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New classes of E3 ligases illuminated by chemical probes

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Abstract

Specificity in the ubiquitin system depends on E3 ligases, largely belonging to a handful of families discovered more than a decade ago. However, the last two years brought a quantum leap in the identification and/or mechanistic characterization of eukaryotic ubiquitin ligases, in part through implementation of activity-based chemical probes and cryo-EM. Here, we survey recent discoveries of RING-Cys-Relay, RZ-finger, and neddylated cullin–RING–ARIH RBR E3–E3 ubiquitin ligase mechanisms. These ligases transfer ubiquitin through unprecedented mechanisms—via novel catalytic domains or domain combinations—and collectively modify unconventional amino acids, non-proteinaceous bacterial lipid targets, and structurally-diverse substrates recruited to numerous cullin–RING ligases. We anticipate major expansion of the types, features, and mechanisms of E3 ligases will emerge from such chemical and structural approaches in the coming years.

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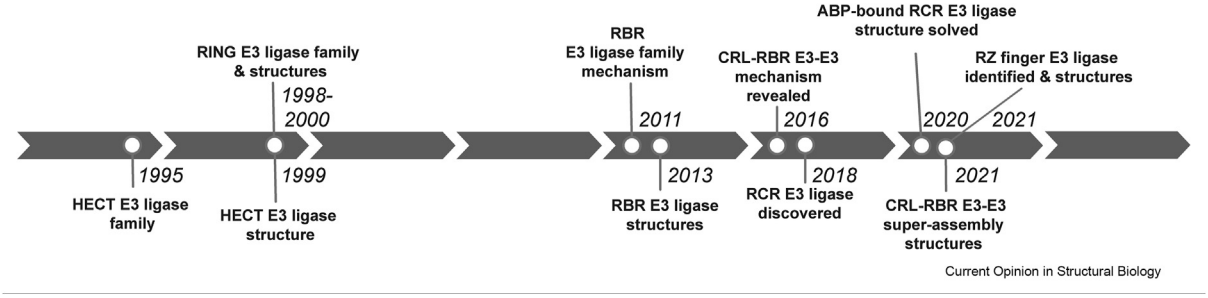
Introduction

Protein ubiquitylation regulates virtually all eukaryotic processes. This depends on E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases collaboratively marking specific proteins with ubiquitin, and in many cases with chains of ubiquitins linked to each other. The vast importance of ubiquitylation is underscored by the estimated numbers of E2 and E3 enzymes—roughly 40 and 600, respectively, in humans, and even more in plants and some other organisms. Different E2 and E3 combinations determine step-by-step progression of biochemical processes in cell division, development, signal transduction, transcriptional regulation, and more [1,2]. Furthermore, mutations in many E3 ligases have severe physiological effects, and either directly cause or contribute to cancers, developmental disorders, neurodegeneration, hypertension, and other pathologies [3–6]. Moreover, viruses and bacteria deploy their own proteins to manipulate host E2s and E3s in ways that contribute to infections [7,8]. Therefore, it of great interest to understand how E3 ligases, together with E2 partners, achieve the fundamental function of transferring ubiquitin to their specific targets. Indeed, small molecule E3 ligase inhibitors and degraders triggering ubiquitin-mediated proteolysis are hot platforms for drug discovery [9,10].

Two major families of E3 ligases, with hallmark catalytic domains called "HECT" (**h**omologous to **E**6AP **C**-**T**erminus) and "RING" (**r**eally **i**nteresting **n**ew **g**ene) were identified in the 1990s [11,12] (Figure 1). For both classes of E3s, substrates are typically recruited to a protein–protein interaction domain, and modified on lysine side-chains, including lysines on ubiquitin during polyubiquitylation. HECT E3s ubiquitylate substrates via a two-step mechanism, with an E3 Cys mediating catalysis: ubiquitin is transferred from the E2 catalytic Cys to that in the E3 HECT domain, and from the HECT E3 Cys to the substrate. These reactions depend on the E2 binding to the N-terminal lobe of the HECT domain, and ubiquitin—initially linked to the E2 and subsequently to the E3 active site—making noncovalent interactions with the C-terminal lobe of the HECT domain [13]. By contrast, RING E3s function indirectly. The RING domain binds both the E2 and the ubiquitin in an E2∼ubiquitin intermediate ("∼" here refers to thioester bond between an enzyme and ubiquitin or a stabilized mimic of this labile bond, and "_" to noncovalent interactions between proteins in multiprotein complexes). The E3 RING domain, E2, and ubiquitin form a tightly-packed "closed" arrangement that allosterically activates the thioester-bond and stimulates ubiquitin discharge from the E2 to lysine [14–16]. As such, RING E3s bridge an activated E2∼ubiquitin intermediate and the remotely-bound substrate. It took more than a dozen years before the discovery of another

