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Immunological Reviews

Roles of linear ubiquitinylation, a crucial regulator of NF- κ B and cell death, in the immune system

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23 Keywords: LUBAC, ubiquitin, linear ubiquitinylation, NF- κ B, inflammation, cell death

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25 Linear ubiquitinylation, a newly identified post-translational modification, is catalyzed by
26 the linear ubiquitin assembly complex (LUBAC), which is composed of three different
27 subunits, HOIL-1L, HOIP, and SHARPIN. LUBAC plays a critical role in the activation of
28 NF- κ B signaling triggered by a variety of stimuli, including TNF- α , IL-1 β , and
29 pathogen-derived components, and in the protection from cell death. Loss of function of
30 SHARPIN in mice triggers chronic inflammation in multiple organs including the skin, as
31 well as immunodeficiency. In humans, mutations in the gene encoding HOIL-1L cause
32 chronic hyperinflammation and immunodeficiency, which are both associated with
33 decreased levels of LUBAC. The linear ubiquitinylation activity of LUBAC is
34 indispensable for B-cell function in mice, and hyperactivation of LUBAC is associated with
35 oncogenesis in certain forms of B-cell lymphoma. In this short article, the current
36 understanding of the biochemistry of LUBAC-mediated linear ubiquitinylation and its
37 involvement in the immune system are discussed.

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Introduction

The ubiquitin conjugation system was identified as a part of an energy dependent protein degradation system (1-4). However, non-degradable roles of the ubiquitin system were identified, and ubiquitin conjugation, termed ubiquitinylation, was recognized as a reversible post-translational modification system that controls the functions of key proteins involved in various physiological processes, such as protein degradation, cell cycle, apoptosis, DNA repair, and signal transduction, in all cell types (1, 5-7). Ubiquitin is a highly conserved 76 amino acid globular protein that is encoded by multiple genes, namely, two ubiquitin-ribosomal fusion genes and two polyubiquitin genes (8-11). Ubiquitin is transferred to a lysine residue of a target protein by a cascade of reactions catalyzed by three types of enzymes, a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (ubiquitin carrier protein) (E2), and a ubiquitin ligase (E3) in an ATP-dependent manner in most cases (12-15) (*Fig. 1*). While conjugation of one ubiquitin to target proteins yields monoubiquitinated proteins, the successive attachment of ubiquitin moieties to ubiquitin-conjugated target proteins results in the generation of polyubiquitinated proteins. E3 ubiquitin ligases determine the specificity of ubiquitinylation by recognizing target proteins, allowing the ubiquitin system to regulate the function of proteins in a timely and specific manner. Ubiquitin can be conjugated to one of seven Lys residues (K6, K11, K27, K29, K33, K48, and K63) or to the amino-terminal Met residue (M1) of ubiquitin

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7 59 conjugated to target proteins, yielding several types of polyubiquitin chains (16). The type
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9 60 of ubiquitin linkage determines the mode of regulation of the protein (17, 18). The K48
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11 61 chain targets conjugated proteins for degradation, whereas the K63 chain serves as a
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14 62 specific binding site for the cellular signaling machinery (19, 20). Each type of linkage has
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17 63 distinct structural flexibility: K48-linked di-ubiquitin forms a relatively compact structure,
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19 64 and the K63-linked di-ubiquitin adopts a flexible and open conformation. Different
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22 65 molecular surfaces generated by distinct ubiquitin linkages are specifically recognized by
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24 66 the corresponding ubiquitin binding domains (UBD) (21, 22) (*Fig. 1*). Conjugated ubiquitin
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27 67 molecules are cleaved by deubiquitinating enzymes (DUBs). More than 90 DUBs exist in
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30 68 humans, and some DUBs are specific for particular chain linkages (23, 24) (*Fig. 1*).
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32 69 This review focuses on a newly identified, M1-linked, linear ubiquitin chain
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35 70 generated by peptide bond formation between the carboxyl group of the C-terminal glycine
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38 71 residue (Gly76) of distal ubiquitin moieties and the α -amino group of the Met1 residue of
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41 72 proximal ubiquitin moieties, and its function in the immune system (25-27). The linear
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44 73 di-ubiquitin has a relatively open conformation of ubiquitin linkages that resembles that of
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47 74 K63-linked di-ubiquitin because the position of M1 is close to that of K63 in ubiquitin (16).
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50 75 Nevertheless, since some UBDs discriminate between the structure of linear and K63
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53 76 linkages, the linear chain has distinct functions that differ from those of the K63-linked
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56 77 ubiquitin chain. The linear chain is involved in the activation of nuclear factor- κ B (NF- κ B)
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59 78 triggered by various stimuli (28-30). Linear chains also contribute to the regulation of cell
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death and several pathogenic conditions related to immune cells (31, 32).

Enzymology of the linear ubiquitin chain

Among multiple ubiquitin genes, the UBB and UBC polyubiquitin genes encode polyubiquitin precursors composed of three and nine ubiquitin moieties. However, translated linear polyubiquitin chains are cleaved into ubiquitin monomers co-translationally by deubiquitinating enzymes (33). Indeed, no linear polyubiquitin can be detected by mass spectrometric analyses, although the presence of ubiquitin linkages via seven Lys residues in ubiquitin was identified in yeast lacking the enzymes that generate linear linkages (34). The linear chain and the enzyme that generates it were first identified in 2006 (27). An E3 ligase complex designated as the linear ubiquitin assembly complex (LUBAC) is the only identified E3 that generates the linear ubiquitin chain. LUBAC is composed of three distinct subunits: HOIL-1L (heme-oxidized IRP2 ligase 1L; also known as RBCK1), HOIP (HOIL-1 interacting protein; also known as RNF31) and SHARPIN (SHANK-associated RH domain-interacting protein) (26, 35, 36) (*Fig. 2*).

Both HOIL-1L and HOIP have the RING-IBR-RING (RBR) domain that is a characteristic feature of ubiquitin ligases; however, only the RBR of HOIP, but not that of HOIL-1L, is indispensable for the catalytic activity of linear ubiquitinylation (37). The RBR ubiquitin ligase is part of a newly identified E3 family and thought to be a hybrid type

of E3 that is homologous to the E6-AP Carboxyl Terminus (HECT) and Really Interesting New Gene (RING) ligases reported previously (38). The RING E3 ligase, which serves as a scaffold for ubiquitin-bound E2 and its substrate, allows the ubiquitin to be directly transferred from the E2 to the substrate. On the other hand, a ubiquitin moiety is usually transferred to the conserved cysteine residue of the HECT domain before it is transferred to a substrate in HECT E3 ligases. The RBR E3 ligase has two RING domains and an amino-terminal classical RING domain (RING1) within the RBR domain that recognize ubiquitin-bound E2, as observed in RING ligases; the ubiquitin molecule is then transferred onto the conserved Cys residue of the RING domain (RING2) to form a thioester intermediate, and ubiquitin bound to the conserved Cys residue is transferred onto a substrate (*Fig. 3*). Thus, the specificity of ubiquitin linkages is thought to be dependent on the final state of the thioester intermediate, namely, by the RBR E3. Indeed, LUBAC specifically generates the linear ubiquitin linkage regardless of the E2s (27) and RING2, and the linear ubiquitin chain determining domain (LDD) is involved in the linkage specificity (39).

HOIL-1L and SHARPIN are accessory molecules that are involved in the stabilization of LUBAC. In cells lacking HOIL-1L or SHARPIN, the other two components of LUBAC are rapidly degraded via an unknown mechanism, leading to a significant decrease in the ability for linear ubiquitinylation (25, 26). Interactions between the ubiquitin-like (UBL) domain of HOIL-1L and ubiquitin-associated (UBA) domain of HOIP

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play a role in the formation of the LUBAC complex, as confirmed by crystallographic analysis of the complex composed of the UBL domain of HOIL-1L and UBA-containing HOIP (40). Interactions between HOIP Npl4-type zinc finger/RanBP2 zinc finger (NZF) 2 and SHARPIN UBL are also involved in LUBAC formation (41, 42); however, HOIP lacking NZF2, and not UBA, can bind to SHARPIN in cells (26). If the two accessory molecules are absent, the ligase activity of HOIP is lost almost completely because of the auto-inhibitory effect of the N-terminal region of HOIP containing UBA domain (41). Thus, HOIL-1L and SHARPIN play crucial roles in enhancing the activity of LUBAC. The molecular weight of LUBAC was estimated at 600 kDa by gel filtration analyses, although HOIL-1L, HOIP, and SHARPIN are 58 kDa, 120 kDa, and 40 kDa, respectively (26, 27) (*Fig. 2*). Thus, the LUBAC complex is thought to contain at least two or three HOIP components, although the precise subunit composition of the complex remains unknown. It is awaiting the resolution of the intact crystal structure of the whole ternary complex of LUBAC.

