THEMES AND VARIATIONS ON UBIQUITYLATION

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Ubiquitylation — the conjugation of proteins with a small protein called ubiquitin — touches upon all aspects of eukaryotic biology, and its defective regulation is manifest in diseases that range from developmental abnormalities and autoimmunity to neurodegenerative diseases and cancer. A few years ago, we could only have dreamt of the complex arsenal of enzymes dedicated to ubiquitylation. Why has nature come up with so many ways of doing what seems to be such a simple job?

UBIQUITIN AND PROTEASOMES

Ubiquitin is a 76-amino-acid globular protein that is highly conserved throughout eukaryotes, with only three amino-acid changes from yeast to human. Its covalent conjugation to other proteins — ubiquitylation (or ubiquitination) — is essential for the degradation of proteins whose levels are regulated either constitutively or in response to changes in the cellular environment. Ubiquitin is integral to myriad processes such as cell-cycle progression; organelle biogenesis; apoptosis; regulated cell proliferation; cellular differentiation; quality control in the endoplasmic reticulum; protein transport; inflammation; antigen processing; DNA repair; and stress responses. In this way, it resembles another post-translational modification — phosphorylation — with which it is intimately intertwined. Phosphorylation can augment or inhibit ubiquitylation, by modifying either the protein destined to be ubiquitylated or the enzymes that catalyse the addition of ubiquitin. So what makes ubiquitin such a great multitasker?

The classical view of ubiquitylation is that it targets proteins for degradation by a multisubunit, ATP-dependent protease termed the proteasome (see the review by Peter Kloetzel on page 179 of this isssue for more information on the proteasome). In addition to its role in proteasomal degradation, ubiquitylation is also emerging as a signal that targets plasma membrane proteins for destruction in vacuoles and/or lysosomes (see the review by Linda Hicke on page 195 of this issue). Thus, ubiquitin targets proteins from topologically distinct locations to fundamentally different proteolytic structures. We are only just beginning to understand the functional diversity of the ubiquitin signal. Although targeting for degradation is undoubtedly one of its key tasks, other cellular functions not directly involving protein degradation, including regulation of translation, activation of transcription factors and kinases, and DNA repair, are controlled in one way or another by this seemingly simple protein.

If ubiquitin can be attached to so many proteins, how is specificity generated? Moreover, how does ubiquitylation of one protein sentence it to destruction in proteasomes when, in another setting, modification with the same polypeptide leads to enhanced translation? We don't have a full solution to this puzzle, but the pieces are falling into place. Specificity is generated largely by the enzymes that recognize substrates and mediate ubiquitylation (FIG. 1). But it is also evident that the fate of the ubiquitylated proteins is determined by the types of ubiquitin conjugate formed. For instance, a single ubiquitin tag does not target a protein for proteasomal degradation, whereas a chain of four or more does[1]. There are also subtly different ways of building a multi-ubiquitin chain — by using different lysine residues of ubiquitin — and these have functional consequences. In addition, intracellular location helps to determine the fate of ubiquitylated proteins: ubiquitylation in the nucleus might not have the same consequence as that in the cytosol, and ubiquitylation of a transmembrane protein at the endoplasmic reticulum (ER) membrane might have a different result from at the plasma membrane.

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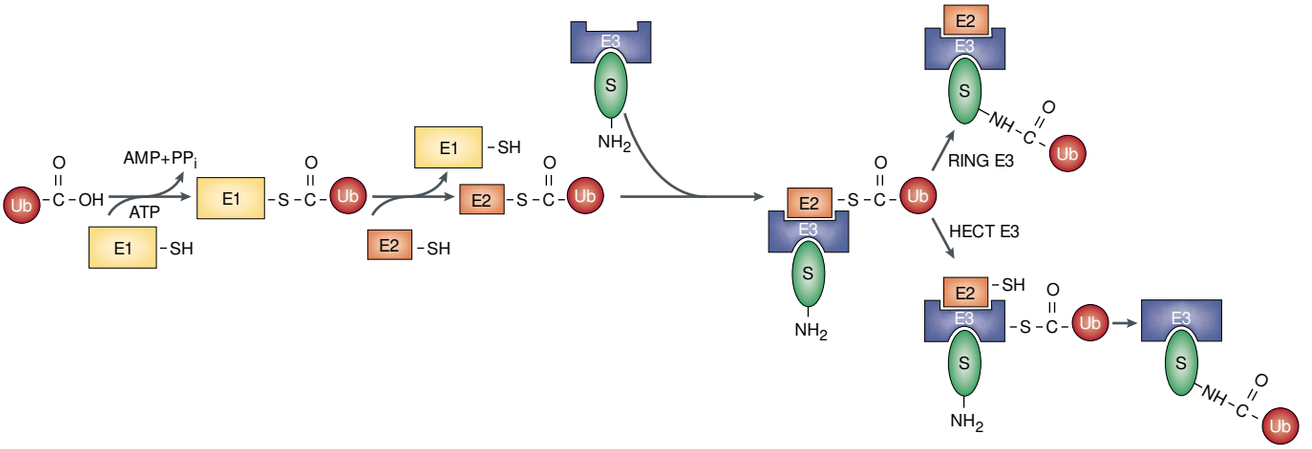

Figure 1 | **The ubiquitylation pathway.** Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s, which might or might not have substrate already bound. For HECT domain E3s, ubiquitin is next transferred to the active-site cysteine of the HECT domain followed by transfer to substrate (S) (as shown) or to a substrate-bound multi-ubiquitin chain. For RING E3s, current evidence indicates that ubiquitin might be transferred directly from the E2 to the substrate.

