Trends in Cell Biology

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Review

Branching Out: Improved Signaling by Heterotypic Ubiquitin Chains

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Ubiquitin chains of distinct topologies control the stability, interactions, or localization of many proteins in eukaryotic cells, and thus play an essential role in cellular information transfer. It has recently been found that ubiquitin chains can be combined to produce branched conjugates that are characterized by the presence of at least two linkages within the same polymer. Akin to their homotypic counterparts, branched chains elicit a wide array of biological outputs, further expanding the versatility, specificity, and efficiency of ubiquitin-dependent signaling. This review discusses emerging understanding of the synthesis and function of branched ubiquitin chains.

Highlights

Branched ubiquitin chains, characterized by a subunit modified by one, two, or more residues, can be an abundant fraction of the total ubiquitin chain pool.

Branched chains can be synthesized via different routes including a single E3, the collaborative effort of two or more E3s, or by the combined action of two or more E2 enzymes.

Branched chains are often detected by effector proteins or complexes that contain multiple ubiquitin-binding domains to read out the distinct linkages or higher ubiquitin concentrations afforded by these conjugates.

Branched chains improve the versatility, complexity, and specificity of ubiquitin-dependent signaling by prioritizing substrate processing, favoring coincidence detection, or enabling editing of ubiquitin functions.

Emerging Evidence for Branched Ubiquitin Chains

The intricate processes that shape the fate and function of eukaryotic cells depend on accurate translation of environmental information into robust cellular responses. To accomplish this task, cells modulate the stability, activity, or localization of key proteins by means of post-translational modifications. These are established by specific enzymes or 'writers', are decoded by selective effectors or 'readers', and are terminated by proteins acting as 'erasers'. Although single modifications can elicit specific responses, signaling is often more complex, and multiple modifications cooperate to sharpen the output of a pathway: for example, phosphorylation, methylation, and ubiquitylation marks can be found on overlapping nucleosomes where they engage in intricate crosstalk to improve the accuracy of gene expression [1]. An emerging example for complex signal transduction is modification of proteins with ubiquitin chains, a reaction that controls the division, differentiation, and survival of all eukaryotic cells [2]. Although cells were known to assemble multiple types of ubiquitin chains [3], it was recently found that the combination of such conjugates to generate a diverse set of branched polymers plays a unique role in cellular information transfer.

The formation of any ubiquitin chain requires that the N terminus or one of the seven Lys residues of substrate-attached ubiquitin molecules is modified with additional subunits. Early work focused on homotypic chains which are connected through the same ubiquitin residue [3]: K11- and K48-linked chains, for example, deliver proteins to the proteasome for degradation [4,5], whereas M1- and K63-linked chains coordinate the assembly of cellular machineries controlling translation, autophagy, immune signaling, and DNA repair [6–10] (Figure 1A). However, many chains are now known to be of more complex heterotypic nature and contain multiple linkages. In mixed chains, all building blocks are modified with a single ubiquitin but are connected through distinct linkages (Figure 1B). By contrast, branched polymers are characterized by at least one subunit that is modified on two or more residues at the same time (Figure 1C). Branched chains can become abundant [11–14], suggesting that they may play a central role in ubiquitin-dependent signaling (Box 1).

As with homotypic conjugates, branched chains can differ in the linkages used for their assembly: following the discovery of a cell-cycle role for K11/K48-branched chains [15], functions were

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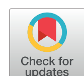

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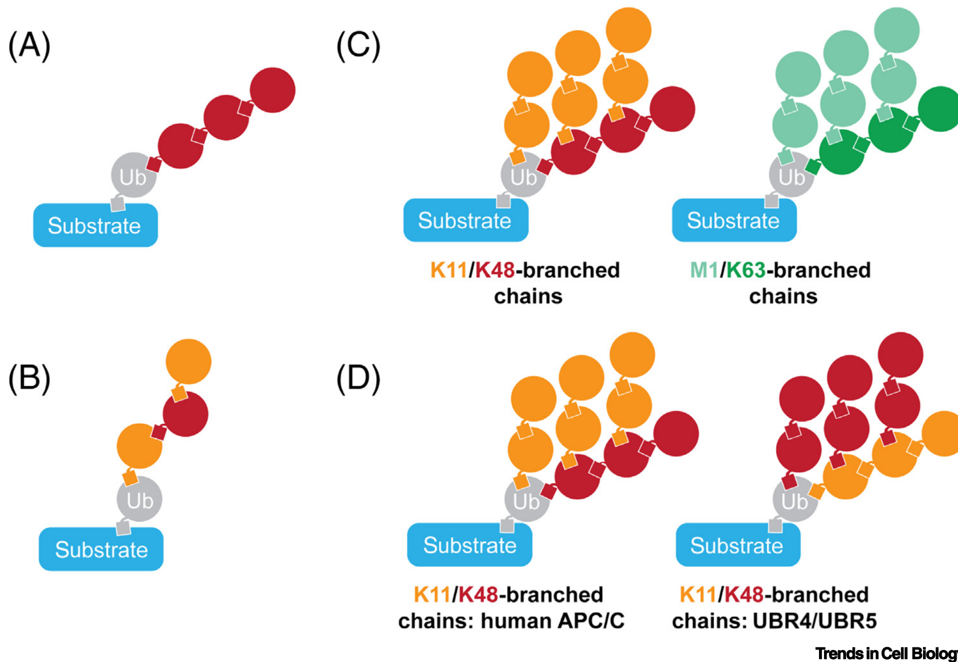

