

A superfamily of protein tags: ubiquitin, SUMO and related modifiers

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The biological functions of many proteins are altered by their covalent attachment to polypeptide modifiers. The best-known example of this type of modification is ubiquitination. Ubiquitin has a well-documented role in targeting proteins for degradation by the proteasome, but additional effects of protein ubiquitination are now being uncovered. Furthermore, multiple polypeptides that are distinct from, but related to, ubiquitin are also enzymatically coupled to target macromolecules, and these ubiquitin-like proteins participate in diverse biological processes such as DNA repair, autophagy and signal transduction.

Cells must be able to respond to rapid changes in both their internal and external environments. A particularly sensitive, rapid and reversible response to environmental stimuli or to a programmatic change in cell state is the post-translational modification of specific proteins. Small-molecule modifications, such as phosphorylation and acetylation, are well-characterized examples of post-translational events that modulate protein function, but there is also a class of larger, proteinaceous modifications that plays an equally important role in protein regulation.

The first example of a protein acting as a post-translational modifier was ubiquitin, but over the past ~15 years, a series of ubiquitin-like modifiers (UBLs) has also been discovered. Compared with small molecule modifiers such as phosphoryl or methyl groups, ubiquitin and UBLs provide much larger and more chemically varied surfaces. Thus, these covalent modifiers can act as flexible adaptor modules for altering protein conformation or protein-protein interactions. UBLs are also more plastic in an evolutionary sense: duplication and diversification of UBL-encoding genes can give rise to multiple related molecules that can acquire new functions and participate in distinct cell regulatory mechanisms.

Ubiquitin conjugation

As the prototypical protein modifier, ubiquitin will be used here to illustrate the general manner by which substrate conjugation and deconjugation take place, but much of this description can be extended to other UBLs [1,2] (Fig. 1). Ubiquitin is synthesized as a precursor that must be processed by de-ubiquitinating enzymes (DUBs) to generate a Gly-Gly sequence at the C terminus, which will be the site of attachment to target molecules. A ubiquitin-activating

enzyme (E1) adenylates the C terminus of ubiquitin, which is then transferred to an E1 cysteinyl side-chain via a thioester linkage. The ubiquitin is subsequently passed to a cysteinyl group on one of the ubiquitin-conjugating enzymes (E2s). Finally, through the action of a third enzyme – a ubiquitin-protein ligase (E3) – ubiquitin and substrate are linked by an amide (isopeptide) bond.

Ubiquitin-protein conjugation *in vivo* is mediated by multiple E2s and an even greater number of E3s. The E3 proteins generally play a dominant role in substrate binding. This multi-step mechanism involving many different enzyme components enables protein ubiquitination to achieve a high degree of specificity and flexibility. E3s can be divided into two broad classes based on whether they contain a HECT (homologous to the E6-AP carboxyl terminus) or a RING (really interesting new gene)-related domain. In HECT E3s, ubiquitin is transferred from the E2 to a conserved cysteine residue in the HECT domain, followed by attack of this thioester by a substrate lysine.

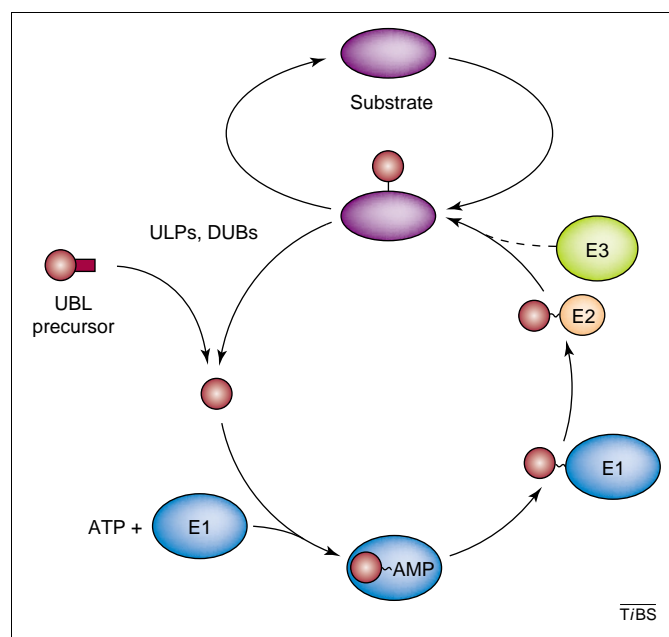


Fig. 1. Generalized ubiquitin-like protein (UBL) conjugation cycle. After UBL precursor cleavage, the C terminus of the mature UBL (red circle) is adenylated and then linked to an activating enzyme (E1) through a high-energy thioester bond. The activated UBL is then passed to a conjugating enzyme (E2). Alone, or in combination with a UBL-protein ligase (E3), the UBL is attached to an amino group of a substrate. E3 enzymes have not been identified for all UBLs, and in some systems, the E2 might be sufficient for substrate modification. In most cases, attachment of a UBL is reversible. De-ubiquitinating enzymes (DUBs) and UBL-specific proteases (ULPs) are responsible for these cleavage reactions.

