Cell. Mol. Life Sci. 65 (2008) 2397 – 2406
1420-682X/08/152397-10
DOI 10.1007/s00018-008-8090-6

Cellular and Molecular Life Sciences

Review

Polyubiquitin chains: functions, structures, and mechanisms

W. Li and Y. Ye*

Building 5, Room 433, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 5 center drive, Bethesda, MD 20892 (USA),
Fax: +1 301 496 0201, e-mail: yihongy@mail.nih.gov

Received 20 February 2008; received after revision 12 March 2008; accepted 1 April 2008
Online First 26 April 2008

Abstract. Ubiquitin is a highly conserved 76-amino-acid polypeptide that is found throughout the eukaryotic kingdom. The covalent conjugation of ubiquitin (often in the form of a polymer) to substrates governs a variety of biological processes ranging from proteolysis to DNA damage tolerance. The functional flexibility of this post-translational modification has its roots in the existence of a large number of ubiquitinating enzymes that catalyze the formation of distinct ubiquitin polymers, which in turn encode different signals. This review summarizes recent advances in the field with an emphasis on the non-canonical functions of polyubiquitination. We also discuss the potential mechanism of chain linkage specification as well as how structural disparity in ubiquitin polymers may be distinguished by ubiquitin receptors to translate the versatile ubiquitin signals into various cellular functions.

Keywords. Ubiquitin, polyubiquitination, p97, UBA, proteasome, autophagy.

Introduction

Ubiquitin and its kin (ubiquitin-like proteins, or Ubls) are a family of highly conserved proteins that share similarity, not only in structure, but also in the way of action [1, 2]. The C terminus of this class of proteins can be ligated to the ε-amino group of a lysine residue or α-amino group of the N-terminal amino acid in a substrate protein. Thiol and hydroxyl groups present in cysteine and serine/threonine residues, respectively, can also serve as acceptors for ubiquitin, albeit less commonly [3–6]. Modification of proteins with Ubls is reversible, as Ubl conjugates can be severed from substrates by the action of a class of protease. Thus, like phosphorylation, modification of proteins with Ubls provides a powerful means to reversibly alter the functional state of the modified proteins.

Although the mechanism of different Ubl conjugation reactions may vary in detail, a common theme has emerged that these reactions are all carried out with the aid of a set of enzymes that usually include an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3). An E1 enzyme activates Ubls in the presence of ATP. As a result, the C-terminal carboxyl group of an Ubl is covalently ligated to the active cysteine residue of the E1 enzyme. Subsequently, the Ubl is transferred to the active cysteine residue in an E2 enzyme, that in turn relays the Ubl molecule to a substrate in the presence of an E3 ligase [7, 8].

Among all the Ubl members, ubiquitin, the founding member of the family, is often conjugated to substrates as a polymer. Specifically, additional ubiquitin molecules can be ligated to one of the seven lysine sites in the previously attached ubiquitin molecule, resulting in the formation of ubiquitin chains containing distinct linkages between the ubiquitin moieties. In *Saccharomyces cerevisiae*, all seven lysine residues can be

* Corresponding author.

used to assemble polyubiquitin chains *in vivo* as revealed by a proteomic analysis [9]. Recent studies also demonstrate the existence of mixed ubiquitin chains that contain more than one type of ubiquitin linkage within a single polymer [10, 11]. In addition, the C terminus of ubiquitin can be linked to the N-terminal amino group of another ubiquitin molecule to form the so-called linear ubiquitin chain [12]. Although the physiological relevance of many ubiquitin chain linkages remains to be demonstrated (see below), modification of substrates with ubiquitin chains of different topologies can, in principle, significantly expand the functional repertoire of polyubiquitin signals.

The complexity of polyubiquitination stems from the existence of a large number of enzymes and auxiliary factors that catalyze the polyubiquitination reactions. Compared with other Ubl family members, which utilize a single E1, E2, and a few E3 ligases in the conjugation reactions, ubiquitin employs two activating enzymes, dozens of E2 enzymes, and hundreds of E3 ligases to fulfill its conjugation requirements [1, 13, 14]. Under certain circumstances, additional factors such as an E4 ubiquitin elongating factor may be 'called in' to the reaction. In conjunction with a ubiquitin ligase, an E4 enzyme can act to further lengthen a ubiquitin oligomer to its full length [15, 16]. Ubiquitin E3 ligases can be classified into three major types on the basis of their 'catalytic' domains: the HECT domain (homologous to E6-associated protein C terminus) E3, the RING finger E3, and the U box E3. The HECT domain was originally identified in E6-AP, a cellular protein that associates with the human papillomavirus E6 gene product to induce the degradation of the tumor suppressor protein p53 [17]. A catalytic cysteine residue present in the HECT domain usually accepts ubiquitin molecules from a cognate E2 enzyme before transferring them to substrates. In contrast, the RING and U box E3s do not form covalent intermediates with ubiquitin. Instead, they appear to function as scaffolds to position substrates in close proximity to an E2-ubiquitin covalent complex, which facilitates the direct transfer of ubiquitin from E2 enzymes to substrates [18, 19]. Interestingly, despite the lack of sequence homology, the RING and the U box domains display remarkable similarity in structure, suggesting a common mechanism of action for these enzymes [20, 21].

It has been well known that polyubiquitin chains bearing different linkages convey distinct structural and functional information. A well-accepted doctrine in the field is that ubiquitin chains linked by Lys48 target substrates to a multi-subunit protease termed the proteasome for degradation. In contrast, Lys63-linked chains perform non-proteolytic functions in at least four pathways: DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis. These canonical functions of polyubiquitination have been a subject of extensive reviews [1, 2, 22–28]. Therefore, in this review, we aim to provide examples of non-canonical functions of polyubiquitination. We will also summarize the recent advance in our understanding of how ubiquitin chain linkages are determined during chain synthesis and discuss how signals encoded by different ubiquitin polymers are interpreted in cells to fulfill the functional diversity of polyubiquitin signals.

Non-canonical functions of polyubiquitination

Non-proteolytic functions for Lys48-linked polyubiquitin chains

The classical view of the function of Lys48-linked polyubiquitination is that a chain consisting of a minimum of four ubiquitin moieties can interact with the proteasome with high affinity to target polyubiquitinated substrates for degradation [29, 30]. However, recent studies also reveal some novel non-proteolytic functions for Lys48-linked polyubiquitin chains.

