POST-TRANSLATIONAL MODIFICATIONS

Atypical ubiquitylation — the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages

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Abstract | Ubiquitylation is one of the most abundant and versatile post-translational modifications (PTMs) in cells. Its versatility arises from the ability of ubiquitin to form eight structurally and functionally distinct polymers, in which ubiquitin moieties are linked via one of seven Lys residues or the amino terminus. Whereas the roles of Lys48- and Lys63-linked polyubiquitin in protein degradation and cellular signalling are well characterized, the functions of the remaining six 'atypical' ubiquitin chain types (linked via Lys6, Lys11, Lys27, Lys29, Lys33 and Met1) are less well defined. Recent developments provide insights into the mechanisms of ubiquitin chain assembly, recognition and hydrolysis and allow detailed analysis of the functions of atypical ubiquitin chains. The importance of Lys11 linkages and Met1 linkages in cell cycle regulation and nuclear factor-κB activation, respectively, highlight that the different ubiquitin chain types should be considered as functionally independent PTMs.

Protein ubiquitylation is a post-translational modification (PTM) in which the 76-amino acid protein ubiquitin (FIG. 1a) is covalently attached via its carboxyl terminus to usually Lys residues in target proteins. Ubiquitin can be attached to proteins as a single entity on one or multiple sites, yielding mono- and multi-monoubiquitylated proteins, respectively (FIG. 1b). Polyubiquitylation is the formation of an ubiquitin chain on target proteins. In a polyubiquitin chain, ubiquitin molecules can be linked through one of the seven ubiquitin Lys residues (which are Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) or through the ubiquitin amino terminal Met1 residue (which generates linear chains) (FIG. 1a). Ubiquitin polymers with a single linkage type are referred to as homotypic chains, whereas heterotypic chains contain mixed linkages within the same chain and can be branched (as a result of ubiquitylation of one ubiquitin at two or more sites; also known as forked chains) or non-branched[1–3] (FIG. 1b).

Ubiquitin chains are assembled by a three-step enzymatic cascade comprising E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligating enzymes[4]. Ubiquitylated substrates are recognized by a large number of proteins containing ubiquitin-binding domains (UBDs), which interact with various surface patches on ubiquitin[5,6] (FIG. 1c), and deubiquitinases (DUBs) reverse this modification by hydrolyzing linkages between ubiquitin moieties or between ubiquitin and the substrate[7,8].

In the current literature, the term polyubiquitin is still a euphemism for only two linkage types, namely Lys48- and Lys63-linked chains. Extensive studies on these chain types have established essential roles in proteasomal degradation for Lys48-linked chains[3] and in cell signalling for Lys63-linked chains[9]. In comparison, very little is known about the remaining 'atypical' chain types linked via Lys6, Lys11, Lys27, Lys29, Lys33 or Met1. However, it is clear that all ubiquitin linkage types coexist in all cell types analysed to date (see below).

Atypical ubiquitin chains are the focus of this Review. We discuss the mechanistic insights into linkage-specific ubiquitin chain assembly, disassembly and binding by ubiquitin-binding proteins and describe the physiological roles of atypical chains that are becoming apparent from recent studies. We further speculate on how to identify linkage-specific enzymes and proteins that regulate atypical chains, as these will be the key to defining the cellular roles of these independently regulated post-translational modifications.

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Ubiquitin chains — what is the difference?

The simplest ubiquitin chain, a diubiquitin, has identical molecular weight, overall charge and chemical composition regardless of how the two ubiquitin moieties are connected. Nevertheless, proteins can distinguish between different types of diubiquitin, posing mechanistic questions about how this can be achieved. It turns out that one key distinguishing feature is the structure of the polymer (FIG. 1d).

To date, five ubiquitin chain types have been structurally characterized by using X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and small angle X-ray scattering (SAXS) (FIG. 1d). The data revealed that different linkage types could be classified as having either 'compact' or 'open' conformations. In the compact conformations of Lys6- (REF. 10), Lys11- (REFS 11–13) and Lys48-linked chains[14,15], the distal (that is, linked via its C terminus) and the proximal (that is, linked via its Lys residue) ubiquitin moieties form an intramolecular interface, whereas in the open conformation of Lys63- (REFS 16,17) and Met1-linked polymers[17] the only contact is the linkage point (FIG. 1d). However, ubiquitin chains are dynamic, and additional structures for Lys48-linked chains[18] and compact forms of Lys63- and Met1-linked chains have been reported[16,19]. Interestingly, most interactions within ubiquitin chains resolved to date can be explained by interactions between two hydrophobic patches, the Ile44 patch and the Ile36 patch (FIG. 1c,d).

The conformational diversity of differently linked ubiquitin chains results in distinct positioning of functional ubiquitin surfaces relative to each other[20] (FIG. 1c). Hence, this conformational diversity might explain how proteins and enzymes discriminate between different linkage types. Other conformations are likely to be revealed with the analysis of Lys27-, Lys29- and Lys33-linked polyubiquitin. These chain types have now been successfully synthesized by using chemical biology approaches (BOX 1), and this will allow their structural characterization. However, the key questions to be addressed remain how DUBs and UBDs bind to polyubiquitin and how they exploit the distinct features of different chains to achieve linkage specificity. This structural information is difficult to obtain, but promises to explain many biological observations.

The abundance of atypical ubiquitin linkages

Initially, Lys48 linkages were assumed to be the only relevant linkage type in cells, as Lys48-linked ubiquitin chains were identified on proteasome substrates[21], and mutation of Lys48, but not of other ubiquitin Lys residues, was found to block the vegetative growth of *Saccharomyces cerevisiae*[22,23]. The presence of Lys63-linked ubiquitin chains in yeast, and the role for this chain type in DNA repair, was first shown in 1995[24], and the non-degradative functions of these polyubiquitin signals in the DNA damage response[25] and in cytokine signalling[26] have now been studied by many groups[9].

The recent application of quantitative mass spectrometry techniques which allow global analysis of total cellular ubiquitin linkages and determination of their relative abundance revealed the surprising observation that indeed all ubiquitin linkages coexist in cells. Although different studies have reported varying abundance for each linkage type (ranging from 10% to 50% for atypical chains)[23,27–30,162], all chain types are present in both yeast and mammalian cells.

Of note, the majority of these studies have been performed on asynchronous cell populations and reflect the distribution of the different linkage types in resting cells. Therefore, these results might not account for specialized functions of individual linkage types that could be regulated in response to particular conditions such as stress. Lys11 linkages are a good example of a linkage type being linked to a particular cellular state.The abundance of this linkage type, which is assembled by the APC/C (anaphase promoting complex; also known as the cyclosome) during mitosis, dramatically rises when cells exit mitosis and the APC/C is activated[13]. In contrast, the abundance of Lys48-linked chains does not change significantly during the cell cycle[13].

This finding highlights that although atypical ubiquitin chains might not be abundant, they may have dedicated physiological roles following activation of their cognate assembly machineries. Moreover, the relative abundance of certain ubiquitin linkages is altered in disease states, including Huntington's disease and Parkinson's disease[27,31]. Hence, it is important to identify and characterize cellular proteins that assemble or utilize atypical ubiquitin chains as well as the circumstances under which these proteins, and their cognate atypical chain, act in cellular regulation.

Assembly of atypical ubiquitin chains

The wealth of data on Lys48- and Lys63-linked polyubiquitin and the recent insights into Lys11- and Met1-linked chains have been facilitated by the identification of assembly systems that generate ubiquitin chains with defined linkages *in vitro* and *in vivo*. For the remaining chain types, specific assembly systems have not been identified, which partly explains why these PTMs have remained poorly characterized.

The minimal requirements for linkage-specific assembly of homotypic ubiquitin chains are processivity in chain assembly and the ability to target a single Lys residue in an acceptor ubiquitin. Targeting of a single Lys residue necessitates donor and acceptor ubiquitin to be spatially close and in the correct orientation. The donor ubiquitin is attached via a thioester linkage to the assembly enzyme, which is either an E2 enzyme or an E3 ligase of the HECT (homologous to the E6-associated protein (E6AP) C terminus) or RBR (Ring between Ring) subfamily. Importantly, the acceptor ubiquitin must also be able to directly bind to the assembly machinery for linkage-specific chain assembly to proceed.

Nevertheless, it is challenging to pinpoint linkage-specific assembly systems from the possible thousands of E2–E3 enzyme combinations that could be formed by the more than 600 E3 ligases and the over 30 E2 enzymes encoded by the human genome. Fortunately, recent insights into the mechanisms of chain assembly allow us to make an educated guess about the most probable candidates for building atypical chains. In particular,

APC/C
(Anaphase promoting complex; also known as the cyclosome). A multi-subunit E3 ligase that targets proteins for proteasomal degradation. Substrates include many cell cycle proteins that need to be degraded during cell cycle progression.

E2-conjugating enzymes and HECT and RBR E3 ligases might be prime suspects to mediate atypical chain assembly.

E2 enzymes. E2-ubiquitin conjugating enzymes are essential for all ubiquitylation events in cells[32,33]. Of the 38 human E2 enzymes, five lack a catalytic Cys, four use ubiquitin-like modifiers[32,33], and at least one (the ubiquitin-conjugating enzyme E2 L3 (UBE2L3; also known as UBCH7)) exclusively discharges on Cys residues[34].

The remaining 28 E2 enzymes can be sub-classified into monoubiquitylating E2s, chain initiating or 'priming' E2s and chain elongating E2s. Monoubiquitylating E2s include UBE2T, which modifies the Fanconi anaemia proteins FANCD2 and FANCI[35–37], and UBE2W, which modifies the E3 ligase CHIP (C terminus of heat shock protein 70 (HSP70)-interacting protein)[32,38].

Priming E2 enzymes attach the first (few) ubiquitin(s) to a substrate prior to chain elongation. UBE2C is a specialized E2 enzyme that acts with the APC/C to prime its substrates[39,40] by recognizing an initiation motif in APC/C substrates[39]. In contrast, the UBE2D (also known as UBCH5) family of enzymes interact with many RING domain-containing E3 ligases and are highly promiscuous as they modify many substrate sequences with all types of linkages[32,40–42].

