Review

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RBR E3-ligases at work

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Abstract

The RING-in-between-RING (RBR) E3s are a curious family of ubiquitin E3-ligases, whose mechanism of action is unusual in several ways. Their activities are auto-inhibited, causing a requirement for activation by protein-protein interactions or posttranslational modifications. They catalyse ubiquitin conjugation by a concerted RING/HECT-like mechanism in which the RING1 domain facilitates E2-discharge to directly form a thioester intermediate with a cysteine in RING2. This short-lived, HECT-like intermediate then modifies the target. Uniquely, the RBR ligase HOIP makes use of this mechanism to target the ubiquitin amino-terminus, by presenting the target ubiquitin for modification using its distinctive LDD region.

Keywords RBR; E3 ligase; TRIAD; autoinhibition; ubiquitination; mechanism
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See the Glossary for abbreviations used in this article.

Introduction

The role of E3-ligases in ubiquitin conjugation is to mediate the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to the target. This step is uncoupled from the first steps in ubiquitination—which involve the E1- and ATP-dependent activation of ubiquitin and its transfer to E2 enzymes—because E1 and E3 use overlapping surfaces of the E2 enzymes. The E3s are therefore involved solely in the chemical transfer of the C-terminus of ubiquitin from a thioester on a cysteine to the isopeptide linkage in the target.

Traditionally, the E3s have been separated into two classes, RING/U-box and HECT type ligases, both of which activate the E2 reaction and recognize the target molecule. The HECT E3-ligases form a thioester-intermediate on an active-site cysteine before transferring it onto its target, whereas RING E3-ligases facilitate the direct transfer of the ubiquitin from the E2 cysteine (Fig 1). More recently, a third class of E3-ligases was identified, the RING-in-between-RING (RBR) E3-ligases, which contain a highly conserved catalytic unit consisting of a RING1, an in-between RING (IBR), and a RING2 domain (Figs 1 and 2) [1].

The RBR class of proteins was first described in 1999 by two separate groups that identified the conservation of a triple-RING/zinc finger motif in eukaryotic species, including animals, plants, fungi and protists [2,3]. Further analysis of the pattern of cysteines and histidines in the RING/zinc fingers indicated that the RBR domain has arisen only once in evolution [4]. There are 14 separate human RBR proteins that have been assigned to 8 distinct subfamilies of the RBR family [5] (Table 1).

The RBR E3-ligases were shown to use both RING and HECT-like mechanisms [1]. The ubiquitin transfer is initiated by the interaction of an E2~ubiquitin with the RBR [1, 6, 7], similar to the interaction between E2s and classical RING E3-ligases [5–7], but in RBRs this interaction is used to facilitate the formation of a HECT-like thioester intermediate between the C-terminus of the ubiquitin and an active-site cysteine on RING2 before it is coupled to its substrate. Furthermore, most, if not all, of these ligases are distinguished by the tight regulation of their enzymatic activity by auto-inhibition [6–11]. In this review, we discuss how RBR E3-ligases transfer ubiquitin to their targets.

Cellular functions of RBRs

The few RBRs that have been analyzed in detail—Parkin, HHARI, TRIAD1, HOIP and HOIL-1L—are involved in important cellular events: transcription and RNA metabolism, translation, subcellular tethering, regulation of posttranslational modifications and protein stability, cellular and stress signalling, cell-cycle control, and response to microbial infection [12]. Consequently, the misregulation of the activity of RBR proteins is important in disease [5, 13–15], which makes them interesting potential drug targets. Here, we briefly discuss their function before a more in-depth analysis of the molecular details of their enzymatic mechanisms.

Parkin

Parkin (PARK2) mutations cause familial autosomal-recessive juvenile Parkinson disease and are a frequent cause of sporadic early- and late-onset Parkinson disease (PD) [16–18]. Parkin is found in many tissues, but is primarily expressed in the brain, including the substantia nigra, indicating a possible involvement in the loss of dopaminergic neurons that form the hallmark of PD [17]. In addition, it is a putative tumour suppressor [19,20].

Parkin recruitment to the outer mitochondrial membrane is dependent on Pink1 [21] and its own ligase activity [22]. The Parkin E3-ligase activity is crucial for its function, and possible targets include α-synuclein [23], CDCrel-1 [24], Pael-R [25], and misfolded

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Glossary

4EHP	Translation initiation factor 4E homologous protein
DUBs	De-ubiquitination enzymes
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugase
E3	Ubiquitin ligase
Gfi1	Growth factor independence 1
HECT	Homologous to the E6-AP carboxy terminus
HHARI	Human homologue of Drosophila Ariadne
HOIL-1L	Heme-oxidized IRP2 ubiquitin ligase-1
IBR	In-between RING
LDD	Linear ubiquitin chain determining domain
LUBAC	Linear ubiquitin chain assembly complex
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NZF	Npl4 zinc-finger
RBR	RING-IBR-RING ligase
RING	Really interesting new gene
UBAN	Ubiquitin-binding domain in ABIN and NEMO

DJ1/PARK7 [26], which accumulate in patients with PD. Therefore, the Parkin E3-ligase activity is thought to be crucial for the prevention of PD pathogenic Lewy body formation, which mainly contain ubiquitin and α-synuclein [27]. Furthermore, Parkin plays important roles in mitophagy upon stimulation by the mitochondrial uncoupler CCCP and possibly in other forms of autophagy in the cell, such as the susceptibility to intracellular bacterial pathogens [21, 28, 29]. A growing number of targets are being identified, including mitofusin-1, mitofusin-2 and HKI, which are involved in maintaining mitochondrial integrity [30–32]; the p38 subunit of aminoacyl-tRNA synthase [33]; RanBP2, which is part of the nucleocytoplasmic transport machinery [34]; transcription factor SIM2 [35]; anti-apoptotic and autophagy inhibitory protein Bcl-2 [36]; and Parkin itself [1, 24]. An in-depth proteomics analysis of the Parkin dependent proteome will be important to validate and identify more direct Parkin targets [37]. Although the understanding of most Parkin interactions is still superficial, a major role for the Parkin E3-ligase function seems to be the protection of cells against the accumulation of misfolded and unfolded proteins.

