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A novel role for RIP1 kinase in mediating TNFα production

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Receptor-interacting protein 1 (RIP1) is a Ser/Thr kinase with both kinase-dependent and kinase-independent roles in death receptor signaling. The kinase activity of RIP1 is required for necroptosis, a caspase-independent pathway of programmed cell death. In some cell types, the inhibition of caspases leads to autocrine production of TNFα, which then activates necroptosis. Here, we describe a novel role for RIP1 kinase in regulating TNFα production after caspase inhibition. Caspase inhibitors activate RIP1 kinase and another protein, EDD, to mediate JNK signaling, which stimulates Sp1-dependent transcription of TNFα. This pathway is independent of nuclear factor κB and also occurs after Smac mimetic/IAP antagonist treatment or the loss of TNF receptor-associated factor 2 (Traf2). These findings implicate cIAP1/2 and Traf2 as negative regulators of this RIP1 kinase-dependent TNFα production pathway and suggest a novel role for RIP1 kinase in mediating TNFα production under certain conditions.

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Receptor-interacting protein 1 (RIP1) is a multi-functional signal transducer involved in mediating nuclear factor κB (NF-κB) activation, apoptosis, and necroptosis. The kinase activity of RIP1 is critically involved in mediating necroptosis, a caspase-independent pathway of necrotic cell death.[1,2] RIP1 kinase and necroptosis are activated by death receptor ligands, such as TNFα and Fas, when apoptosis is blocked.[1,3] Necrostatin-1 (Nec-1), a small molecule inhibitor of RIP1 kinase activity, can also block necroptosis. In some cell types, such as mouse fibrosarcoma L929 cells, necroptosis is activated by inhibition of caspase activity using a pan-caspase inhibitor such as zVAD.fmk.[3] How RIP1 kinase is activated to mediate necroptosis induced by caspase inhibition is not clear.

RIP1 is ubiquitinated by the cellular inhibitor of apoptosis proteins, cIAP1 and cIAP2.[4,5] RIP1 is ubiquitinated by a number of other E3 ubiquitin ligases as well, suggesting that RIP1 ubiquitination might regulate RIP1 activity. Smac mimetics (SMs) are a class of compounds modeled after the N terminus of a cellular protein, Smac/DIABLO, that inhibits the IAPs. SMs are under development as anti-cancer drugs.[6,7] In some cell types, SM treatment can induce autocrine TNFα production and cell death, although the pathway has not been fully elucidated.[7–10]

TNFα is an important pro-inflammatory cytokine involved in mediating cell death and inflammation in many human diseases such as rheumatoid arthritis and cancers. In a genome-wide siRNA screen to identify genes involved in necroptosis, we found that knockdown of *tnfr1* or treatment with a TNFα-neutralizing antibody was protective against zVAD.fmk-induced cell death in L929 cells, indicating that zVAD.fmk was likely inducing autocrine TNFα production.[11] As knockdown of RIP1 protects against zVAD.fmk-induced death,[11] we tested the hypothesis that RIP1 might act upstream of TNFα production after zVAD.fmk and identified a novel function of RIP1 kinase in mediating TNFα production.

EDD/UBR5/hHYD is a putative tumor suppressor and HECT (homologous to E6-AP C-terminus)-domain-containing E3 ubiquitin ligase implicated in cellular pathways including the regulation of gene expression, the DNA damage response, and in necroptosis after it was identified in a siRNA screen.[11,12] EDD regulates gene expression transcriptionally, by forming complexes with transcription factors, and translationally by regulating protein levels of Paip2, a poly-A-binding protein inhibitor.[13,14] EDD is also important in the cellular DNA damage response, mediating ATM phosphorylation of its substrates CHK2 and p53 after DNA damage to control cell cycle arrest.[15–17] Given its multiple functions in mediating cellular processes, EDD likely acts as a chaperone protein, coordinating the various protein complexes involved in different cellular pathways.

In this study, we describe a novel RIP1 kinase-dependent TNFα production pathway occurring in cellular models of necroptosis and apoptosis. We explored this novel TNFα production pathway using a combination of chemical inhibitors and genetic analysis, and defined a protein complex containing EDD, RIP1, and cIAP1 that mediates JNK activation and transcription of TNFα. This TNFα production pathway requires RIP1 kinase and is activated specifically in response to zVAD.fmk stimulation, SM compounds, or TNF receptor-associated factor 2 (Traf2) deficiency.

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Abbreviations: RIP1, receptor-interacting protein 1; NF-κB, nuclear factor κB; NLS, nuclear localization signal; Traf2, TNF receptor-associated factor 2; Nec-1, necrostatin-1; SMs, Smac mimetics; RSV, Rous sarcoma virus; SV40 LT, SV40 large T antigen
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Results

RIP1 and EDD are required for TNFα production in response to zVAD.fmk. To directly examine whether zVAD.fmk stimulates the production of TNFα, we measured TNFα levels after zVAD.fmk treatment. TNFα could be detected in dying L929 cells treated with zVAD.fmk. Nec-1, a RIP1 kinase inhibitor, blocked the increase in TNFα protein levels as well as cell death (Figures 1a and b).

As the knockdown of RIP1 can have differential effects from the inhibition of its kinase activity alone,[11] we used Nec-1 as a tool to examine the role of RIP1 kinase in TNFα production. Although Nec-1 has been shown to be a specific inhibitor of RIP1 kinase,[2] we further tested the specificity of Nec-1 to ensure its suitability for this study. Nec-1 specifically binds RIP1 with K_d 5.6 nM for racemic Nec-1 and K_d 3.1 nM for *R*-Nec-1 (Supplementary Figure S1a). Using KINOMEscan (Ambit Biosciences, San Diego, CA, USA),[18] Nec-1 (10 μM) was tested for activity against 485 kinases and activated mutant kinases. When ranked in order of inhibition, RIP1 is the top kinase inhibited by Nec-1 (Supplementary Figure S1b). Aside from RIP1, no kinases were inhibited greater than 60% by Nec-1. PDGFRβ was inhibited by 72%, however, the K_d of binding of Nec-1 to PDGFRβ was greater than 30 μM, suggesting a false positive (Supplementary Figures S1a and b). Nec-1 is more specific than 35 known kinase inhibitors including imatinib (Gleevec) (Supplementary Figure S1c). As further evidence, Nec-1 can only protect against necroptosis in *Rip1*+/+ MEF cells, but not in *Rip1*−/− MEFs (Supplementary Figure S1d). Thus, we conclude that Nec-1 is a highly specific inhibitor of RIP1 kinase activity and an appropriate tool with which to study the specific role of RIP1 kinase.

