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Research update

Linear ubiquitination in NF-κB signaling and inflammation: What we do understand and what we do not

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ABSTRACT

Despite its small size, ubiquitin is one of the most versatile signaling molecules in the cell and affects distinct cellular processes. It forms the building block of a repertoire of posttranslational modifications of cellular proteins, ranging from the attachment of a single ubiquitin to ubiquitin chains of different linkage. Proteins that contain ubiquitin chain-specific ubiquitin-binding domains recognize different types of ubiquitination and determine the mode of signaling of modified proteins. Polyubiquitin chains were thought to be formed only by the conjugation of the ubiquitin C-terminal Gly to one of the seven internal Lys residues of another ubiquitin. However, the C-terminal Gly was recently shown to also link to the N-terminus of another ubiquitin to form head-to-tail polyubiquitin chains, which is referred to as linear ubiquitination. These linear linkages can be assembled and conjugated to another protein by an E3 ligase complex known as LUBAC, and are recognized by a particular ubiquitin-binding domain known as UBAN. Both have been implicated in the regulation of TNF-induced NF-κB signaling, which induces the expression of a wide range of proteins that mediate many biological processes including inflammation and cell survival. We discuss the molecular players and mechanisms that determine the specificity and outcome of linear ubiquitination in NF-κB signaling, as well as future directions and challenges ahead.

1. Introduction

Ubiquitin is a small protein of 76 amino acids that is encoded as a precursor protein in mammals by 4 different genes and is used by all eukaryotic cells to modify proteins posttranslationally. This ubiquitin label will appoint a new destiny to the ubiquitinated proteins as it influences their activity, interactions and/or stability. As a consequence, the ubiquitin system drives numerous cellular responses including DNA repair, cell cycle progression, signal transduction and membrane protein transport [1]. All these processes can be accomplished by covalent attachment of ubiquitin as a monomer (monoubiquitination) or as a polymer (polyubiquitination) to a target substrate [2]. Ubiquitination is achieved by the concerted action of a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3) [3]. E1 activates the ubiquitin residue by forming an E1–ubiquitin thioester in an ATP-dependent manner [3,4], allowing the transfer of activated ubiquitin to E2. The final step of ubiquitination requires the activity of an E3, which functions as the substrate recognition module of the system and can interact with both E2 and substrate. While only 2 E1s and 38 E2s are known, more than 600 E3 ubiquitin ligases are predicted in the human genome [5]. Ubiquitination can be reversed by deubiquitinases (DUBs) that cleave protein–ubiquitin bonds [6]. The human genome encodes nearly 100 DUBs that can be classified in 4 cysteine protease families and 1 metalloprotease family. Besides reversing the ubiquitination of target proteins, DUBs are also responsible for the processing of ubiquitin precursor proteins and the recycling of ubiquitin from polyubiquitin chains.

Most commonly, the C-terminal Gly76 of ubiquitin is connected via an isopeptide bond to the ε-amino group of a Lys in the substrate [7]. During the last years it has become clear that also non-Lys residues can accept ubiquitin. For example, ubiquitin can be linked to the N-terminal amino acid of a protein [8]. In addition, even non-amino groups such as the sulfhydryl group of Cys [9], or the hydroxyl group of Thr or Ser residues can bind ubiquitin [10,11]. Proteins cannot only be modified by single ubiquitin molecules, but also by polyubiquitin chains. In order to generate these linkages, the C-terminal Gly of the first ubiquitin molecule is linked to the ε-amino group of one of the 7 internal Lys residues of the second ubiquitin moiety (Lys6, Lys11, Lys27, Lys33, Lys48 or Lys63). More recently, binding between Gly76 of one ubiquitin and Met1 of another ubiquitin molecule was described [12]. In this

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way, consecutive ubiquitin molecules are assembled to each other by their N- and C-termini, which is known as linear or head-to-tail ubiquitination. Linear ubiquitin chains are already known for years as *ubiquitin* genes encode linear polyubiquitin precursors that are hydrolyzed to monomers by DUBs [13]. However, it still came as a surprise that a cell would assemble linear polyubiquitin chains from single ubiquitin molecules by means of specific E3 ligases, and use them to modify specific substrates.

The type of linkage and the resulting conformation of the polyubiquitin chain determine the outcome of ubiquitination for a cell. For example, linear, Lys48- and Lys6-linked polyubiquitin chains can target their substrates for proteasomal degradation [14–16]; Lys63- and Lys6-linked polyubiquitin chains contribute to DNA repair [17,18] and innate immunity [19], while Lys11-ubiquitination has mainly been described in cell cycle events in mammals [20,21]. Each type of ubiquitination is recognized by specific ubiquitin-binding domains (UBDs) in other proteins that decode and transmit information conferred by protein ubiquitination to control various cellular events [22].

The prototypic example of a signaling pathway that integrates multiple types of ubiquitination is the NF-κB pathway [23]. Lys63-ubiquitination of signaling intermediates leads to the formation of multi-protein signaling complexes, which ultimately results in activation of the inhibitor of κB (IκB) kinase (IKK) complex. Lys48-ubiquitination of IκBα on the other hand, leads to its proteasomal degradation and sets NF-κB free to translocate to the nucleus. Recently, modification of signaling proteins by linear ubiquitination has been added as a novel regulatory mechanism in NF-κB signaling.

