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NF-κB contributes to Smac mimetic-conferred protection from tunicamycin-induced apoptosis

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Abstract
Smac mimetics that deplete cellular inhibitor of apoptosis (cIAP) proteins have been shown to activate Nuclear Factor-kappa B (NF-κB). Here, we report that Smac mimetic-mediated activation of NF-κB contributes to the rescue of cancer cells from tunicamycin (TM)-triggered apoptosis. The prototypic Smac mimetic BV6 activates non-canonical and canonical NF-κB pathways, while TM has little effect on NF-κB signaling. Importantly, ectopic expression of dominant-negative IκBα super-repressor (IκBα-SR), which inhibits canonical and non-canonical NF-κB activation, significantly reversed this BV6-imposed protection against TM. Similarly, transient or stable knockdown of NF-κB-inducing kinase, which accumulated upon exposure to BV6 alone and in combination with TM, significantly counteracted BV6-mediated inhibition of TM-induced apoptosis. Interestingly, while cIAP2 was initially degraded upon BV6 treatment, it was subsequently upregulated in an NF-κB-dependent manner, as this restoration of cIAP2 expression was abolished in IκBα-SR-overexpressing cells. Interestingly, upon exposure to TM/BV6 apoptosis was significantly increased in cIAP2 knockdown cells. Furthermore, NF-κB inhibition partially prevented BV6-stimulated expression of Mcl-1 upon TM treatment. Consistently, Mcl-1 silencing significantly inhibited BV6-mediated protection from TM-induced apoptosis. Thus, NF-κB activation by Smac mimetic contributes to Smac mimetic-mediated protection against TM-induced apoptosis.

Keywords Apoptosis · Cell death · Smac · NF-κB · Tunicamycin

Introduction

Programmed cell death by apoptosis represents a pivotal cellular mechanism to maintain tissue homeostasis [1]. Persistent cellular stress above a certain threshold impairs cellular resilience and initiates programmed cell death pathways [2]. For example, excessive endoplasmic reticulum (ER) stress can trigger apoptosis [2]. Apoptosis is tightly controlled by various pro- or antiapoptotic factors [3]. For instance, Inhibitor of Apoptosis (IAP) proteins are a family of proteins including cellular cIAP1, cIAP2 and X-linked IAP (XIAP) that prevent apoptosis [4]. Second mitochondria-derived activator of caspases (Smac) is an endogenous antagonist of IAP proteins that is released from the mitochondrial intermembrane space into the cytosol following initiation of apoptosis and supports the induction of cell death by neutralizing IAP proteins [4]. This mechanism has been utilized in oncology by developing small-molecule compounds that mimic the endogenous Smac protein. Smac mimetics not only block the interaction of XIAP and caspases, but also trigger autoubiquitination and subsequent proteasomal degradation of cIAP1 and cIAP2 [4].

Key proteins involved in NF-κB signaling are regulated via the E3 ligase activity of cIAP1 or 2. Therefore, depletion of cIAP proteins by Smac mimetics alters the activation status of NF-κB pathways. Small-molecule Smac mimetics have been shown to activate the non-canonical NF-κB pathway by depleting cIAP proteins, which terminates the cIAP protein-mediated constitutive degradation of NIK [5,

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6]. In addition, exposure to Smac mimetics can alter canonical NF-κB signaling via a crosstalk between the two NF-κB pathways. Transduction of the canonical NF-κB signal is mediated via phosphorylation and subsequent downregulation of Inhibitor of κB (IκB)α and the translocation of NF-κB subunits such as p65 and p50 to the nucleus [7]. Here, NF-κB subunits bind to DNA which results in the transcription of NF-κB target genes [7].

Since NF-κB has been reported to increase cell survival upon ER stress [8], in the present study we explored how Smac mimetics regulate NF-κB activation and ER stress-induced apoptosis.

Materials and methods

Cell culture and chemicals

Cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (both from Biochrom, Berlin, Germany), 1 mM L-glutamine and 1% penicillin/streptomycin (both from Invitrogen), as described previously [9]. The bivalent Smac mimetic BV6 has previously been described [5] and was kindly provided by Genentech Inc. (South San Francisco, CA, USA). TM was purchased from AppliChem (Darmstadt, Germany). If not indicated differently, chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany).

Determination of apoptosis

Apoptosis was determined by analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (FACSCanto II, BD Biosciences, Heidelberg, Germany) as described previously [9].

