0, is ideal for measuring trans interactions using biophysical methods.

Phospho-Parkin activates native Parkin in trans

As the pUbl domain remained dynamic in both native phospho-Parkin and phospho-Parkin K211N (**Fig. 1C**, **Fig. 3A**), we wondered whether a trans-complex was formed between native phospho-Parkin. The latter could also be helpful in the context of activation of various Parkin isoforms lacking either the Ubl domain or RING2 domain (**Extended Data Fig. 6**). To test trans complex formation between native Parkin molecules, we used native phospho-Parkin (1–465) as a pUbl donor and untethered (processed with TEV protease) AUbl-Parkin (TEV) as a pUbl acceptor on RING0. Interestingly, phospho-Parkin formed a stable complex with AUbl-Parkin (77–382) and RING2 (383–465) was displaced from untethered ΔUbl-Parkin (TEV) (**Fig. 5A**, **Extended Data Fig. 7A**), similar to the interaction between phospho-Parkin K211N and untethered ΔUbl-Parkin (TEV) (**Fig. 4B**). The SEC data was confirmed by ITC measurements showing similar affinities between phospho-Parkin or phospho-Parkin K211N and untethered ΔUbl-Parkin (TEV) (**Fig. 5B**, **Fig. 4B**).

We further tested the binding of phospho-Parkin with untethered WT-Parkin (TEV). Similar to untethered ΔUbl-Parkin (TEV), untethered WT-Parkin (TEV) formed a complex with phospho-Parkin, and resulted in the removal of RING2 (383–465) from WT-Parkin (1–382) (**Fig. 5C**, **Extended Data Fig. 7B**). However, unlike untethered WT-Parkin (TEV), untethered Parkin K211N (TEV) failed to form the complex with phospho-Parkin (**Fig. 5C**, **Extended Data Fig. 7C**). This latter findingconfirmed that interactions between pUbl and the basic patch on the RING0 domain form a trans-complex. To further validate this, we also confirmed complex formation using SEC-MALS (size-exclusion chromatography coupled with multi-angle light scattering). MALS analysis further confirmed complex (Phospho-Parkin and WT-Parkin (1–382), Observed M. W. = 94 ± 3 Kda) formation between phospho-Parkin (Observed M. W. = 53 ± 2 Kda) and untethered WT-Parkin (TEV) (Observed M. W. = 52 ± 3 Kda) (**Fig 5D**, **Extended Data Fig. 7B**).

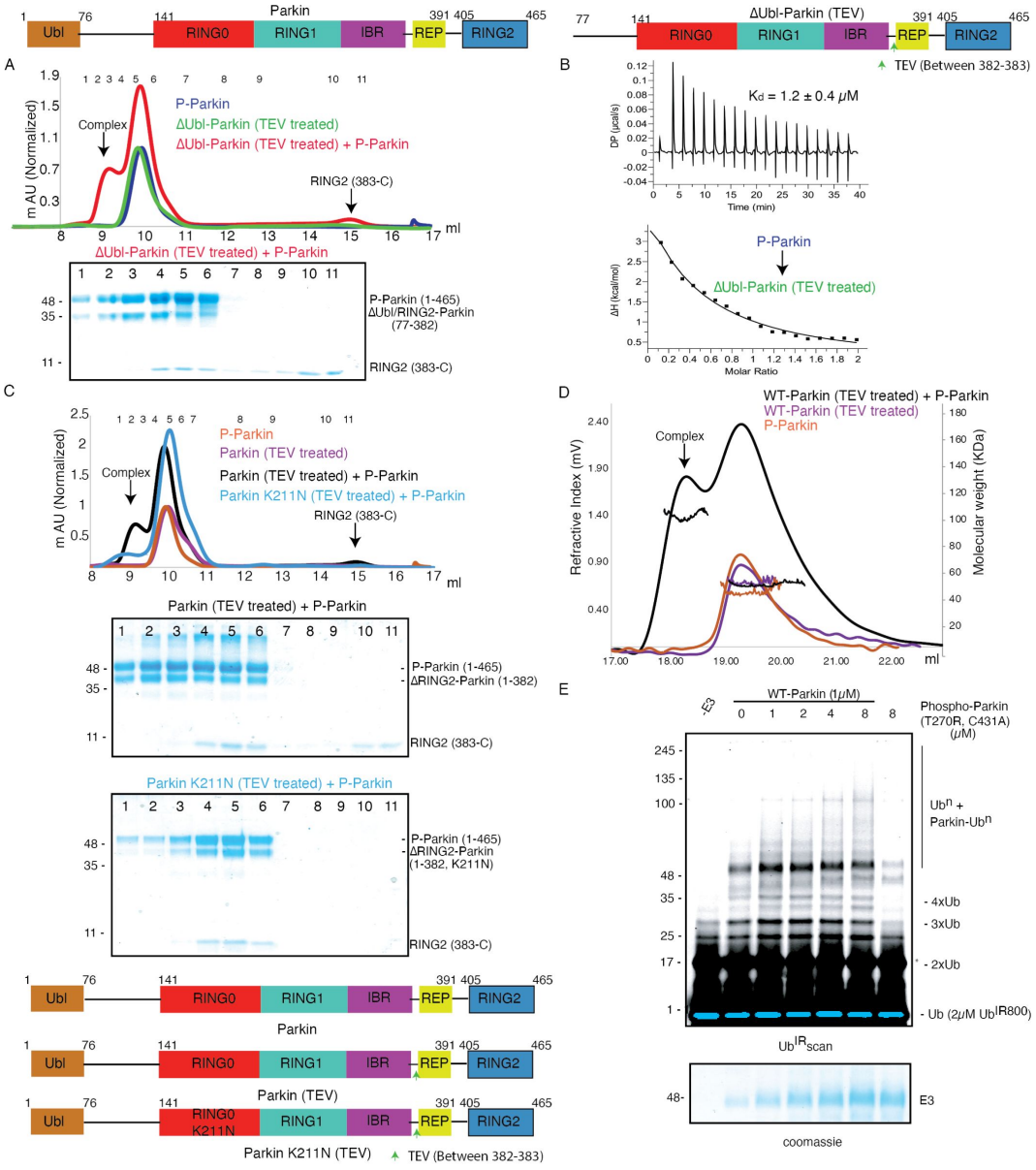

Figure 5

Parkin dimerization and trans-activation of native Parkin are mediated by phosphorylation of the Ubl domain of Parkin

A. SEC assay between phospho-Parkin and untethered AUbl-Parkin. A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown in the lower panel. TEV protein contamination is indicated (*). A schematic representation of the Parkin constructs used for experiments in panels A and B is shown at the top.
B. Isothermal Titration Calorimetry assay between phospho-Parkin and untethered ΔUbl-Parkin (TEV). The dissociation constant (K_d) is shown.
C. SEC assay between phospho-Parkin and untethered WT-Parkin (TEV) (upper panel) or untethered Parkin K211N (TEV) (lower panel). A schematic representation of the Parkin constructs used for experiments in panels C and D is shown at the bottom.
D. SEC-MALS assay to confirm the complex formation between untethered WT-Parkin (TEV) and phospho-Parkin.
E. Ubiquitination assays to check the WT-Parkin activation (right panel) with increasing concentrations of phospho-Parkin T270R/C431A. A non-specific, ATP-independent band is indicated (*). The lower panel shows a Coomassie-stained loading control.

As our binding experiments suggested interaction between phosphorylated Parkin and native Parkin, we next checked whether phosphorylated Parkin can activate native Parkin. To test phospho-Parkin mediated Parkin activation in trans, we used a catalytic-inactive version of phospho-Parkin T270R/C431A with mutations in both the E2 binding site (T270R) and catalytic site (C431A).

Interestingly, we observed that WT-Parkin ubiquitination/autoubiquitination activity was increased with increasing concentrations of phospho-Parkin T270R/C431A (**Fig. 5E**). Although, we were not expecting activation of WT-Parkin by phospho-Parkin as Ubl of WT-Parkin would block the E2 binding site on RING1 in WT-Parkin, activation of WT-Parkin with phospho-Parkin T270R/C431A suggested that a significant inhibition on Parkin is mediated by RING0 blocking RING2, which was released upon pUbl binding.

Further, we wondered whether pUbl would enhance Parkin phosphorylation similar to pUb [32]. To test this, we checked Parkin phosphorylation by PINK1 in the presence of pUbl or pUb. However, unlike pUb, pUbl did not enhance Parkin phosphorylation by PINK1 (**Extended Data Fig. 7D**), confirming that pUbl and pUb binding lead to unique conformational changes in Parkin. Overall, this data demonstrates pUbl-mediated dimerization of Parkin molecules leading to Parkin activation in trans.

Assessment of Parkin activation in cells

It has previously been reported that pUb may interact with the RING0 domain of Parkin and that loss of this interaction underlies loss of Parkin recruitment to the mitochondria in cells expressing Parkin K211N [39]. However, we recently showed that pUb does not bind in the RING0 pocket (comprising K161, R163, and K211), and pUb binds specifically in the RING1 pocket (comprising K151, R305, and H302) [33], unlike phospho-Ubl binding in the RING0 pocket and displacing RING2 in trans (**Fig. 5**). Biophysical assays also revealed that unlike the tight binding of pUb in the RING1, pUbl binding in the RING0 pocket was very transient. Furthermore, K211N mutation in the RING0 pocket resulted in loss of Parkin activity by both loss of pUbl-mediated interactions (**Fig 5**) and by N211-driven conformational changes leading to loss of Parkin activity independent of pUb binding [33]. This loss of Parkin activity would lead to a reduced amount of pUb, resulting in loss of Parkin recruitment to mitochondria. Therefore, we decided to test an activity-independent Parkin recruitment to impaired mitochondria using a Parkin translocation assay in HeLa cells [10,14–16,31]. Consistent with previous studies, [10,14–16,31] full-length wild-type but not catalyticinactive GFP-Parkin C431F was recruited to mitochondria following carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment (**Extended Data Fig. 8A-B**). Similarly, we did not observe the recruitment of GFP-Parkin C431F/H302A or GFP-Parkin C431F/K211N mutants to impaired mitochondria when expressed alone (**Extended Data Fig. 8A-B**).

We observed that co-expression of mCherry-tagged-Parkin WT with GFP-Parkin C431F enabled GFP-Parkin C431F recruitment to the mitochondria, similar to a previous study [31] (**Fig. 6A, D**). Under these assay conditions, we strikingly observed that mutation of the pUb binding pocket in the RING1 completely abolished recruitment of the double mutant GFP-Parkin C431F/H302 to the mitochondria when co-expressed with mCherry-tagged-Parkin WT (**Fig. 6B, D**). This excluded a significant role for the RING0 pocket in pUb binding in the context of full-length parkin expressed in cells following mitochondrial damage (**Fig. 6B, D**). In line with this, mutation of the RING0 binding pocket produced a moderate defect in recruitment of the double mutant GFP-Parkin C431F/K211N to the mitochondria when co-expressed with mCherry-tagged-Parkin WT (**Fig. 6C, D**), suggesting that the transient interaction between pUbl and RING0 of Parkin in trans acts in concert with pUb binding to RING1 pocket for optimal Parkin recruitment to sites of mitochondrial damage (**Fig. 6C, D**). Under all transfection conditions, we did not observe a significant difference in mCherry-tagged Parkin WT (**Extended Fig. 8C**). Furthermore, co-expression of GFP-Parkin C431F or GFP-Parkin C431F/K211Nor GFP-Parkin C431F/H302A with the non-phosphorylatable mCherry-tagged-Parkin S65A failed to rescue recruitment to the mitochondria

(**Fig. 6A, B, C, D**). These findings were in line with our biophysical data and highlight the importance of phospho-Ubl domain-mediated interactions in Parkin recruitment to the mitochondria.

ACT improves enzyme kinetics of Parkin

A previous study identified a small region (101–109) in the linker between Ubl and RING0 as an activator element (ACT) required for Parkin activity [24]. To further explore the role of the ACT, we tested whether the omission of ACT affects the binding of Parkin with the charged state of E2 (E2~Ub). We observed a tight complex formation between phospho-Parkin, pUb, and E2~Ub on SEC assay (**Fig. 7A**). Interestingly, deletion of the ACT did not affect the complex formation with E2~Ub, as phospho-Parkin ΔACT co-eluted with pUb and E2~Ub (**Fig. 7A**). As the displacement of RING2 is a crucial process during Parkin activation, we tested whether the removal of the ACT affects the displacement of the RING2 domain using our TEV-based SEC assay. We observed that phospho-Parkin ΔACT (TEV) after treatment with TEV resulted in a shift where RING2 (383–465) was displaced from the Parkin core (1–382, ΔACT), resulting in the elution of two fragments of Parkin separately on SEC (**Fig. 7B**). As the deletion of ACT did not show any functional defect in Parkin, we hypothesized that the presence of ACT at the interface of RING0 and RING2 might affect the dynamic nature of RING2, thereby regulating the enzyme kinetics. To test this hypothesis, we compared the phospho-Parkin ΔACT ubiquitination activity over different time points. We observed that the deletion of ACT slowed the kinetics of Parkin activity, doubling the time for phosphoParkin ΔACT to reach a similar level of activity as phospho-Parkin (**Fig. 7C**).

ACT is more efficient in cis

The ternary trans-complex of phospho-Parkin (1–140 + 141–382 + pUb) structure in this study was solved at a similar resolution and in the same space group as the previously solved structure of phospho-Parkin (1–382) in complex with pUb [24]. In the previous structure of phospho-Parkin (1–382)-pUb complex (PDBID:6GLC), the ACT region was clearly shown to occupy the hydrophobic pocket on RING0 (**Fig. 8A**). However, we did not see any density of the ACT region in the ternary trans-complex structure of phospho-Parkin (1–140 + 141–382 + pUb) (**Fig. 8A**, **Extended Data Fig. 9A**). Interestingly, we observed that in the ternary trans-complex structure of phospho-Parkin, K48 of the pUbl domain occupied the same pocket that R104 of the ACT region occupied in the structure of phospho-Parkin-pUb complex (**Fig. 8A**, **Extended Data Fig. 9A**). Also, the side-chain of K48 of the pUbl domain was disordered in the previous structure of phospho-Parkin (1–382)-pUb complex (**Fig. 8A**).

We wondered whether the lack of density in the ACT region was due to the preference of ACT to remain associated with the cis molecule rather than to be complemented by the trans molecule. To test this hypothesis, we determined the crystal structure of the ternary trans-complex of phospho-Parkin with cis ACT using phospho-Ubl (1–76) and AUbl-Parkin Q347C (TEV). pUbl formed a stable complex with untethered AUbl-Parkin Q347C (TEV) and resulted in the displacement of RING2 (383–465) (**Fig. 8B**). Fractions containing trans-complex of phospho-Parkin (1–76 + 77–382) with cis ACT were mixed with pUb-3Br to get the crystals of the ternary complex. The ternary trans-complex of phospho-Parkin (1–76 + 77–382 + pUb) with cis ACT was crystallized, and structure was determined at 2.6 Å (**Extended Data Table 1**). Interestingly, in the structure of the ternary trans-complex of phospho-Parkin with cis ACT, we could observe the electron density of the ACT region (**Fig. 8C**, **Extended Data Fig. 9B**). Furthermore, K48, which occupied the ACT region in the ternary trans-complex structure of phospho-Parkin with trans ACT, was disordered in the ternary trans-complex structure of phospho-Parkin with cis ACT, similar to what was seen previously in the phospho-Parkin structure (**Fig. 8A, C**, **Extended Data Fig. 9B**).