The concept that cellular proteins can be targeted for modification by small proteins, resulting in alteration in the fate or function of the targeted protein, extends beyond ubiquitin. A growing list of ubiquitin-like (UBL) proteins is being identified and characterized. As with ubiquitin, the active forms of UBLs include a glycine at the carboxyl terminus that forms an isopeptide bond with ε-amino groups of lysines on target proteins. UBLs include at least five distinct proteins that are related in sequence to ubiquitin as well as two that are not. Of the UBLs that are homologous to ubiquitin, the first to be characterized was a protein that resembles a ubiquitin dimer, known as the ubiquitin cross-reactive protein (UCRP) or ISG15 (REF. 2). Also in this group is *Saccharomyces cerevisiae* RUB1 (which stands for related to ubiquitin) — known as Nedd8 in metazoans (herein referred to as Rub1) and SUMO-1 (small ubiquitin-related modifier; also known as Ubl1, Sentrin or PIC-1 (see the review by Stefan Jentsch and colleagues on page 202 of this issue and REFS 3,4). Modification with Rub1 (rubylation) or with SUMO-1 (sumoylation) can have direct effects on ubiquitylation. Apg12 is a UBL that lacks amino-acid homology with ubiquitin. Apg12 is a central player in a fascinating story in which a multi-enzyme process that parallels ubiquitylation mediates autophagy (see the review by Yoshinori Ohsumi on page 211 of this issue). Although ubiquitin must now share the limelight on the protein modification stage with the UBLs, it alone has the remarkable ability to form a variety of different chains on target proteins — potentiating its capacity to generate a diverse array of signals.

In addition to the UBLs, an increasing number of otherwise structurally unrelated proteins are being found to contain domains homologous to ubiquitin. These ubiquitin-domain proteins (UDPs) have varied cellular functions and, unlike the UBLs, are not known to be covalent modifiers of proteins. Included among the UDPs are ubiquitylation substrates as well as enzymes involved in both the addition and the removal of ubiquitin from proteins. Some UDPs interact with proteasomes as well as with enzymes that are involved in mediating ubiquitylation. Thus, the presence of the ubiquitin domain in otherwise disparate proteins does not simply reflect conservation of a stable structural domain. Instead, the ubiquitin domain probably has an important function in regulating ubiquitin-mediated processes[5] (reviewed in REF. 3).

The ubiquitylation 'toolkit'

Ubiquitylation is a multistep process (FIG. 1), involving at least three types of enzyme. First, a ubiquitin-activating enzyme (also known as E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP-dependent process. Then, a ubiquitin-conjugating enzyme or ubiquitin-carrier enzyme (UBC, also known as E2) accepts ubiquitin from the E1 by a *trans*-thiolation reaction, again involving the carboxyl terminus of ubiquitin. Finally, a ubiquitin protein ligase (E3) catalyses the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue on the substrate. Two distinct E3 families, containing conserved protein domains, have now been identified. HECT domain E3s form thiol-ester intermediates with ubiquitin as part of the process, leading to ubiquitylation of substrates (HECT domain stands for homologous to E6-AP carboxyl terminus, E6-AP being the founder member of this family)[6]. Members of the other class, RING FINGER E3s, are now believed to mediate the direct transfer of ubiquitin from E2 to substrate[7].

There are more E2s than E1s, and more E3s than E2s so, at each step, the number of proteins that can potentially be involved increases, as does the specificity of binding to the next component. It is ultimately the E3, either alone or in combination with its bound E2, that determines the exquisite sensitivity of substrate recognition.

RUB1
(Nedd8 in metazoans). A ubiquitin-like (UBL) protein that is activated by its own E1- and E2-like molecules and modifies cullin family members.

HECT
Stands for homologous to E6-AP carboxyl terminus. The HECT domain is a ~350-amino-acid domain, highly conserved among a family of E3 enzymes.

RING FINGER
Defined structurally by two interleaved metal-coordinating sites. The consensus sequence for the RING finger is: CX2CX(9–39)CX(1–3)HX(2–3)C/HX2CX(4–48)CX2C. The cysteines and histidines represent metal-binding sites with the first, second, fifth and sixth of these binding one zinc ion and the third, fourth, seventh and eighth binding the second.

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BIR REPEAT
(Baculovirus inhibitor of apoptosis repeat). Cysteine-based motif of ~65 amino acids. Inhibitors of apoptosis (IAPs) contain several BIR domains.

c-CBL
Multifunctional protein that modulates signalling through tyrosine-kinase-containing growth factor receptors and tyrosine-kinase-coupled receptors. Has RING-finger-dependent E3 activity.

Before we discuss how these enzymes achieve their high substrate specificities, it is important to appreciate that ubiquitylation is a dynamic and reversible process. De-ubiquitylating enzymes (DUBs) cleave ubiquitin from proteins and from residual proteasome-associated peptides, and disassemble multi-ubiquitin chains. DUBs are also important for processing immature ubiquitin, which is encoded on multiple genes and translated as fusion proteins either with other ubiquitin molecules or as the amino-terminal component of two small ribosomal subunits[8]. These are processed by members of a subfamily of DUBs — the ubiquitin carboxy-terminal hydrolases — resulting in mature ubiquitin (FIG. 2)[9].

E1: the ubiquitylation starter pack

E1 is the product of a single gene with two isoforms arising from alternative translation start sites[10]. Sequences contained within the amino-terminal region of the longer isoform, E1a, allow cell-cycle-regulated nuclear localization and phosphorylation, with an increase in nuclear distribution in G2 phase[11,12]. The finding that cells expressing a temperature-sensitive E1 undergo cell-cycle arrest provided the first evidence for the physiological significance of ubiquitylation[13].

The carboxy-terminal glycine of ubiquitin is essential for activation by E1, and glycines are also found at the carboxyl termini of UBLs (see the review by Stefan Jentsch and colleagues and REFS 3,4). It comes as no surprise, then, that conjugation of UBLs such as Rub1and SUMO-1 to target proteins also requires E1-like enzymes. These have sequence homology to E1, but the E1-like proteins for SUMO-1 and Rub1 are heterodimers, with subunits homologous to the amino- and carboxy-terminal halves of E1.

