Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains

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Polyubiquitin (pUb) chains formed between the C terminus of ubiquitin and lysine 63 (K63) or methionine 1 (M1) of another ubiquitin have been implicated in the activation of the canonical IkB kinase (IKK) complex. Here, we demonstrate that nearly all of the M1-pUb chains formed in response to interleukin-1, or the Toll-Like Receptors 1/2 agonist Pam3CSK4, are covalently attached to K63-pUb chains either directly as K63-pUb/M1-pUb hybrids or indirectly by attachment to the same protein. Interleukin-1 receptor (IL-1R)-associated kinase (IKAR) 1 is modified first by K63-pUb chains to which M1-pUb linkages are added subsequently, and myeloid differentiation primary response gene 88 (MyD88) and TNF Receptor-Associated Factor 6 (TRAF6) (4) can also modify both K63-pUb and M1-pUb chains. We show that the heme-oxidized IRP2 ubiquitin ligase 1 interacting protein (HOIP) component of the linear ubiquitin assembly complex catalyzes the formation of M1-pUb chains in response to interleukin-1, that the formation of K63-pUb chains is a prerequisite for the formation of M1-pUb chains, and that HOIP interacts with K63-pUb but not M1-pUb linkages. These findings identify K63 Ub oligomers as a major substrate of HOIP in cells where the MyD88-dependent signaling network is activated. The TGF-beta-activated kinase 1 (TAK1)-binding protein (TAB) 2 and TAB3 components of the TAK1 complex and the NFkB Essential Modifier (NEMO) component of the canonical IKK complex bind to K63-pUb chains and M1-pUb chains, respectively. The formation of K63/M1-pUb hybrids may therefore provide an elegant mechanism for colocalizing both complexes to the same pUb chain, facilitating the TAK1-catalyzed activation of IKKa and IKKβ. Our study may help to resolve the debate about the relative importance of K63-pUb and M1-pUb chains in activating the canonical IKK complex.

LUBAC | TNF Receptor-Associated Factor 6 | Ubc13 | NF-κB

Interleukin-1 (IL-1) or ligands that activate Toll-Like Receptors (TLRs) initiate “downstream” signaling events by recruiting the adaptase protein MyD88 (1), IL-1 Receptor (IL-1R)-Associated Kinase 4 (IRAK4), IRAK1, and IRAK2 to form an oligomeric structure termed the Myddosome (2, 3). IRAK1s 1 and 2 undergo covalent modification by phosphorylation and ubiquitylation and interact with the E3 ubiquitin ligase TNF Receptor-Associated Factor 6 (TRAF6) (4). TRAF6 can combine with the E2-conjugating complex Ubc13-Uev1a to generate K63-pUb oligomers in vitro (5–7), probably via conformational changes induced by an interaction with the Npl40 Zinc Finger (NZF) domain of TAB2 and TAB3, which bind to K63-pUb chains specifically in vitro (8, 9). Once activated, TAK1 phosphorylates the canonical IkB kinase (IKK) complex and the mitogen-activated protein (MAP) kinase kinases that activate c-Jun N-terminal kinases (JNKs) and p38 MAP kinases, which then trigger “downstream” signaling events. The IL-1-stimulated activation of the canonical IKK complex and MAP kinases fails to occur in mouse embryonic fibroblasts (MEFs) from TRAF6-deficient mice (10) or TAK1-deficient mice (11) or in MEFs from mice that express an inactive, truncated form of TAK1 (12). Moreover, wild-type TRAF6, but not an E3 ligase-deficient mutant, restores IL-1 signaling to TRAF6-deficient MEFs (10). These observations support the hypothesis that TRAF6-generated K63-pUb chains are required for the IL-1-stimulated activation of TAK1 and that TAK1 expression is essential to activate the IKK complex and MAP kinases in MEFs.

NFkB Essential Modifier (NEMO), an integral component of the canonical IKK complex, was reported to bind K63-pUb chains (13, 14), and NF-κB-dependent gene transcription induced by TNFα or IL-1 was restored to NEMO-deficient MEFs by the reintroduction of wild-type NEMO but not by the pUb-binding–defective mutant NEMO[D311N] (13–15). Moreover, the TNFα-stimulated degradation of IkBα and the TNFα or IL-1-stimulated translocation of the p50/p65 NFkB subunits to the nucleus was impaired in fibroblasts from immune-deficient human patients expressing the NEMO[D311G] mutant. Thus, the activation of the canonical IKK complex is attenuated in cells expressing pUb-binding–defective mutants of NEMO (16).

More recently, NEMO was found to bind linear-Ub oligomers with 100-fold higher affinity than K63-Ub oligomers of equivalent length (17, 18), suggesting that NEMO may bind linear-Ub chains preferentially in vivo. These pUb chains, which are produced by the formation of a peptide bond between the α-amino group of the N-terminal methionine (M) of one ubiquitin and the C-terminal glycine of another ubiquitin, will be termed M1-pUb chains hereafter. M1-Ub linkages can be generated in vitro by the linear ubiquitin assembly complex (LUBAC) (19), which is composed of three components: Heme-Oxidized IRP2 ubiquitin Ligase 1 (HOIL-1), HOIL-1 interacting protein (HOIP), and HOIL-1–interacting protein (HIBC).

Significance

Two types of ubiquitin chain are required to switch on one of the major signaling networks of the innate immune system that triggers the production of inflammatory mediators to combat infection by pathogens. This paper reports the unexpected discovery that both types of ubiquitin chain are attached covalently to one another and that the formation of one type of ubiquitin chain is dependent on the prior formation of the other. One function of these hybrid ubiquitin chains is to permit the colocalization of two different kinase complexes, thereby facilitating the activation of one kinase complex by the other to increase the speed of the response to infection.


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Sharpin (20–22). Importantly, the IL-1-stimulated activation of the canonical IKK complex was impaired in MEFs from HOIL-1−/− mice (23), suggesting an important role for M1-pUb chains in this process. Moreover, the readdition of LUBAC to HeLa extracts depleted of this E3 ligase restored the activation of the canonical IKK complex in vitro. Furthermore, LUBAC was reported to activate NFκB-dependent gene transcription when overexpressed in wild-type MEFs, but not in NEMO-deficient MEFs (24). It was inferred from these studies that LUBAC-catalyzed M1-pUb chain formation, rather than K63-pUb chain formation, was critical for the activation of the canonical IKK complex.

Here, we report that nearly all of the M1-pUb chains formed when the MyD88 signaling network is activated are attached covalently to K63-pUb chains. We further show that the IL-1-stimulated formation of K63-pUb chains is a prerequisite for the formation of M1-pUb chains and that HOIP binds specifically to K63-pUb chains in vitro. We suggest that the formation of K63-pUb/M1-pUb hybrids permits the recruitment of TAK1 and the canonical IKK complex to the same pUb chains, facilitating the TAK1-catalyzed phosphorylation and activation of the IKKs.

