Protein regulation: **Tag wrestling with relatives of ubiquitin**

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Ubiquitin modification is a well established way of regulating protein levels and activities. Modification by related ubiquitin-like proteins is turning out to have a diverse range of interesting cellular functions.

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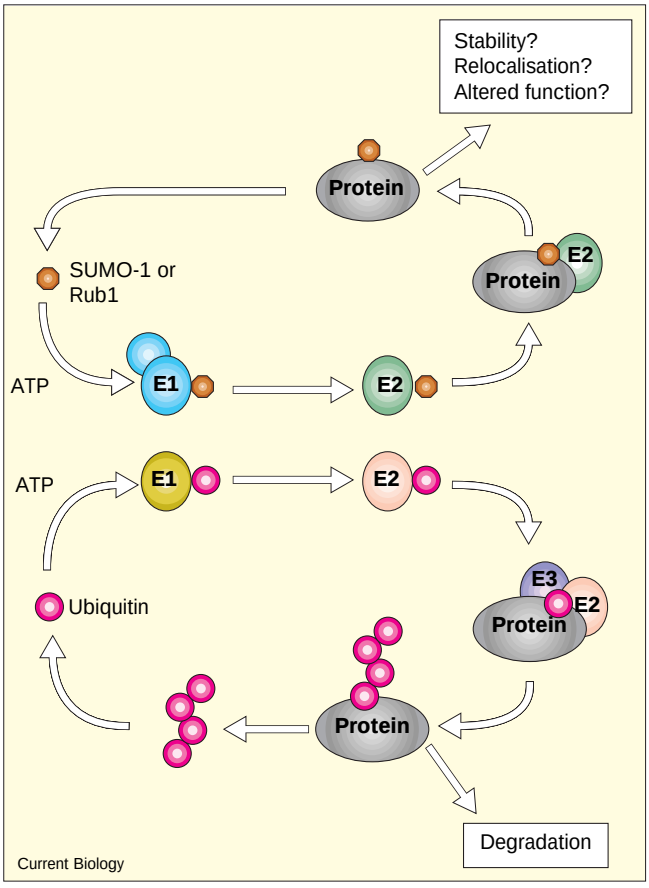
A cell must control the concentrations of its proteins to survive. The rates of synthesis and degradation of functionally critical proteins need to be tightly regulated so that the right concentrations of active molecules are present at the right times. The cell also needs a way of removing specific proteins; for example, cell-cycle progression requires the degradation of cyclin-dependent kinase inhibitors. This process must be carried out efficiently and quickly, and be highly specific. One way of achieving such regulation uses a remarkable molecule called ubiquitin and is known as the ubiquitin-dependant protein degradation pathway. Recent studies have identified a family of proteins related to ubiquitin, which are also turning out to have intriguing roles in cellular regulation — though not by targeted proteolysis.

Ubiquitin is an abundant and highly conserved 76 residue protein, that can exist in cells either as a free monomer or covalently attached to other protein molecules [1]. This covalent modification is required for a variety of processes, including endocytosis, protein targeting to the endosome and the activation of IκBα protein kinase, but its major functional role is in targeting proteins for degradation by the 26S proteasome. Ubiquitin is attached to its target protein via a multi-enzyme ATP-dependent pathway, which results in formation of an isopeptide bond between the ε-amino group of the substrate lysine residue and the carboxyl terminus of ubiquitin (Figure 1). The modification reaction is dependent on ubiquitin's conserved carboxy-terminal sequence arginine–glycine–glycine.

The first step in the pathway is the ATP-dependent formation of a thioester bond between the carboxy-terminal glycine of ubiquitin and the active site cysteine within the ubiquitin activating enzyme E1. Ubiquitin monomers are then transferred to one of several ubiquitin-conjugating (Ubc) E2 enzymes by transesterification. A third enzyme, E3, is sometimes required, and can be either a single protein or a multiprotein complex. E2 and E3 determine the substrate recognition and specificity of ubiquitination. Ubiquitin is then transferred to its target protein and the process repeated, producing a polyubiquitin chain, usually linked via lysine 48. As monoubiquitinated proteins are not degraded, it is likely that polyubiquitin chains act as recognition signals for the 26S proteasome (Figure 1); mutant ubiquitin molecules that cannot be further ubiquitinated actually inhibit proteolysis [1]. The polyubiquitin chains are cleaved from the tagged proteins and disassembled after docking at the proteasome, but before degradation of the target protein.

