Non-canonical ubiquitin-based signals for proteasomal degradation

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Summary

Regulated cellular proteolysis is mediated largely by the ubiquitin–proteasome system (UPS). It is a highly specific process that is time- (e.g. cell cycle), compartment- (e.g. nucleus or endoplasmic reticulum) and substrate quality- (e.g. denatured or misfolded proteins) dependent, and allows fast adaptation to changing conditions. Degradation by the UPS is carried out through two successive steps: the substrate is covalently tagged with ubiquitin and subsequently degraded by the 26S proteasome. The accepted 'canonical' signal for proteasomal recognition is a polyubiquitin chain that is anchored to a lysine residue in the target substrate, and is assembled through isopeptide bonds involving lysine 48 of ubiquitin. However, several 'non-canonical' ubiquitin-based signals for proteasomal targeting have also been identified. These include chains anchored to residues other than internal lysine in the substrates, chains assembled through linking residues other than lysine 48 in ubiquitin, and mixed chains made of both ubiquitin and a ubiquitin-like protein. Furthermore, some proteins can be degraded following modification by a single ubiquitin (monoubiquitylation) or multiple single ubiquitins (multiple monoubiquitylation). Finally, some proteins can be proteasomally degraded without prior ubiquitylation (the process is also often referred to as ubiquitination). In this Commentary, we describe these recent findings and discuss the possible physiological roles of these diverse signals. Furthermore, we discuss the possible impact of this signal diversity on drug development.

This article is part of a Minifocus on Ubiquitin. For further reading, please see related articles: 'Ubiquitin and SUMO in DNA repair at a glance' by Helle D. Ulrich (*J. Cell Sci.* **125**, 249-254). 'Emerging regulatory mechanisms in ubiquitin-dependent cell cycle control' by Annamaria Mocciaro and Michael Rape (*J. Cell Sci.* **125**, 255-263). 'The role of ubiquitylation in receptor endocytosis and endosomal sorting' by Kaisa Haglund and Ivan Dikic (*J. Cell Sci.* **125**, 265-275). 'Cellular functions of the DUBs' by Michael J. Clague et al. (*J. Cell Sci.* **125**, 277-286). 'HECT and RING finger families of E3 ubiquitin ligases at a glance' by Meredith B. Metzger et al. (*J. Cell Sci.* **125**, 531-537). 'No one can whistle a symphony alone – how different ubiquitin linkages cooperate to orchestrate NF-κB activity' by Anna C. Schmukle and Henning Walczak (*J. Cell Sci.* **125**, 549-559).

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Introduction

Ubiquitylation [also known as ubiquitination, as coined by the discoverers of this modification with regard to its connection to proteolysis (Wilkinson, 2005)] is a three-step enzymatic reaction that is carried out by several enzymes: the ubiquitin-activating enzyme (E1), a ubiquitin carrier protein (E2; also known as ubiquitin-conjugating enzyme, UBC) and a ubiquitin-protein ligase (E3). An additional component of the ubiquitylation machinery has been described. This E4 enzyme is involved in elongation of short ubiquitin chains (Koegl et al., 1999). However, the requirement for an E4 activity appears to be limited to a small subset of substrates. Ubiquitylation-dependent proteasomal degradation is involved in the regulation of numerous cellular processes, including cell cycle progression, apoptosis, DNA repair, the maintenance of cellular quality control, autophagy, the regulation of transcription and receptor-mediated endocytosis (Mayer et al., 2005; Mayer et al., 2006; Mayer et al., 2008). In general, modification by ubiquitin serves as a recognition element in trans, whereby different downstream effectors bind to the ubiquitin-modified protein to affect its fate and/or function. In the case of proteasomal degradation, the ubiquitylated protein is recognized by the 26S proteasome and subsequently degraded (Dikic et al., 2009; Su and Lau, 2009).

The widely accepted canonical signal for proteasomal degradation is a polyubiquitin chain that is anchored to the ε-NH_2 group of a lysine residue(s) in the substrate by an isopeptide bond and is assembled through the formation of isopeptide bonds between the C-terminal residue of one ubiquitin moiety (glycine 76) and lysine 48 of the previously conjugated ubiquitin moiety (Chau et al., 1989). Recent studies have reported, however, that other types of ubiquitin chains can also be recognized by the proteasome (Figs 1, 2). These include an ester-based linkage that connects ubiquitin to a threonine or serine residue in the substrate, and a thiolester-based linkage whereby ubiquitin is bound to a cysteine residue in the substrate (McDowell et al., 2010; Tait et al., 2007; Vosper et al., 2009). Ubiquitin can also be conjugated to the α-NH_2 group of the N-terminal residue of the substrate. Instead of using lysine 48 for the linkage, polyubiquitin chains can also be assembled through one of the six additional lysine residues in the molecule. Such homogenous chains based on, for example, lysine 63 (Saeki et al., 2009), or heterogeneous chains in which different ubiquitin–ubiquitin linkages are found, have also been reported to target proteins for proteasomal degradation. Linear chains, in which the ubiquitin links are attached to one another 'head-to-tail', and heterologous chains, in which the links are made of ubiquitin and a ubiquitin-like protein, such as small ubiquitin-like modifier (SUMO), have additionally been shown to target proteins for proteasomal degradation. Surprisingly, it has been demonstrated that the proteasome does not necessarily have to recognize a polyubiquitin chain or tetraubiquitin, which had been described previously as the minimal proteasomal targeting signal (Thrower et al., 2000). The proteasome can also recognize proteins that are modified by a single ubiquitin moiety (monoubiquitylation) or multiple single moieties (multiple monoubiquitylation). Finally, a few exceptional

