## REVIEW ARTICLE

# Getting into position: the catalytic mechanisms of protein ubiquitylation

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The role of protein ubiquitylation in the control of diverse cellular pathways has recently gained widespread attention. Ubiquitylation not only directs the targeted destruction of tagged proteins by the 26 S proteasome, but it also modulates protein activities, protein–protein interactions and subcellular localization. An understanding of the components involved in protein ubiquitylation (E1s, E2s and E3s) is essential to understand how specificity and regulation are conferred upon these pathways. Much of what we know about the catalytic mechanisms of protein ubiquitylation comes from structural studies of the proteins involved in this process. Indeed, structures of ubiquitin-activating enzymes (E1s) and ubiquitin-conjugating enzymes (E2s) have provided insight into their mechanistic details. E3s (ubiquitin ligases) contain most of the substrate specificity and regulatory elements required for protein ubiquitylation. Although several E3 structures are available, the specific mechanistic role of E3s is still unclear. This review will discuss the different types of ubiquitin signals and how they are generated. Recent advances in the field of protein ubiquitylation will be examined, including the mechanisms of E1, E2 and E3. In particular, we discuss the complexity of molecular recognition required to impose selectivity on substrate selection and topology of poly-ubiquitin chains.

Key words: E1, E2, E3, ubiquitin, post-translational modification, RING finger.

## INTRODUCTION

Post-translational protein modifications are used to generate and relay signals in almost every cellular pathway. Protein phosphorylation, the best understood modification, can have numerous consequences including: allosteric activation or inactivation of proteins; alterations in subcellular localization; changes in protein stability; or altered protein–protein interactions [1]. Traditionally other post-translational modifications (including acetylation, ubiquitylation and methylation) are viewed as having more limited and specific roles. For example, ubiquitylation is best known as a signal for controlled protein degradation by the 26 S proteasome. However, recent evidence suggests that the signals generated by ubiquitin may be as far-reaching as those generated by phosphorylation, and different types of ubiquitin modifications are capable of transmitting unique signals (Figure 1). Indeed, as its name would suggest, ubiquitin is involved in many different signalling pathways including cell cycle, endocytosis, transcription, DNA repair, signal transduction, apoptosis and the immune response [2–7]. Ubiquitin is a highly conserved 76 amino acid protein found in all eukaryotes. It is chemically more complex than post-translational modifications such as phosphorylation, as it provides a molecular surface for protein–protein interactions. Thus, conjugation to ubiquitin has the potential to signal diverse outcomes.

The canonical ubiquitin signal, a $Lys^{48}$-linked poly-ubiquitin chain, is recognized by the 26 S proteasome and thereby targets tagged proteins for degradation. Conjugation with $Lys^{48}$-linked poly-ubiquitin chains is a rapid and irreversible method for controlling protein abundance and is often used when an on/off switch-like signal is required. For example, many cell cycle regulatory proteins are controlled by ubiquitin-mediated proteolysis to allow rapid and irreversible transitions between stages of the cell cycle. E3s (ubiquitin ligases) such as the anaphase-promoting complex/cyclosome (APC) and the Skp1-Cdc53/Cul1-F-box protein (SCF), are responsible for targeting these substrate proteins for degradation and these proteolytic events are required for cell cycle control [2,7]. Ubiquitin has six other lysine residues and at least four of these ($Lys^6$, $Lys^{11}$, $Lys^{29}$ and $Lys^{63}$) can function as a linkage for poly-ubiquitin chains [8–12]. $Lys^{11}$- and $Lys^{29}$-linked poly-ubiquitin chains may target proteins to the proteasome [9,11,13]. On the other hand, ubiquitin modifications can also signal nonproteolytic, reversible events such as changes in protein activity, subcellular localization or protein–protein interactions [14]. These modifications include mono-ubiquitin and poly-ubiquitin chains linked through $Lys^6$ or $Lys^{63}$.

Conjugation of a single ubiquitin (mono-ubiquitylation) is a regulatory modification involved in diverse processes including transcription, histone function, endocytosis and membrane trafficking [5,6,15,16]. Mono-ubiquitylation of receptor tyrosine kinases and other plasma membrane proteins recruits proteins of the endocytic pathway and acts as a signal for receptor endocytosis and targeting to the lysosome [17–21]. Interestingly, the sequential attachment and removal of a single ubiquitin molecule on histone H2B is necessary for transcriptional activation, possibly by recruitment of an acetyltransferase complex [22]. Attachment of $Lys^{63}$-linked poly-ubiquitin chains is involved in signalling DNA repair, the stress response, endocytosis and signal transduction, possibly by directly modulating protein functions [23–28]. $Lys^{63}$-linked di-ubiquitin chains have a more extended conformation than $Lys^{48}$-linked di-ubiquitin [29–31]. These structural differences may explain how poly-ubiquitin chains with various

Abbreviations used: APC, anaphase-promoting complex; DUB, deubiquitylating enzyme; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; E4, poly-ubiquitin chain conjugation factor; HAT, histone acetyl transferase; HAUSP, herpes-associated ubiquitin-specific protease; HECT, homologous to E6-AP carboxy-terminus; PCNA, proliferating cell nuclear antigen; RING, really interesting new gene; SCF, Skp1-Cdc53/Cul1-F-box protein; SUMO, small ubiqitin-related modifier; UBA, ubiquitin-associated domain; Ubc, ubiquitin-conjugating enzyme; UBL, ubiquitin-like proteins; UBP, ubiquitin binding protein; UEV, ubiquitin E2 variant; UIM, ubiquitin interacting motif.

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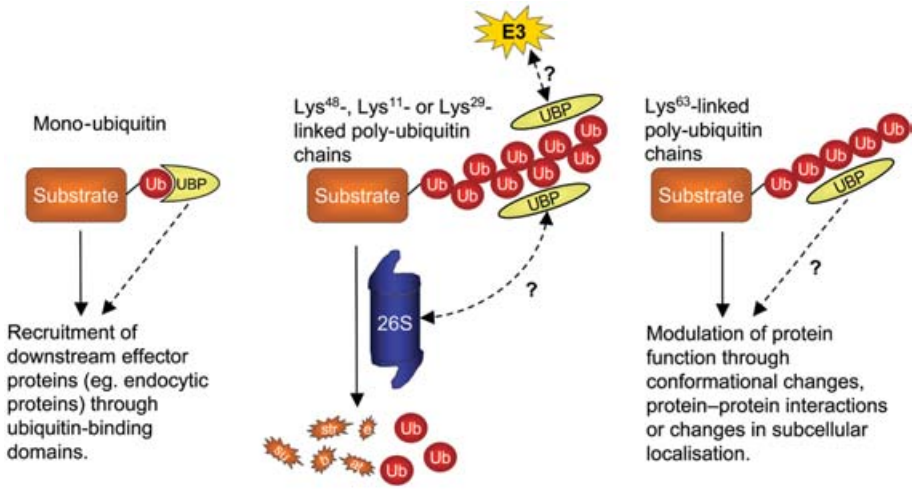


**Figure 1 Ubiquitin modifications**

The consequence of ubiquitylation (the covalent attachment of ubiquitin to a substrate protein) is dependent upon the type of ubiquitin modification. Attachment of a single ubiquitin (Ub) polypeptide may recruit specific UBPs which mediate downstream signalling events. For example, mono-ubiquitylation of membrane proteins can recruit endocytic proteins resulting in internalization. $Lys^{48}$- (or less commonly $Lys^{11}$- or $Lys^{29}$-) linked poly-ubiquitin chains are recognized by the 26 S proteasome which degrades the substrate protein. The ubiquitylation and degradation machinery (E3s and the 26 S proteasome) may be linked through ubiquitin-binding proteins. $Lys^{63}$-linked poly-ubiquitin chains do not signal to the 26 S proteasome and instead seem to modify protein function.

linkages can signal different outcomes. Poly-ubiquitin chains linked through $Lys^{6}$ are disassembled by 26 S proteasomes but their role *in vivo* is not clear [11,32–34].

