MK2/p38/p53 negatively regulate basal IL-1β and non-canonical NF-κB signaling

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Abstract

Interleukin (IL)-1β is a pro-inflammatory cytokine implicated in sterile inflammation and tumor development. Investigating the role of MAPKAP kinase 2 (MK2) in IL-1β processing, we found that *Il1b* mRNA and IL-1β protein levels were elevated in resting *MK2*-knockout (KO) macrophages and in the serum of *MK2/3*-double-KO mice. This was linked to activation of the non-canonical NF-κB pathway in the absence of MK2 or its activator, p38α. Rescue by MK2, its kinase-inactive mutant MK2K79R, or p38α suppressed this pathway and reduced *Il1b* expression. We also observed decreased basal protein levels of tumor suppressor p53 in *MK2*- or *p38α*-deficient cells. Mechanistically, p53 interacts with mitochondrial caspase-3, promoting cleavage of RelB, thereby inhibiting non-canonical NF-κB signaling and subsequent *Il1b* and *TP53* expression. These findings explain elevated basal IL 1β levels in *MK2*-KO macrophages and uncover a new autoregulatory mechanism of *TP53* expression. Additionally, they reveal a new mechanism that contributes to the long-discussed link between cancer and inflammation, wherein the tumor suppressor p53 inhibits cytokine production in parallel.

Key words: Interleukin-1β / MK2 / non-canonical NF-κB pathway / p38α / p53

Introduction

p38 mitogen-activated protein kinases ($p38^{MAPK}$) comprise four distinct isoforms - p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12 or ERK6), and p38δ (MAPK13 or SAPK4) - that are part of highly conserved signaling cascades. They are activated by various cellular stresses, such as inflammatory cytokines, growth factors, bacterial lipopolysaccharides (LPS), osmotic stress, and ultraviolet irradiation. p38 signaling affects gene expression at the levels of transcription, mRNA stability, and translation. It also regulates survival, apoptosis, differentiation, aggregation, and migration (Young, 2013). MK2 and MK3 are the only MAPK-activated protein kinases (MKs) that are exclusively phosphorylated and activated by p38α/β. MK2 and MK3 are closely related in terms of structure and function and share the same substrates. However, MK2 is expressed and active at higher levels than MK3 in most cells and tissues (Ronkina *et al*, 2008). p38α/β binds to a docking motif in the C-terminus of MK2 and phosphorylates MK2 regulatory sites in response to stress. Consequently, MK2 and p38 are co-transported from the nucleus to the cytoplasm, where MK2 substrates are phosphorylated

(Kotlyarov *et al*, 2002). MK2 has several specific substrates, including tristetraprolin (TTP), serum response factor (SRF), heat shock proteins Hsp25/27, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), and RNA-binding motif protein 7 (RBM7). These substrates are involved in the regulation of immediate early gene responses, cytokine expression, cell death and migration (Stokoe *et al*; 1992, Ronkina *et al*, 2011; Tiedje *et al*, 2015; Tiedje *et al*, 2016; Menon *et al*, 2017). Additionally, MK2 stabilizes p38 independently of its catalytic activity through the formation of a binary protein complex (Kotlyarov *et al*, 2002).

p53 is a transcription factor that is responsible for regulating several genes involved in the cell cycle control, apoptosis, and senescence. It also exhibits transcription-factor-independent functions, particularly in the regulation of apoptosis via the mitochondrial pathway. Its medical significance is highlighted by the fact that p53 mutations are present in over 50% of human tumors (Perri *et al*, 2016). In resting cells, p53 is constitutively expressed, but becomes ubiquitinated by Mouse double minute 2 homolog (MDM2) and subsequently degraded by the proteasomes, maintaining low protein levels. Following exposure to DNA-damaging agents or other stress stimuli, p53 undergoes a series of post-translational modifications that stabilize and activate p53 by reducing its interaction with MDM2. These modifications include the phosphorylation of Ser15 and Ser37 (Liu *et al*, 2024; Shieh *et al*, 1997). The stabilization of p53 is also regulated by a broad variety of other interacting proteins. Interestingly, p38 exists in a physical complex with the tumor suppressor p53 and coexpression of p38 stabilizes p53 protein. After UV radiation, p38 phosphorylates p53 at Ser33 and Ser46. These sites are associated with p53-mediated apoptosis and are important for the subsequent phosphorylation of Ser15 and Ser37 of p53 (Bulavin *et al*, 1999).

The transcription factor NF-κB plays a crucial role in regulating the immune responses, cell proliferation and cell death. There are five known NF-κB proteins: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel. These proteins are classified into the canonical and the non-canonical NF-κB pathways. The canonical NF-κB pathway is triggered by a wide variety of receptors that activate TGF-β-activated kinase 1 (TAK1). Then, TAK1 mediates the phosphorylation of the inhibitor of NF-κB kinase (IKK) IKKβ. The activated IKK complex, composed of IKKα, IKKβ and IKKγ, phosphorylates the inhibitor of κB (IκB) IκBα and the IκB-like molecule p105, which subsequently are ubiquitinated and degraded. The cleavage product of the NF-κB1 precursor protein p105, p50, is translocated to the nucleus as a homodimer or a heterodimer together with RelA or c-Rel. There, they bind to specific DNA elements to induce the transcription of canonical target genes. In contrast, the non-canonical NF-κB pathway is activated only by a subset of TNF receptor superfamily members. Under resting conditions, NF-κB-inducing kinase (NIK) is recruited to an ubiquitin ligase complex consisting of a cellular inhibitor of apoptosis (cIAP), tumor necrosis factor (TNF)-receptor-associated factors (TRAF)-2 and TRAF-3. This leads to the constitutive degradation of NIK. After stimulation, TRAF3 is degraded, resulting in the release and stabilization of NIK. NIK then phosphorylates IKKα, which in turn phosphorylates the NF-κB2 precursor protein p100. Subsequently, the cleavage product p52 and RelB are then translocated into the nucleus, where they activate target genes of the non-canonical pathway (Sun, 2012).

Here we demonstrate, that in the absence of MK2 or p38α, basal p53 protein level is reduced and in turn, the non-canonical NF-κB pathway is activated. Subsequently, the pro-inflammatory Interleukin IL-1β coding gene *Il1b* as well as the p53 coding gene *TP53* are upregulated. Accumulation of p53 activates mitochondrial caspase-3, which cleaves RelB. RelB is a direct transcription factor of p53 (Jacque *et al*, 2013). Subsequently, the non-canonical NF-κB pathway and *TP53* transcription itself are inactivated, indicating a novel autoregulatory mechanism of p53 gene expression.

Results

MK2/3-DKO mice display elevated levels of IL-1β

To investigate whether the kinases MK2 and MK3 regulate IL-1β, the *Il1b* mRNA level was analyzed in bone marrow-derived macrophages (BMDMs) from wild-type (WT), *MK2*-KO, *MK2/3*- DKO, and *MK3*-KO mice that were either left untreated or stimulated with either IL-1α or LPS. IL-1α is usually released by dying cells, necrotic cells or stressed cells and occurs in sterile chronic diseases, while LPS is derived from bacteria. The expression and release of IL-1β typically occurs in response to two distinct signals. IL-1α or LPS serves as the first signal, resulting in the expression of *Il1b*. Interestingly, already unstimulated BMDMs of the *MK2/3*-DKO showed elevated *Il1b* mRNA levels (Fig. 1A), which increased further after IL-1α stimulation (Fig. 1B). In contrast, there was no significant difference in the *Il1b* mRNA levels in LPS-treated BMDMs of the different genotypes (Fig. 1C). Consistent with the elevated mRNA levels, *MK2/3*-DKO cells also displayed elevated IL-1β protein levels in unstimulated BMDMs, which increased further after IL-1α stimulation (Fig. 1D). Usually, a second signal, such as ATP or nigericin, is required for the release of IL-1β. Interestingly, the concentration of IL-1β in the supernatant of unstimulated and IL-1α-stimulated MK2/3-DKO-BMDMs was significantly higher than that in WT-BMDMs, even without a second stimulus (Fig. 1E). After the addition of the second stimulus, such as ATP or nigericin, *MK2/3*-DKO BMDMs pretreated with IL-1α released significantly higher levels of IL-1β compared to WT BMDMs (Fig. 1 F, G). In contrast, equal concentrations of IL-1β were released after the addition of ATP or nigericin to LPS-pretreated cells (Fig. 1H, I). Analysis of IL-1 receptor mRNA expression (*IL-1r1*, *IL-1r2*, and *IL-1rn*) revealed no differences among WT, *MK2*-KO, *MK2/3*-DKO, and *MK3*-KO cells (Suppl. 1A-C).

Since unstimulated BMDMs from *MK2/3*-DKO mice exhibit elevated *Il1b* mRNA and IL-1β protein levels and release more IL-1β than unstimulated BMDMs from WT mice, we analyzed the sera of *MK2/3*-DKO and WT mice from the same breeding to determine their basal cytokine levels. *MK2/3*-DKO serum showed significantly higher basal IL-1β levels than WT serum (Fig. 2A). However, there was no significant difference in the basal levels of TNF-α and CXCL-1 (Fig. 2 B, C).

MK2 and MK3 suppress the basal and IL-1α-induced IL-1β level in rescued immortalized *MK2*-KO BMDMs

Next the *MK2*-KO BMDMs were immortalized and transduced with either *MK2* (i*MK2*-KO+*MK2*) or with an empty vector (i*MK2*-KO+*GFP*) as control. Rescuing *MK2* suppressed the elevated *Il1b* mRNA level (Fig. 2D) to a level comparable to the WT BMDMs (Fig. 1A, 2D). In contrast, the basal *Tnf* mRNA level was unaffected in i*MK2*-KO cells, resembling the situation in WT BMDMs (Fig. 2E). IL-1α treatment increased the differences in the *Il1b* mRNA levels in i*MK2*-KO cells as well (Fig. 2F). To investigate, whether MK3 is functionally redundant with MK2, i*MK2*-KO cells were rescued with *MK3*. Indeed, MK3 also repressed the *Il1b* mRNA level in both unstimulated and IL-1α-treated cells (Fig. 2G, H), as well as the basal

IL-1β protein level (Fig. 2I). Therefore, MK2 and MK3 redundantly suppress IL-1β production in these cells.

MK2 suppresses the non-canonical NF-κB pathway

To identify the pathway activated in the absence of MK2, several inhibitors were tested. Actinomycin D, an inhibitor of RNA transcription, was used as a positive control and efficiently suppressed the basal and IL-1α-stimulated *Il1b* mRNA levels (Suppl. 2A). cFos/AP1 and TRAF6 inhibitors did not affect the *Il1b* mRNA levels in i*MK2*-KO cells (Suppl. 2B, C). Suppressing the canonical NF-κB pathway with three specific IKKβ inhibitors (Takinib, sc-514, CAY10657) did not reduce the basal *Il1b* mRNA level in i*MK2*-KO cells. However, they suppressed IL-1α- and LPS-induced *Il1b* mRNA levels (Fig. 3A, Suppl. 2D). In contrast, the simultaneous inhibition of IKKα and IKKβ with HPN-01 also inhibited the basal *Il1b* mRNA expression (Fig. 3A). These results were confirmed with BMS 345541. At low concentrations, BMS 345541 solely inhibits IKKβ, resulting in no reduction of the basal *Il1b* mRNA level. At higher concentrations, where both IKKα and IKKβ are suppressed simultaneously, the basal *Il1b* mRNA level was significantly reduced in i*MK2*-KO cells (Suppl. 2E). Consequently, the specific inhibition of the non-canonical NIK/ IKKα by B022 decreased in particular the basal *Il1b* mRNA and *Map3k14* mRNA expression in i*MK2*-KO cells (Fig. 3A, Suppl. 2F).