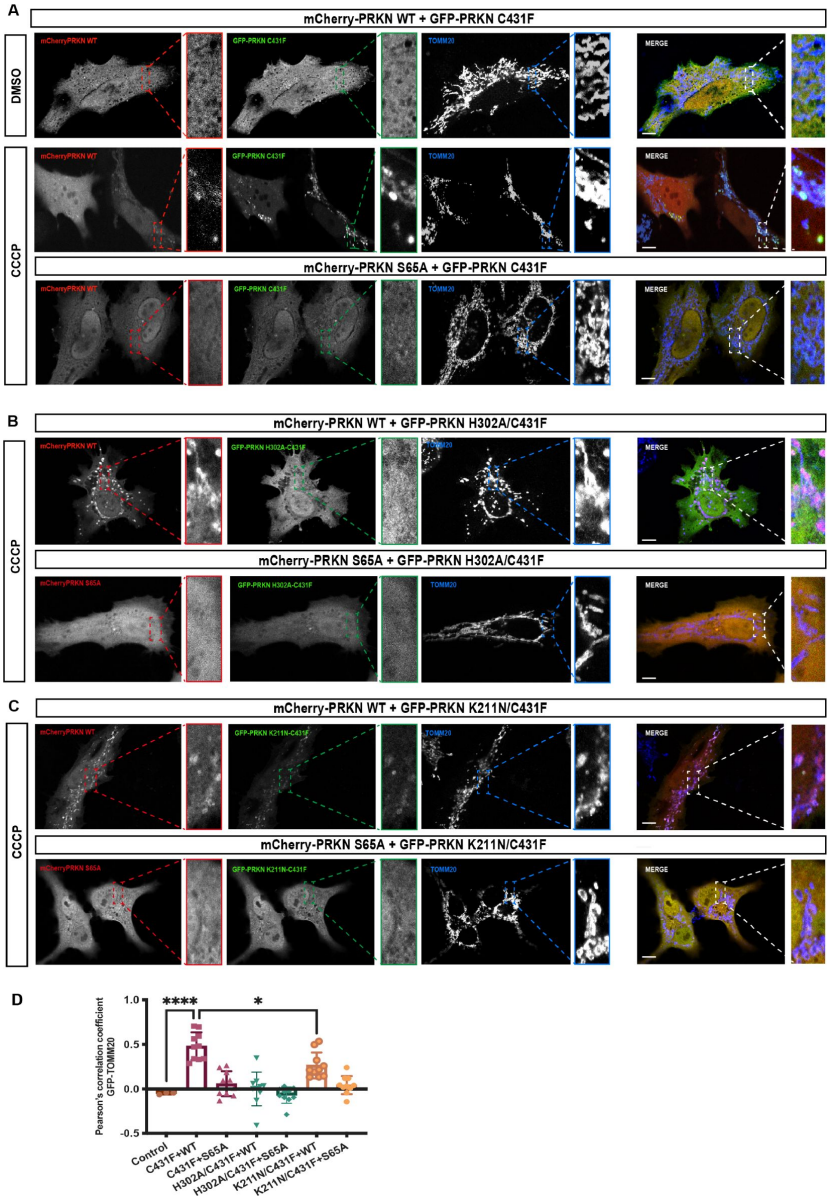

Figure 6

Analysis of Parkin mutant recruitment to mitochondria in HeLa cells

A. Immunofluorescence of HeLa cells co-transfected with either mCherry-Parkin wild-type (WT) or mCherry-Parkin S65A and GFP-Parkin C431F or, B. GFP-Parkin H302A/C431F and, C. GFP-Parkin K211N/C431F. Cells were treated for 1 hour with 10 μM CCCP, and DMSO was used as a control.Mitochondriawere labeled with anti-TOMM20 antibody (blue). Scale bar = 10 μm. D. Quantification of GFP-Parkin (WT and mutants) on mitochondria. The co-localization of GFP-Parkin (WT and mutants) with TOMM20 (mitochondria) was evaluated using Pearson's correlation coefficient. Errors are represented as S.D. Statistical differences in Pearson's correlation coefficient were evaluated using one-way ANOVA and Tukey's multiple comparisons post-test. Statistical significance is as follows: *, $p < 0.05$; ****, $p < 0.0001$.

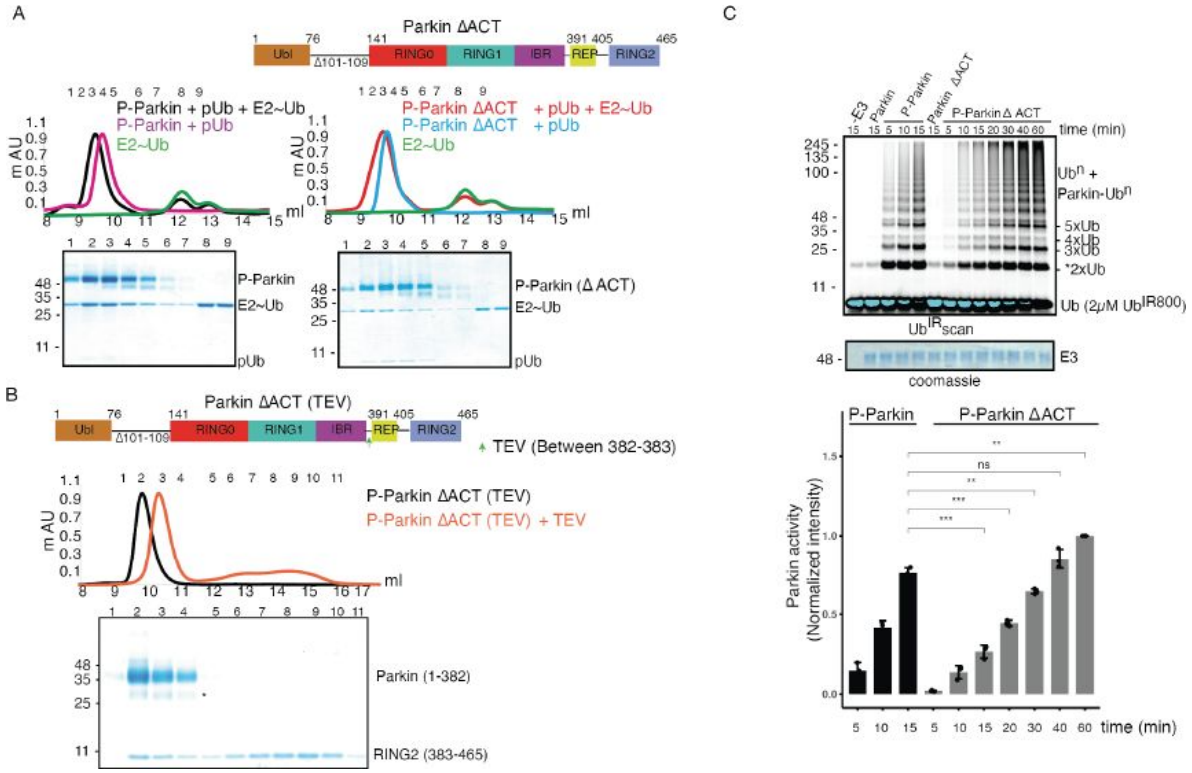

Figure 7

ACT plays a crucial role in enzyme kinetics

A. Size-exclusion chromatography (SEC) assay to test the binding of E2~Ub_{don} with phospho-Parkin (left panel) or phospho-Parkin AACT (right panel). Assays were done using Parkin in complex with pUb. A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown in the lower panel. The upper panel shows a schematic representation of the Parkin ΔACT construct used.

B. Size-exclusion chromatography (SEC) assay to check displacement of the RING2 domain after phosphorylation of Parkin ΔACT. The upper panel shows a schematic representation of the Parkin ΔACT construct used for the RING2 displacement assay. Conformational changes in Parkin, as observed by the SEC experiment, are shown schematically.

C. Ubiquitination assay to check the effect of ACT deletion (ΔACT) on Parkin activity. A non-specific, ATP-independent band is indicated (*). The middle panel shows a Coomassie-stained loading control. In the lower panel, the bar graph shows the integrated intensities of ubiquitin levels from three independent experiments (mean ± s.e.m.). Statistical significance was determined using pair-wise student's t-test (**P < 0.01, ***P < 0.001, ns-nonsignificant).

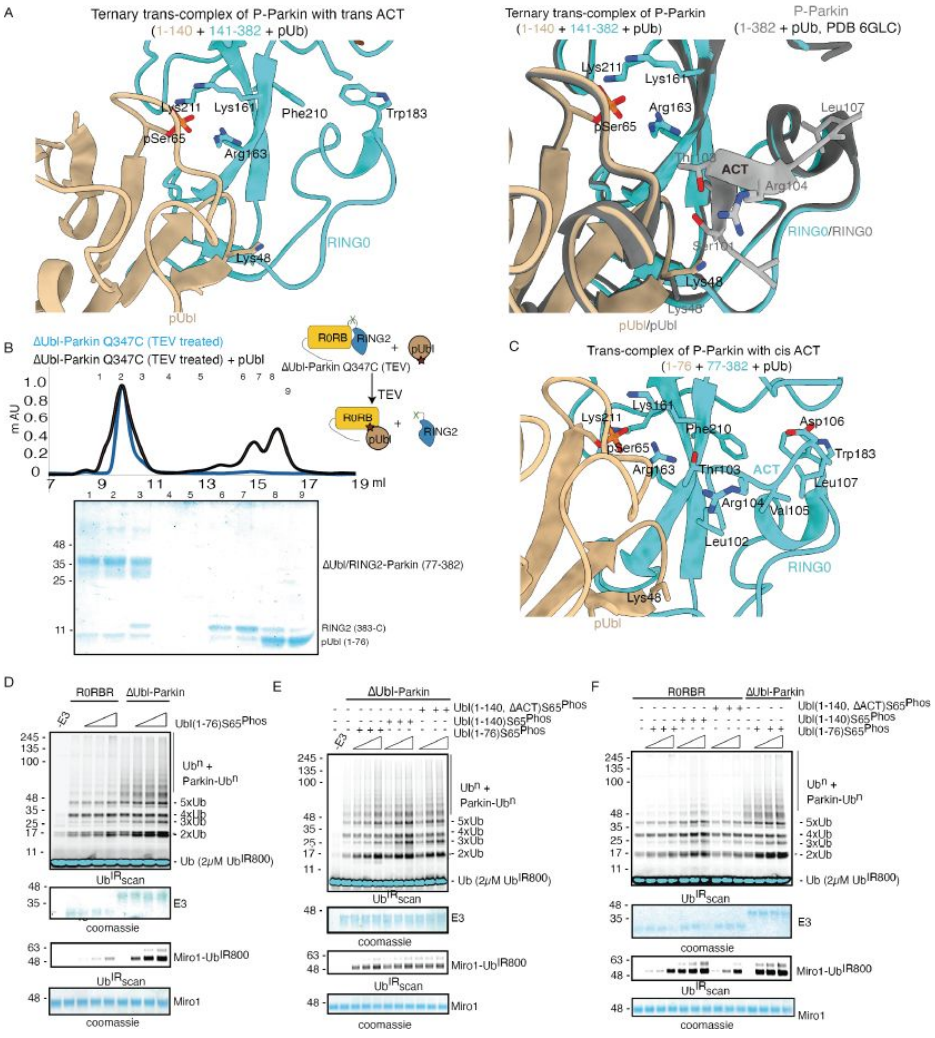

Figure 8

ACT is more efficient in cis

A. Crystal structure of ternary trans-complex of phospho-Parkin with pUb (1–140 + 141–382 + pUb), left panel. pUbl (wheat) and RING0 (cyan) of Parkin are shown. The right panel shows superimposed structures of ternary trans-complex of phospho-Parkin with pUb, colored as the left panel, and the phospho-Parkin complex with pUb (PDB 6GLC) is shown in grey.
B. SEC assay to check the binding between untethered ΔUbl-Parkin (TEV) and phospho-Ubl (1–76). A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown in the lower panel.
C. Crystal structure of ternary trans-complex of phospho-Parkin with cis ACT (1–76 + 77–382 + pUb) shows ACT (cyan) present in the pocket on RING0 (Cyan) and pUbl (wheat) in the vicinity.
D. Comparison of R0RBR and ΔUbl-Parkin activation using the increasing concentrations of pUbl (1–76). A non-specific, ATP-independent band is indicated (*). The middle panel shows a Coomassie-stained loading control. The lower panel shows Miro1 ubiquitination for the respective proteins in the upper lane. Coomassie-stained gel showing Miro1 is used as the loading control of substrate ubiquitination assay.
E. Ubiquitination assay of ΔUbl-Parkin with increasing concentrations of pUbl (1–76), pUbl-linker (1–140), pUbl-linker-ΔACT (1–140, Δ101–109). A non-specific, ATP-independent band is indicated (*). The middle panel shows a Coomassie-stained loading control. The lower panel shows Miro1 ubiquitination for the respective proteins in the upper lane. Coomassie-stained gel showing Miro1 is used as the loading control of substrate ubiquitination assay.
F. Comparison of R0RBR and AUbl-Parkin activation using the increasing concentrations of pUbl (1- 76)/pUbl-linker (1–140)/pUbl-linker-ΔACT (1–140, Δ101–109). A non-specific, ATP-independent band is indicated (*). The middle panel shows a Coomassie-stained loading control. The lower panel shows Miro1 ubiquitination for the respective proteins in the upper lane. Coomassie-stained gel showing Miro1 is used as the loading control of substrate ubiquitination assay.

To validate crystal structures, we compared the ubiquitination activity of R0RBR (141–465) and ΔUbl-Parkin (77–465) in the presence or absence of pUb. The presence of linker (77–140) containing ACT in ΔUbl-Parkin (77–465) made it more active compared to R0RBR (141–465) (**Extended Data Fig. 9C**). We then compared the activation of R0RBR and ΔUbl-Parkin using pUbl (1–76) in trans. We observed that pUbl (1–76) efficiently activated ΔUbl-Parkin (77–465); however, R0RBR (141–465) activation by pUbl (176) was very poor (**Fig. 8D**, **Extended Data Fig. 9D**). Further, we tested whether pUbl-linker (1–140) with or without ACT would affect the activation of ΔUbl-Parkin (77–465) in trans. Interestingly, ubiquitination assays performed using increasing concentrations of pUbl (1–76) or pUbl-linker (1–140), or pUbl-linker-ΔACT (1–140, Δ101–109) showed that ΔUbl-Parkin activation was not affected by the linker (77–140) or ACT region in trans (**Fig. 8E**). However, compared to pUbl (1–76), pUbl-linker (1–140) showed better activation of R0RBR (141–465) (**Fig. 8F**, **Extended Data Fig. 9E**). Also, in contrast to pUbl-linker (1–140), pUbl-linker-ΔACT (1–140, Δ101–109) showed poor activation of R0RBR (141–465) which was similar to pUbl (1–76) (**Fig. 8F**, **Extended Data Fig. 9E**). However, the activity of R0RBR (141–465) complemented with pUbl-linker (1–140) was less than the activity of ΔUbl-Parkin (77–465) complemented with pUbl (176) (**Fig. 8F**, **Extended Data Fig. 9E**). Overall, our data suggested that ACT can be complemented in trans; however, ACT is more efficient in cis.

Crystal structure of pUbl-linker (1–140) depleted R0RBR (R163D/K211N)-pUb complex reveals a new ubiquitin-binding site on Parkin

In the last few years, several structures of Parkin or Parkin complexes were solved in various conditions and from different species. However, the linker (408–415) between REP element and RING2 was mostly disordered, except in structures (PDB 4I1H, 5CAW, 4ZYN) where the above region was modeled in different conformations (**Extended Data Fig. 10A**), highlighting its flexible nature. A pathogenic mutation T415N was also found in the linker (408–415), which abolished Parkin activity. However, the role of this small linker region on Parkin remains elusive. Therefore, we decided to inspect all the structures solved in the present study. In the crystal structure of R0RBR (R163D/K211N)-pUb complex, out of two molecules of Parkin in the asymmetric unit, one molecule of Parkin showed nice electron density of the linker (408–415) region of Parkin (**Fig. 9A, B**). We further noticed conformational changes in the linker (408–415) region in the structure of the R0RBR (R163D/K211N)-pUb complex when compared to the previously solved apo R0RBR structure (PDB 4I1H) (**Extended Data Fig. 10B**). While T410, I411, and K412 were facing outwards in the apo R0RBR structure, in the structure of R0RBR (R163D/K211N)-pUb complex these residues were present in the core (**Fig. 9B**, **Extended Data Fig. 10B**). Interestingly, we noticed interactions between the linker (408–415) of Parkin and pUb from the neighboring molecule of the asymmetric unit (**Fig. 9C**). The core of interactions between the Parkin linker and ubiquitin was mediated by I411, which was involved in hydrophobic interactions with the hydrophobic pocket of ubiquitin (**Fig. 9C**). Other interactions between Parkin and ubiquitin included ionic interactions mediated by K412, and H422 (**Fig. 9C**). Water-mediated interactions between linker (408–415) and ubiquitin included T410 with the carbonyl group of R72 of ubiquitin, and T415 with the carbonyl of G35 of ubiquitin (**Fig. 9C**). Furthermore, E409 formed a salt-bridge with K413 (**Fig. 9C**), which could be required for maintaining the structure of the linker region for ubiquitin binding. Also, residues in the linker region interacting with ubiquitin were highly conserved in Parkin across different species (**Fig. 9D**), suggesting their functional importance. Our data in **Fig. 2** suggested that RING2 was flexible (open and closed states) mediated by pUbl binding in the basic patch. As R0RBR (R163D/K211N)-pUb complex structure was captured in the closed state of RING2, we wondered whether the linker connecting REP and RING2 may adopt an alternate conformation dependent upon RING2 position (open or closed). The crystallization of the open state of phospho-Parkin remains challenging due to the flexible/multiple possible conformations of the REP-RING2 region. Therefore, we used AlphaFold 2 [34] to predict the model of the linker connecting REP and RING2of Parkin. Interestingly, the AlphaFold model predicted helical structure in the linker region of Parkin (**Extended Data Fig. 10C**) in the RING2 open state of Parkin,

indicating the flexible nature of this region under different states (RING2 closed <-> RING2 open) of Parkin. The latter also suggested that the conformation of the linker observed in the crystal structure could be one of the intermediates.

