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REVIEW

"Without Ub I am nothing": NEMO as a multifunctional player in ubiquitin-mediated control of NF-κB activation

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Abstract Ubiquitination has emerged over the years as the most sophisticated way to modify proteins to affect their fate and function. In particular, it has been reported to be instrumental in regulating several steps of the NF-κB signalling pathway which controls inflammation, immunity, adhesion and cell survival. Integrating ubiquitination into NF-κB activation requires the regulatory subunit of IKK, NEMO, which not only displays affinity for polyubiquitin chains, but is also posttranslationally modified by a complex set of reactions involving ubiquitin. Here, we examine how studies of the NEMO/ubiquitin relationship have provided novel insights into the IKK activation process and have uncovered molecular mechanisms that should represent in the future attractive targets for specifically modulating NF-κB function.

Keywords NF-κB · NEMO · Ubiquitin · Signalling

Introduction

Posttranscriptional modification of proteins is crucial for their localization, trafficking, incorporation into higher scale complexes and activation, and for controlling their amount [1]. Until recently, phosphorylation by kinases was considered to be the main process responsible for all these events and a quite exhaustive listing of the components required (kinases, phosphatases), the mechanisms involved and the recognition codes utilized is now available [2]. Nevertheless, an even more complex kind of protein modification, ubiquitination, has recently found its way to the bench of most molecular biologists, leading to fresh ideas for tackling old issues in cell signalling and fuelling the imagination of scientists. This is especially true for a signalling pathway that was identified 25 years ago, the NF-κB pathway. In this review, we describe how ubiquitination has been shown to play an essential and still-expanding role in NF-κB activation, concentrating our discussion on how it influences the mechanism of action of NEMO, which critically controls this process.

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Ubiquitination: players and function

Ubiquitination is a reversible posttranslational modification of proteins, which consists of the covalent attachment of ubiquitin, a small conserved protein of 76 amino acids (8.5 kDa) [3, 4]. Three enzymatic steps are required to add one or several ubiquitins to substrates (Fig. 1). In the first, ubiquitin, which is encoded and expressed as multimeric head-to-tail repeats that are post-translationally cleaved into monomers, is activated by an ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. Then, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2), forming an E2-ubiquitin thioester. Finally, in the presence of a ubiquitin protein ligase (E3), ubiquitin is attached to the target protein through an isopeptide bond between the carboxy terminus of ubiquitin and the ε-amino group of a lysine residue in the target protein. There are only two E1s, tens of E2s and hundreds of E3s, these E3s conferring specificity to the process by recognizing the substrate to be ubiquitinated. Usually, a lysine residue is the ubiquitin acceptor, but other amino acids such as serine, threonine or cysteine are also able to attach ubiquitin.

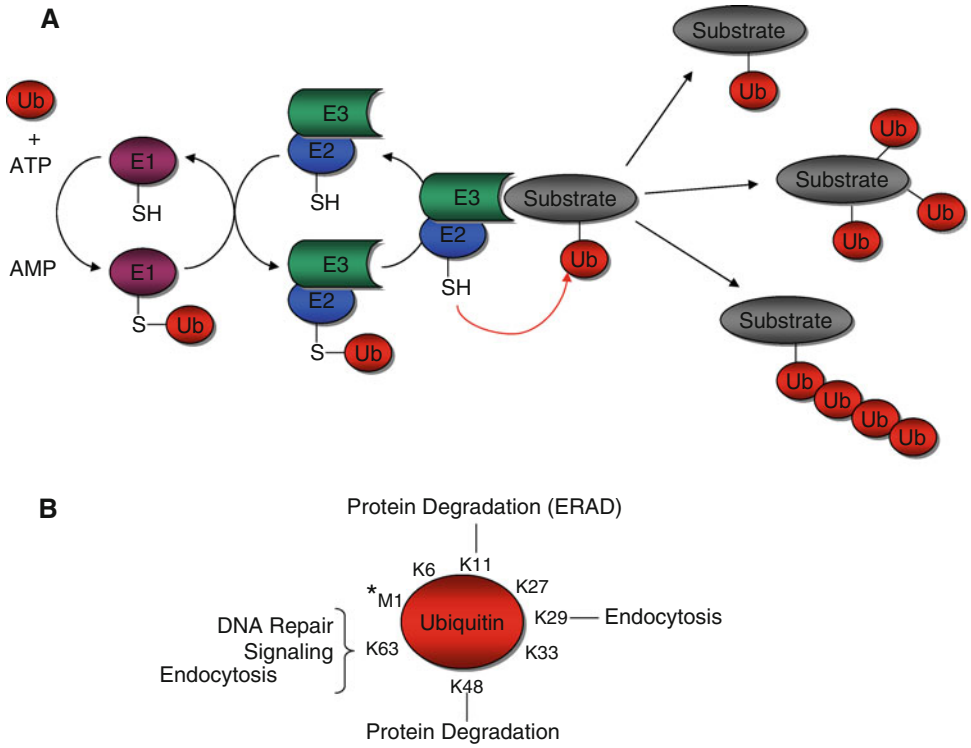

Fig. 1 **a** The ubiquitination process. Substrate modification by ubiquitin involves three distinct components and allows monoubiquitination, multi-monoubiquitination or polyubiquitination. *E1* ubiquitin-activating enzyme, *E2* ubiquitin-conjugating enzyme, *E3* ubiquitin ligase, *Ub* ubiquitin. **b** Ubiquitin linkages. Polyubiquitination is made possible because seven lysine of ubiquitin are available to create linkages with other ubiquitins. The specific biological function associated with each type of linkage is indicated. The *M1 label represents the amino terminus of ubiquitin which is used in case of linear polyubiquitination. *ERAD* endoplasmic reticulum-associated degradation

Ubiquitin-specific peptidases catalyse the reverse reaction, permitting protein deubiquitination and therefore recycling of ubiquitin. Approximately 100 enzymes exhibiting deubiquitinase activity have been identified so far in the human proteome [5].

In cells, proteins can be modified by monoubiquitin or by polyubiquitin chains. Ubiquitin possesses seven lysines (K6, K11, K27, K29, K33, K48 and K63) making possible this polyubiquitination process. The fate of ubiquitinated proteins depends on the linkage type and the length of the ubiquitin chain appended. Originally, K48-linked polyubiquitination was identified as a target for protein degradation by the proteasome 26S subunit, regulating protein levels in cells. In the NF-κB canonical pathway, for instance, the IκB inhibitor is modified with K48-linked chains, inducing its degradation by the proteasome (see below). Since this discovery, many nondegradative cellular processes have also been showed to be regulated by ubiquitination. This is the case for endocytosis, which is controlled by monoubiquitination and K29-linked polyubiquitination, and for activation of signalling pathways, DNA repair and protein trafficking, which can be regulated by K63-linked polyubiquitination [6]. Structural data have revealed significant topological differences between the chains formed, explaining their differential functions. K63-linked chains have an extended conformation, whereas K48-linked chains have a compact structure making them a privileged substrate for recognition by the proteasome [7].

