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Disruption of the autoinhibited state primes the E3 ligase parkin for activation and catalysis

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Abstract

The PARK2 gene is mutated in 50% of autosomal recessive juvenile parkinsonism (ARJP) cases. It encodes parkin, an E3 ubiquitin ligase of the RBR family. Parkin exists in an autoinhibited state that is activated by phosphorylation of its N-terminal ubiquitin-like (Ubl) domain and binding of phosphoubiquitin. We describe the 1.8 Å crystal structure of human parkin in its fully inhibited state and identify the key interfaces to maintain parkin inhibition. We identify the phosphoubiquitin-binding interface, provide a model for the phosphoubiquitin–parkin complex and show how phosphorylation of the Ubl domain primes parkin for optimal phosphoubiquitin binding. Furthermore, we demonstrate that the addition of phosphoubiquitin leads to displacement of the Ubl domain through loss of structure, unveiling a ubiquitin-binding site used by the E2–Ub conjugate, thus leading to active parkin. We find the role of the Ubl domain is to prevent parkin activity in the absence of the phosphorylation signals, and propose a model for parkin inhibition, optimization for phosphoubiquitin recruitment, release of inhibition by the Ubl domain and engagement with an E2–Ub conjugate. Taken together, this model provides a mechanistic framework for activating parkin.

Keywords enzyme mechanism; Parkinson’s disease; phosphorylation; ubiquitination; ubiquitin ligase

Subject Categories Molecular Biology of Disease; Post-translational Modifications; Proteolysis & Proteomics; Structural Biology

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Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder with severe motor and non-motor symptoms (Goedert et al., 2013). There are several genes associated with PD, with autosomal recessive juvenile parkinsonism (ARJP) being the most prevalent familial form of the disease. Mutations in PARK2 and PARK6 genes lead to ARJP, with PARK2 mutations causing > 50% of cases (Bonifati et al., 2002). PARK2 encodes parkin, an E3 ubiquitin ligase, while PARK6 encodes PTEN-induced kinase 1 (PINK1), which phosphorylates Ser65 of parkin and ubiquitin.

Parkin is an RBR-type ubiquitin ligase which is characterized by RING1, inBetweenRING (IBR) and RING2 domains. Recent studies have suggested these names do not accurately reflect the structures or functionality of the domains. As a result, an alternative nomenclature that uses RING1, Benign Rcat (BRcat=IBR) and Required for catalysis (Rcat=RING2) domains has been proposed for all RBR E3 ligases (Spratt et al., 2014) that maintain the familiar RBR scheme. RBR ligases contain domains additional to the signature RBR module that are specific to each member. Parkin has an N-terminal ubiquitin-like domain (Ubl) that shares 65% sequence similarity with ubiquitin, and a zinc-chelating RING0 domain immediately N-terminal to the RBR domains (Hristova et al., 2009). The RING1 domain recruits E2 enzymes, which carry charged ubiquitin and pass it to a catalytic cysteine in the parkin RING2 domain (Wenzel et al., 2011). Parkin can function with multiple E2s and catalyses the formation of several modifications including K63-linked and K48-linked chains, and monoubiquitination. Pathogenic mutations occur throughout Parkin, and mutations in different domains have different effects on activity, solubility and stability (Wang et al., 2005; Hampe et al., 2006; Safadi & Shaw, 2007; Safadi et al., 2011; Spratt et al., 2013). Wild-type parkin exists in an autoinhibited state (Chaugule et al., 2011); subsequently, autoinhibition has been demonstrated to be common to RBR ligases (Smit et al., 2012; Stieglietz et al., 2012; Duda et al., 2013).

The N-terminal Ubl domain inhibits parkin activity, and pathogenic mutations in this domain render parkin active (Chaugule et al., 2011). High-resolution crystal structures of parkin’s RING0-RBR domains, which lack the Ubl domain, revealed multiple domain–domain interactions that regulate parkin activity (Chaugule et al., 2011; Riley et al., 2013; Trempe et al., 2013; Wauer & Komander, 2013). However, the importance of the Ubl domain is highlighted by

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Recent findings that PINK1, the kinase encoded by PARK6, phosphorylates parkin at serine 65, and this phosphorylation enhances parkin activity (Kondapalli et al, 2012; Shibai-Fukushima et al, 2012, 2014). PINK1 also modifies ubiquitin at the equivalent serine 65 (Kane et al, 2014; Kazlauskaite et al, 2014b; Koyano et al, 2014), and this signal is necessary for parkin activation. Together, these phosphorylation signals enable parkin to ubiquitinate multiple substrates, including several involved in mitochondrial homeostasis.

Despite the recent breakthroughs in our understanding of parkin structure and activation, we still do not have a clear picture of how the Ubl domain maintains the tertiary inhibited conformation of wild-type parkin and how this inhibition is disrupted by phosphorylation of both the Ubl domain and ubiquitin to activate parkin. Since many disease-causing mutations are localized to the Ubl domain, understanding how the Ubl controls the inhibition of parkin at the molecular level will likely be the key to developing activator therapeutic molecules that may be useful in treating the disease. In this work, we set out to understand how the Ubl domain regulates parkin function and how the phosphorylation signals impact parkin activity. We report here the 1.8 Å resolution crystal structure of human parkin, complete with the Ubl domain. Our structural and biochemical analysis reveals that the interface between the Ubl and RING1 domains is crucial for parkin’s regulation by remotely altering the E2 promiscuity and present a scaffold for targeting domain interactions to activate parkin.

Results

Although there are crystal structures of truncated parkin comprising the RING0, RING1, IBR and RING2 domains (R0RBR parkin), and a 6.5 Å low-resolution structure of full-length parkin (Riley et al, 2013; Trempe et al, 2013; Wauer & Komander, 2013), there are no high-resolution molecular details of parkin with the Ubl domain intact. Given the importance of this domain, and observations that ARJP mutations within this domain lead to increased ubiquitination activity (Chaugule et al, 2011), we aimed to identify the molecular determinants of the autoinhibition. To re-affirm the autoinhibitory role of the Ubl domain and to test the importance of the linker, we conducted ubiquitination assays using an improved fluorescence-based method (Fig 1A). These experiments repeatedly showed that removal of the Ubl domain (77-C, 111-C) leads to increased parkin autoubiquitination as well as formation of free polyubiquitin chains that was enhanced upon stimulation with phosphoubiquitin. In contrast, more complete removal of the linker (127-C, 141-C (R0RBR)) appears to reverse this process leading to similar ubiquitination levels as the wild-type protein, perhaps indicating that the N-terminus of the linker may play an undiscovered role in ubiquitination by parkin.

