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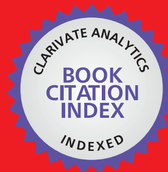

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Chapter

Branching and Mixing: New Signals of the Ubiquitin Signaling System

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Abstract

Posttranslational modifications allow cells and organisms to adapt to their environment without the need to synthesize new proteins. The ubiquitin system is one of the most versatile modification systems as it does not only allow a simple on–off modification but, by forming a chain of ubiquitin molecules, allows conveying multiple signals. The structure of the chains is dependent on the linkage to the previous ubiquitin molecule as every lysine can serve as an acceptor point for this modification. Different chain types code for specific signals ranging from protein degradation to protein targeting different cellular compartments. Recently the code of ubiquitin signals has been further expanded as branching and mixing of different chain types has been detected. As an additional layer of complexity, modifications of the ubiquitin chain by ubiquitin-like modifiers, like NEDD8, SUMO, or ISG15, have been found. Here we will discuss the different chain types and the technical challenges which are associated with analyzing ubiquitin topology-based signaling.

Keywords: ubiquitin, chain topology, ubiquitin-like, branched ubiquitin

1. The ubiquitin-conjugating system

Since its discovery in the 1980s, the ubiquitin signaling system has gained recognition as one of the most versatile, yet complicated, posttranslational signaling systems. The central component of the system is the small protein ubiquitin (76 aa). Ubiquitin itself is always expressed as an immature precursor protein, either fused to a ribosomal protein or as a head-to-tail fusion of five or six ubiquitin moieties. The precursor protein is processed co-translationally and cleaved off the fusion protein right at the ribosome [1] liberating the mature ubiquitin protein. The modification of a target protein with ubiquitin as a PTM utilizes an enzymatic cascade. In the first step, an ubiquitin-activating enzyme (E1) is binding ubiquitin while hydrolyzing one molecule of ATP to AMP forming a thiol ester of ubiquitin's C-terminus with a cysteine residue in its active center. The activated ubiquitin can then be transferred to an ubiquitin-conjugating enzyme (E2) which again forms a thiol ester with a cysteine in its active center. Depending on the cascade which is used, the final transfer is catalyzed by one of three classes of ubiquitin ligases,

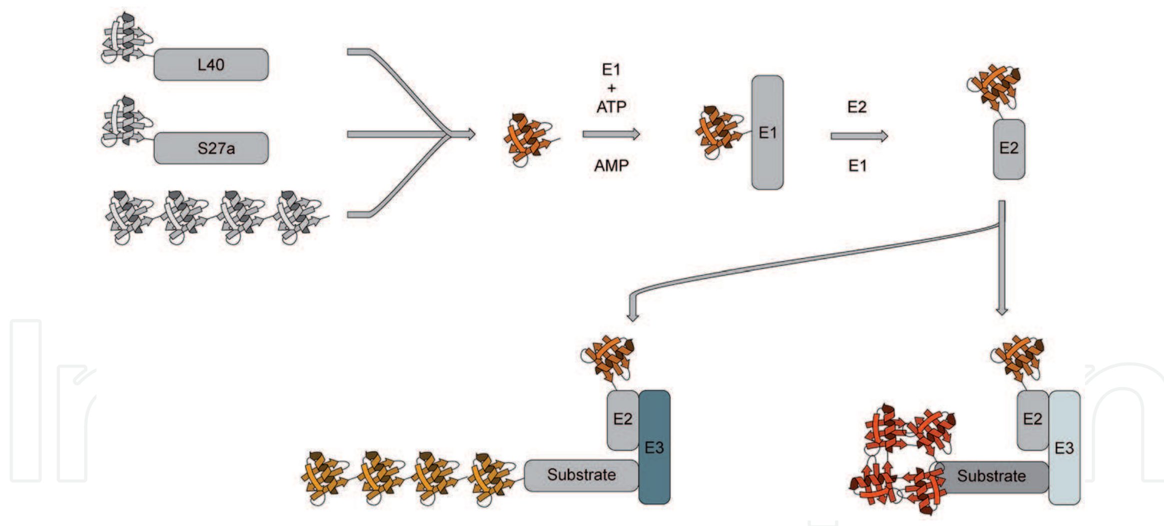

Figure 1.
The ubiquitin-conjugating system. Ubiquitin (gray folded structure) is expressed as fusions either with ribosomal subunits or as ubiquitin multimers, which are cleaved co-translational. The mature ubiquitin (orange) is released and under the consumption of one ATP bound to the activating enzyme (E1). The activated ubiquitin is then passed on to the conjugating enzyme (E2), which finally catalyzes the transfer to a substrate protein involving an E3 ligase. The reaction can then be repeated and catalyzes the formation of a polyubiquitin chain. Depending on the lysine residue in ubiquitin used, the chain can have different structures, as indicated in yellow for a linear ubiquitin chain or in red for a K48-linked chain.

really interesting new gene (RING), ring between ring (RBR), or homologous to E6 C-terminus ligases (HECT ligases) (**Figure 1**). While RING-type ligases are associating with the E2 enzyme and bringing the target protein in close proximity to the E2/E3 complex, RBR and HECT-E3 are able to bind ubiquitin itself. The final transfer of ubiquitin to the target is then catalyzed without the help of an E2 enzyme.

This modification leads to a single modification of the substrate protein with ubiquitin but can also be extended by multiple rounds of modification with ubiquitin itself being the acceptor of the modification, leading to the formation of the ubiquitin chain. The structure of the chain is dependent on which linkage is used within the ubiquitin chain. The chains highly varies in shape from a very compact structure for a K48-linked chain [2–4] to a long stretched shape in the case of K63 [5, 6] and linear ubiquitination [3]. Each of the different chain topologies is associated with different biological functions. Besides the signaling through different chain topologies, ubiquitin signaling can also occur through modifications by a single ubiquitin (monoubiquitin) or multiple monoubiquitinations.

Like for many other PTM systems, the ubiquitin signaling system has possibilities to erase the signal by either disassembling the polyubiquitin chain or by removing ubiquitin from its target. These enzymes are called ubiquitin hydrolases or ubiquitin de-conjugating enzymes (DUBs). Most DUBs belong to the enzymatic class of cysteine hydrolases, which carry a cysteine in their active center. Research on DUB specificity has shown that these enzymes possess a high linkage specificity indicating clear regulatory functions in the cell and are not acting as simple quality check enzymes [7].

2. Ubiquitin-like modifiers (Ubl)

Besides ubiquitin, there are a number of proteins which share significant similarity to ubiquitin. They fall essentially into two groups: proteins with a ubiquitin-like domain and small proteins with a similar size as ubiquitin. The small Ubls like ubiquitin, SUMO, NEDD8, Urm1, Apg8, Apg12, ISG15, Fat10, and Ufm1 are highly

conserved among eukaryotes. Many of these small proteins have been found to be covalently conjugated to a substrate protein, utilizing their own conjugation cascades, which usually consists of an activating enzyme and a conjugating enzyme (see **Table 1**).

2.1 NEDD8

Neuronal precursor cell-expressed developmentally downregulated protein 8 (NEDD8) is structurally the closest relative to ubiquitin. Its E1 enzyme is split into two different parts which are forming the activation enzyme (NAE1/UBA3) [8]. The activated NEDD8 moiety is transferred to the NEDD8-conjugating enzyme E2s (UBC12/UBE2M or UBE2F) and substrate-specific NEDD8 E3 ligase [9]. Unlike the large group of ubiquitin E3 ligases, there are only about 10 different NEDD8 E3 ligases [9], and most of them belong to the group of RING E3 ligases.

The best-characterized physiological neddylation substrates are the cullin proteins (Cul-1, Cul-2, Cul-3, Cul-4A, Cul-4B, and Cul-5) which form the backbone structure of cullin-RING ligases (CRLs). The conjugation of the cullin subunit modulates the activity of E3 ligase [10]. Deconjugation of the NEDD8 is catalyzed by the signalosome complex which removes NEDD8 from the cullin [11–14].

Recently, several other proteins have been identified, which are modified by NEDD8 including ubiquitin itself, p53, mouse double minute 2, and epidermal growth factor receptor (EGFR) [15–17].

2.2 SUMO

Small ubiquitin-related modifier (SUMO) is probably the best-studied Ubl protein. It is highly conserved among eukaryotes with one gene in lower eukaryotes like baker's yeast (Smt3) [18] which developed into three homologs in humans, SUMO-1, SUMO-2, and SUMO-3. SUMO-2 and SUMO-3 are closely related, while SUMO-1 is more divergent. SUMO-1 does not form polymeric chains, while SUMO-2 and SUMO-3 mainly form K11-linked homotypic SUMO chains [19, 20]. SUMO-1 can be linked to the end of a poly-SUMO-2/SUMO-3 chain, effectively terminating chain growth [20]. Like NEDD8, SUMO is activated by a dimeric activating enzyme (SAE1/SAE2). The recognition of the target proteins is done by the conjugating enzyme Ubc9 which recognizes the main SUMOylation motif ΨKxE (Ψ = hydrophobic residue) [21–23]. Some reactions are further enhanced by the action of other E3 ligases, like RANBP2. These E3 ligases catalyze the transfer by recruiting the substrate or catalyzing the transfer of SUMO from Ubc9 [24, 25]. Similar to other Ubls, modification with SUMO can be reversed by specific proteases as summarized in Pichler [26].