In general, the polyubiquitinated chains are homotypic, but some heterotypic chains have been reported [8]. The synthesis of a specific type of polyubiquitinated chain depends on the E2 involved and the couple formed with the E3 partner. For instance, the E2 UBC13, which works in association with Uev1A, is specialized for K63 linkages [9], whereas other E2s, such as UbcH5, can generate various types of chains, sometimes mixed. As a result, a very large spectrum of modifications by ubiquitination can be achieved to variously modify the activity of proteins. To further complicate the picture, it has recently been reported that ubiquitin conjugation is not always required to affect the function of a protein since synthesized unanchored chains can also regulate signalling processes [10].

How does nondegradative ubiquitination influence the function of proteins? During cellular stimulation or adaptive gene regulation, signalling complexes are assembled in which induced ubiquitination often provides docking sites for proteins [11]. The family of proteins carrying ubiquitin-binding domains (UBDs) is large and its members generally bind ubiquitin with low affinity through noncovalent interactions [12, 13]. The ubiquitin-associated (UBA) domain was the first UBD identified. It is composed of three hydrophobic α-helices, recognizing a hydrophobic patch located around Ile 44 on ubiquitin [14]. The UBA domain has also been involved in protein–protein interaction. Currently, around 20 other UBDs are known,

recognizing various surfaces on ubiquitin, not just the patch surrounding Ile 44. More importantly, these UBDs have different affinities for polyubiquitin chain types. For instance, Rpn13, which is an integral component of the 26S proteasome, specifically recognizes proteins modified with K48-linked chains. In contrast, RAP80 targets BRCA1 to foci induced by DNA damage through its two UBDs, which bind K63-linked chains but not K48-linked chains [15].

Very recently, another type of polyubiquitin linkage has been discovered. Ubiquitin can also be attached through its carboxy-terminal glycine to the extreme amino terminus of another ubiquitin, generating head-to-tail linked linear ubiquitin chains [16]. Contrary to usual polyubiquitin chains, linear chains are assembled by a unique E3 complex called linear ubiquitin chain assembly complex (LUBAC) which is composed of two E3 ligases, HOIL-1 and HOIP, with molecular weights of 58 and 120 kDa, respectively (Fig. 2). Both proteins possess multiple domains. The UBA domain of HOIP and the ubiquitin-like (UBL) domain of HOIL-1 are responsible for LUBAC formation, indicating that the UBA domain of HOIP performs a function other than ubiquitin binding. Instead, LUBAC is able to bind ubiquitin via the N-terminal parts of HOIP and HOIL-1 containing the UBL and zinc finger/novel zinc finger (ZF/NZF) domains. The UBL domain of HOIL-1 binds very weakly to the linear chains whereas the HOIP ZF/NZF domains preferentially bind to the K63-linked rather than the linear or K48-linked polyubiquitin chains [17]. In addition, both proteins exhibit a RING in-between-ring RING (RING-IBR-RING) domain with E3 activity, but only the one from HOIP is necessary for the linear polyubiquitination activity of LUBAC. However, linear chain formation is only possible if the two proteins are in a complex.

LUBAC can generate linear polyubiquitin chains with several E2s, including UBCH5s, E2-25K, UBCH17, but is unable to extend chains if ubiquitin is tagged at the N-terminus, confirming specificity in linear linkage [16]. The exact role of linear chains is not fully understood, but they could play role as scaffolding in signal transduction events (see below). In addition, LUBAC may also regulate the degradation of proteins. For instance, it binds and promotes degradation of PKCs [18] and its association with SOCS6 induces stabilization of this protein by degradation of its partners [19].

NEMO in NF-κB signalling

NF-κB transcription factors, a family of dimeric proteins formed by associations between p50, p52, relA, relB and c-rel subunits, are key regulators of inflammation, immunity, adhesion and cell survival. In resting cells, they are kept inactive in the cytoplasm through interaction with inhibitory proteins of the IκB family (IκBα, IκBβ and IκBε) [20, 21]. Following activation, IκBs are phosphorylated at conserved serine residues and this phosphorylation induces their ubiquitination and degradation by the proteasome. As a consequence, released NF-κB proteins translocate to the nuclear compartment where they regulate the activation of a large collection of target genes.

In most situations, NF-κB activation through phosphorylation of IκBs involves a kinase complex, IKK, whose core components are IKK-1/IKKα, IKK-2/IKKβ and NEMO/IKKγ [22]. IKK-1 and IKK-2 are related kinases which phosphorylate IκBs or p105, the precursor of p50. In contrast, NEMO is a regulatory subunit which does not exhibit any catalytic activity. It is a dimeric protein of approximately 50 kDa with a very elongated structure, mostly due to a succession of helical domains (Fig. 3). The first, at the N-terminus, contains both an interface for dimerization and the sequence which recognizes the catalytic subunits of IKK through their C-terminal sequences (NEMO-binding peptides, NBPs). The second is a long coiled-coil (coiled-coil 1, CC1) which extends over approximately 100 amino acids. Located upstream of another coiled-coiled (coiled-coil 2, CC2) is a pseudohelical domain which can become structured upon interaction with a NEMO regulator, such as v-FLIP [23]. Finally, on the C-terminus side of CC2 is a leucine zipper (LZ). In addition to these helical domains, NEMO also contains a

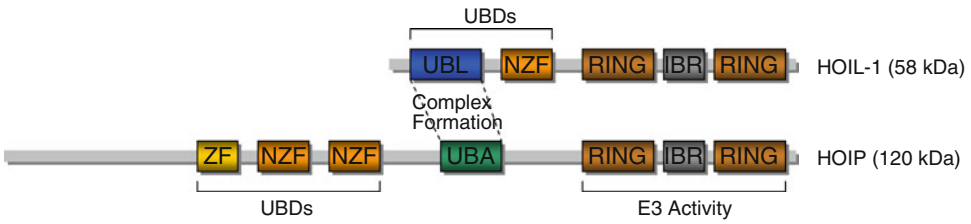

Fig. 2 Structure of LUBAC components. The various structural domains of HOIL-1 and HOIP are presented with their associated functions. The ubiquitin-like (*UBL*) and ubiquitin-associated (*UBA*) domains are responsible for complex formation. The zinc finger (*ZF*) and novel zinc finger (*NZF*) domains are involved in ubiquitin binding. The E3 catalytic activity of LUBAC is mostly dependent on the RING-IBR-RING domain of HOIP

