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Structural basis of generic *versus* specific E2-RING E3 interactions in protein ubiquitination

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Abstract

Protein ubiquitination is a fundamental regulatory component in eukaryotic cell biology, where a cascade of ubiquitin activating (E1), conjugating (E2) and ligating (E3) enzymes assemble distinct ubiquitin signals on target proteins. E2s specify the type of ubiquitin signal generated, while E3s associate with the E2~Ub conjugate and select the substrate for ubiquitination. Thus, producing the right ubiquitin signal on the right target requires the right E2-E3 pair. The question of how over 600 E3s evolved to discriminate between 38 structurally related E2s has therefore been an area of intensive research, and with over 50 E2-E3 complex structures generated to date, the answer is beginning to emerge. The following review discusses the structural basis of generic E2-RING E3 interactions, contrasted with emerging themes that reveal how specificity can be achieved.

Keywords: ubiquitination; E2; RING E3; E2-E3 specificity; structural basis of specificity

Introduction

Dynamic and reversible attachment of the 76-amino-acid modifier ubiquitin (Ub) onto proteins (ubiquitination) constitutes an essential regulatory component in eukaryotic cell biology.[1,2] The highly conserved β-grasp fold of Ub presents two solvent-exposed hydrophobic patches, centred around L8-I44-V70 and I36-L7-L73, which modify the surface and binding property of the ubiquitinated protein. An array of Ub-binding domains (UBDs) have evolved to recognise this invariant fold and trigger specific cellular responses.[3] Conjugation of Ub to single or multiple sites of the target protein, termed mono- or multi-monoubiquitination, is well-documented in protein trafficking and DNA repair pathways.[4–6] Once attached to a substrate, Ub can itself be further modified on any of its seven lysine residues or N-terminal amine to form homotypic or branched Ub chains with structurally distinct topologies.[7–9] Moreover, enzymes with deubiquitinase activities (DUBs) can trim or erase Ub chains, adding greatly to the complexity of Ub signalling.[10]

Target proteins are modified with this so-called "ubiquitin code" by a three-enzyme cascade. Briefly, a Ub-activating enzyme (E1) activates the C-terminus of Ub in an ATP-dependent manner and offloads the activated Ub onto the catalytic cysteine of a Ub-conjugating enzyme (E2) to produce an E2~Ub conjugate (where ~ denotes a thioester bond). Finally, Ub-ligases (E3s) associate with the E2~Ub conjugate, select the substrate for ubiquitination, and mediate formation of an isopeptide bond between the C-terminal carboxyl of Ub and the ε-amine group of the target lysine residue.[11,12] As per the complexity of the ubiquitin code, the number of

enzymes involved in ubiquitination is vast: eukaryotic genome encodes two Ub E1s, 38 E2s, 617 predicted E3s, of which 377 are validated and active, and finally 122 DUBs.[13–16]

On the basis of their mechanism of Ub transfer, E3s have been grouped into three classes.[15] Really Interesting New Gene (RING)-type E3s, characterized by a 30-100 residue RING-finger motif with a "cross-brace" topology coordinating two zinc ions, make up the largest E3 family with 340 validated members.[16] Structurally related U-box E3s are classed together with RINGs, with the U-box fold adopting a similar topology, albeit without zinc ion coordination.[15] RING/U-box E3s recognise E2~Ub conjugate and the substrate and mediate Ub transfer from E2~Ub directly onto the target lysine.[17,18] Distinct from RING/U-box E3s, Homologous to E6-AP C-Terminus (HECT) E3s contain a conserved bilobal catalytic domain, where the N-terminal lobe recruits the E2~Ub and forms a thioester-linked intermediate with Ub prior to ubiquitinating substrates, which are recognized by the C-terminal lobe.[19,20] Finally, the most recently discovered family, RING-between-RING (RBR)-type E3s, use a hybrid, multi-step process where the first RBR RING (RING1) recruits the E2~Ub conjugate in a manner similar to canonical RING E3s, followed by transthiolation of the donor Ub onto the second RBR RING (RING2 or Rcat) prior to ubiquitination of substrates.[21,22]