Several E2 enzymes assemble polyubiquitin processively and with linkage specificity. Five enzymes (namely UBE2K, UBE2R1, UBE2R2, UBE2G1 and UBE2G2) assemble Lys48 linkages, UBE2S assembles Lys11 linkages and UBE2N (also known as UBC13) assembles Lys63 linkages in conjunction with an ubiquitin-binding cofactor, UBE2V1 (also known as UEV1A) or UBE2V2 (also known as MMS2)[32,40,42–44]. Structural studies have revealed distinct mechanisms used by the UBE2N–UBE2V complex and by UBE2S for ubiquitin chain assembly. In the UBE2N–UBE2V complex, the UBE2V cofactor positions the acceptor ubiquitin towards the active site in such a way that only Lys63 is in close proximity to the catalytic Cys residue of UBE2N, thereby determining linkage specificity of the reaction[45–47] (FIG. 2a). UBE2S achieves Lys11 linkage specificity by a distinct mechanism and does not use a cofactor. UBE2S interacts with the TEK box of the acceptor ubiquitin through low-affinity polar contacts. Although this positions the Lys11 side chain towards the active site, the key to achieving specificity lies in the TEK box residue Glu34, which complements the active site of UBE2S in a mechanism of substrate assisted catalysis[46] (FIG. 2b).

Therefore, E2 enzymes are well suited to assemble ubiquitin chains in a linkage-specific manner. Are enzymes mediating the assembly of the elusive atypical chains to be found among the remaining 14 human E2 enzymes? Mass-spectrometric analysis of tagged E2 enzymes that were autoubiquitylated *in vitro* did not reveal clear E2 candidates that mediate the assembly of Lys6-, Lys27-, Lys29- or Lys33-linked chains; however, this analysis also showed UBE2S as being non-specific[48]. It is possible that the less studied E2 enzymes form complexes with ubiquitin-binding proteins, which assist in forming ubiquitin chains, analogous to the UBE2N–UBE2V complex.

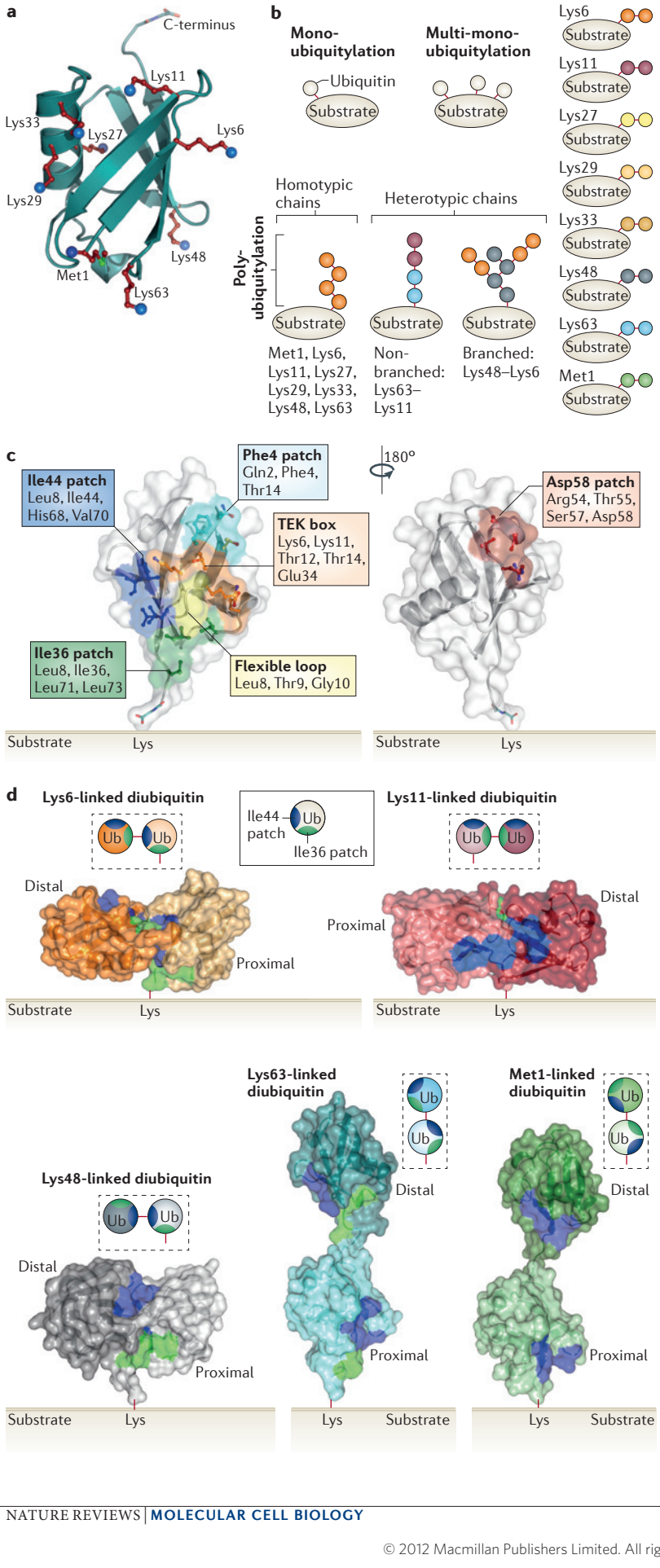
HECT E3 ligases. The 28 human HECT E3 ligases form a small subfamily within the family of E3 ligases, and many of them have important cellular functions[49]. In the ubiquitylation reaction mediated by HECT E3 ligases, a thioester-linked HECT~ubiquitin intermediate is formed by the transfer of ubiquitin from the E2 enzyme to a catalytic Cys of the HECT E3 ligase (FIG. 2c). This process effectively allows HECT-mediated chain assembly to be independent of the E2-encoded linkage specificity. Yet, many HECT E3 ligases have intrinsic linkage specificity. Human E6AP (also known as UBE3A) exclusively assembles Lys48 linkages, whereas yeast Rsp5 (reverses SPT-phenotype 5), its human orthologues NEDD4 (neural precursor cell expressed developmentally downregulated protein 4) and NEDD4L (NEDD4-like) (see below) and human ITCH (E3 ubiquitin protein ligase Itchy homologue) assemble Lys63 linkages *in vitro*[50]. Other members of the HECT E3 ligase family have been associated with the assembly of atypical chain types. UBE3C was implicated in the assembly of Lys29 linkages and Lys48 linkages *in vitro*, whereas ITCH was suggested to ubiquitylate substrates with Lys27-, Lys29- and Lys33-linked chains *in vivo*[51–54]. Many HECT E3 ligases have not been studied with regards to their linkage specificity.

Structurally, HECT domains comprise a larger N-lobe which is connected to a smaller C-lobe by a short linker that provides interdomain flexibility[55]. The C-lobe contains the catalytic Cys residue that receives ubiquitin from the E2 enzyme in a transthiolation reaction. Biochemical analysis has shown that the C-lobe also comprises the determinants for linkage specificity. Domain swapping experiments showed that the 50 C-terminal residues of the HECT domain were sufficient to convert a Lys63-specific ligase into a Lys48-specific ligase[50].

Figure 1 | **Forms of ubiquitylation. a** | The seven Lys residues and Met1 of ubiquitin (Protein Data Bank identifier (PDB ID): 1UBQ[160]) are shown in stick representation, and amino groups that are modified with ubiquitin during chain formation are shown as blue spheres. The ubiquitin carboxyl terminus is also indicated. **b** | Overview of the various forms of ubiquitylation. A substrate can be modified by mono-, multi-mono- or polyubiquitin. Ubiquitin chains are coloured according to linkage-type, and each type of chain represents a distinct post-translational modification. Polyubiquitin can contain a single type of linkage (called homotypic chains, exemplified by a Lys6-linked chain in the schematics) or more than one linkage type (termed heterotypic chains). Heterotypic chains are either branched (also termed 'forked') or non-branched. **c** | The structure and surface of ubiquitin (PDB ID: 1UBQ[160]) is shown in two orientations, and residues involved in forming surface patches that mediate interactions with binding partners (including ubiquitin-binding domains (UBDs) and deubiquitinases (DUBs)) are indicated. These include the Ile44 patch, the Ile36 patch, the Phe4 patch, the Asp58 patch, the TEK box and a flexible loop. **d** | Crystal structures of Lys6-, Lys11-, Lys48-, Lys63 and Met1-linked diubiquitin (PDB IDs: 2XK5, 3NOB, 1AAR, 2JF5 and 2W9N[10,12–14,17]) are shown (coloured as in **b**) and oriented in relation to substrate. Distal and proximal moieties are indicated. Ile44 and Ile36 patches explain all interface interactions.

RING domain
Domain of approximately 70 amino acids with conserved Cys and His residues that coordinate two zinc ions. U-box domains share the RING fold but do not bind zinc.

TEK box
A structural motif on the surface of ubiquitin that consists of Lys6, Lys11, Thr12, Thr14 and Glu34 and is required for UBE2C- and UBE2S-mediated assembly of Lys11-linked ubiquitin chains.



Structural work is getting closer to elucidating the mechanisms underlying chain specificity in HECT domains. The structure of the NEDD4 HECT domain bound to ubiquitin-charged UBE2D revealed a direct interaction between the donor ubiquitin and the HECT domain[56], and this interaction is mediated by the Ile36 patch of ubiquitin and the HECT C-lobe. This complex seems to be poised for transthiolation by placing the HECT and E2 catalytic residues in close proximity[56] (FIG. 2c).

It was already known that the N-lobe of HECT domains can non-covalently interact with ubiquitin, but it was unclear whether this interaction promoted or inhibited chain elongation[57,58]. The crystal structures of Rsp5 and NEDD4L HECT domain in complex with ubiquitin provide a molecular description of the ubiquitin-binding site in the N-lobe, and this non-covalent binding site was shown to be essential for ligase processivity[59,60]. However, the N-lobe ubiquitin-binding site is remote from the catalytic Cys and unlikely to bind the acceptor ubiquitin directly. It is possible that a third ubiquitin bridges the gap between the N-lobe and the donor ubiquitin, and this third ubiquitin, which could interact with the specificity-determining C terminus, might serve as the real acceptor in a chain assembly reaction (FIG. 2c). This is reminiscent of E2 enzymes that harbour an additional ubiquitin-binding site, which is important for chain assembly but is too remote to be targeted by the E2 in *cis*[44]. Further structural insights are required to fully understand HECT domain-mediated specificity.

