

# LUBAC is essential for embryogenesis by preventing cell death and enabling haematopoiesis

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**The linear ubiquitin chain assembly complex (LUBAC) is required for optimal gene activation and prevention of cell death upon activation of immune receptors, including TNFR1<sup>1</sup>. Deficiency in the LUBAC components SHARPIN or HOIP in mice results in severe inflammation in adulthood or embryonic lethality, respectively, owing to deregulation of TNFR1-mediated cell death<sup>2–8</sup>. In humans, deficiency in the third LUBAC component HOIL-1 causes autoimmunity and inflammatory disease, similar to HOIP deficiency, whereas HOIL-1 deficiency in mice was reported to cause no overt phenotype<sup>9–11</sup>. Here we show, by creating HOIL-1-deficient mice, that HOIL-1 is as essential for LUBAC function as HOIP, albeit for different reasons: whereas HOIP is the catalytically active component of LUBAC, HOIL-1 is required for LUBAC assembly, stability and optimal retention in the TNFR1 signalling complex, thereby preventing aberrant cell death. Both HOIL-1 and HOIP prevent embryonic lethality at mid-gestation by interfering with aberrant TNFR1-mediated endothelial cell death, which only partially depends on RIPK1 kinase activity. Co-deletion of caspase-8 with RIPK3 or MLKL prevents cell death in *Hoil-1*<sup>-/-</sup> (also known as *Rbck1*<sup>-/-</sup>) embryos, yet only the combined loss of caspase-8 with MLKL results in viable HOIL-1-deficient mice. Notably, triple-knockout *Ripk3*<sup>-/-</sup> *Casp8*<sup>-/-</sup> *Hoil-1*<sup>-/-</sup> embryos die at late gestation owing to haematopoietic defects that are rescued by co-deletion of RIPK1 but not MLKL. Collectively, these results demonstrate that both HOIP and HOIL-1 are essential LUBAC components and are required for embryogenesis by preventing aberrant cell death. Furthermore, they reveal that when LUBAC and caspase-8 are absent, RIPK3 prevents RIPK1 from inducing embryonic lethality by causing defects in fetal haematopoiesis.**

To determine the physiological role of HOIL-1, we generated HOIL-1-deficient mice by targeting exons 1 and 2 of the *Hoil-1* (also known as *Rbck1*) gene (Extended Data Fig. 1a–d). No mice with homozygous deletion in the *Hoil-1* gene were weaned (Fig. 1a). Analysis of *Hoil-1*<sup>-/-</sup> embryos revealed that they died around embryonic day (E) 10.5 (Fig. 1a, b). This result was confirmed with a strain generated from an independently targeted embryonic stem (ES) cell (C20*Hoil-1*<sup>-/-</sup> mice) (Extended Data Fig. 1e, f). At E10.5, *Hoil-1*<sup>-/-</sup> embryos presented with disrupted vascular architecture and cell death in the yolk sac endothelium (Fig. 1c, d and Extended Data Fig. 1g, h), indicating that the absence of HOIL-1 causes aberrant endothelial cell death. *Hoil-1*<sup>fl/fl</sup> *Tie2-cre*<sup>+</sup> embryos that lack HOIL-1 specifically in endothelial and some haematopoietic cells also died around E10.5 with the same abnormalities (Fig. 1e and Extended Data Fig. 1i, j). Loss of TNF or TNFR1 diminished cell death in the yolk sac and prevented lethality at E10.5 in *Hoil-1*<sup>-/-</sup> embryos (Fig. 1f and Extended Data Fig. 2a–d). As in the *Tnfr1*<sup>-/-</sup> *Hoip*<sup>-/-</sup> (also known as *Tnfrsf1a*<sup>-/-</sup> *Rnf31*<sup>-/-</sup>) double

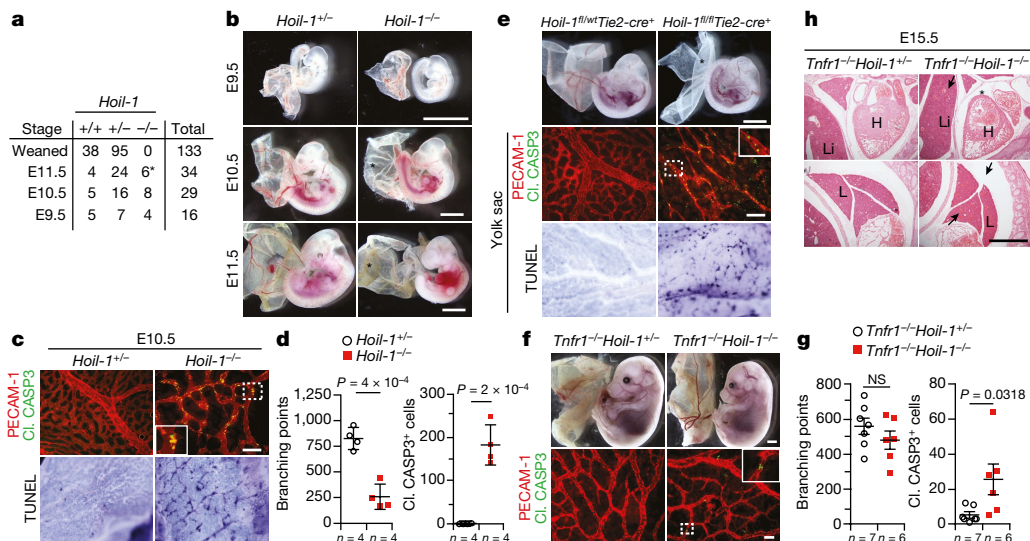
knockouts<sup>8</sup>, *Tnfr1*<sup>-/-</sup> *Hoil-1*<sup>-/-</sup> yolk sacs showed reduced cell death as compared to *Hoil-1*<sup>-/-</sup> embryos (Fig. 1f, g). Although cell death was not completely ablated in *Tnfr1*<sup>-/-</sup> *Hoil-1*<sup>-/-</sup> embryos, it did not appear to significantly affect yolk sac vasculature (Fig. 1f, g and Extended Data Fig. 2e). Nevertheless, *Tnfr1*<sup>-/-</sup> *Hoil-1*<sup>-/-</sup> embryos died at around E16.5 (Extended Data Fig. 2d, f), with heart defects before death (Fig. 1h). Therefore, like HOIP, HOIL-1 is required to maintain blood vessel integrity by preventing TNFR1-mediated endothelial cell death during embryogenesis.

To understand the role of HOIL-1 in LUBAC function, we compared the formation of the TNFR1 signalling complex (TNFR1-SC) in mouse embryonic fibroblasts (MEFs) individually deficient for the LUBAC components. Although TNFR1-SC-associated linear ubiquitination was merely reduced in SHARPIN-deficient MEFs<sup>7</sup>, it was completely absent in *Tnfr1*<sup>-/-</sup> *Hoil-1*<sup>-/-</sup> MEFs, exactly as in *Tnfr1*<sup>-/-</sup> *Hoip*<sup>-/-</sup> MEFs<sup>8</sup> (Fig. 2a). In TNF-stimulated *Tnfr1*<sup>-/-</sup> *Hoil-1*<sup>-/-</sup> MEFs, NF- $\kappa$ B activation was attenuated (Extended Data Fig. 3a) and TNFR1 complex-II formation was enhanced (Fig. 2b), resulting in sensitization to TNF-induced apoptosis and necroptosis (Fig. 2c). Hence, HOIL-1 is as essential as HOIP for linear ubiquitination within the TNFR1-SC.

To determine whether the reduction in HOIP and SHARPIN protein levels in HOIL-1-deficient cells was responsible for the observed loss of linear ubiquitination (Fig. 2a), we reconstituted HOIL-1-deficient MEFs with HOIP, with HOIP plus SHARPIN, or, as a control, with HOIL-1. Reconstitution with HOIP, either alone or with SHARPIN, failed to restore LUBAC recruitment, linear ubiquitination at the TNFR1-SC, or optimal NF- $\kappa$ B activation. Furthermore, the reconstitution of HOIP and/or SHARPIN was unable to prevent TNF-induced complex-II formation and cell death, whereas the re-expression of HOIL-1 corrected all aforementioned defects (Fig. 2d–f and Extended Data Fig. 3b). In the absence of HOIL-1, HOIP was unable to bind to SHARPIN despite both being reconstituted to near endogenous levels (Extended Data Fig. 3c). Thus, HOIL-1 is required for LUBAC assembly and recruitment to the TNFR1-SC, identifying it as an essential component of LUBAC alongside HOIP.

To reveal how HOIL-1 enables LUBAC activity, we generated HOIL-1-deficient MEFs stably expressing full-length wild-type HOIL-1, the UBL domain of HOIL-1 only (HOIL-1-UBL), HOIL-1- $\Delta$ RBR, HOIL-1- $\Delta$ UBL, HOIL-1 with inactivating mutations T201A/R208A in the NZF domain (HOIL-1-NZFmut) or HOIL-1 with a point mutation in the catalytic cysteine of the RBR domain (HOIL-1-C458A) (Fig. 2g). Except for HOIL-1- $\Delta$ UBL, all mutant HOIL-1 proteins bound to HOIP and SHARPIN and stabilized their levels (Fig. 2h). Isolation of the native TNFR1-SC revealed that HOIL-1- $\Delta$ RBR and HOIL-1-C458A fully restored TNF-induced linear ubiquitination in HOIL-1-deficient cells, whereas HOIL-1- $\Delta$ UBL did not (Fig. 2i). HOIL-1-deficient cells

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**Fig. 1 | HOIL-1 deficiency causes embryonic lethality at mid-gestation due to TNFR1-mediated endothelial cell death.** **a**, Mendelian frequencies obtained from inter-crossing *Hoil-1*<sup>+/-</sup> mice. Asterisk denotes dead embryos. **b**, Representative images of embryos from E9.5 to E11.5 quantified in **a**. Asterisks denote poor yolk sac vascularization. Scale bars, 2 mm. **c**, Top, representative images of yolk sac vascularization (PECAM-1, red) and cell death (cleaved (Cl.) CASP3 staining, green) at E10.5 ( $n = 4$  yolk sacs per genotype). Bottom, whole-mount TUNEL staining ( $n = 2$  yolk sacs per genotype). Scale bars, 50  $\mu$ m. **d**, **g**, Quantification of branching points and cleaved CASP3-positive cells in **c** and **f**. Data are mean  $\pm$  s.e.m.  $P$  values from unpaired two-tailed  $t$ -tests are shown. NS, not significant. **e**, Top, representative images of embryos at E10.5 ( $n = 14$  *Hoil-1*<sup>fl/wt</sup>*Tie2-cre*<sup>+</sup> and  $n = 7$  *Hoil-1*<sup>fl/fl</sup>*Tie2-cre*<sup>+</sup> embryos, top). Asterisk

denotes poor yolk sac vascularization. Scale bar, 2 mm. Middle, yolk sac vascularization (PECAM-1, red) and apoptosis (cleaved CASP3, green). Scale bar, 50  $\mu$ m. Bottom, yolk sac whole-mount TUNEL staining ( $n = 6$  *Hoil-1*<sup>fl/wt</sup>*Tie2-cre*<sup>+</sup> and  $n = 2$  *Hoil-1*<sup>fl/fl</sup>*Tie2-cre*<sup>+</sup> yolk sacs per genotype). **f**, Top, representative images of embryos at E15.5 ( $n = 6$  *Tnfr1*<sup>-/-</sup>*HOIL-1*<sup>+/-</sup> and  $n = 19$  *Tnfr1*<sup>-/-</sup>*HOIL-1*<sup>-/-</sup> embryos). Scale bar, 2 mm. Bottom, yolk sac vascularization (PECAM-1, red) and apoptosis (cleaved CASP3, green). Scale bar, 50  $\mu$ m. **h**, Representative images of haematoxylin and eosin (H&E) staining on whole-embryo paraffin sections ( $n = 3$  embryos per genotype). Asterisk denote pericardial effusion, arrows denote congested vessels. H, heart; L, lung; Li, liver. Scale bar, 50  $\mu$ m.

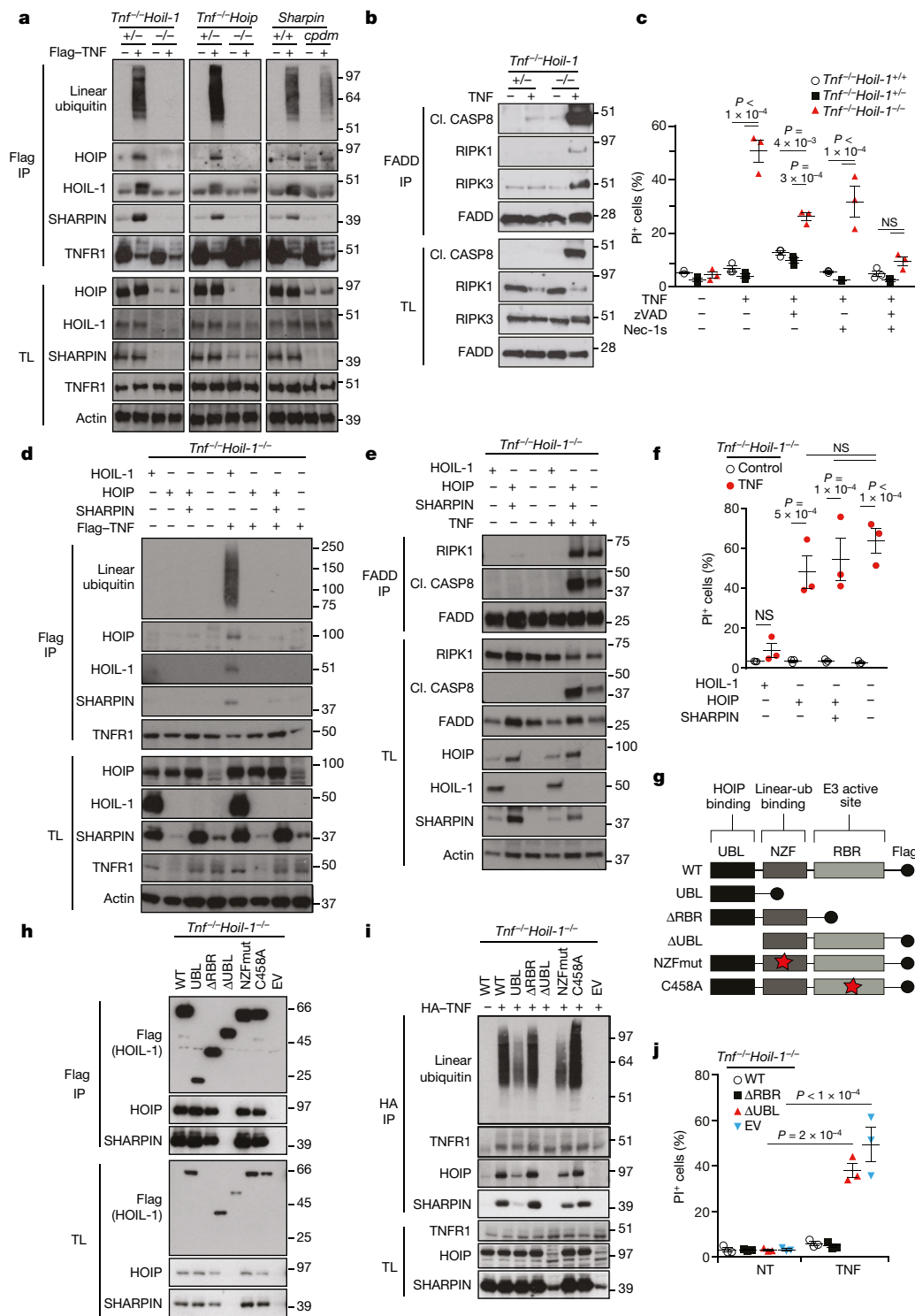
expressing HOIL-1-UBL or HOIL-1-NZFMut only showed partial restoration of linear ubiquitination, correlating with reduced HOIP and SHARPIN levels at the TNFR1-SC (Fig. 2i). Thus, the UBL domain of HOIL-1 is essential for linear ubiquitination at the TNFR1-SC, whereas a functional NZF domain is required for optimal LUBAC presence in the TNFR1-SC. Expression of HOIL-1- $\Delta$ RBR restored optimal NF- $\kappa$ B signalling and prevented aberrant TNF-induced cell killing in contrast to HOIL-1- $\Delta$ UBL (Fig. 2j and Extended Data Fig. 3d). This observation explains why the previously reported mice, regarded as deficient for HOIL-1, are viable as they were generated by targeting exons 7 and 8<sup>11</sup>, probably resembling the HOIL-1- $\Delta$ RBR mutant studied here. Because the UBL of HOIL-1 binds to HOIP, allowing its activation<sup>12</sup>, and the NZF of HOIL-1 binds linear ubiquitin linkages<sup>13</sup> our results provide evidence that HOIL-1 promotes HOIP activation as well as LUBAC assembly and recruitment to the TNFR1-SC via its UBL domain. Once linear ubiquitin chains are formed in the complex, the NZF domain of HOIL-1 promotes LUBAC retention by binding to these chains.

