Intricate mechanism (s) of substrate specificity and loss of function on disease mutation (K211N) of Parkin

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Abstract

Parkin mutations, including K211N, lead to the onset of Parkinson's disease. Ubiquitin-like proteins (NEDD8, Ubiquitin) and Ubiquitin-like (Ubl) domains of Parkin are phosphorylated by PINK1. Parkin is activated by binding of phospho-ubiquitin, phospho-NEDD8, and phospho-Ubl domain on the two distinct pockets of Parkin. While pUb/pNEDD8 binds in the RING1 pocket, pUb/pUbl binding with the RING0 pocket is reported. pUb binding in the RING0 pocket (comprising K211) is believed to rationalize the loss of pUb-mediated Parkin K211N activation. Herein, we demonstrate that the RING0 pocket is specific for pUbl and does not bind with pUb/pNEDD8. In contrast, the RING1 pocket is more suitable for binding with phospho-NEDD8 than phospho-ubiquitin. Also, we demonstrate that the binding of activators in the RING1 and RING0 pockets of Parkin leads to a distinct extent of conformational changes on the RING2. We also show that loss of Parkin activity in disease mutation K211N is independent of Parkin activators (pUb/pNEDD8) binding. Furthermore, the crystal structure of Parkin K211N in complex with pNEDD8 reveals the mechanism of Parkin inactivation due to conformational changes locking RING2 with RING0. This study would help design small-molecule Parkin activators against Parkinson's disease.

Introduction

PARK2 and PARK6 genes expressing Parkin E3 ligase and PINK1 (PTEN-induced putative kinase 1) are found to be heavily mutated in Parkinson's Disease (PD) patients. Parkin and PINK1 control the mitochondrial homeostasis process autophagy (mitophagy) of the damaged mitochondria[1–7].

Parkin is a member of RBR family E3 ligase [8,9] consisting of an N-terminal ubiquitin-like (Ubl) domain followed by four Zn^{2+} binding domains RING0, RING1, inbetweenRING

(IBR), and RING2 domains [10]. Like other members of the RBR family E3 ligase, Parkin catalytic Cys431 (on RING2) of Parkin is charged with ubiquitin in E2 dependent manner before the transfer of ubiquitin on lysines of its substrate[11–14]. Parkin is an autoinhibited cytosolic protein. The E2 binding site on Parkin is blocked by the presence of Ubl on RING1. A short repressor (REP) element between IBR and RING2 also restricts E2 binding. A significant inhibition of Parkin activity is caused by RING0, which occludes catalytic Cys431 on RING2[8,15–17].

Phosphorylation of Ser65 on ubiquitin by PINK1 leads to the recruitment of Parkin on mitochondria-mediated by enhanced interaction between RING1 of Parkin and pUb[11,12,15]. PhosphoSer65 of the Ubl domain of Parkin is phosphorylated by PINK1[12,16–19], leading to fully active Parkin conformation by binding in the RING0 pocket. RING0 pocket (comprising K161, R163, and K211) and RING1 pocket (comprising R305, H302, and K151) bind with phospho-Ser65 of phosphorylated Parkin activators [20–26]. Recently, PINK1-mediated Ser65 phosphorylation on another ubiquitin-like protein, NEDD8, was shown to activate Parkin [27]. While several crystal structures of Parkin with pUb are known [22,23,25,26,28], the structure of pNEDD8 and Parkin complex is not available to reveal its molecular mechanism. Furthermore, it remains unclear whether the two pockets (RING0 and RING1) on Parkin recognize different Parkin activators (pUbl, pUb, and pNEDD8) specifically or promiscuously, as suggested by recent publications [27–29]

Recent publications suggested two pUb binding sites on Parkin, one in the RING1 pocket and another in the RING0 pocket [28,29]. They demonstrated that disease mutation K211N showed loss of Parkin activation in the presence of pUb and loss of Parkin K211N localization to the mitochondria [29]. Parkin construct lacking RING2 domain was used to demonstrate the binding of pUb in both RING0 and RING1 pockets, and K211N mutation leading to loss of pUb binding[28,29]. A new feedforward pathway of Parkin activation was proposed where pUb binding is preferred over pUbl binding in the RING0 pocket [28,29]In light of our recent work demonstrating RING2 negatively affecting pUbl binding in the RING0 pocket, the presence of RING2 could provide specificity [20]. Also, in our recent work, Parkin K211N (C431F), unlike Parkin H302A (C431F), did not show any defect in its translocation to the mitochondria when it was co-expressed with native Parkin[20]. The latter warrants a detailed biophysical study to understand the loss of Parkin activation in disease mutation K211N.

Herein, we solved the structure of Parkin with phospho-NEDD8, which shows the binding of phospho-NEDD8 in the RING1 pocket where phospho-ubiquitin binds. However,

the structure shows new interactions between pNEDD8 mediated by favorable residues on NEDD8 that are not present in pUbl or pUb. Ubiquitination assays further supported the latter to establish a more robust activation of Parkin by pNEDD8 compared to pUb. We also demonstrate that pNEDD8/pUb specifically binds in the RING1 pocket and does not cross-talk with the RING0 pocket. We also demonstrate that specificity in RING0 and RING1 is mediated by unique charge distribution on Parkin surrounding the basic residues that bind phospho-Ser65 of pUbl or pUb/pNEDD8. Similarly, charge distribution around phospho-Ser65 of pUbl, pUb/pNEDD8 is more favorable for specific binding in the RING0 and RING0 pockets, respectively. Our data also suggests that unlike binding in the RING0 pocket, which results in large conformational changes of RING2, binding in the RING1 pocket does not show large conformational changes in RING2. Furthermore, we demonstrate that disease mutation K211N on Parkin perturbs E3 ligase activity independent of Parkin activators (pUb/pNEDD8), unlike previously suggested. We solved the structure of K211N Parkin with pNEDD8, which reveals new conformational changes in RING0, leading to new interactions between RING0 and RING2. These new interactions restrict the movement of RING2 in Parkin K211N, leading to the loss of E3 ligase activity. Overall, this study would help therapeutic efforts to develop small-molecule activators against Parkin, specifically against mutants such as K211N that lead to very strong defects and were proposed to lack binding with activators.

Results

pNEDD8 leads to more robust activation of Parkin

Recent publications suggested two pUb binding sites on Parkin, one in the RING1 pocket and one in the RING0 pocket where pUbl binds [28,29]. Another study showed NEDD8 phosphorylation by PINK1 leading to Parkin activation [27]. While the phosphorylation of different ubiquitin-like proteins by PINK1 and their binding on Parkin is well known, the selectivity and specificity in the above pathway remain elusive. To test the specificity between pUb and pNEDD8, we performed ubiquitination assays on Parkin using increasing concentrations of pUb and pNEDD8. Interestingly, we noticed that pNEDD8-mediated Parkin activation is significantly higher than pUb-mediated Parkin activation (Fig. 1A). Interestingly, assays performed using unphosphorylated NEDD8/Ub also show higher activation of Parkin with NEDD8 (Fig. 1B). Overall, our data suggested that NEDD8 is a better activator of Parkin than Ub.

The crystal structure of Parkin with pNEDD8 suggests optimized binding between Parkin and NEDD8

As our activity assays suggest that NEDD8 is a better substrate over Ub (Fig 1), we wanted to understand the molecular details of NEDD8 and Parkin interaction. Unlike pUb and human Parkin structures [22,23,25], we crystallized the complex of pNEDD8 and human Parkin without any chemical modification/artificial tethering. Human R0RBR and pNEDD8 complex was purified over size-exclusion chromatography and crystallized. The structure R0RBR and pNEDD8 was determined at 2.25 Å. The overall structure of the Parkin and pNEDD8 complex was quite similar to the Parkin and pUb complex (Extended Data Fig. 1A). Although RING2 is not displaced (Extended Data Fig. 1B), we noticed a significantly higher b-factor for RING2 (Fig. 2A) in Parkin and pNEDD8 structure. The latter indicates the flexibility caused in the RING2 region of Parkin due to pNEDD8 binding.

Closer inspection of Parkin structures with pUb or pNEDD8 reveals some minor conformational changes on Helix-1 (H1) of Parkin. For example, Q317 is away from pUb interface in Parkin and pUb (Fig. 2B, 2C), whereas Q317 is present at the interface of pNEDD8 (Fig. 2B, 2D) and forms hydrogen bond with H68 of pNEDD8 (Fig. 2D). Beside the conserved interactions between Parkin and pUb or pNEDD8, new interactions are also seen between Parkin and pNEDD8. For example, S46 of pNEDD8 forms a hydrogen bond with Q316 of Parkin (Fig. 2D). Y45 of pNEDD8 forms hydrogen bonds with Y312 and H302 of Parkin (Fig. 2D). Furthermore, K60 of pNEDD8 forms a hydrogen bond with the carbonyl of L187 of Parkin (Fig. 2D). In addition to the above hydrogen bonds, L62 of pNEDD8 forms hydrophobic interactions with V224 of Parkin. Interestingly, among all known Ubls, which can be phosphorylated by PINK1, the above residues are only present in pNEDD8 and replaced by unfavorable residues in Ubl and Ub (Fig. 2E). These new interactions explain the higher activation by NEDD8 in Figure 1, thus suggesting NEDD8 would be a preferred substrate for binding in the RING1 substrate of Parkin.

