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STATE-OF-THE-ART REVIEW

Linear ubiquitin chain-binding domains

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Ubiquitin modification (ubiquitination) of target proteins can vary with respect to chain lengths, linkage type, and chain forms, such as homologous, mixed, and branched ubiquitin chains. Thus, ubiquitination can generate multiple unique surfaces on a target protein substrate. Ubiquitin-binding domains (UBDs) recognize ubiquitinated substrates, by specifically binding to these unique surfaces, modulate the formation of cellular signaling complexes and regulate downstream signaling cascades. Among the eight different homotypic chain types, Met1-linked (also termed linear) chains are the only chains in which linkage occurs on a non-Lys residue of ubiquitin. Linear ubiquitin chains have been implicated in immune responses, cell death and autophagy, and several UBDs - specific for linear ubiquitin chains - have been identified. In this review, we describe the main principles of ubiquitin recognition by UBDs, focusing on linear ubiquitin chains and their roles in biology.

Introduction

Ubiquitin, an 8.5 kDa protein conserved from yeast to human, is utilized to post-translationally modify substrate proteins [1,2]. Covalent attachment of ubiquitin to a protein (ubiquitination) regulates their activity, stability, and folding, and thus biological functions, in different species [3]. Unlike other post-translational modifications, such as acetylation or phosphorylation, ubiquitination adds a complex protein tag to its substrate. To date, more than 10 000 ubiquitination sites in over 4000 human proteins have been uncovered by mass spectrometry analysis [4].

Like other post-translational modifications such as phosphorylation and methylation, ubiquitination is reversible (Fig. 1A). Substrate ubiquitination is achieved by a multistep ATP-dependent enzymatic cascade catalyzed by an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase [5], whereas deubiquitination is performed in a single step by dubiquitinases (DUBs) (Fig. 1A). In humans, two genes encode E1s, ~ 25 genes encode E2s, and > 600 genes encode E3s. The nature of the ubiquitin modification depends on the E2 enzyme and the E3 ligase. Ubiquitin itself can also be post-translationally modified by phosphorylation, acetylation, sumoylation, and neddylation (Fig. 1B) [6]. The roles of post-translationally modified ubiquitin are not well-understood. However, it is clear that phosphorylation of ubiquitin by PTEN-induced "Putative Kinase 1" (PINK1) plays a critical role in regulating mitophagy, a mitochondrial damage response [7,8].

Abbreviations
ABIN, A20-binding inhibitors of NF-kappaB; CYLD, cylindromatosis; DUB, deubiquitinating enzyme; HOIL-1L, haeme-oxidized IRP2 ubiquitin ligase 1L; LUBAC, linear ubiquitin chain assembly complex; NEMO, NF-kappaB essential modulator; OPTINEURIN, optic neuropathy-inducing protein; OTU, ovarian tumor; OTULIN, OTU deubiquitinase with linear linkage specificity; RIPK1, receptor interacting protein kinase 1; TNF, tumor necrosis factor; UBAN, UBD in ABIN proteins and NEMO; UBD, ubiquitin-binding domain; USP, ubiquitin specific protease; ZF, zinc finger.