Proteins with RING or RING-related motifs represent the more abundant class of E3 and do not appear to form an obligatory thioester intermediate with ubiquitin.

How the different ubiquitination factors bind to substrates and catalyze ubiquitin transfer is beginning to be deciphered, but many questions remain [1]. Here, we consider (1) Is the E1–E2 couple universal among ubiquitin and UBL transfer reactions; (2) do all UBL systems employ E3-like factors; and (3) do all E3-like factors fall into the RING or HECT classes?

The E1–E2 activation and transfer mechanism has been documented for most of the UBLs listed in Table 1. This might reflect the origin of most, if not all, of the ubiquitin and UBL systems from certain bacterial sulfur transferases that operate in enzyme cofactor biosynthesis [3]. However, this does not exclude alternative mechanisms of ubiquitin activation and transfer.

Two very different types of ubiquitin transferases have been reported in the past few years. First, the general transcriptional coactivator TAF_{II}250 appears to be able to catalyze the ATP-dependent mono-ubiquitination of histone H1 [4]. Defects in the apparent histone ubiquitin-ligase transferase activity correlate closely with defects in transcriptional regulation and embryonic development. Second, a ubiquitin transferase activity has been reported for a de-ubiquitinating enzyme, ubiquitin C-terminal hydrolase L1 (UCH-L1) [5]. Here, the ubiquitin-acyl enzyme intermediate that normally forms during ubiquitin-conjugate cleavage by UCH-L1 is thought to be

attacked, not by a water molecule but by a lysine side-chain of a protein, resulting in a new ubiquitin-protein conjugate. UCH-L1 can dimerize and ubiquitinate α -synuclein – a protein that accumulates in Lewy body aggregates in the brains of Parkinson's Disease (PD) patients. Sequence variants of UCH-L1 with increased ubiquitin ligase activity correlate with increased risk of PD [5].

Less controversial is the question of whether all UBL ligation systems require an E3-like activity; the answer is likely to be no. One reason for believing this is that some UBL-ligation systems have just a single target, so additional 'specificity factors' beyond the E2 enzymes might be unnecessary. In the case of small ubiquitin-related modifier (SUMO)-protein ligation, the E2 can directly bind and ubiquitinate substrate *in vitro*, and target-lysine specificity is accounted for by E2 binding alone [6,7]. (Whether SUMO ligation *in vivo* ever occurs without an E3, however, is less certain.)

As noted, E3 proteins have been placed into two classes, HECT or RING. Some proteins, such as U-box factors and a subset of proteins with proposed plant homeodomain (PHD) motifs, such as MEK kinase 1, were thought to fall outside of these classes. However, the U box shares the RING fold [8], and the variant PHDs – which, like RINGs, coordinate a pair of Zn²⁺ ions – are arguably closer to RINGs in structure than to bona fide PHDs [9]. A more provocative example comes from a study of an apparent SUMO-protein ligase activity of the nuclear pore complex

Table 1. Ubiquitin-like proteins for which there is experimental evidence for ligation to other molecules^{a,b}

UBL	Variants	Function	Known Substrates	Ref.
Ubiquitin	Mono	Endocytosis/lysosomal degradation, meiosis, chromatin remodeling	Histones, ion channels, receptors	[51]
	Poly (Lys29)	26S proteasomal degradation?	UFD substrates	[52]
	Poly (Lys48)	26S proteasomal degradation	Many short lived proteins, mis-folded proteins	[53]
	Poly (Lys63)	Post-replicative DNA repair, translation, endocytosis	L28 ribosomal protein, TRAF6, PCNA, plasma membrane proteins	[53]
ISG15 (UCRP)	GATE16 (Golgi-associated ATPase Enhancer of 16 kDa), LC3 (microtubule-associated protein 1 light chain 3), GABARAP	Immune response	Serpin 2a, Stat1, ERK1, others	[54]
AUT7 (APG8)		Autophagy, cytoplasm-to-vacuole targeting, vesicular transport	Phosphatidylethanolamine	[22]
APG12		Autophagy, cytoplasm-to-vacuole targeting	APG5	[22]
NEDD8 (RUB1)		Auxin response, meiosis-to-mitosis transition	Cullins	[2]
SUMO1 (SMT3)	SUMO2, 3	Nuclear transport, chromosome segregation, transcriptional regulation	Many	[21]
HUB1		Cell polarity	Sph1, Hbt1	[55]
FAT10		Apoptosis	Unknown	[56]
URM1 (Ubiquitin Related Modifier 1)		Growth at high temperature	Unknown	[3]
MNSF		T-cell activation	TCR α -like protein	[57]

