# Supporting Information

## Emmerich et al. 10.1073/pnas.1314715110

### 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 by 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 BglII fragment either with or without a C-terminal stop codon leaving the amino acid sequence GGSGGG 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-$Ser^{176}$/$Ser^{180}$ of IκB kinase (IKKα) and phospho-$Ser^{177}$/$Ser^{181}$ of IKKβ (#2697), anti-MyD88 (#4823), anti-GAPDH (#2118), anti-IKKα (#2682), anti-$pSer^{933}$ p105 (#4806), anti-$pThr^{180}$/$pTyr^{182}$ 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-pUb (MABS107, clone 2A3/2E6), and K48-pUb (05-1307, clone Apu2) 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 Team of the Division of Signal Transduction Therapy, Medical Research Council Protein Phosphorylation and Ubiquitylation 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 Otulin 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 ($K48_{2–7}$), K63-linked ubiquitin oligomers ($K63_{2–7}$), or M1-linked ubiquitin oligomers ($M1_{2–7}$ or $M1_9$) were purchased from BostonBiochem. The protein phosphatase from bacteriophage λ (λPPase) 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 antibiotics (100 units/mL penicillin, 0.1 mg/mL streptomycin) and were cultured at 37 °C in a 10% $CO_2$ humidified atmosphere. The human monocyte 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 $Pam_3CSK_4$ 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 deubiquitylase activities and prevent the deubiquitylation of proteins in the cell extracts. Cell lysates were clarified by centrifugation 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.

**Immunoblotting.** Cell extracts or proteins captured by Halo-NEMO or immunoprecipitated by specific antibodies were denatured 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 immunoblotting 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/mL protamine sulfate (Sigma). Fresh media containing 2 μg/mL puromycin was added to select the transduced cells. The sequences of the shRNAs used for Ubc13 knock-down were CCGGCCATAGAAACAGCTAGAGCATCTCGAGATGCTCTAGCTGTTTCTATGGTTTTTG and CCGGCCTTCCAGAAGAATACCCAATCTCGAGATTGGGTATTCTTCTGGAAGGTTTTTG (Sigma MISSION shRNA TRCN0000349623 and TRCN0000318499, respectively). The sequences of the shRNAs used for the HOIP knock-down were CCGGGCGTGGTGTCAAGTTTAATAACTCGAGTTATTAAAC TTGACACCACGCTTTTTTG and CCGGTAATCCTGCAAGTGCTCATTTCTCGAGAAATGAGCACTTGCAGGATTATTTTTTG (Sigma MISSION shRNA TRCN0000168448 and TRCN0000250111, respectively). The sequence of the shRNA used for HOIL-1 knock-down was CCGGCCACAACACTCATCTGTCAAACTCGAGTTTGACAGATGAGTGTTGTGGTTTTTG (Sigma MISSION shRNA TRCN0000007599).

**Treatment with Deubiquitylase 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 $MnCl_2$, 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 λPPase (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 immunoblotted 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 $MgCl_2$. 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 $MgCl_2$, and 2 mM ATP. The reactions were stopped at various times by adding SDS to a final concentration of 1%. The formation of M1-pUb chains was then analyzed 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 $MgCl_2$, 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 mL 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 Cys879Ser knock-in mutation was created in the *Rnf31* gene encoding HOIP using standard techniques. Briefly, a targeting vector was generated using recombinase-mediated cloning techniques. In addition to mutating Cys879 to Ser in exon 16, LoxP sites were introduced in the introns 5′ to exon 12 and 3′ to exon 18. The targeting vector also contained an frt-flanked neomycin resistance gene upstream of exon 12 and an f3-flanked puromycin-resistance gene downstream 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 C57BL/6 ES cells 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*e* 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*e* transgene in subsequent generations. Routine genotyping of the mice was carried out by PCR of ear biopsy samples using the primers CCTTAAGTGTCTGCTCATGGG and CCTTTCTACCTAGAAGGCAAGC for the *Rnf31* gene. These 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*e* transgene was determined by PCR using GGCAGAAGCACGCTTATCG and GACAAGCGTTAGTAGGCACAT as primers, which give a 343-bp band for samples containing the transgene. Mice were maintained on a C57BL/6 background in individually ventilated cages under specific pathogen-free conditions and housed in line with UK and European Union regulations. All procedures were carried out under a UK Government Home Office project license and approved by the University of Dundee Ethical Review Committee.