HHARI

Parkin and HHARI share substantial sequence identity [38]. In PD with mutated Parkin, the E3-ligase activity of Parkin is abolished in all cells. Nevertheless, only the dopaminergic neurons in the brain seem to be sensitive to the loss of Parkin function. Thus, it is likely that other cells express a redundant E3-ligase to compensate for the loss of Parkin. HHARI is a likely candidate, as it binds many of the same protein partners, such as CDCrel-1, synphilin-1, and CASK [39], and may target synphilin-1 and SIM2 for degradation in cells [35]. Interestingly, HHARI is also found in Lewy bodies in dopaminergic neurons in PD, indicating that it cannot compensate the loss of Parkin activity in these cells [39]. Nevertheless, HHARI is not mutated in patients with PD.

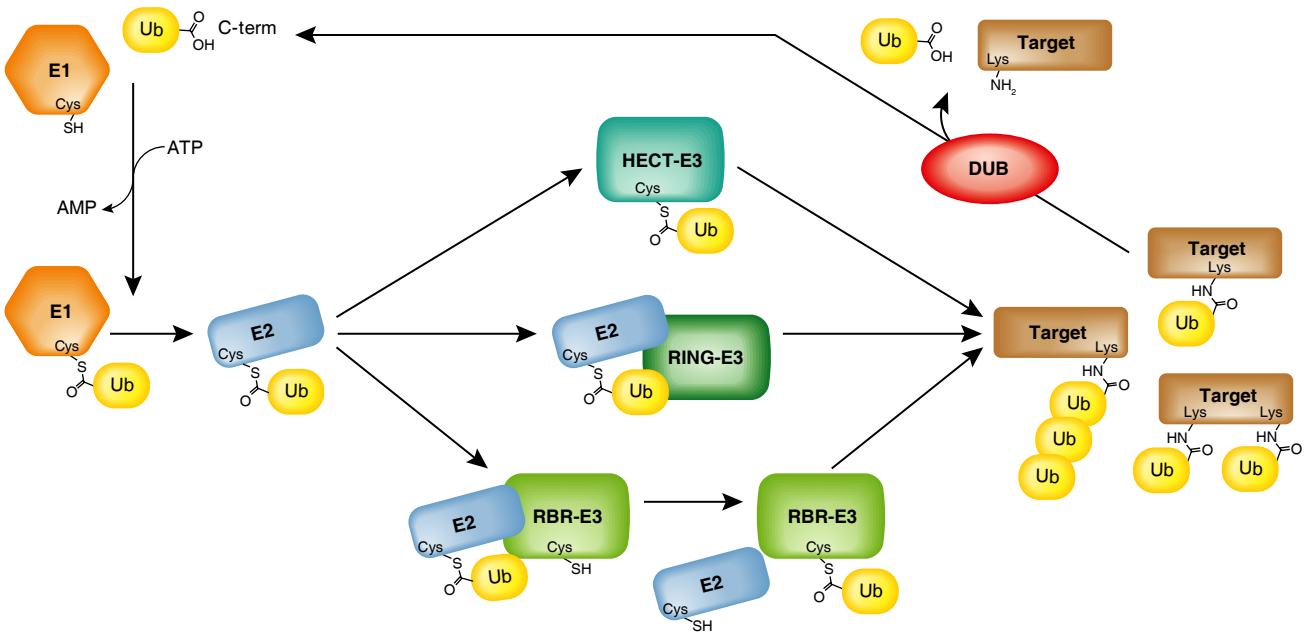


Figure 1. RBR E3-ligases have a unique mode of transferring ubiquitin to a target.
The ubiquitin (Ub) C-terminus is activated in an ATP-dependent manner by an E1 activating enzyme, and is subsequently transferred to form a thioester intermediate on an E2 conjugase. The final transfer of ubiquitin onto its target is mediated by E3-ligases that either form a thioester intermediate with the ubiquitin (HECT E3-ligases), mediate a direct transfer of the ubiquitin from the E2 onto its target (RING E3-ligases), or function as RING/HECT-type hybrids (RBR E3-ligases). Through this cascade of E1, E2 and E3 enzymes, the ubiquitination machinery mediates the formation of mono-ubiquitination, multi-mono-ubiquitination, or ubiquitin chain formation on its targets. The ubiquitin signal can be removed by de-ubiquitination enzymes (DUBs).

In addition to the description of the possible overlapping interactions of HHARI and Parkin, two studies have analyzed the cellular function of HHARI. First, HHARI is suggested to play a role in the regulation of protein translation by targeting the eukaryotic mRNA cap-binding protein 4EHP for proteasomal degradation [40]. Second, HHARI has been shown to positively regulate cellular proliferation, which functionally correlates with its over-expression in head-and-neck squamous cell carcinoma biopsies [41]. Neddylated Cullin-RING ligase complexes have been recently shown to bind and activate HHARI, which has important implications for its cellular function [42].

TRIAD1

Loss of TRIAD1 in mice is associated with embryonic death and causes the degradation of nuclear IκBβ, leading to excessive NF-κB signalling in dendritic cells [43]. Furthermore, the depletion of TRIAD1 causes a defect in membrane trafficking that leads to the accumulation of the growth hormone receptor and the epidermal growth factor receptor in intracellular vesicles and at the plasma membrane [44]. Finally, TRIAD1 plays a role in the regulation of myeloid progenitor cell proliferation, by modulating HoxA10 activity [45] and stabilizing proteins such as Gfi1B and p53 [46,47]. TRIAD1 cellular concentrations seem to be tightly regulated. TRIAD1 levels are up-regulated during granulocytic and monocytic differentiation [48,49]. In contrast, it is negatively controlled by proteasomal degradation, for which it is ubiquitinated by, for example, Mdm2 [46, 48].

