ONLINE SUPPLEMENT

Role of Interferon Regulatory Factor 4 In the Regulation of Pathological Cardiac Hypertrophy

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Running Title: IRF4 modulates cardiac hypertrophy

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Methods and Materials

Reagents

Antibodies against the following proteins were purchased from Cell Signaling Technology: murine IRF4 (#4948), phospho-CREB (#9198) and total-CREB (#9198). The antibody against human IRF4 (#sc56713) was purchased from Santa Cruz Inc. The GAPDH (MB001) antibody was purchased from Bioworld Technology. The BCA protein assay kit was purchased from Pierce. We used IRDye® 800CW-conjugated secondary antibodies (LI-COR Biosciences) for visualization. Platinum Taq DNA polymerase High Fidelity (#11304-029) was purchased from Invitrogen. The pGL3 basic vector (E1751) and the Dual-Luciferase® Reporter (#E1960) Luciferase kit were purchased from Promega. The FUGENE® HD Transfection reagent (#04709713001) was purchased from Roche. Fetal calf serum (FCS) was purchased from Hyclone. Cell culture reagents and all other reagents were purchased from Sigma.

Human heart samples

Left ventricle (LV) samples were collected from human patients with dilated cardiomyopathy (DCM) who were under treatment following heart transplantation. Control samples were obtained from normal heart donors. This study complied with the protocol approved by the Renmin Hospital of Wuhan University Human Research Ethics Committee, and samples were collected after informed consent.

Experimental animal models and aortic banding surgery

The animal protocol was approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. Human IRF4 cDNA was cloned downstream of the cardiac α-myosin heavy chain (α-MHC) promoter. Transgenic mice were generated by microinjecting the α-MHC-IRF4 construct into fertilized mouse embryos and identified by PCR analysis of the tail genomic DNA. IRF4-knockout mice were purchased from the Jackson Laboratory (Stock Number: 009380). Male α-MHC-IRF4 (TG) and non-transgenic (NTG) littermates, IRF4-knockout mice and their wild-type (WT) littermates, ages 8 to 10 weeks (with body weights of 24-27 g), were used in these experiments. Aortic banding (AB) was conducted as described previously[1-5]. Doppler analysis was performed to ensure that adequate constriction of the aorta, and similar pressure overload was achieved. Pressure gradients (mmHg) were calculated from the peak blood velocity (Vmax, m/s, PG=4xVmax2) measured by Doppler analyses across the AB. Control animals underwent the same procedure without aortic banding (sham groups).

Echocardiography and hemodynamics evaluation

Echocardiography was performed using a MyLab 30CV ultrasound (Biosound Esaote Inc.) with a 10-MHz linear array ultrasound transducer. The left ventricle (LV) was assessed in both the parasternal long-axis and short-axis views at a frame rate of 120 Hz. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were

measured from the M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

For hemodynamic measurements, a 1.4-F Millar microtip catheter (SPR-839, Millar Instruments, Houston, Texas) was inserted into the left ventricle through the right carotid artery. After stabilization for 15 minutes, the pressure, volume signals and heart rate were recorded continuously using a pressure-volume conductance system (MPVS-300 Signal Conditioner, Millar Instruments, Inc., USA). The results were analyzed with Chart 5.0 software.

Histological analysis

Hearts were excised and arrested in diastole with 10% potassium chloride solution, fixed by 10% paraformaldehyde and embedded in paraffin. Subsequently, these hearts were sectioned transversely close to the apex to visualize the left and right ventricles at 5 μm. Sections of each heart at the mid-papillary muscle level were stained with hematoxylin-eosin (HE) for histopathology or with picrosirius red (PSR) for the collagen deposition. To determine the myocyte cross-sectional area, sections were stained with FITC-conjugated Wheat germ agglutinin (WGA, Invitrogen Corp) for membranes and with DAPI for nuclei. More than 100 myocytes in sections were outlined in each group. Single myocyte and fibrillar collagen were visualized by microscopy and measured with a quantitative digital image analysis system (Image-Pro Plus 6.0).

