RING Domain E3 Ubiquitin Ligases

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APC, Cbl, CRL, E2, SCF, UPS

Abstract

E3 ligases confer specificity to ubiquitination by recognizing target substrates and mediating transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to substrate. The activity of most E3s is specified by a RING domain, which binds to an E2∼ubiquitin thioester and activates discharge of its ubiquitin cargo. E2-E3 complexes can either monoubiquitinate a substrate lysine or synthesize polyubiquitin chains assembled via different lysine residues of ubiquitin. These modifications can have diverse effects on the substrate, ranging from proteasome-dependent proteolysis to modulation of protein function, structure, assembly, and/or localization. Not surprisingly, RING E3-mediated ubiquitination can be regulated in a number of ways.

RING-based E3s are specified by over 600 human genes, surpassing the 518 protein kinase genes. Accordingly, RING E3s have been linked to the control of many cellular processes and to multiple human diseases. Despite their critical importance, our knowledge of the physiological partners, biological functions, substrates, and mechanism of action for most RING E3s remains at a rudimentary stage.

Contents

INTRODUCTION

Ubiquitination: covalent attachment of ubiquitin to substrates. Typically, ubiquitin's carboxy terminus is joined to the ε-amino group of a substrate lysine

The attachment of ubiquitin and ubiquitin-like polypeptides to intracellular proteins is a key mechanism in regulating many cellular and organismal processes. Ubiquitin is covalently attached to target proteins via an isopeptide bond between its C-terminal glycine and a lysine residue of the acceptor substrate (for general reviews on the ubiquitin system and its enzymes, please consult References 1–3). Assembly of a chain of at least four ubiquitins linked together via their Lys48 residue marks cellular proteins for degradation by the 26S proteasome (4, 5). In contrast, monoubiquitination or polyubiquitination with chains linked together via Lys63 serve as nonproteolytic signals in intracellular trafficking, DNA repair, and signal transduction pathways (for reviews on nonproteolytic roles of ubiquitin, consult References 6 and 7).

Ubiquitination of proteins is achieved through an enzymatic cascade involving

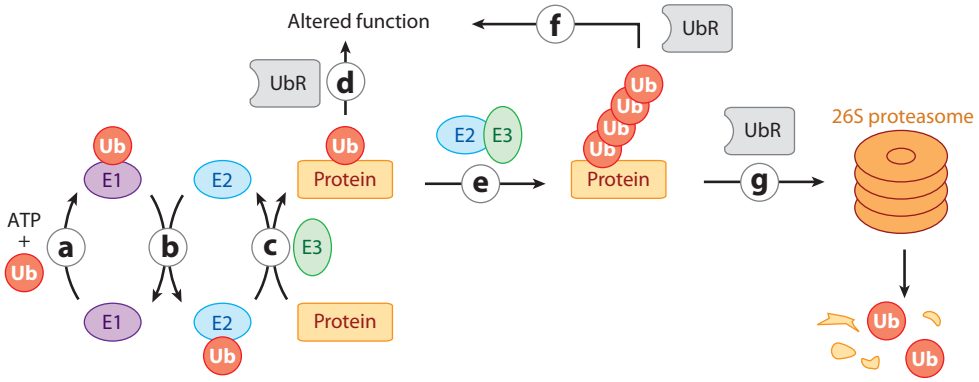


Figure 1

The ubiquitin system. (*a*) Ubiquitin (Ub) and ubiquitin-like proteins are activated for transfer by E1 (ubiquitin-activating enzyme). (*b*) Activated ubiquitin is transferred in thioester linkage from the active-site cysteine of E1 to the active-site cysteine of an E2 ubiquitin-conjugating enzyme. (*c*) The E2∼Ub thioester next interacts with an E3 ubiquitin ligase, which effects transfer of Ub from E2∼Ub to a lysine residue of a substrate. Monoubiquitinated substrate can either dissociate from E3 (*d*) or can acquire additional Ub modifications in the form of multiple single attachments (not shown) or a ubiquitin chain (*e*). The chain can be knit together via different lysine residues of ubiquitin. Whereas monoubiquitin and some types of chains (e.g., those assembled via Lys63 of ubiquitin) serve mainly to alter the function of the modified protein (*f*) (by changing its structure, binding partners, cellular localization, etc.), polyubiquitin chains assembled via the Lys48 residue of ubiquitin typically direct the appended substrate to the proteasome for degradation (*g*). The biological outcome of ubiquitination—be it degradation or signaling—is normally dictated by ubiquitin receptors (UbR) that bind and interpret the ubiquitin signal.

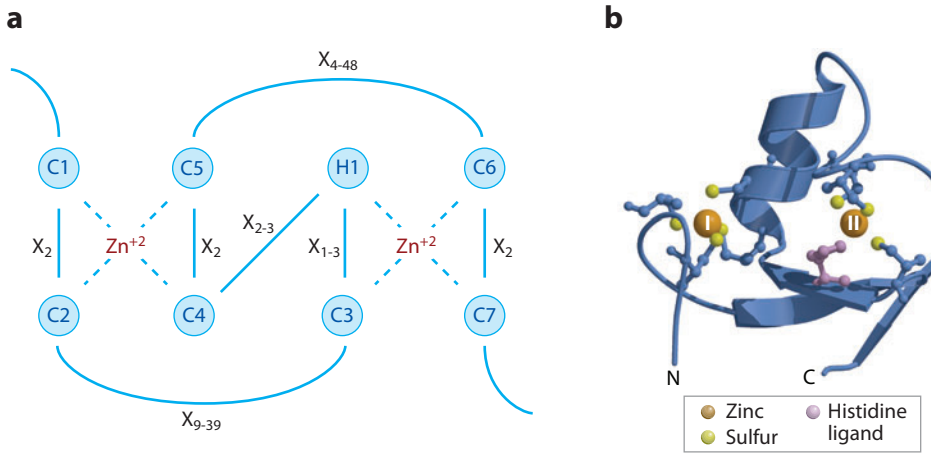
ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes (**Figure 1**). Ubiquitination occurs when an E3 ligase enzyme binds to both substrate and an E2 thioesterified with ubiquitin (E2∼Ub), bringing them in proximity so that the ubiquitin is transferred from the E2 to the substrate, either directly or in a small subset of E3s, via a covalent E3∼ubiquitin thioester intermediate. The pairing of E2s and substrates by E3s determines the specificity in ubiquitination. There are two major types of E3s in eukaryotes, defined by the presence of either a HECT or a RING domain. RING ubiquitin ligases, which are the focus of this review, are conserved from yeast to humans, with about 616 different RING domain ligases potentially expressed in human cells. However, many aspects of these enzymes remain poorly understood. Here, we review what is known about the RING ligase family (including a roster of its members), how RING domains work with E2 enzymes to monoubiquitinate or polyubiquitinate substrates, how these enzymes can be regulated, and what major questions remain to be tackled.

GENERAL FEATURES OF THE RING DOMAIN AND FAMILY

The RING domain was originally described by Freemont and colleagues (8). The basic sequence expression of the canonical RING is Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His-$X_{(2-3)}$-Cys-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys (where X is any amino acid) (**Figure 2*a***). However, the matricial expression used to define the motif in bioinformatics analyses (e.g., Hidden Markov Models) takes into account the frequency of less conserved amino acids in additional positions as well (see **http://ca.expasy.org/prosite/PS50089**). Three-dimensional structures of RING domains (e.g., **Figure 2*b***) revealed that its conserved cysteine and histidine residues are buried within the domain's core, where they help maintain the overall structure through binding two atoms of zinc. Additional

E2: ubiquitin-conjugating enzyme; active-site cysteine forms thioester linkage with the C terminus of ubiquitin

E3 ubiquitin ligase: an enzyme that binds ubiquitin-conjugating (E2) enzyme and substrate and that catalyzes transfer of ubiquitin from E2 to substrate



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RING-HC	Cys - X_2 - Cys - $X_{(9-39)}$ - Cys - $X_{(1-3)}$ - His - $X_{(2-3)}$ - Cys - X_2 - Cys - $X_{(4-48)}$ - Cys - X_2 - Cys
RING-H2	Cys - X_2 - Cys - $X_{(9-39)}$ - Cys - $X_{(1-3)}$ - His - $X_{(2-3)}$ - His - X_2 - Cys - $X_{(4-48)}$ - Cys - X_2 - Cys
$C_2H_2C_4$ (Mdm2)	Cys - X_2 - Cys - X_{10} - His - X_4 - His - X_3 - Cys - X_2 - Cys - X_{10} - Cys - X_2 - Cys
RBQ-1	Cys - X_2 - Cys - X_{11} - Cys - X_2 - Asn - X_2 - Cys - X_2 - Cys - X_{12} - Cys - X_2 - Cys
RBX1	Cys - X_2 - Cys - X_{29} - Cys - X_1 - His - X_2 - His - X_2 - Cys - X_{10} - Cys - X_2 - Asp
LIM	Cys - X_2 - Cys - $X_{(16-23)}$ - His - X_2 - Cys - X_2 - Cys - X_2 - Cys - $X_{(16-23)}$ - Cys - X_2 - Cys
PHD	Cys - X_2 - Cys - $X_{(8-21)}$ - Cys - $X_{(2-4)}$ - Cys - $X_{(4-5)}$ - His - X_2 - Cys - $X_{(12-46)}$ - Cys - X_2 - Cys
C_4H_4 (Cnot4)	Cys - X_2 - Cys - X_{13} - Cys - X - Cys - X_4 - Cys - X_2 - Cys - X_{11} - Cys - X_2 - Cys

Figure 2

The RING finger domain. (*a*) Primary sequence organization of the RING-HC domain. The first cysteine that coordinates zinc is labeled as C1, and so on. H1 denotes the histidine ligand. X_n refers to the number of amino acid residues in the spacer regions between the zinc ligands. (*b*) Ribbon diagram of the three-dimensional crystal structure of the RING domain from c-Cbl. The zinc atoms in sites I and II are numbered. The termini are as marked. (*c*) RING-like sequence variants. LIM and PHD do not form a RING-like interleaved structure and, unlike all the other variants shown, do not possess E3 activity.

semiconserved residues are implicated either in forming the domain's hydrophobic core or in recruiting other proteins. Unlike zinc fingers, the zinc coordination sites in a RING "finger" are interleaved, yielding a rigid, globular platform for protein-protein interaction, hence RING domain (9, 10).

Over time, numerous RING variants have been noted (**Figure 2*c***), including some in which cysteines and histidines are swapped or one of the cysteines is replaced by another residue that can coordinate zinc (e.g., Asp in Rbx1/Roc1) (11). Whether such consensus sequence variations have functional relevance remains unclear. LIM and PHD domains also share a similar pattern of Cys and His residues (12) (**Figure 2*c***), but they fold differently and have not been implicated in ubiquitination (13, 14). The B-box domain of the TRIM subfamily of RING E3s (15) and the U-box domain (16) are structurally related to the RING and function in mediating ubiquitination. The latter can recruit E2 enzymes, whereas the former does not. In the U-box domain, the zinc-binding sites are replaced by conserved charged and polar residues that engage in hydrogen-bonding networks and that are required for maintaining structure and activity (16, 17). The Miz-finger domain, present in E3s (of the PIAS subfamily) that mediate substrate modification with the ubiquitin-like protein, SUMO, has been suggested to be a RING variant as well (17). Perhaps not surprisingly then, a protein completely unrelated to RING or U-box proteins—the

Pseudomonas syringiae inhibitor of host apoptosis AvrPtoB—that was found to possess a RING domain fold when its structure was solved by crystallography was subsequently shown to mediate ubiquitination (18).

A Brief History of the RING

At the time that the RING motif was discovered in Ring1 (really interesting new gene 1), 27 other proteins were shown to possess the motif, and many of them had functions that involved DNA. Thus, it was originally thought that the RING finger might mediate binding to DNA. We now know that the RING domains of many proteins mediate ubiquitin ligase activity instead. However, the first explicit linkage between a RING protein and ubiquitination trailed the description of the RING motif by six years when it was shown that a member of the original cohort of RING proteins, Rad18, can promote ubiquitination of histone (19). This was followed rapidly by discovery of a RING motif in Der3/Hrd1, which is required for endoplasmic reticulum-associated degradation (ERAD) (20), in the Apc11 subunit of the anaphase-promoting complex/cyclosome (21), and in the Prt1 protein of *Arabidopsis thaliana* (22) and the Ubr1 proteins of yeast and mouse (23), which mediate the N-end rule degradation pathway.

Although the studies described above revealed a link between the ubiquitin system and RINGs, the significance of that link was not clarified until 1999, in the *Annus Mirabilis* for the RING domain. Before 1999, few E3s were known, but it was anticipated that many E3s must exist to account for the specificity of what was evidently a major cellular regulatory system. Together, the 1999 papers unambiguously established that the RING domain binds to ubiquitin-conjugating enzymes and promotes a direct transfer of ubiquitin to substrate by a novel mechanism and that many (if not most) of the members of the large family of RING domain proteins are likely to possess this activity.