Interestingly, i*MK2*-KO cells displayed higher basal mRNA expression levels of *Map3k14*, the NIK-coding gene that activates the non-canonical NF-κB pathway (Fig. 3B). Additionally, i*MK2*-KO cells also showed elevated basal levels of the mRNA of non-canonical *Relb* (Fig. 3C). *Relb* and *Nfkb2* mRNA expression increased after IL-1α treatment solely in i*MK2*-KO+*GFP* cells, but not in MK2-rescued i*MK2*-KO+*MK2* cells (Fig. 3D, E). There was no significant difference in canonical *Nfkb1* and *Rela* mRNA expression (Suppl. 2G, H). Furthermore, *Traf2* and *Traf3* were analyzed, as both are known negative regulators of NIK (Grech *et al*, 2004; Sun, 2012). The basal level of *Traf2*, but not *Traf3*, mRNA was significantly reduced in i*MK2*-KO macrophages (Fig. 3F, G).

Next, we analyzed the levels of signaling proteins in the nuclear and cytoplasmic fractions of i*MK2*-KO+*GFP* and i*MK2*-KO+*MK2* BMDMs using Western blotting. Following IL-1α treatment, the nuclear fraction of the i*MK2*-KO+*GFP* cells exhibited higher levels of the non-canonical proteins RelB and NF-κB2, as well as the canonical proteins RelA and NF-κB1 (Fig. 4A). Notably, only the non-canonical NF-κB pathway proteins RelB and NF-κB2 were elevated in the nuclear and cytoplasmic fractions of unstimulated i*MK2*-KO+*GFP* cells, as compared to the same fractions of i*MK2*-KO+*MK2* cells. No differences in basal protein levels were detected among the members of the canonical NF-κB pathway: RelA, NF-κB1, and IκBα (Fig. 4A-C, Suppl.3A-F). Furthermore, whole-cell lysis revealed decreased TRAF2 protein levels in unstimulated i*MK2*-KO cells. However, the basal level of TRAF3 remained unaffected in the absence of MK2. Whole-cell lysis also confirmed an overall reduction of RelB in the presence of MK2 (Fig. 4D, E). Since TRAF2 mediates the proteasome-dependent degradation of the pro-inflammatory transcription factor c-Rel (Jin *et al*, 2015), we also analyzed c-Rel protein

expression. Indeed, i*MK2*-KO+*GFP* cells had higher c-Rel protein levels in the nucleus and cytoplasm than in i*MK2*-KO+*MK2* cells (Fig. 4F, Suppl. 3F).

RNA sequencing of the immortalized BMDMs revealed increased mRNA levels of IL-1β pathway components (e.g., *Il1b* , *Il18*, and *Nlrp3*) and of non-canoncial NF-κB pathway components (e.g., *Chuk*, *Nfkb2*, and *Relb*), as well as a wide range of non-canonical NF-κB pathway targets predicted using known transcription factor target site motifs (e.g., *Birc2*, *Birc3*, *Icam1*, and *Rel*) (Data ref: Xie *et al*, 2009; Data ref: Rouillard *et al*, 2016; Data ref: Diamant *et al*, 2024) in i*MK2*-KO+*GFP* cells (Fig. 5).

MK2-KO macrophages exhibited elevated basal mRNA and protein levels of the non-canonical NF-κB pathway components NIK (*MAP3K14*), RelB, and NF-κB2. Conversely, they displayed decreased mRNA and protein levels of TRAF2, the negative regulator of NIK. Neither the basal mRNA nor basal protein levels of canonical components RelA, NF-κB1, or IκBα are changed in the absence of MK2. In addition, inhibition of the non-canonical, but not the canonical NF-κB pathway, reduced the basal *IL1b* mRNA level in i*MK2*-KO cells. Therefore, MK2 suppresses the upregulation of *IL1b* in unstimulated cells by blocking the non-canonical NF-κB pathway.

Stabilization of the protein kinase p38α by MK2/3 is a mechanism for suppressing *Il1b*

To examine the role of MK2's catalytic activity in suppressing *Il1b*, i*MK2*-KO cells were rescued using the kinase-inactive mutant *MK2K76R*. Catalytic inactivation of MK2 did not affect the suppression of *Il1b* mRNA levels in unstimulated or IL-1α-treated cells (Fig. 6A-B). Beside of its catalytic activity, MK2 also binds and stabilizes its activator, the protein kinase p38α (Smith *et al*, 2000, Lukas *et al*, 2004, White *et al*, 2007). Indeed, i*MK2*-KO+*GFP* BMDMs express significantly less endogenous p38α protein than i*MK2*-KO+*MK2* cells (Fig. 6C, Suppl. 3G). Because the C-terminal extension of MK2 is involved in binding to p38, a mutant lacking this extension (*MK2-Δ365-386*) was transduced in i*MK2*-KO cells. Although this mutant is catalytically active, it could not restore the endogenous p38α protein level and was unable to suppress *Il1b* mRNA levels in untreated or IL-1α-treated cells (Fig. 6C-D). These findings strongly suggest that MK2's inability to stabilize p38 is the primary reason for *Il1b* upregulation.

p38α suppresses the non-canonical NF-κB pathway

Overexpression of *p38α* in i*MK2*-KO cells significantly increased basal TRAF2 levels and reduced basal RelB protein levels (Fig. 7A-C). In addition, *Traf2* mRNA increased and *Map3k14* mRNA decreased (Fig. 7D-E). Also, *Relb* and *Il1b* were significantly reduced in IL-1α-treated i*MK2*-KO cells with overexpressed *p38α* (Fig. 7F, Suppl. 4A). Furthermore, basal *Il1b* mRNA is lowered in i*MK2*-KO+*p38α* cells (Fig. 7G). We then generated two *p38α*-KO RAW 264.7 macrophage cell lines by using CRISPR/Cas9 technology, followed by single-cell sorting. Both *p38α*-KO cell lines showed increased basal mRNA levels of the non-canonical

NF-κB signaling components *Map3k14*, *Relb*, and *Nfkb2* (Fig. 7H-I, Suppl. 4B). Additionally, both *p38α*-KO cell lines exhibited significantly higher basal levels of the non-canonical NF-κB signaling proteins NF-κB2 and RelB compared to the control (Fig. 7J-K). In contrast, the protein level of the canonical NF-κB signaling protein RelA did not change in unstimulated *p38α*-KO RAW 264.7 macrophages (Suppl. 4C-D). *p38α*-KO RAW macrophages had a reduced MK2 protein level (Fig. 7J).

Overexpression of p38α in i*MK2*-KO cells decreased the protein and mRNA levels of components of the non-canonical NF-κB pathway, such as *Map3k14* (NIK) and RelB. Consistent with this finding, knocking out *p38α* in macrophages resulted in elevated levels of these components. Therefore, MK2 suppresses the non-canonical NF-κB pathway and resulting IL-1β production independently of its own kinase activity by stabilizing the protein kinase p38α.

MK2/p38α stabilize p53, which suppresses the non-canonical NF-κB pathway, *Il1b*, and its own expression

Activation of p38 MAPK has been shown to induce rapid degradation of the E3 ubiquitin-protein ligase mouse double minute 2 homolog (MDM2), thereby stabilizing its target, p53 (Park *et al*, 2016). Additionally, p38 has been demonstrated to phosphorylate the tumor suppressor p53 at residues Ser33 and Ser46 in the N-terminus. Furthermore, the co-expression of p38 stabilizes p53 because both proteins exist within the same physical complex (Bulavin *et al*, 1999). Therefore, we analyzed the protein levels of p53 and MDM2 in i*MK2*-KO cells. The basal p53 protein level was indeed significantly reduced in i*MK2*-KO cells. However, the basal MDM2 protein level remained unchanged (Fig. 8A-B, Suppl. 4E). We also analyzed the mRNA level of the p53 gene *TP53*. Unlike the reduced p53 protein levels, the *TP53* mRNA levels increased in resting i*MK2*-KO cells (Fig. 8C). This may be due to the previously described increase in RelB protein levels in i*MK2*-KO cells, since *TP53* is a direct target of RelB (Jacque et al, 2013). Consistent with this finding, *p38α*-KO macrophages exhibited reduced basal levels of p53 protein, but no significant change in MDM2 protein levels (Fig. 8D-E, Suppl. 4F). Similar to i*MK2*-KO cells, *p38α*-KO macrophages also showed elevated *TP53* transcript levels (Fig. 8F). Additionally, inhibiting p38 kinase activity in RAW cells with the specific p38 MAPK inhibitor BIRB 796 did not reduce p53 levels or increase RelB protein levels (Suppl. 4G-I). Therefore, the basal p53 protein level is stabilized by the MK2-p38-p53 protein interaction, and phosphorylation events are not required.

To investigate whether p53 also influences the non-canonical NF-κB pathway, we treated i*MK2*-KO cells with Nutlin-3. Nutlin-3 is an inhibitor that prevents MDM2 from binding to p53, thereby blocking the subsequent degradation of p53 (Vassilev *et al*, 2004). As expected, Nutlin-3 treatment resulted in p53 protein accumulation in i*MK2*-KO cells (Fig. 9A). Interestingly, Nutlin-3 also stimulated RelB protein degradation, as indicated by a weaker RelB band and the appearance of several lower-molecular-weight RelB bands (Fig. 9A-C). Similarly, Nutlin-3-treated RAW 264.7 macrophages also exhibited increased p53 protein levels and significant RelB degradation (Suppl. 5A-B). Although Nutlin-3 treatment significantly reduced

RelB protein levels, no changes in NF-κB2 or TRAF2 protein levels were observed in i*MK2*-KO cells (Fig. 9A). Additionally, *Il1b* and *TP53* mRNA levels were significantly reduced in Nutlin-3-treated i*MK2*-KO cells (Fig. 9D-E). Four hours after Nutlin-3 treatment, *Map3k14*, *Relb*, and *Nfkb2* mRNA levels were also significantly reduced, though *Traf2* levels were not (Suppl. 5C-F). Therefore, p53 suppresses the non-canonical NF-κB pathway by degrading RelB, thereby inhibiting basal *Il1b* and *TP53* expression.

p53 activates mitochondrial caspase-3, which induces RelB degradation

Since RelB can be cleaved by caspase-3 (Kuboki *et al*, 2015; Wu *et al*, 2022), we analyzed the level of this protease in i*MK2*-KO cells. Following Nutlin-3 treatment, i*MK2*-KO cells exhibited decreased levels of full-length caspase-3 protein (procaspase-3) (Fig. 10A-B). Furthermore, the caspase-3-specific inhibitor zDEVDfmk and the pan-caspase inhibitor zVADfmk inhibited Nutlin-3-induced RelB cleavage in i*MK2*-KO macrophages (Fig. 10C-D). Because p53 activates caspases by releasing of mitochondrial cytochrome c (Schuler *et al*, 2000), i*MK2*-KO cells were pre-incubated with either Pifithrin μ (a specific inhibitor of mitochondrial p53 translocation), or Pifithrin α (a p53-specific transcriptional inhibitor). Pifithrin μ, but not Pifithrin α, reduced Nutlin-3-induced RelB cleavage (Fig. 10E-F). Therefore, p53 activates caspase-3 via the mitochondrial pathway, which cleaves the non-canonical RelB protein.

