Supplemental Information

Cooperative Domain Formation by Homologous Motifs
in HOIL-1L and SHARPIN Plays A Crucial Role
in LUBAC Stabilization

Hiroaki Fujita, Akira Tokunaga, Satoshi Shimizu, Amanda L. Whiting, Francisco Aguilar-Alonso, Kenji Takagi, Erik Walinda, Yoshiteru Sasaki, Taketo Shimokawa, Tsunehiro Mizushima, Izuru Ohki, Mariko Ariyoshi, Hidehito Tochio, Federico Bernal, Masahiro Shirakawa, and Kazuhiro Iwai
Figure S1. Generation of HOIL-1L-null mice and their phenotype (Related to Figure 1)

(A) Guide RNA sequences against mHOIL-1L used for generation of knockout mice.

(B) Mutated allele #1 was obtained using guide RNA#1, and mutated allele #2 was obtained using guide RNA#2.

(C) Number of offspring of each genotype obtained by crossing HOIL-1L+/null#2 mice.

(D) Numbers of embryos obtained at each embryonic stage (E9.5, 10.5, 11.5, and 12.5) by crossing HOIL-1L+/null#2 mice.

(E and F) Representative images of TUNEL staining of paraffin-sectioned embryos of the indicated genotypes at E10.5 (E) and quantification of TUNEL-positive cells in embryos of the indicated genotypes (n = 3) (F). Data are expressed as means ± s.e.m. (Significance: **, p < 0.01).

(G) Representative images of whole-mount staining of embryos of indicated genotypes with anti-CD31 antibody at E10.5.

(H) Schematic representation of domains of HOIL-1L and HOIL-N.

(I) RT-PCR analysis of expression levels of the N-terminal and C-terminal parts of HOIL-1L in MEF cells derived from HOIL-1L−/− or WT (+/+) mice.
Figure S2. Role of HOIL-1L and SHARPIN in LUBAC stabilization (Related to Figure 2)

(A) Guide RNA sequences against mHOIP or mHOIL-1L. TKO MEFs carry one allele with a 1 bp deletion and one allele with an 11 bp deletion in the HOIL-1L locus, and one allele with a 2 bp deletion and one allele with a 1 bp deletion in the HOIP locus. The protospacer-adjacent motif (PAM) sequence is depicted in red.

(B) Immunoblot analysis of lysates from TKO MEFs.

(C and D) GST-mHOIP$_{466-630}$ was immobilized on a SPR sensor chip via a GST antibody. Binding between mHOIP$_{466-630}$ and MBP-mSHARPIN UBL$_{163-301}$ (C) or MBP-mHOIL-1L UBL$_{1-140}$ (D) was evaluated.

(E) Conservation residues in N-terminal regions of UBLs of HOIL-1L and SHARPIN.

(F) Binding affinity between mHOIP$_{466-630}$ and UBLs of mSHARPIN or mHOIL-1L containing or lacking N-terminal regions was analyzed.
Figure S3. Purification of the LUBAC ternary complex core, and comparison of the trimeric core and binary complexes (Related to Figure 3)

(A) Elution profile of size-exclusion chromatography of the LUBAC ternary complex core (red line). The elution profile of standard molecular weight markers is presented as a black dotted line. Protein fractions in the main peak (indicated by a blue line) were analyzed by SDS-PAGE (theoretical molecular weights: HOIP, 17,972; HOIL-1L, 15,744; SHARPIN, 19,903). Asterisk indicates an artifact of SDS-PAGE.

(B) Molecular weight estimation of the LUBAC ternary complex core, based on elution volume in analytical size exclusion column chromatography (A). Partition coefficients (K_{av}) of the trimeric LUBAC core and standard proteins were calculated from their elution volumes. The calibration curve was generated by plotting K_{av} of each standard protein against a logarithm of its molecular weight. The molecular weight of the trimeric LUBAC core was estimated as 51.3 kDa, indicating that the three subunits are assembled in a 1:1:1 stoichiometry (theoretical molecular weight: 53,619).

(C) MS spectra of the protein fraction in (A). The three components co-existed in a single fraction.

(D) Comparison of UBL-binding mode between UBA1 and UBA2 in the ternary complex core. The α1 helix in UBA1 binds the surface of the conserved hydrophobic patch on the β-sheet of the mSHARPIN UBL, whereas α7 helix of UBA2 contacts with the opposite surface of the β-sheet of the mHOIL-1L UBL.

(E) UBA–UBL binding modes observed in the crystal structures of the human HOIP/SHARPIN (left panel, PDB:5X0W) and HOIP/HOIL-1L (right panel, PDB:4DBG) binary complexes. The UBAs are colored as in Figure 3E and 3F, whereas the UBLs are in white.

(F) Interface between mHOIP UBA1 and mSHARPIN UBL. Each subunit is colored as in Figure 3. The interface is mainly formed between helices α1–α2 of mHOIP UBA1 and strands β4–β6 of mSHARPIN UBL. mHOIP and mSHARPIN residues involved in the interaction are shown in stick models. Hydrogen bonds are indicated by red dotted lines.
Figure S4. The difference between mouse and human HOIP UBA1 is critical for SHARPIN-mediated HOIP stabilization (Related to Figures 2 and 3)

(A) Cell lysates of TKO MEFs expressing the indicated proteins were probed with indicated antibodies.

(B) Sequence alignment of human and mouse HOIP D-UBA. Amino acid residues of mHOIP Q490 and the corresponding residue of hHOIP are enclosed in a red box.

(C) Schematic representation of mHOIP, hHOIP, and hD-UBA. Cell lysates of TKO MEFs reconstituted with indicated proteins were probed as indicated.

(D) Structural comparison of the HOIP UBA1/SHARPIN UBL interfaces of mouse and human. Helix α1 of mHOIP UBA is superimposed onto that of hHOIP UBA, which is bound to hSHARPIN UBL. Surface charge (red, negative; blue, positive) of hSHARPIN UBL is shown. Side chains of UBAs are shown in stick models. Close-up views around R496 of hHOIP and Q490 of mHOIP are shown in the right panels. Hydrogen bonds and electrostatic interactions are indicated as red dotted lines.

