

## Review

## Using Ubiquitin Binders to Decipher the Ubiquitin Code

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**Post-translational modifications (PTMs) by ubiquitin (Ub) are versatile, highly dynamic, and involved in nearly all aspects of eukaryote biological function. The reversibility and heterogeneity of Ub chains attached to protein substrates have complicated their isolation, quantification, and characterization. Strategies have emerged to isolate endogenous ubiquitylated targets, including technologies based on the use of Ub-binding peptides, such as tandem-repeated Ub-binding entities (TUBEs). TUBEs allow the identification and characterization of Ub chains, and novel substrates for deubiquitylases (DUBs) and Ub ligases (E3s). Here we review their impact on purification, analysis of pan or chain-selective polyubiquitylated proteins and underline the biological relevance of this information. Together with peptide aptamers and other Ub affinity-based approaches, TUBEs will contribute to unraveling the secrets of the Ub code.**

### Complexity of the Ubiquitin Code

Ubiquitylation of proteins is a significant regulatory process that affects almost all cellular functions. Ubiquitin (Ub) is a small, compact, and highly conserved 76 amino acid protein. The C terminus of Ub is attached by an isopeptide bond to the  $\epsilon$ -amino group of lysine residues on target proteins. After Ub is attached to a protein, this proximal Ub can act as a substrate for additional Ubs, which are conjugated to any of its seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) or to its N-terminal methionine (M1) [1]. Protein ubiquitylation was first described as a signal for proteasomal degradation. The so called **ubiquitin–proteasome system (UPS)** (see [Glossary](#)) uses mainly K48 and K11 polyubiquitylation as signals for substrate recognition [2,3]. The 26S proteasome include subunits that contain Ub-binding domains (UBDs) ([Box 1](#)) that participate in the binding of ubiquitin chains [4,5]. The binding and disassembly of many different types of Ub chains vary in both length and linkage specificity [6,7]. Although the presence of one Ub chain type is not by itself indicative of one function, accumulated evidence supports the existence of certain generic roles. For instance, proteasome inhibition leads to accumulation of ubiquitylated proteins containing all seven different types of Ub linkages except for K63 [8], strongly suggesting that K63 chains do not primarily target proteins to proteasomes. K63 Ub chains drive proteolysis primarily by the **autophagy–lysosome system (ALS)** [2] and might mediate DNA repair and other signaling pathways. Not much is known about the functions and mechanisms of more atypical linkages such as Lys6, Lys27, Lys29, Lys33, and Met1 [9]. This Ub chain complexity known as the Ub code includes mono- and multi-mono-Ub modifications, chains mixing multiple Ub linkages and other **ubiquitin-like proteins (UbLs)**. The biological relevance of most of these complex chains remains to be elucidated [10,11].

The Ub code is dynamically regulated by (i) a specific set of enzymes (E1, E2s, and E3s) to generate the chain (writers); (ii) a set of deubiquitylases (DUBs) that cleave Ub completely from

### Highlights

Ub/UbL modification of substrate proteins drives multiple cellular functions through proteolytic and nonproteolytic mechanisms. This complex system consists of conjugation of mono or polymeric chains of various linkages, including heterologous chains of Ub/UbLs. How, when, where, and why this Ub code is written, read, and erased is the subject of intense investigation.

Interpretation of the Ub code is performed by factors that recognize Ub/UbL chains and connect the substrates to downstream effectors. Recognition of chains occurs through discrete domains, specific for each Ub/UbL protein and chain linkage.

Emerging technologies are used to explore the Ub code. Molecular traps are based on Ub-binding domains or affinity binders with specificity for particular Ub/UbLs and linkages. The multimerization of domains achieve high affinity, leading to high purification yields.

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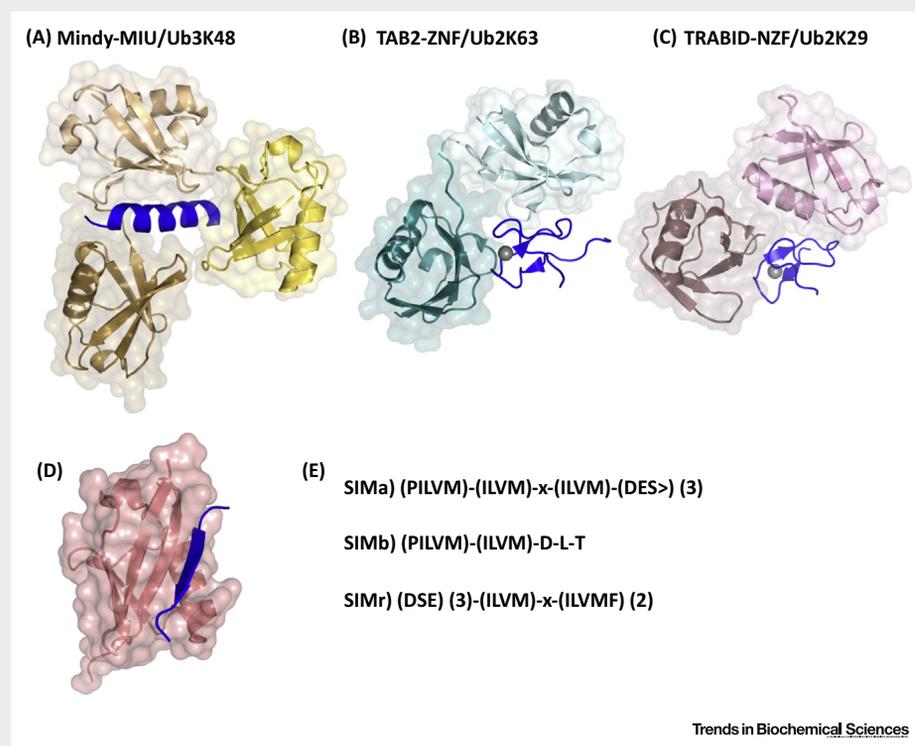
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## Box 1. Ub Family Protein-Binding Domains

Ubiquitylated substrates are recognized by effector proteins that usually contain at least one UBD (Figure IA–C). Recent reviews on UBDs discuss their structural characteristics and how they interact with Ub [26,102,103]. About 20 different families of UBDs have been described so far and are summarized in Table I. Although these UBDs are structurally different, they bind distinct hydrophobic patches contributing to the specificity of the UBDs [26,102]. These UBDs have been grouped according to their diverse underlying structural features. UBDs have been characterized that are composed of single or multiple  $\alpha$ -helices, zinc finger motifs, or pleckstrin-homology domains, each class with multiple members. Another UBD type shares structural similarity to E2 conjugating enzymes, but lacks catalytic activity. A final diverse group of UBDs contains other structural elements (coiled-coil motifs, helix-turn-helix, beta sheets, and SH3). The existence of multiple UBDs is justified by the chain structure diversity and complexity. For instance, K48-, K11-, K33-, and K6-linkages adopt a compact closed conformation where the hydrophobic patches are buried [104,105], while M1, K29, and K63-poly-Ub chains adopt an extended open conformation where hydrophobic patches are accessible and contribute to binding [106]. Furthermore, some binding domains recognize surfaces on two adjacent Ub molecules, conferring chain specificity and producing a cooperative increase in affinity (Figure IA–C) [107–109].

