Ubiquitin and Ubiquitin-Like Proteins in Protein Regulation

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Abstract—The discovery of the ubiquitin system was awarded with the Nobel Prize in Chemistry in 2004. Labeling of intracellular proteins for degradation by a multienzymatic complex, called the proteasome, was identified as the main function of this system. Subsequently, it was discovered that the attachment of ubiquitin to proteins can modify their function without degradation. Finally, a number of other molecules were recognized to be conjugated to proteins in a manner similar to ubiquitin and were henceforth called ubiquitin-like proteins. This review provides an overview of this class of molecules and its implication for function, subcellular location, and half-life of proteins. (Circ Res. 2007;100:1276-1291.)

Key Words: cell cycle ■ inflammation ■ metabolism ■ protein ■ ubiquitin ■ ubiquitin-like proteins

Ubiquitin was discovered in the early 1970s and then recognized to be the molecule that labels intracellular proteins for degradation by a multienzymatic complex, which, in reference to its molecular weight, substrates, and distinction from the lysosome system, was called the 26S proteasome. Subsequently, it was noticed that variations in the attachment of ubiquitin could influence protein function without proteolysis. In addition, molecules other than ubiquitin were identified to confer similar modes of functional protein modification and were henceforth called ubiquitin-like proteins, molecules, or modifiers (ULMs) (Figure 1). Of note, the reversible attachment of these molecules to target proteins is accomplished within a general principle of enzymatic reactions (Figure 2). Remarkably, the number of genes that encode the proteins involved in the enzymatic reactions of ubiquitin and ULMs is similar to the number of genes involved in phosphorylation and dephosphorylation. Hence, the discovery of ubiquitin ultimately led to the identification of another basic type of protein modification and functional regulation.

The Ubiquitin System

It was in 1974 when a 8.5-kDa polypeptide was reported to be successfully purified from bovine thymus, capable of inducing differentiation of T and B cells and expressed in essentially every cell, hence rendering the name “ubiquitous immunopoietic polypeptide.”1 Subsequent studies, however, pointed out that the lymphocyte-differentiating activity was attributable to a contaminating endotoxin rather than the newly identified polypeptide. Instead, the peptide was eventually recognized as an essential component of the ATP-dependent proteolytic system.1 Even though its expression was also not confirmed to be as ubiquitous in the kingdoms of life, “ubiquitin” prevailed as the name for this polypeptide for historic reasons. The related proteolytic system became henceforth known as the "ubiquitin–proteasome system” (UPS).1

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Degradation of proteins via the UPS involves 2 distinct, sequential steps.1 In the first step, the ubiquitin system mediates the modification of proteins via a set of reactions that activate, transfer, and bind ubiquitin to cellular proteins, catalyzed by E1, E2, and E3 enzymes, respectively. Polyubiquitin chains can be stabilized and extended further by the action of E4s.2 E1s use ATP to adenylate ubiquitin at its C terminus, forming a high-energy, mixed anhydride bond that is quickly attacked by the sulfhydryl group of the E1 active-site cysteine. This leads to the formation of a high-energy thioester bond between E1 and ubiquitin and the release of AMP. Subsequently, E1 adenylates a second molecule of ubiquitin, which primes for “uninterrupted” activation and promotes transfer of the activated ubiquitin to E2. In thioester-binding with ubiquitin, E1 readily binds free, uncharged E2 and transfers ubiquitin to the catalytic site of E2, followed by its release. Likewise, free E3 readily binds E2 when bound to ubiquitin but has a much lower affinity for free E2. These differences in relative affinity help drive the transfer of ubiquitin along the enzymatic cascade.3 To allow for efficiency as well as specificity, the structure of the ubiquitin system is hierarchic in quantitative terms, ie, the number of

Figure 1. Polypeptide folds and surface electrostatic potentials of ubiquitin, ISG15, NEDD8, and SUMO-2, highlighting the similarities and unique differences.67,162,163 The images have been used with permission from the American Society for Biochemistry and Molecular Biology67,162 and the Federation of European Biochemical Societies.163
molecules is increasing from 1 ubiquitin molecule and 2 isoforms of 1 E1 to several E2s and an ever-expanding list of E3s. E3s bind to target proteins on the basis of certain recognition patterns, with the most important being a “destabilizing” (free basic, bulky hydrophobic, or uncharged) N-terminal amino acid (so-called N-end-rule). This destabilizing residue can be a standard characteristic of a protein or can be generated via 1 or 2 intermediate steps. Other important substrate recognition patterns include phosphorylation and hydroxylation; on the contrary, acetylation inhibits ubiquitination.

Based on the interaction type with the target protein, 2 major types of ubiquitin E3 enzymes are to be distinguished.4 RING (really interesting new gene) domain-type E3s and structurally related U-box enzymes constitute the first group. They bind the “ubiquitin-loaded” E2 and the protein substrate simultaneously, even position the reactive ubiquitin-E2 thioester bond in close proximity to the nucleophilic lysine residue of the substrate. Furthermore, they trigger subtle conformational changes that facilitate the transfer of ubiquitin. On the contrary, the second group of E3s, called HECT (homologous to E6-associated protein C terminus) domain-type E3s transfer ubiquitin from the E2 to an active-site cysteine in the HECT domain, and the thioester-linked ubiquitin is conjugated to the protein substrate in a second step. The “ligation step” catalyzed by E3s is the formation of a covalent isopeptide bond between a lysine residue of ubiquitin and the ε-amino group of a lysine residue of the substrate or a previously conjugated ubiquitin moiety. On occasion, other residues such as a free ε-amino group of an N-terminal residue or a cysteine side chain can serve as the primary ligation site for the target protein.5 Furthermore, proteomic studies in yeast indicate that all of the 7 lysine residues in ubiquitin can be used for isopeptide bond formation with different and, in some cases, still-to-be-defined functional implications6 (Figure 3). For instance, involvement of ubiquitin lysine residue 48 in the attachment of a polyubiquitin chain to a target protein, ie, K48 polyubiquitination, leads to proteasomal degradation. On the contrary, involvement of lysine residue 63 of ubiquitin, ie, K63 polyubiquitination, leads to modification of protein function but not degradation. The same holds true for the addition of 1 ubiquitin molecule to 1 or several lysine residues in the target protein, ie, monoubiquitination and multiubiquitination.7