The first key breakthrough was reported by four teams who discovered a new subunit of the SCF ubiquitin ligase, which was named Rbx1, Roc1, and Hrt1 by the different groups and which we will refer to hereafter as Rbx1/Roc1 (24–27). These papers together with Reference 190 established the following key points: (*a*) the RING protein and its ability to coordinate zinc are essential for SCF ubiquitin ligase activity in vitro and in vivo; (*b*) Rbx1/Roc1 binds Cul1, and together these proteins bind and activate the E2 enzyme Cdc34; and (*c*) unlike the HECT-domain ubiquitin ligases, ubiquitination mediated by Cul1-Rbx1/Roc1 does not involve an E3-linked ubiquitin thioester intermediate.

The second key breakthrough came from independent work, carried out in parallel in two other laboratories, demonstrating that E2 binding and E3 activity are intrinsic to the RING domains of c-Cbl (28), AO7, and seven other randomly selected RING proteins, which had not been previously implicated in ubiquitination (29). This latter finding was particularly startling as it suggested that a major fraction of the large family of RING proteins might in fact be ubiquitin ligases.

Still in 1999, work from other laboratories demonstrated that the RING domains of c-Cbl (30, 31) and Ubr1 (32) are essential for the ubiquitination and subsequent lysosomal degradation of the epidermal growth factor (EGF) receptor and proteasomal degradation of N-end rule substrates, respectively. These findings were rapidly followed by numerous reports that linked other RING domain proteins to ubiquitination. Together, these reports dramatically expanded the inventory of known E3s.

Bioinformatic Analysis of the RING Family

Bioinformatic analyses have shown that 300 human genes encode RING domain proteins and that 8 genes encode U-box proteins (33). Domains with a RING/U-box fold that are not related by sequence, such as AvrPtoB, cannot be predicted by available methods, and thus we do not know how many such proteins exist. In *Saccharomyces cerevisiae*, RING and U-box

Skp1-Cullin-F-box (SCF): the founding member of a large family of RING-based E3s known as the cullin-RING ligases (CRLs)

CRLs: cullin-RING ubiquitin ligases

domain proteins are encoded by, respectively, 47 and 2 genes. Only six of these are essential for viability in rich medium, which mirrors the fraction of all yeast genes estimated to be essential under these conditions (17%). A greater number—at least 26 yeast RING domain genes—have been under strong selective pressure for conservation throughout eukaryotic evolution, as evidenced by the occurrence of human orthologs (33).

Although in many E3s the substrate-binding site resides in the same polypeptide as the RING domain, certain RING domain proteins belong to complexes where substrate recognition is mediated by a separate subunit, as in the case of Rbx1/Roc1, Rbx2/Roc2, and Apc11. In particular, Rbx1/2 add great diversity to the E3 family by forming SCF and other cullin-RING ubiquitin ligase (CRL) complexes with many alternative substrate-recognition subunits, including those that contain an F box, SOCS box, VHL box, or BTB domain (34). Proteins encoding these domains comprise ~287 human genes (33; S. Batalov, personal communication). In addition, ~20 Ddb1- and Cul4-associated factors (DCAFs) serve as the putative substrate receptors for Cul4-based CRLs (34a). Better predictions of the total number of DCAFs await elucidation of their signature motif(s) (N. Zheng & W. Harper, personal communication). Therefore, up to 616 RING/U-box-dependent E3s are expressed in humans, comprising >95% of all predicted E3s (33). For comparison, 518 human genes encode a protein kinase domain. The number of predicted human E3s, in contrast with the two human ubiquitin E1s and the estimated fewer than 40 ubiquitin E2 genes, is consistent with the role of E3s in conferring specificity and regulation to ubiquitination.

A variety of domains are found among RING proteins, including many involved in signaling, such as SH2, SH3, FHA, ankyrin repeats, PDZ, and ubiquitin like. Three-quarters of the human RING domain proteins exhibit at least one other domain or motif based on bioinformatic primary sequence analyses (33). Indeed, domain composition and protein homology are the best criteria for defining subfamilies of RING domain proteins. The largest subfamily, TRIM/RBCC, has ~76 representatives in humans and is characterized by the presence of a B box and a coiled coil (35, 36). The second largest human subfamily is RBR/TRIAD (37, 38), with at least 14 members exhibiting two RING domains and an in-between RING (IBR) domain.

Most RING Domain Proteins Mediate Ubiquitin Transfer

The avalanche of discoveries reported in 1999 suggested that most of the 300 human RING proteins act as ubiquitin ligases. To date, functional data have been obtained to support the candidacy of nearly half of them (**Supplemental Table 1**. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). Most of the others have not been investigated. However, we do know that not every single RING domain possesses intrinsic E3 activity. For example, the RING domains of Bard1 (39), Bmi1 (40), and MdmX (41) do not exhibit E3 activity by themselves. In each of these cases, however, the RING domain interacts with a second RING domain protein (Brca1, Ring1b, and Mdm2, respectively) that does, and heterodimer formation greatly stimulates E3 activity of the latter. In addition, there are several other cases of well-studied RING domain proteins for which ubiquitin ligase activity has never been conclusively demonstrated. Examples include the Cdk-activating kinase assembly factor Mat1 and the Ste5 and Far1 proteins in budding yeast. Nevertheless, it seems likely that the lion's share of RING domain polypeptides will prove to be ubiquitin ligases or subunits of oligomeric ubiquitin ligases.

RING DOMAINS BIND AND APPEAR TO ACTIVATE E2 ENZYMES

RING domains underlie ubiquitin ligase activity by directly binding ubiquitin-conjugating

enzymes. This key principle was first suggested by the 1999 papers on Rbx1/Roc1, c-Cbl, and AO7, and definitive evidence was provided a year later by the crystal structure of Cbl's RING domain bound to UbcH7 (**Figure 3*a***) (42). The precise nature of RING-E2 interaction has been further probed by NMR analyses of Brca1 and Cnot4 complexed with UbcH5 (43, 44). The structural biology studies combined with mutational analyses highlight residues on the RING and E2 that play a crucial role in sustaining the interface. Key E3-E2 contacts deduced from these efforts are diagrammed in **Figure 3*b***.

The loop regions comprising the zinc coordination sites of the RING domain and the central helix that connects the first and second coordination sites (**Figure 3*a***) together form a shallow cleft on the surface of the RING to which E2s bind (42). Ile383 and Trp408 of c-Cbl and equivalent residues in other RING proteins have most consistently been implicated in interaction with E2s, based on X-ray and NMR data. The side chain of Trp408 in c-Cbl is exposed to solvent in the E2-binding cleft, and bulky, hydrophobic residues are often found in the equivalent position in other RING E3s. Mutation of this residue in c-Cbl, Cnot4, and other E3s eliminates RING-E2 interaction and E3 ubiquitin ligase activity (28, 45). Functional studies of RING E3s typically employ mutations in the zinc-binding residues to inactivate the RING domain (46). We note that such mutations perturb the overall RING domain structure and could potentially render the E3 a substrate for quality control in vivo. We suggest that a better strategy is to mutate the residues equivalent to Trp408 or Ile383 to bulky polar or charged residues.

Systematic two-hybrid and NMR studies with the Brca1-Bard1 heterodimer revealed that a set of Brca1 residues consistently form critical contacts with multiple E2s, with only small differences in the interactions. From the E2 perspective, the first α-helix and loops 1 and 2 typically make important contacts with RING E3s. However, these contacts can vary between different RING-E2 complexes. For example,

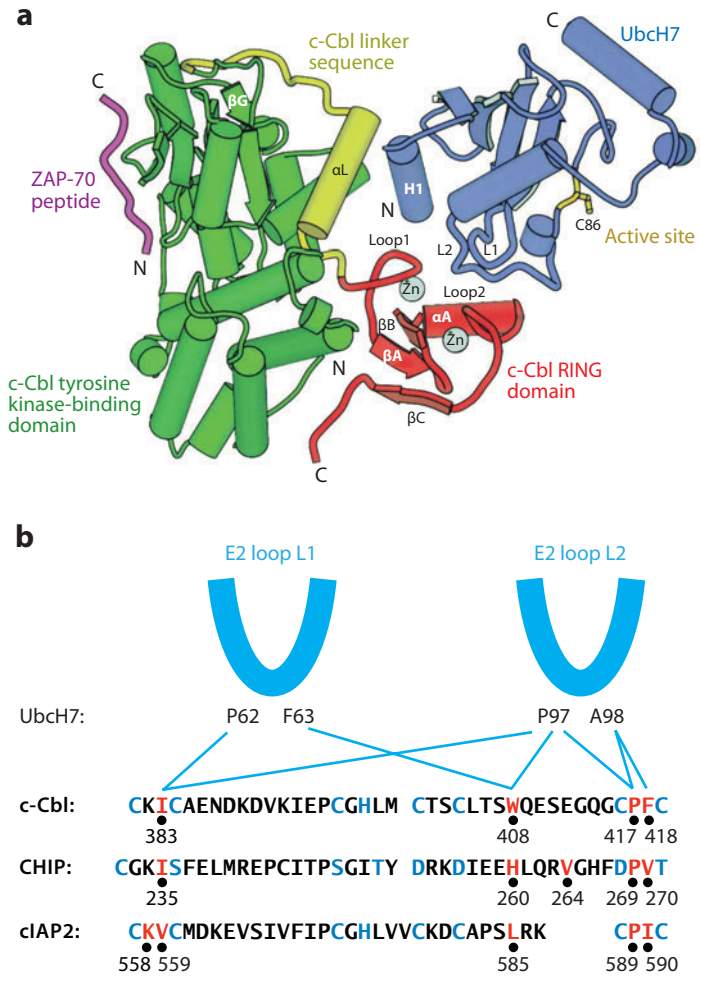


Figure 3

E2 interactions with RING and U-box domains. (*a*) The ternary complex of c-Cbl, UbcH7, and phosphorylated ZAP-70 peptide (reprinted from Reference 42 with permission from Elsevier). (*b*) Contacts between RING/U-box domains and E2s. RING domain sequences from the first to the last pair of zinc-binding residues for c-Cbl and cIAP2 are shown. The sequence of the U box from Chip is shown for comparison. The RING and U-box residues that make the most significant contacts observed in cocrystal structures are shown in red. The zinc-binding residues and equivalent residues in the U box are colored in blue. The information is derived from the following cocrystals: c-Cbl-UbcH7 (42), Chip-UbcH5 (61), Chip-Ubc13 (62), and cIAP2-UbcH5 (63). The E2 residue numbers shown are for UbcH7. The residues are identical in UbcH5 and Ubc13 except for F63, which is a Met in Ubc13. Contacts between the E2 α1 helix and regions outside of the RING domain (e.g., c-Cbl's linker helix) are more variable and are not represented.

UbcH5's loop 1 does not seem to play a major role in binding to Brca1/Bard1. In another example, in the c-Cbl-UbcH7 complex, helix 1 contacts the linker helix of c-Cbl, which is N-terminal to the RING domain. Meanwhile, in a computationally docked Cnot4-UbcH5b complex, UbcH5b's helix 1 contacts residues comprising the first zinc coordination site of Cnot4 (44). The importance of individual residues in helix 1 can vary depending on which E3 is being bound by E2. For example, substitution of Arg5 with Ala in UbcH5b disrupts functional interaction with Cnot4, but not with Apc2-Apc11 (47).

Although it is clear that RING domains recruit ubiquitin-conjugating enzymes, there is often a poor correlation between ubiquitin ligase activity and the ability to bind E2 with high affinity (29). On one hand, Brca1-Bard1 can stably bind UbcH7, but the complex is inactive for ubiquitin transfer (48). On the other hand, some highly active E2-E3 pairs do not exhibit stable association. In fact, the affinity of isolated RING domains for E2s is usually low, with K_D typically in the low micromolar range. In the two best-studied examples of high-affinity E3-E2 complexes, the RING domain is dispensible for complex formation. Tight binding of Ubc2 to Ubr1 is mediated primarily by the BRR (basic rich region) sequence immediately N-terminal to the RING domain (32). Ironically, the RING, but not the BRR domain, is required for Ubr1 function in vivo. Likewise, gp78 has a motif distinct from the RING that recruits the E2, but in this case, the high-affinity E2-binding domain is essential for ubiquitination (49).

Structural and Computational Methods Provide Insight into E3-E2 Interfaces

An interesting development in the study of E3-E2 interfaces is the application of structural and computational methods to manipulate the properties of the interface in a predictable manner. An early success in this arena was reported by Timmers and colleagues (50), who provided evidence that Glu48 of Cnot4 engages in an electrostatic interaction with Lys63 of UbcH5b. Although this particular contact is not essential for the interface to form, charge swap mutations in either partner (e.g., E49K or K63E) eliminate functional interaction. Remarkably, when both mutations are combined, there is complete restoration of binding and activity. In a subsequent study, Kuhlman and colleagues (51) employed protein design software to reengineer the interface of a HECT domain E3-E2 complex. They succeeded in increasing the affinity of the interface, thereby tamping down the dynamic nature of E3-E2 binding. The methods they employed should be equally applicable to RING-E2 interfaces.