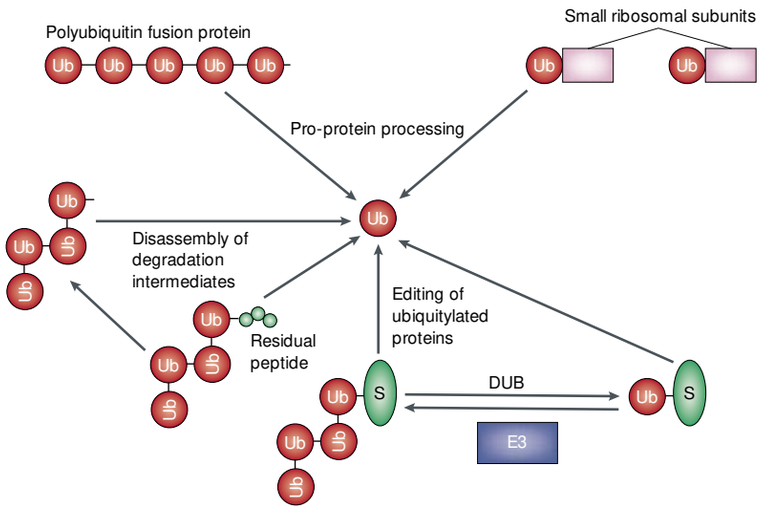

Figure 2 | **The many functions of de-ubiquitylating enzymes.** Ubiquitin is synthesized as fusion proteins of ubiquitin (Ub) monomers (polyubiquitin) or with small ribosomal subunits, which are then processed by cleavage at the carboxy-terminal glycine. After the degradation of protein substrates, ubiquitin must be freed from residual peptides and disassembled. De-ubiquitylating enzymes also reverse the activity of E3s, sequentially removing ubiquitin from substrates (S) . This might occur in specific cellular locations where ubiquitylation is occurring and at the proteasome (adapted from REF. 9).

E2s: take your partner

Enzyme diversity — implying specificity — becomes apparent in the E2s. Even the modest genome of *S. cerevisiae* encodes 13 E2-like products, termed Ubc1–13 (ONLINE TABLE 1), and there are at least 25 mammalian family members. But not all E2-like molecules form thiol-esters with ubiquitin: Ubc9 is dedicated to sumoylation, and Ubc12 functions in rubylation; the mammalian orthologues of these E2-like proteins behave similarly. The identifying characteristic of E2s is a 14–16-kDa core that is ~35% conserved among family members (FIG. 3). Whereas several E2s are limited to this core domain, others have significant amino- or carboxy-terminal extensions. These might facilitate interactions with specific E3s[14,15], or serve as membrane anchors juxtaposing them with specific E3s and substrates[16]. Most known E2s, including all of those in the *S. cerevisiae* genome, are less than 36 kDa, but there are notable exceptions. The most striking of these is the giant 528-kDa polytopic E2 BIR-REPEAT-containing ubiquitin-conjugating enzyme (BRUCE)[17]. The degree of identity among E2s indicates possible redundancy in function. Although there is evidence for this, in some cases quite homologous E2s show considerable differences in their abilities to function with E3s[18,19]. Beware that E2 nomenclature is not standardized across species. For example, *S. cerevisiae* Ubc2, Ubc6 and Ubc7 are not closely related to the human UBCH2, UBCH6 and UBCH7, respectively. Arthur Haas and Thomas Siepmann have tried to make sense of this confusion[20].

The crystal structures of several E2s have been solved, as has the crystal structure of one E2, UBCH7, bound to both the HECT domain of E6-AP and to the RING finger of c-CBL[21,22]. These structures have provided insight into how E2s recognize the two types of E3. Intriguingly, these two E3 domains interact with almost identical regions on the E2, specifically loops designated L1 and L2 (FIG. 3). In addition, the E2 amino-terminal α-helix, also involved in interactions with E1 (REF. 20), has a minor part in E3 interactions. The involvement of this helix in interactions with both E1 and E3s indicates that E2s might dissociate from E3s to receive ubiquitin from E1.

So how do E3s pair up with specific E2s? Amino- and carboxy-terminal E2 extensions are involved, but so are regions within the E2 core. UBCH7 has a phenylalanine at position 63 that provides a point of hydrophobic interaction between UBCH7's L1 loop and regions in the E6-AP HECT domain and in the c-Cbl RING domain. The E2-interacting regions of these two E3s seem to be otherwise unrelated (FIG. 3). Notably, phenylalanine 63 of UBCH7 is conserved in a subset of E2s that interact with HECT E3s (REF. 23), and there is also evidence that, for other E2s, the amino acid in this position helps determine E2–E3 pairs[22]. Interestingly, Ubc3, Ubc7 and their orthologues have 12- and 13-amino-acid insertions, respectively, between the active site and the region that corresponds to the L2 loop in other E2s. On the basis of the Ubc7 crystal structure[24], and viewed in the context of the UBCH7–E3 crystal structures, it is tempting to speculate that these insertions restrict interactions of these E2s to specific E3s.

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WW DOMAIN
Protein interaction domain found in the amino-terminal halves of many HECT E3s, and also in other proteins. Characterized by a pair of tryptophans 20–22 amino acids apart, and an invariant proline within a region of 40 amino acids. WW domains interact with proline-rich regions, including those with phosphoserine or phosphothreonine.

HECT E3s

The discovery of the HECT E3s was a direct consequence of the finding that oncogenic strains of human papillomavirus (HPV) encode isoforms of a protein called E6, which specifically inactivate the tumour suppressor protein p53 (ONLINE TABLE 2; FIG. 4). The breakthrough came when E6-associated protein (E6-AP) — a cellular partner for E6 — was identified. E6 serves as an adaptor between E6-AP and p53, allowing E6-AP to catalyse the ubiquitylation of p53 (FIG. 4)[25]. The characterization of E6-AP led to the identification of a family of proteins that are closely related to E6-AP in a ~350-residue region at their carboxyl termini, the HECT domain. This includes a conserved cysteine that forms a covalent thiol-ester intermediate with ubiquitin[6]. The crystal structure of the E6-AP HECT domain with UBCH7 has a U-shaped appearance with the E2 at one end and the HECT carboxyl terminus at the other (FIG. 3a). The strikingly large distance of 41 Å between the catalytic cysteine of UBCH7 and that of E6-AP leaves much unanswered about how ubiquitin is transferred from E2 to E3. In addition to p53, physiological substrates have now been identified for E6-AP including a UDP, HHR23A (REF. 26), and mutations in the E6-AP gene, including those that effect the HECT domain, give rise to Angelman syndrome, a severe neurological disorder[27] (ONLINE TABLE 2).