L929 cells are exquisitely sensitive to death induced by TNFα treatment, so to directly study the effect of RIP1 on TNFα production we tested different cell types that produce TNFα in response to zVAD.fmk treatment. A mouse macrophage cell line, J774, was found to produce easily measurable TNFα levels in response to zVAD.fmk stimulation (Figure 1c). Both primary macrophages and macrophage cell lines undergo necroptosis in response to zVAD.fmk.[11,19] Although J774 cell treatment with zVAD.fmk induced necroptosis, cell death was not dependent on the production of TNFα. Cell death was observed beginning at least 12 h after the increase in TNFα was first detected and neutralization of TNFα was not sufficient to block zVAD.fmk-induced necroptosis of J774 cells (Figure 1b). Inhibition of RIP1 kinase by Nec-1 completely blocked the production of TNFα in J774 cells, suggesting that RIP1 kinase is required for TNFα production in zVAD.fmk-treated J774 cells (Figure 1c).

The production of TNFα in response to zVAD.fmk treatment was blocked by CHX suggesting that *de novo* protein synthesis is involved (Figure 1d). Consistent with this possibility, our siRNA screen found a significant enrichment of transcription factors and nucleic acid-binding genes among hits protecting against zVAD.fmk-induced necroptosis.[11] Treatment with zVAD.fmk activates *de novo* synthesis of TNFα though a mechanism dependent upon the kinase activity of RIP1.

To find additional components of the RIP1 kinase-dependent pathway of TNFα production, we identified RIP1-binding proteins. 293T cells were transfected with a vector expressing Flag-tagged RIP1 kinase, and RIP1 immunocomplexes were isolated using anti-Flag. The binding proteins were identified by mass spectrometry analysis. This analysis identified FADD, a known RIP1-binding protein, thus validating the

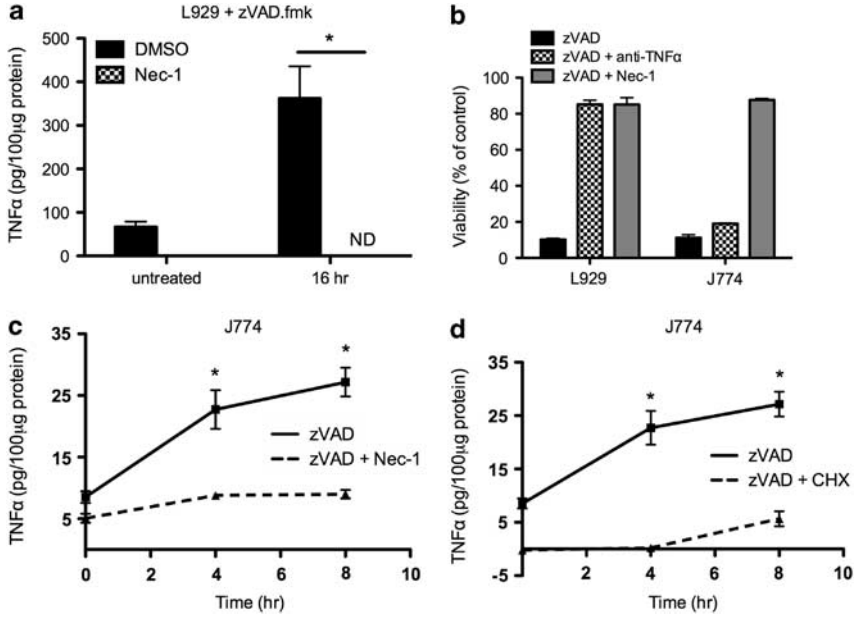

Figure 1 RIP1 kinase activates TNFα production. (**a**) TNFα levels determined by TNFα ELISA and normalized to total protein in lysate from L929 cells treated with 20 μM zVAD.fmk ± 10 μM Nec-1 for 16 h. (**b**) L929 or J774 cells treated with 20 μM zVAD.fmk ± 1.0 μg/ml anti-mTNFα or 10 μM Nec-1. Cell viability measured by ATP assay after 24 h. (**c**) TNFα levels in J774 cells treated with 20 μM zVAD.fmk ± 10 μM Nec-1 for 4 and 8 h were determined as in **a**. (**d**) TNFα levels in J774 cells treated with 20 μM zVAD.fmk ± 0.5 μg/ml CHX. *$P<0.05$, data shown are mean ± S.E.M., $n=3$

experiment. To distinguish binding proteins that have a functional role in mediating the production of TNFα, we compared the list of mass spectrometry-identified proteins with the hits identified in the genome-wide siRNA screen for genes involved in mediating necroptosis of L929 cells in response to zVAD.fmk.[11] EDD, encoded by the gene *edd1*, is both a RIP1-binding protein and a gene whose knockdown blocks zVAD.fmk-induced necroptosis. We confirmed that RIP1 coimmunoprecipitated EDD (Figure 2a). Interestingly, overexpression of EDD consistently increases the levels of exogenously expressed RIP1 protein but has no effect on endogenous RIP1, suggesting that overexpressed RIP1 is stabilized by EDD (Figure 2a, Supplementary Figure S2a). Knockdown of EDD protected against zVAD.fmk but not TNFα-induced necroptosis (Figure 2b), indicating EDD does not have any role in TNFα-induced necroptosis and instead has a likely role in zVAD.fmk-induced TNFα production. To determine whether EDD is required for TNFα production, we generated stable knockdown cell lines using retroviral infection of an shRNA construct against EDD. Knockdown of EDD inhibited zVAD.fmk-induced TNFα, demonstrating that EDD is required for TNFα production (Figure 2c).

To determine how TNFα production is activated, we used real-time PCR to measure TNFα mRNA levels after zVAD.fmk treatment. Stimulation with zVAD.fmk increased TNFα mRNA, and treatment with Nec-1, or knockdown of EDD, was able to block the increase in TNFα mRNA (Figure 2d). Thus, RIP1 and EDD activate the transcription of TNFα after zVAD.fmk treatment.

SM induces TNFα in a manner dependent on RIP1 kinase and EDD. To explore the physiological relevance of this pathway, we looked for other stimuli that could also activate this RIP1 kinase-dependent pathway of TNFα production. SM induces autocrine TNFα production in some cells.[7–10] Thus, we tested whether RIP1 and EDD have role in SM-induced TNFα. Treatment with SM induces the auto-ubiquitination and degradation of cIAP1 and cIAP2, and also blocks XIAP binding to and inhibition of caspases. We tested the effect of the previously described SM-164[20,21] on L929 cells and found that, similar to zVAD.fmk, SM-164 induced TNFα-dependent necrotic cell death that could be inhibited by Nec-1 or a TNFα-neutralizing antibody (Figure 3a). Knockdown of RIP1, EDD, or TNFR1 was able to block SM-induced necroptosis, suggesting that SM might be activating the same RIP1- and EDD-dependent pathway of TNFα production as zVAD.fmk (Figure 3b). ELISA was used to detect an increase in TNFα in the lysate of SM-treated L929 cells. SM-induced TNFα could be inhibited by Nec-1 treatment and in EDD-knockdown cells, confirming that SM activates RIP1 kinase and EDD-dependent TNFα production (Figure 3c).

