CHEMICAL REVIEWS

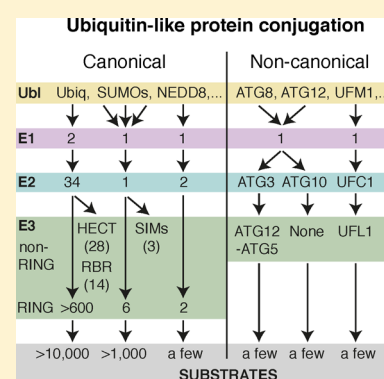# Ubiquitin-like Protein Conjugation: Structures, Chemistry, and Mechanism

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ABSTRACT: Ubiquitin-like proteins (Ubl's) are conjugated to target proteins or lipids to regulate their activity, stability, subcellular localization, or macromolecular interactions. Similar to ubiquitin, conjugation is achieved through a cascade of activities that are catalyzed by E1 activating enzymes, E2 conjugating enzymes, and E3 ligases. In this review, we will summarize structural and mechanistic details of enzymes and protein cofactors that participate in Ubl conjugation cascades. Precisely, we will focus on conjugation machinery in the SUMO, NEDD8, ATG8, ATG12, URM1, UFM1, FAT10, and ISG15 pathways while referring to the ubiquitin pathway to highlight common or contrasting themes. We will also review various strategies used to trap intermediates during Ubl activation and conjugation.

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1. INTRODUCTION

Ubiquitin was isolated in the 1970s as a ubiquitous protein that is conjugated to other proteins through a peptide bond between its C-terminal glycine and a primary amine on the substrate, most typically a lysine residue. Conjugation was subsequently shown to be dependent on the successive activities of enzymes named E1, E2, and E3. In the 1980s, biochemical studies elucidated the chemical reactions catalyzed by these enzymes. Mostly in the 1990s and 2000s, several protein families were discovered that are evolutionarily related to ubiquitin insofar as they share the ubiquitin fold and the capacity to be conjugated to substrates through the concerted action of evolutionarily related E1s, E2s, and E3s (Figure 1).
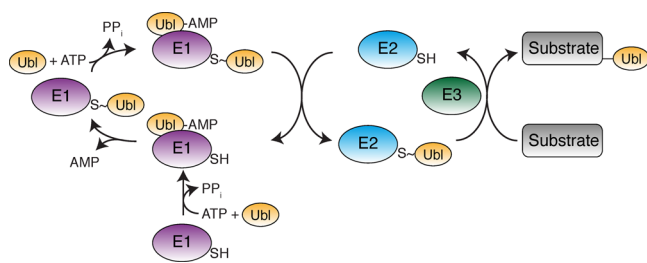

Figure 1. Ubl conjugation cascade where E1, E2, E3, and Ubl designate an E1 conjugating enzyme, an E2 conjugation enzyme, an E3 ligase, and a ubiquitin-like protein, respectively.

These proteins are now collectively referred to as Ubl's, an acronym for ubiquitin-like proteins. Studies on various Ubl conjugation cascades have notably addressed: (i) Ubl recognition by cognate conjugation enzymes, (ii) chemical mechanisms used during conjugation, (iii) substrate specificity, (iv) determinants for substrate modification by one or more Ubl's (chains), and (v) regulation of the conjugation machinery and cross-talk with other post-translational modifications.

In this review, we will focus on structural and mechanistic studies that provided insight into reactions and specificities for the eukaryotic ubiquitin-like conjugation machinery in the past five years, going back further at times to highlight important contributions to the Ubl field. Ubiquitin conjugation cascades have been the subject of numerous recent reviews[1-8] and will not be covered in depth here, but we will refer to mechanisms in the ubiquitin pathway to highlight common or contrasting themes. We will also limit our review to eukaryotic proteins although proteins related to ubiquitin are present in bacteria and archaea (reviewed in Burroughs et al.[9]). In bacteria, the proteins MoaD and ThiS share structural similarity with ubiquitin while MoeB and ThiF share structural and mechanistic similarities with the ubiquitin E1.[10,11] However, these proteins are involved in sulfur metabolism rather than protein conjugation. In archaea, small archaeal modifier proteins (SAMPs) are related to ubiquitin and can be conjugated to proteins (this is also reviewed in Maupin-Furlow[13]).[12] While the conjugation machinery appears limited to SAMPs and proteins similar to eukaryotic E1 in most archaea, an intriguing operon-like cluster was identified in *Candidatus* Caldiarchaeum subterraneum that encodes a Ubl and proteins related to eukaryotic E1, E2, and E3.[14] Ubiquitin and components of its conjugation system predate the emergence of eukaryotes; however, it is clear that eukaryo-genesis resulted in a massive increase in the number of Ub/Ubl genes and pathways,[15,16] the primary focus of this review.

At the end of this review, we will describe strategies that enabled investigators to trap and characterize transient intermediates during conjugation. Concerning nomenclature, we employ a slash (/) to indicate noncovalent interactions, a tilde (∼) to indicate thioester bonds, and a dash (−) to indicate other covalent interactions. The names E1, E2, and E3, will be followed by the name of the protein in subscript (for example, E2$_{UBC9}$). Finally, Ubl's form families of different sizes and the names of the individual family members can vary according to the organism. For simplicity, we will be referring to the Ubl's using generic names such as SUMO, NEDD8, ATG8, ATG12, URM1, UFM1, FAT10, and ISG15.

2. UBIQUITIN-LIKE PROTEINS

Ubl's encompass a family of proteins that share structural and evolutionary relationships with ubiquitin. Each family member possesses a β-grasp fold composed of a five-stranded β-sheet that partially wraps around a central α-helix.[17,18] Ubl's have historically been divided into two types based on whether they are conjugated to substrates: type I Ubl's are conjugated and type II Ubl's are not.[19] Type II Ubl's are generally observed in the context of multidomain proteins, and while they can be found in proteins not directly related to Ubl conjugation cascades, many are observed in certain E1 activating enzymes, E3 ligases, and Ub/Ubl proteases. Hub1 and Esc2, proteins where autonomous Ubl folds are observed, may also be considered as type II Ubl's as they have not been observed conjugated to substrates.[20-22] The protein FUBI is genetically fused with the 40S ribosomal protein S30 as part of the FAU protein.[23] Although FAU undergoes proteolytic processing to release FUBI with a C-terminal diglycine motif, a hallmark of type I Ubl's, it is considered as a de facto type II Ubl because there is no evidence to date that FUBI undergoes conjugation.

Table 1. Ubl's and Their E1 and E2 in Human and Budding Yeast

| family | proteins in *H. sapiens* | | | proteins in *S. cerevisiae* | | |
	Ubl	E1	E2	Ubl	E1	E2
SUMO	SUMO1, SUMO2, SUMO3, SUMO4[a]	UBA2/SAE1	UBC9	Smt3	Uba2/Aos1	Ubc9
NEDD8	NEDD8	UBA3/NAE1	UBC12, UBE2F	Rub1	Uba3/Ula1	Ubc12
ATG8	LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAPL1, GATE-16[a]	ATG7	ATG3	Atg8	Atg7	Atg3
ATG12	Atg12	ATG7	ATG10	Atg12	Atg7	Atg10
URM1	URM1	UBA4	–	Urm1	Uba4	–
UFM1	UFM1	UBA5	UFC1	–	–	–
FAT10	FAT10	UBA6	UBE2Z[b]	–	–	–
ISG15	ISG15	UBA7	UBCH8[b]	–	–	–

[a]SUMO5 and GABARAPL3 were not included in this table as they are likely pseudogenes. [b]UBE2Z and UBCH8 can also work with ubiquitin.

In contrast, type I Ubl's are activated and conjugated to substrates and include the SUMO, NEDD8, ATG8, ATG12, URM1, UFM1, FAT10, and ISG15 protein families. These Ubl's, and the enzymes required for their conjugation, are the primary subject of this review. The Ubl's, E1, and E2 for each Ubl family are presented in Table 1 for *Homo sapiens* and *Saccharomyces cerevisiae*.

2.1. Gene Number and Organization

While some Ubl families can be identified in all eukaryotes, others appear unique to certain phyla. For instance, the ISG15 and FAT10 families appear to function in the immune system of higher eukaryotes and are absent from lower eukaryotes such as fungi. In addition to possessing unique Ubl's, some eukaryotic organisms have expanded the number of genes in a particular Ubl family. While budding yeast possesses one gene for SUMO and one for ATG8, human includes at least four genes for SUMO (SUMO1, SUMO2, SUMO3, and SUMO4) and seven for ATG8 (LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAPL1, and GATE-16). Inclusion of SUMO4 as a bona fide Ubl is controversial as this isoform only appears to be processed and conjugated under stress conditions.[24−26] Two additional Ubl's, SUMO5[27] and GABARAPL3,[28] could represent pseudogenes as the proteins have yet to be detected.

Genes encoding ubiquitin are often observed as head-to-tail concatemers or as fusions with ribosome proteins, and they must be processed by proteases to expose a C-terminal diglycine motif that is required for conjugation. Although encoded as standalone genes, Ubl family members such as UFM1, ISG15, NEDD8, SUMO, and ATG8 encode preproteins that must be processed by proteases to generate a mature form that includes a C-terminal glycine or diglycine motif that is suitable for activation and conjugation. In contrast, genes for ATG12, FAT10, and URM1 are genetically encoded and translated in their mature form. Although all of these Ubl's can be conjugated to substrates, processing and conjugation is not always a prerequisite for function. For example, free ISG15 can function in humans to stabilize USP18 and prevent autoinflammation via noncovalent interactions in a complex that does not require conjugation.[29]

2.2. Structure

The mature form of the Ubl generally includes the β-grasp domain described above and a short flexible C-terminal tail that typically ends with at least one glycine residue. Variations exist, and some Ubl's include additional elements that are conserved and sometimes structured within the particular Ubl family (Figure 2). For example, FAT10 and ISG15 include tandem β-grasp domains separated by a short and sometimes flexible linker. NMR analysis of FAT10 suggests that there is no significant interaction between the two UBL domains,[30] whereas multiple ISG15 crystal structures suggest a defined interface between the two domains.[31−33] Members of the ATG8 family possess two additional α-helices near their N-terminus[34] while ATG12, UFM1, and members of the SUMO protein family include N-terminal extensions that are often missing in available structures, suggesting that they are disordered or at least highly flexible.

2.3. Substrates

Ubiquitin is mainly conjugated to substrates via a peptide bond between the ubiquitin C-terminal glycine and the N$_\zeta$ of lysine residues although it is also possible to conjugate ubiquitin to
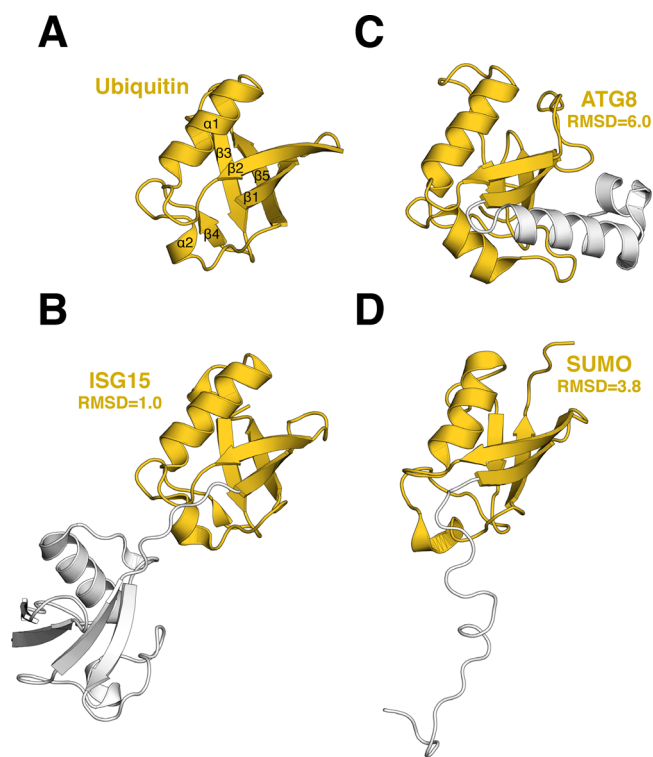

Figure 2. Structure of select Ubl's. (A) Structure of ubiquitin (PDB 1UBQ). (B) Structure of ISG15 (PDB 1Z2M). The first β-grasp domain is colored gray. (C) Structure of ATG8 (GATE-16; PDB 1EO6). The N-terminal extension that contains two α-helices is colored gray. (D) Structure of SUMO (SUMO1; PDB 1A5R). The flexible N-terminal extension is colored gray. Root-mean-square deviations (RMSDs) are calculated between ubiquitin Cα and the Cα of ISG15, ATG8, or SUMO1 that are colored in yellow.

serine, to threonine, to cysteine, or to the N-terminal amine in proteins (reviewed in Stewart et al.[7]). Most Ubl's are ultimately conjugated to proteins with one notable exception being ATG8, which is conjugated to phosphatidylethanolamine (PE)[35] and possibly other lipid head groups.[36] The number of identified substrates conjugated by a particular Ubl varies considerably with tens of thousands of substrates identified for ubiquitin,[37] thousands for SUMO,[38] just a few for URM1,[39] and two for ATG12.[40,41] The capacity to form chains also varies between Ubl's. Chains were first reported for ubiquitin,[42] and it was shown that all of its lysine residues[43] as well as its N-terminal amine group[44] can be used to build chains that differ in structure and function to diversify its signaling capacity.[45] Evidence suggests that some members of the SUMO family form chains in vivo,[46] while chains have also been detected in vivo for NEDD8 and UFM1.[47,48] Some other Ubl's can form chains in vitro; however, convincing data for their existence and function in vivo is still lacking.

3. UBL BINDING MOTIFS

Noncovalent interactions with Ubl's mediate a variety of functions before, during, and after conjugation. Proteins use domains and motifs of varying size and structure to interact with ubiquitin (these are reviewed in Husnjak and Dikic[49]); however, most bind ubiquitin via a hydrophobic patch centered around isoleucine 44 with affinities in the high micromolar range.[49] NEDD8 is closely related to ubiquitin by sequence and structure,[50] and motifs that form noncovalent interactions with

NEDD8 are similar to those that contact ubiquitin.[51,52] FAT10 was also shown to interact with a protein containing UBA domains, perhaps consistent with its high sequence similarity to ubiquitin.[53,54] While noncovalent contacts to Ubl members can encompass similar surfaces, they are generally tailored to form specific Ubl/receptor complexes (Figure 3).
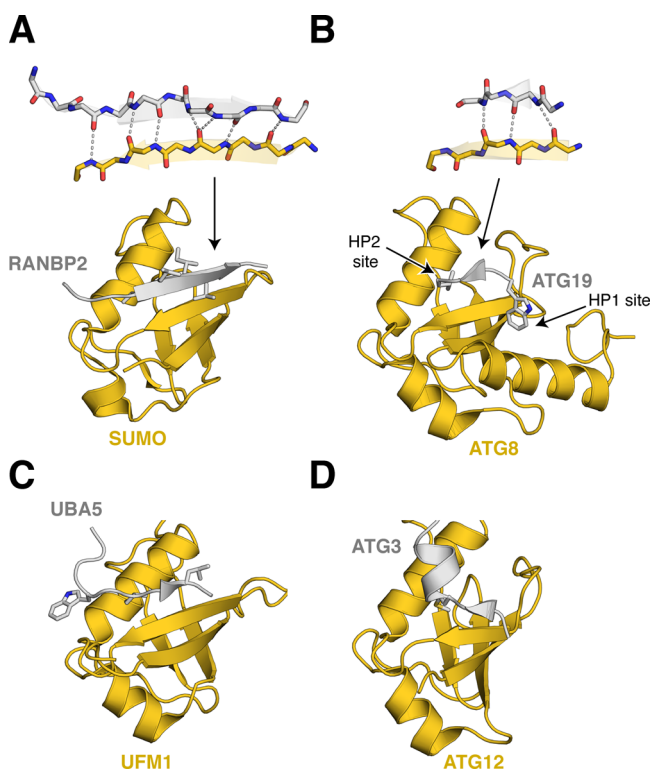

Figure 3. Ubl binding motifs. (A) Structure of SUMO/RANBP2 (PDB 1Z5S). (B) Structure of ATG8/ATG19 (PDB 2ZPN). (C) Structure of UFM1/UBA5 (PDB 5HKH). (D) Structure of ATG12/ATG3 (PDB 4NAW). The Ubl's are in cartoon representation colored yellow, while the Ubl binding motifs are in cartoon representation colored gray. Selected residues of the Ubl binding motifs are presented in stick representation. Backbone interactions that mediate β-strand complementation for SUMO/RANBP2 and ATG8/ATG19 are highlighted at the top.

