ABIN-1 regulates RIPK1 activation by linking Met1 ubiquitylation with Lys63 deubiquitylation in TNF-RSC

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Ubiquitylation of the TNFR1 signalling complex (TNF-RSC) controls the activation of RIPK1, a kinase critically involved in mediating multiple TNFα-activated deleterious events. However, the molecular mechanism that coordinates different types of ubiquitylation modification to regulate the activation of RIPK1 kinase remains unclear. Here, we show that ABIN-1/NF-κB, a ubiquitin-binding protein, is recruited rapidly into TNF-RSC in a manner dependent on the Met1-ubiquitylating complex LUBAC to regulate the recruitment of A20 to control Lys63 ubiquitylation of RIPK1. ABIN-1 deficiency reduces the recruitment of A20 and licenses cells to die through necroptosis by promoting Lys63 ubiquitylation and activation of RIPK1 with TNFα stimulation under conditions that would otherwise exclusively activate apoptosis in wild-type cells. Inhibition of RIPK1 kinase and RIPK3 deficiency block the embryonic lethality of Abin-1–/– mice. We propose that ABIN-1 provides a critical link between Met1 ubiquitylation mediated by the LUBAC complex and Lys63 ubiquitylation by phospho-A20 to modulate the activation of RIPK1.

RIPK1 is a critical regulator of necroptosis and apoptosis, two distinct regulated cell death mechanisms that may be activated under alternative conditions when cells are stimulated by TNFs, an important proinflammatory cytokine involved in mediating a multitude of human diseases. Under apoptotic-deficient conditions, however, the kinase activity of RIPK1 is activated to interact with RIPK3 to induce the formation of a RIPK1/RIPK3 complex, known as complex I or necrosome, which promotes the execution of necrosis. A20 is an important ubiquitin-editing enzyme involved in regulating RIPK1 ubiquitylation. Activation of RIPK1 has been shown to be critical for major human inflammatory degenerative diseases such multiple sclerosis, Crohn’s disease and amyotrophic lateral sclerosis. Human clinical trials on small-molecule inhibitors of RIPK1 are underway in pharmaceutical and biotechnology companies for developing drugs targeting amyotrophic lateral sclerosis, Alzheimer’s disease, rheumatoid arthritis and Crohn’s disease. On the other hand, inactivation of the TNFAIP3 gene, encoding the A20 protein, is associated with inflammatory diseases including multiple sclerosis, rheumatoid arthritis and Crohn’s disease. Understanding the interactive relationship between RIPK1 and A20 may be important for developing effective drugs to treat these conditions. However, the molecular mechanism that regulates the interaction of A20 in TNF-RSC to control the activation of RIPK1 kinase remains unclear.

TNFα stimulation induces the trimerization of TNFR1 to promote the formation of a transient intracellular multi-protein complex, the TNFR1 signalling complex TNF-RSC (also called complex I), which recruits RIPK1 via homotypic interactions of their death domains. TNF-RSC is modified by a complex pattern of ubiquitylation chains with different linkages, including Met1, Lys48 and Lys63 (ref. 14). In TNF-RSC, cIAP1/2 mediate Lys63 ubiquitylation of RIPK1 (ref. 15), which in turn promotes the recruitment of the linear ubiquitin chain assembly complex (LUBAC), composed of HOIP, HOIL-1 and SHARPIN, and subsequent conjugation of Met1 ubiquitin chains. Deficiency in the LUBAC complex leads to impaired activation of NF-κB and increased sensitivity to cell death in response to TNFα stimulation, which can be inhibited by the RIPK1 inhibitor R-7-CI-O-Necrostatin-1 (Nec-1s). Knockdown of HOIL-1 and HOIP leads to a significant reduction in the recruitment of the IKK complex, the kinase critical for the activation of NF-κB. On the other hand, it is not clear how deficiency in LUBAC sensitizes cells to RIPK1-mediated cell death.

ABIN-1 is a ubiquitin-binding protein that can bind to both Lys63 and Met1 ubiquitin chains but not to Lys48 chains. Specific risk haplotypes in the gene Abin-1 (TNFAIP3-interacting protein 1, or Tnip1) are strongly associated with a predisposition for autoimmune inflammatory diseases such as systemic lupus erythematosus and psoriasis. These risk haplotypes produce lower levels of Abin-1 messenger RNA and ABIN-1 protein, suggesting that the susceptibility to autoimmunity is due to their hypomorphic function. Abin-1–/– mice die during late embryogenesis due to fetal liver cell death, anaemia and hypoplasia, which can be blocked by TNFα deficiency.

In this study, we show that ABIN-1 functions as a critical link between Met1/Lys63 ubiquitylation and deubiquitylation of TNF-RSC to control the activation of RIPK1 kinase activity. ABIN-1 deficiency sensitizes cells to necroptosis by promoting the Lys63 ubiquitylation and activation of RIPK1 following TNFα stimulation. The recruitment of ABIN-1 into TNF-RSC requires the LUBAC complex whereas ABIN-1 deficiency inhibits the association of phospho-A20 with TNF-RSC. Importantly, genetic inactivation of
RIPK1 or RIPK3 deficiency prolongs the survival of Abin-1−/− mouse embryonic fibroblasts (MEFs) under various TNFa-mediated paradigms of necroptosis and apoptosis, and rescues the embryonic lethality of Abin-1−/− mice. Thus, ABIN-1 deficiency provides a unique paradigm where necroptosis may be activated by normally pro-apoptotic conditions and blocking necroptosis is sufficient to rescue cell death and embryonic lethality.