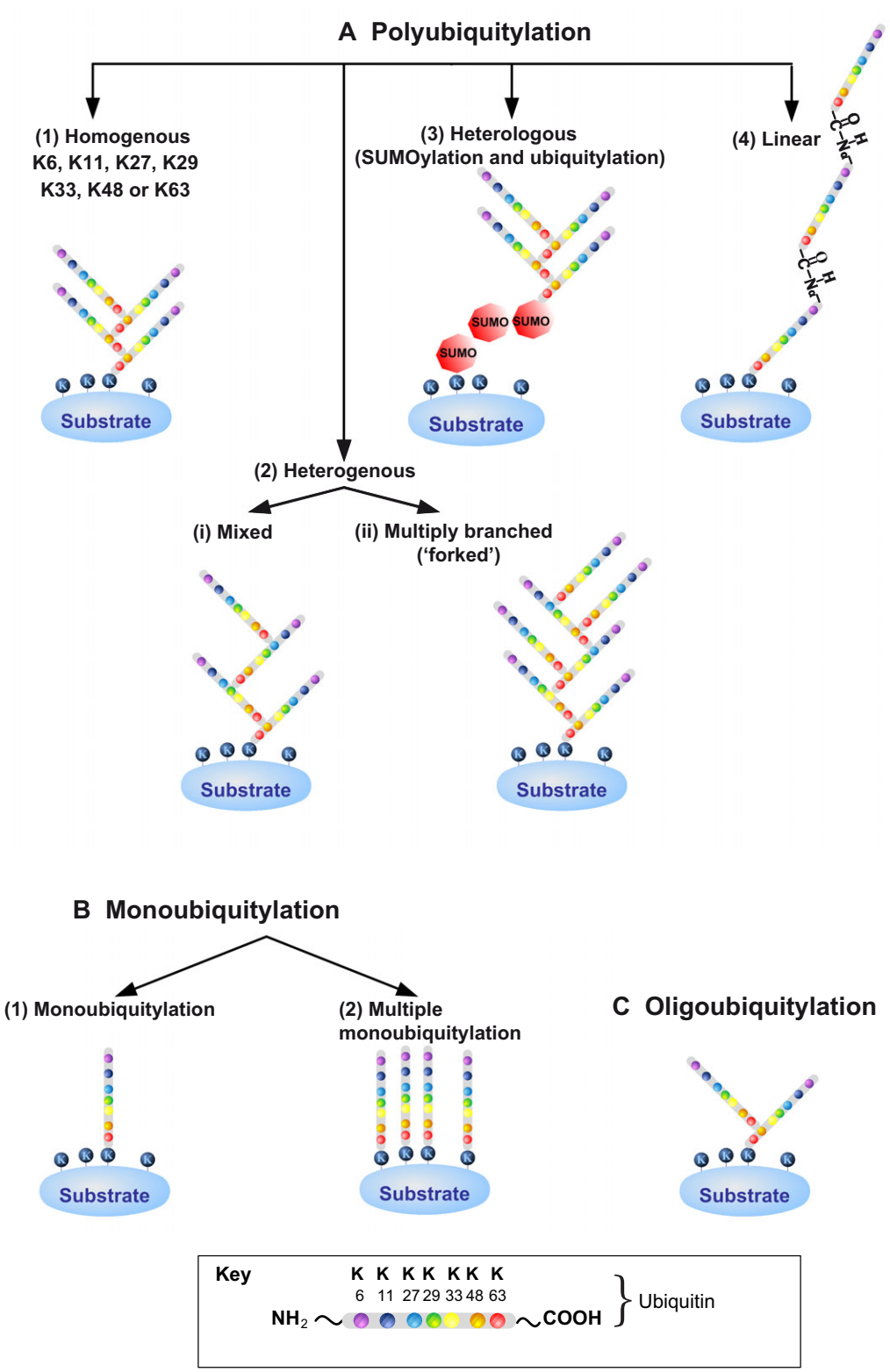

Fig. 1. Different types of ubiquitin chains. (**A**) Polyubiquitylation. Homogeneous chains, namely chains based on linkages involving lysines 6, 11, 27, 29, 33, 48 or 63 (1); heterogeneous chains (2): mixed chains based on linkages involving different lysines (i) and multiply branched (or forked) chains in which several ubiquitin moieties are anchored to distinct lysine residues in a single ubiquitin moiety (ii); heterologous chains are made of both ubiquitin and a ubiquitin-like protein (3); linear chains in which the ubiquitin moieties are linked 'head-to-tail' (the C-terminal residue of the distal moiety is linked to the N-terminal residue of the proximal one) (4). (**B**) Monoubiquitylation representing modification of a protein by: a single ubiquitin (1) or several single ubiquitins (2). (**C**) Oligoubiquitylation, namely the modification of a protein by short ubiquitin chains. Inset: the seven internal lysines of ubiquitin; K, lysine.

cases have been reported, where the proteasome can degrade proteins that have not been modified by ubiquitin at all.

In addition to the different types of ubiquitin modifications described above, recent evidence supports the idea that there are signals that consist of multiply branched ('forked') chains where two (or perhaps even more than two) ubiquitin molecules are linked to a single ubiquitin moiety. In contrast with those chains that target proteins for proteasomal degradation, these chains cannot be efficiently processed by the 26S complex (Kim et al., 2007) and serve non-proteolytic functions (Ben-Saadon et al., 2006).

The broad diversity of ubiquitin-based signals suggests a high level of specificity and selectivity in proteasomal recognition and degradation of proteins. However, our knowledge of the formation

and the biological significance of the variety of these non-canonical proteasomal signals is scarce. It is still unclear which features within the substrate and/or E3 ligase are important for the generation of a specific ubiquitin signal, and whether the existence of numerous signals represents diversity among the acceptor proteins that shuttle substrates to the proteasome and within the proteasome complex itself.

In this Commentary, we describe the evolving repertoire of non-canonical ubiquitin-based proteasomal signals, and review the mechanism of proteasomal degradation of substrates that are degraded in a ubiquitin-independent manner. In particular, we discuss the possible mechanisms that govern the diversity of signals for proteasomal targeting. Importantly, aberrations in the ubiquitin system underlie the pathogenesis of numerous diseases, such as certain forms of neurodegeneration, inflammatory disorders and malignancies. Consequently, the system has served as a platform for novel mechanism-based drug development, including one successful drug that is already in widespread use to combat multiple myeloma (Velcade©). Therefore, one can envision that the broad diversity of proteolytic signals will serve as an even broader platform for the development of specific drugs in the future.