Attempts to crystallize the full-length protein did not generate high-quality crystals, so we undertook a rational approach to provide details of the Ubl domain interaction with the rest of the protein. Previous work has shown that the linker region between the Ubl and RING0 domains (residues 77–141) in parkin is likely poorly structured with little known function (Hristova et al, 2009). In addition, the linker is the most divergent region for parkin when sequences are compared from multiple species (Fig EV1). This suggested that careful deletion of the linker might improve crystalization without eliminating the inhibitory functionality of the Ubl domain. We generated a series of human parkin proteins with modified linker lengths (Fig 1B) between the Ubl domain and RING0–RING1 domain to test their ubiquitination activity and ability to form high-quality crystals. In particular, one construct (UblR0RBR) that included the Ubl (residues 1–83) linked to RING0RBR (residues 144–465) showed autoinhibition similar to the wild-type protein, enhanced ubiquitination with phosphoubiquitin and the ability to ubiquitinate a potential mitochondrial substrate protein, Miro1 (Kazlauskaite et al, 2014a) (Fig 1B).

The structure of parkin reveals a compact, autoinhibited conformation

The crystal structure of UblR0RBR parkin was solved and refined to 1.8 Å, and comprises two molecules in the asymmetric unit (Table 1). The structure has five domains: Ubl (1–76), RING0 (144–216), RING1 (228–327), IBR (328–377) and RING2 (415–465), and a repressor element (REP) (Fig 1C). Residues 354 and 357–360 in the IBR domain are missing in the density, as are the residues 383–390 linking the IBR to the REP, and residues 406–413 linking the REP to the RING2 domain.

The position of the Ubl domain, located between the RING1 and IBR domains, is globally consistent with the low-resolution 6.5 Å structure of full-length rat parkin (Trempe et al, 2013) (Appendix Fig S1). The largest interface in the structure is that formed between the Ubl domain and the rest of parkin, burying ~2,150 Å² accessible surface area (Fig 2A). The primary contact is between the Ubl (β3, β5) and RING1 (helix H1) domains (Fig 2A; Fig EV1). The core interface is stabilized by hydrophobic interactions mediated by I44 and V70 of the Ubl domain and L266, V269 and T270 of RING1 (Fig 2A). This interaction is supported by several key salt bridges and hydrogen bonds including R42 (Ubl) with D262 at the N-terminus of helix H1 (RING1) and R6 and H68 (Ubl) with N273 and D274 at the C-terminus of helix H1. The tail of the Ubl domain is stabilized by interactions between the main chain carbonyl oxygens of R75 and K76 with R245 of RING1. In addition, N8 (Ubl) of the β1–β2 loop sits between E310 and Q311 at the extreme N-terminus of the bent helix (H3) connecting the RING1 and IBR domains (Fig 2A). There are few contacts between the Ubl and IBR domains, the most obvious involving H11 (β1–β2 loop, Ubl) with F333 and K369 (IBR). The intimate Ubl/RING1 association also occurs in trans, based on solution NMR...
Figure 1. Three-dimensional structure of UbIR0RBR parkin.

A Ubiquitination assays show wild-type and UbIR0RBR parkin are inhibited for chain formation (top) and Miro1 ubiquitination (bottom) in the absence of phosphoubiquitin at zero time point and in the presence of phosphoubiquitin at 30 min. Removal of the inhibitory Ubl domain leads to parkin activation dependent on the length of the linker preceding RING0. The middle panel shows a Coomassie-stained loading control. A non-specific, ATP-independent band is indicated (*).

B Ubiquitination assays show effects of varying length of the linker between the inhibitory Ubl domain and R0RBR, in the presence and absence of phosphoubiquitin. A non-specific, ATP-independent band is indicated (*).

C Overall structure of UbIR0RBR parkin depicting the Ubl domain (green), RING0 (blue), RING1 (magenta), IBR (black), REP (yellow) and RING2 (brown). Loops outside domains are in cyan. Zinc atoms are represented as grey spheres. Two views are shown related by a 90° rotation in x.

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Table 1. Crystallographic data collection and refinement statistics.

|                          | UblR0RBR | S65D-UblR0RBR |
|--------------------------|----------|---------------|
| Wavelength (Å)           | 0.9174   | 0.9172        |
| Resolution (Å)           | 56.36–1.79 (1.85–1.79) | 23.36–2.37 (2.46–2.37) |
| Space group              | P2₁2₁2₁  | P2₁2₁2₁       |
| Unit cell abc (Å) ×βγ (%)| 67.3 67.3 206.2 90 90 90 | 66.3 66.3 205.6 90 90 90 |
| Total reflections         | 276,440  | 216,582       |
| Unique reflections        | 87,891   | 37,844        |
| Multiplicity              | 3.1 (2.8) | 5.7 (5.0)     |
| Completeness (%)          | 98.8 (97.6) | 99.8 (98.8)  |
| Mean I/σ(I)               | 11.2 (1.8) | 7.7 (1.5)     |
| Wilson B-factor (Å²)      | 30.7     | 46.0          |
| R-merge                   | 4.9 (56.5) | 17.4 (116.9) |
| R-meas                    | 6.7 (77.0) | 19.1 (130.6) |
| CC1/2                     | 99.6 (75.3) | 99.3 (55.4)  |
| Reflections for R-free    | 4,270    | 1,854         |
| R-work                    | 0.192 (0.224) | 0.220 (0.241) |
| R-free                    | 0.212 (0.227) | 0.241 (0.267) |
| CC (work)                 | 0.951    | 0.912         |
| CC (free)                 | 0.946    | 0.905         |
| Number of non-hydrogen atoms | 6,943  | 6,424         |
| Macromolecules            | 6,180    | 6,055         |
| Ligands                   | 52       | 56            |
| Water                     | 711      | 313           |
| Protein residues          | 784      | 784           |
| RMS bonds Å               | 0.008    | 0.007         |
| RMS angles (°)            | 0.94     | 0.87          |
| Ramachandran favoured (%) | 96.4     | 95.0          |
| Average B-factor (Å²)     | 49.1     | 52.9          |
| Macromolecules            | 48.2     | 53.0          |
| Ligands                   | 44.1     | 62.0          |
| Water                     | 57.3     | 49.6          |

Numbers in parentheses indicate outer shell statistics.

