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XIAP regulates intracellular ROS by enhancing antioxidant gene expression

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ABSTRACT

XIAP (X chromosome-linked inhibitor of apoptosis) is a member of the anti-apoptotic IAP gene family and an inhibitor of caspase-3. We show here that loss of XIAP renders cells highly sensitive to oxidative stress. Stimulation of $XIAP^{-/-}$ MEF with hydrogen peroxide, or other agents that generate reactive oxygen species (ROS) results in increased apoptosis assessed by caspase-3 activity and PARP cleavage. Furthermore, we observed increased levels of ROS and diminished expression of antioxidative genes, e.g., SOD1, -2, NQO1, HO-1, and Txn2 in $XIAP^{-/-}$ cells. In addition, stimulation of $XIAP^{-/-}$ MEF with hydrogen peroxide resulted in enhanced phosphorylation of JNK. Our findings reveal that XIAP, in addition to its well described caspase-inhibitory function, prevents prolonged JNK activation and is critically involved in modulating ROS levels through regulation of antioxidative genes, thereby inhibiting ROS-induced apoptosis.

Reactive oxygen species (ROS) are involved in a variety of pathologies including cancer, cardiovascular, neurodegenerative, and autoimmune diseases [1–5]. However, they also function as second messenger molecules in cellular signal transduction pathways and are important mediators of the innate immunesystem in host-defence [6,7]. Appropriate mechanisms to balance the formation as well as the detoxification of ROS are therefore crucial for the maintenance of cellular integrity, and are mediated by the coordinated activation of genes encoding antioxidant proteins [8,9]. Increased levels of ROS cause membrane damage, protein oxidation and DNA-damage [10]. This damage may either cause direct cell death via necrosis or initiate the self-destruction mechanism of apoptosis [11].

XIAP is a member of the inhibitor of apoptosis protein (IAPs) family that inhibits the activity of caspases-3, -7, and -9 [12], and has been shown to potently protect cells against various apoptotic stimuli [13,14]. However, XIAP deficient mice have no obvious phenotype and no apoptotic abnormalities were found when T cells or fibroblasts were challenged with α-Fas-antibody, TNFα or UV irradiation, respectively [15]. Separated from its anti-apoptotic activity, XIAP also functions in the TGFβ, NF-κB and stress-activated JNK signaling pathways [16–18]. Cytokines such as TGFβ or TNFα have been shown to cause ROS production and stimulate signaling pathways leading to activation of JNK and the transcription factors NF-κB or AP1 [19–22]. Recently, a role of XIAP in regulating intracellular ROS through interaction with AIF has been described [23]. Likewise, the mitochondrial enzymes SOD2 and thioredoxin 2 have been found being upregulated by overexpressed XIAP [24,25]. Here we demonstrate that $XIAP^{-/-}$ fibroblasts (MEF) display a significantly higher sensitivity to ROS-induced apoptosis, resulting in higher rates of apoptotic cell death. Furthermore we show that levels of both NF-κB and Nrf-ARE regulated antioxidant genes are decreased under unstimulated as well as oxidative stress-induced conditions in $XIAP^{-/-}$ MEF. It has been demonstrated that ROS may be mediators of prolonged JNK activation, which is associated with apoptosis [26]. Accordingly, we observed sustained JNK phosphorylation in $XIAP^{-/-}$ MEF, slightly enhanced p38 phosphorylation, while ERK as well as IKK2 were similarly activated. Our results demonstrate that XIAP inhibits apoptosis not only through inhibition of caspases but also through modulation of ROS by regulating antioxidative genes.

Materials and methods

Cell culture, reagents, and antibodies. Mouse embryonic fibroblasts (MEF) were prepared from WT and $XIAP^{-/-}$ mice and were established in culture from E13.5 embryos by spontaneous transformation. Cells were cultured in DMEM (Hyclone, Logan, UT, USA) containing 2 mM L-glutamine, 10% fetal bovine serum (Sigma, St. Louis) 100 U/ml streptomycin and 100 U/ml penicillin (Hyclone, Logan, UT, USA) at 37 °C, and 5% CO_2. For induction of oxidative stress, cells were seeded in 6- or 12-well plates (Nunc) and stimulated 24 h later for the indicated time-points and concentrations with H_2O_2 (Merck), TNFα (R&D System), cycloheximide (CHX), actinomycin D (ActD), phorbol 12-myristate 13-acetate (PMA), *N*-acetylcystein (NAC), $FeSO_4$, and ascorbate (Sigma).