Parkin activators (pNEDD8/pUb) specifically bind in the RING1 pocket

Recent publications suggested that the RING0 pocket (comprising K211) binds with pUb and leads to the feedforward mechanism of Parkin activation [28,29]. In their support, they used constructs lacking RING2 and showed binding of pUB in RING0 along with binding in the RING1 pocket. Our recent work demonstrated that RING2 negatively affects the pUbl binding in the RING0 [20]Therefore, we wondered whether the presence of RING2 would provide specificity to select Parkin activator in the RING0 pocket. To test this, we used

untethered (TEV treated) R0RBR (RING0-RING1-IBR-REP-RING2, TEV site between IBR & REP) protein and performed the RING2 displacement assay similar to our recent research, which shows displacement of RING2 using pUbl/pParkin in trans [20]. We noticed that both pUb or pNEDD8 form complex with R0RBR (Fig. 3A, Extended Data Fig. 2A). However, pUb/pNEDD8 binding failed to displace RING2 from RING0 as suggested by co-elution of REP-RING2 with R0RB (RING0-RING1-IBR) (Fig. 3A, Extended Data Fig. 2A), unlike pUbl/pParkin binding in trans that displace RING2 [20]. Interestingly, pNEDD8, which binds and activates Parkin stronger than pUb, also failed to result in RING2 displacement (Extended Data Fig. 2A). Furthermore, K211N mutation in the RING0 domain did not affect the complex formation between Parkin and pUb/pNEDD8 (Fig. 3B, Extended Data Fig. 2B). However, as expected H302A mutation in the RING1 domain perturbs the complex formation between Parkin and pUb/pNEDD8 (Fig. 3C, Extended Data Fig. 2C). In any of the above conditions, unlike pUbl/pParkin binding in the RING0 pocket displacing the RING2 domain in trans [20], RING2 displacement was not observed suggesting that pUb/pNEDD8 binds specifically in the RING1 pocket. This data also suggests that RING2 domain movement after pUb binding is not as drastic as suggested earlier [28,29]. The latter is consistent with the b-factor analysis of the crystal structure in Fig. 2A, which suggested flexibility, not complete displacement, in the RING2 domain after pNEDD8 binding. Overall, this data suggests the specific nature of two pockets on Parkin and conformational changes to different magnitudes.

Disease mutation K211N perturbs Parkin activity independent of activator (pUb/pNEDD8) binding

Previous data showed that K211N mutation on Parkin leads to loss of pUb-mediated Parkin activation [24,25,29,30]. As our data in Figure 3/Extended Data Figure 2 suggests the specific nature of the two pockets on Parkin and pUb/pNEDD8 not binding with the RING0 pocket, we wanted to understand the role of K211N mutation in Parkin activation. Also, as pNEDD8 activates Parkin stronger than pUb (Fig. 1A), we wondered whether pNEDD8 would rescue the effect of K211N mutation. Interestingly, although, unlike Parkin H302A, Parkin K211N does not show any defect in its interaction with pNEDD8/pUb (Figure 3/Extended Data Figure 2), both Parkin K211N and Parkin H302A are 2-fold less active than native Parkin in the presence of pNEDD8 (Fig. 4A). This observation is consistent with pUb-mediated Parkin activation ([25] and Extended Data Fig. 3). In the lanes without activators (pUb/pNEDD8), we noticed the basal level ubiquitination activity of Parkin K211N was less than WT Parkin (Fig. 4A, Extended Data Fig. 3). Therefore, we decided to test the time-course ubiquitination assay

on Parkin WT/K211N without any activator. Interestingly, we noticed that K211N mutation leads to approximately a 2-fold reduction in basal level of Parkin activity that is independent of any activator (Fig. 4B). Overall, this data suggests that the loss of K211N Parkin activity is not due to its inability to bind with pUb but rather to some unknown reason that needs further exploration.

Unique charge distribution on Parkin determines the specificity of the RING0/RING1 pocket

In contrast to the previous study [28,29], our data suggests that the two pockets (RING0/RING1) on Parkin specifically recognize pUbl and pUb/pNEDD8. Although the sequence comparison in Fig. 2E suggested that key residues are conserved between Ubl, Ub, and NEDD8, differences at some key positions may help determine the specificity. For example, L8 in Ub/NEDD8 is N8 in Ubl, which may not allow binding of pUbl in the RING1 pocket. Also, the difference between key residues mentioned in Fig. 2 helps the specificity between pUb and pNEDD8 for the RING1 pocket. However, the inability of pUb/pNEDD8 to bind the RING0 pocket remains elusive. Interestingly, electrostatic charge surface analysis of Parkin, pUbl, pUb, and pNEDD8 revealed a distinct charge distribution on the ubiquitin-like proteins/domain and the two pockets of Parkin (Fig. 5A-E). pUbl (Fig. 5A) is quite negatively charged compared to pUb (Fig. 5B) or pNEDD8 (Fig. 5C). Furthermore, the surroundings of the basic patch (K161, R163, K211) on the RING0 pocket are made up of electropositive groups (R104/R455) (Fig. 5C). However, the negatively charged (E300) residue is positioned above the basic patch (K151, H302, R305) on the RING1 pocket of Parkin (Fig. 5D). Interestingly, D60 on Ubl is substituted with N60 and K60 on Ub and NEDD8 (Fig. 2E), respectively. An electronegative charge on the 60th position of Ubl would make it more favorable for the RING0 pocket (Fig 5A, 5D). Similarly, N60/K60 on Ub/NEDD8 would not be suitable for interaction with the positively charged residues around the basic patch on the RING0 pocket (Fig. 5B-D).

To validate the structural analysis, we tested Parkin activation assays using pUb (N60D). Interestingly, compared to pUb (WT), pUb (N60D) showed higher activation of Parkin (Fig. 5F, Extended Data Fig. 4), suggesting pUb (N60D) binding in the RING0 pocket, thus leading to a larger displacement of RING2.

To confirm the binding of pUb (N60D) in the RING0 pocket, we performed the RING2 displacement assay. While pUb (WT) did not lead to RING2 displacement and showed binding only in the RING1 pocket (Fig. 6A, Fig. 3), pUb (N60D) resulted in RING2

displacement, and binding in both pockets on Parkin, leading to higher shift on SEC (Fig. 6A). Furthermore, K211N mutation leads to loss of pUb (N60D) binding in RING0 pocket and thus did not displace RING2 pocket (Fig 6B). However, the H302A mutation perturbed the binding of pUb (N60D) in the RING1 pocket while binding with the RING0 pocket and displacement of RING2 remained intact (Fig. 6B). Overall, this data establishes the mechanism of specificity on the RING0 pocket of Parkin.

Disease mutation K211N locks RING2 with RING0 to perturb Parkin activity

As our data clearly showed that the loss of Parkin K211N activity is not due to its inability to bind with pUb/pNEDD8, we wanted to understand the molecular mechanism of the loss of Parkin activity in K211N Parkin. To understand this, we crystallized and determined the structure of the R0RBR (K211N) and pNEDD8 complex. The structure of R0RBR (K211N) and pNEDD8 complex was determined at 1.8 Å (Extended Data Table 1). Similar to R0RBR and pNEDD8 structure, R0RBR K211N and pNEDD8 structure shows only one molecule of pNEDD8 bound in the RING1 pocket, and overall conformation remains the same (Extended Data Fig. 5). However, closer inspection revealed that due to the K211N mutation, the RING0 conformation is changed and pushed towards RING2 (Fig. 7A, Extended Data Fig. 5). N211 forms hydrogen bond with R163 and pulls R163 away from the K161 (Fig. 7A, B). The new conformation of R163 in K211N Parkin forces F209 away from its earlier position. In the new conformation, R163 forms hydrogen bonds with Q165, carbonyl of D219, and N211 (Fig. 7 B). Due to the above conformational changes, RING0 is pushed closer to the RING2 domain (Fig. 7A, B). The latter changes lead to the formation of new hydrogen bonds between K435 (RING2) and carbonyl of T176 (RING0), N428 (RING2) and S181 (RING0) (Fig. 7B). Also, in K211N Parkin structure K161 (RING0) forms salt bridge with the D460 (RING2). Furthermore, H433 (RING2) forms a hydrogen bond with W462 (RING2) in the K211N structure (Fig. 7B). Compared to K211N, in the native Parkin structure distance between atoms (from RING0 and RING2) involved in the above interactions are significantly large and do not form hydrogen bonds/salt bridge (Fig. 7B). The new interactions between RING0 and RING2, in K211N Parkin, would not allow displacement of RING2 and exposure of C431, required for Parkin activation. Furthermore, unlike flexible conformation of RING2 in native Parkin with pNEDD8, lower b-factor of the RING2 in K211N structure confirms that the RING2 flexibility is reduced upon K211N mutation (Fig. 7C). Overall, our data confirms that K211N mutation leads to conformational changes on Parkin restricting RING2

displacement and thus results in loss of Parkin activity that is independent of pUb/pNEDD8 binding.