Ubiquitin-dependent degradation

The diversity of the proteasomal substrates along with that of their targeting signals underlies the versatility and complexity of the UPS. Here, we will describe recent findings on the proteasomal degradation of substrates harboring a variety of non-canonical ubiquitin signals that do not involve lysine-48-based chains.

Ubiquitylation of substrates on lysine residues

Homogenous polyubiquitin chains

The ubiquitin molecule contains seven lysine residues in positions 6, 11, 27, 29, 33, 48 and 63. Studies in yeast have shown that, under certain conditions, any of them can be involved in formation of homogeneous polyubiquitin chains (Peng et al., 2003; Xu et al., 2009; Ziv et al., 2011). Complementing studies in both yeast and mammalian cells have shown that the chains based on linking ubiquitin residues through six out of the seven lysines (namely all but lysine 63) can target proteins for degradation (Bedford et al., 2011; Xu et al., 2009). Corroborating these data, a mass spectrometry analysis has shown that, although the abundance of the different linkages varies, homogeneous polyubiquitin chains based on linkages involving lysines 6, 11, 27, 29 and 48 can all mediate proteasomal degradation (Dammer et al., 2011).

The analysis of ubiquitin chains that target specific substrates for proteasomal degradation has revealed that the E3 ligase C-terminus of HSP70-interacting protein (CHIP) synthesizes polyubiquitin chains that are linked through lysines 6, 11, 48 or 63 on its targets, the molecular chaperones heat-shock proteins HSP70 and HSP90 (Kundrat and Regan, 2010). Studies carried out by other research groups have confirmed the involvement of lysine-63-based chains in targeting proteins for proteasomal degradation. For example, it has been shown in vitro that the modification of the model substrate ubiquitin-dihydrofolate reductase (DHFR) by lysine-63-linked tetraubiquitin chains bound to lysine 48 of the fused ubiquitin moiety, results in its proteasomal degradation (Hofmann and Pickart, 2001). In a cell-free system, troponin I has been shown to be targeted for proteasomal degradation by the muscle-specific ubiquitin RING finger protein 1 (MuRF1, also known as TRIM63) ligase, which, depending on which E2 is used (UBCH1, also known as E2-25kDa, or heterodimeric UBCH13–

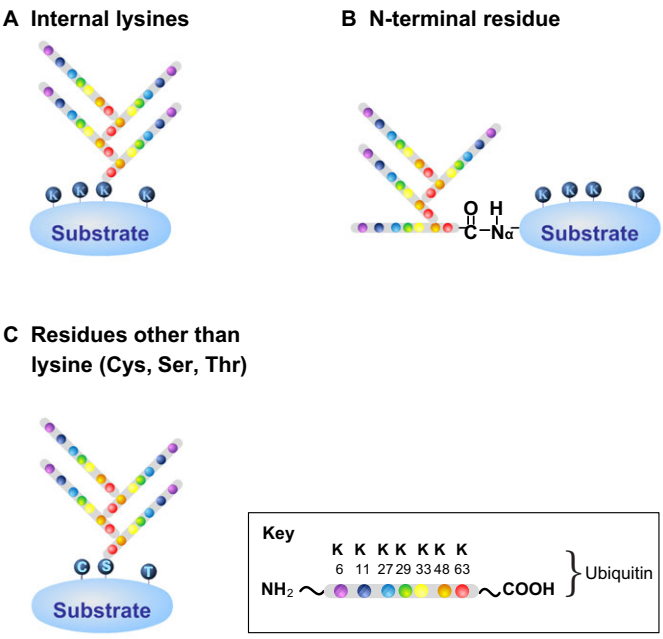

Fig. 2. Different ubiquitylation sites on target substrates. (**A**) Internal lysines. An isopeptide bond is generated between the C-terminal glycine 76 of ubiquitin and an ε-NH_2 group of a lysine residue in the substrate. (**B**) N-terminal residue. A linear peptide bond is created between the C-terminal glycine 76 of ubiquitin and the α-NH_2 group of the N-terminal residue. (**C**) Residues other than lysine (cysteine, serine, threonine). An ester bond is created between the C-terminal glycine 76 of ubiquitin and a serine or a threonine residue in the substrate. A thiolester bond is created between the C-terminal glycine 76 of ubiquitin and a cysteine residue. Cys (or C) denotes cysteine, Ser (or S) denotes serine, and Thr (or T) denotes threonine.

UEV1A, respectively), synthesizes lysine-48- or lysine-63-based ubiquitin chains (Kim et al., 2007). The Rsp5 ubiquitin ligase ubiquitylates the ER membrane-anchored transcription factor Mga2, thereby generating chains that are highly rich in lysine 63 linkages (Saeki et al., 2009). These chains target the substrate for proteasomal processing. Interestingly, lysine-63-linked chains have been detected in various proteasome-bound polyubiquitylated proteins, suggesting that they have a contribution to proteasomal recognition (Saeki et al., 2009). It should be taken into consideration, however, that most of these experiments were carried out using purified components in cell-free assays, and the involvement of such chains in cellular proteasomal degradation is not clear. Furthermore, it should be noted that in both yeast and mammalian cells, lysine-63-based chains have been shown to target mainly membrane proteins for degradation in the vacuole and lysosome, respectively (Lauwers et al., 2010). It appears that these chains are highly specific and cannot be replaced by chains with other linkages. In addition, lysine-63-based chains have important roles in intracellular signaling where the modifications serve non-proteolytic purposes (Chen and Sun, 2009).

The anaphase-promoting complex/cyclosome (APC/C) is an E3 ligase that coordinates progression through the cell cycle by modifying a variety of cell cycle regulators. Several studies have shown that lysine-11-linked chains are crucial regulators of mitotic protein degradation (Jin et al., 2008; Kirkpatrick et al., 2006; Matsumoto et al., 2010), and that this type of ubiquitin modification is upregulated in mitotic human cells (Matsumoto et al., 2010).