(E) The indicated expression plasmids and 5× NF-κB luciferase reporter were transfected into HEK293T cells. NF-κB activity was measured by luciferase assays (mean ± s.e.m., n = 3).

(F) Conserved residues in HOIP UBA1 of various species.
Figure S5. Characterization of LTM mutants (Related to Figures 4 and 5)

(A) Guide RNA sequence against hHOIP. HEK293T HOIP KO cells were homozygous for a 1 bp insertion (depicted in green). The protospacer-adjacent motif (PAM) sequence is depicted in red.

(B) Immunoblot analysis of HEK293T HOIP KO cells. Cell lysates of parent and HEK293T HOIP KO cells were immunoblotted with the indicated antibodies.

(C) The indicated expression plasmids were transfected into HEK293T HOIP KO cells. Cell lysates and anti-FLAG immunoprecipitates were probed as indicated.

(D) The indicated expression plasmids were transfected into HEK293T HOIP KO cells. Cell lysates and anti-FLAG immunoprecipitates were probed as indicated.

(E and F) Conserved residues in the LTM regions of HOIL-1L (E) and SHARPIN (F) from various species.
Figure S6. Therapeutic potential of SHARPIN-LTM peptide against ABC-DLBCL cell lines (Related to Figure 6)

(A–C) HBL1 cells were treated for 2 h with 20 µM of each peptide. After incubation, cells were stimulated with TNF-α (5 ng/ml) for the indicated periods. Cell lysates were analyzed for IκBα levels by immunoblotting (A), NF-κB activity by TransAM® NF-κB p65 assay (B), and secreted IL-8 by ELISA (means ± s.e.m., n = 3) (C).

(D) Reduction of HOIP by destabilization of LUBAC seems to be mediated by ubiquitin-proteolytic pathway. SHARPIN<sup>cpdm/cpdm</sup> MEFs were treated with CHX (20 µg/ml) in the presence of MLN-7243 (10 µM) or E64d/pep (10 µg/ml) for the indicated periods. Cell lysates were probed by immunoblotting.
Table S1  Dissociation constants ($K_d$) from SPR experiments (Related to Figures 4 and S2)

| Interactions                        | $K_d$    |
|-------------------------------------|----------|
| HOIP UBA / SHARPIN UBL              | 16.0±2.3 μM |
| HOIP UBA / HOIL-1L UBL              | 1.34±0.87 μM |
| HOIL-1L LTM-UBL / SHARPIN LTM-UBL   | 2.04±0.29 μM |
Table S2  Data collection and refinement statistics (Related to Figure 3)

| Data collection          |             |
|--------------------------|-------------|
| Wavelength (Å)           | 0.98000     |
| Resolution (Å)           | 48.18–2.40 (2.44–2.40) |
| Space group              | C2          |
| Cell dimensions          |             |
| $a, b, c$ (Å)            | 143.902, 59.732, 58.554 |
| $\alpha, \beta, \gamma$ (°) | 90.00, 97.42, 90.00 |
| Total measurements       | 87,510      |
| Unique cell reflections  | 19,275      |
| Average redundancy       | 4.5 (4.2)   |
| $I/\sigma$               | 14.2 (1.9)  |
| Completeness (%)         | 99.3 (95.5) |
| $R_{\text{merge}}$       | 0.092 (0.520) |

| Refinement               |             |
| Resolution (Å)           | 48.18–2.40  |
| No. reflections          | 17862       |
| $R_{\text{work}}$ (%)    | 18.8        |
| $R_{\text{free}}$ (%)    | 24.4        |
| No. atoms                | 3357        |
| Protein                  | 3326        |
| Water                    | 31          |
| Average B-factor (Å²)    | 64.8        |
| r.m.s.d.                 |             |
| Bond length (Å)          | 0.013       |
| Bond angles (°)          | 1.692       |
| Ramachandran plot        |             |
| Residues in favored region (%) | 96.2       |
| Residues in allowed region (%) | 3.6        |
| Residues in outlier region (%) | 0.2        |