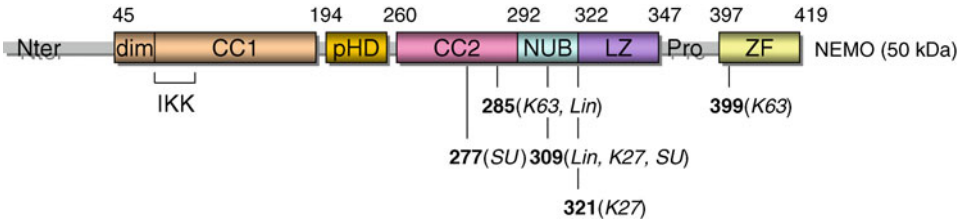

Fig. 3 Structure of NEMO showing the different structural domains. *Nter* amino terminus, *dim* dimerization domain, *CC1* coiled-coil 1, *CC2* coiled-coil 2, *pHD* pseudohelical domain, *NUB* NEMO ubiquitin binding, *LZ* leucine zipper, *Pro* proline-rich region, *ZF* zinc finger. Also indicated is the region binding the catalytic subunits of IKK and the lysine residues that are modified upon cell stimulation (see text for more detail). *Lin* linear ubiquitination, *SU* sumoylation

conserved motif between the CC2 and the LZ (described in detail in the next section), a proline-rich region after the LZ, which is thought to be unstructured, and a ZF at the C-terminus.

A remarkable feature of IKK is its ability to be activated by a wide range of stimuli. Among them are proinflammatory cytokines, such as IL-1β and TNF-α, antigens, bacterial products such as LPS and MDP, virus-derived components such as dsRNA or Tax, and various forms of stress. All these stimuli are dependent upon NEMO to trigger IKK activation and IKK-2 acts as the principal kinase phosphorylating IκB and p105 (canonical pathway) [24]. A subset of stimuli, such as those involved in lymphocyte B organogenesis, specifically target IKK-1 through kinase NIK, but do not require either NEMO or IKK-2 (noncanonical pathway) [25]. In this case, the precursor molecule of p52, p100, is the substrate of IKK-1. Because the NF-κB species activated by the canonical and noncanonical pathways are not identical, the genes that are targeted are distinct and their protein products perform specific functions.

Several signalling pathways of the canonical NF-κB activation process have been characterized in detail, allowing the identification of shared participants and mechanisms during signal transmission to IKK. In particular, a kinase complex composed of TAK1 and its associated subunits TAB1, TAB2 and TAB3, is suspected to play the role of a kinase acting upstream of IKK and to target two phosphoacceptor sites located in the kinase domain of IKK-1 and IKK-2, Ser 176/180 and Ser 177/181, respectively [26]. Moreover, various members of the E3 ligase family are frequently found participating in ubiquitination processes not linked to protein degradation. Among them are members of the TRAF and Pellino families, c-IAPs, and the very recently characterized LUBAC complex.

One of the best-characterized pathways is the TNF-R1 signalling pathway which is activated by TNF-α [17, 27]. Following ligand binding, a recruitment of several proteins to the intracytoplasmic domain of TNF-R1 occurs (Fig. 4). Among them are adaptor TRADD, E3 ligases TRAF2 and cIAPs, and kinases RIP, TAK and IKK. Assembly of this macromolecular signalling complex results in K63-linked ubiquitination of RIP, which is dependent upon TRAF2 and cIAP, the latter E3 ligase being the one that modifies RIP. Ubiquitinated RIP is then thought to attract the TAK complex through the specific affinity of TAB2 and TAB3 for K63-linked chains. Interestingly, LUBAC is also recruited in a cIAP-dependent manner to TNF-R1 upon TNF-α exposure, and plays an essential but still incompletely defined role in the final activation of IKK (this is discussed in the next section).

Similar components, acting quite distinctly, participate in activation of NF-κB by IL-1β and LPS [28] (Fig. 5). Both ligands are recognized by receptors of the same family, which first recruit adaptor MyD88. Binding of kinases IRAK4 and IRAK1 and E3 ligase TRAF6 to MyD88 results in IRAK1 phosphorylation, release of IRAK1/TRAF6 and TRAF6 autoubiquitination. Ubiquitinated TRAF6 then recruits the TAK complex and modifies with K63-linked chains, in concert with Ubc13, both TAK1 and IRAK1. Alternatively, Pellino may be the actual E3 ligase responsible for IRAK1 ubiquitination. Whichever is the case, the final stage of the process involves the recruitment of IKK through NEMO (see more details below) and its phosphorylation/activation by TAK1. In this sequence of events, MEKK3 has recently been introduced as the kinase participating in TAK1 activation. There is also a second wave of MyD88-dependent TAK-independent IKK activation. It require both TRAF6 and MEKK3 but remains incompletely characterized.

The NEMO/ubiquitin connection

NEMO as a ubiquitin binding protein

Wu et al. [29] were the first to report that NEMO exhibits affinity for ubiquitin, based on the isolation of tandem repeats of two or three ubiquitin molecules following a two-hybrid screening using NEMO as bait. Importantly, the domain involved has been shown to include a conserved

