Original Article

Interferon Regulatory Factor 7 Functions as a Novel Negative Regulator of Pathological Cardiac Hypertrophy

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Abstract—Cardiac hypertrophy is a complex pathological process that involves multiple factors including inflammation and apoptosis. Interferon regulatory factor 7 (IRF7) is a multifunctional regulator that participates in immune regulation, cell differentiation, apoptosis, and oncogenesis. However, the role of IRF7 in cardiac hypertrophy remains unclear. We performed aortic banding in cardiac-specific IRF7 transgenic mice, IRF7 knockout mice, and the wild-type littermates of these mice. Our results demonstrated that IRF7 was downregulated in aortic banding–induced animal hearts and cardiomyocytes that had been treated with angiotensin II or phenylephrine for 48 hours. Accordingly, heart-specific overexpression of IRF7 significantly attenuated pressure overload–induced cardiac hypertrophy, fibrosis, and dysfunction, whereas loss of IRF7 led to opposite effects. Moreover, IRF7 protected against angiotensin II–induced cardiomyocyte hypertrophy in vitro. Mechanistically, we identified that IRF7–dependent cardioprotection was mediated through IRF7 binding to inhibitor of κB kinase-β, and subsequent nuclear factor–κB inactivation. In fact, blocking nuclear factor–κB signaling with cardiac-specific inhibitors of κB (IC57B2/3A) super-repressor transgene counteracted the adverse effect of IRF7 deficiency. Conversely, activation of nuclear factor–κB signaling via a cardiac-specific conditional inhibitor of κB kinase-α (S177E/S181E) (constitutively active) transgene negated the antihypertrophic effect of IRF7 overexpression. Our data demonstrate that IRF7 acts as a novel negative regulator of pathological cardiac hypertrophy by inhibiting nuclear factor–κB signaling and may constitute a potential therapeutic target for pathological cardiac hypertrophy. (Hypertension. 2014;63:00:00.) ● Online Data Supplement

Key Words: cardiomegaly ■ fibrosis ■ interferon regulatory factor 7

Cardiac hypertrophy occurs in response to increased biomechanical stress (ie, hypertension, valvular disease, myocardial infarction, or endocrine disorders). It is characterized by enlarged cardiomyocytes, enhanced protein synthesis, accumulation of extracellular matrix, and activation of the fetal cardiac gene program.1–4 Although exercise-induced cardiac hypertrophy can enhance heart function, pathological hypertrophy is an initial compensatory response to increased cardiac workload, which can ultimately result in heart failure, arrhythmias, and sudden death.3,4 During the past few decades, several signal transduction pathways have been suggested to contribute to the development of pathological cardiac hypertrophy, including the epidermal growth factor receptor tyrosine kinase pathway, the mitogen-activated protein kinase pathway, the cAMP-response element binding protein pathway, the protein kinase C pathway, and the calcineurin pathway.5–10 Importantly, these signaling pathways converge in the nucleus to activate various transcriptional regulators, such as nuclear factor–κB (NFκB), activator protein-1, GATA transcription factors, nuclear factor of activated T cells, and myocyte enhancer factor-2, which drive the expression of fetal cardiac and stress response genes that promote pathological cardiac hypertrophy.6–8,11 However, the molecular mechanisms that mediate the critical transition from compensated hypertrophy to decompensated heart failure have remained elusive. Therefore, elucidation of these transcriptional reprogramming processes is essential for identifying potential transcriptional therapies aimed at preventing pathological cardiac hypertrophy and heart failure.

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Interferon regulatory factors (IRFs) are a family of transcription factors comprising 9 members (IRF1-9) in mammalian cells. They were initially found to play an essential role in modulating the expression of type I interferons (IFN-α and -β), but recent studies have indicated that IRFs also play critical roles in antiviral defense, immune response, hematopoietic development, cell growth/apoptosis, cardiac remodeling, and oncogenesis.13–15 Among the 9 family members, IRF7 binds to the DNA consensus sequence GAAWNGAAANY widely existing in genes, which indicates that IRF7 may modulate a more diverse range of target genes. In fact, a recent study by Honda et al17 has described IRF7 as a master transcription factor in the regulation of type 1 IFN-dependent immune responses, suggesting its crucial role in maintaining cellular homeostasis. Furthermore, Barnes et al18 reported that IRF7 overexpression in B-lymphoblastoid cells resulted in an altered transcriptional profile of cellular genes. Notably, IRF7 can function as a transcriptional activator or a repressor, depending on the cell type and its intrinsic transactivation potential.9,20 Accordingly, IRF7 has either oncogenic properties or antitumor effects. For example, IRF7 overexpressing NIH 3T3 (National Institutes of Health 3T3) cells induce tumor formation in nude mice,21 and overexpression of IRF7 inhibits the growth of MCF-7 (Michigan Cancer Foundation-7) breast cancer cells. However, the role of IRF7 in the process of cardiomyocyte remodeling, particularly in response to chronic pressure overload, has not been investigated. Therefore, we sought to determine the impact of IRF7 deficiency and cardiac-specific IRF7 overexpression on aortic banding (AB)–induced cardiac hypertrophy.

Here, we report that IRF7 acts as a novel negative regulator of pathological cardiac hypertrophy through suppression of NFκB signaling. We observed that hypertrophic stress resulted in IRF7 downregulation in both mouse hearts and cardiomyocytes. Moreover, IRF7-deficient mice revealed an aggravated hypertrophic response on chronic AB, whereas the opposite effect occurred in IRF7-overexpressing mice. We also discovered that all regions of IRF7, except for the inhibitory domain (ID: 284-467aa), could interact with the kinase domain (N1/300aa) of inhibitor of κB kinase-β (IKKβ). Furthermore, blocking NFκB signaling counteracted the effects of IRF7 deficiency, whereas constitutively active IKKβ negated the antihypertrophic effects of IRF7. Taken together, our results reveal a previously unrecognized role for IRF7 in the regulation of cardiac hypertrophy.

Methods

All experiments involving animals were approved by the Animal Care and Use Committee of Renmin Hospital, Wuhan University, China. An expanded Methods section is available in the online-only Data Supplement, which includes Reagents, Mice Used in This Study,17 Aortic Banding,1,8,9 Echocardiography and Hemodynamic Measurements,1,8 Histological Analysis, Cultured Neonatal Rat Cardiac Myocytes and Recombinant Adenoviral Vectors,1 Measurement of the Protein/DNA Ratio, Atrial Natriuretic Peptide (ANP) Quantification Assays, Immunofluorescence Analysis, Quantitative Real-Time PCR and Western Blotting,1,8,9 IKKβ Activity Assays, Luciferase Reporter Assays,22,23 Hydroxyproline Assay,25 Immunoprecipitation, Glutathione S-transferase (GST) Pull-down Assay, Confocal Microscopy, and Statistical Analysis.

Results

Expression of IRF7 Is Downregulated in Experimental Hypertrophic Models

IRF7 is expressed constitutively in a variety of tissues, including the heart.26 To determine whether hypertrophic stress can affect IRF7 expression in cardiomyocytes, we first cultured neonatal rat cardiac myocytes (NRCMs) with either Ang II or phenylephrine for 48 hours to induce hypertrophy. Western blotting results revealed that IRF7 protein levels were markedly reduced by 52% (Ang II) and 36% (phenylephrine), respectively. At the same time, we observed a significant increase in 2 hypertrophic markers, β-myosin heavy chain (β-MHC) and ANP (Figure 1A, n=3 independent experiments, P<0.01 versus PBS), and ANP concentrations in culture medium were also remarkably increased (Figure S1A in the online-only Data Supplement, P<0.01 versus PBS). Furthermore, in an experimental mouse model with AB-induced cardiac hypertrophy (evidenced by elevation of β-MHC and ANP levels, Figure 1B), IRF7 expression was downregulated by ≈93% in mouse hearts 4 weeks after AB compared with sham-operated hearts (Figure 1B, n=3 independent experiments, P<0.01 versus sham). Taken together, these results indicate that hypertrophic stress leads to IRF7 downregulation both in vitro (cardiomyocytes) and in vivo (intact mouse hearts), suggesting that IRF7 has a role in stress-induced cardiac remodeling.