Discussion

IL-1β plays an important role in maintaining human homeostasis by regulating nutrition, sleep, and temperature (Dinarello, 1996). However, it is also a potent endogenous pro-inflammatory pyrogen that influences the innate and adaptive immune systems. Prolonged, uncontrolled release of IL-1β causes the pathological effects of chronic inflammation. It is involved in the pathogenesis of various diseases, including arthritis, gout, Alzheimer's disease, diabetes mellitus, obesity, atherosclerosis, and tumor growth (Grebe *et al*, 2018; Guo *et al*, 2015; Menu & Vince, 2011; Strowig *et al*, 2012). The expression of the inactive pro-IL-1β precursor is usually triggered when pattern recognition receptors, such as Toll-like or NOD-like receptors, are stimulated by microbial or viral molecules (Brennan & Bowie, 2010, Kawai & Akira, 2011). However, IL-1β can also stimulate its own expression. The main subunits responsible for activating the NF-κB binding site in the *Il1b* promoter region in response to phorbol 12-myristate 13-acetate (PMA) or LPS stimulation are key components of the canonical NF-κB pathway, including NF-κB1 (p50), RelA (p65), and c-Rel (Hiscott *et al*, 1993; Cogswell *et al*, 1994; Zhang *et al*, 2008). Nevertheless, RelB competes with RelA for binding to κB sites of NF-κB-regulated promoters in dendritic cells (Navarro *et al*, 2025). Additionally, increased *Il1b* levels are detected in *Relb*-KO fibroblasts (Xia *et al*, 1999). In both cases, RelB suppresses LPS-induced inflammatory gene expression. Accordingly, RelB has been shown to prevent RelA from binding to the *Il1b* promoter in LPS-tolerized cells by forming facultative

heterochromatin, which silences *Il1b* transcription (Yoza *et al*, 2006; Chen *et al*, 2009; Yoza & McCall, 2011).

In this study, we demonstrate the activation of *Il1b* transcription via the non-canonical NF-κB pathway in resting i*MK2*-KO macrophages. The non-canonical NF-κB pathway is upregulated in these cells, since basal TRAF2 expression is reduced while basal *Map3k14* (NIK), RelB and NF-κB2 expressions are increased. However, components of the canonical pathway, such as RelA and NF-κB1, remain unaffected. Additionally, mRNA analysis of unstimulated i*MK2*-KO macrophages revealed elevated levels of the targets of the non-canonical NF-κB pathway, including *Il1b*. Inhibiting the non-canonical NF-κB pathway significantly reduced *Il1b* in resting i*MK2*-KO cells, but not in LPS-stimulated cells. In contrast, inhibiting the canonical NF-κB pathway did not lower the basal *Il1b* level in i*MK2*-KO cells, but LPS-induced *Il1b* levels were reduced. These results confirm that LPS activates the canonical NF-κB pathway and *Il1b* transcription. However, the results also reveal an as-yet-unknown activation of *Il1b* transcription via the non-canonical NF-κB pathway in quiescent i*MK2*-KO cells. Similar activation of the *Il1b* promoter, independent of the canonical NF-κB pathway, has only been described for STAT3 so far (Wan *et al*, 2015). Additionally, STAT3 has been shown to activate the non-canonical NF-κB pathway (Nadiminty *et al*, 2006; Jiang *et al*, 2018; Yu *et al*, 2015). Furthermore, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to induce *Il1b* promoter activity in a RelB-dependent manner. However, the underlying molecular mechanisms remain unclear (Vogel *et al*, 2019).

We demonstrate that the activation of the non-canonical NF-κB pathway in resting i*MK2*-KO cells is caused by the destabilization of the p38 and p53 proteins. Accordingly, increased basal levels of non-canonical *Map3k14* (NIK), RelB, and NF-κB2 were detected in *p38α*-KO RAW cells. Levels of the p53 protein were significantly reduced in both, immortalized macrophages derived from *MK2*-KO mice, as well as in CRISPR-edited *p38α*-KO RAW 264.7 cells. MK2 is known to phosphorylate MDM2, leading to reduced p53 protein levels. In *MK2*-KO fibroblasts, p53 protein levels increase following exposure to UV radiation or treatment with anisomycin (Weber *et al*, 2005). These stimuli activate various protein kinases besides p38 and MK2 (Hazzalin *et al*, 1996). Another study found that skin biopsies of *MK2*-KO mice revealed increased p53 protein levels, as well as reduced *Il1b* mRNA and IL-1β protein levels after treatment with 7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA). Additionally, MDM2 phosphorylation was reduced in *MK2*-KO keratinocytes *in vitro* (Johansen *et al*, 2009). While these studies analyzed different cell types (e.g., fibroblasts and keratinocytes) and used different stimuli (e.g., anisomycin or UV radiation), we examined basal levels of IL-1β and p53 in unstimulated *MK2*- or *p38α*-KO macrophages. This explains the opposing effects induced by protein-protein stabilization versus effects dependent on the kinase activity of MK2/p38. Indeed, p38 and p53 are located in the same physical complex, and co-expression of p38 stabilizes p53 (Bulavin *et al*, 1999). On the other hand, p53-stabilizing effects that depend on p38 kinase activity have also been described. In a cell line that is resistant to the chemotherapeutic agent paclitaxel, constitutively activated p38 induces the rapid degradation of MDM2, thereby stabilizing p53 (Park *et al*, 2016). Additionally, p38 phosphorylates p53 at Ser33 and Ser46 following UV exposure, which may contribute to p53 stabilization (Bulavin *et al*, 1999). In conclusion, opposing effects in the stabilization of p53 by

MK2 or p38 have been described, highlighting the complexity and cell-type specificity of p53 regulation.

Adding Nutlin-3 to cells inhibits MDM2 and the subsequent degradation of p53. Nutlin-3 treatment also inhibits the non-canonical NF-κB pathway by degrading RelB in i*MK2*-KO cells and RAW macrophages. RelB is a direct transcription factor of *TP53* (Jacque *et al*, 2013). Indeed, Nutlin-3 treatment also reduced *TP53* mRNA and *Il1b* mRNA. The tumor protein p53 activates caspase-9/-3 via the release of mitochondrial cytochrome c (Drakos *et al*, 2007; Wu & Ding, 2002; Li *et al*, 1997). In addition, activated caspase-3 can cleave RelB (Kuboki *et al*, 2015; Wu *et al*, 2022). Indeed, Nutlin-3 treatment reduces the levels of full-length pro-caspase-3. Inhibiting the p53 mitochondrial pathway or caspase-3 itself prevents RelB cleavage in i*MK2*-KO cells following Nutlin-3 treatment.

After activation, the non-canonical NF-κB pathway has been shown to regulate the expression of its own components. RelB can bind to its own promoter NF-κB sites, thereby autoregulating *Relb* mRNA levels (Bren *et al*, 2001). Furthermore, RelB has been shown to repress *Traf2* expression (Ranuncolo *et al*, 2012). This repression prevents subsequent NIK degradation, which in turn activates the non-canonical NF-κB pathway. Interestingly, decreased levels of the p53 protein and activation of caspase-3 were observed in *Traf2*-deficient cells due to activated JNK signaling (Tsuchida *et al*, 2020). In addition to RelB, NF-κB2 is also positively autoregulated (Liptay *et al*, 1994). To our knowledge, no study has yet been published regarding the positive regulation of RelB or NF-κB2 with respect to their binding to the NIK promoter region or *Map3k14* (NIK) expression. This study describes the significantly reduced *Map3k14* mRNA expression in i*MK2*-KO cells after inhibition of the non-canonical NF-κB pathway. This finding suggests that the non-canonical NF-κB pathway indirectly regulates *Map3k14* mRNA levels. It was shown that p53 suppresses the mRNA expression of *Map3k14* through the miRNA pathway. Knockdown of the tumor suppressor p53 stabilizes NIK, whereas accumulation of p53 protein decreases endogenous NIK protein (Jang *et al*, 2020). The regulation of NIK transcription by p53 could explain our results, as we observed decreased basal p53 protein levels and increased basal *Map3k14* mRNA expression in i*MK2*-KO and *p38α*-KO macrophages. Additionally, we measured significantly reduced Map3k14 mRNA levels in Nutlin-3-treated RAW cells with p53 accumulation.

Here we show for the first time, that p53 negatively regulates the non-canonical NF-κB pathway via caspase-3-dependent degradation of RelB. In i*MK2*-KO and *p38α*-KO macrophages, accompanied by reduced p53 protein levels, the non-canonical NF-κB pathway is activated. This pathway increases the expression of its target genes, including components of the non-canonical NF-κB pathway itself, as well as *Il1b* and *TP53*. Overexpression of *p38α* in i*MK2*-KO cells rescued the protein levels of the non-canonical NF-κB pathway components TRAF2 and RelB. Treatment with Nutlin-3 induced p53 protein accumulation and blocked the non-canonical NF-κB pathway by cleaving RelB with caspase-3. Subsequently, the expression of the targets of the non-canonical NF-κB pathway as well as *Il1b* and *TP53* mRNA, decreased. Thus, p53 can downregulate its own *TP53* expression. This reveals a new autoregulatory mechanism of p53 protein and *TP53* mRNA levels via RelB degradation. Most importantly, these results uncover a new mechanism contributing to the long-discussed link between cancer

and inflammation, in which the tumor suppressor p53 represses cytokine expression. Consequently, the loss of p53 would lead to oncogenic transformation, increased basal cytokine levels and chronic inflammation confirming Virchow's classical observation that inflammation and neoplasia occur coincidentally.

Methods

Cell lines and growth conditions

The wild-type C57BL/6 and MK2-, MK3-, and MK2/3-knockout strains (Kotlyarov *et al*, 1999; Zaru *et al*, 2007) were maintained under specific pathogen-free conditions at the Hannover Medical School animal facility. All animal experiments were approved by the appropriate institutional and state animal welfare committees. Procedures were conducted in accordance with local animal use and care committee guidelines, as well as national animal welfare laws. Both male and female adult mice (ages 7 weeks to 9 months) were used. Blood was collected postmortem from the vena cava and heart. After 30 min of clotting, the serum was collected by centrifugation (1500xg, 10 min, 4°C) and stored at -20°C. Bone marrow cells were isolated and differentiated into bone marrow-derived macrophages (BMDMs) in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific), penicillin/streptomycin (Capricorn Scientific), Gentamycin (10 µg/ml) / Amphotericin B (0,25 µg/ml) solution (CELLnTEC), MEM non-essential amino acids (Gibco), and rM-CSF (80 ng/ml, Wyeth, Boston, MA) for 8 days in a humidified incubator at 37°C and 5% CO_2. The cells were harvested by incubating them for 30 min with non-enzymatic cell dissociation solution (C5789, Sigma-Aldrich), followed by scraping, washing and counting. Experiments were performed the next day.

p38α-KO cells were generated by transfecting RAW 264.7 cells (*M. musculus*, CVCL_0493) with either the *p38α MAPK14* Double Nickase Plasmid (sc-424051-NIC) or the Control Double Nickase Plasmid (sc-437281), according to the Santa Cruz Biotechnology protocol. Then, the cells were sorted by single-cell sorting of GFP-positive cells.

Immortalized MK2-KO (iMK2-KO) macrophages (*M. musculus*) were rescued via viral transduction with an empty vector (pMMP-IRES-GFP), MK3, MK2, a kinase-inactive MK2 mutant (MK2K79R) (Ehlting *et al*, 2011; Ronkina *et al*, 2011), or a mutant lacking an NcoI recognition site and the MK2 C-terminus pMMP-IRES-*MK2-K357R-Δ365-386* (termed as *MK2-Δ365-386)*. The cells were then sorted by the GFP-positive signal. p38α overexpression in iMK2-KO cells was achieved via viral transduction using either an empty pMSC vector (control), or a pMSC vector containing p38α. The cells were selected using 100 µg/mL of hygromycin (Roth) for 9 days. Amphotropic retroviruses were generated by transfecting the 293 GPG packaging cell line (*Homo sapiens*, Ory *et al*, 1996) with the indicated vectors. The RAW 264.7 and iMK2-KO cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin.