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Apoptosis-assays. Caspase-3 activity was measured fluorometrically according to the CASP3F assay kit (Sigma). Hydrolysis of the fluorogenic substrate Ac-DEVD-AMC was followed in 30 min intervals over 3 h in a 96-well microtiter plate reader with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Caspase-3 activity was calculated from the rate of AMC released per min normalized to protein-content (BCA, Pierce).

Analysis of intracellular ROS and superoxide dismutase. MEF were seeded in six-well plates and stimulated 24 h later with 100 μM H_2O_2, 0.2 mmol/l $FeSO_4$/2 mM ascorbate, 0.03 μg/ml actinomycin D or 100 μM xanthine and 100 mU xanthine oxidase in phenol-red-free DMEM. Intracellular ROS levels were determined by incubating cells with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescence diacetate (CM-H_2DCFHDA, Molecular Probes) for 30 min in the stimulation medium at 37 °C and 5% CO_2 in the dark. Cells were detached with trypsin/EDTA, washed, resuspended in PBS and immediately analyzed by flow cytometry with excitation 485 nm and emission 545 nm. Mean fluorescence intensity was obtained by histogram statistics using WinMDI 2.8. Alternatively, ROS were measured in a 96-well microtiter plate reader and mean fluorescence intensity was normalized to protein-content. Enzymatic activity of Superoxide dismutase (SOD) was measured with the Superoxide Dismutase Kit (R&D Systems, #7500-100-K) according to manufacture's instruction. SOD activity was calculated using a SOD standard curve and expressed as units SOD.

RT-PCR. Total RNA was extracted from wild-type and XIAP$^{-/-}$ MEF grown in six-well plates using Trizol (Invitrogen). cDNA was reverse-transcribed using the TaqMan reverse transcription kit (Applied Biosystems) with random hexamers. Real-time PCR was performed in a Roche LightCycler and mRNA levels were normalized to β2microglobulin. Cycling conditions were: denaturating at 95 °C for 10 min, 55 cycles of amplification with 5 s at 95 °C, 5 s at 65 °C, and 15 s at 72 °C, followed by 15 s at 70 °C. Primer sequences are provided in the Supplementary Information.

Immunoblotting. Cells were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM NaF, 0.2 mM Na_3VO_4 protease, and phosphatase inhibitors (Roche). The cell lysates were resolved by SDS–PAGE, transferred to nitrocellulose membranes (Hybond-C, Extra, Amersham, UK), blocked for 1 h in TBS and 5% skim milk, incubated overnight with primary antibodies against p-SAPK/JNK (Thr183/Tyr185), p-p38 (Thr180/Tyr182), p44/42, (ERK) p-IKKβ (Ser181), β-actin (Santa Cruz) or PARP (Biomol, SA-250). Appropriate peroxidase-conjugated antibodies (Amersham) were added for 1 h and detection was performed using SuperSignal West Pico Substrate (Pierce).

Results

XIAP$^{-/-}$ MEF show increased sensitivity to H_2O_2 and TNFα/ActD-induced cell death

Two recent reports have demonstrated that ROS levels are modulated directly [23] or indirectly [24] upon ectopic expression of XIAP. We therefore tested wild-type and XIAP$^{-/-}$ MEF for their sensitivity towards agents that generate ROS. A dose–response curve of wt cells treated with H_2O_2 for 10 h revealed increasing caspase-3 activity that reached a maximum of approx. fivefold over unstimulated conditions at a concentration of 20 mM. In contrast, in XIAP$^{-/-}$ MEF caspase-3 activity reached a maximum already at 1 mM (10-fold) and declined thereafter because of massive cell death at higher concentrations (Fig. 1A) Concomitantly, poly-(ADP-ribose) polymerase (PARP) cleavage was readily detectable at 1 mM H_2O_2 in XIAP$^{-/-}$, but not in wt MEF (Fig. 1A, inset). A similar experiment with lower H_2O_2 concentrations after 6 h of stimulation is shown in Fig. 1B, confirming that caspase-3 activation occurs much earlier in XIAP$^{-/-}$ MEF as in wild-type MEF in a dose-dependent manner.