^aThe ubiquitin superfamily consists of numerous proteins that display structural similarity to ubiquitin and are involved in a wide range of biological activities. Note that ISG15 and FAT10 each consist of a tandem set of ubiquitin-related domains. Ubiquitin shows functional variation depending on how many molecules are added to a substrate and how those molecules are linked to one another. SUMO2/3 can form polymers *in vitro*, and such chains might be able to form *in vivo* at low levels [58].

^bAbbreviations: APG, autophagy defective; AUT, autophagy; FAT, human leukocyte antigen F associated; GABARAP, GABA(A) receptor-associated protein; HUB1, homologous to ubiquitin; ISG15, interferon-stimulated gene; MNSF, monoclonal nonspecific suppressor factor β ; PCNA, proliferating cell nuclear antigen; SUMO, small ubiquitin-related modifier; TCR, T-cell receptor; TRAF6, tumor necrosis factor receptor-associated factor; UCRP, ubiquitin cross-reacting protein.

(NPC) protein RanBP2 [10]. Biochemical studies have shown that a domain isolated from this very large protein, which has neither RING nor HECT motifs, is capable of stimulating the transfer of SUMO from the E2 to a protein, Sp100, that is known to be sumoylated *in vivo*. As yet, however, there are no genetic data demonstrating that RanBP2 stimulates sumoylation of Sp100 in the cell.

Novel functions for ubiquitin

Ubiquitin is best known as a modification that directs proteins to the proteasome, where the proteins are degraded to peptides. Targeting to the proteasome generally requires assembly of a ubiquitin polymer on the substrate, and these ubiquitins are linked to one another by a specific isopeptide bond between Lys48 of one ubiquitin and the C-terminal carboxyl group of the next ubiquitin in the chain. By contrast, ubiquitin chains with non-Lys48 linkages or even a single ubiquitin can sometimes be attached to a substrate.

For example, the pioneering work on ubiquitin conjugation by Harris Busch and colleagues revealed that a single ubiquitin could form a conjugate with histones H2A or H2B that is not subject to rapid degradation in mammalian cells [11]. This functionally mysterious histone modification has recently been revisited in the yeast *Saccharomyces cerevisiae*. Remarkably, histone H2B mono-ubiquitination functions in regulating chromatin structure and transcription by enabling a series of lysine methylations on another core histone, histone H3 [12]. The *trans*-effect of histone H2B ubiquitination presumably works by recruiting the distinct methylases responsible for these histone H3 modifications. H3 methylations (at Lys4 and Lys79) are enriched at transcriptionally active sites, although they are also required, perhaps indirectly, for chromatin-mediated gene silencing [13].

Protein mono-ubiquitination also has important consequences in other cellular contexts. In addition to the established role of membrane receptor ubiquitination in stimulating receptor endocytosis and targeting to the vacuole (lysosome) for degradation, more recent studies have uncovered a requirement for membrane protein mono-ubiquitination in the trafficking of resident vacuolar membrane proteins to the vacuole interior [14]. Surprisingly, components of this same ubiquitin-dependent sorting machinery are apparently commandeered by viruses such as Ebola and HIV-1 to orchestrate the budding of enveloped viruses from the plasma membrane [14]. The mechanistic parallels are intriguing because viral budding is topologically identical to the involution and vesiculation of endosomal membranes that drives proteins to the endosome interior. Current evidence suggests that the observed ubiquitination of viral Gag proteins is not a necessary feature, so precisely which proteins must be ubiquitinated for viral budding and release remains to be determined [14].

Probably relevant to this last issue is the finding that several membrane-protein-sorting factors are also mono-ubiquitinated, sometimes in response to the same ligand-binding events that trigger receptor ubiquitination [14]. Not only are these factors subject to ubiquitination, but they also contain motifs that specifically bind ubiquitin.