To validate the observations from structural analysis, we mutated these residues and compared their ubiquitination activity. In contrast to WT-Parkin, E409A and H422A drastically reduced Parkin activity, whilst I411A, T415N, and K416A resulted in the complete abolishment of Parkin activity (**Fig. 9E**). Further inspection revealed that although the linker region of Parkin is not conserved across different members of RBR family E3-ligases (**Extended Data Fig. 10D**), hydrophobic nature at the corresponding position of I411 on Parkin is conserved among various RBRs except RNF216 (**Extended Data Fig. 10D**). Also, the crystal structures of HOIP, HOIL, HHARI, and RNF216 solved with E2~Ub [35–37] showed interactions between linker region and donor ubiquitin (Ub_{don}) (**Extended Data Fig. 11A**). To test whether the linker between REP and RING2 of Parkin binds with donor ubiquitin (Ub_{don}), we performed binding assays using E2~Ub_{don}. Interestingly, unlike phospho-Parkin, which formed a stable complex with E2~Ub_{don} on SEC and co-eluted with E2~Ub_{don} and phospho-ubiquitin (**Fig. 9F**), phospho-Parkin I411A did not show interaction with E2~Ub_{don} (**Fig. 9F**). Furthermore, the SEC data was confirmed by ubiquitin-vinyl sulfone (Ub-VS) assay [16,24,25] where unlike phospho-Parkin, phospho-Parkin I411A did not react with Ub-VS (**Extended Data Fig. 11B**). We also tested Parkin activity using ubiquitin mutants (L71A or L73A) which would perturb the interactions of ubiquitin and Parkin linker as suggested by structure in **Fig. 9C**. Compared to native ubiquitin, ubiquitin mutants show loss of Parkin activity (**Extended Data Fig. 11C**) which nicely corroborated with our data. Overall, our data showed that the linker region between REP and RING2 interacts with donor ubiquitin and plays a crucial role in Parkin function.

Discussion

Autoinhibition of Parkin is mediated by several mechanisms. Ubl domain and REP element block the E2 binding site on RING1 [8,22,26,27], whereas the RING0 domain occludes the catalytic C431 on RING2. A few years after the discovery of Parkin autoinhibition, various groups discovered PINK1-mediated phosphorylation of S65 on ubiquitin and Ubl domain of Parkin, leading to the activation of Parkin [10,11,12,13]. In the last few years, several structural studies have aimed to understand the conformational changes in Parkin that are driven by phosphorylation leading to Parkin activation. The structure of RING2 truncated phospho-Parkin (1–382) in complex with pUb showed pUbl domain of Parkin bound to the basic patch (comprising K161, R163, K211) on RING0, which led to displacement of RING2 and REP during Parkin activation [24,29]. Previous studies using various biophysical methods reported a K_d of ~2μM between Ubl and RORBR/ΔUbl-Parkin; however, pUbl showed no interaction, which led to the proposed mechanism suggesting displacement of the pUbl domain to activate Parkin [26,27].

Our data show that RING2 and pUbl compete for binding on the basic patch of RING0 (**Fig. 2**). Our data also show that RING2 and REP displacement after Parkin phosphorylation is transient; RING2 and REP return to their original position after removal of the pUbl from phospho-Parkin (**Fig. 2**). Our data explains that due to the net high concentration of the fused domain (RING2 or pUbl), and competitive mode of interaction, binding/displacement of pUbl/RING2 domain in trans couldn't be observed in the previous studies. However, untethering of pUbl/RING2 overcomes the latter issue, and trans interaction between Parkin molecules can be observed. By untethering the linker between RING2 and IBR, after pUbl binding, the displaced RING2 is no longer able to return to the RING0 pocket, thus the binding of pUbl on the basic patch of RING0 is stabilized (**Fig. 2**). Untethered RING2 leads to a strong affinity between phospho-Ubl and core of Parkin with K_d around 1μM (**Fig. 4**,**5**), which is also supported by complex formation on SEC/SEC-MALS using phospho-Parkin and Parkin (**Fig. 5**).

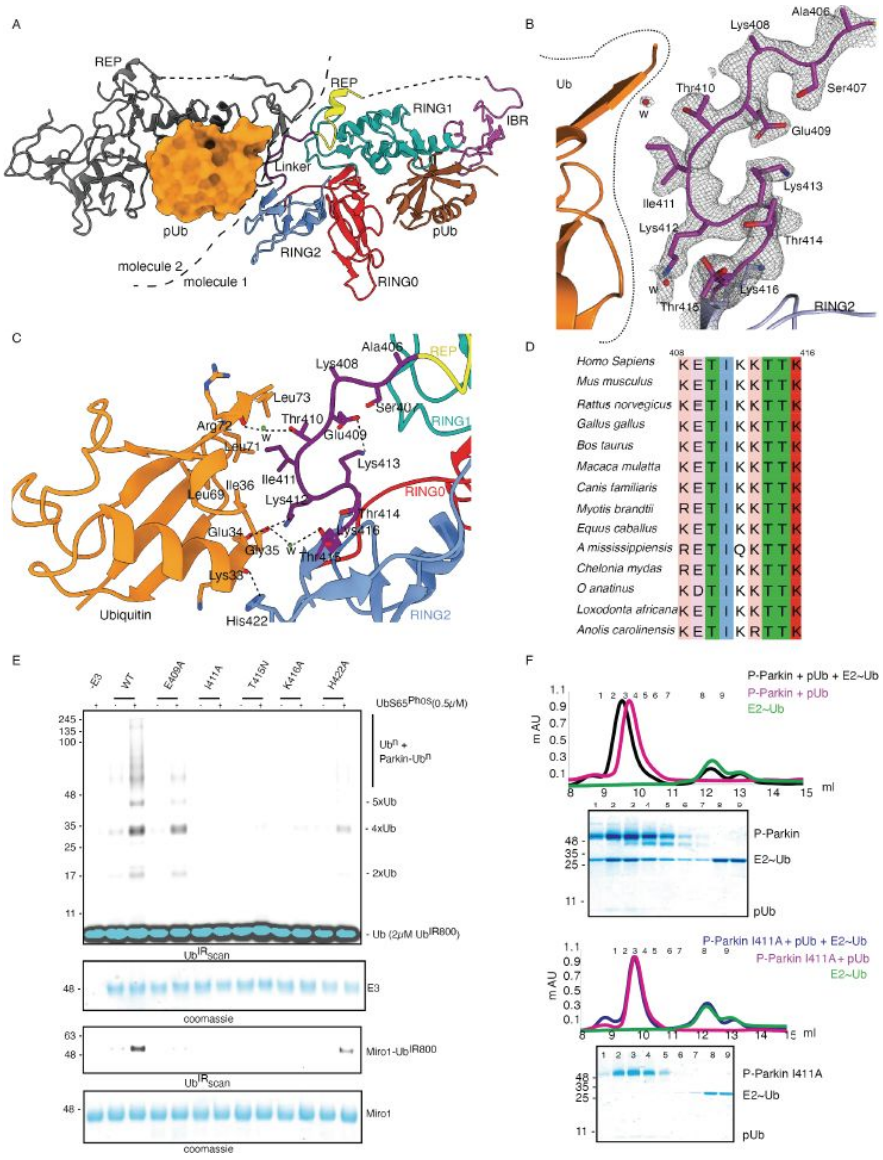

Figure 9

Linker (408–415) of Parkin binds with donor ubiquitin (Ub_{don}) of E2-Ub_{don}

A. The asymmetric unit of the crystal structure of pUbl-linker (1–140) depleted R0RBR (R163D/K211N)- pUb complex. Parkin molecule-1 (domains are shown in different colors) and pUb (brown) are shown. Parkin molecule-2 (grey) and pUb (orange) are shown. The interface of two Parkin molecules is highlighted (dashed line).
B. The 2Fo-Fc map (grey) of the linker region between REP and RING2. 2Fo-Fc map is contoured at 1.5 o. Water molecules are represented as w.
C. Crystal structure shows interactions between the linker (408–415) and ubiquitin. Different regions are colored as in panel A. Hydrogen bonds are indicated as dashed lines.
D. Sequence alignment of Parkin from various species highlighting conservation in the linker (408–415) region. Residue numbers shown on top of sequence alignment are according to human Parkin.
E. Ubiquitination assay of Parkin mutants in the linker region. The middle panel shows a Coomassiestained loading control. The lower panel shows Miro1 ubiquitination for the respective proteins in the upper lane. Coomassie-stained gel showing Miro1 is used as the loading control of substrate ubiquitination assay.
F. Size-exclusion chromatography (SEC) assay to compare the binding of E2~Ub with phospho-Parkin (upper panel) or phospho-Parkin I411A (lower panel). Assays were done using Parkin in complex with pUb. A colored key for each trace is provided. Coomassie-stained gels of indicated peaks are shown.

A feedforward control mechanism was suggested in the PINK1-Parkin pathway wherein PINK1-dependent phosphorylation of ubiquitin and Parkin leads to Parkin activation on mitochondria [11,15,16,38,39]. However, biophysical studies aimed to understand Parkin activity did not show any dimerization of Parkin or Parkin-Parkin association in trans [19–30]. Our data demonstrate that phospho-Parkin and WT-Parkin can form a stable complex in trans to mediate Parkin dimerization (**Fig. 5**). We also show that phospho-Parkin can activate WT-Parkin in trans, reaffirming that a major mode of Parkin autoinhibition is mediated by RING0 blocking the catalytic C431 on RING2 domain. Furthermore, our data suggest an additional feedforward activation model of Parkin wherein fully-activated Parkin (phospho-Parkin bound to pUb) molecules can activate partially-activated Parkin (WT-Parkin bound to pUb) molecules which is mediated by interactions between pUbl and RING0 in trans (**Fig. 10**). The latter can be relevant in the context of healthy carriers of heterozygous mutations on Parkin. The critical role of pUbl supports data showing the importance of Ubl phosphorylation in vivo, as demonstrated by the discovery of Parkinson's patients associated with homozygous S65N Parkin mutation [40]. This data also highlights the importance of various Parkin isoforms that have been identified (**Extended Data Fig. 6**), especially the ones that lack Ubl domain or REP-RING2 domains, as they can complement each other using our proposed trans model in **Fig. 10**.

ACT was proposed to have a role in Parkin activation, as it was shown that the deletion/mutation of ACT leads to the loss of Parkin activity [24]. We demonstrate that ACT plays a key role due to its inherent capacity to bind with the RING0 pocket. Unlike other functional mutations on Parkin affecting interaction with E2 or Ub_{don}, ACT deletion does not affect binding with E2~Ub_{don} (7). We show that ACT plays a crucial role in enzyme kinetics and only slows the Parkin activity, possibly due to affecting the inherently dynamic nature of RING2 (**Fig. 7**). Furthermore, we also demonstrate that although ACT can be complemented in trans, ACT on a cis molecule is more effective (**Fig. 8**).

The linker connecting IBR and RING2 of Parkin comprises two components: a REP element (391–405) and a flexible linker (408–415). Various Parkin structures solved so far show REP element blocking the E2 binding site on RING1; however, linker (408–415) remained flexible in most structures, and its role remained elusive. Interestingly, pathogenic mutation T415N in the linker region was shown to abolish the E3 ligase activity of Parkin [8]. Also, using peptide array analysis, Chaugule and colleagues proposed a Parkin Ubl/ubiquitin-binding (PUB) site in the C-terminal domain of Parkin [8]. Here, we demonstrate that the linker (408–415) interacts with donor ubiquitin (Ub_{don}) of E2~Ub_{don} (**Fig. 9**). Although the linker between IBR-RING2 is not conserved across RBR family E3-ligases, the core of interactions between the linker and Ub_{don} is mediated by hydrophobic residue in the linker region (**Fig. 9**). In the autoinhibited closed state of Parkin, the linker between IBR-RING2 of Parkin is present in a straight conformation, leading to IBR and RING2 occupying diagonally opposite conformation, which is quite similar to what is seen in HOIP RBR and E2~Ub_{don} complex structure (**Fig. 9**, **Extended Data Fig. 11A** [35]). However, the recent structures of RBR family E3-ligases (HHARI, RNF216, HOIL-1) [36,37] show a kinked conformation of the linker connecting IBR-RING2 (**Extended Data Fig. 11A**). Interestingly, the kink in the linker region plays a crucial role in bringing RING2 to the catalytically feasible state (**Extended Data Fig. 11A**). Conversely, under the extended conformation of the linker catalytic feasibility is not possible (**Extended Data Fig. 11A**). The conformational flexibility in the linker (408–415) region of Parkin is also supported by the fact that it is disordered in most Parkin structures, or seen as a loop in couple of Parkin structures, whereas AlphaFold predicts it as a helix similar to other RBR structures (**Fig. 9**, **Extended Data Fig. 10**). Previous data observed the opening of RING2 after the addition of E2~Ub_{don} in R0RBR [30]. The latter observation also suggests that conformational changes might be induced in the linker region after binding with donor ubiquitin or due to the movement of RING2, and needs further investigation. Also, as mentioned above, the conformation of donor ubiquitin and linker captured in the present study

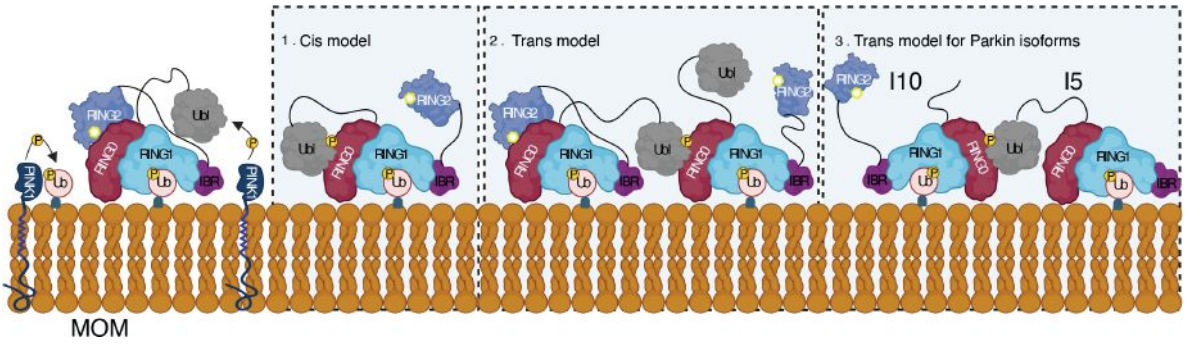

Figure 10

Model shows different modes of Parkin activation

The cis-activation model uses the binding of pUbl in the same molecule, thus resulting in the displacement of RING2 (1). The trans-activation model uses the binding of pUbl of fully-activated Parkin (phospho-Parkin complex with pUb) with partially-activated Parkin (WT-Parkin and pUb complex), thus resulting in the displacement of RING2 in trans (2). Recruitment and activation of Parkin isoforms lacking Ubl (Isoform 10) or RING2 domain (Isoform 5), thus complementing each other using the transactivation model (3). Catalytic cysteine on RING2 is highlighted.

might be one of the possible intermediates. Although the regulatory mechanisms vary across RBR family E3-ligases, the catalytic core (IBR-RING2) undergoes similar conformational changes, leading to a unified catalysis mechanism in various RBR family E3-ligases.

Overall, our new structural and biophysical analysis elaborates new understanding of Parkin activation and regulation that will aid in efforts to develop small molecular activators of Parkin as a therapeutic strategy for PD.

Declarations

Authors' contributions

DRL performed all experiments, solved structures, and analyzed the data. SD purified proteins, performed SEC assays in **Fig. 8**, and analyzed the sequence alignment. OA performed and analyzed IF experiments under the supervision of MM. AP; IF imaging and quantification. PS performed ITC experiments. AK conceived, designed, and supervised the research, refined structures, analyzed the data, and wrote the manuscript with input from all authors.

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Conflict of interest

MM. is a member of the Scientific Advisory Board of Montara Therapeutics Inc and scientific consultant to MSD UK.

Additional Declarations:

The authors declare potential competing interests as follows: MM. is a member of the Scientific Advisory Board of Montara Therapeutics Inc and scientific consultant to MSD UK.