RBR E3 ligases. E3 ligases of the RBR family comprise a canonical RING domain, an in-between RING (IBR) domain and a RING2 domain, and this module is present in 18 human enzymes[61]. The best studied proteins in this family include parkin, an E3 ligase that is involved in Parkinson's disease and mitochondrial maintenance (see below)[62], and HOIP (haeme-oxidized IRP2 ubiquitin ligase 1L (HOIL1L)-interacting protein; also known as RNF31), which is the catalytically active component of the linear ubiquitin chain assembly complex (LUBAC; see below)[63]. LUBAC exclusively assembles linear ubiquitin chains in an E2-independent fashion as it is able to override the intrinsic specificity of, for example, the Lys48-specific E2 enzyme UBE2K[64].

Mechanistic insights into RBR ligase-mediated chain assembly come from the finding that some RBR E3 ligases, including HHARI (human homologue of ariadne; also known as ARIH1), act as RING–HECT hybrids. For these ligases it was found that the first RING domain of the RBR module does not directly discharge an E2-bound ubiquitin onto a substrate but instead onto a Cys residue in the RING2 domain[34] (FIG. 2d). The conservation of the RING2 domain that often contains a suitable acceptor Cys suggests that most RBR domains follow such a hybrid mechanism.

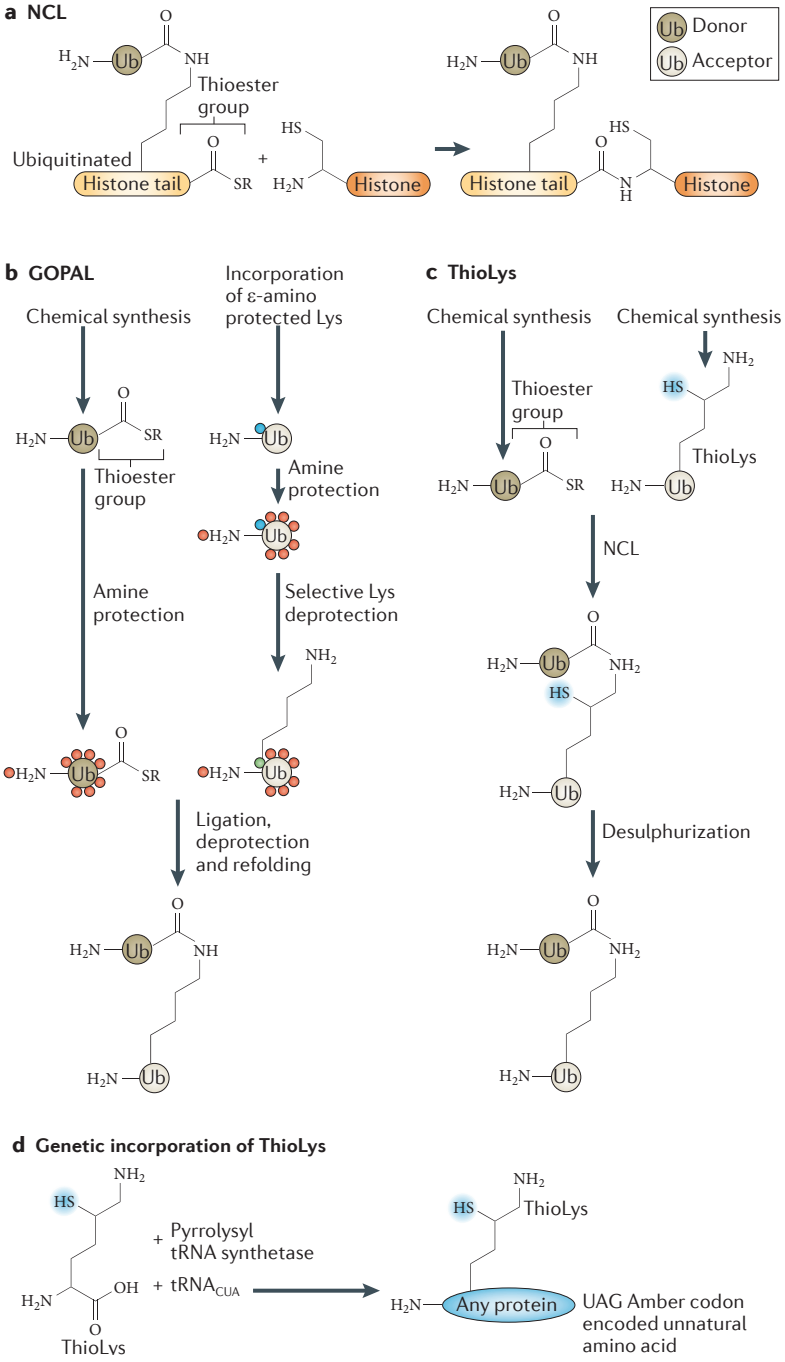
Therefore, an RBR~ubiquitin intermediate can act in an E2-independent fashion just like a HECT domain. We currently lack molecular insights into RBR ligase mechanisms, as members of this ligase

Box 1 | **Chemical biology methods for site-specific protein ubiquitylation**

The chemical stability and folding properties of ubiquitin (Ub), and the fact that it does not contain Cys residues make it amenable to chemical biology techniques. Several methodologies have been reported to direct site-specific ubiquitylation of Lys residues[140–143]. These methods, however, require mutation of the ubiquitin carboxyl terminus or otherwise result in the generation of non-isopeptide linkages between ubiquitin moieties, which could affect polyubiquitin structure.

The first traceless chemical method to generate monoubiquitylated histone tails relied on multiple rounds of native chemical ligation (NCL), in which a histone tail peptide is first ubiquitylated and then ligated to the remaining histone[144,145] (see the figure, part **a**). Two recent methods allow assembly of ubiquitin dimers with isopeptide linkages[10,146–148]. Both methods attach a donor ubiquitin with a C-terminal thioester reactive group (obtained, for example, by chemical synthesis) to a predetermined Lys residue in an acceptor ubiquitin (see the figure, parts **b**,**c**). In the first method termed GOPAL (genetically encoded orthogonal protection and activated ligation) (see the figure, part **b**), the acceptor ubiquitin features a chemically protected Lys residue (depicted in blue) that is encoded by genetic code expansion. Subsequent orthogonal protection of all other amine groups (depicted in orange) and chemoselective deprotection of the encoded Lys generates an ubiquitin with a single acceptor Lys (shown in green) that is ligated to the thioester of the donor ubiquitin, thus creating an isopeptide bond. Deprotection of all remaining amine groups and refolding of the diubiquitin results in native diubiquitin chains[10]. The second method, named ThioLys incorporation [146–148], utilizes a distinct Lys derivative which is obtained by chemical synthesis. The Lys side chain in ThioLys features an additional thiol group on the δ-carbon (highlighted by blue shading)[149] (see the figure, part **c**). In a reaction analogous to NCL, this amine group can be modified by a thioester under protein-friendly conditions and does not require unfolding or refolding. Desulphurization using a mild radical reaction removes the thiol group[146–148], yielding a native isopeptide bond.

Incorporation of ThioLys in ubiquitin has been performed in a tour-de-force to generate the entire ubiquitin molecule by peptide synthesis[146–148]. The recent advance of genetically encoding ThioLys derivatives instead of an Amber stop codon (UAG) in native proteins[150] (see the figure, part **d**), using a modified pyrrolysyl tRNA synthetase–tRNA pair, will allow site-specific ubiquitylation of proteins and help to address open mechanistic and structural questions concerning ubiquitylated proteins.



Coiled coil
Structural motifs that are formed by amino acids arranged in a heptad repeat in which positions 1 and 4 of the repeat are hydrophobic amino acids (Ile, Leu or Val). These domains are present in many proteins and confer them with dimerization or oligomerization propensities.

subfamily have not yet been crystallized. The fact that LUBAC is a processive linear ligase which assembles free chains *in vitro* suggests that this complex carefully positions the acceptor ubiquitin; however, the molecular basis for this ubiquitin-binding event is currently unclear (FIG. 2d).

RING- and U-box-containing E3 ligases. The vast majority of E3 ligases comprise a RING domain or a structurally related U-box domain that promotes discharge of E2~ubiquitin complexes, but usually does not alter E2-intrinsic linkage specificity in chain formation[65]. Despite their abundance and physiological relevance, we still do not fully understand their mechanism. Considering that the RBR subclass of RING E3 ligases was only discovered last year, it is possible that other, mechanistically distinct subclasses of RING E3 ligases exist. For example, the approximately 80 members of the TRIM (tripartite interaction motif) family of E3 ligases comprise a conserved module of a RING domain, a B-box (a zinc-binding fold with RING similarity) and a coiled-coil domain[66], suggesting common and perhaps

