Structure of a HOIP/E2~ubiquitin complex reveals RBR E3 ligase mechanism and regulation

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Ubiquitination is a central process affecting all facets of cellular signalling and function1. A critical step in ubiquitination is the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to a substrate or a growing ubiquitin chain, which is mediated by E3 ubiquitin ligases. RING-type E3 ligases typically facilitate the transfer of ubiquitin from the E2 directly to the substrate2,3. The RING-between-RING (RBR) family of RING-type E3 ligases, however, breaks this paradigm by forming a covalent intermediate with ubiquitin similarly to HECT-type E3 ligases4–6. The RBR family includes Parkin7 and HOIP, the central catalytic factor of the LUBAC (linear ubiquitin chain assembly complex)7. While structural insights into the RBR E3 ligases Parkin and HHARI in their overall auto-inhibited forms are available8–13, no structures exist of intact fully active RBR E3 ligases or any of their complexes. Thus, the RBR mechanism of action has remained largely unknown. Here we present the first structure, to our knowledge, of the fully active human HOIP RBR in its transfer complex with an E2~ubiquitin conjugate, which elucidates the intricate nature of RBR E3 ligases. The active HOIP RBR adopts a conformation markedly different from that of auto-inhibited RBRs. HOIP RBR binds the E2~ubiquitin conjugate in an elongated fashion, with the E2 and E3 catalytic centres ideally aligned for ubiquitin transfer, which structurally both requires and enables a HECT-like mechanism. In addition, three distinct helix–IBR-fold motifs inherent to RBRs form ubiquitin-binding regions that engage the activated ubiquitin of the E2~ubiquitin conjugate and, surprisingly, an additional regulatory ubiquitin molecule. The features uncovered reveal critical states of the HOIP RBR E3 ligase cycle, and comparison with Parkin and HHARI suggests a general mechanism for RBR E3 ligases.

RBR E3 ligases are characterized by an extended RING domain (RING1) followed by an ‘in-between RING’ (IBR) domain and the catalytic domain, which is structurally an IBR domain but is commonly designated RING2 (Extended Data Fig. 1a, b)8–11,14. HOIP, one of the most studied RBRs, is the key E3 ligase of the linear ubiquitin chain assembly complex (LUBAC). It is a prototypical RBR yet contains an extended RING2 domain that includes the LDD (linear ubiquitin chain determining domain, Extended Data Fig. 1c)12–15 and is thus denoted RING2L. The LDD enables the selective formation of linear ubiquitin linkages. The HOIP RBR is kept in an auto-inhibited state by the HOIP UBA domain, whose sequestration by the LUBAC constituent HOIL-1L activates HOIP to trigger, together with SHARPIN, NF-kB signalling and other cellular processes16–20. To obtain the first insight into an active RBR in a key catalytic complex, we generated a stable E2~ubiquitin conjugate (UbcH5B C85K~ubiquitin)21 and isolated its complex with HOIP RBR. The subsequent addition of free ubiquitin proved necessary for crystal formation, allowing us to solve the HOIP RBR/UbcH5B~ubiquitin transfer complex structure at 3.5 Å resolution (Fig. 1a; Extended Data Figs 2 and 3).

The asymmetric unit contains two HOIP RBR molecules interacting with two UbcH5B~ubiquitin conjugates and an additional ubiquitin or E2~ubiquitin conjugate, arranged in a swapped dimer configuration (Extended Data Fig. 3a). While this arrangement could have functional relevance, analysis of interfaces and biophysical examination (Extended Data Fig. 3b–f) indicate a monomeric assembly of the HOIP/E2~ubiquitin loading complex (Fig. 1), represented in the crystal structure by the RING1–IBR module (residues 699–852) from one HOIP molecule and the RING2L (residues 853–1,072) from the second HOIP molecule in the asymmetric unit. In this assembly, the RING1–IBR module forms an elongated arm-like unit (Fig. 2a) that

Figure 1 | Structure of the HOIP RBR/UbcH5B~ubiquitin transfer complex.

a, Structure with key elements annotated. The RBR RING1 and IBR together with two RING1 extension helices (hE1, hE2) form an arm-like unit (magenta; for domain annotations see Extended Data Fig. 1). The RING1–IBR and the catalytic RING2L (green) are connected by two linker helices (hL1, hL2) and together engage the E2~ubiquitin conjugate (UbcH5B~Ubact; orange and cyan, respectively) positioning it for ubiquitin transfer onto the RBR catalytic cysteine (yellow circle). An allosteric ubiquitin molecule (Ubact; blue) binds to the RING1–IBR across from the activated ubiquitin. Zinc ions are shown as grey spheres; hR denotes the RING1 helix. b, Location of the three ubiquitin-binding regions (UBRs, dashed lines) in the complex.
The HOIP RING1–IBR coordinates the UbcH5B~ubiquitin conjugate in a bipartite manner tailored to a HECT-like mechanism. a, Coordination of the UbcH5B~ubiquitin conjugate (orange and cyan) by the RING1–IBR (magenta). HOIP RING2L is indicated schematically. b, The HOIP RING1 coordinates the E2 in a shifted position compared to classic RINGs. Overlay of HOIP RING1/UbcH5B (magenta/yellow) and RNF4 RING/UbcH5A (brown/orange, PDB: 4AP4 (ref. 21)). c, RBR hE2 and IBR form UBR1 binding the activated ubiquitin. A central salt bridge system connects hE2 and ubiquitin. d, Comparison of E2~ubiquitin binding by HOIP RING1 (top) and a classic RING (bottom, RNF4 PDB: 4AP4 (ref. 21)) highlights the differences in E2~ubiquitin thioester positioning (red spheres). The directionality of the thioester attacking residue (active site cysteine of RBR in the HECT-like transfer and lysine of the substrate in RING-mediated transfer) is indicated. e, f, Quantitative thioester transfer (e) (mean activity ± s.e.m. (n = 3); one-way ANOVA followed by Tukey’s post-test; **P < 0.01; ***P < 0.001; Supplementary Fig. 1) and linear polyubiquitination (f) assays of HOIP RBR wild type (WT) and interface mutants, and HOIP catalytic domain (RING2L). g, Thioester transfer and linear ubiquitination assays for UbcH5B wild type and RING1 interaction mutant F62A (Coomassie-stained bands in red).

Together with the RING2L embraces the E2~ubiquitin conjugate in a clamp-like manner (Fig. 1a). This active HOIP RBR conformation is markedly different from previous structures of auto-inhibited RBRs (Extended Data Fig. 1d) and enables an astounding array of features inherent to the active RBR. Most notably, three distinct helix–IBR-fold motifs function as essential discrete ubiquitin-binding regions (UBR) (Fig. 1b).

The HOIP RING1/E2 interaction is tailored towards a HECT-like mechanism, setting it apart from classic RING E3 ligases. While RING/E2 interactions of both classic RING and RBR E3 ligases utilize similar surfaces (Extended Data Fig. 4a)21–26, the position of the HOIP RING1 domain relative to the E2 is shifted compared to classic RING/E2 complexes (Fig. 2b). Therefore, the RBR RING1 and the E2 do not form a composite surface to bind the E2-conjugated activated ubiquitin (Ubact, Extended Data Fig. 4b, c, e), which is key to the mechanism of classic RING E3 ligases21,24,27. Instead, two extension helices (hE1, hE2) link the RBR RING1 to the IBR domain (Figs 1 and 2a)8–11, and helix hE2 with the IBR forms an UBR (UBR1) that engages the activated ubiquitin (Fig. 2c and Extended Data Fig. 5a, b). UBR1 binds ubiquitin in a distinctive mode (mode 1) that utilizes a salt bridge system involving HOIP hE2 residues K783 and E787 and ubiquitin residues K11 and E34, with further support from the HOIP IBR (Fig. 2c and Extended Data Fig. 5b). Thus, in HOIP the entire RING1–IBR arm mediates bipartate binding of the E2~ubiquitin conjugate, with RING1 binding the E2 and the hE2–IBR module binding activated ubiquitin (Fig. 2a). Sequence and structural comparisons with Parkin and HHARI suggest conservation of this mechanism among RBR E3 ligases (Extended Data Figs 4c and 5c). Importantly, the bipartite binding mode results in an elongated conformation of the E2~ubiquitin conjugate with its thioester linkage not suited for direct attack by the amine function of a substrate (Fig. 2d and Extended Data Fig. 4e–f). The consequence is an entirely different catalytic arrangement compared to classic RING-supported catalysis, as emphasized by the observed lack of effect of mutations in UbcH5B L104 and S108, two residues crucial for classic RING/E2 catalysis21,23,26 (Extended Data Fig. 4d). Instead, the E2~ubiquitin thioester is ideally positioned for transfer of the activated ubiquitin onto HOIP RING2L, thus both enabling and requiring a HECT-like mechanism. The importance of each interaction site is demonstrated onto HOIP RING2L, thus both enabling and requiring a HECT-like catalysis21,25,26 (Extended Data Fig. 4d). Instead, the E2...
activity (Fig. 2e, f), indicating a cumulative effect due to a potential role of UBR1 in coordinating Ubact in steps subsequent to its initial transfer to HOIP. However, removal of the salt bridge in HOIP/ubiquitin double mutants and mutation of the HOIP IBR/Ubc recognition domain altered the trajectory of the ubiquitin transfer process (Extended Data Fig. 7).

