Interaction Between NFκB and NFAT Coordinates Cardiac Hypertrophy and Pathological Remodeling

Qinghang Liu, Yi Chen, Mannix Auger-Messier, Jeffery D. Molkentin

**Rationale:** Both nuclear factors of activated T cells (NFAT) and nuclear factor-κB (NFκB) 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 NFκB signaling pathways in cardiomyocytes and its role in cardiac hypertrophy and remodeling.

**Methods and Results:** We identified a novel transcriptional regulatory mechanism whereby NFκB and NFAT directly interact and synergistically promote transcriptional activation in cardiomyocytes. We show that the p65 subunit of NFκB coimmunoprecipitates 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 IκB kinase β (IKKβ) 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, whereas inhibition of NFκB with IκBα-M or dominant negative IKKβ reduces NFAT activity. Importantly, agonist-induced NFκB activation is reduced in p65 null mouse embryonic fibroblasts (MEFs) compared with 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 after pressure overload stimulation, reduces the degree of pathological remodeling, and preserves contractile function.

**Conclusions:** Our results suggest a direct interaction between NFAT and NFκB that effectively integrates 2 disparate signaling pathways in promoting cardiac hypertrophy and ventricular remodeling. (Circ Res. 2012;110:1077-1086.)

Key Words: hypertrophy • signaling • cardiomyocyte • NFAT • nuclear factor-κB • transcriptional regulation • transgenic mice
proliferation, differentiation, immune responses, cell growth, cardiac hypertrophy, and apoptosis. 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, which mediates DNA binding and protein–protein interaction between family members. These proteins associate as homodimers or heterodimers to form transcriptional regulatory complexes. In unstimulated cells, NFκB binds to cytosolic inhibitory proteins, IkBs, mediating cytoplasmic retention. On various types of stimulation, IkBs are phosphorylated by IkB kinase (IKK), causing their degradation and subsequent release of NFκB for nuclear translocation. The IKK complex is composed of 2 catalytic subunits (IKKa and IKKB) and a regulatory subunit (IKKy or NEMO). Recent data also suggest that NFκB can shuttle into the nucleus in nonstimulated cells, although the physiological significance of this finding is unknown. Posttranslational modifications (eg, phosphorylation and acetylation) and physical interaction with coactivators and corepressors have been identified as additional regulatory mechanisms.

NFAT transcription factors are also part of the Rel family, where they bear structural similarity to NFκB and even bind related or overlapping DNA sequence elements. NFAT transcription factors consist of 5 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. 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. 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. To strengthen NFAT-DNA interactions, these factors tend to interact with other transcription factors such as AP-1 (c-Jun/c-Fos), GATA-4, and MEF-2. Here, we determined that NFAT and NFκB family members could interact and assemble “higher-order” transcriptional complexes that synergistically activate hypertrophic gene expression in the heart.

### Methods

An expanded Methods section is available in the online Data Supplement.

### 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. Mice harboring the p65/fl alleles were crossed with mice expressing Cre recombinase under control of the endogenous Nkx2.5 locus. The NFAT-luciferase reporter transgenic mouse as described previously was crossed with a p65/fl/RHD-Myc 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. Pressure gradients (mm Hg) were calculated from the peak blood velocity (Vmax) (m/s) (PG = 4 × Vmax²) 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.

### Luciferase Reporter Assays in Mouse Hearts

In brief, hearts were removed from NFAT-luciferase transgenic mice, some of which were crossed into the p65/fl/RHD-Myc background. Hearts were homogenized in 1 mL of luciferase assay buffer (100 mmol/L KH2PO4, pH 7.8, 0.5% Nonidet P-40, and 1 mmol/L DTT). Homogenates were centrifuged at 3000g for 10 minutes at 4°C, and the supernatants assayed for luciferase activity as described previously.

### 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. After separation from fibroblasts, enriched cardiomyocytes were plated on 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 mmol/L L-glutamine without serum for 24 hours before infection. MEFs were kindly provided by David Baltimore (California Institute of Technology). Adenoviral infections were performed as previously described at a multiplicity of infection of 10–50 plaque-forming units/mL. Cultures were harvested 24 hours after infection, and luciferase assays were performed as described previously. Adβgal, AdΔCnA, Adcain, AdNFAT-luciferase reporter, AdNFATc1-GFP (green fluorescent protein), and AdNFATc3-GFP have been previously described. 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/mL. Cardiomyocytes were prepared for immunocytochemistry as described previously. 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.

### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ΔCnA</td>
<td>activated calcineurin</td>
</tr>
<tr>
<td>fl</td>
<td>loxP-targeted</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein–coupled receptor</td>
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<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
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<tr>
<td>MEF-2</td>
<td>myocyte enhancer factor-2</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor-α</td>
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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 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-NFATc1, anti-p65, anti-p105/p50, and anti-GFP antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA).

Briefly, 20 μg of protein extracts from neonatal cardiomyocytes were incubated in gel shift buffer (12 mmol/L HEPES, pH 7.9, 4 mmol/L Tris, pH 7.9, 50 mmol/L KCl, 12% glycerol, 12 mmol/L EDTA, 1 mmol/L DTT, 0.2 mmol/L PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.7 μg/mL pepstatin) with 0.5 μg poly (dI-dC) and 32P-labeled NFAT or NFkB consensus DNA sequences for 20 minutes at room temperature. For supershift reactions, 1 μL of anti-NFATc1 or anti-p65 antibody was added after 20 minutes of binding reaction. DNA complexes were separated on a 5% nondeaturing polyacrylamide gel in Tris-borate EDTA buffer.

**Immunoprecipitation**

Neonatal rat cardiomyocytes were infected with or without specific adenoviruses for 24 hours. Cells were lysed at 4°C in buffer (50 mmol/L Tris–HCl [pH 7.5], 150 mmol/L NaCl, 0.5% Triton X-100) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL aprotinin). Immunoprecipitation was performed as described previously.21 Whole-cell lysates were cleared by centrifugation at 18 000g for 10 minutes 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 mean±SEM. Paired data were evaluated by Student t test. A 1-way ANOVA with the Bonferroni post hoc test or repeated-measures ANOVA was used for multiple comparisons. P<0.05 was considered statistically significant.

**Results**

**Cross-Talk Between NFAT and NFkB in Cardiomyocytes**

Both calcineurin-NFAT and IKK-NFkB signaling pathways have been implicated as critical regulators of cardiomyocyte hypertrophy.12,26 We tested the hypothesis that these two previously deemed independent signaling pathways may actually cross-talk with one another. We first examined if agonist–induced NFAT and NFkB signaling with Cain or Rcan1 diminished hypertrophic signaling with NFAT and NFkB luciferase activity (Figure 1B). Sim-

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Figure 1. Interdependence of NFAT and NFkB for transcriptional activation. A, NFAT-luciferase activity in neonatal cardiomyocytes infected with recombinant adenoviruses expressing NFAT-luciferase reporter along with β-gal (control), IxBαM, or dnIKKβ and stimulated with phenylephrine (PE, 50 μmol/L), 1% FBS (serum), or angiotensin II (AngII, 100 mmol/L). *P<0.01 versus none; †P<0.05 versus β-gal for each stimulant. B, NFAT-luciferase activity in neonatal cardiomyocytes infected with recombinant adenoviruses expressing NFAT-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.

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E, NFAT-luciferase activity in neonatal cardiomyocytes infected with adenoviruses encoding NFAT-luciferase reporter and other indicated proteins. *P<0.01 versus β-gal; †P<0.05 versus p65−/−, ΔCnA.