Both E2s and E3s display a high level of structural conservation within their respective families. As a result, a given E3 has the potential to recognize numerous E2~Ub conjugates, and *vice versa*.[23,24] At the same time, with the type of Ub signal

generated specified by the E2,[25,26] E3s have to be able to select the right E2 to generate the appropriate Ub signal on the appropriate target protein.[10,27,28] The question of how E3s evolved to discriminate between 38 structurally similar E2s has therefore been an area of intensive research, and with over 50 E2-E3 complex structures deposited in the Protein Data Bank (PDB) to date, the answer is beginning to emerge. Aiming to avoid redundancy with recent, extensive reviews on HECT- and RBR-family E3s,[19,22] the focus will be on recent findings in E2-RING interactions. What follows is a short introduction to the key aspects of E2 and RING E3 morphology, followed by a brief review of the paradigm of generic E2-RING E3 interactions, contrasted with emerging themes that reveal how specificity can be achieved.

E2 morphology

All human E2s can be recognised from their evolutionarily conserved catalytic core, the ubiquitin conjugating (UBC) domain. Some E2s consist only of a UBC domain, while others may have short extensions of typically unstructured regions flanking one or both ends of the UBC domain. In extreme cases, such as in UBE2O and Baculoviral IAP Repeat-containing Protein (BIRC) 6, the extensions to the UBC domain can stretch to up to 4000 residues, and encompass structured regions with E3 activity. A more detailed discussion on classification of E2s on the basis of the presence of such insertions is reviewed elsewhere.[28]

The approximately 150-residue long UBC domain adopts an α/β-fold, typically containing an N-terminal helix (α1), a four-stranded β-meander (β1-4), a short 3_{10}-helix that leads into the central "cross-over" helix (α2), followed by two C-terminal helices (α3-α4) [Fig. 1(A)].[29] The active-site cysteine, nestled in a shallow groove preceding the 3_{10}-helix, is surrounded by well-conserved residues that mediate both thioester and isopeptide bond formation [Fig. 1(A)].[30] Atypically, as in the UBE2G and UBE2R families, the catalytic cleft may harbour functionally important insertions that facilitate linkage-specificity and E3-independent ubiquitination.[38,39]

Across the catalytic cleft over to the N-terminus, α1 and the loops connecting β3 to β4, and the 3_{10}-helix to α2 (hereafter Loop 4 [L4] and Loop 7 [L7]), make up the overlapping E1/E3-recognition interface [Fig. 1(B)]. This overlap ensures that binding to an E1 and to an E3 are mutually exclusive.[33,34] The low-affinity nature of the E2-E3 interaction promotes disengagement of the E2 following ubiquitination, permitting it to be recharged by the E1, and at the same time, allowing the E3 to engage another E2~Ub conjugate.[35–38]

Structure-function studies to date have generated large amounts of information on the E2 family; the PDB contains structures of UBC domains from 30 out of the 38 human E2s. Conservation at the sequence level is moderate, with a mean pairwise sequence identity between the 38 human E2s of 30% (Fig. S1). Despite this, the E2 UBC cores align remarkably well, with a mean pairwise positional shift of 1.1 Å between overlapping C_α atoms of UBE3D2 UBC and that of the other 29 structurally characterised E2s. Substantial divergence is observed only

at the C-terminal α3-α4 region [Fig. 1(A)]. Nevertheless, over the tightly-folded backbone of the UBC core, the surface-exposed residues forming the composite E3-binding site, where RING E3s typically bind, can subtly differ across the E2 family [Fig. 1(B), S2].[38–41] Thus, variation of sequence at α1, L4 or L7 can markedly influence which E3s a given E2 can associate with.

RING E3 morphology

RING domains are characterised by the canonical zinc-coordinating motif C-X_2-C-$X_{(9-39)}$-C-$X_{(1-3)}$-H-$X_{(2-3)}$-C-X_2-C-$X_{(4-48)}$-C-X_2-C, where C is cysteine, H is histidine, and X can be any amino acid.[17]

The 30-100 residue-long RING motif adopts an interdigitating cross-brace fold that coordinates two zinc ions. The compact RING domain typically encompasses two N-terminal β-strands (β1 and β2), a central α-helix (α1) and two bipartite zinc-coordination sites buried within the domain's core: the loops preceding β1 and α1 (ZI_A and ZI_B), and those following β1 and α1 (ZII_A and ZII_B) comprise the first and the second zinc-coordination sites, respectively [Fig. 2(A)]. The central α1 and the flanking finger-like projections of $ZI_{A/B}$ and $ZII_{A/B}$ form a shallow cleft over the domain surface, and make up the E2 interaction site [Fig. 2(B)].