 In cells, ubiquitin conjugation to target proteins must be regulated spatially and temporally and induced only when needed. Ubiquitin chains generated in response to various stimuli are recognized by proteins that have specific UBDs to exert their function. The ubiquitin chains are eventually cleaved by DUBs to abrogate the functions associated with each chain (23, 43). DUBs cleave peptide or isopeptide bonds between ubiquitin and the substrate, and between ubiquitin molecules (inter-ubiquitin linkage) to dissociate

ubiquitin from substrates or ubiquitin, leading to the elimination of the activated state. Some linkage-specific DUBs have been reported, and two DUBs, an ovarian tumor (OTU) DUB with linear linkage specificity (OTULIN, also termed Fam105b or Gumby) and cylindromatosis (CYLD), were shown to cleave linear ubiquitin linkages (44-46). The K63 position is very close to the M1 of ubiquitin, and structural analyses revealed that linear and K63 di-ubiquitins are structurally very similar (47, 48). However, OTULIN binds preferentially to linear di-ubiquitin compared to K63 di-ubiquitin (approximately 100-fold higher affinity for the former) and selectively cleaves linear ubiquitin linkages (45). Another DUB, CYLD, can digest linear linkages. CYLD, which was identified as a tumor suppressor commonly mutated in familial cylindromatosis, is involved in the regulation of NF- κ B activation and was shown to cleave K63 chains specifically (49-53). However, CYLD can digest linear linkages in addition to the K63 linkage, as the structure of the K63 chain is similar to that of linear di-ubiquitin (46).

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153 **Mice lacking LUBAC subunits or linear chain specific DUBs**

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Many combinations of E2s and E3s can generate the same ubiquitin linkages. For example, an E2 complex containing Ubc13 generates K63 chains specifically together with multiple E3s. However, in the case of linear chains, LUBAC is the only E3 identified to date that generates linear chains together with multiple E2s. Thus, the function of linear

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6 159 chains could be probed by ablating the activity of LUBAC. However, phenotypes provoked
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9 160 by ablation of subunits of LUBAC, HOIP, HOIL-1L, and SHARPIN are very much
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11 161 different. First, we describe phenotypes of mice lacking subunits of LUBAC and two DUBs
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14 162 that cleave linear linkages. We then discuss the in vivo roles of these molecules in the
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22 165 a) HOIP

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27 167 Among the three subunits of LUBAC, HOIP is the catalytic subunit. Therefore,
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29 168 we first describe results obtained with mice having genetically engineered HOIP loci. Mice
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32 169 lacking the catalytic activity of HOIP were shown to be embryonic lethal, although a
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35 170 precise phenotype was not described yet (54). The HOIP^{-/-} mouse phenotype was reported
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37 171 recently. HOIP^{-/-} mice die at approximately E10.5 and show disrupted vasculature in the
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40 172 yolk sac (55). This appears to be caused by increased endothelial cell death. Because of its
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43 173 lethality, the function of linear ubiquitin in the immune system could not be addressed
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45 174 without using conditional knockout (KO) mice. Analyses using conditional KO mice are
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47 175 discussed later.

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52 177 b) SHARPIN

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7 179 Mice lacking another subunit of LUBAC, SHARPIN, exhibit different
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9 180 phenotypes. Spontaneous autosomal recessive mutant mice, called chronic dermatitis in
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11 181 mice (*cpdm*), were identified in 1993 as mice exhibiting chronic dermatitis (56). In 2007,
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14 182 SHARPIN was identified as a causative gene product in these mice (26, 35, 36). Two
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17 183 different *cpdm* mouse strains have been described,
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19 184 C57BL/KaLawRij-*Sharpin*^{*cpdm*}/*Sharpin*^{*cpdm*} and CBy.OcB3-*Sharpin*^{*cpdm-Dem*}/*Sharpin*^{*cpdm-Dem*},
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22 185 both of which have a mutation in the first exon resulting in a frame-shift and the absence of
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24 186 functional SHARPIN proteins (56). Because of the lack of SHARPIN, the amounts of the
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27 187 remaining two components of LUBAC, HOIL-1L and HOIP, are drastically reduced, which
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30 188 markedly decreases linear ubiquitinylation activity although the ligase activity of LUBAC
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32 189 is not completely lost. The characteristic phenotype of both strains of mice is severe
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35 190 chronic inflammation of the skin starting at 5–6 weeks after birth. The skin lesions are
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37 191 identified by epidermal hyperplasia, hyperkeratosis, parakeratosis, scattered apoptotic or
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43 193 cells in the dermis and the epidermis (57-60). The development of dermatitis is thought to
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45 194 be independent from acquired immune responses related to lymphocytes because
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47 195 *SHARPIN*^{*cpdm-Dem*} *Rag*^{*-/-*} double mutant mice, which lack T and B cells, show a similar
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50 196 phenotype to that of *SHARPIN*^{*cpdm-Dem*} mice (61). In addition to the dermatitis, infiltration
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53 197 of neutrophils and macrophages is also seen in multiple organs such as the lung, liver, and
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55 198 several joints. The mice also show structural defects of secondary lymphoid organs,
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splenomegaly, a disrupted marginal zone in the spleen, the absence of Peyer's patches in the adult intestine, and reduced levels of serum IgG, IgA, and IgE, but not IgM, which indicates defective class switching in B cells (62, 63).

When *cpdm* mice are crossed with $\text{TNF}\alpha^{-/-}$ mice, the dermatitis, but not the systemic inflammatory effects on other organs, of *cpdm* mice are drastically rescued, even though they carry a heteroallelic deletion of $\text{TNF-}\alpha$ (36). Moreover, intercrossing *cpdm* mice with RIP1 kinase dead mice cures the *cpdm* symptoms almost completely (64). The upregulation of HOIP and HOIL-1L in the skin also ameliorates *cpdm* mice and attenuates NF- κ B activation. Thus, a reduction of LUBAC, and thereby of linear ubiquitylation activity, associated with the loss of function of SHARPIN and its effects on inhibiting NF- κ B and promoting cell death underlie the pathogenesis of *cpdm* mice.

c) HOIL-1L

Loss of HOIL-1L in mice has also been reported (25). Loss of HOIL-1L destabilizes the other two components of LUBAC and reduces the linear ubiquitylation activity of LUBAC. Signal-induced NF- κ B activation is attenuated; however, HOIL-1L KO mice do not exhibit overt phenotypes. This can be attributed to the fact that lack of HOIL-1L enhances cell death only marginally.

d) OTULIN

Homozygous mutant mice carrying a mutation in OTULIN (W96R) called *gumby* mice, which exhibit virtually no DUB activity (65), die at E10.5 and show defects in the organization of branching vascular networks in the head and trunk. Linear ubiquitinated proteins accumulate in *gumby* embryos compared to control embryos (65). The Wnt signaling pathway, which is essential for the sprouting of blood vessels from the pre-existing vasculature during angiogenesis, is activated in *gumby* mice because of impaired linear DUB activity. Indeed, OTULIN interacts with disheveled 2 (DVL2), a critical protein involved in the canonical Wnt pathway, and suppresses canonical Wnt signaling in overexpression studies (46).

f) CYLD

Since CYLD can cleave K63 in addition to linear linkages, its functions are not defined by the cleavage of linear chains alone. Familial cylindromatosis, of which CYLD is a causative gene product, is characterized by multiple benign tumors that develop from skin appendages, such as hair follicles and sweat glands (49, 66, 67). In animal models, *CYLD*^{-/-} mice do not develop spontaneous tumors; however, they are highly susceptible to dextran sulfate sodium (DSS)-induced colitis and azoxymethane (AOM)-induced tumor

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239 development (68, 69). Thus, CYLD plays a critical role in the suppression of tumor
240 proliferation (53). Overexpression of CYLD leads to a decrease in NF-κB activity induced
241 by several receptors including TNFR1, CD40, TLR4, EDAR, and LMP1 (51, 70).
242 Moreover, CYLD interacts with TNFR1-associated factor (TRAF)2 and TRAF6 and
243 impairs their linear ubiquitinylation via its deubiquitinating activity (51). In addition,
244 CYLD was suggested to be involved in the induction of cell death (71-75).

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246 **Mechanistic insight into the functions of linear ubiquitinylation**

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248 Next we discuss the function of linear ubiquitin chains. Accumulating evidence
249 supports the involvement of linear ubiquitin chains in canonical NF-κB activation and the
250 regulation of cell death. Here, we summarize current knowledge on the molecular
251 mechanisms underlying canonical NF-κB activation and cell death regulation mediated by
252 linear ubiquitinylation. Other functions of linear ubiquitin chains are also discussed.