IRF7 Protects Against Angiotensin II–Induced Cardiomyocyte Hypertrophy In Vitro

To define the functional contribution of IRF7 to cardiac hypertrophy, we first performed in vitro studies using primary cultured NRCMs, a well-controlled experimental setting. NRCMs were
infected with either AdIRF7 (adenoviral IRF7) to overexpress IRF7 or AdshIRF7 (adenoviral short hairpin IRF7) to knockdown IRF7 (Figure S1B) and subsequently exposed to Ang II (1 μM) for 48 hours. The cell surface area was then determined by immunostaining with α-actinin. Notably, under basal conditions neither AdIRF7 nor AdshIRF7 affected the size of cultured NRCMs compared with control AdGFP (adenoviral green fluorescent protein) and AdshRNA (adenoviral short hairpin RNA) cells. However, exposure to Ang II significantly reduced the cell surface area of AdIRF7-infected cells by 29% (Figure 2A and 2B), whereas Ang II–induced cardiomyocyte hypertrophy was enhanced by 62% in AdshIRF7-treated NRCMs compared with controls (Figure 2A and 2B). Accordingly, the Ang II–induced expression of hypertrophic hallmarks (ANP and β-MHC) and the ratio of protein/DNA were profoundly suppressed in AdIRF7-infected cardiomyocytes (Figure 2C and 2D), whereas significantly enhanced in AdshIRF7-infected NRCMs (Figure 2C and 2D) compared with controls respectively. These results revealed that IRF7 had an antihypertrophic effect on cardiomyocytes.

Overexpression of IRF7 Attenuates Pressure Overload–Induced Cardiac Hypertrophy

We next sought to examine whether increased IRF7 levels in the heart would attenuate the development of cardiac hypertrophy and failure. Thus, we generated a transgenic (TG) mouse model with cardiac-specific IRF7 expression, using the α-MHC promoter (α-MHCP; Figure S2A). Four germ lines of IRF7-TG mice were created and verified by Western blotting (Figure S2B). At baseline, the IRF7-TG mice displayed normal cardiac morphology and contractile function (Table S1). We selected the highest-expressing IRF7 line (TG21) to be used in our experiments. IRF7-TG mice, along with their WT littermates (referred to as NTG), were subjected to AB surgery or a sham operation, and pressure gradients (mm Hg) were equivalent among all AB-operated mice (Figure S2C). After 8 weeks of AB, NTG mice developed massive cardiac hypertrophy, as indicated by increased heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) ratios (Figure 3A–C). These changes were accompanied by the following features: (1) thickening of the left ventricular (LV) wall (HE staining, Figure 3D); (2) increase cardiomyocyte cross-sectional area (WGA [wheat germ agglutinin] staining, Figure 3D and 3E); and (3) upregulation of the hypertrophic markers ANP, B-type natriuretic peptide, and β-MHC (Figure S2E). Consistent with this, NTG mice exhibited cardiac dilation and dysfunction 8 weeks after AB, as measured by echocardiographic parameters including LV end-diastolic dimension (LVEDd), LV end-systolic dimension (LVEsd), and LV fractional shortening (LVFS; Figure 3F). Hemodynamic analysis also showed impaired systolic and diastolic functioning of the LV in NTG mice, as determined by the minimum rate of pressure (−dP/dt) and the maximum rate of pressure (+dP/dt) in LV isovolumetric contraction (Table S2). However, this pathological cardiac growth, which was induced by chronic pressure overload, was remarkably attenuated in IRF7-TG mice (Figure 3A–F and Figure S2E), and the cumulative survival rate of IRF7-TG mice was increased after 8 weeks of AB surgery (Figure S2D).

Fibrosis is a classical feature of pathological cardiac hypertrophy and is characterized by the accumulation of collagen in the heart.27 To further define the effect of IRF7 overexpression on maladaptive cardiac remodeling, we examined the effect of IRF7 on cardiac fibrosis. The extent of fibrosis was quantified by collagen volume through the visualization of the total amount of collagen present in the interstitial and perivascular spaces. We observed that both interstitial and perivascular fibrosis were dramatically increased in NTG hearts that were subjected to AB, but markedly limited in IRF7-TG hearts that experienced the same treatment (Figure 3G and 3H). Finally, we measured the synthesis of collagen by analyzing the mRNA expression levels of fibrotic markers (eg, connective tissue growth factor, collagen I, and collagen III; Figure S2F).

Our results consistently revealed a decreased fibrotic response
Hypertension

Ablation of IRF7 Exaggerates Pressure Overload–Induced Hypertrophy

To assess the impact of decreased IRF7 levels on the development of cardiac hypertrophy and failure that are induced by chronic pressure overload, we next used an IRF7 knockout mouse model (IRF7−/−), in whom IRF7 expression was absent in the heart (Figure S3A). At baseline, IRF7−/− mice did not show any cardiac abnormalities (Table S1). Our preliminary experiments revealed that almost all IRF7 knockout mice died after 8 weeks of AB surgery (Figure S3C). Therefore, we subjected IRF7−/− mice and their wild-type littermates (referred to as IRF7+/+) to AB surgery for 2 weeks, and pressure gradients (mm Hg) were equivalent among all AB-operated mice (Figure S3B). In contrast to IRF7-TG mice, IRF7−/− mice aggravated AB-induced cardiac hypertrophy, as indicated by greater increases in HW/BW, LW/BW, and HW/TL compared with AB-treated IRF7+/+ mice (Figure 4A–C). Histological examination of heart sections also revealed an increased cross-sectional area of cardiomyocytes in the IRF7−/− mice (Figure 4D and 4E). Consistent with these data, hearts from IRF7−/− mice showed greater hypertrophic marker induction (ANP, B-type natriuretic peptide, and β-MHC) after 2 weeks of AB compared with controls (Figure S3D). Accordingly, IRF7−/− mice exhibited deteriorated cardiac dilation and dysfunction, as observed through echocardiograph and hemodynamic analysis (Figure S3E and Table S3) and decreased cumulative survival rate (Figure S3F). We also assessed the effect of IRF7 deficiency on AB-triggered cardiac fibrosis. Both histological analysis and fibrotic markers analyses consistently demonstrated an increased fibrotic response in AB-operated IRF7−/− mice compared with AB-treated IRF7+/+ mice (Figure 4F and 4G and Figure S3G). Collectively, these loss-of-function data indicate that ablation of IRF7 exaggerates cardiac hypertrophy and fibrosis in response to chronic pressure overload.

IRF7 Suppresses NFκB Signaling

To gain insight into the molecular mechanisms underlying the negative effects of IRF7 on pathological cardiac hypertrophy, we next sought to identify IRF7-regulated targets using a Cignal
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45-Pathway Reporter Array kit (SABiosciences: CCA-901 L). This screening kit provides a comprehensive assay for preliminary monitoring of diverse cell signaling pathways by measuring the activities of downstream transcription factors via a dual-luciferase reporter system. The results showed that the activity of NFκB was significantly blocked by IRF7, which was confirmed by performing an NFκB dual-luciferase reporter assay in hypertrophic cardiomyocytes (in vitro) and hearts (in vivo). NRCMs were infected with either AdIRF7 to overexpress IRF7 or AdshIRF7 to knockdown IRF7. Subsequently, these infected cardiomyocytes were exposed to 1 μM of Ang II for 48 hours. Our results showed that compared with controls, Ang II–induced NFκB activation was significantly reduced in the AdIRF7-infected NRCMs but greatly enhanced in the AdshIRF7-infected cardiomyocytes (Figure S4A). IRF7+/-, NTG, and IRF7-TG mice received Ad-NFκB–Luc injection at ventricular wall immediately after being subjected to AB or sham operation. IRF7 overexpression inhibited whereas the loss of IRF7 promoted NFκB activation induced by AB surgery, which was consistent with data in vitro experiments (Figure 5B). Next, we performed an NFκB Signaling Pathway EpiTect Chip qPCR Array (SABioscience: GM-025A) to determine which genes are regulated by NFκB pathway and further validated the results by real-time polymerase chain reaction. The results revealed that genes (eg, Nfkb2, ikbkb [inhibitor of nuclear factor kappa-B kinase subunit beta], chemokine [C-C motif] ligand 5 [CCL5], CSF2 [colony-stimulating factor 2], Smad3 [mothers against decapentaplegic homolog 3], Bax [B-cell lymphoma 2 associated X protein]) associated with inflammation, apoptosis, and hypertrophy were significantly increased (Figure S4A) in the hearts of AB-subjected IRF7−/− mice. These data indicate that IRF7 may exert anti-inflammatory effects through the inhibition of the NFκB signaling.

It is known that dimeric NFκB (containing p50, p65, and c-Rel) is inactivated by binding to inhibitors of NFκB (IκBα), which sequester NFκB in the cytosol in nonstimulated cells. In the classical pathway, the IκB proteins are regulated by kinases, IKKα, and IKKβ. The latter is particularly important because it phosphorylates IκBα at Ser32/36, leading to ubiquitination/degradation of IκBα by the 26S proteasome and subsequent NFκB nuclear entry. It is also clear that IKKβ activation involves phosphorylation at 2 key serine residues (Ser 177/181). Therefore, to elucidate the ways in which IRF7 inhibits NFκB activity, we next examined phosphorylated levels IKKβ and IκBα in hypertrophic cardiomyocytes (in vitro) and hearts (in vivo). In Ang II–treated NRCMs, IRF7 overexpression significantly limited the kinase activity of IKKβ (Figure S4B) and activation of IKKβ-IκBα pathway, as evidenced by decreased levels of p-IKKβSer181 and p-IκBαSer32/36 compared with control cells (Figure 5C). In line with this, IKKβ–mediated proteosomal degradation of IκBα was attenuated in AdIRF7–myocytes, as indicated by increased levels of total IκBα (Figure 5C). Importantly, similar inhibitory effects of IRF7 on the IKKβ–IκBα signaling cascades were also observed in AB-treated IRF7-TG hearts (Figure 5E). Using loss-of-function strategies, we further demonstrated that decreased IRF7 levels resulted in pronounced activation of IKKβ and IκBα in vitro and in vivo experimental hypertrophic models (Figure 5D and 5F and Figure S4C).