Most SUMO-1 is conjugated to RANGAP1 near the nuclear pore. SUMO-2 is partially cytosolic, while SUMO-3 is mainly located in nuclear bodies. In unstressed cells, most SUMO-2 and SUMO-3 are not conjugated. Upon stress induction

Ubl	E1	E2
NEDD8	UBA3 (UBE1C), APPBP1 (NAE1)	UBE2M
SUMO	SAE1, SAE2	UBE2I
ISG15	UBA7	UbcH8

Table 1.
Ubiquitin-like modifiers that have been found linked to ubiquitin chains and their enzymatic activation cascade.

(e.g., folding stress) both SUMO-2 and SUMO-3 get conjugated to target proteins [27]. SUMO-1 conjugation has been proposed to regulate trafficking between nucleus and cytosol but also change protein–protein interaction [26, 28, 29]. SUMO modification plays an important role in a number of cellular processes like DNA replication, cell cycle regulation, nuclear trafficking, signal transduction, and protein degradation [30–32]. Recently, large-scale studies identified more than 1000 SUMO targets and increased the number of cellular processes even further [33].

2.3 ISG15

Unlike other small ubiquitin-like modifiers interferon-stimulated gene product 15 (ISG15) contains two ubiquitin-fold domains. It is massively induced by interferon treatment, ischemia, DNA damage, and aging as a monomer as well as a conjugated protein (reviewed in [34]. ISGylation requires a three-step enzymatic cascade involving an E1 activating enzyme (Ube1L), an E2 conjugating enzyme (UbcH8), and an E3 ligase (Herc5 or TRIM25/EFP) [35]. ISGylation is reversed by Ub-specific protease USP18 [36].

Several protein targets and cellular functions have been identified, which are regulated by ISGylation. These include the regulation of DNA damage response, autophagy, protein synthesis, the downregulation of the ubiquitin-proteasome system, and the regulation of HIFα in response to hypoxia [37–42].

A particular interest is the finding that modification of nascent proteins by ISGylation occurs after viral infection [43]. Virus infection induces host antiviral responses, including induction of type I interferons [44–47]. The transcription factor IRF3 recruits HERC5 and induces conjugation of ISG15 onto IRF3. This modification attenuates the interaction between Pin1 and IRF3, thus antagonizing IRF3 ubiquitination and degradation. Consistently, host antiviral responses are boosted or crippled in the presence or absence of HERC5, respectively [48–50].

3. Ubiquitin chain topology

Ubiquitination occurs in proteins at one or multiple lysine residues. Ubiquitin itself, containing seven lysine residues, can be ubiquitinated at each one of these lysine residues, as well as at the N-terminal methionine [51, 52]. Proteins can be monoubiquitinated, where a single ubiquitin is conjugated to a lysine residue in the substrate; multi-monoubiquitinated, where a single ubiquitin is conjugated to multiple lysine residues in the substrate; or polyubiquitinated, where the ubiquitin conjugated to the substrate is ubiquitinated itself. Polyubiquitinated chains can be divided into homotypic chains and heterotypic chains, and like for linear chains, different chain topologies lead to different structures and functions in the cell [53–58]. To add to this complexity, ubiquitin chains themselves can also be modified by ubiquitin-like modifiers (**Figure 2**).

3.1 Homotypic chains

Homotypic chains are composed of several ubiquitins linked together through the same lysine or N-terminal methionine residues. This leads to a total of eight possible chain types. Each chain adopts a different conformation: K6, K11, and K48 adopt "compact" conformations, while K27, K29, K33, K63, and M1 adopt "open" conformations, allowing recognition of these chains by different ubiquitin-binding partners implicated in several signaling pathways [53–59]. A short description of the functions of homotypic chains is given below.

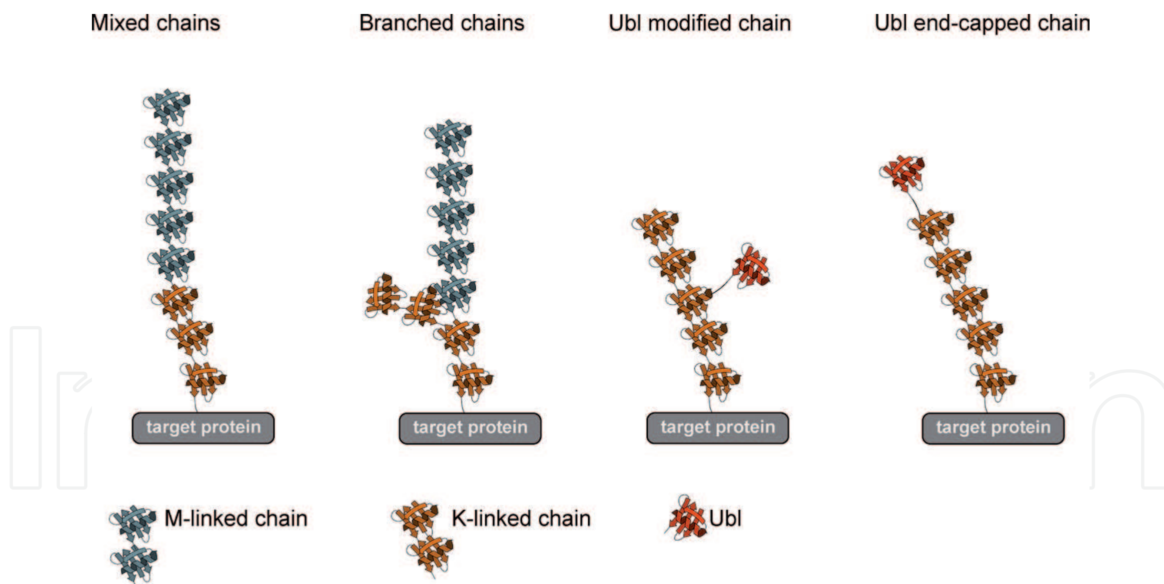

Figure 2.
Complex ubiquitin chains. Homotypic ubiquitin chains can be extended by a different type of chain leading to a mixed ubiquitin chain. If the ubiquitin chain is modified not at the last ubiquitin moiety, a branched ubiquitin chain is created. For mixed ubiquitin/Ubl chains, two attachment points are possible: either as a cap structure, modifying the last ubiquitin, or as a branching point on one of the ubiquitins in the middle.

Studying K6 chains is challenging since this chain is among the less abundant ubiquitin chains [60, 61]. Although its function is still not very clear, K6 has been implicated in mitophagy regulation [62–64] and DNA damage response [65]. More recently Michel et al. showed that HECT E3 ligase HUWE1, previously implicated in cellular processes like DNA repair, stress response, cell death, differentiation, and mitophagy [66, 67], assembles K6 chains [68]. Mitophagy is the process by which cells maintain the energy metabolism by removing damaged mitochondria. During this process, PINK1 accumulates on the surface of the mitochondrial outer membrane and recruits cytosolic PARKIN, an E3 ubiquitin ligase [69]. PARKIN then ubiquitinates mitochondrial proteins by generating canonical (K48 and K63) and noncanonical chains (K6 and K11) eventually leading to mitophagy [63]. USP30 is the only known DUB anchored to the mitochondria outer membrane which has been seen to act as a regulator of mitophagy. Despite having been seen to cleave K6, K11, K48, and K53 chains, USP30 prefers K6 chains [62, 64]. Under normal conditions, USP30 prevents mitophagy of normal mitochondria by maintaining ubiquitination at low levels. Under stress conditions and mitochondrial damage, PARKIN is recruited, highly increasing ubiquitination levels and inducing mitophagy. PARKIN and PINK1 are both mutated in patients with Parkinson's disease [70, 71].

Although not many roles are known for K11 chains, it has been shown that these chains are key players in cell cycle regulation and proteasome degradation. The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase and essential for cell cycle regulation. Along with Ube2C, APC/C targets key players in the cell cycle, like securin and cyclin B1, for proteasomal degradation by assembling K11 chains, thus allowing the transition from metaphase to anaphase [72–75]. In 2013, Mevissen et al. showed the ovarian tumor (OTU) DUBs Cezanne's and Cezanne2's linkage specificity towards K11 chains [76]. In 2014, Bremm and co-workers described Cezanne as a new regulator of HIF1α homeostasis [77], where HIF1α is ubiquitinated with K11 chains. The knockdown of Cezanne increases the amount of K11 polyubiquitin chains and decreases the activity of HIF1α. HIF1α degradation was not disrupted by inhibition of the proteasome suggesting an alternative degradation pathway—possible through chaperone-mediated autophagy—to HIF1α [77]. Cezanne can bind and disassemble K11 chains on APC/C substrates

stabilizing them leading to cell proliferation [78]. Finally, K11 chains were shown to replace K48 chains in the transcription factor Met4 activating it [79], so far only seen to be ubiquitinated with K48 chains leading to transcription repression [80]. Although the exact composition of the newly synthesized K11 chains is still not known, the authors suggest that these chains can either be homotypic K11 chains or heterotypic K11/K48 chains [79].