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254 **a) NF-κB activation**

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256 The first identified function of the linear ubiquitin chain was its involvement in
257 the activation of the canonical NF-κB pathway. NF-κB is a member of a family of dimeric
258 transcription factors composed of five Rel homology domain (RHD)-containing proteins,

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6 259 including RelA (p65), RelB, c-Rel, p105/p50 (NF- κ B1), and p100/p52 (NF- κ B2). The
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9 260 RHD in the amino-terminal region is required for dimerization, nuclear import, and binding
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11 261 to 9–10 base pair NF- κ B-responsive elements referred to as κ B sites. NF- κ B is involved in
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14 262 various biological processes including immune responses, inflammation, and cell survival.
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17 263 Aberrant activation of NF- κ B plays a role in immunological disorders and oncogenesis,
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19 264 including some forms of B-cell lymphoma (76, 77). Two NF- κ B activation pathways have
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21 265 been described, the canonical (classical) and alternative (non-canonical) pathways, which
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24 266 have distinct physiological consequences (78-80). Here, the canonical pathway is
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27 267 introduced because LUBAC-mediated linear ubiquitinylation is involved in the canonical
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29 268 but not the alternative pathway (25, 26, 54) (*Fig. 4*). Various ligands induce the activation
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32 269 of the canonical NF- κ B pathway, which is associated with local inflammatory and immune
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35 270 responses. In the resting state, NF- κ B is restrained in the cytoplasm through binding to
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37 271 inhibitor of NF- κ B (I κ B) proteins. I κ Bs, which include I κ B α , I κ B β , and I κ B ϵ , associate
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40 272 with the RHDs of NF- κ B via their ankyrin-repeat motif. The I κ B kinase (IKK) complex is
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42 273 composed of IKK1 (IKK α), IKK2 (IKK β), and NF- κ B essential modulator (NEMO; also
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45 274 called IKK γ), and is a critical mediator of the canonical NF- κ B pathway (78, 79, 81). Upon
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47 275 activation by various stimuli, the IKK complex is activated by phosphorylation of specific
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50 276 serine residues on IKK2 and induces the phosphorylation of I κ Bs. Phosphorylated I κ Bs are
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52 277 recognized by the SCF ^{β TrCP} ubiquitin ligase, followed by K48-linked ubiquitinylation and
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55 278 degradation via the proteasome (82-85) (*Fig. 4*). LUBAC plays a role in the canonical
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279 NF- κ B pathway triggered by TNF- α , IL-1 β , CD40 ligand (CD40L), and certain Toll-like
280 receptors (TLRs) (25, 35, 54). The current hypothesis for the role of LUBAC-mediated
281 linear ubiquitinylation in canonical NF- κ B activation triggered by ligands of the TNFR
282 family is as follows: upon activation by various stimuli, LUBAC preferentially recognizes
283 and conjugates linear ubiquitin chains on NEMO (25, 86). Mutational analysis revealed that
284 the interaction between the NZF1 domain of HOIP and the coiled-coil 2 and leucine zipper
285 (CoZi) domains of NEMO is involved in LUBAC-mediated linear ubiquitinylation at
286 Lys285 and Lys309 in the CoZi domain of NEMO (25). NEMO possesses a specific
287 ubiquitin binding region referred to as the ubiquitin binding in ABIN and NEMO (UBAN)
288 motif within the CoZi domain (87). The UBAN motif has high affinity for linear
289 di-ubiquitin, but not for K63 di-ubiquitin chains (87, 88). Recognition of the linear
290 di-ubiquitin conjugated to NEMO by the UBAN domain of another NEMO in *trans* may
291 trigger dimerization of the IKK complex, and subsequent trans-autophosphorylation of two
292 specific Ser residues of IKK2, because amino acid residues in the kinase domain of IKK2
293 involved in IKK2 dimerization are required for IKK2 phosphorylation (89) (*Fig. 4*). Upon
294 activation by various stimuli, recognition of NEMO conjugated linear ubiquitin chains by
295 the UBAN domain of NEMO, which is specific for the linear chain, triggers NF- κ B
296 activation. Thus, DUBs that cleave linear chains are thought to block the signal generated
297 by conjugation of linear ubiquitin chains. Recently, two DUBs, OTULIN and CYLD, which
298 can cleave linear chains, were shown to bind continuously to LUBAC via the peptide

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6 299 N-glycosidase (PNGase)/ubiquitin-associated (PUB) domain of HOIP (46, 90, 91). The
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9 300 ligase-DUB interaction is not regulated by any stimulus (46). The activation of NF- κ B by
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11 301 TNF- α is intensified in HOIP null cells expressing a HOIP mutant that is unable to interact
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14 302 with OTULIN or CYLD compared to that in cells expressing wild-type HOIP. Thus, the
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17 303 ligase-DUB interaction may play a role in the optimization of the strength of the signal
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19 304 initiated by stimuli, possibly by modulating the length or number of linear ubiquitin chains.
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22 305 Phosphorylation of a Tyr residue in the PUB-interacting motif of OTULIN abolishes the
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24 306 interaction between the two proteins (90, 91); therefore, signaling mediated by linear chains
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27 307 might be modulated by the activation of tyrosine kinases. The precise role of the interaction
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29 308 between LUBAC and the two DUBs remains to be elucidated.

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32 309 Another DUB, A20, is involved in the suppression of linear chain-mediated
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34 310 NF- κ B activation, although it functions in a DUB-independent manner (92, 93). A20,
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37 311 identified as a suppressive factor for NF- κ B signaling upon TNFR and TLR stimulation (94,
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39 312 95), contains seven Cys₂-Cys₂ zinc finger (ZF) repeats in the carboxyl-terminal region in
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42 313 addition to the N-terminal OTU domain. A20 attenuates NF- κ B activation by cleaving K63
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45 314 chains on RIP1 and conjugating K48 chains onto RIP1, leading to the degradation of the
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47 315 kinase (95). A20 was also shown to suppress LUBAC-mediated NF- κ B activation via the
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49 316 linear chain specific binding activity of the seventh zinc finger (ZF7), possibly by
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52 317 preventing the recruitment of LUBAC and NEMO to the activated receptor complex (92,
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55 318 96). Considering that A20 is a target of NF- κ B and rapidly induced after stimulation, it may
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be a key factor in a negative feedback loop modulating NF- κ B-induced gene expression.

Linear ubiquitinylation is also involved in NF- κ B activation via the nucleotide-binding oligomerization domain-containing (NOD)-like receptors (NLRs) NOD1 and 2, which are cytoplasmic pattern recognition receptors (PRRs) (97, 98). NOD1 and NOD2 selectively recognize bacterial PGN found in both gram-positive and gram-negative bacteria. NOD receptor signaling is initiated by the interaction between NODs and RIP2, which is mediated by the homotypic interaction between their caspase recruitment domains (CARDs). The baculovirus IAP repeat 2 (BIR2) domain of RIP2 then recruits X-linked IAP (XIAP), an E3 ligase with a RING domain, which ubiquitinates RIP2 leading to NF- κ B activation (97). Although RIP2 is modified by K63 polyubiquitinylation in NODs signaling, the ligase activity of LUBAC is also involved in NOD2-mediated NF- κ B activation. The dependence of the interaction between LUBAC and the NOD signaling complex on the ubiquitin ligase activity of XIAP suggests that LUBAC is recruited to the NOD signaling complex via recognition of XIAP-generated ubiquitin chains. The major linear ubiquitinylated protein involved in NOD signaling is RIP2. NOD2 signaling is also limited by OTULIN via cleavage of RIP2-conjugating linear ubiquitin chains. Accordingly, deletion of OTULIN increases the linear ubiquitinylation of RIP2 in response to NOD2 stimulation. However, the role of the linear ubiquitinylation of RIP2 in NOD2 signaling remains unknown. NEMO might also be ubiquitinylated in this setting and involved in NF- κ B activation, despite the fact that NEMO ubiquitinylation has not been convincingly

339 detected (99).

340 Linear ubiquitinylation is also involved in canonical NF- κ B activation mediated
341 by membrane PRRs, TLRs. TLR ligand-induced canonical NF- κ B activation is mediated by
342 Myd88 (100, 101). Upon stimulation with TLR ligands, Myd88 is recruited to activated
343 TLRs, followed by recruitment of the TRAF6 ubiquitin ligase. K63 chains generated by
344 TRAF6 on TRAF6 itself or on other proteins in the activated receptor complex may
345 function as a recruitment signal for LUBAC and induce NF- κ B activation. Linear
346 ubiquitinylation is also involved in IL-1 β -mediated NF- κ B activation, in which Myd88 also
347 plays a role, since linear ubiquitinylation of NEMO was detected in cells stimulated with
348 IL-1 β (25). However, residual NF- κ B activation in cells lacking linear ubiquitinylation
349 activity differs among stimuli and cells: IL-1 β -mediated NF- κ B activation in mouse
350 embryonic fibroblasts (MEFs) is more severely suppressed than that mediated by TLR9
351 ligands in B cells lacking LUBAC's ligase activity (54). Moreover, LUBAC-mediated
352 linear ubiquitinylation is dispensable for NF- κ B activation induced by B-cell antigen
353 receptor, although CD40-mediated NF- κ B activation is impaired almost completely in B
354 cells lacking the catalytic center of HOIP (54). In addition, a ligase-independent role of
355 LUBAC in NF- κ B activation was suggested (102). Thus, current results indicate the
356 existence of several pathways for the activation of IKK.

