Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1695 (2004) 55–72

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Review

Ubiquitin: structures, functions, mechanisms

Cecile M. Pickart[a,*], Michael J. Eddins[b]

[a]Department of Biochemistry and Molecular Biology/Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205, USA

[b]Department of Biophysics and Biophysical Chemistry/School of Medicine, Johns Hopkins University, Baltimore, MD 21205, USA

Available online 6 October 2004

Abstract

Ubiquitin is the founding member of a family of structurally conserved proteins that regulate a host of processes in eukaryotic cells. Ubiquitin and its relatives carry out their functions through covalent attachment to other cellular proteins, thereby changing the stability, localization, or activity of the target protein. This article reviews the basic biochemistry of these protein conjugation reactions, focusing on ubiquitin itself and emphasizing recent insights into mechanism and specificity.

Keywords: E1; E2; E3; Nedd8; Sumo; Ubc; Ubiquitin

1. The ubiquitin family of protein modifiers

Ubiquitin is the prototype of a family of proteins that display remarkably similar structures, but variable sequences (Fig. 1; see Refs. [1–4]). The mature forms of most of these proteins terminate with a signature diglycine sequence, which is usually exposed only after proteolytic processing.

Prokaryotes have no molecule that is functionally analogous to ubiquitin. That is, there is no (known) prokaryotic protein that acts as a signal through covalent attachment to another protein. However, prokaryotes do possess proteins—the evident ancestors of ubiquitin—that display a ubiquitin fold. The bacterial proteins ThiS and MoaD facilitate the insertion of sulfur into the organic cofactors thiamin and molybdopterin, respectively (reviewed in Ref. [3]). As shown for ThiS in Fig. 1 (A versus D), the conformations of these bacterial proteins are similar to that of ubiquitin [5,6]. ThiS and MoaD both carry sulfur in the form of a C-terminal thiocarboxylate that is produced from an initial C-terminal adenylate intermediate (see Refs. [3,7]). As discussed below, the activation of ThiS and MoaD bears a strong mechanistic relationship to the activation of eukaryotic ubiquitin-like proteins.

1.1. Functional diversity in the ubiquitin protein family

The process of evolution has generated a family of eukaryotic proteins that share the ubiquitin fold. These proteins also share a common biochemical mechanism: an isopeptide bond is formed between the modifier's terminal glycine and an amino group of the target protein. (In this article, we will frequently refer to such ubiquitin-like modifier proteins using the generic term "Ubl.") Usually the amino group is contributed by a lysine residue, but N-terminal ubiquitination is also known (see Ref. [8]). Some family members, like Nedd8 and Sumo (Fig. 1B and C), are universally distributed in eukaryotes. Others, such as the linear diubiquitin analogs Fat10 and ISG15, and the single-domain protein Ufm1, arose more recently and are found only in mammals (see Refs. [1,9,10]). Several new eukaryotic members of the ubiquitin family have recently been discovered, including Urm1, which is more closely related to ThiS/MoaD than to ubiquitin [11], and Hub1, which uniquely terminates in a dityrosine motif [12]. In

* Corresponding author. Tel.: +1 410 614 4554; fax: +1 410 955 2926.
E-mail address: cpickart@jhmi.edu (C.M. Pickart).

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.09.019

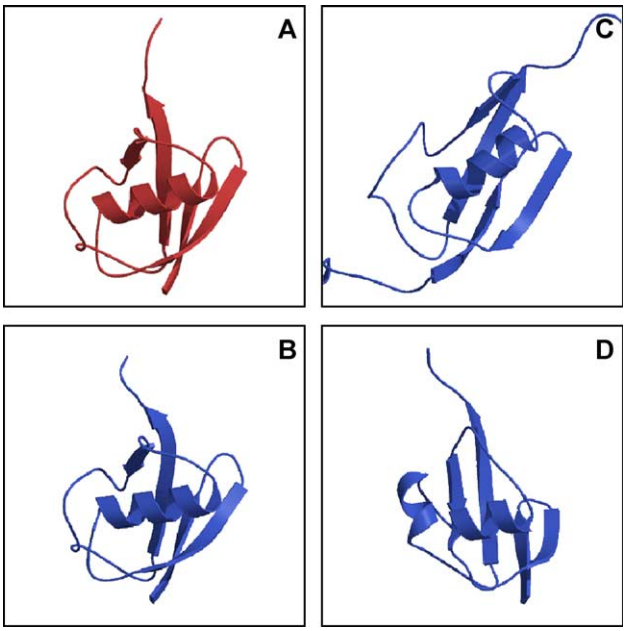

Fig. 1. Members of the ubiquitin protein family: (A) Ubiquitin [138]; (B) Nedd8 [32]; (C) Sumo-1 [139]; (D) ThiS [6]. All ribbon diagrams were generated by MOLSCRIPT [140], and Raster3D [141].

yeast, these two family members have poorly understood roles in target-of-rapamycin (TOR) signaling [13] and cell polarity [12], respectively. In all likelihood, additional ubiquitin family members remain to be discovered since ubiquitin-like ORFs generally fall below the length cutoff for annotation in genome sequencing.

Certain proteins that are mechanistically related to ubiquitin have only been recognized following substantial characterization. Notable examples include Atg8 and Atg12, which are significantly larger than ubiquitin and have no sequence relationship to it, although a structural relationship is likely [14]. Both proteins play important roles in macroautophagy, a process in which regions of cytoplasm are engulfed by a specialized double membrane and delivered to lysosomes so that their contents can be degraded. Atg12 becomes linked to another protein in this pathway, while Atg8 is linked to the amino group of phosphatidylethanolamine to mediate Atg8 association with autophagic membranes [15].

Besides proteins like those shown in Fig. 1, whose biology requires C-terminal chemistry, ubiquitin-like domains also occur as stable elements within other proteins. Because they lack the terminal diglycine motif, these domains cannot be processed or conjugated [3,4]. So-called type II ubiquitin-like domains, which usually have an N-terminal location, bear significant sequence similarity to ubiquitin and display its characteristic fold [16]. There are also stable elements, notably the Ubx domain, that share the ubiquitin fold in the absence of any sequence homology [17]. Stable ubiquitin-like domains often function as targeting elements (see article by Howley and Ref. [17]). In summary, the ubiquitin fold represents a versatile interaction module that has arisen more than once during evolution.

In the rest of this article we discuss only those members of the ubiquitin family that undergo conjugation to other proteins. Strong interspecies sequence conservation indicates that the biological function(s) of each of these proteins is highly conserved. For example, human Sumo-1 is only 20% identical to human ubiquitin, but it is 52% identical to yeast Sumo (called Smt3). Ubiquitin itself presents the most striking case, differing at only 3 of 76 positions between yeast and humans. Ubiquitin's remarkable conservation is believed to reflect strong selective pressure on the entire molecule as a result of its diverse biological functions. Ubiquitin is a well-established, functionally distinct signal in proteasomal and lysosomal proteolysis (see Refs. [18,19] and other articles in this issue). It is also a non-proteolytic signal in subnuclear trafficking [20], DNA damage tolerance [21], and several other processes (see Refs. [19,22]).

Although much remains to be learned, the past several years have seen substantial progress in elucidating the basic functions of eukaryotic ubiquitin-like modifiers. Sumoylation regulates nucleocytoplasmic transport and cell cycle progression by modulating the localization or activity of its substrates (see article by Johnson and Ref. [23]). ISG15 conjugation plays an important role in normal development and in interferon α/β-mediated responses to viral infection [9], although the specific purposes served by ISG15 modification are not yet known. The functions of Fat10 also remain mysterious; this Ubl is encoded in the MHC class I locus and may play a role in cytokine-induced apoptosis [24].

Just as our understanding of the biological functions of ubiquitin family members is still imperfect, so too do we lack a clear understanding of the biochemical mechanisms by which these functions are carried out. The basic scenario is well established: the modifier protein is conjugated to an amino group of a specific target protein and then recognized in a manner that leads to specific downstream events, which vary depending on the identity of the protein modifier and the location and identity of the substrate. In the case of ubiquitin, its polymerization state is also important—ubiquitin can be linked to substrates as a monomer, or in the form of isopeptide-linked polymers called polyubiquitin chains, whose structure can influence the substrate's fate. For example, polyubiquitin chains linked through K48 target substrates to proteasomes, resulting in an essential function for this side chain [25]. Poly-Sumo chains have also been observed within cells, but at least in yeast they do not perform an essential function [26].

Can ubiquitin family members engage in cross-talk? Increasing evidence suggests an answer in the affirmative. For example, the conjugation of Sumo to certain substrates can defend these molecules against modification by ubiquitin and, thus, prevent the consequences that would follow from ubiquitination (for example, Ref. [21]). In a recent unexpected development, UbcH8—a bona fide

conjugating enzyme for ubiquitin—was found to have a second role as a conjugating enzyme for ISG15 [27]. But the best-characterized example of cross-talk is provided by Nedd8/Rub1. This closest relative of ubiquitin modifies one type of ubiquitin-protein ligase (E3) and thereby stimulates these enzymes to become more active in the conjugation of *ubiquitin* to cognate substrates (see Ref. [2] and below).

However, there are many unanswered questions. How is conjugation catalyzed? What molecular principles govern substrate specificity? How is specificity regulated? What factors mediate the recognition of a given substrate-linked Ubl? And how is that recognition translated into specific downstream events? Principles and progress in several of these areas are discussed below.

2. The biochemistry of Ubl conjugation

The conjugation of ubiquitin to substrates usually involves three steps (Fig. 2A): an initial activation step catalyzed by E1, an intermediate step in which the Ubl is covalently linked to a conjugating enzyme (E2), and a final step in which the Ubl reaches its ultimate destination of the substrate amino group. The last step is usually facilitated by a ligase enzyme (E3). Nedd8 and Sumo each have a single E2 and a limited number of E3s. Atg8, Atg12, Ufm1, and ISG15 are each known to have a single E2-like enzyme, but so far these Ubls lack unique E3s. Neither E2s nor E3s are yet known for Urm1, Hub1, and Fat10. The conjugation machinery for ubiquitin differs from these examples mainly in terms of scale: there is a large family of ubiquitin-dedicated E2 enzymes and an even larger set of E3 enzymes (Fig. 2B and below).

2.1. Activating enzymes (E1s)

The first task in protein conjugation reactions is to activate the C-terminus of the Ubl protein for its ultimate fate of attack by the substrate amino group. This reaction is catalyzed by a Ubl-specific E1 enzyme. In eukaryotes, the activation reaction comprises two steps: the initial formation of a Ubl-adenylate intermediate is followed by the reaction of this intermediate with an E1 cysteine residue to form an E1~Ubl thiol ester. In general, each Ubl has a single dedicated E1. This is true even for ubiquitin (except in plants), in spite of its enormous array of downstream

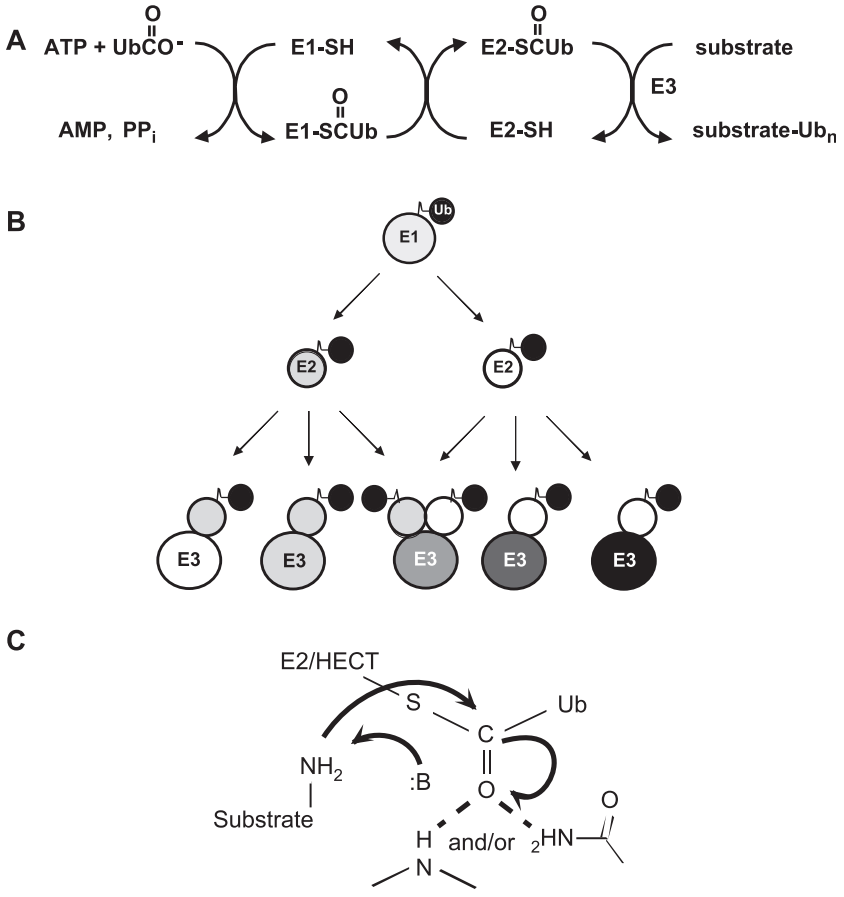

Fig. 2. Conjugation mechanisms and machinery. (A) Basic steps in substrate modification by ubiquitin family members. E3 enzymes have not been identified for all Ubl proteins (see text). (B) Schematic representation of the ubiquitin conjugation cascade. In budding yeast there is one E1, 11 E2s, and more than 20 E3s. Substrate specificity depends mainly on the identity of the E3, but may also be influenced by the identity of the E2 (see text). (C) Anticipated catalysis of protein conjugation, shown for ubiquitin. B: denotes a general base.

conjugating factors. (The only known exceptions are Atg8 and Atg12, which share the same E1 [15].) The formation of a Ubl~adenylate intermediate is the most ancient reaction in ubiquitin-like protein pathways. Thus, *E. coli* MoeB, which is the E1 enzyme for MoaD, catalyzes MoaD adenylation, but not MoeB~MoaD thiol ester formation [3,7].