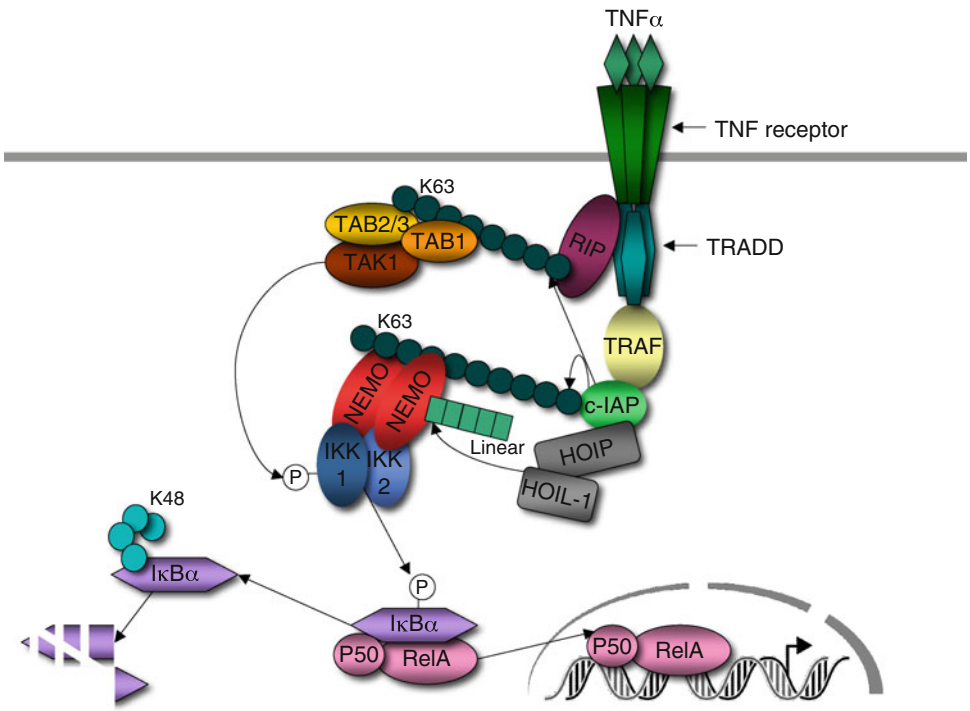

Fig. 4 TNF-R1 signalling pathway. The major proteins involved in NF-κB activation by TNF-α are shown together with their modifications by polyubiquitination (K48-linked, K63-linked and linear) or phosphorylation (see text for more details). *P* phosphate

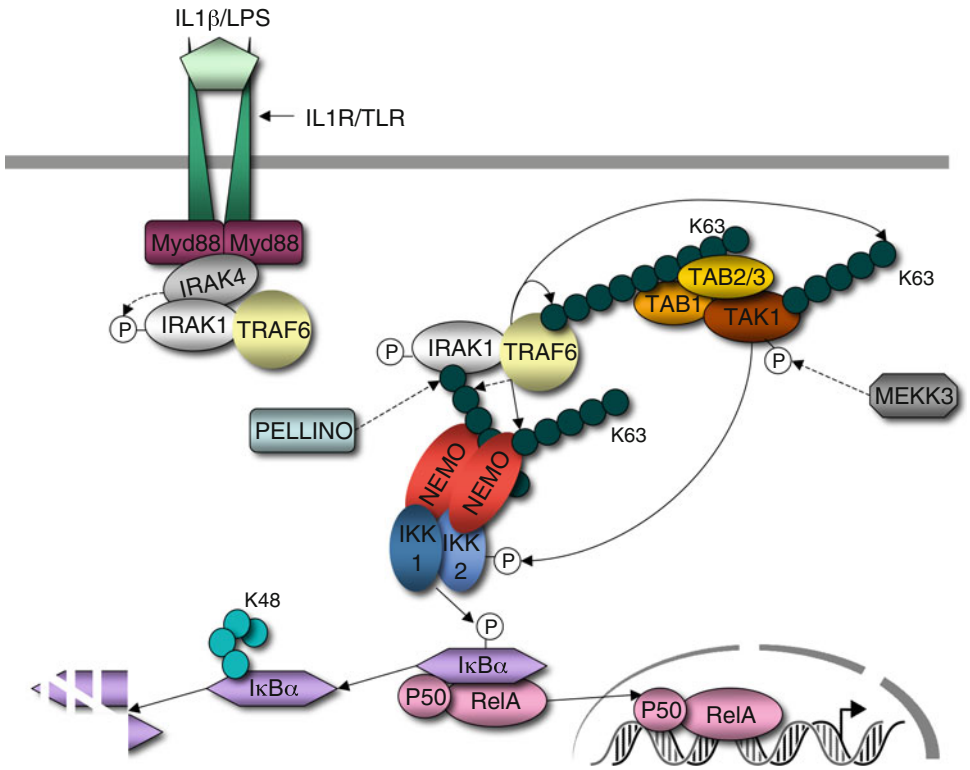

Fig. 5 IL1-R/TLR4 signalling pathway. The major proteins involved in NF-κB activation by IL-1β and LPS are shown together with their modifications by polyubiquitination (K48- and K63-linked) or phosphorylation (see text for more details). *P* phosphate

sequence whose associated function had previously remained unknown for years (Fig. 6). This UBD, called NUB (NEMO ubiquitin) domain, UBAN (ubiquitin binding in ABIN and NEMO), CoZi (CC2 and LZ) or NOA (NEMO, optineurin, ABIN) is also present in four other proteins, ABIN-1, ABIN-2, ABIN-3 and optineurin, whose function is less well understood than that of NEMO. ABIN proteins have been suggested to be negative regulators of the NF-κB signalling pathway [30], whereas optineurin is a Golgi-associated NEMO homologue that plays a role in

negative regulation of NF-κB activation by TNF-α [31] and of IRF signalling through its interaction with TBK1 [32].

In the case of NEMO, the NUB domain encompasses part of CC2, the conserved sequence described above, and part of LZ, whereas in the case of ABINs it is restricted to the conserved motif described above. Mutations at Y308S, F312A and L329P, all impair ubiquitin binding [28, 33]. More importantly, mutations of NEMO causing an inherited disease called anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) [34] (Fig. 7), such as D311N, are also defective in ubiquitin binding, further supporting the view that NEMO binding to ubiquitin is physiologically relevant.

The first studies investigating the NUB domain have indicated that it preferentially recognizes K63-linked polyubiquitinated chains, providing a way to recruit IKK and help its activation in several distinct signalling pathways [28, 33]. In the TNF-R1 signalling pathway, the polyubiquitinated molecule bound by NEMO is RIP which is ubiquitinated by c-IAPs [35] (see above), whereas in the TCR/BCR pathway it is MALT-1 or Bcl10, which can both be modified by K63-linked polyubiquitin chains [36, 37] (see below). In the IL-1R/TLR4 pathways, the same process occurs, but there is still some uncertainty regarding the ubiquitinated protein which recruits IKK through NEMO. TRAF6, which represents an obvious candidate because of its well-known autoubiquitination following cell stimulation, may actually act through IRAK1 [38]. This mode of action takes into account the observation that E3 ligase activity, but not autoubiquitination, of TRAF6, is required for NF-κB activation [39], but is difficult to reconcile this with the supposed role of Pellino as an E3 ligase modifying IRAK through K63-linked polyubiquitination [40]. Finally, ubiquitination of Nod2 and RICK has also been reported upon MDP exposure, providing a putative mechanism for recruiting and activating IKK through NEMO.

