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Role of ubiquitin- and Ubl-binding proteins in cell signaling

Vladimir Kirkin and Ivan Dikic

Besides tagging proteins for degradation, ubiquitin is now recognized as a signaling module for diverse cellular processes, including progression through the cell cycle, DNA repair, gene transcription, receptor trafficking and endocytosis. Recent advances have indicated the existence of a wide variety of ubiquitin-binding proteins that, upon recognition of conjugated ubiquitin moieties, can control assembly of complex signaling networks. Small ubiquitin-like proteins, like SUMO, emerge to play biological roles distinct from ubiquitin, and require specific recognition by a dedicated set of proteins. Identification and characterization of recognition motifs and domains for ubiquitin-like proteins have just begun, promising new insights into the diversity of functions ubiquitin family proteins have in physiological and pathological settings.

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Introduction

Ubiquitin (Ub) is a small versatile protein that has been a focus of active research during the past 30 years. Once freed from its precursor polypeptide by specific proteases, it is subjected to an enzymatic reaction cascade, which involves Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes. This ultimately leads to the covalent attachment of Ub to a lysine (K) residue or the N-terminus of the target protein, referred to as ubiquitylation, or ubiquitination [1]. Through repeated conjugation to itself, Ub can form long chains that, in the case of K48-linkage, constitute a well-recognized proteasomal degradation signal [1]. Alternative Ub chain formation appears to play other important regulatory roles (e.g. K63-linkage in NF-κB signaling [2]). As well as forming polyubiquitin (polyUb) chains, single Ub moieties (monoUb) can also be covalently attached to various proteins. Monoubiquitylation has now surfaced as a major signaling event thought to mediate complex cellular processes, of which endocytosis and DNA repair are the best-studied examples [2]. The ability of different Ub chains and monoUb to signal in different ways seems to depend largely on the specificity and function of proteins that serve as Ub receptors. Ub-binding modules found within these proteins have co-evolved with Ub to recognize and bind their ligand, thereby mediating all known functions of Ub. So far >15 individual ubiquitin binding domains (UBDs) have been identified ([3,4] and references therein) and this number is constantly growing (Table 1).

Besides Ub, 13 other small Ub-like protein modifiers (Ubls) have been described to date (Table 1). All of them share a characteristic β-grasp fold (the 'ubiquitin fold') and can be conjugated to proteins or lipids via their C-terminus [5,6]. Attachment of Ubls to their substrate has been shown to have profound influence on various cellular processes, including transcription, DNA repair, signal transduction and autophagy [6]. By analogy to Ub, recognition of Ubls by specialized protein domains is predicted to drive these cellular responses. Indeed, recent results provide several examples of such novel recognition modules. This review covers major advances made in the last two years in understanding how Ub- and Ubl-binding proteins mediate and control cellular signaling networks.

Ub-binding proteins in proteolysis

The first recognized function of Ub was earmarking proteins for the proteasomal degradation pathway. Consequently, the first Ub-binding protein to be published was a proteasome subunit S5A/RPN10 [7]. In the meantime it has become quite clear that the few constitutive proteasome receptors do receive generous donations from multiple adaptors, such as yeast proteins rad23p and Dsk2p. They possess both UBDs and Ub-like (UBL) domains and, through binding both Ub and the proteasome, dock ubiquitylated cargo to the site of its dismembering (Figure 1a) [8]. The importance of proteasome adaptors for adequate degradation of the ubiquitylated substrate has recently been underscored by the discovery of a novel Ub-binding protein, ZNF216, which can bind polyUb chains via its N-terminal UBD and mediate proteasome-dependent protein degradation. Ablation of *ZNF216* gene in mice led to marked accumulation of polyubiquitylated substrates in the muscle tissue. Interestingly, this deficiency in protein degradation could also protect the muscle tissue from experimentally induced muscle atrophy, a pathological condition associated with increased protein breakdown [9]. Whether ZNF216 represents another member of the UBA-UBL proteasome receptors is unclear. Despite the fact that ZNF216

Table 1

Ubiquitin family of small protein modifiers.

