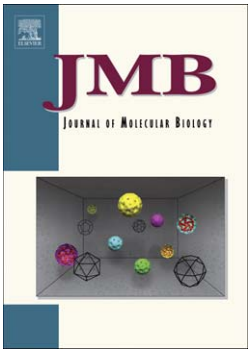


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# The N-terminal extension of UBE2E ubiquitin conjugating enzymes limits chain assembly

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**Abstract**

Protein ubiquitylation depends upon the concerted action of ubiquitin conjugating enzymes (E2s) and ubiquitin ligases (E3s). All E2s have a conserved ubiquitin-conjugating (UBC) domain but many have variable extensions N- and C-terminal to the UBC domain. For many E2s the function of the extension is not well understood. Here we show that the N-terminal extension of the UBE2E proteins regulates formation of polyubiquitin chains by the processive UBC domain. Target proteins are therefore monoubiquitylated by full-length UBE2E, whereas the UBC domain alone polyubiquitylates proteins. Although the N-terminal extension of UBE2E1 is largely disordered in solution, these residues have a critical role in limiting chain building and when fused to the highly processive E2, UBE2D2, ubiquitylation is limited. For some E2s, interaction of ubiquitin with the 'backside' of the UBC domain promotes polyubiquitylation. However, interaction of ubiquitin with the backside of the UBC domain of UBE2E1 does not appear to be important for processivity. This study underscores the importance of studying full-length E2 proteins and not just the highly conserve core domain.

**Keywords**

E2; E3; RING; ubiquitin; chain formation; autoinhibition

**Abbreviations**

APC; Anaphase promoting complex, CARP2; caspase-8 and -10 associated RING protein 2, cIAP2; cellular inhibitor of apoptosis protein 2, C-terminal; carboxy-terminal, E1; Ubiquitin activating enzyme, E2; ubiquitin conjugating enzyme, E3; ubiquitin ligase, IDPR; intrinsically disordered protein region, K0; Lysine-zero, MALS; Multi-angle laser light scattering, MDM2; mouse double minute 2, N-terminal; amino-terminus, SEC; size exclusion chromatography, UBC; ubiquitin-conjugating domain, WT; wild type

**Introduction**

Protein ubiquitylation has a central role in determining protein abundance and in controlling protein interactions. Addition of ubiquitin to substrate proteins depends on a cascade of conserved enzymes.[1] First, the ubiquitin-activating enzyme (E1) activates ubiquitin, resulting in formation of a thioester link between the carboxyl group at the C-terminus of ubiquitin and the catalytic cysteine in the E1. Ubiquitin is then transferred to the catalytic cysteine of the ubiquitin-conjugating enzyme (E2). The third step, which depends on the ubiquitin ligase (E3), results in the formation of an isopeptide bond between the C-terminus of ubiquitin and an amide group, normally a lysine side chain, in the substrate.[2] The hierarchical activity of these enzymes serves to activate ubiquitin; to identify a substrate protein; and to catalyse conjugation to the target.

Substrate proteins can be modified by a single ubiquitin molecule (monoubiquitylation), or by the addition of chains of linked ubiquitin molecules (polyubiquitylation). Polyubiquitin chains are built by the conjugation of one ubiquitin molecule (the donor) to one of the seven lysine residues in the ubiquitin molecule that has already been attached to the substrate (the acceptor).[3] The conformation of the ubiquitin chain, and consequence of the modification, depends on the lysine residue that links the ubiquitin molecules. E2s have a central role in specifying the type of modification.[4] Humans have ~35 E2s, with some E2s able to promote attachment of the first ubiquitin molecule and to build chains, whereas other E2s are only capable of either initiation or chain elongation.[4] The molecular interactions required for chain elongation are not well understood, although several studies have indicated that non-covalent interactions between the E2 and ubiquitin are important.[5; 6] In the case of the promiscuous and widely studied UBE2D family, interaction of ubiquitin with a non-covalent binding site on the opposite side of the E2 to the catalytic site (referred to as the 'backside'), increases chain formation.[6; 7] More recently, a role for interaction between the donor ubiquitin molecule and the E2, UBE2S, has been proposed to promote chain elongation.[5] In this case it is thought that the donor ubiquitin is tethered so that nucleophilic attack by Lys11 (K11) of the acceptor ubiquitin is favoured. This interaction is weak, and could not be

detected by conventional approaches, yet the authors propose that non-covalent interaction of the E2 with ubiquitin is a common feature of processive E2 enzymes.

E2 enzymes lie at the heart of the ubiquitylation cascade and it is not surprising that all E2s have a conserved core ubiquitin conjugating (UBC) domain of ~150 residues. This domain contains the catalytic cysteine to which ubiquitin is attached, as well as conserved features required for interaction with both E1 and E3 proteins.[8] Some E2s have just the core domain and these are referred to as Class I, while others have extensions beyond the core domain.[9] Those with a C-terminal extension are referred to as Class II, an N-terminal extension defines Class III proteins, and those E2s with both N- and C-terminal extensions belong to Class IV. For all E2s the core domain is essential for activity, but several studies have suggested that the N- and C- terminal extensions can modulate ubiquitin transfer. In the case of UBE2C (UbcH10) deletion of the N-terminus resulted in more promiscuous ubiquitin transfer by its cognate E3, APC, leading to the inappropriate ubiquitylation and destruction of substrate proteins.[10] For UBE2R1 (CDC34) the C-terminal extension is required for the formation of polyubiquitin chains,[11] and recent studies suggest this may be due to interaction of the C-terminal residues with the conjugated ubiquitin molecule.[12] These studies highlight the importance of studying the intact E2 proteins, not just the core domain.

Here, we have characterised the UBE2E family of E2s that have an N-terminal extension.[13] The three members of the UBE2E subfamily; UBE2E1, UBE2E2 and UBE2E3 have a highly conserved UBC domain (92% identity), but the N-terminal extension is variable (Fig. 1a). Here we show that the intrinsically disordered N-terminal extension of UBE2E proteins limits formation of ubiquitin chains. In our assays, the full-length UBE2E proteins primarily promote protein monoubiquitylation, whereas the core UBC domain of each can efficiently build polyubiquitin chains.

