

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

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23 The Linear Ubiquitin chain Assembly Complex (LUBAC) is required for optimal gene activation  
24 and prevention of cell death upon activation of immune receptors, including TNFR1<sup>1</sup>. Deficiency  
25 in the LUBAC components SHARPIN or HOIP in mice results in severe inflammation in  
26 adulthood or embryonic lethality, respectively, due to deregulation of TNFR1-mediated cell  
27 death<sup>2-8</sup>. In humans, deficiency in the third LUBAC component, HOIL-1, causes autoimmunity  
28 and inflammatory disease, similar to HOIP deficiency, whereas HOIL-1 deficiency in mice was  
29 reported to cause no overt phenotype<sup>9-11</sup>. By creating HOIL-1-deficient mice, we here show that  
30 HOIL-1 is, however, as essential for LUBAC function as HOIP, albeit for different reasons:  
31 whereas HOIP is LUBAC's catalytically active component, HOIL-1 is required for LUBAC  
32 assembly, stability and optimal retention in the TNFR1-signalling complex (TNFR1-SC), thereby  
33 preventing aberrant cell death. Both, HOIL-1 and HOIP prevent embryonic lethality at mid-  
34 gestation by interfering with aberrant TNFR1-mediated endothelial cell death, which only  
35 partially depends on RIPK1 kinase activity. Co-deletion of Caspase-8 with RIPK3 or MLKL  
36 prevents cell death in *Hoil-1*<sup>-/-</sup> embryos, yet only combined loss of Caspase-8 with MLKL results  
37 in viable HOIL-1-deficient mice. Interestingly, *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos die at late-  
38 gestation due to haematopoietic defects that are rescued by co-deletion of RIPK1 but not MLKL.  
39 Collectively, these results demonstrate that both, HOIP and HOIL-1 are essential LUBAC  
40 components and are required for embryogenesis by preventing aberrant cell death. Furthermore,  
41 they unveil that, when LUBAC and Caspase-8 are absent, RIPK3 prevents RIPK1 from inducing  
42 embryonic lethality by causing defects in foetal haematopoiesis.

43 To determine the physiological role of HOIL-1, we generated HOIL-1-deficient mice by targeting exons  
44 1 and 2 of the *Hoil-1* (*Rbck1*) gene (**Extended Data Fig. 1a-d**). No mice with homozygous deletion in  
45 the *Hoil-1* gene were weaned (**Fig. 1a**). Analysis of *Hoil-1*<sup>-/-</sup> embryos revealed that they died around  
46 embryonic day (E) 10.5 (**Fig. 1a, b**). This result was confirmed with a strain generated from an  
47 independently targeted ES cell (C20*Hoil-1*<sup>-/-</sup> mice) (**Extended Data Fig. 1e, f**). At E10.5, *Hoil-1*<sup>-/-</sup>  
48 embryos presented with disrupted vascular architecture and cell death in the yolk sac endothelium (**Fig.**  
49 **1c, d** and **Extended Data Fig. 1g, h**), indicating that HOIL-1 absence causes aberrant endothelial cell  
50 death. *Hoil-1*<sup>fl/fl</sup>*Tie2-Cre*<sup>+</sup> (endothelium/some haematopoietic cell-specific cre) embryos also died  
51 around E10.5 with the same abnormalities (**Fig. 1e** and **Extended Data Fig. 1i, j**). Loss of TNF or  
52 TNFR1 diminished cell death in the yolk sac and prevented lethality at E10.5 in *Hoil-1*<sup>-/-</sup> embryos (**Fig.**  
53 **1f** and **Extended Data Fig. 2a-d**). As in *Tnfr1*<sup>-/-</sup>*Hoip*<sup>-/-8</sup>, *Tnfr1*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> yolk sacs showed reduced cell  
54 death as compared to *Hoil-1*<sup>-/-</sup> embryos (**Fig. 1f, g**). Although cell death was not completely ablated in  
55 *Tnfr1*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos, it did not appear to significantly affect yolk sac vasculature (**Fig. 1f, g** and  
56 **Extended Data Fig. 2e**). Nevertheless, *Tnfr1*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos died around E16.5 (**Extended Data**  
57 **Fig. 2d, f**) with heart defects prior to death (**Fig. 1h**). Therefore, like HOIP, HOIL-1 is required to  
58 maintain blood vessel integrity by preventing TNFR1-mediated endothelial cell death during  
59 embryogenesis.

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61 To understand the role of HOIL-1 in LUBAC function, we compared TNFR1-SC formation in MEFs  
62 individually deficient for the LUBAC components. Whereas in SHARPIN-deficient MEFs TNFR1-SC-  
63 associated linear ubiquitination was merely reduced<sup>7</sup>, it was completely absent in *Tnf*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> MEFs,  
64 exactly as in *Tnf*<sup>-/-</sup>*Hoip*<sup>-/-</sup> MEFs<sup>8</sup> (**Fig. 2a**). In TNF-stimulated *Tnf*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> MEFs, NF-κB activation  
65 was attenuated (**Extended Data Fig. 3a**) and TNFR1 complex-II formation was enhanced (**Fig. 2b**),  
66 resulting in sensitisation to TNF-induced apoptosis and necroptosis (**Fig. 2c**). Hence, HOIL-1 is as  
67 essential as HOIP for linear ubiquitination within the TNFR1-SC.

68

69 To determine whether the reduction in HOIP and SHARPIN protein levels in HOIL-1-deficient cells  
70 was responsible for the observed loss of linear ubiquitination (**Fig. 2a**), we reconstituted HOIL-1-  
71 deficient MEFs with HOIP, with HOIP and SHARPIN, or, as a control, with HOIL-1. Reconstitution  
72 with HOIP, either alone or with SHARPIN, failed to restore LUBAC recruitment, linear ubiquitination  
73 at the TNFR1-SC, or optimal NF-κB activation. Furthermore, their reconstitution was unable to prevent  
74 TNF-induced complex-II formation and cell death (**Fig. 2d-f** and **Extended Data Fig. 3b**) whilst HOIL-  
75 1 re-expression corrected all aforementioned defects (**Fig. 2d-f** and **Extended Data Fig. 3b**). In the  
76 absence of HOIL-1, HOIP was unable to bind to SHARPIN despite both being reconstituted to near  
77 endogenous levels (**Extended Data Fig. 3c**). Thus, HOIL-1 is required for LUBAC assembly and  
78 recruitment to the TNFR1-SC, identifying it as an essential component of LUBAC alongside HOIP.

79

80 To reveal how HOIL-1 enables LUBAC activity, we generated HOIL-1-deficient MEFs stably  
81 expressing full-length HOIL-1 (WT), the UBL domain of HOIL-1 only (HOIL-1-UBL), HOIL-1-  
82  $\Delta$ RBR, HOIL-1- $\Delta$ UBL, HOIL-1 with inactivating mutations T201A/R208A in the NZF domain (HOIL-  
83 1-NZFmut) or HOIL-1 with a point mutation in the catalytic cysteine of the RBR domain (HOIL-1-  
84 C458A) (**Fig. 2g**). Except for HOIL-1- $\Delta$ UBL, all mutant HOIL-1 proteins bound to HOIP and  
85 SHARPIN and stabilised their levels (**Fig. 2h**). Isolation of the native TNFR1-SC revealed that HOIL-  
86 1- $\Delta$ RBR and HOIL-1-C458A fully restored TNF-induced linear ubiquitination in HOIL-1-deficient  
87 cells, whereas HOIL-1- $\Delta$ UBL did not (**Fig. 2i**). HOIL-1-deficient cells expressing HOIL-1-UBL or  
88 HOIL-1-NZFmut only showed partial restoration of linear ubiquitination, correlating with reduced  
89 HOIP and SHARPIN levels at the TNFR1-SC (**Fig. 2i**). Thus, the UBL domain of HOIL-1 is essential  
90 for linear ubiquitination at the TNFR1-SC, whereas a functional NZF domain is required for optimal  
91 LUBAC presence in the TNFR1-SC. Expression of HOIL-1- $\Delta$ RBR restored optimal NF- $\kappa$ B signalling  
92 and prevented aberrant TNF-induced cell killing in contrast to HOIL-1- $\Delta$ UBL (**Fig. 2j** and **Extended**  
93 **Data Fig. 3d**). This observation elucidates why the previously reported mice, regarded as deficient for  
94 HOIL-1, are viable as they were generated by targeting exons 7 and 8<sup>12</sup>, likely resembling the HOIL-1-  
95  $\Delta$ RBR mutant studied here. Since the UBL of HOIL-1 binds to HOIP, allowing its activation<sup>13</sup> and the  
96 NZF of HOIL-1 binds linear ubiquitin linkages<sup>14</sup>, our results provide evidence that HOIL-1 promotes  
97 HOIP activation as well as LUBAC assembly and recruitment to the TNFR1-SC via its UBL domain.  
98 Once linear ubiquitin chains are formed in the complex, the NZF domain of HOIL-1 promotes LUBAC  
99 retention by binding to these chains.

