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RBR ligase-mediated ubiquitin transfer: a tale with many twists and turns

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Abstract

RBR ligases are an enigmatic class of E3 ubiquitin ligases that combine properties of RING and HECT-type E3s and undergo multi-level regulation through autoinhibition, post-translational modifications, multimerisation and interaction with binding partners. Here we summarize recent progress in RBR structures and function, which has uncovered commonalities in the mechanisms by which different family members transfer ubiquitin through a multi-step process. However, these studies have also highlighted clear differences in the activity of different family members, suggesting that each RBR ligase has evolved specific properties to fit the biological process it regulates.

Main text

Post-translational modification (PTM) of proteins is a crucial mechanism to regulate cellular processes without requiring protein synthesis *de novo*. Ubiquitination is one of the most versatile PTMs and target proteins can be modified by attachment of either a single ubiquitin (Ub) molecule or by chains of Ub molecules that can be linked in many different ways. In addition, ubiquitin itself can be post-translationally modified adding yet another layer of complexity. Ubiquitination is catalysed via an enzymatic cascade involving an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase. E3 ligases select the substrate and promote ubiquitin transfer onto the target either directly from the E2 conjugating enzyme, a mechanism adopted by Really Interesting New Gene (RING)-type ligases, or via an E3-ubiquitin thioester intermediate as observed in Homologous to E6-AP C-terminus (HECT)-type and RING-Between-RING (RBR)-type ligases¹.

HECT-type E3s contain a conserved, bilobal HECT domain that recognises the E2~Ub conjugate and forms the E3~Ub intermediate. In contrast, RBR ligases contain a tripartite domain arrangement, which consists of 3 zinc (Zn)-binding domains: a RING1 domain that is similar to the canonical cross-brace RING fold, followed by IBR (In-Between-Ring) and RING2 domains that adopt highly similar structures, in which the two Zn²⁺ ions are coordinated in a sequential manner².

Substrate ubiquitination by RBR ligases is a multi-step process. It starts with recognition of the E2~Ub conjugate by RING1, followed by transfer of the ubiquitin onto the catalytic cysteine in RING2 to form the thioester intermediate, and finally transfer onto the substrate. The targeted residue can be a lysine residue in a substrate protein to monoubiquitinate or a lysine residue or the N-terminal amino group of ubiquitin to build a poly-Ub chain (Fig. 1). This process combines mechanistic features of RING and HECT-type ligases that are performed by specific sub-domains of the RBR motif: the E2~Ub-recognizing RING1 and the thioester-forming RING2 domain. These domains are separated by the IBR domain and two adjacent flexible linker regions that enable the 3 RBR sub-domains to adopt multiple conformations with respect to one another, which together with intramolecular interactions with regions outside the RBR allow for highly variable mechanisms of inhibition and activation to occur.

Of the 14 RBRs present in humans³, only 3 have been studied in detail, Parkin, HHARI and HOIP, and these will be the focus of this Perspective. While they share commonalities in their mechanism of action, there are also clear differences suggesting that the mechanism of ubiquitin transfer and regulation of RBRs should be regarded as a common catalytic process with many twists to accommodate specific requirements of a given system employing an RBR E3 ligase.

Inhibition

As soon as RBRs were discovered to have a catalytic cysteine, similar to the HECT-type E3 ligases⁴, it was quickly established that most RBRs restrain the enzymatic activity through inhibitory mechanisms⁵⁻⁹(Fig. 2a). However, these modes of inhibition differ between members of the family. For example, in Parkin the proposed E2-binding site is blocked by a small helix (Repressor Element of Parkin-REP), disruption of which promotes enhanced E2 binding¹⁰. However, blocking the E2 binding site is not a universal mechanism for regulating RBR activity, as HHARI is able to recruit UbCH7 with submicromolar affinity, even in its inhibited conformation (Fig. 2a)⁸. Recent structures of HHARI¹¹⁻¹³, and HOIP in complex with E2 loaded with ubiquitin (E2~Ub)¹⁴, confirm that the predicted E2 binding site on the first helix of the RING1 domain is indeed where E2 sits within these complexes (Fig. 2b-d). Thus, in the case of Parkin, it seems likely that some conformational rearrangements occur to expose this site in the productive Parkin-E2 complex.

