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Interaction between NF κ B and NFAT Coordinates Cardiac Hypertrophy and Pathological Remodeling

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Abstract

Rationale—Both nuclear factor of activated T cells (NFAT) and nuclear factor κ B (NF κ B) are Rel homology domain (RHD)-containing transcription factors whose independent activities are critically involved in regulating cardiac hypertrophy and failure.

Objective—To determine the potential functional interaction between NFAT and NF κ B signaling pathways in cardiomyocytes and its role in cardiac hypertrophy and remodeling.

Methods and Results—Here we identified a novel transcriptional regulatory mechanism whereby NF κ B and NFAT directly interact and synergistically promote transcriptional activation in cardiomyocytes. We show that the p65 subunit of NF κ B co-immunoprecipitates with NFAT in cardiomyocytes, and this interaction maps to the RHD within p65. Overexpression of the p65-RHD disrupts the association between endogenous p65 and NFATc1, leading to reduced transcriptional activity. Overexpression of I κ B kinase β (IKK β) or p65-RHD causes nuclear translocation of NFATc1, and expression of a constitutively nuclear NFATc1-SA mutant similarly facilitated p65 nuclear translocation. Combined overexpression of p65 and NFATc1 promotes synergistic activation of NFAT transcriptional activity in cardiomyocytes while inhibition of NF κ B with I κ B α M or dominant negative IKK β reduces NFAT activity. Importantly, agonist-induced NFAT activation is reduced in *p65* null mouse embryonic fibroblasts (MEFs) compared to wild-type MEFs. *In vivo*, cardiac-specific deletion of *p65* using a Cre-loxP system causes a ~ 50% reduction in NFAT activity in luciferase reporter mice. Moreover, ablation of *p65* in the mouse heart decreases the hypertrophic response following pressure overload stimulation, reduces the degree of pathological remodeling, and preserves contractile function.

Conclusions—Our results suggest a direct interaction between NFAT and NF κ B that effectively integrates two disparate signaling pathways in promoting cardiac hypertrophy and ventricular remodeling.

Keywords

hypertrophy; signaling; cardiomyocyte; NFAT; NF κ B

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Disclosures:
None.

Introduction

Cardiac hypertrophy is typically characterized by enlargement of the heart and an increase in myocyte cell volume that occurs in response to hemodynamic stress, acute myocardial injury, infection, or mutations in genes encoding sarcomeric proteins.¹ Pathological hypertrophy of the myocardium temporarily preserves pump function, although prolongation of this state is a leading predictor for the development of arrhythmias and sudden death, as well as dilated cardiomyopathy and heart failure.² Neural, humoral, and intrinsic hypertrophic stimuli directly activate membrane bound receptors that in turn activate nodal intracellular signal transduction pathways such as the mitogen-activated protein kinase (MAPK) cascade, calcineurin-NFAT (nuclear factor of activated T cells), insulin-like growth factor-I-phosphatidylinositol 3-kinase-Akt/protein kinase B, and many others.³ These intracellular signaling cascades then modulate transcriptional regulatory proteins, altering gene expression to facilitate the growth of the heart. For example, transcription factors such as NFAT, myocyte enhancer factor-2 (MEF-2), and GATA4/6 are directly activated by cytoplasmic signaling effectors, which in turn mediate hypertrophic gene expression in cardiomyocytes.^{3,4} Similarly, the transcription factor NF κ B has been implicated as a key regulator of cardiomyocyte hypertrophy both *in vitro* and *in vivo*.⁵ However, the integration amongst these transcriptional effector pathways in the regulation of hypertrophic programming has not been explored in the heart.

The NF κ B/Rel family of transcription factors participates in the regulation of disparate cellular processes such as proliferation, differentiation, immune responses, cell growth, cardiac hypertrophy, and apoptosis.^{6,7} The Rel family proteins include p65 (RelA), p105/p50, p100/p52, RelB, c-Rel and the viral oncoprotein v-Rel. All Rel family members are conserved throughout evolution and share a Rel homology domain (RHD) at their N terminus that mediates DNA binding and protein-protein interaction between family members. These proteins associate as homo- or heterodimers to form transcriptional regulatory complexes. In unstimulated cells, NF κ B binds to cytosolic inhibitory proteins I κ Bs, mediating cytoplasmic retention. Upon various types of stimulation, I κ Bs are phosphorylated by I κ B kinase (IKK) causing their degradation and subsequent release of NF κ B for nuclear translocation.^{8,9} The IKK complex is composed of 2 catalytic subunits (IKK α and IKK β) and a regulatory subunit (IKK γ or NEMO). Recent data also suggests that NF κ B can shuttle into the nucleus in nonstimulated cells, although the physiological significance of this finding is unknown.⁵ Post-translational modifications (e.g., phosphorylation and acetylation) and physical interaction with coactivators and corepressors have been identified as additional regulatory mechanisms.¹⁰

NFAT transcription factors are also part of the RHD family, where they bear structural similarity to NF κ B and even bind related or overlapping DNA sequence elements.⁶ The NFAT family of transcription factors consists of five family members (NFATc1, NFATc2, NFATc3, NFATc4 and NFAT5 [TonEBP]). NFAT transcription factors are normally hyperphosphorylated and sequestered in the cytoplasm, but rapidly translocate to the nucleus after calcineurin-mediated dephosphorylation.¹¹ Genetic and pharmacological studies have shown that the calcineurin-NFAT pathway is both sufficient and necessary for the cardiac hypertrophic response in a number of rodent models.^{12,13} Unlike canonical Rel-containing factors, which often homodimerize on target promoters, NFAT factors share an imperfect RHD that is only capable of weak DNA binding in the monomeric or dimeric state. In order to strengthen NFAT-DNA interactions, these factors prefer to interact with other transcription factors such as AP-1 (c-Jun/c-Fos), GATA-4, and MEF-2.¹⁴ Here we determined that NFAT and NF κ B family members could interact and assemble “higher order” transcriptional complexes that synergistically activate hypertrophic gene expression in the heart.

Materials and Methods

Animal models and procedures

The generation of *RelA* (p65) loxP-targeted (fl) mice, in which exons 7 and 10 were flanked by loxP sites, was previously described.¹⁵ Mice harboring the *p65fl/fl* alleles were crossed with mice expressing Cre recombinase under control of the endogenous *Nkx2.5* locus.¹⁶ The NFAT-luciferase reporter transgenic mouse as described previously,¹⁷ and was crossed with a *p65fl/fl^{Nkx2.5-Cre}* mouse. All experiments involving animals were approved by the Institutional Animal Care and Use Committees at Cincinnati Children's Hospital Medical Center and University of Washington.

