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Linear ubiquitination signals in adaptive immune responses

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Summary

Ubiquitin can form eight different linkage types of chains using the intrinsic Met 1 residue or one of the seven intrinsic Lys residues. Each linkage-type of ubiquitin chain has a distinct three-dimensional topology, functioning as a tag to attract specific signaling molecules, which are so-called ubiquitin readers, and regulates various biological functions. Ubiquitin chains linked via Met 1 in a head-to-tail manner are called linear ubiquitin chains. Linear ubiquitination plays an important role in the regulation of cellular signaling, including the best-characterized Tumor Necrosis Factor (TNF) -induced canonical nuclear factor-kappa B (NF- κ B) pathway. Linear ubiquitin chains are specifically generated by an E3 ligase complex called the linear ubiquitin chain assembly complex (LUBAC) and hydrolyzed by a deubiquitinase (DUB) called ovarian tumor (OTU) DUB with linear linkage specificity (OTULIN). LUBAC linearly ubiquitinates critical molecules in the TNF pathway, such as NEMO and RIPK1. The linear ubiquitin chains are then recognized by the ubiquitin readers, including NEMO, which control the TNF pathway. Accumulating evidence indicates an importance of the LUBAC complex in the regulation of apoptosis, development, and inflammation in mice. In this article, I focus on the role of linear ubiquitin chains in adaptive immune responses with an emphasis on the TNF-induced signaling pathways.

Keywords

linear ubiquitin chain; LUBAC; TNF; NEMO; NF- κ B; apoptosis

Introduction

Ubiquitin is a stable protein of 8.5 kDa in size, and highly conserved from yeast to mammals (1-3). Ubiquitin posttranslationally modifies substrates by covalent attachment through a three-step enzymatic reaction. This ubiquitin conjugation, called ubiquitination typically occurs on lysine (Lys) residues of substrates through the formation of an isopeptide bond. Ubiquitin itself has seven intrinsic Lys residues, which allows the formation of seven different types of homotypic chains (4, 5). More recently, it was shown that not only Lys residues, but also the methionine (Met) 1 residue in ubiquitin can be targeted for the ubiquitin chain formation (6-9). In yeast, all seven Lys-linked ubiquitin chains were identified by multidimensional liquid chromatography coupled with tandem mass

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spectrometry with a relative abundance order of Lys 48 > Lys 63 and Lys 11 >> Lys 33, Lys 27 and Lys 6 (10). Ubiquitin chains are formed by an enzymatic reaction composed of E1 activating enzyme, E2 conjugating enzyme, and E3 ligase (1) and are cleaved by deubiquitinase (DUB) (11-13). Thus, a balance between the formation and removal of particular linkage types of ubiquitin chains on a specific substrate contributes to the regulation of cellular functions.

Depending on the intrinsic residues in ubiquitin used for the polymer formation, the three-dimensional topologies differ (14). Therefore, the ubiquitinated substrates are recruited into particular signaling complexes by recognition of the ubiquitin chains (4, 5, 14). Moreover, recent advanced methods of quantitative mass spectrometry in combination with linkage specific antibodies enabled the determination of the abundance of mixed linkage ubiquitin chain substrates in cultured-mammalian cells (15). Such diversity within polyubiquitin chains is a key element in the regulation of various biological functions including the classical function of ubiquitin, proteasome-dependent protein degradation, as well as adaptive and innate immune signaling cascades, cell death, autophagy, DNA repair, cell cycle, endocytosis, and cancer (4, 5). Met 1-linked ubiquitin chains are conjugated in a tandem-repeated or in a head-to-tail manner, therefore called linear ubiquitin chains. In 2006, Kirisako et al. demonstrated that these ubiquitin chains can be generated by an E3 ligase complex called linear ubiquitin chain assembly complex (LUBAC) *in vitro* (6). They showed that the LUBAC complex consists of two proteins, haem-oxidized IRP2 ubiquitin ligase-1 (HOIL-1L) / RanBP-type and C3HC4-type zinc finger containing 1 (Rbck1), and HOIL-1L interacting protein (HOIP) / Ring Finger Protein 31 (Rnf31). The LUBAC complex specifically generates linear ubiquitin chains with different types of E2s such as E2 - 25K, the human homolog of yeast RAD6B (HHR6B) /ubiquitin-conjugating enzyme E2B (UBE2B), Ubiquitin-conjugating enzyme E2 D1 (UBE2D1) /UbcH5s, and Ubiquitin-conjugating enzyme E2 L3 (UBE2L3) /UbcH7. This was the first discovery that monoubiquitin can form ubiquitin chains via the Met 1 residue and that the HOIP-HOIL-1L complex is the responsible ligase complex (6). However, at that time, the biological function of linear ubiquitination was still obscure besides the stability of ubiquitinated-GFP modified by LUBAC was decreased in cells. In 2009, three groups independently showed a critical role of linear ubiquitination in the regulation of the adaptive immune signaling cascade by taking different approaches (16-18). In 2011, an additional important component of the LUBAC, Sharpin/ shank-interacting protein-like 1 (SIPL1), was found to be crucial in the maintenance of a normal immune system in mice (7-9).

A critical role of different linkage types of ubiquitin chains has been shown in the Tumor necrosis factor (TNF)-induced adaptive immune signaling pathway (19, 20). Upon TNF binding to the TNF receptor 1 (TNFR1), several adapter molecules are recruited to form a signaling complex (Fig. 1). This complex called TNFR complex I, has been shown to consist of TNFR type 1-associated DEATH domain protein (TRADD), TNFR-associated factor 2 (TRAF2), receptor (TNFRSF) -interacting serine-threonine kinase 1 (RIPK1), the cellular inhibitor of apoptosis (cIAP), and ubiquitin-conjugating 13 (UBC13) as an upstream set of proteins. More recently, the LUBAC components, HOIP and HOIL-1L were found to be recruited into TNFR complex I (18, 21). Upon complex formation of the TNFR complex I, downstream kinase complexes such as Transforming growth factor beta-activated kinase 1

(TAK1) / TAK1-binding protein 2 (TAB2) complex and the I κ B kinase (IKK) complex are activated (22). The fact that cIAP, TRAF2 and LUBAC are known to be functional E3 ligases and UBC13 is an E2 enzyme (7-9, 21, 23, 24) highlights the importance of ubiquitin signaling in TNFR1 complex formation. Moreover, it has been shown that multiple molecules, including RIPK1, cIAP, and TRAF2, are polyubiquitinated by Lys 11-linked, Lys 48-linked, Lys 63-linked or linear ubiquitin chains (18, 25, 26). These polyubiquitin chains are essential scaffolds to mediate downstream signaling by forming a signaling complex and in activating the kinase complexes (27, 28). Due to differences in the three-dimensional topologies of the different linkage types of ubiquitin chains, a specific signaling complex is recruited to the ubiquitinated substrates in this signaling cascade. For example, TAB2 has a ubiquitin interaction domain Npl4 zinc finger (NZF), which specifically interacts with Lys 63-linked ubiquitin chains (29, 30). Similarly, linear ubiquitin chains are recognized by NF- κ B essential modifier (NEMO) /IKK- γ via an interaction domain, ubiquitin binding of ABIN proteins and NEMO (UBAN) (16, 31, 32). UBAN-mediated recognition of linear ubiquitin chains in the regulation of TNF signaling cascade will be discussed further.

During this process, ubiquitin - chain editing occurs by DUBs such as A20 (named after its cDNA clone number), cylindromatosis (CYLD) and ovarian tumor (OTU) DUB with linear linkage specificity (OTULIN) (33, 34). A20 is a known inhibitor of NF- κ B activation in response to TNF, Lipopolysaccharide (LPS), Interleukin-1 (IL-1), and IL-17, as well as by receptor activation of NOD1/2 receptor, T cell receptor, and RIG-I/Mda5 receptors (35, 36). A20 contains two different catalytic domains, one for DUB activity and the other for E3 ligase activity. Therefore, A20 plays a dual role in ubiquitin chain editing. It cleaves off Lys 63-linked ubiquitin chains and generates Lys 48-linked ubiquitin chains on its substrate RIPK1, which leads to its proteasomal degradation (35). In both cases, the downstream signaling pathway is inhibited. More recently, the Zinc finger (ZF) 7 domain of A20 was found to disrupt an interaction between LUBAC and NEMO via binding to linear ubiquitin chains, leading to an inhibition of LUBAC-dependent NF- κ B activation (37, 38). In the case of CYLD, it inhibits the TNF-induced downstream signaling cascade by cleaving off Lys 63-linked ubiquitin chains on RIPK1 (33). It was demonstrated by a biochemical study that CYLD has an ability to cleave not only Lys 63-linked ubiquitin chains, but also linear ubiquitin chains (39), which discovery was followed up by a cellular signaling study showing that CYLD inhibits the NF- κ B signaling by forming a complex with LUBAC (40).

