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RIPK1 counteracts ZBP1-mediated necroptosis to inhibit inflammation

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Abstract

Receptor interacting protein kinase 1 (RIPK1) regulates cell death and inflammation via kinase-dependent and -independent functions^{1–7}. RIPK1 kinase activity induces caspase-8-dependent apoptosis and RIPK3/Mixed Lineage Kinase Like (MLKL)-dependent necroptosis^{8–13}. In addition, RIPK1 inhibits apoptosis and necroptosis via kinase-independent functions, which are important for late embryonic development and the prevention of inflammation in epithelial barriers^{14–18}. The mechanism by which RIPK1 counteracts RIPK3/MLKL-mediated necroptosis has remained enigmatic. Here we show that RIPK1 prevents skin inflammation by inhibiting Z-DNA binding protein 1 (ZBP1, also named DAI or DLM1)-mediated activation of RIPK3/MLKL-dependent necroptosis. ZBP1 deficiency inhibited keratinocyte necroptosis and skin inflammation in mice with epidermis-specific RIPK1 knockout. Moreover, mutation of the conserved RIP Homotypic Interaction Motif (RHIM) of endogenous mouse RIPK1 (RIPK1^{mRHIM}) caused perinatal lethality that was prevented by RIPK3, MLKL or ZBP1 deficiency. Furthermore, mice expressing only RIPK1^{mRHIM} in keratinocytes developed skin inflammation that was abrogated by MLKL or ZBP1 deficiency. Mechanistically, ZBP1 interacted strongly with phosphorylated RIPK3 in cells expressing RIPK1^{mRHIM}, suggesting that the RIPK1 RHIM prevents ZBP1 from binding and activating RIPK3. Collectively, these results showed that RIPK1 prevents perinatal

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Author Contributions

J.L. designed and generated the *Ripk1^{mRHIM}* mice. J.L. and C.K. analysed *Ripk1^{mRHIM}/mRHIM* and *RIPK1^{mRHIM}/E-KO* mice and performed genetic crosses to address the role of RIPK3 and ZBP1 in these mice. J.L. and C.K. carried out all immunoblots and immunoprecipitation experiments. S.K. generated and characterised *RIPK1^{E-KO} Zbp1^{-/-}* and *FADD^{E-KO} Zbp1^{-/-}* mice and made the initial discovery that ZBP1 is required for keratinocyte necroptosis in *RIPK1^{E-KO}* mice. S.K. and T.-M.V. conducted immunostainings and qRT-PCR assays in skin samples from *RIPK1^{E-KO}* and *RIPK1^{mRHIM}/E-KO* mice. A.P. generated the *Ripk1^{FL/FL}* mice and L.W. generated *Mkt^{-/-}* mice. M.P. supervised the study, interpreted data and wrote the manuscript together with J.L., C.K. and S.K. J.L., C.K. and S.K. contributed equally and their order of appearance in the author list is random.

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The authors declare no competing financial interest.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the paper. Source data for all figures are provided with the paper. The *Ripk1^{mRHIM}* mice are available from the corresponding author upon request.

death as well as skin inflammation in adult mice by inhibiting ZBP1-induced necroptosis. Furthermore, these findings identify ZBP1 as a critical mediator of inflammation beyond its previously known role in anti-viral defence and suggest that ZBP1 might be implicated in the pathogenesis of necroptosis-associated inflammatory diseases.

Mice with epidermis-specific RIPK1 deficiency (*Ripk1^{FL/FL} K14-Cre^{tg/wt}*, hereafter referred to as RIPK1^{E-KO}) develop skin inflammation due to RIPK3/MLKL-dependent keratinocyte necroptosis¹⁴. We hypothesised that other RHIM-containing proteins may induce RIPK3/MLKL-mediated necroptosis in RIPK1-deficient keratinocytes. In addition to RIPK1 and RIPK3, the only other proteins containing RHIM in humans and mice are TRIF and ZBP1^{13,6,19,20}. We showed previously that TRIF deficiency very mildly ameliorates but does not prevent skin inflammation in RIPK1^{E-KO} mice¹⁴, suggesting that TRIF is not essential for RIPK3 activation and necroptosis in RIPK1-deficient epidermal keratinocytes. ZBP1 is a RHIM-containing protein previously identified as a cytoplasmic DNA sensor capable of inducing type I interferon expression and NF- κ B activation^{19–23}. More recently, ZBP1 was shown to induce necroptosis by activating RIPK3 independently of RIPK1 in response to cytomegalovirus infection²⁴. We therefore hypothesised that ZBP1 might be implicated in triggering RIPK3 activation and necroptosis in epidermal keratinocytes of RIPK1^{E-KO} mice. Immunoblot analysis of epidermal extracts showed that ZBP1 was expressed at low levels in wild type mice but its expression was strongly increased in the epidermis of RIPK1^{E-KO} mice at the age of four weeks (Fig. 1a), supporting that ZBP1 could be involved in triggering keratinocyte necroptosis in this model.

