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Supplemental Information

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

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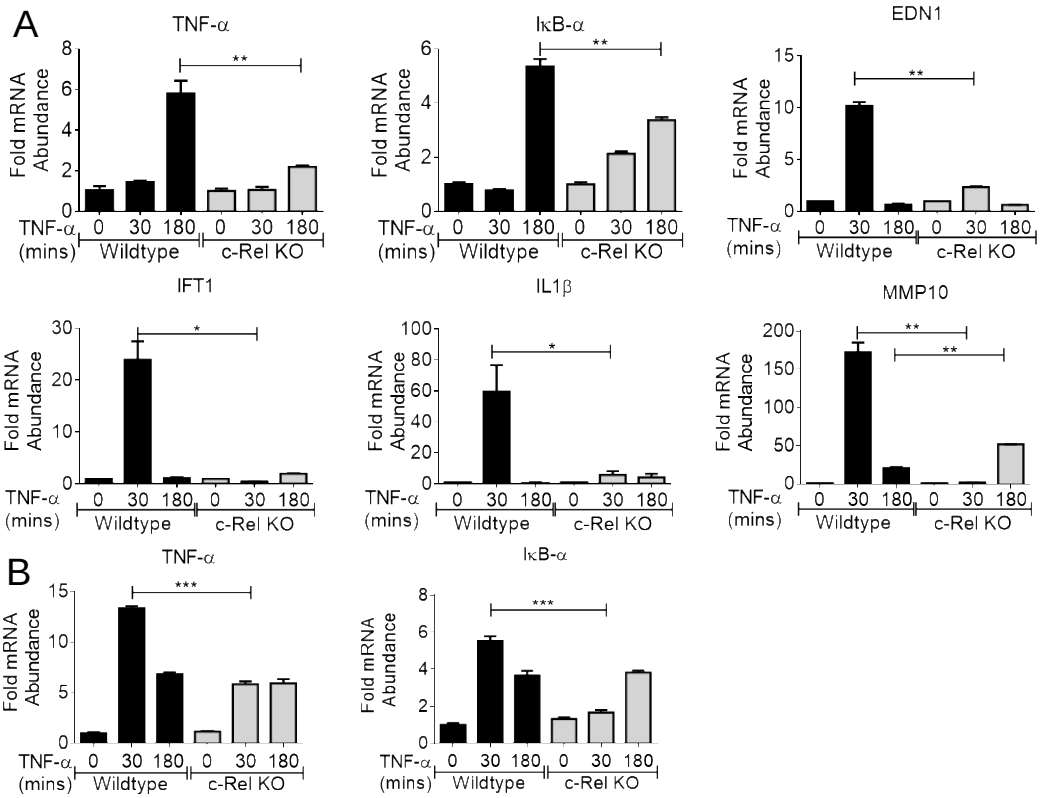

Figure S1. c-Rel Deficiency Decreases the Expression of Selected TNF-Induced Genes. Related to Figure 1. **(A)** Wild-type or c-Rel knockout MEFs (3 x 10^5 at the time of harvest) were treated with 100ng/mL TNF-α for 30 minutes or 3 hours. **(B)** Wild-type or c-Rel knockout BMDMs were treated as above. 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 ± standard error of mean (SEM) (n = 3). p values were obtained by unpaired student t test; *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