**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 unstimulated 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 incubation 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 concentration 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 3 mL 0.1% (vol/vol)

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 $C_{18}$ 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.


1. Cheung PC, Nebreda AR, Cohen P (2004) TAB3, a new binding partner of the protein kinase TAK1. *Biochem J* 378(Pt 1):27–34.
2. Matsumoto ML, et al. (2012) Engineering and structural characterization of a linear polyubiquitin-specific antibody. *J Mol Biol* 418(3–4):134–144.
3. Keusekotten K, et al. (2013) OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell* 153(6):1312–1326.
4. Hjerpe R, et al. (2009) Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep* 10(11):1250–1258.
5. Li X, et al. (1999) Mutant cells that do not respond to interleukin-1 (IL-1) reveal a novel role for IL-1 receptor-associated kinase. *Mol Cell Biol* 19(7):4643–4652.


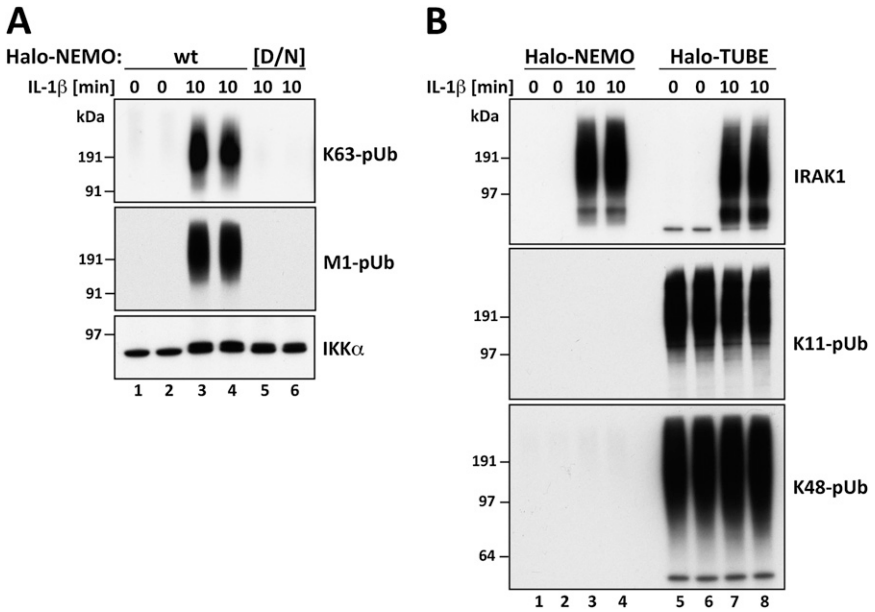


**Fig. S1.** Characterization of the Halo-NEMO pulldown system. (*A*) IL-1R cells were stimulated for 10 min with 5 ng/mL IL-1β and pUb chains, and associated proteins were captured using Halo-NEMO or the ubiquitin-binding defective mutant Halo-NEMO[D311N] as a control. After denaturation in LDS, SDS/PAGE, 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.

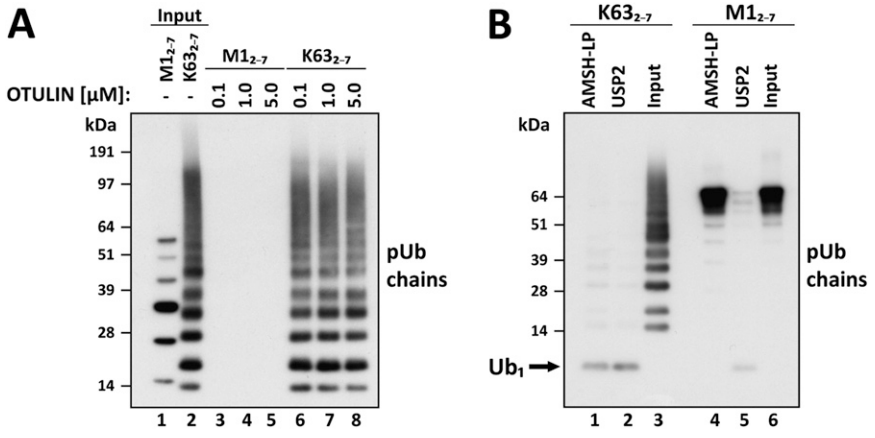


**Fig. S2.** Specificities of the deubiquitylases AMSH-LP, Otulin, and USP2. The ability of Otulin (*A*) and AMSH-LP and USP2 (*B*) to hydrolyze K63-pUb chains (2.0 μg/mL) and M1-pUb chains (1.0 μg/mL) was studied in vitro (*Materials and Methods*). Ubiquitin chains were visualized by immunoblotting with an ubiquitin antibody.