Figure 1. Ubiquitin Chain Architecture. (A) Homotypic ubiquitin (Ub) chains are connected through the same acceptor residue on ubiquitin. Either the α-amino group of Met1 or the ε-amino group of one of the seven Lys residues of ubiquitin is used for chain assembly. (B) Mixed chains contain multiple linkages, but each chain subunit is only modified with one other ubiquitin molecule. (C) Branched ubiquitin chains are characterized by a subunit modified on two or more residues at the same time. Branched conjugates often result from a combination of multiple chain types. Akin to homotypic conjugates, branched chains can differ in the ubiquitin residues used to connect the subunits of the polymer. (D) Branched chains can differ in their architecture even if they contain the same linkage types. K11/K48-branched conjugates assembled by the E3 ubiquitin ligase APC/C contain multiple blocks of K11-linked chains, whereas K11/K48-branched chains produced by the concerted action of the quality-control E3s UBR4 and UBR5 are characterized by blocks of K48-linked polymers.

assigned to K29/K48-, K48/K63-, and M1/K63-branched conjugates [16–18], and biochemical or proteomic analyses pointed to the existence of K6/K48-, K11/K33-, K27/K29-, and K29/K33-branched polymers [19–22]. In theory, 28 distinct branched chains could be built with two linkages, and a larger spectrum of conjugates emerges from the combination of more linkages. Branched chains with the same linkages can still differ in their architecture: whereas K11/K48-branched chains assembled during mitosis contain multiple blocks of K11-linked chains [15], the K11/K48-branched polymers produced in response to proteotoxic stress are characterized by blocks of K48-linked chains [13] (Figure 1D). Although not yet shown, it is possible that differences in the number and/or sequence of branches might affect the outcome of these modifications. Because a large number of different branched chains could be formed, these branched conjugates – if produced in cells and read by selective effectors – could greatly improve the versatility, accuracy, or efficiency of ubiquitin-dependent signaling. How this is accomplished is the focus of this review.

How Are Branched Ubiquitin Chains Synthesized?

A Collaboration between Three Enzymes

As with every ubiquitin mark, formation of branched chains requires the close collaboration of at least three enzymes. Two human E1 enzymes activate ubiquitin in an ATP-dependent manner [23], before they transfer ubiquitin to a Cys residue in one of ~40 E2 enzymes [24]. The E2s then cooperate with ~600 E3 ligases to attach ubiquitin's carboxy-terminus to ε-amino groups

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Box 1. Detection of Heterotypic Ubiquitin Chains

Monitoring branched chains requires detection of ubiquitin molecules that are modified on more than one acceptor residue. This can be achieved indirectly through *in vitro* reconstitution in the presence of ubiquitin mutants. Although mixtures of ubiquitin variants carrying single Lys mutations should support substrate modification, double mutants lacking both attachment sites for branched chain formation should be resistant to ubiquitin transfer [15]. *In vitro* reactions could also be investigated by mass spectrometry, which initially allowed the detection of chains involving neighboring Lys residues, such as K6/K11-, K27/K29-, or K29/K33-branched conjugates [19]. Branched conjugates involving neighboring Lys residues can also be detected in cell lysates by mass spectrometry [22].

Proteomic approaches are more difficult if nonneighboring Lys residues are involved in branched chain assembly or if Lys residues are separated by Arg residues recognized by the trypsin protease during sample preparation; whereas the first issue arises for K11/K48-branched chains, the latter is the case for K48/K63-branched conjugates that play roles in immune signaling [11,15,17]. Under such conditions, middle-down mass spectrometry or ubiquitin-replacement cell lines, which selectively express a ubiquitin variant lacking the Arg54 residue between Lys48 and Lys63, can be implemented to allow proteomic analysis [11,12,74]. Akin to the latter example, expression of a ubiquitin variant carrying a TEV protease cleavage site between Glu53 and Arg54 enables detection of branched chains by western blotting, which allowed researchers to discover such conjugates during mitosis [15].

Linkage-specific antibodies, affimers, and ubiquitin-binding entities have greatly expanded our toolbox to analyze ubiquitin-dependent signaling [13,21,87,93–95]. They can be used to enrich for specific linkages in cell lysates, which can then be followed by treatment with linkage-specific DUBs in a protocol referred to as UbiCrest [89]. This approach has been used to characterize K48/K63- and M1/K63-branched conjugates [11,14,17,84]. Bispecific antibodies that combine arms for each linkage within a branched chain allow such conjugates to be detected by western blotting, immunofluorescence microscopy, or histology [13], which provided key evidence for the role of K11/K48-branched chains in the cell cycle and protein quality control [13,41,60].