**Results**

**IL-1 Stimulates the Formation of M1-pUb and K63-pUb Chains in IL-1 Receptor Cells.** HEK293 cells stably expressing the IL-1 receptor (termed here IL-1R cells) (25) were stimulated with IL-1, and the pUb chains captured from the cell extracts on immobilized Halo-NEMO were analyzed. The Halo-NEMO beads captured M1-pUb and K63-pUb chains from the extracts of IL-1-stimulated cells (Fig. S14), but did not capture K48-pUb chains or K11-pUb chains, in contrast to Halo–tandem-repeated ubiquitin-binding entities (TUBEs) (26), which captured these types of pUb chains (Fig. S1B). M1-pUb or K63-pUb chains captured by Halo-NEMO were incubated in the absence (control) or presence of Otulin, AMSH-LP or both deubiquitylases after SDS/PAGE and immunoblotting with antibodies that recognize K63-pUb or M1-pUb chains specifically (Fig. S2A, B). As shown in A, except that the pUb chains captured by Halo-NEMO were incubated in the absence (control) or presence of Otulin, AMSH-LP or both deubiquitylases before SDS/PAGE and immunoblotting with antibodies that recognize M1-pUb chains (P1), K63-pUb chains (P2), or with an antibody that recognizes M1-pUb and K63-pUb chains equally well (P3). In P3, the lower half of the membrane was developed for 10 min longer than the upper half. (C) Authentic M1-pUb oligomers (30 ng, lane 1; 10 ng, lane 2) and K63-pUb oligomers (3 ng, lane 7 and 6 ng, lane 8) were used as markers to demonstrate that the small Ub oligomers formed in B after treatment with AMSH-LP were linked via M1 of ubiquitin (lanes 3 and 4) and that those formed by treatment with Otulin (lanes 5 and 6) were linked via K63.

**M1-pUb Chains Captured by NEMO Are Linked to K63-pUb Chains.** Otulin is a deubiquitylase that hydrolyzes M1-pUb chains specifically (29, 30) (Fig. S2A), whereas AMSH-LP cleaves K63-pUb chains specifically (Fig. S2B) (9). As expected, Otulin hydrolyzed nearly all of the M1-pUb chains captured from the cell extracts by immobilized NEMO (Fig. 1B, P1, lanes 5 and 6). However, unexpectedly, Otulin treatment reduced the size of the large K63-pUb chains (Fig. 1B, P2; compare lanes 5 and 6 with lanes 5 and 4) and generated a variety of faster-migrating, small pUb oligomers (Fig. 1B, P3, lanes 5 and 6) that comigrated with K63-pUb chains (Fig. 1C, lanes 5–8) and were hydrolyzed by AMSH-LP (Fig. 1B, P3; compare lanes 9 and 10 with lanes 5 and 6). The generation of these small K63-Ub oligomers explains why the amount of the larger K63-pUb chains was reduced after Otulin treatment. Conversely, treatment with AMSH-LP hydrolyzed all of the K63-pUb chains captured by NEMO (Fig. 1B, P2, lanes 7 and 8) and increased the formation of faster-migrating small Ub oligomers (Fig. 1B, P3, lanes 5 and 7) that comigrated with M1-pUb chains (Fig. 1C, lanes 1–4) and could be hydrolyzed by Otulin (Fig. 1B, P3; compare lanes 9 and 10 with 7 and 8). The reduced size of the large pUb chains after incubation with either Otulin or AMSH-LP could be explained if the M1-pUb chains and K63-pUb chains were attached covalently to different lysine residues on the same protein or if the M1-pUb chains were attached directly to K63-pUb chains as K63-pUb/M1-pUb “hybrid” molecules. However, the generation of small K63-Ub oligomers after incubation with Otulin and small M1-Ub oligomers after incubation with AMSH-LP, which are not attached to any other protein, is compatible only with K63/M1-pUb hybrid formation.

**Polyubiquitin Chains Attached to IRAK1 and Captured by NEMO Are K63-pUb/M1-pUb Hybrids.** We identified many proteins by mass spectrometry that were captured by Halo-NEMO from the extracts of IL-1-stimulated IL-1R cells in a ubiquitin-dependent manner because they were not captured by the NEMO[D311N] mutant. Those proteins of relevance to the present study are listed in Table S1. They include many of the proteins known to participate in the MyD88-signaling network (Introduction), namely MyD88 itself, IRAK1, IRAK4, TRAF6, the HOIP, HOIL-1 and Sharpin components of LUBAC and the TAB1, TAB2, TAB3 and TAK1 components of the TAK1 complex (Tables S1 and S2). Proteins captured by Halo-NEMO in a ubiquitin-independent manner...
included, as expected, IKKn and IKKβ (Table S3), the other components of the canonical IKK complex.

We found that IRAK1 became covalently modified by pUb chains in response to IL-1, explaining why it was captured by Halo-NEMO. Incubation with Otulin reduced the size of the pUb chains attached to IRAK1 (Fig. 2A, Upper, lanes 6 and 7), and these Ub chains could now be hydrolyzed by AMSH-LP, indicating that they were linked via K63 of ubiquitin (Fig. 2A, Upper, lanes 8 and 9). Importantly, none of the IRAK1 was reconverted to either the monoubiquitylated or unmodified forms of the protein after incubation with Otulin (Fig. 2A, Upper; compare lanes 6 and 7 with lanes 2 and 3) even though Otulin hydrolyzed virtually all of the M1-Ub linkages captured by NEMO (Fig. 1B, P1, lanes 5 and 6). These results are not compatible with the M1-pUb chains and K63-pUb chains being attached to different lysine residues in IRAK1. They are also not compatible with IRAK1 being modified initially with M1-Ub linkages and subsequently with K63-Ub linkages. The experiments therefore suggested that IRAK1 was modified by K63-pUb chains initially, to which M1-pUb chains were attached subsequently. These results were not specific to this agonist or cell type because similar results were obtained in human THP1 monocytes stimulated with Pam3CSK4, an activator of the TLR1/2 heterodimer that also signals via MyD88 (Fig. 2A, Lower).

**Topology of the pUb Chains Attached to MyD88 and IRAK4.** A significant proportion of the MyD88 and IRAK4 captured by Halo-NEMO from the extracts of IL-1R-stimulated IL-1R cells (Fig. 2B) or Pam3CSK4-stimulated THP1 monocytes (Fig. S3A) underwent polyubiquitylation. Similar to IRAK1, treatment with Otulin or AMSH-LP reduced the size of the large pUb chains attached to MyD88 and IRAK4 (Fig. 2B; compare lanes 4–7 with lanes 2 and 3), whereas treatment with Otulin plus AMSH-LP

converted MyD88 and IRAK4 to monoubiquitylated and unmodified species. The pUb chains attached to MyD88 and IRAK4 therefore also contain both K63-Ub and M1-Ub linkages. However, in contrast to IRAK1, incubation with Otulin generated some monoubiquitylated MyD88 and IRAK4, suggesting that some of the M1-pUb chains may be attached to these proteins without their prior modification by K63-pUb chains. Like the nonspecific deubiquitylase USP2 (Fig. 2A and B, lanes 10 and 11), AMSH-LP not only hydrolyzed K63-pUb chains but also partially reconverted IRAK1 and MyD88, and largely reconverted IRAK4, to the unmodified species (Fig. 2A and B; compare lanes 4 and 5 with lanes 2 and 3). This indicates that AMSH-LP can cleave the isopeptide bond(s) formed between the C terminus of the first ubiquitin and the ε-amino moiety of a lysine residue(s) on these proteins. However, in the case of IRAK1 and MyD88, this isopeptide bond is hydrolyzed more slowly than K63-Ub linkages by AMSH-LP because increased formation of the monoubiquitylated species is also observed.