## Results

*The N-terminal extension of Class III E2s limits ubiquitin transfer*

To investigate the role of the N-terminal residues in UBE2E1 (also known as UbcH6), full-length UBE2E1 ($E2E1^{full}$) and the UBC domain of UBE2E1 ($E2E1^{core}$) were expressed and purified (Figs. 1a and 1b). Initially, the activity of the E2s was assessed with cIAP2, an E3 ligase with a RING domain at its C-terminus.[14; 15] We chose cIAP2 because a protein that comprises the BIR3, UBA, CARD and RING domains (residues 255-604) is readily purified, and it is autoubiquitylated when incubated with UBE2E1 and UBE2D2, which have similar UBC domains (Supplementary Fig. S1a). To compare the activity of different E2 proteins we used purified proteins and *in vitro* assays. Under comparable conditions, $E2E1^{core}$ promoted extensive autoubiquitylation of cIAP2, whereas $E2E1^{full}$ only promoted the addition of 1-2 ubiquitin molecules to cIAP2 (Fig. 1c). Although neither form of UBE2E1 was as active as the highly processive UBE2D2, this result suggested that the N-terminal residues in UBE2E1 limit cIAP2 autoubiquitylation.

To determine if reduced ubiquitin transfer by the full-length protein depended on a specific interaction with cIAP2, or if it was an intrinsic property of UBE2E1, we also assessed ubiquitin transfer together with the E3s, MDM2 and CARP2 (Fig. 1d). The RING domains of cIAP2, MDM2 and CARP2, fused to GST, were used in assays because the isolated RING domains exhibit limited autoubiquitylation, in part due to the absence of lysine residues. GST has 26 lysine residues and the RING domains of MDM2 and CARP2 promoted autoubiquitylation of the GST fusion protein when incubated with $E2E1^{core}$. However, as observed for cIAP2, the ladder due to the addition of ubiquitin is diminished for $E2E1^{full}$ (Fig. 1d). Therefore, attachment of ubiquitin to target proteins by UBE2E1 appears to be impeded.

UBE2E1 belongs to a subfamily of E2s that all have a highly conserved UBC domain, but variable N-terminal extensions (Fig. 1a). To determine if the N-terminal extension of the related E2s, UBE2E2 and UBE2E3, also modulated ubiquitin transfer, comparable core and full-length proteins were purified and characterised. For both UBE2E2 and UBE2E3 the core UBC domain promoted the formation of high molecular weight species that ran near the top of the gel (Fig.

1e). In contrast, the ladders were diminished when full-length UBE2E2 and UBE2E3 were included (Fig. 1e).

These results suggest that the highly conserved UBE2E core domain is capable of promoting extensive ubiquitylation of target proteins. However, the N-terminal extension of all three UBE2E proteins limits efficient ubiquitin transfer.

*Class III E2E proteins are monomeric*

To understand the molecular basis of restricted ubiquitin transfer by full-length UBE2E1, UBE2E2 and UBE2E3, we initially analysed the available structures of full-length UBE2E1 and the core domain of UBE2E2.[16] The core UBC domains of both UBE2E proteins are very similar (Supplementary Fig. 2a) and not surprisingly they resemble other E2s, such as UBE2D2.[16] However, the structure of full-length UBE2E1 (Fig. 2a) has two important differences; (i) helix 1 ($\alpha$1) is extended by two turns, and (ii) residues 21-27, which are part of the N-terminal extension, are resolved. Residues 21-27 interact with the sidechains of Arg116, Asn125 and Gln127, as well the C-terminal carboxyl group of Thr193 on the $\beta$-sheet in the UBC domain. As a consequence the biological unit for full-length UBE2E1 is reported to be a dimer, with dimerisation mediated by interaction of residues 21-27 from one molecule with the core domain of another molecule, as well as by contacts between $\alpha$1 of the two interacting molecules (Fig. 2a). In contrast, like other E2s, the UBC domain of UBE2E2 is a monomer.

To characterise the oligomeric state of E2E1$^{full}$ and E2E1$^{core}$ in solution we analysed the purified proteins using size exclusion chromatography coupled to a multi-angle light scattering detector (SEC-MALS). Both forms of UBE2E1 behaved as monomers, with a calculated mass of 22.4 kDa for E2E1$^{full}$, and 18.4 kDa for E2E1$^{core}$; values that are close to the expected masses of 21.4 kDa and 17.3 kDa, respectively (Fig. 2b and S2b). Likewise, SEC-MALS analysis of purified E2E2$^{full}$ and E2E2$^{core}$ indicated that both these proteins are also monomeric (Fig. 2c and Supplementary Fig. S2c). While the mass of E2E1$^{full}$ and E2E1$^{core}$ differ by only 4 kDa, when separated on a Superdex75 column their elution points are well resolved (Fig. 2d and Supplementary Fig. S2d). When protein mass is determined by reference to protein standards, E2E2$^{full}$ elutes at a volume

expected for a dimer (~40 kDa), whereas E2E2$^{core}$ elutes at a volume consistent with a monomer. It seems likely that the early elution point of E2E1$^{full}$, combined with the crystal contacts, led the authors of the crystal structure to propose that UBE2E1 exists as a dimer. Our SEC-MALS data suggests this is unlikely, and instead UBE2E1 is a monomer. This is consistent with other E2s, such as UBE2K, which have Stoke's radii greater than expected for globular proteins, but have been shown to be monomers.[17; 18]

The early elution of the full-length UBE2E proteins from the size exclusion column could be accounted for by the presence of a disordered region in the full-length proteins as the mass of non-globular proteins, such as those that have regions of extended structure, are often poorly predicted by comparison to globular standard proteins.[19] In the crystal structure of UBE2E1 most of the N-terminal residues are missing suggesting that they were flexible (Fig. 2a) and analysis of the sequence using IUPred indicates that they have features expected for disordered regions (Supplementary Fig. S2e).[20] In support of this, the N-terminal residues of UBE2E1 were sensitive to proteolytic cleavage (data not shown), and Circular Dichroism (CD) spectroscopy (Fig. 2e) showed that the mean residue ellipticity (MRE) for E2E1$^{full}$ is decreased relative to E2E1$^{core}$. Notably, the MRE at 208 and 222 nM are reduced by ~20% in the E2E1$^{full}$ spectra, consistent with the absence of regions of $\alpha$-helical and $\beta$-sheet structure in the N-terminal 40 residues

Together, these results suggest that the N-terminal extension of UBE2E1 is intrinsically disordered and does not serve to mediate E2 dimerisation. As both E2E1$^{full}$ and E2E1$^{core}$ are monomers in solution, it seems unlikely that the oligomeric state of UBE2E1 accounts for the difference in ubiquitin transfer.

