TBK1 and IKKε prevent TNF-induced cell death by RIPK1 phosphorylation

Elodie Lafont1,6, Peter Draber1,2,6, Eva Rieser1,6, Matthias Reichert1,6, Sebastian Kupka1, Diego de Miguel1, Helena Draberova1,2, Anne von Mässenhausen3, Amandeep Bhamra4, Stephen Henderson5, Katarzyna Wojdyla4, Avigayil Chalk2, Silvia Surinova2,4, Andreas Linkermann3 and Henning Walczak2,*

The linear-ubiquitin chain assembly complex (LUBAC) modulates signalling via various immune receptors. In tumour necrosis factor (TNF) signalling, linear (also known as M1) ubiquitin enables full gene activation and prevents cell death. However, the mechanisms underlying cell death prevention remain ill-defined. Here, we show that LUBAC activity enables TBK1 and IKKε recruitment to and activation at the TNF receptor 1 signalling complex (TNFR1-SC). While exerting only limited effects on TNF-induced gene activation, TBK1 and IKKε are essential to prevent TNF-induced cell death. Mechanistically, TBK1 and IKKε phosphorylate the kinase RIPK1 in the TNFR1-SC, thereby preventing RIPK1-dependent cell death. This activity is essential in vivo, as it prevents TNF-induced lethal shock. Strikingly, NEMO (also known as IKKγ), which mostly, but not exclusively, binds the TNFR1-SC via M1 ubiquitin, mediates the recruitment of the adaptors TANK and NAP1 (also known as AZI2). TANK is constitutively associated with both TBK1 and IKKε, while NAP1 is associated with TBK1. We discovered a previously unrecognized cell death checkpoint that is mediated by TBK1 and IKKε, and uncovered an essential survival function for NEMO, whereby it enables the recruitment and activation of these non-canonical IKKs to prevent TNF-induced cell death.

NFR1 signalling involves the formation of two distinct complexes. The plasma membrane-associated TNFR1-SC, also termed complex-I, is responsible for gene activation, whereas the subsequently formed cytosolic complex-II induces cell death. Under physiological conditions, TNFR1 stimulation induces gene activation rather than cell death. However, aberrant TNF-induced signalling causes several autoimmune pathologies and cancer-related inflammation. Therefore, defining the molecular checkpoints that determine the different TNFR1 signalling outputs is critical to understand the biology of inflammation.

Linear ubiquitylation is crucial for multiple immune receptor signalling pathways2,4. LUBAC is the only known E3 ligase capable of forming linear ubiquitin linkages. LUBAC is composed of the following three core proteins: haem-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), SHANK-associated RH-domain-interacting protein (SHARPIN) and the catalytically active HOIL-1-interacting protein (HOIP)6–9. The essence of LUBAC and M1 ubiquitylation has been demonstrated by the embryonic lethality of mice lacking HOIL-1, HOIP or LUBAC activity10–12, which is caused by aberrant TNFR1-mediated signalling10,12.

LUBAC recruitment to complex-I enables full gene activation while preventing cell death13,14. Following the recruitment of LUBAC to the TNFR1-SC, several of its components, including RIPK1, NEMO, TNFR1 and TRADD, are M1 ubiquitylated by LUBAC13,14. In complex-I, M1 ubiquitin acts as a recruitment platform for adaptors such as NEMO, the regulatory subunit of the inhibitor of κB (IkB) kinase (IKK) complex. Although NEMO can be recruited to the TNFR1-SC in the absence of M1 ubiquitin, this process is much weaker than in its presence13. Accordingly, the binding of NEMO to the TNFR1-SC with M1 ubiquitin enables full activation of the canonical IKKs IKKα and IKKβ13. When activated, they phosphorylate IkB, causing the nuclear translocation of nuclear factor-κB (NF-κB), thereby initiating gene transcription. Independently, IKKα and IKKβ prevent TNF-induced cell death by phosphorylating RIPK1. This limits the ability of RIPK1 to promote the formation of complex-II7. Phosphorylation events on RIPK1, mediated by the p38 target MK2, also restrict the capacity of RIPK1 to trigger complex-II formation and, consequently, cell death18–20. Notably, IKKα, IKKβ and p38 and MK2 also mediate expression downstream of TAK1, which is required for TNF-induced gene activation16,21–26.

TBK1 and IKKε are two closely related kinases that are homologous to the canonical kinases IKKα and IKKβ7,18–28. Various adaptors recruit TBK1 and IKKε to distinct immune signalling complexes29. Concomitant TBK1 and IKKε ablation abolishes the activation of interferon signalling by various immune receptors, including TLR3 and TLR4, or after viral infection19,30. Here, we show that by phosphorylating RIPK1 in complex-I, TBK1 and IKKε serve an essential function in TNF signalling, providing a physiologically relevant cell death-restricting checkpoint that depends on M1 ubiquitylation and NEMO.

Results

Effective TBK1 and IKKε recruitment to and activation in complex-I require LUBAC activity. To elucidate how LUBAC modulates TNF signalling, we compared the composition of purified TNFR1-SCs from wild-type and HOIP-deficient A549 cells by mass spectrometry (MS)14. This confirmed that in
HOIP-deficient cells, HOIL-1, SHARPIN, CYLD and SPATA2, as well as the A20–ABIN1–ABIN2 complex, are missing from complex-I, whereas NEMO is still recruited to it, albeit poorly (Fig. 1a; Supplementary Table 2). Importantly, however, TBK1, IKKε and one of their known adaptors, TANK, were detected in complex-I in control but not HOIP-deficient cells (Fig. 1a).

A western blot analysis revealed that TBK1 and IKKε are recruited to and strongly phosphorylated within the native TNFR1-SC on the activatory S172 residue in various HOIP-proficient cell lines, whereas their activation in corresponding HOIP-deficient cells is very weak (Fig. 1b,c; Supplementary Fig. 1a,b). Thus, TBK1 and IKKε are bona fide components of complex-I, and LUBAC enables their recruitment.
Using HOIP-deficient HeLa and A549 cells reconstituted with wild-type HOIP (HOIPWT) or catalytically inactive HOIP (HOIPC885S)39, we determined that effective TBK1 and IKKε recruitment to complex-I requires the M1 ubiquitin-forming activity of LUBAC, as TBK1 and IKKε recruitment was strongly diminished in HOIP-deficient HeLa and A549 cells whether or not HOIPC885S was re-expressed in them (Fig. 1d,e; Supplementary Fig. 1c,d).

The role of TBK1 and IKKε in TNF-induced gene activation is limited. As TBK1 and IKKε are crucial for gene expression by various immune receptor complexes50–52, we evaluated whether these kinases influenced TNF-induced gene activation by generating L929 cells in which TBK1, IKKε and TNF are all knocked out. The absence of TBK1 and IKKε did not significantly affect TNF-induced gene activatory signalling and, if anything, slightly increased IκBα phosphorylation (Supplementary Fig. 2a). This result is in line with the previously proposed roles of TBK1 and IKKε as negative regulators of IKKα and IKKβ activation43. Similarly, in mouse embryonic fibroblasts (MEFs) and A549 cells, treatment with the TBK1 and IKKε-specific inhibitor MRT6730743 (MRT) did not exert any significant effects on the TNF-induced activation of MAPKs or NF-κB (Fig. 2a; Supplementary Fig. 2b).

Next, we evaluated whether TBK1 and IKKε affect gene induction following TNFR1 stimulation. We performed an unbiased RNA sequencing (RNA-seq) analysis following TNF versus TNF plus MRT stimulation, also including TNF and TPCA-1, which, as an IKKα and IKKβ-inhibiting control, is known to profoundly affect TNF-induced gene expression44.