One example of such non-proteolytic functions is regulation of the activity of the transcription factor Met4 in *S. cerevisiae*. Met4 activates expression of genes in the methionine biosynthetic pathway. Like many biosynthetic regulators, the activity of Met4 is tightly controlled by the level of the end products (methionine in this case). When cells encounter medium replete in methionine, Met4 rapidly undergoes polyubiquitination, and this is tightly correlated with the loss of Met4 transcriptional activities. Interestingly, although Met4 is apparently conjugated with Lys48-linked polyubiquitin chains, it remains unexpectedly long-lived [31, 32]. How does polyubiquitinated Met4 escape degradation by the proteasome? It turns out that the transcription factor itself contains a ubiquitin-binding domain that interacts with its own ubiquitin chains to restrict the chain length below a threshold required for proteasome recognition [33]. Although it is still unclear how polyubiquitination governs the transcriptional activity of Met4, these findings clearly demonstrate that under certain conditions, Lys48-linked polyubiquitination can inactivate a modified protein without targeting it to the proteasome for degradation.

Another known non-proteolytic function for Lys48-linked polyubiquitination is the activation of a ubiquitin-selective chaperone termed p97. p97 (also called Cdc48 in *S. cerevisiae*) is a member of the AAA (ATPase associated with various cellular activities)

ATPase family. It can cooperate with distinctive cofactors to differentially act on various substrates conjugated with Lys48-linked polyubiquitin chains [34, 35]. A common theme to emerge is that the recognition of Lys48-linked polyubiquitin chains by a p97 complex somehow activates the ATPase, which extracts ubiquitinated substrates from an immobilized cellular compartment or a large protein complex. Such 'segregase' function of p97 has been well documented for the degradation of misfolded proteins of the endoplasmic reticulum (ER-associated degradation or ERAD) and for the activation of a membrane-bound transcription factor named Spt23 in yeast [36–42]. In the case of ERAD, substrates released from ER membrane still carry polyubiquitin chains and are further shuttled to the proteasome for degradation. In contrast, the released Spt23 appears to lose most of its ubiquitin conjugates and therefore remains stable in cells [42]. Recently, Meyer and colleagues reported a similar 'segregase' function for p97 and its cofactor complex Ufd1-Npl4 in mitosis [43]. They convincingly demonstrated that the p97-Ufd1-Npl4 complex can act on polyubiquitinated Aurora B kinase to extract it from the chromatin during mitosis, which allows chromosome decondensation and the reformation of the nuclear envelope.

Proteolytic function for Lys63-linked polyubiquitin chains

If Lys48-linked polyubiquitination can serve non-degradation purposes, can Lys63-linked polyubiquitin chains act in proteolysis? The answer appears to be 'yes.' Although a survey of the literature only reveals a few scattered reports on Lys63-linked ubiquitin chains being involved in proteasome-dependent degradation [11, 44], other evidence suggests that Lys63-linked polyubiquitination is equally competent in sentencing substrates to a destructive fate, albeit through a proteasome-independent mechanism termed autophagy.

Autophagy is a cellular mechanism whereby damaged organelles or part of the cytosol are engulfed by double-membrane vesicles to form the so-called autophagasome, which subsequently fuses with the lysosomes to degrade the sequestered contents [45]. This process adapts cells to cope with many stress conditions such as amino acid starvation. Moreover, it provides an effective means to eliminate misfolded protein aggregates and damaged organelles that are usually too large to be handled by cellular proteases such as the proteasome.

Although it has been well established that the formation of autophagasomes requires two Ubls (Atg8 and Atg12) that modify the lipid phosphatidylethanolamine and Atg5, respectively, direct involvement of ubiquitin in autophagy-dependent degradation sounds like a far-fetched idea given that no proteins identified in the autophagy pathway have the capacity to interact with ubiquitin. Nevertheless, several recent reports suggest that a protein termed p62 or sequestosome 1 may provides the missing link between ubiquitin and autophagy. These studies highlight an important role for ubiquitin in cargo selection for autophagy-dependent protein turnover, similar to what has been demonstrated for proteasome-mediated proteolysis.

p62 belongs to the Ubl/UBA protein family that contains both a ubiquitin-like fold (Ubl) and an ubiquitin-binding domain (UBA). Many proteins of this family can recognize ubiquitinated proteins via their UBA domain and simultaneously bind the proteasome through an interaction between their Ubl domain and an ubiquitin-interacting motif (UIM) present in the S5a subunit of the proteasome. Thus, it is believed that this class of proteins may shuttle ubiquitinated substrates to the proteasome for degradation. As expected, p62 interacts with the proteasome via its Ubl domain [27]. The Ubl domain of p62 (also called PB1 domain) can also bind to other partners, including some signaling molecules [46], and it mediates the self association of p62, resulting in the formation of a large oligomer. The UBA domain of p62 in isolation displays no preference for Lys48- or Lys63-linked polyubiquitin chains *in vitro* [47, 48]. However, in cells, p62 preferentially recognizes Lys63-linked ubiquitin chains [49]. These observations led Wooten and colleagues to propose that p62 may deliver substrates bearing Lys63-linked polyubiquitin chains to the proteasome for degradation [44]. Although this model may well be true for some substrates, a growing body of evidence suggests that the major destination for p62-bound substrates may be an aggresome-like induced structure (ALIS) [50] that is in turn destroyed by the autophagy pathway. First, p62 directly interacts with LC3, the mammalian homolog of Atg8, which is an essential component of the autophagic machinery. As a result, p62 itself is degraded by the autophagy pathway [51, 52]. Second, p62 can oligomerize to form protein bodies that contain ubiquitinated misfolded proteins. These p62 bodies display partial colocalization with autophagasomes and appear to be cleared by the autophagy pathway because defects in autophagy lead to the accumulation of ubiquitin-positive protein aggregates that also contain p62 [51–55]. Finally, p62 is required for the formation of the ubiquitin-positive protein aggregates as genetic ablation of p62 inhibits the accumulation of such aggregates in autophagy-deficient animals and cells [51, 52]. Together, these studies suggest a model in which misfolded proteins bearing

Lys63-linked ubiquitin chains are recognized by p62, whose polymerization leads to the formation of large protein bodies that sequester these misfolded proteins. The interaction between p62 and LC3 may signal the autophagy pathway to eliminate these p62-containing protein aggregates or ALIS.