Substrates modified by SUMO molecules are also recognized by effector proteins containing motifs that specifically recognize substrates modified by SUMO1, SUMO2, and SUMO3. Recent reviews on SIMs, and how these interact with SUMO molecules, have addressed structurally and biologically relevant aspects (Figure ID) [11,110,111]. At least three types of hydrophobic SIMs contribute to the coordination of SUMO-dependent functions (Figure IE). Typically, SIMs are constituted by a hydrophobic core flanked by one or two clusters of negatively charged amino acid residues [112]. Several SIMs can integrate SUMO-binding domains, optimizing binding, and improving control over processes regulated by protein SUMOylation. Multiple methodologies exploring biochemical, cellular, and molecular aspects of protein SUMOylation have been developed to better understand the role of SIMs in SUMO-regulated processes [61,113–115].



**Figure I. Binding Domains Interacting with Poly-Ub Chains and Small Ubiquitin-like Modifier (SUMO).** Ubiquitin (Ub) Binding Domains, (UBDs) are diverse domains that are able to distinguish between different Ub chain linkages. The binding mechanism depends on the structure of each domain and amino acid composition of each UBD. (A) The MIU motif of MINDY1 (blue) has three distinct Ub interaction sites that enable it to bind to K48-linked polyubiquitin (sand/yellow) (PDB: 5MN9). (B) The NZF domain of TAB2 (blue) binds to K63-linked polyubiquitin (cyan/teal) (PDB: 2WWZ). (C) The NZF domain of TRABID (blue) binds to K29-linked polyubiquitin (pink/violet) (4S1Z). (D) Solution structure of SUMO interacting motif (SIM) bound to SUMO. Cartoon representation of the complex between SUMO1 (pink) and SIM peptide (blue) from the M-IR2 region of RanBP2 (PDB 2LAS). (E) SIMs domains in PROSITE format [112].

## Glossary

**Affimers:** small engineered non-antibody binding proteins designed to mimic the molecular recognition of monoclonal antibodies in various applications.

**Aptamers:** peptides or oligonucleotides with binding specificity for a target protein.

**Autophagy-lysosomal system**

**(ALS):** includes all cellular factors implicated in the regulation of the formation of autophagosomes, fusion with the lysosomes, and proteolytic enzymes.

**Far-western immunoblot:** this technique uses a non-antibody protein that binds the protein of interest. Thus, it is suitable to detect protein-protein interactions.

**Mass spectrometry (MS):** analysis is used to quantify Ub and UbL peptide signatures.

**Post-translational modifications**

**(PTMs):** can be chemical changes such as phosphorylation or acetylation, but also small peptide modifier such as UbLs.

**SUMO-binding entities (SUBEs):** based on the multiplication of SIMs or the SUMO-dependent ubiquitin ligase RNF4.

**Tandem ubiquitin binding entities**

**(TUBEs):** have multiple binding domains to increase the affinity for ubiquitylated proteins.

**Ubiquitin-like proteins (UbLs):** members of the Ub family presenting the  $\beta$ -grasp fold and the C-terminal double glycine that allows its conjugation to target amino-acid residues.

**Ubiquitin-proteasome system**

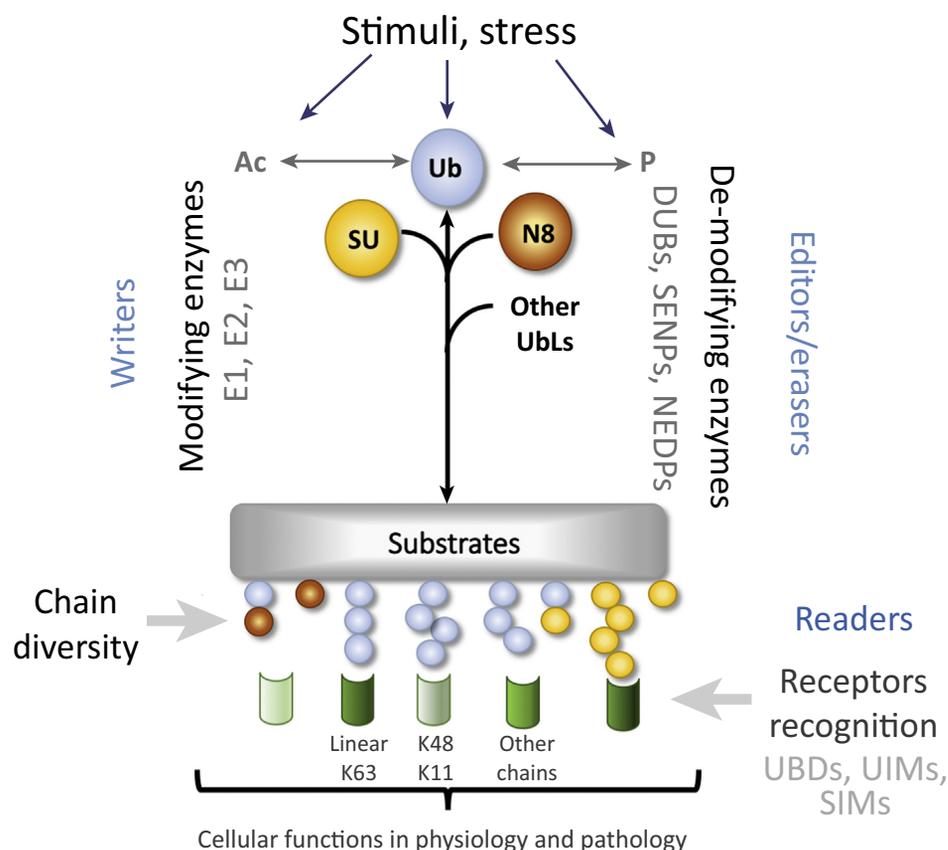
**(UPS):** includes all enzymes and cofactors implicated in protein ubiquitylation and the proteasome.