Crystal structures of several MoeB/MoaD complexes, including one with MoaD in the adenylate form, indicate that the mechanism of this first step in Ubl activation is highly conserved [7]. Key catalytic residues revealed in the structure of the adenylate complex, including an aspartate that binds the ATP-coordinated magnesium ion and three basic residues that provide electrostatic stabilization to the departing pyrophosphate product, are conserved in E1 enzymes from *E. coli* to humans. The C-terminus of MoaD is seen in an extended conformation that permits it to insert into a pocket in MoeB and approach the α-phosphate of bound MgATP. A similarly extended C-terminal conformation is seen in the complex of Nedd8 with its cognate E1 (see below). In higher E1 enzymes, the active site cysteine must subsequently insert into this site to attack the adenylated Ubl (see below). The MoaD/MoeB complex is an $(\alpha\beta)_2$ heterotetramer; unexpectedly, one of the catalytic arginines in each active site is contributed by the other MoeB subunit. This residue is conserved in eukaryotic E1s, where it originates either in a different subunit (Nedd8 and Sumo E1s) or in a distant region of the linear sequence of the same subunit (ubiquitin E1) [7,28].

However, eukaryotic E1s also have unique mechanistic features. The catalysis of thiol ester formation probably requires electrostatic stabilization of the initial oxyanion intermediate, but it is unlikely that any of the catalytic residues seen in the MoeB/MoaD structure performs this role. Furthermore, most higher E1 enzymes bind two molecules of activated Ubl—one as an adenylate and the other as a thiol ester—suggesting that their Ubl binding sites are more complex than the MoaD binding site in MoeB. Also, in contrast to MoeB, which binds MoaD tightly in the absence of ATP [7], the ubiquitin E1 has little affinity for ubiquitin unless MgATP is bound (see Refs. [29,30]). Interestingly, the MoeB/MoaD studies provide a hint of ATP-dependent changes in the orientation of certain active site residues, including the arginine residue discussed above. Finally, the MoaD/MoeB interface is hydrophobic in character [7], whereas two arginine residues on the surface of ubiquitin critically influence recognition by the ubiquitin E1 [31]. Such specific side chain interactions aid in the discrimination by E1s between highly similar Ubls such as ubiquitin and Nedd8. This is important because downstream conjugating factors may lack the capacity to discriminate between different Ubls [32].

Structural studies of APPBP1/Uba3, the heterodimeric E1 for Nedd8, confirm many of these predictions but also hold some surprises. The structure of the unliganded enzyme showed that the adenylate site is indeed very similar to that of MoeB, with conservation of the aspartate and arginine residues discussed above [28]. Several other active site residues are also positioned similarly in both E1s, confirming a conserved mechanism of adenylate formation.

However, Nedd8 interactions with APPBP1/Uba3 are different in character from MoeB/MoaD interactions. The Nedd8 E1 has one ATP binding site per heterodimer. It sits at the base of a long groove whose walls are formed by a domain harboring the catalytic cysteine residue on one side, and a ubiquitin-fold region on the other side (below). The crystal structure of an APPBP1/Uba3/Nedd8/ATP complex shows that Nedd8 binds in this groove with its C-terminal residues in an extended conformation such that the chain passes under a loop of the E1, placing G76 in close proximity to the α-phosphate of ATP [33]. Besides its C-terminus, two other regions of Nedd8 contact the E1 heterodimer: a negatively charged surface of Nedd8's α-helix and a hydrophobic surface on the opposite side of the Nedd8 molecule. Both of these Nedd8 surfaces are highly conserved in ubiquitin, and the APPBP1/Uba3 surfaces that interact with them are conserved in the ubiquitin E1. The specificity of APPBP1/Uba3 for Nedd8 versus ubiquitin therefore reflects interactions with the Nedd8 C-terminus. Nedd8-A72 is a key specificity-determining residue [32]. Although the APPBP1/Uba3 site that interacts with this side chain is largely hydrophobic, discrimination against ubiquitin is mainly due to Uba3-R190, which is positioned at the bottom of the A72 binding pocket. When ubiquitin is modeled into the APPBP1/Uba3/Nedd8 complex in place of Nedd8, ubiquitin-R72 has steric and electrostatic clashes with Uba3-R190. These problems should be alleviated if Uba3-R190 is changed to Q190, as occurs in the ubiquitin E1. Mutational studies confirm these predictions [33]. Therefore, conserved favorable Ubl/E1 interactions provide affinity, while avoidance of unfavorable interactions is a principal basis for specificity.

The catalytic cysteine is ~35 Å away from Nedd8-G76 in the APPBP1/Uba3/Nedd8 complex, indicating that conformational movements of the enzyme and/or Nedd8 accompany thiol ester formation. In the case of ubiquitin, the ultimate product of the activation reaction is a ternary complex containing two molecules of Ubl (above). Although the active site groove of the Nedd8 E1 has enough space to contain two molecules of Nedd8 [28], there is no obvious specific binding site for a second Nedd8. Possibly the second site is cryptic, forming only upon covalent reaction.

A surprising feature of Uba3 is the presence of a domain at its C-terminus that displays a ubiquitin-like fold. The sequence of this region is poorly conserved among eukaryotic E1s, suggesting that it could play a role in Ubl-specific interactions. Although it was suggested that this region helps to recruit the E2 (Ubc12 in this case) to the E1~Nedd8 thiol ester [28], the lack of sequence similarity between Uba3's "ubiquitin-like domain" and Nedd8 is confounding to this model, while functional studies yielded inconclusive results [28]. Still, it is hard to imagine that this

domain is not a Nedd8 mimic. Perhaps movement of this domain is coupled to thiol ester formation, with the E1-linked Nedd8 partially displacing the UbL domain. This could create more room, and perhaps an interaction surface [34], for a second Nedd8 in the active site groove. It might also allow the UbL domain to be more efficiently displayed for purposes of E2 recruitment.

2.2. Conjugating enzymes (E2s)

The next step in the conjugating cascade is transfer of the Ubl from the E1 cysteine residue to an E2 cysteine (Fig. 2A). Nedd8 and Sumo are each passed to a single E2 enzyme (Ubc12 and Ubc9, respectively), but there is a large family of E2s dedicated to ubiquitin, comprising 11 enzymes in *S. cerevisiae* and many more in higher organisms (Fig. 2B and Ref. [29]). Individual ubiquitin E2s dictate specific biological functions of this Ubl because the specificity of the E2/E3 interaction limits the final destinations (substrates) of the ubiquitin carried by a given E2 [18,29].

The large size of the E2 family is often rationalized based on the large number of E3 enzymes, in turn reflecting the large number of substrates. But why do E2s need to exist at all? Why is it that E3s do not acquire ubiquitin directly from E1? At least two possible explanations can be developed, both related to regulatory potential. First, if a given E3 functions mainly with one E2, then the E2 step provides an additional point for regulating the tagging of that E3's substrates, for example by changing E2 activity or concentration [35,36]. Second, having many E2s may further diversify the specificity of ubiquitination. In this model, the identity of the E2 could modulate either target protein selection or the structure of the ubiquitin modification.

Substrates whose ubiquitination involves multiple E2s might exemplify the second strategy. The degradation of the yeast MATα2 transcriptional repressor depends on four different E2s, including Ubc6 and Ubc7 [37]. Although this is an extreme case, the ubiquitination of many substrates targeted by endoplasmic reticulum (ER)-localized E3s requires both Ubc6 and Ubc7, which localize to the ER and associate with one another [37,38]. (MATα2 is targeted by such an E3 [39].) An attractive model to explain why the ubiquitination of certain substrates requires multiple E3s derives from studies of the DNA polymerase processivity factor known as PCNA. The modification of this substrate with a polyubiquitin chain proceeds in two discrete steps: one E3/E2 complex ligates the first ubiquitin, which then serves as the substrate for chain elongation catalyzed by a (different) E3/E2/UEV complex [21]. (The UEV is a specialized conjugating factor, see below.) Perhaps the initiation and elongation phases of polyubiquitin chain synthesis sometimes involve a single E3 acting with distinct E2s. Biochemical studies have shown that the identity of the E2 can influence the structure of a polymeric ubiquitin modification [40].

All E2s, whether dedicated to ubiquitin or other modifiers, share a conserved globular domain of ~150 residues (Fig. 3; see Refs. [29,41]). Some E2s also have N- or C-terminal extensions, which may regulate E3 association, intrinsic E2 activity, or substrate recognition (see Ref. [29]). The E2 active site cysteine, which is positioned within a highly conserved sequence, sits in a shallow cleft on the protein surface. Noncovalent E2/Ubl binding affinities are usually low, highlighting the important role that the E1 plays in bringing the E2 together with the appropriate Ubl protein [29,30].

The lability of E2~Ubl thiol esters has hindered investigation of the molecular mechanisms used by E2s to facilitate ubiquitin conjugation. However, an NMR analysis of chemical shift perturbations during yeast Ubc1~Ub thiol ester formation successfully defined the E2/ubiquitin interface [42]. It includes the C-terminus of ubiquitin (residues 71 through 76) and certain E2 residues proximal to the active site cysteine (Fig. 3E). The results suggest that the C-terminus of ubiquitin adopts a partially extended conformation that wraps around part of the E2 surface, occupying a cleft formed by specific E2 residues. Importantly, the ubiquitin interface on the E2 does not overlap the site where E3 enzymes are expected to bind (Fig. 3E versus F and G). These conclusions agree well with earlier inferences derived from mutational and solution structural studies [42,43]. The high conservation of the E2 core domain suggests that this model will be broadly applicable.

The crystal structures of two E2/E3 complexes have greatly aided in understanding how E3s select their cognate E2s [44,45]. The structures suggest that most E2s contact their cognate E3s through side chains at the C-terminal end of E2 helix 1, the loop connecting β-strands 1 and 2, and the distal end of the active site loop (Fig. 3F and G [29,44,45]). This information provides a rational basis for identifying the cognate E2 of a given E3, and can suggest how to block the formation of specific E2/E3 complexes (for example, Refs. [46,47]). Some E2/E3 complexes have additional specialized interactions, which may involve E2 terminal extensions (for example, Refs. [48,49]).

Where does the E1 contact the E2? A mutational analysis of Ubc9 suggests that the association of free Ubc9 with the free Sumo E1 is mediated by the C-terminal end of Ubc9 helix 1 and the loop between β-strands 1 and 2 (Fig. 3D [50]). The loop contains an insertion that is unique to Ubc9 and several of the residues in the helix are poorly conserved in other E2s, suggesting a potential basis for ensuring that a given Ubl protein is channeled to appropriate downstream conjugating factors. It remains to be confirmed that this interface applies to other E1/E2 pairs, but if it does, then there could be a partial overlap of the E1 and E3 binding surfaces of the E2 [50] (Fig. 3D versus F and G). If this overlap is extensive, then recharging of the E2 may require its release from the E3. In the case of ubiquitin, where multiple rounds of modification are common, this could limit the processivity of substrate conjugation.