Proteins can also be modified by covalent attachment of ubiquitin-like proteins (UBLs), such as Rub1/NEDD8 or SUMO/Sentrin/Smt3/Ubl1, which have structural similarity to ubiquitin, although interestingly UBLs are not known to form polymers on target proteins [35–37]. UBL attachment may also have diverse, non-proteolytic consequences. For example, the conjugation of SUMO (small ubiqitin-related modifier) to histone H4 results in transcriptional repression, probably by recruitment of other proteins such as histone deacetylases [38]. Sumoylation of RanGAP1 targets it to the nuclear pore complex [39,40], the mitotic spindle and kinetochores [41], whereas sumoylation of PML targets it to subnuclear structures called nuclear bodies [42]. UBL conjugation can also inhibit the formation of poly-ubiquitin chains if both SUMO and ubiquitin modifications target the same lysine residue. For instance, the same lysine can be modified with either SUMO or ubiquitin in I$\kappa$B$\alpha$ and proliferating cell nuclear antigen (PCNA) [43,44].

The molecular mechanisms of ubiquitin attachment to target proteins are likely to be similar to UBL attachment: in both cases, the C-terminal glycine residue of the ubiquitin or UBL molecule is attached to a lysine side chain of the substrate protein (or another ubiquitin molecule) to form an isopeptide linkage. However, what distinguishes ubiquitylation from UBL modification of proteins, is the ability to form poly-ubiquitin chains of specific linkage and topology. The process of poly-ubiquitylation therefore requires a solution to two problems of molecular recognition. First, in the initial ubiquitylation reaction, recognition of the appropriate protein substrate and, secondly, recognition of the correct ubiquitin lysine residue within the growing poly-ubiquitin chain. Many of the proteins involved in the ubiquitylation process are defined, and the elucidation of crystal structures of representative components of the ubiquitylation pathway has provided considerable insights into the molecular mechanisms underlying catalysis and specificity. However, despite these advances, detailed questions concerning the molecular mechanisms of ubiquitin transfer remained unanswered. Here, we review the catalytic mechanisms of ubiquitin transfer and discuss recent developments in the field. Each of the components involved in ubiquitin transfer will be examined and discussed with an emphasis on structural aspects. We focus on E3 ubiquitin ligases which provide the specificity for substrate recognition and the topology of poly-ubiquitin chains.

## THE E1/E2/E3 CASCADE

Ubiquitin is attached to target proteins by a three-step mechanism involving the sequential actions of E1, E2 and E3 enzymes (Figure 2) [2,45,46]. First, a ubiquitin-activating enzyme (termed E1) activates ubiquitin: the E1 adenylates the C-terminus of ubiquitin and then forms a thioester bond between the ubiquitin C-terminus and a catalytic E1 cysteine residue [47,48]. To be fully active, the E1 must non-covalently bind to and adenylate a second ubiquitin protein. Secondly, the thioester-linked ubiquitin is transferred from E1 onto the active-site cysteine residue of a ubiquitin-conjugating enzyme (Ubc/E2), where it is again linked by a thioester bond. With the help of a third enzyme, the E3 ubiquitin ligase, ubiquitin is transferred from the E2 to a lysine residue of a substrate protein. This final transfer of ubiquitin results in an isopeptide bond between the $\varepsilon$-amino group of a substrate lysine and the C-terminal carboxylate of ubiquitin. (More rarely, protein ubiquitylation can also occur on the N-terminus of substrate proteins [49–52]). Although the precise roles and mechanisms of different E3s vary, they all promote the transfer of ubiquitin, either directly or indirectly, from a thioester linkage with E2 to an amide linkage with a substrate or another ubiquitin [2]. E3s also provide specificity to the ubiquitin pathway since they recognize and bind to specific substrate sequences or degrons [2,45,46,53]. This specificity allows ubiquitin modifications to be targeted to specific proteins in a temporally and spatially regulated manner. While there exists only one type of E1 enzyme in eukaryotic cells (Uba1) there are 10–30 E2s [45,46]. Each E2 probably interacts with several E3 enzymes. The number of E3s is currently unknown but it is certainly much larger than the number of E2s [46]. Usually, the E3 ligase is the only component in this pathway that is subject to regulation.

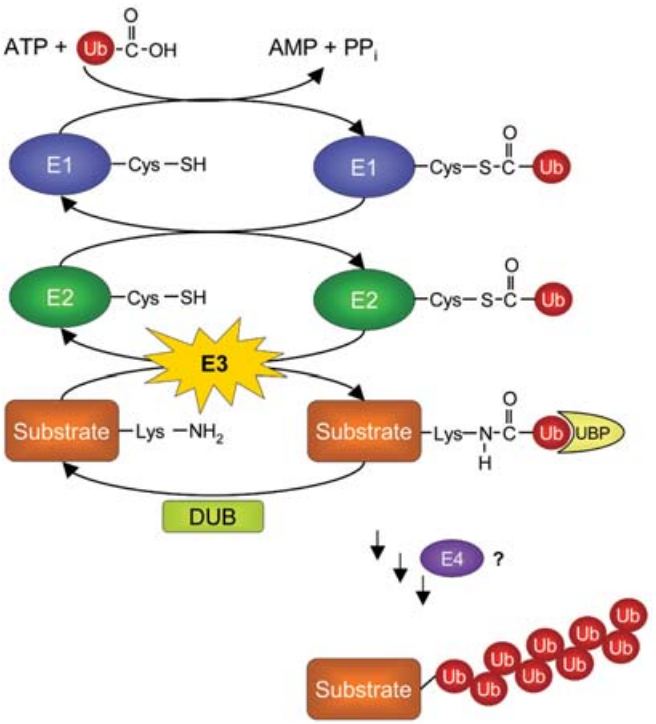


**Figure 2 The ubiquitin-proteasome pathway**

Ubiquitin (Ub) is covalently attached to substrate proteins via a three-step mechanism involving the sequential actions of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) enzymes. The attachment of multiple ubiquitin moieties by E3, perhaps with the help of an E4, results in formation of a poly-ubiquitin chain. Ubiquitin modifications may be removed by DUBs. UBPs interact with ubiquitylated proteins and may prevent the conversion of mono-ubiquitin into poly-ubiquitin chains, protect ubiquitin modifications from DUBs, target proteins to the 26 S proteasome and/or mediate downstream signalling events perhaps through new protein–protein interactions.

