

Title

The HOIL-1L ligase modulates immune signaling and cell death via mono-ubiquitination of LUBAC

Authors

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23 **Abstract**

24 The linear ubiquitin chain assembly complex (LUBAC), which consists of HOIP,
25 SHARPIN, and HOIL-1L, promotes NF- κ B activation and protects against cell death by
26 generating linear ubiquitin chains. LUBAC contains two RING-IBR-RING (RBR)
27 ubiquitin ligases (E3); the HOIP RBR is responsible for catalyzing linear ubiquitination.
28 We found that HOIL-1L RBR plays a crucial role in LUBAC regulation. HOIL-1L RBR
29 conjugates mono-ubiquitin onto all LUBAC subunits, followed by HOIP-mediated
30 conjugation of linear chains onto mono-ubiquitin; these linear chains attenuate LUBAC
31 functions. Introduction of E3-defective HOIL-1L mutants augmented linear
32 ubiquitination, thereby protecting cells against *Salmonella* infection and curing dermatitis
33 caused by reduction in LUBAC levels due to loss of SHARPIN. Our results reveal a
34 regulatory mode of E3s in which the accessory E3 in LUBAC down-regulates the main
35 E3 by providing preferred substrates for auto-linear ubiquitination. Thus, inhibition of
36 HOIL-1L E3 represents a promising strategy for treating severe infections or
37 immunodeficiency.

38

39 Text

40 The ubiquitin system, which is involved in the regulation of various physiological
41 processes¹⁻⁶, requires the sequential transfer of ubiquitin by three enzymes: a ubiquitin-
42 activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase
43 (E3)^{1,7}. The most important feature of the ubiquitin system is that ubiquitin, a small post-
44 translational protein modifier, can be attached not only to its substrates, but also to other
45 ubiquitin molecules, thereby generating ubiquitin chains. Several types of ubiquitin
46 chains exist, and chain type determines the mode of regulation of conjugated proteins^{1,7}.
47 Previous work showed that ubiquitin chains are added to one of seven Lys (K) residues
48 in ubiquitin^{1,7}. However, recent work by our group revealed that a ubiquitin chain can
49 also be generated via the N-terminal Met-1 (M1) of ubiquitin, yielding a linear or M1-
50 linked ubiquitin chain, and that the linear ubiquitin assembly complex (LUBAC)
51 specifically generates such linear chains⁸⁻¹⁰. LUBAC is composed of three subunits: HOIP,
52 HOIL-1L, and SHARPIN; the RING-IBR-RING (RBR) of HOIP is the catalytic center
53 for linear ubiquitination^{9,10}. It has been recently shown that linear ubiquitin chains
54 conjugated onto LUBAC subunits attenuate the linear ubiquitination activity of HOIP,
55 and OTULIN, a linear chain-specific deubiquitinase (DUB) that interacts with LUBAC<sup>11-
56 13</sup>, counteracts this effect by cleaving the chains on LUBAC¹⁴.

57 LUBAC contributes to NF- κ B activation and protects against cell death by
58 generating linear ubiquitin chains^{8-10,15,16}. Promotion of linear ubiquitination is associated
59 with oncogenesis, whereas attenuation of the process is associated with autoinflammation
60 and immunodeficiency¹⁷⁻²¹. Moreover, pathogens such as *Salmonella*, *Shigella*, and

opportunistic *Aspergillus* inhibit LUBAC to promote infection²²⁻²⁶.

LUBAC has two distinct RBR-type ubiquitin ligase activities, one each in HOIP and HOIL-1L, within one ubiquitin ligase complex^{9,10}. The HOIP RBR, as the main catalytic center of LUBAC, specifically generates linear chains. Despite its relatively weak E3 activity²⁷, HOIL-1L RBR has been suggested to be involved in linear ubiquitination of NEMO by HOIP RBR²⁸; however, LUBAC lacking this region can still conjugate linear chains to NEMO²⁹. Thus, the HOIL-1L E3 activity may have roles besides NEMO ubiquitination. HOIL-1L catalyzes oxy-ester bond formation between the C-terminal carboxyl group of ubiquitin and the hydroxy groups of Ser/Thr residues of HOIL-1L and components of the Myddosome^{30,31}. However, the physiological significance of the second ligase of HOIL-1L within LUBAC remains elusive.

Here, we show that the E3 activity of HOIL-1L plays a crucial role in LUBAC regulation. HOIL-1L E3 conjugates mono-ubiquitin onto all LUBAC subunits, followed by HOIP E3-mediated conjugation of linear chains onto the mono-ubiquitin. We found that introduction of a HOIL-1L mutant lacking E3 activity protected cells from *Salmonella* infection by augmenting linear ubiquitination by LUBAC. More importantly, introduction of even one E3-defective HOIL-1L allele dramatically cured dermatitis caused by loss of SHARPIN^{29,32}. These observations reveal a regulatory mode of E3, in which the second, minor ligase activity of an E3 complex negatively regulates the main ligase, and a ligase-associated DUB counteracts this effect. Our results also imply that inhibition of HOIL-1L E3 activity represents a promising strategy for the treatment of severe infections or immunodeficiency.

Results

The HOIL-1L E3 activity down-regulates LUBAC functions

The trimeric LUBAC E3 complex contains two catalytic RBR centers (Fig. 1a), and HOIL-1L RBR is highly conserved throughout evolution (Extended Data Fig. 1a). To investigate the functions of HOIL-1L E3, we introduced HOIL-1L WT or mutants lacking the HOIL-1L RBR, along with HOIP, into mouse embryonic fibroblasts (MEFs) lacking all LUBAC subunits (triple knockout [TKO] MEFs)¹⁶. The HOIL-1L mutants efficiently suppressed activation of caspase-3 triggered by TNF- α and cycloheximide (CHX) (Extended Data Fig. 1b). Cys458 of mouse HOIL-1L forms a thio-ester bond with ubiquitin prior to substrate ubiquitination^{19,27,33}. Ligase-defective HOIL-1L harboring a mutation of Cys458 (C458S or C458A) suppressed caspase-3 activation and protected TKO MEFs from TNF- α -induced cell death (Fig. 1b,c, Extended Data Fig. 1c). Conjugation of linear chains to components of TNFR Complex I, including RIPK1, suppressed cell death by inhibiting formation of the TNF- α -mediated cell death complex containing RIPK1, FADD, and RIPK3 (TNFR Complex II)³⁴⁻³⁶. Consistent with this, loss of HOIL-1L E3 drastically suppressed generation of TNFR Complex II (Fig. 1d). Luciferase assays revealed that ligase-defective HOIL-1L activated NF- κ B to a greater extent than WT (Extended Data Fig. 1d). Transcription of NF- κ B target genes was strongly activated in MEFs expressing the ligase-defective HOIL-1L C458A mutant (Fig. 1e, Extended Data Fig. 1e). Moreover, phosphorylation and degradation of I κ B α , a hallmark of NF- κ B activation, was augmented even in the absence of stimulation (Fig. 1f, Extended Data Fig. 1f). Notably in this regard, HOIP-mediated linear ubiquitination

is involved in suppression of cell death by HOIL-1L RBR, as HOIL-1L C458S failed to inhibit apoptosis of MEFs expressing the ligase-defective HOIP C879A mutant (Fig. 1g).

HOIL-1L E3 attenuates generation of linear ubiquitin chains

Because augmentation of LUBAC function upon loss of HOIL-1L E3 activity depended on HOIP E3, we investigated whether loss of HOIL-1L E3 would affect the amount of linear ubiquitin chains in cells. As expected, introduction of ligase-defective HOIL-1L along with HOIP and SHARPIN dramatically increased the cellular level of linear ubiquitin chains without affecting K48, K63, or other types of chains (Fig. 2a, Extended Data Fig. 2a,b). Consistent with this, LUBAC immunoprecipitated from MEFs expressing ligase-defective HOIL-1L generated more linear chains *in vitro* than LUBAC containing HOIL-1L WT (Fig. 2b). Linear ubiquitination of NEMO, which is involved in NF- κ B activation^{15,37}, was also augmented in non-stimulated TKO MEFs expressing HOIL-1L C458A (Fig. 2c). In addition, the amount of linear ubiquitin, as well as ubiquitinated RIPK1, which is required for protection against cell death, was increased by loss of HOIL-1L E3 (Fig. 2d). These results clearly demonstrated that HOIL-1L E3 down-regulated linear ubiquitination of known substrates, leading to suppression of LUBAC functions, i.e., NF- κ B activation and protection against cell death.

Auto-ubiquitination of HOIL-1L inhibits LUBAC functions, including generation of linear ubiquitin chains

126 During our analyses of ligase-defective HOIL-1L, we realized that slowly migrating
127 HOIL-1L was absent in cells lacking HOIL-1L E3 activity (Fig. 2a,c). The upper HOIL-
128 1L signal appeared to represent auto-ubiquitinated HOIL-1L, as this band was observed
129 in cells lacking HOIP E3 activity³⁸ (Extended Data Fig. 3a). *In vitro* ubiquitination assays
130 confirmed that HOIL-1L auto-monoubiquitinates itself (Fig. 3a). It was recently reported
131 that HOIL-1L can catalyze oxy-ester bond formation between itself and ubiquitin^{30,31}.
132 This oxy-ester bond was cleaved by hydroxylamine (NH₂OH) treatment, but not a DUB,
133 Ub-Specific Protease2 (USP2cc), which cleaves all conventional ubiquitin conjugations³¹
134 (Extended Data Fig. 3b). Isopeptide linkages between Lys residues in substrates and
135 ubiquitin are cleaved by USP2cc, but not NH₂OH³¹. To our surprise, a substantial amount
136 of ubiquitinated HOIL-1L (Ub-HOIL-1L) generated by HOIL-1L E3 *in vitro* was
137 resistant to NH₂OH but could be digested by USP2cc (Fig. 3b), indicating that most Ub-
138 HOIL-1L is generated via Lys residues. As previously observed³¹, however, USP2cc
139 failed to eliminate slower-migrating HOIL-1L immunoprecipitated under non-denatured
140 conditions (Fig. 3c). Because the upper HOIL-1L signal was not detected in MEFs
141 lacking SHARPIN, which contain LUBAC consisting of HOIL-1L and HOIP (Extended
142 Data Fig. 3c), we suspected that USP2cc failed to access ubiquitin in the tight trimeric
143 LUBAC complex¹⁶. To test this possibility, we immunoprecipitated HOIL-1L under
144 denaturing conditions to disrupt the LUBAC complex. USP2cc, but not the linear-specific
145 DUB OTULIN, could efficiently, albeit not completely, eliminate the slower-migrating
146 HOIL-1L immunoprecipitated under denaturing conditions (Fig. 3c). Also, NH₂OH
147 marginally cleaved Ub-HOIL-1L (Extended Data Fig. 3d). We then examined the

ubiquitinated residues by mass spectrometry (MS). These analyses identified Lys residues of HOIP, HOIL-1L, and SHARPIN as residues ubiquitinated in cells expressing HOIL-1L WT. In addition, S107 of human HOIP was identified as ubiquitinated in cells expressing HOIL-1L WT, but not the ligase-defective HOIL-1L C460A mutant (Extended Data Fig. 3e). The MS results strongly indicated that a substantial fraction of Ub-HOIL-1L in cells is generated via Lys residues, whereas oxy-ester ubiquitination also occurs in cells expressing HOIL-1L WT although we could not detect ubiquitinated serine/threonine (S/T) in HOIL-1L.

Hence, we focused on the Lys residues of HOIL-1L. K158 and K174 of mouse HOIL-1L (mHOIL-1L) were identified as ubiquitinated residues in MS analyses³⁹. However, HOIL-1L mutants in which K158 and K174 were replaced with Arg (K158/174R), as well as other mono-K/R mutants, were mono-ubiquitinated (Extended Data Fig. 3f). We then modified all 17 Lys residues of mHOIL-1L to Arg (All-K/R) and found that the resultant HOIL-1L All-K/R mutant was barely ubiquitinated (Fig. 3d). Moreover, the All-K/R mutant increased the level of linear ubiquitin chains in the cell, promoted NF- κ B activation, and protected MEFs against TNF- α -induced apoptosis (Fig. 3e,f, Extended Data Fig. 3g). Introduction of a 3 $\times$ FLAG-tag (3 $\times$ FLAG-HOIL-1L All-K/R), but not a myc-tag, at the N-terminus enabled HOIL-1L All-K/R to auto-ubiquitinate (Fig. 3g, Extended Data Fig. 3h). A Lys residue in the 3 $\times$ FLAG-tag was ubiquitinated, and as expected the ubiquitin on that residue was effectively cleaved by USP2cc, but not NH₂OH (Fig. 3h, Extended Data Fig. 3i). However, we could not detect a ubiquitinated signal in the myc-tag. Given that 3 $\times$ FLAG-HOIL-1L All-K/R C458A did not

ubiquitinate HOIL-1L (Fig. 3g), HOIL-1L All-K/R must have auto-ubiquitinated Lys residues in the N-terminal 3×FLAG-tag on its own. In MS analyses, we could detect ubiquitination of S915 and T955 of mHOIP in HOIL-1L All-K/R cells, implying that the All-K/R mutations did not overtly affect HOIL-1L function (Extended Data Fig. 3j,k). Moreover, introduction of the N-terminal 3×FLAG-tag reversed the increase in linear chain production and the suppression of TNF- α -mediated apoptosis induced by All-K/R, but not the corresponding effects of the ligase-defective mutant (Fig. 3i, Extended Data Fig. 3l). Although we cannot rule out the possibility that loss of mono-ubiquitination or the All-K/R mutations may alter conformation of the LUBAC complex to augment the E3 activity of HOIP completely, these results clearly indicated that auto-ubiquitination of HOIL-1L suppresses LUBAC functions, regardless of the positions of the ubiquitinated residues.

Auto-mono-ubiquitination makes HOIL-1L a preferred target for auto-linear ubiquitination by HOIP

To dissect the molecular mechanism underlying the suppression of linear ubiquitination by LUBAC mediated by auto-ubiquitination of HOIL-1L, we performed *in vitro* ubiquitination assays. Petit-SHARPIN, which consists of fragments of human HOIP (hHOIP) and hSHARPIN, has weak linear ubiquitination activity (Fig. 4a)²³. Addition of mHOIL-1L augmented the linear ubiquitination activity of Petit-SHARPIN, confirming that trimeric LUBAC has efficient linear ubiquitination activity²⁹ (Fig. 4b). Addition of not only ligase-defective mHOIL-1L (C458A) or hHOIL-1L (C460A), but also HOIL-1L

All-K/R, potentiated activity to a greater extent than mHOIL-1L WT (Fig. 4b,c, Extended Data Fig. 4a). Because HOIL-1L C458A was linearly ubiquitinated to a lesser extent than WT (Extended Data Fig. 4b), we generated ligase-defective HOIL-1L with ubiquitin at the N-terminus (Ub-HOIL-1L Δ E3). Addition of ubiquitin reversed HOIL-1L Δ E3-mediated potentiation of linear ubiquitination, and Ub-HOIL-1L Δ E3 was efficiently linearly ubiquitinated (Fig. 4d). These results clearly demonstrated that auto-ubiquitination of HOIL-1L attenuates linear ubiquitination by HOIP E3 by making HOIL-1L a preferred substrate for HOIP RBR. Moreover, addition of Petit-SHARPIN C885A (which lacks linear ubiquitination activity) to HOIL-1L strengthened the auto-ubiquitination activity of HOIL-1L (Fig. 4e), suggesting that formation of trimeric LUBAC also potentiates HOIL-1L E3 activity.

Not only HOIL-1L, but also HOIP and SHARPIN, are mono-ubiquitinated by HOIL-1L E3

OTULIN maintains LUBAC functions by cleaving linear chains conjugated to LUBAC subunits, including HOIP¹⁴. We found that loss of OTULIN increased the levels of slower-migrating bands corresponding to all three components of LUBAC in cells expressing HOIL-1L WT, but not HOIL-1L C458A (Fig. 5a). Modifications of LUBAC subunits in OTULIN KO cells appeared to be linear chains, as all three subunits of LUBAC were efficiently pulled down with M1-TUBE, which selectively binds linear ubiquitin chains (Fig. 5b). OTULIN cleaves only inter-ubiquitin linear linkages, but not ubiquitin-substrate bonds. Treatment of OTULIN KO cells with MBP-OTULIN

increased the levels of mono-ubiquitinated species (Extended Data Fig. 5a), strongly indicating the involvement of HOIL-1L E3 in linear ubiquitination of all LUBAC subunits. Indeed, *in vitro* ubiquitination assays clearly confirmed that HOIL-1L, but not ligase-defective HOIL-1L, mono-ubiquitinated all subunits of LUBAC (Fig. 5c).

RBR E3s bind to E2 at RING1 and transfer ubiquitin from E2 to the conserved Cys in RING2 prior to substrate ubiquitination^{19,40-42}. We generated a HOIL-1L mutant lacking amino acids 251–341, the region containing RING1 (Δ RING1) (Extended Data Fig. 5b). Although all LUBAC subunits were linearly ubiquitinated in cells lacking OTULIN, expression of HOIL-1L Δ RING1 counteracted the increase in their linear ubiquitination induced by OTULIN deletion, implying that E2 bound to HOIL-1L RING1 transfers ubiquitin to HOIL-1L C458 (Fig. 5d, e). Consistent with this, HOIL-1L Δ RING1 or C458A attenuated the augmentation of TNF- α -induced apoptosis induced by OTULIN deletion (Fig. 5f, g, Extended Data Fig. 5c). These results reveal the molecular mechanism underlying HOIL-1L-mediated suppression of LUBAC functions: HOIL-1L E3 mono-ubiquitinates not only HOIL-1L itself but also HOIP and SHARPIN, thereby facilitating HOIP-mediated conjugation of linear chains to all LUBAC subunits by providing a suitable substrate (i.e., acceptor ubiquitin) for HOIP RBR³³. As demonstrated previously¹⁴, this leads to suppression of LUBAC functions. OTULIN counteracts these effects by cleaving linear chains from the LUBAC complex. Considering that loss of HOIL-1L E3 increased the level of linear chains in the cell (Fig. 2a), HOIL-1L E3 may cause HOIP RBR to preferentially recognize LUBAC itself as cis-targets over other substrates (trans-

targets) for linear chain conjugation, thereby contributing to suppression of LUBAC functions. (Fig. 5h).