Eight week-old mice were subjected to transverse aortic constriction (TAC) under isoflurane anesthesia as previously described.¹⁸ Pressure gradients (mm Hg) were calculated from the peak blood velocity (V_{max}) (m/s) ($PG = 4 \times V_{max}^2$) measured by Doppler across the aortic constriction, which was equivalent in all groups of TAC stimulated mice. All mice were anesthetized with 2% isoflurane by inhalation. Echocardiography was performed in M-mode using a Hewlett Packard SONOS 5500 instrument equipped with a 15 MHz transducer as described previously.¹⁹

Luciferase reporter assays in mouse hearts

In brief, hearts were removed from NFAT-luciferase transgenic mice, some of which were crossed into the *p65fl/fl^{Nkx2.5-Cre}* background. Hearts were homogenized in 1 ml luciferase assay buffer (100 mM KH_2PO_4 , pH 7.8, 0.5% Nonidet P-40, and 1 mM DTT). Homogenates were centrifuged at 3,000 *g* for 10 min at 4°C and the supernatants assayed for luciferase activity as described previously.¹⁷

Cell culture, adenoviral infection, and immunocytochemistry

Primary neonatal rat cardiomyocytes were prepared from hearts of 1- to 2-day-old Sprague-Dawley rat pups as previously described.²⁰ After separation from fibroblasts, enriched cardiomyocytes were plated on 1% gelatin-coated 12-well plates for luciferase assays or on 6-cm-diameter dishes for all other experiments. Cells were grown in M199 medium containing 100 U of penicillin-streptomycin/ml and 2 mM L-glutamine without serum for 24 h before infection. p65^{+/+} and p65^{-/-} MEFs were kindly provided by David Baltimore (California Institute of Technology). Adenoviral infections were performed as previously described at a multiplicity of infection of 10 to 50 plaque forming units per ml.²¹ Cultures were harvested 24 h after infection, and luciferase assays were performed as described previously.²¹ Ad β gal, Ad Δ CnA, Adcain, AdNFAT-luciferase reporter, AdNFATc1-GFP (green fluorescent protein), and AdNFATc3-GFP have been previously described.^{17,22-24} AdNF κ B-luciferase reporter was obtained from Vector Biolabs (Philadelphia, PA). Ad-p65 and Ad-p65-RHD-Myc were a gift of Josef Anrather and Hans Winkler (Harvard University). Adenoviral infections were performed as described previously at a multiplicity of infection of 10–100 plaque forming units per ml.²¹

Cardiomyocytes were prepared for immunocytochemistry as described previously.²⁴ Immunocytochemistry was performed using anti-p65 antibody (Santa Cruz Biotechnology), followed by ALEXA Fluor 568 anti-rabbit secondary antibody (Molecular probes). Cells infected with AdNFATc1-GFP or AdNFATc1-SA-GFP were directly visualized by fluorescent microscopy for GFP.

Western blotting and gel shift assays

Protein extraction from mouse heart or cultured cardiomyocytes and subsequent Western blotting followed by enhanced chemiluminescence detection were performed as previously

described.^{17,18} Anti-Myc and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Gel shift assays were performed as previously described.^{12,25} Briefly, 20 μ g nuclear extracts from neonatal cardiomyocytes were incubated in gel shift buffer (12 mM HEPES, pH 7.9, 4 mM Tris, pH 7.9, 50 mM KCl, 12% glycerol, 1.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 0.7 μ g/ml pepstatin) with 0.5 μ g poly (dI-dC) and ³²P-labeled NFAT or NF κ B consensus DNA sequences for 20 min at room temperature. For supershift reactions, 1 μ l of anti-NFATc1 or -p65 antibody was added after 20 min of binding reaction. DNA complexes were separated on a 5% nondenaturing polyacrylamide gel in Tris-borate EDTA buffer.

Immunoprecipitation

Neonatal rat cardiomyocytes were infected with or without specific adenoviruses for 24 h. Cells were lysed at 4°C in buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 μ g/ml aprotinin). Immunoprecipitation was performed as described previously.²¹ Whole cell lysates were cleared by centrifugation at 18,000 \times *g* for 10 min and then incubated with the indicated antibodies and protein A-sepharose beads overnight at 4°C. The beads were washed extensively with binding buffer, and the proteins were resolved on an 8–12% SDS-PAGE for subsequent Western blotting.

Statistics

All results are presented as means \pm SEM. Paired data were evaluated by Student's *t* test. A one-way ANOVA with the Bonferroni's post hoc test or repeated-measures ANOVA was used for multiple comparisons. *P* < 0.05 was considered statistically significant.

Results

Crosstalk between NFAT and NF κ B in cardiomyocytes

Both calcineurin-NFAT and IKK-NF κ B signaling pathways have been implicated as critical regulators of cardiomyocyte hypertrophy.^{12,26} Here we tested the hypothesis that these two previously deemed independent signaling pathways may actually crosstalk with one another. We first examined if NF κ B can influence NFAT transcriptional activity in cultured neonatal cardiomyocytes infected with adenoviruses encoding an NFAT-dependent luciferase reporter cassette. Indeed, NFAT luciferase activity was stimulated by the hypertrophic agonists phenylephrine, serum, and angiotensin II, but this activation was markedly reduced by inhibition of NF κ B signaling with the NF κ B super-suppressor I κ B α M, or dominant negative IKK β (Figure 1A). Similarly, inhibition of NFAT signaling with Cain or Rcan1 diminished hypertrophic agonists-induced NF κ B luciferase activity (Figure 1B). Similar reciprocal regulatory effects between NFAT and NF κ B were observed in cardiomyocytes treated with tumor necrosis factor α (TNF α), a cytokine known to activate NF κ B signaling (Online Figure IA and IB). Activated calcineurin (Δ CnA)-induced NFAT activity was also significantly reduced in NF κ B-*p65* null MEFs compared to wild-type MEFs, while adenoviral-mediated expression of p65 restored NFAT luciferase activity in *p65* null MEFs (Figure 1C). Conversely, NF κ B luciferase activity induced by adenoviral overexpression of IKK β or p65 was also largely blocked in calcineurin-deficient MEFs (lacking CnB1 protein). In addition, calcineurin-deficient MEFs showed lower baseline NF κ B activity compared with wild-type MEFs, suggesting that calcineurin regulates NF κ B equilibrium even without stimulation (Figure 1D). Inhibition of NF κ B signaling with I κ B α M or

dnIKK β also partially blocked Δ CnA-induced NFAT luciferase activity, while inhibition of NFAT signaling with Cain diminished p65 or IKK β -induced NF κ B luciferase activity in cardiomyocytes (Online Figure IC and ID). Consistent with these observations, overexpression of NFATc1 or p65 each cross-stimulated NFAT- and NF κ B luciferase activity in cardiomyocytes, whereas co-expression of NFAT with p65 synergistically activated both NFAT and NF κ B reporter activity (Figure 1E, F). To rule out the involvement of autocrine factors in the observed effects, cardiomyocytes were treated with cultured media collected from cells infected with adenoviruses encoding NFAT, p65, or both, or with IKK β and other inhibitory viruses for NFAT or NF κ B signaling (Online Figure IE, IF, and IG). No significant changes in NFAT- or NF κ B- luciferase activity was detected under these conditions, thus excluding a role for secreted autocrine factors in potentiating NFAT and NF κ B transcriptional activation.

