

XIAP regulates bi-phasic NF- κ B induction involving physical interaction and ubiquitination of MEKK2

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ABSTRACT

The transcription factor NF- κ B is transiently activated by a wide variety of stress signals, including pro-inflammatory mediators, and regulates the expression of genes with e.g., immune, inflammatory, and anti-apoptotic functions. The strength and kinetics of its induction, as well as its ultimate down-regulation is subject to multiple levels of regulation. One such regulatory protein is X chromosome-linked inhibitor of apoptosis (XIAP) that, besides its anti-apoptotic properties, has been shown to enhance NF- κ B activity, however, the underlying molecular mechanism has remained elusive. We show here that following TNF α stimulation XIAP regulates a second wave of NF- κ B activation. XIAP interacts with and ubiquitinates MEKK2, a kinase that has previously been associated with bi-phasic NF- κ B activation. We conclude that, through interaction with MEKK2, XIAP functions in an ubiquitin ligase dependent manner to evoke a second wave of NF- κ B activation, resulting in the modulation of NF- κ B target gene expression.

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1. Introduction

Nuclear factor (NF)- κ B family transcription factors regulate the expression of genes that are essential for immune and acute phase inflammatory responses, and that are associated with biological processes such as cell adhesion, differentiation, proliferation and apoptosis [1–3]. As such, the NF- κ B signaling pathway is subject to crosstalk with other signaling pathways such as JNK, p53, cAMP, JAK/STAT, β -catenin, PDK1/Akt, and estrogen receptor signaling [4]. Moreover, it is tightly regulated in regard to both duration and magnitude of activation, since excessive NF- κ B activity may easily lead to overshooting immune reactions that cause detrimental effects. Therefore, negative feedback loops exist within the pathway itself to ensure appropriate down-regulation, e.g., the expression of its endogenous inhibitor I κ B α , the CYLD tumor suppressor, as well as A20, all of which are NF- κ B dependent [5–7]. On the other hand, positive loops exist that enhance NF- κ B transcriptional activity, such as transcription of AP-1 and ATF family members [8] or of IRF-3 dependent TNF α synthesis following LPS stimulation, leading to prolonged NF- κ B activation [9].

One important aspect of NF- κ B activation concerns its kinetics. Several reports have drawn attention to the fact that not only the magnitude, but also the kinetics of activation of a transcription factor,

including NF- κ B, can have profound effects on the expression of its target genes [10,11]. In several cell types including T cells and skeletal muscle cells, appropriate stimulation results in two or even more phases of NF- κ B nuclear translocation (waves), i.e., a first rapid translocation, then redistribution to the cytoplasm, followed by another phase of nuclear translocation [12]. Depending on the cell type and conditions, several “oscillations” can sometimes be observed. These waves and, in particular, the speed of down-regulation (dampening), is controlled by the three members of the I κ B family, I κ B α , - β , and - ϵ [13]. Thereby I κ B α controls the early, whereas I κ B β mediates the delayed phase of activation. This model was further extended by the observation of complex formation between I κ B α and MEKK3 on one hand, and between I κ B β and MEKK2 on the other hand, such that the two complexes separately control early and delayed NF- κ B activation, respectively [14]. Therefore, all three MEKKs, including MEKK1 participate, besides their regulation of e.g., AP-1, also in the NF- κ B signalling pathway [15,16].

XIAP was originally described as an anti-apoptotic protein of the IAP family [17], but has more recently been implicated in signal transduction, e.g., of the TGF- β and JNK pathways, as well as in cell cycle progression [18–20]. In regard to NF- κ B, XIAP has been shown to enhance its transcriptional activity depending on its RING finger associated ubiquitin ligase but also on its BIR1 domain [21–23], however, the underlying mechanism remained elusive. We show here that XIAP enhances NF- κ B activity specifically by augmenting the later phase of activation, a feature previously reported for MEKK2 [14]. This prompted us to investigate the relationship between the two proteins, and we show that XIAP interacts with and ubiquitinates MEKK2, thereby connecting these hitherto unrelated signaling molecules.

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2. Materials and methods

2.1. Cell culture, plasmids, and transfections

HEK293 cells were obtained from ATCC, wild-type and XIAP deficient mouse embryonic fibroblasts were kindly provided by T. Lindsten [24]. Both cell types were propagated in Dulbecco's modified Eagle's medium (Bio-Whittaker) supplemented with 10% fetal calf serum (Sigma), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM). Bone-marrow derived macrophages were isolated and propagated as described [25]. For determination of NF-κB activity, HEK293 cells were transfected with a 5×NF-κB-luc reporter plasmid (Stratagene) by the calcium phosphate method using 4 µg DNA in total. Luciferase assays were done in triplicates, and levels normalized to β-gal expression from a cotransfected ubiquitin promoter-β-gal expression vector. Human and murine TNFα were from R&D Systems and from Biosource, respectively, and used at a final concentration of 200 U/ml. Expression constructs for ubiquitin were kindly provided by D. Bohmann, K. Haglund and I. Dikic [26], and full-length MEKK1 by Ch. Widmann.