Despite the formidable power of structural and computational approaches, the essence of what constitutes an active RING or RING-E2 pair resists precise description or unambiguous prediction. Five examples illustrate the challenges to predicting E3-E2 pairs. First, the Leu51 residue of Brca1 that is equivalent to the critical Trp408 of c-Cbl is not required for E3 activity, even though it is perturbed by binding of UbcH5 (43). Second, the cleft on the surface of the RING to which E2s bind may not be a prerequisite for E3 activity as was originally proposed (42) because Rag1 apparently lacks this cleft, but nevertheless was subsequently shown to exhibit E3 activity (52, 53). Third, as discussed above, Glu49 of Cnot4 forms an electrostatic pair with Lys63 in loop 1 of UbcH5b. Interestingly, UbcH5c shows high activity with SCF despite the presence of an Arg in Rbx1/Roc1 at the position equivalent to Glu49 of Cnot4. On the basis of the results of Winkler et al. (50) UbcH5c should not productively engage Rbx1/Roc1 owing to an electrostatic clash. Fourth, as noted by Zheng et al. (42), the residues at the core of the c-Cbl-UbcH7 interface are sometimes completely different in other E3-E2 pairs. For example, Trp408 of c-Cbl and Phe63 of UbcH7 form a critical, highly conserved interaction, but in Rad18-Rad6, the corresponding residues are His and Asn. Despite some effort, it was not possible to get c-Cbl to utilize Rad6 by

mutating the E3's critical Trp408 or its interacting residue in the E2. Thus, more extensive modifications may be required to switch E3-E2 specificity (C.A.P. Joazeiro & T. Hunter, unpublished observations). Fifth, as discussed above, the α1 helix of E2s plays an important role in docking to RING proteins but makes radically different contacts in different E3-E2 complexes.

In summary, given the large number of RING E3s and E2s and the variety of chemical strategies that have evolved to mediate their functional contact, uncovering predictive rules for the identification of physiological partners remains a largely unmet challenge.

How the Dynamics of E2-E3 Association Relate to Function

In evaluating the myriad findings that have been reported on E3-E2 association, it is worth bearing in mind that ubiquitin ligases can be formally thought of as bisubstrate enzymes that have two substrates and two products (**Figure 4**). The substrates are E2 thioesterified with ubiquitin (E2∼Ub) and a lysine residue on the target protein (**Figure 4*b***). The two products are the discharged E2 and the target protein linked to ubiquitin via an isopeptide bond (**Figure 4*c***). For an enzyme to operate efficiently, it must bind its products weakly lest it succumb to product inhibition. However, in most published examples, E3-E2 interaction has been probed with E2 that is either naked (i.e., nonthioesterified) or of unknown status, using methods, such as "pull-down" or yeast two-hybrid procedures, poorly suited to the study of a dynamic interface that underlies catalysis.

Pull-down assays involve multiple washes and thus are biased to detect complexes that have half-lives on the order of minutes. Highly

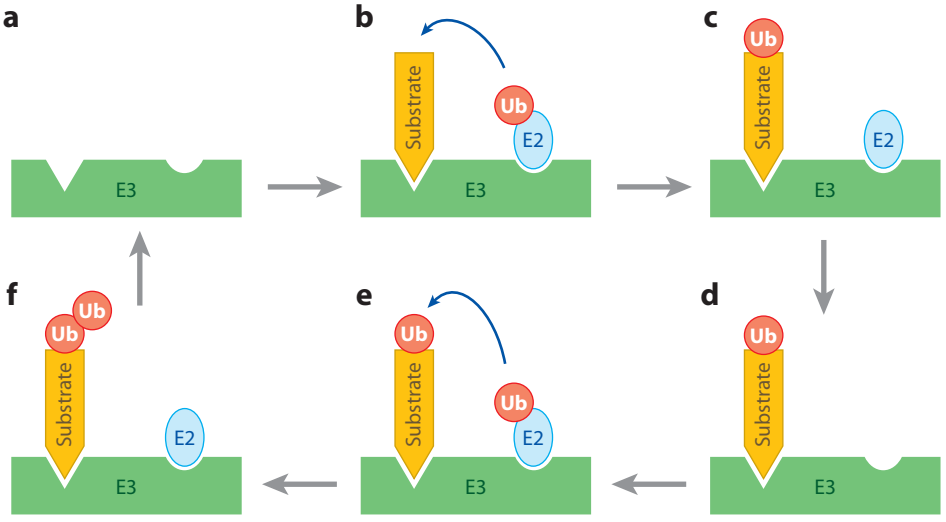


Figure 4

Reaction cycle of a RING E3. RING E3s are bisubstrate enzymes that catalyze the conversion of the reactants E2∼Ub and substrate to the products E2 and substrate-Ub. Unliganded E3 (*a*) binds substrate and E2∼Ub to form the Michaelis complex (*b*). It is generally assumed that the two substrates do not need to bind in a predetermined order. (*c*) Ubiquitin is transferred from E2∼Ub to substrate to yield the products, E2 and substrate-Ub. (*d*) For further ubiquitination to occur, E2 must dissociate to allow a fresh molecule of E2∼Ub to bind (*e*). E2 cannot be recharged on E3 because E1 and E3 use overlapping surfaces to bind E2. The newly recruited E2∼Ub transfers its cargo to yield diubiquitinated substrate (*f*). From this scheme, it is evident that the relative rates of substrate-Ub_n dissociation and E2∼Ub recruitment/E2 dissociation can have a major impact on the number of ubiquitins that a substrate receives every time it binds to E3.

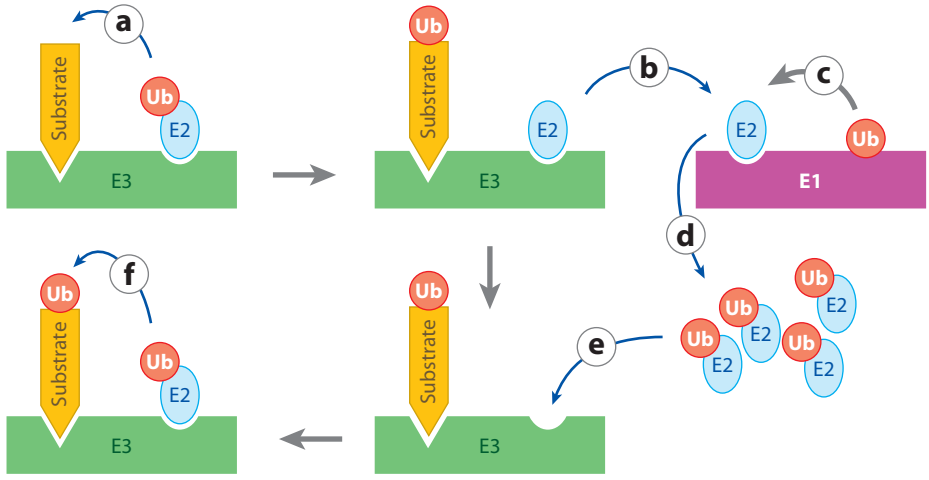


Figure 5

Discharged E2 must dissociate from a RING E3 to be recharged with ubiquitin by E1. After transferring its ubiquitin cargo (*a*), E2 must dissociate from the RING E3 (*b*) so that it can bind to E1 and be recharged (*c*), and return to the cellular pool of E2∼Ub (*d*). Upon its dissociation from E3, the spent E2 makes way for a fresh molecule from this cellular pool to bind (*e*), resulting in a second cycle of ubiquitin transfer (*f*).

active RING ubiquitin ligases can catalyze the transfer of ubiquitin from E2∼Ub to substrate with an apparent rate constant (k_{cat}) of one per second or faster (54, 55). This creates an interesting dilemma given that E2s use overlapping interfaces to bind E1 and E3s (51). Every time an E2 transfers its ubiquitin cargo to substrate, it must dissociate from E3 to make way for a fresh molecule of E2∼Ub (**Figure 5**). The mutually exclusive nature of E1 and RING binding indicate that an E2 cannot be recharged while it remains bound to the RING. Let us reconsider the kinetic data in light of this. A k_{cat} of 1 s^{-1} implies a reaction half time of 0.7 s or less. This sets an upper boundary for the life span of a RING-E2 interface. Thus, one might predict that the E3-E2 interactions that are relevant to catalysis often escape detection because the complexes fall apart during the washing steps in a pull-down experiment.

Despite these arguments, there are multiple examples where E3-E2 interaction is readily detected and appears to be quite strong. Examples include the interactions of Ubc2 with Ubr1 (32) and Ube2g2 with gp78 (49) referred to above. If robust ubiquitination can occur with weakly associating E3 and E2 partners, why do some pairs exhibit tight binding? Moreover, how can stable E3-E2 complexes be effective at catalyzing assembly of ubiquitin chains on substrate if a discharged E2 has to dissociate to be recharged by E1 (51)? In both of these cases, the E3 brandishes an additional domain that mediates tight binding of E2. The interaction surface afforded by these additional elements may enable on-site recharging of E2 by E1. According to this idea, multidentate interaction of a single E2 molecule with E3 would enable dissolution of the RING-E2 interface and recharging of E2 by E1 without complete dissociation of the E2 from E3. Another possibility is that a tight E2-binding site enables sequential assembly of a ubiquitin chain on the active site of the captive E2, as a prelude to en bloc transfer of the chain to substrate (**Figure 6**).

RING E3s Promote Direct Transfer of Ubiquitin from E2∼Ub to Substrate

E6-associated protein (E6-AP) is the first ubiquitin ligase for which detailed mechanistic insight was gained. E6-AP contains a conserved HECT (for homologous to E6-AP carboxy terminus) domain that contains within it an

invariant cysteine that accepts ubiquitin from E2∼Ub to form an intermediate in which ubiquitin is thioesterified to E6-AP (56). This ubiquitin is subsequently transferred to a lysine residue of a substrate molecule (**Figure 7*a***). However, ubiquitin chain assembly catalyzed by SCF plus Cdc34∼Ub is relatively insensitive to the thiol reagent N-ethylmaleimide (27), and the conserved RING Cys residues are not surface exposed, arguing that RING E3s do not form a thiol-based catalytic intermediate with ubiquitin. It is now commonly believed that all RING-based E3s function by catalyzing the direct transfer of ubiquitin from E2∼Ub to substrate (**Figure 7*b***).

Ubiquitin Transfer May Involve a RING-Induced Conformational Change in E2

How does docking of E2∼Ub to a RING domain activate substrate ubiquitination? It is generally accepted that catalysis arises from induced proximity of E2∼Ub and substrate. What remains under debate is whether the E3 plays a more active role. Available structures indicate that the RING domain is too far removed from the E2 active site to play a catalytic role, for example, in deprotonating the substrate lysine or in stabilizing the oxyanion intermediate that develops during formation of an isopeptide bond (57). An alternative possibility is that the RING induces a conformational change in the E2∼Ub that enhances the rate of ubiquitin

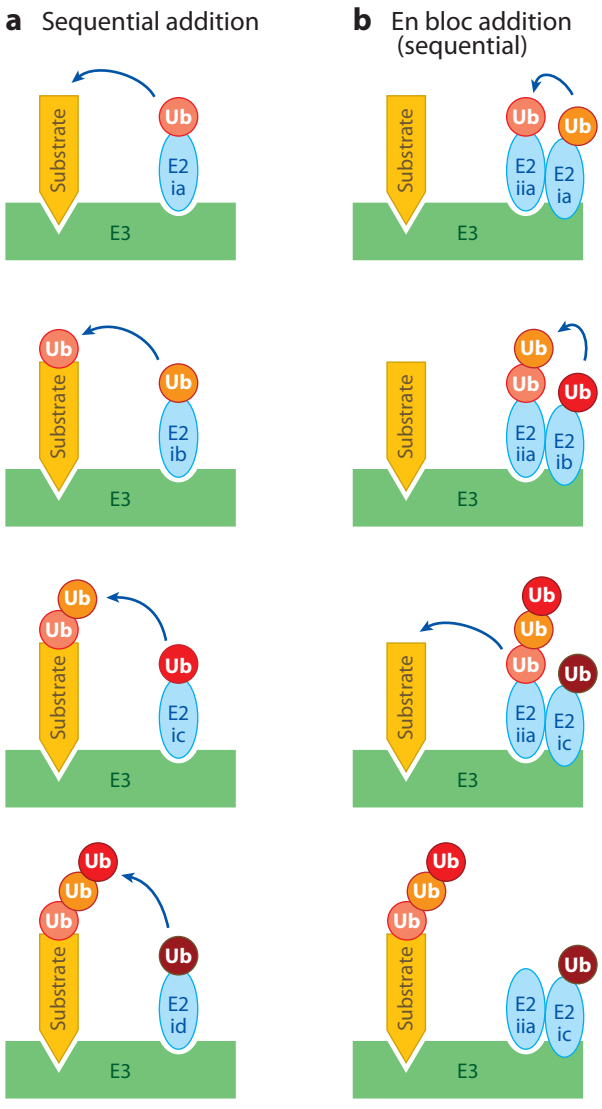


Figure 6

RING E3s may use different mechanisms to catalyze polyubiquitination of substrate. (*a*) The sequential model of chain synthesis postulates that polyubiquitination is achieved by successive addition of ubiquitin molecules to a substrate. In between each round of transfer, the spent E2 dissociates to make way for a fresh molecule of E2∼Ub (e.g., E2ia, E2ib, etc.). (*b*) An alternative possibility is that ubiquitin chains are preassembled on E2 and then transferred en bloc to substrate. Various permutations of this mechanism can be envisioned, including the one shown here (based on the gp78-Ube2g2 E3-E2 pair; Yihong Ye, personal communication) in which the E3 recruits two E2 protomers (E2ia and E2iia). The second protomer remains stably bound at site ii and attacks fresh thioesters that successively bind in the first site (E2ia∼Ub, E2ib∼Ub, etc.), such that a ubiquitin chain is sequentially assembled on the active site of the E2 bound at site ii. Substrate can potentially interrupt this cycle at any stage, resulting in en bloc transfer of ubiquitin chains of varying length. Gp78-Ube2g2 is thought to work this way. En bloc transfer can also occur for chains that are assembled by being ping-ponged between the two E2 active sites, in which case the chain would have opposite polarity (i.e., the most recently added ubiquitin subunit would be most proximal to the E2).