Physical interaction between NFAT and NF κ B

While a direct interaction between NF κ B and NFAT transcription factors has not been demonstrated in cardiomyocytes, a physical association between NFATc1 and c-Rel was previously reported in lymphocytes.²⁷ Hence, we examined if NFAT interacts with p65-NF κ B in cardiomyocytes by immunoprecipitation. The results showed that NFATc1 immunoprecipitated with a p65 antibody but not with a non-specific IgG antibody (Figure 2A). Conversely, immunoprecipitation of NFATc1 with a GFP antibody resulted in the isolation of p65, suggesting an association between these two transcription factors in cardiomyocytes (Figure 2B). Immunoprecipitation experiments were also performed in neonatal cardiomyocytes stimulated with phenylephrine, TNF α , or vehicle control. While the interaction between NFAT-p65 was still observed with these agonists, no changes in intensity were observed (Online Figure II). Since both NFAT and NF κ B contain a Rel homology domain (RHD), which is important for protein-protein interaction and dimerization, cardiomyocytes infected with an adenovirus encoding p65-RHD showed co-immunoprecipitation with NFATc1 (Figure 2C). Importantly, overexpression of p65-RHD disrupted the association between NFATc1 and endogenous p65 (Figure 2C, **middle panel**). Overexpression of p65-RHD also partially inhibited calcineurin-induced NFAT luciferase activity, suggesting that p65-RHD acts as dominant negative for endogenous p65 because it lacks a transcriptional activation domain (Figure 2E). Full length p65 localized primarily in the cytoplasm of unstimulated neonatal cardiomyocytes, similar to NFATc1-GFP (Figure 2D). However, p65-RHD exclusively localized to the nucleus in cultured cardiomyocytes given its lack of N and C-terminal regulatory domains, which remarkably induced constitutive nuclear localization of NFATc1-GFP, presumably due to the strong interaction between these proteins (Figure 2D). Thus p65-RHD is sufficient to retain NFATc1 in the nucleus through their interaction.

A gel shift assay was performed with cardiomyocyte nuclear extracts to determine if NFAT and p65 can form transcriptional complexes. The data show that NFAT DNA-binding activity from this nuclear extract is shifted with an anti-NFAT antibody, as expected, while a p65 antibody partially disrupts the NFAT binding complex (Figure 2F). Conversely, NF κ B DNA-binding activity is completely disrupted with the anti-p65 antibody, as expected, while the anti-NFAT antibody disrupted and supershifted of the NF κ B DNA-binding activity (Figure 2F). These results indicate that NF κ B and NFAT are complexed together in cardiomyocyte nuclear extract at baseline.

NFAT and NF κ B regulate one another's subcellular shuttling

NFAT activity is typically regulated by calcineurin-mediated dephosphorylation within the cytoplasm, resulting in nuclear translocation and the activation of NFAT responsive genes. Similarly, nuclear translocation of NF κ B is required for NF κ B transcriptional activation.

Here we further investigated if NFAT and NF κ B could potentially regulate one another's subcellular shuttling through their ability to complex together. Cardiomyocytes were infected with an adenovirus encoding NFATc1-GFP or the NFATc1 serine/alanine mutant (NFATc1-SA) that is constitutively nuclear (also fused to GFP). In control cells, both NFATc1 and p65 are primarily localized in the cytoplasm (Figure 3A, B, and data not shown). Ionomycin, which activates calcineurin, triggered a robust translocation of NFATc1 to the nucleus (Figure 3A, B). Remarkably, near complete nuclear co-localization of p65 was induced by ionomycin, which is not known to activate NF κ B directly, suggesting it may be due to NFAT translocation (Figure 3A, B). In addition, the GPCR agonist phenylephrine induced both NFATc1 and p65 nuclear translocation in cardiomyocytes (Figure 3A, B). Importantly, cells infected with a constitutively nuclear NFAT mutant, NFATc1-SA, also showed complete translocation of NF κ B, while uninfected cells in the same dish showed cytoplasmic NF κ B (Figure 3A, B; arrowheads versus asterisks). These data suggest that NFAT nuclear shuttling is sufficient to produce NF κ B nuclear translocation in cardiomyocytes.

We also investigated the reciprocal relationship by examining whether activation of NF κ B could influence NFAT subcellular localization. Coinfection of AdNFATc1-GFP with an adenovirus expressing IKK β resulted in robust NFATc1 nuclear translocation (Figure 3C, D). Similarly, cells overexpressing the constitutively nuclear p65-RHD truncation showed complete translocation of NFATc1-GFP (Figure 3C, D). Moreover, TNF α , a known activator of NF κ B, also induced NFATc1 nuclear translocation in cardiomyocytes. Interestingly, overexpression of the calcineurin inhibitory protein Cain, blocked NFATc1 nuclear translocation induced by IKK β or p65-RHD, suggesting that basal calcineurin activity is still required for IKK β or p65-RHD-induced NFAT nuclear shuttling (Figure 3C, D). Consistent with these observations, IKK β -induced NFAT-luciferase activity was blocked by inhibition of calcineurin with Cain or Rcan1 (Figure 3E, F). Importantly, inhibition of NF κ B with the I κ B α M mutant abrogated IKK β -induced NFAT activity, suggesting an NF κ B-dependent action for IKK β in regulating NFAT.

Ablation of p65 diminishes NFAT transcriptional activity in the heart

p65 is one of the major NF κ B subunits expressed in the myocardium. Here we determined that p65 protein expression is increased in pressure overload-induced hypertrophic hearts in the mouse, with no significant change in p50 expression, another prominent NF κ B subunit (Figure 4A). These results suggested that p65 might be a more critical regulator of the hypertrophic response, although its function *in vivo* in accord with NFAT has not been investigated. Standard *RelA* (*p65*) gene-targeted mice perish during early embryonic development precluding an analysis of p65's function in the adult heart.²⁸ To address this limitation here, we used a Cre-loxP-dependent conditional gene targeting approach to permit specific inactivation of *p65* in the heart. Mice homozygous for the *p65-loxP*-targeted allele (*fl/fl*) were crossed with *Nkx2.5-Cre* "knock-in" mice. Western blotting of protein extracts from hearts of 2 month-old *p65fl/fl^{Nkx2.5-Cre}* mice showed > 80% reduction in p65 protein, which did not change endogenous NFAT protein levels (Figure 4B). *p65fl/fl^{Nkx2.5-Cre}* mice were generated at predicted Mendelian ratios and were overtly normal well into adulthood.

To determine the effect of p65 deletion on NFAT transcriptional activity in the heart *in vivo*, we crossed *p65fl/fl^{Nkx2.5-cre}* and NFAT-luciferase reporter mice together.¹⁷ As shown in Figure 4C, deletion of p65 significantly reduced NFAT luciferase activity by ~50% compared to wild-type control mice at baseline (sham groups). Remarkably, pressure overload-induced NFAT-luciferase activity following TAC stimulation was also significantly diminished in *p65* deleted hearts (Figure 4C), consistent with our observation that inhibition of NF κ B can antagonize calcineurin-NFAT signaling in cardiomyocytes (Figure 1A).