Importantly, the type of ubiquitin modification (either mono-ubiquitin or topology of poly-ubiquitin chains) is dictated through the use of both specific E2 ubiquitin-conjugating enzymes and E3 ligases. For example, additional ubiquitin molecules may be attached to $Lys^{48}$ of the preceding ubiquitin (catalysed by the E3) to form a poly-ubiquitin chain linked by $Lys^{48}$-$Gly^{76}$ isopeptide bonds [54]. A fourth enzyme, the E4 (poly-ubiquitin chain conjugation factor), may play a role in chain elongation: Koegl et al. [55] showed that E4 (Ufd2) binds to substrate-conjugated ubiquitin and promotes the E1-, E2- and E3-dependent polymerization of long poly-ubiquitin chains onto a model peptide substrate. E4 is not required for conjugation of the first ubiquitin moieties but may direct specific ubiquitin linkages in poly-ubiquitin chains. Ubiquitin-specific E3 ligases which catalyse the formation of ubiquitin–ubiquitin but not ubiquitin–substrate linkages may play an analogous role in some cases [56,57]. Deubiquitylating enzymes (DUBs) counter the action of the E1/E2/E3 cascade by disassembling poly-ubiquitin chains [58].

*In vivo*, poly-ubiquitin chains are most frequently linked through $Lys^{48}$ [12]. The 26 S proteasome (composed of a 20 S proteolytic core and a 19 S regulatory complex) recognizes $Lys^{48}$-linked poly-ubiquitin chains composed of at least four ubiquitin moieties, degrades the tagged protein and recycles the ubiquitin molecules [59,60]. The context of the poly-ubiquitylated lysine may be important for efficient degradation, as some poly-ubiquitylated proteins are not degraded [52,61,62]. For example, if the lysine is located in a region of the protein which can be readily unfolded, the proteasome would be able to unfold and degrade the protein before it dissociates [62]. Often more than one poly-ubiquitin chain is found on one substrate [62–64]. However, the role of multiple poly-ubiquitin chains is unclear since a single poly-ubiquitin chain is both necessary and sufficient for efficient degradation of at least two physiological substrates, Sic1 and p21 [52,62].

Several protein domains, including the ubiquitin-associated (UBA) domain, ubiquitin interacting motif (UIM), coupling of ubiquitin conjugation to endoplasmic-reticulum degradation (CUE) domain and ubiquitin E2 variant (UEV) domain have been shown to bind to either mono-ubiquitin or poly-ubiquitin chains [14,65,66]. Proteins containing these domains are often responsible for transmitting downstream signals from ubiquitylated proteins. Alternatively, they may inhibit the formation of poly-ubiquitin chains to favour mono-ubiquitylation, possibly by binding to and blocking $Lys^{48}$ [67–71]. Ubiquitin-binding proteins such as Pus1/Rpn10/S5a, Rad23/Rhp23, Dsk2/Dph1/Plic1 and Ddi1 may protect poly-ubiquitin chains from deubiquitylating enzymes [72,73] and/or deliver them to the proteasome [74–77] or other destinations. Some ubiquitin binding proteins (UBPs) bind E3s (for example, Pus1 binds an APC subunit in fission yeast) and/or the 26 S proteasome, suggesting that they may form a direct link between the ubiquitylation and degradation machinery [78,79]. Supporting this proposal, there is evidence that the ubiquitylation machinery, including E2s and E3s such as the APC and SCF, interact with the 26 S proteasome [57,80–84].

## CATALYTIC MECHANISMS OF UBIQUITYLATION

### E1 ubiquitin-activating enzymes

Although the ubiquitin-proteasome pathway has been studied extensively, the molecular details of ubiquitin transfer remain largely unknown. Recent insight into the mechanisms of E1 enzymes has come from crystal structures of both MoeB–MoaD, and APPBP1–UBA3 [85–87]. MoeB–MoaD is a protein complex involved in the molybdenum cofactor biosynthetic pathway and has sequence and mechanistic homologies to E1 (MoeB) and ubiquitin (MoaD) [88]. MoeB activates the MoaD C-terminus (which like ubiquitin contains a C-terminal diglycine motif) by forming an acyl-adenylate intermediate. Thus, the mechanism for adenylation in the MoeB–MoaD complex parallels the activation of ubiquitin by E1. However, in contrast to the E1 reaction, a sulphurtransferase converts the MoaD acyl-adenylate to a thiocarboxylate, and a MoeB–MoaD thioester linkage is not formed. The MoeB–MoaD structure provided molecular details of the adenylation reaction: ATP binds to a nucleotide binding pocket and a conserved aspartic acid residue co-ordinates a $Mg^{2+}$ ion required for the MoaD carboxylate to attack the $\alpha$-phosphate of ATP [85]. The structure also showed that the interface between MoeB and MoaD is primarily mediated by hydrophobic interactions. These features are conserved with ubiquitin and E1 enzymes and therefore suggested a model for how ubiquitin adenylation proceeds.

APPBP1–UBA3 is the heterodimeric E1 enzyme for the ubiquitin-like protein NEDD8 (APPBP1 is homologous to the N-terminal half of the ubiquitin E1, whereas UBA3 is homologous to the C-terminal half [89]). In combination with the MoeB–MoaD structure, the APPBP1–UBA3 structure shows that the three functions of an E1 (adenylation, thioester bond formation and E2 binding) proceed in a co-ordinated 'assembly line' fashion within a single groove where ATP and NEDD8/ubiquitin bind to two adjacent clefts [86,87]. However, the mechanistic details of thioester bond formation and ubiquitin transfer to E2 remain uncertain. In an APPBP1–UBA3–NEDD8–ATP complex, the

C-terminus of NEDD8 is in the adenylation site, 35 Å away from the catalytic cysteine [87]. The flexibility of the C-terminus of NEDD8, as well as substantial rearrangements within the E1, may account for how NEDD8 can move between the two E1 active sites. A conserved threonine residue positioned 3.8 Å from the APPBP1–UBA3 catalytic cysteine may play a role in deprotonating the E1 and/or E2 catalytic cysteines [86]. Interestingly, a region of UBA3 adopts a ubiquitin-like fold and there is some evidence that this domain may be involved in E2 binding [86].

### E2 ubiquitin-conjugating enzymes

Much structural work has been performed on E2 proteins and the structure of their conserved core domain (approx. 150 amino acids) is well established as a central $\beta$-sheet with flanking helices (reviewed in [45,90]). The catalytic cysteine lies in a shallow groove and the E2 structure appears to be relatively inflexible since few structural changes are observed between isolated E2s, E2–E3 complexes and E2–substrate complexes [91–94]. NMR studies have shown that the C-terminal tail of the thioester-linked ubiquitin rests in the shallow groove leading to the active site cysteine [95,96]. The instability of E2–ubiquitin (and E1–ubiquitin) thioester complexes has precluded more detailed structural studies of thioester bond formation. Thus, despite extensive biochemical and structural analysis, the mechanistic details of ubiquitin thioester bond formation and transfer to substrate are unknown. In particular, for isopeptide bond formation, one would expect to find a general base to deprotonate the attacking lysine (Figure 3A) as well as residues that could stabilize the negative charge on the tetrahedral intermediate (Figure 3B). Structural studies did not reveal any obvious catalytic groups near the catalytic cysteine of E2s. Thus it was presumed that any catalytic groups must reside in the less-well characterized E3 'active site' (Figure 3) or that the ubiquitin transfer reaction proceeds spontaneously when the substrate and E2–ubiquitin thioester are correctly positioned.