We then tested TNFα plus cycloheximide (CHX), a combination that promotes apoptosis through blocking the NF-κB-mediated rescue arm of the TNF response. Whereas CHX alone did not result in a detectable response, additional stimulation with TNFα increased caspase-3 activity in a dose-dependent manner. Again, XIAP$^{-/-}$ MEF showed increased sensitivity (Fig. 1C). In contrast and in agreement with previous findings [15] we did not observe enhanced apoptosis in XIAP$^{-/-}$ MEF in time-course experiments with typical CHX/TNFα concentrations (20 ng/ml TNFα plus 10 μg/ml CHX, data not shown). In this regard it has been suggested that cIAP1 and cIAP2, which were originally identified by their association with TNFR2 via TRAF1 and TRAF2 [27,28], most likely take over the anti-apoptotic function of XIAP in the receptor-mediated apoptotic pathway. When using TNFα in combination with low-doses of actinomycin D (a concentration that does not inhibit transcription but inhibits rRNA synthesis) XIAP$^{-/-}$ MEF showed a 10-fold higher sensitivity (Fig. 1D).

In order to test whether antioxidants can compensate for the increased caspase-3 activation in XIAP$^{-/-}$ cells, we treated them with *N*-acetylcystein (NAC). As shown in Fig. 1E, NAC could partially prevent caspase-3 activation in H_2O_2 treated XIAP$^{-/-}$ MEF. Together, this indicates that XIAP$^{-/-}$ MEF display a higher sensitivity towards ROS generating substances as compared to their wt counterparts, and that they can be rescued, at least partially, by antioxidants.

Accumulation of ROS levels in XIAP$^{-/-}$ MEF

Since we observed enhanced sensitivity of XIAP$^{-/-}$ MEF towards oxidative stress, we examined whether accumulation or lack of detoxification of ROS is responsible for increased cell death in XIAP$^{-/-}$ MEF. Wild-type and XIAP$^{-/-}$ MEF were stimulated with 100 μm H_2O_2, iron/ascorbate, ActD or xanthin and xanthine oxidase (X/XO) for 1 or 6 h. Cells were labeled with a cell-permeable fluorescent dye, CM-H2DCFDA and analyzed by flow cytometry or in a fluorescent microtiter plate. As shown in Fig. 2A and B, levels of ROS were higher after 1 h in XIAP$^{-/-}$ MEF under most conditions as compared to wt cells (except after PMA-stimulation), with xanthine/xanthine oxidase showing the most pronounced difference. ROS accumulation was much more evident when cells were stimulated for 6 h (Fig. 2C). Thus, we assumed that XIAP$^{-/-}$ MEF have a decreased capacity to detoxify ROS and therefore show increased sensitivity to ROS-induced cell death.

Expression of NF-κB and Nrf-ARE dependent genes is diminished in XIAP$^{-/-}$ MEF

Next, we analyzed in detail the expression of antioxidant genes in wt and XIAP$^{-/-}$ MEF after stimulation with H_2O_2, TNFα, PMA, or iron/ascorbate (Fe/Asc). Real-time PCR analysis of superoxide dismutase-1 (SOD1) and ferritin heavy chain (Fth1) showed only minor differences under basal and stimulated conditions (with the exception of PMA) between the two cell types, while glutathione peroxidase-1 (GPX1) was lower after stimulation only (Fig. 3A). In contrast, thioredoxin 2 (Txn2), NAD(P)H:quinone oxidoreductase (NQO1) and heme-oxygenase-1 (HO-1) were significantly decreased under basal and stimulated conditions in XIAP$^{-/-}$ MEF as compared to wt cells (Fig. 3B). Similar, mRNA levels of superoxide dismutase-2 (SOD2) were also decreased under basal and stimulated conditions (Fig. 3C, upper panel). To investigate whether decreased SOD mRNA amounts were reflected on the level of biological activity, we assayed for SOD activity, and could confirm that it was diminished in wt and XIAP$^{-/-}$ cells (Fig. 3C, lower panel).