Because both HOIL-1 and HOIP are equally important for LUBAC function and, consequently, for preventing aberrant cell death in vitro and in vivo, we used a genetic strategy to untangle the interaction between HOIL-1 or HOIP and the different cell death components. Inactivation of RIPK1 in *Hoil-1*<sup>-/-</sup> and *Hoip*<sup>-/-</sup> embryos delayed lethality until E14.5 (Fig. 3a and Extended Data Fig. 4a–d). At this time, *Ripk1*<sup>K45A</sup>*Hoil-1*<sup>-/-</sup> and *Ripk1*<sup>K45A</sup>*Hoip*<sup>-/-</sup> embryos had disrupted vascular architecture, excessive cell death in their yolk sacs, hearts, livers and lungs, and presented with heart defects and liver necrosis (Fig. 3b and Extended Data Fig. 4e–h). In accordance, TNFR1 complex-II formation and aberrant apoptosis induced by TNF or lymphotoxin- $\alpha$  (LT- $\alpha$ ) were only partially inhibited in RIP1 kinase-dead *Ripk1*<sup>K45A</sup>*Hoil-1*<sup>-/-</sup> MEFs (Fig. 3c, d and Extended Data Fig. 4i). Thus, although the kinase activity of RIPK1 is essential for excessive TNFR1-induced cell death caused by attenuated LUBAC activity, as previously observed in SHARPIN-deficient mice<sup>4</sup>, this is not the case when LUBAC activity is completely abrogated.

We next tested whether the loss of RIPK3, MLKL or caspase-8 could prevent lethality in *Hoip*<sup>-/-</sup> and *Hoil-1*<sup>-/-</sup> embryos. At E10.5, *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos presented with defects in vascularization, excessive cell death and died at mid-gestation (Extended Data Fig. 5b, c). Owing to the close chromosomal linkage of HOIP and RIPK3, we generated *Mlkl*<sup>-/-</sup>*Hoip*<sup>-/-</sup> mice (Extended Data Fig. 5a). These embryos also died at mid-gestation (Extended Data Fig. 5d). Likewise, neither *Casp8* heterozygosity nor full deletion was sufficient to prevent the mid-gestation lethality of *Hoip*<sup>-/-</sup> and *Hoil-1*<sup>-/-</sup> embryos (Extended Data Fig. 5e, f and data not shown).

As RIPK3-mediated necroptosis may be responsible for the embryonic lethality of *Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> or *Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice<sup>14,15</sup>, we generated *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos and in both cases the lethality was delayed until around E14.5 (Fig. 3e and Extended Data Fig. 6a, b). At this developmental stage, a single intact copy of caspase-8 was sufficient to induce apoptosis-driven loss of yolk sac vascularization (Fig. 3f and Extended Data Fig. 6c, d). Yet, although *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos died around E14.5, yolk sac vascularization was normalized and cell death in the yolk sac and other organs was prevented (Fig. 3f and Extended Data Fig. 6c–f). Moreover, *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> MEFs were resistant to cell death induced by TNF or related cytokines (Extended Data Fig. 6g). Histological examination and microfocus computed tomography scanning revealed the presence of heart defects in both *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> embryos (Extended Data Fig. 6h, i). We therefore conclude that whereas mid-gestation lethality in *Hoil-1*<sup>-/-</sup> embryos is dependent on caspase-8/RIPK3-mediated apoptosis and necroptosis, *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos die at late gestation by a process that is independent of cell death.

In marked contrast to *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice, both *Mlkl*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoip*<sup>-/-</sup> mice were born, albeit at lower than expected Mendelian ratios (Fig. 3g and Extended Data Fig. 7a). These mice were runted and had to be



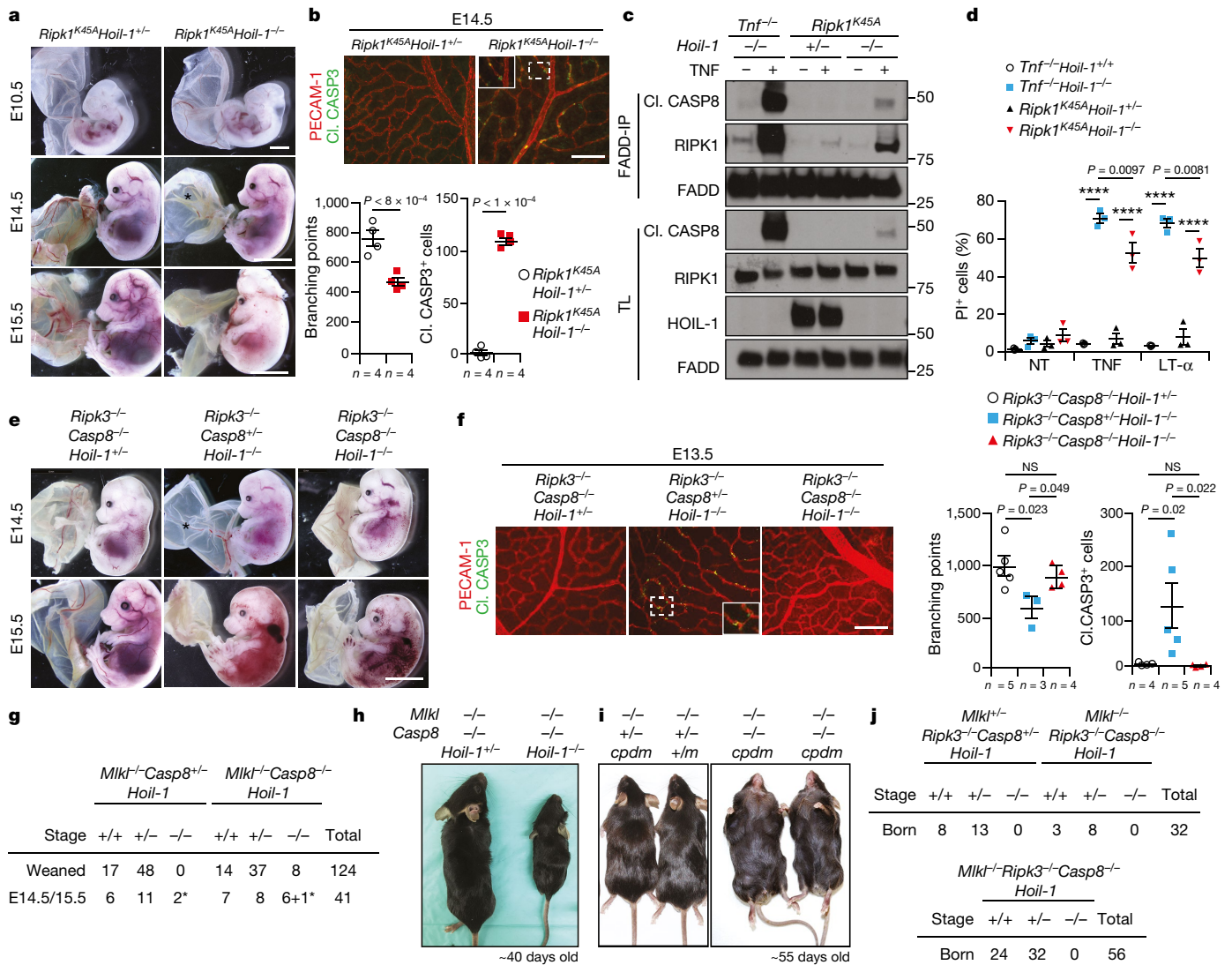
**Fig. 2 | The UBL domain but not the RBR domain of HOIL-1 is essential for LUBAC activity at the TNFR1-SC and to prevent TNF/TNFR1-induced cell death.** **a, d,** TNFR1-SC pull-down by Flag immunoprecipitation (IP) in MEFs derived from mice of the indicated genotypes  $\pm$  Flag-TNF for 15 min ( $n = 2$  independent experiments) (**a**) and reconstituted with HOIL-1, HOIP or HOIP and SHARPIN ( $n = 4$  independent experiments) (**d**). **b, e,** Immunoprecipitation of the adaptor protein FADD in MEFs of the indicated genotypes treated for 4 h with the caspase inhibitor zVAD-fmk  $\pm$  TNF (**b**) and reconstituted as indicated (**e**) ( $n = 2$  independent experiments (**b, e**)). **c, f, j,** Cell death analysed by propidium iodide (PI) staining in MEFs with the indicated

genotypes  $\pm$  TNF  $\pm$  the indicated inhibitors zVAD-fmk (zVAD) and/or necrostatin-1 (Nec-1s) for 24 h (**c**), reconstituted (**f**) or transduced (**j**) as indicated. Mean  $\pm$  s.e.m. ( $n = 3$  independent experiments) and  $P$  values from two-way ANOVA are shown. **g,** Schematic overview of HOIL-1 constructs used to transduce *Tnf<sup>-/-</sup>Hoil-1<sup>-/-</sup>* MEFs. WT, wild type. **h,** Flag immunoprecipitation of indicated HOIL-1 mutants ( $n = 2$  independent experiments). **i,** Endogenous TNFR1-SC pull-down by haemagglutinin (HA) immunoprecipitation in reconstituted *Tnf<sup>-/-</sup>Hoil-1<sup>-/-</sup>* MEFs  $\pm$  HA-TNF for 15 min ( $n = 2$  independent experiments). EV, empty vector; NT, not treated; TL, total lysate. For gel source data (**a, b, d, e, h, i**), see Supplementary Fig. 1.

ethanized by 4–5 weeks of age (Fig. 3h, Extended Data Fig. 7b, c). Histopathological analysis revealed severe inflammation in the liver and lungs (Extended Data Fig. 7d and data not shown). Of note,

*Casp8* heterozygosity resulted in increased apoptosis of endothelial cells, causing lethality in both *Mkl1<sup>-/-</sup>Casp8<sup>+/-</sup>Hoip<sup>-/-</sup>* and *Mkl1<sup>-/-</sup>Casp8<sup>+/-</sup>Hoil-1<sup>-/-</sup>* embryos around E14.5 (Extended Data





**Fig. 3 | Concomitant loss of MLKL and caspase-8, but not loss of RIPK1 kinase activity or combined loss of RIPK3 and caspase-8, promotes survival of LUBAC-deficient mice.** **a**, Representative images of E10.5 ( $n = 6$  embryos per genotype), E14.5 ( $n = 12$  *Ripk1*<sup>K45A</sup>*Hoil-1*<sup>+/+</sup>,  $n = 5$  *Ripk1*<sup>K45A</sup>*Hoil-1*<sup>-/-</sup> embryos per genotype) and E15.5 embryos ( $n = 3$  embryos per genotype). Scale bars, 2 mm (E10.5) and 5 mm (E14.5, E15.5). Asterisk denotes poor yolk sac vascularization. **b**, **f**, Representative images of yolk sac vascularization (PECAM-1, red) and apoptosis (cleaved (CL) CASP3, green) at E14.5 (**b**) or E13.5 (**f**) and quantification. Mean  $\pm$  s.e.m. and  $P$  values from unpaired two-tailed  $t$ -tests (**b**) or one-way ANOVA (**f**) are shown. Scale bar, 50  $\mu$ m. **c**, Immunoprecipitation of the adaptor protein FADD in MEFs treated for 3 h with TNF and zVAD-fmk ( $n = 2$  independent experiments). For gel source data, see Supplementary Fig. 1. **d**, Cell death measured by propidium iodide (PI)

incorporation in MEFs treated with TNF (10 ng ml<sup>-1</sup>) or LT- $\alpha$ , or not treated (NT). Data are mean  $\pm$  s.e.m. ( $n = 3$  independent experiments). \*\*\*\* $P < 0.0001$ , two-way ANOVA. **e**, Representative images of E14.5 ( $n = 11$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/+</sup>, *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/+</sup>*Hoil-1*<sup>-/-</sup> and  $n = 7$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>) and E15.5 ( $n = 5$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/+</sup>,  $n = 4$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/+</sup>*Hoil-1*<sup>-/-</sup> and  $n = 8$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>) embryos. Asterisk denotes poor yolk sac vascularization. Scale bar, 5 mm. **g**, **j**, Mendelian frequencies obtained from intercrossing *Mkl1*<sup>-/-</sup>*Casp8*<sup>+/+</sup>*Hoil-1*<sup>+/+</sup> with *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/+</sup> mice (**g**) or *Mkl1*<sup>+/+</sup>*Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/+</sup> with *Mkl1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/+</sup> mice (**j**, top) or *Mkl1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/+</sup> mice (**j**, bottom). Asterisk denotes dead embryo. **h**, **i**, Representative images of adult mice quantified in **g** (**h**), or  $n = 3$  mice per genotype in **i**.  $m$  denotes *cpdm* mutation.

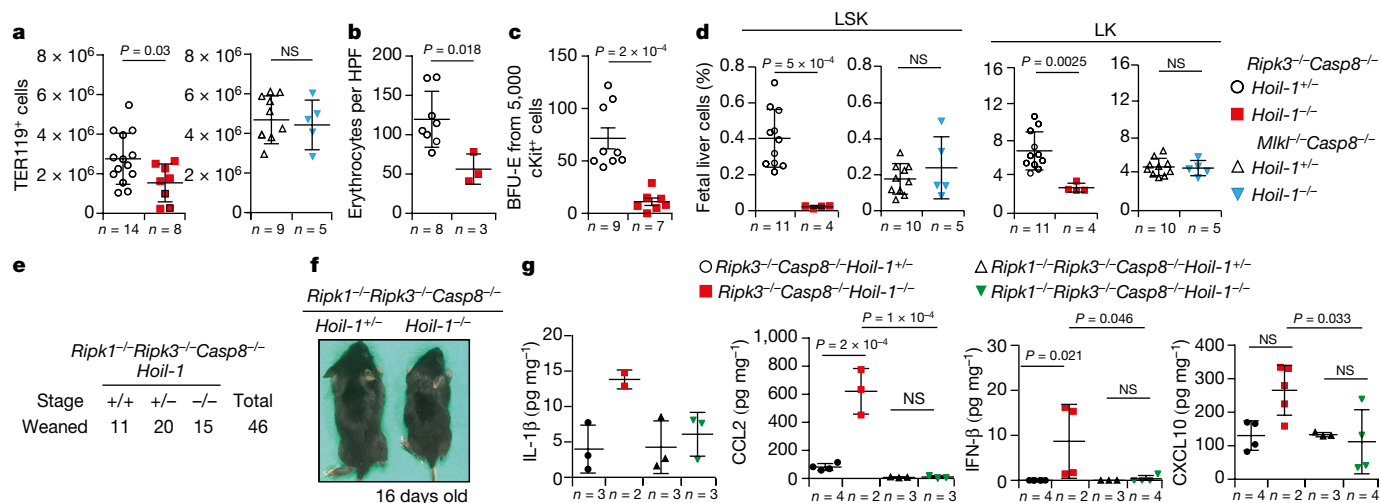
Fig. 7e and data not shown) indicating that caspase-8-driven apoptosis is sufficient to cause embryonic death of LUBAC-deficient embryos. Co-deletion of RIPK3 and caspase-8 causes embryonic lethality in otherwise viable SHARPIN-deficient *cpdm* (chronic proliferative dermatitis mice, also known as *Sharpin*<sup>*cpdm*</sup>) mice<sup>7</sup>. However, *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Sharpin*<sup>*cpdm*</sup> mice were viable and the inflammatory syndrome that characterizes *Sharpin*<sup>*cpdm*</sup> mice was prevented (Fig. 3i and Extended Data Fig. 7f, g), while expectedly<sup>16</sup> developing lymphadenopathy and splenomegaly (Fig. 3i and Extended Data Fig. 7f). Thus, the combined loss of any of the three LUBAC components together with the loss of caspase-8 uncovers a vital functional difference between RIPK3 and MLKL.

