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E2 enzymes: more than just middle men

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Ubiquitin-conjugating enzymes (E2s) are the central players in the trio of enzymes responsible for the attachment of ubiquitin (Ub) to cellular proteins. Humans have ~40 E2s that are involved in the transfer of Ub or Ub-like (Ubl) proteins (e.g., SUMO and NEDD8). Although the majority of E2s are only twice the size of Ub, this remarkable family of enzymes performs a variety of functional roles. In this review, we summarize common functional and structural features that define unifying themes among E2s and highlight emerging concepts in the mechanism and regulation of E2s.

***Keywords*:** E2; ubiquitin-conjugating enzymes; HECT; RING
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Introduction

Nearly every paper on protein ubiquitylation describes the mandatory sequence of enzyme activities required for target modification. The process starts with activation of the ubiquitin (Ub) C-terminus by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction to generate a thioester-linked E1~Ub conjugate. The activated Ub is then handed over to a ubiquitin-conjugating enzyme (E2) via a transthiolation reaction. Finally, a ubiquitin ligase (E3), which can bind both a substrate and an E2~Ub conjugate, mediates the transfer of Ub most commonly onto the ε-amino group of a lysine in the target protein, forming an isopeptide bond. On the basis of their mechanistic strategies, E3s have been classified into three families (i.e., RING-, HECT-, and RING-between-RINGS (RBR)-type E3s) and have been intensely studied. However, the central player in this enzymatic choreography, the E2, is often viewed as simply a carrier of Ub. Although the majority of E2s are only twice the size of Ub, this remarkable family of enzymes performs a variety of functional roles. With only one simple active site, E2s primarily engage in two types of reactions as follows: (1) transthiolation (transfer from a thioester to a thiol group) and (2) aminolysis (transfer from a thioester to an amino group), although additional types of reactivity have been reported (Figure 1B). All E2s interact with an E1 enzyme and one or more E3s. In addition, E2s may directly engage a target protein and so play a role in the determination of where and how a target is modified by Ub [1]. Finally, some E2s also function outside the traditional Ub transfer pathways to regulate the activity of other enzymes [2, 3].

Humans have ~40 E2s that are involved in the transfer of Ub or Ub-like (Ubl) proteins (e.g., SUMO, NEDD8, etc). All E2s contain a core catalytic domain of roughly 150 amino acids, termed the UBC domain (Figure 1A). This domain adopts an α/β-fold typically with four α-helices and a four stranded β-sheet. Important loop regions form part of the E3-binding site (see below) and the E2 active site. Structures of over 32 human E2 proteins (full-length or UBC domain) have been solved and the topologies of most are remarkably consistent with this canonical fold. In a few instances, such as Ube2R1 (Cdc34) or Ube2G2 (Figure 1A), the UBC domain is embellished with functionally important insertions. Although most E2s encompass only a single structural UBC domain, many have short N- and/or C-terminal extensions that can impart important E2-specific functionality (Table 1). Although extensions are often intrinsically disordered, a few adopt secondary structure that then contacts the UBC domain [4]. Furthermore, a few E2s have an additional structured domain linked to their UBC domain (e.g., Ube2K) or are part of large multi-domain proteins (e.g., Ube2O or BIRC6). Thus, E2s have a common fold that has been adapted for specific systems. Rather than discussing what is known about each E2, this review endeavors to identify common functional and structural

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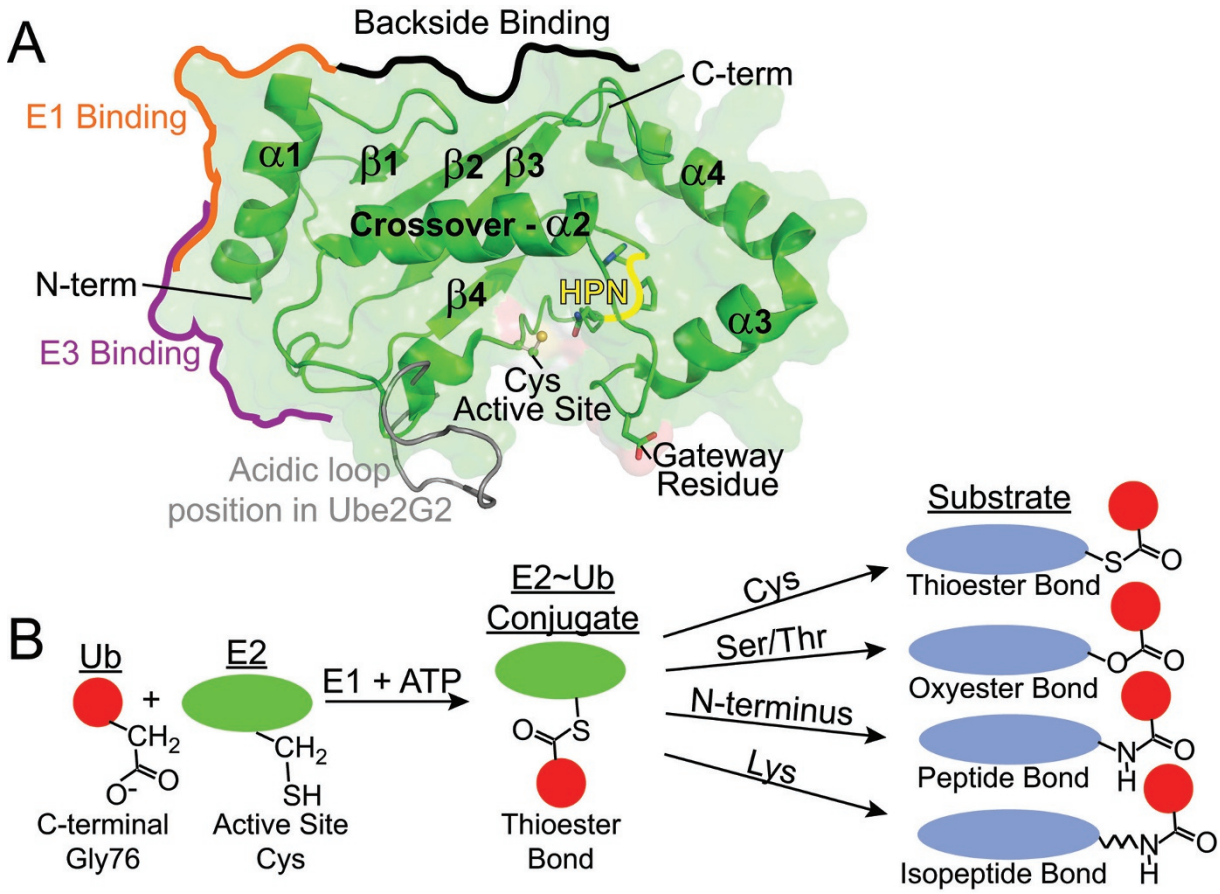

Figure 1 Overview of E2 structure and chemistry. **(A)** Important structural features and common binding surfaces described in the text are labeled on a representative UBC domain (Ube2D3; PDBID: 2FUH) shown in green. The position of the acidic loop in Ube2G2 (PDBID: 2CYX) when the UBC domain is aligned with Ube2D3 is shown in gray. **(B)** The C-terminal carboxylate of Ub is conjugated to the E2 active site cysteine in an E1-catalyzed, ATP-driven reaction. The E2~Ub conjugate reacts with the side chain of a Cys, Ser, Thr, Lys, or N-terminus on a substrate to form the diverse ubiquitin linkages as shown.

features that define unifying themes. We also highlight emerging concepts from the recent literature to help guide future studies.

