The Ubiquitin Code

David Komander[1] and Michael Rape[2]

[1]Division of Protein and Nucleic Acid Chemistry, Medical Research Council Laboratory of Molecular Biology, Cambridge, CB2 0QH, United Kingdom; email: dk@mrc-lmb.cam.ac.uk

[2]Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3202; email: mrape@berkeley.edu

Annu. Rev. Biochem. 2012. 81:203–29

First published online as a Review in Advance on April 10, 2012

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-060310-170328

0066-4154/12/0707-0203$20.00

Keywords

ubiquitin chain, linkage specificity

Abstract

The posttranslational modification with ubiquitin, a process referred to as ubiquitylation, controls almost every process in cells. Ubiquitin can be attached to substrate proteins as a single moiety or in the form of polymeric chains in which successive ubiquitin molecules are connected through specific isopeptide bonds. Reminiscent of a code, the various ubiquitin modifications adopt distinct conformations and lead to different outcomes in cells. Here, we discuss the structure, assembly, and function of this ubiquitin code.

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1. INTRODUCTION

When in 1532 Spanish conquistadores set foot on the Inca Empire, they found a highly organized society that did not utilize a system of writing. Instead, the Incas recorded tax payments or mythology with quipus, devices in which pieces of thread were connected through specific knots. Although the quipus have not been fully deciphered, it is thought that the knots between threads encode most of the quipus' content. Intriguingly, cells use a regulatory mechanism—ubiquitylation—that is reminiscent of quipus: During this reaction, proteins are modified with polymeric chains in which the linkage between ubiquitin molecules encodes information about the substrate's fate in the cell.

Ubiquitylation is brought about by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligase enzymes (E3s) (1–3). These enzymes first catalyze the formation of an isopeptide bond between the C terminus of ubiquitin and usually a substrate lysine, leading to monoubiquitylation (**Figure 1*a***). Monoubiquitylation can occur at a defined residue, such as Lys164 in proliferating cell nuclear antigen (PCNA) (4), or it might be confined to a domain, as in the transcription factor p53 (5). It is possible that multiple lysine residues become modified with one ubiquitin each during multimonoubiquitylation (**Figure 1*b***), with the epidermal growth factor receptor (EGFR) as an example (6).

Modification of the N terminus or one of the seven lysine residues of a substrate-attached ubiquitin leads to formation of polymeric chains. These chains can be short and contain only two ubiquitin molecules or long and incorporate more than ten moieties. Ubiquitin chains are homogenous if the same residue is modified during elongation, as in Met1- (or linear), Lys11-, Lys48-, or Lys63-linked chains (**Figure 1*c***). Chains have mixed topology if different linkages alternate at succeeding positions of the chain (**Figure 1*d***), as seen in NF-κB signaling or protein trafficking (7–10).

If a single ubiquitin is modified with multiple molecules, branched chains of unknown function are generated (**Figure 1*e***).

All possible linkages have been detected in cells (11, 12). For chains linked through Lys6, Lys27, Lys29, or Lys33, few substrates are known, and their significance is poorly understood. However, it has been well established that monoubiquitylation and four homogenous chain types trigger distinct outcomes in the cell, suggesting that ubiquitylation can act as a code to store and transmit information. In this review, we discuss the structure, assembly, and function of this ubiquitin code.

2. STRUCTURE OF THE UBIQUITIN CODE

2.1. Ubiquitin

Ubiquitin is a highly stable protein that adopts a compact β-grasp fold with a flexible six-residue C-terminal tail (**Figure 2*a***) (13). Most of its core residues are rigid, but the β1/β2 loop containing Leu8 shows flexibility that is important for recognition by ubiquitin-binding proteins (**Figure 2*b***) (14). With three conservative changes, ubiquitin is almost invariant from yeast to man. This suggests high evolutionary pressure to conserve the structure of ubiquitin and implies that many of its surfaces are recognized by ubiquitin-binding domains (UBDs).

Ubiquitin is often recognized through a hydrophobic surface that consists of Ile44, Leu8, Val70, and His68 (**Figure 2*a–c***) (15). The Ile44 patch is bound by the proteasome and most UBDs, rendering it essential for cell division (15–17). Another hydrophobic surface is centered on Ile36 and involves Leu71 and Leu73 of the ubiquitin tail (**Figure 2*c***). The Ile36 patch can mediate interactions between ubiquitin molecules in chains, and it is recognized by HECT E3s (18), DUBs (19), and UBDs (20). A surface comprising Gln2, Phe4, and Thr12 is required for cell division in yeast (**Figure 2*c***) (17). This Phe4 patch might function in trafficking (17), and it interacts with the UBAN domain (21) and the ubiquitin-specific protease (USP) domain of DUBs (19). The divergence between Phe4 patches of ubiquitin and its closest homolog Nedd8 enables DUBs to distinguish between these modifiers (22). In higher eukaryotes, the TEK-box of ubiquitin, a three-dimensional motif that includes Thr12, Thr14, Glu34, Lys6, and Lys11, is required for mitotic degradation (**Figure 2*c***) (23). As deamidation of Gln40 by the bacterial protein Cif blocks chain assembly (24), additional surfaces might fulfill as yet unidentified functions.

With respect to the ubiquitin code, the most important features of ubiquitin are its N terminus and its seven lysines, which are the attachment sites for chain assembly. These residues cover all surfaces of ubiquitin and point into distinct directions (**Figure 2*d***). Lys6 and Lys11 are located in the most dynamic region

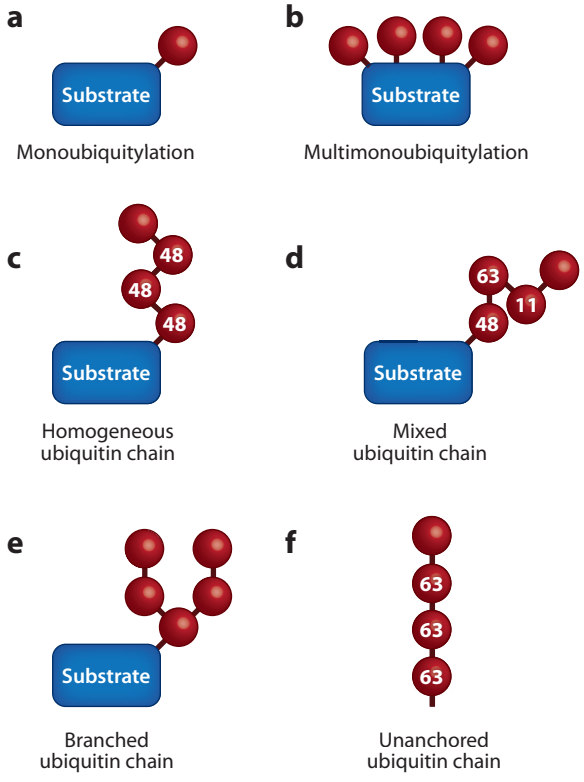


Figure 1

The different topologies of ubiquitylation. (*a*) Monoubiquitylation. (*b*) Multimonoubiquitylation. (*c*) Homogenous ubiquitin chain. (*d*) Mixed ubiquitin chain. (*e*) Branched ubiquitin chain. (*f*) Unanchored ubiquitin chain.