To address the potential role of ZBP1 in triggering keratinocyte necroptosis and skin inflammation in RIPK1^{E-KO} mice we crossed them with *Zbp1^{-/-}* animals²⁵. RIPK1^{E-KO} *Zbp1^{-/-}* mice did not show macroscopic signs of skin disease at the age of four weeks, in contrast to RIPK1^{E-KO} animals that displayed inflammatory skin lesions at this age (Extended Data Fig. 1a). Histological analysis confirmed that 4–5 week-old RIPK1^{E-KO} *Zbp1^{-/-}* animals did not develop skin lesions, as shown by normal epidermal thickness and typical expression of epidermal differentiation markers including Keratins 14, 10 and 6 (Fig. 1b, c). In addition, the skin of RIPK1^{E-KO} *Zbp1^{-/-}* mice did not show increased infiltration of F4/80⁺ myeloid cells and upregulation of inflammatory cytokines and chemokines (Fig. 1b, d). ZBP1 deficiency also reduced the number of TUNEL⁺ keratinocytes in the epidermis of RIPK1^{E-KO} mice (Extended data Fig. 1e, f), suggesting that it prevented keratinocyte necroptosis. RIPK1^{E-KO} *Zbp1^{-/-}* mice remained healthy till the age of 18–20 weeks but subsequently progressively developed inflammatory skin lesions, which however remained milder and focal compared to the severe inflammation affecting the entire skin observed in 4–5 week-old RIPK1^{E-KO} mice (Extended Data Fig. 1b–d). Therefore, ZBP1 deficiency strongly inhibited but did not completely prevent skin inflammation in RIPK1^{E-KO} mice, in contrast to RIPK3 or MLKL deficiency that fully abrogated lesion development in these animals¹⁴. These results suggest that ZBP1 plays a critical role in the induction of RIPK3/MLKL-dependent keratinocyte necroptosis in RIPK1^{E-KO} mice but in its absence alternative mechanisms can activate RIPK3 to trigger necroptosis. Although keratinocyte-specific TRIF deficiency did not considerably inhibit skin inflammation in RIPK1^{E-KO} mice¹⁴, it is possible that TRIF might contribute to skin lesion development in the absence of ZBP1 in

adult RIPK1^{E-KO} *Zbp1*^{-/-} animals. To address whether ZBP1 is generally required for keratinocyte necroptosis we assessed the role of ZBP1 in mice with epidermis-specific FADD deficiency (FADD^{E-KO}), which develop skin inflammation due to RIPK3-mediated keratinocyte necroptosis²⁶. FADD^{E-KO} *Zbp1*^{-/-} mice developed skin inflammation similarly to FADD^{E-KO} mice, showing that ZBP1 deficiency did not inhibit RIPK3-dependent skin inflammation in this model (Extended Data Fig. 2). Thus, ZBP1 induces keratinocyte necroptosis in the absence of RIPK1 but it is not required for keratinocyte necroptosis and skin inflammation caused by epidermal FADD deficiency.

We reasoned that RIPK1 might prevent ZBP1-mediated RIPK3 activation by interacting with these two proteins via its RHIM domain. To specifically address the role of the RHIM domain of RIPK1 *in vivo*, we generated knock-in mice expressing a mutated RIPK1 protein where the QIG conserved amino acids of the RHIM domain at position 529–531 were substituted with alanines (RIPK1^{QIG-AAA}, hereafter referred to as RIPK1^{mRHIM}) using CRISPR/Cas9-mediated gene targeting in mouse zygotes (Extended Data Fig. 3a). Genotyping of progeny obtained from intercrossing heterozygous *Ripk1*^{mRHIM/WT} mice from two independently generated knock-in lines failed to identify any homozygous *Ripk1*^{mRHIM/mRHIM} mice at weaning age (Fig. 2a). Examination of pups obtained from timed matings revealed that *Ripk1*^{mRHIM/mRHIM} mice were alive at 18.5 but died perinatally, similarly to *Ripk1*^{-/-} mice^{15–17,27}. Histological analysis of E18.5 pups revealed epidermal hyperplasia, massive infiltration of F4/80⁺ myeloid cells as well as increased number of TUNEL⁺ and a few cleaved caspase-3 positive (CC3⁺) cells in the dermis of *Ripk1*^{mRHIM/mRHIM} and *Ripk1*^{-/-} pups (Fig. 2b, c). Analysis of intestinal tissues revealed scarce presence of CC3⁺ apoptotic cells in *Ripk1*^{mRHIM/mRHIM} pups compared to the increased number of CC3⁺ cells found in the intestine of *Ripk1*^{-/-} mice (Extended Data Fig. 3b, c). Earlier studies showed that the epidermal hyperplasia observed in *Ripk1*^{-/-} pups depends on RIPK3/MLKL-mediated necroptosis¹⁷, while the intestinal epithelial cell apoptosis is driven by FADD/Caspase-8-dependent apoptosis^{14,15,17,18}. Inhibition of both necroptosis and apoptosis was required to overcome the perinatal lethality of *Ripk1*^{-/-} mice^{15–17}. However, RIPK3 deficiency was sufficient to prevent perinatal lethality of *Ripk1*^{mRHIM/mRHIM} mice, as *Ripk1*^{mRHIM/mRHIM} *Ripk3*^{-/-} animals reached adulthood without showing any signs of pathology at least up to the age of 6 months (Extended Data Table 1 and Extended Data Fig. 4a, b). Interestingly, also *Ripk1*^{mRHIM/mRHIM} *Ripk3*^{wt/-} mice survived to adulthood suggesting that reduction of RIPK3 protein levels by about 50% was sufficient to prevent necroptosis and perinatal lethality in these animals (Extended Data Table 1). In addition, crossing with MLKL-deficient mice (Extended Data Fig. 3d) also rescued perinatal death of *Ripk1*^{mRHIM/mRHIM} animals, with *Ripk1*^{mRHIM/mRHIM} *MLK1*^{-/-} mice surviving at least up to 4 months without signs of disease (Extended Data Table 1 and Extended Data Fig. 4a). Furthermore, ZBP1 expression was upregulated in the skin of *Ripk1*^{mRHIM/mRHIM} pups (Fig. 2d) and ZBP1 deficiency also prevented perinatal lethality of these mice, with *Ripk1*^{mRHIM/mRHIM} *Zbp1*^{-/-} mice surviving at least up to the age of 5 months without showing apparent abnormalities (Extended Data Table 1 and Extended Data Fig. 4a, b). TRIF knockout did not rescue the *Ripk1*^{mRHIM/mRHIM} mice (Extended Data Table 1). Therefore, in contrast to RIPK1 deficiency that causes perinatal lethality due to both caspase-8-mediated apoptosis and RIPK3/MLKL-mediated necroptosis^{15–17}, mutation