Because p65 translocation to the nucleus is important for NFκB activation, we tested the effect of IRF7 on AB-induced p65 nuclear entry via Western blotting. Lamin B was used as a loading control for nuclear extracts. We found that AB-induced phosphorylation of p65 in the nucleus was profoundly attenuated by IRF7 overexpression (Figure 5G) and promoted by IRF7 depletion (Figure 5H), suggesting that IRF7 suppresses p65 nuclear translocation. Collectively, these data indicate that IRF7 negatively regulates the IKKβ–IκBα–NFκB pathway in cardiomyocytes in response to hypertrophic stimuli.

Additionally, given that NFκB activation has been implicated in the regulation of inflammation and apoptosis in many cell types, including cardiac myocytes, we next measured the expression profiles of some NFκB-dependent inflammatory factors (tumor necrosis factor α, monocyte chemotactic protein-1, and interleukin-1β) in animal hearts on chronic AB. Real-time polymerase chain reaction analysis (Figure S4D) and Western blotting (Figure S4F) revealed that IRF7 overexpression remarkably blunted AB-increased increases in tumor necrosis factor α, monocyte chemotactic protein-1, and interleukin-1β. Conversely, loss of IRF7 elicited the opposite effect (Figure S4E and S4G). Terminal deoxynucleotidyl transferase dUTP nick-end labeling analysis and Western blotting showed that the percentage of transferase dUTP nick-end labeling–positive cells, levels of c-Caspase3, and Bax were increased, whereas full-length Caspase3 and Bcl-2 were downregulated in IRF7 knockout mice (Figure S4I and S4K). On the contrary, IRF7 overexpression showed the opposite effects on cardiac apoptosis (Figure S4H and S4J). These results further support the hypothesis that IRF7 is a novel negative modulator of the NFκB signaling pathway in the hypertrophic hearts.

IRF7 Directly Interacts With IKKβ

Because stress-induced IκBα degradation (and thus NFκB activation) is IKKβ dependent, our finding of IRF7-mediated NFκB inhibition prompted us to examine whether IRF7 directly interacted with IKKβ. We first performed coimmunoprecipitation (IP) experiments in HEK293T (human embryonic kidney 293) cells transfected with FLAG-tagged IKKβ and Myc-tagged IRF7. We found that IRF7 could coprecipitate along with IKKβ and vice versa (Figure 6A and 5B) which is consistent with the results of co-IP for endogenous proteins in NRCMs (Figure SSA and SSB). This interaction was further confirmed by the GST–pull-down assay (Figure 6C). We next examined the subcellular localization of IRF7 using immunofluorescent staining. We observed that IRF7 and IKKβ were finely colocalized in the cytoplasm of HEK293T cells (Figure 6D). Similar results were observed in H9C2 cells (Figure S5C). Altogether, these data support that IKKβ can directly interact with and be regulated by IRF7.

To map which regions of IKKβ and IRF7 mediated the interaction, we next generated a series of deletion mutants of IKKβ and IRF7 (Figure 6E). Through IP mapping, we found that only the kinase domain (N1-300aa), located at the N-terminus of IKKβ, was required for the interaction (left, Figure 6E and 6F). However, most regions of IRF7, except for the inhibitory domain (ID: 284-367aa), contributed to IKKβ binding (right, Figure 6E and 6F). Therefore, IRF7 may inhibit IKKβ activity through masking its kinase domain. Importantly, we measured the kinase activity of...
Figure 5. Interferon regulatory factor 7 (IRF7) suppresses nuclear factor (NF) κB signaling. **A**, NFκB-luciferase activity in primary cultured neonatal rat cardiac myocytes (NRCMs) infected with AdIRF7 or AdshIRF7 (n=3, *P<0.05 vs AdGFP/PBS or AdshRNA/PBS, #P<0.05 vs AdGFP/Ang II or AdshRNA/Ang II). **B**, Ad-NFκB–Luc was injected into the ventricular wall of IRF7 transgenic (TG) and IRF7 knockout mice, and NFκB-luciferase activity was determined using a Luciferase Assay System kit (n=4). *P<0.05 vs NTG/sham or WT/sham, #P<0.05 vs NTG/aortic banding (AB) or WT/AB. **C**, Left, Representative Western blots showing the phosphorylation and total protein levels of inhibitor of κB kinase-β (IKKβ), p-IκBα. Right, Quantitative results of the p-IKKβ, p-IκBα, and IκBα in NRCMs infected with recombinant adenoviruses expression IRF7 and treated with Ang II for 60 min (n=3, *P<0.05 vs AdGFP/PBS, #P<0.05 vs AdGFP/Ang II). **D**, Same as shown in **C**, but cells were instead infected with AdshIRF7 and treated with Ang II for 60 min (n=3, *P<0.05 vs AdshRNA/PBS, #P<0.05 vs AdshRNA/Ang II). **E**, Left, Representative Western blots showing that the phosphorylation and total protein levels of IKKβ, p-IκBα. Right, Quantitative results of nucleus p-P65 and P65 protein levels in IRF7 knockout and WT mice 2 weeks after AB surgery (n=3, *P<0.05 vs WT/sham, #P<0.05 vs WT/AB). n indicates number of independent experiments.
IKKβ in NRCMs infected with AdIRF7 or AdIRF7-ID (AdGFP as a control). The results showed that overexpression of wild-type IRF7 significantly inhibited the kinase activity of IKKβ, however, overexpression of IRF7-ID (IRF7-inhibitory domain) did not have any effects on its kinase activity (Figure S5D).

**Inhibitory Role of IRF7 in Pathological Cardiac Hypertrophy Is largely Dependent on the NFκB Signaling Pathway**

Next, we examined whether activation or inactivation of the NFκB signaling cascade would affect the regulatory role of IRF7 in the development of pathological cardiac hypertrophy. To address this issue, we generated an inducible mouse model with the cardiac-specific overexpression of IKKβS177E/S181E, a constitutively active form of IKKβ (Figure S6A). The inducible IKKβS177E/S181E mice were then mated with IRF7-TG mice to create IRF7/IKKβS177E/S181E double transgenic mice (Figure S6B). Cardiac overexpression of IKKβ and IRF7 in these animal models was validated by Western blotting (Figure S6C). After 4 weeks of AB, the double transgenic mice exhibited a remarkable pathological cardiac hypertrophy, as evidenced by higher HW/BW, LW/BW, and HW/TL ratios (Figure S6E–G) compared with AB-operated IRF7-TG mice. Furthermore, histological analysis of hearts from double transgenic mice showed a significant increase in myocyte cross-section and collagen contents compared with AB-operated IRF7-TG mice (Figure S6D and S6H–J), and cardiac function was also dramatically deteriorated (Figure S6M). Additionally, pathological cardiac hypertrophy was remarkably obvious in IKKβS177E/S181E-TG mice after 4 weeks of AB compared with MEM-Cre controls (Figure S6D–M). Taken together, IRF7-elicited inhibitory effects on pathological cardiac remodeling seem to be largely IKKβ dependent.

To further verify the role of the NFκB signaling pathway in the negative effects of IRF7 on cardiac hypertrophy, we overexpressed the nondegradable IκBα super-repressor (IκBαS32A/S36A) in IRF7−/− hearts by crossing cardiac-specific IκBαS32A/S36A TG mice with IRF7−/− mice (Figure S7A/B). We found that IκBαS32A/S36A significantly reversed the detrimental effects of IRF7−/− on AB-induced cardiac hypertrophy, as determined by histological analysis and ratios of HW/BW, LW/BW, and HW/TL (Figure S7C–G). Moreover, the aggravated effects of IRF7−/− on fibrotic response and hypertrophic marker induction were dramatically mitigated by IκBαS32A/S36A overexpression (Figure S7H–K). Importantly, cardiac function was significantly improved (Figure S7L). Collectively, these loss-of-function data confirmed that
NFKB pathway inactivation was responsible for IRF7-elicited negative action on pathological cardiac hypertrophy.

