CMLS, Cell. Mol. Life Sci. 61 (2004) 1546–1561
1420-682X/04/131546-16
DOI 10.1007/s00018-004-4129-5

CMLS Cellular and Molecular Life Sciences

A field guide to ubiquitylation

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Abstract. The capacity for exquisite regulation of ubiquitylation provides eukaryotic cells with a means to fine-tune both protein function and levels. This complex set of processes affects myriad proteins and potentially impacts all cellular processes. Ubiquitylation is brought about through multienzyme processes, with specificity conferred primarily by interactions of substrates with specific ubiquitin protein ligases (E3s) in association with ubiquitin conjugating enzymes (E2s). Regulation of ubiquitylation occurs at multiple levels, including E2-E3 interactions, substrate recognition, chain elongation, binding of ubiquitin to conserved motifs and deubiquitylation. This review presents the fundamentals of the ubiquitin conjugating system.

Key words. Ubiquitin; ubiquitin protein ligase; ubiquitylation; RING finger; HECT; proteasome.

Introduction

The recognition that alteration in protein function by covalent modification with heterologous polypeptides is a common event represents a major conceptual advance in cell biology. While a number of polypeptide modifiers have now been identified [1], by far the most prevalent of these is the one aptly named ubiquitin [2–5]. Ubiquitin is a highly conserved 76-amino acid polypeptide encoded on multiple genes and expressed in all eukaryotes. These genes encode oligomers of ubiquitin or fusions of ubiquitin with other proteins, particularly small ribosomal subunits. When processed to its active monomeric form, ubiquitin can be covalently attached to other proteins through a complex, specific and highly regulated set of processes collectively known as ubiquitylation or ubiquitination. This modification has myriad cellular effects as a consequence of its ability to dramatically alter the fate and function of proteins to which it is attached. In one way or another ubiquitylation regulates almost all cellular processes. Accordingly, alterations of ubiquitylation pathways contribute to the pathogenesis of diseases from cancer to neurodegenerative disorders to viral infections.

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The most well characterized role of ubiquitylation is to render proteins susceptible to degradation by the 26S proteasome. This occurs as a consequence of modification of proteins with chains of four or more ubiquitins linked through lysine 48 (K48) of ubiquitin and the specific recognition of these tagged substrates by the 19S cap of the 26S proteasome [6]. It is now apparent that the proteasome plays important roles beyond simply degrading proteins bearing K48 polyubiquitin chains. Among these functions are specific association with proteins that include ubiquitin domains (UBDs), and certain ubiquitin protein ligases (E3s) [7–9] as well as the targeting of select proteins, such as ornithine decarboxylase, for ubiquitin-independent degradation (see review by Phil Coffino, this issue). The proteasome also includes multiple intrinsic deubiquitylating activities [10–13] (see reviews by Bajorek and Glickman, this issue). Monoubiquitin or polyubiquitin chains linked through either K48 or other lysines, most notably K63, can also have marked effects through proteasome-independent mechanisms including protein kinase activation, DNA repair, modulation of transcription factor activity, and protein trafficking, including endocytosis and lysosomal targeting [14–17].

This review provides an overview of our rapidly evolving understanding of the ubiquitylation system, using specific examples to illustrate fundamental and newly emerging

principles. Other recent reviews that cover specific issues in greater detail are referenced.

Ubiquitylation machinery

In general, ubiquitylation occurs as a result of the sequential action of three classes of enzymes, E1 or ubiquitin activating enzyme, E2 or ubiquitin conjugating enzyme, and E3 or ubiquitin protein ligase (fig. 1). E1, the first enzyme in the ubiquitylation pathway, forms a thiol-ester bond between its active site cysteine and the carboxyl-terminal glycine of ubiquitin. The activated ubiquitin on E1 is subsequently transferred to the active site cysteine of an E2 by transesterification. E3 binds ubiquitin-charged E2 and substrate and facilitates formation of an isopeptide linkage between the carboxyl-terminal glycine of ubiquitin and the ε-amino group of an internal lysine residue on the substrate, or an ubiquitin already attached to the protein [2, 3]. In some cases ubiquitin is attached to the free α-amino group of the substrate rather than to a lysine [18–21].

Substrate specificity is largely determined by the E3. Considering the number of ubiquitylation substrates now known, it is not surprising that database analysis reveals hundreds of predicted E3s. The ability to predict E3s is attributable to the identification of E3 signature motifs, including the HECT (Homologous to E6-AP Carboxyl Terminus) [22], RING (Really Interesting New Gene) finger [23, 24], U-box [25, 26], and PHD (Plant Homeo-Domain) or LAP (Leukemia-Associated Protein) finger domains [27–31].

E1

It is generally believed that a single essential E1 governs ubiquitylation. However, E1- and E2-like domains exist within *Drosophila* dTAF1, and some evidence suggests a role for dTAF1 in histone monoubiquitylation [32]. The significance of this has yet to be established. In mammals utilization of two translation initiation sites results in two E1 isoforms referred to as E1a and E1b [33]. Cells expressing a temperature-sensitive E1 first led to the discovery that ubiquitylation is essential for cell cycle progression and provided in vivo evidence of its role in the proteolysis of short-lived proteins [34, 35]. To activate ubiquitin, E1 binds to MgATP and subsequently to ubiquitin, forming a ubiquitin adenylate that serves as the donor of ubiquitin to the active cysteine in E1 [36, 37]. Each fully loaded E1 carries two molecules of ubiquitin, one as a thiol-ester and the other as an adenylate. The activated ubiquitin is then transferred to the active site cysteine in E2. The carboxyl-terminal glycine of ubiquitin is essential for its activation by E1. The evolutionary conservation in activation for ubiquitin and other ubiquitin-like (UBL) protein modifiers is exemplified both by the presence of a carboxyl-terminal glycine in the active forms of most UBLs, such as SUMO/Pic-1/Sentrin, Nedd8/Rub1, ISG15/UCRP and FAT10 [1, 38], as well as by homology of E1-like molecules that activate UBLs to the ubiquitin E1 [39]. In the case of SUMO and Nedd8 these are heterodimers homologous to the amino and carboxyl portions of the ubiquitin E1 [40, 41].