K27 chains are still the least studied of all ubiquitin chains. E3 protein ligase HACE1 has been shown to assemble K27 chains onto both optineurin and YB-1 [81, 82], indicating a role in secretion through the multivesicular body (MVB) pathway. The ubiquitin ligase RNF168 assembles K27 ubiquitin chains on chromatin linking them to the DNA damage response pathway [83]. During pathogen infection, K27 chain assembly triggers immune response through the recruitment of TBK1 [84, 85]. The NEDD4 family E3 ligases, Itch and WWP2, promote K27 polyubiquitination of SHP-1 enhancing the strength of the T-cell receptor (TCR) signal and in turn negatively regulating in T_H2 cell differentiation [86]. USP19, a deubiquitinating enzyme, removes K27 chains from TRIF, thus inhibiting its recruitment by TLR3/TLR4 and consequently inhibiting TLR3−/TLR4-mediated innate immune response [87]. The E3 ligase Hectd3 assembles K27 chains on Malt1 and Stat3 promoting differentiation of pathogenic TH17 cells in experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis [88].

K29 has been implicated in the Wnt signaling pathway. The E3 ubiquitin ligase, EDD, promotes K29 ubiquitination of β-catenin leading to higher protein levels and enhanced activity [89]. E3 ubiquitin ligase SMURF1 promotes K29 ubiquitination of axin, thus disrupting its association with LRP5/LRP6 and inhibiting the Wnt signaling pathway [90]. The ubiquitin thioesterase ZRANB1, also known as TRABID, preferentially hydrolyzes K29 and K33 chains [4, 55, 57, 91]. Although TRABID/ZRANB1 was proposed to bind and hydrolyze K63 chains from the APC tumor suppressor protein acting as a positive regulator of the Wnt signaling pathway [92], no evidence has been shown, linking TRABID and K29 chains in the Wnt signaling pathway. More recently, TRABID has been implicated in epigenetic regulation, where it regulates Il12 and Il23 genes by TLR. TRABID associates with and stabilizes Jmjd2d by hydrolyzing K29 chains regulating histone modifications and expression of Il12a, Il12b, and Il23a genes [93].

K33 chains have seen to be implicated in autoimmunity. RING-type E3 ligase Cbl-b and HECT-type E3 ligase Itch assemble K33 chains to the TCR-ζ and negatively regulate its phosphorylation and consequently TCR signaling [94]. Later in 2014, Lin et al. reported the inhibition of the type I IFN signaling due to the interaction of the DUB USP38 and TBK1, after viral infection. USP38 hydrolyzes K33 chains from TBK1 promoting K48 polyubiquitination by DTX4/TRIP for its degradation through NLRP4 [95]. During infection with uropathogenic *E.coli*, compartmentalized TLR4 signaling is triggered, and TRAF3 is K33 polyubiquitinated leading to the expulsion of intracellular bacteria by the exocyst complex [96]. After TGF-β stimulation, USP2a removes K33 polyubiquitin chains from the TGFBR promoting the recruitment of R-SMADs and consequently promoting metastasis [97]. A role of K33 chains in trafficking was also suggested. KLHL20, a BTB domain-containing adapter protein, recruits Cul-3 assembling K33 chains to Cm7. The ubiquitination of Cm7 promotes its recruitment to the trans-Golgi network (TGN) [98].

More recently, K33 chains have been implicated in autophagy. E3 ligase RNF166 binds and assembles K33 chains to the autophagy adaptor p62. This mechanism seems to be essential in targeting bacteria to autophagy [99]. SMURF1 induces K29- and K33-linked polyubiquitin of UVRAG, triggering a mechanism that promotes autophagosome maturation and inhibits HCC growth. TRABID/ZRANB1 forms a

complex with UVRAG and cleaves SMURF1-induced K29- and K33-linked polyubiquitin chains from UVRAG inhibiting autophagosome flux and leading to poor prognosis [100].

M1 chains and their importance in signaling pathways and disease have been extensively reviewed [101, 102]. M1 chains are generated by the linear ubiquitin chain assembly complex (LUBAC), composed by HOIP, HOIL-1, and SHARPIN [103–105]. M1 chains play a crucial role in TNF signaling and immune response [104–109]. Formation of complex 1 starts when TNF binds TNFR1, resulting in the recruitment of TRADD and RIPK1. After binding to TNFR1, TRADD recruits TRAF2 which then recruits cIAP1 or cIAP2, two E3 protein ligases which assemble K11, K48, and K63 chains to several components of the complex. LUBAC is then recruited by the complex and assembles M1 chains to several components of the complex including NEMO, an essential modulator of NF-κB, RIPK1, TRADD, and TNFR1. This process then recruits the TAK1-TAB protein complex and the IKK complex (IKKα, IKKβ, and NEMO). While the TAK1-TAB complex activates MAPK cascades triggering JNK and p38 MAPK leading to the activation of the transcription factor AP-1, the IKK complex activates NF-κB signaling. In the absence of LUBAC, complex 2 instead of complex 1 is formed leading to cell death either by apoptosis or programmed necrosis.

K48 is the most abundant and well-studied ubiquitin chains and a major signal for proteasome degradation [60, 61, 110]. It was initially proposed that signaling through a polyubiquitin chain was mandatory for proteasome degradation [111–113]. However, several publications seem to show that monoubiquitination can also target proteins for proteasome degradation [114–116].

K63 is the second most abundant ubiquitin chain in cells [60, 61] and it is implicated in immune response, DNA repair, endocytosis, and protein trafficking (reviewed in [117–119]). K63 chains seem to be essential for the activation of the IKK signaling pathway through TRAF6 [120]. TRAF6 also induces K63 ubiquitination of Akt which is then phosphorylated, activated, and recruited to the membrane [121]. RIG-I is regulated by K63 chains where TRIM25 assembles K63 chains to RIG-I [122]. K63 ubiquitination of IRAK1 is required for the activation of NF-κB signaling [123]. INF-β signaling pathway is activated when STING is ubiquitinated with K63 chains assembled by TRIM56 [124]. K63 ubiquitination and protein trafficking and DNA damage have been extensively reviewed [119, 125]. Two of the most well-studied examples on protein trafficking are the MHC I and EGFR. MHC class I molecules are polyubiquitinated with K63 chains leading to degradation by an endolysosomal pathway [126]. EGFR is also ubiquitinated and promotes its internalization [127, 128]. More recently, it has been shown that K63 chains bind to DNA, enhancing the recruitment of repair factors [129].

3.2 Heterotypic chains

Heterotypic chains are composed of several ubiquitin molecules linked together through different lysines or N-terminal methionine residues and can be classified as mixed chains or branched chains. In mixed chains, each ubiquitin is modified only once by another ubiquitin molecule, while in branched chains each ubiquitin can be modified by two or more ubiquitin molecules. Chains sharing the same linkage types can still have different architectures and thus different structures, resulting in a huge number of possible conjugate combinations affecting different signaling pathways (reviewed in [130–132]). Due to their architecture, the study of heterotypic chains represents a challenge, and their functions in cells are still not clear (**Figure 2**).

K11/K48, K48/K63, and M1/K63 are three of the most studied branched chains.

K11 and K48 both target proteins for degradation. Branched K11/K48 ubiquitin chains seem to increase this signal leading to a more efficient recognition of substrates by the proteasome. The APC/C complex assembles K11 chains to substrates, targeting them for protein degradation. During mitosis, the APC/C complex conjugates K11/K48 branched chains to the kinase Nek2A leading to a more efficient recognition by the proteasome for degradation [133]. The binding efficiency of homotypic K11 chains to the proteasome is much lower than that of homotypic K48 chains and heterotypic K48/K11 chains, and that both K48 chains and K11/K48 chains efficiently target cyclin B1 for proteasome degradation [134]. The development of a bispecific antibody to K11/K48 chains allowed the detection of APC/C complex assembling K11/K48 chains during mitosis. Under proteotoxic stress, leading to the accumulation of newly synthesized and misfolded proteins, these linkages seem to accumulate. These chains seem to have a quality control role where they prevent protein aggregation by proteasomal degradation. Among the effectors of these chains are endogenous p97, BAG6, UBQLN2, p62, UBR5, and HUWE1 [135]. More recently, the structure of branched K11/K48-linked tri-ubiquitin was solved, showing the presence of a novel binding surface exclusive to branched K11/K48-linked polyUb as a result of a unique interface between the branched K11 and K48. This interface binds to Rpn1, one of the proteasomal units able to recognize polyUb, and increases binding efficiency [136].