Figure 2. Illustration of the basic set of reactions in protein modification by ubiquitin and a ULM. Processing of precursor molecules is necessary for the exposure of the conjugation site in most cases. Following its activation by the action of an E1 enzyme, the mature molecule is transferred to an E2 enzyme, which catalyzes the conjugation to the target protein. The latter action may require another “ligating” E3 enzyme. This conjugation process is balanced by deconjugation, which is mediated by a number of different enzymes. The specifics and the cellular localizations vary for the different ubiquitin and ULM systems.
These processes can also direct toward the endosome-lyso-
some pathway. Finally, K29 polyubiquitination seems to labe-
l proteins for lysosomal degradation as well.

The expression of components of the ubiquitin system can be
modulated by hormonal factors such as glucocorticoids.
Furthermore, the activity of E1 and E2 enzymes can be in-
creased and subcellularly directed by phosphorylation. In
addition, E3s can be positively or negatively regulated in
their activity by phosphorylation. Finally, modifications of
the function of ubiquitinating enzymes have been re-
ported in the setting of increased oxidative stress. In
summary, the ubiquitin system is a protein modification
system that operates in different subcellular compartments in
a continuous yet modifiable manner.

The Proteasome System
The 26S proteasome is the proteolytic machinery associated
with the ubiquitin and ULM systems (Figure 4). It is
composed of 19S subunits to either side of the 20S subunit,
which actually yields a sedimentation value of 30 rather
than 26. The 19S complex harbors ATP activity at its base,
allowing unfolding of the protein substrate into the central
tunnel of the 20S proteasome, which is barrel-shaped by 4
stacked rings, 2 outer α and 2 inner β rings, each formed by
7 subunits. The α rings serve as “gatekeepers” for the
“proteolytic core,” which is composed of the β rings. The
subunits of these 2 rings are arranged counter to each other,
allowing spaced distribution of chymotrypsin-like, trypsin-
like, and peptidyl-hydrolase (caspase)-like activities, which
reside with the βi, βs, and β2 subunits, respectively. The 20S
proteasome complex is self-sufficient and degrades misfolded
and oxidized proteins without the requirement of ubiquitina-
tion. On the contrary, proper interaction of some proteins
with the ubiquitin system or the proteasome complex neces-
sitates the facilitating action of chaperones or chaperone-like
molecules. One particular route of delivery of ubiquitinated
proteins to the proteasome has been coined the “escort
pathway” (Figure 4). In addition, the 26S proteasome can
recognize polyubiquitinated proteins directly.

Of note, the 19S activator complex of the 26S proteasome
can be replaced by the 11S regulatory complex in response to
interferon (IFN-γ). Furthermore, IFN-γ can modify the
expression and composition of the proteasome, namely the
replacement of the 3 proteolytic subunits by so-called immu-
noforms, designated βN, βs, and β2. This confers addi-
tional proteolytic activities to the proteasome and favors the
formation of major histocompatibility class I antigens ("immunoproteasome"). These modifications of the proteasome were classically viewed as a transient response that lasts only as long as the stimulus persists. Very recently, however, immunoforms were found to be incorporated in nonimmuno-proteasomes isolated from murine hearts. These findings support the theory of heterogeneous 20S configurations as a more general mode of differential regulation of proteasome function.

In addition, proteasome proteolytic activity can be modified by various posttranslational modifications. At least for the murine cardiac 20S proteasome, protein phosphatase 2A and protein kinase A have been identified as associated partners, mediating the phosphorylation and dephosphorylation of various α and β subunits and thereby up- and downregulation of the 3 proteolytic activities, respectively. Reactive oxygen species, including 4-hydroxynonenal, hydrogen peroxide, and peroxynitrite, have been identified as negative modulators of proteasome function, just like phenolic antioxidants. Also, there are reports on alternate splicing isoforms of the Rpn10 subunit of the 19S complex, indicating additional modes of modulation of ubiquitin/ULM system–linked proteasomal activity. Finally, the 20S proteasome can be capped with either the 11S or the 19S complex on 1 or both sides, or as a hybrid. Hence, the composition of the proteasome is a very dynamic process and subject to different factors that can contribute to species- and organ-specific differences.

Functional Significance of the Ubiquitin–Proteasome System

Degradation of misfolded, damaged, or otherwise malfunctioning proteins is certainly of utmost significance for cellular homeostasis and among the most important functions of the UPS. The endoplasmic reticulum–associated protein degradation (ERAD) system is just one intriguing example in this regard. Furthermore, with or without protein degradation, the ubiquitin system is involved in the regulation of a number of cell signaling pathways. For instance, the UPS takes center stage in the regulation of the stress response and the cellular response to infection, among other functions.
stage in the main activation pathway of nuclear factor kappa B (NF-κB), as detailed in Figure 5.