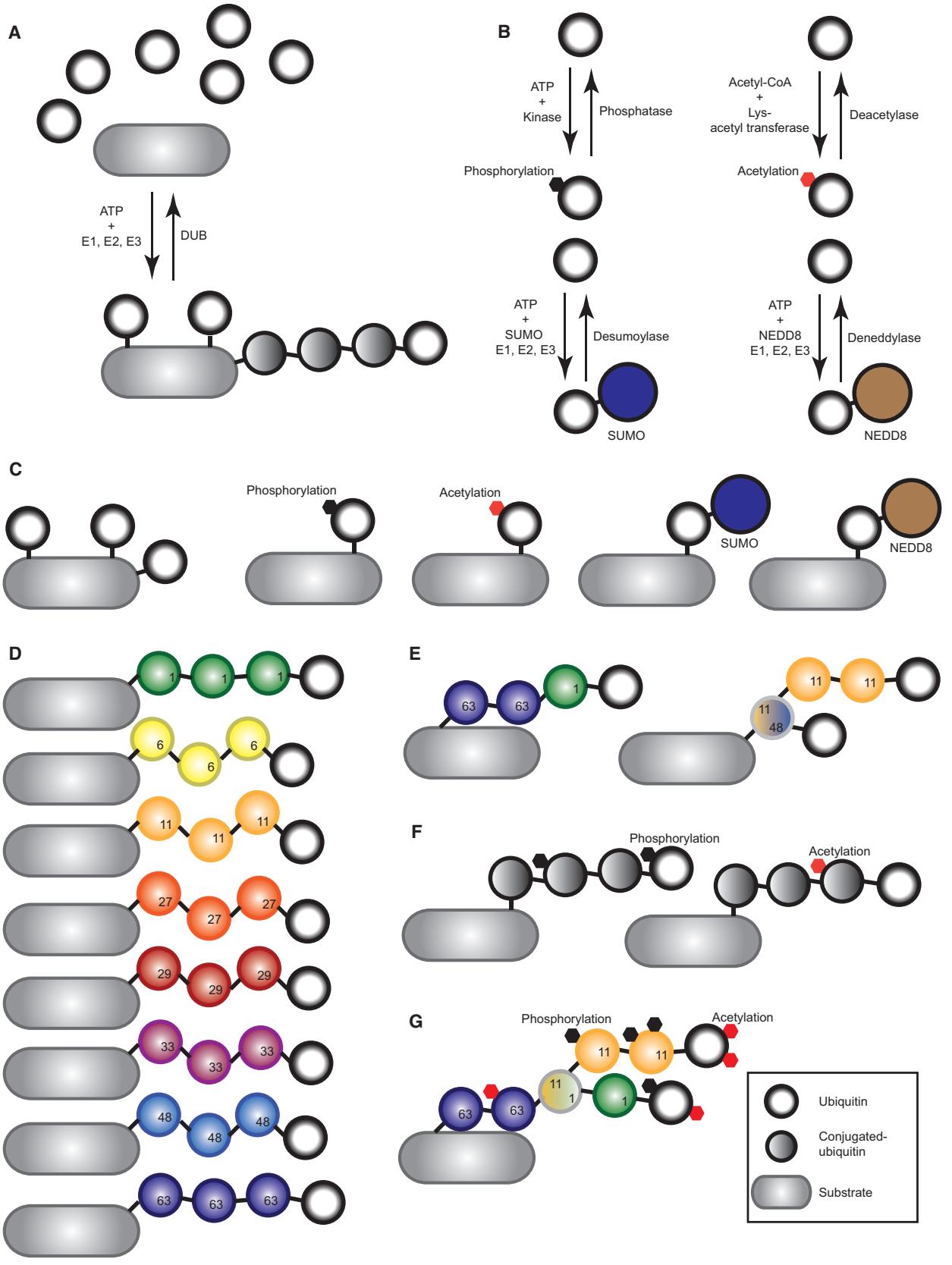


Fig. 1. Ubiquitination of protein substrates. (A) Ubiquitination of a substrate induced by ubiquitin enzymes E1, E2, and E3 in an ATP-dependent manner. The reversible action is regulated by deubiquitinases (DUBs). Depending on an E2–E3 pair, a substrate is ubiquitinated by a monomer or polymer of ubiquitin. (B) Post-translational modifications (PTMs) of ubiquitin by phosphorylation, acetylation, sumoylation, and neddylation. PTMs of ubiquitin can occur on unanchored ubiquitin moieties or ubiquitin conjugated on a substrate in enzyme-dependent manners. Nine phosphorylation sites at Thr (T) and Ser (S) (T7, T12, T14, S20, T22, S57, Y59, S65, T66) and seven acetylation sites at Lys (K) (K6, K11, K27, K29, K33, K48, K63) within ubiquitin have been identified by mass spectrometry. K63 in ubiquitin is used for sumoylation and K48 is used for neddylation. (C) Conjugation of ubiquitin monomers on a substrate as nonmodified or as modified forms (monoubiquitination). (D) A substrate modified with ubiquitin chains linked via an intrinsic residue Met1, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, or Lys63 (polyubiquitination). (E) A substrate modified with a mixed (C) or a branched (D) type of ubiquitin chain. (F) A substrate modified with a post-translationally modified ubiquitin chain, such as phosphorylation of acetylation. (G) A hypothetical ubiquitin chain with various PTMs conjugated on a substrate.

A single ubiquitin moiety can be conjugated on a substrate (monoubiquitination) (Fig. 1C). Modification of ubiquitin by phosphorylation, acetylation, sumoylation, or neddylation can occur before or after the monoubiquitination event is completed (Fig. 1C). Ubiquitin chains are established via conjugation to intrinsic ubiquitin residues, including seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, or Lys63) or the N-terminal Met1 residue [6,9,10]. This process yields eight possible linkage types within homologous, mixed, or branched chains (Fig. 1D,E) [11]. Ubiquitin chains may contain a mixture of modified and nonmodified ubiquitins (Fig. 1F,G). This variation is important for mediating spatiotemporal regulation of cellular signaling. Ubiquitination not only alters the substrate protein's binding partners, but may also directly induce conformational changes within the substrate protein [12,13], influencing critical steps of cellular signaling pathways.

Ubiquitin-binding domains (UBDs)

A wide range of proteins with "Ubiquitin-Binding Domains" (UBDs) recognize diverse ubiquitination types on specific substrate sites, thereby forming distinct signaling complexes (Fig. 2A–D) [14]. Thus far, over 20 different families of UBDs have been identified [15,16]. UBD-containing proteins interact with ubiquitin monomers or chains on substrates. Ubiquitin-UBD interactions may occur in *trans* (between two molecules) (Fig. 2A) or in *cis* (within a molecule) (Fig. 2B). Recruitment of UBD-containing proteins into a signaling complex via ubiquitin-binding regulates downstream signaling pathways [15]. Ubiquitination of a protein with an intrinsic UBD domain may influence its folding [17], which in-turn affects its activity [13] or its interacting partners [16,18,19] to impact signaling cascades.

As mentioned above, recent studies have shown that ubiquitin can be phosphorylated, acetylated, sumoylated, and neddylated. These modifications of ubiquitin potentially alter the UBD-binding surface or chain formation [20–22], therefore it is tempting to speculate that there are distinct UBDs recognizing chains with modified ubiquitins as a monomer or as a chain (Fig. 2C,D). The identification of such UBD-containing proteins would extend our knowledge of ubiquitin biology and its roles in regulating physiology and disease. Alternatively, the post-translational modification of ubiquitin might inhibit canonical ubiquitin-UBD interactions.

UBDs that bind to ubiquitin chains are often selective for a specific type of chain linkage. This selectivity may arise from the recognition of a unique orientation of the chain and distinct surfaces on the ubiquitin moieties, or via direct interaction with the linker region connecting the two ubiquitins. Based on the structural analysis of UBDs, required length of ubiquitin chains to confer specificity to a UBD is suggested to be rather short, either dimeric or trimeric [9,19,23–25]. UBDs in ubiquitin-binding proteins generally occur as single domains or in tandem (Fig. 2A). Tandem "Ubiquitin-Interacting Motifs" (UIMs) in Rap80 and "Arsenite-Inducible RNA-Associated Protein-Like" (AIRAPL) proteins bind cooperatively to Lys63- and Lys48-linked ubiquitin chains respectively [26,27]. An individual UBD may also bind multiple ubiquitin molecules, such as the single "Motif Interacting with Ubiquitin" (MIU) domain within the "MIU-containing Novel DUB family member" (MINDY), which binds to three ubiquitin molecules linked through Lys48 [28]. Also, a double-sided UIM in the "Hepatocyte growth factor-regulated tyrosine kinase substrate" (Hrs) protein is known to bind two independent ubiquitin molecules on either side of an α-helix [29].

Linear ubiquitin chain-specific binding domains and their biological functions

Linear ubiquitin chains linked via Met1 are the only non-Lys residue-dependent type of chain linkage, and thus are biochemically and structurally unique. Linear