*The N-terminal residues of Class III E2E proteins limit chain building*

To investigate the mechanism by which the N-terminal residues limited ubiquitylation we first investigated if ubiquitin loading of the E2 was impeded. Using a charging assay that measures formation of the thioester linked E2~Ub conjugate we showed that E2E1$^{full}$ and E2E1$^{core}$ were both efficiently charged with ubiquitin (Supplementary Fig. S3a). Next, we investigated whether the N-terminal extension of UBE2E1 influenced either addition of the first ubiquitin, or

ubiquitin-chain building. To do this we compared cIAP2 autoubiquitylation with lysine-zero ubiquitin (K0-ubiquitin) and WT-ubiquitin, for both E2E1$^{full}$ and E2E1$^{core}$. For E2E1$^{core}$, the ladder formed with K0-ubiquitin was considerably reduced compared to that formed with WT-ubiquitin (Fig. 3, left panel). In contrast, E2E1$^{full}$ promoted the formation of comparable ladders with both WT- and K0-ubiquitin, and these ladders are similar to those formed by the core domain with K0-ubiquitin (Fig. 3, right panel). This suggests that the N-terminal residues primarily function to limit chain building by UBE2E1. When RNF4 was used as the E3 the activity of E2E1$^{core}$ was also significantly impeded with K0-ubiquitin, whereas the activity of E2E1$^{full}$ with K0- and WT-ubiquitin was similar (Supplementary Fig. S3b).

To determine if the N-terminal residues of UBE2E2 and UBE2E3 also impeded polyubiquitylation we compared their activity with WT-ubiquitin and K0-ubiquitin. As for UBE2E1, the ladders formed by the core proteins were diminished when incubated with K0-ubiquitin, whereas modification by the full-length proteins was comparable with both forms of ubiquitin (Supplementary Fig. 3c). Together, these results show that the core domain of the UBE2E subfamily is capable of promoting the formation of ubiquitin chains. However, the full-length UBE2E proteins do not efficiently promote formation of lysine-linked ubiquitin chains, and monoubiquitylation predominates.

*Characterisation of the N-terminal residues*

The N-terminal residues of UBE2C act independently of the core domain to regulate ubiquitin transfer, and when fused to UBE2D they limited the formation of higher molecular weight species.[10] To investigate if the N-terminal residues of UBE2E1 could also restrict chain formation independently of the core domain we created a chimeric E2 by fusing the 37 N-terminal residues of UBE2E1 to the highly processive E2, UBE2D2 (E2D2-NE2E1) (Fig. 4a, left panel). Following purification of E2D2-NE2E1, autoubiquitylation of cIAP2 was assessed. In assays with WT-ubiquitin, the ladder formed by UBE2D2-NE2E1 was diminished compared to that formed by UBE2D2 alone (Fig. 4a, left panel). In contrast, the activity of the two proteins was more comparable with K0-ubiquitin (Fig. 4b,

right panel). This suggests that like UBE2C, the N-terminal residues of UBE2E1 can inhibit formation of polyubiquitin chains by UBE2D2.

To identify residues within the N-terminal extension that restrict chain building we generated two additional UBE2E1 deletion proteins, missing either 10 or 20 residues from the N-terminus, referred to as E2E1$^{\Delta N10}$ and E2E1$^{\Delta N20}$ respectively. The identity of these proteins was confirmed by mass spectrometry before use in assays because E2E1$^{\Delta N20}$ migrated anomalously when analysed by SDS-PAGE (Fig. 4b). When cIAP2 autoubiquitylation was measured, E2E1$^{\Delta N10}$ was appeared to be equivalent to E2E1$^{full}$, with both E2s promoting limited modification of cIAP2 (Fig. 4b). In contrast E2E1$^{\Delta N20}$ promoted the formation of extended chains, and was more similar to E2E1$^{core}$. This suggested that the most N-terminal 10 residues do not have a critical role in restricting ubiquitin transfer by the core domain of UBE2E1. However, residues 10-20 are required to limit chain formation.

The N-terminal extensions of the three UBE2E proteins are not highly conserved, and the extension on UBE2E3 is slightly longer than that on UBE2E1 (Fig. 1a). Therefore, we generated a set of truncated proteins for UBE2E3 and assessed their activity (Supplementary Fig. S4). When the 11 N-terminal residues were removed to create E2E3$^{\Delta N11}$, ubiquitin transfer was comparable to that of full-length UBE2E3. In contrast, E2E3$^{\Delta N23}$ had an increased ability to promote cIAP2 autoubiquitylation and E2E3$^{\Delta N31}$ was comparable to the core domain, with both promoting extensive polyubiquitylation. This suggested that residues 11-31 of UBE2E3 prevent formation of ubiquitin chains by the core domain of UBE2E3. The sequences of the critical regions in UBE2E1 and UBE2E3 differ but both are rich in Ser/Thr residues and it seems likely that they may have a critical role in modulating chain building.

*Chain building*

Formation of polyubiquitin chains depends upon the linkage of two ubiquitin molecules. For this to occur, the acceptor ubiquitin must be in close proximity to the catalytic site of the E2~Ub conjugate so that one lysine, or a limited number of lysine residues, are positioned for nucleophilic attack. Although the molecular basis of chain formation is not well understood, interactions between the

acceptor ubiquitin molecule and residues surrounding the active site of E2s appear to be important for orienting the acceptor lysine so that chains of a specific linkage form.[5; 21; 22] In addition, interaction of ubiquitin with the backside β-sheet on the opposite face of the E2 to the catalytic cysteine (Fig. 5a) is important for processivity of UBE2D proteins.[6] When this interaction is disrupted, by mutation of Ser22 to Arg (S22R) in UBE2D3, ubiquitin binding and formation of ubiquitin chains is limited.[6; 7] Likewise, disruption of the equivalent Sumo-Ubc9 interaction limits SUMO chain formation.[23]

To understand the role of the 'backside' binding site in our system we first characterized ubiquitin transfer by cIAP2 and the well-characterised UBE2D2 S22R mutant (UBE2D2$^{S22R}$). With K0-ubiquitin and UBE2D2 multiple ubiquitylated species form, indicating that cIAP2 is multimonoubiquitylated (Fig. 5b). To our surprise, although disappearance of the parent cIAP2 band was comparable, the ladder formed by UBE2D2$^{S22R}$ and K0-ubiquitin was slightly diminished, suggesting that UBE2D2$^{S22R}$ had a decreased ability to multi-monoubiquitylate cIAP2. As reported by others,[6; 7] with WT-ubiquitin the UBE2D2$^{S22R}$ mutant had a significantly reduced ability to form very high molecular weight species that correspond to polyubiquitylation of cIAP2 (Fig. 5c). This suggests that in the context of cIAP2, disruption of ubiquitin binding to the 'backside' of UBE2D2 limits both multimonoubiqiutylation and polyubiquitylation.

To investigate if a similar interaction is important for formation of ubiquitin chains by the core domain of UBE2E1 we mutated the equivalent residue, Ser68, to arginine in the core domain of UBE2E1 (E2E1$^{core\text{-}S68R}$). When protein monoubiquitylation was assessed using K0-ubiquitin, E2E1$^{core}$ and E2E1$^{core\text{-}S68R}$ appeared equivalent (Fig. 5d). In addition, with WT-ubiquitin E2E1$^{core}$ and E2E1$^{core\text{-}S68R}$ promoted the formation of similar ladders (Fig. 5e). This suggests that neither mono- nor poly-ubiquitylation of cIAP2 is reduced by mutation of Ser68 in E2E1$^{core}$.

