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Review

Assembly and function of branched ubiquitin chains

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Post-translational modification with ubiquitin is required for cell division, differentiation, and survival in all eukaryotes. As part of an intricate signaling code, ubiquitin is attached to its targets as single molecules or polymeric chains, with the distinct modifications encoding a wide range of outcomes. After early work focused on homotypic ubiquitin chains, such as the K48-linked polymers that drive proteasomal degradation, recent studies noted abundant conjugates that contained ubiquitin molecules modified on two or more sites. Such branched ubiquitin chains are produced in response to specific signals and they exert functions that are critical for cellular and organismal homeostasis. In this review, we will discuss our rapidly evolving understanding of the assembly and function of branched ubiquitin chains.

Highlights

Branched ubiquitin chains are abundant in cells and produced in response to specific signals.

Branched chains can be assembled by multiple mechanisms that often require collaboration between ubiquitylation enzymes.

Branched ubiquitin chains are dynamic, and deubiquitylases can edit, rather than terminate, signaling by branched chains.

Branched ubiquitin chains encode diverse functions that range from improving the efficiency of protein degradation to organizing large signaling complexes.

Small molecule-induced protein degradation often requires branched ubiquitin chains for efficient removal of a therapeutic target.

The basics of ubiquitin chain topology

Most cells in our bodies are primed to rapidly adjust their behavior to changes in their environment. Key to successful adaptation are signaling pathways that respond to outside events by changing the activity or stability of regulatory factors within cells. In most, if not all cases, this information flow relies on posttranslational modifications that are implemented by specific enzymes, or writers, and decoded by dedicated effectors, or readers. An important signaling modality is ubiquitylation, which is essential for cell division, differentiation, and homeostasis in all eukaryotes [1].

Ubiquitylation centers on the highly conserved protein ubiquitin, which between yeast and man differs in only three out of 76 amino acids. Nature left ubiquitin with little room for evolutionary diversification: while the carboxy-terminal tail of ubiquitin is essential for its attachment to targets, its α-amino group, all seven Lys residues, and some of its three Ser or seven Thr residues, serve as sites for ubiquitin polymerization [2,3] (Figure 1A). Surfaces around Phe4, Ile36, Ile44, or Asp58 of the compact ubiquitin molecule are recognized by hundreds of ubiquitin-binding proteins that translate this modification into a specific cellular response [4,5]. Even buried residues are important, as they presumably allow unfolding of the otherwise stable ubiquitin molecule by the effector p97/VCP [6].

A central step in ubiquitin-dependent signaling is the covalent attachment of the modifier, ubiquitin, to proteins [7], although lipopolysaccharides can be targeted as well [8]. When attached to proteins, the carboxy terminus of ubiquitin is most frequently linked to the ε-amino group of a substrate lysine via an isopeptide bond. Proteomic analyses identified thousands of Lys residues that are ubiquitylated in human cells [9,10], a list that could grow as more cell types, tissues, and developmental states are investigated. Ubiquitin can also be connected as an oxyester to the hydroxyl group of Thr or Ser residues [11–13], to a protein's α-amino group via a peptide bond [13–15], or to Cys residues as a thioester [16]. Moreover, bacteria can attach ubiquitin to Ser residues in host proteins through a phosphoribosyl link [17,18]. Although ubiquitylation has long been viewed