Among the substrates that are targeted by APC/C and modified by lysine-11-linked chains are proteins that are required for spindle assembly, such as BRCA1-associated RING domain protein 1 (BARD1), hyaluronan-mediated motility receptor (HMMR), hepatoma upregulated protein (HURP, also known as DLGP5), and nucleolar and spindle-associated protein (NUSAP1) (Song and Rape, 2010). Two E2s are required for APC/C to assemble these specific lysine-11-linked chains: the ubiquitin chain initiator UBCH10 (also known as UBE2C) that primes formation of the chain, and the chain elongator ubiquitin-conjugating enzyme E2S (UBE2S) (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). The formation of these chains depends on the TEK box motif on the ubiquitin surface. Homologous TEK boxes were also found on APC/C substrates. It is therefore possible that recognition of the TEK box on both ubiquitin and the substrate enables APC/C to efficiently synthesize the lysine-11-based chains (Jin et al., 2008). The observation that lysine-11-linked chains are able to compete with lysine 48 chains for binding to the S5 subunit of the proteasome (Baboshina and Haas, 1996), further strengthens the notion that the proteasome has the ability to recognize these chains. In addition to the substrates modified by APC/C, the E2–E3 protein complex comprising the ubiquitin-conjugating enzyme H5a (UBCH5a) and the ligase seven in absentia homolog 1 (SIAH1) can assemble lysine-11-linked ubiquitin chains on β-catenin and thereby cause its proteasomal degradation (Dimitrova et al., 2010).

Taken together, it appears that chains based on all lysine residues in ubiquitin can target substrates for proteasomal degradation. However, one should be cautious in drawing such a broad conclusion, as some of the experiments were carried out in cell-free systems, using purified recombinant components that might not be in physiological concentrations, and might therefore not faithfully represent cellular events. In addition, the involvement of polyubiquitin chains based on lysines 6, 27, 29 and 33 in the degradation of specific cellular proteins has not yet been directly demonstrated, and the conclusion that they are involved in proteasomal degradation is based mostly on mass spectrometry analysis of entire cellular proteomes under non-perturbed and perturbed (i.e. following addition of the proteasome inhibitor MG132) conditions (Xu et al., 2009). Furthermore, cells contain numerous ubiquitin ligases, and the chains that they generate are not necessarily homogenous and might contain more than one type of lysine-based linkage (Fig. 1). The probable presence of mixed ubiquitin chains makes it more difficult to evaluate the precise roles of individual non-canonical homogeneous linkages in targeting substrates for degradation. As a matter of fact, one can argue that a ‘critical mass’ of a certain linkage, for example a segment of four ubiquitins linked through lysine 48, can be sufficient for targeting the substrate for degradation, whereas the other linkages contribute very little, or not at all, to the proteolytic process and were generated spuriously by the primary or secondary ligases. These arguments suggest the possibility of non-specific creation of linkages and indicate that further experiments are required to elucidate the precise relevance of these protein modifications for proteasomal degradation in vivo.

Overall, accumulating experimental evidence suggests that all lysine residues in ubiquitin can be involved in the formation of chains targeting proteins for proteasomal degradation, although the experimental evidence is stronger for certain linkages and weaker for others. It is also difficult to state with certainty that the chains in cells are indeed homogenous. When assuming that different linkages have a role in cellular proteolysis, it should be noted that such chains are similar to lysine-48-based chains in the sense that they are assembled through a single linkage type. Nonetheless, they are clearly different from one another structurally, and therefore might be recognized by different shuttle proteins and proteasomal subunits, or serve to modulate the degradation rate of their target substrates.

Heterogeneous ubiquitin chains

For a long time, it was assumed that polyubiquitin chains are homogeneous, and all moieties are attached to each other through the same internal lysine residue. However, mixed chains, where different moieties are bound through different lysine residues have been identified, mostly through mass spectrometry analysis of cellular and cell-free adducts (e.g. Kirkpatrick et al., 2006). Moreover, multiply branched (or forked) chains in which two (or perhaps even more) ubiquitin moieties are anchored to distinct lysine residues in a single moiety have been described (Ben-Saadon et al., 2006; Kim et al., 2007). In some cases, it has been shown that the ubiquitin moieties are linked to neighboring lysine residues, e.g. lysines 6 and 11, 27 and 29, or 29 and 33 (Kim et al., 2007). However, it should be taken into account that these results are based on mass spectrometry, which involves treatment of samples with trypsin. Therefore, if two ubiquitin moieties were linked to nonadjacent lysines of the proximal ubiquitin, they will be separated, and only the neighboring forks will remain intact and will therefore be detected. In another study, the ubiquitin moieties were shown to be linked to more distant residues (lysines 6, 27 and 48) (Ben-Saadon et al., 2006). Here, the researchers carried out in vitro ubiquitylation assays using different ubiquitin species with point mutations of different internal lysines.

In contrast with branched homogeneous ubiquitin chains, forked chains (Fig. 1) bind to the proteasome substantially less efficiently, and substrates tagged with these chains are degraded more slowly (Kim et al., 2007; Kim et al., 2009). It appears that the function of forked chains in degradation is negligible. In agreement with this, forked chains formed on RING1B (also known as RING2), the ligase component of the polycomb repressive complex 1 (PRC1, also known as PCGF1), serve a non-proteolytic function and instead stimulate the monoubiquitylating ligase activity of RING1B towards its substrate, histone H2A (Ben-Saadon et al., 2006).

Furthermore, APC/C along with UBCH10 monoubiquitylates multiple lysine residues in cyclin B1. These ubiquitin moieties are then converted to mixed polyubiquitin chains (Fig. 1) that are enriched with linkages based on lysine 11, 48 and 63. The elongation reaction is catalyzed by APC/C along with a different E2, UBC4 (also known as UBE2D2). These chains are recognized by the proteasomal ubiquitin receptors, but unlike the forked chains, they target the tagged proteins for degradation (Kirkpatrick et al., 2006).

Taken together, it appears that heterogeneous chains, in which single ubiquitin moieties are linked to one another through different internal lysine residues, are more common than previously thought. Although the physiological significance of this heterogeneity is still elusive, it is clear that forked chains do not target substrates for degradation and instead carry out non-proteolytic functions.