Most recently a DUB specific for linear ubiquitin chains was discovered, called OTULIN / family with sequence similarity 105, member B (FAM105B), which was also shown to be implicated in the regulation of TNF-induced NF- κ B pathway (41, 42). To date, OTULIN is the only known DUB, which has specificity for cleavage of linear ubiquitin chains. A mutation in the OTULIN gene at Trp 96 in mice leads to the Gumby phenotype of embryonic lethality due to the decreased number of secondary and tertiary vessels branching off the internal carotid artery (41). The mutation OTULIN Trp 96 Arg found in Gumby mice affects the catalytic core of ovarian tumor (OTU) domain, which is the DUB domain. In this study, Rivkin et al. demonstrated that Gumby / OTULIN may regulate the Wnt signaling pathway (41). OTULIN was also found to negatively regulate the LUBAC-dependent NF- κ B activation.

Collectively, different types of ubiquitin chains participate in the TNF-induced signaling cascades to give a fine tuning. In this article, the main focus is on the LUBAC-dependent linear ubiquitin chains and how they regulate adaptive immune responses. The latest discoveries that LUBAC is implicated in the regulation of apoptosis, which was examined by cellular biological and mouse genetic approaches, will also be discussed.

Different linkage types of ubiquitin chains in the TNF-induced NF- κ B signaling pathway

Upon binding to the TNFR, TNF activates downstream signaling cascades including the NF- κ B pathway, and the MAP kinase pathways of JNK and p38 (43). Among these pathways, the NF- κ B pathway was shown to include the ubiquitination system as a key regulatory mechanism (Fig. 1). In 1994, Palombella et al. demonstrated that ATP-dependent ubiquitination of an inhibitory factor, an inhibitor of kappaB alpha (I κ B- α) leads to its degradation and it is required for the activation of NF- κ B (44). Later, β TrCP/Slimb protein containing SCF- β TrCP (SKP1-CUL1-F-box ligase containing the F-box protein β TrCP) E3 ligase complex was identified to be the responsible ligase complex for proteasomal degradation of I κ B- α (3, 45, 46).

As well as ubiquitin having a classical function, leading to proteasomal degradation of substrates, it also has other functions dependent on the different linkage types of ubiquitin chains. Such examples have been discovered in the regulation of the NF- κ B signaling pathway. In the upstream signaling events of the NF- κ B pathway, ubiquitin-dependent activation of a protein kinase complex consisting of TAK1 and two adaptor proteins, TAB1 and TAB2 plays a critical role (47). TAB2 has a ubiquitin-chain interacting domain NZF, which specifically interacts with Lys 63-linked diubiquitin chains (29, 30, 48). Based on atomic-level structural studies, TAB2-NZF was shown to directly interact with Lys 63-linked diubiquitin chains (29, 30). The binding surface of the Lys 63-linked diubiquitin chains, consists of two independent surfaces derived from proximal and distal ubiquitin moieties. These structural studies revealed that due to the conformational differences, other linkage types of diubiquitin chains cannot interact with the TAB2-NZF domain. In cells, Lys 63-linked ubiquitin-chain formation induced by E2 conjugating enzymes, UBC13 and Methyl Methanesulfonate Sensitive 2 (MMS2), was shown to be critical for NF- κ B activation (49-51). Together, these studies indicate that alternative polyubiquitin chains function as a signaling module in the regulation of the NF- κ B pathway.

Lately, two additional alternative linkage types of polyubiquitin chains, such as Lys 11-linked ubiquitin chains and Met 1-linked/linear ubiquitin chains were shown to be critical for the NF- κ B activation. First, it was demonstrated that the cellular inhibitor of apoptosis (c-IAP) 1 and UbcH5 promote Lys 11-linked ubiquitin chains on RIPK1 upon TNF stimulation (26). Lys 11-linked ubiquitin chains generated by cIAP and UbcH5 efficiently interact with NEMO (26), suggesting that Lys 11-linked polyubiquitin chains play an independent role from protein degradation and regulate the NF- κ B signaling pathway. Second, Met 1-linked/linear ubiquitin chains were shown to be important for TNF-induced NF- κ B activation (16-18). Linear ubiquitin chains are generated by the LUBAC complex

upon TNF stimulation (7-9, 17, 18). Linear ubiquitin chains are linked via a peptide bond and harbor a particular three dimensional topology (16), which provides the binding surface to NEMO with approximately 100 times higher affinity in comparison to Lys 63-linked diubiquitin chains (16, 32). A role of recognition of the linear diubiquitin chains by NEMO is discussed in greater detail in the following section.

These findings collectively indicate that different linkage types of ubiquitin chains recruit specific signaling complexes via interaction with ubiquitin-chain readers, which fine tune in the regulation of TNF-induced NF- κ B signaling activation.

NEMO as a linear ubiquitin chain reader regulating TNF-induced signaling pathway

Ubiquitin chains on substrates can recruit different signaling complexes via interaction with ubiquitin binding domains (UBDs) (52-54). UBDs can recognize either monoubiquitin or polyubiquitin chains in a non-covalent manner and function as ubiquitin readers. Initial work on the ubiquitin interaction domain of the Pickart lab showed that the ubiquitin associated domain (UBA) of radiation sensitivity abnormal 23 (Rad23) interacts with Lys 48-linked ubiquitin chains with 3.6-fold higher affinity in comparison to Lys 63-linked ubiquitin chains (55). Like other UBAs, the Rad23-UBA domain interacts with monoubiquitin however, with a significant lower affinity than Lys 48-linked ubiquitin chains. This study showed that UBDs can interact with different linkage types of ubiquitin chains in different affinities.

Later, more UBDs were discovered, including within some important molecules in the NF- κ B signaling pathway such as TAB2 (as described in the previous section) (29, 30) and NEMO (16, 32, 56-58), both of which are shown to interact with Lys 63-linked ubiquitin chains. The interaction of those ubiquitin readers with Lys 63-linked ubiquitin chains positively regulates NF- κ B signaling pathway. On the other hand, another type of ubiquitin reader, A20-binding inhibitor of NF- κ B (ABIN) –1 negatively regulates the NF- κ B signaling (31, 59, 60). In the case of ABIN-1, it has a UBD called UBD in ABIN proteins and NEMO (UBAN), which specifically interacts with linear diubiquitin chains (31, 61). Interestingly, bioinformatical analysis revealed that the UBAN domain exists in multiple proteins, such as ABIN-1, ABIN-2, ABIN-3, NEMO and Optineurin (Fig. 2) (31), which are found to play a role in immune signaling (60, 62-64). In our initial study on the UBAN domain in ABIN-1, we demonstrated that ABIN-1 interacts with linear diubiquitin chains, but not with monoubiquitin *in vitro*. Furthermore, the ABIN-1-UBAN domain is critical for its inhibitory function of the NF- κ B signaling cascade demonstrated by cellular assays using the ABIN-1-UBAN mutants, which no longer interact with linear diubiquitin chains (31). However, at that time, we did not understand the biological relevance of the ABIN-1-UBAN domain directly interacting with linear diubiquitin chains *in vitro*, because there was no signaling function of linear ubiquitin chains known yet. Among these proteins, NEMO has the UBAN domain in conjunction with the Zinc Finger (ZF) domain in the C-terminus (Fig. 2). Based on atomic level structural analyses of the UBAN containing region of NEMO, the CoZi forms a homodimer and was shown to directly interact with linear diubiquitin chains

(16). The interaction between the NEMO-CoZi domain and linear diubiquitin chain is 1.4 μM (determined by isothermal titration calorimetry (ITC)) (32) to 1.6 μM (based on the surface plasmon resonance method) (16), while the affinity for Lys 63-linked diubiquitin chain is 131 μM (32), approximately 100 times lower. By performing an *in vitro* interaction assay, it was shown that the recombinant NEMO-UBAN interacts with both linear and Lys 63-linked chains when they are tetramers. However, it still has a preference for interacting with linear tetraubiquitin chains over Lys 63-linked tetraubiquitin chains when it is exposed to the same amount of chains *in vitro* (16, 65). By analyzing the ubiquitin interacting surfaces in the NEMO-UBAN, we found that there are critical amino acid residues for both Lys 63-linked ubiquitin chains and linear ubiquitin chains, or specific for linear ubiquitin chains. Interestingly, mutations at the unique residues of mouse NEMO Arg 309, Arg 312 and Glu 313, which are critical for linear ubiquitin interaction, but not for Lys 63-linked ubiquitin chains, were enough to suppress NF- κB activation induced by various stimuli, including TNF, Interleukin (IL) -1, LPS, CpG, and Ionomycine with phorbol 12-myristate 13-acetate (PMA) (16). These data collectively suggest that the interaction between the NEMO-UBAN and linear diubiquitin chains plays an important role in the regulation of NF- κB signaling pathway. In addition, upon linear ubiquitin chain interaction with the NEMO UBAN/CoZi region, conformational changes occur within the region (65). These conformational changes may affect the interaction of the NEMO-N terminal region with the IKK kinases and regulate the activity of the IKK complex.