E2: ubiquitin-conjugating enzyme

E3: ubiquitin ligase enzyme

UBD: ubiquitin-binding domain

Homologous to E6AP C terminus (HECT): a class of E3s ubiquitin ligases

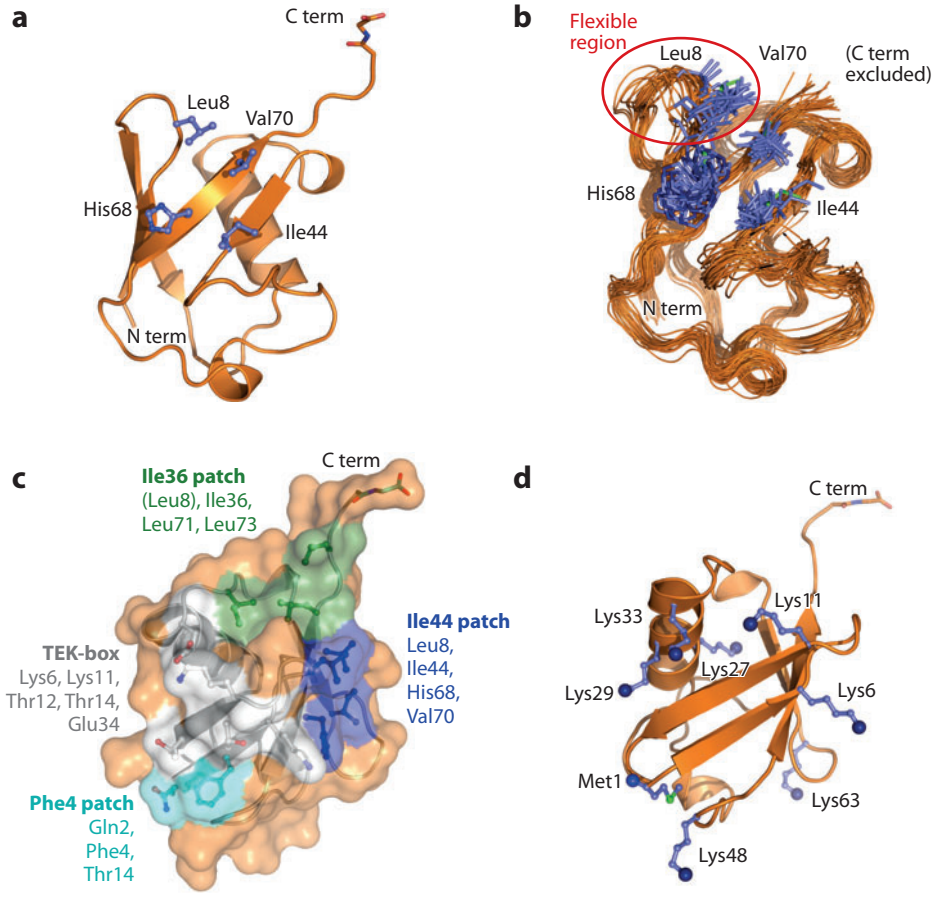


Figure 2

Structural features of ubiquitin. (*a*) Structure of ubiquitin, indicating the C-terminal (C term) tail and residues of the Ile44 patch [Protein Data Bank (pdb) code 1ubq] (13). (*b*) NMR ensemble of ubiquitin on the basis of residual dipolar couplings (14). The first 30 structures of the ensemble (pdb 2k39) are shown. The Ile44 residues are indicated, and the flexible region is highlighted. (*c*) The ubiquitin surface is shown with Ile44 (*blue*), Ile36 (*green*), Phe4 patches (*cyan*), and TEK-box (*white*) highlighted. (*d*) Structure of ubiquitin showing the seven Lys residues and Met1. Blue spheres indicate amino groups used in ubiquitin chain formation. Abbreviation: N term, N terminus.

of ubiquitin that may undergo conformational changes in the context of a chain or upon association with UBDs. As Lys27 is buried, linkage assembly through this residue would require localized changes in ubiquitin structure.

Deubiquitinating enzyme or deubiquitinase (DUB): an enzyme that cleaves the isopeptide bond between a lysine and the C terminus of ubiquitin

2.2. Ubiquitin Chain Structure

Structural characterization of five chain types revealed that different linkages result in distinct chain conformations. Ubiquitin chains adopt either "compact" conformations, where adjacent moieties interact with each other, or "open" conformations, where no interfaces are present except for the linkage site. The canonical Lys48-linked chains adopt compact conformations (**Figure 3*a***) (25–28). In the prevalent model for Lys48-linked diubiquitin, the ubiquitin moieties interact via their Ile44 patches (25–27), and two such diubiquitin modules pack tightly in tetraubiquitin (28). NMR analysis using residual dipolar couplings has identified a

minor population of Lys48-linked diubiquitin in which the Ile36 patch of the distal ubiquitin interacts with the Ile44 patch of the proximal unit (27, 29). This structural flexibility might give binding partners of Lys48-linked chains access to the Ile44 patch, a hot spot for ubiquitin recognition.

Similar to Lys48 linkages, Lys6- and Lys11-linked chains adopt compact conformations, with Lys11-linked chains also displaying structural flexibility (**Figure 3*b*,*c***) (30–32). In one structure of Lys11-linked diubiquitin (30), an asymmetric interface covering the α-helix of ubiquitin is involved, and in another study, the ubiquitin moieties interact symmetrically via Ile36 patches (31). Both conformations are consistent with NMR analysis, suggesting that they coexist in equilibrium (30). Indeed, an analysis of crystal packing revealed a higher-order assembly of Lys11-linked chains that encompasses both conformations (33). In all Lys11-linked chain models, the Ile44 patch is solvent exposed and ready to interact with binding partners.

In contrast to the aforementioned linkages, Met1- and Lys63-linked chains mostly display open conformations (**Figure 3*d*,*e***), as shown by NMR analysis of Lys63-linked ubiquitin (26, 34) and crystal structures of both chain types (35–37). Reminiscent of beads on a string, the extended open conformation endows Lys63 and Met1 linkages with high conformational freedom. Most binding partners of these chains, therefore, likely exploit the distance and flexibility between chain moieties, rather than recognizing a defined geometric assembly of different ubiquitin surfaces (38).

Together, the various structures revealed a large array of geometries that can be utilized by binding partners to distinguish between modifications. As described below, linkage-specific binding proteins might recognize the distance between chain entities or sense the relative orientation of ubiquitin surfaces at successive chain positions. The conformational flexibility of some chain types raises the possibility that UBDs remodel chains to increase interaction interfaces or improve specificity. The structural diversity of the various modifications, therefore, forms the foundation of the ubiquitin code.

HOW TO DISSECT THE UBIQUITIN CODE

Ubiquitin modifications can be analyzed by a plethora of approaches. Reconstitution of cellular pathways in extracts supplemented with ubiquitin mutants revealed the roles of Lys11-linked chains in mitotic degradation (23) and Lys63-linked chains in kinase activation (60, 97). The biochemical and structural analysis of enzymes led researchers to discover Met1-linked chains as regulators of NF-κB signaling (21, 82). In cells, monoubiquitylation is often studied by analyzing linear fusions between ubiquitin and the candidate substrate (141). To dissect roles of chains in vivo, recombinant ubiquitin mutants can be injected into cells or *Xenopus* embryos (23). Mutant ubiquitin can also be overexpressed in cells, which, owing to the tight regulation of endogenous ubiquitin levels, leads only to a modest excess of mutant ubiquitin and often results in weak phenotypes. In a more careful approach, the genes encoding ubiquitin and ubiquitin-ribosome fusions are deleted or their mRNAs depleted by siRNA, and mutant ubiquitin is expressed as a ribosomal fusion (161, 179). The abundance of ubiquitin chain types can be analyzed with antibodies that specifically detect Met1, Lys11, Lys48, or Lys63 linkages (31, 82, 90) or by quantitative proteomics (11, 12, 55, 128). In all cases, assigning a function to a ubiquitin modification requires a combination of these experimental approaches.