of RIPK1 RHIM caused perinatal death exclusively due to ZBP1/RIPK3/MLKL-dependent necroptosis.

To confirm that the RIPK1^{QIG-AAA} mutation disrupted the interaction of RIPK1 with RIPK3, we stimulated primary MEFs from *Ripk1*^{mRHIM/mRHIM} mice with TNF in the presence of cycloheximide (CHX) and zVAD-fmk (TCZ treatment) for different periods of time to induce formation of the necrosome. Immunoblot analysis of RIPK1 immunoprecipitates revealed that RIPK3 strongly interacted with RIPK1 in TCZ-treated wild type but not in *Ripk1*^{mRHIM/mRHIM} primary MEFs (Fig. 3a). Consistently, primary *Ripk1*^{mRHIM/mRHIM} MEFs showed reduced cell death in response to TCZ treatment and foetal liver macrophages (FLMs) from *Ripk1*^{mRHIM/mRHIM} pups were resistant to necroptosis induced by stimulation with TNF + z-VAD-fmk (TZ treatment) (Fig. 3b, c). Notably, we routinely obtained reduced numbers of FLMs from *Ripk1*^{mRHIM/mRHIM} compared to wild type embryos, and the expression levels of ZBP1 were reduced in the *Ripk1*^{mRHIM/mRHIM} cells (Fig. 3d) suggesting that *Ripk1*^{mRHIM/mRHIM} FLMs expressing high levels of ZBP1 may be counter-selected in these cultures. Therefore, disruption of the RHIM-dependent interaction of RIPK1 with RIPK3 protected primary FLMs and MEFs from TNF-induced necroptosis. TNF-induced NF-κB activation was not impaired in *Ripk1*^{mRHIM/mRHIM} MEFs or FLMs (Fig. 3e, f), showing that RHIM-dependent RIPK1 interactions are not required for TNFR1-induced proinflammatory signalling.

To address whether RIPK1 prevents keratinocyte necroptosis and skin inflammation in a RHIM-dependent manner, we crossed *Ripk1*^{mRHIM/wt} with *Ripk1*^{FL/wt} *K14-Cre*^{tg/wt} mice to generate *Ripk1*^{mRHIM/FL} *K14-Cre*^{tg/wt} mice (hereafter referred to as RIPK1^{mRHIM/E-KO}), which express exclusively the mutant RIPK1^{mRHIM} in keratinocytes. In contrast to *Ripk1*^{mRHIM/wt} mice that did not show pathology in their skin or other organs (data not shown), RIPK1^{mRHIM/E-KO} mice developed macroscopically visible signs of skin lesions starting at about 3-4 weeks after birth, which progressively developed to inflammatory skin disease by the age of 9-11 weeks (Fig. 4a-c and Extended Data Fig. 5a-d). Histological analysis showed that the skin lesions in RIPK1^{mRHIM/E-KO} mice resembled those seen in RIPK1^{E-KO} mice, characterised by epidermal hyperplasia and impaired differentiation, increased numbers of dying keratinocytes as well as increased F4/80⁺ myeloid cell infiltration and upregulation of inflammatory cytokine and chemokine expression (Fig. 4a-c and Extended Data Fig. 5c-d). As in RIPK1^{E-KO} mice, homozygous but not heterozygous MLKL deficiency prevented skin lesion development in RIPK1^{mRHIM/E-KO} mice at least up to the age of 22 weeks, showing that the inflammatory skin disease is triggered by MLKL-mediated keratinocyte necroptosis (Fig. 4a-c and Extended Data Fig. 5a-d). RIPK1^{mRHIM/E-KO} mice showed increased expression of ZBP1 in the skin, similarly to RIPK1^{E-KO} mice (Fig. 4d, e). ZBP1 was not expressed in primary keratinocytes from wild type, RIPK1^{E-KO} or RIPK1^{mRHIM/E-KO} mice (Extended Data Fig. 6a), suggesting that its upregulation in the epidermis could be triggered by signals related to the *in vivo* tissue context. Indeed, the increased expression of *Ifnb1* in the skin of RIPK1^{mRHIM/E-KO} mice could be responsible for the upregulation of ZBP1 expression (Fig. 4e) as stimulation with IFNβ induced robust ZBP1 expression in cultured primary keratinocytes from wild type, RIPK1^{E-KO} and RIPK1^{mRHIM/E-KO} mice (Extended Data Fig. 6b). In line with our findings in RIPK1^{E-KO} animals, ZBP1 deficiency prevented the development of skin lesions in

RIPK1^{mRHIM/E-KO} mice at least up to the age of 21 weeks (Fig. 4a-c and Extended Data Fig. 5a, c, d). These results showed that RHIM-dependent RIPK1 function in epidermal keratinocytes is critical to prevent ZBP1-mediated activation of RIPK3/MLKL-driven necroptosis and skin inflammation.