An interesting point about NEMO is that it can be a reader for linear ubiquitin chains or Lys 63-linked chains depending on the cellular context (66-68). To further investigate the dual role of NEMO in terms of recognition of two different linkage types of ubiquitin chains, we generated different NEMO chimera mutants, which interact with 1) both linear and Lys 63-linked chains, 2) exclusively linear chains, or 3) exclusively Lys 63-linked chains (65). By using these NEMO-mutants, we could verify the importance of NEMO as a linear ubiquitin chain reader in the TNF-induced NF- κB signaling cascade in cells. These data collectively indicate that in the TNF-induced NF- κB signaling cascade, NEMO has a critical role as a linear ubiquitin chain reader. However, it is possible that in a different cellular context or in different signaling cascades where Lys 63-linked ubiquitin chains are more abundant, NEMO functions as a Lys 63-linked ubiquitin chain reader to mediate the downstream signaling cascade (27, 67). During our study of the NEMO-UBAN domain as a critical linear ubiquitin chain reader to regulate NF- κB signaling cascade, two important research articles were published at around the same time (17, 18). The authors demonstrated that the LUBAC components, HOIP and HOIL-1L are recruited to the TNFR complex upon TNF stimulation and generate linear ubiquitin chains regulating NF- κB signaling pathway (17, 18). These findings together contribute to a strong indication of the cellular regulatory functions of linear ubiquitin chains in the TNF-induced NF- κB signaling cascade.

Moreover, mutations in the coding region of *IKBKG* (NEMO) were shown to be associated with X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) in male patients (69-71). Among the hypomorphic mutations found in *IKBKG*, mutations Asp 311 Asn, Glu 315 Ala, and Arg 319 Gln (corresponding to Asp 304 Asn, Glu 308 Ala, and Arg 312 Gln in mice) are located in the UBAN region and are the critical residues for the recognition of linear ubiquitin chains. This information suggests a critical role of NEMO

in the clinical features of EDA-ID, which may, in part, depend on the linear ubiquitin chain interaction.

Linear ubiquitin chain formation by the LUBAC complex

As discussed in the previous section, ubiquitin chains are generated by a three-step enzymatic reaction. In the case of Lys-linked ubiquitin chains, the linkage types are mostly determined by the E2 conjugating enzymes (72), whereas in the case of linear ubiquitin chains, the E3 ligase complex LUBAC plays a critical role (6).

The LUBAC complex was initially found to consist of a catalytic protein HOIP and an adaptor molecule HOIL-1L (Fig. 3) (6). More recently, Sharpin was identified to be the third component of the LUBAC complex (Fig. 3). They form a complex of approximately 600 kDa in cells determined by gel filtrations, suggesting that they form oligomers (6, 7). The protein stability of each component seems to depend on the proper LUBAC complex formation of the three components. HOIP and HOIL-1L proteins are destabilized by the lack of Sharpin in mouse embryonic fibroblasts (MEFs) (7), whereas HOIP and Sharpin proteins are destabilized in HOIL-1L deficient MEFs (7) or in immortalized fibroblasts derived from human patients with autosomal recessive *HOIL-1L* deficiency (73). Interestingly, the protein level of HOIL-1L was affected only slightly while Sharpin was drastically decreased in HOIP deficient MEFs or in HOIP knockout mouse embryos (74). Moreover, the modified form of HOIL-1L, which is detectable by western blotting, diminishes when HOIP is depleted (74). The modification of HOIL-1L proteins, which depends on HOIP but not HOIP-E3 ligase activity could be an interesting aspect to delineate how the LUBAC activity may be regulated.

For linear ubiquitination by the LUBAC, the main catalytic center is localized in the RING-in-between-RING (RBR) domain in HOIP, though HOIL-1L also has an RBR domain (Fig. 3) (6). When the HOIP-RBR catalytic Cys residues are mutated, the LUBAC-induced linear ubiquitin chain formation *in vitro* is lost. On the other hand, mutations in the HOIL-1L RBR domain have a minor effect (6). A deletion mutant of HOIP lacking the N-terminus and consisting of the RBR domain and the C-terminal region (Fig. 3) was shown to be capable of generating linear ubiquitin chains without HOIL-1L or Sharpin *in vitro* (75). A longer version of the HOIP deletion mutant additionally containing the NZF2 and UBA domains, both of which are the interaction domains to HOIL-1L or Sharpin (Fig. 3), decreases the catalytic activity. With the longer HOIP mutant containing the NZF2 and the UBA domains, the addition of the other two LUBAC components, Sharpin and HOIL-1L significantly increases the formation of linear ubiquitination *in vitro* (75). Collectively, the information based on the biochemical assays strongly supports a concept that the N-terminal region of full length HOIP conformationally inhibits its catalytic activity. Further biochemical analysis revealed that HOIP has a unique and important C-terminal region adjacent to the RBR domain called the linear ubiquitin chain determining domain (LDD) (Fig. 3) (76). The LDD domain interacts with a single ubiquitin moiety at the $K_D=97\pm7\ \mu\text{M}$ and the interaction is abolished by a point mutation in the LDD at Cys 930. The HOIP Cys 930 Ala mutant no longer generates linear ubiquitin chains *in vitro* nor activates NF- κ B in cells strongly

suggesting that the HOIP-LDD domain coordinates the last step of linear ubiquitin chain formation (76).

Similar to an RBR E3 ligase called human homolog of Ariadne (HHARI), HOIP was shown to be homologous to the E6-AP carboxyl terminus (HECT) -RING hybrid ligase (75, 77). The HECT-RING hybrid E3 ligases bind E2 via the RING1 domain, and then transfer ubiquitin through an obligate thioester-linked ubiquitin, requiring a conserved Cys residue in the RING2 domain. This concept of linear ubiquitin chain formation by HECT-RING hybrid function and holding of the acceptor ubiquitin at the HOIP-LDD domain was further supported by the structural and biochemical analyses of HOIP (77). Moreover, it was shown that the folding of the acceptor ubiquitin moiety at the HOIP-LDD domain is key to determining the linear linkage type of chains created by LUBAC.

Regarding the non-catalytic components of the LUBAC complex, HOIL-1L and Sharpin, they have a redundant role as adaptor molecules of HOIP for the generation of linear ubiquitin chains *in vitro* (6, 8, 17, 75). Both the Sharpin-UBL domain and the HOIL-1L-UBL domain are used as the interaction domains for HOIP (Fig. 3) and contribute to the *in vitro* linear ubiquitin chain formation (75). On the other hand, they overall have a distinct domain organization, which may distinguish the functions of these two molecules (Fig. 3). Sharpin has an N-terminal region called Pleckstrin Homology (PH) Superfold, which is used to form a homodimer *in vitro* determined by the Multi Angle Light Scattering (MALS), Analytical Ultracentrifugation (AUC), and Isothermal titration calorimetry (ITC) experiments suggesting the importance of oligomerization of Sharpin (78). The NZF domains in Sharpin and HOIL-1L are both shown to be UBDs, however, they recognize ubiquitin in different ways (8, 79). The Sharpin NZF domain interacts with monoubiquitin and linear diubiquitin chains while the HOIL-1L NZF domain together with C-terminal tail specifically recognizes linear diubiquitin chains (Fig. 2 and 3) (8, 79). HOIL-1L also has an RBR domain, which is not the major catalytic domain of the LUBAC for linear ubiquitination as mentioned previously (6). However, the recombinant HOIL-1L protein, which contains the UBL, NZF and RBR domains has a residual activity of generating ubiquitin chains *in vitro* (75). In comparison to the HOIP-RBR C-terminus, which efficiently generates linear ubiquitin chains *in vitro*, the RBR domain of HOIL-1L without the UBL and NZF does not have any catalytic activities (79). These differences of Sharpin and HOIL-1L in domain structure may participate in regulating different biological functions. Thereby, it would be critical to elucidate the protein interactome of Sharpin and HOIL-1L, which may reveal the distinct functional roles of these 2 adaptor molecules in the LUBAC.

Functional roles of LUBAC *in vivo*

At the cellular level, LUBAC plays a regulatory role in various immune signaling cascades. Depletion of either Sharpin or HOIL-1L in cells suppresses NF- κ B activation induced by TNF, CD40 ligand (CD40L), IL-1 β , and LPS (7-9). Among these signaling cascades, the TNF-induced pathway is the best characterized one. Upon TNF stimulation, all three LUBAC components were found to be recruited to the TNFR complex I (9). Interestingly, pretreatment of the cells with an IAP inhibitor, called Smac-mimetic compound SM-164, abolishes cIAP protein and at the same time the recruitment of the LUBAC to TNFR

complex I, suggesting that the recruitment of the LUBAC components depends on the E3 ligase activity of cIAP1 (9). These observations raise a possibility that the LUBAC complex may be recruited not only to the TNFR, but also to other receptor complexes. The LUBAC complex may interact with ubiquitin chains generated by a particular E3 ligase in the specific receptor complex, and regulate various cellular signaling pathways. Indeed, LUBAC-induced linear ubiquitination of RIP2 was shown to be important in the nucleotide-binding oligomerization domain containing (NOD) pattern recognition signaling pathway (80). Further cellular functional studies are awaited.