Recent re-examination of E2 structures led to the proposal that a strictly conserved E2 asparagine residue may participate in the reaction [97]. The amide side chain of this asparagine participates in a hydrogen-bond network in E2 structures, suggesting a structural role for this residue. However, Wu et al. [97] present a convincing argument that this residue plays a catalytic role in isopeptide bond formation, specifically to stabilize the oxyanion intermediate. They show that in several different E2/E3 systems, the conserved asparagine is required for ubiquitin transfer from E2 to substrate, but not from E1 to E2 or from E2 to HECT E3 (for homologous to E6-AP carboxy-terminus; a type of E3 which forms an intermediate thioester bond with ubiquitin). This proposal relies on a change in side-chain orientation to reposition the asparagine. It is possible that such allosteric activation occurs only after the E2 has been conjugated to ubiquitin, in a manner reminiscent of the ubiquitin-mediated activation of HAUSP (herpes-associated ubiquitin-specific protease). HAUSP is an enzyme that specifically deubiquitylates p53. Its active site undergoes drastic conformational changes upon ubiquitin binding to realign the catalytic residues into an active conformation [98]. Supporting a similar event in E2s, NMR studies of the E2 Ubc1 suggest that several residues outside the E2–ubiquitin binding surface (including the conserved asparagine) experience perturbations upon E2–ubiquitin thioester formation [96].

Crystal structures of two E3 ligases (E6–AP and c-Cbl) bound to the E2 UbcH7 (described below) show that the specificity of the E2–E3 interaction is dictated by only a few amino acids [91,92]. Both E3s, which are mechanistically and structurally distinct, interact with the E2 in the same manner, using a hydrophobic groove to bind two loops at the end of the E2 $\beta$-sheet (Figures 4 and 5).

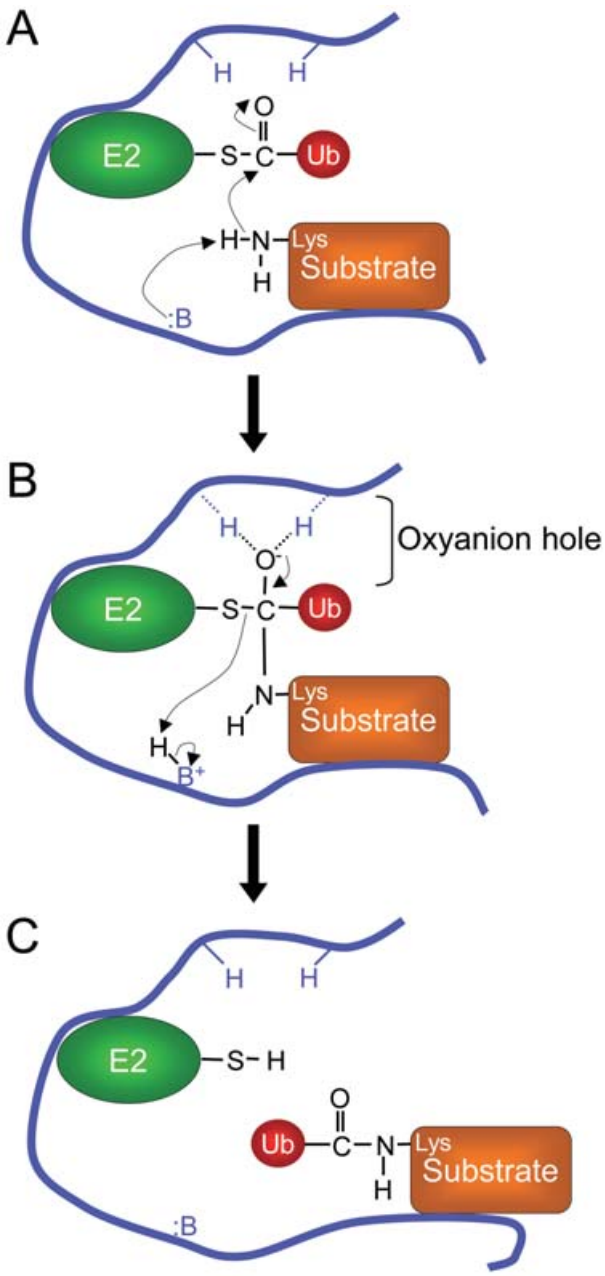


**Figure 3 Proposed mechanism for ubiquitin transfer**

The E3 ligase (shown in blue) has several possible roles. Firstly, it is known to bind both E2 (green) and substrate (orange), and is thought to position them in optimal orientations. Secondly, the E3 may provide a general base to deprotonate the acceptor lysine (**A**). The acceptor lysine is shown here as a substrate lysine; alternatively, it could reside on another ubiquitin (Ub). Thirdly, the E3 may stabilize the negative charge on the oxygen using an oxyanion hole (**B**). Finally, the E3 may reposition the substrate so $Lys^{48}$ of ubiquitin is in the active site and prepared for reaction with a recharged E2 (**C**). Structures of E3s indicate that there are no E3 residues close to the catalytic cysteine of the E2. Therefore, catalytic groups may originate in the E2 or other proteins, or ubiquitin transfer may occur spontaneously due to the highly labile thioester bond.

### E3 ubiquitin ligases

E3 ligases impart selectivity and regulation on the ubiquitylation process by mediating the transfer of the E2-conjugated ubiquitin