The other portion of the RBB/E2{ubiquitin embrace is centred around the catalytic HOIP RING2L (Figs 1a and 3a). Here a helix–IBR motif consisting of helix hL2 from the IBR–RING2L linker and RING2L form a second UBR (Fig. 2b) binding the ubiquitin (Fig. 3a and Extended Data Fig. 6a–c). UBR2, which is conserved in Parkin and HHARI (Extended Data Fig. 6d), uses a hydrophobic pattern in helix hL2 and RING2 to interact with the canonical (I44) and a second hydrophobic patch of ubiquitin (Extended Data Fig. 6b–e). These interactions support the engagement of the ubiquitin R72/R74 and UbcH5B R90, thus facilitating interactions among all three proteins. The second conduit consists of catalytic residues of UbcH5B (N877, D117) and HOIP (H887, Q896) and thus the ubiquitin/E2-thioester transfer complex (Fig. 3b and Extended Data Fig. 6f). Remarkably, in this structure the donor ubiquitin adopts a position identical to the activated ubiquitin in HOIP UBR2 despite lacking the di-Arg motif by polar residues, which is the key characteristic of UBR binding mode 2. This ultimately places the ubiquitin C terminus onto RING2 (Extended Data Fig. 6b, d) and thus the ubiquitin/E2-thioester linkage onto the RBR active site. A previous structure of the isolated HOIP RING2L with two ubiquitin molecules bound in a linear non-covalent arrangement mimics the final HOIP RING2L donor/acceptor ubiquitin transfer complex (Fig. 3b and Extended Data Fig. 6f).

In Importantly, the HOIP RBR/E2{ubiquitin complex structure lacks the spatial gap between the E2 and E3 catalytic centres that is frequently observed in HECT/E2 complex structures and that was also predicted for RBR/E2{ubiquitin transfer complexes 8,11,28,29. Thus, except for the ~3.5 Å spacing due to the C85 to lysine substitution in the E2{ubiquitin conjugate, the HOIP RBR/E2{ubiquitin structure accurately depicts the immediate transfer complex. Here the catalytic centres of HOIP RING2L and E2 come in close proximity via two contact conduits involving all three proteins (Fig. 3c and Extended Data Fig. 7a). The first conduit consists of ubiquitin R72, which interacts with D983 and Q974 in the β5/6-hairpin of HOIP RING2L. Additionally, E976 in this hairpin mediates a salt bridge with ubiquitin R74 and UbcH5B R90, thus facilitating interactions among all three proteins. The second conduit consists of catalytic residues of UbcH5B (N877, D117) and HOIP (H887, Q896) and thus the ubiquitin/E2-thioester transfer complex (Fig. 3b and Extended Data Fig. 6f). These residues appear permissive to close proximity between the reaction centres, yet not crucial for transesterification because, for example, a H887A mutation does not affect the thioester transfer reaction (Extended Data Fig. 7b). Surprisingly, mutation to alanine of UbcH5B D117, a critical residue for classic RING-supported catalysis 21,24, enhances transesterification (Extended Data Fig. 7b), further underlining the vastly different catalytic mechanism of RBR E3 ligases. This finding also points to a trade-off in the E2 active site to support both classic thiolytic activity and efficient ubiquitin transfer.

Figure 4 | An allosteric ubiquitin interacts with UBR3 in the RING1–IBR arm and is crucial for HOIP activity. a, An allosteric ubiquitin (Ub3, blue) binds to UBR3 across the activated ubiquitin. Left, overview depicting UBR3/Ub3 interaction. Top right, UBR3–IBR/Ub3 interaction. Bottom right, magnified view of the HOIP ubiquitin di-Arg binding motif (E809) anchoring a parallel ubiquitin/IBR β-sheet. b, Linear di-ubiquitin enhances HOIP RBR activity. Thiosteer transfer assays of wild-type HOIP and UBR3 mutants (mean activity ± s.e.m. (n = 3); one-way ANOVA followed by Tukey’s post hoc test; ***P < 0.001; NS, not significant; Supplementary Fig. 1). c, Polyubiquitination assays showing release of HOIP UB3–RBR auto-inhibition by HOIL-1L or linear di-ubiquitin. d, Effects of wild-type HOIP or UBR3 mutants in NF-κB reporter assays using HEK293T cells expressing full-length HOIP with or without HOIL-1L (mean activity ± s.e.m. of three biological replicates each with three technical replicates; one-way ANOVA followed by Tukey’s post hoc test; ***P < 0.001; Extended Data Fig. 9f).

Figure 3 | Mechanism of E2{ubiquitin/HOIP RBR ubiquitin transfer. a, UbcH5B–ubiquitin conjugate bound to HOIP RING2L (RING2, light green and the linear ubiquitin chain determining domain (LLD) extension, dark green). UBR1 (schematic of helix–IBR, magenta) cooperates with UBR2 (comprising hL2 and IBR fold of RING2L) to bind the activated ubiquitin. UbcH5B interacts with RING2L in a region designated for the acceptor ubiquitin (displayed in b). b, Positioning of E2{Ubact or donor/acceptor ubiquitin (Ubdon or Ubacc, respectively) onto RING2L. c, Ternary HOIP RBR/E2{ubiquitin catalytic transfer complex. Two main contact conduits (red dashes) position the RBR catalytic cysteine (C885) near the E2 C85-ubiquitin thioester linkage. d, HOIP{ubiquitin thioester formation assay with wild-type or E976A RBR, supports the HOIP/ UbcH5B/Ub link in conduit 1 (mean activity ± s.e.m. (n = 3); two-tailed unpaired Student’s t-test; ***P < 0.001; Supplementary Fig. 1).
RING- and HECT-type RBR E3 ligases. Notably, Ubch7, which is specialized for HECT-like E3 catalysis\(^4\), features a histidine instead of D117 (Extended Data Fig. 7a). Mutational analysis demonstrates a crucial role for conduit 1 and also indicates that the close proximity between the ubiquitin thioester (and thus C85 of Ubch5b) and HOIP catalytic cysteine C885 is the driving factor for E2/RBR E3 ubiquitin transfer (Fig. 3c, d and Extended Data Fig. 7). Analysis of Parkin and HHARI shows conservation of the conduits (Extended Data Fig. 7a). However, while Parkin and HHARI lack the 35/6-hairpin indigenous to HOIP RING2L, they instead possess a pair of conserved polar residues in the RING2 active site loop that are capable of binding the di-Arg motif in conduit 1.

