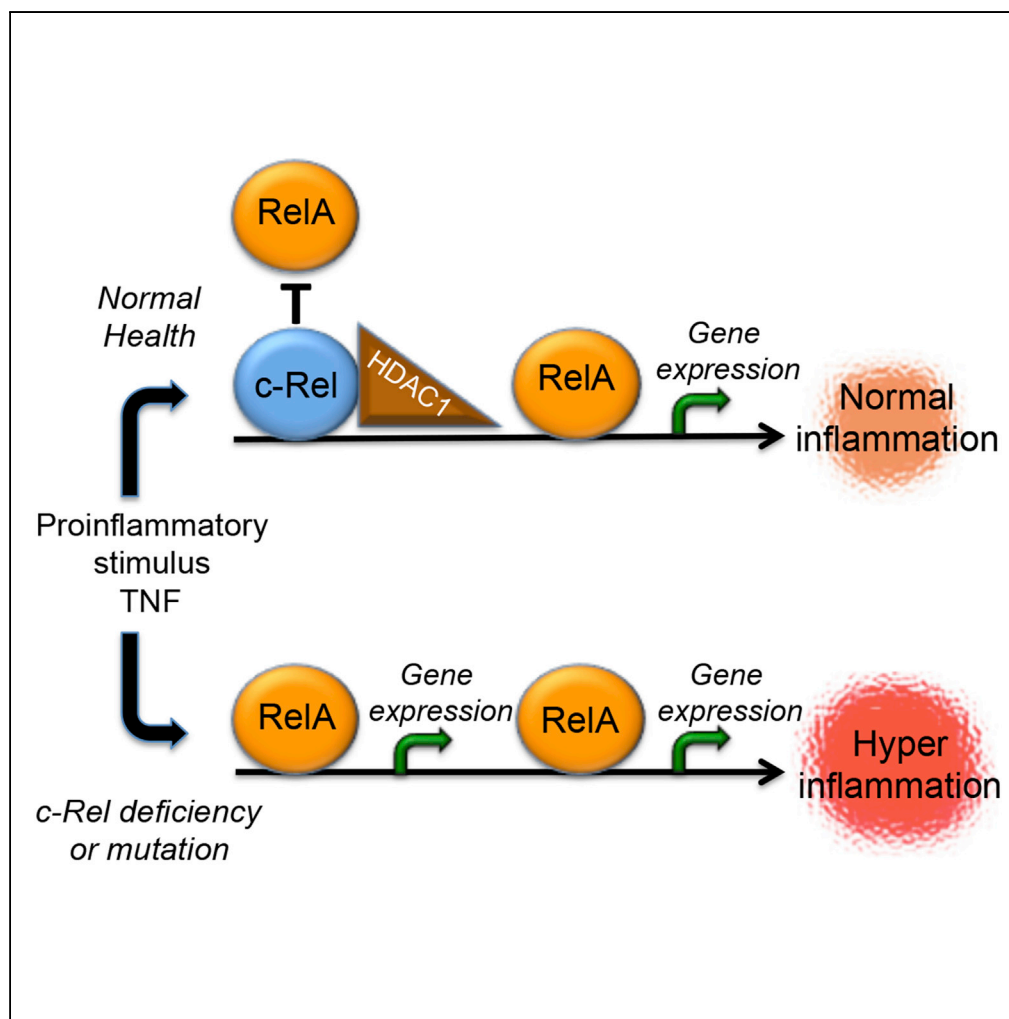


Article

NF- κ B c-Rel Dictates the Inflammatory Threshold by Acting as a Transcriptional Repressor

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HIGHLIGHTS

NF- κ B protein c-Rel act as a repressor of TNF-induced RelA-dependent inflammation

Mutation of Y25, an SNP site in humans, blocks repressor function of c-Rel

c-Rel recruits the co-repressor HDAC1 to the RelA-dependent promoters

Molecular mechanism of anti-inflammatory role of c-Rel

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Article

NF- κ B c-Rel Dictates the Inflammatory Threshold by Acting as a Transcriptional Repressor

Tristan James de Jesús¹ and Parameswaran Ramakrishnan^{1,2,3,4,*}**SUMMARY**

NF- κ B/Rel family of transcription factors plays a central role in initiation and resolution of inflammatory responses. Here, we identified a function of the NF- κ B subunit c-Rel as a transcriptional repressor of inflammatory genes. Genetic deletion of c-Rel substantially potentiates the expression of several TNF- α -induced RelA-dependent mediators of inflammation. v-Rel, the viral homologue of c-Rel, but not RelB, also possesses this repressive function. Mechanistically, we found that c-Rel selectively binds to the co-repressor HDAC1 and competitively binds to the DNA mediating HDAC1 recruitment to the promoters of inflammatory genes. A specific point mutation at tyrosine²⁵ in c-Rel's DNA-binding domain, for which a missense single nucleotide variation (Y25H) exists in humans, completely abrogated its ability to bind DNA and repress TNF- α -induced, RelA-mediated transcription. Our findings reveal that the transactivator NF- κ B subunit c-Rel also plays a role as a transcriptional repressor in the maintenance of inflammatory homeostasis.

INTRODUCTION

The inflammatory response is a multi-phasic, multi-factorial cellular response to a variety of pathological stimuli; a tightly controlled inflammatory response is imperative for the maintenance of homeostasis in mammals (Matzinger, 2002; Medzhitov, 2008). Both defense against infection and cellular responses to injury involve inflammatory response in clearing the infection or to aid in the healing process (Nathan, 2002). However, if inflammation persists following recovery from damage or infection, then this host-friendly defense mechanism turns into a foe and drives a variety of chronic inflammatory and autoimmune diseases such as psoriasis, atherosclerosis, rheumatoid arthritis, and inflammatory bowel disease, as well as leads to inflammation-induced cancer (Netea et al., 2017). Thus the proper initiation, control, and resolution of inflammation is necessary for an organism's health and recovery from pathologies (Serhan et al., 2007).

One of the primary regulators of the inflammatory response is the ubiquitously expressed, inducible transcription factors of REL/NF- κ B family (Ghosh et al., 1998). The NF- κ B family comprises five subunits, RelA (p65), RelB, c-Rel, p105/p50, and p100/p52, that form functional homo- and heterodimers binding to conserved κ B sites in the promoters of genes (Chen et al., 1998; Sen and Baltimore, 1986). In resting cells, NF- κ B dimers are sequestered in the cytoplasm via their interaction with members of the Inhibitor of κ B (I κ B) protein family (Baeuerle and Baltimore, 1988a, 1988b; Beg et al., 1992; Lenardo and Baltimore, 1989; Naumann et al., 1993). NF- κ B activation is typically separated into two signaling pathways: the canonical pathway, which depends on the kinase IKK- β and the regulator NF- κ B essential modulator (NEMO), and the alternative pathway, which depends on the kinases NIK and IKK- α (Pomerantz and Baltimore, 2002). Tumor necrosis factor alpha (TNF- α) is a proinflammatory cytokine that specifically activates the canonical NF- κ B pathway (Ramakrishnan et al., 2004), leading to the expression of a number of cytokines and chemokines mediating inflammation (Sedger and McDermott, 2014). TNF- α signaling leads to IKK β -mediated di-phosphorylation of I κ B- α and its proteasomal degradation freeing the bound NF- κ B (Wajant and Scheurich, 2011) (Gilmore, 1997, 2006). The newly freed NF- κ B dimers translocate to the nucleus and bind to a loose consensus sequence (GGGRNYYCC; R = purine, W = A or T, N = any, Y = pyrimidine) in the open chromatin, which allow binding of different dimers with varying binding strength (Chen et al., 1998).

Previous studies have separated the Rel/NF- κ B family into activating subunits (RelA, RelB, and c-Rel) and repressive subunits (p50 and p52), based on functional studies and the presence or absence of the C-terminal transactivation domains (TAD) (Bours et al., 1993; Schmitz and Baeuerle, 1991). The dimers containing

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TADs are capable of recruiting transcriptional co-activators, which include histone acetyltransferases (HAT), such as p300/CREB-binding protein (CBP), p300/CBP associated factor (PCAF), and Tat-interacting protein 60 (Tip60), which promote gene expression (Bhatt and Ghosh, 2014; Ghizzoni et al., 2011). Homo- and heterodimers of p50 and p52 without TADs act as transcriptional repressors simply by competitively blocking or by preoccupying the κ B sites of activating dimers (Schmitz and Baeuerle, 1991) or by recruiting co-repressors, such as histone deacetylases (HDACs) (Elsharkawy et al., 2010; Rocha et al., 2003).

c-Rel is one of the canonical, transactivating Rel subunits, with major roles in driving many immunological functions (Gilmore and Gerondakis, 2011). It also has a number of key functions in regards to cell cycle regulation and apoptosis across all cell types (Bash et al., 1997; Chen et al., 2003; Hsia et al., 2002; Huguet et al., 1997; Lorenz et al., 2014; Owyang et al., 2001). c-Rel has been shown to recognize a wider range of κ B sites with redundancy allowed at the first and fourth positions (NGGN compared with GGGR), which increases the spectrum of target genes under its regulation (Kunsch et al., 1992). c-Rel exists as homodimer, and it also forms heterodimers with p50, p52, RelA, as well as RelB (Gilmore and Gerondakis, 2011; Marienfeld et al., 2003).

c-Rel knockout mice develop normally but show impaired response to immune challenges and infections as well as significantly reduced T regulatory cell numbers (Kontgen et al., 1995; Tumang et al., 1998; Courtine et al., 2011; Ramakrishnan et al., 2016). Observations made using c-Rel knockout mouse models suggest that c-Rel either promotes or protects from a number of inflammatory and autoimmune diseases. Deficiency of c-Rel has been shown to protect from diseases such as psoriasis (Fan et al., 2016; Fullard et al., 2013), rheumatoid arthritis (Campbell et al., 2000; Eyre et al., 2010; Fan et al., 2018), and experimental autoimmune encephalomyelitis (EAE) (Chen et al., 2011; Hilliard et al., 2002) in mouse models, likely due to the lack of c-Rel-dependent inflammatory gene expression (Gilmore and Gerondakis, 2011). In contrast, c-Rel knockout mice show accelerated pathology of diseases such as type 1 diabetes (Ramakrishnan et al., 2016) and colon inflammation (Courtine et al., 2011). However, the molecular mechanism behind accelerated inflammatory complications, in c-Rel-deficient mice, remains poorly defined.

In this study, we examined the role of c-Rel in regulating proinflammatory gene expression induced by TNF- α . Here, we show that the transcriptional activator c-Rel can act as a selective repressor of TNF- α -induced, proinflammatory gene expression. Absence of c-Rel enhances TNF- α -induced, RelA-driven transcriptional activity. We also show that c-Rel-mediated suppression is dependent on its DNA-binding ability at RelA-binding sites. Additionally, we show that c-Rel specifically binds to the co-repressor HDAC1 and mediates its recruitment to the specific promoters investigated in this study, suggesting a plausible mechanism for the increased expression of inflammatory genes in the absence of c-Rel.

RESULTS

c-Rel Knockout Enhances TNF- α -Induced RelA-Dependent Gene Transcription

c-Rel is suggested to have both pro- and anti-inflammatory roles, and knowledge on the mechanism by which c-Rel restricts inflammation remains elusive. To have a comprehensive understanding on the requirement of c-Rel and its regulatory role in proinflammatory gene expression, we stimulated wild-type and c-Rel knockout mouse embryonic fibroblasts (MEFs) with TNF- α and then studied the expression of selected TNF- α -induced, pro-inflammatory genes. We found that several genes such as CCL2, CCL7, IP-10, CXCL1, A20, IL-6, CXCL2, CCL20, and ZFP36 showed markedly enhanced expression in the c-Rel knockout cells, while maintaining their expression kinetics (Figure 1A). This group of genes was made up largely of inflammatory cytokines and chemokines. Other genes such as ICAM1 and VCAM1 associated with cell migration proteins showed enhanced induction but displayed slower kinetics in c-Rel knockout cells (Figure 1B). The absence of c-Rel does not globally enhance or change kinetics of gene expression as we found that genes such as TNF- α , MMP10, EDN1, IFT1, IL-1 β , and I κ B- α showed a significantly decreased or lack of induction, indicative of dependence on c-Rel for their expression (Figure S1A). To rule out the possibility that the differences in gene expression observed were due to compensatory genetic alterations in immortalized MEFs generated from wild-type and c-Rel knockout mice, we generated c-Rel CRISPR knockout cells using our wild-type MEFs (G1 c-Rel KO) and confirmed the knockdown of c-Rel by western blotting (Figure 1C). Similar to c-Rel knockout MEFs of mouse origin, c-Rel CRISPR knockout cells also showed enhancement of selected TNF- α -induced genes (Figure 1D), demonstrating that the phenotype we observed is indeed specific to c-Rel knockout.