Figure 1



Historical timeline of E3 ubiquitin ligase research. Each bar represents five years, with discoveries of major classes of E3 ligase and their mechanisms highlighted.

major E3 type, the RBR family, named for three sequential hallmark domains originally called **R**ING1, I**B**R and **R**ING2 but now termed **R**ING1, I**B**R, and **R**cat [17] (Figure 1). The RBR mechanism has been referred to as "RING-HECT hybrid" because the RING1 domain binds the E2 portion of an E2∼ubiquitin intermediate much like canonical RING domains bind E2s [17]. However, ubiquitin is splayed apart from the E2 and cradled in a cleft between RING1 and the IBR domain to generate an open E2∼ubiquitin conformation that is conceptually related to but structurally-distinct from that in a HECT E3-E2∼ubiquitin complex [18]. The RBR E3 Rcat domain is a unique zinc-containing fold wherein a non-liganded catalytic Cys mediates 2-step ubiquitin transfer analogous to HECT E3s [17,19].

Whether there are additional types of eukaryotic E3 ligases, or if E3 RING domains always recruit E2s, had remained largely unclear for nearly a decade. Several pathogenic microbes deploy unique bacterial E3 ligases (BELs), which are reviewed elsewhere [20]. Although a number of eukaryotic proteins lacking RING, HECT, or RBR domains have been reported as having E3 ligase activity, for the most part their catalytic mechanisms remain elusive. Nonetheless, the number of structurally-defined ubiquitylation mechanisms has doubled in the past year: identifying and/or structurally characterizing RCR (RING-Cys-Relay), RZ-finger, and E3–E3 ubiquitin ligases depended in part on the development and implementation of reactive activity-based chemical probes mimicking E2∼ubiquitin intermediates (E2∼ubiquitin ABPs) [21–28] (Figure 1). Here, we survey chemical features of E2∼ubiquitin ABPs, and the structures they enabled visualizing that represent fleeting ubiquitylation intermediates.

Activity-based reactive probes for capturing ubiquitylation complexes

One challenge to structural studies of ubiquitylation is that substrates are often modified in less than 1 s [29]. A variety of approaches developed to stabilize the fleeting transition states, including by E2 or E3 mutation [30,31], or chemical biology, have been recently reviewed [32]. Here, we focus on chemical approaches developed based on the hypothesis that fleeting transition states depend on transient but avid protein-protein interactions determining enzymatic architectures. The notion is that reaction with ABPs harness ubiquitin E2s, E3s, and/or substrates in their active conformations. A key breakthrough was an ABP mimicking an E2∼ubiquitin intermediate, but with an electrophile situated between ubiquitin's C-terminus and the active site cysteine of the E2 UBE2L3 [33]. Virdee et al. first developed such an ABP to investigate intricate phosphorylation-dependent allosteric activation of the RBR-type E3 ligase Parkin that is mutated in autosomal recessive juvenile Parkinson's disease. Parkin reactivity with the probe scaled with ubiquitylation activity for purified proteins in vitro, and with endogenous Parkin in cell lysates. Thus, Parkin reactivity with the UBE2L3∼ubiquitin ABP allows screening effects of disease mutations [33].