Values in parentheses correspond to the highest-resolution shell.
Table S3. RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-mouse HOIP     | Tokunaga et al., 2009 | N/A         |
| Anti-human HOIP, 1CB2 | Merck Millipore | Cat# MABE1122 |
| Anti-human HOIP     | Aviva Systems Biology | Cat# ARP43241_P050 |
| Anti-HOIL-1L, 2E2   | Merck Millipore | Cat# AP1140 |
| Anti-HOIL-1L, N-terminal | This study | N/A         |
| Anti-SHARPIN        | Merck Millipore | Cat# ABF128 |
| Anti-SHARPIN        | Abcam   | Cat# ab125188 |
| Anti-phospho IκBα   | Cell Signaling Technology | Cat# 9246 |
| Anti-IκBα           | Cell Signaling Technology | Cat# 4812 |
| Anti-IκBα, C-21     | Santa Cruz Biotechnology | Cat# sc-371 |
| Anti-Caspase3       | Cell Signaling Technology | Cat# 9662 |
| Anti-Ubiquitin, P4D1 | Santa Cruz Biotechnology | Cat# sc-8017 |
| Anti-β-actin        | SIGMA   | Cat# A5316 |
| Anti-β-actin        | SIGMA   | Cat#A2228 |
| Anti-β-tubulin      | CEDARLANE | Cat# CLT9002 |
| Anti-GADPH          | BIOLEGEN Brand | Cat# 631402 |
| Anti-CD31, clone MEC13.3 | BioLegends | Cat# 102502 |
| Anti-HA, Y11        | Santa Cruz Biotechnology | Cat# sc-805 |
| Anti-HA, Tana2      | MBL     | Cat# M180-3 |
| Anti-Myc, 4A6       | Merck Millipore | Cat# 05-724 |
| Anti-Myc, 9E10      | In house | N/A         |
| Anti-FLAG, M2       | SIGMA   | Cat# F3165 |
| Anti-FLAG           | SIGMA   | Cat# F7425 |
| Anti-DDDDK-tag      | MBL     | Cat# PM020 |
| strepMAB-immo       | iba     | Cat# 2-1517-001 |
| Bacterial and Virus Strains |        |            |
| DH5α                | N/A     | N/A        |
| BL21-CodonPlus(DE3)-RIPL Competent Cells | Agilent Technologies | Cat# 230280 |
| BL21(DE3) Competent Cells | Novagen | Cat# 69450 |
| Rosetta 2(DE3) Competent Cells | Novagen | Cat# 71397 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| Cycloheximide       | Calbiochem | Cat# 239674 |
| ATP                 | GE Healthcare | Cat# 272056 |
| puromycin           | SIGMA   | Cat# P9620 |
| G-418               | NacalaiTesque | Cat# 09380-44 |
| Blasticidin         | invivoGen | Cat# ant-bl-1 |
| Dithiothreitol      | Nacalai Tesque | Cat# 14112-94 |
| DNasel              | Roche   | Cat# 10104159001 |
| Complete, EDTA-free | Roche   | Cat# 05056489001 |
| Name                          | Source                        | Code    |
|-------------------------------|-------------------------------|---------|
| IRDye 700 NF-kB consensus oligonucleotide | LI-COR Biosciences           | Cat# 829-07924 |
| MLN-7243                      | Active Biochem                | Cat# A-1384 |
| E-64-d                        | Peptide Institute             | Cat# 4321-v |
| PepstatinA                    | Peptide Institute             | Cat# 4397-v |
| HOIP-N peptide                | Yang et al., 2014             | N/A     |
| SHARPIN unstapled peptide     | This study                    | N/A     |
| SHARPIN-LTM peptide           | This study                    | N/A     |
| Recombinant mouse TNF         | R&D systems                   | Cat# 410-MT |
| Recombinant human TNF         | Promega                       | Cat# G524A |
| Recombinant TNF               | PEPROTECH                     | Cat# 300-01A |
| Recombinant human LUBAC       | Tokunaga et al., 2011         | N/A     |
| Recombinant E1                | Kirisak, et al., 2006         | N/A     |
| Recombinant UbCh7             | Kirisak, et al., 2006         | N/A     |
| Recombinant Ubiquitin         | BostonBiochem                 | Cat# U-100H |
| GST-mHOIP UBA (aa 466-630) WT | This study                    | N/A     |
| GST-mHOIP UBA (aa 466-630) Q607A/L611A/F614A | This study | N/A     |
| GST-mHOIP UBA (aa 466-630) R474A/L483A/V486A | This study | N/A     |
| MBP-mHOIL-1L UBL (aa 1-140) WT | This study                    | N/A     |
| MBP-mHOIL-1L UBL (aa 1-140) L15A/V19A | This study | N/A     |
| MBP-mHOIL-1L UBL (aa 37-161)  | This study                    | N/A     |
| MBP-mSHARPIN UBL (aa 163-301) WT | This study               | N/A     |
| MBP-mSHARPIN UBL (aa 163-301) L176A/I180A | This study | N/A     |
| MBP-mSHARPIN UBL (aa 198-318) | This study                    | N/A     |
| mHOIL-1L UBL (aa 1-189)-strep | This study                    | N/A     |
| His-mHOIP (aa 474-630)        | This study                    | N/A     |
| mHOIL-1L (aa 1-140)           | This study                    | N/A     |
| GST-SHARPIN (aa 163-341)      | This study                    | N/A     |
| ProTEV Plus                   | Promega                       | Cat# V6101 |
| Recombinant HRV3C             | This study                    | N/A     |
| Gel Filtration Standard       | Bio-Rad                       | Cat# 151-1901 |
| MES monohydrate               | Hampton Research              | Cat# HR2-243 |
| Magnesium sulfate hydrate     | Hampton Research              | Cat# HR2-633 |
| Glycerol                      | Nacalai Tesque                | Cat# 17018-83 |