Another feature shared by many HECT E3s, but not E6-AP, is the WW DOMAIN, which is involved in protein–protein interactions and undoubtedly has a role in targeting substrates for ubiquitylation. WW domains occur in groups of two to four in the amino-terminal halves of these proteins (ONLINE TABLE 2; FIG. 4). These tryptophan-based motifs form a hydrophobic pocket for proline-rich sequences as well as certain phosphoserine and phosphothreonine-containing sequences[28,29]. Most WW domain HECT E3s also have an amino-terminal C2 domain that mediates translocation to the plasma membrane in response to increases in intracellular Ca^{2+}. A function for the C2 domain in membrane translocation of a metazoan member of this family, Nedd4, is well established, with evidence to indicate that this domain might mediate interactions with lipid rafts[30].

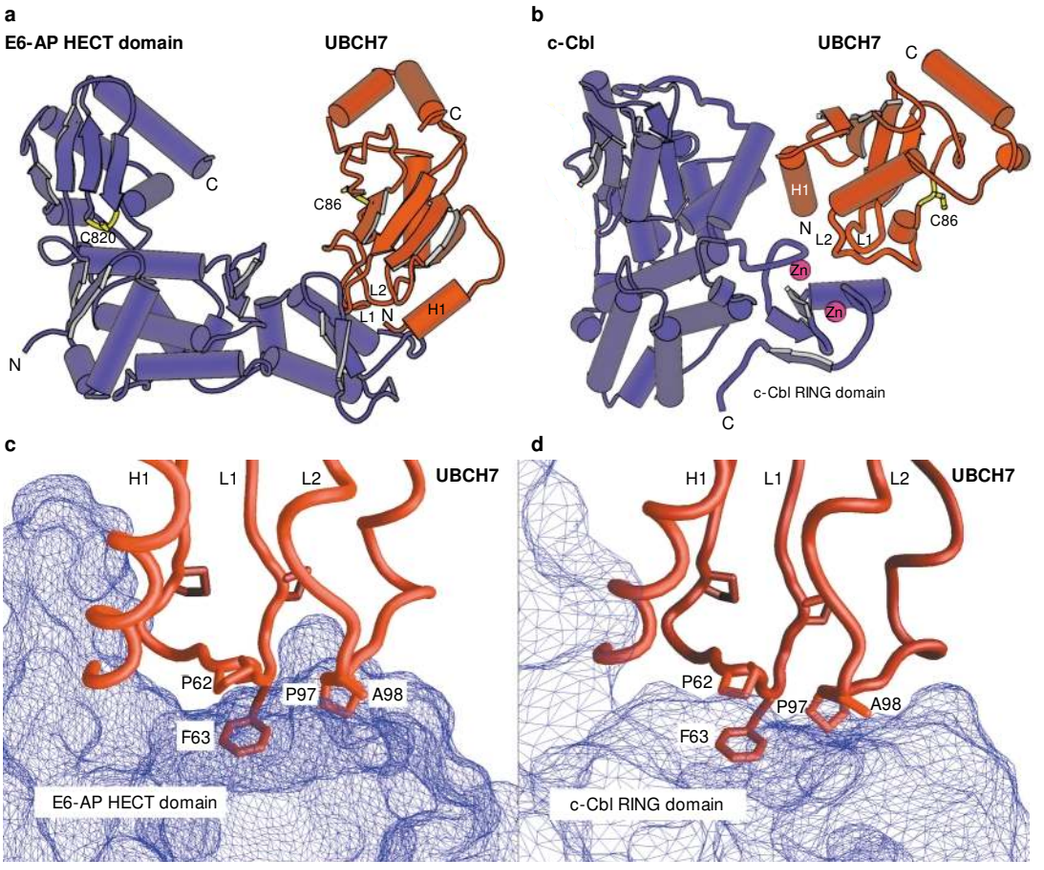

Figure 3 | **E2–E3 interactions.** Model based on the crystal structure of **a** | UBCH7 (red) with the HECT domain of E6-AP (blue) and **b** | with c-Cbl (blue). The structure of UBCH7 is similar to that of other core E2s, which include an amino-terminal α-helix (H1), a 4–5-strand anti-parallel β-pleated sheet (arrows) and a second α-helix that, together with the β-pleated sheet, forms a hydrophobic core. The carboxy-terminal region of E2s is folded into a helix–loop–helix. The conserved catalytic cysteine (C86) is part of a consensus sequence that includes a histidine ten residues upstream of it[20]. **c** | Loops L1 and L2 of UBCH7 are involved in E3 interactions with both the HECT domain and the RING. Phe63 (F63) of UBCH7 inserts into a groove on both E3 enzymes. (Models courtesy of Nicola Pavletich, Lan Huang and Ning Zheng, Memorial Sloan-Kettering Cancer Center, New York, USA. Modified from REFS 21, 22.)

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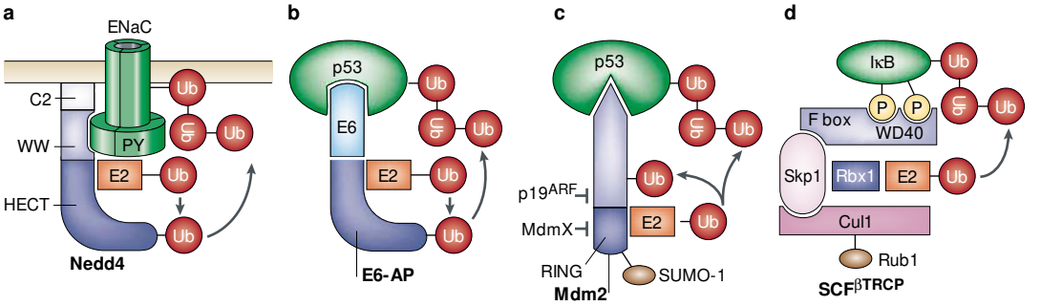

Figure 4 | **Representative E3–substrate interactions a** | Association of Nedd4 with the epithelial sodium channel (ENaC) at the plasma membrane. The C2 domain mediates interactions with the membrane in a Ca^{2+}-dependent manner. The WW domain interacts with PY domains on ENaC, which are deleted in Liddle syndrome. **b** | Ternary complex of p53 with human papillomavirus E6 and E6-AP. **c** | Mdm2 and p53. Both p53 and Mdm2 are substrates for modification by Mdm2 (BOX 1). MdmX blocks binding through the RING finger. $p19^{ARF}$ blocks by binding upstream of the RING and by revealing a nucleolar localization signal. **d** | $SCF^{\beta TRCP}$ as a prototypical cullin-containing E3. Modification of Cul1 with Rub1 increases activity and requires the RING finger protein Rbx1 (BOX 2).