Opposed to K48, K63 chains are non-proteolytic chains playing important roles in different signaling pathways. The combination of K48 and K63 chains in branched chains seems to play an important role in NF-κB signaling [137]. Induced by IL-1βsignaling, HUWE1 cooperates with K63 ubiquitinated TRAF6 to assemble K48 chains to the previously assembled K63 chains. The addition of the K48 chains does not interfere with the recognition by TAB2 but protects K63 chains from deubiquitination by CYLD, enhancing the NF-κB signaling [137]. Interestingly, K63 branched chains seem to target proteins for proteasome degradation. The ubiquitin ligase ITCH is involved in apoptosis regulation through the ubiquitination of TXNIP [138]. ITCH assembles K63 chains to TXNIP that act as a recruitment signal for UBR5 which then assembles K48 branched chains. Because ITCH is a tumor-promoting and anti-apoptotic factor and TXNIP is a tumor suppressor and pro-apoptotic factor, it is possible that during apoptosis, ITCH accelerates TXNIP degradation counteracting its effects [139].

K63/M1 chains, just as M1 and K63 homotypic chains, seem to play a significant role in the innate immune response. Upon activation of MyD88, TNFR1/TRADD, TLR3/TRIF, and NOD1/RIP2 signaling pathways, the formation of K63/M1 branched chains are induced leading to activation of the IKKs [108, 140]. The inflammation-associated protein A20 has both an OTU-type deubiquitinase domain and a ZnF4 motif that binds ubiquitin [141]. After phosphorylation, A20 promotes disassembly of K63 chains during TNFR1 signaling leading to cell death. However, the second step of linear ubiquitination forming branched chains protects TNFR1-associated proteins from K63 disassembling, maintaining the signaling and leading to inflammation [142].

Several other heterotypic chains were found to date. **Table 2** gives an overview of those chains and their biological significance.

3.3 Ubl/ubiquitin chains

Ubiquitin chains can also be modified by ubiquitin-like modifiers (**Figure 2**) [149–153], and although not much is known about these chains, they increase even more the complexity of the ubiquitin code. Ubiquitin chains have been found to be modified by SUMO at K6, K11, K27, K48, and K63. Despite the unclear biological

Chain	Function	Reference
K11/K48	Proteasomal degradation	[133–136]
K48/K63	NF-κB signaling apoptosis	[137, 139]
K63/M1	Innate immune response	[108, 140, 142]
K6/K48	?	[143]
K29/K48	Proteasomal degradation	[55, 144–146]
K11/K63	Endocytosis	[147]
K29/K33	?	[148]

Table 2.
Branched ubiquitin chain types and their associated cellular functions.

role of these modifications, under proteasome inhibition or heat shock conditions, K6 and K27 chains seem to accumulate [151]. ISG15 can form mixed chains with ubiquitin at K29. These chains have a non-proteolytic function and seem to regulate the turnover of ubiquitylated proteins [152]. NEDD8 was found to form branched chains with K48 in human cells acting as a chain terminator [153].

4. Detection methods of ubiquitinated target and chains

4.1 Biochemical and genetic methods

Over the years, different biochemical and genetic methods were developed to detect ubiquitin, ubiquitin-like modifiers, and ubiquitin chains. Although antibodies were available from early on, problems with specificity led to the use of different N-terminally epitope-tagged forms of ubiquitin. Here, antibodies against the epitope tag were used for detection. These constructs were elegantly combined with molecular biological methods, which replaced single lysine residues in ubiquitin with arginine, preventing the formation of ubiquitin chains on these positions. A loss of the chain signal was interpreted as the specific modification by ubiquitin chain with a specific topology. (For a more comprehensive overview see [154]).

For the enrichment of ubiquitin chains of a specific type, biochemical methods and specific antibodies have been developed. While the antibodies were used with varying success due to specificity problems, the use of tandem-repeated ubiquitin-binding entities (TUBE) constructs has gained importance. Here ubiquitin-binding domains with specificity for certain chain topologies are multimerized and used as a pull-down construct [155].

Ubiquitin chain restriction (UbiCRest) is another alternative to identify polyubiquitin chains [76, 156]. In this approach, ovarian tumor family deubiquitinases are incubated with substrates and used as restriction enzymes to detect linkage sites and determine the relative abundance of Ub chains on substrates [76, 156].

4.2 Mass spectrometry-based methods

Mass spectrometry-based proteomics has been used to detect ubiquitination sites for almost 20 years. In 2001, Peng et al. reported that ubiquitinated peptides have a 114 Da mass shift due to the diglycine left behind on a lysine sidechain of ubiquitin from another ubiquitin, after trypsin digestion [157]. In 2003 the same authors, applying their rationale, identified more than 70 ubiquitin-conjugated

proteins and 7 ubiquitination sites (K6, K11, K27, K29, K33, K48, and K63) in ubiquitin itself, being 4 of these sites reported for the first time in vivo [158]. In 2009, Tokunaga et al. reported the identification of linear polyubiquitin in NEMO by mass spectrometry, showing that the NF-κB activation pathway is regulated by LUBAC through the polyubiquitination of NEMO [106]. Due to the low abundance of modified peptides in samples, several enrichment strategies were developed to enrich ubiquitinated peptides and improve ubiquitination identification. The development of an antibody against diglycine linked to the ε-amino group of lysine opened the door to the large-scale identification of ubiquitinated substrate proteins [159]. Although the approach was used very successfully by several laboratories leading to the identification of close to 20,000 ubiquitination sites [160–162] it has some drawbacks. One is that the diglycine remnant left by ubiquitin is not unique, and both NEDD8 and ISG15 leave an identical remnant after trypsin digestion. Additionally, the antibody does not recognize linear ubiquitination. Recently, Akimov et al. developed a new antibody specific to a remnant four-mer peptide of the ubiquitin C-terminus after LysC digestion, identifying 60,000 ubiquitination sites [163]. Other relative quantification methods like stable isotope labeling by/with amino acids in cell culture (SILAC), tandem mass tags (TMT), and label-free quantification have been successfully applied [160, 164–166]; however, these methods are using data-dependent measurements, and although they are most suited for PTMs discovery, they cannot provide information on absolute quantities and/or stoichiometry information.

4.3 Ubiquitin topology analysis

While discovery proteomics based on data-dependent methods (DDA) allows the identification of new proteins and can detect the presence of posttranslational modifications, the reliable identification and quantification of peptides in several samples is hampered by the way how peptides are selected by the mass spectrometer for sequencing. Here, the most intense ion at a very moment is selected, which can lead to the selection of different peptides in consecutive mass spectrometric analysis runs. Contrary to discovery proteomics methods, targeted proteomics tries to identify a given set of peptides in every sample, making this method particularly suited for the analysis of ubiquitin topology experiments [167–169].

The most common techniques for targeted proteomics are selected reaction monitoring (SRM) and parallel reaction monitoring (PRM). Both methods have specific requirements for the mass spectrometer used for the analysis. SRM is bound to a triple-quadrupole mass spectrometer, while PRM measurements require an Orbitrap mass spectrometer. For both methods, a list of peptides is preselected, and the corresponding masses are selected continuously. The selected masses are fragmented in a collision cell, and the fragment masses are monitored. In the case of the ubiquitin topology analysis, the key peptides for the ubiquitin chains are targeted [168]. By comparing this signal with its isotope-labeled version spiked into the mix at a known concentration, it is possible to determine the concentration of the peptide [170]. For the analysis of ubiquitin chain topology, the analysis is focused on unique peptides for each of the ubiquitin chain topologies. By digesting a ubiquitin chain with trypsin under denaturing conditions, ubiquitin peptides are generated. Ubiquitin carries an arginine residue at position 74. Trypsin cleaves after this residue and leaves the double glycine residue on the lysine side chain. This creates a branched peptide with an additional mass of 114 Da at the point of modification. For the targeted analysis are seven branched peptides—for each lysine in ubiquitin one—selected and monitored either by SRM or PRM (reviewed in [154, 171]).

4.4 Detection of branched chains

The detection of heterotypic chains represents a challenge. Specific antibodies can only recognize ubiquitin or one chain at a time [172–174]. Mass spectrometry-based proteomics methodologies are based on the digestion of proteins with a protease, generally trypsin, which cuts after lysines or arginines [175]. Branched chains harbor two or more lysines that are ubiquitinated. Detection of double-ubiquitinated ubiquitin is difficult due to two reasons: first, if branch points are separated by several lysines in ubiquitin, the two branch points are separated into two separate peptides, leading to a loss of the information of the double modification. Second, if the two branch points are on adjacent lysines, the resulting peptide is too long to be measured on a mass spectrometer. An alternative is the coupling of antibody-based enrichment with mass spectrometric analysis [80, 155, 159]. Limited proteolysis associated with middle-down proteomics has been used to characterize polyubiquitin chain structures [143, 145]. Top-down proteomics associated with ultraviolet photodissociation was also applied to determine polyubiquitin chain topology [176]. In 2014, Meyer et al. inserted a TEV cleavage site in ubiquitin which after cleavage allowed for the discrimination between branched and unbranched chains [133]. The authors showed that APC/C synthesizes branched chains that enhance proteasome degradation. Later in 2016, Ohtake et al. used a mutagenesis approach to detect K48/K63 branched chains [137]. The authors replaced ubiquitin's arginine 54 by alanine allowing detection of these chains by mass spectrometry and showed how K48 branched chains protect the deubiquitination of K63. In 2017, Yau et al. developed bispecific antibodies against K11/K48 chains, showing their enhanced signal for protein degradation [135]. More recently, Swatek et al. showed that the leader protease of foot and mouth disease virus cleaves di-ubiquitin between arginine 74 and the C-terminal diglycine, originating one truncated ubiquitin (residues 1–74) and a diglycine modified ubiquitin (residues 1–74) [177]. The authors used the approach coupled to intact MS to identify and quantify ubiquitin chains with one, two, or three branches in whole-cell lysates.