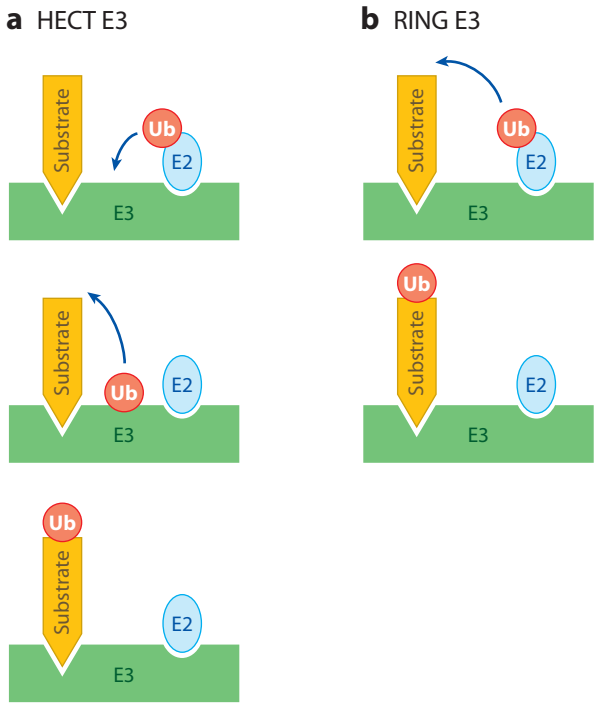


Figure 7

HECT and RING E3s work by different mechanisms. (*a*) HECT E3s have a conserved cysteine residue that accepts ubiquitin from E2∼Ub to form an E3∼Ub thioester. Ubiquitin is transferred from this covalent E3 intermediate to substrate. (*b*) By contrast, RING E3s effect the direct transfer of ubiquitin from E2∼Ub to substrate.

discharge from the active site (27, 190). Arguments against conformational change remain popular (58, 59) because there is no significant change in the three-dimensional structure of various E2s regardless of whether they are bound to a RING domain or a U box (42, 60–63). However, we emphasize that the E2s used in these studies were not esterified with ubiquitin.

By contrast, multiple independent lines of evidence suggest that E2∼Ub undergoes an activating conformational change upon binding to a RING domain. In the first explicit test of whether approximation is sufficient to activate ubiquitination, Cdc34 was fused to its substrate Sic1 (27). This chimera exhibits very low basal autoubiquitination activity but is strongly stimulated by SCF. Second, SCF accelerates discharge of Cdc34∼Ub in the presence of either ubiquitin or hydroxylamine as acceptor (54, 55) (**Figure 8**). Because these acceptors diffuse freely and are not known to bind SCF, it is difficult to envision how SCF could stimulate k_{cat} by induced proximity. Lastly, if the only function of the RING is to dock E2∼Ub in the vicinity of substrate, then arguably the precise nature of the RING-E2∼Ub interface should not matter provided that binding occurs. However, this is clearly not the case because both c-Cbl and Bard1-Brca1 can recruit UbcH5 and UbcH7, but only the UbcH5 complexes are active in ubiquitination (43). Similarly, a mutation in loop 2 of UbcH5b (Trp93 to Tyr) causes a marked reduction in Cnot4-activated ubiquitin discharge, even though binding to Cnot4 is nearly normal (47). Thus, E3-E2 binding is not sufficient for catalysis.

If in fact a RING-induced conformational change underlies E2 activation, then what is the change and how does it occur? A computational approach suggested that the RING-binding and active sites of UbcH5 communicate, even though these sites are ∼15 Å from each other (47). Binding of E2∼Ub to a RING E3 could trigger a rearrangement in the E2-active site that exposes the side chain of an asparagine residue, which is essential for catalysis and is conserved throughout E2s (64). Interestingly, in the available three-dimensional structures of free E2s (i.e., not bound to E3 or ubiquitin), the essential asparagine side chain faces away from the catalytic cysteine and frequently forms hydrogen bonds with the polypeptide backbone. However, in the three-dimensional structure of a SUMO-RANGAP1-Ubc9-Nup358 "product complex," the asparagine side chain rotates toward the active site into the vicinity of the C-terminal glycine of SUMO (65). In this position, the side chain might stabilize the oxyanion transition state intermediate that develops during transfer of ubiquitin to substrate (64). Rotation of this asparagine may function as an "AND" gate that requires that E2 be both thioesterified and bound to E3.

Autoubiquitination: attachment of ubiquitin to either the E2 or E3 subunit within an active E2-E3 complex

HOW DO RING-E2 COMPLEXES INITIATE AND EXTEND POLYUBIQUITIN CHAINS?

In this section, we explore how ubiquitin is transferred from E2 to a substrate molecule bound to E3. Once a pioneer ubiquitin is attached to substrate, it can prime the polymerization of different types of ubiquitin chains that have distinct biological functions.

E2s and Substrates Bind RING E3s at Distant Sites

The chemistry of substrate ubiquitination by RING E3s occurs at the thioester bond that links ubiquitin to E2. Thus, the selection of sites for ubiquitin conjugation boils down to the ability of different lysine residues in the substrate to gain access to the thioester bond. How this occurs remains unclear. In a traditional bisubstrate enzyme, both substrates are held in close apposition to facilitate their chemical reaction (**Figure 9*a***). By contrast, for the RING E3s that have been examined to date by X-ray crystallography, the substrate-docking site on RING E3s is located 50–60 Å from the anticipated location of the thioester bond on docked E2∼Ub (11, 42, 66–68) (**Figure 9*b***). Experiments with peptide substrates that display a target lysine at different distances from where the peptide contacts SCF revealed that a substrate can be ubiquitinated by SCF even if its lysine theoretically cannot "reach" the E2∼Ub based on the relative position of these elements in a computationally docked complex (69). Deffenbaugh et al. (70) argued that E2∼Ub must dissociate from the RING and encounter substrate by diffusion. However, this idea has been disputed (71). As discussed below in the section titled, "RING E3s Can Be Regulated by Conjugation with Ubiquitin Family Proteins," the gap between substrate and E2∼Ub in cullin-RING E3s is most likely bridged by a major conformational change in the E3 structure.

It is commonly assumed that selection of lysine residues within substrates is based largely on their accessibility and not on primary sequence context. This is based in part on the observations that the sites of ubiquitination in SCF substrates, such as IκB (72) and Sic1 (73), reside in supposedly unstructured regions that are located near the degron elements that bind to the E3. Moreover, the sequences that immediately flank ubiquitination sites lack convincing homologies, although few sites have been mapped. This assumption has been challenged by the recent discovery of a sequence element,

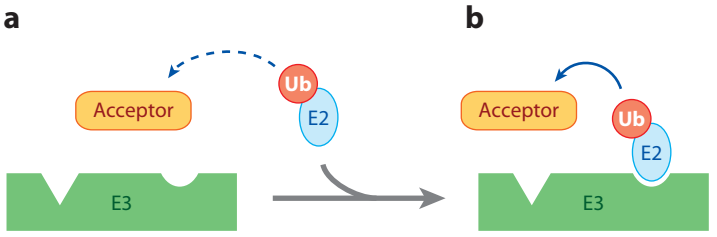


Figure 8

RING E3s activate E2s. (*a*) An E2∼Ub thioester complex has an intrinsic ability to discharge its ubiquitin cargo to a variety of acceptors including ubiquitin itself and low-molecular-weight nucleophiles. However, this reaction typically occurs at a depressed rate (*dotted arrow*). (*b*) The rate of discharge of ubiquitin from E2∼Ub is increased in the presence of E3 (*solid arrow*). The magnitude of increase can be quite large, ranging up to 87-fold for UbcH5b activation by C-NOT4. The mechanism underlying this effect is not known but is thought to be attributed to conformational changes that occur within E2∼Ub upon binding to E3.

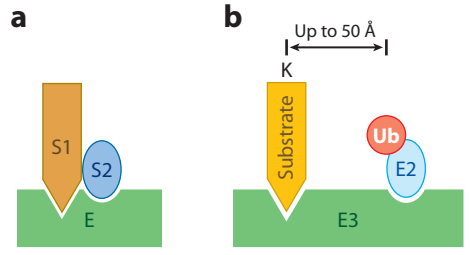


Figure 9

RING E3s comprise an atypical class of bisubstrate enzymes. (*a*) In a conventional bisubstrate enzyme (E), the two substrates (S1 and S2) are held in close approximation to facilitate chemical reaction between them. (*b*) In Skp1-Cullin-F-box (SCF), the RING E3 for which we have the most detailed structural information, the substrate lysine (K) and the ubiquitin (Ub) thioester bond can be separated by many angstroms. The great separation of the reactants implies that conformational changes must occur during the enzymatic cycle to bring them into closer apposition.

Anaphase-promoting complex/cyclosome (APC/C): an E3 that promotes the degradation of numerous regulatory proteins during mitosis

the TEK box, that mediates the assembly of Lys11-linked ubiquitin chains and is found adjacent to sites of ubiquitination in APC/C substrates (74). Functionally equivalent elements could govern selection of substrate lysines by other E3-E2 complexes. Another factor that could contribute to the reactivity of a lysine is the presence of nearby basic residues that could depress its p*K* and thereby enhance its reactivity (75). Vicinal lysines, which are sometimes found near degron sequences (e.g., IκB) (72), may contribute to each other's ubiquitination via p*K* suppression.

Chain Initiation and Elongation Are Separable Processes

Assembly of ubiquitin chains on the Cdc34-SCF substrate Sic1 can be broken down into two steps: chain initiation (**Figure 10*a***) and chain elongation (**Figure 10*b***) (54, 55). The chain initiation step, which corresponds to attachment of ubiquitin directly to substrate, is slow and presumably sequence nonspecific. The chain elongation step corresponds to the formation of a ubiquitin-ubiquitin isopeptide bond to attach the *n+1* ubiquitin to a chain that contains *n* ubiquitins. For Cdc34, this step is 5–30-fold faster than initiation and is highly specific for the Lys48 residue of ubiquitin. Chain initiation and elongation are clearly distinct in that they have distinct molecular requirements (54).

For other RING ligases, chain initiation and elongation may be carried out by separate E2s. The in vitro observation that UbcX is more adept at promoting mono- or oligoubiquitination of an APC/C substrate, whereas Ubc4 assembles long polyubiquitin chains, led to the proposal that these E2s may operate sequentially (76). A similar idea emerged recently to explain the ubiquitination of APC/C substrates in budding yeast (77). Yeast Ubc4 and Ubc1 appear to operate as a "tag team" both in vitro and in vivo to ensure processive ubiquitination and degradation of APC/C substrates, with Ubc1 extending chains initiated by Ubc4. This does not appear to be a general mechanism of action for APC/C, however, because human APC/C appears to work best with UbcH10 (which is not found in budding yeast), and not with Ubc1 (74, 78). Further evidence to support the concept of tag team E2s was provided by a recent report that identified physical and functional interactions between Brca1-Bard1 and six human E2s (48). Four of the E2s direct monoubiquitination of Brca1. In contrast, Ubc13-Mms2 and Ube2k direct the synthesis of ubiquitin chains on Brca1 that has been modified by a monoubiquitin-attaching E2.