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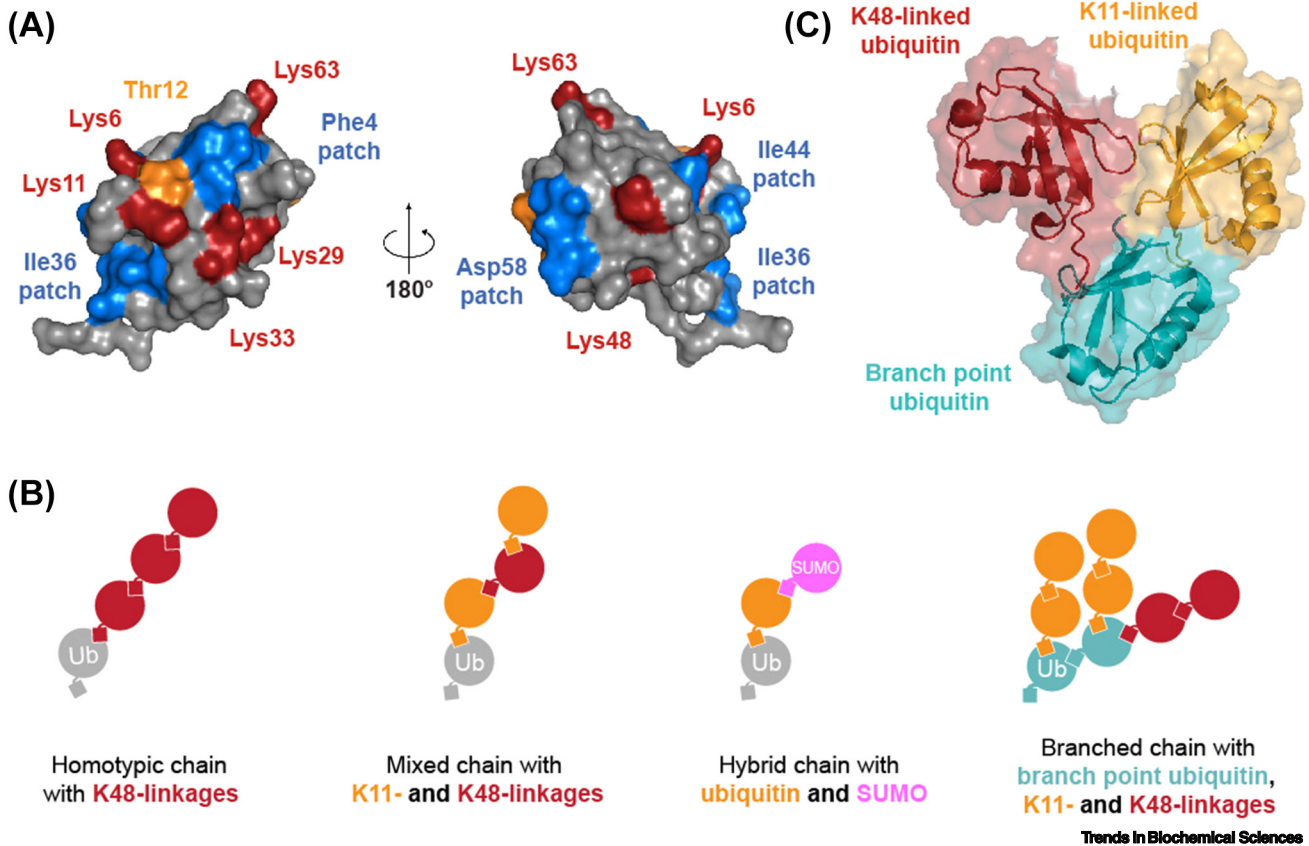
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Figure 1. Branched ubiquitin chains are part of the ubiquitin code. (A) Ubiquitin is a highly conserved protein, with Lys (red) and Ser/Thr (orange) residues that are used as attachment sites for ubiquitin chain formation. Various surfaces of ubiquitin (Phe4, Ile36, Ile44, and Asp58 patches; blue) are recognized by effector proteins, which translate a specific ubiquitin modification into a particular cellular outcome (PDB ID: 1UBQ). (B) Ubiquitin can be assembled into polymeric conjugates of distinct topology and function. Only one example of each chain type is shown. The different colors denote different linkages between ubiquitin molecules (red: K48 linkages; orange: K11 linkages) or distinct modifiers (pink: SUMO1). (C) Structure of a K11/K48-branched ubiquitin trimer (PDB ID: 6OQ1).

as a Lys-directed modification, we now appreciate that multiple acceptors can receive this modification through distinct chemistry.

Reflecting ubiquitin's versatility towards substrate residues, cells also assemble multiple conjugates with distinct structures and functions [2,3,19]. Transfer of a single ubiquitin, or mono-ubiquitylation, can alter the interaction landscape of a target or impact the folding stability of the modified protein [20–23]. In addition, proteins are often decorated with ubiquitin chains that are connected through a side chain in one ubiquitin and the carboxy terminus of another ubiquitin molecule (Figure 1B). In homotypic chains, the same ubiquitin residue is used throughout the polymer. In mixed conjugates, each ubiquitin is modified with one subunit, but different sites serve as acceptors, while hybrid chains contain both ubiquitin and a ubiquitin-like protein, such as SUMO [24]. By contrast, branched chains possess ubiquitin molecules that are modified on two or more sites, which results in a bifurcation of the polymer structure [25–27] (Figure 1B,C). Distinguishing mixed from branched ubiquitin chains can be difficult, as it still requires specialized proteomic or biochemical analyses [25,28,29].

Early work focused on homotypic K11- and K48-linked conjugates that elicit proteasomal degradation [30,31], K63-linked chains that control ribosome biogenesis and DNA repair [32–34], or M1-linked polymers that regulate inflammatory signaling [35]. However, chemical, proteomic, or

immunological detection methods have revealed that heterotypic chains are also abundant in cells [24,28,29,36,37]. These studies showed that up to 20% of ubiquitin molecules in polymers are part of branched conjugates [28,38] and some ubiquitin linkages might even predominantly exist within the context of heterotypic and branched polymers [39,40]. As the abundance of branched chains increases in response to changes in cellular state, such as mitosis or proteotoxic stress [28,36], such conjugates are likely to guide cellular information flow. In this review, we will discuss our rapidly evolving knowledge of the synthesis, function, and disassembly of branched ubiquitin chains.

Synthesis of branched chains

As with every ubiquitin polymer, the assembly of branched chains requires an enzymatic cascade that is composed of E1 ubiquitin activating enzymes, E2 conjugating enzymes, and E3 ligases [41–43]. Human cells possess ~600 E3 ligases, which determine the substrate specificity of this modification. E3 ligases with homologous to E6AP C terminus (HECT), RING-in between-RING (RBR), or RING Cys relay (RCR) domains are charged with ubiquitin before they modify the target. These enzymes often cooperate with the E2 UBE2L3, which can transfer ubiquitin to the catalytic Cys residue in the E3 ligase, but not to Lys residues in substrates [44]. Most E3 ligases, however, contain really interesting new gene (RING) or U-box domains that activate E2 enzymes to directly modify a substrate [45–48]. In contrast to HECT-, RBR-, or RCR-E3 ligases, RING-E3 ligases rely on their E2 enzymes to determine the connectivity of their ubiquitin chains [45,49–51].