We postulated that RIPK1 may bind ZBP1 and prevent its interaction with RIPK3. Since ZBP1 is not expressed in MEFs, we transduced primary wild type or *Ripk1*^{mRHIM/mRHIM} MEFs with a lentiviral vector expressing FLAG-tagged murine ZBP1. Immunoblotting of anti-FLAG immunoprecipitates with anti-RIPK3 antibodies showed that ZBP1 interacted weakly with RIPK3 in wild type MEFs, but this interaction was strongly enhanced in *Ripk1*^{mRHIM/mRHIM} MEFs (Fig. 4f). A slower migrating RIPK3 species was detected in the anti-FLAG immunoprecipitate suggesting that ZBP1 could preferentially associate with phosphorylated RIPK3. Indeed, immunoblotting with monoclonal antibodies specifically recognising RIPK3 phosphorylated at serine 232 (ref 28) revealed that the slower migrating band corresponded to phosphorylated RIPK3 (Fig. 4f). Caspase-8 or MLKL were not detected in the anti-FLAG immunoprecipitate suggesting that these proteins do not interact with ZBP1 under these conditions (Fig 4f). Immunoblotting with anti-RIPK1 antibodies failed to detect RIPK1 in the anti-FLAG immunoprecipitate (Fig. 4f). Moreover, reciprocal immunoprecipitation using anti-RIPK1 antibodies and immunoblotting with anti-FLAG or anti-ZBP1 antibodies also failed to detect an interaction between RIPK1 and ZBP1 (Fig. 4g). Therefore, in contrast to RIPK3, RIPK1 did not interact with ZBP1 in primary MEFs. It is not clear why our results differ from previous studies showing that ZBP1 interacted with RIPK1 in 293T cells^{19,20}, but this could be related to the absence of RIPK3 expression in 293T cells⁹.

Taken together, our results showed that, in the absence of the RIPK1 RHIM domain, ZBP1 strongly interacted with RIPK3 inducing its autophosphorylation triggering downstream activation of MLKL and necroptosis. The inhibitory role of the RIPK1 RHIM domain is particularly important for the maintenance of skin homeostasis during late embryonic life and in adult mice. However, since the lack of RIPK1 or its RHIM specifically in the epidermis triggers keratinocytes necroptosis and inflammation starting few weeks after birth, whereas ubiquitous RIPK1 deficiency or RHIM mutation triggers necroptosis of dermal cells and results in perinatal death, it is likely that the skin hyperplasia during late embryonic life and the associated perinatal lethality are caused by necroptosis of non-epithelial, perhaps stromal or myeloid, cells. Although the precise mechanism of the RIPK1 RHIM-dependent inhibition of ZBP1-mediated RIPK3 activation remains elusive at present, it is possible that RIPK1 associates with RIPK3 to prevent its interaction with ZBP1. At this stage, it is also unclear whether the nucleic acid sensing properties of ZBP1 are involved in activating RIPK3-dependent necroptosis in the absence of the RIPK1 RHIM domain. Taken together, our results revealed an important role of the RIPK1 RHIM domain in counteracting ZBP1-mediated activation of RIPK3/MLKL-dependent necroptosis, which is critical for preventing lethality during late embryogenesis and skin inflammation in adult mice. These findings identify ZBP1 as a potent inducer of inflammation beyond its role in anti-viral defence^{24,29} and suggest that it could be implicated in inflammatory diseases. Future studies will be required to elucidate the mechanism of ZBP1 activation and how RIPK1 inhibits it, but also its potential implication in the pathogenesis of human diseases.

Methods

Mice

Ripk1^{FL/FL} (ref 14) and *Fadd*^{FL/FL} (ref 26), *K14-Cre30*, *Ripk3*^{-/-} (ref 31), and *Zbp1*^{-/-} (ref 25) mice were described previously. Mice were maintained at the SPF animal facilities of the Institute for Genetics and the CECAD Research Center of the University of Cologne, under a 12 h light cycle, and given a regular chow diet (Harlan, diet no. 2918 or Prolab Isopro RMH3000 5P76) *ad libitum*. All animal procedures were conducted in accordance with European, national and institutional guidelines and protocols were approved by local government authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany). Animals requiring medical attention were provided with appropriate care and were sacrificed when they developed macroscopically visible skin lesions to minimize suffering. No other exclusion criteria existed. Mice of the indicated genotype were assigned at random to groups. Mouse studies were performed in a blinded fashion.