3.1. SUMO Binding Domains

SUMO-interacting motifs (SIMs) are the most prevalent and best characterized motifs that bind SUMO proteins. SIMs are found in the SUMO activating enzyme UBA2, in all known SUMO E3s, in some ubiquitin E3s, as well as in some SUMO substrates and receptors. SIMs are typically composed of four hydrophobic residues that are flanked by acidic residues or residues that can be phosphorylated to generate negative charge, although variants include SIMs with an acidic or phosphorylatable residue at the second or third position. SIMs are presumably disordered in solution but adopt a β-strand that complements the SUMO β-sheet in either parallel or antiparallel orientations, thereby complementing a hydrophobic groove on SUMO formed by its central α-helix and second β-strand (Figure 3A). Structures suggest that SIMs can adopt a preferred orientation; however, the binding orientation may not always be an intrinsic characteristic of the SIM sequence as there is at least one case where both orientations were observed in dynamic exchange.[55] Molecular dynamics simulations suggest that the antiparallel arrangement results in a complex

with higher stability compared to the parallel orientation.[56] SIMs that adopt antiparallel orientations also appear to tolerate more changes within their sequence,[56,57] possibly because the antiparallel orientation establishes more backbone-mediated interactions.[57] SIMs typically interact with SUMO with affinities in the micromolar range.[58−62] In higher eukaryotes that possess multiple SUMO isoforms, some SIMs appear to have evolved specificity for certain SUMO isoforms. The structural basis for this preference remains elusive, and the measured differences in affinity between SIMs and different SUMO isoforms rarely exceed 1 order of magnitude.[60,61,63]

As mentioned earlier, SUMO−SIM interactions can be modulated by post-translational modifications such as phosphorylation at positions within or immediately adjacent to the hydrophobic portion of the SIM.[64] In most cases, phosphorylation increases the strength of SUMO−SIM interactions by at least an order of magnitude.[61,62,65] The structural bases for phosphorylation-mediated stabilization of these interactions came from three studies, highlighting contacts between the phosphorylated SIM residues and adjacent SUMO residues, albeit at differing positions.[61,62,65] Comparing binding modes for phosphoSIMs in DAXX and PML suggests some flexibility in recognition of acidic or phosphorylated residues.[65] In addition, differences between SUMO isoforms can result in different contacts to the acidic/phosphorylated residues in the SIM,[60−62,66] suggesting that a particular SIM might gain or lose specificity for a particular SUMO isoform based on its phosphorylated state. In addition to phosphorylation, SUMO−SIM interactions may be modulated by acetylation of certain SUMO lysine residues.[67] Interestingly, SUMO lysine acetylation could counter the effects of SIM phosphorylation as the lysine residues in question constitute those that mediate contacts to the phosphorylated residues in SIMs.[67] Finally, some proteins possess multiple SIMs that can bind multiple SUMO molecules and/or SUMO chains, presumably stabilizing interactions by increasing avidity. With that said, a concept of SIM dominance has emerged based on the observation that certain SIMs within a cluster appear essential for binding SUMO chains whereas others appear dispensable.[68−71]

In addition to SIMs, two other types of SUMO-binding domains have been identified. The ZZ zinc finger domain of HERC2 interacts with SUMO in a zinc-dependent manner.[72] This domain, present in at least 20 human proteins, has a 20-fold preference for SUMO1 over SUMO2.[72] Analysis of the interaction between SUMO1 and the ZZ domain of CBP/p300 by NMR and through mutagenesis reveals that the ZZ domain binds a different surface of SUMO1 than the one employed to engage SIMs.[73] Finally, a MYM-type zinc finger present in at least four human proteins has been shown to interact with SUMO1 in a zinc-independent manner, although in this case MYM interactions appear to use the same SUMO surface for SIM interactions because mutants that destabilize SUMO−SIM interactions also destabilize MYM interactions.[74]

3.2. LC3 Interacting Regions (LIRs)

LIRs, also termed LC3 recognition sequence (LRS) or ATG8-interacting motif (AIM), mediate interactions between multiple proteins within the ATG8 family (reviewed in Birgisdottir et al.[75]). The LIR motif includes a core ΘxxΓ consensus sequence, where Θ is an aromatic residue (typically Trp, Tyr, or Phe) and Γ is an hydrophobic residue (typically Leu, Ile, or Val). An extended consensus called xLIR[76] accounts for the observation that the core LIR is often preceded or intervened by acidic

residues or residues that can be phosphorylated. Numerous structures have been reported that delineate the requisite ATG8–LIR interactions, although it is worth noting that some were obtained through use of a linear fusion strategy where the LIR sequence is fused to the N-terminus of ATG8 resulting in structures that show the LIR from one molecule interacting with ATG8 from the neighboring ATG8 molecule in the crystal lattice.[77−80]

LIR-containing proteins bind a region of ATG8 in a manner that shares some similarity to mechanisms employed in SIM–SUMO interactions as the LIR sequence adopts a β-strand conformation that complements ATG8 β-sheet in a parallel orientation (Figure 3B) with the aromatic and hydrophobic residues of the LIR motif accommodated in two hydrophobic pockets termed the HP1 and HP2 sites (sometimes called the W and L sites). While the HP2 site has a structural equivalent in SUMO, the HP1 site is unique to the ATG8 protein and its formation is partially dependent on residues and surfaces composed by the N-terminal helices that are unique to ATG8 proteins. A structural and kinetic study of the interaction between ATG13 and three LC3 isoforms suggests a sequential binding model.[78] In this model, binding of a hydrophobic residue to the preformed HP2 site tethers the LIR peptide to ATG8 prior to rearrangement of the HP1 site that includes movement of ATG8 Lys49 and Phe52 to allow binding of the aromatic residue.

Acidic residues intervening or preceding the LIR core motif interact with arginine residues on the ATG8 surface and contribute to the specific binding to certain isoforms.[75] Serine/threonine residues are sometimes observed in or adjacent to the LIR core sequence, and their phosphorylation can increase[77,81,82] or decrease[83,84] the strength of the LIR−ATG8 interaction. FUNDC1 constitutes an example where phosphorylation of the LIR motif leads to a decreased interaction with ATG8. Indeed, FUNDC1 acts as a receptor during hypoxia-induced mitophagy that is constitutively phosphorylated on a serine residue close to the LIR motif and the tyrosine residue of the LIR.[83,84] Dephosphorylation of these residues upon hypoxia leads to increased association of FUNDC1 with ATG8.[83,84] The NMR structure of a nonphosphorylated FUNDC1/ATG8 complex suggests that tyrosine phosphorylation decreases ATG8 binding through an electrostatic repulsion, notably with an aspartate residue.[85] This structure further reveals an atypical binding where the HP1 site is simultaneously occupied by the LIR tyrosine residue and an adjacent valine residue.[85]

Natural variations of the LIR motif exist, and extension of the LIR by a helix is observed in FYCO1. In this case, the LIR motif is succeeded by a short helix that positions a glutamate residue to interact with an arginine residue located on the α-helix of ATG8.[86,87] The capacity of ATG8 isoforms to accommodate extended LIR motifs has been proposed as another potential mechanism to explain isoform selectivity.[86,87] In support of this model, the extended LIR binds LC3A with a submicromolar affinity whereas binding of canonical LIRs typically occurs in the micromolar range. The presence of a noncanonical LIR has been hypothesized in the Ubl ATG12 where the aromatic and hydrophobic residues of the LIR are provided by residues that are close in space but not in sequence.[88] The structural details of this interaction remain unknown.

Recently, a small motif in UBA5 capable of interacting with both ATG8 homologues and UFM1 was discovered[89] and termed LIR/UFIM (UFM1-interacting motif). Interaction between this motif and UFM1 is structurally reminiscent of

the interaction between SUMO and an antiparallel SIM (Figure 3C).[89] Notably, a hydrophobic residue occupies a cavity equivalent to HP2 while a tryptophan residue folds over a small hydrophobic region at the start of the central α-helix of UFM1. Although this structure provides a compelling example of cross-reactivity where one sequence can bind more than one type of UBL, the biological consequences of this dual interaction awaits further investigation. Finally, the structure of an ATG3/ATG12−ATG5/ATG16 complex reveals that 13 residues of ATG3 can interact with the second β-strand and the α-helix of ATG12.[90] This sequence forms a short β-strand that runs parallel to the β-strand of ATG12 before continuing as an amphipathic α-helix that provides additional acidic and hydrophobic contacts with ATG12 that contributes to interactions in the midnanomolar range (Figure 3D).[90]

4. E1 ACTIVATING ENZYMES

As the name implies, E1 activating enzymes are required to activate Ub/Ubl's for subsequent steps along the conjugation cascade. E1s have historically been divided between canonical and noncanonical E1s (reviewed in Schulman and Harper[91]). Canonical E1s perform three distinct chemical reactions (Figure 4). In the first reaction, E1s bind ATP, magnesium
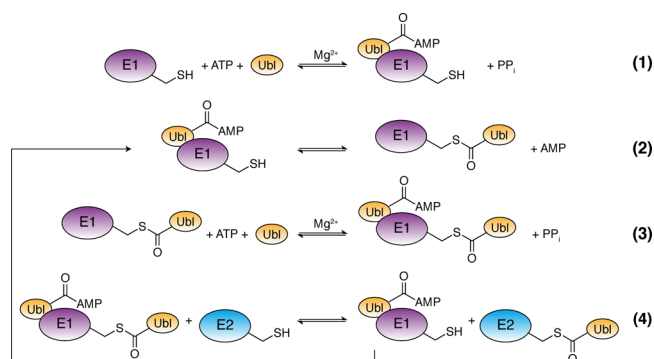

Figure 4. Canonical E1 chemical reactions. (1) The E1 binds a Ubl and ATP and catalyzes adenylation of the Ubl. (2) The E1 catalytic cysteine attacks the Ubl∼AMP resulting in formation of an E1∼Ubl thioester. (3) The E1 adenylates a second Ubl. (4) The Ubl is transferred to an E2 through a transthioesterification reaction.

and the Ubl's to catalyze formation of a high-energy acyl adenylate intermediate with subsequent release of pyrophosphate. In the second reaction, an E1 catalytic cysteine attacks the Ubl∼adenylate to catalyze formation of a high-energy thioester bond between the E1 and Ubl (E1∼Ubl) and release of the AMP product. Finally, the E1∼Ubl catalyzes trans-thioesterification to an E2 to form a high energy thioester-linked E2∼Ubl product. The first structural models for E1 mediated adenylation were gleaned from a MoeB−MoaD complex in 2001,[10] a noncanonical E1/Ubl pair. MoeB and MoaD are evolutionarily related to E1 and ubiquitin, respectively, and participate in the biosynthesis of the molybdenum cofactor in bacteria. While MoeB is capable of adenylating the Ubl MoaD, it lacks a catalytic cysteine that is present in canonical E1s. In 2003, the first structure of a canonical E1 was determined, that of the NEDD8 E1 alone[92] and in complex with NEDD8.[93] Since then, additional structural work has provided snapshots for many of the intermediates during E1 activation. Importantly, information obtained for E1s using different systems and different

organisms can be extrapolated to other E1s due to their high sequence, structural, and functional similarities. As mentioned before, despite these similarities, E1s are often divided into canonical and noncanonical E1 families. Canonical E1s activate the SUMO protein family, NEDD8, ubiquitin, FAT10, and ISG15 and include two pseudosymmetric adenylation domains encoded by one or two genes. In contrast, noncanonical E1s form homodimers similar to that observed for MoeB. These include E1s for the ATG8 and ATG12 protein families, UFM1 and URM1.

4.1. Canonical E1s

Structural information for canonical E1s mainly derives from studies on E1s in the NEDD8, SUMO, and ubiquitin pathways. There is, to date, no structural information available for activation of ISG15 by $E1_{UBA7}$ (also known as $E1_{UBE1L}$) or FAT10 by $E1_{UBA6}$ (also known as $E1_{UBE1L2}$). Canonical E1 enzymes possess a common architecture (Figure 5A) composed of two pseudosymmetric adenylation domains: the inactive adenylation domain (IAD) and the active adenylation domain (AAD). Although some differences exist, each canonical E1 includes an insertion within the IAD that is called the first catalytic cysteine half-domain (FCCH) in the ubiquitin E1 and the NAE1 catalytic cysteine (CC) domain in the NEDD8 E1. The SUMO E1 also includes an insertion at this position, but it was not formally named because of its small size and apparent disorder in available crystal structures. In contrast to the variance observed among insertions in the IAD, each canonical E1 includes a conserved CYS domain inserted within the AAD that contains the E1 catalytic cysteine that varies in size through addition of unique structural elements. Most canonical E1s include a ubiquitin fold domain (UFD) at the C-terminal end of the AAD that assists in selecting cognate E2 ubiquitin conjugating enzymes (UBCs).[94−97]

Structurally, canonical E1s resemble canyons (Figure 5B). The IAD and AAD form the base of the canyon, the UFD domain forms one wall while the FCCH/insertion and CYS domains form the opposing wall.[93] This structural organization is apparent in NEDD8, SUMO, and ubiquitin E1s although differences in the fold of the FCCH and size of the CYS domains exist as mentioned earlier. In all cases, the CYS domain is connected at its N-terminal end to the AAD through a crossover loop that passes over the Ubl C-terminal tail, with the catalytic cysteine located within a helix that immediately follows the crossover loop. The SUMO E1 possesses an α-helix termed the CYS cap that covers its catalytic cysteine prior to thioester bond formation.[98] The CYS cap contains acidic residues that could raise the catalytic cysteine pK_a thereby reducing its reactivity.[98] The CYS cap becomes disordered when the CYS domain rotates to form the thioesterification active site. The IAD and AAD interact via an extended composite interface to form a single ATP binding site. The UFD, which serves to select cognate E2s, is connected to the AAD through a flexible hinge. In the SUMO and NEDD8 systems, this domain may undergo a transition from a partially disordered state to an ordered state upon E2 binding, and it must rotate to bring the E1 and E2 cysteine residues into proximity for thioester transfer.[99,100] Also in the SUMO and NEDD8 E1s, two residues in the UFD hinge and two residues in the crossover loop coordinate a structural zinc ion. Interestingly, the UFD domain of NEDD8 E1 is absent in certain yeast phyla (see below).
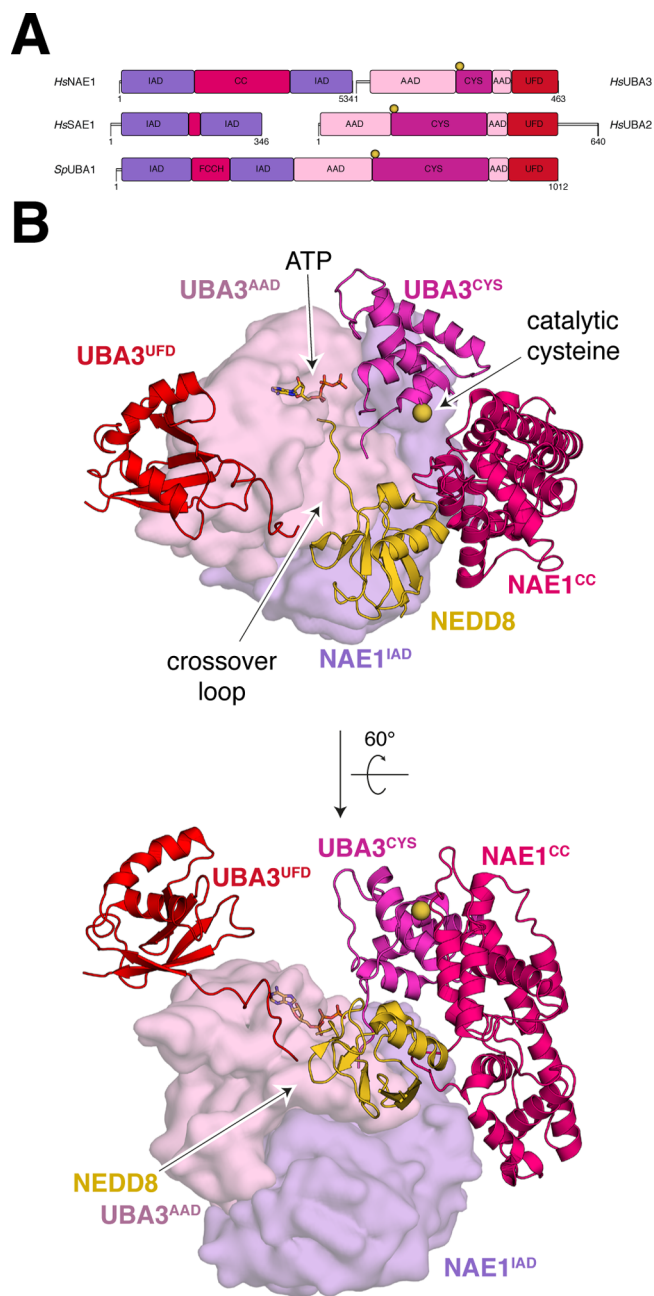
Figure 5. Domain organization of canonical E1s. (A) Primary structure of canonical E1s for NEDD8, SUMO, and ubiquitin. (B) Structure of human NEDD8~$E1_{NAE1/UBA3}$/$E2_{UBC12}$/ATP (PDB 2NVU). Adenylation domains are shown in a Gaussian surface representation, while other domains are in cartoon representation. The sulfur atom of the active site cysteine is in sphere representation colored yellow. Positions for ATP, the crossover loop, and the active site cysteine are highlighted by arrows.