5. Conclusion

Over the last decades, the ubiquitin signaling system has been further and further probed, and today it is clear that it is one of the most complex posttranslational cellular signaling systems. It is involved in almost all cellular processes, and the possibilities in terms of signaling are staggering. Understanding the different signals, which are coded in the ubiquitin chains, is one of the biggest challenges of the ubiquitin field, and the identification of branched and mixed chains and the cross talk with the universe of ubiquitin-like modifiers poses even more challenges. The new tools which are becoming available in combination with new mass spectrometric analysis tools will open the horizon for even more layers of signaling and promise to unravel this hidden chapter of biology.

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Conflict of interest

The authors declare no conflict of interest.

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References

[1] Finley D, Bartel B, Varshavsky A. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature. 1989;**338**:394-401

[2] Varadan R, Walker O, Pickart C, Fushman D. Structural properties of polyubiquitin chains in solution. Journal of Molecular Biology. 2002;**324**: 637-647

[3] Komander D, Reyes-Turcu F, Licchesi JDF, Odenwaelder P, Wilkinson KD, Barford D. Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Reports. 2009;**10**:466-473

[4] Virdee S, Ye Y, Nguyen DP, Komander D, Chin JW. Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase. Nature Chemical Biology. 2010;**6**:750-757

[5] Varadan R, Assfalg M, Haririnia A, Raasi S, Pickart C, Fushman D. Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. The Journal of Biological Chemistry. 2004;**279**:7055-7063

[6] Tenno T, Fujiwara K, Tochio H, Iwai K, Hayato Morita E, Hayashi H, et al. Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains. Genes to Cells. 2004:865-875

[7] Mevissen TET, Komander D. Mechanisms of Deubiquitinase specificity and regulation. Annual Review of Biochemistry. 2017;**86**:159-192

[8] Liakopoulos D, Doenges G, Matuschewski K, Jentsch S. A novel protein modification pathway related to the ubiquitin system. The EMBO Journal. 1998;**17**:2208-2214

[9] Enchev RI, Schulman BA, Peter M. Protein neddylation: Beyond cullin-RING ligases. Nature Reviews. Molecular Cell Biology. 2015;**16**:30-44

[10] Scott DC, Sviderskiy VO, Monda JK, Lydeard JR, Cho SE, Harper JW, et al. Structure of a RING E3 trapped in action reveals ligation mechanism for the ubiquitin-like protein NEDD8. Cell. 2014;**157**:1671-1684

[11] Wolf DA, Zhou C, Wee S. The COP9 signalosome: An assembly and maintenance platform for cullin ubiquitin ligases? Nature Cell Biology. 2003;**5**:1029-1033

[12] Cope GA, Suh GSB, Aravind L, Schwarz SE, Zipursky SL, Koonin EV, et al. Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. Science. 2002;**298**:608-611

[13] Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, et al. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science. 2001;**292**:1382-1385

[14] Zhou C, Seibert V, Geyer R, Rhee E, Lyapina S, Cope G, et al. The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. BMC Biochemistry. 2001;**2**:7

[15] Loftus SJ, Liu G, Carr SM, Munro S, La Thangue NB. NEDDylation regulates E2F-1-dependent transcription. EMBO Reports. 2012;**13**:811-818

[16] Xirodimas DP, Saville MK, Bourdon J-C, Hay RT, Lane DP. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell. 2004;**118**:83-97

[17] Dohmesen C, Koeppel M, Dobbelstein M. Specific inhibition of Mdm2-mediated neddylation by Tip60. Cell Cycle. 2008;**7**:222-231

[18] Schwarz SE, Matuschewski K, Liakopoulos D, Scheffner M, Jentsch S. The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**:560-564

[19] Qin Y, Bao H, Pan Y, Yin M, Liu Y, Wu S, et al. SUMOylation alterations are associated with multidrug resistance in hepatocellular carcinoma. Molecular Medicine Reports. 2014;**9**:877-881

[20] Zhu S, Sachdeva M, Wu F, Lu Z, Mo Y. Ubc9 promotes breast cell invasion and metastasis in a sumoylation-independent manner. Oncogene. 2010;**29**:1763-1772

[21] Lin D, Tatham MH, Yu B, Kim S, Hay RT, Chen Y. Identification of a substrate recognition site on Ubc9. The Journal of Biological Chemistry. 2002;**277**:21740-21748

[22] Bernier-Villamor V, Sampson DA, Matunis MJ, Lima CD. Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. Cell. 2002;**108**:345-356

[23] Reverter D, Lima CD. Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. Nature. 2005;**435**:687-692

[24] Pichler A, Knipscheer P, Saitoh H, Sixma TK, Melchior F. The RanBP2 SUMO E3 ligase is neither HECT- nor RING-type. Nature Structural & Molecular Biology. 2004;**11**:984-991

[25] Pichler A, Gast A, Seeler JS, Dejean A, Melchior F. The nucleoporin RanBP2 has SUMO1 E3 ligase activity. Cell. 2002;**108**:109-120

[26] Pichler A, Fatouros C, Lee H, Eisenhardt N. SUMO conjugation - a mechanistic view. Biomolecular Concepts. 2017;**8**:13-36

[27] Golebiowski F, Matic I, Tatham MH, Cole C, Yin Y, Nakamura A, et al. System-wide changes to SUMO modifications in response to heat shock. Science Signaling. 2009;**2**:ra24

[28] Matunis MJ, Wu J, Blobel G. SUMO-1 modification and its role in targeting the ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. The Journal of Cell Biology. 1998;**140**:499-509

[29] Mahajan R, Gerace L, Melchior F. Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. The Journal of Cell Biology. 1998;**140**:259-270

[30] Cubeñas-Potts C, Matunis MJ. SUMO: A multifaceted modifier of chromatin structure and function. Developmental Cell. 2013;**24**:1-12

[31] Nie M, Boddy MN. Cooperativity of the SUMO and ubiquitin pathways in genome stability. Biomolecules. 2016;**6**:14

[32] Flotho A, Melchior F. Sumoylation: A regulatory protein modification in health and disease. Annual Review of Biochemistry. 2013;**8**:357-385

[33] Hendriks IA, Vertegaal ACO. A comprehensive compilation of SUMO proteomics. Nature Reviews. Molecular Cell Biology. 2016;**17**:581-595

[34] Dzimianski JV, Scholte FEM, Bergeron É, Pegan SD. ISG15: It's complicated. Journal of Molecular Biology. 2019:4203-4216

[35] Dastur A, Beaudenon S, Kelley M, Krug RM, Huibregtse JM. Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. The Journal of Biological Chemistry. 2006;**281**:4334-4338

[36] Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang D-E. UBP43

(USP18) specifically removes ISG15 from conjugated proteins. The Journal of Biological Chemistry. 2002;**277**:9976-9981

[37] Nakashima H, Nguyen T, Goins WF, Chiocca EA. Interferon-stimulated gene 15 (ISG15) and ISG15-linked proteins can associate with members of the selective autophagic process, histone deacetylase 6 (HDAC6) and SQSTM1/p62. The Journal of Biological Chemistry. 2015;**290**:1485-1495

[38] Villarroya-Beltri C, Baixauli F, Mittelbrunn M, Fernández-Delgado I, Torralba D, Moreno-Gonzalo O, et al. ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins. Nature Communications. 2016;**7**:13588

[39] Yeh Y-H, Yang Y-C, Hsieh M-Y, Yeh Y-C, Li T-K. A negative feedback of the HIF-1α pathway via interferon-stimulated gene 15 and ISGylation. Clinical Cancer Research. 2013;**19**:5927-5939

[40] Fan J-B, Miyauchi-Ishida S, Arimoto K-I, Liu D, Yan M, Liu C-W, et al. Type I IFN induces protein ISGylation to enhance cytokine expression and augments colonic inflammation. Proceedings of the National Academy of Sciences of the United States of America. 2015;**112**:14313-14318

[41] Park JM, Yang SW, Yu KR, Ka SH, Lee SW, Seol JH, et al. Modification of PCNA by ISG15 plays a crucial role in termination of error-prone translesion DNA synthesis. Molecular Cell. 2014:626-638