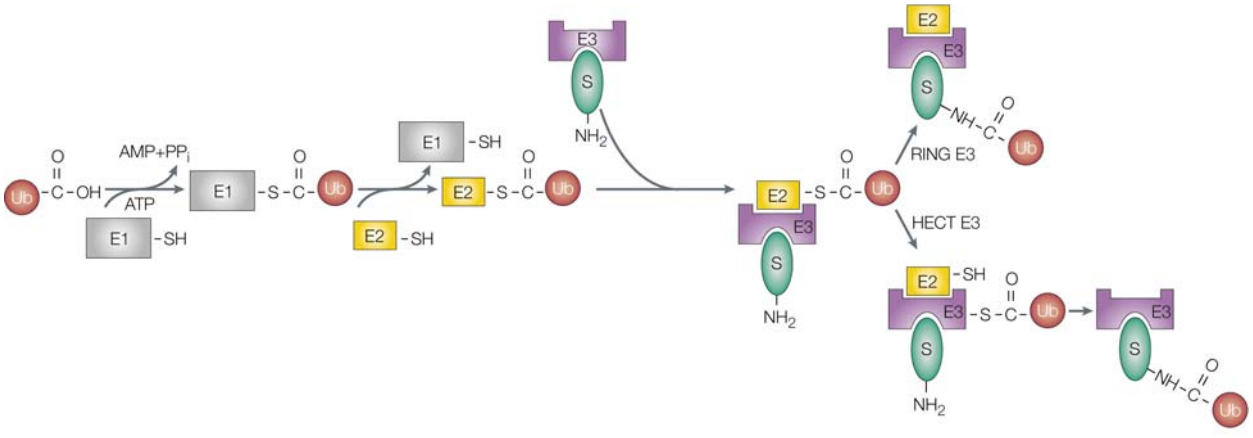

Figure 1. The ubiquitylation pathway. Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s, which may have substrate already bound. For HECT domain E3s, ubiquitin is next transferred to the ac-tive-site cysteine of the HECT domain followed by transfer to substrate (S) (as shown) or to a substrate-bound multi-ubiquitin chain. For RING E3s, current evidence indicates that ubiquitin is transferred directly from E2 to substrate (reprinted with permission from [4]).

E2

The *S. cerevisiae* genome encodes a total of 13 E2-like proteins (Ubc1-Ubc13). Two of these, Ubc9 and Ubc12, are E2s for SUMO and Nedd8, respectively, rather than for ubiquitin [1]. Mammalian genomes include over 30 E2 domains [42]. A conserved ~150-amino acid core domain (UBC) that includes an invariant cysteine that accepts ubiquitin from E1 is the hallmark of E2s. Some E2s have substantial amino- or carboxyl-terminal extensions and some have insertions in the UBC [3]. These sequences may either facilitate or preclude interactions with specific E3s. Similarly, the amino acid composition in predicted or defined regions of contact between E2 and E3 may affect productive E2-E3 interactions. With few exceptions E2s range from 14 to 36 kDa. A striking exception is BRUCE, a 528-kDa polytopic membrane protein that includes multiple BIR repeats and a UBC [43]. Based on the number of potential E3s in the databases, E2s would be predicted to function with multiple E3s. This is borne out by many experimental results. At least in vitro, many E3s also have the capacity to function with multiple E2s; however, there are clear examples of restricted E2-E3 pairings.

E3

E3 domains

The first E3 family characterized was defined by the HECT domain. These were identified as a consequence of the seminal discovery of E6-AP (E6-Associated Protein), as the mediator of HPV E6-dependent ubiquitylation of p53 [44]. It was subsequently recognized that substantial homology to the carboxyl-terminal half of this molecule exists in a number of otherwise unrelated proteins [22]. This highly conserved ~350 amino acid domain is invariably located in the carboxyl-terminal portion of HECT proteins. A cysteine positioned about 35 amino acids upstream of the carboxyl-terminus accepts ubiquitin from bound E2, which is subsequently transferred to substrate. The amino-terminal portions of HECT E3s recognize substrates and regulate subcellular localization [4].

A second E3 family, the largest so far, is defined by the RING finger [23, 24, 45]. RING fingers range from 40 to 100 amino acids. The RING finger is defined by eight conserved cysteines and histidines that together coordinate two zinc ions in a cross-braced fashion [$CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$] [46]. The role of the RING finger in ubiquitylation became apparent several years ago with the determination that a small RING finger protein, Rbx1/Roc1/Hrt1, is essential for multisubunit SCF (Skp1-Cul-F-box) complex E3 activity [47–51], and the demonstration that a number of unrelated RING finger proteins all mediate ubiquitylation [52]. It also became evident that all non-HECT domain E3s that had been identified by this time included a RING finger and that for some of these RING finger-dependent ubiquitylation was demonstrated [53–56]. Since then, numerous RING finger proteins have been shown to mediate ubiquitylation. We are aware of few RING finger proteins that neither mediate E2-dependent ubiquitin nor cooperate with other RING fingers proteins in ubiquitylation when evaluated for autoubiquitylation in vitro with a range of E2s. Whether the RING finger has functions unrelated to ubiquitylation remains to be determined.

Two other motifs related to the RING finger are now implicated in ubiquitylation, the PHD finger and the U-box. The PHD finger is a RING finger variant that includes a cysteine rather than a histidine in the fourth predicted coordinating position and an invariant tryptophan before the seventh zinc-binding residue [57]. Several herpes virus-encoded PHD finger proteins have been implicated in ubiquitylation of major histocompatibility complex (MHC) class I and other membrane proteins in the endoplasmic reticulum and at the cell surface [27, 28, 58]. PHD finger-dependent E3 activities have been demonstrated for mammalian proteins such as MEKK1, which not only activates MAP kinase but also mediates its ubiquitylation [29]. NF-X1, a transcription factor, includes overlapping RING and PHD finger consensus sequences. The PHD but not the RING finger is required for its in vitro activity [31].

The U-box is distantly related to the RING finger in sequence but has no conserved zinc coordinating residues. The first U-box protein implicated in ubiquitylation was UFD2 (discussed below) [59]. Subsequently, CHIP (Carboxyl-terminus of Hsc70 Interacting Protein) was shown to be involved in the degradation of unfolded proteins by functioning as an E3 for Hsp90-interacting proteins [60–62]. Sequence analysis led to the realization that these proteins and others share conserved charged and polar residues and predicted a structure resembling the RING finger [25]. A number of other U-box proteins have now been shown to mediate ubiquitylation in vitro in a manner similar to the RING finger [63].

