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Multitasking with ubiquitin through multivalent interactions

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Abstract

Ubiquitylation - the post-translational modification of proteins with ubiquitin - serves powerful regulatory roles in eukaryotes. It can label proteins for destruction or activate gene transcription. Despite its versatility, ubiquitin is used to signal for cellular events with exquisite specificity. To achieve both versatility and specificity, ubiquitin signaling pathways use multivalency, namely the coordinated use of multiple interaction surfaces. Multivalent interactions regulate each stage of ubiquitin signaling pathways, and appear within the ubiquitin signal, the ubiquitylated substrate, ubiquitin processing enzymes and ubiquitin recognition proteins.

The importance of multivalency in ubiquitin signaling

Ubiquitin is a small 76 amino acid protein that is covalently attached to other proteins to expand their functional repertoire or to control their lifespan. Its regulatory prowess was first described in 1980 owing to its ability to target other proteins for proteolysis [1] and is now known to communicate with >150 proteins [2], via discrete interacting surfaces. This family of proteins is known as ubiquitin receptors and through these, ubiquitylation regulates a vast array of cellular events including protein degradation, protein trafficking, transcription, DNA repair, cell-cycle progression and apoptosis (Figure 1). It is still not fully understood how ubiquitin can signal for a specific cellular component from its large pool of receptors. This article focuses on the factors that determine the outcome of ubiquitylation with an emphasis on the importance of multivalency—the coordinated use of multiple protein–protein interaction surfaces, to propagate a signaling event. Indeed, the ubiquitin signal itself is diverse and often multivalent, as are ubiquitin receptors and substrates.

The ubiquitin signal is diverse and multivalent

Ubiquitin has a C-terminal glycine that is activated in an ATP-dependent manner to form an isopeptide bond with the primary amino group of its substrate, which is usually the ϵ -amino group of a lysine, and also its amino terminus [3]. Serine hydroxyl [4] and cysteine thiol [5,6] groups can also be modified by ubiquitin. Substrates can be monoubiquitylated, via the attachment of a single ubiquitin (Figure 2a), or multiubiquitylated, such that more than one amino acid is modified with monoubiquitin (Figure 2b). Ubiquitin can also be added sequentially to substrates to form ubiquitin chains (polyubiquitylation; Figure 2c). The seven lysines of ubiquitin and its N-terminal methionine (Met1) are used to form ubiquitin chains *in vivo* [7–9]. Ubiquitin chains can be of one linkage type, of mixed linkage or forked with more than one ubiquitin attached to a common moiety (Figure 2c). Forked chains can be formed *in vitro* [10] and are also found *in vivo* [9], but their functional relevance is not yet understood. The multivalency provided by ubiquitin chains can greatly enhance their affinity

for binding partners. For example, the dissociation constant (K_d) values of the NZF (Npl4 zinc finger) domain of TAK1 binding protein 2 (TAB2) for monoubiquitin, Lys63-linked diubiquitin and Lys63-linked tetraubiquitin are $275 \pm 49 \mu\text{M}$, $8 \pm 1.7 \mu\text{M}$ and $0.35 \pm 0.04 \mu\text{M}$, respectively [11].

Thus, ubiquitylation is a type of modification that is highly variable in length and linkage type. Different linkage modes result in different ubiquitin chain conformations and in unique binding epitopes, which can define downstream signaling events. Under physiological conditions, Lys48-linked chains adopt a closed conformation [12], in which the ubiquitin subunits pack against each other (Figure 2c). By contrast, Lys63-linked [13] and Met1-linked chains (commonly referred to as linear) [14] are extended (Figure 2c). A recent molecular modeling study predicted that Lys6, Lys11 and Lys27 linkages promote closed conformations and that Lys29- and Lys33-linked chains are extended [15]. When binding to ubiquitin chains of closed conformation, ubiquitin receptors must compete with the intra-chain ubiquitin packing interactions for access to binding surfaces [16]. This mechanistic feature might enable greater binding stringency and selection against non-specific interactions.