Traf2, an E3 ubiquitin ligase, is constitutively bound to cIAP1/2 within the cell.[22,23] Traf2-knockout mice have elevated levels of serum TNFα,[24] which suggested to us that Traf2 might act similarly to cIAP1/2 to inhibit induction of TNFα. Indeed, knockdown of Traf2 in L929 cells induced TNFα-dependent necroptosis that could be blocked by both TNFα-neutralizing antibody or knockdown of TNFR1 (Figure 3d). Similar to SM- and zVAD.fmk-induced cell death, Traf2 knockdown-induced death could be blocked by Nec-1 and by knockdown of RIP1 or EDD (Figure 3d). Thus, the absence of Traf2, similar to the loss of cIAP1/2 during SM treatment, activates RIP1- and EDD-dependent TNFα production.

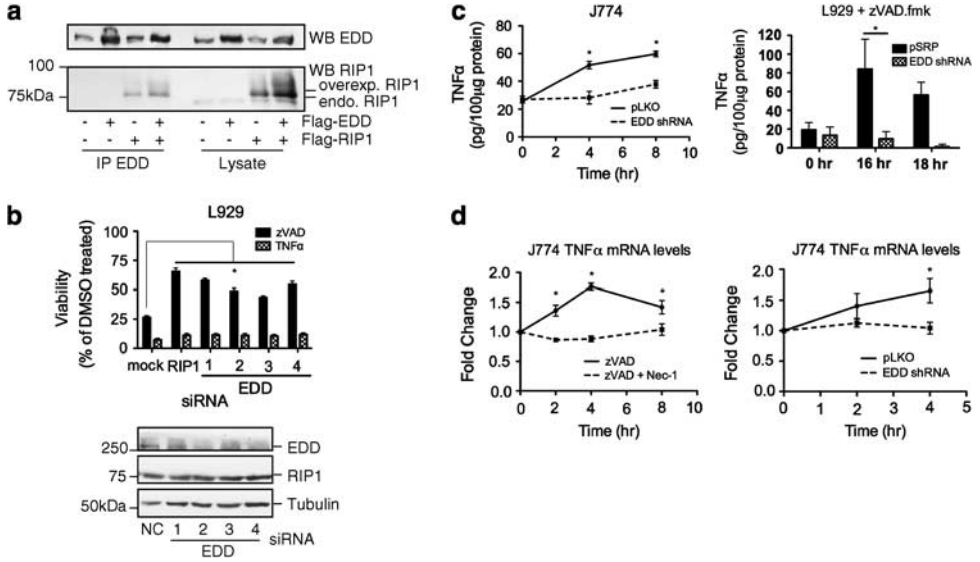

Figure 2 EDD mediates TNFα production. (**a**) 293T cells transfected with Flag-EDD and Flag-RIP1 as indicated. Cell lysates and anti-EDD immunoprecipitates were western blotted with anti-RIP1 and anti-EDD to show both endogenous and overexpressed protein. (**b**) L929 cells transfected for 48 h with siRNA targeting RIP1 or one of the four different siRNAs targeting EDD were treated for 24 h with 20 μM zVAD.fmk or 10 ng/ml hTNFα and cell viability was measured by ATP assay (top) or lysates collected for western blot (bottom) to check protein knockdown. (**c**) J774 cells (left) or L929 cells (right) stably expressing shRNA against EDD or an empty vector control were treated with 20 μM zVAD.fmk and TNFα measured as in **a**. EDD knockdown shown in Figure 6e and Supplementary Figure S3a. (**d**) Real-time PCR of RNA isolated from J774 cells treated with zVAD.fmk with or without Nec-1 (left) or EDD stable knockdown J774 cells treated with zVAD.fmk (right). Relative levels of TNFα transcript were determined compared with GAPDH (left) or 18S rRNA (right) and the fold change was calculated by comparing with DMSO-treated cells. $^*P<0.05$, data shown are mean ± S.E.M., for all experiments $n=3$

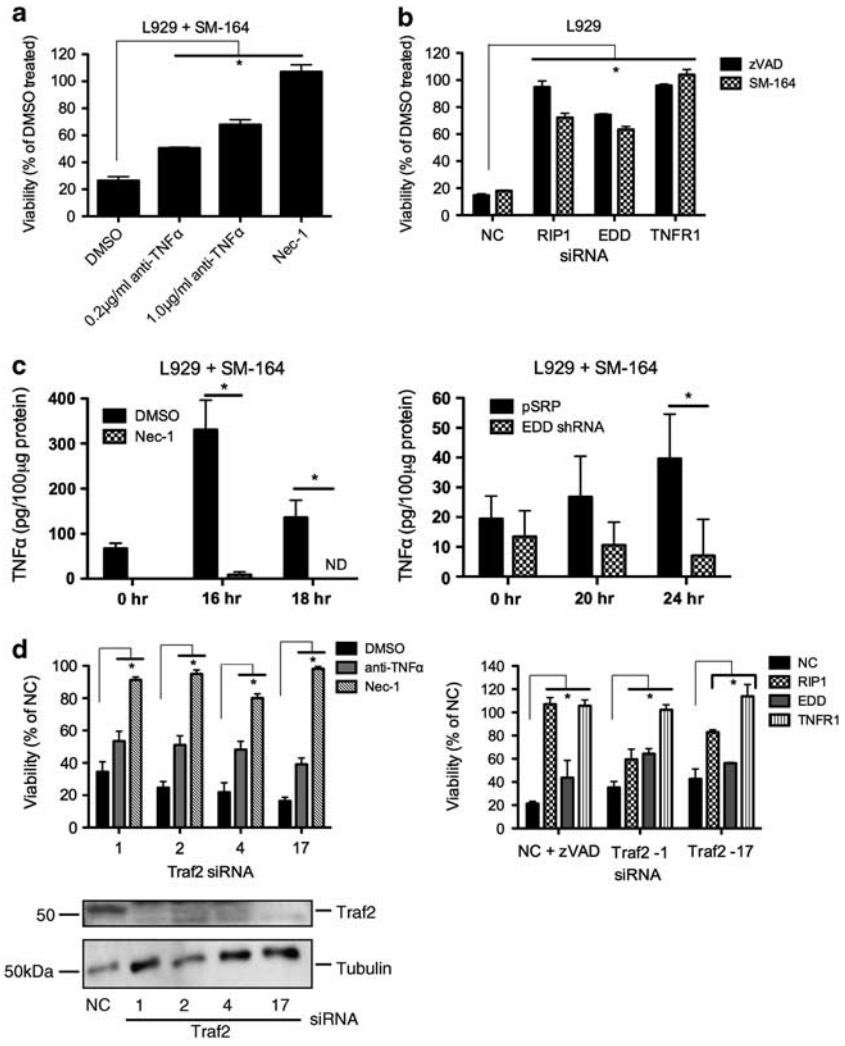

Figure 3 Traf2 and cIAP1 inhibit RIP1- and EDD-dependent TNFα production. (**a**) ATP assay of L929 cells treated with 100 nM SM-164 ± TNFα-neutralizing antibody or 10 μM Nec-1 for 24 h. (**b**) Viability of L929 cells transfected with siRNA against RIP1, EDD, TNFR1, or negative control (NC) for 48 h and treated with 20 μM zVAD.fmk or 100 nM SM-164 for 24 h. (**c**) TNFα levels were measured by ELISA in L929 cells treated with 100 nM SM-164 ± 10 μM Nec-1 (left) or stably expressing EDD shRNA or vector control (pSRP) and treated with 100 nM SM-164 (right). (**d**) ATP assay of L929 cells transfected with siRNA against Traf2 and treated for 72 h, with 1.0 μg/ml anti-mTNFα antibody or 10 μM Nec-1 (left). For western blot, the cells were treated with 10 μM Nec-1 from the start of transfection to block cell death and harvested after 48 h. L929 cells transfected with siRNAs targeting Traf2 and either RIP1, EDD, or TNFR1. NC ± siRNA treated with zVAD.fmk used as positive control. Viability was calculated as the ratio of the luminescence values of Traf2 + siRNA to untreated NC + siRNA (right). $^*P<0.05$, data shown are mean ± S.E.M., for all experiments $n=3$

