Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis

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Ubiquitin modification is mediated by a large family of specificity determining ubiquitin E3 ligases. To facilitate ubiquitin transfer, RING E3 ligases bind both substrate and a ubiquitin E2 conjugating enzyme linked to ubiquitin via a thioester bond, but the mechanism of transfer has remained elusive. Here we report the crystal structure of the dimeric RING domain of rat RNF4 in complex with E2 (UbcH5A) linked by an isopeptide bond to ubiquitin. While the E2 contacts a single protomer of the RING, ubiquitin is folded back onto the E2 by contacts from both RING protomers. The carboxy-terminal tail of ubiquitin is locked into an active site groove on the E2 by an intricate network of interactions, resulting in changes at the E2 active site. This arrangement is primed for catalysis as it can deprotonate the incoming substrate lysine residue and stabilize the consequent tetrahedral transition–state intermediate.

By altering the fate of modified proteins, conjugation with ubiquitin and its homologues has a central role in eukaryotic biology underpinning cell signalling, protein degradation and stress responses. In most cases ubiquitin is transferred to its target proteins from a thioester complex with a ubiquitin conjugating enzyme (E2) by a large family of ubiquitin E3 ligases (E3)1. The RING family of E3s, of which over 600 are encoded in the human genome, possess a conserved arrangement of cysteine and histidine residues that coordinate two zinc atoms2. RING E3 ligases bind both substrate and E2–ubiquitin (E2–Ub) thioester, but the molecular basis by which the RING activates the E2–Ub bond for transfer of ubiquitin to substrate has remained elusive.

RNF4 is a SUMO-targeted ubiquitin ligase3 that has a key role in the DNA damage response4–6 and in arsenic therapy for acute promyelocytic leukaemia7,8. RNF4 contains multiple SUMO interaction motifs, allowing it to engage polySUMO-modified substrates, and a RING domain8 that is responsible for dimerization and catalysis of ubiquitin transfer3,9. Our understanding of RING-catalysed ubiquitination has been hindered by the lack of structures of the key intermediate: a RING bound to E2–Ub. Obtaining this key complex is difficult, as the thioester (or engineered oxyester) bond linking E2 and ubiquitin is highly activated and unstable in the presence of an E3.

Structure of the RING–UbcH5A–Ub complex

We have engineered a mimic of the E2–Ub thioester bond by replacing the active site cysteine of the E2 UbcH5A (also called UBE2D1) with a lysine to generate an isopeptide (amide) bond between the C terminus of ubiquitin and the ε-amino group of the introduced lysine (Supplementary Figs 1 and 2). Isopeptide-linked UbcH5A–Ub bound selectively to the RNF4 RING and acted as a potent inhibitor of RNF4-mediated substrate ubiquitination, confirming that it is an excellent mimic, but crucially, that it is stable in the presence of RNF4 (Supplementary Fig. 3). The E2–Ub mimic was mixed in a 2:1 ratio with a fused RNF4 RING dimer10 and crystallized. A 2.2 Å structure of the resulting complex was determined (Supplementary Table 1). The asymmetric unit contains the central RNF4 RING dimer, two UbcH5A molecules and two ubiquitin molecules related by a two-fold axis (Fig. 1). Each UbcH5A molecule contacts a single RING domain and is linked by an isopeptide bond to ubiquitin (Supplementary Fig. 4) that sits at the RING dimer interface. The complex can be envisaged as a dimer of heterotrimers (RING monomer, UbcH5A and ubiquitin).

Strikingly, ubiquitin is folded back onto the E2, creating an interface that buries approximately 1,800 Å², has 15 hydrogen bonds and 4 salt bridges. L8 of ubiquitin interacts with L97 and K101 of UbcH5A, whereas I44, H68 and V70 in ubiquitin are close to L104, S105 and N114. The side chain of N77 in UbcH5A forms a hydrogen bond with RING residue H160 (zinc ligand) and the main-chain carbonyl of ubiquitin E34 makes a hydrophobic contact with the side chain of Y193 of the RING domain from the other heterotrimer (Fig. 2a). Extensive contacts are evident between the C-terminal 6 residues of ubiquitin and loops surrounding the active site of UbcH5A, particularly residues L86, D87, Q92 and N114. The side chain of N77 in UbcH5A forms a hydrogen bond to the isopeptide carbonyl (Fig. 2b). Mapping conserved E2 residues (Supplementary Fig. 5) shows that highly conserved residues surround the active site and the shallow groove that accommodates the C-terminal region of the linked ubiquitin (Supplementary Fig. 6). The other conserved cluster of E2 residues constitutes the binding site for the E3 ligase.