Western blot analysis

Western blot analysis was performed as described previously [9] using the following antibodies: goat anti-cIAP1 (R&D Systems, Wiesbaden, Germany), mouse anti-p52 (Millipore, Schwalbach, Germany), mouse anti-XIAP (clone 28) (BD Biosciences), rabbit anti-Mcl-1, rat anti-cIAP2 (Enzo Life Sciences, Loerrach, Germany), rabbit anti-p50, rabbit anti-p65 (Santa Cruz Biotechnology), mouse anti-phospho IκBα, rabbit anti-IκBα, rabbit anti-NIK (all from Cell Signaling, Beverly, MA). Mouse anti-α-Tubulin (Calbiochem, San Diego, CA), mouse anti-β-Actin (Sigma-Aldrich), mouse anti-glycerinaldehyd-3-phosphate dehydrogenase (GAPDH) (HyTest, Turku, Finland) or rabbit anti-lamin B1 (Abcam) were used as loading controls. Goat anti-mouse immunoglobulin G (IgG), donkey anti-goat IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown.

Retroviral transduction and RNAi-mediated gene silencing

Overexpression of the dominant-negative IκBα-SR was performed by retroviral transduction using IκBα (S32; 36A) and the pCFG5-IEGZ retroviral vector system as previously described [10]. HEK293T producer cells were transfected with 7.5 µg pGIPZ-shRNAmir vector, 12.5 µg pCMV-dR8.91 and 1 µg pMD2.G using calcium phosphate transfection. All pGIPZ-shRNAmir vectors were purchased from Thermo Fisher Scientific (Waltham, MA, USA): non-silencing control (shCtrl): RHS4346, shMcl_1: GGCAGT CGCTGGAGATTAT, shNIK_1: AGAGTCTGGGTTGTA GAGA and shNIK_2: CAGGACATCTGGTATGTTA. The virus containing supernatant was collected after 48 h and filtered using a 45-µm filter. SH-EP cells were transduced by centrifugation at 1000×*g* for 1 h at room temperature in the presence of 8 µg/ml polybrene and selected for one week with 1 µg/ml puromycin. For transient knockdown by siRNA, cells were reversely transfected with 5 nM SilencerSelect siRNA (Invitrogen) control siRNA (# 4390843) or targeting siRNAs (s17187 for NIK, s1449 for cIAP1, s1452 for cIAP2) using Lipofectamine RNAi Max (Invitrogen) and OptiMEM (Life Technologies).

Nuclear protein extraction, EMSA and determination of NF-κB transcriptional activity

Nuclear protein extraction and EMSA were performed as described previously [10] using the following NF-κB sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (sense). Representative EMSAs of at least two independent experiments are shown. NF-κB transcriptional activity was determined by NF-κB Reporter System pTRH1-NF-κB-EGFP (System Biosciences, Mountain View, CA) in accordance with the manufacturer's instructions.

Statistical analysis

Statistical significance was assessed by Student's *t* Test (two-tailed distribution, two-sample, unequal variance), $*P < 0.05$, $**P < 0.001$.

Results

NF-κB contributes to BV6-conferred protection from TM-induced apoptosis

Initially, we monitored the effects of the Smac mimetic BV6 on the activation status of non-canonical and canonical NF-κB signaling upon treatment with TM as a prototypic inducer of ER stress. BV6 alone and in combination with TM caused accumulation of NIK protein, processing of p100 to p52 and increased phosphorylation of IκBα, while TM had little effect on NF-κB activation (Fig. 1a). Cellular fractionation into cytosolic and nuclear extracts showed that BV6 as well as BV6/TM cotreatment stimulated the translocation of the NF-κB subunits p52, p50 and p65 into the nucleus (Fig. 1b). In addition, BV6 substantially enhanced the binding of NF-κB to the DNA as documented by electrophoretic mobility shift assay (EMSA) (Fig. 1c) and significantly increased NF-κB transcriptional activity (Fig. 1d). In contrast, NF-κB DNA binding or NF-κB transcriptional activity remained largely unchanged upon treatment with TM alone (Fig. 1c, d). These findings demonstrate that BV6 activates non-canonical and canonical NF-κB pathways, whereas TM has little effect on NF-κB signaling.

To investigate the functional impact of NF-κB activation by BV6 on TM-induced cell death, we ectopically expressed dominant-negative IκBα-SR, which blocks canonical and non-canonical NF-κB activation [11]. Control experiments confirmed overexpression of IκBα-SR and showed that it potently suppressed IκBα phosphorylation upon treatment with BV6 alone or in combination with TM (Fig. 2a). In control cells harboring empty vector (EV), BV6 stimulated NF-κB activation (Fig. 2a) and significantly reduced TM-induced DNA fragmentation (Fig. 2b), used as a marker of apoptotic cell death. Importantly, NF-κB inhibition by IκBα-SR completely reversed this BV6-mediated protection against TM resulting in a significant increase in cell death upon TM treatment (Fig. 2b). This set of experiments shows that NF-κB contributes to the BV6-conferred protection from TM-induced apoptosis.