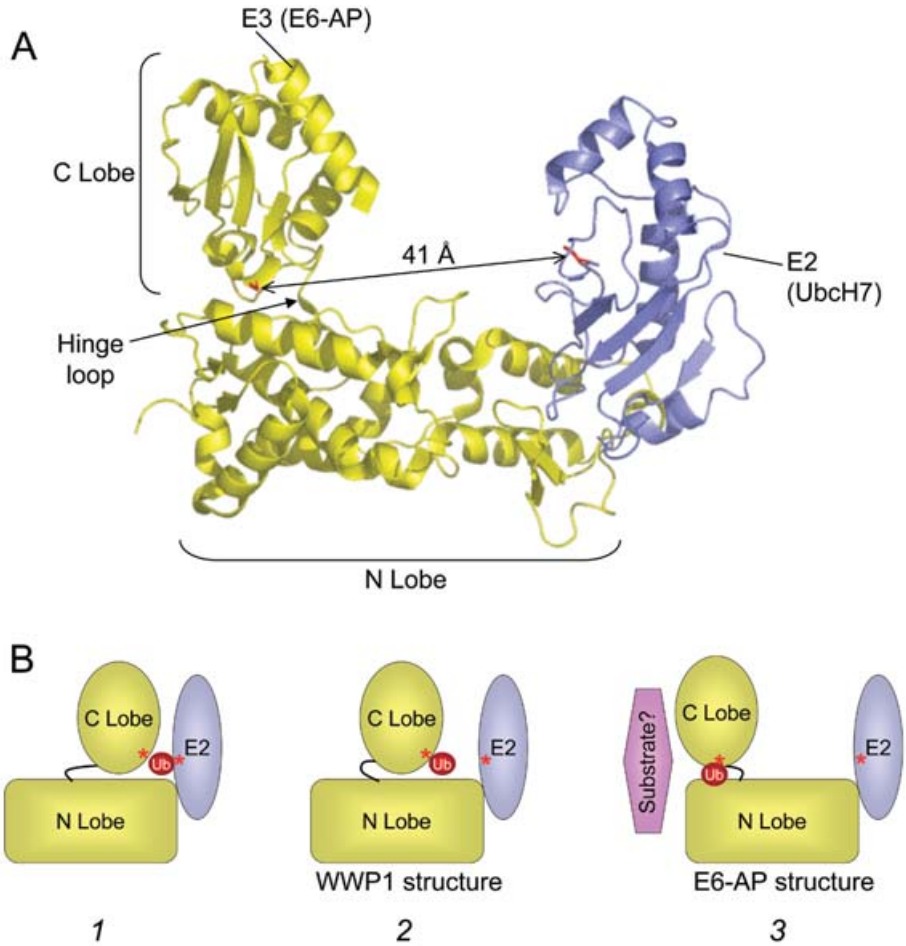


**Figure 4 HECT E3 structures and proposed mechanism**

(**A**) Structure of the E6–AP–UbcH7 complex (Protein Database accession code 1C4Z). The HECT domain of E6-AP (an E3) is shown in yellow, UbcH7 (an E2) is shown in purple and the two active-site cysteines are shown in red stick representation. (**B**) Schematic diagram of the flexibility in HECT E3s. Ubiquitin is shown in red, the HECT E3 is shown in yellow and the E2 is shown in purple. The catalytic cysteines are represented by red asterisks. On the left (1), the C Lobe of the HECT E3 is positioned to accept ubiquitin from the E2 whereas on the right (3; the conformation of the E6-AP structure), it is positioned to transfer the ubiquitin onto substrate. An intermediate between these two extreme states is shown in the centre (2) and is representative of the conformation of the WWP1 structure (Protein Database accession code 1ND7). The proposed site of substrate binding in E6-AP is shown [103].

molecule to substrate proteins specifically recognized by the E3. The E3, in combination with the E2, is also important in determining the topology of the poly-ubiquitin chain. E3 ligases are grouped into two mechanistically distinct categories based on the presence of either a HECT domain or a RING (for really interesting new gene) finger. The minimal role of an E3 ligase is to place the activated ubiquitin in close proximity to lysines of specific substrates. As discussed below, differences in the mechanism of HECT and RING finger E3 ligases suggest that whereas HECT E3 ligases also provide a catalytic contribution, the primary role of a RING finger E3 ligase is to function as a molecular scaffold.

### HECT E3 ubiquitin ligases

The conserved 350 amino acid HECT domain is found in a subset of E3 ligases. HECT E3s form an intermediate thioester bond with ubiquitin using a conserved cysteine residue [99–101]. A crystal structure of the HECT E3 E6-AP complexed with the E2 UbcH7 (Figure 4A) provided the first view of an E3 structure [91]. The HECT domain of E6-AP is composed of two lobes (N and C lobes) which pack together to form an 'L' shape. The E2 binds to the N lobe so that the overall E2–E3 complex is U-shaped with the catalytic cysteines of E6-AP and UbcH7 on opposite sides, approx. 41 Å apart. The catalytic mechanism of ubiquitin transfer is not obvious from this structure and it is unclear how ubiquitin would be transferred from the E2 cysteine to the E3 cysteine.

A recent structure of another HECT E3, WWP1/AIP5, has provided further insight [102]. Instead of adopting an L shape, the HECT domain of WWP1 has the shape of an inverted T (⊥) with the C lobe packing against the middle of the N lobe. The structure within each lobe is conserved with the E6-AP HECT domain. Verdecia et al. [102] show that the flexibility of a hinge loop connecting the N and C lobes is required for catalytic activity and suggest that the five C-terminal residues (which are not present in their structure but are required for ubiquitin transfer [99]) may contain the elusive general base which deprotonates either the HECT catalytic cysteine or the acceptor lysine during ubiquitin transfer. This new structure suggests two alternative mechanisms of ubiquitylation. First, in the 'sequential addition' model, the C lobe picks up a ubiquitin moiety from the E2, rotates around the hinge loop, transfers the ubiquitin onto the substrate and adds additional ubiquitins in a sequential manner. Alternatively, the 'indexation' model proposes that the flexibility of the hinge

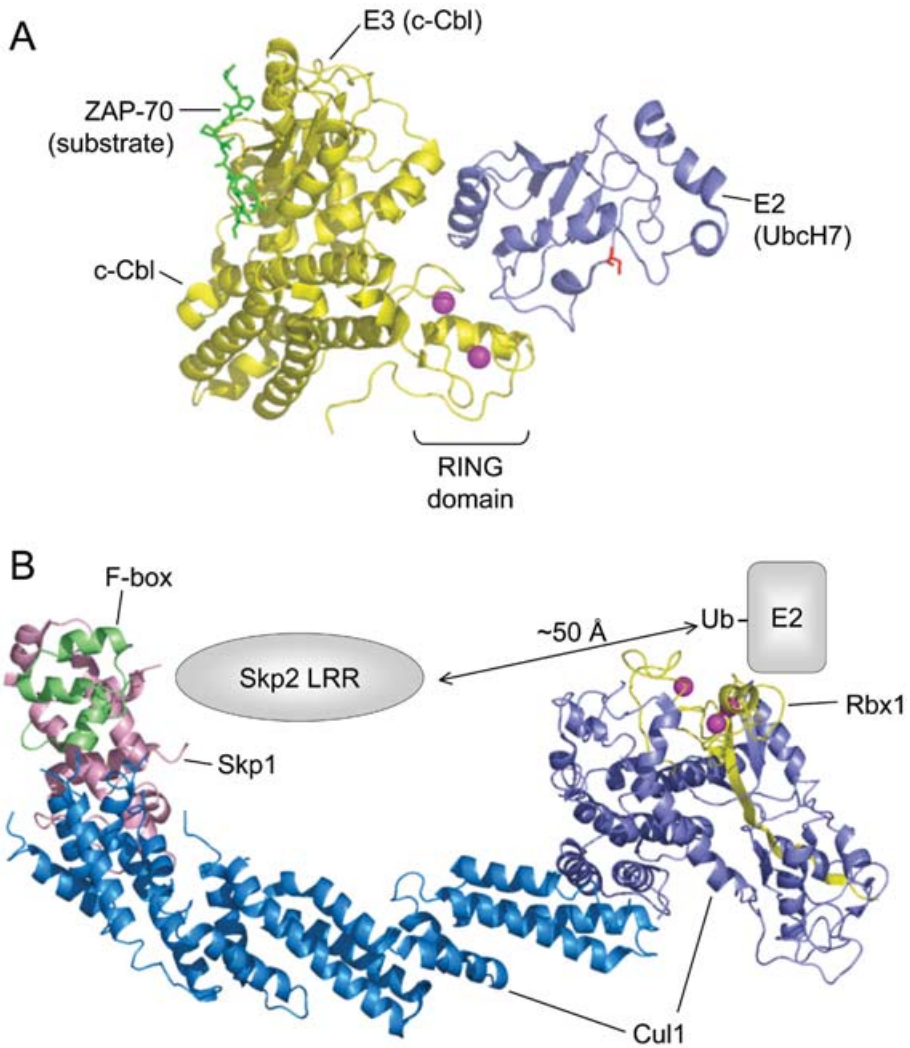


**Figure 5 RING E3 structures**

(**A**) Structure of the UbcH7–c–Cbl complex (Protein Database accession code 1FBV). UbcH7 (an E2) is coloured purple and its active-site cysteine is shown in red, c-Cbl (a single-subunit RING E3) is coloured yellow, the zinc ions are magenta spheres and a ZAP-70 substrate peptide is in green. (**B**) Structure of the Cul1–Rbx1–Skp1–F–box complex (Protein Database accession code 1LDK), a multi-subunit RING E3. Rbx1 (the RING finger protein) is yellow, the zinc ions are magenta spheres, Cul1 C-terminal domain (the cullin homology domain) is purple, Cul1 N-terminal domain is cyan, Skp1 is pink and the F-box of Skp2 is green. The predicted positions of the Skp2 leucine-rich repeats (LRR) and the E2 are shown, based on structures of a Skp1–Skp2 complex [143] and UbcH7–c-Cbl [92].