Another prominent example for proteolytic control of transcriptional activity is hypoxia-inducible factor (HIF), namely its HIF-1 isoform. This transcription factor is composed of 1 regulatory α subunit and 1 stabilizing β subunit, both of which are constitutively expressed. In an environment of high oxygen tension, 2 proline residues in the N-terminal transactivation domain of the α monomer (HIF-1α) are hydroxylated by members of the prolyl hydroxylase protein (PHD) family. This allows the recognition of HIF-1α by the von Hippel–Lindau protein (pVHL), a component of the VCB-Cul-2(pVHL)-elongin C-elongin B Cullin-2) multisubunit ubiquitin ligase complex that mediates polyubiquitination of HIF-1α and thereby its proteasomal degradation.26 Low oxygen tension is a rate-limiting factor for PHDs. Hence, under hypoxic conditions, HIF-1α and HIF-1 transcriptional activity is stabilized, leading to the expression of a number of angiogenesis-, metabolism-, and inflammation-related genes.

Finally, the transcription factor p53, the “guardian of the genome,” is recognized by the RING domain-type ubiquitin ligase Mdm2 in the absence of its phosphorylation.27 Low levels of Mdm2 mediate monoubiquitination and nuclear export of p53, whereas an Mdm2-to-p53 ratio of >3.5 leads to polyubiquitination of p53 and its degradation by the proteasome complex.28 As an aside, monoubiquitinated p53 can be converted to a polyubiquitinated form by action of a protein named p300.29 These modifications of p53 lead to a reduction of the expression of negative cell cycle regulators such as the cyclin-dependent kinase inhibitor p21^{WAF/Cip1} and the proapoptotic mediator Bax. In addition, the half-life of these molecules as well as other cell cycle regulators is directly controlled by the UPS, favoring cell cycle progression.30

DNA repair after replication is facilitated by the radiation gene 6 group of proteins with proliferating-cell nuclear
antigen (PCNA) as a prominent example of a sliding clamp. The activity of PCNA seems to revolve around the lysine residue in position 164 of its amino acid sequence. Mono-ubiquitination of this residue, involving the E2 Rad6 and the E3 Rad18, facilitates the association of PCNA with translation polymerases.\(^{31,32}\) Likewise, K63 polyubiquitination of PCNA contributes to error-free genomic repair and stability.\(^{33}\) Finally, posttranslational modification of histones by ubiquitination points to the involvement of the ubiquitin system in gene transcription, repair, and replication.\(^{34}\)

Internalization and endosomal sorting of plasma-membrane growth factor receptors are additional processes in which an important regulatory role has been attributed to the ubiquitin system. Mono- and multiubiquitination seem to provide recognition signals for endocytotic trafficking of growth factor receptors from the plasma membrane to the lysosome.\(^{35}\)

Taken together, the UPS is involved in a number of central elements of cellular signaling, from the membrane receptor to the transcription factor levels and the related plethora of biological processes, including especially inflammation, proliferation, and DNA repair.

**Ubiquitin Domain Proteins and Ubiquitin-Like Modifiers**

Over time, a number of ubiquitin-like molecules have been identified, which can be divided into 2 separate classes: ubiquitin-domain proteins (UDPs) and ULMs.\(^{3,36,37}\)

UDPs bear a sequence domain that is similar to ubiquitin, but they are not conjugated to proteins. Instead, they fulfill adaptor function, binding noncovalently to ubiquitin or ULMs and its associates via an “ubiquitin-interaction motif” or ubiquitin-associated (UBA) domain. The Rpn10 subunit of the 19S proteasome was the first UDP to be identified, allowing direct recognition of polyubiquitinated proteins by the 26S proteasome. Other UDps are involved in escorting a subset of polyubiquitinated proteins to the 26S proteasome. They fulfill this role by functioning as cofactors or adaptors for the ubiquitin-selective AAA ATPase p97/Cdc48, namely Ubx2 and Ubx3. Alternatively, they function as adaptors to the Rpn1 subunit of the 19S proteasome, namely Rad23, Dsk2, and Ddi1. These latter molecules have an additional ubiquitin-like domain (UBL or UBX).\(^{38,39}\)

**Ubiquitin-Like Protein Modifiers**

The family heritage of ubiquitin-like protein modifiers (ULMs) is not so much by sequence homology but rather by a common 3D structure, the ubiquitin fold, and a C-terminal glycine residue, whose carboxyl group is the site of attachment to the lysine residue of substrates via isopeptide bond formation. Hence, they are conjugated to proteins and function in “ubiquitin-like” manner. The attachment of ULMs creates a protein surface topography in the conjugate that is changed substantially from that of the unmodified protein, at least locally. This may facilitate or inhibit the binding of the protein to another molecule, affect enzymatic activity or subcellular localization, and ultimately determine the half-life of the protein. At least 10 different ULMs exist in mammals (Table), presented in the following paragraphs in historic order.

**Interferon-Stimulated Gene-15**

In 1979, a 15-kDa protein was discovered, the expression of which could be induced by type I interferons (ie, IFN-α and IFN-β), rendering the name interferon-stimulated gene-15 (ISG15).\(^{40,41}\) Almost accidentally, ISG15 was found to be recognized by a rabbit polyclonal antibody against ubiquitin, leading to the name “ubiquitin cross-reactive protein” (URCP).\(^{42}\) The primary sequence of ISG15 consists of 2 domains, each with significant homology to ubiquitin (dimeric ubiquitin).\(^{43}\) As a conjugation trait, ISG15 possesses a C-terminal diglycine motif that is generated by C-terminal processing of its precursor.\(^{43}\) The conjugation of ISG15 to its protein substrates is catalyzed by an enzymatic cascade similar to ubiquitin. All components of this system are upregulated within 2 hours of stimulation with type I IFNs.\(^{44}\) Nevertheless, ISG15 protein conjugates are formed with a delay of 12 to 24 hours, likely reflecting the need for secondary induction of components of the ISG15 system.\(^{45}\)