Diversity and multivalency of ubiquitin processing enzymes

The use of ubiquitin as a diverse signaling mechanism is supported by three enzymes classes, E1 activating enzyme, E2 conjugating enzyme and E3 ligase (Figure 2d), which catalyze substrate ubiquitylation and define the type of ubiquitin chain linkage. Their actions are often regulated by multivalent interactions with each other and other signaling molecules and pathways. An E1 ubiquitin activating enzyme charges ubiquitin in an ATP-dependent manner to form a thioester bond with its catalytic cysteine. This modification induces structural changes in E1 that promote its binding to an E2 conjugating enzyme [17], to which ubiquitin is passed. E2 conjugating enzymes typically require E3 ubiquitin ligases to pass activated ubiquitin to a protein substrate; however, they can play defining roles in the ubiquitin chain linkage type. The E2 Cdc34 (cell division cycle 34 homolog (*Saccharomyces cerevisiae*)) generates predominately Lys48-linked chains [18], whereas the Mms2 (methyl methanesulfonate sensitive 2)–Ubc13 E2 complex catalyzes Lys63-linked chains [19]. In the latter case, Mms2 promotes the selective insertion of the Lys63 side chain of the acceptor ubiquitin into the active site of Ubc13, where it attacks the thioester bond between Gly76 of the donor ubiquitin and the active cysteine of Ubc13 [20].

The human genome is estimated to encode two E1s, ~40 E2s and >600 E3 ligases [21], which generally confer substrate specificity to the enzymatic cascade. E3s are sub-characterized by the presence of RING (Really Interesting New Gene), HECT (homologous to the E6-AP carboxyl terminus) or U-box domains. RING and U-box domains are structurally similar and serve a scaffolding role in which they link a catalytically active E2 enzyme to a protein substrate. Ligases containing HECT domains are catalytically active themselves and charged ubiquitin is transferred from an E2 to their catalytic cysteine for direct transfer to a substrate. E3s can influence or play a defining role in the type of ubiquitin chain linkage used; this is particularly true for HECT domain E3s. Human E6AP, a HECT domain E3, preferentially forms Lys48-linked chains [22], whereas yeast Rsp5 preferentially generates Lys63-linked chains with its cooperating E2s having no influence on chain type specificity [23]. In other cases, a particular E2–E3 combination determines how a substrate is ubiquitylated. For example, proliferating cell nuclear antigen (PCNA) is monoubiquitylated at Lys164 by the E2 Rad6–E3 Rad18 (radiation sensitive) complex [24], whereas the E2 Ubc13–Mms2–E3 Rad5 complex acts on monoubiquitylated PCNA to generate a Lys63-linked chain [24,25].

The timing of substrate ubiquitylation is often relayed through multivalency effects of E3s (Figure 3a). E3s can respond to their own phosphorylation status, to that of their substrate and to interactions with proteins that activate or suppress their activity. The use of multiple interactions to activate or suppress substrate ubiquitylation is exemplified through MDM2, the major E3 ligase for tumor suppressor p53 (reviewed in Ref. [26]), which promotes cell-cycle arrest and apoptosis. In response to DNA damage, the protein kinase ATM (ataxia telangiectasia mutated) phosphorylates the E3 MDM2 at several redundant sites near its RING domain to prevent its oligomerization [27]. Oligomerization of the RING domain of MDM2 is required for its polyubiquitylation of p53, a signaling event that leads to p53 proteolysis [28]; thus ATM-mediated phosphorylation of MDM2 in response to damaged DNA stabilizes p53 protein levels.