In the structures of autoinhibited Parkin and HHARI, the catalytic cysteine (C431 and C357 respectively) is occluded by secondary structure elements unique to each RBR (Fig. 2a)^{8,10,15,16}. In the case of Parkin, this is the RING0 domain. Deletion of RING0, or point mutations in residues securing the RING0:RING2 interface, lead to increased Parkin activity, likely through enhanced access to the catalytic cysteine^{10,15,17}. In HHARI, the catalytic cysteine is more completely occluded by a pair of charged residues in the Ariadne domain (Glu510-Arg511)⁸. However, recent structures of HHARI in complex with E2~Ub show that binding of the Ub-loaded E2 is not sufficient to expose the catalytic cysteine, suggesting that further modifications are needed to release the Ariadne domain from the catalytic site (Fig. 2c)^{11,13}.

The first evidence that the RBRs were regulated at the molecular level by regions outside the RBR module came from the finding that the N-terminal ubiquitin-like (Ubl) domain of Parkin maintained an inhibited conformation, and that removal or disruption of this domain leads to Parkin activation^{5,18,19}. Similarly, removal of domains N-terminal to the RBR from the LUBAC subunit HOIP leads to increased activity of HOIP^{6,7} (however, the multisubunit E3 ligase LUBAC is more complex, because HOIP has not yet been found to exist without HOIL-1 or SHARPIN, which release these autoinhibitory effects within the complex^{6,7,20-22}). Together, these findings suggest that domains outside the RBR module, unique to each family member, play a key role in regulating ligase activity.

Activation

Elucidating how RBRs are activated is key to a mechanistic understanding of these enzymes. Parkin can be activated by binding a ubiquitin moiety that carries a phosphate group at serine 65 (phosphorylation catalysed by the kinase PINK1)²³⁻²⁵. Activation of Parkin also requires phosphorylation of the equivalent serine in the inhibitory Ubl domain of Parkin itself (also catalysed by PINK1)²⁶. Intriguingly, in the cases of both Parkin and HHARI, structures of Parkin bound to its cognate activator phosphoubiquitin (pUb) and of HHARI bound to its cognate E2 do not seem to capture the active forms of the proteins. For example, in both structures of phosphoubiquitin-bound Parkin (one containing the C-terminal RING0-RBR, one containing the Ubl-RING0RBR), the RING0 position remains unaltered, and still partially occludes the catalytic cysteine, while the REP is still occluding the proposed E2 binding site^{27,28}. Similarly, when HHARI is bound to E2~Ub, the Ariadne domain still occludes the catalytic cysteine of HHARI (Fig. 2c)^{11,13}. Therefore, it seems likely that further conformational changes are induced by other mechanisms, likely the phosphorylation of the Ubl domain in Parkin, and/or by substrate binding for other RBRs, however, these states are yet to be

captured. Indeed, full-length NMR analysis of phosphoParkin reveals an extended, less globular conformation, but only in the presence of pUb. This suggests that neither pUb, nor phosphorylation of the Ubl alone are sufficient for Parkin activation, but rather both activation mechanisms are necessary.

The role of ubiquitin and ubiquitin-like proteins

An intriguing observation from the multiple structures of RBRs now available are the apparent multiple binding sites for ubiquitin and ubiquitin-like proteins, and the potential for regulation of activity. For example, in the case of HOIP, a structure of the catalytic RING2-LDD (Linear ubiquitin chain Determining Domain) in complex with ubiquitin clearly shows separate binding sites for both acceptor and donor ubiquitin molecules (Fig. 3a)³⁰. HHARI forms additional interactions with ubiquitin via the loop linking the IBR-RING2 domains¹². The HOIP-E2~Ub structure¹⁴ provides details of the docking site for donor ubiquitin (Fig.3b), a site also predicted by the structure of a Parkin-pUb complex to bind ubiquitin (Fig. 3c)²⁸. This site is along the outside of the helix leading into the IBR domain of each protein.