The signature motif of a branched chain is a ubiquitin molecule that is modified on at least two sites. The synthesis of branched polymers therefore either requires an enzyme with relaxed linkage specificity or a collaboration between enzymes that differ in their preference for acceptor sites in ubiquitin. In an example of the former mechanism, the bacterial E3 ligase NleL produces K6/K48-branched chains based on its ability to synthesize each linkage [52,53], a property that is shared with the E2 enzyme UBE2D3 or the E3 ligases Parkin, HUWE1, WWP1, and HECTD1 [28,37,54–56]. The E3 ligase anaphase-promoting complex (APC/C) is also sufficient to produce branched ubiquitin chains, but requires two E2 enzymes of distinct specificity, UBE2C and UBE2S, to accomplish this [25]. While UBE2C initiates short chains that contain K11, K48, and K63 linkages [57–59], UBE2S branches off specific K11-linked polymers [25,45,60–63] (Figure 2A). This results in (K11, K48, K63)/K11-branched chains that strongly accumulate upon APC/C activation during mitosis [29,36,64] (see Box 1 on the nomenclature of branched chains). The yeast APC/C also produces branched chains by using two E2 enzymes, UBC5 and UBC1, but in this organism, the topology is swapped and K48-linked polymers branch off an initial chain to yield (K11, K48, K63)/K48-branched conjugates [65–67]. The mechanism of APC/C-dependent synthesis of branched chains synthesis requires that the E3 ligase binds two E2s at the same time [68–71] (Figure 2A), which for human APC/C allows the branching enzyme, UBE2S, to stimulate chain initiation by UBE2C [72].

Akin to the engagement of distinct E2 enzymes, two catalytic subunits of a constitutive E3 ligase complex can work together to synthesize branched chains. This approach is used by LUBAC, which relies on its RBR-subunit HOIP to produce M1-linked chains and a second RBR component, HOIL-1L, to branch off oxyester-linked ubiquitin [73] (Figure 2B). HOIL-1L also extends oxyester-linked ubiquitin oligomers [12,73]. To facilitate branching, the LUBAC complex is organized so that the catalytic domains of HOIP and HOIL-1L are in close proximity to each other [73].

In what could be the most prevalent scenario, two E3 ligases come together to decorate a substrate with branched chains. While one partner attaches a first ubiquitin chain to the target, the second E3 ligase branches off conjugates of a distinct topology (Figure 2C). This activity was alluded to in classical yeast studies, where a fused ubiquitin triggered protein degradation
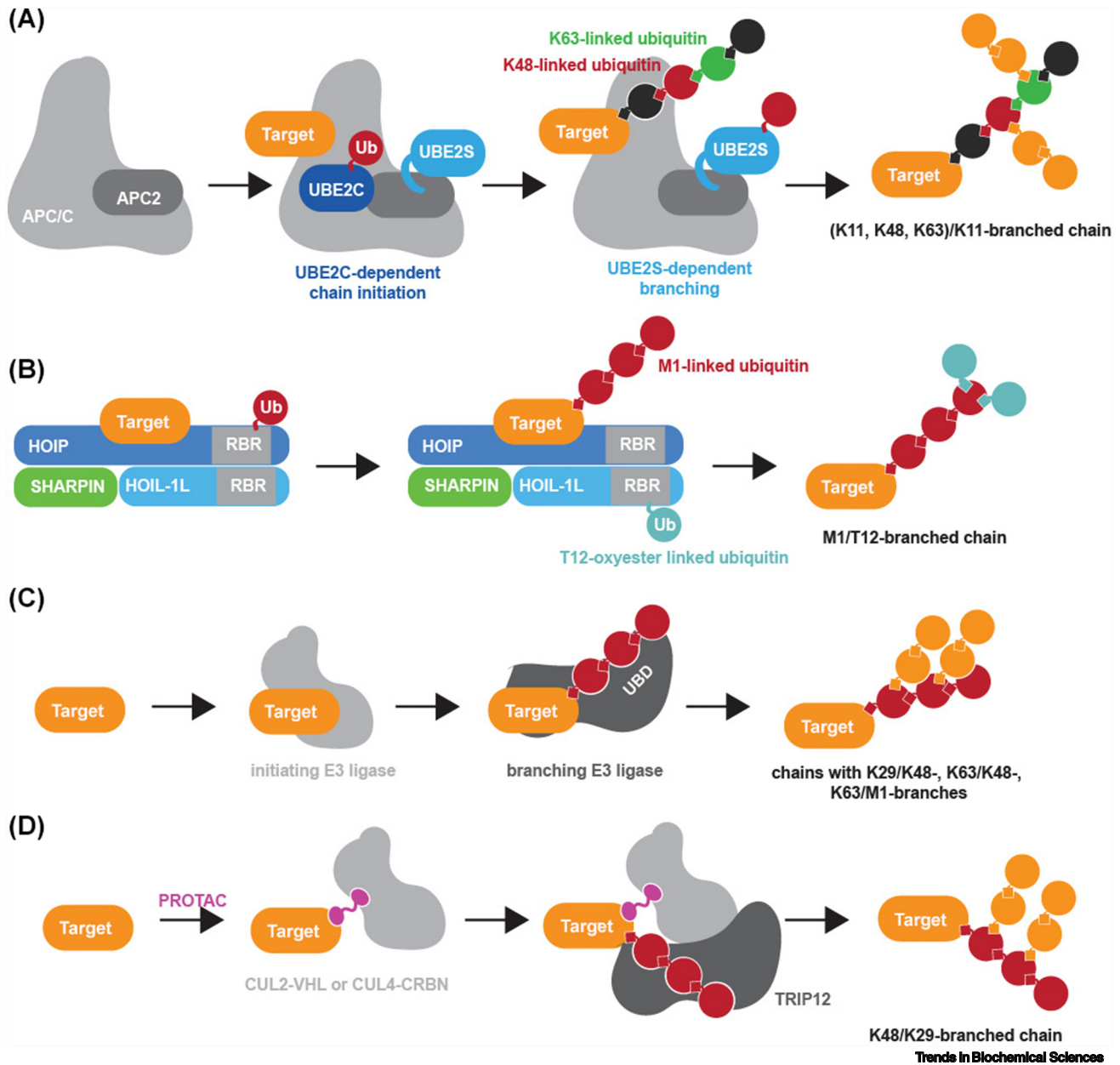
Figure 2. Branched ubiquitin chains are assembled by specific enzymes. (A) The E3 ligase anaphase-promoting complex (APC/C) uses two E2 enzymes with distinct linkage specificity for branched chain assembly. UBE2C initiates short chains containing multiple linkages, while UBE2S branches off specific K11-linked conjugates. Both E2 enzymes bind to the APC2 subunit of the APC/C and are activated by interaction with the really interesting new gene (RING)-domain subunit APC11. (B) LUBAC is a constitutive complex of multiple E3 ligase activities provided by the RING-in between-RING (RBR)-subunits HOIP and HOIL-1L. While HOIP triggers M1-linked chain production, HOIL-1L branches off oxyester-linked ubiquitin molecules. (C) Two E3 ligases can collaborate in producing branched ubiquitin chains. While initiating E3 ligases add the first conjugate, branching E3 ligases bind the chain intermediate through a ubiquitin-binding domain (UBD) and branch off ubiquitin polymers of a distinct linkage. (D) Small molecule-dependent formation of branched ubiquitin chains. PROTAC-dependent formation of K48-linked ubiquitin chains by the E3 ligases CUL2^{VHL} or CUL4^{CRBN} is followed by introduction of K29-linked branches by the homologous to E6AP C terminus (HECT)-E3 ligase TRIP12.