Other MDM2 interactions stimulate p53 degradation, including its phosphorylation at Ser260 by polo-like kinase-1 [29] and its interaction with death-domain-associated protein DAXX [30]. DAXX enhances the intrinsic activity of MDM2 towards p53 and functions as a scaffolding protein to recruit the deubiquitylating enzyme HAUSP, which protects MDM2 from degradation [31] by removing ubiquitin chains that were formed by MDM2 autoubiquitylation [32]. In the nucleus, these actions are counteracted by the tumor suppressor RASSF1A (Ras association domain family protein 1A), which binds MDM2 and DAXX, but displaces HAUSP, thereby destabilizing MDM2 [33]. The MDM2 example highlights the use of multilayered interactions to modulate E3 activity towards specific substrates in response to distinct cellular events. It is worth noting that phosphorylation of a substrate protein can also promote E3 recruitment or displacement [34,35].

In humans, ~95 deubiquitylating enzymes (DUBs) deconjugate ubiquitin chains and remove ubiquitin from substrates (Figure 2d; for a recent review, see Ref. [36]). DUBs seem to be integral to ubiquitin signaling pathways [37] and, like E3s, rely on multivalent interactions to achieve specificity. For example, the polyglutamine disease-associated DUB ataxin-3 contains ubiquitin interacting motifs (UIMs) that impart selectivity to its protease domain for Lys63- and not Lys48-linked chains [38].

As indicated by the MDM2–HAUSP example, DUB and E3 ligase activity can be coordinated to enable tight regulation of ubiquitin signaling events. Indeed, 26 human DUBs are known to bind to one or more proteins involved in attaching ubiquitin to protein substrates [37]. An important example of coordinated ligase and DUB activity is provided by A20, a key inhibitor of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway. NF- κ B transcription factors are essential for immune response, cell survival response and cellular proliferation. A20 harbors both DUB and ubiquitin ligase activity and can convert ubiquitin chains on the receptor-interacting protein (RIP) kinase from Lys63 into Lys48 linkages [39]. This activity shuts down NF- κ B signaling through RIP in two ways; Lys63-linked ubiquitin chains on RIP are required for tumor necrosis factor (TNF)-induced NF- κ B activation [40] and the Lys48-linked chains lead to RIP degradation [41].

Ubiquitin chain variability provides receptor selectivity

The variable length and linkage type of ubiquitin chains provides selectivity to the outcome of ubiquitylation, because some ubiquitin receptors have strong preferences for ubiquitin chains of certain linkage type or size. Receptor specificity for ubiquitin chains of distinct linkage type couples the activities of ubiquitin processing enzymes with downstream signaling events. More than 20 different ubiquitin-binding domain (UBD) families, which exist in >150 human receptor proteins, have been identified to date. The mechanisms that they use to achieve selectivity for specific ubiquitin modifications have been

comprehensively reviewed elsewhere [2]. We discuss a few prominent examples of ubiquitin receptor selectivity below.

Selectivity for ubiquitin chains of certain linkage can be conferred by the interaction of single UBDs with neighboring ubiquitin moieties or with the region that links the ubiquitins together. For example, proteasome-associated ubiquitin receptor hHR23a (human homologue of Rad23 protein a) has a C-terminal ubiquitin-associated (UBA) domain that sandwiches between the two ubiquitin subunits of Lys48-linked diubiquitin to encompass a surface that includes a binding epitope unique to the Lys48 linkage [16].

Alternatively, the coordinated binding of two ubiquitin-binding elements to multiple ubiquitins within a chain can specify chain selectivity. In response to DNA double strand breaks (DSBs), histones H2A and H2AX are ubiquitylated with Lys63-linked chains. This modification triggers DNA-damage response by recruiting to the damage site ubiquitin receptor RAP80 (receptor associated protein 80) and in turn, the breast cancer suppressor protein complex BRCA1 [42], which modulates cell-cycle checkpoints and DNA repair (Figure 1a). RAP80 has a strong preference for Lys63-linked chains over other ubiquitin chains, which is caused by structural features of its two UIMs and their intervening region [43]. This entire region forms a continuous helix that orients the two UIMs in a manner optimal for binding to neighboring ubiquitin moieties of Lys63-linked chains [43,44]. Hence, the Lys63-linked chains on H2A and H2AX serve as a switch to stimulate RAP80 and BRCA1 trafficking to DSBs.