Online Methods

Molecular Biology

The human *PARK2* gene optimized for bacterial expression of FL-Parkin was cloned in the pET15b vector. Various Parkin mutations used in the present study were made using site-directed mutagenesis (SDM). TEV protease site (ENLYFQS) was substituted in the Parkin construct between the 382nd -388th as described in [24], and an HRV 3C protease site (LEVLFQGP) was inserted between residue 140th and 141st residues using site-directed mutagenesis. Ubl (expressing 1–76th amino acids of Parkin) and Ubl-linker (expressing 1–140th amino acids of Parkin) constructs were generated by introducing a stop codon after the 76th and 140th amino acids, respectively, in the FL-Parkin construct. Parkin mutants were generated using site-directed mutagenesis. Mirol (expressing 181st - 592nd amino acid) was amplified from the cDNA of the HEK293T cell line using Phusion polymerase (NEB) and cloned into the pGEX-6P1 vector using EcoRI and BamHI restriction enzymes. To generate fluorescently labeled ubiquitin, ubiquitin (residues 2–76) was cloned in a pGEX-6P vector with an overhang expressing GPLCGS at the n-terminal of ubiquitin. For the generation of ubiquitin-3Br protein, the ubiquitin gene (residues 1–75) was cloned in the pTXB-1 vector. Pediculus humanus corporis PINK1 (115 - 575) was a gift from David Komander [43](Addgene plasmid # 110750). Ube1 was a gift from Cynthia Wolberger [44](Addgene plasmid # 34965).

Protein purification

Parkin constructs were expressed in *Escherichia coli* BL21(DE3)pLysS cells. Cells were grown until OD_{600} reached 0.4; the temperature was reduced to 16 °C, and protein was induced by adding 50 µM IPTG, and media was supplemented with 200 µM $ZnCl_2$. Cells were left to grow overnight at 16 °C. Cells were harvested and lysed using sonication in lysis buffer (25 mM Tris pH 7.5, 200 mM NaCl, 5 mM Imidazole, 1 mM β-mercaptoethanol, and 100 µM AEBSF). Protein was purified over Ni-NTA resin. His-Sumo tag was removed using SENP1 protease. Protein was further purified over Hi-Trap Q HP column (GE Healthcare) followed by a gel-filtration column pre-equilibrated with storage buffer (25 mM Tris pH 7.5, 75 mM NaCl, 250 µM TCEP). Other proteins were also purified using similar protocols. PhPINK1 was purified as published before [43].

Isothermal Titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed using PEAQ ITC (Malvern instruments), and data were analyzed using a single-site binding model and competing binding mode. All titrations were performed at 25°C in 1X PBS buffer containing 250 µM TCEP. In **Fig 4A**, experiments were done using 350 µM of P-Parkin (K211N) in the syringe and 21 µM of ΔUbl-Parkin in the cell. In **Fig. 4B**, experiments were done using 360 µM of P-Parkin K211N in the syringe and 30 µM of untethered ΔUbl-Parkin (TEV)in the cell. In **Fig. 5B**, experiments were done using 260 µM of P-Parkin in the syringe and 24 µM of untethered ΔUbl-Parkin (TEV) in the cell.

Ubiquitination assays

Ubiquitination assays were performed using fluorescently labeled ubiquitin. Ubiquitin labeling was done using Dylight^{TM} 800 Maleimide (Thermo Scientific), as mentioned previously [26], using the manufacturer's specifications. Ubiquitination reactions were performed at 25 □C for 40 minutes in 25 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM DTT, 10 mM ATP. In all reactions, 25 nM Ube1, 250 nM UbcH7 (E2), 1 µM of E3, and 2 µM of Ub^{IR800} were used in 20 µl of the total reaction volume. 0.5 µM of Ub or pUb was used as an allosteric activator for the experiments in **Fig. 3B**, **Fig. 9E**, and **Extended Data Fig. 9C**.

Increasing concentrations of P-Parkin (T270R, C431A) (1 μM, 2 μM, 4 μM, and 8 μM) were used as trans activators in **Fig. 5E**. The transactivation experiments using pUbl, pUbl-linker, and pUbl-linker-ΔACT were carried out with increasing concentrations of 4 μM, 8 μM, and 16 μM in **Fig. 8D, E, F**. Substrate Miro1 ubiquitination reaction was done at 25 □C for 20 minutes with 5 μM Mirol and 0.5 μM of E3. Other conditions were the same as mentioned above for ubiquitination/autoubiquitination assay. The reactions were quenched by SDS loading dye and heated at 95 □C for 5 mins. The samples were resolved on gradient SDS-PAGE and analyzed using Li-COR® Odyssey Infrared Imaging System. Each assay was repeated at least three times. ImageJ software was used to quantify ubiquitination. Bar plots and statistical analysis were done using R.

Cell culture transfection and microscopy experiment

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (vol/vol) FBS, 1% Pen/Strep, and 1% L-Glutamine at 37°C under an atmosphere of 5% CO_2. 24-well cell culture plate (35000 cells/well) was used to seed cells onto borosilicate cover glasses (VWR 631-0148). The following plasmids were generated by MRC Reagent & Services and used to assess Parkin translocation: GFP-Parkin (DU23318), mCherry-Parkin (DU77708), mCherry-Parkin-S65A (DU77709), GFP-Parkin-C431F (DU77645), GFP-Parkin-K211N-C431F (DU77659) and GFP-Parkin-H302A-C431F (DU77713). Transfections were carried out the day after seeding, and plasmids were mixed with PEI (PEI MAX-Polyscience, 24765-1) at a 1:5 ratio in Opti-MEM (Gibco). DNA/PEI mix was left for 45 minutes at room temperature, then added to the cell cultures and incubated for 48 hours before CCCP treatment (10 μM for 1 hour). For immunostaining, cells were fixed with 4 % (wt/vol) paraformaldehyde in PBS for 20 minutes at room temperature and permeabilized with a blocking buffer containing 3 % (wt/vol) Donkey serum and 0.2 % (vol/vol) Triton X-100 in PBS for 1 hour. Cells were incubated with TOMM20 (ab186735) primary antibody overnight at 4 °C, followed by incubation with the anti-mouse Alexa Fluor 405 secondary antibody (ThermoFisher, A-48258) for 1 hour at room temperature. After three washes with PBS and a rinse with Milli-Q water, the cover glasses were mounted onto slides using a Vectashield mounting medium (Vector Laboratories, H-1000). Microscopy was performed on an LSM 880 laser scanning confocal microscope (ZEISS; Plan-Apochromat 63x/NA 1.4) using ZEISS Zen Software. Colocalization was assessed using Volocity Software (version 6.3, Quorum Technologies) and determined as Pearson's correlation coefficient for mitochondrial colocalization of GFP and the mitochondrial marker TOMM20. Images were processed using ImageJ software version 1.51 (100).

Purification of phospho-Ubiquitin (pUb)-3Br

pUb-3Br was purified as published before [28,45]. Briefly, ubiquitin (1–75)-Mxe-intein-chitin binding domain was expressed in *Escherichia coli* BL21(DE3) cells using a pTXB-1 vector. Cells were induced at 0.8 O.D. using 250 pM IPTG and incubated at 22 °C for 12 hrs. Cells were lysed in lysis buffer (20 mM Na_2HPO4 pH 7.2, 200 mM NaCl, 0.1 mM EDTA), and protein was purified using Chitin resin (NEB). The resin was incubated overnight with cleavage buffer (20 mM Na2HPO4 pH 6.0, 200 mM NaCl, 50 mM MESNa, 0.1 mM EDTA) to elute the protein. The eluted protein was reacted with 3-Bromopropylamine hydrobromide (Sigma) at 25 EC for 4 hrs. The reacted protein was purified over Hiload 16/600 Superdex 75pg column (GE Healthcare) pre-equilibrated with 1X PBS. The fractions containing Ub-3Br were concentrated and phosphorylated using PhPINKI. pUb-3Br was purified over Hiload 16/600 Superdex 75pg column preequilibrated with Parkin storage buffer.

Synthesis and purification of UbcH7~Ub

The reaction containing 500 μM of UbcH7 (Cys17Ser/Cys86Ser/Cys137Ser), 15 μM of Ube1, and 2.5 mM of 6xHis-Ub in charging buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM MgCl2, 10 mM ATP) was incubated at 37 °C for 18 hrs. The progress of the reaction was monitored over SDS-PAGE.

The reaction mixture was passed through Ni-NTA resin to capture His-Ub and UbcH7~Ub (His), and the eluted fraction was purified over Hiload 16/600 Superdex 75 pg column (GE Healthcare). Fractions containing UbcH7~Ub were pooled together and stored for further use.

Preparation of Parkin complexes for crystallization

In the present study, Parkin complexes with pUb were captured using pUb-3Br. To capture Parkin complexes with pUb-3Br, human Parkin constructs were mutated to include Q347C, as published before [28], in various constructs for crystallization experiments. For crystallization of pUbl-linker (1–140) depleted Parkin (141–465) and pUbl-linker (1–140) depleted R0RBR R163D/K211N complex with pUb, Parkin Q347C (3C, TEV) and Parkin R163D/K211N/Q347C (3C) constructs were used, respectively. Proteins were expressed and purified as above. Purified proteins were mixed with pUb-3Br, and Parkin was phosphorylated using PhPINK1 in a phosphorylation buffer containing 5 mM ATP and pUb-3Br. GST-HRV 3C protease was added (in a 1:50 ratio), and proteins were left overnight at 4 °C. The proteins were passed through affinity chromatography to remove GST-HRV 3C protease and PhPINK1. Flow-through was further purified over a gel-filtration column. Fractions containing R0RBR with pUb were pooled together and used for crystallization.

Ternary trans-complex of phospho-Parkin (1–140 + 141–382 + pUb) was made using Parkin K211N (3C) construct as the donor of pUbl-linker, and R0RBR Q347C (TEV) construct as the acceptor of pUbl-linker. Purified Parkin K211N (3C) was phosphorylated using PhPINK1 as above. Purified R0RBR Q347C (TEV) was treated with His-TEV followed by His-TEV removal over Ni-NTA resin. 2-fold molar excess of phospho-Parkin K211N (3C) was mixed with TEV-treated R0RBR Q347C (TEV). The complex containing phospho-Parkin K211N (3C) and R0RB Q347C (141–382) was purified over Hiload 16/600 Superdex 200pg column pre-equilibrated with Parkin storage buffer. The latter complex was mixed with pUb-3Br and treated with 3C protease. Protein was further purified over Hiload 16/600 Superdex 75pg column pre-equilibrated with Parkin storage buffer. Fractions containing ternary trans-complex of phosphoParkin (1–140 + 141–382 + pUb) were pooled together, concentrated, and used for crystallization.

Ternary trans-complex of phospho-Parkin with cis ACT (1–76 + 77–382 + pUb) was made using the Ubl (176) domain of Parkin and ΔUbl-Parkin Q347C (TEV). ΔUbl-Parkin Q347C (TEV) was treated with His-TEV, and His-TEV was removed over Ni-resin. A 3-fold molar excess of the pUbl domain was mixed with TEV-treated/RING2 untethered ΔUbl-Parkin Q347C (TEV). The pUbl and ΔUbl-Parkin Q347C (77382) complex was purified over Superdex 75increase 10/300 GL column pre-equilibrated with Parkin storage buffer. The latter trans-complex of phospho-Parkin with cis ACT (1–76 + 77–382) was mixed with pUb-3Br and purified over Superdex 75 increase 10/300 GL column pre-equilibrated with Parkin storage buffer. Fractions containing ternary trans-complex of phospho-Parkin with cis ACT (1–76 + 77–382 + pUb) were pooled together, concentrated, and used for crystallization.

R0RBR (TEV) was purified as stated above. After treatment with TEV, TEV was depleted using Ni-NTA resin, and untethered R0RBR was purified using Hiload 16/600 Superdex 75pg column pre-equilibrated with Parkin storage buffer.

Crystallization and structure determination

Initial crystals of pUbl-linker (1–140) depleted Parkin (141–465) complex with pUb-3Br appeared in 1.6 M Ammonium sulfate, 0.1 M MES monohydrate pH 6.5, and 10% v/v 1,4-Dioxane of HR112 screen (Hampton Research) at 4°C. Seeding was done to grow good-quality crystals in the same condition. The mother liquor containing 20% (v/v) of glycerol was used as a cryoprotectant for freezing crystals in liquid nitrogen. Crystals of pUbl-linker (1–140) depleted R0RBR (R163D/K211N)-pUb complex appeared in 0.15 M Potassium bromide, and 30% w/v Polyethylene glycol monomethyl ether 2,000 of Index screen (Hampton research) at 18°C. The mother liquor containing 20% (v/v) of PEG 400 was used as a cryoprotectant for freezing crystals in liquid

nitrogen. Crystals of ternary trans-complexes of phosphoParkin were obtained in 0.3 M Sodium nitrate, 0.3 Sodium phosphate dibasic, 0.3 M Ammonium sulfate, 0.1 M Tris (base) & BICINE (pH 8.5), 25% v/v MPD, 25% w/v PEG 1000, and 25% w/v PEG 3350 of Morpheus screen (Molecular dimensions). Good quality crystals were grown at 18 °C using microseeding. The mother liquor containing 10% (v/v) of glycerol was used as a cryoprotectant for freezing crystals in liquid nitrogen. Crystals of untethered R0RBR were grown in 0.1 M HEPES, pH 7.5, 8% PEG 4000, 10% isopropanol, and 0.1 M BaCl2 at 4°C. The mother liquor containing 20% (v/v) glycerol was used for vitrification.

Data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Data were processed using XDS [46]. Scaling was done using Aimless, and the structures were determined by molecular replacement using Phaser, as implemented in CCP-7.1 [47]. Structures of pUbl-linker (1–140) depleted Parkin (141–465)-pUb complex or pUbl-linker (1–140) depleted R0RBR (R163D/K211N)-pUb complex were solved by using the structure of Pediculus Parkin-phospho-ubiquitin complex (PDB 5CAW) as a search model. Structures of ternary trans-complex of phospho-Parkin were solved using phosphoParkin structure (PDB 6GLC) as a search model. Untethered R0RBR structure was determined using R0RBR structure (PDB 4I1H) as a search model. The initial model was built and refined using coot[48] and refmac5 [49], respectively.

Purification of phosphorylated proteins

PhPINK1 was used to phosphorylate various Parkin variants used in the study. Phosphorylation buffer contains 50 mM Tris pH 8.5, 100 mM NaCl, 10 mM MgCl2, 10 mM DTT, and 10 mM ATP. The reactions were performed at 25 °C for 4 hrs. Phosphorylation status was checked using Phos-Tag (FUJIFILM) analysis as per the manufacturer's protocol. PINK1 was depleted by affinity chromatography upon completion of the reaction. The phosphorylated proteins were further purified over a gel-filtration column.

Parkin phosphorylation assay

Parkin phosphorylation assay was performed using 5 µM Parkin and 0.25 µM PINK1 in phosphorylation buffer at 25 °C for 15 mins. Increasing concentrations (20 µM, 40 µM, and 80 µM) of pUbl or pUb were added with Parkin to check their effect on Parkin phosphorylation. The samples were analyzed on SDS-PAGE containing Phos-Tag (FUJIFILM) as per the manufacturer's protocol.

Size-exclusion chromatography

For RING2 or Ubl displacement/binding assays, HRV-3C cleavable and TEV cleavable constructs of Parkin were purified and phosphorylated as above. TEV and HRV 3C were added at the molar ratio (protease: Parkin) of 1:5 and 1:15, respectively. After incubation with respective proteases, proteins were purified using affinity chromatography to remove proteases from Parkin. The proteins were loaded onto Superdex 75 increase 10/300 GL column, and fractions were analyzed over SDS-PAGE.

For the trans-complex assays, phospho-Parkin variants were added in 2-fold molar excess. Also, in all trans-complex assays, the TEV site between IBR and RING2 was present only on the target Parkin molecules. Furthermore, before complex formation, TEV was removed by affinity chromatography. Proteins were incubated for 30 minutes at 4 °C before loading onto Superdex 75 increase 10/300 GL column. Fractions were analyzed using SDS-PAGE.

For SEC assay to analyze Parkin interaction with E2~Ub, 10 µM of phospho-Parkin/phospho-Parkin ΔACT/phospho-Parkin I411A was pre-incubated with 15 µM of pUb, followed by the addition of 20 µM of E2~Ub. Proteins were incubated for 1 hr at 4 °C before injecting onto Superdex 75 increase 10/300 GL column. Fractions were analyzed over SDS-PAGE to check the complex formation.