**Polyubiquitylation of Other Proteins.** The ubiquitylated TRAF6 formed in response to IL-1 was not modified by M1-pUb chains because incubation with Otulin had no effect (Fig. S3B). The ubiquitylated forms of TRAF6 detected after treatment with AMSH-LP plus Otulin may arise from monoubiquitylated MyD88 and IRAK4, suggesting that some of the M1-pUb chains may be attached to these proteins without their prior modification by K63-pUb chains.

Minor amounts of HOIP and HOIL-1 were converted to slower-migrating forms in response to IL-1, which disappeared after USP2 treatment, indicating that they were ubiquitylated species (Fig. S3C). Sharpin was partially converted to a USP2-sensitive monoubiquitylated form in response to IL-1 (Fig. S3C). A small amount of the endogenous NEMO was also converted to a monoubiquitylated form in IL-1-stimulated IL-1R cells (Fig. S3D). Trace amounts of polyubiquitylated NEMO were also

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detected, which were hydrolyzed by AMSH-LP but not by Otulin (Fig. S3D; compare lanes 3–8), indicating that NEMO is not modified significantly with M1-pUb chains after IL-1 stimulation.

**Sequential Capture of pUb Chains by Halo-TAB2 and Halo-NEMO.** The Np4 zinc finger (NZF) domain of the TAB2 component of the TAK1 complex binds specifically to K63-pUb chains (8, 9). However, we found that a tandem repeat of the NZF of TAB2 (termed here Halo-TAB2) also captured all of the large M1-pUb chains present in the extracts of IL-1–stimulated cells (Fig. S4) because no high-molecular-mass M1-pUb chains were captured by Halo-NEMO from the supernatant obtained after the first Halo-TAB2 pull-down (Fig. S4, P1; compare lanes 4 and 12). Conversely, no M1-pUb chains were captured from the supernatant of the first Halo-NEMO pull-down by Halo-TAB2 (Fig. S4, P1; compare lanes 2 and 8). Thus, both Halo-TAB2 and Halo-NEMO deplete the cell extracts of M1-pUb chains. On the other hand, Halo-TAB2 captured K63-pUb chains (Fig. S4, P2, lanes 7 and 8) and more pUb-IRAK1 (Fig. S4, P3, lane 8) from the supernatant of the first Halo-TAB2 pull-down, whereas Halo-NEMO did not (Fig. S4, P2 and P3, lanes 5 and 6). The size of pUb-IRAK1 captured by Halo-TAB2 from the supernatant of the first NEMO pull-down was smaller than that captured by Halo-NEMO (Fig. S3B; compare lanes 5 and 6 with 1 and 2). Moreover, unlike the pUb-IRAK1 captured by NEMO (Fig. S3B; compare lanes 7 and 8 with 1 and 2), the pUb-IRAK1 was not reduced in size by treatment with Otulin (Fig. S3B; compare lanes 5 and 6 with 9 and 10). Thus, IL-1 stimulation generates two forms of pUb-IRAK1, one linked to K63/M1-pUb hybrids (captured by Halo-NEMO or Halo-TAB2) and the other linked only to K63-pUb chains (captured by Halo-TAB2 from the supernatant of the first NEMO pull-down).

In contrast to Halo-NEMO, Halo-TAB2 captured K63-pUb chains from the extracts of cells not stimulated with IL-1 (Fig. S4, P2; compare lanes 1 and 3). Taken together, these results establish that NEMO captures M1-pUb chains selectively from the cell extracts, whereas Halo-TAB2 captures only K63-pUb chains. However, both Halo-tagged proteins deplete M1-pUb chains from the extracts of IL-1–stimulated cells because virtually all of the M1-pUb chains are attached covalently to K63-pUb chains.

**LUBAC Is the Only E3 Ubiquitin Ligase That Catalyzes the Formation of M1-pUb Chains in IL-1–Stimulated Mouse Embryonic Fibroblasts.** Although LUBAC catalyzes the formation of M1-pUb chains in vitro, it is not established that it is the only E3 ubiquitin ligase that generates these chains in vivo. We therefore generated knock-in mice in which HOIP was replaced by the E3 ligase-inactive HOIP [C879S] mutant, in which the catalytic cysteine was replaced by serine (Fig. S4A). The human HOIP[C885S] mutant, equivalent to Cys879 of murine HOIP, had no detectable E3 ligase activity (Fig. S4B). The HOIP[C879S] mice displayed embryonic lethality, no embryos being detectable from E11 onward; fibroblasts from mouse embryos that were 10.5 d old (MEFs) were therefore used for all subsequent studies. IL-1 did not induce detectable M1-pUb chain formation in HOIP[C879S] MEFs (Fig. 4A), establishing that HOIP is the catalytic subunit of LUBAC and the only E3 ligase that can produce M1-pUb chains in IL-1–stimulated MEFs. The expression of HOIP, HOIL-1, and Sharpin was similar in MEFs from HOIP[C879S] and wild-type mice (Fig. 4E). The IL-1–stimulated activation of IKKβ was reduced in MEFs from the HOIP[C879S] mice, as judged by reduced...
Enhanced activation of the canonical IKK complex via the MyD88- and IKK

The E2-conjugating complex Ubc13-Uev1a directs the formation of M1-pUb chains. IL-1R cells stably expressing shRNA specific for Ubc13 or an empty vector were stimulated for the times indicated with 5 ng/ml IL-1β, and the cells were lysed in the presence of 100 mM iodoacetamide. The pUb chains captured from the cell extracts with Halo-NEMO were subjected to SDS/PAGE and immunoblotting with antibodies that recognize K63-pUb chains (Top) or M1-pUb chains (Top, second subpanel). The cell extracts (20 μg protein) were also subjected to SDS/PAGE and immunoblotted with the antibodies indicated (Bottom eight subpanels).

phosphorylation of p105, a well-authenticated physiological substrate of IKKβ (27, 28). Thus, M1-pUb chain formation was required for optimal activation of the canonical IKK complex. In contrast, the IL-1-stimulated activation of JNK and p38 MAP kinase was enhanced in HOIP{C879S} MEFs, demonstrating that M1-pUb chain formation is not required for the activation of these protein kinases (Fig. 4A).

The knock-down of both HOIP and HOIL-1 also reduced the IL-1-stimulated formation of M1-pUb chains and the phosphorylation of p105 in IL-1R cells, without affecting the IL-1-stimulated formation of K63-pUb chains significantly (Fig. 4B).

LUBAC Is Active in Unstimulated Cells. Because M1-pUb chains were formed only in response to IL-1 in IL-1R cells or to Pam3CSK4 in THP1 cells, we anticipated that LUBAC would be activated by these agonists. However, unexpectedly, LUBAC immunoprecipitated from IL-1R cell extracts with anti-HOIP was already active in serum grown cells not stimulated with IL-1, and activity did not increase in response to IL-1 (Fig. 4C and Fig. S5 A and B). Similar results were obtained in Pam3CSK4-stimulated THP1 cells. It was possible that HOIP had been activated by interaction with the anti-HOIP antibody, but the same results were obtained if LUBAC was immunoprecipitated from the cell extracts with anti–HOIL-1 or anti-Sharpin (Fig. 4C). It is unlikely that antibodies raised against all three components of LUBAC would activate its E3 ligase activity. We therefore conclude that LUBAC does not undergo a stable covalent modification in response to IL-1 that converts it from an inactive to an active form.