The ubiquitin pathway is hierarchical; most E2s functions with many E3s, so one E2∼ubiquitin ABP could probe much ubiquitylation. Indeed, the UBE2L3∼ubiquitin ABP reacted with catalytic domains of E3 ligases known to harbor catalytic cysteines [23,33]. This set the stage for implementing ABPs mimicking E2∼ubiquitin complexes for new discoveries (Figure 1).

MYCBP2 defines a RING-Cys-Relay (RCR) class of E3

Application of variants of the original E2∼ubiquitin probe (biotinylated for recovery, harboring a more native-like ubiquitin, with two different E2s and mutants in their known E3 binding surfaces) to human cell lysates, followed by affinity purification and mass spectrometry, showed reactivity with a major fraction of all proteins harboring HECT and RBR domains [23]. The RING domain-containing protein MYCBP2 preferentially reacted with the probe harboring an electrophile between ubiquitin's C-terminus and the active site of

the E2 UBE2D3, but not with a mutant in UBE2D3's canonical RING binding site, and to a lesser extent with the ABP having UBE2L3 as E2. This was unexpected, because MYCBP2, which is a neuron-associated E3 ligase responsible for synaptogenesis and axon termination [34], lacks a recognizable domain expected to react with the probe (Figure 2a).

Extensive biochemical characterization revealed a novel catalytic domain, an unprecedented RING-Cys-Relay (RCR) mechanism, and a distinctive amino acid target: the data suggest that ubiquitin is passed in an unorthodox manner, from the catalytic Cys of the RING bound E2 UBE2D3, then between upstream and downstream catalytic cysteines in a MYCBP2 tandem-cysteine (TC) domain, and ultimately to a Thr side-chain hydroxyl group [23].

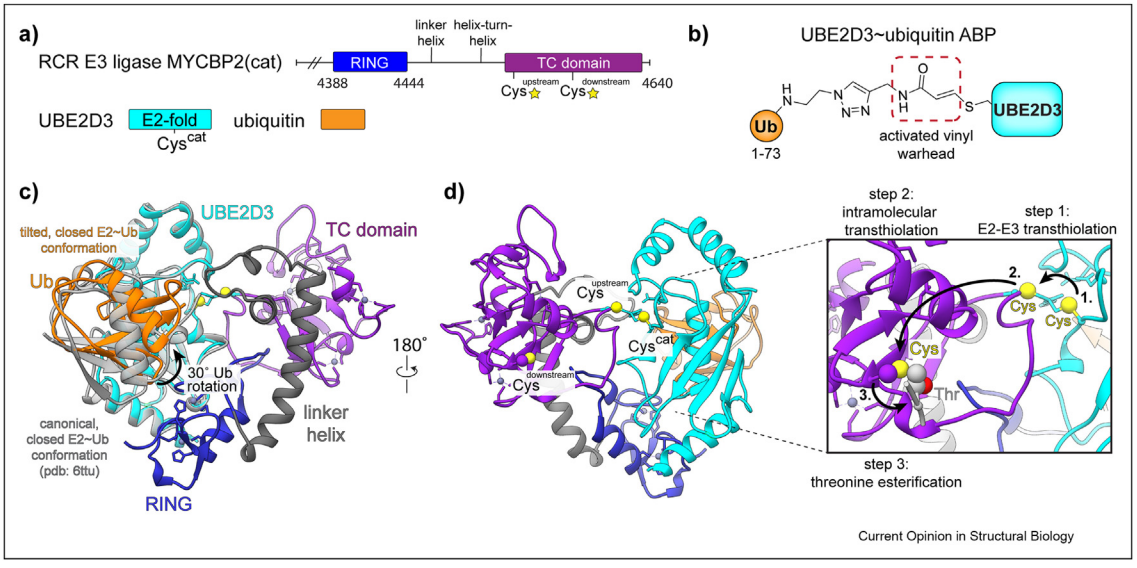
The structural basis for ubiquitin transfer from the E2 to the upstream TC domain catalytic Cys was revealed from crystallographic analysis of the MYCBP2 RCR domain reacted with the UBE2D3~ubiquitin probe [35]. The RING engages the UBE2D3~ubiquitin intermediate in a nearly archetypal closed conformation [35]. However, the orientation between UBE2D3 and its linked ubiquitin is slightly skewed from that formed with a canonical RING (Figure 2b). Also, MYCBP2 lacks a so-called "linchpin arginine," often found in conventional RING E3s, which stabilizes the activated UBE2D3~ubiquitin conformation [14–16]. As a result, the MYCBP2 RING's ability to stimulate ubiquitin discharge from UBE2D3 toward the conventional lysine nucleophile is relatively tempered, and transfer to the TC domain upstream cysteine is favored instead. Although the subsequent reaction has not been visualized structurally, Virdee et al. propose that after ubiquitin is linked to the upstream cysteine, the relay to the downstream cysteine is steered by an entropically driven helix–coil transition of a so-called mediator loop [35] (Figure 2c).

