The role of hybrid ubiquitin chains in the MyD88 and other innate immune signalling pathways

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The adaptor protein MyD88 is required for signal transmission by toll-like receptors and receptors of the interleukin-1 family of cytokines. MyD88 signalling triggers the formation of Lys63-linked and Met1-linked ubiquitin (K63-Ub, M1-Ub) chains within minutes. The K63-Ub chains, which are formed by the E3 ubiquitin ligases TRAF6, Pellino1 and Pellino2, activate TAK1, the master kinase that switches on mitogen-activated protein (MAP) kinase cascades and initiates activation of the canonical \(\kappa B\) kinase (IKK) complex. The M1-Ub chains, which are formed by the linear ubiquitin chain assembly complex (LUBAC), bind to the NEMO (NF-\(\kappa B\) essential modulator) component of the IKK complex and are required for TAK1 to activate IKKs, but not MAP kinases. An essential E3 ligase-independent role of TRAF6 is to recruit LUBAC into the MyD88 signalling complex, where it recognises preformed K63-Ub chains attached to protein components of these complexes, such as IRAK1 (IL-1 receptor-associated kinase), producing ubiquitin chains containing both types of linkage, termed K63/M1-Ub hybrids. The formation of K63/M1-Ub hybrids, which is a feature of several innate immune signalling pathways, permits the co-recruitment of proteins that interact with either K63-Ub or M1-Ub chains. Two likely roles for K63/M1-Ub hybrids are to facilitate the TAK1-dependent activation of the IKK complex and to prevent the hyperactivation of these kinases by recruiting A20 and A20-binding inhibitor of NF-\(\kappa B\)1 (ABIN1). These proteins restrict activation of the TAK1 and IKK complexes, probably by competing with them for binding to K63/M1-Ub hybrids. The formation of K63/M1-Ub hybrids may also regulate the rate at which the ubiquitin linkages in these chains are hydrolysed. The IKK-catalysed phosphorylation of some of its substrates permits their recognition by the E3 ligase SCF\(^{\text{TRCP}}\), leading to their Lys48-linked ubiquitylation and proteasomal degradation. Innate immune signalling is therefore controlled by the formation and destruction of three different types of ubiquitin linkage.

Facts

- The activation of toll-like receptors (TLR) and the interleukin-1 (IL-1) receptor triggers the rapid formation of Lys63-linked and Met1-linked ubiquitin oligomers.
- The Lys63-linked and Met1-linked ubiquitin oligomers are required to activate the TAK1 and canonical \(\kappa B\) kinase complexes, respectively.
- The Met1-linked ubiquitin oligomers are attached covalently to preformed Lys63-linked ubiquitin oligomers, generating Lys63/Met1 ubiquitin hybrids.
- The Lys63/Met1 ubiquitin hybrids can recruit proteins that bind to either Lys63-linked or Met1-linked ubiquitin oligomers.

Open Questions

- Which E3 ligases generate the Lys63-linked ubiquitin chains that are required for the activation of TAK1 and the formation of Lys63/Met1 ubiquitin hybrids?
- In the TLR and IL-1R signalling pathways, how is LUBAC (linear ubiquitin chain assembly complex) able to use Lys63-linked ubiquitin oligomers as its preferred initial substrate, rather than monomeric ubiquitin?
- How is the TLR- or IL-1R-dependent formation of Met1-linked ubiquitin chains regulated?
- What roles do Lys63/Met1-linked ubiquitin hybrids play in innate immune signalling pathways?