loop allows WWP1 to move in a ratchet-like fashion to first form a *tetra*-ubiquitin chain on itself before transferring it to the substrate. The HECT catalytic cysteine is still approx. 16 Å from the proposed site of the E2 cysteine in the WWP1 structure, so additional movements would be required and the WWP1 and E6-AP structures do not represent the extreme endpoints of movement (Figure 4B). Both of these models require substantial movements in the hinge loop, and if this flexibility is a general property of E3 ligases, it would help to explain how they are able to adapt to a constantly changing substrate [102,103].

### RING E3 ubiquitin ligases

All non-HECT E3 ligases, including c-Cbl, APC and SCF, promote the transfer of ubiquitin without forming a covalent intermediate and so far, all of them contain a RING finger domain or a structurally-related domain such as a U-box domain [104–106]. In fact, many isolated RING (and RING-related) domains possess an E2-dependent activity to form poly-ubiquitin chains, either on substrate proteins or on themselves [106,107]. It is unknown how many RING domains have E3 ligase activity; the RING domain is the fifteenth most common domain in the human genome and the twelfth most common in yeast [108]. It contains eight highly conserved $Zn^{2+}$-co-ordinating residues, which bind two $Zn^{2+}$ ions in a 'cross-brace' arrangement [109]. U-box domains have the same fold as the RING domain although the co-ordinated zinc ions are replaced by hydrogen bonds [110].

Structures of RING E3s show that the RING domains interact directly with E2s (Figure 5) [92,111,112]. It was originally assumed that RING fingers contained catalytic groups that participated in ubiquitin transfer (Figure 3). A role for the zinc ions in stabilizing the oxyanion in the tetrahedral intermediate for lysine ubiquitylation (Figure 3B) was proposed, since polycations or zinc ions can stimulate E3-independent, E2-dependent ubiquitin transfer [113,114]. However, in the structures of known E2–E3 complexes, the RING finger is not positioned adjacent to the catalytic cysteine of the E2, and is unlikely to be involved in catalysis [90,92]. Alternatively, RING fingers could allosterically activate

**Table 1 Components of cullin-ring E3 ligases**

BTB, broad complex, tramtrack, bric-a-brac; Doc, destruction of cyclin B; POZ, poxvirus and zinc finger.

| | APC | SCF (SCF1)* | CBC-Rbx1 (SCF2/5) | BTB-Cul3-Rbx1 (SCF3) | SCF4 | SCF7 |
|---|---|---|---|---|---|---|
| Proteins with domains commonly found in E3s | | | | | | |
| RING finger | Apc11 | Rbx1/Roc1/Hrt1 | Rbx1/Roc1/Hrt1 | Rbx1/Roc1/Hrt1 | Rbx1/Roc1/Hrt1 | Rbx1/Roc1/Hrt1 |
| Cullin domain | Apc2 | Cul1/Cdc53 | Cul2 or Cul5 | Cul3 | Cul4 (4A, 4B) | Cul7 |
| Doc domain | Doc1/Apc10 | – | – | – | – | (Cul7)† |
| Other structural and/or adaptor proteins | | | | | | |
| Adaptor proteins | – | Skp1 | Elongin C, Elongin B | BTB/POZ domain proteins‡ | ? | Skp1 |
| TPR motifs | Cdc16/Apc6/Cut9, Cdc27/Apc3/Nuc2, Cdc23/Apc8/Cut23, Apc7§ | – | – | – | – | – |
| Substrate-binding proteins | | | | | | |
| | ? | F-box proteins (eg. Skp2, Cdc4) | BC-box proteins (SOCS box) | BTB/POZ domain proteins‡ | ? | F-box proteins (Fbw29)¶ |
| Activator proteins | | | | | | |
| UBL proteins | – | Nedd8 | Nedd8 | Nedd8 | Nedd8 | Nedd8 |
| WD40 repeats | Cdc20/Fzy/p55$^{CDC}$, Cdh1/Hct1/Fzr or Ama1 | – | – | – | – | – |
| Proteins with unknown structure/function | | | | | | |
| | Apc1/Cut4/Tsg24, Apc4/Cut20, Apc5/Ida, Apc9§, Cdc26/Hcn1, Swm1§, Mnd2§, Apc13§, Apc14§, Apc15§ | – | – | – | – | – |

* SCF-like complexes are named SCF1, SCF2, SCF3 etc. with the number corresponding to the cullin protein [125].
† Cul7 contains both cullin and Doc domains.
‡ BTB/POZ domain proteins fulfil the roles of both adaptor and substrate-binding proteins.
§ Apc7 is present only in metazoans. Apc9, Swm1 and Mnd2 are specific to budding yeast. Apc13, Apc14 and Apc15 are specific to fission yeast.
¶ So far, only one F-box protein has been found to interact with the Cul7 complex.

E2 enzymes. However, the crystal structures of E2–E3 complexes (UbcH7–c-Cbl and Ubc13–Mms2) do not corroborate allosteric E2 activation, since there is little structural difference between bound and free E2 [92,93,115]. In addition, some E2s can assemble poly-ubiquitin chains in the absence of E3 (for example see [13]) suggesting that the E3 is not required for E2 catalytic activity.

While some RING finger proteins such as c-Cbl and Mdm2 function as single-subunit E3s, others function as part of a multiprotein complex. These multi-subunit E3s contain a RING finger, a cullin domain and other subunits, some of which may be responsible for substrate binding. For example, the SCF and related SCF-like complexes are modular E3 ligases composed of four subunits (Table 1) [104,116–128]. So far, all SCF-like complexes have been shown to share the same RING finger protein (Rbx1/Hrt1/Roc1) which binds an E2 and a cullin protein (e.g. Cul1), the cullin binds an adaptor protein (e.g. Skp1), and the adaptor protein binds a substrate binding protein (e.g. F-box protein; Figure 5B) [104,129–131]. Recently, BTB proteins were found to combine the latter two functions [124–127]. Another multisubunit E3, the APC, is much more complex than the SCF. The APC contains thirteen individual subunits (Table 1), eight of which are essential for viability in budding yeast [132–138]. One of the major questions regarding APC structure and function is the arrangement of its subunits and the reason for such complexity. A cryo-electron microscopy reconstruction of the human APC, indicates that it has a complex architecture containing an outer protein wall that adopts a cage-like shape [139]. The authors propose that the inner chamber could be the catalytic centre. Subunit localization and/or molecular detail will provide a deeper understanding of this structure.