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Inhibition of TBK1 and IKKε sensitizes cells to TNF-induced RIPK1-dependent cell death downstream of LUBAC. a, TNF−/− MEFs (a) and L929 cells (b) were treated with TNF (500 ng ml−1 and 50 ng ml−1, respectively) in the presence or absence of MRT and Nec-1s. c, TNF−/− MEFs of the indicated genotype were treated with TNF (500 ng ml−1) in the presence or absence of the indicated compounds. For cell death was measured as a function of time by Sytox Green positivity. The RFU mean of four technical replicates of one representative experiment out of independent experiments. Lysates of untreated cells were analysed by western blotting. Unprocessed original scans of blots are shown in Supplementary Table 1.

A principal component analysis revealed that TNF drastically modulated gene expression, with TNF-treated cells clearly segregated from untreated samples. While the effect of IKKα and IKKβ inhibition on TNF-induced gene expression was substantial, the effect of TBK1 and IKKε inhibition was surprisingly limited (Fig. 2b). The top 100 most altered transcripts were highly similar...
Intriguingly, in both cases, cell death was prevented by the RIPK1 inhibitor Nec-1s (Fig. 3a,b; Supplementary Fig. 3a). Interestingly, the MRT-induced spontaneous death of L929 cells was inhibited by the TNF blocker etanercept (also known as Enbrel; Supplementary Fig. 3b). Next, we created TNF-deficient MEFs and L929 cells in which we further deleted TBK1, IKKε or both kinases to enable us to genetically assess their cell death-preventive function. In line with the results obtained when these kinases were inhibited, concomitant deletion of TBK1 and IKKε strongly sensitized these cells to TNF-induced death (Fig. 3c; Supplementary Fig. 3c,d). Thus, both pharmacological inhibition and genetic ablation of TBK1 and IKKε sensitizes cells to TNF-induced death.

We next investigated the cell death modality induced by TBK1 and IKKε inhibition. While pretreatment with the pan-caspase inhibitor zVAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[(O-methyl)-fluoromethylketone] substantially sensitized MEFs to TNF alone, sensitization to TNF and MRT was significantly higher between TNF-stimulated control cells and TNF and MRT-treated cells, but significantly different in TNF and TPCA-1-treated cells (Fig. 2c; Supplementary Fig. 2c). Notably, the majority of transcripts with significantly altered expression at the 1-h time point in control cells were similarly modulated in TNF and MRT-treated cells but not in TNF and TPCA-1-treated cells (Fig. 2d; Supplementary Table 3). Thus, in contrast to the roles of TBK1 and IKKε as drivers of gene induction in other immune signalling pathways\textsuperscript{47,48}, their role in TNF-induced gene expression is limited.

**TBK1 and IKKε prevent TNF-induced RIPK1-dependent cell death.** We next assessed the role of TBK1 and IKKε in TNF-induced cell death. Strikingly, treatment with MRT or another TBK1 and IKKε-specific inhibitor, BX-795\textsuperscript{45}, drastically sensitized MEFs and L929 cells to TNF-induced death (Fig. 3a,b; Supplementary Fig. 3a). Intriguingly, in both cases, cell death was prevented by the RIPK1 inhibitor Nec-1s (Fig. 3a,b; Supplementary Fig. 3a). Interestingly, the MRT-induced spontaneous death of L929 cells was inhibited by the TNF blocker etanercept (also known as Enbrel; Supplementary Fig. 3b). Next, we created TNF-deficient MEFs and L929 cells in which we further deleted TBK1, IKKε or both kinases to enable us to genetically assess their cell death-preventive function. In line with the results obtained when these kinases were inhibited, concomitant deletion of TBK1 and IKKε strongly sensitized these cells to TNF-induced death (Fig. 3c; Supplementary Fig. 3c,d). Thus, both pharmacological inhibition and genetic ablation of TBK1 and IKKε sensitizes cells to TNF-induced death.

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Fig. 5 | TBK1 and IKKe phosphorylate RIPK1 both in vitro and at the TNFR1-SC, providing a physiologically relevant cell death checkpoint. a, L929 cells pretreated with or without BX-795 were stimulated with TNF (1 µg ml⁻¹) and zVAD-FMK for 15 min before RIPK1 pull down. RIPK1 immunoprecipitate (IP) treated with USP2 and λ-phosphatase (λ-PP) as indicated was analysed by western blotting. b, L929 cells were stimulated with TAP-TNF for 15 min (1 µg ml⁻¹) with or without MRT. TNFR1-SC was FLAG-immunoprecipitated. Samples were first separated by pI on IPG strips pH 3–10 NL, followed by reduction and alkylation and then by 1-dimension SDS–PAGE prior to western blotting. c, GST-tagged RIPK1, Nec-1s and IKKα, β was incubated with GST-tagged RIPK1 in a kinase assay with or without the indicated inhibitors before western blot analysis. d, Phosphosites identified by LC–MS/MS from one kinase assay using GST-tagged TBK1, TAK1 and IKKα, β, USP2 and λ-phosphatase as indicated was analysed by western blotting.

Loss of viability (%)

|     | Control | L929 | L929 + TNF | L929 + TNF + Nec-1s | L929 + TNF + MRT | L929 + TNF + Nec-1s + MRT |
|-----|---------|------|------------|---------------------|-----------------|--------------------------|
|     |         | 0%   | 100%       | 100%                | 80%             | 20%                      |

Loss of viability (%) following TNF and MRT exposure

| Control | L929 | L929 + TNF | L929 + TNF + Nec-1s | L929 + TNF + MRT | L929 + TNF + Nec-1s + MRT |
|---------|------|------------|---------------------|-----------------|--------------------------|
| 0%      | 100% | 100%       | 80%                | 20%             | 20%                      |

Cumulative survival rates of mice following TNF-α-induced shock in the presence of indicated inhibitors were compared using log-rank Mantel–Cox tests. MRT versus TNF + MRT, P < 0.001; TNF versus TNF + MRT, P < 0.001; TNF + MRT versus TNF + MRT + Nec-1s, P = 0.0061. Unprocessed blots are shown in Supplementary Fig. 1.
**Fig. 6 | NEMO acts upstream of the adaptors TANK and NAP1, which recruit TBK1 and IKKe, or TBK1 only, respectively, to the TNFR1-SC.**

- **a.** TANK-deficient A549 cell clones and control cells were treated with TNF (200 ng ml⁻¹) for the indicated times. Lysates were analysed by western blotting.
- **b.** TANK-deficient or corresponding control A549 cells were stimulated with FLAG-TNF (500 ng ml⁻¹) for the indicated times. The purified TNFR1-SC and lysates were analysed by western blotting.
- **c.** NEMO-deficient A549 cells reconstituted with HOIPWT, enzymatically inactive HOIPC885S or vector control were stimulated with FLAG-TNF (500 ng ml⁻¹) for the indicated times. The purified TBK1-associated complex and lysates were analysed by western blotting.

### Table 5.

| Description | Accession | Unique peptides | Sequence coverage (%) |
|-------------|-----------|-----------------|----------------------|
| TBK1        | Q9UHD2    | 9               | 15.1                 |
| NAP1/TBKP1  | Q02645    | 9               | 15.9                 |
| SINTBAD/TBKBP1 | AMWCTY   | 9               | 24.2                 |
| TRAF2       | Q12933    | 8               | 10.9                 |
| TANK        | Q92844    | 8               | 10.9                 |

**Table legend:**

- TNF: 0, 5, 15, 30, 60, 180 min
- p-IKK: 0, 5, 15, 30, 60, 180 min
- p-TBK1: 0, 5, 15, 30, 60, 180 min

**Supplementary Figure 7:**

- Unprocessed original scans of blots are shown in Supplementary Fig. 7.

**Supplementary Information:**

- Raw data can be accessed at the ProteomeXchange Consortium via the PRIDE²⁵ partner repository with the dataset identifier PXD010777; analysed data are provided in Supplementary Table 5.
- A two-step immunoprecipitation process via TAP-tag and analysed by LC–MS/MS. One experiment was analysed. Raw data can be accessed at the ProteomeXchange Consortium via the PRIDE²⁵ partner repository with the dataset identifier PXD010777; analysed data are provided in Supplementary Table 5.