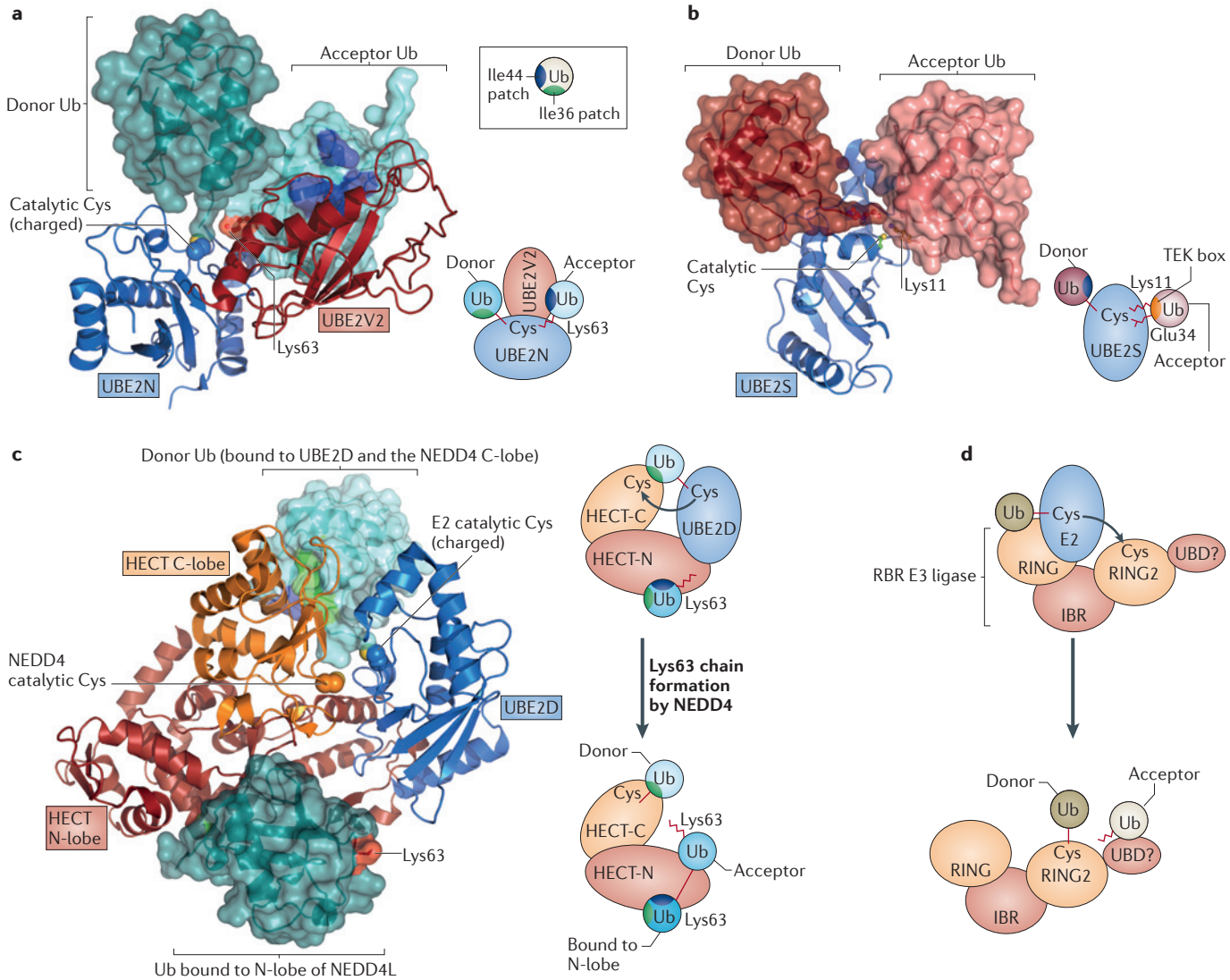


Figure 2 | **Mechanisms of ubiquitin chain assembly.** **a** | Structure of charged ubiquitin-conjugating enzyme E2 N (UBE2N) in complex with ubiquitin-conjugating enzyme E2 variant 2 (UBE2V2; also known as MMS2) (Protein Data Bank identifier (PDB ID): 2GMI[47]) explains how the complex assembles Lys63-linked chains. Crystal lattice contacts reveal the mechanism of chain assembly, in which UBE2V2 binds to the acceptor ubiquitin (Ub), orienting the Lys63 side chain of the acceptor ubiquitin towards the catalytic Cys of UBE2N. The cartoon on the right shows a simplified illustration of the complex. **b** | Nuclear magnetic resonance-derived docking model of UBE2S bound to donor and acceptor ubiquitin[46]. The donor ubiquitin is covalently bound to UBE2S via its Ile44 patch. The acceptor ubiquitin binds through its TEK box, and Glu34 complements the active site of the E2. **c** | Composite model that combines the crystal structure of the E3 ligase NEDD4 (neural precursor cell expressed developmentally downregulated protein 4) bound to ubiquitin-charged UBE2D (PDB ID: 3JW0[56]) via the NEDD4 HECT C-lobe (HECTC, located at the carboxyl terminus), and the crystal structure of NEDD4-like (NEDD4L) with ubiquitin bound to the N-terminal HECT N-lobe (PDB ID: 2XBB[60]). A large gap between donor ubiquitin and N-lobe ubiquitin, and an unfavourable orientation of Lys63 on the N-lobe-bound ubiquitin suggests that perhaps a third ubiquitin molecule is the real acceptor in the Lys63-specific chain assembly reaction, as illustrated in the cartoon to the right. **d** | Model for ubiquitin chain assembly by RBR (Ring between Ring) E3 ligases. Charging of the RBR RING2 domain enables the transfer of ubiquitin molecules to substrates in an E2-independent manner. It is possible that RBR ligases may use UBDs to orient the substrate (which is the acceptor ubiquitin) for specific linkage assembly. IBR, in-between RING; UBD, ubiquitin-binding domain.

distinct mechanisms of ubiquitin chain assembly. In addition, RING E3 ligases harbouring UBDs to position the acceptor ubiquitin could in principle also result in defined chain types. Interestingly, only approximately 30 RING E3 enzymes contain additional UBDs (K. Hofmann, personal communication).

In conclusion, we believe that E2-conjugating enzymes and subclasses of E3 ligases, in particular the HECT and RBR families of enzymes, are good candidates for linkage-specific assembly of atypical chains. Another way to assemble specific ubiquitin chains is to combine a promiscuous ligase with a DUB that cleaves

all but one chain type. Such DUB–E3 complexes could enrich defined (atypical) ubiquitin chains on substrates.

What hydrolyses atypical ubiquitin chains?

Removal of ubiquitin chains from substrates contributes to tight regulation of the ubiquitin system and is functionally important. Roughly 80 genes encoding catalytically active DUBs have been annotated in the human genome[8], and many studies have highlighted their important roles in cellular regulation[7,67] and their great potential in providing future drug targets[68,69]. DUBs belong to five structurally distinct families: ubiquitin specific proteases (USPs; comprising more than 50 members), ovarian tumour proteases (OTU; comprising 14 members), ubiquitin C-terminal hydrolases (UCH; comprising 4 members) and Machado–Joseph disease proteases (also known as Josephins; comprising 4 members)) are cysteine proteases, whereas JAB1/MPN/MOV34 metalloenzymes (JAMMs, comprising 10 members) are zinc metalloproteases[8].

In accordance with the complexity of the ubiquitin modification itself (FIG. 1b), DUBs rely on numerous layers of specificity[8]. With regards to linkage specificity, comprehensive studies have only recently been possible owing to the availability of diubiquitins of all linkage types as *in vitro* reagents. These reagents have since been used to reveal that 12 human USPs cleave most ubiquitin chain types[70], suggesting that USP DUBs are substrate- rather than linkage-specific. For the remaining DUB families, such comprehensive analysis has not been performed, however available evidence suggests that JAMM family DUBs are Lys63-specific[71,72], whereas UCH enzymes are unable to efficiently cleave ubiquitin chains[73].

Several mechanistic concepts have been postulated to explain the molecular basis of DUB linkage specificity, and as for the assembly reaction, careful positioning of the proximal ubiquitin seems to be the key to specificity. The Lys63-specific JAMM enzyme AMSH-like protease (AMSHLP) recognizes the proximal ubiquitin and the isopeptide bond by mediating direct contacts with residues adjacent to Lys63 (REF. 71) (FIG. 3a). As the sequence context of each Lys in ubiquitin is unique, this method could also be used by other DUBs[8].

A particularly interesting family of DUBs, with regards to linkage specificity, is the OTU family of enzymes. Several members seem to have a defined linkage specificity *in vitro*: the OTU domain-containing protein 7B (also known as cezanne 1) cleaves Lys11 linkages[12], tumour necrosis factor receptor (TNFR)-associated factor (TRAF)-binding domain-containing protein (TRABID; also known as ZRANB1) cleaves Lys29- and Lys33-linked chains[74], and OTUB1 cleaves Lys48 linkages[75]. TRABID was initially reported to be Lys63-specific[76], yet it was later found that Lys29 linkages and Lys33 linkages are targeted by this enzyme with 40-fold higher activity than Lys63 (REFS 10,74). The structure of TRABID suggests a new mechanism in which an UBD that contains ankyrin repeats (AnkUBD) and abuts the catalytic core positions the proximal ubiquitin, facilitating cleavage of Lys29- and Lys33-linked chains but not other chain types[74] (FIG. 3b). The mechanism of action of TRABID is conceptually similar to that of the Lys48-specific OTUB1, in which an N-terminal helix that forms following ubiquitin binding helps positioning the proximal ubiquitin relative to the active site[77,78] (FIG. 3c). However, OTUB1 function also requires an extensive proximal ubiquitin-binding site on the OTU domain itself (FIG. 3c).

Further detailed analysis of DUB–substrate complex structures are required to understand how DUBs target atypical ubiquitylation events. Nevertheless, identification of linkage-specific DUBs provides an ideal starting point to study the role of atypical chains in cellular regulation, and linkage-specific DUBs may prove to be valuable tools in ubiquitin research.

Physiological roles of atypical chains

Whereas functions for Lys48- and Lys63-linked ubiquitin chains have been extensively defined, data on the remaining ubiquitin chain types has only recently started to accumulate (FIG. 4). Most of the available data relies on overexpression of ubiquitin mutants in which Arg substitutes either a single Lys or all but one Lys residue. However, Lys mutations affect ubiquitin surfaces and might hence influence ubiquitin chain structure, binding by UBDs and DUBs or even prevent linkage assembly. Lys6 is of particular interest, as it is located within the ubiquitin TEK box[40] (FIG. 1c). Mutation of this residue prevents assembly of Lys11-linked ubiquitin chains by UBE2S[46] (FIG. 2b) and assembly of heterotypic Lys11–Lys63 ubiquitin chains by the Kaposi sarcoma virus E3 ligase K5 (REF. 79). These indirect roles of Lys6 in ubiquitin chain assembly highlight some of the caveats in using ubiquitin mutants to study chain linkages.

Fortunately, several technical advances and new tools (including mass spectrometry, linkage-specific antibodies and ubiquitin chain sensors) are emerging to circumvent these problems, thereby accelerating the study of ubiquitin linkage types (BOX 2). With these new tools at hand, Met1- and Lys11-linked polyubiquitin have been firmly connected to nuclear factor-κB (NF-κB) signalling[80] and cell cycle regulation[81], respectively. Data on the biological roles of the remaining four linkage types are sparse, yet several single reports have associated these PTMs with interesting, independent cellular roles that require further study, as discussed below.

Lys6-linked polyubiquitin — a non-degradative signal?

Little is known about Lys6-linked chains, which are not very abundant in resting cells. The breast cancer type 1 susceptibility (BRCA1)– BRCA1-associated RING domain protein 1 (BARD1) complex, which is a heterodimeric RING E3 ligase complex involved in DNA repair pathways, was reported to assemble Lys6 linkages on itself[82–84] and on its substrates nucleophosmin (NPM)[85], CTIP (also known as RBBP8)[86] and the RNA polymerase subunit RPB8 (REF. 87). Lys6-polyubiquitinylated BRCA1 can be recognized by RAP80 (receptor-associated protein 80)[88], further linking this chain type to the DNA damage response. It is also recognized by p97 (also known as valosin-containing protein (VCP) or Cdc48

Cysteine proteases
Enzyme that use a nucleophilic Cys residue in the catalytic centre. The catalytic triad contains an adjacent His residue to deprotonate the Cys thiol and a polar residue to correctly position the His.