Figure 1



Pathways for modification by ubiquitin and ubiquitin-like proteins. Target protein modification by ubiquitin requires enzymes E1, E2 and sometimes E3, and results in formation of an isopeptide bond between ubiquitin and the target protein. Subsequent ubiquitin molecules can be added on to ubiquitin itself; the resulting polyubiquitinated protein is then degraded by the 26S proteasome, and the ubiquitin recycled for further modification cycles. The SUMO-1 and Rub1 modification pathways are similar to the ubiquitin pathway, but require their own enzymes and only single molecules appear to be attached to target proteins. SUMO-1 and Rub1 modification is not directly linked to proteolysis, but appears to alter the stability and/or localisation of the target protein (see text for details).

There have been a number of recent reports of proteins similar in sequence to ubiquitin, termed ubiquitin-like proteins, that can also form covalent conjugates. These ubiquitin-like modifications have many mechanistic similarities with the ubiquitin pathway, but surprisingly they do not appear to be linked to proteolysis. In particular, the two ubiquitin-like proteins SUMO-1 and NEDD8, and their respective counterparts in the budding yeast *Saccharomyces cerevisiae*, Smt3 and Rub1, have recently attracted considerable attention and appear to have interesting roles in cellular regulation.

SUMO-1 — for 'small ubiquitin-like modifier' — is a small protein that shows only limited sequence similarity to ubiquitin, but does have the conserved carboxy-terminal sequence glycine–glycine. As SUMO-1 was discovered independently by several groups, it has a plethora of alternative names, including PIC1, UBL1, GMP1, Sentrin and SMT3C [2]. SUMO-1 can covalently modify a large number of proteins, of which four have been identified so far; these target proteins, RanGAP1, PML, Sp100 and IκBα [3–7], are considered in more detail below. Like ubiquitin, SUMO-1 is synthesised as a precursor — SUMO-1 has a four residue carboxy-terminal tail that is cleaved off to generate the activate form of the protein [8]. Conjugation of SUMO-1 to target proteins is also ATP-dependent and requires E1 and E2 enzymes; an equivalent of ubiquitin's lysine 48 is lacking, however, so poly-SUMO-1 chains probably cannot be formed.

Does SUMO-1 use the same conjugation enzymes as ubiquitin? The sequence divergence of SUMO-1 from ubiquitin would suggest not, and this is indeed the case. The E1 enzyme for the yeast homologue Smt3, and presumably also for mammalian SUMO-1, is a heterodimer [9]. One subunit, Uba2, is similar in sequence to a carboxy-terminal part of the ubiquitin E1, and contains the active cysteine which forms the thioester with Smt3. The other subunit, Aos1, is similar to the remaining amino-terminal part of ubiquitin E1. An E2 enzyme for SUMO-1/Smt3, Ubc9, is essential for cell-cycle progression and was initially thought to conjugate ubiquitin. Ubc9 turns out to be specific for SUMO-1/Smt3, and uses the E2 conserved active-site residue, cysteine 93, for thioester formation [10–12]. Importantly, conjugation of SUMO-1 to RanGAP1 has an absolute requirement for Ubc9 [12]. Ubc9 also interacts with both IκBα and PML in yeast, and seems likely to be the conjugating enzyme for all SUMO-1/Smt3 modifications.

The first identified SUMO-1 target was the Ran GTPase-activating protein RanGAP1, which appears to be the major SUMO-1 substrate in mammalian cells [3,4]. Ran is a small GTPase of the Ras superfamily, which plays an essential part in nucleocytoplasmic transport. GTP hydrolysis by Ran sustains active nuclear import and export. RanGAP1, which activates the GTPase activity of Ran, is localised predominantly at the nuclear envelope, where it forms a stable complex with the nuclear-pore-complex protein RanBP2/Nup358 [13]. This interaction requires covalent addition of SUMO-1 to RanGAP1 at lysine 526 [8,13]. This modification alone is not sufficient for nuclear-pore-complex binding, however, as a SUMO-1-modified fusion protein containing the carboxyl terminus of RanGAP1 localises to the nucleus, and not to the nuclear pore complex. Covalent attachment of SUMO-1 to RanGAP1 might expose or create a new site for Nup358 binding [13].