The backside of UBE2E1 and UBE2D2 are similar, and 8 of the 12 contact residues are identical, but these results suggest that they are not functionally equivalent. It seems likely that either ubiquitin does not interact with the β-sheet of UBE2E1 or the serine is not essential for binding ubiquitin. In support of the

former, Brzovic *et al* (2006) noted that they did not detect binding of ubiquitin to UBE2E1, and when we evaluated binding of ubiquitin to E2E1$^{core}$ using NMR conditions comparable to those used previously,[6] no interaction was detected (data not shown). Together these results suggest that the molecular basis for polyubiquitylation by UBE2E1 and UBE2D2 differs, and the conserved serine that is present on the 'backside' of many E2s does not have a critical role in UBE2E1. In addition, our results suggest that the 'backside' interaction in UBE2D2 enhances the multimonoubiquitylation of cIAP2, as well contributing to efficient polyubiquitylation.

**Discussion**

The precise pairing of E2 and E3 determines the type of ubiquitin modification each target protein receives. However, our understanding of ubiquitin transfer is limited, and given an E2-E3 pair, in most cases, it is not possible to predict how the target protein will be modified. In addition to the conserved α/β fold UBC domain that defines this class of proteins,[8] many E2s have N- and C-terminal extensions.[9] The role of each extension appears to differ. In the case of UBE2C the N-terminal extension limits polyubiquitylation and is required for correct substrate selection,[10] while the C-terminal extension of UBE2K, which contains a ubiquitin-associated domain (UBA), increases processivity.[24; 25] Here we show that the conserved core domain of the UBE2E family (UBE2E1, UBE2E2 and UBE2E3) can build ubiquitin chains, but the N-terminal extension prevents this activity. As a consequence the full-length UBE2E proteins preferentially monoubiquitylate substrate proteins, whereas the core UBC domain polyubiquitylates them. This observation is likely to be of broad significance because the UBE2E proteins are thought to be *hub* E2s that interact with a large number of E3 ligases.[26]

The UBC domain from UBE2E proteins is conserved (92% sequence identity) and resembles the UBC of other E2 proteins.[16] In contrast, although the sequence of the N-terminal extension of each UBE2E is highly conserved across a range of species, there is considerable variation between the three proteins, and the extension varies between 37 and 52 amino acids in length. However, the N-terminal residues of UBE2E1 are intrinsically disordered,[27] and for all three UBE2E proteins a disordered structure is predicted. Recent evidence suggests that the acidic C-terminal extension of the UBE2R (CDC34) E2s is also intrinsically disordered,[12; 28] and it is likely that other E2s will have regions of disorder.

Disordered regions are prevalent at the termini of proteins where they have a range of functions; including preventing protein aggregation and mediating protein-protein interactions.[29] Conformationally flexible regions, such as the N-terminal residues in UBE2E proteins, offer advantages to proteins because they can bind to multiple targets and they have a large capture area.[30] These features may be advantageous to E2s because ubiquitin-E2 interactions

are weak, and multiple conformations can be adopted, and in fact are required for the assembly of ubiquitin chains with different linkages.[31] Therefore, the flexible nature of the N-terminal residues in UBE2E proteins may allow these resides to inhibit the acceptor ubiquitin molecule from binding at multiple sites on the core domain. More generally, the ability of the disordered regions of E2s to inhibit the formation of ubiquitin chains is consistent with a recent study that highlighted an important role for intrinsically disordered regions in mediating autoinhibition .[32; 33]

For all three UBE2E proteins, deletion studies indicate that the N-terminal extension can limit ubiquitin chain building. Indeed, as reported for UBE2C,[10] fusion of the N-terminal extension of UBE2E1 onto UBE2D2 limits chain building. The molecular features that distinguish E2s that monoubiquitylate substrates, and those with chain building properties, are not well understood.[4; 34] However, residues surrounding the active site of the E2 have been shown to influence donor-acceptor ubiquitin interactions and influence the chain linkage. For example, mutation of residues surrounding the active site of UBE2R1 and UBE2D1 has been shown to influence chain formation.[22; 35] In the case of UBE2D1, it preferentially forms mostly K11-linked chains, but mutation of a single residue near the active site from Serine to Alanine (Ser83Ala) increases formation of K63-linked chains.[22] This indicates that the position of the acceptor ubiquitin plays an important role in specifying the ubiquitin-chain linkage. In a similar manner, noncovalent interactions between UBE2S and the donor ubiquitin molecule orients the acceptor ubiquitin molecule so that formation of K11 linked chains is promoted.[5] It is therefore possible that the N-terminal extension of UBE2E proteins prevents chain building by restricting the donor ubiquitin molecule from accessing the acceptor ubiquitin at the E2 active site. The nature of this interaction is uncertain and it will be important to determine if sequence specific features play an important role, or if just a stretch of disordered residues that have the properties of an 'entropic bristle' are sufficient to inhibit formation of ubiquitin chains.[36] Further studies will be required to define the molecular basis for inhibiting chain extension.

Ubiquitin also interacts with the β-sheet of some E2s (Fig. 5a).[8] For the UBE2D proteins, the non-covalent interaction between ubiquitin and the

backside-binding site of the E2 has been well characterized, and disruption of this interaction by introduction of a mutation (S22R) limits ubiquitin chain formation on target proteins.[6; 7; 37] These studies did not report any changes to the rates of substrate monoubiquitylation and it had been thought that 'backside' binding serves to increase the concentration of E2~Ub conjugate. However, in the case of cIAP2, which is subject to extensive multimonoubiquitylation, both multi-monoubiquitylation and polyubiquitylation are slowed upon disruption of ubiquitin binding to the backside of UBE2D2 (Fig. 5b and 5c). This suggests that the first ubiquitin attached to cIAP2 may be able to recruit an UBE2D2~Ub conjugate, and thereby enhance attachment of subsequent ubiquitin molecules. We therefore propose that interaction of ubiquitin with the backside of UBE2D proteins may act more generally to increase the efficiency of ubiquitin transfer, as opposed to only enhancing ubiquitin chain formation.

