Emerging roles for Lys11-linked polyubiquitin in cellular regulation

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Polyubiquitin chains are assembled via one of seven lysine (Lys) residues or the N terminus. The cellular roles of Lys48- and Lys63-linked polyubiquitin have been extensively studied; however, the cellular functions of Lys11-linked chains are less well understood. Recent insights into Lys11-linked ubiquitin chains have revealed their important function in cell cycle control. Additionally, Lys11 linkages have been identified in the context of mixed chains in many other cellular pathways. In this review, we introduce the specific enzymes that mediate Lys11-linked chain assembly and disassembly, and discuss the diverse cellular processes in which Lys11 linkages participate. Notably, mechanistic insights have revealed how the E2 ubiquitin-conjugating enzyme UBE2S achieves its Lys11 linkage specificity, and two structures of Lys11-linked polyubiquitin highlight the dynamic nature of this compact chain type.

Ubiquitin chains in cellular regulation

Protein ubiquitylation is a versatile post-translational modification in which the small protein ubiquitin is covalently attached to substrate Lys residues. This process is mediated by a three-step enzymatic cascade, in which the ubiquitin C terminus is activated by an E1 enzyme, and transferred onto the active site cysteine of an E2 enzyme. The charged E2 enzyme interacts with E3 ubiquitin ligases to transfer ubiquitin either directly (in case of RING and U-box E3 ligases), or indirectly [via a ubiquitin-modified HECT (homologous to E6-AP C terminus)-E3 ligase intermediate], to a Lys residue of a substrate protein [1]. Ubiquitin chains on substrates are recognized by ubiquitin-binding domains (UBDs) found in many proteins [2], and deubiquitylases (DUBs) reverse this modification [3,4].

A key feature of ubiquitin is its ability to form polymers, in which individual moieties are linked via one of seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 or Lys63 linkages) or the N terminus (linear linkage). In addition to homotypic chains (a chain that comprises only one linkage type), heterotypic chains with two or more linkages also exist [5,6].

Of the eight distinct linkage types, only two have been studied extensively. Lys48-linked polyubiquitin serves as a targeting signal for proteasomal degradation [7,8], which is the most common fate of a ubiquitylated protein. Proteasomal degradation is a key mechanism for protein homeostasis, and is required to clear unfolded/misfolded or damaged proteins [9]. In addition, many cellular processes rely on the precisely organized degradation of specific proteins for their initiation, progression or termination, thus making ubiquitin-dependent degradation essential in eukaryotic organisms [10].

Ubiquitylation can also promote non-degradative outcomes. Lys63-linked ubiquitin chains are involved in cell signaling (e.g. activation of protein kinases), initiate membrane trafficking events, and are essential for the DNA damage response [11].

A third linkage type which is abundantly present in cells are Lys11 linkages [12]. Numerous recent studies have detected this linkage type in diverse cellular pathways, and both degradative and non-degradative roles have been suggested. In this review, we examine these recent insights, and try to provide a comprehensive overview of what is known about this emerging post-translational modification.

Abundance of Lys11-linked ubiquitin chains

Baboshina and Haas discovered the Lys11-specific E2 enzyme UBE2S (also called E2-EPF) in 1996 [13], but it took a further decade to reveal that Lys11 linkages are abundant in vivo [12,14]. Affinity purification of endogenously expressed His-tagged ubiquitin chains from Saccharomyces cerevisiae and subsequent mass-spectrometry (MS) analysis showed that Lys11 linkages were as abundant as Lys48 linkages, each accounting for approximately 30% of the total ubiquitin linkage content [12]. This unexpectedly high abundance in yeast does not seem to be reflected in higher eukaryotes: in mammalian HEK293 cells, Lys11 linkages account for approximately 2% of the total ubiquitin linkage content [15]. A second study in yeast using untagged ubiquitin failed to detect significant quantities of Lys11 linkages [16].