Intriguingly, the opposite side of the same helix seems to mediate various regulatory interactions in the three best studied RBRs. In Parkin, it is the site of pUb binding^{27,28}. In HOIP, it is where additional ubiquitin moieties, suggested to play an allosteric role, are found in the crystal packing of the HOIP-E2~Ub structure¹⁴. In HHARI, this site is occupied by the UBA-like (UBA-L) domain, suggesting a potential regulation by either blocking this site or recruiting a Ubl (Fig. 3d). Two recent structures of HHARI complexed with E2~Ub show contacts between the ubiquitin moiety and the UBA-like domain. The interaction mode however is different in the two structures, suggesting multiple locations can be sampled by the E2~Ub conjugate. Finally, a recent study reports activation of Parkin by the ubiquitin-like protein (UBL) ISG15, through covalent attachment to a lysine in the immediate vicinity of the pUb binding site³¹.

In combination, these studies suggest a role for covalent and noncovalent ubiquitin and UBL binding in the activation and regulation of RBRs (Fig. 3e). Furthermore, the surfaces identified to bind Ub and UBLs, may constitute more general protein-protein interaction sites that could be recognised by other as yet unidentified regulators.

Activation by other proteins

In addition to the ubiquitin-like interactors, several other proteins influence the activity of RBR ligases. This is particularly pertinent in the case of HOIP. In contrast to Parkin and HHARI, the autoinhibited state of HOIP is constitutively released through interaction with its LUBAC partners HOIL-1 and SHARPIN. In effect, HOIP activity is regulated through association with the deubiquitinases (DUBs) OTULIN and CYLD³²⁻³⁵. At present, it is not clear why this fundamental difference between the regulation of activity of LUBAC and Parkin/HHARI exists. However, there are reports of additional interacting partners stimulating activity of Parkin and HHARI. For example, interaction with Eps15 and endophilin A via the Ubl domain of Parkin point towards a possible substrate-induced relief of inhibition^{5,36,37}. Furthermore, the Ariadne domain of HHARI binds to Cullin-RING ligases (CRLs), and this interaction greatly enhances HHARI activity^{38,39}.

Finally, perturbing the intricate domain-domain associations within RBR ligases can lead to activation. For example, it is known that tagging the N-terminus of Parkin with either large globular proteins, or small unstructured epitope tags leads to activation, and in some cases changes substrate preference^{5,40,41}.

The ubiquitin transfer cycle

Over 30 ubiquitin-specific E2 conjugating enzymes exist, most of which carry out both aminolysis and transthiolation reactions and hence work with RING as well as HECT and RBR

ligases⁴². In contrast, UBE2L3 (UbcH7) is strictly cysteine-reactive and unable to transfer ubiquitin in conjunction with RING ligases (though curiously can form stable complexes with some of them). A number of reports suggest that UbcH7 is the physiologically relevant E2 for HOIP and HHARI^{43,44}, yet for most RBR ligases the cognate E2(s) remain unknown and *in vitro* studies often use UBE2D (UbcH5) isoforms, promiscuous E2s that are active with many different E3s .

Interaction with cognate E2 and prevention of accidental ubiquitin discharge

Isolated E2~conjugates undergo aminolysis only slowly and require a mechanism to increase the rate of ubiquitin transfer. This is achieved by complex formation with canonical RING domains that stabilise the otherwise dynamic and flexible conjugate in a closed conformation, in which the I44 hydrophobic patch of ubiquitin contacts the α 2 helix of the E2⁴⁵⁻⁴⁸. Given the high structural similarity of RING1 domains to canonical RINGs this raises the question how ubiquitin discharge onto a lysine residue is prevented upon engagement of E2~Ub conjugates (other than UbcH7) by RBR E3s. Recent structural and biochemical studies provided an explanation for this behaviour: RING1 domains stabilise an open E2~Ub conformation in which lysine reactivity is suppressed, and instead transthiolation, an equilibrium reaction that does not require activation, is promoted¹². Interestingly, the stabilisation of an open E2~Ub conformation is observed for complexes of RBR domains with both UbcH5 and UbcH7, despite the inability of UbcH7 to transfer ubiquitin onto lysine residues. This observation indicates that the prevention of unproductive, and possibly detrimental discharge of ubiquitin onto nearby lysine residues is a key feature determining the mode of the RING1-E2~Ub interaction. Structures of HHARI/UbcH7~Ub complexes show that the RBR-E2~Ub interaction is dominated by the RING1-E2 interface, with minor contacts between ubiquitin and the UBA-like domain of HHARI which is located N-terminal to the RBR

domain (Fig. 2c)^{11,13}. However, these additional contacts do not appear to be functionally important as the isolated RING1 domain is sufficient to engage the E2~Ub in an open conformation¹¹ and the presence of the UBA, IBR and RING2 domains does not increase the affinity of the E2~Ub conjugate for HHARI⁴⁹.