These signature domains may not constitute the whole E3 universe. For example, HPV (human papilloma virus) ICP0 is a RING finger E3 that has a second non-overlapping domain reported to mediate ubiquitylation of both itself and its E2 in vitro [64]. A provocative recent observation is the finding that UCH-L1, a deubiquitylating enzyme implicated in Parkinson's disease, adds ubiquitin to monoubiquitylated α-synuclein through what appears to be the reverse reaction of its deubiquitylating activity. Thus E1 and E2 are not needed [65]. Zinc, independent of defined E3 modules, has been reported to catalyze ubiquitylation [66]. Thus, there may be much to be learned about the means by which substrate ubiquitylation takes place.

Single and multisubunit E3s

E3s can also be classified as either single or multisubunit. Mdm2, a RING finger E3 for p53, has an amino-terminal p53 binding domain and a carboxyl-terminal RING finger. Together with E1 and E2 it is sufficient to ubiquitylate p53 in vitro [56, 67]. Cbl proteins function in a similar fashion for tyrosine kinase-containing receptors [54, 55], and among the HECT domain E3s Nedd4 can directly mediate ubiquitylation of subunits of ENaC (epithelial amiloride-sensitive sodium channel) [68, 69].

Dedicated multi-subunit E3s include the SCF, APC (Anaphase Promoting Complex)/Cyclosome, and CBC (elongin C-elongin B-Cul2). These contain a RING finger subunit (Rbx1 for SCF and CBC; Apc11 for APC), and a member of the cullin family that binds the RING finger protein (Cul1 for SCF; Cul2 or Cul5 for CBC; and Apc2 for APC) [45, 70–72]. They also include structural adaptors that link the cullin to substrate recognition elements. For the SCF, Skp1 serves this role, and for the CBC, this is performed by the elongin C-elongin B dimer [45]. In the case of the APC, which includes at least 11 essential subunits, the precise architecture and role of individual subunits in its structure and regulation are as yet undefined [70, 73]. A common feature of these E3s is that their structural organization provides a means by which a common core ubiquitin ligase activity can associate with multiple substrate recognition elements and thereby target numerous substrates for degradation [71, 72]. This plasticity is most striking for the SCF E3s. Genomes encode many distinct F-box domains that can potentially associate with the core SCF complex (at least 46 in human and 621 in *Arabidopsis thaliana,* for example) [42].

The distinction between single subunit and multi-subunit E3s is, of course, an oversimplification, as some single subunit E3s function in multi-protein complexes depending on the physiological/pathological context and substrate. For example, although E6-AP ubiquitylates physiological substrates as a single subunit E3, in cells infected with certain oncogenic strains of HPV, viral E6 functions as an adaptor redirecting E6-AP to p53 [44, 74]. The RING finger E3 Siah1 interacts directly with substrates, including DCC, Kid, Bob1 and Numb [75–79]. However, it also associates with adenomatous polyposis coli to ubiquitylate β-catenin [80, 81]. As substrate/E3 pairs are further defined, we should expect this to be a recurrent theme.

E3 function

E3-substrate interactions

A commonly asked question is, what is required for a protein to be recognized as an E3 substrate? In principle any interaction that appropriately juxtaposes an E3 with potential ubiquitylation sites could result in a productive interaction. Thus, a variety of protein-protein interactions mediate the association of E3 and substrate. A few representative examples are described below (fig. 2).

Members of the Cbl family, first identified as adaptor proteins, are RING finger-dependent E3s for activated tyrosine kinases. Cbl binds to activated tyrosine kinases through its amino-terminal tyrosine kinase-binding domain and targets them for ubiquitylation [82, 83]. Recruited Cbl apparently also mediates its own ubiquitylation and also targets other components of the activated signaling complex for degradation [84]. In addition to tyrosine kinases, Cbl proteins interact with a variety of other proteins through SH2- and SH3-dependent interactions; thus it is likely that the full range of Cbl substrates is not yet fully realized [82, 83].

For the SCF E3s, F-box proteins function as adaptors that associate the substrate with the E3 in a manner that is largely dependent on substrate phosphorylation. Many F-box proteins include either WD40 repeats or leucine-rich regions that are implicated in substrate recognition [85–87]. In most cases the sequences that mediate F-box-

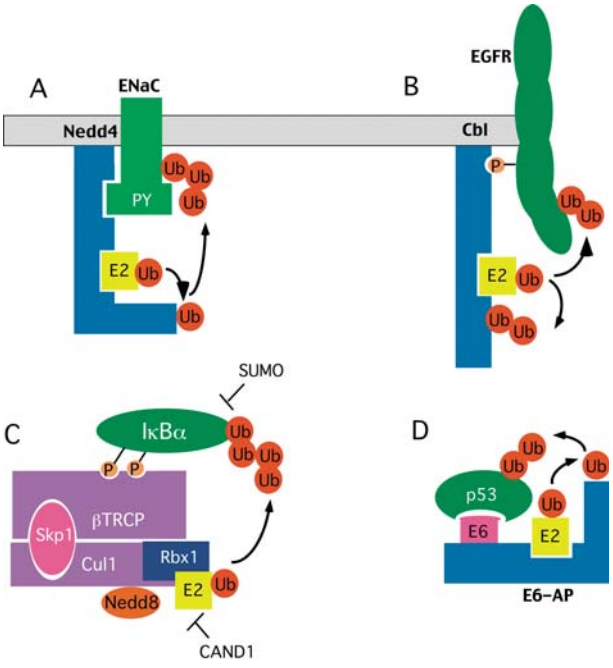

Figure 2. Representative E3-substrate interactions. (*A*) Nedd4 binds to the PY domains of ENaC through its WW domains. Ubiquitin is transferred from E2 to the active site cysteine on Nedd4 and then to acceptor sites on the substrate. (*B*) Cbl is recruited to activated receptors through phospho-tyrosine binding. E2 binds to the RING and transfers ubiquitin to sites both on the receptor and itself. (*C*) $SCF^{\beta TrCP}$ binds to phosphorylated IκBα. Rbx1 recruits E2 and mediates ubiquitylation on specific sites. Ubiquitylation can be blocked by sumoylation. E2 recruitment is enhanced by Nedd8 and CAND1 preferentially binds to un-neddylated complexes, potentially diminishing activity. (*D*) HPV E6 binds E6-AP and redirects it to p53. E6-AP forms intermediates with ubiquitin through its conserved HECT cysteine.

substrate interactions are undefined. However, for $SCF^{\beta TrCP}$, recognition of HIV-Vpu, β-catenin and IκBα all require phosphorylation of the sequence DSGψXS (ψ: a hydrophobic amino acid) on both serines [88–90]. βTrCP also recognizes the NF-κB precursor p105 through a similar motif [91, 92].