**Discussion**

In the present study, we used both gain-of-function and loss-of-function approaches to decipher the potential role of IRF7 in pathological cardiac hypertrophy. For the first time, we observed that overexpression of IRF7 in the heart limited chronic pressure overload–induced cardiac remodeling, whereas loss of IRF7 resulted in detrimental effects on the development of pathological cardiac hypertrophy. We also found that the expression levels of IRF7 were significantly reduced in hypertrophic hearts (Figure 1). Thus, IRF7 upregulation may provide a new therapeutic strategy for the treatment of pathological cardiac hypertrophy.

The mechanisms underlying the negative effects of IRF7 on pathological cardiac remodeling could stem from its direct binding to IKKβ that masks its kinase domain from activation. Except for the inhibitory domain (residues 284–467), which interferes with the binding and transactivation function of IRF7,30 other regions of IRF7 are responsible for the interaction with IKKβ. One of the regions binding to IKKβ is detected between residues 1 to 246, which contains DNA-binding domain and constitutive activation domain. DNA-binding domain, which specifically binds to IFN-stimulated response element to initiate the transcription of downstream genes, is shared by each member of IRF family and has 5 conserved tryptophan repeats.30 Constitutive activation domain is located between residues 151 and 246 and possesses constitutive activation activity.30 Another IKKβ-binding region is found in virus-activated domain and signal response domain of IRF7. Virus-activated domain is an accessory inducibility region on viral infection that is required for increasing basal and inducibility region on viral infection that is required for increasing basal and inducible activity.31 Signal response domain is the site of virus-induced phosphorylation, which regulates the transactivation function of IRF7.32 Only the kinase domain (residues 1–300) of IKKβ interacts with IRF7. IKKβ kinase domain contains several serine residues that can be phosphorylated by upstream kinases, and the phosphorylation is required for activating IKK kinase activity.3134 In our study, IRF7 directly binds to the kinase domain of IKKβ, and then suppresses its phosphorylation level and NFKB signaling. The interaction between IRF7 and IKKβ occurs in the cytoplasm. Hence, besides directly binding to the DNA sequence to modulate target gene expression, IRF7 also functions as a regulator by interacting with other proteins. For instance, it was reported that IRF7 binds to the death domain of MyD88 (myeloid differentiation primary response gene 88) in the cytoplasm to regulate the MyD88-dependent IFN gene expression.3536 Additionally, IRF7 interacts with TRAF6 (tumor necrosis factor receptor-associated factor protein 6), a downstream molecule of MyD88, and activates IFN gene transcription,43 thus our findings further support the notion that IRF7 functions as a critical regulator of IKKβ other than a transcription factor.

On binding to IRF7, the downstream IκB would be inactivated, leading to NFKB retained in the cytosol. In support of this hypothesis, activation of the IKKβ–IκB–NFKB pathway by aortic banding was remarkably limited in IκB-IRF7-overexpressing hearts but dramatically aggravated in IRF7-null hearts (Figure 5E and 5F). Furthermore, restoration of IKKβ activity via overexpression of IKKβS177D/S181E offset IRF7-induced inhibitory effects on pathological hypertrophy (Figure S6). Our findings clearly indicate that the IKKβ–IκB–NFKB signaling cascade is essential for the development of pathological cardiac hypertrophy. It is known that NFKB is a key regulator of gene expression programs downstream of diverse signal transduction cascades during a variety of physiological and pathophysiological processes, such as inflammation, cardiomyocyte growth, and extracellular matrix remodeling.3239 NFKB also directly interacts with other transcription factors that are involved in the development of cardiac hypertrophy. For example, Liu et al40 recently demonstrated a direct interaction between transcription factors, nuclear factor of activated T cells and NFKB, which was suggested to integrate these 2 disparate signaling pathways during cardiac hypertrophy.

Nonetheless, there are some controversial reports on the role of NFKB signaling in the regulation of cardiac hypertrophy. Some investigators have shown that NFKB activation is required for the development of cardiac hypertrophy as well as cardiac dysfunction.4041 For example, cardiac-specific NFKB inhibition mediated by expression of a stabilized IκBα mutant attenuates Ang II–induced cardiac hypertrophy.41 Moreover, cardiac-specific deletion of p65 decreases the hypertrophic response after pressure overload stimulation, reduces the degree of pathological remodeling, and preserves contractile function.40 In contrast, several additional studies have disputed the importance of NFKB in the regulation of cardiac hypertrophy.4243 For example, Hikoso et al42 reported that cardio-specific knockdown of IKKα promoted cardiac hypertrophy, dilatation, and dysfunction in response to pressure overload. NFKB inhibition by cardiac-specific deletion of NEMO (NF-kappa B essential modulator), a regulatory subunit of the IKK complex, augmented cardiac hypertrophy and heart failure after AB.42 In the present study, we observed that the activation of NFKB in the hearts with overexpression of IRF7, suggesting that IRF7 may attenuate cardiac remodeling through negative regulation of NFKB signaling. More importantly, inhibition of the NFKB signaling by overexpression of IκBα combats the deteriorating effects of IRF7 knockout on cardiac dysfunction and dilatation in response to chronic AB, whereas constitutively active IKKβ blocked IRF7-mediated protection from pathological cardiac remodeling. Therefore, the inhibitory effects of IRF7 on cardiac hypertrophy may be largely dependent on inactivation of the NFKB signaling. Our study may add a new molecule to the increasing list that selectively inhibits the activation of transcription factor NFKB in the hypertrophic hearts. Given that NFKB is likely to have multiple divergent effects and may promote or suppress pathogenic processes, future investigation is needed to determine whether IRF7 has a therapeutic potential for the treatment of pathological cardiac hypertrophy.

Given that cardiac hypertrophy is a complicated pathological process that involves multiple molecules, our study cannot rule out other mechanisms that IRF7 is involved. Indeed, as a transcription factor, IRF7 may directly regulate genes related to cardiac hypertrophy. This is actually predicted by the online database. In the present study, we found that IRF7 potentially binds to the regulatory elements of some prohypertrophic and profibrotic genes, such as Myh6, connective tissue growth factor, smad3, smad4, Col11a1, and Col4a2. Therefore, it is likely that IRF7 may have dual roles in regulating cardiac remodeling; however, future work is needed to explore the mechanism by which IRF7 functions as a transcription factor to modulate the development of pathological cardiac hypertrophy.
The limitation of this study is that we used global IRF7 knockout to study the roles of IRF7 in the development of cardiac hypertrophy, and a genome-wide gene expression analysis has not been performed. Considering that global knockout of IRF7 may affect other types of cells other than cardiomyocytes in the heart, we used cultured neonatal rat cardiomyocytes in which IRF7 was knocked down by shIRF7. Our results showed that Ang II–caused hypertrophy was enhanced in IRF7-knockdown myocytes, which seems consistent with the findings observed in knockout mice on chronic pressure overload. Additionally, IRF7/TG mice, which overexpressed IRF7 specifically in the heart, showed significant reduction in hypertrophic response. Taken together, both in vivo and in vitro results consistently reflect the importance of IRF7 in the heart to regulate the development of pathological cardiac hypertrophy.

Notably, interferon regulatory factors (IRFs), consisting of 9 members (IRF1-9) in mammals, represent a group of structurally similar transcription factors characterized by having a well-conserved N-terminal DNA-binding domain and a C-terminal IRF association domain. IRFs exert their function either in cooperation or competition with other factors to play an important role in innate immunity, cell cycle, apoptosis, cancer, metabolic diseases, and cardiovascular diseases.12,13,44,45 Recently, our group intensively reported the role of IRFs in metabolic diseases and cardiovascular diseases.8,15,46-49 IRF3 and IRF9 alleviate hepatic steatosis and insulin resistance by constraining IKKβ/NFκB signaling or activating PPARα (peroxisome proliferator-activated receptor alpha) target genes, respectively.46,47 but IRF7 promotes diet-induced obesity and insulin resistance.48 In cardiovascular system, IRF3 and IRF9 protected against pressure overload–induced cardiac hypertrophy via suppressing ERK1/2 (extracellular signal-regulated kinases 1/2) or myocardin, respectively,8,49 whereas IRF4 aggravated pressure overload–induced hypertrophic response by activating the transcription of cAMP-response element binding protein.8 In this study, we demonstrate that IRF7 protects against pressure overload–induced cardiac hypertrophy by inhibiting IKKβ/NFκB signaling. Therefore, IRFs have the proper response by regulating an identical yet disparate set of targeted genes, and further studies are needed to clarify how these different IRFs interplay each other in the development of cardiovascular diseases and metabolic diseases.

In conclusion, we have found a novel role for IRF7 in regulating stress-induced cardiac hypertrophy and fibrosis. Importantly, IRF7 serves a protective function during pathological cardiac hypertrophy by inhibiting the IKKβ–IkB–NFκB signaling pathway. These observations provide new insights into the mechanisms of cardiac hypertrophy and may have significant implications for the development of novel strategies for the treatment of pathological cardiac remodeling through targeting IRF7.