UbcH5A contacts a single protomer of the RING (Supplementary Fig. 7) and the interface is very similar to that previously described for RING–E2 complexes10,11. At the junction of the three molecules in the heterotrimer a hydrophobic cluster formed by L8, T9 and L71 of ubiquitin, A96 and L97 of UbcH5A, and P137, P178 and R181 of the RING (Fig. 2c and Supplementary Fig. 7). Ubiquitin contacts both protomers of the RING dimer and the interface buries 940 Å² (Fig. 2c). Residues L8 to K11 and L71 with R72 of ubiquitin contact RING residues T179 to R181 within the same heterotrimer, whereas the Q31 to Q40 region of ubiquitin contacts both protomers of the RING dimer. The backbone carbonyl of ubiquitin E34 makes a hydrogen bond with RING residue H160 (zinc ligand) and the main-chain E34 to G35 of ubiquitin stacks with the side chain of Y193 of the RING domain from the other heterotrimer (Fig. 2d). These interfaces explain why dimerization of the RNF4 RING is required for activity9,12. Phylogenetic analysis of RNF4 from a wide range of species and sequence comparison of RNF4 with other dimeric RING and U-box E3 ligases indicate that the bound ubiquitin interacts with conserved features of the RING (Supplementary Fig. 8).

The RING domain does not undergo any major structural change as a result of complex formation (Supplementary Fig. 9a). Ubiquitin

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UbcH5A (Fig. 3a, b) and tested these in a single-turnover substrate ubiquitination assay. Mutations of hydrophobic residues I44 (ubiquitin) and L104 (UbcH5A) at the interface between ubiquitin and UbcH5A abolished ubiquitination activity, whereas mutations K101A, S108A and D112A in UbcH5A and R42A in ubiquitin reduced activity modestly (Fig. 3c, d and Supplementary Figs 10–13). Ubiquitin mutations G35A and I36A (both at the RING interface) substantially (>10X) reduced activity. Significant reductions in ubiquitination were also observed for mutations of L8 and L71 in ubiquitin and L97 in UbcH5A that form a hydrophobic core at the junction of all three molecules in the heterotrimer. In the E2 active site groove, mutations N77A and D87A in UbcH5A abolished activity, whereas D117A severely compromised activity. N114A in UbcH5A and R72A, L73A and R74A in ubiquitin displayed modestly reduced activity (Fig. 3c, d and Supplementary Figs 10–13).

To discriminate between residues in ubiquitin and E2 that influence the ability of the substrate lysine to carry out nucleophilic attack on the E2–Ub thioester and those residues involved in activating the E2–Ub bond, we carried out substrate-independent assays that measure the ability of the RNF4 RING to catalyse hydrolysis of an E2–Ub thioester bond. Using the isopeptide-linked E2–Ub in our crystal structure, we constructed a model of the E2–Ub thioester by replacing K85 in UbcH5A with cysteine and minimizing the geometry. The resulting model shows very minor changes: the Sβ and Cz atoms in C85 are shifted 1.0 Å and 0.2 Å from Cγ and Cz atoms of K85, with smaller changes in I84 and L86. In ubiquitin the Cz atoms of G76 and G75 have moved 0.5 Å and 0.2 Å, respectively. The carbonyl group of the thioester at G76 has moved 0.6 Å and rotated around 45°, resulting in the hydrogen bond with N77 being extended to 3.6 Å (Fig. 5a, b). Coupled with the mutational analysis and evidence that the isopeptide-linked E2–Ub is a competitive inhibitor of ubiquitination, we conclude that the crystal structure is a relevant model for the key E2–Ub–RING heterotrimeric intermediate.

In the absence of an E3 ligase, the ubiquitin thioester linked to the E2 can adopt a wide range of different conformations that also include a ‘folded-back’ conformation. As free ubiquitin has no detectable affinity for the RNF4 RING we suggest that the initial interaction will be between E2 and the RING. In this encounter, with the E2 bound to one RING protomer, the thioester-linked ubiquitin would be engaged by Y193 of the other RING protomer and folded back to contact the α2 helix of UbcH5A, while its C terminus is extended and locked in the active site groove of the E2. This orients the planar thioester bond such that the ubiquitin G76 thioester carbonyl is in the optimal arrangement for nucleophilic attack by the incoming substrate lysine. This arrangement of the E2 active site was not observed in a UbcH5B–Ub thioester complex (Fig. 5c, d). The nucleophilic attack by the substrate lysine would result in formation of a tetrahedral intermediate on the G76 carbonyl carbon. The G76 carbonyl oxygen, with its developing