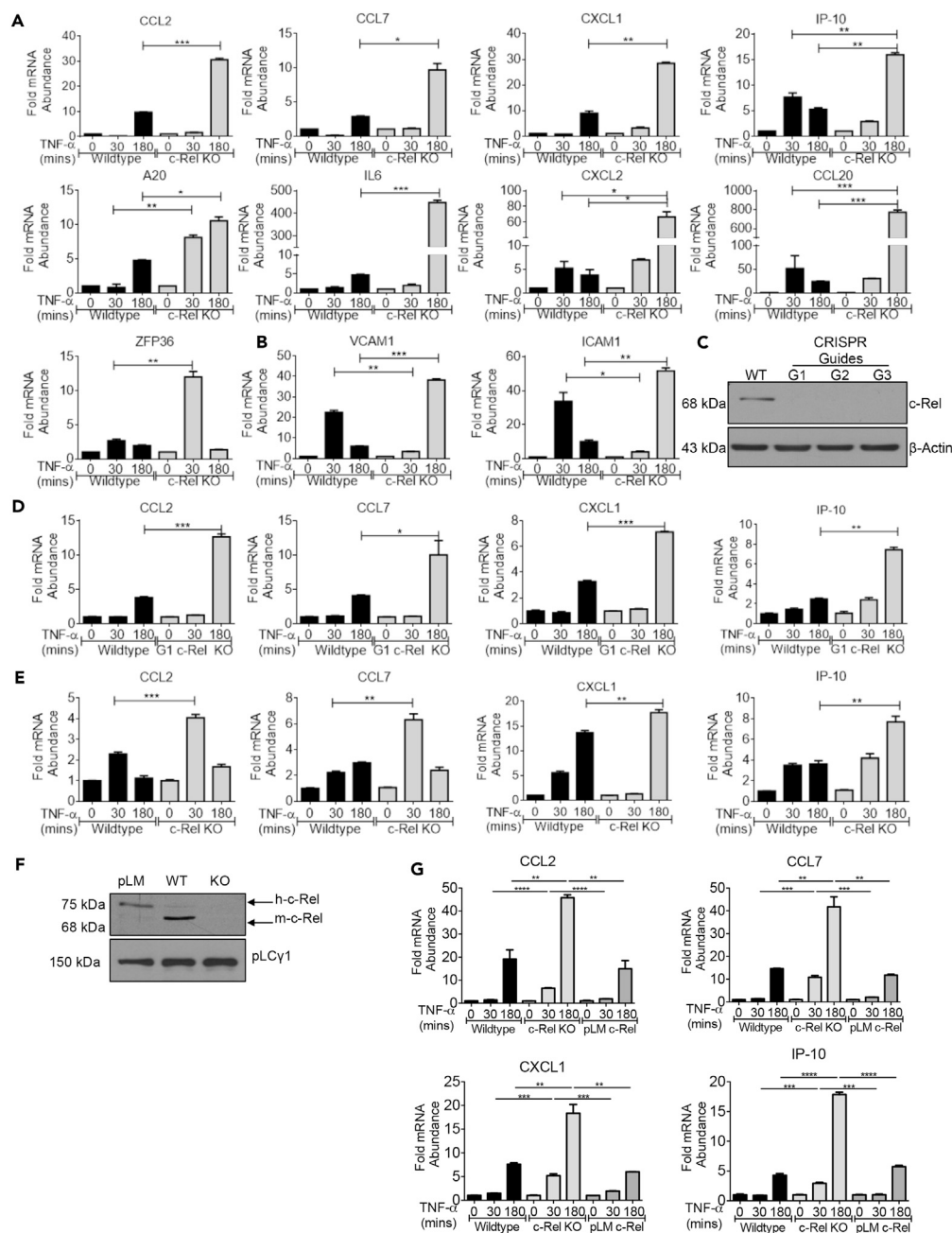


Figure 1. c-Rel Knockout Enhances TNF- α -Induced RelA-Dependent Proinflammatory Gene Expression

(A) Wild-type or c-Rel knockout MEFs (3×10^5 at time of harvest) were treated in a six-well plate with 100 ng/mL TNF- α for 30 min or 3 h.

(B) Wild-type or c-Rel knockout MEFs were cultured and treated as in (A).

(C) Wild-type MEFs were transduced with lentivirus expressing three distinct CRISPR guide sequences against c-Rel, G1, G2 and G3, selected with hygromycin and examined for the knockdown of c-Rel by western blotting.

(D) Pool of four clones from c-Rel CRISPR G1 knockout MEFs was treated as above, and qPCR was performed on indicated genes.

(E) BMDM cells generated from wild-type and c-Rel knockout mice were treated as above, and qPCR were performed on indicated genes.

(F) c-Rel knockout MEFs reconstituted with human-c-Rel by lentiviral transduction (pLM), wild-type (WT), and c-Rel knockout (KO) cell lysates were analyzed by western blotting. pLCy1 was used as a loading control for whole-cell lysates.

Figure 1. Continued

(G) c-Rel knockout MEFs re-expressing human c-Rel (pLM), wild-type, and c-Rel knockout MEFs (4×10^5 at time of harvest) were treated in a six-well plate with 100 ng/mL TNF- α for 30 min or 3 h. Samples were then analyzed by qPCR to determine the abundance of indicated mRNAs relative to that of ribosomal protein L32 (L32).

Data are representative of at least three independent experiments performed in triplicates, presented as mean \pm standard error of mean (SEM) ($n = 3$). p Values were obtained by unpaired Student's t test; ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. See also [Figure S1](#).

To confirm that gene expression changes observed are not cell type specific or associated with transformed MEFs, we generated primary macrophages from bone marrow of wild-type and c-Rel knockout mice. We found that c-Rel knockout murine bone marrow-derived macrophages (BMDM) also showed significant enhancement in the expression of selected TNF- α -induced genes ([Figure 1E](#)). Specifically, critical inflammatory chemokines and cytokines such as CCL2, CCL7, IP-10, and CXCL1 were significantly enhanced both in TNF- α -stimulated c-Rel CRISPR knockout MEF cells and BMDM. Much like in the MEFs ([Figure S1A](#)), genes such as TNF- α and I κ B- α showed markedly reduced expression in c-Rel knockout BMDM suggesting their dependence on c-Rel ([Figure S1B](#)). To further confirm that the enhanced expression of inflammatory genes in c-Rel knockout cells is not due to any secondary alteration in the cells, we re-expressed c-Rel in c-Rel knockout cells by lentiviral transduction ([Figure 1F](#)). We found that transient c-Rel expression reduced the enhanced expression of TNF-induced proinflammatory genes, CCL2, CCL7, CXCL1 and IP-10, in c-Rel knockout cells, indicating that c-Rel is directly involved in the suppression of these genes ([Figure 1G](#)). Taken together, these data show that c-Rel negatively regulates a specific subset of TNF- α -induced inflammatory genes, as well as demonstrates a physiological need for c-Rel for the expression of certain TNF- α -induced inflammatory genes.

c-Rel and Its Homologue, v-Rel, Suppresses TNF- α -Induced RelA Transcriptional Activity

Most of the proinflammatory genes we examined here were previously shown to be regulated by RelA transcriptional activity (Li et al., 2014b; Lim et al., 2007; Tourniaire et al., 2013). Consistent with this, RelA knockout decreased the expression of these inflammatory genes ([Figure S2](#)), demonstrating that c-Rel specifically targets a subset of RelA-dependent genes. To further study the molecular mechanism of c-Rel-mediated suppression of RelA activity, we utilized a reporter gene assay system using classical RelA-dependent promoters. We first examined the individual abilities of RelA and c-Rel to drive transactivation under I κ - κ B, IP-10, and A20 promoters. We found that, as previously reported in several studies (Amir-Zilberstein and Dikstein, 2008; Jin et al., 2017; Kempe et al., 2005), RelA induced substantial activation of the I κ - κ B, IP-10, and A20 promoters. c-Rel showed only nominal ability to activate all the three promoters tested ([Figure 2A](#)). Next, we co-transfected c-Rel with RelA and expected that the transactivating dimer complex, RelA:c-Rel, would show augmented reporter gene activity. To our surprise, we found that addition of c-Rel greatly inhibited RelA-dependent transactivation from I κ - κ B, IP-10, and A20 promoters ([Figure 2B](#)). We also studied the c-Rel-mediated repression of RelA-dependent transactivation under a physiologically meaningful condition of TNF- α stimulation. We utilized TNF- α stimulation in place of RelA overexpression to activate I κ - κ B and IP-10 promoters and found that c-Rel was indeed able to suppress TNF- α -induced transactivation of these promoters ([Figure 2C](#)).

c-Rel stands for the cellular homolog of v-Rel, a protein encoded in avian reticuloendotheliosis virus strain T (Rev T) (Capobianco et al., 1990; Carrasco et al., 1996). It is thought that v-Rel was acquired by Rev T via horizontal transfer of turkey c-Rel and that subsequent mutations resulted in structural alterations in v-Rel ([Figure 2D](#)) and lent v-Rel its high oncogenic potential (Hrdlickova et al., 1994). v-Rel and c-Rel have previously been shown to possess the ability to transcriptionally repress RelA, using HIV- κ B and IL-2- κ B promoter sites (Ballard et al., 1990, 1992). However, the suppressive abilities of v-Rel and c-Rel are not uniformly widespread across promoters as it has been shown that c-Rel activates transcription, whereas v-Rel represses transcription, under κ B sites of mouse H-2K^b promoter (Inoue et al., 1991). v-Rel has been shown to act both as a suppressor and an activator of transcription in the same cell type at different differentiation stages (Walker et al., 1992). Here, we examined the ability of v-Rel to influence inflammation-associated transactivation. We overexpressed v-Rel or RelA in HEK293Ts and found that v-Rel alone had no activity at our selected promoters ([Figure 2E](#)). We then co-expressed v-Rel and RelA and observed the same trend of suppression of RelA-driven transcriptional activity as seen with c-Rel ([Figure 2F](#)). We also examined the ability of v-Rel to inhibit TNF- α -induced transactivation and found that, similar to c-Rel, v-Rel also suppressed TNF- α -induced transcriptional activity ([Figure 2G](#)). Taken together, these data show that v-Rel's

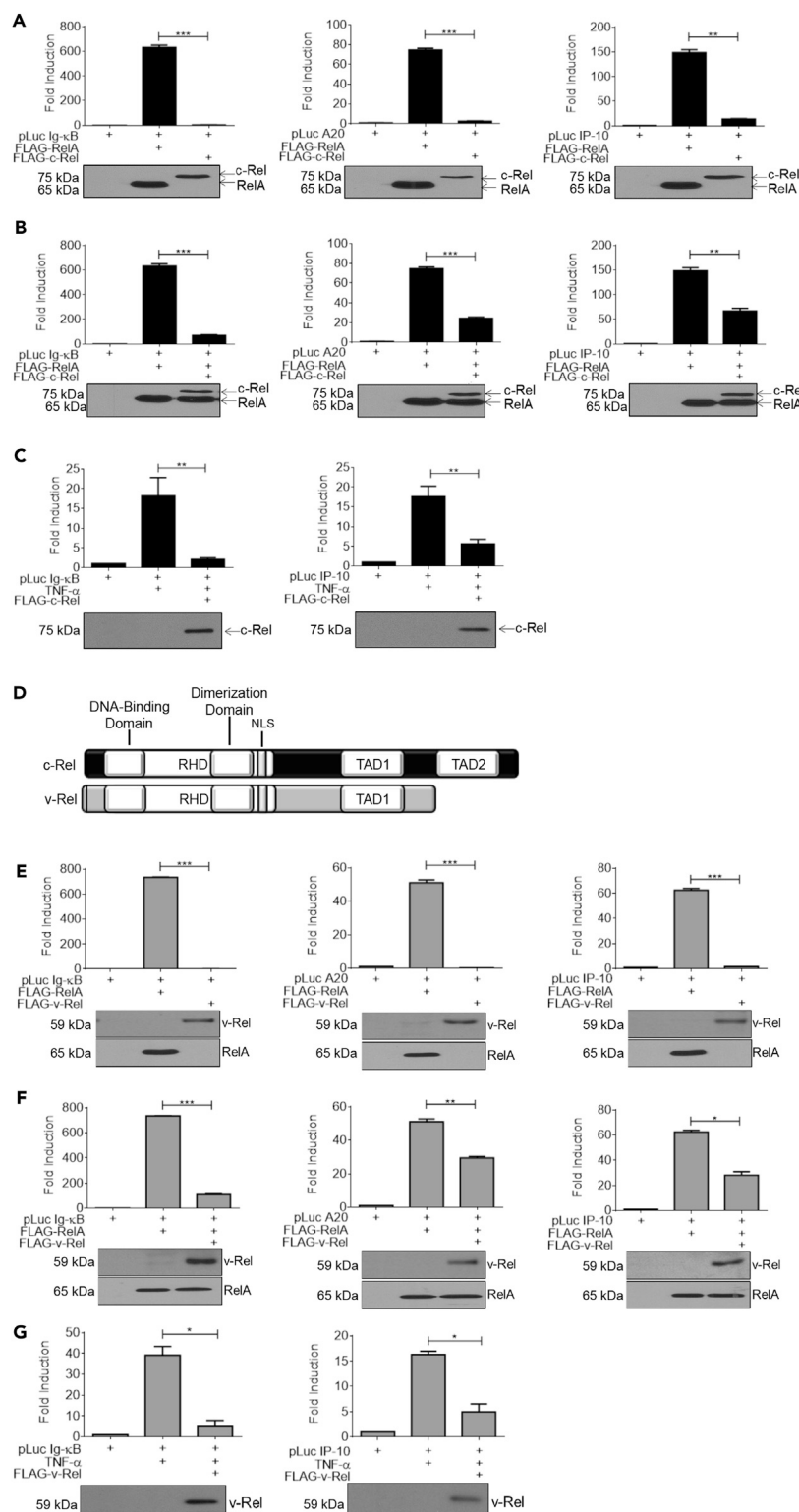


Figure 2. Both c-Rel and v-Rel Suppress TNF- α -Induced RelA-Dependent Transcriptional Activation

(A) HEK 293T cells (3×10^5) were transfected in a six-well plate with plasmids encoding FLAG-tagged RelA or c-Rel together with the indicated luciferase reporter plasmid.