ALIS is morphologically distinct from aggresomes, which are formed in a perinuclear region in proteasome-defective cells to sequester misfolded proteins [56]. Interestingly, Olzmann and colleagues recently reported that misfolded DJ-1 can be modified with Lys63-linked ubiquitin chains, which are subsequently recognized by a ubiquitin receptor termed HDAC6. HDAC6 can interact with dynein, a minus-end-driven microtubule motor, to deliver misfolded DJ-1 to a perinuclear region to promote its sequestration in aggresomes [57]. A similar shuttling function has been previously demonstrated for HDAC6, although its cargo was believed to carry Lys48-linked ubiquitin chains [58]. It was thought that these polyubiquitinated proteins were stationary once they reach the aggresomes. However, Olzmann and colleagues provided evidence that misfolded DJ-1 in the aggresomes is eventually degraded by the autophagy pathway, although their work did not reveal the mechanistic link between aggresome and the autophagy pathway. It is unclear why some substrates bearing Lys63-linked ubiquitin chains are recognized by p62 whereas others are recognized by HDAC6. Furthermore, certain signaling molecules are modified by Lys63-linked polyubiquitin chains, but they manage to escape detection by both of these ubiquitin receptors. It is apparent that ubiquitin chains are not the sole determinant for substrate recognition. The recognition process may be influenced by many other factors, including chain dynamics and accessory proteins. It is conceivable that ubiquitin chains assembled on signaling molecules may be short-lived because they are rapidly disassembled by deubiquitinating enzymes, which serves as an effective means to terminate the signaling activities [59]. Alternatively, they may be preferentially bound by other ubiquitin-binding proteins that prevent these chains from being recognized by either p62 or HDAC6. Despite many unsolved issues, the fact that modification of substrates with Lys63-linked ubiquitin chains targets proteins to either aggresomes or ALIS for clearance by autophagy suggests that these chains can also serve as a destructive signal.

Functions of other types of polyubiquitin linkages

Although most researchers in the ubiquitin field focus their work on either Lys48- or Lys63-linked polyubiquitination, several recent studies have begun to explore the physiological relevance of other types of ubiquitin linkage. For example, Brou and colleagues recently demonstrated that AIP4, a HECT domain E3 ligase, can assemble Lys29-linked polyubiquitin chains on the Notch signaling modulator DTX to target it for lysosomal degradation [60]. Another study showed that two AMPK-related kinases may be modified with Lys29- and/or Lys33-linked ubiquitin chains in cells, and such modification appears to regulate the enzymatic activity of these kinases [61]. Finally, Nishikawa et al. [62] reported that the tumor suppressor BRCA1 E3 complex may assemble Lys6- or Lys29-linked ubiquitin chains on itself, which may regulate the stability of the E3 enzyme. Hopefully, these reports will spark more interest in further investigating the physiological functions of these non-mainstream ubiquitin linkages.

Structural basis for the functional diversity of polyubiquitination

The functional diversity of ubiquitin signals indicates the existence of distinct ubiquitin interpreters in cells. Indeed, one of the most exciting findings in the field is the recent discovery that eukaryotic genomes encode a large number of proteins bearing ubiquitin-binding domains (UBDs) of various kinds [63]. These include UBA, UIM (Ub-interacting motif), DUIM (double-sided ubiquitin-interacting motif), NZF (Npl4 zinc finger), CUE (coupling of Ub conjugation to ER degradation), UEV (Ub-conjugating enzyme variant), GLUE (GRAM-like ubiquitin-binding in Eap45), VHS (Vps27, HRS, STAM), GGA (Golgi-associated gamma-adaptin homologous and TOM1), and PAZ (polyubiquitin-associated zinc finger). UBDs are generally small (20–150 amino acids). Biochemical studies show that most UBDs can bind monoubiquitin with a weak affinity ($Km > 100\ \mu M$), but they usually exhibit a much higher affinity toward polyubiquitin chains [63].

In vitro experiments showed that most UBDs in isolation display no selectivity for ubiquitin chain linkages [48]. Even for the few UBDs that exhibit preference for a given type of ubiquitin chain, replacement of these domains with a more promiscuous UBD often does not alter the function of the UBD-bearing protein in cells [27]. For example, the proteasome adaptor Rad23 mediates the degradation of some proteasomal substrates. Accordingly, its UBA domain preferentially binds to Lys48-linked ubiquitin chains. However, the UBA domain of Rad23 can be replaced with that of Ddi, a domain with no linkage selectivity, without compromising the proteolytic function of Rad23 [64]. Thus, at first glance, it appears that linkage selectivity may not be essential for the

function of many ubiquitin receptors. However, in cells, ubiquitin chain binding by UBDs is often influenced by additional domains present in a ubiquitin receptor or by other subunits within a protein complex that comprises the ubiquitin receptor. These additional factors may turn an otherwise promiscuous UBD into a linkage-specific, chain-binding domain. Additionally, a ubiquitin pathway is usually comprised of a cascade of ubiquitin receptors that hand over ubiquitinated substrates from an upstream component to a downstream effector. In this regard, linkage selectivity likely plays a more critical role at upstream steps, which may explain why substitution of a linkage-specific UBD in a downstream component with a promiscuous UBD may not affect the function of a UBD-bearing protein.

Assuming selective recognition of ubiquitin chains by distinctive ubiquitin receptors can be important for ubiquitin signaling, what is the structural basis for linkage discrimination? Recent nuclear magnetic resonance (NMR) studies demonstrate that at least for Lys48- and Lys63-linked ubiquitin chains, there is a drastic conformational distinction between the two types of polymers. Lys48-linked ubiquitin chains mainly adopt a packed 'closed' conformation wherein the two neighboring ubiquitin subunits form extensive interactions with each other (Fig. 1A). In contrast, Lys63-linked ubiquitin chains form an extended configuration like 'beads on a string,' with no direct contact between the neighboring ubiquitin subunits (Fig. 1B) [65–67].