**Critical Commercial Assays**

| Name                          | Source                        | Code    |
|-------------------------------|-------------------------------|---------|
| GST-Capture Kit               | GE Healthcare                 | Cat# BR-1002-23 |
| Mouse antibody capture kit    | GE Healthcare                 | Cat#   |
| Dual-Luciferase Reporter Assay System | Promega                   | Cat# E1980 |
| High Capacity RNA to cDNA kit | ThermoFisher                  | Cat# 4387406 |
| Topo cloning Kit              | invitrogen                    | Cat# 45-0641 |
| E-Plate L8 PET                | ACEA Biosciences              | Cat# 00300500870 |
| In Situ Cell Death Detection Kit, Fluorescein | Roche                   | Cat# 11684795910 |
| Odyssey Infrared EMSA kit     | LI-COR Biosciences            | Cat# 829-07910 |
| TransAM® p65                  | ACTIVEMOTIF                   | Cat# 40096 |
| IL-8 ELISA                    | PEPROTECH                     | Cat# 900-T18 |
| CellTiter-Glo Luminescent Cell Viability Assay kit | Promega               | Cat# G7570 |
| Deposited Data          | Atomic coordinates, mouse LUBAC ternary complex core | This study | PDB: 5Y3T |
|------------------------|------------------------------------------------------|------------|-----------|
| Experimental Models: Cell Lines | HEK293T | N/A | N/A |
|                         | HEK293T HOIP KO | This study | N/A |
|                         | Jurkat | N/A | N/A |
|                         | Jurkat HOIP KO | Sakamoto et al., 2015 | N/A |
|                         | Immortalized MEF HOIL-1L
null | Tokunaga et al., 2009 | N/A |
|                         | Immortalized MEF HOIL-1L
null | Tokunaga et al., 2009 | N/A |
|                         | Immortalized MEF HOIL-1L
null | This study | N/A |
|                         | Immortalized MEF HOIL-1L
null | This study | N/A |
|                         | Immortalized MEF HOIL-1L
null | Shimizu et al., 2016 | N/A |
|                         | Immortalized MEF SHARPIN
null | Tokunaga et al., 2011 | N/A |
|                         | LUBAC TKO MEF | This study | N/A |
|                         | HBL1 | N/A | N/A |
| Experimental Models: Organisms/Strains | Mouse: HOIL-1L
null | This study | N/A |
|                         | Mouse: HOIL-1L
null | This study | N/A |
| Oligonucleotides | Mouse HOIL-1L primer: Exon4_Fwd, 5’-GGAATGGAGACGGTGCCTATCTC-3’ | This study | N/A |
|                         | Mouse HOIL-1L primer: Exon5_Fwd, 5’-CGAAGCCCCAGGACCCAACCAGGAG-3’ | This study | N/A |
|                         | Mouse HOIL-1L primer: Exon6_Fwd, 5’-TGAGATGTGCTGACTGCAAGA-3’ | This study | N/A |
|                         | Mouse HOIL-1L primer: Exon7_Fwd, 5’-CTGGGCTTCCGAAGACAGGTTTCTC-3’ | This study | N/A |
|                         | Mouse HOIL-1L primer: Exon5_Rev, 5’-CTGAGATGTGCTGACTGCAAGA-3’ | This study | N/A |
|                         | Mouse HOIL-1L primer: Exon9_Rev, 5’-CTCAAGGCTTCCGAAGACAGGTTTCTC-3’ | This study | N/A |
|                         | Mouse HOIL-1L primer: Exon10_Rev, 5’-TCCCTGCAATTCATGTGCTCATG-3’ | This study | N/A |
|                         | Mouse β-actin primer: Fwd, 5’-ATGGATGACGATATCGCTC-3’ | This study | N/A |
|                         | Mouse β-actin primer: Rev, 5’-GATTCCATACCCAGGAAGG-3’ | This study | N/A |
|                         | mHOIL-1L typing primer: Fwd, 5’-TTGCGGACAGGGCTATTTATCG-3’ | This study | N/A |
|                         | mHOIL-1L typing primer Rev, 5’-TCAGAAGGCTATTTATCG-3’ | This study | N/A |
|                         | mHOIL-1L typing primer Fwd, 5’-AGGCCCTGAGGGTTG-3’ | This study | N/A |
|                         | mHOIL-1L typing primer Rev, 5’-GGCCTCAGTATAGGCCATACACC-3’ | This study | N/A |
|                         | hHOIL-1L typing primer Fwd, 5’-TCAGAAGGCTATTTATCG-3’ | This study | N/A |
|                         | hHOIL-1L typing primer Rev, 5’-CTCGAGTGACGATATCG-3’ | This study | N/A |
| Recombinant DNA          |                                                   |            |           |
| Vector  | Description                        | Source                  | Notes |
|---------|-----------------------------------|-------------------------|-------|
| pcDNA3.1 HA-mHOIP WT               | Fujita et al., 2014      | N/A         |
| pcDNA3.1 HA-hHOIP                  | Kisirako et al., 2006    | N/A         |
| pcDNA3.1 HA-hD-UBA                 | This study               | N/A         |
| pcDNA3.1 HA-hD-UBA R490Q           | This study               | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L WT          | Fujita et al., 2014      | N/A         |
| pcDNA3.1 Myc-mHOIL-1L WT           | Fujita et al., 2014      | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L Δaa1-36     | This study               | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L L15A        | This study               | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L V19A        | This study               | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L L15A/V19A  | This study               | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L A31D        | This study               | N/A         |
| pcDNA3.1 FLAG-mHOIL-1L A31F        | This study               | N/A         |
| pcDNA3.1 FLAG-hHOIL-1L WT          | This study               | N/A         |
| pcDNA3.1 FLAG-hHOIL-1L A18P        | This study               | N/A         |
| pcDNA3.1 FLAG-mSHARPIN WT          | Fujita et al., 2014      | N/A         |
| pcDNA3.1 FLAG-hSHARPIN WT          | Tokunaga et al., 2011    | N/A         |
| pcDNA3.1 Myc-mSHARPIN WT           | Fujita et al., 2014      | N/A         |
| pcDNA3.1 FLAG-mSHARPIN Δaa163-197  | This study               | N/A         |
| pcDNA3.1 Myc-mSHARPIN Δaa163-197   | This study               | N/A         |
| pcDNA3.1 FLAG-mSHARPIN L176A       | This study               | N/A         |
| pcDNA3.1 FLAG-mSHARPIN I180A       | This study               | N/A         |
| pcDNA3.1 FLAG-mSHARPIN L176A/I180A| This study               | N/A         |
| pcDNA3.1 Myc-mSHARPIN L176A/I180A | This study               | N/A         |
| pcDNA3.1 Myc-mSHARPIN A192D        | This study               | N/A         |
| pcDNA3.1 Myc-mS(UBL)-HOIL-1L       | This study               | N/A         |
| pcDNA3.1 Myc-mH(UBL)-SHARPIN       | This study               | N/A         |
| pMXs-neo-HA-mHOIP                  | This study               | N/A         |
| pMXs-neo-HA-hHOIP                  | This study               | N/A         |
| pMXs-neo-HA-hD-UBA                 | This study               | N/A         |
| pMXs-IP-FLAG-mHOIL-1L WT           | This study               | N/A         |
| pMXs-IP-FLAG-mHOIL-1L UBL          | This study               | N/A         |
| pMXs-IP-FLAG-hHOIL-1L WT           | This study               | N/A         |
| pMXs-IP-FLAG-mSHARPIN WT           | This study               | N/A         |
| pMXs-IP-FLAG-hSHARPIN WT           | This study               | N/A         |
| pMXs-iRES-Bsr                      | This study               | N/A         |
| pMXs-IRES-Bsr-mSHARPIN             | This study               | N/A         |
| pMXs-puro-IRES-att-FLAG-mSHARPIN WT| This study               | N/A         |
| pMXs-puro-IRES-att-FLAG-mSHARPIN L176A/I180A | This study | N/A |
| pcDNA3.1-MMTV FLAG                 | Tokunaga et al., 2009    | N/A         |
| pcDNA3.1-MMTV FLAG mHOIL-1L WT     | This study               | N/A         |
| pcDNA3.1-MMTV FLAG mHOIL-1L A18P   | This study               | N/A         |
| pGEX 6p1 mHOIP D-UBA (aa 466-630) WT| This study               | N/A         |
| pGEX 6p1 mHOIP D-UBA Q607A/L611A/F614A | This study | N/A |
| pGEX 6p1 mHOIP D-UBA R474A/L483A/V496A | This study | N/A |
| pMAL c2x mHOIL-1L (aa 1-140) WT    | This study               | N/A         |
| pMAL c2x mHOIL-1L (aa 1-140) L15A/V19A | This study | N/A |
| pMAL c2x mHOIL-1L (aa 37-161) WT   | This study               | N/A         |
| Expression System | Description | Source | Cat# |
|-------------------|-------------|--------|------|
| pMAL c2x mSHARPIN (aa 163-301) WT | This study | N/A |
| pMAL c2x mSHARPIN (aa 163-301) L176A/I180A | This study | N/A |
| pMAL c2x mSHARPIN (aa 198-318) WT | This study | N/A |
| pT7-7 mHOIL-1L (aa 1-189)-strep WT | This study | N/A |
| pETDuet-1 mHOIP (aa 474-630) WT, mHOIL-1L (1-140) | This study | N/A |
| pGEX 6p1 mSHARPIN (163-341) | This study | N/A |
| pGEX 6p1 HRV3C | This study | N/A |
| pGL4.32[luc2P/NF-κB-RE/Hygro] | Promega | Cat# E8491 |
| pGL4.74[hRluc/TK] | Promega | Cat# E6921 |
| px330 | Addgene | Cong et al., 2013 |
| px459 | Addgene | Ran et al., 2013 |

