Using Ubiquitin binders to decipher the Ubiquitin Code

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Abstract

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 TUBEs (Tandem-repeated Ubiquitin-Binding Entities). TUBEs allow the identification and characterization of ubiquitin chains, 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 unravelling the secrets of the Ub-code.

The Complexity of the Ubiquitin Code

Ubiquitination 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 Ɛ-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 **Ub-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 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 **Ub-Like molecules (UbL)**. 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 substrates (erasers) or edit the type and linkage of poly-Ub (editors); and (iii) a variety of proteins containing UBDs that recognize the chain type(s) (readers) on the substrate and execute the desired effect **(Figure 1)**. The specificity of the ubiquitylation machinery comes mainly from the E3 ubiquitin ligases, as they participate in the specific recognition of signals present on the target proteins, and the E2 conjugating enzymes which will 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 which help regulate their specificity and activity in distinct signaling events, cellular functions and compartments [15]. Interaction of DUBs with polyUb chains depend on linkage specificity, as shown by structural studies [14, 15].

Protein modification by other UbLs, such as SUMO (Small Ubiquitin-like Modifier), NEDD8 (neural precursor cell expressed, developmentally down-regulated 8), or ISG15 (Interferon-stimulated gene 15), involve specific set 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 ATG8 [24]. Motifs interacting with NEDD8 [25] and 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 a 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 very efficiently [26]. Individual UBDs were first used to isolate ubiquitylated proteins of interest but their low affinity for Ub chains limited their use as purification tools [28]. More recently, other affinity-based technologies, such as **TUBEs**, **aptamers** and **affimers** (see Glossary), 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 will 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 will also highlight how these methods have contributed to evolving fundamental biological concepts and generating translational knowledge.

Methods to Study Protein Modifications by Ub and Ub-Like Proteins

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 Ub-like proteins

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 (e.g. [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 non-covalent 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, 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 by 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 non-modified, co-purifying 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 relatively weak binding affinities of individual UBDs for Ub, with Kd values in the micromolar range [28], suggest that cells employ 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 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 **(Box 1, Table I)** [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 Ubiquitin Interacting Motifs, UIMs) has been employed 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 been also used to develop Ub ligase substrate trapping, a technique that fuse UBDs to an E3 ligase to purify and identify by MS ubiquitylated substrates of a particular E3 ligase [52].

A significant improvement was the multimerization of UBDs coming from various proteins, as is the case for TUBEs (Tandem-repeated Ubiquitin-Binding Entities) [53, 54].

TUBEs consist of tetramerized Ubiquitin 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 Kd range, which approximates that of a very good antibody underlining its potential as a purification tool. It has also been demonstrated that TUBEs 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 mono-ubiquitylated substrates. For example, it was found that multiple mono-ubiquitylated p53 is accumulated in response to chemotherapy [53, 55] and a mono-ubiquitylated pool of IκBα resistant to TNFα-mediated degradation plays a role in the regulation of basal 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 SUMO-Interacting Motifs (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, or **SUBEs**, have been developed [59-61]. SUBEs include four each of SIM2 and SIM3 motifs from RNF4 disposed in tandem (8 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, phage or yeast) [63]. 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^{CNP1} prevents the formation of ectopic centromeres, and that the androgen receptor is a target of the E3 ligase CHIP [71-73]. TUBE2 which is based in 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 [74, 55]. 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 carboxy-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, as K63-linked HIF1A ubiquitylation signaling degradation by the ALS, expanding the known roles of Ub in intracellular proteolysis [77]. TUBEs were also associated with immunoprecipitation protocols (TUBE-IP) to identify ubiquitylated forms of IκBα or p53 [74, 78]. In this way K63-TUBEs were also used to show the timing of 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.

Mass Spectrometry Identification

As mentioned, TUBEs protect Ub-chains from degradation. By performing TUBEs-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 TUBEs-enriched extracts identified a unique interplay between the human host and the UPS of *Plasmodium falciparum* over the course of malaria infection [81]. In addition, whole-proteome methods utilizing TUBEs revealed novel roles of K63 polyubiquitylation in oxidative stress response in *Saccharomyces cerevisiae* **(Figure 3)** [57, 82].

Comparative proteomics studies show 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+ 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 non-selective TUBEs. For instance, TUBEs labelled with fluorescent tags can be used in co-localization 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 Florescence 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 IκBα [89, 90]. In similar assays, TUBE and an antibody against an E3 ligase have been linked to donor and acceptor beads, respectively, showing their co-localization upon E3 ligase autoubiquitylation **(Figure 2B)** [91].

TUBEs have been applied to monitor total cellular Ub in an enzyme-linked immunosorbent assay (ELISA) format, for instance in AlphaLISA or dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) variations [81, 92]. Multiple detection systems and formats can be configured using multi-well plates to search for biomarkers. For those cases wherein the amount of starting material is an issue, TUBEs-based protein arrays have been designed, for example, to detect changes in total protein ubiquitylation after treatment with doxorubicin **(Figure 2B)** [74, 93].