In all these pathways, similar mutations within the NUB domain, for instance those causing EDA-ID in humans, impair the binding of NEMO to its ubiquitinated partners and the subsequent activation of IKK/NF-κB. This demonstrates the pleiotropic function of this domain and provides a likely explanation to the longstanding issue regarding how the regulatory subunit of IKK may integrate so many seemingly disparate signalling inputs.

This most satisfying unified model based on K63-linked chain recognition by NEMO has nevertheless been challenged by the findings of very recent studies aimed at defining, using X-ray and NMR methods, the structure of the NUB domain alone or associated with ubiquitin moieties, usually present as dimers. Such structural studies have shown that the region encompassing amino acids 265–330 forms a parallel intermolecular coiled coil, with a kink at residue Pro299, which marks the boundary between the CC2 and NUB/LZ domains. Using a combination of NEMO mutagenesis and NMR analysis, Lo et al. [41] have proposed structures in which diubiquitin is positioned perpendicular to the coiled-coil, with each ubiquitin contacting both chains of a NEMO dimer. The hydrophobic patch located around Ile44 of ubiquitin is involved in the binding, and each ubiquitin interacts asymmetrically with NEMO. In this study, a comparative analysis of NEMO binding to linear versus K63-linked diubiquitin was performed and indicated a much higher affinity for the linear linkage (dissociation constants of 1.4 vs. 131 mM,

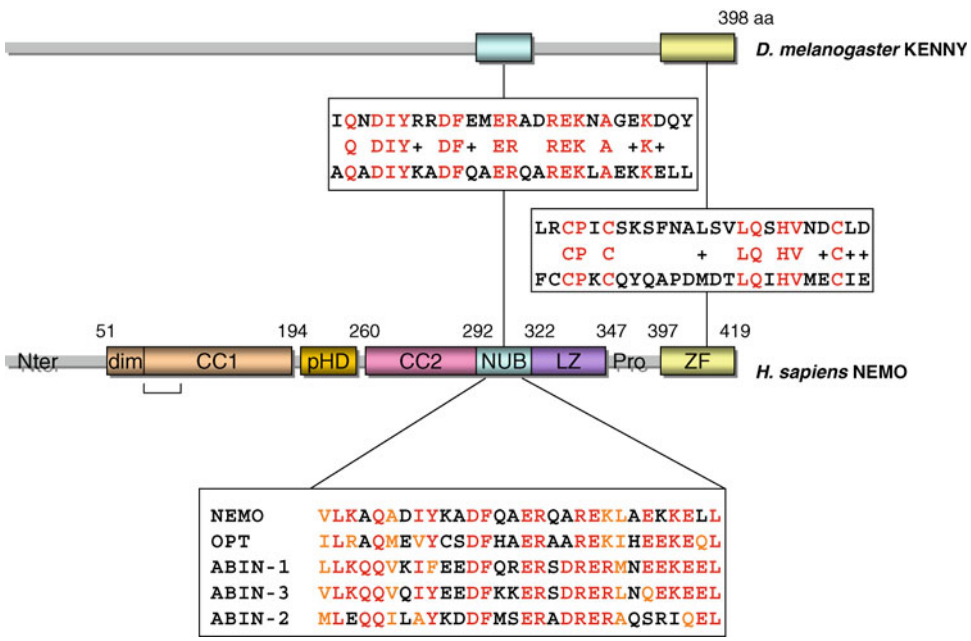

Fig. 6 UBDs of NEMO. *Top* Sequence comparison between *Drosophila melanogaster* KENNY and human NEMO. *Bottom* NUB domain sequences. Identical amino acids are indicated in *red* and conserved ones in *orange*

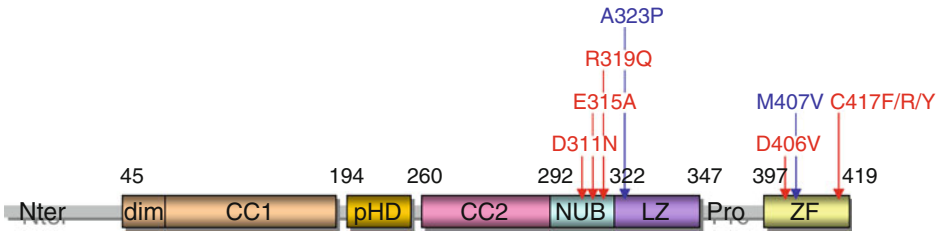

Fig. 7 Pathology-associated missense mutations of NEMO located within the UBDs. Mutations causing incontinentia pigmenti are in *blue* and those causing EDA-ID in *red*. In four instances (D311N, E315A, A323P, M407V), a formal demonstration of an impaired interaction with polyubiquitin has been provided. Short deletions of the NEMO ZF have also been found in patients with incontinentia pigmenti and EDA-ID, and are likely to result in defective ubiquitin binding but are not shown (for review see reference [74])

respectively). This difference might be explained by the presence of interubiquitin interactions in the case of linear diubiquitin, the distal ubiquitin contributing less efficiently to the overall interaction in K63-linked diubiquitin.

This unexpected observation of a higher affinity of the NUB domain for linear diubiquitin has been confirmed by the study of Rahighi et al. [42] who analysed the crystal structure of the 250–339 region of murine NEMO with linear diubiquitin. Although, the present authors disagree with Lo et al. regarding both the stoichiometry of the complex and the exact mode of recognition, they have also observed a much higher affinity of the fragment analysed for linear diubiquitin. More specifically, their crystal lattice revealed that a CC2-LZ dimer binds two diubiquitin chains in a parallel fashion (stoichiometry 1:1), while the NMR model of Lo et al. showed that one diubiquitin chain wraps around the dimeric NUB domain (stoichiometry 2:1). Nevertheless, the interaction with linear diubiquitin also appears almost exclusive, as compared to the interaction with K63- or K48-linked diubiquitin. The second ubiquitin binding site for linear diubiquitin binding revealed by Rahighi et al. does not use the canonical hydrophobic patch of ubiquitin surrounding I44, as does the first one, and mostly binds the proximal ubiquitin through R309, R312 and E313 (R316, R319 and E320 in humans). Supporting the idea that this interaction is physiologically relevant, a series of NEMO mutations affecting residues specifically interacting with linear diubiquitin affect NF-κB activation by TNF-α. For instance, V293A/Y301A/K302A mutations, which impair interaction of NEMO with the distal ubiquitin of the ubiquitin dimer, and R309A/R312A/E313A mutations, which impair interaction with the proximal one, are strongly defective in NF-κB activation.