To date, most MS experiments on total cell lysates have been performed with unstimulated cells. However, the importance of Lys11-linked ubiquitin chains in the cell cycle (see below) was emphasized by immunoblotting using a recently developed Lys11-specific antibody, and a large increase of this linkage type was revealed in synchronized cells exiting mitosis [17]. This finding highlights that the relative abundance of ubiquitin chains can change dramatically when linkage-specific assembly machineries are activated [such as the anaphase-promoting complex/ cyclosome (APC/C) in the cell cycle; see below]. Such changes need to be considered when the biological relevance of barely detectable Lys11-linked (or other atypical) ubiquitin chains is judged.
Cellular processes regulated by Lys11-linked ubiquitin chains

Studies using MS techniques, ubiquitin mutants or the recently developed Lys11 linkage-specific antibody have identified roles for Lys11 linkages in many cellular processes (Figure 1, Tables 1–3). Roles for homotypic Lys11-linked ubiquitin chains in proteasomal degradation of mitotic regulators are now firmly established. Lys11 linkages were also identified in the context of mixed ubiquitin chains on proteins involved in cell signaling, membrane trafficking and the DNA damage response.

Homotypic Lys11-linked ubiquitin chains mediate degradation of cell cycle regulators

The human APC/C is a multisubunit E3 ligase responsible for an orderly progression through mitosis by sequentially targeting cell cycle regulators for destruction [18]. Recent work has revealed that this E3 ligase specifically assembles Lys11-linked ubiquitin chains on APC/C substrates, and that such modified proteins are rapidly degraded by the proteasome during cell cycle progression [19–22]. The APC/C can utilize ‘priming’ E2 enzymes such as UBE2C (also called UBC10) or UBE2D (also called UBC15 or UBC4) [20,23,24] to decorate substrates with mono-ubiquitin and short ubiquitin chains (Figure 1A), and UBE2C seems to be the physiological priming E2 with this ligase [24]. UBE2C appears to recognize a non-linear consensus motif in APC/C substrates (called a TEK box) [20]. This motif is also present in ubiquitin near the Lys11 side chain, allowing UBE2C to assemble short Lys11-linked chains [20]. The Lys11-specific ‘elongating’ E2 enzyme UBE2S extends these short chains into long Lys11-linked ubiquitin polymers on APC/C-bound substrates [19,21,25] (Figure 1A). Ubiquitin chain extension with up to 13 ubiquitin molecules occurs in a single substrate-binding event [26], and the subsequently released substrates are rapidly degraded by the proteasome. Lys11-polyubiquitylation is abrogated by co-depletion of both UBE2C and UBE2S, and results in stabilization of APC/C substrates, and in severe spindle defects and mitotic delay [19].

Interestingly, the APC/C also functions in the nervous system to control, for example, neuronal morphogenesis and synapse development [27]. This raises the question of whether Lys11-linked ubiquitin chains are also involved in APC/C-dependent ubiquitylation events in the brain.

It is striking to note that the role of Lys11 linkages in cell cycle regulation is not conserved in evolution. UBE2C and UBE2S are not present in S. cerevisiae, and yeast APC/C instead uses Ubc4 and Ubc1 to prime and elongate Lys48-linked chains on cell cycle substrates [28]. UBE2S orthologs, which can be easily identified by a highly conserved Lys-rich C-terminal sequence, are present in Dro sophila melanogaster, but not in nematodes including Caenorhabditis elegans, or in fungi (see http://www.ensem bl.org/). It is possible that the more conserved UBE2C could be sufficient to mediate Lys11 ubiquitylation, or alternatively, that a different chain-elongating E2 enzyme might extend Lys11-linked chains with the APC/C if UBE2S is absent. However, a switch from Lys48 (yeast) to Lys11 linkages (mammals) as a degradation signal in the cell cycle suggests that this essential process has evolved to utilize a different ubiquitin chain type. The reason for this switch is unclear, but might be related to the fact that yeast cells undergo a ‘closed’ mitosis (chromosomes segregate in an intact nucleus), whereas human cells developed an ‘open’ mitosis (the nuclear envelope breaks down before chromosome segregation) [29].