357 The involvement of another polyubiquitin chain, the K63-ubiquitin chain, in
358 NF- κ B activation was also suggested (29, 103, 104). In this scenario, K63 chains function

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359 as platforms to recruit both IKK and the TAK1 complex, which is composed of TAK1,
360 TAB1, and TAB2 and/or TAB3, via the ubiquitin binding activities of NEMO and TAB2
361 and/or TAB3, respectively. Then, the TAK1 kinase phosphorylates IKK2, leading to IKK
362 activation. However, since binding of NEMO to K63 is weak because the UBAN domain of
363 NEMO prefers linear di-ubiquitin to K63 di-ubiquitin (87), K63 chains alone may not be
364 enough to activate IKK. Recently, mixed ubiquitin chains, in which both linear and K63
365 linkages co-exist, were identified (105). The TAK1/TAB2/TAB3 and the IKK complex can
366 be recruited to the mixed chain via K63 and linear ubiquitin binding activities, respectively
367 (106). This leads to the phosphorylation of the Ser residues of IKK2 by TAK1 and by
368 trans-autophosphorylation induced by linear chains (89). Linear ubiquitin chains appear to
369 play central roles in canonical NF- κ B activation induced by ligands of the TNF receptor
370 family; however, further analyses are necessary to clarify the roles of linear ubiquitin
371 chains in IKK activation completely. abolishes
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373 b) ERK activation.

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375 In addition to their involvement in NF- κ B activation, linear chains play a role in
376 ERK activation (54). In B cells and macrophages, IKK activates ERK via TPL2
377 phosphorylation (107, 108). Loss of the linear ubiquitinylation activity of LUBAC
378 abolishes CD40- and TLR-mediated ERK activation in B cells almost completely (54).

Thus, linear ubiquitinylation plays a role in ERK activation through IKK, which is activated by linearly polyubiquitinated NEMO (54). However, B-cell receptor (BCR)-mediated ERK activation is not overtly affected in B cells lacking the catalytic center of HOIP, indicating that multiple ERK activation pathways exist in B cells (54).

c) Cell death regulation

Certain TNFR family receptors such as TNF receptor 1 (TNFR1) trigger cell death signals, and LUBAC-mediated linear ubiquitinylation is involved in the regulation of TNFR1-mediated cell death; however, the linearly ubiquitinated substrates involved in cell death regulation remain to be identified (36, 109, 110). TNFR1 is constitutively expressed on the surface of almost all cell types and plays a role in immune and inflammatory responses during infection, cell proliferation, and the regulation of the programmed cell death processes known as apoptosis and necroptosis (programmed necrosis). TNF- α and lymphotoxin alpha (LT- α , also referred to as TNF- β) are the ligands of the receptor. Activation of TNFR1 is initiated by its trimerization on the cellular membrane in response to the binding of extracellular ligands. This leads to a conformational change in the intracellular death domain (DD) of TNFR1 that results in the recruitment of two DD-containing adaptor proteins, TNFR-associated death domain (TRADD) and receptor interacting protein 1 (RIP1), through direct interaction between the

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399 DDs (*Fig. 4*). Certain ubiquitin E3 ligases such as TRAF2, and cellular inhibitor of
400 apoptosis protein 1 and 2 (cIAP1, 2) are recruited to activated TNFR1 via TRADD to
401 assemble a signaling complex known as complex I at the cell membrane (111, 112). In
402 addition to the known components of complex I, LUBAC is also recruited to the activated
403 TNFR1 complex (113). The recruitment of LUBAC to the TNFR1 signaling complex is
404 dependent on the ubiquitin ligase activity of cIAP1 and cIAP2. The NZF domains are
405 UBDs, and the HOIP NZF1 domain can recognize ubiquitin and NEMO simultaneously
406 (89). The ubiquitin binding activity of the NZF1 domain of HOIP is critical for the
407 recruitment of LUBAC to the activated TNF receptor complex and possibly functions by
408 recognizing ubiquitin chains conjugated by cIAPs (*Fig. 5*) (113). cIAPs ubiquitinyrate not
409 only the components of the TNFR1 signaling complex including RIP1, but also undergo
410 auto-ubiquitinylation, eventually leading to the generation of high amounts of various types
411 of ubiquitin chains, including K63 chains. Recognition of K63 chains by HOIP NZF1 may
412 be involved in the recruitment of LUBAC to the activated TNFR1 complex (113).
413 K11-linked chains generated by cIAPs are involved in canonical NF- κ B activation,
414 suggesting that the HOIP NZF binds to K11 chains, although this has not been definitely
415 proven (114). The recruitment of LUBAC to the activated TNFR1 complex may lead to the
416 recruitment of IKK and the linear ubiquitinylation of NEMO, resulting in the activation of
417 NF- κ B.
418 The linear ubiquitinylation activity of LUBAC is required for the protection of

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7 419 cells from TNF- α -mediated cell death. LUBAC can induce the expression of anti-apoptotic
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9 420 genes including Bcl2 and cFLIP by activating NF- κ B. In addition, LUBAC inhibits
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11 421 TNF- α -mediated cell death by a different mechanism. The transition from complex I to
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13 422 complex II triggers cell death. TNFR1 induces two types of programmed cell death,
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15 423 apoptosis and necroptosis, both of which can be initiated by the formation of complex II,
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17 424 which is composed of TRADD, FADD, caspase 8, RIP1, and possibly RIP3 (*Fig. 5*).
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19 425 Caspase 8 is activated by dimerization and digests RIP1 and RIP3, leading to apoptosis.
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21 426 When caspase 8 is inactivated, RIP1 and RIP3 kinases are activated by trans- or
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23 427 auto-phosphorylation, which triggers necroptosis (115-117). The linear ubiquitylation
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25 428 activity of LUBAC plays a role in suppressing the formation of complex II, although the
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27 429 linear ubiquitylation substrates involved in the inhibition of complex II formation have
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29 430 not been convincingly identified yet (36). The removal of K63 linked chains by the A20 or
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31 431 CYLD DUBs from RIP1 is involved in complex II formation (95, 118-121). However,
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33 432 RIP1 is modified by multiple types of chains, including K11 and linear chains in addition to
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35 433 K63 chains. Considering that the linear ubiquitylation activity of LUBAC is required for
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37 434 the inhibition of complex II formation, the linear ubiquitylation of RIP1 might be
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39 435 involved in the inhibition of TNF- α mediated cell death by LUBAC. However, further
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41 436 analyses are needed to clarify the role of LUBAC-mediated linear ubiquitylation in cell
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439 d) Other functions

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441 The involvement of LUBAC-mediated linear ubiquitinylation in other functions
442 was investigated using HOIL-1L^{-/-} or *cpdm* mice.

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444 d)-1 Regulation of inflammasome formation

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446 Pyrin domain-containing 3 (NLRP3), an NLR, functions in the assembly of the
447 inflammasome, which promotes the production of proinflammatory cytokines, mainly
448 IL-1 β and IL-18 (122, 123). NLRP3 senses several pathogen-associated molecular patterns
449 (PAMPs) as well as host damage-associated molecular patterns (DAMPs) released from
450 injured and necrotic cells. The activated NLRP3 and the adaptor protein ASC act together
451 to assemble the inflammasome, which potentiates the cleavage of pro-caspase 1, followed
452 by the release of two active forms, the p10 and p20 domain-containing subunits. The
453 resultant active caspase 1 cleaves pro-IL-1 β and pro-IL-18 to generate the mature
454 proinflammatory cytokines. Thus, NLRP3 is involved in innate immune responses through
455 the rapid production of IL-1 β and IL-18. Formation of the NLRP3-ASC inflammasome is
456 attenuated in bone marrow derived macrophages (BMDMs) from HOIL-1L^{-/-} mice. ASC
457 was identified as a substrate for linear ubiquitinylation associated with inflammasome
458 formation, although the roles of linearly ubiquitinated ASC in inflammasome formation

have not been addressed. Reduction of LUBAC levels affects the activation of the inflammasome, and the secretion of IL-1 β in BMDMs of HOIL-1L^{-/-} mice is suppressed independently of NF- κ B signaling (124). Of note, the RBR domain of HOIL-1L is required for the linear ubiquitinylation of ASC in addition to HOIP RBR, although the RBR domain of HOIL-1L is dispensable for the generation of linear ubiquitin chains (124). The proposed involvement of the RBR domain of HOIL-1L in NEMO ubiquitinylation (86) indicates that the HOIL-1L RBR may be required for ASC linear ubiquitinylation.

d)-2 Inhibition of RIG-I signaling

Retinoic acid-inducible gene-I (RIG-I) belongs to the RIG-I-like receptor (RLR) family among the PRRs, and serves as a cytoplasmic virus RNA sensor that facilitates innate antiviral responses through the upregulation of type I IFNs, IFN- α and IFN- β (122, 125). Upon recognition of viral RNA, RIG-I is conjugated with K63-linked ubiquitin chains generated by TRIM25, which facilitates interaction with an adaptor protein, mitochondrial antiviral signaling protein (MAVS) through their CARD domains. Subsequently, RIG-I interacts with TRAF3 via the TRAF-interacting motifs (TIM) of MAVS, leading to the recruitment of NF- κ B signaling related-proteins including NEMO and IKK-dependent NF- κ B activation (126). Unlike other PRR-mediated signaling mechanisms, LUBAC-mediated linear ubiquitinylation negatively regulates

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RIG-I-mediated signaling (127). NEMO conjugated with linear ubiquitin chains, but not unmodified NEMO, interacts with TRAF3, which interferes with the assembly of the MAVS-TRAF3 complex and eventually inhibits anti-virus IFN secretion (127). Consistent with this concept, the MEFs of SHARPIN-deficient *cpdm* mice show increased IFN mediated antiviral responses when infected with vesicular stomatitis virus (VSV), which is a negative-strand RNA virus (128). Another report suggested that HOIL-1L and HOIP suppress RIG-I signaling induced by Sendai viruses through the inhibition of RIG-I-TRIM25 interaction, which is mainly mediated by TRIM25 degradation in HOIL-1L^{-/-} MEFs with suppressed HOIP expression. In this scenario, TRIM25 is identified as a LUBAC substrate (129). The linear ubiquitin chain may function as a degradation signal, albeit weak (27, 130); however, further investigation is necessary to identify the role of LUBAC in RIG-I signaling.