4.1.1. Adenylation Reaction. Structures of MoeB−MoaD complexes derived from *Escherichia coli* provided the first structural insights to the E1 adenylation reaction (Figure 6A).[10] Although MoeB lacks the ability to transfer its Ubl to E2s, we introduce it here because these structures illustrated the basic mechanism of adenylation, features of which are common to all E1 enzymes. MoeB forms a homodimer of AADs to form two active sites that can accommodate two molecules of MoaD and ATP. The C-terminal diglycine motif of MoaD is held in close proximity to the ATP-binding pocket to position the MoaD

Figure 6. Adenylation domains of E1 and E1-like proteins. (A) Structure of *E. coli* MoaD/MoeB/ATP (PDB 1JWA). (B) Structure of human E1$_{SAE1/UBA2}$/SUMO/ATP (PDB 1Y8R). In both cases, Ubl and adenylation domains are depicted in cartoon and Gaussian surface representations. The ATP and last two residues of the Ubl's are in stick representation. (C) Close-up view of interactions between ATP, the SUMO C-terminus, and the E1 adenylation pocket.
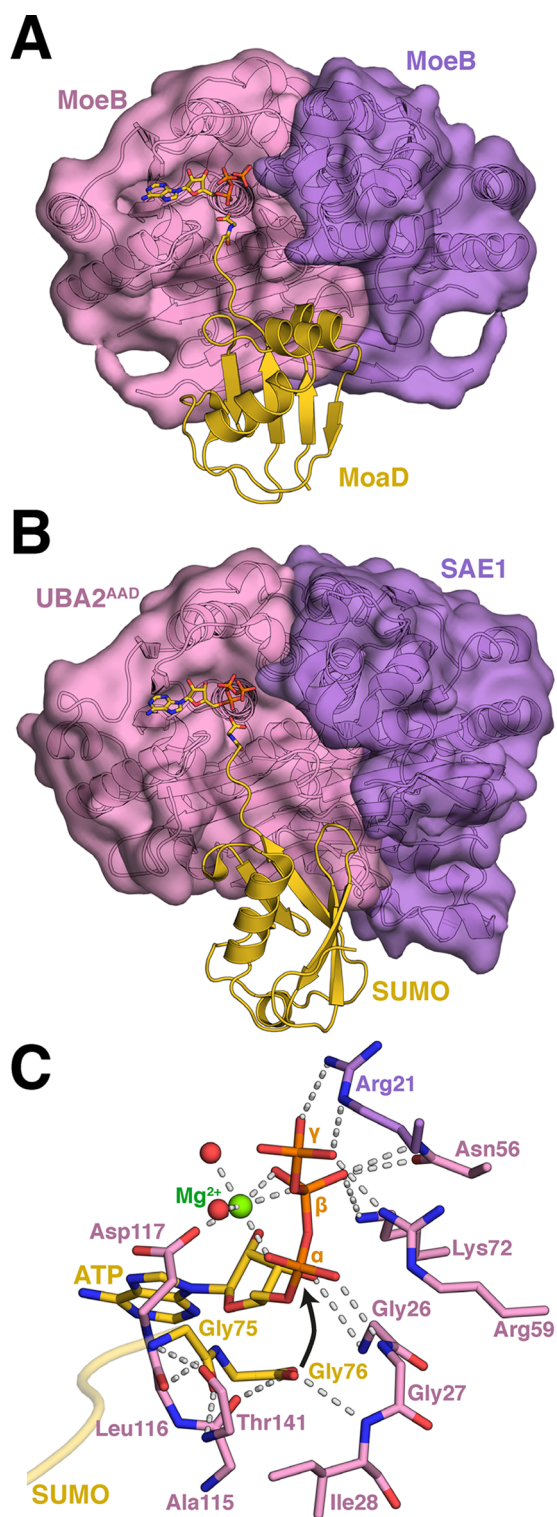
carboxylate terminal group between the AAD and the α-phosphate of ATP. A magnesium ion, held in place by an aspartate residue, is believed to decrease electrostatic repulsion

between the phosphates and the C-terminal carboxylate of MoaD.[10] In NEDD8, SUMO, and ubiquitin E1, the ATP and the C-terminal tail of the Ub/Ubl are positioned next to each other in the AAD adenylation pocket in a manner reminiscent of MoaD and ATP (Figure 6B,C).[93,96,101] Despite being crystallized with ATP and magnesium and poised for adenylation, most E1/Ubl structures reveal the presence of ATP−magnesium and Ubl rather than the acyl adenylate intermediate.[93,97,101] This observation is consistent with prior studies showing that pyrophosphate release is the rate limiting step in adenylation[102] and that the back reaction occurs readily.[103] While most E1s do not undergo adenylation in the crystal, a Ub-adenylate intermediate was observed in a structure of a ubiquitin E1 where it adopts a conformation similar to the one of ubiquitin plus ATP.[104] Although generated artificially, the structure of a SUMO E1 in complex with a SUMO molecule linked to a nonhydrolyzable adenylate mimic revealed a similar configuration.[98]

4.1.2. Thioester Formation. The first E1 structures provided many details pertaining to the adenylation reaction, but the mechanism for pyrophosphate release and subsequent thioester bond formation remained elusive for many years as the E1 active site cysteine and Ubl∼adenylate were separated by as much as ∼35 Å. In 2010, a structure of the SUMO E1 illuminated conformational changes that were required to release pyrophosphate and to bring the active site cysteine into proximity of the Ub/Ubl∼adenylate (Figure 7). This was achieved by linking SUMO and ubiquitin to a nonhydrolyzable adenylate mimic that harbored an electrophile at a position where the thioester bond is formed. Incubation of this mimic with an active E1 resulted in a stable thioether bond and tetrahedral intermediate mimic between the E1 active site cysteine and the Ub/Ubl adenylate mimic.[105] A structure of the resulting SUMO E1 complex and comparison to other SUMO E1 structures revealed several conformational changes that occur during thioester bond formation.[98] One change encompasses expulsion of the N-terminal helix of the IAD, a change that might explain how pyrophosphate is released because it forms part of the adenylation active site and contributes a critical arginine that contacts the ATP γ phosphate (Figure 6). Another major conformational change includes rotation of the CYS domain which brings the catalytic cysteine into proximity of the Ubl∼adenylate and forms a new composite active site that is capable of thioester bond formation. It remains unclear how or if the E1 cysteine is specifically activated for catalysis by neighboring amino acid side chains, but the structure suggests that an α-helix in the AAD, positioned just below the adenylate, could stabilize the transition state during thioester bond formation via hydrogen bonding or perhaps the helix dipole. Following thioester bond formation, the CYS domain is thought to return to its original position through changes that restore the original conformation of the adenylation active site to permit binding of a second Ubl and ATP.

4.1.3. Ubl Transfer to E2s. Thioester transfer between the E1∼Ubl and E2 requires juxtaposition of the E1 and E2 active sites. In the case of the NEDD8 E1, formation of an E1∼NEDD8 thioester triggers a change in the conformation of the UFD that exposes an E2 binding surface.[97] The interaction between NEDD8 E1 and E2$_{UBC12}$ or E2$_{UBE2F}$ is bipartite. One interaction that appears unique to NEDD8 E1/ E2 complexes involves the stabilization of the N-terminal extensions of E2$_{UBC12}$ or E2$_{UBE2F}$ that adopt extended
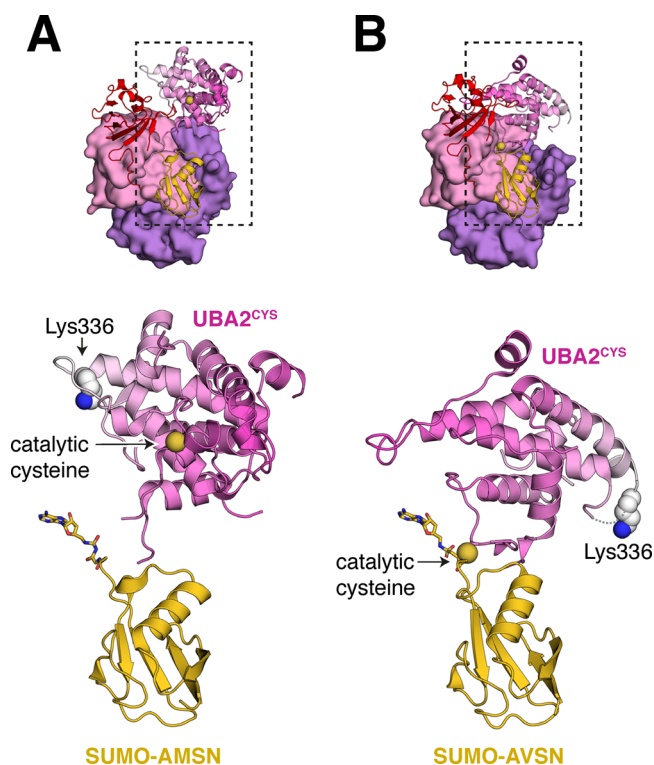
Figure 7. Thioester formation in the SUMO E1. (A) Structure of human E1$_{SAE1/UBA2}$/SUMO−AMSN (PDB 3KYC). (B) Structure of human E1$_{SAE1/UBA2}$/SUMO−AVSN (PDB 3KYD). For simplicity, only the CYS domain and SUMO−AMSN or SUMO−AVSN is presented. The sulfur atom of the active site cysteine is in sphere representation colored in yellow. A color gradient is applied on the CYS domain to highlight the different orientations observed in the two structures. As an additional landmark, Lys336, a lysine residue in the CYS domain, is presented in sphere representation. AMSN and AVSN are non-hydrolyzable AMP mimics with AVSN covalently linked to the E1 catalytic cysteine.

conformations within a groove on NEDD8 E1 (Figure 8A).[106,107] The other interaction involves contacts between the UFD domain of NEDD8's E1 and the core domains of E2$_{UBC12}$ or E2$_{UBE2F}$ in a manner that structurally resembles other ubiquitin−UBD interactions[94] with the β-sheet of the UFD contacting the N-terminal helix and β1−β2 loop of E2$_{UBC12}$ or E2$_{UBE2F}$.[94,107] The structure of E2$_{UBC12}$ in complex with NEDD8 E1 doubly loaded with NEDD8 (one NEDD8 in the adenylation site, another linked to the E1 via a thioester bond) confirmed bipartite recognition of E2$_{UBC12}$ and revealed a conformation where the E1 and E2 active sites faced each other although they remained ∼20 Å apart (Figure 8A).[97] Although this distance is too far to promote transthioesterification, it was hypothesized that the distance could be reduced through a hinge movement of the loop linking the AAD and UFD.[97,108] The structure of a ubiquitin E1−E2 complex where both active sites are cross-linked through a disulfide bond provided insight into additional conformational changes required for transthioesterification.[96] Indeed, further movement in the hinge between the UFD and AAD allows the E2 active site to approach the E1 active site cysteine (Figure 8B). In this arrangement, E2$_{UBC4}$ interacts with the UFD and CYS domain while also contacting the crossover loop and the molecule of ubiquitin that is located in the adenylation site.
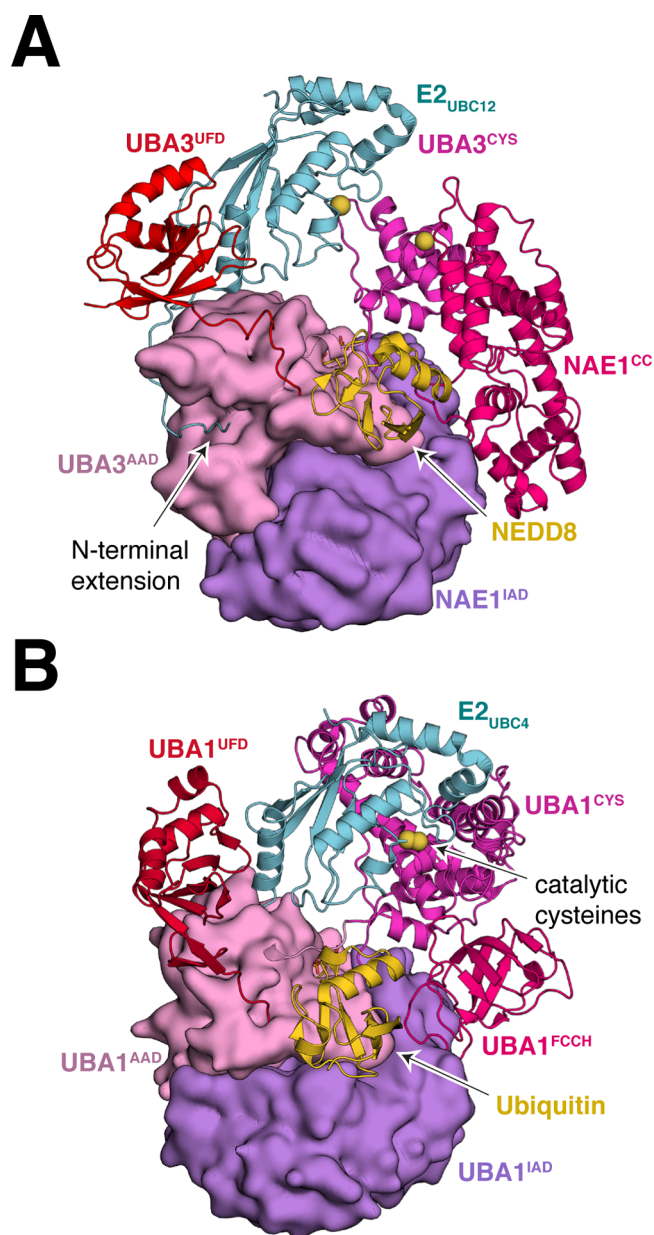
Figure 8. E1−E2 interaction for canonical E1 proteins. (A) Structure of human E1$_{NAE1/UBA3}$/E2$_{UBC12}$/NEDD8/ATP (PDB 2NVU) showing the bipartite binding of the E2$_{UBC12}$ to E1. In this structure, the catalytic cysteine residues of E1 and E2 are separated by ∼20 Å. (B) Structure of *S. pombe* E1$_{UBA1}$/E2$_{UBC4}$/ubiquitin/ATP (PDB 4II2) showing juxtaposition of E1 and E2 active sites. Adenylation domains are shown in Gaussian surface representation. Other domains are in cartoon representation. The sulfur atoms of the active site cysteine residues of E1 and E2 are in sphere representation colored yellow.

While there is currently no structural information available for a good mimic of the transthioesterification reaction, three studies elaborated models for a tetrameric assembly consisting of a doubly loaded ubiquitin E1 with E2 in conformations suitable for thioester transfer.[96,104,109] In each case, the thioester-linked ubiquitin is bordered by the E2, the FCCH domain, and ubiquitin in the adenylation site, although the models differ somewhat. In two cases the thioester-linked ubiquitin contacts the FCCH domain,[104,109] while in another case the thioester-linked ubiquitin contacts the E2.[96] In the later case, ubiquitin was positioned in a similar but not identical

conformation to the closed E2~ubiquitin conformation (see below). The validity of these models, and their generality, remains to be tested.

4.1.4. Directionality of the Reaction.

Each chemical step catalyzed by E1 is reversible, so how does the E1 ensure directionality? First and foremost, ATP is highly abundant, so the reaction could progress by simple mass action. With that said, several mechanisms discussed earlier appear to confer directionality to the overall reaction. Following adenylation, the E1 undergoes large conformational changes that reshape the adenylation active site into one that promotes thioester bond formation. In doing so, residues that contact ATP and are required for adenylation are displaced from the active site to allow the CYS domain to rotate and project the active site cysteine toward the Ubl~adenylate. These conformational changes would facilitate pyrophosphate release, thus inhibiting the back reaction.[98] After thioester bond formation, the CYS domain returns to its original position, allowing reformation of the adenylation active site, a process that is most certainly enhanced by binding another molecule of ATP and Ubl. The presence of ATP and a second Ubl would likely inhibit CYS domain conformational changes, thus preventing the thioester-linked Ubl from undergoing the reverse reaction. This model is consistent with the observation that doubly loaded ubiquitin E1 is most efficient at promoting thioester transfer to E2.[110] Toggling of affinities upon E1:E2 transthioesterification was also proposed as a driving force in directionality.[97] In this case, the Ubl moiety of a Ubl~E1 complex contributes additional surfaces to promote interactions with E2 while Ubl transfer to the E2 reduces the number of contacts between the charged E2 and the E1, thereby facilitating rapid dissociation of the charged E2 and inhibiting attack of the charged E2 by the E1. This is followed in the NEDD8 system by an additional change in the conformation of the UFD that buries E2 binding surfaces to inhibit rebinding between the charged E2 and discharged E1.[97]

4.1.5. Ubl Specificity.

E1s serve as selectivity filters to ensure faithful transfer of cognate Ubl's to cognate E2s because they generally exhibit specificity for one or a few Ubl's. Indeed, reports show that once a noncognate Ubl is loaded on E1, it can be transferred to an E2 without major impediments.[111,112] While many interactions contribute to specificity between cognate E1/Ubl pairings, the identity of the third residue preceding the diglycine motif (position 72 in NEDD8 or ubiquitin) constitutes a major determinant for faithful Ubl activation by its cognate E1.[50,92,93,113] This residue is an arginine in ubiquitin, an alanine in NEDD8, and a glutamine or glutamate in SUMO. Early biochemical work revealed that A72R substitution in NEDD8 increases NEDD8's affinity for ubiquitin E1 by approximately 2 orders of magnitude[50] while the R72A substitution of ubiquitin permits its adenylation by NEDD8 E1.[93] Furthermore, R72L substitution of ubiquitin can be activated and passed to the E2 by NEDD8 E1 while this activity could not be detected for wild type ubiquitin.[111] Structures of cognate E1−Ubl complexes revealed how different residues at this position are recognized by E1. Ubiquitin Arg72 fits into a negatively charged cavity in its E1 forming contacts to multiple residues via hydrogen bonds and hydrophobic interactions.[108] In the case of SUMO, a glutamate residue is stabilized by contacts to an arginine and a tyrosine,[101] while for NEDD8, Ala72 benefits from hydrophobic interactions with adjacent leucine and tyrosine residues.[93] Interestingly, a single R190Q mutation in NEDD8 E1 allows the charging of ubiquitin.[93] Arg190 does not contact NEDD8

Ala72, but it appears that this residue is part of a gating mechanism that prevents NEDD8 E1 from binding ubiquitin.[113] Additional contacts to the Ubl could include contacts to residues from the FCCH or related insertion domains. Similarly, interactions between SUMO and a SIM-like sequence in the C-terminus of its E1[98] could assist in SUMO specificity although this region of the E1 appears dispensable in vivo and its contribution to Ubl specificity has not been assessed. Specificity was also addressed in the case of E1$_{UBA6}$ that activates two different Ubl's, ubiquitin or FAT10,[114−116] and transfers them to E2$_{UBE2Z}$.[115,117] Ubiquitin and FAT10 were reported to undergo adenylation and thioester formation with similar kinetics although FAT10 displays a much tighter binding to E1$_{UBA6}$ than ubiquitin.[118] A Cys-Tyr-Cys-Ile sequence immediately preceding the diglycine motif appears important for the selective activation of FAT10 by E1$_{UBA6}$ and for its transfer to E2$_{UBE2Z}$.[119] Intriguingly, a FAT10 variant with this motif replaced by the Leu-Arg-Leu-Arg motif of ubiquitin displays an increased rate of transfer to E2$_{UBE2Z}$ when compared with the wild type protein.[119]

4.1.6. E2 Specificity.

In addition to E1 specificity for cognate Ubl's, E1s must also select cognate E2s from a large pool of structurally similar proteins. In this regard, E1s appear to select for cognate E2s while also discriminating against noncognate E2s. The UFD appears to be the main specificity determinant for E1:E2 interactions. The structures of five UFD:E2 pairs have been solved,[96,97,107,120,121] and comparison of these structures suggests that conserved insertions and deletions are used to prevent noncognate pairing.[96] Consistent with the idea the UFD domain constitutes the main E2 binding platform for ubiquitin E1, its deletion leads to a sharp decrease in the rate of E2 transthioesterification.[95] For the interaction between NEDD8 E1 and its E2s, specificity also derives from a tight interaction between the N-termini of E2$_{UBE2F}$ or E2$_{UBC12}$ and the residues of a groove in NEDD8 E1[106,107] and deletion of the NEDD8 E1 UFD domain was shown to have a limited effect on the rate of E2 transthioesterification,[95] consistent with the absence of this domain in certain yeast strains. This deletion however increases the rate of noncognate E2 charging in agreement with a role of the UFD domain as a selectivity filter.[95] A study aimed at understanding the bases of E2$_{UBC12}$ specificity for NEDD8 further revealed that certain E2$_{UBC12}$ residues oppose ubiquitin charging and, through alanine mutagenesis, the vestigial preference of E2$_{UBC12}$ for ubiquitin can be partially restored.[112] Finally, structural comparison of E1$_{UBA2}$/E2$_{UBC9}$ pairs for human and yeast suggests a general conservation of the interaction interface between these species.[121]

4.1.7. Regulation.

While regulation of E1s has not been a major focus of investigation, several lines of evidence suggest that E1s could be regulated. For instance, the CYS domain of the SUMO E1 was shown to be targeted by SUMO modification to decrease its interaction with E2$_{UBC9}$, possibly though steric hindrance.[122] SUMO modification on the E1 C-terminal domain has also been shown to affect the subcellular localization of the E1.[123] Perhaps most intriguing are the observations that, under oxidative conditions, the SUMO E1 can form a disulfide bond with its E2 UBC9,[124] thereby inactivating both enzymes. A similar model was proposed for the ubiquitin E1.[125] There are also examples of proteins regulating E1 activity through direct interaction. LMO2 binds the UFD domain of E1$_{UBA6}$ and acts as a competitive inhibitor of E1$_{UBA6}$, thereby decreasing FAT10 loading on E2$_{USE1}$ and

global protein FATylation.[126] Another study reported that glycyl-tRNA synthase (GlyRS) uses its anticodon binding domain to target NEDD8 E1 where it captures NEDD8~E2$_{UBC12}$ through an interaction that depends on the catalytic domain of GlyRS but not on its tRNA synthase activity.[127] GlyRS, but not other tested tRNA synthases, appears to stabilize NEDD8~E2$_{UBC12}$ and was posited as a global regulator of protein neddylation.

