IKK phosphorylates RelB to modulate its promoter specificity and promote fibroblast migration downstream of TNF receptors

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TNFα is a potent cytokine that plays a critical role in numerous cellular processes, particularly immune and inflammatory responses, programmed cell death, angiogenesis, and cell migration. Thus, understanding the molecular mechanisms that mediate TNFα-induced cellular responses is a crucial issue. It is generally accepted that global DNA binding activity of the NF-κB avian reticuloendotheliosis viral (v-rel) oncogene related B (RelB) subunit is not induced upon TNFα treatment in fibroblasts, despite its TNFα-induced nuclear accumulation. Here, we demonstrate that RelB plays a critical role in promoting fibroblast migration upon prolonged TNFα treatment. We identified the two kinases IκB kinase α (IKKα) and IκB kinase β (IKKβ) as RelB interacting partners whose activation by TNFα promotes RelB phosphorylation at serine 472. Once phosphorylated on serine 472, nuclear RelB dissociates from its interaction with the inhibitory protein IκBα and binds to the promoter of critical migration-associated genes, such as the matrix metallopeptidase 3 (MMP3). Further, we show that RelB serine 472 phosphorylation status controls MMP3 expression and promigration activity downstream of TNF receptors. Our findings provide new insights into the regulation of RelB activity and reveal a novel link between selective NF-κB target gene expression and cellular response in response to TNFα.

TNFalpha | NF-kappaB | metalloproteinase | posttranslational modification

The NF-κB transcription factor family consists of five members in mammals—RelA (p65), RelB, cRel (Rel), NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100)—and forms a collection of various homodimeric and heterodimeric complexes (1). NF-κB is essential for the control of immune and inflammatory responses as well as cell proliferation, apoptosis, and migration (2, 3). Aberrant NF-κB activity has been observed in many cancers, including both solid and hematopoietic malignancies, and sustained activation of NF-κB can affect all six hallmarks of cancer including insensitivity to growth inhibitory signals, evasion of apoptosis, induction of angiogenesis, and metastasis (3, 4). Multiple mechanisms have been delineated to achieve the required specificity and selectivity of the NF-κB response, including the regulation of specific target genes by individual NF-κB subunits (5–7), but their influence on cellular responses, such as proliferation, apoptosis, and migration, remains poorly understood.

Tumor necrosis factor α (TNFα) is a potent proinflammatory cytokine that mediates a wide variety of organismal and cellular responses including fever, acute-phase response, cell death and survival, and migration (8). In resting fibroblasts, the most abundant form of NF-κB is the heterodimer RelA/p50, whereas RelB is expressed at low levels and is sequestered in the cytoplasm due to its association with p100 (9). TNFα stimulation triggers the rapid activation of RelA/p50 complexes via the activation of the canonical NF-κB pathway, which relies mostly on the IκB kinase (IKK) complex-dependent-inducible proteolytic degradation of IκBα by the proteasome (10). Once in the nucleus, RelA/p50 activates the transcription of a large number of genes, some of which include cytokines and antiapoptotic proteins. Prolonged TNFα-stimulation results in a marked cytoplasmic and nuclear accumulation of RelB, but it is generally accepted that RelB entering the nucleus in response to TNFα cannot bind to consensus κB sites in fibroblasts (11–13). Several mechanisms of negative regulation of RelB-dependent DNA binding activity upon TNF receptor ligation have been reported, such as trapping in RelA/RelB (14) or p100/RelB complexes (11, 12, 15, 16). However, whether TNFα-induced increase of nuclear RelB protein levels might lead to RelB-dependent activation of a restricted set of endogenous genes in fibroblasts remains an unanswered question.

Analysis of RelB-deficient mice has shown that RelB is essential for the development of medullary epithelium, mature dendritic cell function, and secondary lymphoid organ organization (17). RelB$^{-/-}$ mice also spontaneously develop a multiorgan inflammatory syndrome that contributes significantly to their premature mortality (17). Contrary to RelA, RelB exerts no significant effect on TNFα-inducible apoptosis in fibroblasts (14). However, whether RelB

Significance

TNFα induces chemotaxis of inflammatory cells and fibroblasts, but little is known about the signaling mechanisms controlling this action. It is generally accepted that the avian reticuloendotheliosis viral oncogene (v-rel) related B (RelB) NF-κB subunit is not activated downstream of TNF receptors in fibroblasts. Here, we revealed an activating molecular mechanism leading to RelB transcriptional activation that is critical for the control of TNFα-induced fibroblast migration. We show that the IκB kinase (IKK) phosphorylates RelB on serine 472 in response to TNFα, leading RelB to bind to the promoter of critical migration-associated genes, such as the matrix metallopeptidase 3 (MMP3), thereby controlling MMP3 expression and promigration activity. These findings shed light on a crucial regulatory mechanism controlling selective NF-κB target gene expression and cellular response in response to TNFα.

