Siva1 is a XIAP-interacting protein that balances NFκB and JNK signalling to promote apoptosis

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Summary

XIAP is known as a potent inhibitor of apoptosis, but in addition is involved in cellular signalling, including the NFκB, JNK and TGFβ pathways. Our search for XIAP-interacting partners led us to Siva1, a proapoptotic protein that is known to play a role in T-cell apoptosis through a caspase-dependent mitochondrial pathway. The interaction sites between XIAP and Siva1 were mapped to the RING domain of XIAP and the N-terminal, SAH-containing and death-homology-region-containing domains of Siva1. Co-immunoprecipitation experiments showed that XIAP, Siva1 and TAK1 form a ternary complex in Jurkat T cells. Reporter-gene analysis revealed that Siva1 inhibits XIAP- and TAK1-TAB1-mediated NFκB activation. By contrast, Siva1 increased XIAP- and TNFα-mediated AP1 activity and prolonged TNFα-induced JNK activation, whereas knock down of Siva1 resulted in reduced JNK activation. This suggests that Siva1 differentially modulates signalling by JNK and NFκB and shifts the balance between these pathways towards enhanced JNK activation, a situation that promotes apoptosis. Ectopically expressed Siva1 increased caspase-3 activity, which was inhibited by XIAP in a ubiquitin-ligase-dependent manner. In line with this, Siva1 was lysine-48-linked polyubiquitylated by XIAP. Our findings suggest that, via physical interaction with XIAP and TAK1, Siva1 diminishes NFκB and enhances JNK activity to favour apoptosis.

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Key words: Siva1, XIAP, TAK1, JNK, Apoptosis

Introduction

Programmed cell death or apoptosis is an evolutionarily conserved process that is critically important in embryonic development, tissue homeostasis, in lymphocyte development and immune response. Apoptotic signalling is initiated either through extrinsic (e.g. through death receptors) or intrinsic (mitochondria-dependent) stimulation, resulting in the activation of caspases, the central players within the apoptotic program (Boatright and Salvesen, 2003; Wallach et al., 1999). Dysregulation of apoptosis has been implicated in cancer, neurodegenerative and autoimmune disorders, immunodeficiency and viral infections (Fadeel and Orrenius, 2005). The nuclear factor kappa B (NFκB)/Rel family of transcription factors are important regulators of cell death, survival and proliferation (Karin and Greten, 2005; Karin and Lin, 2002). Various stimuli that include treatment with pro-inflammatory cytokines such as tumor necrosis factor (TNF)α, or phorbol myristyl acetate (PMA), T cell receptor (TCR) crosslinking using either antigen presentation or anti-CD3 antibody treatment, are known to activate NFκB through signal-induced phosphorylation and degradation of the endogenous inhibitors of NFκB, the IκBs, thereby facilitating NFκB translocation to the nucleus and activation of specific gene expression programs (Karin and Lin, 2002). NFκB induces the transcription of anti-apoptotic genes including the cellular inhibitors of apoptosis (IAPs), XIAP, FLIP as well as Bcl2 family members A1, Bcl-xL and Bcl-2, hence dampening caspase activation. In addition to NFκB, the Jun-N-terminal kinase (JNK) group of mitogen-activated protein (MAP) kinases respond rapidly to environmental stress, pro-inflammatory cytokines and TCR ligation, and promote both cell survival and cell death pathways (Davis, 2000; Sabapathy et al., 2001). The mechanisms of proapoptotic signalling by JNK have not yet been clearly established; however, they are known to promote mitochondria dysfunction by increasing the production of reactive oxygen species (ROS) (Ventura et al., 2004). In addition, they are known to activate and regulate both pro- and anti-apoptotic Bcl2 protein family members. Importantly, NFκB inhibits both ROS production and JNK activity through activation of specific target genes including ferritin heavy chain, Mn^{2+} superoxide dismutase, Gadd45β and XIAP (Bubici et al., 2006), resulting in a protective effect towards apoptotic noxae.

The inhibitor of apoptosis (IAP) family is characterized by the presence of at least one BIR (baculovirus IAP repeat) domain that has been identified in IAP1, IAP2, NAIP, survivin and XIAP, and accounts for their ability to inhibit apoptosis (Liston et al., 2003). XIAP is known as the most potent inhibitor of the executor caspase 3 and in addition plays a role in various signalling pathways. For instance, XIAP acts as a cofactor in transforming growth factor-β (TGFβ) signalling (Yamaguchi et al., 1999) through its interaction with TGF-beta-activated kinase 1-binding protein 1 (TAB1; also known as MAP3K7IP1) and activates TGFβ-activated kinase 1 (TAK1), leading to activation of NFκB and JNK signalling pathways (Birkey Reffey et al., 2001; Sanna et al., 2002). Ectopically expressed XIAP is known to enhance phosphorylation of AKT and activate both JNK and NFκB pathways (Asselin et al., 2001; Hofer-Warbinek et al., 2000; Lewis et al., 2004). The C-terminal RING domain of XIAP has E3-ubiquitin-ligase activity, which has been shown to

contribute to its anti-apoptotic activity (Liston et al., 2003) but is also essential for NFκB activation, whereas it is dispensable for JNK signalling (Lewis et al., 2004). Furthermore, XIAP is involved in the regulation of intracellular ROS levels (Bubici et al., 2006; Resch et al., 2008; Wilkinson et al., 2008), and is therefore critically involved in the crosstalk between NFκB and JNK signalling pathways. In animal models, XIAP has been shown to play a key function in maintaining T cell homeostasis of the immune system (Conte et al., 2001). In the human X-linked lymphoproliferative syndrome (XLP), mutations in XIAP lead to enhanced apoptosis in lymphocytes and also result in relatively low numbers of natural killer cells (Rigaud et al., 2006), thereby supporting a regulatory role of XIAP within the homeostasis of the immune system.

Siva1 was originally identified as a protein that interacts with the cytoplasmic tail of CD27, a member of the TNF receptor superfamily (Prasad et al., 1997). It is known to induce apoptosis in T cells through a caspase-dependent mitochondrial pathway (Py et al., 2004). Siva1 is a small (176 amino acids), cysteine-rich protein with a unique N-terminal amphipathic helical region (SAH, residues 36-55), a death domain homology region (DDHR) in the central part (residues 62-100) and a C-terminal cysteine-rich B-box-like or zinc-finger-like structure (residues 136-189) (Prasad et al., 1997). An alternative splice variant of Siva1, Siva2, that lacks most of the DDHR, has been shown to similarly induce apoptosis in T cells (Py et al., 2004). Several studies have shown that Siva1 expression levels are elevated in various pathological situations, such as acute ischemic injury, coxsackievirus B3 infection or HIV-envelope induced apoptosis in T-lymphoid cells (Henke et al., 2000; Padanilam et al., 1998; Py et al., 2007). The mechanism of Siva1-induced apoptosis is known to involve the inhibition of the anti-apoptotic protein Bcl-xL/Bcl2 (Chu et al., 2004) and might also involve inhibition of NFκB activation as recently described during TCR-mediated activation induced cell death (AICD) (Gudi et al., 2006). To obtain further insight into the signalling properties of XIAP and to identify novel interacting proteins that mediate its function(s), we used XIAP and truncated versions of XIAP as baits in a yeast two-hybrid screen and identified the proapoptotic protein Siva1. We found that Siva1 diminished XIAP-induced NFκB activity, whereas AP1 and JNK activation were enhanced. The Siva1-XIAP complex also contained TAK1, which is involved in both NFκB and JNK signalling pathways (Sanna et al., 2002; Sato et al., 2005), suggesting that Siva1 interferes with signalling events leading to both proapoptotic JNK and anti-apoptotic NFκB activity. The balance between XIAP and Siva1 may therefore determine the fate of a cell with regard to apoptosis or survival.

Results

Siva1 interacts with XIAP

In order to identify novel XIAP-interacting proteins we performed a yeast-two hybrid screen using full-length and truncated versions of XIAP as baits (Fig. 1A, schematic representation). Screening of a HeLa cDNA library yielded 75 clones of which one clone contained the entire coding region of Siva1. Back transformation of the prey with the bait constructs confirmed the direct interaction between Siva1 and full-length XIAP as well as the truncated versions. Furthermore, these experiments suggested that the C-terminal RING domain of XIAP is sufficient for interaction with Siva1 (Fig. 1A, right), although the BIR3 domain, or a region between the two domains, might further stabilize the interaction.