Surprisingly, our structure reveals that an additional allosteric ubiquitin molecule (Ub\(_{d\alpha}\)) interacts with a third HOIP UBR. UB3 is located in the RING1–IBR arm immediately upon UB3R and Ub\(_{act}\) (Fig. 1b, 4a and Extended Data Fig. 8a, b). Ub\(_{d\alpha}\) uses a binding mode similar to that of Ub\(_{act}\) with UB2 (mode 2), characterized by hydrophobic interactions and a di-arginine binding clamp (Extended Data Fig. 8a). Ub\(_{d\alpha}\) interacts with helix hE2 of the extended RING1 and with the IBR, and makes additional interactions with helix hE1 (Fig. 4a and Extended Data Fig. 8c). Through this binding, Ub\(_{d\alpha}\) induces a ‘straight’ conformation of helix hE2, locking RING1 and IBR in their relative position, forming UB3 to accommodate the activated ubiquitin (Fig. 4a and Extended Data Fig. 8d–n). Notably, UB3R in the HOIP RBR/UbcH5B~ubiquitin complex binds linear di-ubiquitin (K\(_d\) = 7 μM) better than mono-ubiquitin (K\(_d\) > 50 μM) (Extended Data Fig. 9a). Pre-incubation of HOIP RBR with linear di-ubiquitin leads to improved binding of UbcH5b~ubiquitin (Extended Data Fig. 9b), emphasizing the allosteric function of UB3. Accordingly, HOIP UB3R 1807A and E809A mutants show moderately decreased activity in thioester transfer assays but more pronounced effects in polyubiquitination assays, where linear di-ubiquitin/polylubiquitin are intrinsically produced (Fig. 4b and Extended Data Fig. 9c). Importantly, N- and C-terminally capped linear di-ubiquitin increases HOIP RBR thioester transfer activity in a dose-dependent manner, but cannot activate HOIP UB3R mutants (Fig. 4b and Extended Data Fig. 9d). Moreover, the linear di-ubiquitin I44A mutant also fails to activate HOIP RBR (Extended Data Fig. 9d).

Excitingly, the interaction of Ub\(_{d\alpha}\) with UB3 is structurally similar to that recently reported for phospho-ubiquitin in a tethered complex with ΔUBL. Pediculus humanus Parkin\(^1\) (Extended Data Fig. 8c–e). Binding of phospho-ubiquitin leads to a straight conformation of Parkin helix h2 and an accompanying reorientation of RING1 and IBR, indicating a general role of UB3 and ubiquitin in allosteric regulation of RBR proteins. Functionally, binding of phospho-ubiquitin activates Parkin by counteracting the auto-inhibitory function of the Parkin UBL domain (Extended Data Fig. 8c–e)\(^1\). In HOIP, the UBA domain exerts intramolecular auto-inhibition\(^5,6\). While the structure of auto-inhibited HOIP is unknown, the structure of auto-inhibited HHARI shows binding of its UBA domain to a region analogous to UB3 (Extended Data Fig. 8f–h)\(^11\). To determine if linear di-ubiquitin can overcome HOIP auto-inhibition, we examined its effect on HOIP UBA–RBR. As expected, HOIP UBA–RBR alone exhibits low E3 activity but is activated by HOIL-1 (Fig. 4c). Notably, linear di-ubiquitin can also remove HOIP UBA auto-inhibition and at high concentrations allows the processive formation of polylubiquitin chains by HOIP UBA–RBR (Fig. 4c). Importantly, in HEK293T cells expressing full-length HOIP, the HOIP UB3R 1807A and E809A mutants fail to activate NF-kB regardless of HOIL-1 expression, demonstrating an essential physiological role of UB3 (Fig. 4d). Thus, UB3R probably serves as a critical sensor of ubiquitin chains that regulates LUBAC function. Whether this role is tailored to linear ubiquitin chains or ubiquitin chains in general (Extended Data Fig. 9b, c) needs further investigation in the context of other LUBAC constituents and binding partners.

The features revealed by the HOIP RBR/E2~Ub\(_{act}/Ub\(_{d\alpha}\) complex structure provide the missing links in our understanding of these enigmatic multidomain E3 ligases\(^8,11,14\) and yield a mechanistic model for the RBR E3 ubiquitin ligase cycle, as summarized in Extended Data Fig. 10. Furthermore, the conservation of key mechanistic features in HOIP, HHARI, Parkin and other RBRs (Supplementary Data 2) underlines the general nature of the catalytic RBR cycle revealed in this study.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions B.C.L. designed and carried out all experiments (except for the cell-based experiments), including crystallization, structure solution and refinement, and wrote the manuscript. M.K.D. expressed proteins and performed initial purification. P.D.M. participated in early stages of the study, structure solution and writing of the manuscript. R.S. collected and processed diffraction data. A.R. performed the HEK293T cell experiments under the supervision of C.F.W. S.J.R. oversaw and actively participated in all steps of the study and wrote the manuscript.

Author Information Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 5EDV. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.J.R. (sriedl@SBPdiscovery.org).
No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

**METHODS**

All proteins were expressed in BL21(DE3) E. coli after induction with 0.5 mM IPTG overnight at 20 °C. For expression of HOIP and HOIL-1 constructs, 0.5 mM ZnCl₂ was added to the cultures before induction. Bacteria were harvested by centrifugation, lysed by addition of lysozyme and sonication in the presence of protease inhibitors (PMSF and leupeptin) and harvested by centrifugation. Mutations in UbcH5B, ubiquitin and HOIP were introduced using standard site-directed mutagenesis techniques.

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**Protein expression and purification.** All proteins were expressed in BL21(DE3) E. coli after induction with 0.5 mM IPTG overnight at 20 °C. For expression of HOIP and HOIL-1 constructs, 0.5 mM ZnCl₂ was added to the cultures before induction. Bacteria were harvested by centrifugation, lysed by addition of lysozyme and sonication in the presence of protease inhibitors (PMSF and leupeptin) and harvested by centrifugation. Mutations in UbcH5B, ubiquitin and HOIP were introduced using standard site-directed mutagenesis techniques.
E2 of the published RNF4–RING/UbcH5A~ubiquitin structure (PDB: 4AP4(ref. 21)) with the E2 in our structure and then used this overlay to add the RING1 model generated by Sculptor. This overlay placed the HOIP RING1 Zn2+~coordinating residues near the last remaining free Zn2+ ions found earlier by Phaser MR-SAD, indicating correct placement of the RING1 domain. In the final round of molecular replacement, the two additional ubiquitin (Ub/n) molecules were reinstated at the RING1–IBR interface. At this stage, Refmac4 was used for refinement using settings optimized for low-resolution refinement16 including ‘jelly-bag’ model building and binning. ProSMART44 was used to generate external restraints against high-resolution structures (PDB: 4LJO (ref. 14) for HOIP RING2 and ubiquitin, and PDB: 2E5K (ref. 45) for UbcH5B). After this, clear extra electron density became visible for the unmodelled helical linker regions of the RING1–IBR and RING–RING2L transitions and for other regions omitted in the initial search models. Further model building and refinement was manually performed in Coot46 and Refmac. During refinement additional clear positive difference map electron density became visible and Phaser was used to place one additional UbcH5B molecule (UbcH5B/ad) into this density. TLS restraints were generated using the TLSMD server27 and CNS restraints were used throughout refinement. One overall B-factor was refined in Refmac. In later rounds of refinement the PDB REDO server48 was used for refinement optimization and MolProbity54 was used for structure validation. Data processing and refinement statistics are summarized in Extended Data Fig. 2b. Ramirez-Frank statistics were calculated using MolProbity and 94.8% of all residues are in favoured regions, 4.9% in allowed regions and 0.3% are outliers. The final structure has a MolProbity score of 1.75 (100th percentile). In the final structure the two HOIP RING2 molecules (see also Extended Data Fig. 3) are defined by electron density from residues 699 to 707, 711 to 948, 996 to 991, and 996 to 1011 (chain A) and 699 to 754, 760 to 955, 967 to 1,015, 1,019 to 1,035 and 1,054 to 1,066 (chain B). The catalytic UbcH5B–ubiquitin conjugates are defined from UbcH5B residues 3 to 147 and ubiquitin residues 1 to 76 (chains C and E), and UbcH5B residues 2 to 147 and ubiquitin residues 1 to 76 (chains D and F). The allosteric ubiquitin chains (chains G and H) are defined from residues 1 to 76 and the additional UbcH5B (chain I) is defined from residues 2 to 146. PHENIX was used to calculate simulated annealing (SA) composite omit maps and feature enhanced maps (FEM). All molecular figures were prepared in PyMOL (Schrödinger, LLC).

