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Ubiquitin: not just for proteasomes anymore

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Ubiquitin is a small protein that can be covalently linked to itself or other proteins, either as single ubiquitin molecules or as chains of polyubiquitin. Addition of ubiquitin to a target protein requires a series of enzymatic activities (by ubiquitin-activating, -conjugating and -ligating enzymes). The first function attributed to ubiquitin was the covalent modification of misfolded cytoplasmic proteins, thereby directing proteasome-dependent proteolysis. More recently, additional functions have been ascribed to ubiquitin and ubiquitin-related proteins. Ubiquitin directs specific proteins through the endocytic pathway by modifying cargo proteins, and possibly also components of the cytoplasmic protein trafficking machinery.

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Abbreviations

EGFR	epidermal growth factor receptor
EH	Eps15-homology
Eps15	EGFR pathway substrate clone 15
MVB	multivesicular body
Notch^{IC}	Notch intracellular
RTK	receptor tyrosine kinase
SH2	Src-homology domain 2
SUMO	small Ub-like modifier
Ub	ubiquitin
UBA	Ub-associated
Ubl	Ub-like
UIM	Ub-interaction motif

Introduction: protein modification by ubiquitin and ubiquitin-like proteins

Since its discovery in the mid-1970s, the small protein ubiquitin (Ub) has been associated with cellular housekeeping functions such as eliminating damaged proteins. However, it has recently become clear that Ub is involved in a variety of other vital processes at different places within the cell, ranging from the plasma membrane to the nucleus (Figure 1).

Ub is covalently attached to other proteins through an isopeptide bond between its carboxy-terminal glycine and the epsilon-amino group of lysines in the target protein (reviewed in [1]). This attachment is catalyzed by enzymes that activate and ultimately conjugate the Ub moiety to a lysine residue in the substrate. This can be followed by further additions of Ub to specific lysine residues within the linked Ub itself, to generate a poly-Ub chain (Figure 2). This covalent modification can be reversed by unique proteases specific for the iso-peptide linkage. This general, initially simple, process acquires remarkable versatility and complexity through variations described below.

Nature of the modifier

Although Ub is the most famous and best characterized, other proteins (often referred to as Ub-like [Ubl]) are also conjugated to targets in analogous reactions. Examples of these 'alternative' modifiers are presented in Table 1. Although each of these Ubl proteins exhibits a different degree of sequence homology to Ub, all of them are structurally very similar.

Number of modifier units attached

It has been proposed that different Ub-chain lengths are associated with different processes. Thus, mono- and di-ubiquitination have been implicated in endocytosis [2], whereas chains made up of at least four units seem to be required for efficient proteasomal degradation [3].

