Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP

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Linear ubiquitin chains are important regulators of cellular signalling pathways that control innate immunity and inflammation through nuclear factor (NF)-κB activation and protection against tumour necrosis factor-α-induced apoptosis1–4. They are synthesized by HOIP, which belongs to the RBR (RING-between-RING) family of E3 ligases and is the catalytic component of LUBAC (linear ubiquitin chain assembly complex), a multisubunit E3 ligase5. RBR family members act as RING/HECT hybrids, employing RING1 to recognize ubiquitin-loaded E2 while a conserved cysteine in RING2 subsequently forms a thioester intermediate with the transferred or ‘donor’ ubiquitin. Here we report the crystal structure of the catalytic core of HOIP in its apo form and in complex with ubiquitin. The carboxy-terminal portion of HOIP adopts a novel fold that, together with a zinc-finger, forms a ubiquitin-binding platform that orients the acceptor ubiquitin and positions its α-amino group for nucleophilic attack on the E3–ubiquitin thioester. The C-terminal tail of a second ubiquitin molecule is located in close proximity to the catalytic cysteine, providing a unique snapshot of the ubiquitin transfer complex containing both donor and acceptor ubiquitin. These interactions are required for activation of the NF-κB pathway in vivo, and they explain the determinants of linear ubiquitin chain specificity by LUBAC.

Protein modification with ubiquitin is a key mechanism for the regulation of numerous cellular functions6. The transfer of ubiquitin onto a substrate is catalysed by E3 ligases, which can be classified into RING, HECT and RBR families7,8. Two LUBAC subunits contain RBR domains. However, HOIP (HOIL-1L interacting protein) constitutes the catalytic centre, and its RBR domain, containing C-terminal region (HOIPCBR-C) is sufficient to synthesize linear ubiquitin chains, regardless of E2 (Fig. 1a)9,10,15. Although the RING1 domain of RBRs is assumed to be the primary binding site for E2s, we show that a HOIP construct containing only the catalytic cysteine-carrying RING2 plus a C-terminal extension (HOIPCBR-C) still forms linear ubiquitin chains, roughly sevenfold more slowly than HOIPRRB-C (Fig. 1b and Extended Data Fig. 1)10–12. HOIPCBR-C therefore constitutes the minimal catalytic core. We have determined the crystal structure of this catalytic core in its apo form at 2.4 Å and in complex with ubiquitin at 1.6 Å resolution (Extended Data Table 1). HOIPCBR-C consists of seven α-helices and four zinc-binding modules, and its overall topology seems distinct from other structures (Fig. 1c–e and Extended Data Fig. 2). The helical region forms an elongated structural unit that acts as a platform, the ‘helical base’, to support the zinc-binding modules. The RING2 region of RBRs has recently been shown to adopt the in-between (IBR) domain fold in auto-inhibited Parkin and HHARI (human homologue of ariadne)16–21. This fold is preserved in the structure of active HOIPCBR-C (Fig. 1c–e and Extended Data Fig. 3), and on the basis of this structural and high sequence conservation we suggest that it be renamed CBR (for ‘catalytic IBR’). The second zinc-binding site of the CBR of HOIPCBR-C has a zinc-finger (ZF1) inserted between the second and third Zn2+–coordinating residues. A fourth zinc-binding site is located between helices α3 and α4 (ZF2) and anchors the β-hairpin that is positioned close to the CBR.

In the HOIPCBR-C–ubiquitin complex, ubiquitin makes contacts with residues from the helical base and ZF1 (Fig. 2 and Extended Data Fig. 4). No major conformational changes occur on complex formation, although some disordered regions of apo HOIPCBR-C become ordered (Extended Data Fig. 2d). The ubiquitin bound by the helical base and ZF1 constitutes the acceptor ubiquitin: its α-amino group of M1 is located 3.5 Å away from the thioester-forming C885, poised for nucleophilic attack (Fig. 2). Strikingly, the C-terminal G76 of ubiquitin from a symmetry-related molecule is oriented such that its carboxylate points into the active site of HOIPCBR-C sufficiently close to C885 to promote thioester formation. Thus, the molecular arrangement within the crystal lattice mimics the biologically relevant ubiquitin transfer complex with the donor and acceptor ubiquitin in an orientation consistent with linear chain synthesis (Fig. 2 and Extended Data Fig. 4). We found additional electron density in the active site, which we interpret as a Zn2+ ion. However, mass spectrometry, combined with structural and biochemical analysis, shows that ubiquitin chain synthesis is not a zinc-dependent process (Extended Data Figs 2b and 5).