100

101 Since both HOIL-1 and HOIP are equally important for LUBAC function and, consequently, for  
102 preventing aberrant cell death *in vitro* and *in vivo*, we used a genetic strategy to untangle the interplay  
103 between HOIL-1 or HOIP and the different cell death components. Inactivation of RIPK1 in *Hoil-1*<sup>-/-</sup>  
104 and *Hoip*<sup>-/-</sup> embryos delayed lethality until E14.5 (**Fig. 3a** and **Extended Data Fig. 4a-d**). At this time,  
105 *Ripk1*<sup>K45A</sup>*Hoil-1*<sup>-/-</sup> and *Ripk1*<sup>K45A</sup>*Hoip*<sup>-/-</sup> embryos had disrupted vascular architecture, excessive cell  
106 death in their yolk sacs, hearts, livers and lungs and presented with heart defects and liver necrosis (**Fig.**  
107 **3b** and **Extended Data Fig. 4e-h**). In accordance, TNFR1 complex-II formation and aberrant TNF/LT-  
108  $\alpha$ -induced apoptosis were only partially inhibited in *Ripk1*<sup>K45A</sup>*Hoil-1*<sup>-/-</sup> MEFs (**Fig. 3c, d** and **Extended**  
109 **Data Fig. 4i**). Thus, whilst the kinase activity of RIPK1 is essential for excessive TNFR1-induced cell  
110 death caused by attenuated LUBAC activity, as previously observed in SHARPIN-deficient mice<sup>4</sup>, this  
111 is not the case when LUBAC activity is completely abrogated.

112

113 We next tested whether loss of RIPK3, MLKL or Caspase-8 could prevent lethality in *Hoip*<sup>-/-</sup> and *Hoil-1*<sup>-/-</sup>  
114 embryos. At E10.5, *Ripk3*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos presented with defects in vascularisation, excessive  
115 cell death and died at mid-gestation (**Extended Data Fig. 5b, c**). Due to the close chromosomal linkage  
116 of HOIP and RIPK3, we generated *Mkl1*<sup>-/-</sup>*Hoip*<sup>-/-</sup> mice (**Extended Data Fig. 5a**). These embryos also

117 died at mid-gestation (**Extended Data Fig. 5d**). Likewise, neither Caspase-8 heterozygosity nor  
118 Caspase-8 full deletion was sufficient to prevent the mid-gestation lethality of *Hoip*<sup>-/-</sup> and *Hoil-1*<sup>-/-</sup>  
119 embryos (**Extended Data Fig. 5e, f** and data not shown).

120

121 As RIPK3-mediated necroptosis may be responsible for the embryonic lethality of *Caspase-8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup>  
122 <sup>-/-</sup> or *Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice<sup>15,16</sup>, we generated *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>  
123 *Hoil-1*<sup>-/-</sup> embryos and in both cases the lethality was delayed until around E14.5 (**Fig. 3e** and **Extended**  
124 **Data Fig. 6a, b**). At this developmental stage, a single intact copy of Caspase-8 was sufficient to induce  
125 apoptosis-driven loss of yolk sac vascularisation (**Fig. 3f** and **Extended Data Fig. 6c, d**). Yet, although  
126 *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos died around E14.5, yolk sac vascularisation was normalised and  
127 cell death in the yolk sac and other organs was prevented (**Fig. 3f** and **Extended Data Fig. 6c-f**).  
128 Moreover, *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> MEFs were resistant to cell death induced by TNF or related  
129 cytokines (**Extended Data Fig. 6g**). Histological examination and microfocus CT scanning revealed  
130 the presence of heart defects in both *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup>  
131 embryos (**Extended Data Fig. 6h, i**). We therefore conclude that whereas mid-gestation lethality in  
132 *Hoil-1*<sup>-/-</sup> embryos is dependent on Caspase-8/RIPK3-dependent apoptosis and necroptosis, *Ripk3*<sup>-/-</sup>  
133 *Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos die at late gestation by a process that is independent of cell death.

134

135 In striking contrast to *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice, both *Mkl1*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *Mkl1*<sup>-/-</sup>  
136 *Caspase-8*<sup>-/-</sup>*Hoip*<sup>-/-</sup> mice were born, albeit at lower than expected Mendelian ratios (**Fig. 3g** and  
137 **Extended Data Fig. 7a**). These mice were runted and had to be sacrificed by 4-5 weeks of age.  
138 Histopathological analysis revealed severe inflammation in the liver and lungs (**Fig. 3h, Extended Data**  
139 **Fig. 7b-d** and data not shown). Of note, *Caspase-8* heterozygosity resulted in increased apoptosis of  
140 endothelial cells, causing lethality in both *Mkl1*<sup>-/-</sup>*Caspase-8*<sup>+/-</sup>*Hoip*<sup>-/-</sup> and *Mkl1*<sup>-/-</sup>*Caspase-8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup>  
141 embryos around E14.5 (**Extended Data Fig. 7e** and data not shown) indicating that Caspase-8-driven  
142 apoptosis is sufficient to cause death of LUBAC-deficient embryos.

143

144 Co-deletion of RIPK3 and Caspase-8 causes embryonic lethality in otherwise viable *cpdm* mice<sup>7</sup>.  
145 However, *Mkl1*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*cpdm* mice were viable and the inflammatory syndrome that characterises  
146 *cpdm* mice was prevented (**Fig. 3i** and **Extended Data Fig. 7f, g**), whilst expectedly<sup>17</sup> developing  
147 lymphadenopathy and splenomegaly (**Fig. 3i** and **Extended Data Fig. 7f**). Thus, combined loss of any  
148 of the three LUBAC components together with loss of Caspase-8 uncovers a vital functional difference  
149 between RIPK3 and MLKL.

150

151 We next evaluated whether the lethality of *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice is due to aberrant (RIPK3-  
152 independent) MLKL activation. This was particularly pertinent because MLKL levels were increased  
153 in *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos and MLKL was aberrantly activated in some of them

154 (Extended Data Fig. 7h). However, MLKL co-deficiency did not prevent the death of *Ripk3<sup>-/-</sup>Caspase-*  
155 *8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (Fig. 3j and Extended Data Fig. 7h). Thus, RIPK3 is required for survival of  
156 embryos in the absence of LUBAC by regulating an MLKL-independent process.

157

158 To explore the nature of the pro-survival role of RIPK3, we performed RNAseq on E13.5 *Ripk3<sup>-/-</sup>*  
159 *Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* and *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos and controls (Extended Data Fig. 8a and  
160 Supplementary Table 1). Gene Ontology (GO) enrichment analysis of differentially expressed genes  
161 pointed towards defects in erythropoiesis in *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (Extended Data Fig.  
162 8b). Indeed, reduced levels of erythroid lineage Ter119<sup>+</sup> cells (Fig. 4a), basophilic erythroblasts  
163 (Extended Data Fig. 8c) and mature erythrocytes (Fig. 4b) were observed in of *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
164 *Hoil-1<sup>-/-</sup>* foetal livers. Furthermore, *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* haematopoietic progenitors failed to  
165 differentiate into committed erythroid burst-forming units in culture (Fig. 4e). Further analysis of the  
166 haematopoietic compartment from E13.5 foetal livers revealed abnormally reduced percentages and  
167 total numbers of multipotent progenitors (Fig. 4d and Extended Data Fig. 8d, e) as well as leucocytes,  
168 including granulocytes and macrophages, and myeloid progenitors in the *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*  
169 embryos compared to controls, whereas *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos had normal numbers of  
170 these cells (Extended Data Fig. 8f-k). In addition, the capacity of haematopoietic progenitors to  
171 generate colony-forming myeloid progenitors and multi-potent progenitors was also impaired in the  
172 *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (Extended Data Fig. 8l). Accordingly, the viability of  
173 macrophages obtained from *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* foetal liver cell suspensions in culture was  
174 significantly lower than those of controls and this could not be rescued by inhibiting necroptosis or  
175 apoptosis. *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* foetal liver cells, however, produced normal numbers of  
176 macrophages (Extended Data Fig. 4m). Despite the heart defects of *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*  
177 embryos, blood circulation was normal at E13.5 and the percentages of CD45<sup>+</sup>cKIT<sup>+</sup> cells obtained  
178 from aorta-gonad-mesonephros regions were comparable between *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos  
179 and controls at E11.5 (Extended Data Fig. 8o, p). We therefore conclude that *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-*  
180 *1<sup>-/-</sup>* embryos suffer from intrinsic defects in early haematopoiesis, likely downstream of specification in  
181 the aorta-gonad-mesonephros, resulting in substantial deficiencies in erythroid and myeloid cells.

182

183 Since LUBAC is known to regulate RIPK1<sup>24</sup>, we investigated the role of RIPK1 in the lethality of *Ripk3<sup>-/-</sup>*  
184 *Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos. Lethality of *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos was prevented by  
185 additional loss of RIPK1, despite RIPK1 levels being relatively low in *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*  
186 embryos and RIPK1 deficiency failing to prevent *Hoil-1<sup>-/-</sup>* embryonic lethality (Fig. 4d, e and Extended  
187 Data Fig. 7h and 9a, b). Importantly, the viability of macrophages obtained from *Ripk1<sup>-/-</sup>Ripk3<sup>-/-</sup>*  
188 *Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* foetal livers was comparable to controls (Extended Data Fig. 9c), indicating  
189 normalised haematopoiesis in these mice. The expression of several cytokines, including IL-1 $\beta$ , CCL2,  
190 IFN- $\beta$  and CXCL10, was abnormally increased in *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos but not in *Ripk1<sup>-/-</sup>*

191 *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos (**Fig. 4f and Extended Data Fig. 9d, e**). The function, survival,  
192 differentiation and self-renewal of haematopoietic progenitors are greatly impacted by various of these  
193 cytokines<sup>18,19</sup>. Therefore, our findings suggest that RIPK1-driven deregulated cytokine production in  
194 *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos may impair foetal haematopoiesis. Finally, treatment of pregnant  
195 females with the RIPK1 kinase inhibitor GSK'547A<sup>20</sup> did not prevent lethality of *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
196 *Hoil-1<sup>-/-</sup>* embryos, although it was able to extend the survival of *Ripk3<sup>-/-</sup>Caspase-8<sup>+/-</sup>Hoil-1<sup>-/-</sup>* embryos  
197 (**Extended Data Fig. 9f**). These results suggest that the lethality of *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos  
198 likely depends on the scaffolding function of RIPK1.