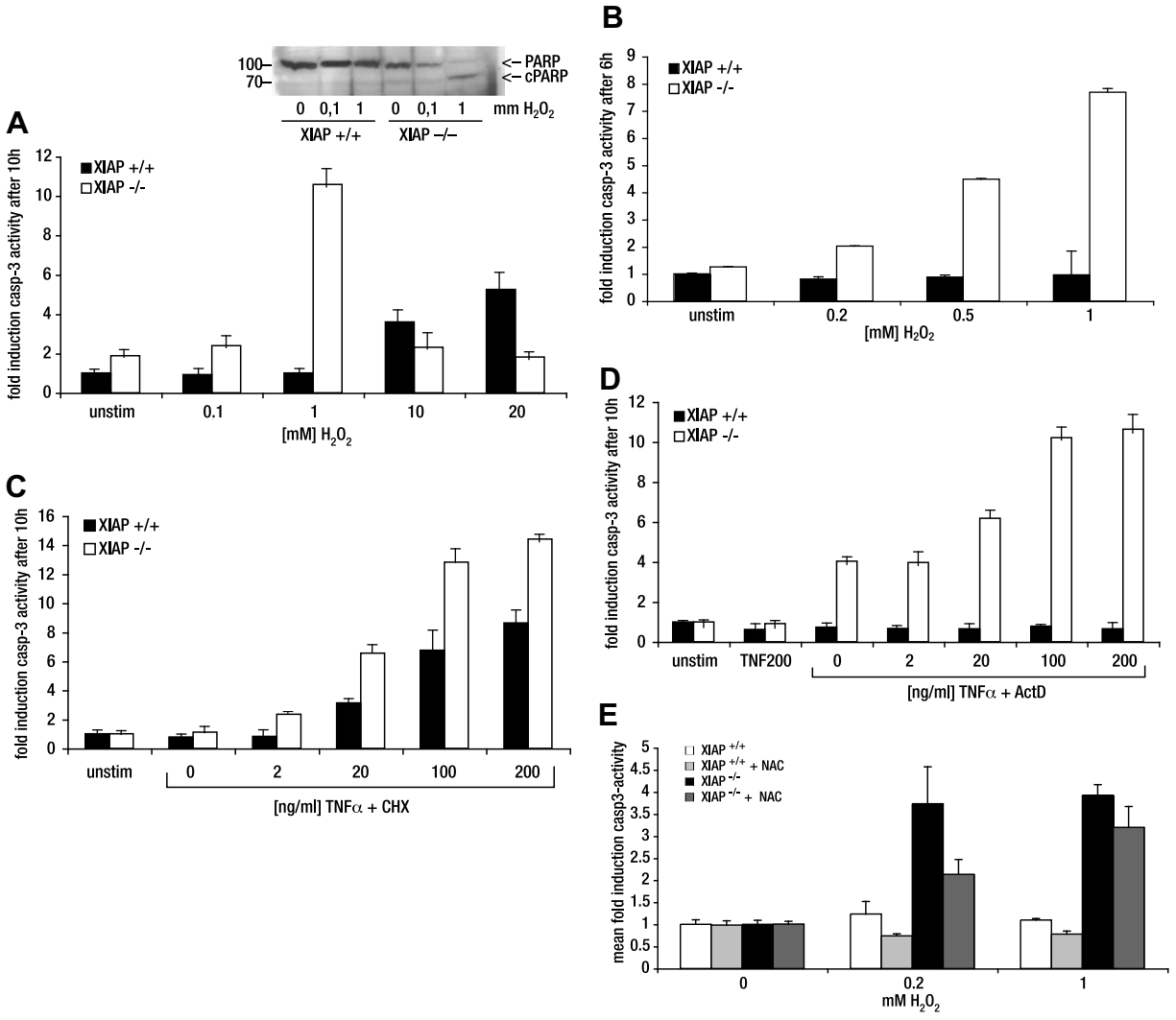

Fig. 1. XIAP$^{-/-}$ MEF show increased sensitivity to H_2O_2 and ActD/TNFα-induced cell death. (A) Wild-type (XIAP$^{+/+}$) and XIAP$^{-/-}$ MEF were treated with increasing concentrations of H_2O_2 for 10 h and caspase-3 activity as well as PARP cleavage was assessed by Western blotting (inset, cPARP indicates the 85 kDa caspase-3 generated cleavage product of PARP). (B) Wild-type and XIAP$^{-/-}$ MEF were treated with increasing concentrations of H_2O_2 for 6 h and caspase-3 activity was measured. Wild-type and XIAP$^{-/-}$ MEF were treated with increasing concentrations of TNFα plus 10 μg/ml cycloheximide (CHX) or 0.03 μg/ml actinomycin D (ActD) and caspase-3 activity was measured after 10 h (C and D, respectively). Fold induction was calculated in relation to unstimulated wild-type MEF. (E) Wild-type and XIAP$^{-/-}$ MEF were incubated with 5 mM NAC 2 h prior to stimulation with indicated concentrations of H_2O_2 and caspase-3 activity was measured after 7 h.

ROS cause prolonged JNK activation in XIAP$^{-/-}$ MEF

Since it has been demonstrated that ROS may be mediators of prolonged JNK activation, which is associated with apoptosis [26], we compared MAP kinase activity in wt and XIAP$^{-/-}$ MEF in response to H_2O_2. Cells were pulsed for 20 min with 100 μM H_2O_2, further cultivated for 40 and 60 min after change of media and extracts analyzed for phosphorylated JNK, p38 and ERK by Western blotting. As shown in Fig. 4 (first panel), we observed strongly elevated and sustained JNK activation in XIAP$^{-/-}$ as compared to wt MEF. Also, phospho-p38 was increased (Fig. 4, second panel). In contrast, no apparent differences were found for ERK and also not for IKK2 (Fig. 4, lower two panels).