2.2. Immunofluorescence

Cells were seeded at 4×10^4 cells/well onto fibronectin-coated Lab Tek II Chamber Slides (Nunc), propagated for 2 days and then stimulated with 200 U/ml murine TNFα (Biosource) for the indicated periods of time. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunostaining for RelA was done with an anti-p65 antibody (sc-109; Santa Cruz Biotechnology; 1:500) followed by goat anti-rabbit Alexa488 (Molecular Probes) secondary reagents at a dilution of 1:5000.

2.3. Protein extraction and Western blotting

Cells were lysed in Triton-buffer (25 mM Tris-HCl pH 7.4, 200 mM NaCl, 1% Triton X-100, protease inhibitors) for 20 min on ice, and insoluble material pelleted at 15 min/13,000 rpm/4 °C. Nuclear extracts were prepared as described [27]. Proteins were separated by SDS-PAGE, transferred electrophoretically onto a PVDF-membrane (Millipore Immobilon-P), and blocked in 5% non fat dry milk in PBS/0.1% Tween-20 for 1 h at room temperature. The following first antibodies were used: α-p65 (sc-109), α-IκBα (sc-371), α-IκBβ (sc-945), α-MEKK2 (sc-1088), α-actin (sc-1616), all from Santa Cruz Biotechnology, α-Flag (F3165, Sigma), and α-XIAP (AF822, R&D Systems). A HRP-conjugated antibody (Amersham Life Technology) was used as secondary reagent, followed by chemiluminescence detection (West Pico, Pierce).

2.4. Co-immunoprecipitation

HEK293 were grown, transfected and lysed in 200 µl Triton-buffer as above. 10 µl of each supernatant were taken as input samples, the remaining supernatant filled up to final volume of 600 µl with Triton-buffer and pre-cleared for 45 min with protein A beads (GE Healthcare) at 4 °C. Immunoprecipitation was done by adding 15 µl anti-Flag matrix (A220, Sigma) for 2 h/4 °C. After extensive washing of the beads with Triton-buffer, the bound proteins were eluted by boiling in 5× Laemmli buffer and analyzed by Western blotting. For endogenous co-immunoprecipitations, an anti-XIAP antibody (AF822, R&D Systems) and Protein A/G Plus beads (Sigma) were used.

2.5. In vivo ubiquitination assay

Cells transfected with expression vectors for 6×His-ubiquitin or mutants as well as MEKKs and XIAP were lysed in a buffer containing 6 M guanidinium HCl, and the His-tagged proteins purified with nickel resins (Ni-NTA) as described [26] followed by Western analysis for MEKKs.

2.6. RNA interference

Validated siRNAs directed against XIAP, MEKK2, and control were obtained from Qiagen, sequences are given in the Supplementary data. Transfections were done using the calcium phosphate method using a final concentration of siRNAs of 50 nM.

2.7. Real-time PCR

Total RNA was isolated from cells grown in 6-well plates using the High Pure RNA isolation kit (Roche). cDNA was reverse-transcribed using the TaqMan reverse transcription kit (Applied Biosystems) with random hexamers. Real-time PCR was performed in a Roche LightCycler. mRNA expression was normalized to β2 microglobulin or PDGB. Cycling conditions were: denaturing at 95 °C for 5 min., 55 cycles of amplification with 5 s at 95 °C, 5 s at 65 °C, and 15 s at 72 °C, followed by 10 s at 95 °C, and 30 s at 70 °C. Primer sequences are given in the Supplementary data.

3. Results

3.1. XIAP is necessary for a second wave of NF-κB nuclear translocation

The stimulatory role of members of the IAP family, including XIAP, towards NF-κB has been well documented [18–22]. However, in contrast to cIAP-1 and -2, which form part of the cytoplasmic TNF receptor complex through binding to TRAF1 and -2, no such association could be demonstrated for XIAP so far [28]. In order to investigate the molecular basis of XIAP mediated NF-κB activation we focused on two aspects, first, the kinetics of induction and second, since the ubiquitin ligase activity of XIAP has been shown to be necessary for this effect [22], the identification of ubiquitination targets of XIAP.