4.2. Noncanonical E1s

Noncanonical E1s form obligate homodimers and do not possess a CYS domain. Instead, their catalytic cysteine is positioned after a crossover loop that is close to the adenylation pocket. Work on E1$_{ATG7}$ and E1$_{UBA5}$ provided considerable structural insight into mechanisms utilized by noncanonical E1s. Comparatively, much less is known concerning E1$_{UBA4}$, which plays a dual role in protein conjugation and sulfur metabolism.[128]

4.2.1. UBA4: URM1 E1.

E1$_{UBA4}$ is a homodimeric protein that includes an adenylation domain followed by a rhodanese-like domain (RLD) and functions as a sulfurtransferase. It is not yet clear how E1$_{UBA4}$ activates URM1 for downstream protein conjugation or if this process even involves the formation of a thioester intermediate,[39,129,130] as is the case for other E1s. Protein modification by URM1 is dependent on a catalytic cysteine located in the RLD, supporting a mechanism where the adenylated URM1 is attacked by a persulfide group on the RLD's catalytic cysteine to yield a disulfide-linked E1$_{UBA4}$−S−S−URM1 intermediate.[130] Attack of this species by a cysteine in the adenylation domain would result in release of a URM1 thiocarboxylate, whereas attack by a substrate lysine residue would rather result in a URM1−protein conjugate.[130]

4.2.2. ATG7: Dual E1 for ATG8 and ATG12.

E1$_{ATG7}$ activates two different Ubl's, ATG12 and ATG8, and respectively pairs them with E2$_{ATG10}$ and E2$_{ATG3}$. E1$_{ATG7}$ includes two domains connected by a short flexible hinge.[131] The N-terminal domain, termed ATG7-NTD, is unique to the E1$_{ATG7}$ family of proteins, whereas its C-terminal domain, termed ATG7-CTD, constitutes the AAD common to all E1s. The structure of full-length E1$_{ATG7}$ resembles a gliding bird where the CTD dimer forms the body while the NTD constitutes the wings.[132] This bipartite organization is important for E1$_{ATG7}$'s ability to pair two different Ubl's with their cognate E2s. Structures of ATG7-CTD−ATG8 complexes show similarity with E1 and E1-like proteins with respect to the positions of the ATP[131] and ATG8 C-terminal tail.[131,133] Interestingly, deletion of the last 13 C-terminal residues of E1$_{ATG7}$ affects ATG8 but not ATG12 conjugation[134] and a peptide encompassing those residues was shown to interact with ATG8 by NMR.[131] However, this interaction is incompatible with the E1$_{ATG7}$/ATG8 conformation captured in the crystal structure where the visible part of the C-terminus of E1$_{ATG7}$ forms a helical pad that interacts with a different region of ATG8. These results suggested a sequential recruitment model where ATG8 first interacts with the E1$_{ATG7}$ C-terminus prior to engaging the adenylation domain.[131] Contrary to canonical E1s, thioester bond formation does not require extensive remodeling of E1$_{ATG7}$ as structures of E1$_{ATG7}$ bound to ATG8 show the catalytic cysteine already poised on a loop just 7 Å from ATG8's C-terminal carboxylate. The conformation required for thioester bond formation has not yet been elucidated.

E1$_{ATG7}$ interacts with two E2s: E2$_{ATG10}$ and E2$_{ATG3}$. An insertion of about 80 residues in the core domain of E2$_{ATG3}$, termed the flexible region, was initially shown to be important for contacting E1$_{ATG7}$.[135] The binding site was later refined to 13 residues that form a helical structure that interacts with ATG7-NTD.[136] Mutation of certain E1$_{ATG7}$ residues that interact with E2$_{ATG3}$'s flexible region only affect E2$_{ATG3}$ but not E2$_{ATG10}$ interaction, suggesting differential binding requirements for each E2.[136] To better understand the bases for dual E2 specificity of E1$_{ATG7}$, structures were determined with the E1$_{ATG7}$ active site cysteine cross-linked to the active site cysteine of E2$_{ATG10}$ and E2$_{ATG3}$ (Figure 9).[132] These structures
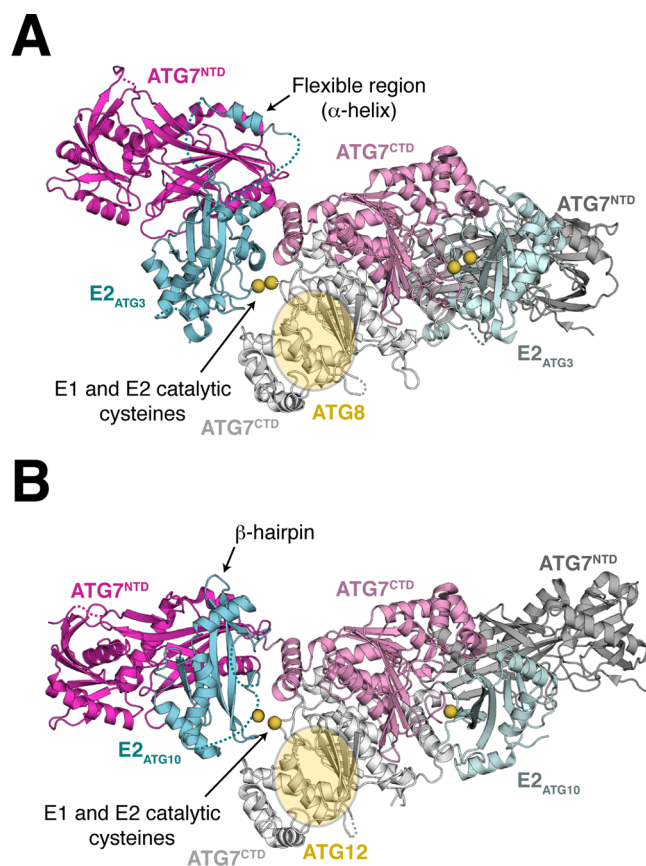
Figure 9. E1−E2 interaction for ATG7. (A) Structure of *S. cerevisiae* E1$_{ATG7}$−E2$_{ATG3}$ (PDB 4GSL). (B) Structure of *S. cerevisiae* E1$_{ATG7}$−E2$_{ATG10}$ (PDB 4GSK). In both cases, E2 binding occurs between the NTD and CTD domains of E1$_{ATG7}$. Dashed lines represent regions missing elements in the crystal structure. Sulfur atoms of the catalytic cysteine residues of E1$_{ATG7}$ and E2$_{ATG3}$ are in sphere representation colored yellow. The catalytic cysteine of E2$_{ATG10}$ is not visible in the structure. The position of ATG8 or ATG12 on the adenylation domain is indicated by a yellow oval.

showed that both E2s bind E1$_{ATG7}$ between the NTD and CTD domain in a region called "under-wing" and that variations in the relative orientation of ATG7-NTD and ATG7-CTD enable unique accommodation of each E2. Furthermore, these structures showed that E2$_{ATG10}$ and E2$_{ATG3}$ use different structural elements to contact ATG7-NTD. While E2$_{ATG3}$ uses an α-helix, E2$_{ATG10}$ uses a β-hairpin. Importantly, these structures confirmed the *trans* mechanism as previously envisioned,[131,133,136] as the E2 bound by one subunit of the E1$_{ATG7}$ dimer is cross-linked to the other subunit. These findings were also corroborated by a contemporaneous study

that showed the same *trans* mechanism using structures of E2$_{ATG3}$/ATG7-NTD and E2$_{ATG10}$/ATG7-NTD.[137] These E1$_{ATG7}$/E2 structures provided considerable insight into E2 selection, although the structural basis for selective pairing of ATG8 with E2$_{ATG3}$ and ATG12 with E2$_{ATG10}$ remains elusive.

4.2.3. UBA5: UFM1 E1. E1$_{UBA5}$ represents a minimalistic E1 composed of a single domain[138] that is necessary and sufficient for UFM1 activation and thioesterification.[139] The structure of an E1$_{UBA5}$/UFM1 complex shows a homodimeric arrangement similar to that observed for MoeB.[140] Comparison of structures of E1$_{UBA5}$ and E1$_{UBA5}$/UFM1 reveals a rearrangement of the crossover loop upon UFM1 binding that repositions the catalytic cysteine closer to the C-terminal end of UFM1.[89,139] This would facilitate the formation of an E1∼UFM1 thioester, a process that is also stimulated by binding of E2$_{UFC1}$.[141] Contrary to canonical E1s, UBA5 does not undergo a second round of adenylation following thioester formation.[141] However, thioester transfer is accelerated by E1$_{UBA5}$ binding of ATP and magnesium.[141] Similar to E1$_{ATG7}$, thioester transfer of UFM1 from E1$_{UBA5}$ to E2$_{UFC1}$ was shown to occur via a *trans* mechanism.[140]

By analogy to canonical E1s, UBA5 was postulated to possess a UFD that would bind E2$_{UFC1}$. This is based on experiments showing interactions between the C-terminus of E1$_{UBA5}$ and E2$_{UFC1}$[142] and observations that deletion of the C-terminus of E1$_{UBA5}$ abrogates UFM1 loading on E2$_{UFC1}$.[139] A region of 23 residues with helical propensity in the E1$_{UBA5}$ C-terminus however appears to be sufficient for E2$_{UFC1}$ interaction, suggesting that the E1$_{UBA5}$ C-terminus does not adopt a ubiquitin fold.[143] The C-terminus of E1$_{UBA5}$ also contains an LIR/UFIM motif that interacts with UFM1. In the context of the homodimeric E1$_{UBA5}$, this motif binds UFM1 in *trans*[140] to facilitate its activation.[89] Mutation or deletion of this motif does not abolish UFM1 activation, suggesting that this motif does not constitute the only determinant for UFM1 selection.[89,139] Furthermore, this motif was shown to interact with certain members from the ATG8 family although this interaction was insufficient to trigger their activation.[89] In addition to UFM1, E1$_{UBA5}$ was also reported to activate SUMO2 in vitro;[144] however, details of this interaction remain unclear.

5. E2 CONJUGATING ENZYMES

E2 conjugating enzymes can be divided into canonical and noncanonical E2s (Figure 10). Canonical E2s include those that carry ubiquitin, SUMO, NEDD8, ISG15, and FAT10 as thioester adducts. They share a common architecture called the UBC fold comprised of approximately 150 residues that typically includes four α-helices and four β-strands with the catalytic cysteine located between β-strand 4 and α-helix 2. In addition to the UBC core, some E2s contain N- and/or C-terminal extensions or large insertions after the catalytic cysteine. As such, canonical E2s have historically been subdivided into four classes according to these criteria.[145] More recent phylogenic analyses have further subclassified canonical E2s into 17 classes.[146] Noncanonical E2s, which comprise those that carry ATG8, ATG12, and UFM1 as thioester adducts, were deemed as too divergent to be included within canonical E2 classifications.[135,146] While these E2s bear some structural resemblance to canonical E2s with respect to their topology, they include more structural variations including a notable lack of the last two C-terminal helices that are observed in canonical E2s. In addition, noncanonical E2s receive their respective Ubl from noncanonical E1s while
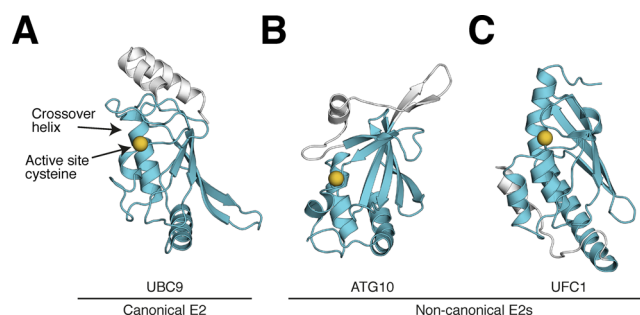

Figure 10. Canonical and noncanonical E2s. (A) A structure of E2$_{UBC9}$ (PDB 1Z5S) was chosen as a representative example of canonical E2s. (B) Structure of E2$_{ATG10}$ (PDB 3VX7), a noncanonical E2. (C) Structure of E2$_{UFC1}$ (PDB 3EVX), a second noncanonical E2 that differs from both E2$_{UBC9}$ and E2$_{ATG10}$. Proteins are in cartoon representation colored cyan except for divergent structural elements that are colored white. The sulfur atoms of the catalytic cysteine residues are in sphere representation to highlight the shift in position of this residue in E2$_{ATG10}$ as compared to E2$_{UBC9}$ and E2$_{UFC1}$.

canonical E2s receive their Ubl from canonical E1s. Canonical E1/E2 interactions are mutually exclusive with E2/E3 interactions,[147] implying that E2s must disengage from E3s to be recharged by E1s. This property appears conserved for noncanonical E2s as illustrated by analysis of E2$_{ATG3}$ activation.[148,149] In this section we will review mechanistic and structural findings on E2s that mediate Ubl conjugation. Complementary information on the functions and structures of E2s involved in ubiquitin conjugation can be found in a recent review.[7]

5.1. Reactions

E1s transfer Ubl's to E2s through a transthioesterification reaction. Following this step, many E2s can transfer their Ubl to a lysine residue through an aminolysis reaction where the primary amine of a deprotonated lysine residue acts as a nucleophile to attack the thioester bond that links the Ubl and E2 (Figure 11). This reaction likely involves formation of a tetrahedral intermediate and ultimately resolves with the formation of an isopeptide (amide) bond between the lysine residue and the Ubl C-terminal glycine. A similar reaction is catalyzed by E2$_{ATG3}$ to conjugate ATG8 to the primary amine of PE.[35]

Several ubiquitin E2s can transfer their ubiquitin moieties to members of the homologous to E6AP C-terminus (HECT) or RING-between-RING (RBR) families of E3 ligases through a transthioesterification reaction that generates an E3 thioester adduct to ubiquitin.[7,150] Once loaded with ubiquitin, these E3s can modify the lysine residue of protein substrates through an aminolysis reaction in the absence of the E2 (reviewed in Buetow and Huang[8]). In Ubl conjugation, this appears to be limited to E2$_{UBCH8}$ that transfers its Ubl ISG15 to HERC5[151] or HHARI.[152]

5.2. Active Site Organization

Canonical E2s share an active site architecture that includes two loops that are supported by multiple intra- or interloop interactions. The catalytic cysteine is located on one of these loops, and its position within the UBC fold is conserved among canonical E2s. While the position of the catalytic cysteine is conserved, the identity and location of other residues that participate in catalysis are often different. Furthermore, and unlike many other enzymes, E2 active sites appear devoid of residues that could participate in general acid/base catalysis
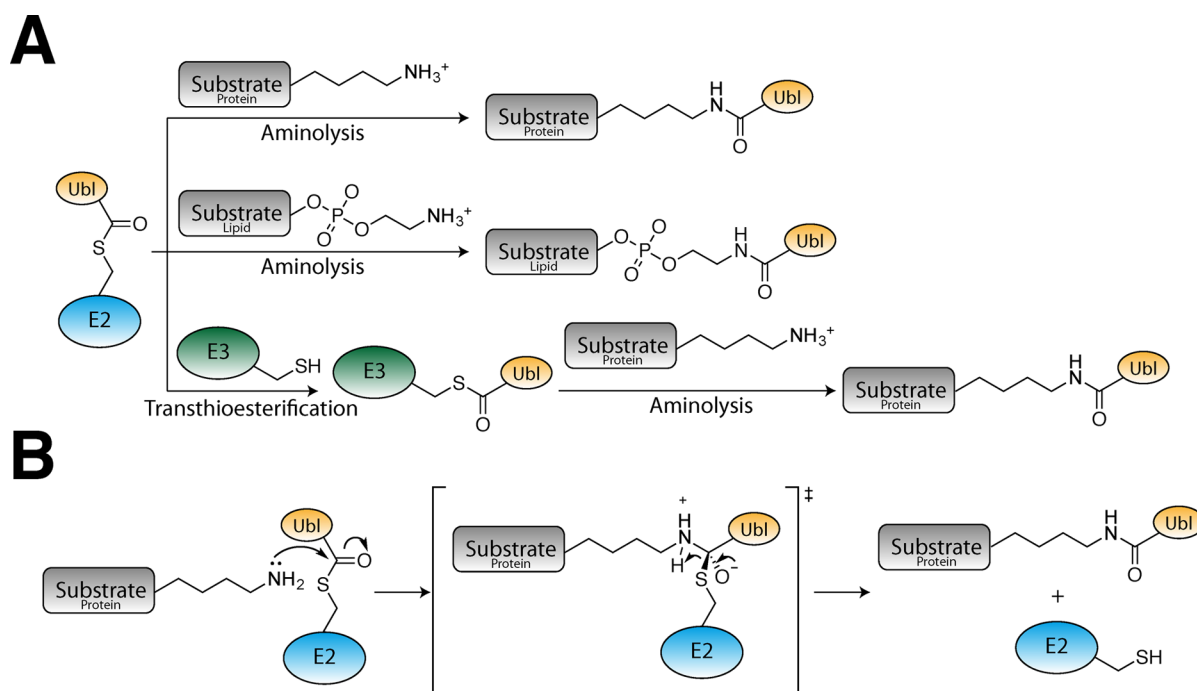
Figure 11. E2 chemical reactions (A) Scheme illustrating how the Ubl moiety of a E2~Ubl thioester can be transferred to the primary amine group of a lysine residue of a protein substrate (top), to the primary amine group of PE (middle), or to a HECT or RBR E3 for subsequent transfer to the lysine residue of a protein substrate (bottom). Transfer to PE is performed by $E2_{ATG3}$. Ubl transfer to E3s of the HECT or RBR families are limited to $E2_{UBCH8}$, and this only allows the ISGylation of a limited number of protein substrates. (B) Mechanism for the aminolysis reaction. In this case, the primary amines are presented in their deprotonated states.

suggesting that they must rely on alternative mechanisms to activate or deprotonate the incoming nucleophile and/or to increase the reactivity of the thioester bond.