Lipopolysaccharide (LPS) and double-stranded RNA are additional stimulators of the ISG15 system and further characterize this system furthermore as “the immunological kin of ubiquitin.”\(^{43}\) Of interest, both UBE1L, the E1 for ISG15, and Ubal1, the E1 for ubiquitin, can use the same E2: UBCH8.\(^{46}\) Very recently, 1 RING-type E3 (estrogen-responsive finger protein) and 1 HECT-type E3 (Herc5) were identified for ISG15, which are also able to function with ubiquitin. Thus, the ISG15 system operates in a mode similar to and even overlapping with the ubiquitin system.\(^{47,48}\)

ISG15 can be secreted by both immune and nonimmune cells and stimulates IFN-γ production from CD3\(^+\) T cells, which, in synergy with other cytokines, results in natural killer cell proliferation and cytotoxicity.\(^{39}\) In line with the functional implications for inflammation, the first molecular target of ISG15 to be identified was serine protease inhibitor 2a, which regulates intracellular proteases in antigen-pres-entng cells.\(^{50}\) Subsequent high-throughput immunoblotting identified key regulators of signal transduction as additional protein substrates for ISG15.\(^{51}\) By mass spectroscopy, 76 proteins were identified as potential targets for the ISG15 system, involved in translation, glycolysis, stress response, and cell motility. Even though ISG15 does not target these substrates for degradation, there has been increasing recognition of an interaction with the proteasome system. For instance, proteasome inhibition increases the level of ISG15 conjugates and modulates the level of the deconjugating enzyme UBP43.\(^{52,53}\) In addition, overexpression of ISG15 is associated with decreased protein polyubiquitination and UBP43 turnover in tumor cells, indicating that ISG15 (and its conjugates) possesses the capability to antagonize the activity of the UPS.\(^{54}\)

The proteasomal target UBP43 was ultimately discovered as a negative regulator of type I IFN signaling, related to its binding to 1 particular interferon receptor subunit.\(^{55}\) UBP43 was originally cloned from mice expressing the leukemia fusion protein AML1-ETO with functional implications for hematopoietic cell differentiation.\(^{56}\) The increase in ISG15
within decidualized endometrial stroma cells and the endometrial synthesis and secretion of ISG15 in response to trophoblast IFN-γ during early pregnancy likewise imply a role different from immunoregulation. Hence, ISG15 is a ULM that has been linked primarily to immune-defense mechanisms, but its functional significance may extend eventually to cell growth and differentiation.

**Fau Ubiquitin-Like Protein**

In 1992, it was reported for the first time that a retrovirus can use an antisense sequence to a cellular gene. The retrovirus was the Finkel–Biskis–Reilly murine sarcoma virus (FBR-MuSV) and the related cellular gene sequence was named FBR-MuSV–associated ubiquitously expressed gene (fau). The corresponding DNA sequence was found to encode for a single 8-kDa ubiquitin-like protein, fused to the small ribosomal subunit protein S30, which was then called Fau ubiquitin-like protein (Fubi, FUB1). Later, it was discovered that the cDNA to monoclonal nonspecific suppressor factor (MNSF) encodes for an almost identical 14.5-kDa fusion product. Even more, FUB1 is cleaved from S30 in the cytoplasm and associates with MNSFα to form a 70-kDa heterodimeric complex that can be released extracellulary to bind to interleukin 11–like receptors. As the expression of MNSF and its receptors is stimulated by IFN-γ, MNSF secretion has been thought to be limited to activated immunoregulatory cells. MNSF inhibits LPS-induced activation of extracellular signal-regulated kinase-1 and tumor necrosis factor production in macrophages, immunoglobulin secretion from lymphocytes, and proliferation of mitogen-activated T and B cells. Hence, a primarily negative role in the regulation of immune and inflammatory processes was ascribed to this ULM. Recently, intracellular linkage of FUB1 to the B-cell lymphoma G protein was found in mitogen-activated murine T cells, implying a proapoptotic role. This is in line with the inhibitory effect on the growth of various tumor cell lines of murine origin. Finally, hNSF (human nonspecific suppressor factor) was identified as the putative human counterpart with CD8+ T cells as the major cellular source and inhibition of B- and T-cell proliferation and immunoglobin secretion from B cells as the prominent cellular effects. Thus, FUB1 has been identified as a ULM.
that exerts antiinflammatory and antiproliferative effects, mainly on leukocyte level.

Neural Precursor Cell-Expressed Developmentally Downregulated-8

In the early 1990s, a set of genes was discovered to be downregulated in neural precursor cells during the development of the murine brain. This included 1 gene that encoded for a 9-kDa protein, which displayed 80% of 81-aa residues and displayed 80% sequence homology with ubiquitin, including a lysine 48 residue, and was named neuronally expressed polyubiquitin class I (UBA3) domain. Remarkably, NEDD8 could be processed by the ubiquitin system and incorporated into polyubiquitin chains if it was not for a difference in 7 residues of their amino acid sequences.\(^{67,68}\) Furthermore, 1 single arginine residue in the heterodimeric E1-activating enzyme APPBP1-UBA3 determines specificity for NEDD8.\(^{69}\) The 3 domains in the 3D structure of APPBP1-UBA3 facilitate the processing of NEDD8 in a way that is similar to an assembly line. This includes the adenylation of the C terminus of NEDD8, the thioester formation with its catalytic site, and the transfer to the catalytic cysteine of the E2 enzyme UBC12, which remains the only E2 to match the binding groove.\(^{70-72}\) UBC12 catalyzes the formation of isopeptide bonds between NEDD8 and lysine residues in target proteins. Furthermore, Roc-1 (regulator of cullins-1) has been suggested as an E3 in the neddylation process just like Mdm2.\(^{73}\) Reversal of this reaction is catalyzed by DEN1/NEDP1, which also processes the precursor molecule to NEDD8 generating a C-terminal conjugation motif. A similar function has been identified for UCH-L1 and UCH-L3, which also operate as deubiquitinating enzymes.\(^{74}\) Again, it is fascinating that a single-residue difference in the C terminus of NEDD8 and ubiquitin allows for the specificity of DEN1/NEDP1.\(^{75}\) Other deubiquitinating enzymes in the family include USP21 and the COP9 signalosome.\(^{76,77}\)