Experiments where the Ubl domain was titrated into R0RBR parkin (residues 141–465). The largest chemical shift changes in R0RBR occur in residues in helix H1, and helix H3 in the RING1 domain (Fig 2B). In addition, chemical shift changes occur for residues A383, S384 and G385 in the tether region following the IBr domain (Fig 2B) that is not observed in the crystal structure, indicating these residues interact with the Ubl domain transiently. Reverse titration experiments that monitored changes in 1H-13C HMQC spectra of the Ubl domain upon R0RBR addition show the methyl groups of residues I2, I44, A46, L61, I66 and V70 have the largest changes, consistent with I44 and V70 facing the RING1 domain as in the crystal structure (Appendix Fig S2) and I2, A46, L61 and I66 facing the tether. Taken together, these data indicate an extensive interface between the Ubl domain and the RING1 domain of parkin that exists both in cis and in trans, in solution and in the crystal.

The extent and nature of the Ubl/RING1 interface suggests it is important for autoinhibition of parkin. Consistent with this, several activating ARJP mutations occur in the Ubl domain including R42P, A46P and R33Q (Chaugule et al., 2011). The R42P mutation would disrupt the central salt bridge between R42 and D262 (Fig 2A). The ARJP mutation of R33Q would abolish a long-range ionic interaction with E370 in the IBr domain. Finally, mutation of A46P would induce a loss of secondary structure in the loop that interacts with the tether region (Appendix Fig S2). Weakening or destabilizing of these interactions that maintain the Ubl position would lead to a loosening of its tight interface with RING1. To test the importance of this interface, we generated several Ubl domain mutants and assayed them for activity (Fig 2C). In contrast to wild-type Parkin, Ubl-interface mutants are more active for chain formation and ubiquitination of Miro1 (Kazlauskaitė et al., 2014a). Taken together, these data show that disruption of the Ubl-RING1 interface leads to constitutively active parkin.

Presence of the Ubl domain maintains a compact RING0–RING1 interface

Several groups reported high-resolution crystal structures of parkin R0RBR domains from human and rat, lacking the Ubl domain (Riley et al., 2013; Trempe et al., 2013; Wauer & Komander, 2013). In order to understand what conformational changes, if any, occur beyond the Ubl/RING1 interface when the Ubl is present, we compared UblR0RBR parkin with the truncated R0RBR structures. The structures agree well (Appendix Fig S3) with root mean squared differences (rmsd) between 1.4 Å (rat R0RBR, 4K7D) and 1.8 Å (human R0RBR, 4I1F). The observed differences are due largely to the positions of the IBr domains in each structure (Appendix Fig S3). Heteronuclear nOe experiments show this domain exhibits the greatest degree of flexibility of any domain in R0RBR parkin (Appendix Fig S4). The REP is located in the equivalent position, and the RING0/RING2 interface is maintained between structures (Appendix Fig S3). Interestingly, when the structures are superimposed using the RING0/RING2 domains as the reference point, the flexibility of the other domains is more pronounced (Fig 3A). In particular, the RING0/RING1 interface widens in the absence of the Ubl domain as the IBr domain swings ~12 Å away from its position when the Ubl domain is attached. These global changes suggest a pivot or hinge in parkin centred near the RING0/RING1 interface. In UblR0RBR, the RING0/RING1 interface buries 730 Å² and involves Y147, Y149, K151, Q155, V157, W183 and L187 of the RING0 domain interacting with H227, I229, A230, P247, E300, H302, R305, I306 and E309 of the RING1 domain (Figs 3B and EV1). Comparison of this interface in UblR0RBR with that in R0RBR reveals a number of changes (Fig 3C). A salt bridge formed by R234 and E404 pinning the domains together is absent in the R0RBR structure, but present when the Ubl domain is attached. A hydrogen bond between H227 and E300 is observed in the presence of the Ubl domain, but not in the R0RBR structures (Fig 3C). In the Ubl-bound structure, the side chains of H302 and E300 (and H227) are facing into the interface, while in the R0RBR structures, these residues are flipped out ~180° away from the interface (Fig 3C). This latter conformation is observed in all R0RBR structures, suggesting this is a consequence...
of the absence of the Ubl domain, rather than an artefact of crystal packing.

A negative charge at position 65 of the Ubl domain causes remodelling of the RING1/RING0 interface

Phosphorylation of S65 in the Ubl domain enhances parkin activity (Kondapalli et al., 2012). In order to understand what conformational changes occur upon phosphorylation of the Ubl domain, we attempted unsuccessfully to crystallize phosphorylated parkin. Therefore, we crystallized a version of parkin carrying a negative charge at position 65 (S65D乌bIR0RBR). This protein, as well as S65E乌bIR0RBR, showed increased ubiquitination activity compared to wild-type parkin that was further enhanced by phosphoubiquitin in a similar fashion to phosphorylated UblIR0RBR (Appendix Fig S5). The S65D乌bIR0RBR crystals grew under the same conditions, with the same unit cell dimensions and crystal packing, as the wild-type UblIR0RBR crystals, suggesting little or no structural changes. The structure of S65D乌bIR0RBR parkin was refined to 2.4 Å (Table 1), with the same residues present in the density as in the wild-type
UbIR0RBR crystals. Superposition of S65UbIR0RBR and UbIR0RBR (rmsd 0.58 Å) reveals no global conformational changes either in the Ubl domain, or in other parts of the protein (Fig 3D). However, close inspection reveals that H227, E300 and H302 of the RING0/RING1 interface are remodelled in the S65UbIR0RBR structure to resemble the interface observed in the truncated R0RBR structures (Fig 3E). Interestingly, the remodelling of these residues gives rise to a continuous basic patch comprising K151, H302 and R305 on the surface of
Parkin, which is not present when the Ubl domain is unmodified (Fig 3F). Since parkin ubiquitination activity is further enhanced through phosphoubiquitin binding, we hypothesized that this basic patch is the phosphoubiquitin-binding site. To test this, we generated H302A parkin and assayed this for interaction with phosphoubiquitin (pUb) (Appendix Fig S6). In contrast to wild-type parkin, H302A parkin does not bind pUb. Furthermore, mutation of residues at the top of the interface that pin the E300 and H302 in the flipped-in conformation is more active than wild-type parkin, while mutation of the residues in the basic patch displays diminished activity compared to wild-type, and cannot be stimulated with pUb (Fig 3G).