The UBC domain of the UBE2E proteins is similar to the UBE2D family (64% sequence identity, 86% similarity) and both families promote the multi-monoubiquitylation and polyubiquitylation of cIAP2. However, we could not detect an interaction between ubiquitin and UBE2E1, and mutation of the conserved serine residue in the 'backside' of the core domain does not impede ubiquitin transfer. In fact cIAP2 autoubiquitylation by the UBC domain of UBE2E1 was comparable to that of UBE2D2 that carries a mutation on its backside ubiquitin-binding site. These observations suggest that the backside of UBE2E1 is not a critical determinant of ubiquitin transfer and this may account for the decreased processivity of the UBC domain compared to UBE2D2.

The N-terminal extensions of the UBE2E family possess a number of serine residues and are intrinsically disordered, properties often associated with post-translational modification and it is possible that in a cellular setting the function of the N-terminal residues could be modulated by modification. Many proteins, including E2s, are acetylated at the N-terminus and this can modulate protein function.[38] Notably, acetylation of the Nedd8 E2, UBC12 (UBE2M), promotes interaction with the Nedd8-E3 DCN1, and as a consequence neddylation of Cullin1 is increased.[39; 40] Acetylation of the SUMO E2 Ubc9 (UBE2I) has also been shown to modulate target preference.[41] Whether the N-terminal extension of UBE2E1 is modified remains an open question, however

these studies highlight how E2 activity can be tuned in unexpected ways by post-translational modifications.

To develop a detailed understanding of the 'ubiquitin code', the behavior of E2s besides the widely studied UBE2D family, is required. Our analysis of the UBE2E proteins reveals an unexpected level of regulation by the N-terminal residues, and highlights the importance of studying the full-length E2 proteins. Future efforts will focus on understanding the molecular mechanisms by which the core domain of UBE2E proteins can promote chain formation and how this can be inhibited by the N-terminal residues.

## Materials and Methods

*Protein Constructs and Cloning*

The E3s used in this study include a truncated form of cIAP2 that includes the BIR3, CARD, UBA and RING domains (residues 255-604) (accession Q13489),[14; 15] as well as the RING domains of cIAP2 (residues 536-605), MDM2 (accession Q00987, residues 417-491) and CARP2 (accession Q8WZ73, residues 319-363). The E2s used include full-length UBE2D2 (accession P62837), UBE2E1 (accession P51965), UBE2E2 (accession Q96LR5) and UBE2E3 (accession Q969T4), as well as the core forms of UBE2E1 ($UBE2E1^{core}$; residues 38-193), UBE2E2 ($UBE2E2^{core}$: residues 47 - 201) and UBE2E3 ($E2E3^{core}$: residues 53 - 207), and other truncations as indicated, for example $E2E1^{\Delta N10}$ is a truncated form of E2E1 that lacks the 10-N-terminal residues. All proteins were expressed as GST fusions and after removal of the N-terminal tag the following residues remain N-terminal to the E2 protein; GPLSGT. In the early forms of UBE2E1 the purified proteins contained a C-terminal extension of 8 residues as a consequence of cloning. These residues were removed in later constructs and shown not to alter activity. Some E2 proteins were also expressed with a C-terminal hexa-His tag, in these constructs the following extra residues, LEHHHHHH, are present C-terminal to the E2. The UBE2D2-UBE2E1 N-terminal residue fusion protein was generated using blunt-end cloning. Residues 1-46 of UBE2E1 precede UBE2D2, and Met1 of UBE2D2 is not coded for. Mutants were generated using QuikChange mutagenesis and confirmed by sequencing.

*Protein Expression and Purification*

All proteins were over-expressed in *E.coli* BL21(DE3). Untagged yeast E1 (accession P20973) was expressed using auto-induction conditions [42]. The cells were lysed in 50 mM Tris pH 8 and the cleared supernatant was bound, in the presence of ATP and $MgCl_2$, to ubiquitin-affinity-resin and following washing purified E1 was eluted by addition of 5 mM DTT. Untagged ubiquitin and lysine zero (K0)-ubiquitin were expressed and purified as described.[43] Briefly, following cell lysis ubiquitin was purified from the clarified lysate using a cation exchange column equilibrated at pH 4.5. Ubiquitin was eluted from the column

at ~350 mM NaCl and the eluted fractions containing ubiquitin were purified further by size exclusion chromatography (SEC).

E3 proteins were expressed at 18°C for 16 hours following addition of 0.1 mM IPTG. Cells were harvested by centrifugation and lysed by sonication. GST-tagged proteins were purified initially by batch affinity chromatography to glutathione sepharose. The GST-tag was then removed using *PreScission* protease and the soluble E3 was purified by SEC using either a Superdex75 or Superdex200 column (GE Healthcare) that had been equilibrated in 1x PBS. 6 x His-tagged proteins were bound to nickel charged 1 mL IMAC columns (GE Healthcare), and eluted by an increasing concentration of imidazole. These proteins were further purified using a Superdex75 column (GE Healthcare) in 1x PBS.

*SEC-MALS analysis*

Purified protein at a concentration of 300 μM was applied to a Superdex75 HR 10/30 column (GE Healthcare) equlibrated in 1x PBS that was coupled in line with a multi angle light scattering (MALS) detector (Wyatt Techonology). Refractive index and light scattering were measured and the data was analysed using ASTRA V software after calibration with a BSA standard. The molecular mass of the monomer was determined using the *ProtParam* online tool.[44]

*Analytical SEC*

Protein standards A-D (67 kDa, 43 kDa, 25 kDa, 13.7 kDa) were applied to a Superdex75 HR 10/30 column equilibrated in 1x PBS. The column void volume was determined by the elution volume of a <200 kDa protein. The void volume was subtracted from the elution volume of the protein standard and plotted against $\log_{10}$ of the molecular mass of the known standards, generating a linear curve. The molecular mass of unknown proteins was then estimated based on their elution volume.

*Circular Dichroism*

The far-UV spectra of UBE2E1 proteins were recorded using an Olis DCM-10 CD Spectrophotometer and a 1 mm quartz cuvette at 20°C. Protein samples at a

concentration of 50 μM in 20 mM Na Phosphate, pH 7.5, 25 mM NaCl were analysed. Measurements were recorded at 1 nm intervals with a 3 s integration time. For each sample the scan shown represents an average of three scans after subtraction of the buffer only measurement.

*Ubiquitylation assays*

Ubiquitylation assays were performed with purified protein components. The final concentration of each protein was; E1 at 5 nM, E2 at 5 μM, E3 at 5 μM and Ub at ~50 μM. Assay reactions also contained, 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$, 5 mM ATP, 2 mM DTT and were incubated at 37°C for the time indicated. Samples were rotated if resin-bound protein was present to ensure mixing of assay components. Reactions were stopped by the addition of 4x Laemmli buffer and analyzed by Coomassie staining after separation by SDS PAGE, or when indicated following gel transfer and immunoblotting.

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**Figure Legends**

Fig. 1.