In the case of the APC, substrate recognition elements/APC activators include Cdc20 and Cdh1/Hct1 [73, 93]. These both contain WD40 repeats implicated in their association with the core APC [73]. Recognition of targets by the APC occurs through substrate recognition motifs that include the destruction box, the KEN box [94–96] and the recently described A box [70, 97].

Among a subfamily of HECT domain E3s a common site of interaction with substrates is WW domains. This tryptophan-based 35-amino acid consensus sequence interacts with multiple proline-containing sequences on target proteins such as PPXY (PY) and PPLP as well as with phosphorylated serine or threonine in appropriate context [98, 99]. WW domain HECT E3s contain two to four WW domains that vary in their relative affinity for different interaction sites [98, 99]. Among the most well-described substrates of WW domain HECT E3s is ENaC [100]. Loss of ENaC PY domains in Liddle's syndrome, an autosomal recessive form of hypertension, is believed to reflect a failure of Nedd4, or other WW HECT E3s, to mediate ENaC ubiquitylation and downregulation [101]. For Nedd4 and its yeast orthologue, Rsp5, there are also examples where ubiquitylation occurs without obvious evidence of interactions with known WW domain binding sites [102, 103]. It is important to note that only a subset of HECT E3s have WW domains. For example, E6-AP recognizes substrates through other interactions sites, and Rsp5 is the only WW domain-containing E3 among the five potential *Saccharomyces cerevisiae* HECT E3s [104, 105].

There are examples where common E3 interaction sites among substrates are not readily apparent based on amino acid sequence. This is the case for the RING finger E3 Siah, which recognizes diverse substrates through its carboxyl-terminal portion [75–79].

E3s involved in ERAD (endoplasmic reticulum-associated degradation) recognize both soluble and transmembrane substrates through a variety of means. ERAD provides quality control in the secretory pathway, targeting misfolded and unassembled proteins for ubiquitin-mediated proteasomal degradation. ERAD also serves to regulate physiological processes, such as cholesterol metabolism, by targeting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase for degradation and in gene transcription by targeting soluble transcription factor Matα2 for degradation [106–108]. ERAD E3s include both integral membrane proteins of the ER as well as cytosolic proteins that are recruited to the ER membrane to ubiquitylate specific proteins. [26, 108–113]. In some instances these recognize epitopes revealed by unfolding of proteins; in other cases they may specifically associate with native substrates; and other interactions are indirect (e.g. via interactions with chaperone molecules) [107, 110, 114, 115]. In the case of HMG-CoA reductase degradation in yeast, the active E3 requires both a RING finger protein, Hrd1/Der3 and a second interacting transmembrane protein, Hrd3 [116]. An example of how viruses make use of ERAD to accomplish their goals by usurping cellular E3s is provided by HIV-Vpu1. This protein serves as an adaptor between $SCF^{\beta TrCP}$ and CD4, targeting the latter for degradation [88]. There are also several examples where viruses encode ERAD E3s within their genomes [28, 30, 58].

E3-E2 interaction and substrate ubiquitylation

E3s interact with E2s and either serve as catalytic intermediates or mediate the direct transfer of ubiquitin from E2 to substrate. HECT domain E3s form obligate catalytic intermediates involving a thiol-ester linkage between ubiquitin and the conserved carboxyl-terminal cysteine [22]. The crystal structure of the E6-AP HECT domain with UbcH7 reveals a U-shaped appearance with E2 on one side and the HECT domain on the other [117]. This structure exhibits a distance of 41 Å between the donor cysteine of the E2 and the HECT domain acceptor cysteine – a distance too great to achieve direct transfer of ubiquitin. A potential resolution to this derives from the crystal structure of another HECT domain E3, WWP1. In this case a different conformation of the HECT domain was found. Modeling of the E6-AP-UbcH7 interaction onto WWP1 results in the E2 and E3 cysteines being within 16 Å [118, 119].

There is no evidence that non-HECT E3s act as catalytic intermediates. The RING finger can directly bind E2 on sites similar to those involved in binding E2 by the HECT domain [120]. Modeling of the c-Cbl-UbcH7 interaction onto the structure of SCF^{Skp2} (Cul1-Rbx1-Skip1-Skp2) suggests a model in which Cul1 serves as a scaffold that organizes the Skp1-Skp2 and Rbx1, enabling Skp2 to present substrate in a favorable orientation to accept ubiquitin from Rbx1-bound E2 [71, 121]. This, together with the capacity of the RING finger to bind E2, is consistent with the RING finger, PHD finger and U-box being primarily docking sites for E2, allowing for efficient transfer of ubiquitin to substrate. However, other more complex roles for the RING finger beyond simply serving as an E2 docking site cannot be excluded. For example, E2 binding by *S. cerevisiae* Cue1p, an integral endoplasmic reticulum (ER)-membrane protein, or by an analogous region of a mammalian ERAD E3, gp78, does not mediate ubiquitylation in the absence of an active RING finger E3 [111, 122]. Additionally, evaluation of E2 binding to the BRCA1-BARD1 dimer reveals that although two E2s, UbcH5C and UbcH7, bind with similar affinities to the

BRCA1 RING, only UbcH5C supports ubiquitylation [123]. Thus, in these cases, E2 binding is not sufficient for E3 activity [123]. Finally, it should be pointed out that all of the structural data on E2-E3 interactions have utilized E2 that is not bound to ubiquitin. There may be surprises that await us when interactions between E2-ubiquitin and RING fingers are evaluated.

While direct binding of the RING finger to E2s has been shown for several E3s, such interactions are often not easily discerned. Although UbcH5B, an E2 that includes little more than the core UBC domain, functions with many RING E3s in vitro, UbcH5B-RING finger binding is often not demonstrable using standard biochemical approaches. Moreover, it is our experience that the strength of binding is not necessarily proportional to in vitro E3 activity as assessed by autoubiquitylation [J. P. Jensen, K.L. Lorick, S. Fang, A. M. Weissman, unpublished observations]. This phenomenon may be related to the recent observation that release of ubiquitin-charged Cdc34 from the RING is essential for ubiquitylation of the SCFCdc4-bound substrate Sic1 [124]. There is clearly much to be learned about the molecular mechanisms by which RING-E2 binding promotes ubiquitylation.

Ubiquitin chain formation: are two E3s better than one?