Recently, the crystal structure of the 249–343 region of murine NEMO complexed with a K63-linked diubiquitin chain has been reported [43]. In this case, the binding of two distinct dimers to only one K63-linked diubiquitin chain is observed. This structure confirms that the second ubiquitin binding site described above is not used for K63-linked diubiquitin binding. Nevertheless, the existence in solution of a complex with a 1:4 stoichiometry remains to be confirmed.

Finally, another model has been proposed based on NMR and mutagenesis, whereby a dimeric NEMO molecule binds two K63-linked chains which run parallel to the NUB domain [44]. In this study, the coupling between NEMO dimerization and ubiquitin binding was more particularly examined since the CC2-NUB-LZ region is also a major participant in dimerization. This is an important issue to consider when mutation of residues is used to confirm or invalidate models of NUB/ubiquitin interaction in the context of full-length NEMO.

In addition to the relatively well-characterized NUB domain, another functionally important part of the NEMO protein, the ZF, has been reported to exhibit affinity for ubiquitin [45]. This suggests the existence of a dual mode of recognition of ubiquitin by NEMO. Its functional importance is supported by the observation that the NUB domain and the ZF are the only two conserved homology blocks detectable when comparing the hNEMO and dNEMO (KENNY) sequences, which diverged more than 800 millions years ago (Fig. 6). Cordier et al. [45] have shown that the NEMO ZF exhibits a large hydrophobic cluster sharing similarities with the ubiquitin-interacting region of the ubiquitin-binding ZF (UBZ) of the human DNA Y-polymerase (pol) η. In isolation, the NEMO ZF interacts with ubiquitin through the hydrophobic pocket located around Ile 44, and an I44A mutation abolishes the interaction. An α-helix on the NEMO side appears to be involved, suggesting a mode of recognition already observed in several other UBDs, as explained above. Importantly, several NEMO mutations causing the inherited diseases incontinentia pigmenti and EDA-ID [46], among them M407V (Fig. 7), exhibit defective ubiquitin binding but no loss of ZF structure, this latter property followed by measuring fluorescence quantum yields in the presence of zinc. This provides a likely molecular explanation for their impaired function.

The presence of two ubiquitin-binding sequences in NEMO is likely to have an important impact on the specific

kind of ubiquitin chains that are actually recognized in vivo by the full-length molecule. Indeed, whereas the isolated NUB domain displays a 100-fold higher affinity for linear chains (see above) and the ZF no specific preference, the whole carboxy-terminus of NEMO containing both domains (amino acids 300–412) interacts 6- to 20-fold more strongly with the K63-linked chains [47], most likely because of cooperative binding and the possibility of recognizing longer multimers. Interestingly, the Pro-rich region located between the NUB domain and the ZF of NEMO can be shortened quite extensively without displaying an obvious deleterious effect on ubiquitin binding. This indicates that this sequence only plays a linker function within a bipartite UBD. There is also a ZF with affinity for ubiquitin in optineurin, which can be functionally exchanged with the one from NEMO, and, more strikingly, in ABIN-2, which is clearly a distinct protein. This emphasizes the high level of similarity regarding ubiquitin recognition by NEMO and ABIN-2, which extends well beyond a single NUB domain. In contrast, the same type of organization apparently does not apply to ABIN-1 or ABIN-3.

In conclusion, despite the accumulation of data summarized above, it is fair to conclude at this stage that, whereas compelling evidence supports the view that NEMO functions as a new member of the family of ubiquitin binding proteins, the specific type of polyubiquitin chains that it recognizes upon cell stimulation still remains uncertain.

NEMO modification through ubiquitination

K63-linked polyubiquitination of NEMO

In two distinct signalling pathways, NEMO is thought to be modified by K63-linked polyubiquitination. The first is the T-cell receptor (TCR) pathway which plays a crucial role in lymphocyte proliferation following antigen exposure. It controls NF-κB activation through a multiprotein complex (CBM complex) composed of CARMA1, Bcl10 and MALT1. The CBM complex is itself activated by PKCθ which, by phosphorylating the linker region of CARMA1, induces a conformational change of this scaffold protein and promotes interaction with Bcl10 and MALT-1. NEMO can be polyubiquitinated upon Bcl10 overexpression and the chains formed are K63-linked [48]. Although the exact identity of the E3 involved remains controversial, TRAF6 represents a likely candidate [49]. One major site of polyubiquitination appears to be K399, located in the NEMO ZF. Nevertheless, mutation of this single residue reduces NF-κB activation following TCR ligation by only approximately 50% in NEMO(−) complemented Jurkat cells, and mice expressing this mutation do not exhibit any defect in T-cell signalling [50]. This suggests that K399 may not be the only modified residue upon antigen exposure.

Polyubiquitination of NEMO has also been reported to occur following Nod2 overexpression [51]. Nod2 is a cytoplasmic sensor of the NLR family which recognizes gram-positive-derived peptidoglycan and plays a pivotal role in innate immunity. It activates NF-κB using RICK and an E3 ligase which has been suggested to be either TRAF6 or TRAF2, depending on the study [52]. Cotransfection of HEK293T cells with Nod2 and various forms of NEMO have indicated a requirement for lysine 285 and 399 in K63-linked polyubiquitination. Complementation of *Nemo*(−) MEFs with K285/399R NEMO does not restore NF-κB activation by MDP, the ligand of Nod2, demonstrating the role of NEMO ubiquitination in NF-κB signalling following detection of bacterial products in the cytosol.

The findings of other studies support the view that modification of NEMO with K63-linked polyubiquitin chains is important for IKK/NF-κB activation. The molecular characterization of a NEMO mutation (A323P) causing incontinentia pigmenti has shown that several lysines located around A323, among them K285, are targeted by TRAF6 upon IL-1 and LPS stimulation [53]. Moreover, the study discussed above using mutated mice [50], although not revealing any obvious defect in TCR signalling, demonstrated an important function for residue K399 in TLR signalling.

All these studies suggest that several distinct signalling pathways which apparently are TRAF6-dependent, at least to some extent, target similar or identical residues on NEMO. Unfortunately, because the acceptor sites for ubiquitination are not always neutralized all at once the various studies reported so far do not often provide a complete view of the phenotype generated by impaired NEMO ubiquitination and do not allow a meaningful comparison between the signalling pathways. Clearly, an identification of the sites of ubiquitination on endogenous NEMO using mass spectrometry is required to get a consistent picture.