Ultimately, the MYCBP2 RCR domain preferentially transfers ubiquitin to side-chain hydroxyls rather than lysine or a protein's N-terminus. A potential docking site for a Thr acceptor was observed in the crystal structure of the RCR domain alone [23], where the downstream catalytic Cys is adjacent to a Thr from a neighboring protein in the crystal lattice. Thus, the relay mechanism enables unique targeting, by virtue of one Cys optimized to receive ubiquitin from the RING-bound E2, while the structural malleability of the second catalytic loop allows receiving ubiquitin from the first Cys, and relocation adjacent to a Thr acceptor (Figure 2d).

ATP-dependent RZ finger E3 ligase RNF213

The E2~ubiquitin probe reactivities also pointed toward the RING domain-containing protein RNF213 as a distinctive E3 ligase [23]. Mutation of RNF213 causes the cerebrovascular disorder Moyamoya disease, and

Figure 2



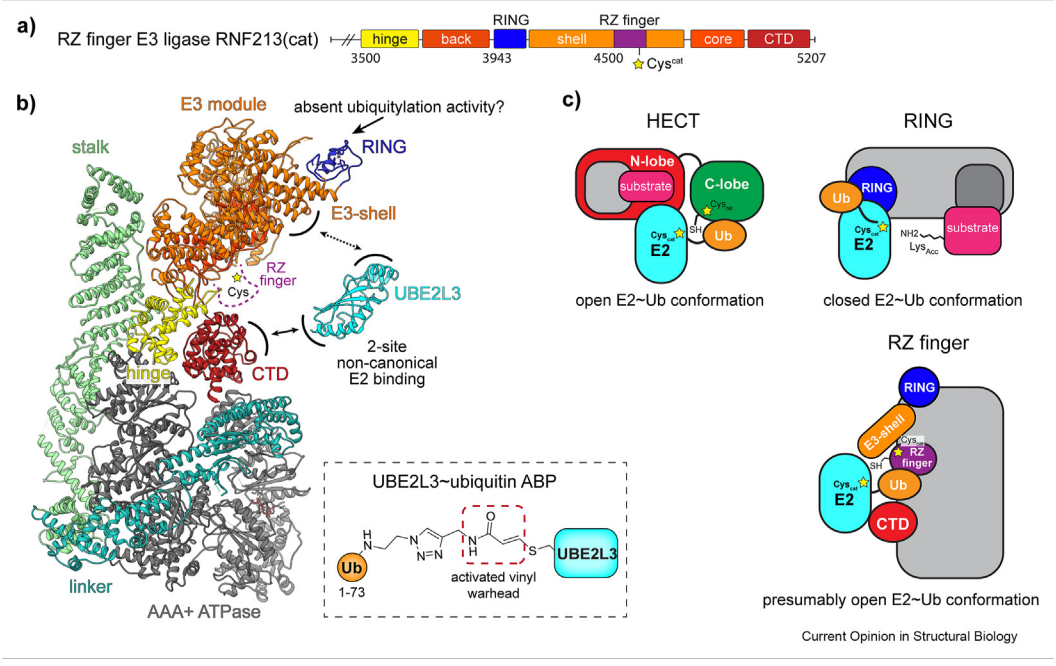
Structural arrangement of ubiquitin transfer by RCR ligase MYCBP2. (**a**) Color-coded schematics of proteins and domains participating in RCR-mediated ubiquitylation [defined by MYCBP2 catalytic (cat) domain], which occurs in 3 steps, from an E2 to the TC domain upstream cysteine, to the downstream cysteine, to a threonine acceptor. Upstream and downstream catalytic cysteines indicated with yellow stars (**b**) Chemical structure of UBE2D3~ubiquitin-ABP employed in this study. The electrophilic warhead is indicated with a dotted red line. (**c**) Comparison of E2~Ubiquitin conformation between UBE2D3~ubiquitin-MYCBP2 (PDB ID: 5O6C, captured with an E2~ubiquitin ABP) and canonical, RING-E2~ubiquitin closed conformation (PDB ID: 4AP4). The relatively skewed conformation of MYCBP2-bound E2~ubiquitin may favor intramolecular ubiquitin transfer rather than discharge to lysine. (**d**) UBE2D3~ubiquitin activity-based probe-captured MYCBP2 rotated horizontally by 180°. Close-up displays threonine from a neighboring molecule in the crystal lattice potentially occupying MYCBP2's ubiquitin acceptor site (PDB ID: 6T7F).