Bispecific antibodies for detection of other branched chain topologies are missing. Moreover, none of the aforementioned technologies allows determination of the precise chain architecture, in other words how many branches are present and where in the conjugate branches are found. The development of new technologies to sequence branched chains more accurately will certainly increase our knowledge of these crucial cellular signaling devices.

of Lys residues, hydroxyl groups of Ser or Thr residues, thiol groups of Cys residues, or to the N-terminal amino groups of other substrates. Really interesting new gene (RING) and U-box family E3s promote the transfer of ubiquitin from the E2 to an acceptor residue in the target [25]. This differs from the homologous to E6AP C terminus (HECT) E3s, which possess an active-site Cys and form an intermediary ubiquitin thioester [26]. The RING-between-RING (RBR) and RING-Cys-relay (RCR) families of E3s are hybrid enzymes that use RING domains to attract charged E2s but transfer ubiquitin to their substrates via an obligate thioester intermediate [27,28].

Separation of Chain Initiation and Branching

These enzymes first modify a substrate residue, followed by the formation of an initial conjugate, and the process is completed when polymers of distinct topology branch off. Although some E3s recognize substrate sequences for efficient initiation [29], global analyses of ubiquitylation sites failed to reveal consensus motifs for rapid modification of target residues [30,31]. By contrast, many E2 enzymes and E3 ligases bind acceptor ubiquitin surfaces to accelerate chain elongation [32–34]. Consistent with this notion, initiation is often slow and inefficient, whereas chain elongation occurs with great speed [29,32,35,36]. Because the same ubiquitin surfaces are recognized for branching and elongation [15], we hypothesize that many E3 ligases will branch ubiquitin chains off an existing conjugate instead of adding additional polymers to different target residues.

Some E3 ligases are able to catalyze both chain initiation and elongation [35], which, as shown for the HECT-E3 WWP1 [37], can lead to the assembly of branched chains (Figure 2A). However, in many cases the distinct steps of chain assembly are delegated to separate enzymes: the RBR-type E3 HHARI, for example, monoubiquitylates proteins that are then decorated with K48-

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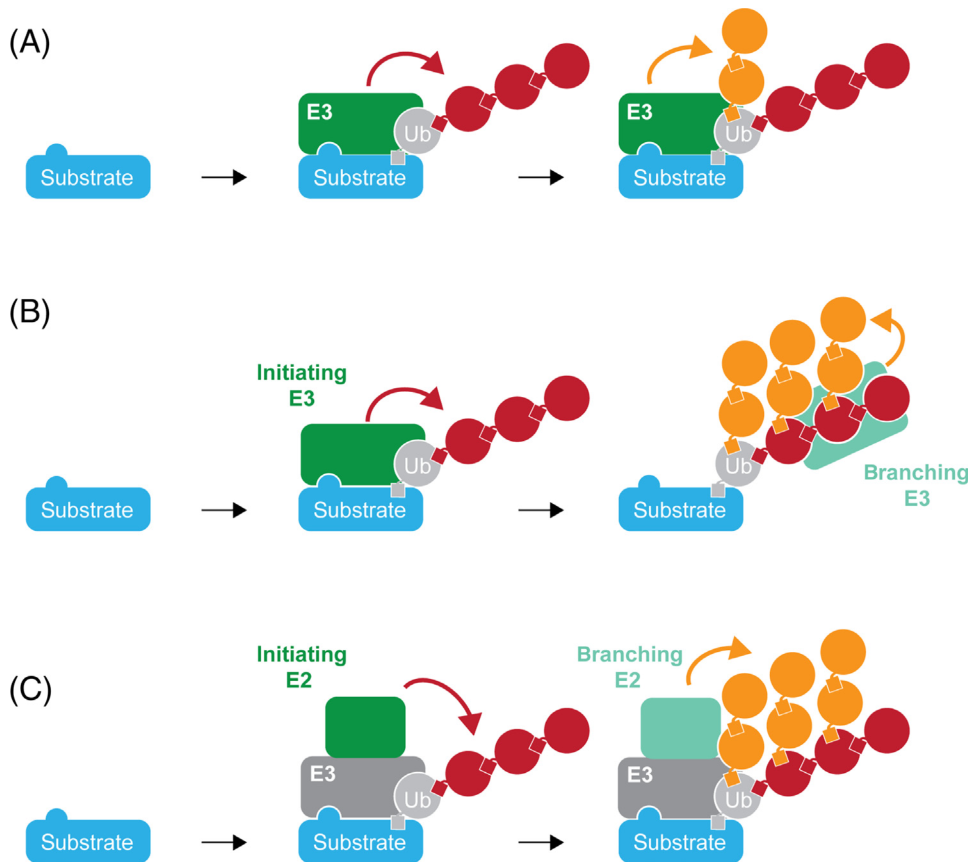

Figure 2. Synthesis of Branched Ubiquitin Chains. (A) A single E3 ubiquitin ligase can produce branched chains if it is able to catalyze chain initiation and elongation, and does not have unique linkage specificity, such as the HECT-E3 WWP1. (B) Branched chains can be produced by two E3 ligases that converge on the same substrate. In many cases, the branching E3 contains a ubiquitin-binding domain (UBD) that recognizes the initial conjugate. The linkage specificity of this UBD and the catalytic specificity of the branching E3 usually differ. (C) Branched chains can also be produced if one E3 cooperates with multiple E2 enzymes that have different linkage specificities. The human APC/C engages the E2s UBE2C or UBE2D to form initial short mixed conjugates on substrates, before the E2 UBE2S branches off multiple K11-linked chains. Abbreviation: Ub, ubiquitin.

linked chains by E3s of the SCF family [38], and the sequential action of the E3s RAD5 and RAD18 is necessary to build K63-linked chains on the DNA polymerase processivity factor PCNA [39]. If the first E3 produces ubiquitin chains, and its successor possesses a different linkage specificity, then branched chains can be synthesized (Figure 2B).