**Software and Algorithms**

| Tool | Website | Version |
|------|---------|---------|
| CRISPR design | http://crispr.mit.edu | N/A |
| PyMol | Pymol | http://www.pymol.org |
| Image J | https://imagej.nih.gov/ij/ | N/A |
| PRISM ver.5 | PRISM ver.5 | http://www.graphpad.com |
| HKL2000 | Otwinowski and Minor, 1997 | http://www.hkl-xray.com/ |
| CCP4 package | Winn et al., 2011 | www.ccp4.ac.uk |
| DSSP | Touw et al., 2015 | http://www.cmbi.ru.nl/xssp/ |
| COOT | Emsley et al., 2010 | https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/ |

**Other**

| Tool | Source | Cat# |
|------|--------|------|
| Ni-NTA Agarose | Qiagen | 30230 |
| Glutathione Sepharose 4B | GE Healthcare | 17-0756-05 |
| HiTrap Q HP | GE Healthcare | 17-1154-01 |
| HiLoad 16/600 Superdex 200 pg | GE Healthcare | 28-9893-35 |
| Superdex 200 10/300 GL | GE Healthcare | 17-5175-01 |
| Amylose Resin | NEB | E8021 |
| rmp proteinA Sepharose Fast Flow | GE Healthcare | 17-5138-02 |
| rProtein A Sepharose Fast Flow | GE Healthcare | 17-1270-02 |
| Glutathione Sepharose 4 Fast Flow | GE Healthcare | 17-5132-02 |
| Strep-Tactin Sepharose | IBA | 2-1201-010 |
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids
cDNAs used in this study were described previously (Fujita et al., 2014; Tokunaga et al., 2011; Tokunaga et al., 2009). The hD-UBA and hD-UBA R490Q [mHOIP (aa 1–473)–hHOIP (aa 480–636)–mHOIP (aa 631–1066)] were generated from the amplified ORFs of human and mouse HOIP. The following proteins were generated from the amplified ORF of mouse HOIL-1L: before NZF (aa 1–189), UBL (aa 1–140), Δα1–36 (aa 37–509), and UBL Δα1–36 (aa 37–161). The following proteins were generated from the amplified ORF of mouse SHARPIN: Δα163–197 (Δα163–197), UBL (aa 163–301 or 163–340), and UBL Δα163–197 (aa 198–318). The following mutants of mHOIL-1L and mSHARPIN, in which the UBLs were exchanged, were generated from the amplified ORFs of mouse HOIL-1L and SHARPIN: S(UBL)-HOIL [SHARPIN (aa 163–301)–HOIL-1L (aa 136–509)] and H(UBL)-SHARPIN [SHARPIN (aa 1–167)–HOIL-1L (aa 7–135)–SHARPIN (aa 302–380)]. Mutants of mHOIP (R474A/L483A/V486A, Q607A/L611A/F614A), mHOIL-1L (L15A, A18P, V19A, A31D, A31F, L15A/V19A), mSHARPIN (Δaa163–197, UBL (aa 163–301 or 163–340), and UBL Δaa163–197 (aa 198–318). The following mutants of mHOIP (R474A/L483A/V486A, Q607A/L611A/F614A), mHOIL-1L (L15A, A18P, V19A, A31D, A31F, L15A/V19A), mSHARPIN (Δaa163–197, UBL (aa 163–301 or 163–340), and UBL Δaa163–197 (aa 198–318). The following mutants of mHOIL-1L and mSHARPIN, in which the UBLs were exchanged, were generated from the amplified ORFs of mouse HOIL-1L and SHARPIN: S(UBL)-HOIL [SHARPIN (aa 163–301)–HOIL-1L (aa 136–509)] and H(UBL)-SHARPIN [SHARPIN (aa 1–167)–HOIL-1L (aa 7–135)–SHARPIN (aa 302–380)]. Mutants of mHOIP (R474A/L483A/V486A, Q607A/L483A/V486A), mHOIL-1L (L15A, A18P, V19A, A31D, A31F, L15A/V19A), mSHARPIN (L176A, I180A, A192D, L176A/I180A), and hHOIL-1L (A18P) were generated by two-step PCR. cDNAs were ligated into the appropriate epitope-tag sequences, and then cloned into pcDNA3.1, pDNA3.1-MMTV, pMAL-c2x, pGEX-6p1, pMXs-IP, pMXs-neo, pMXs-puro-IREs-att (Bochkov and Palmenberg, 2006), and pMXs-IREs-Bsr. pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230) (Cong et al., 2013) and pSpCas9(BB)-2A-Puro (PX459) (Addgene plasmid #48139) (Ran et al., 2013) were obtained from Addgene.