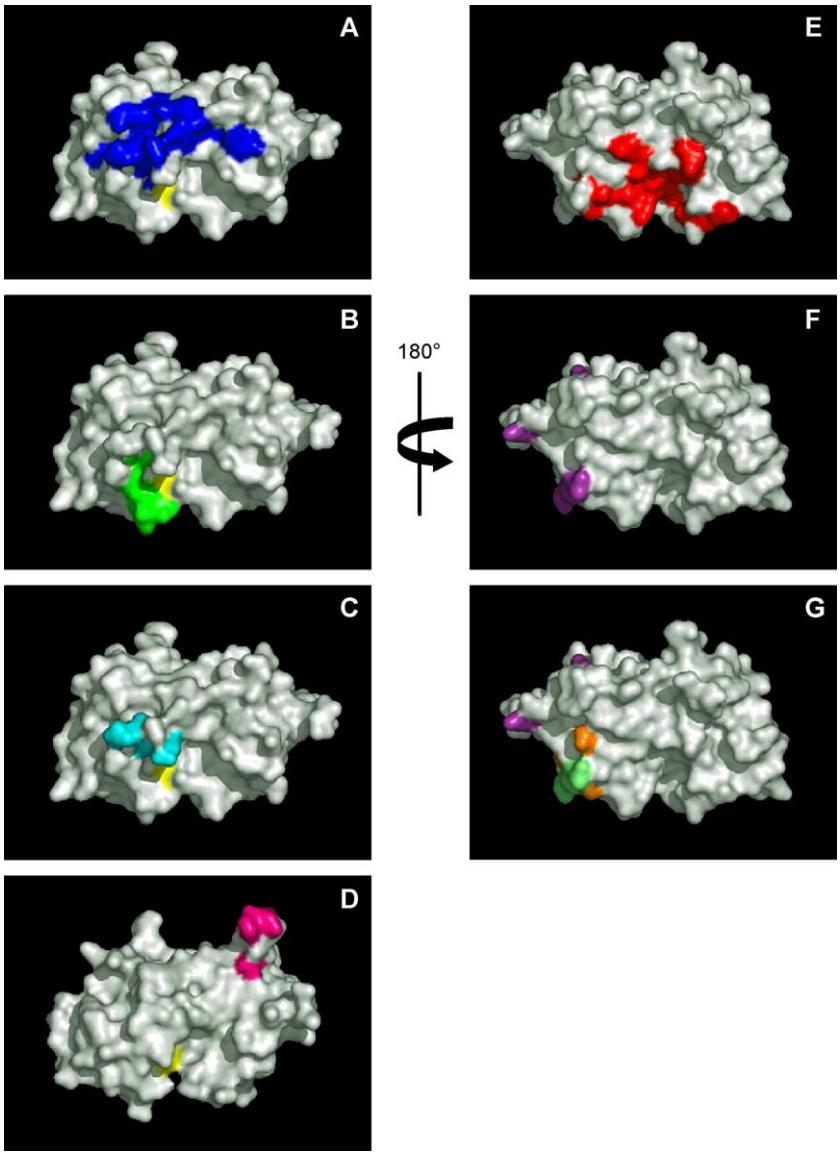

Fig. 3. E2 interaction surfaces. (A) Mms2 (blue [55]); (B) sumoylation site residues (green [51]); (C) acceptor ubiquitin interface in K63-polyubiquitin chain synthesis (teal [55]); (D) E1 (pink [50]); (E) covalently bound ubiquitin (red [42]); (F) RING domain (purple [45]); (G) RING domain (purple) and HECT domain (orange), with overlap in lime [44,45]. Except in panel D, which shows Ubc9, the indicated interfaces have been mapped onto Ubc13 [55]. The models shown in panels E–G were rotated by 180° about the vertical relative to the models in panels A–D. All surface diagrams were generated by PyMOL [142].

As discussed above, we know something about the shadows that are cast by E1, E3s, and (covalently bound) ubiquitin on the E2 surface. How do substrates approach the E2 active site? To date, two studies have shed light on this point. One significant advance is the structure of Ubc9 complexed with RanGAP1 [51]. The sumoylation of this substrate regulates nucleocytoplasmic transport in higher organisms [23]. Unlike ubiquitination, sumoylation is site-specific, usually occurring at a lysine in the motif ΦKXD/E, where Φ is a hydrophobic residue and X is any amino acid. The structure nicely explains this specificity—residues of Ubc9 are seen interacting with each of the RanGAP1 residues in the consensus motif (Fig. 3B), placing the lysine within striking distance (3.5 Å) of the Ubc9 active site cysteine. These interactions are known to contribute significantly to the Ubc9/RanGAP1 binding affinity [52] and should apply in all sumoylation reactions involving a consensus site, as suggested by biochemical studies with two other substrates [51]. There is also a second region of mainly hydrophobic contact between RanGAP1 and Ubc9, accounting for two-thirds of the total interaction surface on Ubc9 [51]. Mutational analyses suggest that this surface is unique to RanGAP1 versus other Ubc9 substrates. RanGAP1 is by far the most efficient Ubc9 substrate known; the existence of a specialized RanGAP1 binding site on Ubc9 can explain this property.

The second study produced a model rather than a structure. Ubiquitin E2 Variant (UEV) proteins resemble E2s but lack the active site cysteine residue. Heterodimeric E2/UEV complexes act as specialized E2s in reactions

where substrates are modified by polyubiquitin chains linked through Ub-K63 [53–56]. In contrast to K48-linked polyubiquitin chains, which target substrates to 26S proteasomes for degradation, K63-linked chains are non-proteolytic signals, most notably in DNA damage tolerance and protein kinase activation (see Ref. [22]). The structural model shows the Ubc13/Mms2 (E2/UEV) complex bound to two ubiquitins. One of them (the donor) is covalently linked to Ubc13; the other (the acceptor) is noncovalently associated with Ubc13/Mms2. The acceptor ubiquitin is poised to insert its K63 side chain into the Ubc13 active site to produce a diubiquitin conjugate [55]. The structural model suggests that the E2/UEV complex selects Ub-K63 as the site of conjugation through a steric exclusion mechanism. Specifically, interactions with substrate surfaces (ubiquitin is the substrate in this reaction) that are distant from K63 result in the selective presentation of this residue to the active site [55,57]. A recent NMR analysis produced a model for the complex of hUbc13/hMms2 bound to a covalent donor ubiquitin and a noncovalently associated acceptor ubiquitin [58] that agrees well with the model proposed previously [55]. This mechanism contrasts with the Ubc9/RanGAP1 example, in which Ubc9 interacts directly with substrate residues near the modification site. Thus, even though the Mms2/Ubc13/Ub example lacks atomic detail, Ub-K63 seems likely to approach the Ubc13 active site by a different route from the one used by the reactive lysine of RanGAP1 to reach the Ubc9 active site (Fig. 3B and C).

One problem in trying to generalize from these results is that both of the above-described conjugation reactions are site-specific—something that is not characteristic of ubiquitin conjugation unless the substrate is ubiquitin itself. Still, the results tend to suggest that there is more than one pathway by which a lysine residue can approach the E2 active site. However, the two examples also share an important property: interactions with regions of the substrate that are distant from its modification site contribute importantly to binding. This property is probably even more relevant in E3s (see below).

What about chemistry? From the point of view of catalysis, the active site landscape seen in the many crystal structures of unliganded E2 enzymes is rather featureless. The E2 has two jobs in the conjugation cascade: to accept the Ubl protein from the E1 enzyme and to pass it to a downstream protein—either an E3 enzyme or the substrate itself (see next section). The transfer of an acyl group (the Ubl protein) between thiols, or from a thiol to an amine, is expected to depend on an "oxyanion hole" that can stabilize the charged intermediate formed during attack of the thiol/amino group (Fig. 2C; see Refs. [59–61]). A general base may also be needed to deprotonate the attacking group [60,61]. The base would be most important when the attacking group is lysine, with its high pK_a.

There are several ways to reconcile these expectations with the apparent dearth of catalytic groups in the E2 active site. First, the general base and/or oxyanion site may simply be absent. A primitive catalytic apparatus could explain the inefficient rates that usually apply when E2s and E2/E3 complexes are assayed in vitro. Second, the active site of the upstream (E1) or downstream (E3) enzyme could provide catalytic elements. Although this explanation is attractive for E1 and certain E3s, it seems unlikely to apply with other E3s, as discussed below. Third, oxyanion holes are often formed from main chain nitrogens [59]; this type of site can be hard to detect biochemically. Finally, the E2 (or E3) active site could contain cryptic groups that adopt a catalytic configuration only after substrate or ubiquitin has bound. Studies with ubiquitin deconjugating enzymes provide precedent for this model. Ubiquitin binding to Hausp, for example, alters the positions of a histidine (general base) and an asparagine (oxyanion hole) so that these side chains become catalytically competent [62]. Indeed, recent studies from our laboratories suggest that a strictly conserved E2 asparagine residue is part of a cryptic oxyanion site [63].

2.3. Ligases (E3s)

The most remarkable feature of the ubiquitin conjugation pathway is the extraordinary diversity of its substrates. This feature, which explains the breadth of ubiquitin's biological functions, is a direct reflection of the large number of E3 enzymes [18]. Just as there are numerous kinases, each with limited suite of substrates, so too are there many E3s, each with a finite set of substrates (Fig. 2B). In both cases, the pairing of specific enzymes with cognate substrates allows for exquisite specificity in regulating substrate modification.

The paradigm of multiple substrate-specific E3s may not apply to other protein modification pathways. So far, only ISG15 and Sumo appear to resemble ubiquitin in being conjugated to a diverse array of substrates [23,64]. Not much is known yet about the mechanics of ISG15 conjugation, but several Sumo-dedicated E3s have recently been discovered (Refs. [23,65]; see also article by Johnson). The recognition of Sumo substrates is not strictly E3-dependent [23], possibly because of the extensive contacts that Ubc9 makes with residues in the sumoylation site (see above). In contrast, substrate modification by ubiquitin is usually strictly E3-dependent [18].

E3s were originally proposed as bridging factors that bring the E2 and substrate together [66]. This has turned out to be a pretty accurate characterization (see below). Increasingly, E3s can also be defined on a bioinformatic basis. Thus, the known ubiquitin E3s belong to just three protein families: *H*omologous to *E*6AP *C*arboxy *T*erminus (HECT), *R*eally *I*nteresting *N*ew *G*ene (RING), and *U*FD2 homology (U-box) proteins. Database mining indicates that the HECT and U-box families, although significant in size, are much smaller than the RING family. Depending on whether all RING proteins are actually E3s, the total number of E3s in higher organisms could range from several hundred to well over a thousand—in either case, a large

number. Moreover, new E3 subfamilies are still being defined [67].

Generating hundreds of E3s from just three protein domains is made possible by the modular construction of E3 enzymes. The HECT, RING, and U-box domains share a common biochemical property of E2 binding. In a given E3, this E2-interacting domain is grafted onto a different domain(s) that is specialized to interact with substrates of that E3 (see Fig. 4). The two domains can be part of the same polypeptide chain, or the substrate- and E2-binding domains can be distinct subunits of a multi-protein complex. Because E3 enzymes have been the subject of several recent reviews [29,41,68], the following sections provide only concise background information before proceeding to discuss selected recent advances. The article by Deshaies in this issue provides an in-depth treatment of one class of RING E3s, known as SCF E3s.

2.4. RING E3s

The RING domain consists of a short motif rich in cysteine and histidine residues, which coordinate two zinc ions [69]. The spacing of these residues is conserved, but the primary sequence conservation among RING domains is limited. The distinctive cross-brace arrangement of the zinc-interacting residues endows the RING domain with a globular conformation, characterized by a central α-helix and variable-length loops separated by several small β-strands (Fig. 5A). Many RING domains have been shown to directly bind E2s (for example, Refs. [70–74]) and there is persuasive evidence that E2/RING interactions are important for the biological functions of RING domain E3s (see Refs. [29,68,75] and below).