Discussion

We demonstrated here that XIAP$^{-/-}$ MEF show increased sensitivity to ROS-induced cell death that could be partially rescued by the antioxidant NAC. Furthermore, we observed an accumulation of intracellular ROS in XIAP$^{-/-}$ MEF after various stimuli. Apart from generation through cellular exposure to ionizing radiation and xenobiotics, a major site of ROS production are the mitochondria, where 1–2% of the oxygen is leaked from complex I (NADH dehydrogenase) and complex II (ubiquinone). Over the past decades it has become clear that mitochondria play a central role in the control of apoptosis. The permeabilization of the mitochondrial outer membrane and release of, e.g., cytochrome *c* from the intermembrane space are the key steps in the activation of the intrinsic apoptotic pathway, while the temporal relationship between ROS production and caspase-activation is still a matter of debate [29,30]. Membrane permeabilization is mediated by proapoptotic BH3-only proteins such as Bax, Bak, Bid or Bim but also occurs after caspase-3 activation and is counteracted by the mitochondrially located Bcl-2 family of proteins. In addition, several antioxidant enzymes, e.g., superoxide dismutase-2 or thioredoxin 2 are localized to the mitochondria, where they scavenge ROS and are involved in the redox-balance of this organelle. XIAP is localized in the cytoplasm as well as in the mitochondria, where it interacts with AIF and synergistically counteracts ROS production [23]. Here we could show that ROS are accumulating in