The antibodies raised against any one component of LUBAC immunoprecipitated the others, confirming that, as in other cells (31, 32), LUBAC is a hetero-trimeric complex composed of HOIP, HOIL-1, and Sharpin, a complex remaining intact after IL-1 stimulation (Fig. 4C, Lower).

Formation of M1-pUb Chains Requires the Formation of K63-pUb Chains. The E2-conjugating complex Ubc13-Uev1a directs the formation of K63-pUb chains in cells. To investigate whether the formation of M1-pUb chains was affected by the formation of K63-pUb chains, we generated IL-1R cells deficient in Ubc13 (Fig. 5). As expected, the IL-1-stimulated formation of K63-pUb chains was almost abolished (Fig. 5, Top) but, interestingly, the IL-1-stimulated formation of M1-pUb chains was also greatly reduced (Fig. 5, Top, second subpanel). Control experiments showed that the expression of HOIP, HOIL-1, and Sharpin was unaltered in the Ubc13-deficient cells (Fig. 5). The phosphorylation of IKKβ, its substrate p105, and JNK was also impaired in the Ubc13-deficient cells (Fig. 5). Importantly, the reexpression of Ubc13 restored the IL-1-stimulated formation of K63-pUb and M1-pUb chains and the activation of the canonical IKK complex and MAP kinases (Fig. S5C). Taken together, these experiments establish that the formation of M1-pUb chains in IL-1R cells requires K63-pUb chain formation.

HOIP Interacts with K63-pUb Chains but Not with M1-pUb Chains. Because most of the M1-pUb chains were attached covalently to K63-pUb chains, this indicated that K63-pUb oligomers were a major substrate for LUBAC in IL-1-stimulated cells. An N-terminal fragment of HOIP containing its two NZF domains was reported to bind weakly to K48-Ub and M1-Ub oligomers but more strongly to K63-Ub oligomers (33). Here, we found that full-length HOIP captured K63-Ub oligomers (Fig. 4D, lanes 2 and 3), but not M1-Ub oligomers (Fig. 4D, lanes 7 and 8) under the experimental conditions that we used. The NZF of HOIL-1 was reported to interact with M1-Ub dimers (34). We confirmed this finding using full-length HOIL-1 (Fig. 4D, lanes 9 and 10), but also detected weak binding to K63-Ub oligomers (Fig. 4D, lanes 4 and 5). Interestingly, HOIP and HOIL-1 captured longer K63-Ub oligomers preferentially, even though they were minor components in the preparation of mixed K63-Ub oligomers used for these binding studies (Fig. 4D, lane 1). The NZF of Sharpin was reported to interact with M1-Ub and K63-Ub oligomers (20, 22). However, full-length Sharpin failed to interact with M1-Ub, K63-Ub, or K48-Ub oligomers under our experimental conditions, indicating that the binding of Ub oligomers to Sharpin is much weaker than to HOIP or HOIL-1.

Discussion

In this paper we show that most of the M1-pUb chains formed in IL-1-stimulated IL-1R cells or Pam3CSK4-stimulated THP1 monocytes, are attached covalently to K63-pUb chains and that significant formation of M1-pUb linkages cannot take place until K63-pUb chains are produced (Fig. 5 and Fig. S5C). Our finding that HOIP interacts specifically with K63-pUb chains may help to explain why the formation of M1-pUb chains depends on the prior formation of K63-pUb chains (Fig. 4D). However, it cannot be the only reason because substantial amounts of K63-pUb chains were present in serum grown cells not stimulated with IL-1 (Fig. 3A), and yet M1-pUb chains did not accumulate under these conditions, despite LUBAC being active under these conditions. We were also unable to accelerate the LUBAC-
catalyzed formation of M1-pUb chains in vitro by adding K63-Ub oligomers to the assays. The recruitment of LUBAC to a MyD88-dependent signaling complex containing the K63-pUb chain-generating E3 ligase TRAF6, and perhaps other K63-pUb chain-generating E3 ligases, would therefore seem to be needed before the M1-pUb chains generated by LUBAC can accumulate and be coupled to K63-pUb chains. Perhaps the interaction of K63-pUb/M1-pUb linkages with one or more proteins in this complex decreases the rate at which they are hydrolyzed by Otulin and/or other deubiquitylases in cells. Alternatively, or in addition, one or more M1-pUb hydrolase(s) may be converted to less active forms when the MyD88-signaling network is activated.

The topology of the K63-pUb/M1-pUb hybrids is clearly hetero-erogeneous because the AMSH-LP-catalyzed hydrolysis of K63-pUb chains or the Otulin-catalyzed hydrolysis of M1-pUb chains generated K63-Ub or M1-Ub oligomers, respectively, of varying length (Fig. 1F and Fig. S6). Working out the precise topology of these hybrid pUb chains will be a challenging project.

It was suggested that the activation of the TAK1 complex results from a conformational change induced by the interaction of K63-pUb chains with its TAB2 and TAB3 subunits (6, 7). In contrast, NEMO binds 100-fold more tightly to M1-Ub dimers than to K63-Ub dimers (17, 18) and captures M1-pUb chains specific for the extracts of IL-1-activated cells (Fig. 3). The formation of K63-pUb/M1-pUb hybrids would therefore appear to be a simple device for colocalizing TAK1 and the canonical IKK complex to the same pUb chain, facilitating the TAK1-catalyzed activation of IKKα and IKKβ (Fig. 6). The failure to form M1-pUb linkages in MEFs from HOIP[C879S] knock-in mice (Fig. 4A, and hence K63-pUb/M1-pUb hybrids, may not only reduce the activation of the canonical IKK complex by preventing colocalization with TAK1, but also divert these TAK1 molecules to the activation of MAP kinase kinases, explaining why the IL-1-activated stimulation of JNK and p38 MAP kinase kinases was enhanced in MEFS from the HOIP[C879S] knock-in mice (Fig. 4A). An enhanced activation of JNK was also reported in MEFS from HOIL-1−/− deficient mice, in which the expression of HOIP was also reduced drastically (23).

It will clearly be of great interest to investigate whether the formation of K63-pUb/M1-pUb hybrids is of more general significance and whether they participate in the regulation of other biological processes dependent on K63-pUb chain formation, such as the cellular response to DNA damage (35).