Modifier	Functions	Ub-/Ubl-binding domains
Ubiquitin	Proteasomal and lysosomal degradation, endocytosis, DNA repair, transcription, chromatin structure	CUE, DUIM, GAT, GLUE, Jab1/MPN, MIU, NZF, PFU, UBA, Ubc, UBM, UBP, UBZ, UEV, UIM, ZnF_A20 Reviewed in [4].
SUMO1, 2, 3	Nuclear localization, transcriptional regulation, DNA repair, antagonizing ubiquitylation	SIM/SBD
NEDD8/Rub1	Regulation of E3 ligases, transcription, proteasomal degradation	UBA
FAT10	Proteasomal degradation, apoptosis	UBA
ISG15	IFN-α/β response	Uncharacterized
Urm1	Nutrient sensing, oxidative stress response	Uncharacterized
Ufm1	Unknown	Uncharacterized
FUB1	T-cell activation	Uncharacterized
Hub1/Ubl5	Pre-mRNA splicing	Uncharacterized
Atg8	Autophagy	Uncharacterized
Atg12	Autophagy	Uncharacterized

physically associates with the proteasome, it lacks a Ub-like domain. More work is therefore needed to pinpoint the nature of this association.

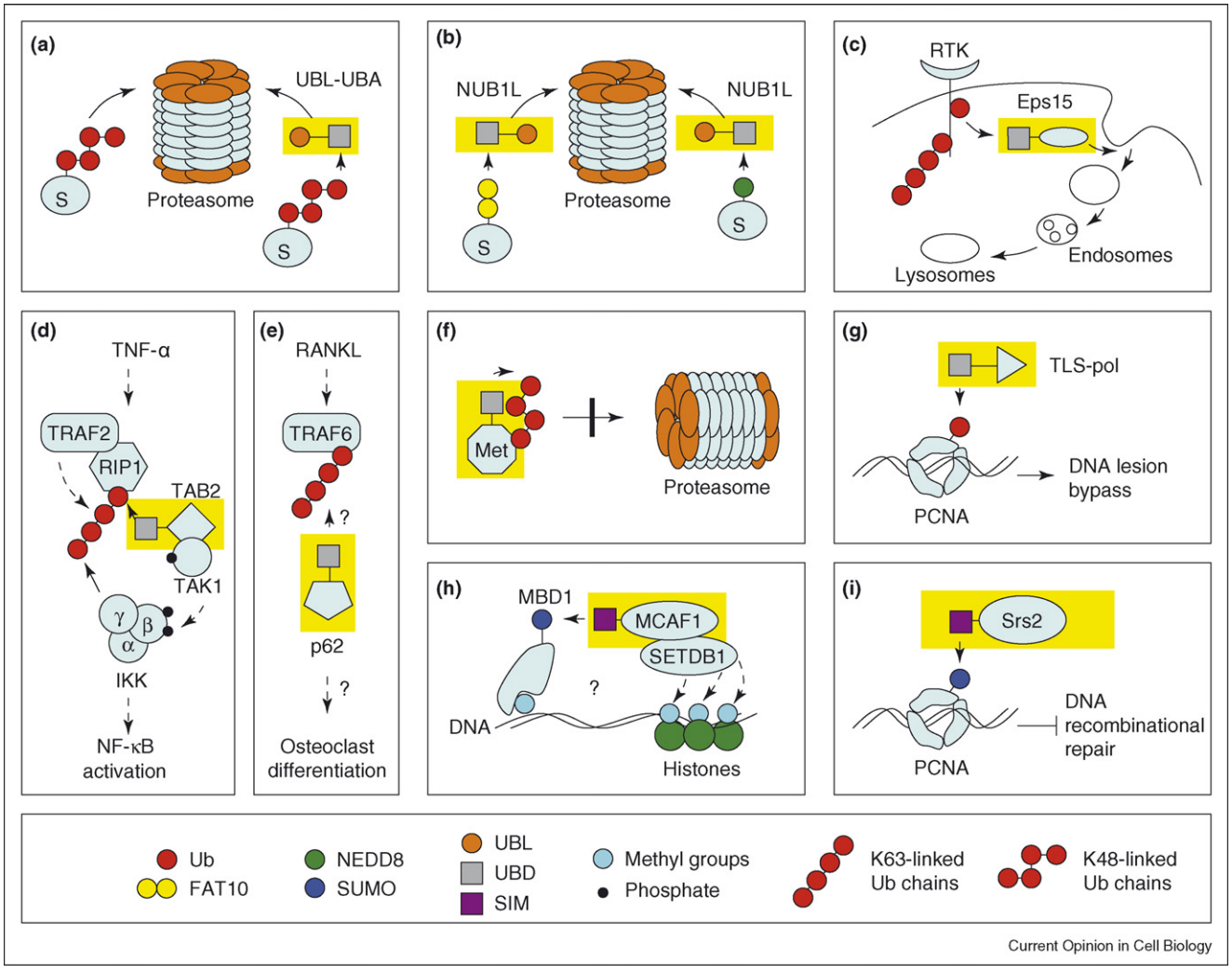
The search for FAT10-interacting proteins has recently resulted in the discovery of an exciting add-on to the Ub-proteasome system. FAT10 is an IFN-γ-inducible Ubl consisting of two Ub-like moieties with proposed roles in cell cycle control and apoptosis [5]. However, it made its debut in proteasome-mediated degradation with the finding that its non-covalently interacting partner, NEDD8 ultimate buster-1L (NUB1L), binds the proteasome via its UBL domain (Figure 1b) [10]. Since NUB1L is also an interferon-inducible protein, FAT10 may function as an immune-response-inducible form of Ub. A recent report has also shown direct binding of FAT10 to the proteasome [11]. Curiously, both NUB1L and its shorter splice variant NUB1 also bind NEDD8, targeting it for degradation ([12] and references therein). Thus, characterization of FAT10- and NEDD8-binding proteins has helped to assign novel functions for these Ubls.

