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1 **Parkin-phosphoubiquitin complex reveals a cryptic ubiquitin binding site**
2 **required for RBR ligase activity**

3

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14 **Abstract**

15 RING-BETWEENRING-RING (RBR) E3 ligases are a class of ubiquitin ligases
16 distinct from RING or HECT E3 ligases. An important RBR is Parkin, mutations in
17 which lead to early onset hereditary Parkinsonism. Parkin and other RBRs share a
18 catalytic RBR module, but are usually autoinhibited and activated via distinct
19 mechanisms. Recent insights into Parkin regulation predict large, unknown
20 conformational changes during activation of Parkin. However, current data on active
21 RBRs are in the absence of regulatory domains. Therefore, how individual RBRs are
22 activated, and whether they share a common mechanism remains unclear. We now
23 report the crystal structure of a human Parkin-phosphoubiquitin complex, which
24 shows that phosphoubiquitin binding induces a movement in the IBR domain to
25 reveal a cryptic ubiquitin binding site. Mutation of this site negatively impacts on
26 Parkin's activity. Furthermore, ubiquitin binding promotes cooperation between
27 Parkin molecules, suggesting a role for interdomain association in RBR ligase
28 | mechanism.
29

30

31 **Introduction**

32 Parkinson's Disease (PD) is a neurodegenerative disorder characterised by the
33 progressive loss of dopaminergic neurons, bradykinesia and tremor ¹. Although
34 primarily a sporadic disorder, mutations in several genes are associated with different
35 Parkinsonism syndromes ², including the genes *PARK2* and *PARK6* which lead to
36 autosomal recessive juvenile Parkinsonism (ARJP) ^{3,4}. *PARK2* and *PARK6* encode
37 Parkin E3 Ubiquitin ligase and PTEN-induced kinase (PINK-1), respectively, and
38 maintain mitochondrial homeostasis ⁵⁻⁸. In addition to its role in mitochondrial protein
39 control, Parkin also regulates protein degradation and induces aggresome formation
40 by K63-linked ubiquitination ⁹⁻¹¹.

41 Parkin belongs to the RBR E3 ubiquitin ligase family, characterised by a RING
42 domain (RING1) followed by an 'in-between RING' (IBR) domain and a catalytic
43 domain (RING2 or Rcat ¹²). RING1 is structurally similar to canonical RING-type E3
44 ligases, and shares the function of binding to E2 ubiquitin conjugating enzymes. In
45 contrast, RING2(Rcat) adopts a linear zinc-binding fold ^{13,14} and possesses a catalytic
46 cysteine capable of forming a thioester bond with activated ubiquitin ^{13,15-19}. The
47 linking 'IBR' domain adopts the same fold as the RING2(Rcat) ²⁰, however its
48 functional role remains unclear. RBR ligases contain additional domains outwith the
49 RBR module. Parkin has an N-terminal ubiquitin-like domain (UBL), which shares
50 65% homology with ubiquitin, and a zinc-binding RING0 or unique parkin domain
51 (UPD) ²¹. Parkin adopts an autoinhibited conformation mediated by multiple domain-
52 domain interactions ²²⁻²⁶. This autoinhibition is released by PINK1-dependent
53 phosphorylation of ubiquitin and the UBL domain of Parkin, leading to activation of
54 Parkin ²⁷⁻³². Additional RBR ligase family members include HOIP, which is a subunit
55 of the linear ubiquitin chain assembly complex (LUBAC) ³³, and the human

56 homologue of Ariadne, HHARI³⁴. The catalytic activity of HOIP is also regulated
57 through autoinhibition, mediated by its UBA domain, which interacts with the other
58 subunits of LUBAC, HOIL-1L and SHARPIN to activate HOIP^{17,33,35,36}. HHARI is
59 also autoinhibited, mediated through its Ariadne domain³⁷. HHARI activation
60 requires interaction of the UBA domain of HHARI with NEDD8^{38,39}. Thus our
61 current understanding of Parkin and other RBRs suggests distinct modes of
62 regulation. In the case of Parkin, we and others have shown that the binding of
63 phosphoubiquitin, leads to Parkin activation via removal of the inhibitory effect of the
64 Ubl domain^{23,24,40-43}. However, all the structural insights into Parkin are in the context
65 of the autoinhibited state²³⁻²⁵, or in the absence of the regulatory Ubl domain^{16,25,26,43}.
66 Insights into HHARI are also based on an inactive conformation³⁷. Recent insights
67 into the catalytic mechanism of the RBR family have come from structural and
68 physical analyses of HOIP^{14,44}. The crystal structure of the catalytic RING2-Like
69 (RING2L) or Rcat domain of HOIP in complex with ubiquitin revealed how linear
70 chains are assembled¹⁴, while a recent complex of the RBR module of HOIP in
71 complex with a charged E2~ubiquitin conjugate revealed a series of allosteric
72 ubiquitin-binding sites coupled to a swapped dimer of the RING2L(Rcat) domain
73 between RBR molecules⁴⁴. Furthermore, the RING2(Rcat) of HHARI has a ubiquitin
74 binding site important for E2~ubiquitin recruitment⁴⁵, and the RING1 of HHARI
75 supports an open conformation of the E2~ubiquitin conjugate. In contrast a structure
76 of the RING0-RBR (R0RBR) domains from *Pediculus humanus corporis* Parkin, in
77 complex with phosphoubiquitin does not show domain swapping between RBR
78 modules, but rather revealed a conformational change in the IBR domain upon
79 phosphoubiquitin binding⁴³. Our current understanding is derived from snapshots of
80 individual domains of large multidomain complexes. However, how RBR ligases

81 function in the context of their regulatory domains remains unclear, and whether they
82 share a universal catalytic mechanism remains unclear.

83 We report here the crystal structures of activated human Parkin (UblR0RBR), and a
84 phosphomimic Parkin (S65DUblR0RBR), in complex with phosphoubiquitin, at 2.7
85 and 2.6 Å resolution respectively. All Parkin domains are present, and in contrast to
86 earlier predictions of large conformational rearrangements upon activation, the Parkin
87 structures reveal subtle, local changes that result in a series of cryptic ubiquitin or
88 ubiquitin-like binding regions that are essential for Parkin function. We find an
89 essential role of the IBR domain in ubiquitin recruitment and Parkin activity.
90 Furthermore, comparative analyses with the HOIP and HHARI modules reveals
91 common ubiquitin or ubiquitin-like binding sites. In particular, comparison with the
92 RBR of HOIP suggests a potential unifying mechanism of cooperation between
93 multiple RBR modules in the mechanism of ubiquitin ligase activity.