Direct targets of the TRIAD1 E3-ligase activity have not been identified *in vivo*, but it seems to regulate the stability of various proteins indirectly, in the sense that it does not directly ubiquitinate them for degradation. For example, TRIAD1 inhibits the proteasome-dependent degradation of Gfi1B in myeloid progenitor cells through an interaction between its RING2 domain and Gfi1B, thereby inhibiting myeloid progenitor cell proliferation [47, 50]. This interaction possibly competes with binding of other E3-ligases that target Gfi1B, or it might recruit DUBs that deubiquitinate Gfi1B. In addition, TRIAD1 has been suggested to compete with Mdm2 for p53 binding, thus preventing the ubiquitin-dependent degradation of p53 [46]. TRIAD1 has also been linked to the regulation of the activity of HoxA10. High expression levels of HoxA10 correlate with a poor prognosis in acute myeloid leukemia (AML) [51,52], and induce TRIAD1 levels in myeloid progenitor cells, which increases total protein ubiquitination levels [45, 53]. Interestingly, TRIAD1 antagonizes HoxA10-induced cellular proliferation, but whether HoxA10 is a direct target for TRIAD1 remains to be determined [51, 52, 54]. Nevertheless, the inhibitory effect of TRIAD1 on the proliferation of myeloid progenitor cells critically relies on its RING domains and is inhibited by proteasome inhibitors [50]. Therefore, the TRIAD1 E3-ligase activity is likely to target HoxA10 or regulators of HoxA10 for proteasomal degradation. Finally, it has recently been shown that TRIAD1 is recruited to and activated by CUL5-RBX2 complexes, which might be critical for identifying additional cellular functions [42].

HOIP and HOIL-1L

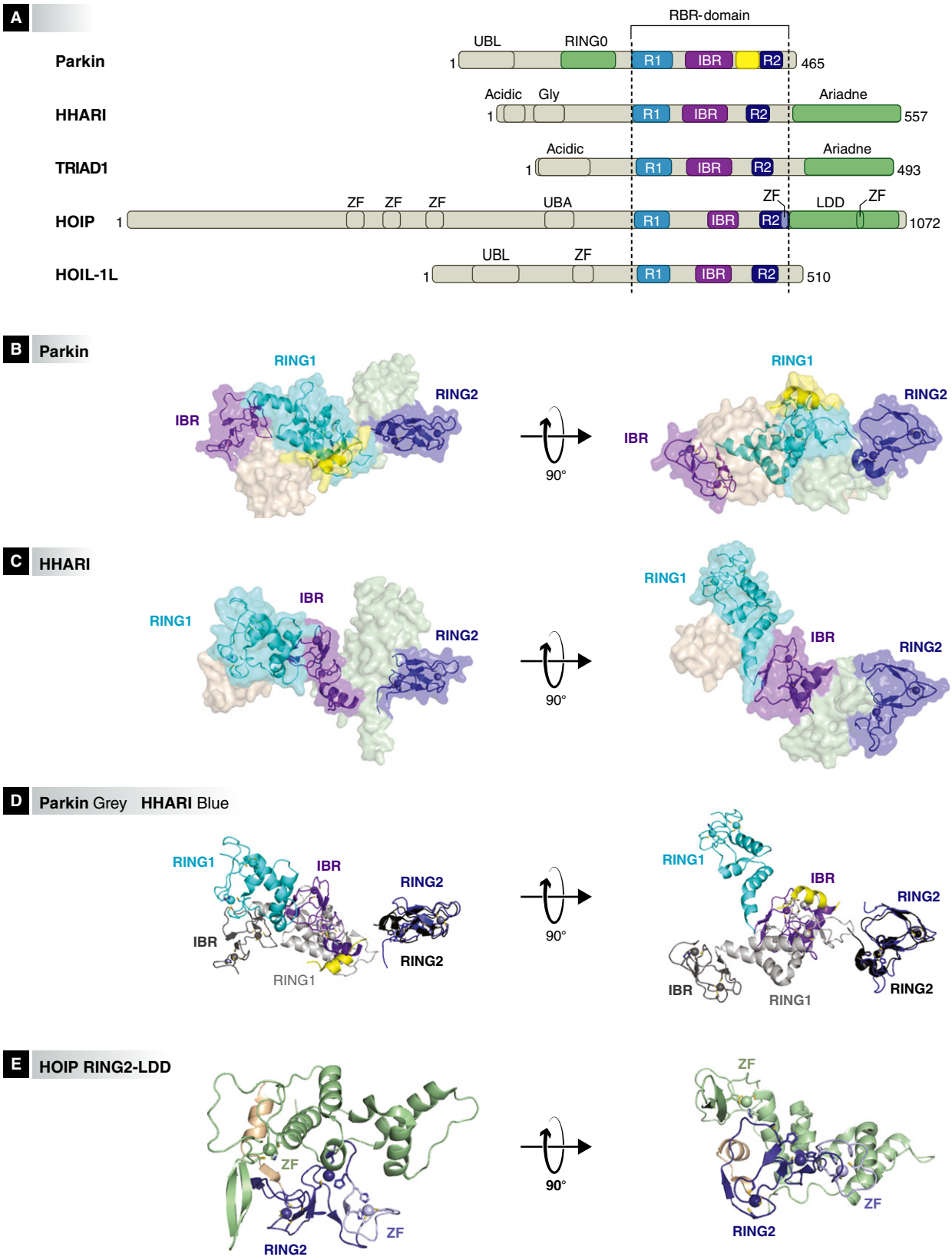
Two RBR E3-ligases, HOIP and HOIL-1L, are part of the linear ubiquitin assembly complex (LUBAC), which is essential for the activation of the NF-κB pathway. LUBAC comprises SHARPIN, HOIP and HOIL-1L, of which HOIP forms a critical catalytic centre [55–59]. LUBAC has the unique capability to mediate the formation of linear ubiquitin chains [57]. Interestingly, HOIP contains the linear ubiquitin chain specificity of the complex [57, 60], but either HOIL-1L or SHARPIN are needed to release its autoinhibited state and direct the activity of the complex towards its targets. Weak linear chain-forming activity was also reported for HOIL-1L [7], based on the fact that His-Ub could not be modified. However, as HOIL-1L requires an intact N-terminus also on the donor ubiquitin [61], further experiments are required to elucidate its independent activity. The LUBAC E3-ligase targets its activity towards NEMO, RIP1, RIP2 and K63-linked ubiquitin chains in the NF-κB pathway [55, 58, 62–64]. Modification of NEMO with linear chains under stress conditions is further regulated by Parkin, which interacts with the LUBAC complex [65]. Mechanistically, besides the RBR of HOIP, the catalytic cysteine in the RBR domain of HOIL-1L is required for the attachment of the first ubiquitin of the linear ubiquitin chain to NEMO [61].