Recognition of Ubch7 by RING1 occurs in a similar manner to canonical E2/E3 complexes and includes residues from loops 4 and 7 of the E2 and from two Zn²⁺-coordinating loops and the central helix of HHARI RING1. However, the second Zn²⁺ loop of most RING1 domains is longer than in canonical RINGs² (by two residues in HHARI) and acts as a steric wedge to prevent Ubch7~Ub from adopting a closed conformation (Fig. 2c)^{11,13}. Furthermore, RING1 domains lack the conserved “linchpin”, a basic residue in canonical RINGs that simultaneously contacts the E2 and ubiquitin. Interestingly, HOIP does not contain an extended Zn²⁺ loop but also binds an Ubch5~Ub conjugate in the open conformation (Fig. 2d)¹⁴. However, in contrast to the predominantly E2/RING1-driven HHARI/Ubch7~Ub interaction, HOIP makes extensive non-covalent contacts with ubiquitin along the entire RBR domain¹⁴. These contacts are crucial for complex formation as the affinity of the HOIP RBR for isolated Ubch5 is very low and ubiquitin conjugation is required to form a stable complex. A similar behaviour is observed with HHARI, suggesting that there might be differences in the manner by which Ubch5 and Ubch7, which only transfers Ub onto cysteine, are recognised by RBRs⁴⁹.

In the case of Parkin, there is no appreciable affinity between wild-type Parkin and any E2 enzyme^{18,19}. Removal of the Ubl domain does not lead to a close association with E2s, neither does mutation of the repressor element of Parkin. However, phosphorylation of Parkin enables binding to Ubch7 with a dissociation constant of ~160 μM ¹⁹ and addition of pUb increases the affinity ~5-fold to a Kd of 20-30 μM ^{18,19,28}. Yet, these are still low affinity interactions, and only when the Ubch7 is loaded with ubiquitin, pUb is present, and Parkin is

phosphorylated does the interaction become sub micromolar^{18,28}, in the same range as the HHARI-UbcH7 interaction. The Parkin/E2~Ub complex is yet to be defined structurally, and therefore it is unclear what conformation will be supported by RING1 of Parkin. However, in contrast to HHARI, Parkin can function with multiple E2s⁵, and in contrast to HOIP, Parkin can catalyse the formation of multiple chain types, suggesting there may be further layers of regulation.

Transfer of ubiquitin from E2 to RBRs

Transfer of ubiquitin from E2 to E3 requires the active site cysteines of both proteins to come into close proximity. The HOIP/UbcH5~Ub complex provides a first glance at how this transthiolation step may occur¹⁴. The crystallised complex shows two molecules of the E2~Ub conjugate bound to two molecules of the RBR such that ubiquitin transfer would occur in trans (Fig. 2d). However, the authors argue that this arrangement is an artefact due to a domain swap and instead ubiquitin transfer occurs in cis within a single RBR chain, which makes extensive contacts with ubiquitin to guide RING2 towards the E2~Ub conjugate. Intriguingly, SAXS analysis of RBR/E2~Ub complexes does not support the existence of a stable compact species, in which the RBR is tightly wrapped around the E2~Ub conjugate, suggesting that this species is highly transient⁴⁹. It is tempting to speculate that the elongated conformation of HOIP-RBR observed in the crystal structure (Fig. 2b) and the domain-swapped compact conformation (Fig. 2d) may represent two species at opposite ends of the reaction pathway: the initial encounter complex and the E2/E3 ubiquitin transfer complex.

An intriguing feature of the compact complex is the occlusion of the acceptor ubiquitin binding site on RING2 by the bound E2 (Fig. 2d). This could suggest that linear ubiquitin chain synthesis by HOIP is not processive as the growing chain will need to dissociate for each round of ubiquitin transfer. The advantage of such a mechanism is not clear at present, but it may

provide a regulatory mechanism to limit activity. In this respect, it is interesting that binding of ubiquitin chains to an allosteric ubiquitin-binding site formed by the IBR domain and preceding linker, opposite the donor ubiquitin-binding site, has been suggested to act as an activator, implying that linear chain synthesis might be tightly controlled by opposing positive and negative regulatory signals.