2. The linear ubiquitination machinery

The linear ubiquitin chain assembly complex (LUBAC) is the only E3 ubiquitin ligase complex known to catalyze the generation of linear polyubiquitin chains in cells [12]. LUBAC was initially identified as a 600 kDa complex containing two related multi-domain proteins: HOIL-1 (heme-oxidized iron-regulatory protein 2 ubiquitin ligase-1; also known as RBCK1) and HOIP (HOIL-1 interacting protein; also known as RNF31), which have masses of 58 and 120 kDa, respectively [12]. Both HOIP and HOIL-1 contain C-terminal RING-IBR-RING (RBR) motifs analogous to those found in E3s such as Parkin. Three recent studies revealed a third component of the LUBAC complex, referred to as SHARPIN (SHANK-associated RH domain-interacting protein; 45 kDa) (Fig. 1) [24–26]. Available evidence suggests that multiple HOIP molecules are present in the 600 kDa LUBAC complex [26,27]. HOIP may function with either SHARPIN or HOIL-1 as an adaptor protein *in vitro* to promote linear ubiquitin chain assembly [24–26], making it possible that HOIP–HOIL-1 and HOIP–SHARPIN may function independently under certain conditions. SHARPIN does not have any enzymatic activity and the two RING fingers of HOIP, but not those of HOIL-1, function as E2-binding sites and mediate E3 ubiquitin ligase activity [12]. To generate linear ubiquitin chains

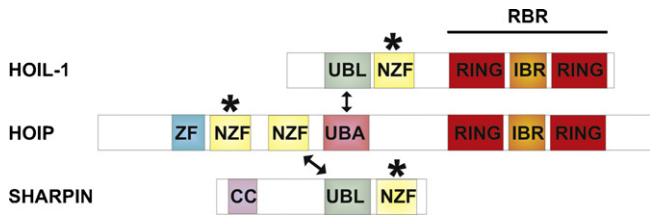

Fig. 1. Schematic overview of the domain structure of the LUBAC components ZF = zinc finger; NZF = Npl4-type zinc finger domain; UBA = ubiquitin-associated domain; IBR = in-between RING-domain, RBR = RING-between-RING, UBL = ubiquitin-like domain; CC = coiled-coil domain. Mutual interactions are indicated by arrows; * = ubiquitin-binding domain.

in vitro, LUBAC can use several E2s, including UBE2D (UbcH5), UBE2L3 (UbcH7), or E2-25K [12], with LUBAC and not the E2 being primarily responsible for determining the linkage specificity. Ubiquitin binding is provided by an Npl4-type zinc finger domain (NZF) that is present in HOIP, HOIL-1 and SHARPIN (HOIP has a second NZF that is however not involved in ubiquitin binding) [24,25]. For the formation of the different LUBAC complexes themselves, HOIL-1 and SHARPIN rely on their ubiquitin-like domain (UBL) to bind to the ubiquitin-associated domain (UBA) or the second NZF domain of HOIP, respectively [12,25]. Furthermore, studies with murine embryonic fibroblasts from *SHARPIN* mutant mice revealed that SHARPIN also stabilizes HOIP and HOIL-1 proteins [24,26].

Although the RING fingers of HOIL-1 do not confer linear ubiquitinating ligase activity to LUBAC, it is of interest that HOIL-1 has been shown to be involved in the Lys48-ubiquitination of a number of proteins such as iron-regulatory protein 2, the transcription factors Bach1 and interferon regulatory factor-3 (IRF3), and TGF-β activated kinase (TAK) 1 binding protein (TAB) 2 and TAB3 [28–31]. In this way, HOIL-1 can negatively regulate tumor necrosis factor (TNF)- and interleukin-1 (IL-1)-induced NF-κB activation by targeting TAB2/3 for proteasomal degradation [31]. It is highly possible that HOIL-1 uses other interacting partners besides HOIP and SHARPIN, as well as other E2 enzymes to achieve this Lys48-chain forming activity.

3. Linear ubiquitination in NF-κB signaling

Ubiquitination plays a key role in NF-κB signaling. From the early days of NF-κB signaling research it is known that SCF-βTrCP-mediated Lys48 polyubiquitination of the NF-κB inhibitor protein IκBα results in its proteasomal degradation, allowing NF-κB to translocate into the nucleus (Fig. 2). In the year 2000, a new regulatory function for ubiquitin was revealed by the finding that IKK is activated through the assembly of Lys63-linked polyubiquitin chains [32]. In the meantime, members of the TRAF and cIAP family were identified as E3 ubiquitin ligases that are capable to modify themselves and other NF-κB signaling proteins with Lys63-linked polyubiquitin chains. The regulatory roles of Lys63-linked chains have been studied extensively [17]. Interestingly, replacement of endogenous ubiquitin with a ubiquitin-Lys63Arg mutant suggested that TNF-induced IKK activation is not restricted to Lys63-linked polyubiquitin chains [33]. More recently, a role for linear ubiquitin chains emerged in NF-κB signaling. The group of Kazuhiro Iwai, who originally identified LUBAC as a ubiquitin ligase that assembles linear polyubiquitin chains [12], examined its effect on transcription from various promotors and found that combined expression of HOIL-1 and HOIP induced NF-κB activity [27]. At the same time Henning Walczak and colleagues identified HOIL-1 and HOIP as functional components of the TNF-receptor 1 (TNF-R1) signaling complex leading to NF-κB activation via a modified tandem affinity purification procedure [34]. Very recently, SHARPIN was identified by three different laboratories as a third component of the LUBAC complex that is essential for linear polyubiquitination in NF-κB signaling [24–26]. Studies in cells defective in one of the LUBAC components, including cells derived from chronic proliferative dermatitis (*cpdm*) mice that carry a spontaneous mutation in the *SHARPIN* gene, confirmed the essential role of LUBAC in NF-κB signaling in response to TNF, IL-1, lymphotoxin-β, CD40-ligand, and lipopolysaccharide (LPS) [24–27,33].