Discussion

Mutations in the gene expressing Parkin E3 ligase lead to a familial form of Parkinson's disease (PD). Due to its key role, Parkin is one of the significant targets from a therapeutic perspective. In the last few years, several groups have extensively contributed to understanding the mechanism of Parkin activation [22,23,25,26]. Many Parkin structures (crystal and NMR structures) have helped immensely toward understanding the Parkin activation mechanism [31]. Disease mutation leading to loss of pUb-mediated Parkin activation [22] was an interesting phenomenon from a therapeutics perspective. Last year, a group suggested pUb binding in the RING0 pocket and proposed a new feedforward mechanism independent of pUbl [28,29]. Disease mutations leading to loss of binding with its substrates make the design of the Parkin activator to cure Parkinson's even more challenging. Also, as proposed earlier, lack of substrate specificity may lead to unwanted catastrophic events in cells.

Unlike previously suggested, we demonstrate that RING0 and RING1 pockets specifically bind with pUbl and pUb/pNEDD8, respectively. pUb and pNEDD8 can bind with RING1; however, pNEDD8 shows optimum binding and higher activation, suggesting a preference for pNEDD8 over pUb binding in the RING1 pocket (Fig. 1 & 2). Unlike previously proposed, pUb/pNEDD8 do not bind in the RING0 pocket (Fig 3 & Extended Data Fig. 2). RING0 pocket on Parkin is recognized explicitly by the pUbl domain of Parkin, which could be utilized to activate Parkin in trans as suggested by our recent work [20]. The higher affinity of the RING1 pocket for NEDD8 could be helpful in the context of previous findings, which showed overexpression of NEDD8 activation enzyme rescue the defects caused by silencing of PINK1 [32].

Several human Parkin and pUb complexes were crystallized by cross-linking of pUb-3Br with Q347C in the IBR domain of domain [20,22,23,25]. The fact that RING2 remains in the closed state in the pUb bound structures could be due to the tethering of pUb and thus restricting the domain movements on Parkin. Unlike the human Parkin and pUb complex, we determine the Parkin and pNEDD8 structure without artifact. However, despite the more robust binding and higher activation of pNEDD8 than pUb (Fig. 1), RING2 was not displaced in the Parkin (Fig. 2). The crystal structure is also supported by the RING2 displacement assay, which confirms RING2 remains associated with the protein core after pUb/pNEDD8 binding (Fig 3 Extended Data Fig. 2). However, N60D mutation on ubiquitin that mimics the

Ubl domain of Parkin results in binding on the RING0 pocket, resulting in RING2 displacement from the core of Parkin (Fig. 5 & 6). pUbl or pUb/pNEDD8 binding due to their specific binding on the two distinct pockets of Parkin leads to larger/faster and smaller/slower displacement of RING2.

While it was believed that disease mutation K211N in the RING0 pocket on Parkin leads to loss of binding with pUb [28,29], we demonstrate that K211N mutation does not affect Parkin interaction with pUb (Fig. 3). Furthermore, we discovered that K211N mutation leads to loss of Parkin activity independent of pUb binding (Fig. 4). The latter was further confirmed by Parkin K211N structure (Fig. 7). The structure of K211N shows conformational changes in the RING0 domain, which restricts the flexibility of RING2 by making locking it with RING0 (Fig. 8) thus leading to loss of activity which is independent of pUb binding. The latter also explains previous studies showing K211N mutation reducing Parkin (lacking Ubl domain or Ser65) recruitment on mitochondria [29]. However, unlike previously believed, the defect in Parkin recruitment is not due to loss of pUb interaction with Parkin K211N. Rather, it is due to the conformational changes that lead to loss of Parkin activity, which would reduce the pUb levels (Fig. 8). This new discovery would be quite useful in the process of small-molecule activators against Parkin by pharmaceutical companies. Also, this study highlights the importance of a detailed structural analysis of various disease mutations on Parkin to help drug design against Parkinson's disease.

Authors' contributions

DRL performed all experiments, solved structures, and analyzed the data. SC purified various proteins. AK designed and supervised the research, refined structures, analyzed the data, and arranged funding. AK wrote the manuscript with input from DRL.

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Conflict of interest

The authors declare no conflict of interest.

Figure legends

Figure 1. Parkin activation assay using various activators

A Ubiquitination assay to check Parkin activation with increasing concentrations of pUb or pNEDD8. The lower panel shows Miro1 ubiquitination. Coomassie-stained gel showing Parkin/Miro1 is used as the loading control. The ATP-independent band is indicated (*). The bar graph (left panel) shows the integrated intensities (mean ± s.e.m.) of ubiquitination levels from three independent experiments as shown in the right panel. Pair-wise student's t-test was used to determine statistical significance (***$P < 0.001$).

B

Figure 2. Crystal structure of Parkin with pNEDD8

A B-factor analysis on the crystal structure of Parkin (R0RBR) complex with pNEDD8. Scale is included to show the color-wise distribution of the b-factor, min (blue) to max (red).

B Conformational changes between structures of Parkin (light grey) with pUb (grey) (pdbid: 5N2W) and Parkin (colored domain-wise) with pNEDD8 (cyan).

C Interactions between Parkin (light grey) and pUb (grey) (pdbid: 5N2W) in the basic patch of RING1. The distance between N60 (pUb) and carbonyl 187 (Parkin) is highlighted.

D Interactions between Parkin (colored domain-wise) and pNEDD8 (cyan) in the basic patch of RING1. Distinct hydrogen bonds between Parkin and pNEDD8 are highlighted with the dashed line.

Figure 3. RING2 displacement assay

A SEC assay between TEV-treated Parkin (R0RBR) and pUB (with CBD-tag). A colored key is provided for each trace. Coomassie-stained gels of indicated fractions are shown in the lower panel.

B SEC assay between TEV-treated Parkin (R0RBR, K211N) and pUB (with CBD-tag). A colored key is provided for each trace. Coomassie-stained gels of indicated fractions are shown in the lower panel.

C SEC assay between TEV-treated Parkin (R0RBR, H302A) and pUB (with CBD-tag). A colored key is provided for each trace. Coomassie-stained gels of indicated fractions are shown in the lower panel.

The lower panel is a schematic representation to explain the SEC data from panels A, B, and C.

Figure 4. Unique charge distribution determining substrate specificity

A Electrostatic charge distribution on phospho-Ubl (pUbl) domain of Parkin.

B Electrostatic charge distribution on phospho-ubiquitin (pUb).

C Electrostatic charge distribution on phospho-NEDD8 (pNEDD8) domain of Parkin.

D Electrostatic charge distribution around the basic patch on the RING0 domain of Parkin.

E Electrostatic charge distribution around the basic patch on the RING1 domain of Parkin.

F Ubiquitination assay to check Parkin activation with increasing concentrations of pUb or pUb (N60D). The bar graph shows the integrated intensities (mean ± s.e.m.) of ubiquitination levels from three independent experiments, as shown in the Extended Data Figure ?. Pair-wise student's t-test was used to determine statistical significance (***$P < 0.001$).

Figure 5. K211N mutation leads to loss of Parkin activity independent of pUb binding

A Ubiquitination assay to check WT/K211N/H302A Parkin activation with increasing concentrations of pNEDD8. The lower panel shows Miro1 ubiquitination. Coomassie-stained gel showing Parkin/Miro1 is used as the loading control. The ATP-independent band is indicated (*). The bar graph shows the integrated intensities (mean ± s.e.m.) of ubiquitination levels from three independent experiments as shown in the lower panel. Pair-wise student's t-test was used to determine statistical significance (***$P < 0.001$).

B Time-course ubiquitination assay to compare the basal activity of WT Parkin or K211N Parkin in the absence of any activator. The lower panel shows Miro1 ubiquitination. Coomassie-stained gel showing Parkin/Miro1 is used as the loading control. The ATP-independent band is indicated (*). The bar graph shows the integrated intensities (mean ± s.e.m.) of ubiquitination levels from three independent experiments, as shown in the lower panel. Pair-wise student's t-test was used to determine statistical significance (***$P < 0.001$).