Analysis of many RING and structurally related U-box domains that have previously been crystallised in complex with E2s shows that primary sequence conservation within the RING/U-box domain core is mediocre, with a mean pairwise sequence identity of only 26% (Fig. S3). Moreover, many RING domains tolerate

various insertions. For instance, several RING E3s including that of RING Finger Protein (RNF) 4, Tripartite Motif Containing (TRIM) 23 and Ring-box protein (RBX) 1 encompass an α-helical insertion between the ZI_A loop and β1, as well as and additional β-strand that runs antiparallel to β1-β2. The central α1 also varies greatly in length, from 5 residues in BIRC2 to 18 residues in RNF25. Finally, the Fanconi anaemia-associated RING E3 FANCL contains an unusually large insertion of three β-strands following α1. In line with this, overall alignment of RING domains is poor, with a mean pairwise positional shift of 2.6 Å between overlapping Cα atoms of RNF4 RING domain and that of the others previously crystallised in complex with E2s [Fig. 2(A)]. Nevertheless, the RING regions α1, $ZI_{A/B}$ and $ZII_{A/B}$, which make up the E2-interaction site, display a much better overlap than the regions not involved in E2-E3 interaction [Fig. 2(A)].

Interestingly, as the UBE2L3-Casitas B-lineage Lymphoma Proto-oncogene (c-Cbl) complex structure revealed for the first time,[24] the typical RING-E2~Ub interaction positions the RING core ~15 Å away from the E2~Ub thioester bond [Fig. 2(B)]. It is now understood that many RING E3s catalyse ubiquitination allosterically via a positively charged “linchpin” residue near the C-terminal end of the RING core.[42] In this model, the linchpin residue interacts with both the E2 and the Ub moieties, shifting the equilibrium of the E2~Ub conjugate from an ensemble of extended conformations, where the Ub moiety is distal to the E2 (opened-state), to a more compact conformation, where the Ub is tethered more tightly to the E2 with the

Ub I44 patch facing E2 α2 (closed-state), thereby allowing the target lysine of the substrate to readily perform nucleophilic attack on the E2~Ub thioester bond.[42,43]

The RING features described above are sufficient for E2~Ub recruitment and substrate ubiquitination,[44–46] with RING E3s such as CCR4-NOT Transcription Complex Subunit (CNOT4) and RNF38 reported to be active as monomers.[45,46] However, an increasing number of RING domains have been found to be functional only when oligomeric. For example, RNF4 and BIRC-family RING E3s form homodimers via their RING domains.[49–52] Similarly, some RING domains that lack intrinsic E2~Ub binding and E3 ligase activity, such as polycomb BMI1 and Double Minute Protein (MDMX), heterodimerize with their respective active, RING domain-containing partners to become functional.[53–55] Regulation of RING activity by homo- or heterodimerisation has recently been reviewed in further detail elsewhere.[56,57]

The canonical E2-RING E3 interaction

Identification of the minimal set of interactions that are required for all E2-RING E3 interactions, which may be referred to as canonical E2-RING E3 interactions, can be achieved by comparison of the structures of the most versatile members of the E2 family. These are the closely related members of the UBE2D family, which build K48-type Ub chains and target many regulatory proteins for destruction,[58] and their distant relative UBE2N, which dimerise with catalytically-dead E2 variants UBE2V1 or UBE2V2 to build K63-type Ub chains and trigger immune responses.[40,59,60] Remarkably, *in vitro* studies have shown that UBE2D1,

UBE3D2, UBE2D3 and UBE2N can individually interact with over 30 RING E3s, with approximately 60% of these RING E3 interaction patterns overlapping.[61] Not surprisingly, 23 out of 29 unique E2-E3 complex structures identified to date are that of UBE2D1, UBE3D2, UBE2D3 or UBE2N.