RNA purification and analysis

BMDM ($5x10^5$ cells/well), i*MK2*-KO or RAW 264.7 cells (both $2x10^5$ cells/well) were seeded, and treated one day later with the indicated concentrations and durations of recombinant murine IL-1α (211-11A, Peprotech), LPS (Escherichia coli 0127:B8; Sigma-Aldrich), Actinomycin D (Cayman Chemicals), B022 (MedChemExpress), BMS 345541 (Axon Medchem), CAY10657 (Cayman Chemicals), SML1160 (Sigma-Aldrich), IKK inhibitor XII (HPN-01, Sigma Aldrich), sc-514 (Cayman Chemicals), T-5224 (Cayman Chemicals), Takinib (MedChemExpress) or Nutlin-3 (Santa Cruz Biotechnology).

RNA was extracted using TRIzol Reagent (Invitrogen) and 0.5 µg RNA (according to the NanoDrop ND-1000 spectrophotometer, peqlab Biotechnologie) was converted to cDNA via reverse transcriptase PCR with Biometra TRIO (analytik jena), contained RevertAid Reverse Transcriptase (EP0442), Oligo(dT)18 Primer (SO132), RiboLock RNase Inhibitor (EO0381) (all from Thermo Fisher Scientific), and Deoxynucleotide (dNTP) Solution Mix (N0447S, New England Biolabs). The cDNA was diluted 1:10, and quantitative PCR was performed using a Rotor-Gene Q (Quiagen), qPCRBIO SyGreen® Mix Separate-ROX (PCR Biosystems), along with the following mouse primers: Gapdh fw 5'-CAT GGC CTT CCG TGT TCC TA-3' , rev 5'-CCT GCT TCA CCA CCT TCT TGA T-3'; Il1b fw 5’-GTG ATA TTC TCC ATG AGC TTT G-3’, rev 5’-TCT TCT TTG GGT ATT GCT TG-3’; Tnf fw 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', rev 5'-TGG GAG TAG ACA AGG TAC AAC CC-3'; Relb fw 5’-TAC GAC AAG AAG TCC ACC-3’, rev 5’-TTT TTG CAC CTT GTC ACA G-3’; Nfkb2 fw 5’-CTT CAG ATT TCG ATA TGG CTG-3’, rev 5’-AGT TAC AGA TCT TGA CAG TAG G-3’; Rela fw 5’-GTA TCC ATA GCT TCC AGA AC-3’, rev 5’-GAA AGG GGT TAT TGT TGG TC-3’; Nfkb1 fw 5’-TGT ATT AGG GGC TAT AAT CCT G-3’, rev 5’-ATG ATC TCC TTC TCT CTG TC-3’; Traf2 fw 5’-GAC CTT GAA AGA ATA CGA GAG-3’, rev 5’CAG ACC TCA TAG TGT ACC TC-3’; Traf3 fw 5’-GAC TCT TCT AAG GAG TGA GG-3’, rev 5’-TGG ATG CTC TTG TTT TTC TC-3’; TP53 fw 5’-TAG GTA GCG ACT ACA GTT AG-3’, rev 5’-GGA TAT CTT CTG GAG GAA GTA G-3’; Il1r1 fw 5’-ACA ATT GTA TGC TGT GTG TG-3’, rev 5’-CGT ATG TCT TTC CAT CTG AAG; il1r2 fw 5’-ACC AGT ACT CAG AGA ATG ATG-3’, rev 5’-AAG ATG ATC AGA GAC AGA GG-3’; Il1rn fw 5’-CAG AAG ACC TTT TAC CTG AG-3’, rev 5’-GGC ACC ATG TCT ATC TTT TC-3’; 18S fw 5'-GTA ACC CGT TGA ACC CCA TT-3', rev 5'-CCA TCC AAT CGG TAG TAG CG-3'; Map3k14 fw 5'-GCT TAC TGA GAA ACT CAA GC -3', rev 5'-CTA GTT CCT CTA CCC GAA AC -3'. The primers were purchased from either Eurofins or Sigma-Aldrich. The results were obtained using the ΔCT method with normalized threshold values relative to *Gapdh* or *18S*. Significances were calculated and graphs were generated using GraphPad Prism.

Additionally, the Research Core Unit Genomics at Hannover Medical School performed 1x 75 bp single-read RNA sequencing of total RNA samples after ribo-depletion using an Illumina NextSeq 550. Heatmaps were generated using Qlucore Omics Explorer 3.9.

Protein analysis via immunoblotting

BMDM ($5x10^5$ cells/well), i*MK2*-KO ($6x10^5$ cells/well), or RAW 264.7 cells ($1x10^5$ cells/well) were seeded for whole-cell lysis. The next day, the cells were treated with IL-1α, Nutlin-3 (sc-45061, Santa Cruz Biotechnology (SCBT)), zDEVDfmk (HY-12466, Hycultec), zVADfmk (4026865, Bachem), Pifithrin μ (HY-10940, Hycultec), or Pifithrin α (506132, Sigma-Aldrich) as indicated, and then lysed using kinase lysis buffer supplemented with protease inhibitors (B14001, Bimake) and phosphatase inhibitors (B15001, Bimake).

To isolate nuclear and cytoplasmic proteins, rescued i*MK2*-KO ($2x10^6$ cells/plate) were seeded and stimulated the next day as indicated. Cyclohexamide (40 µg/ml; Sigma Aldrich), DMSO (Chemsolute) or BIRB 796 (axon medchem) were incubated for 5h prior to nuclear protein extraction. NE-PE Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Scientific) were used for protein isolation and the Pierce BCA Protein Assay Kit (23227, Thermo Scientific) was used to determine protein concentration according to the provided protocols.

20 – 40 µg of protein were mixed with 4× Laemmli's SDS sample buffer, heated at 95°C for 5 min, separated by SDS-PAGE on 7.5% to 16% gradient gels with a PageRuler Prestained Protein Ladder (26616, Thermo Fisher Scientific) and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare) via semidry blotting. The primary antibodies GAPDH (MAB374, Merck Millipore), IκBa (9242, Cell Signaling Technology (CST)), MDM2 (D-7) (sc-13161, SCBT), EF2 (C-9) (sc-166415, SCBT), NF-κB1 p105/p50 (D7H5M) (12540, CST), NF-κB2 p100/p52 (4882, CST), NF-κB p65 (L8F6) (6956, CST), RelB (C1E4) (4922, CST), p53 (1C12) (2524, CST), c-Rel (D4Y6M) (12707, CST), Mouse IL-1β /IL-1F2 (AF-401-SP, R&D systems), Histone H3 (9715, CST), NIK (4994, CST), TRAF2 (C192) (4724, CST), TRAF-3 Isoform 2 (MAB3278, R&D Systems), MAPKAPK-2 (D1E11) (12155, CST), caspase-3 (H-277) (sc-7148, SCBT), or p38α Antikörper (C-20) (sc-535, SCBT) were incubated overnight at 4°C, followed by a 2h incubation with secondary horseradish peroxidase-conjugated antibodies (SCBT) at room temperature. The chemiluminescence (ECL) detection solution (solution A, 1.2 mM luminol in 0.1 M Tris-HCl [pH 8.6]; solution B, 6.7 mM p-coumaric acid in dimethyl sulfoxide; 35% H_2O_2 solution; ratio, 3333:333:1) and the LAS-3000 luminescent image analyzer (Fujifilm) with the associated Image Reader LAS-3000 software were used for detection. The bands were analyzed using Fiji software. Significances were calculated and graphs were generated using GraphPad Prism. Microsoft PowerPoint was used to crop and arrange the Western blots, and to draw the overview image.

Cytokine measurements

BMDMs ($1x10^{\wedge 5}$ cells/well) were stimulated with IL-1α, LPS, ATP (tlrl-atpl, Invivogen), or Nigericin (11437, Cayman Chemicals) as indicated. The Mouse IL-1β uncoated ELISA Kit (88-7013-22, Invitrogen) was used to determine the IL-1β concentration in the supernatant. Absorbance was measured using a Cytation 1 instrument (BioTek). Serum of wild-type or *MK2/3* double knockout mice from the same breeding were analyzed for basal IL-1β, TNF-α and CXCL1 using the Fireplex-96 Inflammation (Mouse) Immunoassay panel (ab235659,

Abcam), and detected using a BD Accuri C6 flow cytometer. The data were analyzed using BD Accuri C6 and GraphPad Prism software.

Quantification and statistical analysis

The number of animals used and the number of times the experiments were repeated are indicated in the figure legends or shown via dot plots. Representative Western blots are depicted. Biological replicates, not technical replicates, are shown. All experiments were non-randomized. The sample size was not determined in advance. The investigators were not blinded. Statistical analyses were performed using Prism, with either a two-sided paired or unpaired t-test, or two-way repeated measures (2W-RM) ANOVA with Bonferroni post-tests. An F-test was used to calculate variance. Samples were excluded from the analysis, if the housekeeping gene (qRNA) or the control protein (Western blot) was a significant outlier, as determined using the GraphPad outlier calculator. The statistical details for each experiment, including the tests performed and the significance values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) are provided in the figure legends and figures. Error bars represent the standard error of the mean (SEM). DeepL was used to improve the style and quality of the texts created by the authors. The abstract was shortened using ChatGPT.

Data availability

All data is available in the source data in the following database record: X-not-provided-yet-X. The RNA-seq data generated in this study are available in the Gene Expression Omnibus (GEO) database under the accession code GSE299040. Any unique materials generated in this study can be requested from the corresponding authors.

Author contributions

Sarah M. Herr: Conceptualization; Investigation; Methodology; Formal analysis; Visualization; Funding acquisition; Writing - original draft. **Diana Stalkopf, Sofie Padaszus, Lukas A. Herbst, Anneke Dörrie, Rainer Niedenthal, Natalia Ronkina, Tatiana Yakovleva:** Investigation. **Alexey Kotlyarov:** Project administration; Supervision. **Matthias Gaestel:** Funding acquisition; Writing - original draft; Project administration, Supervision.

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Disclosure and competing interests statement

The authors state they have no competing interests or disclosures.