Zinc metalloproteases
Proteases that have a zinc-binding motif in the catalytic centre to coordinate zinc. During catalysis the zinc promotes nucleophilic attack.

p97
A hexameric AAA+ ATPase that exerts mechanical force on ubiquitylated cargo to, for example, dislocate proteins from membranes or disassemble complexes.

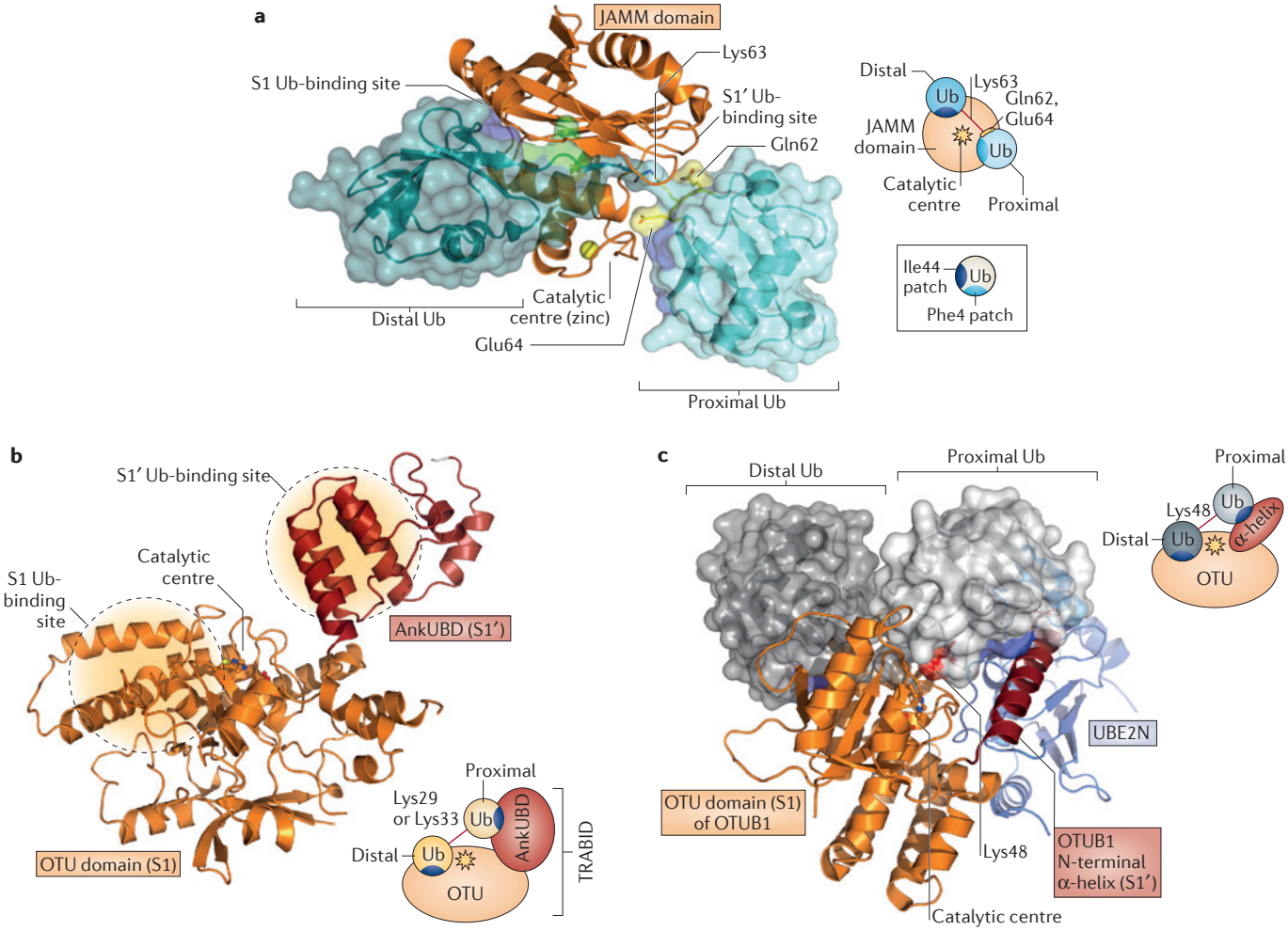


Figure 3 | **Mechanisms of ubiquitin chain hydrolysis. a** | Structure of the deubiquitinase (DUB) AMSHLP (associated molecule with the SH3 domain of STAM-like protein) JAMM domain (orange) bound to Lys63-linked diubiquitin (Protein Data Bank identifier (PDB ID): 2ZNV[71]). Residues adjacent to Lys63, Gln62 and Glu64 are coloured yellow. A cartoon on the right shows the interactions schematically. The interaction of the distal ubiquitin (Ub) with the enzymatic S1 substrate binding site (shown in the structure) through the Ile44 patch and the interaction of the enzymatic S1' site (shown in the structure) with the proximal ubiquitin through the Phe4 patch are indicated. **b** | Structure of the extended TRABID (TRAF-binding domain-containing protein) catalytic domain, showing the adjacent ankyrin-repeat ubiquitin binding domain (AnkUBD; shown in dark red) and the ovarian tumour (OTU) domain (shown in orange) (PDB ID: 3ZRH[74]). Two ubiquitin-binding sites (indicated in yellow) can be inferred, one at the OTU domain (S1) and one at the AnkUBD (S1'). Both sites interact with the Ile44 patch of ubiquitin moieties, suggesting that the AnkUBD helps position Lys29- or Lys33-linked diubiquitin across the active site[74]. **c** | Complex structure of OTUB1 and ubiquitin-conjugating enzyme E2 N (UBE2N), each bound to ubiquitin (PDB ID: 4DHZ[77]). Ubiquitin bound to OTUB1 resides in the S1 site of the enzyme. Direct binding of OTUB1 to ubiquitin-charged UBE2N positions the UBE2N ubiquitin into the S1' ubiquitin-binding site, forming a pseudosubstrate complex. Lys48 of the proximal ubiquitin is adjacent to the active site of OTUB1, explaining the specificity of OTUB1 for this chain type. An amino terminal α-helix (which is absent in the apo structure of OTUB1[75]) forms an interaction interface for the Ile44 patch of the proximal ubiquitin. The positions of the ubiquitin molecules is identical to that of OTUB1 in complex with ubiquitin-charged UBE2D[78]. The star indicates the catalytic centre of the enzymes.

in yeast) cofactor UBXN1 (also known as SAKS1), which negatively regulates the E3 ligase activity of the BRCA1–BARD1 complex[89] (FIG. 4a). A key outstanding experiment that would solidify a link to the DNA damage response is the analysis of the abundance of Lys6 linkages after DNA damage.

Functional consequences of Lys6 modification are currently unclear. Recent mass spectrometry analysis suggests that similarly to Lys63 chains, the abundance of Lys6 linkages does not increase following proteasome inhibition[29,30], suggesting non-proteolytic roles. Our structural analysis suggests that this chain type might form a helical filament with unique binding properties, as the hydrophobic Ile44 and Ile36 patches are engaged in intra-chain contacts[10] (FIG. 1d). Such a unique structure would support non-proteolytic scaffolding roles for Lys6 linkages in cells, however, this hypothesis requires further investigation. As for the other unstudied linkage types, the generation of a linkage-specific antibody (BOX 2) would be most valuable.

Box 2 | **Technical advances in ubiquitin chain research**

Mass spectrometry. Mass spectrometric analysis allows the identification of ubiquitylation sites, as tryptic peptides increase in mass by 110 Da due to the Gly–Gly motif. Ubiquitin absolute quantification (Ubiquitin-AQUA) mass spectrometry applications allow quantification of each ubiquitin linkage[23,151,162]. Combination of two-dimensional gel electrophoresis with mass spectrometry allows identification of chain types present on a protein[110]. Antibodies directed against Gly–Gly-modified Lys peptides allow enrichment of ubiquitylated peptides prior to analysis[29,30]. Some caveats however apply[151,152].

Linkage-specific antibodies . Antibodies detecting Lys11-, Lys48-, Lys63- and Met1-linked chains have been generated[13,153,154] to dissect the biological roles of these chain types. Developing antibodies directed against the remaining ubiquitin chain types will be important to unveil cellular functions for the unstudied atypical chains.

Ubiquitin sensors. Engineered ubiquitin-binding domains (UBDs) that contain tandem ubiquitin binding entities (TUBEs)[155] and that exhibit high affinity for polyubiquitylated proteins can be used as ubiquitin affinity resins. This together with the use of linkage-specific UBDs[118,122,156] allows enrichment of particular linkage types, identification of substrates and linkage type on substrates, as well as monitoring ubiquitin dynamics *in vivo*[157,158].

Ubiquitin replacement strategies. Mutating endogenous ubiquitin is feasible in *Saccharomyces cerevisiae*[22,23] but not in mammalian cells, in which ubiquitin is essential for cell viability. The ubiquitin replacement strategy, which involves knockdown of endogenous ubiquitin in mammalian cells with subsequent replacement by inducible expression of desired ubiquitin mutants[106], will be an exciting tool in the analysis of the remaining atypical ubiquitin chains. However, the cellular stress of depleting ubiquitin limits the application of this method.

Bacteria as a test tube. A synthetic biology approach reconstituting the eukaryotic ubiquitylation cascade in bacteria is a method with great potential to produce large quantities of ubiquitylated proteins and to study minimal requirements to achieve site-specific ubiquitylation[159].

Lys11 linkages — a second degradation signal. Recent work has implicated Lys11-linked chains to act as a proteasomal degradation signal in cell cycle regulation[90]. Indeed, Lys11 linkages are enriched when the proteasome is inhibited[23,29,30]. Long Lys11 polyubiquitin chains (that is, 6–13 moieties) are assembled by the APC/C in a single substrate binding event[40,46,91] (FIG. 4b), and modified proteins are rapidly degraded by the proteasome[40,162]. The importance of Lys11 linkages in cell cycle progression was highlighted by a Lys11-specific antibody[13], which showed that Lys11 linkages but not Lys48 linkages increased when the APC/C is active. It is not yet clear why two independent proteasomal degradation signals (that is, Lys48- and Lys11 chains) have evolved to coexist and whether and how the proteasome distinguishes chain types.