Heterologous chains between ubiquitin and SUMO

SUMO is another small molecule that can also be conjugated to lysine residues of proteins. In certain cases, it forms polymeric chains similar to those formed by ubiquitin. Protein modification with SUMO is involved in many cellular processes, including signal transduction, DNA repair, stress response and targeting of proteins to their subcellular destination (reviewed by Ulrich, 2009).

A crosstalk between SUMOylation and ubiquitylation has been unraveled recently. In one study, it was shown that the conjugates of SUMO2 co-purify with ubiquitin conjugates, and that SUMO2 and/or SUMO3 conjugates accumulate in cells treated with the proteasomal inhibitor MG132. These findings suggest that SUMOylated proteins are also ubiquitylated and targeted for proteasomal degradation (Schimmel et al., 2008). Indeed, it has been shown that proteins that are singly or multiply monoSUMOylated (by SUMO2 or SUMO3) were subsequently polyubiquitylated and degraded by the proteasome, although these experiments did not show that SUMO and ubiquitin are linked to one another in the same chain. Finally, the existence of mixed SUMO–ubiquitin chains could be demonstrated experimentally, but their relevance for proteasomal degradation could not be determined (Schimmel et al., 2008). A different study demonstrated the accumulation of SUMO1-containing conjugates following proteasome inhibition (Matafora et al., 2009). A specific example of heterologous modification of a substrate leading to proteasomal degradation is the promyelocytic leukemia (PML) protein, which is initially polySUMOylated. The SUMO chains then recruit the ubiquitin ligase RING finger protein 4 (RNF4) (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008), which elongates the SUMO chains by adding ubiquitin moieties to them. This, in turn, results in proteasomal degradation of PML (Tatham et al., 2008).

Overall, it appears that proteins that are SUMOylated for various non-proteolytic functions have to be ubiquitylated in order to be targeted for degradation. However, it remains unclear whether the ubiquitylation occurs on a SUMO residue or directly on an internal lysine residue of the substrate. Similarly, further studies are required to elucidate whether SUMOylation affects proteasomal recognition, and whether substrates modified by the two types of conjugates can be degraded.

These findings unravel yet another layer in signaling for proteasomal degradation that involves a ubiquitin-like (UBL) modifier. The UBL might not be involved in the proteolytic process per se but might serve to recruit a ligase that subsequently ubiquitylates the substrate protein.

Linear ubiquitin chains

Besides forming polyubiquitin chains, which are based on isopeptide bonds, ubiquitin can also assemble linear chains in which the ubiquitin moieties are linked to one another ‘head-to-tail’. The chains are generated by the linear ubiquitin chain assembly complex (LUBAC) ubiquitin ligase, which comprises three protein subunits, shank-associated RH domain-interacting protein (SHARPIN), longer isoform of heme-oxidized iron-regulatory protein 2 ubiquitin ligase-1 (HOIL1L or HOIL1) and HOIL1L interacting protein (HOIP, also known as RNF31 and ZIBRA) (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). LUBAC has been shown to promote the degradation of ubiquitin–GFP in cells, by synthesizing a linear ubiquitin chain that is attached to the initial ubiquitin fused to GFP. This suggests a possible role for linear chains in substrate recognition by the proteasome (Kirisako et al., 2006). The modification of the eukaryotic replication clamp protein PCNA [a reaction which is mediated by the AAA ATPase cell division protein 48 (CDC48), nuclear protein localization protein 4 (NPL4) and ubiquitin fusion degradation 1 (UFD1) complex] with linear tetraubiquitin targets the protein for proteasomal degradation (Zhao and Ulrich, 2010). An additional example of a linear ubiquitin chain promoting proteasomal degradation is provided by the model bacterial protein barstar, which is the inhibitor of the ribonuclease barnase. N-terminally tagging barstar with a tetraubiquitin linear chain leads to its efficient degradation by purified proteasomes (Prakash et al., 2009).

Monoubiquitylation

As mentioned above, it has been a dominant paradigm in the ubiquitin field that the minimal ubiquitin oligomer that is required for recognition by the proteasome is a ubiquitin chain made of four moieties (tetraubiquitin) (Thrower et al., 2000). However, several recent studies have demonstrated that monoubiquitylation or multiple monoubiquitylation can be sufficient for efficiently targeting certain substrates for proteasomal degradation. For instance, monoubiquitylation of paired box 3 (PAX3), an important regulator of muscle differentiation, on a specific lysine residue (437 or 475) by the ubiquitin ligase TAF1 (Boutet et al., 2010), targets this protein for proteasomal degradation (Boutet et al., 2007). Similarly, syndecan 4 (SDC4), a cell adhesion receptor that is required for cell migration, becomes monoubiquitylated in its cytoplasmic domain in a WNT- and DSH-dependent manner and is subsequently degraded by the proteasome (Carvallo et al., 2010). Furthermore, proteasomal processing of the NF-κB precursor p105 to the active subunit p50 requires its modification by several single ubiquitin moieties on a cluster of lysine residues that reside in the C-terminal half of the molecule (Kravtsova-Ivantsiv et al., 2009). In addition, proteasomal degradation of phospholipase D (PLD) depends on multiple monoubiquitylation events (Yin et al., 2010).

These examples illustrate the ability of the proteasome to recognize a variety of signals, including different polyubiquitin chains, single monoubiquitin and a cluster of monoubiquitin moieties, which possibly provides an additional level of specificity in targeting proteins for degradation. In addition to the variety of ubiquitin signals and examples of substrates discussed above, it has been reported that ubiquitin fused to the N-terminal residues of peptides that are longer than 20 residues, targets them for rapid proteasomal degradation independent of further ubiquitylation (Shabek et al., 2009). This finding raises the possibility that proteins of a certain size (i.e. above the minimal length of 20 amino acids) can be degraded following monoubiquitylation.