We next evaluated whether the lethality of *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice is due to aberrant (RIPK3-independent) MLKL

activation. This was particularly pertinent because MLKL levels were increased in *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos and MLKL was aberrantly activated in some of them (Extended Data Fig. 7h). However, MLKL co-deficiency did not prevent the death of *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos (Fig. 3j). Thus, RIPK3 is required for the survival of embryos in the absence of LUBAC by regulating an MLKL-independent process.

To explore the nature of the pro-survival role of RIPK3, we performed RNA sequencing (RNA-seq) on E13.5 *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>, *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and control embryos (Extended Data Fig. 8a and Supplementary Table 1). Gene Ontology (GO) enrichment analysis of differentially expressed genes pointed towards defects in erythropoiesis in *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos (Extended Data Fig. 8b). Indeed, reduced levels of erythroid lineage TER119<sup>+</sup> cells





**Fig. 4 | Combined deletion of RIPK3 and caspase-8 causes haematopoietic defects and RIPK1-dependent embryonic lethality in HOIL-1-deficient mice.** **a**, **b**, Number of TER119<sup>+</sup> (erythroid) cells (**a**) and enucleated erythrocytes per high-power field (HPF) (**b**) in E13.5 fetal livers with the indicated genotypes. Data are mean  $\pm$  s.e.m.  $P$  values from unpaired two-tailed  $t$ -tests are shown. **c**, Differentiation of E13.5 fetal liver (c-KIT<sup>+</sup>) progenitors into erythroid burst-forming units (BFU-E). Data are mean  $\pm$  s.e.m.  $P$  values from unpaired two-tailed  $t$ -tests are reported. **d**, Percentage of haematopoietic progenitors negative for

(Fig. 4a), basophilic erythroblasts (Extended Data Fig. 8c) and mature erythrocytes (Fig. 4b) were observed in of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* fetal livers. Furthermore, *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* haematopoietic progenitors failed to differentiate into committed erythroid burst-forming units (BFU-E) in culture (Fig. 4c). Further analysis of the haematopoietic compartment from E13.5 fetal livers revealed abnormally reduced percentages and total numbers of multipotent progenitors (Fig. 4d and Extended Data Fig. 8d, e) as well as leucocytes, including granulocytes and macrophages, and myeloid progenitors in the *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos compared to controls, whereas *Mkl1<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos had normal numbers of these cells (Extended Data Fig. 8f–k). In addition, the capacity of haematopoietic progenitors to generate colony-forming myeloid progenitors and multipotent progenitors was also impaired in the *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (Extended Data Fig. 8l). Accordingly, the viability of macrophages obtained from *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* fetal liver cell suspensions in culture was significantly lower than those of controls and this could not be rescued by inhibiting necroptosis or apoptosis. *Mkl1<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* fetal liver cells, however, produced normal numbers of macrophages (Extended Data Fig. 4m). Despite the heart defects of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos, blood circulation was normal at E13.5 and the percentages of CD45<sup>+</sup>cKIT<sup>+</sup> cells obtained from aorta–gonad–mesonephros (AGM) regions were comparable between *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos and controls at E11.5 (Extended Data Fig. 8o, p). We therefore conclude that *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos have defective early haematopoiesis, probably downstream of specification in the AGM, resulting in substantial deficiencies in erythroid and myeloid cells.

Because LUBAC is known to regulate RIPK1<sup>17,18</sup>, we investigated the role of RIPK1 in the lethality of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos. The lethality of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos was prevented by additional loss of RIPK1, despite RIPK1 levels being relatively low in *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos and RIPK1 deficiency failing to prevent *Hoil-1<sup>-/-</sup>* embryonic lethality (Fig. 4d, e and Extended Data Figs. 7h, 9a, b). Importantly, the viability of macrophages obtained from *Ripk1<sup>-/-</sup>Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* fetal livers was comparable to controls (Extended Data Fig. 9c), indicating normalized haematopoiesis in these mice. The expression of several cytokines, including IL-1 $\beta$ , CCL2, IFN- $\beta$  and CXCL10,

mature lineage markers (Lin<sup>-</sup>) and SCA-1<sup>+</sup>c-KIT<sup>+</sup> (LSK) and SCA-1<sup>-</sup>c-KIT<sup>+</sup> (LK) in E13.5 fetal livers with the indicated genotypes. Data are mean  $\pm$  s.e.m.  $P$  values from unpaired two-tailed  $t$ -tests are reported. **e**, Mendelian frequencies obtained from intercrossing *Ripk1<sup>-/-</sup>Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>+/+</sup>* mice. **f**, Representative images of mice of the indicated genotypes quantified in **e**. **g**, Cytokine levels in embryo homogenates with the indicated genotypes. Data are mean  $\pm$  s.e.m.  $P$  values from one-way ANOVA are reported.

was abnormally increased in *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos but not in *Ripk1<sup>-/-</sup>Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (Fig. 4f and Extended Data Fig. 9d, e). The function, survival, differentiation and self-renewal of haematopoietic progenitors are greatly impacted by several of these cytokines<sup>19,20</sup>. Therefore, our findings suggest that RIPK1-driven deregulated cytokine production in *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos may impair fetal haematopoiesis. Finally, the treatment of pregnant females with the RIPK1 kinase inhibitor GSK3540547A (GSK547A)<sup>21</sup> did not prevent lethality of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos, although it was able to extend the survival of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (Extended Data Fig. 9f). These results suggest that the lethality of *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos probably depends on the scaffolding function of RIPK1.

Although RIPK1 is required for emergency haematopoiesis, RIPK1 might regulate embryonic haematopoiesis differently. Indeed, RIPK1-constitutive or RIPK1-haematopoietic-cell-specific-deficient mice are not embryonically lethal<sup>22,23</sup>. In addition, the absence of LUBAC, RIPK3 and caspase-8 might affect mechanisms during embryogenesis that are different from those perturbed by RIPK1 deficiency alone. Collectively, our findings indicate that in the combined absence of LUBAC and caspase-8, RIPK3 exerts a pro-survival role by regulating RIPK1-mediated signalling (Extended Data Fig. 10). Because *Ripk3<sup>-/-</sup>Casp8<sup>-/-</sup>* mice are viable<sup>14,15,24</sup>, our findings indicate that the control of RIPK1 by either LUBAC or RIPK3 is sufficient to enable proper haematopoiesis in the developing embryo, probably by preventing deregulated cytokine production. Thus, LUBAC and RIPK3 control RIPK1-mediated signalling to allow embryonic haematopoiesis.

## Online content

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at <https://doi.org/10.1038/s41586-018-0064-8>.

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**Author contributions** H.W. conceived the project. N.P. and M.D. performed most experiments. N.P., M.D. and H.W. designed the research and co-wrote the manuscript. A.M., C.B. and T.E. conceived and contributed to the haematopoietic analyses. H.D. and P.D. contributed to in vitro experiments in Fig. 2 and Extended Data Fig. 3. S.K. generated *Mkl1*<sup>-/-</sup> mice, L.T., E.R. contributed to in vivo experiments, T.H. performed cytokine arrays and E.L. and Y.S. contributed with biochemistry data. P.B., T.L.H. and H.W. designed the *Hoil-1*-floxed allele and P.B. generated it. A.F. and W.K. generated and analysed *Ripk1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice. H.D. and A.Sa. performed genotyping. A.B. and J.B. provided GSK'547A and *Ripk1*<sup>K45A</sup> mice. J.R., S.A.-D., A.St. and J.S. performed the *Sharpin*<sup>cdpm</sup> studies. C.H. and M.T.A. performed pathological and microfocus computed tomography analyses. T.E., P.B., A.St., J.S. and E.R. provided scientific insight.

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#### Additional information

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## METHODS

**Mice.** The *Hoil-1*-floxed (*Hoil-1<sup>fl/fl</sup>*) mice were generated by a gene-targeting strategy in ES cells in which the targeting cassette was composed of a hygromycin-resistance cassette flanked by *Frt* sites and exons 1 and 2 of the *Hoil-1* gene flanked by *loxP* sites. Southern blots of C57BL/6 ES cell clones containing the homologous recombination were analysed for the specificity of the recombination and the absence of any unwanted integration. Two ES cell clones were used to generate mutant animals on the C57BL/6 genetic background, corresponding to the two independent *Hoil-1<sup>-/-</sup>* strains (*Hoil-1<sup>-/-</sup>* and C20*Hoil-1<sup>-/-</sup>*). The hygromycin cassette was removed by crossing these mice with C57BL/6 mice expressing the FlpE recombinase and this was followed by a cross with C57BL/6 mice to remove the flpe transgene. *Hoip<sup>-/-</sup>* and *Hoil-1<sup>-/-</sup>* mice were generated by crossing *Hoip<sup>fl/fl</sup>* mice, previously described<sup>17</sup>, and *Hoil-1<sup>fl/fl</sup>* mice (described here) with transgenic mice expressing the *loxP*-deleter Cre recombinase (purchased from JAX: 6054, B6.C-Tg(CMV-Cre)1 Cgn/J). Transgenic mice expressing the Cre recombinase under the control of the *Tie2* (also known as *Tek*) promoter (*Tie2-Cre*) (B6.Cg-Tg(Tek-cre)1Ywa/J)<sup>25</sup> were used to delete floxed genes specifically in endothelial cells. C57BL/6 *Mkl1<sup>-/-</sup>* mice crossed to *Sharpin<sup>cpdm</sup>* mice were previously described<sup>26</sup>. For all other crosses *Mkl1<sup>-/-</sup>* mice were generated using transcription activator-like effector nuclease (TALEN). In brief, TALENs targeting exon 1 of the *Mkl1* gene were cloned via Golden-gate assembly. The RVD sequence of TAL1 against TACCGTTTCAGATGTCA was NIHDHDDNNNGNGHDNINNNINGNNGHDNI, and TAL2 against TCGATCTCCCTGCTGCC was HDNNNINNGHDNNGHDHDDNNGNHDNNGN NHDHD. Capped RNA was produced in vitro using mMACHINE T7 Transcription Kit (Ambion) and poly A tail was added using Poly(A) Tailing Kit (Ambion). Purified transcripts were mixed and adjusted to 25 ng  $\mu\text{l}^{-1}$ . C57BL/6 fertilized eggs were injected into both the cytoplasm and the pro-nucleus. Embryos were transferred into C57BL/6 pseudo-pregnant females. Pups were genotyped by sequencing using genomic DNA obtained from ear punches. One female carrying a 19-base pair (bp) homozygous deletion causing a premature stop codon was selected for further breeding. *Mkl1<sup>-/-</sup>* mice were backcrossed to C57BL/6 mice for two generations. *Sharpin<sup>cpdm</sup>* (C57BL/Ka) and *Tnfr1<sup>-/-</sup>* (2818, B6.129-Tnfr1atm1Mak/J) mice were purchased from JAX. *Tnf<sup>-/-</sup>* mice (C57BL/6;129S6) were provided by W. Kaiser. *Ripk3<sup>-/-27</sup>*, *Casp8<sup>-/-28</sup>*, *Ripk1<sup>K45A4</sup>* and *Ripk1<sup>-/-29</sup>* mice have been reported previously. Timed matings were performed as previously described<sup>8</sup>. All mice were genotyped by PCR, fed ad libitum. All animal experiments were conducted under an appropriate UK project license in accordance with the regulations of UK home office for animal welfare according to ASPA (animal (scientific procedure) Act 1986). The relevant Animal Ethics Committee approved all experiments involving *Sharpin<sup>cpdm</sup>* and the *Ripk1<sup>-/-</sup>* crosses which were maintained under appropriate licenses and subject to ethical review at The Walter and Eliza Hall Institute (Melbourne, Australia) and UT Health Sciences Center San Antonio (TX, USA), respectively.

**Histological analysis, TUNEL and immunofluorescence staining.** Embryos or organs from adult mice were collected and fixed in 10% buffered formalin and paraffin embedded. Sections of 4- $\mu\text{m}$  were stained with haematoxylin and eosin following standard procedures. Necropsy of adult mice or six sagittal serial sections of two different planes of the embryo were used for blinded pathological analysis. For TUNEL staining, sections were treated according to the manufacturer's instructions (DeadEnd Fluorometric TUNEL System, Promega, G3250). For whole-mount TUNEL staining and immunofluorescence staining, samples were processed using the ApoTag plus Peroxidase In situ Apoptosis Detection Kit (Millipore, S7101) according to the manufacturer's instructions and as previously described<sup>8</sup>. Quantification was performed by an experimenter blinded to the genotype of the mice by using ImageJ Software on monochrome images of the whole yolk sac by measuring the area of positive staining. Alternatively, TUNEL-positive cells were counted on five different fields (10 $\times$  magnification). Yolk sacs were stained with antibodies against PECAM-1 (BD Biosciences, 5533370 clone MEC13.3) and cleaved caspase 3 (Cell Signaling, 9664), followed by staining with secondary antibodies, Alexa Fluor 594 goat anti-rat IgG and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A-11007 and A-11034, respectively), and analysed by fluorescent microscopy. Quantification was performed by an experimenter blinded to the genotype of the mice on ten different fields (10 $\times$  magnification) per yolk sac. **Microfocus computed tomography scan.** Embryos were fixed in 4% paraformaldehyde and potassium triiodide (Lugol's iodine/I<sub>2</sub>KI, to impart tissue contrast), with a total iodine content of 63.25 mg  $\text{ml}^{-1}$  (iodine mass of  $2.49 \times 10^{-4}$  mol  $\text{ml}^{-1}$ ), in a 1:1 ratio for 8 h before imaging. Before scanning, the embryos were washed, wrapped in Parafilm M (Bemis) and secured in 3% (w/v) Agar (Sigma-Aldrich), within a low-density plastic cylinder to ensure mechanical stability during scan acquisition. Images were acquired using an XT H 225 ST microfocus-computed tomography scanner with a multimetal target (Nikon Metrology). Scans were reconstructed using modified Feldkamp filtered back projection algorithms with proprietary software (CTPro3D; Nikon Metrology) and post-processed using VG Studio

MAX (Volume Graphics GmbH). Soft tissues were analysed by Phong shading of direct volume renderings and plain projections and the vascular system by maximum intensity projections.

**Cells.** MEFs were isolated from E12.5–E13.5 embryos in accordance with standard procedures and these cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (Sigma). Transformation was performed by lentiviral infection with the SV40 large T antigen. For reconstitution experiments, the coding sequence of mouse HOIP, SHARPIN or HOIL-1 wild-type (WT), the UBL domain of HOIL-1 only (HOIL-1-UBL; amino acids 1–139), HOIL-1- $\Delta$ RBR (amino acids 1–252), HOIL-1- $\Delta$ UBL (amino acids 140–508), HOIL-1 with inactivating mutations T201A/R208A in the NZF domain (HOIL-1-NZFmut) or HOIL-1 with a point mutation in the catalytic cysteine of the RBR domain (HOIL-1-C458A) was inserted in MSCV vector followed by the internal ribosome entry site (IRES)-GFP sequence. These vectors were retrovirally transduced into MEFs and GFP-positive cells were sorted in a MoFlo cytometer (Beckman Coulter).