94

95 **Results**

96 **Crystal structure of UBLR0RBR Parkin in complex with pUb reveals activated** 97 **state of Parkin**

98 Previous studies by ourselves and others suggest that binding of
99 phosphoubiquitin to Parkin relieves the autoinhibition by the UBL domain. Consistent
100 with this, the UBL domain displays weaker binding to the R0RBR fragment of Parkin
101 in *trans* upon phosphoubiquitin binding^{23,24,40-43,46}. In order to understand the
102 allosteric regulation by phosphoubiquitin, we crystallised a complex of Parkin
103 UBLR0RBR (Δ 84-143) with a phosphoubiquitin (pUb) suicide probe (pUb3BR, pUb
104 bromopropylamine). To enable the formation of stable, well diffracting crystals, we
105 generated a covalent Parkin-pUb complex by ligating the pUb3BR probe to a cysteine

106 residue on the surface of Parkin. The cysteine was provided by mutating Gln347, as
107 described previously⁴³. The structure of activated Parkin was refined to 2.7 Å (Table
108 1), with good geometry and refinement statistics. The crystal structure of the Parkin-
109 pUb complex reveals the activated state of Parkin, containing all 5 domains of Parkin
110 UBL, RING0, RING1-IBR-RING2(Rcat) and pUb (Fig 1a). In the asymmetric unit,
111 UBL, RING1-IBR-RING2(Rcat) are contributed from one molecule whereas RING0
112 and pUb are from another molecule, with the biological unit via symmetry related
113 molecules (Supp Fig 1a). Comparison of the activated state of Parkin-pUb with the
114 apo, inactive UBLR0RBR structure²³ shows that although some small
115 rearrangements occur, the UBL-RING1 interface, primarily formed by between β 3
116 and β 5 of the UBL domain, and helix H1 of the RING1 domain, remains largely
117 intact in the presence of phosphoubiquitin (Fig 1b). The largest structural
118 rearrangement upon activation is the movement of the IBR domain. Helix H3 of the
119 RING1 domain straightens, leading to global movement of the IBR domain
120 (henceforth this helix is referred to as H3-IBR), and creating a void between the UBL
121 and IBR domains. This straightening of the H3-IBR helix is also observed in the
122 insect Parkin R0RBR complex with pUb that lacks the UBL domain⁴³. In the apo
123 structure this UBL-IBR interface is formed primarily through interactions of His11 of
124 the UBL domain with Lys369, Glu370 of the IBR domain, and surrounding residues,
125 and these interactions are lost in the phosphoubiquitin-bound complex.
126 Phosphorylation of the UBL domain by PINK-1 has been shown to weaken
127 association of the UBL domain with R0RBR Parkin^{23,24,42,46,47}. Therefore, we
128 wondered whether inclusion of a negative charge at Ser65 to mimic phosphorylation
129 would lead to displacement of the UBL domain in presence of pUb. To test this, we
130 crystallised a covalent complex of S65D-UBLR0RBR Parkin with the pUb suicide

131 probe. In this structure, refined to 2.6 Å resolution, the S65D-UBL domain remains
132 associated with RING1 (Supp Fig.1b). However, there are local conformational
133 rearrangements between UBLR0RBR/S65SD-UBLR0RBR in complex with pUb and
134 the apo structure of UBLR0RBR. In the apo structure of UBLR0RBR, residues 383-
135 390, part of the tether that connects the IBR domain to the Repressor Element of
136 Parkin (REP) are disordered^{23,25}. In contrast, this tether, residues 387-390, is ordered
137 and traceable in the WT Parkin-pUb complex, with Arg392 pointing towards the
138 disordered side-chain of Lys48 in the UBL domain. This Arg392-Lys48 creates an
139 electrostatic repulsion, while Gln389 and Tyr391 cement the association of the UBL-
140 RING1 interface, resulting in flexibility of the 62-65 loop in the UBL domain (Supp
141 Fig 1b). Upon inclusion of a negative charge at position 65, Tyr391 relocates from the
142 UBL-RING1 interface, resulting in disorder of the IBR-REP tether and ordering of
143 the 62-65 loop in the UBL domain (Supp Fig 1c). We wondered whether mutation of
144 these residues would enable the UBL domain to bind ΔUBL-Parkin in the presence of
145 pUb. In the absence of pUb, the UBL domain associates with ΔUBL-Parkin (80-465)
146 with a dissociation constant (Kd) in the low micromolar range^{22-24,43} (Supp Fig 1d).
147 The presence of pUb blocks the UBL interaction with ΔUBL- Parkin (Supp Fig 1d).
148 Gln389Ala, Tyr391Ala and Arg392Ala mutation in ΔUBL-Parkin does not permit
149 UBL re-association in the presence of pUb (Supp Fig 1d). Given that the structures
150 clearly show the UBL-RING1 interface persists in the presence of pUb, we wondered
151 whether the loss of association in solution could be due to a weakened UBL-IBR
152 interface (Fig 1B), rather than UBL-RING1 interface. To test this, we expressed and
153 purified human Parkin lacking the IBR domain, and measured the interaction of the
154 UBL domain with Parkin, using isothermal titration calorimetry (ITC). The UBL
155 domain does not associate with ΔUBL-ΔIBR (80-329,383-465) (Fig 1c). In order to

156 understand the role of the IBR domain we assayed this mutant for E3 ligase activity.
157 Δ UBL-Parkin is active, both in the absence and presence of phosphoubiquitin (Fig
158 1d). In contrast, deletion of the IBR domain in Δ UBL-Parkin leads to complete loss of
159 Parkin activity, which cannot be rescued by the addition of pUb. It is a possibility
160 that removal of the IBR domain results in a constrained version of Parkin that lacks
161 the flexibility needed for maintenance of its catalytic domains. In order to rule this
162 possibility out, we purified Δ UBL- Δ IBR containing a 10-residue (Gly-Thr-Ser-Gly-
163 Thr-Ser-Gly-Ser-Ala-Ser) (Δ UBL- Δ IBRxL10) linker to span the 28 Å distance
164 required, and assayed for ubiquitination activity (Fig 1d). These constructs lacking the
165 IBR domain are folded, monodisperse and migrate at the expected molecular weight,
166 consistent with folded proteins. In contrast to wild-type and Δ UBL-Parkin, both
167 Δ UBL- Δ IBR and Δ UBL- Δ IBRxL10 Parkin lack ubiquitination activity, even in the
168 presence of phosphoubiquitin. Interestingly, the surface of the UBL domain when
169 phosphorylated would result in repulsion with the negatively charged surface of IBR,
170 supporting a weakened UBL-IBR interaction (Supp Fig 1e). The observation that the
171 UBL domain and pUb both interact with IBR, on opposite sides, could explain the
172 competitive mode of binding to Parkin that has previously been observed^{23,24}. Taken
173 together, these results show that activation of Parkin by phosphoubiquitin binding
174 involves loosening of the UBL-IBR interface, caused by a straightening of the H3-
175 IBR helix. Furthermore, these experiments show that the association of the UBL
176 domain with the rest of Parkin depends upon the presence of the IBR domain in
177 addition to the more extensive RING1 surface, and that Parkin activity requires an
178 intact IBR domain.
179

180 **Parkin activation exposes ubiquitin or ubiquitin-like binding surfaces that are**
181 **essential for Parkin activity**