In a different study it has been shown that the primary association of the target substrate with the proteasome depends on its ubiquitylation and is promoted by ATP binding to the 19S subunits. Tighter binding of the ubiquitylated protein to the proteasome requires a loose domain in this protein and is accompanied by hydrolysis of ATP (Peth et al., 2010). One can speculate that the extent of ubiquitylation is increased gradually with the size of the target substrate. The increase might be necessary in order to generate a high enough affinity between the proteasome and the ubiquitylated substrate to ensure its efficient and processive degradation: a low degree of ubiquitylation on a large substrate can destabilize its association with the proteasome, thereby rendering the proteolytic process inefficient. This ‘affinity hypothesis’ could explain, at least in part, the diversity of the proteasomal signals and would suggest that the proteasome has the highest affinity for a ubiquitin chain and the lowest affinity for a single ubiquitin moiety. It is also possible that multiple monoubiquitin moieties that interact simultaneously with several proteasomal recognition sites increase the affinity of the substrate to the proteasome.

Ubiquitylation of substrates on non-lysine sites

Internal sites of ubiquitylation

In some cases, protein residues other than lysine can act as ubiquitin acceptors. For instance, cysteine, serine and threonine residues can

be modified by ubiquitin, although this requires the formation of different chemical bonds between ubiquitin and these residues than ubiquitylation of lysine.

The BH3 interacting-domain death agonist (BID) is a member of the B-cell lymphoma 2 (BCL2) family of anti–apoptotic proteins that has to be cleaved in order to become active. Following cleavage, the N-terminal fragment is ubiquitylated on serine, threonine and cysteine residues and degraded by the proteasome (Tait et al., 2007). Another example is provided by neurogenin (NGN), a transcription factor that has a central role in regulating neuronal differentiation. It is ubiquitylated on canonical (lysine) and non-canonical (cysteine, serine, threonine and the N-terminal residue) sites, and chains with all types of internal linkages can target the protein for proteasomal degradation (McDowell et al., 2010; Vosper et al., 2009).

The cytoplasmic tail of the major histocompatibility complex I (MHC I) heavy chain (HC) in the endoplasmic reticulum (ER) is ubiquitylated on serine, threonine or lysine residues by the mouse γ-herpes virus E3 ligase mK3. It is subsequently degraded by the proteasome through the ER-associated degradation (ERAD) pathway (Wang et al., 2007).

In this context, it is interesting to mention that cell surface MHC class I molecules are ubiquitylated and targeted for what appears to be lysosomal degradation following ubiquitylation on a single cysteine residue (Cadwell and Coscoy, 2005). The reaction is carried out by the MIR1 viral ligase, probably as part of the viral 'strategy' to inactivate the MHC class I system that would otherwise present the viral peptides generated by the UPS to cytotoxic T cells to ensure that the antigen-presenting cell is eliminated.

In most cases ubiquitylation occurs on lysine residues. The ubiquitylation of non-lysine residues might reflect the ability of cells to circumvent the structural limitations of some proteins, whose lysine residues are not exposed, masked or lacking altogether [see Ben-Saadon et al. for an example (Ben-Saadon et al., 2004)]. It appears that the overall low evolutionary conservation of ubiquitylation sites attests to the vitality and adaptability of the UPS, which evolved, among other reasons, in order to remove foreign, mutated and otherwise denatured and/or misfolded proteins.

N-terminal ubiquitylation

In addition to the other 'non-canonical' types of ubiquitylation, it has been reported that several substrates can be ubiquitylated by fusion of the first ubiquitin to the α-NH_2 group of the N-terminal residue in a linear fashion. This linearly conjugated ubiquitin then serves as a target for polyubiquitylation (Breitschopf et al., 1998).

Myoblast determination protein 1 (MyoD) was the first mammalian protein that was identified as a target for N-terminal ubiquitylation (Breitschopf et al., 1998), and a series of independent experiments lent support to this finding. For example, mutation of all lysine residues of MyoD only affects its degradation slightly both in vivo and in vitro, and ubiquitylated forms of lysine-less MyoD accumulate after inhibition of the proteasome in cells. Furthermore, selective chemical modification of the N-terminus or fusing of a Myc tag to the N-terminal residue, while keeping the lysine residues intact, prevents MyoD degradation. These findings support the notion that MyoD lacking lysine residues can nevertheless be ubiquitylated and degraded by proteasome in an N-terminus-dependent manner.

Examples of other proteins that are degraded through N-terminal ubiquitylation are the Epstein-Barr virus latent membrane protein 1 (LMP1) (Aviel et al., 2000), the inhibitor of DNA binding 2 (ID2) protein (Fajerman et al., 2004), and the cyclin-dependent kinase inhibitors p21 (also known as WAF1 and CDKN1A) (Bloom et al., 2003; Coulombe et al., 2004), p19 (also known as ARF) (Kuo et al., 2004) and p16INK4a (Ben-Saadon et al., 2004; Kuo et al., 2004), the extracellular signal-regulated kinase 3 (ERK3) and cyclin G1 (Li et al., 2009). Similarly, the peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α) is primarily degraded through the nuclear N-terminus-dependent ubiquitin proteasome pathway (Trausch-Azar et al., 2010; Wang et al., 2011; Yang et al., 2009).

In all these cases the evidence for N-terminal ubiquitylation is largely indirect, and is based mostly on the observation that mutated proteins lacking lysine residues can nevertheless be degraded by the proteasome and that, in some cases, ubiquitylation still takes place. The first direct evidence for the attachment of the C-terminal residue of ubiquitin to the N-terminal residue of a target substrate came from studies on the degradation of the human papillomavirus oncoprotein-58 E7 (HPV-58 E7), where a fusion peptide representing the two parts of the ubiquitylated protein was identified by mass spectrometry (Ben-Saadon et al., 2004). It is possible that N-terminal ubiquitylation, similar to ubiquitylation on residues other than internal lysine residues, attests for the robustness of a system that had to evolutionarily adapt to the degradation of proteins with different structures and compositions.