By using various genetically modified mouse lines of the LUBAC components (Table 1), biological functions of the LUBAC complex have been analyzed. Tokunaga et al. demonstrated that depletion of HOIL-1L plays rather a minor role *in vivo*. They generated a mouse line targeting the exons coding the RBR domain of the *Hoil* gene (7). These mice have no apparent phenotype in gross appearance and they are fertile. However, the liver tissue of *Hoil*^{-/-} mice showed a significantly higher number of the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells examined by histological analysis, as well as the caspase-3 activity in the liver protein extract suggesting a role of HOIL-1L in the regulation of apoptosis.

On the other hand, Sharpin deficient mice called chronic proliferative dermatitis mouse (Cpdm) with a spontaneous point mutation in the *Sharpin* gene, leading to a premature stop codon, suffer from a systemic inflammatory phenotype (81, 82). In *Sharpin*^{cpdm/cpdm} mice, multiple organs including skin, esophageal, gut, intestine, lung and liver were found to be highly inflamed. The mice develop dermatitis with associated skin lesions starting at two to three weeks of age. Sharpin deficient primary cells derived from *Sharpin*^{cpdm/cpdm} mice are defective in NF- κ B activation induced by various stimuli such as TNF, IL-1 β , LPS and CD40L (7-9). In the skin lesions of *Sharpin*^{cpdm/cpdm} mice, significantly higher signals of the active form of caspase-9 and caspase-3, and TUNEL (8, 9, 83) were detected. In the same line, primary keratinocytes derived from *Sharpin*^{cpdm/cpdm} mice are more sensitive to TNF-induced apoptosis in comparison to the control keratinocytes (9). We also observed that *Sharpin*^{cpdm/cpdm} MEFs behave in a similar way, showing higher sensitivity to TNF-induced apoptosis in comparison to the control MEFs. Initially, we speculated that Sharpin deficiency leads to apoptosis due to suppressed NF- κ B activity, which is known to induce anti-apoptosis genes (84). Interestingly, the apoptosis markers including Propidium Iodide (PI) uptake/Annexin V positivity, cleaved caspase-3 and Poly (ADP-ribose) polymerase (PARP), caspase-8 activity were all significantly higher in Sharpin deficient cells even when we treated the cells with TNF and cycloheximide (CHX) (8, 83). These observations suggest that Sharpin plays a critical role in the regulation of apoptosis at least partially independently from the misregulation of NF- κ B activation. Indeed, exogenous expression of the dominant negative Fas-Associated protein with Death Domain (FADD) or a caspase-8 inhibitory protein called CrmA in *Sharpin*^{cpdm/cpdm} mouse embryonic fibroblasts (MEFs), as well as a pan-caspase inhibitor, zVAD-fmk significantly suppressed the TNF-induced and TNF+CHX-induced apoptosis (Fig. 4) (8, 9). These data strongly suggest that Sharpin plays a role in the apoptosis pathway via FADD and caspase-8 (Fig. 4).

Genetic studies have further supported the anti-apoptosis functions of Sharpin in the TNF signaling pathway (Table 1). For example, deficiencies of TNF, TNFR1, and TRADD (epidermal specific) rescued the skin inflammatory phenotype in *Sharpin^{cpdm/cpdm}* mice (9, 83, 85). IL1-Receptor (IL-1R) deficiency also rescues the skin phenotype of *Sharpin^{cpdm/cpdm}* with lesser extent, but not TNFR2 deficiency (85) suggesting that the skin phenotype of *Sharpin^{cpdm/cpdm}* derives mainly from TNFR1-induced signaling cascade but also regulated in combination with different signaling cascades. In addition, a knockin mouse line of RIPK1 kinase dead mutant (Lys 45 Ala), which itself has no significant developmental phenotype, rescues the inflammatory phenotype of *Sharpin^{cpdm/cpdm}* mice in skin, joint, lung and liver strongly suggesting that the kinase activity of RIPK1 is implicated (86).

Regarding the apoptosis regulator, FADD, the epidermal specific depletion rescued skin inflammation and keratinocyte apoptosis in *Sharpin^{cpdm/cpdm}* mice strongly suggesting that apoptosis and the inflammation phenotype are correlated (83). Interestingly, in the case of caspase-8, which is another apoptosis regulator, heterozygosity rescues the dermatitis phenotype of *Sharpin^{cpdm/cpdm}* mice, but not homozygous knockout of caspase-8 (85). In addition, depletion of RIPK3 has a minor effect on the *Sharpin^{cpdm/cpdm}* phenotype while caspase-8 heterozygosity in addition to RIPK3 depletion markedly delays the *Sharpin^{cpdm/cpdm}* phenotype, and the depletion of both caspase-8 and RIPK3 in the background of *Sharpin^{cpdm/cpdm}* mice induces perinatal lethality (85). These data support a concept that Sharpin regulates cell death and some of the Sharpin deficient mouse phenotype is caused by cell death. Based on the findings by Rickard et al. (85), a balance between the Sharpin, caspase-8 and RIPK3 protein expression levels is critical in the regulation of dermatitis, liver inflammation, and Peyer's patch formation indicating the complex regulatory mechanisms *in vivo*.

In the case of HOIP, *Hoip^{-/-}* mice are embryonic lethal at the stage between embryonic day (E) 10.5 and E11.5 due to the defect in the vascularization of the yolk sac (74). The identical phenotype was observed in the endothelium-specific HOIP knockout, *HOIP^{fl/fl}/Tie2-Cre⁺* mice, indicating the HOIP deficiency in endothelial cells is sufficient to cause embryonic lethality. When HOIP deficient mice are crossed with TNF knockout mice, the embryos survive until E15.5 and suppressed apoptosis was detected at E10.5 in the yolk sac. Moreover, TNFR1 deficiency in the background of HOIP knockout rescued both apoptosis and vascularization in the yolk sac up to E17.5, suggesting that both TNF and lymphotoxin- α (LT- α) play a role in the vascular formation. In contrast, HOIP mutant mice (*Hoip^{linear/linear}*) in which the catalytic domain is deleted are embryonic lethal (87). Similarly, the knockin mouse line of HOIP catalytic dead mutant (Cys 879 Ser) was shown to be embryonic lethal at E10.5, which is identical to the lethality phenotype of HOIP deficient mice (66). Though the detailed analysis of the regulatory mechanism in these mice is awaited, these data collectively suggest that the catalytic activity of HOIP plays a major role during development in mice. B cell-specific deletion of the exons coding the catalytic domains of *Hoip* using Mb1-Cre mice leads to severe impairment of the development of B1 cells in the peritoneal cavity (87). Further, CD40-mediated activation of the canonical NF- κ B and the ERK signaling pathways was severely suppressed in B cells derived from these mice. Collectively, the catalytic activity of HOIP in B cells plays a critical role.

While the gene knockout of HOIL-1L in mice did not show the drastic immune phenotype, *Hoil* gene mutations were found in patients suffering from autoimmune diseases with polyglucosan body myopathy, early-onset, with or without immunodeficiency (73). With the loss of HOIL-1L protein due to the *Hoil* gene mutations in the patient samples, HOIP and Sharpin proteins became unstable, suggesting that the LUBAC complex plays a critical role in the pathogenesis of these patients.

These data indicate an important role of LUBAC in the regulation of various biological functions and strongly suggest that each of the LUBAC components have distinct regulatory roles in biology.

Molecular insights into the LUBAC as an anti-apoptosis regulator

In line with the phenotype observed in keratinocytes of *Sharpin*^{cpdm/cpdm} mice, Sharpin deficiency in cells leads to high sensitivity towards apoptosis. As mentioned in the previous section, loss of function studies using Sharpin deficient MEFs or keratinocytes showed significant increases of TNF-induced apoptosis. Apoptosis markers including caspase-8 activity, caspase-3 activity, PARP cleavage, TUNEL, and Annexin V significantly increased in these cells (83, 86). These data indicate a critical role of Sharpin in anti-apoptosis signaling. We further analyzed an involvement of other LUBAC components in the regulation of apoptosis. We generated a stable-HOIP knockdown line using a human keratinocyte cell line called HaCaT (83). By treatment of shHOIP-HaCaT cells with TNF alone or TNF+CHX significantly increased apoptosis markers suggesting that Sharpin and HOIP may function as a complex. However, as recent studies showed, apoptosis observed in *Sharpin*^{cpdm/cpdm} and HOIP knockout mice are in different tissues leading to distinct phenotypes (74). Moreover, apoptosis was observed only in the liver tissue derived from HOIL-1L knockout mice, but not in other organs (17). Based on these observations, it is speculated that the LUBAC complex regulates apoptosis signaling cascades, but this regulation could be in a tissue- or a condition-specific manner.

Based on the mouse genetic analysis and cellular biological analysis, we started to understand more about an involvement of the LUBAC complex in the regulation of cell death signaling cascades. However, there are still important open questions. Firstly, does linear ubiquitination induced by LUBAC play a role in anti-apoptosis function (Fig. 4)? For this question, because loss of catalytic activity of HOIP in mice led to embryonic lethality at the same stage of E10.5 as HOIP depletion mice, which die due to abnormal apoptosis (Table 1) (66), it was speculated that linear ubiquitination plays a role in anti-apoptosis. Peltzer et al. demonstrated that HOIP deficient cells reconstituted with a catalytic dead HOIP-Cys 885 Ser mutant are significantly more sensitive to TNF-induced apoptosis determined by PI positivity (74) indicating a critical role of HOIP catalytic activity.