F, NFAT-luciferase activity in neonatal cardiomyocytes infected with adenoviruses encoding NFAT-luciferase reporter and the other indicated proteins. *P<0.01 versus β-gal; †P<0.05 versus corresponding CnB1−/−. NFkB signaling pathways have been implicated as critical regulators of cardiomyocyte hypertrophy.12,26
duced in NFκB-p65 null MEFs compared with wild-type MEFs, whereas adenoviral-mediated expression of p65 re-
stored NFAT luciferase activity in p65 null MEFs (Figure 1C). Conversely, NFκB luciferase activity induced by adeno-
viral overexpression of IKKβ or p65 was also largely blocked in calcineurin-deficient MEFs (lacking CnB1 protein). In 
addition, calcineurin-deficient MEFs showed lower baseline NFκB activity compared with wild-type MEFs, suggesting 
that calcineurin regulates NFκB equilibrium even without stimulation (Figure 1D). Inhibition of NFκB signaling 
with 1μM B or dnIKKβ also partially blocked ΔCnA-induced NFAT luciferase activity, whereas inhibition of NFAT 
signaling with Cain diminished p65- or IKKβ-induced NFκB luciferase activity in cardiomyocytes (Online Figure I, C 
and D). Consistent with these observations, overexpression of NFATc1 or p65 each cross-stimulated NFκB and NFAT-
luciferase activity in cardiomyocytes, whereas coexpression of NFAT with p65 synergistically activated both NFAT and 
NFκB reporter activity (Figure 1E and 1F). To rule out the involvement of autocrine factors in the observed effects, 
cardiomyocytes were treated with cultured media collected from cells infected with adenovirus encoding NFAT, p65, or 
both, or with IKKβ and other inhibitory viruses for NFAT or NFκB signaling (Online Figure I, E, F, and G). No significant 
changes in NFAT- or NFκB-luciferase activity was detected under these conditions, thus excluding a role for secreted 
autocrine factors in potentiating NFAT and NFκB transcriptional activation.

Physical Interaction Between NFAT and NFκB
Whereas a direct interaction between NFκB and NFAT transcription factors has not been demonstrated in cardiomy-
cytes, a physical association between NFATc1 and c-Rel was previously reported in lymphocytes.27 Hence, we examined if 
NFAT interacts with p65-NFκB in cardiomyocytes by immuno-
precipitation. The results showed that NFATc1 immunoprecipitated with a p65 antibody but not with a nonspecific 
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 cardiomy-
cytes stimulated with phenylephrine, TNFα, or vehicle 
control. Although the interaction between NFAT-p65 was still 
observed with these agonists, no changes in intensity were 
observed (Online Figure II). Because both NFAT and 
NFκB contain a Rel homology domain (RHD), which is 
important for protein-protein interaction and dimerization, 
cardiomyocytes infected with an adenovirus encoding p65-
RHD showed coimmunoprecipitation 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 
hindered calcineurin-induced NFAT luciferase activity, sug-
gest that p65-RHD acts as dominant negative for endog-
enous p65 because it lacks a transcriptional activation domain 
(Figure 2D). Full-length p65 localized primarily in the cytoplasm of unstimulated neonatal cardiomyocytes, similar 
to NFATc1-GFP (Figure 2E). However, p65-RHD exclu-

duly 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 2E). Thus, p65-RHD is 
sufficient to retain NFATc1 in the nucleus through their 
interaction.

A gel shift assay was performed with cardiomyocyte protein 
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, whereas a p65 antibody partially disrupts the NFAT 
DNA-binding activity completely disrupted with the anti-p65 antibody, as expected, whereas the anti-NFAT antibody disrupted and super-
shifted of the NFκB DNA-binding activity (Figure 2F). These 
results indicate that NFκB and NFAT are complexed together in 
cardiomyocyte nuclear extract at baseline.

NFAT and NFκB 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 NFκB is required for 
NFκB transcriptional activation. We further investigated if 
NFAT and NFκB could potentially regulate one another’s 
subcellular shuttling through their ability to complex to-
gether. Cardiomyocytes were infected with an adenovirus 
encoding NFATc1-GFP or the NFATc1 serine/alanine mu-
ant (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 and 3B and data not 
shown). Ionomycin, which activates calcineurin, triggered a 
robust translocation of NFATc1 to the nucleus (Figure 3A 
and 3B). Remarkably, near complete nuclear colocalization 
of p65 was induced by ionomycin, which is not known to 
activate NFκB directly, suggesting it may be due to NFAT 
translocation (Figure 3A and 3B). In addition, the G-protein– 
coupled receptor (GPCR) agonist phenylephrine induced both 
NFATc1 and p65 nuclear translocation in cardiomyocytes 
(Figure 3A and 3B). Importantly, cells infected with a 
constitutively nuclear NFAT mutant, NFATc1-SA, also 
showed complete translocation of NFκB, whereas uninfected 
cells in the same dish showed cytoplasmic NFκB (Figure 3A 
and 3B; arrowheads versus asterisks). These data suggest that 
NFAT nuclear shuttling is sufficient to produce NFκB nu-

clear translocation in cardiomyocytes.