We next verified this interaction in mammalian cells by co-immunoprecipitation after transient transfection in HEK293 cells. As shown in Fig. 1B, immunoprecipitation of FLAG-Siva1 co-precipitated XIAP as well as the E3 ligase mutant $XIAP_{H467A}$. Of note, binding of endogenous XIAP to Siva1 became evident with increasing amounts of ectopically expressed Siva1 (supplementary material Fig. S1A). Concomitantly with the observed increased complex formation, we found higher molecular mass species of Siva1 in the whole cell lysates as well as in the immunoprecipitates. To confirm the interaction on the endogenous level, we utilized a cell line that expresses relatively high levels of Siva1 (Prasad et al., 1997), namely SW480 epithelial cells (supplementary material Fig. S1B). We then investigated the interaction in Jurkat T cells, since Siva1 has been shown to regulate apoptosis in T cells (Prasad et al., 1997). Cells were stimulated with PMA/Iono [phorbol-12-myristate-13-acetate (PMA) and ionomycine (Iono)], a well documented treatment that bypasses the proximal TCR signalling events by directly activating protein kinase C and inducing a strong and sustained increase in intracellular ionized Ca^{2+}. As shown in Fig. 1C, and in supplementary material Fig. S1C, significant amounts of XIAP were detected in Siva1 immune complexes under unstimulated and stimulated conditions, confirming the presence of natural complexes of XIAP and Siva1. Complex formation increased over time following stimulation, as did Siva1 on both the protein (Fig. 1C, second panel; supplementary material Fig. S1C, third panel) and mRNA levels (data not shown), suggesting a function in activation-induced signal transduction.

The three BIR domains of XIAP are known to bind to caspases 3, 7 and 9 and inhibit apoptosis (Liston et al., 2003) and they are also bound to XAF1, Smac/DIABLO or Omi/HtrA2, which act as XIAP antagonists (Creagh et al., 2004; Liston et al., 2003). In order to delineate the domains involved in the interaction between XIAP and Siva1, various truncated forms of XIAP, including E3-ligase-deficient mutants, were coexpressed as Myc fusion proteins (Fig. 1D) along with FLAG-tagged Siva1 in HEK293 cells. As shown in Fig. 1E, Siva1 co-immunoprecipitated with the BIR3 domain plus the RING domain (B3+R and $B3+R_{H467A}$), and the RING domain only (R and R_{H467A}). Furthermore, Siva1 did not bind to an XIAP construct lacking the RING domain (ΔRING) (Lewis et al., 2004) (supplementary material Fig. S1D), thus confirming the yeast two-hybrid findings. Co-precipitations with the E3 ligase mutants were consistently more efficient, suggesting that inactivation of the E3 ligase activity of XIAP may stabilize the interaction between them.

Next we investigated which structural domains of Siva1 are involved in XIAP binding. The proapoptotic property has been attributed to the SAH domain (Chu et al., 2004; Xue et al., 2002). We therefore tested whether an N-terminal deletion construct containing the SAH-domain and the DDHR of Siva1 (termed SivaΔC) can bind to XIAP and its truncated versions. HEK293 cells were transiently transfected with FLAG-SivaΔC, together with XIAP or $XIAP_{H467A}$. As shown in Fig. 1F, the SivaΔC construct readily co-immunoprecipitated with the full-length XIAP and $XIAP_{H467A}$ and with the truncated versions of XIAP lacking the BIR1 and 2 domains (Fig. 1G). Probably because of the lower stability of the isolated RING domain in the complex, the interaction was only detectable for the E3 ligase mutant version. Taken together, the above studies demonstrated that the N-terminal region of Siva1 containing both the SAH and the DDH region interacts with the RING-finger domain of XIAP. It would be interesting to see whether XIAP is also able to interact with the alternative splice variant Siva2, which lacks most of the DDHR.

Siva1 negatively regulates NFκB activity

Siva1 has been shown to bind to the intracellular domain of CD27, a member of the TNF-receptor superfamily, thereby promoting apoptosis. Ligation of CD27 to its ligand CD70 activates both NFκB and JNK signalling pathways via TNF receptor associated factor (TRAF) 2 and 5 and NFκB-inducing kinase (NIK) (Akiba et al., 1998). It was shown that Siva1 binds to the same region of CD27 as TRAF2 (Nestler et al., 2006), suggesting that it might act as a