There are now many examples for how teamwork between E3 ligases results in the formation of branched chains. Substrates of the yeast ubiquitin-fusion degradation (UFD) pathway are initially modified by UFD4, before UFD2 assembles K29/K48-branched chains [16,36,40]. Several quality-control E3 ligases, such as yeast DOA10, HRD1, UBR1, and SAN1, or metazoan UBR4 and UBR5, collaborate to produce K11/K48-branched chains [13,41]. During cell death signaling, the thioredoxin interacting protein TXNIP1 is modified with K63-linked chains by the E3 ITCH, before UBR5 and HUWE1 synthesize K63/K48-branched conjugates [11,17]. A similar cooperation can occur between E2 enzymes that engage the same E3 (Figure 2C): the human anaphase-promoting complex (APC/C) relies on two E2s with limited linkage preference, UBE2C and UBE2D, for

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initiation [29,42]. It then employs UBE2S to branch off blocks of K11-linked chains to produce K11/K48-branched conjugates [15]. The yeast APC/C uses a similar strategy, built around the E2s UBC4 and UBC1, to synthesize K11/K48-branched chains characterized by blocks of K48 linkages [43].

Integrating the Distinct Ubiquitylation Activities

How multiple ubiquitylation enzymes can converge on the same substrate, a key step in building branched chains, is not fully understood. It might involve a scaffold, such as the APC/C, which binds substrates, initiating E2, and branching E2 through distinct motifs, thus bringing all players of branched chain formation into close proximity with each other [44]. E3 ligases can similarly engage each other to form hubs of chain formation, as observed in the N-end rule pathway [45]. In other cases, branching E3s are directed to substrates by detecting the initial mark: HUWE1 and UBR5 use UBA domains to bind K63-linked chains, before they synthesize K48 linkages to build K48/K63-branched chains [11,13,17,21], and UFD2 detects K29 linkages through N-terminal loops before it produces K29/K48-branched conjugates [16]. Similar mechanisms are put in place by E2s: the yeast branching E2 UBC1 contains a UBA domain [46], and UBE2S even depends on ubiquitin recognition for completing its active site and catalyzing K11-linked chain formation [32]. The ability of ubiquitylation enzymes to bind to conjugates of linkages distinct from their catalytic preference is therefore a recurrent theme of branched chain formation.

The separation of chain initiation and elongation raises the possibility that each step can be regulated independently. Indeed, the E2 UBE2C, which initiates branched chain formation by the APC/C, is degraded during the G1 phase of the cell cycle [47], whereas association of UBE2S with the APC/C is regulated by cell cycle-dependent phosphorylation [48]. Restricting the availability of UBE2C and UBE2S for APC/C limits K11/K48-branched chain formation to a brief period between mitosis and early G1 [13]. Given that phosphorylation can stimulate ubiquitin recognition by specific effectors [49], it is possible that association of branching E3s with the initial chains could be regulated, as could the formation of complexes between E3s. However, the latter two modes of regulation have not yet been observed in cells, illustrating how future work will be necessary to dissect the molecular mechanisms and regulation of branched chain formation.

How Are Branched Chains Decoded?

Once branched chains have been synthesized, they are recognized by effectors that translate the ubiquitin mark into a specific outcome for the modified protein. Most effectors contain one or more of ~20 distinct modular ubiquitin-binding domains (UBDs) [3]. These include ubiquitin-associated (UBA) domains, as in the proteasomal shuttle HHR23A and the autophagy receptor p62/SQSTM [50,51]; UBAN domains, that control immune signaling [52]; and ubiquitin-interacting (UIM) motifs in RPN10, a component of the proteasomal lid [53]. Branched chains differ from homotypic polymers by the presence of at least two linkage types as well as the branch itself. Branched chains also increase the ubiquitin concentration close to the substrate, which allows distinct modes of cellular recognition.

Because most UBDs bind ubiquitin with low micromolar affinity [50], effectors often need to find ways to improve the detection of a modified substrate. Some effectors accomplish this feat by using multiple UBDs that can interact with successive chain moieties at the same time [54]. If their UBDs possess distinct linkage specificities, effectors could engage branched chains by recognizing both linkages present in the conjugate (Figure 3A). This detection mode likely accounts for the strong ability of HHR23A to bind to a set of branched chains centered around K48 linkages [15]: although the first UBA domain of this proteasomal shuttle associates with ubiquitin without much linkage specificity, its second UBA domain shows preference for K48 linkages [50,54]. In a

