

Cardiovascular, Pulmonary, and Renal Pathology

The NF- κ B Subunit c-Rel Stimulates Cardiac Hypertrophy and Fibrosis

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Cardiac remodeling and hypertrophy are the pathological consequences of cardiovascular disease and are correlated with its associated mortality. Activity of the transcription factor NF- κ B is increased in the diseased heart; however, our present understanding of how the individual subunits contribute to cardiovascular disease is limited. We assign a new role for the c-Rel subunit as a stimulator of cardiac hypertrophy and fibrosis. We discovered that c-Rel-deficient mice have smaller hearts at birth, as well as during adulthood, and are protected from developing cardiac hypertrophy and fibrosis after chronic angiotensin infusion. Results of both gene expression and cross-linked chromatin immunoprecipitation assay analyses identified transcriptional activators of hypertrophy, myocyte enhancer family, *Gata4*, and *Tbx* proteins as *Rel* gene targets. We suggest that the p50 subunit could limit the prohypertrophic actions of c-Rel in the normal heart, because p50 overexpression in H9c2 cells repressed c-Rel levels and the absence of cardiac p50 was associated with increases in both c-Rel levels and cardiac hypertrophy. We report for the first time that c-Rel is highly expressed and confined to the nuclei of diseased adult human hearts but is restricted to the cytoplasm of normal cardiac

tissues. We conclude that c-Rel-dependent signaling is critical for both cardiac remodeling and hypertrophy. Targeting its activities could offer a novel therapeutic strategy to limit the effects of cardiac disease. (Am J Pathol 2012, 180:929–939; DOI: 10.1016/j.ajpath.2011.11.007)

Cardiovascular disease underpins the development of cardiac hypertrophy and heart failure and is the primary cause of death in the developed world.¹ During periods of acute physical and metabolic stress, the heart employs hemodynamic coping mechanisms, including increasing stroke volume and heart rate to meet the increased demand. In response to prolonged stress, the heart undergoes a physiological compensatory mechanism whereby it becomes enlarged (ie, cardiac hypertrophy). This process is governed by a series of biochemical and molecular changes in the heart, including cardiac remodeling and the reactivation of a group of genes collectively known as the fetal gene program.^{2–4} Recent discoveries in both animal models and the clinic suggest that cardiac hypertrophy is a dynamic process that may be reversible.^{5–7} Nonetheless, despite considerable research efforts, the complex signaling events regulating development and reversion of cardiac hypertrophy are not fully understood.

Nuclear factor- κ B (NF- κ B) is a pleiotropic transcription factor that, in addition to playing fundamental roles in immunity, also regulates the expression of genes controlling cell survival and fate.⁸ NF- κ B activity is elevated during cardiovascular disease, and its signaling is strongly implicated in the development of cardiac remodel-

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eling (fibrosis), hypertrophy, and heart failure.^{9–13} The NF- κ B/Rel family comprises five members: RelA (p65), NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), c-Rel, and RelB. These are divided into two classes. Class I subunits are synthesized as precursors p105 and p100; these proteins are then proteolytically processed, yielding the p50 and p52 subunits, respectively. The full-length proteins contain ankyrin repeat domains and can act as inhibitory κ B proteins. RelA (p65), c-Rel, and RelB proteins comprise the class II subunits. Importantly, only class II subunits contain a transactivation domain in the C-terminus allowing them to interact with the transcriptional machinery. The five subunits combine either as a homodimer or as heterodimers that bind to a decameric DNA consensus sequence known as the κ B site to modulate gene transcription.⁸ NF- κ B is activated via two pathways, the canonical (classical) and noncanonical pathway. Canonical signaling uses the RelA, p50, and c-Rel subunits, whereas activation of the noncanonical pathway is mediated by RelB and p100/p52. Clinical studies have linked canonical NF- κ B signaling with susceptibility and progression of cardiac disease, in that increased nuclear RelA has been observed in failing human hearts,^{14,15} whereas *NFKB1* gene polymorphisms are associated with an increased susceptibility to developing dilated cardiomyopathy.^{16,17}

Global inhibition of this pathway using either pharmacological NF- κ B inhibitors, transgenic mice, or expression of dominant negative I κ B under the control of a cardiac specific promoter in rodent models of heart disease is cardioprotective.^{18–21} These data highlight NF- κ B as a potential therapeutic target. However, recent data generated from studies using cardiac-specific Nemo (or IKK γ), a regulatory subunit of NF- κ B,²² or IKK β knockout mice,²³ the upstream kinases regulating canonical NF- κ B signaling, revealed that activation of RelA is critical for promoting myocyte survival and cardiac homeostasis. This suggests that long-term pan-blockade of IKK/RelA-dependent NF- κ B signaling in the diseased heart is likely to be detrimental. However, NF- κ B biology is complex, with multiple levels of control (including regulatory stimuli, activating kinases, post-translational modifications, and subunit composition).^{24–27} Each subunit is functionally distinct and has discrete biochemical characteristics. Growing evidence and knowledge now suggest that the Rel subunit is important in study of NF- κ B signaling in the context of disease.^{28–33} Given that the RelA subunit is critical for cardiac homeostasis and therefore unlikely to be a good therapeutic target, we decided to investigate the roles of the two other canonical NF- κ B subunits, c-Rel and NF- κ B/p50, in cardiac disease. These may provide more selective therapeutic targets, leaving the cardioprotective actions of RelA signaling intact.

In the present study, we began by comparing cardiac expression of c-Rel in normal versus end-stage cardiomyopathic human hearts. We show for the first time that c-Rel was found in the nucleus of diseased but not normal hearts. We discovered that gene deletion of the c-Rel subunit in mice protects against development of stress-induced cardiac hypertrophy and fibrosis. Finally, we

suggest that the p50 subunit may act to antagonize the prohypertrophic effects of c-Rel. With this report, we unravel some of the complexities of NF- κ B system within heart pathophysiology and identify a new role for the c-Rel subunit as a positive regulator of cardiac hypertrophy. We suggest that the disease-related shift in balance from p50 toward c-Rel-dependent signaling has a positive influence on the expression of genes controlling the adaptive response of the stressed heart and that, unlike RelA, the c-Rel subunit may prove to be a good therapeutic target for the treatment of cardiovascular disease.

Materials and Methods

Mice

Experiments were performed on either mixed C57Bl/6; 129PF2/J *Nfkb1*^{-/-} and F2 hybrid *Nfkb1*^{+/+} wild-type (WT) control mice (Jackson Laboratory, Bar Harbor, ME) or genetically modified mice lacking *Nfkb1* or *Rel* on a pure C57Bl/6 background and C57Bl/6 WT controls.^{34,35} *Rel*-deficient mice were a kind gift of Hsiou Chi-Liou (Weill Cornell Medical College). Mice were housed in pathogen-free conditions. Age-matched, 12-week-old or newborn (day 1) male *Nfkb1*^{-/-}, *Rel*^{-/-}, and WT control mice were weighed, then euthanized by cervical dislocation; the hearts were removed and weighed.