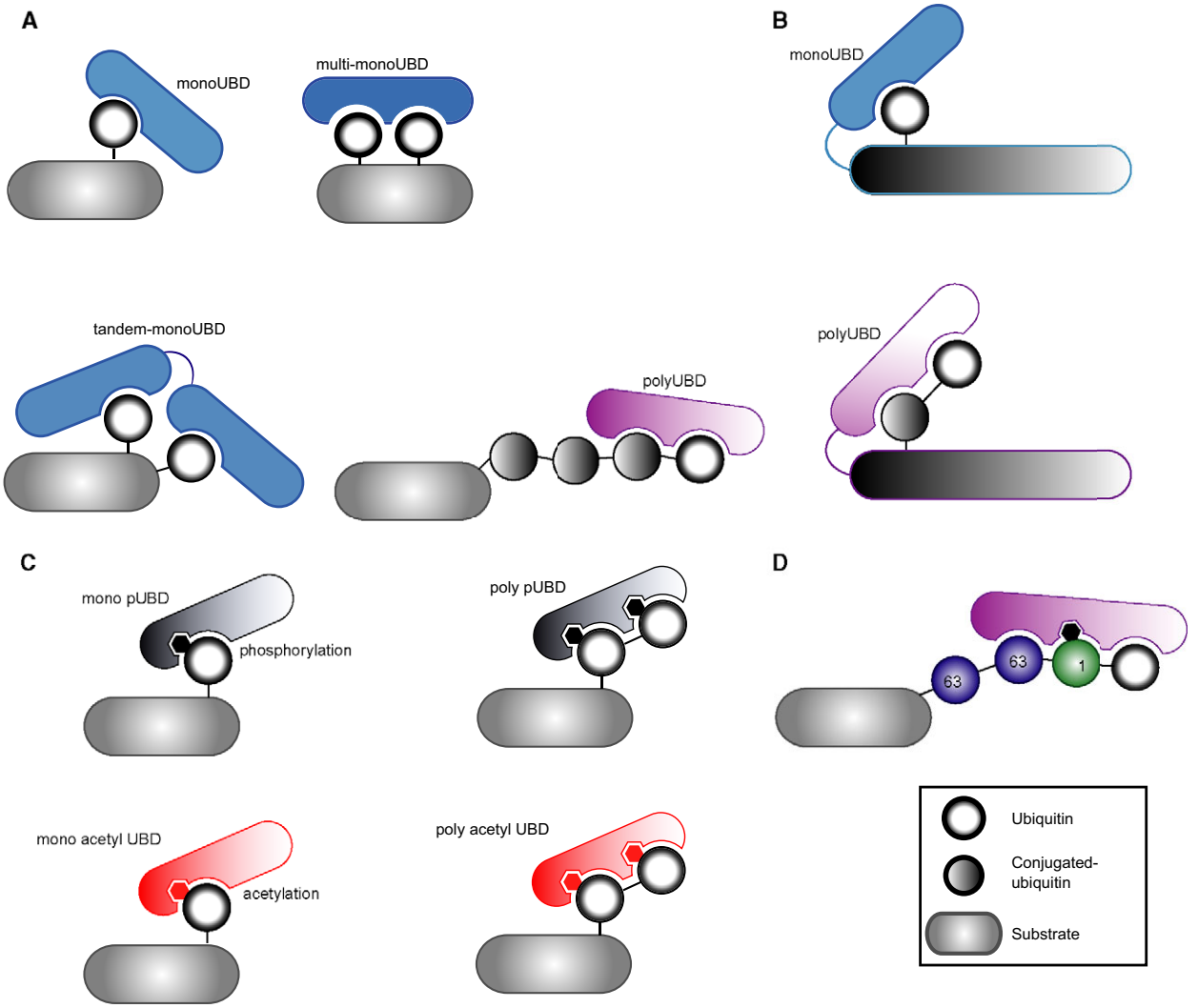


Fig. 2. Types of ubiquitin-binding domains. (A) Ubiquitin-binding domains (UBDs) recognizing monoubiquitin, multiple monoubiquitin or a ubiquitin chain conjugated on substrates in *trans*. (B) UBDs recognizing monoubiquitin or ubiquitin chains conjugated within the same molecule (in *cis*). (C) UBDs binding to post-translationally modified ubiquitin moieties such as phosphorylation and acetylation. (D) A hypothetical UBD interacting with a hypothetical type of mixed-ubiquitin chains.

ubiquitination plays important roles in regulating immunity and cell death signaling cascades, as well as autophagy (Fig. 3A–B) [30–37]. Thus far, the only E3 ligase complex known to generate linear ubiquitin chains is the "Linear Ubiquitin Chain Assembly Complex" (LUBAC), which consists of "Haeme-Oxidized IRP2 ubiquitin Ligase 1L" (HOIL-1L), "HOIL-1-Interacting Protein" (HOIP), and "Shank-Associated RH Domain-Interacting Protein" (SHARPIN) (Fig. 3A,B) [38–40]. HOIP is a "RING-in-Between-RING" (RBR)-type E3 ligase, and its C-terminal "Linear ubiquitin chain Determining Domain" (LDD) is critical for the formation of linear/Met1-linked ubiquitin chains [41]. Like many other RBR-type E3 ligases, HOIP is autoinhibited, and becomes catalytically active upon binding to HOIL-1L or SHARPIN, presumably due to conformational changes in HOIP [42]. Interestingly, the HOIP-RBR domain does not require HOIL-1L or SHARPIN to catalyze at least unanchored linear ubiquitin chains *in vitro* [42]. Linear ubiquitin chains can be hydrolyzed by the DUBs "OTU DUB with linear linkage specificity" (OTULIN) [43,44] and Cylindromatosis (CYLD) (Fig. 3A,B) [45,46].

Ubiquitin chains with linear linkages establish distinct interactions with specific UBDs. Thus far, seven proteins harboring linear ubiquitin chain-specific UBDs are known, including HOIL-1L, OTULIN, and