Cooperation between RBR modules

LUBAC functions as at least a dimer of 2 RBR ligases, HOIP and HOIL-1, with an additional subunit, SHARPIN. HOIP has also been reported to function with Parkin under cellular stress, with Parkin increasing LUBAC activity⁵⁰. In the structure of the HOIP RBR module bound to E2~Ub, the donor ubiquitin carried by the E2 bound to one molecule of HOIP, interacts with the RING2 domain of a second molecule of HOIP¹⁴, meaning that the RING2 of one RBR completes the second RBR moiety (Fig. 2d). It is tempting to speculate that this arrangement may mimic a potential interaction between the RBR modules of HOIP and HOIL-1, thereby adding yet another layer of regulation. Intriguingly, the crystal structure of Parkin in complex with pUb also shows a potential coupling of multiple RBR modules, whereby the IBR domain cradling the donor ubiquitin in one molecule of Parkin could transfer that ubiquitin to the RING2 domain of a second Parkin molecule²⁸. Rescue experiments mixing RBR mutants lend some support to this notion. RNF144, which is an RBR ligase that contains only the RBR module and a short transmembrane domain, has been suggested to function only as a dimer, with oligomerisation through the transmembrane domain, further supporting the notion that RBR domains may regulate each other⁵¹. However, this does not seem to be the case in HHARI^{11,13} where transfer from E2 to E3 occurs in cis. Whether RBRs can cooperate in trans, perhaps through the multiple ubiquitin docking sites, remains an open question.

Substrate selection and chain linkage specificity

Our knowledge of the structural features underlying substrate selection by RBR ligases is currently limited to ubiquitin itself during linear chain synthesis by HOIP, which is bound by RING2 and a C-terminal region referred to as the linear chain determining domain (LDD)(Fig. 3a)³⁰. In contrast, nothing is known about how the hundreds of proposed Parkin substrates may be recognised⁵² or how HHARI may select CRL substrates to prime them by monoubiquitination³⁹. Substrate selection by HHARI may be driven by recognition of neddylated CRLs³⁸.

Linkage specificity of the polyubiquitin chain is generally believed to be determined by the last thioester-forming enzyme of the ubiquitination cascade, implying that E2 enzymes adopt this role in conjunction with RING E3s whereas HECT and RBR ligases themselves control chain type. At present, HOIP is the only RBR member which strictly follows this rule and only synthesises linear (M1-linked) chains. This activity requires a specific region of HOIP that binds the acceptor ubiquitin, the LDD, which is partially integrated within RING2 (Fig. 2b). In contrast, Parkin forms multiple types of polyUb chains, whereas HHARI primarily monoubiquitinates its substrates to work with Cullin E3s, but can also mediate polyUb chain synthesis in autoubiquitination assays. For other RBR family members details of linkage specificity are still largely unexplored. This apparent lack of chain linkage specificity, at least with Parkin, raises the question why the reaction would need to proceed via an E3-thioester intermediate, which is generally assumed to provide linkage specificity.

We speculate that the multi-step mechanism adopted by RBR ligases, using 3 domains tethered to one another by flexible linkers may be important to allow the E3 to retain

selection of the lysine residues (or the N-terminal methionine) to be modified during the initiation and chain extension process, regardless of the E2 they are working with. Furthermore, the flexible tripartite domain structure of RBRs allows multiple levels of regulation including post-translational modifications, interaction with ubiquitin, pUb, UBLs and other binding partners or association with membranes.

Future directions

At present, we only have a very incomplete picture of the dynamic range of these proteins and many questions remain unanswered, particularly with respect to substrate selection, chain linkage specificity, possible cooperation between RBR modules and regulation of activity *in vivo*. There are undoubtedly many more twists in the RBR tale to be uncovered!