Blood Pressure Measurements

WT, *Nfkb1*^{-/-}, and *Rel*^{-/-} mice were anesthetized by intraperitoneal injection of a water solution with a fentanyl and fluanisone combination (Hypnorm; VetaPharma, Leeds, UK) and midazolam at the following doses, per gram of body weight: 3.8 μ g fentanyl, 120 μ g fluanisone, and 60 μ g midazolam. A 1.4-French Millar Mikro-Tip pressure-volume catheter (ADInstruments, Oxford, UK) was introduced into the right carotid artery and placed in the ascending aorta. Systolic and diastolic pressures were recorded using PowerLab Chart5 software and analyzed using Millar PVAN data analysis software version 3 (ADInstruments). Data are reported as means \pm SEM of five mice per group.

Angiotensin Infusion

Age-matched adult WT or *Rel*^{-/-} mice were anesthetized with isoflurane, and Alzet 2004 mini-osmotic pumps (Durect, Cupertino, CA) containing either 0.9% sterile saline (vehicle) or angiotensin (700 μ g/kg per day) were placed in the flank of the mouse. After 4 weeks of infusion, mice were weighed, then euthanized by cervical dislocation; the hearts were removed and weighed. All data are reported as means \pm SEM of six WT and five *Rel*^{-/-} mice per group.

Cell Culture

The rat embryonic cardiomyocyte cell line H9c2 (a kind gift from Deborah Henderson, Newcastle, UK) was cul-

tured on plastic in Dulbecco's modified Eagle's medium, supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 10% fetal calf serum, and was maintained at 37°C in an atmosphere of 5% CO₂.

Human Left Ventricular Myocardium

Human left ventricular (LV) tissue was obtained from male patients undergoing cardiac transplantation for end-stage heart failure. Normal nondonor suitable human LV tissue was obtained from healthy male individuals involved in road traffic accidents. Tissue was formalin-fixed, processed, and embedded in paraffin blocks.

Histology, Immunohistochemistry, and Image Analysis

Sirius Red and H&E staining was performed as described previously.³¹ Photomicrographs were taken using a Leica DMR and JVC camera system (Leica Microsystems, Wetzlar, Germany). Densitometry and cell areas were calculated using the Leica Qwin image analysis system in at least six $\times 20$ fields in the left ventricle. Immunohistochemical staining for mouse α -sarcomeric actin, laminin, and c-Rel was performed as follows. Slides were deparaffinized, and then citric saline antigen retrieval was performed. For c-Rel, a combination of citric saline and trypsin antigen retrieval was performed. Endogenous peroxidase activity was blocked using hydrogen peroxide, and further inhibition of nonspecific binding was achieved using an avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK) followed by incubation with 20% swine serum (Dako, Ely, UK). Antibody specific to α -sarcomeric actin or laminin (Abcam, Cambridge, UK) was diluted 1:250 in PBS and c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in PBS with 20% swine serum, then applied to the slides and incubated overnight at 4°C. Slides were washed and amplification of the antigen was achieved using a quick kit (Vector Laboratories) for α -sarcomeric actin or biotinylated swine anti-rabbit diluted 1:200 (Dako), followed by ABC complex (Vector Laboratories) for c-Rel and laminin. Slides were then washed and presence of α -sarcomeric actin, laminin, or c-Rel was visualized with diaminobenzidine staining. Slides were then counterstained with Mayer's hematoxylin, dehydrated, cleared in Clearene solvent (Leica Microsystems), and mounted in Pertex mounting medium (Leica Microsystems).

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from 10 to 15 μ g of heart tissue or $\sim 5 \times 10^6$ H9c2 cells using a total RNA purification kit (Qiagen, Valencia, CA; Crawley, UK). Heart tissue was homogenized using a sterile pestle in Qiagen RLT lysis buffer, then 0.01% v/v proteinase K was added to the homogenate, followed by incubation at 55°C for 10 minutes. RNA was then isolated according to the manufacturer's instructions. RNA was treated with 1 μ L DNase

(Promega, Madison, WI) for 30 minutes at 37°C and first-strand cDNA was produced via incubation with random hexamer primer [p(dN)₆] and 100 units MMLV reverse transcriptase, as described previously.³¹

SDS-PAGE and Immunoblotting

Murine heart tissue was lysed in Laemmli buffer, heated at 95°C for 10 minutes, sonicated, and centrifuged for 30 minutes at 16,000 $\times g$. Total protein (10 to 30 μ g) was fractionated by 9% SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 0.1% TBS/Tween 20 containing 3% nonfat dried milk before overnight incubation with primary antibodies; c-Rel (rabbit, 1:1000; Santa Cruz Biotechnology), α -sarcomeric actin (mouse, 1:1000; Abcam), p105/p50 (rabbit, 1:1000; Abcam), or GAPDH (rabbit, 1:2000; Abcam). Membranes were washed in TBS/Tween 20 and incubated with either mouse anti-rabbit horseradish peroxidase conjugate (1:2000; Cell Signaling Technology, Danvers, MA) or goat anti-rabbit horseradish peroxidase conjugate (1:5000; Sigma-Aldrich, St. Louis, MO) for 2 hours. Blots were washed and antigen detected by enzymatic chemiluminescence (Amersham; GE Healthcare, Little Chalfont, UK).

SYBR Green Quantitative RT-PCR

Primers amplifying the mouse and rat target gene sequences of interest were designed using OLIGO software version 4.0 (Molecular Biology Insights, Cascade, CO) (Table 1). Gata4-specific primers were as described previously.³⁶ Relative level of transcriptional difference between knockout and control mice was calculated as $[1/(2^A)] \times 100$, where A is the difference between mean wild-type CT and mean knockout CT after the GAPDH value has been deducted from the target gene for each animal.

Plasmid DNA, Cell Transfection, and Reporter Assays

Overexpression plasmids encoding respiratory syncytial virus β galactosidase (RSV- β -gal), RSV-p50, and RSV-c-Rel, described previously,³⁷ were a gift from Neil D. Perkins (Newcastle University, UK). Transfection of H9c2 cells with 3 μ g of plasmid DNA was achieved using linear polyethylenimine (m.w., $\sim 25,000$; Polysciences, Warrington, PA; Eppelheim, Germany) or Effectene (Qiagen) reagent, as described previously.^{37,38} An 828-bp sequence from the Gata4 promoter was amplified from rat genomic DNA using primers rat Gata4 PR Forward 5'-TTAGCTAGCTCAAACCTCTAATCTCAGCAA-3' and rat Gata4 PR Reverse 5'-AACGATATCTGAAGTTGGATTACTGGAACAAT-3'. The forward primer contains a recognition site for NheI at the 5' end and the reverse primer contains a cut site for EcoRV at the 3' end. The PCR product was digested with NheI and EcoRV, purified, and then cloned into an NheI-EcoRV digested pGL4.17 vector (Promega) using T4 DNA ligase. The cloned promoter sequence was then verified using sequencing. H9c2 cells were cotransfected with 1 μ g Gata4-luciferase and