The role of LUBAC in NF-κB signaling has been documented in most detail in the case of TNF-R1 signaling. Upon TNF stimulation, LUBAC is recruited to and stabilizes the TNF-R1 signaling complex [34] (Fig. 2). Taking into account that this complex is packed with ubiquitinated proteins such as RIP1, TRAF2, NEMO and cIAP1/2, LUBAC recruitment and stabilization of the TNF-R1 signaling

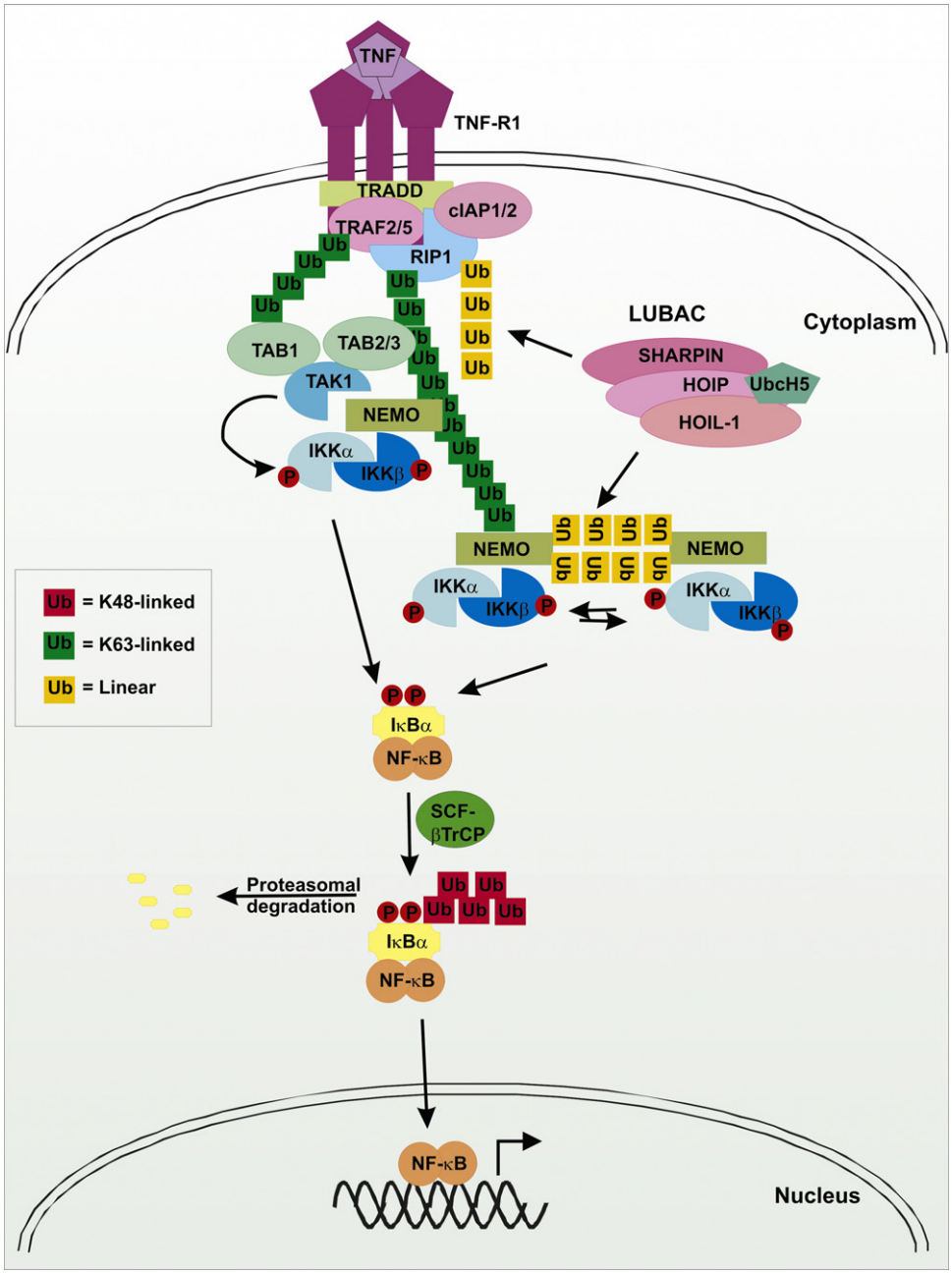

Fig. 2. Linear ubiquitination in TNF-induced NF-κB signaling. Triggering of TNF-R1 by TNF initiates the recruitment of several cytosolic proteins, including the E3 ubiquitin ligases TRAF2, TRAF5, cIAP1, cIAP2, and the kinase RIP1 to the receptor complex. Many of these signaling proteins are then modified by cIAP1/2 with Lys63-linked polyubiquitin chains, which are recognized by specific ubiquitin-binding proteins (TAB1, TAB2, TAB3 or NEMO) that function as scaffolds for TAK1 and IKKα/IKKβ. Induced proximity results in the TAK1-induced phosphorylation and activation of IKK kinases, which can also transphosphorylate each other. LUBAC (composed of SHARPIN, HOIP and HOIL-1) is also recruited to the TNF-R1 signaling complex and adds linear polyubiquitin chains to NEMO and RIP1. Intermolecular binding between the linear polyubiquitin chains of NEMO and the UBAN domain of another NEMO molecule leads to multimerization or conformational changes of the IKK complex that facilitate trans-autophosphorylation and activation of the IKK kinases. These phosphorylate IκBα at Ser32 and Ser36, which creates a binding site for the SCF-βTrCP E3 ligase complex that is responsible for the Lys48-linked polyubiquitination of IκBα, resulting in its proteasomal degradation. NF-κB dimers then translocate to the nucleus, where they bind to the κB sites of promoters of various genes.