Western blotting and quantitative real-time PCR

Cardiac tissue and cultured cardiac myocytes were lysed using a RIPA buffer, and the protein concentration was determined with a BCA protein assay kit. Protein extracts (50 μg) were separated by SDS-PAGE and were transferred to nitrocellulose membranes and probed with various primary antibodies. After incubation with a secondary IRDye® 800CW-conjugated antibody, signals were visualized with an Odyssey Imaging System. The specific protein expression levels were normalized to GAPDH on the same nitrocellulose membrane.

For real time-PCR, total RNA was extracted from the frozen mouse tissue using a TRIzol (Invitrogen) and reverse-transcribed into cDNA using oligo (dT) primers with a Transcriptor First Strand cDNA Synthesis Kit. PCR amplifications were quantified using the SYBR Green PCR Master Mix (Applied Biosystems) and normalized to GAPDH gene expression.

CREB-luciferase reporter assays

Nucleotides -1310 to +51 of the human CREB gene were amplified from the genome of 293T cells by PCR with Platinum Taq DNA polymerase High Fidelity and inserted into the pGL3 basic vector as a CREB-F1 vector. H9C2 cells (2×10^6 per well) were seeded on 24-well plates the day before transfection. Using a FUGENE® HD Transfection reagent, cells at 50-80% confluence were transfected with 1 μg of pGL3-CREB. A 50 ng of the expression plasmid (pRL-Thymidine Kinase, pRL-TK)

was co-transfected as a transfection efficiency control, and luciferease activities were normalized based on TK activities. After 24 hours, cells were harvested, washed three times with phosphate-buffered saline (PBS) and lysed in 100 µl of the passive lysis buffer (PLB) according to the manufacturer's instructions (Promega). Cell debris was removed by centrifugation, and the supernatant was used for the luciferase assay using Single-Mode Microplate Readers SpectraMax® (Molecular Devices).

Cardiomyocyte culture and infection with recombinant adenoviral vectors

The replication-defective adenoviral vectors expressing the full-length IRF4 gene and a control adenoviral vector expressing the GFP protein were used in this study. The shIRF4 that led to the greatest decrease in IRF4 levels was selected for further experiments and the shRNA virus was used as a control. We infected cardiac myocytes with Ad-shRNA, Ad-shIRF4, Ad-GFP and Ad-IRF4. The cultures of cardiac myocytes were prepared as described in our previous studies with a minor revision[2, 3]. Cardiomyocytes isolated from hearts of 1- to 2-day-old Sprague-Dawley rats were seeded at a density of 3×10^5/well onto six-well culture plates in MEM supplemented with 10% FCS and penicillin/streptomycin. Subsequently, these myocytes were infected with AdIRF4, Ad-shIRF4 and/or AdCREB, Addn-CREB for 12 hours with 10 MOIs. The culture medium was then replaced with serum-free medium for 12 hours, followed by stimulation with 1 µM Ang II for 48 hours. AdCREB was purchased from Applied Biological Materials Inc. (Cat. # 000407A). Addn-CREB was constructed in our lab with the CREB Dominant-Negative Vector which was ordered from Clontech (Cat. # 631925).

Measurement of the Protein/DNA Ratio

For the quantitative analysis of total cellular protein and DNA content, neonatal rat cardiomyocytes were washed twice with PBS (phosphate-buffered saline) after stimulated with Ang II or PBS for 48 hours. Then, 0.2N perchloric acid (1 ml) was added to cells. The samples were centrifuged for 10 min at 10,000 ×g. The precipitates were incubated for 20 min at 60 °C with 250 µl of 0.3 N KOH. Protein content was analyzed by the BCA method using BCA Protein Assay Kit (Pierce, CAT: 23225). DNA content was detected using Hoechst dye 33258 (Invitrogen, CAT: H3569) with salmon sperm DNA (Sigma, CAT: D1626) as a standard.