Table 1. SYBR Green Quantitative RT-PCR Primers

Gene	Forward primer	Reverse primer
Rat/mouse <i>Gapdh</i>	5'-GCACAGTCAAGGCCGAGAAT-3'	5'-GCCTTCTCCATGGTGGTGAA-3'
mouse <i>RelA</i>	5'-GCCAGACCCGAGTATCC-3'	5'-GTCCCGCACTGTCACCTG-3'
mouse <i>RelB</i>	5'-CTGGCTCCCTGAAGAACC-3'	5'-CGCTCTCCTTGTGATTC-3'
mouse <i>Rel</i>	5'-CTCTGCCTCCCATTTTCTA-3'	5'-GGTCCCAGTCATCAACAC-3'
mouse <i>p52</i>	5'-CTATCTGGTGATTGTGGA-3'	5'-GGCCGGTCCCTCATAGTT-3'
mouse <i>Mef 2A</i>	5'-TACAAATCACACGCATAA-3'	5'-GAAATGATGAGTCTAT-3'
mouse <i>Mef 2B</i>	5'-GCAACGCCTCTTCCAGTA-3'	5'-CAAGGAGCAGCGGGTAGG-3'
mouse <i>Mef 2C</i>	5'-ATGAGCGTAACAGACAGG-3'	5'-TTGGTGCCTGTAAGATG-3'
mouse <i>Mef 2D</i>	5'-ATGGGGAGGAAAAAGATT-3'	5'-GGAGTGGTTGAAGATGAT-3'
mouse <i>Nkx2-5</i>	5'-GCCAACAGCAACTTCGTGAACT-3'	5'-ACTCTGCACGGTGTCAAGTCC-3'
mouse <i>Tbx-5</i>	5'-CTGGCCTTAATCCAAAAACA-3'	5'-GCCAGTTACGGACCATTTGTATC-3'
mouse <i>Tbx-20</i>	5'-GAGCAGCTCCTCAACAGATGG-3'	5'-CCGTGGCTGGTACTTATTCATT-3'
mouse <i>Foxm1b</i>	5'-CCTGTCTCCTCCACTCCTA-3'	5'-ACTGGGCTGAAATCTAAC-3'
mouse <i>ANP</i>	5'-GCTCCTTCTCCATCACCTCG-3'	5'-ACCGGCATCTTCTCCTCCA-3'
mouse <i>BNP</i>	5'-TATCTGTACCCGCTGGGAGG-3'	5'-TTGTGAGGCCTTGGTCTTC-3'
mouse <i>Nkx2-5</i>	5'-ACGGCAGGACCACTGATC-3'	5'-CGTAGGCCGGGAGCTAGG-3'
rat <i>Tbx5</i>	5'-GGTCCGTAACCTGGTAAAG-3'	5'-ATTTTCGTCTGCTTTTCAC-3'
rat <i>Tbx20</i>	5'-CAGATGGTGTCTTTTGA-3'	5'-CCGTGTGGTCTTTCTTCT-3'
rat <i>Mef 2A</i>	5'-CAACATTAGCAGAGTCCA-3'	5'-TATTCGCACCAGTATTTTC-3'
rat <i>Mef 2B</i>	5'-AGGGGACTGGGCACATC-3'	5'-GTGGTTAGGAAGGCGAAGC-3'
rat <i>Mef 2C</i>	5'-GCAGACGATTTCAGTAGGT-3'	5'-CCTTTGTTTTCTTTCTTG-3'
rat <i>Mef 2D</i>	5'-TGTCCCAAGTCGTGAAC-3'	5'-GCCGCATCTCTTCACAG-3'
rat <i>GATA4</i>	5'-GCAGGGCGGAGGCAGTGG-3'	5'-AGGCCTGGGGTGTAAAGC-3'
rat <i>ANP</i>	5'-ACCTGGAGGAGAAGATGCCG-3'	5'-TGTTGCAGCCTAGTCCGCTC-3'
rat <i>BNP</i>	5'-GTGCTGCCCCAGATGATT-3'	5'-GGTCTATCTCTGCCCAAAG-3'
rat <i>Rel</i>	5'-TCGGTGTGTAAAGAAAA-3'	5'-TCCATAGACTCGCTGACT-3'
rat <i>p50</i>	5'-TGGTCAAAATGGA AAAA-3'	5'-ATAGGCAAGGTGAGAATG-3'
rat <i>RelB</i>	5'-AGCGAAAGCGGGACTGC-3'	5'-GATGGTGGGAGGAACAGG-3'
rat <i>RelA</i>	5'-TCACCAAAGACCCACTC-3'	5'-AGGGGTATTTGTTGGTCT-3'
rat <i>p52</i>	5'-ATGCGGAGAAATGAAGAGC-3'	5'-GCATCCCCAGTGACAGC-3'

The annealing temperature was 55°C for all primers.

3 µg of either RSV-β-gal, RSV-p50, or RSV-c-Rel and 10 ng of Renilla (pRLTK) vector using an Effectene transfection kit (Qiagen). Cells were lysed in passive lysis buffer 48 hours after transfection, and luciferase and Renilla activity was measured using a Stop & Glo kit (Promega).

Cross-Linked Chromatin Immunoprecipitation Assay

A cross-linked chromatin immunoprecipitation (ChIP) assay was performed using 100 µg cross-linked chromatin prepared from H9c2 cells as described previously.³⁸ In each ChIP reaction, 10 µg of antibody raised against c-Rel (Santa Cruz Biotechnology), p50 (Abcam) or appropriate irrelevant antibody control was used. The 3500-bp sequence upstream of ATG for each gene was taken from the ENSEMBL database, and putative transcription factor binding sites were predicted *in silico* using Promo (available via the ALGGEN server, Polytechnic University of Catalonia, Barcelona, Spain) Transfac 8.3 and TFSearch software (see Supplemental Table S1 at <http://ajp.amjpathol.org>). ChIP primers for rat brain natriuretic peptide (BNP), rat atrial natriuretic peptide (ANP), rat Gata4, rat Mef2A promoters, and Tgfβ1 exon 1-intron 1 boundaries were specific. The sequences were as follows. Rat BNP site 1 Forward 5'-CTATACAAGGCCT-GCGGTTT-3' and reverse 5'-TGCCTCTGCTTTATCCTG-3'; rat ANP forward 5'-GAGAGGAGCTGGACCATGAG-3' and reverse 5'-CCCAGCATCCACATAAAAAGC-3'; rat Mef2A forward 5'-TCTGCCCTGACATCCCTAC-3' and

reverse 5'-AGAGCTCCCAGGAACTACCC-3'; rat Gata4 sites 1 to 4 forward 5'-AGAGGGGCTTTTCGGTAGAA-3' and reverse 5'-GAAAGTCCGAAGCAGTGTCC-3'; rat Gata4 site 5 forward 5'-GTGCTCAGTCCTTCTCTGG-3' and reverse 5'-TGGAATACCACCCGGTAAGA-3'; and rat Tgfβ1 exon 1-intron 1 forward 5'-GACCTGCTGGCA-ATAGCTTC-3' and reverse 5'-ACGGGAGTGGGAGCA-GAA-3'. The annealing temperature was 55°C. Primers recognizing the PPARγ exon 1-intron 1 boundaries have been described previously.³⁹ Each PCR reaction was performed in triplicate, and the analysis was repeated in at least three independent ChIP experiments. A signal intensity value for each sample was calculated from the mean of the experiments.