Results
ABIN-1 deficiency promotes necroptosis. ABIN-1 deficiency has been proposed to promote caspase-8-mediated apoptosis in TNFa-stimulated cells23. However, the caspase inhibitor zVAD.fmk did not inhibit, but rather further sensitized Abin-1−/− MEFs to cell death induced by TNFa and cycloheximide (CHX), and the level of cell death for both treatments was significantly higher than in the wild-type (WT) MEFs (Fig. 1a,b). Furthermore, Abin-1−/− MEFs showed increased sensitivity to cell death induced by TNFa/zVAD compared with WT controls (Fig. 1c). Similar data were obtained using both immortalized and primary Abin-1−/− MEFs. In contrast, Abin-1−/− MEFs showed no difference compared with WT MEFs in their sensitivity to apoptosis induced by staurosporine, suggesting no change in sensitivity to apoptosis in general. In addition, we tested the effect of ABIN-1 knockdown in murine fibrosarcoma L929 cells, which undergo necroptosis by stimulation with TNFa alone24. We found that knockdown of ABIN-1 in L929 cells significantly sensitized them to cell death induced by TNFa (Fig. 1d,e). Similarly, knockdown of ABIN-1 also sensitized the death of RGC-5 cells to TNFa/CHX/zVAD-induced cell death (Fig. 1f). Finally, when treated with TNFa/zVAD combined with SM-164, an IAP antagonist25, Abin-1−/− MEFs died earlier than WT MEFs (Supplementary Fig. 1a).

Treatment with TNFa/CHX/zVAD or TNFa/zVAD can induce necroptosis independently of caspases, which can be effectively inhibited by the RIPK1 inhibitor Nec-1s and by the genetic inactivation of RIPK1, PARP-1, caspase-3 activation in Abin-1−/− MEFs (Fig. 1g,h). Since TNFa/CHX/zVAD-induced cell death involves the activity of RIPK1 and RIPK3, which can be inhibited by Nec-1s, and by the genetic inactivation of RIPK1, PARP-1, and caspase-3 activation in Abin-1−/− MEFs (Fig. 1g,h), we detected increased interaction of p-Ser166 RIPK1 with FADD in Abin-1−/− MEFs stimulated by TNFa/CHX/zVAD compared with cells in the WT control (Fig. 2c).

When MEFs were stimulated with TNFa/CHX/zVAD.fmk, higher levels of phospho-MLKL, a marker for MLKL activation30, were detected in Abin-1−/− MEFs in WT (Fig. 2f,g). Furthermore, we found that when treated with TNFa/zVAD.fmk, Abin-1−/− MEFs also showed increased p-MLKL and p-Ser166 RIPK1 compared with WT MEFs (Fig. 2h). Importantly, the formation of complex IIb and p-MLKL in both Abin-1−/− and WT MEFs was effectively blocked by Nec-1s, as well as in Abin-1−/−; Ripk1D138N/D138N MEFs (Fig. 2c,i). On the basis of these results, we conclude that ABIN-1 deficiency promotes the activation of RIPK1 to mediate necroptosis.

Inhibition of necroptosis prolongs the survival of Abin-1−/− cells with activated caspases. Nec-1s can inhibit necroptosis of WT MEFs induced by TNFa/CHX/zVAD but not apoptosis induced by TNFa/CHX26. Surprisingly, we found that treatment with Nec-1s also reduced the death of Abin-1−/− MEFs induced by TNFa/CHX in the absence of zVAD.fmk, a condition that normally induces apoptosis independently of RIPK1. Similar data were obtained using both immortalized and primary Abin-1−/− MEFs (Fig. 3a-c). TNFa/CHX-induced death of RGC-5 cells was also sensitized by knockdown of Abin-1 and inhibited by Nec-1s (Fig. 3d).

TNFa stimulation under certain conditions may induce the activation of RIPK1 to mediate apoptosis, termed RIPK1-dependent apoptosis. However, RIPK1-dependent apoptosis induced by TNFa stimulation combined with cIAP1/2 deficiency, TAK1 inhibition or IKKα/IKKβ deficiency cannot be blocked by knockdown of RIPK3 or MLKL30,44 (Supplementary Fig. 2a). Unexpectedly, we found that the survival of Abin-1−/− MEFs stimulated by TNFa/CHX was prolonged by knockdown of RIPK3 or MLKL (Fig. 3e) and by genetic mutations in Abin-1−/−; Ripk1D138N/D138N mutant MEFs or in Abin-1−/−; Ripk3−/− MEFs (Fig. 3f). Thus, blocking key mediators of necroptosis can prolong the survival of TNFa/CHX-treated Abin-1−/− cells, a condition that normally induces RIPK1-independent apoptosis.

Since TNFa/CHX-induced apoptosis involves the activation of caspases, we next characterized the biochemical evidence for caspase activation in Abin-1−/− cells. Although the binding of RIPK1 with caspase-8 was not different in Abin-1−/− MEFs stimulated by TNFa/CHX compared with that of WT MEFs (Supplementary Fig. 2b), the stimulation of Abin-1−/− MEFs treated with TNFa/CHX led to not only earlier and stronger cleavage of PARP-1, caspase-3 and RIPK1, mediated by caspase-8 (ref. 45), but also the simultaneous appearance of phosphorylated MLKL, which, as expected, was not detected in Abin-1−/− MEFs (Fig. 4a). In addition, we also detected the formation of complex IIb, defined by the interaction between RIPK1/RIPK3 that is normally found only under necroptotic conditions after inhibition of caspases in Abin-1−/− MEFs stimulated by TNFa/CHX (Fig. 4b). The formation of complex IIb and phosphorylation of MLKL induced by TNFa/CHX in Abin-1−/− MEFs were blocked by Nec-1s and in Abin-1−/−; Ripk1D138N/D138N MEFs (Fig. 4c,d).