Ubiquitin-binding proteins in endocytosis

The majority of cell-surface receptors undergo endocytosis either constitutively or as a result of their activation. Conjugation with monoUb [13] or oligo-Ub chains [14•] functions as an endosomal sorting signal for receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs), transporters and ion channels [15,16]. Elaborate molecular machinery is responsible for recognition and sorting of monoubiquitylated cargo from the cell membrane and, further along the endocytic route, for targeting it for lysosomal degradation. UBD-containing endocytic adaptor proteins, such as Eps15, epsins, Hrs and Tsg10, play a key role in this process (Figure 1c). They act as receptors for monoUb and bridge Ub-modified proteins to the supramolecular complexes responsible for sorting cargo into the lumen of endosomal vesicles [15–17]. The study of the mode of Ub recognition by endocytic adaptors has brought unexpected findings that underscore the unique complexity of the signal provided by Ub [4]. For instance, structural analysis of the Hrs-UIM (Ub-interacting motif) in complex with Ub revealed that this particular UBD can simultaneously bind two Ub molecules [18•]. Such a double-sided UIM is thought to make the Hrs-Ub interaction highly efficient, which is crucial for lysosomal degradation of RTKs. Future work is likely to address the possibility that oligomeric K63-linked Ub chains found to be associated with activated RTKs [14•] possess Hrs-UBD binding properties different from those displayed by multiple monoUb. Should this be the case, it will help delineate biological roles of the two Ub modifications in recruitment of endocytic factors. Importantly, UIMs of other endocytic adaptors, such as Eps15, are also predicted to interact with two Ub molecules [18•], which suggests a broader role for this type of interaction in endocytosis. Other UBDs, such as GAT (GGA and Tom1) domain, may also bind two Ub moieties at a time [19,20].

Many endocytic adaptors carry more than one UBD [17]. This alone may explain the ability of Ub to regulate assembly of large protein complexes controlling endocytosis. Yet a higher level of complexity in the interaction between Ub and Ub-binding endocytic proteins exists. Rabex-5 is a GTPase exchange factor (GEF) that is essential for activation of the small GTPase Rab5, which regulates endosome fusion. Like many other proteins involved in endocytosis, Rabex-5 gets recruited to activated cell-surface receptors upon their monoubiquitylation [21•]. Three independent reports have now shown that Rabex-5 possesses two novel UBDs; one of these, MIU (motif interacting with ubiquitin), binds the canonical Ile44 patch of Ub (to which the majority of other UBDs bind [3]), whereas the other, ZnF_A20 (A20 zinc finger), interacts with a novel Ub hydrophobic interface centered on Asp58 [21•,22•,23]. These studies provide an example of combinatorial recognition of a single Ub molecule by multiple UBDs.