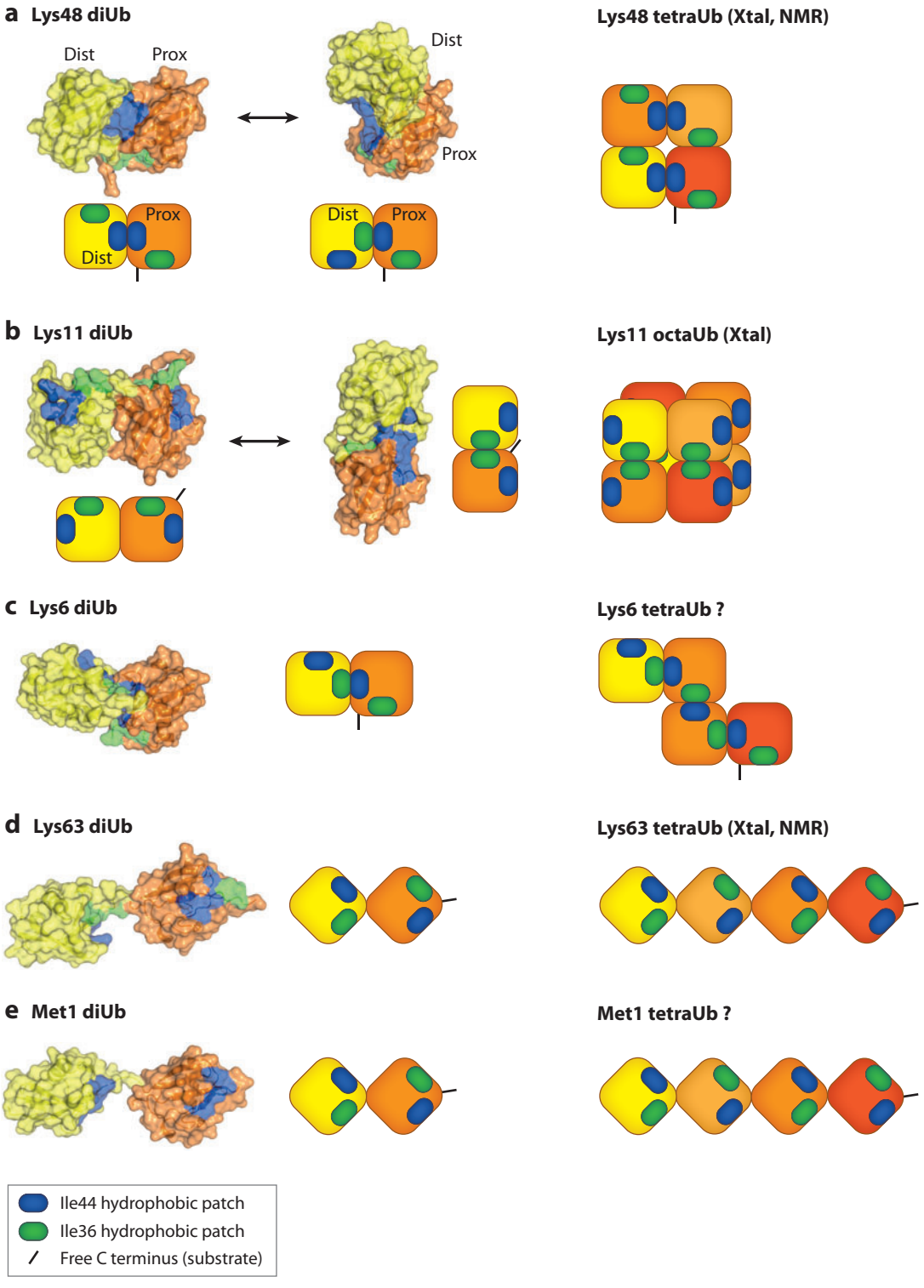
3. WRITING THE UBIQUITIN CODE

Any functional code requires its specific assembly—just as review articles make sense only if letters are arranged in a sequence that gives meaning to the resulting words. In a similar manner, ubiquitylation will trigger specific outcomes only if the responsible enzymes catalyze formation of largely the same product each time they act on their substrate.

3.1. Monoubiquitylation

The enzymes catalyzing monoubiquitylation have to recognize substrate lysine residues, while sparing those of ubiquitin from modification, a specificity that can be

Distal ubiquitin: last ubiquitin moiety in a chain without a modified lysine



determined by the E2, the E3, or a particular substrate-E3 complex.

In an example of the latter approach, the polycomb E3 ligase complex Bmi1-RING1 monoubiquitylates histone H2A on Lys119 (39), even though it collaborates with Ube2D/UbcH5, a nonspecific E2 that usually modifies multiple substrate and ubiquitin lysine residues (40). Bmi1-RING1 binds to both DNA and nucleosomes, which results in a stiff substrate-E3 complex that exposes the active site of Ube2D toward Lys119 of H2A. Owing to the rigidity of this assembly, ubiquitylation of Lys119 introduces a steric impediment to further modification, thereby restricting the reaction to monoubiquitylation.

Alternatively, E3 enzymes can block the ability of E2s to catalyze chain formation, as seen with Rad18 and its E2 Rad6. Although Rad6 can synthesize mixed or Lys48-linked chains (41, 42), it promotes monoubiquitylation of PCNA when collaborating with Rad18 (4, 43). Similar to other E2s, Rad6 depends on a noncovalent ubiquitin-binding site for chain formation; Rad18 occupies this site, thereby blocking chain formation without interfering with monoubiquitylation (41).

In some cases, the E2 determines monoubiquitylation, yet the molecular basis for this specificity is poorly understood. For example, the E2s Ube2W and Ube2T, together with the E3 FANCL, decorate the DNA repair protein FANCD2 with a single ubiquitin (44, 45). Ube2W also catalyzes monoubiquitylation with other E3s, such as Brca1-Bard1 and CHIP (46, 47). If these E3s utilize the nonspecific Ube2D instead of Ube2W, substrates are modified with ubiquitin chains, showing that in this case it is the E2, Ube2W, that encodes the information for monoubiquitylation.

3.2. Ubiquitin Chain Assembly by RING Domain and U-Box Ligase Enzymes

The enzymes that catalyze chain formation face a different specificity issue: They need to modify specific lysine residues of ubiquitin. For E3s containing a RING or U-box domain, this linkage specificity is likely determined by the E2 (3). This hypothesis is supported by the observation that RING or U-box E3s can synthesize different chain types depending on the E2: Brca1-Bard1 or Murf, for example, assembles Lys63 linkages with the heterodimeric E2 enzyme Ube2N-Uev1A, but Lys48 linkages when bound to Ube2K (46, 48). Similarly, CHIP synthesizes Lys63-linked chains with Ube2N-Uev1A but is unspecific with Ube2D (49). Conversely, RING E3s that interact with a single E2 generally display the specificity of this E2: Multi-subunit E3 ligases of the SCF family decorate substrates with Lys48-linked chains by using the Lys48-specific E2 Ube2R1 (50); gp78, a regulator of endoplasmic reticulum–associated degradation, assembles Lys48-linked chains with the Lys48-specific E2 Ube2G2 (51); and the anaphase promoting complex (APC/C) produces Lys11-linked chains using the Lys11-specific Ube2S (52).

RING E3s and their E2s initiate chain formation on a substrate lysine (**Figure 4*a***), which can occur at random positions or in preferred sequence environments, referred to as chain initiation motifs (53). The initiating E2s often assemble short chains, which can

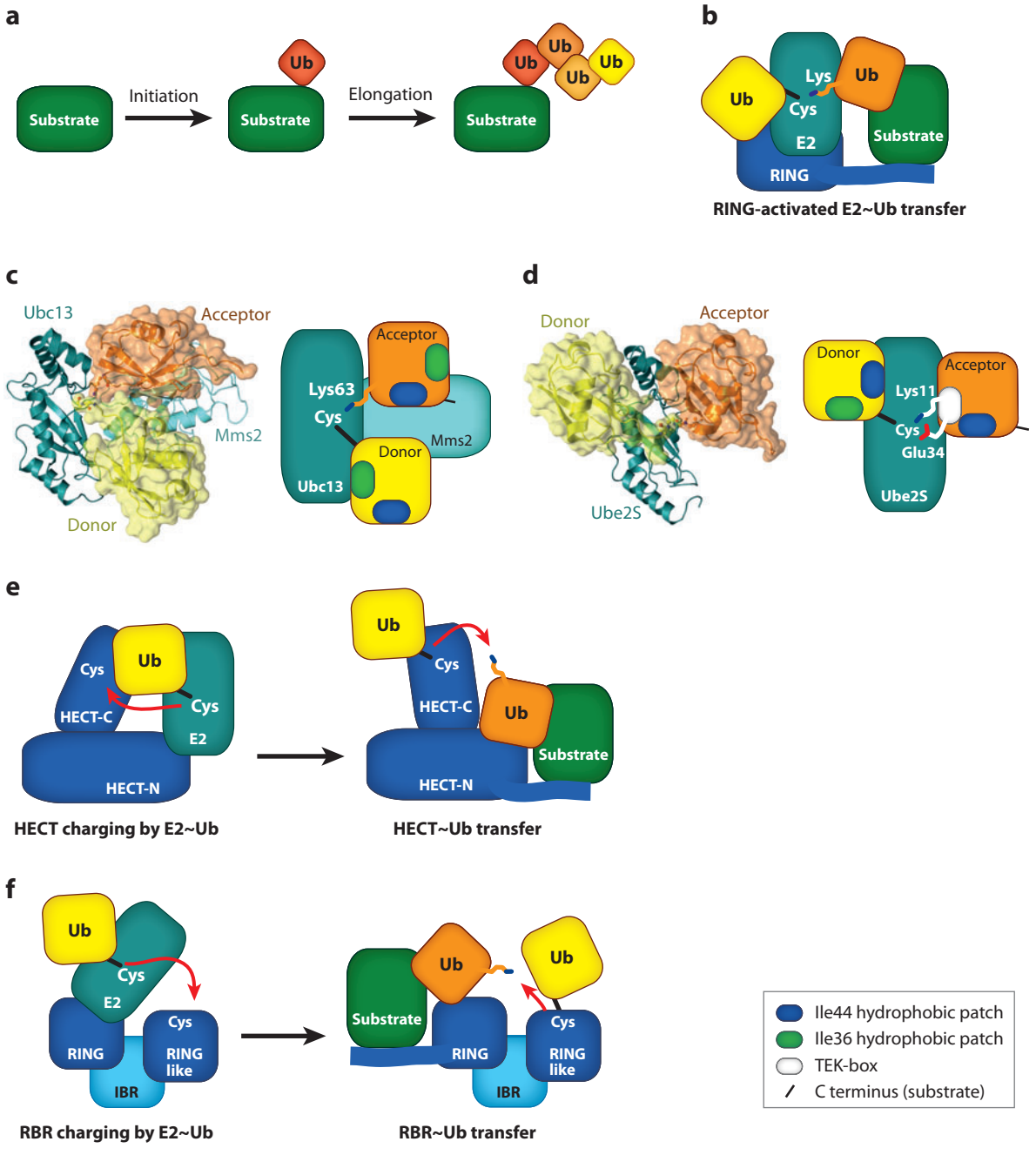
Really interesting new gene (RING): a class of E3 enzymes