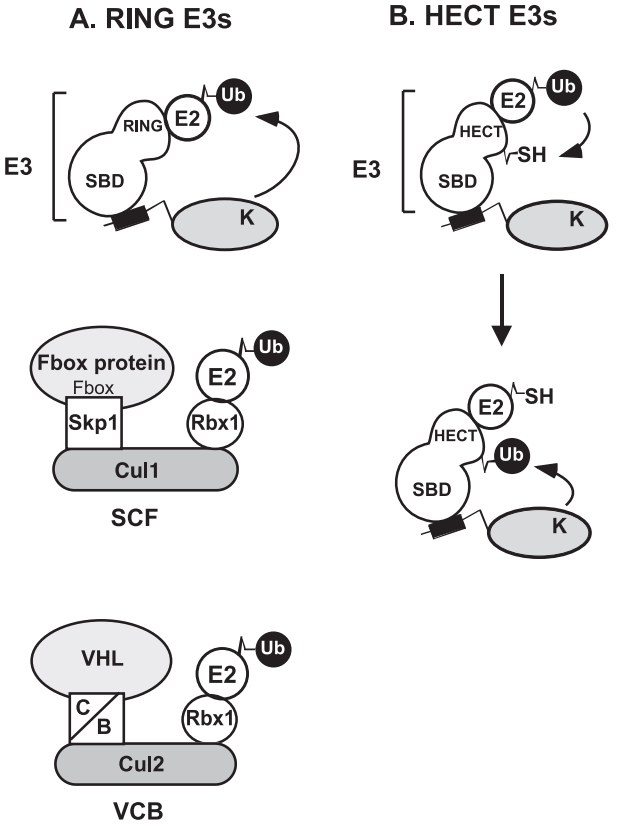

Fig. 4. E3 enzymes. (A) RING domain E3s: top, single subunit enzyme; middle, SCF complex; bottom, VCB (C and B denote elongins C and B). K denotes a substrate lysine residue; the black rectangle represents a degron. Not shown: Cul3-based E3s, in which BTB domain proteins carry both adaptor and substrate-interaction functions, or Cul4A-based E3s [67]. (B) HECT domain E3s. SBD denotes substrate binding domain.

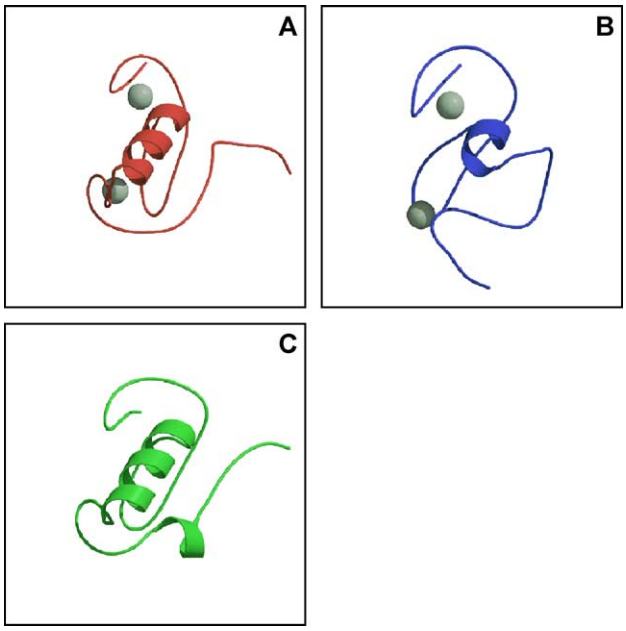

Fig. 5. Conformational of multiple E2-binding domains. (A) RING domain [45]; (B) PHD [92]; (C) U-box [110]. The gray spheres denote zinc ions.

RING E3s best epitomize the original E3 mechanistic model (Fig. 4A). These enzymes come in two flavors, single-subunit and multi-subunit, but much of the following discussion applies to both families. The crystal structure of a c-Cbl/UbcH7 complex [44] shows that the E2 interacts with the RING through the packing of hydrophobic residues from the E2 (see above) into a shallow groove on the RING surface. There are also peripheral electrostatic contacts. It remains uncertain how much these individual contacts contribute to affinity and specificity in RING/E2 interactions.

The most surprising feature of this structure was its revelation that no RING domain side chain comes closer than ~15 Å to the E2 active site cysteine [45]. Based on this and other structures [73,76], it is believed that RING E3s practice "catalysis by proximity." In other words, the principal role of a RING domain E3 is to increase the probability of reaction by bringing together the substrate lysine and the E2~Ub intermediate (see Ref. [41]). What is puzzling is that even if the E2~Ub intermediate comes equipped with a minimal catalytic apparatus [63], there is no evidence for specific interactions that can rigidly fix the substrate lysine residue in a favorable orientation for

reaction. In fact, there is evidence against this type of specific interaction: despite a few counter-examples [77,78], most substrates appear capable of being modified on several or even many lysines (see Refs. [29,79]). And in contrast to sumoylation, there is no consensus site for ubiquitination.

Recent studies with BRCA1 suggest that RING E3s might also employ more subtle mechanisms of catalysis. The N-terminus of BRCA1, which contains a RING domain, is a hotspot for mutations that predispose to breast cancer. At least six such mutations involve zinc-binding cysteines in the RING domain, indicating that BRCA1's E3 activity is relevant to its role as a tumor suppressor, although the relevant substrates remain to be identified (see Refs. [80,81]). BRCA1 forms a heterodimeric complex with another RING domain protein, BARD1. This association is required for robust ligase activity [81] even though the BRCA1/BARD1 interface does not include a significant contribution from either RING domain [80]. Mutations that abrogate the BRCA1/BARD1 interaction [82] instead map to regions of each protein that associate in a four-helix bundle [80]. Theoretically, this arrangement leaves each RING domain free to bind an E2, but in fact the E2 UbcH5c associates exclusively with the RING domain of BRCA1 [74]. The UbcH5c binding interface on BRCA1, while encompassing the hydrophobic groove seen in the c-Cbl/UbcH7 structure, also includes several more distant contacts with the BRCA1 helical domain [74]. That these contacts play a significant role in ubiquitin conjugation is suggested by comparative studies with a different E2, UbcH7, which binds well to BRCA1/BARD but does not enjoy the noncanonical contacts, and does not support conjugation [74].

A large subset of RING E3s differs from the family members discussed above by virtue of their complex structures. Most of these enzymes feature a common RING domain subunit, Rbx1 (also known as Roc1 or Hrt1), which functions in E2 recruitment (see Refs. [68,75] and article by Deshaies). A member of the cullin protein family serves as a scaffold that binds Rbx1; the cullin/Rbx1/E2 subassembly possesses a core ligase activity that is manifested in E2 or cullin autoubiquitination or the assembly of free polyubiquitin chains [72,83,84]. This activity is directed toward a specific substrate through the properties of a specificity subunit that (usually) binds to the cullin via one or more adaptor proteins (Fig. 4A). The cullin family comprises several members, most of which are already known to serve as the organizing subunits for subfamilies of multi-subunit RING E3s [67].

Cul1/Cdc53 is the scaffold of the SCF E3s, which recognize their substrates through an Fbox protein (so called because of the Fbox motif, which interacts with the adaptor). There are 38 Fbox proteins in humans, suggestive of a large set of SCF E3s (see Ref. [73]). Known SCF E3s feature prominently in regulation of the G1/S cell cycle transition [68]. Cul2 is the scaffold of an E3 whose specificity subunit binds through different adaptors (discussed below). Cul3 is the scaffold of a large E3 family whose specificity subunits bind directly to the cullin [67]. The anaphase promoting complex (APC) is a complex multi-subunit E3 that plays a pivotal role in the regulation of mitosis [85]. Two of its subunits, APC2 and APC11, are distant members of the cullin and RING domain families, respectively (see Refs. [68,73]).

The multi-subunit RING E3s provide a spectacular example of how specificity in ubiquitin conjugation is diversified through combinatorial mechanisms (see Ref. [68] and article by Deshaies). One can easily envision the SCF E3 specificity being regulated through the exchange of subunits in response to intracelluar needs. Indeed, certain substrate-binding subunits are autologous substrates, suggesting that the "old" specificity subunit is degraded in order to effect efficient recruitment of the new one (see Ref. [29]).

The molecular mechanisms used by multi-subunit RING E3s to facilitate ubiquitin conjugation remain mysterious. The same issues apply as with the single-subunit RING E3s (above), but with additional complications introduced by the scale of these protein machines. The crystal structure of the SCF^{Skp2} complex exemplifies the problems [73]. It shows that Cul1 adopts a highly extended (110 Å long), rigid conformation that includes three repeats of a novel folding unit. Rbx1 binds at one end of Cul1, while the adaptor subunit Skp1 binds at the other end. The specificity subunit Skp2 is also elongated [86]. It binds to Skp1/Cul1 so that the free end of Skp2 points toward the Rbx1-bound E2. A 50-Å gap separating Skp2 and the E2, which is also predicted from a different Skp1/Fbox structure [76], is presumably occupied by the bound substrate. The SCF^{Skp2} structure reveals that Rbx1 engages in an intimate, intermolecular β-sheet interaction with Cul1. Relative to canonical RING domain E3s, Rbx1 has an insertion that coordinates an extra zinc, but unlike the canonical sites, it is dispensable for catalytic activity [73]. The same is true of APC11 [87].

The cullin-based E3s are subject to a novel mode of regulation by a different UbL—the modification of a specific cullin lysine residue by Nedd8 is necessary in order for these E3s to display optimal activity (see Refs. [29,68]). What neddylation does to achieve this effect is not fully understood; it may stimulate E2~Ub binding to Rbx1 [88]. Consistent with this model, the SCF^{Skp2} structure shows that the Nedd8 conjugation site is favorably positioned to influence the properties of the Rbx1/E2 complex [73]. Although Nedd8 has its own E1 and E2, there is no known Nedd8-specific E3. Therefore, neddylation may represent a specialized auto-modification reaction of cullin-based E3s [89,90].

In the SCF E3s, the proposed job of the Cul1 scaffold is to create a rigid separation between the substrate bound to the Fbox subunit and the E2~Ub intermediate bound to Rbx1 [73]. Consistent with this model, introducing a

flexible linker into the center of Cul1 inhibits the ligase activity of SCF^{Skp2} without affecting substrate (p27) binding [73]. It was suggested that the rigid architecture helps to display a substrate lysine residue in a conformation appropriate for reaction with the E2~Ub intermediate. In principle, rigidity could be an important element of a proximity-based mode of catalysis. In practice, a rigid orientation should depend on interactions with residues surrounding the conjugation site, which appear to be absent in most cases (see above). The model that rigidity is important for SCF E3 function contrasts with the conclusion of recent structural studies of a HECT E3 [91]. In this case, flexibility was deemed to be important (see below).

A small group of E3s was previously suggested to be defined by the presence of a PHD domain (*P*lant *H*omeo*D*omain). PHD domains resemble RING domains in that folding relies on the coordination of two zinc ions in a cross-brace arrangement [92,93]. The RING and PHD domains differ in the details of metal ligands and core residue packing, but the defining zinc atoms of each domain are positioned an identical 14 Å apart and the overall conformations of the two domains are quite similar (Fig. 5A, B). However, recent bioinformatic studies suggested that the "PHD" E3s are actually RING E3s [94]. Whether these domains in known E3s are specialized PHD domains or RING domains is still being debated.

Among the proteins containing this domain are the herpesvirus MIR1/2 (*M*odulator of *I*mmune *R*ecognition) proteins, which conjugate ubiquitin to host cell MHC class I molecules, leading to endocytosis and degradation in lysosomes [95,96]. Ubiquitin conjugation and MHC class I down-regulation is an important tool used by viruses to evade host defenses [97]. If indeed the E2 binding domain is a PHD, then both activities require an intact PHD, suggesting that this domain acts to recruit an E2~Ub intermediate. Interestingly, substrate binding by MIR2 relies on an interaction of transmembrane regions of the MIR and class I molecules [95,96,98].

In another striking example, the MEKK1 kinase, which harbors this domain near its N-terminus, conjugates ubiquitin to the ERK1/2 MAP kinases, leading to ERK1/2 degradation by 26S proteasomes [99]. The destructive outcome of this MEKK1/ERK encounter, which is important for stress-induced apoptosis, contrasts with the ERK activation that ensues following a conventional (kinase) interaction of these molecules [99]. This example is particularly interesting given how frequently PHDs occur in conjunction with other functional domains. The PHD family has several hundred members. Many of these proteins have been implicated in transcriptional regulation, and mutations in PHD proteins are observed in a number of human diseases (see Refs. [92,93,97]). These relationships gain added interest because of recent advances in our understanding of how ubiquitin conjugation regulates transcription (see Refs. [100,101]).