only if it contained both Lys29 and Lys48 [74,75]. It was ultimately found that the E3 ligases UFD4, TOM1, and UFD2 mark the ubiquitin-fusion substrate with K29/K48-branched chains [76,77]. The combination of E3 ligases determines the resulting chain topology: if UBR5 teams up with

Box 1. The nomenclature of branched ubiquitin chains

The explosion in the discovery of distinct branched chain types necessitates that we develop a nomenclature to describe these conjugates more precisely. This should acknowledge our limited understanding of the exact architecture of branched chains and allow for incorporation of new findings without having to reinvent how we refer to such conjugates. In the following text, we propose such a nomenclature of branched ubiquitin chains.

To synthesize a branched chain, enzymes first ubiquitylate a substrate Lys residue and produce a short conjugate that we describe as the initiating chain. An internal subunit of the initiating chain is then modified to produce the branch point ubiquitin, which is often extended into a polymer that we refer to as the branching chain. A branched conjugate is the combination of an initiating and at least one branching chain.

The initiating chain can either be homotypic or it might be of mixed topology and consist of multiple linkages. Branching chains often have a single topology. For example, the initiating E2 enzyme of human APC/C, UBE2C, produces short mixed chains that contain K11, K48, and K63 linkages, while the branching E2 UBE2S adds homotypic K11-linked polymers. To denote all linkages in the initiating chain, we propose to refer to the resulting conjugates assembled as (K11, K48, K63)/K11-branched chains. This differentiates such chains from those produced by the yeast APC/C, which uses the E2 UBC4 to assemble initiating conjugates containing K11, K48, and K63 linkages and the E2 UBC1 to branch off K48-linked conjugates. These conjugates would be best described as (K11, K48, K63)/K48-branched chains. If a branching chain possesses multiple linkages, this could easily be incorporated: $(K_{initiating\ 1}, K_{initiating\ 2}, ...)/(K_{branching\ 1}, K_{branching\ 2}, ...)$. The linkage(s) within the initiating chain should always be noted before those in the branching chain.

In most cases, the precise topology of branched chains has not been determined and we do not know all linkages within initiating or branching chains. As additional linkages are discovered, they can be added to the description of a branched conjugate. Until the architecture of a branched chain has been delineated more precisely, we propose to refer to such conjugates as ubiquitin chains with K_X/K_Y branches, with K_X and K_Y referring to the linkages detected on the branch point ubiquitin. Based on our current knowledge, ubiquitin conjugates assembled on misfolded proteins by UBR4 and UBR5 would thus be best described as chains with K11/K48-branches. If UBR4 would also produce K27-linkages on such substrates, as it has recently been suggested to do on oxidized proteins [125], the resulting conjugates would be described as (K11, K27)/K48-branched chains.