There are a number of outstanding issues regarding the mechanics of substrate ubiquitylation, including understanding those factors that determine sites of ubiquitylation and the means by which polyubiquitylation occurs. Initial studies on model proteins suggested a high degree of specificity in lysines targeted for ubiquitylation. It is now evident that some proteins exhibit seemingly random ubiquitylation, such as c-Jun and T-cell-antigen receptor subunits, while for others, such as IκBα, there is a great deal of specificity. Furthermore, in some cases, the amino terminus rather than a lysine is targeted for ubiquitylation [4, 18–21].

After the first ubiquitin attaches to a substrate, the next step is often to generate polyubiquitin chains. Ubiquitin has seven internal lysine residues to choose from (K6, K11, K27, K29, K33, K48 and K63). Despite the potential for enormous complexity, polyubiquitin chains are generally linked either through K48 or K63 or, in some cases, K29 [3, 4]. The precise molecular basis by which chain elongation occurs and the means by which it is regulated are largely unknown. A study on UFD4, a *S. cerevisiae* HECT E3, suggested that UFD2 functions with UFD4 (a HECT domain E3) as a ubiquitin chain elongation factor or E4 [59] (fig. 3). UFD2 is now known to be

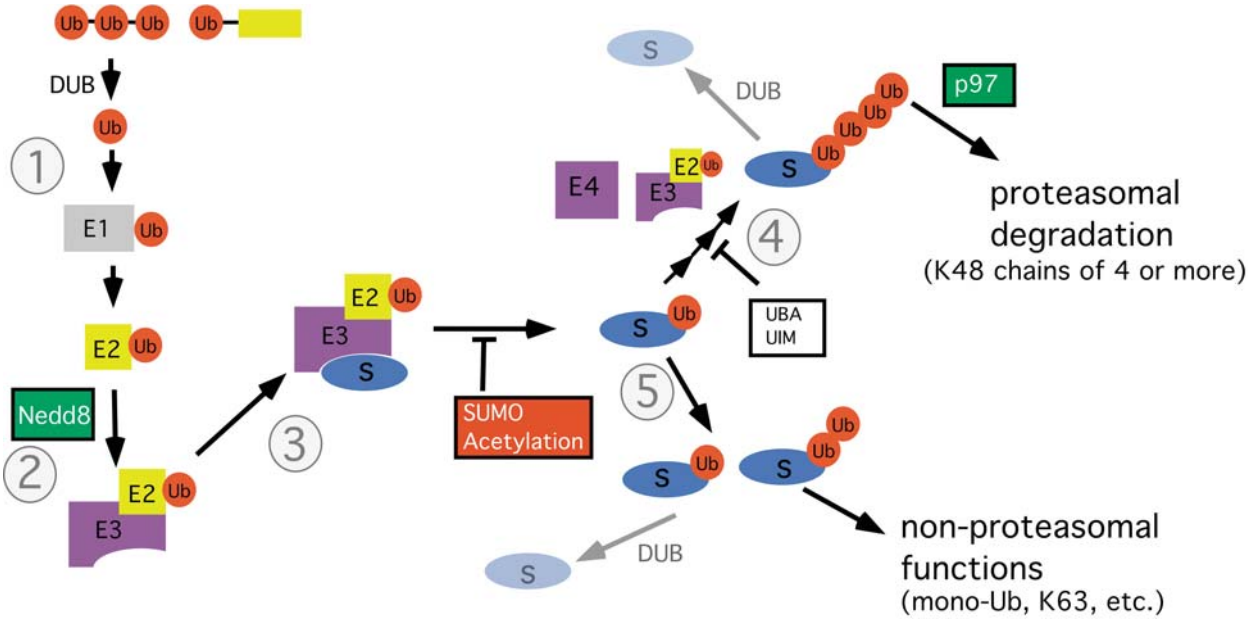

Figure 3. Regulation of ubiquitylation. (1) Ubiquitin is synthesized as polyubiquitin or as fusions with small ribosomal subunits and cleaved to its active form by DUBS. E1 activates ubiquitin followed by its transfer to E2 (see also fig. 1). (2) For SCF and CBC E3s there is evidence that Nedd8 enhances E2 recruitment to E3s. This is not the case for most E3s. (3) The mechanisms involved in substrate (S) recognition and specificity in ubiquitylation vary substantially, as discussed in the text. In some instances SUMO and acetylation have been shown to antagonize ubiquitylation. (4) Generation of polyubiquitin chains can be facilitated by E4 factors. The distinction between E4s and involvement of multiple E3s is unclear and may be a matter of semantics. A number of factors can enhance proteasomal targeting, most notably p97 (aka VCP or CDC48). In some cases ubiquitin binding domains such as the UBA and the UIM may preclude efficient polyubiquitin chain formation. (5) The net effect of preventing K48-linked chains may be to favor mono-ubiquitylation or in some cases perhaps favor non-K48 linked chains (e.g. K63). This may provide the cell with a means of preventing proteasomal degradation of certain substrates and thereby allow non-proteasomal function for ubiquitylation to be realized. Throughout the process DUBs play important roles.

a U-box protein, and mammalian orthologs mediate ubiquitylation independent of other E3s [25, 63].

While the E4 issue awaits resolution, the concept of cooperation among proteins containing E3 modules brings up a recurring theme, that of E3 oligomerization. RING finger protein homo- and heterodimerization is quite common, either through the RING finger or other domains. Dimers of SINAT5, an *A. thaliana* Siah1 ortholog, are required for efficient ubiquitylation [125]. Heterodimerization has also been shown to enhance ubiquitylation. BRCA1 by itself exhibits a low level of autoubiquitylation [52]. However, when it dimerizes with BARD1, one of the few RING finger proteins not shown to have ubiquitylating activity, the heterodimer exhibits robust autoubiquitylation [126, 127]. In this case there is no evidence that the BARD1 RING finger binds E2 or plays an active role in the ubiquitylation process; rather BARD1 facilitates the activity of BRCA1 through dimerization of the RINGs [123]. A newly identified E3 heterodimer is Parkin and CHIP. Parkin belongs to a RING finger protein subfamily that contains two RING fingers flanking a cysteine-rich IBR (in between RING) motif. Dimerization with CHIP significantly enhances Parkin-mediated in vitro ubiquitylation of Pael receptor and its ability to inhibit cell death [128]. Dimerization of Mdm2 and MdmX has also been found [129]. Like BARD1, MdmX was one of the few RING E3s for which there was little evidence for intrinsic activity. However, there is recent evidence that MdmX may have a low level of E3 activity in vitro [130] and enhances the activity of Mdm2 toward p53 [131]. The importance of oligomerization in E3 activity is indirectly supported by the observation that a number of known or suspected E3s, like Parkin, contain multiple RING or related motifs. These may function as intramolecular oligomers and by extension provide a mechanism to simultaneously involve multiple E2s in ubiquitin chain elongation. Alternatively, some of these domains might function in a manner analogous to BARD1, enhancing the activity of a heterologous RING finger. Almost all known RING finger and some HECT domain E3s are capable of mediating their own ubiquitylation. The extent to which dimerization generally plays a role in this remains to be determined.