Lys63-Linked Ubiquitin Oligomers Activate the Protein Kinase TAK1

The role of Lys48-linked ubiquitin (K48-Ub) oligomers in triggering protein degradation by the proteasome was worked out between 1978 and 1989,1,2 but it was not until the mid 1990s that cellular roles for other types of Ub oligomers began to emerge. In 1995, yeast expressing the Ub\(^{[K63R]}\) mutation were shown to be hypersensitive to ultraviolet irradiation and failed to make a particular subset of ubiquitin conjugates.3 This provided indirect evidence that K63-Ub oligomers were involved in DNA repair, but direct evidence was only obtained 4 years later when Ubc13, a protein known to be required for DNA repair, was shown to assemble K63-Ub chains specifically in the presence of yeast orthologues of the mammalian protein Uev1a.4 The same paper also provided genetic evidence that the enhanced UV sensitivity of yeast expressing Ubc13 mutants was caused by the failure to synthesise these ubiquitin chains.

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Soon after, tumour necrosis factor receptor-associated factor 6 (TRAF6), a protein whose expression is essential for interleukin-1 (IL-1) and toll-like receptor (TLR) signalling, was shown to possess E3 ubiquitin ligase activity, which in combination with the Ubc13-Uev1a E2-conjugating complex catalysed the specific formation of Lys63-linked ubiquitin (K63-Ub) chains. Even more importantly, the TRAF6-generated K63-Ub chains were found to activate a protein kinase in vitro, which could in turn activate the canonical IκB kinase (IKK) complex and MAP kinase 6 (MKK6) in vitro. It was purified and identified as the TAK1 (transforming growth factor-activated kinase 1) complex, comprising the TAK1 catalytic subunit, TAK1-binding protein 1 (TAB1) and TAB2. Subsequently TAB3, a TAB2-related protein, was identified and it became clear that two TAK1 complexes (TAB1–TAK1–TAB2 and TAB1–TAK1–TAB3) are expressed in cells. The essential role of TAK1 in mediating the IL-1-dependent activation of the canonical IKK complex and MAP kinases was demonstrated by studies in embryonic fibroblasts from TAK1 knockout (KO) mice or knock-in mice lacking TAK1 catalytic activity.

The C-termini of TAB2 and TAB3 possess Npl4 zinc fingers (NZF) that bind to K63-Ub chains specifically and are essential for the activation of TAK1 in vitro. Thus the idea emerged that TRAF6-generated K63-Ub chains interact with TAB2 or TAB3 inducing a conformational change that activates the TAK1 catalytic subunit. This allosteric mechanism is similar to the activation of cyclic AMP-dependent protein kinase (PKA) by cyclic AMP or calmodulin-dependent protein kinases by calcium ions.

How MyD88 Signalling Activates the E3 Ligases TRAF6 and Pellino

Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein required for signal transduction by TLRs and receptors of the IL-1 family of cytokines (IL-1, IL-18 and IL-33). The interaction of components of microbial pathogens with TLRs or IL-1 with the IL-1R recruits MyD88 to the receptors, and then members of the IL-1 receptor-associated kinase (IRAK) family to MyD88 through a series of death domain (DD) interactions. The DD of IRAK4 interacts with the DD of MyD88 and the DDs of IRAK1 and/or IRAK2 with the DD of IRAK4, forming oligomeric complexes termed Myddosomes. TRAF6 interacts with Pro-Xaa-Glu motifs in the C-terminal regions of IRAK1 and IRAK2, triggering the dimerisation of the RING domain of TRAF6, activating its E3 ligase function. IRAK1 also phosphorylates Pellino1 and Pellino2 converting them from inactive to active E3 ubiquitin ligases. In the presence of E1-activating enzyme and the E2-conjugating complex Ubc13-Uev1a, TRAF6 and Pellinos 1 and 2 both contribute to the formation of K63-Ub chains, which become attached to IRAK1 and other components of the Myddosome. One essential role of TRAF6 is to recruit LUBAC into the IL-1 signalling complex where it interacts with K63-Ub-substrates, such as IRAK1, to produce K63/M1-Ub-IRAK1. In the human cell lines we have studied, the expression of IRAK1 is essential for IL-1 signalling and the IRAK2 in these cells cannot compensate for the loss of IRAK1. Protein kinases are highlighted in red and E3 ligases in green human cells, but not in TRAF6 KO cells that also lack expression of Pellino1 and Pellino2. Moreover, we have generated a knock-in mouse in which TRAF6 is replaced by an E3 ligase-inactive mutant, and shown that MyD88 signalling is reduced, but not abolished in primary macrophages and embryonic fibroblasts from these mice. These and other findings indicate that TRAF6, Pellino1 and Pellino2 contribute to the MyD88-dependent formation of K63-Ub chains in some cells (Figure 1), and that the essential roles of TRAF6 in this pathway are independent of its E3 ligase activity.