**Immunoprecipitation.** For isolation of the TNFR1-SC, transformed MEFs were stimulated with 3 $\times$  Flag-2 $\times$  Strep-TNF at 0.5  $\mu\text{g ml}^{-1}$  for 15 min, and controls were left untreated. Cells were subsequently solubilised in lysis buffer (30 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM KCl, 10% glycerol, 1% Triton X-100, EDTA-free proteinase inhibitor cocktail (Roche, 5056489001) and 1 $\times$  phosphatase-inhibitor cocktail 2 (Sigma, P5726-1ML)) at 4 $^{\circ}\text{C}$  for 30 min. The lysates were cleared by centrifugation, and 3 $\times$  Flag-2 $\times$  Strep-TNF (0.5  $\mu\text{g ml}^{-1}$  per sample) was added to the untreated samples. Subsequently, the lysates were subjected to anti-Flag immunoprecipitation using M2 antibody coupled sepharose beads (Sigma, A2220-5ML) for 16 h. For immunoprecipitation of FADD, transformed MEFs were treated with 20  $\mu\text{M}$  zVAD-fmk (Abcam, ab120487) in the presence or absence of 100 ng  $\text{ml}^{-1}$  6 $\times$  His-TNF for 3 h. Cells were lysed as described above and FADD was immunoprecipitated using anti-FADD antibody (Santa Cruz, sc-5559) and protein G Sepharose Beads (GE healthcare, 17-0618-01) at 4 $^{\circ}\text{C}$  for 4 h. For SHARPIN immunoprecipitation, anti-SHARPIN antibody (ProteinTech, 14626-1-AP) was used. For all immunoprecipitations, the beads were washed three times with lysis buffer. Proteins were eluted in 50  $\mu\text{l}$  of LDS buffer (NuPAGE, Invitrogen) containing 50 mM dithiothreitol (DTT). Samples were analysed by western blotting.

**Western blot analysis and antibodies.** Whole embryos were snap-frozen and homogenized in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40 and 1 $\times$  EDTA-free proteinase inhibitor cocktail (Roche, 5056489001) or RIPA buffer with 6 M urea for the experiment in Extended data Fig. 7h. Alternatively, cells were washed twice with ice-cold PBS before lysis in lysis buffer. Protein concentration of lysates was determined using BCA protein assay (Thermo Scientific). Lysates were subsequently denatured in reducing sample buffer at 95 $^{\circ}\text{C}$  for 10 min before separation by SDS-PAGE (NuPAGE) and subsequent analysis by western blotting using antibodies against HOIL-1<sup>30</sup>, HOIP (custom-made, Thermo Fisher Scientific), SHARPIN (ProteinTech, 14626-1-AP), TNFR1 (Abcam, ab19139), actin (Sigma, A1978), pIKB $\alpha$  (Cell Signaling, 9246), I $\kappa$ B $\alpha$  (Cell Signaling, 9242), phospho p65 (Cell Signaling, 3033) cleaved caspase-8 (Cell Signaling, 9429), linear ubiquitin (Merck Millipore, MABS199), RIPK1 (BD, 610459), RIPK3 (Enzo, ADI-905-242-100), FADD (Assay Design, AAM-121), MLKL (Millipore, MABC604), phospho-MLKL (Abcam, ab196436) and tubulin (Sigma, T9026).

**Cell death analysis by propidium iodide staining.** Cells were seeded to 80% confluence and were then incubated with 100 ng  $\text{ml}^{-1}$  His-tagged TNF, 1  $\mu\text{g ml}^{-1}$  CD95L-Fc, 1  $\mu\text{g ml}^{-1}$  isoleucine zipper tagged murine TRAIL (iz-mTRAIL), 100  $\mu\text{g ml}^{-1}$  poly(I:C) HMW (InvivoGen, thrl-pic), 20 ng  $\text{ml}^{-1}$  IFN- $\gamma$  (Peprotech, 315-05) or 100 ng  $\text{ml}^{-1}$  LT- $\alpha$  (Thermo Fisher Scientific, 10270-HNAE) for 24 h, unless otherwise indicated. When indicated the following inhibitors were used: 20  $\mu\text{M}$  zVAD-FMK (Abcam, ab120487), 10  $\mu\text{M}$  necrostatin-1 (Biovision, 2263-5). Supernatants and adherent cells were collected and resuspended in PBS containing 5  $\mu\text{g ml}^{-1}$  propidium iodide. Propidium iodide-positive cells were enumerated by FACS (BD Accuri).

**RNA-sequencing analysis.** E13.5 embryos were snap frozen and RNA was prepared using the RNeasy minikit (Qiagen, 74104) according to the manufacturer's instruction. To generate the library, samples were processed using the KAPA mRNA HyperPrep Kit (KK8580) according to the manufacturer's instructions. In brief, mRNA was isolated from total RNA using Oligo dT beads to pull down poly-adenylated transcripts. The purified mRNA was fragmented using chemical fragmentation (heat and divalent metal cation) and primed with random hexamers. Strand-specific first strand cDNA was generated using reverse transcriptase in the presence of actinomycin D. The second cDNA strand was synthesized using dUTP in place of dTTP, to mark the second strand. The resultant cDNA was then 'A-tailed' at the 3' end to prevent self-ligation and adaptor dimerization. Truncated adaptors, containing a T overhang were ligated to the A-tailed cDNA. Successfully ligated cDNA molecules were then enriched with limited cycle PCR. Libraries to be multiplexed in the same run were pooled in equimolar quantities, calculated from Qubit



and Bioanalyser fragment analysis. Samples were sequenced on the NextSeq 500 instrument (Illumina) using a 43-bp paired-end run. Run data were de-multiplexed and converted to fastq files using the Illumina bcl2fastq Conversion Software v2.18 on BaseSpace. Fastq files were then aligned to a reference genome using STAR on the BaseSpace RNA-Seq alignment app v1.1.0. Reads per transcript were counted using HTSeq and differential expression was estimated using the BioConductor package DESeq2 (BaseSpace app v1.0.0). Next, four groups of differentially regulated genes were analysed: low and high abundance *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> versus *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> embryos and low and high abundance in *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> versus *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos. To identify genes that were specifically altered in the absence of HOIL-1, the Venny 2.1 software was used to exclude genes that were differentially expressed between *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> embryos from those between *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos. Genes that were already differentially expressed between the corresponding HOIL-1-expressing controls (that is, *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> embryos) were excluded from the differentially expressed genes between *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos. The resulting list of genes (33/85) was entered in the STRING software (<https://string-db.org>) to assess for functional enrichment in biological networks. Gene Ontology (GO) terms with false discovery rate below 1% are shown.

#### Flow cytometry analysis, colony-forming unit assay and macrophage culture.

For phenotypic analysis, single-cell suspensions from mechanically dissociated E13.5 fetal livers or a pool of aortas (AGM region) from three embryos were stained for 30 min on ice with various antibody cocktails. The antibodies against the surface markers examined were: CD16/32, clone 93 and 2.4G2 (eBioscience, 45-0161-82 and BD553141), CD135, clone A2F10.1 (BD, 553842), Ly-6A/E, clone D7 (Sca-1) (BD, 558162), CD117 (c-Kit), clone 2B8 (BD, 560185), CD34, clone RAM34 (BD, 562608), mouse lineage cocktail, clones 17A2/RB6-8C5/RA3-6B2/Ter-119/M1/70 (Biolegend, 133313 and BD, 561301), CD16/32, clone 2.4G2 (BioXcell, CUS-HB-197), CD11b, clone M1/70 (Biolegend, 101228 and eBioscience, 15-0112-81), CD11c, clone HL3 (BD, 561241), F4/80, clone BM8 (Biolegend, 123110), GR-1, clone RB6-8C5 (Biolegend, 108416 and 108410), CD45, clone 30-F11 (Biolegend, 103128 and Biolegend, 103112), CD3ε, clone, 145-2C11 (Biolegend, 100310), B220, clone RA3-6B2, (Biolegend, 103210), CD71, clone RI7217 (Biolegend, 113807), TER-119, clone TER-119 (Biolegend, 116234) and fixable viability dye (eBioscience, 65-0864-18 and 65-0867-14). The myeloid progenitors were identified in the LK population as CD34<sup>+</sup>CD16/32<sup>-</sup> (CMP), CD34<sup>+</sup>CD16/32<sup>+</sup> (GMP); CD34<sup>-</sup>CD16/32<sup>-</sup> (MEP). Fluorescence minus one (FMO) was used as a gating control. For quantification of absolute number of cells, a defined number of flow cytometric reference beads (Invitrogen) were mixed with the samples before acquisition. Samples were processed either using LSR Fortessa (BD Biosciences) or sorted in a FACSAria FUSION cell sorter (BD Biosciences). Data were analysed with FlowJo 7.6.1 software (Treestar). Cytospin preparations of 10,000 cells per slide of E13.5 fetal liver homogenates were stained by May-Grünwald Giemsa staining and enucleated erythrocytes were quantified blindly as number of cells per HPF using ImageJ Software. For growth of primitive erythroid progenitor cells or all haematopoietic stem cells, 5,000 sorted Lin<sup>-</sup>c-KIT<sup>+</sup> E13.5 liver cells were cultured in MethoCult SF containing cytokines, including EPO (Stem Cell, M3436) or Mouse Methylcellulose Complete Media (R&D, HSC007), respectively. Colonies were enumerated after 14 days of incubation. For preparation of fetal liver-derived macrophages, equal amounts of E13.5 single cell suspensions were cultured and differentiated for 5 days in DMEM supplemented with 10% FCS plus

20% L929-conditioned medium (as a source of M-CSF) supplemented or not with the indicated inhibitors. Cells were imaged using EVOS Auto cell imaging system and viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7572). Alternatively, cells were stained with Hoechst dye and enumerated using Citation cell imaging platform.

**Cytokine analysis.** Embryo homogenates prepared as described above ('Western blot and antibodies' section) were analysed with Proteome Profiler Arrays (Mouse Angiogenesis Array, ARY015, and Mouse Cytokine array Panel A, ARY006 both R&D). ELISA kits used were the CXCL4 (R&D, DY595), CXCL11 (Abcam, ab204519), CXCL10 (R&D, DY466-05), IFN lambda 2/3 (Pbl assay science, 62830-1), IL-1β (ThermoFisher, BMS6002) and IFN-β ELISA (ThermoFisher, 424001).

**Epidermal thickness quantification.** Per mouse, 1–2 pieces of skin were taken and epidermal thickness was measured by microscopy using a 20× magnification. Quantification was performed by an experimenter blinded to the genotype of the mice by using the CellSens software with at least 20 measurements per mouse.

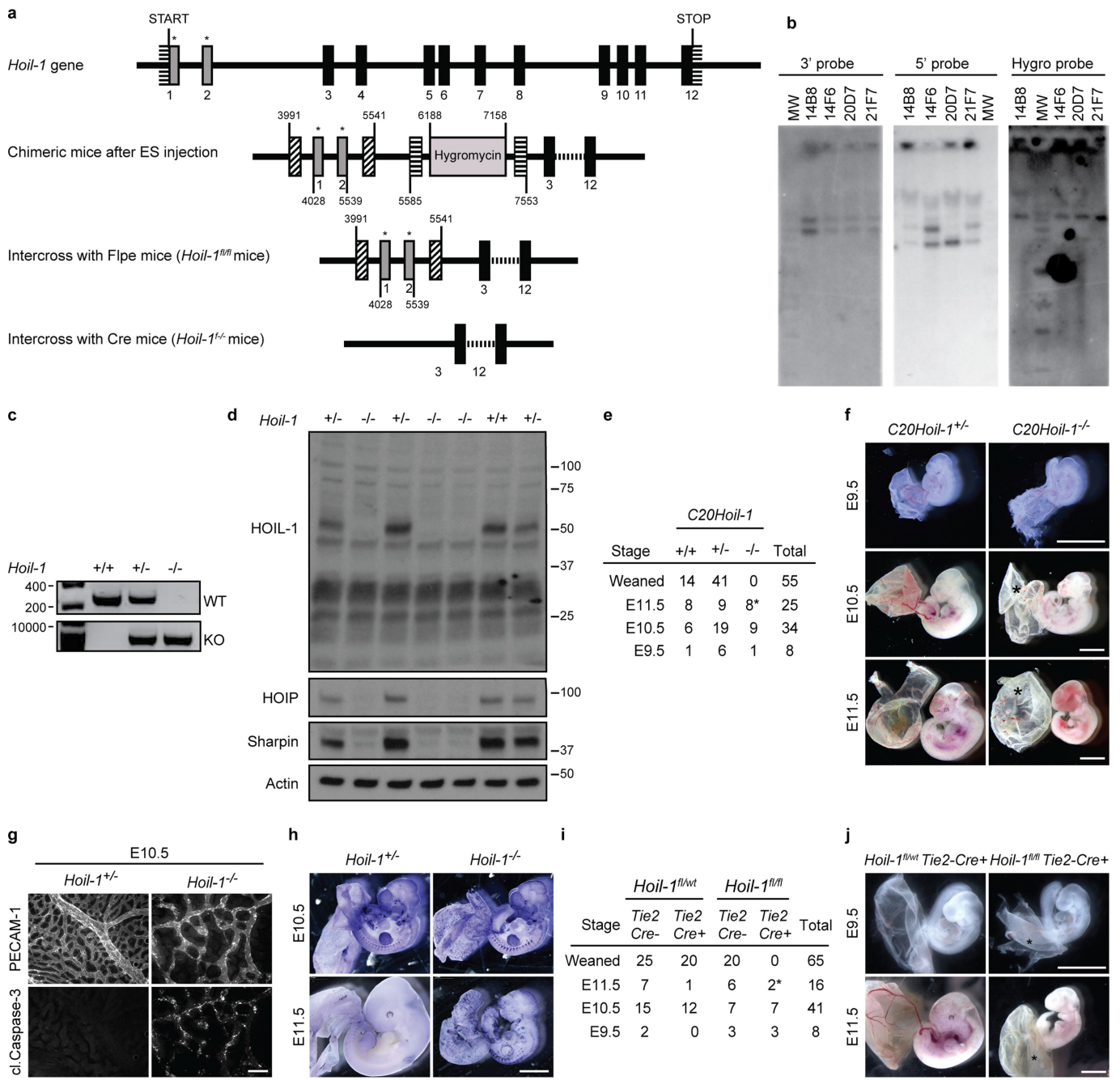
**Pharmacological inhibition of RIPK1 kinase activity.** Mice were fed with rodent chow containing 100 mg kg<sup>-1</sup> of the RIPK1 kinase inhibitor GSK3540547A (GSK'547A) (GlaxoSmithKline LLC) starting a week before mating and kept on this diet throughout pregnancy until caesarean section at the indicated time points.

**Statistics and reproducibility.** Group size was determined based on preliminary datasets. Statistical significance was determined using unpaired, two-tailed parametric Student's *t*-test. One- or two-way ANOVA with Tukey's multiple comparisons test was applied. 95% Confidence interval was considered for statistics and *P* < 0.05 was considered significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. Multiplicity-adjusted *P* values are reported for multiple comparisons. All statistical analyses were performed using Graphpad Prism 6. Statistical transformations for RNA-seq were performed with DESeq2 and adjusted *P* values used the Benjamini–Hochberg test. All in vitro experiments were performed at least twice with similar results. Unless indicated in figure legends in vivo experiments were performed with at least two embryos per genotype. At least three embryos were considered for statistical testing. The experiments were not randomized.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability.** RNA-sequencing analysis data are available from the Sequence Read Archive (SRA) database SRP134865 (BioProject accession PRJNA437851) and comparative datasets including genes differentially regulated genes between embryo homogenates with different mutations are displayed in Supplementary Table 1.