E2 reactivity

In a cell, E2s exist mainly as E2~Ub conjugates and are therefore poised to react [5]. However, E2~Ub conjugates have low rates of Ub transfer in the absence of an E3 ligase, presumably to avoid energy-costly cycles of conjugation and off-target ubiquitylation. A long-standing question in the field is "What keeps the reactivity of an E2~Ub low?" or put another way "How is reactivity stimulated by E3s?". Studies over the past several years provide insights into both of these fundamental questions. A full understanding requires characterization of E2 reactivity *per se* and of how an E3 alters it.

Intrinsic reactivity of E2s

The ability of E2~Ub conjugates to transfer Ub can be assessed by gauging their intrinsic reactivity towards small-molecule nucleophiles such as free lysine (aminolysis) or cysteine (transthiolation) [6].This assay strips away complications inherent in other standard *in vitro* assays such as the highly popular auto-ubiquitylation assays or assays that use "universal" substrates such as histone 2A [6]. Use of a single (small) substrate that carries the nucleophilic functional group of interest allows the reactivity of different E2~Ub conjugates to be compared directly in the presence and absence of an E3. Presumably, all E2s can transfer Ub via transthiolation, a requirement for activation by an E1. In the most common example of a transthiolation reaction, an E2 transfers its thioester-linked Ub to the active-site cysteine residue of a HECT-type E3 ligase (for a review of E3 mechanisms,

Table 1 Summary of structural, biochemical, and functional properties of E2-conjugating enzymes

Human gene name	Other names[1]	Domain organization	Backside interaction	Closed conformation	Collaborating E3 ligase types[2]	Functional information
Ubiquitin E2 enzymes						
Ube2A	*Ubc2*, *Rad6*, Rad6A	UBC	with Ub [116] and E3 [71, 117][3]	NA	RING, HECT, RBR	E2 in DNA damage repair [118]
Ube2B	*Ubc2*, *Rad6*, Rad6B					
Ube2C	UbcH10	Ext – UBC	with E3 [79][3], but not with Ub [119][4]	No (suspected) [79][3]	RING, HECT, RBR	chain-initiating E2 of APC/C [59, 120]
Ube2D1	*Ubc4/5*, UbcH5A	UBC	with Ub [65, 120] and E3 [75][3, 4]	Yes [31, 69][3,4]	RING, HECT, RBR	promiscuous lysine- and cysteine-reactive E2 [10]
Ube2D2	*Ubc4/5*, UbcH5B					
Ube2D3	*Ubc4/5*, UbcH5C					
Ube2D4	*Ubc4/5*, UbcH5D					
Ube2E1	UbcH6	Ext – UBC	not with Ub [87][4]	Yes [31][4]	RING, HECT, RBR	monoubiquitylating E2s by virtue of their N-terminal extensions [87]
Ube2E2	UbcH8[5]		NA	NA		
Ube2E3	UbcH9, UbcM2		with Ub and E3 [74][4]	NA		
Ube2G1	*Ubc7*, Ube2G	UBC + insert	NA	Yes [121][3]	RING, HECT [28]	K48 chain-building enzyme even in the absence of an E3 [122]
Ube2G2	*Ubc7*	UBC + insert	with Ub [123], E3 [50, 124], Cue1 [81] and itself [125][3, 4]	Yes [49][3]	RING, HECT, RBR	K48 chain-building in dependence of an E3 [84]; E2 involved in the ERAD pathway [126]
Ube2H	*Ubc8*, UbcH2, E2-20K	UBC – Ext	NA	NA	RING, HECT, RBR	
Ube2J1	*Ubc6*	UBC + insert – Ext	NA	NA	RING, HECT, RBR	E2 involved in the ERAD pathway [127]
Ube2J2	*Ubc6*	UBC + insert – Ext	NA	NA	RING, HECT, RBR	E2 with hydroxyl specificity (serine/threonine) [22]
Ube2K	*Ubc1*, E2-25K	UBC – UBA	N/A	Yes [31, 35][3, 4]	RING, HECT, RBR	K48 chain-building enzyme even in the absence of an E3 [128]
Ube2L3	UbcH7, UbcM4	UBC	not with Ub [65][4]	NA	HECT, RBR, not with RING	cysteine-only reactive E2 [10]
Ube2N	*Ubc13*	UBC	not with Ub [29]	Yes [29, 31][3, 4]	RING, HECT, RBR	E2 collaborating with proteins of the Ube2V family to build K63 Ub-chain [129]
Ube2NL		UBC	NA	NA	NA	no catalytic cysteine
Ube2O	E2-230K	Ext – UBC – Ext	NA	NA	its own N-terminus, RING, RBR	large multidomain E2/E3 hybrid [130-132]

Ube2Q1	Ube2Q	Ext – UBC + insert	NA	NA	RING	
Ube2Q2		Ext – UBC + insert	NA	NA	RING, HECT	
Ube2QL		UBC + insert	NA	NA	RING	
Ube2R1	*Cdc34*, *UbcH3*	UBC + insert – Ext	NA	Yes [133][3, 4]	RING, HECT, RBR	K48 Ub chain-building enzyme and cognate E2 of SCF E3 ligases [134]
Ube2R2	Cdc34B, Ubc3B	UBC + insert – Ext	NA	NA	RING, HECT, RBR	
Ube2S		UBC – Ext	NA	Yes [36][3, 4]	RING, HECT, RBR	K11 Ub chain-building enzyme [59, 60]
Ube2T		UBC – Ext	NA	NA	RING, HECT, RBR	E2 of the Fanconi Anemia DNA damage pathway specific for FANCL [54]
Ube2U		UBC – Ext	NA	NA	NA	
Ube2V1	Ube2V, Uev1, Uev1A	Ext – UBC	with Ub [41, 135][3]	inactive	RING, HECT (in combination with Ube2N)	catalytically inactive E2-like proteins interacting with Ube2N for K63 chain formation [41]
Ube2V2	*Mms2*, Uev2	UBC				
Ube2W	*Ubc16*	UBC	NA	Yes [13][4]	RING, HECT, RBR	specificity for N-terminal amine group [14, 15]
BIRC6	Bruce, Apollon	Ext – UBC – Ext	with C-terminal extension [28][3]	NA	its own N-terminus, HECT (isolated UBC)	giant multidomain E2/E3-hybrid [136]
Ubl E2 enzymes						
Ube2F		Ext – UBC	NA	NA	RING	NEDDylation E2 [137]
Ube2I	*Ubc9*	UBC	with SUMO [73] and E3 [77, 78][3,4]	Yes [78][3]	SP-RING, RanBP2	SUMO E2 [138]
Ube2L6	UbcH8[5]	UBC	NA	Yes [139][3]	HECT, RBR	bispecific E2 for ISG15 and Ub [140]
Ube2M	*Ubc12*	Ext – UBC	NA	Yes [141][3, 4]	RING	NEDDylation E2 [137]
Ube2Z		Ext – UBC – Ext	with C-terminal extension [4][3]	NA	NA	bispecific E2 for FAT10 and Ub [142]
ATG10		UBC-like	NA	NA	NA	ATG12-specific E2 [143]
ATG3		Ext – UBC-like + insert	with ATG12 via loop at backside [144][3]	NA	NA	ATG8 lipidation-specific E2 [145]

Abbreviations: Ext, N- or C-terminal extension; RBR, RING-between-RINGS; NA, not applicable; UBC, UBC domain.