Defective HOIL-1L E3 activity protects cells against *Salmonella typhimurium* infections

We next examined the pathophysiological impacts of HOIL-1L E3. LUBAC protects cells against infection by invasive bacteria, including *Salmonella*^{22,24}. Loss of HOIL-1L E3 efficiently restricted proliferation of *S. typhimurium*, as well as infection-induced cell death (Fig. 6a-c, Extended Data Fig. 6a). Structured illumination microscopy (SIM) revealed that infecting *S. typhimurium* were covered with high levels of linear ubiquitin (Fig. 6d, Extended Data Fig. 6b–d). Moreover, infection strongly induced expression of NF- κ B target genes in MEFs expressing HOIL-1L C458A (Fig. 6e). Considering that the levels of LUBAC subunits were virtually identical between MEFs expressing WT and HOIL-1L C458A (Fig. 2a–d), we concluded that loss of HOIL-1L E3 augmented LUBAC activity, resulting in effective clearance of *S. typhimurium*.

Loss of HOIL-1L E3 protects mice from apoptotic liver failure and cures dermatitis caused by lack of SHARPIN

HOIL-1L Δ RING1 augmented NF- κ B activation, suppressed apoptosis, and generated linear ubiquitin chains at a level comparable to the C458A mutant (Extended Data Fig. 7a–d). Hence, to examine the effects of HOIL-1L E3 knockout in mice, we generated conditional knockout (KO) mice in which exons 7 and 8 of HOIL-1L (which encode

amino acids 251–341) were deleted (Δ RING1) (Extended Data Fig. 5b, 7e). Expression levels of HOIL-1L Δ RING1 were relatively low, whereas the levels of the other components of LUBAC were almost the same as in WT (Extended Data Fig. 7f). HOIL-1L can exist alone without forming LUBAC²⁹. Anti-SHARPIN immunoprecipitation revealed that almost all HOIL-1L Δ RING1 formed a complex with SHARPIN and HOIP, whereas a substantial amount of HOIL-1L WT was not present in LUBAC and could be detected in the unbound fraction (Extended Data Fig. 7g). Accordingly, we used HOIL-1L Δ RING1 for subsequent analyses because deletion of RING1 did not overtly affect the amount of LUBAC, although catalytic inactive point-mutation (C458A) might be preferred to delete HOIL-1L E3 activity. Mice with whole-body deletion of HOIL-1L RING1 (HOIL-1L Δ RING1/ Δ RING1) were viable up to the age of 12 months without overt phenotypes (Fig. 7a), although loss of HOIL-1L E3 significantly increased the amount of linear ubiquitin in all organs (Fig. 7b, Extended Data Fig. 7h). Immunostaining revealed linear chains in lung tissue, indicating that LUBAC functions were augmented in HOIL-1L Δ RING1/ Δ RING1 mice (Fig. 7c). However, HOIL-1L Δ RING1 mice did exhibit some mild phenotypes. For example, the mice exhibited mild splenomegaly; infiltration by inflammatory cells in most organs, especially the lungs; and elevated serum levels of immunoglobulin (Fig. 7d–f). Moreover, flow cytometry revealed that the percentage of activated B cells and CD4⁺ T cells increased (Fig. 7g,h, Extended Data Fig. 7i).

HOIL-1L Δ RING1/ Δ RING1 MEFs and primary hepatocytes were resistant to TNF- α -induced apoptosis (Extended Data Fig. 8a–c). Hence, we examined the effects of loss of HOIL-1L E3 in disease models. Intraperitoneal injection of lipopolysaccharide (LPS)

and D-galactosamine (D-GalN), a specific inhibitor of hepatic transcription, induces acute hepatocytic apoptosis^{43,44}; we investigated whether loss of HOIL-1L E3 would protect mice against this effect. Although we observed no apparent differences in livers not injected with LPS/D-GalN (Extended Data Fig. 8d), LPS and D-GalN induced massive hepatocyte apoptosis in WT livers 7 hours after injection, whereas virtually no overt apoptosis could be detected in HOIL-1L Δ RING1/ Δ RING1 livers (Fig. 8a, Extended Data Fig. 8e,f). Loss of HOIL-1L E3 also protected mice from death triggered by LPS/D-GalN injection (Fig. 8b). These results indicated that deletion of HOIL-1L E3 protects against apoptotic cell death.

Loss of SHARPIN causes chronic autoinflammation in the skin (chronic proliferative dermatitis in mice: cpdm) due to augmented TNF- α -induced death of keratinocytes, a result of the decrease in LUBAC ligase activity caused by reduced levels of HOIL-1L and HOIP^{29,32,45}. To increase LUBAC activity in cpdm mice, we introduced the HOIL-1L Δ RING1 alleles. To our great surprise, introduction of even one HOIL-1L Δ RING1 allele dramatically ameliorated cpdm dermatitis and suppressed keratinocyte apoptosis without affecting the amount of HOIP (Fig. 8c–e, Extended Data Fig. 8 g,h). These results clearly show that augmentation of linear ubiquitination activity of HOIP E3 upon loss of HOIL-1L E3 ameliorates cpdm. Moreover, the findings indicate that cpdm is caused mainly by attenuated HOIP E3 activity rather than altered composition of LUBAC subunits.

Discussion

The LUBAC ubiquitin ligase complex promotes NF- κ B activation and suppresses cell death by conjugating linear ubiquitin chains^{9,10}. Experiments involving deletion or mutation of OTULIN have revealed that LUBAC conjugates linear ubiquitin chains to its subunits, thereby down-regulating its own functions¹⁴. LUBAC contains two ubiquitin ligase centers, the RBRs of HOIP and HOIL-1L; the former catalyzes formation of linear ubiquitin chains^{8,9}. In this study, over the course of our analyses for HOIL-1L RBR, we identified an unexpected regulatory mode of the LUBAC ubiquitin ligase, in which two different E3s work coordinately to regulate LUBAC functions (Fig. 5h). The accessory E3 center HOIL-1L RBR mono-ubiquitinates LUBAC subunits, which makes the subunits preferred auto-linear ubiquitination (cis-linear ubiquitination) substrates for the main E3 of LUBAC, HOIP RBR, thereby suppressing linear chain-mediated LUBAC functions by attenuating linear ubiquitination of other targets (trans-linear ubiquitination). Notably, since ubiquitin is the preferred target of linear ubiquitination by HOIP E3⁴⁶, loss of auto-mono-ubiquitination activity of HOIL-1L E3 renders LUBAC subunits undesirable substrates for linear ubiquitination by HOIP RBR. Therefore, HOIL-1L Δ RING1 or the C458A mutant leads to enhancement, but not suppression, of LUBAC function, which underlies the benign phenotype of mice lacking E3 activity of HOIL-1L as compared to the HOIL-1L null mice and the reversal of the SHARPIN null phenotype by the HOIL-1L Δ RING1. OTULIN counteracts it by digesting linear chains from LUBAC, which are conjugated by coordinating function of HOIL-1L and HOIP E3s, via a process that depends on a constitutive interaction between the deubiquitinase and the

complex¹⁴. Coordination between the functions of two different E3s has been reported previously; however, in those cases, the first E3 mono-ubiquitinates the target proteins, and the second E3 polyubiquitinates the mono-ubiquitin residues introduced by the first to efficiently conjugate ubiquitin chains to substrates^{47,48}. By contrast, in the case of LUBAC, the coordinate functions of two E3s regulate the main catalytic activity by decorating the ligase complex itself, and two E3s, HOIL-1L and HOIP, are present in the same ligase complex.

We also provided solid evidence that deletion of HOIL-1L E3 potentiates LUBAC functions *in vitro* and *in vivo*. Augmented LUBAC activity has been implicated in lymphomagenesis^{18,49} and resistance to chemotherapeutic agents such as cisplatin^{50,51}. The MALT1 paracaspase, which removes HOIL-1L RBR from LUBAC by cleaving HOIL-1L between Arg165 and Gly166^{52,53}, is upregulated in most activated B cell-like diffuse large B cell lymphoma (ABC-DLBCL)^{53,54}. Although it was suggested that MALT1-mediated cleavage of HOIL-1L suppresses LUBAC functions⁵², we have observed that the HOIL-1L ‘before NZF’ mutant, which resembles HOIL-1L (1–165), augmented LUBAC function (Extended Data Fig. 1b). Because augmented LUBAC activity is involved in the pathogenesis of ABC-DLBCL¹⁸, MALT1-mediated cleavage of HOIL-1L may augment the function of LUBAC, which plays critical roles in lymphomagenesis. Although HOIL-1L mutations play causative roles in diseases associated with suppressed LUBAC functions^{21,55-57}, our results imply that some mutations in HOIL-1L may augment LUBAC activity by suppressing HOIL-1L E3. Given that loss of even one allele of HOIL-1L RING1 can augment LUBAC function in

344 mice sufficiently to cure cpdm (Fig. 8c), some HOIL-1L mutations may be involved in
345 oncogenesis by augmenting LUBAC function. Further analyses of cancer genomes will
346 reveal whether or not HOIL-1L mutations are involved in oncogenesis. Pathogens such
347 *Shigella* and opportunistic *Aspergillus* inhibit LUBAC to promote infection^{23,26}. Here, we
348 showed that inhibition of HOIL-1L E3 augmented clearance of the intracellular pathogen
349 *Salmonella* (Fig. 6). Thus, our results identify HOIL-1L E3 as a promising target for
350 potentiating clearance of pathogens, especially in immunocompromised hosts.

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Author Contributions

Y.F., H.F., and K.I. conceived and designed the project. Y.F. performed most of the experiments. M.K. and A.N. performed bacterial infection experiments. F.O., Y.S., and K.T. performed mass spectrometry. K.S. performed immunostaining of lungs of mice and generation of M1-TUBE. R.T. provided advice on the project. Y.F. and K.I. wrote the manuscript with contributions from all other authors.

Competing Financial Interests

The authors declare no competing financial interests.

REFERENCES

1. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-479 (1998).
2. Komander, D. & Rape, M. The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203-229 (2012).
3. Husnjak, K. & Dikic, I. Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu. Rev. Biochem.* **81**, 291-322 (2012).
4. Mukhopadhyay, D. & Riezman, H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* **315**, 201-205 (2007).
5. Wickliffe, K., Williamson, A., Jin, L. & Rape, M. The multiple layers of ubiquitin-dependent cell cycle control. *Chem. Rev.* **109**, 1537-1548 (2009).
6. Zinngrebe, J., Montinaro, A., Peltzer, N. & Walczak, H. Ubiquitin in the immune system. *EMBO Rep.* **15**, 28-45 (2014).
7. Kornitzer, D. & Ciechanover, A. Modes of regulation of ubiquitin-mediated protein degradation. *J. Cell Physiol.* **182**, 1-11 (2000).
8. Kirisako, T. *et al.* A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* **25**, 4877-4887 (2006).
9. Iwai, K., Fujita, H. & Sasaki, Y. Linear ubiquitin chains: NF- κ B signalling, cell death and beyond. *Nat. Rev. Mol. Cell Biol.* **15**, 503-508 (2014).
10. Sasaki, K. & Iwai, K. Roles of linear ubiquitinylation, a crucial regulator of NF- κ B and cell death, in the immune system. *Immunol. Rev.* **266**, 175-189 (2015).
11. Takiuchi, T. *et al.* Suppression of LUBAC-mediated linear ubiquitination by a specific interaction between LUBAC and the deubiquitinases CYLD and OTULIN. *Genes Cells* **19**, 254-272 (2014).
12. Damgaard, R.B. *et al.* The Deubiquitinase OTULIN Is an Essential Negative Regulator of Inflammation and Autoimmunity. *Cell* **166**, 1215-1230 e1220 (2016).
13. Elliott, P.R. & Komander, D. Regulation of Met1-linked polyubiquitin signalling by the deubiquitinase OTULIN. *FEBS J.* **283**, 39-53 (2016).
14. Heger, K. *et al.* OTULIN limits cell death and inflammation by deubiquitinating LUBAC. *Nature* **559**, 120-124 (2018).
15. Fujita, H. *et al.* Mechanism underlying I κ B kinase activation mediated by the linear ubiquitin chain assembly complex. *Mol. Cell. Biol.* **34**, 1322-1335 (2014).

- 402 16. Fujita, H. *et al.* Cooperative Domain Formation by Homologous Motifs in HOIL-
403 1L and SHARPIN Plays A Crucial Role in LUBAC Stabilization. *Cell Rep.* **23**,
404 1192-1204 (2018).
- 405 17. Boisson, B. *et al.* Human HOIP and LUBAC deficiency underlies
406 autoinflammation, immunodeficiency, amylopectinosis, and lymphangiectasia. *J.*
407 *Exp. Med.* **212**, 939-951 (2015).
- 408 18. Yang, Y. *et al.* Essential role of the linear ubiquitin chain assembly complex in
409 lymphoma revealed by rare germline polymorphisms. *Cancer Discov.* **4**, 480-493
410 (2014).
- 411 19. Elton, L., Carpentier, I., Verhelst, K., Staal, J. & Beyaert, R. The multifaceted role
412 of the E3 ubiquitin ligase HOIL-1: beyond linear ubiquitination. *Immunol. Rev.*
413 **266**, 208-221 (2015).
- 414 20. Brazee, P., Dada, L.A. & Sznajder, J.I. Role of Linear Ubiquitination in Health
415 and Disease. *Am. J. Respir. Cell Mol. Biol.* **54**, 761-768 (2016).
- 416 21. Boisson, B. *et al.* Immunodeficiency, autoinflammation and amylopectinosis in
417 humans with inherited HOIL-1 and LUBAC deficiency. *Nat. Immunol.* **13**, 1178-
418 1186 (2012).
- 419 22. Noad, J. *et al.* LUBAC-synthesized linear ubiquitin chains restrict cytosol-
420 invading bacteria by activating autophagy and NF- κ B. *Nat. Microbiol.* **2**, 17063
421 (2017).
- 422 23. Sakamoto, H. *et al.* Gliotoxin suppresses NF- κ B activation by selectively
423 inhibiting linear ubiquitin chain assembly complex (LUBAC). *ACS. Chem. Biol.*
424 **10**, 675-681 (2015).
- 425 24. van Wijk, S.J.L. *et al.* Linear ubiquitination of cytosolic Salmonella Typhimurium
426 activates NF- κ B and restricts bacterial proliferation. *Nat. Microbiol.* **2**, 17066
427 (2017).
- 428 25. MacDuff, D.A. *et al.* Phenotypic complementation of genetic immunodeficiency
429 by chronic herpesvirus infection. *Elife* **4** (2015).
- 430 26. de Jong, M.F., Liu, Z., Chen, D. & Alto, N.M. Shigella flexneri suppresses NF-
431 κ B activation by inhibiting linear ubiquitin chain ligation. *Nat. Microbiol.* **1**,
432 16084 (2016).
- 433 27. Stieglitz, B., Morris-Davies, A.C., Koliopoulos, M.G., Christodoulou, E. &
434 Rittinger, K. LUBAC synthesizes linear ubiquitin chains via a thioester

intermediate. *EMBO Rep.* **13**, 840-846 (2012).

28. Smit, J.J. *et al.* Target specificity of the E3 ligase LUBAC for ubiquitin and NEMO relies on different minimal requirements. *J. Biol Chem* **288**, 31728-31737 (2013).

29. Tokunaga, F. *et al.* SHARPIN is a component of the NF- κ B-activating linear ubiquitin chain assembly complex. *Nature* **471**, 633-636 (2011).

30. Cohen, P., Kelsall, I.R., Nanda, S.K. & Zhang, J. HOIL-1, an atypical E3 ligase that controls MyD88 signalling by forming ester bonds between ubiquitin and components of the Myddosome. *Adv. Biol. Regul.*, 100666 (2019).

31. Kelsall, I.R., Zhang, J., Knebel, A., Arthur, J.S.C. & Cohen, P. The E3 ligase HOIL-1 catalyses ester bond formation between ubiquitin and components of the Myddosome in mammalian cells. *Proc. Natl. Acad. Sci. U S A* **116**, 13293-13298 (2019).

32. Ikeda, F. *et al.* SHARPIN forms a linear ubiquitin ligase complex regulating NF- κ B activity and apoptosis. *Nature* **471**, 637-641 (2011).

33. Dove, K.K. & Klevit, R.E. RING-Between-RING E3 Ligases: Emerging Themes amid the Variations. *J. Mol. Biol.* **429**, 3363-3375 (2017).

34. Peltzer, N., Darding, M. & Walczak, H. Holding RIPK1 on the Ubiquitin Leash in TNFR1 Signaling. *Trends Cell Biol.* **26**, 445-461 (2016).

35. Ting, A.T. & Bertrand, M.J.M. More to Life than NF-kappaB in TNFR1 Signaling. *Trends Immunol.* **37**, 535-545 (2016).

36. Wertz, I.E. *et al.* Phosphorylation and linear ubiquitin direct A20 inhibition of inflammation. *Nature* **528**, 370-375 (2015).

37. Tokunaga, F. *et al.* Involvement of linear polyubiquitylation of NEMO in NF- κ B activation. *Nat. Cell Biol.* **11**, 123-132 (2009).

38. Peltzer, N. *et al.* HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death. *Cell Rep.* **9**, 153-165 (2014).

39. Kim, W. *et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* **44**, 325-340 (2011).

40. Reiter, K.H. & Klevit, R.E. Characterization of RING-Between-RING E3 Ubiquitin Transfer Mechanisms. *Methods Mol. Biol.* **1844**, 3-17 (2018).

41. Walden, H. & Rittinger, K. RBR ligase-mediated ubiquitin transfer: a tale with many twists and turns. *Nat. Struct. Mol. Biol.* **25**, 440-445 (2018).