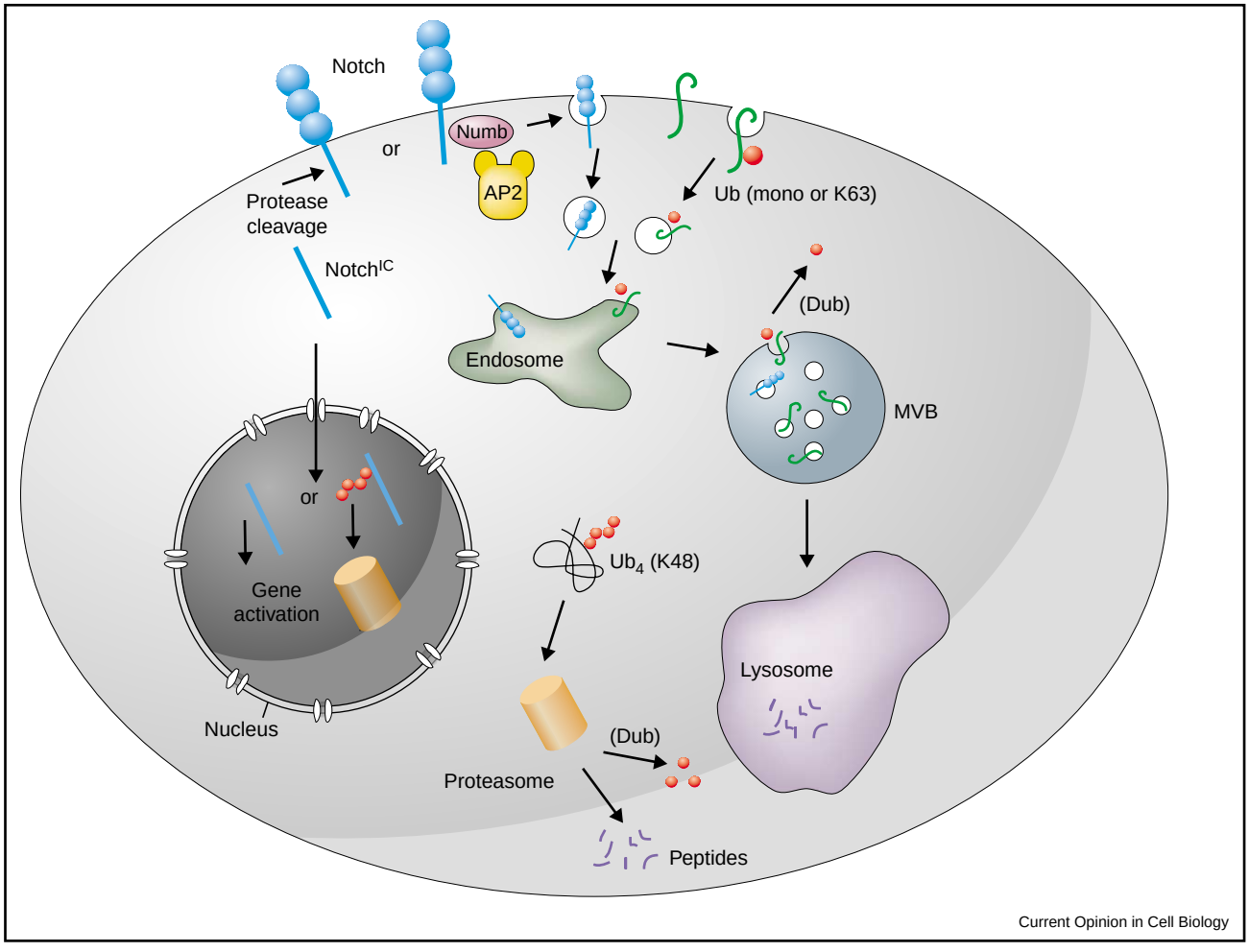
The linkage formed

Since the resulting conformation of the poly-Ub chain depends on which lysines within Ub are used for the iso-peptide bond formation (Figure 2), the linkage also provides an additional layer of specificity. Thus, whereas K48-linked chains are usually associated with proteasomal degradation, the K63 linkage is involved in a variety of other processes including endocytosis and DNA repair [4]. The functional roles of poly-Ub chains formed through K11 and K29 linkages are less clear.

Most functions of Ub can be understood in the context of this small protein acting as a tag. The information transmitted by this tag depends on the nature of the modification, as described above, which defines the specificity of the tag for different cellular machinery and commits the target protein to different fates. Thus, when the tag is recognized by subunits of the 26S proteasome, the cytosolic protein is targeted for degradation (the 'classical' role of Ub). This degradation is associated with either housekeeping functions or other specific purposes such as regulation of protein level [5•] or antigenic-peptide generation [6].

In this review, we will focus on the 'unconventional' roles of Ub in cell regulation; that is, when Ub/Ubl-recognition

Figure 1



An overview of some of the cellular compartments where Ub functions. The proteasome (orange barrel) degrades cytosolic or nuclear proteins containing a chain of four Ub molecules joined by K48 linkages. The transmembrane receptor Notch has several modes of regulation: one in which Notch internalization is promoted by association with Numb and AP2, and another involving proteolytic cleavage of Notch^{IC}, followed by transport of Notch^{IC} to the nucleus, where it either activates transcription or is degraded. Finally, transmembrane proteins at the plasma membrane (green) can use Ub (red) as a signal for inclusion in endocytic vesicles. Moreover, a Ub signal can direct sorting of transmembrane proteins into the lumenal vesicles of the MVB. De-ubiquitinating enzymes (Dub) remove covalently attached Ub to allow its reuse.

systems other than the proteasome are involved. These include protein-trafficking machinery, as well as processes where the function of Ub/Ubl as a tag is not clearly established, such as transcription factor activation.