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Box 1:

14 RBRs are present in humans, but only 3 have been studied in any detail so far: Parkin, HHARI and HOIP. Parkin is important for the maintenance of mitochondrial homeostasis and mutations in the Parkin genes are associated with autosomal juvenile Parkinsonism^{53,54}. HHARI (and its homologs in other species) have been associated with a number of cellular functions including the regulation of developmental processes, of protein translation and of cellular proliferation^{44,55,56}. In contrast to Parkin and HHARI that function as single polypeptides, HOIP is a subunit of the linear ubiquitin chain assembly complex (LUBAC), a constitutive complex of two RBR-domain containing proteins, HOIP and HOIL-1, plus SHARPIN. LUBAC was initially shown to play a crucial role in the regulation of immune and inflammatory signalling, but has since been linked to the regulation of multiple cellular functions including apoptosis and cancer^{20-22,57}.

Figure Legends

Figure 1. The catalytic cycle of RBR ligases

Schematic representation of ubiquitin transfer mediated by RBR ligases starting with binding of an E2~Ub conjugate by the RING1 (red), followed by transfer of the ubiquitin from the E2 (yellow) to the catalytic cysteine in RING2 (blue) and onto a substrate, (here a ubiquitin molecule to form a ubiquitin chain; the donor ubiquitin is highlighted in purple and the acceptor ubiquitin in cyan). Arrows indicate the likely flexibility of the linkers connecting the RBR subdomains. Regions outside the RBR are indicated in grey.

Figure 2. Domain arrangement during autoinhibition and in the active state of RBRs

(a) The autoinhibited structures of Parkin (left, PDB 5C1Z) and HHARI (right, PDB 4KBL) in the same orientation, aligned via helix α 1 of RING1 (red), with IBR in green, RING2 in blue and regions outside the RBR in grey. The relative domain arrangement of the RBR subdomains is shown beneath to illustrate the differences in the position of the RING2 domains. The positions of the catalytic cysteine in RING2 is indicated. **(b)** The RBR domain of a single polypeptide of HOIP as seen in the active HOIP/UbcH5-Ub-bound structure (PDB 5EDV) in the same orientation as Parkin and HHARI. The LDD is highlighted in cyan. **(a,b)** The composition of domains outside the RBR modules is shown as schematics underneath the crystal structures. HOIP contains a PUB domain, 3 zinc finger domains and a UBA domain N-terminal to the RBR ("PUB-NZFs-UBA"). **(c)** Structure of the HHARI/UbcH7~Ub complex (PDB 5UDH), showing that RING2 does not contact the donor ubiquitin (purple) and that the catalytic cysteine is still occluded after E2~Ub binding. **(d)** Structure of the HOIP RBR module in its closed, domain-swapped form, bound to UbcH5-Ub. The RING1 (red) and IBR (green) domains are contributed by molecule 1 and RING2 (blue) and LDD (grey) by molecule 2. The acceptor

ubiquitin binding site that is occluded by the E2 (yellow) is highlighted in cyan. The proposed allosteric ubiquitin is shown in orange.

Figure 3. Multiple Ub and Ubl-binding sites determine the activity of RBRs

(a) Complex between the HOIP RING2/LDD fragment and a donor (purple) and acceptor (cyan) ubiquitin (PDB 4LJO) showing how HOIP recognizes the acceptor ubiquitin in such a manner that only a linear (M1-linked) chain can be built. (b) Complex of HOIP RBR with E2~Ub (PDB 5EDV), with RING1 (red), linker helix (grey) and IBR (green) shown. E2 (yellow) positions the donor ubiquitin (purple), and the proposed allosteric ubiquitin (orange) binds to the linker helix and IBR domain. The schematic above is coloured accordingly. (c) Parkin-pUb complex (PDB 5N2W) with RING1 (red), linker helix (grey) and IBR (green) shown. Phosphoubiquitin (orange) binds to the linker helix, and the proposed donor ubiquitin binding site, occupied by a Ubl domain in the structure, is shown in purple. The schematic above is coloured accordingly. (d) HHARI structure (PDB 5UDh) with RING1 (red), linker helix (grey) and IBR (green) shown. The UBA-like domain (orange) packs against the linker helix. The schematic above is coloured accordingly. (e) Schematic of the positions in RBR domains identified so far that can binding ubiquitin or ubiquitin-like molecules. The donor ubiquitin is shown in purple with multiple shaded molecules indicating that the donor ubiquitin can make contacts across all domains of the RBR. The acceptor ubiquitin identified in HOIP is shown in cyan and the position of allosteric ubiquitin and ubiquitin-like binders is indicated in orange.

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