To address the first question, we analyzed XIAP deficient mouse embryonic fibroblasts (MEF) for nuclear translocation of NF-κB p65/RelA. Immunostaining revealed two distinct phases of NF-κB translocation in wt MEF in response to TNFα, the first peaking at 15 min, and the second one starting after approx. 1 h (Fig. 1A). In XIAP^{-/-} cells, the first phase of NF-κB translocation occurred at the same time-point and even more pronounced, however, the second phase was strongly diminished. This could be confirmed by Western analysis of nuclear extracts (Fig. 1B, upper panel), and also in HUVEC transfected with siRNAs directed against XIAP (see Supplementary data, Fig. 1). In addition, we analyzed IκB levels in the corresponding cytoplasmic extracts. Whereas IκBα degradation and re-synthesis was virtually identical between wt and XIAP^{-/-} MEF, there was a tendency for higher IκBβ levels in XIAP^{-/-} cells at later times (Fig. 1B, lower panels), corroborating previous findings that IκBα controls the early, whereas IκBβ controls later phases of NF-κB activation [13,14].

In order to assay whether these differences in nuclear translocation of NF-κB also translate into biological activity, we performed reporter gene analysis. In HEK293 cells, knock-down of XIAP by an antisense construct resulted in diminished NF-κB activity (as measured by a cotransfected 5×NF-κB-luc reporter) at later, but not earlier times after TNFα stimulation (Fig. 1C). Since the ubiquitin ligase activity of XIAP has previously been shown to be necessary for the stimulatory effect towards NF-κB [22], we also used an ubiquitin ligase-deficient mutant (XIAP^{H467A}). As shown in Fig. 1D, XIAP^{H467A} could act as a dominant-negative molecule, and diminished the TNFα induced NF-κB activation at 16, but only very weakly at 4 h post stimulation. Together, these results indicate that XIAP influences the later phase of NF-κB activation by controlling a second wave of NF-κB nuclear translocation, and that its activity is dependent on the XIAP ubiquitin ligase activity in accordance with previous reports [22].

3.2. XIAP interacts with and ubiquitinates MEKK2

Consequently, we sought to identify the molecule(s) that are ubiquitinated by XIAP. Ubiquitination plays an important role in the