K48-linked and K63-linked ubiquitin chain formation. K48-linked and K63-linked ubiquitin chains were formed through a linkage-specific enzymatic reaction using Cdc34 and UbcH13/Uev1a E2 ubiquitin-conjugating enzymes, respectively, as described in the literature36. Ubiquitin chains were separated using ion-exchange chromatography as described above for purification of mono-ubiquitin. Purified K48-linked di-ubiquitin was directly desalted into protein buffer using PD-10 desalting columns, whereas K63-linked di-ubiquitin was further purified on a Superdex 75 10/300 GL size-exclusion chromatography column equilibrated in protein buffer. Native ubiquitin without additional residues was used to generate di-ubiquitin chains for ITC experiments, whereas N-terminally blocked ubiquitin (32 μM) and N- and C-terminally blocked di-ubiquitin solution were injected into the cell containing 40–50 μM HOIP RBR or HOIP RBR/di-ubiquitin complexes. The experiments were performed at 23 °C in buffer containing 10 mM HEPES pH 7.9, 100 mM NaCl. For titrations of UbcH5B~ubiquitin into HOIP RBR/di-ubiquitin complexes, HOIP RBR was pre-incubated with an equimolar amount of di-ubiquitin before the ITC experiments. Data were analysed using the Origin software (Microcal). Analytical ultracentrifugation (AUC), Sedimentation equilibrium experiments were performed in a ProteomeLab XL-1 (Beckman Coulter) analytical ultracentrifuge. HOIP RBR/UbcH5B~ubiquitin as used for crystallization was loaded into a 6-channel equilibrium cell at 5.0, 2.5 and 1.25 μM concentration and centrifuged at 100,000 rpm, 20 °C in an An-SO Ti 8-plate rotor until equilibrium was achieved. Data were analysed using HeteroAnalysis software (J. L. Cole and J. W. Lary, University of Connecticut; http://www.biotech.uconn.edu/aut/).

Isothermal titration calorimetry (ITC). ITC experiments were performed on an ITC200 calorimeter (Microcal). Aliquots (2 μL each) of 500–650 μM UbcH5B~ubiquitin or di-ubiquitin solution were injected into the cell containing 5–60 μM HOIP RBR or HOIP RBR/di-ubiquitin complexes. The experiments were performed at 23 °C in buffer containing 10 mM HEPES pH 7.9, 100 mM NaCl. For titrations of UbcH5B~ubiquitin into HOIP RBR/di-ubiquitin complexes, HOIP RBR was pre-incubated with an equimolar amount of di-ubiquitin before the ITC experiments. Data were analysed using the Origin software (Microcal). NF-κB luciferase assay. Human embryonic kidney (HEK) 293T cells (ATCC) were co-transfected with NF-κB-luc reporter plasmid that contains an NF-κB response element upstream of the promoter driving the luciferase reporter gene, pGL4.74/hBluc/Tk (Promega) and epoGene tagged Flag-HOIP or myc-HOIL–HL plasmid in 6-well plates in triplicates using Lipofectamine 2000 transfection reagent. Since this assay could be carried out in a variety of cellular contexts, HEK293T cells were used because they are easy to transfet and suitable for the assay. The cells tested negative for mycoplasma contamination. Empty pcDNA3.1(+) vector was used as control. After 36h, cells were lysed and 20 μL cell lysates were used to measure firefly luciferase and Renilla luciferase (transfection control) signals using the dual luciferase reporter assay system according to the manufacturer’s protocol (Promega). Data were analysed in GraphPad Prism and one-way ANOVA followed by Tukey’s post hoc tests were used for statistical analysis. Immunoblotting was performed with anti-Flag (clone M2, Sigma-Aldrich) and anti-myc (clone 9E10, Sigma-Aldrich) antibodies, to confirm equivalent wild-type and mutant protein expression levels.

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Extended Data Figure 1 | HOIP domain organization and nomenclature. a, Domain organization of HOIP as commonly outlined in the literature. HOIP consists of a PNGase/ubiquitin-associated (PUB) domain followed by a B-box zinc-finger (B-box) domain, NPL4 zinc-fingers (NZF), the auto-inhibitory UBA domain and the RING-between-RING module (RBR, grey background). The HOIP RBR module contains the typical RING1, in-between RING (IBR) and RING2 domains, and a HOIP-specific additional linear ubiquitin chain determining domain (LDD). A yellow circle indicates the RBR catalytic cysteine (C885) forming the HECT-like thioester intermediate with ubiquitin. The binding sites of the other LUBAC constituents HOIL-1L and SHARPIN are also indicated. b, The RBR RING2 domain has the topology of an IBR domain. The individual HOIP RING1, IBR and RING2 domains from the HOIP RBR/E2~Ub/Ub structure are shown to enable direct comparison of their folds. This illustrates that the zinc-finger domain designated RING2 in fact adopts the topology of an IBR, as multiple groups have reported for various RBR E3 ligases previously. The terms RBR and RING2 however are used in this study for consistency with the widely accepted nomenclature. c, The HOIP RING2–LDD region. HOIP features an extension of its catalytic RING2 domain termed LDD, which adds two zinc-fingers and a helical arrangement to the RING2. The LDD is usually denoted as a domain following RING2. However, Rittinger and colleagues showed that the LDD is intertwined with the HOIP RING2 to form a single extended domain that contains a central canonical RBR RING2 with the additional features of the LDD ensuring the linear ubiquitin chain formation characteristic of HOIP. This domain will thus be designated RING2L (for RING2–LDD). The RING2L from the current HOIP structure is displayed with RING2 in light green and LDD in dark green. d, The structural arrangement of active HOIP RBR in the HOIP/E2~ubiquitin complex is markedly different from that of auto-inhibited RBRs. Left, active HOIP RBR from the HOIP/E2~ubiquitin complex. The RING1–IBR region and the RING2L are coloured magenta and green respectively. The individual RBR domains are also highlighted: RING1, yellow circle; IBR, orange circle; RING2, red circle. The RING1 extension helices (hE1, hE2) and IBR–RING2 linker helices (hL1 and hL2) are labelled. Middle and right, analogous representations of auto-inhibited Parkin and HHARI (PDB: 4I1H (ref. 8) and PDB: 4KBL (ref. 11)). Additional domains and regions besides the RBR of Parkin and HHARI are coloured grey.
Extended Data Figure 2 | Quality of crystallographic data and electron density maps. a, Final 2Fo-Fc (left) and simulated annealing (SA) composite omit (right) electron density maps of select interfaces of the HOIP/UbcH5B--Ub/Ub complex contoured at 1σ. Proteins are shown in sticks and coloured according to Fig. 1. b, Data collection and refinement statistics.

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Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Complexity of the crystallographic asymmetric unit and structure of the HOIP/UbcH5B~ubiquitin/ubiquitin E2–E3 transfer complex. a, The asymmetric unit contains two transfer complexes. Left, colour schematic of the proteins present in the asymmetric unit. Middle, structure of the asymmetric unit. The asymmetric unit contains two HOIP RBR/UbcH5B~ubiquitin complex arrangements (complex 1, 2). The two UbcH5B~ubiquitin conjugates are coloured orange and cyan, respectively, and are bound to two HOIP RBR molecules (magenta and green), which cross over between the complexes. Additional allosteric ubiquitin (Ub_{allo} blue) and UbcH5B~ubiquitin (Ub_{H5B}~Ub_{allo}, yellow and blue, respectively) molecules are bound to the HOIP RBRs in complex 1 and complex 2, respectively. Since Ub_{allo} makes all contacts with the RBRs and the UbcH5B of the UbcH5B~Ub_{allo} conjugate solely mediates crystal contacts (bottom left), only Ub_{allo} of complex 1 is displayed and discussed in the text and figures in terms of the additional ubiquitin binding. The black oval indicates an additional HOIP hL1/Ub act inter-complex interaction discussed in panels e and f.