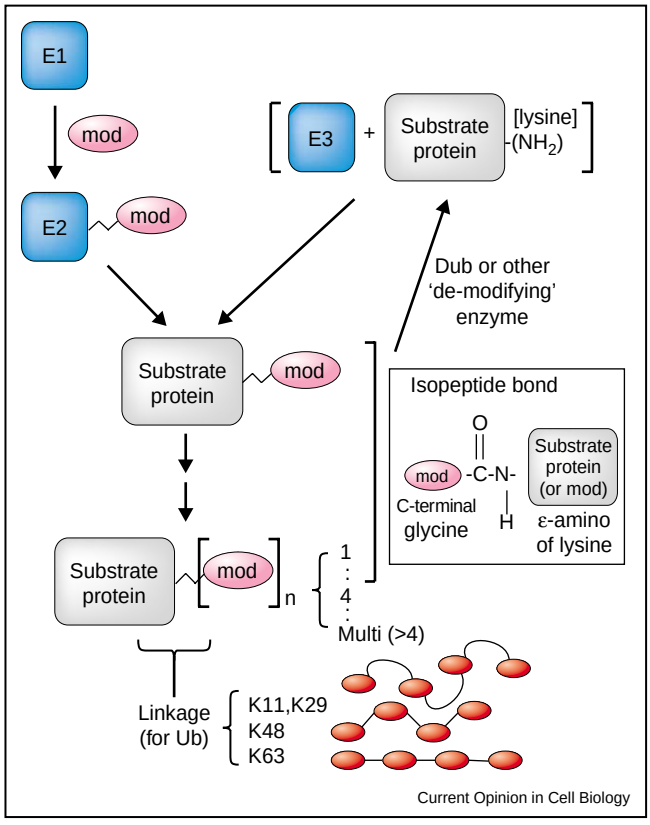
The Ub/Ubl tag recognized by protein-trafficking machinery

One area where Ub/Ubl-research has experienced significant and very exciting advances is in different steps of the membrane protein transport system: Ub participates in targeting proteins to endosomal compartments either from the plasma membrane [2] or from the *trans*-Golgi network [7]. Ub is also involved in protein sorting from endosomes to multivesicular bodies (MVBs) and in delivery of transmembrane proteins to the interior of the lysosomal/vacuolar compartment (reviewed in [8]). Transport across the nuclear envelope through nuclear pores is another event in which Ubl proteins (particularly SUMO [small Ub-like modifier]; Table 1) play increasingly well-characterized roles [9]. A common denominator in these processes is the presence of a single Ub/Ubl molecule, or in a few cases a short Ub chain (less than four units) linked through Ub K63 [2]. Distinct readouts are expected from recognition of the tag by different components of the protein-trafficking machinery.

Plasma membrane protein internalization

The list of membrane proteins that are ubiquitinated before internalization has expanded enormously in recent times. The classical examples are the yeast G-protein-

Figure 2



An overview of the cycle of Ub and Ubl modifier protein metabolism. The attachment of modifier proteins (Ub or Ubl proteins, [mod]) to a substrate protein begins with an E1 enzyme that activates the modifier so it can be linked through a thioester bond to an E2 conjugating enzyme. E2 enzymes can directly transfer the modifier onto a substrate protein, usually in cooperation with an E3 ligase enzyme that selects particular substrates. The modifiers themselves can also be substrates. In the case of Ub, one of four lysine residues can be used in formation of the iso-peptide bond, leading to a chain of Ub proteins (red). De-ubiquitinating enzymes (Dub) cleave covalently attached Ub to allow its reuse.

coupled receptors Ste2 and Ste3 [10,11], and the ABC transporter proteins [12]. Now, many other plasma membrane proteins of yeast are known to require Ub for their internalization [2], suggesting that the Ub tag functions as an internalization signal in *Saccharomyces cerevisiae*. Although the role played by Ub in protein internalization in higher eukaryotes seems to be more complex and not as general as in yeast, several well-studied examples are described below.

Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) family member activated by ligand binding. EGFR is ubiquitinated by the E3 ligase c-Cbl following ligand-dependent phosphorylation of the receptor [13••] as a pre-requisite for its downregulation (see the review by Dikic in this issue). This process involves the recruitment of the adaptor protein CIN85 [14] and the endocytic regulatory protein endophilin [15••,16]. Similar mechanisms involving c-Cbl in hepatocyte growth factor receptor internalization have also been reported [17••], whereas distinct Cbl-related Ub-ligases have been implicated in downregulating other membrane proteins [18,19].

An interesting twist to the Ub/EGFR story comes from studies of the protein Eps15 (EGFR pathway substrate clone 15). Eps15 was originally identified as a phosphorylation substrate of EGFR and was later shown to be essential for endocytosis [20]. Eps15 is made up of three Eps15-homology (EH) domains and several peptide motifs, including two copies of the recently described UIM (Ub-interaction motif) [21•]. Polo *et al.* [22••] showed that the Eps15 UIMs interact with Ub. These UIMs are also required for the mono-ubiquitination of Eps15 at lysine residues outside of the UIM, possibly mediated by the Ub-ligase Nedd4 [22••,23,24]. The UIMs of other endocytic proteins such as epsin are similarly required for their own mono-ubiquitination reactions [22••,24]. Interestingly, the adaptor protein CIN85 (which lacks UIMs) is also mono-ubiquitinated, but in this case c-Cbl itself seems to be involved the Ub modification [14].