The canonical E2-RING E3 interaction is mediated by conserved hydrogen-bonding and van der Waals interactions tethering E2 α1, L4 and L7 to RING E3 ZI_A, $\alpha1/ZI_B$ and ZII_B, respectively (Fig. 3). The UBE2D1-RNF4 complex can be used to illustrate each of these interactions.[49] UBE2D1 α1 carries two surface-exposed residues, R5 and K8. With their sidechains projecting into the first zinc-coordinating centre of RNF4, UBE2D1 R5 and K8 form salt bridges with the I134 and M136 backbone carbonyls on RNF4 ZI_A, respectively. Meanwhile, the M136 sidechain slots into the contact area formed in-between R5 and K8. UBE2D1 L4 approaches the same zinc-coordinating centre of RNF4 from the other side, with the bulky F62 side chain buried within van der Waals radii of the RNF4 $a1/ZI_B$ residues C162 and D165. UBE2D1 L7 also engages RNF4 at both zinc-coordination centres via its well-conserved SPA motif: S94 forms a hydrogen-bond with the backbone carbonyl of P174 on RNF4 ZII_B, and P95 and A96 side chains form a pocket fitting I134 side chain on RNF4 ZI_A. Finally, the linchpin arginine coordinates UBE2D1 Q91 backbone carbonyl and is also poised to form a salt bridge with the donor ubiquitin (Fig. 3).

The interactions described above generally hold true for all E2-RING E3 pairs structurally characterised to date and do indeed position the RING core on the E2 with remarkable precision (Fig. 4). Nevertheless, variations are tolerated. For

example, the cCBL-UBE2L3 and RNF8-UBE2N complexes lack one of the salt bridges and the stacking van der Waals interaction that tether the E2 α1 to RING E3 ZI_A.[24,62] In contrast, some RINGs such as TRIM23, RNF13, RNF2 and Glycoprotein 78 (GP78) have a carboxylate-containing residue on their ZI_A and form an additional salt bridge with E2 α1 (Fig. S3).[63–65] Additionally, RINGs such as RNF165, RNF13, RNF2, GP78 and Zinc and Ring Finger 1 (ZNRF1) have a bulky tryptophan side chain, buttressing the pocket on ZI_B where the hydrophobic side chain of E2 residue on L4 sits (Fig. S3).[59,64–66] Carboxyl Terminus of HSC70-interacting Protein (CHIP) is able to interact with UBE2D and UBE2N, two E2s that have the SPA motif on L7, but fails to recruit those E2s that lack this motif, UBE2K, UBE2H, UBE2C and UBE2L3.[40] Such variations within the RING and UBC domains influence how and to what extent a given E3 interact either with one or a spectrum of E2s.

The apparent robustness of the canonical E2-RING E3 interaction can be misleading. The UBE2L3:cCBL complex, which was the first E2-RING E3 complex structure to be solved, is such an example. As expected due to the presence of the canonical E2-RING E3 interactions described above, UBE2L3 can recruit cCBL *in vitro*, however, the resulting complex lacks apparent ubiquitination activity.[23,24] The reason for this became clear when UBE2L3 was found to be able to carry out only a transthiolation reaction.[21] Hence, UBE2L3 can promote ubiquitination with HECT/RBR type E3 ligases, which involve a transthiolation step, but not with RING E3s, which mediate transfer of the donor Ub directly onto target lysine residues.

Thus, there is more to a fruitful E2-E3 partnership than the ability of both partners to recognise one another.

Achieving specificity beyond the canonical E2-RING E3 interaction

The focus of the following sections will be on the known examples of specialised E2-E3 interactions. Where possible, biological context will be provided to better understand the function provided by the insofar rare structural features that mediate E2-E3 specificity.

Specialised E2-RING E3 interaction facilitated by accessory regions

An emerging theme in specific E2-E3 complexes is that the E3 encodes a specialised E2-binding helix distal to its RING core. The E2-binding helix occupies a deep contact area generated outside the canonical E2-E3-interaction site, at the reverse side of the UBC core, the so called UBC "backside". Currently there are three well-characterised cases, where GP78, RING E3 RAD18 and RNF25 bind to UBE2G2, UBE2B and UBE3D2/UBE2E3 in this manner, respectively. Of note, in each case the backside binding helix occupies hydrophobic clusters unique to its cognate E2 (Fig. 5). Details of these interactions are discussed below.