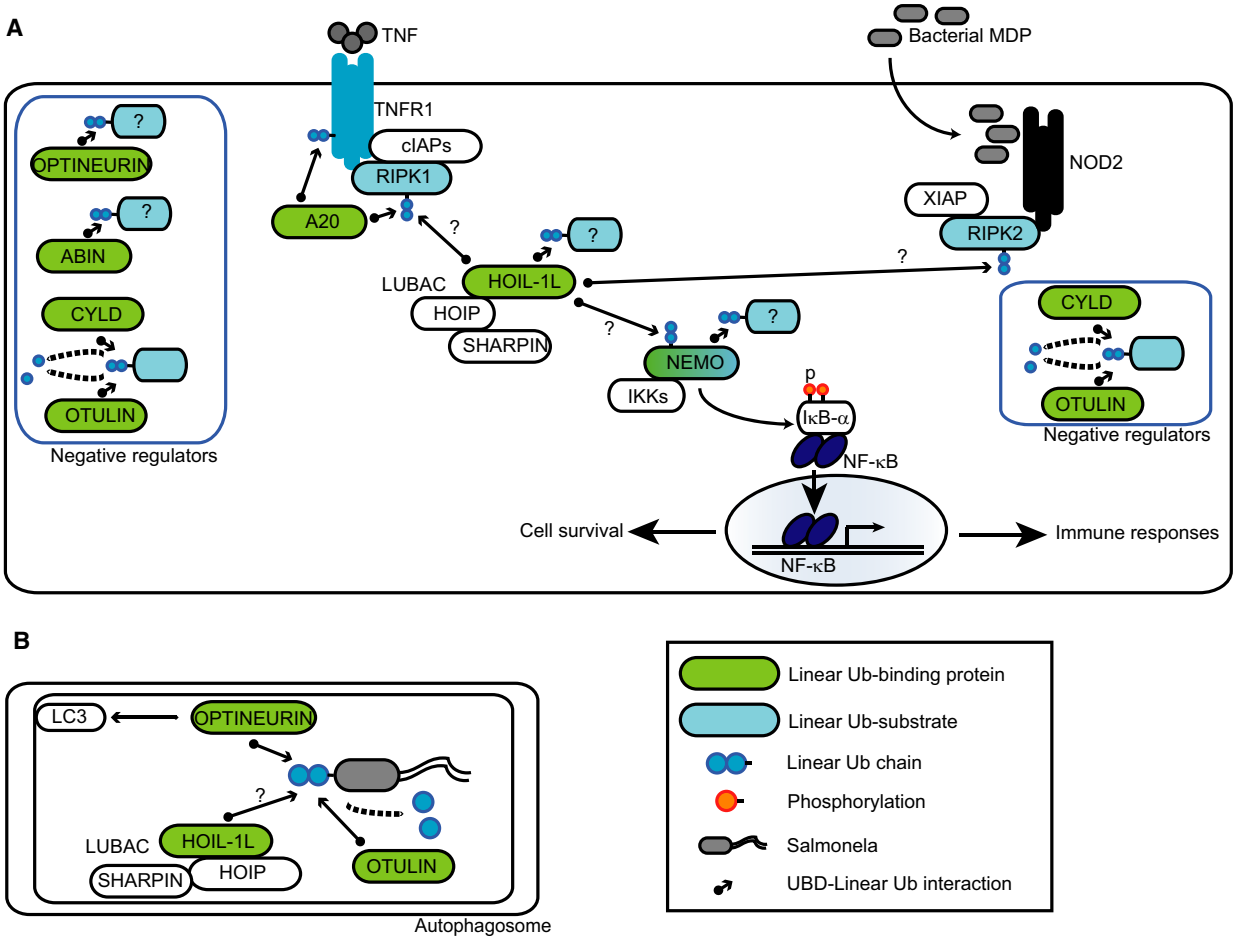


Fig. 3. Linear ubiquitin chain-specific UBDs in the immune signaling cascade and autophagy. (A) UBD-containing proteins known to regulate the TNF- and NOD2-dependent NF-κB signaling cascades. Linear ubiquitin chain-specific UBD-containing proteins (in green) regulate the signaling cascades in a positive or in a negative manner. TNFR1, RIPK1, NEMO, and RIPK2 (in blue) are ubiquitinated with linear ubiquitin chains. Whether UBDs in OPTINEURIN, ABIN-1, HOIL-1L and NEMO or DUBs (CYLD and OTULIN) selectively recognize linear ubiquitin chains conjugated on a specific substrate remains open. (B) Linear ubiquitin chain-binding proteins, OPTINEURIN and HOIL-1L, as well as a DUB OTULIN play a role in the selective autophagy pathway targeting ubiquitin-conated salmonella (xenophagy). OPTINEURIN interacts also with the LC3 family members. Lipidated forms of LC3 are a part of the autophagosome membrane. HOIL-1L together with a ubiquitin E3 ligase HOIP generates linear ubiquitin chains and regulate xenophagy. It remains open if interaction between HOIL-1L-NZF and linear ubiquitin chains play a role in this process.

CYLD (Fig. 4A–G). All of these proteins have been implicated in regulating the signaling pathways that involve linear ubiquitination (Fig. 3A–B) [31–34].

The "UBD in ABIN proteins and NEMO" (UBAN) domain

NEMO-UBAN

The UBAN domain in "NF-κB essential modulator" (NEMO)/"IκB kinase gamma" (IKKγ) was the first linear ubiquitin chain-specific binding domain identified [47] (Fig. 4A), and has been structurally analyzed by x-ray crystallography (Fig. 5A) [19,48]. The C-terminal zinc finger (ZF) of NEMO co-immunoprecipitates with polyubiquitinated signaling components, and thus NEMO was speculated to interact with Lys63-linked ubiquitin chains. Unexpectedly, NEMO-UBAN interacts with linear diubiquitin chains with approximately 100-fold higher affinity compared to Lys63-linked diubiquitin chains [19,49]. NEMO-UBAN forms a parallel homodimer that adopts a highly symmetric coiled-coil structure, resulting in the simultaneous binding of two linear diubiquitin chains along NEMO-UBAN (Fig. 5A). NEMO binds to the distal and proximal ubiquitins within a linear