Cellular processes regulated by Lys11 linkages in the context of mixed chains

Recent reports have identified Lys11 linkages in the context of mixed ubiquitin chains in several cellular processes. It is currently not clear whether the substrates modified with mixed ubiquitin chains comprise several homotypic chains with distinct linkages, or whether they are modified with chains that contain multiple linkage types sequentially or in branched/forked structures. New methods to

Table 1. Enzymes associated with Lys11-linked chain assembly

<table>
<thead>
<tr>
<th>E2 enzyme</th>
<th>E3 ligase (type)</th>
<th>Linkage types</th>
<th>Substrate</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBE2C / UBE2S</td>
<td>APC/C (RING)</td>
<td>Lys11</td>
<td>Many</td>
<td>Degradation</td>
<td>[19–21]</td>
</tr>
<tr>
<td>UBE2D</td>
<td>cAP1 (RING)</td>
<td>Lys11, Lys48, Lys63</td>
<td>RIP1, others?</td>
<td>Kinase activation?</td>
<td>[38]</td>
</tr>
<tr>
<td>UBE2D / UBE2N</td>
<td>K5 (RING)</td>
<td>Lys11, K63</td>
<td>MHC I</td>
<td>Endocytosis</td>
<td>[34,35]</td>
</tr>
<tr>
<td>Ubc6 (yeast)</td>
<td>Doa10 (RING)</td>
<td>Lys11, others?</td>
<td>ERAD substrates</td>
<td>ERAD</td>
<td>[12]</td>
</tr>
<tr>
<td>unknown</td>
<td>EDD (HECT)</td>
<td>Lys11, Lys29</td>
<td>β-catenin</td>
<td>Protein stabilization?</td>
<td>[45]</td>
</tr>
</tbody>
</table>

Table 2. Enzymes associated with Lys11-linked chain disassembly

<table>
<thead>
<tr>
<th>DUB</th>
<th>Family</th>
<th>Specificity</th>
<th>Substrates</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cezanne (also called OTUD7B)</td>
<td>OTU</td>
<td>Lys11</td>
<td>RIP1?</td>
<td>NFκB inhibition</td>
<td>[41]</td>
</tr>
</tbody>
</table>