The transmembrane RING E3 GP78 is resident on the outer membrane of the endoplasmic reticulum (ER), where it works in parallel with several other E3s to prevent deleterious accumulation of misfolded proteins through ER-associated degradation (ERAD).[67,68] Towards this end GP78 sequester UBE2G2 to the ER via a

specialised helix distal to its RING core, termed the UBE2G2-binding region (G2BR). G2BR binds to UBE2G2 at UBC backside, extending the canonical E2-E3 contact area and markedly increasing UBE2G2-GP78 interaction affinity.[69]

In this non-canonical E2-E3 interaction, R578 and Q579 on G2BR coordinates E31 and N30 on the UBE2G2 β1-β2 loop, while at the other end of G2BR, K600 coordinates E45/E50 on the UBE2G2 β2-β3 loop, clamping the G2BR helix on the UBC backside. Meanwhile, several hydrophobic residues project towards the UBC backside. G2BR L582 presses into G27 on UEB2G2 β1. In most other E2s, this position is occupied by bulkier residues (W in UBE2T; T in UBE2R1/2 Q/E in UBE2J1/2) and would cause steric hindrance with G2BR L582 side chain (Fig. S1). At the same time, G2BR L589, L590 and L598 are within van der Waals radii of UBE2G2 L40, V53 and L165, respectively [Fig. 6(A)].[65,68]

UBE2B is involved in DNA damage tolerance (DDT), where it interacts with the E3 ligase RAD18 to specifically monoubiquitinate Proliferating Cell Nuclear Antigen (PCNA).[70] Interestingly UBE2B is known to have intrinsic processive ubiquitination activity, which is promoted allosterically by a free Ub noncovalently binding to UBE2B backside.[71] Therefore, UBE2B interaction with RAD18 via the UBC backside binding RAD18 helix (hereafter UBE2B Binding Region; 2BBR) serves the dual purpose of achieving specificity between this E2-E3 pair, and competitively inhibiting the allosteric activation of processive ubiquitination.[71]

In the case of UBE2B:RAD18, the 2BBR helix is held in place with an extensive hydrogen-bonding network and several key hydrophobic contacts [Fig.

6(B)]. Crucially, N-terminus of 2BBR occupies the cleft between UBE2B β1-β2, where Y342, R343, H346 and E349 on 2BBR form salt bridges with the polar UBE2B residues making this cleft. At the same time, from 2BBR C-terminus through to N-terminus, Y361, A357, L353 and F350 are within van der Waals radii of V22, F41, V39 and V56, respectively, which traverse the UBE2B β-meander [Fig. 6(B)]. This arrangement ensures a tight and specific binding between UBE2B-RAD18.

The RING E3 RNF25 also contains a specialised helix that that binds to UBC backside.[72,73] Unlike GP78 and RAD18, RNF25 is reported to work with two E2s, UBE3D2 and UBE2E3, with comparable tight affinity,[72,73] suggesting that the specialised RNF25 helix (hereafter UBE3D2 and UBE2E3 Binding Region; D2E3BR) may have dual-specificity. Moreover, D2E3BR binding has different effect on UBE3D2 and UBE2E3. When bound to UBE3D2, D2E3BR abolishes processive ubiquitination, via a mechanism similar to that seen in UBE2B-RAD18.[41,48] Though, the biological significance of this is not yet known.[73] On the other hand, D2E3BR binding to UBE2E3, which normally monoubiquitinates its substrates, promotes processivity by forcing UBE2E3~Ub in close-state, thereby increasing UBE2E3~Ub:RNF25 reactivity.[72]

D2E3BR is tethered to the RING core by a short ~20 residue linker, ensuring that RNF25 makes a tight clamp around the E2s that it interacts with. Complex structure of UBE3D2:RNF25 supports this model: M222 on RNF25-D2E3BR linker fits in-between K4 and H7 side chains on UBE3D2 α1 (K5 and Q7 in UBE2E3, respectively; Fig. S1), while R244 and R248 at the C-terminal end of the UBE2E3

helix coordinates the terminal UBE3D2 carboxylate, and finally, the D2E3BR tail runs antiparallel to UBE3D2 β4-α2 loop, forming series of hydrogen bonds [Fig. 6(A)].[73]