- 468 42. Dove, K.K. *et al.* Structural Studies of HHARI/UbcH7 approximately Ub Reveal
 469 Unique E2 approximately Ub Conformational Restriction by RBR RING1.
 470 *Structure* **25**, 890-900 e895 (2017).
- 471 43. Maes, M., Vinken, M. & Jaeschke, H. Experimental models of hepatotoxicity
 472 related to acute liver failure. *Toxicol. Appl. Pharmacol.* **290**, 86-97 (2016).
- 473 44. Mignon, A. *et al.* LPS challenge in D-galactosamine-sensitized mice accounts for
 474 caspase-dependent fulminant hepatitis, not for septic shock. *Am. J. Respir. Crit.*
 475 *Care. Med.* **159**, 1308-1315 (1999).
- 476 45. Kumari, S. *et al.* Sharpin prevents skin inflammation by inhibiting TNFR1-
 477 induced keratinocyte apoptosis. *Elife* **3** (2014).
- 478 46. Stieglitz, B. *et al.* Structural basis for ligase-specific conjugation of linear
 479 ubiquitin chains by HOIP. *Nature* **503**, 422-426 (2013).
- 480 47. Dove, K.K. *et al.* Two functionally distinct E2/E3 pairs coordinate sequential
 481 ubiquitination of a common substrate in *Caenorhabditis elegans* development.
 482 *Proc. Natl. Acad. Sci. U S A* **114**, E6576-E6584 (2017).
- 483 48. Scott, D.C. *et al.* Two Distinct Types of E3 Ligases Work in Unison to Regulate
 484 Substrate Ubiquitylation. *Cell* **166**, 1198-1214 e1124 (2016).
- 485 49. Dubois, S.M. *et al.* A catalytic-independent role for the LUBAC in NF- κ B
 486 activation upon antigen receptor engagement and in lymphoma cells. *Blood* **123**,
 487 2199-2203 (2014).
- 488 50. MacKay, C. *et al.* E3 ubiquitin ligase HOIP attenuates apoptotic cell death induced
 489 by cisplatin. *Cancer Res.* **74**, 2246-2257 (2014).
- 490 51. Niu, J., Shi, Y., Iwai, K. & Wu, Z.H. LUBAC regulates NF- κ B activation upon
 491 genotoxic stress by promoting linear ubiquitination of NEMO. *EMBO J.* **30**, 3741-
 492 3753 (2011).
- 493 52. Klein, T. *et al.* The paracaspase MALT1 cleaves HOIL1 reducing linear
 494 ubiquitination by LUBAC to dampen lymphocyte NF- κ B signalling. *Nat.*
 495 *Commun.* **6**, 8777 (2015).
- 496 53. Douanne, T., Gavard, J. & Bidere, N. The paracaspase MALT1 cleaves the
 497 LUBAC subunit HOIL1 during antigen receptor signaling. *J. Cell Sci.* **129**, 1775-
 498 1780 (2016).
- 499 54. Hailfinger, S. *et al.* Essential role of MALT1 protease activity in activated B cell-
 500 like diffuse large B-cell lymphoma. *Proc. Natl. Acad. Sci. U S A* **106**, 19946-

19951 (2009).

55. Wang, K. *et al.* Whole-genome DNA/RNA sequencing identifies truncating mutations in RBCK1 in a novel Mendelian disease with neuromuscular and cardiac involvement. *Genome Med.* **5**, 67 (2013).

56. Nilsson, J. *et al.* Polyglucosan body myopathy caused by defective ubiquitin ligase RBCK1. *Ann. Neurol.* **74**, 914-919 (2013).

57. Krenn, M. *et al.* Mutations outside the N-terminal part of RBCK1 may cause polyglucosan body myopathy with immunological dysfunction: expanding the genotype-phenotype spectrum. *J. Neurol.* **265**, 394-401 (2018).

Figure Legends

Fig. 1 | HOIL-1L E3 activity negatively regulates LUBAC functions, including suppression of apoptosis and NF- κ B activation.

a, Schematic representation of the domains of LUBAC subunits. **b, c**, Suppression of apoptosis by ligase-defective HOIL-1L. **b**, Lysates from LUBAC TKO MEFs stably reconstituted with the indicated proteins and stimulated with TNF- α (2.5 ng ml⁻¹) and CHX (20 μ g ml⁻¹) were probed as indicated. The experiments were repeated twice, independently, with similar results. **c**, Cell death of the indicated MEFs following treatment with TNF- α (10 ng ml⁻¹) and CHX (20 μ g ml⁻¹) was monitored by assaying for lactate dehydrogenase (LDH) activity. Mean $\pm$ S.D. is shown; n=3 independent experiments. *P*-values are from one-way ANOVA. **d**, Generation of TNFR Complex II (anti-FADD immunoprecipitates: IP) in MEFs stably expressing the indicated proteins. The experiments were repeated twice, independently, with similar results. **e**, Expression of NF- κ B target genes, as determined by qPCR, in MEFs expressing the indicated proteins. Mean $\pm$ S.D is shown; n=5 independent experiments; *P*-values are from two-tailed Student's *t*-test. **f**, Phosphorylation and degradation of I κ B α in LUBAC TKO MEFs expressing the indicated proteins and treated with CHX (20 μ g ml⁻¹). The experiments were repeated three times, independently, with similar results. **g**, Caspase-3 cleavage in MEFs expressing the indicated proteins treated with TNF- α (2.5 ng ml⁻¹) and CHX (20 μ g ml⁻¹). The experiments were repeated three times, independently, with similar results. Statistical source data are provided in Statistical Source Data Fig. 1 and unprocessed immunoblots are provided in Unprocessed Blots Figure 1.

Fig. 2 | HOIL-1L E3 activity negatively regulates generation of linear ubiquitin chains by LUBAC.

a, Augmented generation of linear ubiquitin chains in HEK293T cells expressing ligase-defective HOIL-1L. Lysates from HEK293T cells transfected with the indicated expression plasmids were probed as indicated. **b**, Augmented linear ubiquitination by LUBAC containing ligase-defective HOIL-1L. LUBAC containing the indicated HOIL-1L proteins was immunoprecipitated from LUBAC TKO MEFs with anti-HA antibody. The samples were subjected to *in vitro* ubiquitination assays, and lysates of the cells were probed with the indicated antibodies. **c**, Linear ubiquitination in anti-NEMO immunoprecipitates from LUBAC TKO MEFs stably expressing the indicated proteins, not subjected to any stimulation. Cells were lysed with lysis buffer containing 10 mM N-ethylmaleimide (NEM). **d**, Linear ubiquitination and RIPK1 ubiquitination in anti-FLAG immunocomplexes from LUBAC TKO MEFs stably reconstituted with the indicated proteins, stimulated with FLAG-TNF- α for the indicated periods.

The data shown were repeated three times, independently, with similar results. Unprocessed immunoblots are provided in Unprocessed Blots Figure 2.

Fig. 3 | Auto-ubiquitination of HOIL-1L inhibits LUBAC functions by suppressing generation of linear ubiquitin chains.

a, Modification of HOIL-1L in the presence of indicated HOIL-1L proteins in *in vitro* ubiquitination assays, which were conducted at 37°C for the indicated times. **b**,

Generation of upper (modified) HOIL-1L signal in *in vitro* ubiquitination assays (37°C, 18 h), and digestion of the upper (modified) HOIL-1L signal generated *in vitro* with USP2cc or NH₂OH (37°C, 1 h). **c**, Digestion of the upper (modified) HOIL-1L signal with USP2cc or OTULIN (37°C, 1 h) in anti-FLAG immunoprecipitates obtained under non-denaturing or denaturing conditions from LUBAC TKO MEFs expressing 3×FLAG–HOIL-1L. The amount of modified HOIL-1L was assessed by immunoblotting (**a–c**). **d**, **e**, Upper HOIL-1L signal (**d**) and linear ubiquitin chains (**e**) in HEK293T cells expressing the indicated HOIL-1L proteins, as determined by immunoblotting. **f**, Caspase-3 cleavage in LUBAC TKO MEFs stably reconstituted with the indicated HOIL-1L proteins, stimulated with TNF-α (5 ng ml⁻¹) and CHX (20 μg ml⁻¹). **g**, LUBAC TKO MEFs stably expressing the indicated proteins were probed as indicated to estimate the amount of modified HOIL-1L. **h**, LUBAC TKO MEFs stably reconstituted with mHOIP, SHARPIN, and 3×FLAG-HOIL All-K/R were subjected to mass spectrometry to assess the ubiquitination sites in the 3×FLAG residues of HOIL All-K/R. **i**, Viability of LUBAC TKO MEFs expressing the indicated HOIL-1L proteins, stimulated with TNF-α (10 ng ml⁻¹), was evaluated using the iCELLigence system. The data shown were repeated three times, independently, with similar results. Unprocessed immunoblots are provided in Unprocessed Blots Figure 3.

Fig. 4 | HOIL-1L E3 activity negatively regulates LUBAC functions *in vitro*.

a, Schematic representation of the domains of Petit-SHARPIN and experimental protocols for **b–e**. **b–d**, Generation of linear chains in the presence of the indicated HOIL-

1L proteins in *in vitro* ubiquitination assays of Petit-SHARPIN, as assessed by immunoblotting with the indicated antibodies. **e**, Modification of the indicated HOIL-1L proteins in *in vitro* ubiquitination assays with Petit-SHARPIN C885A and HOIL-1L WT or the indicated mutants, as assessed by immunoblotting.

The data shown were repeated three times, independently, with similar results. Unprocessed immunoblots are provided in Unprocessed Blots Figure 4.

Fig.5 | HOIL-1L ubiquitinates all LUBAC subunits, followed by HOIP-mediated linear ubiquitination onto mono-ubiquitin, and OTULIN counteracts this effect.

a–e Modification of LUBAC subunits in WT or OTULIN KO MEFs expressing the indicated HOIL-1L proteins. **a,d**, The indicated proteins in the lysates were probed as indicated. **b,e**, Linear ubiquitin-specific tandem ubiquitin binding entity (M1-TUBE). Material pulled down from the indicated MEFs with M1-TUBE were probed as indicated. **c**, Modification of all LUBAC components in the presence of the indicated HOIL-1L and Petit-SHARPIN C885A proteins in *in vitro* ubiquitination assays, as assessed by immunoblotting with the indicated antibodies. **f**, Introduction of ligase-defective HOIL-1L C458A together with HOIP and SHARPIN restored the OTULIN KO phenotype induced by treatment with TNF- α (1 ng ml⁻¹) plus CHX (20 μ g ml⁻¹). **g**, Viability of WT or OTULIN KO MEFs expressing the indicated HOIL-1L proteins, stimulated with TNF- α (10 ng ml⁻¹), was assessed using the iCELLigence system. **h**, Schematic of the mechanism underlying HOIL-1L E3-mediated suppression of LUBAC activity. HOIL-1L mono-ubiquitinates all LUBAC subunits (SHARPIN, HOIL1 and HOIP), and HOIP

further conjugates linear ubiquitin chains to mono-ubiquitin, which is conjugated to LUBAC by HOIL-1L, as cis-linear ubiquitination (second, left). HOIL-1L E3 renders HOIP RBR able to preferentially recognize LUBAC over other substrates including NEMO or RIPK1 for linear chain conjugation as trans-linear ubiquitination, thereby suppressing LUBAC functions. OTULIN counteracts cis-linear ubiquitination of LUBAC, as reported previously. Loss of mono-ubiquitination of LUBAC following deletion of HOIL-1L E3 profoundly suppresses cis-linear ubiquitination of LUBAC and increases its linear ubiquitination activity towards substrates (trans-linear ubiquitination). The data shown were repeated three times, independently, with similar results. Unprocessed immunoblots are provided in Unprocessed Blots Figure 5.

Fig. 6 | Defective HOIL-1L E3 activity protects against *Salmonella typhimurium* infections by activating LUBAC.

a–e, *S. typhimurium* infections of LUBAC TKO MEFS stably expressing HOIP, SHARPIN, and 3×FLAG–HOIL-1L WT or C458A. Quantification of intracellular *S. typhimurium* proliferation, presented as mean colony forming units (CFUs) per well. Mean ± S.D. is shown; n=3 independent experiments; *P*-values are from one-way ANOVA followed by Tukey's multiple comparison test (**a**). Microscopic images of the indicated MEFs infected with *S. typhimurium*. Scale bars, 100 μm. The experiments were repeated three times, independently, with similar results. (**b**). Cell death induced by *S. typhimurium* was monitored by assaying for LDH activity. Mean ± S.D. is shown; n=3 independent experiments; *P*-values from one-way ANOVA followed by Tukey's multiple

comparison test are shown (c). **d**, Structured illumination micrographs. The indicated MEFs were infected with *S. typhimurium* and stained for linear ubiquitin, 3×FLAG–HOIL-1L, or Hoechst 33342 at 6 h post-infection. Scale bars, 2 μm. The experiments were repeated three times, independently, with similar results. **e**, qPCR analyses of expression levels of the NF-κB target genes *Il6* and *Tnf* in cells infected with *S. typhimurium* at 4 h post-infection. Mean is shown; n=3 independent experiments; *P*-values are from one-way ANOVA followed by Tukey’s multiple comparison test. Statistical source data are provided in Statistical Source Data Fig. 6.

Fig. 7 | Generation of HOIL-1L E3–defective ΔRING1 mice.

a, Macroscopic pictures of the indicated littermate mice at 18 weeks old (left panel) and 12 months old (right panel). **b**, Lysates from organs of 9-week-old littermate mice of the indicated genotypes were subjected to immunoblotting for linear ubiquitin. **c**, Immunostaining for linear ubiquitin in 12-week-old littermate mice. Linear ubiquitin, green; DAPI, blue. Scale bars, 50 μm. **d**, Macroscopic appearance of spleens of the indicated mice at 18 weeks old. Scale bars, 1 cm. **e**, Histological analysis, performed by H&E staining, of tissue sections from 9-week-old mice of the indicated genotypes. The data shown in a-e were repeated twice, independently, with similar results. **f**, Antibody titers of serum of the indicated mice were determined by ELISA. Mean ± S.D. is shown; n=5 independent experiments; *P*-values were obtained by a two-tailed Student’s t-test. n.s. means not significant. **g,h**, Splenocytes of littermate mice of the indicated genotypes at 15 weeks old were examined by flow cytometry. CD19⁺ B cells of splenocytes were

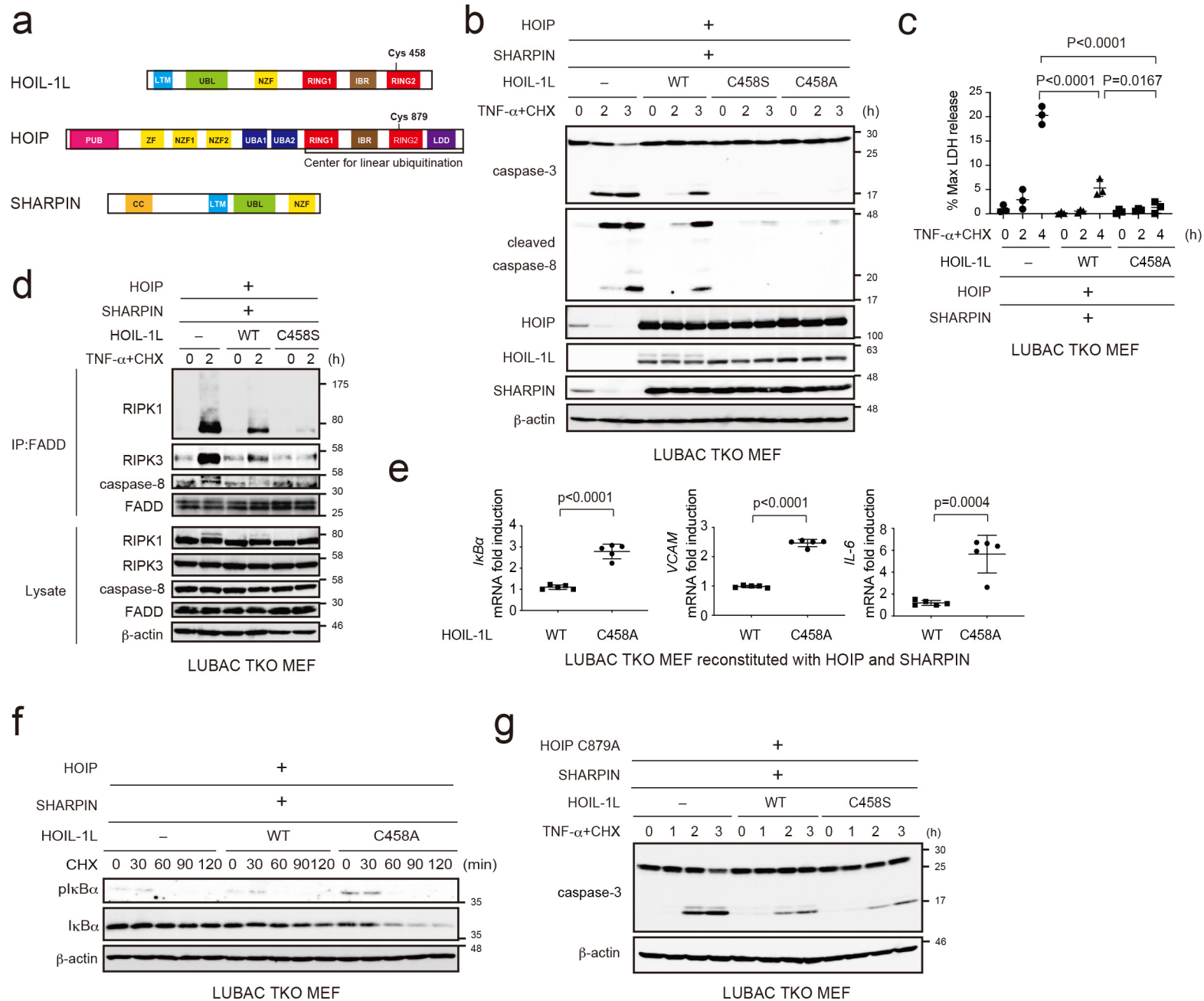
analyzed for surface expression of CD38 and PNA (**g**, upper panel), FAS and PNA (**g**, lower panel), and TCR β +CD4⁺ T cells of splenocytes were analyzed for surface expression of CD25 (**h**), all hallmarks of activated lymphocytes, are indicated. Figures exemplifying the gating strategy for flow cytometry experiments were provided in Supplementary Figure 1a,b. The data shown in a,b, d,e, g,h were repeated three times, independently, with similar results and the data shown in c were repeated twice, independently, with similar results.