Figure 6. RING2 displacement assay to determine the role of N60D mutation

A SEC assay between TEV-treated Parkin (R0RBR) and pUB (with CBD-tag)/pUb N60D (with CBD-tag). A colored key is provided for each trace. Coomassie-stained gels of indicated fractions are shown in the lower panel.

B SEC assay between TEV-treated Parkin (R0RBR)/Parkin (R0RBR, K211N)/Parkin (R0RBR, H302A) and pUb N60D (with CBD-tag). A colored key is provided for each trace. Coomassie-stained gels of indicated fractions are shown in the lower panel.

The lower panel is a schematic representation to explain the SEC data from panels A, B, and C.

Figure 7. Crystal structure of Parkin (R0RBR, K211N) and pNEDD8 reveal mechanism of loss of Parkin activity

A Conformational changes between structures of native Parkin (apo or bound with pUb/pNEDD8) and K211N Parkin bound with pNEDD8. RING0 and RING2 domains from various native Parkin structures are shown in shades of grey. RING0 (orange) and RING2 (cyan) domains of K211N Parkin are shown. Conformational shifts in the RING0 domain are highlighted with the black arrow. Conformational shifts between RING0 and RING2 are highlighted with red arrow (K211N Parkin) or green arrow (native Parkin). Residues undergoing conformational changes due to K211N mutation are also highlighted.

B Standalone structures of K211N Parkin (upper panel) and native Parkin (lower panel) to highlight conformational changes. Hydrogen bonds are shown with a dashed line (upper panel), and the distance between the same atoms is highlighted with an arrow (lower panel).

C B-factor analysis on the crystal structure of Parkin (R0RBR, K211N) with pNEDD8. Scale is included to show the color-wise distribution of the b-factor, min (blue) to max (red).

Figure 8. Model depicting mechanisms of pUb/pNEDD8-mediated Parkin activation, and K211N mutation leading to loss of Parkin activity

Extended Data Figure 1. Analysis of Parkin and pNEDD8 structure

A Superimposition of Parkin (R0RBR, different domains are colored) and pNEDD8 (cyan) structure with Parkin (grey) and pUB (grey) structure (pdbid: 5N2W).

B The 2Fo-Fc map (grey) of the R0RBR (colored as panel A) and pNEDD8 (cyan) structure. The 2Fo-Fc map is contoured at 1.2 σ.

C B-factor analysis on the crystal structure of R0RBR (pdbid: 4I1H). Scale is included to show the color-wise distribution of the b-factor, min (blue) to max (red).

Extended Data Figure 2. SEC assay to check RING2 displacement in the presence of pNEDD8

A SEC assay between TEV-treated Parkin (R0RBR) and pNEDD8 (with His-SUMO tag). A colored key is provided for each trace. Coomassie-stained gels of indicated fractions are shown in the lower panel.

B SEC assay between TEV-treated Parkin (R0RBR, K211N) and pNEDD8 (with His-SUMO tag).

C SEC assay between TEV-treated Parkin (R0RBR, H302A) and pNEDD8 (with His-SUMO tag).

Extended Data Figure 3. Ubiquitination assay on Parkin mutants in presence of pUb

Ubiquitination assay to check WT/K211N/H302A Parkin activation with increasing concentrations of pUb. The lower panel shows Miro1 ubiquitination. Coomassie-stained gel showing Parkin/Miro1 is used as the loading control. The ATP-independent band is indicated (*).

Extended Data Figure 4. Ubiquitination assay with pUb or pUb N60D

The representative gel image used for quantification in Fig. 5 F

Extended Data Figure 5. Analysis of Parkin K211N and pNEDD8 structure

Superimposition of various Parkin (both apo and complex with pUb) structures (different shades of grey) with the structure of R0RBR K211N (domains colored) and pNEDD8 (green).

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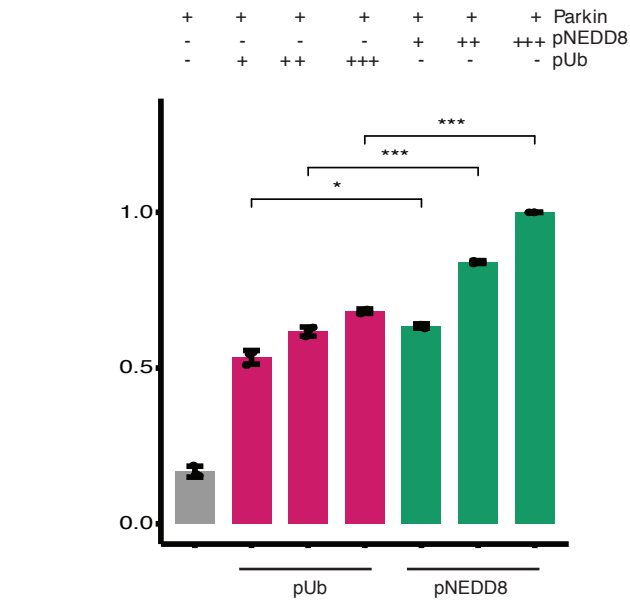
Extended Data Table 1
Data collection and Refinement statistics

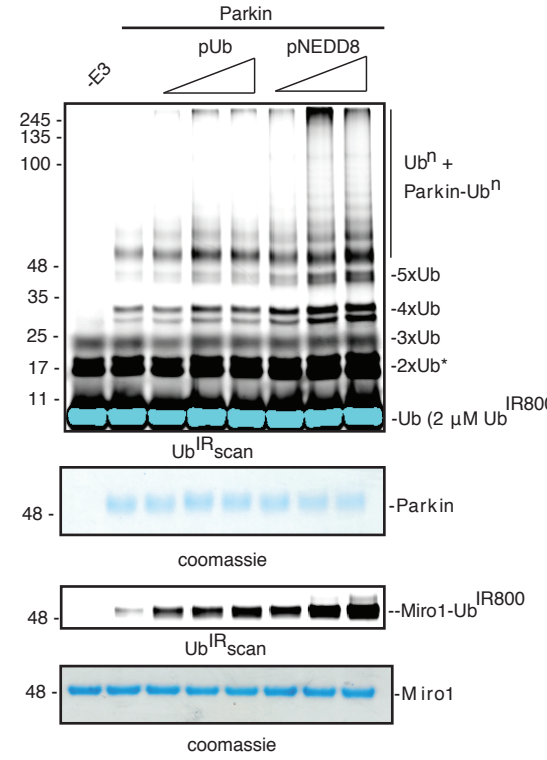
	Parkin and pNEDD8	Parkin K211N and pNEDD8
Data collection		
Resolution range	40.91 - 2.25 (2.331 - 2.25)	39.83 - 1.8 (1.864 - 1.8)
Space group	I 1 2 1	P 21 21 21
a, *b*, *c* (Å)	77.976, 47.747, 127.859	58.249, 74.603, 80.067
α, β, γ (°)	90, 105.38, 90	90, 90, 90
Total reflections	93740 (9602)	146049 (15003)
Unique reflections	21812 (2149)	32755 (3236)
Multiplicity	4.3 (4.5)	4.5 (4.6)
Completeness (%)	99.67 (99.95)	99.17 (99.32)
I/σ(I)	9.23 (1.76)	10.25 (2.98)
Wilson B-factor	35.54	17.67
R-merge	0.1159 (0.84)	0.09746 (0.4231)
CC1/2	0.993 (0.619)	0.996 (0.826)
Refinement		
Reflections used in refinement	21766 (2149)	32750 (3236)
R-work/R-free	0.2116/ 0.2509	0.1712/ 0.2090
No of Atoms		
macromolecules	2929	3045
ligands	20	29
solvent	174	324
RMS deviations		
Bond length (Å)	0.008	0.011
Bond angles (°)	1.25	1.76
Ramachandran favored (%)	95.64	96.55
Ramachandran outliers (%)	0	0.53
B-factors		
macromolecules	45.86	22.19
ligands	47.2	39.68
solvent	45.05	32.07

Accession code	8WZO	8WZN

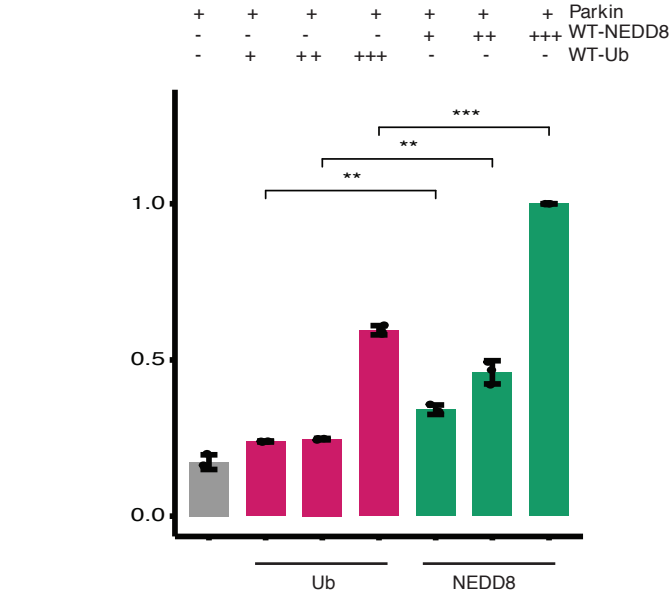
Statistics for the highest-resolution shell are shown in parentheses