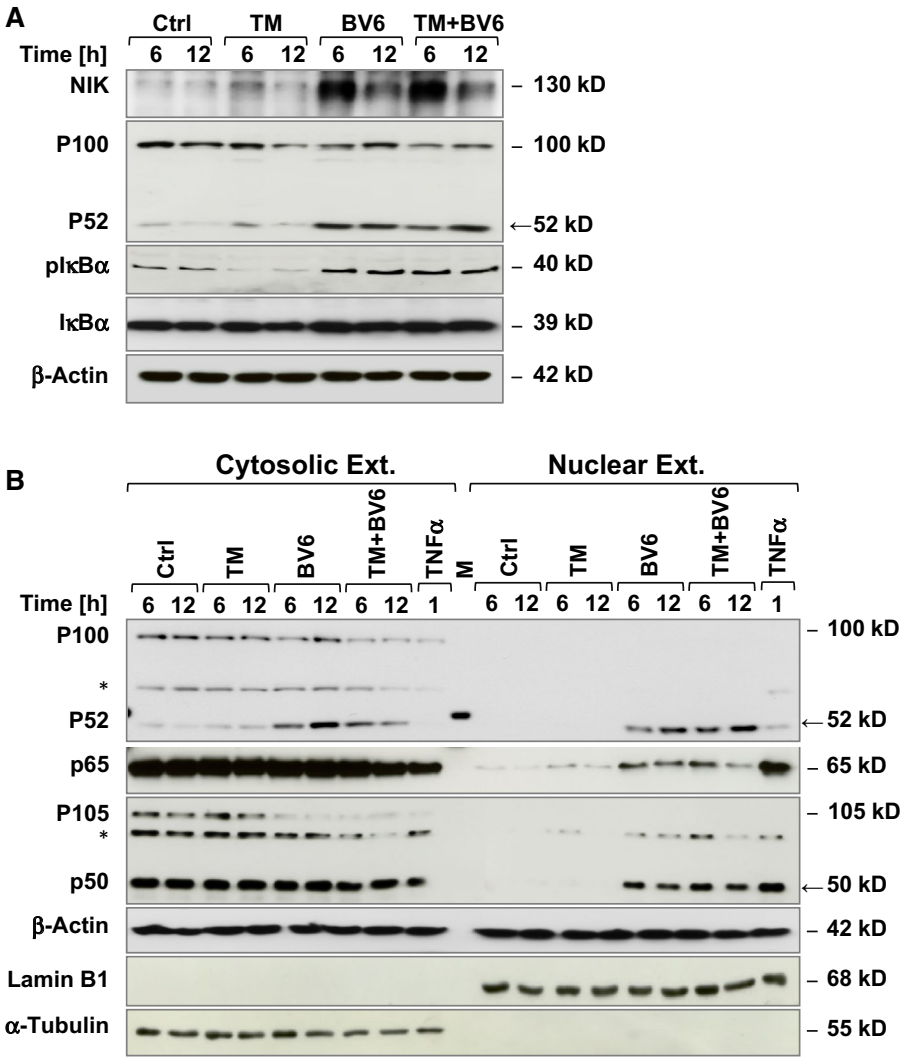
NIK contributes to BV6-conferred protection against TM-induced apoptosis

Since BV6-mediated depletion of cIAP proteins has been reported to lead to accumulation of NIK [5], a key upstream kinase in the non-canonical NF-κB pathway, we explored the contribution of NIK to BV6-mediated protection against TM. To this end, we both stably and transiently silenced NIK. Stable knockdown of NIK using two distinct shRNAs was confirmed by Western blotting (Fig. 3a); the proteasome inhibitor MG132 was used as positive control to trigger NIK accumulation in addition to BV6 (Fig. 3a). Importantly, knockdown of NIK significantly inhibited BV6-mediated protection against TM-induced apoptosis, resulting in a significantly higher percentage of DNA fragmentation in TM/BV6-treated NIK knockdown cells compared to TM/BV6-treated control cells (Fig. 3b). Similarly, transient silencing of NIK significantly increased TM/BV6-induced apoptosis compared to TM/BV6-treated control cells (Fig. 3c, d). Together, these findings demonstrate that NIK contributes to BV6-conferred protection against TM-induced apoptosis.