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might be involved in other TNFα-triggered biological responses, particularly cell migration, is currently unknown. Limited activation of RelB was reported in fibroblasts upon stimulation with growth factors involved in proper control of cell migration, such as serum and PDGFβ (18). The IKK complex has emerged as being a critical mediator for cell migration upon TNFα stimulation (19), but whether IKK exerts its migratory function via RelB is currently unknown.

In the study presented here, we have revealed a key role for RelB in promoting TNFα-induced fibroblast migration via its inducible phosphorylation on serine 472 by IKK and subsequent dissociation from its nuclear interaction with the inhibitory protein IκBα. We have found that the metalloproteinase MMP3 is a direct target of RelB upon TNFR ligation, and showed that RelB exerts its serine 472-dependent promigration activities by inducing MMP3 expression and consequently MMP3 activity.

Results

IKK Phosphorylates RelB Serine 472 upon Prolonged TNFα Treatment in Fibroblasts. Although it is generally accepted that RelB is not activated downstream of the classical NF-κB pathway inducer TNFα in fibroblasts, nonetheless we and others have observed that TNFα treatment (at least 6 h) induces RelB nuclear accumulation in these cells (11–14). Thus, we have investigated the possible existence of alternative activating molecular mechanisms leading to RelB transcriptional activation upon prolonged TNFα stimulation in fibroblasts. To address this question, we undertook to identify new RelB-interacting partners by a biochemical approach based on specific biotinylation in vivo using RelB as a bait (*SI Materials and Methods*), taking advantage of the very high affinity of avidin/streptavidin for biotinylated templates and efficient single-step purification, and identified all three subunits of the IKK complex as RelB-interacting partner candidates in fibroblasts. To examine further the significance of this RelB–IKK complex interaction, we performed coimmunoprecipitation (co-IP) experiments using whole cell extracts from WT mouse embryonic fibroblasts (MEFs) either left untreated or treated by TNFα for 6 h. As a control, similar IP experiments were performed by using nonimmune serum to verify the specificity of the interaction. Reciprocal experiments with RelB-, IKKα-, IKKβ-, and IKKγ-specific antibodies showed that all three subunits of the IKK complex coimmunoprecipate with RelB and at a similar level in resting and TNFα-induced cells (Fig. 1*A*). The IKK complex being a serine/threonine kinase (20), we next assessed whether the IKK complex directly phosphorylates RelB upon prolonged TNFα stimulation. First, we performed in vitro IKK immunocomplex kinase assays using IKKα that was immunoprecipitated from WT MEFs stimulated with TNFα for 6 h and either GST–RelB full length (FL) or its C-terminal (C-ter) or its N-terminal halves (Fig. S1*A*, *Left*) as a substrate. The GST–RelB FL protein was significantly phosphorylated by IKK in its C-ter half (Fig. S1*A*, *Right*). Five GST–RelB deletion mutants containing a subdomain of the RelB C-ter half coding region (Δ1–Δ5 mutants) (Fig. S1*B*, *Left*) were used as substrates in IKK kinase assays. Only mutant Δ4 was efficiently phosphorylated by IKK (Fig. S1*B*, *Right*). We have mutated to alanine each of the four serine residues into Δ4 GST–RelB (Fig. S1*C*, *Left*), and IKK kinase assays revealed that a point mutation at serine 472 abolished phosphorylation of the Δ4 RelB deletion mutant (Fig. S1*C*, *Right*). TNFα inducibility of the IKK-dependent RelB serine 472 phosphorylation was further confirmed by IKK kinase assays using whole cell extract from WT MEFs either left untreated or treated with TNFα upon IP of either IKKα (Fig. 1*B*), IKKβ (Fig. 1*C*), or IKKγ (Fig. S1*D*). Importantly, sequence alignment reveals a high degree of conservation of this serine residue across species (Fig. S1*E*), and murine serine 451 (equivalent to human serine 472) is phosphorylated by the IKK complex upon TNFα stimulation in MEFs (Fig. S1*F*).

To go one step further, we have generated a custom antiphospho serine 472-specific RelB antibody and shown by immunoblotting