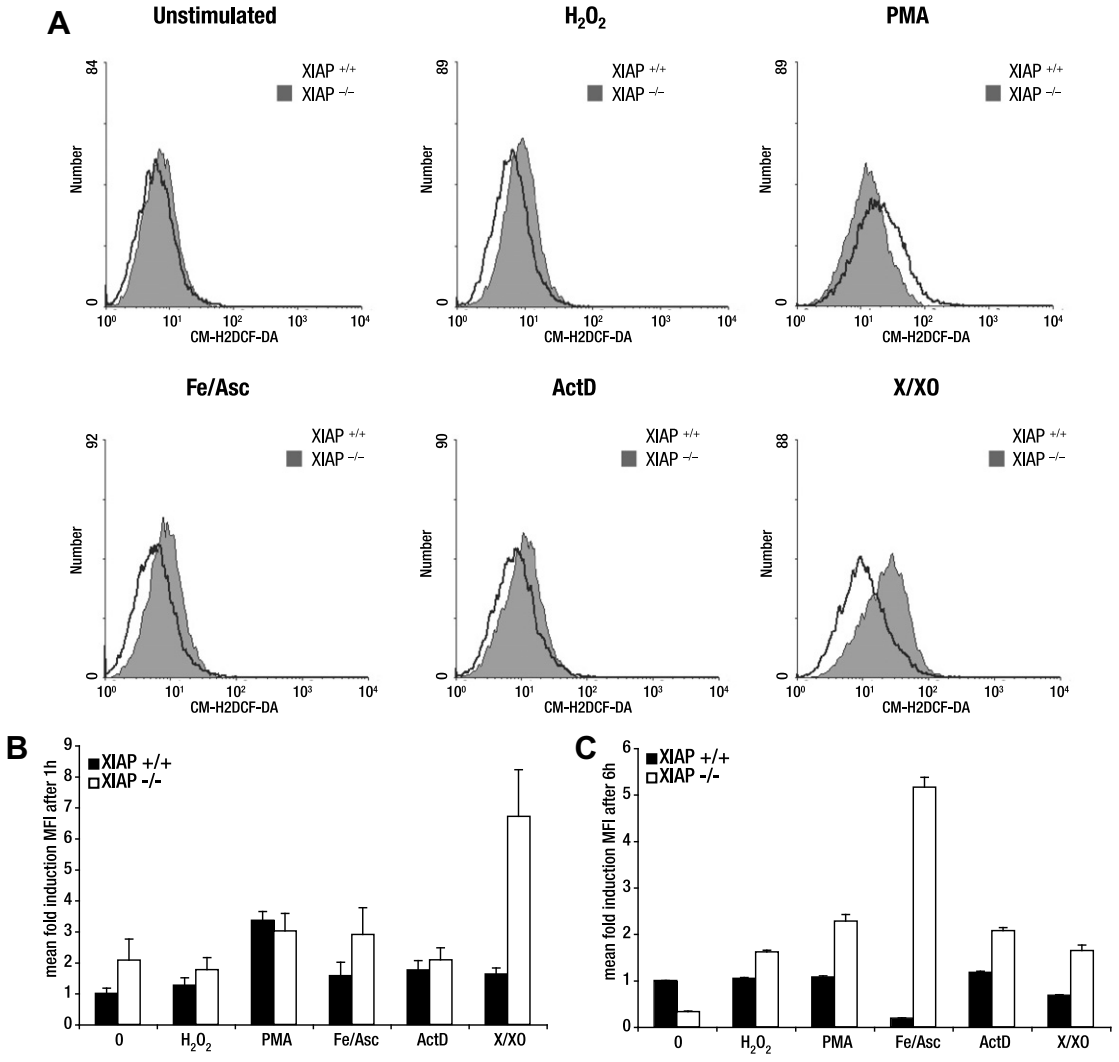

Fig. 2. Accumulation of ROS levels in XIAP$^{-/-}$ MEF. (A) Wild-type (XIAP$^{+/+}$) and XIAP$^{-/-}$ MEF were stimulated with either 100 μM H_2O_2, 2.5 μg/ml PMA, 0.2 mmol/2 mmol Fe/Asc, 0.03 μg/ml ActD or 100 μM xanthine/100 mU xanthine oxidase (X/XO) for 1 h in phenol-red-free DMEM. CM-H_2DCFHDA (10 μM) was added for 30 min and ROS production was measured by FACS-analysis. Alternatively, ROS production was measured in a 96-well microtiter plate reader and fold induction was calculated in relation to unstimulated wild-type MEF (B). (C) Cells were stimulated as described above and ROS production was measured after 6 h.

XIAP$^{-/-}$ MEF, indicating that XIAP is critically involved in the detoxification of ROS. Accordingly, we found mRNA levels of various antioxidant enzymes being diminished under basal and stimulated conditions in XIAP$^{-/-}$ MEF.