*One experiment representative of two (a, b, d–g) or three independent experiments (h, i) is shown. Unprocessed original scans of blots are shown in Supplementary Fig. 7.*
TBK1 and IKKe prevent TNF-induced RIPK1 activation and ensuing complex-II formation. TNFR1-SC assembly precedes the formation of complex-II, which contains RIPK1, FADD, FLIP, caspase-8, RIPK3 and MLKL. Depending on the cellular context and the relative expression of pro- and anti-apoptotic and pro- and anti-necroptotic proteins, complex-II can trigger apoptosis or necroptosis, which depends on the ability of a given cell type to undergo death by the respective modality. Importantly, in either case, cell death induction requires the activity of RIPK1.

Performing in vitro kinase assays to test whether TBK1 and IKKe can directly phosphorylate RIPK1 revealed that both kinases were absent or inhibited, we assessed whether TBK1 and IKKe prevent cytotoxicity by preventing RIPK1 from mediating complex-II formation. We treated A549 cells expressing TAP (tandem affinity purification)-tagged RIPK3 with TNF and zVAD-FMK or a combination of TNF, zVAD-FMK and BX-795 before immunoprecipitating RIPK3. TBK1 and IKKe inhibition markedly enhanced the association of RIPK3 with caspase-8 and FADD, phosphorylated MLKL (p-MLKL) and phosphorylated RIPK1 (p-RIPK1) (Fig. 4a). Enhanced RIPK3 binding to caspase-8 and p-MLKL was also observed in RIPK3-expressing HeLa cells and, decisively, concomitant RIPK1 inhibition prevented complex-II formation (Fig. 4b).

In accordance with the increased cell death observed following TBK1 and IKKe inhibition, L929 cells and MEFs also displayed increased RIPK1 activation and complex-II formation following TNF, zVAD-FMK and MRT co-treatment (Fig. 4c,d). Importantly, genetic TBK1 and IKKe co-ablation in MEFs (Fig. 4e) and TNF and MRT treatment of primary BMDMs (Fig. 4f) also substantially enhanced complex-II formation. In all cases, complex-II formation was dependent on RIPK1 kinase activity (Fig. 4b–f). Hence, TBK1 and IKKe prevent TNF-induced cell death by restricting RIPK1 autoactivation and consequent complex-II formation.

TBK1 and IKKe mediate phosphorylation of RIPK1 in the TNFR1-SC. We hypothesized that the negative effect of TBK1 and IKKe on RIPK1 autophosphorylation might be achieved via TNF-induced RIPK1 phosphorylation mediated by these kinases. Thus, we determined whether we could detect any TBK1- and IKKe-dependent phosphorylation of RIPK1. After immunoprecipitating RIPK1 from L929 cells treated with TNF or TNF and BX-795 in the presence of zVAD-FMK, we incubated the resulting immunoprecipitates with or, as a control, without the pan-deubiquitinase USP2 to completely deubiquitylate RIPK1. This was done with or without λ-phosphatase to uncover any RIPK1 phosphorylation that requires TBK1 and IKKe activity (Fig. 5a). USP2 treatment resulted in the collapse of most modified high-molecular-weight forms onto low-molecular-weight forms of RIPK1. The intermediate-molecular-weight forms remaining after USP2 treatment completely collapsed onto the band of unmodified RIPK1 following treatment with λ-phosphatase. This result demonstrates that phosphorylation accounts for the TBK1 and IKKe-dependent mobility shift of deubiquitylated RIPK1. Importantly, TNF and BX-795 co-treatment reduced the RIPK1 mobility shift compared to TNF treatment. Most importantly, this shift was not seen following λ-phosphatase treatment, demonstrating that TBK1 and IKKe inhibition specifically prevents RIPK1 phosphorylation (Fig. 5a).

To assess whether TBK1- and IKKe-dependent RIPK1 phosphorylation occurs in the TNFR1-SC, we immunoprecipitated complex-I following TNF versus TNF and MRT treatment before evaluating the status of RIPK1 phosphorylation by two-dimensional (2D) gel electrophoresis. This revealed that TBK1 and IKKe inhibition reduced the low isoelectric point (pI) forms of RIPK1 after TNF stimulation in complex-I (Fig. 5b). Importantl, RIPK1 phosphorylation in complex-I was also reduced in MEFs and L929 cells in which TNF, TBK1 and IKKe were knocked out, and in MEFs in which TNF and TBK1 were knocked out (Fig. 5c; Supplementary Fig. 5a). We therefore conclude that TBK1 and IKKe promote RIPK1 phosphorylation within the native TNFR1-SC.

Performing in vitro kinase assays to test whether TBK1 and IKKe can directly phosphorylate RIPK1 revealed that both kinases were able to do so (Fig. 5d,e). A MS analysis of the in vitro kinase assay employing recombinant IKKe and RIPK1 in the presence of Nec-1s showed that RIPK1 was phosphorylated on numerous residues, as also confirmed by 2D gel electrophoresis (Fig. 5f; Supplementary Fig. 5b; Supplementary Table 4). Notably, several of these residues have been reported to be phosphorylated by other kinases and other kinases.

Together, these results show that TBK1 and IKKe mediate RIPK1 phosphorylation on multiple residues in complex-I, thereby preventing RIPK1 autophosphorylation and the consequent formation of complex-II.

TBK1 and IKKe phosphorylate RIPK1 independently from IKKα, IKKβ and MK2. TNF-induced RIPK1 activation is controlled by phosphorylation that is mediated by IKKα, IKKβ and MK2, all acting downstream of TAK1. These phosphorylation events function as checkpoints to inhibit RIPK1-dependent complex-II formation. We next determined whether the TBK1- and IKKe-mediated RIPK1 phosphorylation represents a distinct cell death-preventing checkpoint. We purified the TNFR1-SC from cells treated with TNF together with different inhibitors or combinations thereof targeting TBK1 and IKKe (MRT), IKKα and IKKβ (TPCA-1), MK2 (PF-3644022) and TAK1 (7-oxozeanol) before treating the immunoprecipitated complexes with USP2 to visualize phosphorylation events. This analysis confirmed that inhibition of TAK1, MK2 and/or IKKα and IKKβ reduces RIPK1 phosphorylation in complex-I. Importantly, however, TBK1 and IKKe phosphorylated RIPK1 independently of MK2 and of IKKα and IKKβ, as only the combined inhibition of TBK1 and IKKe, either with that of TAK1 or with those of MK2 and IKKα and IKKβ, abrogated RIPK1 phosphorylation (Fig. 5g).

A 2D gel analysis of complex-I further revealed that several low-pI forms of RIPK1 disappeared after the addition of MRT in conditions under which all other known phosphorylation events are inhibited (Fig. 5h). Thus, TBK1 and IKKe activity in complex-I promotes the phosphorylation of RIPK1 at multiple sites that are not phosphorylated by other RIPK1-inhibiting kinases. In line with...
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a distinct checkpoint mediated by TBK1 and IKKe, their inhibition sensitized MEFs and L929 cells to RIPK1-dependent TNF-induced death even in the presence of MK2 and/or IKKα and IKKβ inhibitors (Fig. 5i,j). Thus, TBK1 and IKKe-mediated RIPK1 phosphorylation constitutes a cell death checkpoint that is different from previously described ones. Furthermore, these results strongly suggest that TBK1 and IKKe directly phosphorylate RIPK1 in the TNFR1-SC.

**TBK1 and IKKe protect against TNF-induced RIPK1-dependent lethal shock in vivo.** To reveal whether the cell death checkpoint identified herein is functionally relevant in vivo, we employed TNF at an established sublethal dose7,20 in a murine model of TNF-induced shock that requires RIPK1-dependent cell death49,50. As expected, none of the mice succumbed following treatment with TNF alone. Strikingly, however, TNF and MRT co-treatment resulted in a highly significant reduction in survival, with 50% lethality only 8.5 h after injection and an overall survival of less than 20%. Importantly, concomitant RIPK1 inhibition completely prevented TNF and MRT-induced lethality (Fig. 5k). Hence, inhibition of RIPK1 by TBK1 and IKKe is essential for protection against TNF-induced lethal shock.