HOIPCBR-C employs residues from helices α2 and α6 in the helical base to contact T14, E16, D32 and K33 of the acceptor ubiquitin, whereas ZF1 rests against helix α1 and the preceding loop (Fig. 3a and Extended Data Fig. 6a), ensuring that the N-terminal amino group is positioned closest to the active site and thus specifying linear chain synthesis. The side chain of M1 points away from the active site, indicating that its selection is driven stereochemically. To analyse the role of individual residues in ligase activity we used a combination of steady-state and single-turnover assays (Fig. 3c, d and Extended Data Fig. 6). Contributions from the helical base are crucial for ubiquitin chain synthesis, especially R935 and D936, but mutation of these residues does not impede thioester formation (Extended Data Fig. 6c). In ubiquitin the most severe effect was seen when K33 was mutated, and E16A and D32A showed decreased activity. No point mutation could be identified at the ZF1/ubiquitin interface that decreased chain synthesis; however, deletion of ZF1 resulted in an almost complete loss of activity (Fig. 3c). This impairment was not caused by protein misfolding, because ubiquitin–thioester formation was unaffected (Extended Data Fig. 6c). Instead, ZF1 deletion abolished transfer of the donor to the acceptor ubiquitin. These data suggest that the helical base constitutes the primary binding site for the acceptor ubiquitin, which is further supported by ZF1.

HOIPCBR-C residues that contact the acceptor ubiquitin are located in a region termed the ‘linear ubiquitin chain determining region’ (LDD; residues 910–1082)22. Our structure now shows that this is not an independent ubiquitin-binding module, but together with the CBR it forms a superdomain that contacts donor and acceptor ubiquitin to create a platform that promotes linear chain synthesis.

Contacts between HOIPCBR-C and the donor ubiquitin primarily involve the C-terminal tail of ubiquitin, which is guided towards the...
active site through a channel created by the N-terminal antiparallel \( \beta \)-strands of the CBR and a \( \beta \)-hairpin formed by \( \beta F \) and \( \beta E \) (Figs 2 and 3b). These structural elements restrict tail mobility, ensuring that the carboxylate of G76 is located next to the catalytic cysteine. Contacts between Q974 and D983 in the \( \beta \)-hairpin and R72 and R74 from ubiquitin are crucial for donor ubiquitin binding (Fig. 3b–d and Extended Data Fig. 6). The \( \beta \)-hairpin is largely disordered in the apo structure, suggesting that together with the CBR it could act as a flexible clamp locking the donor ubiquitin into place. Ubiquitin is further sandwiched by the N-terminal \( \beta \)-sheets of the CBR that form a hydrophobic pocket accommodating L73 and contacting L71. Mutation of either residue results in a severe loss of activity (Fig. 3c). Most of the hydrophobic

Figure 1 | Structure of the catalytic core of HOIP. a, Composition of LUBAC. SHARPIN, SHANK-associated RH domain-interacting protein; HOIL-1L, longer isoform of haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1. Boxed: the crystallized catalytic core HOIP\(_{CBR} C\), biochemical assays employed HOIP\(_{RBR} C\). Below, diagram of new elements identified: CBR, ZF1, ZF2 and helical base. b, Single-turnover assays showing that lack of RING1 decreases activity 6.8-fold (HOIP\(_{CBR} C\), 0.050 min\(^{-1}\), compared with HOIP\(_{RBR} C\), 0.341 min\(^{-1}\)). c–e, Ribbon representations of HOIP\(_{CBR} C\) with the helical base in grey, CBR in purple, ZF1 in cyan, ZF2 and \( \beta \)-hairpin in green, \( \text{Zn}^{2+} \) ions as spheres, coordinating residues in ball-and-stick representation and the catalytic cysteine in yellow. The structure represents HOIP\(_{CBR} C\) from the ubiquitin complex and includes regions disordered in the apo form.

Figure 2 | The HOIP\(_{CBR} C\)–ubiquitin transfer complex containing donor and acceptor ubiquitin. a, Ribbon representation of HOIP\(_{CBR} C\) in complex with acceptor (orange) and donor (yellow) ubiquitin. HOIP\(_{CBR} C\) is shown in the same orientation as in Fig. 1e. The positions of C885, donor G76 and acceptor M1 are indicated. Inset: contacts made by HOIP\(_{CBR} C\) with donor and acceptor ubiquitin. The arrow shows the proximity between G76 of the donor and S\(_7\) of C885. b, The HOIP\(_{CBR} C\)–ubiquitin complex with HOIP\(_{CBR} C\) shown in a surface representation to emphasize the spatial relationship between the three molecules.
indicating that domains outside HOIPRB-R do not affect chain linkage trends seen in ubiquitination assays using different ubiquitin mutants, present in all three subunits, there was a strong correlation with the possibly as a result of regulatory roles of ubiquitin-binding domains.

Consonant with our structural and biochemical affect interaction with the donor (D983A) and acceptor (R935A, p65 nuclear translocation assays employing HOIP mutants shown to physiological context, we performed in vivo specificity (Extended Data Fig. 7). To validate our conclusions in a general property of RBRs (Extended Data Fig. 3).