Taken together, these data suggest that the addition of a negative charge at position 65 of the Ubl domain alters the surface of parkin at the RING0/RING1 interface to facilitate optimal pUb binding.

**Phosphorylated ubiquitin binding induces a structural change in parkin**

PINK1-mediated phosphorylation of ubiquitin at S65 (pUb) enhances parkin activity. In order to understand how this is achieved, we performed chemical shift perturbation experiments monitored by $^1$H-^{15}N TROSY spectroscopy. Addition of pUb to
Table 2. Thermodynamic properties for parkin activation.

| Protein          | Activator | $K_a$ (µM) | N  | $\Delta H$ (kJ/mol) | $\Delta S$ (J/molK) |
|------------------|-----------|------------|----|---------------------|---------------------|
|                  |           |            |    |                     |                     |
| **A. R0RBR Parkin with Ubl and Ub** |           |            |    |                     |                     |
| R0RBR            | Ubl       | 3.8 ± 1.3  | 0.98| $-44 \pm 3$         | $-44$              |
|                  | Ubl S65D  | 5.0 ± 0.9  | 1.03| $-38 \pm 1$         | $-29$              |
|                  | Ubl S65E  | 5.3 ± 1.2  | 0.98| $-46 \pm 2$         | $-54$              |
|                  | pUbl      | 26 ± 10    | 1.04| $-16 \pm 9$         | $33$               |
| R0RBR            | Ub        | 67 ± 1.0   | 1.05| 31 ± 1              | 184                |
|                  | Ub S65D   | 66 ± 0.3   | 1.01| 25 ± 1              | 184                |
|                  | Ub S65E   | 6.6 ± 0.1  | 1.10| 48                  | 261                |
|                  | pUb       | 0.016 ± 0.002| 0.93| 32                  | 261                |
| **B. Full-length Parkin with Ubl and Ub** |           |            |    |                     |                     |
| Parkin           | Ubl       | N.O.       | –   | –                   | –                   |
|                  | Ub        | 45 ± 9     | 0.90| 10 ± 1              | 115                |
|                  | pUbl      | 0.16 ± 0.02| 1.09| 50                  | 293                |
|                  | pParkin   | 0.37 ± 0.04$^*$ | –   | –                   | –                   |
|                  | pUb       | 0.021 ± 0.006 | 0.017 ± 0.005$^*$ | – | – |

Average values from a minimum of two experiments.

$^*$Data from Ordureau et al (2014). N.O., binding not observed.

R0RBR results in a cluster of residues with significant chemical shift changes including Y149, C150, K151 and G152 (RING0); R275, F277, V278, D280, Q282, G284, Y285 and S286 (β16–β17 in RING1); and I306, G308, E309, Y312 and N313 (helices H2, H3 in RING1) (Fig 4A; Appendix Fig S7). These residues surround the hinge region at the RING0/RING1 interface that is optimized for pUb binding (Fig 3B, C, E and F). There are no significant chemical shift changes including Y149, C150, K151 and G152 (RING0); R275, R305, surrounds the phosphoSer65 of ubiquitin. The pUb orientation is governed by the short β16–β17 region in RING1, appears to be absent in all other RING domain protein structures. Further, the position of the β16–β17 loop is absent in the structure of the RBR E3 ligase HHARI (Duda et al, 2013) where it is replaced by a ubiquitin-associated-like domain. The pUb-binding site is remote from the typical E2-binding site expected to reside on the opposite end of the RING1 domain.

Since the crystal structures of UblR0RBR and NMR titration experiments show that the RING0/RING1 interface is altered upon Ubl binding, we hypothesized that a similar allosteric event might occur through pUb binding to parkin. Consistent with this, several chemical shift changes occur in residues at the junction of the C-terminus of helix H3 and the IBR domain upon pUb addition (Fig 4A). The isolated IBR domain shows no chemical shift perturbations when S65EUb is titrated in, suggesting the chemical shift changes at the helix H3/IBR domain interface might result from a structural change that accompanies pUb binding. In order to test this, we used isothermal titration calorimetry (ITC) to measure thermodynamic changes in enthalpy (ΔH) and entropy (ΔS) that occur upon binding of R0RBR with pUb, S65DUb or S65EUb (Fig 4C, Table 2). As a control, we measured the binding of pUb and phosphomimetic Ubl domains to R0RBR where remodelling of the RING0/RING1 interface is the most notable structural change. As expected from the crystal structures, pUb interacts exothermically with R0RBR with negative enthalpy (−16 kJ/mol) and small entropy (33 J/molK) changes consistent with new ionic and hydrophobic interactions formed and minimal overall structural change (Fig 4C and D). The data also show that Ubl binding to R0RBR is enthalpically driven regardless of whether the phosphomimetics (S65DUb, S65EUb) or pUb is used, indicating the phosphomimetic Ubl and pUb proteins have similar binding modes to R0RBR. Remarkably, the interaction of pUb or phosphomimetic ubiquitin (S65DUb, S65EUb) with R0RBR, or pUb with full-length parkin, has opposite enthalpy and entropy changes (Fig 4C and D, Table 2). In contrast to Ubl binding, the binding of pUb to R0RBR is an endothermic binding event marked by large positive enthalpy (+32 kJ/mol) and
entropy (+261 J/mol/K) changes (Fig 4C and D). The large positive entropy change signifies that the pUb–R0RBR interaction is driven by an increase in disorder in the system, a result of a loss of structure in parkin that accompanies pUb binding (Fig 4C). Interestingly, three-dimensional structures of the isolated IBR domain show little structure in the region that comprises the C-terminus of helix H3 of the RING1 domain, the site of several chemical shift changes upon R0RBR interaction with pUb (Beasley et al., 2007). This is in contrast to the crystal structures (Fig 1C) that show a well-defined, albeit bent helix that interacts with the first Zn\(^{2+}\)-binding site of the IBR domain and the C-terminus of helix H1 in the RING1 domain. Together, these data show that pUb binding to the RING0/RING1 hinge interface results in a concomitant loss of structure near the C-terminus of helix H3 (RING1) and the IBR domain.