**Mechanism of RING–Mediated Ubiquitination**

Using the isopeptide-linked E2–Ub in our crystal structure, we constructed a model of the E2–Ub thioester by replacing K85 in UbcH5A with a cysteine and minimizing the geometry. The resulting model shows very minor changes: the Sβ and Cz atoms in C85 are shifted 1.0 Å and 0.2 Å from Cγ and Cz atoms of K85, with smaller changes in I84 and L86. In ubiquitin the Cz atoms of G76 and G75 have moved 0.5 Å and 0.2 Å, respectively. The carbonyl group of the thioester at G76 has moved 0.6 Å and rotated around 45°, resulting in the hydrogen bond with N77 being extended to 3.6 Å (Fig. 5a, b). Coupled with the mutational analysis and evidence that the isopeptide-linked E2–Ub is a competitive inhibitor of ubiquitination, we conclude that the crystal structure is a relevant model for the key E2–Ub–RING heterotrimeric intermediate.

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**E2 and Ubiquitin Residues Required for Activity**

Previous mutational analysis revealed the importance of the RING residue R181—which contacts both E2 and ubiquitin in the present structure (Fig. 2)—in the ubiquitination activity of RNF4 (ref. 3). Moreover, Y193 in the RING plus L8 and I44 in ubiquitin were shown to be required for activity. Although it was thought that these residues might interact directly, the present structure emphasizes their importance but shows that they are not in direct contact. To validate our structure further, we introduced mutations into ubiquitin and
negative charge, would move down below the plane of the original thioester bond and form a hydrogen bond to N77, stabilizing the tetrahedral intermediate. In fact the atoms would move towards the experimental orientation of the carbonyl in the isopeptide bond that makes a 2.8 Å hydrogen bond with N77. The role of UbcH5A D117, which sits above the thioester and is re-positioned by ubiquitin binding, has been clarified by analysis of the D117A mutant. Of the mutants which are defective in the ubiquitination assay, only D117A retains wild-type levels of oxyester hydrolysis (Fig. 3f).

Because the E2–Ub oxyester bond is hydrolysed in the presence of E3 (no transfer to substrate) 3, only a residue with the sole function to position and/or activate the incoming lysine nucleophile should possess activity in oxyester hydrolysis assays but be inactive in ubiquitination.

Implications for transfer of ubiquitin and related modifiers

This is the first structure of a RING E3 ligase bound to a ubiquitin-loaded E2, but the mechanism proposed here for ubiquitin transfer to substrate is consistent with previous work. Key roles for residues N77 (ref. 20) and D117 (ref. 21) in E2 catalytic activity have been suggested previously. Evidence that activation of the thioester bond requires both ubiquitin/ubiquitin-like modifier (Ubl) and E2 to be bound by the E3 comes from previous work on RNF4 (ref. 3), the SIZ1 (ref. 22) and RanBP2 (ref. 23) SUMO E3 ligases, and the NEDD4L HECT E3 ligase19. The folded-back conformation where the I44 hydrophobic patch of ubiquitin (or equivalent region of SUMO) engages the α2 helix of the E2 has been suggested as an intermediate in ubiquitin/Ubl transfer based on NMR models15,17, mutagenesis coupled with modelling22,24, and from the structure of a SUMO substrate–E2–E3 product complex23,25. Comparing the NMR model of UBC1 (also called UBE2K)–Ub thioester 15 with the present structure shows that although ubiquitin in the UBC1–Ub complex is in the folded-back conformation, it is different from the present structure where interactions between ubiquitin and the RING extend and exert tension on the ubiquitin C terminus, locking it down into the E2 active site groove. In the absence of its cognate E3 the ubiquitin C-terminal tail in the UBC1–Ub complex is not locked down in the UBC1 active site groove and the thioester is thus not activated (Supplementary Fig. 16).

The folded-back conformation was also observed in the structure of SUMO-modified RanGAP1 in complex with UBC9 (also called UBE2I) and the SUMO E3 ligase RanBP2 (ref. 25) (trapped product complex). The position of the SUMO C-terminal tail and hydrogen bonding interactions within the active site groove of UBC9 are remarkably similar to those seen for UbcH5A–Ub bound to the RNF4 RING (Supplementary Fig. 17). Although both RNF4 and RanBP2 interact with ubiquitin/SUMO to lock it into this conformation, it is different from the present structure where interactions between ubiquitin and the RING extend and exert tension on the ubiquitin C terminus, locking it down into the E2 active site groove. In the absence of its cognate E3 the ubiquitin C-terminal tail in the UBC1–Ub complex is not locked down in the UBC1 active site groove and the thioester is thus not activated (Supplementary Fig. 16).