This mode of donor ubiquitin presentation to the substrate may be a residues contacting L71 and L73 are conserved in CBRs, indicating that it might be able to activate the incoming -amino group or stabilize the transition state. To test its contribution to catalysis, we measured the activity of H887A, which was decreased more than 1,000-fold at 15°C. This is due to lack of transfer to the acceptor ubiquitin rather than impairment of thioester formation (Figs 3d and 4a). Thus, H887 is not required for transthiolation from acceptor ubiquitin contacts Helical base ZF1 Contacts with CBR β-hairpin

Ubiquitin chain synthesis involves the nucleophilic attack of an amino group from a lysine or the N terminus of ubiquitin onto a ubiquitin-thioester formed by E2 or the HECT-type E3s. The reaction requires a general base to deprotonate the nucleophile and a mechanism to stabilize the transition state. In the HOIPRB-R-ubiquitin complex, H887 of HOIP CBR-C forms a hydrogen bond with M1 of ubiquitin, indicating that activity could be restored at increased pH, which would aid deprotonation of the nucleophile. Indeed, ubiquitin transfer assays with H887A showed activity could be rescued at pH 9.0, although this required a higher temperature and higher substrate concentrations (Fig. 4b). Accordingly, enzyme activity was lost below pH 6.0, the approximate pKₐ of an imidazole side chain (Extended Data Fig. 8). Taken together, these

Ubiquitin WTWT

Figure 3 | Contacts between HOIPCBR-C and ubiquitin required for ubiquitin transfer. a, Close-up of the HOIPCBR-C/acceptor ubiquitin interface focusing on the helical base and ZF1. b, Details of the HOIPCBR-C/donor ubiquitin (yellow) interface. Positions of C885 and acceptor ubiquitin M1 are indicated. c, Steady-state ubiquitination assays. Mutants that target the acceptor interface are boxed in orange, those with donor in yellow. Ub, ubiquitin; WT, wild type.

d, Single-turnover assays to determine the rate of tetraubiquitin formation. e, Luciferase assays showing that the NF-kB pathway is not efficiently activated by HOIP mutants H887A, R935A, D936A and D983A in comparison with wild type. f, p65 translocations assay showing impaired p65 nuclear translocation on expression of HOIP ligase-deficient mutants. Three independent experiments were performed using triplicate samples. Results were analysed by ANOVA1 followed by Tukey post-tests. Error bars represent s.e.m. Two asterisks, P < 0.01; three asterisks, P < 0.001 compared with wild-type HOIP.

Ubiquitin WTWT

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observations support a model for ubiquitin transfer in which H887 acts as a general base to activate the nucleophile (Fig. 4c). This mechanism is maintained in vivo as indicated by the decrease in NF-xB activation and p65 translocation by the H887A mutant (Fig. 3e, f). A histidine residue in this position is conserved in a number of RBRs and has recently been shown to be important for activity in Parkin and HHARI\textsuperscript{16,17,20}, suggesting that the mechanism of nucleophile activation may be conserved.

The structure of the HOIP\textsubscript{CBR-C}-ubiquitin complex presented here provides the first insights into how an E3 ligase directs the synthesis of specific ubiquitin chains: a non-covalent ubiquitin-binding site orients the acceptor so that only the \( \alpha \)-amino group of M1 is presented to the active site, in a similar manner to the mechanism used by linkage-specific E2s\textsuperscript{24-26} (Extended Data Fig. 9). M1 is part of a \( \beta \)-sheet and is less flexible than the \( \varepsilon \)-amino group of lysine, perhaps explaining why HOIP has evolved a single structural unit that integrates the CBR domain with the donor and acceptor ubiquitin-binding regions. Comparison of CBR structures from active HOIP with auto-inhibited Parkin and HHARI suggests that the overall mechanism of donor ubiquitin presentation is conserved in the RBR family (Extended Data Fig. 3c)\textsuperscript{16-21}. Further studies are now required to reveal the mechanism that promotes the formation of the active ligase complex and explain how chain linkage specificity is achieved in other RBR ligases.

**METHODS SUMMARY**

Proteins were expressed in *Escherichia coli* and purified by standard procedures. Steady-state ubiquitination, thioester formation and transfer assays were performed as described\textsuperscript{41}. Diffraction data were collected at 100 K at Diamond Light Source, beamlines IO2 and IO4-1. The apo HOIP\textsubscript{CBR-C} structure was solved by SAD, and the HOIP\textsubscript{CBR-C}-ubiquitin complex was solved by molecular replacement.

**Online Content** Any additional Methods, 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.S., R.R.R. and M.G.K. purified proteins and performed structural and biochemical analysis. A.C.M.-D. and N.R.B. purified proteins and conducted biochemical analysis. V.S. performed in vivo studies. E.C. produced expression plasmids. S.H. performed MS analysis. I.D. coordinated experimental work and contributed ideas. K.R. contributed to structural analysis and wrote the paper. All authors contributed to data analysis, experimental design and paper writing.