complex is most likely facilitated by the NZF domain that is present in all LUBAC components and which has been shown to bind linear, Lys48- and Lys63-linked polyubiquitin chains [24,34]. LUBAC recruitment still occurs in RIP1- and NEMO-deficient cells, but not in cIAP1/2-, TRADD- or TRAF2-deficient cells. In addition, LUBAC recruitment needs E3 ligase activity of cIAP1/2, but not of TRAF2 [34]. Taken together, these data indicate that LUBAC is recruited to a polyubiquitin chain platform generated by cIAP1/2 in the TNF-R1 signaling complex. TRADD and TRAF2 most likely function to connect cIAP1/2 to the TNF-R1 signaling complex.

Once recruited to the TNF-R1 signaling complex, LUBAC modifies NEMO with linear polyubiquitin chains at Lys285 and Lys309, which is required for efficient activation of NF-κB [27,34]. Because cIAP1/2 and NEMO bind linear polyubiquitin chains via their UBA and UBAN (also called NUB or CoZi) domain, respectively, linear ubiquitination of NEMO enhances the recruitment of cIAP1/2 and NEMO and stabilizes the TNF-R1 signaling complex. In addition, binding of NEMO to linear ubiquitin chains may trigger multimerization or conformational changes of the IKK complex, facilitating the transphosphorylation and concomitant

activation of the IKK kinase subunits. Besides NEMO, also RIP1 is subjected to linear ubiquitination in response to TNF, although to a much lesser extent [24]. The role of linear ubiquitination of RIP1 is still unknown but, similar to linear ubiquitinated NEMO, it may contribute to the recruitment and retention of other signaling proteins in the TNF-R1 signaling complex. Interestingly, whereas only linear ubiquitinated NEMO could be identified in the TNF-R1 signaling complex, also Lys63-, Lys48- and Lys11-ubiquitinated forms of RIP1 were found. Lys11-linked ubiquitination of RIP1 by cIAP1/2 has recently been reported and would lead to the degradation of RIP1 in the TNF-R1 signaling complex [35]. Lys63-ubiquitination of RIP1 contributes to NF-κB activation, whereas absence of this type of RIP1 ubiquitination allows the formation of death signaling complexes leading to apoptosis or necroptosis [36]. Similarly, cells deficient in one of the components of the LUBAC system show enhanced TNF-induced apoptosis, indicating that also linear ubiquitination of RIP1 may prevent it from signaling cell death [34]. As LUBAC is required for NF-κB signaling in response to multiple different receptors, it is likely that other proteins, in addition to RIP1 and NEMO, can be targeted by LUBAC. In contrast to TNF-induced NF-κB activation, IL-1-induced IKK activation is abolished upon replacement of endogenous ubiquitin with a Lys63Arg mutant that can no longer be used for Lys63-ubiquitination [33], suggesting non-redundant roles for Lys63-linked and linear ubiquitination.

Linear ubiquitin chains attached to NEMO apparently do not promote degradation. However, it has been shown that artificial fusion of tetraubiquitin to the N- or C-terminus of other proteins results in their degradation [16], suggesting that the positioning of the chain on the target may affect its efficiency as a degradation signal. Whether linear polyubiquitin chains naturally occurring on endogenous substrates act as degradation signals remains an open question. In this context, HOIP has already been shown to target specific proteins (TRIM25, IRF3) for proteasomal degradation but it is still unclear whether this is due to linear or K48-linked ubiquitination (see below). Recognition of linear ubiquitin chains by dedicated ubiquitin-binding domains of other proteins might shield linear ubiquitinated proteins from proteasomal targeting factors (see also below). As association of linear ubiquitin chains with the proteasome has been observed [37], it is also possible that inefficient processing rather than targeting is responsible for the inefficient degradation of linear ubiquitinated proteins.

4. Role of linear ubiquitination in other signaling pathways

Besides NF-κB activation, TNF-R1 triggering also leads to the activation of MAP kinases such as JNK and ERK. A role for linear ubiquitination in their activation has been suggested but current evidence is rather controversial. Two independent studies showed reduced TNF-induced JNK activation in SHARPIN mutant (*cpdm*) MEFs [24,25], whereas Tokunaga and colleagues demonstrated higher JNK and ERK activity in these cells and in *HOIL-1*-deficient MEFs [26,27]. Studies with B cells or peritoneal macrophages derived from these mice also showed reduced JNK and ERK signaling in response to CD40L and LPS [24,25,38]. The JNK/ERK signaling proteins that are regulated by linear ubiquitination remain to be identified.