182 The straightening of the H3-IBR helix caused by phosphoubiquitin binding
183 results in a void being created between the UBL and IBR domains (Supp Fig 2a).
184 Intriguingly, analysis of the crystal packing of activated Parkin reveals that this void
185 is occupied by the UBL domain of another molecule of Parkin (Supp Fig 2b). The
186 UBL domain of Parkin shares 65% sequence similarity with ubiquitin. We therefore
187 wondered whether the void created by the loosening of the UBL-IBR interaction,
188 occupied in our crystal structures by the UBL domain of a second molecule of Parkin,
189 could accommodate ubiquitin. To explore this idea, we modelled ubiquitin into the
190 void (Fig 2a). This arrangement reveals potential surfaces on Parkin that could
191 interact with ubiquitin, referred to as Ubiquitin Binding Region (UBR) 1,2, and 3,
192 respectively (Fig 2). UBR1 is contributed by the UBL domain of Parkin, mediated by
193 His11, Phe13, Arg33, Gln34 (Fig 2b). Mutation of UBR1 residues on Parkin results in
194 a minor reduction in ubiquitin chain formation, but no observable decrease in
195 substrate ubiquitination, in this case Miro1 (Fig 2b). UBR2 and UBR3 are formed on
196 the straightened helix (H3) of RING1 and the IBR of Parkin, respectively. The
197 potential ubiquitin binding surfaces are mediated by Arg275, Gln317, Tyr318, Glu321
198 on H3 of RING1, and Arg334 and Pro335 of the β -hairpin on IBR (Fig 2c). Mutations
199 in UBR2 (R275A, Y318A, E321A) and UBR3 (R334A) result in loss of E3 ligase
200 activity, both in ubiquitin chain formation and Miro1 ubiquitination (Fig 2c).
201 Therefore, UBR2 and UBR3, but not UBR1, are important for Parkin activity. In
202 order to further clarify which regions of activated Parkin interact with ubiquitin, we
203 performed NMR chemical shift perturbation experiments of the R0RBR fragment of
204 Parkin in complex with pUb (Fig 2d). Upon addition of unlabelled Ub to the R0RBR-

205 pUb complex it was clear that the IBR domain was a major site of interaction. The
206 largest chemical shift changes occur for residues G329-R334 of the β -hairpin and the
207 adjoining loop (G361-A363, R366) of the IBR domain. Changes were also observed
208 in the straightened helix (H3) of RING (R314, V324) near those observed in the
209 crystal structure. However, several of these resonances are broadened by pUb binding
210 and are therefore difficult to visualise in the NMR spectra. Nevertheless, this analysis
211 shows a large ubiquitin interaction surface in activated Parkin that is consistent with
212 that for UBR2 and UBR3 sites predicted from the crystal packing analyses. These
213 findings suggest a role for ubiquitin (or ubiquitin-like protein) binding at surfaces
214 exposed upon pUb binding in the regulation of Parkin activity. Consistent with this, a
215 recent study shows that modification of the IBR domain at Lys349 and Lys369 by the
216 ubiquitin-like protein ISG15, positively regulates Parkin activity⁴⁸. Interestingly,
217 Lys349 is also a target of Parkin autoubiquitination activity¹³. Therefore, we
218 wondered whether Parkin modification by ubiquitin can influence Parkin activity. To
219 explore this possibility, we generated a covalently linked Parkin carrying a ubiquitin
220 chain (UBLR0RBR347Cys~pUb-M1-(UBS65A)₃, to mimic polyubiquitinated Parkin.
221 In comparison with UBLR0RBR347Cys~pUb or providing pUb to UBLR0RBR in
222 *trans*, UBLR0RBR347Cys~pUb-M1-(UBS65A)₃ activity is dramatically enhanced
223 (Supp Fig 2c). Taken together, these data suggest an important role for the
224 displacement of the UBL-IBR interface, revealing that modification of the IBR,
225 phosphorylation of Ser65 in the UBL-IBR interface, and pUb binding to the IBR
226 domain all contribute to the creation of a ubiquitin/ubl binding region, and that
227 ubiquitin binding stimulates Parkin activity.

228

229 **The UBL-IBR ubiquitin binding site recruits the donor ubiquitin**

230 The arrangement of phosphoubiquitin of one molecule of Parkin, and the UBL
231 domain of a second molecule, is reminiscent of the activator ubiquitin and donor
232 ubiquitin recently described in the structure of the RBR of HOIP in complex with a
233 charged E2⁴⁴ (Fig 3a). Furthermore, UbcH7 interaction with phosphorylated Parkin is
234 enhanced 20 fold by the addition of phosphoubiquitin and charging of the E2 with
235 ubiquitin^{23,49}. Thus we wondered whether the ubiquitin binding site created from the
236 activation of Parkin by phosphoubiquitin could accommodate the charged
237 E2~ubiquitin conjugate. To test this, we measured UbcH7 or UbcH7~ubiquitin
238 interaction with phosphoparkin (pParkin) or a UBR2 mutant pParkinE321A, in the
239 presence of pUb. ITC experiments show a similar affinity of pParkin or
240 pParkinE321A for UbcH7 (Kd of 19 and 23.5 μ M respectively) in the presence of
241 pUb, suggesting that mutation of the ubiquitin binding region of Parkin does not
242 interfere with E2 binding (Fig 3b). In contrast, while phosphoParkin binds to
243 UbcH7~ubiquitin with a 30-fold higher affinity, this enhanced binding is diminished
244 for phosphoParkinE321A (~2.5 fold) (Fig 3b). In addition, size exclusion
245 chromatography of these complexes reveals that mutation of E321 to alanine
246 abolishes formation of the phosphoParkin UbcH7~Ub complex in the presence of
247 phosphoubiquitin (Supp Fig 3a). We ruled out the possibility that mutation of E321
248 leads to loss of phosphoubiquitin binding by ITC measurement (Supp Fig 3b). These
249 data suggest that the ubiquitin carried by charged E2 binds at the UBR regions in
250 activated Parkin. Previous studies have shown that when the only source of ubiquitin
251 is phosphoubiquitin, Parkin cannot catalyse ubiquitin chains^{49,50}. However, UbcH7
252 can be charged with pUb⁵⁰. Furthermore, measurements of Parkin fragments
253 interacting with either ubiquitin or pUb showed a similar isotherm profile, but with
254 much tighter binding to pUb²³. Both these observations suggest that the pUb-binding

255 pocket on the surface of Parkin is the dominant ubiquitin binding site. Therefore we
256 wondered whether loading of E2 with pUb redirects the conjugate to the pUb binding
257 site on the Parkin, rather than going to the donor ubiquitin binding site. To test this,
258 we “activated” Parkin using either pUb, or UbcH7 charged with pUb (UbcH7~pUb).
259 Interestingly, UbcH7~pUb, but not UbcH7~ubiquitin, activates Parkin to a similar
260 extent as pUb alone (Fig 3c). Taken together, these data suggest that the cryptic
261 ubiquitin binding site created between the UBL-IBR domains upon Parkin activation,
262 recruits the donor ubiquitin as carried by the E2.