NF-κB-mediated upregulation of cIAP2 contributes to BV6-conferred protection from TM-induced apoptosis

Having established that NF-κB is required for BV6-conferred protection from TM-induced apoptosis, we next wanted to gain insights into the underlying molecular mechanisms. As Smac mimetics have been well described to cause depletion of cIAP proteins by stimulating their autoubiquitination and subsequent proteasomal degradation [5, 6], we analyzed the effect of BV6 on expression levels of IAP proteins. As expected, BV6 caused rapid downregulation of cIAP1 and cIAP2, while expression of XIAP remained unchanged (Fig. 4a). Interestingly, while cIAP2 was initially degraded one hour after addition of BV6, it was upregulated again after six hours of BV6 treatment (Fig. 4a). As cIAP2 is a prototypic NF-κB target gene, we explored the role of NF-κB in this upregulation. Therefore, we monitored cIAP2 expression upon exposure to BV6 and/or TM in IκBα-SR-overexpressing and EV control cells. Noticeably, NF-κB inhibition in IκBα-SR-overexpressing cells abolished cIAP2 upregulation after six hours of BV6 treatment (Fig. 4b). In contrast, cIAP2 was re-expressed again after six hours of BV6 treatment after its initial depletion in EV control cells (Fig. 4b). We also noted that cIAP2 expression was constitutively suppressed in IκBα-SR-overexpressing cells compared to EV control cells (Fig. 4b), consistent with its regulation by NF-κB.

To address the contribution of cIAP2 to BV6-conferred protection against TM we knocked down cIAP2 expression. Control experiments confirmed that cIAP2 silencing suppressed basal cIAP2 expression as well as BV6-stimulated upregulation of cIAP2 (Fig. 4c, d). Importantly, upon exposure to TM/BV6 apoptosis was significantly increased in cIAP2 knockdown cells compared to control cells (Fig. 4e). In addition, cIAP2 silencing slightly enhanced cell death triggered by TM alone (Fig. 4e). These data indicate that NF-κB-mediated upregulation of cIAP2 after its initial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◂**Fig. 1** BV6 activates non-canonical and canonical NF-κB signaling. SH-EP cells were treated for indicated times (**a**–**c**) or for 24 h (**d**) with 0.4 µg/ml TM and/or 4 µM BV6. Expression levels of NIK, p100, p52, IκBα and phospho-IκBα were analyzed by Western blotting (**a**). Expression levels of p100, p52, p65, p105, and p50 were analyzed in cytoplasmic and nuclear fractions by Western blotting; stimulation with 10 ng/ml Tumor necrosis factor (TNF)α for 1 h was used as positive control; M, molecular weight marker; asterisks, unspecific bands (**b**). Nuclear extracts were analyzed for NF-κB DNA-binding activity by EMSA; stimulation with 10 ng/ml TNFα for 1 h was used as positive control (**c**). NF-κB transcriptional activity was determined using a Green Fluorescent Protein (GFP)-labeled NF-κB reporter construct and fold increase in NF-κB transcriptional activity is shown with mean + SEM of three independent experiments performed in triplicate; $^{**}P < 0.001$ (**d**)

depletion contributes to BV6-conferred protection from TM-induced apoptosis.

In addition to these knockdown studies, we employed a second approach to test the role of cIAP2 in mediating BV6-conferred protection from TM-induced apoptosis. To mimic the effect of BV6, which initially causes depletion of cIAP1/2 (Fig. 4a) followed by upregulation of cIAP2 via

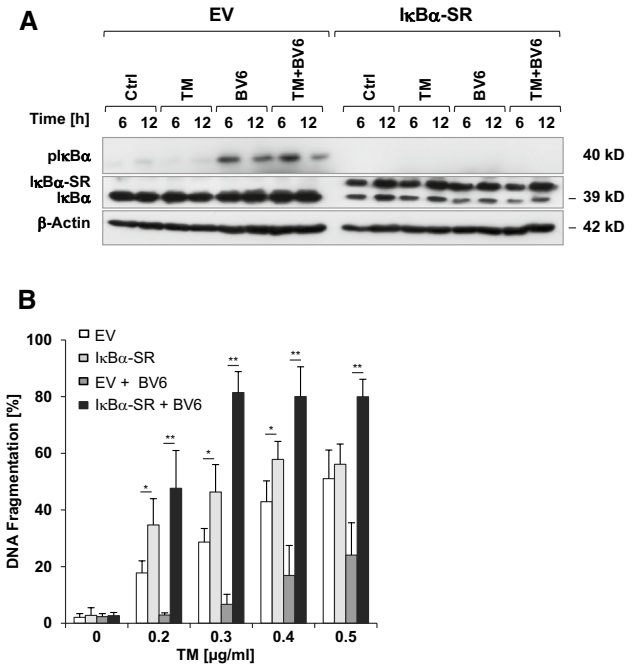

Fig. 2 NF-κB contributes to BV6-conferred protection from TM-induced apoptosis. SH-EP cells stably expressing IκBα-SR or EV control were treated for indicated times (**a**) or for 72 h (**b**) with 0.4 µg/ml TM and/or 4 µM BV6. Expression levels of NIK, p100, p52, IκBα and phospho-IκBα were analyzed by Western blotting (**a**). Apoptosis was determined by flow cytometric analysis of DNA fragmentation of PI-stained nuclei (**b**). Mean + SEM of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{**}P < 0.001$

NF-κB (Fig. 4b), we used cIAP1/2 double knockout (DKO) mouse embryonic fibroblasts (MEFs) with tamoxifen-inducible expression of cIAP2 [12]. Intriguingly, reconstitution of cIAP2 into cIAP1/2 DKO MEFs significantly reduced TM-induced cell death (Fig. 4f). This emphasizes that cIAP2 contributes to BV6-mediated inhibition of TM-induced apoptosis.