This leads to the following questions; what are the substrates for the linear ubiquitination by the LUBAC complex and which readers are recruited to the linear ubiquitin chains. It is essential to answer these questions to further elucidate the molecular mechanisms of the LUBAC-dependent apoptosis pathway. Interestingly, ABIN-1, which has the UBA domain and specifically interacts with linear ubiquitin chains, was shown to be a negative regulator

of apoptosis (88). ABIN-1 inhibits the TNFR complex II formation, which is dependent on the ubiquitin binding ability of ABIN-1, suggesting a possible role of ABIN-1 as a reader of linear ubiquitin chains in the LUBAC-dependent anti-apoptosis pathway.

The other critical point is regarding the molecular mystery of the LUBAC in the apoptosis regulation. Even though both Sharpin and HOIL-1L function as adaptor molecules for HOIP catalytic activity *in vitro* and they both regulate TNF-induced NF- κ B signaling cascade in a redundant manner, Sharpin and HOIL-1L seem to have a different impact on apoptosis. This leads to the question of what are the molecular mechanisms which distinguish Sharpin and HOIL-1L in the regulation of apoptosis. The speculations would be; 1) they have distinct binding partners through the distinct protein interacting domains (Fig. 3), giving selectivity towards substrate determination in apoptosis signaling, or 2) expression patterns and levels of HOIL-1L and Sharpin in various tissues are different leading to distinct apoptosis phenotypes in mice. To elucidate these points, it is necessary to further investigate the cellular and molecular functions of HOIL-1L and Sharpin. It would be also interesting to analyze the HOIL-1L depletion in mice by targeting exons containing the N-terminus of *Hoil* gene. Another aspect related to apoptosis regulation is that Sharpin plays a role in the apoptosis signaling cascade through TNFR complex II, which includes FADD, caspase-8, RIPK1, and TRADD (8). Based on this, it is of great interest to test if the LUBAC complex plays a role in the apoptosis signaling induced by other cell-death ligands such as FasL and TNF-related apoptosis-inducing ligand (TRAIL), which induce apoptosis through a signaling complex called the death-inducing signaling complex (DISC) containing FADD and caspase-8 (89-91).

Lastly, how the activity of LUBAC is regulated in the TNF signaling cascade remains totally elusive. Especially because LUBAC may function both in NF- κ B activation and in the anti-apoptosis cascade downstream of the TNFR (Fig. 4), linear ubiquitination of the substrate by LUBAC must be tightly regulated to give a fine tuning in these signaling cascades. The recombinant LUBAC complex purified using the *E. coli* expression system is catalytically active (9), and there is no obvious change in protein expression level after TNF stimulation of cells (7-9). Based on these observations, it is attempting to speculate that 1) cellular localization of LUBAC is critical, 2) post translational modification of the LUBAC components regulates the activity, or 3) recruitment of the specific signaling complex plays a role. Since all LUBAC components are ubiquitinated in cells when deubiquitinase is inhibited (42, 92), it would be interesting to examine if ubiquitination of HOIP, HOIL-1L and Sharpin plays a role in the regulation of signaling cascades and participates in the regulation of catalytic activity.

Recently, several groups have reported that LUBAC components are involved in cancer biology (93-106). In all cases, high expression of LUBAC components promotes cancer progression and the depletion of the LUBAC components suppresses cancer development. It seems that there is also a case that the LUBAC regulates cancer in a catalytic independent manner (94), suggesting a new layer of versatility in the regulation of cellular signaling. Together with the role of the LUBAC complex in the apoptosis signaling cascade, there might be a strategy to suppress cancer development by targeting the LUBAC components.

Negative regulation of linear ubiquitin chains by DUBs

Like any other linkage types of ubiquitin chains, linear ubiquitin chains are cleaved by different types of DUBs, such as OTULIN (41, 42) and CYLD (40). In comparison to the Lys-linked ubiquitin chains, which are conjugated via an isopeptide bond, linear ubiquitin chains are linked by a peptide bond. Keusekotten et al. demonstrated that among 14 known OTU family members, OTULIN is the only DUB, which specifically hydrolyzes peptide bond and not an isopeptide bond, giving OTULIN a specificity towards linear ubiquitin chains (42). OTULIN also has a high selectivity in interacting with linear ubiquitin chains (Fig. 2) and this interaction is 100 times stronger than the interaction with Lys 63-linked ubiquitin chains, which are the most structurally similar chains to linear chains. Although studies on the biological functions of linear ubiquitin chains in different signaling cascades are emerging, its role in the regulation of the TNF-induced NF- κ B pathway is most well characterized. Therefore, it was speculated that OTULIN controls LUBAC-dependent NF- κ B signaling cascade. Indeed, at the cellular level, OTULIN inhibits LUBAC-induced NF- κ B activity dependently on the catalytic activity of OTULIN (42). Moreover, OTULIN was shown to restrict NOD2-induced innate immune signaling by cleaving linear ubiquitin chains on RIPK2 while depletion of OTULIN in cells increased linear ubiquitination of RIPK2 (92). Based on these observations, an OTULIN-deficient cell line would be a great tool to identify new linear ubiquitination targets.

Interestingly, OTULIN was found to interact directly with an N-terminus domain of HOIP called Peptide: N-glycanase/UBA or UBX-containing proteins (PUB) (Fig. 3) (40, 107, 108). Interaction between HOIP and OTULIN may balance the amount of linear ubiquitin chains on the substrate, hence regulating the cellular functions.

The biological relevance of OTULIN *in vivo* was demonstrated by Rivkin et al. in the recessive, embryonically lethal *gumby* mutant mouse lines (41). They identified two mouse lines with a distinct mutation in the *Fam105b/Otulin* gene *Gum^{Trp96Arg}* or *Gum^{Asp336Glu}* both of which cause embryonic lethality. Gumby mice die between E12.5-E14 due to angiogenic deficits indicating a critical role in mouse embryonic development (Table 1). Furthermore, based on the yeast two hybrid screening data, Rivkin et al. identified disheveled (DVL2) as a direct interacting partner of gumby and demonstrated that gumby acts as a modulator of canonical Wnt signaling during angiogenesis (41). They speculated that because both DVL2 and HOIP binds to gumby via the N-terminus, and the interaction with HOIP requires the catalytic activity of gumby, the interactions may regulate overall complex activity, substrate selection or specific signaling pathway (41).

While the overexpression of OTULIN Cys 129 Ser mutant together with LUBAC in cells significantly increases the amount of linear ubiquitin chains in comparison to when LUBAC alone is overexpressed, LUBAC-induced NF- κ B activation is not enhanced by co-expression of the OTULIN Cys 129 Ser mutant (42, 92, 108). These observations suggest that linear ubiquitin chains in cells need to be just at the right amount to activate NF- κ B, because excess linear ubiquitin chains does not simply activate the signaling pathway.

Another DUB, which was found to cleave linear ubiquitin chains is CYLD (39). CYLD was originally identified as a gene mutated in familial cylindromatosis and inhibits the NF- κ B signaling pathway by hydrolyzing Lys 63-linked ubiquitin chains on RIPK1 or TRAF2 (109). CYLD was later shown to hydrolyze also linear ubiquitin chains *in vitro* (39) and interacts with HOIP via the PUB domain (40). Overexpression of CYLD inhibits NF- κ B activation induced by LUBAC similarly to OTULIN suggesting its redundant roles in the regulation of the signaling pathway. An involvement of OTULIN and CYLD in the regulation of linear ubiquitination-dependent apoptosis signaling pathway remains elusive (Fig. 4).

Together, a balance between linear ubiquitin chain formation by LUBAC and cleavage by the DUBs including OTULIN and CYLD is critical for maintaining homeostasis of cellular signaling. Understanding further how the activity of the LUBAC complex and DUBs are controlled under certain circumstances would be critical in elucidating their biological and pathological functions.

Conclusions and outstanding questions

Linear ubiquitin chains have been shown so far to be critical in the regulation of the adaptive and innate immune signaling pathways, the apoptosis signaling pathway and the Wnt signaling pathway. Similarly to other linkage types of ubiquitin chains, linear ubiquitin chains function as a protein modifier to recruit signaling complexes which adjust and control cellular functions. There are several important players involved, including E3 ligase (s), DUBs, substrates and readers.

Enzymes for linear ubiquitination

To date, LUBAC is the only E3 ligase complex known to generate specifically linear ubiquitin chains, while OTULIN is the only known DUB, which has a specificity to hydrolyze linear ubiquitin chains. It would be of great interest to identify other enzymes, which can generate linear ubiquitin chains and DUBs which can specifically cleave linear ubiquitin chains.

Substrates for linear ubiquitination

Another question is whether there are other targets of linear ubiquitination by LUBAC. NEMO, RIPK1 and RIPK2 are the only substrates found to be linearly ubiquitinated so far. To understand further the biological functions of linear ubiquitin chains, it is crucial to identify the substrates of LUBAC. Furthermore, these ubiquitinated substrates can be then recognized by different readers, which may be the key molecules regulating biological functions.