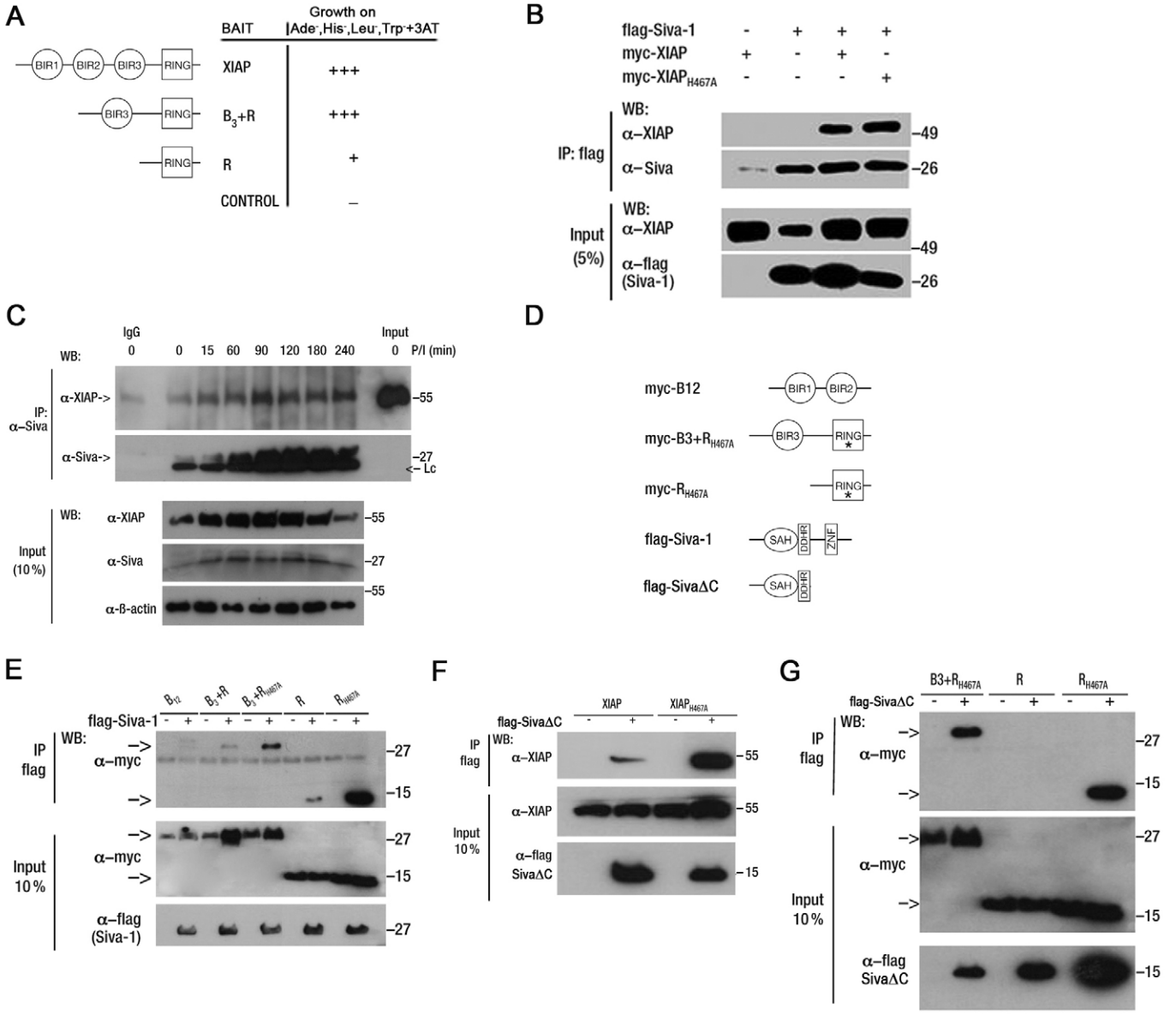

Fig. 1. Siva1 interacts with XIAP. (A) Schematic representation of the XIAP bait constructs used for yeast two-hybrid screening (left) and verification of the interaction after back transformation and growth on –Ade, –His, –Leu, –Trp +3AT medium (+++, strong growth; +, weak growth; –, no growth) (right). (B) Co-immunoprecipitation of XIAP and Siva1 in HEK293 cells that were transfected with FLAG-Siva1, Myc-XIAP or the E3-ubiquitin-ligase mutant $XIAP_{H467A}$. Siva1 was immunoprecipitated (IP) with FLAG-matrix and the presence of XIAP or $XIAP_{H467A}$ as well as Siva1 in the precipitates was determined by western blotting with antibodies against XIAP or Siva (IP, upper two panels). Expression levels of XIAP (transfected and endogenous) and Siva1 are shown (Input). (C) Endogenous co-immunoprecipitation of Siva1 and XIAP. Jurkat T cells were stimulated with PMA-ionomycin (P/I) for the indicated times and immunoprecipitations were performed with anti-Siva antibody or IgG control. Precipitates were immunoblotted and membranes probed with XIAP and Siva antibodies (IP, upper panels, LC indicates the light chain). Expression levels of XIAP, Siva and β-actin (loading control) are shown (Input; lower three panels). Note that Siva1 levels increase upon stimulation, whereas XIAP levels remain constant. (D) Expression constructs of XIAP and Siva1 used for interaction mapping. (E) Siva1 interacts with the XIAP RING domain. HEK293 cells were transfected with Myc-tagged wild-type or ubiquitin ligase mutant XIAP constructs along with FLAG-Siva1 (+) or empty vector (–). Upper panel: immunoprecipitation (IP) of FLAG-tagged Siva1 and detection of Myc-tagged XIAP constructs with α-Myc antibody in western analysis; lower two panels (Input): expression of the Myc-tagged XIAP truncations and FLAG-Siva1 in the cell extracts. (F) XIAP interacts with the N-terminal part of Siva1. HEK293 cells were transfected with XIAP or $XIAP_{H467A}$ along with a truncated version of Siva1, FLAG-SivaΔC (+) or empty vector (–). SivaΔC was immunoprecipitated (IP) and XIAP was detected in precipitates with α-XIAP antibody (upper panel). Expression levels of co-transfected proteins are shown in the lower two panels (Input). (G) SivaΔC interacts with the XIAP RING domain. HEK293 cells were transfected with Myc-tagged XIAP truncations, as shown schematically in 1D, along with FLAG- SivaΔC (+) or empty vector (–). SivaΔC was immunoprecipitated (IP) and binding of XIAP truncations was detected with α-Myc antibody using western analysis (upper panel). Expression levels of co-transfected proteins are shown in the lower two panels (Input). Numbers on right of the panels are molecular masses in kilodaltons.

negative regulator of NFκB activation and hence promote an apoptotic program, e.g. during TCR-mediated activation-induced cell death (AICD) (Gudi et al., 2006). In the light of these reports, as well as the previous finding that XIAP enhances NFκB signalling (Hofer-Warbinek et al., 2000; Lewis et al., 2004; Winsauer et al., 2008), we next investigated whether Siva1 interferes with XIAP-induced NFκB activation. Siva1 reduced XIAP-induced NFκB activation in a dose-dependent fashion as measured by reporter gene assays in HEK293 cells (Fig. 2A) and in Jurkat T cells (data not shown). Western blot analysis of cell lysates (Fig. 2A, insert) revealed that both XIAP and Siva1 are stabilized when coexpressed. The higher expression levels of both Siva1 and XIAP in these samples were not caused by higher transfection efficiency as assessed by a co-transfected β-Gal expression plasmid. One described mechanism of how XIAP mediates NFκB activation involves its interaction with TAB1, which in turn activates TAK1

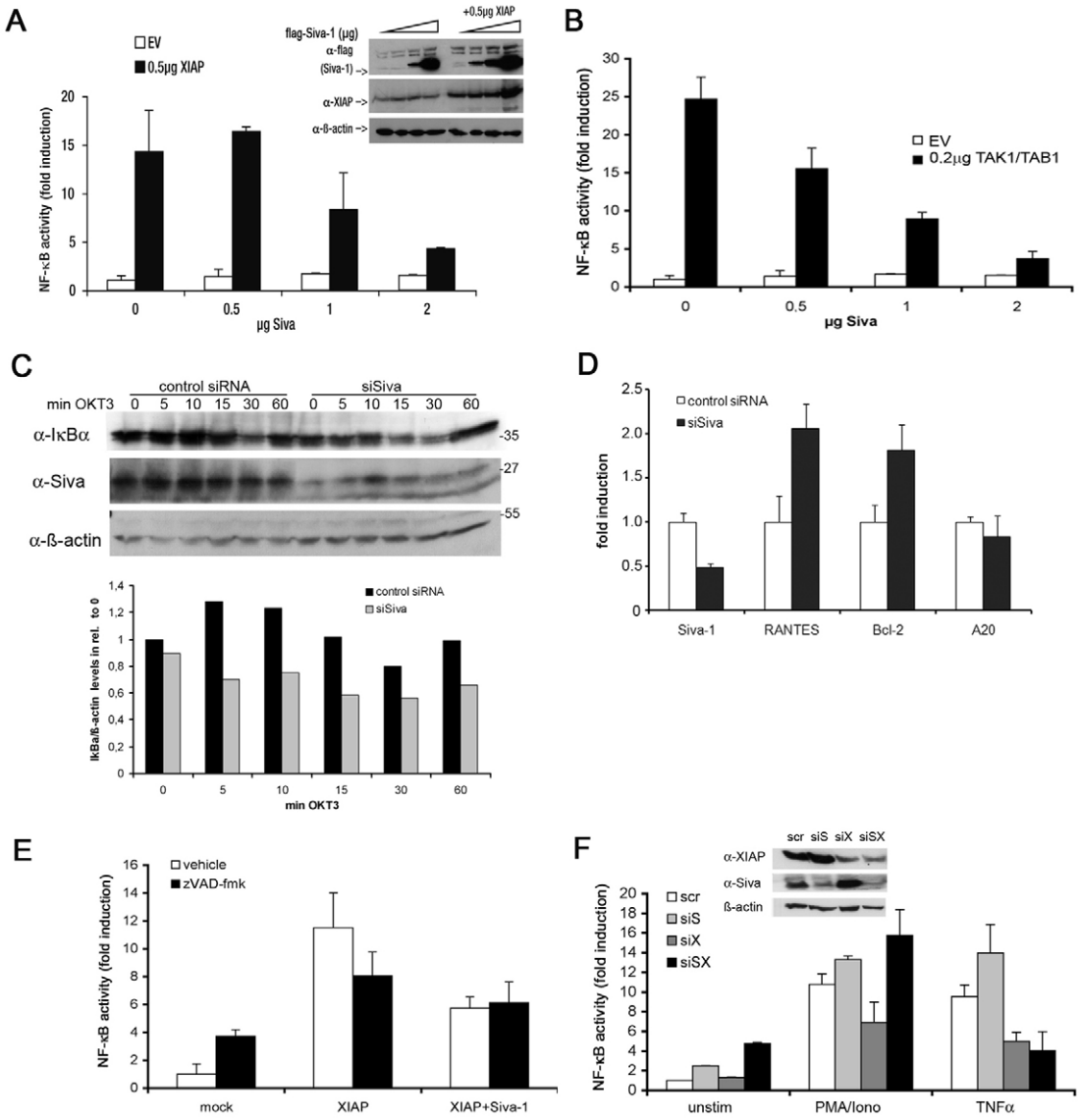

Fig. 2. Siva1 negatively regulates NFκB activity. HEK293 cells were co-transfected with a 5×NFκB-luc reporter along with increasing amounts of FLAG-Siva1 in the absence (empty vector, EV) or presence of XIAP (A) or TAK1-TAB1 (B). Transfection efficiency was monitored by β-Gal expression from a co-transfected UBT–β-Gal vector and used to normalize luciferase activity. Fold induction was calculated in relation to EV without Siva1. Expression levels of Siva1 and XIAP were determined by western blotting (A, insert). (C) Jurkat T cells stably expressing lentiviral-delivered siSiva1 (siSiva) or control siRNA were stimulated with anti-CD3 antibody (OKT3, 5 μg/ml) for the indicated times and whole cell extracts were subjected to western blot analysis for IκBα degradation (upper panel). Siva levels and anti-β-actin as loading control are shown in the lower two panels. Densitometric measurements of IκBα levels normalized to β-actin in relation to control siRNA cells before stimulation (0 minutes) were performed using ImageJ software (lower bar graph). (D) Siva1 differentially affects the expression of NFκB-dependent genes. Real-time PCR analysis of RNA isolated from control and siSiva Jurkat T cells that were stimulated for 24 hours with PMA/Iono. (E) Inhibition of NFκB by Siva1 is independent of caspase activation. HEK293 cells were transfected with 5xNFκB-luc reporter along with XIAP (0.5 μg) alone or in combination with Siva1 (1 μg). 12 hours post-transfection, cells were treated with the pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethylketone (zVAD-fmk, 20 μM in DMSO) or vehicle. 36 hours post transfection, cells were lysed and luciferase activity was measured. (F) Knock down of Siva1 enhances NFκB activity. Jurkat T cells were transfected with siRNAs directed against Siva (siS), XIAP (siX), the combination (siSX), or scrambled control (scr), along with a 5xNFκB-luc reporter. 12 hours post-transfection cells were stimulated with PMA/Iono or TNFα (20 g/ml) and NFκB activity was determined by luciferase assay 12 hours later. Knock down efficiency was analyzed by western blotting (insert). Luciferase values were normalized to β-Gal activity. Values are means ± standard deviation of triplicate experiments.