Generation of *Ripk1*^{mRHIM} and *Mik1*^{-/-} mice using Crispr/Cas9-mediated gene targeting in mouse zygotes

For the generation of *Ripk1*^{mRHIM} mice Cas9 mRNA (TriLink) together with the 129bp ssDNA repair oligo (IDT) and the short guide RNA (sgRNA) targeting the RHIM domain of the murine *Ripk1* gene were microinjected into the pronucleus of fertilized oocytes obtained from C57BL/6 mice. For the generation of the *Mik1*^{-/-} allele Cas9 mRNA together with the sgRNA targeting the *Mik1* gene were microinjected into the pronucleus of fertilized oocytes obtained from C57BL/6 mice. On the next day, the injected embryos were transferred to foster mothers and allowed to develop to term. Mutations in the genome of progeny were determined by analysis of genomic DNA using the T7 endonuclease I assay (NEB) and sequencing. For the analysis of the *Mik1* locus an additional ApaI digest was performed. The sequence of the ssDNA oligo used as a repair template for the RipK1 RHIM domain is: 5' - TATCTCTTTTCTATTTCAGATGACCTCATAAAATATACTATATTCAATAGTTCTGGTAT TGCAGCAGCTAACCACAATTATATGGATGTTGGACTGAATTCACAACCACCAAACA ATACTTGCAAAGAA-3'. sgRNA was generated by *in vitro* transcription (NEB, E2040S) from the px330 vector (42230, Addgene) containing the *Ripk1* targeting sequence: 5' - aatagtctgtgattcagat-3' or the *Mik1* targeting sequence: 5' - cgtctaggaaaccgtgtgca-3'. An *Mik1* allele shown to have a 2bp deletion that causes a frameshift and a premature stop was propagated as the *Mik1* knockout allele used for this study.

Histological analysis of tissue sections

Skin and intestine tissues were embedded in paraffin or snap frozen in OCT compound. Antigen retrieval for paraffin sections was performed in citrate buffer, pH6 for the skin sections from *RIPK1*^{E-KO} and *RIPK1*^{mRHIM/E-KO} mice and in Tris-EDTA buffer, pH9 or Proteinase K for the skin and intestine sections from *Ripk1*^{mRHIM} and *Ripk1*^{-/-} mice. Anti-active caspase 3 (9661, Cell signalling), anti-F4/80 (clone A3-1, homemade or MCA497G, BIO-RAD), anti-Keratin 14 (MS-115, Neomarkers), anti-Keratin 6 (PRB-169P, Covance), anti-Keratin 10 (PRB-159P, Covance) were used for the staining. Stainings were visualized with ABC Kit Vectastain Elite (Vector Laboratories) or Streptavidin-HRP (Millipore) and

DAB substrate (DAKO and Vector Laboratories) or Alexa-488 and Alexa-549 fluorescence conjugated secondary antibody. F4/80 staining was done on cryo sections. All sections were counterstained with haematoxylin or stained with DAPI. TUNEL assay was performed using DeadEnd™ Fluorometric TUNEL System (G3250, Promega) as per manufacturer's instructions. Quantification of epidermal thickness was performed by measurement of epidermal thickness in five optical fields per section. In each field, four measurements were performed. Percentage of inflamed area was determined as the percentage of inflamed versus total number of optical fields at 20x on individual skin sections. TUNEL and CC3 positive cells were quantified under 3-4 optical fields and normalized over the visual area counted.

Immunoblotting and immunoprecipitation

Antibodies against the following proteins were used for Western blot analysis: RIPK3 (ADI-905-242-100, Enzo or 95702, Cell signaling), RIPK1 (610459, BD or 3493, Cell signaling), Tubulin (T6074, Sigma), p-IκBα (9246, Cell signaling), IκBα (sc-371, SantaCruz), p-p65 (3033, Cell signaling), Flag (F7425, Sigma), ZBP1 (AG-20B-0010, Adipogen or custom-made, Eurogentec), p-RIPK3 (ab195117, Abcam), Caspase 8 (4790, Cell signaling), MLKL (MABC604, Millipore), GAPDH (NB300-221, Novus). The signals were detected by SuperSignal West Pico Chemiluminescent substrate (34080, Thermo). The membranes were reprobed after incubation in Restore Western Blot stripping buffer (21059, Thermo).

In order to co-immunoprecipitate ZBP1 interacting proteins, ZBP1 cDNA was generated from IFNβ (97265, Biomol) stimulated wild type primary MEFs using Superscript III first-strand kit (18080-051, Invitrogen) and the sequence confirmed DNA was cloned into lentiviral pBOB-Flag vector (provided by Dr. J. Han, Xiamen University). Primary wild type and *Ripk1*^{mRHIM/mRHIM} MEFs were transduced with recombinant lentiviruses. Four days after infection, the cell lysates were prepared in immunoprecipitation buffer (20 mM HEPES-KOH (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 10 % Glycerol). FLAG tag immunoprecipitation was performed by anti-DYKDDDDK antibody magnetic beads (017-25151, Wako) and RIPK1 was precipitated by antibody against RIPK1 (610459, BD) which was BS3-crosslinked to Dynabeads (10004D, Life Technologies).

Cell death assay

Primary MEFs and foetal liver-derived macrophages were seeded in 96 well plates (1×10^4 cells/well for MEF and 2×10^4 cells/well for FLM) one day before TNF treatment. On the experiment day, indicated amounts of recombinant murine TNF (VIB Protein Service Facility, Ghent), CHX (A0879, Applichem), z-VAD-fmk (N-1560, Bachem) and Nec-1 (BML-AP309, Enzo) were added to cells. Eighteen hours after incubation, cell viability was determined by neutral red assay as described³².

Keratinocytes isolation, culture and stimulation

Keratinocytes from newborn pups were isolated using dispase II (D4693, Sigma). The skin was incubated in dispase II overnight at 4° C. After incubation, epidermis was separated and

incubated with TrypLE (12605-010, Gibco) for 20 minutes and flushed with medium, centrifuged and cultured in low Ca^{2+} DMEM/Ham's F12 medium (F 9092-0.46, Biochrom) with 10% chelax treated FCS and supplements. For immunoblot analyses, 4×10^5 cells were seeded in collagen coated 6 well plates and three hours before stimulation the medium was replaced by fresh medium without EGF. Keratinocytes were stimulated by 20ng/ml TNF or 1000U/ml IFN β for eighteen hours.