Met1-Linked Ubiquitin Oligomers and the MyD88-Dependent Activation of the Canonical IKK Complex

The canonical IKK complex comprises the related protein kinases IKKα and IKKβ, and a regulatory component called NF-κB essential modulator (NEMO). NEMO possesses an ubiquitin-binding domain that was initially shown to interact with K63-Ub oligomers. Mutations in this domain, such as NEMO[D311N], prevent binding to ubiquitin oligomers, impair activation of the canonical IKK complex and cause X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency in humans. These findings highlighted the
importance of ubiquitin binding to NEMO for the activation of the canonical IKK complex in vivo. They also suggested that K63-Ub chains might function as molecular scaffolds to co-recruit the IKK and TAK1 complexes, thereby facilitating the TAK1-dependent activation of the IKKs.26

An unexpected twist to the regulation of the IKK complex emerged with the discovery of the linear ubiquitin chain assembly complex (LUBAC), a heterotrimeric complex comprising HOIP (the catalytic subunit), HOIL-1 and Sharpin.30–33 LUBAC is a unique E3 ubiquitin ligase, which catalyses the formation of Met1-linked ubiquitin (M1-Ub) chains (also called linear ubiquitin chains) in which the N-terminal methionine of one ubiquitin forms a peptide bond with the C-terminal carboxylate of another ubiquitin molecule. Significantly, NEMO was found to bind to M1-Ub dimers with 100-fold higher affinity than K63-Ub dimers.34,35 Moreover, the IL-1-dependent formation of M1-Ub chains was abolished (Figure 2a) and activation of the canonical IKK complex greatly reduced (Figure 2b) in embryonic fibroblasts from knock-in mice expressing the E3 ligase-inactive HOIP[C879S] mutant.36 The IL-1-dependent activation of the canonical IKK complex was similarly reduced in embryonic fibroblasts from knock-in mice expressing the NEMO[D311N] mutant (Figure 2b). Thus, the LUBAC-catalysed formation of M1-Ub chains was abolished (Figure 2a) and the formation of M1-Ub chains in embryonic fibroblasts from wild-type (WT) mice, but not in knock-in mice expressing the E3 ligase-inactive HOIP[C879S] mutant. Thus, the formation of M1-Ub chains and their interaction with NEMO are both needed for TAK1 to initiate activation of the IKK complex.37 The interaction of M1-Ub chains with NEMO may induce a conformational change that permits TAK1 to phosphorylate Ser176/177 of IKKβ.

The formation of M1-Ub chains is not required for the IL-1-dependent activation of p38β MAP kinase, c-Jun N-terminal kinase 1 (JNK1) and JNK2.36

The Role of Lys48-Linked Ubiquitylation in MyD88 Signalling

IKKβ activates the transcription factor NF-κB by phosphorylating its inhibitory (IκB) components. For example, the dual phosphorylation of IκBα at Ser32 and Ser36 recruits the SCFβTRCP E3 ligase, leading to Lys48-linked ubiquitylation and proteasomal degradation of IκBα. The p65 and p50 components of NF-κB can then translocate to the nucleus as a heterodimer to stimulate the transcription of NF-κB-dependent genes. IKKβ also phosphorylates the transcription factor IRF5 (interferon regulatory factor 5) at Ser462, inducing its dimerisation and nuclear translocation where it switches on the transcription of genes encoding major inflammatory cytokines, such as IL-12 and interferon-β.42,43 Thus IKKβ switches on two of the ‘master’ transcription factors required for inflammatory mediator production (Figure 3).