Table 3. Ubiquitin binding proteins for linkages including Lys11

<table>
<thead>
<tr>
<th>Ub binding protein</th>
<th>Type</th>
<th>Specificity</th>
<th>Substrate</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subunit 5A</td>
<td>Tandem UIM</td>
<td>Lys11, Lys48, others</td>
<td>Proteasome bound</td>
<td>Proteasome recognition</td>
<td>[13]</td>
</tr>
<tr>
<td>RPN10, RAD23, DSK2</td>
<td>UBA</td>
<td>Lys11, Lys48, Lys63, (mixed linkages)</td>
<td>Proteasome targets</td>
<td>Proteasome shuttling</td>
<td>[47]</td>
</tr>
<tr>
<td>SAKS1, other UBA-UBX</td>
<td>UBA</td>
<td>Lys11, (also Lys48, Lys63, Lys33)</td>
<td>CDC48 targets</td>
<td>CDC48 shuttling</td>
<td>[32]</td>
</tr>
<tr>
<td>NEMO</td>
<td>UBAN and ZnF</td>
<td>Linear (UBAN), Lys11, Lys48, Lys63 (ZnF)</td>
<td>RIP1</td>
<td>Kinase activation?, degradation?</td>
<td>[38]</td>
</tr>
</tbody>
</table>
Figure 1. Functions of Lys11-linked chains in cells.
Lys11-linked ubiquitin chains are involved in different cellular pathways by mediating degradative and non-degradative processes. (a) The RING E3 ligase complex APC/C targets its substrates for (iii) proteasomal degradation by assembling Lys11-linked ubiquitin chains using the E2 enzymes (i) UBE2C for chain initiation [20] and (ii) UBE2S for subsequent chain elongation [19,25]. (b) In S. cerevisiae, the RING E3 ligase Doa10 and Ubc6 have been suggested to assemble Lys11-linked ubiquitin chains on ERAD substrates to mark misfolded proteins for destruction by the proteasome [12]. Mammalian UBA-UBX proteins link ubiquitylated proteins to the AAA+ ATPase CDC48 that escorts ubiquitylated substrates to the proteasome [32]. (c) WRNIP1 is modified with short mixed ubiquitin chains that serve as a proteasomal degradation signal [49] (d) MHC class I is ubiquitylated by the viral RING E3 ligase K5 and subsequently internalized. Endocytosis of MHC I depends on Lys11 and Lys63 linkages with mixed or branched topology [34,35]. (e) Activation of the TNF receptor results in RIP1 polyubiquitylation and subsequent activation of downstream kinases. The RING E3 ligase cIAP, together with UBE2D, assembles K11- and K63-linked chains on RIP1 [38]; these modifications might activate the signaling cascade. (f) β-catenin is stabilized upon WNT stimulation and translocates to the nucleus to promote transcription. The HECT E3 ligase EDD might assemble Lys11- and Lys29-linked ubiquitin chains on β-catenin, resulting in the accumulation and stabilization of β-catenin in the nucleus [45].
study chain topology are required to resolve this question. In addition, the functional relevance of K11 linkages within mixed ubiquitin chains needs to be analyzed in more detail in future studies.

Endoplasmic reticulum-associated degradation
Endoplasmic reticulum-associated degradation (ERAD) is a cellular quality-control mechanism that ensures that only correctly folded and glycosylated proteins are displayed on the cell surface [30], and Lys11 linkages have been associated with this process [12]. An E2–E3 pair involved in ERAD, namely Ubc6 and Doa10, synthesizes Lys11 linkages in S. cerevisiae (Figure 1b). Deletion of UBC6 reduces the total cellular amount of Lys11 linkages by approximately 40%, whereas Lys48 or Lys63 linkages are not affected. Moreover, Lys11 linkages accumulate in the presence of endoplasmic reticulum (ER) stress [12]. An important cellular machine involved in ERAD is the CDC48 (cell division control 48; also called p97) ubiquitin-dependent chaperone, a hexameric AAA+ ATPase that escorts ubiquitylated proteins to the proteasome [31]. Adaptor proteins of the UBA–UBX family mediate ubiquitin recognition by CDC48, and a recent proteomic screen identified Lys11 linkages as being enriched in UBA–UBX protein complexes [32]. This finding lends further support to the idea that K11 linkages function in ERAD and downstream processes, which deserves further characterization (Figure 1b).

Endocytosis
Internalization and intracellular trafficking of transmembrane receptors depends on ubiquitylation, and roles for mono- and Lys63-linked polyubiquitin chains have been firmly established [33]. Additionally, two recent reports associated Lys11-linked ubiquitin chains with this process [34,35]. Major histocompatibility complex (MHC) class I molecules display pathogen-derived peptides on the cell surface to alert the immune system to viral or bacterial infection and to attract cytotoxic T lymphocytes. Kapoši’s sarcoma-associated herpesvirus encodes the membrane-bound RING E3 ligases K3 and K5, which serve to down-regulate MHC class I [36]. Both E3 ligases mediate ubiquitylation of MHC class I using UBE2D and UBE2N (also called UBC13). However, whereas K3 assembles Lys63-linked chains [37], the related K5 ligase assembles ubiquitin chains containing both Lys11 and Lys63 linkages on MHC class I, and these hetertypic ubiquitin chains contain mixed linkages or branches in the polymer [34,35] (Figure 1D). Functionally, MHC class I endocytosis is more rapid upon assembly of homotypic Lys63-linked chains by K3, and proceeds with slower kinetics when mixed/branched chains are attached by K5 [34].