In contrast to their mostly positive contribution in NF-κB and JNK/ERK signaling, HOIL-1 and HOIP were recently shown to negatively regulate the activation of the transcription factor IRF3 in response to retinoid acid-inducible gene I (RIG-I) stimulation [39,40]. RIG-I is one of the intracellular receptors that sense viral RNA and subsequently initiates signaling cascades leading to type I interferon (IFN) production, thereby establishing an antiviral state [41,42]. Triggering of RIG-I initiates its Lys63-linked polyubiquitination by the E3 ubiquitin ligase TRIM25 (tripartite motif containing 25) [43]. This facilitates the interaction of RIG-I with the adaptor protein MAVS (also known as VISA, IPS-1 or Cardif), and the subsequent recruitment of signaling molecules such as TANK-binding kinase (TBK1) (Fig. 3) [44–46]. This IKK-related kinase then phosphorylates and activates the transcription factor IRF3 to induce the production of type I IFN and other antiviral proteins. The LUBAC proteins HOIP and HOIL-1 were shown to independently target RIG-I and TRIM25 to effectively suppress virus-induced IFN production [39]. Both LUBAC components compete with TRIM25 for interacting with RIG-I by directly binding TRIM25 and RIG-I, thus preventing TRIM25-mediated RIG-I ubiquitination and its downstream signaling. In addition, HOIL-1 and HOIP can modify TRIM25 with linear and Lys48-linked polyubiquitin chains, resulting in its proteasomal degradation. Consequently, genetic deletion or depletion of HOIL-1 and HOIP in MEF cells enhances virus-induced IFN-β production and impairs viral replication [39]. HOIL-1 overexpression has been shown to also downregulate RIG-I signaling by inducing the proteasomal degradation of IRF3 [40]. However, Inn and colleagues were unable to reproduce these findings and did not find reduced expression of IRF3 in HOIL-1 and HOIP double deficient MEF cells [39], suggesting that IRF3 may not be a direct substrate for LUBAC. Taken together, these findings implicate an important role for LUBAC in tempering an excessive and prolonged antiviral response.

5. Sensing and modulation of linear ubiquitination

The recognition of different types of polyubiquitin chains by chain specific UBDs that are present in other proteins determines the outcome of ubiquitination [22]. At least three different UBDs: UBAN, NZF and UBA were identified as linear polyubiquitin sensors [24,34,47,48]. As already mentioned, the UBAN domain of NEMO recognizes linear ubiquitin chains attached to RIP1 and NEMO in the TNF-R1 signaling complex, which enhances its recruitment and stabilizes the TNF-R1 signaling complex. In addition, linear ubiquitination of NEMO and its recognition by UBAN domains in other NEMO molecules also leads to NEMO oligomerization, which may enhance IKK cross-phosphorylation and activation [27,34]. It should be mentioned that although NEMO preferentially binds linear polyubiquitin, it also recognizes Lys63-linked polyubiquitin chains by means of a dipartite UBD consisting of UBAN and a more C-terminal zinc finger [49]. The UBAN domain of NEMO is highly related to the UBAN domain that is present in ABINs and Optineurin [50]. These proteins were previously identified as negative regulators of NF-κB signaling and their activity was shown to strictly depend on an intact UBAN domain [24,34,47,48,51,52]. Interestingly, ABIN-1 is also recruited to the TNF-R1 signaling complex upon TNF stimulation [34]. The mechanism by which these proteins interfere with TNF signaling is still unclear. Optineurin was previously shown to compete with NEMO for binding to Lys63-ubiquitinated RIP1 [52], and one might speculate that it also competes with NEMO for binding to linear ubiquitinated RIP1. Alternatively, ABINs and Optineurin might also act as adaptor proteins that recruit specific DUBs to ubiquitinated signaling proteins. In this context, Optineurin was recently shown to be required for CYLD-mediated inhibition of TNF-induced NF-κB activation [53]. CYLD is one of the few DUBs that show protease activity towards linear polyubiquitin chains [54], making it a likely candidate to reverse linear ubiquitination in NF-κB signaling. Other DUBs, including ubiquitin specific protease (USP) 2, USP5 (also known as isopeptidase T) and USP15, were also shown to cleave linear polyubiquitin chains, among other chain types [54,55]. Future studies that investigate the effect of these and other DUBs on linear ubiquitination in NF-κB signaling will be of high interest.