[1]For consistency, the human gene name is used for E2s discussed in the body of this review. Here we list other common names and the yeast homologs (italic).

[2]References for this column are beyond the scope of this manuscript.

[3]Structural evidence

[4]Biochemical evidence

[5]UbcH8 may refer to both Ube2E2 and Ube2L6

see Metzger *et al*. [7]). There is, however, one reported example of an E2 transferring Ub to a cysteine residue on a substrate. The yeast peroxisome E2 Pex2 modifies the peroxisomal receptors Pex5p and Pex10p on conserved and functionally essential cysteine residues [8, 9]. Although a complete assessment of all human E2s has not yet been reported, most Ub E2s examined to date also show some propensity to transfer Ub to free lysine, consistent with their ability to work as E2s with the largest class of E3 ligases, the RING-type E3 (see discussion below).

The intrinsic reactivity assay has uncovered unexpected features of some E2s that have changed the way we think about E2s and E3s. Notably, Ube2L3 (UbcH7), the E2 used in many early structural studies with RING-type E3s, is not reactive towards lysine and only exhibits reactivity towards cysteine [10]. The implication is that Ube2L3, although it binds to many RING domains, is only functional as an E2 with HECT-type E3s. This observation and its implication were key in redefining an entire class of E3s, the RBRs that were known to function with Ube2L3. RBRs, such as Parkin and HHARI, have RING domains, but also contain a conserved cysteine residue that forms an obligatory E3~Ub intermediate. Thus, RBRs are functional hybrids that exploit elements found in both RING and HECT E3s [10].

Another example of unusual intrinsic E2 reactivity is Ube2W. Although known to work with E3s typically associated with product lysine ubiquitylation such as BRCA1/BARD1 and FANCL [11, 12], Ube2W exhibits no intrinsic activity towards free lysine [13]. Instead, Ube2W attaches Ub to the N-terminal α-amino group of proteins to form a Ub-fusion protein product [14, 15]. While still an aminolysis reaction and therefore not fundamentally different from the reaction with lysine, intrinsic reactivity assays revealed that Ube2W can transfer Ub to the α-amino group of small lysine-less peptides but not to free lysine, whereas Ube2D3, for example, can transfer Ub to lysine but not to the α-amino group [13]. This feature distinguishes Ube2W as fundamentally different in its reactivity (and therefore, its substrates) from all other characterized E2s. Along with its unique reactivity profile, Ube2W has an unusual UBC domain, in which its C-terminal region is disordered and dynamic but hovers near the active site [13, 16]. Ube2W recognizes and modifies disordered N-termini independently of substrate sequence through interactions between its own disordered C-terminal region and the substrate backbone [13]. The requirement of a disordered N-terminus on its substrate explains the strict monoubiquitylating activity of Ube2W, as the N-terminus of Ub is highly structured and is therefore not a good substrate for Ube2W. There are recent reports that the preference for N-terminal modification by Ube2W may not be absolute, as the retroviral restriction RING E3 TRIM5α is monoubiquitylated by Ube2W despite being acetylated on its N-terminus [17]. This suggests that Ube2W may also facilitate isopeptide bond formation, possibly if an N-terminus is blocked. Nevertheless, the preference of Ube2W for disordered N-termini gives it a (so far) unique target selection mechanism for a primary modification event that can subsequently be exploited by other E2 enzymes to form Ub chains (discussed below).

In an intriguing twist, a non-protein primary amine is targeted by ATG3, an E2 that plays a role in the expansion of autophagosomal membranes. ATG3 conjugates Ubl proteins of the LC3/ATG8 family to phosphatidylethanolamine in the outer phagosome membrane [18, 19]. The aminolysis reaction catalyzed by ATG3 results in the formation of an amide bond, which covalently links a Ubl protein to a phospholipid to anchor it to the membrane.

In addition to the documented reactivity of E2s with thiol and amino groups, other chemical reactivities have been reported for some (unusual) E2s [20]. Ube2J2 has been reported to attach Ub to the major histocompatibility complex via hydroxyl groups (serine/threonine) in collaboration with a viral RING E3 ligase, murine K3 (mK3) [21, 22]. Consistent with this proposal, Ubc6, a yeast homolog of Ube2J2, is essential for degradation of a lysine-less substrate [23]. Ube2J2 ubiquitylation products are sensitive to treatment with strong base, which hydrolyzes oxyesters but not amide bonds. Normally, the hydroxyl groups on serine, threonine, and tyrosine are not particularly nucleophilic, implying that the active site of Ube2J2 may possess additional catalytic residue(s) beyond those in most E2s. To date, the intrinsic ability of the Ube2J2~Ub conjugate to react with serine or threonine has not been demonstrated, so neither the structural nor chemical determinants for hydroxyl attachment of Ub have been identified. In summary, the growing examples of non-canonical Ub transfer by E2s highlight the need to explicitly test for ubiquitylation on non-lysine residues in substrates. To date, there are no examples where an E3 alters the chemical reactivity profile of an E2, so the intrinsic reactivity of a given E2 will likely be predictive of the nature of its products.

Enhancement of E2 reactivity by E3 ligases

There are two main classes of E3s with which E2s function: RING-type and HECT-type E3s. Together, these E3s comprise ~700 proteins in the human genome. A third smaller class, the RBRs, combines elements of both RING and HECT E3s. E3s from pathogenic bacteria

that operate in eukaryotic cells and use the host ubiquitylation machinery (including E2s) may constitute other classes. Although the E3 classes are topologically distinct, some E2s have been shown to cross class boundaries and function with multiple types of E3s (Table 1). Much of the early structural work aimed at understanding E2 function focused on how they recognize E3s. Most E2/E3 complexes are characterized by moderate to weak affinity and cannot be co-purified. Nevertheless, NMR and crystallography studies helped define a canonical E3-binding surface on the UBC domain formed by residues in helix 1, and loops 4 and 7 [24, 25]. This surface is involved in binding to both RING and HECT domains and also overlaps with the region recognized by E1 enzymes (Figure 1A) [26, 27]. That this one region is responsible for multiple protein interactions may explain why many Ub-specific E2s exhibit similar electrostatic properties in this region of the UBC domain [28].

E3/E2 complexes, however, represent the complex that exists after the Ub transfer reaction has occurred. Substantial progress has been made in understanding the properties of E2~Ub conjugates and the structural changes that occur when they interact with an E3 and are poised for Ub transfer. The C-terminal residues of Ub (amino acids 72-76) are highly flexible and allow a Ub molecule that is covalently tethered to an E2 active site to swing by its tail, sampling an ensemble of conformations relative to the E2 domain. Solution studies of Ube2D3~Ub and Ube2N~Ub conjugates show an array of orientations that involve little or no contact between the E2 and ubiquitin ("open states") and some conformations ("closed states") that involve contacts between the Ub hydrophobic patch centered on Ub I44 and residues in the E2 crossover helix (Figure 2). Remarkably, although Ube2D3~Ub and Ube2N~Ub conjugates are highly dynamic, the ensembles of conformations adopted by them are different in terms of the relative fraction of "closed" versus "open" states [29].