Interestingly, Lys6, Lys11 and Lys63 of ubiquitin are required for K5-mediated endocytosis of MHC class I, as shown by rescue experiments using ubiquitin mutants [34]. The requirement for Lys6 might be explained by recent mechanistic insights into Lys11 linkage assembly [26] (see below).

Cell signaling
Recent reports associate Lys11 linkages with cytokine signaling [38,39], and suggest non-degradative roles for this chain type. Cytokines such as tumor necrosis factor-α (TNF-α) result in rapid ubiquitin-mediated activation of protein kinase cascades and transcription factors including nuclear factor κB (NF-κB) [40]. Receptor-interacting protein 1 (RIP1) is a signaling molecule that is Lys63-ubiquitylated within minutes of cytokine stimulation. The combination of linkage-specific antibodies and MS led to the finding that RIP1 is modified with Lys11-linked polymers in vivo, and that the RING E3 ligase cellular inhibitor of apoptosis (cIAP) together with UBE2D, assembles these chains [38] (Figure 1E). Consistent with this observation is a recent, elegant study that detected Lys11 linkages, among others, on ubiquitylated RIP1 [39]. Lys11 and Lys63 linkages bind the protein kinase adaptor NF-κB essential modulator (NEMO) with similar affinity, thus indicating non-degradative signaling roles for ubiquitin chains containing Lys11 linkages [38]. Interestingly, the only known Lys11-specific DUB Cezanne [41] was reported to be a negative regulator of NF-κB signaling [42], which is consistent with a role for Lys11 linkages in this pathway. However, the timing of Lys11 modifications on RIP1 requires further analysis, as it is known that RIP1 undergoes sequential Lys63 and Lys48 ubiquitylation, with Lys63-linked chains activating the pathways, and Lys48-linked chains degrading RIP1 and hence switching off signaling (a process termed ‘ubiquitin editing’) [43]. Given that Lys11-linked chains are efficient degradation signals in the cell cycle (see above), it remains possible that this chain type mediates RIP1 degradation in this pathway.

WNT/β-catenin signaling is important for embryonic development, and affects cell proliferation, cell polarity and cell fate [44]. Results obtained from ectopic expression of ubiquitin mutants in human HEK293T cells suggest that the HECT E3 ligase EDD modifies β-catenin with ubiquitin chains containing Lys29 and Lys11 linkages [45] (Figure 1F). However, whether this modification leads to the stabilization [45] or degradation of β-catenin [46] is not clear, and it remains to be proven whether Lys11 linkages play a functional role in these processes.

Other events
UBE2C- and UBE2D-mediated in vitro ubiquitylation of proteins often leads to the assembly of short mixed polyubiquitin chains linked via various Lys residues, including Lys11, Lys48 and Lys63 [47,48]. These ubiquitylated species are degraded by the proteasome with comparable kinetics to Lys48-polyubiquitylated proteins, and they are recognized by the proteasome shuttling factors RAD23, DSK2 and RPN10 [47]. The degradation of the DNA damage-response factor Werner helicase-interacting protein 1 (WRNIP1) is mediated by a similar mixture of polyubiquitin chains in vivo [49]. Hence, multiple short homo- or hetertypic ubiquitin chains, including Lys11 linkages, could be physiologically relevant alternative signals for proteasomal degradation (Figure 1C).

Implication of Lys11-linked polyubiquitin in human disease
Many of the described cellular pathways that utilize Lys11 linkages are involved in human disease, and overexpression of the Lys11-specific E2 enzymes UBE2C and UBE2S has
been associated with cancer. In addition, Lys11 linkages have also been found in the protein aggregates that lead to neurodegenerative diseases.