Statistical Analysis

P values were calculated using a two-tailed unpaired Student's *t*-test or one-way analysis of variance with Tukey's post hoc test or Bonferroni's test for individual subgroup comparison if required. In all tests, *P* < 0.05 was used as the criterion for statistical significance.

Ethics

All animal experiments were approved by the local ethical review committee and were conducted under a United Kingdom Home Office license. All human LV tissue was collected by a protocol approved by the Papworth (Cambridge) Hospital Tissue Bank review board

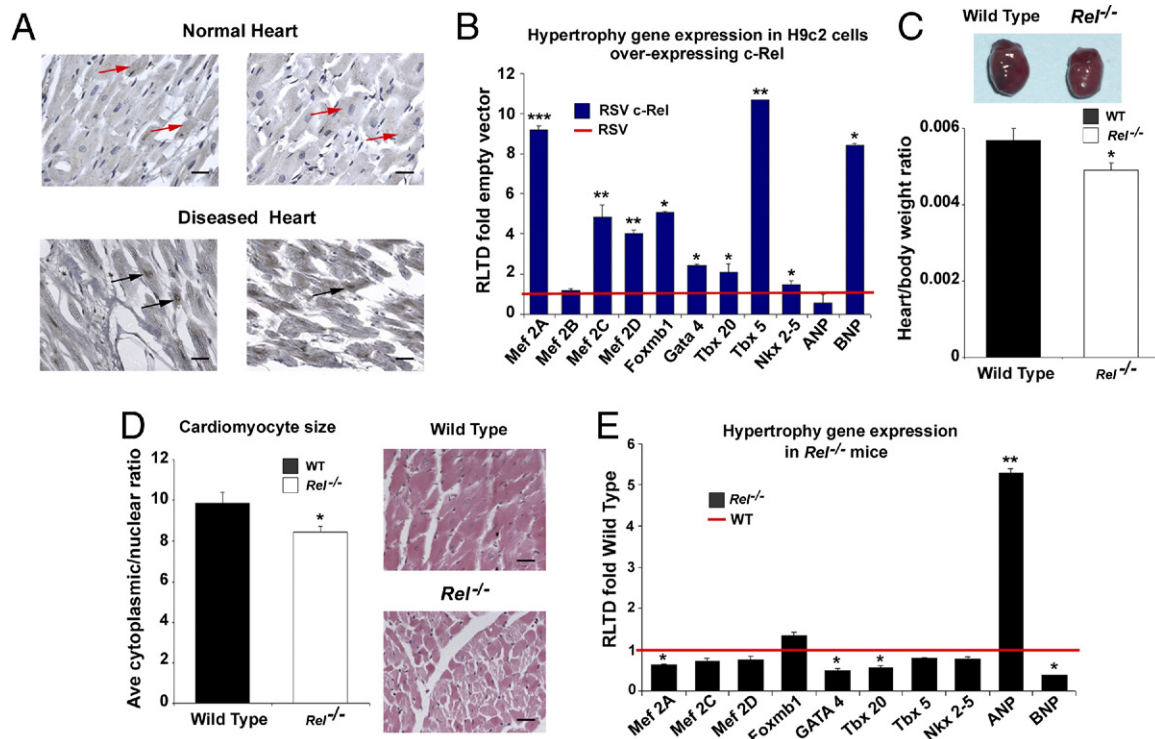


Figure 1. c-Rel regulates cardiac growth in mouse and human. **A:** Human LV tissue from normal and diseased hearts was stained for c-Rel protein. **Black arrows** indicate nuclear staining; **red arrows** indicate cytoplasmic staining. Photomicrographs are representative of five normal and five diseased human hearts. H9c2 cells were transiently transfected with RSV- β -gal (control plasmid) or RSV-c-Rel. **B:** Quantification of mRNA showed that c-Rel positively regulates cardiac expression of transcriptional activators of hypertrophy and hypertrophic markers. Relative level of transcriptional difference RLTD was expressed as mean fold change \pm SEM, compared with RSV, of five independent transfections. **C:** Representative images of hearts isolated from adult WT (**left**) and *Rel*^{-/-} mice (**right**) show decreased heart size in the knockout mice. Cardiac hypertrophy is further highlighted by a decrease in heart/body weight ratio in mice lacking *Rel*, compared with WT. Results are expressed as mean ratio change in *Rel*^{-/-} mice compared with WT \pm SEM; *n* = 20 mice/group. **D:** Cardiomyocyte size is reduced in *Rel*^{-/-} mice, compared with controls. Photomicrographs show heart sections from WT and *Rel*^{-/-} mice, stained with hematoxylin and eosin. Original magnification, $\times 400$. Scale bars: 100 μ m. Image analysis was used to calculate mean cytoplasmic/nuclear area ratios. Results are expressed as mean ratio change compared with WT \pm SEM; *n* = 5 mice/group. **E:** Eight of the 10 genes measured were down-regulated in *Rel*^{-/-} mice, compared with WT. Data are expressed as mean RLTD fold change \pm SEM, relative to WT; *n* = 5 mice/genotype. All *P* values were calculated using a one-way analysis of variance or an unpaired two-tailed Student's *t*-test. **P* = 0.05, ***P* = 0.01, and ****P* = 0.001.

and the Cambridgeshire Research Ethics Committee (Reference: 06/Q0104/64). Written consent was obtained from every individual according to the Papworth Tissue Bank protocol.

Results

c-Rel Positively Regulates Cardiac Growth in Mouse and Human

The canonical or classical NF- κ B signaling pathway uses three of the five family members: RelA, c-Rel, and p50. Of these, only RelA and c-Rel contain a transactivation domain.²⁶ The p50 subunit lacks a transactivation domain, and therefore p50 homo-dimers are unable to stimulate gene transcription unless complexed with a coactivator.^{27,32} Previous studies have reported an increase in RelA activation in the diseased heart,^{14,15} but studies using genetically modified mice suggest that therapeutic targeting of this subunit or its upstream kinases could be detrimental, given that RelA signaling plays a vital role in preventing cardiomyocyte apoptosis in response to cardiac damage.^{22,23} However, the role of the other trans-

activating subunit, c-Rel, in cardiac pathology has not been characterized previously.

To ascertain whether c-Rel-dependent signaling plays a role in the pathogenesis of cardiac disease, we performed immunohistochemical staining for c-Rel in LV tissue from normal and diseased human hearts (Figure 1A). c-Rel was confined primarily to the nucleus in cardiomyocytes in patients with end-stage ischemic or idiopathic dilated cardiomyopathy. Conversely, in normal, nondiseased heart tissue we observed comparatively lower levels of c-Rel, restricted to the cytoplasm. We hypothesize that the nuclear localization of c-Rel in the diseased heart alters the transcriptional activity of target genes and contributes to disease pathogenesis. To identify candidate prohypertrophic target genes under the control of c-Rel, we overexpressed c-Rel in the neonatal rat cardiac cell line H9c2. These experiments revealed that c-Rel induces multiple genes that promote cardiac hypertrophy, including transcriptional stimulators of hypertrophy (myocyte enhancer factor proteins Mef2A, Mef2C, and Mef2D, Gata4, and Tbx5) and the cardioprotective factor BNP (Figure 1B). Levels of Foxm1b, a transcription factor reported to regulate lung and heart cell size and promote liver regeneration^{40,41} were also elevated when c-Rel was overexpressed. These

data led us to hypothesize that cardiac deletion of c-Rel would result in a reduction in heart size.