Chain initiation: modification of a substrate lysine residue with the first ubiquitin

Figure 3

Ubiquitin chain structure. Diubiquitin (diUb) molecules of different linkages are shown with the distal (dist) molecule in yellow and the proximal (prox) molecule in orange, alongside a schematic representation. Ile44 (*blue*) and Ile36 (*green*) patches are indicated. (*a*) Lys48-linked diUb, Protein Data Bank (pdb) code 1aar (*left*) (25), pdb 2pe9 (*right*) (185). The tetramer model (tetraUb) is based on data in Reference 28. (*b*) Lys11-linked diubiquitin, pdb 2xew (*left*) (30), pdb 3nob (*right*) (31). The model of Lys11-linked octaubiquitin (octaUb) is based on crystal packing of both structures (33). (*c*) Lys6-linked diubiquitin, pdb 2xk5 (32). (*d*) Lys63-linked polyubiquitin, pdb 2jf5 (35). The model of a longer chain is based on Reference 186. (*e*) Met1-linked polyubiquitin, pdb 2w9n (35).

be connected nonspecifically as for Ube2D (54); contain a favored linkage, as seen for the Lys11-preferring Ube2C (23, 55); or are homogenous, as for Lys48-specific Ube2R1 (50). In most cases, the initiating E2s cooperate with a specific chain-elongating E2 (**Figure 4*b***). This allows for assembly of Lys11-linked chains by Ube2S (52, 56), Lys48-linked chains by Ube2K/Ubc1 or Ube2R1 (46, 50, 57), and Lys63-linked chains by Ube2N-Uev1A



(46, 58). E2s with specificity for Lys6, Lys27, Lys29, or Lys33 have not been reported (59).

Selection of the appropriate lysine used for chain formation requires recognition of a specific acceptor ubiquitin surface by the E2 donor ubiquitin complex. The Lys63-specific Ube2N achieves this feat by teaming up with an auxiliary subunit, Uev1A (**Figure 4*c***) (60). Uev1A contains a UBC-E2 variant domain that has lost its catalytic cysteine but has retained its capacity to noncovalently bind ubiquitin (58). This interaction orients the acceptor ubiquitin such that Lys63 faces the active site of charged Ube2N. By contrast, monomeric E2s directly recognize the acceptor ubiquitin. Ube2S, for example, binds the TEK-box of ubiquitin through a surface close to its active site (**Figure 4*d***) (61). A similar interaction is required for Lys11 linkage formation by Ube2C (23) and Ube2D (62), suggesting that the TEK-box is of broad importance for Lys11 linkage formation. The Lys48-specific E2s, Cdc34, Ubc1, or Ube2G2, employ an acidic loop or residues close to their active site for acceptor recognition, yet the corresponding ubiquitin surface is not well defined (50, 51, 57, 63).

In most cases, the acceptor ubiquitin is bound with very low affinity: Even Ubc13-Mms2 binds acceptor ubiquitin with a K_m of only 437 μM (64). Such low affinities suggest that additional mechanisms contribute to the linkage specificity of E2s. Previous work with the SUMO-E2 Ube2I/Ubc9 identified an aspartate of the E2 that deprotonates the acceptor lysine, thereby turning it into an efficient nucleophile (65). An acidic residue is also required in ubiquitin E2s that show activity toward lysine, while it is not required for activity toward cysteine, as the thiol group does not need to be deprotonated at physiological pH (66). Importantly, the Lys11-specific Ube2S lacks an acidic residue in its active site and, instead, utilizes the Glu34 of the acceptor ubiquitin for Lys11 activation (**Figure 4*d***) (61). Other lysine residues of ubiquitin are not paired up with properly oriented acidic residues, explaining why Ube2S does not modify them with high efficiency. As the Lys48-specific yeast Ubc1 requires Tyr59 of ubiquitin for linkage formation, similar mechanisms of substrate-assisted catalysis might also contribute to formation of other chain types (57).

3.3. Chain Formation by HECT E3s

A different class of enzymes, the HECT E3s, contain a catalytic cysteine (67). E2s charge this cysteine with ubiquitin before this ubiquitin is used for modification. HECT E3s display a wide range of linkage specificities: Yeast Rsp5 and human Nedd4 assemble Lys63-linked chains (68, 69); E6AP (E6-associated protein) synthesizes Lys48 linkages (68, 70); KIAA10/UBE3C promotes formation of Lys29 and Lys48 linkages (70); a bacterial HECT-like E3 triggers assembly of Lys6- and Lys48 linkages (71); and HUWE1 appears to be nonspecific (68).

Figure 4

Mechanism of linkage-specific ubiquitin (Ub) chain assembly by E2s. (*a*) Ubiquitin chain formation proceeds through an initiation step, during which a substrate lysine residue is modified, and elongation, during which ubiquitin molecules are added to the growing chain. (*b*) Proposed mechanism of RING E3-catalyzed ubiquitin transfer from a charged E2 to a substrate or ubiquitin lysine. ~Ub indicates a covalent ubiquitin thioester intermediate. (*c*) Heterodimeric Ubc13-Mms2 recognizes acceptor ubiquitin through the UBC-variant domain of Mms2. This positions the acceptor Lys63 to the active site of charged Ubc13. (*d*) Monomeric Ube2S catalyzes linkage formation through substrate-assisted catalysis. Ube2S recognizes the TEK-box of acceptor ubiquitin. Activation of the acceptor Lys11 requires a ubiquitin residue, Glu34. Thus, the correct surface of the acceptor ubiquitin, which includes Lys11 and Glu34, must be exposed to the catalytic cysteine of charged Ube2S in order for the active site to be completed and for linkage formation to occur. (*e*) Mechanism of HECT ubiquitin chain formation. The E2 charges a cysteine in the HECT domain C-terminal (HECT-C) lobe forming a HECT-ubiquitin thioester. The HECT domain presumably positions the acceptor for linkage-specific chain assembly. (*f*) Mechanism of RING-in-between-RING (RBR) ubiquitin chain formation. The RING domain binds to and discharges the E2 to a cysteine in the C-terminal RING-like domain. Abbreviations: HECT-N, HECT N-terminal lobe; IBR, in-between-RING.

The catalytic domain of HECT E3s consists of an N-terminal lobe, which binds the E2, and a C-terminal lobe, which contains the active-site cysteine (**Figure 4*e***) (72, 73). As the acceptor lysine attacks the thioester between the cysteine of the E3 and ubiquitin, linkage specificity should be determined by the HECT E3 and not the E2. Indeed, Rsp5 and E6AP assemble Lys63- or Lys48-linked chains, respectively, despite using the nonspecific E2 Ube2D; many HECT E3s promote chain formation with Ube2L3/UbcH7, a thiol-reactive E2 that does not modify lysine residues (66), and HECT domain swaps are sufficient to change linkage specificity despite using the same E2s (68).

To determine linkage specificity, HECT domains must orient and activate the acceptor lysine. Indeed, Rsp5, Smurf2, and Nedd4 engage in noncovalent interactions with an acceptor ubiquitin through residues of their N-terminal lobe (69, 74, 75). KIAA10 binds to acceptor ubiquitin through sequences that are N-terminal to the HECT domain (70), and E6AP requires both E2 and ubiquitin surfaces for acceptor recognition (70). However, although this acceptor binding turned out to be required for processive chain formation, it does not determine linkage specificity (69, 74). Thus, how HECT domains synthesize chains of defined topologies remains poorly understood.