Sec-Mals

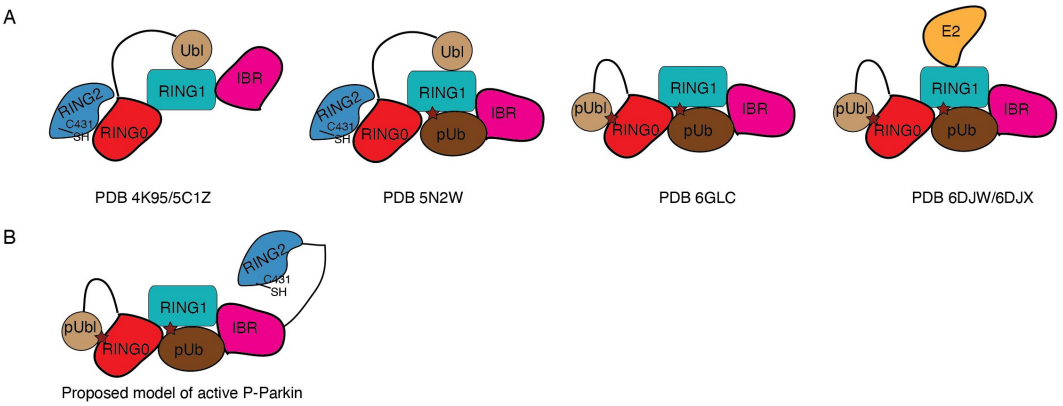
Size-exclusion chromatography (SEC) was performed with inline multi-angle light scattering (MALS) using the Viscotek SEC-MALS 20 system. Protein at 4–6 mg/mL (100 µL) was loaded on P2500-P4000 columns (Malvern) at a flow rate of 0.3 mL/min in buffer containing 20 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.25 mM TCEP. The data were analyzed using OmniSEC 5.11 software.

Supplementary Files

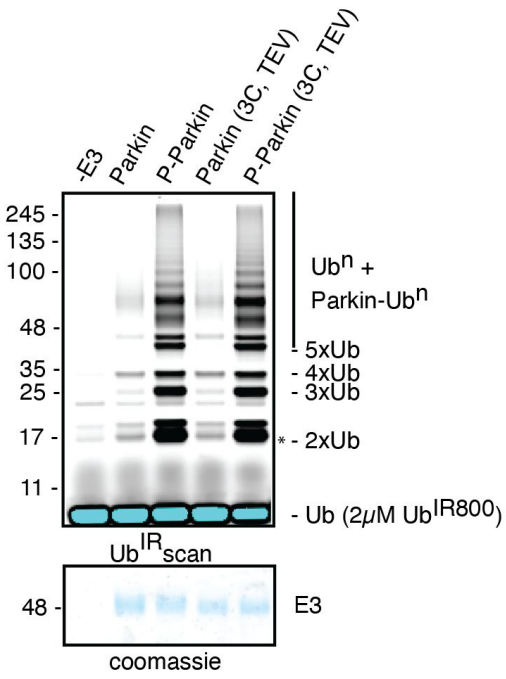
	ternary trans-complex of phospho-parkin with pUb	pUbl-linker depleted parkin (141-465) complex with pUb	Untethered R0RBR	ternary trans-complex of phospho-parkin with cis ACT and pUb	pUbl-linker depleted parkin (141-465, R163D, K211N) complex with pUb
Data collection					
Resolution range	34.30 - 1.92 (1.98 - 1.92)	39.15 - 3.3 (3.41 - 3.3)	48.28 - 2.9 (3.004 – 2.9)	35.84 - 2.6 (2.69 - 2.6)	37.47 - 2.35 (2.43 - 2.35)
Space group	P 32 2 1	P 64 2 2	C 2 2 21	P 32 2 1	P 1 21 1
Cell dimensions					
a, *b*, *c* (Å)	83.804, 83.804, 105.033	187.805, 187.805, 141.857	86.672, 132.579, 64.692	82.764, 82.764, 103.494	45.45, 76.426, 114.329
α, *β*, *γ* (°)	90, 90, 120	90, 90, 120	90, 90, 90	90, 90, 120	90, 100.485, 90
Total reflections	186235 (17323)	373278 (38759)	32244 (3362)	170918 (17328)	97886 (9139)
Unique reflections	32991 (3234)	22705 (2115)	8492 (843)	13023 (1273)	31073 (3105)
Multiplicity	5.6 (5.4)	16.4 (17.4)	3.8 (4)	13.1 (13.6)	3.2 (2.9)
Completeness (%)	99.55 (99.35)	91.65 (92.19)	98.84 (99.29)	99.17 (98.82)	96.28 (96.61)
I/σ(I)	14.09 (1.41)	11.75 (0.72)	11.83 (3.78)	17.69 (1.94)	12.45 (2.55)
Wilson B-factor	43.26	132.82	49.22	46.5	40.73
R-merge	0.05746 (0.9399)	0.1928 (3.632)	0.09615 (0.3465)	0.1416 (1.696)	0.0735 (0.52)
CC1/2	0.993 (0.738)	0.998 (0.48)	0.993 (0.921)	0.999 (0.763)	0.996 (0.695)
Refinement					
Reflections used in refinement	32964 (3229)	20836 (2053)	8492 (843)	12933 (1258)	31062 (3105)
R-work/R-free	0.2031/0.2368	0.2360/0.2750	0.2180/0.2438	0.2141/0.2355	0.1952/0.2119
No of Atoms					
macromolecules	2937	5457	2396	3006	6033
ligands	19	59	21	30	37
solvent	96	2	19	52	258
RMS deviations					
Bond length (Å)	0.008	0.009	0.008	0.009	0.007
Bond angles (°)	1.20	1.24	1.64	1.20	1.17
B-factors					
macromolecules	61.01	150.78	44.37	64.69	44.87
ligands	70.26	196.7	45.47	79.76	48.68
solvent	60.53	118	38.72	65.63	47.36
Accession code	8IKM	8IK6	8JWV	8IKT	8IKV

Table 1

Data collection and Refinement statistics



ExtendedDataFigure-1



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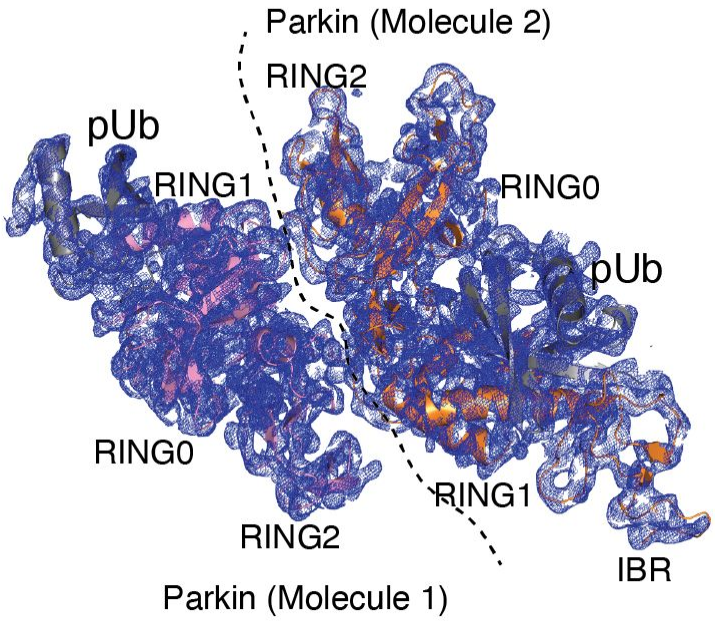

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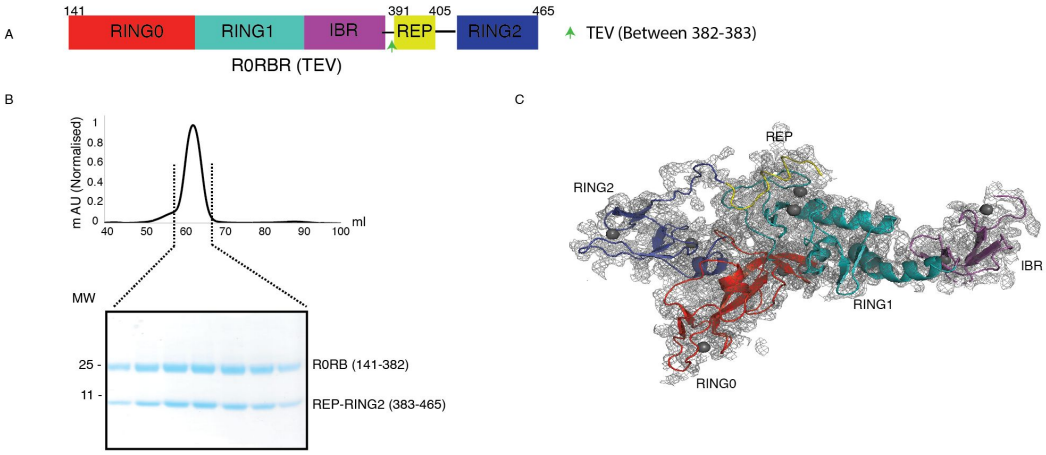

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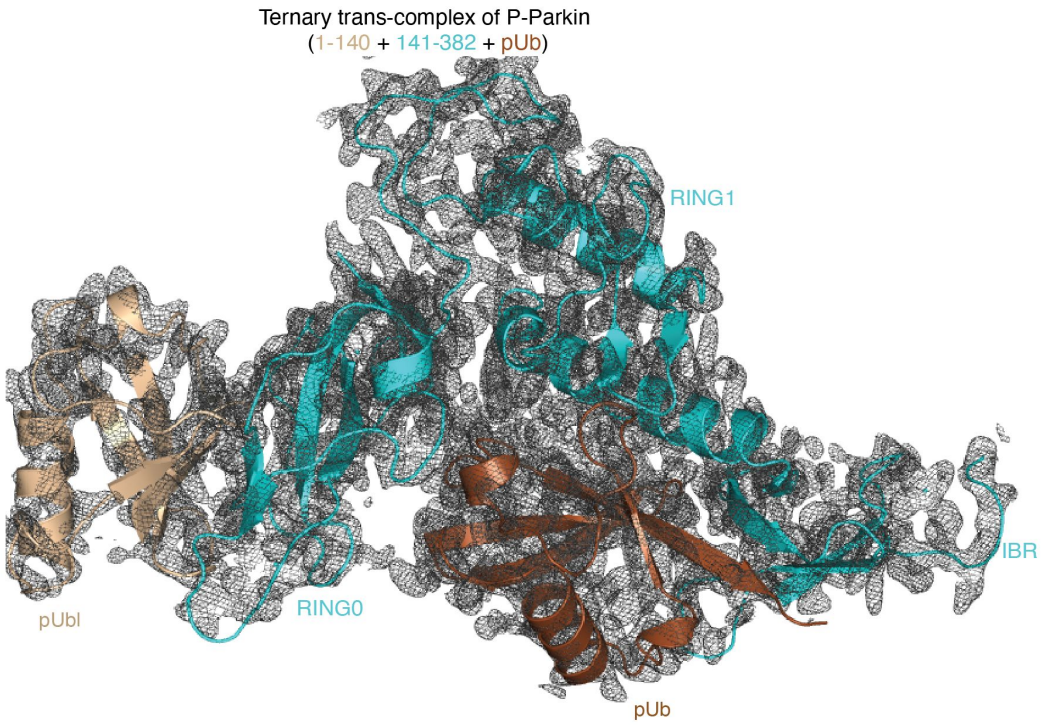

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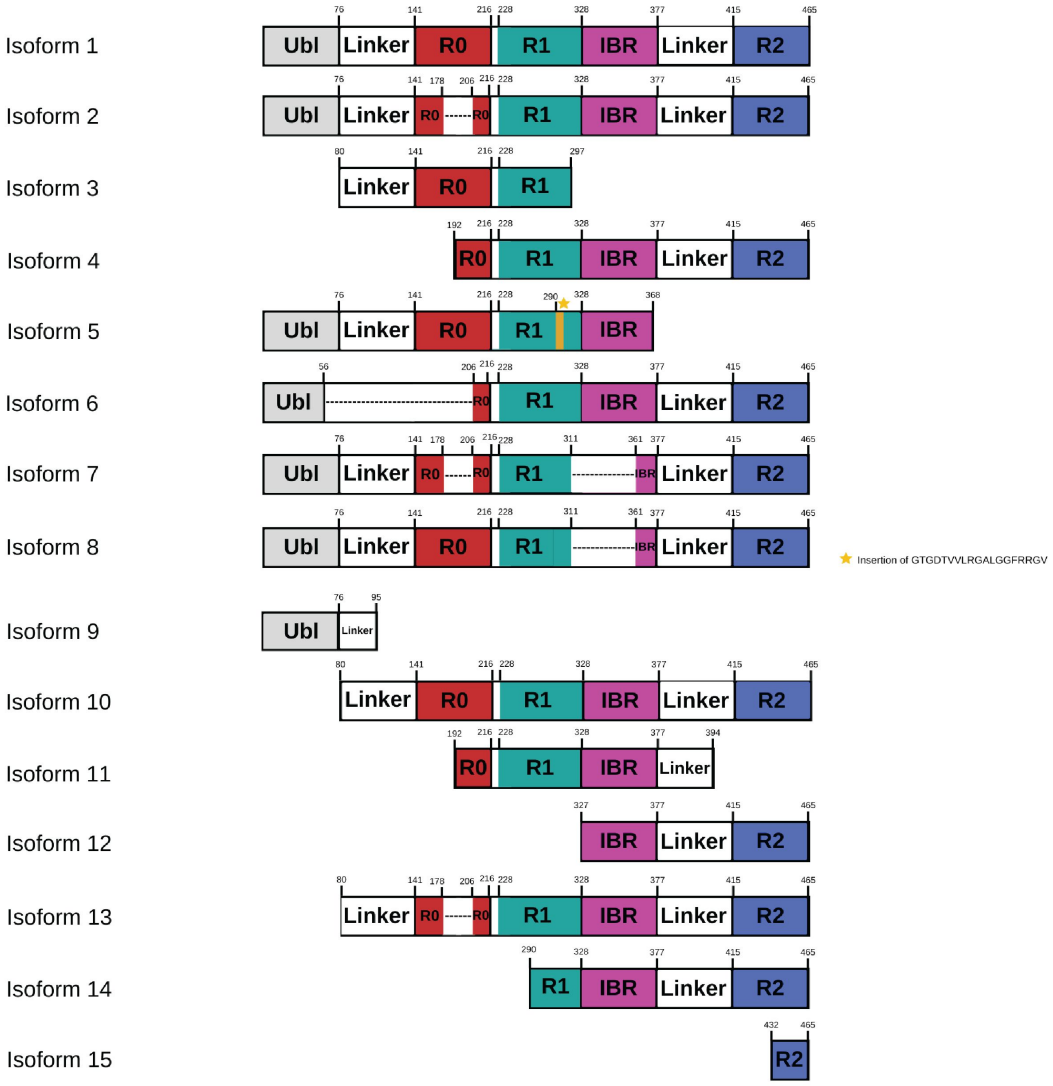

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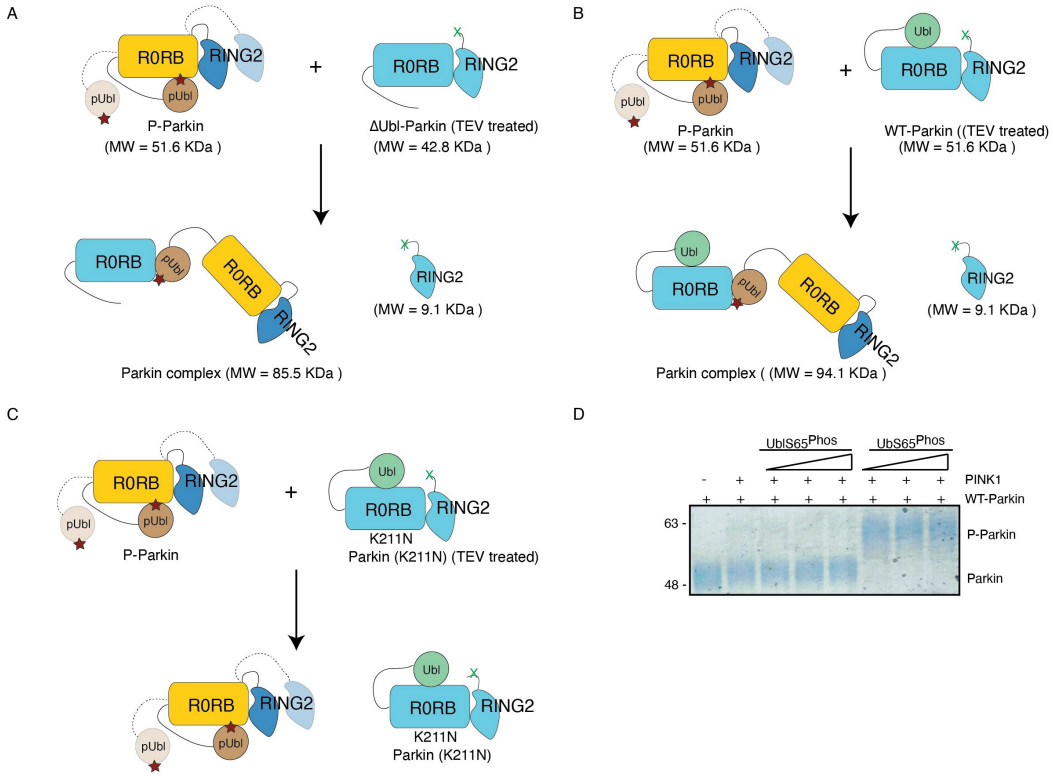