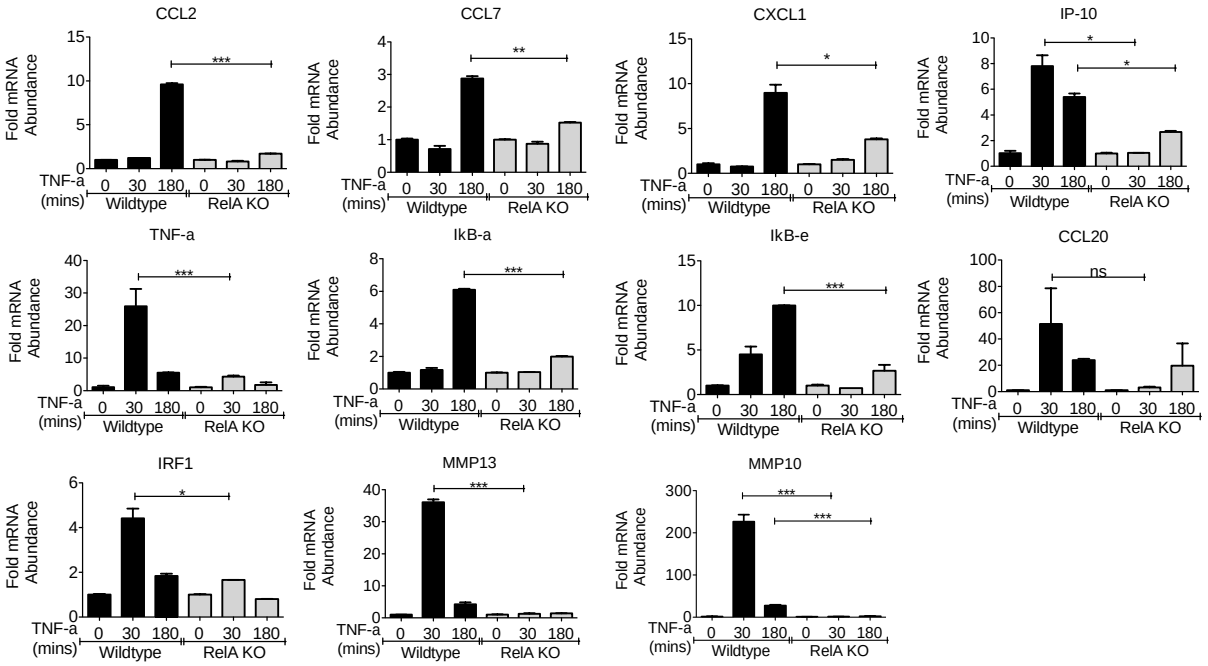

Figure S2. Genes Suppressed by c-Rel are RelA Targets. Related to Figure 2. Wild-type or RelA knockout MEFs (3 x 10^5 at the time of harvest) were treated in a 6-well plate with 100ng/mL TNF-α for 30 minutes or 3 hours. Samples were then analyzed by qPCR to determine the abundance of RelA-dependent mRNA 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 ± standard error of mean (SEM) (n = 3). p values were obtained by unpaired student t test; *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