The N-terminal residues of UBE2E proteins restrict ubiquitin transfer. (a) Sequence alignment of human UBE2E1, UBE2E2 and UBE2E3 generated using ClustalW. For the core domains only sequence differences relative to UBE2E1 are indicated. The start of the core constructs is indicated. (b) Schematic representation of the E2E1$^{full}$, E2E1$^{core}$ and E2D2 proteins used in this study. (c) Autoubiquitylation of cIAP2 was used to compare the activity of UBE2D2, E2E1$^{full}$ and E2E1$^{core}$. Purified cIAP2, E1, ubiquitin, and the indicated E2 were mixed in ATP-containing assay buffer and incubated at 37˚C for the time indicated. Reactions were stopped by addition of Laemmli buffer and visualized by Coomassie Blue staining of SDS-PAGE. (d) E2E1$^{full}$ has a reduced ability to promote the autoubiquitylation of GST-cIAP2 RING, GST-MDM2 and GST-CARP2. The assays were as described in (c) except an anti-ubiquitin antibody was used to visualize the upper panel. (e) The activity of equivalent core and full-length versions of the related E2s, UBE2E2 and UBE2E3 was also assessed by monitoring the autoubiquitylatin of cIAP2.

Fig. 2

UBE2E1 is a monomeric protein with a flexible N-terminal extension. (a) The structure of UBE2E1 (PDB:3bzh) is shown as a ribbon, with one molecule coloured grey and the other cyan. The surface is also shown for the grey molecule. The termini of each chain are indicated. (b) SEC MALS analysis of E2E1$^{full}$ indicates that this protein is a monomer (observed mass of 22.4 kDa; predicted mass of 21.4 kDa). A 300 μM sample of E2E1$^{full}$ was separated on a 24 mL S75 column coupled to a Wyatt Dawn 8+ detector (Wyatt Technology, Santa Barbara, CA). The refractive index trace is shown (line) as well as the determined protein mass (boxes). The expected mass of the monomer and of the dimer are indicated by dashed lines. (c) A similar analysis of E2E2$^{full}$ indicated that this protein is also a monomer. Determined molecular mass of 23.8 kDa and a predicted monomer mass of 22.25 kDa. (d) Analytical SEC analysis of E2E1$^{full}$ and E2E1$^{core}$. A sample of both E2E1$^{full}$ and E2E1$^{core}$ was applied to a 24 mL S75

column (GE Healthcare). The resulting chromatogram as well as that obtained from protein standards (GE Healthcare) is shown (Bovine Serum Albumin of 67 kDa; Ovalbumin of 43 kDa; Chymotrypsinogen A of 25 kDa; Ribonuclease A of 13.7 kDa) (See Supplementary Fig. S3a). (e) Circular dichroism spectra of $E2E1^{full}$ (blue line) and $E2E1^{core}$ (black line). The spectra were recorded using an Olis DCM-10 spectrophotometer.

Fig. 3

The N-terminal extension of UBE2E E2s limits ubiquitin chain building. cIAP2 autoubiquitylation assays were performed as described in Fig. 1 except that either wild type (WT) or lysine zero (K0) ubiquitin was used to assess lysine-linked chain building. Assays were incubated at 37˚C for 60 min. cIAP2 activity assays with UBE2D2 were included as an activity control. Note that equal amounts of K0- and WT-ubiquitin were included in assays, but on these gels the K0-ubiquitin stacks as a tight band, while the WT-ubiquitin band is more diffuse.

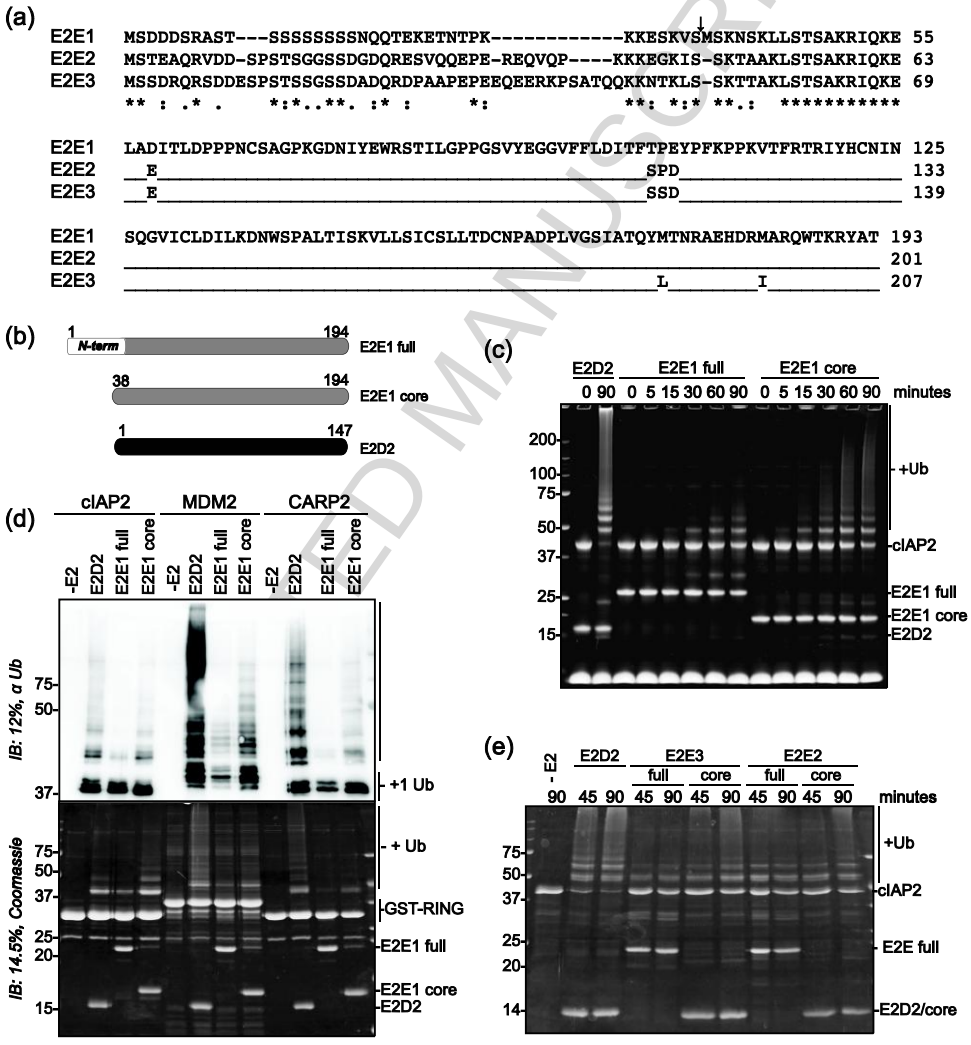
Fig. 4

The N-terminal extension of UBE2E1 can limit ubiquitin transfer by UBE2D2. (a) The N-terminal extension of UBE2E1 was fused to UBE2D2 using blunt end cloning. Autoubiquitylation assays with cIAP2 and the indicated E2 proteins were carried out to compare the activities of the proteins. Assays were performed as described in Fig. 1C using WT-ubiquitin. (b) To identify residues that limit ubiquitin transfer a series of N-terminal truncation were made as described in the methods. These proteins were purified to homogeneity and utilized in autoubiquitylation assays with cIAP2. Note $E2E1^{\Delta N20}$ runs anomalously when separated by SDS-PAGE.