Materials and Methods

NEMO, the ubiquitin-binding-defective mutant NEMO[D311N], a protein expressing two copies of the NZF domain of TAB2, and TUBEs (26) were expressed in Escherichia coli as Halo-tagged proteins. The bacteria were harvested; lysed in 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF); and sonicated. The bacterial cell lystate was centrifuged to remove debris, and the supernatant was coupled to the HaloLink resin (Promega) by incubation for 5 h at 4 °C as described by the manufacturer. The resin was washed extensively with 50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.1 mM EDTA, 270 mM sucrose, 0.03% (wt/vol) Brij 35, 0.1% (vol/vol) 2-mercaptoethanol, 0.2 mM PMSF, and 1 mM benzamidine and stored at 4 °C. To check coupling efficiency, an aliquot of the resin was resuspended in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT containing 0.1 μg/ml TEV protease to release covalently bound NEMO, NEMO[D311N], and the NZF domains of TAB2 or TUBEs followed by SDS/PAGE and staining with Coomassie Blue. All experimental procedures are described in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Xiaoxia Li for IL-1R cells, the DNA Sequencing Service of the Medical Research Council Protein Phosphorylation and Ubiquitinylation Unit (MRC-PPU), and the MRC-PPU’s teams for DNA cloning, protein production, tissue culture, and for outstanding technical support. The work was supported by a Wellcome Trust Senior Investigator Award (to P.C.); by grants from the Medical Research Council, AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck KGaA, Janssen Pharmaceutica, and Pfizer (to P.C.); and by grants from the Medical Research Council (UK) to the Institute for Cancer Research (ICR UK) and the Institute of Cancer Research (ICR UK) and the Wellcome Trust. This work was supported by the National Institutes of Health (DK029978) to W.G. and in part by a grant from the Canadian Institutes of Health Research (CIHR) to R.P. This work is dedicated to the late Tony Pawson whose discoveries inspired the research described in this paper.

11. Sato S, et al. (2005) Essential function for the kinase TAK1, but also divert these TAK1 molecules to the activation of MAP kinase kinases, explaining why the IL-1-activated stimulation of JNK and p38 MAP kinase kinases was enhanced in MEFS from the HOIP[C879S] knock-in mice (Fig. 4A). An enhanced activation of JNK was also reported in MEFS from HOIL-1−/− deficient mice, in which the expression of HOIP was also reduced drastically (23).
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SI Materials and Methods

Materials. HaloLink Resin and the pFN18A HaloTag T7 Flexi Vector were purchased from Promega. All DNA constructs and expression vectors were generated through the DNA cloning team, Division of Signal Transduction Therapy, Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, United Kingdom. DNA encoding the Npl4 Zinc Finger (NZF) domain of TAB2 (TAB2[644-692]) (NCBI NM_015093) was amplified from pGEX6p-1 TAB2 (1) as a BamHI BgIII fragment either with or without a C-terminal stop codon leaving the amino acid sequence GGSGGGG between the HALO tag and the first TAB2 domain and the sequence GGGRS between the first and second TAB2 NZF domains. The sources of antibodies are given below.

Antibodies. An antibody that recognizes phospho-Ser176/Ser180 of IkB kinase (IKKβ) and phospho-Ser177/Ser181 of IKKβ (#2697), anti-MyD88 (#4823), anti-GAPDH (#2118), anti-IKKα (#2682), anti-pSer533 p105 (#4806), anti-pThr180/pTyr182 p38 MAP kinase (#9211), and NfκB Essential Modifier (NEMO) (#2695), for immunoblotting were obtained from Cell Signaling Technology and anti-α-tubulin (F2168) from Sigma. A phospho-specific antibody that recognizes c-Jun N-terminal kinases (JNKs) phosphorylated at Thr183 and Tyr185 (#44682) and anti-Ubc13 (#37–1100) were obtained from Invitrogen. Anti-ubiquitin was from Dako (#Z0458), and anti-IRAK1 (H-273) and anti-TRAF6 (H-274) from Santa Cruz Biotechnology. Antibodies that recognize K63-Ub (HWA4C4), K11-Ub (MAB107, clone 2A3/2E6), and K48-Ub (05-1307, clone Aup2) linkages and anti-IRAK4 (#07-418) were from Merck-Millipore. The M1-pUb antibody (2) has been described previously. Antibodies against Heme-Oxidized IRP2 ubiquitin Ligase 1 (HOIL-1) (S150D, second bleed), HOIL-interacting protein (HOIP) (S174D, fourth bleed), SHARPIN (S209D, second bleed), and NEMO (S190C, second bleed) for immunoprecipitation were raised in sheep, and the anti-sera were affinity-purified on antigen-agarose columns by the Antibody Production Unit, University of Dundee, United Kingdom.

Proteins. Proteins were expressed as the full-length human sequences unless stated otherwise. The HOIP (and HOIP[C885S] mutant), HOIL-1, and Sharpin components of linear ubiquitin assembly complex (LUBAC), TRAF6, and IL-1β were expressed in Escherichia coli as GST-fusion proteins separated by a PreScission protease cleavage site and purified by chromatography on glutathione-Sepharose (GE Healthcare). GST-IL-1β was cleaved with PreScission protease to release IL-1β[117-268] and purified by gel filtration on Superdex G200. Murine IL-1α was purchased from Sigma-Aldrich. The ubiquitin-like modifier-activating enzyme (UBE1), the E2 ubiquitin-conjugating enzymes Ubc13-Uev1a (also called UBE2N-UBE2V1) and UbcH7 (also called UBE2L3), and the deubiquitylases AMSH-Like Protein (AMSH-LP[264-436]), and isoform B of USP2 were purchased from Ubiquigent Ltd. The deubiquitylase Outilin was expressed and purified as described (3). Ubiquitin was expressed and purified by the Protein Production Team of the Protein Phosphorylation and Ubiquitylation Unit, University of Dundee. K48-linked ubiquitin oligomers (K482-7), K63-linked ubiquitin oligomers (K632-7), or M1-linked ubiquitin oligomers (M12-7 or M18) were purchased from BostonBiochem. The protein phosphatase from bacteriophage λ (PPase) was obtained from New England Biolabs and trypsin (sequencing grade modified) was obtained from Promega.

Preparation of Halo-Tagged Proteins and Pull-Down Assays. NEMO, the ubiquitin binding-defective mutant NEMO[D311N], a protein expressing two copies of the NZF domain of TAB2, and tandem-repeated ubiquitin-binding entities (TUBEs) (4) were expressed in E. coli as Halo-tagged proteins. The bacteria were harvested; lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM benzamidine and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated. The bacterial cell lysate was centrifuged to remove debris, and the supernatant was coupled to the HaloLink resin (Promega) by incubation for 5 h at 4 °C as described by the manufacturer. The HaloLink resin was added at a ratio of 1 mL of resin per 10 mL of cleared cell lysate. The resin was washed extensively with 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1 mM EDTA, 270 mM sucrose, 0.03% (wt/vol) Brij 35, 0.1% (vol/vol) 2-mercaptoethanol, 0.2 mM PMSF, and 1 mM benzamidine and stored at 4 °C. To check coupling efficiency, an aliquot of the resin was resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT containing 0.1 μg/μL tobacco ETCH virus (TEV) protease to release covalently bound NEMO, NEMO[D311N], and the NZF domains of TAB2 or TUBEs followed by SDS/PAGE and staining with Coomassie Blue.

To capture M1-pUb and/or K63-pUb chains from the cell extracts, as well as the proteins to which these pUb chains were attached covalently or noncovalently, 2–3 mg [IL-1 receptor (IL-1R) cells) or 3 mg (THP1 cells) of cell extract protein were incubated for 4 h at 4 °C with Halo-linked ubiquitin-binding proteins (20 μL packed volume). The beads were washed three times with 1 mL of lysis buffer containing 500 mM NaCl and once with 1 mL of 10 mM Tris-HCl, pH 8.0. Proteins captured by the immobilized NEMO were released by denaturation in SDS and analyzed by immunoblotting.