This has led to the idea that these cytosolic factors might form dynamic protein networks wherein ubiquitination of one factor enables binding to other trafficking molecules, and the density or character of these membrane-localized protein complexes helps in sorting decisions, such as whether a protein should be sent to the lysosome or recycled to the cell surface [14].

Alternative ubiquitin chains

Discrimination between ubiquitination events that target a protein to the proteasome rather than to another fate is probably not determined solely by whether the protein is mono- or poly-ubiquitinated. Poly-ubiquitin chains with different ubiquitin-ubiquitin isopeptide linkages are also formed *in vivo*, and these chains might have different targeting capacities. Most notably, ubiquitin polymers involving ubiquitin-Lys63 linkages have been connected to several different signaling pathways. In mammalian cells, activation of the nuclear factor- κ B (NF κ B) transcription factor requires formation of Lys63-linked ubiquitin chains (probably on the TRAF6 signaling protein) [15]. This eventually leads to the degradation of I κ B, thereby releasing active NF κ B. Interestingly, a heterodimer of the E2 Ubc13 and an E2 variant (Mms2 or UEV) that lacks a catalytic cysteine is responsible for assembling these Lys63-linked ubiquitin chains [1]. In yeast, the equivalent E2 heterodimer is required for formation of Lys63 chains on proliferating cell nuclear antigen (PCNA) in response to DNA damage [16]. PCNA is a processivity factor for DNA polymerases and is essential for processes such as DNA replication and replication-linked DNA repair. The Rad6 E2 is required for attachment of an initial ubiquitin to PCNA, whereas Ubc13-Mms2 helps extend the ubiquitin monomer into Lys63-linked ubiquitin polymers. Strikingly, these different forms of PCNA ubiquitination appear to channel DNA lesions into different DNA repair pathways, with mono-ubiquitination leading to error-prone repair and the polymeric modification resulting in error-free repair [16].

The crystal structure of the Ubc13-UEV dimer has been solved, and the probable arrangement of two ubiquitins on the surface of this complex can, in principle, account for its ability to assemble ubiquitin chains with Lys63 linkages [17]. It is tempting to presume that nature has exploited structural differences between alternative ubiquitin chains to generate different types of signals [18]. However, one can reasonably ask whether the different fates of ubiquitin conjugates with different ubiquitin chain linkages are not due to any major difference in their structural properties *per se*, but might reflect the geometry of the conjugating enzymes that happen to work on a particular substrate. The C-terminal ends of ubiquitin are highly flexible [19], so it is possible that the precise sites of linkage are not always crucial to chain function. Indeed, ubiquitin chains of various linkages have been reported to bind with comparable affinity to the proteasome [20].

Ubiquitin-like proteins

Here we focus on examples that highlight the broad regulatory sweep of ubiquitin-like protein modification

pathways and describe some of their mechanistic nuances. For more comprehensive treatments see [1,21,22].

At least ten UBL-ligation pathways that parallel the ubiquitin pathway in mechanism are now known to transfer small protein moieties onto various substrates (Table 1). Each of these ubiquitin-like pathways has distinct cellular functions. Some, such as the SUMO and related to ubiquitin (RUB) pathways, have been identified in all eukaryotes examined, whereas others, such as the ISG15 pathway, have a more restricted phylogenetic distribution. These UBLs all share structural similarity with ubiquitin.

SUMO

Of the UBLs, SUMO has probably received the most attention over the past few years. SUMO conjugation is essential for viability, at least in budding yeast [21]. This process, termed sumoylation, is highly regulated in all eukaryotes and participates in diverse events such as nuclear transport, transcriptional regulation, chromosome segregation and cell-cycle control [21].

The first identified target of sumoylation was RanGAP1, the GTPase-activating protein for the Ran GTPase, which is required for nucleocytoplasmic trafficking [21]. Sumoylation of RanGAP1 is necessary for the enzyme to bind to the RanBP2 subunit of the NPC. It is thought that NPC localization of RanGAP1 facilitates nuclear protein import [21]. In yeast, neither RanBP2 nor the sumoylation domain of RanGAP1 is present, but a recent study nonetheless implicates SUMO conjugation in nuclear trafficking in this organism [23].