Another important substrate of IKKβ is the protein kinase Tpl2 (tumour promotion locus 2). Two heterotrimeric Tpl2 complexes are expressed in cells, comprising the Tpl2β, or Tpl2L (tumour promotion locus 2L) at Ser400, which is followed by the Tpl2-catalysed phosphorylation of Ser176 and its interaction with 14-3-3 transcription factors required for alternative initiation start sites at Met1 (Tpl2L) or Met30 (Tpl2S).45 IKKβ has a dual role in the activation of Tpl2. First it phosphorylates p105 at Ser927 and Ser932, leading to the SCFβTRCP-catalysed Lys48-linked ubiquitylation of p105 and its proteasomal degradation;46,47 second it phosphorylates Tpl2S at Ser400, which is followed by the Tpl2-catalysed phosphorylation of Ser443 and its interaction with 14-3-3

![Figure 2](image-url)

Figure 2 The formation of M1-Ub chains and their interaction with NEMO are required for TAK1 to initiate the activation of the canonical IKK complex. (a) IL-1α induces the formation of M1-Ub chains in embryonic fibroblasts from wild-type (WT) mice, but not in knock-in mice expressing the E3 ligase-inactive HOIP[C879S] mutant. (b) The IL-1α-dependent, TAK1-catalysed phosphorylation of IKKβ at Ser177 is suppressed in embryonic fibroblasts from HOIP[C879S] knock-in mice that do not form M1-Ub chains, or in embryonic fibroblasts from NEMO[D311N] knock-in mice in which M1-Ub chains are formed, but are unable to interact with NEMO. Adapted from Emmerich et al.26 and Zhang et al.37
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Figure 3 The formation of K63/M1-Ub hybrids facilitates the activation of TAK1 and IKK complexes, and the phosphorylation of their substrates. The K63/M1-Ub hybrids produced by innate immune signalling pathways recruit the TAK1 and IKK complexes. The interaction of K63-Ub chains with TAB2 and TAB3 activates TAK1 allowing it to phosphorylate and activate MAP kinase kinases 4 and 7 (MKK4, MKK7), which activate the MAP kinases, termed TAK1 and IKK2. The binding of M1-Ub chains to NEMO induces a conformational change that permits TAK1 to phosphorylate IKKβ at Ser177. IKKβ also activates the TAK2 kinase complex, which activates MEK1 and MEK2, the protein kinases that activate ERK1 and ERK2. TAK2 additionally phosphorylates MKK3 and MKK6. MKK3 and MKK6 operate redundantly with MKK4 to phosphorylate and activate p38α in some cells. Protein kinases are highlighted in red, ubiquitin-binding proteins in purple and ubiquitin (Ub) molecules in blue. Further details are given in the text.

How is LUBAC Regulated?

The activation of the MyD88 signalling pathway triggers the formation of M1-Ub chains within minutes, but the intrinsic E3 ligase activity of LUBAC is not increased. Observations indicate that LUBAC is not converted from an inactive to an active form by a stable covalent modification, such as phosphorylation. Instead, as discussed later, the formation of M1-Ub chains is likely to be triggered by the recruitment of LUBAC into the MyD88 signalling complex where it is targeted to its substrates by TRAF6. However, the formation of M1-Ub chains may be facilitated by the inhibition of Otulin and/or CYLD (the product of the Cylindromatosis gene), which appear to be the major deubiquitylases that hydrolyse M1-Ub chains, as discussed elsewhere in this issue. Interestingly, Otulin binds to the PUB (N-terminal peptide: N-glycanase/UBA- or UBX-containing protein) domain of HOIP, while CYLD is also recruited to the PUB domain of HOIP, but indirectly via the protein SPATA2 (spermatogenesis-associated protein 2). The formation and destruction of M1-Ub chains is therefore catalysed by a single multi-enzyme complex.