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Figure 2 | Molecular interfaces in the RNF4 RING–UbcH5A–Ub complex. a, Detail of the interaction between ubiquitin (orange) and the α2 helix of UbcH5A (green). b, Detail of the interaction interface between ubiquitin (orange) and UbcH5A (green) in the E2 active site groove. The side chain of K85 in UbcH5A that forms the isopeptide bond with ubiquitin is coloured violet. c, The hydrophobic cluster at the centre of the ubiquitin (orange), UbcH5A (green) and RING (cyan) heterotrimer. d, Stacking interaction between the main chain of ubiquitin (orange) in one heterotrimer and Y193 of the RING (blue) from the other heterotrimer.
Figure 3 | Mutational analysis of the RNF4 RING–UbcH5A–Ub complex. a. Side chains of altered residues in ubiquitin contacting RNF4 (blue), UbcH5A (green), or both RNF4 and UbcH5A (yellow). b. Side chains of altered residues in UbcH5A contacting RNF4 (blue), ubiquitin (orange), both RNF4 and ubiquitin (yellow), or neither (green). c. Reaction rates were determined (mean ± s.d. of duplicates) for single-turnover, RNF4-dependent substrate ubiquitination assays with mutant forms of ubiquitin. Wild-type ubiquitin is in grey and mutants are colour coded as in a. d. Assays with UbcH5A mutants quantified as in c and colour coded as in b. e. RNF4-mediated hydrolysis of UbcH5A(C85S)–Ub oxyesters with mutations in ubiquitin. Rates are mean ± s.d. of duplicates. f. As in e, with mutations in UbcH5A.

Figure 4 | The same interfaces in E2 and ubiquitin are important for CHIP and RNF4 activity. a. Autoubiquitination activity of CHIP (top panel) and RNF4 (bottom panel) with ubiquitin mutants. Western blots probed with anti-ubiquitin antibody are shown. Longer exposure is shown for I36A and L71A ubiquitin, as binding of the antibody is affected by these mutations. b. Autoubiquitination activity as in a, but with UbcH5A mutants.
Figure 5 | E3-mediated structural changes associated with the catalytically primed form of UbcH5A–Ub. a. Model of UbcH5A–Ub thioester (grey) compared with isopeptide-linked UbcH5A(C85K)–Ub (K85 is violet). b. Comparison of modelled thioester with isopeptide linkage. Hydrogen bonds are black (isopeptide) or grey (modelled thioester) dashes, with distances shown in Å. c. Comparison of the position of ubiquitin relative to E2 in the UbcH5A–Ub–RING complex reported here with the UbcH5B–Ub–RING–UbcH5A(S22R/C85K)–Ub complex. d. E2–Ub conjugate was prepared by incubating UbcH5A(S22R/C85K) (200 µM) with His6-tagged ubiquitin (200 µM) in a 2:1 molar ratio. Crystals grew from a 1:1 sitting-drop with a reservoir solution containing 3 mM ATP, 5 mM MgCl₂, 50 mM Tris pH 10.0, 150 mM NaCl and 0.8 mM TCEP. The E2–Ub conjugate was purified by Ni²⁺-affinity chromatography. His₆-tag was removed using TEV protease and the conjugate was further purified by Ni²⁺-affinity chromatography and gel filtration chromatography. The RNF4 RING–UbcH5A(S22R/C85K)–Ub complex was prepared by mixing the UbcH5A(S22R/C85K)–Ub with a linear fusion of two RNF4 RING domains in a 2:1 molar ratio. Crystals grew from a 1:1 sitting-drop with a reservoir solution containing 18% (w/v) PEG 3,000, 0.1 M Tris (pH 7.2), and 0.2 M calcium acetate. The structure was solved by molecular replacement to a resolution of 2.2 Å using in house X-rays. A single-turnover substrate ubiquitination assay for RNF4 has been described previously⁹.