In addition to our incomplete knowledge about linkages, we do not understand the number or sequence of branches within complex ubiquitin chains. If multiple blocks of branching chains are added to an initial conjugate, this could be denoted through a subscript. For example, UBE2S appears to add multiple blocks of K11-linked chains to an initial chain. If this would be two blocks, then the description of branched chains assembled by APC/C, UBE2C, and UBE2S would evolve to $(K11, K48, K63)/(K11)_2$. However, expanding this nomenclature in this way will have to await the development of methods to sequence branched ubiquitin chains.

UBR4, it synthesizes K11/K48-branched chains [36], yet its collaboration with ITCH results in K63/K48-branched polymers [78]. Moreover, TRAF6 works with HUWE1 to build K63/K48-branched chains [79], while it engages LUBAC to assemble K63/M1-linked chains [40,80,81]. Such crosstalk between E3 ligases was also observed in a therapeutic setting, where the E3 ligases CUL4^{CRBN} or CUL2^{VHL} partner with TRIP12 to modify targets of small molecule-induced protein degradation with K48/K29-branched chains [82] (Figure 2D). Given these observations, we anticipate that complex formation between E3 ligases, as it was noted in the N-end rule pathway [83], facilitates assembly of branched conjugates.

Irrespective of its initiating partner, the branching enzyme acts on a ubiquitin polymer and all known branching enzymes accordingly harbor ubiquitin-binding motifs. In line with assembling chains containing K48/K11 branches, the APC/C engages K48-linked ubiquitin for exposure to K11-specific UBE2S [84]. UFD2 uses amino-terminal loops to detect K29-linked ubiquitin for the synthesis of K29/K48-branched chains [76]. The yeast E2 UBC1 and its human counterpart UBE2K rely on a UBA domain to bind K63-linked chains for K63/K48-branched chain production [85], while the E3 UBR5 uses a UBA domain to assemble branched chains of the same topology [78]. Some branching E3 ligases possess multiple ubiquitin-binding domains, as seen with the UBA and UIM domains of HUWE1 [36,56,86,87], or the UBA and NZF domains of LUBAC [73]. Even if a responsible domain has not been identified, the binding of a branching E3 ligase to ubiquitin has still been noted [88].

Together, these studies converge on a model in which many branched ubiquitin chains are assembled through a collaboration of ubiquitylation enzymes with distinct linkage specificity. While the two partners can engage each other either transiently or constitutively, the branching enzyme typically contains a ubiquitin-binding domain to coordinate chain initiation with branching. Both identifying E3 ligase complexes or finding enzymes that bind ubiquitin could thus point to new branching enzymes [75].

Disassembly of branched chains

As ubiquitylation is a reversible modification, it did not come as a surprise that deubiquitylases (DUBs) edit or erase branched ubiquitin chains. Akin to the Ovarian Tumor (OTU)-family DUBs that show specificity for homotypic chain types [89], some DUBs preferentially cleave branched polymers. This includes UCH37, which is activated by binding to the proteasomal lid subunit RPN13 [90,91] (Figure 3A). UCH37 removes K48 linkages from branched ubiquitin molecules, while leaving the variable second linkage intact [92,93] (Figure 3B). Although it is not fully understood how UCH37 gains its specificity for branched chains, it does recognize both moieties that are attached to the shared ubiquitin molecule [93] (Figure 3C). Debranching by UCH37 is required for continued proteasomal activity, potentially by keeping ubiquitin receptors accessible for further rounds of substrate engagement [92,93].

In addition to UCH37, other DUBs cleave branched ubiquitin conjugates. At least *in vitro*, mitochondrial USP30 shows preference for chains with K6/K48 branches over homotypic conjugates
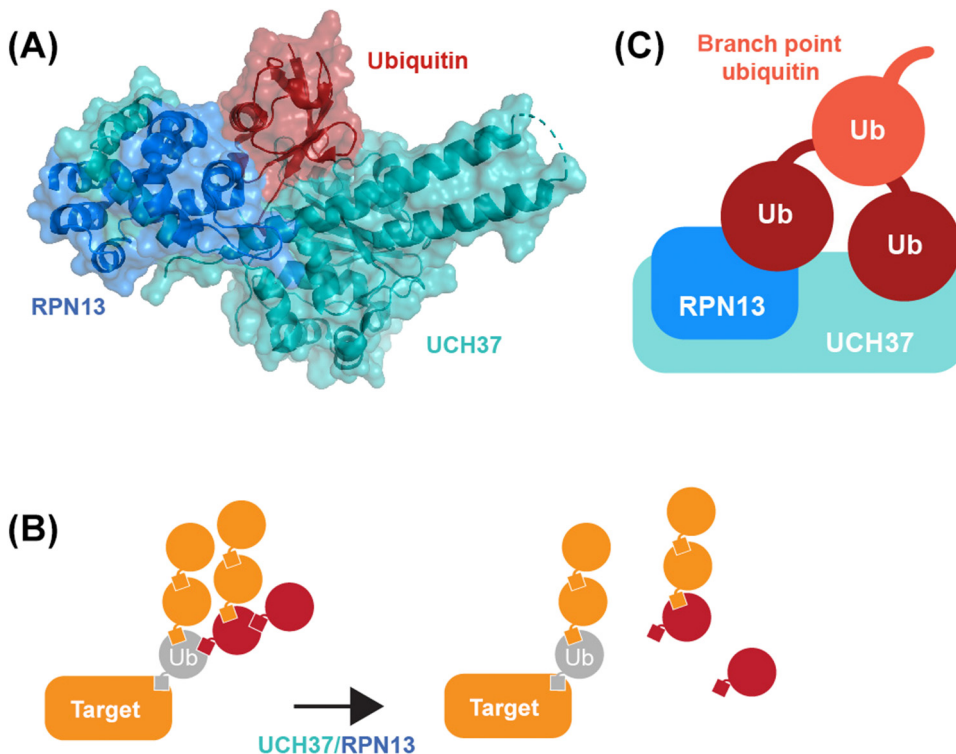
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Figure 3. Branched ubiquitin chains can be specifically dismantled by deubiquitylases. (A) Structure of the debranching enzyme UCH37 bound to its activator, the proteasomal lid subunit RPN13, and a single ubiquitin molecule (PDB ID: 4WLR). (B) UCH37 specifically cleaves the K48 linkage of branched ubiquitin molecules. (C) UCH37 can bind both distal ubiquitin molecules that are attached to a central branch point ubiquitin.