Many different transcriptional regulators have also been shown to be sumoylated, although the precise functional consequences are still under debate. A major SUMO substrate in mammalian cells is the promyelocytic leukemia (PML) protein [24]. Once PML is modified by SUMO, it is directed to PODs (PML oncogenic domains), where it functions as a key structural component and acts to recruit several proteins, including transcription factors. POD localization of these factors can activate or inhibit transcription [24].

The importance of transcription-factor sumoylation is highlighted by Sp3 [25,26]. This protein binds the GC box, which is present in many mammalian gene promoters, and can act both as an activator and a repressor of transcription. The inhibitory activity of Sp3 is due to a short element that contains an hKxE sumoylation motif. Mutation of this lysine, which is modified by SUMO *in vivo*, to arginine (K539R) abolishes the transcriptional inhibitory activity of Sp3 without affecting DNA binding. Translational fusion of Sp3 to SUMO can override the transcriptional inhibition defect associated with the K539R mutation [26], indicating that even in this unnatural linkage, attachment to SUMO is sufficient to turn Sp3 into a repressor. The challenge now will be to show how SUMO modification of Sp3 leads to repression of its transcriptional targets.

Sumoylation also plays a role in chromosome structure and dynamics. For instance, the *Drosophila* PIAS (protein inhibitor of activated STAT) protein dPIAS – a presumptive SUMO ligase – is required for normal mitotic chromosome condensation and interphase chromosome

organization [27]. More recently, studies using mutations of the SUMO protease Ulp2 suggest a function for SUMO in controlling chromosome dynamics in yeast cells [28]. In the *ulp2* mutant, the centromere-proximal regions of sister chromatids are more frequently separated than in wild-type cells, and the separation extends more distally from the centromere. Part of this defect was traced to a failure to desumoylate Top2, the yeast topoisomerase II protein, which is sumoylated specifically in metaphase. Although this modification appears to be important for the role of Top2 in centromere cohesion dynamics, it is not required for its essential catalytic activity.

SUMO modification can also directly modulate signaling pathways. A revealing example is the transient sumoylation of a *Dictyostelium* MAP kinase in response to a differentiation signal [29]. Upon nutrient starvation, amoeboid *Dictyostelium* cells produce cAMP, triggering chemotaxis and aggregation of surrounding cells. One lysine residue within the MEK1 kinase of this signaling cascade is a target for both sumoylation and subsequently ubiquitination. In response to cAMP, nuclear MEK1 is rapidly sumoylated and transported to the cell cortex where a crucial MEK1 function is presumably fulfilled. Within a few minutes, MEK1 is desumoylated, enabling its return to the nucleus where it is ubiquitinated and degraded [29].

Rub1 or NEDD8

Among the ubiquitin-like molecules, Rub1 or NEDD8 (here referred to simply as RUB) is the most similar in sequence to ubiquitin. Its only known targets are the cullins, most or all of which are subunits of SCF (Skp1–cullin–F-box protein) or SCF-related ubiquitin ligases. The SCF-like family of E3s, which include a RING protein in their catalytic core, ubiquitinate a wide array of substrates [1]. Substrate recognition is mediated primarily by the F-box protein.

RUB attachment to the cullin subunit of SCF complexes is necessary for the activity and/or specificity of the ubiquitin ligase (Fig. 2). For the SCF ligase that ubiquitinates I κ B α , attachment of RUB to the cullin subunit enhances recruitment of the E2–ubiquitin intermediate to the SCF complex, thereby stimulating SCF-dependent ubiquitination [30]. Two models have been proposed to explain this observation. First, RUB attachment might cause a conformational change in the cullin that favors formation of an E2–E3 complex capable of more efficient ubiquitination. Alternatively, RUB could enhance an electrostatic interaction between the SCF and E2; this might transiently stabilize their association, providing the time necessary to poly-ubiquitinate I κ B α [31]. Recently, an SCF ligase inhibitor, known as CAND1 (cullin-associated and neddylation-dissociated), was found to bind to immature SCF complexes, perhaps preventing inappropriate ubiquitination activity. RUB conjugation to the cullin acts to dissociate the CAND1 protein, enabling the formation of a mature SCF complex capable of ubiquitinating its substrate [32,33].