Perspectives

By using IRF7 knockout mice and cardiac-specific transgenic mice, we have provided evidences sufficient to demonstrate that IRF7 negatively regulates pressure overload–induced cardiac remodeling via suppressing the IKKβ–IkB–NFκB signaling pathway. These observations suggest that increasing of IRF7 expression could serve as a lead target for the prevention and treatment of cardiac hypertrophy and failure.

 Acknowledgments

We thank Dr Taniguchi Tadatsugu (The University of Tokyo) for generously providing the IRF7 knockout mice. Rui Zhang, Li-Hua Gan, Ya-Fen Lin, Xue-Yong Zhu, Zhang-Li Li, Miao Yin, and Xin Zhang are gratefully acknowledged for providing technology help.

Sources of Funding

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Disclosures

None.

References

17. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, Taniguchi T. IRF-7 is the master...


**Novelty and Significance**

**What Is New?**

- Interferon regulatory factor 7 (IRF7) is downregulated in murine hypertrophic hearts and neonatal rat cardiomyocytes treated with Ang II or phenylephrine.

- IFR7 is a negative regulator of pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction.

- IFR7 regulates the development of cardiac hypertrophy via interacting with inhibitor of xB kinase-β and suppressing inhibitor of xB kinase-β–nuclear factor-xB signaling pathway.

**What Is Relevant?**

- Cardiac remodeling induced by pressure overload is close to what occurs in hypertension.

- The molecular mechanisms of hypertension-induced cardiac remodeling are not elucidated.

- This finding provides additional information for further understanding the effect of IRF family proteins on cardiac remodeling and implications for the development of strategies for the treatment of cardiac remodeling and heart failure.

**Summary**

The absence of interferon regulatory factor 7 (IRF7) promotes pressure overload–induced cardiac remodeling and dysfunction, whereas cardiac-specific overexpression of IFR7 inhibits the hypertrophic response by interacting with inhibitor of xB kinase-β and then suppressing the inhibitor of xB kinase-β–nuclear factor-xB signaling. Based on the results of the present study, we propose that IRF7 could be considered a therapeutic target for the prevention of cardiac hypertrophy.
Interferon Regulatory Factor 7 Functions as a Novel Negative Regulator of Pathological Cardiac Hypertrophy

Ding-Sheng Jiang, Yu Liu, Heng Zhou, Yan Zhang, Xiao-Dong Zhang, Xiao-Fei Zhang, Ke Chen, Lu Gao, Juan Peng, Hui Gong, Yingjie Chen, Qinglin Yang, Peter P. Liu, Guo-Chang Fan, Yunzeng Zou and Hongliang Li

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Interferon Regulatory Factor 7 Functions as a Novel Negative Regulator of Pathological Cardiac Hypertrophy

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Running title: IRF7 regulates cardiac hypertrophy

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Supplemental Methods

Reagents

Antibodies against the following proteins were purchased from Cell Signaling Technology: IKKβ (2370, 1:1000 dilution), p-IκBαser32/36 (9246, 1:1000 dilution), IκBα (4814, 1:1000 dilution), p-p65ser33 (3033, 1:1000 dilution), p65 (4764, 1:1000 dilution), TNFα (3707, 1:1000 dilution), MCP-1 (2029, 1:1000 dilution), Bcl2 (2870, 1:1000 dilution), Caspase3 (9662, 1:1000 dilution), C-Caspase3 (9661, 1:1000 dilution) and p-IKKαser180/βser181 (2681, 1:1000 dilution). Antibodies against β-MHC (sc53090, 1:200 dilution), ANP (sc20158, 1:200 dilution), IRF7 (sc9083, 1:200 dilution), and Lamin B (sc6217, 1:200 dilution), were purchased from Santa Cruz Biotechnology. We also used antibodies against GAPDH (Bioworld Technology; MB001, 1:10000 dilution), Bax (Bioworld Technology; BS2538, 1:500 dilution), IL-1β (R&D Systems; AF-401-NA, 1:1000 dilution), Flag (Sigma; F3165, 1:1000 dilution), and Myc (Roche; 1:1000 dilution). The BCA protein assay kit was purchased from Pierce. We used IRDye® 800CW-conjugated secondary antibodies (LI-COR Biosciences) or Peroxidase-conjugate d secondary antibodies (Jackson ImmunoResearch Laboratories) for visualization. Fetal calf serum (FCS) was purchased from Hyclone, all other reagents were purchased from Sigma.

Mice Used in This Study

All experiments involving animals were approved by the Animal Care and Use Committee of Renmin Hospital, Wuhan University. The animal models used for this study are described below.

IRF7 Knockout and Cardiac-Specific IRF7 Overexpressing Mice

Full-length mouse IRF7 cDNA (Origene, MC216147) was cloned downstream of the cardiac α-miosin heavy chain (α-MHC) promoter. The construct was microinjected into fertilized mouse embryos to generate cardiac-specific IRF7 transgenic (TG, C57BL/6J background) mice, the successful generations of these mice were confirmed via PCR analyses of tail genomic DNA using specific forward (5’-ATCTCCCCCATAAGAGTTTGAGTC-3’) and the reverse (5’-CCAGACTCGGATTCCAGTATGTG-3’) primers. IRF7 knockout mice (IRF7-/-, C57BL/6J background, RBRC01420) were generously provided by Dr Taniguchi Tadatsugu (The University of Tokyo) and shipped by REKEN1. IRF7-/- mice were identified by PCR analysis of tail genomic DNA using primer 1 (5’-GTGGTACCCAGTCCTGCCCTTATCTCTTACT-3), primer 2 (5’-TCGGTCTTATCGGCTCCGATTC-3) and primer 3 (5’-AGTAGATCCAGCTCCCCGGCTAAGTTGAC-3). The IRF7-/-, IRF7 TG mice and their wild-type littermates (aged 8 to 10 weeks old, with body weights of 24-27 g) were used in the experiments.

Cardiac-Specific IκBαS32A/S36A-Overexpressing Mice and IκBαS32A/S36A/IRF7+/− Mice

The hemagglutinin-tagged full-length IκBαS32A/S36A super repressor (Addgene, 15264) cDNA was cloned downstream of the cardiac α-MHC promoter. The construct was microinjected into fertilized mouse embryos to generate cardiac-specific IκBαS32A/S36A super repressor transgenic mice which were identified by PCR analysis of tail genomic DNA with forward (5’-ATCTCCCCCATCAGTGGTTGAGTC-3’) and reverse (5’-TTCTGGCTGGTTGATGAT-3’) primers. We crossed

1
IRF7 knock-out with IκBαS32A/S36A transgenic to produce the IκBαS32A/S36A/IRF7−/− mice, which were then crossed back with IRF7−/− mice to obtain IκBαS32A/S36A/IRF7−/− mice. The IκBαS32A/S36A transgene and IκBαS32A/S36A/IRF7−/− mice were C57BL/6J background.

Cardiac-Specific Conditional IKKβS177E/S181E-Cre Transgenic and IKKβS177E/S181E-Cre/IRF7 Double Transgenic Mice

To get the IKKβS177E/S181E flox mice, we subcloned Flag-tagged, constitutively active, IKKβS177E/S181E (Addgene, 11105) into the CAG-CAT-LacZ construct. This construct contains a CMV enhancer and the chicken β-actin gene (CAG) promoter, which are linked to the chloramphenicol acetyltransferase (CAT) gene and flanked by loxP sites. In the transgenic mice, IKKβS177E/S181E is blocked by the presence of the CAT gene if the CAT sequence is excised by Cre recombinase, IKKβS177E/S181E is expressed. The IKKβS177E/S181E flox mice were crossed with α-MHC–MerCreMer transgene mice (MEM-Cre-TG) (Myh6-cre/Esr1, Jackson Laboratory, 005650) to obtain the IKKβS177E/S181E-Cre TG mice. For cardiac specific-overexpression of IKKβS177E/S181E, tamoxifen (Sigma, T-5648) was injected into 6-week old mice that were positive for the IKKβS177E/S181E gene that contained MEM-Cre-TG. In particular, 200 mg of tamoxifen was dissolved in 1 ml of ethanol. Approximately 9.0 ml of corn oil (Sigma, C-8267) was added to this solution to attain a final tamoxifen concentration of 20mg/ml. The emulsion was sonicated prior to its injection into mice, and 80 mg/kg/day of the emulsion was injected intraperitoneally (i.p.) daily for 5 consecutive days. IRF7-TG mice were crossed with IKKβS177E/S181E-Cre TG mice to produce the IKKβS177E/S181E-Cre/IRF7 double transgenic (DTG) mice. MEM-Cre-TG mice were used as controls. The MEM-Cre-TG, IKKβS177E/S181E-Cre TG and DTG mice were C57BL/6J background. All experiments were performed in male mice with body weights of 24-27g (8 to 10 weeks old).