263

264 **Cooperation between multiple Parkin molecules promotes ubiquitin transfer**

265 Using the position of the UBL domain, the HOIP RBR/E2~Ub structure, and
266 the chemical shift perturbations on Parkin as guides, we modelled an activated Parkin-
267 donor ubiquitin complex (Supp Fig 4a). In the model, with only one molecule of
268 Parkin, it is difficult to envision how the E2 of the E2~Ub conjugate would reach the
269 proposed E2 binding site at the base of the RING1. In addition, it is difficult to
270 determine how the ubiquitin would reach the catalytic cysteine in the RING2(Rcat).
271 Indeed, despite many efforts to understand how the catalytic cysteine in the
272 RING2(Rcat) domain mechanistically directs Parkin activity, previous Parkin
273 structures show that the Cys431 of the RING2(Rcat) domain is ~35 Å away (using the
274 sulfur of Cys431, and the side-chain oxygen of Thr240 as the reference points) from
275 the predicted E2 binding site on RING1²⁵. Furthermore, multiple groups have
276 reported that Cys431 is occluded by RING0^{16,25,26}. In our crystal structure of the
277 Parkin-pUb complex, one molecule of activated Parkin accommodates a UBL domain
278 from a neighbouring molecule, which we model as donor ubiquitin. This modelled
279 donor ubiquitin packs against the catalytic RING2(Rcat) domain of a neighbouring

280 Parkin molecule (Fig 4a). This quaternary arrangement of Parkin molecules can also
281 accommodate an E2, with proposed interactions with the RING1 and REP of
282 molecule 2, satisfying observed E2 interaction sites in HOIP and HHARI^{44,45} (Fig
283 4a). Therefore, we wondered whether the UBR2 donor ubiquitin binding site would
284 affect the loading on ubiquitin onto the catalytic cysteine (C431). In order to test this,
285 we designed an *in vitro* assay that evaluates the transfer of donor ubiquitin from the
286 E2 to the RBR catalytic residue. First, we generated a Parkin RING2(Rcat) mutant,
287 able to trap the catalytic Parkin~Ub intermediate^{13,17}, referred to as R0RBR^{CH}. This
288 species has a serine in place of the catalytic cysteine (C431S) and a His433Ala
289 mutation in order to trap an ester-bound ubiquitin and impede any subsequent
290 discharge. This R0RBR^{CH} Parkin species can be charged with ubiquitin and was
291 sensitive to sodium hydroxide hydrolysis thus confirming the RING2(Rcat)-ubiquitin
292 ester link. The addition of phosphoubiquitin in this setup greatly enhances the
293 RING2(Rcat) charging by around 7-fold while a catalytic Cys431Ala mutant (R0RBR
294 C431A) Parkin cannot be charged with ubiquitin in either scenario (Fig 4b). We then
295 generated a UBR2 patch mutant of Parkin in this background, R0RBR^{CH} E321A. In
296 contrast to R0RBR^{CH}, this mutant is defected in ubiquitin charging even in the
297 presence of phosphoubiquitin (26-fold less). Finally, a combined mutant of the
298 phosphoubiquitin patch and the UBR2 patch (R0RBR^{CH} H302A+E321A) was
299 drastically reduced (60-fold less) in ubiquitin charging of the RING2(Rcat) (Fig 4b).
300 Recent studies have suggested a physiological role for self-association of Parkin
301 molecules. For example, in 2013, two independent studies reported the observation
302 that catalytically compromised Parkin (C431 mutants) could not translocate to the
303 mitochondria after PINK1 activation^{15,19}. However, co-expression of C431 mutant
304 Parkin with either wild-type Parkin, or other mutations including the R275W that

305 would affect the UBR2 binding site, could rescue this translocation defect. These
306 observations raise the possibility of cooperation between Parkin molecules. We have
307 demonstrated that mutation of the donor ubiquitin binding site UBR2 in Parkin results
308 in loss of Miro1 ubiquitination (Fig 2c). Thus we wondered whether a constitutively
309 phosphoubiquitin-bound form of Parkin could support the activity of otherwise
310 inactive Parkin, in this case phosphoParkinE321A. To test this, we took the
311 crystallised species of Parkin UblR0RBR covalently linked to phosphoubiquitin, and
312 titrated it into phosphoParkinE321A (Fig 4c). We find that wild-type phosphoParkin
313 can ubiquitinate Miro1 (Fig 4c). Addition of UblR0RBR, or UblR0RBR covalently
314 complexed with pUb does not further enhance this activity. In contrast,
315 phosphoParkinE321A is defective in Miro1 ubiquitination (Fig 4c). Addition of
316 autoinhibited UblR0RBR does not significantly enhance Miro1 ubiquitination (Fig 4c,
317 Supp Fig 4b). However, addition of equimolar amounts of active UblR0RBR
318 complexed with pUb rescues Miro1 ubiquitination to a greater extent than having the
319 UblR0RBR.pUb complex alone (Fig 4c). These data suggest that the activity of
320 inactive Parkin molecules can be stimulated by the presence of activated Parkin
321 molecules. Taken together, these data demonstrate that Parkin molecules can function
322 together to ligate ubiquitin.

323

324

325 **Discussion**

326 Previous extensive characterisation of Parkin has shown that Parkin exists in
327 an autoinhibited state, mediated through multiple domain-domain interactions,
328 including the UBL-RING1 interface, the REP blocking the proposed E2 binding site,
329 and the proposed occlusion of the catalytic cysteine, Cys431, by the RING0 domain

330 ^{16,22-26}. All these inhibitory mechanisms are relieved by the activation of Parkin by
331 pUb ^{27,28,30}. pUb and the phosphorylated UBL domain are unable to simultaneously
332 bind to R0RBR Parkin in solution, *in trans*, suggesting an allosteric regulation of
333 Parkin by pUb binding ^{23,24,42,46}. In addition, previous studies have led to predictions
334 of large conformational changes in Parkin upon activation. Interestingly, a
335 computational analysis of Parkin suggest that phosphorylation of the UBL domain
336 initiates a large change, mediated by the 65 amino acid linker between the UBL
337 domain and RING0 (residues 77-140) ⁴⁷. An important caveat of our current
338 understanding of the mechanism of Parkin activity is that structures are either of
339 fragments of Parkin (R0RBR) ^{16,25,26}, or all domains but lacking the UBL-RING0
340 linker ^{23,24}. However, in a low resolution structure of full length rat Parkin, the linker
341 is present in the protein, but is completely disordered in the crystal and can't be
342 modelled ²⁵. The UBL domain and R0RBR domains remain as they are in the absence
343 of the linker ^{23,24}. Interestingly, while the linker length, although not the composition,
344 is conserved down to *Drosophila* ^{23,24}, Parkin from nematodes does not have a long
345 linker between the UBL and RING0 domains, therefore the functional importance of
346 this linker is still unclear. In this study, we present a modified model for Parkin
347 regulation, based on the first structures of an RBR ligase in the activated state,
348 complete with regulatory domains. Our structures reveal that in contrast to the large
349 conformational changes that have been predicted for Parkin function, activation of
350 Parkin results in local rearrangements of Parkin domains to reveal ubiquitin binding
351 sites. These ubiquitin binding sites recruit molecules of ubiquitin, or ubiquitin carried
352 by an E2, to bridge Parkin molecules and allow the utilisation of catalytic domains
353 from neighbouring molecules. In this model, the UBL-IBR interaction (Fig 5a) is
354 perturbed by UBL phosphorylation or pUb binding, displacing the IBR from the UBL

355 domain (Fig 5b/c). This IBR displacement opens a ubiquitin binding pocket on the
356 helix (H3)-IBR surface. A (donor) ubiquitin on loaded E2 occupies this new pocket
357 while E2 occupies the proposed sites on RING1, and the RING2(Rcat) of the
358 neighbouring molecule of Parkin (Fig 5d).