Mcl-1 upregulation via NF-κB contributes to BV6-mediated rescue of TM-induced apoptosis

Since Mcl-1 is another NF-κB target gene [13] that has been reported to protect cells from ER stress-induced apoptosis [14], we next investigated the role of Mcl-1. While TM decreased Mcl-1 expression, the addition of BV6 antagonized this TM-stimulated downregulation of Mcl-1 (Fig. 5a). Of note, NF-κB inhibition partially prevented BV6-stimulated expression of Mcl-1 upon TM treatment (Fig. 5b), indicating that NF-κB contributes to sustain Mcl-1 expression by BV6. To test the functional relevance of Mcl-1 for BV6-mediated protection against TM we silenced Mcl-1 (Fig. 5c). Importantly, knockdown of Mcl-1, which attenuated Mcl-1 accumulation upon cotreatment with TM/BV6 (Fig. 5d), significantly inhibited BV6-mediated protection from TM-induced apoptosis (Fig. 5e). In addition, Mcl-1 silencing significantly increased sensitivity towards TM alone (Fig. 5e). Together, this set of experiments indicates that BV6-stimulated, NF-κB-mediated upregulation of Mcl-1 contributes to BV6-mediated rescue of TM-induced apoptosis.

Discussion

In the present study, we demonstrate that NF-κB activation by Smac mimetic contributes to Smac mimetic-mediated protection against TM-induced apoptosis. This conclusion is supported by a number of independent elements of evidence. We showed that NF-κB was required for BV6-conferred protection from TM-induced apoptosis, as NF-κB inhibition by dominant-negative IκBα-SR reversed this BV6-mediated protection against TM. In addition, our data demonstrated that NIK, a key upstream kinase in the non-canonical NF-κB pathway, contributed to BV6-conferred protection from TM. Upon the induction of ER stress, BV6 supported the expression of NF-κB target genes with antiapoptotic functions such as cIAP2 and Mcl-1 that were involved in the rescue from TM-induced apoptosis by BV6.

Cancer cells have been reported to escape from Smac mimetic-induced apoptosis by upregulating cIAP2, which is no longer subject to BV6-stimulated proteasomal degradation, as cIAP1, which is required for ubiquitination of cIAP2, is simultaneously depleted by BV6 [15]. We have shown here that NF-κB activation by BV6 consistently restored expression levels of cIAP2 after its initial degradation by BV6. Intriguingly, this NF-κB-mediated restoration of cIAP2 expression contributed to BV6-conferred protection from TM-induced apoptosis. Downregulation of Mcl-1 during ER stress has been linked to inhibition of translation [16–18]. Our findings indicated that BV6-stimulated maintenance of Mcl-1 levels was mediated in part by NF-κB, since it was attenuated in IκBα-SR cells. This finding is in line with the reported transcriptional control of Mcl-1 by NF-κB [13].

NF-κB has been described to exert both anti- and proapoptotic functions, dependent on the cellular context. We previously reported that Smac mimetics prime glioblastoma or pancreatic carcinoma cells to chemotherapy- or irradiation-stimulated apoptosis in an NF-κB-dependent manner [19–21]. Moreover, NF-κB was found to be involved in several non-apoptotic functions of Smac mimetics, including Smac mimetic-stimulated migration and invasion of glioblastoma cells as well as differentiation of cancer stem-like cells upon exposure to Smac mimetics [22, 23]. Together, these studies highlight the context-dependent role of NF-κB in the cellular response to Smac mimetics. The reported antiapoptotic function of NF-κB during the ER stress response upon treatment with TM has implications for developing Smac mimetics as cancer therapeutics.