Although many E2s have been characterized at the biochemical and structural level, several characteristics of the E2 active site can be appreciated from studies in the SUMO system that identified $E2_{UBC9}$ residues adjacent to the catalytic cysteine that contribute to a microenvironment that suppresses the pK_a of the incoming lysine nucleophile while optimally positioning the thioester bond and incoming lysine residue for catalysis (Figure 12).[153−155] One particular study highlighted the contribution of Asn85, Tyr87, and Asp127 to isopeptide bond formation using discharge assays, model substrates, and structural analysis of mutations at each position in complex with the substrate RANGAP1.[155] Importantly, mutation of individual residues to alanine resulted in a decreased rate of discharge with little effect on substrate binding. Asn85 lies within the HPN motif that is highly conserved among E2s,[156,157] and its side chain is within hydrogen bonding distance to the carbonyl oxygen of the C-terminal glycine in structures of E2 thioester mimics or product complexes.[154,158−166] As such, this residue appears optimally positioned to stabilize the thioester bond prior to catalysis and was in fact originally proposed to stabilize the oxyanion intermediate.[156] While Asn85 is certainly important for catalysis, later studies suggested that it was also important for stabilizing the loop that contains Asp127.[155,167] Unlike Asn85 that contacts the thioester, Tyr87 and Asp127 appear to contribute to catalysis by forming an environment that orients and desolvates the incoming lysine nucleophile.[155] Asp127 is often present in other E2s as a serine or aspartate residue,[155,168] and in some cases phosphorylation of the serine residue has been shown to increase catalytic efficiency[155,168,169] although

downregulation has also been reported.[170] While Tyr87 is less well conserved, the hydrophobic property of amino acids at the analogous structural position appears widely conserved.[155]

In cases where equivalents of Asp127 or Tyr87 are missing, structural equivalents appear to be contributed by the substrate itself. For instance, $E2_{UBC12}$ lacks a residue equivalent to Tyr87 in $E2_{UBC9}$. In a structure of a charged $E2_{UBC12}$/CUL1 complex, a tyrosine residue in the CUL1 substrate acts as a structural mimetic of Tyr87.[171] While the equivalent of Asp127 in $E2_{UBC9}$ exists in $E2_{UBC12}$, mutation of Asp143 had little effect on NEDD8 conjugation. In this case, the backbone carbonyl of Asp143 appears closer to the incoming lysine and may functionally replace its carboxylic group.[171] Another example was illustrated for $E2_{UBC1}$, an E2 that lacks a structural equivalent to Tyr87.[172] In this case, the authors suggested that ubiquitin Tyr59 contributes to the formation of a hydrophobic microenvironment that assists in activating the attacking lysine residue, in this case Lys48 of ubiquitin. $E2_{UBE2S}$ constitutes a case of substrate-assisted catalysis as Glu34, a glutamate residue in the ubiquitin substrate, is predicted to interact with the target lysine Lys11, thereby functionally mimicking Asp127 in $E2_{UBC9}$.[173]

The location of the active site is not strictly conserved for all E2s. In $E2_{ATG3}$ and $E2_{ATG10}$, despite being present in topologically equivalent locations, the position of the active site cysteine is shifted by ~12 Å when compared to canonical E2s such as $E2_{UBC9}$. Despite this difference, the catalytic cysteine of $E2_{ATG10}$ is located adjacent to Tyr56 and Asn114, two residues that could function in manners similar to those reported for Tyr87 and Asp127 in $E2_{UBC9}$. Consistent with this idea, mutation of these residues to alanine leads to a defect on k_{cat} with little effect on K_m.[174]
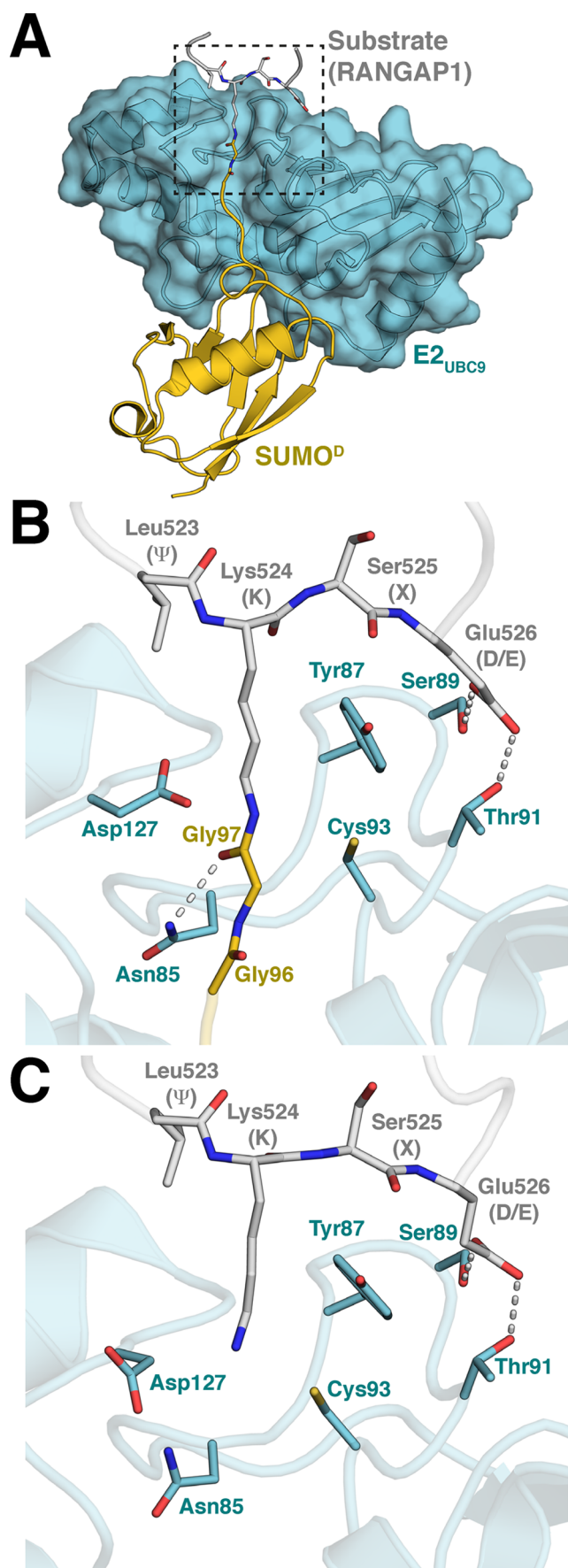
Figure 12. E2 active site. (A) Overall view and (B) close-up view of E2$_{UBC9}$/SUMO−RANGAP1 (PDB 1Z5S) illustrating how the

Figure 12. continued

RANGAP1 substrate and SUMO are positioned in the E2$_{UBC9}$ active site. This state represents a product complex after conjugation where SUMO, colored yellow, has been transferred to a lysine of RANGAP1 colored gray. SUMO^D designates a SUMO protein in donor (D) configuration. E2$_{UBC9}$ is in cartoon representation colored cyan. Certain residues of the E2 active site are in stick representation. The consensus sequence for substrate recognition by E2$_{UBC9}$ is indicated on top. (C) Close-up of E2$_{UBC9}$/RANGAP1 (PDB 1KPS) representing RANGAP binding prior to catalysis in the absence of SUMO.

5.3. Target Selection

Most E2s do not exhibit specificity and consequently require an E3 to promote selective interactions with their substrates. In contrast, E2$_{UBC9}$ can directly recognize substrates that contain a SUMO consensus motif comprised by Ψ-Lys-X-Asp/Glu where Ψ is a hydrophobic residue, Lys is the target lysine to which SUMO is attached, and X is any residue.[175] While SUMO can be conjugated to other sites, mass spectrometry analyses of SUMO targets show a clear enrichment of this motif or related motifs at sites of conjugation (see Hendriks and Vertegaal[38] for review). The structural basis for recognition of this motif was first illustrated by a structure of RANGAP1/E2$_{UBC9}$ (Figure 12).[153] In this complex, the hydrophobic residue contacts a somewhat featureless hydrophobic surface formed by residues Pro128-Ala129-Gln130, an observation consistent with accommodation of hydrophobic residues that differ in size. As discussed in section 5.2, the substrate lysine residue is positioned via aliphatic contacts to Tyr87 and hydrogen bonding interactions with Asp127 to place its primary amine next to the catalytic cysteine of E2$_{UBC9}$. The X residue is located above Tyr87, enabling interactions between the Asp/Glu residue that includes aliphatic contacts to Tyr87 as well as hydrogen bonding contacts to Ser89 and Thr91. Two lysine residues (Lys74, Lys76) are also proximal to the Asp/Glu residues. Although other E2s may harbor intrinsic substrate specificities, the SUMO system appears to be the only one where in vitro and in vivo substrate specificities appear well reconciled by available structural and biochemical data. Other examples include the noncanonical E2s E2$_{ATG10}$ and E2$_{ATG3}$. Indeed, no E3 has been found for E2$_{ATG10}$ for the modification of the ATG5 substrate on a specific lysine of its helical domain.[176,177] NMR and cross-linking analyses of the ATG5/E2$_{ATG10}$ interaction is consistent with the idea that direct recognition of ATG5 by E2$_{ATG10}$, notably through ATG5 C-terminal ubiquitin-like domain, is sufficient for mediating its conjugation to ATG12.[174] E2$_{ATG3}$ was also reported to be sufficient for recognizing its PE substrate in vitro, although this process is stimulated by E3$_{ATG12−ATG5}$.[178]

Formation of Ubl chains can be considered as a special case of target selection as the target is the Ubl itself. In the ubiquitin system, multiple strategies are employed for chain formation. For example, the heterodimeric E2$_{MMS2}$/E2$_{UBC13}$ uses the inactive component E2$_{MMS2}$ to bind ubiquitin and position Lys63 in the active site of E2$_{UBC13}$[179] while the monomeric E2$_{UBE2S}$ exploits a noncovalent interaction with ubiquitin to promote synthesis of Lys11 chains.[173] In the case of SUMO, certain SUMO isoforms contain one or more SUMO consensus motifs within their N-terminal extensions that can be used to form chains.[46,180] It was also proposed that assembly of two or more E2$_{UBC9}$ enzymes can favor formation of SUMO chains.[181] In this case, one E2$_{UBC9}$ would scaffold its backside bound

SUMO such that the SUMO consensus site could reach the active site of a second SUMO~E2$_{UBC9}$ complex. The association of at least two E2$_{UBC9}$ molecules is needed as the SUMO consensus site of a backside bound SUMO cannot reach the active site of the same E2$_{UBC9}$. The concept that E2/E2 interactions might promote chain formation was also proposed based on packing of E2s in certain crystal forms.[182−184] In yet another study, SUMO modification of E2$_{UBC9}$ at Lys153 was shown to decrease E2 activity; however, SUMO-modified E2$_{UBC9}$ increased chain formation by unmodified E2$_{UBC9}$ suggesting that association of two or more E2s, directly or indirectly, can result in increased chain formation.[185] In the NEDD8 system, one study suggested that NEDD8 chains can be formed on the active site cysteine of the E2$_{UBC12}$ and then transferred en bloc to the substrate.[186] In this case, NEDD8 chains appear to be facilitated by association of two or more E2s. Formation of mixed chains has been observed for ubiquitin and some Ubl's with ubiquitination of SUMO chains by SUMO-targeted ubiquitin ligases being possibly the best understood process.[187,188] Evidence also exists for formation of mixed chains containing NEDD8 and ubiquitin under stress conditions, perhaps by misprocessing of NEDD8 by the ubiquitin conjugation machinery.[189,190] Finally, the ISGylation of Lys29 of ubiquitin was recently reported.[191] While multiple mechanisms have been proposed for formation of chains, the underlying structural bases for these activities remain unclear.

5.4. Dynamics

The dynamics of the E2~Ubl thioester adduct are best understood in the ubiquitin system where comparisons between multiple E2~ubiquitin structures or their mimics have revealed that ubiquitin can adopt a variety of orientations relative to the E2.[158,166,179,192] NMR studies have further suggested that different E2s can populate different conformational states.[193] One conformation with particular significance was termed the "closed" conformation. In this case, ubiquitin packs against the crossover helix of the E2, thereby positioning the ubiquitin C-terminus in a shallow groove that leads to the E2 active site cysteine where it is stabilized by multiple interactions. The first structure of an E2 thioester-linked to ubiquitin, determined by NMR, revealed ubiquitin in a state similar to the closed conformation.[158] A subsequent structure of E3$_{RANBP2}$/E2$_{UBC9}$/SUMO−RANGAP1 showed SUMO in a closed conformation (Figure 13A) in work that proposed that the closed conformation was required for activation of the thioester bond.[154] Closed conformations have since emerged as a hallmark for E2~Ubl activation in the ubiquitin, SUMO, and NEDD8 pathways for aminolysis[160,161,171,194,195] because the closed conformation positions the thioester bond in the E2 active site in an orientation that enhances reactivity. As described in section 6, E3 ligases can enhance reactivity by stabilizing the closed conformation (see below).

5.5. Backside Binding by Ubl's

Studies in the ubiquitin and SUMO pathways have revealed that proteins, including ubiquitin and SUMO, can interact with E2s on a surface termed the E2 "backside" that is opposite to the active site (Figure 13B). Backside binding between E2s and ubiquitin or SUMO contributes to chain building activities[181,196−198] or to regulation of E2 activities via allostery.[162] Although backside E2/Ubl complexes utilize similar surfaces in ubiquitin or SUMO pathways, the strength of these interactions can vary. Ubiquitin and certain ubiquitin E2s interact with
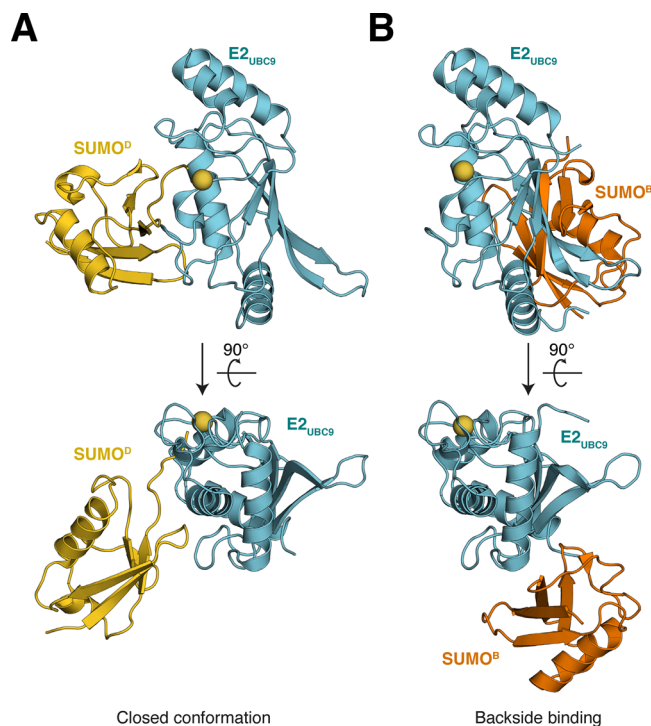

Figure 13. E2~Ubl complexes. (A) Structure of E2$_{UBC9}$ in complex with SUMO in a closed conformation (PDB 1Z5S). (B) Structure of E2$_{UBC9}$ in complex with SUMO that binds E2$_{UBC9}$ on the E2 backside (PDB 2PE6). Proteins are in cartoon representation with catalytic cysteine residues in sphere representation. SUMO^D and SUMO^B represent SUMO proteins in donor (D) and backside (B) configurations, respectively.

affinities in the high micromolar range,[162,196] while interactions between SUMO and E2$_{UBC9}$ occur in the nanomolar range.[164,181,199,200] Other proteins can also bind to E2s via the backside surface. For instance, a membrane-anchored ubiquitin-fold protein (MUB) that is targeted to membranes following prenylation of its C-terminus was shown to bind the backside of certain ubiquitin E2s; however, binding affinity in this case was in the nanomolar range due to the presence of a longer loop that extends the binding interface.[201] In addition to the canonical ubiquitin interaction on the backside of an E2, a second "noncanonical" binding site for ubiquitin has been found on E2$_{RAD6}$.[202] There is limited data on the prevalence of this alternate ubiquitin interaction surface among other E2s.

In addition to Ubl's, several proteins can bind the backside of E2 to modulate E2 activities. The SUMO E3 ligase RANBP2 contacts E2$_{UBC9}$ through its backside.[154] In the ubiquitin system, the protein CUE1 contacts the K48 chain-building E2$_{UBC7}$ on its backside though an α-helical U7BR domain.[203] CUE1 also possesses a Cue domain that displays preference for binding the proximal penultimate ubiquitin moiety of a chain,[204] presumably to increase its chain extending activities. Collectively, these studies reveal that the E2 backside can act as a versatile platform to modulate E2 activities or localization.