Quantitative RT-PCR

Total RNA from skin tissue was extracted with Trizol Reagent (Life Technologies) and RNeasy Columns (Qiagen) and cDNA was prepared with Superscript III cDNA-synthesis Kit (Life Technologies). qRT-PCR of *Il1-b*, *Il-6*, *Il-33*, *Tnf*, *Cxcl3*, *Ccl3*, *Ccl5* and *Ccl4* genes was performed with TaqMan probes (Life Technologies). The expression of *Zbp1*, *Ifnb1* and *Ppia* in Fig 4.e were analysed using SYBR Green master mix (4367659, Thermo) with the following primer sets, Zbp1-F: GCTATGACGGACAGACGTGG, Zbp1-R: TGTTGACCGGATTGTGCTGA, Ifnb1-F: AGCTCCAAGAAAGGACGAACA, Ifnb1-R: GCCCTGTAGGTGAGGTTGATCT, Ppia-F: ATGGTCAACCCACCGTGT, Ppia-R: TTTCTGCTGTCTTTGGAACCTTGTC. HPRT and Tbp for TaqMan and PPIa for SYBR Green were used as reference genes. Data were analysed according to the $\Delta\Delta\text{CT}$ method.

Statistical analysis

Data shown in column graphs represent mean $\pm$ SEM. To determine group size necessary for adequate statistical power, power analysis was performed using preliminary data sets. For statistical analysis of data from qRT-PCR, quantification of epidermal thickness and inflamed area, when data did not fulfil the criteria for Gaussian distribution, nonparametric Mann-Whitney test was performed. Unpaired Student's t-test was performed for the quantification of TUNEL and CC3 staining. Paired Student's t-test was performed for statistical analysis of results obtained from cell viability assays. * P 0.05; ** P 0.01; *** P 0.005. Statistical analysis was performed using Graphpad Prism and Microsoft Excel.

Extended Data

Extended Data Figure 1. ZBP1 deficiency strongly delays and ameliorates skin inflammation in RIPK1^{E-KO} mice.

a, Photographs of mice with the indicated genotypes at the age of 4 weeks. Images shown are representative of n 60 RIPK1^{E-KO} and n 40 RIPK1^{E-KO} *Zbp1^{-/-}* mice.

b, Photographs of mice with the indicated genotypes and age. Images shown are representative of n 4 RIPK1^{E-KO} mice at the age of 5-7 weeks and n 20 RIPK1^{E-KO} *Zbp1^{-/-}* mice at the age of 17-35 weeks.

- c**, Table summarizing the macroscopically observed skin lesions and time of sacrifice of 21 aged RIPK1^{E-KO} *Zbp1*^{-/-} mice.
- d**, Representative images of skin sections from RIPK1^{E-KO} *Zbp1*^{-/-} mice and their respective controls stained with H&E (n = 18) or immunostained with the indicated antibodies (n = 4) at the age of 17-35 weeks. Nuclei stained with DAPI. Scale bars, 50 μ m.
- e**, Representative images of skin sections from 4-5 week old RIPK1^{E-KO} (n = 6) and RIPK1^{E-KO} *Zbp1*^{-/-} (n = 3) and their respective control mice stained with TUNEL or immunostained with anti-CC3 antibodies. Nuclei stained with DAPI. Scale bars, 50 μ m.
- f**, Microscopic quantification of CC3 and TUNEL positive cells on skin sections from 4-5 week old mice with the indicated genotypes. Epi, Epidermis; Der, Dermis.

Extended Data Figure 2. ZBP1 is not required for keratinocyte necroptosis and skin inflammation in mice with epidermis-specific FADD deficiency.

a, Representative photographs depicting the macroscopically observed phenotype of FADD^{E-KO} (n = 10) and FADD^{E-KO} Zbp1^{-/-} (n=5) mice at the age of 6 days.

b, Representative images of skin sections from 6 day old mice with the indicated genotypes stained with H&E (n=6) or immunostained with the indicated antibodies (n=3). Nuclei stained with DAPI. Scale bars, 50 μm.

c, qRT-PCR analysis of the mRNA expression of the indicated cytokines and chemokines in total skin RNA from 6 day old mice with the indicated genotypes.

Extended Data Figure 3. CRISPR/Cas9 mediated generation of *Ripk1^{mRHIM}* and *Mkl1^{-/-}* mice.

a, Schematic depiction of the generation of *Ripk1^{mRHIM/mRHIM}* mice indicating the sequence of the sgRNA and the single stranded oligo used for mutating the RHIM domain that were introduced by pronuclear injection into mouse zygotes and the sequencing result of one of the two obtained founders.

b, Small intestinal sections from E18.5 pups were stained with H&E or TUNEL or immunostained with anti-CC3 antibodies. Representative images shown (WT n=6 for H&E, n=3 for TUNEL and anti-CC3; *Ripk1*^{mRHIM/mRHIM} n=5 for H&E and n=3 for TUNEL and anti-CC3; *Ripk1*^{-/-} n=3 for H&E, TUNEL and anti-CC3). Nuclei stained with DAPI. Scale bars, 50 μ m.

c, Microscopic quantification of CC3 and TUNEL positive cells on gut sections from E18.5 pups with the indicated genotypes.

d, Diagram indicating the sgRNA target sequence (capital letters) used to generate a mutation in exon 2 downstream of the ATG of the *Mkl1* gene. The PAM sequence is indicated in red. Sequencing showing the 2 base pair deletion found in #97 at position chr8:111,333,648-111,333,649 (mm10), which results in a frameshift after amino acid 34 and a premature stop codon at amino acid position 55 of MLKL. This *Mkl1* knockout allele was used throughout this study.