In the NF-κB pathway, conjugated linear ubiquitin chains are selectively recognized by the UBAN domain of NEMO and the NZF domains of HOIL-1L and SHARPIN [55, 66–69], which are believed to stabilize the TNF-R1/NEMO/LUBAC signalling complex, co-localize LUBAC with the TAK1-complex, recruit additional NEMO molecules, and facilitate NF-κB-dependent gene expression. However, the DUBs A20 and OTULIN/Gumby are also recruited to the linear ubiquitin chains to negatively regulate LUBAC-induced NF-κB activation by preventing the interaction between NEMO and LUBAC [64, 70–72]. The role of LUBAC in the immune response is reviewed in this *EMBO reports* ubiquitin series [73].

The RBR mechanism

RBR E3-ligases follow a two-step mechanism whereby the interaction of a ubiquitin-charged E2-enzyme with RING1 promotes the transfer of ubiquitin to a cysteine on RING2, to form a thioester

Figure 2. Domain arrangements in RBR E3-ligases.
A) Domain organization of the RBR E3-ligases Parkin, HHARI, TRIAD1, HOIP and HOIL-1L. The domain borders are drawn to scale according to Uniprot definitions (www.uniprot.org). Ubiquitin like domain (UBL), acidic region (Acidic), glycine-rich region (Gly), zinc finger (ZF), ubiquitin-associated domain (UBA). The RBRs are represented in RING1:cyan (R1), purple (IBR) and blue (R2). The Parkin RING0, C-terminal Ariadne domain and linear ubiquitin determining domain (LDD) are represented in pale green and the Parkin linker/tether helix (also called PUB or REP) is yellow. B) Crystal structure of full-length Parkin (surface representation) (PDB 4k95). The RBR (cartoon) is autoinhibited by the N-terminal regions of Parkin. The colors correspond to the colors in the schematic representation in Figure 2A. C) Surface representation of the crystal structure of full length HHARI (PDB 4kbl). The RBR is shown as cartoon. D) A cartoon of superposed RBR domains of Parkin and HHARI (SSM (WinCoot 0.7.1) superposition of RING2 domain of HHARI on the RING2 domain of Parkin). Parkin RING1:light grey80, IBR:dark grey40, PUB domain: yellow, RING2:black. HHARI RING1:cyan, IBR:purple, RING2:blue. E) Crystal structure of HOIP RING2-LDD (PDB 4ljo).


A
RBR-domain
Parkin
UBL
RING0
R1
IBR
R2
1
465
HHARI
Acidic
Gly
Ariadne
557
TRIAD1
493
HOIP
ZF
UBA
LDD
1072
HOIL-1L
510
B Parkin
RING1
RING2
IBR
90°
C HHARI
D Parkin Grey HHARI Blue
E HOIP RING2-LDD

intermediate, prior to the transfer of ubiquitin to its target protein. Since the thioester intermediate is reminiscent of the HECT E3-enzymes, the mechanism has been named HECT-like, although it should be noted that the structures of these domains do not resemble HECT domains (see below). This combined RING/HECT-like molecular mechanism underlying the RBR-mediated ubiquitination of proteins was uncovered in HHARI based on a study on Parkin and HHARI in 2011 [1], and confirmed for HOIP in 2012 [6,7] and Parkin in 2013 [22]. As several RBR E3-ligases have been shown to function via this two-step mechanism, it is likely that all RBR E3-ligases function in this manner.

The RBRs share a number of features that distinguish them from other RING E3s. First, the requirement for an E2 *in vitro* is less strict than in most other E3-ligases. RBRs are able to use the E1 as the donating thioester directly, in a step that is also independent of the RING1 domain of the RBR [6, 7, 74]. Second, the thioester bond between ubiquitin and the cysteine on the RING2 domain is very transient and difficult to detect, indicating that this step is not rate-limiting in the reaction. Finally—unlike classical RING E3-ligases in which the interaction between the RING domain and the E2 activates the E2~ubiquitin thioester [75,76]—the RBR RING1 is not sufficient to allow the discharge of ubiquitin from the E2, but additionally requires the presence of the RING2 catalytic cysteine [1, 6, 77]. Altogether, this suggests that the RING1 domain may not facilitate the allosteric activation of the E2~ubiquitin as occurs in classical RINGs [78–80], but possibly catalyzes the transfer of the ubiquitin onto RING2 by a different mechanism.

RBR structures

Recent structural information of the RBR regions of Parkin, HHARI and HOIP provides insight into the molecular details of the architecture of the RBR proteins (Fig 2) [9–11, 77, 81]. The crystal structures of Parkin and HHARI show that the relative orientation of the RING1, IBR and RING2 domains of the RBR unit is highly variable. The RBR of Parkin was crystallized in its auto-inhibited form as a compact structure with extensive inter-domain interactions [9–11], while the RBR of HHARI adopts an extended conformation in its autoinhibited state [77]. A HOIP construct lacking RING1 and the IBR reveals the tight interaction between RING2 and the C-terminal linear ubiquitin chain determining domain (LDD) [82].

In the crystal structures of Parkin, the RING2 domain of the RBR is positioned at the opposite side of the protein from the IBR, placing the two domains 49 Å apart by a linker. In this conformation, the E2~ubiquitin bound to RING1 is positioned >50 Å away from the active- site C431 in RING2, too far for trans-thiolation of the ubiquitin from the E2 onto the E3 [9–11]. In HHARI, the C-terminal Ariadne domain blocks the active-site cysteine in RING2, preventing the transfer of the ubiquitin from an E2 onto the RBR RING2 [77]. Thus, the RBRs require conformational changes for their activation. Additional studies are needed to reveal the precise orientation of the RING1, IBR and RING2 domains in catalytically active forms of the RBR proteins, and it remains to be seen how long-lived such states will be.