(Lu et al., 2007; Yamaguchi et al., 1999). Based on this, we investigated whether Siva1 had an influence on TAK1-TAB1-induced NFκB activation as well. The results in Fig. 2B show that increasing amounts of Siva1 efficiently diminish TAK1-TAB1-induced activation of NFκB, and similar results were obtained with the SivaΔC construct (supplementary material Fig. S2A). In addition, TNFα-induced NFκB activation was also slightly reduced by Siva1 (supplementary material Fig. S2B). Because NFκB activity measurements by reporter gene assays give only limited information about the kinetics of NFκB activation, we next performed a series of experiments in Jurkat T cells in which Siva1 expression was silenced by lentiviral-delivered siRNA directed against Siva1. Stimulation of these cells by TCR crosslinking with anti-CD3 (OKT3) antibody resulted in a more pronounced IκBα degradation compared with control siRNA-transduced cells (Fig. 2C, upper panel and densitometric evaluation of IκBα levels, lower panel). IκBα levels were slightly lower in unstimulated siSiva cells, but differences to control cells increased upon stimulation. Similar results were obtained after TNFα or PMA/Iono stimulation (data not shown). This is in agreement with a previous report showing enhanced nuclear NFκB p65 following TCR stimulation after Siva1 knock down as well as higher expression levels of anti-apoptotic proteins such as Bcl-xL and FLIP (Gudi et al., 2006). To further extend these findings, we analyzed gene expression of several known NFκB-regulated genes and found that mRNA levels of Bcl2 and RANTES (regulated upon activation of normal T cells expressed and secreted) were elevated in siSiva cells, whereas expression levels of A20 were similar to that in control siRNA cells after PMA/Iono treatment (Fig. 2D).

During apoptosis, the activation of caspases can lead to inactivation of the NFκB (rescue) pathway, e.g. through cleavage of IKKγ/NEMO (Frelin et al., 2008). Therefore, we tested whether the reduction of NFκB activation by Siva1 might be a consequence of caspase activation and assessed NFκB activity in the presence of a pancaspase inhibitor, zVAD-fmk. As shown in Fig. 2E and supplementary material Fig. S2C, inhibition of XIAP- or TNFα-induced NFκB activation by Siva1 was independent of caspase activities.

To investigate the role of both XIAP and Siva1 in activation-induced signal transduction in more detail, we performed siRNA-mediated knock down experiments in Jurkat T cells. NFκB activity was increased after knock down of Siva1 under unstimulated conditions and after PMA/Iono and TNFα stimulation (Fig. 2F). Knock down of XIAP reduced both PMA/Iono- and TNFα-stimulated NFκB activity. When both Siva1 and XIAP were knocked down, the two stimuli behaved differently: whereas basal and PMA/Iono-stimulated NFκB activity was increased, TNFα-induced NFκB activity was decreased. Thus, Siva1 blocks TNFα-mediated NFκB signalling involving XIAP in Jurkat T cells, and knock down of Siva1 does not rescue the loss of XIAP after TNFα treatment. By contrast, after PMA/Iono stimulation knock down of Siva1 can compensate for the loss of XIAP, suggesting the existence of an additional, XIAP-independent, Siva1-sensitive pathway.

Siva1, XIAP and TAK1 form a ternary complex

It is well known that NFκB activation involves the MAP3K TAK1 upon TCR stimulation as well as after PMA/Iono stimulation (Sato et al., 2005; Wan et al., 2006). Based on the results above, we speculated that Siva1 might also interact with TAK1. Indeed, we found that Siva1 associated with TAK1 as well as with XIAP in Jurkat T cells under unstimulated conditions (Fig. 3A) and complex formation was increased after PMA/Iono treatment (Fig. 3B). When testing the contribution of TAB1 to the complex formation, we found that it was dispensable for the Siva1-TAK1 interaction (Fig. 3C, lane 4). Furthermore, coexpressed XIAP did not result in substantially more TAK1 in the Siva1 immunoprecipitate (compare lane 4 and 10). Together, these results suggest that the NFκB activation mediated by the TAK1-TAB1-XIAP complex is attenuated by Siva1.

Siva1 increases AP1 and JNK activity

Stimulation of cells with TNFα as well as TCR ligation activates not only NFκB, but also JNK signalling pathways through MAP3K isoforms, including TAK1 (Davis, 2000; Sabapathy et al., 2001; Sato et al., 2005; Su et al., 1994). Activated JNKs in turn enhance AP1 transcriptional activity. We therefore investigated whether Siva1 could stimulate AP1 activity. As shown by reporter gene assays, AP1 activity induced by either XIAP or TNFα treatment was increased by co-transfected Siva1 in a dose-dependent fashion (Fig. 4A and B, respectively). Of note, Siva1-increased AP1 activity was independent of the XIAP E3 ligase (supplementary material Fig. S3A). Accordingly, knock down of Siva1 led to decreased AP1 activity upon PMA/Iono stimulation (Fig. 4C) and after XIAP overexpression (supplementary material Fig. S3B).

As has been shown for NFκB, JNK is activated in a biphasic manner after TNFα treatment and is critical for TNFα-stimulated AP1-dependent gene expression (Ventura et al., 2004; Ventura et al., 2006). Since we observed increased XIAP and TNFα-mediated AP1 activity upon Siva1 coexpression, we investigated the influence of Siva1 on JNK activation. As shown in Fig. 4D, ectopic expression of Siva1 prolonged phosphorylation of JNK upon TNFα stimulation in HEK293 cells (first panel), whereas IκBα degradation (second panel) and nuclear translocation of NFκB-p65 (fourth panel) was delayed, in accordance with the results shown in Fig. 2. In agreement with this, knock down of Siva1 in Jurkat T cells diminished JNK phosphorylation upon treatment with PMA/Iono (Fig. 4E). Together, these results suggest that Siva1 modulates signalling events leading to activation of both NFκB and AP1, as they occur after, for example, TNF receptor or TCR ligation. Because enhanced and prolonged JNK activation has been implicated in apoptosis (Bubici et al., 2006; Chen and Tan, 2000; Ventura et al., 2004), the Siva1-mediated attenuation of NFκB and concomitant JNK activation are suggestive of a proapoptotic scenario.

XIAP counteracts Siva1-induced apoptosis

Overexpression of Siva1 and the alternative splice variant Siva2 have been shown to induce apoptosis in T cells and in neurons (Jacobs et al., 2007; Py et al., 2007; Py et al., 2004) whereas knock down of Siva1 reduces apoptosis (Gudi et al., 2006; Jacobs et al., 2007). Because XIAP is a potent inhibitor of apoptosis, we next investigated the biological consequence of the XIAP-Siva1 interaction with regard to apoptosis. We silenced Siva1 and XIAP by conventional siRNA in Jurkat T cells, stimulated them with plate-bound anti-CD3 antibody (OKT3) to mimic TCR ligation and measured caspase-3 activity as a read-out for apoptosis. Knock down of Siva1 reduced, whereas knock down of XIAP increased, OKT3-induced caspase-3 activity (Fig. 5A). Double knock down resulted in decreased caspase-3 activation, in line with the increased NFκB activation observed previously (Fig. 2F).