The internalization of growth hormone receptor (GHR) has also been connected to Ub [25]. However, unlike other signaling receptors, GHR internalization requires the recruitment of the Ub-conjugation machinery, but not

Table 1

Ubl proteins and their functions.

'Alternative' modifier	Function	References
SUMO (SMT3 in yeast, Ubl1, sentrin, GMP1 or PIC-1)	Targets proteins to the nucleus; frequently involved in regulating transcription	[42]
Nedd8 (Rub1 [related to Ub] in yeast)	Regulates the SCF (Skip1/Cullin/F-box) Ub ligases	[43]
Hub1 (homologous to Ub)	Plays a role in cell polarity processes in yeast	[44]
ISG15 (interferon stimulated gene of 15 kDa) or UCRP (Ub cross-reactive protein)	Implicated in the regulation of interferon signaling	[45]
Apg12 (autophagy-12)	Regulates the 'cytoplasm-to-vacuole' targeting and autophagy pathways	[46]

ubiquitination of the receptor itself [25]. This is consistent with a more general requirement for ubiquitination of associated endocytic machinery in regulating receptor trafficking and signaling.

Notch

The transmembrane receptor Notch is a key component of a signaling pathway involved in cell-to-cell communication that controls cell differentiation and pattern formation (reviewed in [26]). Notch receptor interaction with its ligand Delta triggers the clipping of the cytoplasmic part of Notch (Notch intracellular [Notch^{IC}]), which translocates to the nucleus to activate gene transcription (Figure 1) [26]. Given the profound consequences of activating this pathway, cells have devised several mechanisms to regulate it, including ubiquitination and endocytosis. One strategy involves the endocytic protein Numb bridging Notch to the endocytic adaptor complex AP-2, thereby triggering Notch internalization (Figure 1) [27]. In fact, AP-2 is required for Numb-mediated asymmetric cell division in *Drosophila*, a process that involves Notch downregulation within specific subcellular domains [28•].

Another intricate mechanism for Notch pathway control requires the ubiquitination of pathway components, mediated by different Ub ligases. For example, Nie *et al.* [5•] found that the ring-finger-containing protein LNX (ligand of Numb-protein X) ubiquitinates Numb to target it for proteasomal degradation. Therefore, expression of LNX enhances Notch signaling by decreasing its Numb/AP2-dependent internalization. Notch itself is the target of two Ub-ligases: Itch, which ubiquitinates Notch at the plasma membrane [19], and Sel-10, which regulates the nuclear levels of Notch^{IC} [29]. As expected, each enzyme negatively regulates the Notch signaling pathway.

Finally, three papers [30•–32•] show that the ring-finger-containing Ub-ligase Neuralized (Neur) specifically promotes the mono-ubiquitination and internalization of Delta (the ligand for Notch) in *Xenopus* and *Drosophila*. Further investigation is necessary to establish the concerted effects of each of these pathways that regulate Notch. It is clear, however, that, similarly to the EGFR, cells rely on Ub-mediated internalization to regulate the Notch signaling pathway. Studies of the Notch pathway also show that Ub can downregulate ligands (such as Delta) in addition to receptors.

Finding molecules capable of recognizing the Ub tag within the protein trafficking system continues to be the object of intensive research. Evidence indicates that key endocytic molecules like Eps15 (and its yeast homolog Ede1) and the epsins bind Ub through motifs such as UIMs and UBA (Ub-associated) domains [21•,22••,33•]. How interactions between these endocytic proteins and Ub control the internalization of membrane proteins has not been clearly established, but models can be proposed. For example, ubiquitinating enzymes such as c-Cbl might modify plasma membrane proteins (e.g. RTKs) and also directly mediate association of endocytic proteins required for coated-pit/vesicle formation (e.g. the CIN85–endophilin complex). The resulting ubiquitinated cargoes and/or endocytic machinery might in turn recruit proteins such as Eps15 and epsins through their UIM and UBA domains. Finally, these Ub-binding proteins could subsequently interact with other components of the endocytic machinery, such as endocytic accessory proteins and clathrin. Regulatory mechanisms involving post-translational modifications such as phosphorylation or additional ubiquitination (e.g. Nedd4-mediated) can add extra layers of complexity to this scheme. This cascade of binding events would result in the efficient formation of the endocytic multimolecular complexes essential for internalization in the vicinity of ubiquitinated receptors.