There are only few transcription factors, including NF-κB and the NF-E2-related factors (Nrf), that are activated by ROS and/or electrophiles generated from xenobiotics. NF-κB activates transcription of antioxidant enzymes such as superoxide dismutase-2 (SOD2) or ferritin heavy chain (FTH1), thereby protecting cells from increased oxidative stress and apoptosis [31,32]. Another group of genes such as glutathione *S*-transferase, γ-glutamylcystein synthetase, NAD(P)H quinone reductase (NQO1), heme-oxygenase-1 (HO-1), thioredoxin 2 (Txn2) as well as FTH1 are activated in cells by xenobiotics such as oxidants, carcinogens and electrophilic compounds [33]. The promoters of these genes contains a cis-acting element, termed the antioxidant response element [34] and the transcription factor Nrf2 alone or in combination with AP1-family members such as JunB, JunD or c-Jun has been shown to regulate these genes. We and others have demonstrated that ectopic expression of XIAP can activate both NF-κB and AP1 [17,35,36]. However, XIAP is also regulated on the post-transcriptional level via an IRES-element, and XIAP protein-levels might therefore be increased in stress-situations, e.g., under oxidative/electrophilic stress [37]. In accordance with a recent report [24,25] we found that the transcription and enzymatic activity of mitochondrially located SOD2, that catalyzes dismutation of superoxide anion into hydrogen peroxide, is strongly impaired in XIAP$^{-/-}$ MEF. Similar, basal and stimulated levels of mitochondria-localized thioredoxin 2 are greatly diminished in the absence of XIAP. In contrast, ferritin heavy chain (Fth1), which is the primary iron sequester that counteracts the formation of hydroxyl radicals was not affected in XIAP$^{-/-}$ MEF (with the exception after PMA-stimulation). In addition, we found impressive differences in mRNA levels of NQO1 and HO-1, genes that are regulated by Nrf-ARE. These genes can be coordinatively regulated by AP1-family members, which are themselves activated by, e.g., the stress-activated kinase JNK [8]. To our knowledge, the involvement of XIAP in the regulation of NQO1 and HO-1 has not been described before. Whether XIAP directly mediates transcription of these two genes (e.g., by activating AP1-transcription factors) or indirectly through modulating ROS levels remain to be investigated. Finally, we found sustained JNK activation in XIAP$^{-/-}$ MEF after hydrogen peroxide stimulation, supporting the notion that XIAP inhibits prolonged JNK activation after, e.g., TNFα stimulation, thereby inhibiting