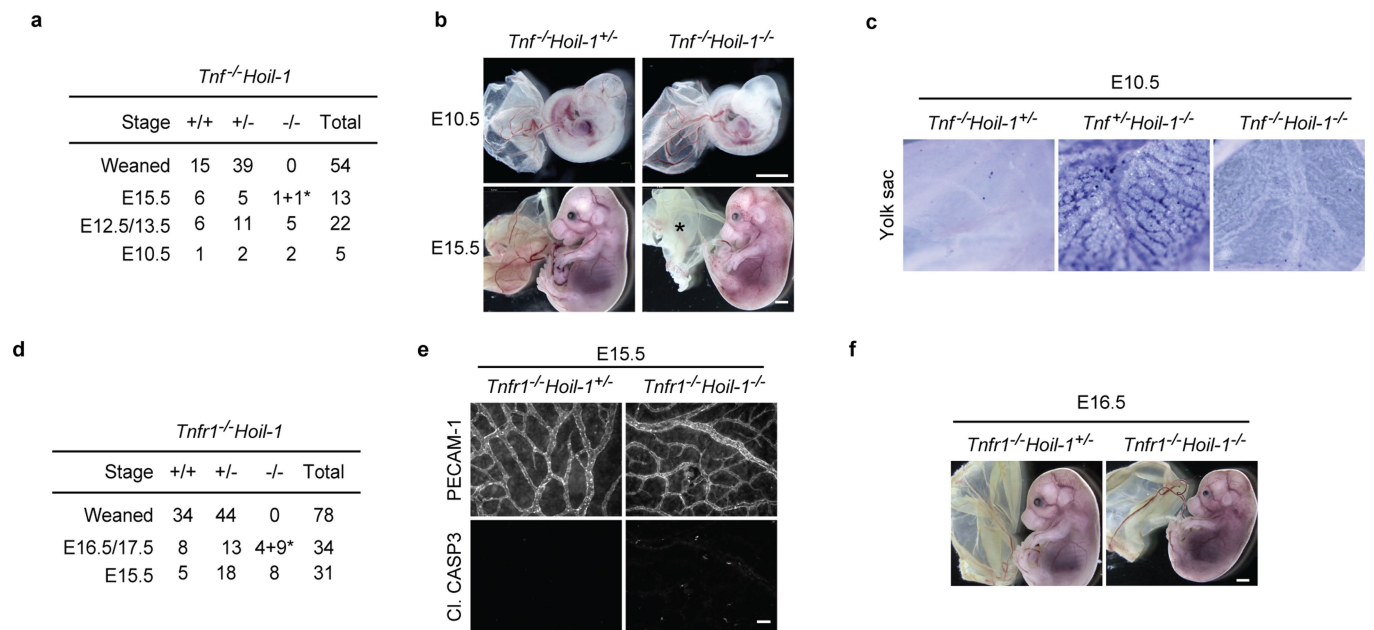
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**Extended Data Fig. 1 | HOIL-1-deficient mice die at mid-gestation.**

**a**, Schematic representation of the *Hoil-1*-knockout strategy. Solid boxes represent *Hoil-1* exons and grey boxes with a star indicate the targeted exons. Boxes with diagonal and horizontal strips represent *loxP* and *Frt* sites, respectively. **b**, Specificity of gene recombination was assessed by Southern blotting with 5' and 3' probes external to the construct in four clones (14B8, 14F6, 20D7 and 21F7). Digest of the DNA with *Apa*I, followed by hybridization with the 3' probe was expected to show a 5,700-bp band for the wild-type allele and a 7,700-bp band for the mutant allele. All four clones appeared to have the correct recombination on the 3' side. Digest of the DNA with *Sph*I and hybridization with the 5' probe was expected to show a 4,500-bp wild-type band and a 6,200-bp band for the mutated allele. Clones 14B8 and 14F6 appeared to be correctly recombined on the 5' side. Finally, cutting the DNA with *Apa*I and hybridizing with a hygromycin probe showed a single band in all clones, indicative of a single integration of the construct in all four ES clones. Clones 14B8 and 14F6 were selected for generation of the two

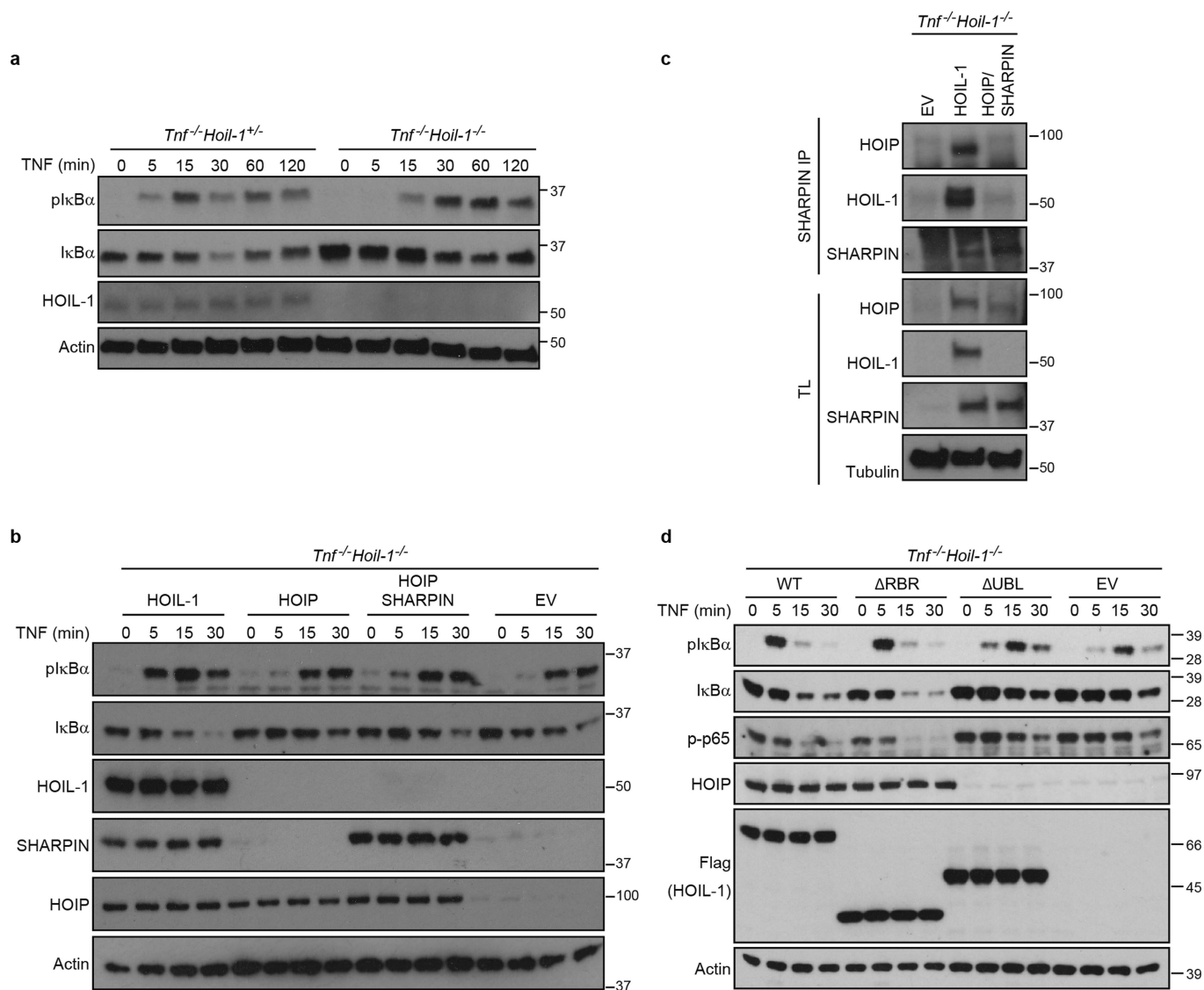
*Hoil-1*<sup>-/-</sup> strains. **c**, PCR analysis of *Hoil-1* wild-type, heterozygous and knockout mice. **d**, Protein levels of HOIL-1, HOIP and SHARPIN in whole embryo lysates ( $n = 3$  for *Hoil-1*<sup>+/-</sup> and *Hoil-1*<sup>-/-</sup> embryos and  $n = 1$  for *Hoil-1*<sup>+/+</sup> embryos). For gel source data (c, d), see Supplementary Fig. 1. **e**, Quantification of genotypes of animals obtained from intercrossing C20*Hoil-1*<sup>+/-</sup> mice. Asterisk indicates dead embryo. **f**, Representative images of C20*Hoil-1*<sup>+/-</sup> and C20*Hoil-1*<sup>-/-</sup> embryos from E9.5 to E11.5 as quantified in e. Scale bars, 2 mm. **g**, Single staining showing vascularization (PECAM-1, top) and apoptosis (cleaved CASP3, bottom) of yolk sacs. Merged image is shown in Fig. 1c. **h**, Whole-mount TUNEL staining of embryos at the indicated stages (embryo per genotype  $n = 2$  at E10.5,  $n = 8$  for *Hoil-1*<sup>+/-</sup> and  $n = 5$  for *Hoil-1*<sup>-/-</sup> at E11.5). Scale bar, 2 mm. **i**, Quantification of genotypes of animals obtained from intercrossing *Hoil-1*<sup>fl/wt</sup>*Tie2-cre*<sup>+</sup> with *Hoil-1*<sup>fl/fl</sup>*Tie2-cre*<sup>-</sup> mice. Asterisk indicates dead embryo. **j**, Representative images of embryos with conditional deletion of *Hoil-1* in *Tie2-Cre*-expressing cells as quantified in i. Scale bar, 2 mm. Asterisks denote poorly vascularized yolk sac.



**Extended Data Fig. 2 | TNFR1 signalling drives cell death and lethality of HOIL-1-deficient mice at mid-gestation. a, d,** Quantification of genotypes of animals obtained from intercrosses of *Tnf<sup>-/-</sup>Hoil-1<sup>+/-</sup>* (a) and *Tnfr1<sup>-/-</sup>Hoil-1<sup>+/-</sup>* (d) mice. Asterisk denotes dead embryo. **b,** Representative images of embryos quantified in a at E10.5 and E15.5. Asterisk denotes poor yolk sac vascularization. **c,** Cell death as detected

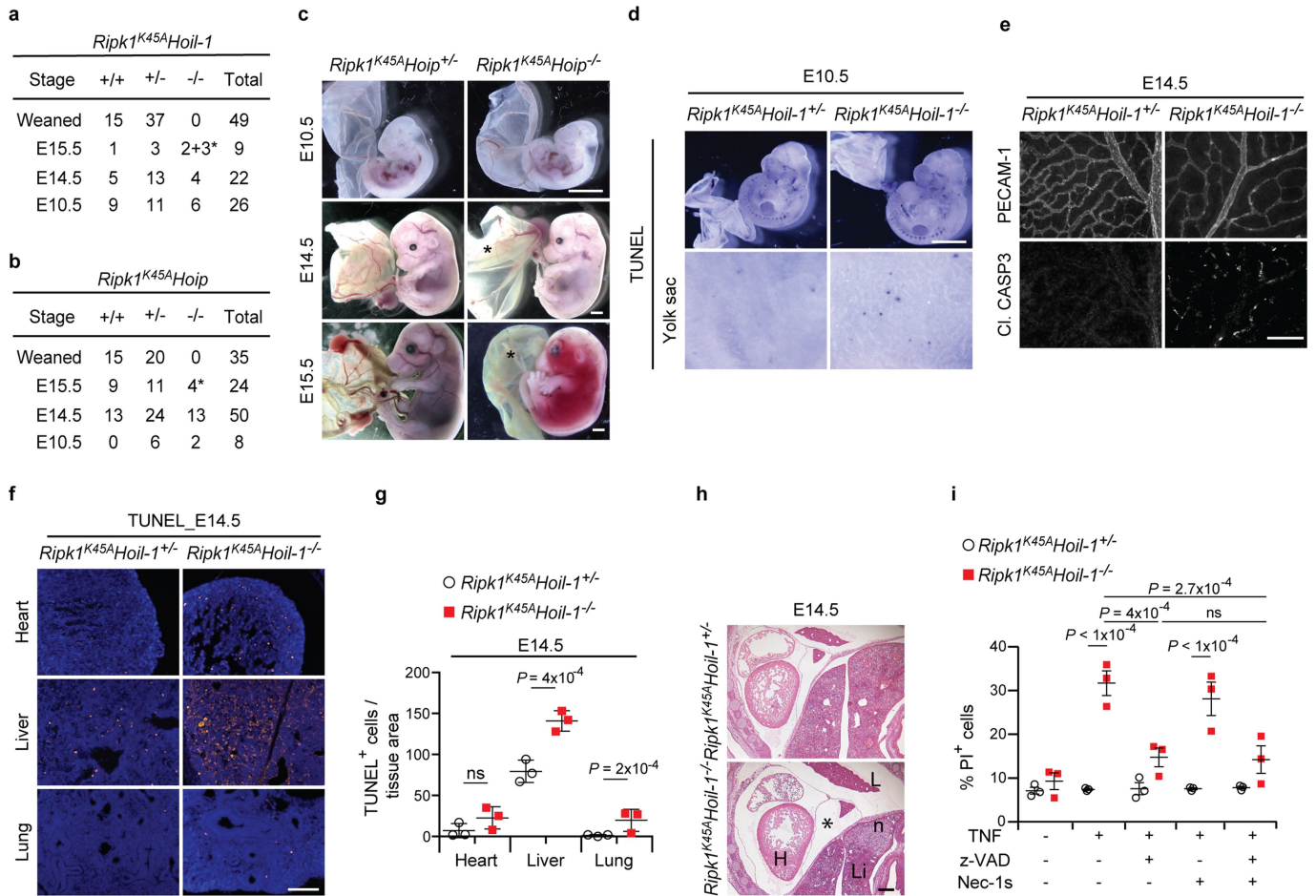
by whole-mount TUNEL staining in yolk sacs at E10.5 ( $n = 3$  embryos per genotype). **e,** Single staining showing vascularization (PECAM-1, top) and apoptosis (cleaved CASP3, bottom) of yolk sacs. Merged image is shown in Fig. 1g. Scale bar, 50  $\mu\text{m}$ . **f,** Representative images of embryos at E16.5 ( $n = 2$  for *Tnfr1<sup>-/-</sup>Hoil-1<sup>+/-</sup>* and  $n = 4$  for *Tnfr1<sup>-/-</sup>Hoil-1<sup>-/-</sup>*).





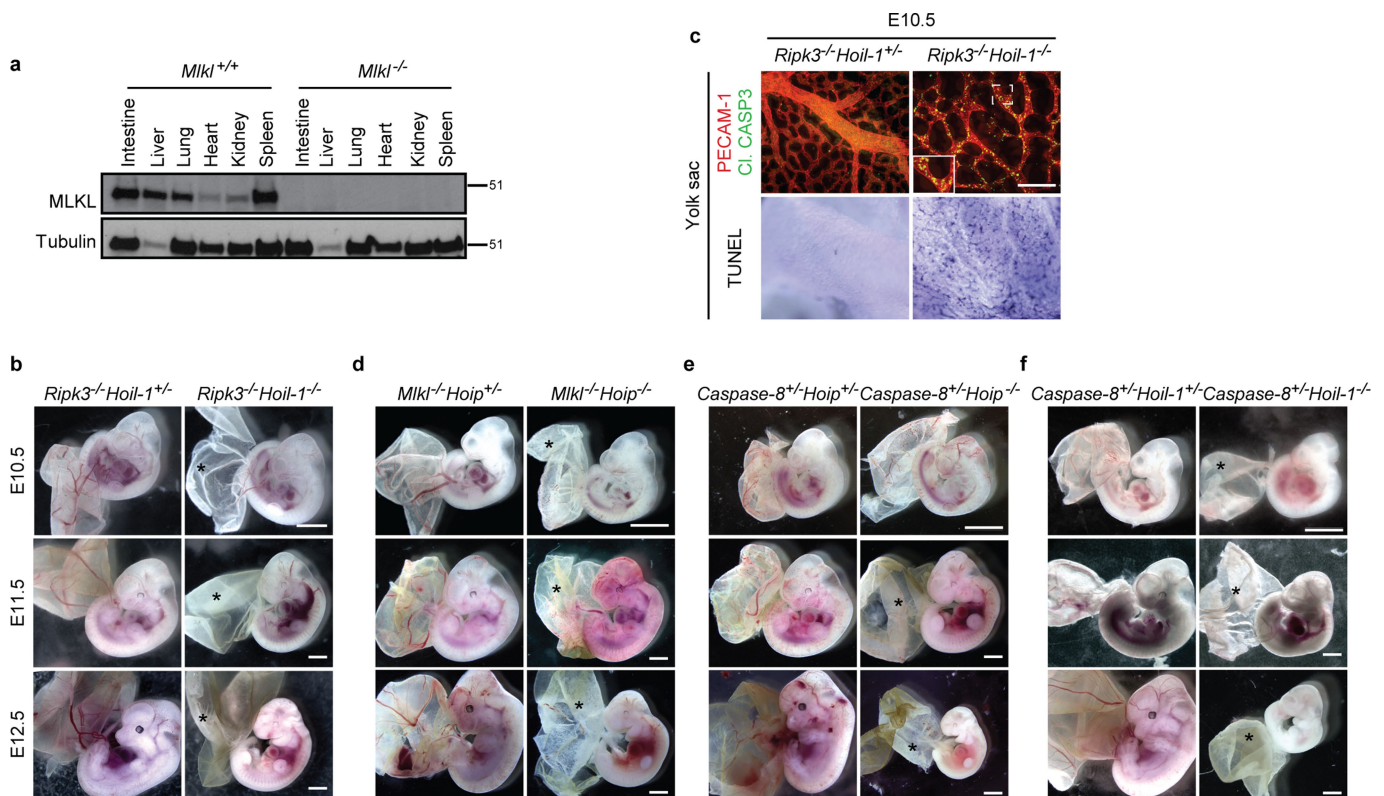
**Extended Data Fig. 3 | HOIL-1 is required for optimal TNF-induced NF- $\kappa$ B activation independently of its RBR domain. a, b, d,** Western blot analysis of the indicated proteins in whole-cell lysates from MEFs of the indicated genotypes after they had been stimulated with TNF (or left untreated) for the indicated time points (a), after overexpression of the different LUBAC components (b; HOIL-1, HOIP, SHARPIN) or

after expression of the indicated mutant forms of HOIL-1 (d) ( $n = 2$  independent experiments). c, SHARPIN immunoprecipitation was performed in *Tnf<sup>-/-</sup>Hoil-1<sup>-/-</sup>* MEFs reconstituted with HOIL-1 or a combination of HOIP and SHARPIN and analysed by western blotting ( $n = 2$  independent experiments). For gel source data, see Supplementary Fig. 1.