We next investigated the effect of inhibiting RIPK1 kinase activity on the hallmark of caspase activation in Abin-1−/− MEFs. Interestingly, Nec-1s treatment, genetic inactivation of RIPK1 kinase, and RIPK3 deficiency each blocked MLKL phosphorylation and prolonged the survival of Abin-1−/− MEFs treated with TNFa/CHX, without affecting caspase-3 activation or cleavage of its substrate, PARP. Thus, the normally pro-apoptotic stimulus TNFa/CHX can activate necroptosis in Abin-1-deficient cells and, furthermore, inhibition of RIPK1 kinase activity or RIPK3 deficiency can promote their survival without blocking caspase activation. Thus, inhibition of RIPK1 kinase activity and RIPK3 can promote the survival of Abin-1−/− MEFs stimulated by TNFa/CHX without blocking caspase activation.
Fig. 1 | ABIN-1 deficiency sensitizes cells to necroptosis. a–c. Immortalized (a,c) and primary (b) WT (Abin-1+/+) or Abin-1−/− MEFs were treated for 7 h (a) and 8 h (b) with TNFα/CHX (TC), TNFα/CHX/zVAD.fmk (TCZ) or staurosporine (STS) (a,b), in the presence or absence of Nec-1s, or TNFα/zVAD.fmk, or TNFα/zVAD.fmk (c), as indicated. d–f. 1,292 cells (d,e) or RGC-5 cells (f) were transfected with siRNAs targeting ABIN-1 to knockdown ABIN-1, or control (NC), for 48 h. The cells were then treated with TNFα (d,e) or TNFα/CHX/zVAD.fmk (f) with or without Nec-1s for 8 h. Knockdown efficiency is shown on the right of d–f. g. WT (Abin-1+/+) and Abin-1−/− MEFs were transfected with control non-targeting (NC), RIPK3 or MLKL siRNAs to knock down RIPK3 and MLKL. The death of MEFS treated with or without Nec-1s was measured. The knockdown efficiency is shown by western blotting with indicated antibodies on the right. h,i. The death of MEFS with the indicated genotypes induced by TNFα/CHX/zVAD.fmk with or without Nec-1s for 8 h was measured. TNFα (T): 10 ng ml−1; cycloheximide (CHX or C): 1 μg ml−1 (a,e–i) or 10 μg ml−1 (b,f); zVAD.fmk (Z): 20 μM; Nec-1s: 10 μM; staurosporine (STS): 1 μM. The graphs (a–i) depict the mean (± s.e.m.) of n = 4 independent biological experiments. P values were calculated by two-tailed Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001). Cell death was measured by ToxiLight (a,b,e–i) or CellTiter-Glo (c,d) assays. Source data together with precise P values can be found in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 7.

Recruitment of ABIN-1 into TNF-RSC is dependent on LUBAC and RIPK1. We next investigated whether ABIN-1 was recruited into TNF-RSC (complex I) of TNFα-stimulated cells. ABIN-1 was rapidly recruited into TNF-RSC within 5 min of TNFα stimulation (Fig. 5a). Although ABIN-1 deficiency had no effect on the recruitment of RIPK1 into TNF-RSC, the high-molecular-weight ubiquitination of RIPK1 was increased in Abin-1−/− MEFs. Furthermore, we found that significantly increased phosphorylation of Ser166
RIPK1 was associated with TNF-RSC in Abin-1−/− MEFs (Fig. 5b). Knockdown of ABIN-1 in RGC-5 cells had a similar effect (Supplementary Fig. 3a). Inhibition of RIPK1 by Nec-1s blocked the appearance of phospho-Ser166 RIPK1, but had no effect on the high-molecular-weight-ubiquitylated RIPK1 (Fig. 5c).

Since RIPK1 is known to be predominantly modified by Lys63 ubiquitylation in TNF-RSC, we next examined the impact of ABIN-1 deficiency on RIPK1 Lys63 ubiquitylation using tandem immunoprecipitation mass spectrometry (Tandem-MS). We found that Lys63 ubiquitylation of TNFR1-associated RIPK1 was increased in Abin-1−/− MEFS compared with that of WT (Fig. 5d). The increased Lys63 ubiquitylation levels of RIPK1 in Abin-1−/− MEFS were further confirmed by directly immunoprecipitating Lys63-ubiquitylated proteins in cells lysed in 6 M urea and followed by western blotting for RIPK1 (Supplementary Fig. 3b). In addition, we also analysed the Lys63-ubiquitylation pattern of RIPK1 in complex IIb; consistently, increased Lys63 ubiquitylation of RIPK1 in Abin-1−/− MEFS stimulated by TCZ was detected before the activation of RIPK1, detected by anti-p-Ser166 RIPK1 (Supplementary Fig. 3c).

Since ABIN-1 deficiency had no effect on the recruitment of HOIP and SHARPIN (Supplementary Fig. 3d), the key components of the LUBAC complex and cIAP1/2 in TNF-RSC, we next investigated whether there was a relationship between ABIN-1 deficiency and the recruitment of ABIN-1 into TNF-RSC. We found that the recruitment of ABIN-1 into TNF-RSC was affected by LUBAC. We found that the recruitment of ABIN-1 depends on the presence and/or output of the LUBAC complex and cIAP1/2 in TNF-RSC.

ABIN-1 stabilizes the recruitment of phospho-A20 in TNF-RSC. As HOIP-deficient cells are unable to recruit ABIN-1 or A20 to TNF-RSC, we next investigated whether there was a relationship...
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Abin-1, the recruitment of A20 to TNF-RSC. While the expression of A20 that ABIN-1 might control the activation of RIPK1 by regulating by TNFα/CHX/zV AD was enhanced compared with that of WT knockdown control is shown in Supplementary Fig. 1c.

62 1-deficient cells, the p-Ser166 RIPK1 in A20 –/– MEFs stimulated between the recruitment of these two proteins. Like that of ABIN-1-deficient cells, the p-Ser166 RIPK1 in A20 +/- MEFs were treated for 7 h (a) and 8 h (b) with TNFα/CHX (TC) in the presence or absence of Nec-1s as indicated. The knockdown control is shown in Supplementary Fig. 1c. WT and Abin-1 +/- MEFs were transfected with siRNA for RIPK3 or MLKL, or control non-targeting (NC) siRNA, to knock down RIPK3 and MLKL. The knockdown control is shown in Fig. 1d, f, g. MEFs with the indicated genotypes were treated with TNFα alone, CHX alone and TNFα/CHX as indicated. Depicted P values were calculated for Abin-1 +/- and Abin-1 +/- Ripk1D138N (f) or Abin-1 +/- and Abin-1 +/- Ripk3siRNA MEFs (g) treated with TC. Cell death was measured by ToxiLight (a-e) or CellTiter-Glo (f, g) assays. TNFα (T): 10 ng ml^-1; cycloheximide (CHX or C): 1 μg ml^-1; (a, c, e, g) or 10 μM (b, d); Nec-1s: 10 μM. The graphs depict the mean (±s.e.m.) of n = 4 independent biological experiments. P values were calculated by two-tailed Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001). Source data together with precise P values can be found in Supplementary Table 2.