Notably, all multisubunit RING E3s contain a cullin domain and *in vitro* experiments suggest that the cullin–RING complex recruits and activates the E2 to form ubiquitin chains, but does not provide substrate specificity [113,114,140–142]. In a Cul1–Rbx1–Skp1–F-box crystal structure (Figure 5B), Cul1 has an elongated $\alpha$-helical N-terminal domain and a globular C-terminal region containing the cullin homology domain [112]. Cul1 and the RING finger Rbx1 are intimately associated through two mechanisms: first Cul1 and Rbx1 form an intermolecular $\beta$-sheet and, secondly, the cullin homology domain forms a V-shaped groove where the RING domain binds. Rbx1 has an insertion within its RING finger to form a third zinc co-ordination site and, although the function of this third zinc is unknown, it may be important since it is also present in the APC [112,114]. Modelling of E2 and Skp2 into the Cul1–Rbx1–Skp1–F-box structure suggests that the SCF is a Cul1-based scaffold whose main function is to correctly position ubiquitin-charged E2 bound by Cul1–Rbx1, and substrate bound by Skp2 [92,112,143].

## A QUESTION OF POSITIONING?

Instead of providing a catalytic function, the favoured view is that RING E3s act as scaffolds to bring ubiquitin-charged E2 and substrate into close proximity [45,90–92,94,109,112]. In support

of this idea, many substrates can be ubiquitylated on any lysine within an acceptable distance, negating the need to precisely position specific acceptor lysines [45,46,62,144,145]. Indeed, the combination of several structures of SCF subcomplexes suggests that the role of a RING E3 is to increase the effective concentration of lysines in the vicinity of the E2 active site [92,112,143,145,146]. Furthermore, in the only two structures of RING E3s (c-Cbl and SCF), the substrate binding sites are distant from the E2 ubiquitylation sites (approx. 60 Å and 50 Å respectively) and there is a distinct lack of E3 residues in close proximity to the reaction site (Figure 5) [92,112].

Examination of the structures of other E3-like proteins can give further insight into the mechanisms of E3s. Structures of Mms2–Ubc13, a complex of an E2 (Ubc13) and a UEV-containing protein (Mms2), also support a non-catalytic role for E3s [93,115,147]. This complex catalyses the formation of unanchored (i.e. unattached to substrate) $Lys^{63}$-linked poly-ubiquitin chains (in which case Mms2 plays an E3-like role) or, in combination with a RING E3, substrate-linked $Lys^{63}$ poly-ubiquitin chains [24,25,148]. Mms2–Ubc13 appears to act by providing a scaffold to optimally position the donor ubiquitin to react with $Lys^{63}$ (and not $Lys^{48}$) of an acceptor ubiquitin without providing any catalytic residues [93,115,147]. The donor ubiquitin, acceptor ubiquitin and E3 are expected to bind in three separate channels which converge at the Ubc13 catalytic cysteine residue.

Crucial insights into the mechanism of lysine ubiquitylation have been obtained by investigations of sumoylation processes and, in particular, from the crystal structure of a Ubc9–RanGAP complex [94]. RanGAP1 is sumoylated by the SUMO-conjugated E2 Ubc9, without the involvement of an E3 ligase. The catalytic site of Ubc9 shares many similarities with E2s that mediate ubiquitylation, and therefore the structure of the Ubc9–RanGAP complex serves as a model system for understanding mechanisms of lysine ubiquitylation. The lysine substrate on RanGAP is accommodated within a shallow groove leading to the catalytic cysteine of Ubc9, with the $\varepsilon$-amino group within 3.5 Å of the thiol sulphur [94]. This places the $\varepsilon$-amino group in an ideal position to attack the activated electrophilic carbon of the SUMO-E2 thioester bond. Interestingly, the structural data argue against enzyme-mediated deprotonation of the $\varepsilon$-amino group, and it is likely that the optimal orientation and proximity of the $\varepsilon$-amino group and reactive thioester bond is sufficient to promote catalysis. Significantly, residues of Ubc9 in the vicinity of the substrate lysine $\varepsilon$-amino group that have the potential to function as a general base ($Asn^{85}$, $Tyr^{87}$ and $Asp^{127}$) are not conserved with other E2s, and mutation of these residues does not abolish catalytic activity [94].

Together, all structural work on RING finger E3s to date suggests that they function to provide a platform that positions charged E2 in close proximity to the substrate. The mechanism of catalysis remains unclear since enzymic groups have not been identified (Figure 3). It appears that ubiquitin transfer may occur spontaneously when the highly labile E2–ubiquitin thioester bond is presented to a substrate lysine in a favourable conformation.

### Lysine ubiquitylation and acetylation are mechanistically related reactions

As well as being the target of ubiquitylation, lysine side chains can also be modified by acetylation and methylation, raising the interesting possibility that ubiquitylation, acetylation and methylation, which are mutually exclusive reactions, are inter-dependent in certain biological processes [149–153]. Interestingly the chemical modification of the $\varepsilon$-amino group of a lysine side chain by ubiquitylation is mechanistically equivalent to the acetylation

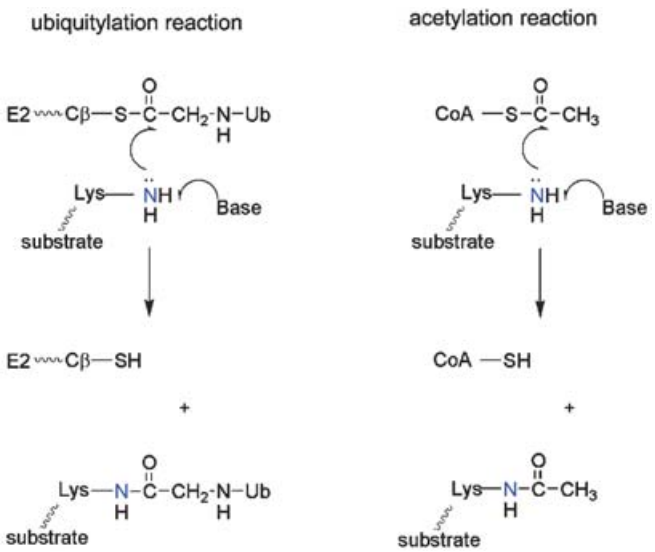


**Figure 6 Schematic of lysine ubiquitylation and acetylation reactions**

Similarities between the ubiquitylation catalysed by E2 ubiquitin-conjugating enzymes in association with E3 ligases, and acetylation reactions catalysed by histone acetyl transferases are shown.

reaction: an isopeptide bond is generated in both situations resulting from the reaction of the lysine $\varepsilon$-amino group with a high-energy thioester bond (Figure 6). This would suggest that knowledge of the catalytic mechanism and basis for substrate specificity of histone acetyl transferases (HATs) could provide insights into mechanisms of lysine ubiquitylation. HATs use acetyl-CoA as the acetyl donor for transfer to the acceptor lysine residue. In acetyl-CoA, the acetyl group transferred to the substrate lysine residue is activated via its high-energy thioester linkage to coenzyme A, exactly analogous to the activation of the C-terminus of ubiquitin by a thioester bond. There is also a sub-class of HATs where the acetyl group is first transferred from acetyl-CoA to a catalytic site cysteine residue, before transfer to the substrate lysine, directly analogous to the transfer of ubiquitin from E1 to E2 (or from E2 to HECT E3) [154]. Like the ubiquitylation reaction, the acetylation reaction proceeds via nucleophilic attack by the lysine $\varepsilon$-amino group onto the electrophilic carbon centre of the thioester bond of acetyl-CoA (Figure 6). In the structure of the *Tetrahymena* HAT GCN5 in complex with a histone H3 peptide and coenzyme A, the general base that abstracts a proton from the attacking $\varepsilon$-amino group was proposed to be a water molecule, activated via a hydrogen bond to a conserved glutamate, whereas a main-chain amide group in the protein stabilizes the transition state tetrahedral reaction intermediate [155].