Figure 2. Continued

(B) HEK 293T cells (3×10^5) were transfected in a six-well plate with FLAG-tagged RelA with or without FLAG tagged c-Rel plasmid and the indicated luciferase reporter plasmid.

(C) HEK 293T cells (3×10^5) were transfected in a six-well plate with the indicated luciferase reporter and c-Rel plasmids. Eighteen hours following transfection, cells were stimulated with 100 ng/mL TNF- α for 6 h.

(D) Schematic representation of c-Rel (top) and v-Rel (bottom) structures.

(E) HEK 293T cells (3×10^5) were transfected in a six-well plate with plasmids encoding FLAG-tagged RelA or v-Rel together with indicated luciferase reporter plasmid.

(F) HEK 293T cells (3×10^5) were transfected in a six-well plate with FLAG-tagged RelA and v-Rel along with luciferase reporter plasmids as indicated.

(G) HEK 293T cells (3×10^5) were transfected in a six-well plate with indicated luciferase reporter and v-Rel plasmids as indicated. Eighteen hours following transfection, cells were stimulated with 100 ng/mL TNF- α for 6 h. Luciferase activity was assessed using dual luciferase assay system. RelA alone values in the pairs Figures 2A, 2B, 2E, and 2F were from the same representative experiment for accurate comparison. Luciferase activity was assessed using dual luciferase assay system.

Data are presented as mean \pm standard error of mean (SEM). p Values were obtained by unpaired Student's t test;

***p < 0.001, **p < 0.01, *p < 0.05. (A–H) Top: Data in bar graphs are representative of three independent experiments performed in triplicates (n = 3). RelA alone values in Figures 2A and 2B were from the same representative experiment for accurate comparison. (A–H). Bottom: Western blotting analysis of total cell lysates of luciferase assay using anti-FLAG-tag antibody. See also Figure S2.

specific repressive ability is likely conserved from c-Rel and the various mutations and structural changes that it acquired did not compromise its repressive function.

Suppressive Ability of c-Rel on TNF- α -Induced Signaling Is Not Conserved in RelB

Since the c-Rel and RelB subunits are structurally similar, we also tested the ability of RelB, with transactivation domain, to act as a repressor of RelA-induced transactivation. We found that RelB, like c-Rel and v-Rel, did not induce transcriptional activation under Ig- κ B, IP-10, or A20 promoters (Figure 3A). Unlike c-Rel and v-Rel, RelB was unable to suppress RelA-induced transactivation of Ig- κ B and A20 promoters, and it caused only a modest decrease in IP-10 promoter activation (Figure 3B). This also holds true for TNF- α -induced transcriptional activity, with RelB being unable to suppress Ig- κ B or IP-10 promoters (Figure 3C).

We studied whether deficiency of RelB at physiological conditions affects expression of TNF- α -induced RelA-dependent genes, whose expression was increased in the absence of c-Rel (Figure 1), using wild-type and RelB KO cells. Interestingly, RelB knockout MEFs did not show enhanced TNF- α -induced gene expression, suggesting that RelB does not play an inhibitory role like c-Rel. Expressions of genes such as CCL2, CCL7, and IP-10 were found decreased in RelB knockout cells following TNF stimulation, whereas no significant effect was found on CXCL1 expression (Figure 3D). Expressions of several other inflammatory genes were compromised in RelB-deficient cells, indicating a possible role requirement of RelB in TNF- α -induced expression of these genes (Figure S3A). We found that RelB showed transient binding to Ig- κ B sequences (Figure S3B top panels) and strong sustained binding to CCL2- κ B site (Figure S3B middle panels) in oligonucleotide pull-down assays, which is consistent with the significant reduction of CCL2 expression in RelB knockout cells (Figure 3D). We also confirmed that the decreased gene expression in RelB knockout cells was not due to a defect in TNF- α -induced classical NF- κ B activation. We found that TNF- α -induced RelA nuclear translocation and I κ B α degradation occurred to a similar extent in wild-type and RelB knockout cells (Figure 3E). This shows that repression of TNF- α -induced gene expression is a unique characteristic of c-Rel, and the RelB subunit, with a conserved REL homology domain and transactivation domain, is unable to execute repressive role like c-Rel.

c-Rel Knockout Enhances TNF- α -Induced Nuclear Translocation and DNA Binding of RelA

To further dissect the molecular events that result in enhanced TNF- α -induced RelA-dependent gene expression, we examined RelA protein amounts, its nuclear translocation, and DNA binding in control and c-Rel knockout cells. First, we examined whether a deficiency of c-Rel or other NF- κ B subunits affect RelA protein amounts and found comparable RelA in all the cell types tested (Figure 4A). This confirms that the observed increase in RelA-dependent gene expression in c-Rel knockout cells is not due to an increase in the total protein amounts of RelA. Next, we examined the nuclear translocation of RelA following TNF- α stimulation in control and c-Rel knockout cells and found a substantial increase in rapidly translocated RelA in the nucleus of c-Rel knockout cells (Figure 4B). To exclude the possibility that the enhanced

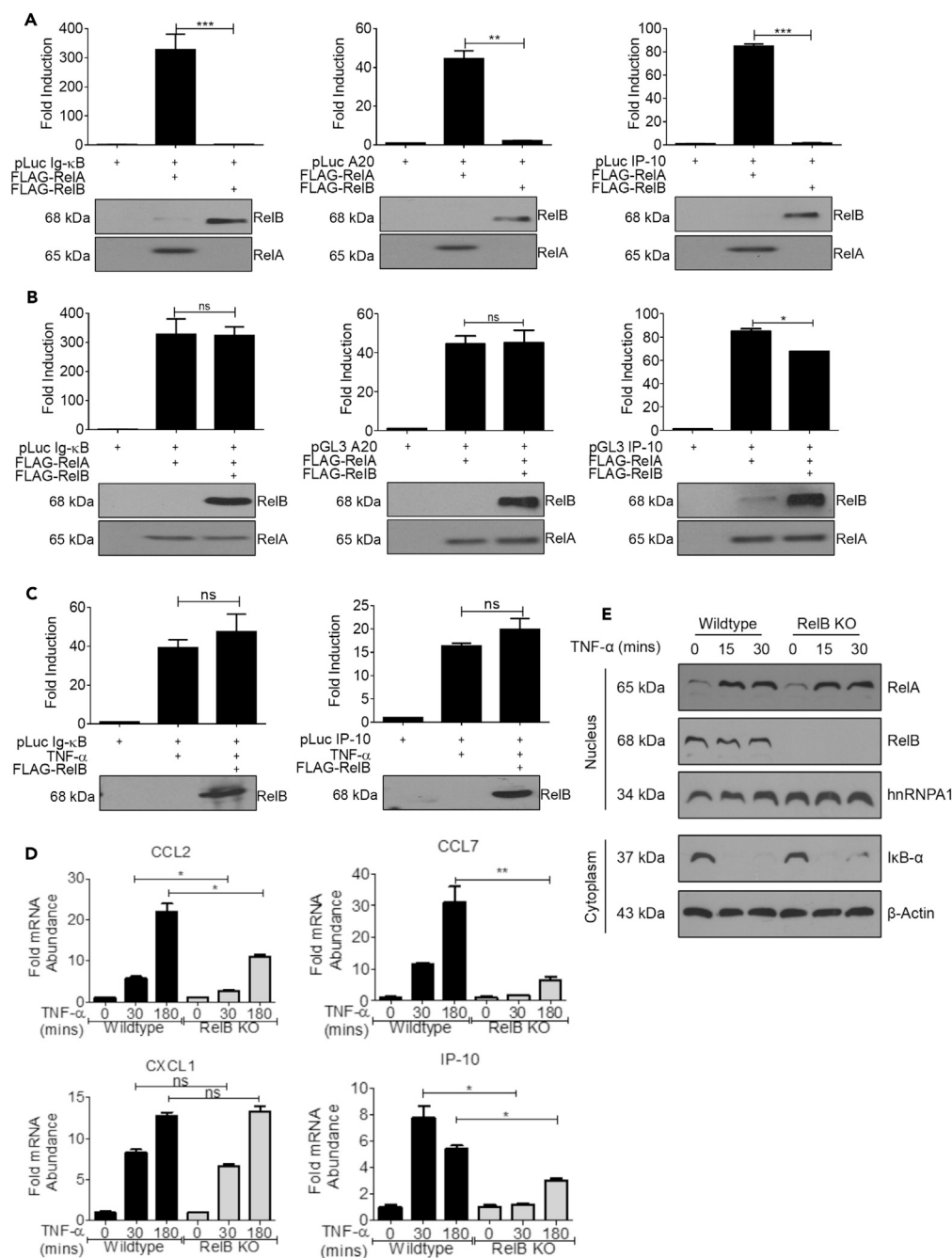


Figure 3. Suppressive Ability of c-Rel on TNF- α -Induced Signaling is Not Conserved in RelB

(A) HEK 293T cells (3×10^5) were transfected in a six-well plate with plasmids encoding FLAG-tagged RelA or RelB together with indicated luciferase reporter plasmid.

(B) HEK 293T cells (3×10^5) were transfected in a six-well plate with FLAG-tagged RelA with or without FLAG tagged RelB plasmid and the indicated luciferase reporter plasmid.

(C) HEK 293T cells (3×10^5) were transfected in a six-well plate with indicated luciferase reporter and RelB plasmids as indicated. Eighteen hours following transfection, cells were stimulated with 100 ng/mL TNF- α for 6 h. Luciferase activity was assessed using dual luciferase assay system. (A–C) Top: Data in bar graphs are representative of three independent experiments performed in triplicates. Bottom: Western blotting analysis of total cell lysates of luciferase assay using anti-FLAG-tag antibody. Blots are representative of three independent experiments. RelA alone values in Figures 3A and 3B were from the same representative experiment for accurate comparison.

Figure 3. Continued

(D) Wild-type or RelB knockout MEFs were treated with 100 ng/mL TNF- α for 30 min or 3 h. Samples were then analyzed by qPCR to determine the abundance of indicated mRNA relative to that of ribosomal protein L32 (L32). Data are presented as mean \pm standard error of mean (SEM) (n = 3). p Values were obtained by unpaired Student's t test; ***p < 0.001, **p < 0.01, *p < 0.05, ns not significant.

(E) Wild-type or RelB knockout MEFs were treated with 100 ng/mL TNF- α for 15 or 30 min. Nuclear and cytoplasmic extracts were analyzed with antibodies against the indicated proteins. hnRNPA1 was used as the loading control for nuclear fraction, and actin was used as the loading control for cytoplasmic fraction.

See also [Figure S3](#).

response to TNF- α is due to a change in the surface expression of TNF receptor (TNFR), we examined the TNFR cell surface expression in control and c-Rel knockout MEFs and found comparable TNFR levels in both the cell types ([Figure S4A](#)). To confirm that the enhanced RelA activation observed in c-Rel knockout MEFs is not a cell-type-dependent phenomenon, we also studied the TNF- α -induced RelA nuclear translocation in primary BMDM. We found that, similar to MEFs, c-Rel knockout primary BMDM also showed enhanced TNF- α -induced RelA nuclear translocation ([Figure 4C](#)). However, nuclear translocation of NF- κ B is insufficient to prove its transcriptional potential, which requires its binding to cognate DNA elements. To determine whether the additional RelA in the nucleus of c-Rel deficient cells was DNA binding competent, we performed oligonucleotide pull-down assays using biotinylated Ig- κ B oligos. In line with the enhanced RelA-dependent gene expression observed in c-Rel-deficient cells, we found considerable increase in RelA DNA binding at the early TNF- α stimulated time point ([Figure 4D](#)). Furthermore, we also found an enhancement of p50 DNA binding along with RelA, suggesting TNF- α -induced robust activation of prototypical NF- κ B dimers containing RelA and p50 subunits in c-Rel-deficient cells ([Urban et al., 1991](#)). We also examined whether the increase in RelA/p50 dimer binding to the DNA results from an increase in the preexisting RelA/p50 complex in the absence of c-Rel. We immunoprecipitated RelA and p50 from the cytoplasmic fractions of wild-type and c-Rel KO cells and found no significant changes in the proportion of RelA or p50 ([Figure 4E](#)). However, c-Rel deficiency resulted in enhanced I κ B α binding of both RelA and p50 ([Figure 4E](#)). We further explored the mechanism of enhanced transactivation in c-Rel KO cells; however, we did not observe any substantial change in the binding of the co-activator p300 to the Ig- κ B oligonucleotides in c-Rel knockout cells at the same time point when enhanced RelA and p50 binding was observed ([Figure S4B](#)).