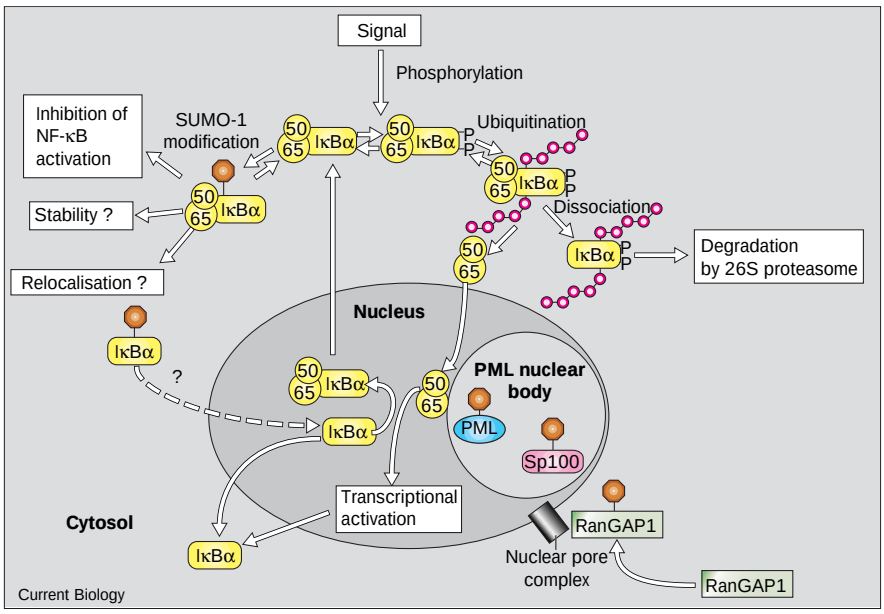
An intriguing new paper by Desterro *et al.* [14] reports that SUMO-1 can also modify IκBα, the inhibitor of NF-κB. NF-κB is a transcription factor that plays a key role in regulating the inducible expression of a number of genes, particularly those involved in immune and inflammatory responses [15]. The transcriptional activity of NF-κB is primarily controlled by a signalling pathway which affects the intracellular localisation of NF-κB (Figure 2). Inactive NF-κB exists in a cytoplasmic complex with its inhibitor IκBα. Upon stimulation by various factors, such as cytokines or stress, IκBα is phosphorylated and then degraded by the ubiquitin-dependent pathway (Figure 2). This allows NF-κB to translocate to the nucleus, bind to specific DNA target sequences and activate transcription. Interestingly, as part of an autoregulatory loop, NF-κB also activates IκBα transcription and newly made IκBα transiently accumulates in the nucleus, where it mediates the transport of the inactive NF-κB–IκBα complex back to the cytoplasm (Figure 2).

Desterro *et al.* [14] found that the non-phosphorylated, but not the phosphorylated, form of IκBα can be modified by SUMO-1, and that the modified form is resistant to signal-induced degradation. The most extraordinary feature of the SUMO-1 modification of IκBα is that the same lysine residue is conjugated by either SUMO-1 or ubiquitin (Figure 2). SUMO-1-modified IκBα cannot be ubiquitinated and would therefore be resistant to proteasomal degradation. In support of this idea, Desterro *et al.* [14] demonstrated that SUMO-1 expression inhibits NF-κB-dependent transcription and that the modification of IκBα by SUMO-1 is inhibited by phosphorylation. This strongly points to SUMO-1 playing a major role in regulating the transcriptional activity of NF-κB, further illustrating the cellular importance of SUMO-1 modification.

The two other identified proteins that have been found to be subject to modification by SUMO-1, promyelocytic leukaemia protein (PML) and Sp100 [6,7,16], are both found in large nuclear multi-protein complexes known as 'PML nuclear bodies' [17]. Unlike RanGAP1 and IκBα, multiple conjugates of PML with SUMO-1 have been seen, probably resulting from the conjugation by SUMO-1 on several lysine residues [5]. The SUMO-1 modification

Figure 2

Activation of NF-κB and the role of SUMO-1 modification. Phosphorylation of the NF-κB inhibitor IκBα triggers its polyubiquitination and degradation, allowing NF-κB, a p50–p65 heterodimer, to translocate to the nucleus and activate transcription of specific genes, including that for its inhibitor IκBα. SUMO-1 modification of non-phosphorylated IκBα prevents ubiquitin attachment, thus inhibiting NF-κB activation. The other known SUMO-1 target proteins – RanGAP1, PML and Sp100 – are also shown.



appears to be important for the intranuclear compartmentalisation of PML and Sp100 — unmodified PML and Sp100 localise to the soluble nucleoplasm and cytoplasm, respectively, whereas SUMO-1-modified PML and Sp100 partition to the insoluble nuclear matrix PML nuclear bodies [5,6]. It is tempting to speculate that a dynamic equilibrium exists between these two states, although it remains to be determined which is biologically relevant, and what cellular signals regulate this partitioning.

What are the functions of SUMO-1 modification? A couple of main themes are emerging from these recent studies. One is that, like ubiquitin, SUMO-1 plays a major role in targeting protein molecules to particular subcellular compartments. This is true of RanGAP1, the modified form of which is targeted to nuclear pore complexes, and perhaps true of PML and Sp100, modified forms of which are partitioned to PML nuclear bodies. Another plausible role for SUMO-1 is in the regulation of ubiquitin-mediated proteolysis, as in the case of IκBα. The modification of the same lysine residue on a target protein by either SUMO-1 or ubiquitin means that SUMO-1 addition can block ubiquitin attachment and thus ubiquitin-dependent proteolysis. This competition may not occur with all substrates, but perhaps only to a small number of proteins that require an extra level of regulation. The identification of further SUMO-1 target proteins will greatly help our future understanding of SUMO-1 function.