Rsp5 is the only *S. cerevisiae* C2–WW domain HECT E3 and exemplifies the capacity of a single E3 to ubiquitylate distinct proteins in several cellular compartments. Rsp5 interacts with one of its substrates, the large subunit of RNA polymerase II (LsPolII), directly through its WW domains, which bind the proline-rich carboxyl terminus of LsPolII (REF. 29). A second function of Rsp5 is to activate two transcription factors, Spt23 and Mga2, by facilitating the ubiquitin- and proteasome-dependent cleavage of the soluble components of these proteins from their ER-membrane-bound precursors[31]. This concept of limited cleavage by proteasomes has a precedent in the maturation of a metazoan transcription factor, NF-κB[32]. Rsp5 is best known for its ability to ubiquitylate at least 13 plasma membrane transporters and receptors. Surprisingly, however, there is little evidence for direct interactions between these targets and Rsp5. This suggests that these interactions are indirect and perhaps facilitated by C2-mediated membrane targeting. In contrast to Rsp5, a direct WW domain–substrate interaction is important for the ubiquitylation of at least one membrane protein: Nedd4 binds and ubiquitylates subunits of the epithelial sodium channel (ENaC) through its WW domains, leading to downregulation of the number of active channels (FIG. 4a). Mutation of proline-rich regions on ENaC causes Liddle syndrome, an inherited form of hypertension in which ENaC activity is enhanced, presumably owing to the inability of Nedd4 to downregulate ENaC[29,33,34] (also see the review by Linda Hicke.)

RING finger E3s

Unlike the HECT domain, the RING finger was described in the early 1990s, years before any suspicion of a role in ubiquitylation. RING fingers include eight metal-binding residues that coordinate two zinc ions, arranged in an interleaved pattern[35]. This distinguishes them from the tandem arrangement of metal-coordinating residues characteristic of zinc fingers. The realization that the RING finger has a general role in ubiquitylation has come about during the past two years from the convergence of a number of lines of investigation that included: first, the discovery that a small RING finger protein, Rbx1 (Ring box protein-1; also known as ROC1 or Hrt1), was a requisite component of the multisubunit SCF (Skp1/Cul1/F-BOX protein) family E3s[36–40]; second, the finding that many otherwise unrelated RING finger proteins mediate ubiquitylation[41]; and last, the realization that all known or suspected E3s that are not HECT proteins include a RING finger[15,42–48]. We do not know how many of the hundreds of RING finger proteins have the capacity to mediate ubiquitylation. Nonetheless, a sample of otherwise unrelated members of this family predicts that it will be a large percentage[41] (ONLINE TABLE 3). So far, most RING finger proteins that have been shown to interact with E2s and to mediate ubiquitylation in *in vitro* systems lack defined substrates other than themselves. Prominent among these is the product of the breast and ovarian cancer susceptibility gene 1 (BRCA1)[41]: mutations in this protein — including one in the RING finger — are found in familial forms of breast and ovarian cancer[49]. Ascertaining which E2-interacting RING finger proteins are bona fide E3s for heterologous substrates, and which are primarily substrates for regulated, E2-dependent, 'auto-ubiquitylation' is an exciting challenge.

Unlike the HECT-domain E3s, where roles for thiolester intermediates with ubiquitin are well established, there is little evidence to indicate the existence of similar intermediates between ubiquitin and RING finger proteins. So is the RING finger simply an E2-docking site that passively juxtaposes the carboxyl terminus of ubiquitin bound to E2 with lysines on substrates, or does the RING allosterically activate E2 bound to ubiquitin and thereby enhance transfer? Although there is some experimental evidence for a possible activating function[15], a comparison of the structure of an E2 (UBCH7) bound to c-Cbl to that of E2s by themselves[22] (FIG. 3) provides little support for this. So, at present there is no clear answer to this question.

A convenient way to think about RING finger proteins is to divide them into single and multisubunit E3s. Single-subunit E3s contain the substrate recognition element and the RING finger on the same polypeptide.

F-BOX
A conserved ~50-residue region found in proteins that associate with Skp1 and potentially form the SCF E3s. There are over a hundred distinct members of this family.

CULLIN FAMILY
Proteins with homology to Cul1, which was first shown to be involved in cell-cycle exit in *Caenorhabditis elegans*.

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Box 1 | Mdm2 and ubiquitylation

As might be expected for a protein whose task is normally to destroy the 'guardian of the genome', there are numerous safety mechanisms that prevent Mdm2 from running amok. First, Mdm2 has intrinsic RING-finger-dependent E3 activity towards itself, as well as p53 (REFS 42,43)**. Second, phosphorylation of p53 blocks its interaction with Mdm2** (REFS 85, 86)**. Third, an Mdm2-binding protein, p19^{ARF}, binds upstream of its RING finger and exposes a cryptic nucleolar localization signal co-linear with the Mdm2 RING finger. This sequesters Mdm2 away from p53, preventing the Mdm2-mediated degradation of p53** (REFS 87, 88)**. Binding of p19^{ARF} also inhibits the intrinsic activity of Mdm2** (REFS 89,90) ***in vitro*****. Last, Mdm2 activity is similarly inhibited by dimerization with a related RING finger protein, MdmX** (REFS 91–93)**. Nevertheless, there is evidence that Mdm2 is also subject to positive regulation by modification with SUMO-1, which seems to enhance ubiquitylation of p53 by Mdm2 while diminishing auto-ubiquitylation[94].**

Mdm2 also illustrates the substrate specificity of RING finger E3s. The p53 family member p73 binds Mdm2 but is stabilized by this interaction rather than targeted for degradation[95–97]. Substitution of a heterologous RING for that of Mdm2 reconstitutes auto-ubiquitylation and proteasomal targeting of the chimeric molecule; however it does not ubiquitylate p53 or target it for degradation[42].