Analysis of the pathophysiological roles of linear ubiquitylation using LUBAC mutant mice

The phenotypes of mice lacking components of LUBAC are drastically diverse because of differences in the residual amount of functional LUBAC mainly. No LUBAC complex or linear ubiquitylation activity is observed in HOIP^{-/-} mice. No linear ubiquitylation activity can be detected in mice expressing C-terminal deleted HOIP or a

ubiquitin binding deficient HOIP mutant in HOIP null mice despite the presence of the LUBAC complex. Mice lacking HOIL-1L (HOIL-1L^{-/-}) or SHARPIN (*cpdm*) express a LUBAC complex composed of the other two subunits, although the amount of residual LUBAC is drastically reduced because of the destabilization of LUBAC provoked by the loss of one subunit. The linear ubiquitylation activity is reduced but present in mice lacking SHARPIN or HOIL-1L. In addition, SHARPIN or HOIL-1L that does not form a complex with HOIP exists in cells. Indeed, LUBAC independent functions of SHARPIN or HOIL-1L were reported. SHARPIN controls lymphocyte migration via direct interaction with β 1-integrin or lymphocyte-function-associated antigen-1 (LFA-1) (131, 132). HOIL-1L targets protein kinase C ζ for degradation under hypoxic conditions to promote tumor survival (133). Therefore, results obtained using HOIL-1L^{-/-} or *cpdm* mice, which are not linear chain null mice, need to be interpreted with caution.

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512 a) Mechanism underlying *cpdm* pathology

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SHARPIN-deficient *cpdm* mice and HOIL-1L^{-/-} mice are both defective in NF- κ B activation because of instability of the LUBAC complex. Suppressed NF- κ B activation is characterized by delayed phosphorylation of I κ B α and decreased expression of NF- κ B-targeting genes in response to TNF- α stimulation in both types of mutant mice (25, 26). Nevertheless, *cpdm* mice, but not HOIL-1L^{-/-} mice, develop chronic dermatitis as

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described above (29). TNF- α signaling plays a role in the pathogenesis of *cpdm*, as intercrossing *cpdm* mice with TNF- α null mice ameliorates chronic dermatitis (35). The main functions of LUBAC-mediated linear ubiquitinylation are its contribution to canonical NF- κ B activation and cell death regulation. NF- κ B activation is suppressed in both *cpdm* and HOIL-1L^{-/-} mice at comparable levels (25, 26). MEFs from *cpdm* mice are more sensitive to TNF- α -induced cell death than those from HOIL-1L^{-/-} mice despite the fact that TNF- α -induced cell death is strongly induced in HOIL-1L^{-/-} cells (25). TNF- α induces two types of programmed cell death, apoptosis and necroptosis, and the latter is more prone to induce inflammation than the former (134, 135). To investigate the role of necroptosis and apoptosis in *cpdm* pathology, *cpdm* mice are crossed with mice lacking molecules involved in these two types of programmed cell death. Deletion of RIP3 or mixed lineage kinase domain-like protein (MLKL), which are both involved in necroptosis, only partially ameliorates *cpdm* symptoms (109, 110). Caspase 8 or FADD is involved in the induction of apoptosis. However, their deletion promotes necroptosis. Deficiency of caspase 8 or FADD specifically in keratinocytes combined with RIP3 ameliorated *cpdm* symptoms almost completely (109, 110). Moreover, crossing *cpdm* mice with mice expressing kinase-negative RIP1, which impairs necroptosis and certain forms of apoptosis, cured *cpdm* dermatitis completely (64). Thus, both apoptosis and necroptosis are involved in *cpdm* pathology.

A recent report showed that the *Rnf31* gene, which encodes HOIP, is located

between the *Psme2* and *Irf9* genes, both of which are induced by interferon- γ (IFN- γ). In addition, the *Rnf31* gene was shown to have an IFN regulatory factor 1 and two IFN- γ -responsive regulatory elements on the upstream and downstream regions of the genes, respectively (136). As expected, mRNA transcription of HOIP was increased after IFN- γ treatment. Increased protein levels of both HOIL-1L and SHARPIN in addition to HOIP were observed in MEFs, BMDMs, and primary keratinocytes in response to type I and type II IFN, which are induced mainly by immune cells including activated lymphocytes after RNA or DNA virus infection leading to enhanced activation of NF- κ B (136). Moreover, intradermal injection of type I or type II IFN ameliorated *cpdm* dermatitis by upregulating HOIP expression in the skin, indicating that reduction of residual LUBAC underlies *cpdm* pathology. Therefore, attenuated NF- κ B activation may also underlie *cpdm* pathology although NF- κ B is known as a proinflammatory transcription factor (137). It is, thus, of interest to determine the phenotype of mice with further reduction of LUBAC composed of SHARPIN and HOIP in HOIL-1L^{-/-} mice.

b) Investigating the role of LUBAC-mediated linear ubiquitinylation in B lymphocytes

To dissect the functions of linear ubiquitin chains, it is indispensable to use mice lacking HOIP or the linear ubiquitinylation activity of HOIP. However, complete loss of the linear ubiquitinylation activity of LUBAC is associated with embryonic lethality. Therefore,

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559 the roles of linear ubiquitin chains in the immune system should be probed using tissue or
560 cell type specific deletion of LUBAC ligase activity. Mice lacking the C-terminal part of
561 HOIP, which contains the catalytic center for linear ubiquitinylation (B-HOIP Δ linear mice),
562 were used to investigate the roles of LUBAC-mediated linear ubiquitinylation in B
563 lymphocytes (54). The development of conventional B cells, both follicular and marginal B
564 cells, was not affected by the loss of linear ubiquitinylation activity of LUBAC. However,
565 the effector functions of B cells were heavily affected, with almost complete suppression of
566 responses to thymus-dependent antigen and thymus-independent antigen II. Moreover,
567 differentiation of B1 cells, which reside in the peritoneum, was heavily impaired by the loss
568 of LUBAC ligase activity, and the serum titers of all subclasses of antibodies in resting
569 stages were suppressed. These results indicated that the linear polyubiquitinylation activity
570 of LUBAC plays crucial roles in B cells. Biochemical analyses revealed that canonical
571 NF- κ B and ERK activation mediated by the TNFR family receptors CD40 and
572 transmembrane activator and CAML interactor (TACI) is attenuated in B cells from B-HOIP
573 Δ linear mice. CD40-induced JNK or alternative NF- κ B activation is not overtly affected in
574 HOIP Δ linear B cells. Activation of canonical NF- κ B and ERK by TLR4 and TLR9 is also
575 attenuated albeit less significant than that mediated by CD40. In this study, BCR-mediated
576 signaling, which plays an important role in B1 cell development (138), was not affected by
577 the loss of linear ubiquitinylation activity. These results suggest that receptors other than
578 BCR play a role in B1-cell development through the activation of the canonical NF- κ B

pathway. However, the involvement of LUBAC in BCR-mediated canonical NF- κ B activation via a ligase-independent mechanism was suggested in another study (139).

Loss of HOIL-1L in patients and mice

Patients with bi-allelic mutations in HOIL-1L genes have been reported (31, 140, 141). Fibroblast cells from these patients decreased protein levels of the other LUBAC components, HOIP and SHARPIN, and impaired TNF- α and IL-1 β induced NF- κ B activation. In one study, chronic hyperinflammation, invasive bacterial infections associated with mild immunodeficiency, cardiomyopathy, and muscular amylopectinosis were detected in three patients, whereas the remaining 13 showed cardiomyopathy and muscular amylopectinosis, but no immunological symptoms (31).

HOIL-1L^{-/-} mice do not show any overt phenotypes under pathogen-free conditions, although deposition of amylopectin-like materials could be observed in the aging heart (142). On the other hand, HOIL-1L^{-/-} mice are susceptible to acute infections by several pathogens including *Listeria monocytogenes*, indicating that HOIL-1L^{-/-} mice are immunodeficient (142). HOIL-1L^{-/-} mice are resistant to chronic infection by murine γ -herpesvirus 68 (MHV68), suggesting that chronic herpesvirus infection generates signs of auto-inflammation. These results imply that the lack of immunological symptoms in HOIL-1L^{-/-} mice is due to the pathogen-free conditions.