Regulation of ubiquitylation

Regulation by E3 autoubiquitylation

The most sensitive indicator of in vitro activity for a potential E3 is E2-dependent autoubiquitylation or self-ubiquitylation. Similarly, there is increasing evidence for autoubiquitylation as part of the normal physiological function of E3s such as Siah, Mdm2, E6-AP and Cbl family members. For Siah and Mdm2, the result is rapid degradation [132, 133]. Despite their instability, these E3s manage to efficiently target heterologous substrates. Undoubtedly, substrate association has the potential to affect ligase autoubiquitylation in a manner that is likely to vary substantially. For some E3s autoubiquitylation can be affected by other proteins such as ARF, which stabilizes Mdm2 [134], or by HPV E6, which facilitates both E6-AP oligomerization and intramolecular autoubiquitylation [135–36]. In other instances, such as Cbl, apparent autoubiquitylation is correlated with recruitment to activated receptor complexes [84].

Regulation of ubiquitylation by E2-like proteins

UEV (Ubiquitin E2 variant) proteins include regions that exhibit a high degree of homology to E2s in the UBC domain but lack the canonical active site cysteine [137]. A role for a UEV in enhancing ubiquitylation was established for Uev1A in TRAF6-mediated ubiquitylation. Uev1A binds ubiquitin and, together with Ubc13, they catalyze K63 polyubiquitin chains in a TRAF6 RING finger-dependent manner, which leads to the activation of IκB kinase [14, 17, 138]. Another UEV protein, Tsg101 (tumor susceptibility gene 101), is an essential protein, loss of which results in cell cycle arrest and embryonic lethality [139, 140]. This protein has been implicated either directly, or as a consequence of its similarity to yeast Vsp23, in a number of cellular processes. Tsg101 has now been shown to enhance HIV-2 gag ubiquitylation, without evidence for gag degradation [141]. Whether, analogous to Uev1A, Tsg101 functions together with an active E2 and E3 to mediate ubiquitylation and whether this results in K63-linked chains remains to be determined.

A reasonable prediction is that UEV proteins would function as naturally occurring ‘dominant negatives’ for cellular E2s. Evidence consistent with this derives from the finding that Tsg101 binds Mdm2 and in cells Tsg101 overexpression inhibits Mdm2 autoubiquitylation and increases stability [142]. Curiously, however, this is associated with enhanced p53 downregulation. The basis for this dichotomy is not clear. Additionally, while one study suggests that cells from Tsg101$^{-/-}$ mice accumulate p53 [140], another on mice specifically lacking Tsg101 expression in the mammary gland failed to support a major role in p53 regulation [143]. While the concept of UEVs as dominant negatives is attractive, overall direct experimental support for such a function is lacking.

Regulation of ubiquitylation by proteins that bind ubiquitin

For ubiquitylation to exert its numerous cellular functions in a specific manner, ubiquitylated proteins must be recognized by adaptor/effector molecules (fig. 3). Some of the more well-described ubiquitin binding proteins/motifs are described briefly below.

Ubiquitin binding proteins are included in the 19S proteasome cap. The first of these to be identified was the S5a subunit of the lid of the 19S complex. Initial enthusiasm for this as the primary polyubiquitin binding component of the proteasome diminished with the observation that yeast negative for this subunit were viable [144]. More recently, one of the ATPases included in the base of the 19S, S6′ (aka Rpt5) was shown to bind multi-ubiquitin chains (see accompanying articles this issue for more detail) and may function as the primary recognition site for ubiquitylated proteins by the 26S proteasome [145].

In addition to proteasome subunits, a number of other proteins have now been identified that bind to either polyubiquitin chains or to mono-ubiquitylated proteins. Among these is a protein referred to variously as p97, VCP (valosin-containing protein) or Cdc48 [146]. Interestingly, this protein, like S6′, is a member of the family of AAA ATPases that, reminiscent of the heterohexameric arrangement of ATPase subunits of the base of the 19S proteasome cap, forms a homohexamer [145] (see also articles by Bajorek and Glickman, this issue). p97 was first implicated in ubiquitylation with the determination that it bound polyubiquitylated IκBα and, through interactions with the proteasome, facilitated degradation [147]. Subsequently, binding to a number of polyubiquitylated proteins has been demonstrated, and it has been determined that VCP-mediated proteasomal degradation is dependent on its ATPase activity [148, 149]. There are now a number of lines of evidence that this ATPase also plays an essential role, either by itself or in complex with other proteins, in promoting the energetically unfavorable removal of ubiquitinated proteins from the endoplasmic reticulum [149, 150]. Other proteins with which p97 interacts include proteins that can themselves bind ubiquitin either through a UBA (ubiquitin-associated domain) (see below) in the case of p47 or through a motif that resembles a zinc finger, as is the case for Npl4 [151]. p97 is also implicated in the ubiquitin-dependent activation by proteasomal cleavage of a transcription factor SPT23, which exists as an integral membrane protein of the ER in its precursor form [152, 153]. p97 likely has other cellular roles yet to be uncovered.

The UBA domain is a motif of 55 amino acids, and the first motif specifically associated with ubiquitin binding [7, 154, 155]. The binding of the UBA domain to ubiquitin has been demonstrated for proteins of diverse function [7, 155]. The UBA domains of Rad23/Rhp23 are perhaps the most thoroughly characterized. Rad23 has two UBA domains and an amino-terminal UBD (ubiquitin domain). Each UBA binds ubiquitin, and having either one intact inhibits polyubiquitin chain formation [156]. Based on these findings, UBAs might be expected to stabilize ubiquitylated proteins with which they interact by preventing the generation of proteasome targeting signals, and could favor monoubiquitylation. On the other hand, the UBD in Rad23 binds to RPN1 of the proteasome, thereby potentially facilitating proteasomal targeting of interacting ubiquitylated proteins [157]. The overall function of UBA domains is likely complex and dependent on the protein in which it is contained [158].