Specific biological functions of each LUBAC component

Based on the different phenotypes of mouse knockouts of each LUBAC component, it is speculated that catalytic dependent and independent roles of the LUBAC complex exist. Moreover, there is a possibility that the LUBAC complexes consisting of only two components exist, and this may also be in a tissue specific manner. It would be interesting to delineate if the LUBAC complex can regulate distinct biological functions depending on the

composition of the complex, whether with only two components (HOIP and Sharpin, or HOIP and HOIL-1L) or with all three known components (HOIP, Sharpin and HOIL-1L).

Molecular regulatory mechanisms of the LUBAC

At the molecular level, the recombinant LUBAC complex has been shown to be active in *in vitro* ubiquitination assays. However, its activity must be regulated at the cellular level. In this regulation, one can speculate that 1) post translational modifications of HOIP play a role, or 2) interaction and dissociation of HOIP with DUBs or other regulators control the linear ubiquitination of the substrate. Further analysis in this aspect is required.

Control of chain length in linear ubiquitin chains

Another open question is related to the chain length of linear ubiquitin chains, which are conjugated on the substrate. For the readers such as NEMO, it has been shown that linear diubiquitin chain gives a sufficient binding surface to be recognized, suggesting that the dimer is the functional unit (16). However, once the chain length becomes longer, it seems that the readers recognize the chains in a more flexible manner. Examination of the mechanism how the length of the linear ubiquitin chains is regulated, and whether if the control of the length is important in a particular condition, would be the key to delineating the complex mechanisms of cell signaling regulated by the ubiquitin system overall.

Concluding remark

As recent reports have indicated that the LUBAC components are involved in the regulation of inflammation and cancer development, it is critical to elucidate the molecular regulatory mechanism of how LUBAC activity is regulated which may contribute to the development of possible therapies.

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References

1. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998; 67:425–479. [PubMed: 9759494]
2. Ozkaynak E, Finley D, Varshavsky A. The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature.* 1984; 312:663–666. [PubMed: 6095120]
3. Hershko A. Ubiquitin: roles in protein modification and breakdown. *Cell.* 1983; 34:11–12. [PubMed: 6309404]
4. Kulathu Y, Komander D. Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat Rev Mol Cell Biol.* 2012; 13:508–523. [PubMed: 22820888]
5. Ikeda F, Dikic I. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep.* 2008; 9:536–542. [PubMed: 18516089]
6. Kirisako T, et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* 2006; 25:4877–4887. [PubMed: 17006537]

7. Tokunaga F, et al. SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex. *Nature*. 2011; 471:633–636. [PubMed: 21455180]
8. Ikeda F, et al. SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. *Nature*. 2011; 471:637–641. [PubMed: 21455181]
9. Gerlach B, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature*. 2011; 471:591–596. [PubMed: 21455173]
10. Peng J, et al. A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol*. 2003; 21:921–926. [PubMed: 12872131]
11. Reyes-Turcu FE, Ventii KH, Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu Rev Biochem*. 2009; 78:363–397. [PubMed: 19489724]
12. Komander D, Clague MJ, Urbe S. Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol*. 2009; 10:550–563. [PubMed: 19626045]
13. Nijman SM, et al. A genomic and functional inventory of deubiquitinating enzymes. *Cell*. 2005; 123:773–786. [PubMed: 16325574]
14. Komander D, Rape M. The ubiquitin code. *Annu Rev Biochem*. 2012; 81:203–229. [PubMed: 22524316]
15. Phu L, et al. Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals. *Mol Cell Proteomics*. 2011; 10 M110 003756.
16. Rahighi S, et al. Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell*. 2009; 136:1098–1109. [PubMed: 19303852]
17. Tokunaga F, et al. Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat Cell Biol*. 2009; 11:123–132. [PubMed: 19136968]
18. Haas TL, et al. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. *Mol Cell*. 2009; 36:831–844. [PubMed: 20005846]
19. Verhelst K, Carpentier I, Beyaert R. Regulation of TNF-induced NF-kappaB activation by different cytoplasmic ubiquitination events. *Cytokine Growth Factor Rev*. 2011; 22:277–286. [PubMed: 22119011]
20. Bianchi K, Meier P. A tangled web of ubiquitin chains: breaking news in TNF-R1 signaling. *Mol Cell*. 2009; 36:736–742. [PubMed: 20005838]
21. Li X, Yang Y, Ashwell JD. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature*. 2002; 416:345–347. [PubMed: 11907583]
22. Emmerich CH, Schmukle AC, Walczak H. The emerging role of linear ubiquitination in cell signaling. *Sci Signal*. 2011; 4:re5. [PubMed: 22375051]
23. Varfolomeev E, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell*. 2007; 131:669–681. [PubMed: 18022362]
24. Xia ZP, Chen ZJ. TRAF2: a double-edged sword? *Sci STKE*. 2005; 2005:pe7. [PubMed: 15728425]
25. Vucic D, Dixit VM, Wertz IE. Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. *Nat Rev Mol Cell Biol*. 2011; 12:439–452. [PubMed: 21697901]
26. Dynek JN, et al. c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. *EMBO J*. 2010; 29:4198–4209. [PubMed: 21113135]
27. Xu M, Skaug B, Zeng W, Chen ZJ. A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. *Mol Cell*. 2009; 36:302–314. [PubMed: 19854138]
28. Chen ZJ. Ubiquitination in signaling to and activation of IKK. *Immunol Rev*. 2012; 246:95–106. [PubMed: 22435549]
29. Kulathu Y, Akutsu M, Bremm A, Hofmann K, Komander D. Two-sided ubiquitin binding explains specificity of the TAB2 NZF domain. *Nat Struct Mol Biol*. 2009; 16:1328–1330. [PubMed: 19935683]
30. Sato Y, Yoshikawa A, Yamashita M, Yamagata A, Fukai S. Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by NZF domains of TAB2 and TAB3. *EMBO J*. 2009; 28:3903–3909. [PubMed: 19927120]

31. Wagner S, et al. Ubiquitin binding mediates the NF-kappaB inhibitory potential of ABIN proteins. *Oncogene*. 2008; 27:3739–3745. [PubMed: 18212736]
32. Lo YC, et al. Structural basis for recognition of diubiquitins by NEMO. *Mol Cell*. 2009; 33:602–615. [PubMed: 19185524]
33. Harhaj EW, Dixit VM. Regulation of NF-kappaB by deubiquitinases. *Immunol Rev*. 2012; 246:107–124. [PubMed: 22435550]
34. Fiil BK, Gyrd-Hansen M. Met1-linked ubiquitination in immune signalling. *FEBS J*. 2014; 281:4337–4350. [PubMed: 25060092]
35. Wertz IE, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature*. 2004; 430:694–699. [PubMed: 15258597]
36. Catrysse L, Vereecke L, Beyaert R, van Loo G. A20 in inflammation and autoimmunity. *Trends Immunol*. 2014; 35:22–31. [PubMed: 24246475]
37. Verhelst K, et al. A20 inhibits LUBAC-mediated NF-kappaB activation by binding linear polyubiquitin chains via its zinc finger 7. *EMBO J*. 2012; 31:3845–3855. [PubMed: 23032186]
38. Tokunaga F, et al. Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-kappaB regulation. *EMBO J*. 2012; 31:3856–3870. [PubMed: 23032187]
39. Komander D, Reyes-Turcu F, Licchesi JD, Odenwaelder P, Wilkinson KD, Barford D. Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep*. 2009; 10:466–473. [PubMed: 19373254]
40. Takiuchi T, et al. Suppression of LUBAC-mediated linear ubiquitination by a specific interaction between LUBAC and the deubiquitinases CYLD and OTULIN. *Genes Cells*. 2014; 19:254–272. [PubMed: 24461064]
41. Rivkin E, et al. The linear ubiquitin-specific deubiquitinase gumbly regulates angiogenesis. *Nature*. 2013; 498:318–324. [PubMed: 23708998]
42. Keusekotten K, et al. OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell*. 2013; 153:1312–1326. [PubMed: 23746843]
43. Karin M, Gallagher E. TNFR signaling: ubiquitin-conjugated TRAFs control stop-and-go for MAPK signaling complexes. *Immunol Rev*. 2009; 228:225–240. [PubMed: 19290931]
44. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell*. 1994; 78:773–785. [PubMed: 8087845]
45. Yaron A, et al. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature*. 1998; 396:590–594. [PubMed: 9859996]
46. Kanarek N, Ben-Neriah Y. Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBs. *Immunol Rev*. 2012; 246:77–94. [PubMed: 22435548]
47. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*. 2001; 412:346–351. [PubMed: 11460167]
48. Kanayama A, et al. TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell*. 2004; 15:535–548. [PubMed: 15327770]
49. Andersen PL, et al. Distinct regulation of Ubc13 functions by the two ubiquitin-conjugating enzyme variants Mms2 and Uev1A. *J Cell Biol*. 2005; 170:745–755. [PubMed: 16129784]
50. Zhou H, et al. Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. *Nature*. 2004; 427:167–171. [PubMed: 14695475]
51. Yamamoto M, et al. Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling. *Nat Immunol*. 2006; 7:962–970. [PubMed: 16862162]
52. Buchberger A. From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol*. 2002; 12:216–221. [PubMed: 12062168]
53. Randles L, Walters KJ. Ubiquitin and its binding domains. *Front Biosci (Landmark Ed)*. 2012; 17:2140–2157. [PubMed: 22652769]
54. Dikic I, Wakatsuki S, Walters KJ. Ubiquitin-binding domains - from structures to functions. *Nat Rev Mol Cell Biol*. 2009; 10:659–671. [PubMed: 19773779]
55. Raasi S, Varadan R, Fushman D, Pickart CM. Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat Struct Mol Biol*. 2005; 12:708–714. [PubMed: 16007098]