Specialised E2-RING E3 interaction facilitated by the RING domain itself

The poorly characterised RING E3 ligase ZNRF1 was found to tightly interact with UBE2N in cells,[74] a broad-specificity E2 that associates with many other E3s to build K63-type Ub chains.[75] ZNRF1 is known to bind UBE2Ds and UBE2W, but it appears to be specialised to interact with UBE2N in particular, with its binding affinity against UBE2N being ~50 nM.[59,75] *In vitro* data suggests that ZNRF1 might inhibit UBE2N activity via binding it tightly.[59] The UBE2N:ZNRF1 complex structure illustrates the details of this astonishingly tight E2-E3 interaction and reveals that it is mediated by specialisation of ZNRF1 within its canonical E2-E3 binding interface.[59]

In this specialised E2-E3 interaction, ZNRF1 ZI_A displays not only L188, which stacks in-between R7 and K7 side chains, but also two additional carboxylate-containing residues that form an extensive hydrogen-bonding network not seen in other E2-E3 pairs. ZNRF1 ZI_A E183 and E189 forms salt bridges with UBE2N R14 and R6, while V185, I186 and L188 backbone carbonyls are coordinated by UBE2N R7 and K10 (Fig. 7). Notably, while the specialisation at ZNRF1 ZI_A offers UBE2N:ZNRF1 interaction unusually high affinity, it does not hamper either partner to interact with others.

The Fanconi anaemia pathway mediates DNA interstrand crosslink repair, where site-specific monoubiqutination of FANCD2 acts as the key signalling event

that triggers recruitment of the required downstream DNA repair factors.[27] The RING E3 ligase FANCL interacts with UBE2T to mediate FANCD2 monoubiqutination.[39] With the UBE2T-FANCL binding affinity being in the sub-μM range, FANCL exclusively select UBE2T from a pool of E2s.[39] The reason for the remarkable specificity between UBE2T and FANCL lies within two-fold speciation of the UBE2T-FANCL binding interface, which display a more extensive interaction network than the typical E2-RING E3 pair.

The canonical E2-RING interaction involves stacking of a variable RING ZI_A residue in-between two branched residues on E2 α1. In UBE2T:FANCL, however, this variable residue is tyrosine and extends hydrophobic contacts within the E2/α1-RING/ZI_A interface further. FANCL Y311 fits in-between UBE2T R6 and R7, with its aromatic sidechain forming π-π stacking interaction with R6 and R9 guanidium groups and its hydroxyl group forming an additional salt bridge with N103 on UBE2T L7. Moreover, S5 on UBE2T α1, which is typically a bulky residue like lysine or glutamine in other E2s (Fig. S1), acts a negative selectivity factor. UBE2T/L4-FANCL/α1 interface also displays extended contact area, with R60 on N-terminal end of UBE2T L4 coordinating E340, an additional residue on FANCL ZII_B (Fig. 8). In other E2s, the position equivalent to UBE2T R60 frequently carries the opposite charge, with only UBE2W sharing this variation (Fig. S1, S2). Reversing the charge on this residue by mutating R60 alone abolishes UBE2T binding to FANCL.[39] Finally, FANCL ZII_B carries S363 instead of the linchpin arginine seen in most other RING E3s (Fig. S2). UBE2T forms a salt bridge with the ZII_B S363 hydroxyl via R99 on L7

(Fig. 8), which is substituted with different residues all other E2s except UBE2I and AKTIP (Fig. S1), and adds another layer of specificity to UBE2T:FANCL interaction.

Concluding remarks

The human genome encodes 38 E2s and over 600 predicted RING E3s. Current evidence suggests that both E2 and E3 family enzymes align most closely at the structural level in the regions that comprise the E2-E3 binding interface. The high structural conservation of this canonical interface suggests all E2s could potentially bind all E3s indiscriminately, and vice versa. However, experimentally this is not the case, suggesting there is some control over specificity.

Over the past two decades the question of how E2-E3 pairs interact and how some E2-E3 pairs achieve specificity have been extensively studied, generating 29 E2-E3 complex structures with a unique RING E3 component. Among these, 23 out of the 29 contain only broad-specificity E2s, namely UBE2D1, UBE3D2, UBE2D3 or UBE2N. Thus, the structural basis of the canonical E2-E3 interaction is well understood. Structurally-characterised examples of E2-E3 pairs where each partner shows specificity for the other, however, are only beginning to emerge. The list includes UBE2G2:GP78, UBE2B:RAD18 and UBE3D2/UBE2E3:RNF25, all of which achieve specificity via regions on the E3 that are outside the RING domain. Another RING E3, ZNRF1 has adaptations within its RING domain that allows a tight interaction with UBE2N, but not with other E2s. UBE2T and FANCL, on the other

hand, show exquisite specificity towards each other, which is mediated by two-fold speciation of the canonical E2-E3 interface.