Immunofluorescence Staining

Neonatal rat cardiomyocytes (NRCM) were cultured on cover slips, infected with Ad-IRF4 or Ad-shIRF4, and treated with Ang II (1 µM) for 48 hours. Cardiomyocytes were fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature; washed three times; permeabilized with 0.1% Triton X-100 in PBS for 40 minutes; and stained with α-actinin (1:100 dilution), using standard immunofluorescence staining techniques. For the tissue section staining, the procedure was the same as that used for the NRCM staining after de-parafinisation step (as described above).

Statistical analysis

Data are shown as means ± SEM. Differences among groups were assessed by ANOVA followed by Tukey's *post hoc* test. Comparisons between two groups were performed by Student's *t*-test. A value of $P<0.05$ was considered to be a statistically significant difference.

References

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Supplemental Tables

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

Parameter	IRF4+/+ mice (n=12)	IRF4-/- mice (n=13)	NTG mice (n=10)	TG mice (n=12)
BW(g)	27.26±0.68	27.32±0.33	26.93±0.44	27.56±0.42
HW/BW(mg/g)	4.38±0.10	4.22±0.11	4.28±0.10	4.17±0.10
LW/BW(mg/mg)	5.35±0.12	5.17±0.10	5.24±0.13	5.15±0.10
HW/TL(mg/mm)	6.49±0.09	6.26±0.12	6.42±0.12	6.22±0.12
HR (beats/min)	549±15	525±9	558±22	512±17
LVEDD(mm)	3.84±0.09	3.76±0.06	3.64±0.07	3.63±0.08
LVESD(mm)	2.10±0.08	2.12±0.05	1.96±0.04	2.08±0.10
FS (%)	45.2±1.5	43.4±0.9	46.0±1.1	43.0±1.8

BW=body weight; HW=heart weight; LW=lung weight; TL=tibia length; HR=heart rate; LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; FS=fractional shortening, All values are presented as means ± SEM.

Table S2 Parameters in IRF4-/- and IRF4+/+ mice at 8 weeks after sham operation or AB

Parameters	Sham		AB	
	IRF4+/+ mice (n=15)	IRF4-/- mice (n=11)	IRF4+/+ mice (n=12)	IRF4-/- mice (n=14)
BW(g)	27.43±0.40	27.62±0.55	28.72±0.48	28.95±0.25
HW/BW(mg/g)	4.14±0.07	4.08±0.08	8.25±0.21*	6.31±0.13*†
LW/BW(mg/g)	4.89±0.01	4.81±0.11	11.47±1.03*	6.92±0.17*†
HW/TL(mg/mm)	6.35±0.10	6.24±0.13	12.93±0.31*	9.99±0.19*†
HR(beats/min)	547±12	536±18	496±19*	498±8
LVEDD(mm)	3.67±0.10	3.55±0.04	5.30±0.15*	4.58±0.05*†
LVESD(mm)	2.02±0.11	2.00±0.05	4.15±0.18*	3.21±0.07*†
FS (%)	45.3±1.7	43.7±1.2	22.0±1.5*	29.9±1.3*†
LVESP (mmHg)	111.76±3.46	110.02±4.37	126.90±8.07	158.66±4.41
LVEF (%)	55.1±2.2	57.4±1.4	29.0±1.5*	45.2±3.8*†
dp/dt max (mmHg/sec)	9780.5±318.4	10216.5±446.8	6150.2±226.8*	8267.0±311.9*†
dp/dt min (mmHg/sec)	-8339.7±320.5	-8864.8±311.2	-4835.2±223.2*	6907.3±439.3*†

LVESP=Left ventricular End-systolic Pressure. LVEF=Left ventricular ejection fraction.

*P<0.05 versus IRF4+/+/ sham operation. †P<0.05 versus IRF4+/+ AB after 8 weeks AB.

All values are presented as means ± SEM.

Table S3 Parameters in IRF4 transgenic mice (TG) and wild type littermates (NTG) at 4 weeks after sham operation or AB.