Ablation of p65 reduced pressure overload-induced cardiac hypertrophy

Calcineurin-NFAT signaling has been implicated as a central regulator of pathological cardiac hypertrophy,¹² and deletion of NFATc3 or c2 each reduced the hypertrophic response in gene-targeted mice.^{29,30} Based on our observations that ablation of p65 antagonized calcineurin-NFAT signaling, we hypothesized that p65 is also required for efficient cardiac hypertrophy in response to pressure overload stimulation *in vivo*. Indeed, TAC stimulation for 4 weeks in *p65fl/fl^{Nkx2.5-Cre}* mice showed reduced cardiac hypertrophy (whole organ and cellular) and fibrosis compared with *p65fl/fl* and *Nkx2.5-Cre* control mice (Figure 5A and Online Figure IIIA–D). Measurement of lung weight normalized to body weight also showed reduced pulmonary congestion in *p65fl/fl^{Nkx2.5-Cre}* mice after 4 weeks of TAC compared with controls (Figure 5B). Echocardiographic assessment of ventricular chamber dimensions and fractional shortening also showed preserved cardiac function and less dilation after TAC in *p65fl/fl^{Nkx2.5-Cre}* mice compared with controls (Figures 5C,D,E). Taken together, these results indicate that p65 ablation reduces pressure overload-induced cardiac hypertrophy and ventricular remodeling, consistent with less hypertrophy and remodeling observed previously in NFATc3 or c2 deleted mice, supporting the hypothesis that these 2 pathways functionally intersect in the heart.

Discussion

We demonstrated for the first time the interdependence between NFAT and NF κ B signaling in mediating hypertrophic gene expression in the heart. Indeed, hypertrophic agonist-induced NFAT transcriptional activity was partially blocked by inhibition of NF κ B with the I κ B super-suppressor, dominant negative IKK β , or genetic deletion of *p65*. This result suggests that full transcriptional activation of NFAT requires intact NF κ B signaling and p65 transcriptional activity. Antithetically, genetic inhibition of calcineurin-NFAT showed compromised NF κ B transcriptional activation in cardiac myocytes. Consistent with these observations, the potent NF κ B inhibitor, PDTC, promoted rapid nuclear export of NFAT from the nucleus of stimulated cells leading to reduced NFAT transcriptional activity, although the mechanism underlying this effect was not identified.³¹

Mechanistically, we showed that NFAT directly interacts with p65 to form a complex that promotes transcriptional synergy. Cooperative interactions between NF κ B and other transcription factors have been suggested in the regulation of gene expression. For instance, interactions between NF κ B and the signal transducer and activator of transcription (STAT) factors are the basis for synergistic transcriptional activation of genes after TNF α and interferon- γ co-stimulation.³² Other transcription factors that interact with NF κ B include AP-1, CREB, NF-IL, myc, and IRF-1.⁶ Unlike canonical Rel-containing factors, which often homodimerize on target promoters, NFAT factors share an imperfect Rel homology domain that is only capable of weak DNA binding in the monomeric or dimeric state. In order to strengthen DNA interaction on target promoters, NFAT factors prefer to interact with other nuclear transcription factors such as AP-1, GATA-4, MEF-2, and now p65-NF κ B.¹⁴

Under basal conditions, NFAT transcription factors are excluded from the nucleus by phosphorylation of the N-terminal regulatory domain. NFAT transcription factors are prototypical effectors of calcineurin signaling.³³ Calcineurin directly interacts with NFAT through a conserved docking motif, and upon activation by calcium/calmodulin, calcineurin dephosphorylates NFAT unmasking a nuclear localization signal. NFAT subsequently enters the nucleus and activates gene transcription.³³ Our data indicate that intracellular shuttling of NFAT is regulated by NF κ B signaling, in part, by direct binding of the transcription factors themselves. Thus, direct interaction between NFAT and NF κ B provides a new regulatory mechanism for NFAT transcriptional activation by affecting the nuclear import

and export equilibrium of each factor. NF κ B nuclear translocation induced by IKK β or p65-RHD enhances NFAT nuclear localization. However, basal calcineurin activity is required for IKK β or p65-RHD-induced NFAT nuclear retention since this effect was blocked by the calcineurin inhibitory protein Cain. Thus, some level of calcineurin-mediated basal dephosphorylation of NFAT is necessary for NFAT to periodically transit through the nucleus to have the opportunity to bind p65-NF κ B and be retained.

NF κ B plays important roles in cardiac hypertrophy and remodeling. For example, *in vitro* studies have shown that NF κ B is required for hypertrophic growth of cardiomyocytes in response to G protein-coupled receptor (GPCR) agonists such as phenylephrine, endothelin-1 and angiotensin II.^{26,34,35} In addition, *in vivo* studies using different animal models of NF κ B inactivation by I κ B α M mutant overexpression, p65 silencing, or p50 deletion also showed reduced cardiac hypertrophy in response to aortic banding,³⁶ chronic infusion of GPCR agonists,^{37,38} and transgenic overexpression of myotrophin.^{39,40} However, inhibition of NF κ B signaling in cardiac specific IKK β or NEMO knockout mice showed enhanced hypertrophy and pathological remodeling.^{41,42} Thus manipulation of different components of the NF κ B signaling pathway may have distinct functional consequences in the heart.

Intriguingly, we observed upregulation of p65 but not p50 in the heart following pressure overload. In addition, we were not able to detect association of p50 with NFAT by immunoprecipitation in cardiomyocytes (data not shown). These results suggest that different NF κ B subunits may have distinct roles in signal transduction and cellular function. Indeed, p65 nuclear translocation and cytokine expression were observed in p50 deficient cardiomyocytes upon stimulation, suggesting that p65 functions independent of p50.⁴³ In addition, embryonic fibroblasts from *p65* null mice were deficient in activating certain TNF α -inducible genes with NF κ B binding sites,⁴⁴ while *p50* null cells showed normal activation.⁴⁵

Our results also provide new insights into how NF κ B induces cardiac hypertrophy and fetal gene expression. No NF κ B binding sites have been identified in the promoter regions of adult or fetal cardiac genes associated with cardiac hypertrophy. Here we showed that NF κ B affected cardiac hypertrophy and remodeling through a physical interaction with the well-defined hypertrophic transcription factor NFAT, for which many direct promoter interaction sites have been identified in hypertrophic genes.^{12,30} Indeed, overexpression of a constitutively activated NFAT mutant protein resulted in cardiac hypertrophy in transgenic mice, suggesting that NFAT activation is sufficient to promote the hypertrophic response of the heart.¹² In addition, genetic inhibition of NFAT isoforms attenuated the hypertrophic response to hypertrophic agonists and pressure-overload.^{29,30,46}