How do UBDs distinguish structural variations in different ubiquitin polymers? Structural analyses demonstrate that the recognition of monoubiquitin by various UBDs allways involves a similar hydrophobic surface on ubiquitin that is composed of three residues, Leu8, Ile44, and Val70 [68, 69]. Chemical shift perturbation experiments show that these residues also contribute to the interaction of UBDs with both Lys48- and Lys63-linked ubiquitin chains. The interaction of Lys63-linked ubiquitin chains with a UBD is thought to be similar to that of a monoubiquitin because each ubiquitin subunit can independently bind a UBD. By contrast, the compact Lys48-linked ubiquitin chains bury Leu8, Ile44, and Val70 residues at the interface between the two neighboring ubiquitin subunits (Fig. 1A). Thus, the interaction of Lys48-linked polymers with a UBD must involve a conformational transition to expose these hydrophobic residues. A recently modeled structure of a Rad23 UBA domain in complex with Lys48-linked di-ubiquitin based on NMR data provides some important insights on how Lys48-linked ubiquitin chains may be selectively recognized by some UBDs [70]. In this model, the UBA domain is situated between the two ubiquitin subunits, which are spatially positioned such that the UBA makes simultaneous contacts with both of them (Fig. 1C). The interaction of the UBA with the proximal ubiquitin (the one closer to a substrate) is reminiscent of that with a mono-ubiquitin, that is, the same hydrophobic patch on ubiquitin is employed to bind to helix2 of the UBA. In contrast, the contact between UBA and the distal ubiquitin is made via the Gly-Lys linker and several residues close to the linker (residues 70–73) of the distal ubiquitin. These residues, together with the hydrophobic surface on the proximal ubiquitin, form an extended hydrophobic pocket that embraces the UBA domain. The additional interactions between the UBA and the linker not only explain why Rad23 UBA binds more tightly to polyubiquitin chains than monoubiquitin, but also reveal the molecular basis of linkage selectivity for Rad23.

How are Lys48-linked ubiquitin chains converted from the 'closed' to an 'open' conformation to accommodate a UBD? Crystallographic analyses of Lys48-linked diubiquitin and tetraubiquitin reveal several distinct geometries including both closed and open conformations, indicating conformational flexibility for Lys48-linked ubiquitin chains [71–74]. In solution, a switch from a closed to an open conformation can occur when the pH is decreased [66, 75]. These observations suggest that the two neighboring ubiquitin subunits in Lys48-linked chains are not rigidly locked in the closed conformation. The two geometries of Lys48-linked chains may exist in equilibrium in cells, and the presence of a UBD may shift the equilibrium in favor of an open configuration.

Mechanism of linkage specification

Given the important role played by chain linkages in various biological processes, a central question in the field is how different ubiquitin linkages are specified. In principle, the formation of a ubiquitin linkage must involve two ubiquitin molecules, one being donor and the other acceptor. The donor should be covalently linked to the active cysteine of a ubiquitin-conjugating enzyme (E2) or to that of a HECT domain E3 enzyme before being ligated to a lysine residue in an acceptor ubiquitin molecule. Existing evidence suggests that the formation of a given ubiquitin linkage requires specific interactions between the donor ubiquitin-bound E2 or E3 enzymes and the acceptor ubiquitin molecule. Such interactions precisely orient the two ubiquitin molecules in a spatial geometry that allows only one of the seven lysine residues in the acceptor to be ligated with the donor ubiquitin (Fig. 2A).

An example in support of the above-mentioned model is the synthesis of Lys63-linked ubiquitin chains by a