Parameter	NTG- Sham mice (n=13)	TG -Sham mice (n=13)	NTG-AB mice (n=13)	TG -AB mice (n=11)
BW (g)	27.62±0.33	27.80±0.39	28.03±0.28	26.18±0.70
HW/BW(mg/g)	4.00±0.13	4.19±0.09	6.48±0.11*	8.40±0.35*†
LW/BW(mg/mg)	4.95±0.14	5.05±0.13	7.13±0.10*	10.87±1.30*†
HW/TL(mg/mm)	6.12±0.15	6.46±0.13	10.03±0.19*	11.94±0.37*†
HR (beats/min)	522±36	500±13	491±8	480±10
LVEDD(mm)	3.57±0.06	3.62±0.06	4.61±0.04*	5.00±0.10*†
LVESD(mm)	1.99±0.06	2.07±0.02	3.14±0.02*	3.61±0.08*†
FS (%)	44.3±0.8	42.8±1.1	31.9±0.5*	27.6±1.2*†
LVESP (mmHg)	107.02±1.94	100.97±1.49	171.92±3.85*	122.83±2.51†
LVEF (%)	61.3±0.9	57.3±2.0	37.1±1.5*	26.7±1.4*†
dp/dt max (mmHg/sec)	10370.6±427.0	10698.4±328.0	7825.7±352.5*	5959.4±227.5*†
dp/dt min (mmHg/sec)	-8330.9±273.5	-8956.1±411.7	-6949.7±282.0*	-4534.2±275.6*†

*P<0.05 versus NTG mice after 4 weeks sham operation. †P<0.05 versus NTG mice after 4 weeks AB operation. All values are means ± SEM.