[37]. Befitting this specificity, USP30 counteracts the E3 ligase Parkin that induces mitophagy by plastering defective mitochondria with a ubiquitin carpet containing many branched subunits [28,94,95]. TRABID erases K29/K48-branched chains to protect the E3 ligase HECTD1 from autoubiquitylation and degradation [54], while another OTU family member, the usually K11-specific Cezanne, also cleaves oxyester-linked branches [73]. It will be interesting to determine whether other linkage-specific DUBs of the OTU family can act on branched ubiquitin chain types [89].

Similar to E3 ligases, debranching DUBs could provide unique regulatory features. As they cleave off branched molecules while leaving the remainder of the ubiquitin mark intact, they edit, rather than terminate, ubiquitin signaling. This differentiates debranching enzymes from members of the USP family, which do not show much linkage preference and erase entire chains [96]. On the flipside, inhibition of DUBs through branching can prolong ubiquitin-dependent signaling [79,80]. The discovery of debranching DUBs therefore suggests that cells can toggle between distinct ubiquitin modifications, revealing a ubiquitin code that is much more dynamic than previously appreciated.
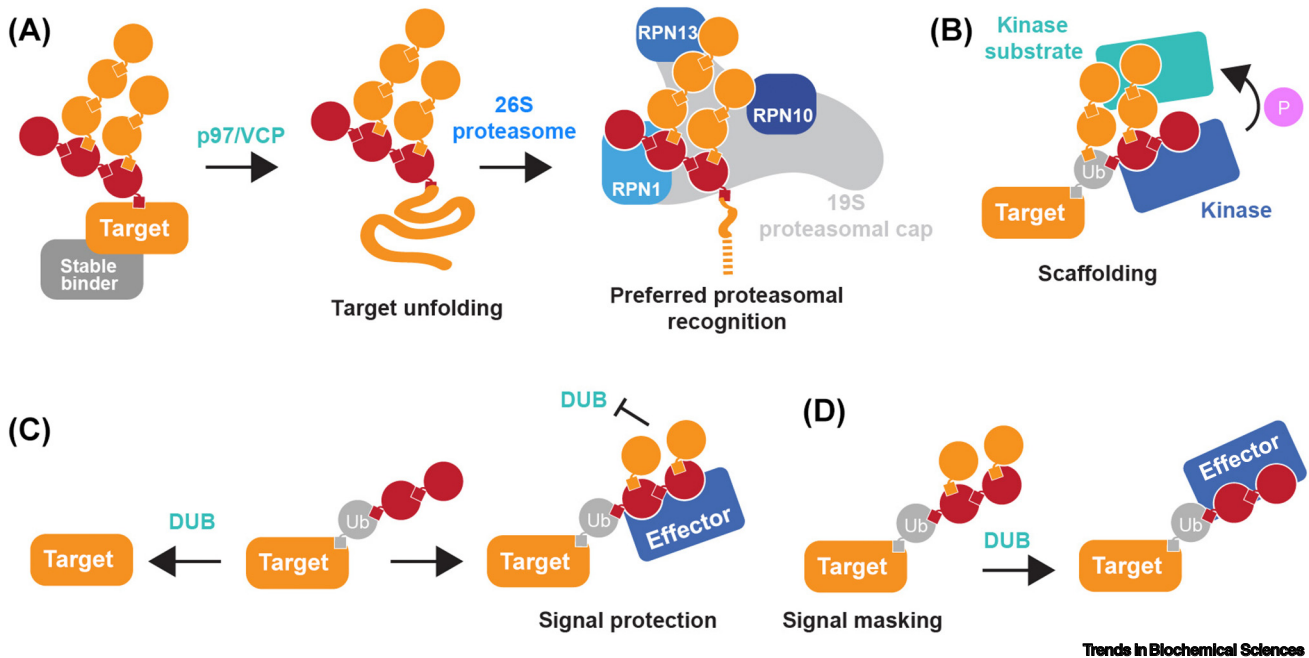
Functions of branched chains

Akin to homotypic ubiquitin chains, branched polymers exert specific functions that are dependent on their topology. Branched conjugates can elicit qualitatively different outcomes, as some induce degradation and others stabilize protein interactions. In addition, branched chains can amplify the signal of a homotypic polymer and thereby play a quantitatively distinct role. As we are yet unable to sequence ubiquitin chains, it is unclear whether the number or sequence of branches impact the output of these modifications. With research into branched chains still in its infancy, the full spectrum of cellular outcomes encoded by these conjugates remains unknown and we expect that many functions of branched ubiquitin chains are awaiting discovery.