Hybrid Ubiquitin Chains Containing Both M1-Ub and K63-Ub Linkages

Most of the M1-Ub chains formed in response to IL-1β or the TLR1/2 agonist Pam3CSK4 are attached covalently to K63-Ub chains, forming ubiquitin chains containing both types of linkage, hereafter called K63/M1-Ub ‘hybrids’. Some of the K63/M1-Ub hybrids present in cell extracts are ‘anchored’ covalently to the components of the Myddosome, and most strikingly to IRAK1 (Figure 1). However, K63/M1-Ub hybrids that are not ‘anchored’ covalently to any other protein can also be detected in extracts prepared from IL-1-stimulated cells. Whether these ‘unanchored’ K63/M1-Ub hybrids are formed de novo or generated by cleavage from ‘anchored’ K63/M1-Ub hybrids is unknown. The relative importance of anchored and unanchored K63/M1-Ub hybrids in activating the TAK1 and IKK complexes remains to be clarified.

When the M1-Ub chains present in cell extracts are hydrolysed by incubation with Otulin, the size of the Ub chains attached to IRAK1 is reduced, but no mono-ubiquitylated IRAK1 is generated. This finding, together with the reduced formation of M1-Ub chains in Ubc13-deficient cells, indicates that IRAK1 is first modified by Lys63-linked ubiquitylation, the M1-Ub linkages being added subsequently. Therefore, although LUBAC can assemble M1-Ub chains from monomeric ubiquitin in vitro, it is K63-Ub oligomers and not monomeric ubiquitin, which are the substrates first used by LUBAC when it is recruited into the MyD88 signalling complex. Interestingly, the MyD88-dependent formation of M1-Ub chains is drastically reduced in TRAF6 KO cells, and can be restored by the re-expression of wild-type or E3 ligase-inactive mutants of TRAF6. The specific recognition of K63-Ub-substrates by LUBAC may be facilitated by the NZF domains present in HOIP, which interact specifically with K63-Ub chains in vitro. The specific hydrolysis of the M1-Ub chains present in K63/M1-Ub hybrids liberates small K63-Ub oligomers of varying length and, conversely, the specific hydrolysis of K63-Ub chains liberates small M1-Ub oligomers of varying length. These experiments demonstrate that the topology of the K63/M1-Ub hybrids is complex. They also indicate that once the first M1-Ub linkage has been made to a preformed K63-Ub oligomer, LUBAC can then use monomeric ubiquitin as a substrate to add additional M1-Ub linkages. The HOIL-1 component of LUBAC also possesses NZF domains but, in
contrast to those present in HOIP, they bind to M1-Ub oligomers more strongly than to K63-Ub oligomers.\textsuperscript{36,54} This could operate as a positive feedback loop to recruit more molecules of LUBAC to the MyD88 signalling complex once the initial M1-Ub linkages have been formed.

Potential Roles of the K63/M1-Ub Hybrids

The formation of hybrid ubiquitin chains containing both K63-Ub and M1-Ub linkages is not confined to the MyD88 signalling network, but is a feature of at least three other innate immune signalling pathways. These include the nucleotide oligomerisation domain 1 (NOD1) and NOD2 pathways that are activated by components of bacterial peptidoglycans, the TLR3 pathway, which responds to viral double-stranded RNA and signals via the adaptor protein TRIF (TIR domain-containing adapter-inducing interferon-β), and the TNF (tumour necrosis factor) signalling pathway.\textsuperscript{55,56} These observations suggest that the formation of K63/M1-Ub hybrids is of general significance for the regulation of these signal transduction pathways, raising the question of what their specific roles might be.