Another recently identified ubiquitin-like protein is Rub1 — for 'related to ubiquitin 1' — which was initially identified through the yeast genome project [18] and is the functional orthologue of NEDD8, a developmentally regulated human protein [19]. Rub1 and NEDD8 are the closest relatives of ubiquitin identified so far. Both have the conserved carboxy-terminal arginine–glycine–glycine motif and the lysine residue that in ubiquitin provides the link for polyubiquitination. Both can also covalently modify a number of nuclear proteins, though there appear to be fewer target proteins for Rub1/NEDD8 than for ubiquitin and SUMO-1 [20–22]. E1 and E2 enzymes specific for Rub1 have been identified. The Rub1 E1 is a heterodimer of Enr2, also known as Ula1, and Uba3. Enr2 and Uba3 are similar to amino-terminal and carboxy-terminal parts, respectively of ubiquitin E1. The Rub1 E2 enzyme is Ubc12; it is not known whether an E3 enzyme is involved in Rub1 modification [22].

What are the targets for Rub1 modification? Two recent papers have reported that Rub1 covalently modifies Cdc53 [21,22], a protein required for G1–S cell-cycle progression in *S. cerevisiae*. Cdc53 is a component of a ubiquitin ligase complex — an E3 enzyme — that catalyses the addition of ubiquitin to a variety of target proteins using substrate-specific 'adaptors'. The adaptors are so-called F-box proteins; one such F-box adaptor is Cdc4, which directs specific degradation of the cyclin-dependent kinase inhibitor Sic1. The mammalian homologue of Rub1, NEDD8, has recently been shown to conjugate to cullin-4A, which is related to Cdc53 [23]. The involvement of Cdc53 in both the Rub1 and ubiquitin modification pathways raises the interesting question of whether there is interplay between the two systems. Rub1 might alter the substrate specificity of the ubiquitin ligase complex or affect the partitioning of Cdc53 to different complexes. Alternatively, Rub1 conjugation might simply

be required for optimal assembly of the ubiquitin ligase complex. It appears unlikely that Rub1 modification has an essential role in cell-cycle regulation, as *rub1*, *uba3*, *enr2* or *ubc12* deletions leave yeast surprisingly healthy.

There are clearly many parallels between the ubiquitin and ubiquitin-like conjugation systems, although ubiquitin-like modifiers appear to act on a more specialised set of substrates than ubiquitin itself. It is difficult at present to know whether there is a general function for all ubiquitin-like modifications. SUMO-1 and Rub1 both appear to act in a way that is connected with ubiquitin-dependent protein degradation, although modification by SUMO-1 and Rub1 *per se* does not alter the stability of their respective target proteins. Could ubiquitin-like modification act as general inhibitor of ubiquitin-dependent proteolysis? This cannot be answered yet from available data, but it is an attractive theory. Ubiquitin-like modification might provide an additional, dynamic way of regulating protein concentration in the cell. Such modifications might be reversible, for example by cleavage with specific hydrolases. The ubiquitin-specific hydrolase HAUSP, which localises to PML nuclear bodies but does not hydrolyse SUMO-1-modified conjugates, may promote SUMO-1 modification by cleavage of ubiquitin from ubiquitin–PML conjugates, allowing conjugation by SUMO-1 instead [24].

Do ubiquitin-like modifications regulate the cell cycle? It is known that the E2 enzyme Ubc9 is essential for progression from G2 to early M phase, but it is unable to form a thioester with ubiquitin and is specific for SUMO-1. The implication is that SUMO-1 modifications are essential for cell-cycle progession. The modification of Cdc53 by Rub1 also points towards a link to cell-cycle regulation, as Cdc53 is required for G1–S progression. The obvious speculation would be that these ubiquitin-like modifications stabilise cell-cycle control proteins, perhaps transiently, before they are actively degraded.

Another recurring theme is that the subcellular localisation of a target protein is often affected by ubiquitin-like modification. SUMO-1 appears to be involved in nuclear or perinuclear targeting, and another ubiquitin-like family member, UCRP — 'ubiquitin cross-reactive protein', also known as ISG15 — has been reported to target substrates to the cytoskeleton [25]. Ubiquitin-like modifications may not only inhibit ubiquitin-mediated degradation, but also relocalise target proteins. Whether there is a common function for all ubiquitin-like modifications is not clear. One thing that is clear, however, is that the ubiquitin-like modification field is advancing rapidly and it will not be long before we have answers to all of these and other interesting questions.

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