RING-type E3s, which include RINGs and the structurally related U-box proteins, make up the vast majority of E3 ligases (for a recent review, see Metzger *et al*. [7]). Unlike HECT and RBR E3 ligases that form E3~Ub intermediates during Ub transfer, RING E3s do not participate chemically in Ub transfer. Instead, RING E3s bind substrate and an E2~Ub conjugate to facilitate Ub transfer directly from the E2 active site to a substrate. In this respect, a RING-type E3 functions as a required protein co-factor for E2~Ub conjugates. Although E2~Ub conjugates may bind some E3s tighter than the free E2 counterpart, NMR studies show that RING E3/E2~Ub complexes are still dynamic [30]. Nonetheless, interaction with RING E3s dramatically enhances the intrinsic reactivity of many, although not all, E2~Ub conjugates towards aminolysis. As a paradigm example, members of the Ube2D family of E2s react with lysine slowly in the absence of an E3 but rapidly in the presence of a RING domain [10]. Solution studies found that RING interactions promote E2~Ub closed states [31]. Snapshots of closed conformations have been captured in recent crystal structures [32-34].

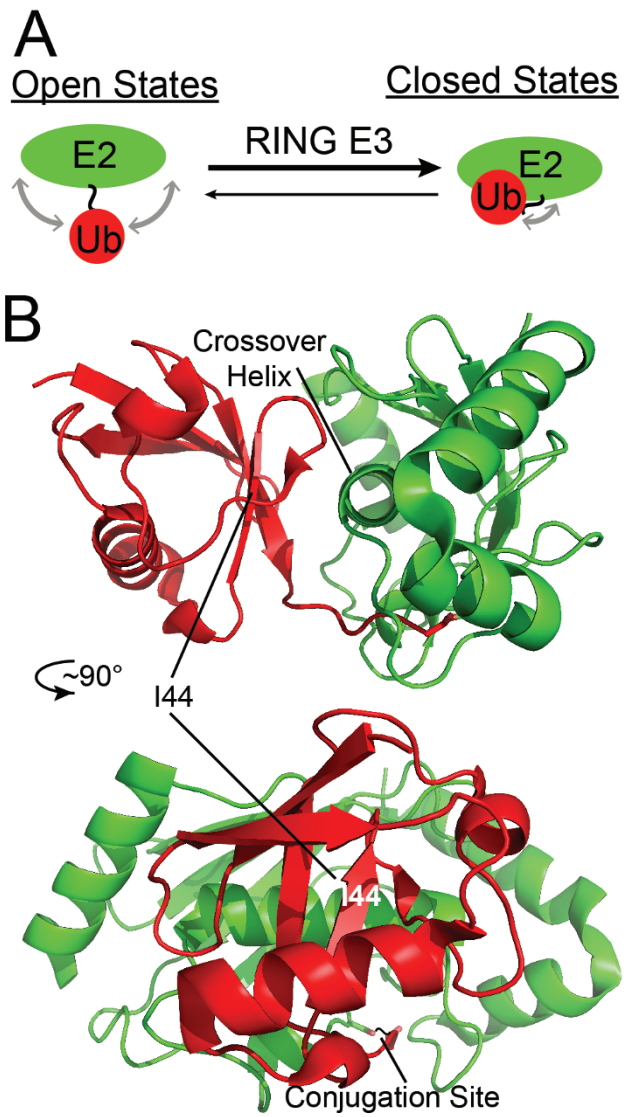

Figure 2 Ubiquitin positioning in the E2~Ub conjugate. **(A)** A cartoon depiction of the dynamic "open states" in which Ub samples a range of various positions relative to the E2, but shifts towards population of more "closed states" upon binding of a RING-type E3. **(B)** Crystal structure of an E2~Ub oxyester conjugate (Ube2D2) in a closed state showing the interface formed between the E2 (green) crossover helix and the I44 surface of Ub (red) upon binding a RING E3 (BIRC7 (not shown); PDBID: 4AUQ). The two representations are the same structure rotated ~90º about the vertical axis. The E2 is in the same orientation as in Figure 1 in the bottom representation.

Surprisingly, the E2/E3 interaction critical for promoting the closed E2~Ub conformation occurs over 15 Å away from the E2 active site. E3/E2~Ub structures reveal that a conserved RING residue donates a hydrogen bond to an E2 backbone carbonyl in loop 7 and one or more backbone groups in the tail of Ub [32, 33]. Structurally, the conserved RING residue (usually an arginine, lysine, or asparagine) appears to position the Ub C-terminus for nucleophilic attack. Substitution of the RING residue with a non-hydrogen-bonding residue abrogates activation of the E2~Ub by RING E3s, so it was dubbed the "allosteric linchpin" residue [31]. Furthermore, destabilization of closed E2~Ub states by mutations in the E2/Ub interface also decreases the ability of RING E3s to activate the E2~Ub for Ub transfer. Taken together, these results have led to the notion that closed E2~Ub states are activated states for aminolysis. Unfortunately, use of stable mimics of the thioester linkage (e.g., oxyester- or isopeptide-linked E2~Ub species), required to obtain crystals of E2~Ub conjugates, yields structures that do not contain the atoms that undergo the relevant chemistry. So the underlying activation mechanism remains unclear. Studies on a growing number of E2s and E3s confirm that promotion of E2~Ub closed states by RINGs is a common mechanistic strategy shared among many, although not all, E2/RING E3 pairs (Table 1). Finally, consistent with the notion that closed states are more reactive in aminolysis, the intrinsic lysine reactivity of an E2~Ub conjugate in the absence of a RING E3 appears to correlate with its natural tendency to populate closed states, although more examples are required to confirm this prediction [29, 35, 36].

As more E2s undergo thorough investigation, exceptions and/or variations to the general mechanism are emerging. An intriguing example is Ube2S, the dedicated E2 for the multi-subunit APC/C E3 that regulates cell cycle progression. On its own, Ube2S~Ub populates closed states to a considerable extent and can catalyze formation of free polyUb chains in the absence of an E3 [36]. Notably, mutations designed to disrupt canonical E2/RING interactions that would involve the APC/C RING domain subunit (Apc11) do not affect activity [37, 38]. On the other hand, two non-RING subunits, Apc2 and Apc4, contribute to Ube2S activation in a poorly understood mechanism that may involve the C-terminal helix of the Ube2S UBC domain [37]. It is tempting to speculate that the novel mechanism used by APC/C to activate its dedicated E2 ensures that APC/C substrates are not ubiquitylated at the wrong place or time. Undoubtedly, new variations of the general mechanism will continue to emerge in the future.

In contrast to the dependence of aminolysis on closed E2~Ub conformations, E2~Ub conjugates readily undergo transthiolation reactions in the absence of E3s. This implies that E3s that use a HECT-type mechanism (i.e., progress via an E3~Ub conjugate intermediate) do not need to promote closed states. This prediction is supported by structures of E2~Ub bound to a HECT (NEDD4L) or RBR (HOIP) E3, which reveal a Ube2D2~Ub conjugate in an open conformation, poised for transthiolation to the E3 active-site cysteine [39, 40]. Consistent with the notion that aminolysis depends on closed E2~Ub states, intrinsic reactivity assays show that neither HECTs nor RBRs enhance the intrinsic lysine reactivity of Ube2D3 [31]. A corollary is that, by trapping open E2~Ub, HECT-type E3s position their active-site cysteine for transthiolation and inhibit serendipitous transfer to any nearby lysines. This prediction has been confirmed in the complex of a pathogenic bacterial effector, OspG, that traps Ube2D3~Ub in an open conformation similar to that observed in the NEDD4L complex [3]. In this high-affinity complex, Ube2D3~Ub is highly reactive towards free cysteine, but shows greatly reduced reactivity towards free lysine when compared with free Ube2D3~Ub.