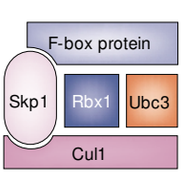
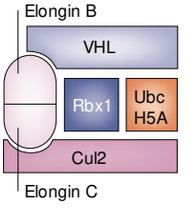
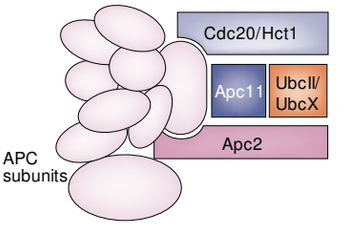
Multisubunit E3s all include a small RING finger protein and a member of the CULLIN family of proteins as well as other subunits, some of which recognize substrates (FIG. 3). Single-subunit RING finger proteins include well-studied E3s such as the oncoprotein Mdm2, which ubiquitylates p53 (REFS 42, 43), the proto-oncoprotein c-Cbl, which ubiquitylates growth factor receptors[44–46], and the inhibitors of apoptosis (IAPs)[50,51]. Parkin is a RING finger E3 that has two RINGs at its carboxyl terminus separated by an IBR (in-between RING), a region common to proteins that have two RING fingers. Parkin also has an amino-terminal ubiquitin domain, making it a member of both the RING and UDP families. Mutations in Parkin's RINGs are associated with juvenile Parkinson's disease, and a synaptic-vesicle-associated protein (CDCrel-1) has been identified[52,53] as a substrate for this E3.

The compact RING finger is found in diverse, otherwise unrelated, proteins. It therefore follows that the sites of substrate interaction for RING proteins will be highly varied. For example, the interactions of c-Cbl depend on its atypical SH2 domain, and for the IAPs the BIR domain probably facilitates binding of some substrates. For Mdm2, interactions with p53 occur through its amino-terminal domain, whereas the RING is located at its carboxyl terminus. Mdm2 is illustrative of the complex regulation that can be a feature of single-subunit RING finger E3s (BOX 1).

Multisubunit cullin-containing RING E3s

Exploration into the intricacies of the cell cycle led to the discovery of multisubunit SCF E3s (TABLE 1, FIG. 4) and to the discovery of the anaphase-promoting complex (APC) or cyclosome, which includes at least 12 distinct subunits. A missing link in the function of SCF E3s was provided in 1999 with the identification of a non-canonical RING finger protein, Rbx1, as a component of both SCF and the structurally related von Hippel–Lindau–Cul2/elongin B/elongin C (VHL–CBC) complex[36–40]. In retrospect, it became obvious that the small RING finger protein Apc11 functions in a similar capacity in the APC, and indeed this subunit has activity towards substrates *in vitro*[48,54,107]. An emerging concept is that the cullin family proteins intrinsic to these E3 complexes (Apc2 in the APC) interact with linker proteins that recruit the substrate-recognition components (see

Table 1 | **Multisubunit, Cullin-containing RING E3s***

	SCF	VCB-CUL2	APC
			
RING	Rbx1 (Hrt1/Roc1)	Rbx1 (Hrt1/Roc1)	Apc11
Cullin	Cdc53 (Cul1)‡	Cul2‡	Apc2
Adaptors	Skp1	Elongin B: homologous to amino terminus of Skp1. Elongin C: a UDP.	Multiple APC subunits (pink), some with tetratricopeptide repeats. These presumably have adaptor functions.
E2	Ubc3 (Cdc34)	UbcH5A, others?	Ubc11, UbcX
Substrate recognition	F-box proteins. These include those with WD40 repeats, leucine-rich domains and others.	VHL, possibly other SOCS box-containing proteins.	Cdc20 (Fizzy) and Hct1 (Fizzy-related); both contain WD40 repeats.
Substrates (partial list)	Sic1, IκBα, β-catenin, G1 cyclins, CD4 bound to phosphorylated HIV Vpu, others.	HIF1α	Mitotic cyclins, Pds1, Cut2, Ase1, Scc1, Securin, others.

*See REFS 55–57 for comprehensive reviews on the multisubunit RING finger E3s.
‡Modified with Rub1, which is mediated by Ubc12 and Rbx1 — evidence suggests that this increases E3 activity. Other cullin family members are similarly modified[99–106] (reviewed in REF. 3).

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Box 2 | Examples of de-ubiquitylating enzymes

- **Fat facets: De-ubiquitylating enzyme (DUB) implicated in *Drosophila melanogaster* photoreceptor development.**
- **DUB1 and DUB2: Products of immediate-early response genes expressed in response to cytokines (IL-3, IL-5, and GM-CSF in the case of DUB1, and IL-2 in the case of DUB2).**
- **BAP1: Binds to the amino-terminal RING finger domain of BRCA1.**
- **UBPY: Human growth-regulated DUB.**
- **Ap-UCH: *Aplysia* neuronal DUB implicated in long-term facilitation.**
- **Ubp-M: Implicated in cell-cycle progression, phosphorylated in a cell-cycle-dependent manner.**
- **Ubp4: Mutants defective in degradation of the yeast mating-type factor MATα2.**
- **Ubp3: Regulation of gene silencing.**
- **D-UBP-64E: Position effect variegation in *Drosophila*.**
- **UCH-L1: Mutations associated with neurological disorders.**
- **TRE-2: A mammalian proto-oncogene homologous to yeast Doa4. The latter is a proteasome-associated DUB that is implicated in removing ubiquitin from post-proteolyic proteasome-bound peptides[68].**
- **Isopeptidase T: Disassembles free multi-ubiquitin chains beginning with the most proximal ubiquitin. A free carboxy-terminal glycine is required. In Alzheimer's disease, a frameshift in translation results in the generation of 'ubiquitin+1', which lacks this glycine. Ubiquitin+1 can serve as a substrate for generation of isopeptidase-T-resistant multi-ubiquitin chains[98].**

DUBs and their functions are reviewed in REFS 9, 67.

REFS 55–57 for reviews on the multisubunit RING finger E3s) (ONLINE TABLE 2).

SCF E3s recognize and ubiquitylate a diverse group of phosphoproteins, with substrate specificity conferred by members of the large family of F-box proteins[58]. SCF substrates are generally phosphoproteins, but phosphorylation is not an inherent requirement for ubiquitylation by SCF E3s, as shown through the use of engineered F-box proteins[59]. There are examples in which one F-box protein is responsible for recognizing several substrates, as is the case for β-transducin-repeat-containing protein (βTRCP). $SCF^{\beta TRCP}$ recognizes phosphorylated β-catenin and IκBα. Additionally, reminiscent of the E6-AP and p53 story, nascent forms of the HIV receptor CD4 are indirectly targeted for ubiquitylation in the ER membrane by $SCF^{\beta TRCP}$ owing to the binding of CD4 to HIV-encoded Vpu, which has phosphorylation sites akin to those of β-catenin and IκBα[55]. Some F-box proteins are themselves ubiquitylated and targeted for degradation. Whether this downregulates their levels or facilitates proteasomal targeting of associated phosphoproteins awaits determination. A recent provocative observation is that ubiquitylation of the transcription factor Met4 by SCF^{Met30} leads to the functional inactivation of Met4, but not to its proteolysis[60].