The RUB-cullin conjugate is dynamic, and a major RUB-cleaving activity for this substrate has been traced to the COP9 signalosome (CSN), a 500 kDa protein complex

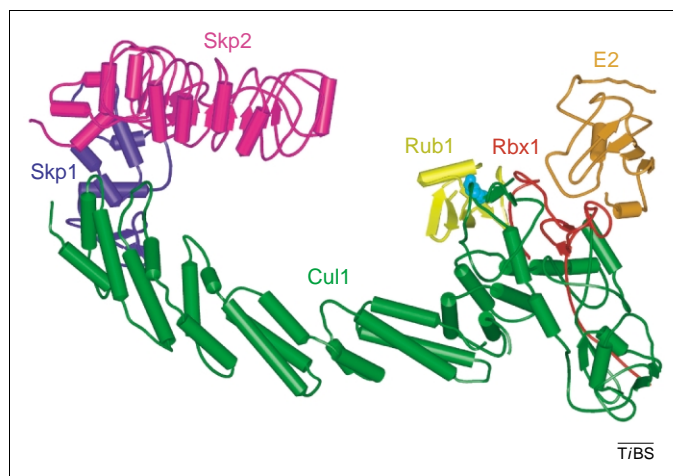


Fig. 2. Structure of a Skp1–cullin–F-box (SCF)-class ubiquitin-protein ligase. Structure of the Cul1–Rbx1–Skp1–Skp2–E2 complex based on the crystal structures of several subcomplexes (adapted from [59] using PDB ID files: 1C4Z, 1LDK and 1FSZ). Cul1 is a cullin and Skp2 an F-box protein. The RUB (related to ubiquitin) structure [60] (PDB ID: 1BT0) is positioned to reflect the position of the lysine to which it becomes ligated (shown in aquamarine). Substrate binding might occur within the cleft formed between the F-box protein (Skp2) and the E2.

originally identified from its role in photomorphogenesis in plants [34]. The Csn5 subunit of the CSN is the key catalytic component of this isopeptidase activity, and it contains a putative metalloprotease sequence signature also seen in the related Rpn11 (POH1) proteasome subunit that acts on ubiquitin conjugates [35]. Loss of COP9 RUB isopeptidase activity leads to phenotypic aberrations similar to those associated with mutations in RUB-ligating enzymes. A possible explanation for this is that removal of RUB enables release of the E2 from the E3 or E3–Ub–substrate complex, which might be required for polyubiquitination by the ligase and/or for recycling the cullin–RING protein subcomplexes for use in other SCF-like ligase complexes.

As with Rpn11, Csn5 is only active when in a larger complex. Interestingly, all the subunits of the CSN show homology to subunits in a subcomplex of the proteasome called the lid, suggesting a close evolutionary relationship between the two complexes [34]. The SCF ubiquitin ligases can bind both to proteasomes and the CSN [36,37]. Perhaps the CSN evolved from what had originally been (and possibly still could be) an alternative proteasome-lid complex, but developed a distinct specificity for RUB-cullins that evolved from its ability to bind SCFs.

Interferon-stimulated gene 15

The first UBL to be identified was the product of interferon-stimulated gene 15 (ISG15) [38], yet it has remained one of the most enigmatic of the UBLs. ISG15 is induced by treatment with interferon, lipopolysaccharides or upon viral infection of human cells [39]. An ISG15-activating (E1-like) protein has been identified [40], as has the first ISG15-specific isopeptidase, UBP43, which acts to remove ISG15 from at least a subset of conjugates [39]. Based on western blot analysis following interferon stimulation, multiple proteins are conjugated to ISG15. Among them are the JAK and STAT proteins, suggesting that ISG15 participates in JAK-STAT signaling in

response to interferon, which leads to suppression of cell proliferation and enhancement of apoptosis [41].

What is clear about ISG15 at this point is that mutationally induced increases or decreases in the levels of ISG15 conjugates correlate with dramatic phenotypic changes. Increased levels of ISG15 conjugates, due to a deletion of UBP43, lead to neurologic disorders and premature death in mice [42], whereas decreased levels of ISG15 conjugation result in improper monoblast differentiation [43] and possibly to tumorigenic progression of lung tumors [44]. ISG15 conjugation is likely to contribute to the immune response against many viruses. Certain viruses have evolved to overcome this response. For example, infection by influenza B virus strongly induces ISG15 expression, yet no ISG15-protein conjugates accumulate. This is owing to NS1B, a viral protein that binds to ISG15 and prevents its activation by the UBE1L E1-like enzyme [40].