Cell Culture and Cell Lysis. Mouse embryonic fibroblasts (MEFs) and HEK293 cells stably expressing the IL-1R (kindly provided by Xiaoxia Li and George Stark, Case Western Reserve University, Cleveland) (5) were maintained in DMEM supplemented with 10% (vol/vol) FBS, 2 mM l-glutamine and antibodies (100 units/mL penicillin, 0.1 mg/mL streptomycin) and were cultured at 37 °C in a 10% CO2 humidified atmosphere. The human monocye cell line THP1 was maintained in RPMI medium supplemented with 10% (vol/vol) FBS.

The IL-1R cells were stimulated with 5 ng/mL human IL-1β, MEFs with IL-1α, and THP1 cells with 1 μg/mL Pam3CSK₄ for the times indicated in the figure legends. Cells were rinsed in ice-cold PBS and extracted in lysis buffer [50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (vol/vol) Triton X-100, 0.27 M sucrose, 10 mM sodium 2-glycerophosphate, 0.2 mM PMSF, 1 mM benzamidine], and the protein phosphatase inhibitors 1 mM sodium ortho-vanadate, 50 mM NaF, 5 mM sodium pyrophosphate to prevent the dephosphorylation of proteins in the cell extracts. Unless otherwise indicated, 100 mM iodoacetamide was also included to inactivate deubiquitylation activities and prevent the deubiquitylation of proteins in the cell extracts. Cell lysates were centrifuged at 14,000 × g for 30 min at 4 °C and the supernatants (cell extracts) were collected and their protein concentrations determined by the Bradford procedure.

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Immuno blotting. Cell extracts or proteins captured by Halo- 
NEMO or immunoprecipitated by specific antibodies were dena- 
tured in lithium dodecyl sulfate (LDS), and the samples were run on 
4–12% gradient polyacrylamide gels (NuPAGE, Invitrogen). After 
transfer to PVDF membranes, and blocking with 5% (wt/vol) nonfat 
dry milk in TBST buffer [50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% (vol/vol) Tween 20], proteins were visualized by immu- 
noblotting using the ECL system (GE Healthcare).

**Generation of IL-1R Cells Stably Expressing shRNA.** The cells were generated by retroviral transduction using a Murine Moloney Leukemia virus-based system prepared with VSV-G envelope 
protein. Retrovirus particles were prepared according to the 
manufacturer’s instructions (Clontech). Viruses encoding the shRNA plasmids were harvested 24 and 48 h after transfection and incubated with IL-1R cells for 24 h in the presence of 2 
µg of 
20-probes. Correctly targeted 
0.01% (wt/vol) Brij-35 with or without USP2 
stimulated or un-
RNA TRCN0000168448 and 
μ-loxP site is inserted and generate 
transmitting chimeric mice were then crossed to 
to exon 18. The targeting vector also 
TTG and CCGGCCTCCAG- 
to exon 12 and 3 ' 
knock-in allele was bred away from the Flp 
μ-loxP site. The sequence of the targeting vector is available on request. The vector was electroporated into 57BL/6 
and, and following positive and negative selection, resistant clones 
were screened by PCR. Positive clones were confirmed by Southern blotting using both 5' and 3' probes. Correctly targeted 
ES cells were injected into blastocysts to generate chimeric mice. Germ-line–transmitting chimeric mice were then crossed to 
C57BL/6 Flp transgenic mice to remove the puromycin and neomycin resistance genes. Following deletion of the resistance 
genes, the Rnf31 knock-in allele was bred away from the Flp 
transgene in subsequent generations. Routine genotyping of the 
mice was carried out by PCR of ear biopsy samples using the 
primers CCTTTAAGTGTCTGCATGGG and CCTTTCTA- 
CCTAGAAGCCAAGC for the Rnf31 gene. These primers 
primers amplify the region where the 3' loxP site is inserted and generate 
a wild-type band of 183 bp and a knock-in band of 385 bp. The 
presence of the Flp transgene was determined by PCR using 
GCCAGAAGCGCTATTG and GACAAGCGTTAGTGTGTGTG- 
GTTTGG (Sigma MISSION shRNA TRCN0000168448 and 
and TRCN0000318499, respectively). The sequences of the shRNAs used for the HOIP knock-down were GCCGGCG- 
TGGTGCAGTTTATAACCTGAGATTAAAC TTG- 
ACACACCGTITTGTTGAGACATGAGTGTTGTGTTTGG (Sigma MISSION shRNA TRCN0000007599).

**Treatment with Deubiquitylating and Phosphatase.** The pUb chains and pUb proteins captured by Halo-NEMO 
beads were washed three times with 1 mL of lysis buffer containing 0.5 M NaCl and once with 1 mL of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM DTT. Following the last wash, the beads were resuspended in 30 µL of 50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM DTT, 1 mM MnCl2, 0.01% (wt/vol) Brij-35 with or without USP2 (1 µM), AMSh-LP (5 µM) or Otulin (1 µM), and with or without the addition of JPPase (100 units per reaction). After incubation for 60 min at 30 °C, incubations were terminated by denaturation in 1% (wt/vol) LDS. Eluted proteins were separated from the beads using Spin-X columns and subjected to SDS/PAGE. The protein gels were transferred to PVDF membranes and immu
noblotted with the appropriate antibodies.

**LUBAC E3 Ligase Activity Assay.** One microgram of anti-HOIP, anti- 
HOIL-1, or anti-Sharpin was incubated for 2 h at 4 °C with 
Protein G-Sepharose (10 µL packed beads) in 500 µL of 50 mM 
Tris-HCl, pH 7.5, and 0.2% (vol/vol) Triton X-100. The beads 
were washed three times with cell lysis buffer and incubated for 16 h at 4 °C with 1 mg of cell extract protein (cells lysed 
without iodoacetamide). The beads were collected by brief 
centrifugation, washed three times with 0.5 mL of 50 mM 
Tris-HCl, pH 7.5, 1% (vol/vol) Triton X-100, 0.05% (vol/vol) 
2-mercaptoethanol, and 0.2 M NaCl and once with 50 mM 
Tris-HCl, pH 7.5, and 5 mM MgCl2. The immunoprecipitated 
LUBAC complex was incubated at 30 °C for 3 min, and the E3 
ligase reaction was initiated by the addition of 30 µL of 20 mM 
Tris-HCl, pH 7.5, 2 mM DTT, 0.1 µM UBE1, 0.4 µM UbcH7, 
10 µM ubiquitin, 5 mM MgCl2, and 2 mM ATP. The reactions 
were stopped at various times by adding SDS to a final concen 
tration of 1%. The formation of M1-pUb chains was then ana 
yzed by immunoblotting with anti-ubiquitin.