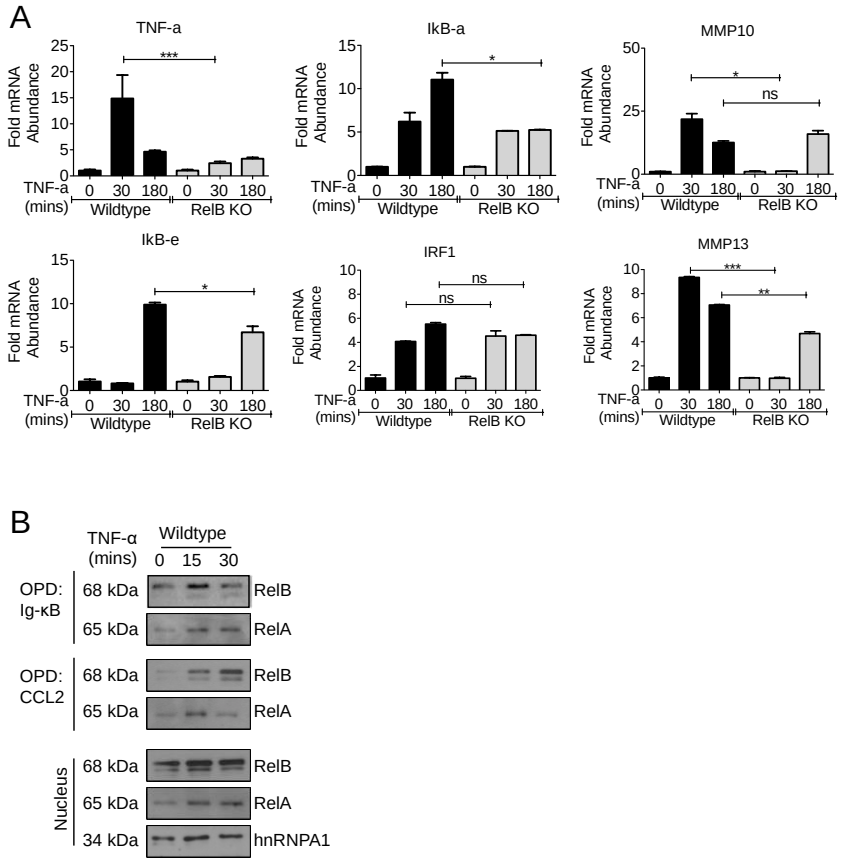

Figure S3. **RelB knockout does not enhance RelA-dependent gene expression**. Related to Figure 3. **A**. Wild-type or RelB knockout MEFs (3 x 10^5 at the time of harvest) were treated in a 6-well plate with 100ng/mL TNF-α for 30 minutes or 3 hours. Samples were then analyzed by qPCR to determine the 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 ± 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-κB and CCL2-κB sites**. Wild-type MEFs were treated with 100ng/mL TNF-α for 15 or 30 minutes. Nuclear lysates equivalent to 200µg of nuclear proteins per sample were utilized for in vitro pulldown assay using the Ig-κB or CCL2- κB 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.

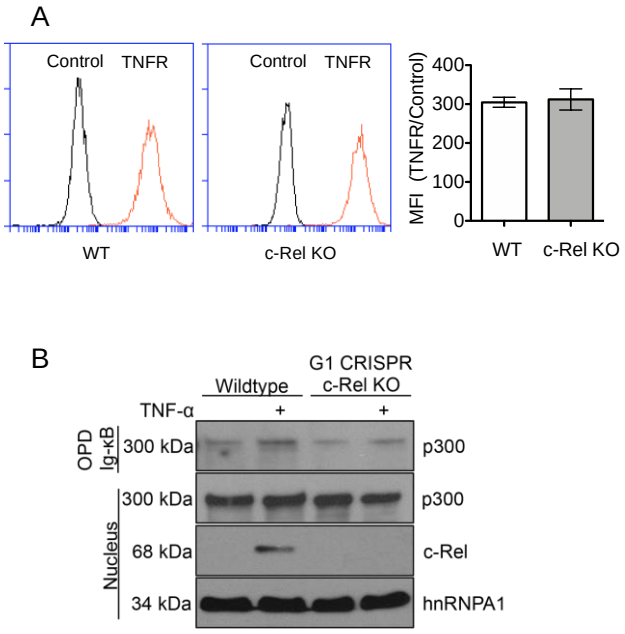

Figure S4. Related to Figure 4. (**A) Cell surface expression of TNFR1 in WT or c-Rel KO MEFs.** Single cell suspensions of wild-type or knockout mouse embryonic fibroblasts were stained with PE conjugated anti-TNFR1 antibody (Biolegend) and analyzed using flow cytometry (n = 3). (**B**) **c-Rel deficiency 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 400µg 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).