Proteolytic functions

In the first example of signaling by branched ubiquitin chains, (K11, K48, K63)/K11-branched conjugates were found to act as proteasomal priority signals that allow for rapid and efficient protein clearance [25,36] (Figure 4A). These chains rise in abundance during mitosis or upon proteotoxic stress, when many proteins must be quickly turned over [36]. Substrates of ubiquitin chains with K11/K48 branches are often caught in very stable interactions, such as histones that are tightly bound to each other yet need to be rapidly removed from transcription start sites in early mitosis [64]. In addition to acting on cytosolic and chromatin-bound proteins, the same ubiquitin chain types help eliminate proteins of the endoplasmic reticulum [97,98], which upon translocation into the cytosol become prone to aggregation [99]. The ability of branched chains to induce efficient degradation during mitosis and proteotoxic stress has been conserved from yeast to humans [67,100].

Other branched polymers also drive efficient proteasomal degradation. Having noted that most K29 linkages are part of conjugates that contain K48 connections [39], polymers with K29/K48 branches were found to trigger the elimination of long-studied model substrates of the ubiquitin proteasome system [76,97]. Similar chain topologies also allow for proteasomal recognition of the VPS34 kinase [101], a crucial regulator of proteotoxic stress, and they elicit the efficient removal of PROTAC-dependent substrates of CUL2^{VHL} and CUL4^{CRBN} [82]. The K29-specific branching E3 ligase TRIP12 also helps degrade the FBW7 substrate adaptor of SCF E3 ligases [88]. Akin to chains with K11/K48 or K29/K48 branches, K63/K48-branched conjugates assembled by the E3 ligases ITCH and UBR5 trigger proteasomal degradation [78]. It is interesting to note that all proteolytic branched conjugates contain blocks of K11 or K48 linkages, which within

Figure 4. Branched chains encode diverse functions. (A) Branched ubiquitin chains act as proteasomal priority signals that elicit preferential recognition by the ubiquitin-selective segregase p97/VCP and by multiple receptor subunits of the proteasomal cap. (B) Branched chains can act as signaling scaffolds through independent recognition of the different linkages by distinct effector proteins. (C) Branched chains can protect a ubiquitin signal from deubiquitylase (DUB)-mediated disassembly, while still allowing for recognition by downstream effector proteins. (D) Branched chains can prevent effector engagement, requiring prior debranching for efficient signaling.

homotypic chains also instigate proteasomal degradation [30,31]. Branched chains are therefore not only a very strong, but also a broadly used, proteasomal delivery signal.

The improved ability of branched chains to elicit degradation might in part be due to their enhanced detection by proteasomal receptors, as seen with K11/K48-branched ubiquitin trimers that are recognized by RPN1 with tenfold higher affinity than their linear counterparts [102] (Figure 4A). As the proteasome contains three ubiquitin receptors with slightly distinct linkage preferences [103–105], simultaneous recognition of multiple linkages in a branched chain, as well as the high local ubiquitin concentration at the substrate, could also increase the affinity of a modified protein for the proteasome. Because multivalent interactions often delay complex dissociation, the synergistic recognition of branched chains might explain the need to trim such conjugates at the proteasome to preserve the degradation capacity of this essential machine [92,93].

In addition to efficient proteasomal recognition, branched chains are preferred substrates of the p97 segregase [36] (Figure 4A). This enzyme unfolds proteins starting with the ubiquitin tag and thereby separates them from stable partners at chromatin, membranes, or aggregates [6,106]. While yeast CDC48 can unfold substrates decorated with very long homotypic chains, human p97 strongly prefers branched conjugates [25,36,107,108]. Unfolding by p97 prepares proteins for proteasomal degradation [109], which is likely relevant for substrates of small molecule-induced degradation that lack evolved proteasomal initiation sites [82,110]. p97 further improves substrate turnover by recruiting E3 ligases that add more ubiquitin molecules to ensure persistent proteasomal recognition [111,112] and by handing over ubiquitylated proteins to shuttle factors of the 26S proteasome [113]. Thus, processing by p97 likely contributes in multiple ways to the improved ability of branched chains to elicit proteasomal degradation.

As proteasomal degradation is a key defense mechanism against aggregation, an event that often wreaks havoc in the brain, mutations in writers or readers of branched chains cause neurological disease. *UBR4* is mutated in episodic ataxia [114], while *UBR5* is within a genomic island determining the age of onset of Huntington's disease [115]. *TRIP12* has been associated with Clark-Baraitser syndrome and Parkinson's disease [116] and mutations in *HUWE1* cause Turner type mental retardation and X-linked intellectual disability [117]. *VCP*, which encodes p97, is mutated in amyotrophic lateral sclerosis, frontotemporal dementia, multisystem proteinopathy, and tauopathies [118]. These observations highlight the role of branched chains in preserving organismal homeostasis and they raise the exciting possibility that increasing branched chain production or recognition may yield new therapeutic approaches against protein aggregation diseases.