199

200 Although RIPK1 is required for emergency haematopoiesis, RIPK1 might regulate embryonic  
201 haematopoiesis differently. Indeed, RIPK1-constitutive or haematopoietic-cell-specific-deficient mice  
202 are not embryonically lethal<sup>21,22</sup>. In addition, absence of LUBAC, RIPK3 and Caspase-8 might affect  
203 mechanisms during embryogenesis that are different from those perturbed by RIPK1 deficiency alone.  
204 Collectively, our findings imply that in the combined absence of LUBAC and Caspase-8, RIPK3 exerts  
205 a pro-survival role by regulating RIPK1-mediated signalling (**Extended Data Fig. 10**). Since *Ripk3<sup>-/-</sup>*  
206 *Caspase-8<sup>-/-</sup>* mice are viable<sup>15,16,23</sup>, our findings indicate that control of RIPK1 by either LUBAC or  
207 RIPK3 is sufficient to enable proper haematopoiesis in the developing embryo, likely by preventing  
208 deregulated cytokine production. Thus, LUBAC and RIPK3 control RIPK1-mediated signalling to  
209 allow embryonic haematopoiesis.

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## 299 **Author Contributions**

300 H.W. conceived the project. N.P. and M.D. performed the majority of the experiments. N.P., M.D. and  
301 H.W. designed the research and co-wrote the manuscript. A.M., C.B. and T.E. conceived and  
302 contributed to the haematopoietic analyses. H.D. and P.D. contributed to *in vitro* experiments in Figure

303 2 and Extended Data Fig. 3, S.K. generated *Mlkl*<sup>-/-</sup> mice, L.T., E.R. contributed to *in vivo* experiments,  
304 T.H performed cytokine arrays and E.L. and Y.S. contributed with biochemistry data. P.B., T.L.H. and  
305 H.W. designed the *Hoil-1* floxed allele and P.B. generated it. A.F. and W.K. generated and analysed  
306 *Ripk1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice. H.D. and A.S. performed genotyping. A.B. and J.B. provided  
307 GSK'547A and *Ripk1*<sup>K45A</sup> mice. J.R., S.A.D., A.St. and J.S. performed the *cdpm* studies. C.H. and  
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314

### 315 Figure Legends

316

#### 317 **Figure 1: HOIL-1 deficiency causes embryonic lethality at mid-gestation due to TNFR1-mediated** 318 **endothelial cell death**

319 **a**, Mendelian frequencies obtained from inter-crossing *Hoil-1*<sup>+/-</sup> mice, \*: dead embryos. **b**,  
320 Representative images of embryos from E9.5 to E11.5 quantified in (a), \*: poor yolk sac vascularisation.  
321 Scale bar: 2 mm. **c**, Representative images of yolk sac vascularisation (PECAM-1, red) and cell death  
322 (cleaved (cl.) Caspase-3 staining, green) at E10.5 (top panel) (*n*=4 yolk-sacs/genotype), and whole-  
323 mount TUNEL staining (bottom, panel) (*n*=2 yolk-sacs/genotype). Scale bar: 50 μm. **d**, **g**,  
324 Quantification of branching points (g) and cleaved Caspase-3 positive cells (g). Mean ± s.e.m. values  
325 and *P* values from unpaired two-tailed *t*-tests are shown. **e**, Representative images of embryos at E10.5  
326 (*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 panel). \*: poor yolk sac  
327 vascularisation. Scale bar: 2 mm. Yolk sac vascularisation (PECAM-1, red) and apoptosis (cleaved  
328 Caspase-3, green) (middle panel). Scale bar: 50 μm. Yolk sac whole-mount TUNEL staining (*n*=6 *Hoil-*  
329 *1*<sup>fl/wt</sup>*Tie2-Cre*<sup>+</sup> and *n*=2 *Hoil-1*<sup>fl/fl</sup>*Tie2-Cre*<sup>+</sup> yolk-sacs/genotype, bottom panel). **f**, Representative  
330 images of embryos at E15.5 (top panel, *n*=6 *Tnfr1*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> and *n*=19 *Tnfr1*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> embryos), scale  
331 bar: 2 mm, and yolk sac vascularisation (PECAM-1, red) and apoptosis (cleaved Caspase-3, green)  
332 (bottom panel), Scale bar: 50 μm. **h**, Representative images of H&E staining on whole-embryo paraffin  
333 sections (*n*=3 embryos/genotype). \*: pericardial effusion, arrows; congested vessels. H, heart; L, lung;  
334 Li, liver. Scale bar: 50 μm.

335

336 **Figure 2: The UBL domain but not the RBR domain of HOIL-1 is essential for LUBAC activity**  
337 **at the TNFR1-SC and to prevent TNF/TNFR1-induced cell death.**

338 **a, d**, TNFR1-SC pull-down by FLAG- immunoprecipitation (IP) in MEFs derived from mice of the  
339 indicated genotypes  $\pm$  FLAG-TNF for 15 min ( $n=2$  independent experiments) (a) and reconstituted with  
340 HOIL-1, HOIP or HOIP and SHARPIN ( $n=4$  independent experiments) (d). **b, e**, FADD-IP performed  
341 in MEFs of the indicated genotypes treated for 4 h with the caspase inhibitor zVAD-fmk  $\pm$  TNF (b) and  
342 reconstituted as indicated (e) ( $n=2$  independent experiments (b,e)). **c, f, j**, Cell death analysed by  
343 propidium iodide (PI) staining in MEFs with the indicated genotypes  $\pm$  TNF  $\pm$  the indicated inhibitors  
344 for 24 h (c), reconstituted (f) or transduced (j) as indicated (f, j). Mean  $\pm$  s.e.m. ( $n=3$  independent  
345 experiments) and  $P$  values from two-way ANOVA are shown. **g**, Schematic overview of HOIL-1  
346 constructs used to transduce *Tnf<sup>-/-</sup>Hoil-1<sup>-/-</sup>* MEFs. **h**, Flag-IP of indicated HOIL-1 mutants ( $n=2$   
347 independent experiments). **i**, Endogenous TNFR1-SC pull-down by HA-IP in reconstituted *Tnf<sup>-/-</sup>Hoil-1<sup>-/-</sup>*  
348 MEFs  $\pm$  HA-TNF for 15 min ( $n=2$  independent experiments). TL: total lysate, NT: not treated, EV:  
349 empty vector. For gel source data (a,b,d,e,h,i), see Supplementary Figure 1.

350

351 **Figure 3: Concomitant loss of MLKL and Caspase-8, but not loss of RIPK1 kinase activity or**  
352 **combined loss of RIPK3 and Caspase-8, promotes survival of LUBAC-deficient mice**

353 **a**, Representative images of E10.5 ( $n=6$  embryos/genotype), scale bar: 2 mm, E14.5 ( $n=12$   
354 *Ripk1<sup>K45A</sup>Hoil-1<sup>+/-</sup>*,  $n=5$  *Ripk1<sup>K45A</sup>Hoil-1<sup>-/-</sup>* embryos/genotype) and E15.5 embryos ( $n=3$   
355 embryos/genotype). Scale bar: 5 mm. \*: poor yolk sac vascularisation. **b, f**, Representative images of  
356 yolk sac vascularisation (PECAM-1, red) and apoptosis (cleaved (cl.) Caspase-3, green) at E14.5 (b) or  
357 E13.5 (f) and quantification. Mean  $\pm$  s.e.m. and  $P$  values from unpaired two-tailed  $t$ -tests (b) or one-  
358 way ANOVA (f) are shown. Scale bar: 50  $\mu$ m. **c**, FADD-IP in MEFs treated for 3 h with zVAD-fmk  $\pm$   
359 TNF ( $n=2$  independent experiments). For gel source data, see Supplementary Figure 1. **d**, Cell death by  
360 PI incorporation in MEFs  $\pm$  TNF (10 ng/ml) or LT- $\alpha$ . Mean  $\pm$  s.e.m. ( $n=3$  independent experiments)  
361 and  $P$  values (\*\*\*\* $P<0.0001$ ) from two-way ANOVA are reported. NT: not treated. **e**, Representative  
362 images of E14.5 ( $n=11$  *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>*, *Ripk3<sup>-/-</sup>Caspase-8<sup>+/-</sup>Hoil-1<sup>-/-</sup>* and  $n=7$  *Ripk3<sup>-/-</sup>*  
363 *Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*) and E15.5 embryos ( $n=5$  *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>*,  $n=4$  *Ripk3<sup>-/-</sup>Caspase-8<sup>+/-</sup>*  
364 *Hoil-1<sup>-/-</sup>* and  $n=8$  *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*). \*: poor yolk sac vascularisation. Scale bar: 5 mm. **g, j**,  
365 Mendelian frequencies obtained from inter-crossing *Mkl1<sup>-/-</sup>Caspase-8<sup>+/-</sup>Hoil-1<sup>+/-</sup>* with *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
366 *Hoil-1<sup>+/-</sup>* mice (g) or *Mkl1<sup>+/-</sup>Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* with *Mkl1<sup>-/-</sup>Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* mice  
367 (top) or *Mkl1<sup>-/-</sup>Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* mice (bottom) (j). \*: dead embryos. **h, i**, Representative  
368 images of adult mice quantified in (g) for (h) or  $n=3$  mice/genotype in (i). m: *cpdm* mutation.