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LUBAC in diseases

In addition to the role of mutations in HOIL-1L genes, the involvement of LUBAC-mediated linear ubiquitinylation in disease was revealed recently. Aberrant NF-κB activation plays a critical role in the oncogenesis of activated B-cell-like diffuse large B-cell lymphomas (ABC DLBCL) (139). Yang et al. reported that SHARPIN and HOIP are essential for the growth of ABC DLBCL cells but not germinal center B-cell-like (GCB) DLBCL cells through the suppression of NF-κB activation. They also showed that two rare SNPs of the HOIP gene are highly enriched in ABC DLBCL (139). Mutations of the A20 DUB that abrogate linear ubiquitin binding activity of the protein were reported in Hodgkin and non-Hodgkin B-cell lymphomas (92). Attenuated LUBAC expression suppresses lung metastases of osteosarcomas in mice (143). Moreover, downregulation of HOIP or SHARPIN expression promoted apoptosis induced by platinum-based genotoxins, including cisplatin and carboplatin, which are widely used anti-cancer drugs (144). Thus, LUBAC activity might be a suitable target to control malignant tumors. Gliotoxin, a major virulence factor of the opportunistic pathogen *Aspergillus fumigatus*, was recently identified as a specific inhibitor of LUBAC-mediated linear ubiquitinylation (145), which highlights the crucial roles of linear ubiquitinylation in the protection from infectious agents and the possibility of isolating LUBAC inhibitors from natural products.

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620 **Concluding remarks**

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622 The present short article introduces the roles of the recently identified ubiquitin
623 modification, linear ubiquitinylation, in the immune system. Linear polyubiquitin chains
624 and LUBAC were identified in 2006 during analyses aimed at elucidating the molecular
625 mechanism underlying the generation of polyubiquitin chains (27). To date, linear
626 ubiquitinylation has been shown to play a role in inflammation, immune responses, and
627 oncogenesis, as expected from its involvement in NF- κ B activation. Inhibitors of
628 LUBAC-mediated linear ubiquitinylation were suggested as suitable candidates for the
629 treatment of malignant tumors. In addition, the finding that the ubiquitin ligase activity of
630 LUBAC is induced by type I and type II IFNs implies a novel connection between the IFN
631 and NF- κ B pathways. The fact that phosphorylation of a Tyr residue in OTULIN, a linear
632 chain specific DUB, suppresses the interaction between OTULIN and LUBAC, indicates
633 crosstalk between Tyr phosphorylation and NF- κ B activation. Thus, the roles of linear
634 ubiquitinylation might not be limited to NF- κ B activation or cell death regulation. In
635 addition to NEMO and A20, proteins containing linear chain specific UBDs were reported.
636 For example, the UBAN motif of Optineurin, which is a product of a mutated gene in
637 glaucoma and amyotrophic lateral sclerosis, can recognize linear di-ubiquitin (146-148).
638 Moreover, Optineurin is an autophagy receptor involved in the autophagic clearance of

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cytosolic Salmonella (146). The study of linear ubiquitin chains is just beginning. Further analyses may reveal additional unexpected roles of linear ubiquitin chains in the near future.

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1032 **Figure legends**

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1034 **Figure 1. The ubiquitin conjugating system and ubiquitin chain lineage-specific**

1035 **behavior.**

1036 Ubiquitylation is an enzymatic process mediated by a ubiquitin activating enzyme (E1), a

1037 ubiquitin conjugating enzyme (ubiquitin carrier protein) (E2), and a ubiquitin ligase (E3).

1038 First, ubiquitin is ATP-dependently transferred to E1 via formation of a thioester bond

1039 between the C-terminal carboxyl group of ubiquitin and the cysteine residue in the active

1040 site of E1. Ubiquitin on the E1 is then transferred to the cysteine residue of E2, which also

1041 forms a thioester bond. Finally, E3 associates with the ubiquitin-bound E2 and a substrate,

1042 and catalyzes the conjugation of ubiquitin to the substrate via an isopeptide bond between

1043 the ϵ -amino group of a lysine residue of the substrate and the C-terminal carboxyl group of

1044 ubiquitin. There are three classes of E3s: HECT, RING, and RBR E3s. HECT and RBR E3s

1045 bind to ubiquitin before conjugating ubiquitin to substrates, as described in Figure 3. Once

1046 the first ubiquitin is conjugated to the substrate, additional ubiquitins are conjugated

1047 successively onto the terminal ubiquitin on the substrate to form polyubiquitin chains.

1048 Polyubiquitin chains are generated by isopeptide bond formation between the C-terminal

1049 carboxyl group of one ubiquitin and an ϵ -amino group of one of seven Lys (K) residues in

1050 another ubiquitin molecule, thus generating seven types (K6, K11, K27, K29, K33 aK48,

1051 and K63) of linkages. A linear (M1) linkage, in which the C-terminal carboxyl group of one

ubiquitin forms a peptide bond with an α -amino group of the N-terminal Met (M) residue of another ubiquitin, was reported. Four types of polyubiquitin chains [K11-linked, K48-linked, K63-linked, and Linear (M1-linked)] are shown. Each chain is recognized by ubiquitin binding proteins containing a specific ubiquitin binding domain (UBD). The polyubiquitin chains are cleaved by deubiquitinating enzymes (DUB). DUBs can be specific for certain types of ubiquitin linkage. Ubiquitin monomers newly-trimmed by DUBs are integrated into the ubiquitin pool to be used for further ubiquitination of other proteins.

Figure 2. Schematic representation of LUBAC components.

The linear ubiquitin chain assembly complex (LUBAC) is composed of heme-oxidized IRP2 ligase 1L (HOIL-1L), HOIL-1 interacting protein (HOIP), and SH3 and multiple ankyrin-repeat domains protein (SHANK)-associated RBCK1 homology (RH) domain-interacting protein (SHARPIN). Arrows indicate protein-protein interactions to form LUBAC's ternary structure. Interactions between the HOIP NZF2 and SHARPIN UBL are also involved in LUBAC formation; however, HOIP lacking NZF2, and not UBA, binds to SHARPIN in cells. HOIP contains the catalytic center for ubiquitin E3 ligase activity in its carboxyl-terminal region. UBL, ubiquitin-like domain; UBA, ubiquitin-associated domain; NZF, nuclear protein localization 4(Npl4)-type zinc finger; RING, really interesting new gene; IBR, in-between-RING domain; ZF, zinc finger; PUB,

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6 1072 peptide N-glycosidase (PNGase)/ubiquitin-associated domain; LDD, linear ubiquitin chain
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9 1073 determining domain.
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14 1075 **Figure 3. Different ubiquitin conjugation modes mediated by three E3 ligase families.**

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17 1076 A. Homology to E6-AP C-terminus (HECT) E3 ligases forms an intermediate complex via
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19 1077 a thioester bond between a cysteine residue of E3s and the C-terminus of ubiquitin
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22 1078 activated by E2 before transfer to the substrate. B. Really Interesting New Gene (RING) E3
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24 1079 ligases catalyze the conjugation of ubiquitin from E2 to the substrate directly. C. The
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27 1080 RING-In-Between-RING (IBR)-RING (RBR) E3 ligase family, to which LUBAC belongs,
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29 1081 uses the hybrid ubiquitin conjugation mechanism of the RING and the HECT E3s. In this
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32 1082 mechanism, the RING2 domain of RBR E3s binds to the C-terminus of ubiquitin to form
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35 1083 an intermediate, as seen in a RING-like manner after the binding of ubiquitin-conjugated
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37 1084 E2 to the RING1 domain of the RBR E3s. The activated ubiquitin on the RING2 domain is
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40 1085 subsequently transferred to a substrate in a HECT-like manner.

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45 1087 **Figure 4. LUBAC-mediated linear ubiquitylation in the canonical NF-κB activation**
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47 1088 **pathway.**

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50 1089 The contribution of LUBAC to the tumor necrosis factor (TNF)-α-induced activation of
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53 1090 nuclear factor-kappa B (NF-κB) pathway is well-established. TNF-α engagement to TNF
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55 1091 receptor 1 (TNFR1) allows its conformational change resulting in the recruitment of RIP1
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6 1092 and TRADD via homotypic interactions between two death domains; TRAF2 and cellular
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8 1093 inhibitor of apoptosis proteins (cIAPs) are also incorporated into the TNFR1 complex.
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10 1094 RIP1 is often detected as a ubiquitin-conjugated protein after TNF- α stimulation. The E3
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12 1095 ligases, cIAPs, ubiquitinate components of the activated TNFR1 complex, including RIP1,
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14 1096 with K63 and K11-linked ubiquitin chains, and themselves by auto-ubiquitination. These
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16 1097 generated ubiquitin chains on components of the TNFR1 complex were suggested to serve
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18 1098 as a platform to recruit the TAK1 complex, which is composed of TAK1, TAB1, and TAB2
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20 1099 and/or TAB3, and the recruitment of TAK1 facilitates the phosphorylation of IKK2
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22 1100 necessary for the activation of the IKK complex. LUBAC is recruited to the ubiquitin
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24 1101 chains within the TNFR1 complex via the K63 (possibly also K11) ubiquitin chain-binding
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26 1102 property of the NZF1 domain of HOIP. The HOIP NZF1 domain can interact with NEMO
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28 1103 in the IKK complex in addition to ubiquitin chains, and conjugate the linear ubiquitin chain
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30 1104 on NEMO. The linear ubiquitin chain conjugated to NEMO recruits another IKK complex
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32 1105 because NEMO contains a specific binding region for linear ubiquitin chains called the
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34 1106 ubiquitin binding in ABIN and NEMO (UBAN) motif, which triggers dimerization of IKK
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36 1107 complexes and subsequent trans-autophosphorylation of IKK2. The activated IKK complex
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38 1108 phosphorylates I κ B α , leading to its K48-linked ubiquitination and proteasomal degradation.
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40 1109 This process activates the cytosolic NF- κ B transcription factor, facilitating its translocation
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42 1110 to the nucleus and the induction of the expression of target genes.
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1112 **Figure 5. Inhibition of TNF- α mediated cell death by linear ubiquitylation**