**UBE2S and UBE2C are involved in cancer**

Gene-expression profiling and immunohistochemistry studies have shown that UBE2C and UBE2S are over-expressed in many human cancer types, and are associated with tumor progression [50–52]. More recently, a transgenic mouse model has revealed that UBE2C overexpression leads to chromosome instability and tumor initiation [53]. In addition, the role of UBE2C as a predictive marker and as a new molecular target for therapeutic intervention in cancer cells has been discussed intensively [54–56].

In addition to the established role of UBE2S in cell cycle regulation, this enzyme has also been linked to hypoxia signaling [57,58]. The response to hypoxic stress (i.e. low oxygen levels) is regulated by the transcription factor hypoxia-inducible factor 1 (HIF1), which under normoxic conditions (i.e. normal oxygen levels), is degraded in a ubiquitin-dependent fashion [59]. The tumor suppressor von Hippel–Lindau protein (VHL) is part of an E3 ligase complex that mediates ubiquitylation and subsequent degradation of HIF1α. UBE2S was reported to associate with VHL and to target it for proteasomal degradation, thereby stabilizing HIF1α [57]. In addition, the promoter region of UBE2S contains hypoxia-response elements, and UBE2S expression is induced by hypoxia [58]; this could constitute a positive feedback loop to further increase the level of HIF1α under hypoxic conditions. Because the Lys11 specificity of UBE2S is well established, homotypic Lys11-linked ubiquitin chains might also regulate VHL stability.

**Lys11 linkages in proteinopathies**

Abnormal deposition of insoluble protein aggregates in neurons can lead to neurodegenerative disorders such as Alzheimer’s disease (AD) or Huntington’s disease (HD) [60]. The protein aggregates are often enriched in ubiquitin, suggesting a dysfunction or blockage of the ubiquitin–proteasome system [61]. HD is a polyglutamine (polyQ) disease in which the polyQ stretch of the huntingtin gene is extended, leading to depositions of aggregated huntingtin fragments. Such fragments, taken from brains of a HD transgenic mouse model, are strongly modified with Lys11, Lys48, and Lys63 linkages [62].

Neurofibrillary tangles (NFTs) form a pathological feature of AD, and comprise aggregates of the microtubule-associated protein tau, which is modified with ubiquitin chains containing at least three different linkages (Lys6 and Lys11 and Lys48) in AD patient samples [63]. Lys11, Lys48 and Lys63 linkages are significantly increased in brains from human patients with AD [15]. Interestingly, the polyubiquitin pattern of samples from patients with late-stage AD is best mimicked by heat-shock treatment of neuronal cultures [15], thus representing a valuable advance in the study of these proteinopathies.

**Mechanisms of Lys11 linkage assembly**

The detailed mechanisms of ubiquitylation remain elusive, and specific chain assembly is best understood for the Lys63-specific UBE2N–UEV1A E2 system [64]. NMR and docking studies have recently unraveled the mechanisms of linkage specificity for UBE2S [26] (Figure 2A), a single domain monomeric E2 with intrinsic Lys11 specificity [13,26]. The catalytic Ubc domain of UBE2S interacts non-covalently with the hydrophobic patch of the ‘donor’ ubiquitin (i.e. the ubiquitin molecule that is also covalently attached to the active site Cys residue). This bipartite interaction restricts the conformational freedom of the donor ubiquitin on the E2 surface, and is crucial for providing chain-assembly processivity [26]. A similar mechanism has been suggested for Lys48-linked ubiquitin chain

![Figure 2. Mechanism of Lys11-linked chain assembly.](image)