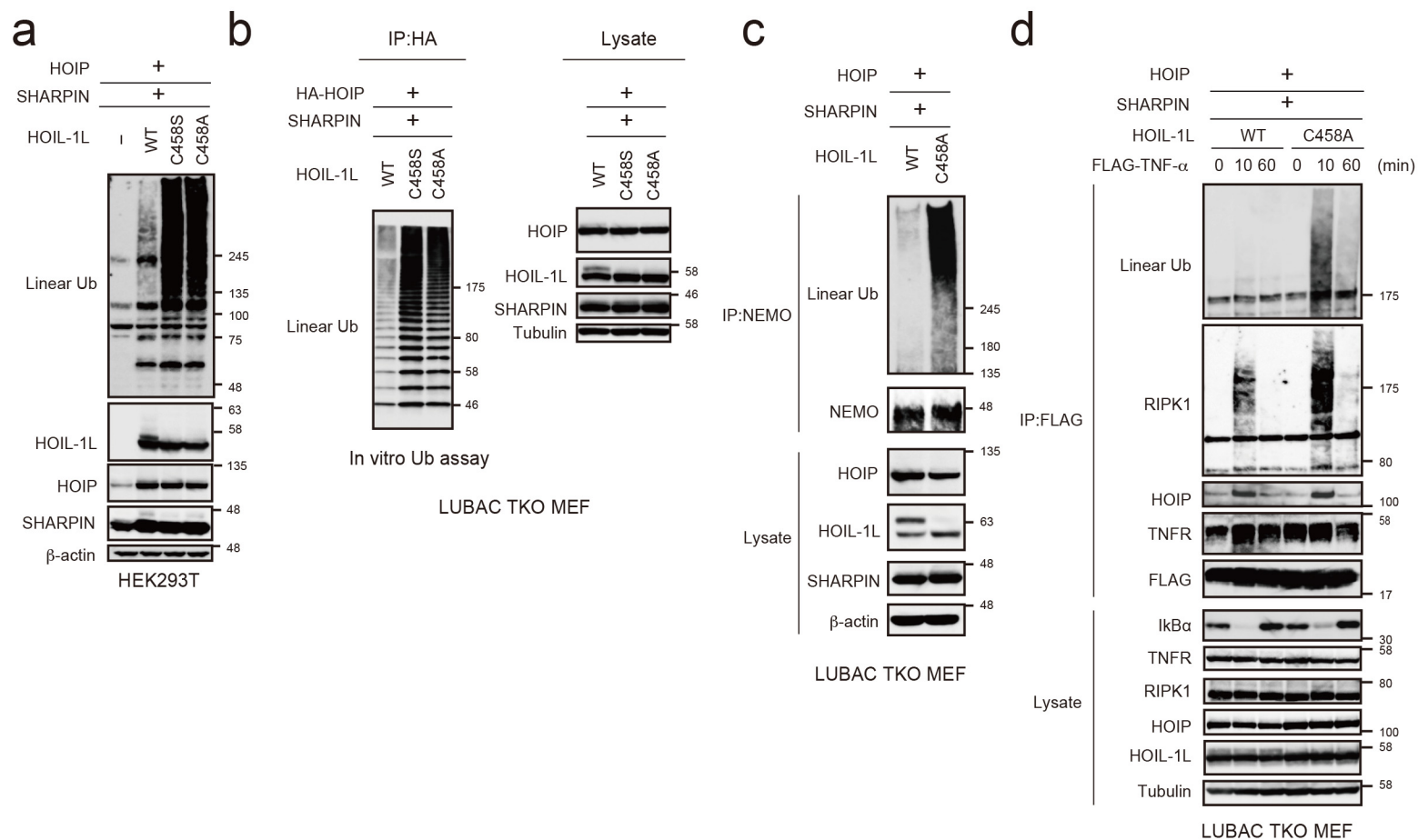
Statistical source data are provided in Statistical Source Data Fig. 7 and unprocessed immunoblots are provided in Unprocessed Blots Figure 7.

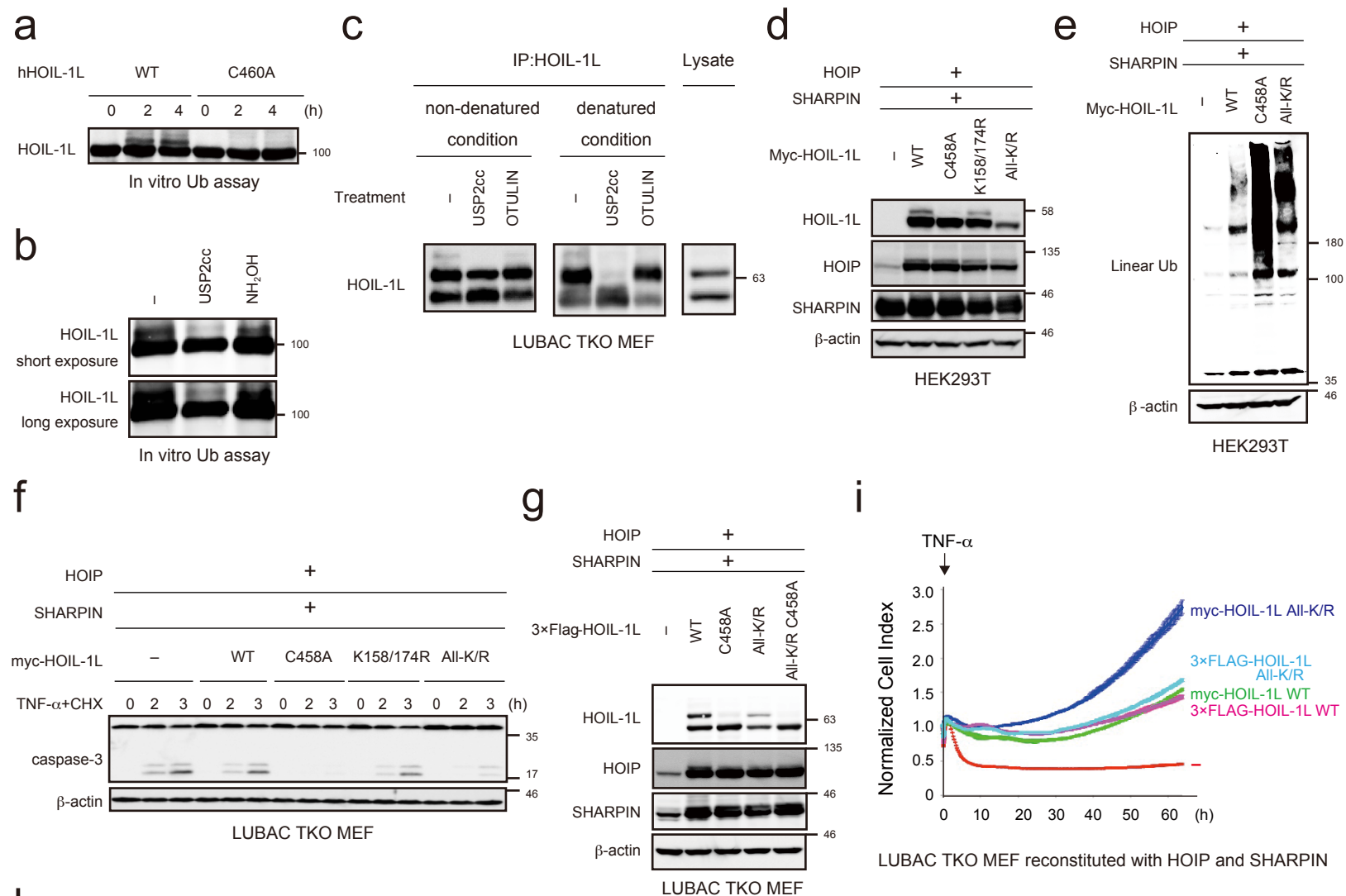
Fig. 8 | Pathophysiological roles of HOIL-1L E3-defective mice.

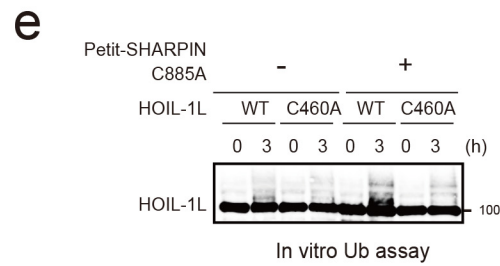
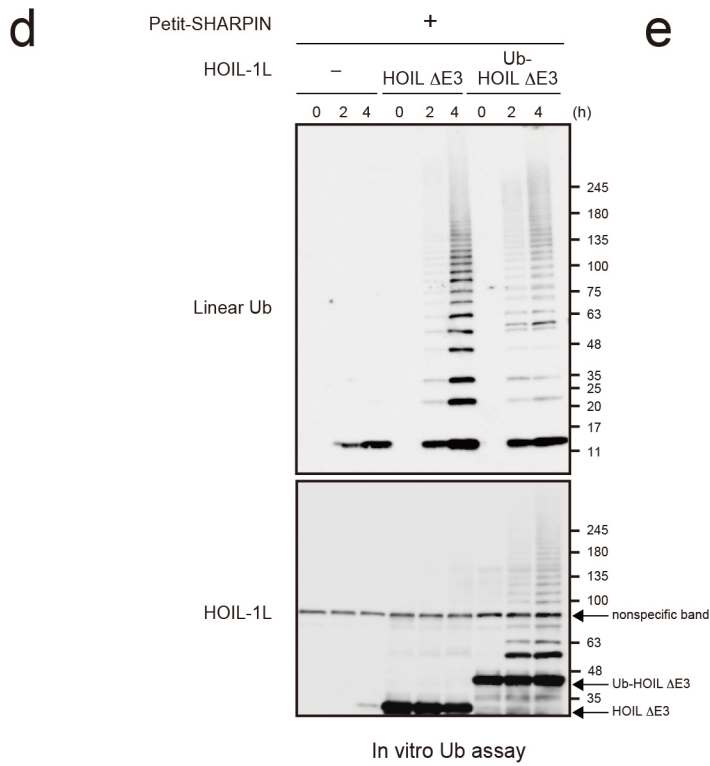
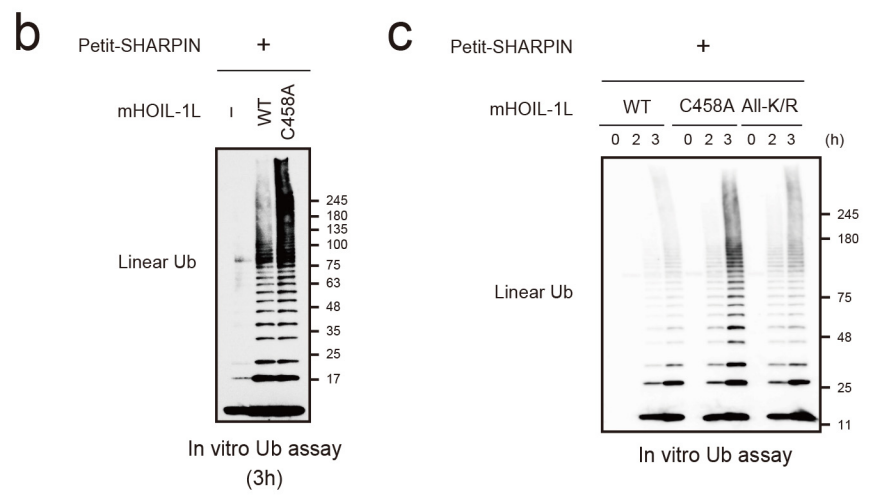
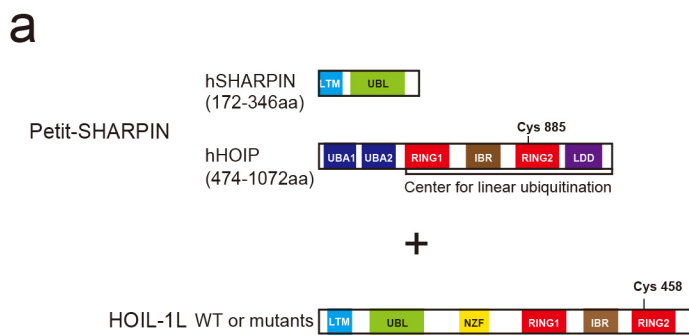
a, Hepatocyte apoptosis induced by D-GalN (700 mg per kg) and LPS (10 μ g per kg) in mouse livers of the indicated genotypes. Acquisition of macroscopic images, H&E staining, immunostaining for cleaved caspase-3 and TUNEL staining in livers of the indicated genotypes were performed 7 h after i.p. injections. The experiments were repeated twice, independently, with similar results. **b**, Kaplan–Meier survival graph comparing the two strains of mice up to 24 h after i.p. injections of LPS/D-Gal-N. *P*-values were obtained by a two-tailed log–rank test. **c**, Acquisition of macroscopic pictures, H&E staining, and immunostaining for cleaved caspase-3 and cleaved caspase-8 were performed in skin sections from 10-week-old mice of the indicated genotypes. The experiments were repeated three times independently, with similar results. **d**, **e**, Severity scores of dermatitis in mice of the indicated genotypes from 3 weeks to 10 weeks.

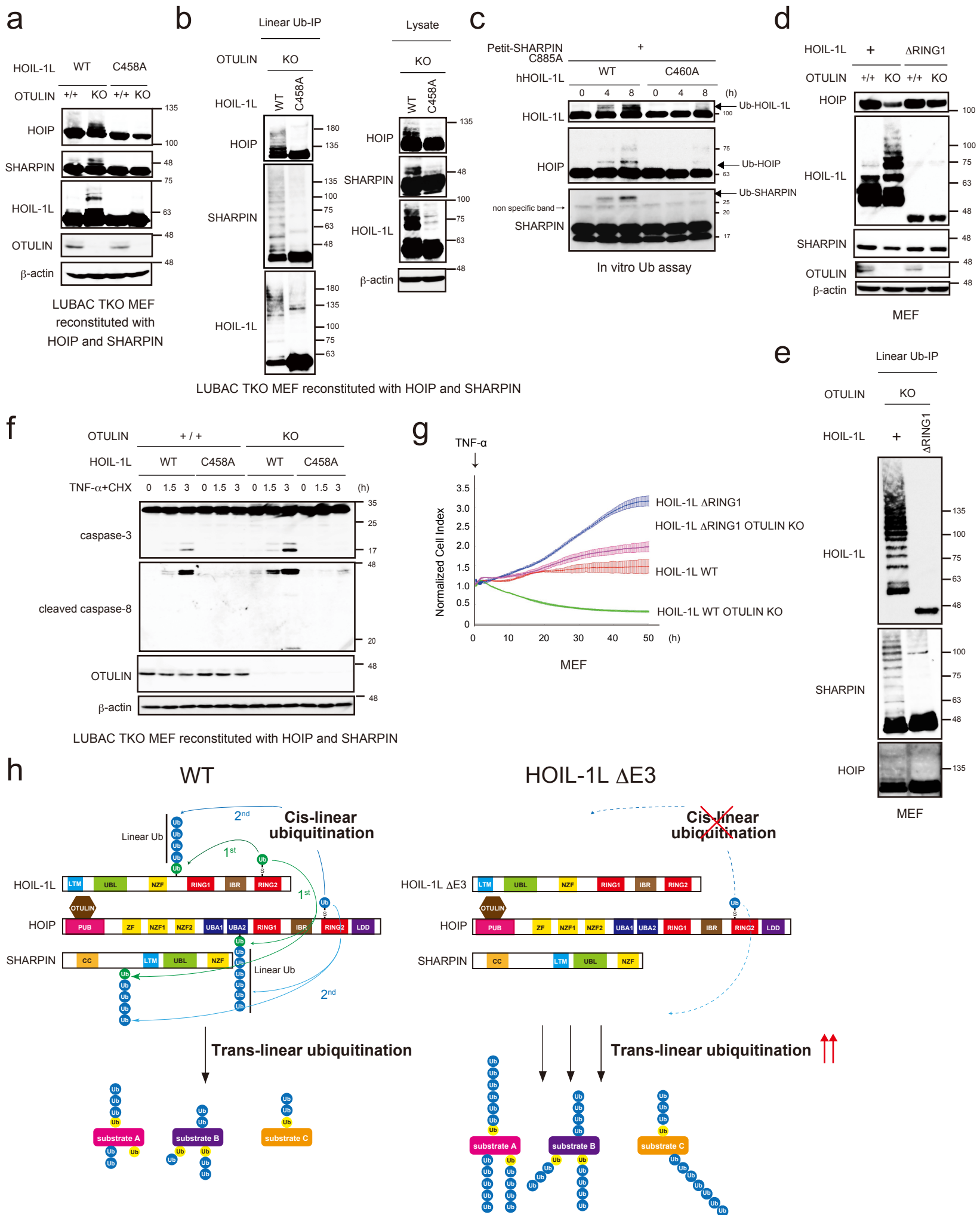
666 SHARPIN_{cpdm/cpdm}HOIL-1L^{+/+} (n=14), SHARPIN_{cpdm/cpdm}HOIL-1L^{ΔRING1/+} (n =20),
667 SHARPIN_{cpdm/cpdm}HOIL-1L^{ΔRING1/ΔRING1} (n =13). Data are means ± S.D.; *P*-values are
668 from one-way ANOVA followed by Tukey's multiple comparison test. Statistical
669 source data are provided in Statistical Source Data Fig. 8.