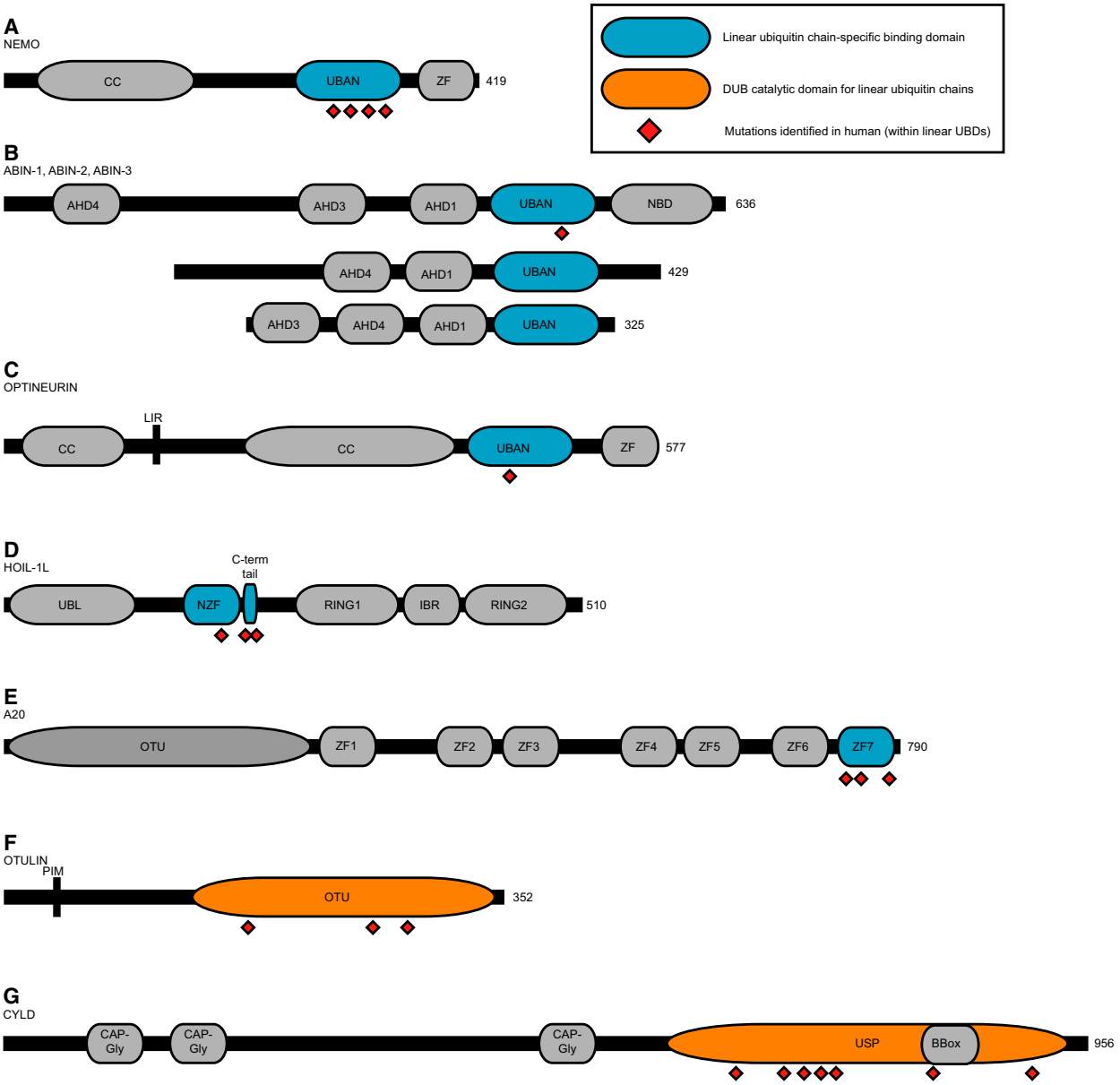


Fig. 4. Linear ubiquitin chain-specific UBD-containing proteins. (A–G) Schematics of the domain structures of the UBAN-containing proteins, NEMO (A), ABIN-1, ABIN-2, ABIN-3 (B) and OPTINEURIN (C), the Zinc Finger domain-containing proteins, HOIL-1L (D) and A20 (E), and deubiquitinases for linear ubiquitin chains, OTULIN (F) and CYLD (G). Linear ubiquitin chain-interacting domains are indicated in blue and the DUB catalytic domains are in orange. Positions of disease-associated human gene mutations are indicated with red squares. CC, coiled-coil, UBAN, ubiquitin binding domains, ZF, zinc finger, AHD, ABIN homology domain, NBD, NEMO-binding domain, LIR, LC3-interacting region, NZF, Npl4 zinc finger, RING, really interesting new gene, IBR, in between RING, OTU, ovarian tumor, PIM, PUB-interacting motif, CAP, cytoskeleton-associated protein, USP, ubiquitin specific protease.

diubiquitin via distinct surfaces centered on Ile44 and Phe4 respectively (Fig. 5A). Furthermore, tight interactions between NEMO-UBAN and the C-terminal tail of the distal ubiquitin (residues Leu71-Arg74) that forms a linker between two ubiquitin molecules ensures its specificity for linear ubiquitin chains.

NEMO plays an important role as part of the "IκB Kinase" (IKK) complex in the "Nuclear Factor-KappaB" (NF-κB) activation signaling cascade, both as a linear ubiquitination substrate and linear ubiquitin chain binding protein (Fig. 3A). The interaction of linear ubiquitin chains with NEMO-UBAN alters the

conformation of NEMO [17,19], which may have an allosteric effect, regulating IKK kinase activity and downstream signaling. Genetic [19] and pharmacological [50] inhibition of the interaction between NEMO-UBAN and linear ubiquitin chains preclude the full activation of NF-κB, revealing an important role for the NEMO-linear ubiquitin chain interaction in regulating the NF-κB pathway. Whether NEMO-UBAN binds to linear ubiquitination formed on NEMO itself, and whether this interaction affects the oligomeric state of the IKK complex, remain unclear.

Patients suffering from incontinentia pigmenti and "Ectodermal Dysplasia with Immuno Deficiency" (EDA-ID) display mutations in the *NEMO* gene, including at positions within the UBAN domain (Fig. 4A, Fig. 5A, Table 1) [51–53]. Interestingly, some of these mutations (Asp311Asn, Glu315Ala, and Arg319Gln, corresponding to Asp304Asn, Glu308Ala and Arg312Gln in mouse NEMO as in Fig. 5A) prevent the interaction between NEMO-UBAN and linear ubiquitin chains, suggesting that loss of this interaction might underlie this disease [19].

ABIN-1, 2, 3-UBAN

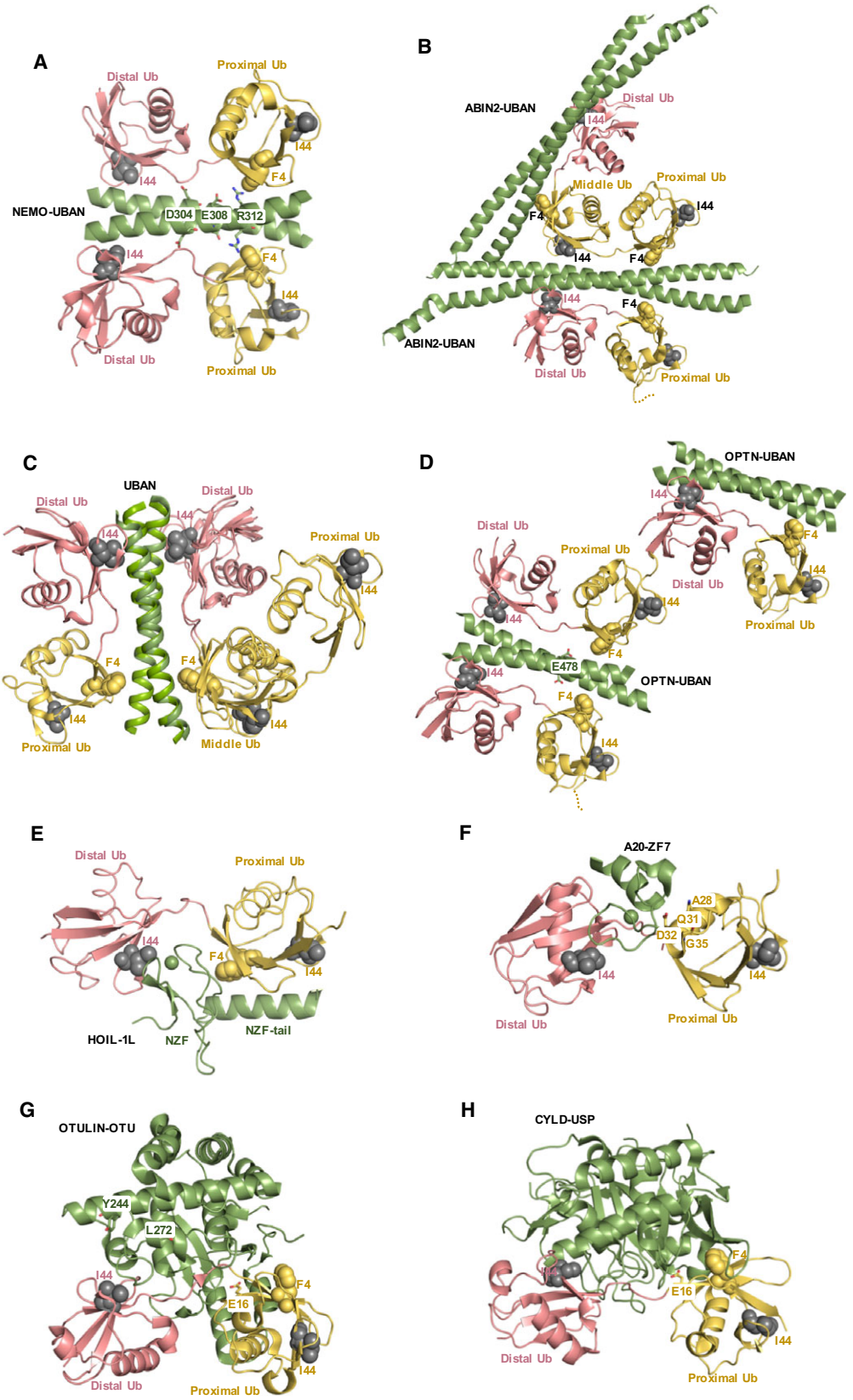
The three "A20-Binding Inhibitors of NF-κB" (ABIN) proteins are known to inhibit the NF-κB pathway and the ABIN-UBAN domains are important for this regulation (Fig. 3A and 4B) [54]. The ABIN proteins are implicated in immunity and cell death based on the analysis of genetically modified mouse models and cellular assays [47]. The three ABIN proteins appear to play nonredundant roles in NF-κB signaling. For example, NF-κB activation induces the expression of ABIN-1 and ABIN-3 but not ABIN-2 [47], suggesting distinct functions in cell signaling regulation.