Supplemental Figures

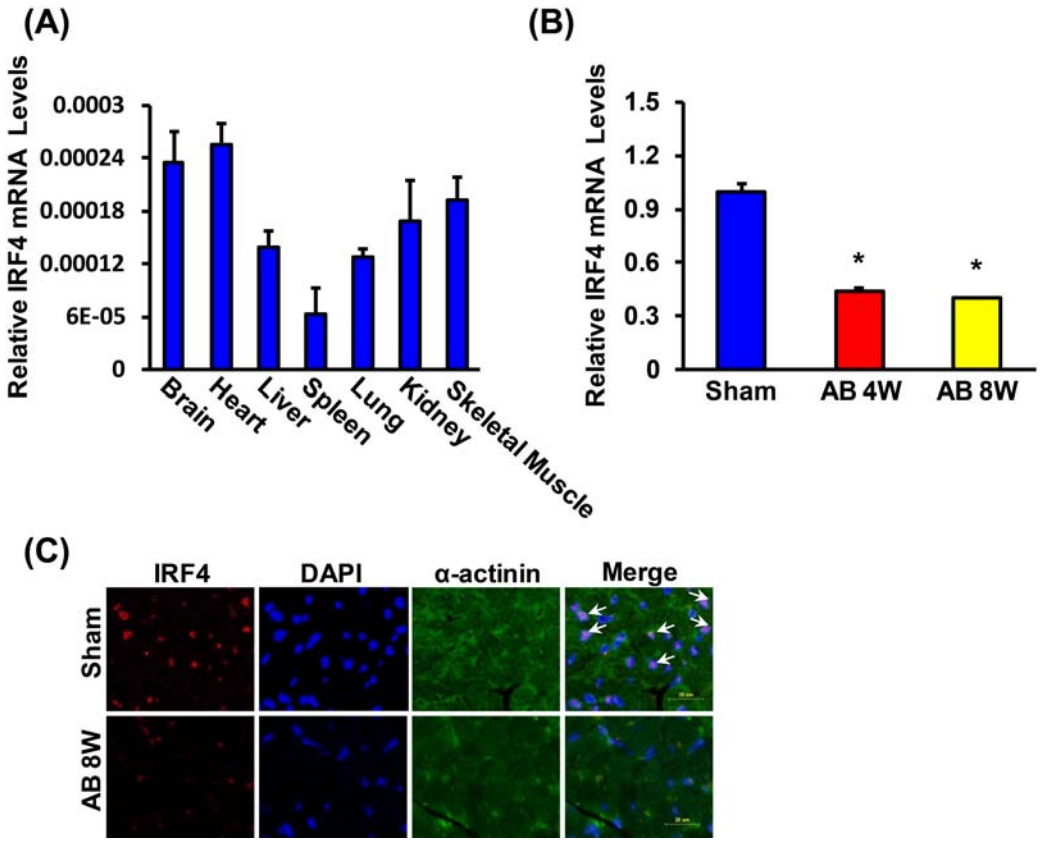

Figure S1 IRF4 is expressed and downregulated in murine hearts upon hypertrophic stimuli. (A) The levels of IRF4 mRNA in different tissues of C57BL/6 mice under normal conditions. (B) The levels of IRF4 mRNA in hearts of C57BL/6 mice after AB or Sham operation. (C) The immunofluorescence results showed that IRF4 was expressed in the nuclei of cardiomyocytes and downregulated in hypertrophic murine hearts (red: IRF4; blue: nucleus; green: α-actinin). **P*<0.05 *vs*. sham

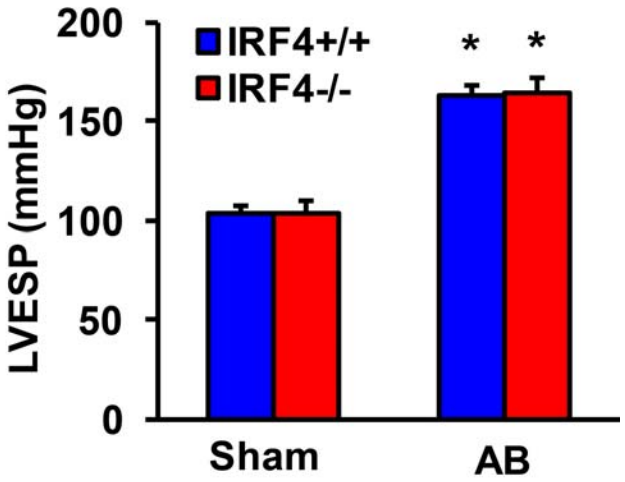

Figure S2 The left ventricular end-systolic pressure (LVESP) in IRF4+/+ and IRF4-/- mice 1 week after AB, and there is no significant difference between these two group neither upon Sham nor AB surgery. *P<0.05 *vs*. sham