The UIM (ubiquitin-interacting motif) was recently identified as a motif capable of binding monoubiquitin [155, 159]. Notably, this conserved 20-amino acid consensus is included within S5a [155]. The presence of UIMs in a number of different proteins implicated in endocytosis is providing insights into the means by which ubiquitylation serves to mediate endocytosis and endosomal sorting [159–163]. Accordingly, as with the UBA, there is now evidence to suggest that binding of monoubiquitin to UIM may sterically inhibit polyubiquitin chain formation, and thus may in part explain the lack of polyubiquitylation frequently seen with membrane proteins. UIM-containing proteins implicated in endocytosis, such as Eps15, Eps15R, epsins and Hrs are themselves substrates for ubiquitylation. The molecular basis by which these proteins are targeted for ubiquitylation has not yet been established. It is speculated that UIM-containing endocytic proteins are recruited to the ubiquitylated tails of membrane proteins. The signaling-dependent monoubiquitylation of UIM-containing proteins themselves may further facilitate assembly of networks of UIM proteins for endocytosis and sorting.

The Cue domain is the most recently identified ubiquitin-binding motif. This 43-amino consensus sequence is contained in the Cue1p protein of yeast and also in the mammalian ERAD E3 gp78 and was recognized as a motif conserved in a number of proteins by Ponting [164]. Recently, a role for the Cue domains as a monoubiquitin binding domain also capable of facilitating its own monoubiquitylation was established. Yeast Vps9, a protein implicated in endocytosis in yeast, binds ubiquitin through its Cue domain and is ubiquitylated by a yeast HECT E3, Rsp5 [165]. Structurally, the Cue domain bears significant resemblance to the UBA [166].

Regulation by E3-interacting partners

In addition to E2-like proteins and ubiquitin binding proteins, the activity of ubiquitin ligases can be regulated by other E3-interacting partners. There are an increasing number of examples of such heterologous regulation, including the enhancement of BRCA1 activity by BARD1, as discussed above. In the case of the RING finger E3 COP1, interactions with the coiled-coil domain protein SPA1, which is a negative regulator of phyA signaling during plant development, stimulates the E3 activity of residual nuclear COP1 towards LAF1, thereby desensitizing phyA signals [167].

This heterologous regulation is perhaps best exemplified by Mdm2 (reviewed in [168]). The activity of Mdm2 towards both itself and p53 is modulated in a variety of

ways, including through its dimerization with the closely related RING finger protein MdmX [131]. A well-studied, yet poorly understood, modulator of Mdm2 activity is ARF. ARF and, more recently, several ribosomal proteins have been shown to bind Mdm2, affecting both its intrinsic activity and subcellular localization [169–171]. Interestingly, it has been reported that the Mdm2 RING finger is able to bind to RNA specifically, leading to inhibition of Mdm2 dimerization and auto-ubiquitylation in vitro [172]. Of unclear relation to this observation is the finding that the Mdm2 RING includes a Walker A or P loop motif through which it can bind adenine-containing nucleotides. ATP-bound Mdm2 is preferentially localized to the nucleolus, and mutation of the binding site abolishes ARF-independent nucleolar localization and adenine nucleotide-dependent upregulation of Mdm2 E3 activity. Given the ubiquitous cellular distribution of RNA and ATP, it will of course be very interesting to further examine the physiological relevance of these observations [173].

Regulation of ubiquitylation by post-translational modifications

Phosphorylation

As the two most prominent regulated post-translational modifications, the relationships between phosphorylation and ubiquitylation are predictably numerous and highly complex. One major role for phosphorylation is to create binding sites for E3s on substrates. For example, serine/threonine phosphorylation results in binding sites for F-box proteins and for some WW domains [94]. Ligand-stimulated tyrosine phosphorylation of receptor tyrosine kinases allows for recognition by Cbl proteins [82]. Similarly, ligand-induced tyrosine phosphorylation of JAK2 (Janus kinase 2) leads to recruitment of SOCS-1 through its SH2 domain [174]. SOCS-1 has a region that is homologous to the region of the VHL (von-Hippel-Lindau) protein that mediates its incorporation into the CBC core, allowing SOCS-1 to assemble into $CBC^{SOCS\text{-}1}$ and target JAK2 for ubiquitylation [174, 175]. Phosphorylation can also stabilize proteins through inhibition of E3s interactions, as is the case with *c-Jun* [176]. For p53, stress-induced phosphorylation within its amino terminus provides stabilization by preventing Mdm2 binding [177]. However, phosphorylation of Mdm2 by AKT may promote its nuclear transport and thereby potentially enhance interactions with p53 [178–182]. For the multisubunit APC, not surprisingly, the role of phosphorylation in activation and ubiquitylation is complex. Multiple kinases are implicated in playing both inhibitory and stimulatory roles [70, 73]. It is also clear that ubiquitylation can regulate phosphorylation both directly as well as indirectly. One of the most striking examples is the activation of IκB kinase by TRAF6-mediated K63 ubiquitylation, which in turn leads to the phosphorylation of IκBα and its subsequent targeting for degradation by $SCF^{\beta TRCP}$ [14, 17]. It is also now apparent that kinase and ubiquitin ligase activity can co-exist in the same proteins, as discussed above for MEKK1 [29].

Oxidation

One class of protein modifications that has been suspected of being associated with targeting for ubiquitylation is oxidative changes. There are now two clear examples where oxidative changes serve as physiological sensors, directing ubiquitylation of specific substrates. The first of these is HIF-1α, which is a hypoxia-inducible angiogenic factor. Above certain threshold levels of oxygen, HIF-1α is targeted for ubiquitylation by CBC^{VHL} as a consequence of HIF-1α proline hydroxylation [183–187], which creates a recognition site for VHL. The second example relates to the regulation of intracellular iron. A RING finger E3, HOIL-1, has recently been identified as a factor that recognizes and targets a key sensor of intracellular iron stores, IRP2, for ubiquitylation and proteasomal degradation upon oxidation. In this case, the precise recognition signal for the E3 has yet to be discerned [188].