c-Rel Recruits the Co-repressor HDAC1 to the RelA-Dependent Promoters

To further validate the enhanced DNA binding of RelA in c-Rel knockout cells under physiologically relevant endogenous levels, we performed chromatin immunoprecipitation (ChIP) using anti-RelA antibody. We examined the enrichment of RelA-dependent promoters, IP-10 and CXCL1, by qPCR and found that TNF- α -induced RelA binding at both promoters was significantly enhanced in c-Rel knockout cells ([Figure 5A](#)). We also performed ChIP using anti-c-Rel antibody to confirm that c-Rel indeed binds to these RelA-dependent promoters. Our results show that c-Rel is present at the IP-10 and CXCL1 promoters at the basal state. Moreover, c-Rel occupancy at these promoters decreased, whereas RelA occupancy increased following TNF- α stimulation ([Figure 5B](#)), suggesting that c-Rel recruited to the promoters of selected RelA-dependent genes may repress transcriptional activation. Next, we studied the mechanism of c-Rel-mediated transcriptional repression by examining the binding of c-Rel to co-repressors. We found that c-Rel was bound to HDAC1 at basal state and this binding was enhanced by TNF stimulation ([Figure 5C](#)). c-Rel also showed weak binding to HDAC3, and no c-Rel binding was observed with HDAC2 and HDAC4 ([Figure 5C](#)). We performed oligonucleotide pull down with Ig- κ B oligos and found that c-Rel deficiency completely blocked HDAC1 binding to the DNA ([Figure 5D](#)), suggesting that c-Rel is critical for HDAC1 recruitment to the promoter. Similar to co-immunoprecipitation experiments, oligonucleotide pull down also showed no HDAC2 and HDAC4 binding to the c-Rel containing complex. We did not observe any change in basal HDAC3 binding to the DNA in wild-type of c-Rel KO cells, and TNF stimulation decreased HDAC3 in the DNA bound complexes ([Figure 5D](#)). We also performed ChIP using anti-HDAC1 antibody to study the c-Rel-dependent HDAC1 recruitment at physiological conditions. We found that absence of c-Rel significantly decreased HDAC1 occupancy at IP-10 and CXCL1 promoters ([Figure 5E](#)), suggesting that lack of co-repressor recruitment may contribute to the enhanced transactivation of these promoters.

c-Rel Competitively Blocks DNA Binding of RelA

All members of the NF- κ B family possess the ability to bind to the DNA through their N-terminal DNA-binding domains ([Ghosh and Hayden, 2008](#)). The N-terminal half of c-Rel homodimer has been crystallized

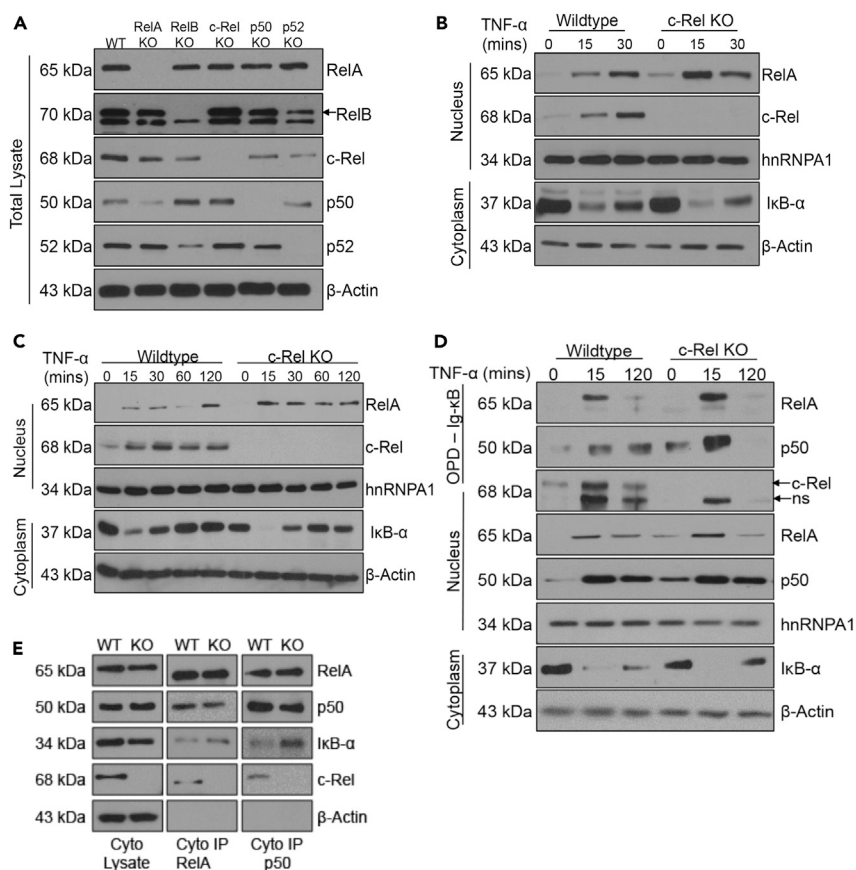


Figure 4. c-Rel Knockout Enhances TNF- α -Induced Nuclear Translocation and DNA Binding of RelA

(A) Total cell lysates of wild-type and the knockouts of RelA, RelB, c-Rel, p50, and p52 MEFs were analyzed with antibodies to each of the indicated NF- κ B proteins. Actin was used as the loading control.

(B) Wild-type or c-Rel knockout MEFs were left untreated or treated with 100 ng/mL TNF- α for 15 or 30 min. Nuclear and cytoplasmic extracts were analyzed with antibodies against the indicated proteins. hnRNPA1 was used as the loading control for nuclear fraction, and actin was used as the loading control for cytoplasmic fraction.

(C) Primary bone marrow-derived macrophages generated from wild-type or c-Rel knockout mice were left untreated or stimulated with 100 ng/mL TNF- α for the indicated time points. Nuclear and cytoplasmic extracts were analyzed with antibodies against the indicated proteins.

(D) Wild-type or c-Rel knockout MEFs (3×10^6 at time of harvest) were left untreated or treated with 100 ng/mL TNF- α for 15 or 120 min. Nuclear and cytoplasmic extracts were prepared, and 100 μ g of nuclear proteins per sample was utilized in an *in vitro* pull-down assay using biotinylated Ig- κ B oligonucleotide. The precipitated proteins were separated in SDS/PAGE gel and probed for RelA, p50, and c-Rel. ns indicates non-specific band. Nuclear and cytoplasmic extracts were also probed using the indicated antibodies to examine the extent of their nuclear translocation as well as the degradation of I κ B in the cytoplasm.

(E) Cytoplasmic extracts were prepared from wild-type or c-Rel knockout MEFs. Cytoplasmic lysates and immunoprecipitates of RelA and p50 antibodies were analyzed with the antibodies against indicated proteins.

Data for (A), (B), (D), and (E) are representative of four independent experiments, and C is representative of three independent experiments. See also Figure S4.

with the CD28 response element in the IL-2 promoter. It was shown that five critical, highly conserved residues in the DNA-binding domain of chicken c-Rel (arginine²¹, arginine²³, tyrosine²⁴, glutamic acid²⁷, and arginine¹⁷⁸) mediate direct base contact (Huang et al., 2001). Tyrosine²⁴ in chicken c-Rel was also shown to mediate van der Waals interaction with the methyl groups of the DNA as well as directly interact with the DNA backbone (Huang et al., 2001). Therefore, we chose to mutate tyrosine²⁵ in human c-Rel (the corresponding residue of tyrosine²⁴ in chicken c-Rel), which is conserved also in v-Rel (Figure 6A), to disrupt the DNA binding of c-Rel. We introduced a conventional point mutation in the DNA-binding domain of c-Rel, tyrosine²⁵ to phenylalanine (Y25F), and found that c-Rel Y25F failed to inhibit RelA-mediated

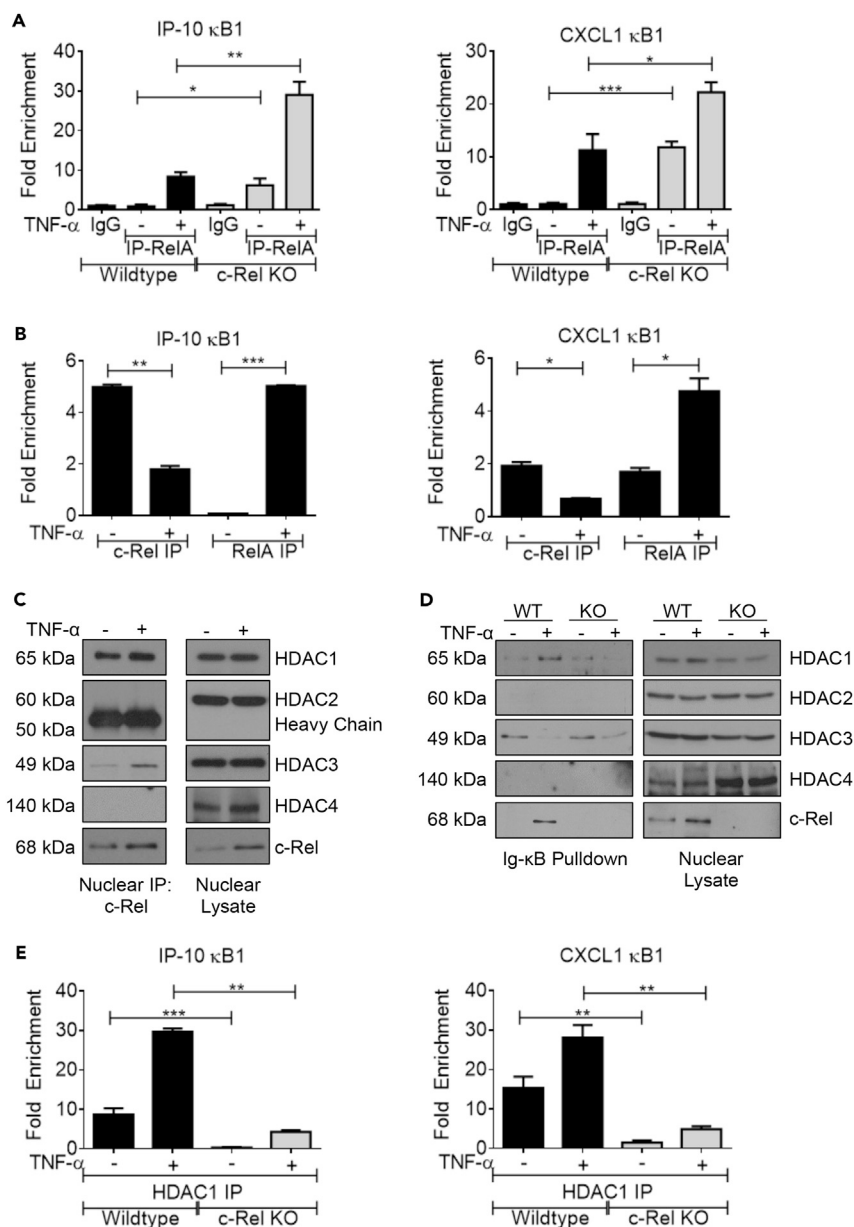


Figure 5. c-Rel Recruits the Co-repressor HDAC1 to the RelA-Dependent Promoters

(A) Wild-type or c-Rel knockout MEFs (30×10^6 cells/condition at time of harvest) were left untreated or treated in 3×15 -cm plates per condition with 100 ng/mL TNF- α for 15 min. Chromatin immunoprecipitation (ChIP) was performed using control IgG or RelA antibody.

(B) Wild-type MEFs were treated as in (A) and ChIP was performed using anti-c-Rel or anti-RelA antibodies. Enrichment of IP-10 and CXCL1 promoter regions in the ChIP samples were examined by qPCR in triplicates.

(C) Wild-type MEFs were treated with TNF- α for 15 min, and nuclear lysates were immunoprecipitated using anti-c-Rel antibody and examined for binding to the indicated HDACs.

(D) Oligonucleotide pull-down assay was performed as in Figure 4D, and the precipitated proteins were separated in SDS/PAGE gel and probed for indicated HDACs and c-Rel.

(E) Wild-type and c-Rel KO cells were treated as in (A), and ChIP was performed using anti-HDAC1 antibody.