Phosphorylated ubiquitin binds to parkin and displaces the Ubl domain

Our crystallographic studies show that phosphorylation of the Ubl domain optimizes the RING0/RING1 interface for pUb binding, while NMR and ITC experiments show pUb binding causes a loss of structure near the IBR domain. These allosteric structural changes infer that pUb and pUb cannot be bound to parkin simultaneously. In order to test this, we performed a competition experiment monitored by NMR spectroscopy. We titrated R0RBR parkin into \(^{13}\)C-labelled S65EUbl (Fig 5A, top) and followed this in \(^{1}\)H–\(^{13}\)C-HMQC spectra such that signals from both the unbound and bound forms of S65EUbl were visible. For example, changes in the positions of A46 and L61 of the Ubl domain show a clear interaction with R0RBR. Upon addition of S65EUb, the signals for the bound form of S65EUbl revert back to those of the unbound S65EUbl state (Fig 5A, bottom). This experiment shows that phosphomimetic Ub binding displaces the bound S65EUbl domain from parkin. In a reciprocal experiment, we titrated R0RBR parkin into \(^{13}\)C-labelled S65EUb to form the activated R0RBR–S65EUbl complex (Fig 5B, top). Upon addition of S65EUbl, the signals for the bound form of S65EUb remain (Fig 5B, bottom) despite S65EUb and S65EUbl having similar dissociation constants for R0RBR (Table 2). This indicates that phosphomimetic Ubl domain is unable to bind to an activated R0RBR–S65EUbl complex. Together, these experiments show that simultaneous binding of S65EUbl and S65EUb to R0RBR is not possible. Further, the data show that although S65EUbl optimizes the pUb-binding site, the binding of S65EUb fully releases S65EUbl from parkin, consistent with the proposed allosteric loss of structure to the C-terminus of helix H3 and IBR domain that would interfere with the Ubl-binding site. This would also indicate that activation of parkin through pUb binding would prevent re-engagement with the Ubl domain until the pUb is released.

![Figure 5. pUb binding displaces the Ubl domain.](image-url)
Activation by phosphoubiquitin uncovers a ubiquitin recognition site

The residues involved in the Ubl/RING1 interface are largely conserved between ubiquitin and the Ubl domain (Fig 6A and B). Thus, displacement of the Ubl domain by phosphoubiquitin binding may lead to exposure of a surface competent for interaction with ubiquitin. We and others have previously reported an interaction between active parkin and ubiquitin (Chaugule et al., 2011; Zheng & Hunter, 2013). Therefore, we wondered if the consequence of Ubl displacement is to provide a ubiquitin-binding surface for recruitment of the charged E2–ubiquitin complex. In order to test this hypothesis, we generated mutations in helix H1 of the RING1 domain and assayed them for ubiquitin chain formation and Miro1 ubiquitination. Single-point mutations D262A, T270R and D274R are deficient in both ubiquitin chain formation and Miro1 ubiquitination (Fig 6C), in contrast to corresponding Ubl domain mutations that are activating (Fig 2C). Interestingly, the single L266K mutant is active for ubiquitin chain formation but deficient in Miro1 ubiquitination. This suggests that although L266K would be expected to repel the Ubl domain, it is still able to form a productive complex with the E2–Ub. In contrast, a triple mutant along the Ubl-binding surface of helix 1, D262A/L266K/D274R, is unable to form ubiquitin chains or ubiquitinate Miro1.

Figure 6. pUb binding to parkin uncovers a ubiquitin binding site.

A. Structure-based alignment of Ubl domain and ubiquitin. Conserved residues are shown in green, and conservative substitutions are shown in blue. Residues that interact with the RING1 domain (magenta), IBR (black) and tether (cyan) are denoted with circles.

B. Close-up of the interface between helix H1 in the RING1 domain and the Ubl domain based on the structure shown in Fig 1C. Key residues for the interaction are highlighted. The structure of ubiquitin is superimposed with Ubl domain including the corresponding residues found at the RING1 interface.

C. Ubiquitination assay of RING1 mutations in the Ubl/RING1 interface. Effects of single-point mutations and a triple mutation on both ubiquitin chain formation (top) and Miro1 ubiquitination (bottom) are shown. A Coomassie-stained loading control is shown in between. A non-specific, ATP-independent band is indicated (*).

D. Size-exclusion chromatography of phosphoparkin (pParkin), phosphohub and phosphoubiquitin (pUb), phosphoparkin, phosphoubiquitin and UbcH7, and phosphoparkin, phosphoubiquitin and UbcH7 isopeptide linked to ubiquitin (UbcH7–Ub) (left) versus the same experiment using the triple mutant of parkin (right). A coloured key of each trace is provided, and Coomassie-stained gels of the indicated peaks are shown below the trace.

E. Isothermal titration calorimetry of phosphoparkin complexed with phosphohububiquitin titrated with either UbcH7 alone (left) or UbcH7–Ub (right). Dissociation constants for each titration are shown.
Recent data suggest that in the presence (but not absence) of phosphoubiquitin, parkin forms a discrete complex with E2\textsubscript{Ub} (Ordureau et al., 2015). Therefore, we assayed the ability of the wild-type phosphoparkin/phosphoubiquitin complex to interact with E2, or E2\textsubscript{Ub} (Fig 6D). Phosphoparkin and phosphoubiquitin form a complex as measured by size-exclusion chromatography consistent with the tight binding observed in ITC experiments (Table 2). Addition of the E2 UbcH7 does not produce a further shift consistent with the observed moderate binding affinity of UbcH7 for phosphoparkin in the presence of phosphoubiquitin (K\textsubscript{d} = 19 \mu M, Fig 6E). In contrast, the addition of UbcH7–Ub leads to the formation of a higher molecular weight complex that has an apparent 20-fold tighter affinity than UbcH7 alone (Fig 6D and E), suggesting the ubiquitin molecule is recognizing an additional binding site on parkin. To test whether this ubiquitin site includes the D262/L266/D274 residues along helix 1, similar size-exclusion chromatography experiments were conducted using the D262A/L266K/D274R triple mutant protein. In contrast to wild-type parkin, the RING1 triple mutant is unable to form the pParkin/pUb/UbcH7–Ub complex (Fig 6D). Taken together, these data suggest that the consequence of Ubl displacement is the creation of a ubiquitin-binding site on RING1 to facilitate E2\textsubscript{Ub} recruitment.