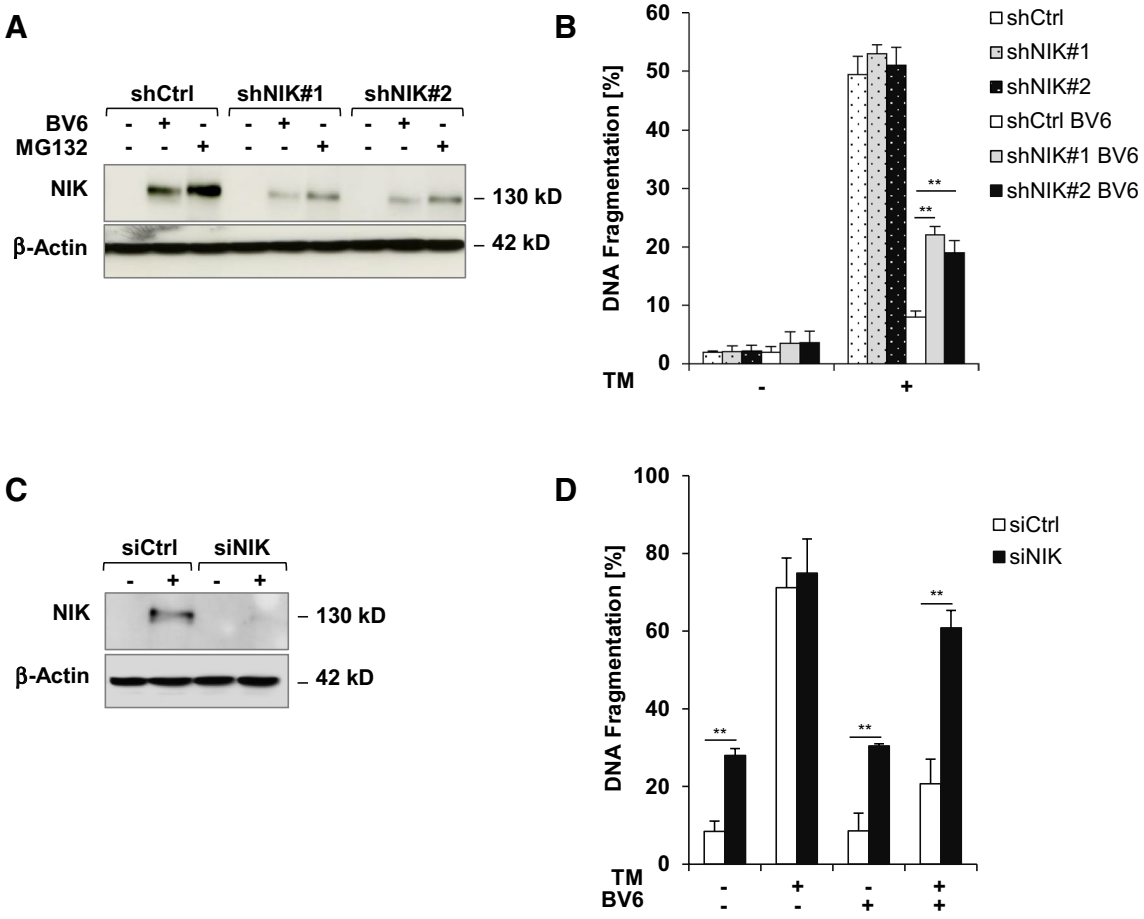

Fig. 3 NIK contributes to BV6-conferred protection against TM-induced apoptosis. **a, b** SH-EP cells were stably transduced with shRNA against NIK (shNIK) or vector control (shCtrl). Expression of NIK was analyzed by Western blotting after treatment with 4 μM BV6 or 1 μg/ml MG132 for 3 h; the proteasome inhibitor MG132 was used as control to trigger NIK accumulation (**a**). Apoptosis was determined by flow cytometric analysis of DNA fragmentation of PI-stained nuclei after treatment with 0.4 μg/ml TM and/or 4 μM BV6 for 72 h (**b**). Mean + SEM of three independent experiments performed in triplicate are shown; $^{**}P < 0.001$. **c, d** SH-EP cells were transiently transfected with siNIK or siCtrl. NIK expression was analyzed by Western blotting after treatment with 4 μM BV6 for 3 h (**c**). Apoptosis was measured by flow cytometric analysis of DNA fragmentation of PI-stained nuclei after treatment with 0.4 μg/ml TM and/or 4 μM BV6 for 48 h (**d**). Mean + SEM of three independent experiments performed in triplicate are shown; $^{**}P < 0.001$