Blocking of NF κ B activity could represent a novel approach to attenuate cardiac hypertrophy and adverse remodeling. We demonstrated that *p65* ablation reduced pressure overload-induced cardiac hypertrophy with preserved cardiac function, possibly through diminished NFAT signaling. In addition, cardiac deletion of *p65* also showed reduced cardiac hypertrophy and ventricular remodeling with improved contractile function in response to myocardial infarction (data not shown), and similar effects were observed in a transgenic mouse expressing I κ B super-suppressor.⁴⁷ Great efforts have been made for the development of highly specific NF κ B inhibitors for treating autoimmune diseases and different types of cancer, some of which are being evaluated in phase II clinical trials. Although the calcineurin inhibitors cyclosporine A and FK506 dramatically attenuates cardiac hypertrophy in most animal models,⁴⁸ severe side effects preclude their use for heart disease in humans.⁴⁹⁻⁵¹ Thus, targeting NF κ B may represent a valid alternative therapeutic

strategy in treating hypertrophy or heart failure, which secondarily would reduce the effectiveness of NFAT activity in the heart.

Novelty and Significance

What is known

- Calcineurin-NFAT signaling is a central regulator of pathological cardiac hypertrophy.
- NF κ B is a key regulator of cardiomyocyte hypertrophy both *in vitro* and *in vivo*.

What new information does this article contribute?

- We provide the first evidence showing a direct interaction between NFAT and NF κ B that effectively integrates two disparate hypertrophic signaling pathways in the heart.
- We identified a novel transcriptional regulatory mechanism whereby NF κ B and NFAT directly interact and synergistically promote transcriptional activation of one another in cardiomyocytes.
- Targeting NF κ B may represent a novel therapeutic strategy in treating cardiac hypertrophy or heart failure by secondarily diminishing NFAT signaling in the heart.

This study was designed to determine the potential functional interaction between NFAT and NF κ B signaling pathways in cardiomyocytes and its role in cardiac hypertrophy and remodeling. Our findings provide new mechanistic insight into how NF κ B and NFAT synergistically induce cardiac hypertrophy and pathological remodeling. We demonstrate that NF κ B-p65 ablation reduced pressure overload-induced cardiac hypertrophy with preserved cardiac function, possibly through diminished NFAT signaling. Therefore, targeting NF κ B may represent as a valid alternative therapeutic strategy in treating hypertrophic heart failure, which secondarily would diminish NFAT signaling in the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

ΔCnA	activated calcineurin
fl	loxP-targeted
GFP	green fluorescent protein
IKK	I κ B kinase
MEFs	mouse embryonic fibroblasts
MEF-2	myocyte enhancer factor-2

NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κ B
RHD	Rel homology domain
TAC	transverse aortic constriction
TNFα	tumor necrosis factor α

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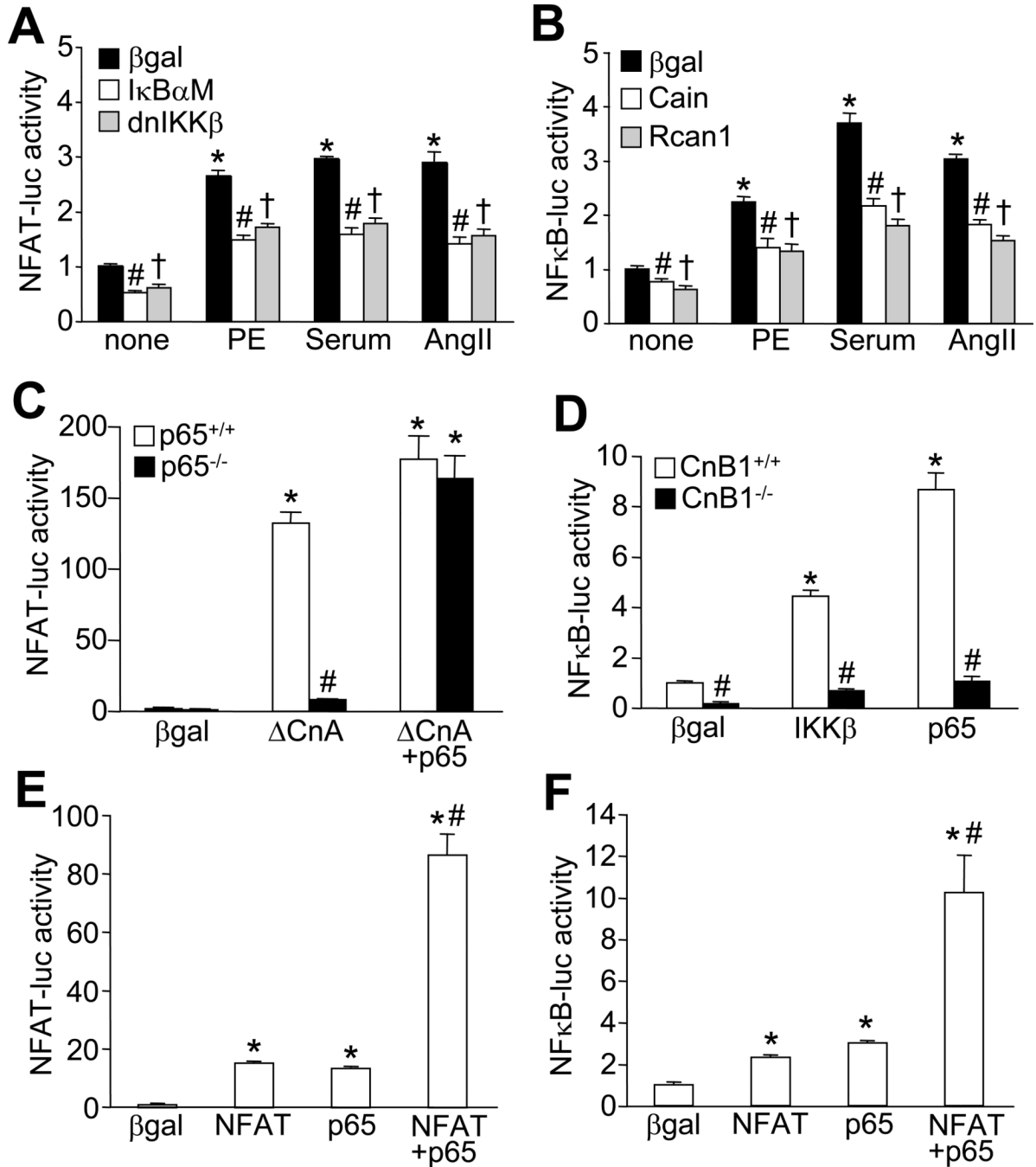