**Discussion**

Until 2011, parkin was thought to be constitutively active. Since then, it has become clear that parkin is regulated in multiple ways. The Ubl domain inhibits parkin activity (Chaugule et al., 2011), the REP element blocks an E2-binding site (Trempe et al., 2013), and it has been proposed that the catalytic cysteine, C431, is occluded by the RING0 domain (Riley et al., 2013; Trempe et al., 2013; Wauer & Komander, 2013). An important observation from the structures of UblR0RBR and S65DUblR0RBR parkin is that the catalytic cysteine (C431) in the RING2 domain remains in a similar environment as that found in truncated parkin structures lacking the Ubl domain. Further, NMR chemical shift perturbation experiments show
minimal changes for C431 or to residues at the RING0/RING2 interface near the catalytic site, indicating this region of parkin does not undergo any large structural change that might expose C431. Interestingly, analysis of the UblR0RBR, S65UbR0RBR and R0RBR structures all show the thiol group of C431 is partially exposed and capable of accepting a ubiquitin molecule from the E2-Ub conjugate. Thus, the increased ubiquitination reactivity observed upon phosphoubiquitin binding likely results from enhanced recruitment of the E2-Ub conjugate due to exposure of an E2-Ub-binding site following release of the Ubl domain (Fig 6), rather than a change in structure near the catalytic C431 site.

The discovery of autoinhibition propelled efforts to uncover activators of parkin, the most compelling of which is the kinase, PINK1, upstream of parkin (Clark et al., 2006; Park et al., 2006). PINK1 phosphorylates the Ubl domain and ubiquitin at the equivalent residue S65, leading to parkin activation (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014b; Koyano et al., 2014). Recent studies report effects of each phosphorylation signal on the activity of parkin. For example, while parkin can be phosphorylated by PINK1 in the absence of phosphoubiquitin, the addition of pUb enhances parkin phosphorylation, suggesting that parkin bound to pUb is a better substrate for PINK1 than parkin alone (Kazlauskaite et al., 2015). However, phosphorylation of parkin is independent of parkin catalytic activity and parkin associates tightly with ubiquitin chains only when both ubiquitin and parkin are phosphorylated (Ordureau et al., 2014), leading to the proposal of a feed-forward mechanism. Furthermore, in cells where the only ubiquitin source is a non-phosphorylatable S65A mutant, parkin is still phosphorylated (Ordureau et al., 2015), albeit GFP-tagged parkin, which is an important caveat since it is not established that N-terminally tagged parkin species can be interpreted as wild-type (Chaugule et al., 2011; Burchell et al., 2012). We present here a mechanistic model of parkin inhibition and allosteric activation based on our structural, biochemical and biophysical insights (Fig 7). First, the Ubl domain inhibits parkin activity in the absence of phosphoubiquitin signal (Fig 7, step 1). In this inhibited conformation, the E300 and H302 are facing into the RING0/RING1 interface. Upon activation, PINK1 phosphorylates ubiquitin and the Ubl domain of parkin (Fig 7, step 2) to induce a remodelling of the RING0/RING1 interface (Fig 3C–E). Although phosphorylation of ubiquitin alone leads to a dramatic increase in its affinity for parkin (Table 2, Fig 4D), this optimized parkin conformation exhibits a further 10-fold greater affinity for pUb (Ordureau et al., 2014) (Table 2) mediated by the H302/R305/K151 basic patch (Fig 7, step 3, Fig 3B). Our model of the pUb-binding site along helix H3 of RING1 is confirmed by the recent crystal structure of pUb bound to Pediculus humanus R0RBR parkin (Wauer et al., 2015). Phosphoubiquitin binding leads to an entropically driven loss of structure in the RING1 bent helix (H3) (Fig 7, step 3). This remodelling leads to release of the Ubl domain, exposing the binding surface on RING1. Since the residues in the Ubl domain that bind the RING1 domain are well conserved with ubiquitin (Fig 6A and B), our model predicts that this exposed RING1 surface will engage ubiquitin in an E2-Ub conjugate to form a ‘charged’ parkin (Fig 7, step 4). This accounts for observations that parkin can function with different E2s. Finally, the tightly associated inhibited structure, small conformational changes, interface remodelling and multiple binding sites explain why different patient mutations have different effects on parkin activity. Although there is some debate on the ‘order’ of activating events, our study supports a model whereby each phosphorylation event enhances the activation of parkin, which is likely a major mechanism of parkin regulation in the context of low concentrations of pUb in cells (Koyano et al., 2014). The mechanistic model presented here provides important insights into parkin regulation that will be key to designing therapeutics that can control the activity of parkin and may be useful in the treatment of early-onset forms of Parkinson’s disease.

Materials and Methods

Protein purification and crystallization

UblR0RBR Parkin (residues 1–83, 144–465), phosphomimetic S65UbR0RBR and R0RBR (residues 141–465, lacking Ubl domain and linker) constructs were expressed as His-Smt3 fusion proteins in BL21 E. coli cells as previously described (Chaugule et al., 2011; Spratt et al., 2013). Media were supplemented with 50 mM ZnCl2, and cells were induced at 0.6 OD600 with 120 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated overnight at 16°C. Cells were lysed, and protein was purified over Ni-NTA resin. Beads were incubated overnight at 4°C with ULP1 protease to remove the His-Smt3 tag. The flow through from Ni-NTA incubation was collected and passed through a Mono-Q column and then loaded on Superdex-75 equilibrated with Buffer A (20 mM Tris pH 7.5, 75 mM NaCl and 250 μM TCEP). Fractions containing purified protein were pooled and concentrated using Viva spin 10-kDa cut-off concentrators. UblR0RBR (6 mg/ml) and S65UbR0RBR (2.8 mg/ml) parkin were used for sitting drop crystallization using commercial screens. Protein crystals were obtained for both parkin forms at 4°C using 200 mM LiSO4, 100 mM Bis-TRIS pH 5.5 and 20% PEG 3350 by sitting drop vapour diffusion method. Crystals appeared in 2–3 days and then allowed to grow for a week. Crystals were flash-frozen using 20% glycerol as cryoprotectant. Native data sets were collected at Diamond Light Source, beamline IO4-1 and IO4.