3.4. Chain Formation by RING-In-Between-RING E3 Ubiquitin Ligases

A distinct set of E3s contains a sequence of a RING, a RING-in-between-RING (RBR), and a RING-like domain. These RBR E3s are RING/HECT hybrids: They utilize the RING domain to recruit an E2, which transfers ubiquitin to a cysteine in the RING-like domain, to form a thioester intermediate (**Figure 4*f***) (66). RBR E3s display linkage specificity: The linear ubiquitin chain assembly complex (LUBAC) assembles Met1-linked chains (8, 76–78), and parkin catalyzes monoubiquitylation as well as Lys63-, Lys48-, and Lys27-linked chain formation (79, 80). As RBR E3s function with Ube2L3, an E2 without reactivity against lysine (66), linkage specificity of chain formation must be determined by the E3, and not the E2. Consistent with this notion, RBR E3s can synthesize chains that differ from the inherent specificity of a cooperating E2: Whereas Ube2K usually assembles Lys48-linked chains (81), LUBAC and Ube2K produce Met1-linked chains (82). How RBR E3s determine their linkage specificity, however, is not known.

4. READING THE CODE: CONCEPTS IN UBIQUITIN BINDING

Once the code has been written, effector proteins with UBDs translate the modifications into specific outcomes (15). Although many UBDs have been described, their potential for linkage-specific ubiquitin recognition has rarely been assessed comprehensively, and limited structural and mechanistic insight is available. For example, a crystal structure of Lys48-linked chains in complex with a protein has not been reported. Fortunately, Lys63-linked diubiquitin has been caught in complex with UBDs, revealing several concepts of ubiquitin recognition.

4.1. Exploiting the Distance Between Ubiquitin Molecules

In the various chain types, the distance between successive ubiquitin moieties can differ considerably. Proteins with multiple UBDs exploit this property by introducing a defined spacer between the UBDs, as seen in proteins that contain tandem repeats of ubiquitin-interacting motifs (UIMs) (38, 83). UIMs consist of an α-helix with a hydrophobic binding site for the Ile44 patch of ubiquitin (84). In Rap80, a subunit of the Brca1-E3, two UIMs are separated by a seven-residue helix that positions the UIMs to recognize extended Lys63-, but not compact Lys48-, linked chains (**Figure 5*a***) (38, 83). Conversely, the two UIMs of Ataxin-3 are separated by a short linker of two residues, which

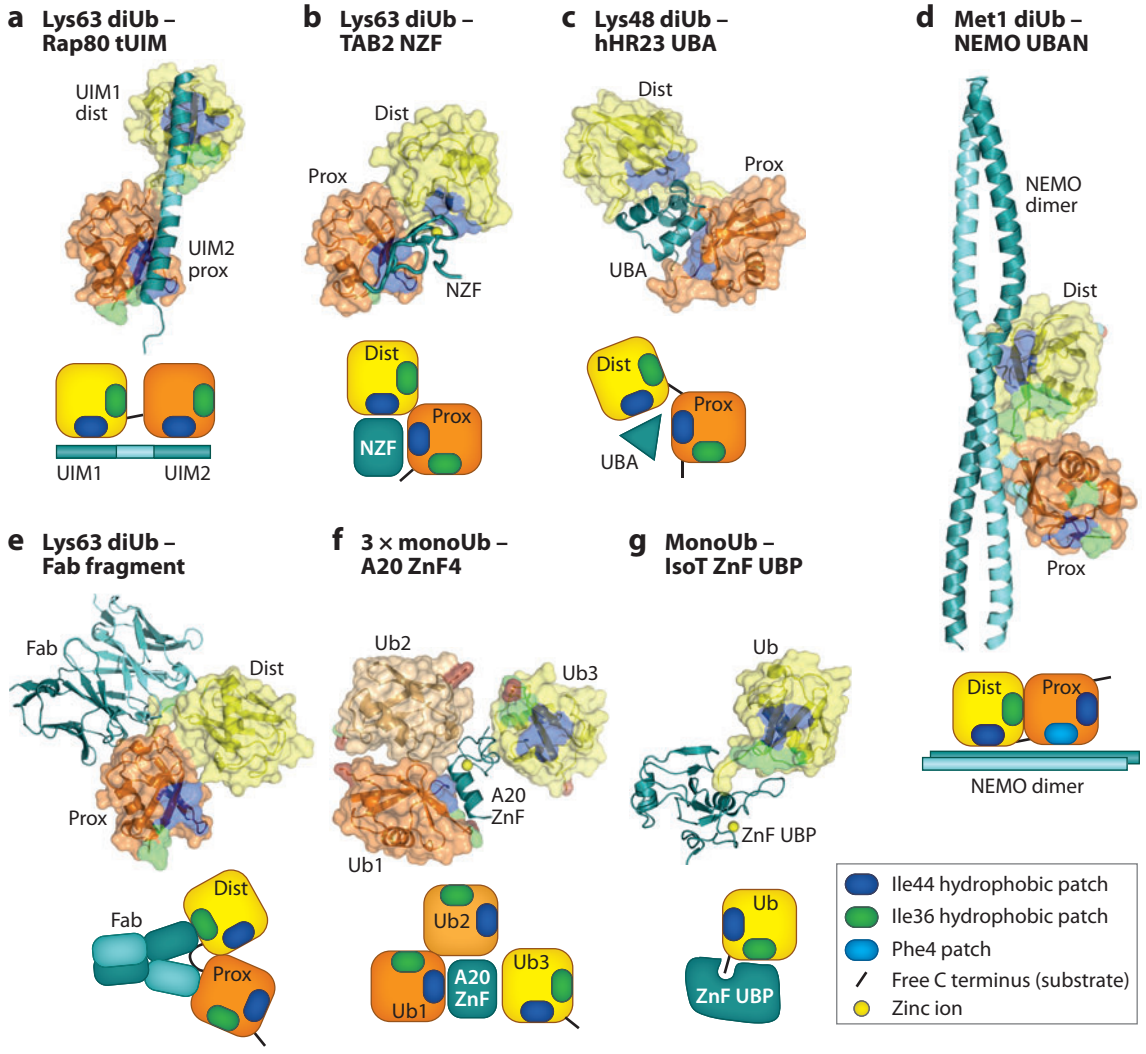


Figure 5

Concepts in ubiquitin binding. Ubiquitin binding domains are shown in cyan/teal, and the ubiquitin chains appear as in **Figure 3**. Zinc ions are shown as yellow spheres. (*a*) Crystal structure of the RAP80 tandem ubiquitin-interacting motif (tUIM) bound to Lys63-linked diubiquitin (diUb), Protein Data Bank (pdb) code 3a1q (86). (*b*) Crystal structure of the TAB2 NZF domain bound to Lys63-linked diubiquitin, pdb code 2wwz (87). (*c*) NMR model of the hHR23 UBA domain bound to Lys48-linked diubiquitin, pdb code 1zo6 (89). (*d*) Crystal structure of the NEMO UBAN domain bound to Met1-linked diubiquitin, pdb code 2zvn (21). (*e*) Crystal structure of the Lys63-specific antibody bound to Lys63-linked diubiquitin, pdb code 3dvg (90). Only part of the Fab fragment is shown. (*f*) Crystal structure of the A20 ZnF domain bound to three ubiquitin molecules, pdb code 3oj3 (94). (*g*) Crystal structure of the Usp5/IsoT ZnF UBP domain bound to ubiquitin, pdb code 2g45 (20). Abbreviations: dist, distal; prox, proximal.

results in preferential recognition of more compact Lys48 linkages. Swapping the linkers made Ataxin-3 Lys63 and Rap80 Lys48 selective, emphasizing how the length of the linker, the "ruler," determines binding specificity (38).

4.2. Exploiting Chain Flexibility

TAB2 and TAB3, adaptors of the TAK1 kinase complex, contain Npl4-like zinc fingers (NZFs) (85). These NZF domains are able to distinguish between structurally similar

Proximal ubiquitin: the ubiquitin moiety attached to a substrate or with a free C terminus in unanchored chains

Unanchored ubiquitin chains: ubiquitin chains that are not attached to substrates

Lys63- and Met1-linked chains, a specificity enabled by the dynamic nature of these chain types (**Figure 5*b***). As seen in crystal structures of NZF domains bound to Lys63-linked diubiquitin (86, 87), the Ile44 patches of each ubiquitin interact with a perpendicular surface on the same NZF domain. This "bending" of Lys63-linked dimers displaces Met1 of the proximal ubiquitin from the C terminus of the distal unit, thereby preventing an equivalent binding mode for Met1-linked chains (86, 87).