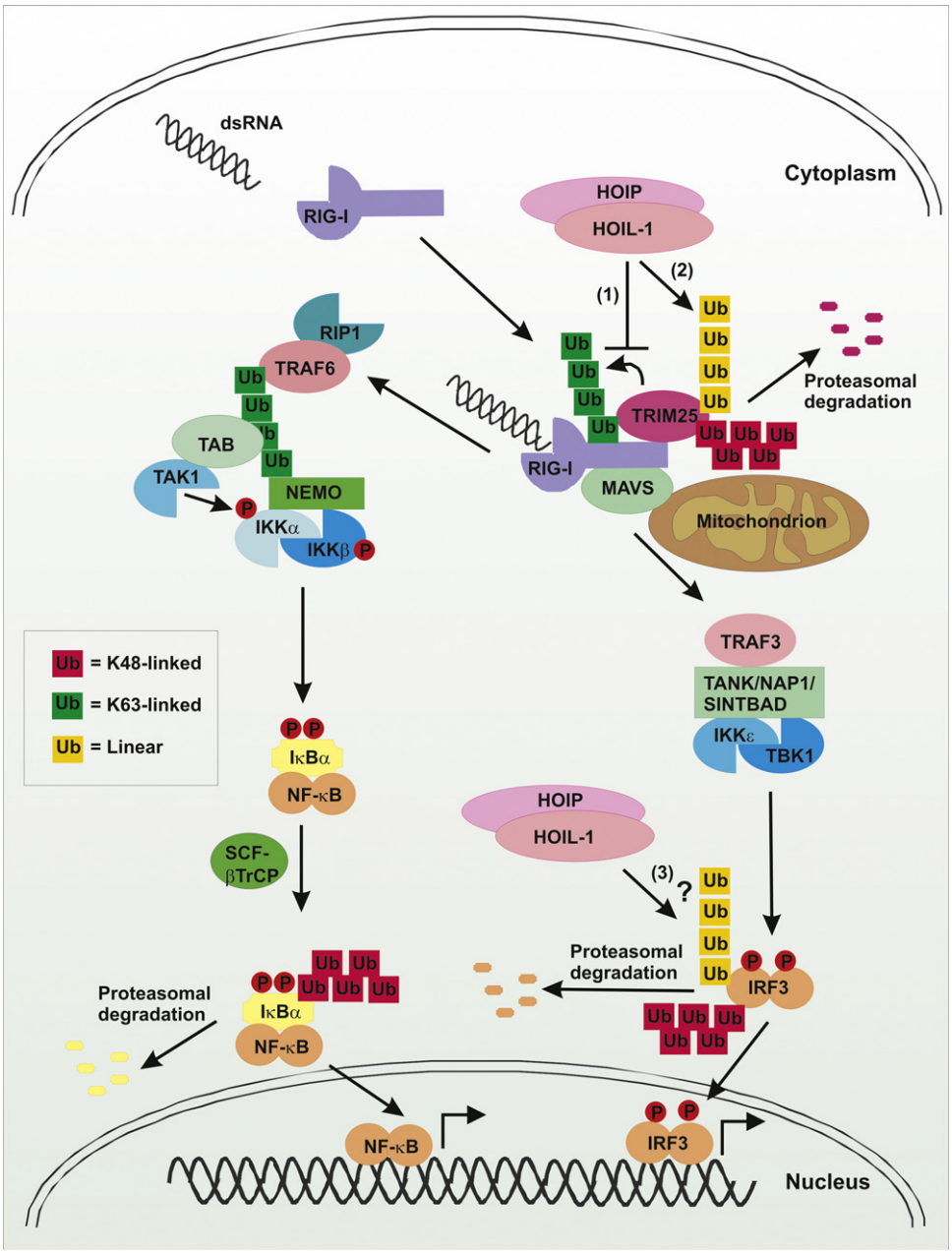

Fig. 3. Linear ubiquitination in RIG-I-induced IRF3 signaling. Viral double stranded RNA (dsRNA) is recognized in the cytoplasm by RIG-I, which is Lys63-ubiquitinated by TRIM25. This facilitates the recruitment of RIG-I to the adaptor protein MAVS, which resides at the mitochondrial membrane and initiates NF-κB and IRF3 signaling. The activation of NF-κB is mediated by Lys63-ubiquitination of the RIP1/TRAF6 complex, which facilitates the recruitment of the TAB/TAK1 and NEMO/IKK kinase complexes, leading to the phosphorylation and activation of IKK. The IKK-mediated phosphorylation of IκBα and its concomitant SCF-βTrCP-mediated Lys48-ubiquitination triggers the proteasomal degradation of IκBα, which frees NF-κB dimers to translocate to the nucleus. The activation of IRF3 starts with the recruitment of TRAF3 and the TBK1/IKKε kinase complex (various adaptors have been described: TANK, NAP1, SINTBAD) to MAVS, leading to the TBK1/IKKε-mediated phosphorylation of IRF3, which then translocates to the nucleus. HOIL-1 and HOIP can inhibit RIG-I-induced IRF3 activation by three different mechanisms: (1) by competing with TRIM25 for binding to RIG-I, hereby preventing TRIM25-mediated RIG-I ubiquitination; (2) by targeting TRIM25 for proteasomal degradation; (3) by targeting IRF3 for proteasomal degradation (the latter is still controversial). If proteasomal degradation of TRIM25 or IRF3 is mediated by linear or Lys48-linked ubiquitination is not known yet.

Additional to the UBAN domain, also other linear polyubiquitin chain sensing UBDs are present in proteins that contribute to NF-κB signaling. For example, the NZF domain of SHARPIN is essential for binding to linear polyubiquitin chains and activation of NF-κB [25]. The NZF of the adaptor protein TAB2 interacts with both Lys63-linked and linear polyubiquitin chains [27], and may be involved in the recruitment of the TAK1 kinase complex to the NEMO/IKK complex, resulting in the TAK1-mediated activation of IKK kinases. Finally, cIAP1/2 also interact with both linear and Lys63-linked polyubiquitin chains via their UBA domain [56].

Recently, significant advances towards understanding the mechanisms that determine specificity in ubiquitin signaling have been made. Binding specificity is determined by multimeric interactions in which different UBDs synergistically bind multiple ubiquitin molecules, and form contacts with regions that link ubiquitin molecules into a polymer [22]. In this context, the hydrophobic patch in ubiquitin surrounding three central residues (Leu8, Ile44 and Val70) or around Asp58 plays an important role. This patch is buried in Lys48-linked, but accessible in Lys63-linked or linear polyubiquitin chains [57,58]. In addition, UBDs often show specificity for a certain chain

length and linkage pattern [22]. The affinity between ubiquitin and its respective UBD is nevertheless relatively low. However, affinity is increased by additional protein interaction domains and increased local concentrations of interaction partners in signaling complexes [22]. DUBs not only use their own UBD or UBD-containing adaptor proteins to recognize specific polyubiquitin chains, but also recognize the isopeptide linkage and its chemical environment that is different in each type of polyubiquitin chain. Although linear chains adopt a similar extended conformation as Lys63-linked polyubiquitin chains, differences are identified in the surrounding of the isopeptide linkage [54]. The linkage of linear ubiquitin chains is more restrained due to small shifts of the distal Gly76 residue and the vicinity of the Lys63-side chain and Met1. In contrast to linear and Lys63-linked chains, Lys48-linked polyubiquitin chains have a more compact conformation with additional interactions between different ubiquitin molecules. Hence, this conformation is less flexible than the linear or Lys63-linked polyubiquitin chains.