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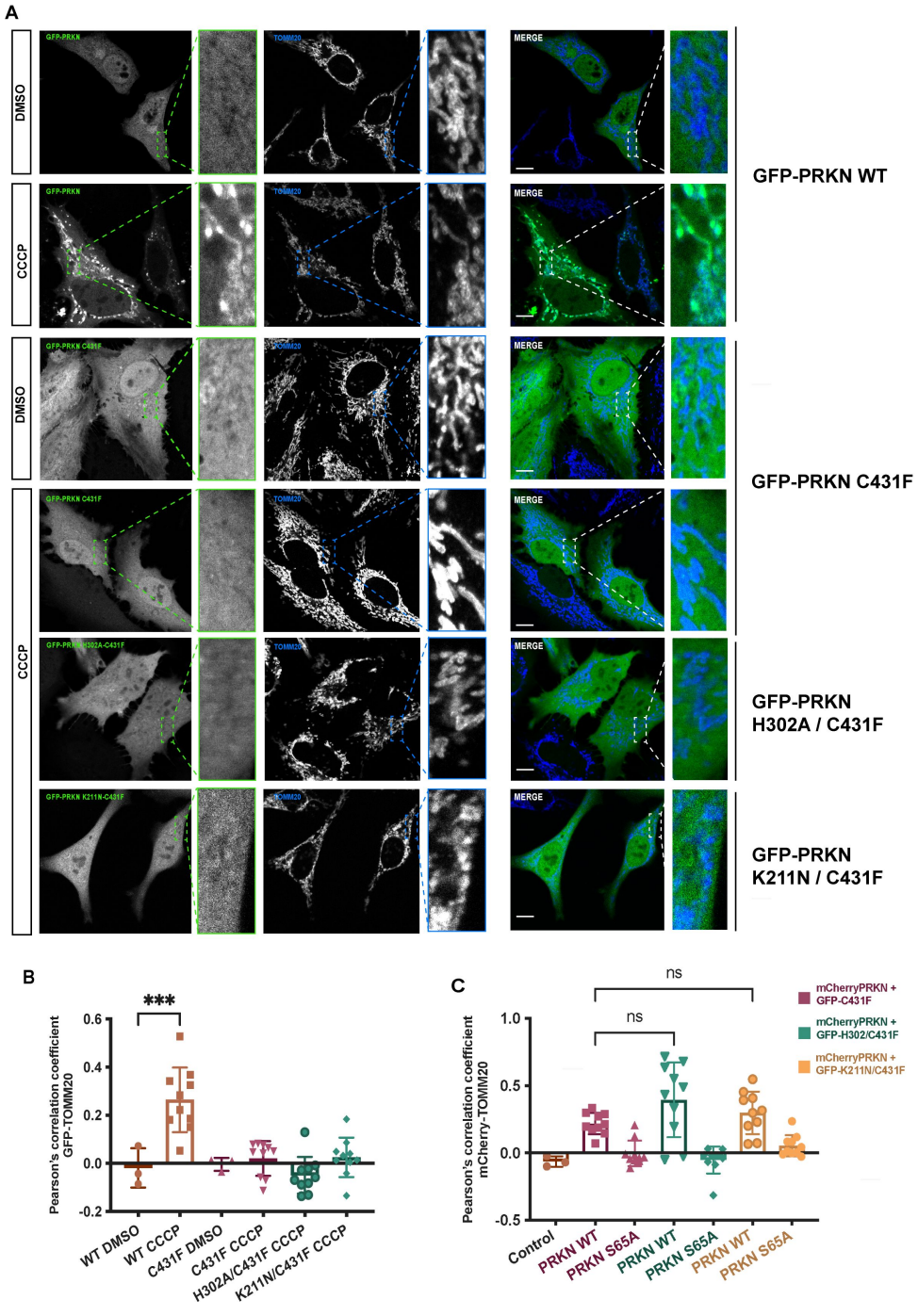

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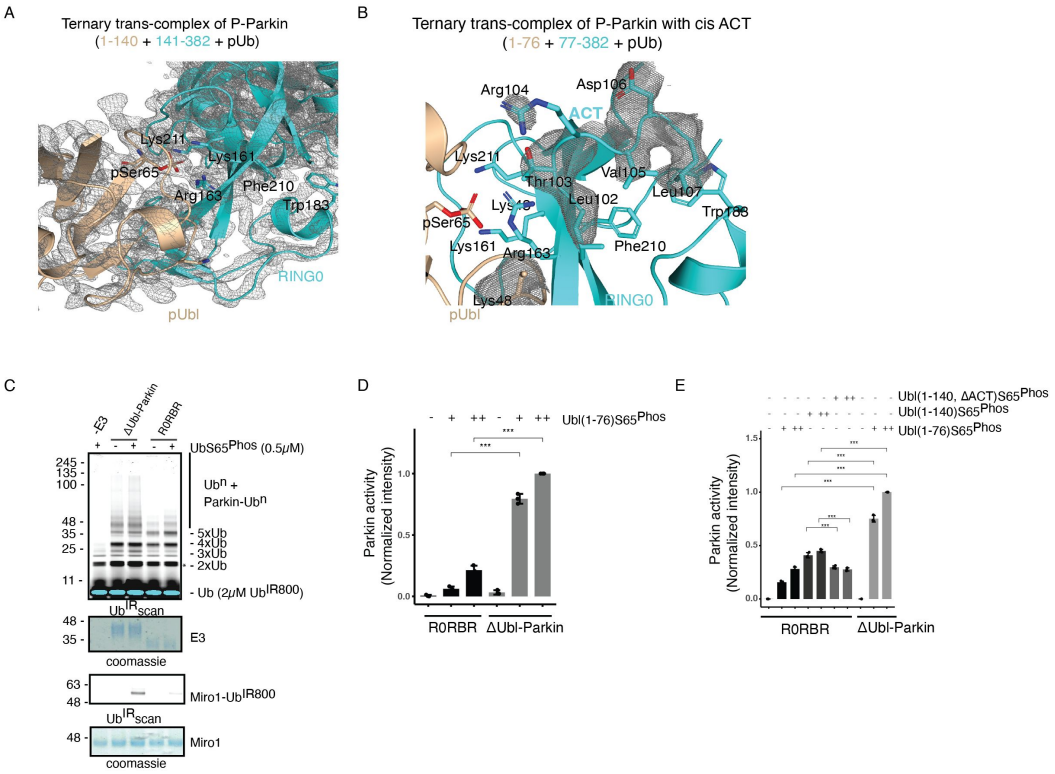

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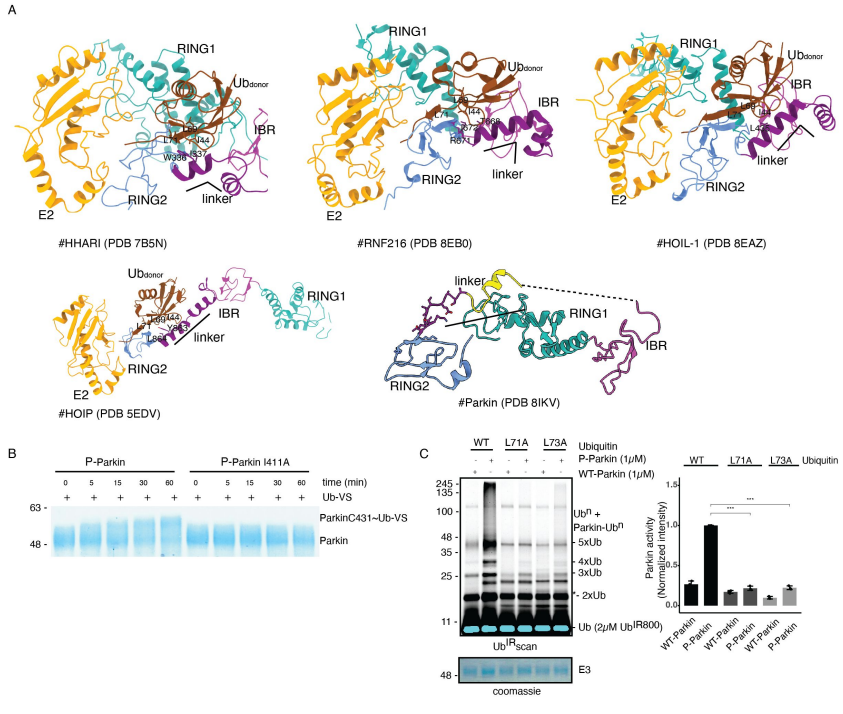

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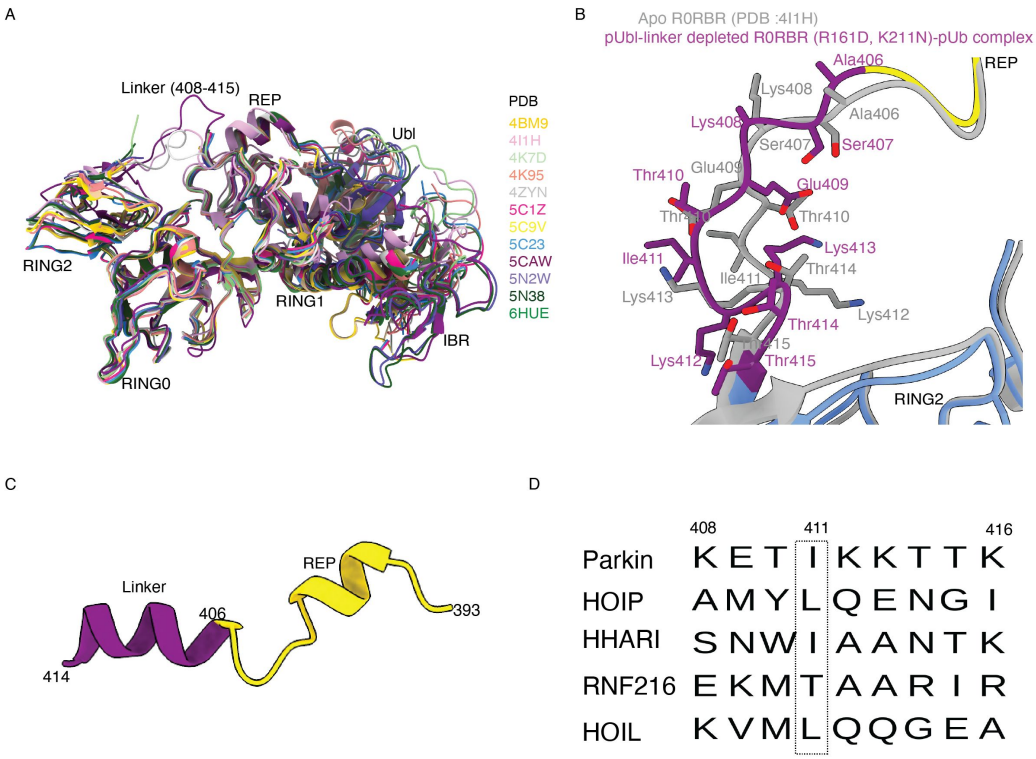

ExtendedDataFigure-11

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Editors

Reviewing Editor
Luke Wiseman
Scripps Research Institute, La Jolla, United States of America

Senior Editor
Amy Andreotti
Iowa State University, Ames, United States of America

Reviewer #1 (Public Review):

Summary:

The authors used structural and biophysical methods to provide insight into Parkin regulation. The breadth of data supporting their findings was impressive and generally well-orchestrated.

Strengths:

(1) They have done a better job explaining the rationale for their experiments thought-out.

(2) The use of molecular scissors in their construct represents a creative approach to examine inter-domain interactions. Appropriate controls were included.

(3) From my assessment, the experiments are well-conceived and executed.

(4) The authors do a better job of highlighting the question being addressed experimentally.

https://doi.org/10.7554/eLife.96699.2.sa3

Reviewer #2 (Public Review):

In the revised manuscript, the authors tried to address some of my comments from the previous round of review. Notably, they have performed some additional ITC experiments where protein precipitation is not an issue to probe interactions between PARKIN and different domains. In addition, they have toned down some of the language in the text to better reflect their data and results. However, I still feel that the manuscript lacks some key answers regarding the relative interactions between p-PARKIN and different domains, as discussed in my previous review. A deeper dive into the underlying biophysical and biochemical features that drive these interactions is important to fully understand the importance of their work. However, this manuscript does provide some interesting potential insights into the mechanisms of PARKIN activation that could be useful for the field moving forward.

https://doi.org/10.7554/eLife.96699.2.sa2

Reviewer #3 (Public Review):

Summary:

In their manuscript, Lenka et al present data that could suggest an "in trans" model of Parkin ubiquitination activity. Parkin is an intensely studied E3 ligase implicated in mitophagy, whereby missense mutations to the PARK2 gene are known to cause autosomal recessive

juvenile parkinsonism. From a mechanistic point of view, Parkin is extremely complex. Its activity is tightly controlled by several modes of auto-inhibition that must be released by queues of mitochondrial damage. While the general overview of Parkin activation has been mapped out in recent years, several details have remained murky. In particular, whether Parkin dimerizes as part of its feed-forward signaling mechanism, and whether said dimerization can facilitate ligase activation, has remained unclear. Here, Lenka et al. use various truncation mutants of Parkin in an attempt to understand the likelihood of dimerization (in support of an "in trans" model for catalysis).

Strengths:

The results are bolstered by several distinct approaches including analytical SEC with cleavable Parkin constructs, ITC interaction studies, ubiquitination assays, protein crystallography, and cellular localization studies.

Weaknesses:

As presented, however, the storyline is very confusing to follow and several lines of experimentation felt like distractions from the primary message. Furthermore, many experiments could only indirectly support the author's conclusions, and therefore the final picture of what new features can be firmly added to the model of Parkin activation and function is unclear.

Following peer review and revision, the claims are still not fully supported by direct evidence. While the experimental system may be necessary and/or convenient given the unique challenges in studying Parkin, it does not directly speak toward the conclusions that the authors make, nor does it provide an accurate representation of biology.

https://doi.org/10.7554/eLife.96699.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors used structural and biophysical methods to provide insight into Parkin regulation. The breadth of data supporting their findings was impressive and generally well-orchestrated. Still, the impact of their results builds on recent structural studies and the stated impact is based on these prior works.*
>
> *Strengths:*
>
> *(1) After reading through the paper, the major findings are:*
>
> *- RING2 and pUbl compete for binding to RING0.*
>
> *- Parkin can dimerize.*
>
> *- ACT plays an important role in enzyme kinetics.*
>
> *(2) The use of molecular scissors in their construct represents a creative approach to examining inter-domain interactions.*
>
> *(3) From my assessment, the experiments are well-conceived and executed.*

We thank the reviewer for their positive remark and extremely helpful suggestions.

> *Weaknesses:*
>
> *The manuscript, as written, is NOT for a general audience. Admittedly, I am not an expert on Parkin structure and function, but I had to do a lot of homework to try to understand the underlying rationale and impact. This reflects, I think, that the work generally represents an incremental advance on recent structural findings.*
>
> *To this point, it is hard to understand the impact of this work without more information highlighting the novelty. There are several structures of Parkin in various auto-inhibited states, and it was hard to delineate how this is different.*

For the sake of the general audience, we have included all the details of Parkin structures and conformations seen (Extended Fig. 1). The structures in the present study are to validate the biophysical/biochemical experiments, highlighting key findings. For example, we solved the phospho-Parkin (complex with pUb) structure after treatment with 3C protease (Fig. 2C), which washes off the pUbl-linker, as shown in Fig 2B. The structure of the pUbl-linker depleted phospho-Parkin-pUb complex showed that RING2 returned to the closed state (Fig. 2C), which is confirmation of the SEC assay in Fig. 2B. Similarly, the structure of the pUbl-linker depleted phospho-Parkin R163D/K211N-pUb complex (Fig. 3C), was done to validate the SEC data showing displacement of pUbl-linker is independent of pUbl interaction with the basic patch on RING0 (Fig. 3B). In addition, the latter structure also revealed a new donor ubiquitin binding pocket in the linker (connecting REP and RING2) region of Parkin (Fig. 9). Similarly, trans-complex structure of phospho-Parkin (Fig. 4D) was done to validate the biophysical data (Fig. 4A-C, Fig. 5A-D) showing trans-complex between phospho-Parkin and native Parkin. The latter also confirmed that the trans-complex was mediated by interactions between pUbl and the basic patch on RING0 (Fig. 4D). Furthermore, we noticed that the ACT region was disordered in the trans-complex between phospho-Parkin (1-140 + 141-382 + pUb) (Fig. 8A) which had ACT from the trans molecule, indicating ACT might be present in the cis molecule. The latter was validated from the structure of trans-complex between phospho-Parkin with cis ACT (1-76 + 77-382 + pUb) (Fig. 8C), showing the ordered ACT region. The structural finding was further validated by biochemical assays (Fig. 8 D-F, Extended Data Fig. 9C-E).