369

370 **Figure 4: Combined deletion of RIPK3 and Caspase-8 causes haematopoietic defects and RIPK1-**  
371 **dependent embryonic lethality in HOIL-1-deficient mice.**

372 **a, b**, Number (No.) of the TER119<sup>+</sup> (erythroid) cells (a) and enucleated erythrocytes/high-power field  
373 (HPF) (b) in E13.5 foetal livers with the indicated genotypes. Mean  $\pm$  s.e.m. and *P* values from unpaired  
374 two-tailed *t*-tests are shown. **c**, Differentiation of E13.5 foetal liver (c-KIT<sup>+</sup>) progenitors into burst  
375 forming units-erythrocyte (BFU-E). Mean  $\pm$  s.e.m. and *P* values from unpaired two-tailed *t*-tests are  
376 reported. **d**, Percentage of haematopoietic progenitors negative for mature lineage markers (Lin<sup>-</sup>) and  
377 SCA-1<sup>+</sup>c-KIT<sup>+</sup> (LSK) and SCA-1<sup>-</sup>c-KIT<sup>+</sup> (LK) in E13.5 foetal livers with the indicated genotypes.  
378 Mean  $\pm$  s.e.m. and *P* values from unpaired two-tailed *t*-tests are reported. **e**, Mendelian frequencies  
379 obtained from inter-crossing *Ripk1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> mice. **f**, Representative images of mice  
380 of the indicated genotypes quantified in (e). **g**, Cytokine levels in embryo homogenates with the  
381 indicated genotypes. Mean  $\pm$  s.e.m and *P* values from one-way ANOVA are reported.

## 382 MATERIALS AND METHODS

### 383 *Mice*

384 The *Hoil-1* floxed (*Hoil-1<sup>fl/fl</sup>*) mice were generated by a gene targeting strategy in ES cells in which the  
385 targeting cassette was composed of a hygromycin resistance cassette flanked by *Frt* sites and exons 1  
386 and 2 of the *Hoil-1* gene flanked by *loxP* sites. Southern blots of C57BL/6 ES cell clones containing  
387 the homologous recombination were analysed for the specificity of the recombination and the absence  
388 of any unwanted integration. Two ES cell clones were used to generate mutant animals on the C57BL/6  
389 genetic background, corresponding to the two independent *Hoil-1<sup>-/-</sup>* strains (*Hoil-1<sup>-/-</sup>* and C20*Hoil-1<sup>-/-</sup>*).  
390 The hygromycin cassette was removed by crossing these mice with C57BL/6 mice expressing the FlpE  
391 recombinase and this was followed by a cross with C57BL/6 mice to remove the flpe transgene. *Hoip<sup>-/-</sup>*  
392 and *Hoil-1<sup>-/-</sup>* mice were generated by crossing *Hoip<sup>fl/fl</sup>*, mice, previously described<sup>24</sup>, and *Hoil-1<sup>fl/fl</sup>*  
393 mice (described here) with transgenic mice expressing the loxP-deleter Cre recombinase (purchased  
394 from JAX: 6054, B6.C-Tg(CMV-Cre)1 Cgn/J). Transgenic mice expressing the Cre recombinase under  
395 the control of the Tie2 promoter (*Tie2-Cre*) (B6.Cg-Tg(Tek-cre)1Ywa/J)<sup>25</sup> were used to delete floxed  
396 genes specifically in endothelial cells. C57BL/6 *Mikl<sup>-/-</sup>* mice crossed to *cpdm* mice were previously  
397 described<sup>26</sup>. For all other crosses *Mikl<sup>-/-</sup>* mice were generated using Transcription activator-like effector  
398 nuclease (TALEN). In brief, TALENs targeting exon 1 of the *Mikl* gene were cloned via Golden-gate  
399 assembly. The RVD sequence of TAL1 against TACCGTTTCAGATGTCA was NI HD HD NN NG  
400 NG NG HD NI NN NI NG NN NG HD NI and TAL2 against TCGATCTTCCTGCTGCC was HD NN  
401 NI NG HD NG NG HD HD NG NN HD NG NN HD HD. Capped RNA was produced *in vitro* using  
402 mMESAGE mMACHINE® T7 Transcription Kit (Ambion) and poly A tail was added using Poly(A)  
403 Tailing Kit (Ambion). Purified transcripts were mixed and adjusted to 25 ng/μL. C57BL/6 fertilised  
404 eggs were injected into both the cytoplasm and the pro-nucleus. Embryos were transferred into  
405 C57BL/6 pseudo-pregnant females. Pups were genotyped by sequencing using genomic DNA obtained  
406 from ear punches. One female carrying a 19 bp homozygous deletion causing a premature stop codon  
407 was selected for further breeding. *Mikl<sup>-/-</sup>* mice were backcrossed to C57BL/6 mice for two generations.  
408 *Sharpin<sup>mm</sup>* (C57BL/Ka, *cpdm*) and *Tnfr1<sup>-/-</sup>* (2818, B6.129-Tnfrsf1atm1Mak/J) mice were purchased  
409 from JAX. *Tnf<sup>-/-</sup>* mice (C57BL/6;129S6) were provided by William Kaiser. *Ripk3<sup>-/-</sup>*<sup>27</sup>, *Caspase-8<sup>-/-</sup>*<sup>28</sup>,  
410 *Ripk1<sup>K45A</sup>*<sup>4</sup> and *Ripk1<sup>-/-</sup>*<sup>29</sup> mice have been reported previously. Timed matings were performed as  
411 previously described<sup>8</sup>. All mice were genotyped by PCR, fed *ad libitum*. All animal experiments were  
412 conducted under an appropriate UK project license in accordance with the regulations of UK home  
413 office for animal welfare according to ASPA (animal (scientific procedure) Act 1986). The relevant  
414 Animal Ethics Committee approved all experiments involving *cpdm* and the *Ripk1<sup>-/-</sup>* crosses which were  
415 maintained under appropriate licenses and subject to ethical review at The Walter and Eliza Hall  
416 Institute (Melbourne, Australia) and UT Health Sciences Center San Antonio (TX, USA), respectively.

417 *Histological analysis, TUNEL and immunofluorescence staining*

418 Embryos or organs from adult mice were collected and fixed in 10% buffered formalin and paraffin  
419 embedded. Sections of 4  $\mu\text{m}$  were stained with haematoxylin and eosin following standard procedures.  
420 Necropsy of adult mice or six sagittal serial sections of two different planes of the embryo were used  
421 for blinded pathological analysis. For TUNEL staining, sections were treated according to the  
422 manufacturer's instructions (DeadEnd™ Fluorometric TUNEL System, Promega, G3250). For whole  
423 mount TUNEL staining and immunofluorescence staining, samples were processed using the ApoTag  
424 plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, S7101) according to the manufacturer's  
425 instructions and as previously described<sup>8</sup>. Quantitation was performed by an experimenter blinded to  
426 the genotype of the mice by using ImageJ Software on monochrome images of the whole yolk sac by  
427 measuring the area of positive staining. Alternatively, TUNEL-positive cells were counted on five  
428 different fields (10x magnification). Yolk sacs were stained with antibodies against PECAM-1 (BD  
429 Biosciences, 5533370 Clone MEC13.3) and cleaved caspase-3 (Cell Signaling, 9664), followed by  
430 staining with secondary antibodies, Alexa Fluor 594 Goat anti-Rat IgG and Alexa Fluor 488 Goat anti-  
431 Rabbit IgG (Invitrogen, A-11007 and A-11034, respectively), and analysed by fluorescent microscopy.  
432 Quantification was performed by an experimenter blinded to the genotype of the mice on ten different  
433 fields (10x magnification) per yolk sac.

434 *Microfocus CT scan*

435 Embryos were fixed in 4% paraformaldehyde and potassium triiodide (Lugol's iodine/I<sub>2</sub>KI, to impart  
436 tissue contrast), with a total iodine content of 63.25 mg/mL (iodine mass of  $2.49 \times 10^{-4}$  mol/mL), in a  
437 1:1 ratio for 8 h before imaging. Prior to scanning, the embryos were washed, wrapped in Parafilm M  
438 (Bemis, Oshkosh, WI, USA) and secured in 3% w/v Agar (Sigma-Aldrich, UK) within a low-density  
439 plastic cylinder to ensure mechanical stability during scan acquisition. Images were acquired using an  
440 XT H 225 ST microfocus-CT scanner with a multimetal target (Nikon Metrology, Tring, UK). Scans  
441 were reconstructed using modified Feldkamp filtered back projection algorithms with proprietary  
442 software (CTPro3D; Nikon Metrology) and post-processed using VG Studio MAX (Volume Graphics  
443 GmbH, Heidelberg, Germany). Soft tissues were analysed by Phong shading of direct volume  
444 renderings and plain projections and the vascular system by maximum intensity projections.