If one analyzes the individual domains in these structures, the RING1 domain in HHARI and Parkin has the typical C3HC4 topology of classical RING domains, coordinating two zinc-ions in a cross-brace structure that contains all the necessary features for the interaction with E2s. Neither the IBR domain nor the RING2 domain has a RING fold, but they are zinc fingers that coordinate two zinc ions in a similar manner. They share a common IBR-fold that is also found in the APC/C inhibitors Emi1 (FBXO5) and Emi2 (FBXO43) [83]. Interestingly, the RING2 of HOIP differs from other RBR proteins, since it has an additional zinc finger incorporated near the end of RING2 (Fig 2E), which is structurally important for the positioning of the target ubiquitin in linear ubiquitin chain formation [82].

Table 1. Human RBR E3-ligases

Subfamily	Name	Alternative names	Uniprot
Ariadne	ARIH1	ARI1, HHARI	Q9Y4X5
Ariadne	ARIH2	ARI2, TRIAD1	O95376
Ariadne	CUL9	PARC, H7-AP1, KIAA0708	Q8IWT3
Ariadne	ANKIB1	KIAA1386	Q9P2G1
Parkin	PARK2	PRKN, PARKIN	O60260
RNF144	RNF144A	KIAA0161, hUIP4, UBCE7IP4	P50876
RNF144	RNF144B	P53RFP, IBRDC2	Q7Z419
XAP3	RBCK1	HOIL-1L, RNF54, XAP3	Q9BYM8
Dorfin	RNF19A	DORFIN	Q9NV58
Dorfin	RNF19B	NKLAM, IBRDC3, DJ174N9.1	Q6ZMZ0
Paul	RNF31	HOIP, PAUL, ZIBRA	Q96EP0
TRIAD3	RNF216	ZIN, TRIAD3, UBCE7IP1	Q9NWF9
ARA54	RNF14	ARA54	Q9UBS8
Not assigned	RNF217	C6orf172, IBRDC1, FLJ16403	Q8TC41

The crystal structures of Parkin and HHARI show that the IBR forms multiple interactions with RING1, as well as with regions N- and C-terminal of the RBR unit. Furthermore, models of the E2-bound RBR unit suggest that parts of the IBR contribute to the RING1 binding surface of the E3 on which the E2~ubiquitin docks [11]. Therefore, the IBR forms an important structural part of the RBR that is probably involved in the regulation of the activity and accessibility of the RBR unit.

The RING2 domain is the catalytic unit of the RBR. It contains a conserved cysteine residue, which forms the active site with which the ubiquitin C-terminus can form a thioester bond during the transfer of the ubiquitin from the E2 onto the substrate. The final transfer of the ubiquitin from the E2 cysteine onto the E3 RING2 domain is suggested by the crystal structure of Parkin and HHARI to be facilitated by a catalytic triad that consists of C431 (catalytic cysteine), H433 and E444 (numbering according to the Parkin sequence) [9–11, 77]. These residues are not conserved throughout the RBR family and the details of the reaction of the subsequent step, the transfer of a donor ubiquitin from the RING2 cysteine onto a target ubiquitin, seem to be subtly different between Parkin (or HHARI) and HOIP. In the latter, the arrangement of the catalytic site is different, due to the addition of a second zinc finger at this position, and only the corresponding RING2 C885 and H887 of the proposed catalytic triad are essential, whereas Q896 (which aligns with Parkin E444) is not involved in the chain-forming reaction [6, 11]. In addition, the residues around the N-terminus on the target

ubiquitin, to which the C-terminus of the donor ubiquitin is attached, may contribute to the chemistry of the reaction [61, 82]. Despite these differences in the catalytic site, all three RBRs can react with ubiquitin that is modified with a reactive group on its C-terminus, such as VME or propargyl [11], indicating that they share features that differ from other thioester forming molecules such as E2s and HECT E3s.

Autoinhibition and regulation of the RBR domain

The activity of the different RBR E3-ligases is highly regulated (Fig 3). The proteins can be regulated at the transcriptional level, but are also activated and inactivated by posttranslational modifications and protein-protein interactions.

All of the characterized RBR E3-ligases are autoinhibited by domains surrounding the RING1, IBR and RING2 domains. The E3-ligase activity of Parkin is embedded within the RBR domain in its C-terminus. Its catalytic activity was reportedly auto-inhibited by its N-terminal ubiquitin-like domain (UBL) [8], which binds to a linker/tether region between the IBR and RING2 of the RBR domain. This interaction is important for Parkin function, as multiple pathogenic mutations are found in the UBL [8]. At least three targets of Parkin contain domains that interact with the auto-inhibitory UBL domain, suggesting a target-induced Parkin activation [84,85]. Structural studies reveal that the linker/tether region obstructs E2 enzyme access to the RING1 domain even in the absence of the UBL, indicating that this linker region is critical to relieve the autoinhibition [9–11, 77] (Fig 3). In addition, RING0 was found to obstruct access to the catalytic cysteine and ligase activation requires its rearrangement [9–11].

Alternative activation may occur through the posttranslational modification of Parkin with Nedd8, which was reported to induce its E3-ligase activity [86]. Also phosphorylation of Ser65 by PINK1 activates Parkin [87–89], in a mechanism where the ubiquitin-ligase Fbxo7 is important [28]. In contrast, tyrosine-phosphorylation by c-Abl seems to inhibit Parkin [90]. Furthermore, Parkin activity is regulated by the modification of multiple cysteines within its IBR with nitric oxide (s-nitrosylation) [91,92], which is possibly induced