Similarly to E2s, HATs are single domain proteins composed of a mixed $\alpha/\beta$-structure, with a catalytic site formed from two pronounced clefts that engage the acetyl-CoA and peptide substrate. These deep clefts, that together form the catalytic site of the HAT, are in marked contrast to the catalytic site of E2, located within a shallow surface channel. In addition, the secondary structure topology of the E2s is distinct from HATs, and there is no evidence for any evolutionary relationship between these two families of enzymes. Since a direct correspondence between the catalytic sites of HATs and E2s does not exist, the activation of the substrate lysine $\varepsilon$-amino group, and stabilization of the tetrahedral transition state intermediate in these two classes of enzymes is probably achieved via different mechanisms.

## Formation and topology of poly-ubiquitin chains

To make matters more complicated, the discussion above focuses mainly on catalysis of the initial ubiquitin–substrate bond and does not consider how the E3 is able to poly-ubiquitylate substrates. Formation of poly-ubiquitin chains of defined topology on a specific protein substrate represents a formidable problem of molecular recognition. Specific ubiquitylation of one or more lysine residues on a protein substrate is followed by ubiquitin chain elongation involving processive modification of a particular lysine residue on the growing ubiquitin chain. Since the fate of a protein depends on both its ubiquitylation state and the topology of the ubiquitin chain, specificity of both reactions is critical. How the E2/E3 complex is capable of recognizing two different substrates with high selectively is not understood. The participation of E4-like factors or multimerization of E2s or E3s may play a role in chain elongation [55,129]. Otherwise large conformational changes would be required or the E3 would have to translate down the ubiquitin chain as it is formed. There is increasing evidence that multimerization of components of the ubiquitin pathway, including E2s and F-box proteins, may be important for their functions and may facilitate the formation of poly-ubiquitin chains [129,156–161]. For example, NEDD4 (an E3) has two independent E2 binding sites, both of which are able to bind E2 and both are required for activity [162]. The SCF E2 Cdc34p forms multimers [163] and this multimerization is dependent on the formation of an E2–ubiquitin thioester bond [164]. This Cdc34 self-association is required for poly-ubiquitin chain formation [164]. Although it is clear that in some cases multimerization plays a role in protein ubiquitylation, further studies will be required to determine its importance and whether it is a general feature of ubiquitylation.

A recent study on the ubiquitylation of substrates by the SCF has suggested an intriguing alternative mechanism for the poly-ubiquitylation process. Deffenbaugh et al. [165] showed that the formation of an E2–ubiquitin thioester increases the E2–SCF dissociation rate and that release of ubiquitin-charged E2 is essential for ubiquitylation of an SCF substrate. In fact, a mutant E2 (Cdc34) that binds the SCF with higher affinity than wild-type E2 (but otherwise appears to function normally) does not ubiquitylate substrates. They propose a 'hit and run' model in which ubiquitin-charged E2 is recruited to the SCF and released in close proximity to the SCF-bound substrate. This dynamic release of E2 by E3 may explain why multiple substrate lysine residues are ubiquitylated. This model, although explaining a mechanism for poly-ubiquitin chain formation, raises the question of how specificity for $Lys^{48}$ linkages is determined.

In some instances, separate E2/E3 ligase complexes are responsible for catalysing the two components of this process; protein recognition, and ubiquitin lysine recognition. For example, the mono-ubiquitin attached to p53 by the E3 ligase Mdm2 is extended to form a poly-ubiquitin chain by the E3 ligase activity of p300 [166]. Similarly, the Ubc13–Mms2 E2 complex, in association with the E3 Rad5, catalyses formation of a $Lys^{63}$-linked poly-ubiquitin chain onto the single ubiquitin molecule attached to PCNA via the Rad6-Rad18 E2/E3 ligase activity [44]. In addition to modifying mono-ubiquitylated PCNA, the Ubc13–Mms2 complex participates in the nuclear facton-$\kappa$B signalling pathway, catalysing $Lys^{63}$-linked poly-ubiquitylation of TRAF2 [28,167]. Significantly, Ubc13–Mms2 is capable of synthesizing $Lys^{63}$-linked poly-ubiquitin chains *in vitro* without an E3 ligase [24], demonstrating that specificity for ubiquitin's $Lys^{63}$ resides in the E2 itself. These data, together with the Ubc13–Mms2 crystal structure described above, suggest that at least part of the specificity for $Lys^{63}$ ubiquitin chain topology is imparted by the E2 itself, and this may represent a general property of poly-ubiquitylation reactions including $Lys^{48}$-linked ubiquitin chains. In other poly-ubiquitylation reactions, such as those catalysed by E2–SCF complexes, both substrate recognition and chain elongation are mediated via the same E2–E3 complex [132]. A survey of the functions of E2s suggests that their activities are usually restricted to a certain class of ubiquitylation reaction (i.e. mono-, $Lys^{48}$- or $Lys^{63}$-linked poly-ubiquitin chains [45]). It is therefore likely that while specificity for the target protein is conferred by the E3 ligase, synthesis of a specific poly-ubiquitin linkage will be determined from a combination of the E2 and E3.

## Concluding remarks

Protein ubiquitylation clearly plays a fundamental role in cellular function. While $Lys^{48}$-linked poly-ubiquitin chains are required for controlled protein degradation, other ubiquitin modifications have diverse functions in numerous cellular processes. In fact, it is now becoming evident that ubiquitylation may be as pervasive as phosphorylation as a mechanism for controlling protein function. Importantly, post-translational modifications are highly interdependent processes. Phosphorylation is known to regulate most ubiquitylation events, and likewise ubiquitylation plays an essential role in regulating cell cycle kinases and activation of the I$\kappa$B kinase. As a testament to the importance of ubiquitylation, the number of E3 ubiquitin ligases is expected to be extremely large. There are over 200 RING-finger-containing genes in the human genome [108]. It remains to be determined how many of these function as E3 ubiquitin ligases but in a random screen of six RING fingers, all were able to catalyse the formation of poly-ubiquitin chains [107]. Despite the increasing number of studies, details of the catalytic mechanisms of ubiquitylation are still unresolved. In particular, the role of E3 ubiquitin ligases and how they adapt to a constantly changing substrate in order to maintain a specific poly-ubiquitin chain topology is a mystery. As has been suggested previously, the only function of the RING finger E3 may be to provide a scaffold, bringing ubiquitin-charged E2 and substrate into close proximity. It is also possible that E3 flexibility, dynamic E2 binding and/or the formation of multimers is key to their function. Resolving these questions by conventional structural analyses, such as X-ray crystallography and single particle cryo-electron microscopy, presents considerable challenges, and will require the application of time-resolved studies to capture enzymic intermediates. However, elucidation of the catalytic mechanism of ubiquitylation will give considerable insight into the regulation of many diverse cellular pathways, and provide opportunities for the development of drugs that modulate specific ubiquitylation pathways to either promote or suppress protein degradation.

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