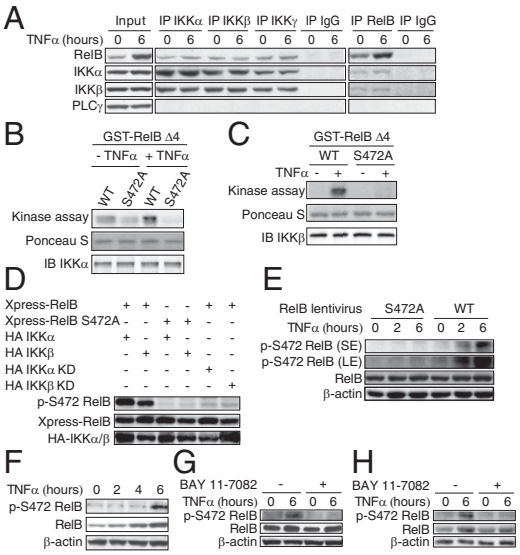

Fig. 1. IKK phosphorylates RelB at serine 472 upon prolonged TNFα treatment in vivo. (*A*) RelB constitutively interacts with the IKK complex in vivo. Whole cell extracts from WT MEFs were subjected to IP with anti-IKKα, IKKβ, IKKγ, RelB, or nonimmune control antibody, and analyzed by immunoblotting for the indicated proteins. (*B* and *C*) The IKK complex phosphorylates RelB serine 472 in vitro upon TNFα treatment. Whole cell extracts from WT MEFs stimulated or not by TNFα for 6 h were subjected to IP with anti-IKKα (*B*) or IKKβ (*C*), and IKK kinase assays were performed using the indicated substrates. RelB Δ4, 441–504 aa. Immunoprecipitated IKKα or IKKβ was detected by immunoblotting. (*D*) IKK phosphorylates RelB serine 472 in vivo. Whole cell extracts from HEK293 cells transiently transfected with the indicated cDNA were analyzed by immunoblotting for RelB serine 472 phosphorylation. (*E* and *F*) TNFα induces RelB serine 472 phosphorylation in vivo. Whole cell extracts from murine RelB-deficient MEFs transduced with the indicated RelB lentiviruses (*E*) or human HTB-126 cells (*F*) were analyzed for RelB serine 472 phosphorylation. SE/LE, short/long exposure. (*G* and *H*) TNFα-induced RelB serine 472 phosphorylation depends on IKK activity. RelB-deficient MEFs expressing WT RelB (*G*) or HTB-126 cells (*H*) were pretreated with Bay-11–7082 at 5 μM and treated with TNFα for the indicated periods of time.

that RelB serine 472 phosphorylation could be detected when WT RelB was coexpressed with HA–IKKα or HA–IKKβ but not catalytically inactive K44M IKKα and IKKβ mutants (Fig. 1*D*), indicating that a functional IKK complex is required for induced RelB phosphorylation. Not surprisingly, no RelB phosphorylation was observed upon expression of the RelB S472A mutant. Next, it was important to determine whether RelB serine 472 phosphorylation is increased by TNFα stimulation in vivo. Because it appeared that our custom RelB serine 472 phospho-specific antibody could only recognize the human but not the murine form of phosphorylated RelB (Fig. S2), a lentiviral vector was used to stably express human WT RelB or RelB S472A mutant in RelB-deficient MEFs. Although basal RelB serine 472 phosphorylation was observed in WT RelB-expressing cells, 6 h of TNFα stimulation led to induction of RelB serine 472 phosphorylation, whereas no such phosphorylation was observed in RelB S472A-expressing cells (Fig. 1*E*). RelB S472 phosphorylation was also induced upon PDGFβ treatment (Fig. S3*A*). Importantly, a RelB S472A-infected cell line expressed RelB at levels similar to that of WT RelB (Fig. S4*A*), and expression levels were similar to that of endogenous murine RelB in WT MEFs (Fig. S4*B*). Moreover, protein expression levels for RelA, p100, p105, p52, and p50 were comparable in RelB-deficient MEFs reconstituted with WT and S472A RelB (Fig. S4*A*), and WT and S472A RelB underwent similar nuclear translocation upon TNFα treatment (Fig. S4*C*). Further, TNFα-inducible phosphorylation of endogenous RelB at serine 472 was confirmed in human HTB-126 cells (Fig. 1*F*). Notably, TNFα-induced RelB

serine 472 phosphorylation was abrogated by the IKKα/β inhibitor Bay 11–7082 both in RelB-deficient MEFs expressing human WT RelB (Fig. 1*G*) and in human HTB-126 cells (Fig. 1*H*), and was significantly reduced by the two IKKβ inhibitors PS-1145 (Fig. S5*A*) and TPCA-1 (Fig. S5*B*). Altogether, these results indicate that the major IKK-dependent TNFα-inducible phosphorylation site of RelB is serine 472.