The structure of TEV-treated R0RBR (TEV) (Extended Data Fig. 4C) was done to ensure that the inclusion of TEV and treatment with TEV protease did not perturb Parkin folding, an important control for our biophysical experiments.

> *As noted, I appreciated the use of protease sites in the fusion protein construct. It is unclear how the loop region might affect the protein structure and function. The authors worked to demonstrate that this did not introduce artifacts, but the biological context is missing.*

We thank the reviewer for appreciating the use of protease sites in the fusion protein construct. Protease sites were used to overcome the competing mode of binding that makes interactions very transient and beyond the detection limit of methods such as ITC or SEC. While these interactions are quite transient in nature, they could still be useful for the activation of various Parkin isoforms that lack either the Ubl domain or RING2 domain (Extended Data Fig. 6, Fig. 10). Also, our Parkin localization assays also suggest an important role of these interactions in the recruitment of Parkin molecules to the damaged mitochondria (Fig. 6).

> *While it is likely that the binding is competitive between the Ubl and RING2 domains, the data is not quantitative. Is it known whether the folding of the distinct domains is independent? Or are there interactions that alter folding? It seems plausible that conformational rearrangements may invoke an orientation of domains that would be incompatible. The biological context for the importance of this interaction was not clear to me.*

This is a great point. In the revised manuscript, we have included quantitative data between phospho-Parkin and untethered ΔUbl-Parkin (TEV) (Fig. 5B) showing similar interactions using phospho-Parkin K211N and untethered ΔUbl-Parkin (TEV) (Fig. 4B). Folding of Ubl domain or various combinations of RING domains lacking Ubl seems okay. Also, folding of the RING2 domain on its own appears to be fine. However, human Parkin lacking the RING2 domain seems to have some folding issues, majorly due to exposure of hydrophobic pocket on RING0, also suggested by previous efforts (Gladkova et al.ref. 24, Sauve et al. ref. 29). The latter could be overcome by co-expression of RING2 lacking Parkin construct with PINK1 (Sauve et al. ref. 29) as phospho-Ubl binds on the same hydrophobic pocket on RING0 where RING2 binds. A drastic reduction in the melting temperature of phospho-Parkin (Gladkova et al.ref. 24), very likely due to exposure of hydrophobic surface between RING0 and RING2, correlates with the folding issues of RING0 exposed human Parkin constructs.

From the biological context, the competing nature between phospho-Ubl and RING2 domains could block the non-specific interaction of phosphorylated-ubiquitin-like proteins (phospho-Ub or phospho-NEDD8) with RING0 (Lenka et al. ref. 33), during Parkin activation.

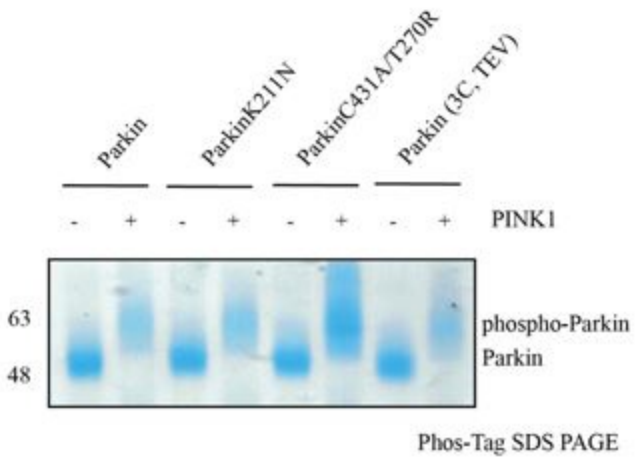
> *(5) What is the rationale for mutating Lys211 to Asn? Were other mutations tried? Glu? Ala? Just missing the rationale. I think this may have been identified previously in the field, but not clear what this mutation represents biologically.*

Lys211Asn is a Parkinson's disease mutation; therefore, we decided to use the same mutation for biophysical studies.

> *I was confused about how the phospho-proteins were generated. After looking through the methods, there appear to be phosphorylation experiments, but it is unclear what the efficiency was for each protein (i.e. what % gets modified). In the text, the authors refer to phospho-Parkin (T270R, C431A), but not clear how these mutations might influence this process. I gather that these are catalytically inactive, but it is unclear to me how this is catalyzing the ubiquitination in the assay.*

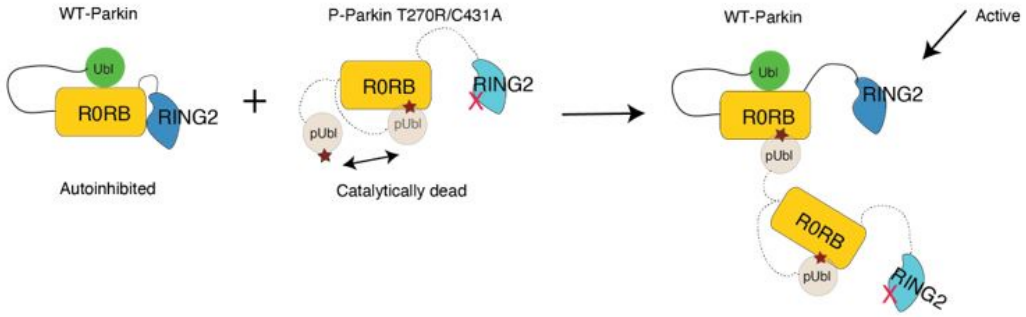
This is an excellent question. Because different phosphorylation statuses would affect the analysis, we ensured complete phosphorylation status using Phos-Tag SDS-PAGE, as shown below.

Author response image 1.



Our biophysical experiments in Fig. 5C show that trans complex formation is mediated by interactions between the basic patch (comprising K161, R163, K211) on RING0 and phospho-Ubl domain in trans. These interactions result in the displacement of RING2 (Fig. 5C). Parkin activation is mediated by displacement of RING2 and exposure of catalytic C431 on RING2. While phospho-Parkin T270R/C431A is catalytically dead, the phospho-Ubl domain of phospho-Parkin T270R/C431would bind to the basic patch on RING0 of WT-Parkin resulting in activation of WT-Parkin as shown in Fig. 5E. A schematic figure is shown below to explain the same.

Author response image 2.



> *(7) The authors note that "ACT can be complemented in trans; however, it is more efficient in cis", but it is unclear whether both would be important or if the favored interaction is dominant in a biological context.*

First, this is an excellent question about the biological context of ACT and needs further exploration. While due to the flexible nature of ACT, it can be complemented both in cis and trans, we can only speculate cis interactions between ACT and RING0 could be more relevant from the biological context as during protein synthesis and folding, ACT would be translated before RING2, and thus ACT would occupy the small hydrophobic patch on RING0 in cis. Unpublished data shows the replacement of the ACT region by Biogen compounds to activate

Parkin (https://doi.org/10.21203/rs.3.rs-4119143/v1). The latter finding further suggests the flexibility in this region.

> *(8) The authors repeatedly note that this study could aid in the development of small-molecule regulators against Parkin to treat PD, but this is a long way off. And it is not clear from their manuscript how this would be achieved. As stated, this is conjecture.*

As suggested by this reviewer, we have removed this point in the revised manuscript.

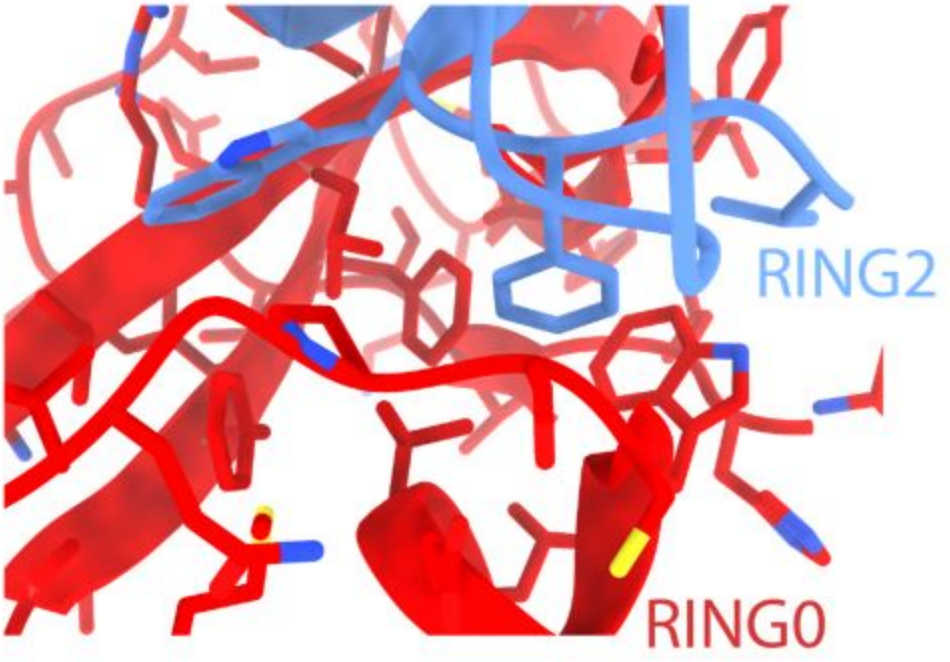
> ***Reviewer #2 (Public Review):***
>
> *This manuscript uses biochemistry and X-ray crystallography to further probe the molecular mechanism of Parkin regulation and activation. Using a construct that incorporates cleavage sites between different Parkin domains to increase the local concentration of specific domains (i.e., molecular scissors), the authors suggest that competitive binding between the p-Ubl and RING2 domains for the RING0 domain regulates Parkin activity. Further, they demonstrate that this competition can occur in trans, with a p-Ubl domain of one Parkin molecule binding the RING0 domain of a second monomer, thus activating the catalytic RING1 domain. In addition, they suggest that the ACT domain can similarly bind and activate Parkin in trans, albeit at a lower efficiency than that observed for p-Ubl. The authors also suggest from crystal structure analysis and some biochemical experiments that the linker region between RING2 and repressor elements interacts with the donor ubiquitin to enhance Parkin activity. Ultimately this manuscript challenges previous work suggesting that the p-Ubl domain does not bind to the Parkin core in the mechanism of Parkin activation. The use of the 'molecular scissors' approach to probe these effects is an interesting approach to probe this type of competitive binding. However, there are issues with the experimental approach manuscript that detract from the overall quality and potential impact of the work.*

We thank the reviewer for their positive remark and constructive suggestions.

> *The competitive binding between p-Ubl and RING2 domains for the Parkin core could have been better defined using biophysical and biochemical approaches that explicitly define the relative affinities that dictate these interactions. A better understanding of these affinities could provide more insight into the relative bindings of these domains, especially as it relates to the in trans interactions.*

This is an excellent point regarding the relative affinities of pUbl and RING2 for the Parkin core (lacking Ubl and RING2). While we could purify p-Ubl, we failed to purify human Parkin (lacking RING2 and phospho-Ubl). The latter folding issues were likely due to the exposure of a highly hydrophobic surface on RING0 (as shown below) in the absence of pUbl and RING2 in the R0RB construct. Also, RING2 with an exposed hydrophobic surface would be prone to folding issues, which is not suitable for affinity measurements. A drastic reduction in the melting temperature of phospho-Parkin (Gladkova et al.ref. 24) also highlights the importance of a hydrophobic surface between RING0 and RING2 on Parkin folding/stability. A separate study would be required to try these Parkin constructs from different species and ensure proper folding before using them for affinity measurements.

Author response image 3.



> *I also have concerns about the results of using molecular scissors to 'increase local concentrations' and allow for binding to be observed. These experiments are done primarily using proteolytic cleavage of different domains followed by size exclusion chromatography. ITC experiments suggest that the binding constants for these interactions are in the μM range, although these experiments are problematic as the authors indicate in the text that protein precipitation was observed during these experiments. This type of binding could easily be measured in other assays. My issue relates to the ability of a protein complex (comprising the core and cleaved domains) with a Kd of 1 μM to be maintained in an SEC experiment. The off-rates for these complexes must be exceeding slow, which doesn't really correspond to the low μM binding constants discussed in the text. How do the authors explain this? What is driving the Koff to levels sufficiently slow to prevent dissociation by SEC? Considering that the authors are challenging previous work describing the lack of binding between the p-Ubl domain and the core, these issues should be better resolved in this current manuscript. Further, it's important to have a more detailed understanding of relative affinities when considering the functional implications of this competition in the context of full-length Parkin. Similar comments could be made about the ACT experiments described in the text.*

This is a great point. In the revised manuscript, we repeated ITC measurements in a different buffer system, which gave nice ITC data. In the revised manuscript, we have also performed ITC measurements using native phospho-Parkin. Phospho-Parkin and untethered ΔUbl-Parkin (TEV) (Fig. 5B) show similar affinities as seen between phospho-Parkin K211N and untethered ΔUbl-Parkin (TEV) (Fig. 4B). However, Kd values were consistent in the range of 1.0 ± 0.4 μM which could not address the reviewer's point regarding slow off-rate. The crystal structure of the trans-complex of phospho-Parkin shows several hydrophobic and ionic interactions between p-Ubl and Parkin core, suggesting a strong interaction and, thus, justifying the co-elution on SEC. Additionally, ITC measurements between E2-Ub and P-Parkin-pUb show

similar affinity (Kd = 0.9 ± 0.2 μM) (Kumar et al., 2015, EMBO J.), and yet they co-elute on SEC (Kumar et al., 2015, EMBO J.).

> *Ultimately, this work does suggest additional insights into the mechanism of Parkin activation that could contribute to the field. There is a lot of information included in this manuscript, giving it breadth, albeit at the cost of depth for the study of specific interactions. Further, I felt that the authors oversold some of their data in the text, and I'd recommend being a bit more careful when claiming an experiment 'confirms' a specific model. In many cases, there are other models that could explain similar results. For example, in Figure 1C, the authors state that their crystal structure 'confirms' that "RING2 is transiently displaced from the RING0 domain and returns to its original position after washing off the p-Ubl linker". However, it isn't clear to me that RING2 ever dissociated when prepared this way. While there are issues with the work that I feel should be further addressed with additional experiments, there are interesting mechanistic details suggested by this work that could improve our understanding of Parkin activation. However, the full impact of this work won't be fully appreciated until there is a more thorough understanding of the regulation and competitive binding between p-Ubl and RIGN2 to RORB both in cis and in trans.*

We thank the reviewer for their positive comment. In the revised manuscript, we have included the reviewer's suggestion. The conformational changes in phospho-Parkin were established from the SEC assay (Fig. 2A and Fig. 2B), which show displacement/association of phospho-Ubl or RING2 after treatment of phospho-Parkin with 3C and TEV, respectively. For crystallization, we first phosphorylated Parkin, where RING2 is displaced due to phospho-Ubl (as shown in SEC), followed by treatment with 3C protease, which led to pUbl wash-off. The Parkin core separated from phospho-Ubl on SEC was used for crystallization and structure determination in Fig. 2C, where RING2 returned to the RING0 pocket, which confirms SEC data (Fig. 2B).

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In their manuscript "Additional feedforward mechanism of Parkin activation via binding of phospho-UBL and RING0 in trans", Lenka et al present data that could suggest an "in trans" model of Parkin ubiquitination activity. Parkin is an intensely studied E3 ligase implicated in mitophagy, whereby missense mutations to the PARK2 gene are known to cause autosomal recessive juvenile parkinsonism. From a mechanistic point of view, Parkin is extremely complex. Its activity is tightly controlled by several modes of auto-inhibition that must be released by queues of mitochondrial damage. While the general overview of Parkin activation has been mapped out in recent years, several details have remained murky. In particular, whether Parkin dimerizes as part of its feed-forward signaling mechanism, and whether said dimerization can facilitate ligase activation, has remained unclear. Here, Lenka et al. use various truncation mutants of Parkin in an attempt to understand the likelihood of dimerization (in support of an "in trans" model for catalysis).*
>
> *Strengths:*
>
> *The results are bolstered by several distinct approaches including analytical SEC with cleavable Parkin constructs, ITC interaction studies, ubiquitination assays, protein crystallography, and cellular localization studies.*

We thank the reviewer for their positive remark.

> *As presented, however, the storyline is very confusing to follow and several lines of experimentation felt like distractions from the primary message. Furthermore, many experiments could only indirectly support the author's conclusions, and therefore the final picture of what new features can be firmly added to the model of Parkin activation and function is unclear.*

We thank the reviewer for their constructive criticism, which has helped us to improve the quality of this manuscript.