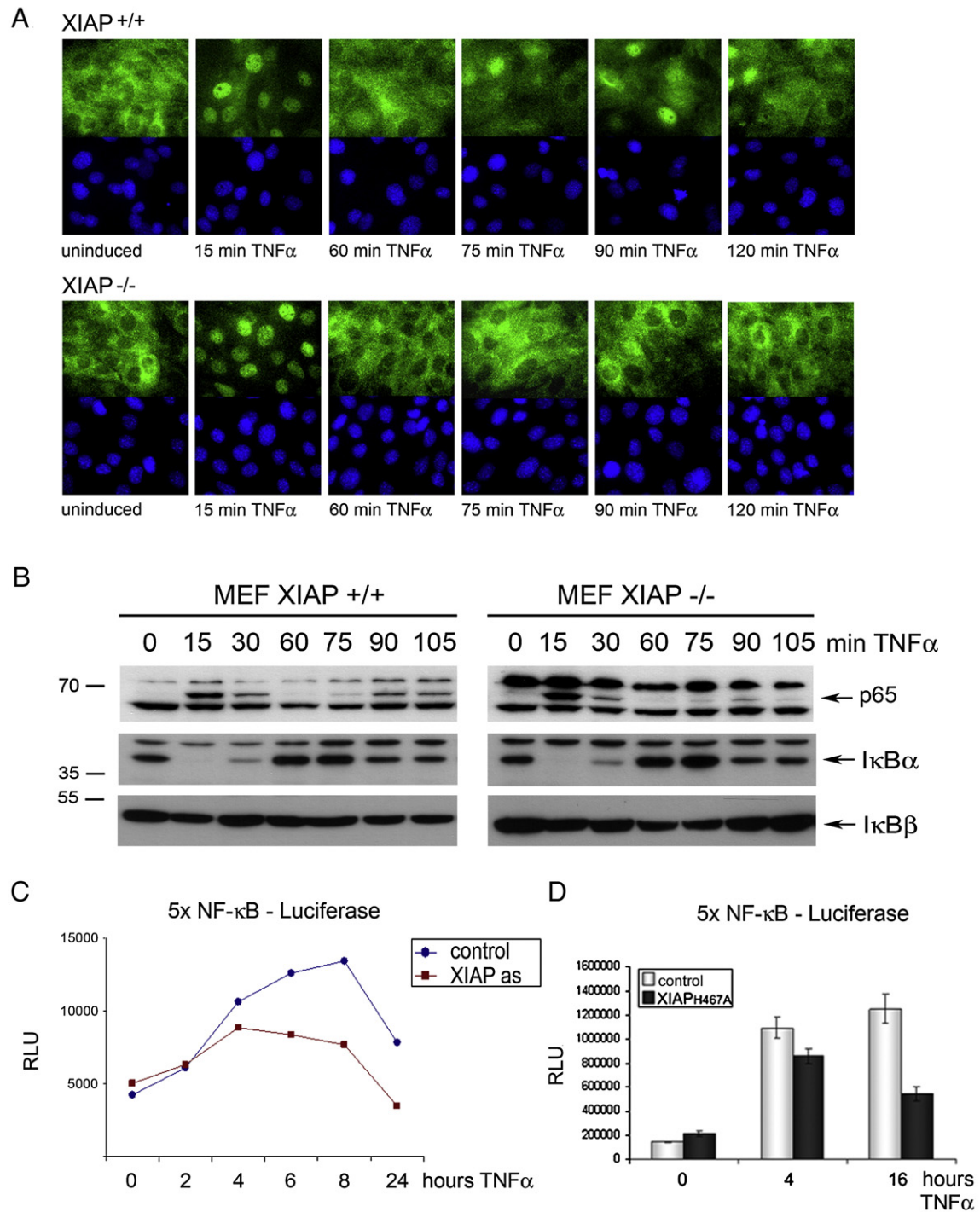


Fig. 1. XIAP prolongs NF- κ B activation by evoking a second wave of nuclear translocation. (A) wt (upper) and XIAP^{-/-} (lower panels) MEF were stimulated with TNF α , and nuclear translocation of NF- κ B determined by immunostaining with an anti-RelA antibody (green). Nuclei were stained with Hoechst 33258 (blue). Note that in wt cells, NF- κ B nuclear translocation occurs in two distinct phases, whereas in XIAP^{-/-} cells the second phase is strongly diminished. (B) Western analysis of TNF α stimulated wt and XIAP^{-/-} MEF. In the upper panel, nuclear extracts were analyzed for RelA/p65, which is indicated by an arrow. The ratio of RelA/p65 levels for the 15 and 90 min time points were determined by densitometry and are 1:0.56 for wt, and 1:0.24 for XIAP^{-/-} cells. The lower two panels show I κ B α and I κ B β (indicated by arrows) in the corresponding cytoplasmic extracts. (C) XIAP expression affects later time points of activation. HEK293 cells were transfected with an antisense XIAP or control vector and a 5 \times NF- κ B-luc reporter, and luciferase measured at the indicated times after TNF α stimulation. (D) An ubiquitin ligase-deficient XIAP mutant inhibits NF- κ B activity at later stages of activation. HEK293 cells were transfected with XIAP^{H467A} or control vector plus a 5 \times NF- κ B-luc reporter, and luciferase measured 4 and 16 h after TNF α stimulation. Error bars indicate standard deviation (SD).

NF- κ B signaling pathway, including one of the key regulatory steps, the proteasomal degradation of I κ B α following K48-linked poly-ubiquitination [3]. However, several other ubiquitination events, including addition of K63-linked chains (e.g., to TRAF2, -6, NEMO, RIP) have been reported to have a regulatory function by recruiting the respective proteins into complexes [29]. We therefore chose several cognate

components of the NF- κ B signaling pathway and tested whether they could bind to XIAP and serve as substrates for ubiquitination in an in vivo assay (see Supplementary data, Fig. 2). One of the proteins bound by XIAP was MEKK2, as first shown in co-immunoprecipitations after transient transfection (Fig. 2A). Binding of MEKK2 to XIAP^{H467A} appeared to be more robust than to the wt protein suggesting that

XIAP release from its substrate is facilitated after completion of the reaction. To further confirm the interaction on the endogenous level and to determine whether TNF α stimulation has an influence on the interaction we immunoprecipitated XIAP from wt MEF and analyzed for co-precipitated MEKK2; XIAP^{-/-} cells served as negative control (Fig. 2B). MEKK2 was found to be associated with XIAP in unstimulated cells as well as 5 min after stimulation with TNF α , but the interaction appeared to be reduced at 70 min after stimulation. This reduction was only seen in the co-precipitate, total MEKK2 levels were not affected (Fig. 2B, lowest panel). MEKK2 levels were also similar in TNF α stimulated wt and XIAP^{-/-} MEF (see Supplementary data, Fig. 3), and did not change after knock-down of XIAP in HEK293 cells (Fig. 3B). In a subsequent *in vivo* ubiquitination assay, wt XIAP but not the ubiquitin ligase-deficient mutant XIAP^{H467A} could ubiquitinate MEKK2 (Fig. 2C).