The ubiquitin-E3-ligase activity of XIAP has been demonstrated to enhance its anti-apoptotic property (Liston et al., 2003). To

determine the necessity of the XIAP ubiquitin-E3-ligase activity in Siva1-induced apoptosis, we performed experiments in *Xiap*$^{-/-}$ mouse embryonic fibroblasts (MEF) to exclude a potential interference with endogenous XIAP. Cells were transfected with increasing amounts of Siva1 in the absence or presence of co-transfected XIAP or XIAP$_{H467A}$. Under these experimental conditions, Siva1 induced caspase-3 activation could be inhibited by co-transfected XIAP but not by its ubiquitin ligase mutant, suggesting that XIAP counteracts Siva1-induced apoptosis in an ubiquitin-ligase-dependent manner (Fig. 5B). To test whether XIAP could ubiquitylate Siva1, cells were transfected with FLAG-Siva1, His-tagged ubiquitin and XIAP or XIAP$_{H467A}$, and ubiquitylated material was pulled down under denaturating conditions using nickel-nitrilotriacetic acid (Ni-NTA) and analyzed by western blotting. As shown in Fig. 5C, polyubiquitylated Siva1 was detected after coexpression of XIAP, but not of XIAP$_{H467A}$ (see also higher molecular mass species in supplementary material Fig. S1A). Furthermore, ubiquitylation of Siva1 was enhanced in the presence of the proteasome inhibitor MG132, suggesting a degradative (lysine-48-linked) polyubiquitylation of Siva1. To verify this we used the respective mutated versions of ubiquitin (K48R and K63R). Polyubiquitylated Siva1 was detected in the presence of the wild type as well as the K63R-ubiquitin mutant, but not in the presence of the K48R-ubiquitin construct, indicating that ubiquitylation is predominantly K48 linked (supplementary material Fig. S4).

Discussion

Various extracellular stimuli – including growth factors, cytokines, antigen encounter or stress – trigger signalling cascades that result in the activation of NFκB and AP1, which are nuclear transcription factors that regulate the expression of multiple genes involved in cell growth, differentiation and apoptosis. In addition, stimulation of cells induces transient protein-protein interactions involved in specific signal transduction pathways, and the strength and duration of stimulation is critically involved in the life or death decision of cells. XIAP is known as a potent inhibitor of apoptosis not only through its direct inhibition of caspases 3, 7 and 9, but also through modulation of cellular signalling within the TGFβ, NFκB and JNK pathways (Birkey Reffey et al., 2001; Hofer-Warbinek et al., 2000; Lewis et al., 2004; Sanna et al., 2002; Winsauer et al., 2008; Yamaguchi et al., 1999). By using a yeast two-hybrid screening approach, we identified the proapoptotic protein Siva1 as a novel XIAP-interacting protein. Interaction mapping with truncated versions of both proteins revealed that the C-terminal RING domain of XIAP is sufficient for binding to the N-terminal, SAH and DDHR-containing domain of Siva1. Several interaction partners of Siva1 are known so far, including CD27, the CVB3 capsid protein VP2 and the peroxisomal membrane protein PMP22, which interact with the N-terminal part of Siva1, whereas the lysophosphatidic acid (LPA) receptor 2 and the cytoplasmatic domain of CD4 interact with the C-terminal part of Siva1 (Henke et al., 2000; Lin et al., 2007; Nestler et al., 2006; Prasad et al., 1997; Py et al., 2007). Furthermore, the SAH domain of Siva1 has been shown to interact with and inhibit the anti-apoptotic protein Bcl-xL, thereby sensitizing cells to UV-radiation-induced apoptosis (Xue et al., 2002). Based on

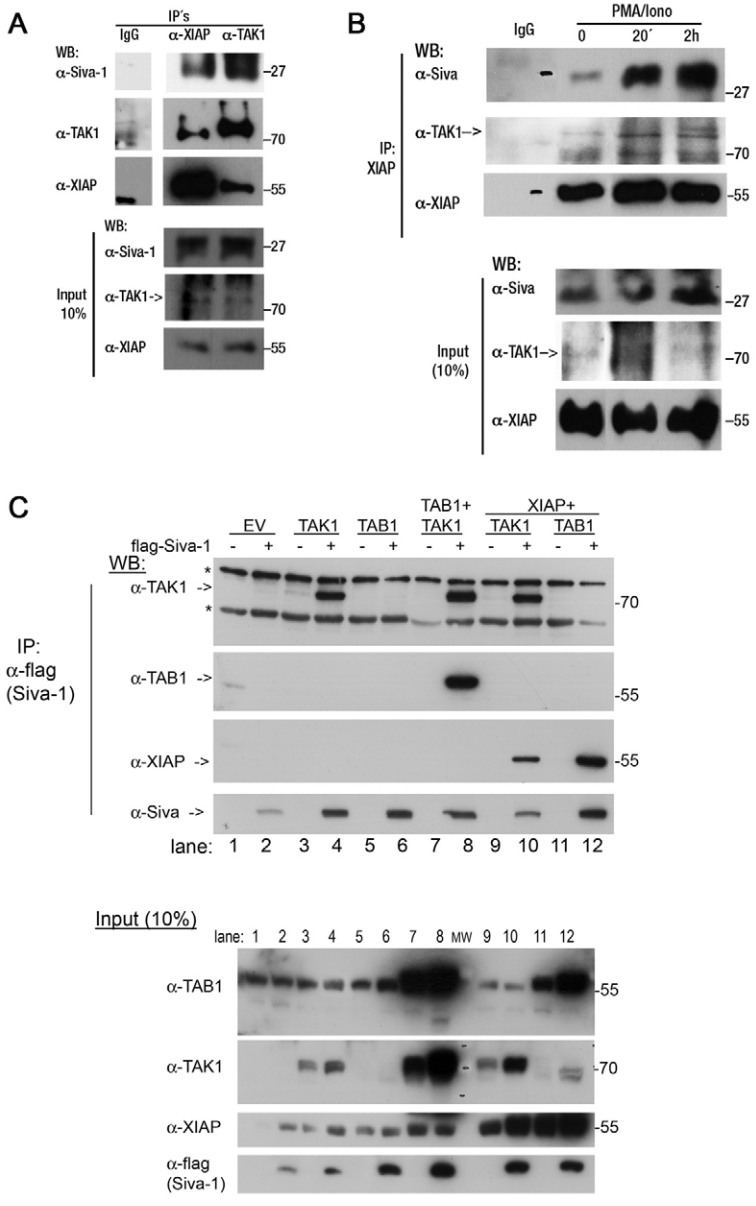

Fig. 3. Siva1, XIAP and TAK1 form a ternary complex. (A) Immunoprecipitations (IPs) of XIAP or TAK1 in Jurkat T cells show that Siva1 interacts with both XIAP and TAK1 (upper panel). Expression levels of Siva1, TAK1 and XIAP are shown in the lower three panels (Input, 10%). (B) Jurkat T cells were stimulated with PMA/Iono for the indicated times. XIAP was precipitated with a XIAP antibody, and Siva1 as well as TAK1 in the complex was revealed by western blotting (IP, upper three panels). Expression levels of Siva1, TAK1 and XIAP in the whole cell extracts are shown in the lower three panels (Input). Numbers on right of the panels are molecular mass markers in kilodaltons. (C) Siva1 interacts with TAK1 independently of XIAP and TAB1. HEK293 cells were transfected with TAK1, TAB1 and XIAP, either alone or in combination, and either empty vector (–) or FLAG-Siva1 (+). Siva1 was immunoprecipitated with anti-FLAG antibody and the presence of TAK1, TAB1, XIAP as well as Siva1 in the precipitate was assessed by western blotting (IP, upper four panels; TAK1 corresponds to the 70 kDa band in the upper panel, arrow; *unspecific band). Expression levels of transfected proteins are shown in the lower four panels (Input). Note that the input samples were run on a separate gel (MW, molecular mass marker). Numbers on the right of the panels are molecular masses in kilodaltons.

previous findings showing that the RING domain of XIAP is not involved in binding to caspases, our interaction-mapping studies suggest that Siva1 may not directly interfere with the binding of XIAP to caspases, Smac/DIABLO or AIF (Liston et al., 2003; Wilkinson et al., 2008), which are known interacting partners of the BIR1 and 2 domains.