**Extended Data Fig. 4 | Ablation of the kinase activity of RIPK1 in HOIL-1- or HOIP-deficient embryos prevents cell death and lethality at mid-gestation but not at late gestation.** **a, b**, Quantification of genotypes of animals obtained after intercrossing *Ripk1<sup>K45A</sup>Hoil-1<sup>+/-</sup>* (**a**) and *Ripk1<sup>K45A</sup>Hoip<sup>+/-</sup>* (**b**) mice. Asterisk indicates dead embryo. **c**, Representative images of embryos quantified in **b**. Asterisks denote poor yolk sac vascularization. Scale bar, 2 mm. **d**, Whole-mount TUNEL staining of embryos ( $n = 2$  embryos). Scale bar, 2 mm. **e**, Single staining showing vascularization (PECAM-1, top) and apoptosis (cleaved CASP3, bottom) of yolk sacs. Merged image is shown in Fig. 3b. **f, g**, Representative

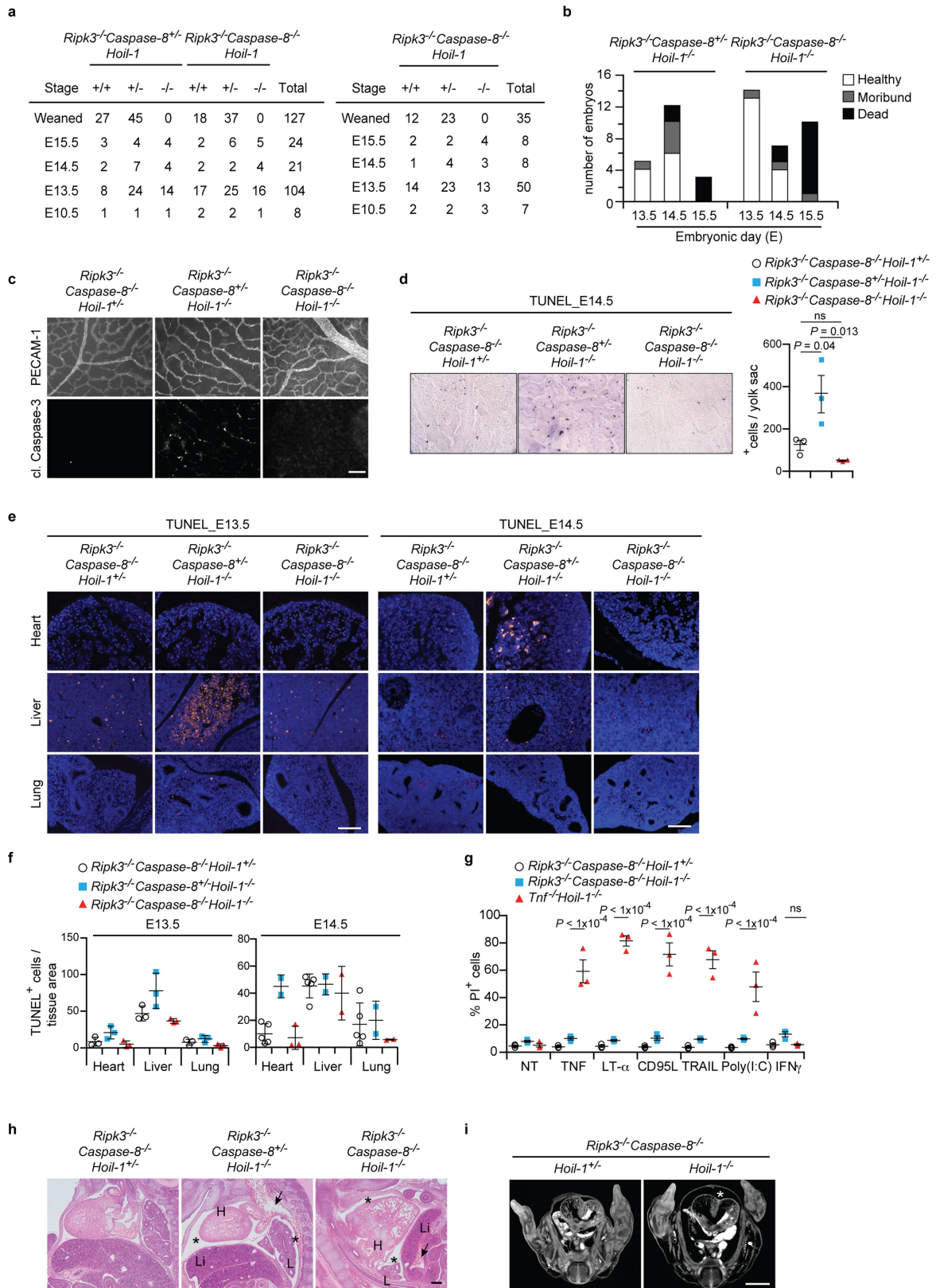
images of cell death in different organs (**f**) and quantification (**g**) as detected by TUNEL staining at E14.5 ( $n = 3$  embryos per genotype). Scale bar, 50  $\mu\text{m}$  (**f**). Mean  $\pm$  s.e.m. ( $n = 3$  embryos per genotype).  $P$  values from one-way ANOVA are reported. **h**, Representative images of H&E staining on whole embryo paraffin sections ( $n = 3$  embryos per genotype). Asterisk denotes pericardial effusion. n, necrotic area. Scale bar, 200  $\mu\text{m}$ . **i**, Cell death was analysed by propidium iodide (PI) staining in MEFs stimulated with TNF for 24 h plus the indicated cell death inhibitors. Mean  $\pm$  s.e.m. ( $n = 3$  independent experiments).  $P$  values from two-way ANOVA are reported.



**Extended Data Fig. 5 | Individual deletion of mediators of apoptosis or necroptosis does not prevent cell death and lethality at mid-gestation of HOIL-1- or HOIP-deficient embryos.** **a**, Western blot analysis of MLKL expression in the indicated organs derived from *Mlkl*<sup>-/-</sup> mice ( $n = 2$  mice per genotype), as control. For gel source data, see Supplementary Fig. 1. **b**, **d**–**f**, Representative images of embryos at different stages of gestation (E10.5:  $n = 7$  for *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 5$  for *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>; E11.5:  $n = 5$  for *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 2$  for *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>; E12.5:  $n = 9$  for *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 2$  for *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> (**b**), E10.5:  $n = 16$  for *Mlkl*<sup>-/-</sup>*Hoip*<sup>+/-</sup> and  $n = 6$  for *Mlkl*<sup>-/-</sup>*Hoip*<sup>-/-</sup>; E11.5:  $n = 8$  for *Mlkl*<sup>-/-</sup>*Hoip*<sup>+/-</sup> and  $n = 6$  for *Mlkl*<sup>-/-</sup>*Hoip*<sup>-/-</sup>; E12.5:

$n = 10$  for *Mlkl*<sup>-/-</sup>*Hoip*<sup>+/-</sup> and  $n = 5$  for *Mlkl*<sup>-/-</sup>*Hoip*<sup>-/-</sup> (**d**), E10.5:  $n = 5$  for *Casp8*<sup>+/-</sup>*Hoip*<sup>+/-</sup> and  $n = 4$  for *Casp8*<sup>+/-</sup>*Hoip*<sup>-/-</sup>; E11.5:  $n = 6$  for *Casp8*<sup>+/-</sup>*Hoip*<sup>+/-</sup> and  $n = 3$  for *Casp8*<sup>+/-</sup>*Hoip*<sup>-/-</sup>; E12.5:  $n = 3$  for *Casp8*<sup>+/-</sup>*Hoip*<sup>+/-</sup> and  $n = 2$  for *Casp8*<sup>+/-</sup>*Hoip*<sup>-/-</sup> (**e**), E10.5:  $n = 2$  for *Casp8*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 4$  for *Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup>; E11.5:  $n = 2$  for *Casp8*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 5$  for *Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup>; E12.5:  $n = 6$  for *Casp8*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 3$  for *Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> (**f**). Asterisks denote poor yolk sac vascularization. Scale bars, 2 mm. **c**, Representative images of yolk sac vascularization and cell death at E10.5 as detected by PECAM-1 (red) and cleaved CASP3 staining (green) (top) and whole-mount TUNEL staining (bottom) ( $n = 4$  per genotype). Scale bar, 50  $\mu\text{m}$ .

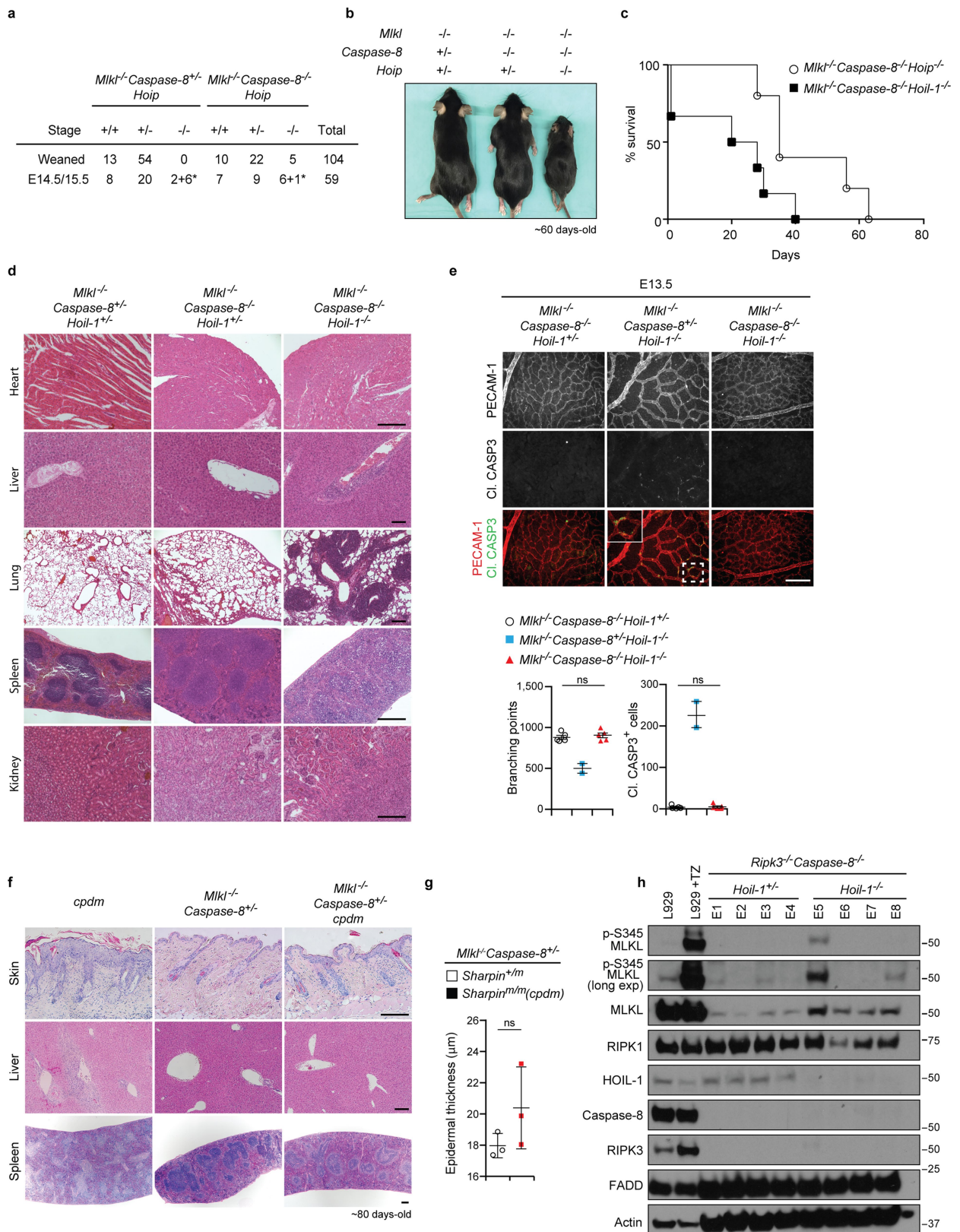




Extended Data Fig. 6 | See next page for caption.

**Extended Data Fig. 6 | Combined deletion of RIPK3 and caspase-8 prevents cell death but not embryonic lethality at late gestation that is caused by the loss of HOIL-1.** **a**, Quantification of genotypes of animals obtained from inter-crosses of *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> with *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> mice (left) or *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> mice (right). **b**, Health status of *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos at different developmental stages. **c**, Single staining showing vascularization (PECAM-1, top) and apoptosis (cleaved CASP3, bottom) of yolk sacs. Merged image is shown in Fig. 3f. Scale bar, 50  $\mu$ m. **d**, Cell death as detected by whole-mount TUNEL staining in yolk sacs at E14.5 (left) and respective quantification (right). Mean  $\pm$  s.e.m. ( $n = 3$  embryos per genotype). *P* values from one-way ANOVA are reported. **e, f**, Representative images (**e**) and quantification (**f**) of cell death in different organs as detected by TUNEL staining at E13.5 ( $n = 3$  embryos per

genotype) and E14.5 ( $n = 5$  for *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup>,  $n = 2$  for *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> lung and liver and  $n = 3$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> heart). Scale bars, 50  $\mu$ m. Data are mean  $\pm$  s.e.m. **g**, Cell death was analysed by propidium iodide (PI) staining in MEFs stimulated or not with the indicated ligands for 24 h. Data are mean  $\pm$  s.e.m. ( $n = 3$  independent experiments). *P* values from two-way ANOVA are reported. **h**, Representative images of H&E staining on E13.5 whole embryo paraffin embedded sections ( $n = 3$  for *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and  $n = 2$  for *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup>). Asterisks denote pericardial effusion. Arrows denote congested vessels. Scale bar, 200  $\mu$ m. **i**, Representative images of microfocus computed tomography scan images of whole E13.5 embryos ( $n = 3$  embryos per genotype). Asterisks denote pericardial effusion.