Figure 1. Interdependence of NFAT and NF κ B for transcriptional activation

A, NFAT-luciferase activity in neonatal cardiomyocytes infected with recombinant adenoviruses expressing NFAT-luciferase reporter along with β -gal (control), I κ B α M, or dnIKK β and stimulated with phenylephrine (PE, 50 μ M), 1% FBS (serum), or angiotensin II (AngII, 100 nM). * P < 0.01 versus none; #, † P < 0.05 versus β -gal for each stimulant. **B**, NF κ B-luciferase activity in neonatal cardiomyocytes infected with recombinant adenoviruses expressing NF κ B-luciferase reporter along with β -gal (control), Cain, or Rcan1 and stimulated with PE, FBS, or AngII. * P < 0.01 versus none; #, † P < 0.05 versus β -gal for each stimulant. **C**, NFAT-luciferase activity from p65^{+/+} or p65^{-/-} MEFs infected

with adenoviruses expressing the NFAT-luciferase reporter with the other indicated proteins. * $P < 0.01$ versus β -gal; # $P < 0.05$ versus p65^{+/+} Δ CnA. **D**, NF κ B-luciferase from CnB1^{+/+} or CnB1^{-/-} MEFs infected with adenoviruses encoding NF κ B-luciferase reporter and other indicated proteins. * $P < 0.01$ versus β -gal; # $P < 0.05$ versus corresponding CnB1^{+/+}. **E**, NFAT-luciferase activity in neonatal cardiomyocytes infected with adenoviruses encoding NFAT-luciferase reporter and other indicated proteins. * $P < 0.05$ versus β -gal; # $P < 0.05$ versus NFAT or p65 only. **F**, NF κ B-luciferase activity in neonatal cardiomyocytes infected with adenoviruses encoding the NF κ B-luciferase reporter with the other indicated proteins. * $P < 0.05$ versus β -gal; # $P < 0.05$ versus NFAT or p65 only.

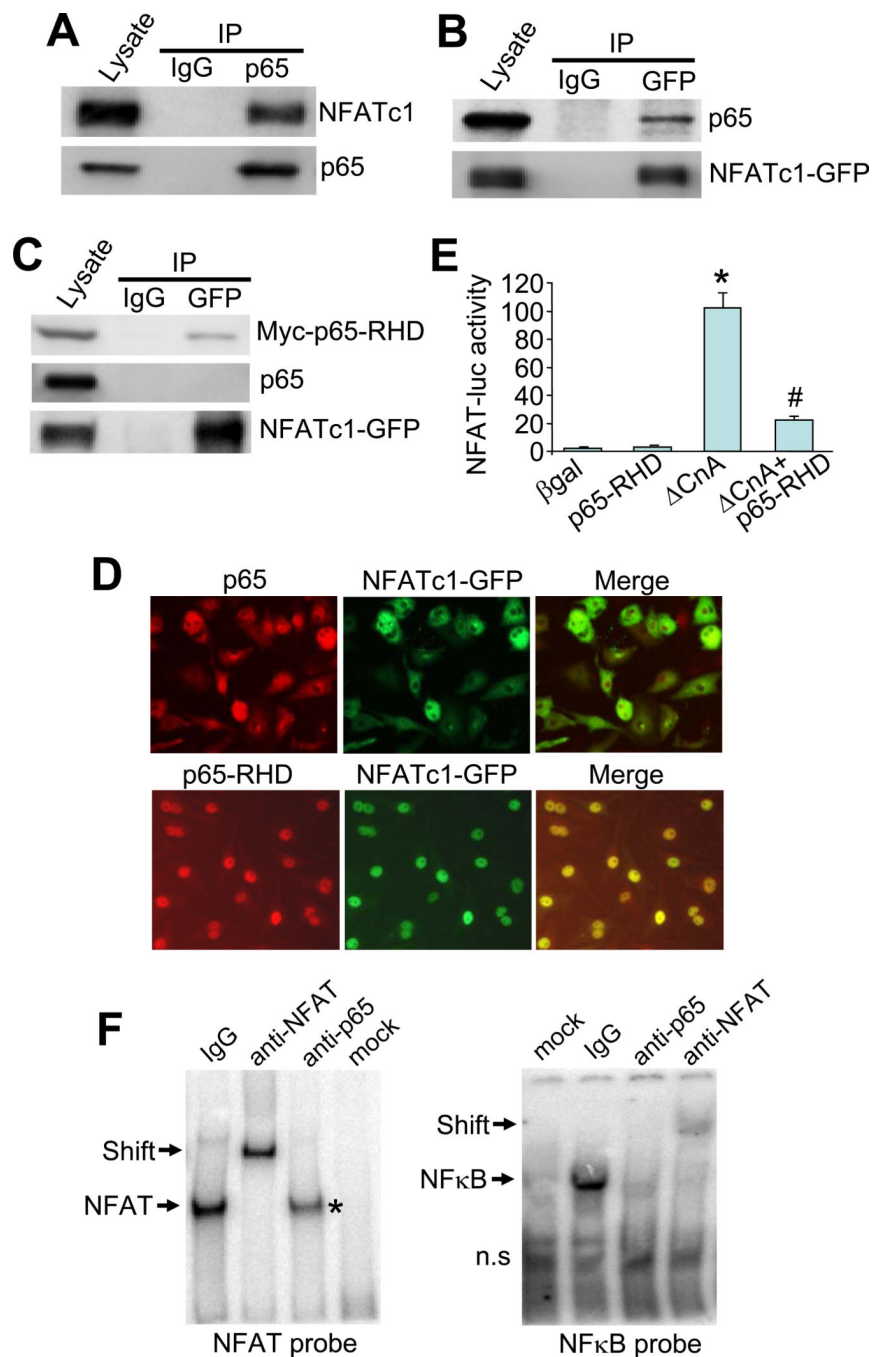


Figure 2. Physical interaction between NFAT and p65 in cardiomyocytes

A, Western blots for NFATc1 and p65 following immunoprecipitation (IP) with anti-p65 antibody or pre-immune IgG from neonatal cardiomyocytes infected with AdNFATc1-GFP. **B**, Western blots for NFATc1 and p65 following immunoprecipitation with an anti-GFP antibody or pre-immune IgG from neonatal cardiomyocytes infected with AdNFATc1-GFP. **C**, Western blots for Myc, p65, and GFP following immunoprecipitation with an anti-GFP antibody or pre-immune IgG from neonatal cardiomyocytes infected with p65-RHD-Myc and NFATc1-GFP adenoviruses. **D**, Immuno-staining for p65 or Myc (p65-RHD) in red and GFP for NFAT in neonatal cardiomyocytes infected with p65, p65-RHD-Myc and NFATc1-

GFP adenoviruses. **E**, NFAT-luciferase activity in neonatal cardiomyocytes infected with an adenovirus encoding NFAT-luciferase reporter along with the other indicated adenoviruses. Results were summated from 3 independent experiments. * $P < 0.05$ versus β -gal; # $P < 0.05$ versus Δ CnA only. **F**, Gel shift for NFAT or NF κ B DNA binding activity in cardiomyocyte nuclear extracts incubated with anti-NFAT, anti-p65, or pre-immune IgG. Arrows indicate specific NFAT or NF κ B binding activity. n.s. = non-specific.