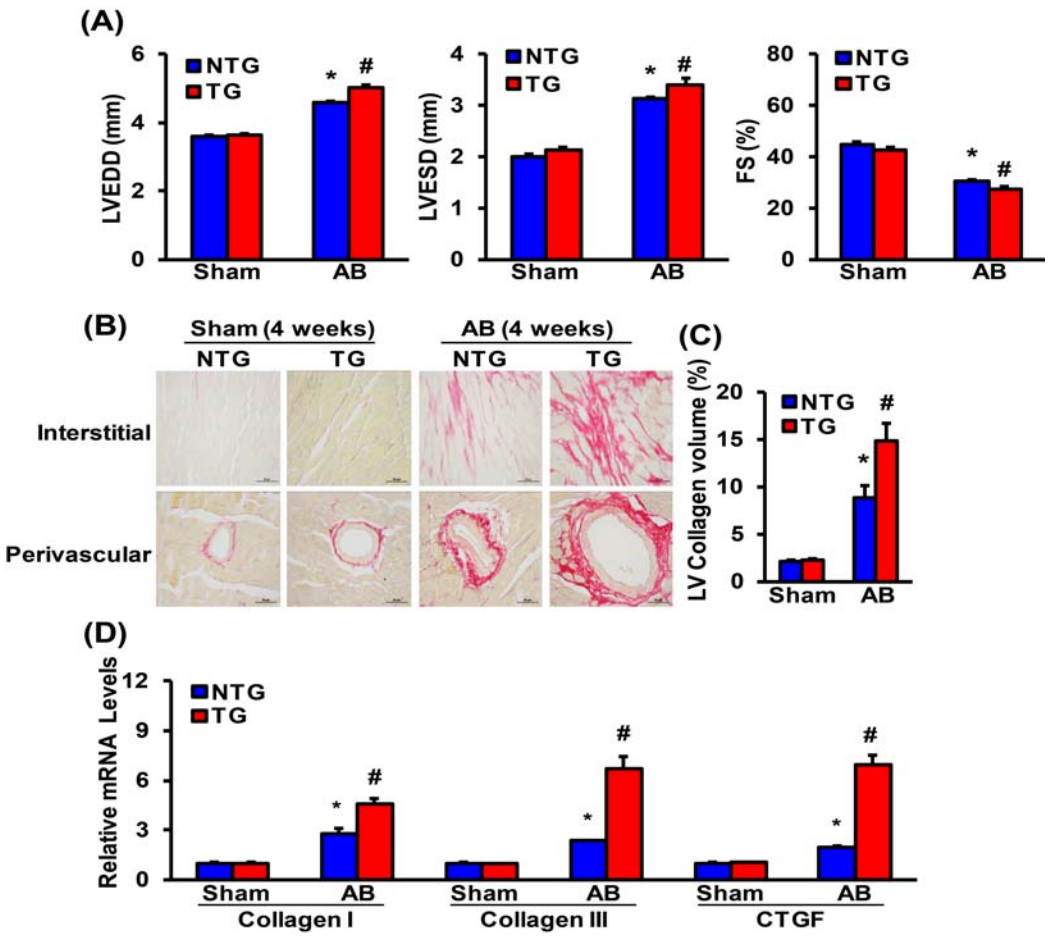

Figure S3 Overexpression of IRF4 aggravates pressure overload-induced cardiac hypertrophy. A, Parameters of echocardiographic results for NTG and IRF4-TG mice upon 4-week aortic banding (AB) or sham operations (n=6-9 mice per experimental group, **P*<0.05 *vs.* NTG/sham; #*P*<0.05 *vs.* NTG/AB). LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; FS=fractional shortening. B, PSR staining of LV sections in the indicated groups upon 4-week AB (scale bar=50μm). C, Quantification of the total collagen volume in AB-treated NTG and IRF4-TG mice (n=25+ fields per experimental group, **P*<0.05 *vs*. NTG/sham; #*P*<0.05 *vs.* NTG/AB). D, Real-time PCR analyses of the fibrotic markers (collagen I, collagen III and CTGF) in the indicated mice. n=4 mice per experimental group, **P*<0.05 *vs*. NTG/sham; #*P*<0.05 *vs.* NTG/AB.

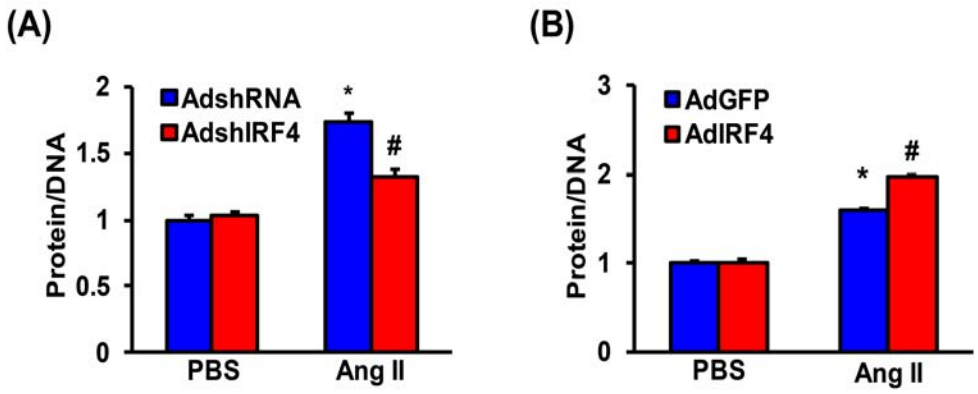

Figure S4 IRF4 regulates angiotensin II-induced cardiomyocyte hypertrophy *ex vivo.* The ratio of protein/DNA was determined in AdshRNA- and AdshIRF4-infected neonatal rat cardiomyocytes (A), as well as in AdGFP- and AdIRF4-myocytes (B) upon the treatment with either PBS or Ang II (1μM) for 48 h.