Synthesis and purification of phosphorylated ubiquitin and Ubl domain

Pediculus humanus GST-PINK1 (128-C) was purified on a GSTrap FF column (GE Healthcare) using 50 mM Tris, 250 mM NaCl, 1 mM DTT at pH 7.5 and eluted with freshly prepared 50 mM Tris, 250 mM NaCl, 1 mM DTT and 10 mM glutathione at pH 7.5. GST-PINK1 (10 μM) was incubated with either Ub (500 μM) or UblD (500 μM) and dialysed against buffer containing 50 mM Tris, 0.5 mM DTT, 10 mM MgCl2 and 5 mM ATP (pH 7.5) for 2 h at 25°C. The reaction was monitored to completion using Phos-Tag SDS-PAGE. Following the reaction, GST-PINK1 was removed from the reaction using a GSTrap FF column, collecting the flow-through fractions containing pUb or the pUbl domain. The phosphorylated proteins were separated from unphosphorylated species on a HiTrap Q column using 20 mM Bis-Tris propane (pH 8.7) and a 2-h 0–100% 0.5 M NaCl elution gradient. Unphosphorylated species were found in the flow through, while pure pUb and pUblD eluted around 20–40% of the salt gradient.
Synthesis and purification of UbcH7–Ub

The isopeptide-linked UbcH7–Ub conjugate was synthesized following the method of Plechanova et al (Plechanova et al., 2012). His-tagged Uba1 (25 μM, not TEV-cleavable), His-TEV-Ub (200 μM) and UbcH7 (C17S/C86K/C137S, 400 μM) were incubated at 37°C for up to 16 h in 50 mM CHES, 150 mM NaCl, 10 mM ATP and 10 mM Mg2+ at pH 9.0 until UbcH7–Ub conjugate formation reached a stable level as viewed by SDS–PAGE electrophoresis. Purification of the UbcH7–Ub conjugate was performed with a HisTrap column (GE Healthcare) initially to remove His-Uba1, and following TEV cleavage to remove His-Uba1. Purified UbcH7–Ub was isolated using a HiLoad Superdex (GE Healthcare) size-exclusion column to separate UbcH7–Ub from Ub.

Structure determination

Data were indexed, processed and scaled using Mosflm and Scala as implemented in CCP4 (CCP4, 1994). The UbiR0RBR structure was determined by molecular replacement. Full-length rat parkin structure (4K95) was used as a search model. The initial model was further built and refined using coot (Emsley & Cowtan, 2004) and autobuster (Bricogne et al, 2011). The phosphomimetic S65Dubiquitin UblR0RBR structure was solved using UbiR0RBR as the search model, and refined as UbiR0RBR. Both models have excellent geometry with statistics listed in Table 1.

Isothermal titration calorimetry

All calorimetry experiments were performed using either a Microcal VP-ITC system (GE Healthcare) or a NanoITC (TA Instruments) at 25°C. All experiments were completed 2–3 times using freshly prepared proteins in either 50 mM HEPES, 50 mM NaCl and 250 mM tris(carboxymethyl)phosphine (TCEP) at pH 7.5 or 50 mM HEPES, 150 mM NaCl and 0.5 mM TCEP at pH 8.0. The concentrations of each protein in the experiments were as follows: 50 μM R0RBR parkin titrated with 1.25 mM ubiquitin, 750 μM S65D ubiquitin, 400 μM phosphoubiquitin, 750 μM Ubl domain and 1 mM phosphoUbl domain. Full-length parkin (50 μM) was titrated with 1.75 mM ubiquitin, 1.75 mM S65D ubiquitin or 400 μM phospho-ubiquitin. Phosphoparkin (35 μM) was titrated with 2.4 μM UbcH7 or UbcH7–Ub. Data were analysed using single-site binding models.

Ubiquitination assays

Ubiquitination reactions were performed at 30°C in 50 mM DL-malic acid, MES monohydrate, Tris (MTr) pH 7.5, 50 mM NaCl, 2.5 mM MgCl2, 5% (v/v) glycerol and 0.75 mM diethytheritol (DTT) buffer system. All reactions contained 25 nM of recombinant human E1, 500 nM of Ube2L3 (UbcH7), 1 μM E3 and 5 mM ATP in a 20 μl final reaction volume.

Complete ubiquitination profiles were analysed using fluorescently labelled ubiquitin (ULIR800). Ubiquitin (residues 2–76) was expressed and purified bearing a GPLCGS overhang at the N-terminus. The cysteine residue in the overhang was targeted for site-specific incorporation of a DyLight™ 800 Maleimide (Life Technologies, 46621) dye following the manufacturer's protocol. Labelled species were purified by cation exchange chromatography and stored at −20°C as single-use aliquots. Reactions to monitor ubiquitination profiles contained 2 μl of ULIR800 and supplemented with either phosphorylated ubiquitin or unlabelled ubiquitin as indicated. Reactions were terminated with SDS loading buffer, resolved by SDS–PAGE and analysed by direct fluorescence monitoring using Li-COR® Odyssey Infrared Imaging System.

Glutathione S-transferase (GST)-human Miro1 (residues 1 to 592) was expressed and purified using standard protocols. GST-Miro1 ubiquitination reactions were carried out as above except with a final ubiquitin concentration of 20 μM (inclusive of indicated amount phosphorylated ubiquitin). Reactions were subjected to immunoblotting using anti-GST rabbit monoclonal primary antibody (1H14L28, Life Technologies, 1/3,000 dilution) and fluorescent-labelled secondary antibody (926-32213, Li-COR®, 1/10,000 dilution). Blots were visualized using Li-COR® Odyssey Infrared Imaging System.

Size-exclusion chromatography–multiAngle light scattering analysis

Size-exclusion chromatography and multiangle light scattering (SEC–MALS) experiments were performed on a Dionex Ultimate 3000 HPLC system with an inline Wyatt miniDAWN TREFS MALS detector and Optilab T-rEX refractive index detector. SEC–MALS experiments were performed on a Superdex 75S CL 10/300 (GE Healthcare) column in buffer containing 50 mM HEPES with 100 mM NaCl at pH 7.5. Phosphoubiquitin (140 μM) and phosphoparkin proteins (20 μM) were incubated for 30 min before injections. Molar masses spanning elution peaks were calculated using ASTRA v6.0.0.108 (Wyatt). For the size-exclusion analysis with UbcH7 or UbcH7–Ub, phosphoparkin proteins (20 μM) were incubated with phosphoubiquitin (40 μM) and either UbcH7 (40 μM) or UbcH7–Ub (40 μM) for 30 min prior to injections.