Ubiquitin-independent degradation

In addition to several different types of ubiquitin modifications that target proteins for degradation, certain proteins appear to be degraded by the proteasome in a ubiquitin-independent manner. For instance, it has been reported that myeloid cell leukemia 1 (MCL1) (Li et al., 2007; Stewart et al., 2010) and CCAAT/enhancer-binding protein δ (C/EBPδ) (Zhou and Dewille, 2007) can be proteasomally degraded without prior modification by ubiquitin.

Another example is provided by the ubiquitin-independent proteasomal degradation of ornithine decarboxylase (ODC) (Bercovich et al., 1989; Murakami et al., 1992). Instead of relying on ubiquitylation, this process requires a specific 'chaperoning' protein, antizyme-1 (Murakami et al., 1992). Antizyme-1 binds to the ODC monomer, which results in exposure and recognition of the C-terminal domain of the enzyme by the 26S proteasome. It should be mentioned, however, that ODC can be degraded by the proteasome in the absence of antizyme-1, albeit at a substantially reduced rate. Antizyme-1 itself is not degraded along with ODC and is recycled: it is degraded independently in a ubiquitin-dependent manner. Antizyme-1 has also been demonstrated to associate and stimulate the proteasomal degradation of cyclin D1 (Newman et al., 2004) and the aurora A kinase (Lim and Gopalan, 2007a; Lim and Gopalan, 2007b). However, unlike ODC, these proteins are also degraded in a ubiquitin-dependent manner. Therefore, the significance and contribution of the ubiquitin-independent pathway and antizyme-1 for their degradation is not clear.

It has also been reported that ODC (Asher et al., 2005a), and the tumor suppressors p53 and p73 (Asher et al., 2005b) can be degraded by the 20S proteasome. This process is regulated by NADH quinone oxidoreductase (NQO1), which stabilizes these proteins: once NQO1 is inhibited by its inhibitor dicuomarol or its expression is silenced, ODC, p53 and p73 are destabilized. The mechanism of action of NQO1 is still not clear, but its function

Table 1. Ubiquitin ligases (E3) that synthesize 'non-canonical' ubiquitin chains that subsequently target substrates for proteasomal degradation

E3 ligase (E2 enzyme)	Type of ubiquitin chain	Substrates	References
CHIP	Chains based on lysine 6, 11, 48 and 63	HSP70 and HSP90	(Kundrat and Regan, 2010)
MuRF1 (UBCH1 or heterodimer UBCH13–UEV1)	Chains based on lysine 48 or 63	Troponin I	(Kim et al., 2007)
RSP5	Lysine-63-based chain	Mga2	(Saeki et al., 2009)
APC/C (UBCH10, UBE2S)	Lysine-11-based chain	BARD1, HMMR, HURP, NUSAP1	(Song and Rape, 2010)
APC/C	multiple monoubiquitylation, followed by formation of mixed chains	Cyclin B1	(Kirkpatrick et al., 2006)
SIAH1 (UBCH5a)	Lysine-11-based chain	β-catenin	(Dimitrova et al., 2010)
RNF4	Heterologous SUMO-ubiquitin chains	PolySUMOulated PML	(Lallemand-Breitenbach et al., 2008; Tatham et al., 2008)
LUBAC	Linear	Ubiquitin–GFP	(Kirisako et al., 2006)
CDC48–NPL4–UFD1	Linear tetraubiquitin	PCNA	(Zhao and Ulrich, 2010)
TAF1	Monoubiquitin	PAX3	(Boutet et al., 2010)
mK3	Ubiquitylation on serine, threonine or lysine residues	MHC I heavy chain (HC)	(Wang et al., 2007)

appears to be mediated by its binding to the substrate. As NQO1 generates NAD^+ from NADH, it is possible that the mechanism of NQO1-dependent protein stabilization is linked to either the redox state of the cell and/or to the availability of NAD^+ for ADP ribosylation. It should be noted that these proteins are degraded by the 20S complex in a ubiquitin-independent process. Because all these proteins are also degraded by the 26S proteasome in highly regulated processes, degradation through the NQO1-dependent pathway must occur under unique and still to be determined conditions. Another protein that has been reported to be degraded by the 20S proteasome without prior ubiquitylation is BIM-extra long [BIM(EL)], an intrinsically disordered protein that is a member of the BCL2 family (Wiggins et al., 2011). Inhibitor of κ light chain gene enhancer in B cells alpha (IκBα) has also been shown to be degraded by the core 20S proteasome in a ubiquitin-independent manner, and its degradation could be protected by the expression of the p65 subunit of NF-κB (Alvarez-Castelao and Castano, 2005; Kroll et al., 1997). Again, IκBα is also degraded in a signal- and phosphorylation-dependent manner (Alkalay et al., 1995; Yaron et al., 1997) by the UPS. Thus the 20S- and ubiquitin-independent pathway must be active, if at all, under unique, still to be studied, conditions.

Taken together, these studies provide evidence that the 20S proteasome can degrade proteins in a ubiquitin-independent manner. However, the role the 20S proteasome has in cellular proteolysis – if it has any – is highly controversial. For example, it is difficult to explain specific substrate recognition by the 20S proteasome. Furthermore, the 20S proteasome is inactive proteolytically, as the N-terminal domains of the α-rings interlace with one another, thereby blocking entry of substrates into the proteolytic chamber. Supporting this notion is the finding that the 26S proteasome dissociates and releases free 20S complexes during the stationary phase in *S. cerevisiae*, but at the same time, proteolysis rates decrease, probably in order to protect the cell from self-digestion during starvation. Moreover, degradation rates increase dramatically, when the α-subunits of the 20S proteasome are mutated so that their N-terminal domains cannot interlace and thus form a proteasome with a permanently opened gate (Bajorek et al., 2003). It should be noted that most of the experiments suggesting independent 20S proteasomal activity used oxidatively damaged proteins and were carried out in cell-free systems (e.g. Davies and Goldberg, 1987; Fagan et al., 1986; Grune et al., 2003). There is no convincing experimental evidence to support the notion that the 20S proteasome does indeed have a role in the degradation of proteins, even damaged ones, in cells. By contrast, there are ample lines of experimental evidence that suggest that the degradation of damaged proteins is dependent on the UPS. For instance, it has been shown that the degradation of cellular proteins damaged by heat, cadmium or paraquat requires the E2s UBC4 and UBC5, the CDC48–UFD1–NPL4 ligase complex and the proteasome (Medicherla and Goldberg, 2008). In addition, because degradation of all types of proteins – native as well as denatured – requires metabolic energy in vivo, it seems that the process must be mediated by an intact 26S proteasome complex, the assembly and maintenance of which are dependent on ATP. In addition, ubiquitylation also requires energy, whereas degradation by the 20S complex is independent of energy (reviewed by Weissman et al., 2011).