Right, close-up of the region where the two RBRs of HOIP cross over between the complexes. The close-up shows that residues D852 and P853 of the respective RBRs come in 6 Å proximity suggesting a continuity in the biological complex in which the two residues from the respective RBRs are linked (as indicated by the grey background), resulting in the monomeric complex schematically illustrated underneath and discussed in panels c–f. b, The RING1–IBR and RING2L form two distinct entities to bind E2~ubiquitin. The monomeric complex as displayed for complex 1 assumes a flexible linkage between the autonomous units of the RING1–IBR arm and the RING2L from the two different RBR molecules in the asymmetric unit. This linkage is formed by residues D852 and P853 connecting IBR and RING2L (schematically illustrated in the cartoon). To test the structural integrity of the assumed link and the autonomy of the RING1–IBR arm on one side and the RING2L on the other side, we introduced a spacer comprising five alanine residues between D852 and P853 in HOIP RBR (see cartoon) and measured the activity of the RBR D852-Ala5-P853 insertion mutant in polyubiquitination assays. The assays show that the mutant (right) retains an activity similar to the wild-type RBR (left) indicating that indeed RING1–IBR and RING2L act as autonomous units. The dramatically reduced activity of HOIP RING2L alone (residues P853 to end) is also shown for reference (middle). c, The HOIP RBR/UbcH5B~ubiquitin complex is monomeric at concentrations of 1.25–5 μM. To determine if the HOIP RBR/UbcH5B~ubiquitin complex is indeed monomeric in solution, we analysed the isolated HOIP RBR/UbcH5B~ubiquitin complex protein material that was used for crystallization by sedimentation equilibrium analytical ultracentrifugation (SE-AUC). SE-AUC provides an absolute, shape-independent measurement of molecular weight, thus allowing accurate determination of the oligomeric state. The three SE-AUC experiments performed on the HOIP RBR/UbcH5B~ubiquitin complex yielded an absolute molecular weight (MW) of 71,658 Da, indicating a monomeric complex. At an order of magnitude higher concentrations (12.5–50 μM), SE-AUC results indicate the formation of a dimer with a MW of ~144 kDa, although curve fitting residuals also show substantial presence of aggregates (data not shown). These results indicate that the biological complex in solution is monomeric at physiological low μM concentrations such as those used for the thioester transfer assays and polyubiquitination assays. However, the dimeric arrangement observed in the crystal structure might be relevant in a high concentration setting such as within the LUBAC complex. Here, a high local concentration of HOIP RBR could favour binding of the E2~ubiquitin between the RING1–IBR and RING2L of two neighbouring molecules. Importantly, all mechanisms depicted in this article hold true for both the monomeric and dimeric states (as illustrated in d). This means that the deduced mechanism is in principle applicable to different RBR E3 ligases of which some might function as dimers in local high concentration assemblies (such as within the LUBAC), whereas others might be active in a monomeric setting. d, Schematic illustration of the dimeric arrangement as observed in the asymmetric unit. The schematic shows that all features deduced (Figs 1–4 and Extended Data Figs 4–10) are also valid for the dimeric case (binding of Ub_{allo} is omitted for clarity). e, Asymmetric unit dimer-related interactions between HOIP hL1 and UbcH5B. The dimeric arrangement contains no additional protein–protein interfaces compared to the monomeric assemblies with the exception of hL1 residues W847, M850 and N851, which in the asymmetric unit contact the activated ubiquitin of the other complex (indicated by an oval in a). f, Mutational analysis of HOIP hL1/Ub act interactions. Mutations of HOIP hL1 residues that interact with Ub act have no effect on thioester transfer activity (Goomassie-stained bands in red), indicating that this ‘trans’ complex interaction is not critical for the RBR mechanism, in line with the model of a monomeric arrangement.
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | The HOIP RING1–IBR applies an altered binding mode compared to classic RING E3s necessitating a HECT-like mechanism. a, The HOIP RBR RING1 uses an E2 interaction pattern similar to classic RINGs, but which results in a shifted binding. Shown are the details of the RING/E2 interaction in the HOIP RING/RING1/Ubch5B–ubiquitin complex (left), the RNF4 RINGclassic/UbcH5A–ubiquitin complex (middle; PDB: 4AP4 (ref. 21)), and the BIRC7 RINGclassic/UbcH5B–ubiquitin complex (right; PDB: 4AUQ (ref. 24)). The HOIP RBR-type RING1 uses a pattern of hydrophobic residues as the core of the interaction with E2 that is similar to that in classic RING E3 ligases. Subtle differences however support a shifted binding mode (see also Fig. 2b). The main features of the RING and E2 as well as HOIP residues mutated in Fig. 2e–g are displayed in bold. Zinc-finger (ZF) 1 and 2 of the RING domains and the SPA-loop of the E2 containing a conserved Ser-Pro-Ala motif are annotated. For the following panels the same structures and colour codes as in a are used. b, The shift in binding and altered surface residues in HOIP RING1 do not support the composite RING/E2 binding site for activated ubiquitin used by classic RING/E2 complexes. UbcH5A/B E2s are rendered as surface representation and the activated ubiquitin in surface representation. The bipartite binding mode used by the HOIP RING1–hE2–IBR arm (see also Fig. 2a) results in an elongated E2–ubiquitin formation (left, only the RING1 domain of HOIP is depicted) while formation of a composite RING/E2 binding surface in the case of classic RING E3 ligases (middle, right) results in binding of the activated ubiquitin in a compact manner with a bent E2–ubiquitin conformation. Importantly, this bent conformation places the thioester link in a specific position relative to the catalytic machinery of the E2, allowing direct attack by the lysine/amine function of a substrate or growing ubiquitin chain. The Lys85/Ser85 residues mediating the E2–ubiquitin linkage and mimicking UbcH5A/B catalytic cysteine C85 are displayed as red spheres. In the elongated E2–ubiquitin conformation propagated by the HOIP RBR, this attack is not possible. The linkage is however ideally positioned for the attack by the RBR catalytic cysteine in a HECT-like mechanism (see also Fig. 3 and Extended Data Fig. 7a). f, Close-up of the catalytic centres in E2–ubiquitin linkages. Details of the catalytic centres resulting from the E2–ubiquitin conjugate conformations outlined in e and, for comparison, the HECT-type E3 NEDD4L/UbcH5B–Ub structure (PDB: 3JW0 (ref. 28)), with the directionality of an attacking amine indicated as previously proposed21. In the HECT-like RBR arrangement, the UbcH5B–ubiquitin linkage is not aligned correctly relative to the E2 catalytic machinery for a direct attack by an amine function. This is similar in the HECT-type arrangement in the NEDD4L complex but completely different from the arrangement in classic RING-supported E2 catalysis. Additionally, the ubiquitin C-terminal residues G75–G76 reside in a position that would overlap with the attacking amine. The available structure of the BIRC7/UbcH5B–Ub complex (PDB: 4AUQ (ref. 24)) features an UbcH5B N77A mutant and the remainder of the Asn side-chain has been manually added based on wild-type UbcH5B from PDB: 2ESK (ref. 45) (right).