2.5. U-box E3s

An E2-binding domain called the U-box defines a relatively small family of E3s. The U-box was first identified in yeast Ufd2 [102]. Ufd2 is unusual—studies to date suggest that it lacks its own substrate and instead promotes the polyubiquitination of another E3's substrate. This property caused Ufd2 to be classified as an "E4" [102]. At least one other U-box protein, *C*-terminus of *H*sc70 *I*nteracting *P*rotein (CHIP), also displays E4-like activity [103]. However, CHIP can also behave as a conventional E3. The E3/E4 boundary is somewhat blurry. For example, Rad5 (a RING E3) seems to recognize as its "substrate" a ubiquitin that has been linked to a target protein by another E3 [21]. Although Rad5 is considered to be an E3, some of its properties are rather E4-like.

The first hint that U-box proteins might act as conventional E3s came from bioinformatics. Aravind and Koonin [104] predicted that the U-box would adopt a RING domain-like conformation in which electrostatic interactions, rather than metal binding, provide the organizing principle. In turn this suggested that the U-box would bind E2s and facilitate ubiquitin conjugation. Experimental confirmation soon followed. In these studies, several U-box proteins were found to interact directly with E2s and to be subject to autoubiquitination [105–108].

Prp19 is a yeast pre-mRNA splicing factor with an N-terminal U-box that is important for biological activity [109]. The recently reported solution structure of the Prp19 U-box is remarkably similar to the structure of the RING domain [110]. Both domains feature a central α-helix and several small β-strands that are separated by variable loops; the main secondary structural elements are almost superimposable (Fig. 5A and C). In fact, the structure of the Prp19 U-box resembles the structures of several RING domains as closely as the latter structures resemble one another [110].

How is the U-box stabilized? The structure shows a pronounced hydrophobic core, along with two "internal interaction centers" comprising multiple, dynamic hydrogen bonds and salt bridges [110]. The characters of the residues involved in these interactions are largely conserved among different U-box proteins, and their spacing is similar to that of the zinc-coordinating residues of the RING domain. Because of this conserved spacing, the principal interactions that stabilize the U-box and RING domains occur in the same spatial locations [110], as predicted by the original bioinformatic analysis [104]. Moreover, just as the mutation of a zinc-coordinating residue leads to RING domain unfolding, so also does the disruption of a key electrostatic interaction cause U-box unfolding [110].

As in the RING domain, the folding of the U-box creates a shallow groove on one face of the domain that is largely hydrophobic in character [110]. Some of the residues in this groove have been mutated in U-box E3s, and this has been

found to abolish ligase activity [105] even though the mutations do not destabilize the domain's overall conformation [110]. On the other hand, the *prp19-1* mutation, which changes a valine residue in the hydrophobic core to isoleucine, causes a complete loss of folding in vitro [110] and inhibition of pre-mRNA splicing in vivo [109]. Thus, proper conformation of the Prp19 U-box is vital for pre-mRNA splicing. These results suggest that Prp19 regulates splicing through a mechanism involving ubiquitin conjugation. Consistent with this model, the Prp19 U-box displays an in vitro autoubiquitination activity that is dependent on U-box integrity [110]. The physiological substrates of Prp19 remain to be identified.

The best-studied member of the U-box E3 family is CHIP. Many of CHIP's known substrates are misfolded proteins whose recognition is dependent on the association of CHIP with the Hsc70 or Hsp90 chaperones (see Ref. [111]). CHIP substrates that conform to this paradigm include the cystic fibrosis transmembrane receptor [112], the glucocorticoid receptor [113], and tau [114,115]. In these targeting events, the chaperone appears to serve as a specificity factor that recognizes the (unfolded) protein substrate. By relegating recognition to a chaperone, CHIP can target diverse proteins that resemble one another only by virtue of their unfolded states. This is one of several mechanisms by which damaged or misfolded proteins are selectively targeted for degradation by proteasomes (see Refs. [38,111]).

2.6. HECT E3s

RING, PHD, and U-box E3s all facilitate ubiquitin conjugation by acting as bridging factors. The HECT E3s employ a mechanism that is unique among E3s, but similar to many other ubiquitin-handling enzymes—they form a thiol ester intermediate with ubiquitin [116]. In HECT E3-dependent reactions, the E3 cysteine, not the E2 cysteine, is the last stop for activated ubiquitin (Fig. 4B).

*E6 A*ssociated *P*rotein (E6AP) is the founding member of the HECT E3 family. E6AP is (in)famous because it acquires, upon binding of the E6 protein of an oncogenic human papillomavirus, the ability to bind and ubiquitinate the host cell p53 protein, resulting in p53 degradation, viral DNA replication, and attendant deleterious consequences for the host cell [117]. E6AP also recognizes a number of substrates in normal cells (see Ref. [29]) and the E6AP locus is mutated in a human disease known as Angelman syndrome (see Ref. [44]). HECT E3s are defined by the presence of a region of ~350 amino acids that is homologous to the C-terminus of E6AP [118].

The N-terminus of E6AP mediates substrate recognition, while its HECT domain binds the E2~Ub intermediate and accepts ubiquitin at a conserved cysteine residue (see Ref. [29]). The crystal structure of the E6AP-HECT/UbcH7 complex showed that the HECT domain is L-shaped, with the active site positioned near the bend in the L [44]. The area around the active site is a hotspot for mutations that cause Angelman syndrome, indicating that loss of E3 activity is relevant in the disease. Although the active site lacks well-positioned candidates for the anticipated general base and oxyanion hole, there are a number of suitable side chains that are not too far away. It is therefore possible that a more competent active site is organized once ubiquitin (or substrate) is bound. The E2-binding site is at the end of the base of the L. Even though there is no structural similarity between the HECT and RING domains, both domains bind the E2 in a similar way—residues at the C-terminal end of the first E2 helix and in the loops between two E2 β-strands make hydrophobic and electrostatic contacts with the E3 surface (Fig. 3F and G [44,45,73]).

A startling feature of this structure is that the E2 and E3 cysteines are separated by a whopping 41 Å. The inference that catalytic ubiquitin transfer involves large-scale conformational transitions is confirmed in the recent crystal structure of the HECT domain of another family member, WWP1 [91]. Here the HECT domain is folded into an inverted T shape rather than an L shape. Consequently, the HECT cysteine and the (modeled) E2 cysteine are only 16 Å apart. Modeling shows that the two HECT structures can be interconverted by rotation and translation about a three-residue hinge-loop region positioned near the bend of the L in the E6AP-HECT structure. Continuing along this trajectory, a conformation is reached in which the E2 and HECT cysteines are only 5 Å apart. Functional studies support the idea that these modeled conformational transitions are biologically relevant. Deleting the hinge-loop residues, or mutating them in a manner that should reduce their rotational freedom, inhibits WWP1-HECT-catalyzed autoubiquitination, while a four-residue insertion into the hinge-loop region is well tolerated [91].

Results obtained with the WWP1-HECT domain suggest that flexible movements of different HECT subdomains are important for catalysis. This contrasts strongly with SCF^{Skp2}, where a similar experimental strategy (changing the flexibility of a linker) led to the conclusion that rigidity is paramount [73]. This qualitative difference between the RING and HECT families seems likely to reflect their fundamentally different mechanisms. One can speculate that the HECT active site has elements that carry out conventional chemical catalysis (above). The advantage could be spectacular: in the serine protease family, the combined rate enhancement attributable to the general base and oxyanion hole is about 10^9-fold [61]. It appears unlikely that E2s have this kind of complete active site (above). Thus, it is attractive to think that RING E3s overcome the attendant disadvantage by rigidly fixing the lysine and the E2~Ub intermediate in a favorable orientation [73]. The missing link in this model, as discussed above, is a site on the E2 or RING that interacts with the environs of the substrate lysine residue so as to immobilize it.

If the HECT and E2 cysteines can approach within 5 Å of each other, then why are they so far apart in both HECT structures? Verdecia and co-workers propose a model that explains the need for a varying HECT–E2 distance and, at the same time, addresses a long-standing problem in ubiquitin conjugation. Many, perhaps most, E3s modify their substrates with polyubiquitin chains. Exactly how this happens is poorly understood. Does the E3 add ubiquitins one at a time? The problem with this mechanism is that the E3 has to change its specificity after the first transfer, so that ubiquitin (versus substrate) is recognized. Is there a change from a substrate- to a ubiquitin-recognizing E3 after the first transfer? This model may apply in certain cases (above [21]). Or is the chain built up on the E2 or E3, and then transferred in an already-assembled state to the substrate? This last mechanism demands two sites where the activated ubiquitin (chain) can reside during its assembly, so RING E3s cannot employ it unless they have two E2 binding sites. However, there are two sites for activated ubiquitin binding in HECT E3/E2 complexes.

The specific model [91] postulates that the first or proximal ubiquitin of the chain (the one whose C-terminus will ultimately be linked to the substrate lysine residue) remains bound to the HECT cysteine through successive rounds of ubiquitin transfer from the E2~Ub intermediate. Thus, the distal terminus of the growing chain will gradually approach the bound E2, and the optimal HECT–E2 distance will increase as the chain grows. At some point, steric impediments will slow the chain elongation rate and favor capture of the chain by a substrate lysine residue. This attractive model makes several predictions, which have yet to be tested.

3. Molecular basis of substrate specificity in protein modification reactions

Substrate recognition is the purview of the E3 enzyme. The substrate-interacting domain, which is distinct from the E2-binding domain, recognizes a specific sequence or structural element (a ubiquitination signal) in the cognate substrate (Fig. 4). The E3–substrate interaction is often regulated, frequently through covalent modification of the substrate. Modifications that are known to modulate the recognition of substrates by their cognate E3s include phosphorylation (especially in cell cycle regulation), glycosylation, acetylation, and hydroxylation (see Refs. [68,119–123]). The underlying principles of substrate recognition are well established and have been reviewed previously [66,68,124], although new examples are discovered on a regular basis. What was missing until recently was a view of an E3/signal interface at atomic resolution. Three recent structures reveal the molecular interactions of authentic E3s with their cognate substrates. In two cases, the substrate must be covalently modified in order to be recognized.

3.1. VHL/HIF-1α interaction

*V*on Hippel Lindau/Elongin *B*/Elongin *C* (VBC) is a Cul2-based E3 whose specificity subunit, VHL, is mutated in the Von Hippel Lindau hereditary cancer syndrome ([125]; Fig. 4A). Elongins B and C are adaptors that link VHL to Cul2. The RING domain subunit is Rbx1. Members of the *H*ypoxia *I*nducible *F*actor (HIF) family of transcription factors are cancer-relevant substrates of the VCB E3 [126]. The recognition of these substrates is oxygen-regulated: HIF-1α is rapidly ubiquitinated and degraded in normoxic conditions, but it is refractory to ubiquitination in hypoxic conditions. HIF-1α stabilization (i.e., hypoxia) leads to the induction of genes that regulate angiogenesis and glucose metabolism [125].

Oxygen regulates HIF-1α stability through the enzymatic hydroxylation of a specific proline residue, which makes HIF-1α recognizable by VHL [120,121,127,128]. Recently, lysine acetylation was identified as a further mechanism that regulates the ubiquitination of HIF-1α [123]. The molecular mechanism of this regulation remains to be fully elucidated. The HIF-1α lysine residue that becomes acetylated is not particularly close to the hydroxylated proline(s), so perhaps acetylation is necessary for the recognition of HIF-1α by the prolyl hydroxylase.

An earlier structure of the VHL/B/C complex revealed that many mutations which predispose to VHL disease are clustered on a solvent-exposed hydrophobic surface of VHL, leading to the speculation that this surface binds a specific substrate (HIF-1α had not been identified at that time) [129]. This proposal was confirmed by structures of a hydroxylated HIF-1α peptide bound to VHL [130,131]. The structures show that the peptide binds in an extended conformation along a β-sandwich region of VHL (Fig. 6A). Although there are β-sheet-like interactions between VHL and main chain groups of the substrate peptide, interactions with the hydroxyproline residue generate the lion's share of the affinity and specificity. This side chain is deeply buried, with its hydroxyl group hydrogen-bonded to buried serine and histidine residues of VHL. If HIF-1α bound to VHL in an unmodified state, these buried VHL side chains would have to be desolvated without forming compensatory favorable contacts. Thus, both positive and negative elements help to generate the appropriate specificity. The VHL/hydroxy-HIF-1α interface embodies a simple mode of recognition in which specific contacts with the modifying (hydroxyl) group are central to discrimination by the E3. This interface is an attractive drug target—blocking the interaction would stabilize HIF-1α independent of oxygen status, allowing for induction of angiogenesis following heart attack or stroke.