Serine 472 Phosphorylation Status Regulates RelB Promigration Function in Fibroblasts upon Prolonged TNFα Treatment. It was important to further explore the functional relevance of RelB serine 472 phosphorylation upon TNFR ligation. Because we have previously reported that RelB knockdown does not significantly affect TNFα-induced apoptosis in fibroblasts (14), we speculated that RelB might influence other TNFα-triggered biological responses, particularly cell migration, via its inducible serine 472 phosphorylation. To test this hypothesis, we first developed a stable RelB knockdown approach by RNA interference in WT MEFs using either a lentivirus carrying a shRNA-targeting RelB or a scrambled control (Fig. 2*A*, *Upper* panels), and analyzed fibroblasts migration by scratch-wound assays on arrested cells by serum starvation exposed or not to TNFα for 18 h (Fig. 2 *A* and *B*). RelB knockdown resulted in a marked and significant decrease in wound closure under unstimulated conditions, and strikingly the effect was even more pronounced on TNFα-induced fibroblast migration. Importantly, the reduced fibroblast migration upon RelB knockdown was rescued by expression of human WT RelB (Fig. 2*C*). Further, to examine whether phosphorylation of RelB on serine 472 was required for RelB-dependent fibroblast migration, we compared wound closure in RelB-deficient MEFs reconstituted with either an empty lentivirus or WT RelB or RelB S472A mutant either left untreated or stimulated with TNFα for 18 h. Reintroduction of WT RelB but not the S472A mutant markedly increased wound closure under the unstimulated conditions, and remarkably the difference was even more pronounced at 18 h post-TNFα stimulation (Fig. 2 *D* and *E*). Next, we evaluated whether the serine 472 phosphorylation status of RelB affected cell migration by transwell assays. As shown in Fig. 2*F*, expression of WT RelB but not the S472A mutant increased the migration rate of fibroblasts compared with that seen in empty lentivirus-infected RelB-deficient MEFs. Serum promigration activity is also in part exerted in a RelB–S472-dependent manner (Fig. S3*C*). Because cell migration is a highly coordinated cellular event that is powered by actin filament polymerization and remodeling, we also examined the effect of RelB serine 472 phosphorylation status on the organization of actin cytoskeleton. Reintroduction of WT RelB but not the S472A RelB mutant markedly and significantly increased the number of stress fibers compared with that seen in empty lentivirus-infected RelB-deficient MEFs upon TNFα treatment (Fig. 2 *G* and *H*). Taken together, these results strongly suggest that RelB serine 472 phosphorylation promotes fibroblast migration downstream of TNFRs.

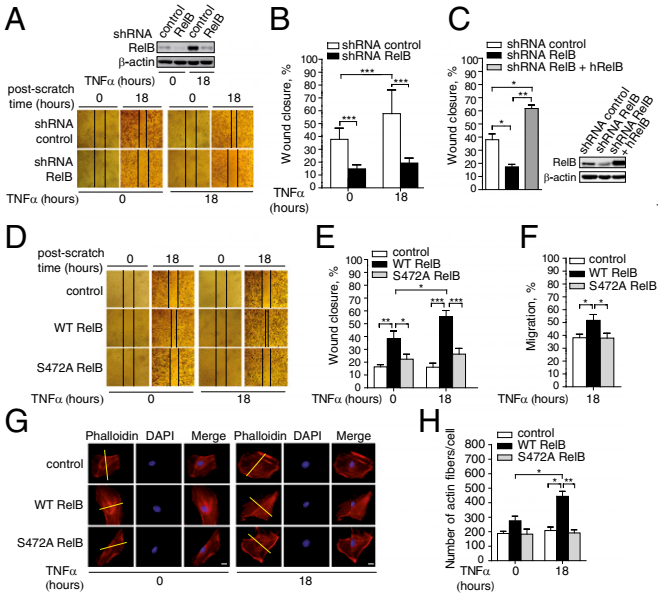

Fig. 2. RelB serine 472 phosphorylation status controls TNFα-induced fibroblast migration. (*A* and *B*) RelB knockdown by RNAi impairs TNFα-induced cell migration in WT MEFs. WT MEFs transduced with lentiviruses encoding either a shRNA-targeting RelB or a scrambled control were analyzed for cell migration by scratch-wound assays. Efficient knockdown of RelB protein levels was analyzed by immunoblotting. Wound closure of one representative experiment is shown (*A*), and the percentage of wound closure is represented (*B*); $n = 4$. (*C*) Inhibition of fibroblast migration by RelB knockdown was rescued by expression of human RelB. MEFs transduced with the indicated lentiviruses were analyzed for cell migration as in *A*; $n = 3$. Efficient RelB rescue was analyzed by immunoblotting. (*D*–*F*) RelB exerts a serine 472-dependent promigration function upon TNFR ligation. RelB-deficient MEFs transduced with the indicated lentiviruses were analyzed for cell migration by scratch assays ($n = 7$) (*D* and *E*) or by transwell assays ($n = 6$) (*F*). (*G* and *H*) RelB serine 472 is critical for organization of actin fibers. Filamentous actin stress fibers from RelB-deficient MEFs transduced with the indicated lentiviruses were detected by fluorescence microscopy using rhodamine-conjugated phalloidin. Staining of one representative cell is shown (*G*). (Magnification: 63×.) Statistical analysis of the number of stress fibers per cell is represented in *H*; $n = 3$. Means ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