Large numbers of predicted RING E3 sequences are yet to be characterised, while many that have been characterised were studied using truncated proteins lacking the regions outside the RING scaffold. The future, therefore, is likely to reveal myriad other mechanisms that E2-E3 pairs employ to achieve specificity.

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Figure legends

Figure 1: The conserved UBC fold of E2s and variation in the E3-binding region

(A) Ribbon representation of UBE3D2 (PDB: 2ESK),[52] showing the residues involved in catalysis as ball-and-sticks, and highlighting the E1-/E3-binding sites. The ribbon diagram is coloured to reflect the average pairwise positional shift of each overlapping C_α atom between UBE3D2 and UBE2A (PDB: 6CYO),[76] UBE2B (PDB: 1JAS),[77] UBE2C (PDB: 1I7K),[78] UBE2D1 (PDB: 2C4P), UBE2D3 (PDB: 5EGG),[79] UBE2E1 (PDB: 5BZH), UBE2E2 (PDB: 1Y6I), UBE2G1 (PDB: 2AWF), UBE2H (PDB: 2Z5D), UBE2J2 (PDB: 2F4W), UBE2K (PDB: 1YLA), UBE2Q1 (PDB: 2QGX), UBE2Q2 (PDB: 1ZUO), UBE2R1 (PDB: 2OB4), UBE2S (PDB: 1ZDN), UBE2T (PDB: 1YH2), UBE2U (PDB: 1YRV), UBE2V1 (PDB: 2A4D), BIRC6 (PDB: 3CEG),[80] UBE2F (PDB: 2EDI), UBE2G2 (PDB: 2CYX),[81] UBE2I (PDB: 1A3S),[82] UBE2L3 (PDB: 5TTE),[82] UBE2L6: (PDB: 1WZW), UBE2M (PDB: 1Y8X),[33] UBE2N (PDB: 2C2V),[40] UBE2V2 (PDB: 4ONM),[83] UBE2W (PDB: 2MT6) and UBE2Z (PDB: 5A4P).[84] **(B)** UBE3D2, UBE2N, UBE2L3 and UBE2T E3-binding sites, made up of their respective helix 1 (α1), Loop 4 (L4) and Loop 7 (L7), shown as surface representation, with the surface coloured using the "Yellow-Red-Blue" script,[84] which colours surface exposed hydrophobic, negatively charged and positively charged residues yellow, red and blue, respectively. The composite nature of the E3-binding site is highlighted by demarcating individual components with black lines. Orientation

of the UBE3D2 is rotated 45 degrees clockwise on x and y axes relative to that in panel A.

Figure 2: The conserved cross-brace fold of RING E3s

(A) Ribbon representation of a single RNF4 monomer from UBE2D1~Ub:RNF4 complex (PDB: 4AP4)[49] showing the zinc ions as spheres, the residues involved in zinc-coordination and the allosteric linchpin residue as ball-and-sticks, and demarcating the E2 -binding site. The ribbon diagram is coloured to reflect the average pairwise positional shift of each overlapping C_α atom between RNF4 and unique RING cores from all available E2-RING E3 complex structures including RNF146 (PDB: 4QPL),[85] TRIM25 (PDB: 5FER),[86] BIRC2 (PDB: 6HPR),[50] BIRC3 (PDB: 3EB6),[51] BIRC7 (PDB: 4AUQ),[52] RNF38 (PDB: 4V3K),[48] RNF25 (PDB: 5D1M),[73] RNF165 (PDB: 5D0M),[66] MDM2 (PDB: 5MNJ),[87] TRIM23 (PDB: 5VZW),[63] RNF13 (PDB: 5ZBU), E4B (PDB: 3L1Z),[88] RNF2 (PDB: 3RPG),[64] GP78 (PDB: 2LXP),[65] SIZ1 (PDB: 5JNE),[89] c-CBL (PDB: 1FBV),[24] RBX1 (PDB: 4P5O),[90] TRAF6 (PDB: 3HCT),[91] TRIM5 (PDB: 4TKP),[48] RNF8 (PDB: 4WHV),[62] ZNRF1 (PDB: 5YWR),[59] LNX1 (PDB: 5H7S),[92] CHIP (PDB: 2C2V),[40] FANCL (PDB: 2CCG).[39] **(B)** UBE2D1~Ub:RNF4 complex highlighting position of the RING domain relative to the E2, with the allosteric linchpin residue coordinating E2 as well as the donor Ub shown as ball-and-sticks. RING, E2 and Ub are shown as blue-red, white, and grey