NMR spectroscopy

Uniformly 2H,13C,15N-labelled or 2H,12C,15N-labelled parkin R0RBR was expressed in minimal media supplemented with 15NH4Cl (1 g) and either 2H,13C-glucose (2 g) or 2H,12C-glucose (2 g)(Cambridge Isotope Laboratories). Cells were initially grown in 70% D2O until the OD600 reached 0.9 units. Cells were pelleted, re-suspended in minimal media containing 99.8% D2O supplemented with 0.25 mM ZnCl2 and grown until the OD600 reached 0.8 units. Following the addition of a further aliquot of ZnCl2 (0.25 mM), the temperature was lowered to 16°C and cells were induced with 100 μM IPTG for 20 h. Assignment of R0RBR was accomplished using 1H,13C,15N-labelled protein (300–500 μM) in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP and 90% H2O/10% D2O at pH 7.0 using DSS as an internal reference. All NMR data were collected at 25°C on a Varian Inova 600 MHz NMR spectrometer equipped with a triple resonance cryogenic probe and z-field gradients or an 850 MHz Bruker Avance III spectrometer with TCI cryoprobe. All 1H,15N-HSQC spectra were collected in TROSY mode (Pervushin et al., 1997). Triple resonance pulse sequences (HNCa, HN(CO)Ca, HN(CA)CB, HN(COCA)CB, HNCO, HN(CA)CO) (Yang & Kay, 1999) were collected in TROSY mode using deuterium decoupling as described for fully deuterated proteins. Heteronuclear NOE NMR experiments (Farrow et al, 1994)
were performed at 600 MHz using 500 μM uniformly deuterated parkin 141-C. Proton saturation was achieved through a 5-s irradiation following an 11-s relaxation delay. The equivalent non-saturated experiment contained a 16-s relaxation delay. Both saturated and non-saturated experiments were conducted in duplicate. All data were processed using NMRPipe and NMRDraw (Delaglio et al., 1995) and analysed using NMRViewJ (Johnson & Blevins, 1994).

Protein interaction experiments were conducted using 100–200 μM 2H,13C,15N-labelled R0RBR and either 13C-labelled or 2H,13C-labelled Ub or Ub1D proteins. 1H,15N-TROSY spectra were collected to monitor chemical shifts of backbone amides in R0RBR. 1H,13C-HMQC spectra (Tugarinov et al., 2004) were collected to monitor chemical shifts of side chain methyl groups in Ub or Ub1D. Chemical shift perturbations were quantified using the following weighted formula: \((0.2 \times Δ\Delta\text{N}) + \Delta\Delta\text{H}/2\) and plotted as a function of residue using Prism5 (Graphpad).

Molecular modelling of phosphoubiquitin bound to parkin R0RBR

Docking of pUb with R0RBR used chemical shift perturbations from NMR experiments (described above). Interacting residues in R0RBR were defined as those that shifted greater than the average shift + 0.5 standard deviations and had greater than 20% side chain accessible surface area. Due to the limited number of methyl-containing residues, residues in pUb were chosen where methyl resonances shifted regardless of accessible surface area. An upper distance limit of 5.0 Å was set for ambiguous distance restraints. A single unambiguous distance restraint of 3.0 Å was set based on mutagenesis experiments that indicated p665 (Ub) was near H302 (R0RBR) that was also supported by chemical shift changes of neighbouring residues. The coordinates for S65DUblR0RBR was used as the starting structure following the removal of the coordinates corresponding to the Ub domain and adjoining linker region. Missing regions, where chemical shift changes were observed upon pUb interaction, were modelled in using the Modeller (Eswar et al., 2006) plug-in for UCSF Chimera (Pettersen et al., 2004). Docking of pUb with R0RBR used HADDOCK (Dominguez et al., 2003). A total of 1,000 initial complexes were calculated, and the best 100 structures were water refined. Standard parameters were used except inter_rigid (0.1) which was set to allow tight packing of the two proteins, and the unambiguous force constants were increased by five-fold compared to the unambiguous constants. The location and orientation of the pUb with respect to R0RBR were similar in all 100 water-refined complexes, having a backbone rmsd of 0.37 ± 0.02 for the best 20 complexes. Paramagnetic relaxation enhancement experiments (PRE) were used to confirm the orientation of the pUb molecule with respect to the R0RBR protein. Briefly, 12C/14N/2H-labelled cysteine-substituted Ub (LSC or K48C) was expressed in minimal media, purified and phosphorylated using Pediculus humanus GST-PINK1 as described above. Proteins were spin-labelled with 3-(2-iodoacetamido)-PROXYL (Sigma Aldrich) by incubating each protein with 10 molar equivalents for 2 h at room temperature and dialysed exhaustively at 4°C. ESI-MS confirmed quantitative (> 95%) phosphorylation and successful introduction of spin label (~90 and ~60% spin label incorporation for LSC and K48C, respectively). 1H,13N TROSY spectra were collected monitoring 400 μM 13C/15N/2H-R0RBR before and after the addition of 1.2 molar equivalents of each nitroxide-labelled pUb variant. Identical spectra were collected following the addition of 10 molar equivalents of ascorbic acid to quench the nitroxide spin tag. PRE factors in R0RBR were determined in NMRViewJ by measuring peak intensities with an active and quenched nitroxide spin tag.

Data and reagents availability

The PDB files of the structures determined in this study have been deposited with the RCSB Protein Data Bank (www.rcsb.org) with accession codes 5C1Z (UbR0RBR) and 5C23 (S65DUblR0RBR). NMR assignments have been deposited to the BMRB (www.bmrw.wisc.edu) under accession codes 25708 (S65UEUb), 25707 (Ub bound to R0RBR parkin), 25709 (S65UEUb bound to R0RBR parkin) and 26605 (Ub domain bound to R0RBR parkin). Recombinant proteins and plasmids generated for the present study are available to request on the MRC-PPU reagents website (https://mrcppureagents.dundee.ac.uk/).

Author contributions

AK crystallized and solved the Ub1R0RBR and S65DR0RBR structures, performed the complex experiments in Fig 6, analysed data and wrote the manuscript. JDA collected NMR experiments for assignment, conducted NMR and ITC experiments for Ub, Ub56SE and pUb R0RBR interactions and pUb-modelling experiments (PRE and heteronuclear nOe), analysed data and wrote the manuscript. TECC assigned NMR spectra, conducted NMR and ITC experiments for Ub, Ub56SE and pUb R0RBR interactions, and analysed data and wrote the manuscript. RJMT performed ITC experiments and analysis, and expressed and purified multiple proteins. VKC designed and performed ubiquitination assays. RT cloned several constructs. RS performed SEC-MALS analysis in Appendix Fig S6. PM assigned NMR spectra of R0RBR. AK produced phosphoproteins. DES completed preliminary Ub interaction experiments with R0RBR by NMR and synthesized spin-labelled pUb proteins for pUb–R0RBR interaction experiments. KRB expressed and purified numerous isotopically labelled proteins for NMR experiment. GSS and HW conceived of the study, designed experiments, analysed data and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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