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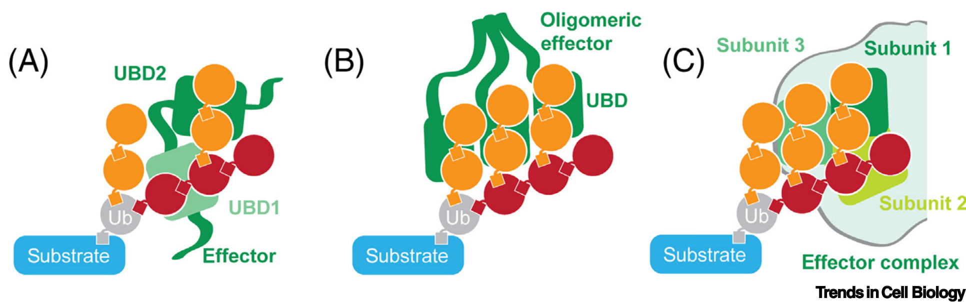

Figure 3. Cellular Detection of Branched Ubiquitin Chains. (A) Effectors of branched chains typically have multiple ubiquitin-binding domains (UBDs). In some cases, as with the proteasome shuttle HHR23A, these UBDs are within the same polypeptide chain. (B,C) Effectors can also engage each other, which results in higher-order complexes containing multiple UBDs. Examples for such effectors of branched chains are the p47 or UBXD1 adaptors that engage the p97/VCP segregase as trimers, providing multiple ubiquitin-binding sites in a single effector complex (B) or different ubiquitin-binding subunits of the 26S proteasomal cap (C). Abbreviation: Ub, ubiquitin.

similar manner, the A20 and NEMO proteins contain distinct domains for reading out M1- and K63-linked ubiquitin [18,55], making them attractive candidates to detect the M1/K63-heterotypic chains implicated in the activation of transcription factor NF-κB [14,18].

In addition to combining UBDs within the same polypeptide, ubiquitin chains could also be detected with higher affinity if multiple effectors engage each other. By being in the same complex, collaborating UBDs are able to read out the distinct linkages or increased ubiquitin concentrations afforded by branched conjugates (Figure 3B). A variation on this theme is played by hexameric p97/VCP, which recognizes its targets through dedicated adaptors [56]. Some of these adaptors, such as p47 or UBXD1, engage p97/VCP as trimers, and thus provide multiple ubiquitin-binding sites for a single effector complex. p97/VCP accordingly binds K11/K48-branched chains with higher affinity than homotypic K11- or K48-linked conjugates containing the same number of ubiquitin molecules [13,15], and it is the most strongly enriched effector in purifications of K11/K48-branched chains from human cells [13].

Akin to p97/VCP, the proteasomal cap contains multiple binding sites for ubiquitylated targets, namely the regulatory particle non-ATPase subunits RPN1, RPN10, and RPN13 [57], which display slightly different preferences towards ubiquitin linkages: RPN1 associates most effectively with K6- and K48-linked chains [58], RPN10 prefers K11- and K48 linkages [53], whereas RPN13 detects monoubiquitin and K48-linked polymers [59]. Although it has not been formally tested, the presence of several slightly distinct UBDs within the proteasomal lid could explain why branched chains trigger degradation more efficiently than do homotypic polymers [15] (Figure 3C). Together, these results suggest that combining UBDs with distinct linkage preference, either within the same polypeptide or effector complex, generates an effective means for reading out branched chains. Whether there are UBDs that directly bind to the ubiquitin branch is not known.

What Information Can Branched Ubiquitin Chains Encode?

Because effectors couple ubiquitin signals to a particular consequence for the modified protein, they determine the function of branched chains in cells. In the following we discuss the roles of branched chains that have recently emerged. It is important to note that this is often work in progress, and crucial details about how branched chains control cellular information flow remain to be discovered.

Specific Recognition: Protein Unfolding and Complex Dissociation by p97/VCP

The first branched polymers associated with a specific function, K11/K48-branched chains, dramatically increase in abundance during mitosis and in response to proteotoxic stress [13,41,60],

and cell division regulators or misfolded proteins of the cytoplasm and endoplasmic reticulum (ER) are accordingly labeled with such conjugates [13,15,41,60,61]. Bispecific antibodies against K11/K48-branched chains allowed the identification of p97/VCP as a crucial effector of K11/K48-branched chains [13], which agreed with the known cell-cycle and protein quality-control roles of p97/VCP [62]. These findings also confirmed early *in vitro* results that revealed preferential binding of p97/VCP to substrates modified with branched, rather than homotypic, chains [15]. Targeting to p97/VCP has therefore emerged as a central signaling function of branched ubiquitin conjugates (Figure 4A).

The AAA+ ATPase p97/VCP translates the energy of ATP hydrolysis into protein unfolding [63], which prepares well-structured ubiquitylated proteins for degradation by the 26S proteasome [64,65]. p97/VCP shows much higher unfolding activity towards proteins decorated with branched chains than for substrates modified with homotypic polymers [65]. Cells also use p97/VCP to segregate proteins from binding partners, a function that was originally found to be required for activation of a membrane-bound transcription factor and for ER-associated degradation [66,67]. Its segregase activity is also at the heart of the role of p97/VCP in quality control, mitochondria-associated degradation, DNA recombination and repair, and cell-cycle control [62]. Many of these processes involve proteins modified with branched chains, suggesting that p97/VCP-dependent protein unfolding and complex dissociation are frequent events following modification with such ubiquitin conjugates.