Author Information

Coordinates and structure factors are deposited in the Protein Data Bank under accession codes 4LJQ (apo HOIPCBR-C structure), 4LJO (wild-type HOIPCBR-C–ubiquitin complex) and 4LJP (H889A HOIPCBR-C–ubiquitin complex). 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 K.R. (katrin.rittinger@nimr.mrc.ac.uk).
METHODS
Cloning, expression and protein purification. Cloning, expression and purification of Ube1, UbcH5A (UBE2D1), HOIP-RBR-C and mutants thereof, HOIP (residues 300–1072), HOIL-1L, SHARPIN and His6-M1C-ubiquitin have been described14. HOIP-RBR-C was expressed and purified using the same procedure as for HOIP-RBR-C. Point mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene). The ZF1 of HOIP (residues 906–923) was deleted in HOIP-RBR-C and replaced with the sequence PG using Overlap Extension-PCR7. Untagged ubiquitin and mutants were prepared according to ref. 28. Selenomethionine (SeMet)-substituted proteins were produced by standard procedures. Ubiquitin used for crystallization was purchased from Sigma and further purified by gel filtration. All plasmids were verified by DNA sequencing. Protein molecular mass was verified by electrospray ionization mass spectrometry. The fold of all proteins was analysed by circular dichroism spectroscopy.

Ubiquitination assays. Ubiquitination assays were performed using 1 μM E1, 5 μM UbcH5A, 5 μM HOIP (or 5 μM each: HOIP residues 300-1072, HOIL-1L and SHARPIN) and 200 μM ubiquitin14. Reactions were incubated at 30 °C for 1 h and samples taken at 0, 5, 15, 30 and 60 min (HOIP-RBR-C) or 0.5, 1, 2 and 4 h (HOIP-RBR-C). Reactions were stopped by the addition of SDS sample buffer containing 40 mM N-ethylmaleimide. For LUBAC assays an additional precipitation step using 50 mM sodium acetate pH 4.0 at 60 °C was introduced. Samples were analysed by SDS-PAGE and visualized with Coomassie Brilliant blue.

Thioester formation and ubiquitin transfer assays. Labelling of His6-Cys-ubiquitin with Cy5-Maleimide mono-Reactive Dye (GE Healthcare) and transfer assays were performed as described, with minor modifications14. Cy5-ubiquitin (1 μM) was mixed with 2 μM E1 and 1 mM ATP. After 5 min 10 μM UbcH5A was added and after further 5 min 20 μM HOIP-RBR-C. To monitor ubiquitin transfer, 10 μM Ub-His6 was added. Samples were taken before each addition and analysed by SDS-PAGE in the absence and presence of dithiothreitol.

Single-turnover fluorescence resonance energy transfer assays. Ubch5A was charged with Cy5-labelled linear diubiquitin and purified by gel filtration. E2–thioester (0.3 μM or (for pH-dependent assays) 3.0 μM) was mixed with 0.3 μM or 3.0 μM Cy3-labelled di-ubiquitin. After addition of 3.0 μM of HOIP-RBR-C or HOIP-RBR-C, tetra-ubiquitin chain synthesis was observed by fluorescence resonance energy transfer between Cy3 and Cy5 using excitation and emission wavelengths of 540 and 670 nm, respectively. Samples were incubated at 15 °C in 50 mM HEPES pH 7.4, 150 mM NaCl or at 25 °C for pH-dependent assays using phosphate (pH 6–8) and CHES (pH 8.5–10.0) buffers. Data were analysed by single exponential curve fitting. Data for constructs with very low activity were analysed using Genejuice. After 36 h of transfection, lysates were prepared and subjected to luciferase assays in accordance with the manufacturer’s protocol (Roche). The stereochemistry of the final models was analysed with Procheck. The model of apo HOIP-RBR-C has 94.8% of its residues in favoured regions, 4.2% in allowed regions and 1% outliers. The final models of wild-type HOIP-RBR-C–ubiquitin and HOIP-RBR-C H899A–ubiquitin have 97.6% and 95.5% of their residues in the favoured regions of the Ramachandran plot, respectively. Structural figures were prepared in PyMol.

Mass spectrometry. For zicz content analysis by native mass spectrometry, purified proteins were dialysed at 4 °C against 20 mM ammonium acetate pH 7.4. Molecular mass was determined by electrospay ionization (ESI) on a microTOFQ mass spectrometer (Bruker Daltonics, Coventry, UK). Protein was infused into the mass spectrometer at 3 μl min−1 using an electrospay voltage of 4.5 kV. Inductively coupled plasma mass spectrometry (ICP–MS) was used to determine the concentration of Ca and Zn (as 44Ca and 65Zn) in the protein samples using an Agilent 7700x instrument in helium (He) collision mode.