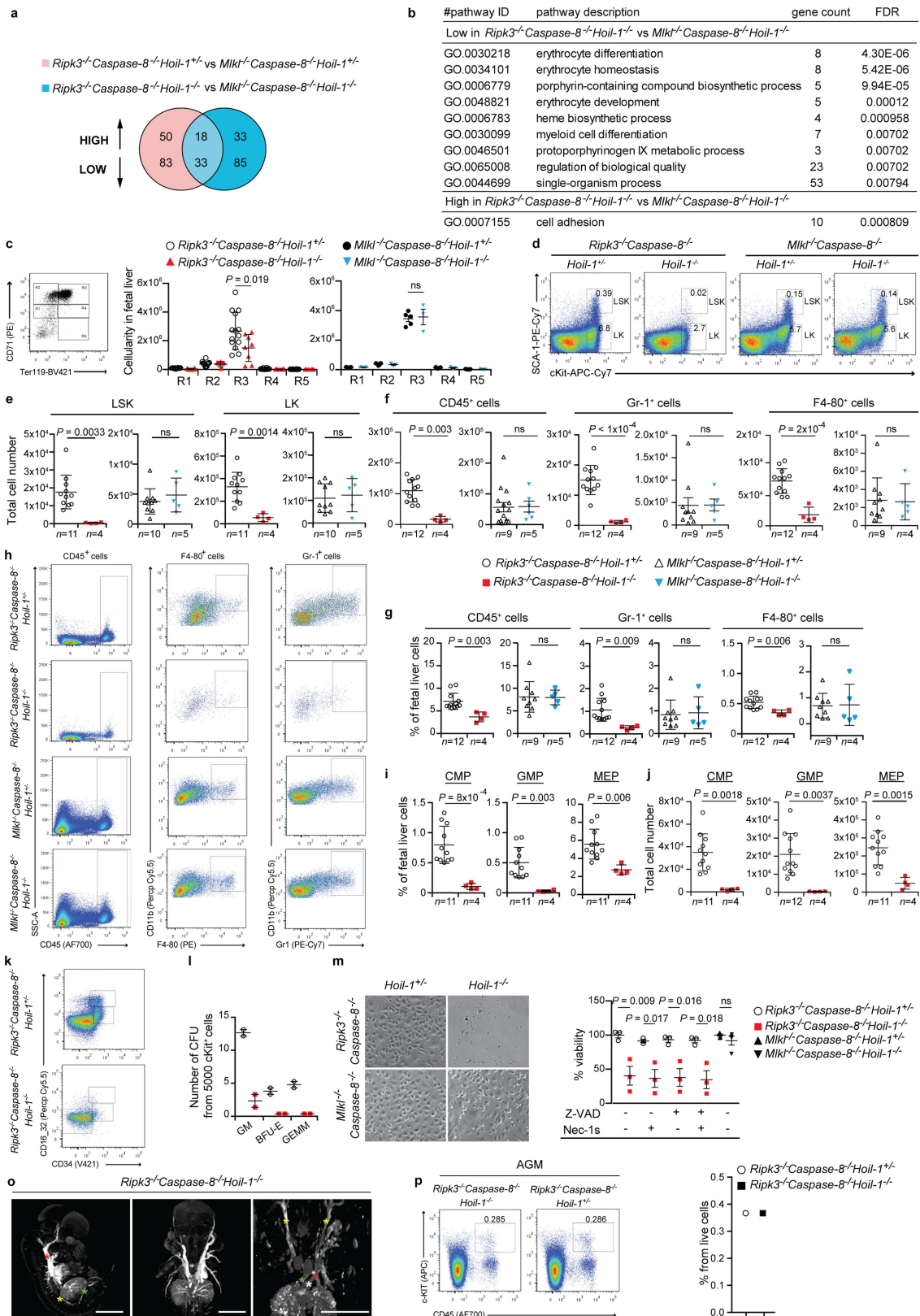
Extended Data Fig. 7 | See next page for caption.



**Extended Data Fig. 7 | Combined deletion of MLKL and caspase-8 promotes survival of LUBAC-deficient mice.**

**a.** Quantification of genotypes of animals obtained from intercrosses of  $Mkl^{-/-}Casp8^{+/-}Hoip^{+/-}$  with  $Mkl^{-/-}Casp8^{-/-}Hoip^{+/-}$  mice. Asterisk denotes dead embryo. **b.** Representative images of adult mice as quantified in **a**. **c.** Kaplan–Meier plot of mouse survival ( $n = 6$  for  $Mkl^{-/-}Casp8^{-/-}Hoip^{-/-}$  and  $n = 9$  for  $Mkl^{-/-}Casp8^{-/-}Hoil-1^{-/-}$  mice). **d.** Representative images of H&E staining of the indicated organs ( $n = 3$  mice per genotype). Scale bars,  $200\ \mu\text{m}$ . **e.** Representative images of yolk sac vascularization (PECAM-1, red) and apoptosis (cleaved CASP3, green) (top) at E13.5 and respective quantifications (bottom). Data are mean  $\pm$  s.e.m. ( $n = 5$  for  $Mkl^{-/-}Casp8^{-/-}Hoil-1^{+/-}$  and

$Mkl^{-/-}Casp8^{-/-}Hoil-1^{-/-}$  and  $n = 2$  for  $Mkl^{-/-}Casp8^{+/-}Hoil-1^{-/-}$ ). Statistical significance was determined with unpaired two-tailed *t*-tests comparing  $Mkl^{-/-}Casp8^{-/-}Hoil-1^{+/-}$  and  $Mkl^{-/-}Casp8^{-/-}Hoil-1^{-/-}$  embryos. **f.** Representative H&E staining images of the indicated organs ( $n = 3$  embryos per genotype). Scale bars,  $200\ \mu\text{m}$ . **g.** Epidermal thickness quantification of mice of the indicated genotypes in **f**. Data are mean  $\pm$  s.e.m. ( $n = 3$  mice per genotype). Statistical significance was determined with unpaired two-tailed *t*-tests. **h.** Western blot analysis of lysates from whole E13.5 embryos of the indicated genotypes and L929 cells treated with/without TNF plus zVAD-fmk (TZ) for 2 h as antibody validation ( $n = 4$  embryos per genotype performed twice). For gel source data, see Supplementary Fig. 1.

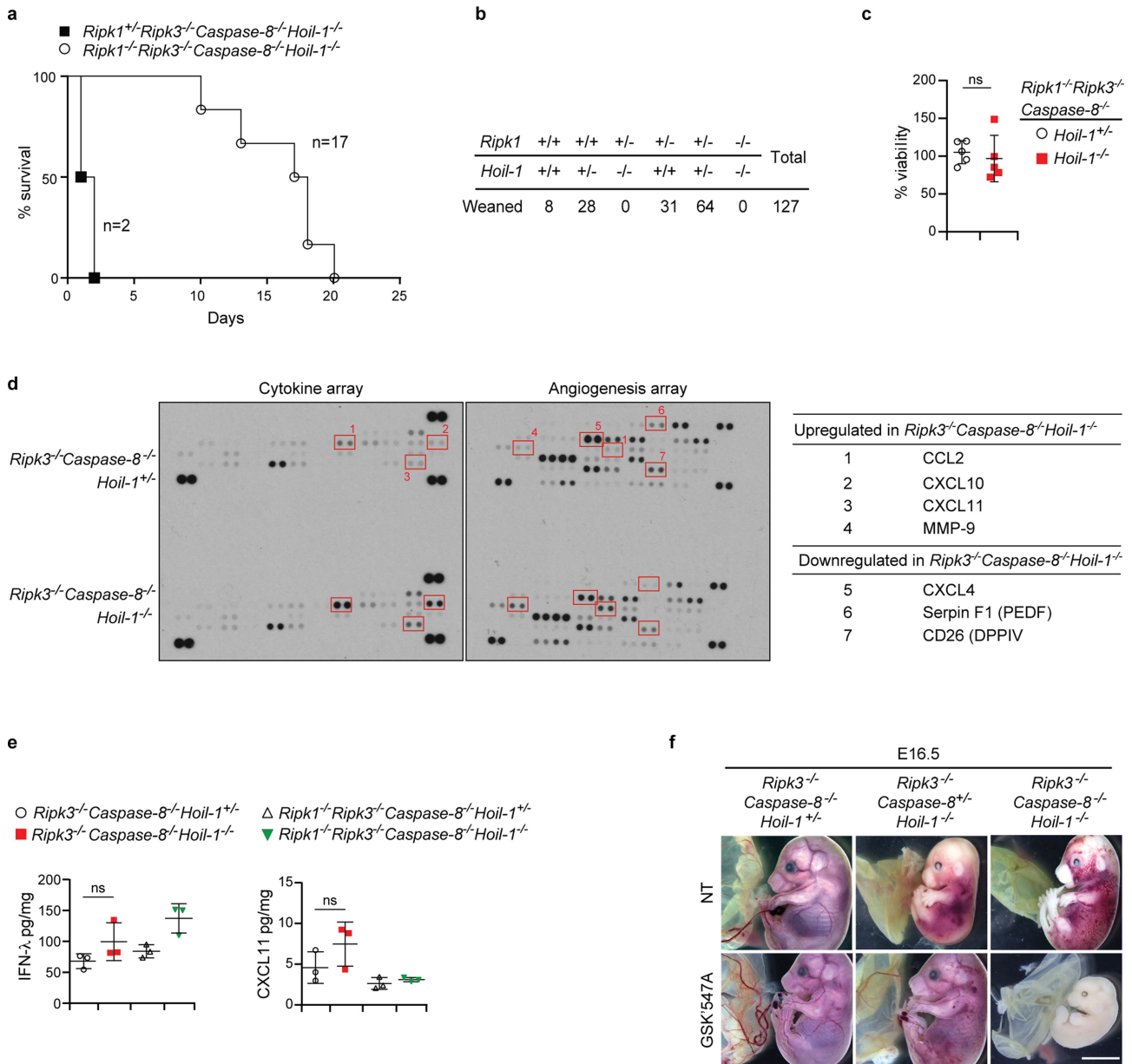


Extended Data Fig. 8 | See next page for caption.

**Extended Data Fig. 8 | Combined deletion of RIPK3 and caspase-8 causes haematopoietic defects and RIPK1-dependent embryonic lethality in HOIL-1-deficient mice.** **a**, Venn diagram depicting genes differentially expressed by RNA-seq analysis between E13.5 embryos of the indicated genotypes. **b**, Gene Ontology (GO) enrichment analysis of differentially expressed genes (85 low and 35 high in **a**). FDR, false discovery rate. **c**, Representative FACS profile of E13.5 fetal liver cells with different erythroblast populations gated according to their CD71 and TER119 expression levels (R1–R5) and quantification. R1 contains immature red blood cell progenitors, including primitive and later-stage erythroid progenitor cells (erythroid burst-forming unit (BFU-E) and colony-forming unit (CFU-E), respectively); R2 comprises mainly pro-erythroblasts and early basophilic erythroblasts; R3 contains both early and late basophilic erythroblasts; R4 is composed of chromatophilic and orthochromatophilic erythroblasts; and R5 consists of late orthochromatophilic erythroblasts and reticulocytes. Data are mean  $\pm$  s.e.m. ( $n = 14$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup>,  $n = 8$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>,  $n = 5$  for *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and  $n = 3$  for *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> fetal livers). *P* values from two-way ANOVA are reported. **d**, **h**, **k**, Representative FACS profile of E13.5 fetal liver cells for the indicated haematopoietic populations (sample size specified in **e–g**, **i**, **j**). **e**, **f**, **j**, Total cell number of the different haematopoietic cell subsets in fetal liver cell suspensions from E13.5 embryos of the indicated genotypes gated as in **d**, **h** and **k**, respectively. Total number of multipotent progenitors (LSK and LK cells) (**e**), mature CD45<sup>+</sup> blood cells, including granulocytes (GR-1<sup>+</sup>) and macrophages (F4-80<sup>+</sup>) (**f**) and myeloid progenitors (common myeloid progenitor (CMP), granulocyte–monocyte progenitor (GMP) and megakaryocyte–erythrocyte progenitor (MEP)) (**j**). Data are mean  $\pm$  s.e.m. *P* values

from unpaired two-tailed *t*-tests are shown. **g**, **i**, Percentages of mature CD45<sup>+</sup> leucocytes, GR-1<sup>+</sup> and F4-80<sup>+</sup> cells (**g**) and CMP, GMP and MEP cells (**i**). Data are mean  $\pm$  s.e.m. *P* values from unpaired two-tailed *t*-tests are shown. **l**, Differentiation of E13.5 fetal liver (c-KIT<sup>+</sup>) progenitors into CFU-granulocytes and macrophages (GM), BFU-E and/or CFU-granulocyte, erythroid, macrophage, megakaryocyte (GEMM). Mean  $\pm$  s.e.m. ( $n = 2$  fetal livers). **m**, Micrographs of differentiated macrophages ( $n = 3$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>,  $n = 5$  *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 4$  *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> fetal livers) and percentage viability of macrophages from E13.5 fetal liver cell suspensions from embryos of the indicated genotypes in the presence or absence of the indicated inhibitors. Data are mean  $\pm$  s.e.m. ( $n = 3$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>,  $n = 5$  *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 4$  *Mkl1*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> fetal livers). *P* values from two-way ANOVA are shown. **o**, Microfocus computed tomography scan images of *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos showing maximum intensity projections, with windowing applied to highlight vasculature (high contrast). No anatomical defects that would explain destruction of red blood cells or poor distribution of blood to the peripheries were found ( $n = 3$  embryos). In the left image, yellow star denotes distal aorta, green star denotes umbilical vessels, and red star indicates descending thoracic aorta. In the right image, yellow star denotes carotid artery, red star denotes descending thoracic aorta, white star denotes ductus arteriosus, and blue star denotes ascending thoracic aorta. **p**, Representative FACS profile of a pool of three E11.5 dorsal aortas, containing the AGM region, per indicated genotype and quantification. This experiment was performed once with three embryos per genotype.