We also investigated the reciprocal relationship by exami-
ning whether activation of NFκB could influence NFAT 
subcellular localization. Coinfection of AdNFATc1-GFP 
with an adenovirus expressing IKKβ resulted in robust 
NFATc1 nuclear translocation (Figure 3C and 3D). Similarly, 
cells overexpressing the constitutively nuclear p65-RHD 
truncation showed complete translocation of NFATc1-GFP 
(Figure 3C and 3D). Moreover, TNFα, a known activator of 
NFκB, also induced NFATc1 nuclear translocation in cardio-
myocytes. Interestingly, overexpression of the calcineurin 
inhibitory protein Cain blocked NFATc1 nuclear transloca-
tion induced by IKKβ, p65-RHD, or TNF suggesting that basal calcineurin activity is still required for IKKβ or p65-RHD-induced NFAT nuclear shuttling (Figure 3C and 3D). Consistent with these observations, IKKβ-induced NFAT-luciferase activity was blocked by inhibition of calcineurin with Cin or Rcan1 (Figure 3E and 3F). Importantly, inhibition of NFκB with the IκBα/M mutant abrogated IKKβ-induced NFAT activity, suggesting an NFκB-dependent action for IKKβ in regulating NFAT.

Ablation of p65 Diminishes NFAT Transcriptional Activity in the Heart

p65 is one of the major NFκB subunits expressed in the myocardium. 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 NFκB 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, we used a Cre-loxP–dependent conditional gene targeting approach to permit specific inactivation of p65 in the heart. Mice homozygous for the p65loxP-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/flNkx2.5-Cre mice showed 80% reduction in p65 protein, which did not change endogenous NFAT protein levels (Figure 4B). p65fl/flNkx2.5-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/flNkx2.5-Cre and NFAT-luciferase reporter mice together.17 As shown in Figure 4C, deletion of p65 significantly reduced
NFAT luciferase activity by ~50% compared with wild-type control mice at baseline (sham groups). Remarkably, pressure overload–induced NFAT-luciferase activity after TAC stimulation was also significantly diminished in p65 deleted hearts (Figure 4C), consistent with our observation that inhibition of NF

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 NFATc2 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/flNkx2.5-Cre mice showed reduced cardiac hypertrophy (whole organ and cellular) and fibrosis compared with controls (Figure 5A and Online Figure III, A through D). Measurement of lung weight normalized to body weight also showed reduced pulmonary congestion in p65fl/flNkx2.5-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/flNkx2.5-Cre mice compared with controls (Figure 5C, 5D, and 5E). 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 NFATc2-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 NFkB signaling in mediating hypertrophic gene expression in the heart. Indeed, hypertrophic agonist-induced NFAT transcriptional activity was partially blocked by inhibition of NFkB with the IκBα supersuppressor, dominant negative IKKβ, or genetic deletion of p65. This result suggests that full transcriptional activation of NFAT requires intact NFkB signaling and p65 transcriptional activ-
potent NF

NFAT transcriptional activity is reduced in p65-deleted hearts in vivo. A, Western blots for p65, p50, and glyceraldehyde-3-phosphate dehydrogenase (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/flNkx2.5-Cre) hearts. C, Measurement of NFAT luciferase activity from p65fl/fl and p65fl/flNkx2.5-Cre mice containing the NFAT luciferase reporter transgene after 2 weeks of TAC or sham operation. *P<0.05 versus sham; #P<0.05 versus corresponding p65fl/fl. Number of mice analyzed is shown in the bars.

Figure 4. NFAT transcriptional activity is reduced in p65-deleted hearts in vivo. A, Western blots for p65, p50, and glyceraldehyde-3-phosphate dehydrogenase (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/flNkx2.5-Cre) hearts. C, Measurement of NFAT luciferase activity from p65fl/fl and p65fl/flNkx2.5-Cre mice containing the NFAT luciferase reporter transgene after 2 weeks of TAC or sham operation. *P<0.05 versus sham; #P<0.05 versus corresponding p65fl/fl. Number of mice analyzed is shown in the bars.