Extended Data Figure 5 | Helix–IBR-fold motifs constitute new ubiquitin-binding regions (UBR) in active RBR proteins: binding of the activated ubiquitin by UBR1 using binding mode 1. a, Schematic illustrating binding mode 1 used by hE2–IBR to bind the activated ubiquitin in the RING1–IBR arm. The general principle of this binding mode is that the RING1 extension helix 2 (hE2) preceding the IBR presents a pattern of charged/polar residues (indicated by blue and red squares, which symbolize K, R, H and E, Q, N residues respectively) that interact with ubiquitin E34 and K11. These interactions are supported by the IBR surface, with a particular contribution of hydrophobic residues (yellow square) flanking the salt bridge system. b, Coordination of the activated ubiquitin by HOIP hE2–IBR in mode 1. HOIP hE2 residues K783 and E787 bind ubiquitin residues E34 and K11 and are flanked by hydrophobic residues M791 and W798 from HOIP IBR. c, Structurally equivalent residues in Parkin and HHARI. Displayed are the hE2–IBR modules from auto-inhibited Parkin (PDB: 5C1Z (ref. 12)) and HHARI (PDB: 4KBL (ref. 11)) with residues equivalent to HOIP residues in b depicted, illustrating the general conservation of UBR1. It should be noted that these structures feature auto-inhibited forms of the RBR proteins, which exhibit a kink in hE2 of UBR1. This kink would sterically hinder ubiquitin binding to UBR1 and probably participates in the RBR auto-inhibition mechanism (see also Extended Data Fig. 8). d, Thioester-transfer assays of ubiquitin and UBR1 salt bridge mutants. In agreement with the observed four-residue salt bridge system in b, the single K11A or E34A ubiquitin mutations show only a slight to moderate effect since the remaining charged residue can still coordinate the two oppositely charged residues of HOIP. In contrast, elimination of both similarly charged residues in the complex by combining the HOIP K783A and ubiquitin K11A or HOIP E787A and ubiquitin E34A mutations results in a more dramatic loss of activity (mean activity ± s.e.m. (n = 3), one-way ANOVA followed by Tukey’s post hoc test; **P < 0.01; ***P < 0.001; NS, not significant; representative gels shown in Supplementary Fig. 1). e, f, Role of the IBR in UBR1. Close-up of the additional IBR/Ub act interactions in stick representation (e) shows that HOIP S803 and ubiquitin K6 coordinate the backbone carbonyl functions of ubiquitin T12 and HOIP A800/K829, respectively. W798, which is involved in hydrophobic interactions, is also displayed in sphere representation. Quantitative thioester transfer assays (f) show that alanine mutants of residues outlined in e cause a significant loss of activity (mean activity ± s.e.m. (n = 3), left: one-way ANOVA followed by Tukey’s post hoc test, right: two-tailed unpaired Student’s t-test; **P < 0.01; ***P < 0.001; representative gels shown in Supplementary Fig. 1).
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | Binding of the activated ubiquitin by UBR2 using binding mode 2 and exclusive binding of E2 and acceptor ubiquitin. a, Schematic illustrating binding mode 2 used by a helix–IBR-fold motif (hL2–RING2) to bind the activated ubiquitin and position the thioester linkage for the transfer reaction. The second helix (hL2) of the linker between the IDR domain and the catalytic RING2 domain uses a pattern of two or three hydrophobic residues (yellow squares) to interact with the ubiquitin canonical hydrophobic patch surrounding I44 (ref. 28; not shown). Hydrophobic residues of the RING2 IDR-fold complete the hydrophobic interaction network by coordinating residues L71 and L73 in the second hydrophobic patch of ubiquitin (not shown). The central hydrophobic interaction network by coordinating residues L71 and L73 not shown). Hydrophobic residues of the RING2 IRB-fold complete the hydrophobic interaction network by coordinating residues L71 and L73 in the second hydrophobic patch of ubiquitin (not shown). The central hallmark of this binding mode is the coordination of the characteristic di-Arg (R72, R74) motif in the ubiquitin C terminus, resulting in a firm placement of the C terminus on ZF1 of RING2. b, Structure of the interaction of the helix–IBR-fold in HOIP hL2–RING2 (UBR2) with the activated ubiquitin. Left, hL2 residues L860, Y863 and L864 (yellow spheres) interact with ubiquitin residues L8, I44 and V70 (not shown). Additionally, hydrophobic residues F876 and Y878 (yellow spheres) from the IDR-fold of the minimal catalytic RING2 (light green, see also Extended Data Fig. 1c) coordinate ubiquitin residues L8, L71 and L73 (not shown). Right, display of the full HOIP RING2 including the LDD insertion (RING2L). The coordination of the ubiquitin di-Arg motif is achieved by HOIP residues D983 and E976 from the LDD insertion that is part of the catalytic HOIP RING2L (dark green). This results in the placement of the E2–Ub-thioester linkage (K85 replacing UbcH5B C85 is shown as orange sticks) in the vicinity of the catalytic HOIP C885. Bottom, alternatively oriented views of the interaction. c, The two hydrophobic patches of ubiquitin engaged by UBR2. The hydrophobic residues of HOIP RING2 interacting with ubiquitin as highlighted in b are shown as yellow sticks. The interaction residues on the canonical hydrophobic patch of ubiquitin (L8, I44, V70) and the second hydrophobic patch (L71, L73) are displayed as grey sticks. d, The RING2 domains of Parkin and HHARI also contain a helix–IBR-fold (hL2–RING2) module with patterns of residues consistent with the formation of a UBR2. Left, helical predictions for the region preceding the RING2 domains of HOIP, Parkin and HHARI. The structures of Parkin and HHARI in their auto-inhibited forms do not display a helix equivalent to hL2 because this region is either not defined (in the crystal structures of HHARI and most Parkin structures) or adopts an extended conformation (in two other Parkin structures)4–11. However, a helical prediction reliability score (with 1 lowest to 9 highest score) calculated using JPred436 shows a strong helical probability for the segment of Parkin and HHARI preceding the RING2 domain. In fact, the score is similar to that of HOIP; which is displayed with the observed helical secondary structure, pointing to the presence of an equivalent of hL2 in active forms of Parkin and HHARI.