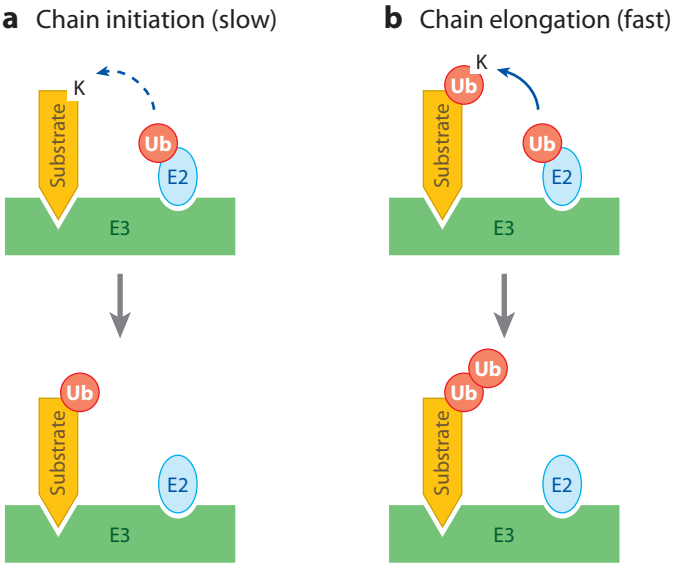


Figure 10

(*a*) Substrate polyubiquitination by RING E3s can be resolved into a slow chain initiation step (*dashed arrow*) followed by rapid chain elongation (*b*) (*solid arrow*). Specific recognition of conjugation sites within ubiquitin and closer approximation of the acceptor lysine (K) and E2~Ub may both contribute to the enhanced rate of the second step.

Ubiquitin Chains Can Be Built on Substrate or Transferred En Bloc

Although most studies assume that ubiquitin chains are built upon substrate by the successive addition of ubiquitin molecules to the distal end of a growing chain (a.k.a. the sequential mechanism) (**Figure 6*a***), ubiquitin chain assembly can potentially proceed by several different mechanisms (79). Ube2g2 can attach

ubiquitin chains to substrates by a mechanism that differs strikingly from the classic sequential mechanism (80). The Lys48 residue of ubiquitin thioesterified to Ube2g2 can attack a second Ube2g2~Ub to form Ube2g2 thioesterified to diubiquitin (81; Y. Ye, personal communication). Repeated cycles of attack on fresh Ube2g2~Ub molecules by the distal Lys48 of a growing chain leads to long thioester-linked polyubiquitin that can be transferred en bloc to substrate (**Figure 6*b***). This mode of assembly, which requires proximity of two molecules of Ube2g2, could explain the existence of an essential E2-binding element in gp78 in addition to the RING domain (49). The applicability of this mechanism to ubiquitination reactions within cells is uncertain because, in budding yeast, polyubiquitin thioesterified to the orthologous Ubc7 does not detectably accumulate except under circumstances where Ubc7 is unstable, in which case the thioester-linked chain may serve as an autologous degradation signal to rid the cell of excess Ubc7 (82).

Roles of E2s and RING E3s in Generation of Ubiquitin Chain Topology

The RING ligases that have been characterized to date yield different patterns of substrate ubiquitination. A number of RING ligases primarily monoubiquitinate or oligoubiquitinate their substrates. Prominent examples include ubiquitination of p53 by Mdm2 (83) and histone H2B by Bre1-Rad6 (84, 85). Ubiquitination may terminate after the transfer of only one or a few ubiquitins because the substrate simply falls off the E3 before the reaction can proceed further. However, in the case of Met4, its oligoubiquitination is specified by an internal ubiquitin-binding domain that caps the growing chain and prevents its further extension (86). Yet other E3-E2 pairs transfer multiple ubiquitins to substrate, but in the form of several monoubiquitins rather than a polyubiquitin chain. Quantitative mapping of ubiquitin attachment sites by mass spectrometry revealed that the first few ubiquitins transferred to cyclin B by Ubc4-APC/C are distributed over several lysines (87). Multiple monoubiquitination can directly promote degradation of cyclin B in vitro (R. King, personal communication) but most often probably does not function as a proteasome-targeting signal in vivo, as exemplified by modification and lysosomal targeting of the EGF receptor by c-Cbl (88). Other RING-E2 complexes transfer multiple ubiquitins to substrate preferentially in the form of a polyubiquitin chain. RING-E2 complexes can assemble chains linked exclusively via the Lys6 (89, 90), Lys48 (4, 91), and Lys63 (92) residues of ubiquitin. Ultimately, all seven lysine residues of ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) can form chain linkages in vivo (93). Thus, the number of topological isomers is potentially huge if chains of mixed linkage are produced. For example, if an E3-E2 complex can elongate a ubiquitin chain via any lysine of ubiquitin, chains with a length of four would comprise 243 (7^3) different isomers, and the number would increase even further if branched chains (94) are considered.

The nature of ubiquitin conjugation by E2-E3 complexes is critical because the outcome of ubiquitination is usually determined by the topology of the conjugate. Thus, it is important to understand how E2-E3 complexes build different types of ubiquitin chains. Pure Ubc13-Mms2 assembles free Lys63-linked chains (92) and pure Cdc34 (95), E2-25K/Ubc1 (96), or Ubc7/Ube2g2 (97) assemble free Lys48-linked chains even in the absence of an E3, and thus chain linkage specificity is an intrinsic property of these E2s (**Figure 11**). Several examples have been described where a single RING can recruit different E2s that have different linkage specificities. In each case, the output of the reaction is determined by the known specificity of the E2 (48, 94). This is the opposite of what occurs with HECT domain ligases, where the chain linkages of the product are determined by the E3 (98). Taken together, these observations suggest that the nature of the reaction product that is formed, be it a substrate-ubiquitin or ubiquitin-ubiquitin linkage, is determined by the identity of the UPS enzyme that forms

UPS: ubiquitin-proteasome system

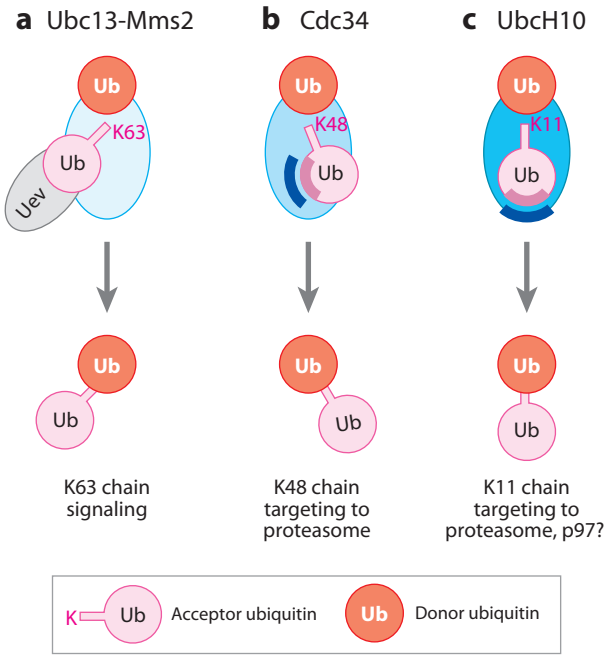


Figure 11

Assembly of different ubiquitin linkages by E2s. (*a*) Ubc13-Mms2 is a heterodimeric E2. The acceptor ubiquitin binds to the catalytically inactive Mms2 subunit such that its Lys63 is positioned to attack the thioester bond that links the donor ubiquitin to Ubc13. The Lys63-linked chains that are formed function in signaling. (*b*) Acceptor ubiquitin binds via its hydrophobic patch (*dark pink crescent*) to Cdc34, such that its Lys48 is positioned to attack the thioester. The "acidic" loop of Cdc34 (*dark blue crescent*) may help position acceptor ubiquitin on the surface of Cdc34 but is not required for binding. The Lys48-linked chains that are formed target substrates to the proteasome. Ube2g2 is very similar to Cdc34 and most likely functions the same way. (*c*) Acceptor ubiquitin binds via its TEK box (*dark pink crescent*) to an unknown site (*blue crescent*) on UbcH10, such that its Lys11 is positioned to attack the thioester. Modification of anaphase-promoting complex/cyclosome substrates with Lys11-linked chains is essential for their turnover by the proteasome. Curiously, p97 complexes are enriched for Lys11-linked chains.

the last thioester intermediate with the ubiquitin that is being transferred. However, findings discussed below suggest that this principle may not extend to all E2s.

It is striking to contrast the behavior of Ubc13-Mms2, Cdc34, E2-25K/Ubc1, and Ube2g2 with that of Ubc4, UbcH5, and UbcH10. Whereas the former readily synthesize diubiquitin, preferentially polyubiquitinate substrates, and build chains of a single predetermined linkage regardless of their E3 partner, Ubc4 and UbcH5 have low activity in diubiquitin synthesis (55, 99) and preferentially attach multiple monoubiquitins to cyclin B (87). What is remarkable about UbcH5 in particular is that its capacity to make different linkages can vary. UbcH5c produces a mixture of Lys11, Lys48, and Lys63 linkages with APC/C, Murf, Chip, and Mdm2 (87, 94). However, when assayed in conjunction with Brca1-Bard1, it synthesizes predominantly Lys6-linked chains (89, 90), and with the heterodimeric TRIAD subfamily E3 Hoil-1L-Rbck1/Hoip, it generates linear (i.e., linked together via the N terminus) ubiquitin chains (100). A few possibilities can account for the unusually promiscuous and variable behavior of these E2s. It is possible that there exist multiple distinct, isoenergetic, ubiquitin-E2∼Ub noncovalent complexes for UbcH5 et al. that favor different chain linkages. Alternatively, the linkages that form may reflect the potential of each individual lysine of ubiquitin to collide randomly with the thioester. The latter seems likely for Ubc4 and UbcH5 based on the observation that the k_{cat}/K_m for ubiquitination of an APC/C or SCF substrate is the same regardless of whether the substrate is unmodified or monoubiquitinated (55, 77, 101), which is in stark contrast to what is seen for Cdc34-SCF and Ubc1-APC/C (54, 55, 77) and argues against the existence of a strong noncovalent binding site that positions the attacking ubiquitin. Ironically, UbcH5b does, in fact, possess a noncovalent binding site for ubiquitin that can enhance ubiquitin polymerization in a model system with Brca1 (102). However, UbcH5's similar k_{cat}/K_m values for unmodified and monoubiquitinated SCF substrates argue against a role for this site in substrate polyubiquitination reactions (55). Lastly, RING E3s could influence chain topology by providing noncovalent docking sites that orient the acceptor ubiquitin for attack. Known and putative ubiquitin-binding domains are, in fact, present in many E3s (33, 103–105). Such domains are particularly prevalent in the TRIAD subfamily of RING ligases to which Hoil-1L belongs; many E3s in this subfamily contain at least one type of ubiquitin-binding domain, such as UBA, NZF, UIM, and UEV (Kay Hofmann, personal

communication). If Ubc4 and UbcH5 do not possess a high-affinity noncovalent binding site for the attacking (i.e., acceptor) ubiquitin, there would be minimal energetic barrier for E3 to overcome to impose a particular linkage specificity by providing its own ubiquitin-binding domain to orient the acceptor ubiquitin.

Whereas UbcH10 is similar to UbcH5 in forming multiple different ubiquitin-ubiquitin linkages (87), it differs in an important respect. Formation of Lys11 linkages by UbcH10 requires a TEK-box sequence within ubiquitin (74), suggesting the existence of a noncovalent binding site for ubiquitin on UbcH10 that positions Lys11 to attack the thioester. It remains uncertain why UbcH10—unlike Cdc34, Ube2g2, and E2-25K/Ubc1—is not more restricted in the type of ubiquitin linkages that it forms.

Returning to the E2s that make ubiquitin chains of specific linkage, a key question is, how do they do it? The genesis of ubiquitin chain linkage specificity is best understood for the synthesis of Lys63-linked chains by the heterodimeric E2 Ubc13-Mms2 (92). The basis for this activity was revealed by the crystal structure of the Ub~Ubc13-Mms2 complex (106). In the crystal, ubiquitin oxyesterified to the Ubc13 subunit of one complex binds noncovalently to Mms2 in an adjacent complex, such that the ε-amino group of Lys63 is within 3 Å of the oxyester bond. This work forged what is likely to be a general principle for ubiquitin chain synthesis by RING E3s: Chain linkage specificity arises because there exists a noncovalent binding site for ubiquitin on E2 that positions a particular lysine residue of the acceptor ubiquitin to attack the thioester bond. Although the mechanisms are less well understood, the same principle almost certainly underlies the abilities of Cdc34 (95), Ube2g2/Ubc7 (97), and E2-25K/Ubc1 (96) to specifically assemble Lys48-linked chains. For Cdc34, linkage specificity involves the acidic loop immediately C-terminal to the active-site cysteine as well as hydrophobic patch residues on ubiquitin (54). Processive chain synthesis by E2-25K/Ubc1 requires the C-terminal ubiquitin-binding UBA domain, but chain linkage specificity is intrinsic to the catalytic domain (77, 99).