Analytical ultracentrifugation. Sedimentation velocity experiments were performed in a Beckman XL-A analytical ultracentrifuge. Samples were dialysed against the buffer blank, 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine pH 7.5. Centrifugation was performed at 50,000 r.p.m. (201,240g) and 293 K in an An50-Ti rotor at 125 μM sample concentration. Data were analysed in terms of the size distribution function C(S) using the program SEFIT24.

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Extended Data Figure 1 | Comparison between the catalytic activities of the crystallized HOIP_{CBR-C} construct and the HOIP_{RBR-C} construct. a, Steady-state ubiquitination assays comparing the catalytic activity of the RBR-C construct of HOIP (HOIP_{RBR-C}) with the CBR-C construct (HOIP_{CBR-C}), showing that activity is reduced in HOIP_{CBR-C} with only short ubiquitin chains formed after 1 h. For this reason, all steady-state assays were performed with HOIP_{RBR-C}. To confirm that HOIP_{CBR-C} had retained the ability to specifically synthesize linear chains, a ubiquitination assay was performed with ubiquitin containing an N-terminal His_{6} tag that was no longer able to produce linear chains (right-hand gel). All gels were stained with Coomassie blue and converted to black and white. b, To ensure that any effects seen in ubiquitination assays were not due to inefficient loading of ubiquitin mutants onto E1 or E2 enzymes, the mutants were tested for their ability to form a thioester quantitatively with E1 and UbcH5A within 5 min. Samples were analysed on SDS gels and loaded in sample buffer in the absence or presence of dithiothreitol to detect the thioester.
Extended Data Figure 2 | Topology of HOIP_{CBB-C} content of the asymmetric unit of the apo crystals, solution behaviour of this fragment and comparison with the ubiquitin bound structure. 

a, Topology diagram of HOIP_{CBB-C} maintaining the same colour scheme as in Fig. 1. b, The apo HOIP_{CBB-C} structure contains four molecules of HOIP_{CBB-C} in the asymmetric unit that overlap with root mean squared deviation values of 0.8–1.0 Å. They are related by a local two-fold axis, and two molecules form a disulphide bond (shown in cyan and blue) and coordinate a fifth Zn^{2+} ion. Boxed: details of disulphide formation and zinc coordination by H887 and H889 of each monomer. Some of the loop regions in the apo structure are disordered. These include: residues 866–868 in two monomers, 880–882 in all monomers, 960–964 in all monomers and 974–983 in the respective monomers. c, Sedimentation velocity run of the crystallized construct at a sample concentration of 125 μM and derived C(S) distribution (S_{20,w} = 2.38; S_{20,sm} = 2.56), indicating that it exists as a monomer in solution. d, Overlap of the apo (blue) and ubiquitin-bound (cyan) structures highlighting the regions that become ordered on complex formation (indicated by a dotted line) with ubiquitin (shown in grey). C885, H887 and H889 are shown as sticks and the Zn^{2+} ions as spheres.
Extended Data Figure 3 | Comparison of CBR domains from different RBR family members. a, Sequence alignment of different CBR domains. Conserved cysteine and histidine residues in the CBR involved in coordinating zinc 1 and zinc 2 are highlighted in yellow, and residues making hydrophobic contacts with the C-terminal tail of the donor ubiquitin are highlighted in green. The sequence forming ZF1 in HOIP has been removed for clarity and is indicated in cyan. The region around the catalytic C885 and H887, which are both crucial for catalytic activity, is highlighted in red. The glycine preceding C885 is conserved in other RBRs and might be important to allow the ubiquitin-loaded E2 access to the catalytic cysteine. The sequence variation of site 2 is much higher than for site 1, and in particular the sequence between zinc-coordinating residues 2 and 3, which in HOIP accommodates ZF1, varies significantly in length. b, This sequence variation is reflected in the structures of CBRs from HOIP, Parkin (PDB 4K7D) and HHARI (PDB 4KC9), which overlap well in site 1 and the following first two zinc-coordinating residues of site 2 but subsequently diverge significantly. Nevertheless, the positions of zinc 2 overlap well. The IBR of HOIP (pink; PDB 2CT7) is shown for comparison in the overlap. The ZF1 is shown in cyan and the Zn$^{2+}$ ions are shown as spheres. c, HOIP-CBR, in complex with the donor ubiquitin, overlapped with the CBRs of Parkin and HHARI, showing conserved hydrophobic residues that contact L71 and L73 of ubiquitin in ball-and-stick representation as well as the catalytic cysteine of the ligase. The C-terminal portion of the CBR (site 2) has been omitted for clarity. This overlap shows clearly that the C-terminal tail of the donor ubiquitin could be accommodated in a similar manner in Parkin and HHARI.
Extended Data Figure 4 | The HOIP<sub>CBR-C</sub>–ubiquitin complex in the asymmetric unit and arrangement in the crystal lattice. 