**NEMO-recruited TANK and NAP1 engage TBK1 and IKKe to the TNFR1-SC.** Having identified that the control of TNF-induced cell death by TBK1 and IKKe is essential, we determined the biochemical mechanism of their recruitment to complex-I. TBK1 and IKKe are known to associate with various adaptors that recruit them to different signalling platforms59. One such adaptor is TANK1, which is recruited to complex-I in a HOIP-dependent manner (Fig. 1). Using TANK-deficient cells, we observed that IKKe recruitment was abrogated in these cells and that TBK1 recruitment was reduced but still occurred (Fig. 6a,b). Thus, IKKe recruitment entirely relies on TANK, whereas TBK1 is recruited by TANK and at least one additional adaptor.

After ruling out optineurin, a suggested TNFR1-SC component60, as the protein responsible for TNF-dependent TBK1 recruitment to complex-I (Supplementary Fig. 6a), we sought to identify the additional adaptor (or adaptors) for TBK1. We undertook this in an unbiased manner by studying the TBK1 interactome using TBK1-deficient cells re-expressing TAP-tagged TBK1 and performing an MS analysis on the affinity-purified TBK1. With the aim of elucidating the mechanism of how TBK1 and IKKe exert their function, we made the surprising discovery that NEMO mediates TBK1 and IKKe activity exerts only limited effects on TNF-induced gene expression. However, preventing TBK1 and IKKe activity exerts only limited effects on TNF-induced gene expression. Instead, we discovered that by phosphorylating RIPK1 on multiple sites within complex-I, TBK1 and IKKe control a physiologically essential cell death checkpoint. Accordingly, their pharmacological inhibition or genetic ablation resulted in TNF-induced cell death, both in cell lines and in vivo, as a consequence of unleashed RIPK1 activity and aberrant complex-II formation.

**Discussion**

A major focus of current research on TNF-induced cell death is placed on the regulation of RIPK1, the central kinase in this process. Various post-translational modifications of RIPK1 are at the core of this regulation56–58.

Currently, RIPK1 is thought to be kept in check in the cytosol under unstimulated conditions54. Following TNF stimulation, RIPK1 is recruited to complex-I45, where it is rapidly post-translationally modified, including by cIAP1- and cIAP2-mediated K63-, K11- and K48-linked ubiquitylation and LUBAC-catalysed M1 ubiquitylation. M1 ubiquitin promotes the complex-I recruitment of NEMO together with its associated kinases, IKKα and IKKβ. Apart from activating NF-κB, IKKα and IKKβ inactivate RIPK1 by phosphorylation, preventing its translocation to complex-II. Recently, the existence of an additional cell death checkpoint involving the p38 MAPK target MK2 was described59–60.

While TBK1 and IKKe were shown to be activated after TNF stimulation56–58, their function in TNF signalling remained enigmatic. Here, we identified a NEMO-dependent checkpoint that controls TNF-induced cell death by the TBK1- and IKKe-mediated phosphorylation of RIPK1, which is largely dependent on M1 ubiquitylation and functionally independent from known cell death checkpoints (Supplementary Fig. 6h). It was recently proposed that the TBK1-mediated phosphorylation of RIPK1 on T189 impairs its substrate-binding capacity59. Our results, however, show that TBK1 and IKKe phosphorylate RIPK1 on multiple residues. It therefore appears that the TBK1- and IKKe-mediated regulation of RIPK1 is more complex. Further investigation will be required to define the contribution of the different TBK1- and IKKe-mediated phosphorylation events in keeping RIPK1 in check.

Interestingly, TBK1-deficient mice are embryonically lethal at embryonic day 14.5 due to aberrant TNFR1-induced cell death49,50, a phenotype similar, but not identical, to that of NEMO-deficient animals44,46. Hence, it was initially assumed that TBK1 activates TNF-induced NF-κB-dependent gene expression. However, preventing TBK1 and IKKe activity exerts only limited effects on TNF-induced gene expression. Instead, we discovered that by phosphorylating RIPK1 on multiple sites within complex-I, TBK1 and IKKe control a physiologically essential cell death checkpoint. Accordingly, their pharmacological inhibition or genetic ablation resulted in TNF-induced cell death, both in cell lines and in vivo, as a consequence of unleashed RIPK1 activity and aberrant complex-II formation.

With the aim of elucidating the mechanism of how TBK1 and IKKe exert their function, we made the surprising discovery that NEMO mediates TBK1 and IKKe activation in complex-I. Mechanistically, NEMO enables the recruitment of TANK, which brings both TBK1 and IKKe to the complex, and of NAP1, which brings in additional TBK1. These results are striking, as they demonstrate that NEMO serves an essential survival function beyond regulating IKKα and IKKβ. Intriguingly, NEMO was previously shown to exert cell death-preventing functions independently of NF-κB57,62. Our results provide an explanation for this observation, as the combined activities of IKKα and IKKβ, TAK1 and MK2, and TBK1 and IKKe are required to keep RIPK1 in check. Of note, NEMO mutations are causative for a spectrum of diseases, including incontinentia pigmenti, ectodermal dysplasia and...
imunodeficiency, which are thought to be caused by a deficiency in activating IKKα and IKKβ. On the basis of our results, one may consider that aberrant TNF-induced cell death caused by the lack of TBK1 and IKKε activity may participate in disease progression in patients with certain NEMO mutations.

Our results prompt the question as to what could be the evolutionary advantage of having three distinct checkpoints all focused on RIPK1, a single component in the TNFR1 pathway. TNF is induced as one of the first cytokines in response to various cellular stressors. It is tempting to speculate that this triple safeguard mechanism might serve to ensure that, should anything go wrong—perhaps as the result of a pathogen-mediated targeted intervention—with any of three of the arguably most crucial signalling pathways for innate and adaptive immunity, this is sensed as early as possible during innate immune signalling. The inability to properly phosphorylate RIPK1 in response to TNF would serve this purpose, with the resulting outcome of TNF signalling, the untoward death of the cell, triggering an alternative route to inflammation.

We herein identified a crucial role for TBK1 and IKKε in preventing TNF-induced cell death. So far, these kinases have mainly been considered as modulators of gene expression, mostly in interferon responses and autophagy. However, certain pathologies have been associated with mutations or altered expression of TBK1 and IKKε, including neuroinflammatory diseases, for example amyotrophic lateral sclerosis or frontotemporal dementia, and various types of cancer. Based on our results, diseases caused by NEMO deficiency should be added to this list. It will be interesting to evaluate to which extent aberrant TNF-induced, RIPK1-mediated cell death caused by deregulated TBK1 and IKKε activity participates in the initiation or progression of these diseases.

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Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability, and associated accession codes are available at https://doi.org/10.1038/s41556-018-0229-6.

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Author contributions

H.W. conceived the project. E.L., P.D., E.R., M.R., S.K., A.v.M. and A.L. designed and performed the experiments and analysed the obtained data. S.S. and K.W. performed the MS experiments. S.S., A.B. and K.W. performed the MS experiments and analysed the obtained data. H.D. and S.S. supervised the MS experiments and analysed the obtained data. S.S. supervised the MS experiments. H.D. and A.C. performed experiments. D.d.M. generated essential tools for the study. S.H. analysed the RNA-seq data. E.L., E.R., P.D. and H.W. wrote the manuscript.

Competing interests

H.W. is a co-founder and shareholder of Apogenix AG. All other authors have no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to H.W.