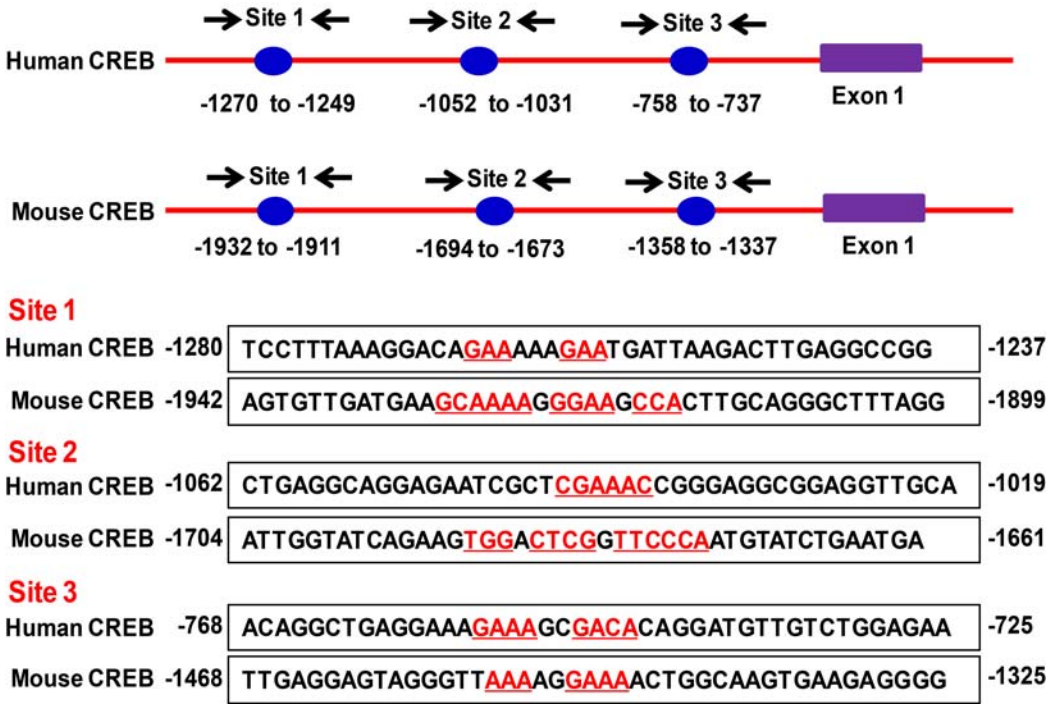

Figure S5 Phylogenetic foot-printing identified three conserved IRF4-binding sites (sites 1, 2 and 3) in the promoter region of human CREB and mouse CREB orthologs.