SM-induced TNFα production has been studied in human cancer cell lines including breast, ovarian, and lung cancer cells. To validate the role of this RIP1- and EDD-dependent pathway in a previously established model of autocrine TNFα production, we tested the effect of Nec-1 and EDD knockdown on SM-induced apoptosis in the human breast cancer MDA-MB-231 cells. MDA-MB-231 cells undergo TNFα-dependent apoptosis with 100 nM SM-164.[20] As has been previously shown, knockdown of RIP1 is able to block SM-induced apoptosis (Figure 4a).[7] However, neither Nec-1 treatment nor knockdown of EDD inhibited 100 nM SM-induced apoptosis (Figures 4a and b). Surprisingly, we found that a 1000-fold lower dose of SM-164 (0.1 nM) is sufficient to induce TNFα-dependent apoptosis, with death blocked by a TNFα-neutralizing antibody or by caspase inhibition. This 'low dose' of SM induces RIP1 kinase-dependent cell death that is blocked by Nec-1 or by knockdown of EDD (Figure 4c).

To determine whether RIP1 kinase induces TNFα transcription in the MDA-MB-231 cell model, we measured TNFα mRNA levels by real-time PCR after treatment with either 0.1 nM or 100 nM SM-164. Both doses of SM increased TNFα transcription, but Nec-1 was only able to inhibit TNFα transcription in the cells stimulated with the low dose of SM (Figure 4d). The high, 100 nM dose of SM might be activating additional or non-specific pathways that contribute to TNFα transcription and cell death in a RIP1 kinase-independent manner. Low-dose SM activates a specific RIP1 kinase-dependent mechanism of TNFα transcription.

A complex of EDD, RIP1, and the E3 ligase cIAP1 regulates TNFα production. To confirm the complex of

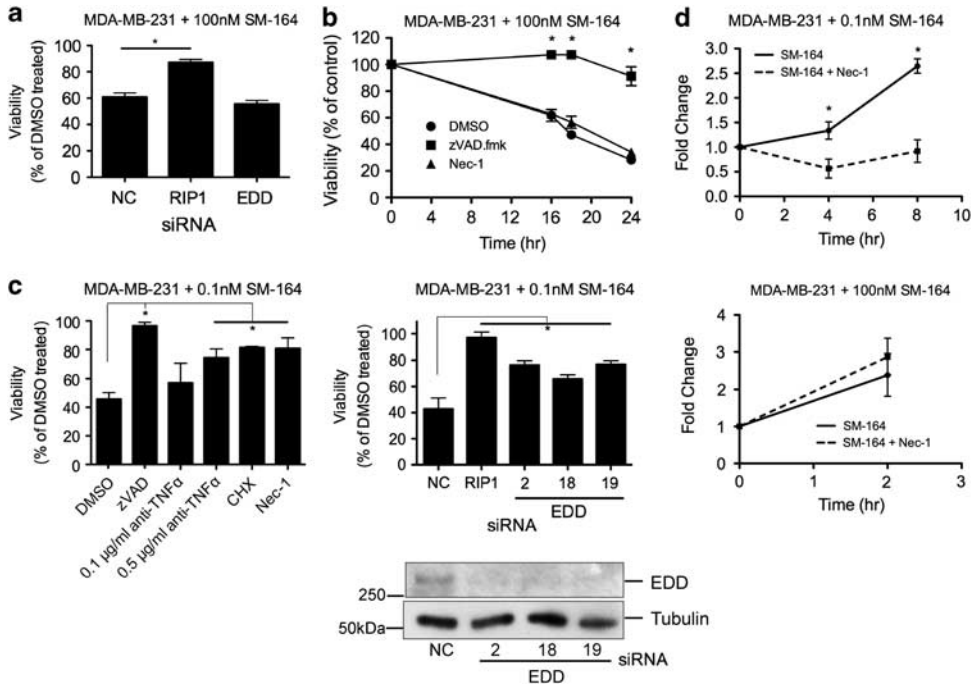

Figure 4 Smac mimetic induces RIP1 kinase- and EDD-dependent TNFα production in MDA-MB-231 cells. (**a**) ATP assay of MDA-MB-231 cells transfected with NC, RIP1, or EDD siRNA for 48 h and treated with 100 nM SM-164 for 24 h. (**b**) Time course of MDA-MB-231 cells treated with 100 nM SM-164 ± 20 μM zVAD.fmk or 10 μM Nec-1. Cell viability was measured by ATP assay. (**c**) ATP assay of MDA-MB-231 cells treated with 0.1 nM SM-164 ± 20 μM zVAD.fmk, anti-hTNFα-neutralizing antibody, 10 μg/ml CHX, or 10 μM Nec-1 for 24 h (left). MDA-MB-231 cells transfected with the indicated siRNA for 48 h and treated with 0.1 nM SM-164 for 24 h or harvested for western blot (right). (**d**) RT-PCR of RNA from MDA-MB-231 cells treated with 0.1 nM SM-164 (top) or 100 nM SM-164 (bottom) ± 10 μM Nec-1 for 2, 4, or 8 h as indicated. Relative levels of TNFα transcript determined by normalizing to 18S rRNA transcript and fold change calculated in comparison with DMSO-treated cells. $^*P<0.05$, data shown are mean ± S.E.M., for all experiments $n=3$, western blots were repeated two times with similar results

EDD with RIP1, we examined the interaction of EDD and RIP1. We found that RIP1 coimmunoprecipitates with endogenous EDD in a constitutive manner and this binding is unaffected by RIP1 kinase activation with zVAD.fmk stimulation (Figure 5a) or after SM treatment (Figure 5b). Additionally, cIAP1, the target of SM, also coimmunoprecipitates with EDD unaffected by zVAD.fmk treatment (Figure 5c).

To understand the interactions between the proteins in this complex, we determined the binding domains of RIP1 and cIAP1 to EDD. We tested the ability of EDD to bind RIP1 truncation mutants lacking the kinase domain (ΔKD), the death domain (ΔDD), and both the intermediate and death domains (ΔC). Each of these RIP1 truncation mutants expressed similarly, except for RIP1 ΔKD, which expressed as several bands. This is not due to loss of its kinase activity as the kinase inactive RIP1 K45M does not show this expression pattern (Supplementary Figure S2b). Each of the RIP1 truncation constructs was able to coimmunoprecipitate with EDD, however, the amount of ΔKD in the immunocomplex was enriched compared with that of ΔC or ΔDD, suggesting that EDD predominantly interacts with the non-kinase domain of RIP1 (Figure 5d).