359 Based on current data, Parkin is unique in the RBR family in that it has a
360 distinct structural arrangement of RING1-IBR-RING2(Rcat) domains, where
361 RING2(Rcat) and RING1 are in juxtaposition. In contrast, in HOIP and HHARI
362 RING2(Rcat) and RING1 are separated by IBR (Supp Fig 5a)^{37,44}. Although RBRs
363 share a conserved catalytic RING1-IBR-RING2(Rcat) module, they are usually
364 autoinhibited and activated via distinct mechanisms. pUb binding or UBL
365 phosphorylation releases Parkin autoinhibition²⁷⁻³², HOIP autoinhibition is released
366 by UBA mediated interactions with LUBAC constituents HOIL-1L or SHARPIN
367^{22,29,30,33,36,38,51} and HHARI autoinhibition is released by NEDD8 binding at the UBA
368³⁸. In the structure of full-length HHARI, the proposed NEDD8 binding pocket (Supp
369 Fig 5b) corresponds to the pocket is occupied by pUb or allosteric ubiquitin (Ub_{allo}) in
370 active Parkin or HOIP, respectively. This suggests that NEDD8 binding to HHARI
371 would have a similar effect as pUb or Ub_{allo} binding has on Parkin and HOIP. Inactive
372 HHARI and Parkin have a compact H3-IBR region (Supp Fig 5c). Active Parkin and
373 HOIP structures enable pUb or Ub_{allo} binding on one side of the H3-IBR region which
374 in the case of Parkin is occluded when inactive, and allows donor ubiquitin binding on
375 the opposite side of the H3-IBR (Supp Fig 5d). Therefore, we speculate that blocking
376 the donor ubiquitin binding site on one side of the H3-IBR, relieved upon pUb or
377 Ub_{allo} binding on the opposite side via opening of the H3-IBR to allow donor
378 ubiquitin binding, could be a common mechanism of regulation in RBR ligases.

379 The structure of HOIP-RBR in complex with loaded E2 also suggests that the
380 RING1 and RING2(Rcat) of the same molecule cannot crosstalk as they are not close
381 enough to allow catalysis of thioester formation, mediated by the catalytic cysteines
382 of E2 and RING2(Rcat) (Supp Fig 5e). In the active state of HOIP-RBR the
383 processive unit is formed by the RING2(Rcat) of molecule 1, and the RING1-IBR of
384 a second molecule, where E2 interacts with the RING2(Rcat) of molecule 1 and
385 RING1 of molecule 2 whereas the donor ubiquitin interacts with the RING2(Rcat) of
386 molecule1 and the H3-IBR of molecule 2 (Supp Fig 5e). In contrast, in Parkin the
387 processive unit is formed by RING1-RING2(Rcat) of molecule 1, and the IBR of
388 molecule 2 (Fig 4d). This difference in the arrangement of domains between Parkin
389 and HOIP, in the active state, is consistent with the differences in RING1-IBR-
390 RING2(Rcat) structural organisation in three dimensions (Supp Fig 5a). Interestingly,
391 inter/intra-molecular pairing of RING1-RING2(Rcat)-IBR domains of RBRs with E2
392 and donor ubiquitin explains the previous observations that Parkin and other RBRs
393 favour the extended ‘open’ conformation of loaded E2^{44,45}. Furthermore, a role for
394 Parkin oligomerisation or self-association has previously been reported¹⁵, along with
395 reports of Parkin having ubiquitin-binding activity^{19,22}. Our structures of active
396 Parkin support the idea that PINK1 induces structural changes in Parkin that stimulate
397 ubiquitin binding. In the context of the mitochondrial membrane environment, where
398 Parkin functions in mitophagy⁵²), high local concentrations of Parkin, recruited by
399 PINK1-catalysed phosphorylation of ubiquitin, could result in the feed-forward
400 amplification of Parkin-mediated ubiquitination observed at the mitochondria^{52,53}.
401 Furthermore, it has been reported that Parkin functions with the linear chain assembly
402 complex, LUBAC, comprising two RBR proteins in HOIP and HOIL-1L⁵⁴. The
403 structures reported here open up the possibility of cooperation between RBR modules,

404 and therefore we speculate that there is a possibility that RBR ligases can function in
405 concert.

406 Within the RBR module, it has long been predicted that RING1 recruits the E2
407 ⁵⁵, recently formally demonstrated for HHARI and HOIP ^{44,45}; and that the
408 RING2(Rcat) domain, harbouring the catalytic cysteine, is the catalytic intermediate
409 domain ¹⁸. However, a functional role for the IBR domain has been less clear. Our
410 observation that covalent modification in the IBR enhances Parkin activity, and that
411 deletion of IBR or mutations in IBR leads to loss of Parkin activity, coupled with a
412 recent report of increased Parkin activity after ISG15ylation on K349 and K363 of the
413 IBR ⁴⁸, suggest a crucial role for the IBR in Parkin function. Our study suggests that
414 the role of the IBR, together with H3 (helix connecting RING1 and IBR), is to
415 mediate interactions with the inhibitory UBL domain in the inactive state, and the
416 donor ubiquitin in the active state, respectively. Furthermore, the IBR and
417 RING2(Rcat) of RBRs are structurally similar folds, each containing two β -hairpin
418 turns. Comparison of Parkin and HOIP structures in the active state reveals that the
419 IBR can interact with 2 ubiquitin molecules (pUb or Ub_{allo}, and donor ubiquitin)
420 spanning across both β -hairpins (Supp Fig 5a). Similar to the IBR, the RING2(Rcat)
421 of HOIP also interacts with 2 ubiquitin molecules (donor and acceptor ubiquitin) ^{14,44}
422 mediated by both β -hairpins (Supp Fig 5f). Interestingly this observation furthers the
423 idea that the ubiquitination process is facilitated by the inherent ability of E1, E2 and
424 E3 proteins to interact with ubiquitin ⁵⁶. In our Parkin structures, only one ubiquitin
425 binding site is captured, predicted to be the donor ubiquitin binding site, while the
426 acceptor ubiquitin may occupy the opposite surface, in a manner similar to HOIP
427 (Supp Fig 5g); However, we cannot rule out that there may be subtle differences in

428 the acceptor ubiquitin or substrate recognition between RBRs that result in different
429 type of modifications by different RBRs.