Membrane-protein delivery to multivesicular bodies

The function of Ub as tag in the MVB-sorting pathway is another area within the protein trafficking field that has advanced impressively (reviewed in [8]). Once at endosomes, some protein cargoes are included in vesicles that bud away from the cytoplasm, inward into the endosomal lumen, to generate the MVB. After the MVB fuses with the lysosome/vacuole, the vesicles and their contents are degraded. The cytosolic tails of proteins to be sorted into the MVB are labeled with Ub moieties, and the molecular machinery involved in the recognition of this tag has been recently identified. The yeast protein Vps27 (vacuolar protein sorting 27; this is a homolog of mammalian Hrs [HGF-regulated tyrosine kinase substrate]) binds ubiquitinated cargoes via its UIMs [33•] and recruits the Ub-binding complex ESCRT-I (endosomal sorting complex required for transport I), which is composed of three other Vps proteins [34••]. This initial step is followed by the recruitment of two more ESCRT complexes (II and III) that sequentially interact with the ubiquitinated cargoes and deliver them into budding areas (reviewed in [8]).

Intriguingly, certain viruses such as HIV take advantage of this system and recruit the mammalian ESCRT-I complex (and subsequently the rest of the machinery) to the plasma membrane through ubiquitinated viral envelope proteins [35••]. There, the endosomal sorting system is utilized in a topologically analogous process, but this time to help the virus to bud away from the cytosol into the extracellular media [35••].

Nuclear transport

The translocation of the MEK1 kinase between cytosol and nucleus in *Dictyostelium* is an interesting case of antagonistic regulation by Ub and a Ubl [36•]. As a result of chemoattractant stimulation, inactive MEK1 is SUMOylated in the nucleus (see Table 1), translocated

to the cytosol, and then moves to the plasma membrane where it becomes active. Opposing this process, signaling can also promote ubiquitination and nuclear retention of MEK1 by the Ub-ligase Mip1 (MEK1-interacting protein-1), which downregulates MEK1 [36•].

Ub/Ubl and transcription regulation

Another expanding area of research in the Ub/Ubl field is the control of gene expression. Different strategies by which Ub affects nuclear activity include a 'conventional' mechanism, where proteasomal degradation either destroys or activates (converting inactive into active forms) transcription factors, as well as proteasome-independent ('unconventional') mechanisms (reviewed in [37]).

Taking one example, the yeast transcription factor Met4 is ubiquitinated by SCF^{Met30} and inactivated either by degradation or by a proteolysis-independent mechanism [38••]. Thus, Ub–Met4 is degraded when cells are grown in minimal medium, whereas cells grown in rich medium do not degrade Ub–Met4. In rich medium, Ub–Met4 is excluded from methionine-regulated promoters, but maintains active transcription at other promoters.

Inhibitory modification by Ub also applies to histones [39]. Control of histone activity is further complicated by cross-talk between methylation and ubiquitination of histones, in which Rad6-mediated ubiquitination of histone-2B is required for histone-3 methylation. These modifications are linked to regulation of transcriptional silencing [40•].

Another interesting effect exerted by Ub comes from the observation that activity of transcription activator domains (TADs) inversely correlates with TAD levels [37]. It is clear now that ubiquitination not only regulates TAD concentration in a proteasome-dependent manner, but also is necessary for TAD activation [41•]. Salghetti and co-workers clearly showed that deletion of SCF^{Met30} renders TADs inactive and the lack of this Ub-ligase can be circumvented by the fusion in frame of Ub [41•].

Conclusions

The recent advances in studies of Ub and Ubl proteins have been rapid and wide-ranging in their implications. The future holds a better understanding of the physiological consequences of these modifications, including the regulation of subcellular localization, reversible allosteric conformations, recruitment of binding partners, and protein half-life. It is clear that one must consider the possibility that Ub and Ubl modifications can have either positive or negative regulatory effects; Ub is not simply an 'off' switch! Another very interesting subject is the identification of the enzymes that can add or remove Ub and Ubl from target proteins, and importantly, how the activity of these enzymes is controlled. Since approximately 2% of the genome of *S. cerevisiae* is devoted to Ub and Ubl metabolism, it is clear that we've only begun to scratch the surface of the many critical functions that depend on these small proteins.

Update

While this manuscript was in press, three reports on the American Society for Cell Biology summer meeting, entitled *Non-traditional functions of ubiquitin and ubiquitin-like proteins* (Colorado Springs, CO, USA: August 11–14 2002), were published [47–49]. These interesting articles also highlight some of the latest findings on unconventional functions for Ub and Ubl proteins in cell regulation.

Acknowledgements

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