MMP3 Is a Novel RelB Target Gene That Depends on RelB Serine 472 Phosphorylation upon TNFα Treatment. To gain further insights into the mechanism that controls the serine 472-dependent RelB activating function on fibroblast migration, we examined whether RelB serine 472 phosphorylation status has an impact on the RelB-mediated gene expression program, particularly those that could mediate the promigration function of RelB in fibroblasts. Microarray analysis of RNAs isolated from resting and TNFα-stimulated RelB-deficient MEFs expressing either WT RelB or S472A mutant were performed (Fig. S6), and selected genes were further validated by quantitative RT-PCR. Strikingly, we revealed the requirement for RelB serine 472 in activating gene expression of MMP3, a member of the matrix metalloproteinase family with documented promigration and invasive function in cancer cells (21) downstream of TNFR ligation (Fig. 3*A*). In contrast, although expression of previously described RelB-dependent genes was indeed modulated upon introduction of WT RelB into RelB-deficient cells, mRNA expression levels of all these genes appeared to be independent of the serine 472 phosphorylation status in fibroblasts (Fig. 3*A* and Fig. S7). Importantly, RelB knockdown by RNA interference in WT MEFs also resulted in a marked decrease in TNFα-induced MMP3 mRNA levels (Fig. 3*B*). In contrast, as a control, no significant effects on RelA expression were seen.

To further assess underlying mechanisms, we examined the possible involvement of RelB serine 472 phosphorylation in classical NF-κB signaling in response to TNFα treatment. Introduction of S472A into RelB-deficient cells induced the characteristic biphasic time course of NF-κB DNA binding activity upon TNFα treatment to a level similar to that seen with WT RelB (Fig. S8*A*), and the composition of the NF-κB DNA binding complexes, mainly RelA–p50, was unaffected in S472A cells (Fig. S8*B*). Similarly, RelB S472A mutation did not affect NF-κB DNA binding activity upon PDGFR (Fig. S3*B*) and LTβR ligation (Fig. S8*C*). Moreover, IκBα and IκBβ degradation were unaffected in S472A cells compared with WT RelB cells (Fig. S8*D*), as was expression of the classical NF-κB target genes IκBα, IL6, CCL2/MCP-1, CCL5/RANTES, MnSOD, and Icam1 (Fig. S8*E*). Taken together, these results strongly suggest that RelB serine 472-mediated MMP3

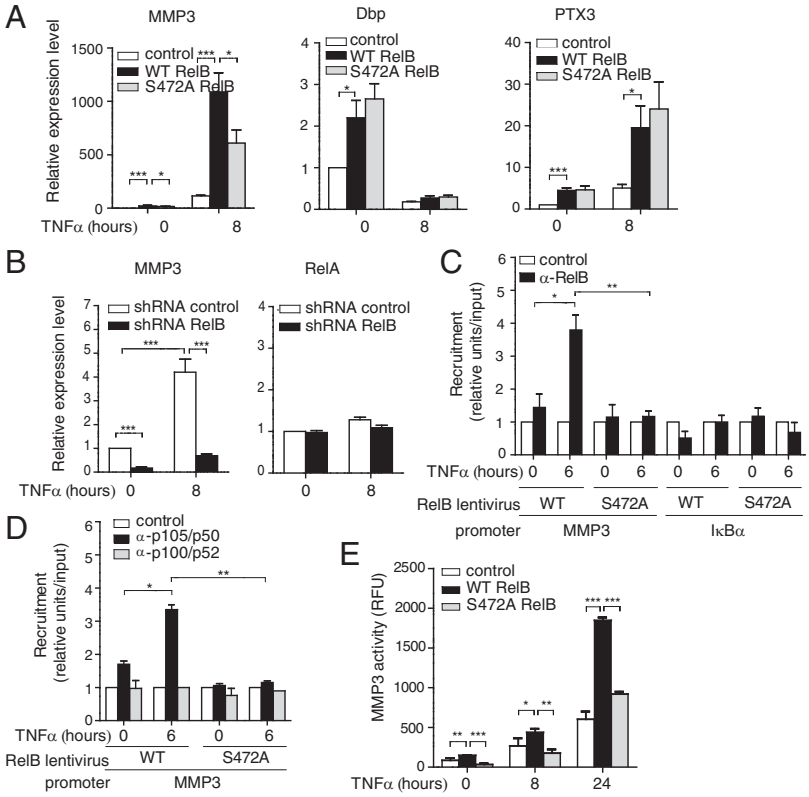

Fig. 3. RelB serine 472 is critical for TNFα-induced MMP3 expression and activity. (*A*) RelB exerts a serine 472-dependent activation of MMP3 expression in response to TNFα. RT-qPCR was performed for the indicated genes using total RNAs prepared from RelB-deficient MEFs transduced with the indicated lentiviruses ($n = 3$), normalized to the level of hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. (*B*) Stable RelB knockdown decreased TNFα-induced MMP3 protein expression levels in WT MEFs. WT MEFs were transduced with lentiviruses encoding either a shRNA-targeting RelB or a scrambled control, and RT-qPCR was performed as in *A*; $n = 3$. (*C*) TNFα-induced recruitment of RelB to the *MMP3* promoter depends on serine 472 phosphorylation status. Recruitment of WT RelB and S472A mutant to *MMP3* and *IκBα* promoters was examined by ChIP assays followed by qPCR analysis; $n = 3$, normalized to inputs. (*D*) p50 recruitment to the *MMP3* promoter is RelB serine 472 dependent. Recruitment of p50 and p52 was examined by ChIP assays as in *C*. (*E*) RelB serine 472 is critical for TNFα-induced MMP3 activity. MMP3 activity is represented as relative fluorescence units (RFUs); $n = 4$. Means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression is dependent on IKK complex kinase activity independently of classical NF-κB activation downstream of TNFRs.