Product determination by E2s

A major question is how residue specificity is achieved during ubiquitylation. For HECT-type enzymes that transfer Ub via an E3~Ub intermediate prior to the ultimate transfer to substrate, specificity is enforced primarily by the E3. However, for RING-type E3s, which represent the vast majority of E3s, and for situations that are E3-independent, the E2 plays a determining role. With this in mind, we coin the new old adage: "The last guy holding the activated Ub/Ubl gets to determine the product". In this regard, it is useful to categorize E2s in terms of their general product proclivities: (1) E2s that transfer Ub/Ubl onto a target protein residue (monoubiquitylating or priming E2s); (2) E2s that transfer Ub/Ubl onto another Ub/Ubl (chain-building E2s); and (3) promiscuous E2s that can do either. The distinction between transferring Ub to a lysine on a target protein versus a lysine on Ub itself implies different determinants for specificity. While there are numerous examples of each type of E2, current understanding is most advanced for chain-building E2s, although progress is being made on all fronts.

It is easy to imagine that an E2 that catalyzes formation of a linkage-specific polyUb chain will have evolved determinants to recognize the specified lysine residue presented within its structural and/or sequence context. Whereas K63-specific Ube2N uses a tightly bound E2-like subunit (either Ube2V1 or Ube2V2) to position the K63 side chain of the incoming (acceptor) Ub [41],

other linkage-specific E2s are able to specify their product using only their UBC domains. Ube2K has a unique region near its active site that interacts with a tyrosine near K48 in the acceptor Ub to provide K48-linkage specificity. Ube2S uses acidic residues in the final UBC domain helix to interact with the acceptor Ub and orient K11 towards the C-terminus of the donor Ub bound to its active site [36, 42, 43]. A family of K48-specific E2s that includes Ube2R1, Ube2R2, Ube2G1, Ube2G2, and yeast Ubc7 has a short ~12 amino acid insertion proximal to the E2 active site that determines specificity (Figure 1A). This short, highly flexible element appears to dictate product formation using a number of strategies (discussed below).

Ube2R1 and its yeast counterpart Cdc34 are dedicated E2s for the large multi-subunit SCF (Skp/Cullin/F-Box) E3s that target proteins to the proteasome for degradation. Both of these enzymes also have an acidic C-terminal extension that interacts with a "basic canyon" on the cullin subunit of an SCF complex, helping to position the E2 near the RING subunit while allowing for rapid association and turnover in chain building [44]. In these E2s, the aforementioned short insertion, termed the acidic loop, affects function in several ways. First, the loop of Cdc34 interacts directly with the SCF complex [44, 45]. Use of additional, special structural features for E3 binding could serve to limit the ability of other cellular E2s to bind and be activated by the SCF, possibly generating products other than the desired K48-linked polyUb chains on SCF substrates. Second, the acidic loop helps orient the donor Ub for attack by the incoming K48 on the acceptor Ub. For the Cdc34~Ub conjugate, positioning the donor Ub appears to set the stage for binding of the acceptor Ub, allowing recognition of Ub residues in a loop located between residues E51 and Y59 to properly position the acceptor K48 within the E2 active site [46]. Third, the acidic loop appears to play a role in lowering the pK_a of an as yet unidentified ionizable group on the E2, Ub, or even possibly the incoming K48, which must be deprotonated to become nucleophilic [45]. Altogether, these interactions promote the efficiency and fidelity of K48-linked polyUb chain synthesis by Ube2R1 and its homologues [45, 47, 48]. In the case of Ube2G2, binding of a non-RING region ("G2BR") of its E3, gp78, to the backside of the UBC domain (see Figure 1A and discussed in more detail below) alters the acidic loop conformation, which is helical in the free E2 structure but is unwound in the G2BR-bound structure [49, 50]. The unwinding generates a series of interactions among E2, E3, and Ub that help stabilize a closed E2~Ub conformation to increase aminolysis reactivity. Thus, similar to the "extra" E2/E3 interaction between the Cdc34 acidic loop and its RING E3, the requirement of an extra, allosteric interaction between Ube2G2 and gp78 ensures that the K48 chain-building E2 cannot work with any RING E3 it happens to contact.

Although our understanding of how product specificity is dictated is fairly detailed when Ub serves as the incoming source of lysine, the same question regarding non-Ub substrates remains a thorny one. There are likely several reasons for this. First is the sheer number of E2s that can transfer Ub in cells and the difficulty of defining relevant E2/E3/target ensembles. Second is the even larger number of potential substrates. Third is the confounding situation that only a tiny minority of all targets are modified on specific residues. This is likely because in the case of polyubiquitylation, it is the chain itself that serves as the recognition element for further actions, so exactly where on a target protein that chain is attached is of little importance. This provides a rationale for the observation that most of the targets in which a specific residue is modified have only a single Ub (or Ubl) attached and therefore represent a very different type of product and outcome.

By far, the best-characterized E2 that transfers a Ub or Ubl moiety directly to a target residue is the sole SUMO E2 Ube2I (Ubc9). In the absence of an E3, Ube2I can modify targets that contain a lysine in a ψ-K-X-D/E consensus motif (with ψ being a bulky hydrophobic and X being any amino acid), if the motif is present in a loop or unstructured region [51, 52]. The active site of Ube2I positions the incoming lysine in this sequence context optimally for the nucleophilic attack on the activated SUMO thioester bond [51, 53]. Although the consensus sequence is not an absolute requirement for Ube2I-dependent SUMOylation, this example illustrates the intimate contact between an E2 and a target that occurs during direct transfer reactions. Much less is known about the Ub E2s that target specific lysine residues and how this is achieved. For instance, Ube2T monoubiquitylates its substrate FANCD2 on a specific lysine with its RING E3 FANCL in the Fanconi Anemia DNA repair pathway [54]. The yeast E2 Rad6, with help from its cognate RING E3 ligase Rad18, transfers a single Ub molecule to a specific lysine (K164) on PCNA [55], a signal that promotes the switching from normal replicative polymerases to Y-family translesion polymerases to bypass a DNA lesion [56, 57].

Among the E2s that show little or no preference regarding the residues to which they transfer the Ub moiety, the ubiquitous Ube2D family (a.k.a UbcH5) is paramount. Family members show remarkably low rates of reaction with lysine in the absence of an E3, but are highly activated upon binding to a vast array of RING-

type E3s. The Ube2D family is essentially indiscriminate and, once activated, will transfer Ub to any lysine residue that comes near its active site. Although this property makes Ube2D family members popular reagents for *in vitro* studies, it also has made identification of their biologically relevant partners and substrates challenging.

Finally, it is important to appreciate that some or all linkage-specific chain-building E2s can only perform their function on substrates that have previously been acted upon by another E2, a priming E2. This is because the highly specific chain builders such as Ube2N, Ube2S, and Ube2R1 can only transfer their conjugated Ub to another Ub molecule. This leads to a division of labor among E2s in which one E2 initiates or primes chain synthesis and a second E2 builds and extends the polyUb chain [11, 58]. Such a strategy is used for substrates of the APC/C E3 ligase complex during cell cycle progression. Either Ube2C or a Ube2D family member transfers the first Ub onto human APC/C substrates and Ube2S then builds the K11-linked polyUb chains that are a hallmark of APC/C-mediated proteasomal degradation [59, 60]. Interestingly, the APC/C appears to repurpose its RING subunit to bind and track the growing Ub chain during Ube2S-mediated catalysis, presumably inhibiting incorrect chain building by the promiscuous Ube2D E2s [37, 38].