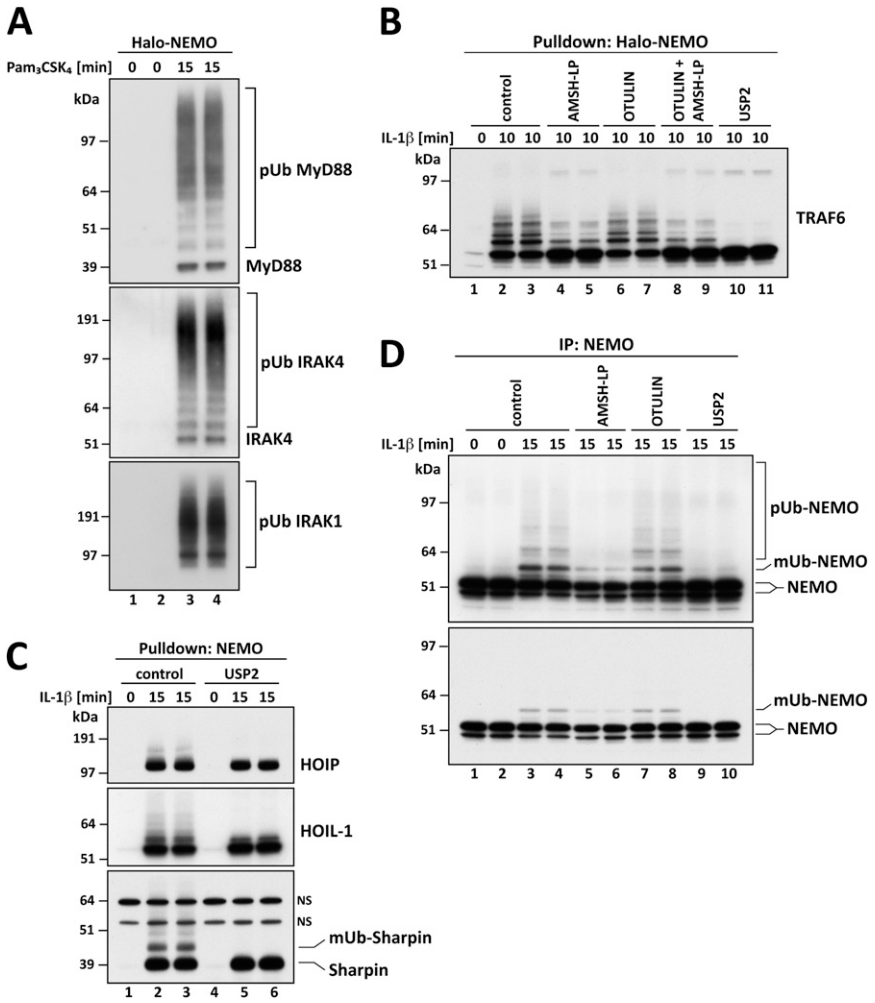


**Fig. S3.** Characterization of proteins captured by Halo-NEMO from the extracts of THP1 monocytes and IL-1R cells. (*A*) THP1 monocytes were stimulated with 1 μg/mL $Pam_3CSK_4$, and the pUb chains were captured from the cell extracts with Halo-NEMO. After denaturation in LDS, followed by SDS/PAGE 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, Otulin, or USP2 and immunoblotted for NEMO. The immunoblot shown in the upper panel was exposed for 10 times longer than in the lower panel.