Further in vivo evidence for a direct role for RelB serine 472 phosphorylation in regulating MMP3 transcription was obtained by chromatin IP (ChIP) analysis. As shown in Fig. 3*C*, TNFα stimulation induced efficient recruitment of WT RelB but not the S472A mutant to the *MMP3* promoter. As a control, neither WT RelB nor the S472A mutant was found to bind to the *IκBα* promoter. Recruitment of p50 to the *MMP3* promoter was also seen to be dependent on RelB serine 472 phosphorylation status upon TNFα treatment (Fig. 3*D*). In contrast, p52 was not seen to bind to the MMP3 promoter either in WT RelB or RelB S472A-expressing cells. Altogether, it suggests that RelB–p50 complexes bind to the *MMP3* promoter. We next evaluated whether RelB serine 472-dependent up-regulation of MMP3 gene expression affects MMP3 activity downstream of TNFR. MMP3 activity was markedly and significantly increased upon TNFα stimulation in WT RelB but not S472A-expressing cells (Fig. 3*E*). Collectively, these results suggest that TNFα-mediated RelB serine 472 phosphorylation up-regulates MMP3 expression, and consequently its activity, through direct transcriptional control in fibroblasts.

RelB Serine 472 Phosphorylation Status Controls the Association of Nuclear RelB with the Inhibitory Protein IκBα. It was important to determine how RelB serine 472 may control MMP3 expression. Because RelB requires p50 or p52 as a dimerization partner to bind DNA, we first compared the cellular distributions of p105/p50 and p100/p52 in TNFα-induced RelB S472A and WT RelB-expressing MEFs (Fig. 4*A*). A fraction of p105 and p50 was constitutively present in the nucleus of WT RelB-infected cells and their levels were comparable in S472A RelB-expressing MEFs. In addition, p50 levels increased slightly and at similar levels in the nucleus of TNFα-treated RelB-deficient MEFs expressing either WT or S472A RelB. Within 8 h of TNFα treatment, p100 and p52 protein expression levels and nuclear accumulation upon TNFR ligation are not affected by RelB serine 472 phosphorylation status (Fig. 4*A*). Thus, the marked reduction of TNFα-induced RelB S472A mutant recruitment to the *MMP3* promoter compared with that seen with WT RelB, despite similar RelB accumulation in the nucleus (Fig. 4*A* and Fig. S4*C*), is not due to lack of production of its heterodimerization partner p50 and p52, nor to increased p100

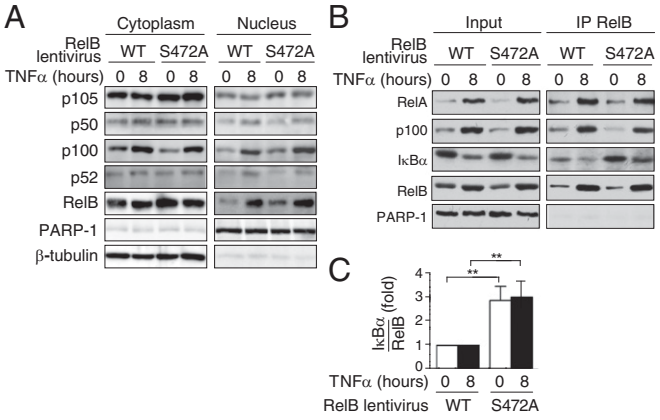

Fig. 4. RelB serine 472 phosphorylation status controls the association of nuclear RelB with the inhibitory protein IκBα. (*A*) Protein expression levels and cellular distribution of NF-κB members in RelB-deficient MEFs expressing WT RelB or the S472A mutant. Cytoplasmic and nuclear extracts of RelB-deficient MEFs transduced with the indicated lentiviruses, and treated or not with TNFα for 8 h, were analyzed by immunoblotting for the indicated proteins. (*B* and *C*) RelB serine 472 phosphorylation status controls the association of nuclear RelB with IκBα. Nuclear extracts prepared as in *A* were subjected to IP with anti-RelB antibody and analyzed for associated RelA, p100, and IκBα. The results of one representative experiment are shown (*B*), and quantification of RelB–IκBα complexes is represented in *C*. Means ± SEM; $n = 5$; $^{**}P < 0.01$. Signals were graphed relative to immunoprecipitated RelB and respective to WT RelB–IκBα complexes.