EDD was expressed with cIAP1 or a related family member, XIAP, to confirm the specificity of binding. Antibodies against EDD coimmunoprecipitated cIAP1, but not XIAP (Figure 5e). Testing the binding of cIAP1 deletion constructs indicated that BIR1 and BIR2 of cIAP1 are the minimal regions to coimmunoprecipitate with EDD, and are pulled down with the same efficiency as full-length cIAP1, suggesting that BIR1-BIR2 is sufficient for cIAP1 to bind EDD (Figure 5f). There was no binding observed of the BIR3, CARD, or RING-containing constructs with EDD, indicating these domains do not interact with EDD. The BIR domains of cIAP1 also mediate its interactions with other proteins such as Traf2, Smac, and RIP1.[25,26] EDD may act as a scaffold and bind to both cIAP1 and RIP1 kinase. This complex is important for regulating downstream signaling and the activation of TNFα transcription after zVAD.fmk or SM treatment.

A NF-κB independent mode of TNFα production. Both the canonical and non-canonical NF-κB pathways (NF-κB1 and NF-κB2, respectively) are activated by SM treatment; inhibition of NF-κB1 blocks TNFα production and cell death.[9,10] Loss of either cIAP1/2 or Traf2 stabilizes NIK, inducing the proteasomal processing and activation of NF-κB2.[9,10,27] Indeed, NF-κB2 is processed and activated within hours of SM-164 treatment in both 100 nM SM-164-treated MDA-MB-231 cells and SM-treated L929 cells (Supplementary Figure S2c, Figure 6a). Low-dose SM (0.1 nM) induced NF-κB2 processing in MDA-MB-231 cells as well, albeit with slower kinetics, likely due to the slower rate of cIAP1 degradation (Supplementary Figure S2c). Importantly, Nec-1 had no effect on NF-κB2 processing in

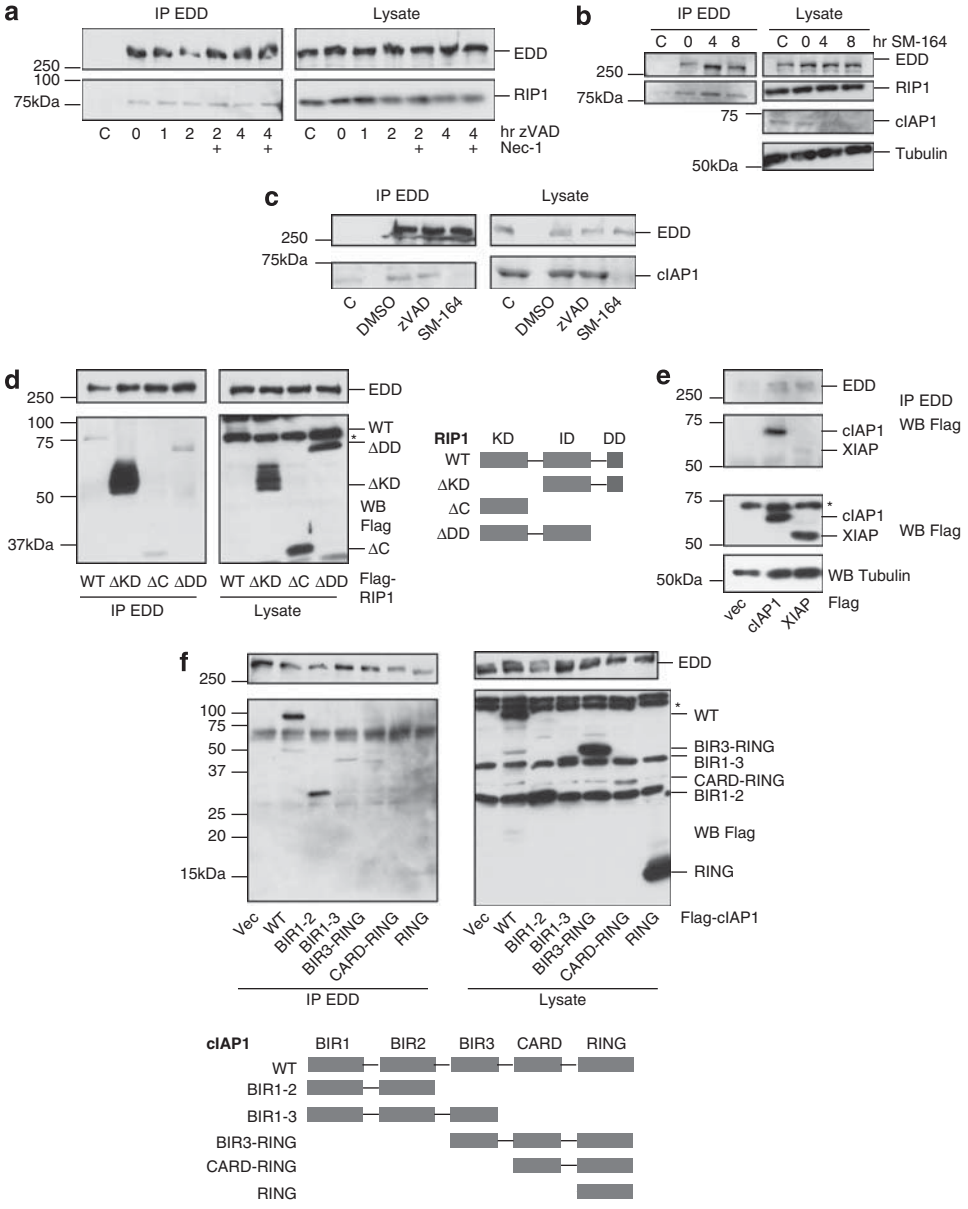

Figure 5 Complex of EDD, RIP1, and cIAP1 activate TNFα production. (**a**) J774 cells treated with 20 μM zVAD.fmk ± 10 μM Nec-1 and immunoprecipitated with anti-EDD or an isotype-matched control antibody (C). The immunoprecipitated proteins were western blotted with anti-RIP1 (representative of three independent experiments). (**b**) L929 cells were treated with 100 nM SM-164 and EDD immunoprecipitated from cells and western blotted for RIP1. (**c**) L929 cells treated with 20 μM zVAD.fmk or 100 nM SM-164 for 7 h and EDD immunoprecipitated and western blotted for cIAP1 interaction. (**d**) EDD immunoprecipitated from 293T cells expressing EDD and the indicated Flag-RIP1 construct. IP was western blotted with anti-Flag to detect RIP1 interaction. (**e**) 293T cells expressing EDD and Flag-cIAP1, Flag-XIAP, or vector control. EDD immunoprecipitated and western blotted with anti-Flag to detect IAP binding. (**f**) EDD immunoprecipitated from 293T cells expressing indicated Flag-cIAP1 construct and western blotted with anti-Flag to detect interaction of cIAP1 construct. All experiments were repeated at least two times. * Indicates a non-specific band

either low-dose SM-treated MDA-MB-231 cells or SM-stimulated L929 cells, indicating that RIP1 kinase does not mediate NF-κB2 activation (Figure 6a, Supplementary Figure S2c). We were unable to detect the degradation of the inhibitor protein IκBα, a marker for NF-κB1 activation, under any stimulation (Figure 6a, Supplementary Figures S2c and d). As NF-κB is not activated in a RIP1 kinase-dependent manner after either zVAD.fmk or SM stimulation, NF-κB is unlikely to have a role in cell death or TNFα production as induced by zVAD.fmk or SM in L929 and J774 cells.