Recently, Siva1 has been shown to negatively regulate NFκB activity in T cells after TCR stimulation suggesting a regulatory role in AICD with implications for peripheral tolerance, T cell homeostasis and cancer (Gudi et al., 2006). In accordance with these results, we found that Siva1 could inhibit NFκB activation mediated by XIAP, TAK1-TAB1 or TNFα. Based on our previous analysis of XIAP-regulated genes (G.W., unpublished data), we analyzed NFκB-dependent gene expression and observed that genes such as Bcl2 and RANTES were increased in siSiva cells. By contrast, the expression of the XIAP-independent gene A20 was similar in siSiva and control siRNA cells, further supporting the contention that XIAP and Siva1 are functionally linked. Inhibition of caspases did not abolish diminished NFκB activity in the presence of Siva1, confirming that reduced NFκB activity is not secondary to caspase activation. XIAP activation of NFκB during TGFβ signalling has been shown to involve its interaction with TAB1, which further activates TAK1 (Birkey Reffey et al., 2001; Lu et al., 2007; Yamaguchi et al., 1999). Recently, we found that XIAP is important for the biphasic activation of NFκB upon stimulation with TNFα and involves the interaction with, and ubiquitylation of, another MAP3K, MEKK2 (Winsauer et al., 2008). Likewise, the ubiquitin ligase activity of XIAP is essentially

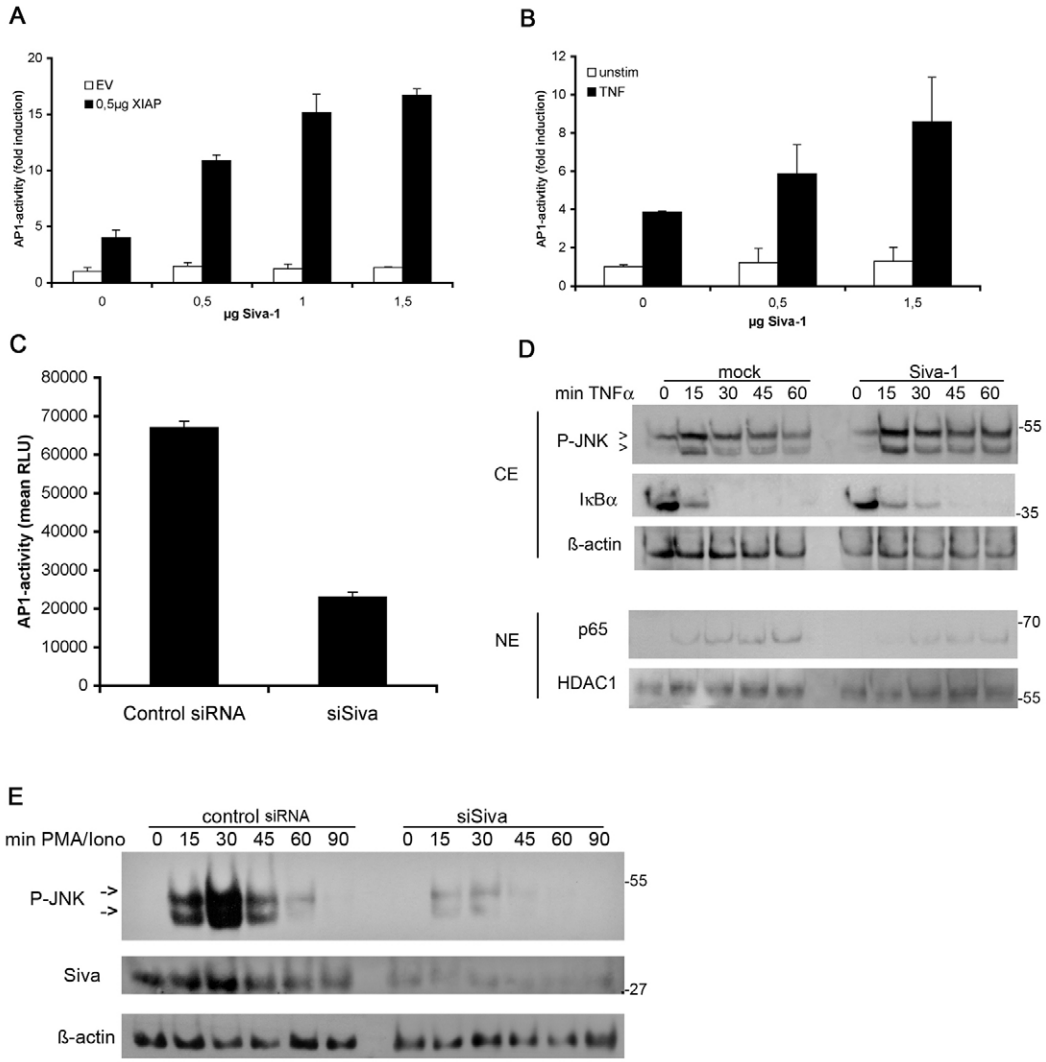

Fig. 4. Siva1 increases AP1 and JNK activity. HEK293 cells were co-transfected with an AP1-luc reporter (1 µg) and increasing amounts of FLAG-Siva1, either in the absence (EV) or presence of XIAP (A), or were stimulated with TNFα (B). Luciferase activity was normalized to β-Gal activity and fold induction was calculated in relation to EV-transfected cells. (C) Jurkat T cells stably expressing lentiviral-delivered siSiva or control siRNA were transfected with an AP1-luc reporter and UBT-β-Gal and stimulated for 10 hours with PMA/Iono. Luciferase activity was normalized to β-Gal activity (RLU: relative light units). (D) Siva1 increases activation of JNK and decreases IκBα degradation as well nuclear translocation of p65. HEK293 cells were transfected with Siva1 or empty vector (mock) and stimulated for the indicated times with TNFα (20 ng/ml) 24 hours after transfection. Cellular and nuclear fractions were subjected to western blot analysis with the indicated antibodies: P-JNK, IκBα and β-actin (CE, upper three panels) or p65 and HDAC1 (NE, lower two panels). (E) Knock down of Siva1 reduces JNK activation. Jurkat T cells stably expressing lentiviral-delivered siSiva or control siRNA were stimulated with PMA/Iono for the indicated times. Whole cell extracts were subjected to western blot analysis for P-JNK (upper panel). Protein levels of Siva and β-actin as loading control are shown in the lower two panels.