The UBAN domains in the ABIN proteins were first identified in 2008 [54] and shown to negatively regulate NF-κB signaling. However, a structural understanding of the ABIN-UBAN domain remained unclear until recently. A crystal structure of the ABIN-2-UBAN domain in complex with a linear triubiquitin chain indicated that this domain forms a coiled-coiled homodimer that provides binding sites for two of the ubiquitins, similar to NEMO-UBAN (Fig. 5B,C) [24]. From each triubiquitin chain, the distal ubiquitin binds the UBAN domain through its Ile44 surface. The middle ubiquitin employs both the Phe4- and Ile-44 centered surfaces, acting as the proximal ubiquitin for the same UBAN domain and the distal ubiquitin for the second UBAN dimer from the neighboring asymmetric unit. The third ubiquitin in the chain forms the proximal ubiquitin for binding to the second UBAN dimer (Fig. 5B). Although each ABIN2-UBAN dimer appears to bind one linear triubiquitin in solution as well as in each asymmetric unit of the crystal, the crystal packing reveals a second triubiquitin chain on the other side of the ABIN2-UBAN dimer (Fig. 5B). Thus, high local concentrations of linear ubiquitin chains might lead to the formation of a complex in which two linear ubiquitin chains bind either side of a ABIN2-UBAN dimer. It would be important to understand how triubiquitin chains may support oligomerization of UBAN-containing proteins like ABIN-2. For instance, diubiquitin versus triubiquitin chain binding to ABIN-2 might trigger differential readouts in downstream signaling activation, since only the latter would be predicted to induce oligomerization.

The *TNIP1* gene encoding ABIN-1 is associated with susceptibility to psoriasis in humans [55]. Moreover, genome-wide association studies (GWAS) reveal links between *TNIP1* mutations and systemic lupus erythematosus (SLE) [56,57] and asthma [58]. Finally, recurrent somatic mutations in the UBAN domain of ABIN-1 were found in Diffuse large B-cell lymphoma (DLBCL) (Fig. 4B, Table 1) [59], suggesting an involvement of linear ubiquitin chains.

OPTINEURIN-UBAN

The "Optic Neuropathy-Inducing" (OPTINEURIN)-UBAN domain was initially predicted by bioinformatic analysis together with other UBANs in ABIN proteins and NEMO (Fig. 4C) [54]. A structural study of OPTINEURIN-UBAN in complex with linear tetraubiquitins revealed a binding mode similar to that of linear ubiquitin with NEMO and ABIN-2 [23]. In this crystal structure, two tetraubiquitin chains within neighboring asymmetric units interact with opposing faces of an UBAN dimer, with two ubiquitins from each chain interacting with one face of a UBAN dimer (Fig. 5D). OPTINEURIN is a multifunctional protein, involved in protein trafficking by vesicles, autophagy and signal transduction. OPTINEURIN functions as an autophagy receptor to regulate selective autophagy at least partially through its UBAN domain, and its "microtubule associated protein 1 light chain 3 (LC3)-interacting region" (LIR) (Fig. 3B, Fig. 4C) [60]. Furthermore, the OPTINEURIN-UBAN/linear ubiquitin chain interaction regulates the selective autophagy pathway through "TANK Binding Kinase 1" (TBK1) kinase-dependent phosphorylation [35]. OPTINEURIN is also a negative regulator of the NF-κB signaling cascade (Fig. 3A).



Some of the patients suffering from glaucoma or amyotrophic lateral sclerosis (ALS) display mutations in *OPTINEURIN* (*OPTN*). A Glu478Gly mutation within the UBAN domain of OPTINEURIN (Fig. 4C, Fig. 5D, Table 1) [61], which recapitulates a mutation in ALS patients, disrupts its interaction with linear tetraubiquitin chains and abolishes OPTINEURIN-mediated inhibition of NF-κB signaling [23]. Together, these data suggest that linear ubiquitin chain recognition by OPTINEURIN is important for human health.

The UBAN domains in NEMO, ABINs and OPTINEURIN (Fig. 4A–C) display a similar interaction mode with linear ubiquitin chains (Fig. 5A–D), yet they have either positive or negative roles in TNF-induced immune signal regulation (Fig. 3A). It will be of interest to determine how their specific UBAN-dependent interacting partners and biological functions are precisely regulated to activate or inhibit the downstream signaling cascade.