Linear polyubiquitination of NEMO

As discussed above, linear ubiquitination is also thought to occur in cells, and involves the LUBAC complex composed of HOIP and HOIL-1L. Tokunaga et al. [54] have made the seminal observation that overexpression of LUBAC activates NF-κB. This effect may be explained, at least in part, by what is described above in the TNFR signalling section. More interestingly, they have shown that NEMO is a direct target for LUBAC-dependent linear polyubiquitination. Indeed, HOIP and HOIL-1L bind to

NEMO, with a stronger affinity for HOIP, through the ZF/NZF domains of HOIP and HOIL-IL and the CC2/LZ portion of NEMO. In vitro or following its overexpression in cells, LUBAC is able to join linear chains of ubiquitin to NEMO, and K285 and K309 represent the targeted residues. Importantly, the process does not involve Ubc13, which is the E2 conjugating enzyme required for K63-linked chain synthesis. It remains to be elucidated what may be the exact contribution of NEMO linear polyubiquitination to pathways other than the TNFR pathway, especially the ones involving TRAF6 (see above), and how modifications by linear and K63-linked polyubiquitinations, respectively, control NEMO function, especially considering the fact that the same K285 residue is involved in both processes.

Pathogen-induced degradation of NEMO through ubiquitination

Cytoplasm-invading bacteria, such as *Shigella*, release components inducing an inflammatory response during multiplication. To fight inflammation, these bacteria express highly efficient effectors that usually down-modulate key signalling molecules involved in host defence [55]. Among these effectors, which are delivered into the host cell cytoplasm and nucleus through a type III secretion system [56], are a class of proteins belonging to the IpaH family. Recently, IpaH proteins have been shown to exhibit E3 ligase activity [57], suggesting that they may control cellular processes through ubiquitination. Nevertheless, the identity of the targeted protein(s) remains unclear.

Ashida et al. [58] have demonstrated that *Shigella*-derived IpaH9.8 is an inhibitor of NF-κB activity and blocks IκBα degradation. In contrast, it is unable to inhibit MAPK signalling. Importantly, NEMO and ABIN-1 were identified as IpaH9.8 binding partners during a two-hybrid screening. In the case of NEMO, amino acids responsible for the interaction are located between residues 347 and 396, whereas residues 351–367 of ABIN-1 are required. In an in vitro assay and following cotransfection, IpaH9.8 appeared able to ubiquitinate NEMO. This ubiquitination involves residue K27 of ubiquitin and leads to NEMO degradation by the proteasome. Two lysine residues (K309 and K321) are targeted by IpaH9.8 and their mutation results in protection against IpaH9.8-induced degradation.

In addition to the non-orthodox type of chains used to modify NEMO, participation of ABIN-1 in the ubiquitination process has also been observed. Down-modulating ABIN-1 expression through the use of siRNAs reduces IpaH9.8-induced NEMO degradation. Moreover, NEMO ubiquitination by IpaH9.8 is increased in vitro upon addition of ABIN-1. Therefore, ABIN-1 appears to act as an adaptor for IpaH9.8/NEMO interaction. Since a version of ABIN-1 mutated in its NUB domain is less effective in promoting NEMO degradation by IpaH9.8 than wt ABIN-1, one may imagine that upon *Shigella* infection, Nod-1 recognizes bacterial peptidoglycans and induces NEMO ubiquitination through K63-linked chains (proinflammatory process). At the same time, ubiquitinated NEMO would also attract ABIN-1, favouring its interaction with IpaH9.8 and its subsequent degradation (antiinflammatory process) (Fig. 8).

So far, this study is the only one that has demonstrated regulation of NEMO function through ubiquitination-induced degradation. It will now be interesting to determine whether other members of the IpaH family in other bacterial species also target NEMO and whether NEMO represents a frequent target to shut off inflammation following microbial infection.

Coupling of NEMO ubiquitination to sumoylation in the response to DNA damage

Exposure of DNA to damaging agents is a trigger for NF-κB activation, and this process has been reported to involve a complicated sequence of events requiring a pool of free NEMO protein shuttling between the cytoplasm and the nucleus [59]. It has been demonstrated that sumoylation of NEMO plays a critical role in this translocation event. Although describing this process in detail here is beyond the scope of this review, it is worth mentioning that, following sumoylation, a monoubiquitination event takes place in the nucleus [60]. Although the machinery involved remains undefined, the function of this modification appears to be linked to the export of NEMO from the nucleus, allowing IKK activation. Interestingly, the

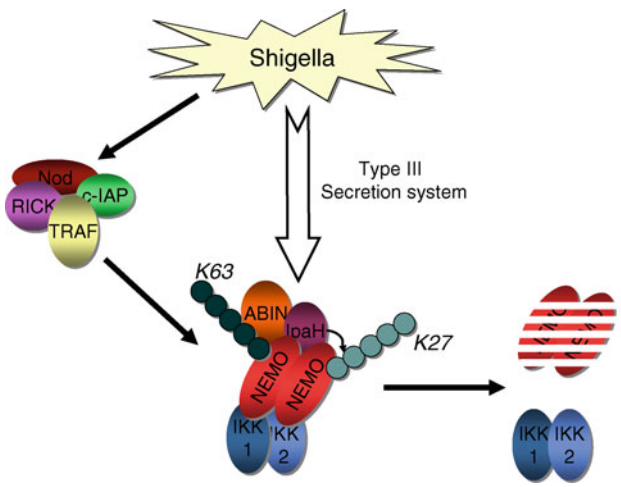

Fig. 8 Inhibition of IKK activation by *Shigella* effector IpaH9.8. *Shigella* infection detected by Nod1 activates IKK but, at the same time, synthesis of microbial IpaH9.8 shuts off IKK activity by inducing NEMO degradation (see text for more details)

modified lysine residues have been identified as K277 and K309, which are also the sites for NEMO sumoylation. This suggests the existence of a coupling between the sumoylation and ubiquitination processes, something reminiscent of what has been previously shown to occur in the nucleus, for instance concerning the proliferating cell nuclear antigen [61].

Negative regulation of NEMO ubiquitination

NF-κB activation needs to be tightly regulated to avoid any deleterious effect, such as excessive inflammation, autoimmunity and carcinogenesis. Several modes of negative regulation participating in IKK shut-off have been reported, among them deubiquitination. Using a two-hybrid screening with NEMO as bait, two laboratories have identified the protein CYLD [62, 63]. This tumour suppressor was originally discovered as mutated in cylindromatosis, a genetic disease characterized by tumours developing during adulthood and exclusively derived from skin adnexae [64]. Importantly, CYLD is a member of the deubiquitinase family which exclusively targets substrates modified with K63-linked, or possibly linear chains, but not K48-linked chains. This restricted specificity has been explained after analysing the structure of the CYLD catalytic domain [65]. Compared to other ubiquitin-specific processing proteases, which cleave K48-linked chains, the domain that binds the proximal ubiquitin exhibits very little amino acid conservation. Instead, a unique extended β12/β13 loop provides the cleavage specificity for K63-linked chains.