3.2. Cdc4/phospho-Sic1 interaction

The interaction of phosphorylated substrates with Cdc4 epitomizes a more complex mode of recognition. Yeast

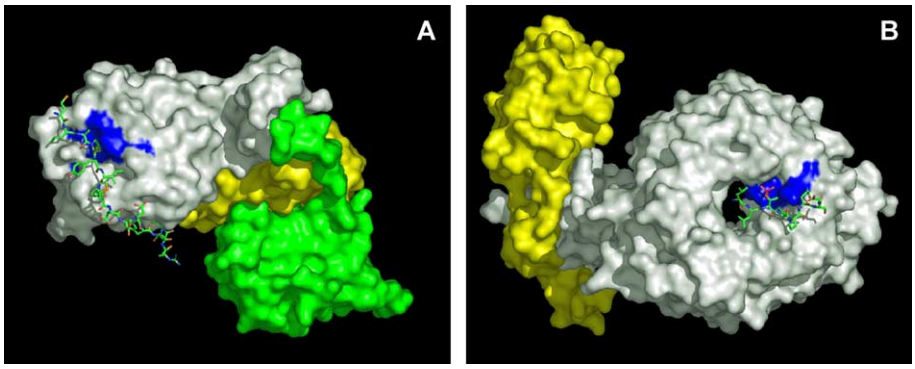

Fig. 6. Molecular basis of substrate specificity: E3/signal interactions. (A) Hydroxylated HIF-1α peptide bound to VHL/elongin B/elongin C [130]. VHL is colored white with residues colored blue that interact with the HIF-1 hydroxyproline. Elongin B is colored green with elongin C colored yellow; (B) phospho-CPD bound to Skp1/Cdc4 [76]. Cdc4 is colored white with residues colored blue that interact with the CPD phosphate; Skp1 is colored yellow.

SCF^{Cdc4} targets a number of cell cycle regulatory factors for degradation by proteasomes, in a manner that is invariably regulated by substrate phosphorylation [76,132]. One key substrate, Sic1, must be eliminated by 26S proteasomes in order for cells to pass from G1 into S phase and initiate DNA replication. The recognition of Sic1 by Cdc4 requires Sic1 phosphorylation by the G1 cyclin-dependent kinase (CDK). The properties of this regulatory modification are unusual: Sic1 must carry a large number of phosphates (five or six) before it can be recognized by Cdc4, but the phosphates can be distributed among any of nine potential sites [132,133]. Each of the phosphorylated sites of Sic1 has a low affinity for Cdc4 relative to an optimized phosphopeptide, the so-called Cdc4-phosphodegron or CPD, that was discovered through a combinatorial approach [132]. This mode of phospho-Sic1 recognition by Cdc4 can lead to a switch-like regulation of Sic1 degradation, ensuring that exit from G1 phase is delayed until G1 CDK activity is appropriately high. In this way, premature DNA replication is avoided (see Refs. [122,132]).

How does Cdc4 accomplish this unusual mode of recognition? Cdc4 is a WD40 protein, indicating that it folds into a β-propeller. Sequence- and structure-based considerations suggested that the Cdc4 WD40 domain harbors a single phosphopeptide binding site defined by a trio of conserved arginine residues [132]. The structure of a CPD/Cdc4 complex (Fig. 6B) shows that this is indeed the sole site of interaction with the optimized degron [76]. The structure explains several features shared by the CPD and the natural phosphodegrons. For example, preferences for leucine and proline at the −1 and +1 positions, respectively, reflect the presence of appropriate pockets for these side chains. The structure also explains why the natural phosphodegrons of Sic1 bind with a low affinity: each of them has at least one feature that hinders optimal interaction with the recognition site of Cdc4—usually, a nearby basic residue that will experience electrostatic repulsion from the arginine residues of Cdc4. The current hypothesis for Sic1 recognition invokes multiple weakly-interacting phosphodegrons that interact with a single site. Kinetic modeling suggests that the observed switch-like behavior derives mainly from cooperative effects on the rate of degron escape [134]. When considering Sic1 degradation (versus simple E3/Sic1 binding), the rate of ubiquitin conjugation is also part of the equation, and could contribute to a threshold effect because the relative rates of ubiquitin transfer and substrate dissociation could change from ineffective to effective with a modest decrease in the substrate dissociation rate.

The Cdc4/CPD interaction has interesting ramifications for the recognition of other Cdc4 substrates. Some Cdc4 targets have suboptimal phosphodegrons, but at least one has a site very like the optimal CPD. The results in hand, and the properties of these substrates, suggest that distinct substrates can sequentially access a single recognition site on Cdc4, depending on factors such as the timing and number of phosphate modifications, the contexts of the surrounding residues, etc. Thus, the degradation of different substrates may be subject to sophisticated regulation even when ostensibly identical degrons bind to a single E3 site.

3.3. Mdm2/p53 interaction

Mdm2 is a RING E3 that controls levels of the tumor suppressor p53 through several mechanisms [135]. Mdm2 binds p53 and blocks p53's transcriptional activation function. Mdm2 has also been linked with p53 nuclear export, which inhibits p53-mediated transcription. The *mdm2* gene is itself induced by p53, leading to an autoregulatory negative feedback loop. Regulating p53 protein levels via ubiquitination and degradation is another mode of control. The structure of the N-terminal substrate binding domain of Mdm2 bound to a p53 peptide comprising the ubiquitination signal shows the mode of binding [136]. Mdm2 contains a hydrophobic cleft in which the p53 peptide binds via its hydrophobic face, utilizing in

particular three p53 residues that are also used in p53 transactivation.

Mdm2 is overexpressed in many human tumors, and the inhibition of Mdm2 expression has been shown to lead to the activation of p53 [137]. Disruption of the Mdm2/p53 interaction also leads to p53 activation. Recently, a small-molecule antagonist has been identified [137] that binds Mdm2 in the p53-binding pocket (above), thus preventing p53 ubiquitination and leading to the activation of the p53 pathway. This line of research may eventually lead to a novel therapy for cancers characterized by transcriptionally active p53 alleles.

4. Concluding remarks

The past several years have seen tremendous advances in elucidating the basic biology and biochemistry of the ubiquitin protein family, but a great deal remains to be learned. In terms of biology, many of the recently discovered modifier proteins remain poorly characterized. Thus, we still do not know the functional consequences of substrate modification by ISG15, Fat10, Urm1, Ufm1, or Hub1; this is also true in many instances of sumoylation. Our knowledge of the conjugating machinery for these proteins is very incomplete. And it is almost certain that new members of the ubiquitin protein family have yet to be discovered. On the other hand, it is now clear that biochemical mechanisms of protein conjugation are highly conserved. This property will facilitate ongoing and future investigations.

By far, the greatest strides have been made with the founding family member. We now have a much higher resolution view of the protein–protein interactions that mediate ubiquitin activation, transfer, and conjugation. What is missing, as discussed above, is a clear understanding of how these reactions are catalyzed. Answering this question will be an important advance in basic knowledge. But it is also important for other purposes—successful drugs are often designed to interact with and neutralize catalytic groups. The E3/substrate interface, which is likely to be idiosyncratic, is also an attractive drug target [137]. So far, only a few such interfaces have been characterized in full molecular detail, but more are sure to follow. Given the extreme biomedical importance of protein conjugation, new discoveries about specificity and mechanism should be forthcoming on a regular basis.

Once conjugation has occurred, the protein modifier must be recognized as a preface to downstream consequences. There has been a recent acceleration in the identification of ubiquitin-recognizing motifs and domains (see Refs. [17,19]), but functional understanding of these domains is still at an early stage. The mechanisms by which other family members are recognized remain essentially unknown. This area, too, should provide plenty of exciting results over the next few years.

Acknowledgements

We are grateful for support from the NIH (grants DK46984 and GM60372). We apologize to the many colleagues whose work could not be cited directly due to space limitations. We thank W. Chazin, R. Klevit, and B. Schulman for communicating results in advance of publication.

References

[1] C.M. Pickart, Polyubiquitin chains, in: J. Peters, R. Harris, D. Finley (Eds.), Ubiquitin and the Biology of the Cell, Plenum Press, New York, 1998, pp. 19–63.
[2] M. Hochstrasser, There's the Rub: a novel ubiquitin-like modification linked to cell cycle regulation, Genes Dev. 12 (1998) 901–907.
[3] M. Hochstrasser, Evolution and function of ubiquitin-like protein-conjugation systems, Nat. Cell Biol. 2 (2000) E153–E157.
[4] S. Jentsch, G. Pyrowolakis, Ubiquitin and its kin: how close are the family ties? Trends Cell Biol. 10 (2000) 335–342.
[5] M.J. Rudolph, M.M. Wuebbens, K.V. Rajagopalan, H. Schindelin, Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation, Nat. Struct. Biol. 8 (2001) 42–46.
[6] C. Wang, J. Xi, T.P. Begley, L.K. Nicholson, Solution structure of ThiS and implications for the evolutionary roots of ubiquitin, Nat. Struct. Biol. 8 (2001) 47–51.
[7] M.W. Lake, M.M. Wuebens, K.V. Rajagopalan, H. Schindlein, Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB–MoeD complex, Nature 414 (2001) 325–329.
[8] J. Bloom, V. Amador, F. Bartolini, G. DeMartino, M. Pagano, Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation, Cell 115 (2003) 71–82.
[9] K.J. Ritchie, D.E. Zhang, ISG15: the immunological kin of ubiquitin, Semin. Cell Dev. Biol. 15 (2004) 237–246.
[10] M. Komatsu, T. Chiba, K. Tatsumi, S.-i. Iemura, I. Tanida, N. Okazaki, T. Ueno, E. Kominami, T. Natsume, K. Tanaka, A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier, EMBO J. 23 (2004) 1977–1986.
[11] K. Furukawa, N. Mizushima, T. Noda, Y. Ohsumi, A protein conjugation system in yeast with homology to biosynthetic reaction in prokaryotes, J. Biol. Chem. 275 (2000) 7462–7465.
[12] G.A.G. Dittmar, C.R.M. Wilkinson, P.T. Jedrzejewski, D. Finley, Role of a ubiquitin-like modification in polarized morphogenesis, Science 295 (2002) 2442–2446.
[13] A.S. Goehring, D.M. Rivers, G.F. Sprague, Urmylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast, Mol. Biol. Cell 14 (2003) 4329–4341.
[14] Y. Paz, Z. Elazar, D. Fass, Structure of GATE-16, membrane transport modulator and mammalian ortholog of autophagocytosis factor Aut7p, J. Biol. Chem. 275 (2000) 25445–25450.
[15] Y. Ohsumi, Molecular dissection of autophagy: two ubiquitin-like systems, Nat. Rev., Mol. Cell Biol. 2 (2001) 211–216.
[16] K.J. Walters, M.F. Kleijnen, A.M. Goh, G. Wagner, P.M. Howley, Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a, Biochemistry 41 (2002) 1767–1777.
[17] A. Buchberger, From UBA to UBX: new words in the ubiquitin vocabulary, Trends Cell Biol. 12 (2002) 216–221.
[18] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[19] L. Hicke, R. Dunn, Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins, Annu. Rev. Cell Dev. Biol. 19 (2003) 141–172.