Although the mechanisms of Lys11-chain assembly and the substrates of the APC/C are becoming well defined, virtually nothing is known about proteins that recognize this chain type. A linkage-specific UBD for Lys11-chains has not yet been identified. We have described the first Lys11-specific DUB, cezanne[12]. Cezanne does not seem to be involved in cell cycle regulation but has been linked to epidermal growth factor receptor (EGFR) trafficking and cell motility, although Lys11 linkages were not implicated in these processes[92].

Several reports have identified Lys11 linkages in other cellular processes, including endoplasmic reticulum associated degradation (ERAD), membrane trafficking[79,93] and TNFα signalling[41] (see below), in which these linkages mostly act in conjunction with other chain types[2,11]. We anticipate that Lys11-linked ubiquitin chains will become important players in ubiquitin biology (FIG. 4b).

Lys27 linkages in mitochondrial biology. Chains that are formed by Lys27, Lys29 and Lys33 of the ubiquitin helix are more challenging to detect by mass spectrometry and show low abundance in resting cells. Linkage-specific antibodies or other tools have not been developed, and ligases that assemble these chain types *in vitro* are unknown. Nevertheless, these atypical chains have been assigned independent and intriguing cellular roles; however, all of these identified functions require further study.

Several findings suggest a role for Lys27 linkages in mitochondrial biology. Mitochondrial damage leads to mitochondrial depolarization, resulting in translocation and activation of the RBR E3 ligase parkin, which assembles Lys27-linked polyubiquitin on several mitochondrial proteins, including voltage-dependent anion-selective channel protein 1 (VDAC1)[94,95]. Ubiquitylated VDAC1 is recognized by the autophagy adaptor p62, thus triggering clearance of damaged mitochondria by mitophagy (FIG. 4c). Interestingly, the Josephin DUB ataxin 3 is able to hydrolyse the atypical chains assembled on parkin[96]. Dysfunctional mitochondria are central to neurodegenerative disorders, and mutations in parkin cause early-onset Parkinson's disease. It would be exciting if Lys27-linked polyubiquitin had dedicated roles in mitochondrial maintenance or mitophagy.

ITCH and Lys27, Lys29 and Lys33 linkages in T cells. In T cells, the ζ-chain of TCR (T cell antigen receptor) undergoes ITCH- and CBLB (casitas B-lineage lymphoma proto-oncogene B)-dependent Lys33-linked polyubiquitylation[53]. Lys33-linked ubiquitylation of TCRζ leads to reduced phosphorylation of the receptor subunit and inhibits association of the activating kinase ZAP70 (ζ-chain-associated protein of 70 kDa) with the receptor, thus dampening TCR signalling through a non-degradative mechanism (FIG. 4e). Mice lacking both ITCH and CBLB show increased T cell activation and develop spontaneous autoimmunity as a result[53].

ITCH has further roles in T cells as it was reported to assemble Lys27-linked ubiquitin chains on the transcription factor TIEG1 (transforming growth factor-β (TGFβ)-inducible early growth response protein 1). TIEG1 is essential for the development of TGFβ-induced regulatory T cells (Treg). Phosphorylation of TIEG1 induces its ubiquitylation with Lys27 linkages, which inhibits its nuclear translocation[52] (FIG. 4c). ITCH has also been suggested to catalyse Lys29-linked chains on Deltex and Notch receptor to target them to lysosomes[54].

The reported roles for ITCH in assembling atypical ubiquitin chains are in contrast to its *in vitro* specificity for Lys63-linkage assembly, perhaps suggesting further layers of regulation of linkage specificity in cells.

Lys29 linkages and Lys33 linkages — degradative or not? Several reports have identified roles for both Lys29- and Lys33-linked chains in different processes, such as the regulation of the AMPK (AMP-activated protein

Mitophagy
The selective degradation of mitochondria by autophagy.

kinase)-related protein kinases. Members of this family are polyubiquitylated with Lys29 linkages and Lys33 linkages, and although these modifications inhibit kinase activity, they do not lead to degradation of the kinase[97]. The assembly machinery that mediates this process is still unknown.

Interestingly, TRABID shows specificity for hydrolyzing the same Lys29 and Lys33 linkage-combination[10,74] (see above). Expression of an inactive version of TRABID in cells results in its localization to cytosolic puncta enriched in atypical chains[74]. As the appearance of these puncta was unaffected by proteasome inhibitors[74], this may also indicate that these linkage types are inefficient proteolytic signals.

TRABID had been connected to the WNT–β-catenin signalling pathway[76], but substrates remain poorly defined. The HECT E3 ligase EDD (E3 ubiquitin ligase identified by differential display; also known as UBR5) was suggested to ubiquitylate β-catenin with Lys29- or Lys11-linked ubiquitin chains[98], however, whether this leads to stabilization[98] or destabilization[99] of β-catenin is unclear.

Lys29 linkages are likely to also signal to the proteasome, as the chain type is enriched following proteasome inhibition[29,30]. In addition, ubiquitin chains containing Lys29 linkages are formed on substrates of the ubiquitin-fusion degradation (UFD) pathway by the cooperative action of the HECT ligase Ufd4 and the RING ligase Ubr1 (REFS 100,101) (FIG. 4d). Furthermore, Ufd2 functions as an E4 enzyme, which elongates ubiquitin chains on UFD substrates with Lys29 linkages[102], implying a role for Lys29 linkages in cellular proteostasis. Thus, Lys27-, Lys29- and Lys33-linked chains seem to have many roles in cells, but all functions require further characterization.

Atypical ubiquitylation in TNFR signalling. TNFα is a multifaceted cytokine that, in a context dependent manner, evokes different responses in cells, including innate immune responses, cell survival, cell death and inflammation[103]. The different outcomes originate from the TNFR signalling complex (TNFRSC), which consists of adaptor proteins, ubiquitin ligases and protein kinases and which is assembled at the activated TNFR[104]. NF-κB activation downstream of the TNFRSC requires polyubiquitylation of several substrates, and the ubiquitylated substrates then serve as a platform for the recruitment and activation of protein kinase complexes, including TAK1 (TGFβ-activated kinase 1) and IKK (inhibitor of κB kinase)[105] (FIG. 5).

Until recently, it was thought that Lys63-linked chains are the only chain type required for NF-κB activation downstream of TNFR. An elegant approach using a ubiquitin replacement strategy (BOX 2) demonstrated that TNFα-mediated NF-κB activation did not solely depend on the Lys63 residue of ubiquitin[106]. This finding, combined with the observation that the only known Lys63-specific E2 enzyme UBE2N was not required for TNFα-dependent NF-κB activation, suggests that additional chain types are required for NF-κB activation[106,107]. Data from several laboratories showed that in addition to Lys63-linkages, several atypical chains contribute to NF-κB signalling[41,108], and recent work has highlighted important roles for Met1-linked chains in NF-κB activation[63].

Met1-linked ubiquitin chains in NF-κB activation. Met1-linked ubiquitin chains are the precursors of polyubiquitin in all eukaryotic cells, as ubiquitin is first translated as a linear polymer and then post-translationally cleaved to generate monoubiquitin. Surprisingly, a 600 kDa E3 ligase complex was shown to specifically assemble this chain type[64]. This complex, called LUBAC, comprises catalytically active HOIP and two adaptor proteins, termed HOIL1L (also known as RBCK1)[64] and, as was shown recently, sharpin (SHANK-associated RH domain-interacting protein)[109–111] (FIG. 5).

The exquisite specificity of LUBAC suggests unappreciated physiological roles for Met1-linked ubiquitin chains[64], and LUBAC and this chain type have now been firmly linked to NF-κB activation. It was shown that LUBAC is recruited to TNFα and CD40 receptor complexes following receptor activation. The generation of Lys63-, Lys11- and Lys48-linked ubiquitin chains on receptor components by the E3 ligases cIAP1 (cellular inhibitor of apoptosis 1) and cIAP2 (REFS 110,112), and their recognition by the UBDs of LUBAC are a prerequisite for this recruitment event[41] (FIG. 5). In this complex, LUBAC is thought to ubiquitylate NEMO (NF-κB essential modulator; also known as IKKγ) and other proteins[110,111].

The significance of linear ubiquitylation mediated by LUBAC in TNFα signalling is underscored by genetic studies. Loss of sharpin deregulates linear chain formation in tissues, leading to cell death[110,111]. Mice with chronic proliferative dermatitis (*cpdm*)[113], an inflammatory disorder that arises from defective activation of the NF-κB pathway, harbor a mutation in the *Sharpin* gene. Importantly, *cpdm* mice could be rescued by simultaneous deletion of TNFR, revealing the interplay between TNFα signalling and linear ubiquitin chains in this pathway[110]. Although several questions still need to be addressed, it seems that removing linear linkages in this signalling pathway switches the cellular response from cell survival to cell death and inflammation.