reducing RNF213 levels by CRISPR ameliorates lipotoxicity, but little was known of RNF213 ubiquitin ligase activity or its direct targets [36,37]. Unlike MYCBP2 whose reactivity was specific to only one of the four original E2∼ubiquitin probes, RNF213 reacted equally well with probes generated with different E2s (UBE2D2 and UBE2L3), and with mutants in canonical RING binding residues. These properties hearkened the astonishing target—and extraordinary ubiquitylation mechanism not involving any known E3 ligase domain - revealed by three recent studies. Notably, a cryo EM structure of this unusually large (586 kDa) protein, from Clausen et al., revealed an N-terminal stalk, a dynein-like AAA-type ATPase domain, and an E3-ligase module containing the RING and other domains, none of which explained reactivity with the probe [24] (Figure 3a, b).

Randow et al. discovered RNF213 as the E3 ligase responsible for coating cytosol-invading Salmonella with ubiquitin—but not on a proteinaceous target [25]. Elegant deductive studies, including employing Salmonella mutants defective in generating portions of bacterial lipopolysaccharide (LPS), showed RNF213 ubiquitylates lipid A, most likely via ester linkages between one or more of its hydroxyl groups and ubiquitin's C-terminus. However, the RING domain is neither required for intrinsic E3 ligase activity as monitored by autoubiquitylation [24], nor for LPS esterification activity [25]. Unexpectedly, a 27-residue "RZ-finger" was required for E3 ligase activity.

So, how does this giant novel E3 work? Cryo EM data for a complex reacted with the UBE2L3∼ubiquitin probe, which represents the transition state for ubiquitin transfer from this E2 to RNF213, confirmed that the RING domain does not bind E2 [27]. Instead, UBE2L3 is recruited to two distinct sites by the RNF213 "E3 shell" and C-terminal domain, for ubiquitin linkage to the RZ-finger catalytic cysteine (Figure 3b, c). The specific interactions are unique to RNF213, and are not found in HECT or RING E3s. Nonetheless, other E3s including anaphase-promoting complex/cyclosome (APC/C) and neddylated cullin–RING ligase E3s likewise bind their cognate E2s through multivalent interactions