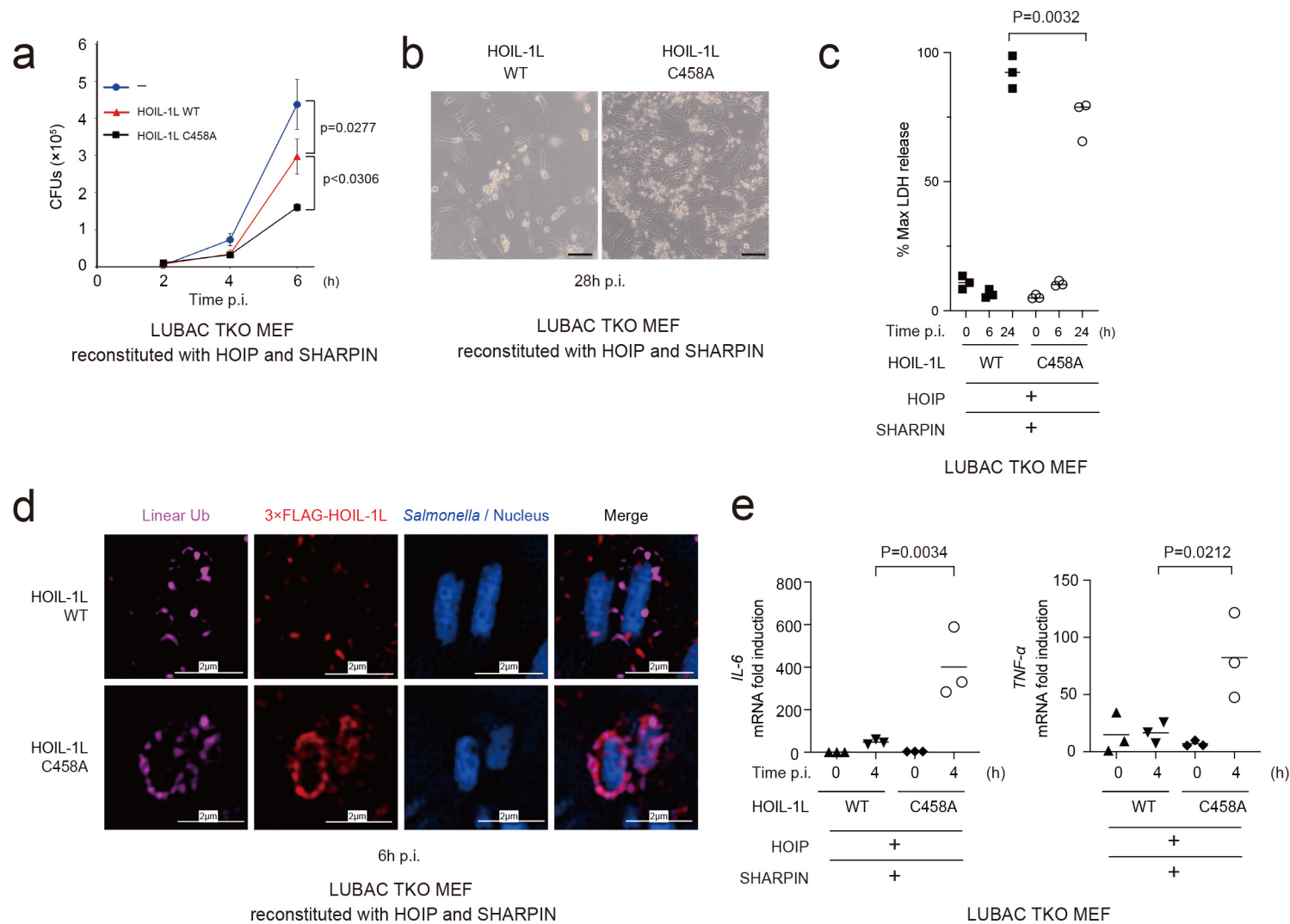


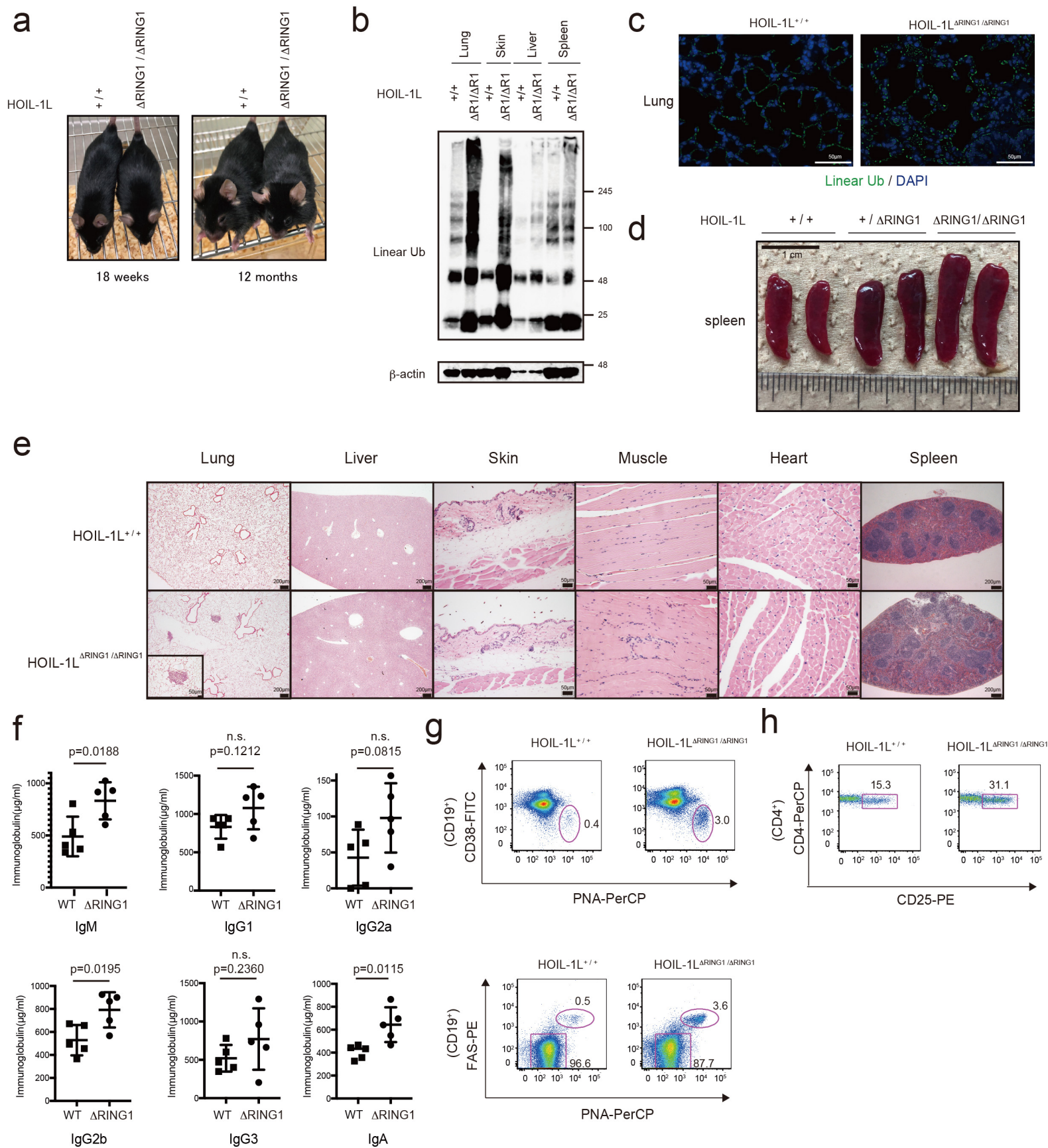


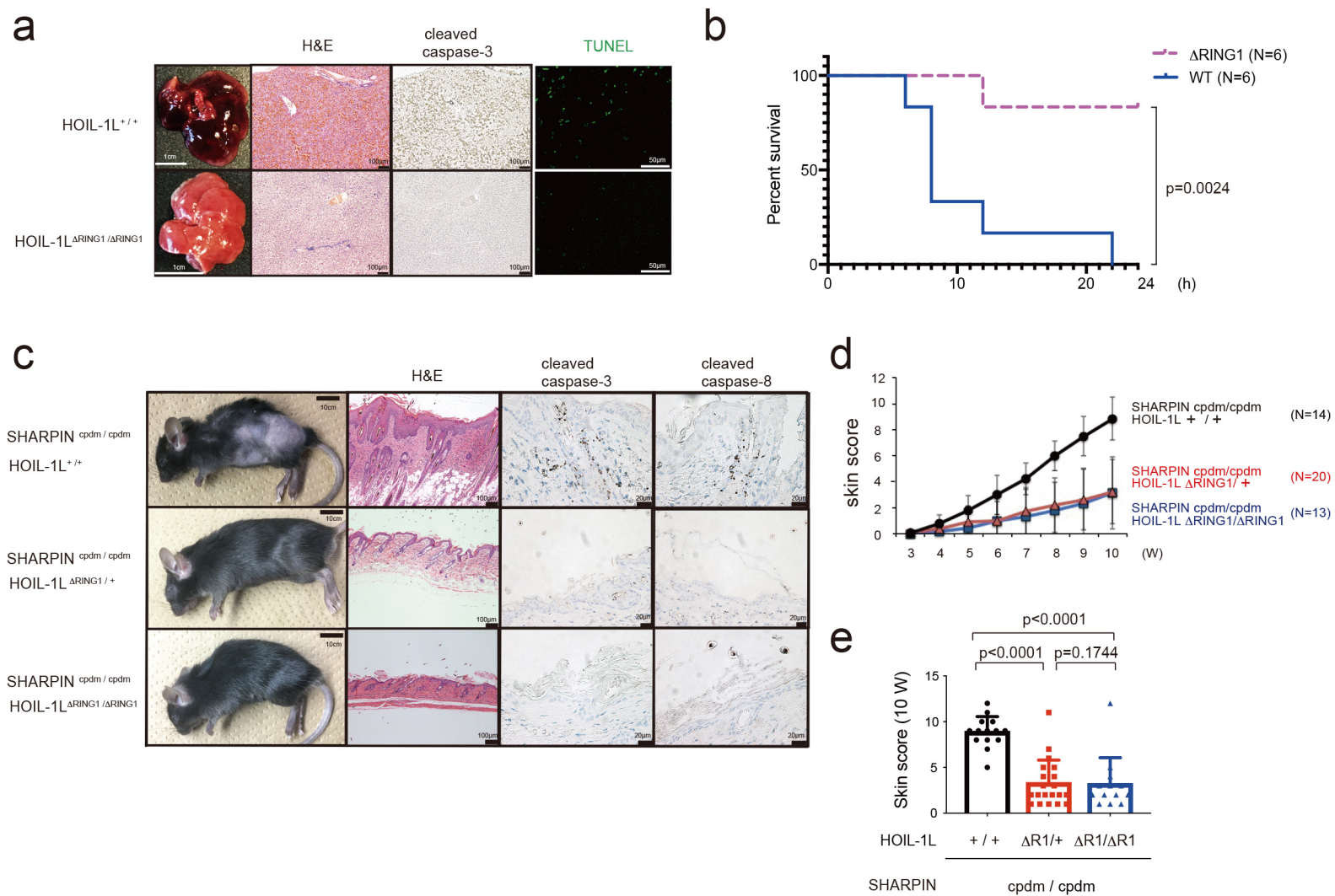




Fuseya et al. Figure 5







Fuseya et al. Figure 8

Methods

Cell cultures, transfection, and retroviral expression

MEFs and HEK293T cells were grown in DMEM containing 10% FBS, 100 IU ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Keratinocytes were cultured in serum-free human keratinocyte medium (DS Pharma Biomedical) supplemented with bovine pituitary extract. Primary hepatocytes were grown in William's E medium (Gibco) supplemented with 10% FBS, GlutaMAX (Gibco/Thermo Fisher Scientific), penicillin, streptomycin, 2 µg ml⁻¹ insulin, and 100 nM dexamethasone. Transfections were performed using Lipofectamine 2000 (Invitrogen). For retroviral expression, pMXs-IP, pMXs-neo, or pMXs-IRES-Bsr encoding LUBAC components was transfected into Plat-E packaging cells as described previously¹⁶. The resultant viruses were used to infect LUBAC TKO cells, and stably transduced cells were selected using puromycin, G-418, or blasticidin.

Plasmids, antibodies, and reagents

cDNAs used in this study were described previously^{16,29,37}. Two deletion mutants were generated from the amplified ORF of mouse HOIL-1L: before RING1 (aa 1–279) and before NZF (aa 1–189). Mutants of mHOIP (C879A) and mHOIL-1L (C458A, C458S) were generated by two-step PCR. cDNAs were ligated into the appropriate epitope-tag sequences and then cloned into pcDNA3.1, pMAL-c2x, pT7-7, pMXs-IP, pMXs-neo, pMXs-IRES-Bsr, or pSpCas9(BB)-2A-Puro (PX459) (#48139, Addgene)⁵⁸. A synthetic cDNA encoding HOIL-1L All-K/R was purchased from Eurofins. Antibodies are listed in

Supplementary Table 1.

qRT-PCR

For real-time quantitative reverse transcription PCR (qRT-PCR), total RNA was isolated using the RNeasy Mini Kit (Qiagen). DNase-treated RNA (100 ng) was reverse-transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI ViiA7 Real-Time PCR system (Applied Biosystems). Primers are listed in Supplementary Table 2. All gene expression levels were normalized against the corresponding levels of *Actb* (encoding β -actin).

Immunoprecipitation, immunoblotting, and deubiquitination analysis

Cells were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma-Aldrich) with or without 10 mM N-ethylmaleimide (NEM). Lysates were clarified by centrifugation at 15,000 rpm for 20 min at 4°C. For lysates of mouse tissues, 50 mg samples of tissue were homogenized and lysed with the lysis buffer described above. For denaturing conditions, cells were lysed in phosphate-buffered saline (PBS) containing 1% SDS and then heated at 95°C for 10 min to disrupt noncovalent interactions. After heating, lysates were sheared with a 25-gauge needle and

centrifuged at 15,000 rpm for 5 min at room temperature; the resultant supernatant was diluted to 0.1% SDS with lysis buffer mentioned above. For immunoprecipitations, Protein G beads (Thermo Fisher Scientific) were prepared by washing three times with PBS and then incubated with the appropriate antibodies for 1 h at 4°C. The antibody-bead conjugates were then incubated with lysates for 2 h at 4°C, followed by five washes with lysis buffer. For deubiquitination analysis, USP2cc (5 µg) or OTULIN (5 µg) in 20 µl buffer containing 20 mM Tris-HCl (pH 7.5) and 5 mM DTT was added to immunoprecipitated beads or 30 µg cell lysate, and then incubated at 37°C for 60 min.

Immunoprecipitation of TNFR Complex I

After treatment with FLAG-His-TNF-α (1 µg ml⁻¹), cells were lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 2 mM PMSF, and protease inhibitor cocktail; centrifuged at 10,000 *g* for 20 min at 4°C; and immunoprecipitated with anti-FLAG M2 antibody. Immunoprecipitated TNFR1 complex was eluted by incubation at 37°C for 40 min in 30 µl TBS buffer containing 400 ng µl⁻¹ of 3×FLAG peptide (Sigma-Aldrich), and then analyzed by immunoblotting.

Immunoprecipitation of TNFR Complex II

Cells were pretreated with Z-VAD-FMK (10 µM) (MBL) for 60 min, and then

stimulated with TNF- α (2.5 ng ml⁻¹) and CHX (20 μ g ml⁻¹) for the indicated periods. Cells were lysed with lysis buffer containing 30 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM PMSF, and protease inhibitor cocktail, followed by centrifugation at 10,000 *g* for 10 min at 4°C. The cleared lysates were immunoprecipitated with anti-FADD antibody.

Luciferase assays

HEK293T cells were transfected with pGL4.32 (Luc2p/NF- κ B-RE/Hygro) and pGL4.74 (hRLuc/TK) (Promega) along with plasmids encoding WT or mutant LUBAC components. Twenty-four hours 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 using real-time cellular analysis (RTCA) technology

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. The next day, cells were treated with TNF- α (10 ng ml⁻¹), and the cell index was continuously monitored. Data were normalized against cell indices at the time of TNF- α treatment.

Measurement of lactate dehydrogenase (LDH) release

LDH release was measured using the Cytotox96 Non-Radioactive

Cytotoxicity Assay kit (Promega). Briefly, cells were seeded on a 24-well plates at 1×10^5 or 2×10^4 cells per well, and then treated with TNF- α (10 ng ml⁻¹) plus CHX (20 μ g ml⁻¹) or *S. typhimurium*, respectively. After culture for the indicated periods, the media were collected. LDH level in culture media was determined by measuring absorbance at 490 nm on a SpectraMax M5 (Molecular Devices).

***In vitro* ubiquitination assay**

Anti-HA immunoprecipitates from cells expressing HA-HOIP, Petit-SHARPIN, or Petit-SHARPIN C885A along with recombinant HOIL-1L (WT or mutant) were incubated at 37°C for the indicated periods with E1 (100 ng), UbcH7/UBE2L3 (400 ng), ubiquitin (5 μ g), and 2 mM ATP in 20 μ l buffer containing 20 mM Tris Tris-HCl (pH 7.5), 5 mM MgCl₂, and 1 mM DTT. For deubiquitination analysis of HOIL-1L, recombinant HOIL-1L WT or mutants were incubated overnight at 37°C with E1 (100 ng), UbcH7/UBE2L3 (400 ng), ubiquitin (5 μ g), and 2 mM ATP in 20 μ l buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 1 mM DTT. After the overnight incubation, NH₂OH (0.5–1.5 M) or USP2cc (5 μ g) was added, and the sample was incubated at 37°C for 60 min. After incubation, reactions were terminated by addition of SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting.

Expression and purification of recombinant proteins

MBP-mHOIL-1L (WT, C458A, and All-K/R), MBP-hHOIL-1L (WT, C460A), hHOIL-1L Δ E3-Strep, [aa 1–281], Ub-hHOIL-1L Δ E3-Strep, MBP-hOTULIN, His₆-USP2cc, and Petit-SHARPIN C885A were expressed in *E. coli* BL21 cells (CodonPlus DE3 #230280). MBP-fusion, Strep-tagged, and His₆-tagged proteins were purified using amylose resin (New England BioLabs), Strep-Tactin XT Superflow High Capacity (IBA Lifesciences), and Ni-NTA Agarose (QIAGEN), respectively. Petit-SHARPIN, recombinant E1, and recombinant Ub_{CH7}/UBE2L3 were prepared and purified as described previously^{8,37,23}.