> *Major concerns:*
>
> *(1) This manuscript solves numerous crystal structures of various Parkin components to help support their idea of in trans transfer. The way these structures are presented more resemble models and it is unclear from the figures that these are new complexes solved in this work, and what new insights can be gleaned from them.*

The structures in the present study are to validate the biophysical/biochemical experiments highlighting key findings. For example, we solved the phospho-Parkin (complex with pUb) structure after treatment with 3C protease (Fig. 2C), which washes off the pUbl-linker, as shown in Fig. 2B. The structure of pUbl-linker depleted phospho-Parkin-pUb complex showed that RING2 returned to the closed state (Fig. 2C), which is confirmation of the SEC assay in Fig. 2B. Similarly, the structure of the pUbl-linker depleted phospho-Parkin R163D/K211N-pUb complex (Fig. 3C), was done to validate the SEC data showing displacement of pUbl-linker is independent of pUbl interaction with the basic patch on RING0 (Fig. 3B). In addition, the latter structure also revealed a new donor ubiquitin binding pocket in the linker (connecting REP and RING2) region of Parkin (Fig. 9). Similarly, trans-complex structure of phospho-Parkin (Fig. 4D) was done to validate the biophysical data (Fig. 4A-C, Fig. 5A-D) showing trans-complex between phospho-Parkin and native Parkin. The latter also confirmed that the trans-complex was mediated by interactions between pUbl and the basic patch on RING0 (Fig. 4D). Furthermore, we noticed that the ACT region was disordered in the trans-complex between phospho-Parkin (1-140 + 141-382 + pUb) (Fig. 8A) which had ACT from the trans molecule, indicating ACT might be present in the cis molecule. The latter was validated from the structure of trans-complex between phospho-Parkin with cis ACT (1-76 + 77-382 + pUb) (Fig. 8C), showing the ordered ACT region. The structural finding was further validated by biochemical assays (Fig. 8 D-F, Extended Data Fig. 9C-E).

The structure of TEV-treated R0RBR (TEV) (Extended Data Fig. 4C) was done to ensure that the inclusion of TEV and treatment with TEV protease did not perturb Parkin folding, an important control for our biophysical experiments.

> *(2) There are no experiments that definitively show the in trans activation of Parkin. The binding experiments and size exclusion chromatography are a good start, but the way these experiments are performed, they'd be better suited as support for a stronger experiment showing Parkin dimerization. In addition, the rationale for an in trans activation model is not convincingly explained until the concept of Parkin isoforms is introduced in the Discussion. The authors should consider expanding this concept into other parts of the manuscript.*

We thank the reviewer for appreciating the Parkin dimerization. Our biophysical data in Fig. 5C shows that Parkin dimerization is mediated by interactions between phospho-Ubl and RING0 in trans, leading to the displacement of RING2. However, Parkin K211N (on RING0) mutation perturbs interaction with phospho-Parkin and leads to loss of Parkin dimerization and loss of RING2 displacement (Fig. 5C). The interaction between pUbl and K211 pocket on RING0 leads to the displacement of RING2 resulting in Parkin activation as catalytic residue

C431 on RING2 is exposed for catalysis. The biophysical experiment is further confirmed by a biochemical experiment where the addition of catalytically in-active phospho-Parkin T270R/C431A activates autoinhibited WT-Parkin in trans using the mechanism as discussed (a schematic representation also shown in Author response image 2).

We thank this reviewer regarding Parkin isoforms. In the revised manuscript, we have included Parkin isoforms in the results section, too.

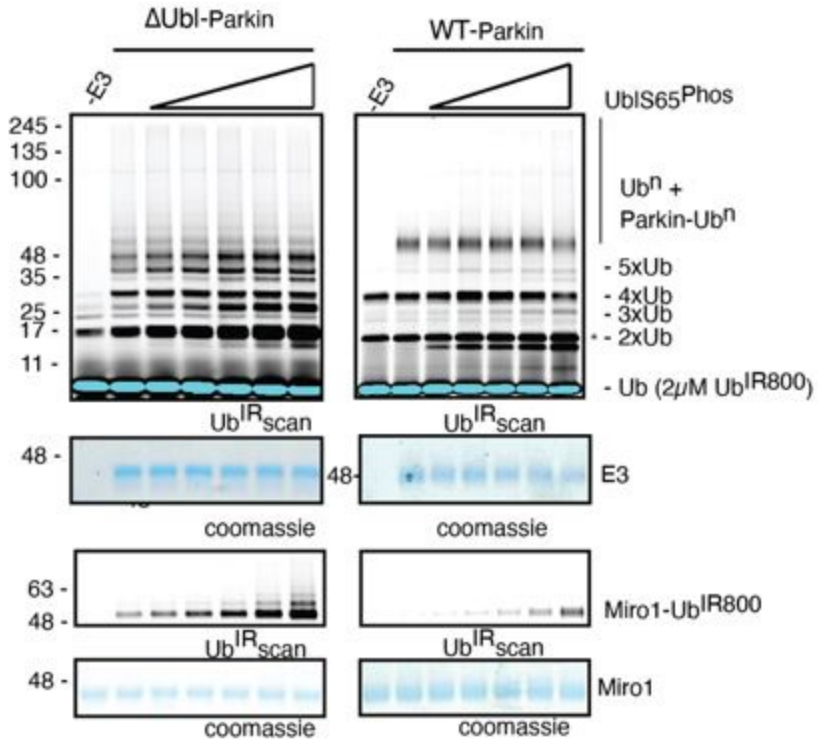
> *(2a) For the in trans activation experiment using wt Parkin and pParkin (T270R/C431A) (Figure 3D), there needs to be a large excess of pParkin to stimulate the catalytic activity of wt Parkin. This experiment has low cellular relevance as these point mutations are unlikely to occur together to create this nonfunctional pParkin protein. In the case of pParkin activating wt Parkin (regardless of artificial point mutations inserted to study specifically the in trans activation), if there needs to be much more pParkin around to fully activate wt Parkin, isn't it just more likely that the pParkin would activate in cis?*

To test phospho-Parkin as an activator of Parkin in trans, we wanted to use the catalytically inactive version of phospho-Parkin to avoid the background activity of p-Parkin. While it is true that a large excess of pParkin (T270R/C431A) is required to activate WT-Parkin in the in vitro set-up, it is not very surprising as in WT-Parkin, the unphosphorylated Ubl domain would block the E2 binding site on RING1. Also, due to interactions between pParkin (T270R/C431A) molecules, the net concentration of pParkin (T270R/C431A) as an activator would be much lower. However, the Ubl blocking E2 binding site on RING1 won't be an issue between phospho-Parkin molecules or between Parkin isoforms (lacking Ubl domain or RING2).

> *(2ai) Another underlying issue with this experiment is that the authors do not consider the possibility that the increased activity observed is a result of increased "substrate" for auto-ubiquitination, as opposed to any role in catalytic activation. Have the authors considered looking at Miro as a substrate in order to control for this?*

This is quite an interesting point. However, this will be only possible if Parkin is ubiquitinated in trans, as auto-ubiquitination is possible with active Parkin and not with catalytically dead (phospho-Parkin T270R, C431A) or autoinhibited (WT-Parkin). Also, in the previous version of the manuscript, where we used only phospho-Ubl as an activator of Parkin in trans, we tested Miro1 ubiquitination and auto-ubiquitination, and the results were the same (Author response image 4).

Author response image 4.



> *(2b) The authors mention a "higher net concentration" of the "fused domains" with RING0, and use this to justify artificially cleaving the Ubl or RING2 domains from the Parkin core. This fact should be moot. In cells, it is expected there will only be a 1:1 ratio of the Parkin core with the Ubl or RING2 domains. To date, there is no evidence suggesting multiple pUbls or multiple RING2s can bind the RING0 binding site. In fact, the authors here even show that either the RING2 or pUbl needs to be displaced to permit the binding of the other domain. That being said, there would be no "higher net concentration" because there would always be the same molar equivalents of Ubl, RING2, and the Parkin core.*

We apologize for the confusion. “Higher net concentration” is with respect to fused domains versus the domain provided in trans. Due to the competing nature of the interactions between pUbl/RING2 and RING0, the interactions are too transient and beyond the detection limit of the biophysical techniques. While the domains are fused (for example, RING0-RING2 in the same polypeptide) in a polypeptide, their effective concentrations are much higher than those (for example, pUbl) provided in trans; thus, biophysical methods fail to detect the interaction. Treatment with protease solves the above issue due to the higher net concentration of the fused domain, and trans interactions can be measured using biophysical techniques. However, the nature of these interactions and conformational changes is very transient, which is also suggested by the data. Therefore, Parkin molecules will never remain associated; rather, Parkin will transiently interact and activate Parkin molecules in trans.

> *(2c) A larger issue remaining in terms of Parkin activation is the lack of clarity surrounding the role of the linker (77-140); particularly whether its primary role is to tether the Ubl to the cis Parkin molecule versus a role in permitting distal interactions to a trans molecule. The way the authors have conducted the experiments presented in*

> *Figure 2 limits the possible interactions that the activated pUbl could have by (a) ablating the binding site in the cis molecule with the K211N mutation; (b) further blocking the binding site in the cis molecule by keeping the RING2 domain intact. These restrictions to the cis parkin molecule effectively force the pUbl to bind in trans. A competition experiment to demonstrate the likelihood of cis or trans activation in direct comparison with each other would provide stronger evidence for trans activation.*

This is an excellent point. In the revised manuscript, we have performed experiments using native phospho-Parkin (Revised Figure 5), and the results are consistent with those in Figure 2 (Revised Figure 4), where we used the K211N mutation.

> *(3) A major limitation of this study is that the authors interpret structural flexibility from experiments that do not report directly on flexibility. The analytical SEC experiments report on binding affinity and more specifically off-rates. By removing the interdomain linkages, the accompanying on-rate would be drastically impacted, and thus the observations are disconnected from a native scenario. Likewise, observations from protein crystallography can be consistent with flexibility, but certainly should not be directly interpreted in this manner. Rigorous determination of linker and/or domain flexibility would require alternative methods that measure this directly.*

We also agree with the reviewer that these methods do not directly capture structural flexibility. Also, rigorous determination of linker flexibility would require alternative methods that measure this directly. However, due to the complex nature of interactions and technical limitations, breaking the interdomain linkages was the best possible way to capture interactions in trans. Interestingly, all previous methods that report cis interactions between pUbl and RING0 also used a similar approach (Gladkova et al.ref. 24, Sauve et al. ref. 29).

> *(4) The analysis of the ACT element comes across as incomplete. The authors make a point of a competing interaction with Lys48 of the Ubl domain, but the significance of this is unclear. It is possible that this observation could be an overinterpretation of the crystal structures. Additionally, the rationale for why the ACT element should or shouldn't contribute to in trans activation of different Parkin constructs is not clear. Lastly, the conclusion that this work explains the evolutionary nature of this element in chordates is highly overstated.*

We agree with the reviewer that the significance of Lys48 is unclear. We have presented this just as one of the observations from the crystal structure. As the reviewer suggested, we have removed the sentence about the evolutionary nature of this element from the revised manuscript.

> *(5) The analysis of the REP linker element also seems incomplete. The authors identify contacts to a neighboring pUb molecule in their crystal structure, but the connection between this interface (which could be a crystallization artifact) and their biochemical activity data is not straightforward. The analysis of flexibility within this region using crystallographic and AlphaFold modeling observations is very indirect. The authors also draw parallels with linker regions in other RBR ligases that are involved in recognizing the E2-loaded Ub. Firstly, it is not clear from the text or figures whether the "conserved" hydrophobic within the linker region is involved in these alternative Ub interfaces. And secondly, the authors appear to jump to the conclusion that the Parkin linker region also binds an E2-loaded Ub, even though their original observation from the crystal structure seems inconsistent with this. The entire analysis feels very preliminary and also comes across as tangential to the primary storyline of in trans Parkin activation.*

We agree with the reviewer that crystal structure data and biochemical data are not directly linked. In the revised manuscript, we have also highlighted the conserved hydrophobic in the linker region at the ubiquitin interface (Fig. 9C and Extended Data Fig. 11A), which was somehow missed in the original manuscript. We want to add that a very similar analysis and supporting experiments identified donor ubiquitin-binding sites on the IBR and helix connecting RING1-IBR (Kumar et al., Nature Str. and Mol. Biol., 2017), which several other groups later confirmed. In the mentioned study, the Ubl domain of Parkin from the symmetry mate Parkin molecule was identified as a mimic of “donor ubiquitin” on IBR and helix connecting RING1-IBR.

In the present study, a neighboring pUb molecule in the crystal structure is identified as a donor ubiquitin mimic (Fig. 9C) by supporting biophysical/biochemical experiments. First, we show that mutation of I411A in the REP linker of Parkin perturbs Parkin interaction with E2~Ub (donor) (Fig. 9F). Another supporting experiment was performed using a Ubiquitin-VS probe assay, which is independent of E2. Assays using Ubiquitin-VS show that I411A mutation in the REP-RING2 linker perturbs Parkin charging with Ubiquitin-VS (Extended Data Fig. 11 B). Furthermore, the biophysical data showing loss of Parkin interaction with donor ubiquitin is further supported by ubiquitination assays. Mutations in the REP-RING2 linker perturb the Parkin activity (Fig. 9E), confirming biophysical data. This is further confirmed by mutations (L71A or L73A) on ubiquitin (Extended Data Fig. 11C), resulting in loss of Parkin activity. The above experiments nicely establish the role of the REP-RING2 linker in interaction with donor ubiquitin, which is consistent with other RBRs (Extended Data Fig. 11A).

While we agree with the reviewer that this appears tangential to the primary storyline in trans-Parkin activation, we decided to include this data because it could be of interest to the field.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations For The Authors):***
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> *(1) For clarity, a schematic of the domain architecture of Parkin would be helpful at the outset in the main figures. This will help with the introduction to better understand the protein organization. This is lost in the Extended Figure in my opinion.*

We thank the reviewer for suggesting this, which we have included in Figure 1 of the revised manuscript.

> *(2) Related to the competition between the Ubl and RING2 domains, can competition be shown through another method? SPR, ITC, etc? ITC was used in other experiments, but only in the context of mutations (Lys211Asn)? Can this be done with WT sequence?*

This is an excellent suggestion. In the revised Figure 5, we have performed ITC experiment using WT Parkin, and the results are consistent with what we observed using Lys211Asn Parkin.

> *(3) The authors also note that "the AlphaFold model shows a helical structure in the linker region of Parkin (Extended Data Figure 10C), further confirming the flexible nature of this region"... but the secondary structure would not be inherently flexible. This is confusing.*

The flexibility is in terms of the conformation of this linker region observed under the open or closed state of Parkin. In the revised manuscript, we have explained this point more clearly.

> *(4) The manuscript needs extensive revision to improve its readability. Minor grammatical mistakes were prevalent throughout.*

We thank the reviewer for pointing out this and we have corrected these in the revised manuscript.

> *(5) The confocal images are nice, but inset panels may help highlight the regions of interest (ROIs).*

This is corrected in the revised manuscript.

> *(6) Trans is misspelled ("tans") towards the end of the second paragraph on page 16.*

This is corrected in the revised manuscript.

> *(7) The schematics are helpful, but some of the lettering in Figure 2 is very small.*

This is corrected in the revised manuscript.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) A significant portion of the results section refers to the supplement, making the overall readability very difficult.*

We accept this issue as a lot of relevant data could not be added to the main figures and thus ended up in the supplement. In the revised manuscript, we have moved some of the supplementary figures to the main figures.

> *(2) Interpretation of the experiments utilizing many different Parkin constructs and cleavage scenarios (particularly the SEC and crystallography experiments) is extremely difficult. The work would benefit from a layout of the Parkin model system, highlighting cleavage sites, key domain terminology, and mutations used in the study, presented together and early on in the manuscript. Using this to identify a simpler system of referencing Parkin constructs would also be a large improvement.*

This is a great suggestion. We have included these points in the revised manuscript, which has improved the readability.

> *(3) Lines 81-83; the authors say they "demonstrate the conformational changes in Parkin during the activation process", but fail to show any actual conformational changes. Further, much of what is demonstrated in this work (in terms of crystal structures) corroborates existing literature. The authors should use caution not to overstate their original conclusions in light of the large body of work in this area.*

We thank the reviewer for pointing out this. We have corrected the above statement in the revised manuscript to indicate that we meant it in the context of trans conformational changes.

> *(4) Line 446 and 434; there is a discrepancy about which amino acid is present at residue 409. Is this a K408 typo? The authors also present mutational work on K416, but this residue is not shown in the structure panel.*

We thank the reviewer for pointing out this. In the revised manuscript, we have corrected these typos.

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