**a**, The asymmetric unit contains one molecule of HOIP<sub>CBR-C</sub> (blue) and one molecule of ubiquitin (orange). The ubiquitin bound to HOIP<sub>CBR-C</sub> represents the acceptor ubiquitin; its α-amino group of M1 is located in close proximity to the thioester-forming C885, which is shown in ball-and-stick representation and the Zn<sup>2+</sup> ions are shown as grey spheres, including the Zn<sup>2+</sup> ion found in the active site. Inset: details of the active site highlighting the proximity of M1 and C885 and H887. The Zn<sup>2+</sup> ion found in the active site has been removed for clarity.

**b**, HOIP<sub>CBR-C</sub> and ubiquitin that constitute the asymmetric unit are shown in blue and orange, respectively. A symmetry-related complex that contributes the donor ubiquitin is shown in yellow (ubiquitin) and light blue (HOIP). All other complexes in the lattice are shown in grey (HOIP) and red (ubiquitin).
Extended Data Figure 5 | Active site arrangement including coordination of the fifth Zn$^{2+}$ ion and ubiquitination assays with wild-type HOIP$_{RBR-C}$ and the H889A mutant. 

a, We found residual electron density in the active site of the wild-type HOIP$_{CBR-C}$–ubiquitin complex (shown in transparent grey), which adopts a tetrahedral coordination and which we interpret as a Zn$^{2+}$ ion. This Zn$^{2+}$ is coordinated by the catalytic cysteine, H887, the α-amino group of ubiquitin and an imidazole from the crystallization solution (Imd). The observation that apo and substrate-bound HOIP$_{CBR-C}$ contain a metal ion close to the active site prompted us to investigate a possible role in catalysis. Metal binding was examined by native electrospray mass spectrometry and ICP–MS, which indicated the presence of roughly five Zn$^{2+}$ ions in the wild-type protein. Mutation of either of the histidines H887A and H889A, which coordinate the additional zinc in the apo structure, decreased the number to four. The structure of the H889A mutant in complex with ubiquitin (in blue and orange, respectively) lacks additional electron density in the active site, while retaining full catalytic activity, indicating that the catalytic step is not metal-dependent. Instead we believe that high reactivity of the active site induces disulphide bond formation and subsequent zinc coordination across the interface during crystallization of apo HOIP$_{RBR-C}$, whereas in the substrate-bound complex the active site cysteine itself coordinates a metal ion with the help of an imidazole molecule from the crystallization buffer.

b, Sigma-A weighted omit map contoured at 1.5σ showing the Zn$^{2+}$ ion in the active site and its coordination by the α-amino group of ubiquitin M1, C885 and H887 from HOIP$_{CBR-C}$, and an imidazole molecule from the crystallization buffer.

c, Ubiquitination assays comparing the activity of wild-type HOIP$_{RBR-C}$ and the H889A mutant that no longer coordinates a fifth Zn$^{2+}$ ion but retains full catalytic activity.

d, The molecular mass of each construct listed (in daltons) was determined in its native and denatured forms by ESI–MS. The difference in mass of native and denatured proteins was used to calculate the number of Zn$^{2+}$ ions present (63.4 Da per Zn$^{2+}$). The calculated mass of the constructs under investigation contains the additional sequence GPG that remains after removal of the glutathione S-transferase tag with PreScission protease. Metal analysis by ICP–MS did not reveal the presence of significant amounts of metal ions apart from Zn$^{2+}$. 

Extended Data Figure 6 | Active site arrangement including coordination of the fifth Zn$^{2+}$ ion and ubiquitination assays with wild-type HOIP$_{RBR-C}$ and the H889A mutant. 

a, We found residual electron density in the active site of the wild-type HOIP$_{CBR-C}$–ubiquitin complex (shown in transparent grey), which adopts a tetrahedral coordination and which we interpret as a Zn$^{2+}$ ion. This Zn$^{2+}$ is coordinated by the catalytic cysteine, H887, the α-amino group of ubiquitin and an imidazole from the crystallization solution (Imd). The observation that apo and substrate-bound HOIP$_{CBR-C}$ contain a metal ion close to the active site prompted us to investigate a possible role in catalysis. Metal binding was examined by native electrospray mass spectrometry and ICP–MS, which indicated the presence of roughly five Zn$^{2+}$ ions in the wild-type protein. Mutation of either of the histidines H887A and H889A, which coordinate the additional zinc in the apo structure, decreased the number to four. The structure of the H889A mutant in complex with ubiquitin (in blue and orange, respectively) lacks additional electron density in the active site, while retaining full catalytic activity, indicating that the catalytic step is not metal-dependent. Instead we believe that high reactivity of the active site induces disulphide bond formation and subsequent zinc coordination across the interface during crystallization of apo HOIP$_{RBR-C}$, whereas in the substrate-bound complex the active site cysteine itself coordinates a metal ion with the help of an imidazole molecule from the crystallization buffer.

b, Sigma-A weighted omit map contoured at 1.5σ showing the Zn$^{2+}$ ion in the active site and its coordination by the α-amino group of ubiquitin M1, C885 and H887 from HOIP$_{CBR-C}$, and an imidazole molecule from the crystallization buffer. 