A different type of chain flexibility can be important for recognition of compact chains, as seen for UBA domains of proteasomal shuttling factors that bind Lys48-linked ubiquitin. These UBA domains slot into a Lys48-linked ubiquitin dimer to interact with Ile44 patches of both ubiquitin molecules (**Figure 5*c***) (88, 89), an event that requires dynamic opening of compact Lys48-linked chains (88, 89). Thus, similar to NZF domains, certain UBA domains require ubiquitin chain flexibility to allow for recognition of a particular linkage.

4.3. Recognizing the Linkage Context

The simplest way of recognizing a particular ubiquitin modification is to directly bind the isopeptide bond or the sequence context of the linkage. As an example, the Lys63-linkage-specific antibody interacts with the C terminus of the distal ubiquitin as well as with its Ile36 patch (**Figure 5*e***) (90). Although Lys63 on the proximal Ub is not contacted, residues closely preceding Lys63 mediate antibody binding. Interestingly, this interaction requires a compact Lys63-linked diubiquitin in which the linkage is accessible to the antibody (90).

The linkage context is also recognized by the UBAN domain of NEMO, a subunit of IκB kinase (91), which binds linear diubiquitin (21, 35, 92). NEMO forms a symmetric dimer that has two adjacent ubiquitin-binding sites, with one recognizing the distal Ile44 patch and the second binding to the proximal Phe4 patch (**Figure 5*d***) (21). Although the peptide bond between ubiquitin molecules is not directly contacted, Gln2 of the proximal ubiquitin makes key interactions (21). Similar interactions are not accessible for the proximal unit in Lys63-linked diubiquitin, which can only bind the distal site with weaker affinity (93). NEMO, therefore, illustrates how UBDs can recognize the linkage context to distinguish between structurally related chain topologies.

4.4. Combining Binding Sites

A single UBD can also achieve specificity by recognizing distinct surfaces in multiple ubiquitin molecules of a chain. This interaction mode is illustrated by a regulator of inflammatory processes, A20, which binds Lys63-linked chains. NMR and crystallographic analysis revealed distinct interactions between one A20 zinc-finger domain and three ubiquitin molecules (**Figure 5*f***) (94). A20 binds the Ile44 patch, the TEK-box, and a surface surrounding Asp58 of distinct ubiquitin moieties. Although the ubiquitin molecules were not covalently linked, all Lys63 side chains were in proximity to an adjacent ubiquitin C-terminus, suggesting that this structure represents a model for the interaction of Lys63-linked tri-ubiquitin with one A20 zinc-finger domain.

4.5. Detecting the Free C Terminus of Unanchored Chains

Unanchored ubiquitin chains are generated during ubiquitin biosynthesis as products of the *UBB* and *UBC* genes (95), by DUBs that internally cleave ubiquitin chains or release entire chains from substrates (96), or by ubiquitylation enzymes that synthesize unanchored polymers for signal transduction (97, 98). Unanchored chains contain at their proximal end a free ubiquitin C terminus, which is normally masked by attachment of ubiquitin to substrates. Using its N-terminal ZnF-UBP domain, the DUB USP5/IsoT binds this free C terminus of ubiquitin, including the Gly-Gly sequence, with nanomolar affinity (**Figure 5*g***) (20, 99). This interaction mediates substrate binding, converts the active site of USP5 into a catalytically competent state, and allows USP5 to

preferentially disassemble unanchored chains from their proximal end (20, 99). Because of the latter property, USP5 has been used to validate the role of unanchored chains in signaling (97, 98); however, it should be noted that USP5 can act on attached chains with an activity that is comparable to other USP enzymes (30).

5. ERASING THE CODE

Any useful code should be carefully employed only at times of need. Indeed, to prevent ubiquitylation from being constitutively on, modifications are reversed by DUBs. Human cells contain ~55 USPs, 14 ovarian tumor DUBs (OTUs), 10 JAMM family DUBs, 4 ubiquitin C-terminal hydrolases (UCHs) and 4 Josephin domain DUBs (96). To specifically control ubiquitin-dependent signaling, these enzymes have to deal with chains of distinct linkage, topology, and length.

5.1. Housekeeping and Substrate-Specific Deubiquitinating Enzymes

Several DUBs, referred to as housekeeping enzymes, play important roles in establishing the ubiquitin code. For example, proteasome-bound DUBs, such as USP14, UCH37/UCH-L5, and RPN11/POH1, protect ubiquitin from degradation (100). This process is vital for keeping sufficient levels of free ubiquitin that can be used for chain assembly. Similar functions might be performed by DUBs that interact with ubiquitin-processing complexes, such as the COP9 signalosome (USP15) (101), or the p97 segregase [YOD1 (102), VCIP135 (103), Ataxin-3 (104)].

Another large group of DUBs disassembles chains independently of the linkage, yet these enzymes gain specificity by being targeted to a select set of substrates. These DUBs include most members of the USP family, which regulate many cellular reactions, including splicing, protein trafficking, or chromatin remodeling. Many USP DUBs are recruited to substrates through interaction domains (96) or adaptor subunits (105). Although a comprehensive analysis has not been reported, most USPs are active against all linkages (22, 32, 35) and also hydrolyze the isopeptide bond between the substrate and the first ubiquitin. An exception from this nonspecificity is CYLD, which prefers Met1- and Lys63-linked chains (35, 98, 106). Hence, most USPs can be considered nonspecific with regard to the ubiquitin code but specific with respect to their substrates.

5.2. Linkage-Specific Deubiquitinating Enzymes

In contrast to the aforementioned examples, several DUBs respond to the ubiquitin code and display specificity toward one or a few linkages. JAMM family DUBs are often Lys63 specific, as seen for AMSH (107), AMSH-LP (108), BRCC36, and POH1 (109). In addition, linkage-specific OTU DUBs have been described; these descriptions showed that OTUB1 is specific for Lys48 linkages (110, 111), Cezanne is specific for Lys11 linkages (30), and Trabid is specific for Lys29 and Lys33 linkages (32, 112). As linkage-specific DUBs may not be able to cleave off the last ubiquitin (30), their activity might generate monoubiquitylated substrates with distinct signaling properties.

The structure of AMSH-LP with Lys63-linked diubiquitin revealed the basis for the linkage specificity displayed by JAMM family DUBs (108). AMSH-LP binds to the open conformation of the Lys63-linked diubiquitin and contacts Gln62 and Glu64 of the proximal ubiquitin. Thus, reminiscent of some UBDs, JAMM DUBs might recognize the sequence context of the Lys63-isopeptide bond. The structure of Trabid revealed a different mechanism to achieve specificity; this enzyme uses an Ankyrin-repeat UBD directly upstream of the catalytic OTU domain to position the proximal ubiquitin (112). However, for the OTU and remaining DUB families, structures are available only in complex with a single ubiquitin (19, 113–115), which revealed high-affinity binding sites for the distal ubiquitin, while leaving the

interaction sites for the proximal ubiquitin that provides the modified lysine unclear. These structures did imply, however, that compact chain conformations should not be recognized by DUBs unless they undergo extensive remodeling to expose the isopeptide bond.

5.3. Ubiquitin Chain Editing

Ubiquitin chain editing is perhaps the most sophisticated utilization of the ubiquitin code. During this process, one chain type is replaced by a chain of different topology, which changes the fate of the modified substrate. Editing could be achieved by complexes between sequentially acting DUBs and E3s, as they are often observed in cells (105). Alternatively, single proteins might combine DUB and E3 activity, as seen in a pathway that regulates the transcription factor NF-κB (116). Activation of NF-κB relies on Lys63-specific ubiquitylation of proteins, whereas its inactivation includes a negative feedback loop centered on A20, a protein that combines DUB and E3 domains. It has been proposed that A20 first deubiquitylates Lys63-modified proteins and then modifies them with Lys48-linked chains to trigger their degradation. In this manner, A20 not only stops signaling through Lys63-linked chains, but it also removes the signal transducers that need to be resynthesized before signaling can resume. Although many experiments, including linkage-specific antibodies, support this view of A20 action (90, 94, 116), additional layers of regulation might contribute. Indeed, A20 prefers to cleave Lys48-linked ubiquitin chains in vitro (117, 118), and it was shown to interact with ubiquitin-binding adaptors, such as TAX1BP1 (119) or ABINs (120); E3 ligases, such as ITCH (121) and RNF11 (122); and E2 enzymes (123).