RING Domain E3s Often Function as Oligomers

RING domain proteins multimerize in a bewildering number of ways. Some RING domain proteins form heterodimers, such as Mdm2-MdmX (41), Ring1b-Bmi1 (40), and Brca1-Bard1 (39). In these particular cases, only Mdm2, Ring1b, and Brca1 have significant E2 binding and E3 activity, and in the case of Brca1-Bard1, Bard1 may serve solely a structural role as it does not contact the E2 (43). In this case, the RING-RING interaction would conceptually be no different than any other interaction that might stabilize a RING domain's structure or tether it to a larger complex (e.g., cullin-RING interaction). However, we note that, because the E2s that have been used are not thioesterified with ubiquitin, there may be more than meets the eye. RING E3s can also homooligomerize, as has been reported for Traf (107), Siah (108), cIAP (63), and c-Cbl (109) RING ligases, as well as for the Prp19 (110) and CHIP (62) U-box proteins. Yet more complexity is introduced by the ability of heterodimeric RING ligases, such as Mdm2-MdmX, to oligomerize (59), and of certain RING domains to self-assemble into supramolecular structures (58). In addition, some multisubunit SCF-type E3s also dimerize (111).

The molecular basis for RING E3 dimerization is quite variable. For example, CHIP homodimerizes via hydrophobic patches that mediate U-box-U-box interaction as well as via N-terminal helical hairpins in each protomer that pack to form a four-helix bundle (62). Although both the U-box and helical hairpin interfaces have local twofold axes of symmetry, the axes are tilted 30° with respect to each other. As a consequence, the substrate-binding TPR domains have markedly different dispositions with respect to the U-boxes. The TPR domain of one subunit interacts with its cognate U-box, occluding the recruitment of E2. Thus, the intact dimeric complex can only

recruit a single molecule of E2. Other RING E3s dimerize via domains that are directly adjacent to the RING. In these cases, dimerization could mask one of the RING domains as observed for CHIP, or there may be direct interplay between two E2s bound to a dimeric complex. By contrast, some E3s dimerize via domains that are distal from the RING in the primary sequence, such as in the case of c-Cbl's C-terminal UBA domain (109). An extreme example is provided by the SCF heterotetramer, which dimerizes via a small peptide segment within the substrate receptor subunit, placing the Rbx1 subunits at opposite ends of an extended, ~300-Å long dimer (111).

The functional impact of RING E3 oligomerization remains poorly understood. The best defined case is the SCF^{Cdc4} complex. The F-box subunit Cdc4 contains a short dimerization domain (i.e., D domain) that mediates dimerization of the entire tetrameric complex (111). Dimerization of SCF^{Cdc4} is important; monomeric SCF^{Cdc4} complexes that contain inactivating point mutations in the D domain retain E3 activity, but the pattern of substrate ubiquitination is less processive, and dimerization-defective *CDC4* does not complement a *cdc4*Δ mutant. Dimerization of SCF complexes can also be modulated by phosphorylation of residues adjacent to the D domain as a means to regulate activity (112). The molecular basis of why dimerization of SCF^{Cdc4} is important remains controversial. Tang and colleagues (111) emphasize the potential advantage of having two E2s at distal ends of the dimer focused on the ubiquitination of a single substrate bound near the center of the complex, whereas others (67, 113) have proposed that dimerization increases affinity for substrates bearing multiple Cdc4 ligands via an avidity effect. The latter proposal seems more appealing, especially in light of the observation that some F-box proteins, such as Skp2, lack a D domain, and consequently, the SCF^{Skp2} heterotetramer is monomeric.

One interesting possibility is that dimerization of E3s in some signaling pathways may be functionally equivalent to dimerization of receptor tyrosine kinases in that a signal induces formation of a dimer in which one protomer ubiquitinates the other, yielding a mark that serves as a platform to assemble a signaling complex. Consistent with this idea, autoubiquitination of TRAF6 Lys124 is essential for the ability of TRAF6 to signal to its downstream target in the NF-κB pathway (114).

In the case of other E3s, the functional significance and molecular rationale for multimerization is not as well defined. Dodecamers of Brca1-Bard1 have dramatically elevated E3 activity compared to the Brca1-Bard1 heterodimer in an autoubiquitination-type assay (58). Likewise, higher-order Mdm2 complexes appear to have a reduced K_m for E2 (59). Unambiguous interpretation of these results awaits assays with physiological substrates for these ligases.

REGULATION OF RING E3s

The mechanistic insights that have been gleaned from biochemical investigations on RING domain ubiquitin ligases provide a framework for investigating regulatory mechanisms that govern ligase activity. In this section, we describe how the activity of RING domain ligases is controlled posttranslationally by covalent modifications such as phosphorylation or conjugation with ubiquitin-like proteins, by noncovalent binding of protein or small-molecule ligands, or by competition among substrates.

Ubiquitination Is Often Regulated by Substrate Modification

Phosphorylation regulates almost every biological process in which its role has been tested, and ubiquitination by RING E3s is no exception. Broadly speaking, phosphorylation influences the action of these enzymes either through effects on the substrate, E2, or E3 itself. Of these, substrate phosphorylation is the most prominent and well-understood mechanism. The general paradigm for control of ubiquitination by substrate phosphorylation emerged

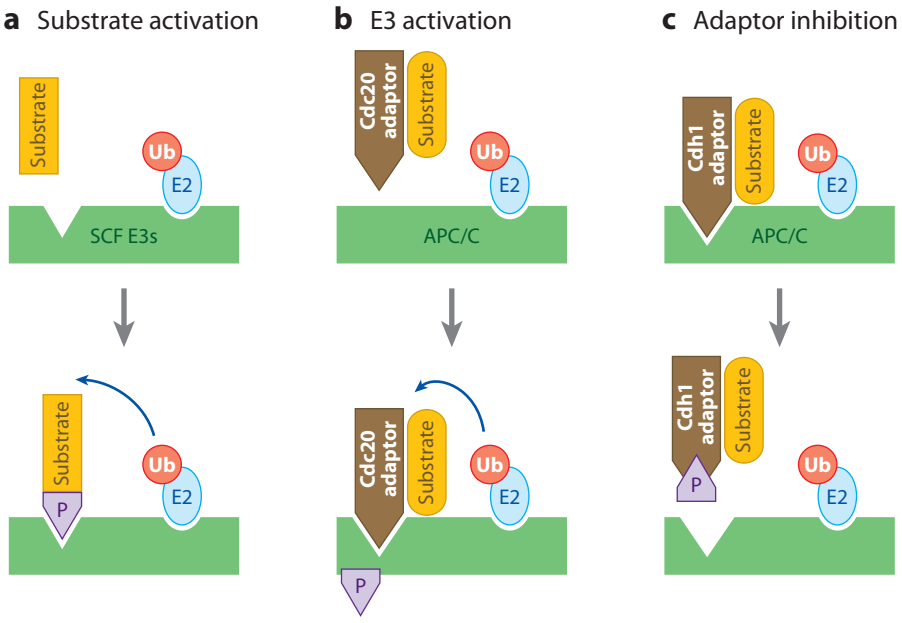


Figure 12

Phosphorylation regulates RING E3s by diverse mechanisms. (*a*) Substrates of Skp1-Cullin-F-box (SCF) and other cullin-RING ligases typically must be modified—most often by phosphorylation—before they can bind the E3 and be subject to ubiquitination. (*b*) Anaphase-promoting complex/cyclosome (APC/C)-Cdc20 is activated by phosphorylation during mitosis. Although the mechanism is not fully understood, phosphorylated APC/C binds more tightly to the substrate activator Cdc20. (*c*) Conversely, APC/C-Cdh1 is inhibited by phosphorylation during mitosis because phosphorylated substrate activator Cdh1 is unable to bind APC/C.

from studies on Sic1 ubiquitination by the ubiquitin ligase SCF^{Cdc4} in budding yeast (91, 115, 116). Sic1 is stable during the early G1 phase of the cell cycle. When G1 cyclin-Cdk is activated in late G1 phase, it phosphorylates Sic1 on nine or more sites, a subset of which are important for its ubiquitination (115). Phosphorylated Sic1 (but not the unmodified protein) binds the Cdc4 subunit of SCF^{Cdc4}, which positions it for ubiquitination by the E2 enzyme Cdc34 (**Figure 12*a***) (91, 116). The phosphodegron of another SCF^{Cdc4} substrate, cyclin E, has two phosphorylated residues that each make direct contacts with basic pockets on the surface of Cdc4 (66, 67). Similar findings have been made for the phosphodegrons from β-catenin (69) and p27 (68), both of which make direct contact with the receptor subunits of their respective SCF ubiquitin ligases. Indeed, the vast majority of CRL substrates are targeted to their respective ligase by a covalent modification, including glycosylation (117) and proline hydroxylation (118, 119). In an interesting twist, sumoylation of some proteins recruits the heterodimeric RING E3 Slx8-Rfp, which ubiquitinates the sumoylated protein (120–123). For a detailed review of substrate degrons, please consult Reference 124.

The observation that Sic1 ubiquitination requires multiple phosphorylation sites suggests the possibility that Sic1 turnover is ultrasensitive to levels of its kinase, G1 cyclin-Cdk (115). Indeed, Sic1 must possess any combination of six phosphorylation sites to bind tightly to Cdc4, and as a consequence, Sic1 turnover may behave in a switch-like fashion as G1 cyclin–CDK activity increases during the late G1 phase (125). The basis of how Cdc4 "measures" the multisite phosphorylation of Sic1 remains unclear (67, 126). Tyers and colleagues favor the

idea that the individual phosphorylation sites constitute low-affinity ligands, each of which can interact with a single binding pocket on Cdc4 (125, 127, 128). Multiple low-affinity ligands when present on the same molecule may behave equivalently to a single high-affinity ligand. By contrast, Pavletich's group noted that Cdc4 has two basic pockets that bind the phosphates in the bisphosphorylated cyclin E degron (67). The presence of two molecules of Cdc4 within each dimeric SCF holoenzyme (111) yields four phosphate-binding pockets per complex, each of which may need to be occupied to stabilize the association of Sic1 with SCF^{Cdc4}. Further work is needed to resolve the exact role of multisite phosphorylation.

E2s and RING E3s Can Be Regulated by Phosphorylation

In addition to regulating substrate competence, phosphorylation can also directly regulate the activity of E2s and RING-based E3s. The first such example emerged from the study of APC/C (129). Phosphorylation of budding yeast APC/C subunits by mitotic Cdk enhances ubiquitin ligase activity by a mechanism that appears to involve recruitment of the activator protein Cdc20 (**Figure 12*b***) (130). Conversely, phosphorylation of the APC/C activator Hct1/Cdh1 by mitotic Cdk inhibits its activity by preventing its binding to APC/C (**Figure 12*c***) (131). More detailed accounts of APC/C regulation by phosphorylation can be found elsewhere (132, 133). No doubt, many other RING E3s are also regulated positively and/or negatively by cycles of phosphorylation and dephosphorylation.

E2s that collaborate with RING E3s are regulated by phosphorylation. Cdk-mediated phosphorylation of Ser120 activates human and yeast Rad6/Ubc2 (134). Human and yeast Cdc34 are also phosphorylated, in this case by casein kinase 2 on up to five sites scattered throughout the catalytic and C-terminal tail domains (135–140). Phosphorylation of Cdc34 has a variety of effects on activity and localization.

In addition to phosphorylation, other chemical modifications can influence E3 activity. For example, acetylation of residues within the RING domain of Mdm2 by CBP/p300 inhibits ligase activity in vitro by an unknown mechanism (141).

RING E3s Can Be Regulated by Conjugation with Ubiquitin Family Proteins

Perhaps the most pervasive means by which RING E3s are regulated posttranslationally is through conjugation of ubiquitin or ubiquitin-like proteins. Many RING E3s are known to be ubiquitinated, often by an autocatalytic process. Autocatalytic ubiquitination can be a simple consequence of E3 activity with no functional impact. Alternatively, it could lead to downregulation of E3 activity owing to degradation by the proteasome. Autocatalytic regulation of this sort was noted for the multisubunit SCF complex wherein E2 bound to the RING subunit catalyzes ubiquitination of the F-box subunit, resulting in its degradation (142, 143). Autoubiquitination might serve as a homeostatic mechanism wherein bound substrate shields an F-box from ubiquitination, resulting in accumulation of the F-box protein only when its substrate is present (144, 145). Negative regulation via autoubiquitination has also been noted for Mdm2 (83). Whereas p53 is clearly a key target of Mdm2, autocatalytic turnover of Mdm2 has been thought to play a critical role in titrating its activity. Indeed, a deubiquitinating enzyme that binds and deubiquitinates Mdm2, Hausp, plays an important role in the Mdm2-p53 circuitry (146, 147). Mutation of Mdm2's RING domain eliminates E3 activity but does not block its turnover in vivo, suggesting that Mdm2 stability can also be regulated in *trans* by other E3s (46). Another prominent example of negative regulation by autoubiquitination is provided by the inhibitor of apoptosis (IAP) proteins (148). In cell death signaling, the RING domain of XIAP serves two functions: It promotes turnover of caspase-3 and other putative substrates and functions as

a *cis* degron that can be activated by proteins that promote cell death (149–151). Conversely, in some cases, autoubiquitination has the opposite effect and switches on ubiquitin ligase activity, as has been reported for Bard1-Brca1 (152) and Bmi1-Ring1b (153), although the underlying mechanism remains unknown. Autoubiquitination may also be nonproteolytic and activate signaling, as in the case of Traf6 (see RING Domain E3s Often Function as Oligomers, above). Regulation of RING E3 activity by ubiquitin conjugation can also be mediated by a separate E3. In addition to the Mdm2 example cited above, SCF^{Skp2} is downregulated in early G1 phase by APC/C-dependent turnover of Skp2 (154, 155).