Some ubiquitin receptors recognize different chain linkages with remarkable specificity to discriminate between highly similar structures. Lys63 and Met1 are within 7 Å of each other in ubiquitin and linkage through these amino acids results in an almost identical ubiquitin chain conformation [14]. However, the UBAN (Ubiquitin binding in ABIN and NEMO) domain of NEMO (NF-κB essential modulator) differentiates between these two linkage types, as it binds Met1- and Lys63-linked diubiquitin with K_d values of 1.6 μM [45] and 131 μM [46], respectively. The higher affinity for Met1-linked diubiquitin is caused by the binding of the UBAN domain to both of its ubiquitin moieties and their linker region [45]; this UBD binds to only one ubiquitin moiety of Lys63-linked diubiquitin [47]. The TAB2 NZF domain also discriminates between Met1- and Lys63-linked chains; it binds Met1- and Lys63-linked diubiquitin with K_d values of 148 μM and 8 μM, respectively [11]. This UBD binds adjacent ubiquitin moieties of Lys63-linked diubiquitin via two distinct binding surfaces [11]. Such tandem binding is not possible for Met1-linked chains, because they cannot adopt the required configuration [11].

Ubiquitin chain multivalency enables simultaneous interaction with multiple ubiquitin-binding domains

The binding of multiple UBDs to ubiquitin chains provides a general mechanism for enhancing the binding affinity of ubiquitin receptors for ubiquitylated substrates (Figure 3b). In humans, the proteasome component S5a has two UIMs (although an embryo-specific splice variant exists that lacks one of them [48]), which are separated by flexible linker regions causing their relative orientation to be undefined [49]. This flexibility is markedly restricted when S5a binds to Lys48-linked diubiquitin because each UIM binds a ubiquitin moiety of the same molecule simultaneously [50]. The outcome of this coordinated binding is significantly increased affinity between S5a and diubiquitin with a K_d decrease from 73 μM with monoubiquitin to 8.9 ± 0.6 μM with Lys48-linked diubiquitin [50], suggesting that its two UIMs are not used to recruit multiple substrates to the proteasome simultaneously, but rather to increase affinity for each ubiquitylated substrate. It is worth noting that S5a and hHR23a can bind a common ubiquitin chain [51], as can S5a and Rpn13 [50], the other

intrinsic ubiquitin receptors of the proteasome [52]. The biological significance of these interactions is not yet clear; however, complexes of multiple ubiquitin receptors with a ubiquitylated substrate provide additional levels of multivalency, which most likely leads to a greater binding affinity. Such complexes also seem to operate during endocytic processes to enhance binding affinity. Although monoubiquitylation is sufficient for receptor internalization during endocytosis [53], quantitative mass spectrometry indicates that more than half of all ubiquitylated epidermal growth factor receptor (EGFR) is conjugated with ubiquitin polymers (largely connected by Lys63) [54]. Most likely, modification with a polymeric ubiquitin chain enables more interactions with the UIMs of endocytic adaptors and in turn, interactions of higher affinity compared with that possible with only one ubiquitin subunit.

A recent study demonstrated the importance of the avidity that results from simultaneous binding of multiple UBDs to a ubiquitin chain for Lys63 selectivity [55]. In particular, previously published Lys63 specificities for various UBA domains were found to arise from avidity artifacts, because the UBDs being analyzed were fused to the dimeric protein glutathione-S-transferase (GST) [55]. Hence, UBD oligomerization can lead to ubiquitin chain specificities that arise from avid combinations of intrinsically nonselective interactions.