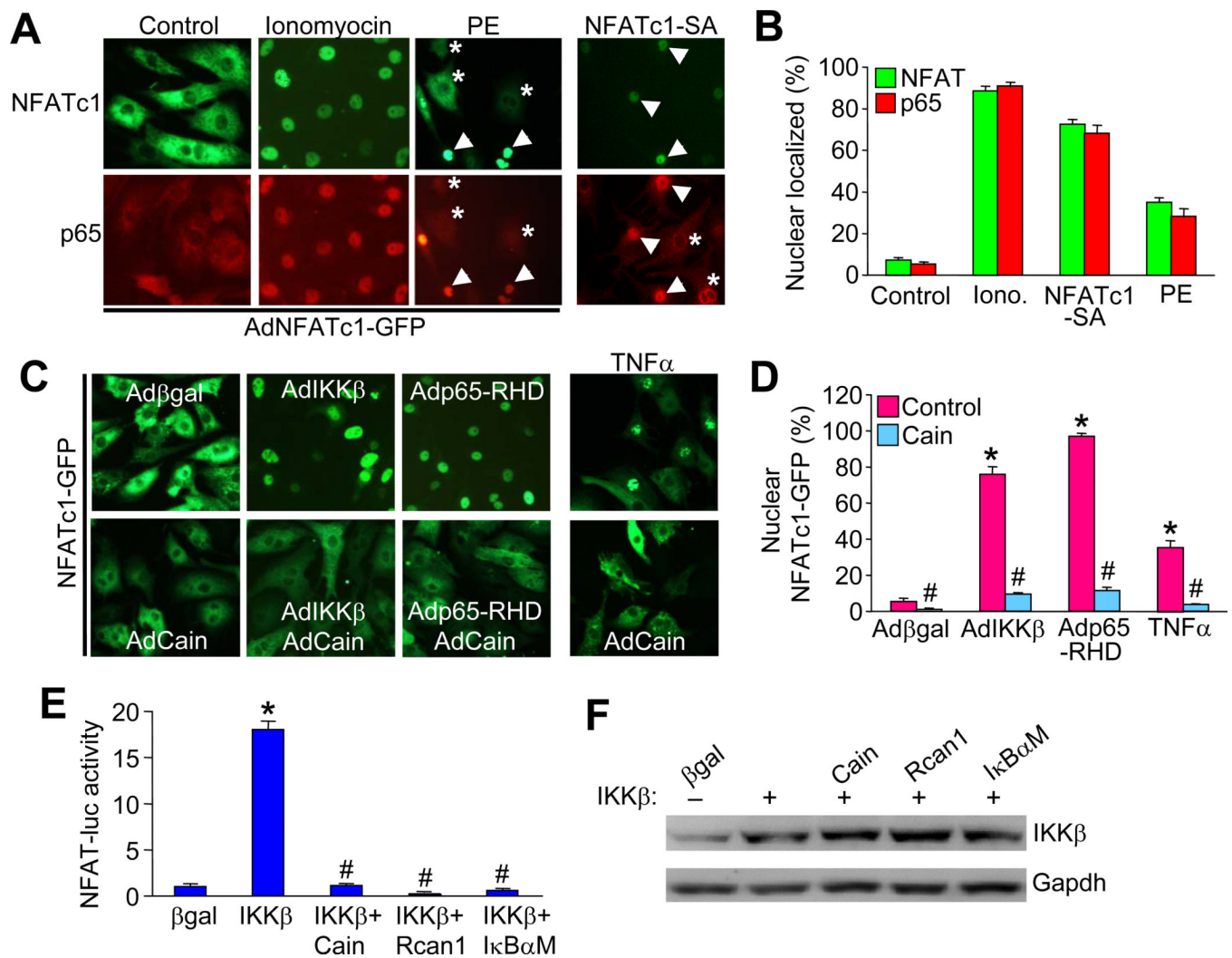


Figure 3. Co-localization of NFAT and NFκB in cardiomyocytes

A, Immunofluorescent images from cultured neonatal cardiomyocytes infected with AdNFATc1-GFP (green) and stimulated with 1 μM ionomycin, 50 μM phenylephrine, or vehicle control, or infected with an NFATc1-SA-GFP adenovirus. Cells were also immunostained for endogenous p65 (red). Arrowheads indicate nuclear localization while asterisks show cytoplasmic localization. **B**, Quantification of nuclear localized NFATc1 and p65 in cells treated as indicated in **A**. **C**, Immunofluorescent images from cultured neonatal cardiomyocytes infected with AdNFATc1-GFP along with the other indicated adenoviruses or stimulated with 20 ng/ml TNFα. **D**, Quantification of nuclear localized NFATc1 in cells treated as indicated in **C**. **E**, NFAT-luciferase activity in neonatal cardiomyocytes infected with the AdNFAT-luciferase reporter and the other indicated adenoviruses. **P* < 0.05 versus β-gal; #*P* < 0.05 versus IKKβ only. Results were summated from 3 independent experiments. **F**, Control western blot to show equal levels of IKKβ overexpression for the experiment shown in **E**.