TNFα stimulation has also been shown to lead to phosphorylation of A20 and CYLD by IKK complex as well as activation of p38 (refs. 39–42). While the phosphorylation of A20, CYLD and p38 induced by TNFα was largely unaffected by ABIN-1 deficiency (Supplementary Fig. 4a), using an anti-phospho-Ser381 A20 antibody the recruitment of these two proteins. Like that of ABIN-1-deficient cells, the p-Ser166 RIPK1 by regulating the recruitment of A20 to TNF-RSC. While the expression of A20 was largely normal in Abin-1 +/- MEFs, the levels of A20 in TNF-RSC were significantly reduced by ABIN-1 deficiency (Fig. 6a). On the other hand, the recruitment of IKKβ and p38 (refs. 39–42). While the phosphorylation of A20, CYLD and p38 induced by TNFα was largely unaffected by ABIN-1 deficiency (Supplementary Fig. 4a), using an anti-phospho-Ser381 A20 anti-body, the recruitment of phospho-A20 to TNF-RSC was significantly reduced in Abin-1 +/- MEFs compared with that of the WT (Fig. 6a). On the other hand, the recruitment of IKKβ and p38 (refs. 39–42). While the phosphorylation of A20, CYLD and p38 induced by TNFα was largely unaffected by ABIN-1 deficiency (Supplementary Fig. 4a), using an anti-phospho-Ser381 A20 anti-body, the recruitment of phospho-A20 to TNF-RSC was significantly reduced in Abin-1 +/- MEFs compared with that of the WT (Fig. 6a). On the other hand, the recruitment of IKKβ and p38 (refs. 39–42). While the phosphorylation of A20, CYLD and p38 induced by TNFα was largely unaffected by ABIN-1 deficiency (Supplementary Fig. 4a), using an anti-phospho-Ser381 A20 anti-body, the recruitment of phospho-A20 to TNF-RSC was significantly reduced in Abin-1 +/- MEFs compared with that of the WT (Fig. 6a).

Fig. 3 | Inhibition of RIPK1, RIPK3 and MLKL prolongs the survival of apoptotic ABIN-1-deficient cells induced by TNFα/CHX. a–c. Immortalized (a, c) and primary (b) WT (Abin-1+/+) or Abin-1 +/- MEFs were treated for 7 h (a) and 8 h (b) with TNFα/CHX (TC) in the presence or absence of Nec-1s as indicated. The knockdown control is shown in Supplementary Fig. 1c. WT and Abin-1 +/- MEFs were transfected with siRNA for RIPK3 or MLKL, or control non-targeting (NC) siRNA, to knock down RIPK3 and MLKL. The knockdown control is shown in Fig. 1d, f, g. MEFs with the indicated genotypes were treated with TNFα alone, CHX alone and TNFα/CHX as indicated. Depicted P values were calculated for Abin-1 +/- and Abin-1 +/- Ripk1D138N (f) or Abin-1 +/- and Abin-1 +/- Ripk3siRNA MEFs (g) treated with TC. Cell death was measured by ToxiLight (a–e) or CellTiter-Glo (f, g) assays. TNFα (T): 10 ng ml^-1; cycloheximide (CHX or C): 1 μg ml^-1; (a, c, e, g) or 10 μM (b, d); Nec-1s: 10 μM. The graphs depict the mean (±s.e.m.) of n = 4 independent biological experiments. P values were calculated by two-tailed Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001). Source data together with precise P values can be found in Supplementary Table 2.

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NEMO to TNF-RSC in Abin-1−/− MEFs was not altered (Fig. 6a,b). Importantly, the increased RIPK1 ubiquitylation in the TNF-RSC of Abin-1−/− MEFs was not affected by the treatment with Nec-1s (Fig. 6b). Thus, regulating the recruitment of phosphorylated A20 into TNF-RSC by ABIN-1 affects the ubiquitylation of RIPK1.

We next asked whether phosphorylation of A20 played a role in its recruitment into TNF-RSC. Using A20−/− MEFs complemented with WT or S381A A20 mutant, we found that the stimulation of TNFα induced the recruitment of WT, but not the S381A A20 mutant, into TNF-RSC (Fig. 6c). Furthermore, the recruitment of the S381D mutant in the WT cells was highly effective but remained deficient in Abin-1−/− MEFs (Fig. 6d). To directly test whether ABIN-1 might be able to interact with phosphorylated A20, we immunoprecipitated ABIN-1 from TNFα-stimulated cells and found that A20 interacting with ABIN-1 was effectively phosphorylated and, furthermore, the binding of ABIN-1 with the S381A mutant was significantly reduced (Fig. 6e,f), suggesting that a critical function of Ser381 phosphorylation of A20 is to promote its recruitment into TNF-RSC by interacting with ABIN-1.
Truncated ABIN-1 (amino acids 1–420) was unable to bind to A20 (Supplementary Fig. 4b). Consistently, unlike full-length ABIN-1, truncated ABIN-1 (amino acids 1–420) was unable to suppress the enhanced sensitivity of Abin-1−/− MEFs to necroptosis induced by TNFα/CHX/zVAD (Supplementary Fig. 4c). Furthermore, the truncated ABIN-1 (amino acids 1–420) could not be recruited into