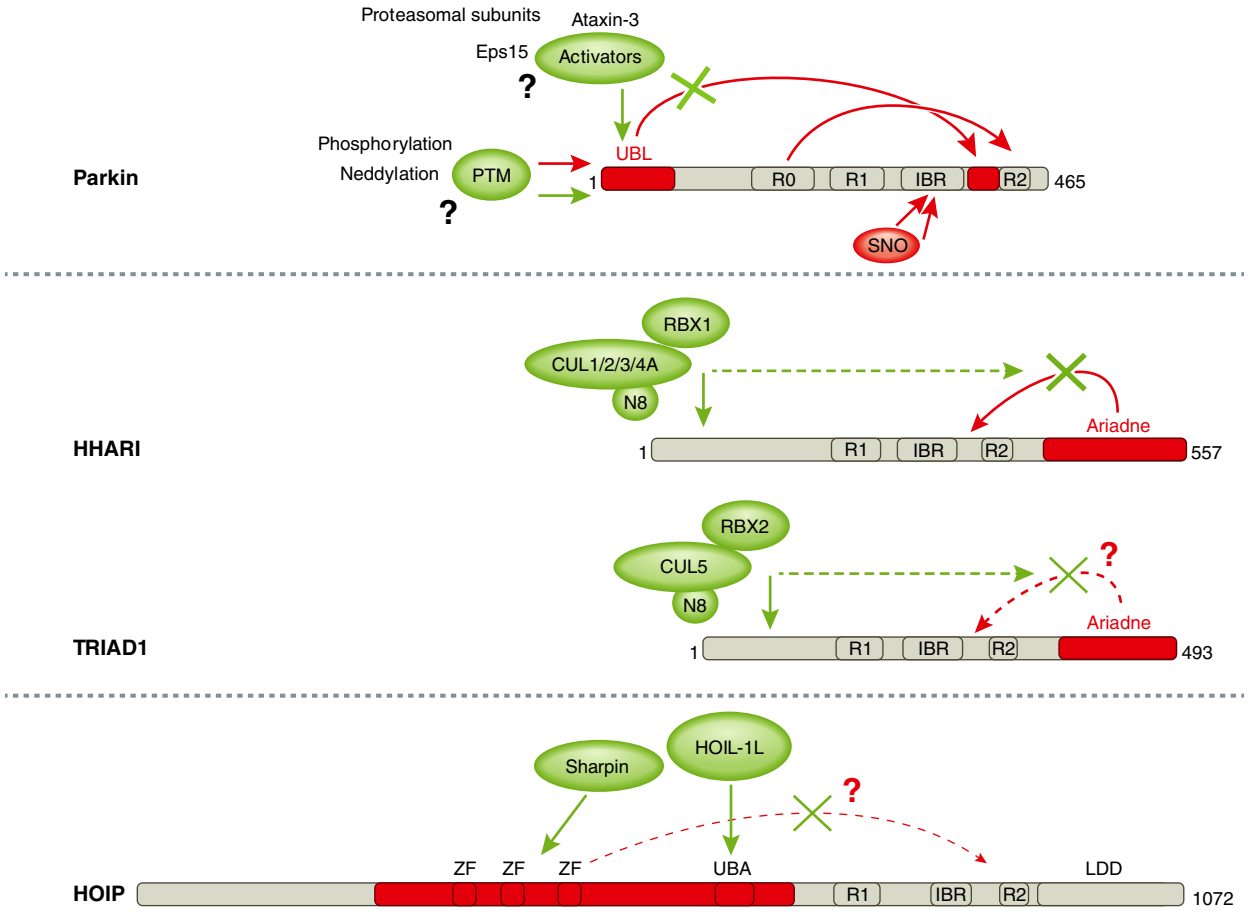


Figure 3. Regulation of the E3-ligase activity of Parkin, HHARI, TRIAD1 and HOIP.
Schematic representation of the RBR E3-ligases. The domains involved in autoinhibitory interactions are shown in red, whereas activators are shown in green. Autoinhibitory interactions between domains are depicted by the red arrows, and green arrows indicate interactions and domains that are influenced by the activators. Dotted lines are used for interactions that have not been fully characterized yet. See text for details. IBR = in between ring; LDD = linear-ubiquitin-chain determining domain; N8 = Nedd8; PTM = post-translational modification; SNO = s-nitrosylation; R = RING; UBA = ubiquitin associated domain; ZF = zinc finger.

by the increased levels of nitrosative stress in PD [93]. However, the precise effects of the s-nitrosylation on Parkin activity remain to be resolved.

In HHARI, the Ariadne domain that is C-terminal to its RBR provides an auto-inhibitory function. This autoinhibition is released upon interaction of the N-terminus of HHARI with a NEDD8-CUL1-RBX1 Cullin-RING complex [42] (Fig 3). The Ariadne RBR E3-ligase TRIAD1 is also strongly activated upon an interaction with a Neddylated Cullin-RING complex consisting of NEDD8-CUL5-RBX2 (Fig 3) [42]. The interaction with Neddylated Cullin-RING complexes might be a common feature of Ariadne RBR E3-ligases, which would link their function to that of Cullin-RING complexes. Interestingly, the details of the autoinhibition of HHARI and TRIAD1 must be different from Parkin autoinhibition, as E2 interaction is not impeded [42].

The RBR of HOIP is inhibited by domains that are located N-terminally to the RBR [6,7]. HOIP activation takes place through interactions between its UBA-domain and/or NZF2 domains with the UBL-domain of either HOIL-1L or Sharpin, its partners in the LUBAC complex (Fig 3) [7,56,57,59]. In the absence of its N-terminal domains, the RBR unit is constitutively active and HOIL-1L no longer provides additional activation, indicating that the N-terminal domains mediate the auto-inhibition. However, NEMO ubiquitination still requires the presence of additional LUBAC components [61]. The precise mechanism by which the HOIP N-terminus blocks the RBR activity is uncertain, as the RBR is not inhibited when the N-terminus is added to *in vitro* reactions *in trans* and there are no crystal structures of inhibited HOIP available. Consequently, the exact mechanism by which the catalytic domain is kept in an inactive state in full length HOIP remains to be resolved.

Interestingly, in both Parkin and HOIP, the domains N-terminal and/or within the RBR autoinhibit the catalytic activity [6–11], whereas in HHARI the C-terminal domain constrains the active conformation of the RBR unit by blocking the RING2 domain [77]. Thus, regulation of RBR activity by domains outside the RBR is a general feature of this class of E3 ligase, but the specific mechanism by which the catalytic activity is inhibited varies for each individual protein. Nevertheless, the interactions of Parkin, HOIP, HHARI and TRIAD1 N-termini with other proteins may release the auto-inhibitory state of the RBRs [42,84,85].

Chain formation specificity by RBRs

The formation of the HECT-like ubiquitin~E3 intermediate by RBR proteins would suggest that the target specificity of the RBRs resembles that of the HECT E3-ligases, in which the E2s do not play a role in the final transfer of the ubiquitin onto its target. Indeed, this is the case for HOIP, for which the linear-chain-forming ability overrules the E2 chain-type specificity [57].