[42] Okumura F, Okumura AJ, Uematsu K, Hatakeyama S, Zhang D-E, Kamura T. Activation of double-stranded RNA-activated protein kinase (PKR) by interferon-stimulated gene 15 (ISG15) modification down-regulates protein translation. Journal of Biological Chemistry. 2013;**288**:2839-2847

[43] Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**:10200-10205

[44] Hsiang T-Y, Zhao C, Krug RM. Interferon-induced ISG15 conjugation inhibits influenza a virus gene expression and replication in human cells. Journal of Virology. 2009;**83**:5971-5977

[45] Lenschow DJ, Giannakopoulos NV, Gunn LJ, Johnston C, O'Guin AK, Schmidt RE, et al. Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection in vivo. Journal of Virology. 2005;**79**:13974-13983

[46] Okumura A, Lu G, Pitha-Rowe I, Pitha PM. Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. Proceedings of the National Academy of Sciences of the United States of America. 2006;**103**:1440-1445

[47] Okumura A, Pitha PM, Harty RN. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. Proceedings of the National Academy of Sciences. 2008;**105**:3974-3979

[48] Shi H-X, Yang K, Liu X, Liu X-Y, Wei B, Shan Y-F, et al. Positive regulation of interferon regulatory factor 3 activation by Herc5 via ISG15 modification. Molecular and Cellular Biology. 2010;**30**:2424-2436

[49] Kim YJ, Kim ET, Kim Y-E, Lee MK, Kwon KM, Kim KI, et al. Consecutive inhibition of ISG15 expression and ISGylation by cytomegalovirus regulators. PLoS Pathogens. 2016;**12**:e1005850

[50] González-Sanz R, Mata M, Bermejo-Martín J, Álvarez A, Cortijo J, Melero JA, et al. ISG15 is upregulated in respiratory syncytial virus infection and reduces virus growth through protein ISGylation. Journal of Virology. 2016;**90**:3428-3438

[51] Terrell J, Shih S, Dunn R, Hicke L. A function for monoubiquitination in the internalization of a G protein-coupled receptor. Molecular Cell. 1998;**1**:193-202

[52] Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nature Cell Biology. 2003;**5**:461-466

[53] Ye Y, Blaser G, Horrocks MH, Ruedas-Rama MJ, Ibrahim S, Zhukov AA, et al. Ubiquitin chain conformation regulates recognition and activity of interacting proteins. Nature. 2012;**492**:266-270

[54] Bremm A, Freund SMV, Komander D. Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne. Nature Structural & Molecular Biology. 2010;**17**:939-947

[55] Kristariyanto YA, Abdul Rehman SA, Campbell DG, Morrice NA, Johnson C, Toth R, et al. K29-selective ubiquitin binding domain reveals structural basis of specificity and heterotypic nature of k29 polyubiquitin. Molecular Cell. 2015;**58**:83-94

[56] Castañeda CA, Dixon EK, Walker O, Chaturvedi A, Nakasone MA, Curtis JE, et al. Linkage via K27 bestows ubiquitin chains with unique properties among Polyubiquitins. Structure. 2016;**24**:423-436

[57] Michel MA, Elliott PR, Swatek KN, Simicek M, Pruneda JN, Wagstaff JL, et al. Assembly and specific recognition of k29- and k33-linked polyubiquitin. Molecular Cell. 2015;**58**:95-109

[58] Liu Z, Gong Z, Jiang W-X, Yang J, Zhu W-K, Guo D-C, et al. Lys63-linked ubiquitin chain adopts multiple conformational states for specific target recognition. eLife. 2015;**4**:1-19

[59] Zhang X, Smits AH, van Tilburg GBA, Jansen PWTC, Makowski MM, Ovaa H, et al. An interaction landscape of ubiquitin signaling. Molecular Cell. 2017;**65**: 941-955.e8

[60] Dammer EB, Na CH, Xu P, Seyfried NT, Duong DM, Cheng D, et al. Polyubiquitin linkage profiles in three models of proteolytic stress suggest the etiology of Alzheimer disease. The Journal of Biological Chemistry. 2011;**286**:10457-10465

[61] Ziv I, Matiuhin Y, Kirkpatrick DS, Erpapazoglou Z, Leon S, Pantazopoulou M, et al. A perturbed ubiquitin landscape distinguishes between ubiquitin in trafficking and in proteolysis. Molecular & Cellular Proteomics. 2011;**10**:M111.009753

[62] Cunningham CN, Baughman JM, Phu L, Tea JS, Yu C, Coons M, et al. USP30 and parkin homeostatically regulate atypical ubiquitin chains on mitochondria. Nature Cell Biology. 2015;**17**:160-169

[63] Ordureau A, Sarraf SA, Duda DM, Heo J-M, Jedrychowski MP, Sviderskiy VO, et al. Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. Molecular Cell. 2014;**56**:360-375

[64] Gersch M, Gladkova C, Schubert AF, Michel MA, Maslen S, Komander D. Mechanism and regulation of the Lys6-selective deubiquitinase USP30. Nature Structural & Molecular Biology. 2017;**24**:920-930

[65] Elia AEH, Boardman AP, Wang DC, Huttlin EL, Everley RA, Dephoure N, et al. Quantitative proteomic atlas of Ubiquitination and acetylation in the DNA damage response. Molecular Cell. 2015;**59**:867-881

[66] Kao S-H, Wu H-T, Wu K-J. Ubiquitination by HUWE1 in tumorigenesis and beyond. Journal of Biomedical Science. 2018;**25**:67

[67] Strappazzon F, Di Rita A, Peschiaroli A, Leoncini PP, Locatelli F, Melino G, et al. HUWE1 controls MCL1 stability to unleash AMBRA1-induced mitophagy. Cell Death and Differentiation. 2019. Epub ahead of print

[68] Michel MA, Swatek KN, Hospenthal MK, Komander D. Ubiquitin linkage-specific affimers reveal insights into K6-linked ubiquitin signaling. Molecular Cell. 2017;**68**:233-246.e5

[69] Palikaras K, Lionaki E, Tavernarakis N. Mechanisms of mitophagy in cellular homeostasis, physiology and pathology. Nature Cell Biology. 2018;**20**:1013-1022

[70] Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998;**392**:605-608

[71] Valente EM, Abou-Sleiman PM, Caputo V, Muqit MMK, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004;**304**:1158-1160

[72] Kirkpatrick DS, Hathaway NA, Hanna J, Elsasser S, Rush J, Finley D, et al. Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nature Cell Biology. 2006;**8**:700-710

[73] Jin L, Williamson A, Banerjee S, Philipp I, Rape M. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell. 2008;**133**:653-665

[74] Matsumoto ML, Wickliffe KE, Dong KC, Yu C, Bosanac I, Bustos D, et al. K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. Molecular Cell. 2010;**39**:477-484

[75] Min M, Mevissen TET, De Luca M, Komander D, Lindon C. Efficient APC/C substrate degradation in cells undergoing mitotic exit depends on K11 ubiquitin linkages. Molecular Biology of the Cell. 2015;**26**:4325-4332

[76] Mevissen TET, Hospenthal MK, Geurink PP, Elliott PR, Akutsu M, Arnaudo N, et al. OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. Cell. 2013;**154**:169-184

[77] Bremm A, Moniz S, Mader J, Rocha S, Komander D. Cezanne (OTUD7B) regulates HIF-1α homeostasis in a proteasome-independent manner. EMBO Reports. 2014;**15**:1268-1277

[78] Bonacci T, Suzuki A, Grant GD, Stanley N, Cook JG, Brown NG, et al. Cezanne/ OTUD 7B is a cell cycle-regulated deubiquitinase that antagonizes the degradation of APC /C substrates. The EMBO Journal. 2018;**37**(16):1-17

[79] Li Y, Dammer EB, Gao Y, Lan Q, Villamil MA, Duong DM, et al. Proteomics links ubiquitin chain topology change to transcription factor activation. Molecular Cell. 2019;**76**: 126-137.e7

[80] Flick K, Ouni I, Wohlschlegel JA, Capati C, McDonald WH, Yates JR, et al. Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. Nature Cell Biology. 2004;**6**:634-641

[81] Liu Z, Chen P, Gao H, Gu Y, Yang J, Peng H, et al. Ubiquitylation of autophagy receptor Optineurin by HACE1 activates selective autophagy for tumor suppression. Cancer Cell. 2014;**26**:106-120

[82] Palicharla VR, Maddika S. HACE1 mediated K27 ubiquitin linkage leads to YB-1 protein secretion. Cellular Signalling. 2015;**27**:2355-2362

[83] Gatti M, Pinato S, Maiolica A, Rocchio F, Prato MG, Aebersold R, et al. RNF168 promotes noncanonical K27 ubiquitination to signal DNA damage. Cell Reports. 2015;**10**:226-238

[84] Wang Q, Liu X, Cui Y, Tang Y, Chen W, Li S, et al. The E3 ubiquitin ligase AMFR and INSIG1 bridge the activation of TBK1 kinase by modifying the adaptor STING. Immunity. 2014;**41**:919-933