The cell cycle inhibitor p21 is degraded through two pathways (reviewed by Lu and Hunter, 2010). Its cell-cycle-regulated degradation is ubiquitin-dependent (Abbas et al., 2008; Amador et al., 2007; Bornstein et al., 2003; Kim et al., 2008; Shibata et al., 2011; Wang et al., 2005), whereas its degradation during resting conditions is ubiquitin-independent (Chen et al., 2007; Chen et al., 2004; Jin et al., 2003; Sheaff et al., 2000; Zhang et al., 2004). Likewise, a dual proteasomal pathway eliminates the hepatitis C virus (HCV) core protein. The first one is ubiquitin-dependent (Suzuki et al., 2009) and the other is ubiquitin-independent (Yuksek et al., 2009) but requires the proteasome activator PA28γ (Suzuki et al., 2009). However, the conclusion that the second pathway does not require ubiquitin is based on the utilization of a mutant form of the viral protein that does not contain lysine residues. Therefore, the possibility that other degradation mechanisms, such as ubiquitylation on non-lysine residues within the molecule, cannot be excluded.

Taking into consideration these different examples, we believe that the single well-established case of proteasome-dependent, yet ubiquitin-independent, degradation is that of ODC, and that it is possible there is cell-cycle-independent degradation of p21. Further studies are required to substantiate other modes of ubiquitin-independent degradation, in particular degradation mechanisms that are dependent on the 20S proteasome.

Conclusions and perspectives

Whereas non-canonical ubiquitin chains appear to serve diverse non-proteolytic functions, among them activation of the NF-κB signaling pathway, regulation of DNA damage response pathways, activation of transcriptional repressive complexes and endocytosis (reviewed by Ikeda and Dikic, 2008), little is known about their role in targeting proteins for proteasomal degradation.

The countless substrates of the ubiquitin system, and the processes regulated by it, probably resulted in the evolution of a broad repertoire of signals that can be recognized by different ubiquitin binding domain (UBD)-containing proteins, including shuttle proteins and the proteasome. As for ubiquitin, the complexity and diversity of its code depend on different conformations of the chains, which are dependent in turn on the particular lysine residue involved in the internal linkages, the chain length and/or different ubiquitin acceptor sites within the protein substrate. In addition, the code might depend on other small molecule modifiers such as ubiquitin-like proteins that can also be part of the chain.

One important, yet unsolved, question relates to the diversity of the internal linkages. If chains made of ubiquitin moieties that are linked through all internal lysine residues can target proteins for degradation, why is this diversity needed? It is possible that the different structures of the individual chains determines the strength of their interaction with the proteasome and/or shuttle proteins, as well as the recognition sensitivity of different DUBs, which might in turn affect degradation priorities and rates among different substrates. For example, the closed conformation of lysine-48-based chains is clearly different from the extended conformation of lysine-63-based and linear chains, a difference that, as noted, might govern specific degradation characteristics. The structure of other homogeneous chains, not to mention mixed and forked chains, has not been resolved but can be partially predicted using molecular modeling (Fushman and Walker, 2010). The compact structures of lysine-11- (Bremm et al., 2010) and lysine-6-based (Virdee et al., 2010) diubiquitin moieties has been determined and suggests that these have a completely different structure and/or protein surface, and hence different characteristics, than lysine-48- and lysine-63-based chains. Clearly, additional structural analyses and isolation and characterization of the interactomes of the different chains are required to take the first steps towards understanding the meaning of this diversity. It is also possible that the functional redundancy among the various chains provides the system with the robustness it needs to recognize the countless protein substrates targeted by the system, including an infinite number of conformations of even a single misfolded protein.

With regards to the E2s and E3s involved in the formation of these chains (Table 1), studies carried out using cell-free assays have shown that some enzymes can synthesize chains containing all linkages, whereas others are specific, catalyzing the formation of homogeneous chains (Kim et al., 2007; Kirkpatrick et al., 2006). However, most of these analyses were carried out using recombinant purified proteins in vitro, and it is not clear whether they faithfully represent cellular events. Compared with experimental systems, the cell contains all the E2s and E3s of the UPS, and these can act sequentially to generate all types of chains without one enzyme having to synthesize them all. Importantly, and as noted above, it is possible that in the cell the different linkages are generated spuriously rather than intentionally, and that they do not have a physiological role in targeting the respective substrates for degradation. Instead, it is possible that proteins could be targeted to the proteasome by only a short stretch of a lysine-48-based segment that is present somewhere within the chain.

The detailed analysis of the structures of the individual chains, and the identification of the specific proteins interacting with different chains and their role in targeting the different substrates for degradation, could pave the road towards the development of specific modulators that can regulate specific substrates and therefore processes. Thus, the inhibition of DUBs that only process specific linkages could accelerate the degradation of substrates tagged with such chains (by ensuring that the substrates remain modified with long chains that are recognized more efficiently by the proteasome), such as, for example, aggregated proteins [see Lee et al. for an example (Lee et al., 2010)]. The inhibition of a different subset of DUBs, however, could inhibit the proteasome by not allowing removal of the chain, and thereby keeping the tagged substrates bound to the proteasome and blocking the access for other ubiquitylated substrates (e.g. D'Arcy et al., 2011).

An additional unsolved question that needs to be addressed in future studies is the identity of proteasomal subunits and shuttle proteins. Because these proteins are responsible for the recognition of the numerous ubiquitin chains, and possibly the recognition of multiple single ubiquitin moieties, their identification and characterization will provide additional insight into the regulation of this complex system.

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Journal of Cell Science

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