The zinc fingers (ZFs)

HOIL-1L-NZF

HOIL-1L is a component of the LUBAC complex responsible for linear ubiquitination [62]. HOIL-1L itself is not considered to have the central catalytic 'center' of LUBAC, but HOIP, for generating linear ubiquitin chains. Linear ubiquitin-binding by HOIL-1L is mediated by its "Npl4 zinc finger" (NZF) domain and its C-terminal α-helical tail extension, referred to as the NZF-tail (Fig. 4D) [63]. The two domains are linked via a loop region, which itself does not interact with the ubiquitin chain (Fig. 5E) [63]. The NZF domain binds the canonical Ile44 hydrophobic surface on the distal ubiquitin within a diubiquitin chain, whereas the NZF tail binds the Phe4 surface of the proximal ubiquitin (Fig. 5E). Unlike the UBAN domains, HOIL-1L does not make extensive interactions with the C-terminal tail of the distal ubiquitin. Thus, the specificity of HOIL-1L-NZF for linear ubiquitin chains appears to be determined by the relative spatial orientation and the spacing between the distal and proximal ubiquitins. The physiological relevance of the HOIL-1L NZF is not fully understood besides its implication in NF-κB activation [63]. The interaction between linear ubiquitin chains and the HOIL-1L-NZF might be required for LUBAC-dependent linear ubiquitination. Alternatively, this interaction may protect chains from DUB-dependent hydrolysis, resulting in positive regulation of downstream signaling cascades. Furthermore, HOIL-1L might be recruited to the TNFR complex via the NZF domain.

As part of LUBAC, HOIL-1L regulates immune signaling cascades as well as selective autophagy by linearly ubiquitinating key signaling components, namely NEMO and RIPK2 (Fig. 3A). HOIL-1L NZF mutants that no longer interact with linear ubiquitin chains cannot fully activate NF-κB signaling in gene reporter assays [63], strongly suggesting that binding between HOIL-1L and linear ubiquitin chains is critical for regulation of the NF-κB signaling pathway. Mutations in the *RBCK1* gene encoding HOIL-1L have been identified in patients suffering from polyglucosan body myopathy with immunodeficiency (Fig. 4D, Table 1) [64–66].

A20-ZF7

A20 protein was initially found to regulate NF-κB and cell death signaling pathways [67]. A20 harbors multiple functionally distinct domains that are important for regulating ubiquitin-dependent signaling cascades (Fig. 4E). For instance, A20 was first shown to acts as a ubiquitin E3 ligase to target "Receptor Interacting Protein Kinase 1" (RIPK1) for proteasomal degradation, and also as a DUB to hydrolyze Lys63-linked ubiquitin chains on RIPK1 and negatively regulate NF-κB activation [68]. Later, it was shown that A20 interacts with RNF11 and an E3 ligase Itch to negatively regulate NF-κB signaling [69], demonstrating its role as an E3 ligase may depend on these E3 ligases. More recently, A20-ZF7 (Fig. 4E) has been shown to have catalytically independent mechanisms of action [70,71] by supporting interactions between A20 and linear ubiquitin chains on TNFR1 and RIPK1 within a signaling complex (Fig. 3A) [72]. A20 ZF7 simultaneously binds the Ile44 hydrophobic patch on the distal ubiquitin within diubiquitin, and forms a hydrogen bonding network with a region on the α-helix of

Fig. 5. Structure of linear ubiquitin chain-specific UBDs. (A–G) UBDs (NEMO-UBAN [PDB 2ZVO] (A), ABIN-2-UBAN [PDB 5H07] (B), Superimposition of NEMO UBAN/LUb2 and ABIN-2 UBAN/LUb3 crystal structures (C), OPTN-UBAN [PDB 5B83] (D), HOIL-1L-NZF [PDB 3B08] (E), A20-ZF7 [PDB 3VUY] (F), OTULIN-OTU [PDB 3ZNZ] (G), and CYLD-USP [PDB 3WXE] (H). UBAN domains are colored green, distal and proximal ubiquitins are shown in pink and gold respectively. Ile44 and Phe4 (F4) residues are indicated as gray and gold spheres. Residues from proximal ubiquitin that interact with A20-ZF7, and residues from NEMO, OPTN, and OTULIN that are involved in binding to ubiquitin and their mutations associate with various diseases are labeled and shown as sticks.

Table 1. Mutations identified in human genes encoding linear ubiquitin chain-binding domains.

Protein	Domain	Mutation (Nucleotide)	Mutation (Amino acid)	Mutation type	Associated disease	References
NEMO/IKKγ	UBAN	c.863C > G	p.A288G	Missense	Anhidrotic ecdermal dysplasia with immune deficiency (EDA-ID)	[51]
		c.932A > G	p.D311G	Missense	EDA-ID	[52]
		c.931G > A	p.D311N	Missense	EDA-ID	[51]
		c.944A > C	p.E315A	Missense	Immunodeficiency, susceptible to mycobacterial disease	[53]
		c.956G > A	p.R319Q	Missense	Immunodeficiency, susceptible to mycobacterial disease	[53]
ABIN-1	UBAN	c.2015G > A	p.E476K	Missense	Gastrointestinal diffuse large B cell lymphoma (DBLCL)	[59]
Optineurin	UBAN	c.1743A > G	p.E478G	Missense	Amyotrophic Lateral Sclerosis (ALS)	[61]
HOIL-1L	NZF	c.553C > T, c.ex1_ex4del	p.Q185X	Nonsense, deletion	Invasive bacterial infections, systemic autoinflammation, amylopectinosis	[64]
		c.90C > T, c.68_69ins AGGAGCG	p.Q222X, p.E190 fs	Nonsense, frameshift insertion	Muscle weakness, cardiomyopathy, Amylopectinosis	[65]
		c.727G > T, c1160A > G	p.E243X, p.N387S	Nonsense, missense	Muscle weakness, polyglucosan storage, cardiomyopathy	[66]
A20	ZF7	c.2665C > T	p.P765P	Synonymous codon	Autoimmune disease	[74]
		c.2666G > C	p.A766P	Missense	Autoimmune disease	[75]
		c.2317G > R[a]	p.M778I	Missense	Hodgkin's Lymphoma	[73]
OTULIN	OTU	c.517delC	p.G174Dfs*2	Missense, premature stop codon	Autoinflammation, panniculitis, dermatosis syndrome	[82]
		c.731A > G	p.Y244C	Missense	Autoinflammation, panniculitis, dermatosis syndrome	[82]
		c.815T > C	p.L272P	Missense	Autoinflammation, panniculitis, dermatosis syndrome	[81,82]
CYLD	USP	c.1776delA	p.G593Afs/p.K603X	Frameshift mutation	Familial cylindromatosis	[88]
		c.1787G > A	p.G596D	Missense	Multiple familial trichoepithelioma (MFT)	[89]
		c.1893_1906del ATATTATAGTGAAA	p.E631_T636DfsX10	14 bp deletion, premature stop codon	Familial cylindromatosis	[91]
		c.2172delA	p.K734X	Frameshift, premature stop codon	Brooke-Spiegler syndrome	[90]
		c.2240A > G	p.E747G	Missense	Brooke-Spiegler syndrome	[92]
		c.2241_2242delAG	p.E747fsX[c]763	Frameshift, premature stop codon	Multiple familial trichoepithelioma (MFT)	[93]
		c.2272C > T	p.R758X	Nonsense	Familial cylindromatosis	[88]

For A20, R[a] = sequence variation present in non tumor cells suggesting a polymorphism [73].

proximal ubiquitin, consisting of residues Gln31/Asp32 and the backbones of residues Ala28/Gly35 (Fig. 5F). Moreover, Leu71, Arg72, and Leu73 from the C-terminal tail of distal ubiquitin interact with A20 ZF7.