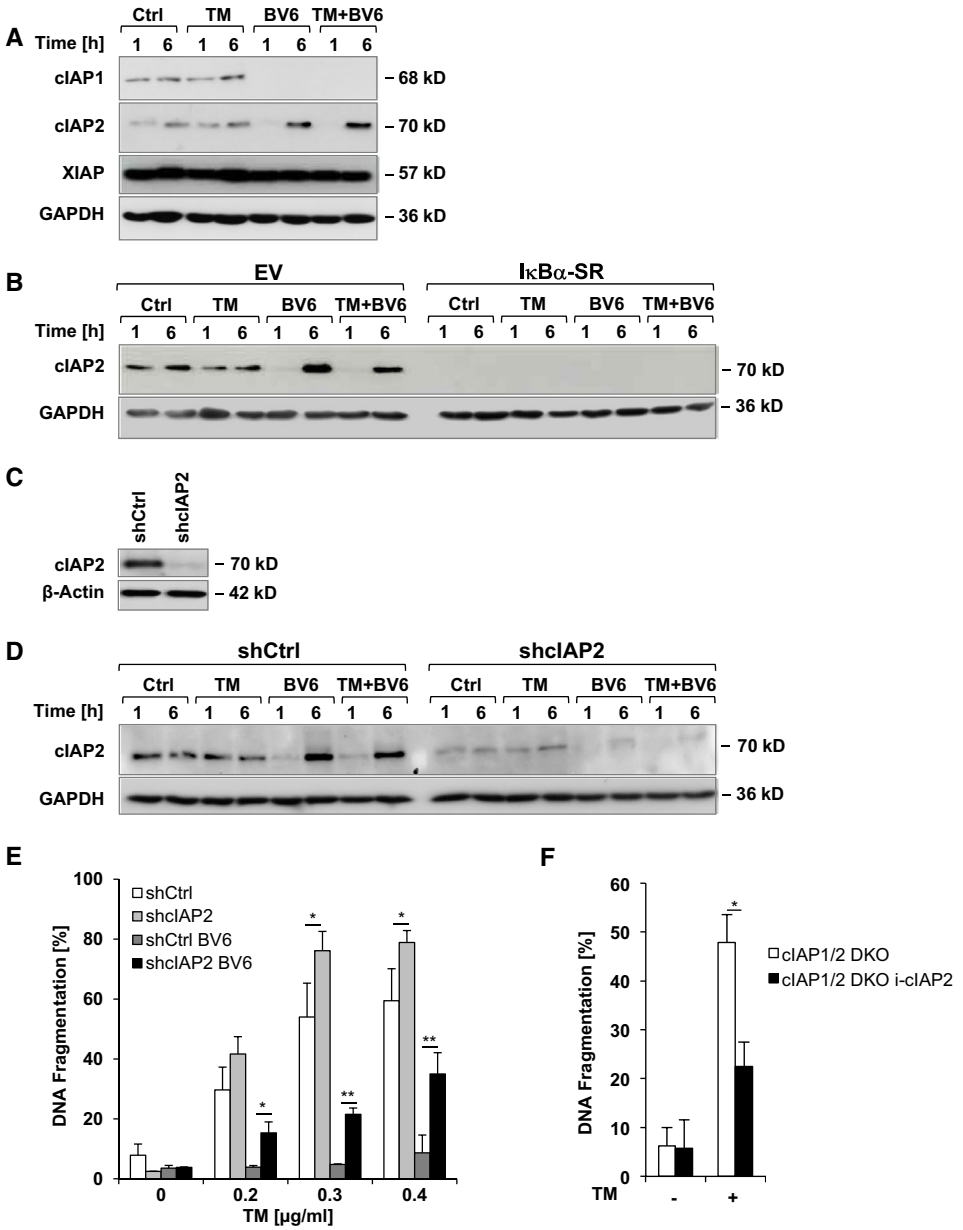

Fig. 4 NF-κB-mediated upregulation of cIAP2 contributes to BV6-conferred protection from TM-induced apoptosis. **a** SH-EP cells were treated for indicated times with 0.4 µg/ml TM and/or 4 µM BV6 and expression levels of cIAP1, cIAP2 and XIAP were assessed by Western blot analysis. **b** SH-EP cells stably expressing IκBα-SR or EV were treated for indicated times with 0.4 µg/ml TM and/or 4 µM BV6 and expression of cIAP2 was analyzed by Western blotting. **c-e** SH-EP cells were transduced with shCtrl or vector containing a shRNA sequence against cIAP2 (shcIAP2). Expression of cIAP2 was analyzed by Western blotting (**c**). Expression of cIAP2 was assessed by Western blot analysis after treatment for indicated times with 0.4 µg/ml TM and/or 4 µM BV6 (**d**). Apoptosis was determined after treatment for 72 h with 0.4 µg/ml TM and/or 4 µM BV6 by flow cytometric analysis of DNA fragmentation of PI-stained nuclei; mean + SEM of three independent experiments performed in triplicate are shown; $*P<0.05$; $**P<0.001$ (E). **f** cIAP1/2 DKO MEFs with tamoxifen-inducible expression of cIAP2 (i-cIAP2) were treated with 50 nM 4-hydroxytamoxifen for 48 h, followed by treatment with 0.25 µg/ml TM and/or 4 µM BV6 for 48 h. Apoptosis was determined by flow cytometric analysis of DNA fragmentation of PI-stained nuclei. Mean + SEM of three independent experiments are shown, $*P<0.05$