METHODS SUMMARY

Recombinant proteins were expressed in Escherichia coli cells and purified by standard methods. For structural analysis of a stable mimic of the UbcH5A–Ub thioester, mutations C85K and S22R²⁸ were introduced into UbcH5A (UbcH5A(S22R/C85K)). The isopeptide bond-linked UbcH5A(S22R/C85K)–Ub conjugate was prepared by incubating UbcH5A(S22R/C85K) (200 µM) with His₆-tagged ubiquitin (200 µM) at 35 °C for 26 h in a buffer containing 3 mM ATP, 5 mM MgCl₂, 50 mM Tris pH 10.0, 150 mM NaCl and 0.8 mM TCEP. The E2–Ub conjugate was purified by Ni²⁺-affinity chromatography. His₆-tag was removed using TEV protease and the conjugate was further purified by Ni²⁺-affinity chromatography and gel filtration chromatography. The RNF4 RING–UbcH5A(S22R/C85K)–Ub complex was prepared by mixing the UbcH5A(S22R/C85K)–Ub with a linear fusion of two RNF4 RING domains in a 2:1 molar ratio. Crystals grew from a 1:1 sitting-drop with a reservoir solution containing 18% (w/v) PEG 3,000, 0.1 M Tris (pH 7.2), and 0.2 M calcium acetate. The structure was solved by molecular replacement to a resolution of 2.2 Å using in house X-rays. A single-turnover substrate ubiquitination assay for RNF4 has been described previously⁹.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions A.P. cloned, expressed and purified proteins, carried out structural analysis, conducted biochemical experiments and interpreted the data. E.G.J. purified recombinant proteins and carried out biochemical analysis. M.H.T. carried out mass spectrometry analysis. J.H.N. contributed to structural analysis and data analysis. A.P., J.H.N. and R.T.H. wrote the paper. R.T.H. conceived the project and contributed to data analysis.

Author Information Coordinates and structure factors of the RNF4 RING–UbcH5A(S22R/C85K)–Ub complex were deposited in the Protein Data Bank under accession code 4AP4. 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 this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.T.H. (r.t.hay@dundee.ac.uk).
METHODS

Cloning, expression and purification of recombinant proteins. Expression and purification of Rattus norvegicus RNf4, human UbcH5A, and His6-tagged linear fusion of four SUMO2 molecules (4 × SUMO2-2) has been described previously 3. Mutations S22R and C85K were introduced into UbcH5A using PCR-based site-directed mutagenesis and the mutant protein was expressed and purified as described for wild-type UbcH5A. A linear fusion of two RNf4 RING domains was generated by sub-cloning the first RING domain (RING1, residues 131–194 of R. norvegicus RNf4) into pLouv vector using Ncol and BamHI restriction sites. The second RING domain (RING2, residues 131–194) was inserted using BamH and HindIII restriction sites with a single glycine residue as a linker between the two RINGS. The RING1–RING2 linear fusion was expressed and purified as described for wild-type RNf4. Human ubiquitin (residues 1–76) was sub-cloned into pHIS-TEV-30a vector using Ncol and BamHI restriction sites. The final model has good resolution. The crystals were limited by our ability to resolve the long cell edge due to a Rigaku Saturn CCD with X-rays generated from a Rigaku 007 HF generator. The resolution structure was used to solve this data. The models were adjusted manually using COOT 23, the isopeptide bond and the missing ubiquitin residues were substituted iodoacetamide for chloroacetamide to limit false identifications of ubiquitin. Pull-down assay. Binding between MBP-tagged RNf4 and ubiquitin-loaded UbcH5A was analysed by a pull-down assay as described previously 24. Mass spectrometry. UbcH5A(S22R/C85K)–Ub conjugate (both 5 μg) were fractionated by 10% SDS–PAGE. Coomassie-stained bands were excised and tryptic peptides extracted as described previously 25, substituting iodoacetamide for chloroacetamide to limit false identifications of ubiquitination sites 26. Peptide samples were analysed by LC-MS/MS using a Quadrupole mass spectrometer (Thermo Scientific) using high-resolution HCD fragmentation. Peptides were identified and quantified by MaxQuant (v 1.2.2.5) running the Andromeda search engine 23 using both a human proteome and paralog-specific small ubiquitin-like modifier processing. Protein sequences were analysed using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences) and quantified using the LI-COR software. Reactions were performed in duplicate and reaction rates are shown as mean ± s.d. Pull-down assay. Binding between MBP-tagged RNf4 and ubiquitin-loaded UbcH5A was analysed by a pull-down assay as described previously 24, 25. Mass spectrometry. UbcH5A(S22R/C85K)–Ub conjugate (both 5 μg) were fractionated by 10% SDS–PAGE. Coomassie-stained bands were excised and tryptic peptides extracted as described previously 25, substituting iodoacetamide for chloroacetamide to limit false identifications of ubiquitination sites 26. Peptide samples were analysed by LC-MS/MS using a Quadrupole mass spectrometer (Thermo Scientific) using high-resolution HCD fragmentation. Peptides were identified and quantified by MaxQuant (v 1.2.2.5) running the Andromeda search engine 23 using both a human proteome (Human IPI v3.68) and the recombinant protein sequence databases. Both Gly-Gly and Lys-Arg-Gly-Gly variable modifications to lysine were included in the search to detect ubiquitination by two methods.

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