Multivalent interactions involving ubiquitin chains and multiple ubiquitin receptors can also transduce signals, as exemplified by the kinase activation mechanism in the NF- κ B pathway. Many signaling pathways that activate NF- κ B converge on ubiquitin-dependent TGF β -activated kinase 1 (TAK1) activation of I- κ B kinase kinase (IKK). IKK phosphorylation by TAK1 requires the regulatory subunit of the IKK complex NEMO and the presence of ubiquitin chains. The nature of ubiquitylation in this pathway is complex and seems to involve both Met1- and Lys63-linked chains [45]; however, the ubiquitin chains probably function as a scaffold to nucleate TAK1 and IKK through their UBDs (TAB2 and NEMO, respectively; Figure 1b), because IKK activation is supported even by unanchored Lys63-linked chains *in vitro* [56].

Multivalent interactions of ubiquitin receptors influence the fate of ubiquitylated substrates

Surfaces of ubiquitin receptors that do not bind ubiquitin play key roles in the trafficking and processing of ubiquitylated substrates (Figure 3c). The first signaling role discovered for ubiquitylation was its targeting of proteins to the 26S proteasome (a 2.5 MDa proteolytic machine, described in Ref. [57]) for degradation (Figure 1c). Ubiquitin receptors associated with proteasomal degradation have regions that dock them into the proteasome or that enable transient interaction with its components. The UBD of one such receptor, Rpn13, assembles into the proteasome via a surface opposite to its ubiquitin-binding region [52,58] and this protein contains another domain that binds [59–61] and activates [60,61] Uch37, one of the three DUBs of the proteasome. Before their degradation by the proteasome, substrates are deubiquitylated and unfolded for passage through a narrow chamber leading to the catalytic center of the proteasome. Rpn13 might perform a dual functionality in the capture and deubiquitylation of proteasome substrates through its multivalent interactions with ubiquitylated substrates and Uch37.

Some protein aggregates or inclusions that are refractory to proteasomal degradation can be removed by the autophagy - lysosome pathway, in which autophagic vesicles engulf components to be degraded before fusion with a lysosome (Figure 1d). New findings suggest that ubiquitin might serve as a selective degradation signal for autophagic targeting through ubiquitin receptors p62 (also called SQSTM1) and NBR1 (neighbor of BRCA1).

These two proteins bind LC3 (Atg8 (autophagy related gene 8) in yeast) [62,63], which is covalently attached to and defines the autophagic membrane. Bacteria that enter the cytosol of mammalian cells are also ubiquitylated and targeted for destruction by autophagy; recent findings show that the ubiquitin receptor NDP52 (nuclear dot protein 52) can bind simultaneously to ubiquitin-coated bacteria and LC3 [64].

The integration of multivalent ubiquitin-dependent interactions with ubiquitin-independent interactions is used extensively for endocytic trafficking to direct proteins from the plasma membrane to multi-vesicular bodies (MVBs) (Figure 1e). Endocytic adaptors EPS15 (epidermal growth factor receptor substrate 15) and epsins (EPS15-interacting proteins) are recruited to ubiquitylated cell-surface receptors through their multiple UIMs. Epsins also bind to clathrin [65] and induce membrane curvature [66], and the coordinated action of these independent interactions mobilizes ubiquitylated substrates into the central parts of clathrin-coated pits for receptor internalization into vesicles called early endosomes [67]. Once at the early endosome, ubiquitylated proteins require ESCRT (endosomal sorting complexes required for transport) complexes for sorting into MVBs [68] (Figure 1e), and this trafficking similarly relies on multivalent ubiquitin-dependent and ubiquitin-independent interactions. HGS (hepatocyte growth factor-regulated tyrosine kinase substrates; yeast Vps27) and STAM (signal transducing adapter molecule) of ESCRT-0 bind ubiquitin, clathrin and PI3P (phosphatidylinositol 3-phosphate). These interactions recruit and concentrate cargo at specific clathrin-coated endosomal subdomains (reviewed in Ref. [69]). HGS recruits ESCRT-I through its interaction with TSG101 (tumor susceptibility gene 101), which also binds ubiquitin through a UEV (ubiquitin-conjugating enzyme E2 variant) domain. Multivalent interactions with PI3P, ubiquitin and ESCRT-I similarly recruits ESCRT-II and, in turn, ESCRT-III to sites of MVB biogenesis. Hence, trafficking through this pathway occurs through the coordinated actions of many ubiquitylation-dependent protein-protein interactions.