Mutations in the linear ubiquitin-binding A20-ZF7 domain were found to be associated with B cell lymphomas (Fig. 4E, Table 1) [71,73–75]. Mutations within the catalytic "Ovarian Tumor" (OTU) domain and the ZF4 domain of A20 have been identified in patients with autoinflammatory syndrome, Hodgkin's and non-Hodgkin's lymphoma, and diabetes [73,76,77].

UBAN and ZF domains use distinct mechanisms to interact with linear ubiquitin chains, however, they

both recognize the same surface on the distal ubiquitin within a linear ubiquitin chain, suggesting that one linear ubiquitin chain can be captured by only one linear UBD at a time. The physiological relevance of linear ubiquitin chain recognition by the UBAN or the ZF domains in the immune responses and in other biological functions needs to be further clarified in the future.

The DUB catalytic domains for linear ubiquitin chains

OTULIN-OTU

Although the E3 ligase complex LUBAC specific for linear ubiquitination was discovered in 2006 [62], linear ubiquitin chain-specific DUBs were only recently discovered in 2013 [43,44]. The catalytic domain within the DUB OTULIN recognizes the linear ubiquitin dimer and specifically hydrolyzes linear ubiquitin chains (Fig. 4F and 5G) [43,44]. OTULIN binds to distinct surfaces on distal and proximal ubiquitins within diubiquitin, and specifically recognizes residues at the C-terminal tail of the distal ubiquitin that link the two ubiquitin molecules [43]. Whereas the distal ubiquitin uses its Ile44 hydrophobic surface for binding, the proximal ubiquitin binds the OTU domain via its α-helix and Phe4-centered surface (Fig. 5F). Importantly, Glu16 from the proximal ubiquitin takes part in the activation of OTULIN, revealing a substrate-assisted catalysis mechanism [27].

OTULIN negatively regulates various signaling cascades in which linear ubiquitination plays a role, such as immune signaling cascades and the cell death pathway mediated by the "TNF Receptor" (TNFR) or "Nucleotide-binding Oligomerization Domain-containing protein 2" (NOD2) (Fig. 3A). OTULIN is also known to directly interact with HOIP [78–80]. Interestingly, loss-of-function OTULIN mutations in mice lead to embryonic death between E12.5-E14 due to angiogenic deficits, indicating a role for OTULIN in vascularization [44].

Mutations within the catalytic OTU domain in OTULIN were identified in autoinflammation and dermatosis syndrome patients (Fig. 4F, Table 1) [81,82]. The autoimmune disease-related OTULIN mutations Leu272Pro and Tyr244Cys are not directly involved in interactions with linear ubiquitin chains but they are located on the helix arm that forms part of the binding pocket for the distal ubiquitin [43] (Fig. 5G). The Leu272Pro mutation of OTULIN suppresses the DUB-mediated hydrolysis of linear ubiquitin chains *in vitro*, supporting the physiological relevance and molecular function of linear ubiquitin binding by OTULIN [81].

CYLD-USP

CYLD is a tumor suppressor DUB that utilizes its catalytic specific protease (USP) domain to recognize ubiquitin chains and regulate immune signaling cascades (Fig. 4G). CYLD displays dual specificity for, and thus hydrolyzes, linear and Lys63-linked ubiquitin chains [80,83]. In the crystal structure of CYLD-USP in complex with linear diubiquitin, distal ubiquitin uses a hydrophobic patch centered on Ile44, as well as its C-terminal tail including residues Val70-Gly76, to interact with the USP domain (Fig. 5H). The CYLD-USP binding region on proximal ubiquitin involves residues from the Phe4 patch (Fig. 4E). Moreover, similar to the OTU domain, Glu16 of the proximal ubiquitin is shown to contribute to the hydrogen bonding with the CYLD-USP domain and thus to the catalytic activity of the CYLD enzyme. CYLD functions as an inhibitor in the TNFR and NOD2 signaling cascades by deubiquitinating both linear and Lys63-linked ubiquitin chains, which are important for mediating these signaling cascades (Fig. 3A) [46]. Interestingly, CYLD makes a signaling complex with HOIP via "Spermatogenesis Associated 2" (SPATA2) at TNFR [84–87]. The interplay between the deubiquitinase CYLD or OTULIN and the ubiquitin E3 ligase HOIP in a complex is an interesting aspect to consider for the regulation of downstream signaling cascades. Several mutations in *CYLD*, mostly within the USP domain, have been identified in patients with familial cylindromatosis (Fig. 4G, Table 1) [88–93].

Although OTULIN and CYLD have overlapping functions in the regulation of immune signaling cascades (Fig. 3A), their roles *in vivo* seem to be distinct based not only on their association with different human diseases but also on the distinct phenotypes of their genetically modified mouse models. $Cyld^{-/-}$ mice show no gross defects and are born at the expected Mendelian ratios [94], whereas OTULIN loss-of-function mutant mice (Gumby mice) are embryonic lethal [44]. Their distinct functions in biology likely depend on their different noncatalytic functions and interacting partners, as well as the regulation of their expression and activity.

Concluding remarks

The first linear ubiquitin chain-specific UBD was discovered in NEMO and shown to regulate the inflammatory NF-κB signaling cascade [19]. Since that discovery, roles for linear ubiquitination have been demonstrated in various diverse signaling pathways. Thus far, all proteins with linear ubiquitin chain

binding domains have been established as critical regulators of immune-related signaling cascades. Many gene mutations associated with autoimmune diseases are within linear ubiquitin chain-specific UBDs or DUB catalytic domains, clearly indicating a role for disrupted linear ubiquitination in human disease.

Outstanding questions

- Do linear ubiquitin chain-specific UBDs equally recognize free ubiquitin chains and substrate-conjugated ubiquitin chains?
- What are the contributions of ubiquitin-UBD interaction in enzymatic complexes? For example, does the HOIL-1L-NZF interaction with linear ubiquitin chains regulate LUBAC catalytic activity? Does the NEMO-UBAN interaction with linear ubiquitin chains affect the activity of the IKK kinases? Do these interactions have an allosteric effect?
- Does the interaction of the linear UBDs with ubiquitin chains protect them from hydrolysis mediated by OTULIN or CYLD? Does this interaction regulate linear ubiquitin chain length?
- Are there unknown UBDs that specifically recognize linear-Lys linked mixed chains, or post-translationally modified linear ubiquitin chains?

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Author contributions

LMF, SR, and FI wrote the manuscript and made figures and a table.

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