(A)–(E) $n = 4$. Data are presented as mean \pm standard error of mean (SEM). p Values were obtained by unpaired Student's t test; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

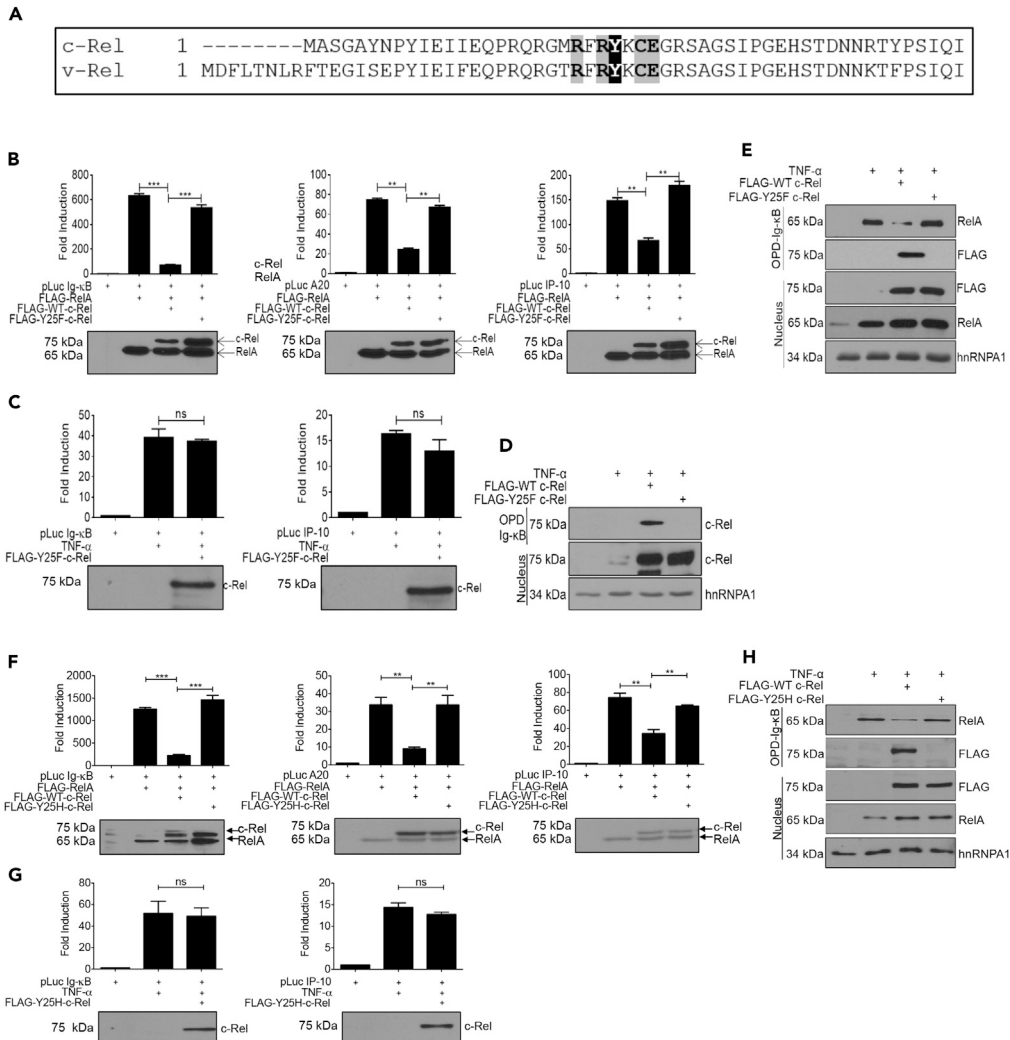


Figure 6. A Point Mutation, Y25F or Y25H, Disrupting c-Rel's DNA-Binding Ability Compromises Its Repressive Function on RelA-Dependent Transactivation

(A) Amino acid sequence alignment of N-terminal DNA-binding regions of c-Rel and v-Rel, with critical residues for DNA binding highlighted.

(B) HEK 293T cells (3×10^5) were transfected in a six-well plate with plasmids encoding FLAG-tagged RelA and WT or Y25F c-Rel together with luciferase reporter plasmids as indicated in the figure.

(C) HEK 293T cells (3×10^5) were transfected in a six-well plate with FLAG-tagged Y25F c-Rel and the indicated luciferase reporter plasmid. Eighteen hours following transfection, cells were stimulated with 100 ng/mL TNF- α for 6 h. Luciferase activity was assessed using a dual luciferase assay system. Data are presented as mean \pm standard error of mean (SEM). p Values were obtained by unpaired Student's t test; ***p < 0.001, **p < 0.01, ns non significant. (B and C) Data in bar graphs are technical triplicates representative of three independent experiments (n = 3). RelA alone value in Figure 2A was from the same representative experiment in Figure 2A for accurate comparison. Bottom: Western blotting analysis of total cell lysates of luciferase assay using anti-FLAG-tag antibody. Blots are representative of three independent experiments.

(D) c-Rel knockout MEFs (3×10^6) were transfected with FLAG-tagged wild-type or Y25F c-Rel. Eighteen hours following transfection, MEFs were left untreated or treated with 100 ng/mL TNF- α for 15 min. Nuclear lysates equivalent to 200 μ g of nuclear proteins per sample were utilized for *in vitro* pull-down assay using the Ig- κ B oligonucleotide. Nuclear lysates were probed with indicated antibodies. hnRNPA1 was used as loading control.

(E) HEK 293T cells (2×10^6 /10-cm plate) were transfected with FLAG-tagged wild-type or Y25F c-Rel. Cells were stimulated and analyzed as in (D).

(F) Cells were transfected with c-Rel Y25H, and experiments were performed as in (B).

(G) Cells were transfected with c-Rel Y25H, and experiments were performed as in (C).

(H) HEK 293T cells were transfected with FLAG-tagged wild-type or Y25H c-Rel as in (E). Cells were stimulated and analyzed as in (D).

Data for (D), (E), and (H) are representative of three independent experiments. See also Figure S5.

activation of Ig- κ B, A20, or IP-10 promoters (Figure 6B). We also examined the ability of c-Rel Y25F to inhibit TNF- α -induced transactivation and found that the Y25F mutation completely blocked the ability of c-Rel to suppress TNF- α -induced transcriptional activity (Figure 6C). We also found that the Y25F mutation in c-Rel completely abolishes c-Rel's DNA-binding ability (Figure 6D). To determine whether c-Rel disrupts DNA binding of RelA by competitively binding to the same κ B sites, we overexpressed either wild-type or Y25F mutant c-Rel, which were expressed at similar levels, and studied RelA binding to the Ig- κ B promoter. We found that overexpression of wild-type c-Rel substantially decreased DNA binding of RelA, whereas c-Rel Y25F neither bound DNA nor blocked DNA binding of RelA (Figure 6E). We confirmed that the Y25F mutation in c-Rel does not cause any gross structural defect, as it did not affect c-Rel's ability to dimerize with RelA or its ability to homodimerize with wild-type c-Rel (Figure S5A).

Intriguingly, a missense single nucleotide variation has been reported in human c-Rel, a change of the base T to C, that results in the codon change TAC to CAC, resulting in the amino acid substitution of tyrosine²⁵ to histidine. This SNP was identified in the c-Rel locus in a whole-genome sequencing study of five indigenous southern African Khoisan hunter-gathers (Schuster et al., 2010); however, no clinical significance has been correlated with this SNP yet. We studied the functional effect of histidine substitution at Y25 of c-Rel by transiently expressing c-Rel Y25H in cells. We found that, similar to Y25F mutation, c-Rel Y25H mutation also compromised c-Rel's ability to suppress RelA-induced (Figure 6F) and TNF- α -induced (Figure 6G) transactivation. c-Rel Y25H neither showed the ability to bind to the DNA nor interfered with the DNA binding of RelA (Figure 6H).

We also examined the relative affinity of c-Rel to bind to the Ig- κ B site in comparison with RelA. To do this, we transiently expressed FLAG-tagged wild-type c-Rel and RelA in HEK 293 cells, stimulated the cells with TNF- α to induce their nuclear translocation, and examined their affinity to bind Ig- κ B promoter. We found that both c-Rel and RelA were translocated to the nucleus at comparable levels, yet c-Rel showed significantly enriched binding to the Ig- κ B promoter at physiological salt concentration and its binding was retained even at high salt concentration of 250 mM, showing that c-Rel has high binding affinity as well as high avidity to bind RelA-binding sites in the DNA (Figure S5B).

c-Rel Represses TNF- α -Induced Inflammatory Gene Expression In Vivo

TNF- α injection into mice provides an experimental model to study acute inflammation *in vivo* (Van Bogaert et al., 2011), and it has been shown to enhance the expression of proinflammatory genes in the liver (Cattrysse et al., 2016). To investigate the *in vivo* relevance of our findings, we injected wild-type and c-Rel knockout mice with TNF- α and studied its effect on the *in vivo* DNA binding of c-Rel and RelA by ChIP as well as on the expression of proinflammatory genes in the liver. We found that c-Rel indeed binds to the RelA-dependent promoters, IP-10 and CXCL1, *in vivo* (Figure 7A) and its binding was found increased at the time point when RelA binding was decreased at these promoters (Figure 7B). Consistent with the *ex vivo* cellular experiments, liver cells of c-Rel knockout mice showed enhanced and sustained RelA DNA binding at IP-10 promoter (Figure 7C left). RelA binding at CXCL1 promoter showed a difference in kinetics in c-Rel knockout liver cells, with decreased binding at early time point and substantially increased binding at later time point (Figure 7C right). We also examined the expression of IP-10 and CXCL1 in the liver cells and found that c-Rel deficiency significantly enhanced the acute *in vivo* expression of TNF- α -induced IP-10 (Figure 7D top). Analysis of CXCL1 expression revealed that, unlike IP-10, the early expression level of CXCL1 was not enhanced, but its expression was sustained with high levels maintained at 3 h post TNF- α injection (Figure 7D bottom).

Taken together, these results demonstrate that c-Rel is a physiologically relevant repressor of TNF- α -induced RelA-dependent transactivation and that it can recruit HDAC1 and competitively bind RelA binding regions in the DNA and thereby selectively repress proinflammatory gene expression (Schematic model, Figure 7E).

DISCUSSION

Here, we identified a role for c-Rel as a repressor of TNF- α -induced, RelA-dependent, pro-inflammatory gene transcription. Based on the canonical function of transactivation domains, we expected that all the NF- κ B subunits with transactivation potential, when overexpressed in cells, would behave promiscuously in their ability to induce transcription from prototypical NF- κ B promoters. Counterintuitively, c-Rel not only showed poor transactivation potential at Ig- κ B, IP-10, and A20 promoters but also significantly

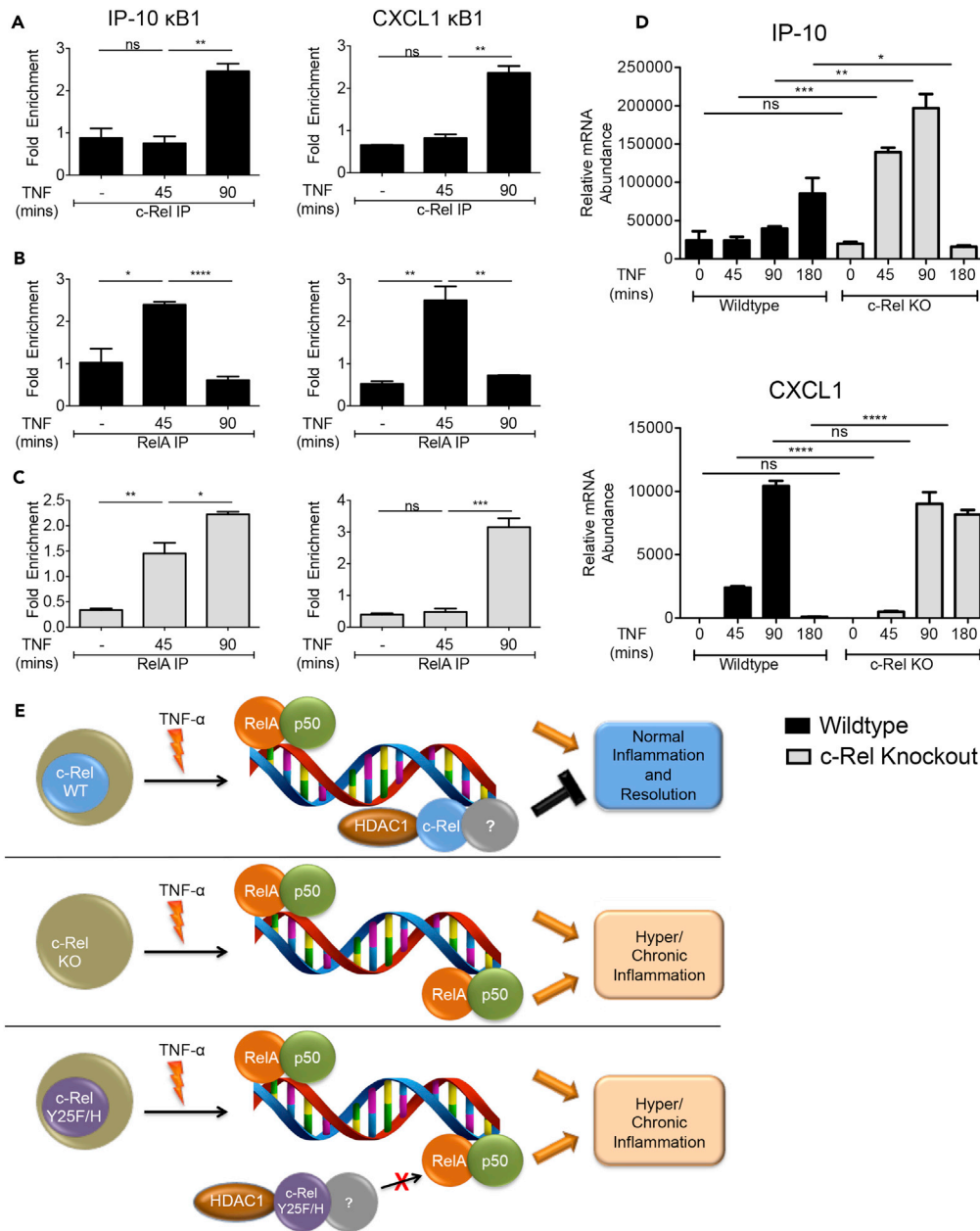


Figure 7. c-Rel Represses RelA DNA Binding and Transactivation In Vivo

(A–D) Wild-type or c-Rel knockout mice (n = 3) were injected with PBS or TNF- α (5 μ g/mouse). Mice were euthanized at 45, 90, or 180 min after injection, and single-cell suspension of liver was processed for chromatin immunoprecipitation and qPCR. ChIP was performed using control IgG and either c-Rel (A) or RelA (B and C) antibodies. Enrichment of IP-10 and CXCL1 promoter regions in the ChIP samples were examined by qPCR in triplicate. (D) TNF- α -induced *in vivo* expression of IP-10 (top) and CXCL1 (bottom) relative to that of ribosomal protein L32 in the liver was examined by qPCR in triplicate. Data are presented as mean \pm standard error of mean (SEM). p Values were obtained by unpaired Student’s t test; ****p < 0.0001, ***p < 0.001, **p < 0.01, ns non-significant.