430 Taken together, our data suggest a common mechanism of regulation in RBRs.
431 First, autoinhibition via blocking a donor ubiquitin binding pocket on H3-IBR.
432 Second, release of autoinhibition by a UBL, be that phosphoubiquitin, NEDD8,
433 ISG15, or ubiquitin itself, opening the H3-IBR binding site. Third, donor ubiquitin
434 binding on the H3-IBR accompanied by a bridging of RBR molecules to facilitate
435 access to the catalytic cysteine. Given the importance of Parkin function in mitophagy
436 and PD, understanding the multiple regulatory modes required for function will
437 provide a framework for the design of small molecules to modulate Parkin activity.

438

439

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447

448 **Author contributions**

449 AK designed and performed experiments, solved the crystal structures, analysed data
450 and wrote the manuscript. VKC purified Miro1, made labelled ubiquitin and
451 developed the labelled ubiquitin based assay, and performed the RING2(Rcat) loading
452 experiments. TEC performed the NMR CSP experiments, KRB did the AUC

453 experiments. RT cloned several constructs. CJ, RS and AK purified various reagents
454 for assays. GSS and HW designed the experiments, analysed data and wrote the
455 manuscript.

456

457 **Conflict of interest**

458 I declare that the authors have no competing interests as defined by Springer Nature,
459 or other interests that might be perceived to influence the results and/or discussion
460 reported in this paper.

461

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604

605 **Figure Legends**

606 **Figure 1 Overall structure of Parkin-phosphoubiquitin complex in the activated** 607 **state**

- 608 a) Crystal structure of UBLR0RBR and pUb complex showing UBL, RING0 ,
609 RING1, IBR, REP, and RING2(Rcat) of Parkin, in complex with
610 phosphoubiquitin (blue). The phosphate group of phosphoubiquitin is shown in
611 stick representation.
- 612 b) Comparison of UBLR0RBR-pUb complex (coloured) with the apo structure of
613 UBLR0RBR (grey) (PDB code 5C1Z²³). UBLR0RBR superimposed on the
614 UBLR0RBR-pUb complex structure shows conformational changes between the
615 IBR and the UBL domain, and the void formed (marked with arrow), helix 1 (H1)
616 and helix 3 (H3) of RING1 are marked. UBL (green) remains associated with H1
617 of RING1 (cyan) in both apo and complex structures.
- 618 c) Isothermal Titration Calorimetry assays showing UBL interaction with Δ UBL
619 Parkin (left panel), and that deletion of IBR in Δ UBL Parkin leads to loss of UBL
620 and Parkin interaction (right panel).
- 621 d) Deletion of the IBR in Δ UBL Parkin leads to loss of E3 ligase activity of
622 Parkin in ubiquitin chain formation, Parkin autoubiquitination, and Miro1

623 ubiquitination. FL (autoinhibited) or Δ UBL (active) Parkin comparison is shown
624 with Δ UBL- Δ IBR Parkin, or Δ UBL- Δ IBR with a 10-residue linker in the presence
625 or absence of pUb. Uncropped gel images are shown in Supplementary Data Set
626 1.

627

628 **Figure 2 Identification of potential ubiquitin binding regions important for**
629 **Parkin function.**

630 **a)** Model of ubiquitin (grey surface) binding to Parkin, based on crystallographic
631 packing of a second Parkin molecule (shown in Supp Fig 2b). The boundary of
632 one UBLR0RBR-pUb complex (domains coloured as in Figure 1) is represented
633 by a dashed line.

634 **b)** The UBL (green) and forms ubiquitin binding region 1 (UBR1) (left panel).
635 For clarity, UBL (green), modelled ubiquitin (grey), RING1 helices H1, H3
636 (cyan), IBR (brown) are schematically represented below, with molecule 1
637 boundary of Parkin marked with a dashed line, and UBR1 marked as a shaded
638 box. The activity of UBR1 mutants was monitored by fluorescently-labelled
639 ubiquitin incorporation into ubiquitin chains and Parkin autoubiquitination, and
640 Miro1 ubiquitination (right panel). Coomassie-stained gel is shown as loading
641 control. In this and in all subsequent ubiquitination assays, a non-specific, ATP-
642 independent band is indicated (*).

643 **c)** H3 of RING1 (cyan) and IBR (brown) make 2 important interfaces with
644 ubiquitin (grey) (left upper panel), shown as UBR2 (purple box) and UBR3 (red
645 box), respectively (left lower panel). Mutations in UBR2 and UBR3 of Parkin
646 lead to loss of E3 ligase activity of Parkin in ubiquitin chain formation, Parkin

647 autoubiquitination, and Miro1 ubiquitination (right panel). Assay conditions are as
648 in panel b.

649 **d)** Chemical shift perturbation map obtained from NMR titration experiments
650 where ^2H , ^{14}N -labelled Ub was titrated into a solution of ^2H , ^{12}C , ^{15}N -labelled
651 R0RBR Parkin in complex with ^2H -labelled pUb, and followed by ^1H , ^{15}N TROSY
652 experiments. Residues that experienced chemical shift changes greater than 1
653 standard deviation above the average shift in the absence of Ub are indicated in
654 yellow.

655

656 **Figure 3 Parkin activation promotes donor ubiquitin (Ub_{don}) binding at H3-IBR**
657 **interface**

658 **a)** Nature of interactions (red sphere: electrostatic, black sphere: hydrophobic)
659 between pUb (blue)/UB (grey) and H3 (cyan)/IBR (brown) of Parkin are shown
660 schematically (left panel). Residues on H3 (cyan)-IBR (brown) of Parkin
661 interacting with pUb (blue) and Ub (grey surface) (modelled by superposition on
662 UBL of molecule 2 in the UBLR0RBR-pUb complex structure) are shown as
663 sticks (middle panel). Helix-connecting-RING1-IBR and IBR of HOIP interaction
664 with activator ubiquitin (Ub) and donor ubiquitin (Ub_{don}) (pdb code 5EDV⁴⁴).

665 **b)** Isothermal Titration Calorimetry assays showing Glu321Ala mutation does not
666 affect Parkin interaction with UbcH7 (upper panel), but does reduce Parkin
667 interaction with the UbcH7~Ub iso-peptide (lower panel). ITC measurements
668 were performed using phosphorylated Parkin in the presence of pUb.

669 **c)** Parkin can be activated by UbcH7C86K~pUb conjugate similarly to pUb.
670 Ubiquitination assay was performed with 1 μM of ParkinS65A. Parkin was
671 activated with 1 μM of UbS65A/pUb/UbcH7C86K~Ub/UbcH7C86K/

672 UbcH7C86K~pUb (Left panel), coomassie-stained gel is shown as loading control
673 (lower panel). A model depicting distinct ubiquitin (UbcH7~Ub or pUb/
674 UbcH7C86K~pUB) binding regions on Parkin is shown in the right panel.

675

676 **Figure 4 Parkin molecules cooperate to facilitate ubiquitin transfer**

677 **a)** Model of crystal packing between molecules of Parkin. The modelled donor
678 ubiquitin (grey surface) sits above the catalytic cysteine (C431) of the
679 RING2(Rcat) (red) of a neighbouring Parkin molecule. For clarity, the rest of the
680 Parkin molecules are shown in slate, except for the UBL domain (green), and IBR
681 (brown). The predicted E2 binding region on each molecule is shown.