The MyD88, TRIF, NOD1/2 and TNF signalling pathways all lead to the rapid, TAK1-dependent activation of the canonical IKK complex. Since the TAB2 and TAB3 components of TAK1 complexes interact specifically with K63-Ub oligomers, and the NEMO component of the canonical IKK complex binds selectively to M1-Ub oligomers, the formation of K63/M1-Ub hybrids may permit the co-recruitment of these two kinase complexes to the same ubiquitin chains (Figure 3). One role for the K63/M1-Ub hybrids may therefore be to facilitate the TAK1-dependent activation of the IKK complex.\textsuperscript{37}

However, K63/M1-Ub hybrids are likely to control the activation of innate immune signalling in other ways. For example, the protein ABIN1 possesses a ubiquitin-binding domain similar to that present in NEMO.\textsuperscript{51} ABIN1 restricts the activation of TAK1 and the canonical IKK complex, probably by competing with TAB2/3 and NEMO for binding to K63/M1-Ub hybrids (Figure 4a). The genetic evidence supporting this hypothesis comes from studies with mice in which the gene encoding ABIN1 was replaced by the ubiquitin-binding-defective ABIN1[D485N] mutant. This variant is equivalent to the NEMO[D311N] mutation discussed earlier. The MyD88-dependent activation of TAK1, IKKs and MAPKs, and the production of inflammatory mediators, was enhanced several fold in dendritic cells and B cells from the ABIN1[D485N] mice\textsuperscript{57} (Figure 4b). Interestingly, the ABIN1[D485N] knock-in mice spontaneously developed an autoimmune disease similar to Type III and Type IV systemic lupus erythematosus (SLE) in humans, which can be prevented by crossing the ABIN1[D485N] mice to MyD88 KO mice.\textsuperscript{57} This demonstrates that enhanced MyD88 signalling causes the disease. SLE was
also prevented by crossing ABIN1(D485N) mice to knock-in mice expressing kinase-inactive mutants of IRAK1 or IRAK4.\(^{58}\) ABIN1 polymorphisms have been shown to predispose to lupus, psoriasis and other autoimmune diseases in eight human populations (see Nanda et al.\(^{58}\) for more information), suggesting that drugs inhibiting IRAK1 and/or IRAK4 could have therapeutic potential for the prevention and perhaps the treatment of lupus caused by hyperactivation of the MyD88 signalling network.

The ABIN1-interacting protein A20 is an immediate early gene whose expression is increased when the MyD88 pathway is activated, and which also prevents the overproduction of inflammatory mediators by this pathway.\(^{52}\) The C-terminal domain of A20 contains seven zinc fingers. The fourth and seventh zinc fingers bind to K63-Ub\(^{59–60}\) and M1-Ub oligomers,\(^{51,62}\) respectively, making A20 a prime candidate to interact with K63/M1-Ub hybrids and compete with the TAK1 and IKK complexes for binding to these molecules. Since ABIN1 and A20 both interact with K63/M1-Ub hybrids and with each other,\(^{63}\) ternary complexes formed between ABIN1, A20 and K63/M1-Ub hybrids may restrict the activation of the TAK1 and IKK complexes more effectively than either ABIN1 or A20 alone (Figure 4). These observations can explain why A20 has been described as a ‘central gatekeeper’ in inflammation and immunity\(^{64}\) and it also prevents some types of lymphoma.\(^{65}\)

The N-terminal domain of A20 possesses deubiquitylase activity. It was recently reported that the covalent attachment of M1-Ub oligomers to K63-Ub oligomers reduces the rate at which A20 can hydrolyse K63-Ub linkages \textit{in vitro}, suggesting a novel role for K63/M1-Ub hybrids in regulating the rate of Ub chain hydrolysis.\(^{66}\) It was also reported that the IKK\(\beta\)-catalysed phosphorylation of A20 enhances the rate at which A20 hydrolyses K63-Ub chains.\(^{67}\) However, the physiological significance of the A20 deubiquitylase activity merits further attention, because other investigators reported that knock-in mice expressing a deubiquitylase-inactive mutant of A20 appeared normal, with no signs of inflammation. The mice also had normal proportions of B, T, dendritic and myeloid cells. Moreover lipopolysaccharide and TNF responses, such as NF-\(\kappa\)B activation, were unaffected.\(^{67}\)

**Conflict of Interest**

The authors declare no conflict of interest.

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