56. Windheim M, Stafford M, Peggie M, Cohen P. Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaBalpha kinase. *Mol Cell Biol.* 2008; 28:1783–1791. [PubMed: 18180283]
57. Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. *Nat Cell Biol.* 2006; 8:398–406. [PubMed: 16547522]
58. Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell.* 2006; 22:245–257. [PubMed: 16603398]
59. Heyninck K, Kreike MM, Beyaert R. Structure-function analysis of the A20-binding inhibitor of NF-kappa B activation, ABIN-1. *FEBS Lett.* 2003; 536:135–140. [PubMed: 12586352]
60. Verstrepen L, Carpentier I, Verhelst K, Beyaert R. ABINs: A20 binding inhibitors of NF-kappa B and apoptosis signaling. *Biochem Pharmacol.* 2009; 78:105–114. [PubMed: 19464428]
61. Ikeda F, Rahighi S, Wakatsuki S, Dikic I. Selective binding of linear ubiquitin chains to NEMO in NF-kappaB activation. *Adv Exp Med Biol.* 2011; 691:107–114. [PubMed: 21153314]
62. Zhu G, Wu CJ, Zhao Y, Ashwell JD. Optineurin negatively regulates TNFalpha-induced NF-kappaB activation by competing with NEMO for ubiquitinated RIP. *Curr Biol.* 2007; 17:1438–1443. [PubMed: 17702576]
63. Wild P, et al. Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. *Science.* 2011; 333:228–233. [PubMed: 21617041]
64. Boyle KB, Randow F. The role of ‘eat-me’ signals and autophagy cargo receptors in innate immunity. *Curr Opin Microbiol.* 2013; 16:339–348. [PubMed: 23623150]
65. Kensche T, Tokunaga F, Ikeda F, Goto E, Iwai K, Dikic I. Analysis of nuclear factor-kappaB (NF-kappaB) essential modulator (NEMO) binding to linear and lysine-linked ubiquitin chains and its role in the activation of NF-kappaB. *J Biol Chem.* 2012; 287:23626–23634. [PubMed: 22605335]
66. Emmerich CH, et al. Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc Natl Acad Sci U S A.* 2013; 110:15247–15252. [PubMed: 23986494]
67. Tarantino N, et al. TNF and IL-1 exhibit distinct ubiquitin requirements for inducing NEMO-IKK supramolecular structures. *J Cell Biol.* 2014; 204:231–245. [PubMed: 24446482]
68. Yoshikawa A, Sato Y, Yamashita M, Mimura H, Yamagata A, Fukai S. Crystal structure of the NEMO ubiquitin-binding domain in complex with Lys 63-linked di-ubiquitin. *FEBS Lett.* 2009; 583:3317–3322. [PubMed: 19766637]
69. Filipe-Santos O, et al. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. *J Exp Med.* 2006; 203:1745–1759. [PubMed: 16818673]
70. Doffinger R, et al. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. *Nat Genet.* 2001; 27:277–285. [PubMed: 11242109]
71. Hubeau M, et al. New mechanism of X-linked anhidrotic ectodermal dysplasia with immunodeficiency: impairment of ubiquitin binding despite normal folding of NEMO protein. *Blood.* 2011; 118:926–935. [PubMed: 21622647]
72. Mattioli F, Sixma TK. Lysine-targeting specificity in ubiquitin and ubiquitin-like modification pathways. *Nat Struct Mol Biol.* 2014; 21:308–316. [PubMed: 24699079]
73. Boisson B, et al. Immunodeficiency, autoinflammation and amylopectinosis in humans with inherited HOIL-1 and LUBAC deficiency. *Nat Immunol.* 2012; 13:1178–1186. [PubMed: 23104095]
74. Peltzer N, et al. HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death. *Cell Rep.* 2014; 9:153–165. [PubMed: 25284787]
75. Stieglitz B, Morris-Davies AC, Koliopoulos MG, Christodoulou E, Rittinger K. LUBAC synthesizes linear ubiquitin chains via a thioester intermediate. *EMBO Rep.* 2012; 13:840–846. [PubMed: 22791023]
76. Smit JJ, Monteferrario D, Noordermeer SM, van Dijk WJ, van der Reijden BA, Sixma TK. The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension. *EMBO J.* 2012; 31:3833–3844. [PubMed: 22863777]

77. Stieglitz B, et al. Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP. *Nature*. 2013; 503:422–426. [PubMed: 24141947]
78. Stieglitz B, Haire LF, Dikic I, Rittinger K. Structural analysis of SHARPIN, a subunit of a large multi-protein E3 ubiquitin ligase, reveals a novel dimerization function for the pleckstrin homology superfold. *J Biol Chem*. 2012; 287:20823–20829. [PubMed: 22549881]
79. Sato Y, et al. Specific recognition of linear ubiquitin chains by the Npl4 zinc finger (NZF) domain of the HOIL-1L subunit of the linear ubiquitin chain assembly complex. *Proc Natl Acad Sci U S A*. 2011; 108:20520–20525. [PubMed: 22139374]
80. Damgaard RB. The ubiquitin ligase XIAP recruits LUBAC for NOD2 signaling in inflammation and innate immunity. *Mol Cell*. 2012; 46:746–758. [PubMed: 22607974]
81. HogenEsch H, Gijbels MJ, Offerman E, van Hooft J, van Bekkum DW, Zurcher C. A spontaneous mutation characterized by chronic proliferative dermatitis in C57BL mice. *Am J Pathol*. 1993; 143:972–982. [PubMed: 8362989]
82. Seymour RE, et al. Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. *Genes Immun*. 2007; 8:416–421. [PubMed: 17538631]
83. Kumari S, et al. Sharpin prevents skin inflammation by inhibiting TNFR1-induced keratinocyte apoptosis. *Elife*. 2014:3.
84. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*. 2003; 114:181–190. [PubMed: 12887920]
85. Rickard JA, et al. TNFR1-dependent cell death drives inflammation in Sharpin-deficient mice. *Elife*. 2014:3.
86. Berger SB, et al. Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. *J Immunol*. 2014; 192:5476–5480. [PubMed: 24821972]
87. Sasaki Y, et al. Defective immune responses in mice lacking LUBAC-mediated linear ubiquitination in B cells. *EMBO J*. 2013; 32:2463–2476. [PubMed: 23942237]
88. Oshima S, et al. ABIN-1 is a ubiquitin sensor that restricts cell death and sustains embryonic development. *Nature*. 2009; 457:906–909. [PubMed: 19060883]
89. Lavrik IN, Krammer PH. Regulation of CD95/Fas signaling at the DISC. *Cell Death Differ*. 2012; 19:36–41. [PubMed: 22075988]
90. Shirley S, Morizot A, Micheau O. Regulating TRAIL receptor-induced cell death at the membrane: a deadly discussion. *Recent Pat Anticancer Drug Discov*. 2011; 6:311–323. [PubMed: 21756247]
91. Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol*. 2007; 39:1462–1475. [PubMed: 17403612]
92. Fiil BK, et al. OTULIN restricts Met1-linked ubiquitination to control innate immune signaling. *Mol Cell*. 2013; 50:818–830. [PubMed: 23806334]
93. Donley C, et al. Identification of RBCK1 as a novel regulator of FKBPL: implications for tumor growth and response to tamoxifen. *Oncogene*. 2014; 33:3441–3450. [PubMed: 23912458]
94. Dubois SM, et al. A catalytic-independent role for the LUBAC in NF-kappaB activation upon antigen receptor engagement and in lymphoma cells. *Blood*. 2014; 123:2199–2203. [PubMed: 24497531]
95. Ehrlund A, Jonsson P, Vedin LL, Williams C, Gustafsson JA, Treuter E. Knockdown of SF-1 and RNF31 affects components of steroidogenesis, TGFbeta, and Wnt/beta-catenin signaling in adrenocortical carcinoma cells. *PLoS One*. 2012; 7:e32080. [PubMed: 22427816]
96. Gustafsson N, Zhao C, Gustafsson JA, Dahlman-Wright K. RBCK1 drives breast cancer cell proliferation by promoting transcription of estrogen receptor alpha and cyclin B1. *Cancer Res*. 2010; 70:1265–1274. [PubMed: 20103625]
97. Gustafsson Sheppard N, Heldring N, Dahlman-Wright K. Estrogen receptor-alpha, RBCK1, and protein kinase C beta 1 cooperate to regulate estrogen receptor-alpha gene expression. *J Mol Endocrinol*. 2012; 49:277–287. [PubMed: 23042805]
98. He L, Ingram A, Rybak AP, Tang D. Shank-interacting protein-like 1 promotes tumorigenesis via PTEN inhibition in human tumor cells. *J Clin Invest*. 2010; 120:2094–2108. [PubMed: 20458142]