1113 Upon binding TNF- α , TNFR1 interacts with TRADD, RIP1, TRAF2, and cIAP1 and 2 to
1114 form complex I. LUBAC is also recruited to the activated TNFR1 complex via recognition
1115 of ubiquitin chains in the TNFR1 complex possibly by cIAP1 and 2. LUBAC protects cells
1116 from death by inducing the expression of various NF- κ B target genes, including Bcl-2 and
1117 cFLIP. In addition to the induction of anti-apoptotic genes, LUBAC inhibits
1118 TNF- α -mediated cell death by a different mechanism, the inhibition of complex II. The
1119 transition from complex I to complex II triggers cell death. TNFR1 induces two types of
1120 programmed cell death, apoptosis and necroptosis, and formation of complex II, which is
1121 composed of TRADD, FADD, caspase 8, RIP1, and possibly RIP3, precedes both types of
1122 cell death. After the formation of complex II, caspase 8 is activated by dimerization and
1123 digests RIP1 and RIP3, leading to apoptosis. When caspase 8 is inactivated, RIP1 and RIP3
1124 kinases are activated and trigger necroptosis. The linear ubiquitination activity of LUBAC
1125 is involved in the suppression of complex II formation, although the linear ubiquitylation
1126 substrates involved in the inhibition of complex II formation have not been convincingly
1127 identified to date.

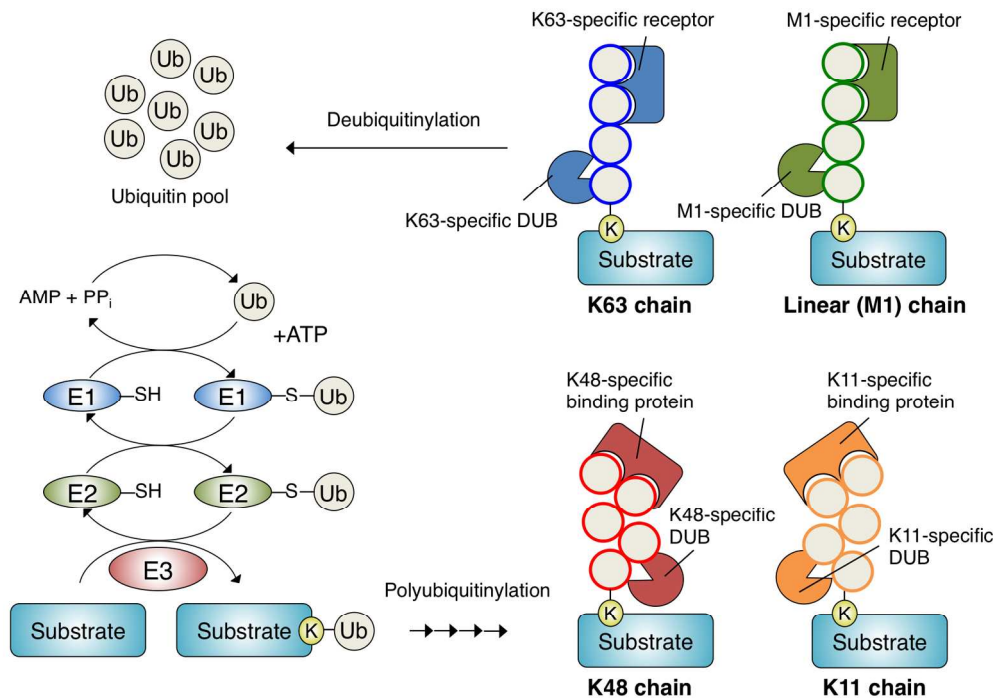


Figure 1. The ubiquitin conjugating system and ubiquitin chain lineage-specific behavior.

Ubiquitylation is an enzymatic process mediated by a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (ubiquitin carrier protein) (E2), and a ubiquitin ligase (E3). First, ubiquitin is ATP-dependently transferred to E1 via formation of a thioester bond between the C-terminal carboxyl group of ubiquitin and the cysteine residue in the active site of E1. Ubiquitin on the E1 is then transferred to the cysteine residue of E2, which also forms a thioester bond. Finally, E3 associates with the ubiquitin-bound E2 and a substrate, and catalyzes the conjugation of ubiquitin to the substrate via an isopeptide bond between the ϵ -amino group of a lysine residue of the substrate and the C-terminal carboxyl group of ubiquitin. There are three classes of E3s: HECT, RING, and RBR E3s. HECT and RBR E3s bind to ubiquitin before conjugating ubiquitin to substrates, as described in Figure 3. Once the first ubiquitin is conjugated to the substrate, additional ubiquitins are conjugated successively onto the terminal ubiquitin on the substrate to form polyubiquitin chains. Polyubiquitin chains are generated by isopeptide bond formation between the C-terminal carboxyl group of one ubiquitin and an ϵ -amino group of one of seven Lys (K) residues in another ubiquitin molecule, thus generating seven types (K6, K11, K27, K29, K33, K48, and K63) of linkages. A linear (M1) linkage, in which the C-terminal carboxyl group of one ubiquitin forms a peptide bond with an α -amino group of the N-terminal Met (M) residue of another ubiquitin, was reported. Four types of polyubiquitin chains [K11-linked, K48-linked, K63-linked, and Linear (M1-linked)] are shown. Each chain is recognized by ubiquitin binding proteins containing a specific ubiquitin binding domain (UBD). The polyubiquitin chains are cleaved by deubiquitinating enzymes (DUB). DUBs can be specific for certain types of ubiquitin linkage. Ubiquitin monomers newly-trimmed by DUBs are integrated into the ubiquitin pool to be used for further ubiquitination of other proteins.

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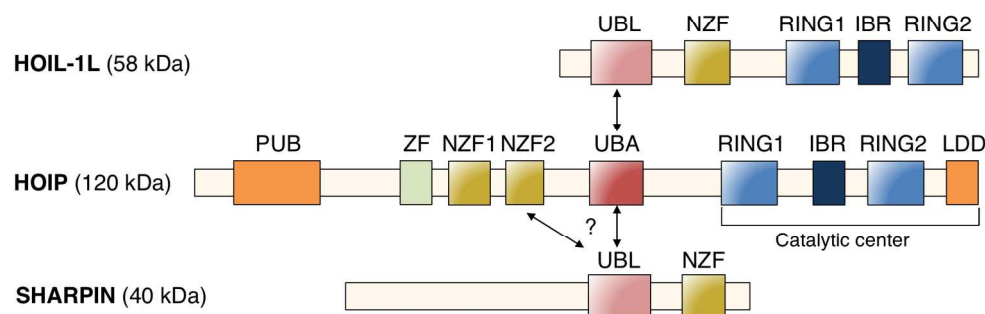


Figure 2. Schematic representation of LUBAC components. The linear ubiquitin chain assembly complex (LUBAC) is composed of heme-oxidized IRP2 ligase 1L (HOIL-1L), HOIL-1 interacting protein (HOIP), and SH3 and multiple ankyrin-repeat domains protein (SHANK)-associated RBCK1 homology (RH) domain-interacting protein (SHARPIN). Arrows indicate protein-protein interactions to form LUBAC's ternary structure. Interactions between the HOIP NZF2 and SHARPIN UBL are also involved in LUBAC formation; however, HOIP lacking NZF2, and not UBA, binds to SHARPIN in cells. HOIP contains the catalytic center for ubiquitin E3 ligase activity in its carboxyl-terminal region. UBL, ubiquitin-like domain; UBA, ubiquitin-associated domain; NZF, nuclear protein localization 4(Npl4)-type zinc finger; RING, really interesting new gene; IBR, in-between-RING domain; ZF, zinc finger; PUB, peptide N-glycosidase (PNGase)/ubiquitin-associated domain; LDD, linear ubiquitin chain determining domain.