Although we did not observe RIP1 kinase-dependent activation of NF-κB, to verify that NF-κB does not have a role in zVAD.fmk- or SM-induced cell death, we knocked down NF-κB1 and NF-κB2 and tested the effect on cell death. Consistent with previously published work,[28] knockdown of NF-κB1 and NF-κB2 were not protective against necroptosis

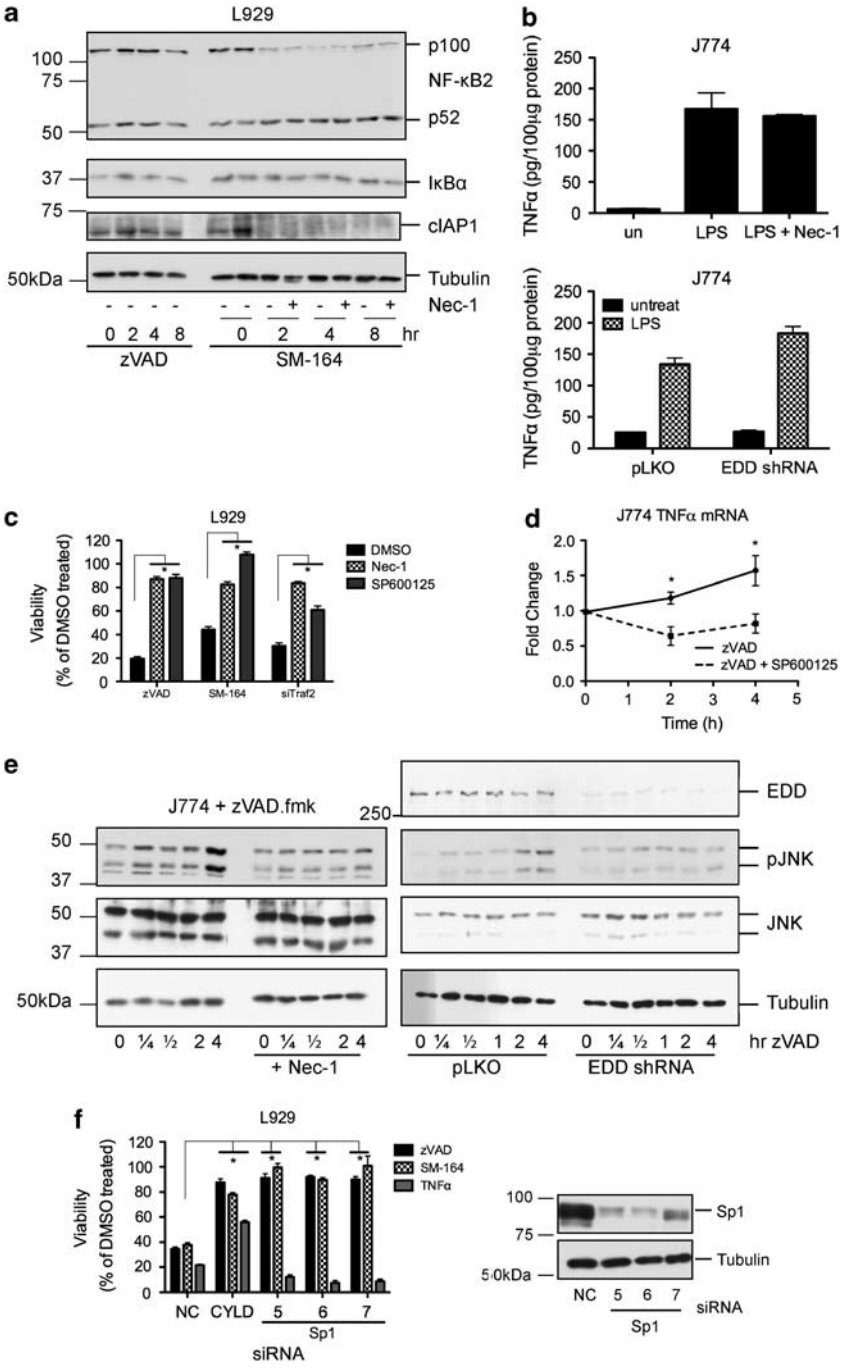

Figure 6 RIP1 and EDD activate JNK signaling and TNFα production. (**a**) L929 cells treated with 20 μM zVAD.fmk or 100 nM SM-164 ± 10 μM Nec-1 for 2, 4, and 8 h. Cells lysates were western blotted to determine NF-κB activation (representative of two independent experiments). (**b**) WT J774 cells (top) or stable EDD knockdown cells (bottom) treated with 1 μg/ml LPS ± Nec-1 for 2 h and TNFα levels measured by ELISA in the cell lysate (NS). (**c**) ATP assay of L929 cells treated as indicated ± 10 μM Nec-1 or 1 μg/ml JNK inhibitor SP600125 for 24 h. (**d**) J774 TNFα mRNA levels determined by RT-PCR after treatment with 20 μM zVAD.fmk ± 1 μg/ml SP600125 for 2 and 4 h. Fold change calculated by comparing with GAPDH control and normalizing to untreated cells. (**e**) J774 cells (left) or J774 EDD stable knockdown cell lines (right) were treated with zVAD.fmk ± Nec-1 as indicated for 15 min, 30 min, 1, 2, or 4 h and western blotted with anti-phospho-JNK (pJNK) (representative of three independent experiments). (**f**) ATP assay of L929 cells transfected with siRNA targeting Sp1 for 48 h and treated with 20 μM zVAD.fmk, 100 nM SM-164, or 10 ng/ml hTNFα for 24 h. Cell lysates western blotted to confirm knockdown of Sp1. $^*P<0.05$, data shown are mean ± S.E.M., for all experiments $n=3$

(Supplementary Figure S2e). An inhibitor of NF-κB, SN50,[29] did not block zVAD.fmk-induced TNFα release in J774 cells (Supplementary Figure S2f). Thus, we conclude that TNFα production induced by zVAD.fmk or SM is independent of NF-κB activity.

Lipopolysaccharide (LPS) is a pro-inflammatory stimulus found on the outer membrane of bacteria that activates TNFα transcription. Although LPS-induced TNFα is dependent on NF-κB, we tested the role of RIP1 and EDD in this pathway of TNFα production. Neither Nec-1 nor EDD knockdown was

able to block LPS-induced TNFα (Figure 6b). Thus, RIP1 and EDD activation of TNFα production is distinct from NF-κB-dependent pathways such as LPS-induced TNFα.