NEDD8-conjugated proteins appear to reside predominantly in the nucleus, and until recently it was thought that members of the cullin family of proteins were the only substrate class for the neddylation system (with the exception of the anaphase-promoting complex 2).\(^{78}\) Cullins function as scaffolding subunits of ubiquitin–protein ligases such as SCF (SKP1-CUL1-F-box) and interact with RING finger proteins (Roc-1 for SCF) to enable the recruitment of E2s. In this particular interaction, the conjugation of NEDD8 to a specific lysine residue in cullin-1 (CUL1) by Roc-1 prevents the association of CUL1 with the inhibitory protein CAND1 (cullin-associated and neddylation-dissociated-1), hence sustaining SCF activity for ubiquitin-mediated proteolysis.\(^{79}\) On the other hand, the COP9 signalosome de neddylates CUL1, which allows CAND1 to bind and to displace the SKP1–F-box heterodimer, thereby turning off SCF activity.\(^{80}\) The VCE ligase complex and p53 were identified as additional substrates for the NEDD8 system. Impaired neddylation of CUL2 impairs assembly of the VCB-Cul-2 complex and thereby efficient HIF-1α ubiquitination and degradation.\(^{81}\) Mdm2 is the E3 that mediates the conjugation of NEDD8 to p53, resulting in an inhibition of transcriptional activity.\(^{82}\)

Conjugation of NEDD8 to proteins can also lead to their degradation via the proteasome. This is mediated by the adaptor proteins NEDD8 ultimate buster-1 (NUB1) and NUB1 ligand (NUB1L).\(^{83,84}\) Contrary to other UDPs, NUB1 interacts with the 19S subunit Rpn10/S5a through the C terminus and not through the UBL domain, which is nevertheless required for NUB1 function.\(^{85}\)

NEDD8 is expressed in proliferating cells and downregulated during cellular differentiation.\(^{86}\) An essential role for NEDD8 in cell cycle control and embryogenesis has become evident in gene knockout studies.\(^{87}\) This role relates to the contribution of NEDD8 to the degradation of p27\(^{kip1}\), p57\(^{kip2}\), and cyclin E, which facilitates the transition of cells from the G\(_1\) to the S phase of the cell cycle.\(^{87}\) Moreover, NEDD8 targets the microtubule-serving complex katanin for degradation during the transition form meiosis to mitosis, which allows the assembly of a large mitotic spindle.\(^{88}\) In addition to cell cycle regulation, it was very recently shown that the ubiquitin ligase c-Cbl also mediates the conjugation of NEDD8 to membrane growth factor receptors, enhancing their ubiquitination and metabolism.\(^{89}\) In human diseases, accumulation of NEDD8 has been observed in a number of different neurodegenerative disorders, namely in neurofibrillary tangles in Alzheimer’s disease, in Lewy bodies in Parkinson’s disease, and in neuronal and glial inclusions in multiple system atrophy.\(^{90}\) Moreover, NUB1 suppresses the formation of Lewy-body-like inclusions by proteasomal degradation of synphilin-1.\(^{91}\)

In summary, NEDD8 primarily functions as a regulator of ubiquitin–protein ligases and secondly as a decoy for proteins to undergo proteasomal degradation. Cell cycle progression is the primary biological process NEDD8 has been associated with but the knowledge of this ULM system is extending as will be the knowledge of its biological significance.

F-Adjacent Transcript-10

Genetic studies on the human major histocompatibility complex class I region led to the discovery of so-called human leukocyte antigen–F-adjacent transcripts (FATs), among them being FAT10, as reported in 1995.\(^{92}\) This 18-kDa protein is composed of 2 tandem head-to-tail UBL domains and possesses a free C-terminal diglycine motif. Interestingly, FAT10 can interact directly with the 26S proteasome via either one of its UBL domains and hence provides a ubiquitin-independent road for protein degradation.\(^{93,94}\) In further distinction from ubiquitin, the absence of FAT10-specific deconjugating enzymes makes FAT10 conjugation seemingly irreversible. Still, the detection of FAT10 conjugates is difficult, likely because of interaction with the proteasome. In this context, proteasomal degradation of FAT10 and its conjugates is facilitated by NUB1L by a factor of 8 (Figure 4).\(^{95}\)

FAT10 is expressed constitutively in lymphoblastoid cell lines and dendritic cells and can be stimulated in others. FAT10 expression undergoes cell cycle–specific changes,
with the highest and lowest expression during the S phase and G2/M phase, respectively.\textsuperscript{96} Proapoptotic factors such as p53 can downregulate the expression of FAT10.\textsuperscript{97} Interestingly, forced overexpression of FAT10 can lead to apoptosis of murine fibroblasts. Vice versa, lymphocytes of FAT10 knockout mice are more prone to spontaneous apoptotic death.\textsuperscript{98} Cytokines such as IFN-γ and tumor necrosis factor α can (synergistically) upregulate FAT10 expression. Also, FAT10 is among the most upregulated genes in HIV-infected cells and has been associated with HIV-induced cell death.\textsuperscript{44,99} Overexpression of FAT10 has also been noted in several epithelial cancers, including gastrointestinal and gynecological malignancies, in association with increased chromosome instability and mitotic nondisjunction.\textsuperscript{100} It has been suggested that FAT10 may modulate carcinogenesis through its viability and apoptosis.