Mass spectrometric analysis

LUBAC TKO MEFs stably reconstituted with human SHARPIN, myc-human HOIP, and 3 $\times$ FLAG-human HOIL-1L WT or C460A were subjected to MS. HOIL-1L was immunoprecipitated with anti-DDDDK (FLA-1), followed by separation with SDS-PAGE and staining with Bio-Safe Coomassie (Bio-Rad). Gel regions corresponding to molecular weights of interest were excised. For reduction and alkylation of cysteine residues (Fig. 3h and S3e), the gels were incubated for 1 h with 5 mM TCEP and subsequently for 10 min with 5 mM methyl methanethiosulfonate (MMTS) in 50 mM ammonium bicarbonate (AMBC). The gel pieces were washed in 50 mM AMBC/30% acetonitrile (ACN) for 2 h, and then with 50 mM AMBC/50% ACN for 1 h. The gel pieces were then dehydrated in 100% ACN for 15 min. Proteins were digested with 20 ng

μl⁻¹ sequence grade trypsin (Promega) in 50 mM AMBC/5% ACN, pH 8.0, at 37°C for 16 h. The digested peptides were extracted four times with 0.1% trifluoroacetic acid (TFA)/70% ACN. Peptides were concentrated by vacuum centrifugation and resuspended in 0.1% TFA.

For MS analysis, an Easy nLC 1200 (Thermo Fisher Scientific) was connected online to an Orbitrap Fusion LUMOS (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded onto a C18 analytical column (IonOpticks, Aurora Series Emitter Column, AUR2-25075C18A 25 cm × 75 μm 1.6 μm FSC C18 with nanoZero fitting) and separated using an 80 or 90 min gradient (solvent A, 0.1% FA; solvent B, 80% ACN/0.1% FA). For parallel reaction monitoring, the Orbitrap Fusion LUMOS instrument was operated in targeted MS/MS mode by the Xcalibur software (Thermo Fisher Scientific), and the peptides were fragmented by higher-energy collisional dissociation (HCD) with a normalized collision energy of 30. MS/MS resolution, target AGC values, and isolation windows were set to 30,000, 5E4, and 2.0 *m/z*, respectively. For data-dependent acquisition of MS/MS spectra, the most intense ions (Cycle Time: 1 sec) with charge states from +2 to +7 were selected for fragmentation by HCD with a normalized collision energy of 30, and fragment ions were detected using an Ion Trap. AGC target and isolation window were set to 1E4 and 1.6 *m/z*, respectively. Easy nLC 1000 (Thermo Fisher Scientific) and Q Exactive (Thermo Fisher Scientific) instruments were used for acquisition of the data shown in Fig. S3e, for which peptides were separated on C18 analytical columns (Reposil-Pur 3 μm, 75 μm i.d. × 12 cm packed

tip column, Nikyo Technos Co., Ltd) with a 90 min gradient (solvent A, 0.1% FA; solvent B, 100% ACN/0.1% FA). The Q Exactive instrument was operated in data-dependent mode using the Xcalibur software, and the top 10 most intense ions with charge states from +2 to +4 were selected for fragmentation by HCD with a normalized collision energy of 28. The data were analyzed using SEQUEST in Proteome Discoverer 2.2 (Thermo Fisher Scientific). The mass tolerances for the precursor and fragment ions were 10 ppm and 20 mmu (for Orbitrap) or 0.6 Da (for Ion Trap), respectively, and peptide identification was filtered at FDR < 0.01.

Absolute quantification (AQUA) of ubiquitin linkages was performed essentially as previously described⁵⁹ with some modifications. Whole-cell lysates (24 µg) were separated by SDS-PAGE, and gel regions > 75 kD were excised for in-gel trypsin digestion as described above. After trypsin digestion, AQUA peptides (15 fmol/injection) were added to the extracted peptides. Concentrated peptides were diluted with 20 µL 0.1% TFA containing 0.05% H₂O₂ to oxidize methionine residues and then incubated at 4°C overnight. Easy nLC 1200 and Orbitrap Fusion LUMOS were operated as described above, and peptides were separated using a 45 min gradient. The data were processed using the PinPoint software 1.3 (Thermo Fisher Scientific), and peptide abundance was calculated based on the integrated area under the curve of the selected fragment ions.

Generation of knockout cells by CRISPR/Cas9

Generation of LUBAC TKO MEFs was described previously¹⁶. To knock out OTULIN, wild-type, ΔRING1, or TKO MEFs reconstituted with HOIP and SHARPIN were

transfected with pX459 encoding a gRNA sequence targeting OTULIN (ACTTCCATAAGGCGAGTCCG). Transfections were performed using Lipofectamine 2000, followed by selection with puromycin for 2 days. Isolated colonies were verified as OTULIN KO by immunoblotting with anti-OTULIN antibody and by genomic PCR using the following primers:

OTULIN typing_Fwd: 5'- TGGGGGTCCCATACTAGATA -3'

OTULIN typing_Rev: 5'- ACACTGCATGTAACACCTTC-3'

Tandem ubiquitin binding entity (TUBE) assay

Halo-tagged linear ubiquitin chain-specific tandem ubiquitin binding entity (M1-specific TUBE) was purified as described previously^{60,61}. To measure linear ubiquitination in MEFs, 2 mg cell lysates were incubated at 4°C for 3 h with 2 µg M1-specific TUBE coupled with 20 µl of equilibrated Magne HaloTag beads (Promega) in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100. The precipitates were washed five times with 0.005% Nonidet P40 Substitute in PBS, boiled in SDS sample buffer, and analyzed by immunoblotting.

***S. typhimurium* infection**

S. typhimurium (NBRC13245) was purchased from the National Institute of Technology and Evaluation, Biological Resource Center, Japan. Before each infection experiment, *S. typhimurium* strains were freshly streaked from glycerol stocks on Luria-Bertani (LB) plates supplemented with the appropriate antibiotics. The next day, a single colony was

inoculated into LB supplemented with antibiotics, grown overnight, and subcultured (1:30) in fresh LB for 3 h at 37°C to reach an optical density at 600 nm (OD₆₀₀) of 1. MEFs were maintained in DMEM without antibiotics. Infections were performed at a multiplicity of infection (MOI) of 100 for 30 min at 37°C in pre-warmed DMEM lacking antibiotics. Following three washes with warm PBS, medium was replaced with DMEM supplemented with 100 µg ml⁻¹ gentamycin (Sigma-Aldrich), and cells were maintained for the indicated time periods at 37°C. To count intracellular bacteria, cells from quadruplicate wells were lysed with 0.1% Triton X-100 (v/v) in sterile saline, and serial dilutions were spread on LB plates and incubated at 37°C.

Microscopy

S. typhimurium or GFP-expressing *S. typhimurium*-infected cells were fixed in 4% paraformaldehyde PBS (Fujifilm) for 15 min at 37°C. Cells were washed three times with PBS, followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min at room temperature. Cells were subsequently blocked with 2% BSA in PBS for 30 min at room temperature. Cells were incubated with the indicated primary antibodies for 16 h at 4°C. Cells were washed three times with PBS and then incubated with the appropriate secondary antibodies. After washing, nuclei and *S. typhimurium* were counterstained with Hoechst 33342 (Invitrogen) and mounted in VECTASHIELD antifade reagent (Vector Laboratories) on microscopic glass supports. Confocal images were obtained on a Zeiss 710 confocal microscope (ZEISS). SIM images were obtained using a Nikon N-SIM system equipped with a 100×/1.49 TIRF oil immersion objective lens (Nikon). Image

processing, including three-dimensional reconstruction and co-localization analysis, were carried out using the NIS-Element advanced research software (Nikon).

Mice and preparation of primary cells

SHARPIN^{cpdm/cpdm} mice were obtained from the Jackson Laboratory. To generate KO mice harboring a conditional deletion of exons 7 and 8 of the gene encoding HOIL-1L (HOIL-1L Δ RING1 cKO), the targeting vector shown in Extended Data Fig. 7e was transfected into ES cells (TT2), and G418-resistant colonies were selected. Homologous recombinants were microinjected into 8-cell embryos of ICR mice. The resultant chimeric mice were intercrossed with C57BL/6 mice. Mice lacking HOIL-1L RING1 throughout the body (HOIL-1L Δ RING1) were generated by mating HOIL-1L Δ RING1 cKO with CAG-Cre mice (RBRC No. RBRC01828)⁶². Preparation of primary keratinocytes, hepatocytes, and MEFs were described previously^{16,37,63}. All mouse protocols were approved by Kyoto University.

Acute liver injury model

Mice intraperitoneally injected with D-GalN (700 mg kg⁻¹) and LPS (10 μ g kg⁻¹) or with PBS were euthanized at 7 h after treatment, and liver and serum samples were collected. For DNA extraction, 50 mg liver was lysed for 2 h at 55°C with lysis buffer containing 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 40 mM NaCl, and proteinase K (0.1 mg ml⁻¹), followed by centrifugation at 13,000 rpm for 10 min. Genomic DNA was precipitated with isopropanol, and dried pellets were dissolved in TE buffer.

241

242 **Histology, TUNEL assay, and immunohistochemistry**

243 Samples were fixed in 4% buffered paraformaldehyde, followed by paraffin embedding.
244 Sections were subjected to TUNEL staining using the In Situ Cell Death Detection Kit,
245 Fluorescein (Roche Life Science). For immunohistochemistry, after antigen retrieval for
246 10 min at 99°C in citric buffer (pH 6), slides were blocked for 30 min with PBS containing
247 2% BSA, 5% goat serum, and 0.1% Triton X-100; incubated with primary antibody for
248 16 h at 4°C; and incubated for 1 h at room temperature with the appropriate Alexa Fluor
249 488-conjugated secondary antibody at a 1:200 dilution. After washing, the samples were
250 mounted with a coverslip using ProLong gold antifade reagent with DAPI (Thermo Fisher
251 Scientific) and imaged on a BZ-900 fluorescence microscope (Keyence) or FV1000D
252 confocal laser scanning fluorescence microscope (Olympus). For peroxidase staining,
253 slides were treated with 3% H₂O₂ for 10 min at room temperature, washed, blocked, and
254 incubated with a primary antibody as mentioned above. After washing, the slides were
255 incubated for 20 min with Simple Stain Mouse MAX-PO (Nichirei Biosciences), washed,
256 incubated with DAB (3,39-diaminobenzidine tetrahydrochloride) (Pierce), and
257 immediately washed and counterstained with hematoxylin.

258

259 **ELISA**

260 To measure total immunoglobulins, anti-mouse IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3
261 antibodies (SouthernBiotech; all at 1 µg ml⁻¹) were added to 96-well ELISA plates (Nunc
262 MaxiSorp) and incubated at 4°C overnight. The wells were then blocked with 1% BSA

in PBS at 37°C for 75 min. Appropriately diluted serum was incubated at room temperature for 60 min, and then HRP-conjugated anti-mouse IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 antibodies (SouthernBiotech) were added. BD OptEIA (BD Biosciences) was used as the substrate, and absorbance at 450 nm was measured using a microplate reader (Molecular Devices).

Flow cytometry analysis

Single-cell suspensions prepared from the spleen of 8- to 15-week-old mice of the indicated genotype were stained with fluorochrome-conjugated antibodies. All samples were acquired using a FACSCanto II (BD Biosciences), and the results were analyzed using the FlowJo software (Tree Star).

Evaluation of dermatitis severity

Severity of dermatitis was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). Areas evaluated were the face, chest, abdomen, rostral back, and caudal back. The skin score was the sum of each score obtained.

Statistical analysis and Reproducibility

Statistical analyses were performed using GraphPad Prism 8 version 8.4.0 (GraphPad Software). Statistical significance was determined using, a two-tailed Student's t-test, one-way ANOVA followed by Tukey's multiple comparison test or a two-tailed log-rank

test. The exact sample sizes (n) used to calculate statistics are provided in the figure legends. P values are provided in the figure legends. No data points were excluded. *P*-values < 0.05 were considered statistically significant. All experiments were reproduced with similar results at least twice.

Data Availability

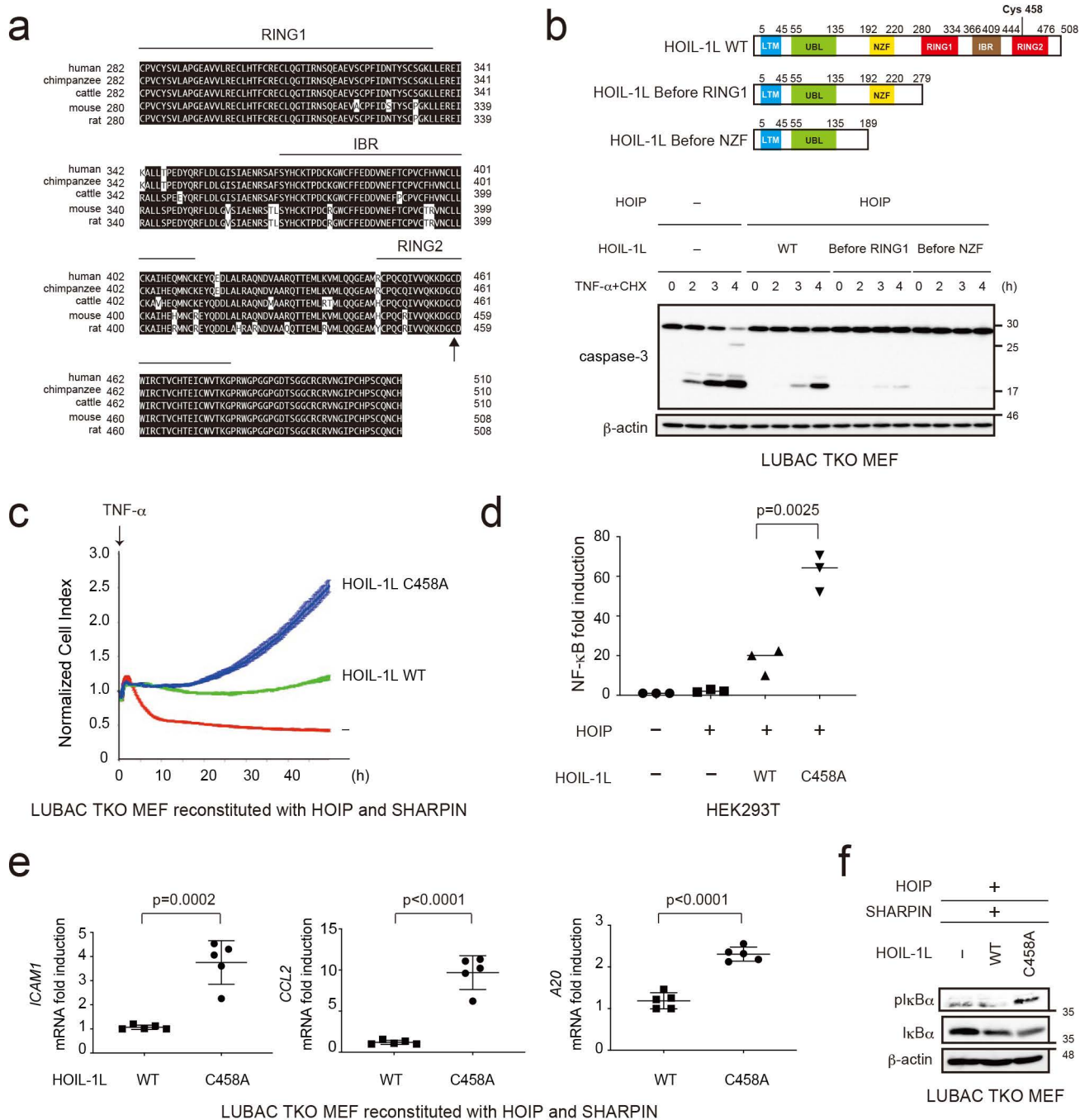
Source data for Figs.1-8, Extended Data Figs.1-5,7,8 have been provided as Statistical Source data or Unprocessed Blots. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Supplementary information is available in the online version of this paper.