Figure 3



Structure of RZ-finger E3 ligase RNF213 and E3 ligase models. (**a**) Schematic of RNF213 color-coded by domains. RZ-finger catalytic Cys4462 is depicted as yellow star. CTD, C-terminal domain. (**b**) Structure of RNF213 with domains colored as in (**a**) RZ-finger, poorly visible by cryo-EM, is depicted with dotted line. Two sites on RNF213—the CTD and E3-shell-mediated non-canonical interactions with E2 UBE2L3. Dashed lines approximate sites of UBE2L3-E3-shell interaction, which are not clearly resolved in cryo-EM density. Box indicates chemical structure of UBE2L3∼ubiquitin-ABP. The electrophilic warhead is highlighted with a dotted red line. (**c**) Top left-working model for ubiquitin transfer from an E2 to a HECT E3 ligase. The HECT domain N-lobe binds E2 while its linked ubiquitin extends away in an open conformation to contact HECT C-lobe for transfer to the catalytic cysteine. Top right-working model for ubiquitin transfer from an E2 bound to a RING E3. A closed, canonical conformation, in which the E2, ubiquitin, and the RING domain all contact each other, promotes ubiquitin transfer to a substrate's lysine residue. Ultimately, the closed arrangement stimulates the discharge from E2 to lysine. Bottom-working model for ubiquitin transfer to RZ-finger E3 ligase. Instead of binding to RNF213's RING domain, E2∼Ubiquitin engages the RNF213 CTD and E3-shell in an open-like conformation to discharge ubiquitin onto the RNF213 catalytic cysteine.

[30,38]. Ubiquitin linked to the RZ-finger was poorly visible in the map, perhaps reflecting functionally-important conformational heterogeneity. Notably, ATP, stimulates RNF213-dependent discharge of ubiquitin from UBE2L3 [27]. Future studies will undoubtedly reveal how these and yet other atypical E3 features activities are coordinated to ubiquitylate Lipid A, and potentially other unconventional human targets regulating lipid levels.

CRL–RBR E3–E3 ligase super-assemblies

The largest E3 family—Cullin–RING ligases (CRLs)—play essential roles in health and disease, and are major platforms for therapeutic development. Substrates are recruited to interchangeable receptor subunits connected to one end of an elongated cullin subunit. A RING-domain-containing "RBX" protein bound to the other end of the cullin mediates E3 ligase activity [39–41]. The impact of CRLs derives from numerous receptors and several cullin–RBX complexes, for example, in humans, ≈70 CRL1s comprising F-box protein substrate receptors and CUL1-RBX1, and ≈40 CRL5s with BC-box protein substrate receptors and CUL5-RBX2. CRL assembly and ubiquitylation activity are stimulated by site-specific cullin modification by the ubiquitin-like protein NEDD8 [39–41]. Some neddylated CRLs function like conventional RING E3s, binding an E2∼ubiquitin intermediate in the closed conformation, which is activated and steered to substrate by the cullin's linked NEDD8. However, many neddylated CRLs employ an ARIH-family RBR-type E3 as the ubiquitin carrying enzyme [22]. Co-assembly of neddylated CRL and ARIH E3s into E3–E3 ligases is specific: some RBX1-containing CRLs partner with ARIH1, CRL5s that harbor the RING subunit RBX2 partner with ARIH2, and CUL9 is a single polypeptide encompassing both a cullin and an RBR amongst other domains [21,22,42,43]. Genetic studies have implicated CRL-ARIH E3–E3 ligases as regulating translation in yeast, development of nematodes, and diverse processes ranging from mRNA decay to HIV infectivity in human cells [42,44–46].

The structural mechanisms of E3–E3-mediated ubiquitin ligation were revealed by cryo-EM of complexes generated with three ABPs: (1) a UBE2L3∼ubiquitin ABP reacted with neddylated CRL1-bound ARIH1 captured the conformation representing ubiquitin transfer between E2 and ARIH1 E3; (2) an ABP with an electrophile at ubiquitin's C-terminus enabled generation of a complex representing a neddylated CRL1-bound ARIH1∼ubiquitin intermediate; and (3) an ABP with an electrophile between ubiquitin's C-terminus and a peptide substrate captured the conformation representing substrate ubiquitylation [26].

The structures reveal that formation of the complex transforms the CRL1 and ARIH1 E3s. Although each E3 is inactive on its own, together they form an active neddylated CRL–ARIH1 E3–E3 supercomplex. The E3–E3 complex adopts radically different conformations for the two transition states [26]. First, interactions with CUL1-linked NEDD8 allosterically activate ARIH1 to properly bind the UBE2L3∼ubiquitin intermediate. Meanwhile, ARIH1's autoinhibitory "Ariadne" domain binds RBX1's RING domain and portions of CUL1, releasing ARIH1's Rcat domain to capture ubiquitin from UBE2L3. The ubiquitin-linked Rcat domain relocates to hover near F-box-protein bound substrates. This arrangement allows ubiquitylation of diverse substrates recruited to diverse CRL receptors [26].