Mechanistically, we showed that NFAT directly interacts with p65 to form a complex that promotes transcriptional synergy. Cooperative interactions between NFkB and other transcription factors have been suggested in the regulation of gene expression. For instance, interactions between NFkB and the signal transducer and activator of transcription (STAT) factors are the basis for synergistic transcriptional activation of genes after TNFα and interferon-γ costimulation.32 Other transcription factors that interact with NFkB 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. 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-NFkB.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 on 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 NFkB signaling, in part, by direct binding of the transcription factors themselves. Thus, direct interaction between NFAT and NFkB provides a new regulatory mechanism for NFAT transcriptional activation by affecting the nuclear import and export equilibrium of each factor. NFkB nuclear translocation induced by IKKβ or p65-RHD enhances NFAT nuclear localization. However, basal calcineurin activity is required for IKKβ- 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-NFkB and be retained.

NFkB plays important roles in cardiac hypertrophy and remodeling. For example, in vitro studies have shown that NFkB is required for hypertrophic growth of cardiomyocytes in response to GPCR agonists such as phenylephrine, endothelin-1 and angiotensin II.26,34,35 In addition, in vivo studies using different animal models of NFkB inactivation by IkBα or IkBα knockout mice showed enhanced hypertrophy and pathological remodeling.41,42 Thus manipulation of different components of the NFkB signaling pathway may have distinct functional consequences in the heart.

Intriguingly, we observed upregulation of p65 but not p50 in the heart after 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 NFkB subunits may have distinct roles in signal transduction and cellular function. Indeed, p65 nuclear translocation and cytokine expression were observed in p50-deficient cardiomyocytes on stimulation, suggesting that p65 functions independent of p50.43 In addition, embryonic fibroblasts from p65 null mice were deficient in activating certain TNFα-inducible genes with NFkB binding sites,44 whereas p50 null cells showed normal activation.45

Our results also provide new insights into how NFkB induces cardiac hypertrophy and fetal gene expression. No NFkB binding sites have been identified in the promoter regions of adult or fetal cardiac genes associated with cardiac
hypertrophy. We showed that NFκB 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 NFκB 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 IκB supersuppressor.47 Great efforts have been made for the development of highly specific NFκB 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 attenuate cardiac hypertrophy in most animal models,48 severe side effects preclude their use for heart disease in humans.49–51 Thus, targeting NFκB 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.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- Calcineurin-NFAT signaling is a central regulator of pathological cardiac hypertrophy.
- NFκB 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 NFκB that effectively integrates two disparate hypertrophic signaling pathways in the heart.
- We identified a novel transcriptional regulatory mechanism whereby NFκB and NFAT directly interact and synergistically promote transcriptional activation of one another in cardiomyocytes.
- Targeting NFκB 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 NFκB signaling pathways in cardiomyocytes and its role in cardiac hypertrophy and remodeling. Our findings provide new mechanistic insight into how NFκB and NFAT synergistically induce cardiac hypertrophy and pathological remodeling. We demonstrate that NFκB-p65 ablation reduced pressure overload–induced cardiac hypertrophy with preserved cardiac function, possibly through diminished NFAT signaling. Therefore, targeting NFκB may represent as a valid alternative therapeutic strategy in treating hypertrophic heart failure, which secondarily would diminish NFAT signaling in the heart.
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SUPPLEMENTAL MATERIAL

Online Materials and Methods Supplement

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. Mice harboring the *p65fl/fl* alleles were crossed with mice expressing Cre recombinase under control of the endogenous Nkx2.5 locus. The NFAT-luciferase reporter transgenic mouse as described previously, 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 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. Cardiac hypertrophy was induced by transverse aortic constriction (TAC) to produce pressure overload using a 27 g needle as previously described. Pressure gradients (mm Hg) across the aortic constriction were calculated from the peak blood velocity (*Vmax*) (m/s) (PG = 4 x *Vmax*^2) measured by Doppler, which was equivalent in all groups of TAC stimulated mice. Fractional shortening (FS) from echocardiographic measurements was calculated using left ventricle dimensions in end of systole and diastole (LVES and LVED, respectively): FS = [(LVED - LVES)/LVED] x 100 (%).

Luciferase reporter assays in mouse hearts
Luciferase reporter assays from mouse hearts were performed as described previously. Briefly, 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 KH2PO4, 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.