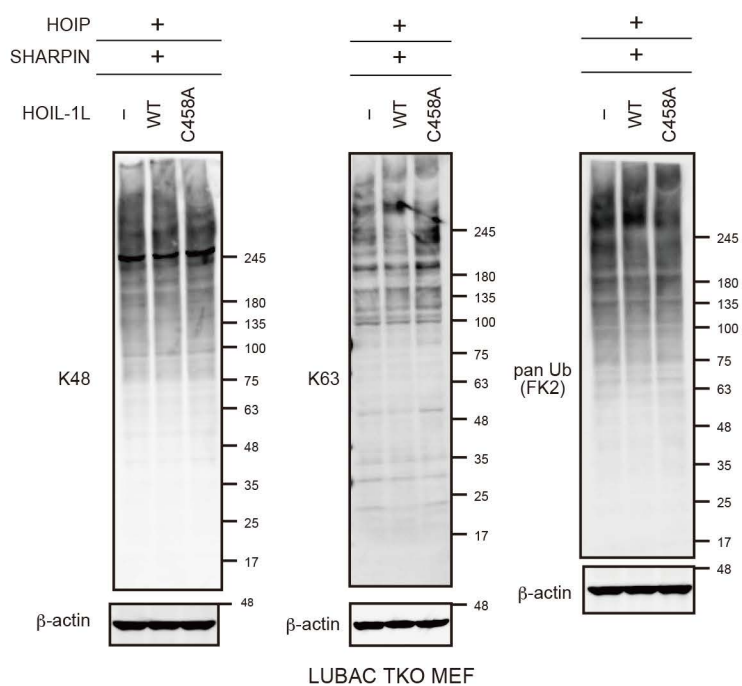
REFERENCES

58. Ran, F.A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281-2308 (2013).
59. Ohtake, F., Saeki, Y., Ishido, S., Kanno, J. & Tanaka, K. The K48-K63 Branched Ubiquitin Chain Regulates NF- κ B Signaling. *Mol. Cell* **64**, 251-266 (2016).
60. Hjerpe, R. *et al.* Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep.* **10**, 1250-1258 (2009).
61. van Wijk, S.J., Fiskin, E. & Dikic, I. Selective monitoring of ubiquitin signals with genetically encoded ubiquitin chain-specific sensors. *Nat. Protoc.* **8**, 1449-1458 (2013).
62. Matsumura, H., Hasuwa, H., Inoue, N., Ikawa, M. & Okabe, M. Lineage-specific cell disruption in living mice by Cre-mediated expression of diphtheria toxin A chain. *Biochem. Biophys. Res. Commun.* **321**, 275-279 (2004).
63. Tamiya, H. *et al.* IFN- γ or IFN- α ameliorates chronic proliferative dermatitis by

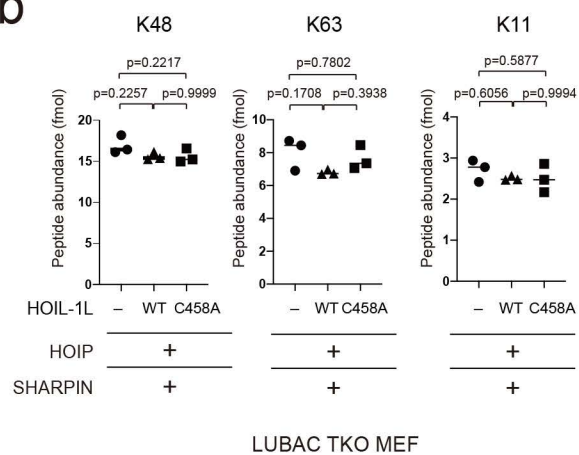
311 inducing expression of linear ubiquitin chain assembly complex. *J. Immunol.* **192**,
312 3793-3804 (2014).
313

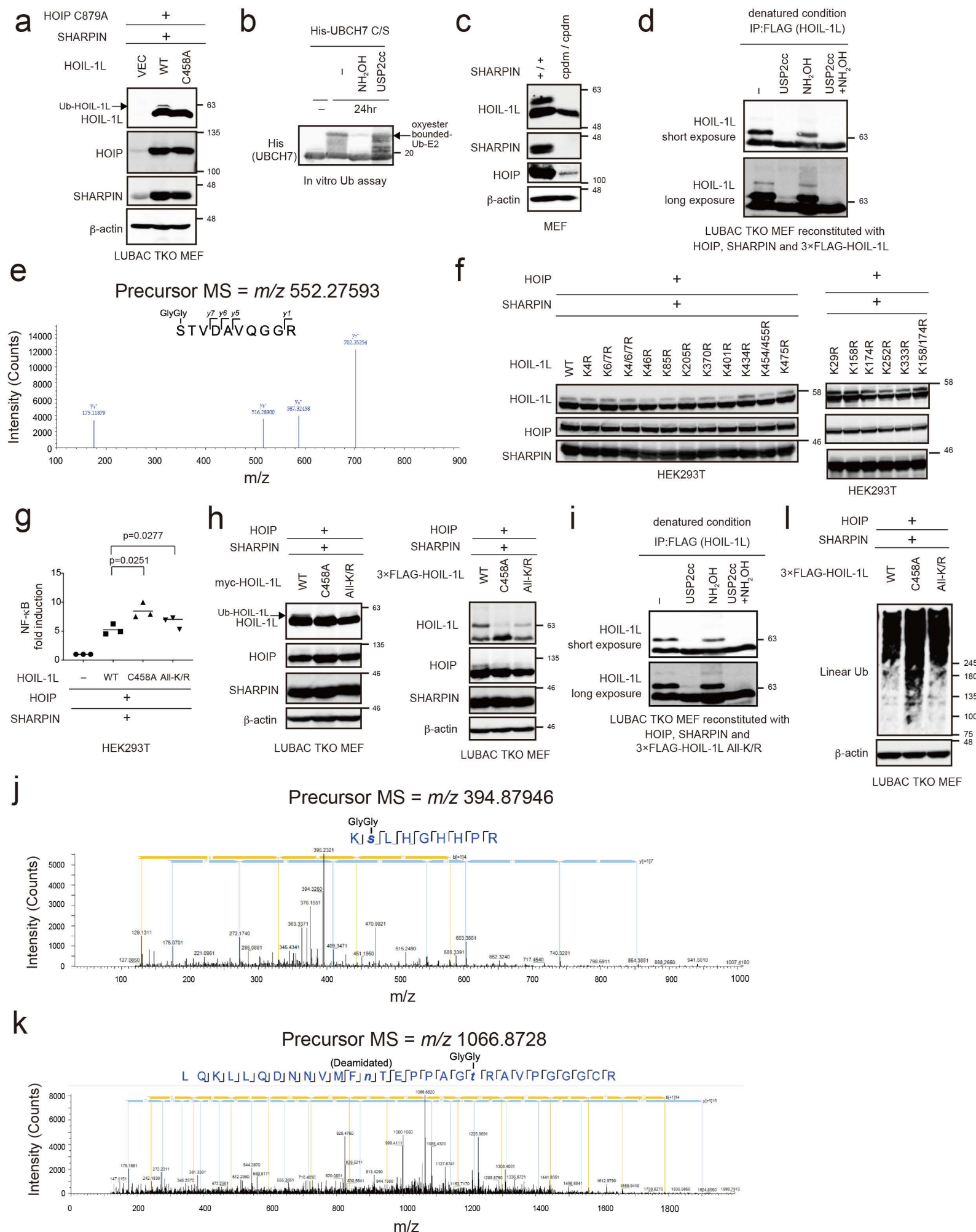


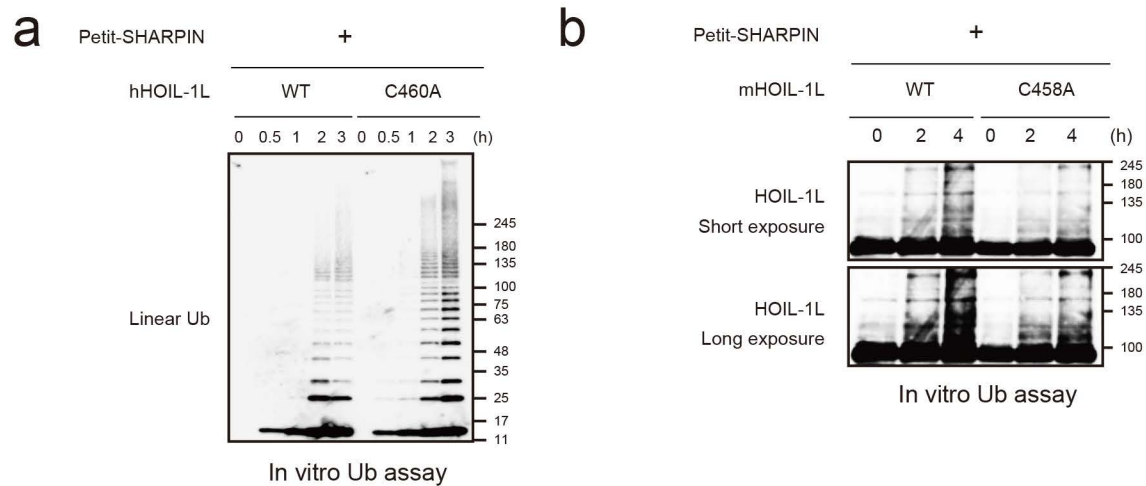
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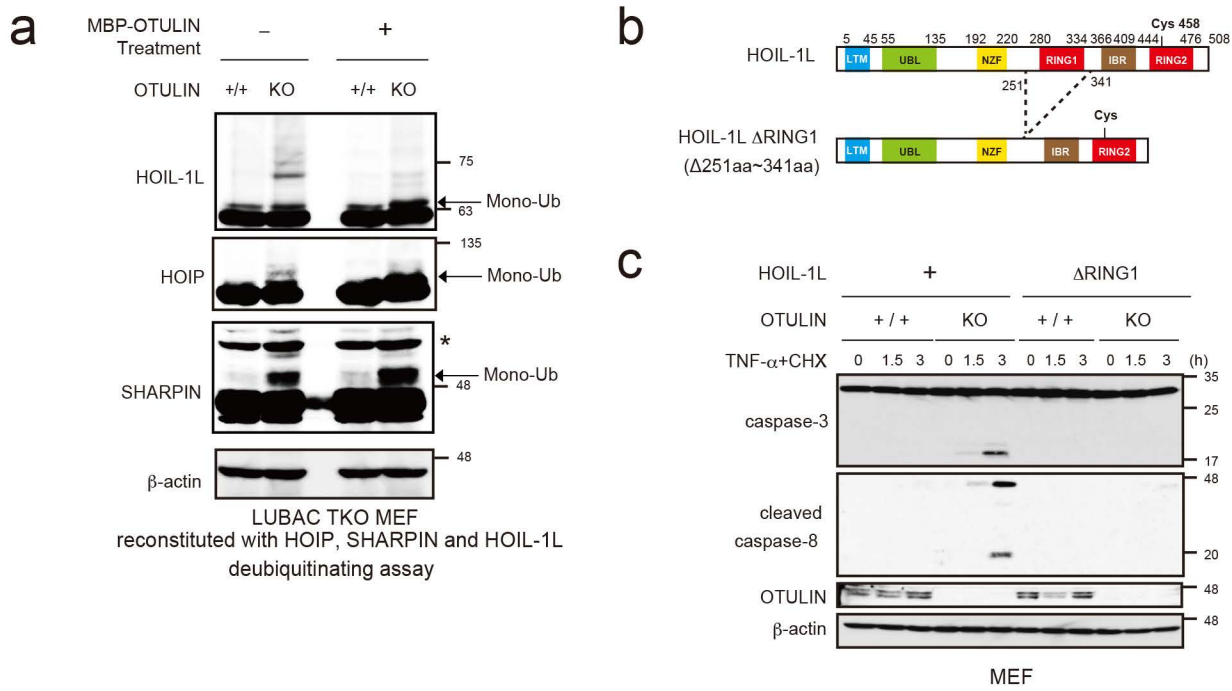


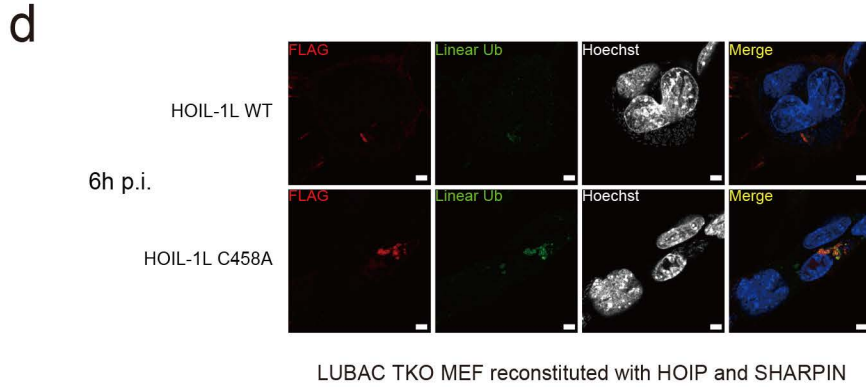
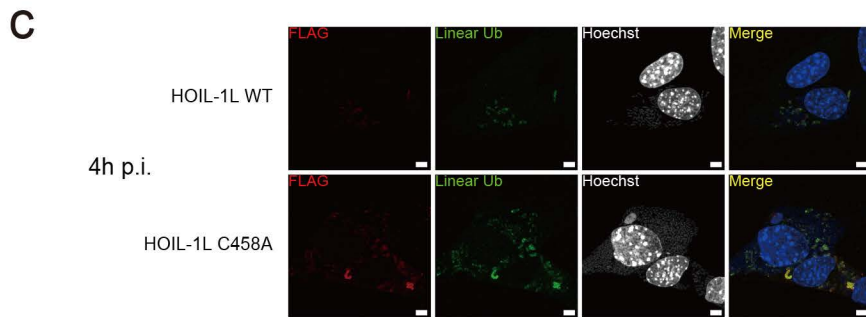
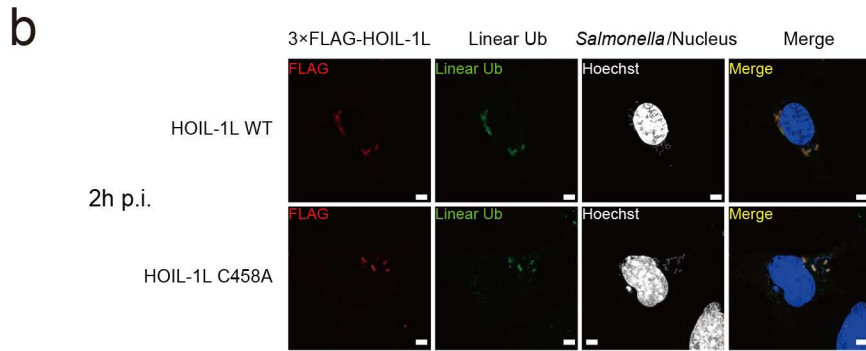
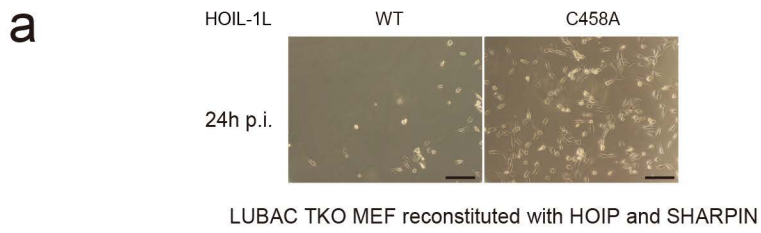
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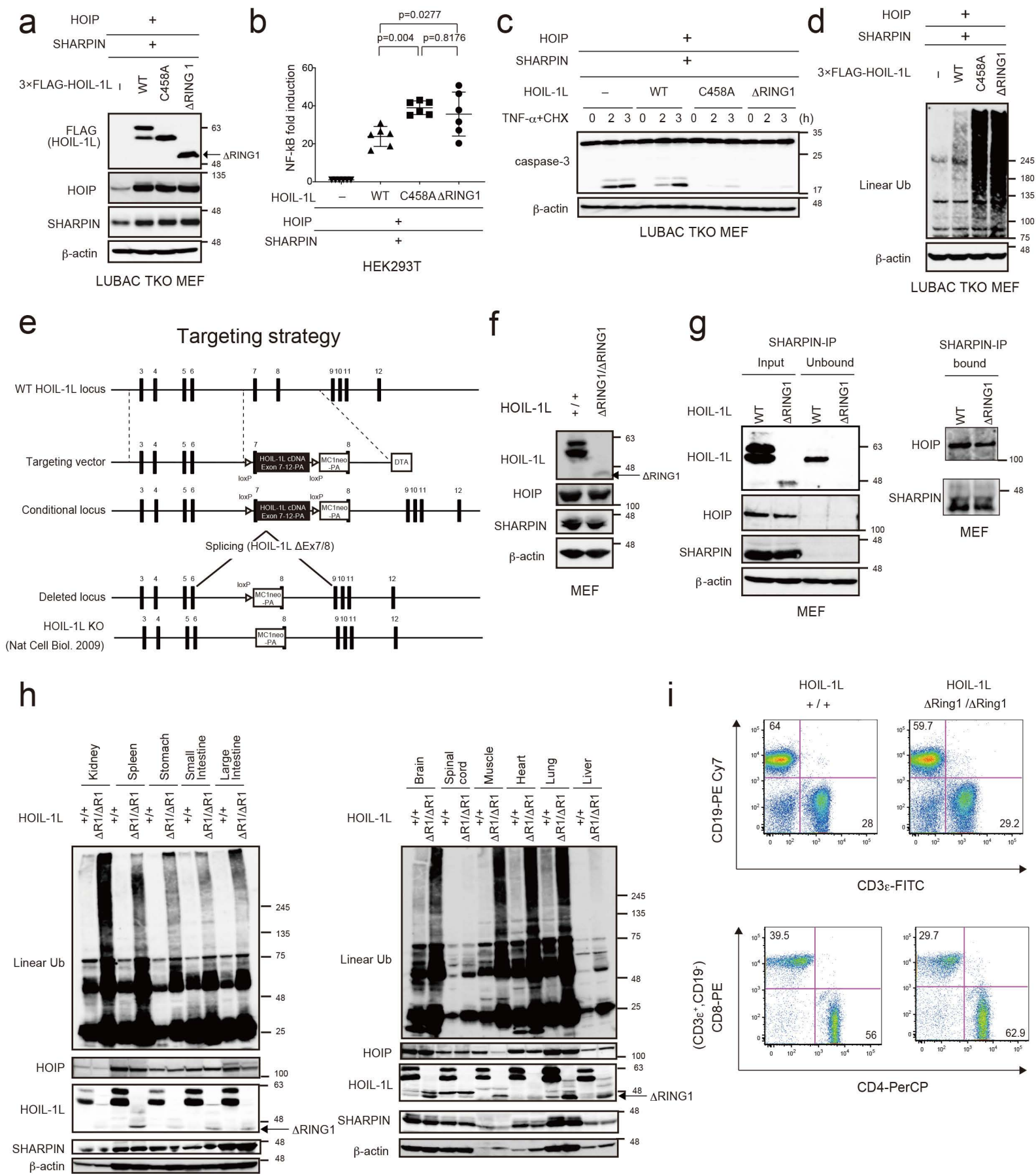