In addition to regulation by ubiquitin, RING E3s are controlled via conjugation of ubiquitin-like proteins. A prominent example is the activation of cullin-RING ligases by attachment of Nedd8 to a conserved lysine on the cullin subunit (**Figure 13**). The Nedd8 conjugation pathway has been reviewed elsewhere and is not covered here (156, 157). The exact mechanism by which conjugation of Nedd8 activates CRLs has been debated. Nedd8-modified Cul1 binds more tightly to Ubc4 (158), possibly owing to direct interaction between Ubc4 and Nedd8 (159). However, the effect of Nedd8 conjugation on E2 recruitment is modest (55). The crystal structure of a Nedd8-modified Cul5-Rbx1 complex reveals that Nedd8 conjugation brings about a massive conformational change (160). In the unmodified complex, Rbx1 is cradled between the winged helix B and four-helix bundle of Cul5 (**Figure 13*b***). Upon its conjugation, Nedd8 binds at the interface of these domains and levers them apart. This frees the RING domain of Rbx1, which springs forth from the

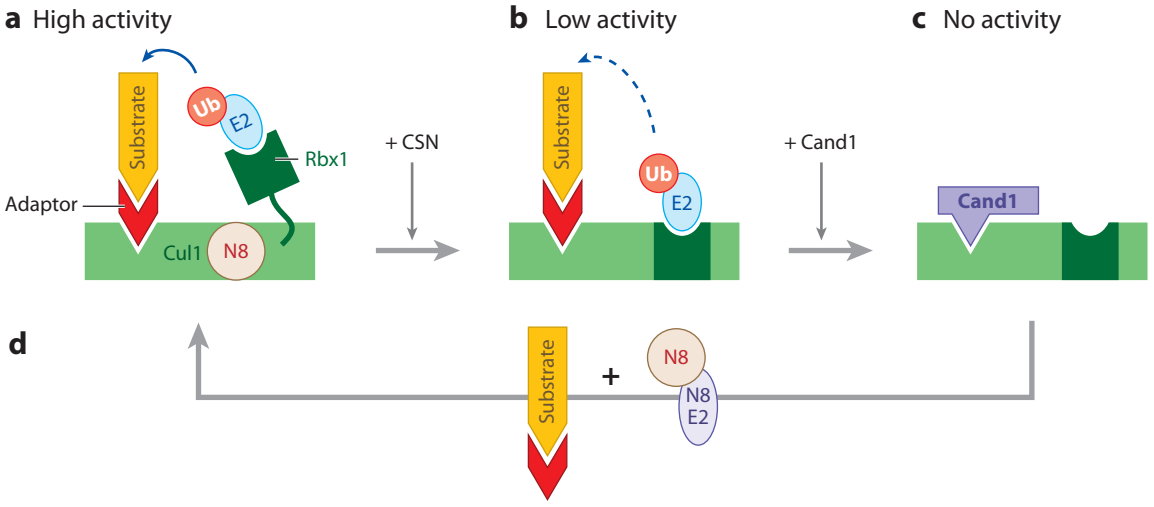


Figure 13

Cullin-RING ligases are regulated by a complex cycle involving covalent modification and a noncovalent binding partner. (*a*) Cullin-RING ubiquitin ligases (CRLs) exhibit maximal activity when the cullin subunit is conjugated with the ubiquitin (Ub)-like protein Nedd8 (N8) on a conserved lysine. This induces a major conformational change that dramatically reconfigures the interface with RING subunit, such that the RING domain extends from the surface of cullin on a flexible tether and can adopt multiple orientations. (*b*) Highly active CRL complexes are converted to a low-activity state by the Cop9 signalosome (CSN), which is an isopeptidase that cleaves the bond that links Nedd8 to cullin. Unmodified cullin forms multiple contacts with the RING subunit, which restricts the mobility of the RING domain. (*c*) Low-activity CRL complexes can be mothballed in an inactive state by binding of the cullin-RING complex to the Cand1 protein. Binding of Cand1 is mutually exclusive with binding of the adaptor-substrate complex and Nedd8 conjugation. (*d*) A functional CRL can be reassembled by the concerted action of adaptor-substrate complexes and the Nedd8 conjugating enzyme, which together can displace Cand1, clearing the way for recruitment of E2∼Ub.

surface of Cul5, remaining tethered by a short linker that can adopt at least two distinct conformations (**Figure 13*a***). This should have a dramatic impact on the relative distance and range of orientations that an E2∼Ub bound to Rbx1/Roc1 can adopt with respect to a substrate bound to the F-box subunit. In support of this, Nedd8 conjugation enhances the k_{cat} for ubiquitin transfer to substrate (55, 160). The major effect of Nedd8 conjugation on SCF foreshadows the potential impact of autoubiquitination that is often seen with other RING E3s.

RING E3 Activity Can Be Controlled by Binding Partners

Binding proteins are emerging as important players in the repertoire of RING ligase regulators. An example of this form of regulation is conferred by the Cand1 protein, which binds to cullins and sequesters them in an inactive state (**Figures 13*c*** and **14*a***) (161, 162). The mechanism of Cand1's action was clarified by the crystal structure of Cand1 bound to Cul1-Rbx1/Roc1. Cand1 forms an extended structure that cradles Cul1 while binding to its N- and C-terminal domains (163). Bound Cand1 simultaneously obscures the Skp1-binding and neddylation sites on Cul1. In the simultaneous presence of a Skp1-F-box complex and Nedd8 conjugation enzymes, Cand1 is displaced from Cul1, and an active SCF complex is formed (**Figure 13*d***) (164).

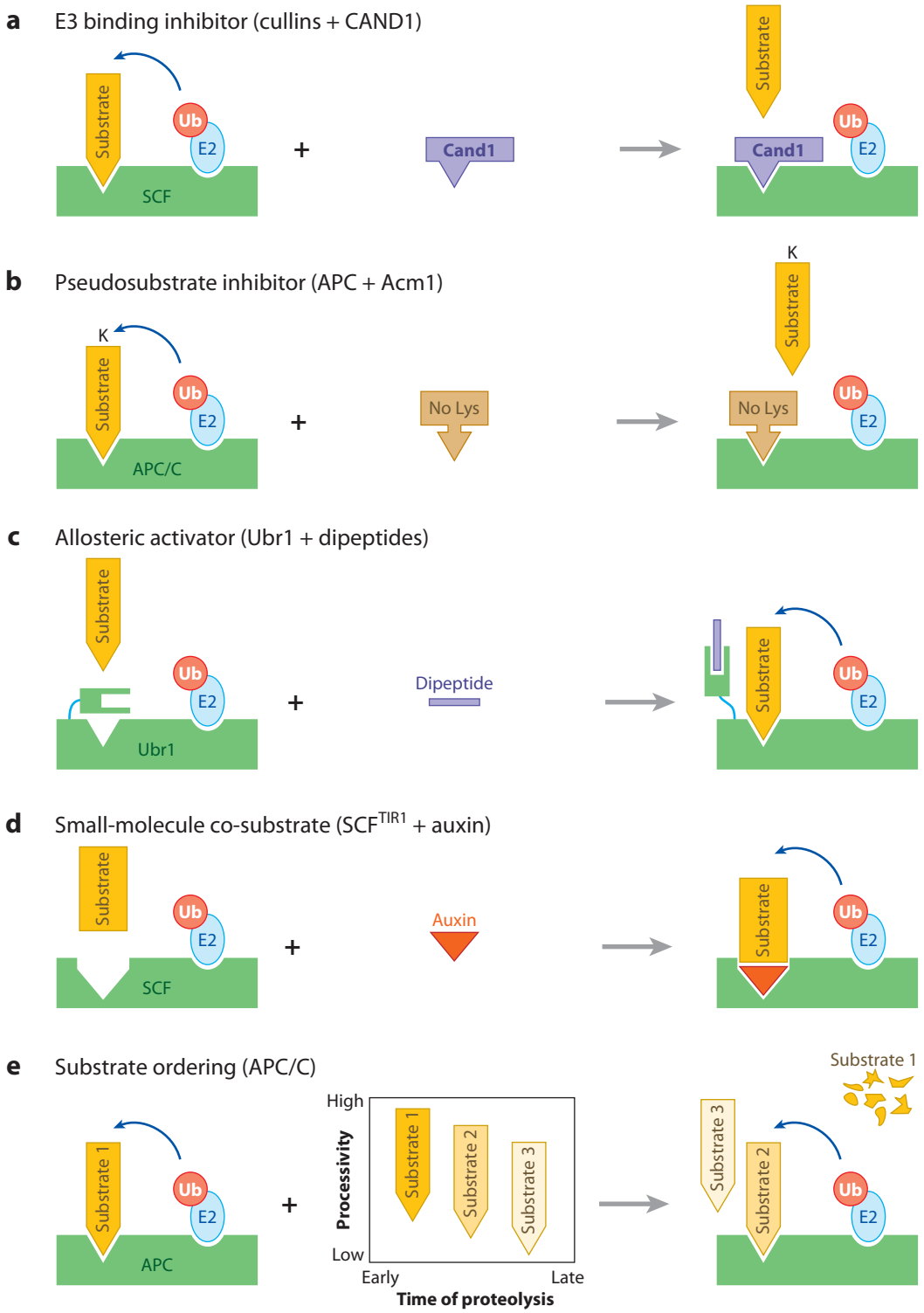
Very recently, pseudosubstrates have emerged as a major new theme in the regulation of RING ligases. Pseudosubstrate regulation was first described for $SCF^{\beta\text{-}TrCP}$, which binds the abundant ribonuclear protein hRNP-U but does not ubiquitinate it (165). hRNP-U competes with authentic substrates for access to β-TrCP, and it was suggested that the ribonuclear protein comprises an "affinity gate" that prevents the spurious ubiquitination of proteins that have poor affinity for the ligase complex. Although the importance of hRNP-U's pseudosubstrate function remains uncertain, this mode of regulation appears to be a major mechanism for control of APC/C activity. The negative regulators Emi1 (166), Mad3 (167, 168), and Acm1 (169–171) all employ destruction box and/or KEN-box motifs normally found in substrates to bind to and inhibit the APC/C activators Cdc20 and Cdh1. Emi1 and Acm1 are specific for Cdh1, whereas Mad3 is specific for Cdc20. An interesting possibility is that these proteins act as substrate imposters that bind APC/C and the activator proteins very tightly but do not present a suitable lysine for modification (**Figure 14*b***). Thus, they remain stuck on APC/C, unable to dissociate to make way for normal substrates (169).

RING E3s Can Be Regulated by Small Molecules

In addition to control by protein ligands, the activity of RING ligases can also be controlled

Figure 14

RING E3 activity can be controlled by multiple distinct noncovalent regulatory mechanisms. (*a*) Skp1-Cullin-F-box (SCF) and other cullin-RING ubiquitin ligases (CRLs) are retained in an inhibited state by the binding protein Cand1. (*b*) Pseudosubstrate proteins that bind E3 competitively with substrate but are not subject to ubiquitination can block activity toward conventional substrates. This mechanism has been observed for both anaphase-promoting complex/cyclosome (APC/C) and SCF complexes. (*c*) Ubr1 possesses an autoinhibitory domain that blocks substrate access. Binding of dipeptide to Ubr1 relieves this inhibition, allowing substrate to bind. (*d*) The plant signaling hormone auxin induces substrate turnover by filling a cavity in the substrate-binding pocket of SCF^{Tir1}. Auxin bound to Tir1 makes contacts with bound substrate and thereby provides binding energy that stabilizes the substrate-E3 interaction. (*e*) APC/C degrades its various cell cycle substrates in a specific temporal pattern. This substrate ordering arises from the differential processivity of the substrates. Highly processive substrates acquire a ubiquitin chain rapidly and consequently are degraded early in anaphase, whereas substrates with poor processivity must rebind APC/C multiple times to acquire a ubiquitin chain and, as a result, are not degraded until G1 phase when all of the more processive substrates have been eliminated.



by small-molecule ligands. The paradigmatic example is the control of Ubr1 by dipeptides (**Figure 14*c***) (172). Dipeptides bind Ubr1 and allosterically enhance its ubiquitin ligase activity toward Cup9, a transcriptional repressor that blocks expression of the mRNA that encodes the dipeptide transporter Ptr2. This creates a positive feedback loop wherein dipeptides enhance expression of Ptr2 and consequently increase the cell's capacity for dipeptide uptake. In contrast to this positive regulation, electrophilic compounds react with Cys151 on Keap1 (kelch-like ECH-associated protein 1, the substrate-binding subunit of a Cul3 complex). This causes Keap1 to dissociate from Cul3 (173, 174), leading to accumulation of the Cul3-Keap1 substrate Nrf2, which induces transcription of a set of genes involved in the antioxidant response.