**Small Ubiquitin-Related Modifier**

In 1996, the activating protein for the nuclear Ras-like GTPase Ran (RanGAP1), which is required for the bidirectional transport of proteins and ribonucleoproteins across the nuclear core complex, was found to be modified by reversible, covalent conjugation to a 11.5-kDa ubiquitin-like protein, rendering the name “GAP modifying protein 1” (GMP1) or “small ubiquitin-related modifier 1” (SUMO-1).\textsuperscript{103,104}

In lower eukaryotes, only 1 SUMO gene is expressed, whereas vertebrates express 3 to 4 paralogs.\textsuperscript{105} SUMO-1 has a uniquely long and highly flexible N terminus that protrudes from the core of the protein. Furthermore, SUMO-1 does not have a consensus sumoylation motif at the N-terminal region, which allows polychain formation for SUMO-2 and SUMO-3.\textsuperscript{106,107} SUMO-4 was recently added to the SUMO family and characteristically lacks posttranscriptional processing and the capability to form covalent interactions with substrates.\textsuperscript{108}

SUMO-1 is processed by limited proteolysis to expose its C-terminal glycine residues and is activated in an ATP-dependent manner by an enzyme composed of SAE1 and SAE2 (AOS1-UBA2 in yeast).\textsuperscript{37,105,109} This heterodimer catalyzes the formation of adenylated SUMO in which the C-terminal carboxyl group of SUMO is covalently linked to AMP. Breakage of the SUMO-AMP linkage is followed by formation of a covalent intermediate in which the C-terminal carboxyl group of SUMO forms a thioester bond with the sulfhydryl group of a specific cysteine residue of SAE2. The activated SUMO-1 is then transesterified from SAE2 to a cysteine residue in the active-site of the SUMO conjugating enzyme Ube9. As a distinguishing feature, Ube9 can recognize the substrate and directly transfer the activated SUMO-1 by the formation of an isopeptide bond between the C-terminal carboxyl group of SUMO and the ε-amino group of lysine in the substrate protein, provided that the lysine is part of the SUMO conjugation motif. The primary site of SUMO-1 modification seems to be the hydrophobic residue/lysine/any residue/glutamine motif. This sequence can bind directly to Ube9 and may serve as a signature sequence that identifies SUMO substrates to the conjugation machinery without an E3. However, not all proteins with this motif undergo sumoylation, and sites not conforming to this motif can still be conjugated to SUMO.\textsuperscript{37,110} Phosphorylation-dependent sumoylation motifs exist, e.g., with heat-shock factors. Also, acetylation can be a preparation step for sumoylation, as in the case of histone H4. Subcellular localization and appropriate sequence presentation may likewise be of significance. Hence, the structural context seems to dictate sumoylation.

Three classes of SUMO-related E3-like enzymes exist: Ran BP2, polycomb-group protein-2, and the superfamily of the protein inhibitor of activated STAT (PIAS).\textsuperscript{37,105,109} Of interest, these 3 E3 classes have distinct subcellular localizations and mediate the modification of specific substrates. Desumoylation is catalyzed by cysteine proteases, termed ubiquitin-like-protein-specific protease 1 and -2 (Ulp1 and Ulp2) in yeast.\textsuperscript{37,105,109} Ulp1 also cleaves 4 amino acids from the C terminus of the primary translation product to generate the mature 97-aa form of SUMO-1. Ulp1 is essential for cell cycle progression, whereas Ulp2, although not essential, is required for normal chromosome stability and for recovery from cell cycle checkpoint arrest. Ulp1 colocalizes with nuclear pore proteins and Ulp2 localizes to the nucleoplasm. In humans, 7 sentrin/SUMO-specific proteases (SENP) have been described, and 4 in particular. SENP-1 and -2 are able to process all 3 sumoylating isoforms seemingly without distinction, whereas SENP-3 and SENP-5 display a preference for SUMO-2 and SUMO-3. Of interest, all of these proteases are located at distinct subcellular localizations, dictated by the N-terminal region. SENP-1 localizes to the nucleus; SENP-3 and SENP-5 to the nucleolus; and SENP-2 to the cytoplasm, nuclear pore, or nuclear body, depending on splicing.\textsuperscript{111–113} This matches with the following patterns of their substrates: SUMO-1 to the nuclear membrane, SUMO-2 to the nuclear bodies and nucleoli, and SUMO-3 to the cytoplasm and nucleoli.\textsuperscript{109} As for the conjugating enzyme, Ubc9 is predominantly nuclear but also appears to be associated with filaments of the nuclear pore complex projecting into both the nucleus and the cytoplasm.\textsuperscript{109} These distribution patterns suggest that sumoylation is involved in nucleocytoplasmic transport, and RanGAP1 and promyelocytic leukemia protein (PML) are prominent examples in this regard.\textsuperscript{109} Whereas the 70-kDa unmodified form of RanGAP1 is exclusively cytoplasmic, the 90-kDa SUMO-1–conjugated form of RanGAP1 associates with cytoplasmic fibers of the nuclear pore complex and the mitotic spindle apparatus during mitosis.\textsuperscript{103,104} As very recently delineated, it is necessary for PML to be sumoylated at 3 sites, and namely lysine 160 in interphase, to allow nuclear body formation and the concentration of other sumoylated proteins such as Sp100 via a network of covalent and noncovalent SUMO interactions.\textsuperscript{114} This nuclear body may then serve as a “molecular anchor” for transcription factors, the inventory of which is regulated by sumoylation. This would be in line with the function of these subnuclear structures in the regulation of transcription and DNA repair. In addition to SUMO-1, SUMO-3 seems to be important for this process.\textsuperscript{115}
To date, more than 100 proteins have been identified as substrates for the SUMO system. Proteomic studies revealed that SUMO-1 and SUMO-2/3 have distinct and overlapping sets of target proteins and that nearly one-third of the identified target proteins are putative transcriptional regulators, with the other two-thirds being composed of signaling molecules, nuclear envelope proteins, and cell membrane proteins. The heterodimeric transcription factor activator protein-1 can be modified by SUMO-1, -2, or -3 at 1 of 2 acceptor sites on c-Jun or 1 site on c-fos, leading to a reduction in transcription activation, most likely attributable to intranuclear compartmentalization. SUMO modification also seems to limit the extent of transcriptional synergy, as characteristically noted for steroid receptors, including the glucocorticoid, the mineralocorticoid, the progesterone, and the androgen receptor. As yet another mode, sumoylation of transcription factors can recruit corepressor molecules to promoters and influence the assembly of transcription factors on promoters. Furthermore, sumoylation can influence the recruitment of chromatin-modifying enzymes and induce changes in the chromatin structure, consistent with repression. Rather as an exception, sumoylation can increase transcriptional activity, as shown for p53. Under conditions of DNA damage, increase in p53 activity is furthermore facilitated by SUSP4, a newly identified SUMO-specific protease that competes for the binding site of Mdm2 and desumoylates Mdm2, which promotes Mdm2 self-ubiquitination and degradation. Of additional note, HIF-1 is yet another of the few transcription factors whose stability and activity is increased by SUMO-1.