682 **b)** Mutation of UBR2 and phosphoubiquitin patches of Parkin leads to defects in
683 formation of a RING2(Rcat)-Ub ester intermediate, monitored using fluorescently-
684 labelled ubiquitin (left top). Sodium hydroxide sensitivity confirms the
685 RING2(Rcat)-ubiquitin ester bond (left bottom). Coomassie-stained gels are shown
686 as total Parkin levels. Integrated intensities of Parkin-Ub ester levels from three
687 independent experiments were plotted as mean \pm SEM (right). Statistical
688 significance was determined by one-way analysis of variance with Bonferroni's
689 multiple-comparison test. (n = 3, ****P < 0.0001).

690 **c)** Activated UblR0RBR complexed with phosphoubiquitin can stimulate the
691 activity of inactive Parkin. The activity of phosphoParkin mutated in the ubiquitin
692 donor binding site (E321A) is enhanced by the addition of UblR0RBR Parkin
693 covalently complexed with phosphoubiquitin. Addition of UblR0RBR Parkin
694 alone does not enhance the activity of pParkinE321A. Ubiquitination of Miro1 is
695 monitored using fluorescently-labelled ubiquitin (left top). Coomassie stained gel
696 shows total Miro1 levels (left bottom). Integrated intensities of Miro1-Ub levels

697 from three independent experiments were plotted as mean \pm SEM (right).
 698 Statistical significance was determined by one-way analysis of variance with
 699 Bonferroni's multiple-comparison test. (n = 3, ****P < 0.0001, **P < 0.01)
 700

701 **Figure 5 Model of Parkin regulation**

702 a) Parkin is autoinhibited by UBL blocking IBR (brown) and H3 (red) of RING1
 703 (cyan).

704 b) Phosphorylation (marked with red asterisk) of UBL/pUb binding creates a
 705 pocket at H3-IBR and UBL interface, leading to activated Parkin.

706 c) Activated Parkin shows small rearrangements triggered by the straightening of
 707 the H3-IBR helix. Loaded E2 (E2~Ub) is recognised at this new interface, E2
 708 occupying interface with REP and RING1 of activated Parkin, Ub_{don} (grey) sitting
 709 on the interface of IBR (brown box) and H3 (red helix) of RING1 of neighbouring
 710 molecule of Parkin (marked with a dashed line).

711

712 **Table 1**

713 **Table 1 Data collection and refinement statistics (molecular replacement)**
 714

	UBLR0RBR+pUb (5N2W)	S65DUBLR0RBR+pUb (5N38)
Data collection		
Space group	P63 2 2	P63 2 2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	147.3 147.3 87.5	146.5 146.5 88.4
α , β , γ (°)	90 90 120	90 90 120
Resolution (Å)	56.34-2.68 (2.81- 2.68) ^a	72.56-2.60 (2.76- 2.60) ^a
<i>R</i> _{merge}	10.7 (71.9)	8.2 (48.4)
<i>I</i> / σ (<i>I</i>)	8.9 (1.9)	9.1 (2.2)
<i>CC</i> _{1/2}	99.4 (66.8)	99.5 (64.0)
Completeness (%)	93.6 (93.7)	96.4 (88.4)
Redundancy	4.6 (4.2)	3.5 (3.2)
Refinement		
Resolution (Å)	56.34 (2.68)	72.56 (2.60)
No. reflections	15007	16984
<i>R</i> _{work} / <i>R</i> _{free}	19.93/24.17	18.16/22.86

No. atoms		
Protein	3619	3565
Ligand/ion	23 ^a	17 ^b
Water	29	46
<i>B</i> factors		
Protein	60.2	55.9
Ligand/ion	84.4	79.1
Water	53.4	37.8
R.m.s. deviations		
Bond lengths (Å)	0.01	0.01
Bond angles (°)	1.14	1.17

715 Single crystals were used for structure determination.

716 ^a8 Zinc ions, 1 Chloride ion, 10 trimethyloxide atoms, 4 aminopropane atoms

717 ^b8 Zinc ions, 1 Chloride ion, 4 polyethylene glycol atoms, 4 aminopropane atoms

718 **Online Methods**

719 **Protein expression and purification**

720 UBLR0RBRGln347Cys (resi 1-83, 144-465) and S65DUBLR0RBRgln347Cys (resi
721 1-83, 144-465) were expressed as His-Smt-3 fusion. FL, various mutants and
722 truncated constructs of Parkin were expressed as His-SUMO fusion. Constructs were
723 expressed in BL21 (DE3) *E. coli* cells and purified as previously described (21,22).
724 Parkin or ubiquitin were phosphorylated using *Pediculus humanus* PINK1 (126-C) or
725 *Tribolium castaneum* PINK1 (TcPINK1), expressed and purified as previously
726 described^{23,57}. Miro1 (181-592) and fluorescently labelled ubiquitin (Ub^{IR800}) were
727 prepared as previously described²³.

728

729 **Preparation of phospho-Ubiquitin-3BR probe**

730 Ubiquitin was expressed as ubiquitin 1-75-Mxe-intein/chitin binding domain using
731 pTXB-1 vector ((DU49003)) in BL21 *E. coli* cells. Cells were induced at 0.5 OD₆₀₀
732 with 300μM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and incubated at 20°C
733 overnight. Cells were lysed in lysis buffer (20mM Na₂HPO₄ pH7.2, 200mM NaCl and
734 0.1mM EDTA). Clear lysate was incubated with Chitin resin, after washing with 2-
735 column volume of lysis buffer protein was eluted in 20mM Na₂HPO₄ pH6.0, 200mM
736 NaCl and 0.1mM EDTA and 0.1mM MESNA (Sodium 2-mercaptoethanesulfonate).
737 Eluted material was reacted with 3-Bromo-propyl amine hydrobromide (SIGMA) as
738 described before^{58,59}. After reaction, protein was purified on size-exclusion column
739 pre-equilibrated with PBS. Fractions containing ubiquitin were collected and
740 phosphorylated using *Pediculus humanus* GST-PINK1, and further purified by size-
741 exclusion chromatography. M1-(UbS65A)₃-pUb-3BR was prepared similarly by
742 expressing M1 linked linear tetra-ubiquitin, in the same vector, with distal ubiquitins'
743 Ser65 mutated to Ala.

744

745 **Preparation of UBLR0RBR347Cys~pUb/S65DUBLR0RBR347Cys~pUb**

746 We first mutated UBLR0RBR Parkin to contain a cysteine residue at position 347.

747 The corresponding residue in *Pediculus humanus* Parkin is a cysteine, and this

748 enabled R0RBR Parkin to react with the phosphoubiquitin-3BR probe, as described

749 previously⁴³. UBLR0RBRGln347Cys/S65DUBLR0RBRGln347Cys was reacted

750 with 3 fold molar excess of pUb-3BR probe at room temperature for 2hrs. Complex

751 was purified by size-exclusion chromatography in 20mM Tris pH7.5, 75mM NaCl

752 and 250μM TCEP buffer. UBLR0RBR347Cys~pUb-(M1-UbS65A)₃ was prepared

753 using the same method.