[20] I. Garcia-Higuera, T. Taniguchi, S. Ganesan, M.S. Meyn, C. Timmers, J. Hejna, M. Grompe, A.D. D'Andrea, Interaction of the Fanconi Anemia proteins and BRCA1 in a common pathway, Mol. Cell 7 (2001) 249–262.
[21] C. Hoege, B. Pfander, G.-L. Moldovan, G. Pyrowolakis, S. Jentsch, RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO, Nature 419 (2002) 135–141.
[22] C.M. Pickart, Back to the future with ubiquitin, Cell 116 (2004) 181–190.
[23] J.S. Seeler, A. Dejean, Nuclear and unclear functions of SUMO, Nat. Rev., Mol. Cell Biol. 4 (2003) 690–699.
[24] S. Raasi, G. Schmidtke, M. Goettrup, The ubiquitin-like protein FAT10 forms covalent conjugates and induces apoptosis, J. Biol. Chem. 276 (2001) 35334–35443.
[25] D. Finley, S. Sadis, B.P. Monia, P. Boucher, D.J. Ecker, S.T. Crooke, V. Chau, Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant, Mol. Cell. Biol. 14 (1994) 5501–5509.
[26] G.R. Bylebyl, I. Belichenko, E.S. Johnson, The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast, J. Biol. Chem. 278 (2003) 44113–44120.
[27] C. Zhao, S.L. Beaudenon, M.L. Kelley, M.B. Waddell, W. Yuan, B.A. Schulman, J.M. Huibregtse, R.M. Krug, The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an INF-α/β-induced ubiquitin-like protein, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7578–7582.
[28] H. Walden, M.S. Podgorski, B.A. Schulman, Insights into the ubiquitin transfer cascade from the structure of the E1 for NEDD8, Nature 422 (2003) 330–334.
[29] C. Pickart, Mechanisms underlying ubiquitination, Annu. Rev. Biochem. 70 (2001) 503–533.
[30] D.T. Huang, H. Walden, D. Duda, B.A. Schulman, Ubiquitin-like protein activation, Oncogene 23 (2004) 1958–1971.
[31] T. Burch, A.L. Haas, Site-directed mutagenesis of ubiquitin: differential roles for arginine in the interaction with ubiquitin-activating enzyme, Biochemistry 33 (1994) 7300–7308.
[32] F.G. Whitby, G. Xia, C.M. Pickart, C.P. Hill, Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes, J. Biol. Chem. 273 (1998) 34893–34991.
[33] H. Walden, M.S. Podgorski, D.T. Huang, D.W. Miller, R.J. Howard, D.L. Minor, J.M. Holton, B.A. Schulman, The structure of the APPBP1–UBA3–NEDD8–ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1, Mol. Cell 12 (2003) 1427–1437.
[34] R. Varadan, O. Walker, C.M. Pickart, D. Fushman, Structural properties of polyubiquitin chains in solution, J. Mol. Biol. 324 (2002) 637–647.
[35] B. Sarcevic, A. Mawson, R.T. Baker, R.L. Sutherland, Regulation of the ubiquitin-conjugating enzyme hHR6A by CDK-mediated phosphorylation, EMBO J. 21 (2002) 2009–2018.
[36] A. Yamanaka, S. Hatakeyama, K. Kominami, M. Kitagawa, M. Matsumoto, K. Nakayama, Cell cycle-dependent expression of mammalian E2-C regulated by the anaphase-promoting complex/cyclosome, Mol. Biol. Cell 11 (2000) 2821–2831.
[37] P. Chen, P. Johnson, T. Sommer, S. Jentsch, M. Hochstrasser, Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MATa2 repressor, Cell 74 (1993) 357–369.
[38] T. Sommer, D.H. Wolf, Endoplasmic reticulum degradation: reverse protein flow of no return, FASEB J. 11 (1997) 1227–1233.
[39] R. Swanson, M. Locher, M. Hochstrasser, A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Mata2 repressor degradation, Genes Dev. 15 (2001) 2660–2674.
[40] K. Wu, A. Chen, P. Tan, Z.-Q. Pan, The Neddi-conjugated ROC1–CUL1 core ubiquitin ligase utilizes Nedd8 charged surface residues for efficient polyubiquitin chain assembly catalyzed by Cdc34, J. Biol. Chem. 277 (2002) 516–527.
[41] A.P. VanDemark, C.P. Hill, Structural basis of ubiquitylation, Curr. Opin. Struct. Biol. 12 (2002) 822–830.
[42] K.S. Hamilton, M.J. Ellison, K.R. Barber, R.S. Williams, J.T. Huzil, S. Mckenna, C. Ptak, M. Glover, G.S. Shaw, Structure of a conjugating enzyme-ubiquitin thiol ester intermediate reveals a novel role for the ubiquitin tail, Structure 9 (2001) 897–904.
[43] T. Miura, W. Klaus, B. Gsell, C. Miyamoto, H. Senn, Characterization of the binding interface between ubiquitin and class I human ubiquitin-conjugating enzyme 2b by multidimensional heteronuclear NMR spectroscopy in solution, J. Mol. Biol. 290 (1999) 213–228.
[44] L. Huang, E. Kinnucan, G. Wang, S. Beaudenon, P.M. Howley, J.M. Huibregtse, N.P. Pavletich, Structure of an E6AP–UbcH7 complex: insights into ubiquitination by the E2–E3 enzyme cascade, Science 286 (1999) 1321–1326.
[45] N. Zheng, P. Wang, P.D. Jeffrey, N.P. Pavletich, Structure of a c-Cbl–UbcH7 complex: RING domain function in ubiquitin-protein ligases, Cell 102 (2000) 533–539.
[46] H.D. Ulrich, Protein–protein interactions in an E2–RING finger complex: implications for ubiquitin-dependent DNA damage repair, J. Biol. Chem. 278 (2003) 7051–7058.
[47] G. Martinez-Noel, U. Muller, K. Harbers, Identification of molecular determinants required for interaction of ubiquitin-conjugating enzymes and RING finger proteins, Eur. J. Biochem. 268 (2001) 5912–5919.
[48] Y. Xie, A. Varshavsky, The E2–E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain, EMBO J. 18 (1999) 6832–6844.
[49] K. Madura, R.J. Dohmen, A. Varshavsky, N-recognin/Ubc2 interactions in the N-end rule pathway, J. Biol. Chem. 268 (1993) 12046–12054.
[50] K.P. Bencsath, M.S. Podgorski, V.R. Pagala, C.A. Slaughter, B.A. Schulman, Identification of a multifunctional binding site on Ubc9p required for Smt3p conjugation, J. Biol. Chem. 277 (2002) 47938–47945.
[51] V. Bernier-Villamor, D.A. Sampson, M.J. Matunis, C.D. Lima, Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1, Cell 108 (2002) 345–356.
[52] D.A. Sampson, M. Wang, M.J. Matunis, The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification, J. Biol. Chem. 276 (2001) 21664–21669.
[53] R.M. Hofmann, C.M. Pickart, Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair, Cell 96 (1999) 645–653.
[54] T.F. Moraes, R.A. Edwards, S. McKenna, L. Pastushok, W. Xiao, J.N.M. Glover, M.J. Ellison, Crystal structure of the human ubiquitin conjugating complex, hMms2–hUbc13, Nat. Struct. Biol. 8 (2001) 669–673.
[55] A.P. VanDemark, R.M. Hofmann, C. Tsui, C.M. Pickart, C. Wolberger, Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer, Cell 105 (2001) 711–720.
[56] L. Deng, C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, Z.J. Chen, Activation of the IkB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain, Cell 103 (2000) 351–361.
[57] S. McKenna, L. Spyracopoulos, T. Moraes, L. Pastushok, C. Ptak, W. Xiao, M.J. Ellison, Non-covalent interaction between ubiquitin and the human DNA repair protein Mms2 is required for the Ubc13-mediated poly-ubiquitination, J. Biol. Chem. 276 (2001) 40120–40126.
[58] S. McKenna, T. Moraes, L. Pastushok, C. Ptak, W. Xiao, L. Spyracopoulos, M.J. Ellison, An NMR-based model of the ubiquitin-bound human ubiquitin conjugation complex Mms2–Ubc13: the

structural basis for lysine 63 chain synthesis, J. Biol. Chem. 278 (2003) 13151–13158.
[59] N.D. Rawlings, A.J. Barrett, Families of cysteine peptidases, Methods Enzymol. 244 (1994) 461–486.
[60] P. Kursula, J. Ojala, A.-M. Lambeir, R.K. Wierenga, The catalytic cycle of biosynthetic thiolase: a conformational journey of an acetyl group through four binding modes and two oxyanion holes, Biochemistry 41 (2002) 15543–15556.
[61] P. Carter, J.A. Wells, Dissecting the catalytic triad of a serine protease, Nature 332 (1988) 564–568.
[62] M. Hu, P. Li, M. Li, W. Li, T. Yao, J.-W. Wu, W. Gu, R.E. Cohen, Y. Shi, Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde, Cell 111 (2002) 1041–1054.
[63] P.-Y. Wu, M. Hanlon, M. Eddins, C. Tsui, R. Rogers, J.P. Jensen, M.J. Matunis, A.M. Weissman, C. Wolberger, C.M. Pickart, A conserved catalytic residue in the E2 enzyme family, EMBO J. 22 (2003) 1–10.
[64] M.P. Malakhov, K.I. Kim, O.A. Malakhova, B.S. Jacobs, E.C. Borden, D.-E. Zhang, High-throughput immunoblotting: ubiquitin-like protein ISG15 modifies key regulators of signal transduction, J. Biol. Chem. 278 (2003) 16608–16613.
[65] E.S. Johnson, A.A. Gupta, An E3-like factor that promotes SUMO conjugation to the yeast septins, Cell 106 (2001) 735–744.
[66] A. Hershko, H. Heller, S. Elias, A. Ciechanover, Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown, J. Biol. Chem. 258 (1983) 8206–8214.
[67] L. Pintard, A. Willems, M. Peter, Cullin-based ubiquitin ligases: Cul3–BTB complexes join the family, EMBO J. 23 (2004) 1681–1687.
[68] R.J. Deshaies, SCF and cullin/RING H2-based ubiquitin ligases, Annu. Rev. Cell Dev. Biol. 15 (1999) 435–467.
[69] K.L. Borden, RING domains: master builders of molecular scaffolds? J. Mol. Biol. 295 (2000) 1103–1112.
[70] T.K. Albert, H. Hanzawa, Y.I.A. Legtenberg, F.A.J. van den Heuvel, M.A. Collart, R. Boelens, H.T.M. Timmers, Identification of a ubiquitin-protein ligase subunit within the CCR4–NOT transcription repressor complex, EMBO J. 21 (2002) 355–364.
[71] K.L. Lorick, J.P. Jensen, S. Fang, A.M. Ong, S. Hatakeyama, A.M. Weissman, RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 11364–11369.
[72] J.H. Seol, R.M. Feldman, W. Zachariae, A. Shevchenko, C.C. Correll, J.S. Lyapina, Y. Chi, M. Galova, J. Claypool, S. Sandmeyer, K. Nasmyth, A. Shevchenko, R.J. Deshaies, Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34, Genes Dev. 13 (1999) 1614–1626.
[73] N. Zheng, B.A. Schulman, L. Song, J.J. Miller, P.D. Jeffrey, P. Wang, C. Chu, D.M. Koepp, S.J. Elledge, M. Pagano, R.C. Conaway, J.W. Conaway, J.W. Harper, N.P. Pavletich, Structure of the Cul1–Rbx1–Skp1–F box^{Skp2} SCF ubiquitin ligase complex, Nature 416 (2002) 703–709.
[74] P.S. Brzovic, J.R. Keeffe, H. Nishikawa, K. Mayamoto, D. Fox, M. Fukuda, T. Ohta, R. Klevit, Binding and recognition in the assembly of an active BRCA1–BARD1 ubiquitin ligase complex, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 5646–5651.
[75] C.A.P. Joazeiro, A.M. Weissman, RING finger proteins: mediators of ubiquitin ligase activity, Cell 102 (2000) 549–552.
[76] S. Orlicky, X. Tang, A. Willems, M. Tyers, F. Sicheri, Structural basis for phosphodependent substrate selection and orientation by the SDFCdc4 ubiquitin ligase, Cell 112 (2003) 243–256.
[77] V. Chau, J.W. Tobias, A. Bachmair, D. Marriott, D.J. Ecker, D.K. Gonda, A. Varshavsky, A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein, Science 243 (1989) 1576–1583.
[78] D.C. Scherer, J.A. Brockman, Z. Chen, T. Maniatis, D.W. Ballard, Signal-induced degradation of IkBa requires site-specific ubiquitination, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1125–11263.
[79] M.D. Petroski, R.J. Deshaies, Context of multiubiquitin chain attachment influences the rate of Sic1 degradation, Mol. Cell 11 (2003) 1435–1444.
[80] P.S. Brzovic, P. Rajagopal, D.W. Hoyt, M.-C. King, R.E. Klevit, Structure of a BRCA1–BARD1 heterodimeric RING–RING complex, Nat. Struct. Biol. 8 (2001) 833–837.
[81] R. Hashizume, M. Fukuda, I. Maeda, H. Nishikawa, D. Oyake, Y. Yabuki, H. Ogata, T. Ohta, The RING heterodimer BRCA1–BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation, J. Biol. Chem. 276 (2001) 14537–14540.
[82] J.R. Morris, N.H. Keep, E. Solomon, Identification of residues required for the interaction of BARD1 with BRCA1, J. Biol. Chem. 277 (2002) 9382–9386.
[83] T. Ohta, J.J. Michel, A.J. Schottelius, Y. Xiong, ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity, Mol. Cell 3 (1999) 535–541.
[84] P. Tan, S.Y. Fuchs, A. Chen, K. Wu, C. Gomez, Z. Ronai, Z.-Q. Pan, Recruitment of a ROC1–CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IkBa, Mol. Cell 3 (1999) 527–533.
[85] J.M. Peters, The anaphase-promoting complex: proteolysis in mitosis and beyond, Mol. Cell 9 (2002) 931–943.
[86] B.A. Schulman, A.C. Carrano, P.D. Jeffrey, Z. Bowen, E.R. Kinnucan, M.S. Finnin, S.J. Elledge, J.W. Harper, M. Pagano, N.P. Pavletich, Insights into SCF ligases from the structure of the Skp1–Skp2 complex, Nature 408 (2000) 381–386.
[87] Z. Tang, B. Li, R. Bharadwaj, H. Zhu, E. Ozkan, K. Hakala, J. Deisenhofer, H. Yu, APC2 cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex, Mol. Biol. Cell 12 (2001) 3839–3851.
[88] T. Kawakami, T. Chiba, T. Suzuki, K. Iwai, K. Yamanaka, N. Minato, H. Suzuki, N. Shimbara, Y. Hidaka, F. Osaka, M. Omata, K. Tanaka, NEDD8 recruits E2-ubiquitin to SCF E3 ligase, EMBO J. 20 (2001) 4003–4012.
[89] T. Kamura, M.N. Conrad, Q. Yan, R.C. Conaway, J.W. Conaway, The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2, Genes Dev. 13 (1999) 2928–2933.
[90] D. Liakopoulos, T. Busgen, A. Brychzy, A. Jentsch, A. Pause, Conjugation of the ubiquitin-like protein NEDD8 to cullin-2 is linked to von Hippel–Lindau tumor suppressor function, Proc. Natl. Acad. Sci. U. S. A. 11 (1999) 5510–5515.
[91] M.A. Verdecia, C.A.P. Joazeiro, N.J. Wells, J.-L. Ferrer, M.E. Bowman, T. Hunter, J.P. Noel, Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase, Mol. Cell 11 (2003) 249–259.
[92] J. Pascual, M. Martinez-Yamout, H.J. Dyson, P.E. Wright, Structure of the PHD zinc finger from human Williams–Beuren Syndrome transcription factor, J. Mol. Biol. 304 (2000) 723–729.
[93] A.D. Capili, D.C. Schultz, F.J. Rauscher, K.L.B. Borden, Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains, EMBO J. 20 (2001) 165–177.
[94] L. Aravind, L.M. Iyer, E.V. Koonin, Scores of RINGS but no PHDs in ubiquitin signaling, Cell Cycle 2 (2003) 123–126.
[95] L. Coscoy, D.J. Sanchez, D. Ganem, A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition, J. Cell Biol. 155 (2001) 1265–1273.
[96] E.W. Hewitt, L. Duncan, D. Mufti, J. Baker, P.G. Stevenson, P.J. Lehner, Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation, EMBO J. 21 (2002) 2418–2429.