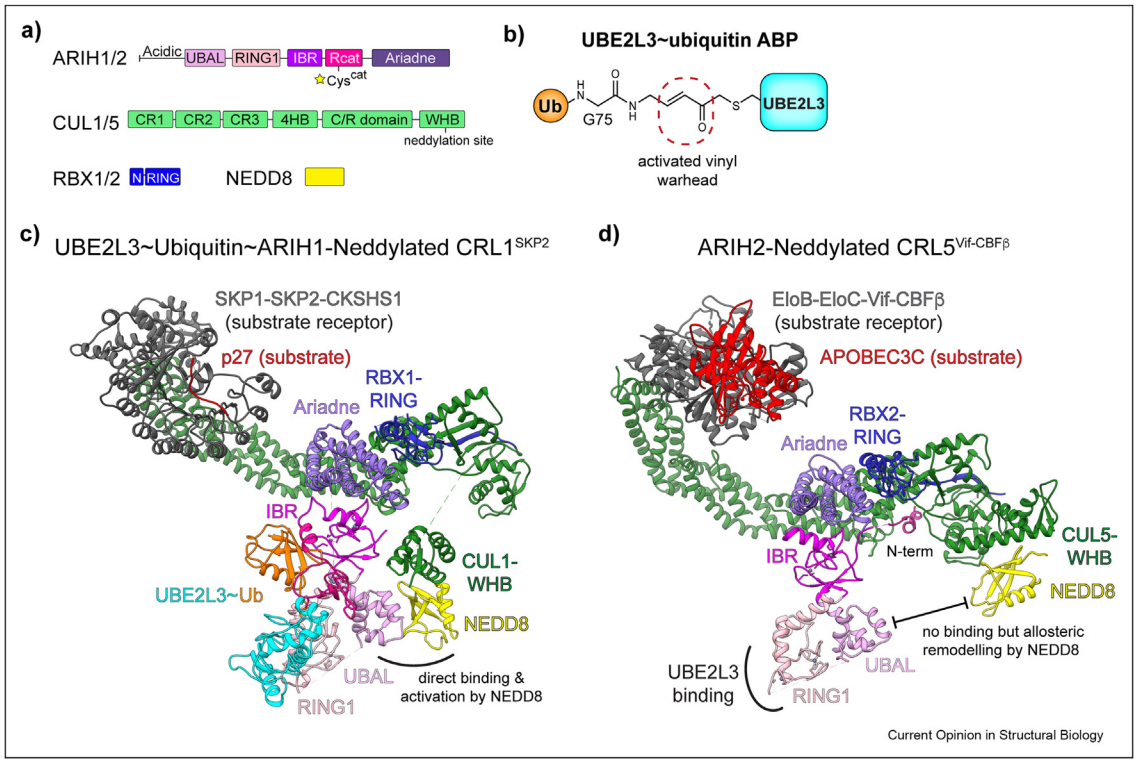
Due to high homology, it was anticipated that ARIH2 and ARIH1 alone, and their the neddylated CRL-bound assemblies would superimpose, and that specificity would be dictated by sequence differences at ARIH–CRL interfaces. Comparing structures of ARIH1 and ARIH2 revealed distinct autoinhibition complementing their cognate neddylated CRL partners [26,28] (Figure 4a–d). ARIH1 and ARIH2 Ariadne domains both restrain the Rcat domain, which is released upon Ariadne domain binding to its specific partner CUL-RBX complex. However, auto-inhibited ARIH2 is poised to properly engage UBE2L3∼ubiquitin, thus obviating the need to directly engage NEDD8. Instead, NEDD8 binds two domains from CUL5. The resultant remodeling generates a groove that binds ARIH2's otherwise intrinsically disordered N-terminus (Figure 4a–d). The role of ARIH1's homologous N-terminal sequence remains elusive.