5.6. E2 Modifications

Covalent modification of an E2 by its own Ubl has been suggested as a mechanism to regulate E2 activities, specificities, or levels of the respective E2. For example, E2$_{UBE2Z}$ is FATylated,[117] a modification that targets the E2 for proteasomal degradation.[205] In the case of metazoan E2$_{UBC9}$, SUMO modification on the first α-helix has been shown to alter

substrate specificity with little effect on enzyme activity.[206] $E2_{ATG3}$ can be modified with ATG12 on a specific lysine, and this modification requires the $E2_{ATG3}$ catalytic cysteine and was shown to occur in *cis*.[41] The resulting ATG12−$E2_{ATG3}$ plays a role in mitochondrial homeostasis.[41]

6. E3 LIGASES

E3 protein ligases are generally considered as factors that increase the rate of ubiquitin or Ubl conjugation to substrates. This is often accomplished by recruiting the E2∼Ubl thioester and substrate into a complex. While colocalization is critical, the E3 can also enhance the rate of isopeptide bond formation by templating the charged E2∼Ubl thioester into a "primed" conformation that positions or aligns the ubiquitin or Ubl thioester bond for nucleophilic attack. While the ubiquitin system includes more than 600 E3s and dozens of E2s that combine in unique configurations to dictate substrate specificity,[207] only a few E3s have been reported thus far for SUMO, NEDD8, and ISG15 Ubl's. Furthermore, only one E3 has been identified to date for ATG8 and UFM1 and no E3s have been identified to date for FAT10, URM1, and ATG12. In the ubiquitin system, really interesting new gene (RING), HECT, and RBR proteins constitute the three main E3 families (recently reviewed in Buetow and Huang[8]). Those E3s that do not belong to one of these families are generally referred to as atypical. In this section, we will review RING E3s that facilitate Ubl conjugation, emphasizing common and contrasting properties with those of the ubiquitin E3 RING family. We will also describe mechanisms underlying atypical Ubl E3 ligase activity. While important, we will not describe HECT and RBR ligases or E3 ligases for ISG15 conjugation[151,152,208,209] as most insights gained for these E3s relied on characterizations with ubiquitin and not ISG15. We refer the reader to Buetow and Huang for their detailed review of ubiquitin E3s.[8]

6.1. RING Ligases

6.1.1. RING Domain and its Variants. The RING domain contains cysteine and histidine residues that coordinate two zinc ions with a cross-braced topology (reviewed in Deshaies and Joazeiro[210]). In a landmark study, Lorick et al.[211] noted that several RING domain-containing proteins that interact with E2 also displayed ubiquitin E3 ligase activity. U-boxes and SP-RING domains were later shown to be structurally and functionally related to the RING domain. Indeed, the U-box domain shares a similar topology as the RING domain except that the canonical RING cysteine and histidine residues that coordinate the two zinc ions are replaced by side chains that interact to stabilize the cross-braced architecture.[212] The SP-RING domain is hybrid between RING and U-box domains in that it possesses one zinc coordination site with the other replaced by side chains that interact to stabilize the cross-braced architecture.[213] Proteins with U-box domains are known to catalyze ubiquitin E3 ligase activity, whereas proteins with SP-RING domains catalyze SUMO E3 ligase activity. Collectively, the RING domain and its structural variants increase the rate of ubiquitin, SUMO, NEDD8, and ISG15 conjugation by binding the charged E2∼Ubl thioester adduct and activating it for chemistry.[211,214−216]

6.1.2. E2 Binding. The first atomic insights into E2 recognition by RING E3s came from the structure of $E3_{CBL}$/$E2_{UBCH7}$ in the absence of ubiquitin.[217] This structure showed how a surface on the RING domain composed of two E3 loops and an α-helix between the two respective zinc coordination

sites contacts two loops and the N-terminal helix of the E2. While this structure showed how the RING binds E2, direct involvement of the RING domain in E2-mediated conjugation remained a puzzle because the E2 active site was ~15 Å from the RING domain.[217] Similar interactions were later observed for several E3/E2 pairs (Figure 14), including those for the
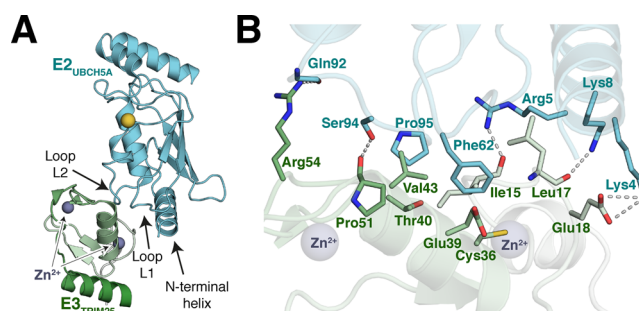

Figure 14. Representative RING E3/E2 interaction. (A) Overall view and (B) close-up view of human $E3_{TRIM25}$/$E2_{UBCH5A}$ (PDB 5FER). Both proteins are in cartoon representation. A white-to-green gradient running from the N- to C-terminus has been applied to $E3_{TRIM25}$. Two zinc ions are depicted as gray spheres. Residues contributing to the $E3_{TRIM25}$/$E2_{UBCH5A}$ interaction are in stick representation.

NEDD8 and SUMO pathways.[171,200] In addition to canonical E3/E2 interactions, some RING-containing proteins use additional domains to increase affinity for a particular E2 or to alter E2 properties. For example, $E3_{RNF125}$ includes a zinc finger that folds back on the RING domain to stabilize the RING and to extend interaction surfaces with the E2,[218] while other proteins include extensions that contact the backside of the E2, frequently through the formation of an α-helix.[203,219−222] In the case of $E3_{RAD18}$/$E2_{RAD6}$ interaction, the $E3_{RAD18}$ C-terminal α-helix binds the backside of $E2_{RAD6}$ to prevent ubiquitin from binding the same surface, thus decreasing the ability of this E3/E2 pair to form ubiquitin chains.[220] To illustrate yet another variation, the RING of $E3_{FANCL}$ uses a short N-terminal extension and additional hydrophilic contacts to achieve specific interaction with $E2_{UBE2T}$.[223] Additional domains can also be used for allosteric regulation. For example, binding of an $E3_{GP78}$ C-terminal α-helix on the backside of $E2_{UBE2G2}$ was reported to stimulate E2 activity through an allosteric mechanism.[219] To provide an example of ligand induced regulation, binding of one unit of a poly(ADP-ribose) chain by the WWE domain of $E3_{RNF146}$ triggers a conformational change in this RING-containing protein that increases E2 binding and stimulates E3 activity.[224] In the SUMO system, the SP-RING domain is frequently followed by a SIM with evidence pointing toward a role for this SIM in contacting SUMO conjugated substrates or a second molecule of SUMO that is bound to the backside of the $E2_{UBC9}$∼SUMO thioester adduct.[200,225] In the NEDD8 system, DCN1 is often viewed as a co-E3 for RBX1 because it further stimulates $E3_{RBX1}$-mediated Cullin neddylation.[226] In this case, DCN1 recognizes the Cullin substrates and the acetylated N-terminal extension of $E2_{UBC12}$ to facilitate $E2_{UBC12}$∼NEDD8 recruitment.[171,227,228]

6.1.3. E2∼Ubl Binding and Activation. While structures of multiple E2/E3 pairs provided clues as to how RING E3s might increase the rate of E2-mediated catalysis, these complexes represented product complexes because they lacked ubiquitin or the E2∼ubiquitin thioester (or mimics). Indeed,

structural studies of RING E3/E2∼ubiquitin complexes revealed that catalytic activation was achieved because the RING domain stabilizes a closed E2∼ubiquitin conformation that aligns the thioester bond for nucleophilic attack and ubiquitin discharge.[160−163,165,194,195,229,230] This mechanism also appears prevalent in the NEDD8 and SUMO pathways where RING E3s stabilize the closed conformation of E2$_{UBC9}$∼SUMO and E2$_{UBC12}$∼NEDD8 (Figure 15).[171,200]
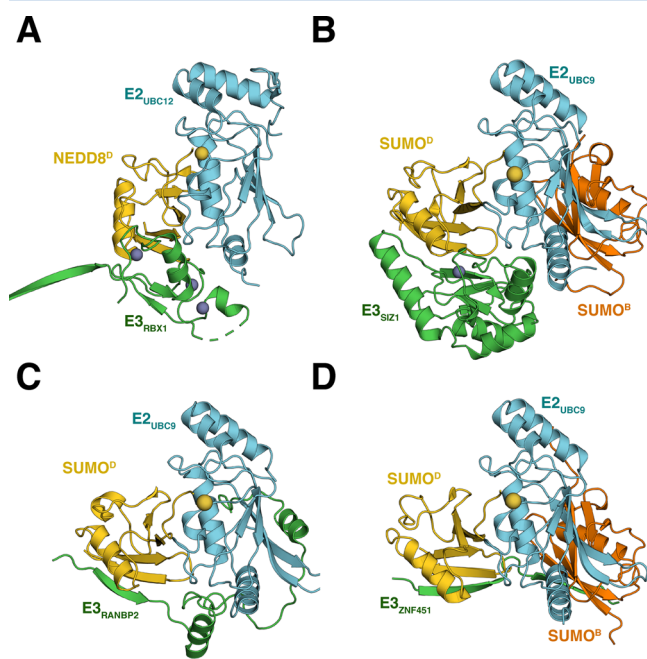

Figure 15. E3 stabilization of a closed E2∼Ubl conformation. (A) Structure of human NEDD8∼E2$_{UBC12}$/E3$_{RBX1}$ (PDB 4P5O). The position of the catalytic cysteine (a serine residue in the structure) is indicated by a yellow sphere. (B) Structure of yeast SUMO∼E2$_{UBC9}$/E3$_{SIZ1}$ (PDB 5JNE). (C) Structure of human SUMO∼E2$_{UBC9}$/E3$_{RANBP2}$ (PDB 1Z5S). (D) Structure of human SUMO∼E2$_{UBC9}$/E3$_{ZNF451}$ (PDB 5D2M). Zinc atoms are in gray sphere representations. SUMO^D and SUMO^B represent SUMO proteins in donor (D) and backside (B) configurations, respectively. NEDD8^D designates a NEDD8 protein in donor (D) configuration.

Formation of a closed conformation remains dependent on canonical E2/E3 interactions but now also depends on contacts between the RING or ancillary motifs and the ubiquitin or the Ubl moiety. In the case of dimeric RINGs, one RING binds the E2 while the other RING provides vital contacts to ubiquitin.[161,163,165,195,229,230] For monomeric RINGs, additional structural elements contribute interactions that are functionally analogous to those provided by dimeric RINGs. In the case of E3$_{CBL-B}$, ubiquitin is contacted by an N-terminal helix that contains a phosphotyrosine residue,[160] interactions that increase E2 catalytic efficiency by more than 2 orders of magnitude.[160] The monomeric RING of E3$_{ARK2C}$ binds a second molecule of ubiquitin on its backside to form a complex that stabilizes E2∼ubiquitin in a closed conformation through ubiquitin−ubiquitin interactions.[231]

Early studies in the SUMO pathway suggested the importance of interactions between an acidic region of E3$_{SIZ1}$ and a basic patch on SUMO that was derived by docking E2$_{UBC9}$∼SUMO in a closed conformation to the E3$_{SIZ1}$ SP-RING domain,[213] but the true nature of contacts was not revealed until a structure of E3$_{SIZ1}$/E2$_{UBC9}$∼SUMO/PCNA was

determined.[200] This structure showed that SUMO does interact with E3$_{SIZ1}$; however, contacts were more extensive than anticipated as the E3$_{SIZ1}$ contains an additional domain termed the SP-CTD that includes an embedded SIM-like motif that stabilizes donor SUMO in the closed conformation.

Stabilization of the closed E2∼Ubl conformation is also observed in the NEDD8 pathway. In this system, the RING-containing E3$_{RBX1}$ interacts with its obligate partner CUL1 via an N-terminal β-strand extension of E3$_{RBX1}$ that intercalates in a β-sheet of CUL1.[232] A short linker between the N-terminal extension of E3$_{RBX1}$ and its RING domain enables a hinge motion of the RING domain relative to CUL1. While E2$_{UBC12}$ binds the RING domain of E3$_{RBX1}$, the NEDD8 moiety of E2$_{UBC12}$∼NEDD8 binds E3$_{RBX1}$ through the linker region. This freezes the conformation of the linker and orients the E3$_{RBX1}$ active site relative to the lysine substrate, in this case a lysine in CUL1 itself. E3$_{RBX1}$ binding to NEDD8 thus fulfills multiple roles, it maintains NEDD8 in the closed conformation and it orients the entire complex for substrate recognition.[171] E3$_{RBX1}$ also binds E2$_{UBCH5}$ and E2$_{CDC34}$ to promote substrate ubiquitination. An NMR study showed minimal interaction between isolated E3$_{RBX1}$ and E2$_{CDC34}$ while interactions with E2$_{CDC34}$∼ubiquitin revealed a dissociation constant in the midmicromolar range[233] underscoring the importance of contacts to ubiquitin in a system that is likely optimized for rapid release of the E2 product upon ubiquitin discharge.[233]

An unusual case of RING-mediated ubiquitin binding is seen in the anaphase promoting complex/cyclosome (APC/C) complex, a multisubunit E3. In this case, the RING-containing protein APC2 interacts with E2$_{UBE2C}$ through canonical E2/E3 interactions to prime the substrate by ubiquitination; however, efficient substrate polyubiquitination is dependent on replacement of E2$_{UBE2C}$ by E2$_{UBE2S}$. In this latter step, E2$_{UBE2S}$ does not contact the canonical RING surface of APC2, but instead uses its C-terminal tail to contact other APC/C surfaces. This allows APC2 to interact with ubiquitin in the context of a ubiquitinated substrate to catalyze E2$_{UBE2S}$-mediated chain elongation.[234]

One noted hallmark of ubiquitin RING activation is a "linchpin" arginine within the RING that contacts both E2 and ubiquitin to lock ubiquitin in the closed conformation (reviewed in Buetow and Huang[8]).[194] While this residue is clearly important in a variety of E3s, it may not be a universal requirement as at least one case reported little effect on activity upon substitution to alanine.[229] Interestingly, this residue is notably absent in E3$_{SIZ1}$[200] and, in E3$_{RBX1}$, alanine substitution of the topologically equivalent residue (Asn98) does not affect conjugation while an N98R substitution impairs neddylation.[171] Instead, another arginine, Arg46, functions in an analogous manner by interacting with E2$_{UBC12}$ and NEDD8, although it is present on a distinct surface of E3$_{RBX1}$. While there are many ways in which RINGs or related domains interact with their E2∼Ubl thioester substrates, a common mechanism emerges, namely that RING and RING-like domains bind the E2∼Ubl thioester, activating it for conjugation by stabilizing the closed conformation.

6.1.4. Substrate Interaction.

Structural studies on E3/E2∼Ubl/substrate complexes are scarce, but the ones that exist provide vital clues as to how E3/E2∼Ubl complexes target their substrates (Figure 16). One example pertains to E2$_{UBC12}$-mediated NEDD8 modification of residue Lys720 of CUL1, a subunit of the E3 ligase complex. The structure of a co-E3$_{DCN1}$/E3$_{RBX1}$/E2$_{UBC12}$∼NEDD8/CUL1 complex[171] revealed few
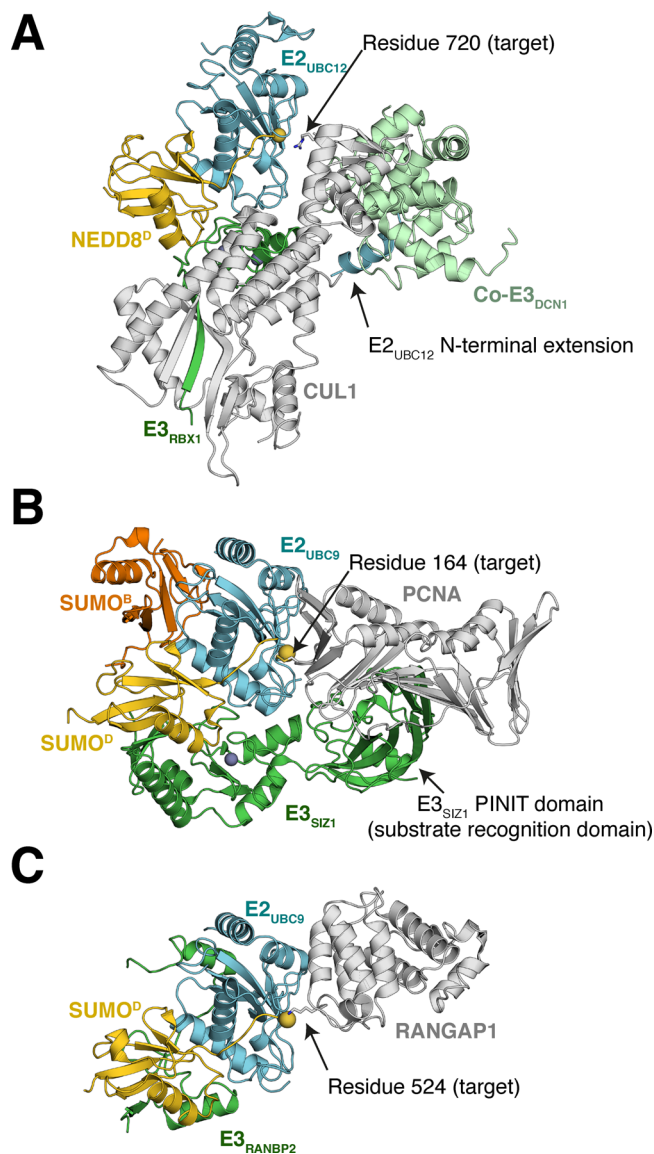
Figure 16. E3/E2∼Ubl/substrate complexes. (A) Structure of human co-E3$_{DCN1}$/E3$_{RBX1}$/E2$_{UBC12}$∼NEDD8/CUL1 (PDB 4P5O). The target residue 720 (an arginine in the structure) is in stick representation. NEDD8^D designates a NEDD8 protein in donor (D) configuration. (B) Structure of yeast SUMO−E3$_{SIZ1}$/SUMO∼ E2$_{UBC9}$−PCNA (PDB 5JNE). The target residue 164 (a cysteine in the structure) and its linkage to the E2 catalytic cysteine via ethanedithiol are presented in stick representation. SUMO^D and SUMO^B represent SUMO proteins in donor (D) and backside (B) configurations, respectively. (C) Structure of human SUMO−RANGAP1/E2$_{UBC9}$/E3$_{RANBP2}$ (PDB 1Z5S). The isopeptide linkage between the target residue 524 and the C-terminal glycine of SUMO is in stick representation.