Fig. 5:

Contribution of the 'backside' ubiquitin binding site to ubiquitin transfer. (a) Overlay of the core domain of UBE2E1 (3GBZ) onto the structure of UBE2D2~Ub conjugate (PDB: 3A33). The interaction between ubiquitin from one molecule, shown as a ribbon (teal) and a surface, and the backside of UBE2D2 (teal ribbon) is shown. UBE2E1 is shown as a grey ribbon. The side-chain of Ser22 (UBE2D2)

and Ser68 (UBE2E1) are shown as red sticks. (b) Comparison of initial ubiquitin transfer for UBE2D2 (E2D2) and the backside mutant (E2D2 S22R). Assays were performed as described in Fig. 1C except that K0-ubiquitin was included in the assay. (c) Assays are as in (b) except that WT-ubiquitin was used, allowing the chain building capacity of UBE2D2 (E2D2) and the 'backside' binding mutant (E2D2 S22R) to be compared. (d) The initial rate of ubiquitin transfer by $E2E1^{core}$ (E2E1 core) and the backside mutant (E2E1 core S68R) was analysed in a similar manner. (e) Likewise chain building was assessed using WT-ubiquitin.



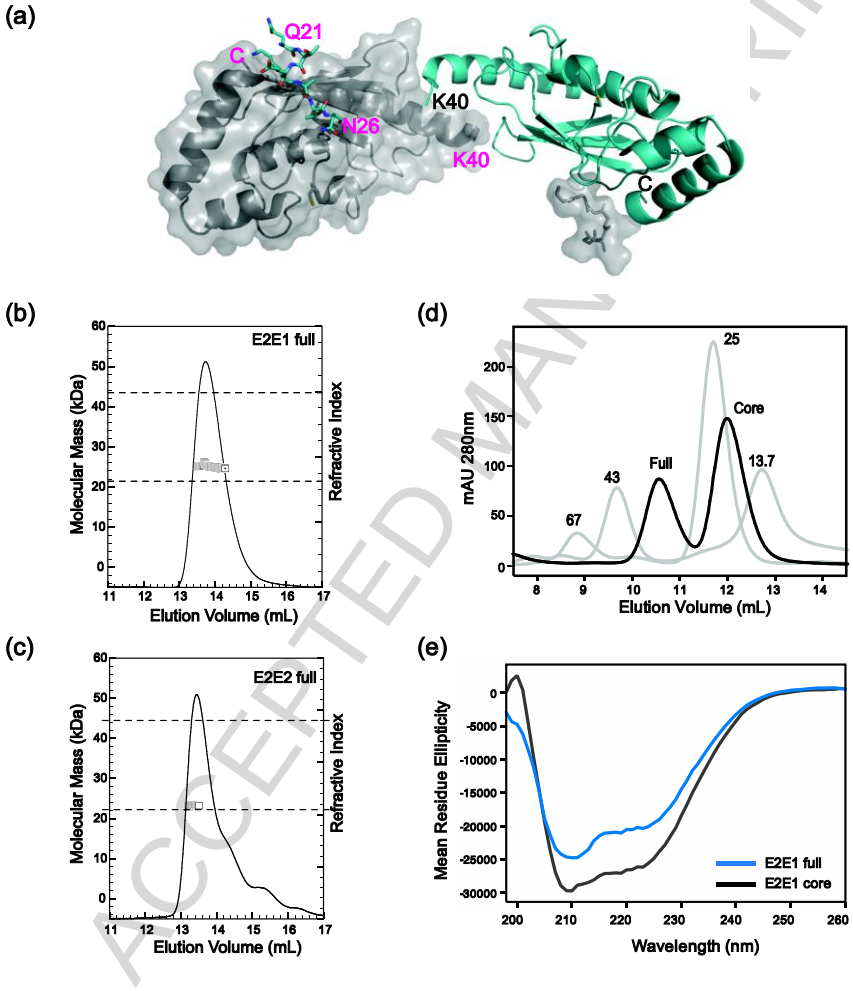
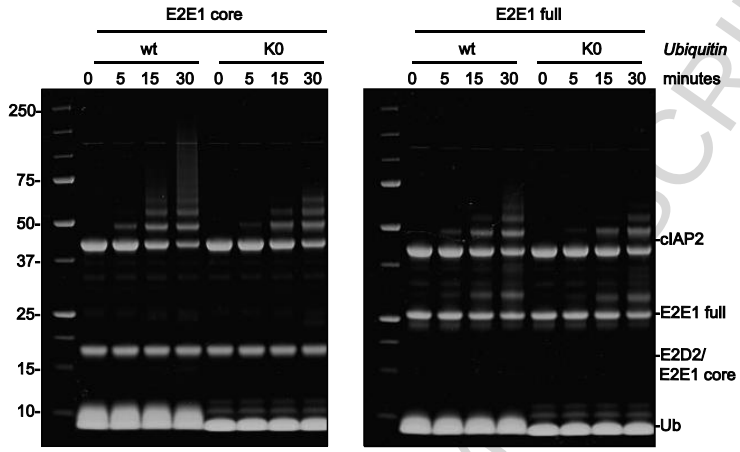