In yeast, gene mutations affecting the E1 and E2 in the SUMO system lead to cell cycle arrest at the G1/M transition point. Modification of DNA topoisomerase II by SUMO-1 and SUMO-2/3 is required for centromere cohesion and proper chromosome segregation, respectively. Sister chromatid separation and exit from mitosis require the anaphase-promoting complex and its ubiquitin E3 ligase activity, which is stimulated by sumoylation. Some proteins can also undergo successive modification by the SUMO and the ubiquitin system (Figure 5). Finally, contrary to these synergistic actions, protein ubiquitination and sumoylation can lead to antagonistic consequences. Taken together, the SUMO system regulates nucleocytoplasmic translocalization, protein–protein interactions, protein–DNA binding activity, and/or protein stability and is involved in genome organization, repair, and transcription.

**Apg8 and Apg12**

Autophagy is a cellular degradation mechanism which involves the formation of a double-membraned vesicle around cytoplasmic sections. The resulting autophagosome then fuses with the lysosome, followed by hydrolysis of its contents. Studies on autophagy-defective (Apg) mutants in yeast led to the discovery of 2 ubiquitin-like systems, which were confirmed to exist in human cells in 1998. As for the first of these 2 systems, Apg12 is conjugated to Apg5 by the action of the E1- and the E2-like enzyme Apg7 and Apg10, respectively. Conjugation of Apg12 to Apg5 is constitutive and required for the elongation of the isolation membrane to form a complete spherical autophagosome. This process can be facilitated by Apg16-mediated cross-linking of 2 or more Apg12-Apg5 conjugates to form a large protein complex that localizes to autophagosome precursors. Apg12-Apg5 conjugates also positively affect the second ubiquitin-like Apg system. Unlike Apg12, Apg8 is expressed as a precursor molecule, whose C-terminal arginine residue is removed by the cysteine protease Apg4/Aut2 to yield a C-terminal glycine residue, which allows covalent conjugation to the amino group of phosphatidylethanolamine, a common membrane phospholipid. Apg7 again is the E1 involved in this system; in fact, it has remained the only example of an E1 that can activate 2 different sets of ULMs. Apg3 is the E2 catalyzing the conjugation of Apg8, and Apg4/Aut2 has been identified as the deconjugating enzyme. In concert, these reactions allow the covalent but transient binding of Apg8 to membranes despite its hydrophilic nature, and thereby the membrane dynamics so important for autophagosome formation. One and 4 mammalian homologs to Apg12 and Apg8, respectively, exist with similar and different cellular activities. In summary, the 15.1-kDa Apg12 and the 14.3-kDa Apg8 are involved in the membrane process of autophagy and, more specifically, in directing membrane docking and fusion at the lysosome or vacuole.

**Figure 6.** A, One lysine residue of a protein can undergo a variety of modifications, including the attachment of ubiquitin, SUMO, or NEDD8. (modified from 140) B, The transcription factor p53 can be modified in its activity by ubiquitin or the above-mentioned ULMs. Likewise, the activity of the ligase murine double minute clone 2 (Mdm 2, which catalyzes the conjugation of ubiquitin or NEDD8 to p53) can be altered negatively or positively by ubiquitination or sumoylation, respectively. Similarly, a number of other proteins can be conjugated to either ubiquitin or SUMO with antagonistic effects, including HIF-1α, IκBα, CREB (cAMP-response element-binding protein), PCNA, and Huntingtin.
Ubiquitin-Related Modifier-1
The specific search for yet another ULM in yeast was rewarded with the report of the discovery of the 11-kDa “ubiquitin-related modifier-1” (Urm1) in 2000. However, rather than with ubiquitin it shares 20% and 23% sequence homology with the Escherichia coli sulfur carrier proteins ThiS (involved in thiamin biosynthesis) and MoaD (molybdopterin synthase small subunit), respectively.133 Urm1 localizes to the cytoplasm and to punctuate spots within the cytoplasm.134 In Saccharomyces cerevisiae, supported by action of the E1-like enzyme Uba4, Urm1 can be conjugated via its C-terminal glycine residue to Ahp1 (alkyl hydroperoxide reductase-1). Ahp1 is the only identified target protein so far with implications for a potential role in the oxidative stress response.134,135 Most of all, the Urm1 system may be unique in the lack of a need for a conjugating or ligating enzyme. Indeed, Uba4 possesses a rhodanese-homology domain that may serve as a built-in E2. Still, most of the function and regulation of this system is to be discovered.136