445 *Cells*

446 MEFs were isolated from E12.5-E13.5 embryos in accordance with standard procedures and these cells  
447 were maintained in DMEM medium supplemented with 10% foetal bovine serum (Sigma).  
448 Transformation was performed by lentiviral infection with the SV40 large T antigen. For reconstitution  
449 experiments, the coding sequence of murine HOIP, SHARPIN or HOIL-1 wild-type (WT), the UBL  
450 domain of HOIL-1 only (HOIL-1-UBL; AA 1-139), HOIL-1- $\Delta$ RBR (AA 1-252), HOIL-1- $\Delta$ UBL (AA

451 140-508), HOIL-1 with inactivating mutations T201A/R208A in the NZF domain (HOIL-1-NZFmut)  
452 or HOIL-1 with a point mutation in the catalytic cysteine of the RBR domain (HOIL-1-C458A) was  
453 inserted in MSCV vector followed by the internal ribosome entry site (IRES)-GFP sequence. These  
454 vectors were retrovirally transduced into MEFs and GFP-positive cells were sorted in a MoFlo  
455 cytometer (Beckman Coulter).

#### 456 *Immunoprecipitation*

457 For isolation of the TNFR1-SC, transformed MEFs were stimulated with 3xFlag-2xStrep-TNF at 0.5  
458 µg/mL for 15 min, and controls were left untreated. Cells were subsequently solubilised in lysis buffer  
459 (30 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 2 mM KCl, 10% Glycerol, 1% Triton X-100,  
460 EDTA-free proteinase inhibitor cocktail (Roche, 5056489001) and 1x phosphatase-inhibitor cocktail 2  
461 (Sigma, P5726-1ML) at 4°C for 30 min. The lysates were cleared by centrifugation, and 3xFlag-  
462 2xStrep-TNF (0.5 µg/mL/sample) was added to the untreated samples. Subsequently, the lysates were  
463 subjected to anti-Flag immunoprecipitation using M2 antibody coupled sepharose beads (Sigma,  
464 A2220-5ML) for 16 h. For FADD immunoprecipitation, transformed MEFs were treated with 20 µM  
465 zVAD-fmk (Abcam, ab120487) in the presence or absence of 100 ng/mL 6xHis-TNF for 3 h. Cells  
466 were lysed as described above and FADD was immunoprecipitated using anti-FADD antibody (Santa  
467 Cruz, sc-5559) and protein G Sepharose Beads (GE healthcare, 17-0618-01) at 4°C for 4 h. For Sharpin  
468 immunoprecipitation, anti-Sharpin antibody (ProteinTech, 14626-1-AP) was used. For all  
469 immunoprecipitations, the beads were washed three times with lysis buffer. Proteins were eluted in 50  
470 µL of LDS buffer (NuPAGE, Invitrogen) containing 50 mM DTT. Samples were analysed by Western  
471 blotting.

#### 472 *Western blot analysis and antibodies*

473 Whole embryos were snap-frozen and homogenised in RIPA buffer (50 mM Tris pH 8.0, 150 mM  
474 NaCl, 0.5% sodium deoxycholate, 1% NP-40 and 1xEDTA-free proteinase inhibitor cocktail (Roche,  
475 5056489001) or RIPA buffer with 6M Urea for the experiment in Extended data Fig 7h. Alternatively,  
476 cells were washed twice with ice-cold PBS prior to lysis in lysis buffer. Protein concentration of lysates  
477 was determined using BCA protein assay (Thermo Scientific). Lysates were subsequently denatured in  
478 reducing sample buffer at 95°C for 10 min before separation by SDS-PAGE (NuPAGE) and subsequent  
479 analysis by Western blotting using antibodies against HOIL-1<sup>30</sup>, HOIP (custom-made, Thermo Fisher  
480 Scientific), SHARPIN (ProteinTech, 14626-1-AP), TNFR1 (Abcam, ab19139), Actin (Sigma, A1978),  
481 pIκBα (Cell Signaling, 9246), IκBα (Cell Signaling, 9242), cleaved caspase-8 (Cell Signaling, 9429),  
482 linear ubiquitin (Merck Millipore, MABS199), RIPK1 (BD, 610459), RIPK3 (Enzo, ADI-905-242-  
483 100), FADD (Assay Design, AAM-121), MLKL (Millipore, MABC604), phospho-MLKL (Abcam,  
484 ab196436) and Tubulin (Sigma, T9026).

485 *Cell death analysis by PI staining*

486 Cells were seeded to 80% confluence and were then incubated with 100 ng/mL His-tagged TNF, 1  
487 ug/mL CD95L-Fc, 1 µg/mL isoleucine zipper tagged murine TRAIL (iz-mTRAIL), 100 µg/mL  
488 Poly(I:C) HMW (InvivoGen, tlr1-pic), 20 ng/mL IFN-γ (Peprotech, 315-05) or 100 ng/mL LT-α  
489 (Thermo Fisher Scientific, 10270-HNAE) for 24 h, unless otherwise indicated. When indicated the  
490 following inhibitors were used: 20 µM Z-VAD-FMK (Abcam, ab120487), 10 µM Necrostatin-1s  
491 (Biovision, 2263-5). Supernatants and adherent cells were harvested and resuspended in PBS containing  
492 5 µg/mL propidium iodide (PI). PI-positive cells were enumerated by FACS (BD Accuri).

493 *RNA sequencing analysis*

494 E13.5 embryos were snap frozen and RNA was prepared using the RNeasy minikit (Qiagen, 74104)  
495 according to the manufacturer's instruction. To generate the library, samples were processed using the  
496 KAPA mRNA HyperPrep Kit (p/n KK8580) according to the manufacturer's instructions. Briefly,  
497 mRNA was isolated from total RNA using Oligo dT beads to pull down poly-adenylated transcripts.  
498 The purified mRNA was fragmented using chemical fragmentation (heat and divalent metal cation) and  
499 primed with random hexamers. Strand-specific first strand cDNA was generated using Reverse  
500 Transcriptase in the presence of Actinomycin D. The second cDNA strand was synthesised using dUTP  
501 in place of dTTP, to mark the second strand. The resultant cDNA was then "A-tailed" at the 3' end to  
502 prevent self-ligation and adapter dimerisation. Truncated adaptors, containing a T overhang were  
503 ligated to the A-Tailed cDNA. Successfully ligated cDNA molecules were then enriched with limited  
504 cycle PCR. Libraries to be multiplexed in the same run were pooled in equimolar quantities, calculated  
505 from Qubit and Bioanalyser fragment analysis. Samples were sequenced on the NextSeq 500 instrument  
506 (Illumina, San Diego, US) using a 43bp paired end run. Run data were de-multiplexed and converted  
507 to fastq files using Illumina's bcl2fastq Conversion Software v2.18 on BaseSpace. Fastq files were  
508 then aligned to a reference genome using STAR on the BaseSpace RNA-Seq alignment app v1.1.0.  
509 Reads per transcript were counted using HTSeq and differential expression was estimated using the  
510 BioConductor package DESeq2 (BaseSpace app v1.0.0). Next, 4 groups of differentially regulated  
511 genes were analysed: low and high abundance *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/+</sup>* versus *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
512 *Hoil-1<sup>+/+</sup>* embryos and low and high abundance in *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* versus *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
513 *Hoil-1<sup>-/-</sup>* embryos. To identify genes that were specifically altered in the absence of HOIL-1, the Venny  
514 2.1 software was used to exclude genes that were differentially expressed between *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
515 *Hoil-1<sup>+/+</sup>* and *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/+</sup>* embryos from those between *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* and  
516 *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos. Genes that were already differentially expressed between the  
517 corresponding HOIL-1-expressing controls (i.e. *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/+</sup>* and *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
518 *Hoil-1<sup>+/+</sup>* embryos) were excluded from the differentially expressed genes between *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
519 *Hoil-1<sup>-/-</sup>* and *Mkl1<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos. The resulting list of genes (33/85, dark green) was



520 entered in the STRING software (string-db.org) to assess for functional enrichment in biological  
521 networks. Gene ontology (GO) terms with false discovery rate (FDR) below 1% are shown.