The amount of linear ubiquitination is not only determined by the expression and activity of DUBs and linear polyubiquitin binding proteins that might shield modified proteins from DUB recognition, but also by the specific activity of the E3 ubiquitin ligase. Cross regulation was shown between LUBAC and members of the conventional protein kinase C (cPKC) family [59]. LUBAC ubiquitinates activated cPKCs, which marks them for proteasomal degradation, while cPKCs inhibit the E3 ligase activity of LUBAC by inducing its proteolytic cleavage. The responsible protease and physiological conditions leading to LUBAC cleavage remain to be identified.

6. Functional role of linear ubiquitination in immunity and inflammation

Information on the physiological role of linear ubiquitination is still very limited. Mice harboring a mutation in the *SHARPIN* gene develop a chronic proliferative dermatitis (mutation symbol, *cpdm*) phenotype. The skin of these mice displays epidermal hyperplasia with infiltration of inflammatory cells and keratinocyte cell death [25,60]. Inflammatory lesions are also found in other epithelial layers like those of the mouth, oesophagus and forestomach, as well as in the liver, lungs and several joints [60]. In addition, *cpdm* mice show defective development of lymphoid tissues, characterized by the absence of Peyer's patches, poorly defined follicles and no germinal centers and follicular dendritic cells in spleen, lymph nodes and nasal-associated lymphoid tissues [25], resulting in a defective B-cell population, but normal T-cell responses. Analysis of MEFs, B cells or keratinocytes derived from *cpdm* mice revealed defects in NF-κB activation in response to TNF, CD40 ligand, IL-1β or lymphotoxin, consistent with reduced IKK activation. Cells lacking SHARPIN are also highly sensitive to TNF-induced death. Breeding of *cpdm* mice in a TNF negative background rescued their inflammatory phenotype in the skin and liver, whereas the majority of developmental phenotypes remained unaffected, including the absence of Peyer's patches and altered leukocyte populations [24]. Thus *cpdm* mice show a TNF-dependent inflammatory phenotype and a TNF-independent developmental phenotype. Crossing *cpdm* mice with IL-1 receptor accessory protein-deficient mice also ameliorated the skin phenotype, but to a lesser extent than TNF deficiency [61]. These results suggest that mutations in components of the LUBAC system and its interacting partners could contribute to autoimmunity. Interestingly, patients with mutations in the UBAN domain of NEMO manifest a similar phenotype as *cpdm* mice [47,62–64].

In contrast to mutant SHARPIN (cpdm) mice, HOIL-1-deficient mice show no abnormal phenotype [26,27], suggesting possible redundancy between the three LUBAC components and implicating additional functions of SHARPIN independent of HOIP and HOIL-1.

7. Targeting linear ubiquitination for therapeutic purposes

Persistent NF-κB activation is central to the pathogenesis of many inflammatory diseases and cancer [65]. Therefore, targeting NF-κB activation is of high therapeutic interest and several inhibitors have been described, including antioxidants, peptides, decoy oligodeoxynucleotides, dominant-negative or constitutive active proteins, natural products, and small synthetic compounds [66]. Several of these molecules target specific steps such as IKK activity, nuclear translocation or DNA binding. Also various steroids and nonsteroid anti-inflammatory drugs such as aspirin have been found to block NF-κB, but their effects are highly pleiotropic. Many pharmaceutical companies are developing small molecule inhibitors of IKK and IKK-related kinases. Although the therapeutic potential of IKK inhibitors and many other NF-κB inhibitory products has been demonstrated in a number of animal models of inflammation and cancer, yet no specific NF-κB blocker has been approved for human use. A major problem is that molecules that block IKK activation lack specificity and thus interfere with NF-κB's physiological roles in immunity, inflammation, and cellular homeostasis. Long-lasting general NF-κB inhibition is therefore associated with non-tolerable adverse effects due to immunodeficiency. It is believed that cell type-specific or receptor-specific NF-κB inhibitors may be less prone to side effects, illustrating the need for a better knowledge of NF-κB signaling pathways.

As ubiquitination plays a key role in NF-κB signaling, also ubiquitination inhibitors are believed to hold promise for the development of novel treatments. Interestingly, enhanced expression of SHARPIN or HOIL-1 is seen in a diversity of human cancers and is correlated with the anti-apoptotic function of NF-κB activation [67,68]. Given the important role of ubiquitination in several processes one may wonder whether ubiquitination inhibitors can have a therapeutic window. However, confidence is gained from the fact that despite the important role of the proteasome in maintaining cellular homeostasis, proteasome inhibitors have already demonstrated clinical efficacy in the treatment of multiple myeloma and mantle cell lymphoma and are under evaluation for the treatment of other malignancies [69]. Bortezomib (Velcade®, Millenium Pharmaceuticals, Inc) is the only reversible 26S proteasome inhibitor that has been approved by the Federal Drug Administration (FDA) and the European Regulatory Agency (EMEA) for the treatment of multiple myeloma. Although bortezomib affects other signaling pathways, its efficacy may in part be due to inhibition of NF-κB activity. Interestingly, bortezomib has proven to be beneficial to reduce skin inflammation observed in *cpdm* mice by blocking NF-κB activation [70]. Nevertheless, hypereosinophilia remained and other organs were still affected. Unfortunately, the widespread clinical use of bortezomib continues to be hampered by the appearance of dose-limiting toxicities and drug-resistance. The pharmaceutical industry therefore makes great efforts to develop proteasome inhibitors that act through mechanisms distinct from that of bortezomib and several next generation proteasome inhibitors are in advanced clinical trials.