To test this idea, we isolated hearts from normal, non-stressed adult *Rel*^{-/-} and WT mice. *Rel*-deficient mice are viable and apparently normal, apart from some modest immune defects, attenuated responses to liver injury, and certain bacterial infections. We observed that the *Rel*^{-/-} hearts appeared to be smaller than the WT controls (Figure 1C). This was verified by a significant reduction in heart/body weight ratio in *Rel* knockout mice, compared with WT controls. Blood pressure did not differ significantly between *Rel* knockout and WT mice (see Supplemental Table S2 at <http://ajp.amjpathol.org>), suggesting that the cardiac phenotype is not a consequence of hemodynamic changes. The reduction in overall heart size and heart/body weight ratio was accompanied by a decrease in the cytoplasmic/nuclear ratio of cardiomyocytes in *Rel*-deficient hearts (Figure 1D). To determine whether the reduction in heart size is a feature of development or a postnatal phenotype, we measured the heart/body weight ratio in newborn mice. This ratio was also significantly reduced in *Rel*^{-/-} newborn mice, confirming that c-Rel can control cardiac growth during development as well as in adult life (see Supplemental Figure S1A at <http://ajp.amjpathol.org>).

To determine whether c-Rel controls genes that regulate cardiomyocyte size, we next measured expression of hypertrophy-associated genes in both genotypes. The transcription factors Mef2A, Mef2C, Mef2D, Gata4, Tbx5, and Tbx20 were expressed at reduced levels in *Rel*-deficient heart (Figure 1E). Of note, all of these genes were significantly up-regulated by overexpression of *Rel* (Figure 1B). BNP was expressed at reduced levels in *Rel*^{-/-} hearts (Figure 1E); by contrast, ANP levels were elevated, which may indicate that a compensatory mechanism operates to ensure appropriate physiological levels of this natriuretic peptide.

Gene Deletion of c-Rel Protects Against Angiotensin-Induced Cardiac Hypertrophy and Fibrosis

Our data show that c-Rel stimulates cardiac growth under normal physiological conditions and that it is confined to the nucleus in human diseased hearts. We therefore asked whether c-Rel-dependent gene transcription is required for the development of cardiac hypertrophy. To test this hypothesis, we subjected both *Rel*^{-/-} and WT mice to 4 weeks of angiotensin or saline (vehicle) infusion via a mini-osmotic pump. Prolonged exposure to angiotensin causes hypertension and chronic pressure overload, triggering cardiac remodeling, which in turn results in cardiac hypertrophy and fibrosis. Calculation of heart/body weight ratio confirmed the reduction in heart size in saline-infused *Rel*^{-/-} mice, compared with the WT (Figure 2A). Chronic angiotensin infusion caused a 27% increase in heart/body weight ratio in WT mice. In *Rel*^{-/-} mice, however, the ratio was increased by 9%, only a third of that observed in WT animals. This cardioprotective effect was further supported by a statistically signif-

icant reduction in the cytoplasmic/nuclear area ratio of LV cardiomyocytes in *Rel*^{-/-} mice, compared with WT mice, treated with angiotensin, as shown by laminin immunostaining (Figure 2B; see also Supplemental Figure S1B at <http://ajp.amjpathol.org>).

Cardiac remodeling and fibrosis are features of this model. Morphometric analysis of Sirius Red staining for collagen revealed that mice lacking *Rel* develop significantly less fibrosis than control mice (Figure 2C). We also noted a failure to up-regulate expression of key prohypertrophic transcription factors, including Mef2A and Nkx2-5, and a suppression of basal Gata4 levels in chronic angiotensin-treated *Rel*^{-/-} mice. The inability to induce Mef2A expression after angiotensin treatment in these mice is the likely explanation for the observed increase in levels of the cardioprotective peptide ANP; Naya et al⁴² reported that ANP expression is elevated in Mef2A-deficient mice. Up-regulation of ANP is a normal physiological response to protect the heart from damage caused by pressure overload. Elevated expression of ANP could be another mechanism conveying protection to *Rel*-deficient mice from chronic angiotensin infusion-induced hypertrophy (Figure 2D).

c-Rel Directly Binds Hypertrophy-Related Gene Promoters

To determine whether c-Rel directly regulates genes that promote cardiac hypertrophy or whether the effects observed *in vitro* and *in vivo* are indirect, we performed ChIP assays. With this technique, we were able to demonstrate a physical interaction between *Rel* and κ B regions of the Gata4 (Figure 3A) and Mef2A (Figure 3B) gene promoters, providing further evidence for c-Rel as a transcriptional regulator of prohypertrophic genes. To determine whether c-Rel or p50 can transactivate the Gata4 promoter, we cotransfected H9c2 cells with a Gata4-luciferase construct and either RSV- β -gal, RSV-p50, or RSV-c-Rel. Overexpression of c-Rel increased activity of the Gata4-luciferase reporter construct by twofold, whereas overexpression of p50 was without effect, suggesting that c-Rel but not p50 is a critical transcriptional regulator of the Gata4 gene (see Supplemental Figure S2A at <http://ajp.amjpathol.org>).

To rule out nonspecific interaction of the c-Rel antibody with the transcriptional machinery, we performed ChIP analysis on the exon 1-intron 1 boundaries of *Tgfb1* and *Pparg*, two control genes that lack c-Rel binding sites. We did not observe an interaction of p50 or c-Rel with either gene, suggesting the interactions with Gata4, Mef2A, and the natriuretic peptides are specific (Supplemental Figure S2B at <http://ajp.amjpathol.org>). This identifies c-Rel but not p50 as the critical NF- κ B subunit regulating the Mef2A promoter. These data are consistent with our observations in the angiotensin infusion model, in which *Rel* gene-deficient mice fail to induce expression of Mef2A in response to angiotensin. c-Rel was also recruited to the gene promoters of the cardioprotective peptides ANP and BNP (Figure 3C). These results verify that c-Rel not only promotes transcription of hypertrophy-associated genes but also directly binds their promoters.