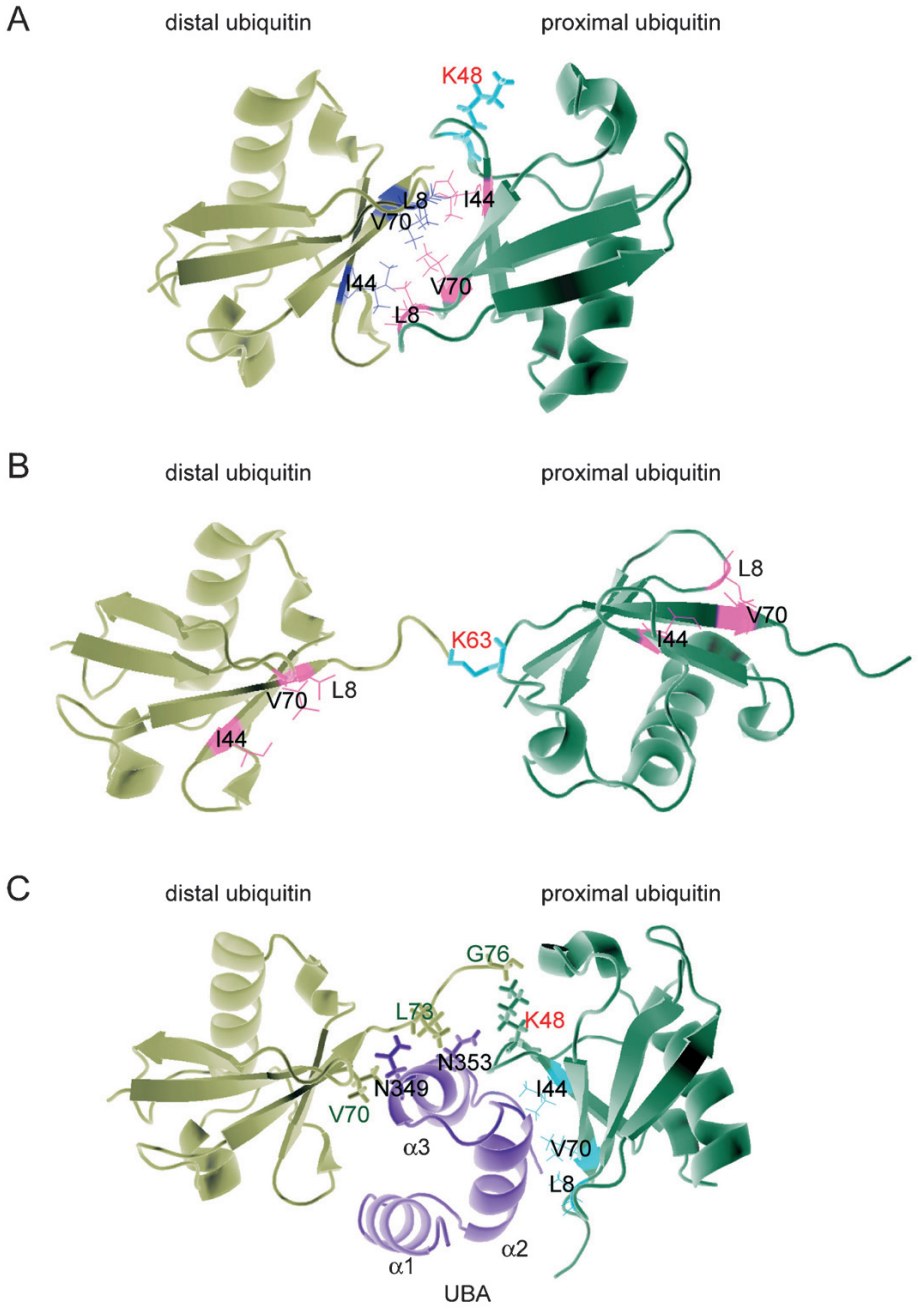

Figure 1. Structural basis for linkage selectivity. (*A*), Lys48-linked di-ubiquitin mainly forms a closed conformation. Side chains of residues Leu8, Ile44 and Val70 are buried between the two packed ubiquitin moieties. (*B*), Lys63-linked di-ubiquitin adopts an open configuration, in which the hydrophobic patch formed by Leu8, Ile44 and Val70 on each ubiquitin moiety can independently interact with a ubiquitin-binding domain. (*C*), Interaction of a Rad23 UBA domain with Lys48-linked di-ubiquitin. Note that the hydrophobic patch on the proximal ubiquitin molecule binds helix 2 of the UBA, whereas helix 3 of the UBA interacts with the C terminus of the distal ubiquitin as well as the linker region. Models were generated using PDB coordinates 2PEA, 2JF5 [D. Komander, P. Odenwaelder, and D. Barford, unpublished data], and 1ZO6, respectively.

heterodimeric E2 complex Ubc13-Mms2/UEV. An unusual feature of Ubc13-Mms2/UEV-mediated polyubiquitination is its independence on E3 ubiquitin ligases. Structural studies reveal that the Ubc13-Mms2/UEV complex is capable of binding to two ubiquitin molecules: one (the donor) is covalently linked to the catalytic cysteine in Ubc13, whereas the other (the acceptor) is non-covalently associated with Mms2/UEV. The interaction of Mms2/UEV with a surface distant from the Lys63 on the acceptor ubiquitin molecule helps to position the acceptor such that only its Lys63 residue can readily attack the thioester bond that connects the donor ubiquitin with Ubc13 (Fig. 2B) [76–78]. Another example is the linkage specification by the HECT domain E3 ubiquitin ligase KIAA10. Unlike RING finger E3s, HECT domain E3s contain an active cysteine residue to which a donor ubiquitin must be first covalently conjugated before being transferred to a lysine residue on an acceptor ubiquitin. KIAA10 can catalyze the formation of both Lys48- and Lys29-linked ubiquitin chains *in vitro*. Using an elegant *in vitro* assay, Pickart and colleagues systematically analyzed the residues on the acceptor ubiquitin molecule that are critical for