contacts between the NEDD8-bound E2$_{UBC12}$ and its CUL1 substrate, suggesting substrate recognition relied on multiple binding interactions that collectively lead to correct positioning of the activated E2 relative to the target lysine. While the co-E3 DCN1 binds to and bridges the acetylated N-terminal extension of E2$_{UBC12}$ and CUL1, the UBC domain of E2$_{UBC12}$ is recognized by E3$_{RBX1}$ through a classical RING E3/E2 interaction. NEDD8 also plays an important role in the active complex as the closed conformation of NEDD8 bends the E3$_{RBX1}$ linker between the N-terminal extension of E3$_{RBX1}$ and its RING domain to bring the E2$_{UBC12}$ active site into proximity

of CUL1 Lys720. Structural studies of ubiquitin modification of histone H2A at Lys119 provided another example where the E2 and substrate barely interact.[235] In this case, the structure of an E3$_{RING1B−BMI1}$/E2$_{UBCH5C}$/nucleosome complex revealed few contacts between the E2$_{UBCH5C}$ and the region surrounding Lys119. Instead, the E3 binds to a region centered on the acidic patch of the nucleosome and this interaction, combined with E2$_{UBCH5C}$/DNA interactions, results in positioning of Lys119 close to the E2$_{UBCH5C}$ active site. Any role for ubiquitin in this complex remains unclear as it was absent from this structure. The last example draws from E3$_{SIZ1}$ ligase catalyzed SUMO modification of yeast PCNA, a substrate that includes two SUMO modification sites: a primary site at Lys164 and a secondary site at Lys127.[236] While Lys127 modification can be enhanced by E3$_{SIZ1}$, it lies within a SUMO consensus motif and can be modified by the SUMO E2$_{UBC9}$ in the absence of E3$_{SIZ1}$. In contrast, Lys164 modification appears strictly dependent on the E3$_{SIZ1}$.[237] Subsequent structural and biochemical studies revealed that the SP-RING domain was important for modification of Lys127 and Lys164, but Lys164 modification relied on interactions between PCNA and the N-terminal PINIT domain of E3$_{SIZ1}$.[213] The structure of an SUMO−E3$_{SIZ1}$/SUMO∼E2$_{UBC9}$−PCNA complex provided a rationale for Lys164 modification in that E3$_{SIZ1}$/PCNA interactions position the substrate within the activated E3/E2∼Ubl complex to force-feed Lys164 into the E2$_{UBC9}$ active site.[200]

6.2. Atypical E3 Ligases

6.2.1. SIM-Based SUMO E3 Ligases.
E3$_{RANBP2}$ is an atypical SUMO E3 ligase[238] whose catalytic domain(s) reside in a 30 kDa fragment called the IR1-M-IR2 repeat.[239] The structure of an E3$_{RANBP2}$/E2$_{UBC9}$/SUMO−RANGAP1 complex that corresponds to a product complex after conjugation revealed that the E3$_{RANBP2}$ IR1-M domain uses a combination of loops and helices to contact the E2$_{UBC9}$ backside while a SIM binds the donor SUMO to position it in a closed conformation analogous to that described above for RING-mediated E2∼Ubl activation (Figure 15C).[154] This early study suggested that E3 ligase activity was due to coordination of the Ubl∼E2 thioester through stabilization of the Ubl∼E2 closed conformation. E3$_{ZNF451}$ is another recently identified SUMO E3 whose catalytic module includes two SIMs that are separated by an intervening Pro-Leu-Arg-Pro sequence.[164,240] The structure of an E3$_{ZNF451}$/E2$_{UBC9}$/RANGAP1−SUMO complex revealed that E3$_{ZNF451}$ uses its N-terminal SIM to maintain the donor SUMO in a closed conformation[164] while its C-terminal SIM engages a second SUMO molecule that is bound on the backside of E2$_{UBC9}$ (Figure 15D). This places the Pro-Leu-Arg-Pro sequence under the E2 to enable direct contacts between the arginine residue and E2$_{UBC9}$. E3$_{ZNF451}$ is a target of extensive SUMO modification,[38] and SUMO modification of an E3$_{ZNF451}$ fragment at a site close to the catalytic module can increase SUMO E3 activity, presumably because SUMO modification provides a second SUMO in *cis* for backside interactions with E2∼SUMO. This mechanism was also proposed in the context of E3$_{RNF38}$/E2$_{UBCH5B}$ interactions where ubiquitination of the E3 or a substrate results in an increased catalytic activity.[162] In this case, however, the authors proposed that ubiquitin binding in *cis* on the backside of the E2 can also trigger allosteric activation of the E2.[162] In summary, E3$_{RANBP2}$ and E3$_{ZNF451}$ use SIMs to maintain a donor SUMO in the closed conformation while employing unique strategies to recognize the E2. The idea that SIMs are necessary, although not sufficient, for SUMO

E3 ligase activity appears to be a unifying theme among SUMO E3 ligases, including proteins with a SP-RING domain. While SIMs can play a role in SUMO E3 ligases, their ability to interact with SUMO can result in spurious in vitro artifacts and misidentification of SIM-containing proteins as bona fide SUMO E3 ligases as discussed in Parker and Ulrich.[241]

6.2.2. UFL1. $E3_{UFL1}$ is the only protein identified thus far for which UFM1 E3 activity has been reported.[242,243] $E3_{UFL1}$ increases the rate of UFM1 conjugation to UfBP1[242] and ASC1[48] although it shares no sequence similarity with E3s from other systems and its mode of action remains unclear. It has been suggested that $E3_{UFL1}$ could bridge $E2_{UFC1}$ and its substrate UfBP1 to promote UFM1 conjugation.[242] Interestingly, modification of ASC1 is dependent on prior UFMylation of UfBP1 and likely involves formation of an ASC1/UFM1–UfBP1/$E3_{UFL1}$ complex.[48] This suggests that UFM1–UfBP1 might act as a co-E3, and phenotypic similarities between *UfBP1*, *UFL1*, and *UBA5* knockouts in mice is consistent with this idea.[244,245]

6.2.3. ATG12–ATG5. The ATG12–ATG5 conjugate functions in at least two ways in vivo. First, it acts as an E3 for $E2_{ATG3}$-mediated conjugation of ATG8 to PE.[178,246] Second, through its association with ATG16 and ATG8–PE, it forms a two-dimensional mesh that organizes associated membranes.[88] ATG12–ATG5 is formed by conjugation of the Ubl ATG12 to a specific lysine residue of ATG5.[176] Conjugation appears irreversible, as proteases capable of cleaving ATG12–ATG5 have not been identified to date. Structural analyses of the ATG12–ATG5 complex reveals an extended interface between ATG12 and ATG5 that extends beyond the isopeptide linkage, presumably to maintain ATG12 in a fixed orientation relative to ATG5 (Figure 17).[90,247,248] This interface appears important for the E3 activity of the ATG12–ATG5 conjugate as E3
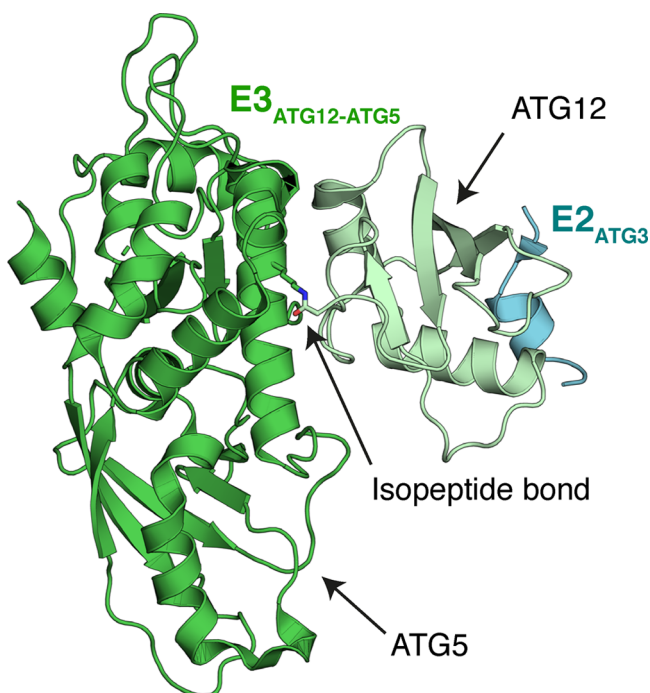

Figure 17. E3/E2 complex in the ATG8 system. Structure of $E3_{ATG12–ATG5}$/$E2_{ATG3}$ (PDB 4NAW). Proteins are in cartoon representation with the isopeptide linkage between ATG12 and ATG5 in stick representation.

activity is compromised by mutations that disrupt the ATG12/ATG5 interface or a conserved composite surface that includes elements from both ATG5 and ATG12.[247] Also, strategies that bring ATG12 and ATG5 together by means other than native isopeptide linkage failed to reconstitute E3 activity.[247]

ATG8 conjugation to PE can be recapitulated in vitro using $E1_{ATG7}$, $E2_{ATG3}$, mature ATG8, ATP, and liposomes containing a high proportion of PE, suggesting that $E2_{ATG3}$~ATG8 can recognize its PE substrate in an E3-independent manner.[249] While this reaction is inefficient in the context of liposomes with low PE content,[249] the ATG12–ATG5 conjugate, but not ATG12 and/or ATG5 alone, increases $E2_{ATG3}$-mediated ATG8 conjugation.[178] Since ATG12 binds $E2_{ATG3}$ with nanomolar affinity without substantive contributions by ATG5,[90,247] it appears that the ATG12–ATG5 conjugate might be required to activate the ATG8~$E2_{ATG3}$ thioester. Consistent with this model, Sakoh-Nakatogawa et al.[250] observed differences in available $E2_{ATG3}$ structures around its catalytic cysteine that correlated with active and inactive conformations. Precisely, they showed that $E3_{ATG12–ATG5}$, or mutation of a phenylalanine residue that physically supports the catalytic loop in some structures, induces a conformational change in $E2_{ATG3}$ that results in its activation. How the $E3_{ATG12–ATG5}$ conjugate binds $E2_{ATG3}$ to modulate its conformation remains unknown. It also remains unclear if the $E3_{ATG12–ATG5}$ conjugate maintains a stable scaffold[90,247] or if it undergoes conformational changes[248] upon ATG8~$E2_{ATG3}$ binding. There is no evidence to date to suggest that $E3_{ATG12–ATG5}$ promotes formation of a closed E2~Ubl conformation via contacts to ATG8. With that said, ATG12 possesses a noncanonical LIR motif formed by two residues that are distant in sequence but close in space[88] that when mutated reduce ATG8 conjugation.[88] Thus, it is tempting to speculate that the ATG12 LIR may contact ATG8 moiety within ATG8~$E2_{ATG3}$ to activate the ATG8~$E2_{ATG3}$ thioester.

Finally, evidence suggests that $E3_{ATG12–ATG5}$ and $E2_{ATG3}$ activities can be spatially regulated through a series of protein–protein and protein–lipid interactions. The ATG5 moiety of a ATG12–ATG5 conjugate associates with the N-terminus of ATG16,[177] a protein that dimerizes through a coiled coil region,[251] interactions that contribute to membrane targeting of $E3_{ATG12–ATG5}$.[177,252] Proteins from the PIWI family also contribute to targeting ATG8 and ATG12–ATG5/ATG16 to PI3P-containing membranes where ATG8 conjugation occurs.[253,254] Sakoh-Nakatogawa et al.[255] further showed that $E2_{ATG3}$ is recruited to preautophagosomal structures in a LIR-dependent manner, consistent with the idea that ATG3/ATG8–PE interactions serve as a positive feedback loop to increase ATG8–PE production and membrane expansion. Membrane curvature may also act as a positive feedback loop as it is induced by ATG8–PE and sensed by an amphipathic helix in the N-terminus of $E2_{ATG3}$.[256,257] Taken together, these studies depict $E3_{ATG12–ATG5}$ as an atypical E3 under multiple layers of regulation.

7. TRAPPING INTERMEDIATES IN CONJUGATION CASCADES

The activation and conjugation cascades for ubiquitin or Ubl's often employ interactions that are sometimes weak, often transient, and nearly always chemically labile. As such, structural studies often rely on stabilization of intermediates through chemical or artificial means. In this section we will review some of the strategies used to trap and structurally
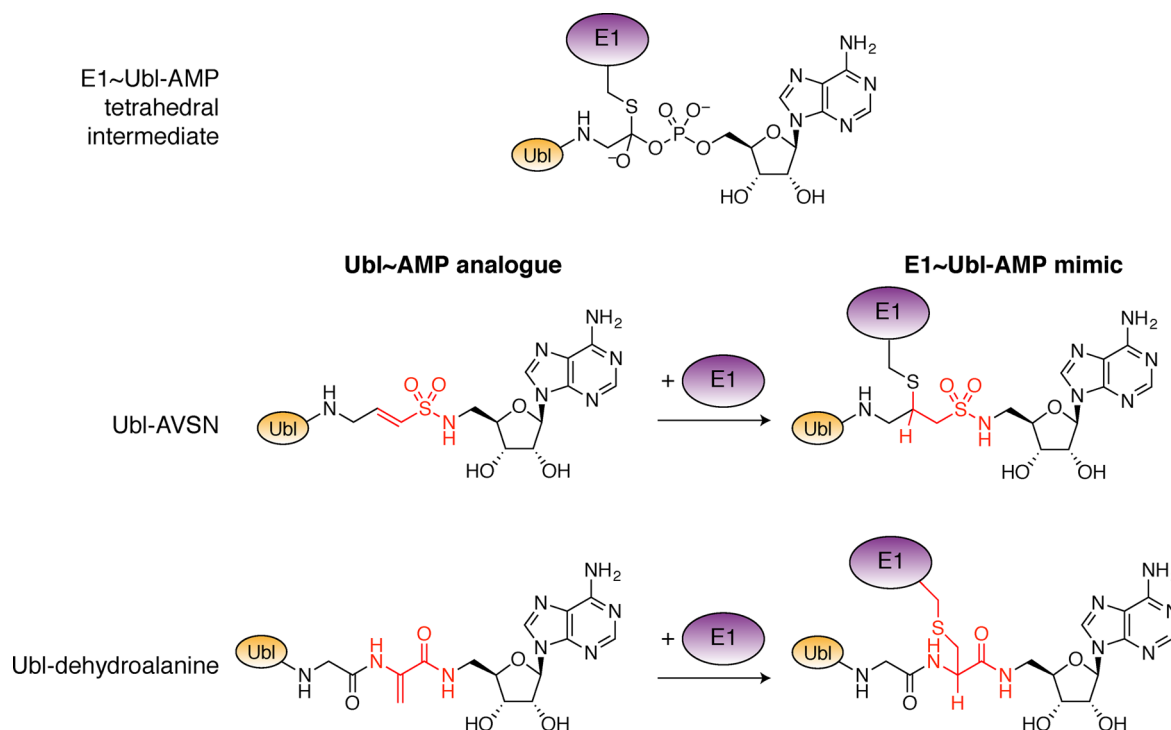
Figure 18. Chemical structures representing methods for trapping the E1 thioesterification step. Non-native linkages are colored red.

characterize various intermediates during Ubl activation and conjugation. As many of these methods are general and can be applied to ubiquitin and Ubl proteins, we will describe examples from various systems.

7.1. E1 Activation Intermediates

Most structures of canonical E1s in complex with ubiquitin or Ubl proteins revealed similar E1 conformations, with the C-terminal Ubl tail locked in place under the α-phosphate of the bound ATP−magnesium complex, and the catalytic cysteine far from the adenylation active site.[92,93,96,97,101,104,106,108] To trap complexes after adenylation or during thioester bond formation, Lu et al.[105] synthesized tripeptides containing 5′-(sulfonylaminodeoxy)adenosine (AMSN) or a 5′-(vinylsulfonylaminodeoxy)adenosine (AVSN) that could be ligated to the C-terminus of SUMO or ubiquitin using native chemical ligation. While both result in nonhydrolyzable adenylate Ubl intermediates, the AVSN moiety possesses an eletrophilic center that reacts with the cognate E1 catalytic cysteine to generate a stable thioether bond (Figure 18). This strategy was used to determine the structure of an E1∼AVSN−SUMO complex with the catalytic cysteine covalently attached to the AVSN within the adenylation pocket.[98] An analogous chemical strategy was recently described to trap E1 activation intermediates where a nonhydrolyzable AMP analogue was ligated to the C-terminus of a Ubl through native chemical ligation resulting in the formation of a cysteine residue that can be converted to dehydroalanine using 2,5-dibromohexanediamide.[258] In this case, the dehydroalanine residue contains the electrophilic center that reacts with the E1 catalytic cysteine. This strategy was employed to trap E1$_{UBA1}$∼5′-(dehydroalanine-aminodeoxy)adenosine−ubiquitin and E1$_{ATG7}$∼5′-(dehydroalanine-aminodeoxy)adenosine−LC3 complexes.[258]

7.2. Transthiolation Intermediates for E1/E2

Huang et al.[97] obtained a structure of a NEDD8∼E1/E2 complex by mutating the E2 catalytic cysteine to alanine to

prevent the transfer of NEDD8 from the E1 to the E2. This structure provided some of the first important details on how the E2 is recognized by E1, but it was unable to provide a mechanism for transthiolation as the two active sites remained separated by an ∼20 Å. To trap conformations that would allow the E1 and E2 active sites to come into close proximity, two sulfhydryl-to-sulfhydryl cross-linking strategies were used (Figure 19). In the first case, Kaiser et al.[132] used
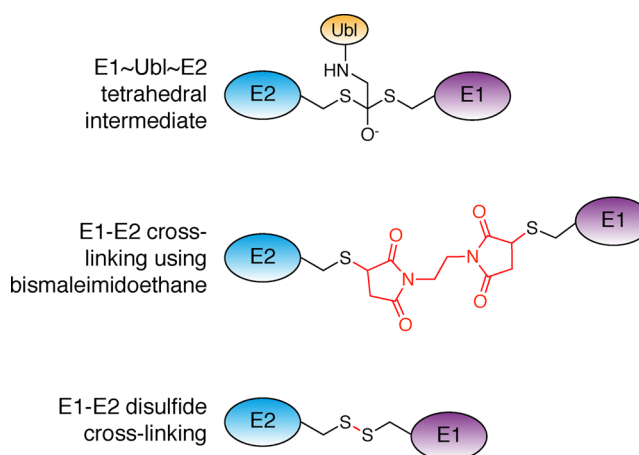
Figure 19. Chemical structures representing methods for trapping E1/E2 complexes. Non-native linkages are colored red.

bismaleimidoethane, a bifunctional cross-linker, to bring together the E1 and E2 active sites. Although this strategy successfully trapped both E1$_{ATG7}$−E2$_{ATG3}$ and E1$_{ATG7}$−E2$_{ATG10}$ complexes, the E2 loops containing the catalytic cysteine were partially disordered suggesting conformational flexibility,[132] perhaps because the maleimide-based cross-linker is longer and more complex when compared to the predicted tetrahedral intermediate. An alternative strategy utilized the *S. pombe*

Figure 20. E2∼Ubl mimics. Non-native linkages or amino acid residues are colored red.