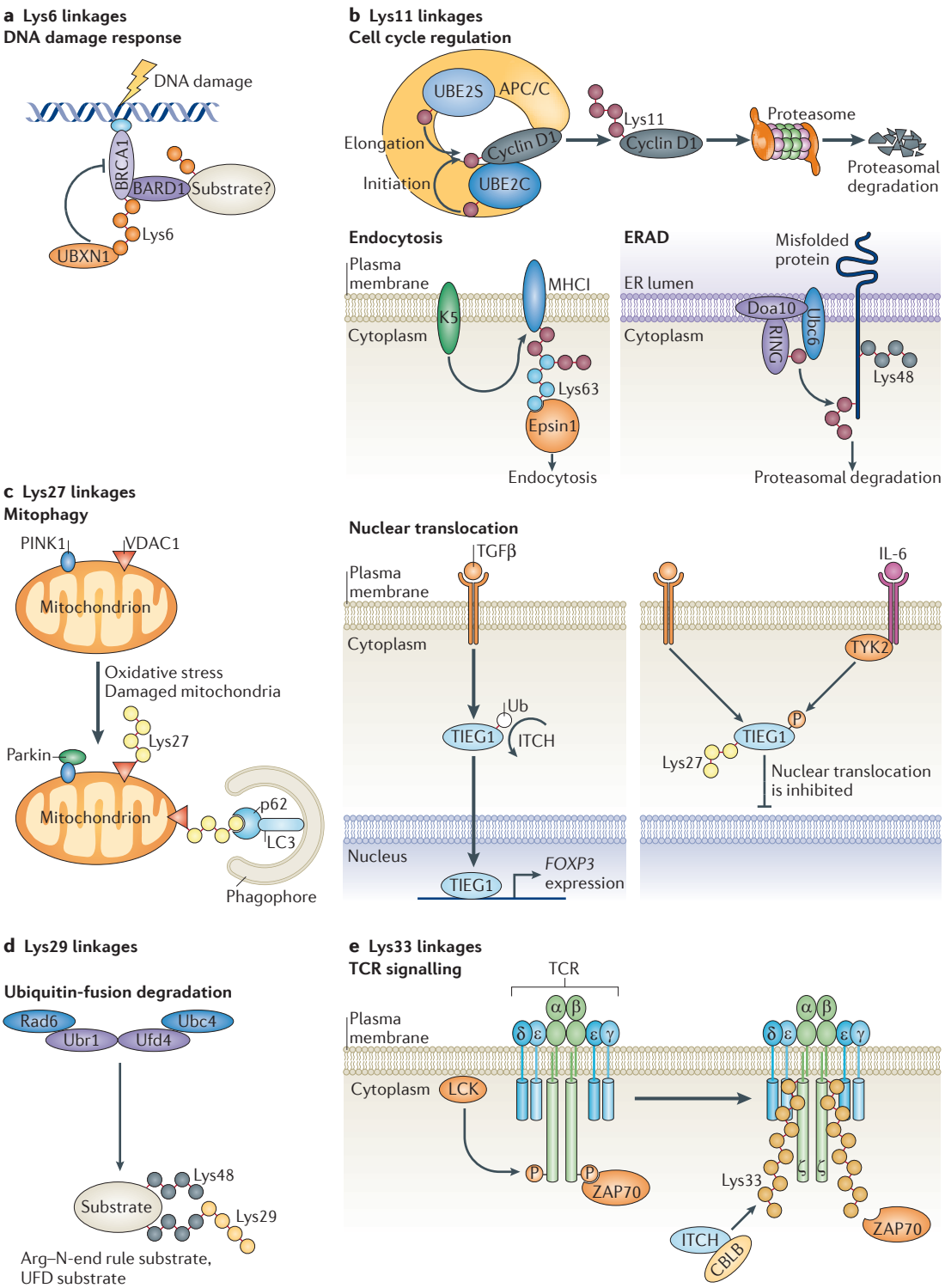
Linkage-specific chain recognition in NF-κB signalling. The different ubiquitylation events leading to NF-κB activation downstream of cytokine receptor complexes ultimately activate two key ubiquitin-dependent kinase complexes, TAK1 and IKK, which contain essential UBDs in their subunits TAB2 (TAK1-binding protein 2) and NEMO, respectively[114–116]. Importantly, these UBDs can distinguish between structurally similar Lys63- and Met1-linked chains (FIG. 1d,5b).

Nuclear protein localization 4 zinc finger (NZF) domains are small zinc-binding folds of approximately 35 residues that can bind to mono- and polyubiquitin[117]. Despite its small size, the NZF domain of TAB2 has two ubiquitin interaction surfaces that interact with the Ile44 patches on adjacent Lys63-linked ubiquitin moieties, bending the ubiquitin chain in the process[118,119]. Structural studies revealed that linear chains are unable

to adopt a similar conformation, which enables TAB2 to distinguish Lys63- from Met1-linked chains[118] (FIG. 5b).

HOIL1L also uses an NZF domain to bind ubiquitin chains, but intriguingly its NZF domain is specific for Met1-linked chains[120]. Like TAB2, the NZF domain of HOIL1L binds to the Ile44 patch on a distal ubiquitin. The Phe4 patch on the proximal ubiquitin is bound by the NZF domain[120] and, in addition, by a specificity-providing α-helical extension that also binds the proximal ubiquitin, thereby enhancing binding affinity[120] (FIG. 5). The example of the NZF domain highlights how small UBDs have evolved to become highly linkage-specific.

We anticipate that many future insights into the biology of atypical chains will arise from studying UBDs. Considering the differences in ubiquitin chain



structure (FIG. 1d) we would expect to identify novel classes of UBDs for atypical chains. An interesting example was the discovery of the UBAN (ubiquitin binding in ABIN and NEMO) domain in NEMO as a linkage-specific UBD for Met1-linked polyubiquitin.

Signal integration by NEMO. NEMO is part of the IKK complex and has a role in coupling upstream ubiquitylation events with IKK activation and in directing the kinase activity towards its substrate[121]. How exactly ubiquitin and NEMO regulate IKK activation is still unclear, and the matter is complicated by the ability of NEMO to bind various chain types and by the fact that NEMO is ubiquitylated itself.

NEMO shows highest affinity for linear ubiquitin chains as compared to other chain types and interacts with Ile44 and Phe4 patches of Met1-linked diubiquitin through its UBAN domain[122]. The UBAN domain forms a symmetric coiled-coil dimer, and Met1-linked chains bind alongside the helix, thereby forming a linear-specific interface with both moieties[122] (FIG. 5). Other chain types, including Lys63-linked chains, can only utilize one binding site in NEMO[123], and this explains the higher affinity of NEMO for Met1 linkages[17,122–124].

Whereas the UBAN domain of NEMO is specific for Met1-linked chains, the full-length protein also binds Lys63- and Lys11-linked chains[41,124], albeit with lower affinity. This is partly due to an additional ubiquitin-binding zinc finger[125] that increases the affinity of NEMO for Lys63 linkages and may work in conjunction with the UBAN domain[126].

Linear-ubiquitin chain binding to the NEMO UBAN domain is peculiar. Despite forming a symmetric complex in the crystal structure, biophysical data clearly show that NEMO binds only one linear diubiquitin molecule with high affinity[122,127,128], suggesting some form of negative cooperativity that is not understood structurally. Importantly, NEMO undergoes a conformational change in the UBAN domain when bound to two linear ubiquitin dimers, in comparison to the unbound molecule[122]. In addition to ubiquitin binding, NEMO is targeted by various E3 ligases that assemble a variety of linkages on a fairly small number of Lys residues in NEMO. Most significantly, LUBAC modifies NEMO on at least two sites (Lys285 and Lys309), and this modification was shown to be important for TNFα-induced and essential for genotoxic stress-induced IKK and NF-κB activation[129,130]. The TRIM E3 ligase TRIM23 catalyses Lys27 ubiquitylation of NEMO on four Lys residues (Lys165, Lys309, Lys325, Lys326), and this modification is required for antiviral defence signalling but not for TNFα signalling[131]. The *Shigella* spp. effector protein IpaH9.8 (invasion plasmid antigen H9.8) hijacks innate immune signalling in the host by mediating Lys27 ubiquitylation of NEMO on Lys309 and Lys321 to inhibit NF-κB activation[132] (FIG. 5c). TRAF7 (TNFR-associated factor 7) was reported to catalyse Lys29-linked polyubiquitylation on NEMO, thereby targeting it for lysosomal degradation[108].

The accumulated data suggest a refined model for IKK activation (FIG. 5d). In a first step, the IKK complex is localized to activated TNFR complexes by virtue of Met1-, Lys63- and Lys11-linked polyubiquitin or other chain types that can interact with NEMO. However, this interaction cannot trigger the conformational changes that are required for full IKK activation. Once at the complex, LUBAC ubiquitylates NEMO and this provides the UBAN domain with high local concentration of linear chains. This enables NEMO to bind two linear chains simultaneously and thereby triggers conformational changes in NEMO that lead to IKK activation. This would explain how single point mutants can activate the IKK complex without disturbing ubiquitin binding or ubiquitylation[115], that is by triggering a similar conformational change within NEMO. Ubiquitylation of NEMO by LUBAC may release the IKK complex from TNFRSC as the highest affinity ubiquitin-binding sites of NEMO are now occupied in *cis*. Clearly, NEMO ubiquitylation will continue to be an exciting field in the years to come.

◀ Figure 4 | **Physiological roles of atypical ubiquitylation.** **a** | The DNA repair-associated E3 ligase complex consisting of breast cancer type 1 susceptibility (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1) assembles Lys6-linked ubiquitin chains on itself or its substrates. UBXN1 (ubiquitin regulatory X (UBX) domain-containing protein 1) binds to Lys6 ubiquitylated BRCA1 to inhibit the ligase activity of the complex. **b** | The E3 ligase complex APC/C (anaphase promoting complex; also known as the cyclosome) uses the E2 ubiquitin-conjugating enzyme E2 C (UBE2C) for chain initiation and UBE2S for Lys11-specific ubiquitin chain elongation on cell cycle substrates (for example, cyclin D1) to target them for proteasomal degradation (top). Major histocompatibility complex (MHC) class I is endocytosed when it is ubiquitylated by the RING E3 ligase K5, encoded by the Kaposi sarcoma virus (bottom left), with heterotypic or branched ubiquitin chains containing Lys11 linkages and Lys63 linkages enabling Epsin1-mediated endocytosis of MHCI. The yeast E2 enzyme Ubc6 and the E3 ligase Doa10, which contains a RING domain, ubiquitylate misfolded endoplasmic reticulum associated degradation (ERAD) substrates with Lys11-linked and Lys48-linked chains to target them for proteasomal degradation (bottom right). **c** | Mitochondrial depolarization triggers translocation of the RBR E3 ligase parkin to the mitochondria where it is phosphorylated by the kinase PINK1 (phosphatase and tensin homologue (PTEN)-induced putative kinase protein 1). This activates parkin to ubiquitylate VDAC1 (voltage-dependent anion-selective channel protein 1) with Lys27-linked ubiquitin chains that are recognized by components of the autophagy machinery (p62, LC3) to initiate mitophagy (left). Monoubiquitylation of TIEG1 by the E3 ligase ITCH induces the translocation of TIEG1 to the nucleus where it binds to *FOXP3* promoter and induces FOXP3 expression. However, in the presence of IL-6 tyrosine kinase TYK2 phosphorylates TIEG1, and phosphorylated TIEG1 becomes a target for Lys27 polyubiquitination, which prevent its nuclear translocation (right). **d** | The ligases Ubr1 and Ufd4 together with their cognate E2 enzymes Rad6 and Ubc4, respectively, target substrates of the Arg–N-end rule pathway and the ubiquitin-fusion degradation (UFD) pathway for ubiquitylation with Lys48- and Lys29-linked chains. **e** | T cell receptor (TCR) stimulation results in the phosphorylation of Tyr in the cytoplasmic tail of the ζ-chain by the kinase LCK (lymphocyte cell-specific protein Tyr kinase), which then leads to recruitment and amplification of TCR signalling by the kinase ZAP70 (ζ-chain-associated protein of 70 kDa). The ligases ITCH and CBLB (casitas B lineage lymphoma proto-oncogene B) negatively regulate TCR signalling by ubiquitylating the ζ-chain of the TCR with Lys33-linked ubiquitin chains, which leads to reduced association of ZAP70 with the receptor.