[97] L. Coscoy, D. Ganem, PHD domains and E3 ubiquitin ligases: viruses make the connection, Trends Cell Biol. 13 (2003) 7–12.
[98] D.J. Sanchez, L. Coscoy, D. Ganem, Functional organization of MIR2, a novel viral regulator of selective endocytosis, J. Biol. Chem. 277 (2002) 6124–6130.
[99] Z. Lu, S. Xu, C. Joazeiro, M.H. Cobb, T. Hunter, The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2, Mol. Cell 9 (2002) 945–956.
[100] S.E. Salghetti, A.A. Caudy, J.G. Chenoweth, W.P. Tansey, Regulation of transcriptional activation domain function by ubiquitin, Science 293 (2001) 1651–1653.
[101] R.C. Conaway, C.S. Brower, J.W. Conaway, Emerging roles of ubiquitin in transcription regulation, Science 296 (2002) 1254–1258.
[102] M. Koegl, T. Hoppe, S. Schlenker, H.D. Ulrich, T.U. Mayer, S. Jentsch, A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly, Cell 96 (1999) 635–644.
[103] Y. Imai, M. Soda, S. Hatakeyama, T. Akagi, T. Hasikawa, K.-I. Nakayama, R. Takahashi, CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity, Mol. Cell 10 (2002) 55–67.
[104] L. Aravind, E.V. Koonin, The U box is a modified RING finger—a common domain in ubiquitination, Curr. Biol. 10 (2000) R124–R132.
[105] S. Hatakeyama, M. Yada, M. Matusmoto, N. Ishida, K.-I. Nakayama, U-box proteins as a new family of ubiquitin-protein ligases, J. Biol. Chem. 276 (2001) 33111–33120.
[106] E. Pringa, G. Martinez-Noel, U. Muller, K. Harbers, Interaction of the RING finger-related U-box motif of a nuclear dot protein with ubiquitin-conjugating enzymes, J. Biol. Chem. 276 (2001) 19617–19623.
[107] S. Murata, Y. Minami, M. Minami, T. Chiba, K. Tanaka, CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein, EMBO Rep. 2 (2001) 1133–1138.
[108] J. Jiang, C.A. Ballinger, Y. Wu, Q. Dai, D.M. Cyr, J. Hohfeld, C. Patterson, CHIP is a U-box dependent E3 ubiquitin ligase, J. Biol. Chem. 276 (2001) 42938–42944.
[109] H.R. Chen, S.P. Jan, T.Y. Tsao, Y.J. Sheu, J. Banroques, S.C. Cheng, Snt309p, a component of the Prp19p-associated complex that interacts with prp19p and associates with the spliceosome simultaneously with or immediately after dissociation of U4 in the same manner of Prp19p, Mol. Cell. Biol. 18 (1998) 2196–2204.
[110] M.D. Ohi, C.W. Vander Kooi, J.A. Rosenberg, W.J. Chazin, K.L. Gould, Structural insights into the U-box, a domain associated with multi-ubiquitination, Nat. Struct. Biol. 10 (2003) 250–255.
[111] D.M. Cyr, J. Hohfeld, C. Patterson, Protein quality control: U-box-containing E3 ubiquitin ligases join the fold, Trends Biochem. Sci. 27 (2002) 368–375.
[112] G.C. Meachem, C. Patterson, W. Zhang, J.M. Younger, D.M. Cyr, The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation, Nat. Cell Biol. 3 (2001) 100–105.
[113] P. Connell, C.A. Ballinger, J. Jiang, Y. Wu, L.J. Thompson, J. Hohfeld, C. Patterson, The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins, Nat. Cell Biol. 3 (2001) 93–96.
[114] H. Shimura, D. Schwartz, S.P. Gygi, K.S. Kosik, CHIP–Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival, J. Biol. Chem. 279 (2004) 4869–4876.
[115] L. Petrucelli, D. Dickson, K. Kehoe, J. Taylor, H. Snyder, A. Grover, M. De Lucia, E. McGowan, J. Lewis, G. Prihar, J. Kim, W.H. Dillmann, S.E. Browne, A. Hall, R. Voellmy, Y. Tsuboi, T.M. Dawson, B. Wolozin, J. Hardy, M. Hutton, CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation, Hum. Mol. Genet. 13 (2004) 703–714.
[116] M. Scheffner, U. Nuber, J.M. Huibregtse, Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade, Nature 373 (1995) 81–83.
[117] J.M. Huibregtse, M. Scheffner, P.M. Howley, E6-AP directs the HPV E6-dependent inactivation of p53 and is representative of a family of structurally and functionally related proteins, Cold Spring Harbor Symp. Quant. Biol. 59 (1994) 237–245.
[118] J.M. Huibregtse, M. Scheffner, S. Beaudenon, P.M. Howley, A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 2563–2567.
[119] Y. Yoshida, T. Chiba, F. Tokunaga, H. Kawasaki, K. Iwai, T. Suzuki, Y. Ito, K. Matsuoka, K. Yoshida, K. Tanaka, T. Tadashi, An E3 ubiquitin ligase that recognizes sugar chains, Nature 418 (2002) 438–442.
[120] P. Jaakkola, D.R. Mole, Y.-M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. von Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-alpha to the von HippelLindau ubiquitylation complex by O_2-regulated prolyl hydroxylation, Science 292 (2001) 468–472.
[121] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin, HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing, Science 292 (2001) 464–468.
[122] R.J. Deshaies, J.E. Ferrell, Multisite phosphorylation and the countdown to S phase, Cell 107 (2001) 819–822.
[123] J.-W. Jeong, M.-K. Bae, M.-Y. Ahn, S.-H. Kim, T.-K. Sohn, M.-H. Bae, M.-Q. Yoo, E.J. Song, K.-J. Lee, K.-W. Kim, Regulation and destabilization of HIF-1α by ARD1-mediated acetylation, Cell 111 (2002) 709–720.
[124] J.D. Laney, M. Hochstrasser, Substrate targeting in the ubiquitin system, Cell 97 (1999) 427–430.
[125] W. Kim, W.G. Kaelin, The von Hippel–Lindau tumor suppressor protein: new insights into oxygen sensing and cancer, Curr. Opin. Genet. Dev. 13 (2003) 55–60.
[126] K. Kondo, W.Y. Kim, M. Lechpammer, W.G. Kaelin, Inhibition of HIF2α is sufficient to suppress pVHL-defective tumor growth, PLoS Biol. 1 (2003) 439–444.
[127] F. Yu, S.B. White, Q. Zhao, F.S. Lee, HIF-1a binding to VHL is regulated by stimulus-sensitive proline hydroxylation, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 9630–9635.
[128] N. Masson, C. William, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation, EMBO J. 20 (2001) 5197–5206.
[129] C.E. Stebbins, W.G. Kaelin, N.P. Pavletich, Structure of the VHL–ElonginC–ElonginB complex: implications for VHL tumor suppressor function, Science 284 (1999) 455–461.
[130] J.-H. Min, H. Yang, M. Ivan, F. Gertler, W.G. Kaelin, N.P. Pavletich, Structure of an HIF-1a–pVHL complex: hydroxyproline recognition in signaling, Science 296 (2002) 1886–1889.
[131] W.C. Hon, M.I. Wilson, K. Harlos, T.D. Claridge, C.J. Schofield, C.W. Pugh, P.H. Maxwell, P.J. Ratcliffe, D.D. Stuart, E.Y. Jones, Structural basis of the recognition of hydroxyproline in HIF-1 alpha by pVHL, Nature 417 (2002) 975–978.
[132] P. Nash, X. Tang, S. Orlicky, Q. Chen, F.B. Gertler, M.D. Mendenhall, F. Sicheri, T. Pawson, M. Tyers, Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication, Nature 414 (2001) 514–521.
[133] R. Verma, R.S. Annan, M.J. Huddleston, S.A. Carr, G. Reynard, R.J. Deshaies, Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase, Science 278 (1997) 455–460.
[134] P. Klein, T. Pawson, M. Tyers, Mathematical modeling suggests cooperative interactions between a disordered polyvalent ligand and a receptor site, Curr. Biol. 13 (2003) 1669–1678.
[135] Y. Yang, C.-C.H. Li, A.M. Weissman, Regulating the p53 system through ubiquitination, Oncogene 23 (2004) 2096–2106.
[136] P.H. Kussie, S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, N.P. Pavletich, Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain, Science 274 (1996) 948–953.

[137] L.T. Vassilev, B.T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, E.A. Liu, In vivo activation of the p53 pathway by small-molecule antagonists of MDM2, Science 303 (2004) 844–848.
[138] S. Vijay-Kumar, C.E. Bugg, W.J. Cook, Structure of ubiquitin refined at 1.8 A resolution, J. Mol. Biol. 194 (1987) 531–544.
[139] P. Bayer, A. Arndt, S. Metzger, R. Mahajan, F. Melchior, R. Jaenicke, J. Becker, Structure determination of the small ubiquitin-related modifier Sumo-1, J. Mol. Biol. 280 (1998) 275–286.
[140] P.J. Kraulis, MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures, J. Appl. Crystallogr. 24 (1991) 946–950.
[141] E.A. Merritt, D.J. Bacon, Raster 3D: photorealistic molecular graphics, Methods Enzymol. 277 (1997) 505–524.
[142] W.L. DeLano, The PyMOL molecular graphics system, DeLano Scientific, San Carlos, CA, USA, 2002.