Intramolecular interactions between ubiquitin-binding regions and covalently attached ubiquitin can inhibit ubiquitin receptor activity

Ubiquitin receptors can be conjugated with ubiquitin, which in turn binds to their UBDs (Figure 3d). Such intramolecular interactions can inhibit intermolecular interactions with their ubiquitylated substrates. This mode of regulation exists in the endocytic pathway. HGS, EPS15 and epsin undergo coupled monoubiquitylation, such that their UBD mediates their own ubiquitylation by binding to a ubiquitylated E3 [70] or to an E3 with a ubiquitin-like domain [71]. This modification leads to *cis* interactions with the attached ubiquitin, which inhibits *trans* interaction with ubiquitylated targets [72]. The role of this so-called coupled monoubiquitylation in endocytosis remains poorly understood. It is possible that it weakens the interaction with cargo to enable ready passage of the substrate. The DUB UBPY (ubiquitin-specific protease Y) could relieve this autoinhibition by removing the conjugated ubiquitin from receptors [73,74], thus activating them towards new substrates.

Substrates modulate the effects of their ubiquitylation

Although the fate of a ubiquitylated substrate is largely determined by its interaction with ubiquitin receptors, some substrate features can modulate the effects of ubiquitylation even after their recognition by a receptor of a designated function. For example, interaction with the proteasome typically culminates in the degradation of ubiquitylated substrates; this mechanistic pathway is an effective means to control protein lifespan. Such degradation, however, seems to require substrates to harbor, or be 'complexed' with, a protein containing an unstructured region [75,76], and proteins that are not ubiquitylated can be proteolyzed

simply by associating with those that are, so long as they contain an unstructured region [76].

By contrast, folded domains within ubiquitylated proteins appear to protect substrates from degradation, as exemplified by the NF- κ B precursors p105 (NF- κ B1) and p100 (NF- κ B2). These NF- κ B precursor proteins contain C-terminal I κ B-like ankyrin repeats that inhibit NF- κ B transcriptional activity [77]. This inhibitory effect is alleviated by the proteasome, which cleaves p105 and p100 to generate their active, DNA-binding forms, namely p50 and p52, respectively [78,79]. One of the contributing factors for the partial proteolysis of these precursor proteins is the stability of the N-terminal Rel homology domain [80], because residues essential for Rel homology domain stability are also essential for p105 processing [81].

Between the Rel homology domain and ankyrin repeats lies a glycine rich region, which is also required for processing of these NF- κ B precursor proteins [82]. Regions of low sequence complexity have similarly evolved in various viral proteins and impair their proteasome-mediated degradation. Epstein-Barr nuclear antigen 1 (EBNA1) is ubiquitylated and recognized by the proteasome, but a glycine-alanine tract interacts inefficiently with proteasomal ATPases, thereby impairing its unfolding and translocation into the 20S catalytic core [83,84]. Similarly, Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen 1 (LANA1) has a highly repetitive acidic sequence, which impairs its degradation [85].

Ubiquitylation can change binding affinities by adding multivalency to already existing interactions

In the nucleus, ubiquitylation is widely used to change the affinity of already existing interactions by adding multivalency (Figure 3e). For example, PCNA encircles DNA to serve as a 'sliding clamp' for DNA polymerases during DNA replication. When a damaged site is encountered, replication is stalled and PCNA is monoubiquitylated at Lys164 (Figure 1a). This modification is recognized by UBDs of trans-lesion polymerases to increase their affinity for PCNA and to promote their error-prone trans-lesion synthesis mode of replication [86,87]. After bypassing the lesion, the error-free, processive polymerase takes over. This switch might be due to PCNA deubiquitylation because the exchange back to the processive polymerase is prohibited when PCNA is monoubiquitylated at Lys164 [88].