Conclusions and future perspectives

In this Review we summarize what is known about atypical ubiquitin chains, which are chain types that are not linked via Lys48 or Lys63. It is becoming increasingly clear that all chain types are regulated independently, and that they regulate distinct cellular processes. Regardless of whether multiple chain types result in the same cellular outcome (for example, proteasomal degradation), the fact

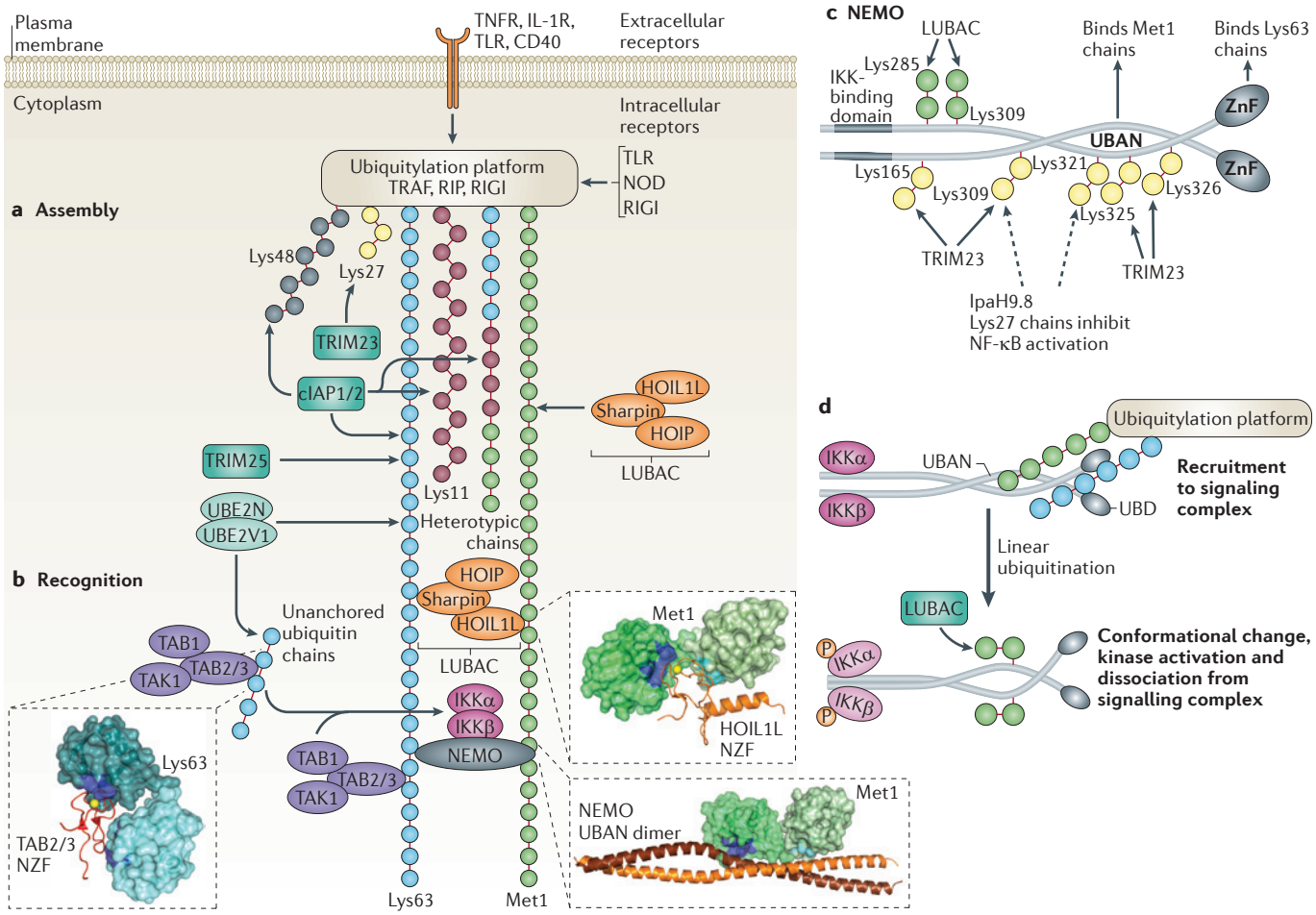


Figure 5 | **NF-κB signalling regulated by atypical linkages. a** | Assembly of signalling complexes. Several stimuli, both extracellular and intracellular, are recognized by receptors (for example, tumour necrosis factor receptor (TNFR), interleukin-1 receptor (IL-1R), Toll-like receptor (TLR), CD40, nucleotide oligomerization domain (NOD) and RIGI (retinoic acid inducible gene I)) to trigger nuclear factor-κB (NF-κB) signalling. Receptor activation leads to the assembly of signalling complexes as ubiquitylation platforms that also contain components of the ubiquitin assembly machinery, such as E2 enzymes (for example, the UBE2N–UBE2V complex and UBE2D (not shown)) and E3 ligases (for example, TNFR-associated factors (TRAFs), tripartite interaction motif (TRIM) proteins, cellular inhibitor of apoptosis (cIAP) proteins and the linear ubiquitin chain assembly complex (LUBAC)). This leads to ubiquitylation of different substrates with differently linked ubiquitin chains, heterotypic chains or to the formation of free unanchored chains[161]. Substrates for ubiquiylation that function in NF-κB signalling include, for example, the TRAF family of E3 ligases, receptor interacting proteins (RIPs) and RIGI. The length of ubiquitin chains shown here are for illustrative purpose only and do not necessarily reflect chain length in cells (which is unknown). **b** | Recognition of ubiquitin chains. Ubiquitin chains are recognized by kinase signalling complexes, such as TAK1 (transforming growth factor-β (TGFβ) activated kinase 1) and IKK (inhibitor of κB kinase) complexes. The TAK1 and IKK complexes bind polyubiquitin through their subunits TAB2 (TAK1-binding protein 2) and NEMO (NF-κB essential modifier), respectively. The TAB2 NZF (nuclear protein localization 4 zinc finger) domain remodels Lys63 chains to a 'bent' conformation (shown in cyan in the structure-inset to the left), by binding both Ile44 patches (PDB ID: 2WWZ[118]). LUBAC interacts with ubiquitin chains via several UBDs including a Met1-linkage specific NZF domain in HOIL1L (haeme-oxidized IRP2 ubiquitin ligase 1L). The structure of the HOIL1–linear diubiquitin complex (structure-inset on top right) reveals how a helical extension of the HOIL1L NZF domain makes additional contacts with the Phe4 patch of the proximal ubiquitin to provide linkage specificity (PDB ID: 3B08 (REF. 120). The dimeric NEMO UBAN domain (shown in brown and orange in the bottom structure-inset) contacts the same patches on linear diubiquitin (PDB ID: 2ZVO[122]). The Ile44 (blue) and Phe4 (cyan) patches of ubiquitin are highlighted. **c** | NEMO is shown in grey and its Met1-specific UBAN and the C-terminal zinc-finger (ZnF) ubiquitin-binding domains are indicated. Ubiquitylation sites mediated by E3 ligases (such as TRIM23 and LUBAC) are highlighted, and different chain types are colour-coded. The *Shigella* spp. effector protein IpaH9.8 (invasion plasmid antigen H9.8) hijacks innate immune signalling by mediating Lys27 ubiquitylation of NEMO. **d** | A model for how ubiquitin binding and ubiquitylation of NEMO leads to IKK activation. NEMO (shown in grey) is recruited to intracellular receptors by binding to Met1- and Lys63-linked ubiquitin chains via its UBAN and zinc-finger ubiquitin-binding domains, respectively. Subsequent linear ubiquitylation of NEMO by LUBAC induces a conformational change in NEMO that might promote activation of the IKK kinase complex and its dissociation from the receptor complex. HOIP, HOIL1L-interacting protein. UBAN, ubiquitin binding in ABIN and NEMO.

that the upstream machinery is different for each chain type allows for many layers of additional, independent control, which was not anticipated until recently. To identify and understand these mediators of independent control, in particular the proteins that detect the linkage type of a chain, is the key challenge for the future and will unlock new areas of biology.

Many questions remain. We know very little about chain topology. What is the abundance of heterotypic chains, and how complicated can these structures become? Are there specialized functions for heterotypic chains? We still lack tools and techniques to study the topology of ubiquitin chains, which is essential to understand the abundance and function of heterotypic chains. Here, linkage-specific DUBs could become important future research tools, and we envisage their appearance in 'ubiquitin chain sequencing' applications.

Another completely unresolved, yet important issue, is ubiquitin chain length. It is unclear how long ubiquitin chains are *in vivo*, and how long they need to be in order to generate a response. Few techniques are available to address this looming question, and available data are ambiguous.

On one hand, it is clear that many UBDs and DUBs can distinguish between differently linked diubiquitin molecules, and assembling this shortest of chains would be energy efficient. Some proteins are known to be modified with diubiquitin[133], and mass spectrometry data have suggested a predominant occurrence of short chains[28]. Indeed, the appearance of a polyubiquitylated 'smear' on a protein gel may arise from multiple short chains or multi-monoubiquitylation events.

On the other hand, the processivity of E3 ligase reactions *in vitro* and in cells is remarkable, and long chains can be formed within seconds by HECT and RBR ligases[59,134]. The APC/C assembles up to 13 Lys11 linkages on a substrate during a single substrate-binding event[46].

For Lys48 chains, tetraubiquitin is required to signal to the proteasome[135]. Why tetraubiquitin? The architecture of the proteasome lid[136], shuttling factors[137] and the improved stability towards DUBs[138] have been used to explain this observation. However, these length requirements could be different for other chain types.

Indeed, it is unclear to what extent atypical chains are proteasomal degradation signals and how they are shuttled to and bound by the proteasome. It will be important to study whether the kinetics of substrate degradation depend on the linkage type and how atypical chains are processed by the many editing enzymes present in the proteasome lid[139].

We think that to address these questions, 'designer' ubiquitin chains and ubiquitylated substrates will be essential, and new quantitative assays to study kinetics of chain assembly, binding and disassembly need to be developed. These methods will eventually illuminate the mechanics of the ubiquitin system and provide the basis for assigning biological roles to atypical chains.

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Competing interests statement
The authors declare competing financial interests; see Web version for details.

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