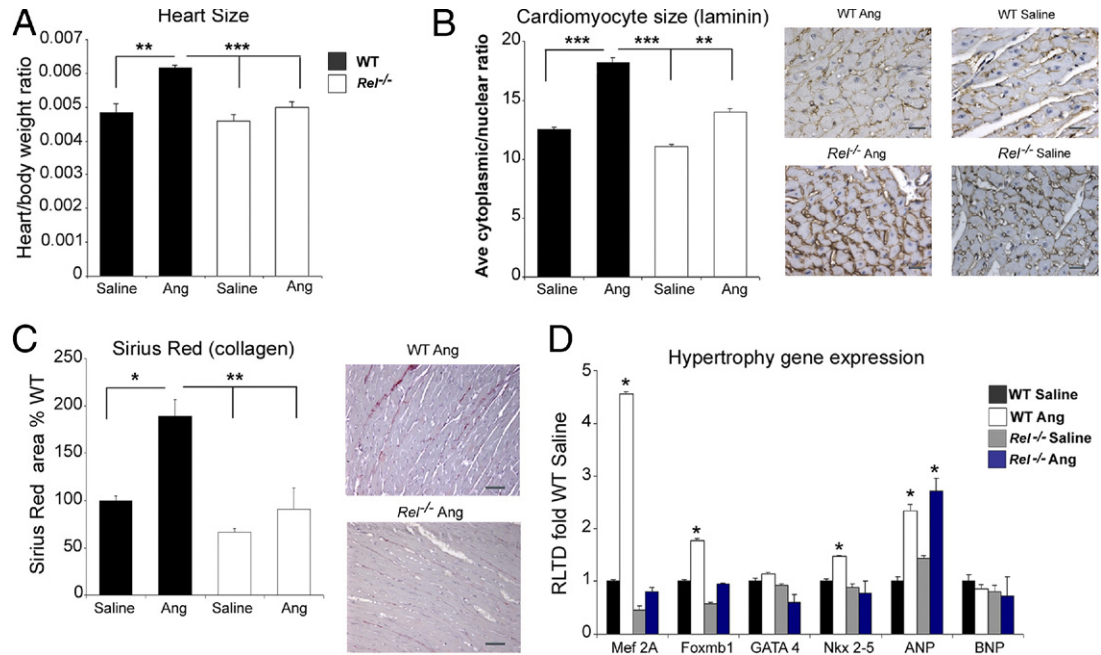


Figure 2. *Rel* knockout mice are protected from angiotensin-induced cardiac hypertrophy and fibrosis. **A:** The heart/body weight ratio was calculated in *Rel*^{-/-} and WT mice after 4 weeks of saline vehicle or angiotensin (Ang) infusion. **B:** Mean cytoplasmic/nuclear area ratio was calculated using image analysis of laminin-stained hearts. Data are expressed as mean percentage change, compared with WT. Cardiomyocyte size was increased in WT mice after angiotensin, indicative of a hypertrophic response. By contrast, cardiomyocytes of angiotensin-treated *Rel*^{-/-} mice were smaller than in the WT, suggesting a cardioprotective effect. Representative photomicrographs show heart sections from angiotensin-infused WT and *Rel*^{-/-} mice immunostained with anti-laminin antibodies. Cardiac remodeling and fibrosis was reduced in angiotensin-infused *Rel*^{-/-} mice, compared with WT. Original magnification, ×400. Scale bars: 100 μm. **C:** Densitometric analysis of Sirius Red-stained heart tissues showed a statistically significant increase in collagen deposition in WT mice after angiotensin infusion; however, less collagen was observed in both the saline and angiotensin-infused *Rel*^{-/-} mice. Representative photomicrographs show Sirius Red-stained heart sections from angiotensin-treated WT and *Rel*^{-/-} mice. Data are expressed as mean ratio or percentage change ± SEM; *n* = 5 (*Rel*^{-/-}) and *n* = 6 (WT) mice/group. **D:** Relative mRNA levels of hypertrophy-associated genes were determined in *Rel*^{-/-} and WT mice after angiotensin infusion. Angiotensin infusion induces cardiac expression of transcriptional regulators of hypertrophy; however, this response is attenuated in *Rel*^{-/-} mice. The RLTD was calculated between WT and *Rel*^{-/-} mice and expressed as a mean fold change ± SEM, relative to WT; *n* = 4 mice/genotype. All *P* values were calculated using a one-way analysis of variance. **P* = 0.05, ***P* = 0.01, and ****P* = 0.001.

The p50 Subunit of NF-κB Represses c-Rel Expression and Limits Cardiac Hypertrophy and Fibrosis

Regulation of c-Rel at the transcriptional level is poorly defined. To determine whether overexpression of either p50 or c-Rel affects endogenous expression of the five

NF-κB subunits, we overexpressed p50 and c-Rel in H9c2 cells and measured each family member at the RNA level. Our data revealed that p50 can repress the expression of c-Rel, whereas overexpression of c-Rel leads to its own positive regulation (Figure 4A). These data suggest that a function of p50 in the normal heart may be to limit expression of c-Rel and limit its influence

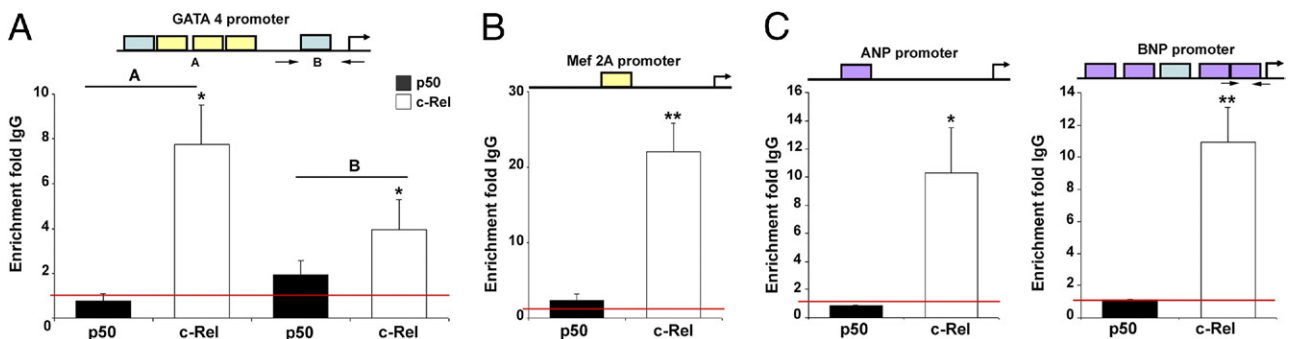


Figure 3. c-Rel binds to hypertrophy-related gene promoters. Cross-linked ChIP analysis revealed binding of c-Rel to the promoters of transcriptional activators of hypertrophy (A and B) and to cardioprotective factors (C). In the promoter schematics, blue boxes indicate NF-κB-c-Rel consensus sequences, yellow boxes indicate preferential c-Rel consensus sequences, and purple boxes indicate NF-κB consensus sequences. H9c2 cells were formalin-fixed. Chromatin was then isolated and sheared by sonication, and 100 μg of chromatin was incubated with an anti-c-Rel antibody, anti-p50 antibody, or irrelevant IgG isotype control. Immunoprecipitation reactions were performed, proteins were digested, and cross-links were reversed before purification of genomic DNA and qRT-PCR amplification of promoters of interest. Binding was normalized to total input genomic DNA and is expressed as fold IgG control. Data are representative of at least three separate experiments. All *P* values were calculated using one way analysis of variance. **P* = 0.05, ***P* = 0.01.