Protein ubiquitylation is also used to alter the DNA-binding affinity of nucleotide excision repair (NER) factor xeroderma pigmentosum group C (XPC). NER targets DNA damage throughout the genome by the coordinated action of the UV-damaged DNA binding protein1 (DDB1)–DDB2 complex and the XPC–HR23B complex. Upon UV exposure, a complex formed by DDB1–DDB2, E3 ligase cullin 4A, and regulator of cullins-1 (ROC1) is recruited to damage sites and ubiquitylates DDB2 and XPC [89]. Whereas DDB2 ubiquitylation culminates in its degradation [90], XPC ubiquitylation enhances its DNA binding affinity [89]. These modifications enable damaged DNA to be transferred from DDB2 to XPC, which otherwise exhibits stronger affinity for damaged DNA [91]. This switch is important because downstream NER factors are recruited through XPC–HR23B [92].

Ubiquitylation is also used to weaken substrate interactions with binding partners. Histones H3 and H4 are ubiquitylated in response to UV-induced DNA damage, facilitating the recruitment of NER machinery to damaged sites by weakening histone–DNA interactions [93]. H2AX ubiquitylation is similarly used to signal for its release from chromatin at DSBs [94]. Histone ubiquitylation is also implicated in the regulation of gene transcription, although less is known of the mechanistic details (Figure 1f). Nucleosomes are dynamically

evicted and reassembled during transcript elongation to provide RNA polymerase II transient access to DNA. H2B ubiquitylation plays a part in nucleosome dynamics [95] and histone H3 methylation [96] by affecting nucleosome stability [97].

Concluding remarks

Ubiquitin-mediated signaling is enabled by a large repertoire of enzymes that control the timing of modification, create diversity in the ubiquitin signal itself and enable dynamic alteration of the modification throughout a signaling pathway or in response to new stimuli. These enzymes communicate through ubiquitin to downstream receptors that operate within a larger context to enable signaling specificity. Versatility and specificity become congruent in ubiquitin signaling pathways through multivalency. Ubiquitin-binding regions are typically just one of many functional surfaces present in the receptor, which can contribute to the binding interaction, subcellular localization or link ubiquitin signaling with other post-translational modifications, such as phosphorylation. Although we have not discussed them in this review, ubiquitin belongs to a family of ubiquitin-like proteins that resemble ubiquitin structurally and perform their own distinct signaling, which can cross-talk with ubiquitin signaling. The ubiquitin signaling network is of therapeutic importance because parts of it are hijacked by pathogens or compromised in human diseases. It therefore is likely to have yet uncharted therapeutic potential and the manipulation of ubiquitin-mediated protein degradation is actively being pursued for such purposes. Currently, the proteasome inhibitor bortezomib (Velcade™) is used to treat multiple myeloma and mantle cell lymphoma; this inhibitor preferentially induces apoptosis in tumor cells [98]. The underlying mechanisms of its greater cytotoxicity in tumor cells are complex, ranging from the specific accumulation of proapoptotic proteins such as NOXA (NADPH oxidase activator 1) [99] to the activation of apoptosis through an endoplasmic reticulum (ER) stress response [100]. Perhaps not surprisingly, drugs targeting the proteasome suffer from unwanted side effects, because many physiologically important processes are regulated by proteasomal proteolysis. Targeting of specific E3s, DUBs or ubiquitin receptors might afford clinical efficacy with fewer side effects. It is foreseeable that the multivalent interactions that regulate E3 and ubiquitin receptor activities could ultimately be used to target ubiquitin signaling for specifically restricting viral budding, stabilizing tumor suppressors or promoting DNA repair.

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Figure 1.