Figure 1



Ub- and Ubl-binding proteins in cell signaling. **(a)** Polyubiquitylated substrate (S) proteins destined for degradation can be delivered to the proteasome via binding to either constitutive Ub proteasomal receptors or the UBA-UBL family of proteasome adaptors, which possess Ub-like (UBL) and Ub-binding (UBA) domains. **(b)** Two Ubls, FAT10 and NEDD8, may also serve proteasome-targeting role. The proteasome adaptor protein NUB1L is envisaged to link FAT10- and NEDD8-conjugated cargo to the proteasome. **(c)** Ub-binding endocytic adaptors, such as Eps15 and Hrs, induce formation of protein complexes that mediate sorting of ubiquitylated cell-surface receptors into the lumen of endocytic vesicles and intraluminal vesicles of the multivesicular body that are destined for lysosomal degradation. **(d)** In response to TNF-α receptor activation, a dynamic signaling complex is assembled that includes the E3 ligase TRAF2 and the kinase RIP1. Conjugation of RIP1 to K63-linked polyUb chains provides binding surfaces for both TAK1 kinase and IKK kinase complexes. IKK recruitment via the IKKγ (NEMO) subunit and its TAK1-mediated phosphorylation activates IKK and leads to the propagation of the signal that finally results in NF-κB activation. **(e)** Scaffold protein p62 possesses a UBD, which is crucial for its function in osteoclast differentiation in response to RANKL. The exact mechanism of p62 action is however elusive. **(f)** UBD of the transcription factor Met is engaged in an intramolecular interaction with nascent polyUb chains, thus blocking their growth and recognition by the proteasome machinery. **(g)** Translesion polymerases (TLS-pol) polη and polι bind mono-Ub moieties covalently attached to PCNA and thereby get recruited to the sites of certain types of DNA damage, allowing error-prone DNA synthesis across the lesion site. **(h)** Recruitment of MCAF1 to the sumoylated methyl-CpG-binding protein MBD1 is proposed to be at least in part dependent on MCAF-1-SIM. This SUMO-SIM interaction is thus suggested to ensure formation of a chromatin-remodeling complex containing the histone methyltransferase SETDB1. **(i)** SIM-containing Srs2 helicase is recruited to SUMOylated PCNA during S phase of the cell cycle, thereby preventing untimely DNA recombination events. Yellow boxes highlight Ub- and Ubl-binding domain-containing proteins.

As an important regulatory mechanism, Ub-binding endocytic adaptor proteins are themselves subject to monoubiquitylation. The ability of UBD-containing endocytic adaptors to bind ubiquitylated cargo proteins is profoundly inhibited by self-monoubiquitylation, whereby the intramolecular interaction between their UBDs and monoUb precludes their binding *in trans* to ubiquitylated targets [24••]. Moreover, it has recently been discovered that the UIM of Eps15 can recruit the monoubiquitylated NEDD4 and the UBL-containing Parkin

E3 ligases, thereby promoting its own monoubiquitylation [25•,26•]. These findings underscore the importance of UBD–Ub interactions in both propagation and modulation of the Ub signal.

Ub signaling in transcription and DNA repair

Gene transcription is a fundamental process whose regulation is the end point of many signaling pathways in the cell. Given the fact that histones were the first published substrate for Ub-conjugation, the role of Ub in chromatin regulation has long been suggested. Today it is largely believed that monoubiquitylation is able to influence transcription by causing changes in the other post-translational modifications of histones, such as methylation and acetylation, and hence altering chromatin structure [27]. Although the involvement of Ub-binding proteins in this process is confidently predicted and a few candidates have already been named [27], major players in the Ub-triggered chromatin remodeling await discovery.

The role Ub-binding proteins play in the regulation of transcription factor activity is much better understood. For example, the complete activation cascade of the major inflammatory transcription factor NF-κB is tightly controlled by Ub, involving formation of both K48- and K63-linked poly-Ub chains [2]. In addition to the well-established tumor necrosis factor (TNF)-induced IκB kinase (IKK) activation mechanism, whereby the TAK1 kinase complex is recruited to K63-polyubiquitylated RIP and then phosphorylates and stimulates IKK, several reports have now described the direct recruitment of the IKKγ (NEMO) subunit to K63-polyubiquitylated RIP (Figure 1d). This binding is mediated via a novel UBD found in NEMO and is necessary for IKK and NF-κB activation in response to TNF-α [28•,29•]. It remains unclear, however, whether ubiquitylated RIP is the sole substrate for NEMO binding and hence IKK recruitment to the TNF-induced NF-κB activation complex, and clearly more work is required to put these findings into a physiological context.