Table 2. E2 interactions with human RBR E3-ligases

Name	E3 Activity[a]	Y2H[b]	Pull down/interactions[c]	Functional[d]	Ubiquitin Chain types[e]	References
HHARI	Yes	L3, L6	L3 (R1+20AA), L6 (R1,IBR)	D3, L3		[1, 38, 40, 103, 106]
TRIAD1	Yes	D1, D2, D3, D4, E1, E2, E3, L3, L6, R2, T, V1	L3 (R1), E1 (R1), N (R2)	D3, E1, E2, L3, N/V2	K48 (L3), K63 (N/V2), K* (D3)	[42, 47, 48, 50, 107, 108]
PARC	Yes			L3		[109]
ANKIB1	nd					
PARKIN	Yes		L3(R2), L6(R2)	A, D2, D3, L3, L6	K48, K63 (N/2V)	[1, 24–26, 36, 94, 95, 99, 100, 110]
RNF144A	nd	H, L3, L6, V1, V2	L3(R1), L6(R1)			[108, 111]
RNF144B	Yes	I, L3, L6, T, U, Z	L3 (RBR), L6(RBR)			[108, 112–114]
HOIL-1L	Yes	D4, G1, L3, L6, N, S, U		D3		[108, 115]
RNF19A	Yes		L3(RBR), L6(RBR)			[116–118]
RNF19B	Yes		L3(FL), L6(FL)			[119,120]
HOIP	Yes		L6 (FL)	D3, L3	Linear (all tested E2s)	[6, 121]
RNF216	Yes		L3, L6		K48 (E2 not identified)	[122]
RNF14	Yes	D1, D2, D3, D4, E1, E2, E3, U, V1,W	E1(R1), E2(R1), E3(R1)	E1, E2, E3		[108, 114, 123, 124]
RNF217	nd					
LUBAC	Yes			B, D1, D2, D3, K, L3	Linear (all tested E2s)	[55, 57, 58, 62]

[a]Experimental evidence for E3-ligase activity yes/not done.
[b]E2 interaction-partners identified by yeast-two-hybrid, Ube2 names are shown.
[c]E2 interaction-partners identified by pull down assays or by other methods, the column contains the Ube2 names and the RBR interaction-sites between brackets.
[d]Ube2 names of the E2s that have been shown to be functionally active with the RBR E3.
[e]Ubiquitin chain types formed in cooperation with specific E2s of which the Ube2 name is shown between brackets.
K* various different ubiquitin chain types formed.

HOIP ligase activity, either in the context of LUBAC or as an N-terminal deletion construct that is constitutively active ($HOIP^{RBR\text{-}LDD}$), is induced by a variety of E2-enzymes. All E2s induce the selective formation of linear ubiquitin chains, indicating that the E2s do not contribute to the chain formation specificity of HOIP (Table 2). Instead, the transfer of the ubiquitin from the E3 onto the N-terminal amine-group of the target ubiquitin is mediated by the specific positioning of the target ubiquitin by the HOIP C-terminal LDD [6,7] (Fig 4). The structure of the HOIP RING2-LDD region in complex with ubiquitin was recently solved [82], and interestingly it reveals that the LDD embraces the RING2 domain. The RING2 and LDD together create the binding site for the target ubiquitin and position it to present the amino-terminus to the RING2~donor ubiquitin thioester for the formation of a peptide bond between the two ubiquitins (Fig 4). This LDD extension to the RBR domain is not found in other RBR E3-ligases, suggesting that this mechanism of chain formation is unique to HOIP.

The targeting of a lysine in NEMO by the HOIP/HOIL-1L complex requires NEMO recognition sites in LUBAC [58] as well as the presence of both an active HOIP RBR and an active HOIL-1L RING2 catalytic site [61]. Thus, also in this case the E3 determines which lysines are modified. Understanding the details of this two RBR mechanism will require further studies but, intriguingly, the process also depends on an intact amino terminus of the donor ubiquitin [61], as modifications at the N-terminus or mutation of the adjacent E16 affect the process. If other RBRs have such a requirement, studies using tagged ubiquitin may not reveal their true activity. Lysine targeting by LUBAC could be rare in cells, as most of the linear chains on NEMO are attached to K63 chains, conjugated to the ubiquitin amino-termini in these chains [63].

In contrast to the E3-dependent targeting of LUBAC, the current literature suggests that ubiquitin chain formation by the RBRs ARIH2, Parkin, and RNF216 relies on the ubiquitin chain formation specificity of the E2 that is used in the reaction (Table 2).

The Parkin RBR domain is functionally active with the E2s Ube2A [94], Ube2D2 [95], Ube2D3 [1,24,96], Ube2L3 [86,97–99], and Ube2L6 [24], with which it mediates the formation of various ubiquitin signals (Table 2). Parkin mediates the formation of K63-linked ubiquitin chains in cooperation with the K63-specific E2 Ube2N/Ube2V2 [100,101], to target misfolded DJ1 for dynein-mediated transport to aggresomes [26]. It also catalyses the formation of K48-linked ubiquitin chains [100,101], and works with Ube2L3 to target RanBP2 for degradation [86]. Finally, Parkin has been suggested to mediate the mono-ubiquitination of targets in an IBR-RING2 dependent manner in cooperation with Ube2L3 and Ube2N/Ube2V2 [98], targeting p38 [97], Hsp70/Hsc70 [102], and Bcl-2 [36]. A comparison of the Parkin-mediated ubiquitin chain formation activities in the various studies suggests that the E2s play a major role in the specificity of ubiquitin chain formation. Consequently, the E2 enzyme that is used by Parkin strongly determines the cellular outcome of the proteins that are targeted.

The E3-ligase HHARI has been shown to interact through its RING1/IBR domains to the E2s Ube2L3 and Ube2L6 (Table 2) [38, 40,103]. However, only the functionality of the E2/HHARI interaction with Ube2L3 has been validated [1]. Interestingly, also Ube2D3 has been shown to be functionally active with HHARI, even though is has not been identified as a binding partner in yeast two-hybrid studies [1,38,40]. Unfortunately, there are no data available about the ubiquitin chain formation specificity and target selection by this E3, leaving the precise mechanism by which HHARI ubiquitinates its targets to be resolved.