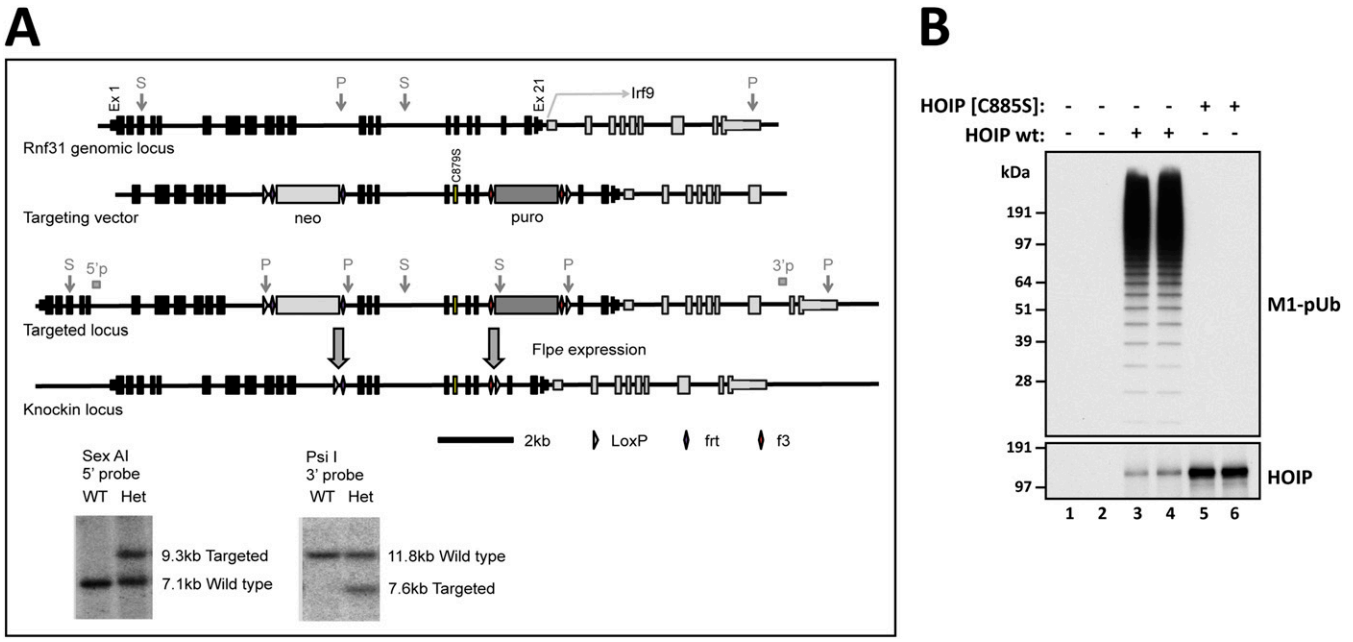


**Fig. S4.** Generation of knock-in mice expressing the HOIP[C879S] mutant. (*A*) HOIP knock-in mice expressing the HOIP[C879S] mutant instead of the wild-type protein were generated by mutating Cys879 to Ser in exon 16 of the Rnf31 gene encoding mouse HOIP. A diagram of the targeting strategy is shown, 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 AI (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*.