Architecturally related to the SCF E3s is the VHL–CBC complex (TABLE 1). In this complex, the adaptor Skp1 is replaced by the dimer of elongin B, which has homology to Skp1, and elongin C, which is a UDP. Notably, VHL mutants that fail to assemble with the CBC core are associated with the malignancies of von Hippel–Lindau disease[61,62]. An important substrate for VHL–CBC is hypoxia-inducible transcription factor 1α (HIF1α), which positively regulates vascular endothelial growth factor (VEGF), providing an explanation for the highly vascular nature of the clear cell renal carcinomas seen in VHL disease[63–65]. Analogous to the F box, the VHL protein contains a suppressor of cytokine signalling (SOCS) box that interacts with the core of this E3. It might be that other SOCS-containing proteins can replace the VHL and allow for recognition of other specific substrates[66].

The most complicated of the multisubunit E3s is the APC. The first identified substrates for this E3 were mitotic cyclins, but the list of substrates is growing (TABLE 1). In *S. cerevisiae*, at least 12 essential APC components have been identified. Although the intricacies of the APC's architecture are largely unknown, there are substantial parallels to the SCF and VHL–CBC E3s (TABLE 1). Phosphorylation and dephosphorylation are known to be important regulators of APC activity[56].

Several functions for DUBs

One lesson learned from studying phosphorylation is that the removal of phosphate groups can be as tightly regulated as their addition. Knowing that ubiquitylation is a reversible process, we might expect similarly tight controls for removal of ubiquitin. It should come as no surprise, then, that there are at least 19 yeast DUBs and substantially more in mammals. DUBs come in two flavours — ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin-specific processing enzymes (UBPs) — both of which are thiol proteases. UCHs catalyse the removal of carboxy-terminal fusion proteins from ubiquitin (recall that ubiquitin is always translated as a fusion protein), with a preference for substrates in which ubiquitin is fused to small peptides. UBPs are generally larger, thought of as being responsible for removing ubiquitin from larger proteins, and are involved in the disassembly of multi-ubiquitin chains[9,67].

At the proteasome, DUBs cleave multi-ubiquitin chains from residual peptides[68] and shorten protein-bound multi-ubiquitin chains by sequentially removing the terminal ubiquityl group[69]. This 'proof-reading' function ensures that highly ubiquitylated proteins preferentially remain associated with the proteasome. Another important function of DUBs is to prevent the accumulation of residual multi-ubiquitin chains at proteasomes. Failure to disassemble these chains has the potential to wreak havoc upon the normal movement of ubiquitylated proteins to and through the proteasome (FIG. 2). Moreover, DUBs are constitutively active in the removal of ubiquitin from substrates, as inhibition of proteasome function causes the accumulation of mostly non-ubiquitylated proteins. It is clear that DUBs have crucial cellular roles, but beyond general housekeeping, is there any evidence that certain DUBs have functions in specific cellular processes? It is early days, but there are examples in both yeast and higher eukaryotes (BOX 2).

Different types of ubiquitin signal

The way in which ubiquitin is linked to proteins has the potential to alter their fate (FIG. 5). A single protein can be modified on one or more lysines with a single

IκBα
Inhibitory subunit of the NF-κB transcription factor. It is phosphorylated, ubiquitylated and degraded in response to stimuli that activate NF-κB.

SOCS BOX
Suppressor of cytokine signalling box first identified in an inhibitor of Jak family kinases.

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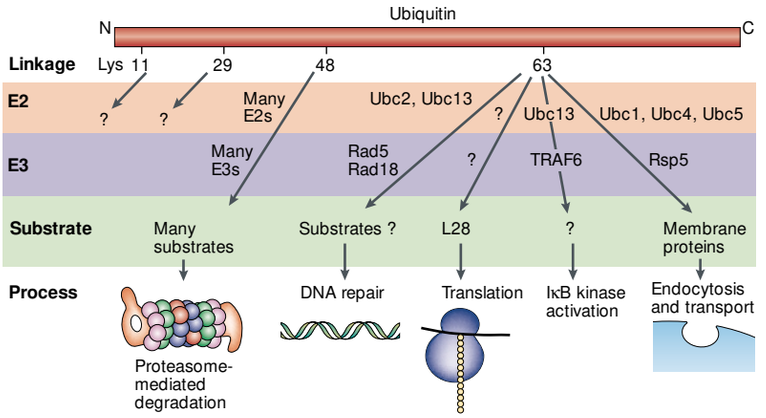

Figure 5 | **Different functions for different ubiquitin linkages.** Schematic representation of ubiquitin with lysines and roles of different linkages. Ubiquitin has several lysine residues and, *in vivo*, can form multi-ubiquitin chains linked through positions 11, 29, 48 and 63. The functions of Lys11 and Lys29-linked chains are unknown. Lys48-linked chains target proteins to the proteasome but might have other functions, and Lys63-linked chains have a range of fates.

ubiquitin (monoubiquitylation; see the review by Linda Hicke), with lysine-linked chains of ubiquitin (multi-ubiquitylation) or combinations of the two. As mentioned above, only multi-ubiquitin chains target proteins for proteasomal degradation, with multi-ubiquitin chains of four or more ubiquitin molecules linked through lysine 48 (K48) being adequate as a proteasome-targeting signal. Such chains are formed by isopeptide linkages between a lysine on the last ubiquitin of a growing chain with the carboxy-terminal glycine of a new ubiquitin molecule. How E3s mediate both the transfer of ubiquitin to a lysine on a substrate and also add ubiquitin to a growing end of a multi-ubiquitin chain of more than ten ubiquitins is poorly understood, but an accessory factor (E4) that facilitates the formation of multi-ubiquitin chains for one yeast E3 has been identified[70]. However, E4s are not generally required for the formation of multi-ubiquitin chains.