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Methods

Recombinant proteins, cells and cell lines. Human full-length GST (glutathione S-transferase)-RIPK3 was from Anova, GST-IKKε was from ThermoFisher and GST-TBK1 was from Sigma-Aldrich. Untagged TNF-HA (human influenza hemagglutinin)-TNF and TAP-TNF were produced and purified as previously described. Ectenasept was purchased from Pfizer.

Wild-type cancer cell lines were purchased from the American Type Culture Collection. The generation and reconstitution of cancer cell lines with HOIP, TANK, NAP1, optineurin, NEMO, TBK1 and IKKβ, or TNF knocked out, and the generation of the different knockout MEFs were performed as previously described. The single guide RNA sequences used are presented in Supplementary Table 6. Newly generated knockout cells were validated by sequencing. A549 and HeLa cells were transduced to express a TAP-tagged form of RIPK3. Briefly, we used a carboxy-terminal-TAP-tag consisting of a 2x Streptag II sequence followed by a PreScission scission site and 1x Flag tag. The coding sequence of human RIPK3 was inserted into the retroviral MSCV vector, followed by an internal ribosome entry site and the open reading frame of enhanced green fluorescent protein (EGFP). This vector was transfected using Lipofectamine 2000 in Phoenix-AMPHO cells. One day after transfection, the medium was replaced, and viral supernatants were collected at days 2 and 3. Viral supernatants were passed through a 0.45-µm filter, added to HeLa or A549 cells at 60% confluence in the presence of polybrene (6 µg/ml) before cells were subjected to spinfection (1300 x g, 5 min, 30°C). EGFP-positive cells were isolated using MoFlo FACS (Beckman Coulter) to more than 95% purity 2 days after infection. HOIP knockout HeLa and A549 cells re-expressing HOIP(2x), HOIP(3x), Empty pBabe or the MSCV vector as well as TBK1 knockout A549 cells expressing TBK1(1x) or TBK1(2x)130 were generated as previously described. A549 NEMO knockout cells were transduced with pBabe-puro to re-express NEMO(1x) or NEMO(3x) (200-250 amino acid deletion). Corresponding primers can be found in Supplementary Table 6. All cell lines were regularly tested for mycoplasma using a MycoAlert Mycoplasma Detection kit (Lonza).

Inhibitors and antibodies. The following inhibitors were used at the indicated final concentration in vitro, unless otherwise specified in the figure or figure legends: MRT (2 µM; Sigma-Aldrich); BX-795 (1 µM; Invitrogen); TP-53A-1 (5 µM; Tocris Bioscience); PF-3644022 (1 µM; Tocris Bioscience); 7-oxoazanol (1 µM; Tocris Bioscience); Nec-1s (10 µM; Biovision); xVAD-FMK (20 µM; Abcam); cycloheximide (0.5 µg/ml; Sigma-Aldrich). All antibodies used in this study are listed in Supplementary Table 7, including information regarding dilutions and validation.

Retrotransduction of cells. Coding sequences of HOIP(2x), HOIP(3x) or TBK1(1x) or TBK1(2x130) were inserted into the retroviral MSCV vector containing GFP as the selection marker. Following infection, cells were sorted using MoFlo FACS (Beckman Coulter).

Tandem affinity purification. Samples were directly lysed in the case of TBK1-TAP-expressing cells or corresponding control cells. For A549 cells expressing HOIP(1x) or with HOIP knocked out (7.5 × 10⁶ cells each), cells were first stimulated with TAP-TNF for 15 min. Cells were subsequently solubilized in IP lysis buffer (30 mM Tris-HCl pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 10% glycerol, 1% n-dodecyl-maltoside, 50 mM NaF, 5 mM Na₃VO₄, 1 mM DTT) at 4°C overnight at 4 °C and eluted with 5 mM biotin. Proteins were precipitated using protein-A beads (Sigma) and washed three times with IP lysis buffer (1 ml), and proteins were eluted by 5 mM TEAB at ambient temperature for 20 min in the dark, and digested with 1:10 trypsin to protein ratio in-gel at 37°C for 4 h. Protein gel bands were redigested to extract any remaining peptides. Samples were evaporated to dryness at 30°C and resolubilized in 0.1% formic acid.

Mass spectrometry. nLC-MS/MS was performed using a Q Exactive Orbitrap Pro mass spectrometer interfaced to a NanoFlex ion source and coupled to an Easy-nLC 1000 (Thermo Scientific). Peptides were separated on a 24-cm fused silica emitter, 75-µm diameter, packed in-house with Reprosil-Pur C18-AQ 2.4-µm (Dr. Maisch) using a linear gradient from 5% to 30% acetonitrile/0.1% formic acid over 10 min (for TBK1 affinity purification (AP)-MS) or 30 min (for TNFR-SC AP-MS and kinase assays) at a flow rate of 250 nl/min. Precursor ions were mass selected in the higher-energy collisional dissociation mode in the orbitrap analyzer at a resolution of 70,000 and a target value of 3 × 10⁴ ions. The ten most intense ions from each MS1 scan were isolated, fragmented in the higher-energy collisional dissociation cell, and measured in the orbitrap at a resolution of 17,500. The proteomic raw data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD008497 (TNFR-SC-AP-MS and kinase assays) and PXD008477 (TBK1 analysis) and PXD008518 (TBK1 kinase assay). Analyses of these data are provided in Supplementary Tables 2, 4 and 5.

Protein and phosphosite identification. Raw data were analysed using MaxQuant v.1.5.3.8, where they were searched against the human IPI human database (http://www.uniprot.org, downloaded 22 Oct 2015 (for TNFR-SC AP-MS) and 5 Oct 2017 (for TBK1 AP-MS and kinase assays)) using default settings. Carboxymethylation of cysteine residues was set as the fixed modification, and oxidation of methionine residues and acetylation at amino termini were set as the variable modifications. Phosphorylation (STY) was set as a variable modification for the kinase assays. Enzyme specificity was set to trypsin with maximally two missed cleavage allowed. To ensure high confidence identifications, peptide-spectrum matches, peptides and proteins were filtered at a less than 1% false discovery rate. Proteins identified with a single peptide were filtered out. Label-free quantification in MaxQuant was used to quantify the AP-MS data.

Processing of TNFR-SC AP-MS data. Proteins quantified in unstimulated controls were regarded as contaminants and filtered out. Proteins detected in more than 15 experiments in the Contaminant Repository for Affinity Purification Mass Spectrometry Data (CRAPome) were filtered out. Proteins not present in the TNF- stimulation data were excluded. A protein–protein interaction network was generated in the STRING protein–protein interaction database using known interactions from curated databases and experimentally determined using default settings. Proteins not connected in the interaction network were filtered out.

Processing of TBK1 AP-MS data. Proteins quantified in TBK1 knockout cells were filtered out. Proteins detected in more than 15 experiments in the CRAPome were filtered out. A protein–protein interaction network was generated in the STRING protein–protein interaction database using known interactions from curated databases and experimentally determined using default settings. Proteins not connected in the interaction network were filtered out.

Processing of RIPK1 kinase assay data. Three raw files for each sample type (50% of the first digestion, 10% of the second digestion and 90% of the second digestion) were searched and grouped in MaxQuant to produce one output file. Non-RIPK1 phosphoproteins were filtered out from the 'phosphoSTYtxt' results file. Phosphorylation profiles of RIPK1 were obtained from the 'STY txt' file obtained from the RPK1 kinase assay. Phosphorylated sites detected in RIPK1-1x IKK (that were absent from RIPK1 alone) were taken forward. Phosphosite localization was carried out using the 'Phospho (STY) Probabilities' column. Serine, threonine or tyrosine residues with the following probability values were identified as follow: probabilities of <0.1 were considered not likely to be phosphorylated; probabilities of ≥0.1 and <0.75 were considered to be possible phosphorylations; and probabilities of ≥0.75 were considered to be likely phosphorylations.