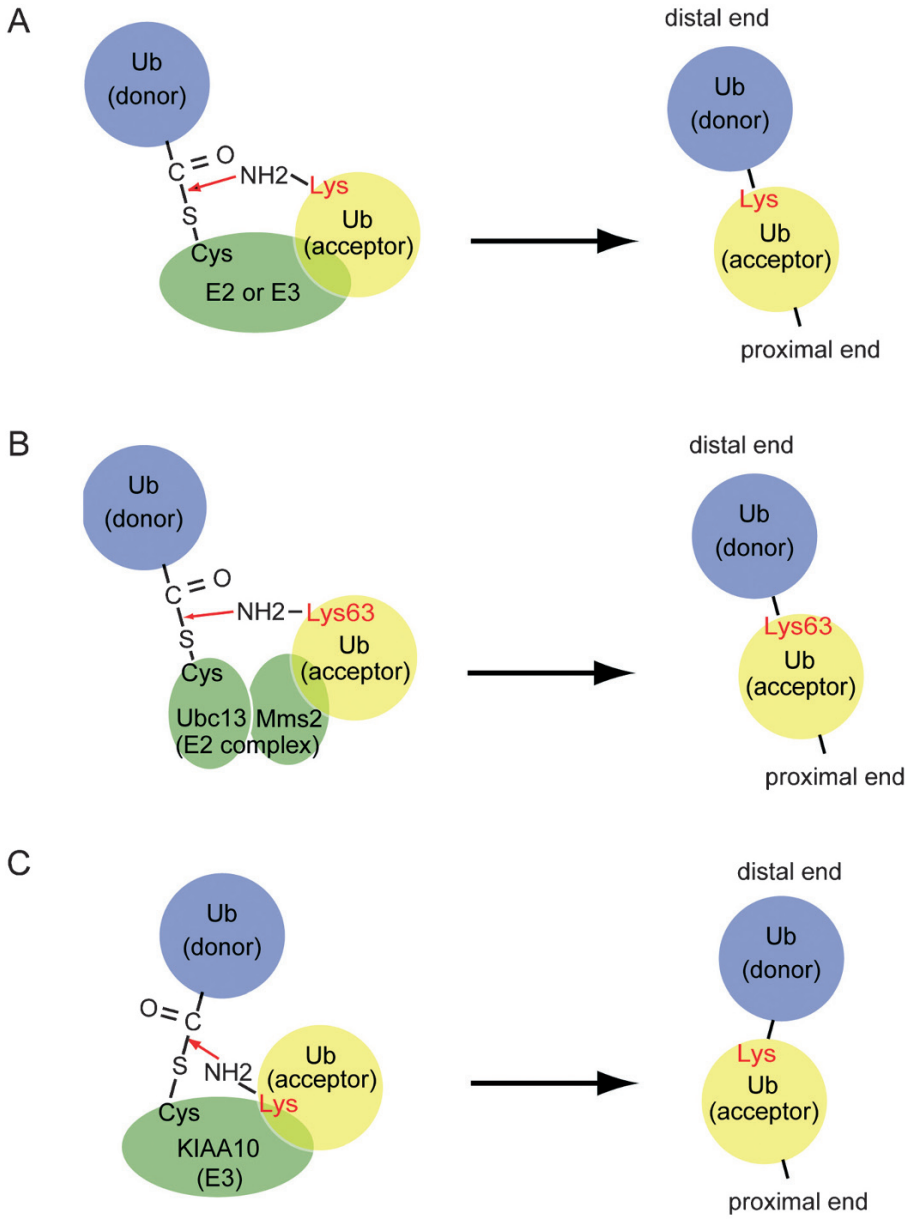

Figure 2. Molecular mechanism of linkage specification. (*A*), General outline of linkage specification. Interactions between the acceptor ubiquitin and an E2 or E3 enzyme (indicated by the overlap between the two molecules) position a specific lysine residue in the acceptor in close proximity to the donor ubiquitin. Red arrow indicates the attack of the donor ubiquitin thioester linkage by the acceptor lysine side chain. The distal and proximal ends of the final product are labeled. (*B*), Mms2 in the heterodimeric E2 complex interacts with a surface on the acceptor ubiquitin that is distant from the acceptor lysine residue (Lys63), which helps to properly orient the attacking lysine side chain. (*C*), KIAA10 likely interacts with residues near the attacking lysine residue (Lys48 or Lys29) on the acceptor ubiquitin to promote the ligation of donor ubiquitin to a specific lysine residue in the acceptor ubiquitin.

linkage specification. Interestingly, they found that residues critical for the formation of Lys29-linked chains are clustered on a surface near Lys29 of the acceptor ubiquitin, whereas residues essential for Lys48 linkage are all located near Lys48. These results suggest that residues close to an acceptor lysine residue may participate in some kind of interactions with the donor ubiquitin-E3 complex to position the nearby acceptor lysine residue in close proximity to the donor ubiquitin (Fig. 2C) [79].

The above-mentioned examples illustrate a principle that may be applicable to other ubiquitin-conjugating enzymes such as Cdc34 and Ube2g2/Ubc7. Although Cdc34 and Ube2g2/Ubc7 appear to employ distinctive mechanisms to polymerize Lys48-linked ubiquitin chains on substrates (Cdc34 may add ubiquitin molecules one at a time to a substrate whereas Ube2g2/Ubc7 may first polymerize ubiquitin chains on its catalytic cysteine residues before transferring the chains 'en bloc' to a substrate) [80–82], the formation of Lys48-linked ubiquitin chain by these enzymes seems to involve interactions between a conserved acidic loop of these E2 enzymes and the acceptor ubiquitin molecules analogous to either KIAA10 or Ubc13/Mms2. Mutations in the acidic loop severely inhibit chain formation for both Cdc34 and Ube2g2/Ubc7. For Cdc34, deletion of the acidic loop turns Cdc34 into a promiscuous E2 enzyme as it can now catalyze the formation of non-Lys48-linked ubiquitin chains. These results suggest that the acidic

loop of these E2 enzymes may be involved in positioning the acceptor ubiquitin molecule such that only the Lys48 on the acceptor ubiquitin becomes the favorable site for chain elongation.

Our proposed model may also explain why certain E2 enzymes such as Ubc5 are capable of synthesizing ubiquitin chains containing all seven possible linkages and even bifurcated ubiquitin forks (two ubiquitin moieties are linked to two lysines in a single ubiquitin molecule) in an *in vitro* assay [11]. Perhaps Ubc5 does not interact with the acceptor ubiquitin molecule or only forms promiscuous interactions with it. Consequently, multiple lysine residues in the acceptor ubiquitin may all have the opportunity to be ligated with the donor. Since proteins carrying forked ubiquitin chains are not favored substrates of the proteasome [11], it is possible that auxiliary factors may exist in cells to prevent the formation of such non-productive ubiquitin signals.

Perspective

Standing in sharp contrast to our understanding of the functional repertoire of polyubiquitination, our knowledge of the molecular basis of linkage specification and recognition is scarce. In the past few years, many scientists have started to use a combination of structural and biochemical tools to address these open questions. As a result, some fundamental principles are beginning to emerge. However, to obtain a thorough mechanistic view on polyubiquitination, more structural work would be required to elucidate the relative geometry of the components in an enzyme/substrate complex that recapitulates the transition state of a ubiquitin ligation reaction. We also need to analyze more polyubiquitination reactions using well-defined *in vitro* biochemical assays. An important question is how general the above described principles are. Given the large number of E2 and E3 enzymes that have been utilized by nature to accommodate the diverse functional needs of cells, complexity is something that an ubiquitin biologist cannot avoid. We can confidently predict that new concepts will emerge as we attempt to extend findings learned from one ubiquitin conjugating system to another.

Acknowledgements. We thank M. Krause for critical reading of the manuscript and apologize for not being able to cite all the papers relevant to this topic due to space constraints. Our research is supported by an intramural research program of the NIDDK at the National Institutes of Health.