#### 522 *Flow Cytometry analysis (FACS), colony forming unit assay and macrophage culture*

523 For phenotypic analysis, single-cell suspensions from mechanically dissociated E13.5 foetal livers or a  
524 pool of aortas (AGM region) from 3 embryos, were stained for 30 min on ice with various antibody  
525 cocktails. The antibodies against the surface markers examined were: CD16/32, clone 93 and 2.4G2  
526 (eBioscience, 45-0161-82 and BD553141), CD135, clone A2F10.1 (BD, 553842), Ly-6A/E, clone D7  
527 (Sca-1) (BD, 558162), CD117 (c-Kit), clone 2B8 (BD, 560185), CD34, clone RAM34 (BD, 562608),  
528 mouse Lineage Cocktail, clones 17A2/RB6-8C5/RA3-6B2/Ter-119/M1/70 (Biolegend, 133313 and  
529 BD, 561301), CD16/32, clone 2.4G2 (BioXcell, CUS-HB-197), CD11b, clone M1/70 (Biolegend,  
530 101228 and eBioscience, 15-0112-81), CD11c, clone HL3 (BD, 561241), F4/80, clone BM8  
531 (Biolegend, 123110), GR-1, clone RB6-8C5 (Biolegend, 108416 and 108410), CD45, clone 30-F11  
532 (Biolegend, 103128 and Biolegend, 103112), CD3 $\epsilon$ , clone, 145-2C11 (Biolegend, 100310), B220, clone  
533 RA3-6B2, (Biolegend, 103210), CD71, clone RI7217 (Biolegend, 113807), TER-119, clone TER-119  
534 (Biolegend, 116234) and Fixable Viability Dye (eBioscience, 65-0864-18 and 65-0867-14). The  
535 myeloid progenitors were identified in the LK population as CD34<sup>+</sup>CD16/32<sup>-</sup> (CMP), CD34<sup>+</sup>CD16/32<sup>+</sup>  
536 (GMP); CD34<sup>-</sup>CD16/32<sup>-</sup> (MEP) Fluorescence minus one (FMO) were used as a gating control. For  
537 quantification of absolute number of cells, a defined number of flow cytometric reference beads  
538 (Invitrogen) were mixed with the samples before acquisition. Samples were processed either using LSR  
539 Fortessa (BD Biosciences) or sorted in a FACSAria FUSION cell sorter (BD Biosciences). Data were  
540 analysed with FlowJo 7.6.1 software (Treestar). Cytospin preparations of 10.000 cells/slide of E13.5  
541 foetal liver homogenates were stained by May-Grunwald Giemsa staining and enucleated erythrocytes  
542 were quantified blindly as number of cells per HPF using ImageJ Software. For growth of primitive  
543 erythroid progenitor cells or all haematopoietic stem cells, 5000 sorted Lineage<sup>-</sup>c-KIT<sup>+</sup> E13.5 liver cells  
544 were cultured in MethoCult™ SF containing cytokines, including EPO (Stem Cell, M3436) or Mouse  
545 Methylcellulose Complete Media (R&D, HSC007), respectively. Colonies were enumerated after 14  
546 days of incubation. For preparation of foetal liver-derived macrophages, equal amounts of E13.5 single  
547 cell suspensions were cultured and differentiated for 5 days in DMEM supplemented with 10% FCS  
548 plus 20% L929-conditioned medium (as a source of M-CSF) supplemented or not with the indicated  
549 inhibitors. Cells were imaged using EVOS Auto cell imaging system and viability was measured using  
550 the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7572). Alternatively, cells were  
551 stained with Hoechst dye and enumerated using Citation cell imaging platform.

#### 552 *Cytokine analysis*

553 Embryo homogenates prepared as described above (*Western blot and antibodies*) were analysed with  
554 Proteome Profiler Arrays (Mouse Angiogenesis Array, ARY015, and Mouse Cytokine array Panel A,

555 ARY006 both R&D). ELISA kits used were the CXCL4 (R&D, DY595), CXCL11 (Abcam, ab204519),  
556 CXCL10 (R&D, DY466-05), IFN Lambda 2/3 (Pbl assay science, 62830-1), IL-1 $\beta$  (ThermoFisher,  
557 BMS6002) and IFN- $\beta$  ELISA (ThermoFisher, 424001).

#### 558 *Epidermal thickness quantification*

559 Per mouse, 1-2 pieces of skin were taken and epidermal thickness was measured by microscopy using  
560 a 20x magnification. Quantification was performed by an experimenter blinded to the genotype of the  
561 mice by using the CellSens software with at least 20 measurements per mouse.

#### 562 *Pharmacological inhibition of RIPK1 kinase activity*

563 Mice were fed with rodent chow containing 100 mg/kg of the RIPK1 kinase inhibitor GSK3540547A  
564 (GSK'547A) (GlaxoSmithKline LLC) starting a week prior to mating and kept on this diet throughout  
565 pregnancy until cesarean section at the indicated time points.

#### 566 *Statistics and reproducibility*

567 Group size was determined based on preliminary data sets. Statistical significance was determined using  
568 unpaired, two-tailed parametric Student's t-test. One- or two-way ANOVA with Tukey's multiple  
569 comparisons test was applied. 95% Confidence interval was considered for statistics and  $P$ -values ( $P$ )  
570 of  $<0.05$  was considered significant and indicated with  $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$  and  
571  $****P<0.0001$ . Multiplicity adjusted  $P$  values are reported for multiple comparisons. All statistical  
572 analyses were performed using Graphpad Prism 6. Statistical transformations for RNAseq was  
573 performed with DESeq2 and adjusted  $P$  values utilised the Benjamini Hochberg test. All in vitro  
574 experiments were performed at least twice with similar results. Unless indicated in figure legends in  
575 vivo experiments were performed with at least 2 embryos per genotype. At least 3 embryos were  
576 considered for statistical testing.

#### 577 *Data availability statement*

578 Additional information on this manuscript can be found in the Extended Data files, Supplementary  
579 Figure 1 and Source Data (gels and graphs). RNA sequencing analysis data is available from SRA  
580 database SRP134865 (BioProject ID: PRJNA437851) and comparative data sets including genes  
581 differentially regulated genes between embryo homogenates with different mutations are displayed in  
582 Supplementary Table 1.

583

584 **Extended Data Figure Legends**

585

586 **Extended data Figure 1. HOIL-1-deficient mice die at mid-gestation**

587 **a**, Schematic representation of the *Hoil-1* knockout strategy. Solid boxes represent *Hoil-1* exons and  
588 grey boxes with a star indicate the targeted exons. Boxes with diagonal and horizontal strips represent  
589 LoxP and Frt sites, respectively. **b**, Specificity of gene recombination was assessed by Southern blotting  
590 with 5' and 3' probes external to the construct in four clones (14B8, 14F6, 20D7 and 21F7). Digest of  
591 the DNA with ApaI, followed by hybridisation with the 3' probe was expected to show a 5700 bp band  
592 for the WT allele and a 7700 bp band for the mutant allele. All four clones appeared to have the correct  
593 recombination on the 3' side. Digest of the DNA with SphI and hybridisation with the 5' probe was  
594 expected to show a 4500 bp WT band and a 6200 bp band for the mutated allele. Clones 14B8, 14F6  
595 and 21F7 appeared to be correctly recombined on the 5' side. Finally, cutting the DNA with ApaI and  
596 hybridising with a hygro probe showed a single band in all clones, indicative of a single integration of  
597 the construct in all four ES clones. Clones 14B8 and 14F6 were selected for generation of the two *Hoil-*  
598 *I*<sup>-/-</sup> strains. **c**, PCR analysis of *Hoil-1* wild-type, heterozygous and knockout mice. **d**, Protein levels of  
599 HOIL-1, HOIP and SHARPIN in whole embryo lysates (*n*= 3 for *Hoil-1*<sup>+/-</sup> and *Hoil-1*<sup>-/-</sup> embryos and  
600 *n*=1 for *Hoil-1*<sup>+/+</sup> embryos). For gel source data (c, d), see Supplementary Figure 1. **e**, Quantification of  
601 genotypes of animals obtained from inter-crossing *C20Hoil-1*<sup>+/-</sup> mice. \*indicates dead embryos. **f**,  
602 Representative images of *C20Hoil-1*<sup>+/-</sup> and *C20Hoil-1*<sup>-/-</sup> embryos from E9.5 to E11.5 as quantified in  
603 (e). Scale bar: 2 mm. **g**, Single staining showing vascularisation (PECAM-1, top panel) and apoptosis  
604 (cleaved Caspase-3, bottom panel) of yolk sacs. Merged image is shown in Fig. 1c. **h**, Whole-mount  
605 TUNEL staining of embryos at the indicated stages (embryo/genotype *n*=2 at E10.5, *n*=8 for *Hoil-1*<sup>+/-</sup>  
606 and *n*=5 for *Hoil-1*<sup>-/-</sup> at E11.5). Scale bar: 2 mm. **i**, Quantification of genotypes of animals obtained  
607 from inter-crossing *Hoil-1*<sup>fl/wt</sup>*Tie2-Cre*<sup>+</sup> with *Hoil-1*<sup>fl/fl</sup>*Tie2-Cre*<sup>-</sup> mice. \*indicates dead embryos. **j**,  
608 Representative images of embryos with conditional deletion of *Hoil-1* in *Tie2-Cre* expressing cells as  
609 quantified in (i). Scale bar: 2 mm. \*: poorly vascularised yolk sac.

610

611 **Extended data Figure 2. TNFR1 signalling drives cell death and lethality of HOIL-1-deficient**  
612 **mice at mid-gestation.**

613 **a, d**, Quantification of genotypes of animals obtained from inter-crosses of *Tnfr1*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> (a) and *Tnfr1*<sup>-</sup>  
614 *Hoil-1*<sup>+/-</sup> (d) mice. \*: dead embryos. **b**, Representative images of embryos quantified in (a) at E10.5  
615 and E15.5, \*; poor yolk sac. **c**, Cell death as detected by whole-mount TUNEL staining in yolk sacs at  
616 E10.5 (*n*= 3 embryos/genotype). **e**, Single staining showing vascularisation (PECAM-1, top panel) and  
617 apoptosis (cleaved Caspase-3, bottom panel) of yolk sacs. Merged image is shown in Fig. 1g. Scale bar:  
618 50 µm. **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-*  
619 *I*<sup>-/-</sup>).