A


+ + + + + + + Parkin
- - - - + ++ +++ pNEDD8
- + ++ +++ - - - pUb
***
***
*
1.0
0.5
0.0
pUb
pNEDD8


Parkin
pUb
pNEDD8
-E3
245
135
100
48
35
25
17
11
Ub^n +
Parkin-Ub^n
-5xUb
-4xUb
-3xUb
-2xUb*
-Ub (2 µM Ub^IR800)
Ub^IR scan
48
-Parkin
coomassie
48
--Miro1-Ub^IR800
Ub^IR scan
48
-Miro1
coomassie

B


+ + + + + + + Parkin
- - - - + ++ +++ WT-NEDD8
- + ++ +++ - - - WT-Ub
***
**
**
1.0
0.5
0.0
Ub
NEDD8

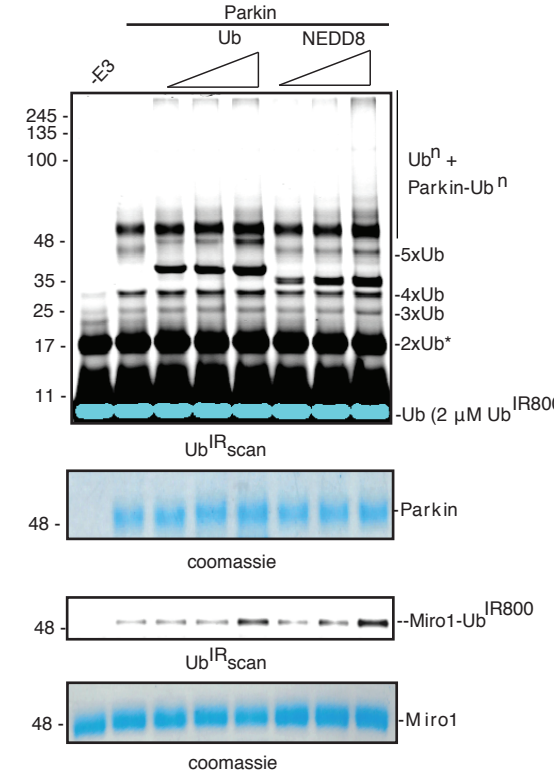

Parkin
Ub
NEDD8
-E3
245
135
100
48
35
25
17
11
Ub^n +
Parkin-Ub^n
-5xUb
-4xUb
-3xUb
-2xUb*
-Ub (2 µM Ub^IR800)
Ub^IR scan
48
-Parkin
coomassie
48
--Miro1-Ub^IR800
Ub^IR scan
48
-Miro1
coomassie

Figure 1

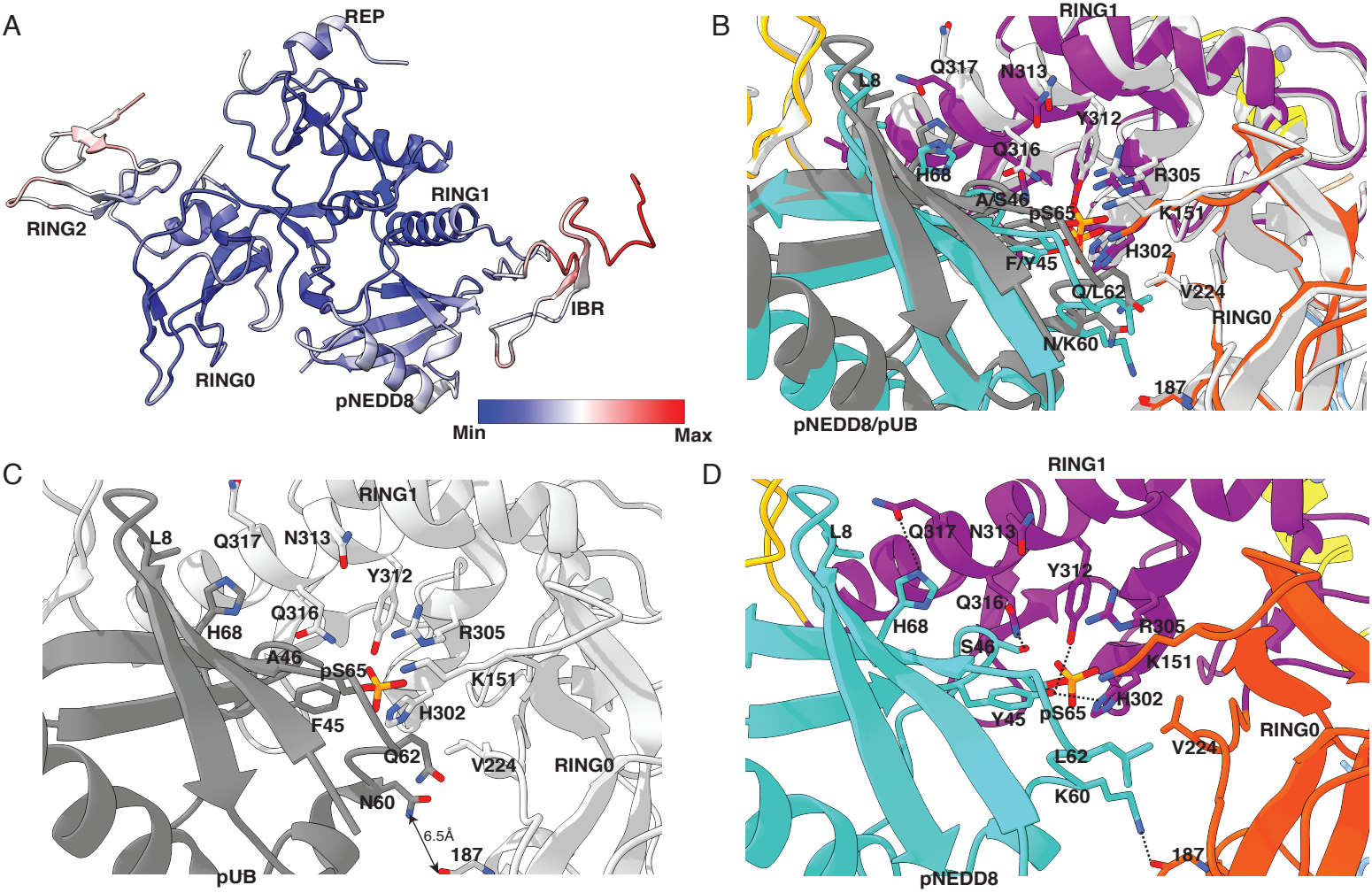
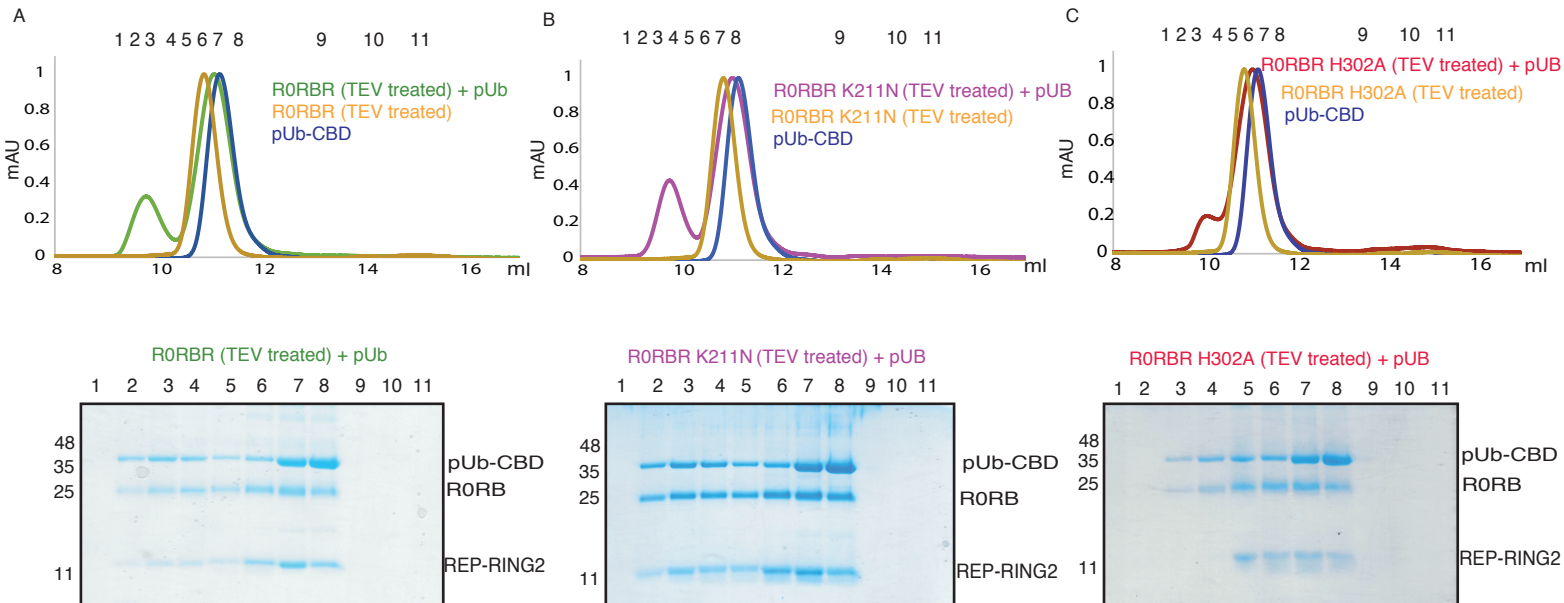
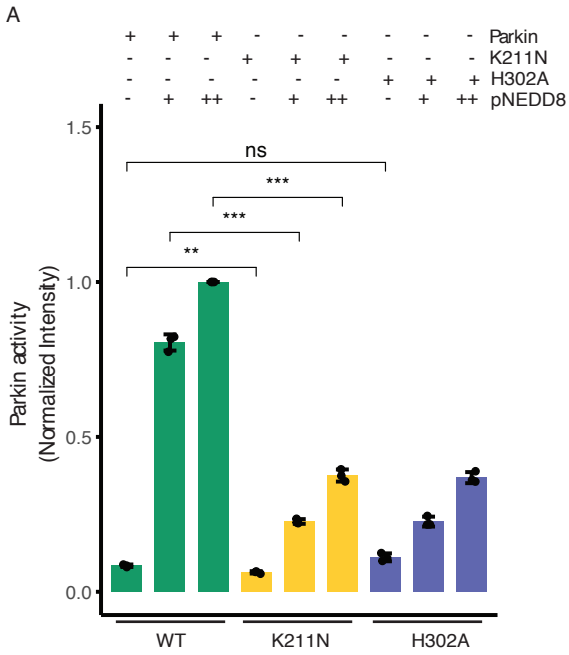