Capture of Ubiquitin Oligomers by Polyubiquitin-Binding Proteins. 
GST-tagged proteins (4 µg) were immobilized on glutathione- 
Sepharose [10 µL packed beads equilibrated in 25 mM Hepes (pH 7.5), 1 mM EGTA, 2 mM MgCl2, 0.5% (vol/vol) Triton 
X-100] plus 150 mM NaCl and incubated for 20 min at 4 °C with 
0.5 mL of the same buffer. The beads were washed three times 
and then incubated for 1 h at 20 °C with 0.3 µL of purified pUb 
oligomers (2 µg) in the same buffer. The beads were washed five 
times with buffer plus 250 mM NaCl and once with buffer 
without NaCl and Triton X-100. Bound proteins were released 
by denaturation with 1% SDS or LDS, the beads were removed by 
centrifugation for 1 min at 2,000 × g, and the supernatants 
were heated for 5 min at 70 °C and subjected to SDS/PAGE.

**Generation of HOIP Knock-In Mice.** A Cys789Ser knock-in mutation was created in the Rnf31 gene encoding HOIP using standard 
techniques. Briefly, a targeting vector was generated using re-
combine-mediated cloning techniques. In addition to mutating 
Cys879 to Ser in exon 16, LoxP sites were introduced in the in-
trons 5' to exon 12 and 3' to exon 18. The targeting vector also 
contained an Ir-flanked neomycin resistance gene upstream of 
exon 12 and an E3-flanked paracrine-resistance gene down-
stream of exon 18 to allow for dual positive selection of targeted 
ES cells. The sequence of the targeting vector is available on request. The vector was electroporated into 57BL/6 
ES cells and, and following positive and negative selection, resistant clones 
were screened by PCR. Positive clones were confirmed by Southern blotting using both 5' and 3' probes. Correctly targeted 
ES cells were injected into blastocysts to generate chimeric mice. Germ-line–transmitting chimeric mice were then crossed to 
57BL/6 Flp transgenic mice to remove the puromycin and neomycin resistance genes. Following deletion of the resistance 
genes, the Rnf31 knock-in allele was bred away from the Flp 
transgene in subsequent generations. Routine genotyping of the 
mice was carried out by PCR of ear biopsy samples using the 
primers CCTTTAAGTGTCTGCATGGG and CCTTTCTA- 
CCTAGAAGCCAAGC for the Rnf31 gene. These primers 
primers amplify the region where the 3' loxP site is inserted and generate 
a wild-type band of 183 bp and a knock-in band of 385 bp. The 
presence of the Flp transgene was determined by PCR using 
GCCAGAAGCGCTATTG and GACAAGCGTTAGTGTGTGTG- 
GTTTGG (Sigma MISSION shRNA TRCN0000168448 and 
and TRCN0000318511, respectively). The sequence of the shRNA used for the HOIP knock-down was GCCGGCG- 
TGGTGCAGTTTATAACCTGAGATTAAAC TTG- 
ACACACCGTITTGTTGAGACATGAGTGTTGTGTTTGG (Sigma MISSION shRNA TRCN0000007599).

In-Solution Tryptic Digest and Mass Spectrometric Analysis. To identify NEMO-captured proteins by mass spectrometry, 50 mg of 
cell extract protein obtained from IL-1β–stimulated or un-
stimulated IL-1R cells were incubated for 16 h at 4 °C with 
0.4 mL immobilized Halo-NEMO or Halo-NEMO[D311N] as 
a control. The beads were washed three times with lysis buffer 
containing 500 mM NaCl and washed 10 times with 50 mM 
Tris-HCl, pH 7.5. Bound proteins were eluted from beads by 
icubation for 10 min with 50 mM Tris-HCl, pH 7.5, and 6 M 
guanidine hydrochloride. Samples were then diluted with 0.1 M 
ammonium bicarbonate to a final guanidine hydrochloride con-
centration of 1 M, incubated for 30 min at 45 °C with 5 mM 
DTT, and alkylated for 30 min at 20 °C in the dark with 50 mM 
iodoacetamide. Trypsin was then added to a final concentration 
of 3.75 µg/mL, and after incubation for 12 h at 37 °C the digests 
were made 1% (vol/vol) in trifluoroacetic acid (TFA) to terminate 
the reaction. The samples were desalted using Sep-Pak cartridges (WAT 
023501) equilibrated in 0.1% (vol/vol) TFA. Tryptic digests 
were applied to the Sep-Pak column and washed with 5 mL 0.1% (vol/vol) 
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TFA, and peptides were eluted with 1 mL 70% (vol/vol) acetonitrile/0.1% (vol/vol) TFA solution and taken to dryness. The dried peptides were resuspended in 20 μL 0.1% (vol/vol) TFA and separated on a Proxeon EASYn-LC system (Thermo Scientific) using a 15-cm long C18 column. Mass spectra were acquired on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) operating in data-dependent mode. After conversion to mzXML, the raw data were searched using X!Tandem with the K-score plug-in against version 3.87 of the International Protein Index human protein database using static carboxamidomethylation of cysteine residues and accounting for tryptic peptides with up to two missed cleavages. The Trans-Proteomic Pipeline was used to assign peptide and protein probabilities and to filter results at a 1% false discovery rate.


Fig. 51. Characterization of the Halo-NEMO pulldown system. (A) IL-1R cells were stimulated for 10 min with 5 ng/mL IL-1β and pUb chains, and associated proteins were captured using Halo-NEMO or the ubiquitin-binding defective mutant Halo-NEMO[D311N] as a control. After denaturation in LDS, SDS/PAGE, and transfer to a PVDF membrane, immunoblotting was carried out with antibodies that recognize K63-pUb chains, M1-pUb chains, and IKKα. (B) As in A, except that pUb chains in the cell extracts were captured using Halo-TUBE as well as Halo-NEMO. The gels were immunoblotted to detect IRAK1, K11-pUb chains, and K48-pUb chains.

Fig. 52. Specificities of the deubiquitylases AMSH-LP, Otulin, and USP2. The ability of Otulin (A) and AMSH-LP and USP2 (B) to hydrolyze K63-pUb chains (2.0 μg/mL) and M1-pUb chains (1.0 μg/mL) was studied in vitro (Materials and Methods). Ubiquitin chains were visualized by immunoblotting with an ubiquitin antibody.
Fig. S3. Characterization of proteins captured by Halo-NEMO from the extracts of THP1 monocytes and IL-1R cells. (A) THP1 monocytes were stimulated with 1 μg/mL Pam3CSK4, and the pUb chains were captured from the cell extracts with Halo-NEMO. After denaturation in LDS, followed by SDS/PAGE and transfer to PVDF membranes, the membranes were immunoblotted with antibodies that recognize MyD88, IRAK4, and IRAK1. (B) Characterization of the pUb chains attached covalently to TRAF6. The experiment was performed as in Fig. 2 B except that the proteins captured by Halo-NEMO were immunoblotted for TRAF6. (C) The experiment was performed as in A, except that IL-1R cells were stimulated with 5 ng/mL IL-1β. The captured proteins were incubated without (control) or with USP2, and, after their release by denaturation in SDS, the HOIP, HOIL-1, and Sharpin components of LUBAC were identified by immunoblotting. The monoubiquitylated form of Sharpin is denoted by mUb-Sharpin. NS, nonspecific bands. (D) As in B, except that the endogenous NEMO was immunoprecipitated from 2 mg of cell extract protein with a specific antibody (3 μg). The immunoprecipitates were incubated without (control) or with AMSH-LP, Otulins, or USP2 and immunoblotted for NEMO. The immunoblot shown in the upper panel was exposed for 10 times longer than in the lower panel.
Fig. S4. Generation of knock-in mice expressing the HOIP[C879S] mutant. (A) HOIP knock-in mice expressing the HOIP[C879S] mutant instead of the wild-type protein were generated by mutating Cys879 to Ser in exon 16 of the Rnf31 gene encoding mouse HOIP. A diagram of the targeting strategy is shown, and further details are given in SI Materials and Methods. Correct targeting was confirmed by Southern blot analysis using 5′ and 3′ probes on Sex A1- or Psi I-digested DNA, respectively. The location of the probes and Sex A1 (S) and Psi I (P) sites and representative Southern blots are shown. (B) Cys885 in human HOIP is equivalent to Cys879 in the murine protein. The full-length, wild-type human HOIP and the HOIP[C885S] mutant were expressed as GST-tagged proteins in E. coli, and their E3 ligase activities were measured using the ubiquitylation assay described in Materials and Methods.