The strikingly distinct CRL–ARIH assemblies demonstrate closely related E3 ligase pairs have their own mechanisms to amalgamate and jointly ubiquitylate substrates [26,28]. Remarkably, a ubiquitin-like protein alters the properties of homologous targets completely differently. Unlike previously-characterized assemblies where ubiquitin or a ubiquitin-like protein directly mediates interactions, the neddylated CRL5–ARIH2 structure shows NEDD8-mediated allostery indirectly driving interactions [28].

Future perspectives

The RCR, RZF, and E3–-E3 structures revealed unexpected mechanisms of ubiquitin ligation by proteins harboring known E3 ligase motifs, answered longstanding questions including how hydroxyl groups are modified and how CRLs accommodate diverse substrates, and defined functions of NEDD8. A common theme is that conventional—or even novel-enzymatic activities are tempered such that ubiquitylation depends on each unique mechanism: MYCBP2-bound UBE2D3∼ubiquitin disfavors lysine targeting and thus favors threonine via the tandem Cys relay [23,35]; RNF213 is relatively inactive in the absence of nucleotide binding its dynein-like domain

Figure 4



Comparison of different neddylated CRL–RBR E3–E3 super-assemblies. (**a**) Color-coded schematics of domains and proteins in E3–E3 ubiquitin ligases. Catalytic cysteines in ARIH1 (Cys357) and ARIH2 (Cys310) Rcat domains are illustrated with a yellow star. Neddylation site (K720 for CUL1, K724 for CUL5) is indicated on WHB domain. C/R, intermolecular cullin–RBX domain; N, N-terminus. (**b**) Chemical ABP structure used as stable mimics of the first transition states in the E3–E3 ubiquitylation cascade, with electrophilic warhead highlighted with dotted red line. (**c**) Structure of neddylated $CRL1^{SKP2}$-ARIH1 reacted with UBE2L3~ubiquitin probe, representing ubiquitin transfer to ARIH1's catalytic cysteine. (**d**) Neddylated $CRL5^{Vif–CBFβ}$-ARIH2 assembly with domains colored according to (**a**) UBE2L3 binding site on ARIH2's RING1 domain is indicated. In contrast to the ARIH1–CRL1 assembly, NEDD8 modification of CRL5 promotes conformational changes in CRL5 that create new binding sites for ARIH2's N-terminus and Ariadne domain.

[27]; and neddylated CRLs and ARIH RBRs are inactive until they bind and reshape each other [26,28].

Several new principles underlying E2 activities have also emerged from recent structural studies. Lysine reactive E2s sense not only the amine nucleophile, but also its display from the precise side-chain hydrocarbon linker to the backbone [47]. This latter activity can be modulated: for example, a structure showed how an E3 can promote force-feeding of a suboptimal lysine into the E2∼SUMO active site to promote SUMOylation [31]. For another E2, UBE2W, an atypical structure contributes to an unconventional active site specialized for modifying protein N-termini [48,49]. Another distinctive E2 is UBE2S, which collaborates with APC/C E3 to generate K11-linked polyubiquitin chains. However, distinctive E2 surfaces bind unique APC/C domains and not the RING. Nonetheless, the RING is required for polyubiquitylation, by binding and positioning the acceptor ubiquitin adjacent to the UBE2S active site [38]. Other RING domains have also been found to bind ubiquitin [50]. Perhaps RNF213's RING domain binds ubiquitin, or serves other functions outside of those required to ubiquitylate Lipid A. On top of these examples of eukaryotic E2s and E3s, increasing numbers of bacterial ubiquitylating enzymes mediating entirely new types of ubiquitylation —not even involving E1 or ubiquitin's C-terminal tail—are being reported. Thus, we look forward to future identification of E2 and E3 enzymes that catalyze ubiquitylation in ways that are so unprecedented that we cannot even anticipate them based on our present knowledge.

Conflict of interest statement

B.A.S. is on the scientific advisory boards of Interline Therapeutics and BioTheryX, and is co-inventor of intellectual property related to DCN1 small molecule inhibitors licensed to Cinsano.

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- • of special interest
- •• of outstanding interest

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