AUT7 and APG12

Two of the most divergent UBLs are AUT7 (APG8) and APG12. These proteins share ~20% sequence identity [3], but neither shows obvious sequence similarity to ubiquitin. Nevertheless, AUT7 has been shown to have the same tertiary fold as ubiquitin [45]. As with other UBLs, AUT7 and APG12 utilize an E1–E2 enzyme couple for their covalent linkage to substrates (Fig. 3). Interestingly, the two modifiers share a single E1-like enzyme but have different E2-like proteins [22]. This is the only known example in which a single E1 can activate two different UBLs. The ability of the E1 to transfer AUT7 and APG12 to distinct E2s suggests that each E2 binds to the E1–UBL complex in a way that is only productive for transfer of the cognate UBL. The E1-like protein for AUT7 and APG12 shows sequence conservation with other E1 and E1-related proteins, but the similarity between the two autophagy E2s is barely detectable, and neither E2 gives a significant match to any other E2s [3]. The high sequence divergence of these UBLs and their E2s leaves open the possibility of more UBL conjugation pathways not yet discovered.

Both AUT7 and APG12 function in the starvation response known as autophagy. AUT7 is necessary for formation of intermediate membrane structures that arise during formation of the autophagosome. Remarkably, the target of AUT7 ligation is not a protein, but a phospholipid – the triglyceride phosphatidylethanolamine (PtdEtn) [22]. The head group of PtdEtn is a primary amine, which could function in AUT7 ligation analogously to the ϵ -amino group of lysine in UBL-protein attachment. One intriguing possibility for the function of AUT7–PtdEtn conjugation is that multiple rounds of AUT7 attachment and release deposit phospholipids into the newly forming membrane of the autophagosome precursor [22].

APG12 conjugation is required for either autophagosome precursor elongation or completion of autophagosome formation. There is a single known target for APG12, the APG5 protein. This conjugation appears to be constitutive and there is no known protease for cleaving APG12 from its substrate [22]. For higher eukaryotes, it has been speculated that counterparts of yeast AUT7 and