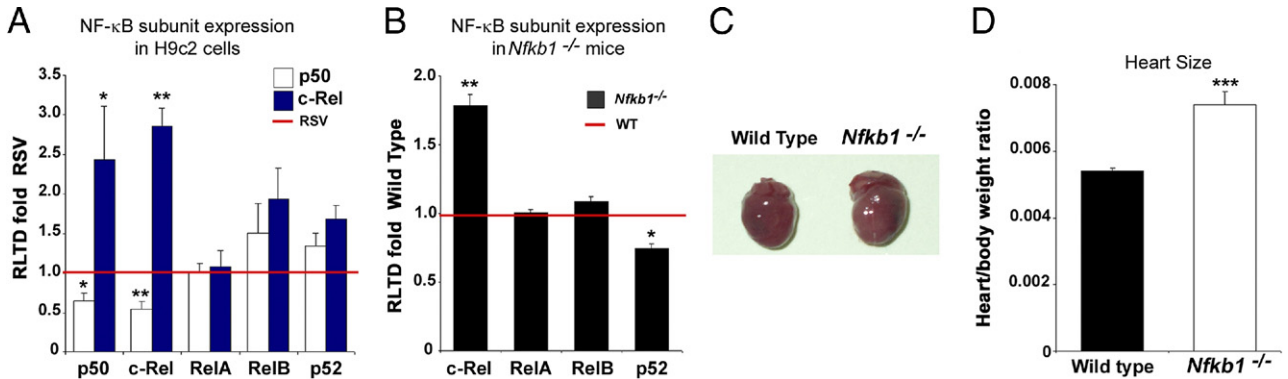


Figure 4. p50 suppresses the prohypertrophic effects of c-Rel. H9c2 cells were transiently transfected with expression plasmids (control RSV-β-gal, RSV-p50, or RSV-c-Rel). RNA was isolated, and cDNA was generated and used as a template for qRT-PCR. The RLTD was calculated and expressed as mean fold change ± SEM, relative to RSV, of five independent transfections. **A:** Gene expression levels of the individual NF-κB subunits revealed that overexpression of p50 repressed expression of c-Rel. **B:** RNA was isolated from the hearts of adult WT and *Nfkb1*^{-/-} mice, and mRNA levels of the NF-κB subunits RelA, NF-κB2, c-Rel, and RelB were quantified using qRT-PCR. c-Rel was the only NF-κB subunit to be up-regulated in *Nfkb1*^{-/-} mice. The RLTD between WT and *Nfkb1*^{-/-} mice was calculated and expressed as a mean fold change ± SEM relative to WT; *n* = 5 mice/genotype. **P* = 0.05, ***P* = 0.01. **C:** Representative photographs of hearts isolated from adult WT and *Nfkb1*^{-/-} mice show increased heart size in *Nfkb1*^{-/-} mice. **D:** The heart/body weight ratio was calculated in *Nfkb1*^{-/-} and WT control mice on a pure C57Bl/6 background; data are expressed as mean percentage change ± SEM, compared with WT; *n* = 17 mice/genotype. An increase in heart/body weight ratio was observed in male *Nfkb1*^{-/-} mice, compared with WT controls, in both backgrounds. ****P* < 0.001.

on cardiac physiology. To investigate this further, we compared NF-κB subunit expression in the hearts of WT and *Nfkb1* (p50) knockout mice. Our data confirmed that c-Rel is repressed by p50 (Figure 4B; see also Supplemental Figure S3A at <http://ajp.amjpathol.org>), given that mice lacking this subunit have elevated cardiac levels of c-Rel. We then predicted that hearts lacking p50 may be enlarged, because the repressive effects of p50 have been lost.

To test this idea, we isolated hearts from *Nfkb1* (p50^{-/-}) gene knockout and WT mice. Hearts from *Nfkb1*^{-/-} mice were indeed larger than the WT controls, as confirmed by heart/body weight ratios (Figure 4, C and D). This prohypertrophic phenotype was conserved in two different mouse genetic backgrounds (Figure 4D; see also Supplemental Figure S3B at <http://ajp.amjpathol.org>). Hypertrophy of *Nfkb1*^{-/-} hearts was confirmed by a statistically significant increase in the number of sarcomeric units and cardiac sarcomeric actin levels (Figure 5A; see also Supplemental Figure S3A at <http://ajp.amjpathol.org>), as well as by an increase in the cytoplasmic/nuclear ratio (Figure 5B; see also Supplemental Figure S3C at <http://ajp.amjpathol.org>). There was no difference in blood pressure between the two genotypes, suggesting that the spontaneous development of cardiac hypertrophy in *Nfkb1*^{-/-} mice is not driven by pre-load or after-load effects on the heart (see Supplemental Table S1 at <http://ajp.amjpathol.org>). For newborn mice, however, we found no evidence of difference in heart/body ratio, suggesting that hypertrophy develops with adulthood (see Supplemental Figure S3D at <http://ajp.amjpathol.org>).

Cardiac remodeling and fibrosis are common features of heart damage and disease. Sirius Red staining revealed spontaneous development of fibrosis in the hearts of 12-week-old *Nfkb1*^{-/-} mice (Figure 5C). We next sought to determine whether transcription factors that drive cardiac hypertrophy are up-regulated in the hearts of *Nfkb1*^{-/-} mice. Levels of cardiac gene expression of

Mef2 isoforms 2A, 2C and 2D were increased in *Nfkb1*^{-/-} mice, compared with controls, and all were down-regulated in *Rel*-deficient mice. Gata4 is a key transcriptional regulator linked to the development of cardiac hypertrophy, and its expression was also elevated in these mice (Figure 5D). We also observed an increased expression of Foxm1b in *Nfkb1*^{-/-} mice (Figure 5D), which may be highly relevant, given the role previously suggested for this factor in organ hypertrophy. Consistent with these data, markers of cardiac hypertrophy Nkx2-5 and TBx20 were also elevated in *Nfkb1*^{-/-} hearts.

The vasoactive hormones BNP and ANP are produced by cardiomyocytes and are cardioprotective.⁴³ BNP levels were modestly elevated in *Nfkb1*^{-/-} heart; by contrast expression of ANP was markedly suppressed. Of note, genes for Mef2A, Gata4, and the natriuretic peptide BNP were not only elevated in the *Nfkb1*^{-/-} hearts, but were also identified as c-Rel target genes in the ChIP analysis.

Discussion

Activation of NF-κB is strongly linked with the development of cardiac growth and hypertrophy, and blockade of this pathway has been proposed as a promising approach for prevention of adverse cardiac remodeling.^{18–20} This idea is supported by clinical observations and in rodent models of heart disease. Clinically, increased nuclear translocation of the RelA subunit has been observed in end-stage dilated cardiomyopathy. Furthermore, Santos et al¹⁶ recently reported a polymorphism in the *Nfkb1* gene that leads to reduced gene activation, which is associated with an increase in onset and progression of cardiac remodeling, cardiac deterioration, and heart failure in patients with dilated cardiomyopathy. In rodent models of cardiac hypertrophy involving angiotensin II, isoproterenol, and aortic-banding, significant cardioprotective effects were observed either in mice expressing an NF-κB super-repressor or in ro-