Table 1. UBDs

UBD structural class	UBD examples (abbreviations)	Representative proteins	Refs
α-Helical	UBA	RAD23, DSK2	[116,117]
	CUE	Vps9	[118,119]
	UIM	S5a, RAP80, EPS15	[120–123]
	MIU/IUIM	Rabex5	[124]
	DUIM	Hrs	[125]
	VHS	STAM	[126]
	GAT	GGA3, TOM1	[127–129]
Zinc-finger	NZF	Npl4	[130], HOIP
	ZnF-A20	A20, Rabex-5	A20, [124]
	ZnF-UBP	USP5, HDAC6	[131,132]
	UBZ	Polymerase/Rad18	[133,134]
Pleckstrin-homology	PRU	Rpn13	[135]
	GLUE	EAP45	[136,137]
Ub-conjugation-related	UEV	VPS23, TSG101	[138,139]
Diverse fold class	Jab1/MPN	Prp8p	[140]
	PFU	Doa1/PLAA	[141]
	SH3(variant)	CIN85, amphiphysin	[142,143]
	UBAN	NEMO, optineurin	[144]

substrates (erasers) or edit the type and linkage of poly-Ub (editors); and (iii) a variety of proteins containing UBDs that recognize the chain types (readers) on the substrate and execute the desired effect (Figure 1). The specificity of the ubiquitylation machinery comes mainly from the E3 Ub ligases, as they participate in the specific recognition of signals present on the target proteins, and the E2 conjugating enzymes that work only with a limited set of E3 enzymes [12,13]. However, there are no consensual lysines to attach Ub on target proteins as occurs with other UbLs [12,13]. Removal of the Ub chains is mediated by ~100 DUBs identified in humans [14]. DUBs have UBDs that help regulate their specificity and activity in distinct signaling events, cellular functions, and compartments [15]. Interaction of DUBs with poly-Ub chains depends on linkage specificity, as shown by structural studies [14,15].

Protein modification by other UbLs, such as the small ubiquitin-like modifier (SUMO), the neural precursor cell expressed, developmentally downregulated 8 (NEDD8), or the interferon-stimulated gene (ISG)15, involve specific sets of enzymes for conjugation to and deconjugation from substrates [16]. Like Ub, SUMO2/3 paralogs form polymeric chains that are often terminated by SUMO1, which lacks a consensus SUMOylation site [17]. NEDD8 has been shown to form chains *in vitro* [18]. Heterologous Ub/NEDD8 chains are formed in response to stress conditions [10,19]. SUMO chains are targets of polyubiquitylation [11], and proteomic studies suggest extensive cross-modifications of unknown function among Ub, NEDD8, SUMO, and other UbLs [20–22]. Therefore, Ub and UbL modifications function as a signaling system with a complex form of regulation. Like Ub, UbLs are signals interpreted by decoder proteins containing motifs/domains that specifically recognize them (Box 1). The best-studied motifs are those interacting with SUMO, known as SUMO-interacting motifs (SIMs) [23] and autophagy-related protein ATG8 [24]. Motifs interacting with NEDD8 [25] and



Trends in Biochemical Sciences

**Figure 1. The Complexity of the Ubiquitin Code.** The elaborated architecture of the ubiquitin (Ub) chains adopting distinct conformations is due to the integration of multiple Ub linkages, such as the Ub-like molecules Small Ubiquitin-like Modifier, SUMO (SU), or neural precursor cell expressed, developmentally downregulated 8, NEDD8 (N8), but also other post-translational modifications, including phosphorylation (P) and acetylation (Ac). Those modifications might respond to specific stimuli or stress. The formation of highly dynamic Ub and Ub-like chains is regulated by modifying (Writers: E1 activating, E2 conjugating, and E3 ligase enzymes for Ub family members) and demodifying (Editors/erasers: Deubiquitylases or DUBs, SUMO-specific proteases and isopeptidases or SENPs, NEDD8 Proteases or NEDPs) enzymes that are specific for each modification. The resultant chains are recognized by receptor proteins connecting with effector functions (Readers: Ubiquitin Binding Domains or UBDs, Ubiquitin Interacting Motifs or UIMs, SUMO interacting motifs or SIMs). Chain remodeling occurs after cell activation in response to distinct physiological or pathological events.

other UbLs exist, but additional efforts are required to fully characterize them and understand their roles in driving specific functions. Deciphering the Ub code has become a priority in research and biomedicine because of its potential to identify specific biomarkers for multiple diseases and new targets for drug development (Box 2).

UBDs recognize diverse Ub chains on specific protein substrates [26]. UBDs described to date recognize a specific patch of hydrophobic residues on one Ub molecule, called the Ile44 patch (L8, I44, and V70) [12,27]. Structural studies have demonstrated that UBDs selectivity arises from the recognition of a unique orientation of an Ub chain and distinct surfaces on an Ub moiety or through the direct interaction with the linker region connecting two Ub molecules (Box 1) [27]. The required length of Ub chains binding to a UBD is short since di- or tri-Ub chains bind efficiently [26]. Individual UBDs were first used to isolate ubiquitylated proteins of interest but

### Box 2. Druggability of the Ub System

Given the parallel between the Ub and Ub-Like (Ub/Ubl) regulation and other PTMs, such as phosphorylation, the UPS became an obvious target for developing drugs having an impact on its regulatory enzymes. For this reason, it was somehow surprising that the first drug developed targeted a catalytic subunit of the proteasome, previously thought to be a conserved part of the UPS and thus not a selective target for drug development. Currently, the proteasomal inhibitors bortezomib and carfilzomib are prescribed as first-line therapies for drug-refractory multiple myeloma [145], garnering ~US\$17 billion per year. These impressive numbers have heightened pharmaceutical interest in Ub/Ubl pathways. DUBs and E3 ligases have always been recognized as prime targets from which to develop selective therapies. The only E3 ligase drugs presently marketed, however, are thalidomide and its derivatives (called immunomodulatory imides or IMiDs), being prescribed for multiple myeloma and mantle cell lymphomas [146]. The IMiDs bind cereblon, the substrate binding subunit of cullin ligase, and modulate E3 ligase function [147]. This results in ubiquitylation of Ikaros and other C2H2 zinc finger transcription factors (as well as other  $\beta$ -loop-containing degrons) and activation of antitumor responses by the immune system [148,149]. Thalidomide was previously used for treating morning sickness in pregnant mothers, but tragically resulted in eye, heart, and especially limb defects among many children. Only recently the transcription factor SALL4 was identified as a likely target for thalidomide in causing these birth defects [150,151].