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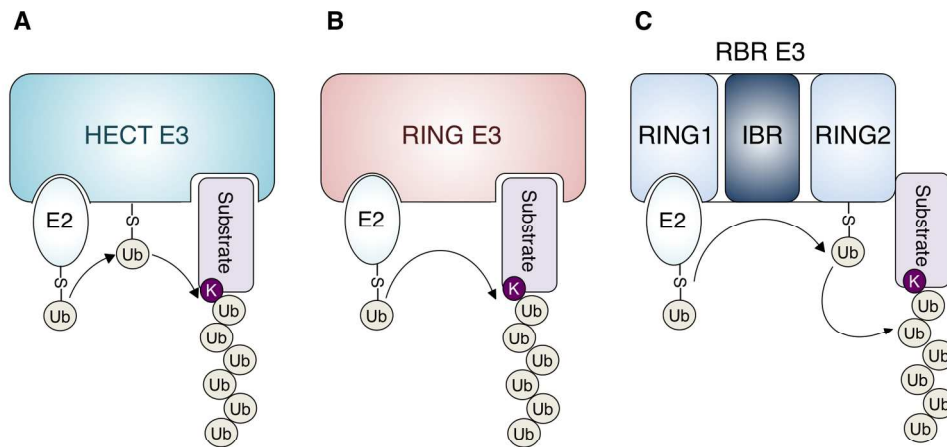


Figure 3. Different ubiquitin conjugation modes mediated by three E3 ligase families.

A. Homology to E6-AP C-terminus (HECT) E3 ligases forms an intermediate complex via a thioester bond between a cysteine residue of E3s and the C-terminus of ubiquitin activated by E2 before transfer to the substrate. B. Really Interesting New Gene (RING) E3 ligases catalyze the conjugation of ubiquitin from E2 to the substrate directly. C. The RING-In-Between-RING (IBR)-RING (RBR) E3 ligase family, to which LUBAC belongs, uses the hybrid ubiquitin conjugation mechanism of the RING and the HECT E3s. In this mechanism, the RING2 domain of RBR E3s binds to the C-terminus of ubiquitin to form an intermediate, as seen in a RING-like manner after the binding of ubiquitin-conjugated E2 to the RING1 domain of the RBR E3s. The activated ubiquitin on the RING2 domain is subsequently transferred to a substrate in a HECT-like manner.

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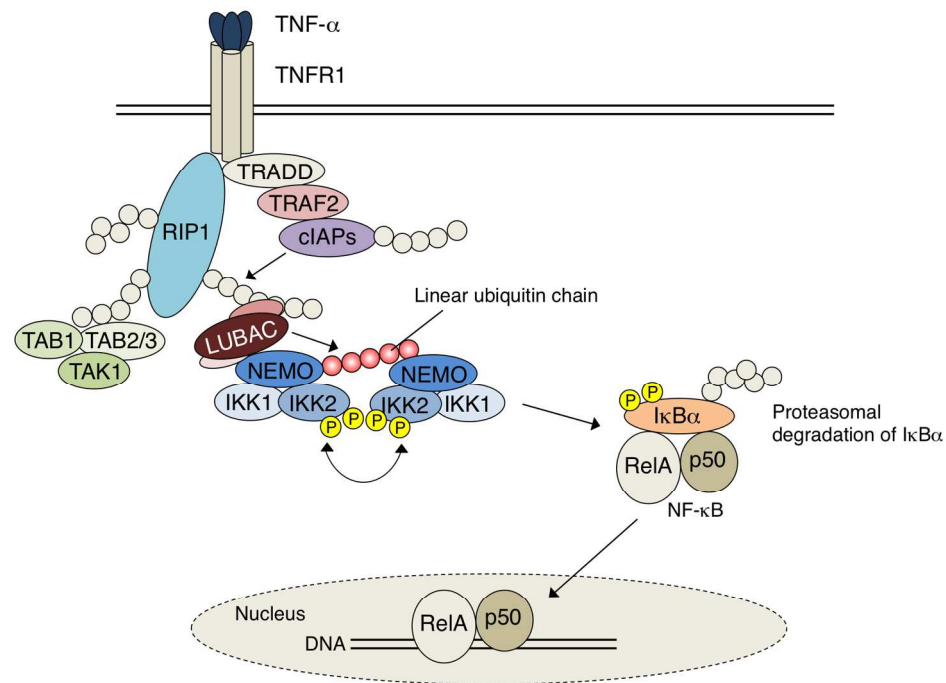


Figure 4. LUBAC-mediated linear ubiquitinylation in the canonical NF-κB activation pathway. The contribution of LUBAC to the tumor necrosis factor (TNF)-α-induced activation of nuclear factor-kappa B (NF-κB) pathway is well-established. TNF-α engagement to TNF receptor 1 (TNFR1) allows its conformational change resulting in the recruitment of RIP1 and TRADD via homotypic interactions between two death domains; TRAF2 and cellular inhibitor of apoptosis proteins (cIAPs) are also incorporated into the TNFR1 complex. RIP1 is often detected as a ubiquitin-conjugated protein after TNF-α stimulation. The E3 ligases, cIAPs, ubiquitinate components of the activated TNFR1 complex, including RIP1, with K63 and K11-linked ubiquitin chains, and themselves by auto-ubiquitination. These generated ubiquitin chains on components of the TNFR1 complex were suggested to serve as a platform to recruit the TAK1 complex, which is composed of TAK1, TAB1, and TAB2 and/or TAB3, and the recruitment of TAK1 facilitates the phosphorylation of IKK2 necessary for the activation of the IKK complex. LUBAC is recruited to the ubiquitin chains within the TNFR1 complex via the K63 (possibly also K11) ubiquitin chain-binding property of the NZF1 domain of HOIP. The HOIP NZF1 domain can interact with NEMO in the IKK complex in addition to ubiquitin chains, and conjugate the linear ubiquitin chain on NEMO. The linear ubiquitin chain conjugated to NEMO recruits another IKK complex because NEMO contains a specific binding region for linear ubiquitin chains called the ubiquitin binding in ABIN and NEMO (UBAN) motif, which triggers dimerization of IKK complexes and subsequent trans-autophosphorylation of IKK2. The activated IKK complex phosphorylates IκBα, leading to its K48-linked ubiquitination and proteasomal degradation. This process activates the cytosolic NF-κB transcription factor, facilitating its translocation to the nucleus and the induction of the expression of target genes.

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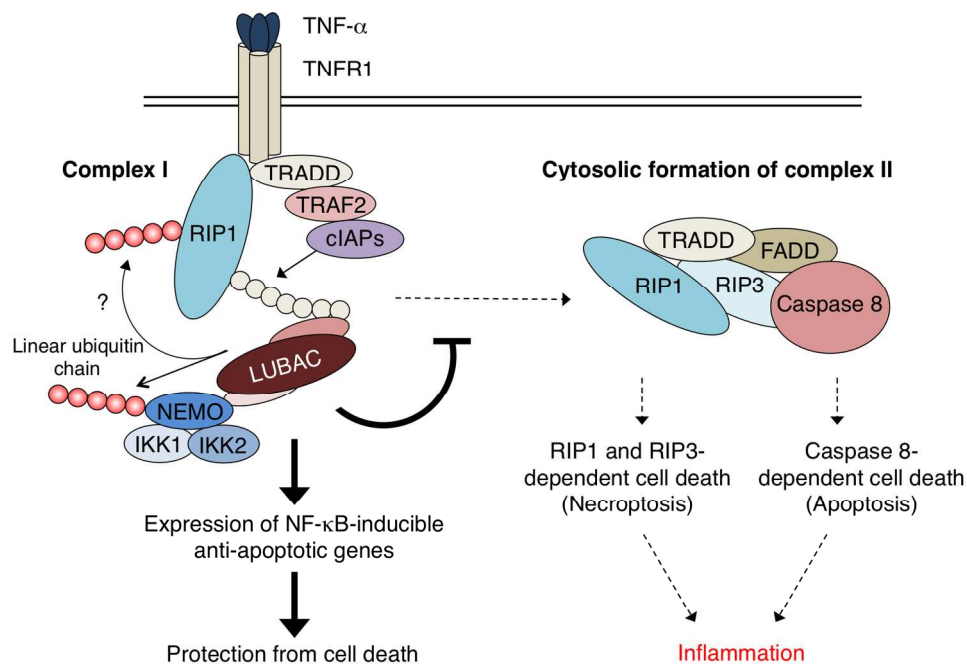


Figure 5. Inhibition of TNF- α mediated cell death by linear ubiquitinylation

Upon binding TNF- α , TNFR1 interacts with TRADD, RIP1, TRAF2, and cIAP1 and 2 to form complex I. LUBAC is also recruited to the activated TNFR1 complex via recognition of ubiquitin chains in the TNFR1 complex possibly by cIAP1 and 2. LUBAC protects cells from death by inducing the expression of various NF- κ B target genes, including Bcl-2 and cFLIP. In addition to the induction of anti-apoptotic genes, LUBAC inhibits TNF- α -mediated cell death by a different mechanism, the inhibition of complex II. The transition from complex I to complex II triggers cell death. TNFR1 induces two types of programmed cell death, apoptosis and necroptosis, and formation of complex II, which is composed of TRADD, FADD, caspase 8, RIP1, and possibly RIP3, precedes both types of cell death. After the formation of complex II, caspase 8 is activated by dimerization and digests RIP1 and RIP3, leading to apoptosis. When caspase 8 is inactivated, RIP1 and RIP3 kinases are activated and trigger necroptosis. The linear ubiquitination activity of LUBAC is involved in the suppression of complex II formation, although the linear ubiquitinylation substrates involved in the inhibition of complex II formation have not been convincingly identified to date.

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