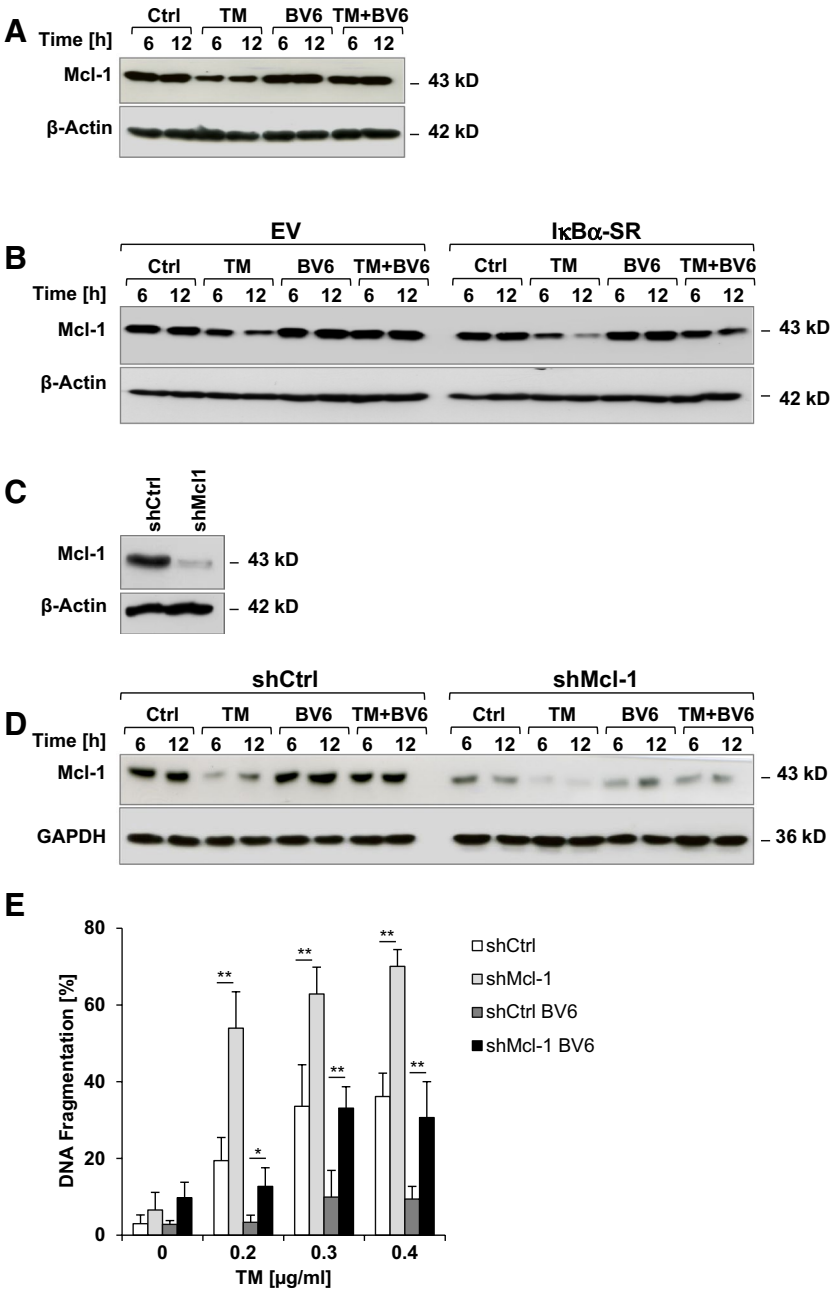

Fig. 5 Mcl-1 upregulation via NF-κB contributes to BV6-mediated rescue of TM-induced apoptosis. **a** SH-EP cells were treated for indicated times with 0.4 µg/ml TM and/or 4 µM BV6 and expression of Mcl-1 was assessed by Western blot analysis. **b** SH-EP cells stably expressing IκBα-SR or EV were treated for indicated times with 0.4 µg/ml TM and/or 4 µM BV6 and expression of Mcl-1 was analyzed by Western blotting. **c**–**e** SH-EP cells were transduced with shCtrl or vector containing a shRNA sequence against Mcl-1 (shMcl-1). Expression of Mcl-1 was analyzed by Western blotting (**c**). Expression levels of Mcl-1 were assessed after treatment for indicated times with 0.4 µg/ml TM and/or 4 µM BV6 by Western blot analysis (**d**). Apoptosis was determined after treatment for 72 h with 0.4 µg/ml TM and/or 4 µM BV6 by flow cytometric analysis of DNA fragmentation of PI-stained nuclei; mean + SEM of three independent experiments performed in triplicate are shown; $*P < 0.05$; $**P < 0.001$ (E)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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