In the case of APC/C, the monoubiquitylated substrate is an intermediate that most likely does not disassociate from the APC/C complex. Here different E2s work with a common E3. An interesting contrast is PCNA, whose mono- and polyubiquitylation represent separate signals that have different functional outcomes. As discussed above, PCNA is monoubiquitylated by the Ube2A/B (Rad6) E2s and the RING E3 Rad18 during postreplicative DNA damage repair [55-57]. A second E2/E3 pair, namely, Ube2N/Ube2V2 and the RING E3 Rad5, together builds a K63-linked chain at the same site, to create a signal that promotes template-switching and engagement of the homologous recombination machinery [55, 61]. Our survey of the literature is consistent with an interpretation where Ube2N can only transfer Ub to the K63 of another Ub, implying that monoubiquitylated PCNA serves as the substrate in this reaction. An unusual alternative mechanism for the modification of PCNA with K63-linked chains has recently been proposed in which K63-linked polyUb is built directly onto the active site cysteine of Ube2N. The K63-linked chain is subsequently transferred via transthiolation to another E2, Rad6, which then transfers the preformed chain *en bloc* to K164 on PCNA [62]. Regardless of which mechanism prevails, the process requires a division of labor between two E2s.

Our final example involves the E2 Ube2W, with its unique ability to monoubiquitylate proteins on their N-termini. Early *in vitro* studies demonstrated that a substrate that is modified by Ube2W can serve as the template for chain building by Ube2N and Ube2K [11]. Several recent studies indicate that Ube2W may work as a chain-initiating E2 in the innate immune response where K63-linked chains play a critical role. The functional importance of Ube2W in the innate immune system was recently established *in vivo* using knockout mice [63]. Ube2W appears to monoubiquitylate the RING E3 ligases TRIM5α and TRIM21, a prerequisite for their K63 polyubiquitylation by Ube2N/Ube2V2 [17, 64].

These examples demonstrate that, whether on their own or in tandem, E2s (activated by RING-type E3s) dictate the nature of the product and therefore the ultimate signal generated and its biological outcome.

Regulation of E2 activity

The central role that E2s play in Ub signaling makes them attractive targets for regulatory control. Although an understanding of E2 regulation is still emerging, it is clear that E2s can be regulated by multiple mechanisms. We have already described the most general mechanism whereby E2 reactivity is regulated via interaction with a RING-type E3 to promote a closed, more reactive E2~Ub conformation. Progress is being made on other aspects of E2 regulation. Here we give an overview of the regulatory mechanisms identified to date and highlight emerging themes.

Non-covalent modulation of E2 activity by backside binding

Modulation of activity through non-covalent interactions is a general mechanism of enzyme regulation. On E2s, the site used most frequently for regulatory non-covalent binding events is the so-called 'backside' surface (Table 1). Located on the face opposite from the catalytic pocket, the surface is made from UBC domain residues on the β-sheet, C-terminal end of helix α1 and the following loop, and may also include the C-terminus of helix 4 (Figure 1A). The earliest reported backside interaction was of Ub non-covalently bound to Ube2D3 via the hydrophobic patch centered on I44, a Ub surface used in many different protein-protein interactions [65]. Although weak in affinity (~300 μM K_D), the interaction promotes an increase in processivity of polyUb chain building by Ube2D3 [66-69]. The modulator can be free Ub or Ub that is conjugated to another Ube2D3 molecule (in a piggyback manner), which may serve to increase the local concentration of E2~Ub conjugates near an E3

(and therefore, a substrate) [65]. Backside binding by Ub increases the intrinsic lysine reactivity of Ube2D2~Ub [70], indicating an allosteric effect, although the details of how the binding at a distal site affects active site reactivity remain to be defined experimentally.

The chain-building activity of other E2s is enhanced by Ub backside binding as well. The interaction is critical for the E3-independent ability of Ube2B to build K11-linked polyUb chains [71]. Similarly, intrinsic chain-building ability of the yeast homolog of Ube2B, Rad6, is enhanced by Ub binding [72]. Finally, the Ubl SUMO binds to the backside of its E2, Ube2I, where the interaction also has a positive effect on SUMO chain building [73]. Although the structural details of these interactions may vary, they define a general regulatory mechanism by which non-covalent binding of a Ub/Ubl to the distal backside surface has a positive effect on an E2's chain-building efficiency.

Somewhat paradoxically, non-covalent binding of Ub to certain E2s negatively affects chain building and processivity. The observed preference of Ube2E3 to generate monoubiquitylated products arises from specific interactions involving K48 on Ub and backside residues of the E2 [74]. Disruption of this interaction by mutation of either Ub or E2 backside residues results in the rapid generation of K63-linked Ub chains by Ube2E3. Thus, this E2 can be thought of having an intrinsic ability to build polyUb chains that is inhibited by Ub binding on its backside. The two opposite effects of backside Ub binding suggest that it can act as either a throttle or a brake for chain building.

Some RING-type E3s have accessory regions that bind to the backside surface of E2s and modulate their activity. As the examples below demonstrate, this E3 region is frequently an α-helical segment outside the RING domain, and the binding results in either increased affinity for E2~Ub conjugates and/or allosteric affects on E2~Ub activity. Notably, the E2s that have been reported to engage in backside binding with an E3 also bind Ub (or Ubl) on their backside (see Table 1). This sets up a competition between the two possible non-covalent interactions. Consistent with this notion, the backside-binding element of the E3 AO7 decreases the processivity of chain building by the E2 Ube2D2 [70, 75]. Similarly, backside binding by accessory elements of the RING E3 Rad18 inhibits the intrinsic chain-forming activity of Ube2B, thus promoting monoubiquitylation of PCNA and histone 2B [71, 72].

In an interesting variation on the theme, the SUMO E3 ligase, RanBP2, binds to the backside of Ube2I. Lacking a RING domain, RanBP2 contains a natively unfolded region known as internal repeat region 1 (IR1) that exhibits SUMO E3 ligase activity [76, 77]. IR1 appears to stabilize a closed conformation of the Ube2I~SUMO1 conjugate via contacts on the canonical E3-binding surface of Ube2I, the backside of Ube2I, and by interactions with SUMO [78]. Precisely how IR1 enhances the transfer of SUMO remains mechanistically unclear [78].

Multi-subunit E3s also make use of the E2 backside surface. The APC/C engages Ube2C using both a RING subunit Apc11 and the WHB domain of a cullin subunit Apc2, the latter interaction being via the E2's backside [79]. While use of two subunits to recruit the E2 serves to ensure specificity for Ube2C, the additional interaction also appears to direct substrate ubiquitylation by reducing the degrees of freedom available for the E2/RING assembly. Cue1, a transmembrane subunit of the yeast ERAD RING E3 complex, has an α-helix that binds to the backside of the ERAD E2, Ubc7, a homologue of human Ube2G2. Cue1 binding tethers Ubc7 to the membrane and increases the affinity of Ubc7 for its ERAD E3 [80, 81]. But in addition to localizing the E2, Cue1 binding modulates Ubc7 activity by enhancing Ub loading of the E2 by E1 and by allosterically opening loops around the E2 active site. Analogously, the aforementioned human ERAD E3 ligase, gp78, uses the non-RING G2BR to interact with the backside of its E2, Ube2G2 [49]. Remarkably, even G2BR binding *in trans* can increase the affinity of Ube2G2~Ub for the gp78 RING and can enhance both E3-dependent and E3-independent Ub transfer activity, suggesting cooperative allosteric interactions between RING and G2BR binding [49, 50]. The situation is different in the case of uncharged Ube2G2, which binds the E3 with lower affinity due to loss of some G2BR contacts, an effect that promotes dissociation of reacted (or inactive) E2 from the E3, allowing for exchange with active E2~Ub conjugate [49, 50, 82]. In sum, these reciprocal sets of allosteric interactions represent an exquisite example of how a RING and an accessory feature of an E3 ligase can work synergistically through an E2 to ensure processive rounds of ubiquitylation.