Cell activation and immunoprecipitation. For TNFR1-SC preparation, cells were washed with PBS, resuspended in serum-free medium and stimulated with FLAG-TNF or TAP-TNF for the indicated times. Cells were lysed in IP lysis buffer at 4°C for 1 h. FLAG-TNF or TAP-TNF (500 ng) was added to the lysates of non-stimulated control samples. Subsequently, the lysates were centrifuged at 13,300 r.p.m. for 20 min and the TNFR1-SC was immunoprecipitated using M2-Agarose beads (Sigma) overnight at 4°C. The following day, the beads were washed three times with IP lysis buffer (1 ml), and proteins were eluted by
boiling in reducing sample buffer. Samples were analysed by western blotting. For the isolation of retrovirally expressed TAP-tagged proteins, cells were stimulated with untagged TNF as indicated and subjected to immunoprecipitation with biotinylated M2-Agarose. Samples of immunoprecipitation with biotinylated M2-Agarose for RIPK1 (BD, 610439) or FADD (Santa Cruz, H-181) was performed by antibody-coupling to protein A/G-Agarose beads (Santa Cruz) for 3 h at room temperature. Deubiquitylation and/or dephosphorylation was performed on some immunoprecipitates as indicated in the figure legends. For deubiquitylation, beads were resuspended in DUB buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM DTT) and 1 μM of the recombinant deubiquitinating USP2 (Boston Biochem) was added. The assay was performed at 30°C for 1 h. For samples in which the additional removal of phosphorylation was carried out, 400 U of λ-phosphatase was added where indicated and reactions were further incubated at 37°C for 30 min.

**SDS–PAGE and western blotting.** Proteins were separated using 4–15% Mini- or Midi-Protein-TGX gels (Bio-Rad) with Tris–glycine–SDS running buffer. Proteins were transferred on Midi or Midi 0.2-μm nitrocellulose membranes (Bio-Rad transfer packs) using a Trans-Blot Turbo Transfer System from Bio-Rad. Proteins were detected using antibodies as indicated.

**Isolation of BMDMs.** For the preparation of BMDMs, 6–12-week-old mice were killed. Hind legs were removed and bones were separated from muscle tissue. Femur and tibia were opened on each side, and bone marrow was flushed using a 25-gauge needle and syringe. Cells were then resuspended in RPMI medium containing 10% FCS, 1% penicillin, 1% streptomycin (Invitrogen), and 10% conditioned medium from L929 cells and passed through a cell strainer. Subsequently, cells were plated in a 12- or 24-well plate. The conditioned medium was replaced every 2 days, and cells were incubated for 7 days before the experiment.

**RNA-seq analysis.** A349 wild-type cells were pretreated with vehicle (dimethylsulfoxide (DMSO)), MRT or TPCA-1 followed by TNF stimulation (200 ng/ml) for 0, 1 or 4 h. Triplicates for each stimulation time and type of treatment were performed. Cells were then lysed and their total RNA extracted using a RNeasy Mini kit (Qiagen, 74104) according to the manufacturer's instructions. To generate the library, samples were processed using a KAPA mRNA HyperPrep kit (KK8580) according to the manufacturer's instructions. Briefly, mRNA was isolated from total RNA using Oligo dT beads to pull down polyadenylated transcripts. The purified mRNA was fragmented using chemical hydrolysis (heat and divalent metal cations) and purified with random hexamers. Strand-specific first strand complementary DNA was generated using reverse transcriptase in the presence of actinomycin D. The second cDNA strand was synthesized using dUTP in place of dTTP to mark the second strand. The resultant cDNA was then ‘A-tailed’ at the 3’ end to prevent self-ligation and adapter dimerization. Truncated adaptors, containing a T overhang, were ligated to the A-tailed cDNA. Successfully ligated cDNA molecules were then enriched by limited cycle PCR. All pipetting steps and incubations were performed by automation on a Hamilton StarLet liquid handler, with the exception of the high temperature fragmentation and the limited cycle PCR. Libraries to be multiplexed in the same run were pooled in equimolar quantities, calculated from Qubit and Bioanalyser fragment analyses. Samples were sequenced on a NextSeq 500 instrument (Illumina) using either a 43- or 81-bp paired-end run. Raw data were demultiplexed and converted to fastq files using Illumina’s bc2fastq Conversion software v.2.19. Next, the expression of Illumina paired RNA-seq transcript counts was quantified using kallisto software and a GRCh38 transcript model. The data were imported to the R statistical environment and summarized at the gene level as specified for each panel in the legends. Survival curves for the in vivo experiment were compared using log-rank Mantel–Cox tests. The significance between the samples is indicated in the figure. For the RNA-seq experiments, statistical analyses are specified in the “RNA-seq analysis” section.

**Statistics and reproducibility.** For the vast majority of the western blot analyses, results shown are representative of at least two independent experiments, as indicated in each legend. All statistical analyses were performed using GraphPad Prism v.6 software (Graphpad). Cell death data are presented as relative fluorescent units (RFU) mean of technical replicates of Sytox Green-positive cells for one representative experiment out of three independent experiments, as specified in the legends. For the loss of viability data, values are expressed as the mean percentage of loss of viability ± s.d. for three to four independent experiments as specified for each panel in the legends. Survival curves for the in vivo experiments were compared using log-rank Mantel–Cox tests. The significance between the samples is indicated in the figure. For the RNA-seq experiments, statistical analyses are specified in the “RNA-seq analysis” section.

**Code availability.** For the RNA-seq analysis, the data were imported to the R statistical environment and summarized at the gene level (that is, transcript counts summed) using tximport. Statistical transformations (for example, the log used in Fig. 2c and Supplementary Fig. 2c) and analyses of differential expression were performed using DESeq2. Multiple testing adjustments of differential expression utilized the Benjamini–Hochberg and independent hypothesis weighting (IHW) adjustments. Transcripts that were significantly changed at 1 h (corresponding to Fig. 2d) is provided in Supplementary Table 3. The raw data for RNA-seq analysis (Fig. 2; Supplementary Fig. 2c; Supplementary Table 3) and the proteomic raw data (Figs. 1a and 3f; Supplementary Tables 2, 4 and 5) are available as described below. The RNA-seq raw dataset generated during the current study is available in the SRA repository and can be accessed using the BioProject accession number PRJNA422567 or the SRA accession number SRP126844 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRR126844). The specific analysis of transcripts that significantly changed at 1 h (corresponding to Fig. 2d) is provided in Supplementary Table 3.

**Cell death analysis.** Cells were seeded the day before the experiment at 90,000 cells per well in a 24-well plate or at 150,000 cells per well in a 12 well plate. The following day, cells were pretreated with the indicated inhibitors and treated with recombinant human TNF (50 ng/ml) for RIPK1 (BD, 610439) or FADD (Santa Cruz, H-181) was performed by antibody-coupling to protein A/G-Agarose beads (Santa Cruz) for 3 h at room temperature. Deubiquitylation and/or dephosphorylation was performed on some immunoprecipitates as indicated in the figure legends. For deubiquitylation, beads were resuspended in DUB buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 5 mM DTT) and 1 μM of the recombinant deubiquitinating USP2 (Boston Biochem) was added. The assay was performed at 30°C for 1 h. For samples in which the additional removal of phosphorylation was carried out, 400 U of λ-phosphatase was added where indicated and reactions were further incubated at 37°C for 30 min.