CYLD overexpression has been shown to inhibit NF-κB activation by TNF-α or IL-1β. Conversely, *CYLD* expression knocked down with RNA interference in cells [63] and *Cyld* invalidation in the mouse [66] results in a quantitative increase in NF-κB activation, without affecting the overall kinetics of the process. How CYLD exactly controls the IKK activation step nevertheless remains incompletely defined, since it can downregulate the activity of several components of NF-κB signalling in addition to NEMO, and among these are TRAF2, TRAF6, RIP, TAK1 and RIG-1 [66].

How the catalytic activity of CYLD is itself regulated is also poorly understood, but may involve phosphorylation [67]. Indeed, CYLD is phosphorylated upon cell stimulation by TNF-α or antigen exposure and mutation of a serine cluster located between amino acids 418 and 444 results in decreased TRAF2 ubiquitination and NF-κB activation. Since IKK has been suggested to be a kinase for CYLD, this phosphorylation event may represent a negative regulatory mechanism. It is worth noting that Ser 418 of CYLD has recently been shown also to be targeted by IKKε, an IKK-related kinase [68]. Again, phosphorylation of CYLD at serine 418 decreases its deubiquitinase activity and appears necessary for IKKε-driven transformation.

Another important negative regulator of NF-κB signalling is A20 which has been shown to play a critical role in restraining inflammation [69]. A20 is a member of the otubain family of deubiquitinases exhibiting dual enzymatic activity. Indeed, it is not only able to deubiquitinate substrates modified by K63-linked polyubiquitins but can also induce their K48-linked polyubiquitination and degradation through its E3 ligase activity. One of the main targets of A20, acting within the so-called A20 ubiquitin-editing complex [70], has been shown to be ubiquitinated RIP which is first deubiquitinated then degraded by A20. This allows the shut-off of NF-κB activation in response to TNF-α. A20 can also affect NEMO ubiquitination via ABIN-1 [71]. In this case, ABIN-1, which is an A20-binding protein, recognizes NEMO via a bipartite mechanism. Its NUB domain interacts with the polyubiquitinated chains of NEMO and its NEMO-binding domain directly contacts NEMO.

It remains unclear how CYLD and A20 both control the ubiquitinated status of NEMO. A plausible explanation is that CYLD acts primarily to limit the extent of NEMO ubiquitination, keeping in check the level of IKK activation, whereas A20, which is a well-known transcriptional target of NF-κB, acts secondarily to shut off IKK activity. Indeed, in contrast to what is observed with Cyld(−) MEFs (see above), A20(−) MEFs do not show increased levels of IKK activation when treated with TNF-α or IL-1β, but display a long-lasting IKK activity.

Concluding remarks

There is no doubt now that the regulatory function of NEMO within the IKK complex is heavily associated with ubiquitination. Indeed, on the one hand, NEMO displays affinity for polyubiquitin chains through a new dual mode of recognition, and, on the other hand, its activity is itself regulated by ubiquitination. This ubiquitin addiction nevertheless remains incompletely understood, especially regarding its exact relationship to the IKK activation process.

Binding of NEMO to ubiquitin represents an obvious way to attract the IKK complex to upstream activators. It has been proposed, for instance, that NEMO binding to ubiquitinated RIP would permit colocalization of IKK with the TAK complex, which also exhibits affinity for K63-linked chains. This induced promiscuity would result in phosphorylation of IKK by TAK1. Variations around this theme are, of course, possible since it has recently been reported that TAK1 is itself ubiquitinated. In other pathways, ubiquitinated TRAF6, IRAK, Bcl10, MALT1, Nods and RICK

may also be attractors for IKK activation by TAK1. Alternatively, ubiquitinated NEMO might be recognized by the TAK complex, allowing IKK activation. Sorting out these sequences of events will be a challenging task since most of the molecules described above are both ubiquitin-binding proteins and templates for ubiquitination. Unfortunately, the identification of lysine residues that are required for ubiquitination and the nature of the appended chains, using mass spectrometry, remains difficult. Moreover, mutagenesis of these residues, which are rarely unique, complicates the analysis since it can also affect the structure of the proteins. Nevertheless, progress has been made recently concerning the identification in vivo of the type of polyubiquitin chains formed. Indeed, monoclonal antibodies specifically recognizing the ubiquitin linkages, especially the K63-linked one, have been engineered [72, 73].

The participation of linear ubiquitination in NF-κB activation also complicates the picture described above, especially regarding the precise role of such modification in NEMO function. The discovery of ubiquitination by linear chains will require a re-evaluation of numerous published studies, which were based on protein overexpression and, more problematically, on the use of HA-ubiquitin which is not recognized by LUBAC. As mentioned above, antibodies specific for K63-linked chains are available. Since antibodies specific for linear polyubiquitinated chains have also been produced [54], it should now be possible to make substantial progress on the following important issues. First, are NEMO partners also targets for linear ubiquitination by LUBAC? Although LUBAC has recently been identified as an integral component of the TNF-R1 signalling pathway [17], it remains uncertain whether it participates in other NF-κB-activating pathways as well. In all these cases, identifying new LUBAC substrates, in addition to NEMO, will be interesting since this may furnish new levels at which to act to specifically modulate NF-κB activation. One may imagine using antilinear polyubiquitin antibodies to pull them down and to identify them after western blotting. Second, does NEMO recognize K63-linked ubiquitin chains, linear chains or both? As mentioned above, this question, which is linked to the previous one, remains unsolved since no protein modified by a linear chain has been identified so far, besides NEMO itself. Third, what is the exact function of NEMO ubiquitination (K63-linked, linear or both)? Although recruitment of IKK activators such as the TAK complex is an attractive hypothesis, one can also imagine that NEMO ubiquitination induces its own multimerization, triggering IKK activation. In this case, linear ubiquitination may be specifically dedicated to that task.

In conclusion, data accumulated over the years have revealed the pleiotropic role played by ubiquitin in the NF-κB signalling pathway and have shown that activation of IKK, which is the key event in this pathway, is highly dependent upon ubiquitination through NEMO. Learning more about the processes regulated by ubiquitination in NF-κB signalling will not only provide new putative targets to act on for therapeutic purposes but also furnish a wealth of information regarding processes involving ubiquitin. This may, in turn, be exploited to understand other signalling pathways and physiological events.

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