The other MEKKs (1 and 3) were not XIAP-specific ubiquitinated (Supplementary Fig. 4 online). To determine whether ubiquitination is K48 or K63 linked, we used mutant ubiquitin constructs where either of these lysines had been replaced by arginines. As shown in Fig. 2D, both mutant ubiquitin chains could be attached to MEKK2 (the quantitative differences as compared to wt ubiquitin are due to lower expression levels of the mutants). Together, these experiments demonstrate that XIAP can bind to MEKK2 (although we cannot formally demonstrate at this point that the interaction is direct, or exclude that it requires additional proteins), and that XIAP promotes K48 and K63 linked ubiquitination of MEKK2. This suggests that these modifications may be linked both to degradation (in accordance with the findings of Yang et al. showing that XIAP can interact with UbcH5 [30]) but also serve to promote the interaction with other proteins.

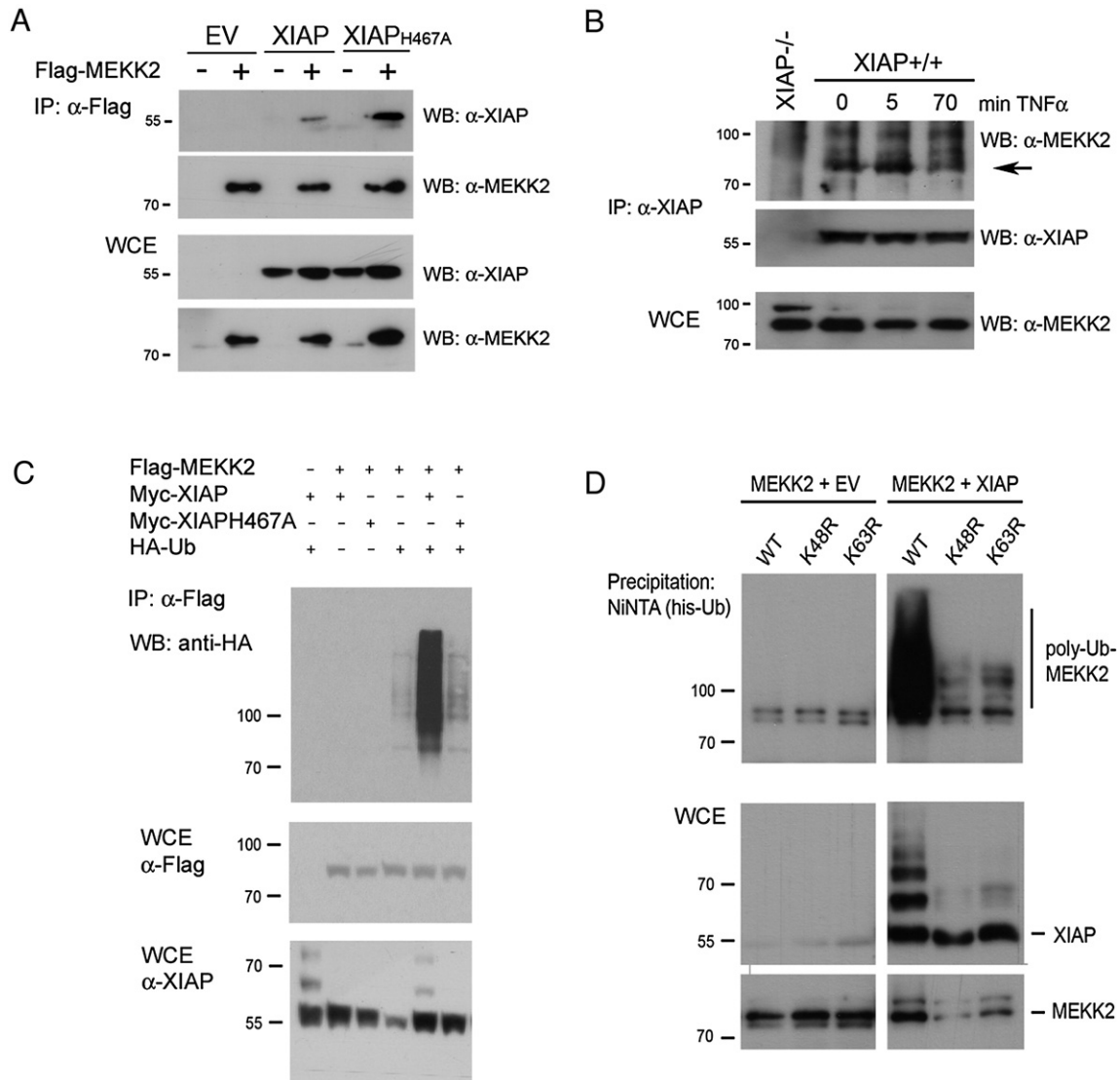


Fig. 2. XIAP interacts with and ubiquitinates MEKK2. (A) Flag-MEKK2, XIAP or the ubiquitin ligase-deficient mutant XIAP^{H467A} were transfected into HEK293 cells, MEKK2 was precipitated with anti-Flag matrix, and XIAP as well as MEKK2 in the precipitate determined by Western blotting (upper two panels). Amounts of XIAP and MEKK2 in the whole cell extracts (WCE) are shown in the lower two panels. (B) Co-immunoprecipitation of endogenous proteins. XIAP was precipitated from TNF α stimulated wt (XIAP^{+/+}) MEF extracts with an anti-XIAP antibody, and co-precipitated MEKK2 analyzed by Western blotting (upper panel, MEKK2 is indicated by an arrow). Middle panel: Western blot of XIAP in the precipitate. Lower panel: MEKK2 in WCE. The first lane shows XIAP^{-/-} MEF as control. Note that MEKK2 levels are similar in WCEs of wt and XIAP^{-/-} MEF. The nature of the additional higher molecular weight band in XIAP^{-/-} MEF is unknown. WCEs represent 5% of the amounts used for precipitations. (C) XIAP ubiquitinates MEKK2. HEK293 cells were transfected with flag-MEKK2, XIAP or XIAP^{H467A}, and HA-tagged