1 Welchman, R.L., Gordon, C. and Mayer, R.J. (2005) Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat. Rev. Mol. Cell Biol 6, 599–609.
2 Kerscher, O., Felberbaum, R. and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu. Rev. Cell. Dev. Biol. 22, 159–180.
3 Cadwell, K. and Coscoy, L. (2005) Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. Science 309, 127–130.
4 Wang, X., Herr, R.A., Chua, W.J., Lybarger, L., Wiertz, E.J. and Hansen, T.H. (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. J. Cell Biol. 177, 613–624.
5 Williams, C., van den Berg, M., Sprenger, R.R. and Distel, B. (2007) A conserved cysteine is essential for Pex4p-dependent ubiquitination of the peroxisomal import receptor Pex5p. J. Biol. Chem. 282, 22534–22543.
6 Tait, S.W., de Vries, E., Maas, C., Keller, A.M., D'Santos, C.S. and Borst, J. (2007) Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its N-terminal fragment. J. Cell Biol. 179, 1453–1466.
7 Weissman, A.M. (2001) Themes and variations on ubiquitylation. Nat. Rev. Mol. Cell Biol. 2, 169–178.
8 Pickart, C.M. (2001) Mechanisms underlying ubiquitination. Annu. Rev. Biochem. 70, 503–533.
9 Peng, J., Schwartz, D., Elias, J.E., Thoreen, C.C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S.P. (2003) A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. 21, 921–926.
10 Ben-Saadon, R., Zaaroor, D., Ziv, T. and Ciechanover, A. (2006) The polycomb protein Ring1B generates self atypical mixed ubiquitin chains required for its in vitro histone H2A ligase activity. Mol. Cell 24, 701–711.
11 Kim, H.T., Kim, K.P., Lledias, F., Kisselev, A.F., Scaglione, K.M., Skowyra, D., Gygi, S.P. and Goldberg, A.L. (2007) Certain pairs of ubiquitin-conjugating enzymes (E2 s) and ubiquitin-protein ligases (E3 s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. J. Biol. Chem. 282, 17375–17386.
12 Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K. and Iwai, K. (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J. 25, 4877–4887.
13 Jin, J., Li, X., Gygi, S.P. and Harper, J.W. (2007) Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. Nature 447, 1135–1138.
14 Chiu, Y.H., Sun, Q. and Chen, Z.J. (2007) E1-L2 activates both ubiquitin and FAT10. Mol. Cell 27, 1014–1023.
15 Hoppe, T. (2005) Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. Trends Biochem. Sci. 30, 183–187.
16 Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U. and Jentsch, S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell 96, 635–644.
17 Scheffner, M., Huibregtse, J.M., Vierstra, R.D. and Howley, P.M. (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75, 495–505.
18 Hatakeyama, S. and Nakayama, K.I. (2003) U-box proteins as a new family of ubiquitin ligases. Biochem. Biophys. Res. Commun. 302, 635–645.
19 Fang, S. and Weissman, A.M. (2004) A field guide to ubiquitylation. Cell. Mol. Life Sci. 61, 1546–1561.
20 Aravind, L. and Koonin, E.V. (2000) The U box is a modified RING finger – a common domain in ubiquitination. Curr. Biol. 10, R132–134.
21 Tu, D., Li, W., Ye, Y. and Brunger, A.T. (2007) Structure and function of the yeast U-box-containing ubiquitin ligase Ufd2p. Proc. Natl. Acad. Sci. USA. 104, 15599–15606.
22 Pickart, C.M. and Eddins, M.J. (2004) Ubiquitin: structures, functions, mechanisms. Biochim. Biophys. Acta. 1695, 55–72.
23 Hochstrasser, M. (2004) Ubiquitin signalling: what's in a chain? Nat. Cell Biol. 6, 571–572.

24 Ulrich, H.D. (2004) How to activate a damage-tolerant polymerase: consequences of PCNA modifications by ubiquitin and SUMO. Cell Cycle 3, 15–18.
25 Pickart, C.M. and Fushman, D. (2004) Polyubiquitin chains: polymeric protein signals. Curr. Opin. Chem. Biol. 8, 610–616.
26 Chen, Z.J. (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat. Cell Biol. 7, 758–765.
27 Kim, I. and Rao, H. (2006) What's Ub chain linkage got to do with it? Sci. STKE 2006, pe18.
28 Mukhopadhyay, D. and Riezman, H. (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 315, 201–205.
29 Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K. and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 243, 1576–1583.
30 Thrower, J.S., Hoffman, L., Rechsteiner, M. and Pickart, C.M. (2000) Recognition of the polyubiquitin proteolytic signal. EMBO J. 19, 94–102.
31 Kaiser, P., Flick, K., Wittenberg, C. and Reed, S.I. (2000) Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. Cell 102, 303–314.
32 Kuras, L., Rouillon, A., Lee, T., Barbey, R., Tyers, M. and Thomas, D. (2002) Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. Mol. Cell 10, 69–80.
33 Flick, K., Raasi, S., Zhang, H., Yen, J.L. and Kaiser, P. (2006) A ubiquitin-interacting motif protects polyubiquitinated Met4 from degradation by the 26S proteasome. Nat. Cell Biol. 8, 509–515.
34 Ye, Y. (2006) Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase. J. Struct. Biol.156, 29–40.
35 Jentsch, S. and Rumpf, S. (2007) Cdc48 (p97): a 'molecular gearbox' in the ubiquitin pathway? Trends Biochem. Sci. 32, 6–11.
36 Bays, N.W., Wilhovsky, S.K., Goradia, A., Hodgkiss-Harlow, K. and Hampton, R.Y. (2001) HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Mol. Biol. Cell 12, 4114–4128.
37 Ye, Y., Meyer, H.H. and Rapoport, T.A. (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414, 652–656.
38 Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D.H. and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat. Cell Biol. 4, 134–139.
39 Braun, S., Matuschewski, K., Rape, M., Thoms, S. and Jentsch, S. (2002) Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J. 21, 615–621.
40 Rabinovich, E., Kerem, A., Frohlich, K.U., Diamant, N. and Bar-Nun, S. (2002) AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol. Cell. Biol. 22, 626–634.
41 Ye, Y., Meyer, H.H. and Rapoport, T.A. (2003) Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. J. Cell Biol. 162, 71–84.
42 Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H. and Jentsch, S. (2001) Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. Cell 107, 667–677.
43 Ramadan, K., Bruderer, R., Spiga, F.M., Popp, O., Baur, T., Gotta, M. and Meyer, H.H. (2007) Cdc48/p97 promotes reformation of the nucleus by extracting the kinase Aurora B from chromatin. Nature 450, 1258–1262.
44 Babu, J.R., Geetha, T. and Wooten, M.W. (2005) Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation. J. Neurochem. 94, 192–203.
45 Reggiori, F. and Klionsky, D.J. (2005) Autophagosomes: biogenesis from scratch? Curr. Opin. Cell Biol. 17, 415–422.
46 Wooten, M.W., Geetha, T., Seibenhener, M.L., Babu, J.R., Diaz-Meco, M.T. and Moscat, J. (2005) The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. J. Biol. Chem. 280, 35625–35629.
47 Cavey, J.R., Ralston, S.H., Hocking, L.J., Sheppard, P.W., Ciani, B., Searle, M.S. and Layfield, R. (2005) Loss of ubiquitin-binding associated with Paget's disease of bone p62 (SQSTM1) mutations. J. Bone Miner. Res. 20, 619–624.
48 Raasi, S., Varadan, R., Fushman, D. and Pickart, C.M. (2005) Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nat. Struct. Mol. Biol. 12, 708–714.
49 Seibenhener, M.L., Babu, J.R., Geetha, T., Wong, H.C., Krishna, N.R. and Wooten, M.W. (2004) Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol. Cell. Biol. 24, 8055–8068.
50 Lelouard, H., Ferrand, V., Marguet, D., Bania, J., Camosseto, V., David, A., Gatti, E. and Pierre, P. (2004) Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. J. Cell Biol. 164, 667–675.
51 Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G. and Johansen, T. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. 282, 24131–24145.
52 Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J.I., Ezaki, J., Murata, S., Hamazaki, J., Nishito, Y., Iemura, S.I., Natsume, T., Yanagawa, T., Uwayama, J., Warabi, E., Yoshida, H., Ishii, T., Kobayashi, A., Yamamoto, M., Yue, Z., Uchiyama, Y., Kominami, E. and Tanaka, K. (2007) Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131, 1149–1163.
53 Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H. and Johansen, T. (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. 171, 603–614.
54 Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E. and Tanaka, K. (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441, 880–884.
55 Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H. and Mizushima, N. (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441, 885–889.
56 Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998) Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. 143, 1883–1898.
57 Olzmann, J.A., Li, L., Chudaev, M.V., Chen, J., Perez, F.A., Palmiter, R.D. and Chin, L.S. (2007) Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. J. Cell Biol. 178, 1025–1038.
58 Kawaguchi, Y., Kovacs, J.J., McLaurin, A., Vance, J.M., Ito, A. and Yao, T.P. (2003) The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 115, 727–738.
59 Heyninck, K. and Beyaert, R. (2005) A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. Trends Biochem. Sci. 30, 1–4.
60 Chastagner, P., Israel, A. and Brou, C. (2006) Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. EMBO Rep. 7, 1147–1153.
61 Al-Hakim, A.K., Zagorska, A., Chapman, L., Deak, M., Peggie, M. and Alessi, D.R. (2008) Control of AMPK-related kinases by USP9X and atypical Lys29/Lys33-linked polyubiquitin chains. Biochem. J.