The identification of a central role for linear ubiquitination in NF-κB signaling offers additional opportunities for therapeutic exploitation and drug discovery. For example, targeting the binding between linear polyubiquitin and UBDs with peptidomimetics or small molecules may be an attractive approach. Proof-of-principle has been provided with ubistatins, which are small-

molecule inhibitors that were originally identified in a chemical genetic screen in Xenopus extracts to identify inhibitors of cell-cycle progression. Ubistatins were shown to bind the hydrophobic interface of Lys48-linked polyubiquitin and in this way prevent the recognition of ubiquitinated substrates by the UBDs of the ubiquitin receptor proteins RPN10 and RPN13 of the proteasome, thus preventing their degradation [71]. Due to their inability to pass the cell membrane, these components were unfortunately excluded for clinical use. Nevertheless, ubistatins reinforce the notion that it is indeed possible to disrupt protein–protein interactions between polyubiquitin chains and UBDs with small molecules. However, as mostly the same patches of the ubiquitin molecule are recognized by many different UBDs in many proteins, it will be necessary to unravel the specific binding characteristics of linear polyubiquitin-specific UBDs in more detail, so binding with other polyubiquitin linkage types is not affected. Another possibility to modulate linear ubiquitination might be to target the E3 ubiquitin ligase activity of LUBAC. MLN4924 (Millennium Pharmaceuticals, Inc.) was recently discovered as a small molecule inhibitor of NEDD8-activating enzyme and prevents the essential NEDD8 modification of the cullin subunit of cullin-containing RING-finger E3s, which represent a subclass of multisubunit E3 ligases that control the ubiquitination and turnover of many key substrates including the NF-κB inhibitor protein IκBα. MLN4924 is currently being tested in phase I clinical trials for the treatment of different types of cancer [72]. Although neddylation is not known to be required for LUBAC activity, these data illustrate the therapeutic potential of E3 ligase inhibition. cIAP1/2 are another type of E3 ligases that are actively targeted by a number of companies and several cIAP1/2 antagonists have entered clinical trials for cancer treatment [73]. Small-molecule antagonists that prevent the interaction between different LUBAC components or between LUBAC and other proteins such as E2s and specific substrates such as NEMO or RIP1 may be an alternative option. In this context, the potential of therapeutic targeting of the interaction between E3 ligases and a specific substrate has already been nicely illustrated with the development of cis-imidazoline derivatives (nicknamed "Nutlins"; Roche) that specifically disrupt protein–protein interactions between the E3 ubiquitin ligase HDM2 and the tumor suppressor p53. Therefore, it may be worthwhile to invest in defining the hotspots of interaction between different LUBAC components and other proteins. Clearly, more basic research on the biology of linear ubiquitination will undoubtedly direct the drug discovery efforts surrounding this target in the coming years.

8. Outlook

Although the use of cells deficient in one of the LUBAC components clearly illustrates the contribution of LUBAC and linear ubiquitin in NF-κB activation and in resistance towards TNF-induced apoptosis, further studies are required to unravel the role of this modification in other pathways or processes. As the existence of linear ubiquitination and LUBAC is a rather recent finding, still much has to be (re)investigated. Are NEMO, RIP1 and TRIM25 the only substrates for LUBAC in the NF-κB and IRF3 pathways, respectively? And what is the physiological relevance of the LUBAC-mediated proteasomal degradation of TAB2/TAB3? In addition, LUBAC is the only E3 ligase complex which can generate linear ubiquitin chains, detected so far. However, with the only recent notion of the existence of linear ubiquitination, other E3 ligases have to be reexamined for their ability to generate this ubiquitin linkage type. The identification of other E3s that generate linear ubiquitin chains would broaden the significance of linear ubiquitination beyond the NF-κB pathway. The same must be considered for the specificity of DUBs. Moreover, ubiquitin chain specificity of most of the DUBs has been tested *in vitro*, but linkage specificities may differ *in vivo*. For example, A20 shows DUB activity towards Lys48-linked polyubiquitin chains *in vitro*, but towards Lys63-linked polyubiquitin chains *in vivo* [74–77]. This is most probably due to secondary modifications or interacting proteins and it is not unlikely that similar mechanisms determine DUB specificity for linear ubiquitin chains. Also the function and mechanism of action of linear ubiquitin binding proteins such as ABINs, which are also recruited to the TNF receptor signaling complex, and Optineurin is still incompletely known. In addition, several other linear ubiquitin interacting proteins have recently been identified using protein arrays and some of these were already linked with NF-κB activation [78]. To fully understand the function of linear ubiquitination in NF-κB signaling and beyond, more detailed studies on the dynamics of ubiquitination and UBD-ubiquitin recognition, as well as on the effect of ubiquitin chain length, will be essential. It is not unlikely that a single protein is modified with different polyubiquitin chains dependent on its subcellular localization or presence in larger protein complexes. Also, different types of ubiquitination may function independently to direct the same protein in distinct signaling cascades. It is clear that we have only scratched the surface of the complex mechanisms that regulate NF-κB signaling in inflammation and immunity, and it can be expected that new and unexpected functions of linear ubiquitination will be revealed in the near future.

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