ubiquitin, MEKK2 precipitated with an anti-flag antibody followed by Western blotting using anti-HA for detection of the attached ubiquitin chains. The lower two panels show the presence of MEKK2 and XIAP in the cell extracts (WCE). (D) Ubiquitination of MEKK2 by K63 and K48 mutated ubiquitin. Cells were transfected with MEKK2 in the presence or absence of XIAP as indicated, plus His-tagged wt and mutant ubiquitin constructs (K48R and K63R). The stronger ubiquitination by the wt ubiquitin constructs is caused by higher expression levels due to a different vector backbone. EV: empty vector. WCEs represent 2% of the total amount for C and D. Molecular weight markers in kDa are shown on the left.

In order to further support these findings on a functional level, we performed knock-down of XIAP and MEKK2, either individually, or in combination. We reasoned that, if the two proteins would function independently from each other, the effect of the combined knock-down would be expected to be additive. In contrast, no further reduction would be expected in case they were functionally linked. As shown in Fig. 3A, knock-down of XIAP diminished NF- κ B activity, as did MEKK2. Knock-down of both generated a very similar level of NF- κ B activity, suggesting that the two proteins are indeed closely linked also on a functional level. The knock-down efficiency of both proteins by the respective siRNAs is shown in Fig. 3B.

Given the crosstalk between NF- κ B and JNK [31] as well as the involvement of MEKK2 in AP-1 regulation [32], we have hypothesized that in the absence of XIAP also the latter pathway would be influenced. We and others have previously shown that ectopically expressed XIAP modulates MAP kinase (JNK and p38) activity [20,33]. However, no changes in JNK activity were observed in the *in vivo* situation [24]. Here, we found that MEKK2 can activate AP-1 in a reporter gene assay in wt MEF to a higher extent as compared to XIAP^{-/-} cells (see Supplementary data, Fig. 5), suggesting that XIAP also influences MEKK2-mediated AP-1 activity. This will be subject of further studies.