Antibodies and reagents
Antibodies against the following proteins were purchased from Cell Signaling Technology and used at the indicated dilutions for western blot analysis: p-IκBα (9246, 1:2000), IκBα (4812, 1:2000), and caspase-3 (9662, 1:2000). Antibodies against ubiquitin (P4D1) (sc-8017, 1:2000) and IκBα (C-21) (sc-371, 1:2000) were purchased from Santa Cruz Biotechnology. Antibodies against β-actin (A5316, 1:10,000), β-actin (A2228, 1:10,000), and FLAG (M2) (F3165, 1:2000) used for western blot analysis, and anti-FLAG antibody (F7425, 1:150) used for immunoprecipitation, were purchased from Sigma. Anti-GAPDH (631402, 1:2000) used for western blot analysis, and anti-CD31 (MEC13.3) (102502, 1:100) used for immunohistochemistry, were purchased from BioLegend. Antibodies against HA (Tana2) (M180-3, 1:2000) and DDDDK-tag (PM020, 1:2000) were purchased from MBL and used for western blot analysis. Anti-Myc (4A6) (05-724, 1:2000) was purchased from Merck Millipore, anti-β-tubulin (CLT9002, 1:10,000) was purchased from CEDARLANE, anti-HOIP (ARP43241_P050, 1:2000) was purchased from Aviva Systems Biology, anti-SHARPIN (ab125188, 1:2000) was purchased from Abcam, and StrepMAB-Immo (2-1517-001) was purchased from IBA. Antibodies against the following proteins were made in-house (Fujita et al., 2014; Tokunaga et al., 2011; Tokunaga et al., 2009): mouse HOIP (1:2000), human HOIP (1CB2) (1:2000), HOIL-1L (2E2) (1:2000), and SHARPIN (1:2000). To generate the anti-mouse HOIL-1L N-term antibody, strep-tagged mouse HOIL-1L N-terminus (aa 1–189) was expressed in Escherichia coli (Agilent Technologies), and then purified using Strep-Tactin Sepharose (IBA). Purified protein was used to immunize rabbits, and IgG was purified from antisera using Protein A–Sepharose
(GE Healthcare). SHARPIN peptide synthesis, reverse-phase high-performance liquid chromatography purification, and amino acid analysis were performed as described previously (Bernal et al., 2007).

**Cell lines**

HEK293T cells, HEK293T HOIP KO cells, MEFs derived from WT, cpdm, HOIP Δlinear, HOIL-1L−/−, or HOIL-1L–null mice, and LUBAC TKO MEFs were grown in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) with 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37°C under 7.5% CO₂. HBL1 cells were grown at 37°C under 7.5% CO₂ in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

**Transfection and retroviral expression**

Transfections were performed using Lipofectamine 2000. For retroviral expression, pMXs-IP, pMXs-puro-IRES-att, pMXs-neo, or pMXs-IRES-Bsr containing LUBAC components were transfected into Plat E packaging cells. The resultant viruses were used to infect LUBAC TKO MEFs, cpdm MEFs or HOIL-1L–null MEFs, and stably transduced cells were selected using puromycin, G-418, or blasticidin.

**Cell lysis and immunoprecipitation**

Cells were lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich)], and the lysates were clarified by centrifugation at 15,000 rpm for 20 min at 4°C. For immunoprecipitation, lysates were incubated with the appropriate antibodies for 90 min on ice, and then immobilized on rmp–Protein A–Sepharose beads (GE Healthcare). The beads were washed five times with lysis buffer.

**Immunoblotting and EMSA**

Samples were separated by SDS-PAGE, and then transferred to PVDF membranes. After blocking in Tris-buffered saline (TBS) containing 0.1% Tween-20/5% (w/v) nonfat dry milk, the membrane was incubated with the appropriate primary antibodies, followed by the corresponding secondary antibodies. The membranes were visualized by enhanced chemiluminescence and analyzed on a LAS4000mini or LAS3000 instrument (GE Healthcare). EMSAs for NF-κB activity were performed using the Odyssey Infrared EMSA kit (LI-COR Biosciences) and IRDye 700 NF-κB consensus oligonucleotide (LI-COR Biosciences), and visualized on an Odyssey 9120 Infrared Imaging System (LI-COR Biosciences).

**Purification of the LUBAC ternary complex**

cDNA fragments encoding mouse HOIP D-UBA (aa 474–630) and mouse HOIL-1L LTM-UBL (aa 1–140) were inserted between the EcoRI and NotI sites and the NdeI and EcoRV sites of pET Duet-1 (Novagen), respectively. A Tobacco Etch Virus (TEV) protease cleavage site was inserted between the N-terminal hexa-histidine tag (His tag) and HOIP D-UBA. A cDNA fragment of mouse SHARPIN LTM-UBL (aa 163–341) was inserted between the EcoRI and NotI sites of pGEX-6p-1 (GE Healthcare), yielding recombinant mSHARPIN LTM-UBL with extra amino acid residues (LGSPEF) at the N-terminus after GST-tag cleavage with HRV3C protease. The
binary complex of mHOIP D-UBA and mHOIL-1L LTM-UBL was co-expressed in *E. coli* strain BL21 (DE3). Cells were grown at 37°C until OD$_{600}$ reached 0.6; protein expression was induced by addition of IPTG to a final concentration of 0.5 mM, and culture was continued at 18°C overnight. Cells were harvested by centrifugation. The cell pellet was resuspended in Buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% Glycerol, and 1 mM TCEP) and lysed by sonication. The lysate was clarified by centrifugation, and applied to a Ni-NTA agarose column (Qiagen). After extensive column washing, bound proteins were eluted in Buffer A containing 400 mM imidazole. Fractions containing the binary complex were pooled and co-purified with mSHARPIN LTM-UBL as described below. GST-mSHARPIN LTM-UBL was overexpressed in *E. coli* strain Rosetta2 (DE3) (Novagen) in LB medium. Cells were cultured and processed in the same manner as for purification of the HOIP–HOIL-1L complex. The clarified lysate containing GST-mSHARPIN LTM-UBL was applied to a glutathione-Sepharose affinity column (GE Healthcare), and subsequently His-tagged mHOIP D-UBA–mHOIL-1L LTM-UBL complex was added to form the ternary complex. After incubation at 4°C for 30 min, unbound proteins were removed by extensive column washing. The ternary complex was eluted from the column by cleavage of the GST tag with 400 µM HRV3C protease. To cleave the His tag from mHOIP D-UBA, TEV protease (Promega) was added at a final concentration of 250 µM. The ternary complex was applied to a HiTrap Q HP anion exchange column (GE Healthcare) and eluted with a linear NaCl gradient from 50 to 600 mM. The complex was further purified by size-exclusion chromatography using HiLoad Superdex 16/60 200 pg (GE Healthcare) in a buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1 mM TCEP. Peak fractions containing the stoichiometric ternary complex were combined, concentrated (typically to 7.0 mg/ml), and stored at 4°C until further use.