[85] Xue B, Li H, Guo M, Wang J, Xu Y, Zou X, et al. TRIM21 promotes innate immune response to RNA viral infection through Lys27-linked polyubiquitination of MAVS. Journal of Virology. 2018;**92**(14):1-19

[86] Aki D, Li H, Zhang W, Zheng M, Elly C, Lee JH, et al. The E3 ligases itch and WWP2 cooperate to limit TH2 differentiation by enhancing signaling through the TCR. Nature Immunology. 2018;**19**:766-775

[87] Wu X, Lei C, Xia T, Zhong X, Yang Q, Shu H-B. Regulation of TRIF-mediated innate immune response by K27-linked polyubiquitination and deubiquitination. Nature Communications. 2019;**10**:4115

[88] Cho JJ, Xu Z, Parthasarathy U, Drashansky TT, Helm EY, Zuniga AN, et al. Hectd3 promotes pathogenic Th17 lineage through Stat3 activation and Malt1 signaling in neuroinflammation. Nature Communications. 2019;**10**:701

[89] Hay-Koren A, Caspi M, Zilberberg A, Rosin-Arbesfeld R. The EDD E3 ubiquitin ligase ubiquitinates and up-regulates β-catenin. Molecular Biology of the Cell (MBoC). 2011;**22**:399-411

[90] Fei C, Li Z, Li C, Chen Y, Chen Z, He X, et al. Smurf1-mediated Lys29-linked nonproteolytic polyubiquitination of axin negatively regulates Wnt/β-catenin signaling. Molecular and Cellular Biology. 2013;**33**:4095-4105

[91] Licchesi JDF, Mieszczanek J, Mevissen TET, Rutherford TJ, Akutsu M, Virdee S, et al. An ankyrin-repeat ubiquitin-binding domain determines TRABID's specificity for atypical ubiquitin chains. Nature Structural & Molecular Biology. 2011;**19**:62-71

[92] Tran H, Hamada F, Schwarz-Romond T, Bienz M. Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. Genes & Development. 2008;**22**:528-542

[93] Jin J, Xie X, Xiao Y, Hu H, Zou Q, Cheng X, et al. Epigenetic regulation of the expression of Il12 and Il23 and autoimmune inflammation by the deubiquitinase Trabid. Nature Immunology. 2016;**17**:259-268

[94] Huang H, Jeon M-S, Liao L, Yang C, Elly C, Yates JR 3rd, et al. K33-linked polyubiquitination of T cell receptor-zeta regulates proteolysis-independent T cell signaling. Immunity. 2010;**33**:60-70

[95] Lin M, Zhao Z, Yang Z, Meng Q, Tan P, Xie W, et al. USP38 inhibits type I interferon signaling by editing TBK1 Ubiquitination through NLRP4 signalosome. Molecular Cell. 2016;**64**:267-281

[96] Miao Y, Wu J, Abraham SN. Ubiquitination of innate immune regulator TRAF3 orchestrates expulsion

of intracellular Bacteria by exocyst complex. Immunity. 2016;**45**:94-105

[97] Zhao Y, Wang X, Wang Q, Deng Y, Li K, Zhang M, et al. USP2a supports metastasis by tuning TGF-β signaling. Cell Reports. 2018;**22**:2442-2454

[98] Yuan W-C, Lee Y-R, Lin S-Y, Chang L-Y, Tan YP, Hung C-C, et al. K33-linked polyubiquitination of Coronin 7 by Cul3-KLHL20 ubiquitin E3 ligase regulates protein trafficking. Molecular Cell. 2014;**54**:586-600

[99] Heath RJ, Goel G, Baxt LA, Rush JS, Mohanan V, Paulus GLC, et al. RNF166 determines recruitment of adaptor proteins during antibacterial autophagy. Cell Reports. 2016;**17**:2183-2194

[100] Feng X, Jia Y, Zhang Y, Ma F, Zhu Y, Hong X, et al. Ubiquitination of UVRAG by SMURF1 promotes autophagosome maturation and inhibits hepatocellular carcinoma growth. Autophagy. 2019;**15**:1130-1149

[101] Walczak H, Iwai K, Dikic I. Generation and physiological roles of linear ubiquitin chains. BMC Biology. 2012;**10**:23

[102] Dittmar G, Winklhofer KF. Linear ubiquitin chains: Cellular functions and strategies for detection and quantification. Frontiers in Chemistry. 2020;**7**:915

[103] Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. The EMBO Journal. 2006;**25**:4877-4887

[104] Ikeda F, Deribe YL, Skånland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, et al. SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature. 2011;**471**:637-641

[105] Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. Nature. 2011;**471**:591-596

[106] Tokunaga F, Sakata S-I, Saeki Y, Satomi Y, Kirisako T, Kamei K, et al. Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nature Cell Biology. 2009;**11**:123-132

[107] Haas TL, Emmerich CH, Gerlach B, Schmukle AC, Cordier SM, Rieser E, et al. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Molecular Cell. 2009;**36**:831-844

[108] Emmerich CH, Ordureau A, Strickson S, Arthur JSC, Pedrioli PGA, Komander D, et al. Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**:15247-15252

[109] Müller-Rischart AK, Pilsl A, Beaudette P, Patra M, Hadian K, Funke M, et al. The E3 ligase parkin maintains mitochondrial integrity by increasing linear ubiquitination of NEMO. Molecular Cell. 2013;**49**:908-921

[110] Kwon YT, Ciechanover A. The ubiquitin code in the ubiquitin-proteasome system and autophagy. Trends in Biochemical Sciences. 2017;**42**:873-886

[111] Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, et al. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science. 1989;**243**:1576-1583

[112] Finley D, Sadis S, Monia BP, Boucher P, Ecker DJ, Crooke ST, et al. Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. Molecular and Cellular Biology. 1994;**14**:5501-5509

[113] Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. The EMBO Journal. 2000;**19**:94-102

[114] Boutet SC, Disatnik M-H, Chan LS, Iori K, Rando TA. Regulation of Pax3 by proteasomal degradation of monoubiquitinated protein in skeletal muscle progenitors. Cell. 2007;**130**:349-362

[115] Kravtsova-Ivantsiv Y, Cohen S, Ciechanover A. Modification by single ubiquitin moieties rather than polyubiquitination is sufficient for proteasomal processing of the p105 NF-kappaB precursor. Molecular Cell. 2009;**33**:496-504

[116] Braten O, Livneh I, Ziv T, Admon A, Kehat I, Caspi LH, et al. Numerous proteins with unique characteristics are degraded by the 26S proteasome following monoubiquitination. Proceedings of the National Academy of Sciences of the United States of America. 2016;**113**:E4639-E4647

[117] Komander D, Rape M. The ubiquitin code. Annual Review of Biochemistry. 2012;**81**:203-229

[118] Swatek KN, Komander D. Ubiquitin modifications. Cell Research. 2016;**26**:399-422

[119] Erpapazoglou Z, Walker O, Haguenauer-Tsapis R. Versatile roles of K63-linked ubiquitin chains in trafficking. Cell. 2014;**3**:1027

[120] Deng L, Wang C, Spencer E, Yang L, Braun A, You J, et al. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell. 2000;**103**:351-361

[121] Yang W-L, Wang J, Chan C-H, Lee S-W, Campos AD, Lamothe B, et al. The E3 ligase TRAF6 regulates Akt ubiquitination and activation. Science. 2009;**325**:1134-1138

[122] Gack MU, Shin YC, Joo C-H, Urano T, Liang C, Sun L, et al. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature. 2007;**446**:916-920

[123] Ordureau A, Smith H, Windheim M, Peggie M, Carrick E, Morrice N, et al. The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys63-linked polyubiquitination of IRAK1. The Biochemical Journal. 2008;**409**:43-52

[124] Tsuchida T, Zou J, Saitoh T, Kumar H, Abe T, Matsuura Y, et al. The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. Immunity. 2010;**33**:765-776

[125] Schwertman P, Bekker-Jensen S, Mailand N. Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers. Nature Reviews. Molecular Cell Biology. 2016;**17**:379-394

[126] Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, et al. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. The EMBO Journal. 2006;**25**:1635-1645

[127] Huang F, Kirkpatrick D, Jiang X, Gygi S, Sorkin A. Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Molecular Cell. 2006;**21**:737-748

[128] Bertelsen V, Sak MM, Breen K, Rødland MS, Johannessen LE, Traub LM, et al. A chimeric pre-ubiquitinated EGF receptor is constitutively endocytosed in a clathrin-dependent, but kinase-independent manner. Traffic. 2011;**12**:507-520

[129] Liu P, Gan W, Su S, Hauenstein AV, Fu T-M, Brasher B, et al. K63-linked polyubiquitin chains bind to DNA to facilitate DNA damage repair. Science Signaling. 2018;**11**(533):1-12

[130] Yau R, Rape M. The increasing complexity of the ubiquitin code. Nature Cell Biology. 2016;**18**:579-586

[131] Ohtake F, Tsuchiya H. The emerging complexity of ubiquitin architecture. Journal of Biochemistry. 2017;**161**:125-133