Although the identification of p97/VCP as a major effector of branched chains has been an exciting development, it is not known how p97/VCP engages branched polymers, in other words whether it recognizes the proximity of ubiquitin chains or binds to the branch itself. It also remains to be determined whether p97/VCP prefers specific branched chain topologies and whether only select coadaptors are involved in processing such conjugates. Given that mutations in branching E3 ligases and p97/VCP result in protein aggregation and neurodegeneration [68], it will be important to improve our understanding of the interplay between heterotypic conjugates and the p97/VCP machinery.

Signal Amplification: Proteasomal Priority Signals

Because many p97/VCP-substrates are delivered to the proteasome, it was not surprising that degradation emerged as another function of branched chains. Previous work had found that the efficiency of proteasomal turnover is improved if more ubiquitin molecules are attached to a substrate [69,70]. To achieve this goal, E3 ligases could assemble longer chains, but many enzymes have difficulties in accomplishing this feat, and only add ~6–7 molecules to a growing chain [32,35,71,72]. Alternatively, it was shown that adding multiple ubiquitin chains improved proteasomal substrate processing [69,70]. However, the model substrates used in these studies were intrinsically disordered and contained many Lys residues, suggesting that folded proteins demand different strategies.

Filling this gap, branching E3s can add more ubiquitin molecules to substrates without the need to overcome slow initiation or difficult elongation. K11/K48-branched chains accordingly improve substrate recognition by the proteasome receptor RPN10 or the shuttle HHR23A, and they strongly accelerate substrate clearance by proteasomes in purified systems or extracts [15]. These conjugates are difficult to detect in interphase, suggesting that K11/K48-branched chains might trigger immediate degradation in cells, unless proteasome inhibition results in their accumulation [13,60]. K11/K48-branched chains are also synthesized in response to challenging conditions that require rapid protein elimination, including the need to degrade aggregation-prone proteins in the cytoplasm or ER [13,41,60]. Similar roles in mediating efficient proteasomal

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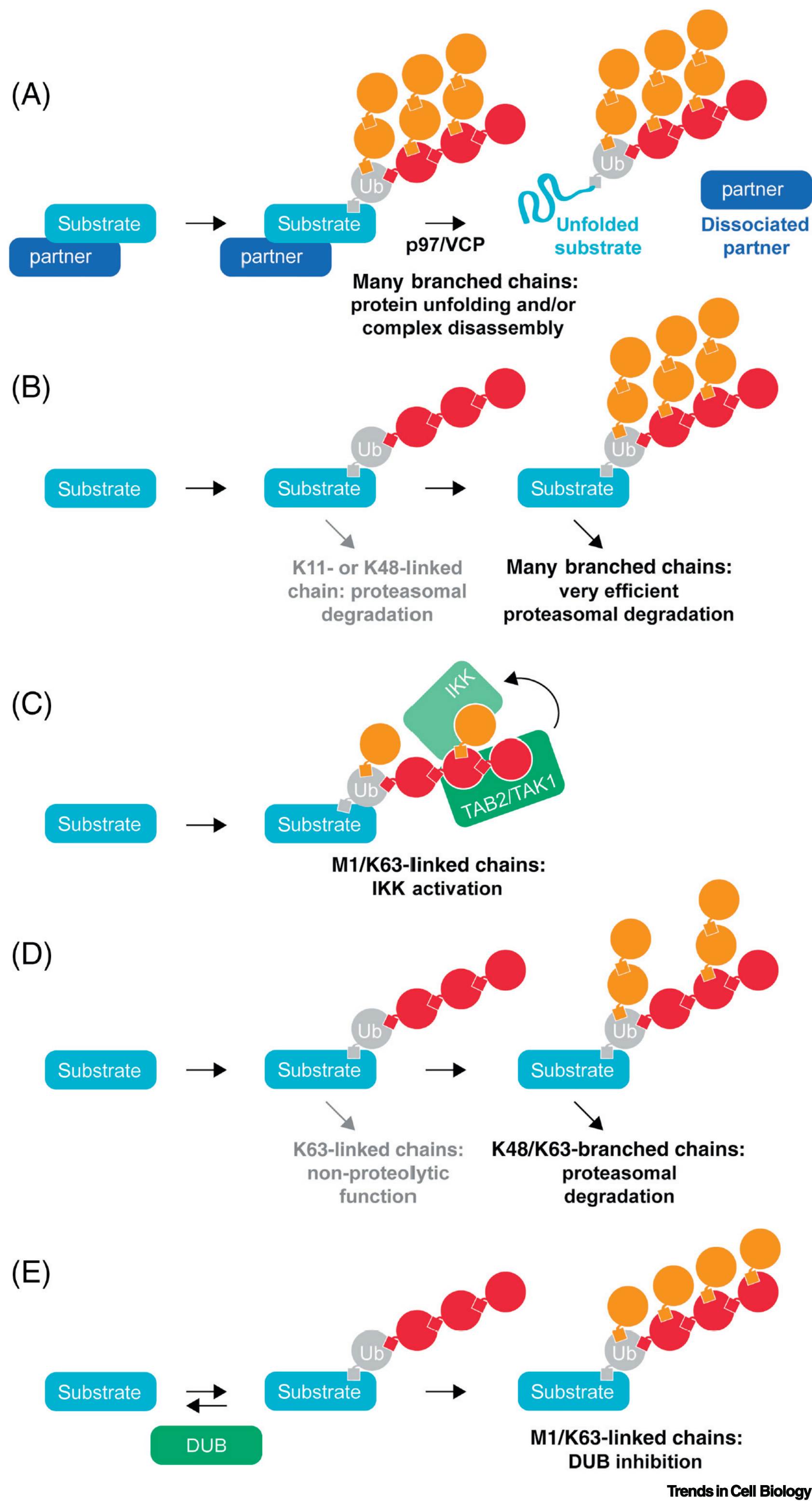