ubiquitin E1 and E2$_{UBC4}$ and activation of the E2 cysteine by 2,2′-dipyridyldisulfide to catalyze formation of a disulfide bond between the E1 and E2 active site cysteine residues.[96] While a disulfide also fails to mimic a bona fide tetrahedral intermediate, the disulfide bond reduces the distance between E1 and E2 when compared to t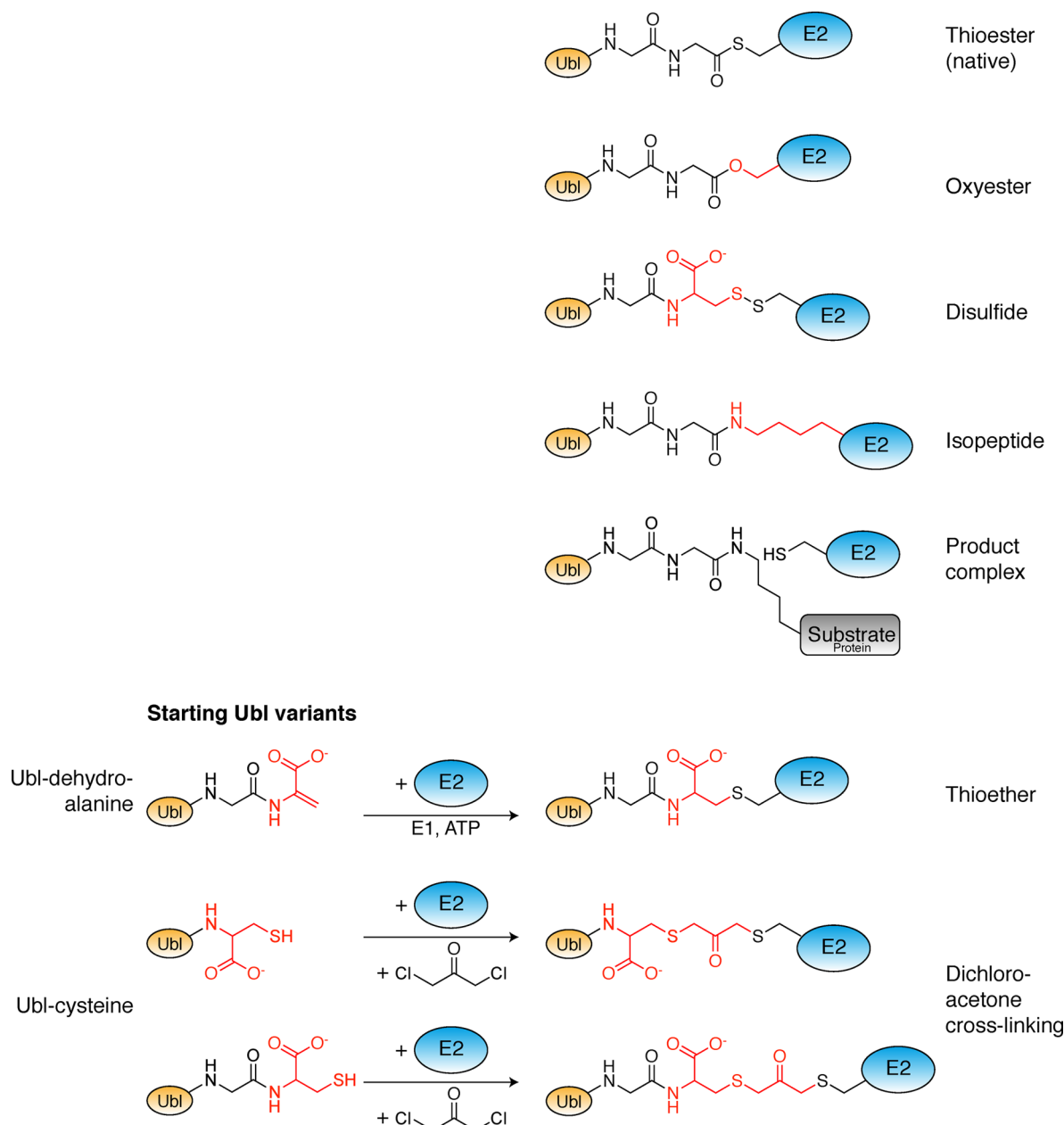he maleimide-based strategy, and the structure of the resulting complex revealed new surfaces between the E1 and E2 that are important for transthiolation activity. Furthermore, the E1−E2 disulfide complex was shown to occur in vivo when cells are placed under oxidizing conditions.[124,125] Additional strategies will be required to capture better mimics of Ubl∼E1/E2 transthiolation intermediates.

7.3. E2∼Ubl Mimics

E2∼Ubl is an intermediate in the conjugation cascade and an essential component of intact E3 ligase complexes; however, the thioester bond between the E2 active site cysteine and Ubl C-terminus is labile and short-lived, especially in the presence of E3s. A pioneering NMR study first observed the E2$_{UBC1}$∼ubiquitin thioester in situ by having unlabeled E1, ATP, and magnesium chloride present in the NMR tube to reiteratively generate the E2∼ubiquitin species.[158] This method was subsequently employed to study E2$_{UBC13}$∼ubiquitin, E2$_{MMS2}$/E2$_{UBC13}$∼ubiquitin, and E2$_{UBCH5C}$∼ubiquitin complexes by NMR,[196,259] where it was estimated that thioester bond formation proceeded to ∼90% completion.[158,196] This method has clear advantages in that it results in native thioester bonds, but its use is limited to select NMR studies. Therefore, other strategies were required to overcome the labile nature of the E2∼Ubl thioester bond to generate stable E2∼Ub mimics (Figure 20). Mimics using the oxyester, disulfide, or isopeptide strategies were detailed in a recent methods article.[260]

7.3.1. Oxyester. Early studies using E2$_{RAD6}$ and E2$_{UBC1}$ showed that mutation of the E2 catalytic cysteine to serine

allowed for replacement of the thioester bond by a more stable oxyester bond.[261,262] This strategy enabled the first NMR study of an E2~ubiquitin complex,[263] and it has been used since to structurally investigate many different E2~ubiquitin complexes, sometimes in the presence of E3s.[166,173,179,184,193–195,264–269] This strategy was also used to study E2$_{UBC12}$~NEDD8[171] and closed conformations of E2~ubiquitin and E2~NEDD8.[171,173,193–195,266,269] The main advantage of the oxyester mimic is that this single atom substitution results in a bond that is structurally similar to the native thioester. However, the single atom substitution can also change chemical reactivity with some surprising results. Scott et al.[171] reported that an E2~NEDD8 oxyester conjugate has a higher propensity to undergo hydrolysis versus aminolysis when compared to a native thioester-linked conjugate. Although the oxyester mimic is more stable, it still undergoes hydrolysis or aminolysis[179,264] and half-lives of 5−20 h have been reported for complexes between ubiquitin and E2$_{UBE2G1}$ and E2$_{UBE2R1}$, respectively.[269] Furthermore, E3s can decrease the half-life of the oxyester conjugate. For example, an E2$_{UBCH5C}$~ubiquitin complex has a half-life of 58 h in the absence of an E3 and a half-life of 10 h in the presence of E3$_{E4BU}$.[194] Therefore, additional mutations are often required to trap complexes that contain E3s and E2~Ubl oxyester-based conjugates. In an E3$_{BIRC7}$/E2$_{UBCH5B}$~ubiquitin complex, the oxyester bond is cleaved after 1−3 days in the absence of a stabilizing E2$_{UBCH5B}$ N77A mutation.[195] In the case of E3$_{NEDD4L}$/E2$_{UBCH5B}$~ubiquitin complexes, the HECT E3 catalytic cysteine was mutated to serine or alanine to prevent decomposition of the complex.[264] For the E3$_{RBX1}$/E2$_{UBC12}$~NEDD8/CUL1/DCN1 complex where E2$_{UBC12}$~NEDD8 is linked by an oxyester bond, Asn103 was mutated to a serine and the target lysine was mutated to arginine.[171] Efforts have also been made to identify experimental conditions that stabilize the oxyester bond. The use of a citrate buffer at pH 5.75 was reported to increase the lifetime of an E2$_{UBCH5C}$~ubiquitin oxyester in the presence of E3$_{E4BU}$ from a few hours to several days.[266]

7.3.2. Disulfide. A disulfide bond can be formed between the E2 catalytic cysteine and a ubiquitin variant where the last glycine residue is replaced by a cysteine residue.[192,269–272] This often requires nonreducing conditions and mutation of other E2 cysteine residues to prevent formation of disulfides at other sites. This strategy was used to isolate E2$_{UBC1}$~ubiquitin and E2$_{UBCH8}$~ubiquitin conjugates that were reported to be stable for weeks[270] or months,[192] respectively. Adding to the utility of this approach, NMR studies observed few differences between the thioester and disulfide-linked E2$_{UBC1}$~ubiquitin complexes, suggesting that the disulfide-linked E2$_{UBC1}$~ubiquitin was a good mimic for its thioester linked counterpart.[270] In contrast, another NMR report suggested that a disulfide-linked E2~Ubl was not as good a mimic as an oxyester linked E2~Ubl as there was less evidence for interactions between E2$_{UBE2R1}$ and ubiquitin in the case of a disulfide conjugate.[269] This discrepancy could be due to interference between the carboxylate group introduced by the disulfide strategy, or because E2$_{UBE2R1}$ contains an extended acidic loop close to the active site that is absent in E2$_{UBC1}$. Taken together, these studies suggest that the disulfide strategy may not work for all E2s. A variation of the disulfide strategy employs dichloroacetone, a bifunctional cross-linker, to bridge the E2 catalytic cysteine and the last residue of G75C or G76C ubiquitin variants.[273]

7.3.3. Isopeptide. Plechanovová et al.[161] reported the successful isolation an E2~ubiquitin mimic by replacing the catalytic cysteine with a lysine residue that can be conjugated to ubiquitin at high pH directly from E1~ubiquitin thioester resulting in a stable peptide bond between the E2 and ubiquitin. Isolation of the E2−Lys−ubiquitin adduct enabled structure determination of an E3$_{RNF4}$/E2$_{UBCH5A}$~ubiquitin complex, and isolation of a complex that mimics the activated state prior to conjugation with E2−Lys−ubiquitin in the closed conformation. Since then, multiple studies have used the same strategy to characterize various E2~ubiquitin complexes.[160,162,163,165,202,229−231,274−276] A variation of this strategy was recently reported where a residue proximal to the E2 catalytic cysteine was mutated to lysine.[200] In this case, the E2 is charged by E1 to generate an E2~SUMO thioester with subsequent attack by the engineered lysine to generate a stable peptide bond that leaves the E2 catalytic cysteine available for additional modifications (see below).

7.3.4. Thioether. Mulder et al.[277] formed a thioether bond between an E2 and a ubiquitin variant where the last glycine residue is replaced by an electrophilic dehydroalanine residue. This variant requires activation by E1, a reaction that also generates thioether-linked E1−ubiquitin as a byproduct. This technique was used to isolate E2~ubiquitin and E2~NEDD8 complexes resulting in the structure determination of an E2$_{UBCH5C}$~ubiquitin complex. Comparison of this structure to a previously determined complex containing an oxyester revealed quasi-identical E2 active site organization with the exception of increased disorder of Arg90, presumably to accommodate the carboxylate group introduced by this strategy.

7.3.5. Substrate and Product Complexes. Substrates or conjugated products that fail to dissociate from the E2 can sometimes be used to trap complexes that closely resemble conformations for the substrates prior to peptide bond formation. As an example, the C-terminal domain of mammalian RANGAP1 interacts with E2$_{UBC9}$ prior to and after conjugation to SUMO via an extended interface that includes a canonical SUMO modification motif.[153] Structural and biochemical analyses of several RANGAP1/E2$_{UBC9}$ complexes suggested that the substrate lysine was positioned close to where it would be in an E2$_{UBC9}$~SUMO thioester complex and that residues surrounding the E2 active site were positioned to facilitate the reaction.[154,159,164] Subsequent isolation of product inhibited complexes enabled purification and structural characterization of two E3 ligase domains from RANBP2 and ZNF451 by combining the E3 domains with E2$_{UBC9}$, and SUMO conjugated RANGAP1 to isolate E3$_{RANBP2}$/E2$_{UBC9}$~SUMO complex mimics[154,159] and an E3$_{ZNF451}$/E2$_{UBC9}$~SUMO complex mimic.[164] In each of these complexes, the RANGAP1 lysine was conjugated to SUMO via an isopeptide bond, SUMO was observed in the closed and activated conformation, and the C-terminal SUMO glycine reside was positioned just above the active site cysteine with its C-terminal carbonyl oxygen pointing toward the conserved E2 asparagine residue suggesting that the product complexes resemble the substrate complexes immediately after conjugation.

7.4. Trapping E2/E3 Complexes

Unlike the examples described above for RANGAP1, most E2/E3 complexes are unstable and are not easily isolated for structural or biochemical studies. Stabilization of transient E2/E3 interactions can be achieved by linear fusion of these

proteins. For example, a weak interaction between E2$_{UBCH5C}$ and E3$_{RING1B}$ was overcome by using an E3—E2 linear fusion and this fusion was instrumental in forming a complex between a nucleosome, E2$_{UBCH5C}$, E3$_{RING1B}$, and E3$_{BMI1}$.[235] Although successful, this approach prevented loading of the E2 with ubiquitin because the fused E3 masks E2 regions important for E1 interaction.[235] In a second example, fusing a SUMO molecule to the C-terminus of E3$_{SIZ1}$ enhanced interactions between the SUMO E3$_{SIZ1}$ and E2$_{UBC9}$ by positioning the fused SUMO for interaction with the backside of E2$_{UBC9}$.[200] In a last example, genetically fusing E2$_{UBE2T}$ at the C-terminus of the E3$_{FANCL}$ RING domain was reported to be critical for obtaining a high-resolution structure of the complex.[223]

7.5. Trapping Ubl~E2/Substrate Complexes

Successful strategies for trapping Ubl~E2/substrate structures include use of a three-way cross-linking strategy that was developed to trap a complex consisting of the HECT E3$_{RSP5}$, ubiquitin, and a substrate (Figure 21).[278] A similar approach
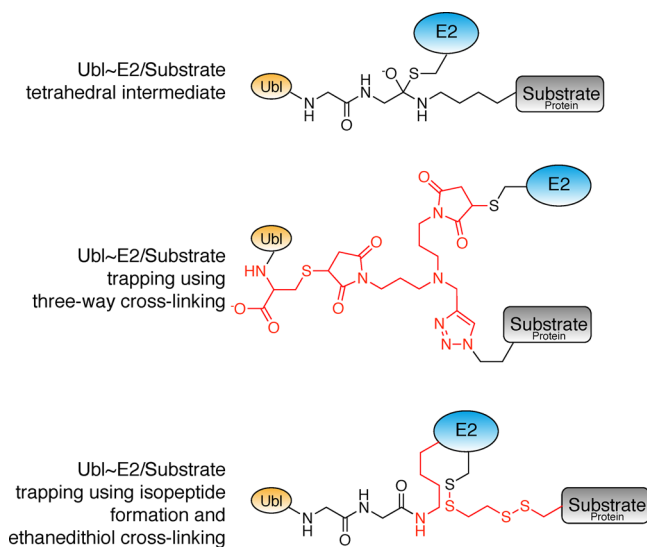

Figure 21. Chemical structures representing methods for trapping Ubl~E2/substrate complexes. Non-native linkages or residues are colored red.

was then used to tether E2$_{UBCH10}$, ubiquitin, and a substrate within an APC complex.[279] In this case, the substrate was synthesized with an azidohomoalanine in lieu of a target lysine. This allowed the addition of a bifunctional maleimide-based cross-linker via click chemistry. This cross-linker was in turn reacted with E2$_{UBCH10}$ and a ubiquitin variant possessing a cysteine residue at position 75. A second method for cross-linking a Ubl~E2/substrate complex involved use of an E2$_{UBC9}$ variant where a residue proximal to E2 catalytic cysteine was mutated to lysine so that it could be conjugated to SUMO while leaving the E2 active site cysteine intact.[200] By combining the conjugated E2 variant with a substrate that substituted the target lysine to cysteine, the authors were able to cross-link the E2 and substrate cysteine residues using ethanedithiol, a molecule that closely resembles the lysine side chain with respect to the number of bridging atoms when compared to the predicted tetrahedral intermediate.[200]

8. CONCLUSION AND FUTURE CHALLENGES

Structural and mechanistic studies depict Ubl conjugation as a highly dynamic process under multiple layers of regulation. Indeed, many of the proteins involved in conjugation pathways can undergo conformational changes that are often integral to their function. Multiple studies laid the groundwork for understanding basic mechanisms underlying conjugation enzymes; however, recent work has highlighted the importance of continued investigation as we continue to uncover important contributions of noncanonical adaptations in enzymes and factors involved in Ub/Ubl conjugation cascades.

Strategies that take advantage of cross-linking and/or fusions to isolate and structurally characterize mimics for intermediates during conjugation cascade have been instrumental to our understanding of the Ubl conjugation machinery. However, these strategies can sometimes lead to deformations, as they are not always isosteric to their native counterparts and often require extensive structure—function analysis to verify the structural contacts in these complexes. Future challenges should be focused on isolation of chemical adducts that more closely resemble the true intermediates or development of methods that extend the lifetime of these intermediates so that better mimics for the transient states can be isolated for investigation.

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Santé. C.D.L. is an Investigator of the Howard Hughes Medical Institute.

ABBREVIATIONS

AAD   active adenylation domain
ADP   adenosine diphosphate
AIM   ATG8-interacting motif
AMP   adenosine monophosphate
AMSN   5′-(sulfonylaminodeoxy)adenosine
APC/C   anaphase promoting complex/cyclosome
ATP   adenosine triphosphate
AVSN   5′-(vinylsulfonylaminodeoxy)adenosine
CTD   C-terminal domain
FCCH   first catalytic cysteine half-domain
GlyRS   glycyl-tRNA synthase
HECT   homologous to E6AP C-terminus
IAD   inactive adenylation domain
LIR   LC3-interacting region
LRS   LC3 recognition sequence
NTD   N-terminal domain
PE   phosphatidylethanolamine
RBR   RING-between-RING
RING   really interesting new gene
RLD   rhodanese-like domain
SAMP   small archaeal modifier proteins
SIM   SUMO-interacting motif
UBC   ubiquitin conjugating enzyme
Ubl   ubiquitin-like protein
UFD   ubiquitin fold domain
UFIM   UFM1-interacting motif

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