Figure 3



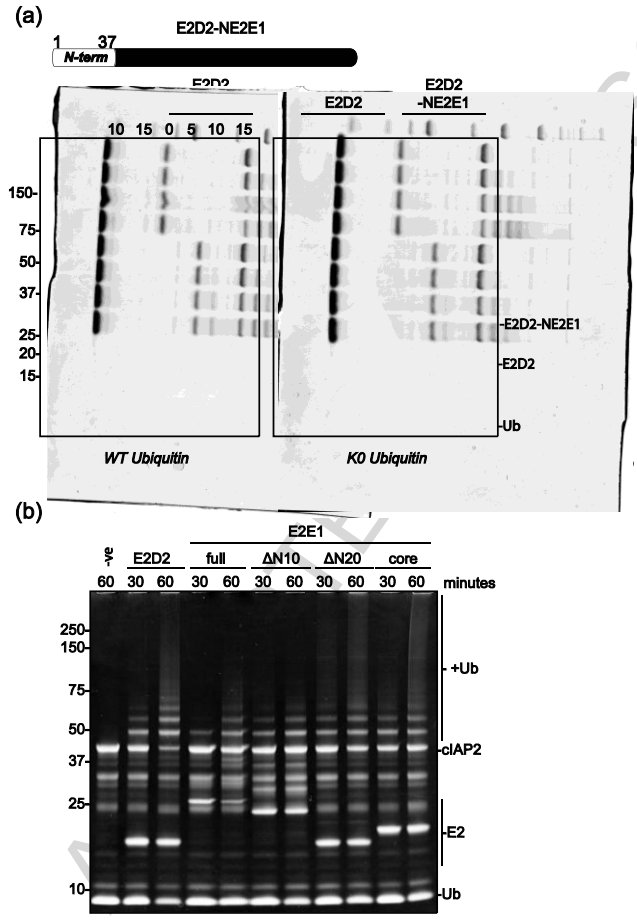
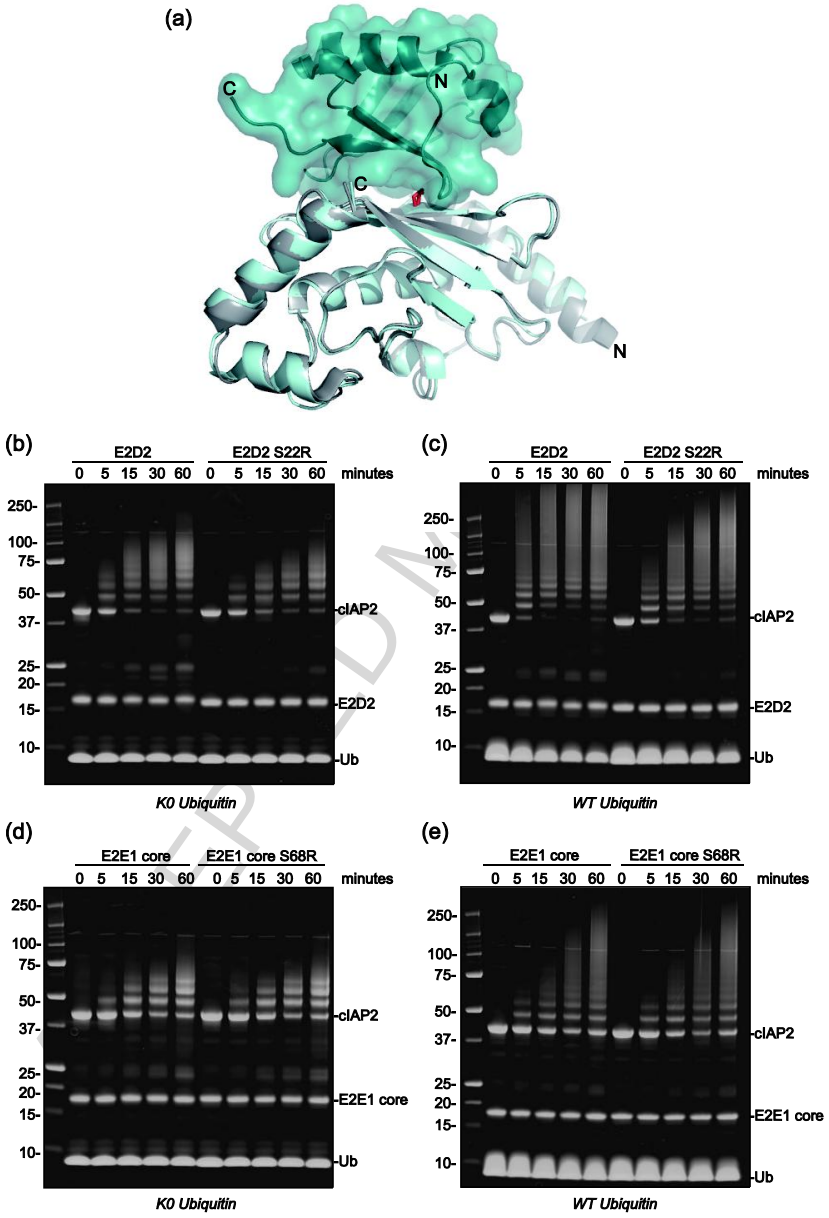
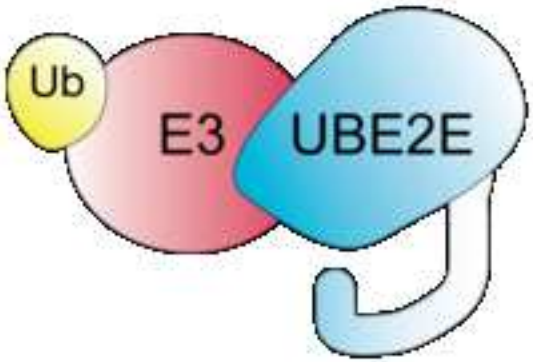
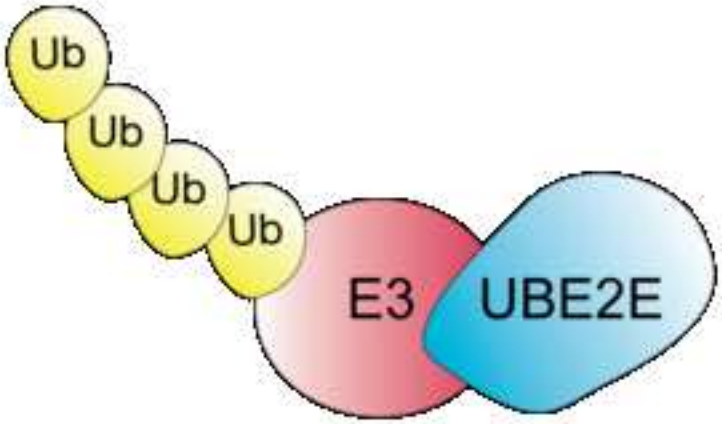


Figure 5






Monoubiquitylation




Polyubiquitylation

**Highlights**

Background: E2 conjugating enzymes have a central role in the ubiquitylation cascade and can determine the architecture of ubiquitin modifications. Some E2s comprise just the conserved core domain, whereas others have N- and C-terminal extensions.

Result: Here we show that the UBC domain of UBE2E proteins can build polyubiquitin chains, but the N-terminal extension of this family of E2 proteins limits polyubiquitylation. As a consequence the unmodified full-length proteins preferentially monoubiquitylate target proteins.

Significance: The UBE2E family of proteins interact with numerous E3 ligases, including many that also interact with the chain building UBE2D family, therefore the selective recruitment of these two E2s may influence the nature of the modification attached to target proteins.