99. Li J, et al. SHARPIN overexpression induces tumorigenesis in human prostate cancer LNCaP, DU145 and PC-3 cells via NF-kappaB/ERK/Akt signaling pathway. *Med Oncol.* 2015; 32:444. [PubMed: 25550157]
100. Pouwels J, et al. SHARPIN regulates uropod detachment in migrating lymphocytes. *Cell Rep.* 2013; 5:619–628. [PubMed: 24210817]
101. Queisser MA, et al. HOIL-1L functions as the PKCzeta ubiquitin ligase to promote lung tumor growth. *Am J Respir Crit Care Med.* 2014; 190:688–698. [PubMed: 25118570]
102. Thompson HG, Harris JW, Lin L, Brody JP. Identification of the protein Zibra, its genomic organization, regulation, and expression in breast cancer cells. *Exp Cell Res.* 2004; 295:448–459. [PubMed: 15093743]
103. Tomonaga M, et al. Activation of nuclear factor-kappa B by linear ubiquitin chain assembly complex contributes to lung metastasis of osteosarcoma cells. *Int J Oncol.* 2012; 40:409–417. [PubMed: 21947385]
104. Yang Y, et al. Essential role of the linear ubiquitin chain assembly complex in lymphoma revealed by rare germline polymorphisms. *Cancer Discov.* 2014; 4:480–493. [PubMed: 24491438]
105. Zhang Y, et al. Activation of nuclear factor kappaB pathway and downstream targets survivin and livin by SHARPIN contributes to the progression and metastasis of prostate cancer. *Cancer.* 2014; 120:3208–3218. [PubMed: 24925528]
106. Zhu J, et al. The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor alpha and modulates estrogen-stimulated breast cancer cell proliferation. *Oncogene.* 2014; 33:4340–4351. [PubMed: 24441041]
107. Elliott PR, et al. Molecular basis and regulation of OTULIN-LUBAC interaction. *Mol Cell.* 2014; 54:335–348. [PubMed: 24726323]
108. Schaeffer V, Akutsu M, Olma MH, Gomes LC, Kawasaki M, Dikic I. Binding of OTULIN to the PUB domain of HOIP controls NF-kappaB signaling. *Mol Cell.* 2014; 54:349–361. [PubMed: 24726327]
109. Sun SC. CYLD: a tumor suppressor deubiquitinase regulating NF-[kappa]B activation and diverse biological processes. *Cell Death Differ.* 2010:25–34. [PubMed: 19373246]

Figure 1. Ubiquitination in the TNF-induced NF-κB signaling cascade

Different linkage types of ubiquitin chains including Met 1-, Lys 11-, Lys 48-, and Lys 63-linked ubiquitin chains participate in the TNF-induced NF-κB signaling pathway and have roles in fine-tuning the regulation of the downstream cascade. Ubiquitin chains are generated by E3 ligases (shown in orange), such as cIAP, HOIP containing LUBAC complex, and SCF-βTrCP. These ubiquitin chains are hydrolyzed by DUBs (in yellow) such as OTULIN and CYLD. A20 has a dual role as an E3 ligase as well as a DUB. Ubiquitination of the substrates including cIAPs, RIPK1, NEMO, and IκB-α have shown to be critical for the signaling pathway.

Figure 2. Linear ubiquitin interaction domains in different proteins

Schematic domain structures of the linear ubiquitin chain interacting proteins are shown. Linear ubiquitin interacting domains, such as the UBAN domains in NEMO, ABIN proteins and Optineurin, A20-ZF7, OTULIN-OTU and HOIL-1L-NZF-tail, are indicated in blue. Helix; HLX, Coiled-coil; CC, ABIN homology domain; AHD, NEMO-binding domain; NBD, leucine zipper; LZ, LC3-interacting region; LIR, ubiquitin-like; UBL, really interesting new gene; RING, in between ring; IBR.

Figure 3. Schematic domain structures of the LUBAC components and OTULIN

The LUBAC complex consists of HOIP, Sharpin, and HOIL-1L. Schematic domain structures, interactions with other components and interactions with ubiquitin molecules are indicated. The PH fold in Sharpin is used for homo-oligomerization. The UBL domains of Sharpin and HOIL-1L interact with HOIP-NZF2 and HOIP-UBA, respectively. OTULIN interacts with HOIP via the PUB domain. Sharpin-NZF, HOIP-NZF1 and HOIP-LDD interact with monoubiquitin (Protein Data Bank (pdb) code 1UBQ). OTULIN-OTU and HOIL-1L-NZF-C terminal tail bind to linear diubiquitin chain (pdb code 2W9N).

Figure 4. TNFR complex II-mediated apoptosis signaling cascades

The LUBAC complex participates not only in the TNFR complex I-mediated NF-κB signaling pathway, but also in the TNFR complex II-dependent apoptosis pathway. Sharpin inhibits TNFR complex II directly. HOIP and HOIL-1L play a role in the apoptosis regulation. An involvement of linear ubiquitinated substrates, DUBs such as OTULIN and CYLD is elusive.

Table 1
Genetically modified mouse models of LUBAC components and OTULIN

Gene	Genotype	Phenotype and comments	Reference
<i>Hoip</i>	<i>Hoip</i> ^{-/-}	Lethal at E10.5, vascularization defect and increased apoptosis in yolk sac at E10.5	(74)
	<i>Hoip</i> ^{-/-} ; <i>Tnfr</i> ^{-/-}	Lethal at E15.5, vascularization defect of yolk sac, decreased apoptosis in yolk sac at E10.5	(74)
	<i>Hoip</i> ^{-/-} ; <i>Tnfr1</i> ^{-/-}	Lethal at E17.5	(74)
	<i>Hoip</i> ^{fl/fl} ; <i>Tie2-Cre</i> ⁺	Very similar to <i>Hoip</i> ^{-/-}	(74)
	<i>Hoip</i> ^{linear/linear}	Embryonic lethal, Deletion of exons which targets the RBR catalytic domain	(87)
	<i>Hoip</i> ^{fl/fl} ; <i>Mb1-Cre</i> ⁺	Defects in development of B1 cells, defects of CD40L-induced NF-κB and ERK activation in B cells (87) Deletion of exons, which targets the RBR catalytic domain, in B cells	
	<i>Hoip</i> ^{C879S/C879S}	Lethal at E10.5	(66)
<i>Sharpin</i>	<i>Sharpin</i> ^{cpdm/cpdm}	Systemic inflammation (skin, gut, lung, liver, joint), apoptosis (skin, lung, liver), Splenomegaly, loss of Peyer patch formation, dermatitis, increased level of serum IgM	(7-9, 81, 82, 85, 86)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Tnfr1</i> ^{-/-}	Inflammation and apoptosis in different tissues and splenomegaly are inhibited, no Peyer patch	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Tnfr2</i> ^{-/-}	No significant effect on the <i>Sharpin</i> ^{cpdm/cpdm} phenotype	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Il1r</i> ^{-/-}	Onset of the skin dermatitis is delayed	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Tnfr</i> ^{-/-}	Dermatitis and liver inflammation are blocked	(9)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Rip</i> ^{K45A/K45A}	Survival is completely rescued, inflammation (skin, liver, joint, lung) is inhibited The IgM level becomes normal	(86)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>FADD</i> ^{fl/fl} ; <i>K14-Cre</i> ⁺	Dermatitis and apoptosis in the skin is inhibited	(83)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>TRADD</i> ^{fl/fl} ; <i>K14-Cre</i> ⁺	Dermatitis and apoptosis in the skin is inhibited	(83)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Rip3</i> ^{-/-}	Onset of the skin dermatitis is mildly delayed, liver inflammation and spleen size are decreased	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Mkl1</i> ^{-/-}	Very similar to <i>Sharpin</i> ^{cpdm/cpdm} ; <i>Rip3</i> ^{-/-}	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Casp8</i> ^{gw/-}	Onset of the skin dermatitis is delayed	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Casp8</i> ^{-/-}	Not tested, embryonic lethality expected	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Casp8</i> ^{gw/-} ; <i>Rip3</i> ^{-/-}	Markedly cpdm phenotype is inhibited including Peyer patch formation defect	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Casp8</i> ^{-/-} ; <i>Rip3</i> ^{-/-}	Most of the pups are lethal at Day 2 after birth, surviving mice have no dermatitis phenotype	(85)
	<i>Sharpin</i> ^{cpdm/cpdm} ; <i>Bid</i> ^{-/-}	No significant effect on the <i>Sharpin</i> ^{cpdm/cpdm} phenotype	(85)
<i>Hoil</i>	<i>Hoil</i> ^{-/-}	No apparent phenotype, increased apoptosis detected in liver	(17)
<i>Otulin</i>	<i>Otulin</i> ^{gun/gun}	Lethal at E12.5-E14, vascularization defect, Wnt signaling defect	(41)