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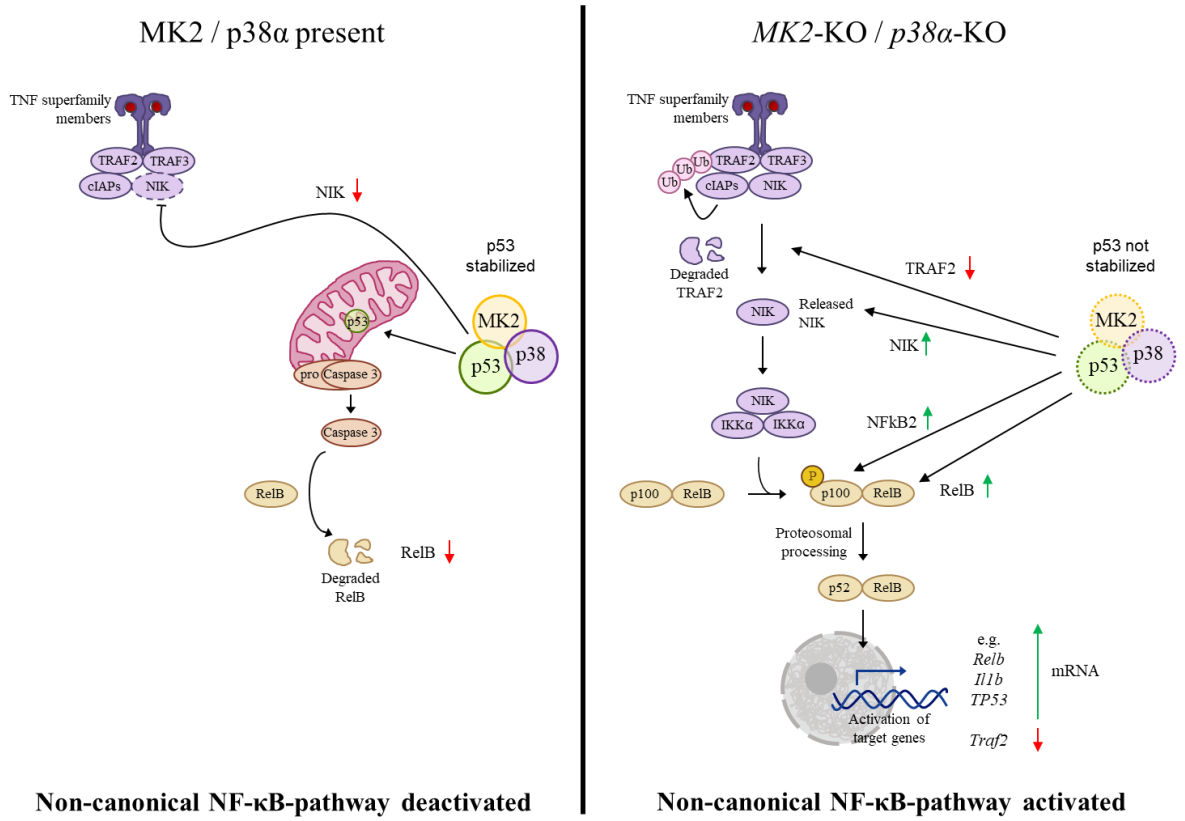
Synopsis:



The tumor suppressor p53 is known to negatively regulate the non-canonical NF-κB pathway by suppressing the expression of *Map3k14* (NIK) mRNA. This study identifies a novel mechanism by which p53 inhibits the non-canonical NF-κB pathway in macrophages: p53 activates caspase-3, which subsequently cleaves RelB. Absence of the protein kinase complex MK2/p38α destabilizes p53, leading to the basal activation of the non-canonical NF-κB pathway.

- The protein kinases MK2 and p38α stabilize the level of the p53 protein in macrophages.
- p53 activates mitochondrial caspase-3, which cleaves the non-canonical NF-κB component RelB.
- Resting *MK2*-KO and *p38α*-KO macrophages display reduced basal levels of p53 and increased activation of the non-canonical NF-κB pathway.
- In resting *MK2*-KO cells, the non-canonical NF-κB pathway suppresses TRAF2 expression and induces RelB transcription, as well as that of the cytokine IL-1β and p53 itself.

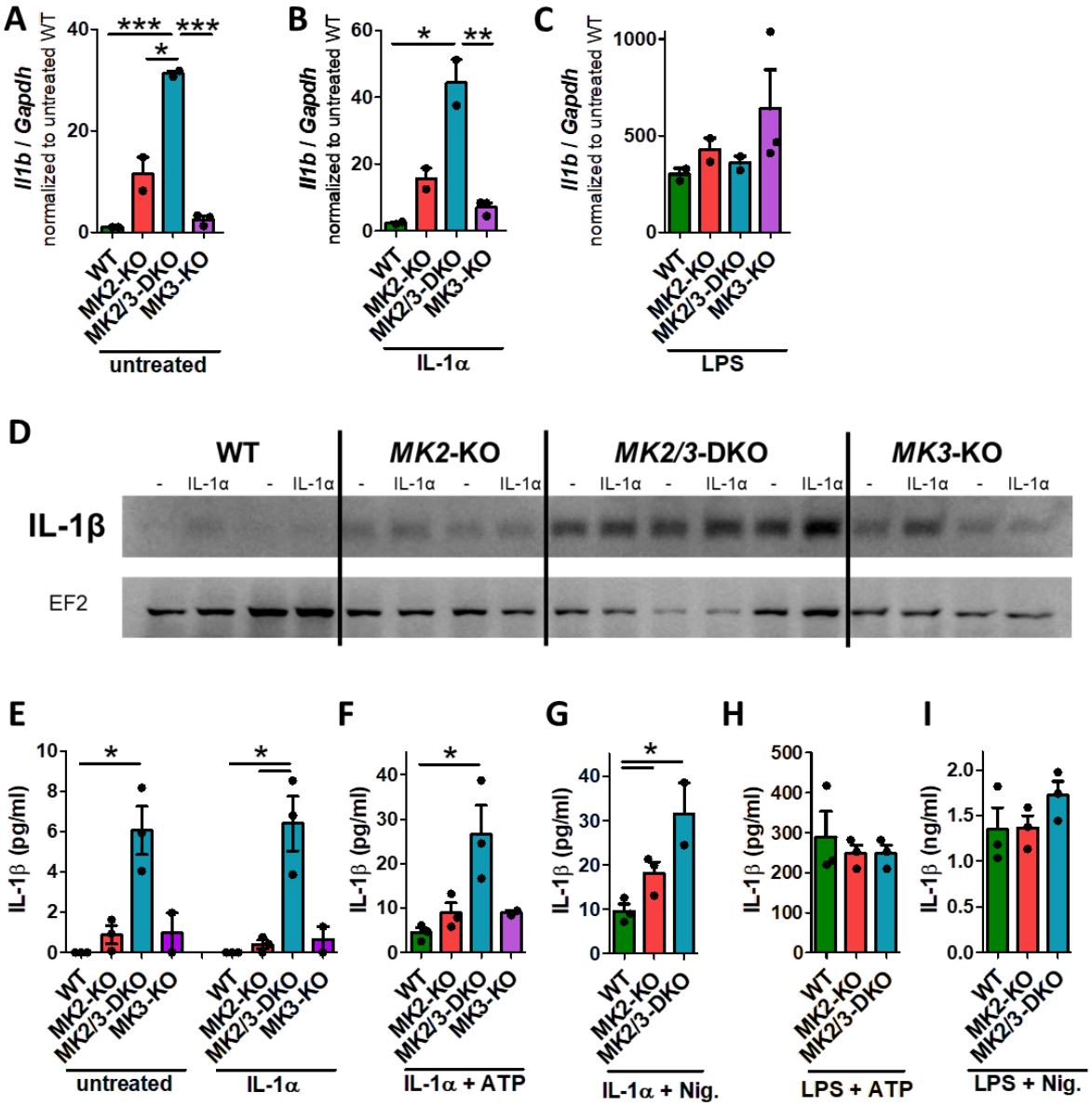


Figure 1: Interleukin (IL)-1β are elevated in *MK2/3* double knockout (DKO) mice

Il1b mRNA levels are increased in **(A)** untreated and **(B)** IL-1α-treated (5 ng/ml, 1h) *MK2/3*-DKO bone marrow-derived macrophages (BMDMs) compared to wild type (WT), but not in **(C)** LPS-treated (100 ng/ml, 1h) BMDMs. **(D)** Basal and IL-1α-stimulated (5 ng/ml, 2h) IL-1β protein levels are elevated in *MK2/3*-DKO BMDM. Elongation factor 2 (EF2) serves as a control. **(E)** The concentration of IL-1β is higher in the supernatant of untreated and IL-1α-treated (5ng/ml, 4h) *MK2/3*-DKO BMDM than in WT, also when **(F)** ATP (5 mM, 5.5h) or **(G)** Nigericin (20 µM, 8h) is added to IL-1α. The IL-1β concentration in LPS-treated (1 µg/ml, 2h) *MK2/3*-DKO BMDM is similar to that in WT BMDM after the addition of **(H)** ATP (3 mM, 8h) or **(I)** Nigericin (20 µM, 8h). N=2-3 mice/group, mean ± SEM, students t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

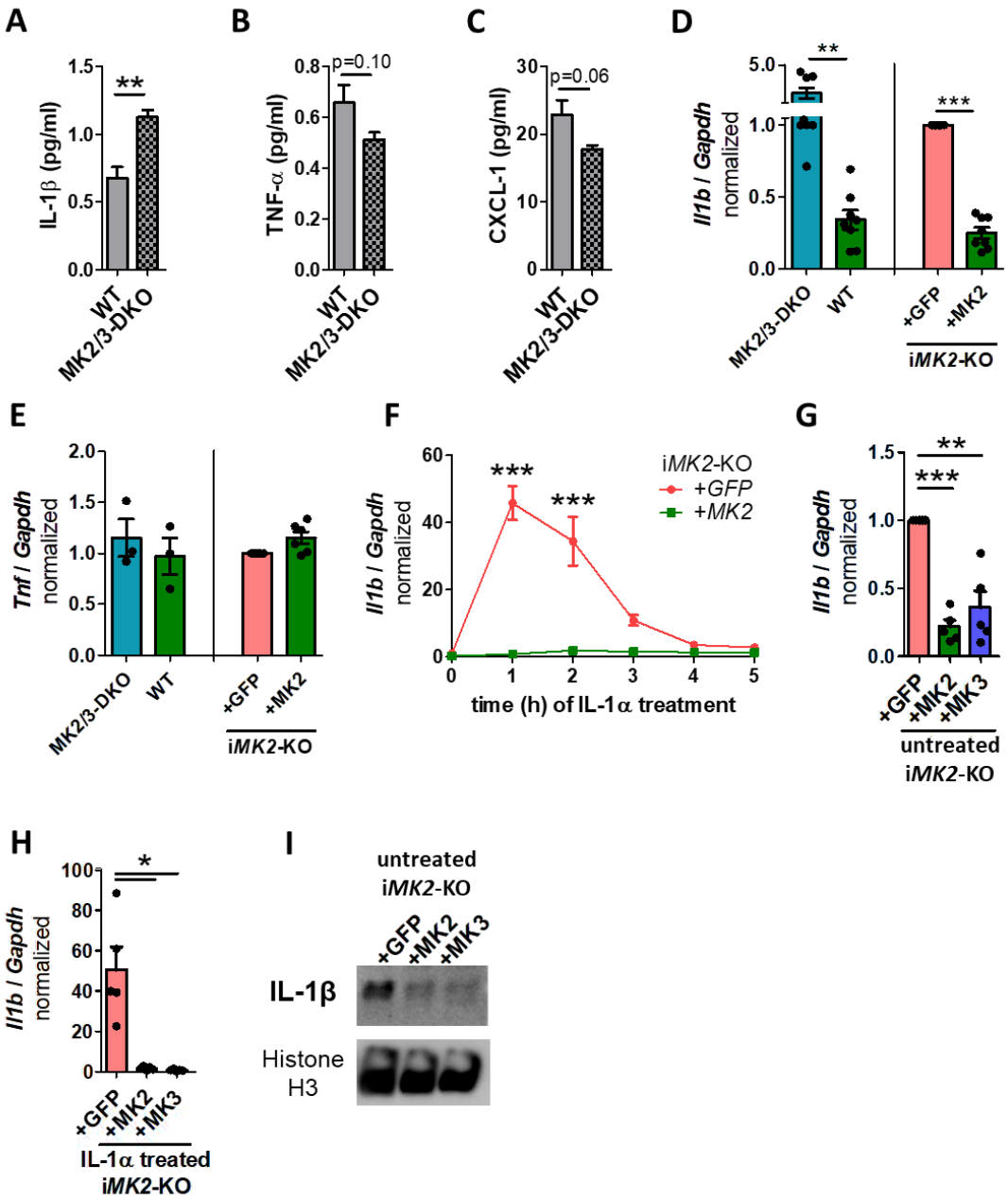


Figure 2: The level of IL-1β is increased in immortalized *MK2*-KO (i*MK2*-KO) cells

(A) The basal concentration of IL-1β is elevated in the serum of *MK2/3*-double knockout (DKO) mice, whereas levels of **(B)** Tumor necrosis factor (TNF)-α and **(C)** chemokine C-X-C motif ligand-1 (CXCL-1) are not increased. **(D)** i*MK2*-KO cells transduced with an empty vector (+*GFP*) showed elevated levels of *Il1b* mRNA compared to *MK2*-rescued cells (+*MK2*) (right), similar to those observed in *MK2/3*-DKO and WT bone-marrow-derived macrophages (BMDMs) (left). **(E)** *Tnf* mRNA is neither affected in resting BMDM (left) nor i*MK2*-KO cells (right). **(F)** i*MK2*-KO+*GFP* cells show increased *Il1b* mRNA after IL-1α treatment (5 ng/ml) compared to *MK2*-rescued cells. Similar to *MK2*, rescuing *MK3* decreases the level of *Il1b* mRNA in **(G)** resting or **(H)** IL-1α (5 ng/ml, 1h)-treated i*MK2*-KO cells, **(I)** as well as the basal level of IL-1β protein. Histone H3 serves as a control. **A-E, G-H)** N=3-7, mean ± SEM, students t-test, **F)** N=3, mean ± SEM, 2W-RM-ANOVA with Bonferroni posttests, * p<0.05, ** p<0.01, *** p<0.001.