**Analytical size-exclusion chromatography**

Analytical size-exclusion chromatography for the LUBAC ternary complex core was performed at 4°C using Superdex 200 10/300 GL (GE Healthcare) in a buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1 mM TCEP. Fractions were analyzed by SDS-PAGE, and visualized by CBB and mass spectroscopy.

**SPR analysis**

GST-HOIP UBA (aa 466–630) WT, UBA2mut (Q607A/L611A/F614A), UBA1mut (R474A/L483A/V486A), MBP-HOIL-1L UBL (aa 1–140, 37–161), MBP-HOIL-1L UBL L15A/V19A (aa 1–140), MBP-SHARPIN UBL (aa 163–301, 198–318) and MBP-SHARPIN UBL L176A/I180A (aa 163–301) were expressed in *E. coli* (Agilent Technologies). Expression of GST-HOIP UBA was induced by addition of 0.2 mM IPTG, and culture was continued at 15°C overnight. Expression of MBP-HOIL-1L UBL or SHARPIN UBL was induced by 0.3 mM IPTG, followed by a further 6 h incubation at 25°C. Cells were collected by centrifugation and frozen rapidly. Subsequently, the cells were resuspended in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT (Nacalai Tesque), 200 µg/ml lysozyme chloride (Nacalai Tesque), 10 µg/ml DNase (Roche), 2 mM PMSF, and protease inhibitor cocktail (Roche) at 4°C for 30 min, and then lysed in the presence of 0.2% Triton X-100 at 4°C for 20 min. Insoluble material was removed by centrifugation at 14,000 rpm for 20 min at 4°C. GST-HOIP UBA was purified from the supernatant using glutathione–Sepharose beads (GE Healthcare), and MBP-HOIL-1L UBL and MBP-SHARPIN UBL were purified using Amylose Resin (NEB). Binding affinities
between UBA and the UBLs were measured on a Biacore 3000 (GE Healthcare). GST-HOIP UBA was immobilized on a CM5 sensor chip with an anti-GST antibody using the GST capture kit (GE Healthcare) in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 0.05% (v/v) surfactant P20 at 25°C. Binding between GST-HOIP UBA and MBP-UBLs was measured in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 0.05% (v/v) surfactant P20 at 25°C. The $K_d$ of HOIL-1L UBL–HOIP UBA or SHARPIN UBL–HOIP UBA was calculated by steady-state affinity analysis. In Figures 2G and 2I, 4E–4G, and S2F, the binding affinities of HOIP UBA for HOIL-1L UBL alone (500 µg/ml), SHARPIN UBL alone (500 µg/ml), and the mixture of HOIL-1L and SHARPIN UBL (500 µg/ml each) were measured. In Figure 4C and 4D, to immobilize HOIL-1L (aa 1–189)-strept, anti-mouse antibody was first immobilized on the sensor chip using the mouse antibody capture kit (GE Healthcare). StrepMAB-Immo (IBA Lifesciences) was then applied to capture HOIL-1L (aa 1–189)-strept. Binding between HOIL-1L (aa 1–189)-strept and MBP-SHARPIN UBLs was measured in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 0.05% (v/v) surfactant P20 at 25°C.

**Generation of cell lines**

To generate HOIP–HOIL-1L–SHARPIN TKO MEFs, MEFs from mice with cpdm were electroporated using a NEPA21 electroporator (NEPAGENE) with pX330 plasmid containing a gRNA sequence against mHOIP or mHOIL-1L. After 4 days of culture, cells were seeded at low density. Colonies were picked, and the expression level of LUBAC was analyzed by immunoblotting as a primary screen. Next, genomic regions of HOIL-1L or HOIP were amplified by PCR using the following primers: mHOIL-1L typing_FWD (5′-TTGCCAACAGGCCAATTTGATG-3′) and typing_REV (5′-TGCGGTGATGCAACAATATCCTG-3′); mHOIP typing_FWD (5′-AGCGCCCTGAGGTGGGATT-3′) and typing_REV (5′-GGCCTCCTCAGTATAGCCATACAACC-3′). PCR products were cloned into the TOPO cloning vector (Invitrogen), and mutations were identified by sequencing. To generate HEK293T HOIP KO cells, HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen) with a pX459 plasmid containing a gRNA sequence against hHOIP. Starting on the following day, cells were selected with puromycin for 2 days. Following selection, the cells were seeded at a low density, and isolated colonies were picked. To identify HOIP KO cells, the expression level of HOIP was analyzed by immunoblotting, and the HOIP locus was amplified by PCR using the following primers: hHOIP typing_FWD (5′-TTCCGGGCAGGCCGCTTTCCCTG-3′) and typing_REV (5′-CTCTGTGTAGCCATATACTCG-3′) PCR products were analyzed by sequencing. Primary MEFs from HOIL-1L–null or WT littermate mice were immortalized with SV-40 large T antigen. In Figure 5F, HOIL-1L-null MEFs were transfected with pDNA3.1 MMTV-FLAG mHOIL-1L or A18P using Lipofectamine 2000 (Invitrogen). The next day, cells were seeded at a low density and selected with G-418, and single colonies were picked.