The multiple roles of protein ubiquitylation. **(a)** In the nucleus, ubiquitylation signals for proteasome-independent regulation of DNA repair. Histones and PCNA are examples of nuclear targets of ubiquitylation. **(b)** Ubiquitylation functions in kinase activation by acting as a scaffold to bring kinases and their substrates together to enable the activation of kinase cascades, as exemplified by the activation of IKK by TAK1. The E3 TRAF6 (TNF receptor-associated factor 6) ubiquitylates several proteins including NEMO and itself upon ligand stimulation. TAK1 and IKK complexes interact with each other upon binding these ubiquitin chains with their ubiquitin-binding subunits (TAB2 and NEMO, respectively). IKK is thus activated by TAK1 and competent to phosphorylate IκB, which leads to its ubiquitylation and proteasomal degradation **(i)**. IκB degradation releases NF-κB into nucleus, thereby activating gene transcription. **(c)** Proteasome-mediated degradation **(i)** regulates many cellular processes through targeted degradation of key regulatory proteins. **(d)** Ubiquitylation signals for protein quality control to degrade misfolded proteins. Protein quality control of ER proteins occurs through the ER-associated protein degradation (ERAD) pathway by proteasomal degradation **(i)**, whereas some large protein aggregates are eliminated through the autophagy (Av, autophagic vesicles)-lysosome route **(ii)**. Polyubiquitylation acts as a signal for autophagy targeting. **(e)** Ubiquitylation signals for vesicular trafficking between membrane compartments. **(iii)** Ligand-bound plasma membrane proteins internalized into early endosomes (EE). **(iv)** Proteins sorting into

multivascular bodies (MVB). **(v)** Newly synthesized membrane proteins sorting from the trans-Golgi network (TGN) to the MVB. **(f)** Ubiquitin signaling regulates gene transcription.

Figure 2.

Different forms of protein ubiquitylation. Protein substrates (grey) can be (a) monoubiquitylated with a single ubiquitin (ub, blue), (b) multiubiquitylated or (c) polyubiquitylated. (c) Ubiquitin chains can form (i) extended or (ii) closed conformations of one or more linkage type or (iii) even forked chains with multiple ubiquitins attached to a common moiety. (d) Ubiquitin is activated by an E1 activating enzyme (brown) and conjugated to its substrate (grey) through the coordinated action of E2 conjugating (orange) and E3 ligase (yellow) enzymes. Substrate recognition is typically conferred by E3s, whereas ubiquitin chain linkage specificity can be determined by E2, E3 or E2–E3 pairs. DUBs (purple) can trim ubiquitin moieties from chains to reverse the effects of substrate ubiquitylation and recycle the ubiquitin, or to edit the chain length or linkage type.

Figure 3.

The diversity and specificity of ubiquitin signaling is relayed through different layers of coordinated protein–protein interactions (multivalency). **(a)** E3 ligases are tightly regulated to target a specific substrate at a specific time and location through multiple protein–protein interactions. Autoubiquitylation is a common way to regulate E3 ligase activity and is counteracted by a specific DUB. Phosphorylation is another mechanism used to activate or suppress E3 activity toward a substrate. Some E3 ligases also bind accessory proteins that can suppress their activity or mediate other interactions. **(b)** The moieties of a ubiquitin chain coordinate the simultaneous binding of multiple ubiquitin-binding domains (UBDs) from the same protein or from different ubiquitin receptors. This mode of multivalency can lead to preferences for chains of specific linkage type, to increased binding affinity and to the bridging of multiple ubiquitin receptors. **(c)** The consequence of a ubiquitin receptor binding to a ubiquitylated substrate is determined through multiple coordinated protein interactions. Ubiquitin receptors usually contain functional domains that direct localization to specific sites or complexes within a cell. They can also contain elements that interact with enzymes, such as kinases, DUBs or E3s. **(d)** Some ubiquitin receptors can be ubiquitylated and their UBDs can bind intramolecularly to ubiquitin. Such interactions relay an inhibitory effect such that the receptor no longer binds to its substrates; this effect is relieved by DUB activity. **(e)** In some cases, ubiquitylation is used to change binding affinities between protein–protein and protein–DNA interactions. In this example, interaction between covalently attached ubiquitin and a UBD leads to an increased binding affinity.