This example highlights a critical bottleneck in developing drugs that focus on DUBs and Ub ligases – the ability to comprehensively identify targets of their activity to avoid deleterious off-target effects. If this knowledge is available, more thorough screenings can lead to improved drug candidates. A better characterization of molecular mechanisms underlying Ub-mediated regulation will be essential to progress in this area.

their low affinity for Ub chains limited their use as purification tools [28]. More recently, affinity-based technologies, such as **tandem-repeated Ub-binding entities (TUBEs)**, **aptamers**, and **affimers**, coupled with proteomic analysis, have emerged as promising methods to identify UbL-modified proteins. The crucial roles that Ub/Ubl modifications have in diverse cellular processes and the burgeoning interest in Ub/Ubl pathways as sources of new drug targets (Box 2), has motivated the continuing development of a broad toolbox for studying UbL-modified proteins.

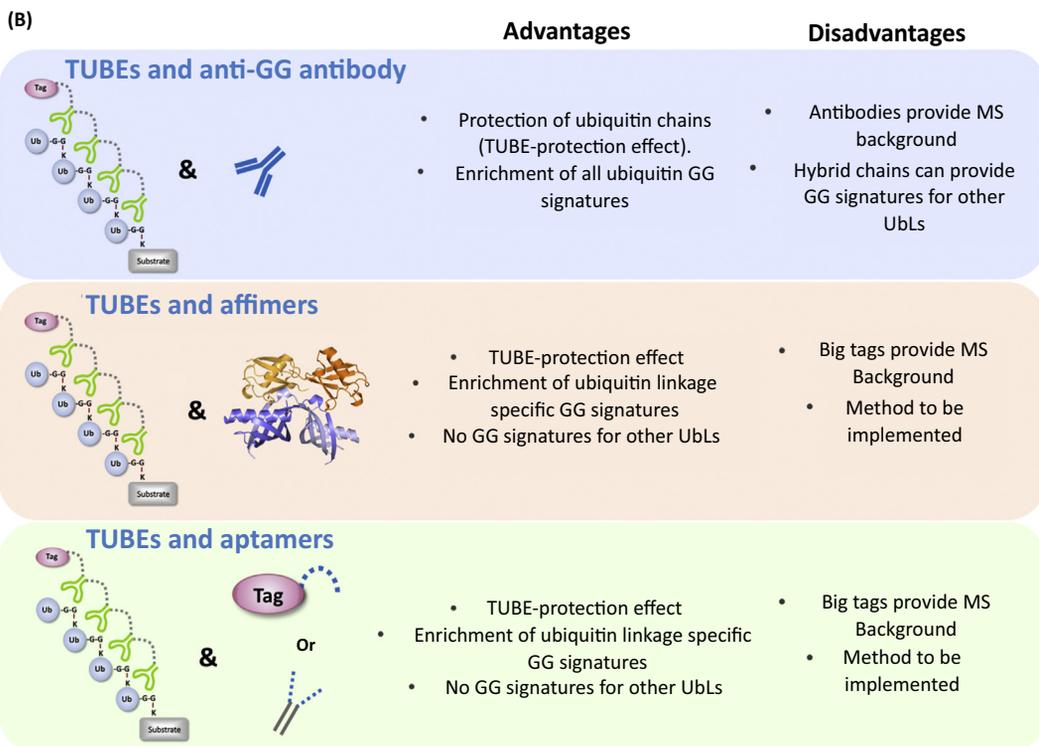
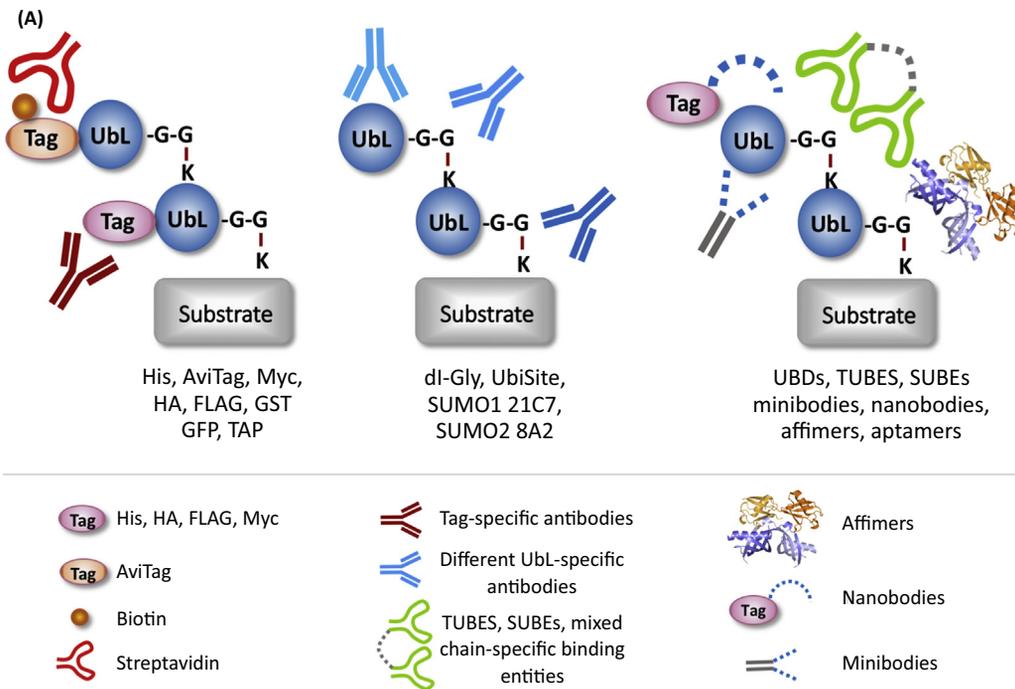
In this review we discuss the latest advances on the use of these technologies aiming to illustrate advantages and disadvantages of various applications, so researchers can decide which are the most appropriate for their experiments. We also highlight how these methods have contributed to evolving fundamental biological concepts and the generation of translational knowledge.

### Methods to Study Protein Modifications by Ub and UbLs

Owing to the central role of Ub/Ubl modifications in proteostasis, the isolation and identification of modified proteins via **mass spectrometry (MS)** has become a fundamental step toward understanding cellular processes and disease. However, this presents difficulties as the proportion of modified versus the total pool of a given protein is usually low, modifications being transient and sensitive to the action of DUBs. Nonetheless, a number of techniques have been developed to overcome these obstacles (Figure 2A).