A
REP
RING2
RING1
IBR
RING0
pNEDD8
Min
Max
B
RING1
L8
Q317
N313
Y312
Q316
H68
R305
A/S46
pS65
K151
H302
F/Y45
Q/L62
V224
RING0
N/K60
187
pNEDD8/pUB
C
RING1
L8
Q317
N313
Y312
Q316
H68
R305
A46
pS65
K151
H302
F45
Q62
V224
RING0
N60
6.5Å
187
pUB
D
RING1
L8
Q317
N313
Y312
Q316
H68
R305
S46
K151
H302
Y45
pS65
RING0
V224
L62
K60
187
pNEDD8
E
1 10 20 30 40 50 60 70
Ubl(Parkin) MIVFVRFNSSHGFPVEVDSDTSIFQLKEVVAKRQGVPADQLRVIFAGKELRNDWTVQNCDLDQQSIVHIVQRPWRK
Ubiquitin MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
NEDD8 MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQMNDEKTAADYKILGGSVLHLVLALRGG

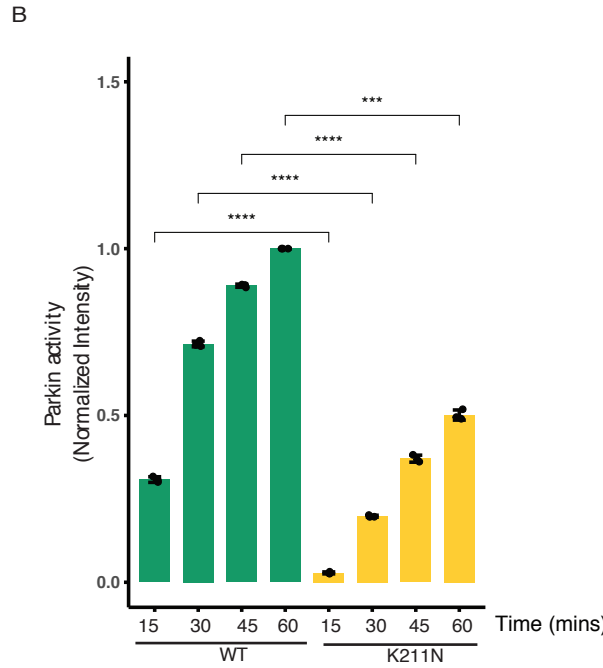
Figure 2

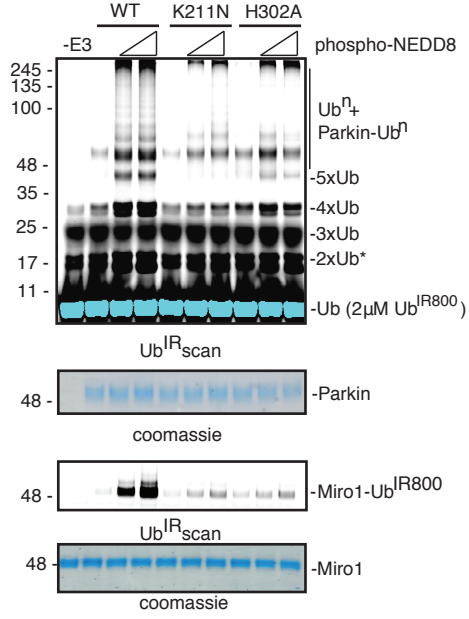

A
1 2 3 4 5 6 7 8 9 10 11
R0RBR (TEV treated) + pUb
R0RBR (TEV treated)
pUb-CBD
mAU
ml
B
R0RBR K211N (TEV treated) + pUB
R0RBR K211N (TEV treated)
pUb-CBD
C
R0RBR H302A (TEV treated) + pUB
R0RBR H302A (TEV treated)
pUb-CBD
R0RBR (TEV treated) + pUb
R0RBR K211N (TEV treated) + pUB
R0RBR H302A (TEV treated) + pUB
48
35
25
11
pUb-CBD
R0RB
REP-RING2

RING2
C431
RING1
IBR
RING0
pUB/pNEDD8
pUb/NEDD8

Figure 3


A
Parkin
K211N
H302A
pNEDD8
Parkin activity
(Normalized Intensity)
ns
***
***
**
WT
K211N
H302A


B
Parkin activity
(Normalized Intensity)
***
****
****
****
Time (mins)
WT
K211N


WT
K211N
H302A
-E3
phospho-NEDD8
Ub^n+
Parkin-Ub^n
-5xUb
-4xUb
-3xUb
-2xUb*
-Ub (2µM Ub^IR800)
Ub^IR scan
-Parkin
coomassie
-Miro1-Ub^IR800
Ub^IR scan
-Miro1
coomassie

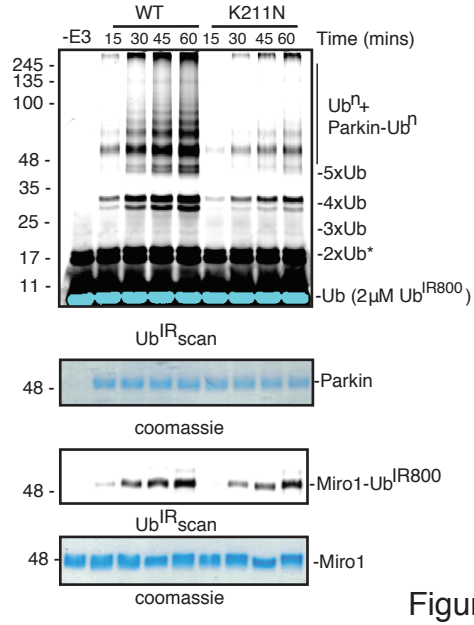

WT
K211N
-E3
Time (mins)
Ub^n+
Parkin-Ub^n
-5xUb
-4xUb
-3xUb
-2xUb*
-Ub (2µM Ub^IR800)
Ub^IR scan
-Parkin
coomassie
-Miro1-Ub^IR800
Ub^IR scan
-Miro1
coomassie