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targeting were documented for K29/K48- and K48/K63-branched chains [11,16,73]. Branched chains therefore elicit particularly efficient degradation, and this prompted us to refer to these conjugates as proteasomal priority signals (Figure 4B).

Why then are branched chains produced so abundantly during mitosis, where they were initially discovered [13,15,74]? The responsible E3, the APC/C, targets >100 substrates during the brief period of mitotic exit [75], and thus needs to induce degradation with high efficacy. Moreover, APC/C-dependent formation of branched chains likely serves another role in complex disassembly: the APC/C branching E2, UBE2S, is necessary to silence a drug-induced mitotic checkpoint [76,77], a reaction that involves ubiquitin-dependent dissociation of the APC/C from checkpoint proteins [78–80]. It is also possible that other complexes are disassembled during mitosis in a manner dependent on APC/C-dependent modification with branched ubiquitin chains. These findings suggest that the APC/C installs branched chains to combine complex disassembly with rapid degradation, thereby meeting the unique needs of remodeling the proteome at the beginning of a new cell cycle. Although more work is needed, we speculate that the potential to integrate protein unfolding and complex disassembly with proteasomal delivery may have contributed to the emergence of branched chains as central proteolytic devices in eukaryotic cells.

Combinatorial Signaling: Regulation of the NF-κB Transcription Factor

Although these examples illustrate how branched chains can drive protein degradation, there is also evidence that they can act nonproteolytically. Setting them apart from homotypic chains, branched polymers contain at least two linkages. Because two linkages can be recognized independently of each other by UBDs [81], branched chains allow combinatorial signaling through coincidence detection, a feature that often increases the specificity of signal transduction.

The ability of heterotypic chains to foster coincidence detection has been documented in the events leading to activation of the NF-κB transcription factor, a key modulator of the immune response. Following pathogen or cytokine detection, the E3s TRAF6 and Pellino 1/2 produce K63-linked chains [82]. Even if not attached to any substrate [83], these chains recruit the TAB2/TAB3–TAK1 kinase, which then switches on the IKK kinase to drive NF-κB activation [7]. Recent work showed that K63-linked chains assembled by TRAF6 are decorated with M1-linked ubiquitin by the E3 LUBAC that is composed of HOIL-1, HOIP, and SHARPIN [14,84,85]. The LUBAC-subunit HOIP interacts with K63 linkages, but not with M1 linkages, which likely facilitates the production of M1/K63-linked chains [14]. It is unknown, however, whether these conjugates are branched, where M1 linkages decorate internal K63-linked subunits, or mixed, where M1 linkages top off K63-linked conjugates.

Irrespective of their architecture, M1/K63-linked chains provide binding sites for upstream and downstream components of the NF-κB pathway (Figure 4C): whereas M1 linkages are detected

Figure 4. Functions of Branched Ubiquitin Chains in Cellular Signal Transduction. (A) Protein unfolding and complex disassembly. Branched chains target proteins to p97/VCP, which is an AAA+ ATPase that unfolds proteins to prepare them for proteasomal degradation or dismantles protein complexes in a ubiquitin-dependent manner. (B) Proteasomal priority signals. Branched chains are more effective in targeting proteins for proteasomal degradation than are homotypic chains that contain the same number of ubiquitin subunits and are attached to the same substrate. (C) Coincidence detection. The distinct linkages in branched chains can be read out independently of each other by ubiquitin-binding domains (UBDs). This allows branched chains to bridge components of signal transduction pathways to increase the speed or accuracy of cellular information flow. (D) Signal editing. If there is a time-delay between attachment of the first chain and branching off of the second conjugate, the branching polymer could alter the function of the first chain. (E) Signal protection. Branching impairs recognition of ubiquitin chains by linkage specific deubiquitylases (DUBs), thus extending the lifetime of a ubiquitylation signal. Abbreviation: Ub, ubiquitin.

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by the UBAN domain in the IKK subunit NEMO [52], the NZF-domain in TAB2 of the TAK1 complex binds K63 linkages [86]. The M1/K63-linked chains produced by the concerted action of TRAF6 and LUBAC thus provide a scaffold that brings TAK1 and IKK into close proximity with each other to allow rapid activation of IKK. Because multiple cytokine signaling pathways rely on M1/K63-linked hybrid chains [84], coincidence detection of branched chains by successive components of a signaling pathway might be a frequent means to increase the speed or accuracy of ubiquitin-dependent signal transmission.