(E) Hypothetical schematic model describing DNA-binding-dependent suppression of RelA-dependent transcription by c-Rel. Top. c-Rel-containing dimers with co-repressor, HDAC1, occupy certain RelA-binding sites limiting RelA-induced gene expression. Middle. Absence of c-Rel exposes sites repressed by c-Rel for RelA binding and enhanced inflammatory gene expression. Bottom. Mutation of Y25 in c-Rel blocks c-Rel’s DNA binding allowing enhanced RelA binding and inflammatory gene expression.

suppressed RelA-induced transactivation of these promoters as well as several RelA-induced proinflammatory genes. The repression by c-Rel was not a generic effect as several genes showed decreased induction in the absence of c-Rel (Figures S1A and S1B), suggesting that c-Rel has both activator and suppressor roles following TNF- α stimulation.

The suppressor role of c-Rel was found to be conserved in v-Rel as well, an ability that would be highly advantageous to the reticuloendothelial virus when trying to survive long enough to replicate in a host. Interestingly, RelB was unable to suppress RelA as well as TNF- α -induced gene expression. Both homodimer and heterodimer of RelB with p50 have been shown to bind DNA in co-crystallization studies with consensus NF- κ B sequences (Huang et al., 2005; Moorthy et al., 2007). We also found RelB binding to κ B sites (Figure S3B), consistent with the suggested ability of RelB containing heterodimers to possess similar DNA binding specificity like c-Rel and RelA-containing heterodimers (Siggers et al., 2011). It has also been shown that stably overexpressed RelB can inhibit RelA activity by sequestering RelA and preventing its DNA binding (Marienfeld et al., 2003). Despite its abilities to bind consensus NF- κ B sequences and to inhibit RelA in an enforced expression system, why RelB does not interfere with RelA-induced transactivation under these conditions remains to be addressed. Furthermore, RelB has been shown to bind DNA poorly *in vivo*, most likely due to the sequestration of RelB-containing complexes by NF- κ B p100 (Derudder et al., 2003) or their repression by RelA binding (Jacque et al., 2005), which may restrict its DNA binding ability. This suggests that, although c-Rel and RelB are generally classified as transactivating members of NF- κ B family, there are inherent differences in the manner in which they form functional activating or repressing dimers and bind DNA, which warrants further structure-function studies.

The repressor function of c-Rel on RelA-dependent transactivation was found to be dependent on its DNA binding ability. The higher affinity of c-Rel to bind to the κ B promoters (Figure S5B), along with increased RelA DNA binding in the absence of c-Rel and decreased RelA DNA binding upon c-Rel overexpression, points to a competitive blockade of RelA binding by c-Rel. However, more comprehensive studies are necessary to delineate whether the c-Rel-containing dimers preoccupy the RelA site preventing RelA's access or they competitively displace RelA complexes from the DNA based on c-Rel's higher affinity for the κ B sites. In the transient co-expression experiments using FLAG-tagged proteins, despite the lower expression levels (Figures 2B and 6B), c-Rel was able to cause significant suppression at the studied promoters and showed higher binding strength to the Ig- κ B-site. This is logically sound, especially outside of the lymphoid and myeloid compartments, where RelA expression has been shown to be much higher in most of these cell types than c-Rel (Oeckinghaus and Ghosh, 2009); an activated system still needs to suppress an over-stimulated cell's response even with a paucity of c-Rel.

Our results show that lack of c-Rel does not lead to an increase in RelA:p50 dimers; however, we found an increase in both RelA:I κ B- α and p50:I κ B- α complexes (Figure 4E). This indicates that c-Rel deficiency may result in an enhancement of RelA and p50 homodimers or other heterodimers containing RelA and p50 that may account for enhanced RelA nuclear translocation observed in c-Rel knockout cells. Our results also show that the enhanced RelA-dependent transactivation in c-Rel knockout cells is not due to increased p300 binding in the transcription complex at the minimal promoter sequence (Figure S4B). However, it is possible that p300 or other co-activators may show enhanced binding at a distal locus in the promoter region and enhance the gene expression. Whether the repressor dimer containing c-Rel is a homodimer or a heterodimer with p50 or other NF- κ B subunit is an interesting question to address. Our Ig- κ B oligonucleotide pull-down data show the presence of c-Rel and p50 in the wild-type cells at 120 min of TNF- α stimulation (Figure 4D). However, DNA-binding p50 is absent at the 120-min time point in c-Rel knockout cells, implying a need for c-Rel/p50 heterodimers to bind DNA at this later time point. This may very well be the dimer that represses RelA transcriptional activity.

In addition to its role in DNA binding, c-Rel also plays a role in the recruitment of co-repressor HDAC1 to an open promoter site, with no substantial effect on HDAC3 binding (Figures 5D and 5E). The increased TNF-induced NF- κ B-dependent HDAC1 recruitment is consistent with previous studies, which show the enhancement of both HATs and HDACs at active transcription sites (Li et al., 2014a; Peserico and Simone, 2011; Wang et al., 2009). c-Rel contains two transactivating domains and does in fact drive transcriptional activation at several κ B sites (Rao et al., 2003; Sanjabi et al., 2000; Siebenlist et al., 1994). In spite of the presence of two TADs, why c-Rel acts as an inhibitor at selected sites and whether this is a sequence-specific phenomenon remain to be determined. It appears that c-Rel is imperative for proper immune function

as well as to fine-tune the quantitative output of RelA-induced transactivation by setting a threshold limiting the immune response and keeping smaller insults from generating disproportionately large inflammatory responses.

c-Rel-deficient mice have normal immunological development but poor immunological function (Gilmore and Gerondakis, 2011). c-Rel deficiency has been shown to result in an increased systemic inflammatory response as a result of polymicrobial sepsis in a cecal ligation and puncture mouse model (Courtine et al., 2011). The increased mortality in this model could stem from an increased proinflammatory cytokine storm or decreased expression of c-Rel-dependent genes regulating survival or compromised adaptive immune response. We also found an enhanced inflammatory response in a c-Rel deficient non-obese diabetic (NOD) mouse model that showed greatly accelerated diabetogenesis, a tell-tale consequence of enhanced pancreatic inflammation (Ramakrishnan et al., 2016). In contrast to these anti-inflammatory roles of c-Rel, several studies have also reported a proinflammatory role of c-Rel in systemic inflammation in collagen-induced arthritis (Campbell et al., 2000) and ovalbumin-induced pulmonary inflammation (Donovan et al., 1999). How is it possible that c-Rel displays both pro- and anti-inflammatory roles, in a cell type- or disease-dependent manner? We show here that c-Rel acts as a repressor of only a specific subset of TNF- α -induced RelA-driven proinflammatory genes (Figure 1). We and others have shown that c-Rel also positively regulates the expression of several proinflammatory genes such as IL-2, GM-CSF, IFNG, IP10, ICAM1, and TNF (Carmody et al., 2007; Chen et al., 2010; Gilmore and Gerondakis, 2011; Ramakrishnan et al., 2013); immunosuppressive FOXP3 in T regulatory cells (Isomura et al., 2009); and feedback regulators of NF- κ B activation such as I κ B- α (Figure S1). We show here that c-Rel is not a global suppressor of TNF- α -induced gene expression, and in fact several inflammatory genes are indeed dependent on c-Rel, including TNF- α itself (Figure S1), and several others show altered expression kinetics in the absence of c-Rel (Figure 1B). Specifically, c-Rel was found required for auto-induction of TNF- α following TNF- α stimulation (Figure S1), and this dampening of TNF- α expression may limit self-sustaining chronic inflammatory cascade. This decrease in TNF- α expression in c-Rel knockout cells is in line with the protective effect seen in c-Rel knockout mice for TNF- α -dependent inflammatory diseases such as rheumatoid arthritis (Campbell et al., 2000).

Providing a direct correlation of our findings with human disease relevance, a recent study reported a patient with a homozygous mutation causing functional knockout of c-Rel protein expression and combined immunodeficiency (Beaussant-Cohen et al., 2019). B cell responses to CD40L, IL-2 production by T cells, and myeloid-derived production of IL-12 family members IL-12, IL-21, and IL-23 were all impaired in this patient, enhancing susceptibility to opportunistic infections. These defects are consistent with those reported in c-Rel knockout mice (Gilmore and Gerondakis, 2011), and it will be interesting to study whether altered inflammatory gene expression observed in c-Rel knockout mouse recapitulates in c-Rel-deficient humans. Further studies are also required to study the striking role of c-Rel Y25H SNP in human population (Schuster et al., 2010), which compromises suppressive effect of c-Rel on RelA-dependent inflammatory gene expression (Figure 6). It also remains unknown whether Y25 is a phosphorylation site that may influence c-Rel function. Thus, our findings showing the role of c-Rel in the regulation of inflammation as a suppressor, an inducer, and a modulator of kinetics of gene expression provide a conceivable mechanism for the paradoxical observations of c-Rel-driven resistance and susceptibility to various inflammatory and autoimmune pathologies. These finding also pose c-Rel as a potential target to develop therapeutics to control the deleterious effects of uncontrolled inflammation.

Limitations of the Study

We showed a possible mechanism for the anti-inflammatory role of c-Rel in which it represses TNF-induced RelA transcriptional activity by competitive κ B-site binding and HDAC1 recruitment. However, the exact c-Rel containing canonical dimer(s) (c-Rel:c-Rel homodimer or heterodimer of c-Rel with RelA, RelB, p50 or p52) mediating the repressive function is currently unknown. Additionally, we do not know whether post-translational modification of c-Rel is involved in this process. Although we showed the repressor role of c-Rel in four cell types, i.e., mouse fibroblasts, human embryonic kidney cells, mouse primary macrophages, as well as *in vivo* in mouse liver cells using proinflammatory cytokine TNF, whether this happens in other cell types and in response to other inflammatory stimuli remains to be investigated.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

DATA AND CODE AVAILABILITY

All the data and methods necessary to reproduce this study are included in the manuscript and [Supplemental Information](#). Reagent request will be readily fulfilled following the materials transfer policies of Case Western Reserve University.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100876>.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.R.; Methodology, T.J.D. and P.R., Investigation, T.J.D. and P.R., Validation, T.J.D., Writing – Original Draft, T.J.D., Writing – Review and Editing, P.R., Supervision, P.R., Project Administration, P.R., Funding Acquisition, P.R.

DECLARATION OF INTERESTS

Authors declare no competing financial interests.

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Supplemental Information

NF- κ B c-Rel Dictates the Inflammatory Threshold by Acting as a Transcriptional Repressor

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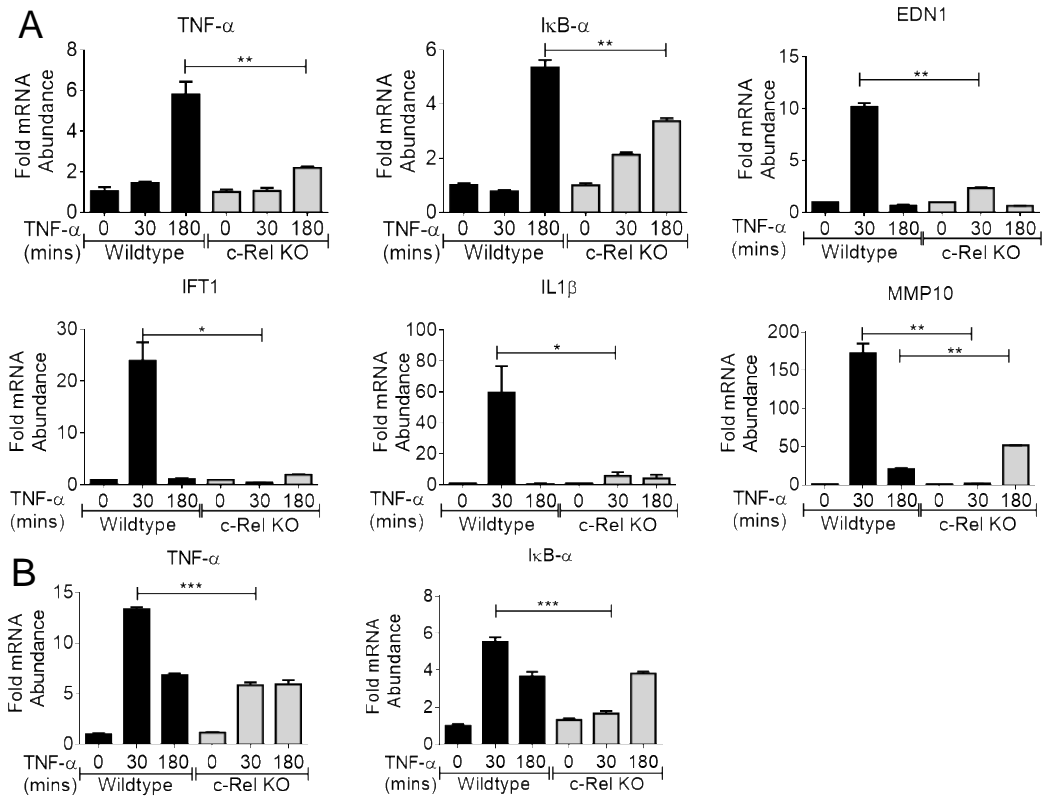


Figure S1. c-Rel Deficiency Decreases the Expression of Selected TNF-Induced Genes.