Fig. S5. LUBAC activity is not increased by stimulation with IL-1. (A) IL-1R cells were stimulated for 15 min without (−) or with (+) 5 ng/mL human IL-1β, and the cells were lysed in the absence of iodoacetamide. LUBAC was immunoprecipitated from the cell extracts with anti-HOIP, and the LUBAC-catalyzed formation of M1-Ub oligomers at 30 °C was monitored at the times indicated by immunoblotting with anti-ubiquitin. Aliquots of the cell extract (25 μg protein) were subjected to SDS/PAGE and immunoblotted with anti-tubulin (as a loading control) and with an antibody that recognizes the phosphorylated form of the IKKβ substrate p105. (B) As in A, except that preimmune IgG was used for immunoprecipitation (lane 4) as well as anti-HOIP (lanes 1–3). Lane 5, same as lane 2, except that the ubiquitylation reaction was not performed. The immunoprecipitates were also immunoblotted with anti-HOIP (lower). (C) IL-1R cells stably expressing shRNA specific for Ubc13 (Fig. 5) were retransfected with shRNA-resistant DNA encoding FLAG-Ubc13, which restored the IL-1-stimulated formation of K63-pUb and M1-pUb chains and the phosphorylation of p105 and JNK. Further details are given in the legend to Fig. 5.

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Fig. S6. How K63/M1-pUb hybrid chains may be formed by IL-1–stimulated signaling complexes. IL-1 stimulation leads to the activation of TRAF6, which forms a productive complex with Ubc13-Uev1a to produce K63-pUb chains. LUBAC is recruited to the IL-1–signaling complex and via a specific interaction of its catalytic component HOIP with K63-ub linkages catalyzes the transfer of ubiquitin to a K63-ub oligomer, thereby forming a K63/M1-ub hybrid. The M1-pUb linkages can then be elongated by LUBAC. The lengths of both the M1-ub oligomers and the K63-ub oligomers within the hybrids are variable, and whether every ubiquitin molecule is linked via both K63 and M1 or whether K63-pUb oligomers can be added to preformed M1-ub linkages is unknown. The K63-pUb/M1-pUb hybrids may not be attached to any other protein as shown in the figure or may be attached covalently to IRAK1 and perhaps other proteins.

Table S1. Proteins captured by NEMO from the extracts of IL-1β–stimulated cells: Identification of proteins binding only to Halo-NEMO upon IL-1β stimulation

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<th>IL-1β</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>NEMO</td>
<td>NEMO(D311N)</td>
</tr>
<tr>
<td>HOIL-1</td>
<td>0</td>
<td>21 (52)</td>
<td>0</td>
</tr>
<tr>
<td>HOIP</td>
<td>0</td>
<td>9 (13.5)</td>
<td>0</td>
</tr>
<tr>
<td>IRAK1</td>
<td>0</td>
<td>51 (64.7)</td>
<td>0</td>
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<tr>
<td>IRAK4</td>
<td>0</td>
<td>26 (57.8)</td>
<td>0</td>
</tr>
<tr>
<td>MyD88</td>
<td>0</td>
<td>12 (55.7)</td>
<td>0</td>
</tr>
<tr>
<td>Sharpin</td>
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<td>7 (15.5)</td>
<td>0</td>
</tr>
<tr>
<td>TRAF6</td>
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<td>24 (58.8)</td>
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</tr>
</tbody>
</table>

The number of unique peptides identified in each protein are shown for two independent experiments (second experiment highlighted in boldface type). Numbers in parentheses represent the percentage of the amino acid sequence of each protein that was identified in the mass spectrometric analysis. 0, no peptides from these proteins were detected.
Table S2. Proteins captured by NEMO from the extracts of IL-1β–stimulated cells: Identification of proteins binding much more strongly to Halo-NEMO upon IL-1β stimulation

<table>
<thead>
<tr>
<th>Protein name</th>
<th>no IL-1β</th>
<th>IL-1β</th>
<th>IL-1β[D311N]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEMO</td>
<td>NEMO</td>
<td></td>
</tr>
<tr>
<td>TAB1</td>
<td>0</td>
<td>3 (7.1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5 (15)</td>
<td>25 (69.8)</td>
<td>7 (24.2)</td>
</tr>
<tr>
<td>TAB2</td>
<td>1 (2.8)</td>
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<tr>
<td></td>
<td>2 (4.9)</td>
<td>19 (40.3)</td>
<td>2 (6.4)</td>
</tr>
<tr>
<td>TAB3</td>
<td>0</td>
<td>4 (10.2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 (18.6)</td>
<td></td>
</tr>
<tr>
<td>TAK1</td>
<td>1 (5.4)</td>
<td>8 (18.8)</td>
<td>2 (9.6)</td>
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<tr>
<td></td>
<td>1 (4.2)</td>
<td>26 (43.4)</td>
<td>3 (12.7)</td>
</tr>
</tbody>
</table>

The number of unique peptides identified in each protein are shown for two independent experiments (second experiment highlighted in boldface type). Numbers in parentheses represent the percentage of the amino acid sequence of each protein that was identified in the mass spectrometric analysis. 0, no peptides from these proteins were detected.

Table S3. Proteins captured by NEMO from the extracts of IL-1β–stimulated cells: Identification of proteins binding similarly to Halo-NEMO and to Halo-NEMO [D311N]

<table>
<thead>
<tr>
<th>Protein name</th>
<th>no IL-1β</th>
<th>IL-1β</th>
<th>IL-1β[D311N]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEMO</td>
<td>NEMO</td>
<td></td>
</tr>
<tr>
<td>IKKα</td>
<td>0</td>
<td>6 (9.9)</td>
<td>7 (10.1)</td>
</tr>
<tr>
<td></td>
<td>13 (20.7)</td>
<td>55 (56.5)</td>
<td>38 (48.3)</td>
</tr>
<tr>
<td>IKKβ</td>
<td>10 (15)</td>
<td>13 (19)</td>
<td>24 (36.1)</td>
</tr>
<tr>
<td></td>
<td>23 (35)</td>
<td>45 (64.3)</td>
<td>37 (48.5)</td>
</tr>
</tbody>
</table>

The number of unique peptides identified in each protein are shown for two independent experiments (second experiment highlighted in boldface type). Numbers in parentheses represent the percentage of the amino acid sequence of each protein that was identified in the mass spectrometric analysis. 0, no peptides from these proteins were detected.