nuclear accumulation. Because we have reported that trapping of nuclear RelB into RelA or p100 complexes is an important mechanism of regulation of RelB DNA binding upon TNFα treatment, we next compared the nuclear association of WT and S472A RelB with p100 and RelA. No significant difference in WT and S472A RelB association with either p100 or RelA was observed (Fig. 4*B*). Further, because IκBα has recently emerged as a negative regulator of RelB activity in dendritic cells upon TLR stimulation (22), we speculated that serine 472 phosphorylation-dependent association of RelB with IκBα might control RelB DNA binding ability upon TNFR ligation in fibroblasts. To test this hypothesis, we compared WT and S472A RelB binding to IκBα in the nucleus of TNFα-stimulated MEFs. Remarkably, IκBα coimmunoprecipitates with WT RelB at much lower levels compared with S472A RelB in the nuclear compartment of either unstimulated or TNFα-treated cells (Fig. 4 *B* and *C*). Importantly, WT RelB- and S472A-infected MEFs expressed similar levels of nuclear IκBα. In contrast, no significant difference of RelB interaction with IκBα was seen in the cytoplasmic compartment (Fig. S9). Because phosphorylation of RelB on serine 472 is increased by TNFα stimulation (Fig. 1 *E* and *F*), these results suggest that serine 472 phosphorylation is required for nuclear RelB to dissociate from its inhibitory interaction with IκBα, thereby allowing recruitment to critical genomic targets, such as MMP3.

MMP3 Activity Is Required for RelB Serine 472-Mediated Fibroblast Migration upon TNFR Ligation. Because we have characterized the promigration gene MMP3 as a serine 472-dependent RelB target gene in fibroblasts, we examined the direct contribution of MMP3 in TNFα-induced RelB serine 472-mediated cell migration by a direct RNA interference approach. Two different siRNAs directed against MMP3 efficiently repressed MMP3 mRNA expression levels (Fig. S10*A*), and both basal and TNFα-induced MMP3 activity was dramatically reduced upon MMP3 knockdown (Fig. S10*B*). Most importantly, MMP3 knockdown markedly decreased TNFα-induced cell migration of WT RelB-expressing cells (Fig. 5 *A* and *B*). Not surprisingly, it has little effect on S472A RelB-expressing cells. Taken together, our data suggest that TNFα-induced serine 472 phosphorylation of RelB promotes cell migration by up-regulating the expression of selective NF-κB target genes, such as MMP3.

Discussion

In the study presented here, we have uncovered a role for RelB in inducing fibroblast migration via its TNFα-inducible phosphorylation at serine 472 by IKK and subsequent selective gene expression of promigration genes, such as MMP3. Knowing that there is unequivocal evidence indicating that global RelB DNA binding activity is not induced upon TNFα treatment in fibroblasts (11–14), our findings constitute a significant advance in understanding the fine-tuned regulation of RelB once in the nucleus upon TNFα treatment.

IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (20). IKKβ and IKKγ are essential for rapid and transient NF-κB induction by proinflammatory signaling cascades, such as the one triggered by TNFα, via the classical NF-κB pathway that mainly relies on IκBα degradation and results in activation of RelA-containing complexes (1). In contrast, IKKα is required for the late and sustained activation of NF-κB in response to a specific subset of TNF family members via the alternative NF-κB pathway that relies on the inducible proteolysis of cytoplasmic p100 and results in RelB transcriptional activation (23, 24). Here, we show that RelB can interact with the tripartite IKKα/IKKβ/IKKγ complex, and both endogenous IKKα- and IKKβ-containing IKK complexes can phosphorylate RelB on serine 472 upon TNFα treatment. Moreover, ChIP experiments have shown that RelB–serine 472 phosphorylation status is critical for RelB to be recruited to the *MMP3* promoter in fibroblasts. Thus, altogether it strongly suggests a role for the IKKγ-dependent IKKα/β-containing complex in controlling RelB–DNA binding activity through its TNFα-induced phosphorylation of RelB at serine 472. Nevertheless it cannot be excluded that RelB might also be phosphorylated by the alternative IKKα-containing complex independently of IKKβ and IKKγ (12). RelB S472 phosphorylation is observed upon prolonged TNFα stimulation. It most probably reflects the late phase of TNFα-induced IKK activity that was reported to be critical for dynamic NF-κB activation (25, 26). Phosphorylation of other NF-κB subunits by the IKK complex has been reported (27), including phosphorylation of RelA on serine 536 (28, 29). However, whether phosphorylation at these sites might affect the gene expression program controlling cell motility remains elusive. Interestingly, in contrast to RelB serine 472 phosphorylation that promotes fibroblast migration, which we report here, RelA phosphorylation at threonine 505, which is inducible by the Chk1 kinase, has been implicated as a negative regulator of constitutive fibroblast migration (30). Importantly, contrary to RelB serine 472, RelA threonine 505 phosphorylation is not inducible upon TNFα treatment (31). Altogether, these observations suggest nonredundant function of RelA and RelB subunits in the control of cell motility.