Aortic Banding

Aortic banding (AB) was performed in accordance with previously described methods. Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Sigma). The left chest was opened to identify the thoracic aorta by blunt dissection at the second intercostals space after absence of reflexes was observed. AB was achieved by tying the descending thoracic aorta against a 27G (in mice with body weights of 24-25g) or 26G (in mice with body weights of 25-27g) needle with a 7-0 silk suture, and then removing the needle. In addition, Doppler analysis was performed to ensure that adequate constriction of the aorta had been achieved. Pressure gradients (mmHg) were calculated from the peak blood velocity (Vmax, m/s, PG=4xVmax²) measured by Doppler analyses across the AB, which was equivalent in all groups of AB-operated mice. A similar sham operation was performed, but without aortic constriction.

Echocardiography and Hemodynamic Measurements

Mice were anesthetized by using 1.5-2% isoflurane and echocardiography and hemodynamic measurements were performed at the indicated times in accordance with previously described methods. Cardiac function and structure were determined by echocardiography using a Mylab30CV (ESAOTE) machine with a 15-MHz probe. 2-D M-mode measurements of LV internal diameter were obtained from at least three beats and then averaged. LV end-diastolic dimension
(LVEDD) and LV end-systolic dimension (LVESD) were measured at the time of largest and smallest LV areas, respectively. LV fractional shortening (LVFS) was calculated using the following formula: LVFS (%) = (LVEDD-LVESD)/LVEDD×100%. For hemodynamic measurements, a 1.4-French Millar catheter-tip micromanometer catheter (SPR-839; Millar Instruments) was inserted through the right carotid artery into the left ventricle. The pressures and dP/dt were recorded continuously with an Aria pressure-volume conductance system that was coupled with a Powerlab/4SP A/D converter and stored, and displayed on a personal computer.

**Histological Analysis**
Hearts were excised from anesthetized (pentobarbital sodium; 50 mg/kg, i.p.) mice, fixed for > 24 hours in 10% formalin after being arrested with 10% potassium chloride solution, and then embedded in paraffin. Subsequently, these hearts were sectioned transversely at 5µm. Sections were stained with either hematoxylin-eosin (HE) for histopathology or picrosirius red (PSR) for the collagen deposition. The myocyte cross-sectional area (CSA) was measured with a quantitative digital image analysis system (Image-Pro Plus 6.0) using images that were captured from FITC-conjugated wheat germ agglutinin (WGA, Invitrogen Corp) stained sections. More than 100 myocytes in the examined sections were outlined for each group of mice.

**Cultured Neonatal Rat Cardiac Myocytes and Recombinant Adenoviral Vectors**
Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared in accordance with previously described methods. Briefly, cardiomyocytes from 1- to 2-day-old Sprague-Dawley rats were isolated in phosphate-buffered saline (PBS) containing 0.03% trypsin and 0.04% type II collagenase. After the fibroblasts were removed using a differential attachment technique, NRCMs were seeded at a density of 2×10^5 cells/well onto six-well culture plates coated with gelatin in a plating medium that consisted of DMEM/F12 medium supplemented with 20% FCS, BrdU (0.1 mM, an inhibitor of the proliferation of fibroblasts) and penicillin/streptomycin. After 48 hours, the culture medium was replaced with serum-free DMEM/F12 for 12 hours prior to stimulation with angiotensin II (Ang II, 1 µM) or phenylephrine (PE, 100 µM). To over-express IRF7, the entire coding region of the rat IRF7 gene under the control of the cytomegalovirus promoter was encompassed by replication-defective adenoviral vectors. A similar adenoviral vector encoding the GFP gene was used as a control. To knock-down IRF7 expression, three rat shIRF7 constructs were obtained from SABiosciences (KR54505G), and the construct that produced the most significant decrease in IRF7 levels was selected from three generated AdshIRF7 adenoviruses for further experiments. AdshRNA was used as the non-targeting control. The NRCMs were infected with adenovirus in diluted media at a multiplicity of infection (MOI) of 100 for 24 hours.

**Measurement of the Protein/DNA Ratio**
For determining total cellular protein and DNA content, NRCMs were washed twice with PBS after stimulated with Ang II or PBS for 48 hours. Then, 0.2N perchloric acid (1 ml) was added to each well. The samples were centrifuged for 10 min at 10,000 g at 4°C. The precipitates were incubated for 20 min at 60 °C with 250 µl of 0.3 N KOH. BCA Protein Assay Kit (Pierce, CAT: 23225) was used to assess protein content, and Hoechst dye 33258 (Invitrogen, CAT: H3569) was used to detect
DNA content with salmon sperm DNA (Sigma, CAT: D1626) as a standard.

**Atrial Natriuretic Peptide (ANP) Quantification Assays**
Enzyme-linked immunosorbent assay (ELISA) was used to measure the production of ANP released into the cell culture medium by using a Synergy HT reader (Bio-Tek). The cell culture supernatant was analyzed for ANP using AssayMax Rat ANP ELISA Kit (Assay Pro, Winfield, MO, and Catalog No. ERA7010-1) following the instructions of the supplier after centrifuging at 2000g for 10 min to remove any debris. The absorbance was read at 450 nm immediately after the addition of reagents.

**Immunofluorescence Analysis**
The NRCMs stained with α-actinin antibody to determine cell surface areas by immunofluorescence staining. In brief, cardiac myocytes were infected with different adenoviruses for 24 hours and subsequently stimulated with 1 µM Ang II for 48 hours. The cells were then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 40 min, and stained with α-actinin at a dilution of 1:100 dilution using standard immunofluorescence staining techniques.

**Quantitative Real-Time PCR and Western Blotting**
Total mRNA was extracted from ventricles and primary cells using TRIzol (Invitrogen), and cDNA was synthesized with oligo (dT) primers using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Selected gene differences were confirmed by quantitative real-time PCR using SYBR Green (Roche) and results were normalized against GAPDH gene expression. Total protein and nuclear protein were extracted from the ventricles and primary cells in accordance with previously described approaches. Protein concentrations were determined using Pierce® BCA Protein Assay Kit (Pierce). A total of 50 µg (for fluorescence) or 15 µg (for chemiluminescence) of protein was used for SDS-PAGE (Invitrogen) followed by transfer to PVDF membrane (Millipore), which was then incubated with various primary antibodies overnight at 4°C. After incubation with a secondaryIRDye® 800CW-conjugated antibody (Li-Cor Biosciences, at 1:10000 dilution) or Peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, at 1:10000 dilution), signals were visualized with an Odyssey Imaging System (Li-Cor Biosciences) or FluorChem E (Cell Biosciences). The specific protein expression levels were normalized to GAPDH for total lysate or to Lamin B for nuclear proteins on the same nitrocellulose membrane.

**IKKβ Activity Assays**
NRCMs infected with AdIRF7 (AdGFP as a control) or AdshIRF7 (AdshRNA as a control) or AdIRF7-ID grown in 60-mm dishes were lysed in extract buffer (20 mM Tris, pH 7.4, 10 mM NaF, 150 mM NaCl, 1 mM Na-orthovanadate, 1% [wt/vol] Triton X-100, 10 µg/ml leupeptin, 10 mM Na-pyrophosphate, 1 mM PMSF, 1 mM glycerophosphate, and 5 µg/ml aprotinin). The cell lysates were centrifuged at 10,000g for 30 min at 4°C after incubated for 10 min on a rotating wheel (at 4°C). 250 µl of supernatants were incubated with 1 µg IKKβ antibodies (Cell Signaling Technology, #8943) and 20 µl Protein A/G-agarose beads (11719394001, 11719386001, Roche) for 2h at 4°C to immunoprecipitate endogenous IKKβ. The pelleted beads were washed three times with extract
buffer and twice with a buffer containing 5 mM MgCl₂ and 50 mM Tris (pH 7.4) after centrifugation on a benchtop centrifuge. 0.5 µg of purified GST-1kBα-1-55 was incubated with immunoprecipitates containing IKKβ. Reactions were carried out in 5mM MgCl₂, 50mM Tris (pH7.4), and 1mM ATP-Na₂ for 30 min at 30°C and ended by the addition of SDS-PAGE sample buffer and then separated in SDS-PAGE followed by western blotting with indicated antibodies.

Luciferase Reporter Assays
A Cignal 45-Pathway Reporter Array (SABiosciences, CCA-901L) was used to quickly identify relevant pathways for further analysis. Briefly, 50 µl Opti-MEM® with 200 ng GFP or IRF7 plasmid was added to each well of the Cignal Finder Array plate, and the reporter assay constructs were resuspended by gently tapping the side of the plate. Subsequently, 0.6 µl of Attractene Transfection Reagent (QIAGEN) in 50 µl of Opti-MEM® was incubated at room temperature for 5 min and then added to each well that contained 50 µl of the diluted nucleic acids. After a 20-minute incubation form complex formation was completed, 50 µl of a prepared cell suspension (8 × 10⁴ cells in Opti-MEM® containing 10% of fetal bovine serum) was added to each well that contained constructs-Attractene complexes. After 16 hours of transfection, the medium was changed to complete growth medium (DMEM with 10% FBS, 0.1 mM NEAA, 1 mM Sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin). At 24 hours after co-transfection, the luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega).