Figure 4

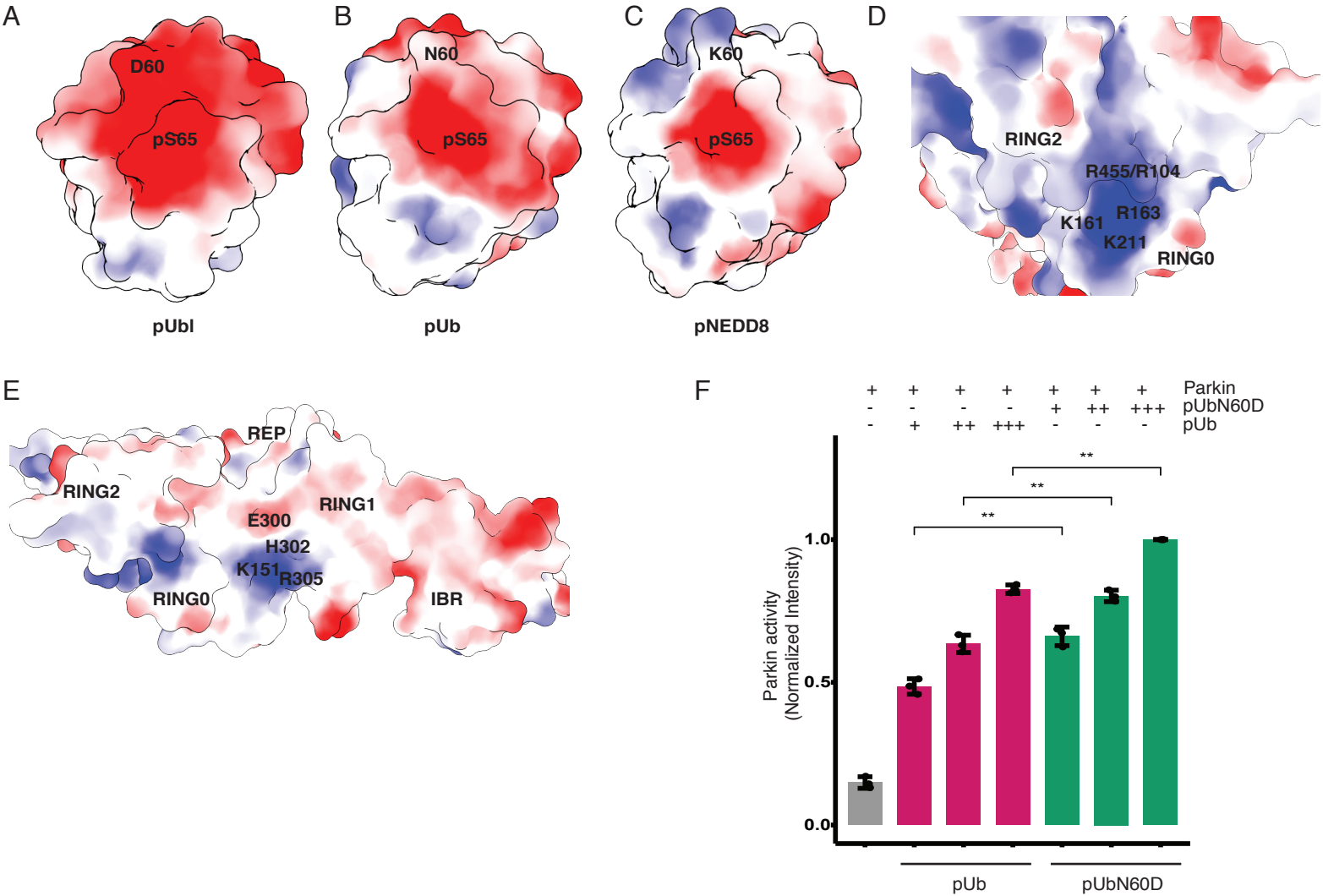

A
D60
pS65
pUbl
B
N60
pS65
pUb
C
K60
pS65
pNEDD8
D
RING2
R455/R104
R163
K161
K211
RING0
E
REP
RING2
RING1
E300
H302
K151
R305
RING0
IBR
F
Parkin
pUbN60D
pUb
Parkin activity
(Normalized Intensity)
1.0
0.5
0.0
**
pUb
pUbN60D

Figure 5

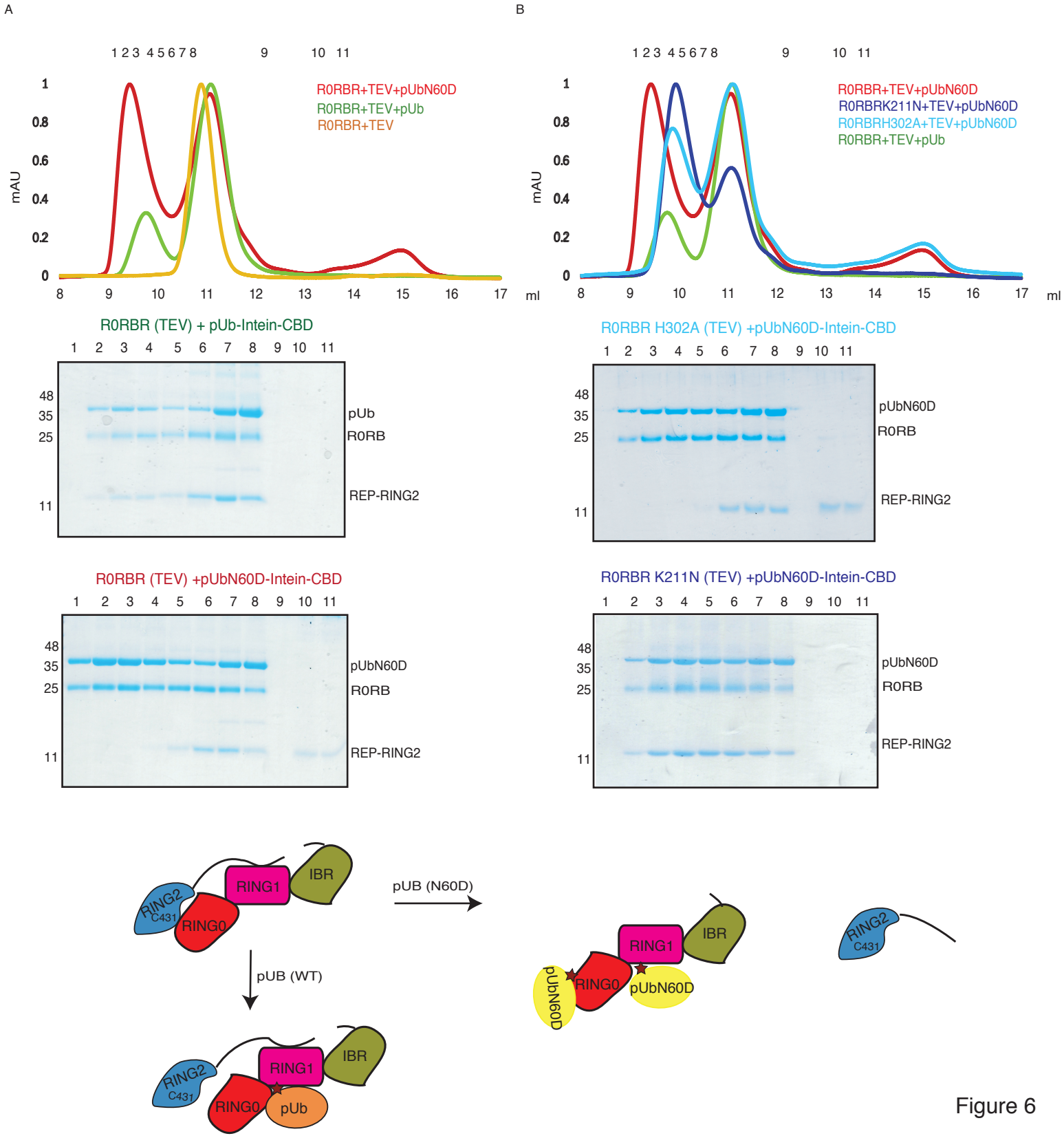
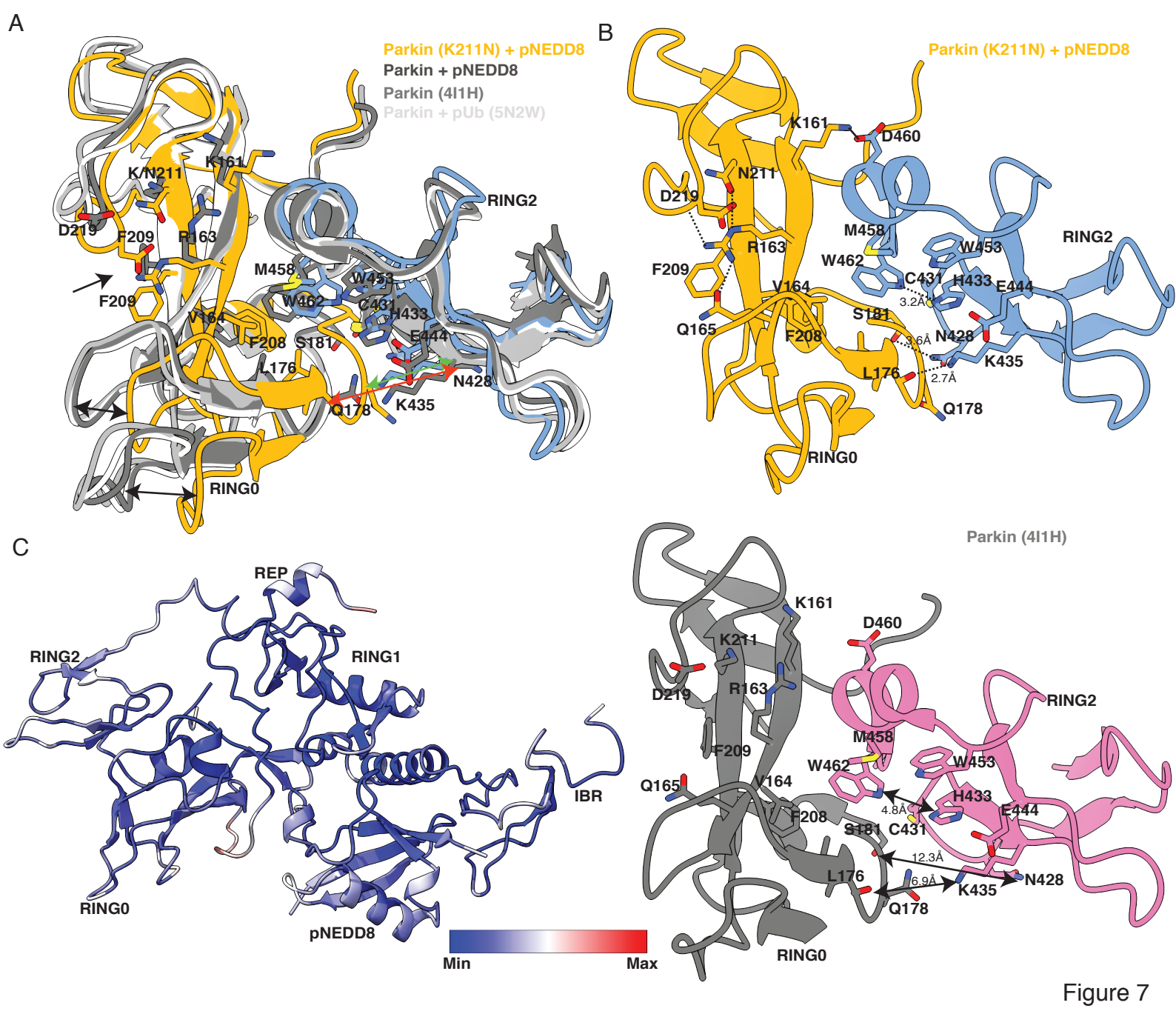