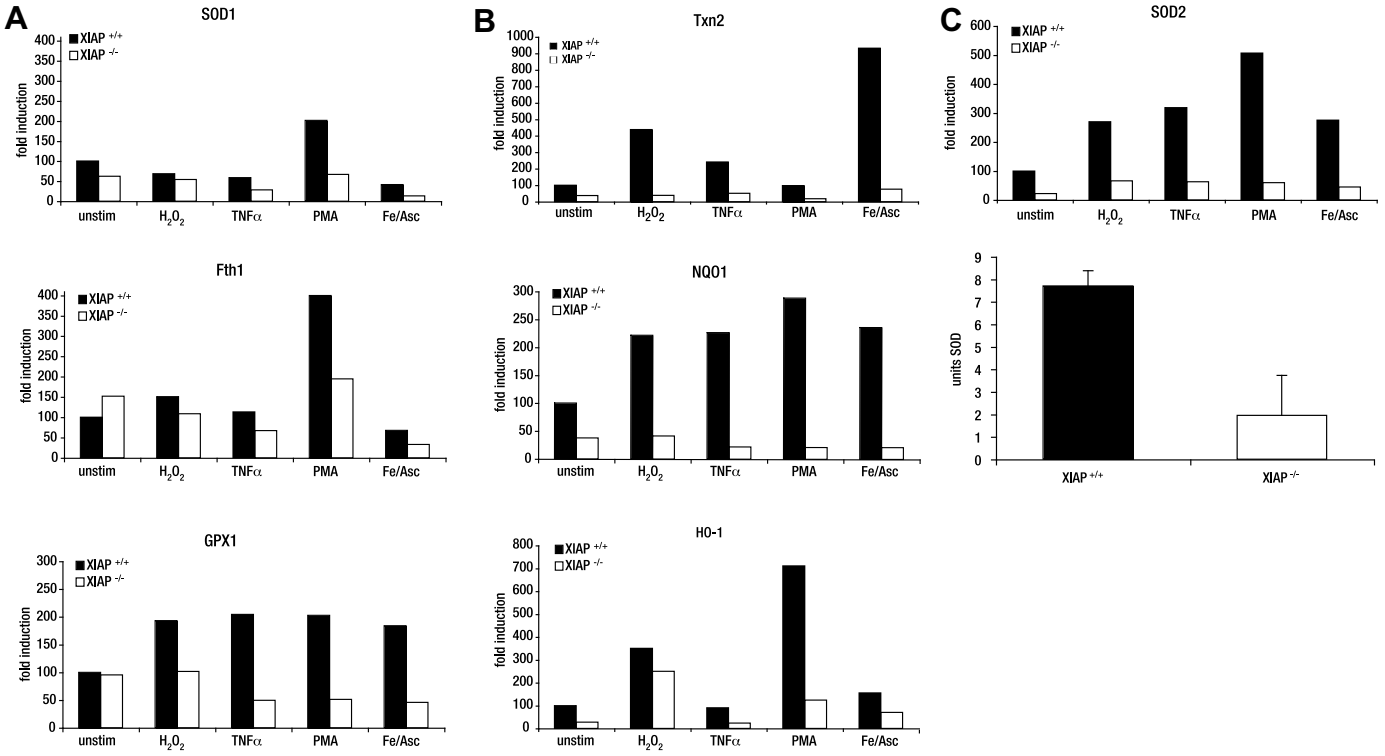

Fig. 3. Expression of NF-κB and Nrf-ARE dependent antioxidant genes is diminished in XIAP$^{-/-}$ MEF. Wild-type (XIAP$^{+/+}$) and XIAP$^{-/-}$ MEF were stimulated 90 min with either 100 μM H_2O_2, 10 ng/ml TNFα, 2.5 μg/ml PMA, or 0.2 mmol/2 mmol Fe/Asc, and mRNA levels of SOD1, Fth1, GPX1 (A) as well as Txn2, NQO1, and HO-1 (B) were measured by real-time PCR and normalized to β2microglobulin. Fold induction was calculated in relation to unstimulated, wild-type MEF. (C) Basal and stimulated SOD2 mRNA levels (upper panel) were measured as described above. Enzymatic activity (U/ml) of superoxide dismutase (SOD) was assessed from 3×10^5 untreated wild-type (XIAP$^{+/+}$) and XIAP$^{-/-}$ MEF using SOD standard. (lower panel) A representative experiment of three is shown.

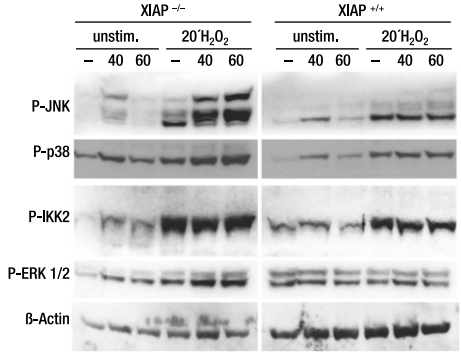

Fig. 4. ROS cause prolonged JNK activation in XIAP$^{-/-}$ MEF. XIAP$^{-/-}$ and wild-type (XIAP$^{+/+}$) MEF were stimulated for 20 min with 100 μM H_2O_2 followed by medium change and further incubated for 40 and 60 min. To control for effects of handling and media changes, control cells were treated as above but without H_2O_2 stimulation. Cells were harvested and phosphorylation of JNK, p38, IKK2, and ERK was assessed by Western blotting.

apoptosis [26,38]. Based on our findings we propose a model where XIAP modulates transcription of NF-κB and Nrf-ARE-dependent antioxidative genes, thereby reducing ROS accumulation and prolonged JNK activation and inhibits apoptosis in further consequence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.142.

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