Covalent modulation of E2 reactivity

A second common mechanism of enzyme regulation is through covalent post-translational modifications that modulate activity. There are a growing number of examples of E2s that are themselves the target of ubiquitylation. The stability of yeast Ubc7 is dependent on the presence of its binding partner, Cue1, a subunit of the ERAD complex (see above). Under limiting Cue1 conditions, a polyUb chain is synthesized on the Ubc7 active-site cysteine that targets it for proteasomal degradation [83]. This provides a highly effective way to

regulate E2 activity, namely, by destroying the E2. Chain building directly on E2 active sites has only been reported in limited cases (e.g., Ubc7 and its human homolog Ube2G2 [84], and Ube2D in collaboration with the bacterial effector SspH2 [85]). However, considering that their detection requires special conditions to inhibit the loss of thioester-linked chains during sample handling, it is still unclear how general the feature might be among E2s.

Ubiquitylation of E2s on lysine residues also occurs. Ube2T, the E2 involved in the Fanconi Anemia DNA repair pathway, transfers Ub to a lysine near its active site and two lysines located in its C-terminal extension [54]. Ubiquitylated Ube2T has been observed *in vitro* and in cells, and its production is enhanced by the E3, FANCL. However, unlike Ubc7 autoubiquitylation, (multi)-monoubiquitylated Ube2T does not signal for its degradation, but has decreased Ub transfer activity *in vitro*. Ube2E1 provides a third example of E2 regulation by autoubiquitylation. Similar to Ube2T, ubiquitylation results in inhibition of E2 activity [86]. Modification occurs on a lysine near the active site and on lysines in the unstructured N-terminal extension of Ube2E1. Notably, it is ubiquitylation of Ube2E1's N-terminal extension that gives rise to the inhibition. Deletion of the N-terminal extension of Ube2E family members switches their (*in vitro*) activity from mono- to polyubiquitylation, although a molecular mechanism for the inhibitory function of the N-terminus has yet to be been defined [87]. In summary, the examples to date demonstrate that, like ubiquitylation of target proteins, ubiquitylation of E2s themselves can regulate cellular E2 activity either by controlling E2 protein levels or by controlling E2 activity *per se*. The first mechanism, degradation, is irreversible and it remains to be determined whether regulation via monoubiquitylation of E2s can be reversed by one or more of the myriad of deubiquitylating enzymes in the cell.

The Ubl SUMO can also act as a covalent modulator of E2 activity. The SUMO E2 Ube2I is itself regulated by SUMOylation, although the modification likely occurs *in trans* as the modified lysine, in human Ube2I on helix α1, is too distant from the active site. The consequence of Ube2I SUMOylation appears to be context-dependent: it can increase, decrease, or have no effect on SUMO transfer activity, depending on the location of SUMO attachment and types of E2 contacts made by the E3 or the substrate [88, 89]. Targets that contain SUMO-interacting motifs (SIMs) are more readily recruited to SUMO-Ube2I with a concomitant increase in (E3-independent) SUMO transfer by Ube2I, but the modification appears to block binding interfaces important for some E3s and (non-SIM-containing) target substrates [89]. Thus, autoSUMOylation is a mechanism by which cells may simultaneously tune SUMOylation of multiple substrates, increasing some, decreasing some, and allowing others to remain at a steady state. A second example of SUMO modification of an E2 is the polyUb chain-building E2, Ube2K [52]. Like Ube2I, the site of modification is on helix α1, but on a residue in the E1 and E3 binding interface (Figure 1), consistent with the decreased activity observed *in vitro*.

There is growing evidence that E2s can be modulated by other covalent modifications, including those associated with oxidative stress. The E2 Ube2E3 regulates the activity of Nrf2, a transcription factor that induces expression of anti-oxidant genes to neutralize reactive oxygen species and restore redox homeostasis [90]. Alkylation of non-catalytic C136 of Ube2E3 (to mimic its oxidation) results in constitutive binding of the E2 to Nrf2, increasing its half-life and thus its transcriptional activity [90]. Although the mechanistic details of this regulation are not fully understood, this regulation also depends on the catalytic activity of Ube2E3. Intriguingly, Ube2E3's C136 replaces the proline in a conserved HPN triad (Figure 1A), which has been reported to be required for E2 activity [51]. In a second example, Ube2I is reversibly inhibited by disulfide bond formation between its catalytic cysteine and that of the SUMO1 E1 subunit Uba2 [91]. While very high levels of oxidative stress can thereby lead to global deSUMOylation, this pathway may serve highly specific functions in the context of redox signaling, e.g., upon macrophage activation [91]. Intriguingly, several chemotherapeutic drugs used in the treatment of acute myeloid leukemia induce the SUMO E1 and E2 disulfide bond formation and cause loss of SUMOylation [92]. Similarly, a disulfide bond formed between the Ub E1 Uba1 and the E2 Ube2R1 upon oxidative stress is associated with increased Ube2R1 substrate stability and delayed cell cycle progression [93]. In summary, these E2 modifications all seem to function as redox sensors that chemically alter the E2 active site and therefore its activity.

Our final example of modulation by covalent modification of E2s involves pathogenic bacteria that invade eukaryotes. By introducing effector proteins directly into the host cell, they target a variety of pathways to facilitate invasion, inhibit innate immune response, and establish an environment for replication. Not surprisingly, such pathogens have evolved a variety of ways to exploit the Ub signaling pathways, and the regulation of specific E2s is one such strategy. An effector protein from the enteric pathogen *Shigella*, Osp1, deamidates a glutamine residue in Ube2N, resulting in inhibition of NF-κB signaling and the host immune response [94]. Although Osp1 can effectively compete with E3s for Ube2N

binding, the primary mode of inhibition by Osp1 is the deamidation of Q100 to yield a glutamate residue [95]. This irreversible modification places a negative charge at the beginning of the Ube2N crossover helix, a structural feature known to be involved in the closed E2~Ub conformation and activation by RING-type E3s [29, 31].

Examples of the most common covalent modification to modulate protein activity, phosphorylation, are strikingly underrepresented among E2s. Early studies reported functionally important phosphorylation in E2 extensions [96-98], but there are a growing number of identified phosphosites that are within UBC domains. Of particular interest are modifications near the active site. For example, CK2-mediated phosphorylation on a crossover helix residue of yeast Cdc34 (S130) appears to repulse the acidic loop that can occlude the catalytic cleft, resulting in its opening [99, 100]. This affects E2 charging as well as reactivity of the Cdc34~Ub thioester in a mechanism that may apply to all E2s featuring an acidic loop [100]. Furthermore, phosphorylation by Cdk-9 on a serine referred to here as the "gateway residue" (Figure 1) activates Ube2A [101-103]. The gateway residue is in a loop that forms the opening of the E2 active-site cleft and is most often an aspartate or serine followed by proline (the Cdk-9 consensus site) in human E2s. Mutation of the gateway aspartate in the Ube2D family severely reduces Ub transfer to lysine side chains [33]. Simulations have also suggested that E2s with a gateway aspartate (but not a glutamate) are constitutively active, whereas the activity of E2s with a gateway serine (but not a threonine) may be controlled by phosphorylation [104]. The hypothesis awaits experimental confirmation, but provides the possibility of a unifying mechanism that links E2 activity and phosphorylation near the active site.