Histological analysis and cell size measurement
For histological analysis, adult hearts were fixed in 10% formalin/phosphate-buffered saline and dehydrated for paraffin embedding. Fibrosis was detected with Masson's Trichrome staining on 5-µm paraffin sections. Blue collagen staining was quantified using Metamorph software. For cell surface area measurements, membranes were stained with TRITC- or FITC-labeled lectin from Triticum vulgaris (Sigma), and nuclei were labeled with TO-PRO 3 iodine (Molecular Probes, Carlsbad, CA). Cellular areas were quantified with ImageJ 1.33 software (Scion Corp., Frederick, MD).

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. 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. Cultures were harvested 24 h after infection,
and luciferase assays were performed as described previously.\textsuperscript{7} Ad\textsuperscript{β}gal, AdΔCnA, Adcain, AdNFAT-luciferase reporter, AdNFATc1-GFP (green fluorescent protein), and AdNFATc3-GFP have been previously described.\textsuperscript{3,8-10} AdNFκB-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.\textsuperscript{7}

Neonatal rat cardiomyocytes were prepared for immunocytochemistry as described previously.\textsuperscript{10} 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.\textsuperscript{3,5} Anti-Myc and anti-GFP antibodies were 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.\textsuperscript{11} Briefly, 20 µg 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 µg/ml aprotinin, 2 µg/ml leupeptin, 0.7 µg/ml pepstatin) with 0.5 µg poly (dI-dC) and \textsuperscript{32}P-labeled NFAT or NFκB consensus DNA sequences for 20 min at room temperature. For supershift reactions, 1 µl of anti-NFATc1 or anti-p65 antibody was added after 20 min of binding reaction. DNA complexes were separated on a 5% non-denaturing 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 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml aprotinin). Immunoprecipitation was performed as described previously.\textsuperscript{7} Whole cell lysates were cleared by centrifugation at 18,000 \textit{x} 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 ± SEM. Paired data were evaluated by Student's \textit{t} test. A one-way ANOVA with the Bonferroni's post hoc test or repeated-measures ANOVA was used for multiple comparisons. \textit{P} < 0.05 was considered statistically significant.


Online Figure I. NFAT- and NFκB-luciferase activity assays in neonatal myocytes

A, NFAT-luciferase activity in neonatal cardiomyocytes infected with recombinant adenoviruses expressing NFAT-luciferase reporter along with βgal, IκBαM, or dnIKKβ and stimulated with 20 ng/ml TNFα or vehicle control. *P < 0.05 vs. None; # P < 0.05 vs. TNFα βgal. B, NFκB-luciferase activity in neonatal cardiomyocytes infected with recombinant adenoviruses expressing NFκB-luciferase reporter along with βgal, Cain, or Rcan1 and stimulated with 20 ng/ml TNFα or vehicle control. *P < 0.05 vs. None; # P < 0.05 vs. TNFα βgal. C, NFAT-luciferase activity in neonatal cardiomyocytes infected with adenoviruses expressing NFAT-luciferase reporter and the other indicated proteins. *P < 0.01 vs. βgal; #P < 0.05 vs. Con ΔCnA. D, NFκB-luciferase in neonatal cardiomyocytes infected with adenoviruses encoding NFκB-luciferase reporter and the other indicated proteins. *P < 0.01 vs. βgal; #P < 0.05 vs. corresponding Con. E, F, and G, NFAT or NFκB-luciferase activity in neonatal cardiomyocytes infected with adenoviruses expressing NFAT- or NFκB-luciferase reporter, and then treated with cultured media collected from cells infected with the indicated indicated adenoviruses. No significant difference between the groups were observed for E, F, and G.
Online Figure II. Western blots for p65 and GFP following immunoprecipitation (IP) with anti-GFP antibody or pre-immune IgG from neonatal cardiomyocytes infected with AdNFATc1-GFP and then stimulated with 50 μM phenylephrine, 20 ng/ml TNFα, or vehicle control.
Online Figure III. p65 is required for pressure overload-induced cardiac hypertrophy and remodeling. A and B, interventricular septal thickness at diastole (IVSd) and left ventricular posterior wall thickness at diastole (LVPWd) in the indicated genotypes 4 weeks after TAC or sham operation, measured by M-mode echocardiography. C, Myocyte surface area (µm²) from cardiac histological sections of the mice shown in A and B. D, Fibrosis quantification from Masson’s trichrome stained cardiac histological sections. *P < 0.05 vs. Sham. #P < 0.05 versus p65fl/fl or Nkx2.5-Cre with TAC. †P < 0.05 versus p65fl/fl TAC.