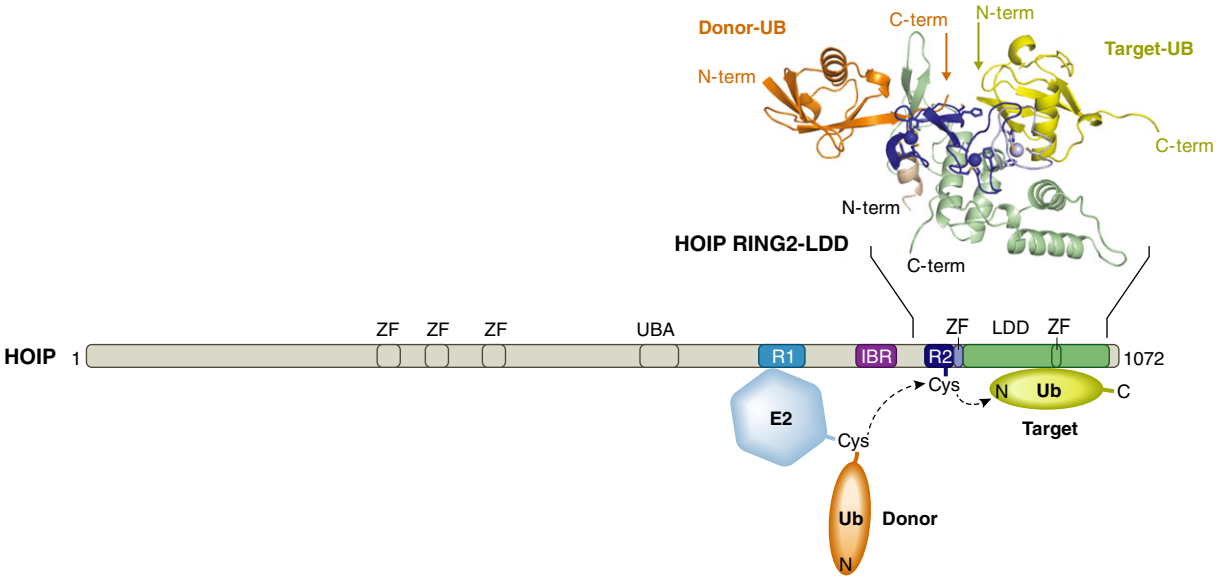


Figure 4. Linear ubiquitin chain formation by HOIP.
The positioning of the target ubiquitin (yellow) by the HOIP LDD (green) is critical for the transfer of the donor-ubiquitin (orange) from the E2 (blue), via a cysteine on RING2 onto the N-terminus of the target ubiquitin. The crystal structure of HOIP RING2-LDD in complex with a donor and a target ubiquitin (PDB 4ljo) shows a snapshot of the orientation of the proteins just before the two ubiquitins are linked together by HOIP. IBR = in between ring; LDD = linear-ubiquitin-chain determining domain; R = RING; UBA = ubiquitin associated domain; ZF = zinc finger.

Sidebar: In need of answers

The past 2 years have seen a rapid increase in insight into the structure and function of RBR E3-ligases, revealing how these ligases perform the ubiquitination reaction. The field now appreciates the importance of their two-step RING/HECT-like mechanism, their autoinhibition and its release by protein partners, and the first insights into the transfer to different target amino groups have emerged.
Nevertheless, important mechanistic and functional questions remain: how do RBRs achieve E2 activation, given that the process is different from other RING domains? What do the RBRs look like in the activated state? Does the chain-specificity in the activated state follow E2 or E3 preferences? As the amino-group specificity of HOIP is regulated by its unique LDD, it is unlikely to be mirrored in other RBR ligases. When studying the chain type and lysine specificity of RBRs, it may be valuable to keep in mind the surprising importance of an intact donor-ubiquitin N-terminus for lysine targeting by HOIL-1L.
Ultimately, insights into mechanism and activation may help to address the most important question of all: what are the genuine targets of these RBR ligases in cells. The identification of these targets is a prerequisite for full appreciation of their function.

The E3-ligase activity of TRIAD1 in cells can potentially be mediated by a large variety of E2s that have been found as TRIAD1 interaction partners (Table 2). Of these E2s, Ube2L3 and Ube2E1 have been shown to interact with the TRIAD1 RING1 domain, and Ube2N binds to TRIAD1 RING2 [50]. The functional relevance of the interaction has been validated for several E2s in auto-ubiquitination and free ubiquitin chain formation assays, showing that TRIAD1 mediates the formation of K48-ubiquitin chains with Ube2L3, K63-ubiquitin chains in cooperation with Ube2N/Ube2V2, and various different chains in cooperation with Ube2D3 [42, 47, 48]. Thus, the E3-ligase activity of TRIAD1 follows the ubiquitin chain formation specificity of the E2 that is used in the reaction. Nevertheless, it is not clear if TRIAD1 follows the E2 chain-type choice on its cellular targets.

So far, many of these assays rely on autoubiquitination as a read-out for activity. Moreover, most have been performed without the full activation of the E3 ligase, as details of the activation mechanisms of RBRs have only recently been revealed. It will be interesting to reconstitute reactions with the different E2 enzymes in the activated states of the RBR enzymes and follow the behaviour on genuine targets. Thus far, it appears as if the final transfer of the ubiquitin by these RBR E3-ligases is mediated by the E2 determining the chain type, despite the dependence on a HECT-like transfer to the target. This suggests that the E2s stay in the complex to facilitate chain formation, which puts an interesting mechanistic constraint on the reaction. Possibly, the E2s cooperate directly with RING2 to position the target ubiquitin close to the active site cysteine of the RBR E3-ligase. Alternatively, the role for the E2 in the ubiquitin chain formation specificity might be explained by a mechanism in which the E2 and E3 collaborate to mediate the specific formation of ubiquitin chains on the E2 active site cysteine [104], before the RBR RING2 mediates the en-bloc transfer of the ubiquitin chain from the E2 onto the target protein. These options require further studies. In addition, the precise role of the RING domains in the RBR needs further analysis, as interaction studies identified that some RBRs interact with E2s through their RING2 domains (Table 2), or bind possible targets via the RBR [24, 35, 105]. Consequently, the combined RING/HECT type mechanism might be modulated in these reactions, and further studies are needed to elucidate their functional significance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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