Fig. 5 | Increased Lys63 ubiquitylation of RIPK1 in Abin-1−/− MEFs stimulated by TNFα. a–c, WT (Abin-1+/+) and Abin-1−/− MEFs were stimulated by Flag-TNFα (100 ng ml−1) for the indicated periods of time, and Nec-1s was added in selected samples as indicated (c). TNF-RSC (Complex I) was immunoprecipitated using anti-FLAG resin, and the recruitment of ABIN-1 and RIPK1 was analysed by western blotting using the indicated antibodies (a). The activation of RIPK1 was analysed by western blotting using anti-p-RIPK1(p-Ser166) antibody (b). β-Actin is a loading control. d, WT (Abin-1+/+) and Abin-1−/− MEFs were stimulated by Flag-TNFα (100 ng ml−1) for the indicated periods of time and the cell lysates were immunoprecipitated using anti-FLAG resin. The immunocomplexes were then denatured in 6 M urea. Lys63-chain-specific ubiquitin antibody was used in the second immunoprecipitation in 3 M urea to analyse RIPK1 ubiquitylation in TNF-RSC by western blotting using anti-RIPK1. e, Hoip+/+ and Hoip−/− MEFs were stimulated by TNFα, and anti-TNFFR1 was used to immunoprecipitate TNF-RSC. The immunocomplexes were analysed by western blotting using anti-ABIN-1, anti-A20, anti-RIPK1, anti-p-Ser166-RIPK1, anti-SHARPIN and anti-TNFR1. f, WT (Abin-1+/+) MEFs were pre-treated with SM-164 for 4 h, followed by TNFα for the indicated periods of time. Anti-TNFFR1 was used to immunoprecipitate TNF-RSC. The immunocomplexes were analysed by western blotting using anti-ABIN-1, anti-HOIP, anti-SHARPIN and anti-TNFR1. TNFα (T): 100 ng ml−1; Nec-1s: 10 μM; SM-164: 200 nM. Experiments were repeated independently with similar results twice (d) or at least three times (a–f). Unprocessed original scans of blots are shown in Supplementary Fig. 7.
Fig. 6 | ABIN-1 is important for the recruitment of p-A20 into TNF-RSC. a, WT (Abin-1+/+) and Abin-1−/− MEFs were stimulated by Flag-TNFα (100 ng ml−1) for the indicated periods of time. TNF-RSC (Complex I) was immunoprecipitated using anti-FLAG resin, and the recruitment of A20, p-A20(p-S381) and IKKβ was analysed using the indicated antibodies by western blotting analysis. b, WT (Abin-1+/+) and Abin-1−/− MEFs were stimulated by Flag-TNFα (100 ng ml−1) for the indicated periods of time in the presence or absence of Nec-1s as indicated, and TNF-RSC (Complex I) was immunoprecipitated using anti-FLAG resin and analysed using the indicated antibodies. c, A20−/− MEFs complemented with FLAG-A20 WT (FLAG-A20) or FLAG-A20-S381A mutant (S381A) were stimulated by TNFα (100 ng ml−1) for the indicated periods of time. Complex I was immunoprecipitated using anti-TNFα antibody from the lysates and analysed by anti-A20 and anti-TNFα.

d, TNF-RSC in WT (Abin-1+/+) and Abin-1−/− MEFs expressing FLAG-A20-S381D mutant was analysed by anti-TNFα. e, WT (Abin-1+/+) MEFs were stimulated by TNFα (100 ng ml−1) for the indicated periods of time. Complex I was immunoprecipitated from cell lysates using anti-ABIN-1 and the immunocomplex was analysed by anti-p-A20(p-S381) and IKKβ. f, WT (Abin-1+/+) MEFs were stimulated by TNFα (100 ng ml−1) for the indicated periods of time. The cell lysates were analysed by anti-A20 immunoprecipitation followed by western blotting using anti-A20 and anti-ABIN-1. β-Actin is a loading control.

Taking these results together, we conclude that the recruitment of phospho-A20 by ABIN-1 into TNF-RSC may play a critical role in regulating Lys63 ubiquitylation and activation of RIPK1.

Blocking necroptosis rescues the embryonic lethality of Abin-1−/− mice. Since Abin-1−/− deficiency in mice leads to TNFα-mediated embryonic lethality after post-coital day E18.5 (ref. 20), we considered the possibility that necroptosis might contribute to the embryonic lethality of Abin-1−/− mice. We generated Abin-1−/−; Ripk3−/− double-knockout (DKO) mice and Abin-1−/−; Ripk1D138N/D138N mice. As expected, we were unable to obtain viable Abin-1−/−; Ripk3−/− or Abin-1−/−; Ripk1−/− mice. Consistent with the activation of both apoptosis and necroptosis following loss of Abin-1, Abin-1−/− E16.5 embryonic tissues exhibited hallmarks of apoptosis, including the cleavage of caspase-3 and PARP-1, and necroptosis, including the increased levels of RIPK1, RIPK3 and MLKL in the

complex I; nor could the expression of truncated ABIN-1 rescue the ability of Abin-1−/− MEFs to recruit A20 (Supplementary Fig. 4d). Thus, the domain of ABIN-1 that is required for binding with A20 is also required for the recruitment of A20 and ABIN-1 into complex I.

Taking these results together, we conclude that the recruitment of phospho-A20 by ABIN-1 into TNF-RSC may play a critical role in regulating Lys63 ubiquitylation and activation of RIPK1.
insoluble fraction, which is known to occur when cells undergo necroptosis— as well as the phosphorylation of MLKL (Fig. 7a). Interestingly, these biochemical hallmarks of both apoptosis and necroptosis were completely absent in E16.5 Abin-1−/−; Ripk3−/− DKO and Abin-1−/−; Ripk1D138N/D138N embryos (Fig. 7b). Furthermore, Abin-1−/−; Ripk3−/− DKO mice and Abin-1−/−; Ripk1D138N/D138N mice were born at expected Mendelian ratios (Supplementary Table 1) and were indistinguishable from their WT littermates when examined at ~8 weeks of age (Supplementary Fig. 5). Furthermore, histopathological analysis and TUNEL staining of embryonic E18 livers revealed massive necrosis in Abin-1−/− mice that was suppressed by the RIPK1D138N mutation (Fig. 7c-e). Thus, inhibiting necroptosis and apoptosis effectively rescues the embryonic lethality and allows the normal development of Abin-1−/− mice.