JNK signaling activates TNFα transcription downstream of RIP1 and EDD. Our siRNA screen identified a number of transcription factors as hits, including c-Jun and Sp1, both of which can be activated by JNK and MAPK signaling.[11,30,31] The importance of c-Jun/AP-1 in mediating TNFα transcription and cell death in response to zVAD.fmk has already been confirmed.[19,28] Inhibition of JNK signaling blocks zVAD.fmk-, SM-, or Traf2 knockdown-induced necroptosis (Figure 6c). The JNK inhibitor SP600125 blocks TNFα transcription induced by zVAD.fmk (Figure 6d). Consistently, increased phosphorylation of JNK, indicating JNK kinase activation, was observed shortly after zVAD.fmk treatment. A definitive role for RIP1 kinase in activating JNK signaling has not been previously shown. We found that JNK phosphorylation after zVAD.fmk was attenuated in Nec-1-treated and EDD-knockdown cells (Figure 6e, Supplementary Figure S3a). SM stimulation also activates JNK signaling in a manner dependent on RIP1 kinase and EDD (Supplementary Figure S3b). Thus, RIP1 kinase and EDD activate JNK signaling to induce TNFα transcription.

TNFα itself can also activate JNK. Nec-1, however, has no effect on TNFα-induced JNK phosphorylation (Supplementary Figure S3c). AIP1/Dab2IP, an ASK1 (JNK MAP3K) interacting protein, is reportedly a substrate of RIP1 after TNFα stimulation.[32] However, neither knockdown of AIP1/Dab2IP nor knockdown of ASK1 in our siRNA screen[11] protected against zVAD.fmk-induced cell death (Supplementary Figure S3d). TNFα-induced JNK activation is RIP1 kinase independent and is activated by a separate pathway from zVAD.fmk treatment.

Analysis of our siRNA screen showed a significant enrichment of screen hits with Sp1-binding sites in their promoters, suggesting that Sp1 regulates transcription of genes required for necroptosis.[11] Consistent with this possibility, we found that knockdown of Sp1 specifically inhibits zVAD.fmk- or SM-induced cell death but not TNFα-induced necroptosis, similar to EDD knockdown (Figure 6f). Sp1 is able to synergize with c-Jun/AP-1 to activate TNFα transcription.[33] It is likely that both of these transcription factors are activated by JNK signaling downstream of RIP1 kinase and EDD to promote transcription of TNFα and possibly other genes in response to zVAD.fmk or SM stimulation.

Discussion

RIP1 kinase has been previously shown to specifically mediate TNFα-induced necroptosis downstream of TNFR1 by regulating the formation of complex IIb.[34,35] In this study, we demonstrate a novel function of RIP1 kinase involving its interaction with EDD to regulate JNK activation and TNFα production. This pathway of TNFα production is activated specifically in response to treatment with zVAD.fmk or SM, or by knockdown of Traf2 and is distinguishable from the TNFα production pathway regulated by NF-κB that can be activated by TLR signaling (Figure 7). We show that activation of this

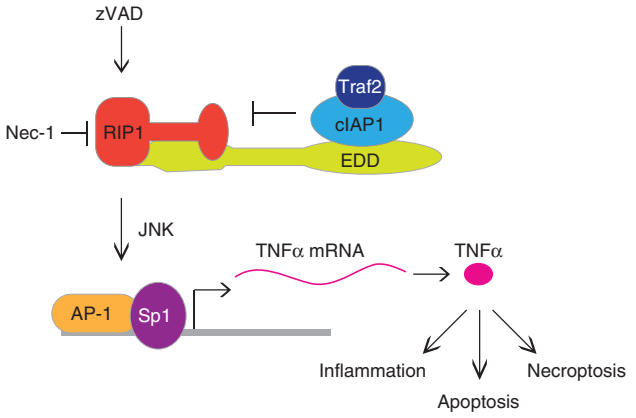

Figure 7 Model of RIP1- and EDD-mediated pathway of TNFα production. RIP1 kinase-dependent TNFα production can be activated by the treatment with zVAD.fmk, SM to degrade cIAP1/2, or by knockdown of Traf2. EDD, RIP1, and cIAP1 are constitutively bound and RIP1 kinase and EDD activate JNK signaling after stimulation. Traf2 and cIAP1 also constitutively interact. JNK activates the transcription factors AP-1 and Sp1, which transcribe TNFα mRNA. Nec-1 inhibits the activation of JNK and production of TNFα. TNFα produced can elicit inflammatory responses and induce cell death in the surrounding cells

RIP1 kinase-dependent pathway leads to TNFα transcriptional activation. Our examination of this pathway indicates that RIP1 kinase and EDD mediate a common pathway of JNK activation and TNFα production in mouse and human systems, cell types such as macrophages and breast cancer cells, and in cells capable of undergoing either apoptosis or necroptosis. Our study suggests that RIP1 kinase not only regulates necroptosis downstream of TNFR1 signaling, but also has an important role in mediating the production of TNFα.

The role of RIP1 kinase in activating TNFα production is distinct from its role in mediating necroptosis. The function of RIP1 in TNFα production may provide a possible explanation for situations where Nec-1 was found to protect against apoptosis. MDA-MB-231 cells treated with a low dose of SM undergo typical TNFα-dependent apoptosis that can be blocked by Nec-1. This is due Nec-1 inhibiting the production of TNFα, which is required for apoptosis to occur after SM treatment, rather than a role of RIP1 kinase in mediating apoptosis itself. SM are currently in clinical trials as an anti-cancer treatment, indicating the role of RIP1 kinase in mediating the production of TNFα may be relevant for SM activity *in vivo*.

Both cIAP1/2 and Traf2 have been implicated as E3 ubiquitin ligases targeting RIP1.[5,36] The finding that loss of either cIAP1/2 or Traf2 can activate RIP1 suggests that these proteins normally function to keep RIP1 inactive. *traf2*−/− mice are normal at birth but become progressively runted and die prematurely with elevated serum TNFα levels.[24] The lethal phenotype is rescued by the loss of TNFα or TNFR1 in double-knockout *traf2*−/− *tnf*−/− or *traf2*−/− *tnfr1*−/− mice.[37] Our study suggests the possibility that RIP1 kinase regulated JNK activation mediates the production of TNFα in these mouse models of human diseases. The identification of SM and Traf2 knockdown as inducers of RIP1 kinase-mediated

TNFα production suggests that RIP1 kinase activation might be regulated directly by ubiquitination by cIAP1/2 and/or Traf2.

SMs, in addition to inducing the degradation of cIAP1 and cIAP2, and inhibit the activity of XIAP.[6,20] Many of the cellular effects of SM in sensitizing cells to apoptosis have been attributed to the loss of caspase inhibition by XIAP.[8,20] However, the effect of SM on RIP1 is likely due to the degradation of cIAP1/2, as the effect of SM on TNFα production was recapitulated in cIAP1-knockout cells but not XIAP-deficient cells.[10] Furthermore, EDD specifically interacts with cIAP1, so it is likely that they act in the same pathway.