The choice of lysine is also an important decision when building up a multi-ubiquitin chain because ubiquitin itself has seven conserved lysine residues, all of which are potential sites of isopeptide linkage to the carboxyl terminus of another ubiquitin. *In vivo*, K11, K29, K48 and K63 all can form ubiquitin–ubiquitin linkages. K48-linked multi-ubiquitin chains are potent targeting signals that lead to recognition and degradation of proteins by proteasomes. K63 linkages, however, are apparently not proteasome-targeting signals: instead, they are important for DNA repair[71] and other functions. A specific E2, Ubc13, functions together with Mms2, a molecule that structurally resembles an E2 but lacks the canonical cysteine, to generate K63 chains[72]. These proteins are implicated in DNA repair together with Ubc2 (Rad6) and two RING finger proteins, Rad5 and Rad18 (REFS 73, 74). A recent and surprising observation is that a ribosomal subunit, L28, is a major substrate for modification with K63-linked chains. L28 ubiquitylation, which is most prominent during S phase of the cell cycle, is stimulated by irradiation, is reversible, and enhances translation[75]. Activation of K63 multi-ubiquitin chain formation by a RING finger E3, TRAF6 (TNF-receptor-associated factor 6, where TNF stands for tumour necrosis factor), results in the activation of IκB kinase[76]. K63-linked chains also have roles in the endocytosis and targeting for vacuolar degradation of yeast transporters (reviewed in REFS 29, 33; see the review by Linda Hicke).

Most proteins have many lysine residues, so can the position of a ubiquitin signal on a protein affect its fate in different ways? In some cases, specific lysines on proteins are ubiquitylation targets[77,78], whereas in others there is little specificity[79,80]. Furthermore, there are now several examples where the amino termini of proteins, rather than lysines, can serve as ubiquitylation sites[81]. We do not know how or whether the site of ubiquitylation on a protein, like the nature of the multi-ubiquitin linkages, affects its eventual fate.

Destination: proteasome

Alluring as these variations on a theme are, to our knowledge most ubiquitylated proteins have K48-linked chains and are recognized by 26S proteasomes. The catalytic component of this remarkable and highly complex structure is a cylindrical chamber of 28 subunits (the 20S core) that includes two copies each of subunits with trypsin, chymotrypsin, and peptidylglutamyl peptidase-like activities (see the review by Peter Kloetzel). The 20S core is capped at each end by a multisubunit regulatory complex, the 19S cap. This multisubunit cap fulfils several roles, including recognition of multi-ubiquitin chains and some UDPs, and also allows for the ubiquitin-independent proteasomal targeting of ornithine decarboxylase (see the review by Philip Coffino on page 188 of this issue). There is compelling evidence for the ubiquitin-independent, proteasome-dependent degradation of at least one other protein, $p21^{Cip1}$ (REF. 82). Whether the 19S cap has a function in the proteasomal targeting of $p21^{Cip1}$ remains unknown. A cap component that recognizes multi-ubiquitin chains — S5A — has been identified, but genetic evidence indicates that there might be other multi-ubiquitin recognition elements within the cap[83]. The 19S cap also contains DUBs and multiple ATPases. It should be appreciated that the proteasome is a dynamic structure that is modified, for example, in response to the inflammatory cytokine interferon-γ. Proteasomal degradation is not limited to ubiquitylated cytosolic and nuclear proteins. Ubiquitylation also targets ER lumenal and membrane proteins for degradation. Specific E2s and a yeast RING finger protein are among the proteins involved in this process[33,84]. Understanding how ubiquitylation and proteasomal degradation — processes that do not occur in the ER lumen — contribute to the retrograde movement of proteins out of the ER and their concomitant degradation is a topological puzzle that awaits resolution.

From proteolysis to proteomics

Ubiquitin-mediated regulated protein degradation is essential to virtually all aspects of eukaryotic cell biology. We now know that HECT domains and a substantial number of RING fingers are E3 modules and that F-

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box and possibly SOCS-box proteins are substrate-recognition elements for multisubunit E3s. These insights have coincided with a massive increase in the rate at which deduced protein sequences are becoming available through the genome projects — a resource that we will need to define the complex network of components, substrates and regulators of the ubiquitylation system. This information should prove especially useful as we develop more sophisticated tools, such as protein arrays, to probe differences in cellular protein levels, allowing us to identify proteins that undergo accelerated or delayed degradation in disease in much the same way that we now use DNA microarrays to probe for differences in gene expression.

It is now clear that ubiquitylation is much more than a proteasomal targeting signal. How it mediates responses to DNA damage, facilitates endosomal transport, and increases the efficiency of translation are all open questions, as is the role of UDPs in ubiquitin-mediated processes. The realization that UBLs are also conjugated to proteins using similar tools, and that some UBLs modulate ubiquitylation, makes it evident that cells have evolved multidimensional networks of regulated protein modifications to fine-tune protein levels and activity in ways that are yet to be fully appreciated.

Links

DATABASE LINKS **Ubiquitin | UCRP | RUB1 | Nedd8 | SUMO-1 | Apg12 | ubiquitin domain | HECT domain | RING finger | E1 | Ubc9 | Ubc12 | BRUCE | Ubc7 UBCH7 | E6-AP | c-Cbl | Ubc3 | p53 | HHR23A | Angelman syndrome | WW domains | C2 domain | Nedd4 | Rsp5 | SPT23 | MGA2 | NF-κB | Liddle syndrome | Rbx1 | Skp1 | Cull | BRCA1 | familial forms of breast and ovarian cancer | Mdm2 | parkin | IBR | juvenile Parkinson's disease | BIR domain | von Hippel Lindau | Cul-2 | elongin B | elongin C | Apc11 | F Box | βTRCP | β-catenin | CD4 | von Hippel–Lindau | HIF1α | SOCS | Ubc13 | Mms2 | Ubc2 | Rad5 | Rad18 | L28 | TRAF6 | S5A**

FURTHER INFORMATION **Weissman lab | Nottingham University ubiquitin site | Wilkinson lab | Ubiquitin and the biology of the cell**

ENCYCLOPEDIA OF LIFE SCIENCES **Ubiquitin pathway | Proteins: postsynthetic modifications**

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