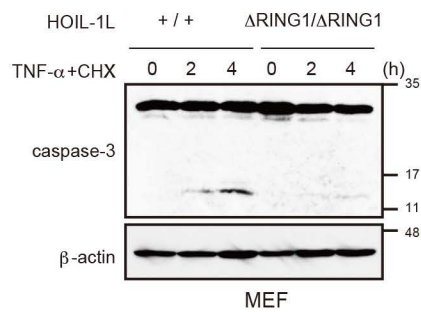




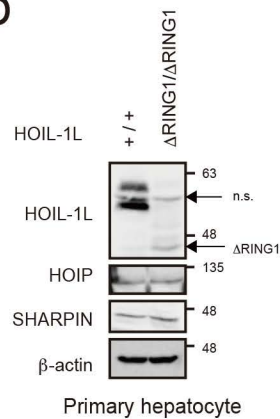




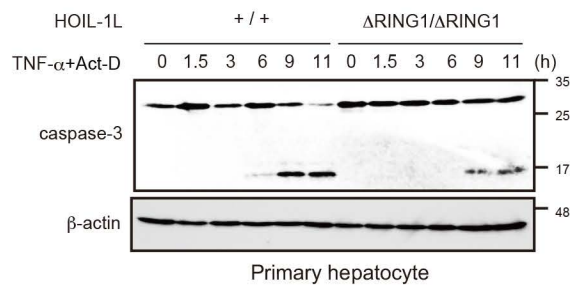
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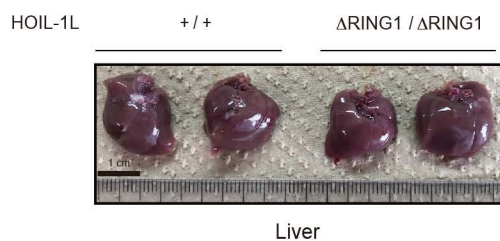
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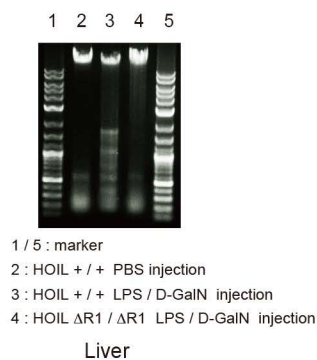
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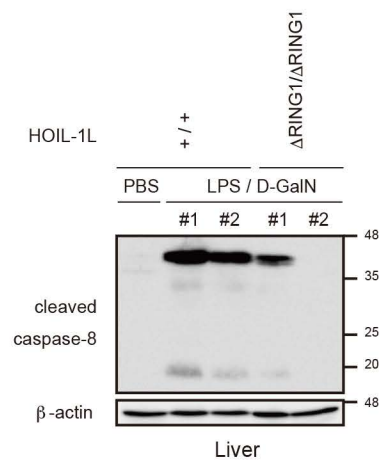
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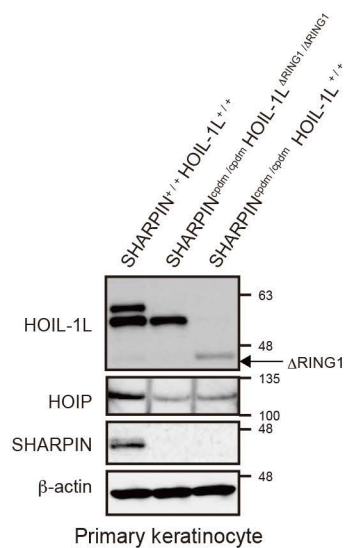
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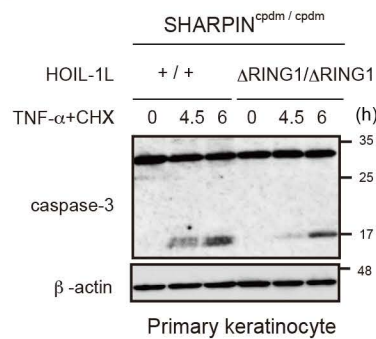
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Extended Data Fig. 1 | The highly conserved HOIL-1L RBR ubiquitin ligase negatively regulates LUBAC functions.

a, Conserved residues in the RING1-IBR-RING2 domains of HOIL-1L in the indicated species. Arrow indicates the catalytic cysteine of HOIL-1L. **b**, Schematic representation of mouse HOIL-1L and its mutants. Cleavage of caspase-3 in LUBAC TKO MEFs stably reconstituted with HOIP and the indicated HOIL-1L protein, stimulated with TNF- α (3 ng ml⁻¹) and CHX (20 μ g ml⁻¹), was assessed by immunoblotting. The experiments were repeated twice, independently, with similar results. **c**, Viability of LUBAC TKO MEFs stably reconstituted with HOIP, SHARPIN, and the indicated HOIL-1L protein, stimulated with TNF- α (10 ng ml⁻¹), was measured using the iCELLigence system. The experiments were repeated three times, independently, with similar results. **d**, NF- κ B activation in HEK293T cells transfected with the indicated expression plasmids and 5 $\times$ NF- κ B luciferase reporters was measured by luciferase assay. Mean is shown; n=3 independent experiments; *P*-values are from a two-tailed Student's *t*-test. **e**, Quantitative PCR (qPCR) analyses of expression levels of NF- κ B target genes in LUBAC TKO MEFs stably reconstituted with HOIP, SHARPIN, and the indicated HOIL-1L protein. Mean $\pm$ S.D. is shown; n=5 independent experiments; *P*-values are from a two-tailed Student's *t*-test. **f**, Phosphorylation and degradation of I κ B α in LUBAC TKO MEFs stably reconstituted with HOIP and SHARPIN. The experiments were repeated three times, independently, with similar results. Data and unprocessed blots are available as source data.

Extended Data Fig. 2 | Loss of HOIL-1L E3 does not overtly affect K48, K63, or other types of chains.

a, Amounts of the indicated types of ubiquitin chains in LUBAC TKO MEFs stably reconstituted with HOIP and SHARPIN, and the indicated HOIL-1L protein. Lysates from TKO MEFs expressing the indicated HOIL-1L proteins were probed with the indicated antibodies. The experiments were repeated three times, independently, with similar results. **b**, Assessment of ubiquitin linkages in lysates of LUBAC TKO MEFs stably reconstituted with HOIP, SHARPIN, and the indicated HOIL-1L protein using MS analyses. Mean is shown; n=3 independent experiments; *P*-values were obtained by one-way ANOVA followed by Tukey's multiple comparison test. Data and unprocessed blots are available as source data.

Extended Data Fig. 3 | Auto-ubiquitination of HOIL-1L inhibits LUBAC functions.

a, Lysates of LUBAC TKO MEFs stably reconstituted with HOIP C879A, SHARPIN, and the indicated HOIL-1L protein were probed as depicted. Arrow shows the upper band of HOIL-1L. **b**, *In vitro* ubiquitination reactions were performed at 37°C for 24 h with His-UBCH7 C/S to generate an oxy-ester bond between ubiquitin and UBCH7, followed by digestion of ubiquitinated UBCH7 with NH₂OH or USP2cc at 37°C for 1 h. **c,h,i** Lysates of indicated MEFs were probed as depicted. **d,i** Digestion of the upper (modified) HOIL-1L signal with USP2cc or NH₂OH at 37°C for 30 min anti-FLAG immunoprecipitates obtained under denaturing conditions from LUBAC TKO MEFs expressing 3×FLAG–HOIL-1L WT (**d**) or the All-K/R mutant (**i**). **e,j,k**, LUBAC TKO

MEFs stably reconstituted with HOIP, SHARPIN and 3×FLAG-HOIL WT (e) or the All-K/R mutant (j,k) were subjected to mass spectrometry to assess ubiquitination sites in LUBAC subunits. S107 of hHOIP (e), S915 of mHOIP (j), and T955 of mHOIP (k) were identified as ubiquitination sites. f, Lysates of HEK293T cells transfected with the indicated expression plasmids were probed as indicated. g, NF-κB activation in HEK293T cells transfected with the indicated expression plasmids and 5×NF-κB luciferase reporters was assessed by luciferase assay. Mean is shown; n=3 independent experiments; *P*-values are from a two-tailed Student's t-test. The data shown were repeated twice, independently, with similar results. Data and unprocessed blots are available as source data.

Extended Data Fig. 4 | Loss of HOIL-1L E3 activity increases LUBAC activity *in vitro*.

a, b, Linear ubiquitin (a) or modification of indicated HOIL-1L proteins (b) in *in vitro* ubiquitination assays with Petit-SHARPIN and HOIL-1L WT or the indicated mutants, as assessed by immunoblotting. The data shown were repeated three times, independently, with similar results. Unprocessed blots are available as source data.

Extended Data Fig.5 | HOIL-1L ubiquitinates all LUBAC subunits, HOIP conjugates linear ubiquitin onto the ubiquitin, and OTULIN counteracts this effect.

a, Modification of LUBAC subunits in WT or OTULIN KO MEFs treated or not treated with lysates containing MBP-OTULIN at 37°C for 1 h. * shows non-specific bands. The

experiments were repeated three times, independently, with similar results. **b**, Schematic representation of HOIL-1L WT and Δ RING1 (lacking aa 251–341). **c**, Cleavage of caspase-8 and caspase-3 induced by TNF- α (1 ng ml⁻¹) plus CHX (20 μ g ml⁻¹) in WT or OTULIN KO MEFs expressing the indicated HOIL-1L proteins. The experiments were repeated twice, independently, with similar results.

Unprocessed blots are available as source data.

Extended Data Fig. 6 | Loss of HOIL-1L E3 protects against *Salmonella typhimurium* infection, and infecting *S. typhimurium* are covered with high levels of linear ubiquitin in MEFs expressing ligase-defective HOIL-1L.

a-d, *S. typhimurium* infections of LUBAC TKO MEFs stably expressing HOIP, SHARPIN, and 3 $\times$ FLAG–HOIL-1L WT or C458A. Microscopic images of indicated MEFs were collected 24 hours after infection. Scale bars, 500 μ m (**a**). Confocal micrographs of LUBAC TKO MEFs stably expressing HOIP, SHARPIN, and 3 $\times$ FLAG–HOIL-1L WT or C458A were infected with *S. typhimurium* and stained for linear ubiquitin (1E3), FLAG (M2), or Hoechst 33342 at 2 h (**b**), 4 h (**c**), and 6 h (**d**) post-infection. Scale bars, 5 μ m (**b–d**).

The data shown were repeated three times, independently, with similar results.

Extended Data Fig. 7 | Generation of HOIL-1L Δ RING1 mice as a model for ligase-defective HOIL-1L mice.

a, LUBAC TKO MEFs stably reconstituted with the indicated proteins were probed as shown. **b**, NF- κ B activation in HEK293T cells transfected with the indicated expression plasmids and 5 $\times$ NF- κ B luciferase reporters was assessed by luciferase assay. Mean $\pm$ S.D. is shown; n=6 independent experiments; *P*-values are from one-way ANOVA followed by Tukey's multiple comparison test. **c**, Cleavage caspase-3 in LUBAC TKO MEFs stably reconstituted with the indicated proteins and stimulated with TNF- α (2.5 ng ml⁻¹) plus CHX (20 μ g ml⁻¹) was assessed by immunoblotting. **d**, Level of linear ubiquitin in LUBAC TKO MEFs stably reconstituted with the indicated proteins. **e**, Schematic representation of conditional and deleted loci of HOIL-1L conditional Δ RING1 mice. **f**, Cell lysates of primary MEFs from WT and HOIL-1L Δ RING1 mice were probed as indicated. **g**, Indicated MEFs were immunoprecipitated with anti-SHARPIN antibody, and bound and unbound fractions were probed as indicated. **h**, Lysates of the indicated organs from 8-week-old littermate mice of the indicated genotypes were probed as depicted. **i**, Splenocytes of 15-week-old littermate mice of the indicated genotypes were examined by flow cytometry. Splenocytes were analyzed for surface expression of CD3 and CD19 (upper panels) and T cell subpopulations of splenocytes were analyzed for surface expression of CD4 and CD8 (lower panels). Experiments were repeated independently with similar results twice (a,c,g,h,i) or at least three times (d,f). Data and unprocessed blots are available as source data.

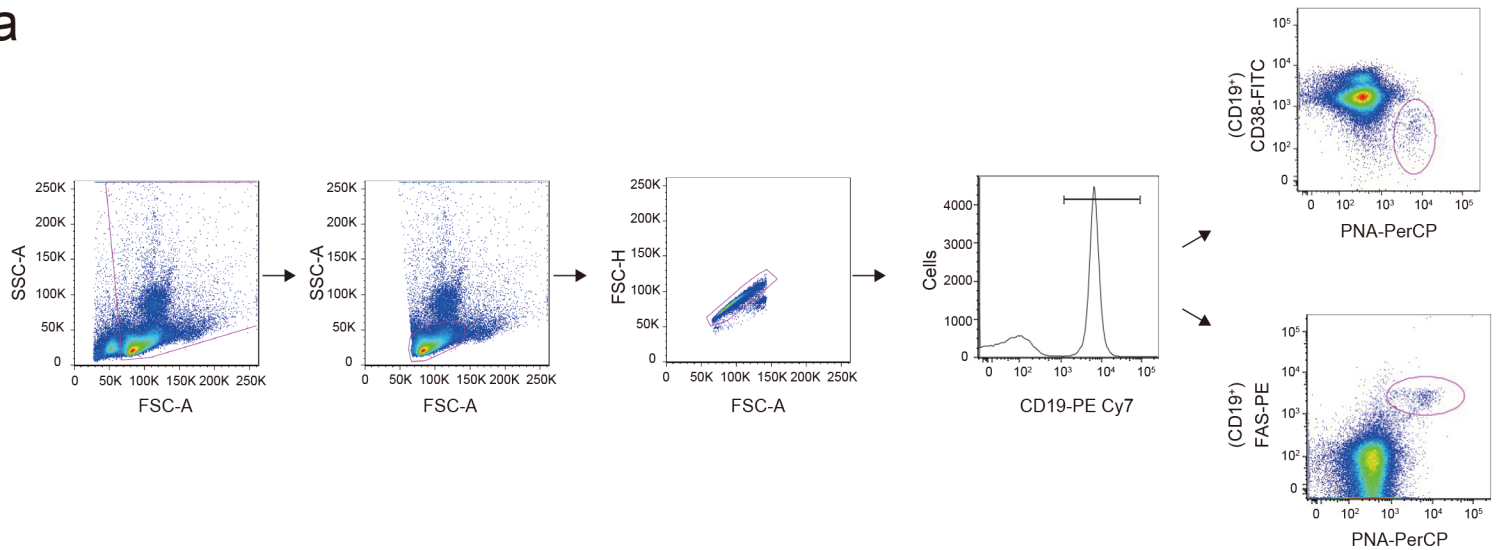
Extended Data Fig. 8 | Pathophysiological roles of HOIL-1L Δ RING1 in mice.

a, Primary MEFs from WT and HOIL-1L Δ RING1 mice were probed as depicted after

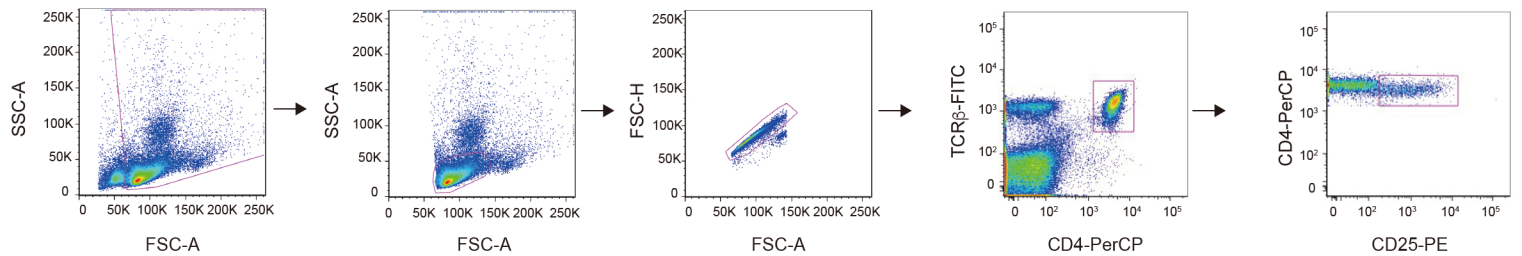
stimulation with TNF- α (5 ng ml⁻¹) plus CHX (20 μ g ml⁻¹) for the indicated periods. **b**, Lysates of primary hepatocytes from 12-week-old WT or HOIL-1L Δ RING1 mice were probed as indicated. **c**, Cleavage of caspase-3 in primary hepatocytes from 12-week-old WT or HOIL-1L Δ RING1 mice after stimulation with TNF- α (10 ng ml⁻¹) plus actinomycin D (Act-D) (100 ng ml⁻¹) was assessed by immunoblotting. **d**, Macroscopic appearance of livers of the indicated mice at 18 weeks old. Scale bar, 1 cm. **e,f**, Mice of the indicated genotypes were injected intraperitoneally with LPS/D-GalN or PBS. Seven hours after injection, DNA ladder (**e**) or cleaved caspase-8 (**f**) in liver lysates of the indicated mice was probed as depicted. **g**, Primary keratinocytes from 7-week-old WT or HOIL-1L Δ RING1 mice were probed as indicated. **h**, Cleavage of caspase-3 in primary keratinocytes from 7-week-old WT or HOIL-1L Δ RING1 mice after stimulation with TNF- α (10 ng ml⁻¹) plus CHX (20 μ g ml⁻¹) was assessed by immunoblotting. Experiments were repeated independently with similar results twice (a,c,e-h) or at least three times (b,d). Unprocessed blots are available as source data.

Supplementary Figure1: Gating strategy for flow cytometry analysis

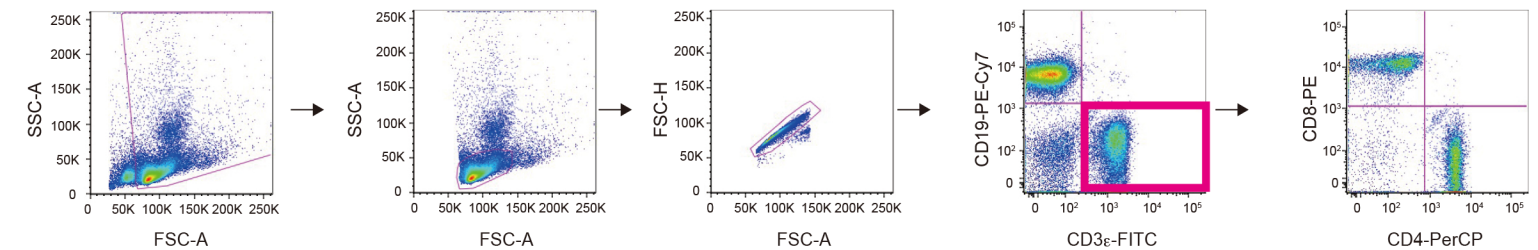
a



b



c



a-c, Single-cell suspensions prepared from the spleen of 8- to 15-week-old mice of the indicated genotype were stained with fluorochrome-conjugated antibodies. Live lymphocytes were gated with forward-scatter (FSC) and side-scatter (SSC), and FSC-area (FSC-A) and FSC-height (FSC-H) were used to discriminate single cells from doublet and multiplet cells. CD19⁺ B cells of splenocytes were analyzed for surface expression of CD38 and PNA, or FAS and PNA (a). TCRβ⁺CD4⁺ T cells of splenocytes were analyzed for surface expression of CD25 (b). Splenocytes were analyzed for surface expression of CD3ε and CD19, and CD19⁻CD3ε⁺ cells were analyzed for surface expression of CD4 and CD8 (c).