Discussion
Understanding the molecular mechanism that regulates the dynamics of ubiquitylation-deubiquitylation of TNF-RSC has been a critical goal of TNFα research. We show here that the dominant function of ABIN-1 in TNF-RSC is to connect the Met1-ubiquitylating
complex LUBAC in TNF-RSC with the recruitment of A20, a deubiquitinating enzyme that modulates Lys63 ubiquitylation of RIPK1 (Supplementary Fig. 6). Phosphorylation of A20 has been shown to promote its deubiquitylation activity46–49; however, the mechanism by which TNF-RSC recruits this important modulator was unclear. The recruitment of ABIN-1 into TNF-RSC requires the LUBAC complex, whose recruitment depends on cIAP1/2, which provides Lys63, as well as Lys11, ubiquitylation of TNF-RSC74,75. The ability of ABIN-1 to bind to both Lys63 and Met1 ubiquitin chains76 fits perfectly with its role as a modulator of TNF-RSC ubiquitylation to connect the TNFR1 complex decorated with both Lys63 and Met1 ubiquitylations with the phospho-A20 deubiquitylating enzyme to modulate the activation of RIPK1. Thus, ABIN-1 is a key missing piece in connecting Met1/Lys63-ubiquitylated TNFR1 with A20-mediated deubiquitylation.

A20 was identified as a regulator of NF-κB66,67. Recently, it was reported that A20 deficiency sensitized cells to necroptosis67. Thus, the inability to effectively recruit A20 provides an important mechanism for the increased sensitivity of ABIN-1-deficient cells and Abin-1−/− mice to both apoptosis and necroptosis. These results also suggest that a critical mechanism mediated by the NF-κB pathway in suppressing the activation of RIPK1 and cell death is through upregulation of A20 expression and phosphorylation of A20 mediated by the activated IKK complex to promote its recruitment into TNF-RSC by interacting with ABIN-1 that in turn modulates the ubiquitylation pattern of RIPK1 to prevent its activation. Importantly, since ABIN-1 deficiency has no effect on the activation of NF-κB mediated by TNF, these results suggest that the dominant functions of ABIN-1 and A20 in the TNF-RSC are to control the activation of RIPK1.

LUBAC-mediated Met1 ubiquitylation is important for efficient recruitment of NEMO and activation of the NEMO/IKK/IKK (NEMO/IKK) complex to promote the downstream NF-κB pathway7. Our results suggest that mediating the recruitment of ABIN-1 might be an additional role of LUBAC-mediated Met1 ubiquitylation in TNF-RSC, independent of its role in regulating NF-κB activation. On the other hand, inactivation of LUBAC not only blocks the recruitment of ABIN-1, but also inhibits the phosphorylation of A20 mediated by the IKK complex. Thus, a common consequence of deficiency in either LUBAC or ABIN-1 is to lead to an imbalanced ubiquitylation pattern that includes dysregulated Lys63 ubiquitylation of RIPK1. Since deficiencies in HOIP, ABIN-1 and A20 all promote the activation of RIPK1, we propose that an imbalanced ubiquitylation of RIPK1 that includes increased Lys63 ubiquitylation and/or decreased Met1 ubiquitylation of RIPK1 may be critical in mediating its activation to promote cell death. Future studies are needed to identify the sites of RIPK1 modification by different E3 ubiquitin ligases, characterize the types of ubiquitin linkages and elucidate their functional significances in regulating the activation of RIPK1.

Our study described here demonstrates a unique paradigm that ABIN-1 deficiency sensitizes cells to both apoptosis and necroptosis. Furthermore, blocking RIPK1 or RIPK3 is sufficient to offer complete protection against embryonic lethality of Abin-1−/− mice. Necroptosis maybe activated in ABIN-1-deficient cells stimulated by TNFα/CHX, which is a classical pro-apoptotic stimulus in WT cells. Furthermore, blocking necroptosis might allow cell survival with activated caspases. The ability of cells to tolerate certain levels of activated caspases is consistent with the increasing acknowledgement of caspases mediating non-apoptotic functions, such as cytokine production, cell migration and neuronal function68.

Our results suggest that a key function of ABIN-1 during embryonic development is to suppress cell death by regulating TNFR1 signalling. Since suppression of necroptosis by RIPK1DSMN mutation or RIPK3 deficiency is sufficient to protect embryonic survival of Abin-1−/− mice, our results also suggest that the activation of necroptosis is highly detrimental to embryonic development. Since the activation of necroptosis mediates the lethality of mice with mutations in multiple genes such as caspase-8, FADD or the non-catalytically active caspase-8 homologue cFLIP and ABIN-1 during embryonic development9–12, we propose that necroptosis may be evoked as a key quality-control mechanism during embryogenesis to ensure the development of normal vertebrate animals.

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Methods

Animals. Rip3−/− mice were kindly provided by V. Dixit of Genentech. Rip3−/−/ab1 mice were kindly provided by M. Pasparakis of University of Cologne, Germany, and M. Kelliher of University of Massachusetts, USA. Eight- to ten-week-old Abin-1−/- and Rip3−/−/Abin-1−/- mice and female mice were mating in pairs. All animals were maintained in a pathogen-free environment, and experiments on mice were conducted according to the protocols approved by the Harvard Medical School Animal Care Committee.