Sumoylation

Post-translation modifications can also regulate ubiquitylation through blocking ubiquitylation sites. A well-established example is IκBα. Phosphorylated IκBα binds to $SCF^{\beta TRCP}$, leading to ubiquitylation, specifically on two residues, K21 and K22. K21 sumoylation blocks ubiquitylation, thereby leading to IκBα stabilization [189]. Modification of the same lysine of PCNA by mono-ubiquitin, polyubiquitin and by SUMO has been found during DNA repair. The three modifications differentially affect resistance to DNA damage [15]. However, in this case the role of sumoylation is apparently not to prevent proteasomal degradation of PCNA, as PCNA polyubiquitin chains are linked through K63. p53 is also sumoylated on a lysine within its carboxyl-terminal cluster of ubiquitylation sites; however, there is little evidence that this functions to inhibit Mdm2-mediated p53 ubiquitylation [190].

Acetylation

Acetylation can also compete with ubiquitylation in modification of ubiquitylation sites. In the case of Smad7, a substrate for the WW HECT E3 Smurf1, acetylation occurs on the same sites as ubiquitylation, thereby stabilizing this TGFβ receptor-interacting protein [191]. Similarly for p53, ubiquitylation mediated by Mdm2 occurs on a cluster of carboxyl-terminal lysines that can be acetylated by p300/CBP. Acetylation inhibits Mdm2-mediated ubiquitination of p53 [192].

Neddylation

Nedd8 is a UBL whose only well-characterized substrates are the cullin subunits of multi-subunit E3s including the

SCF and CBC [193-196]. Although not essential in *Schizosaccharomyces pombe*, Neddylation plays important roles in both cell cycle progression and morphogenesis in mammals [197], and is implicated in the ubiquitylation of p27, IκBα and the NF-κB precursor by SCF E3s [198, 199]. Neddylation increases SCF E3 activity by promoting the recruitment of the ubiquitin-loaded E2 [195]. Recently, CAND1 has been identified as a protein that selectively binds to un-neddylated Cul1-Rbx1, preventing recruitment of Skp1 [196, 200]. Neddylation of this CAND1-bound Cul1 may promote formation of an active SCF not only by allowing for E2 recruitment but also by affecting CAND-1 association and the association of Skp1 with the Cul1-Rbx1 complex.

Recent provocative findings related to deneddylation come from study of the COP9 signalosome (CSN), which was originally identified as a suppressor of light-dependent development of *A. thaliana* but also found in mammals [201]. The eight-subunit core of this complex bears significant resemblance to the lid section of the 19S proteasome cap, with each subunit having a proteasome paralogue. In *A. thaliana*, the CSN is implicated as a positive regulator of SCF^{TIR1} [202]. A key observation is that a metalloprotein-based deneddylating activity is encoded in the CSN5 subunit [201]. How a dynamic process of neddylation and deneddylation affects SCF activity now becomes of great interest.

Regulation by deubiquitylating enzymes

The work of E2s and E3s can be reversed by the action of deubiquitylating enzymes. Two major classes of DUBs have been described: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs); both are cysteine hydrolases [203]. In general, UCHs hydrolyze primarily carboxyl-terminal ester and amide bonds of ubiquitin and are capable of cleaving ubiquitin precursors to generate active ubiquitin. Among other functions, UBPs cleave and disassemble polyubiquitin chains. In addition to playing housekeeping roles, specific DUBs have been linked to disease pathogenesis and are implicated in embryonic development, signal transduction, tumor suppression and growth. All of this is consistent with the idea that some DUBs are substrate specific. One example of this is Fat facets, which is a *Drosophila melanogaster* DUB implicated in patterning the compound eye that specifically interacts with and deubiquitylates liquid facet, an epsin homolog. This leads to liquid facet stabilization [204]. Recently, HAUSP (herpes virus-associated ubiquitin-specific protease) was identified as an interacting protein for p53. HAUSP deubiquitylates p53 in vitro and in vivo and stabilizes p53 even in the presence of excess Mdm2 [205]. Other DUBs that may have specificity for particular substrates include BAP1, which has been shown to bind to the amino terminal RING finger-containing region of BRCA1 [206, 207], although BAP1 does not deubiquitinate BRCA1 in vitro [208]. As we learn more about cognate E3 substrate pairs, there will undoubtedly be an explosion in knowledge about DUBs and their specific substrates.

In addition to cysteine protease DUBs, it now appears that analogous to the metalloprotease deneddylating activitiy of the CSN, a similar metalloprotease activity exists within the paralogous 19S proteasome subunit, Rpn11 [11, 13, 201]. The release of polyubiquitin chains from proteasome-bound substrates by the action of this DUB facilitates degradation, underscoring the tight linkage between flux through the proteasome and the removal of polyubiquitin chains.

Glancing back … looking ahead

Less than a decade ago only a handful of naturally occurring ubiquitylation substrates had been identified, and by the mid-1990s the amino acid sequences of only a few established E3s were known. As recently as 4 years ago the only known E3 signature was the HECT domain, and the paradigm for ubiquitylation was, more or less, E1 to E2 to E3 to proteasome, with the understanding that DUBS could act to counter this process before irreversible proteolysis. We now find ubiquitylation everywhere we look. There is an ongoing explosion of findings leading to the emergence of new principles, and we are beginning to appreciate the incredible complexities and intricacies involved in regulating ubiquitylation and the fate of ubiquitylated proteins. For the RING and related structures, their compact nature provides the opportunity for them to be one element in multi-functional proteins. It is now apparent that there are collectively many hundreds of HECT domains, RINGs, PHD fingers and U-boxes encoded in mammalian genomes. The utilization of adaptors such as F-box proteins result in the targeting of many substrates by a single core E3 complex. Thus, we can now begin to understand how ubiquitylation regulates such a large number of proteins. Further, the existence of multiple ubiquitin binding motifs not only provides a means for recognition and trafficking of ubiquitylated species, but in some cases also serves to modulate polyubiquitin chain formation, thereby potentially altering the fate of substrates.

If things were not complex enough, it has also become evident that nature makes use of lysines in other ways that intersect with ubiquitylation in the multi-dimensional matrix of the cell. In the case of neddylation, this modification facilitates ubiquitylation. However, for other UBLs, particularly SUMO, as well as for acetylation, steric effects generated by binding to neighboring lysines or direct competition for ubiquitin sites presents an elegant and reversible means for dynamically fine-tuning

protein function. The challenge for us now is to map this intricate circuitry to understand how the cellular signals that contribute to these regulatory modifications intersect and diverge. This is a task that will require novel approaches in structural and temporal-spatial analysis of protein assemblies. Aside from the obvious challenges and rewards, understanding the role of ubiquitylation and its complex regulation will be reflected in an enhanced understanding of diseases associated with perturbation of these crucial cellular processes.

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