620

621 **Extended data Figure 3. HOIL-1 is required for optimal TNF-induced NF- $\kappa$ B activation**  
622 **independently of its RBR domain.**

623 **a, b, d**, Western blot analysis of the indicated proteins in whole-cell lysates from MEFs of the indicated  
624 genotypes after they had been stimulated with TNF (or left untreated) for the indicated time points in  
625 minutes (min) (a), overexpressing the different LUBAC components (b) or the indicated mutant forms  
626 of HOIL-1 (d) ( $n=2$  independent experiments). **c**, SHARPIN-IP was performed in *Tnf<sup>-/-</sup>Hoil-1<sup>-/-</sup>* MEFs  
627 reconstituted with HOIL-1 or a combination of HOIP and SHARPIN and analysed by Western blotting  
628 ( $n=2$  independent experiments). TL: total lysate, EV: empty vector. For gel source data, see  
629 Supplementary Figure 1.

630

631 **Extended data Figure 4. Ablation of the kinase activity of RIPK1 in HOIL-1- or HOIP-deficient**  
632 **embryos prevents cell death and lethality at mid-gestation but not at late gestation.**

633 **a, b**, Quantification of genotypes of animals obtained after inter-crossing *Ripk1<sup>K45A</sup>Hoil-1<sup>+/-</sup>* (a) and  
634 *Ripk1<sup>K45A</sup>Hoip<sup>+/-</sup>* (b) mice. \*indicates dead embryos. **c**, Representative images of embryos quantified in  
635 (b) \*; poor yolk sac vascularisation. Scale bar: 2 mm. **d**, Whole-mount TUNEL staining of embryos  
636 ( $n=2$  embryos). Scale bar: 2 mm. **e**, Single staining showing vascularisation (PECAM-1, top panel) and  
637 apoptosis (cleaved (cl.) Caspase-3, bottom panel) of yolk sacs. Merged image is shown in Fig. 3b. **f, g**,  
638 Representative images of cell death in different organs (f) and quantification (g) as detected by TUNEL  
639 staining at E14.5 ( $n=3$  embryos/genotype). Scale bar: 50  $\mu$ m (f). Mean  $\pm$  s.e.m. ( $n=3$  embryos/genotype)  
640 and *P* values from one-way ANOVA are reported (g). **h**, Representative images of H&E staining on  
641 whole-embryo paraffin sections ( $n=3$  embryos/genotype). \*, pericardial effusion, n, necrotic area. H,  
642 heart; L, lung; Li, liver. Scale bar: 200  $\mu$ m. **i**, Cell death was analysed by PI staining in MEFs stimulated  
643 or not with TNF for 24 h plus the indicated cell death inhibitors. Mean  $\pm$  s.e.m. ( $n=3$  independent  
644 experiments) and *P* values from two-way ANOVA are reported.

645

646 **Extended data Figure 5. Individual deletion of mediators of apoptosis or necroptosis does not**  
647 **prevent cell death and lethality at mid-gestation of HOIL-1- or HOIP-deficient embryos.**

648 **a**, Western blot analysis of MLKL expression in the indicated organs derived from control *Mlkl<sup>-/-</sup>* mice  
649 ( $n=2$  mice/genotype). For gel source data, see Supplementary Figure 1. **b, d, e, f**, Representative images  
650 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>*  
651 ; 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  $n=2$   
652 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>*  
653 *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:  
654  $n=5$  for *Caspase-8<sup>+/-</sup>Hoip<sup>+/-</sup>* and  $n=4$  for *Caspase-8<sup>+/-</sup>Hoip<sup>-/-</sup>*; E11.5:  $n=6$  for *Caspase-8<sup>+/-</sup>Hoip<sup>+/-</sup>* and  
655  $n=3$  for *Caspase-8<sup>+/-</sup>Hoip<sup>-/-</sup>*; E12.5:  $n=3$  for *Caspase-8<sup>+/-</sup>Hoip<sup>+/-</sup>* and  $n=2$  for *Caspase-8<sup>+/-</sup>Hoip<sup>-/-</sup>* (e),  
656 E10.5:  $n=2$  for *Caspase-8<sup>+/-</sup>Hoil-1<sup>+/-</sup>* and  $n=4$  for *Caspase-8<sup>+/-</sup>Hoil-1<sup>-/-</sup>*; E11.5:  $n=2$  for *Caspase-8<sup>+/-</sup>*  
657 *Hoil-1<sup>+/-</sup>* and  $n=5$  for *Caspase-8<sup>+/-</sup>Hoil-1<sup>-/-</sup>*; E12.5:  $n=6$  for *Caspase-8<sup>+/-</sup>Hoil-1<sup>+/-</sup>* and  $n=3$  for *Caspase-*

658  $8^{+/+}Hoil-1^{-/-}$  (f)). \*: poor yolk sac vascularisation. Scale bar: 2 mm. **c**, Representative images of yolk sac  
659 vascularisation and cell death at E10.5 as detected by PECAM-1 (red) and cleaved (cl.) Caspase-3  
660 staining (green) (top panel) and whole mount TUNEL staining (bottom panel) ( $n=4$  per genotype). Scale  
661 bar: 50  $\mu$ m.

662

663 **Extended data Figure 6. Combined deletion of RIPK3 and Caspase-8 prevents cell death but not**  
664 **embryonic lethality at late gestation that is caused by the loss of HOIL-1.**

665 **a**, Quantification of genotypes of animals obtained from inter-crosses of  $Ripk3^{-/-}Caspase-8^{+/+}Hoil-1^{+/+}$   
666 with  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{+/+}$  mice (left panel) or  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{+/+}$  mice (right panel). **b**,  
667 Health status of  $Ripk3^{-/-}Caspase-8^{+/+}Hoil-1^{-/-}$  and  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{-/-}$  embryos at different  
668 developmental stages. **c**, Single staining showing vascularisation (PECAM-1, top panel) and apoptosis  
669 (cleaved (cl.) Caspase-3, bottom panel) of yolk sacs. Merged image is shown in Fig. 3f. Scale bar: 50  
670  $\mu$ m. **d**, Cell death as detected by whole-mount TUNEL staining in yolk sacs at E14.5 (left panel) and  
671 respective quantification (right panel). Mean  $\pm$  s.e.m. ( $n=3$  embryos/genotype) and  $P$  values from one-  
672 way ANOVA are reported. **e, f** Representative images (e) and quantification (f) of cell death in different  
673 organs as detected by TUNEL staining at E13.5 ( $n=3$  embryos/genotype) and E14.5 ( $n=5$  for  $Ripk3^{-/-}$   
674  $Caspase-8^{-/-}Hoil-1^{+/+}$ ,  $n=2$  for  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{-/-}$  and  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{-/-}$  lung and  
675 liver and  $n=3$   $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{-/-}$  heart). Scale bar: 50  $\mu$ m (e). Mean  $\pm$  s.e.m. values are shown  
676 (f). **g**, Cell death was analysed by PI staining in MEFs stimulated or not with the indicated ligands for  
677 24 h. Mean  $\pm$  s.e.m. ( $n=3$  independent experiments) and  $P$  values from two-way ANOVA are reported.  
678 **h**, Representative images of H&E staining on E13.5 whole-embryo paraffin embedded sections ( $n=3$   
679 for  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{+/+}$  and  $Ripk3^{-/-}Caspase-8^{-/-}Hoil-1^{-/-}$  and  $n=2$  for  $Ripk3^{-/-}Caspase-8^{+/+}Hoil-$   
680  $1^{-/-}$ ). \*: pericardial effusion, arrows; congested vessels H, heart; L, lung; Li, liver. Scale bar: 200  $\mu$ m. **i**,  
681 Representative images of micro-focus CT scan images of whole E13.5 embryos ( $n=3$   
682 embryos/genotype). \*: pericardial effusion

683

684 **Extended data Figure 7. Combined deletion of MLKL and Caspase-8 promotes survival of**  
685 **LUBAC-deficient mice.**

686 **a**, Quantification of genotypes of animals obtained from inter-crosses of  $Mlkl^{-/-}Caspase-8^{+/+}Hoip^{+/+}$  with  
687  $Mlkl^{-/-}Caspase-8^{-/-}Hoip^{+/+}$  mice. \*: dead embryos. **b**, Representative images of adult mice as quantified  
688 in (a). **c**, Kaplan-Meier plot of mouse survival ( $n=6$  for  $Mlkl^{-/-}Caspase-8^{-/-}Hoip^{-/-}$  and  $n=9$  for  $Mlkl^{-/-}$   
689  $Caspase-8^{-/-}Hoil-1^{-/-}$  mice). **d**, Representative images of H&E staining of the indicated organs ( $n=3$   
690 mice/genotype). Scale bar: 200  $\mu$ m. **e**, Representative images of yolk sac vascularisation (PECAM-1,  
691 red) and apoptosis (cleaved (cl.) Caspase-3, green) (top panel) at E13.5 and respective quantifications  
692 (bottom panel). Mean  $\pm$  s.e.m ( $n=5$  for  $Mlkl^{-/-}Caspase-8^{-/-}Hoil-1^{+/+}$  and  $Mlkl^{-/-}Caspase-8^{-/-}Hoil-1^{-/-}$  and  
693  $n=2$  for  $Mlkl^{-/-}Caspase-8^{+/+}Hoil-1^{-/-}$ ) are shown and results were analysed with unpaired two-tailed  $t$ -

694 tests comparing *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* and *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* embryos. **f**, Representative  
695 images of H&E staining of the indicated organs ( $n=3$  embryos/genotype). Scale bar: 200  $\mu\text{m}$ . **g**,  
696 Epidermal thickness quantification of mice of the indicated genotypes in (f). Mean  $\pm$  s.e.m values ( $n=3$   
697 mice/genotype) are shown and results were analysed with unpaired two-tailed *t*-tests. **h**, Western blot  
698 analysis of lysates from whole E13.5 embryos of the indicated genotypes as well as L929 cells treated  
699 or not with TNF plus zVAD-fmk for 2 h as antibody validation ( $n=4$  embryos/genotype performed  
700 twice). For gel source data, see Supplementary Figure 1.