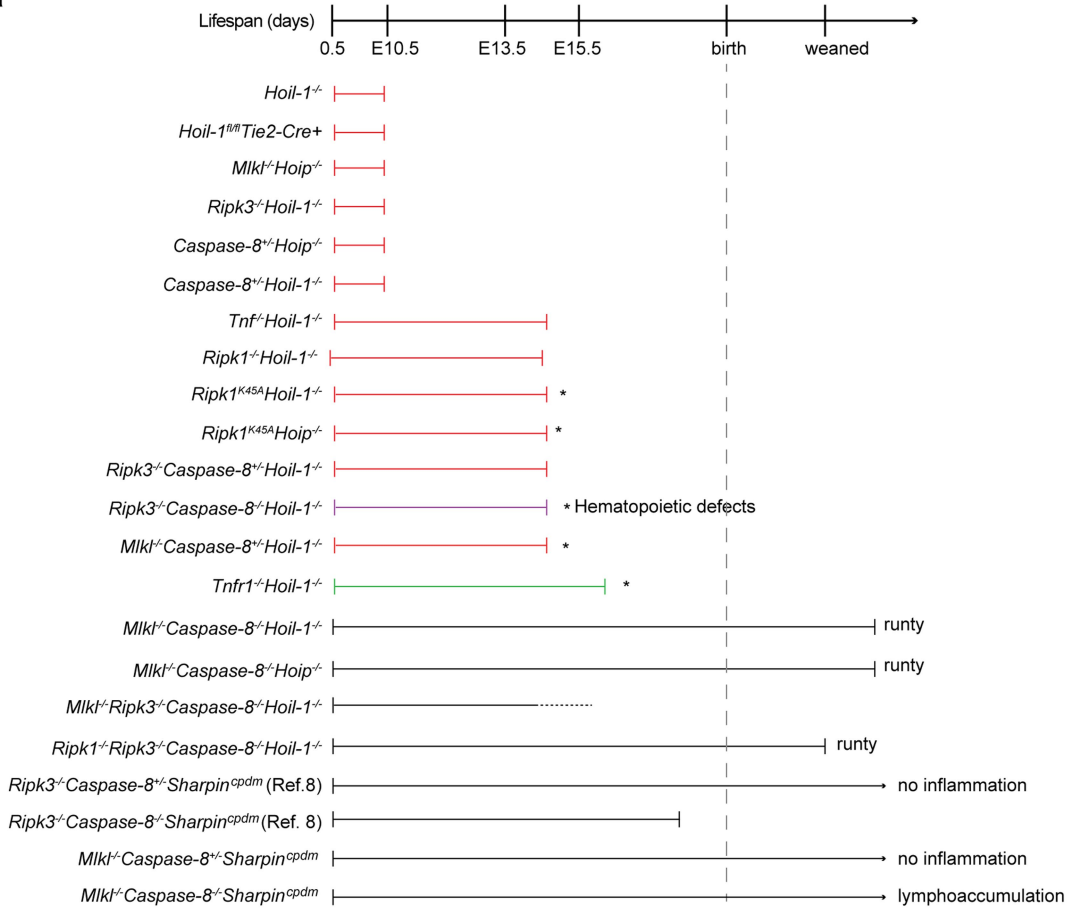


**Extended Data Fig. 9 | Concomitant deletion of RIPK1 prevents embryonic lethality of *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice.**

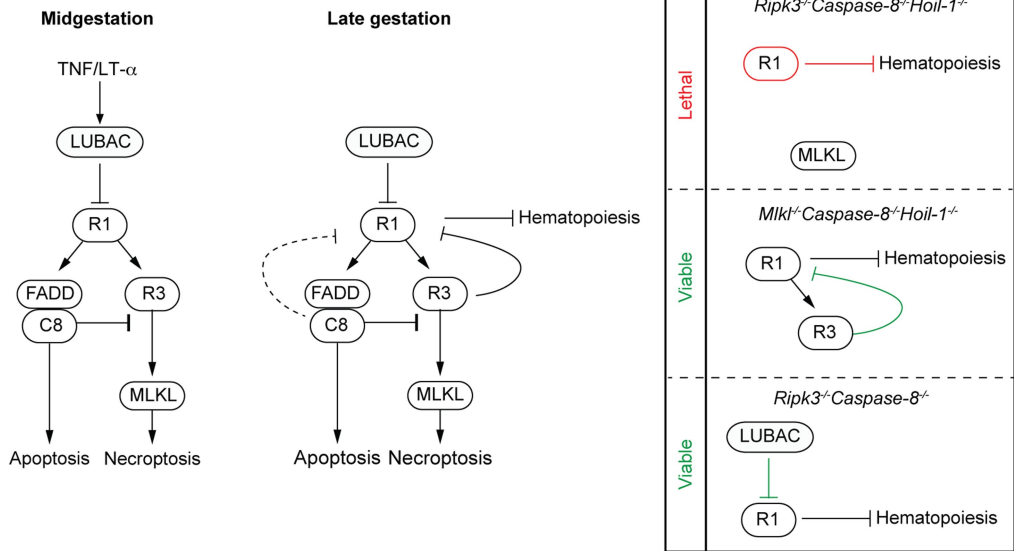
**a**, Kaplan–Meier plot of mouse survival ( $n = 17$  for *Ripk1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and  $n = 2$  for *Ripk1*<sup>+/-</sup>*Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice). **b**, Quantification of genotypes of animals obtained from intercrosses of *Ripk1*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> mice. For simplicity not all possible genotypes are represented. **c**, Percentage viability of macrophages from E13.5 fetal liver cell suspensions from embryos of the indicated genotypes. Data are mean  $\pm$  s.e.m. ( $n = 5$  fetal livers/genotype). Statistical significance was determined with unpaired two-tailed *t*-tests. **d**, Cytokine arrays from *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>

embryos (left) and table listing the altered cytokines (right). Red squares highlight the differences ( $n = 1$  for each genotype). For gel source data, see Supplementary Fig. 1. **e**, Cytokine analysis in homogenates from embryos of the indicated genotypes. Data are mean  $\pm$  s.e.m. ( $n = 3$  embryos per genotype). *P* values from one-way ANOVA are reported. **f**, Representative images of E16.5 embryos from control mothers or mothers fed with the RIPK1 kinase inhibitor GSK'457A from mating and throughout gestation (embryos treated with GSK'457A  $n = 5$  for *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and  $n = 7$  *Ripk3*<sup>-/-</sup>*Casp8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> and  $n = 3$  for *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup>). Scale bar, 5 mm.

a



b



Extended Data Fig. 10 | See next page for caption.

**Extended Data Fig. 10 | Schematic representation of findings in this study. a,** Diagram indicating extent of viability and phenotypes of single, double, triple and quadruple knockout mice. Red lines indicate cell death and loss of yolk sac vascularization phenotype. Green line indicates mild cell death phenotype without loss of yolk sac vascularization. Asterisk indicates that heart defects were observed. **b,** Proposed model of LUBAC function during embryogenesis. At mid-gestation (left), LUBAC maintains vascular tissue integrity by preventing aberrant TNF/LT- $\alpha$ -mediated caspase-8- and RIPK3/MLKL-induced cell death. At late gestation, LUBAC is required not only to prevent aberrant cell death but also to prevent severe defects in haematopoiesis that are driven by RIPK1 but can be prevented by RIPK3 (middle). Genetic ablation of

LUBAC and of different components of the cell death machinery indicates that (right): (1) in the absence of LUBAC, caspase-8 and RIPK3, RIPK1 provokes lethality, probably by depleting multipotent progenitors in the haematopoietic compartment; (2) in the absence of caspase-8 and MLKL, cell death induced by loss of LUBAC is prevented and RIPK3 is present to exert its protective role on fetal haematopoiesis by precluding aberrant RIPK1 signalling; and (3) in the absence of caspase-8 and RIPK3, the presence of LUBAC is sufficient to prevent RIPK1 from causing severe defects in haematopoiesis and lethality since *Ripk3*<sup>-/-</sup>*Casp8*<sup>-/-</sup> mice are viable<sup>14,15,24</sup>. This indicates that RIPK3 and LUBAC can compensate for each other to block aberrant RIPK1 signalling.



## Life Sciences Reporting Summary

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### ► Experimental design

#### 1. Sample size

Describe how sample size was determined.

Sample size was determined empirically and was based on our previous work using HOIP-deficient. We aimed for a number of at least 3 animals per group to allow basic statistical analysis while using a justifiable number of mutant mice/embryos.

#### 2. Data exclusions

Describe any data exclusions.

Exceptional embryos (either wildtype or knockout) found dead in utero were excluded from the analyses

#### 3. Replication

Describe whether the experimental findings were reliably reproduced.

Preliminary data showed that the phenotype observed in the study was consistent and that every single mouse bearing a mutation carried the exact same phenotype. Thus, a sample size of n=3 was sufficient to reach statistical significance. More mice were however included and reliably reproduced

#### 4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

animal experiments were randomised since the genotypes of the mice were unknown at the time of experimentation. Genotyping was performed at the end of each experiment except when a pool of embryos was used for AGM FACS analysis.

#### 5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

In all experiments with the exceptions mentioned below the genotypes of the mice were unknown at the time of experimentation and therefore the analyses were blinded. Quantifications in figure 4, TUNEL and branching points was performed by a scientist who was blinded to the identity of the samples. RNA seq and AGM FACS analysis as well as ELISAs, Western blotting and in vitro experiments were not blinded.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

## 6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a Confirmed

- The exact sample size ( $n$ ) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g.  $P$  values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

## ► Software

Policy information about [availability of computer code](#)

### 7. Software

Describe the software used to analyze the data in this study.

Quantifications were performed with Excell, ImageJ and GraphPad and statistical analysis with GraphPad. For FACS analysis FlowJo 7.6.1 software (Treestar) was used.

For RNA seq analysis samples were sequenced on the NextSeq 500 instrument (Illumina, San Diego, US) using a 43bp paired end run. Run data were de-multiplexed and converted to fastq files using Illumina's bcl2fastq Conversion Software v2.18 on BaseSpace. Fastq files were then aligned to a reference genome using STAR on the BaseSpace RNA-Seq alignment app v1.1.0. Reads per transcript were counted using HTSeq and differential expression was estimated using the BioConductor package DESeq2 (BaseSpace app v1.0.0). Functional analysis was performed using Venny 2.1 and STRING (string-db.org) softwares

Microfocus CT scans were reconstructed using CTPro3D; Nikon Metrology and analysed with VG Studio MAX (Volume Graphics GmbH, Heidelberg, Germany). Images and figures were processed with Adobe Photoshop and Illustrator CS6, respectively.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

## ► Materials and reagents

Policy information about [availability of materials](#)

### 8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Mice generated in this study as well as unique reagents are available upon request. GSK'547A is property of GSK, RIPK3 KO mice are from Genentech, Caspase-8 KO mice are from Razq Hakem and RIPK1 KO from Philip Leder.

## 9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The following antibodies were used for immunofluorescence staining: PECAM-1 (BD Biosciences, 5533370 Clone MEC13.3, dilution 1/250 lot n 2251541, validation <http://www.bdbiosciences.com/eu/applications/research/stem-cell-research/cancer-research/mouse/purified-rat-anti-mouse-cd31-mec-133/p/550274>), cleaved caspase-3 (Cell Signaling, 9661, dilution 1/250, lot ns 43 and 12, validated by immunohistochemical analysis of paraffin-embedded mouse embryo, using Cleaved Caspase-3 (Asp175) Antibody preincubated with control peptide or Cleaved Caspase-3 (Asp175) Blocking Peptide #1050 in website). Alexa Fluor 594 Goat anti-Rat IgG (Invitrogen, A-11007, lot n 1241432), Alexa Fluor 488 Goat anti-Rabbit IgG (Invitrogen, A-11034, lot n 1408830). All secondary antibodies were used at a 1/500 dilution. The following antibodies were used for Western blot analysis: HOIP (custom-made, Thermo Fisher Scientific, validated using HOIP deficient cells in Peltzer et al. 2014 Cell Reports), SHARPIN (ProteinTech, 14626-1-AP, lot n 00005729, validated using cpdm mice in Figure 2), HOIL-1 (home-made, validated using HOIL-1 deficient MEFs in Figure 2), TNFR1 (Abcam, ab19139, lot n GR3187980-1), Actin (Sigma, A1978, lot n 026M4780V), plkB $\alpha$  (Cell Signaling, 9246, lot 19, validated in MEFs stimulated with TNF in this work), I $\kappa$ B $\alpha$  (Cell Signaling, 9242, lot n 10, validated in MEFs stimulated with TNF in this work), cleaved caspase-8 (Cell Signaling, 9429, validated in Caspase-8 deficient embryo homogenates in Extended Data Fig. 7h), linear ubiquitin (Merck Millipore, MABS199, clone 1E3, lot n 2980771, validated using recombinant linear polyubiquitin chains in website), RIPK1 (BD, 610459, clone 38, lot n 14414, validated in human endothelial cell lysates in website), RIPK3 (Enzo, ADI-905-242-100, lot n 06041509, validated in mouse 3T3 whole cell lysate in the absence or presence of blocking peptide with RIP3 antibody in website), FADD (Assay Design, AAM-121, lot n 04201742), MLKL (Millipore, MABC604, clone 3H1, validated using MLKL KO mice in Extended Data Fig. 5a), phospho-MLKL (Abcam, ab196436, lot n GR246882-13, validated in L929 cells treated with TNF and ZVAD Extended Data Fig. 7h), Tubulin (Sigma, T9026) and FADD (Santa Cruz, sc-5559, clone H181). All antibodies were used at a 1/1000 dilution. HRP coupled antibodies for western blotting were the following, Goat anti-IgG1 (catalogue n., 1070-05, lot n G1013-5075E), Goat anti-Rabbit (catalogue n., 4050-05, lot n., I15144-T365L), Goat anti-Rat (catalogue n., 3050-05, lot n., G8212-PK146), Goat anti-Mouse (catalogue n., 1031-05, lot n., FO415-NB76G). All antibodies were purchased and validated by Southern Biotech and used at a 1/10.000 dilution.

The following antibodies were used for Flow Cytometry analysis: CD16/32, clone 93 and 2.4G2 (eBioscience, 45-0161-82, lot n 11-0161-82 and BD553141), CD135, clone A2F10.1 (BD, 553842, lot n 7272953/2342831), Ly-6A/E, clone D7 (Sca-1) (BD, 558162, lot n 38503), CD117 (c-Kit), clone 2B8 (BD, 560185, lot n B249344), CD34, clone RAM34 (BD, 562608, lot n 7187754), mouse Lineage Cocktail, clones 17A2/RB6-8C5/RA3-6B2/Ter-119/M1/70 (Biolegend, 133313, lot n B227259 and BD, 561301), CD16/32, clone 2.4G2 (BioXcell, CUS-HB-197), CD11b, clone M1/70 (Biolegend, 101228, lot n B223927 and eBioscience, 15-0112-81), CD11c, clone HL3 (BD, 561241), F4/80, clone BM8 (Biolegend, 123110), GR-1, clone RB6-8C5 (Biolegend, 108416, lot n B188267 and 108410), CD45, clone 30-F11 (Biolegend, 103128, lot n B241345 and Biolegend, 103112), CD3 $\epsilon$ , clone, 145-2C11 (Biolegend, 100310), B220, clone RA3-6B2, (Biolegend, 103210), CD71, clone RI7217 (Biolegend, 113807, lot n B213759) and TER-119, clone TER-119 (Biolegend, 116234, lot n B244218). Antibodies were used at a 1/100 dilution except for the lineage cocktail which was used at a 8/100 dilution. Validation for antibodies can be found in manufacturer's website.

## 10. Eukaryotic cell lines

- State the source of each eukaryotic cell line used.
- Describe the method of cell line authentication used.
- Report whether the cell lines were tested for mycoplasma contamination.
- If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

No eukaryotic cell lines were used

No eukaryotic cell lines were used

No eukaryotic cell lines were used

No eukaryotic cell lines were used



## ► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

### 11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Males and females from 6 weeks up to 24 weeks were used for time matings. Embryos were analysed at every embryonic days from E10.5-E16.5, regardless of gender. MEFs were obtained from E13.5 embryos. Embryos older than E14.5 were humanely culled by cervical dislocation. Adult MLKL/Caspase-8/HOIL-1- or HOIP-deficient or RIPK1/RIPK3/Caspase-8/HOIL-1-deficient mice were analysed around 20-40 days depending on the health status. MLKL/Caspase-8/cpdm mice were analysed at around 60 days. The genetic background of mice used in this study is C57BL6. All animal experiments were conducted under an appropriate UK project license in accordance with the regulations of UK home office for animal welfare according to ASPA (animal (scientific procedure) Act 1986). A summary of all strains used in this study is detailed in Extended Data Fig.10a.

Policy information about [studies involving human research participants](#)

### 12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.

## Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

### ► Data presentation

For all flow cytometry data, confirm that:

- 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- 3. All plots are contour plots with outliers or pseudocolor plots.
- 4. A numerical value for number of cells or percentage (with statistics) is provided.

### ► Methodological details

- |  |   |
|--|---|
| 5. Describe the sample preparation.  | Single-cell suspensions from mechanically dissociated E13.5 foetal livers or a pool of E11.5 aortas (AGM region) from 3 embryos in PBS supplemented with 10% FCS, were stained for 30 min on ice with antibodies described.   |
| 6. Identify the instrument used for data collection.                                   | LSR Fortessa (BD Biosciences) or FACSAria FUSION cell sorter (BD Biosciences).  |
| 7. Describe the software used to collect and analyze the flow cytometry data.          | FlowJo 7.6.1 software (Treestar)  |
| 8. Describe the abundance of the relevant cell populations within post-sort fractions. | Sorted samples were confirmed for purity post-sort via flow cytometry. Sorted populations were of ~98% purity.  |
| 9. Describe the gating strategy used.  | Fluorescence minus one (FMO) were used as gating controls. Compensation was performed with compensation beads (eBioscience). For quantification of absolute number of cells, a defined number of flow cytometric reference beads (Invitrogen) were mixed to the samples before acquisition.<br>All cells were first gated in FSC/SSC according to cell size and granularity. This population was then gated according to their viability using cell viability dye (eBioscience) (negative population). Subsequently cells were gated according to their lineage positivity or negativity to finally arrive gate according to the specific surface markers as shown in the extended figures for the desired cell population. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.