#### Expression of Tagged Ub and UbLs

Among the most common methodologies is the use of tagged forms of Ub and UbLs. Poly-histidine tagged (His)-Ub and UbLs have been used frequently for MS in cultured cells and model organisms [28,29]. The isolation of His-Ub by nickel affinity chromatography can be performed under denaturing conditions, protecting targets from the action of DUBs, but background can be expected from proteins containing natural His stretches. Alternatively, a short peptide (AviTag) that can be biotinylated by the enzyme BirA can be used as a tag. Biotin-streptavidin interaction allows denaturing purification procedures in cultured cells and transgenic animals [30–33]. Background from a small number of endogenously biotinylated proteins



can be expected. Other epitope tags such as Myc, hemagglutinin (HA), FLAG, glutathione S-transferase (GST), or green fluorescent protein (GFP), require immunoprecipitation under mild conditions, leading to the isolation of noncovalent interactors as well as modified substrates (Figure 2A). To increase the purity of the samples, two different tags following two sequential purification steps can be used [34], although the increased size of the tag can potentially interfere with conjugation/deconjugation rates.

One advantage of tagged UbLs is that they can be easily modified for specific goals, for example, mutations can be introduced to optimize MS identification [35,36] or to prevent formation of certain chain types [37,38]. In addition, UbL-modified proteins can be captured in a tissue- or temporal-specific manner in genetically tractable organisms [31,33,39]. One disadvantage is the need for exogenous expression by transfection or transgenesis, which might alter cellular dynamics. This can be minimized by generating stable transformants with low expression levels or fusing the tag endogenously by genetic manipulation [29]. Notably, the need for transgenesis and overexpression for tagged UbLs largely precludes their use with human patient-derived tissues, which is especially relevant in the study of pathologies.

#### Antibody-Based Strategies

Monoclonal antibodies have been developed that recognize the C-terminal di-Gly motif of Ub that remains attached to target lysines after digestion with trypsin, facilitating purification of these branched peptides (also known as Ub signatures) and identification by MS (Figure 2A) [22]. However, other UbLs (NEDD8 and ISG15) also leave a di-Gly signature on their target peptides, complicating the analysis. To avoid this, UbiSite antibodies have been recently developed, which recognize a longer Ub-specific branched peptide generated by digestion with LysC [21]. Antibodies have also been developed that recognize specifically the signatures of SUMO1 and SUMO2/3 [40,41], or M1-, K48-, and K63-linkage-specific Ub chains [42–46]. Advantages include the use of almost any starting material and, of particular importance, the analysis of endogenously modified proteins. However, conditions for capture may not solubilize all potential targets, and nonmodified, copurifying proteins may lead to background. Moreover, larger amounts of starting material may be necessary, and antibodies can be costly. These reasons motivated the development of alternative procedures to explore Ub-linkage specific functions.

### Approaches Based on Ub/UbL-Binding Peptides to Study Protein Modifications

#### Molecular Traps Based on Ub/UbL-Binding Domains

Another interesting technology recently developed is the use of peptides or small proteins that interact with single or various Ub/UbL chain types (see Box 1 for the description of different UBDs and SIMs). The weak binding affinities of individual UBDs for Ub, with  $K_d$  values in the micromolar range [28], suggest that cells use a multivalent binding mechanism that would facilitate dynamic and transient interactions with multiple Ub moieties to execute activities linked to poly-Ub signaling. Beyond the Ub–UBD interaction, binding of ubiquitylated proteins

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**Figure 2. Methods to Purify Ubiquitylated Proteins for Study by Mass Spectrometry.** (A) Multiple strategies have been developed to identify ubiquitylated proteins by mass spectrometry (MS). Tagged versions of ubiquitin (Ub) or Ub-Like proteins (UbLs) have been extensively used in proteomic studies. Some of the most popular tags include poly-(His), AviTag, Myc, hemagglutinin (HA), FLAG, glutathione S-transferase (GST), green fluorescent protein (GFP), or tandem affinity purification with more than one tag (TAP) (left panel). Antibodies against the di-Gly signature recognizing various Ub family members or specific antibodies for UbLs are among the most used (middle panel). Binding tools of different natures based in small proteins/peptides with affinity for Ub or UbLs have been used in distinct proteomic studies (right panel). (B) Tandem ubiquitin binding entities (TUBEs) can be potentially combined with other technologies to optimize detection of chain linkages [84]. TUBEs have been successfully used in combination with anti-di-Gly (anti-GG) antibodies with positive results. TUBEs could be combined with peptide affimers (middle panel) or aptamers (lower panel) to improve detection of chain linkages, although no reports have been published. All these combinations might have advantages and disadvantages that are listed in a nonexhaustive way in this figure. Abbreviation: SUBEs, SUMO-binding entities.

and their receptors likely involves conformational changes, non-UBD sequences, specificity for a particular linkage, and increased avidity due to either protein oligomerization or the existence of multiple Ub-binding surfaces in single UBDs (see Table I in Box 1) [47–49].

Considering their specificity for poly-Ub, UBDs can be used in affinity purification of poly-ubiquitylated proteins. Initial attempts involved full-length Ub-binding proteins. For example, a GST-tagged full-length human S5a [containing two tandem Ub-interacting motifs (UIMs)] has been used to enrich the ubiquitylated proteins from brain and placental tissue [50]. Similarly, the Ub-binding properties of Rad23 and Dsk2 have been utilized to screen the substrates of the 26S proteasome in budding yeast [51]. UBDs have also been used to develop Ub ligase substrate trapping, a technique that fuses UBDs to an E3 ligase to purify and identify ubiquitylated substrates of a particular E3 ligase by MS [52].

A significant improvement was the multimerization of UBDs coming from various proteins, as is the case for TUBEs [53,54]. TUBEs consist of tetramerized Ub-associated (UBA) domains from the proteins UBQLN1 (TUBE1) or HR23A (TUBE2) separated by flexible linkers and fused to multiple tags to facilitate purification and detection (Figure 2A) [53]. TUBEs bind preferentially to polyubiquitylated proteins, with affinities in the low nanomolar K<sub>d</sub> range, which approximates to that of a good antibody, underlining its potential as a purification tool. It has also been demonstrated that TUBE binding shields the poly-Ub chains from DUB cleavage and from proteasome recognition and degradation [53].

While TUBEs display a preference for poly-Ub chains, they can also be used to isolate highly abundant mono or multiple monoubiquitylated substrates. For example, it was found that multiple monoubiquitylated p53 is accumulated in response to chemotherapy [53,55], and a monoubiquitylated pool of IκBα resistant to (tumor necrosis factor-α) TNFα-mediated degradation plays a role in the regulation of basal nuclear factor-κB (NF-κB) signaling [56].

Since some UBDs show preference for certain linkages, TUBEs can be designed to capture substrates enriched in particular linkage types. In an illustrative example, a K63-selective TUBE was used to enrich K63-linked polyubiquitylated proteins and analyze their role in oxidative stress response by MS-based proteomics [57].