2D gel electrophoresis. TNFR1-SC was eluted from M2-Agarose beads in 300 μl lysis buffer containing 5 U ml−1 PreScission (GE Healthcare Life Sciences) and 250 μg ml−1 3X FLAG-peptide (Sigma) for 12 h at 4°C. A second elution step was carried out for 6 h, and both elution volumes were pooled. Eluted TNFR1-SC was then prepared for 2D electrophoresis using a 2-D Clean-Up kit (GE Healthcare Life Sciences). The pellet from the second elution step was resuspended in an 85 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM 2-mercaptoethanol (GIBCO) solution (7 μM urea, 2 M thiourea, 4% CHAPS (3-(cholamidopropyl) dimethylammonio)-1-propanesulfonate), 0.5% carrier ampholyte (IPG buffer of respective pH), 20 mM DTT, 0.002% bromophenol blue), before being loaded by cup loading onto either pH 3–10 NL or pH 4–7 rehydrated 7-cm Immobiline Drystrips (GE Healthcare) and run on an Ettan IPGphor 3 system using the manufacturer's recommended settings. The Immobiline strip was then washed with equilibration buffer (GE Healthcare) containing 65 mM DTT and 135 mM iodoacetamide for 15 min each. Equilibrated gel strips were then loaded and run on NuPAGE 4–12% Bis-Tris ZOOM Protein Gels, followed by western blotting.

**Mice injections and monitoring for TNF-α induced shock.** Six- to eight-week-old female C57BL/6N mice each received two intraperitoneal injections (total volume of 300 μl per mouse) at 15 min intervals of vehicle, Nec-1s (120 μg), MRT (300 μg) or a combination thereof, followed by one intravenous injection (total volume of 150 μl per mouse) of vehicle or murine TNF (5 μg). Inhibitors and TNF were reconstituted and diluted in endotoxin-free PBS. Animals were under permanent observation. Six to ten mice were used per group, as specifically indicated for each experiment. The study protocol used for the animal experiments and for the use of the TNF-induced shock model was obtained from the German Approval of Animal Act committee (application no. V244-7224.121-1). All in vivo experiments were performed according to institutional, national and European ethical animal regulations (Protection of Animals Act).

**Data availability.** The raw data for RNA-seq analysis (Fig. 2; Supplementary Fig. 2c; Supplementary Table 3) and the proteomic raw data (Figs. 1a and 3f; Supplementary Tables 2, 4 and 5) are available as described below. The RNA-seq raw dataset generated during the current study is available in the SRA repository and can be accessed using the BioProject accession number PRJNA422567 or the SRA accession number SRP126844 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRR126844). The proteomic raw data have been deposited in the ProteomeXchange Consortium via the PRIDE® partner repository with the dataset identifiers PXD008497 (TNFR1-SC analysis), PXD010777 (TBI1 analysis) and PXD058518 (RIPK1 kinase assay). Source data for the graphs of all the other experiments in this study are available in Supplementary Table 1, and unprocessed scans for western blots are displayed in Supplementary Fig. 7. Publicly available tools were used for RNA-seq analysis as specified in the online methods, and the corresponding computational code is available upon request directly with the authors.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection

Cell death was recorded with Incucyte and Cell Titer Glo assays were run on the Mithras LB 940.

For RNA seq libraries to be multiplexed in the same run were pooled in equimolar quantities, calculated from Qubit and Bioanalyzer fragment analysis. Samples were sequenced on the NextSeq 500 instrument (Illumina, San Diego, US) using a either a 43bp or 81bp paired end run.

LC-MS/MS was performed on a QExactive Orbitrap Plus interfaced to a NANOSPRAY FLEX ion source and coupled to an Easy-nLC 1000 (Thermo Scientific). Peptides were separated on a 24 cm fused silica emitter, 75 μm diameter, packed in-house with Reprosil-Pur 200 C18-AQ, 2.4 μm resin (Dr. Maisch) using a linear gradient from 5% to 30% Acetonitrile/0.1% Formic acid over 10min (for TBK1 AP-MS) or 30 min (for TNFR-SC AP-MS and kinase assay), at a flow rate of 250 nL/min. Precursor ions were measured in a data-dependent mode in the orbitrap analyser at a resolution of 70,000 and a target value of 36 ions. The ten most intense ions from each MS1 scan were isolated, fragmented in the HCD cell, and measured in the orbitrap at a resolution of 17,500.

Data analysis

Quantifications were performed with Excel, ImageJ and GraphPad and statistical analysis with GraphPad. Images and figures were processed with Adobe Photoshop and Illustrator CS6, respectively.

For RNAseq analysis data were demultiplexed and converted to fastq files using Illumina’s bcl2fastq Conversion Software v2.19. Next, the expression of Illumina paired RNA-Seq transcript counts was quantified using kallisto software65 and a GRCh38 transcript model. The data was imported to the R statistical environment and summarised at the gene level (i.e transcript counts summed) using tximport.
Statistical transformations and analysis of differential expression were performed with DESeq271. Relevant transcripts were illustrated using BioVenn software.

For protein and phosphosite identification raw data were analysed with MaxQuant version 1.5.2.8 where they were searched against the human UniProt database (http://www.uniprot.org/, downloaded 22/10/2015 (for TNFR-SC AP-MS) and 05/10/2017 (for TBK1 AP-MS and kinase assay) using default settings. Label-free quantification in MaxQuant was used to quantify the AP-MS data.

For processing of the TNFR1-SC and TBK1 AP-MS data a protein-protein interaction network was generated in the STRING protein-protein interaction database using known interactions from curated databases and experimentally determined at default settings.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The RNA-Seq dataset generated during the current study are available in the SRA repository and can be accessed by using the following BioProject accession: PRJNA422567 or SRA accession: SRP126844 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP126844).

The proteomic data has been deposited on the ProteomeXchange Consortium via the PRIDE(ref) partner repository with the dataset identifier PXD008497 (TNFR1-SC analysis), PXD010777 (TBK1 analysis), and PXD008518 (RIPK1 kinase assay).

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences  □ Behavioural & social sciences  □ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size           | No statistical method was used to determine the correct sample size. Instead the sample sizes were determined based on our experiences from previous studies using similar methodologies. |
|-----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions       | No data was excluded from the study.                                                                                                                                                               |
| Replication           | All biological experiments were carried out under clearly defined and standard conditions and were repeated at least twice whenever possible. All replication attempts were successful.                  |
| Randomization         | Mice were randomly allocated to experimental groups.                                                                                                                                              |
| Blinding              | In vivo experiment was done by a scientist blinded to the treatment schedule. The in vitro experiments were not carried out blinded but most of them were done in parallel by at least two researchers.             |

Reporting for specific materials, systems and methods
Materials & experimental systems

Methods

| Involved in the study | Method involved in the study |
|-----------------------|-----------------------------|
| Unique biological materials | ChiP-seq |
| Antibodies | Flow cytometry |
| Eukaryotic cell lines | MRI-based neuroimaging |
| Palaeontology | |
| Animals and other organisms | |
| Human research participants | |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Knockout cell lines were generated using the CRISPR-Cas9 system and are readily available under request upon publication of the study.

Antibodies

**Antibodies used**

- p-TBK1 (Cell Signaling, 5483; TBK (Cell signaling, 3013); p-IKKe (Cell Signaling, 8766); IKKe (Cell signaling, 2905); p-IKKa/b (Cell signaling, 2697); RIPK1 (Cell signaling, 3493); p-IkBα (Cell signaling, 9242); IkBa (Cell signaling, 4670); ERK1/2 (Cell signaling, 4671); JNK (Cell signaling, 9258); p-p38 (Cell signaling, 9215); p-RIPK1 S166 (Cell signaling, 65746); murine p-RIPK1 S166 (Cell signaling, 31122); cleaved murine Caspase-8 (Cell signaling, 9429); HOIP (Ubiquigent, 68-0013-100); SHARPIN (Proteintech, 14626-1-AP); RIPK1 (BD, 610459); TNFR-1 (Santa Cruz, SC-8436); GAPDH (Abcam, ab8245); Actin (Sigma, A1978); M1-Ubiquitin (Merck Millipore, MABS199); p38 (Santa Cruz Biotech, sc-728); murine caspase-8 (Enzo Life Sciences, C15); p-MLC (Abcam, ab187091); murine p-MLC (Abcam, ab196436); murine RIPK3 (Enzo Life Sciences, ADI-905-242-100); FLAG (Sigma, M2); FADD (Enzo Life Sciences, 1F7); FADD (Santa Cruz, H-181); TANK (R&D Systems; AF4755); AZI2 / NAP1 (abcam, ab192253); Optineurin (abcam, ab151240); IKKγ (Santa Cruz, FL-419; sc-8330); SINTBAD (Cell Signaling, 8605); HOIL-1 (made in house); IkKa (Santa Cruz, B-8; sc-7606); TRAF2 (Enzo Life Sciences, ADI-AAP-422-D); FADD (Santa Cruz Biotech, sc-6036/M-19).