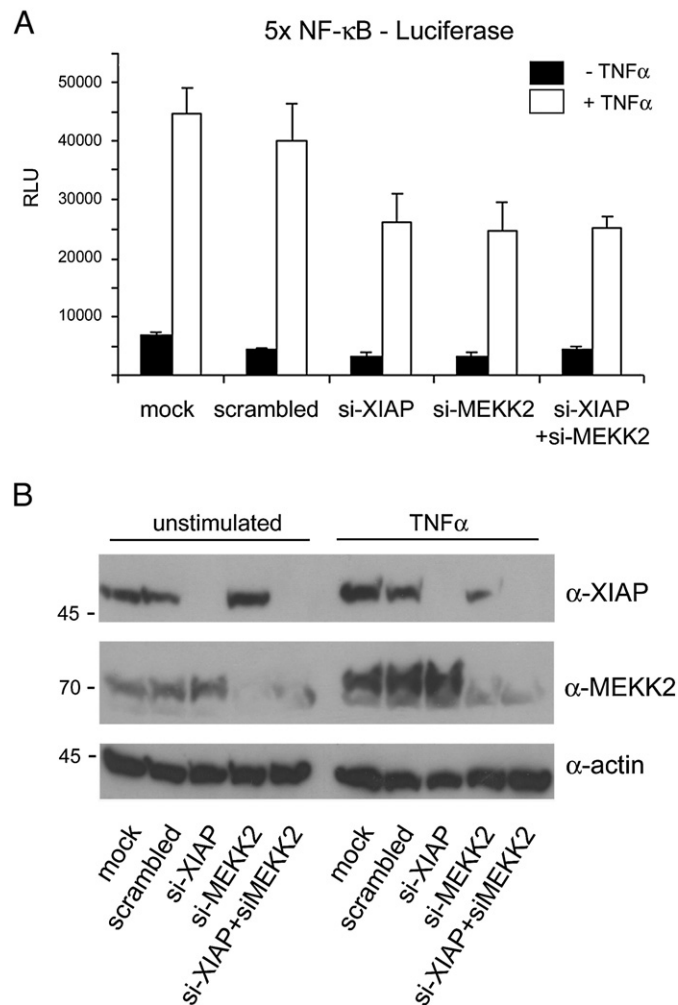


Fig. 3. Lack of synergistic NF- κ B attenuation by combined XIAP and MEKK2 knock-down indicates a close functional linkage of the two proteins. (A) HEK293 cells were transfected with siRNAs directed against either XIAP (si-XIAP) or MEKK2 (si-MEKK2) alone or in combination, a scrambled control, or mock transfected, plus a 5 \times NF- κ B-luc reporter, and 36 h later stimulated with TNF α . Experiments were done in triplicates, and luciferase was measured after 10 h. Error bars indicate SD. (B) knock-down efficiency was analyzed by Western blotting using anti-XIAP, anti-MEKK2, and anti-actin (loading control) antibodies.

3.3. XIAP dependent changes in NF- κ B kinetics result in altered gene expression

Next, we set out to investigate the biological consequences of altered NF- κ B activity. We analyzed the expression of selected NF- κ B target genes in TNF α stimulated macrophages, a cell type that plays a key role in the innate immune system. As shown by real-time PCR in Fig. 4, three types of genes could be distinguished. Group A comprises genes (IL-12 α , Gro- α) with similar kinetics but generally higher levels in XIAP wt cells at all time points, including unstimulated conditions. Genes in group B (Cox-2, IL-6) showed higher expression at later time points in wt cells but also weaker initial inducibility (e.g., at 1 h). Group C comprises genes (IL-12 β , I κ B α) displaying no significant differences between the two cell types. In addition, we analyzed a set of genes in wt and XIAP^{-/-} MEF, and also found both affected and non-affected (NF- κ B dependent) genes. Also, striking differences in IL-6 protein expression were observed in these cells (see Supplementary data, Figs. 6 and 7). This indicates that, although all genes tested are known to be NF- κ B dependent, a subset (group B) responds to the bi-phasic activation of the transcription factor with higher expression preferentially at later time points.

4. Discussion

Bi-phasic activation of NF- κ B subunits has been observed soon after the discovery of this family of transcription factors, and described in various cell types and different situations, including activation of T cells and monocytes [34,35], and I κ B β was found to be responsible for the late phase of activation [36]. More recently, genetic studies revealed that the (sometimes even multiple) phases of nucleo-cytoplasmic shuttling of the p65/RelA subunit (oscillations) can be attributed to the presence or absence of the three I κ B members - α , - β , and - ϵ [13]. Thereby, rapid degradation and NF- κ B dependent re-synthesis of I κ B α is responsible for strong negative feedback regulation of the initial phase, resulting in shut-down of NF- κ B activity and removal from the nucleus approx. 1 h after stimulation. In contrast, I κ B β and - ϵ function to dampen the NF- κ B oscillatory behavior and stabilize NF- κ B responses during longer stimulations. In extension of these findings, Schmidt et al. [14] found that members of the MEKK family regulate these two phases, in the sense that I κ B α forms a complex with MEKK3, and I κ B β with MEKK2. Therefore, the current model of NF- κ B activation suggests that the I κ B α -MEKK3 complex is responsible for the early and the I κ B β complex for the late phase of induction of the transcription factor.

When studying the previously reported stimulatory effect of XIAP on NF- κ B in more detail [21], we observed that following TNF α stimulation, XIAP deficient MEF show a greatly diminished second phase of NF- κ B activation, reminiscent of the situation after MEKK2 knock-down [14]. We therefore investigated the relationship between XIAP and MEKK2 in more detail and found that the two proteins can physically interact. Moreover, XIAP is capable of ubiquitinating MEKK2, which is in line with the previous observation of Lewis et al. [22] and ourselves that the ubiquitin ligase activity of XIAP is necessary for its effect on NF- κ B. When dissecting the nature of the XIAP mediated ubiquitination of MEKK2, we found that both K48 and K63-linked chains could be attached; this opens the intriguing possibility that K63-linked ubiquitination serves to recruit the MEKK2 containing protein complex to other structures to further transmit the signal. Such a scenario has been reported for K63 poly-ubiquitinated NEMO and RIP which are recognized by TAB2/3 and recruitment and activation of the IKK complex [37], however, in our case the nature of the protein(s) that potentially recognize ubiquitinated MEKK2 remains to be determined.