Modulation of E2 activity by transcriptional/translational control

As discussed above, protein degradation signaled by polyubiquitylation is a mechanism by which the ubiquitylation machinery can control protein levels. E2 transcription and translation are also emerging as important strategies for long-term regulation of Ub/Ubl transfer. Both intrinsic and external forces take advantage of this strategy. Ube2I expression is increased in response to 17β-estradiol under the control of the estrogen receptor (ERα) and nuclear factor Y [105]. Ube2L3 undergoes transcriptional regulation in response to aryl hydrocarbon receptor (AHR) signaling [106]. The resulting increase in Ube2L3 levels leads to degradation of cell cycle control proteins, identifying a connection between AHR signaling and the previously established role that Ube2L3 plays in cell cycle regulation [107]. Epstein-Barr virus (EBV) targets E2s to control host cells by regulating translation of the E2 BIRC6. The latter is an inhibitor of apoptosis protein and its degradation by Nrdp1 induces apoptosis in response to apoptotic stimuli [108]. An EBV-encoded microRNA (BART15-3p) that leads to host cell apoptosis specifically targets the E2, BIRC6, leading to a decrease in translation of the BIRC6 mRNA without affecting the mRNA stability [109].

Modulation of E2 activity by small molecules

Targeting specific E2s with small molecules is becoming more feasible thanks to the large amount of structural and biochemical information available on E2s, both on their own and in active complexes. Once discovered, selective small-molecule inhibitors can serve as powerful tools to study the function and physiological roles of E2s and, possibly, as leads for drug discovery. Due to its role in the immune response, there is interest in Ube2N as a potential drug target. A known NF-κB inhibitor (NSC697923) covalently modifies the catalytic cysteine of Ube2N and binds to a cleft in the active site that is not accessible in other E2s, thus providing specificity [110]. Structure-based mutations confirmed that NSC697923 inhibition of NF-κB signaling and DNA damage response in cells is due specifically to the small molecule's action on Ube2N. Another small-molecule inhibitor (CC0651) selectively inhibits Ube2R1, the specialized E2 for SCF E3 ligases, which builds K48-linked polyUb chains on its targets for proteasomal degradation. In a co-crystal structure of Ube2R1, Ub, and CC0651, the inhibitor is sandwiched between the E2 and Ub [111]. Remarkably, the resulting non-covalent complex resembles the closed, activated E2~Ub conjugate seen for other E2s (Figure 2). The inhibitor increases RING affinity for the E2, but decreases E2~Ub hydrolysis rates perhaps by acting as a molecular glue. Notably, while the CC0651-binding surfaces on both the E2 and Ub are rather flat and therefore not obvious candidates for small-molecule binding, the insight that the two proteins together form a targetable interface may guide future efforts to design or screen for small-molecule inhibitors specific to other E2s. Development of small-molecule inhibitors will allow researchers to tie together the idiosyncratic roles E2s play biologically with what is known mechanistically about how they are activated and regulated.

Expanding the realm: E2s as direct regulators of enzyme activity

OTUB1 is a deubiquitinase (DUB) that selectively hydrolyzes K48-linked polyUb chains and, therefore, can play a regulatory role in modulating Ub signaling. Rather

unexpectedly, OTUB1 DUB activity is enhanced by interaction with free E2s. Binding of Ube2D2 stabilizes the disordered OTUB1 N-terminus in an α-helical conformation, which completes the binding site for K48-linked diUb. The E2-mediated conformational change decreases the K_m of OTUB1 for diUb by over 35-fold, thereby enhancing the rate of OTUB1-dependent polyUb degradation [2]. This is an example of an E2 acting as an effector protein to stimulate enzyme (DUB) activity. But this is not the only regulatory interaction involving E2s and OTUB1. OTUB1 can also simultaneously bind free Ub and certain E2~Ub conjugates (e.g., Ube2D(1/2/3)~Ub, Ube2E1~Ub, or Ube2N~Ub). The two Ub moieties occupy both Ub-binding sites on OTUB1 and effectively inhibit its DUB activity. Furthermore, the E2~Ub conjugate in the OTUB1 complex is bound in a less reactive open conformation and the E3-binding surface of the E2 is occupied by OTUB1 binding. On this basis, it has been proposed that OTUB1 inhibits the ability of E2~Ub conjugates to participate in Ub transfer reactions and in building polyUb chains [2]. Thus, OTUB1/E2~Ub/Ub complexes may inhibit both E2~Ub activity and DUB activity, whereas OTUB1/E2 complexes can stimulate cleavage of K48-linked chains. These findings suggest a model where OTUB1 is poised to regulate either polyUb chain elongation or degradation and the type of regulation depends on the levels of free Ub and the relative levels of free E2.

A final example comes from the world of pathogenic bacteria. The effector protein OspG, is found in several pathogenic strains of *Shigella*, *Yersinia* and *E. coli*, plays a role in tempering innate immune responses. *Shigella* lacking OspG induce a much stronger inflammatory response than wild-type *Shigella* [112]. Sequence analysis suggested that OspG is a kinase, so it was surprising when a two-hybrid screen found that OspG selectively interacts with E2~Ub conjugates [112]. Rather than serving as substrates for phosphorylation by OspG, E2~Ub conjugates regulate its kinase activity [113]. A co-crystal structure of OspG and Ube2D3~Ub revealed that the E2~Ub conjugate binds in an open (inactive) conformation while stabilizing OspG in an active conformation with its kinase active site fully accessible to substrates [3]. Thus, although OspG by itself is a poor kinase, it uses a clever strategy whereby it recognizes and binds tightly to E2~Ub conjugates present at relatively high concentration in its host cell to stimulate its enzymatic activity.

Concluding remarks

We have endeavored here to define the current state of understanding for the central enzymes in the trio required to attach Ub to proteins. Three decades after their initial discovery and characterization [6, 114], there are finally answers to the questions posed earlier in this review, namely, "What keeps the reactivity of an E2~Ub low?" and "How is reactivity stimulated by E3s?". There is a growing understanding of the ways in which E2s can limit themselves to monoubiquitylating substrates or building chains efficiently and with fidelity. But we still lack fundamental information such as the intrinsic reactivity (and therefore side chain specificity) of some human E2s, and the identification of biological E2/E3/substrate sets remains an enormous experimental hurdle. A combination of biochemical approaches and use of emerging gene-editing approaches will allow progress on these and other fronts in the near future.

It is not surprising that E2s are regulated by other components of the ubiquitylation machinery, although the diversity of strategies utilized is unexpected. Less predictable is the ability of E2s to serve as regulators of other proteins and enzymes. Although the number of defined examples is still small, we expect this to be an emerging theme. In this regard, it is worth noting that a large-scale two-hybrid screen involving over 40 human E2 and E2-like proteins identified > 200 unique interacting proteins [115]. Of these, 30% are E3 ligases and another ~10% are proteins involved in the Ub transfer pathways or deubiquitylation. But what of the remaining 60% of identified proteins? We expect that the list contains numerous additional examples where the small and versatile UBC domain functions outside of canonical E1/E2/E3 Ub transfer pathways, likely in a regulatory capacity. Apparently, there is still much we do not know about E2s.

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