62 Nishikawa, H., Ooka, S., Sato, K., Arima, K., Okamoto, J., Klevit, R.E., Fukuda, M. and Ohta, T. (2004) Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. J. Biol. Chem. 279, 3916–3924.
63 Hicke, L., Schubert, H.L. and Hill, C.P. (2005) Ubiquitin-binding domains. Nat. Rev. Mol. Cell. Biol. 6, 610–621.
64 Kim, I., Mi, K. and Rao, H. (2004) Multiple interactions of rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. Mol. Biol. Cell 15, 3357–3365.
65 Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C. and Fushman, D. (2004) Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J. Biol. Chem. 279, 7055–7063.
66 Tenno, T., Fujiwara, K., Tochio, H., Iwai, K., Morita, E.H., Hayashi, H., Murata, S., Hiroaki, H., Sato, M., Tanaka, K. and Shirakawa, M. (2004) Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains. Genes Cells 9, 865–875.
67 Ryabov, Y.E. and Fushman, D. (2007) A model of interdomain mobility in a multidomain protein. J. Am. Chem. Soc. 129, 3315–3327.
68 Kang, R.S., Daniels, C.M., Francis, S.A., Shih, S.C., Salerno, W.J., Hicke, L. and Radhakrishnan, I. (2003) Solution structure of a CUE-ubiquitin complex reveals a conserved mode of ubiquitin binding. Cell 113, 621–630.
69 Mueller, T.D., Kamionka, M. and Feigon, J. (2004) Specificity of the interaction between ubiquitin-associated domains and ubiquitin. J. Biol. Chem. 279, 11926–11936.
70 Varadan, R., Assfalg, M., Raasi, S., Pickart, C. and Fushman, D. (2005) Structural determinants for selective recognition of a Lys48-linked polyubiquitin chain by a UBA domain. Mol. Cell 18, 687–698.
71 Cook, W.J., Jeffrey, L.C., Carson, M., Chen, Z. and Pickart, C.M. (1992) Structure of a diubiquitin conjugate and a model for interaction with ubiquitin conjugating enzyme (E2). J. Biol. Chem. 267, 16467–16471.
72 Cook, W.J., Jeffrey, L.C., Kasperek, E. and Pickart, C.M. (1994) Structure of tetraubiquitin shows how multiubiquitin chains can be formed. J. Mol. Biol. 236, 601–609.
73 Phillips, C.L., Thrower, J., Pickart, C.M. and Hill, C.P. (2001) Structure of a new crystal form of tetraubiquitin. Acta. Crystallogr. D Biol. Crystallogr. 57, 341–344.
74 Eddins, M.J., Varadan, R., Fushman, D., Pickart, C.M. and Wolberger, C. (2007) Crystal structure and solution NMR studies of Lys48-linked tetraubiquitin at neutral pH. J. Mol. Biol. 367, 204–211.
75 Varadan, R., Walker, O., Pickart, C. and Fushman, D. (2002) Structural properties of polyubiquitin chains in solution. J. Mol. Biol. 324, 637–647.
76 VanDemark, A.P., Hofmann, R.M., Tsui, C., Pickart, C.M. and Wolberger, C. (2001) Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer. Cell 105, 711–720.
77 McKenna, S., Moraes, T., Pastushok, L., Ptak, C., Xiao, W., Spyracopoulos, L. and Ellison, M.J. (2003) An NMR-based model of the ubiquitin-bound human ubiquitin conjugation complex Mms2.Ubc13: the structural basis for lysine 63 chain catalysis. J. Biol. Chem. 278, 13151–13158.
78 Eddins, M.J., Carlile, C.M., Gomez, K.M., Pickart, C.M. and Wolberger, C. (2006) Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. Nat. Struct. Mol. Biol. 13, 915–920.
79 Wang, M., Cheng, D., Peng, J. and Pickart, C.M. (2006) Molecular determinants of polyubiquitin linkage selection by an HECT ubiquitin ligase. EMBO J. 25, 1710–1719.
80 Petroski, M.D. and Deshaies, R.J. (2005) Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. Cell 123, 1107–1120.
81 Li, W., Tu, D., Brunger, A.T. and Ye, Y. (2007) A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. Nature 446, 333–337.
82 Ravid, T. and Hochstrasser, M. (2007) Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. Nat. Cell Biol. 9, 422–427.