When assaying the biological consequences of altered NF- κ B inducibility in XIAP deficient cells, we observed that NF- κ B dependent genes do not respond uniformly to the altered kinetics of the transcription factor. In particular, both genes with higher expression at later time points (as it might be expected), but also with generally higher levels at all time

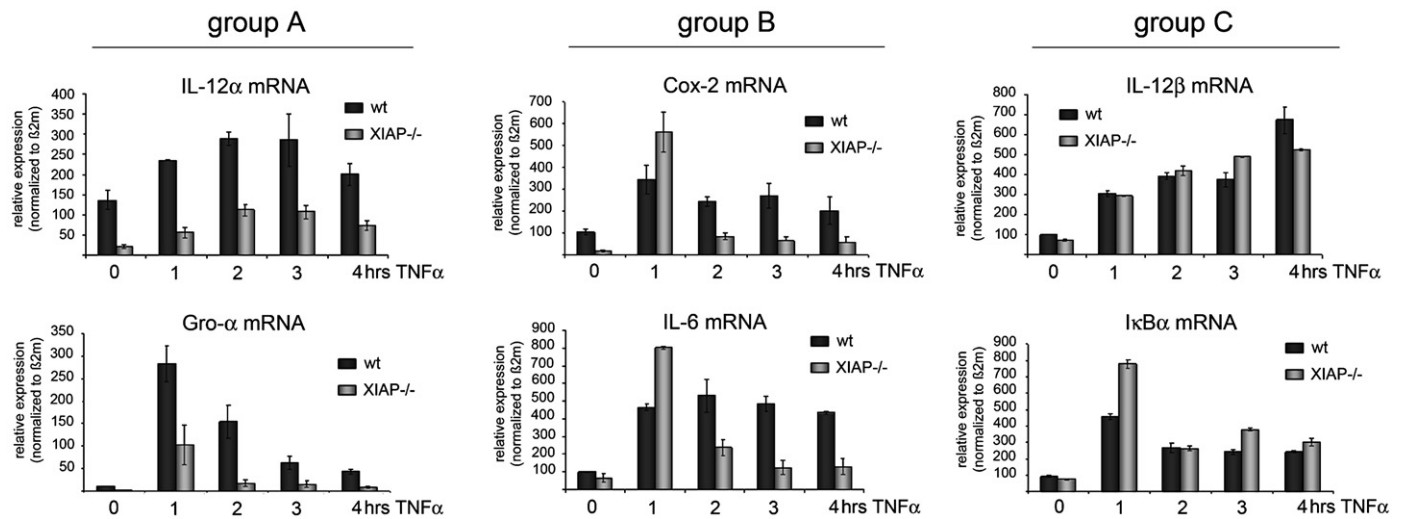


Fig. 4. Expression of NF- κ B dependent genes in wt and XIAP^{-/-} macrophages. Bone-marrow derived macrophages were isolated and differentiated from wt and XIAP^{-/-} mice, stimulated with TNF α for the indicated time points and mRNA analyzed by real-time PCR. Expression levels were normalized to β 2 microglobulin. Group A: genes with similar kinetics and generally higher expression levels; group B: genes with lower inducibility and higher expression at later time points; group C: no difference. Additional genes that were analyzed but not shown are Saa3 and iNOS (behaving like the genes in group A), and IL-18 (group C, no difference). Data are representative of two independent isolates, and error bars indicate SD.

points were found in XIAP wt cells. Also, several (NF- κ B dependent) genes did not display significant differences between the two cell types. This might be attributed to the additional influence of AP-1 family members such as JNK which appear to be influenced by XIAP as well. Their behavior may also be explained on the level of NF- κ B itself, since e.g., the number and nature of NF- κ B binding sites and their immediate versus late accessibility by NF- κ B has been shown to influence the responsiveness of different genes to this transcription factor [38].

5. Conclusion

In summary, we provide mechanistic insight how XIAP functions to regulate NF- κ B activity, namely by controlling a second wave of nuclear translocation that leads to prolonged activation. XIAP thereby interacts with MEKK2, a kinase that has been proposed to be responsible for delayed NF- κ B activation through complex formation with I κ B β [14]. XIAP ubiquitinates MEKK2 both on -K48 and K63-linked sites, opening the possibility that the attached ubiquitin chains promote the association with other factors, similar to the situation where K63-linked poly-ubiquitinated proteins are recognized by TAB2/3, resulting in the assembly of a higher order structure that facilitates the recruitment and activation of the IKK complex [37]. With the help of XIAP mediated ubiquitination, the MEKK2/I κ B β complex might undergo a similar recruitment to become activated and evoke late phase NF- κ B translocation.

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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.cellsig.2008.08.004.

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