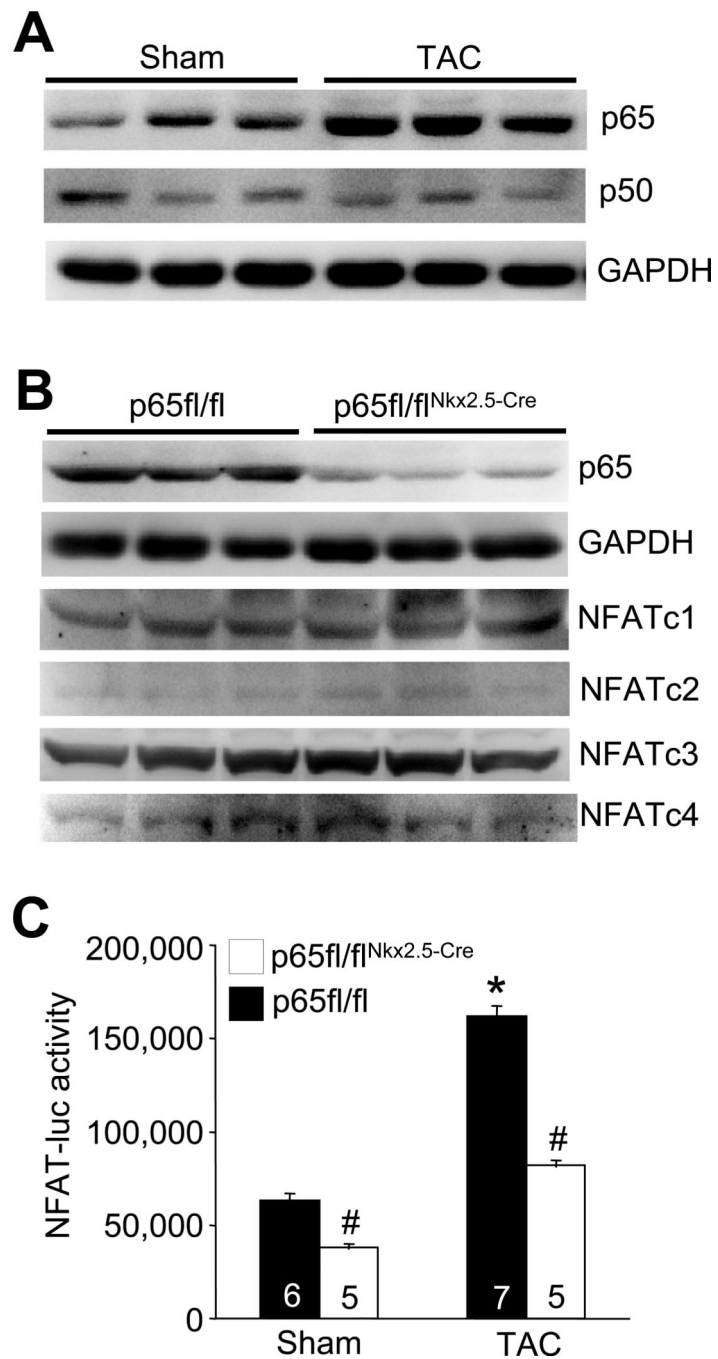


Figure 4. NFAT transcriptional activity is reduced in *p65* deleted hearts *in vivo*

A, Western blots for p65, p50, and GAPDH in heart extracts from sham- and TAC-operated wild-type mice. **B**, Western blot for p65, GAPDH, and NFAT isoforms in control (*p65fl/fl*) and *p65*-deleted (*p65fl/fl^{Nkx2.5-Cre}*) hearts. **C**, Measurement of NFAT luciferase activity from *p65fl/fl* and *p65fl/fl^{Nkx2.5-Cre}* mice containing the NFAT luciferase reporter transgene after 2 weeks of TAC or a sham operation. **P* < 0.05 versus sham; #*P* < 0.05 versus corresponding *p65fl/fl*. Number of mice analyzed is shown in the bars.

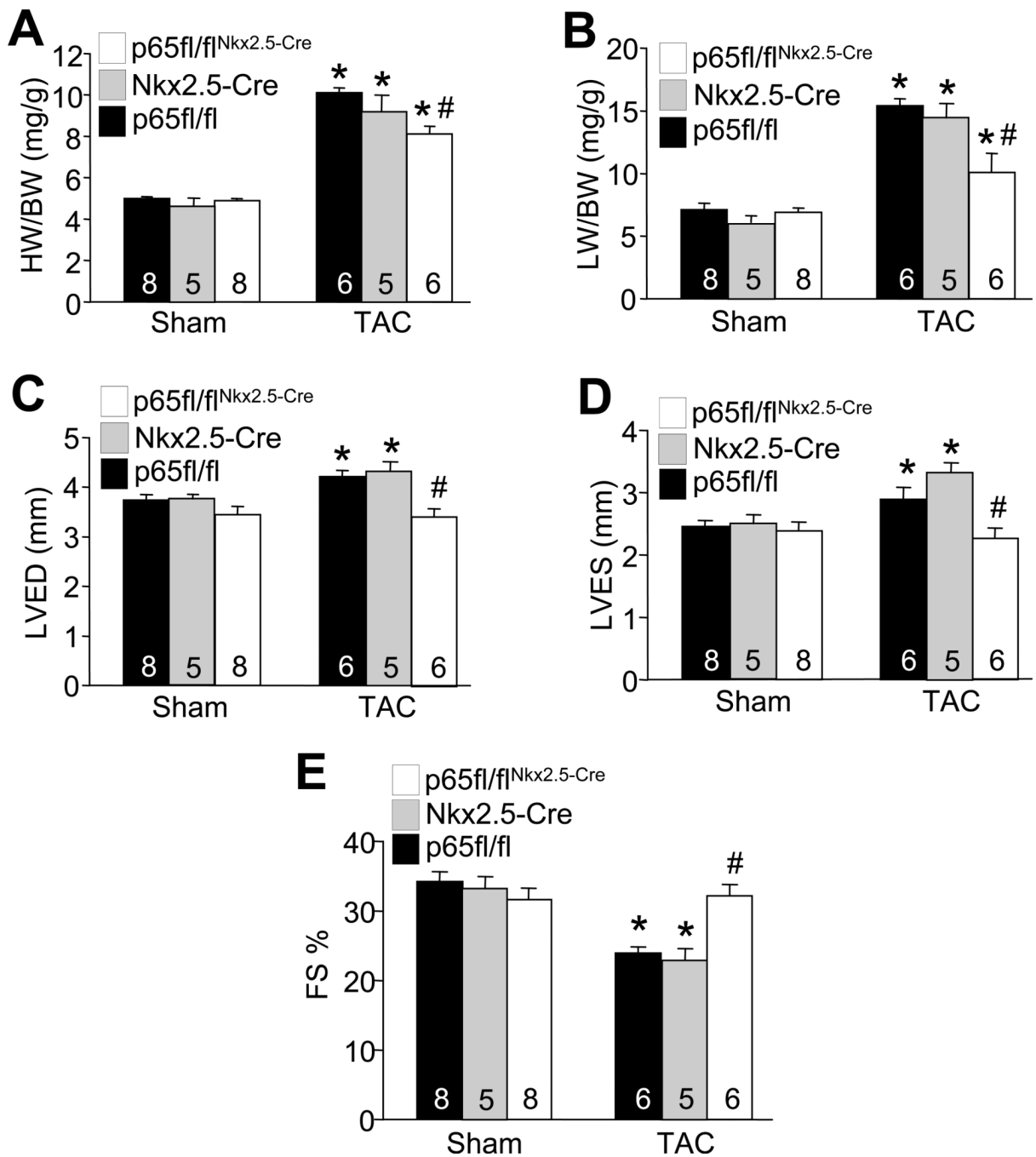


Figure 5. p65 is required for pressure overload-induced cardiac hypertrophy

A and B, Heart weight to body weight ratio (HW/BW) and lung weight to body weight ratio (LW/BW) in the indicated genotypes 4 weeks after TAC or sham operation. * $P < 0.05$ vs. *p65fl/fl* sham or *p65fl/fl*^{Nkx2.5-Cre} sham. # $P < 0.05$ versus *p65fl/fl* or Nkx2.5-Cre TAC. **C, D, and E,** Left ventricular end diastolic dimension (LVED), left ventricular end systolic dimension (LVES), and cardiac fractional shortening percentage (FS) from sham- and TAC-operated mice after 4 weeks of pressure overload, measured by M-mode echocardiography. * $P < 0.05$ vs. *p65fl/fl* sham or *p65fl/fl*^{Nkx2.5-Cre} sham. # $P < 0.05$ versus *p65fl/fl* or Nkx2.5-Cre TAC. Number of mice analyzed is shown in the bars.