Ubiquitin-Like Protein-5
UBL5 was discovered as an 8.5-kDa gene product in screening studies of human adult iris cDNA, as reported in 2001.137 It contains a ubiquitin superfold with an electrostatic surface that is different from ubiquitin.138 Uniquely, the C terminus contains a dityrosine rather than a diglycine, and the final tyrosine residue is part of a final B-sheet structure. Even though argued against by these facts, conjugation products have been reported in Saccharomyces cerevisiae with homologous to ubiquitin-1 (Hub1) as the yeast ortholog. Unrelated to conjugation, a role in mRNA and pre-mRNA splicing has been implicated in yeast.139 In higher-order animals, available data indicate a potential role in the central nervous system regulation of food uptake and body weight via the hypothalamus/limbic system, in the context of which it has become known as “beacon.”140 The underlying molecular mechanism is yet to be revealed, and it may not involve protein modification in the typical mode of a ULM but rather a hormone-like action. As outlined in the original study, UBL5 is expressed in every tissue studied so far with cytoplasmic localization, hence indicating a much broader and yet to be discovered biological role.139

Ubiquitin-Fold Modifier-1
In the search for molecules that interact with the human Apg8 homolog GATE16, an E1-like protein was discovered and subsequently its substrate. This 9.1-kDa protein substrate is composed of 85 aa and possesses a ubiquitin-like fold, hence rendering the name ubiquitin-fold-modifier 1 (Ufm1) in 2004.141 Similar to other ULMs, Ufm1 is processed at the C terminus to expose a glycine residue, which allows the conjugation to target proteins via the E1-like enzyme Uba5 and the E2-like enzyme Ufc1.142 However, in distinction from ubiquitin, a cluster of acidic residues is absent in Ufm1.142 Also, Ufm1 has 6 lysine residues, and future studies are needed to characterize their involvement in protein conjugation, including chain formation. Ufm1 and its conjugates are expressed in all tissues examined so far, including brain, heart, lung, liver, and kidney. As for subcellular distribution patterns, Ufm1 was noted to be predominantly localized to the nucleus and diffusely in the cytoplasm. With regard to target proteins and biological processes, these remain to be identified, but recent studies imply a potential role in the endoplasmic reticulum stress response.143 Therefore, as with other newer members of the ULM family of proteins, main aspects of the Ufm1 system are still to be discovered.

Implications for Cardiovascular Diseases
Over the past years, ubiquitin and ULMs have been linked to cardiovascular diseases (Figure 7). For instance, the ubiquitin ligase NEDD4L is involved in the ubiquitination and internalization of epithelial sodium channels (ENaCs) in the distal nephron. A defect in the interaction motif of ENaCs leads to persistent increase in their surface expression and thereby increased sodium retention and blood pressure elevation. This constitutes the molecular basis for the Liddle syndrome.144 The UPS also modulates the activity of endothelial nitric oxide synthase (eNOS) and the expression of endothelin-1 (ET-1).145,146 Sumoylation of the transcription factor GATA-2 is of additional significance. Most notably in this regard, conjugation of SUMO-2 has been shown to suppress the activity of the GATA-2–dependent ET-1 promoter in endothelial cells.147 An alteration of the balance between nitric oxide and ET-1 in favor of the latter may contribute not only to vascular tone and blood pressure but also to atherosclerosis.30 Recent studies confirm increased ubiquitination of proteins in complicated coronary and symptomatic carotid artery plaques but disagree on the pathophysiological significance of the proteasome.148–150

Ischemic myocardial injury has been associated with the stimulation of the ubiquitin system; for instance, deficiency of the ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) seems to be detrimental to the injury response.151 Upregulation of the UPS has also been shown in atrophic and hypertrophic cardiac remodeling.152,153 Moreover, sumoylation can contribute to the differentiation and hypertrophy of cardiomyocytes by enhancing GATA-4 transcriptional activity.154 As a general theme in cardiomyopathies, dysregulation of the UPS with accumulation of ubiquitinated protein may ultimately impair cardiomyocyte function and contribute to myocyte loss by various mechanisms, including apoptosis and autophagy.155–157

Figure 7. Overview of the involvement of the ubiquitin system in the spectrum of cardiovascular diseases.
Finally, the UPS influences the density of cardiac voltage-gated sodium and potassium channels, with implications for cardiac arrhythmias. Recent data further highlight the role of SUMO, namely SUMO-1, in silencing plasma membrane potassium channels. Thus, mainly the ubiquitin system has been recognized across the broad spectrum of cardiovascular diseases, but data are emerging for ULM systems as well. Future studies will reveal more details regarding their pathophysiological role and, importantly, their therapeutic potential.

Summary

Great advances have been made in the identification of the ubiquitin and ubiquitin-like systems. These systems mediate the attachment of ubiquitin and ULMs to proteins, affecting their half-life subcellular location, and function. With regard to some target proteins and biological processes, overlapping and even antagonistic effects between the different systems are noteworthy. Discoveries on the potential implications of these systems for cardiovascular diseases are emerging, extending from hypertension to atherosclerosis and various forms of heart disease and arrhythmias.

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Disclosures

None.

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