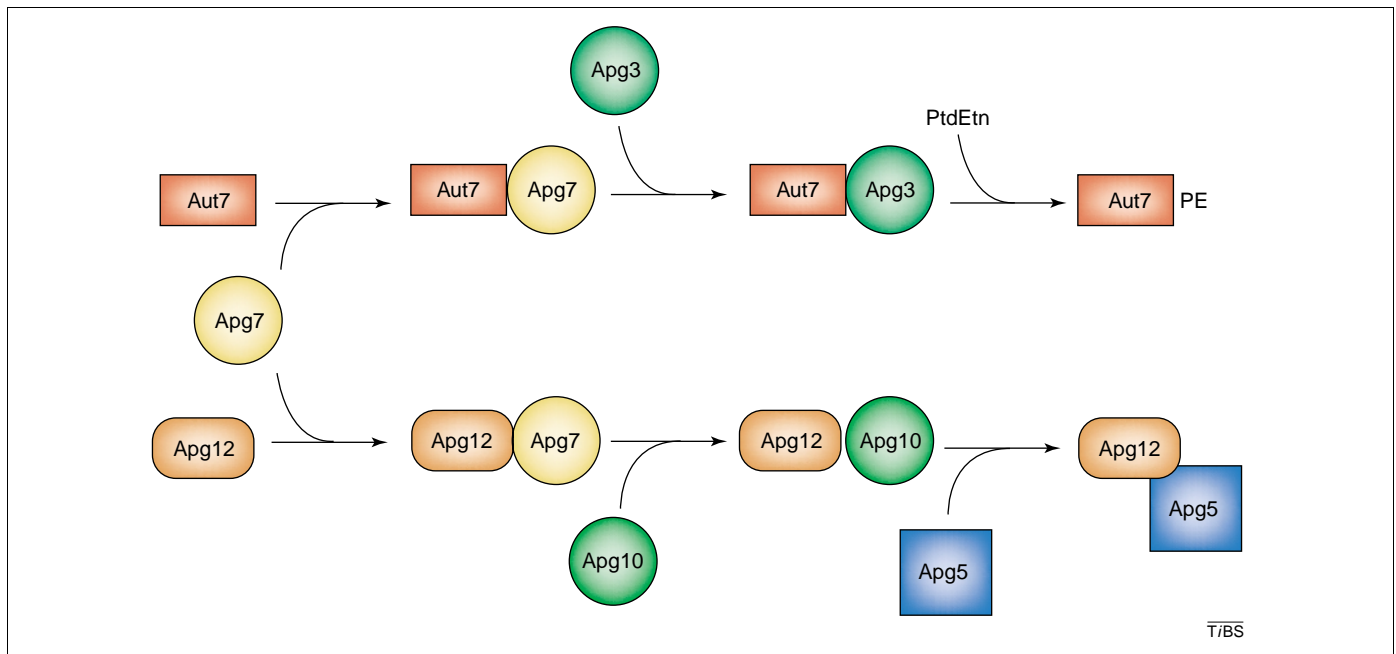


Fig. 3. The APG12 and AUT7 conjugation systems are required for autophagy. Both the AUT7 and APG12 conjugation pathways begin with the APG7 enzyme (E1). Activated AUT7 is passed to the APG3-conjugating enzyme (E2), which subsequently attaches AUT7 to phosphatidylethanolamine (PtdEtn). Activated APG12 utilizes a different E2, APG10, and is ligated to the APG5 protein substrate.

APG12 have more diverse roles in membrane dynamics. For instance, one of the human AUT7 paralogs is an apoptosis-specific protein and another is localized to the Golgi [22].

Connections between the UBLs and ubiquitin

The ubiquitin family of protein modifiers is thought to derive from a single ancestral conjugation system. This raises the question of whether, after their multiplication and divergence during evolution, some of the UBL conjugation systems retain any functional overlap or cross-regulation. The modification of ubiquitin E3s by RUB is one obvious example of such cross-regulation. Functional isolation of UBL ligation pathways might have involved relatively small changes in the UBLs and their co-evolved ligation enzymes. For example, RUB is only poorly activated by the ubiquitin E1, but a single change in RUB – at residue 72 – to the amino acid found in ubiquitin, makes RUB virtually indistinguishable in its ability to bind the ubiquitin E1 [46]. The mutant RUB is readily transferred to at least some ubiquitin E2s, and can bind the proteasome when in mixed chains with ubiquitin [46]. It is possible, therefore, that some factors can bind both RUB and ubiquitin conjugates.

Recently, a mammalian RUB1-binding protein named NUB1 has been found that might provide a link between RUB conjugates and the proteasome [47]. NUB1 contains an N-terminal ubiquitin-like domain (UbD) which allows it to bind to the proteasome regulatory cap. Overexpression of NUB1 leads to decreased levels of RUB and its conjugates. Proteasome inhibitors prevent these decreases, suggesting that the proteasome might recognize and degrade certain RUB-linked substrates with the aid of an ‘adaptor’ such as NUB1. Interestingly, a similar adaptor mechanism might operate with some ubiquitinated proteins [48].

Another type of cross-regulation between UBLs, for which considerable evidence is now accruing, is the modification of the same protein by more than one UBL, sometimes at the same residue. For instance, both SUMO and ubiquitin can modify the same residues of I κ B α , MEK1 and PCNA. Sumoylation can protect a protein from degradation by preventing ubiquitin ligation [2] or it can prevent some other effect of ubiquitin ligation, such as the stimulation of DNA repair in the case of PCNA [16]. It is possible that the ISG15 protein can also protect certain proteins from proteasomal degradation by an analogous mechanism. Proteasome inhibitors dramatically increase the levels of ISG15 conjugates following interferon stimulation. In the favored model, ISG15 conjugation was proposed to compete with poly-ubiquitination and, therefore, proteasomal degradation of the (unidentified) substrates [49]. Slowing proteasome activity with inhibitors would enable more of these substrates to shift into relatively long-lived ISG15-conjugated forms, accounting for the large increase in their levels.

Not surprisingly, there are other post-translational modifications that can also directly modulate ubiquitin or UBL ligation. For instance, the balance between acetylation and ubiquitination of the same pair of lysine residues in the Smad7 signal-transduction protein determines the intracellular levels of this inhibitory Smad protein [50]. We can anticipate a rich and intricate regulatory interplay among different UBL modifications and also between UBL conjugation and chemically distinct modifications of the same proteins.

Concluding remarks

Modification of proteins with UBLs has emerged as a widely exploited mechanism for regulating diverse biological processes. Although some of the biological functions of these modifications are starting to be deciphered, in

most cases we have yet to learn exactly how the UBL modification elicits a particular change in protein activity. Attachment of a UBL could alter substrate conformation, or the UBL-protein conjugate might have a different affinity for a ligand than does either component alone. These models are consistent with the findings that modification by at least some UBLs can change substrate-protein localization, presumably by enabling interaction with different targets. The identification of new substrates for each UBL will certainly provide additional clues to the function of the modifier, but many more biochemical, genetic and structural studies will be required before we obtain a clear picture of the precise structural and functional consequences of protein ligation for each of the UBLs.

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