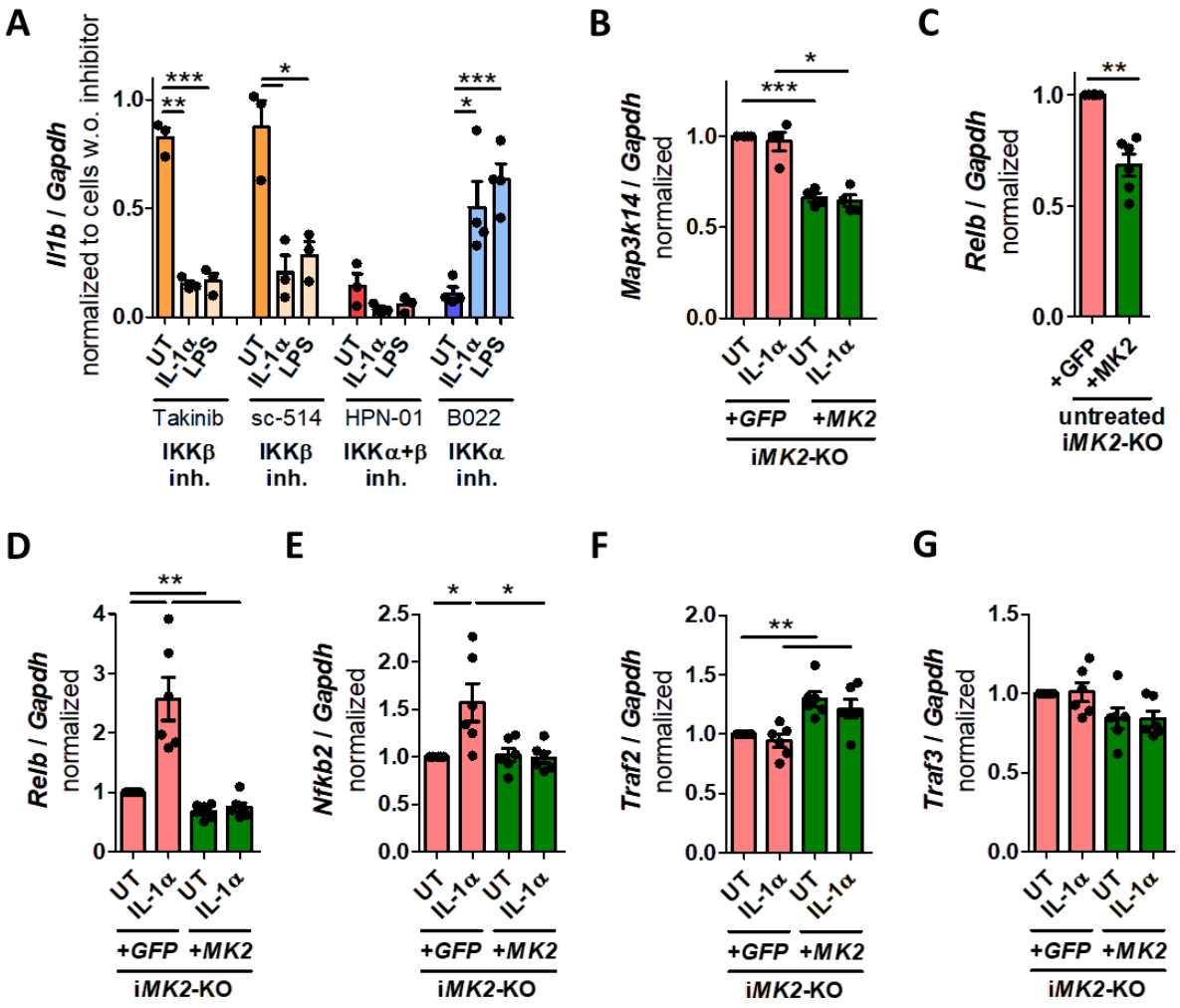


Figure 3: The non-canonical NF-κB pathway is activated in i*MK2*-KO cells (mRNA)

(A) Inhibition of the canonical NF-κB pathway using the IKKβ inhibitors Takinib (10 µM, 2h) and sc-514 (50 µM, 2h) reduced *Il1b* mRNA levels in IL-1α- and LPS-treated i*MK2*-KO cells, but did not affect basal *Il1b* levels. Inhibiting IKKα and IKKβ with HPN-01 (10 µM, 2h) reduced *Il1b* mRNA levels in untreated (UT) and IL-1α (5 ng/ml, 1h)- or LPS-stimulated cells (100 ng/ml, 1h). Inhibition of the non-canonical NF-κB pathway by IKKα inhibitor B022 (5 µM, 2h) reduced mainly basal *Il1b* mRNA. **(B)** The level of *Map3k14* mRNA is increased in i*MK2*-KO+*GFP*. **(C)** *Relb* mRNA level is increased in UT i*MK2*-KO+*GFP* cells. **(D)** *Relb* and **(E)** *Nfkb2* mRNA levels are increased in IL-1α (5ng/ml, 1h)-stimulated i*MK2*-KO+*GFP* cells. **(F)** Basal *Traf2* mRNA is reduced in i*MK2*-KO+*GFP* cells. **(G)** The *Traf3* mRNA level is not changed significantly. mean ± SEM, students t-test, * p<0.05, ** p<0.01, *** p<0.001.

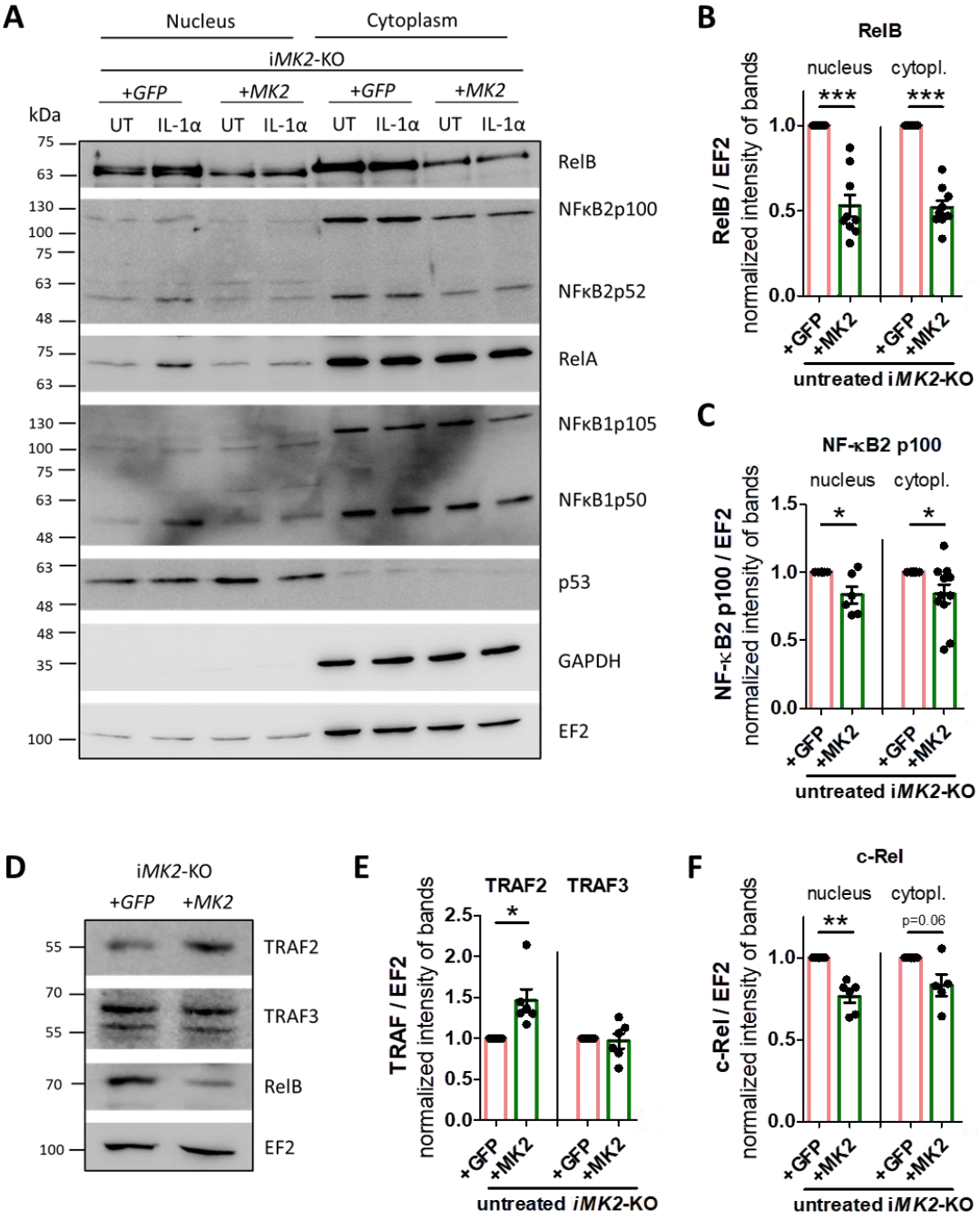


Figure 4: The non-canonical NF-κB pathway is activated in i*MK2*-KO cells (protein)

(A-C) Compared to i*MK2*-KO+*MK2* cells, untreated (UT) i*MK2*-KO+*GFP* cells have higher levels of the non-canonical proteins RelB and NF-κB2 in nuclear and cytoplasmic fractions, but not of the canonical RelA and NF-κB1 proteins. Following IL-1α treatment (5 ng/ml, 2h), the nuclear fraction of i*MK2*-KO cells showed elevated protein levels of RelB, NF-κB2, RelA, and NF-κB1. p53 serves as a nuclear control, and GAPDH as a cytoplasmic control. EF2 acts as a general control, used for normalization. **(D-E)** The basal protein level of TRAF2 is reduced in whole cell lysis of i*MK2*-KO+*GFP* cells, whereas TRAF3 is not changed. **(F)** The protein level of c-Rel is increased in i*MK2*-KO+*GFP* cells. N=5-11, mean ± SEM, students t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