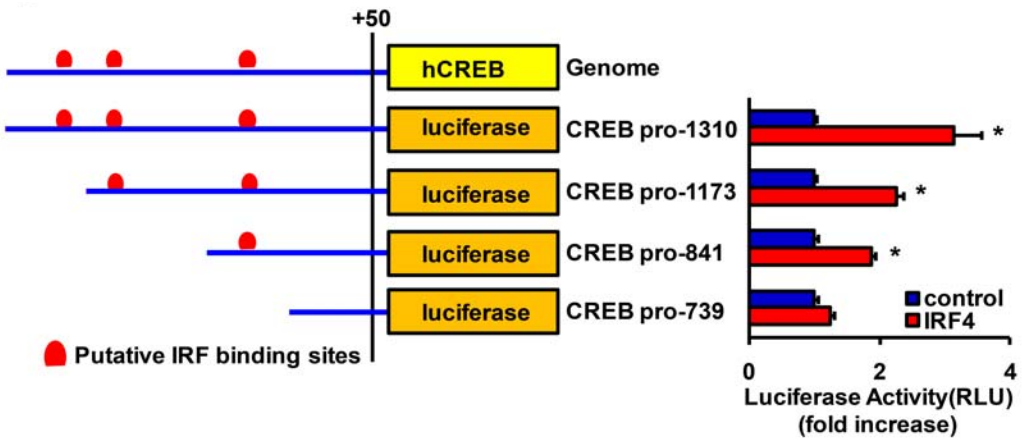

Figure S6 Schematic constructs of various deletions in the human CREB promoter. Putative IRF4 binding sites are indicated and intercepted in order (left). H9c2 cells were co-transfected with each deletion mutant of the CREB promoter and pcDNA3.1-GFP or pcDNA3.1-IRF4. After 24 h, cells were harvested for a luciferase assay. Values of the corresponding luciferase activity are shown for four deletion mutants of the CREB promoter (right). **P*<0.05 *vs*. controls. Similar results were observed in 3 additional independent experiments.

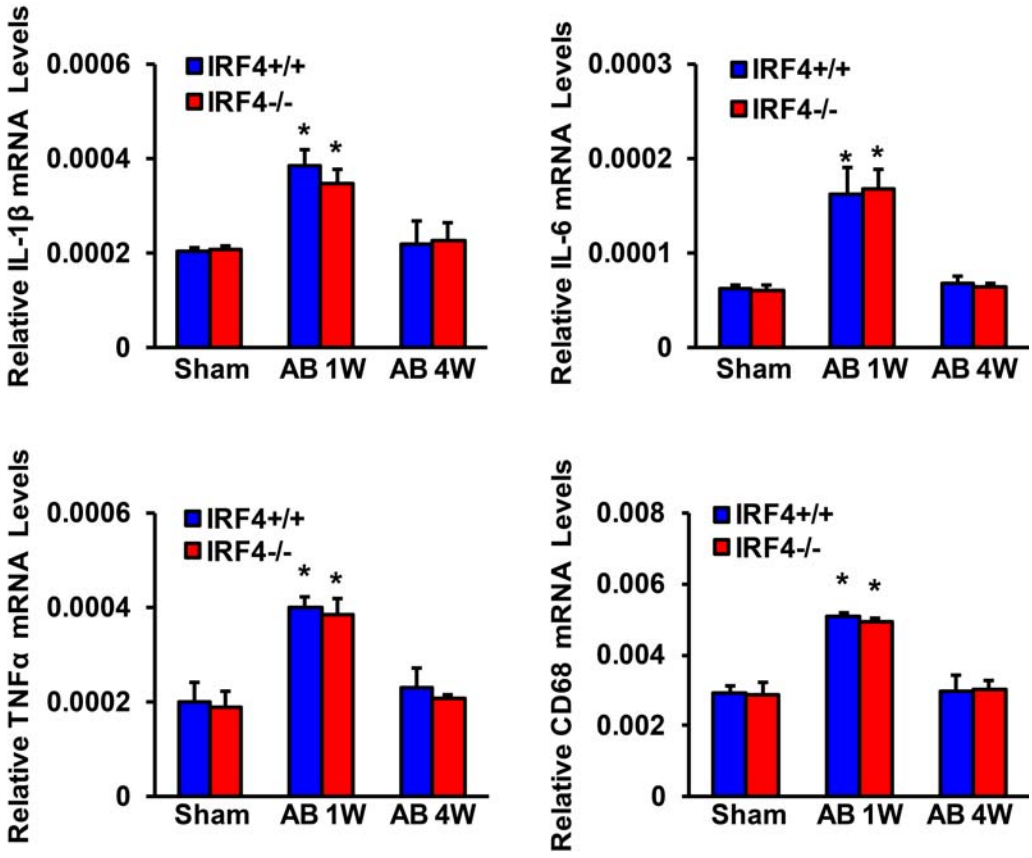

Figure S7 The status of inflammation/ inflammatory cell infiltration was examined in WT vs. IRF4 KO hearts upon 1-week and 4-week AB, as determined by the mRNA levels of IL-1β, IL-6, TNFα, and CD68. The results showed that mRNA levels of IL-1β, IL-6, TNFα, and CD68 were significantly increased at 1 week after AB and decreased to basal levels at 4 weeks after AB, but the status of inflammation/ inflammatory cell infiltration has no significance difference between WT and IRF4 KO hearts upon AB.