754

755 **Crystallisation and Structure determination**

756 UBLR0RBRGln347Cys/S65DUBLR0RBRGln347Cys~pUb were crystallised at 4°C

757 in sitting drop plates by mixing 1:1 of protein (7mg/ml) and mother liquor (100mM

758 Tris pH 8.5, 200mM TMAO, PEG MME 2000). Crystals were flash frozen in liquid

759 nitrogen using 20% of PEG400 as cryo-protectant in the original mother liquor. Data

760 were collected at Diamond Light Source, wavelength 0.979Å. Data were solved by

761 molecular replacement program Phaser in CCP4⁶⁰ by using UBL (1-76), RING0

762 (142-216), RING1 (229-328), IBR (329-377) and RING2(Rcat) (415-465) domains of

763 apo Parkin structure (5c1z) and pUb (4wzp) as ensembles. Solution obtained by

764 phaser was built and refined in iterative cycles by using coot⁶¹ and autobuster⁶²,

765 respectively. Ramachandran values were calculated using Molprobit⁶³. Both

766 structures have excellent geometry with ~95% of residues in the most favoured

767 regions. The structure coordinates and structure factors have been deposited in the

768 Protein Data Bank, with the accession codes 5N2W and 5N38.

769

770 **Size-exclusion chromatography**

771 Size-exclusion chromatography experiment was performed on analytical column
772 superdex-75, pre-equilibrated with 50mM HEPES pH 7.5, 200mM NaCl, 250μM of
773 TCEP. 10μM of Phospho-Parkin was incubated with 2 fold molar excess of pUb or
774 pUb and Ubch7~Ub for 1 hr prior to loading on column. Ubch7~Ub was prepared as
775 described previously²³.

776

777 **Isothermal titration calorimetry**

778 ITC experiments were performed using PEAQ-ITC (Malvern instruments), and data
779 were analysed using single-site binding model. 30μM of WT-Phospho-
780 Parkin/Phospho-ParkinGlu321Ala (premixed with 1.2 fold molar excess of pUb) were
781 titrated using 1.6mM or 1.48mM of Ubch7 in the syringe, respectively. 21.4μM of
782 ΔUBL, 40μM of ΔUBL (Gln389Ala, Tyr291Ala, Arg392Ala) premixed with 1.2 fold
783 molar excess of pUb, and 19μM of ΔUBL-ΔIBR were titrated using 1.14mM, 750μM,
784 500μM of WT UBL domain in the syringe, respectively. Ubch7 and UBL titrations
785 were performed at 20°C in 50mM HEPES pH 7.5, 200mM NaCl, 250μM of TCEP
786 and PBS, 500μM of TCEP, respectively.

787

788 **Ubiquitination assay**

789 Ubiquitination reactions were performed at 30°C in 50mM Tris pH 7.5, 100mM
790 NaCl, 2.5mM Mgcl₂, 5% glycerol, 500μM TCEP. Ubiquitin chain extension reactions
791 contained 25nm of recombinant human E1, 500nM of Ubch7, 1μM E3 and 5mM of
792 ATP in 20 μl of final reaction volume. 0.5 μM of pUB/ WT-UB was used as an
793 allosteric activator in various reactions. Ubiquitination reaction was analysed using

794 3 μ M of fluorescently labelled ubiquitin (Ub^{IR800}). Ubiquitin labelling was performed
795 using DyLightTM 800 Maleimide as described before²³. Miro1 ubiquitination was
796 performed in the similar setup using 5 μ M of Miro1 (181-592) and 12.5nm of
797 recombinant human E1, 250nM of UbcH7, 0.5 μ M E3 and 5mM of ATP in 20 μ l of
798 final reaction volume. For Figure 3d, UBS65A/WT-
799 Ub/UbcH7C86K/UbcH7C86K~Ub were phosphorylated in separate reactions with
800 GST tagged TcPINK1; UbcH7C86K~Ub with no PINK1 was used as control. Prior to
801 addition to Ubiquitination reaction, PINK1 was depleted using GST resin.
802 Miro1 ubiquitination was performed in the similar setup using 5 μ M of Miro1 (181-
803 592) and 12.5nm of recombinant human E1, 250nM of UbcH7, 0.5 μ M (unless
804 otherwise specified) E3 and 5mM of ATP in 20 μ l of final reaction volume. All
805 assays were repeated at least 3 times.

806

807 **Parkin ubiquitin loading**

808 Reactions monitoring RING2(Rcat)-ubiquitin ester formation were performed at 30°C
809 for 90 min in 50mM Tris pH 7.5, 100mM NaCl, 5mM MgCl₂, 1mM TCEP and 0.5%
810 polyethylene glycol 6000 reaction buffer. Reactions contained 50nm E1, 10 μ M
811 UbcH7, 10 μ M Ub^{IR800}, 3 μ M E3 and 5mM ATP in final reaction volume of 10 μ l. Non-
812 activatable pUb-6His (3 μ M) was used as an allosteric activator where indicated.
813 Reactions were stopped using NuPAGE LDS Sample Buffer (Invitrogen) that
814 contained reducing agents and boiled for 5min. To hydrolyse ester linkages the boiled
815 samples were cooled and further treated with 0.4M NaOH for 20 min at 42°C. The
816 samples were resolved by SDS-PAGE and analyzed by direct fluorescence
817 monitoring using Li-COR Odyssey Infrared Imaging System. Integrated intensities of
818 Parkin-Ub ester species from three independent experiments were obtained using

819 Image Studio (Odyssey) imaging software, plotted as mean \pm standard error of mean
820 (SEM) and statistically analysed using GraphPad Prism7.

821

822 **Chemical Shift Perturbations**

823 A complex of ^2H , ^{12}C , ^{15}N -labelled R0RBR with ^2H -labelled pUb (prepared as above)
824 was purified to homogeneity using gel filtration chromatography in 25 mM HEPES,
825 100 mM NaCl, 0.5 mM TCEP at pH 7.0. Chemical shift perturbation experiments
826 were measured using ^1H , ^{15}N -TROSY spectra of 205 μM ^2H , ^{12}C , ^{15}N -R0RBR/ ^2H -pUb
827 in the absence and presence of ^2H , ^{14}N -labelled Ub. These experiments were
828 quantified using the following weighted formula: $((0.2 \times \Delta\delta\text{N}^2) + \Delta\delta\text{H}^2)^{1/2}$ and
829 plotted as a function of residue. All NMR experiments were collected using a triple
830 resonance cryogenic probe on a Varian Inova 600 MHz NMR spectrometer at 25°C.

831

832 **Accession codes**

833 The protein structure files reported in this manuscript are available from the Protein
834 Data Bank with accession codes 5N2W, and 5N38.

835

836 **Data availability statement**

837 All constructs are available on request from the MRC Protein Phosphorylation and
838 Ubiquitylation Unit reagents Web page (<http://mrcppureagents.dundee.ac.uk>). All
839 other data are available from corresponding author upon reasonable request.

840

841 **Methods-only references**

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