As for Ub, similar tandem affinity binding entities have been designed for other UbLs, including SUMO. The four SIMs from the SUMO-dependent E3 ligase RNF4 were used to isolate and identify 300 polySUMO conjugates from cultured eukaryotic cells [58]. Similar to TUBEs, **SUMO-binding entities (SUBEs)**, have been developed [59–61]. SUBEs include four each of SIM2 and SIM3 motifs from RNF4 disposed in tandem (eight SIMs in total) fused to a GST or biotin tag. They specifically interact with polySUMO chains and do not bind to poly-Ub chains or free SUMO moieties [59]. Creating new tools to isolate substrates modified by heterologous or hybrid Ub/UbL chains would perhaps reveal processes regulated in this manner [62].

### Other Binding Strategies

In addition to the strategies based on existing Ub/UbL-binding domains, other approaches based on novel binding proteins are capable of isolating endogenous conjugates (Figure 2A and Box 3). Peptide aptamers, such as affimers, adnectins (also known as monobodies), and darpins are characterized by small size, a stable folding core domain, and variable domains or peptide loops that can be randomized. Likewise, camelid-derived nanobodies can be derived from *in vivo* sources by immunization or synthetic design. Large libraries can be generated and screened using display and panning methods (using ribosomes, phages, or yeast) [63].

**Box 3. Tools Recognizing Ub Chain Diversity**

Multiple tools have been developed to isolate and/or identify individual Ub chain types. The specificity and applications of these tools could be distinct; however, redundancy might exist and parallel comparisons under the same conditions are lacking in most cases. In many scenarios, these tools are complementary and can be used to avoid cross-reactions (e.g., between antibodies) or to unravel a higher level of molecular complexity as soon as it is verified that the binding sites are not competing with each other. In many cases, and depending on the nature of the interaction, tools containing multiple binding domains (such as TUBEs) have higher affinity for Ub chains than those having single binding sites. In [Table 1](#) chains recognized by each tool are indicated, including the names of the tools and the name of distributors or references associated to them.

**Table 1. Specific Binding Tools for Ub Chain Diversity**

Tool	Linkage recognition	Names	Distributor	Refs
Antibodies	M1	LUB9/1E3	Sigma, Millipore	[152]
	K11	2A3/2E6	Sigma, Millipore	
	K27	EPR17034	Abcam	
	K48	EP8589, 4289, Apu2	Abcam, Cell Signaling, Millipore	
	K63	EPR8590-448, D7A11, Apu3, HWA4C4.	Abcam, Cell Signaling, Millipore, Thermo Fisher	
	Mono- and Poly-Ub	FK2	Millipore	
	PolyUb	FK1	Millipore	
TUBEs	All chains	TUBE1, TUBE2	Lifesensors, Boston Biochem	[53]
	K63	TUBEK63	Lifesensors	
	K48	TUBEK48	Lifesensors	[153]
SUBEs	PolySUMO2/3	RNF4 based SUMO-affinity matrices	Boston Biochem, Lifesensors	[58]
		SUMO-traps		[59]
Aptamers	K48 K63	Minibodies and Nanobodies	Hybrigenics	[154]
Affimers	K6 K33 SUMO1 SUMO2	Linkage-specific affimers	Avacta	[64]

Affimers have been described that discriminate K6 or K33/K11 di-Ub linkages [64]. These tools can be used in a variety of applications including MS studies to identify specific targets of these modifications [64,65]. Aptamer technology can also identify binders that inhibit Ub/UbL pathways [66–68]. Nanobodies can be used for targeted degradation via E3 ligase fusion or recruitment [69,70] ([Figure 2A](#) and [Box 3](#)).

To date, most published studies use the UBD-based molecular traps, so we now provide more detailed examples of their use in research and in the clinic.

**Applications for TUBEs**

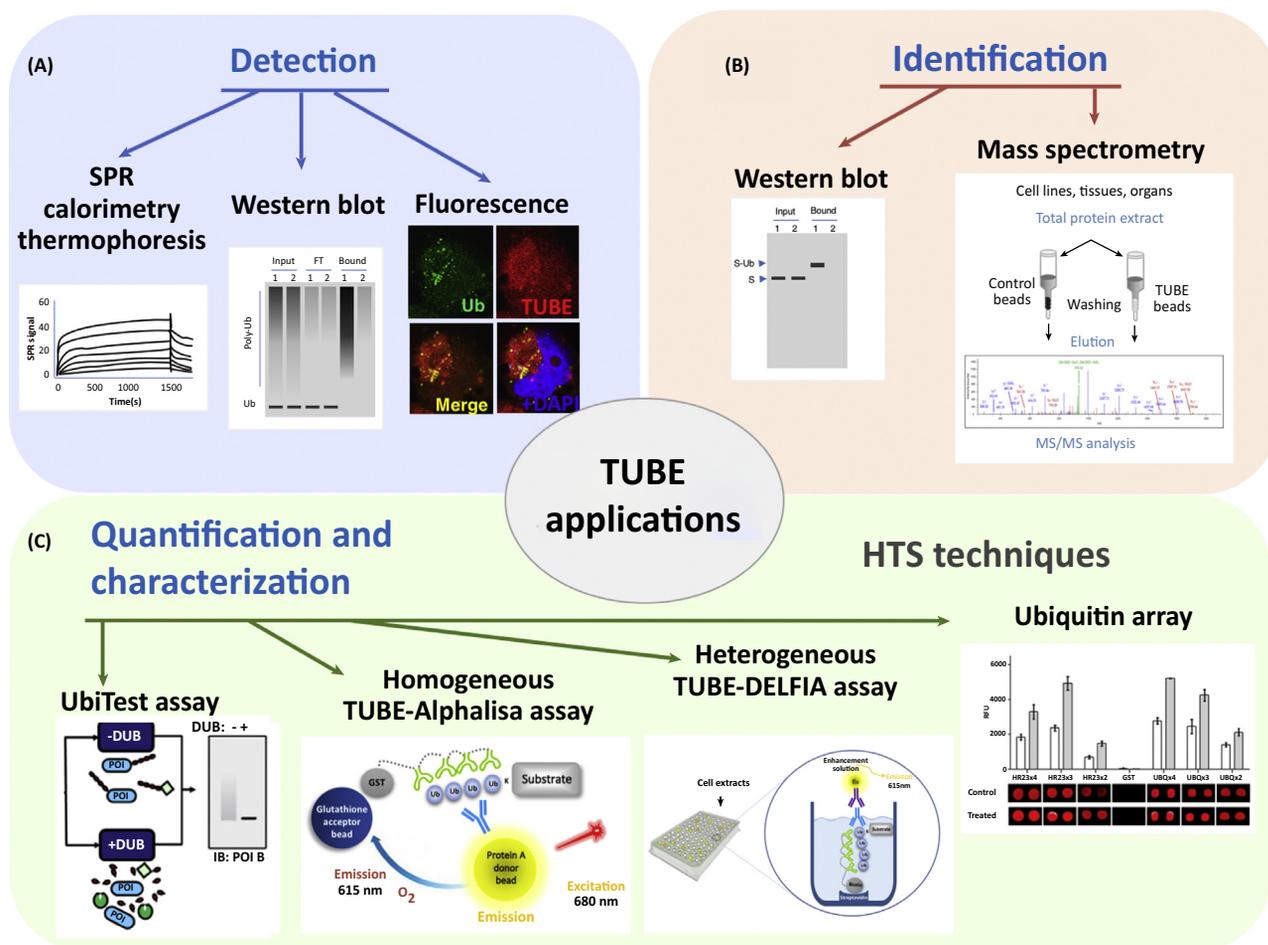
TUBEs and other molecular traps such as SUBEs, alone or in combination with other techniques ([Figure 2B](#)), have been used for a wide variety of applications ([Figure 3](#), [Key Figure](#)). We highlight here some of their published uses.