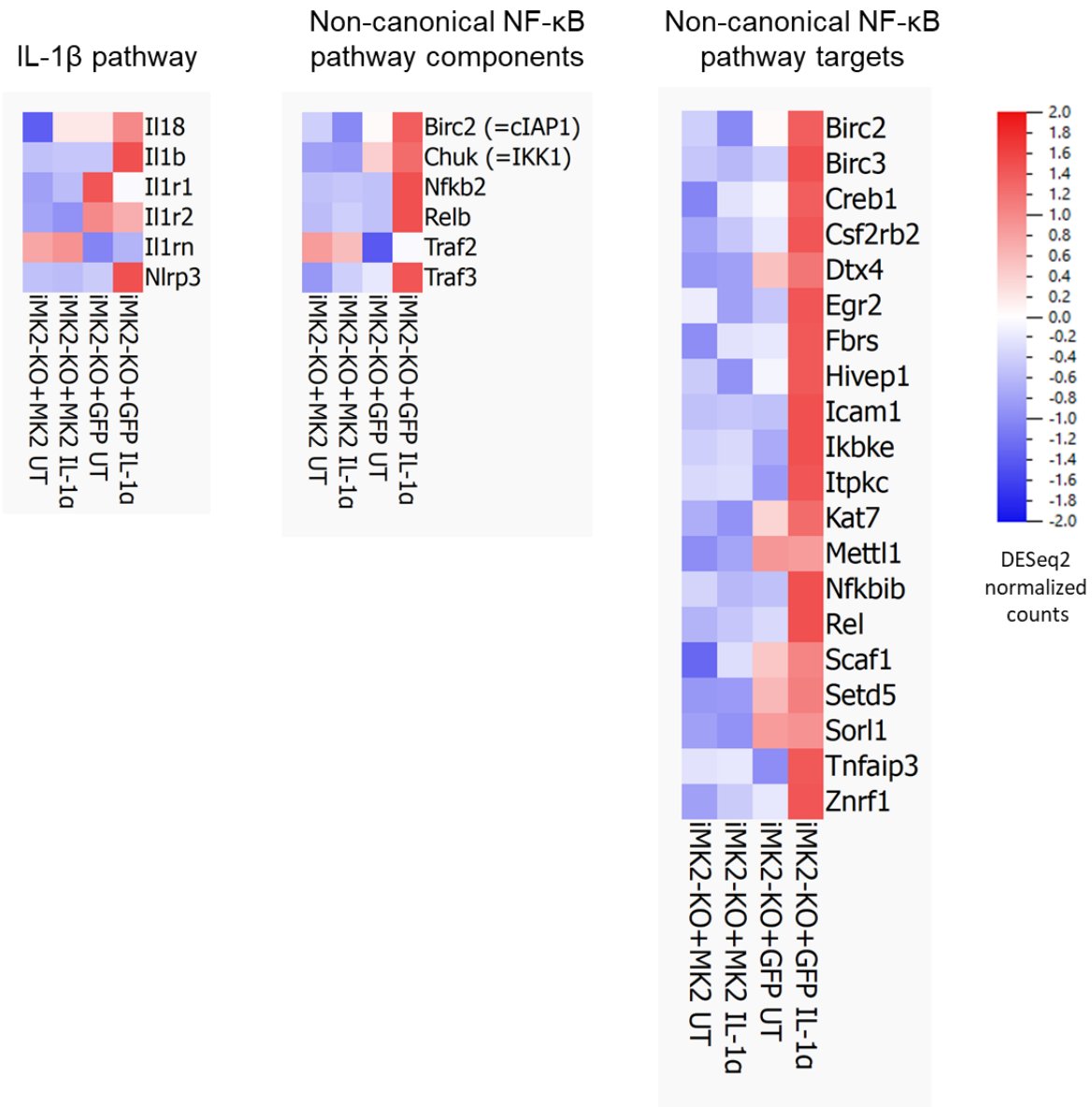


Figure 5: The non-canonical NF-κB pathway is activated in i*MK2*-KO cells

RNA sequencing revealed increased levels of mRNA for components of the IL-1β and non-canonical NF-κB pathways, as well as for targets of the non-canonical NF-κB pathway, in i*MK2*-KO+*GFP* cells. This finding was reinforced by IL-1α treatment (5 ng/ml, 1h).

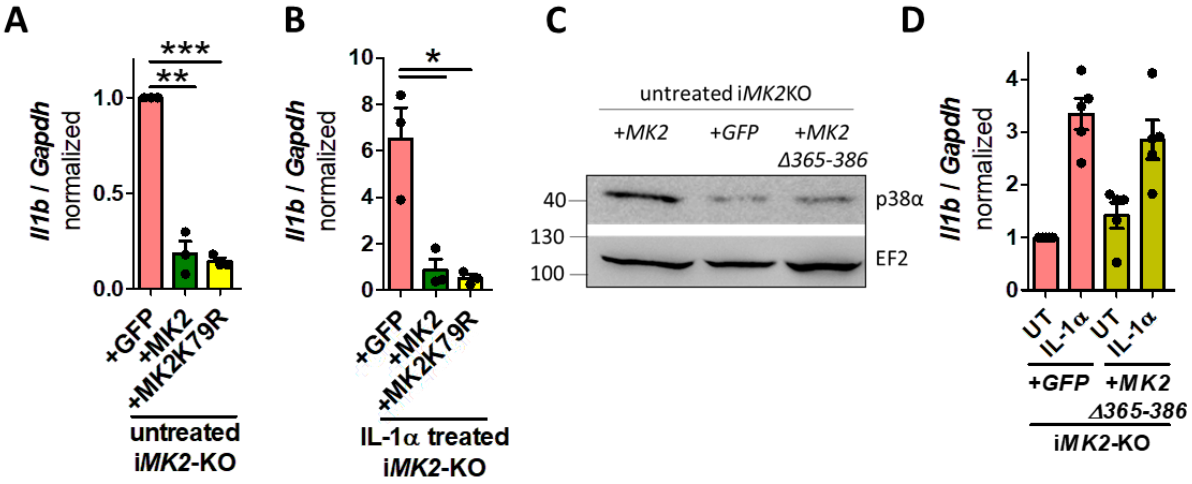


Figure 6: The MK2 kinase activity is not involved, but the MK2 C-terminus is important

(A) The rescued MK2 kinase-inactive mutant, MK2K79R, reduces *Il1b* mRNA levels to a degree comparable to that of the rescued MK2 in untreated (UT) and **(B)** IL-1α-treated (5 ng/ml, 1h) i*MK2*-KO macrophages. **(C)** i*MK2*-KO cells have lower levels of the p38α protein. These levels can be restored by rescuing *MK2*, but not by rescuing a MK2 mutant lacking the C-terminus *MK2-Δ365-386*. **(D)** MK2-Δ365-386 does not affect the *Il1b* mRNA levels in UT or IL-1α-treated cells. N=3-5, mean ± SEM, students t-test, * p<0.05, ** p<0.01, *** p<0.001.

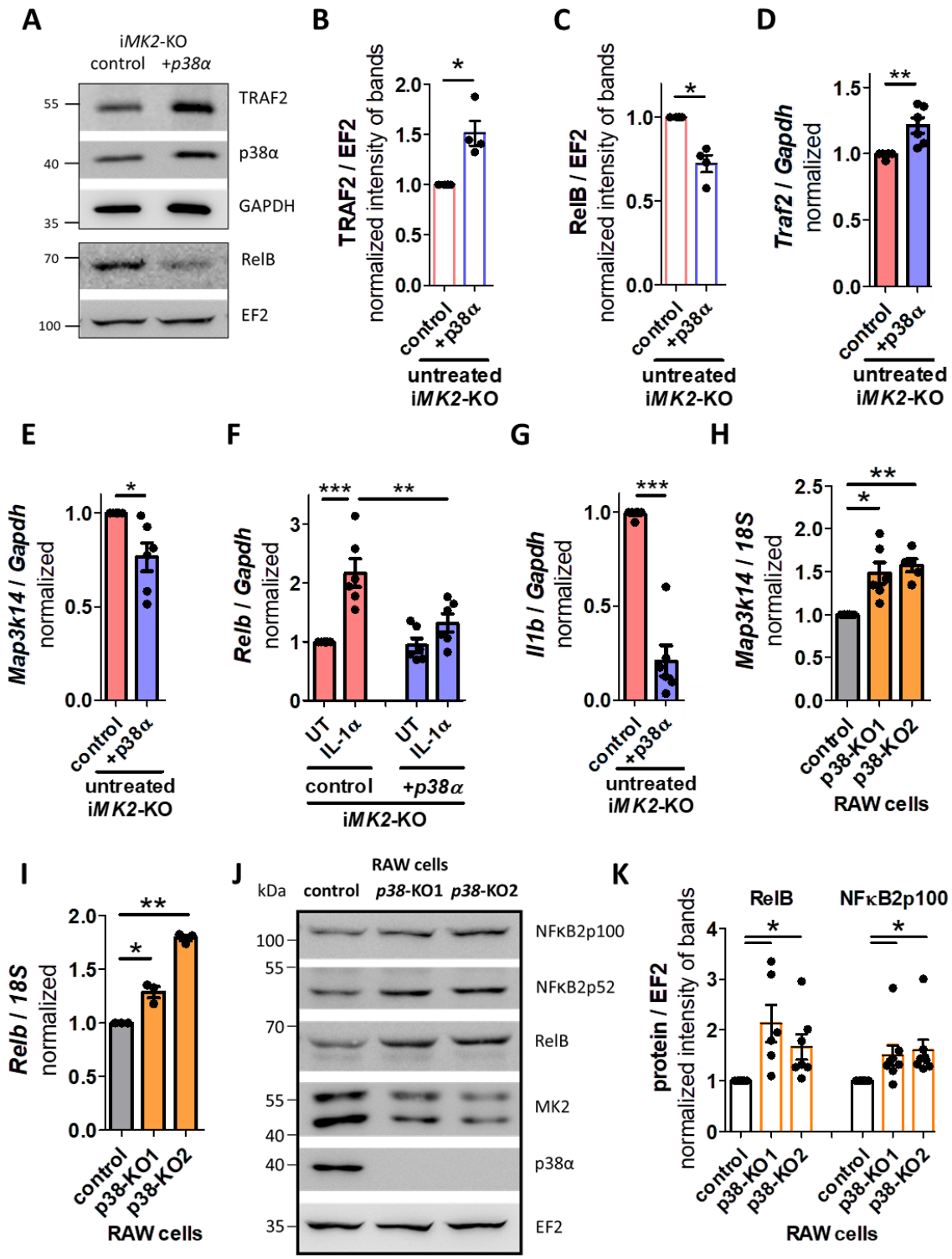


Figure 7: p38α inactivates the non-canonical NF-κB pathway in resting cells

(A-C) Overexpression of *p38α* in i*MK2*-KO cells increases basal TRAF2 and reduces basal RelB protein levels. **(D)** Overexpression of *p38α* increases basal *Traf2* mRNA and **(E)** reduces basal *Map3k14* mRNA as well as **(F)** *Relb* in IL-1α-treated cells (5 ng/ml, 1h). **(G)** Overexpression of p38α reduces basal *Il1b* mRNA. **(H)** Resting *p38α*-KO RAW cells harbor increased *Map3k14* mRNA and **(I)** *Relb* mRNA levels. **(J-K)** Resting *p38α*-KO RAW cells have elevated RelB and NF-κB2 protein levels. N=3-7, mean ± SEM, students t-test, * p<0.05, ** p<0.01, *** p<0.001.