**Luciferase assay**

HEK293T cells were transfected with pGL4.32 (Luc2p/NF-κB–RE/Hygro) and pGL4.74 (hRLuc/TK) (Promega) along with WT or mutant LUBAC components. At 21–24 h after transfection, cells were lysed, and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) on a Lumat Luminometer (Berthold).
Cell viability assay

Cell viability was continuously monitored as an impedance-based cell index using the iCELLigence system (ACEA Bioscience). For each sample, 20,000 cells were plated onto an E-Plate L8 PET (ACEA Bioscience). The next day, cells were treated with TNF-α (10 ng/ml), and cell index was monitored continuously. Plots show data normalized against cell indices at the time of TNF-α treatment. Viability of HBL1 cells in Figure 6H was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega).

Immunohistochemistry

For whole-mount immunohistochemical analyses of CD31, embryos at E10.5 were fixed for 2–4 h with 4% paraformaldehyde in PBS at 4°C, followed by three 30 min washes with PBS-T (PBS with 0.2% Triton X) at 4°C. Next, samples were blocked in 1% BSA in PBS for 1 h at room temperature, and then incubated for 2 days at 4°C with anti-CD31 primary antibody diluted 1:100 in blocking buffer. Samples were washed three times with PBS-T for 30 min at 4°C, followed by two 30 min washes at room temperature, and then incubated overnight at 4°C with Alexa Fluor 546–conjugated secondary antibody diluted 1:1000 in blocking buffer. After five 30 min washes with PBS-T at room temperature, samples were dehydrated with a graded concentration of methanol, and then incubated overnight at 4°C in a graded concentration of methyl salicylate and benzyl alcohol/benzyl benzoate to achieve optimal optical clearing. TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche) as described previously (Shimizu et al., 2016). Samples were analyzed under a stereoscopic fluorescence microscope.

Ubiquitin and kinase assay

Recombinant His6-HOIP/HOIL-1L/SHARPIN, E1, and UbcH7 were prepared as described previously (Kirisako et al., 2006; Tokunaga et al., 2011). Trimeric LUBAC (0.2 μM) was incubated on ice for 3 h with DMSO, HOIP-N (80 μM), SHARPIN unstapled (80 μM), and SHARPIN-LTM (80 μM) peptides in buffer containing 20 mM Tris-HCl (pH 7.5) and 1 mM DTT. Next, a mixture of E1 (100 ng), UbcH7 (400 ng), and Ub (5 μg) with or without 2 mM ATP was added [in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, and 1 mM DTT], and the sample was incubated at 37°C for 30 min.

To prepare S100 lysates (Sakamoto et al., 2015), Jurkat HOIP KO cells were lysed with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 2 mM PMSF, 50 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were centrifuged at 15,000 rpm at 4°C for 15 min, and 0.11 volume of buffer containing 0.3 mM Tris-HCl (pH 7.5), 1.4 M KCl, and 30 mM MgCl2 was added to the supernatant. The sample was then centrifuged at 100,000 g at 4°C for 1 h. The resultant supernatant was dialyzed against buffer containing 20 mM Tris-HCl (pH 7.2) and 1 mM DTT. S100 lysates of Jurkat HOIP KO cells (10 μg) and trimeric LUBAC (0.1 μM) were incubated on ice for 3 h with DMSO, HOIP-N (80 μM), SHARPIN unstapled (80 μM), and SHARPIN-LTM (80 μM) peptides in buffer containing 20 mM Tris-Tris-HCl (pH 7.5), 1 mM DTT, and 10 μg/ml leupeptin. Next, a mixture of E1 (100 ng), UbcH7 (400 ng), Ub (5 μg), and creatine phosphokinase (Sigma-Aldrich) in buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, 10
mM creatine phosphate, and phosphatase inhibitor cocktail (Nacalai Tesque) with or without 2 mM ATP was added, and the sample was incubated at 37°C for 30 min.

**RT-PCR**

RNA was isolated from HOIL-1L or WT MEFs using the RNeasy Mini Kit (QIAGEN) and reverse transcribed using the high-capacity RNA-to-cDNA Kit (Applied Biosystems). RT-PCR of HOIL-1L was performed using the following primers:

- Exon4_Fwd, 5' - GGAATGGAGACGGTGCTATCTC-3';
- Exon5_Fwd, 5' - CGAAGCCAGCAACAACCAGGAG-3';
- Exon6_Fwd, 5' - TGTGAGATGTGCTGCGTCAAG-3';
- Exon8_Fwd, 5' - CATTGACAGCACCTACTCATGCC-3';
- β-actin_Fwd, 5' - ATGGATGACGATATCGCTC-3';
- Exon5_Rev, 5' - CTGGGCTTCGAAGGACAGGTGTC-3';
- Exon9_Rev, 5' - CTCAGGTGTTCTGTCTTCTG-3';
- Exon10_Rev, 5' - TCCCTGCAATTCTATGTGCTCATG-3';
- β-actin_Rev, 5' - GATTCCATACCCAGGAAGG-3'.

**ELISA and TransAM® NF-κB p65 assay**

HBL1 cells were seeded at 300,000 cells/ml in RPMI 1640 plus 10% FBS, and then treated for 2 h with SHARPIN unstacked (20 µM) or SHARPIN-LTM (20 µM) peptides. The cells were stimulated with TNF-α (5 ng/ml) for 0, 5, 10, 30, or 60 min for determination of NF-κB p65 activation, and for 4 or 24 h for measurement of secreted IL-8. Cells were pelleted by centrifugation (250 × g, 5 min, 4°C). Supernatants and pellets were stored separately at -80°C until use. Secreted IL-8 in cell supernatants was measured using the Human IL-8 (CXCL8) Standard TMB ELISA Development kit (PEPROTECH). Pelleted cells were lysed and analyzed for NF-κB p65 activity using the TransAm NF-κB p65 ELISA kit.
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