**Study of the Ubiquitylation of Specific Proteins**

TUBEs are most commonly used as affinity-purification reagents for polyubiquitylated proteins. GST or biotinylated TUBEs can be conjugated either to solid supports such as agarose or magnetic beads for affinity purification or be included during the lysis and used as capture reagents. K63-selective TUBEs have been used to show that Parkin-derived K63-Ub chains are dispensable for mitophagy, that ubiquitylation of CENP-A<sup>CNP1</sup> prevents the formation of ectopic centromeres, and that the androgen receptor is a target of the E3 ligase CHIP [71–73].

## Key Figure

## Applications for TUBE-Based Technology



**Figure 3.** Tandem ubiquitin binding entities (TUBEs) are versatile tools that have been used in multiple applications. These applications include: (A) detection methods as reagents for western blotting or fluorescence and also to measure binding affinity or specificity (surface plasmon resonance or SPR, calorimetry, or thermophoresis). (B) Identification methods when TUBEs are used to affinity purify (pull down) ubiquitylated proteins that can be detected with target-specific antibodies by western blotting or mass spectrometry. (C) Quantification of total or individual ubiquitylated proteins and characterization of the functions associated with them using various formats such as protein arrays or High Throughput Screening (HTS) techniques such as TUBE-AlphaLISA (amplified luminescent proximity homogeneous assay linked to immunosorbent assay) and TUBE-DELFI (dissociation-enhanced lanthanide fluorescent immunoassay) or to identify specific deubiquitylases (DUBs) for a given substrate (UbiTest assay). Abbreviation: POI, protein of interest.

TUBE2, which is based on the UBA domain of the UV excision repair protein HR23A, has been used to demonstrate the role of Ub in multiple types of stress, including chemotherapy [55,74]. Pan-specific TUBEs have been used to show that Rac1 is ubiquitylated by the HECT E3 Hace1 [74,75], and to discriminate the ubiquitylation pattern of cells that are sensitive or resistant to

doxorubicin chemotherapy, underlining the potential use of TUBEs in the search for biomarkers [55,74,75].

Ub-mediated degradation of essential enzymes may have particular importance in the central nervous system. Using TUBEs, it was shown that Ub C-terminal hydrolase 1 (UCHL1) antagonizes ubiquitylation of choline transporter (CHT) in a cholinergic neuron model [76]. While K48-linked polyubiquitylation is typically considered to target proteins for degradation, TUBEs identified noncanonical Ub-dependent degradation mechanisms, such as K63-linked HIF1A ubiquitylation signaling degradation by the ALS, expanding the known roles of Ub in intracellular proteolysis [77]. TUBEs are also associated with immunoprecipitation protocols (TUBE-IP) to identify ubiquitylated forms of  $\text{I}\kappa\text{B}\alpha$  or p53 [74,78]. In this way K63-TUBEs are also used to show the timing of receptor interacting serine/threonine kinase 2 (RIPK2) ubiquitylation in NOD2 signaling [79]. Thus, TUBE-IP can be used to investigate kinetics of Ub chain formation or remodeling in response to multiple stimuli.

#### MS Identification

As mentioned, TUBEs protect Ub chains from degradation. By performing TUBE-based isolation under native conditions, it is also possible to preserve interactors and complexes built around ubiquitylated proteins, such as the proteasome in an example from adriamycin-treated MCF7 cells [74,80]. MS analysis of TUBE-enriched extracts identified a unique interplay between the human host and the UPS of *Plasmodium falciparum* over the course of a malaria infection [81]. In addition, whole-proteome methods utilizing TUBEs have revealed novel roles of K63 polyubiquitylation in oxidative stress response in *Saccharomyces cerevisiae* (Figure 3) [57,82].

Comparative proteomics studies have shown the utility of TUBEs for complementary MS studies. For instance, when using stable isotope labeling with amino acids in cell culture (SILAC) to study the effects of Ndfip1/Ndfip2 knockout in mouse CD4<sup>+</sup> T cells, biotin-TUBE enrichment yielded higher numbers of identified proteins compared with the di-Gly antibody [83]. The combined use of both technologies significantly increased the identified number of di-Gly peptides when a specific F-box protein (FBXO21) was overexpressed (Figure 2B) [84].

#### Detection Methods

Given their specificity, TUBEs have been used in place of antibodies to detect ubiquitylated proteins. Distinct applications have been implemented with distinct tagged versions of nonselective TUBEs. For instance, TUBEs labeled with fluorescent tags can be used in colocalization experiments in combination with antibodies recognizing specific Ub-target proteins (Figure 3) [85]. One innovative example is a method for studying inflammasome formation in relation to Ub modification of NLRP3 (nucleotide-binding oligomerization domain-like receptor P3) [86]. Chain-selective TUBEs can similarly be used in **far-western immunoblots**. For instance, use of the biotin-labeled K63-TUBE revealed K63-polyubiquitylation of PINK1 kinase by the E3 ligase TRAF6 [87].

#### High-Throughput Screens, Arrays, and Drug Discovery

TUBEs have been incorporated into high-throughput screens (HTS) for monitoring E3 ligase and DUB activities. Time resolved fluorescence resonance energy transfer (TR-FRET)-based assays monitor autoubiquitylation of E3s, in which biotinylated TUBE is labeled with acceptor fluorophore, while E3 antibody is labeled with donor fluorophore [88]. Examples include the characterization of Ndfip-dependent activation of Nedd4-family HECT E3 ligases and the ubiquitylation of GFP-fused  $\text{I}\kappa\text{B}\alpha$  [89,90]. In similar assays, TUBE and an antibody against

an E3 ligase have been linked to donor and acceptor beads, respectively, showing their colocalization upon E3 ligase autoubiquitylation (Figure 2B) [91].