The mechanism of UBE2S-mediated assembly of Lys11 linkages was recently revealed by NMR analysis [26]. Binding sites for both the donor and acceptor ubiquitin are present on UBE2S. A residue in ubiquitin, Glu34, complements the catalytic site of UBE2S in a mechanism of substrate-assisted catalysis. (b) Cartoon structure of ubiquitin with residues of the TEK box shown in ball-and-stick representation. Glu34 in the TEK box forms a salt bridge with Lys11 (indicated by purple dotted lines).
assembly by UBE2R (also called CDC34) [65]. Linkage specificity of UBE2S is acquired by a simultaneous, transient interaction with the ‘acceptor’ ubiquitin (i.e. the ubiquitin providing the Lys11 side chain for isopeptide formation) (Figure 2A). The low-affinity interaction with the acceptor ubiquitin does not allow UBE2S to distinguish between the seven Lys residues on ubiquitin. Instead, this exquisite specificity is achieved by a mechanism of substrate-assisted catalysis, in which Glu34, a residue within the TEK box in the acceptor ubiquitin, complements the catalytic site of UBE2S. Glu34 reduces the $pK_a$ of the nearby Lys11 side chain in cis, and singles out this Lys on ubiquitin to be selected by an otherwise catalytically less efficient E2 enzyme. No other ubiquitin Lys provides an active site complementing residue that can be used by UBE2S in a similar fashion [26].

The ubiquitin TEK box comprises residues Lys6, Thr12, Thr14, and Glu34 on the ubiquitin surface (Figure 2B), and this patch is also used in the UBE2C-mediated assembly of short Lys11-linked chains on APC/C substrates [20]. The TEK box residues on the β1/β2 strand reside in the region of highest conformational flexibility [66], and can potentially adopt a range of different conformations to form a contiguous interface for binding to the E2 enzyme (Figure 2B).

It is less clear how other E2 enzymes or HECT E3 ligases assemble Lys11-linked chains. UBE2D family members that assemble K11-linked chains with K5 in endocytosis [34], or with cellular inhibitor of apoptosis (cIAP) in TNF signaling [38], are intrinsically nonspecific enzymes, which will also assemble Lys48 and Lys63 linkages [38, 48, 67]. They also do not seem to restrict the position of the donor ubiquitin on the Ubc domain [68]. However, in context of the K5 E3 ligase, UBE2D requires the ubiquitin TEK box residue Lys6 for Lys11-linked chain assembly [34]. This finding also suggests that other, non-specific E2 enzymes utilize the TEK box to assemble Lys11 linkages.

### Structural features of Lys11-linked polyubiquitin

The structure of Lys11-linked ubiquitin chains has been studied by X-ray crystallography and NMR spectroscopy [17, 41]. All data indicate that this chain type adopts compact conformations in which neighboring isopeptide-linked ubiquitin moieties interact with each other.

The two crystal structures [17, 41] are at first sight non-identical, and display markedly different overall conformations. In one conformation (crystal structure 1, CS1, pdb-id 2xew [41]) (Figure 3A), ubiquitin molecules interact via a previously unreported interface on the ubiquitin helix, whereas in CS2, a region known as the Ile36 patch is involved in the interface (pdb-id 3nob [17]). Lys11-linked ubiquitin dimers hence seem to adopt two distinct compact conformations, a finding that is also consistent with NMR analysis of this chain type [41]. A dynamic equilibrium between (at least) two conformational states highlights the dynamic features of ubiquitin chains (Figure 3C).

However, closer inspection of the crystal packing reveals that in fact, both conformations could be observed in either crystal structure (Figure 3C, D, E), forming an identical higher-order structure in the crystals (Figure 3D, E) [41].

Eight ubiquitin moieties formed a compact barrel-shaped structure in which all molecules can be linked via Lys11 (Figure 3F). This seemingly stable arrangement might be a feasible model for a long Lys11-linked ubiquitin chain attached to a substrate [41].