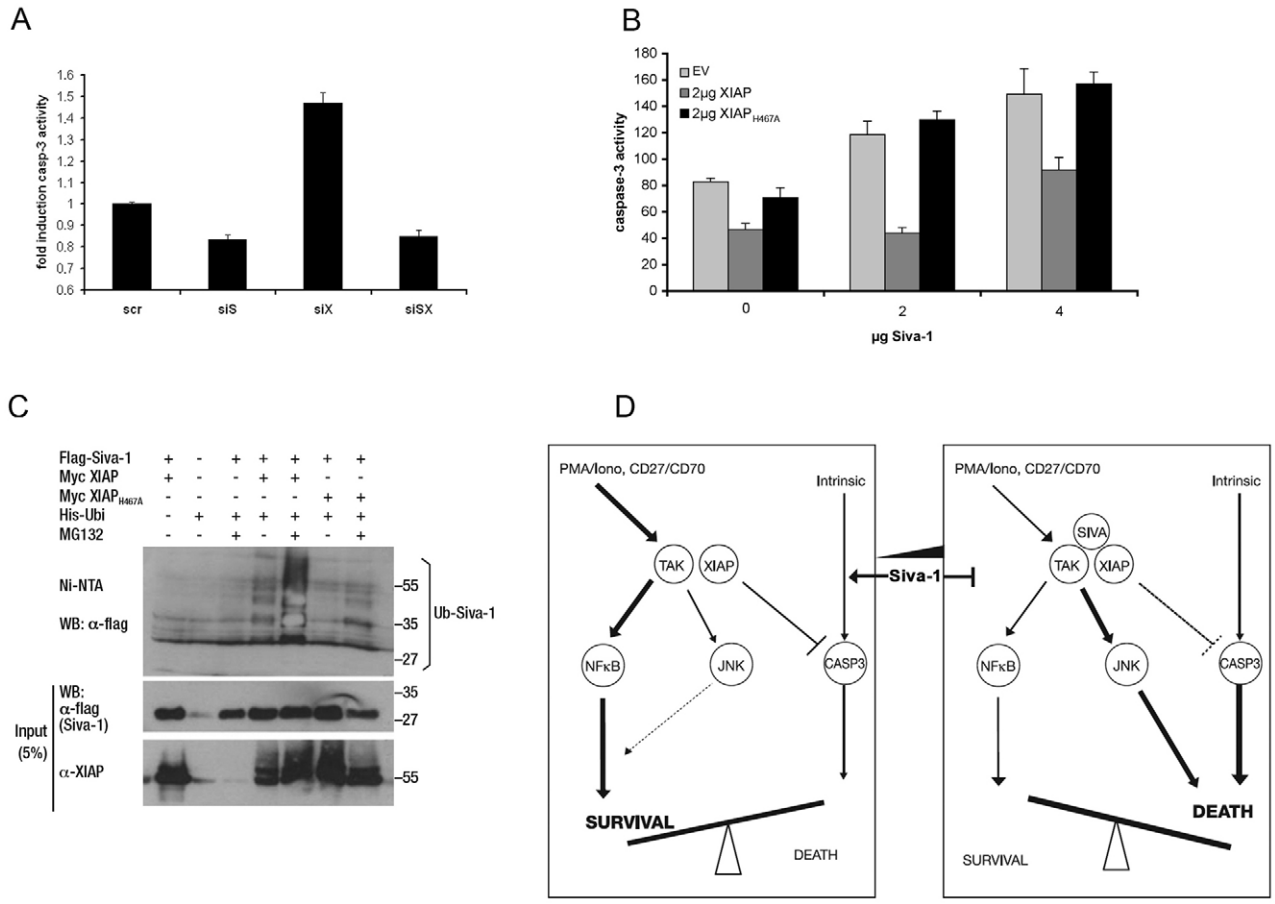

Fig. 5. XIAP ubiquitylates and counteracts Siva1-induced apoptosis. (A) Jurkat T cells were transfected with siRNAs directed against Siva (siS), XIAP (siX), both (siSX), or a scrambled control (scr), and 24 hours later stimulated with plate-bound anti-CD3 antibody (OKT3, 5 µg/ml) for 6 hours. Caspase-3 activity was measured and normalized to protein levels (Bradford assay). Values shown are the means ± standard error of mean. (B) XIAP inhibits Siva1-induced apoptosis in a ubiquitin-ligase-dependent manner. *Xiap*$^{-/-}$ mouse embryonic fibroblasts were transfected with 2 and 4 µg of Siva1 along with empty vector, 2 µg XIAP or $XIAP_{H467A}$. Caspase-3 activity was measured 36 hours post-transfection and normalized to protein levels. (C) Siva1 is a target for XIAP-mediated ubiquitylation. HEK293 cells were transfected with FLAG-Siva1, His-tagged ubiquitin, empty vector (–), XIAP, or the ubiquitin-ligase mutant $XIAP_{H467A}$. Cells were treated for 6 hours with the proteasome inhibitor MG132 (+) or vehicle alone (–). Ubiquitylated proteins were precipitated under denaturating conditions using Ni-NTA beads, and the presence of polyubiquitylated Siva1 was assessed by immunoblot analysis with anti-FLAG antibody (upper panel). Expression levels of Siva1 and XIAP are shown in the lower two panels (Input). (D) Siva1 balances NFκB and JNK signalling to promote apoptosis. Model showing how Siva1 shifts the balance from NFκB-mediated pro-survival towards JNK-mediated apoptotic signalling: stimulation of TNF-receptor family members (e.g. CD27, TNF-RI/II) as well as TCR (or PMA/Iono) stimulation can activate both survival and apoptotic signalling cascades. Thereby, XIAP and TAK1 promote survival through activation of NFκB and inhibition of caspase 3 (left). This can be counterbalanced by Siva1, which promotes apoptosis through inhibition of NFκB and leads to prolonged JNK signalling (right). Fine tuning is achieved by the relative abundance of the single proteins: high amounts of XIAP account for ubiquitylation and degradation of Siva1, resulting in NFκB-mediated survival. Increased levels of Siva1 shift the balance, resulting in a functional switch towards prolonged JNK signalling and cell death.

involved in activation of NFκB, whereas it is dispensable for activation of stress-responsive pathways, such as the JNK pathway (Lewis et al., 2004; Sanna et al., 2002). Because Siva1 was found to interact with the RING domain of XIAP (Fig. 1E), we suggest that Siva1 interferes with XIAP-mediated ubiquitylation events, resulting in diminished NFκB activation. Knock down studies in Jurkat T cells revealed that both XIAP and Siva1 differentially regulate NFκB activity, dependent on the stimulus. Upon TNF receptor stimulation, XIAP is of central importance for NFκB activation, because knock down of XIAP could not be rescued by knock down of Siva1. By contrast, the lower PMA/Iono-mediated NFκB activation in XIAP knock down cells was compensated by reduction of Siva1, suggesting an inhibitory function towards an additional signalling component(s), such as the MAP3K TAK1. Co-immunoprecipitation studies revealed that Siva1 indeed interacts with TAK1 in addition to XIAP. Previous studies demonstrated that the C-terminal part of TAB1 binds to TAK1 (Yamaguchi et al., 1999), whereas the N-terminal part binds to the XIAP BIR1 domain (Lu et al., 2007). Furthermore, XIAP has been shown to interact with TAK1, but the sites of interaction have not been mapped (Kaur et al., 2005; Sanna et al., 2002). In order to investigate whether the interaction of Siva1 with TAK1 occurs through TAB1 or XIAP, we performed additional association studies in HEK293 cells. Our data suggest that both TAB1 and XIAP are dispensable for the interaction of Siva1 with TAK1, thus supporting the notion that Siva1 is able to bind to both TAK1 and XIAP simultaneously. These findings might have further consequences for inhibition of NFκB activation by Siva1, since binding of Siva1 to the XIAP RING domain might interfere with its ability to mediate oligomerization and activation of, for example, TAK1 or MEKK2. Similarly, binding of Siva1 to TAK1 might interfere with its proximity-induced transphosphorylation

and activation. Nevertheless, it is important to note that our association studies do not distinguish between direct and indirect association between Siva1 and TAK1 since the experiments were performed using cell lysates. Hence, we cannot rule out that the formation of the ternary Siva1-TAK1-XIAP complex is mediated by other proteins.

Although decreasing XIAP and TNFα mediated NFκB activity, AP1 activity was enhanced by Siva1 and reduced in Siva1 knock down cells that were stimulated with PMA/Iono. AP1 transcription factor components, such as Jun and JunB, are activated through phosphorylation by JNKs, which in turn are activated upon environmental stress and by cytokines such as TNFα, or after TCR ligation. It has been shown that XIAP stimulates AP1 activity in an ubiquitin-ligase-independent manner (Lewis et al., 2004), but the role of XIAP in activation of JNKs is contradictory: selective activation of JNK1 by ectopically expressed XIAP has been shown to protect cells from caspase-3-induced apoptosis (Sanna et al., 2002) and involves TAK1. However, XIAP inhibits JNK1 activation upon TGFβ stimulation through proteasomal degradation of TAK1 (Kaur et al., 2005). Similarly, XIAP has been shown to modulate the crosstalk between NFκB and JNK signalling pathways upon TNFα stimulation (Karin and Lin, 2002), thereby protecting cells from apoptosis. These discrepancies might originate from the different experimental approaches, stimuli and cell types used. In addition, JNKs have both pro- and anti-apoptotic activities, depending on the duration of signalling: prolonged JNK activation is associated with apoptosis, whereas transient activation can signal cell survival (Bubici et al., 2006; Chen and Tan, 2000; Davis, 2000; Sabapathy et al., 2001; Ventura et al., 2004). The mechanism that accounts for proapoptotic signalling by JNK has not been established, but might involve increased ROS generation (Ventura et al., 2004), phosphorylation-induced inhibition of anti-apoptotic Bcl2 family members including Bcl2, as well as activation of proapoptotic BH3-only proteins such as Bax and Bim (Lei et al., 2002; Yamamoto et al., 1999). Likewise, we have recently demonstrated that XIAP is an important negative regulator of ROS generation and duration of JNK activation during oxidative stress conditions (Resch et al., 2008). We have demonstrated that ectopic expression of Siva1 enhances JNK activation whereas knock down of Siva1 diminishes JNK activation. Together, these results support the notion that Siva1 shifts the balance towards enhanced JNK signalling, which in turn might trigger mitochondrial-dependent cell death through activation of, for example, Bim or Bax. It is tempting to speculate that JNK-mediated activation of these BH3-only molecules is the underlying mechanism of the originally described mitochondrial-dependent, Siva1-induced cell death (Prasad et al., 1997); this will be subject of further investigations. Finally, we showed that Siva1 is K48 ubiquitylated by XIAP, thereby inhibiting Siva1-induced apoptotic signalling. Based on our results we propose the following model. Stimulation of TNF or T-cell receptor (PMA/Iono) activates both survival and apoptotic signalling cascades. TAK1 and XIAP promote survival through activation of NFκB and inhibition of caspase 3 (Fig. 5D, left). This is counterbalanced by Siva1, promoting apoptosis through inhibition of NFκB and prolonging JNK activation (Fig. 5D, right).

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Materials and Methods

Yeast two-hybrid screen

The Matchmaker Two-Hybrid System 3 (Clontech, Palo Alto, CA) was used to screen a HeLa cDNA library (Clontech) with full-length as well as truncated versions of an E3 ligase mutant of XIAP as bait. Full-length XIAP (1500 bp), BIR3+RING (797 bp) and the RING-finger only (275 bp) were subcloned *Nde*I-*Eco*RI, *Nco*I-*Eco*RI and *Eco*RV-*Bam*HI, respectively, into the pGBKT7 vector (Fig. 1A, schematic representation). Co-transformation of the bait and prey plasmids was performed according to the lithium acetate transformation protocol using yeast strain AH109 under highly stringent conditions (medium lacking Ade, His, Leu and Trp, supplemented with 35 mM 3-amino-1,2,4-triazole, 3-AT). A total of 1×10^6 transformants was screened. Colonies were analyzed by PCR and the cDNA insert sequenced. DNA was isolated and transformed into electrocompetent *Escherichia coli* (*Stbl2*), and interactions of bait and prey were confirmed by back transformation into yeast. Clones growing in the presence of the specific bait construct but not with the empty vector were considered as putative positive interaction partners.