A
B
1 2 3 4 5 6 7 8 9 10 11
mAU
ml
R0RBR+TEV+pUbN60D
R0RBR+TEV+pUb
R0RBR+TEV
R0RBRK211N+TEV+pUbN60D
R0RBRH302A+TEV+pUbN60D
R0RBR (TEV) + pUb-Intein-CBD
R0RBR H302A (TEV) +pUbN60D-Intein-CBD
R0RBR (TEV) +pUbN60D-Intein-CBD
R0RBR K211N (TEV) +pUbN60D-Intein-CBD
pUb
pUbN60D
R0RB
REP-RING2
RING2
C431
RING0
RING1
IBR
pUB (N60D)
pUB (WT)

Figure 6


A
Parkin (K211N) + pNEDD8
Parkin + pNEDD8
Parkin (4I1H)
Parkin + pUb (5N2W)
K/N211
K161
D219
F209
R163
M458
W462
W453
C431
H433
E444
V164
F208
S181
L176
Q178
K435
N428
RING2
RING0
B
Parkin (K211N) + pNEDD8
K161
D460
N211
D219
R163
M458
W462
W453
RING2
F209
V164
C431
H433
E444
3.2Å
S181
Q165
F208
3.6Å
N428
K435
L176
2.7Å
Q178
RING0
C
REP
RING2
RING1
IBR
RING0
pNEDD8
Min
Max
Parkin (4I1H)
K161
D460
K211
D219
R163
RING2
M458
F209
W462
W453
Q165
V164
H433
4.8Å
E444
F208
S181
C431
12.3Å
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6.9Å
K435
N428
Q178
RING0

Figure 7

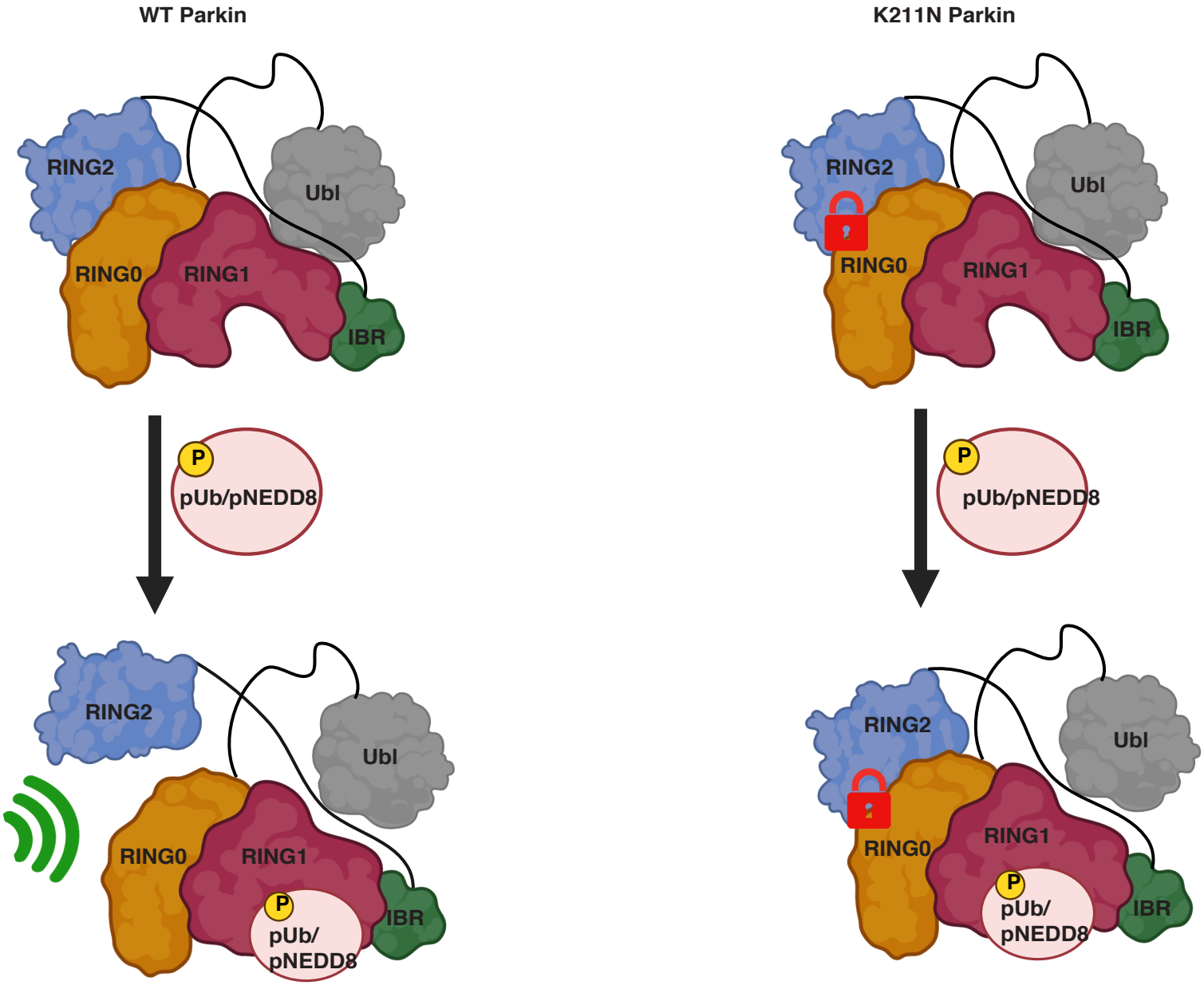
WT Parkin
RING2
Ubl
RING0
RING1
IBR
P
pUb/pNEDD8
RING2
Ubl
RING0
RING1
P
pUb/
pNEDD8
IBR
K211N Parkin
RING2
Ubl
RING0
RING1
IBR
P
pUb/pNEDD8
RING2
Ubl
RING0
RING1
P
pUb/
pNEDD8
IBR

Figure 8