6. CELLULAR FUNCTIONS OF THE UBIQUITIN CODE

The combinatorial action of ubiquitylating, deubiquitylating, and ubiquitin-binding proteins determines the modified protein's fate; it carries the meaning of the code. Historically, the idea of a ubiquitin code emerged from the distinct consequences of proteolytic Lys48-linked and nonproteolytic Lys63-linked chains, a view that might be too simplified: Multiple chain types, including Lys63-linked chains, are now known to drive degradation, whereas Lys48-linked chains can function nonproteolytically, for example, in transcription factor regulation (124). This suggests that the functions of ubiquitylation depend on chain topology, but also on other factors, such as the timing and reversibility of the reaction, enzyme or substrate localization, or interactions between E3s and effectors. Proteomic analyses found thousands of proteins that act in almost all signaling pathways to be modified with ubiquitin (125, 126). To pay tribute to this complexity, we focus our discussion on selected roles of ubiquitylation that are brought about by distinct chain types and influenced by additional cellular inputs.

6.1. Proteolytic Functions of the Ubiquitin Code

6.1.1. Regulation of proteasomal degradation. It is well established that ubiquitin chains can target proteins to the 26S proteasome, a protease required for cell division in all eukaryotes (100). Consistent with Lys48 being the only essential lysine of ubiquitin in yeast, the role in proteasomal targeting was first assigned to Lys48-linked chains (127). Many E3s, including the SCF, gp78, or E6AP, trigger substrate turnover by synthesizing Lys48-linked chains (50, 51, 68). As a result, Lys48 linkages are the most abundant linkage in all organisms subjected to quantitative proteomic analysis, and their levels increase rapidly when the proteasome is inhibited (11, 12, 125, 128).

However, early experiments had already indicated that other linkages could also be recognized by the proteasome (129, 130). These atypical linkages accumulate upon proteasome inhibition (12), suggesting that

they also contribute to protein degradation. Indeed, Lys11-linked chains bind proteasomal receptors and trigger degradation of cell cycle regulators during mitosis (23, 31, 52). In human cells, Lys11 linkages accumulate dramatically upon activation of the responsible E3, the APC/C, and inhibition of Lys11-linked chain formation stabilizes APC/C-substrates and leads to cell cycle arrest (23, 31). Lys11-linked chains are, therefore, proteolytic signals that are particularly important during mitosis.

Other chain types mediate proteasomal degradation less frequently. Lys29-linked chains contribute to substrate turnover in the ubiquitin-fusion-degradation pathway (131, 132), and in a few cases, Lys63-linked or mixed chains were held accountable for triggering degradation (48, 55, 133). Thus, Lys11-, Lys29-, Lys48-, and Lys63-linked chains might all have roles in proteasomal degradation, a diversity in targeting signals that is reflected by the plasticity in substrate recognition by proteasomal subunits: Rpn13 binds monoubiquitin and Lys48-linked diubiquitin with similar affinity (134), S5a/Rpn10 interacts with chains of multiple topologies (135), and various proteasomal shuttling factors show only modest preference for Lys48 compared to Lys63 linkages (136).

Why Lys11- and Lys48-linked chains trigger degradation more frequently than other modifications is not entirely clear. It is possible that enzymes synthesizing Lys11- and Lys48-linked chains are less likely to introduce branches, which can impede degradation (48). In addition, Lys11- and Lys48-specific enzymes, such as the APC/C or SCF, often interact with the proteasome to efficiently couple ubiquitylation and degradation (137, 138). For some E3s, binding to the proteasome is required for sending substrates to degradation; deletion of a proteasome-binding domain in Ufd4 does not affect ubiquitylation, but inhibits substrate turnover (139). It is also possible that atypical linkages are more prone to deubiquitylation, although our limited understanding of DUB biology has not allowed this hypothesis to be rigorously tested.

6.1.2. Regulation of lysosomal degradation. The degradation of plasma membrane proteins occurs in lysosomes, and substrates are targeted to this proteolytic compartment through monoubiquitylation or Lys63-linked chains (140). Ubiquitylation can be initiated at the membrane and lead to endocytosis, as seen for yeast membrane receptors that are substrates of the HECT E3 Rsp5 (141). In such cases, an in-frame fusion of ubiquitin to the substrate is often sufficient for internalization, even if the ubiquitylation machinery is disrupted (142). Alternatively, ubiquitylation can occur at endosomal membranes to control localization after internalization. This was demonstrated with EGFR for which mutation of all ubiquitylation sites did not block endocytosis but strongly affected its routing to lysosomes (143, 144). As a result, deubiquitylation by Usp8 or AMSH can lead to recycling of EGFR to the plasma membrane (145, 146).

Ubiquitylated membrane proteins are recognized by different ESCRT complexes, which bind ubiquitin with a modest preference for Lys63 linkages (147, 148). In addition to ubiquitin-binding motifs, ESCRT-complexes recognize the coat of endocytic vesicles or lipids of endosomal membranes (147). Thus, the UBDs that read out the ubiquitin modification are enriched in proximity of their substrates, suggesting that colocalization of substrates and effectors helps determine the consequences of a ubiquitylation event.

Although ubiquitylated protein aggregates are also degraded in lysosomes, they pass through autophagosomes on their route to elimination. The aggregates are coupled to autophagosomes by adaptor molecules, which bind to substrates with a moderate preference for Lys63-linked chains (149). Reminiscent of ESCRT-complexes, autophagy receptors are enriched in proximity to their substrates through interactions with autophagosomal membranes. Thus, Lys63-linked chains can trigger proteolysis, yet efficient targeting to lysosomes may also require additional inputs, such as the specific localization of effector proteins.

6.2. Nonproteolytic Functions of the Ubiquitin Code

Ubiquitylation is also able to regulate signaling nonproteolytically as it can be used to recruit proteins to participate in particular signaling pathways, to attract trafficking factors that change a substrate's localization, or to control a substrate's activity. In most cases, the ubiquitin conjugate is recognized with low affinity, but the multivalent recognition of both substrate and ubiquitin allows for tight regulation. These nonproteolytic functions of the ubiquitin code are often the consequence of monoubiquitylation or Met1- and Lys63-linked chain formation.

6.2.1. Regulation of protein interactions. The attachment of a single ubiquitin often suffices to recruit binding partners as seen for PCNA, a processivity factor for DNA polymerases (4, 150). In response to DNA damage, PCNA is monoubiquitylated (4, 151), which recruits Y family DNA polymerases (152, 153). These polymerases recognize PCNA through a PCNA-interaction motif, the PIP-box, and ubiquitin through UBZ or UBM domains, leading to a high-affinity interaction that replaces replicative polymerases from PCNA. In this manner, monoubiquitylation of PCNA contributes to a ubiquitin-dependent polymerase switch that rescues stalled replication forks from collapsing. Following the successful repair of the damaged DNA, the recruitment signal for Y family polymerases is turned off by Usp1-dependent deubiquitylation, allowing the replication machinery to return to its normal state (154). Similarly, monoubiquitylation of FANCD2 and FANCI, two proteins involved in DNA repair, recruits the FAN1 nuclease, and this signaling event is also turned off by Usp1 (155–158). Thus, monoubiquitylation is a tool for the reversible recruitment of an enzyme to a particular cellular location.

Protein interactions can also be regulated by Lys63-linked chains: The modification of a spliceosomal protein with Lys63-linked chains stabilizes an snRNP complex, which is reorganized upon Usp4-dependent deubiquitylation (159, 160). Similarly, modification of a ribosomal protein with Lys63-linked chains stabilizes polysomes to promote translation (161). The scaffolding role of Lys63-linked chains is most apparent during the response that cells mount to DNA damage, an event that is dependent on a series of E3 enzymes (162–166). The recruitment of several E3s to sites of DNA damage depends on Lys63-linked chains that are probably attached to histone proteins. The E3s Rnf8 or Rnf168 directly recognize Lys63 linkages through MIU or UBZ domains, whereas Brca1 depends on a binding partner, Rap80. Together, these E3s generate ubiquitin-rich foci that act as stable recruitment platforms for DNA repair enzymes and for checkpoint molecules that inhibit cell cycle progression in the face of damage.

An interesting concept has been introduced with unanchored Lys63-linked chains acting as transient mediators of protein interactions (98). Rather than being attached to a substrate, unanchored chains function as recruitment platforms that attract and cluster multiple recognition factors. Because of the high activity of DUBs that recognize unanchored chains, such as USP5, the half-life of these signaling intermediates is likely short, and some of their specificity might be gained by being synthesized in proximity to their binding partners. Unanchored Lys63-linked ubiquitin chains mediate the activation of TAK1 and IKK kinases and the RIG-I antiviral protein (97, 98, 167). RIG-I binds Lys63-linked, but not Lys48- or Met1-linked, chains through tandem CARD domains (97), which leads to RIG-I dimerization and facilitates downstream signaling events (167).