Related to Figure 1. **(A)** Wild-type or c-Rel knockout MEFs (3×10^5 at the time of harvest) were treated with 100ng/mL TNF- α for 30 minutes or 3 hours. **(B)** Wild-type or c-Rel knockout BMDMs were treated as above. Samples were analyzed by qPCR to determine the abundance of indicated mRNAs relative to that of ribosomal protein L32 (L32). Data in bar graphs are representative of three independent experiments performed in triplicates. Data are presented as mean \pm standard error of mean (SEM) ($n = 3$). p values were obtained by unpaired student t test; *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

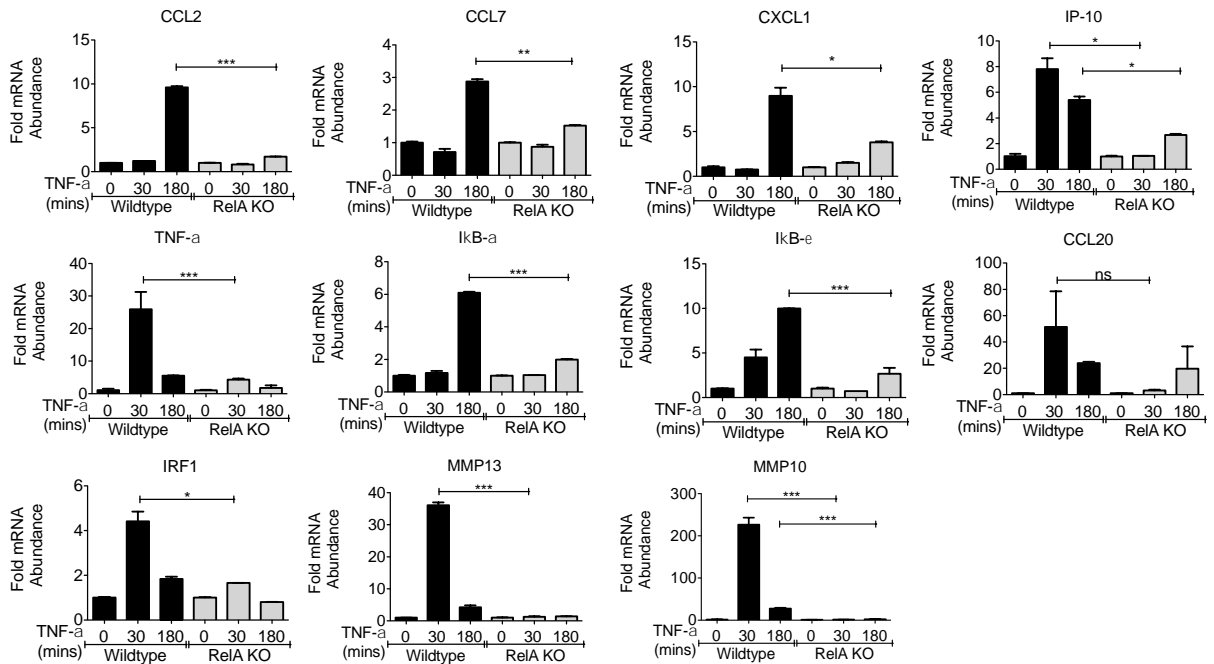


Figure S2. Genes Suppressed by c-Rel are RelA Targets. Related to Figure 2. Wild-type or RelA knockout MEFs (3×10^5 at the time of harvest) were treated in a 6-well plate with 100ng/mL TNF- α for 30 minutes or 3 hours. Samples were then analyzed by qPCR to determine the abundance of RelA-dependent mRNA expression relative to that of ribosomal protein L32 (L32). Wild-type qPCR values were from the same representative experiment in Figure 4 for accurate relative comparison. Data are representative of three independent experiments performed in triplicates, presented as mean \pm standard error of mean (SEM) ($n = 3$). p values were obtained by unpaired student t test; *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

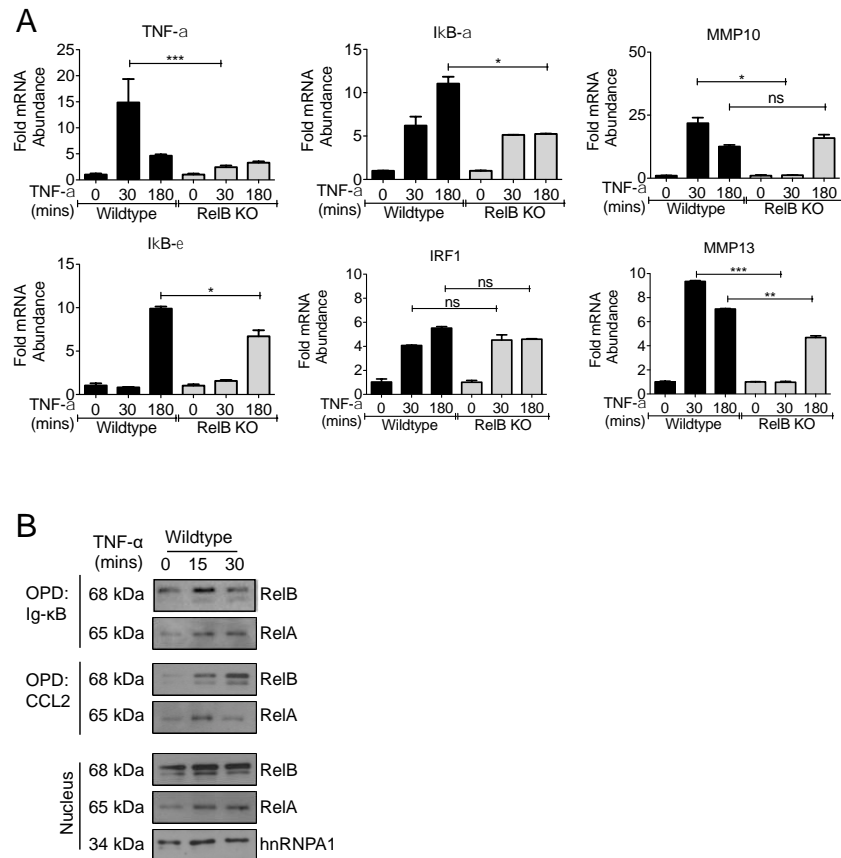


Figure S3. RelB knockout does not enhance RelA-dependent gene expression. Related to Figure 3. **A.** Wild-type or RelB knockout MEFs (3×10^5 at the time of harvest) were treated in a 6-well plate with 100ng/mL TNF- α for 30 minutes or 3 hours. Samples were then analyzed by qPCR to determine the abundance of indicated mRNA expression relative to that of ribosomal protein L32 (L32). Data in bar graphs are representative of three independent experiments performed in triplicates. Data are presented as mean \pm standard error of mean (SEM) ($n = 3$). p values were obtained by unpaired student t test; *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$. **B. RelB binds to Ig- κ B and CCL2- κ B sites.** Wild-type MEFs were treated with 100ng/mL TNF- α for 15 or 30 minutes. Nuclear lysates equivalent to 200 μ g of nuclear proteins per sample were utilized for in vitro pulldown assay using the Ig- κ B or CCL2- κ B oligonucleotides (top and middle panels). Nuclear lysates were probed with indicated antibodies. hnRNPA1 was used as loading control (bottom panels). Data is representative of two independent experiments.

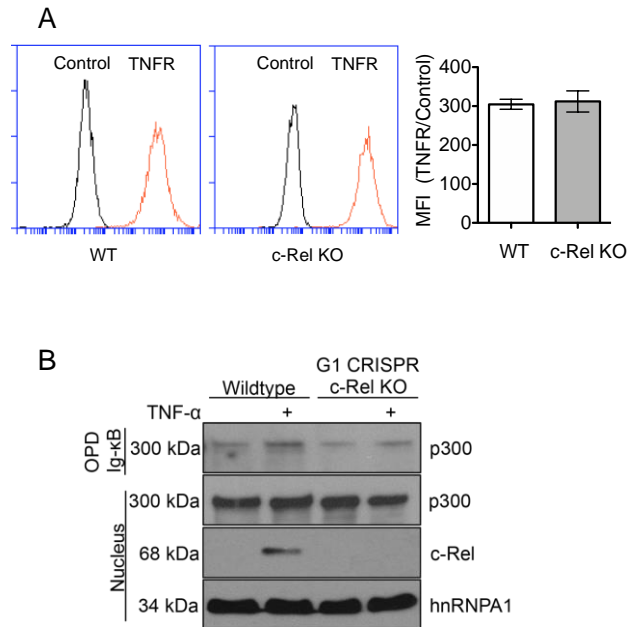


Figure S4. Related to Figure 4. **(A) Cell surface expression of TNFR1 in WT or c-Rel KO MEFs.** Single cell suspensions of wild-type or knockout mouse embryonic fibroblasts were stained with PE conjugated anti-TNFR1 antibody (Biolegend) and analyzed using flow cytometry ($n = 3$). **(B) c-Rel deficiency does not enhance p300 binding at the NF-κB promoter.** Wild-type or c-Rel CRISPR knockout MEFs were left untreated or treated with 100ng/mL TNF- α for 15 minutes. Nuclear lysates equivalent to 400 μ g of nuclear proteins per sample were utilized for in vitro pulldown assay using the Ig-κB oligonucleotide. The pulldown precipitates and nuclear extracts were probed with antibodies against the indicated proteins ($n = 3$).

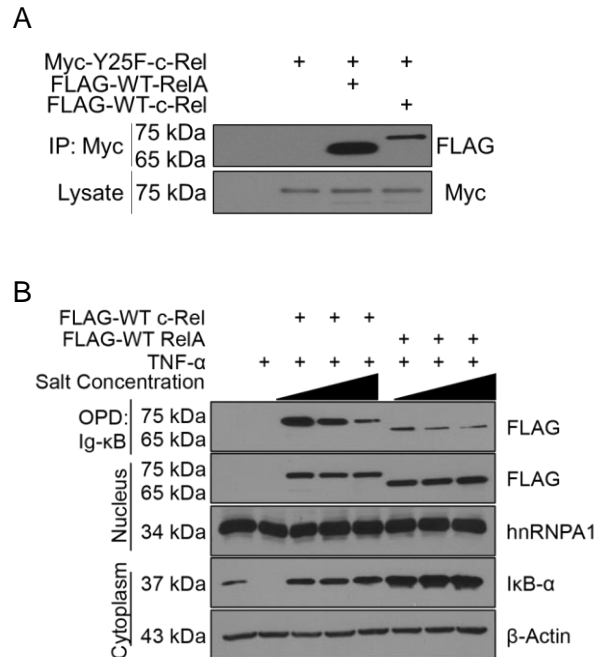


Figure S5. (A) Y25F Mutation Does Not Disrupt c-Rel's Homo- or Hetero- Dimerization.

Related to Figure 6. HEK293T cells (2×10^6) were transfected with myc-tagged Y25F c-Rel and FLAG-tagged RelA or c-Rel plasmids. Myc-tagged Y25F c-Rel was immunoprecipitated and the samples were analyzed by Western blotting with anti-myc- and anti-FLAG antibodies. Data is representative of two independent experiments. **(B) c-Rel Shows High Affinity for RelA Binding Site.**

HEK 293Ts (5×10^6) were transfected with FLAG-tagged wild-type c-Rel or RelA. Eighteen hours following transfection, cells were left untreated or treated with 100ng/mL TNF- α for 15 minutes. Nuclear lysates equivalent to 100 μ g of nuclear proteins per sample were utilized for in vitro pulldown assay using biotinylated Ig-kB oligonucleotide. The neutravidin beads with the pulldown precipitates were washed with buffer containing 150mM, 200mM, or 250mM, three times, separated on a gel and probed with anti-FLAG antibody to detect FLAG-tagged RelA and c-Rel. The total nuclear extracts were also probed with anti-FLAG and anti-hnRNPA1 antibody and cytoplasmic extracts were probed with I κ B- α and β -actin antibodies. Data are representative of three independent experiments.

Supplemental Table 1: qPCR Primer Sequences. Related to Figures 1, 3, 7, S1, S2, and S3.