Plasmid constructs

An expression plasmid containing the coding sequence of Siva1 was kindly provided by S. Benichou and subcloned by PCR (5′-*Bam*HI, 3′-*Not*I) into pcDNA3.1-Flag (Invitrogen). The Siva1 truncation containing the SAH domain and the death domain homology region (DDHR; 1-309) was prepared by PCR. The XIAP cDNA was subcloned by PCR into a CMV-myc expression vector (Clontech). XIAP deletion constructs (BIR12 1-780, BIR3+RING 780-1495, RING 1224-1495) were prepared by PCR. The RING deletion construct of XIAP (ΔRING) has been described previously (Lewis et al., 2004). Integrity of the constructs was confirmed by sequencing and western blot analysis. The 5xNFκB-luc and AP1-luc reporter constructs were purchased from Stratagene. The expression vectors for β-galactosidase driven by a ubiquitin promoter (UBT–β-Gal), TAK1 and TAB1 have been described previously (Hofer-Warbinek et al., 2000). His-tagged ubiquitin (pMT107-his-ubiquitin) was kindly provided by L. Schmitz. XIAP and Siva siRNAs and scrambled control were from Qiagen (XIAP: #1027400, Siva: #1027416, control: #1022076). Lentiviral vectors (SIN-GFP) expressing siSiva or control siRNA were kindly provided by R. Gudi and K. V. S. Prasad (Gudi et al., 2006).

Cell culture, transfections and inductions

HEK293 cells and $XIAP^{-/-}$ mouse embryonic fibroblasts (MEF prepared from mice provided by C. Duckett) were cultured in DMEM (BioWhitthaker, Belgium) and Jurkat cells (clone E6.1) were maintained in RPMI 1640 (Sigma, St Louis), both supplemented with 10% FCS (Sigma), 1% penicillin-streptomycin and L-glutamine (BioWhittaker, Belgium) at 37°C under 5% CO_2. HEK293 cells and MEF were seeded into 6-, 12- or 24-well plates coated with 1% gelatine and transiently transfected using the calcium phosphate co-precipitation method. Transfection of Jurkat cells was performed with a Nucleofector electroporation device (Amaxa Biosystems, Köln, Germany). 2×10^6 Jurkat cells were harvested by centrifugation and the cell pellet was resuspended in 100 µl cytomix solution, with 2-4 µg DNA or 100 pmol siRNA, and nucleofected as suggested by the manufacturer. Cells were washed once with culture medium and subsequently cultured for the indicated period of time. To generate lentivirus, 293T cells were transfected with lentiviral vectors along with viral packaging vectors using the calcium phosphate method. Jurkat T cells were infected with viral supernatant for 8 hours and then returned to culture media. Four days after transfection, knock down efficiency was assessed by western blotting and FACS analysis of GFP-positive cells. Stimulations and transfections were done with cells that were approximately 70-80% GFP positive.

For reporter gene assays, the 5xNFκB-luc or AP1-luc reporter construct was co-transfected with FLAG-Siva1 and XIAP or TAK1/TAB1 along with an expression plasmid encoding UBT-β-gal as an internal control. Empty vector (CMV-myc) was added to 4 µg. At 48 hours after transfection, cells were washed with PBS and lysed in 1× passive cell lysis buffer (Promega). Luciferase activity was measured from 10 µl of lysate in a luminometer (Lumat, LB9507; EG&G Berthold, Bad Wildbad, Germany) and the results recorded as relative light units (RLU). RLUs were normalized to β-gal activity, which was measured photometrically at 570 nm using CPRG as the chromogenic substrate. Experiments were repeated at least three times and each experiment was done in triplicate. For induction of cells, phorbol-12-myristate-13-acetate (PMA 10 nM) and ionomycin (0.5 µM; both from Sigma) dissolved in DMSO were used. TNFα (20 ng/ml) was from R&D Systems. Monoclonal anti-CD3 (OKT3) was from Orthoclone.

Western blot analysis and immunoprecipitation

Samples from either whole-cell pellets, nuclear fractions or cytosolic fractions were prepared as described previously (Resch et al., 2008; Winsauer et al., 2008), resolved by SDS-PAGE and subjected to western blot analysis. The primary antibodies used were anti-IκBα, anti-p65, anti-HDAC1, anti-β-actin anti-HA (both mouse and rabbit), which were from Santa Cruz, and anti-SAPK/JNK (Thr183/Tyr185), which was from Cell Signaling. Molecular mass markers were from Fermentas or PeqLab. In some cases, the density of blots was quantified using ImageJ software. For co-immunoprecipitations, HEK293 cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate,

1% Triton X-100 and protease inhibitors (Roche) for 30 minutes on ice and centrifuged at 4°C at 12,000 ***g***. Clarified lysates were immunoprecipitated with anti-FLAG–Matrix (Sigma) for 3 hours at 4°C by end-over-head rotation. Precipitates were washed three times with lysis buffer and proteins were eluted by boiling the beads for 5 minutes in 2× Laemmli buffer. Eluted proteins or indicated amounts of whole cell extracts (Input) were separated by 12% SDS-PAGE, transferred to Hybond membranes (Amersham), blocked and incubated with goat or mouse anti-XIAP (R&D Systems), goat or mouse anti-TAK1, goat anti-TAB1, goat anti-Siva (recognizing both Siva1 and Siva2; Santa Cruz), or mouse anti-FLAG (Sigma) antibodies. After washing, membranes were incubated with HRPO-linked secondary antibodies (Amersham), washed and developed using WestPico substrate (Pierce).

For endogenous co-immunoprecipitations 2×10^6 Jurkat cells were stimulated for the indicated periods of time and lysed in PBS supplemented with 1% Triton X-100 and protease inhibitors (Roche). Lysates were pre-cleared with protein A/G-beads (Santa Cruz) for 30 minutes at 4°C and then incubated with anti-Siva, anti-XIAP or anti-TAK1 antibodies or control IgG prebound to protein A/G beads overnight. Immunoprecipitates were washed four times with lysis buffer and analyzed by western blotting. For precipitation of XIAP and TAK1, monoclonal antibodies were used (from BD and Santa Cruz, respectively).

Quantitative RT-PCR

Total RNA was isolated from cells using the High Pure RNA isolation kit (Roche) according to the manufacturer's protocol. cDNA was reverse-transcribed using the TaqMan reverse transcription kit (Applied Biosystems) with random hexamers. Real-time PCR of cDNA was performed in a Roche LightCycler. mRNA expression was normalized to β2-microglobulin. Cycling conditions were: denaturating at 95°C for 10 minutes, 55 cycles of amplification with 5 seconds at 95°C, 5 seconds at 65°C and 15 seconds at 72°C, followed by 15 seconds at 70°C. Primer sequences were as follows: Siva1: forward: 5′-GTC CAT TGC CTG TTC CTC AT-3′, reverse: 5′-AGG TCT CGA ACA TGG CAC AG-3′; RANTES: forward: 5-CCA TAT TCC TCG GAC ACC AC-3′, reverse: 5′-GGG TGA CAA AGA CGA CTG CT-3′; Bcl-2: forward: 5′-GAG GAT TGT GGC CTT CTT TG-3′, reverse: 5′-TTC AGA GAC AGC CAG GAG AAA-3′; A20: forward: 5′-CAT GCA TGC CAC TTC TCA GT-3′, reverse: 5′-CAT GGG TGT GTC TGT GGA AG-3′; β2-microglobulin: forward: 5′-GAT GAG TAT GCC TGC CGT GTG-3′, reverse: 5′-CAA TCC AAA TGC GGC ATC T-3′.

Ubiquitylation assays

In vivo ubiquitylation assay with His-tagged ubiquitin was performed according to a protocol available from Tansey Lab Protocols (http://tanseylab.cxhl.edu/protocols.html). Where indicated, cells were treated with the proteasome inhibitor MG132 (5 μM) or vehicle (DMSO).

Apoptosis assays

Jurkat or MEF cells were electroporated or transfected as described above, 24 hours later cells were harvested and caspase 3 activity was assessed fluorimetrically by the hydrolysis of the peptide substrate Ac-DEVD-AMC (Sigma-Aldrich, CASP3F) in a fluorometric multiwell microplate reader with excitation set at 360 nm and emission at 460 nm.

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