Nonproteolytic functions

As K11- and K48-linked chains themselves trigger proteasomal degradation [30,31], it might not be surprising that a combination of these linkages produces a strong proteolytic signal. However, branching can also involve chain topologies that provoke nonproteolytic outcomes and, in these cases, the distinct ubiquitin linkages can be detected by effector proteins independently of each other to allow for combinatorial decoding [119]. As an example, ubiquitin chains with K63/M1 branches help activate the NF-κB transcription factor by bringing a kinase, which binds to K63-linked chains, into proximity of its target that is recruited via the M1 linkages [40,81] (Figure 4B).

In addition to scaffolding, branching can impact the persistence of a ubiquitin signal, as it was observed when ubiquitin chains with K63/K48 branches were first detected [79]. These chain types are assembled on the E3 ligase TRAF6 in a reaction that requires the activity of TRAF6 itself as well as the branching enzyme HUWE1. Rather than driving degradation, K48 branches protect K63-linked chains on TRAF6 from recognition by the DUB CYLD, but they do not prevent the same conjugates from recruiting downstream kinases that activate NF-κB [79] (Figure 4C). M1-linked ubiquitin molecules that branch off K63-linked chains similarly protect these polymers from disassembly by A20 [80]. By inhibiting a DUB without impacting a downstream effector, branching effectively preserves the ubiquitin signal. In a variation on this mechanism, it can be the removal of a branch that allows for recognition of ubiquitin conjugates by linkage-specific effector proteins, as seen during DNA repair [120] (Figure 4D). Branching can therefore modulate, rather than amplify, the information flow through ubiquitin-dependent pathways.

Such a function could be at play in yeast methionine metabolism, which requires the MET4 transcription factor. When methionine is abundant, modification of MET4 with a short K48-linked chain blocks expression of genes involved in methionine biogenesis [121]. This nonproteolytic function of a K48-linked chain relies on a ubiquitin-binding domain in MET4 that detects the conjugate on the same molecule and generates a closed transcription factor conformation. Attaching K11-linked ubiquitin to MET4 disrupts this autoinhibitory conformation and thus allows transcription to proceed [122]. Although more work is required to dissect the MET4 modifications, this study further suggests that branching can rewire ubiquitin-dependent signaling and it raises the provocative idea that some ubiquitin chains with K11/K48 branches might fulfill nonproteolytic functions.

Concluding remarks

Driven by novel technologies, such as bispecific antibodies, middle-down mass spectrometry, or ubiquitin clipping [28,29,36,52,92], we have witnessed an explosion in our understanding of branched ubiquitin chains. However, without being able to sequence ubiquitin chains in the same way as we do with nucleotide polymers, we are still unable to decipher the number and sequence of branches in such conjugates. The architecture of branched chains therefore remains somewhat mysterious and what is currently described as a single chain type might turn out to be

Outstanding questions

Can we sequence complex ubiquitin chains to decipher their precise topology, including the number and sequence of branches?

How many types of branched ubiquitin chains are produced in cells and how many of these encode unique biological information?

Do cells toggle between ubiquitin modifications to establish a much more dynamic ubiquitin code than previously appreciated?

Are there E3 ligases that are specialized on producing branched ubiquitin chains and how are these regulated?

Are there effector proteins that directly bind to branched ubiquitin molecules to provoke a particular cellular response?

a diverse collection of distinct conjugates. In fact, ubiquitin chains with a signature K11/K48 branch can either contain blocks of K11-linked polymers, as seen for substrates of the APC/C, or possess multiple K48-linked polymers, as noted for misfolded proteins [36]. It remains unknown whether such differences in the architecture of branched chains are read out by dedicated effectors and provoke unique outcomes in cells.

Understanding the precise topology of branched chains will be critical, as the complexity of these conjugates can be staggering: only taking the eight major ubiquitin linkages into account, 28 branches can be produced and many more branched ubiquitin molecules or chain types might be formed if an enzyme can use three or four linkages to assemble a conjugate. The complexity of branched chains further increases as we begin to consider oxyester-linked or phosphorylated ubiquitin [123]. In addition, ubiquitin molecules modified on more than two sites have been detected in cells [28] and *in vitro* [26], and whether triple branches serve signaling roles will be an interesting question for future studies.

While enriching branched polymers of a specific architecture is still challenging, purifying E3 ligase complexes could provide a route to discovering new functions of branched chains. Recent proteomic approaches to systematically map protein interactions have uncovered many E3 ligases that work together and might assemble branched ubiquitin marks on their substrates [124]. Some E3 ligase combinations are bound to produce new branched chain types and fulfill still unknown roles in signaling. Even if such enzymes provoke the most canonical output for branched chains and drive rapid proteasomal turnover, their discovery will be of high interest for improving induced protein degradation as a therapeutic approach. Deciphering signaling by branched chains is therefore not only bound to increase our understanding of how information travels in cells, but it might also produce tangible outcomes for the many patients of neurodegenerative diseases that could benefit from efficient protein degradation to improve their condition (see Outstanding questions).

Declaration of interests

M.R. is co-founder and SAB member to Nurix Therapeutics and Zenith Therapeutics, SAB member for Monte Rosa Therapeutics, and iPartner at The Column Group Ventures.

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