All antibodies used in this study were diluted 1:1000. Only p-IKKe (Cell signaling, 8766) was used 1:500 and Actin (Sigma, A1978) was diluted 1:10000.

**Validation**

- p-TBK1 (Cell Signaling, 5483; validation https://www.cellsignal.com/products/primary-antibodies/phospho-tbk1-nak-ser172-d52c2-2xp-rabbit-mab/5483
- TBK (Cell signaling, 3013); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-tbk1-nak-ser172-d52c2-2xp-rabbit-mab/5483?
- p-IKKe (Cell Signaling, 8766); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-ikk-ser172-d1b7-rabbit-mab/8766
- IkKe (Cell signaling, 2905); validation: https://www.cellsignal.com/products/primary-antibodies/ikk-d2og4-rabbit-mab/2905
- p-IKKa/b (Cell signaling, 2697); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-ikk-bser176-180-16ah-rabbit-mab/2697
- RIPK1 (Cell signaling, 3493); validation: https://www.cellsignal.com/products/primary-antibodies/rip-d94c12-2xp-rabbit-mab/3493
- p-IkBα (Cell signaling, 9242); validation: https://www.cellsignal.com/products/primary-antibodies/pikba-antibody-rodent-specific/9246
- IkBa (Cell signaling, 9242); validation: https://www.cellsignal.com/products/primary-antibodies/ikkba-antibody/9242
- p-ERK1/2 (Cell signaling, 4670); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-p44-42-mapkerk1-2-thr202-tyr204-d13-14-4e-xp-rabbit-mab/4670
- ERK1/2 (Cell signaling, 4695); validation: https://www.cellsignal.com/products/primary-antibodies/p44-42-mapk-erk1-2-137fs-rabbit-mab/4695
- p-JNK (Cell signaling, 4671); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-sapk-jnk-thr183-tyr185-98fs-rabbit-mab/4671
- JNK (Cell signaling, 9258); validation: https://www.cellsignal.com/products/primary-antibodies/jnk2-56g8-rabbit-mab/9258
- p-p38 (Cell signaling, 9215); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-p38-mapk-thr180-tyr182-3d7-rabbit-mab/9215
- p-RIPK1 S166 (Cell signaling, 65746); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-rip-s166-d13s-rabbit-mab/65746
- murine p-RIPK1 S166 (Cell signaling, 31122); validation: https://www.cellsignal.com/products/primary-antibodies/phospho-rp-166-antibody-rodent-specific/31122
- cleaved murine Caspase-8 (Cell signaling, 9429) validation: https://www.cellsignal.com/products/primary-antibodies/cleavedcaspase-8-asp387-antibody-mouse-specific/9429
- HOIP (Ubiquigent, 68-0013-100); validated by using ctr versus HOIP KO cells
- SHARPIN (Proteintech, 14626-1-AP); validated by using ctr versus SHARPIN KO cells
- RIPK1 (BD, 610459); validation: http://www.bdbiosciences.com/us/applications/research/apoptosis/phosphorylated-antibodies/purifiedmouse-anti-rip-38/rip/p/610459
- TNFR-1 (Santa Cruz, SC-8436); validation: https://www.scbt.com/scbt/product/tfn-r1-antibody-h-5
GAPDH (Abcam, ab8245); validation: https://www.abcam.com/gapdh-antibody-6c5-loading-control-ab8245.html
Actin (Sigma, A1978); validation: https://www.sigmaaldrich.com/catalog/product/sigma/a1978?lang=en&region=GB&gclid=EAIaIQobChMIgrjX1I2A3QIVBbSbTCh1HrQlQEAYASAEgJlhUFDBwE
M1-Ubiquitin (Merck Millipore, MABS199); validation: http://www.merckmillipore.com/GB/en/product/Anti-Linear-UbiquitinClone-1E3-Antibody,MM, MF-MABS199

p38 (Santa Cruz Biotech, sc-728); validation: https://www.scbt.com/scbt/product/p38alpha-antibody-n-20
murine caspase-8 (Enzo Life Sciences, C15); validation by using Caspase-8 KO MEFs versus wt MEFs
p-MLKL (Abcam, ab187091); validation: https://www.abcam.com/mlkI-phospho-s358-antibody-epr9514-ab187091.html
murine p-MLKL (Abcam, ab196436); validation: https://www.abcam.com/mlki-phospho-s345-antibody-epr95152-ab196436.html
murine RIPK3 (Enzo Life Sciences, ADI-905-242); validation: http://www.enzolifesciences.com/ADI-905-242/rrip3-polyclonalantibody/

FLAG (Sigma, M2); validation: https://www.sigmaaldrich.com/catalog/product/sigma/f1804?lang=en&region=GB&gclid=EAIaIQobChMIgrjX1I2A3QIVBbSbTCh1HrQlQEAYASAEgJlhUFDBwE
FADD (Enzo Life Sciences, 1F7); validation: http://www.enzolifesciences.com/ADI-AAM-212/fadd-monoclonal-antibody-1F7/
FADD (Santa Cruz, H-181); validation: https://www.scbt.com/scbt/product/fadd-antibody-h-181

TANK (R&D Systems; AF4755); validation: http://www.rndsystems.com/products/human-mouse-tank-antibody_af4755
AZI1 / NAP1 (abcam, Ab192253); validation: https://www.abcam.com/azi2-antibody-epr14698-c-terminal-ab192253.html
Optineurin (abcam, ab151240); validation: https://www.abcam.com/optineurin-antibody-ab151240.html

IKKT (Santa Cruz, FL-419; sc-8330); validation: https://www.scbt.com/scbt/product/ikkgamma-antibody-fl-419
SINTBAD (Cell Signaling, 8605); validation: https://www.cellsignal.co.uk/products/primary-antibodies/sintbad-d1a5-rabbitmab/8605

HOIL-1 (Home made; validated by comparing lysates from HOIL-1 KO to control cells)
IKKa (Santa Cruz, B-8; sc-7606); validation: https://www.scbt.com/scbt/product/ikkalpha-antibody-b-8#
TRA2 (Enzo Life Sciences, ADI-AAP-422-D); validation: http://www.enzolifesciences.com/ADI-AAP-422/tra2-polyclonal-antibody/
FADD (Santa Cruz Biotech, sc-6036/M-19); validation: https://www.scbt.com/scbt/product/fadd-antibody-m-19

Most of the listed antibodies have been used in our laboratory for many years and have been carefully validated using knockout MEFs or more recently via different techniques including siRNA ko, CrispR, TALEN or ZNF approach to achieve knockout cells.
Many antibodies were also validated by the provider as stated on their website.

### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | AS49 and HeLa cells were sourced from ATCC, IKKa/b KO MEFs were provided by Matthieu Bertrand, NEMO KO MEFs were provided from Manolis Pasparakis. HDIP KO MEFs were generated in our lab as well as TNF KO MEFs. All other knockout cells presented in this study were generated in our lab using the CRISPR-Cas9 system. |

| Authentication | Authentication was not performed as none of the cells used have been listed in the commonly misidentified lines. |

| Mycoplasma contamination | All cell lines were regularly tested for mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (LONZA) |

| Commonly misidentified lines (See [ICLAC register](https://www.iclarc.org)) | No misidentified lines were used in this study |

### Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

| Laboratory animals | Six- to eight-week old female C57BL/6N mice were used in this study |

| Wild animals | No wild animals were used in this study |

| Field-collected samples | No field-collected samples were used in this study |