TUBEs can also be used to assist in qualitative assessment of ubiquitylation. To develop USP7 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 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 towards *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 mono-ubiquitylated proteins is still challenging. Since 50% of conjugated Ub exists in the mono-ubiquitylated 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 mono-ubiquitylated substrates. This has been achieved by traditional antibody methods (e.g., mono-ubiquitylated 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 TUBEs 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 diGly-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 greatly 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 TUBEs-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.

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 like phosphorylation or acetylation, but also small peptide modifier such as UbLs.

SUMO Binding Entities (SUBEs): are based on the multiplication of SUMO-Interactive motifs (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 β-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.

BOX 1: Ubiquitin Family Proteins-Binding Domains

Ubiquitylated substrates are recognized by effector proteins that usually contain at least one ubiquitin (Ub)-binding domain (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 quite 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, 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]. Further, 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 SUMO-interacting motifs (SIMs), and how these interact with SUMO molecules, have addressed structural and biological relevant aspects **(Figure ID)** [11, 110, 111]. At least 3 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 (SBDs), 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].

Table I. Ubiquitin-binding domains (UBDs)

UBD structural class	UBD examples (abbreviations)	Representative proteins	Refs
Alpha-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 eta/Rad18	[133, 134]
Pleckstrin-	PRU	Rpn13	[135]
homology	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]

Figure I: Binding domains interacting with poly-Ub chains and SUMO. Ubiquitin (Ub) Binding Domains, UBDs, are very diverse domains that are able to distinguish different Ub chain-linkages. The binding mechanism depends on the structure of each domain and amino acid composition of each UBDs. (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 bound to SUMO. Cartoon representation of the complex between SUMO1 (pink) and SUMO interacting motif (SIM) peptide (blue) from the M-IR2 region of RanBP2 (PDB 2LAS). (E) SIMs domains in PROSITE format [112].

BOX 2: Druggability of the Ubiquitin System

Given the parallel between the ubiquitin (Ub) and Ub-Like (Ub/UbL) regulation and other post-translational modificaitons (**PTMs**), such as phosphorylation, the Ub Proteasome System (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 Carlfizomib are prescribed as first line therapies for drug refractory multiple myeloma [145], garnering ~$17 billion per year. These impressive numbers heightened pharmaceutical interest in Ub/UbL pathways. Deubiquitylases (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.

BOX 3: Tools Recognizing Ub Chain Diversity

Multiple tools have been developed to isolate and/or identify individual ubiquitin (Ub) chain types. The specificity and applications of these tools could be very distinct; however, redundancy might exist and parallel comparisons under the same conditions are lacking in most of the 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 of 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 I** chains recognized by each tool are indicated, including the names of the tools and the name of distributors or references associated to them.

Table I. Specific binding tools for ubiquitin chain diversity

Tool	Linkage recognition	Name (s)	Distributor	Refs
Antibodies	M1	LUB9/1E3	Sigma, Millipore	[152]
	K11		Sigma, Millipore	
		2A3/2E6		
	K27		Abcam	
		EPR17034		
	K48		Abcam, Cell Signaling,	
		EP8589, 4289, Apu2		
	K63		Millipore	

	Mono- & Poly-Ub PolyUb	EPR8590-448, D7A11, Apu3, HWA4C4. FK2 FK1	Abcam, Cell Signaling, Millipore, Thermo Fisher Millipore Millipore	
TUBEs	All chains K63 K48	TUBE1, TUBE2 TUBEK63 TUBEK48	Lifesensors; Boston Biochem Lifesensors Lifesensors	[53] [153]
SUBEs	PolySUMO2/3	RNF4 based SUMO-affinity matrices SUMO-traps	Boston Biochem, Lifesensors	[58] [59]
Aptamers	K48 K48	Minibodies and Nanobodies	Hybrigenics	[154]
Affimers	K6 K33 SUMO1 SUMO2	Linkage-specific affimers	Avacta	[64]

Figure 1. The complexity of the Ub-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 down-regulated 8, NEDD8 (N8), but also other PTMs including phosphorylation (P) and acetylation (A). 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 de-modifying (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 physiologic or pathologic events.

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. 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-histidine (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 nature 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 antibodies with very 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 non-exhaustive way in this figure.

Figure 3, Key Figure. Applications for TUBEs-based technology. 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-blot or florescence, but 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-blot or by mass spectrometry, MS. (C) Quantification of total or individual ubiquitylated proteins and characterization of the functions associated to them using various formats such as protein arrays or High Throughput Screening (HTS) techniques like TUBE-AlphaLISA and TUBE-DELFIA (dissociation-enhanced lanthanide fluorescent immunoassay) or to identify specific DUBs for a given substrate (UbiTest assay).

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