Mouse genotyping. Lengths of 1–3 mm of mouse tails were cut and digested overnight at 55 °C in tail lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM NaCl, 0.5% SDS and 20 μg ml−1 Proteinase K). Cell debris was removed by centrifugation in a microfuge for 30 min at 4 °C. DNA was precipitated by isopropanol (mixed 1:1), washed with 50% ethanol and dried in a SpeedVAC. Pellets of DNA were resuspended in 50 mM Tris-HCl, pH 8.0 for 2–4 h. Abin-1 genotyping used the following primers (5’-3’): ATGGTGGTTAGGCATGCAGGATAG (common), CCTCCAAACAGCAGAGGAAAGC (WT) and TTGGATCCCTGGCCTACACCG (KO). For Rip3 (5’-3’): CGCTTCTAGAACCCCTAATGGTCAG (common), CGACGAGCTTGGTCCATG (KO) and CCAGAGCCCTAGCTTGGAC (KO). For Rip3−/−/Abin-1−/-: TACCTTCTAAAAAGCTTTCC (common), AATGGGAACCACAGCATTGGC (WT) and CCCTCGAAGAGGTCTCAG (KO).

Cell lines, plasmids, siRNA and cell culture. I.929, immortalized MEFs, HEK293T and RGC-5 were cultured under 5% CO2 at 37 °C in Dulbecco’s modified Eagle medium (DMEM) (Corning Cellgro, cat. no. 10-017-CV) containing 10% fetal bovine serum (FBS) (Gibco, cat. no. 10370-028), 1% penicillin-streptomycin (Gibco, cat. no. 15140-122) and 1% l-glutamine. Human kidney HEK293T cells were transfected using PEI (Polysciences, cat. no. V116) was dissolved in dimethylsulfoxide (DMSO) at a concentration 10 μM. Custom-synthesized (Selleckchem) Smac mimetic SM-164 (ref. 54) was dissolved in DMSO at a concentration of 10 μM and used at a final concentration of 500 nM. SZ-7-Oxazoeacon (Sigma, cat. no. 09899) was dissolved in DMSO at a concentration of 25 μM and used at final concentration of 500 nM.

Cell death assays. Cell viability was determined by using CellTiter-Glo Luminescent Cell Viability Assay (Promega, cat. no. G7571) and cell death by using ToxiLight Non-destructive Cytotoxicity BioAssay Kit (Lonza, cat. no. LT07). All experiments were conducted on 96-well plates using at least four biological replicates. Data were collected using Synergy 2: multimode plate reader from BioTek.

Antibodies, immunoprecipitation and immunoblotting. The following primary antibodies were used: ABIN-1 (1:1,000 Ubiquitin, cat. no. 68-0001-00), p-RIPK1 (1:1,000, 20 min), p-MLC (1:1,000), MLKL (1:1,000) and Abin-1 (1:500). Secondary antibodies were used: anti-rabbit (1:5,000 SouthernBiotech, cat. no. 4050-05), anti-mouse (1:5,000 SouthernBiotech, cat. no. 1031-05) and anti-β-actin (1:5,000 SouthernBiotech, cat. no. 10010-01).

For experiments not involving immunoprecipitation (IP), cell pellets were lysed in the RIPA lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 150 mM NaCl, 0.1% SDS, 0.2% sodium deoxycholate, 50 mM NaF, 10 mM Na3VO4, 5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM Na2VO4, complete Protease Inhibitor Cocktail (Sigma-Aldrich, cat. no. 11679498001), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM N-ethylmaleimide (NEM) (Sigma-Aldrich, cat. no. E3876). The protein concentration in the collected supernatants was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, cat. no. 23225) and normalized to the lowest concentration. Samples were diluted with 4× SDS-PAGE sample loading buffer (240 μM Tris-HCl pH 6.8, 40% (v/v) glycerol, 8% (v/v) SDS, 0.04% bromophenol blue and 5% (v/v) β-mercaptoethanol)

For Complex I IP, cells were harvested in NP-40 buffer (25 mM HEPES-KOH pH 7.5, 0.2% NP-40, 120 mM NaCl, 0.27 μM sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM Na2VO4, 2 μM Protease Inhibitor Cocktail, 2 mM PMSF and 20 mM NEM) and incubated either with FLAG-tagged beads (Sigma, cat. no. A2220) for 4 h, or overnight with TNFR1 antibody followed by 4 h incubation with Protein A/G ultra link resin (Thermo Scientific, cat. no. 35333). Complex IIb was immunoprecipitated by incubation with protein G-Sepharose, washed with lysis buffer and eluted in sample buffer for 4°C with rotation followed by 4 h incubation with Protein A/G ultra link resin. For Lys63 ubiquitin IP, cells were harvested in 6 M urea lysis buffer (6 M urea, 0.5 M NaCl, 0.1 M EDTA, 1 M EGTA, 50 mM Na2VO4, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM Na2VO4, 2 μM Protease Inhibitor Cocktail, 2 mM PMSF and 20 mM NEM) and flash frozen in liquid nitrogen. Thawed lysates were centrifuged at 16,000 g for 15 min at 4°C and supernatants were diluted with lysis buffer (without urea) to bring the urea concentration to 3 M. For Lys63 IP, 3 μg of the chain-specific antibody was incubated at 4°C overnight followed by 4 h incubation with Protease A/G ultra link resin (Pierce, cat. no. 26101). Beads were washed and eluted in 70 μl with 2× SDS-PAGE sample buffer using Thermomixer (Eppendorf).

The primary antibodies were incubated in 2% BSA overnight at 4°C. Membranes were washed three times (8 min each time) with TBST. Appropriate HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. After application of ECL solution membranes were exposed to X-ray film. The results were analyzed with GelPro Analyzer software.
Statistics and reproducibility. Each individual experiment was repeated independently with similar results at least three times, except the experiments shown in Figs. 2e, 4d, 5d, 6c, 6d and 7a,b and Supplementary Figs. 2b and 3c, which were performed twice. Statistical analysis was performed using Microsoft Excel 2011. Prism 7 software was used to generate graphs. Statistical significance was evaluated by using two-tailed Student’s t-test. Differences were considered statistically significant if \( P < 0.05 (^{*}) \); \( P < 0.01 (** \) or \( P < 0.001 (*** \). Data are expressed as the mean ± standard error of the mean (s.e.m.). Exact \( P \) values for each experiment (where applicable) can be found in Supplementary Table 2.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. All data that support the conclusions are available from the authors on reasonable request. Source data for Figs. 1a–i and 3a–g and Supplementary Figs. 1a–c, 2a, 4c and 5a,b have been provided as Supplementary Table 2.