Recently, the scaffold protein p62/sequestosome-1, which participates in the NGF- and RANKL-induced NF-κB activation, has become a focus of intense research owing to its prominent role in the pathogenesis of Paget's disease, a skeletal disorder associated with increased bone resorption by the osteoclasts [30]. Interestingly, the majority of the pathogenic mutations within the p62 protein affect its Ub-binding UBA domain, suggesting that the Ub–UBA interaction is important for p62 function. Indeed, a UBA-deficient form of p62 has been shown to significantly enhance osteoclastogenesis *in vitro* [30]. The fact that complete ablation of p62 leads instead to impaired osteoclast formation both *in vitro* and *in vivo* [31] suggests a fine regulatory role for the UBD of p62. Although the UBA domain of p62 is necessary for its binding to the RANKL-induced signaling complex that also contains TRAF6 (Figure 1e) ([30] and references therein), it is unclear which component of this complex carries the Ub-modification that recruits p62 specifically via its UBA. Intriguingly, Wooten *et al.* have demonstrated that TRAF6 K63-linked auto-polyubiquitylation is dependent on functional p62 [32].

Regulated degradation of transcription factors is an important mechanism of transcriptional control. Accordingly, many activated transcription factors are unstable as a consequence of polyUb conjugation [27]. An interesting twist to this paradigm has been added recently with the discovery that the yeast transcription factor Met4, which is responsible for induction of genes required for synthesis of sulfur-containing polypeptides, contains a UBD. Flick *et al.* showed that the Met4 UIM is engaged in an intramolecular interaction with nascent polyUb chains being conjugated to Met4, thereby preventing both polyUb chain growth and Met4 recognition by the proteasome (Figure 1f) [33••]. Although the formal proof is missing, the described mechanism may be responsible for the maintenance of a pool of inactive but ready-to-use transcription factor, which could be mobilized to mediate a rapid transcriptional response.

DNA repair is another intricate cellular process that involves signaling via Ub-binding proteins [34]. Of various DNA repair pathways, the mechanism of translesion DNA synthesis (TLS) has recently gained special attention because of the emerging role of Ub and SUMO in this process [35]. TLS has evolved to relieve DNA-replication stress inflicted by the presence of DNA damage, and allows DNA replication across DNA lesions to take place. TLS is finely tuned by ubiquitylation and sumoylation of proliferating cell nuclear antigen (PCNA), which normally acts as a sliding clamp on replicating DNA to recruit DNA polymerases [35]. Ubiquitylated PCNA can specifically recruit TLS-specific DNA polymerases to induce bypass of the DNA lesion in the TLS pathway ([35,36••] and references therein). While searching for proteins that bind Ub independently of its Ile44 patch, Bienko *et al.* identified novel UBDs (UBM and UBZ) in the TLS-specific Y-family polymerases polη and polι [36••]. These UBDs mediate polη and polι recruitment into replication foci and are indispensable for the tight binding of the TLS polymerases to PCNA (Figure 1g). Importantly, the UBZ of polη is able to restore the response of DNA repair-deficient cells to UV irradiation [36••]. More recently, this has been corroborated by two other reports that highlighted the evolutionarily conserved role of the Ub–TLS polymerase interactions [37,38]. The recruitment of DNA-repair-specific polymerases to the replication foci via Ub–UBD interactions may provide an important insight into the etiology of a variant form of the malignancy-associated Xeroderma Pigmentosum disease. Deficiency in polη has been linked to Xeroderma Pigmentosum [39] and it is tempting to speculate that

mutations in UBDs of polη and other TLS polymerases might be causal to the inability of affected cells to repair UV-induced DNA damage.

Signaling role of SUMO-binding proteins

Modification of proteins with SUMO has long been known to regulate various cellular processes, such as nuclear transport, cell cycle, transcription and DNA repair [40]. In a great number of cases, however, the exact mechanism by which sumoylation is translated into a biological effect is unknown. However, by analogy to Ub, it is safe to speculate that SUMO-binding proteins will act as sensors for this modification, thereby providing the basis for protein–protein interaction platforms. Indeed, we and others have characterized SUMO-interacting motifs (SIMs), also known as SUMO-binding domains (SBDs), that are present in a great variety of proteins [41–44]. Interestingly, despite overall structural similarity, SUMO reveals binding surfaces that are distinct from Ub [44], which enhances the specificity of SUMO signaling.