These RBR E3 ligases also contain residues capable of interacting with the hydrophobic patch in ubiquitin in positions equivalent to HOIP Y863 and L864 (highlighted in yellow). Right, structures of the RING2 domains of PARKIN (PDB: 4I1H (ref. 8)) and HHARI (PDB: 4KBL (ref. 11)) showing hydrophobic residues (yellow sphere representation) in structurally equivalent positions to HOIP F876 and Y878 and residues (labelled red) capable of interacting with the di-Arg motif in their catalytic RING2. Helix hL2 with the conserved hydrophobic residues not present in the crystal structures as discussed above is indicated schematically. Bottom, different orientations with the putative placement of the ubiquitin C terminus indicated schematically. e, The effect of UBR2 alanine mutations in thioester transfer assays increases with their proximity to the di-Arg motif. Left, mutation of HOIP UBR2 hydrophobic residues to alanine. The hL2 L860A and L864A mutations show little effect on activity, while the Y863A mutation and particularly the RING2L F876 and Y878 mutations, which reside proximal to the di-Arg binding motif formed by D983 and E976 (see also Extended Data Fig. 7) show a marked reduction in activity. Middle, right, mutation of complementary ubiquitin residues involved in UBR2 binding. Similarly to the HOIP mutations, the ubiquitin I44A, L71A and L73A mutations show increasing effects with a closer location to the di-Arg motif. Furthermore, the ubiquitin R74A mutation shows a strong effect on activity, emphasizing the importance of its interaction with HOIP and of the resulting placement of the ubiquitin C terminus linked to the E2. The ubiquitin R72A mutant failed to form an UbcH5B–ubiquitin conjugate, thus preventing analysis. Coomassie-stained bands are in red. f, Overlay of the UbcH5B binding site on HOIP RING2L with the binding site of the acceptor ubiquitin. Left, UbcH5B–ubiquitin acceptor (orange/cyan) interaction with RING2L (green) from the HOIP RBR/E2–ubiquitin complex. Right, RING2L interaction with two ubiquitin molecules arranged in linear fashion, mimicking the HOIP RING2L–ubiquitin donor to ubiquitin acceptor (Ub donor, Ub acceptor) transfer complex (PDB: 4LJP (ref. 14)). Despite the fact that the placement of the donor ubiquitin in the 4LJP structure results from a crystal contact, this ubiquitin exhibits a position identical to that of the activated ubiquitin bound to UBR2 in the HOIP RBR/UbcH5B–Ub complex. It should be noted that the UBR2 interaction with hL2 is missing because the RING2L from the crystal neighbour presenting the donor ubiquitin pushes hL2 into a different conformation. Importantly, in the HOIP RBR/UbcH5B–ubiquitin complex (left) the E2 binds RING2L in a region that overlaps with the binding site for the acceptor ubiquitin (Ub donor, dark blue) in the RING2L–ubiquitin donor to ubiquitin acceptor transfer complex (right). This highlights how the E2–ubiquitin conjugate and the acceptor ubiquitin (which is the substrate of the E3 reaction) cannot bind the RBR at the same time, thus making a HECT-like transfer a requirement in the E3 ligase mechanism of RBR proteins.
Extended Data Figure 7 | Catalytic centre of the E2~ubiquitin/HOIP RBR E3 transfer complex. a, Close-up view of the catalytic centre of the transfer complex shows conservation of the contact conduits. Top left, close-up view of the catalytic centre in the HOIP/UbcH5B~ubiquitin transfer complex. Contact conduits 1 and 2 are highlighted with grey backgrounds. HOIP catalytic cysteine C885 is depicted in sphere representation. K85 replacing the catalytic cysteine (C85) in UbcH5B and ubiquitin G76 are displayed in stick representation, featuring the UbcH5B~ubiquitin linkage. Top right, model of the conduits in a Parkin/UbcH5B~ubiquitin complex. The structure of Parkin RING2 (from auto-inhibited Parkin, PDB: 4I1H (ref. 8)) was overlaid on that of HOIP RING2 indicating equivalent contact conduits. Bottom left, analogous model for HHARI using RING2 from auto-inhibited HHARI (PDB: 4KBL (ref. 11)). Bottom right, model of the conduits in a HOIP/UbcH7~ubiquitin complex. The model was generated from PDB entry 4Q5E (ref. 55) with UbcH7 (with the free catalytic cysteine C86 displayed) overlaid on Ubch5B of the HOIP/UbcH5B~ubiquitin transfer complex. The structure of the HOIP/UbcH5B~ubiquitin transfer complex and the other models depicted indicate a conservation of the contact conduits. Mechanistically, the conduits allow for the RBR catalytic cysteine and the E2 catalytic cysteine~ubiquitin linkage to be in close proximity, which serves as main driving force of the transesterification reaction. A reaction driven mainly by proximity is also in agreement with the chemical nature of the catalytic cysteine, which has a pKₐ of ~8 for the free amino acid. This allows the cysteine to naturally deprotonate, without an absolute need for HOIP H887 (refs 10, 14), before attack of the ubiquitin G76 carbonyl function. In addition, the thioester linkage is far more labile than for example an amide bond, thus further facilitating a proximity-mediated reaction⁶⁶. However, in light of the geometric arrangement observed, additional subtle catalytic contributions of H887 in supporting the transition state of the reaction and/or re-protonation of the E2 catalytic cysteine are in principle possible. This prospect is particularly intriguing because UbcH7 exhibits a potential break in conduit 2 (between H887 and H119), yet provides its own histidine (H119) to the catalytic centre. b, Thioester transfer assays for HOIP contact conduit mutants. Left, thioester transfer assays show that the D983A and E976A mutations strongly affect activity. This is consistent with D983 forming the di-Arg binding motif in UBR2 (see Extended Data Fig. 6) and E976 bridging the three proteins in the complex. In contrast, the H887A mutation does not have a marked effect, in agreement with published results¹⁰,¹⁴. The Q974A mutation also does not have a strong effect, pointing to a weak auxiliary function of this residue in support of the critical D983 (Coomassie-stained bands in red). Right, the UbcH5B R90A mutation shows a moderate yet significant effect, in line with the structure, which suggests a more pronounced effect for HOIP E976A than for UbcH5B R90A. Surprisingly, mutation of the catalytic D117 in UbcH5B, which is essential for classic RING-supported catalysis, shows a positive effect on the HECT-like thioester transfer, further emphasizing a separate mechanism for RBR HECT-like E2/E3 mechanisms (as outlined in Fig. 2 and Extended Data Fig. 4). The gain of function of the D117A mutation also points to a trade-off for this E2 residue to participate in the classic RING-supported versus RBR HECT-like E2/E3 mechanisms (mean activity ± s.e.m. (n = 3), two-tailed unpaired Student’s t-test; **P < 0.01; representative gels shown in Supplementary Fig. 1). c, Polyubiquitination assays for HOIP contact conduit alanine mutants. These assays show similar activity profiles as the thioester transfer assays except for the H887A mutation, which is essential for amide bond formation in the second transfer reaction¹⁰,¹⁴.
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | The h$_{22}$–IBR module contains an additional UBR (UBR3) that binds an allosteric ubiquitin using binding mode 2. a, UBR3 binds the allosteric ubiquitin (Ub$_{allo}$) using binding mode 2. Cartoon depicting the overall features of binding mode 2 in UBR3. The binding of Ub$_{allo}$ is largely analogous to the binding of the activated ubiquitin by UBR2 in the helix–IBR-fold of h$_{22}$–RING2L (Extended Data Fig. 6a). Yellow squares indicate hydrophobic patches. b, Location of the UBR3 Ub$_{allo}$ interface in the overall complex and its relation to the UBR1/Ub$_{act}$ interface. The additional ubiquitin (Ub$_{allo}$) binds to UBR3 in the h$_{22}$–IBR module immediately across UBR1 and the activated ubiquitin. The electron density map (feature-enhanced map) bound to Ph-Parkin (grey blue) interacts with the ubiquitin canonical hydrophobic patch (L8, I44 and V70/not shown) and a second hydrophobic patch (L71 and L73/not shown), with the critical HOIPUb$_{allo}$ interaction. The di-Arg binding motif is also depicted, with E809 coordinating ubiquitin R72 and R74 and aligning the ubiquitin C terminus in a parallel manner with sheet β3 of the IBR. A red arrow indicates that UBR3 sterically allows the binding of di-ubiquitin/polyubiquitin chains on the C-terminal side of the bound ubiquitin (see also Extended Data Fig. 9). Ubiquitin Ser65 is indicated for comparison with the puUb/Ph-Parkin structure (bottom). Top middle, close-up on the di-Arg binding motif. Top right, close-up on the additional contacts between h$_{22}$ and Ub$_{allo}$. HOIP R770 interacts with D766, which makes contacts to Y778, and the backbone carbonyl functions of ubiquitin K63 and E64. Bottom, the recent structure of phospho-ubiquitin bound to Ph-Parkin (PDB: 5CAW (ref. 13)) reveals a similar mechanism. Left, close-up with the residues corresponding to those in HOIP depicted. Middle, close-up of the di-Arg motif. Ph-Parkin D346 coordinates R72 similar to HOIP UBR3/Ub$_{allo}$. The chemical tether introduced in the puUb/Ph-Parkin structure between the phospho-ubiquitin C terminus and a non-conserved Cys in Ph-Parkin (C349) shifts ubiquitin R74 away from Ph-Parkin D346 indicating that the di-Arg binding motifs of HOIP and Parkin undergo a similar interaction with ubiquitin or phospho-ubiquitin respectively. Right, the Ph-Parkin interaction equivalent to the HOIP h$_{22}$ interaction involves a ubiquitin phospho-serine 65 (pSer65) binding pocket in Ph-Parkin. Phospho-serine 65 is directly coordinated by R307 and Y314 and also H304, which is positioned similarly to HOIP D766. d, The binding of phospho-ubiquitin propagates the formation of UBR1 in Parkin. Overlay of Ub$_{allo}$ ( slate) bound to HOIP (magenta) and phospho-ubiquitin (light blue) bound to Ph-Parkin (grey blue). Ubiquitin binding propagates a straight conformation of h$_{22}$ and an opening of the IBR relative to the extended RING1 (only h$_{23}$ is shown). The tether introduced in the phospho-ubiquitin/Ph-Parkin