701

702 **Extended data Figure 8. Combined deletion of RIPK3 and Caspase-8 causes haematopoietic**  
703 **defects and RIPK1-dependent embryonic lethality in HOIL-1-deficient mice.**

704 **a**, Venn diagram depicting genes differentially expressed by RNAseq analysis between E13.5 embryos  
705 of the indicated genotypes. **b**, Gene Ontology (GO) enrichment analysis of differentially (85 low and  
706 35 high in (a)) expressed genes. FDR: false discovery rate. **c**, Representative FACS profile of E13.5  
707 foetal liver cells with different erythroblast populations gated according to their CD71 and TER119  
708 expression levels (R1-R5). R1 contains immature RBC progenitors, including BFU-E and CFU-E; R2  
709 comprises mainly pro-erythroblasts and early basophilic erythroblasts; R3 contains both early and late  
710 basophilic erythroblasts; R4 is composed of chromatophilic and orthochromatophilic erythroblasts; and  
711 R5 consists of late orthochromatophilic erythroblasts and reticulocytes and **quantification**. Mean  $\pm$   
712 s.e.m. ( $n=14$  *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>*,  $n=8$  *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*,  $n=5$  for *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
713 *Hoil-1<sup>-/-</sup>* and  $n=3$  for *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>* foetal livers) and *P* values from two-way ANOVA are  
714 reported. **d, h, k** Representative FACS profile of E13.5 foetal liver cells for the indicated haematopoietic  
715 populations (sample size specified in (e-g, i, j)). **e, f, j** Total cell number of the different haematopoietic  
716 cell subsets in foetal liver cell suspensions from E13.5 embryos of the indicated genotypes gated as in  
717 (d), (h) and (k), respectively. Total number of multipotent progenitors (LSK and LK cells) (e), mature  
718 CD45<sup>+</sup> blood cells, including granulocytes (GR-1<sup>+</sup>) and macrophages (F4-80<sup>+</sup>) (f) and myeloid  
719 progenitors (CMP, GMP and MEP) (j). Mean  $\pm$  s.e.m. and *P* values from unpaired two-tailed *t*-tests are  
720 reported. **g, i**, Percentages of mature CD45<sup>+</sup> leucocytes, GR-1<sup>+</sup> and F4-80<sup>+</sup> cells (g) and CMP, GMP and  
721 MEP (i). Mean  $\pm$  s.e.m. and *P* values from unpaired two-tailed *t*-tests are reported. **l**, Differentiation of  
722 E13.5 foetal liver (c-KIT<sup>+</sup>) progenitors into CFU-granulocytes and macrophages (GM), burst forming  
723 units-erythrocyte (BFU-E) or and CFU-granulocyte, erythroid, macrophage, megakaryocyte (GEMM).  
724 Mean  $\pm$  s.e.m. ( $n=2$  foetal livers). **m**, Micrographs of differentiated macrophages ( $n=3$  *Ripk3<sup>-/-</sup>Caspase-*  
725 *8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* and *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>-/-</sup>*,  $n=5$  *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* and  $n=4$  *Mlkl<sup>-/-</sup>Caspase-8<sup>-/-</sup>*  
726 *Hoil-1<sup>-/-</sup>* foetal livers) and percentage viability of macrophages from E13.5 foetal liver cell suspensions  
727 from embryos of the indicated genotypes in the presence or absence of the indicated inhibitors as  
728 measured by Cell Titer Glo. Mean  $\pm$  s.e.m. ( $n=3$  *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>Hoil-1<sup>+/-</sup>* and *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>*

729 *Hoil-1*<sup>-/-</sup>, *n*=5 *Mkl1*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *n*=4 *Mkl1*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> foetal livers) and *P* values  
730 from two-way ANOVA are reported. **o**, MicroCT scan images of *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos  
731 showing maximum intensity projections, with windowing applied to highlight vasculature (high  
732 contrast). No anatomical defects that would explain destruction of RBCs or poor distribution of blood  
733 to the peripheries were found (*n*=3 embryos). Left image: yellow star: distal aorta, green star: umbilical  
734 vessels and red star: descending thoracic aorta. Right image, yellow star: carotid artery, red star:  
735 descending thoracic aorta, white star: ductus arteriosus, blue star: ascending thoracic aorta. **p**,  
736 Representative FACS profile of a pool of three E11.5 dorsal aortas, containing the AGM region, per  
737 indicated genotype and quantification. This experiment was performed once with 3 embryos per  
738 genotype.

739

740 **Extended data Figure 9. Concomitant deletion of RIPK1 prevents embryonic lethality of *Ripk3*<sup>-/-</sup>**  
741 ***Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> mice.**

742 **a**, Kaplan-Meier plot of mouse survival (*n*=17 for *Ripk1*<sup>-/-</sup>*Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoip*<sup>-/-</sup> and *n*=2 for *Ripk*<sup>+/-</sup>  
743 *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoip*<sup>-/-</sup> mice). **b**, Quantification of genotypes of animals obtained from inter-crosses  
744 of *Ripk1*<sup>+/-</sup>*Hoil-1*<sup>+/-</sup> mice. For simplicity not all possible genotypes are represented. **c**, Percentage  
745 viability of macrophages from E13.5 foetal liver cell suspensions from embryos of the indicated  
746 genotypes as measured by Cell Titer Glo. Mean ± s.e.m. (*n*=5 foetal livers/genotype) are reported and  
747 results were analysed with unpaired two-tailed *t*-tests. **d**, Cytokine arrays from *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-*  
748 *1*<sup>+/-</sup> and *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>-/-</sup> embryos (left panels) and table listing the altered cytokines (right  
749 panel). Red squares highlight the differences (*n*=1 for each genotype). For gel source data, see  
750 Supplementary Figure 1. **e**, Cytokine analysis in homogenates from embryos of the indicated genotypes.  
751 Mean ± s.e.m. (*n*=3 embryos/genotype) and *P* values from one-way ANOVA are reported. **f**,  
752 Representative images of E16.5 embryos from control mothers or mothers fed with the RIPK1 kinase  
753 inhibitor GSK'457A from mating and throughout gestation (embryos treated with GSK'457A *n*=5 for  
754 *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-1*<sup>+/-</sup> and *n*=7 *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>+/-</sup>*Hoil-1*<sup>-/-</sup> and *n*=3 for *Ripk3*<sup>-/-</sup>*Caspase-8*<sup>-/-</sup>*Hoil-*  
755 *1*<sup>-/-</sup>). Scale bar: 5 mm.

756 **Extended data Figure 10. Schematic representation of findings in this study**

757 **a**, Diagram indicating extent of viability and phenotypes of single, double, triple and quadruple  
758 knockout mice. Red lines indicate cell death and loss of yolk sac vascularisation phenotype. Green line  
759 indicates mild cell death phenotype without loss of yolk sac vascularisation. Asterisk (\*) indicates that  
760 heart defects were observed. **b**, Proposed model of LUBAC function during embryogenesis. At mid-  
761 gestation (left panel), LUBAC maintains vascular tissue integrity by preventing aberrant TNF/LT- $\alpha$ -  
762 mediated Caspase-8- and RIPK3/MLKL-induced cell death. At late gestation, LUBAC is not only  
763 required to prevent aberrant cell death but also to prevent severe defects in haematopoiesis that are

764 driven by RIPK1 but can be prevented by RIPK3 (middle panel). Genetic ablation of LUBAC and of  
765 different components of the cell death machinery indicates that (right panel): 1) in the absence of  
766 LUBAC, Caspase-8 and RIPK3, RIPK1 provokes lethality most likely by depleting multipotent  
767 progenitors in the haematopoietic compartment; 2) in the absence of Caspase-8 and MLKL, cell death  
768 induced by loss of LUBAC is prevented and RIPK3 is present to exert its protective role on foetal  
769 haematopoiesis by precluding aberrant RIPK1 signalling; and 3) in the absence of Caspase-8 and  
770 RIPK3, the presence of LUBAC is sufficient to prevent RIPK1 from causing severe defects in  
771 haematopoiesis and lethality since *Ripk3<sup>-/-</sup>Caspase-8<sup>-/-</sup>* mice are viable<sup>15,16,23</sup>. This indicates that RIPK3  
772 and LUBAC can compensate for each other to block aberrant RIPK1 signalling.

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