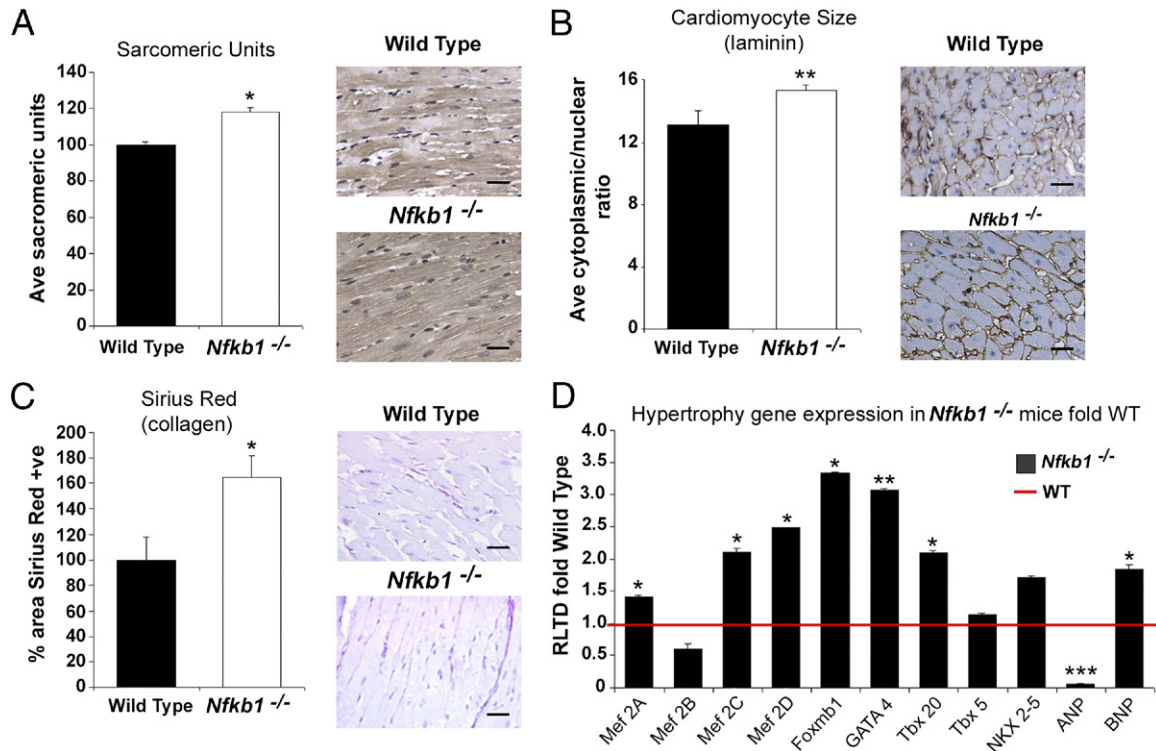


Figure 5. Features of cardiac hypertrophy and fibrosis are observed in *Nfkb1* knockout mice. **A:** Heart sections were stained for α -sarcomeric actin and sarcomeric units were counted. The mean number (Ave) of sarcomeric units in *Nfkb1*^{-/-} mice was significantly increased, compared with WT. **P* = <0.05. **B:** Representative photomicrographs of laminin-stained WT and *Nfkb1*^{-/-} mouse hearts. Mean cardiomyocyte cytoplasmic/nuclear area ratios were calculated using image analysis software. The ratio was significantly increased in *Nfkb1*^{-/-} hearts, compared with WT. ***P* < 0.01. **C:** Representative photomicrographs of Sirius Red-stained heart sections from WT and *Nfkb1*^{-/-} mice. Densitometric analysis revealed a statistically significant increase in collagen deposition (red fibers) in *Nfkb1*^{-/-} mice. **P* = <0.05. Data are expressed as means \pm SEM of eight WT and six *Nfkb1*^{-/-} mice. +ve, positive. **D:** Relative mRNA levels of the transcriptional regulators of hypertrophy myocyte enhancer factor 2 (Mef2) A, C, and D (but not Mef2B), Gata4, and Foxm1b were elevated in *Nfkb1*^{-/-} mice, compared with WT. Deletion of *Nfkb1* was associated with an increase in gene expression of Nkx2-5 and of Tbx20. Cardiac mRNA levels of the cardioprotective proteins brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) were quantified. BNP expression was increased in the *Nfkb1*^{-/-} mice, compared with WT; however, expression of ANP was barely detectable in *Nfkb1*^{-/-} mice. RLTD was calculated between WT and *Nfkb1*^{-/-} mice and expressed as mean fold change \pm SEM, relative to WT; *n* = 5 mice/genotype. Original magnification, \times 400. Scale bars: 100 μ m.

dents treated with pan NF- κ B or IKK inhibitors.^{44,45} Other studies, however, have indicated that NF- κ B signaling is cardioprotective in heart disease, particularly those linked to the RelA subunit, because inhibiting the actions of this subunit is likely to severely affect cardiomyocyte homeostasis and survival.^{22,23,27,46} Hence, long-term pan-blockade of NF- κ B signaling could be detrimental. An alternative approach would be to target one or more of the other four NF- κ B subunits. Here, we have described for the first time a pivotal role for c-Rel as a stimulator of cardiac hypertrophy and remodeling.

Our data suggest that c-Rel is predominantly found in the cytoplasm of cardiomyocytes in the normal human heart, whereas its localization is primarily nuclear in the diseased human heart. Mice lacking c-Rel develop with reduced heart size and are protected from angiotensin-induced cardiac hypertrophy and fibrosis. We further suggest that the p50 subunit limits the influence of c-Rel on cardiac physiology by suppressing its expression. The importance of this crosstalk between the two subunits was highlighted by our observation of spontaneous development of cardiac hypertrophy and fibrosis in mice lacking p50. Grumont et al⁴⁷ identified three putative NF- κ B binding sites in the *Rel* gene promoter. Their electrophoretic mobility shift assay analysis revealed that Re-

IA:p50 heterodimers and p50:p50 homodimers bind the κ B1 and κ B3 sites; however, these sites exhibit a higher affinity for the p50 homodimer, whereas dimers containing RelA, c-Rel, and p50 are bound to the κ B2 site. Expression of c-Rel is regulated at the transcriptional level in immune cells, and control of c-Rel has been shown to vary greatly depending on stimulus, cell type and cell function.⁴⁸ Future investigation into the mechanism by which p50 regulates c-Rel expression in the normal and diseased heart is therefore warranted, given that targeting the crosstalk between p50 and c-Rel may reveal therapeutic opportunities.

Alternatively, the interaction of c-Rel with its target hypertrophy-related genes (*Mef2a* and *Gata4*) may offer strategies to inhibit cardiac remodeling. Activation of Mef2A-dependent transcription is required for cardiac development and growth and for initiating the phenotypic reprogramming of myocytes that results in cardiac remodeling and hypertrophy.^{2,42,49,50} Mef2A appears to be a direct target of c-Rel, and therefore a plausible explanation for the reduction in *Rel* knockout heart size at birth and in adulthood could be insufficient production of Mef2A (although this would require further investigation). Furthermore, deficiency of c-Rel leads to a failure to induce Mef2A expression after chronic angiotensin infu-

sion, which was associated with protection from cardiac fibrosis and enlargement. Mef2A expression was also induced in cardiac cells overexpressing c-Rel and in the hearts of mice lacking p50. Defining the nature of the c-Rel-containing NF- κ B complexes assembled at the promoters of *Mef2a* and other c-Rel target genes identified in the present study will be an important step toward improving our understanding of the transcriptional mechanisms that regulate stress-induced remodeling of the heart. Moreover, the regulatory events that lead to nuclear accumulation of c-Rel in the diseased human heart will be of great interest and could be a potential source of therapeutic targets.

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