Our study clearly shows that RelB activation downstream of TNFR involves its serine 472-dependent selective gene expression program via the serine 472 phosphorylation-dependent dissociation of nuclear RelB from its interaction with the inhibitory protein IκBα, indicating that this modification helps in providing the specificity and selectivity required to induce a context-dependent RelB transcriptional response. Nonetheless, additional factors most probably contribute to the control of RelB-dependent gene activation in response to TNFα. RelB DNA binding was induced in TNFα-stimulated NF-κB2–deficient cells (11, 14), and Fbxw7α- and GSK3-mediated proteosomal degradation of nuclear p100 has recently emerged to account for the control of RelB activity (32), indicating that nuclear p100 accounts for the control of RelB DNA binding activity. Further, because selective recruitment to *Blc* and *Elc* promoters depends on a different κB site whose consensus sequence is distinct from the classical κB site (33), κB site specificity might exist. Finally, other mechanisms, such as overall ReB protein expression levels (34, 35) and chromatin remodeling (36), most likely also influence RelB recruitment to specific promoters. Whether these mechanisms are intertwined with the status of RelB serine 472 phosphorylation is currently unknown. Nonetheless, our results clearly demonstrate that an important mechanism of RelB activity regulation downstream of TNFR is based on its specific TNFα-inducible phosphorylation that allows nuclear RelB to dissociate from its inhibitory interaction with IκBα.

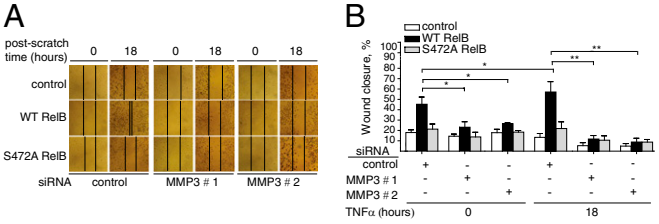

Fig. 5. RelB serine 472 phosphorylation status promotes cell migration in a MMP3-dependent manner. (*A* and *B*) MMP3 knockdown by RNAi inhibits RelB serine 472-mediated cell migration upon TNFα treatment. RelB-deficient MEFs transduced with the indicated lentiviruses were transfected with either a scrambled control or two different siRNA oligonucleotides targeting MMP3 and analyzed for cell migration by scratch-wound assays. Means ± SEM; $n = 5$; $^{*}P < 0.05$, $^{**}P < 0.01$.

We have found that RelB promotes cell migration in fibroblasts in a serine 472-dependent manner via the induction of promigration gene expression, such as MMP3 (but also MMP9 and MMP10), downstream of TNFR. It has been recently reported that RelB can promote the more invasive phenotype of ERα-negative breast cancer cell lines (37), and RelB increases the incidence of metastatic tumors in a mice xenograft model of prostate cancer (38). Furthermore, RelB knockdown strongly reduces glioma cell migration and invasion (39). However, whether RelB serine 472 phosphorylation can participate in the invasiveness of cancer cells is currently unknown but is worth further investigation. TNFα is critical for tumor promotion and progression (8). MMPs are well reported to play a crucial role in the control of cell motility, invasion, and morphological changes (40). Therefore, a careful evaluation of the status of RelB serine 472 phosphorylation is certainly required to define the role of RelB in the various stages of malignant transformation and metastasis development, and blocking RelB-specific phosphorylation might have great potential for therapeutic intervention.

In conclusion, we established RelB and its phosphorylation on serine 472 by IKK as an important positive regulatory mechanism of TNFα-induced fibroblast migration. Our data are of great functional importance because they constitute a significant advance in the understanding of RelB regulation and associated physiological function and shed light on a novel positive link between selective NF-κB target gene expression and cellular responses downstream of TNFRs.

Materials and Methods

Cells Culture. RelB-deficient MEFs (passage 3–5) and -immortalized fibroblasts and HTB-126 and HEK293 cells were grown in DMEM with 10% (vol/vol) FBS and antibiotics.

MMP3 Activity Assays. Cell culture supernatant was collected, incubated with 1 mM of amino-phenyl mercuric acetate for 24 h at 37 °C, and MMP3 activity measured by using the SensoLyte 490 MMP3 Fluorometric Assay Kit (AnaSpec).

Scratch-Wound Assays. Cells were grown to a confluent monolayer for 24 h in DMEM with 10% FBS, and then FBS was lowered to 0.5% for an additional 24 h before scratches were made using a 0.5-mm-diameter pipette tip. Scratch area was monitored over 18 h. TNFα (30 ng/mL) was added right after scratches were made.

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