interaction appears to exert a strain on IBR loop 2 and thus the IBR. This suggests that in the absence of the artificial tether phospho-ubiquitin can propagate the formation of a fully functional UBR1 (binding Ub$_{allo}$) in Parkin concomitantly to relieving the UBL autoinhibition, as elegantly demonstrated by Komander and colleagues. e, The interaction of phospho-ubiquitin with a site analogous to UBR3 in Ph-Parkin causes a straight conformation of h$_{22}$ and reorientation of the IBR relative to RING1 as prerequisite to accommodate the activated ubiquitin. Left, full-length auto-inhibited human Parkin (PDB: 5C1Z (ref. 12)). Right, tethered phospho-ubiquitin in complex with ΔUBL-Ph-Parkin (PDB: 5CAW (ref. 13)). f, Conservation of UBR3 in HHARI. HHARI features a helix–IBR module similar to that of HOIP, with conserved hydrophobic patches and a polar residue (Q288) in the position of HOIP E809 that is in principle capable of binding the ubiquitin di-Arg motif. The h$_{22}$–IBR region from auto-inhibited HHARI (PDB: 4KBL (ref. 11)) is displayed. As for auto-inhibited Parkin, a key difference from active HOIP is the kink in helix h$_{22}$ of auto-inhibited HHARI (see also panel g and Extended Data Fig. 5c). Taken together, these features indicate an overall similarity and the existence of a UBR3 that is allosterically linked to UBR1 in HOIP. Parkin and HHARI and potentially also other RBR proteins (see alignment in Supplementary Data 2). g, HHARI UBA domain (pink) binds HHARI RBR (light orange) in an equivalent position as Ub$_{allo}$ binding to HOIP, but promotes an inhibitory conformation of RING1–IBR that cannot bind the activated ubiquitin (PDB: 4KBL (ref. 11)). h, UBR3 is a regulatory hotspot and UBR3/UBR1 crosstalk. Parkin auto-inhibition is facilitated by an UBL domain and is inherently linked to the equivalent of UBR3 and counteraction by phospho-ubiquitin binding. Different to Parkin, no structure of auto-inhibited HOIP RBR is available and thus the conformation of auto-inhibited HOIP is not known. However HHARI is, like HOIP, auto-inhibited by its UBA domain and the auto-inhibited structure of HHARI has been solved previously (PDB: 4KBL (ref. 11)). This structure reveals that unlike the UBL of Parkin, the UBA of HHARI directly utilizes the region of UBR3 for binding and auto-inhibition, which includes the kink in h$_{22}$ and a relative RING1–IBR positioning that is incompatible with binding of Ub$_{act}$. Therefore, in this study, further underlying a regulatory ‘hotspot’ function of the RBR UBR3/UBR1. Left, the UBA domain (pink) of HHARI utilizes an anti-parallel 3-sheet anchor with strand 32 of the HHARI IBR (light orange) positioning the UBA to induce a kink in helix h$_{22}$ compared to its conformation in active HOIP (magenta) and counteracting the formation of a productive UBR3 and UBR1. Right, binding of the Ub$_{allo}$ to UBR3 in mode 2 utilizes a parallel 3-sheet anchor (centred around the di-Arg binding interaction) with strand 32 of the IBR in active HOIP (magenta) inducing a straight conformation of h$_{22}$ and a conformation of UBR1 suited to bind the activated ubiquitin of the E2–Ub$_{act}$ conjugate. Putative shifts for an analogous UBR1 formed in HHARI are indicated. Of note, the structure of auto-inhibited HOIP is not known and therefore the placement of the UBA in the schematic illustration of Extended Data Fig. 10 is deduced based on the similar auto-inhibition of HOIP and HHARI by their UBA domains, which still needs to be demonstrated.
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | UBR3 interacts with di-ubiquitin and allosterically promotes E2–ubiquitin binding and HOIP RBR activation. a, ITC experiments analysing the binding of mono-ubiquitin or linear di-ubiquitin to an isolated HOIP RBR/E2–ubiquitin complex. While the binding of mono-ubiquitin is below the sensitivity of the experimental setting ($K_d > 50 \mu\text{M}$), the binding of linear di-ubiquitin exhibits a $K_d$ of 7.1 $\mu\text{M}$. b, ITC experiments analysing the binding of UbcH5B–ubiquitin to HOIP RBR in the absence and presence of different di-ubiquitin chains. Top left, the binding stoichiometry of UbcH5B–ubiquitin and wild-type HOIP RBR is $n = 1.8$, indicating that two UbcH5B–ubiquitin molecules interact with one RBR through UBR1/2 (catalytic binding) and UBR3 (binding of the ubiquitin moiety of the E2–ubiquitin conjugate) with a combined overall $K_d$ of 1.6$\mu\text{M}$. The graph shows a single titration step, indicating ‘crosstalk’ between UBR3 and UBR1. Top right, the presence of K48-linked di-ubiquitin leads to 1:1 binding ($n = 0.9$) of UbcH5B–ubiquitin to HOIP RBR, indicating that di-ubiquitin occupies UBR3 and limits UbcH5B–ubiquitin binding to only its bona fide catalytic binding site (with ubiquitin binding to UBR1/2 and UbcH5B binding to RING1/RING2L). However, the presence of K48-linked di-ubiquitin results in the lowest affinity ($K_d = 3.4 \mu\text{M}$) for the binding of the conjugate to the RBR, indicating a negative effect of this linkage compared to the other di-ubiquitin entities tested. Bottom left, K63-linked di-ubiquitin has a more favourable effect on UbcH5B–ubiquitin binding ($K_d = 2.0 \mu\text{M}$). Bottom right, the strongest allosteric effect is observed in the presence of linear di-ubiquitin, which enables sub-micromolar binding ($K_d = 600 \text{nM}$) of UbcH5B–ubiquitin. These results show that linear di-ubiquitin functions as a potent activator of HOIP RBR by binding to UBR3 (see also below and Fig. 4). While the structure depicts the interactions of one ubiquitin unit with UBR3, a second ubiquitin C-terminal to the UBR3-interacting ubiquitin may undergo further interactions with the IBR (as indicated by the arrow in Extended Data Fig. 8c). The cartoon representations summarize the configuration of each ITC experiment. c, Polyubiquitination assays of UBR3 mutants. The HOIP RBR I807A, E809A and R770A mutants exhibit a marked reduction in activity, supporting the importance of UBR3 in HOIP function. d, Activation of HOIP RBR by di-ubiquitin. While wild-type HOIP RBR is activated by the presence of increasing concentrations of wild-type linear di-ubiquitin, wild-type linear di-ubiquitin only has a weak effect on activation of the UBR3 R770A mutant (similar to the I807A and E809A mutants in Fig. 4b). Additionally the di-ubiquitin mutant I44A, which is mutated at a critical UBR3-interacting residue in both ubiquitin units, does not have an activating effect on wild-type HOIP RBR thioester activity (mean activity ± s.e.m. ($n = 3$), one-way ANOVA followed by Tukey’s post hoc test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant; representative gels shown in Supplementary Fig. 1). e, Effect of linear versus K48-linked di-ubiquitin on HOIP RBR thioester transfer activity. In contrast to the ITC binding studies in b, linear and K48-linked di-ubiquitin are both able to increase the thioester transfer activity of HOIP RBR (although the experimental setup necessary to investigate the K48-linkage resulted in larger error; see Methods; mean activity ± s.e.m. ($n = 3$), one-way ANOVA followed by Tukey’s post hoc test; *$P < 0.05$; **$P < 0.01$; NS, not significant; representative gels shown in Supplementary Fig. 1). These results show an UBR3-dependent activating effect of di-ubiquitin, and thus potentially of polyubiquitin chains. However, whether HOIP UBR3 acts as a universal ubiquitin sensor or has a preference for linear ubiquitin over other types of linkage needs to be further examined through careful investigations also including full-length proteins of the LUBAC in cellular settings. Additionally, although there is a substantial gap between UBR3 and the position of the acceptor ubiquitin, longer acceptor ubiquitin chains might be able to bridge this gap and mediate a cooperative effect between the two sites. This would be consistent with a recent publication showing that the presence of K63-linked ubiquitin chains is frequently necessary for the formation of linear polyubiquitin chains$^{38}$. f, Protein expression levels for the NF-κB reporter assays in cells shown in Fig. 4d. Shown are anti-Flag immunoblots of wild-type HOIP and mutants and anti-myc immunoblots of HOIL-1L, demonstrating similar protein expression levels in different cell lysates. Lysates were also probed by immunoblotting for actin as a loading control. Uncropped blots are shown in Supplementary Fig. 1. g, Time course of HOIP–ubiquitin thioester transfer assay. Left, SDS–PAGE showing time course of HOIP–ubiquitin thioester transfer assay. Coomassie-stained bands in red visualized using LI-COR Odyssey at 700 nm. Right, plot of quantified HOIP–ubiquitin thioester transfer assay time-course (mean ± s.e.m., $n = 2$). The 10-s time point used in the end-point assays throughout the study is highlighted in red.
Extended Data Figure 10 | Schematic of RBR mechanism: HOIP RBR activation and E3 ligase cycle. HOIP RBR is initially auto-inhibited by its UBA domain. Sequestration of the auto-inhibitory HOIP UBA domain by HOIL-1L releases the conformational restraint exerted by the UBA, allowing formation of UBR1 and UBR3. Binding of a ubiquitin entity such as a linear ubiquitin chain to UBR3 stabilizes the active conformation of UBR1 and the RING1–IBR arm, facilitating binding of the E2~ubiquitin conjugate. In the subsequent HOIP/E2~ubiquitin transfer complex, the E2~ubiquitin conjugate is engaged in a clamp-like manner bringing the RBR active cysteine and the E2~ubiquitin thioester in close proximity, ultimately leading to the transfer of the ubiquitin to the RBR cysteine. The E2 then vacates the complex, freeing the site for binding of the acceptor ubiquitin, the N-terminal amine of which attacks the RBR thioester. Once the ubiquitin chain linkage is formed, the ubiquitinated substrate/growing ubiquitin chain must exit RING2L to enable binding of a new E2~ubiquitin conjugate for the next loading of the RBR in the HECT-like E3 ligase cycle. The growing ubiquitin chain could be retained near the RBR by the HOIP NZF domains, HOIL-1L or SHARPIN, directly linking the HECT-like mechanism to co-operative processes within the LUBAC.