Extended Data Figure 4. Rescue of perinatal lethality of *Ripk1*^{mRHIM/mRHIM} mice by deficiency of ZBP1, MLKL, or RIPK3.

a, Representative photographs and body weights of the indicated mice.

b, Representative H&E stainings of skin, liver, spleen, colon and small intestine sections from 5-month-old *Ripk3*^{wt/-} (n=4), *Ripk1*^{mRHIM/mRHIM} *Ripk3*^{-/-} (n=4) and *Ripk1*^{mRHIM/mRHIM} *Zbp1*^{-/-} mice (n=3).

Extended Data Figure 5. MLKL or ZBP1-deficiency prevents skin inflammation in *Ripk1*^{mRHIM/E-KO} mice.

a, Representative photographs of RIPK1^{mRHIM/E-KO} (n=9), RIPK1^{mRHIM/E-KO} *Mkl1*^{wt/-} (n=11), RIPK1^{mRHIM/E-KO} *Mkl1*^{-/-} (n=16) and RIPK1^{mRHIM/E-KO} *Zbp1*^{-/-} (n=7) at the age of 9-11 weeks.

b, Representative images of skin sections from 9-11 week old RIPK1^{mRHIM/E-KO} *Mkl1*^{wt/-} (n=11) and the respective control mice stained with H&E or immunostained with the indicated antibodies. Nuclei stained with DAPI. Scale bars, 50 μ m.

c, Representative images of skin sections from 9-11 week old $RIPK1^{mRHIM/E-KO}$ (n = 6), $RIPK1^{mRHIM/E-KO} Mlt^{-/-}$ (n=3), $RIPK1^{mRHIM/E-KO} Zbp^{-/-}$ (n=3) and their respective control mice stained with TUNEL or immunostained with anti-CC3 antibodies. Nuclei stained with DAPI. Scale bars, 50 μ m.

d, Microscopic quantification of CC3 and TUNEL positive cells on skin sections from 4-5 week old mice with the indicated genotypes. Epi, Epidermis; Der, Dermis.

Extended Data Figure 6. Expression of ZBP1 in primary keratinocytes.

a, Immunoblot analysis of lysates of primary keratinocytes derived from mice with the indicated genotypes. Each lane represents keratinocytes from individual mice. Cell lysates of wildtype FLM was used as a positive control. For gel source data, see Supplementary Figure 1.

b, Immunoblot analysis of primary keratinocytes derived from mice with the indicated genotypes were left untreated (Medium) or stimulated with TNF or IFN β for 18h. For gel source data, see Supplementary Figure 1.

Extended Data Table 1
Numbers of progeny obtained at weaning age from rescue crosses of *Ripk1*^{wt/mRHM} mice with *Ripk3*^{-/-}, *Mlkl*^{-/-}, *Zbp1*^{-/-} and *Trif*^{-/-} mice

Genotype of offspring Genotype of parents	<i>Ripk3</i> ^{-/-}				<i>Ripk3</i> ^{wt/-}			
	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM
Line 1 <i>Ripk1</i> ^{wt/mRHM} <i>Ripk3</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Ripk3</i> ^{wt/-}	6	12	9	12	27		13	
Line 2 <i>Ripk1</i> ^{wt/mRHM} <i>Ripk3</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Ripk3</i> ^{-/-}	16	32	10	18	33		5	

Genotype of offspring Genotype of parents	<i>Mlkl</i> ^{-/-}				<i>Mlkl</i> ^{wt/-}			
	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM
Line 2 <i>Ripk1</i> ^{wt/mRHM} <i>Mlkl</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Mlkl</i> ^{-/-}	17	41	13	10	32		0	
<i>Ripk1</i> ^{wt/mRHM} <i>Mlkl</i> ^{-/-} <i>Ripk1</i> ^{wt/mRHM} <i>Mlkl</i> ^{-/-}	16	25	20					

Genotype of offspring Genotype of parents	<i>Zbp1</i> ^{-/-}				<i>Zbp1</i> ^{wt/-}			
	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM
Line 2 <i>Ripk1</i> ^{wt/mRHM} <i>Zbp1</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Zbp1</i> ^{wt/-}	4	8	5	10	14		0	
<i>Ripk1</i> ^{wt/mRHM} <i>Zbp1</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Zbp1</i> ^{-/-}	10	19	7	4	23		0	

Genotype of offspring Genotype of parents	<i>Trif</i> ^{-/-}				<i>Trif</i> ^{wt/-}			
	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	WT	<i>Ripk1</i> ^{wt/mRHM} /wt	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM	<i>Ripk1</i> ^{wt/mRHM} /mRHM
Line 2 <i>Ripk1</i> ^{wt/mRHM} <i>Trif</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Trif</i> ^{wt/-}	7	12	0	14	27		0	
<i>Ripk1</i> ^{wt/mRHM} <i>Trif</i> ^{wt/-} <i>Ripk1</i> ^{wt/mRHM} <i>Trif</i> ^{-/-}	11	24	0	16	12		0	

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ZBP1 induces keratinocyte necroptosis and skin inflammation in RIPK1^{E-KO} mice.