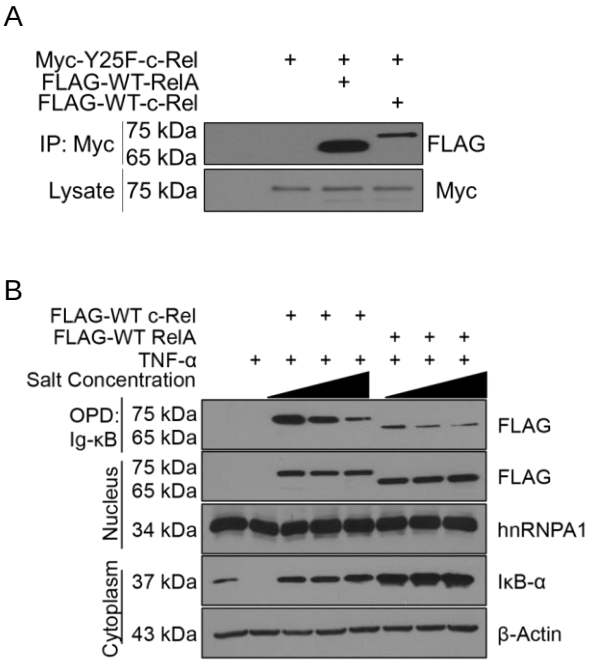

Figure S5. (A) Y25F Mutation Does Not Disrupt c-Rel's Homo- or Hetero- Dimerization. Related to Figure 6. HEK293T cells (2 x 10^6) were transfected with myc-tagged Y25F c-Rel and FLAG-tagged RelA or c-Rel plasmids. Myc-tagged Y25F c-Rel was immunoprecipitated and the samples were analyzed by Western 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 x 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 100µg of nuclear proteins per sample were utilized for in vitro pulldown assay using biotinylated Ig-κB 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 IκB-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	CATTCTCACTGGCCCGTCAT
CXCL2	CCCTCAACGGAAGAACCAAAG	TTCCCGGGTGCTGTTTGT
EDN1	GCACCGGAGCTGAGAATGG	GTGGCAGAAGTAGACACACTC
ICAM1	TGTCAGCCACTGCCTTGGTA	CAGGATCTGGTCCGCTAGCT
IFIT1	CTGAGATGTCACTTCACATGGAA	GTGCATCCCCAATGGGTTCT
IκB-α	CTGCAGGCCACCAACTACAA	CAGCACCCAAAGTCACCAAGT
IκB-ε	TGGACCTCCAACTGAAGAACT	TTCCTCTGCAATGTGGCAATG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IRF1	AGGCCGATACAAAGCAGGAGA	GCTGCCCTTGTTCCTACTCTG
c-JUN	ACTCGGACCTTCTCACGTC	CGGTGTAGTGGTGATGTGCC
MMP-10	AACACGGAGACTTTTACCCTTTT	GGTGCAAGTGTCCATTTCTCAT
MMP-13	ACCTCCACAGTTGACAGGCT	AGGCACTCCACATCTTGGTTT
MMP-3	TGTCCCGTTTCCATCTCTCTC	TGGTGATGTCTCAGGTTCCAG
TNF-α	CTACTCCCAGGTTCTCTTCAA	GCAGAGAGGAGGTTGACTTTC
VCAM1	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC
ZFP36	TCTCTGCCATCTACGAGAGCC	CCAGTCAGGCGAGAGGTGA
L32	ACGTCCCAAAAATAGACGCAC	TTCATAGCAGTAGGCACAAAGG

Transparent Methods

Cells

Control and all the NF-κB 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 – **CACCG**TGTCTGTGCTGCGCTCCCCT;

Rev – **AAAC**AGGGGAGCGCAGCACAGACA**C**.

Guide 2: For – **CACCG**AGCGCAGCACAGACAACAACCGG;

Rev – **AAAC**CCGGTTGTTGTCTGTGCTGCGCT**C**.

Guide 3: For – **CACCG**TAATTGAACAGCCAAGGCAG;

Rev – **AAAC**CTGCCTTGGCTGTTCAATTA**C.**

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 µg/mL) and incubated for 48 hours. Transduced cells were selected with 350 µg /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 Peprotech. 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, v-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-κB, 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-κB subunits c-Rel or RelA (1 μg/well). Total DNA concentration in each well was normalized to 3 μg/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 ± 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 4ºC. 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-κBα (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-κBα (1:1000).

Oligonucleotide Pulldown Assay

Oligonucleotide pulldown assays using biotinylated Ig-κB sequence were performed as previously described (Ramakrishnan et al., 2013).

The oligonucleotides used for CCL2-κB site were as follows:
Fwd: Biotinylated 5' AGAATGGGAATTTCCACGCTC 3'

Rev: 5' GAGCGTGGAAATTCCCATTCT 3'. In brief, annealed, biotinylated Ig-κB site and CCL2-κB site oligos were used to isolate active, DNA-binding NF-κB 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 1µg 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), $MgCl_2$ final concentration: 2.5 mM, Dye: SYBR Green I, Fwd/Rev Primer final concentration: 500 nM, Machine used: CFX96 (Bio-Rad), Reaction volume: 10 µL, 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

ΔΔCt method. All values were normalized to the housekeeping gene L32. Data are presented as mean ± 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 $5x10^6$ 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 (+$MgCl_2$) 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 µL 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 1µg 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 µL IP elution buffer, and incubated on a heated shaker at 65˚C at 900 RPM overnight. Extraction of DNA was performed using phenol/$CHCl_3$/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 μg/mouse in 200 μl) and sacrificed after 45, 90, or 180 minutes by CO_2 inhalation. Liver was harvested and processed into a single cell suspension through a 70 μM 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 10^6 cells) or chromatin immunoprecipitation (180 x 10^6 cells) as described above.

Supplemental References

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