Mouse Gene	Forward 5'-3'	Reverse 5'-3'
A20	GAACAGCGATCAGGCCAGG	GGACAGTTGGGTGTCTCACATT
CCL20	CTGAAGACCTTAGGGCAGAT	AAGGAATGGGTCCAGACATAC
CCL7	GCTGCTTTCAGCATCCAAGTG	CCAGGGACACCGACTACTG
CXCL1	CTGGGATTCACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC
IP-10	AGGACGGTCCGCTGCAA	CATTCTCACTGGCCCCTCAT
CXCL2	CCCTCAACGGAAGAACCAAAG	TTCCCGGGTGCTGTTTGT
EDN1	GCACCGGAGCTGAGAATGG	GTGGCAGAAGTAGACACACTC
ICAM1	TGTCAGCCACTGCCTTGTA	CAGGATCTGGTCCGCTAGCT
IFIT1	CTGAGATGTCACCTCACATGGAA	GTGCATCCCAATGGTTTCT
I κ B- α	CTGCAGGCCACCAACTACAA	CAGCACCCAAAGTCACCAAGT
I κ B- ϵ	TGGACCTCCAAGTGAAGAAGT	TTCTCTGCAATGTGGCAATG
IL-1 β	GCAACTGTTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IRF1	AGGCCGATACAAAGCAGGAGA	GCTGCCCTTGTTCCTACTCTG
c-JUN	ACTCGGACCTTCTCACGTC	CGGTGTAGTGGTGATGTGCC
MMP-10	AACACGGAGACTTTTACCCTTTT	GGTGCAAGTGTCCATTTCTCAT
MMP-13	ACCTCCACAGTTGACAGGCT	AGGCACTCCACATCTTGTTTT
MMP-3	TGTCCCGTTTCCATCTCTCTC	TGGTGATGTCTCAGGTTCCAG
TNF- α	CTACTCCAGGTTCTCTTCAA	GCAGAGAGGAGGTTGACTTTC
VCAM1	AGTTGGGGATTTCGGTTGTTCT	CCCCTCATTCTTACCACCC
ZFP36	TCTCTGCCATCTACGAGAGCC	CCAGTCAGGCGAGAGGTGA
L32	ACGTCCCAAAAATAGACGCAC	TTCATAGCAGTAGGCACAAAGG

Transparent Methods

Cells

Control and all the NF- κ B knockout mouse embryonic fibroblasts (MEF) and Human Embryonic Kidney 293T cells (HEK293Ts) were grown in DMEM media supplemented with 100 U/ml penicillin/streptomycin, 4 mM L-glutamine and 10% fetal bovine serum. Bone marrow was isolated from wild type and c-Rel knockout mice. Bone marrow derived macrophages (BMDM) was prepared by culturing bone marrow cells in DMEM media supplemented with 10% J558 conditioned media for 8 days as previously described (Winzler et al., 1997).

Generation of CRISPR/Cas9 mediated c-Rel knockout MEFs

The CRISPR/Cas9-mediated knockdown of c-Rel in MEF cells was performed as previously described (Shalem et al., 2014; Tomalka et al., 2017). We designed three different guide RNAs that were cloned in LentiCrisprV2 system and expressed in HEK 293 cells.

Guide 1: For – **CACCGTGTCTGTGCTGCGCTCCCCT**;

Rev – **AAACAGGGGAGCGCAGCACAGACAC**.

Guide 2: For – **CACCGAGCGCAGCACAGACAACAACCGG**;

Rev – **AAACCCGGTTGTTGTCTGTGCTGCGCTC**.

Guide 3: For – **CACCGTAATTGAACAGCCAAGGCAG**;

Rev – **AAACCTGCCTTGGCTGTTCAATTAC**.

Wild-type MEFs were spinfected at 3000 RPM for 90 minutes at 30°C with the HEK 293 cells viral supernatants in the presence of Polybrene (10 μ g/mL) and incubated for 48 hours. Transduced cells were selected with 350 μ g /mL hygromycin and four individual c-Rel knockout clones were then pooled to generate stable knockout pools for each of the three guide RNAs.

Mice

The c-Rel knockout mouse line was kindly provided by H.C. Liou (Weill Medical College of Cornell University, New York). C57BL/6 mice were from a colony maintained in-house. Mice

were housed and handled in accordance with the National Institutes of Health (NIH) guidelines under protocols approved by the Institutional Animal Care and Use Committee.

Reagents

Lipofectamine 2000 was obtained from Life Technologies. Recombinant human TNF- α was obtained from Peprtech. Protein A and protein G agarose used for immunoprecipitation was obtained from GE Healthcare Biosciences. Magnetic protein A/G beads for CHIP and Neutravidin beads for oligo pulldown were from ThermoFisher Scientific.

Plasmids

The complementary DNAs (cDNAs) for human wild-type c-Rel, RelA, RelB, ν -Rel, and the mutant Y25F and Y25H c-Rel with N-terminal FLAG or Myc tag were cloned into the pcDNA4 vector for transient expression. The Y25F and Y25H mutations were generated by PCR-based site-directed mutagenesis. FLAG tagged human wild-type c-Rel was cloned into the pLM lentiviral vector.

Luciferase Assay

HEK293T cells plated in 6-well plates were transfected with pGL3 firefly luciferase vector (500 ng/well) containing promoters of Ig- κ B, IP-10, or A20. Renilla luciferase was expressed under HSV-thymidine kinase promoter in the pRL-TK vector (100 ng/well). Cells were co-transfected with FLAG-tagged NF- κ B subunits c-Rel or RelA (1 μ g/well). Total DNA concentration in each well was normalized to 3 μ g/well using pcDNA4 empty vector. Twenty-four hours following transfection, cells were harvested and firefly and renilla luciferase activities were analyzed using the Dual Luciferase Reporter Assay System following manufacturer's instructions (Promega). Luminescence were read on a Spectramax 3000 plate reader and plotted as fold relative light units. Data are presented as mean \pm standard error of mean (SEM) (n = 3). p values were obtained by unpaired student t test; *** p < 0.001, ** p < 0.01 * p < 0.05.

Immunoprecipitation and Western Blotting

Cells were lysed in hypotonic cytoplasmic lysis buffer plus protease inhibitor cocktail (10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT) for 15 minutes on ice. A final concentration of 0.625% NP40 was added to the lysate and vortexed immediately for 10 seconds to disrupt remaining cellular membranes. Lysates were spun at 10,000g for 30 seconds at 4°C and supernatants were used as cytoplasmic lysate. Pellets were washed once in 2 times original volume of cytoplasmic buffer without NP40 and spun at 10,000g for 5 seconds at 4°C to remove any remaining cytoplasmic proteins. Nuclear pellets were lysed for 30 minutes on ice in nuclear lysis buffer plus protease inhibitor cocktail (20 mM HEPES pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM DTT). Pellets were vortexed for 5 seconds, three times during lysis to ensure complete disruption. Lysates were spun at 10,000g for 10 minutes at 4°C and supernatants were used as nuclear lysates. Protein levels were normalized using BCA assay (ThermoFisher Scientific). The cytoplasmic lysates were supplemented with NaCl and nuclear lysates were diluted with salt free lysis buffer to obtain a final salt concentration of 150mM in the lysates. Immunoprecipitations were carried out at 4°C. For Western blot analysis, cytoplasmic/nuclear lysates as well as immunoprecipitates were resolved through 9% SDS-PAGE gels. Proteins from the gel were transferred onto nitrocellulose membranes, probed using the antibodies described below, and visualized by enhanced chemiluminescence assay.

Details of the antibodies used in Immunoprecipitation and Western blotting: FLAG (Sigma; M2), Myc (Sigma; 4A6), Actin (SCBT; AC-15), human c-Rel (SCBT; SC-71), mouse c-Rel (Biolegend; 655894), RelB (SCBT; C-19), RelA (SCBT; F-6), hnRNPA1 (SCBT; 4B10), I- κ B α (SCBT; C-21), p50 (CST; 13586), p52 (CST; 4882), HDAC1 (Biolegend; 815101), HDAC2 (Biolegend; 680104), HDAC3 (SCBT; H-99), HDAC4 (SCBT; H-92), p300 (CST; 86377), and pLCy1 (SCBT; SC-81). Most of the primary antibodies were used at 1:2000 dilutions, except actin and hnRNPA1 (1:10,000), pLCy1 (1:5000), FLAG and Myc (1:4000) and I- κ B α (1:1000).

Oligonucleotide Pulldown Assay

Oligonucleotide pulldown assays using biotinylated I κ B sequence were performed as previously described (Ramakrishnan et al., 2013).

The oligonucleotides used for CCL2- κ B site were as follows:

Fwd: Biotinylated 5' AGAATGGGAATTTCCACGCTC 3'

Rev: 5' GAGCGTGGAAATTTCCATTCT 3'. In brief, annealed, biotinylated I κ B site and CCL2- κ B site oligos were used to isolate active, DNA-binding NF- κ B dimers from nuclear lysates using neutravidin beads (ThermoFisher Scientific).

Quantitative Real-Time PCR

Total RNA was isolated from cells using the DNAaway RNA miniprep kit (Bio Basic). For liver samples, 50% ethanol was used instead of 100% ethanol at initial precipitation step to enhance RNA yield as suggested by the Qiagen RNeasy mini handbook. RNA yields were quantified by NanoDrop spectrophotometer and 1 μ g of total RNA was converted to cDNA using the Applied Biosystems High Capacity Reverse Transcription kit (Cambridge). qPCR was performed with cDNA corresponding to approximately 20-30ng of RNA in triplicate for the genes of interest. The details of the qPCR in compliance with the recommendations provided at www.rdml.org/miqe.html, were as follows: Program- Step 1- 95°C, 3min, Step 2- 95°C, 4 sec, Step 3- 60°C, 60 sec, Read, then Go To Step 2 x 40 times. Master Mix - KAPA SYBR Fast Universal Master Mix (Kapa Biosystems) Polymerase: KAPA SYBR Polymerase (Proprietary engineered version of Taq polymerase), MgCl₂ final concentration: 2.5 mM, Dye: SYBR Green I, Fwd/Rev Primer final concentration: 500 nM, Machine used: CFX96 (Bio-Rad), Reaction volume: 10 μ L, Consumables used: Hard-Shell PCR plate, 96-Well, Thin wall (Catalog #: HSP9601, Bio-Rad), Transparency: Clear and Sealing method: Adhesive (Catalog #: 236366, ThermoFisher Scientific). Gene names and sequences of primers used for qPCR are given in supplementary table 1. Experimental triplicate samples were run for biological replicates for all stimulation conditions. Gene expression was quantified as fold induction over control, using the

$\Delta\Delta\text{Ct}$ method. All values were normalized to the housekeeping gene L32. Data are presented as mean \pm standard error of mean (SEM) (n = 3). p values were obtained by unpaired student t test; **** p < 0.0001, *** p < 0.001, ** p < 0.01 * p < 0.05.

Chromatin Immunoprecipitation

Wild-type or c-Rel KO MEFs were plated at 5×10^6 cells/plate in 3 x 15 cm dishes per stimulus condition. The following day, plates were left untreated or treated with 100 ng/ml TNF- α . Plates were washed with warm PBS and incubated with 2 mM DSG in PBS (+MgCl₂) for cross-linking proteins on an orbital shaker for 45 minutes at room temperature. Cells were then washed with warm PBS and incubated for 15 minutes at room temperature in 1% formaldehyde for cross-linking DNA. Formaldehyde was quenched with 2.5 M glycine for 5 minutes and the plates were washed with PBS. All crosslinking steps for the liver cells were performed in 50 mL conical tubes on a rocker at room temperature. Cells were removed from plate by scraping and pelleted at 2,000 RPM for 5 minutes at 4°C. Cells were lysed in Farnham cell membrane lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, with protease inhibitors) for 15 minutes, and nuclear pellets were spun down at 10,000 RPM for 10 minutes at 4°C. The nuclear pellets were resuspended in 100 μ L of RIPA buffer and chromatin was sheared at 4°C in a Sonicator® 3000 Ultrasonic Liquid Processor for 80 cycles of 8 seconds ON and 40 seconds OFF at output 5. Magnetic dynabeads were washed in 5 mg/mL BSA in PBS for blocking using a magnetic block 5 times, and then incubated with 1 μ g of anti-RelA antibody (Cell Signaling Technology), anti-HDAC1, or anti-c-Rel antibody (Biolegend) per sample in an end-over-end rotator at 4°C overnight. Sonicated supernatant and antibody-coupled magnetic beads were incubated in an end-over-end rotator at 4°C overnight. Beads were pelleted and then washed with 1 mL LiCl IP wash buffer containing 10 mM Tris, pH 8.1, 0.25 M LiCl, 1% IGEPAL-CA 630, 1% Deoxycholic acid, and 1 mM EDTA, five times for 10 minutes at 4°C. Beads were then washed once with 1mL TE and resuspended in 200 μ L IP elution buffer, and incubated on a heated shaker at 65°C at 900 RPM overnight. Extraction of DNA was performed using phenol/CHCl₃/isoamyl alcohol

and purified using Qiagen PCR Cleanup Kit. Quantitative RT-PCR was then performed on the eluates to amplify IP-10 and CXCL1 promoters. The following primer pairs were used: IP-10; Fwd. 5'-tcc aag ttc atg ggt cac aa-3' and Rev. 5'-gat gtc tct cag cgg tgg at-3'. CXCL1; Fwd. 5'-cta atc ctt ggg agt gga g-3' and Rev. 5'-ccc ttt tat gct cga aac-3'.

Details of the antibodies used in ChIP: c-Rel (Biolegend; 655894), RelA (CST; 6956), and HDAC1 (Biolegend; 815101).

In Vivo TNF Injections

Recombinant mouse TNF- α was dissolved in sterile phosphate buffered saline (PBS) prior to injection. Wild-type and c-Rel knockout mice (7-10 weeks old males and females, sex and age matched, n = 3 per condition) were intraperitoneally injected with sterile PBS or TNF- α (5 μ g/mouse in 200 μ l) and sacrificed after 45, 90, or 180 minutes by CO₂ inhalation. Liver was harvested and processed into a single cell suspension through a 70 μ M cell strainer with a 3 mL syringe plunger. Following RBC lysis, the cell pellets were washed three times with 15 ml of cold PBS and immediately processed for RNA extraction (40 x 10⁶ cells) or chromatin immunoprecipitation (180 x 10⁶ cells) as described above.

Supplemental References

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