c, Ubiquitination assays comparing the activity of wild-type HOIP$_{RBR-C}$ and the H889A mutant that no longer coordinates a fifth Zn$^{2+}$ ion but retains full catalytic activity. 

d, The molecular mass of each construct listed (in daltons) was determined in its native and denatured forms by ESI–MS. The difference in mass of native and denatured proteins was used to calculate the number of Zn$^{2+}$ ions present (63.4 Da per Zn$^{2+}$). The calculated mass of the constructs under investigation contains the additional sequence GPG that remains after removal of the glutathione S-transferase tag with PreScission protease. Metal analysis by ICP–MS did not reveal the presence of significant amounts of metal ions apart from Zn$^{2+}$. 

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Extended Data Figure 6 | Diagram of the HOIP<sub>CBR-C</sub>–ubiquitin complex interface and complete set of steady-state ubiquitination assays performed with HOIP<sub>RBR-C</sub>.  

**a**, Diagram of the interface between HOIP<sub>CBR-C</sub> and donor and acceptor ubiquitin, respectively. The colour scheme used in Fig. 1 has been maintained, with a cut-off of 3.5 Å for polar and 4.0 Å for hydrophobic interactions. The C terminus of the donor ubiquitin is oriented towards the catalytic cysteine through multiple interactions with HOIP. The carbonyl of G76 forms a hydrogen bond with the C885 backbone amide and is 3.5 Å distant from its S<sub>c</sub>. The backbone NH of G76 makes further contact with the loop carrying C885, whereas an extended conformation for the rest of the tail is maintained by interactions with the CBR and β-hairpin. This arrangement is reminiscent of RING ligases that lock the E2—Ub in a folded-back conformation<sup>35–37</sup>, indicating that this might be a general mechanism to activate a ubiquitin thioester intermediate.  

**b**, The interface between HOIP and the acceptor ubiquitin includes residues from the helical base of HOIP that interact with T14, E16, D32 and K33. Mutation of T14 in HOIP<sub>RBR-C</sub> to alanine has only a modest effect on the steady-state synthesis of linear ubiquitin chains, whereas E16 and D32 show significantly decreased activity; the strongest effect was seen with the K33A mutant. In HOIP<sub>RBR-C</sub>, mutation of R935 and D936 almost completely abrogates activity, whereas the R1032A mutant has only a minor effect.  

**c**, E3-thioester formation and ubiquitin transfer assays. The diagram illustrates the set-up: lanes 1–4, donor Cy5-ubiquitin loading onto E1 and E2, followed by addition of wild-type HOIP<sub>RBR-C</sub> or mutants to form an E3—thioester (lanes 1 and 2 of each experiment). To monitor ubiquitin transfer, a C-terminally blocked ubiquitin was added (lanes 3 and 4 of each experiment).  

**d**, Mutations that disrupt the interaction of the C-terminal tail of the donor ubiquitin with the conserved region of the CBR and the β-hairpin strongly decrease the ability to produce linear ubiquitin chains. In contrast, mutation of E866, which is not conserved in other RBR family members, and its contact R42 has no effect on ubiquitin chain synthesis. Some of the gels included in this figure are also shown in Fig. 3c and are included for comparison.
Extended Data Figure 7 | In vitro ubiquitination assays with heterotrimeric LUBAC and in vivo co-immunoprecipitation of HOIP mutants with SHARPIN and HOIL-1L. a, In vitro ubiquitination assays with heterotrimeric LUBAC, showing that the overall activity of LUBAC is lower than that of isolated HOIPRBR-C, possibly as a result of regulatory roles of the ubiquitin-binding domains that are present in all three LUBAC subunits. Nevertheless, the trends observed with isolated HOIPRBR-C are conserved with LUBAC; those mutations that had only a minor or no effect on chain synthesis (T14A and R42) show the same behaviour, whereas the E16A, K33A, L71A, L73A and R74A mutants show a significant decrease in ubiquitin chain synthesis. b, Co-immunoprecipitation assays show that mutations in HOIP that interfere with the binding of donor or acceptor ubiquitin and interfere with ubiquitin chain synthesis have no effect on complex formation between LUBAC subunits, and hence any effects seen in NF-κB activation and p65 translocation assays are not due to impaired complex formation.
Extended Data Figure 8 | pH dependence of ubiquitin transfer with wild-type HOIP<sub>RBR-C</sub> and the H887A mutant. Ube1 (0.5 μM) was charged with 1 μM His<sub>Cy5</sub>-ubiquitin (Ub) using 5 mM ATP at pH 7. a, The pre-charged E1-ubiquitin thioester (E1−Ub) was subsequently mixed with 10 μM UbcH5A and incubated for 5 min under different buffer conditions ranging from pH 7 to pH 11. Complete ubiquitin transthiolation from the E1 onto UbcH5A (E2−Ub) can be observed at pH 7–9, is impaired at pH 10 and abolished at pH 11. Wild-type HOIP<sub>RBR-C</sub> (20 μM; E3(wt), top row) or HOIP<sub>RBR-C</sub> H887A (20 μM; E3(H887A), bottom row) were added to each sample and incubated for a further 5 min. Under these conditions, a thioester intermediate for both wild-type (E3(wt)−Ub) and mutant HOIP<sub>RBR-C</sub> (E3(H887A)−Ub) can be detected at pH 7–9. All samples were finally mixed with 10 μM C-terminal His<sub>Cy5</sub>-tagged ubiquitin (Ub) at pH 7–9 and to some extent at pH 10. In contrast, product formation is absent at pH 7 and pH 8 for HOIP<sub>RBR-C</sub> H887A, indicating that histidine 887 is required for catalysis under physiological pH conditions. The assay was performed in 5 mM MgCl<sub>2</sub>, 150 mM NaCl and 200 mM buffer (HEPES pH 7.0, HEPES pH 8.0, CHES pH 9.0, CHES pH 10.0 and CAPS pH 11.0).