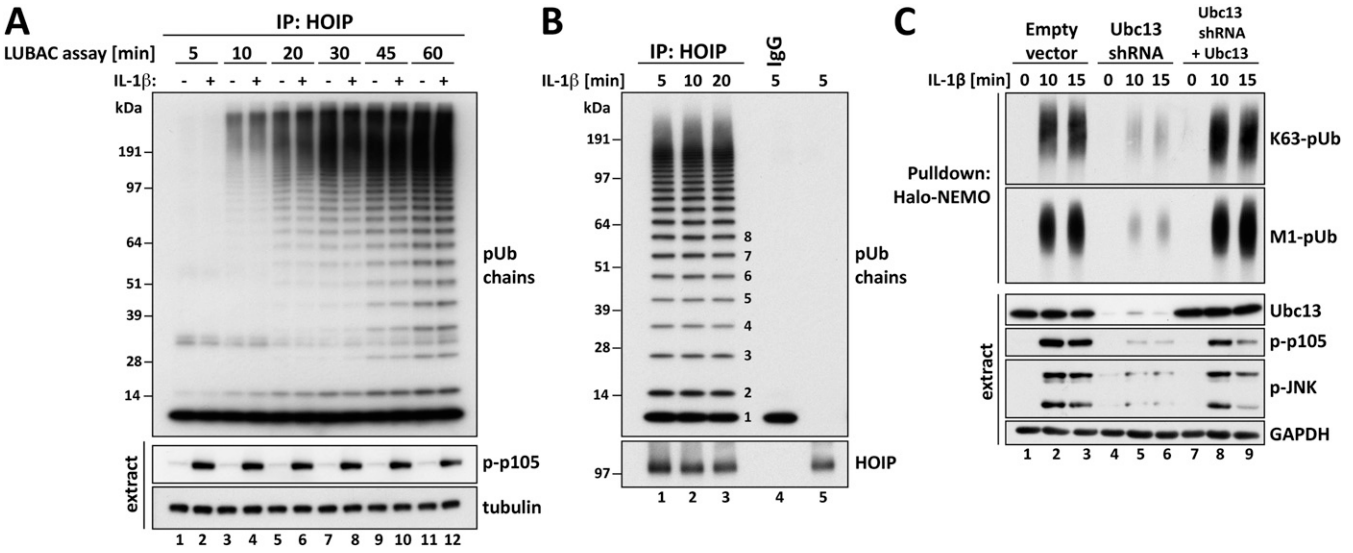


**Fig. S5.** LUBAC activity is not increased by stimulation with IL-1. (*A*) IL-1R cells were stimulated for 15 min without (–) or with (+) 5 ng/mL human IL-1β, and the cells were lysed in the absence of iodoacetamide. LUBAC was immunoprecipitated from the cell extracts with anti-HOIP, and the LUBAC-catalyzed 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.

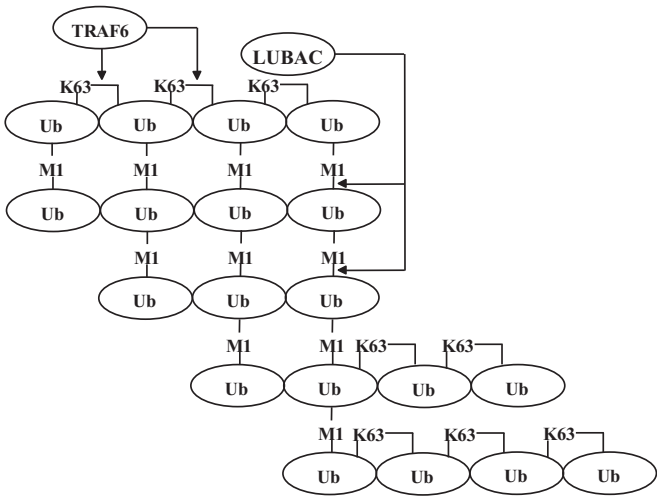


**Fig. S6.** How K63/M1-pUb hybrid chains may be formed by IL-1–stimulated signaling complexes. IL-1 stimulation leads to the activation of TRAF6, which forms a productive complex with Ubc13-Uev1a to produce K63-pUb chains. LUBAC is recruited to the IL-1–signaling complex and via a specific interaction 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**

| Protein name | no IL-1β NEMO | IL-1β NEMO | IL-1β NEMO[D311N] |
|---|---|---|---|
| HOIL-1 | 0 | 21 (52) | 0 |
| | | **19 (59.2)** | |
| HOIP | 0 | 9 (13.5) | 0 |
| | | **46 (53.4)** | |
| IRAK1 | 0 | 51 (64.7) | 0 |
| | | **57 (57.7)** | |
| IRAK4 | 0 | 26 (57.8) | 0 |
| | | **23 (60)** | |
| MyD88 | 0 | 12 (55.7) | 0 |
| | | **22 (65)** | |
| Sharpin | 0 | 7 (15.5) | 0 |
| | | **22 (66.1)** | |
| TRAF6 | 0 | 24 (58.8) | 0 |
| | | **52 (71)** | |

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**

| Protein name | no IL-1β NEMO | IL-1β NEMO | IL-1β NEMO[D311N] |
|---|---|---|---|
| TAB1 | 0 | 3 (7.1) | 0 |
| | **5 (15)** | **25 (69.8)** | **7 (24.2)** |
| TAB2 | 1 (2.8) | 4 (8.1) | 2 (4.9) |
| | **2 (4.9)** | **19 (40.3)** | **2 (6.4)** |
| TAB3 | 0 | 4 (10.2) | 0 |
| | | **8 (18.6)** | |
| TAK1 | 1 (5.4) | 8 (18.8) | 2 (9.6) |
| | **1 (4.2)** | **26 (43.4)** | **3 (12.7)** |

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]**

| Protein name | no IL-1β NEMO | IL-1β NEMO | IL-1β NEMO[D311N] |
|---|---|---|---|
| IKKα | 0 | 6 (9.9) | 7 (10.1) |
| | **13 (20.7)** | **55 (56.5)** | **38 (48.3)** |
| IKKβ | 10 (15) | 13 (19) | 24 (36.1) |
| | **23 (35)** | **45 (64.3)** | **37 (48.5)** |

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.