EDD has been implicated in diverse cellular processes such as the DNA damage response and gene expression. EDD constitutively binds RIP1 kinase and the E3 ubiquitin ligase cIAP1. We propose that EDD functions as a scaffold protein in this pathway and interacts with the critical regulatory proteins. Similar to its role in mediating ATM phosphorylation of its substrates p53 and CHK2,[15–17] EDD may also mediate RIP1 phosphorylation of its substrate(s) in this pathway. We propose that EDD and RIP1 kinase mediate the activation of JNK signaling, potentially via recruitment of a RIP1 substrate that activates JNK to mediate multiple signaling pathways that are regulated by JNK. Future work is needed to determine how RIP1 kinase and EDD activate the JNK signaling pathway.

Materials and Methods

Gene knockdown experiments. L929 and MDA-MB-231 cells were reverse transfected with 25-50 nM siRNA (Dharmacon, Lafayette, CO, USA) in 384-well plates (Corning, Lowell, MA, USA) using HiPerfect transfection reagent (Qiagen, Gaithersburg, MD, USA), according to the manufacturers protocol. After 48 h of transfection, the cells were treated with zVAD.fmk, hTNFα, or SM-164. After 18–24 h, cell viability was determined by ATP assay using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI, USA). Stable knockdown cell lines were generated using a retroviral or lentiviral expression system to infect L929 or J774 cells, respectively. Expression of pSRP or pLKO.1 and packaging vectors were transfected into 293T cells to generate virus. L929 or J774 cells were infected with the virus and selected in medium containing 10 μg/ml or 2 μg/ml puromycin, respectively.

TNFα ELISA. J774 cells were plated 1×10^5 cells/well in 24-well plates or L929 cells plated 4×10^6 cells/10-cm plate, treated, and the cell supernatant collected or cells lysed in 1% Triton X-100 in PBS supplemented with Complete Protease Inhibitor (Roche, Indianapolis, IN, USA). Total protein levels were determined by Bradford protein assay (Bio-rad, Hercules, CA, USA). The levels of TNFα in cell lysate or supernatant were quantified using the mTNFα ELISA kit according to the manufacturers instructions (R&D Systems, Minneapolis, MN, USA).

Real-time PCR. To determine mRNA levels of TNFα in the cell, RNA was harvested according to the manufacturers protocol using the Qiagen RNeasy kit. One microgram of RNA was reverse transcribed with random hexamers and SuperScript II First Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA). The qPCR performed using 2× SYBR green master mix on the ABI 7900HT qPCR machine (Applied Biosystems, Carlsbad, CA, USA). Fold change in RNA was calculated using the comparative *C*t method, normalizing to 18S rRNA or GAPDH control. Taqman probes were used for mTNFα and GAPDH (Applied Biosystems). Primers used are as follows: mTNFα (forward: 5′-CTTCTCAT TCCTGCTTGTGG-3′, reverse: 5′-ATGAGAGGGAGGCCATTTG-3′), hTNFα (forward: 5′-GAGGCCAAGCCCTGGTATG-3′, reverse: 5′-CGGGCCGATTGATCT CAGC-3′ PrimerBank ID 25952110b2),[38] or 18S rRNA (foward: 5′-CCTGCGGCTT AATTTGACTC-3′, reverse: 5′-AGACAAATCGCTCCACCAAC-3′).

Coimmunoprecipitation. 293T cells were transfected by the calcium phosphate method and lysed after 24 h in 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, and protease inhibitor cocktail (Roche). L929 cells were lysed in the buffer described except with 50 mM NaCl and 0.5% NP-40. The indicated antibody was used with protein A/G sepharose beads (Thermo Scientific, Waltham, MA, USA) for immunoprecipitation. IP was analyzed by SDS-PAGE and western blot.

Mass spectrometry of RIP1 interacting proteins. 293T cells were transfected by the calcium phosphate method with Flag-tagged RIP1 for 48 h. The cells were lysed (20 mM HEPES (pH 7.3), 5 mM EDTA, 150 mM NaCl, 5 mM NaF, 0.2 mM $NaVO_3$, 1% Triton X-100, complete protease inhibitor cocktail) and immunoprecipitated using anti-Flag M2 agarose (Sigma, St. Louis, MO, USA). The beads were washed 5× with lysis buffer and the bound proteins eluted using 150 ng/μl Flag peptide. The eluted proteins were TCA precipitated and identified by mass spectrometry.

Expression vectors. The following plasmids were used: pcDNA3-Flag-RIP1 WT, ΔKD, ΔC, and ΔDD. RIP1 ΔKD was constructed using the following primers: 5′-CGAATCCGGAATTCCGGCCGACATTT-3′, and 5′-TGCAGACTCGAGGTTC TGGCTGACGTAAAT-3′ to PCR a fragment from nt 843-2007 (NM_003804). RIP1 ΔDD was constructed using the forward primer 5′-ACGATGACGATAAAG AATTCAGGATGCAA-3′, and the reverse primer 5′-AGGTGCTCGAGCGTCAG ACTAGTGGTATT-3′ to PCR a fragment from nt 1-1751 (NM_003804). RIP1 ΔC was constructed using the same forward primer as the ΔDD construct and the reverse primer 5′-TTCTTCTAATTGCTCGAGATAAAAAGGCCT-3′ to PCR a fragment from nt 1-885 (NM_003804). Each of the PCR fragments was cloned into pcDNA3 using *Eco*RI and *Xho*I. pCMV-Tag2b-Flag-EDD (courtesy of C.K.W. Watts); Flag-XIAP; Flag-cIAP1, WT and the truncation constructs BIR1-2, BIR1-3, BIR3-RING, CARD-RING, RING. The shRNAs targeting EDD were inserted into the empty vector backbone (sequences: 5′-TGACAGCAGAACAACATAATT-3′ in pSRP; 5′-GCTCGTCTTGATCTACTTTAT-3′ in pLKO.1) (courtesy of K.P. Lu).

Antibodies and reagents. The primary antibodies used were anti-RIP1 (BD Biosciences, San Jose, CA, USA), anti-EDD (Novus Biologicals for IP, rat monoclonal raised against aa 459–794 for WB), anti-pJNK, anti-JNK, and anti-NF-κB2 (Cell Signaling), anti-NF-κB1, anti-IκBα, and anti-Traf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cIAP1, anti-Flag M2 (Sigma), and anti-mouse and anti-human TNFα-neutralizing antibodies (R&D Systems). The compounds used were zVAD.fmk, hTNFα (Cell Sciences, Canton, MA, USA), SM-164 (kindly provided by Dr. Xiaomeng Wang),[20,21] 7-Cl-O-Nec-1,[3] SP600125 (A.G. Scientific, San Diego, CA, USA), SN50/SN50M (Calbiochem, Darmstadt, Germany), and LPS (Sigma).

Tissue culture. L929, J774, and 293 T cells were maintained in DMEM supplemented with 10% FBS and penicillin and streptomycin (Invitrogen). MDA-MB-231 cells were grown in RPMI with 10% FBS, penicillin, and streptomycin (Invitrogen).

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)