A further interesting fact about the dimer and higher-order structures is that the common interaction site of ubiquitin known as the hydrophobic patch, comprising residues Leu8, Ile44, His68 and Val70, is largely solvent-exposed (with the exception of Val70 in CS2 that is buried at the interface). This finding suggests that ubiquitin interactions via the hydrophobic patch can occur without dramatic remodeling of the chain. The distinct hydrophobic interfaces exposed by longer chains (Figure 3F), further suggest the presence of specialized binding domains that recognize such higher-order structures. In conclusion, structurally distinct Lys11-linked ubiquitin chains create novel possibilities for ubiquitin recognition by interacting proteins.

### Concluding remarks and future perspectives

In 2003, Gygi and colleagues demonstrated that all seven Lys residues in ubiquitin are used to assemble polyubiquitin chains in vivo [14], and that Lys11 linkages were among the most prominent atypical linkage types identified. Since then, much has been learned about the function of Lys11-linked ubiquitin chains, which seem to take part in essential cellular processes such as proteasomal degradation, and potentially also in non-degradative contexts.

An alternative degradation signal based on a structurally distinct ubiquitin chain could have many advantages in a physiological setting. Firstly, specialized proteasomal shuttling factors could exist that direct the degradation of Lys11-modified substrates. There have been reports of possible functionally analogous Lys11-selective shuttling systems for CDC48-mediated processes [32], although these data need to be further analyzed and validated.

A second powerful contribution to physiological linkage specificity exists at the level of deubiquitylation. Lys48-selective DUBs would not interfere with the degradation of Lys11-modified substrates, and these enzymes would therefore not interfere with, for example, the cell cycle [20, 41]. By contrast, multiple chains with mixed linkages or even branches could prevent deubiquitylation by linkage-specific DUBs, thereby efficiently resisting DUB action.

However, the largest family of DUBs, the ubiquitin-specific proteases (USPs), comprise enzymes that target particular substrates, but show little specificity with regard to the lysine linkage within the ubiquitin chain [41, 69]. By contrast, the ovarian tumor (OTU) family of DUBs comprises members with exquisite linkage specificity. Among the OTU enzymes, we have recently described the first Lys11-specific deubiquitylase, Cezanne [41]. Defining the cellular roles of Cezanne might reveal novel processes involving Lys11 linkages, or strengthen our understanding of the roles of Cezanne in cytokine signaling [42]. Further analysis is needed to determine whether Cezanne inhibits non-degradative Lys11-mediated signaling events [38] or perhaps prevents RIP1 degradation. A further tantalizing question is how Cezanne achieves its
specificity, especially because the related enzymes TNF-associated factor protein-binding domain (TRABID) and A20 do not cleave Lys11 linkages efficiently [41].

Finally, once a Lys11-modified protein arrives at the proteasome, it might be processed differently by proteasomal subunits compared with a Lys48-modified substrate. Both Lys11- and Lys48-linked chains are recognized by the tandem ubiquitin-interacting motif (UIM) domains of the proteasomal subunit S5A [13], but a comparison of binding affinities has not yet been performed. Additionally, other subunits might provide Lys11 binding, for example, the pleckstrin-like receptor for ubiquitin (Pru) domain in RPN13 [70]. Proteasomes contain at least three DUBs that remove ubiquitin chains from substrates. Their intrinsic linkage preference might therefore affect the residence time of a substrate at the proteasome lid [9]. Finally, ubiquitin chain editing by proteasomal E3 ligases might change the topology of the ubiquitin signal [71].

What seems to be important is a quantitative kinetic measurement of the degradation rate of Lys11- versus Lys48- (versus other chain types, versus mixed Lys-) modified substrates, to understand if a linkage-dependent hierarchy of degradation is imposed at the proteasome level. Considering that Lys11-linked chains are used to
direct tightly regulated processes such as the cell cycle, such a hierarchy might indeed exist.

Many other questions about the roles of Lys11 linkages in cellular regulation, and also tantalizing mechanistic and structural questions, remain. Of special note, the identification of linkage-specific ubiquitin binding domains and DUBs could serve as a valuable entry point to unveil additional biological roles of this abundant cellular post-translational modification.

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