[132] Haakonsen DL, Rape M. Branching out: Improved signaling by heterotypic ubiquitin chains. Trends in Cell Biology. 2019;**29**(9):704-716

[133] Meyer H-J, Rape M. Enhanced protein degradation by branched ubiquitin chains. Cell. 2014;**157**:910-921

[134] Grice GL, Lobb IT, Weekes MP, Gygi SP, Antrobus R, Nathan JA. The proteasome distinguishes between heterotypic and Homotypic Lysine-11-linked Polyubiquitin chains. Cell Reports. 2015;**12**:545-553

[135] Yau RG, Doerner K, Castellanos ER, Haakonsen DL, Werner A, Wang N, et al. Assembly and function of heterotypic ubiquitin chains in cell-cycle and protein quality control. Cell. 2017;**171**:918-933.e20

[136] Boughton AJ, Krueger S, Fushman D. Branching via K11 and K48 bestows ubiquitin chains with a unique interdomain interface and enhanced affinity for proteasomal subunit Rpn1. Structure. 2020;**28**:29-43.e6

[137] Ohtake F, Saeki Y, Ishido S, Kanno J, Tanaka K. The K48-K63 branched ubiquitin chain regulates NF-κB signaling. Molecular Cell. 2016;**64**:251-266

[138] Zhang P, Wang C, Gao K, Wang D, Mao J, An J, et al. The ubiquitin ligase itch regulates apoptosis by targeting thioredoxin-interacting protein for ubiquitin-dependent degradation. The Journal of Biological Chemistry. 2010;**285**:8869-8879

[139] Ohtake F, Tsuchiya H, Saeki Y, Tanaka K. K63 ubiquitylation triggers proteasomal degradation by seeding branched ubiquitin chains. Proceedings of the National Academy of Sciences of the United States of America. 2018;**115**:E1401-E1408

[140] Emmerich CH, Bakshi S, Kelsall IR, Ortiz-Guerrero J, Shpiro N, Cohen P. Lys63/Met1-hybrid ubiquitin chains are commonly formed during the activation of innate immune signalling. Biochemical and Biophysical Research Communications. 2016;**474**:452-461

[141] Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature. 2004;**430**:694-699

[142] Wertz IE, Newton K, Seshasayee D, Kusam S, Lam C, Zhang J, et al. Phosphorylation and linear ubiquitin direct A20 inhibition of inflammation. Nature. 2015;**528**:370-375

[143] Valkevich EM, Sanchez NA, Ge Y, Strieter ER. Middle-down mass spectrometry enables characterization of branched ubiquitin chains. Biochemistry. 2014;**53**:4979-4989

[144] Liu C, Liu W, Ye Y, Li W. Ufd2p synthesizes branched ubiquitin chains to promote the degradation of substrates modified with atypical chains. Nature Communications. 2017;**8**:14274

[145] Xu P, Peng J. Characterization of polyubiquitin chain structure by middle-down mass spectrometry. Analytical Chemistry. 2008;**80**:3438-3444

[146] Saeki Y, Tayama Y, Toh-e A, Yokosawa H. Definitive evidence for Ufd2-catalyzed elongation of the ubiquitin chain through Lys48 linkage. Biochemical and Biophysical Research Communications. 2004;**320**:840-845

[147] Boname JM, Thomas M, Stagg HR, Xu P, Peng J, Lehner PJ. Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. Traffic. 2010;**11**:210-220

[148] Hospenthal MK, Freund SMV, Komander D. Assembly, analysis and architecture of atypical ubiquitin chains. Nature Structural & Molecular Biology. 2013;**20**:555-565

[149] Galisson F, Mahrouche L, Courcelles M, Bonneil E, Meloche S, Chelbi-Alix MK, et al. A novel proteomics approach to identify SUMOylated proteins and their modification sites in human cells. Molecular & Cellular Proteomics. 2011;**10**:M110.004796

[150] Lamoliatte F, Bonneil E, Durette C, Caron-Lizotte O, Wildemann D, Zerweck J, et al. Targeted identification of SUMOylation sites in human proteins using affinity enrichment and paralog-specific reporter ions. Molecular & Cellular Proteomics. 2013;**12**:2536-2550

[151] Hendriks IA, D'Souza RCJ, Yang B, Verlaan-de Vries M, Mann M, Vertegaal ACO. Uncovering global SUMOylation signaling networks in a site-specific manner. Nature Structural & Molecular Biology. 2014;**21**:927-936

[152] Fan J-B, Arimoto K-I, Motamedchaboki K, Yan M, Wolf DA, Zhang D-E. Identification and characterization of a novel ISG15-ubiquitin mixed chain and its role in regulating protein homeostasis. Scientific Reports. 2015;**5**:12704

[153] Singh RK, Zerath S, Kleifeld O, Scheffner M, Glickman MH, Fushman D. Recognition and cleavage of related to ubiquitin 1 (Rub1) and Rub1-ubiquitin chains by components of the ubiquitin-proteasome system. Molecular & Cellular Proteomics. 2012;**11**:1595-1611

[154] Beaudette P, Popp O, Dittmar G. Proteomic techniques to probe the ubiquitin landscape. Proteomics. 2016;**16**:273-287

[155] Hjerpe R, Aillet F, Lopitz-Otsoa F, Lang V, England P, Rodriguez MS. Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. EMBO Reports. 2009;**10**:1250-1258

[156] Hospenthal MK, Mevissen TET, Komander D. Deubiquitinase-based analysis of ubiquitin chain architecture using ubiquitin chain restriction (UbiCRest). Nature Protocols. 2015;**10**:349-361

[157] Peng J, Gygi SP. Proteomics: The move to mixtures. Journal of Mass Spectrometry. 2001;**36**:1083-1091

[158] Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, et al. A proteomics approach to understanding protein ubiquitination. Nature Biotechnology. 2003;**21**:921-926

[159] Xu G, Paige JS, Jaffrey SR. Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. Nature Biotechnology. 2010;**28**:868-873

[160] Wagner SA, Beli P, Weinert BT, Nielsen ML, Cox J, Mann M, et al. A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. Molecular & Cellular Proteomics. 2011;**10**:M111.013284

[161] Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, et al. Systematic and quantitative assessment of

the ubiquitin-modified proteome. Molecular Cell. 2011;**44**:325-340

[162] Emanuele MJ, Elia AEH, Xu Q, Thoma CR, Izhar L, Leng Y, et al. Global identification of modular cullin-RING ligase substrates. Cell. 2011;**147**:459-474

[163] Akimov V, Barrio-Hernandez I, Hansen SVF, Hallenborg P, Pedersen A-K, Bekker-Jensen DB, et al. UbiSite approach for comprehensive mapping of lysine and N-terminal ubiquitination sites. Nature Structural & Molecular Biology. 2018;**25**:631-640

[164] Isasa M, Rose CM, Elsasser S, Navarrete-Perea J, Paulo JA, Finley DJ, et al. Multiplexed, proteome-wide protein expression profiling: Yeast Deubiquitylating enzyme knockout strains. Journal of Proteome Research. 2015;**14**:5306-5317

[165] Swaney DL, Beltrao P, Starita L, Guo A, Rush J, Fields S, et al. Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. Nature Methods. 2013;**10**:676-682

[166] Ordureau A, Münch C, Harper JW. Quantifying ubiquitin signaling. Molecular Cell. 2015;**58**:660-676

[167] Shi T, Su D, Liu T, Tang K, Camp DG 2nd, Qian W-J, et al. Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. Proteomics. 2012;**12**:1074-1092

[168] Picotti P, Aebersold R. Selected reaction monitoring-based proteomics: Workflows, potential, pitfalls and future directions. Nature Methods. 2012;**9**:555-566

[169] Gillette MA, Carr SA. Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. Nature Methods. 2013;**10**:28-34

[170] Picotti P, Lam H, Campbell D, Deutsch EW, Mirzaei H, Ranish J, et al. A database of mass spectrometric assays for the yeast proteome. Nature Methods. 2008;**5**:913-914

[171] Longworth J, Dittmar G. Assessment of ubiquitin chain topology by targeted mass spectrometry. Methods in Molecular Biology. 1977;**2019**:25-34

[172] Fujimuro M, Sawada H, Yokosawa H. Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. FEBS Letters. 1994;**349**:173-180

[173] Newton K, Matsumoto ML, Wertz IE, Kirkpatrick DS, Lill JR, Tan J, et al. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell. 2008;**134**:668-678

[174] Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. The Journal of Biological Chemistry. 1983;**258**:8206-8214

[175] Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocols. 2006;**1**:2856-2860

[176] Cannon JR, Martinez-Fonts K, Robotham SA, Matouschek A, Brodbelt JS. Top-down 193-nm ultraviolet photodissociation mass spectrometry for simultaneous determination of polyubiquitin chain length and topology. Analytical Chemistry. 2015;**87**:1812-1820

[177] Swatek KN, Usher JL, Kueck AF, Gladkova C, Mevissen TET, Pruneda JN, et al. Insights into ubiquitin chain architecture using Ub-clipping. Nature. 2019;**572**(7770):533-537