To demonstrate the change in NFκB signaling pathway, 40 µl of PBS containing 6x10¹¹ pfu of Ad-NFκB-Luc (VETOR BOLABS, Catalog No. 1740) or 6x10¹¹ pfu of Ad-pRL-Luc (VETOR BOLABS, Catalog No.1671, served as an internal control) was injected using a 30 G needle at multiple locations in the ventricular wall of IRF7⁺⁻, IRF7⁻⁻, non-transgenic (NTG), and IRF7 TG mice according to our published methods ⁶. The luciferase reporter assay in vivo was done as previously described⁷. Briefly, after 2 weeks of AB or sham surgery, mice infected Ad-NFκB-Luc were euthanized with an overdose of pentobarbital solution (200 mg/kg intraperitoneal). The heart was harvested, homogenized and lysed using tissue lysis buffer from Promega according to the manufacturer’s protocol. Then, the lysates were centrifuged for 20 min at 10,000 g, and the supernatant was used to determine both luciferase activity and protein content. Luciferase activity was measured using a Luciferase Assay System kit (Promega), and a Single-Mode SpectraMax® Microplate Reader (Molecular Devices). Protein content was determined using the BCA protein assay kit and was used to normalize luciferase activity per sample.

Hydroxyproline Assay
Hydroxyproline assay was performed to measure total collagen content in ventricular tissue of indicated group by modified method of Neuman and Logan⁸. Briefly, followed by vacuum drying of the samples, the tissue samples were digested with 6N hydrochloric acid overnight at 110 °C. After resuspending the samples in citric acetate buffer, a colored reaction was done by adding chloramine T, isopropyl alcohol, and Ehrlich’s reagent. The samples were incubated at 25 °C for 18 h, and intensity of the red color was measured at 558 nm using Synergy HT (Bio-Tek). Hydroxyproline content in the unknown samples was calculated with the help of a standard curve. The amount of collagen was calculated by multiplying hydroxyproline content by a factor of 8.2.
**Immunoprecipitation**

Full-length and deletion mutant human IKKβ expression vectors were constructed by cloning the human IKKβ cDNA into pcDNA5/FRT/TO-FLAG mammalian expression vector, which was modified from pcDNA5/FRT/TO (Invitrogen). For immunoprecipitation (IP), HEK293T cells co-transfected with pcDNA5-Flag-IKKβ and pSicoR-EGFP-Myc-IRF7 for 48 hours were collected, and lysed in 250 µl IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche). After being centrifuged at 13,000 g for 15 minutes, 250 µl of the sample was incubated with 1 µg specific antibodies and 10 µl Protein A/G-agarose beads (11719394001, 11719386001, Roche). Subsequently, IP products were washed with cold IP buffer for 5 to 6 times, and then separated by SDS-PAGE, and western blotted using the indicated primary antibodies.

**Glutathione S-transferase (GST) Pull-down Assay**

Purification of GST-IRF7 from *Escherichia. Coli* lysates was performed and the pull-down assay was carried out using glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences AB) according to the manufacturer’s instructions. Briefly, the GST-IRF7 beads were incubated with Flag-IKKβ-transfected HEK293T cell lysates (5 µg) in IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40 supplemented with protease inhibitor cocktail) for 4 hours at 4 °C. After being washed four times with IP lysis buffer (no cocktail), precipitated proteins were separated in SDS-PAGE followed by western blotting with anti-Flag antibodies (Sigma).

**Confocal Microscopy**

HEK293T cells were plated on gelatin-coated coverslips in 24-well plates. Upon cotransfection with pEGFP-myc-IRF7 and pCherry-IKKβ for 48 hours, cells were washed three times in PBS, fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes, then incubated in Image-IT™ FX signal enhancer (I36933, Invitrogen) for 30 minutes. After being washed with TBST for three times and stained with DAPI (1 g/ml, 15 minutes), the coverslides were mounted with mounting solution (D2522, Sigma) and images were obtained with a confocal laser-scanning microscope (Fluoview 1000; Olympus).

**Statistical analysis**

Data are represented in terms of the mean ± SD. A two-tailed Student’s *t*-test was used to compare the means of two groups of samples, and one-way ANOVA test with LSD (equal variances assumed) or Tamhane’s T2 (equal variances not assumed) was applied for multiple groups. A level of *P*<0.05 was considered statistical significance.
References


### Supplemental Tables

#### Table S1. Anatomic and Echocardiographic Analysis in 10-12 Week Old Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IRF7+/+ mice (n=13)</th>
<th>IRF7-/- mice (n=12)</th>
<th>IRF7 NTG mice (n=10)</th>
<th>IRF7 TG mice (n=12)</th>
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<tr>
<td>BW (g)</td>
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</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.60±0.14</td>
<td>3.56±0.21</td>
<td>3.56±0.09</td>
<td>3.58±0.18</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.94±0.19</td>
<td>1.96±0.17</td>
<td>1.96±0.11</td>
<td>1.94±0.15</td>
</tr>
<tr>
<td>FS (%)</td>
<td>45.80±4.60</td>
<td>45.20±3.49</td>
<td>45.40±2.30</td>
<td>45.00±4.12</td>
</tr>
</tbody>
</table>

BW=body weight; HW=heart weight; LW=lung weight; TL=tibial length; HR=heart rate; LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; FS=fractional shortening, All values are presented as the mean ± SD.
**Table S2** Parameters in IRF7 transgenic mice (TG) and wild type littermates (NTG) at 8 weeks after sham operation or AB

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NTG- Sham mice</th>
<th>TG -Sham mice</th>
<th>NTG-AB mice</th>
<th>TG -AB mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>28.84±1.20</td>
<td>27.96±2.06</td>
<td>29.08±1.64</td>
<td>28.20±1.88</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>510.86±41.69</td>
<td>492.29±31.11</td>
<td>477.86±39.85</td>
<td>495.57±22.24</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>92.24±5.33</td>
<td>109.53±8.33</td>
<td>128.14±10.07*</td>
<td>157.13±7.45*†</td>
</tr>
<tr>
<td>EF (%)</td>
<td>58.32±7.35</td>
<td>54.97±5.68</td>
<td>24.63±2.38*</td>
<td>36.85±6.92*†</td>
</tr>
<tr>
<td>dp/dt max (mmHg/sec)</td>
<td>10298.57±1041.11</td>
<td>10287.75±605.18</td>
<td>6203.33±729.32*</td>
<td>8098.00±755.75*†</td>
</tr>
<tr>
<td>dp/dt min (mmHg/sec)</td>
<td>-8947.00±771.72</td>
<td>-8855.00±1118.43</td>
<td>-4577.67±702.93*</td>
<td>-6809.25±1157.63*†</td>
</tr>
</tbody>
</table>

LVESP=Left ventricular End-systolic Pressure. LVEF=Left ventricular ejection fraction. *P<0.05 vs. NTG sham operation. †P<0.05 vs. NTG AB after 8 weeks AB. All values are the mean ± SD.
Table S3 Parameters in IRF7-/- and IRF7+/+ mice at 2 weeks after sham operation or AB surgery.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th></th>
<th>AB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IRF7+/+ mice</td>
<td>IRF7-/- mice</td>
<td>IRF7+/+ mice</td>
<td>IRF7-/- mice</td>
</tr>
<tr>
<td></td>
<td>( n=12 )</td>
<td>( n=14 )</td>
<td>( n=12 )</td>
<td>( n=13 )</td>
</tr>
<tr>
<td>BW (g)</td>
<td>26.86±1.35</td>
<td>26.74±1.56</td>
<td>26.87±1.18</td>
<td>26.62±1.64</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>516.29±26.64</td>
<td>503.22±20.32</td>
<td>479.29±27.58</td>
<td>500.00±15.92</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>93.86±7.25</td>
<td>111.28±8.54</td>
<td>157.72±6.86*</td>
<td>152.35±13.02*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>59.52±6.26</td>
<td>54.69±5.57</td>
<td>49.90±5.05*</td>
<td>27.05±4.48†</td>
</tr>
<tr>
<td>dp/dt max (mmHg/sec)</td>
<td>10498.14±755.89</td>
<td>10093.67±749.81</td>
<td>9639.29±1402.39</td>
<td>7294.88±898.34†</td>
</tr>
<tr>
<td>dp/dt min (mmHg/sec)</td>
<td>-9149.29±667.78</td>
<td>-8998.22±817.50</td>
<td>-8445.71±790.90</td>
<td>-5996.13±1023.53†</td>
</tr>
</tbody>
</table>