b, The pre-charged E1-ubiquitin thioester (E1−Ub) was mixed with 10 μM UbcH5A (E2) and incubated for 5 min in 150 mM sodium acetate buffer ranging from pH 5.2 to pH 6.6 with 0.1 increments. All samples display the same amount of charged UbcH5A (E2−Ub). Similarly, the addition of 20 μM HOIP<sub>RBR-C</sub> (E3) shows formation of the thioester charged intermediate (E3−Ub) to the same extent. Each sample was mixed with 10 μM C-terminal His<sub>Cy5</sub>-tagged diubiquitin (Ub<sub>2</sub>) as acceptor to allow product formation (Ub<sub>3</sub>). The discharge of E3-thioester ubiquitin onto the acceptor is clearly impaired at pH values below 5.8. Gels were run under non-reducing conditions.
Extended Data Figure 9 | The structural basis of chain linkage specificity. Surface representation of HOIP, with the acceptor ubiquitin in orange ribbon representation, indicating the position of all seven lysine residues present in ubiquitin, as well as the N-terminal methionine and the catalytic cysteine, C885, of HOIP. The figure clearly shows that the \( \alpha \)-amino group of methionine 1 is closest to the active-site cysteine, explaining the specificity for linear chains.
Extended Data Table 1 | Data collection and refinement statistics

| Data collection                  | Apo HOIP            | HOIP WT/ubiquitin | HOIP H889A/ubiquitin |
|---------------------------------|---------------------|-------------------|----------------------|
| Space group                     | P1                  | P 3₁              | P 3₁                 |
| Cell dimensions                 |                     |                   |                      |
| $a$, $b$, $c$ (Å)               | 44.21, 47.77, 111.14| 45.95, 45.95, 133.01| 46.0, 46.0, 133.37   |
| $\alpha$, $\beta$, $\gamma$ (°) | 101, 90, 99         | 90, 90, 120       | 90, 90, 120          |
| Resolution (Å)                  | 29.61 (2.44-2.59)*  | 44.34-1.56 (1.60-1.56)* | 38.17-2.15 (2.21-2.15)* |
| $R_{sym}$ or $R_{merge}$        | 0.129 (0.576) *     | 0.039 (0.581) *   | 0.094 (0.614) *      |
| $I/\sigma I$                    | 10.07 (2.64) *      | 12.2 (2.1) *      | 9.3 (2.4) *          |
| Completeness (%)                | 91.7 (71.0) *       | 99.2 (98.8) *     | 99.5 (99.5) *        |
| Redundancy                      | 3.33 (2.80) *       | 3.4 (3.4) *       | 5.1 (5.4) *          |

Reﬁnement

| Resolution (Å)                  | 29.61-2.44          | 44.34-1.56         | 38.17-2.15           |
| No. reflections                 | 30946               | 84025             | 33376               |
| $R_{work}$, $R_{free}$          | 20.64/24.27         | 18.12/21.24       | 17.57/21.61         |
| No. atoms                       |                     |                   |                      |
| Protein                         | 5849                | 2306              | 2292                |
| $Zn^{2+}$                       | 18                  | 6 (+imidazole)    | 4                   |
| Water                           | 46                  | 222               | 120                 |
| B-factors                       |                     |                   |                      |
| Protein                         | 44.61               | 38.7              | 50.3                |
| $Zn^{2+}$                       | 43.88               | 30.6              | 37.0                |
| Water                           | 36.88               | 41.9              | 42.7                |
| R.m.s deviations                |                     |                   |                      |
| Bond lengths (Å)                | 0.0130              | 0.006             | 0.008               |
| Bond angles (°)                 | 1.790               | 1.032             | 1.115               |

One crystal was used for each of the data sets.  
* The highest resolution shell is shown in parenthesis.