More recently, a novel mode of E3 regulation by small molecules has been unraveled. The plant hormone auxin fills a cavity in the substrate-binding site of its receptor protein, the F-box protein Tir1, thereby creating additional molecular surface to stabilize the binding of SCF^{TIR1} substrates (**Figure 14*d***) (175). The ability of Tir1 to use a small molecule as a coreceptor to stabilize binding of substrates may be a general theme in signaling by plant hormones because similar mechanisms appear to operate for perceiving jasmonate and gibberellins (176). Small molecules may play a much broader role in regulation of E3 function than is currently appreciated.

Regulation by Substrate Competition

Perhaps the most unorthodox mode of regulating RING ligases is the substrate ordering mechanism proposed by Rape et al. (177). APC/C mediates the turnover of its substrates in a particular order, with securin and cyclin B degraded soon after APC/C is abruptly activated at the metaphase/anaphase transition, and degradation of Aurora A and Plk1 following later. This particular order is crucial; it would be disastrous if Aurora A and Plk1 were degraded before chromosome segregation was initiated by degradation of securin because these proteins play important roles in anaphase. Rape et al. demonstrated that APC/C degrades substrates in sequential order on the basis of their processivity, with the most processive substrates degraded first, and the least processive degraded last (**Figure 14*e***). The most processive substrates have a high probability of acquiring a degradation-competent chain every time they bind APC/C, whereas the least processive substrates must shuttle on and off APC/C multiple times before acquiring a ubiquitin chain of a length sufficient to attract the proteasome. Each time a low-processivity substrate dissociates from APC/C, it runs the risk of being deubiquitinated before it can bind again. Therefore, the least processive substrates must wait until the more processive substrates are cleared before they can repetitively access APC/C with sufficient speed to acquire a degradation-competent chain. Although this model was based on in vitro systems, results in mouse oocytes demonstrate that substrate competition can occur between cyclin B and securin in vivo (178).

NEW DIRECTIONS IN RING E3 RESEARCH

Two major challenges will dominate research on RING E3s in the immediate future. The first of these will be to elucidate the broad physiological context in which each RING E3 operates, ranging from characterizing their biological functions and the defects that arise in their absence, to identifying relevant E2 partners and critical substrates. The second major challenge will be to decipher from a structural and enzymological perspective how RING E3s can catalyze different types of ubiquitin modifications with a high degree of specificity and efficiency.

Functional Screens Identify RING E3s That Regulate Specific Processes

Hundreds of RING E3s confer specificity to ubiquitination and are involved in almost every

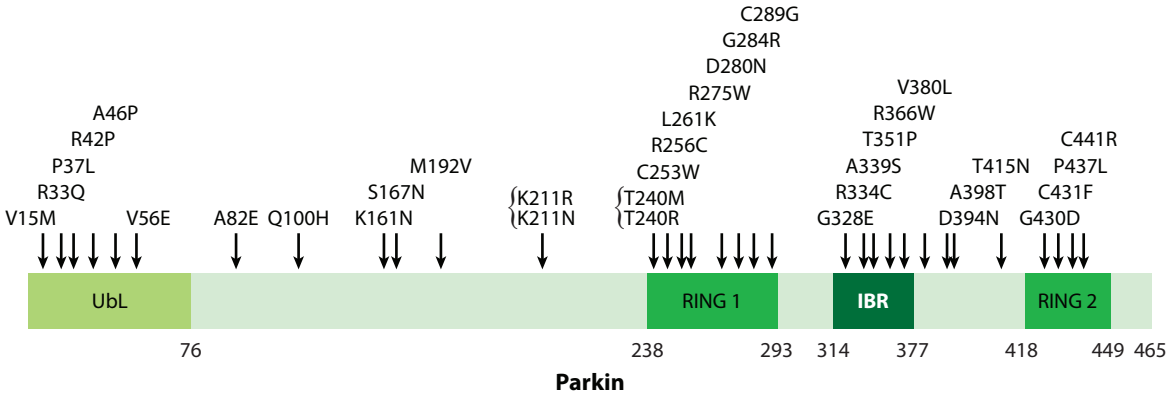


Figure 15

Domain architecture and familial mutations of Parkin (*PARK2*) associated with Parkinson's Disease. Not all mutations associated with familial forms of the disease are indicated (188). Missense mutations are distributed throughout the gene's 12 exons, and the respective amino acid positions are indicated by arrows. UbL stands for the ubiquitin-like domain, and IBR is the in-between RING domain. The domains' amino acid boundaries are shown at the bottom. Courtesy of Ted Dawson, John Hopkins University (189).

cellular process that has been examined. Their involvement in many diseases is also apparent, as illustrated by findings of myriad Parkin mutations in Parkinson's disease (**Figure 15**) and of mutation or dysregulation of Mdm2, VHL, Brca1, and other E3s in cancer. As a substantial fraction of predicted human E3s has not yet been studied at any level, generating a complete inventory of human E3s represented a significant first step to accelerate their characterization (see Bioinformatic Analysis of the RING Family, above) (33). Accelerated progress in this area is also being driven by the development of high-throughput methodologies that allow ubiquitin ligases to be identified on the basis of their role in turnover of a particular protein or their ability to elicit a specific cellular phenotype. For example, a high-throughput RNAi screen identified $SCF^{\beta\text{-}TrCP}$ as a key mediator of REST turnover during neuronal differentiation (179). In a complementary approach, a mitochondria-anchored RING E3, MULAN, was discovered using E3-focused cDNA and siRNA collections to screen for genes that regulate mitochondrial dynamics (33). Widespread use of RNAi and cDNA screens has tremendous potential to help link E3s to specific cellular regulatory pathways and functions and to validate E3s for drug targeting.

Identification of E3 Substrates Is Essential for Understanding Mechanism and Physiology

In many instances, a ubiquitin ligase that affects a specific process is identified in a functional screen, but the substrates that account for the biology are not known. Identifying these substrates is a major challenge, and this is important to understand not only the biology of what the enzyme does, but also the biochemistry of how it works. Unless there is evidence to support that the E3 targets itself in vivo, biochemical studies that monitor autoubiquitination (or other nonphysiological readouts) may arrive at incorrect deductions about the mechanism and regulation of ubiquitination of physiological substrates. For example, Nedd8 conjugation has only a very modest effect on synthesis of free ubiquitin chains by SCF but a much more dramatic effect on substrate ubiquitination (55). Tellingly, small molecules can distinguish between autoubiquitination versus substrate ubiquitination, further suggesting that the former is not necessarily a good model for the latter (180).

Specific ubiquitin ligase substrates have been sought by several distinct approaches, but no single method has cornered the market,

and we are still in need of efficacious, fast, and cheap proteome-wide methods. Conventional protein-protein interaction screens, such as the yeast two-hybrid screen, have succeeded in identifying multiple E3-substrate pairs. Using a clever modification of a conventional pull-down assay, Pagano's group (181) has had considerable success in identifying substrates for the ubiquitin ligase $SCF^{\beta\text{-}TrCP}$. The essence of their approach is to immunoprecipitate β-TrCP, add a ubiquitin-conjugation system plus tagged ubiquitin, and then recover those proteins that incorporate tagged ubiquitin for analysis by mass spectrometry. The advent of green fluorescent protein (GFP) fusion libraries has enabled powerful new methods for substrate identification. In one approach, a collection of yeast strains that each express a different open reading frame (ORF) fused to GFP is screened in a wild-type and mutant background to identify those proteins that accumulate when a specific ubiquitin ligase is absent (182). In another approach, a bar-coded retroviral ORF-GFP library that coexpresses an internal red fluorescent protein (RFP) standard is transfected into cells, which are then subjected to various treatments (e.g., inhibition of a particular E3), sorted by a fluorescence-activated cell sorter according to GFP/RFP ratio, and tracked by microarray hybridization to identify those ORFs whose normalized abundance changes in response to depletion of a specific ligase (183).

A hazard associated with identifying UPS substrates lies in the fact that an important function of the system is general garbage disposal. It is widely known that a significant fraction of proteins can misfold during synthesis, rendering them substrates for the system (184). As a consequence, any proteome-wide effort to identify UPS substrates is bound to uncover a very large number of proteins, including ones that are normally stable and abundant (185). Some RING E3s have been implicated in nonspecific quality control, and some of these same E3s (e.g., gp78 and Ubr1) mediate the regulated turnover of specific substrates (186, 191).

Deeper Insight into Mechanisms Is Urgently Needed

Despite the passage of 10 years since the discoveries that the RING domain mediates E2 binding and ubiquitination, we still know vexingly little about how these fascinating proteins work. E3s are complicated enzymes that carry out different reactions (chain initiation and elongation of chains of different linkages) involving multiple steps, any one of which can potentially be subject to regulation. To understand them, it is imperative that we develop sophisticated assays that allow us to visualize and measure the rates of each step in the reaction cycle (**Figure 4**). It is also essential that we solve more complete structures of RING E3s with all the relevant parts in place as well as in various complexes with E2 and ubiquitin. The more fine-grained our understanding of RING E3 mechanisms becomes, the more accurately we will be able to interpret the impact of drugs, covalent modifications, and mutations on activity.

Another aspect of E3 mechanism that remains challenging is that in many cases it is not known what the physiological E2 partner is. This exposes a critical lacuna in our understanding because the E2 often determines the pattern of ubiquitin conjugates that are formed, which in most cases governs the biological outcome of ubiquitination. Most often, assignment of an E2-E3 pair is approached by screening a limited battery of purified E2s to find the one that works best in vitro with the E3 in question. This approach typically covers at most one-quarter of the known E2s and moreover may bias unfairly in favor of E2s that do not depend on covalent modification of either the E2 or E3 for activity or that survive recombinant expression and storage. The risks inherent in this approach are underscored by recent data suggesting that neither Ubc4 nor UbcH5 is a physiological partner of human APC/C, and their use can obscure a key regulatory control that restricts APC/C activity in the presence of unattached kinetochores (78). It is clear from this example that use of inappropriate E2-E3 pairs could have broad ramifications for a number of studies on

the mechanism and regulation of E2-E3 complexes, as well as in screens for small-molecule inhibitors of these enzymes.

Because E2s are typically not identified in pull-down/mass spectrometry analyses of E3s, how can partners be identified and validated? One approach to identify E3-E2 pairs is to perform two-hybrid experiments. In the case of Brca1, this yielded six potential E2s in addition to the two that were already known (48). It remains unclear whether Brca1 collaborates in vivo with all of these E2s or only a subset of them. It is critical to seek genetic confirmation that a particular E2 functions with a given E3, but this is infrequently done and does not exclude the potential for indirect effects, which loom large for E2s, such as UbcH5, that have been implicated in many pathways. Genetic approaches are also susceptible to redundancy given that the human genome codes for ~40 E2s. Establishing cognate E2-E3 pairs may ultimately require methods such as in vivo cross-linking (187). Determining the proper physiological pairing of E3s and E2s is a difficult but important problem that demands urgent attention.

SUMMARY POINTS

1. RING-based ubiquitin ligases comprise one of the largest families of enzymes in human cells, with over 600 members. These proteins have been linked to the regulation of innumerable cellular processes and multiple human diseases.
2. RING domains recruit E2s that are thioesterified with ubiquitin and also activate E2 to discharge its ubiquitin cargo to a substrate.
3. RING E3s catalyze monoubiquitination or synthesis of polyubiquitin chains assembled via different lysine residues of ubiquitin. These modifications have a range of biological effects, from proteasome-dependent proteolysis (Lys48- and Lys11-linked polyubiquitin) to posttranslational control of protein function, structure, assembly, and/or localization (Lys63 and other linkages).
4. A bewildering array of mechanisms regulate ubiquitination of substrates by RING E3s, including various covalent modifications to the substrates and ligases themselves, allosteric control by small molecules, sequestration by binding proteins and pseudosubstrates, and competition among substrates.

FUTURE ISSUES

1. We need more reliable methods to identify physiological E2-E3 pairs. Identifying the correct E2 partner is key to understanding what the E3 does and how it works.
2. We need better, faster, and cheaper proteome-wide methods to identify E3 substrates and to assign function to known and orphan E3s.
3. We need assays that will allow us to quantify every step in the E3 reaction cycle, which will yield a deeper understanding of enzyme mechanism.
4. We need to capture three-dimensional structures of RING E3s and their coconspirators in all stages of the reaction cycle.
5. We need to capture a greater diversity of RING E3 structures, including intact TRIM and TRIAD proteins and RING E3s with accessory domains, such as those involved in noncovalent ubiquitin binding.

DISCLOSURE STATEMENT

R.J.D. is a founder and shareholder in Proteolix, which is developing drugs against components of the ubiquitin system to treat cancer and inflammation, and is a paid consultant to Genentech. C.A.P.J. is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Annual Review of Biochemistry

Volume 78, 2009

Contents

Gene Expression

Lipid and Membrane Biogenesis

Recent Advances in Biochemistry

Indexes

Errata

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