*P<0.05 vs. IRF7+/+ sham operation. †P<0.05 vs. IRF7+/+ AB after 2 weeks AB.
All values are presented as the mean ± SD.
Figure S1 (A) The ANP content in culture medium of NRCMs treated with Ang II, PE or PBS (n=3 independent experiments. *P<0.05 vs. PBS. (B) Confirmatory western blot for IRF7 knock-down and overexpression in AdshIRF7-infected cardiomyocytes and in AdIRF7-infected cardiomyocytes. Left: Representative blots. Right: Quantitative results.
Figure S2 Overexpression of IRF7 Attenuates Pressure Overload-Induced Cardiac Hypertrophy. (A) Schematic diagram of the construction of transgenic mice with full-length mouse IRF7 cDNA under the control of α-myosin heavy chain promoter. (B) Representative blots and quantitative results for determination of transgenic IRF7 levels expressed in the heart tissue from four lines of TG and their control WT mice. (C) Pressure gradients (mmHg) were calculated from the peak blood velocity (Vmax, m/s, PG=4xVmax2) measured by Doppler analyses across the AB, which were equivalent among NTG and TG AB-operated mice. (D) The cumulative survival rate of IRF7 NTG and TG mice after 8 weeks of AB (n=12-16 mice per experimental group). (E) Real-time PCR analyses of the hypertrophic markers ANP, BNP and β-MHC induced by AB in the indicated mice (n=4 mice per experimental group). (F) Real-time PCR analyses of the fibrotic markers CTGF, Collagen I, and Collagen III in the indicated mice (n=4 mice per experimental group). *P<0.05 vs. NTG/sham; #P<0.05 vs. NTG/AB.
Figure S3 Ablation of IRF7 in Hearts Exaggerates Pressure Overload-induced Hypertrophy.

(A) Deletion of IRF7 was confirmed by western blot. IRF7<sup>+/+</sup> stands for wild-type (WT) hearts, and IRF7<sup>-/-</sup> represents knockout (KO) hearts. (B) Pressure gradients (mmHg) were equivalent among IRF7<sup>+/+</sup> and IRF7<sup>-/-</sup> AB-operated mice. (C) The cumulative survival rate of IRF7<sup>+/+</sup> and IRF7<sup>-/-</sup> mice after 8 weeks of AB (n=13-19 mice). (D) Real-time PCR analyses of the hypertrophic markers ANP, BNP and β-MHC induced by AB in IRF7 KO and WT mice (n=4). (E) Parameters of echocardiographic results for IRF7 KO and WT mice (n=7-9). (F) The cumulative survival rate of IRF7<sup>+/+</sup> and IRF7<sup>-/-</sup> mice after 2 weeks of AB (n=13-17 mice). (G) Real-time PCR analyses of the fibrotic markers CTGF, collagen I, and collagen III in the indicated mice (n=4). *P<0.05 vs. WT/sham; #P<0.05 vs. WT/AB. n indicates number of mice per experimental group.
Figure S4 IRF7 Suppresses NFκB Signaling. (A) The results of NFκB Signaling Pathway EpiTect Chip qPCR Array were validated by Real-time PCR analyses (n=4 mice). *P<0.05 vs. WT/AB. (B) The kinase activity of IKKβ after overexpression IRF7 in NRCMs, Left, Representative Western blot, Right, Quantitative results; (n=3 independent experiments). (C) The kinase activity of IKKβ after knockdown IRF7 in NRCMs, Left, Representative Western blot, Right, Quantitative results; (n=3 independent experiments). (D-E) Real-time PCR analyses of TNFα, MCP-1 and IL-1β induced by
AB in the (D) IRF7 TG and NTG mice and (E) IRF7 KO and WT mice (n=4 mice, *P<0.05 vs. NTG or WT/Sham, #P<0.05 vs. NTG or WT/AB). (F) Left, Representative western blots; Right, Quantitative results of the TNFα, MCP-1 and IL-1β protein levels in IRF7 TG and NTG mice 2 weeks after AB (n=3 independent experiments, *P<0.05 vs. NTG/Sham #P<0.05 vs. NTG/AB). (G) Left, Representative western blots; Right, Quantitative results of the TNFα, MCP-1 and IL-1β protein levels in IRF7 KO and WT mice 2 weeks after AB (n=3 independent experiments, *P<0.05 vs. WT/Sham #P<0.05 vs. WT/AB). (H-I) The TUNEL staining of heart tissue of NTG and TG (H); or IRF7+/+ and IRF7−/− (I) at the indicated time point, TUNEL positive cells: Green; α-actinin: Red; DAPI: Blue (n=4 mice). *P<0.05 vs. WT/AB or NTG/AB. (J-K) Upper, Representative western blots showing the protein levels of Caspase3, C-Caspase3; Bax and Bcl2; Bottom, Quantitative results of the Caspase3, C-Caspase3; Bax and Bcl2 in IRF7 NTG and TG (J) or WT and IRF7 KO mice (K) after AB or Sham operation (n=3 independent experiments). *P<0.05 vs. NTG/Sham or WT/Sham, #P<0.05 vs. NTG/AB or WT/AB.
Figure S5 IRF7 Directly Interacts with IKKβ. (A) Western blot with IRF7 or IKKβ antibody after co-IP of IRF7 from NRCMs whole cell lysates using IRF7 antibody. (B) Western blot with IRF7 or IKKβ antibody after co-IP of IKKβ from NRCMs whole cell lysates using IKKβ antibody. (C) Representative confocal images showing co-localization of IRF7 and IKKβ in H9C2 cells. (D) The kinase activity of IKKβ after overexpression IRF7 or IRF7-ID in NRCMs, Left, Representative Western blot, Right, Quantitative results. All the above results repeated at least three times.
**Figure S6 Overexpression of IKKβ^{S177E,S181E} Eliminate the Protective Effects of IRF7 Transgenic after Aortic Banding.**

(A) TG constructs and Cre-mediated recombination event. The structures of the target gene (CAG-CAT- IKKβ^{S177E,S181E}) and their recombined allele are shown. (B) The breeding strategy for the production of IKKβ^{S177E,S181E}/IRF7 DTG mice. (C) Representative western blots for determination of IRF7 and IKKβ levels in the heart of MEM-Cre, IKKβ^{S177E,S181E}-TG, IRF7-TG, and DTG mice. (D) Histological analyses of HE and PSR staining of MEM-Cre, IKKβ^{S177E,S181E}-TG, IRF7-TG, and DTG mice 4 weeks after the AB surgery (n=6). (E-G) Results for the ratios of (E) HW/BW; (F) HW/TL; and (G) LW/BW in the indicated groups (n=8-13).
(H) Cell sectional area (n=100+ cells); (I) LV collagen volume (n=25+ fields); (J) Hydroxyproline content per 100mg heart tissue (n=6 mice) in the indicated groups. (K) Real-time PCR analyses of the hypertrophic markers ANP, BNP and β-MHC induced by AB in the indicated mice (n=4). (L) Real-time PCR analyses of the fibrotic markers CTGF, collagen I, and collagen III in the indicated mice (n=4). (M) Parameters of the echocardiographic results for the indicated groups (n=6-10). n indicates number of mice per experimental group. n indicates number of mice per experimental group.
Figure S7 Overexpression of IκBα<sup>S32A,S36A</sup> Super-repressor Rescues the Phenotype of IRF7 Knock-out after Aortic Banding. (A) Breeding scheme for the production of IκBα<sup>S32A,S36A</sup> -TG/IRF7<sup>-/-</sup> mice. (B) Representative western blots for determination of IRF7 and IκBα levels in the heart of NTG, IκBα<sup>S32A,S36A</sup> transgenic, IRF7<sup>-/-</sup>, and IκBα<sup>S32A,S36A</sup> -TG/IRF7<sup>-/-</sup> mice. (C) Histological analyses of HE and PSR staining of NTG, IκBα<sup>S32A,S36A</sup> transgenic, IRF7<sup>-/-</sup>, and IκBα<sup>S32A,S36A</sup> -TG/IRF7<sup>-/-</sup> mice 4 weeks after the AB surgery (n=6). (D-F) Statistical results for the ratios of (D) HW/BW; (E) HW/TL; and (F) LW/BW in the indicated groups (n=11-14). (G) Cell sectional area (n=100+ cells); (H) LV collagen volume (n=40+ fields); (I) Hydroxyproline content.
per 100mg heart tissue (n=6 mice) in the indicated groups. **(J)** Real-time PCR analyses of the hypertrophic markers ANP, BNP and β-MHC induced by AB in the indicated mice (n=4). **(K)** Real-time PCR analyses of the fibrotic markers CTGF, collagen I, and collagen III in the indicated mice (n=4). **(L)** Parameters of the echocardiographic results for the indicated groups (n=6-11). n indicates number of mice per experimental group.