Many of the known SIM-containing proteins identified to date have nuclear functions, which is consistent with the general role sumoylation plays in nuclear processes. One of the widely accepted functions of SUMO is inhibition of transcription. Covalent binding of SUMO to various transcription factors has been shown to promote their interaction with co-repressors [45]. In line with this, Uchimura *et al.* have recently proposed involvement of a SIM in heterochromatin formation. Their experimental data suggest that sumoylation of MBD1, a methyl-CpG-binding protein, is at least in part responsible for the recruitment of the SIM-containing MCAF1 (MBD1-containing chromatin-associated factor 1), which is a component of a chromatin-remodeling complex also containing a histone methyltransferase, SETDB1 [46]. Although direct association of SETDB1 with sumoylated MBD1 via MCAF1 could not be demonstrated, the SIM–SUMO interaction was suggested to be important for linking DNA methylation to the chromatin-remodeling machinery (Figure 1h). This mechanism remains unconfirmed, however, since sumoylated MBD1 bound to methylated DNA has not been found to associate with SETDB1 and instead inhibited MBD1–SETDB1 complex formation [47]. In addition, MBD1 is known to bind SETDB1 independently of its sumoylation status ([47] and references therein).

A role for SUMO-binding proteins in DNA repair pathways has also been suggested. Thus, covalent attachment of SUMO to PCNA has long been known to play an important role in control of DNA replication and repair, where the major function of SUMOylation was suspected to be that of antagonizing ubiquitylation [40]. However, two recent publications have changed this view by demonstrating that PCNA SUMOylation is responsible for the recruitment of a SIM-containing Srs2 to replication forks (Figure 1i). Presence of Srs2 helicase is proposed to block unwanted homologous recombination, thereby preserving genome integrity [48,49]. Since Srs2 can itself be sumoylated ([50] and references therein) it seems likely that intramolecular SIM–SUMO interaction will regulate Srs2 recruitment to PCNA in the fashion seen with the TLS polymerases [36••].

Conclusions and perspectives

Characterization of non-covalent interactions between Ub and Ub-binding proteins using both biochemical and structural analyses has provided an important insight into the mechanistic link between ubiquitylation and the processes it regulates. The variety and specificity of cellular responses to Ub can now be attributed to the diversity of UBD-containing proteins, each of which displays a unique binding repertoire. Although UBD–Ub interactions are typically weak (K_d>50 μM [4]), the latest research has shown how binding of a single UBD to multiple Ub moieties or vice versa can lead to a net increase in the binding efficacy. Use of genetic models will now be essential to provide an *in vivo* validation for the major biochemical findings and test their physiological relevance, while detailed knowledge of the structure of UBDs should aid in designing specific mutants for such studies.

Importantly, discovery of Ub-binding proteins has fueled the search for proteins that can mediate signaling by other members of the Ubl family, including SUMO, Nedd8 and FAT10. As more Ubls join in, it becomes more challenging to discern the protein networks that are regulated by Ub and its kin. However, given the versatility of the Ubl protein signals, it is clear that their deciphering will have an important clinical impact. Various pathological conditions, including cancer and neurodegenerative disorders, have repeatedly been linked to defects in the Ub-proteasome system [51]. Autophagy is another prominent degradation system linked to tumorigenesis and neurodegeneration [52]. Since this mode of cellular degradation is critically dependent on conjugation with autophagy-specific Ubls (Atg8 and Atg12), identification of their binding partners is mandatory for understanding this cellular pathway. Implementation of experimental knowledge gained from Ubl protein/Ub-binding protein networks in drug design will be a challenging task. Given the huge number and relatively low affinity of the Ub–UBD interactions, drug specificity will surely become an issue. An additional problem will be posed by larger interaction surfaces between proteins, which will make these interactions less amenable to inhibition with small chemicals. Since Ubls share common three-dimensional structure and display partially overlapping functions (e.g. Ub and Nedd8), it is envisaged that a certain degree of cross-talk between the different Ubl-mediated signaling pathways exists [53,54]. Identification of proteins that can bind more than one Ubl is likely to unveil molecular hubs

for Ubl signaling networks and help with the design of therapeutic strategies.

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