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- **Experimental design**

1. **Sample size**
   - Describe how sample size was determined.
   - No statistical method was used to predetermine sample size.

2. **Data exclusions**
   - Describe any data exclusions.
   - No data were excluded from the analysis.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - Each individual experiment was repeated independently with similar results at least three times, except experiments shown in Figs 1c, 2e, 3f, g, 4d, 5d, 6c, d, f and Supplementary Figs 2b, and 3c twice.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Randomization was not relevant to this study as only appropriate cell lines were tested.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Investigators were not blinded to the group allocation during data collection and analysis.

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

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

MetaMorph image acquisition software, ImageJ, Graph Pad Prism 8, Adobe Illustrator and Microsoft Excel 2011

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

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

All plasmids created in this study are available from the authors on request. All other materials used are commercially available.
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 primary antibodies were used: ABIN-1 (1:1,000 Ubiquigent, Cat# 68-0001-100; validation: https://www.ubiquigent.com/images/uploads/68-0001-100-30238-v1-0-0.pdf), p-RIPK1 (1:1,000 Cell Signaling, Cat#65746; validation: https://media.cellsignal.com/pdf/65746.pdf), p-MLKL (1:1,000 Abcam, Cat# ab196436; validation: http://www.abcam.com/mlk-phospho-s345-antibody-epr95152-ab196436.html), MLKL (1:1,000 Abcam, Cat# ab67942; validation: http://www.abcam.com/mlk-antibody-ab67942.html), PARP-1 (1:1,000 Cell Signaling, Cat# 9542; validation: https://media.cellsignal.com/pdf/9542.pdf), cleaved Caspase-3 (1:1,000 Cell Signaling, Cat# 9661; validation: https://media.cellsignal.com/pdf/9661.pdf), Caspase-8 (1:2,000 Enzo, Cat# ALX-804-447-C100; validation: http://www.enzolifesciences.com/ALX-804-447/caspase-8-mouse-monoclonal-antibody-1g12/), SHARPIN (1:1,000 Proteintech, Cat# 14626-1-AP; validation: http://www.ptglab.com/products/SHARPIN-Antibody-14626-1-AP.htm), HOIP (1:1,000 Abcam, Cat# ab46322; validation: http://www.abcam.com/mf31hoip-antibody-chip-grade-ab46322.html), NEMO (1:1,000 Cell Signaling, Cat# 2685S; validation: https://www.cellsignal.com/products/primary-antibodies/ikkg-antibody/2685), p-CyLD (1:1,000 Cell Signaling, Cat# 4500S; validation: https://www.cellsignal.com/products/primary-antibodies/phospho-cyld-ser418-antibody/4500), CYLD (1:1,000 Cell Signaling, Cat# 8462; validation: https://www.cellsignal.com/products/primary-antibodies/cyld-d1a10-rabbit-mab/8462), p-p38 (1:1,000 Cell Signaling, Cat# 9212; validation: https://media.cellsignal.com/pdf/9211.pdf), K63 ubiquitin antibody (kindly provided by Genentech; validation: https://www.ncbi.nlm.nih.gov/pubmed/18724939), RIPK1 (1:1,000 Cell Signaling, Cat# 3493; validation: https://media.cellsignal.com/pdf/3493.pdf and 1:1,000 BD Biosciences, Cat# 610459; validation: http://www.bdbiosciences.com/us/applications/research/apoptosis/purified-antibodies/purified-mouse-anti-rip-38rip/p/610459), RIP3 (1:1,000 Serotec, Cat# AHP1797; validation: https://www.bio-rad-antibodies.com/mouse-rip3-antibody-m-19.html and 1:300 Santa Cruz, Cat# sc-374639; validation: https://datasheets.scbt.com/sc-374639.pdf), TNFR1 (1:1,000 Cell Signaling, Cat# 133775; validation: https://media.cellsignal.com/pdf/13377.pdf and R&D Systems, Cat# AF-425-PB, validation: https://www.rndsystems.com/products/mouse-tnfr1-tnfrs1a-antibody-af-425-pb), A20 (1:1,000 Cell Signaling, Cat# 5630; validation: https://media.cellsignal.com/pdf/5630.pdf), IKKp (1:1,000 BD Technologies, Cat# 611254; validation: https://www.bdbiosciences.com/us/applications/research/apoptosis/purified-antibodies/purified-mouse-anti-rip-38rip/p/610459), FLAG-M2 (1:5,000 Sigma, Cat# F3165, validation: http://www.molecularinfo.com/MTM/I/I3/FlagAb.pdf), HA-probe (1:1,000 Santa Cruz, Cat# sc-805; validation: http://datasheets.scbt.com/sc-805.pdf), β-actin (1:5,000 Santa Cruz, Cat# sc-81178; validation: https://datasheets.scbt.com/sc-81178.pdf). The following secondary antibodies were used: anti-rabbit (1:5,000 SouthernBiotech, Cat# 4050-05), anti-mouse (1:5,000 SouthernBiotech, Cat# 1031-05), anti-rat (1:5,000 SouthernBiotech, Cat# 3010-05) and anti-sheep (1:5,000 SouthernBiotech, Cat# 2018-02).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. 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.

Abin1, Ripk3 and Ripk1 D138N MEFs were isolated from the appropriate mice embryos as described in Methods section. L929, HEK239T and RGC-5 were purchased from ATCC

MEFs were authenticated by genotyping and western blot.

All of the cell lines were tested regularly for mycoplasma contamination.

No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample
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.

Abin-1+/- and Ripk1-D138N (C57BL/6), Ripk3-/- (C57BL/6N), males and females, 8 weeks old. For MEFs generation 12-14 weeks old.

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.

The study did not involve human research participants