ribbons, respectively. Orientation of the UBE2D1~Ub:RNF4 complex is arranged so that the UBE2D1 is superposed on the UBE3D2 in Figure 1(A).

Figure 3: Canonical E2-RING interactions illustrated on RNF4:UBE2D1 complex

Ribbon representation of a UBE2D1~Ub:RNF4 complex dimer, with the donor Ub and the second E2-RING E3 dimer in the asymmetric unit hidden from view for clarity (PDB: 4AP4).[49] Zinc ions, residues involved in the canonical E2-RING E3 interaction and hydrogen-bonding interactions are shown as spheres, ball-and-sticks and dashed lines, respectively.

Figure 4: Superposition of several canonical E2:RING complexes

Superposition of the 20 available E2-RING E3 complexes, where the E2 is UBE2D1, UBE3D2 or UBE2N [see Fig. 2(A) legend for the full list], illustrating the precision of RING recruitment by the UBC fold. E2 and RING E3 in each complex is shown as ribbons and wires, respectively. For clarity, where present, regions outside the the first E2-RING E3 complex, such as the E2-RING E3 dimer. E2:RING E3 complexes are positioned so that the E2 component is superposed on the UBE3D2 in Figure 1(A).

Figure 5: Backside comparison of UBE2G2, UBE2B and UBE3D2

UBE2G2-GP78 (PDB: 2LXP),[65] UBE2B-RAD18 (PDB: 1YBF)[71] and UBE3D2-RNF25 (PDB: 5D1M)[73] complex structures, where the E2 component and the backside

binding E3 helix are shown as surface and ribbon representations, respectively. To highlight the differences seen in the different UBC backside regions, surface residues were coloured using the "Yellow-Red-Blue" script,[84] which colours surface exposed hydrophobic, negatively charged and positively charged residues yellow, red and blue, respectively. Orientation of the structures are arranged so that the E2s are rotated 120 degrees anti-clockwise relative to that in Figure 1(A).

Figure 6: Specialised E2-RING E3 interactions facilitated accessory regions

(A) Ribbon representation of UBE2G2-GP78 (PDB: 2LXP),[65] showing the zinc ions as spheres, residues involved the specialised E2-RING E3 interaction as ball-and-sticks, and hydrogen-bonding interactions as dashed lines. **(B)** Ribbon representation of UBE2B-RAD18 (PDB: 1YBF),[71] showing the zinc ions as spheres, residues involved the specialised E2-RING E3 interaction as ball-and-sticks, and hydrogen-bonding interactions as dashed lines. **(C)** Ribbon representation of UBE3D2-RNF25 (PDB: 5D1M),[73] showing the zinc ions as spheres, residues involved the specialised E2-RING E3 interaction as ball-and-sticks, and hydrogen-bonding interactions as dashed lines.

Figure 7: Specialised UBE2N-ZNRF1 interaction facilitated by the RING domain itself

Ribbon representation of a single UBE2N:ZNRF1 dimer, with the second E2-RING E3 dimer hidden from view for clarity (PDB: 5YWR).[59] Zinc ions, residues involved in E2-RING E3 interactions and hydrogen-bonding interactions are shown as spheres,

ball-and-sticks and dashed lines, respectively. The orientation of the complex is the same as in Figure 3.

Figure 8: Specialised UBE2T-FANCL interaction facilitated by the RING and the E2

Ribbon representation of a single UBE2T:FANCL dimer, with the second E2-RING E3 dimer hidden from view for clarity (PDB: 4CCG).[39] Zinc ions, residues involved in E2-RING E3 interactions and hydrogen-bonding interactions are shown as spheres, ball-and-sticks and dashed lines, respectively. The orientation of the complex is the same as in Figure 3.