a, Immunoblot analysis of ZBP1, RIPK1 and GAPDH in epidermal protein extracts from wild type (*Ripk1^{FL/WT}*) and RIPK1^{E-KO} mice at postnatal day 3 (P3) and P28. Lanes represent samples from individual mice. For gel source data, see Supplementary Figure 1.

b, Skin sections from 4 – 5 week old mice were stained with H&E or immunostained with the indicated antibodies. Representative images shown (RIPK1^{E-KO} n=9 for H&E and n 6 for immunostainings; RIPK1^{E-KO} *Zbp1^{-/-}* n=10 for H&E and n 3 for immunostainings). Scale bars, 50 μm.

c, Microscopic quantification of epidermal thickness (Epi. th.) and inflamed skin area (Infl. area) in 4 – 5 week old mice with the indicated genotypes.

d, qRT-PCR analysis of the mRNA expression of the indicated cytokines and chemokines in RNA isolated from total skin from 4 – 5 week old mice with the indicated genotypes.

Figure 2. Mutation of the RIPK1 RHIM domain causes perinatal lethality and inflammatory skin hyperplasia in mice.

a, Table showing the numbers of weaned offspring of *Ripk1^{mRHIM/wt}* parents from two independently generated knock-in lines (L1 & L2).

b, Skin sections from E18.5 pups were stained with H&E or TUNEL or immunostained for CC3 or F4/80. Representative images shown (WT n=6 for H&E, n=3 for TUNEL, anti-CC3 and anti-F4/80; *Ripk1^{mRHIM/mRHIM}* n=5 for H&E and anti-CC3, n=4 for TUNEL and n=3 for anti-F4/80; *Ripk1^{-/-}* n=3 for H&E, TUNEL, anti-F4/80 and anti-CC3). Scale bars, 50 μm.

c, Microscopic quantification of CC3 and TUNEL positive cells on skin sections from E18.5 pups with the indicated genotypes. Epi, Epidermis; Der, Dermis.

d, Immunoblot analysis of total skin lysates from E18.5 pups of the indicated genotypes. Lanes represent samples from individual embryos. For gel source data, see Supplementary Figure 1.

Figure 3. Mutation of the RIPK1 RHIM domain prevents TNF-induced association of RIPK1 with RIPK3 and necroptosis.

a, Immunoblot analysis with the indicated antibodies of RIPK1 immunoprecipitates and total lysates from primary WT and *Ripk1*^{mRHIM/mRHIM} MEFs treated with TNF (20ng/ml), CHX (1μg/ml) and z-VAD-fmk (20μM) for the indicated periods of time. h, hours. Representative data shown from two independent experiments. For gel source data, see Supplementary Figure 1.

b, c, Primary MEFs (**b**) or FLMs (**c**) from mice with the indicated genotypes were treated with combinations of TNF (20ng/ml), CHX (1μg/ml), z-VAD-fmk (20μM) and Nec-1 (30μM) for 18 hours. Cell viability was determined by neutral red assay. Graphs show mean ± SEM from pooled data from 5 (**b**) and 3 (**c**) independent experiments. **P* 0.05; ***P* 0.01; ****P* 0.005.

d, Immunoblot analysis of primary MEFs or FLMs from WT or *Ripk1*^{mRHIM/mRHIM} mice with the indicated antibodies. Lanes represent primary cells from individual mice. For gel source data, see Supplementary Figure 1.

e, f, Primary MEFs (**e**) or FLMs (**f**) from WT or *Ripk1*^{mRHIM/mRHIM} mice were stimulated with TNF (20ng/ml) for the indicated periods of time and NF-κB activation was assessed by immunoblotting with the indicated antibodies. Representative data shown from three independent experiments. For gel source data, see Supplementary Figure 1.

Figure 4. RHIM-dependent RIPK1 function prevents MLKL/ZBP1-mediated necroptosis and skin inflammation.

a, Skin sections from 9-11 week old mice were stained with H&E or immunostained with the indicated antibodies. Representative images shown (*RIPK1*^{mRHIM/E-KO} *n*=9 for H&E and *n*=3 for immunostainings; *RIPK1*^{mRHIM/E-KO} *Mlkl*^{-/-} *n*=5 for H&E and *n*=3 for immunostainings; *RIPK1*^{mRHIM/E-KO} *Zbp1*^{-/-} *n*=4 for H&E and *n*=3 for immunostainings). Scale bars, 50 μ m.

b, c, Microscopic quantification of epidermal thickness (Epi. th.) and inflamed skin area (Infl. area) (**b**) and qRT-PCR analysis of the mRNA expression of the indicated cytokines and chemokines (**c**) in 9-11 week old mice with the indicated genotypes.

d, e, Immunoblot analysis of ZBP1 and MLKL expression in epidermal lysates (**d**) and qRT-PCR analysis of *Zbp1* and *Ifnb1* mRNA levels in total skin (**e**) from 4 week-old mice of the indicated genotypes. Lanes represent samples from individual mice. For gel source data, see Supplementary Figure 1.

f, g, Immunoblot analysis with the indicated antibodies of anti-FLAG (**f**) or anti-RIPK1 (**g**) immunoprecipitates and total lysates from primary WT and *Ripk1*^{mRHIM/mRHIM} (mR) MEFs transduced with lentiviruses expressing FLAG or FLAG-tagged ZBP1. Representative data shown from two independent experiments. For gel source data, see Supplementary Figure 1.