TUBEs have been applied to monitor total cellular Ub in an ELISA format, for instance in AlphaLISA or dissociation-enhanced lanthanide fluorescent immunoassay (DELFA) variations [81,92]. Multiple detection systems and formats can be configured using multiwell plates to search for biomarkers. For those cases in which the amount of starting material is an issue, TUBE-based protein arrays have been designed, for example, to detect changes in total protein ubiquitylation after treatment with doxorubicin (Figure 3) [74,93].

TUBEs can also be used to assist in qualitative assessment of ubiquitylation. To develop Ub-specific peptidase (USP)7 inhibitors, an assay comprised of TUBE enrichment of ubiquitylated proteins, followed by their elution and treatment with or without a broad-spectrum DUB was used [94]. In this way the effects of USP7 inhibitor treatment on Tip60 and forkhead box P3 (FoxP3) ubiquitylation in mouse T-regulatory cells were demonstrated [94]. This approach was further adapted using chain-linkage specific DUBs OTUB1 (K48-specific) and AMSH (K63-specific) to demonstrate that USP7 is modified predominantly by K48-linked poly-Ub chains [95]. These applications of TUBEs validated USP7 inhibitor P217564 in cells and enabled advancement of this compound toward *in vivo* animal model studies.

### Limitations and Challenges for Ub/UbL-Binding Based Approaches

While TUBEs have a number of advantages over other technologies, there are limitations. Development of new TUBES can be challenging and, although screening for Ub/UbL-binding elements can be performed in an unbiased, high-throughput manner, the validation steps require considerable planning and effort.

Most TUBEs recognize poly-Ub chains and isolation of low abundant monoubiquitylated proteins is still challenging. Since 50% of conjugated Ub exists in the monoubiquitylated state [96,97], a priority is to develop a molecular trap or affinity binders that can selectively identify a single conjugated Ub/UbL or even specific monoubiquitylated substrates. This has been achieved by traditional antibody methods (e.g., monoubiquitylated histones [98]).

Ub chains exist in branched and heterotypic forms comprised of Ub and UbLs. TUBEs have not been extensively evaluated for specific binding in these scenarios, while at least one antibody shows specificity for branched chains [99]. The chimeric K11/K48 antibody employs variable regions from K11- and K48-specific antibodies.

Most TUBE variants were first tested *in vitro* to analyze their specificity and affinity for distinct chain types. Chain composition *in vivo*, however, is more complex than initially suspected [62], questioning whether chains containing only a single type of linkage would be a general feature or an exception. It is therefore likely that even linkage-specific TUBEs will capture a small percentage of other linkage types. Moreover, correct titration is important, since it has been observed that an excess of K63-specific TUBEs can allow weak binding to K48-linked poly-Ub [100]. Likewise, saturating a solid support such as a plate surface or an agarose bead with chain-selective TUBE may lead to poor selectivity, perhaps due to high density or avidity effects. Optimization is often required and recommended. Furthermore, some data support the notion that TUBEs may have affinity for heterotypic chains containing other UbL proteins [78,101], but better characterization is needed since these are stimulus-dependent events.

For imaging and cellular applications, TUBEs must be introduced into cells by protein transduction or transfection of expression constructs [84,85]. Cellular overexpression of TUBEs could result in aberrant effects, due to the inherent ability of TUBEs to interfere with the action of DUBs and proteasomal degradation, so inducibility or titration is recommended.

Regarding the combined used of TUBEs with di-Gly-specific antibodies, some studies suggest that incompatibilities may exist [80,81], but in some cases the combination of both technologies has provided better results for the detection of Ub signatures [83,84].

### Concluding Remarks and Future Perspectives

Ub/Ubl-binding based strategies can contribute to improving our knowledge of the role of these modifications in molecular and cellular processes. A wider panel of tools to enrich Ub/Ubl chain types would help in studying their respective biological roles. For example, K6-specific TUBEs will be helpful to identify the individual proteins modified during various selective autophagy events. Little is known about the biological roles of K27-, K29-, and K33-linked poly-Ub chains, so isolating substrates and interactors of these rare Ub linkages may provide insight into their functions (see Outstanding Questions). The unique properties of TUBEs to protect ubiquitylated proteins from degradation will certainly help to increase the purification yield and detection of scarce ubiquitylated proteins by MS. Custom aptamers, alone or in combination with UBDs, may generate new molecular traps with increased affinity or new specificities. The reduced protein size of aptamers might be advantageous for some applications.

Novel TUBEs and TUBE-based assays are currently under development. The full potential of TUBEs has yet to be applied to fluorescence-based techniques such as flow cytometry/fluorescence activated cell sorting (FACS) and various types of microscopy to study the direct localization of Ub chains or their interactions by FRET inside live, fixed, or permeabilized cell and tissue samples.

The progression of UPS-targeted drugs to the clinic will require a battery of assays to validate these compounds and TUBEs are well suited owing to their adaptability to HTS formats. Moreover, TUBEs can be adapted for the discovery of diagnostic markers and drug targets by targeted or global MS studies of differential protein ubiquitylation in disease. Their use to monitor ubiquitylation of downstream substrates of DUBs and E3 ligases will be particularly important for determining the efficacy of potential drugs for these emerging target classes. In summary, TUBEs and other Ub binders will certainly continue to play a role in Ub drug discovery.

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### Outstanding Questions

How and when are target proteins driven to one or the other proteolytic pathway? Ub and Ubls are important signals to regulate the degradation mediated by the two major intracellular proteolytic pathways, the UPS and ALS. Apart from a few cases, it is not clear how this decision is taken. This question will be crucial to understand protein homeostasis in physiology and pathology.

How can we determine the correct order in which Ub and Ubl moieties are disposed within a chain? Recent technologies such as TUBEs in combination with MS techniques should contribute to decode the biological meaning of distinct chain types. The use of the actual tools and development of new technology will be crucial to progress in this sense.

What is the impact of post-translationally modified Ub or Ubls on the function, localization, and stability of target proteins? Recent evidence indicates that Ub and Ubls such as SUMO are modified by other PTMs. Gain or loss of function could be expected with respect to the target proteins. Case-by-case studies will be necessary to address this question.

How consistent are ubiquitylation profiles from healthy tissue samples compared with disease tissues samples? Is the Ub code too complex to be a reliable biomarker? Recent evidence indicates that ubiquitylation defects are observed in certain pathologies. However, it is not clear whether these defects are also present in other diseases and just indicative of aberrant cellular processes, or if they are unique to a specific disease. These aspects may become clearer as more diseases are examined.

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