Ubiquitylation can also impair interactions. For example, monoubiquitylation of Smad4 blocks its association with the transcriptional cofactor Smad2 (168). By deubiquitylating Smad4, USP9X relieves the impediment for cofactor binding and triggers transcriptional activation. Similar results are achieved in EGFR signaling through coupled monoubiquitylation (169–171). Monoubiquitylation of substrate adaptors of the endocytic machinery

leads to an intramolecular interaction between the conjugate and the adaptor's UBD and blocks the ability of the adaptor to recognize its ubiquitylated cargo (170). The transcription factor Met4 shows that such regulation does not rely on monoubiquitylation. Met4 is modified by SCF^{Met30} with Lys48-linked chains, which bind to an internal UIM in Met4 and block its ability to engage with coactivators (124). These examples underscore that the topology of the conjugate and the context of the modification can determine the outcome of ubiquitylation.

6.2.2. Regulation of protein activity. Ubiquitylation can affect a protein's activity by different means. In a straightforward mechanism of activation, an inhibitor is sent for degradation. Among many examples, the inhibitor of the NF-κB transcription factor, IκBα, is degraded after modification with Lys48-linked chains by $SCF^{\beta TrCP}$ (172, 173). The proteasome can also activate a protein by cleaving off inhibitory domains, as seen for proteasomal cleavage of an NF-κB precursor (174). Similar reactions are observed in budding yeast, where proteasomal processing of the transcription factor SPT23 is a prerequisite for its release from the endoplasmic reticulum membrane (175), or in fission yeast, where activation of the membrane-bound transcription factor SREBP requires ubiquitin-dependent cleavage (176). Proteasomal processing might involve atypical linkages, as cleavage of NF-κB might require multimonoubiquitylation (177), whereas SPT23-processing is brought about by Rsp5, an E3 that catalyzes monoubiquitylation or Lys63-linked chain formation (175). The substrates also have to withstand unfolding through proteasomal ATPases (178), again underscoring how the outcome of ubiquitylation can be determined, at least in part, by the sequence context of the modification.

Activation of NF-κB usually occurs in response to external stimuli, such as the tumor necrosis factor α. Binding of the tumor necrosis factor α to its membrane receptor initiates a variety of ubiquitylation events, such as formation of Lys63-linked chains by TRAF6 (60), mixed Lys11/Lys63-linked chains by the RING E3 ligase cIAP1 (7, 179), or Met1-linked chains by LUBAC (82). LUBAC modifies NEMO, a subunit of the IκBα kinase (IKK) complex. Intriguingly, the Met1-linked chains on NEMO are recognized by the UBAN domain of NEMO itself, which may cause a conformational change in the intertwined helices of NEMO dimers (21). As NEMO is a core regulatory subunit of IKK, these conformational changes might lead to allosteric activation of IKK. NEMO also associates with Lys63-linked chains (180) and with mixed Lys11/Lys63-linked chains that are detected on the receptor interacting protein 1, RIP1 (7). Lys63-linked chains activate IKK by promoting its binding to the upstream TAK1 kinase complex (181). These findings suggest that distinct types of ubiquitin topologies, i.e., Met1-, Lys11/Lys63-, Lys63-, or Lys48-linked chains, regulate signaling through inhibitor degradation, proteasomal processing, allosteric activation, or recruitment of upstream activating enzymes.

6.2.3. Regulation of protein localization. The role of ubiquitylation in regulating localization serves as a final example for the diverse functions of the ubiquitin code. Ubiquitin-dependent changes in localization had originally been observed in yeast, where internalization of plasma membrane proteins can be brought about by monoubiquitylation (141, 142). Interestingly, ubiquitin can determine the intracellular location, even if it is not connected to a substrate through an isopeptide bond; the E2 Ube2E3/UbcM2 is transported into the nucleus only when it is charged with a thioester-linked ubiquitin (182). The ubiquitin-loaded Ube2E3 is bound by a transport factor of the importin family, but whether these importins also interact with other cargoes in a ubiquitin-dependent manner has not been determined.

Ubiquitylation can also indirectly affect protein localization. Following its multimonoubiquitylation, the transcription factor p53 is exported out of the nucleus (183). p53 is modified on several C-terminal lysine residues, and in-frame fusions of ubiquitin to p53 are

Chain elongation: extension of chains by addition of additional ubiquitin molecules

sufficient to drive its nuclear export (5). Because cytoplasmic accumulation of these fusions depends on a nuclear export signal in p53, monoubiquitylation likely changes the accessibility of the nuclear export sequence in p53 to the export machinery, rather than being an export signal itself. As opposed to p53 degradation, monoubiquitylation of p53 can be reversed by USP10, which allows reimport and reactivation (184). Thus, by affecting intra- or intermolecular binding events, ubiquitylation can lead to many different consequences that result from a combinatorial recognition of the ubiquitin conjugate and additional factors, such as membrane lipids, binding partners, or substrate domains.

7. CONCLUSIONS

Groundbreaking work with Lys48- and Lys63-linked chains suggested that different ubiquitin linkages result in unique consequences for the modified proteins. During the past years, much of this ubiquitin code hypothesis has been confirmed: Different chain topologies adopt unique compact or open conformations; they are synthesized by enzymes that assemble the specific modifications; they are recognized by linkage-specific ubiquitin-binding proteins that couple the modification to a particular outcome; they are disassembled by enzymes that act as erasers of the code; and they function in a wide range of different processes.

Several questions, however, remain open. We know little about the physiological relevance of Lys6, Lys27, Lys29, and Lys33 linkages or more complex structures, such as branched chains. Moreover, it is possible that substrates are modified with multiple chain types at the same time, but whether this is associated with a particular function has not been tested. Even for better-understood topologies, fundamental questions need to be addressed: Why, for example, does the APC/C assemble Lys11- instead of Lys48-linked chains to drive degradation? Is there physiological relevance to differences in chain length? Do proteins that restrict chain elongation serve roles other than preventing the waste of ubiquitin? How dynamic are ubiquitin chains? And can the flexibility of chain structures be regulated to modulate recognition by ubiquitin-binding proteins?

In a broader sense, the systems biology of the ubiquitin code has to be analyzed in more detail. Given the importance of E2s in determining specificity, it is surprising how little we know about physiological E2-E3 pairs. The same holds true for DUBs, which are often found in complexes with E3s and, hence, have a lot of potential in modulating the ubiquitin code. Finally, only a few E3s have been studied in sufficient detail to allow a somewhat comprehensive assessment of their substrates. More insight into substrate modifications and their cellular consequences will undoubtedly uncover more functions for the ubiquitin code.

A major breakthrough in understanding the Inca quipus came with the discovery that certain combinations of knots encode numbers, yet most of the information found between those numeric knots remains mysterious. It is the numbers of the ubiquitin code, i.e., the residues used for assembling chains, that became better understood in recent years; what all topologies mean, however, has not been completely unraveled. Thus, as it is the case for those trying to decipher the code of an ancient empire, there is much to be learned before we can crack the ubiquitin code in its entirety.

SUMMARY POINTS

1. Proteins can be modified with a single ubiquitin or with polymeric chains that differ in the connection between ubiquitin molecules.
2. The different ubiquitin modifications adopt distinct structures.

3. Ubiquitin-binding proteins exploit various strategies to specifically interact with particular types of ubiquitin modifications.
4. Ubiquitin chains can be disassembled by nonspecific or linkage-specific DUBs.
5. The various ubiquitin modifications trigger a wide range of biological reactions, including protein degradation, activation, and localization.
6. The consequences of ubiquitylation are determined by the chain topology in combination with additional factors, such as substrate localization or sensitivity to deubiquitylation.

FUTURE ISSUES

1. What are the functions of Lys6, Lys27, Lys29, and Lys33 linkages or branched ubiquitin chains?
2. Are there more complex structures, such as multiple chain types, attached to a single substrate, and what are their functions?
3. How important is chain length for signaling?
4. Can the dynamics of ubiquitin chains be regulated to modulate signaling?
5. Can we dissect the network of enzymes and substrates to discover novel functions of the ubiquitin code?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We apologize to all whose work could not be cited owing to page limitations. D.K. would like to thank Yogesh Kulathu for comments on the manuscript. D.K. is funded by the Medical Research Council and the EMBO Young Investigator Program. M.R. is grateful to Julia Schaletzky for many discussions and for critically reading this manuscript, and to Gary Urton of Harvard University for his introduction into Inca archeology. M.R. is funded by grants from the National Institutes of Health, the Pew Foundation, the March of Dimes Foundation, and the California Institute for Regenerative Medicine. Both authors thank their labs for continued discussions and for their enthusiasm toward unraveling the ubiquitin code.

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