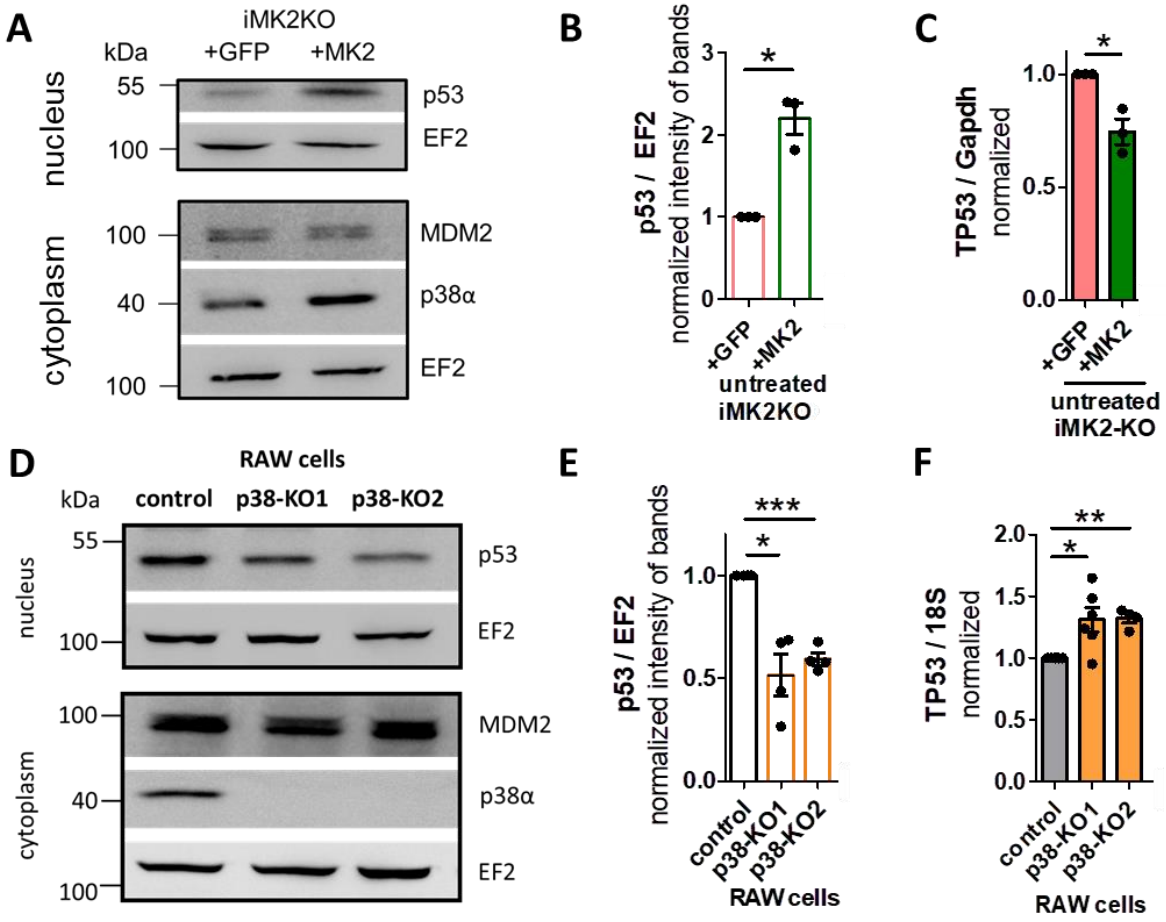


Figure 8: MK2/p38α stabilize p53 protein

(A-B) Resting i*MK2*-KO+*GFP* cells have less p53 protein level in the nucleus fraction, but similar MDM2 protein level in the cytoplasmic fraction compared to i*MK2*-KO+*MK2* macrophages. **(C)** *TP53* mRNA is increased in i*MK2*-KO cells. **(D-E)** Resting *p38α*-KO RAW macrophages have lower levels of p53 protein in the nuclear fraction, but similar levels of MDM2 protein in the cytoplasmic fraction compared to the control cells. **(F)** *TP53* is increased in *p38α*-KO cells. N=3-6, mean ± SEM, students t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

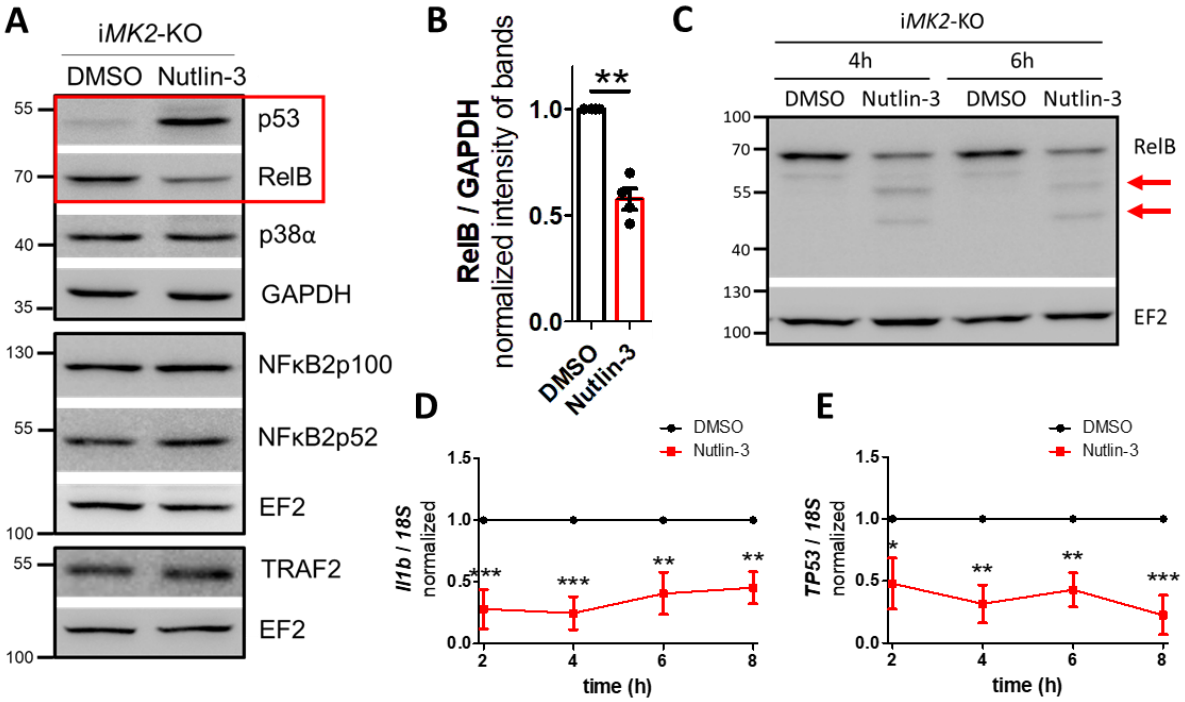


Figure 9: p53 inactivates the non-canonical NF-κB pathway via RelB degradation

(A-B) Nutlin-3 (20 µM, 4h) treated i*MK2*-KO cells accumulate p53 and harbor reduced RelB protein, but not NF-κB2, TRAF2 or p38α. **(C)** RelB cleavage products appear in Western blots after Nutlin-3 (20 µM) treatment in i*MK2*-KO cells. **(D)** *Il1b* mRNA and **(E)** *TP53* mRNA are reduced in Nutlin-3 (20 µM)-treated i*MK2*-KO cells. N=3-4, mean ± SEM, **B)** students t-test, **D-E)** 2W-RM-ANOVA with Bonferroni posttests, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

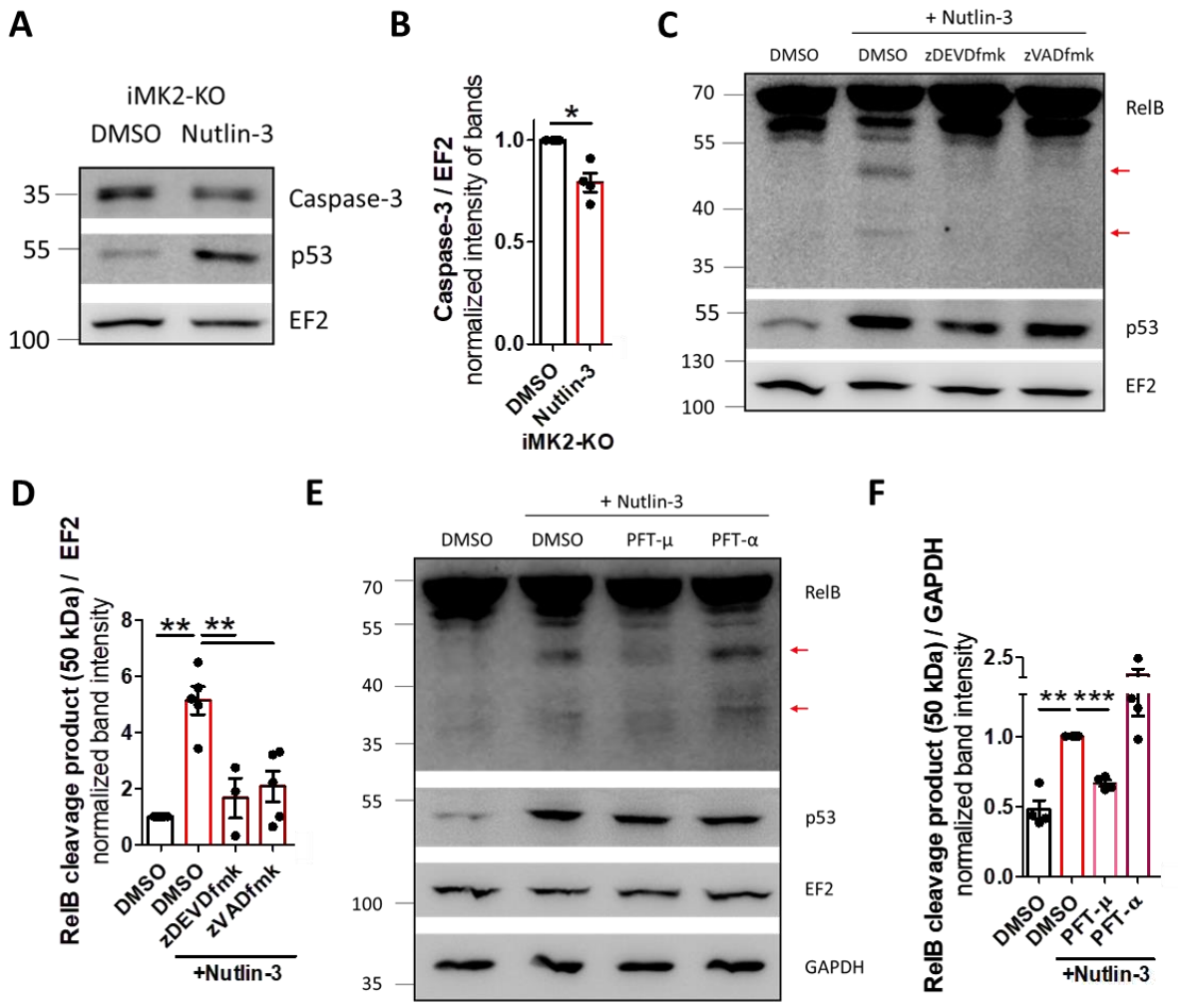


Figure 10: p53 activates mitochondrial caspase-3, which cleaves RelB

(A-B) Nutlin-3 (20 µM, 4h) treated i*MK2*-KO cells have reduced levels of full-length Pro-caspase-3 protein. **(C-D)** The appearance of RelB cleavage products after Nutlin-3 (20 µM, 4h) treatment in i*MK2*-KO cells can be blocked by the caspase-3 inhibitor z-DEVD-fmk (100 µM, 1h), the pan-caspase inhibitor z-VAD-fmk (25 µM, 1h), and **(E-F)** the inhibitor of p53 mitochondrial translocation Pifithrin-µ (10 µM, 1h), but not by the p53 transcriptional inhibitor Pifithrin-α (10 µM, 1h) in i*MK2*-KO cells. **A-F)** N=3-5, mean ± SEM, students t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.