Signal Editing: Turning off Nonproteolytic Ubiquitin Marks

If there is a delay between the addition of the initial chain and the branching off of a second conjugate, both ubiquitin marks could signal independently of each other. This could allow a branched chain to edit the role of the first conjugate, a function that complements previous models of chain editing dependent on successive deubiquitylase (DUB) and E3 ligase action [87]. It has recently been shown that TXNIP, which regulates the redox-controller thioredoxin as well as cell survival, is initially modified with nonproteolytic K63-linked chains by ITCH and WWP1, before UBR5 and HUWE1 branch off K48-linked polymers [17]. Because K63-linked conjugates control nonproteolytic signaling, they could turn on a particular function of TXNIP before branching of K48-linked chains results in TXNIP degradation. K48/K63-branched chains, which can become very abundant in cells [11], could thus encode signaling functions that are restricted in time (Figure 4D). However, it is unclear whether K63-linked chains regulate TXNIP independently of its stability. Whether chain editing is a crucial function of branched chains will therefore require more work in understanding TXNIP modification and the identification of additional substrates of K48/K63-branched conjugates.

Signal Protection: Preventing DUBs from Accessing Their Targets

As a final example in the growing list of functions for branched chains, we turn to DUBs that terminate or prevent ubiquitin-dependent signaling [88]. Most DUBs are members of the family of ubiquitin-specific proteases that cleave ubiquitin with little linkage specificity and effectively dismantle branched chains [14,15]. Some DUBs, such as those of the ovarian tumor OTU family, are specific for a particular linkage, which only allows them to cleave one linkage within branched conjugates. It is conceivable that some DUBs target the branched structure itself, and their discovery would reveal an interesting facet of regulation for these heterotypic conjugates.

Importantly, DUBs can encounter problems in targeting specific linkages if these are embedded in branched chains. This has been described in the pathways that lead to activation of the aforementioned NF-κB transcription factor, an event that is tightly controlled by the linkage-specific DUBs A20 and CYLD [89–91]. When phosphorylated, A20 disassembles homotypic K63-linked chains, but it is unable to do so if these linkages are found within M1/K63-linked conjugates [18]. LUBAC-dependent branching of M1 linkages from K63-linked conjugates thus extends the signaling lifetime of the latter polymer. In a similar manner, CYLD cleaves K63 linkages, but only if these are not part of K48/K63-branched chains [11]. In this case, branching of K48 linkages protects the target, K63-modified TRAF6, from inactivation. The presence of nonpreferred linkages can thus prevent linkage-specific DUBs from accessing their substrates, which impairs chain disassembly and prolongs ubiquitin-dependent signaling (Figure 4E).

Concluding Remarks and Future Perspectives

The examples described in this review illustrate that branched chains elicit a wide spectrum of specific outcomes for their modified targets. Branched chains can exert functions that are qualitatively or quantitatively different from those encoded by their homotypic building blocks. However, despite much progress, many important questions in this thriving field of study remain

Outstanding Questions

What are the cellular contexts and functions of understudied branched chains, including K6/K48, K11/K33, and K29/K33? Proteomics and biochemical assays have suggested the existence of such branched conjugates, but their functions remain unknown.

How do E3 ligases cooperate to synthesize branched conjugates? How elongating E3 enzymes are recruited to a substrate to form a branch chain, and what provides specificity to branched chain synthesis, constitute important open questions.

How does branched chain architecture affect effector recognition? Understanding how the number of branches, the arrangement of the linkages within a chain, the presence of a branch itself, and the proximity of different chain types might influence effector recognition is an exciting area of future research.

Because branched chains constitute priority signals for degradation, could increased production of branched conjugates help to target difficult substrates for degradation, and thereby provide a therapeutic avenue for neurodegenerative diseases?

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open (see Outstanding Questions). Much needs to be learned about the architecture of branched chains and whether the number or sequence of branches matters in terms of signaling. This will require the development of new technologies to 'sequence' linkages within a branched conjugate. Concomitantly with understanding chain topology, we will need to further dissect molecular mechanisms of branched chain assembly and detection, and this will provide a foundation for understanding how branching is regulated within the context of complex cellular signaling networks.

It is interesting to note that branched chains have been implicated in the degradation of aggregation-prone proteins that cause neurodegenerative diseases. Several branching E3s or effectors, such as UBR4, UBR5, HUWE1, and p97/VCP, are mutated in neurodegenerative or neurodevelopmental disorders, and K11/K48-branched chains decorate disease-linked proteins, such as Huntingtin variants with extended polyQ tracts [13]. How inhibition of branched chain synthesis contributes to neuronal death, and whether increased production of such conjugates could protect neurons during aging or in disease, has not been determined. Given the dramatic impact of neurodegenerative diseases on our society, developing a better understanding of the physiology of branched ubiquitin conjugates should be a focus of future studies.

Ubiquitin-dependent signaling has been described as a code in which different linkages elicit distinct functions [3,92]. In such a code, homotypic chains might be compared with words, but branched chains that result from the combination of different linkages could be thought of as short cellular sentences. As in human communication, cellular sentences appear to encode more specific types of information that are needed by cells to face the demands of daily life. Learning more about the increasingly complex ubiquitin code, namely its words, grammar, and sentences, will help us to uncover fundamental principles of information transfer in eukaryotic cells, a goal worthwhile for both basic and applied science.

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