Supplement Material

Methods

Primary cultures of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl:(WI) BR-Wistar rats (Charles River Laboratories). A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described [1].

Construction of adenoviruses

Recombinant adenovirus vectors were constructed, propagated, and titered as previously described [2]. Briefly, pBHGloxΔE1,3 Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC316 shuttle vector containing the gene of interest into HEK293 cells using LipofectAMINE 2000 (Life Technologies). Through homologous recombination, the test genes were integrated into the E1-deleted adenoviral genome. The viruses were propagated in HEK293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring myc-MurF1. Adenovirus harboring β-galactosidase (Ad-LacZ) was used as a control.

Construction of short hairpin RNA (shRNA) adenoviral expression vector

The pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). A hairpin-forming oligo corresponding to bases 616-638 (5'-GGAGGAACTGAGCCACAAG<u>TTCAAGAGA</u>CTTGTGGCTCAGT TCCTCCTTTTTTGGAAA-3') of the rat MuRF1 cDNA and its antisense were synthesized with ApaI and HindIII overhangs, annealed, and subcloned distal to the U6 promoter. The loop sequence is underlined. A recombinant adenovirus was made by homologous recombination in HEK293 cells as described above.

Immunoblot analysis

For immunoblot analyses, heart homogenates were prepared in RIPA lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% deoxycholic acid, 10 mM $Na_4P_2O_7$, 5 mM EDTA, 0.1 mM Na_3VO_4, 1 mM NaF, 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.5 mg/ml aprotinin, and 0.5 mg/ml leupeptin. Immunoblots were performed as previously described [3]. The antibodies used include anti-MuRF1 (ECM Biosciences), anti-CnA (BD Biosciences), anti-NFAT1 (Abcam), anti-Histone H3 (Cell Signaling Technologies), anti-creatine kinase (CK)-MB, anti-Troponin-I and anti-ubiquitin (Santa Cruz), anti-β-myosin heavy chain (MYH) and anti-GAPDH (SIGMA) antibodies.

Nuclear and cytosol extract preparation

Cells were washed in PBS and resuspended in hypotonic buffer, containing10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 100 μM Na_3VO_4, 1 mM DTT, and 500 μM PMSF. The cells were then lysed by adding 10% IGEPAL CA-630 and vortexing vigorously. Nuclei were pelleted by centrifugation at 800 X g for 30 min. The supernatant (cytosol) was saved for analysis. The nuclei were then resuspended in hypertonic buffer, containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 100 μM Na_3VO_4, 1 mM DTT, and 500 μM PMSF, and rocked for 30 min on a shaking platform at 4ºC. The samples were centrifuged at 16,000 X g for 5 min, and the supernatant (nuclear extracts) was saved.

Immunoprecipitation

Cardiac myocytes were lysed with IGEPAL CA-630 buffer (50 mM Tris-HCl (pH 7.4), 1% IGEPAL CA-630, 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 μM leupeptin, and 0.1 μM aprotinin). Primary antibody was covalently immobilized on protein A/G agarose using the

Pierce® Crosslink Immunoprecipitation Kit according to the manufacturer's instructions (Thermo Scientific). Samples were incubated with immobilized antibody beads for at least 2 h at 4°C. After immunoprecipitation, the samples were washed with TBS five times. They were then eluted with glycine-HCl (0.1 M, pH 3.5), and the immunoprecipitates were subjected to immunoblotting using specific primary antibodies and a conformation-specific secondary antibody that recognizes only the native IgG (Cell Signaling).

Luciferase assay

Hearts were harvested and weighed, and equal amounts of the heart homogenates were subjected to luciferase assays using a luciferase assay system (Promega) [4].

Calcineurin activity assay

Phosphatase activity was measured by using a calcineurin assay kit (Enzo Life Sciences) according to the manufacturer's instructions. Heart homogenates from Tg-ZAKI, *Murf1*$^{-/-}$, *Murf1*$^{-/-}$-Tg-ZAKI and NTg mice were prepared, and calcineurin activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide) in the presence or absence of EGTA. The amount of PO_4 release was determined photometrically using the Biomol Green reagent (Enzo Life Sciences). The activity of calcineurin in each sample was normalized to the amount of total protein in the sample.

Calcineurin turnover assay

Cycloheximide (CHX) chase for evaluating calcineurin turnover was performed as described previously with modification [5]. Briefly, cultured cardiomyocytes were transduced with either Ad-sh-MuRF1 or Ad-sh-scramble. Ninety-six hours following transduction, cells were incubated with 50 ng/ml CHX for the indicated periods of time, after which lysates were immediately collected into lysis buffer.

Transverse aortic constriction (TAC)

The method used to impose PO on mice has been described [6]. As controls, sham operations were performed without constricting the aorta. After 2 or 4 weeks, echocardiography and hemodynamic measurements were performed as described [7].

Echocardiography

Mice were anesthetized with 12 μl/g of body weight of 2.5% Avertin (Sigma), and echocardiography was performed with ultrasonography (Acuson Sequoia C256, Siemens Medical Solutions) as previously described [8].

Histological analyses

Heart specimens were fixed with formalin, embedded in paraffin, and sectioned into 6 mm-thick slices. Interstitial fibrosis was evaluated by Masson's Trichrome staining [9]. Myocyte cross-sectional area was measured from images taken of wheat germ agglutinin (WGA)-stained 1 mm-thick sections [9]. The outline of 100-200 myocytes was traced in each section, using the MetaMorph image system. DNA fragmentation was detected *in situ* using TUNEL staining [10].

Immunofluorescence microscopy

Cardiomyocytes were fixed with 3.7% paraformaldehyde in PBS for 10 min and incubated with PBS containing 0.5% Triton X-100 for 15 min. After being washed with PBS, the cells were first incubated with primary antibodies in PBS, and then incubated with secondary antibodies in PBS.

Analysis of messenger RNA (mRNA) expression

Total RNA from the heart was extracted using TRIzol (Life Technologies) and first-strand cDNA was generated using a first-strand cDNA synthesis kit (GE Healthcare Biosciences) [11]. Real-time PCR was carried out using specific primers as described [11].

References

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Online Table 1

Postmortem pathologic measurements in control and *Murf1*$^{-/-}$ mice 2 weeks after TAC

	WT		***Murf1*$^{-/-}$**	
	Sham	**TAC**	**Sham**	**TAC**
N	7	6	6	8
BW (g)	25.6±1.1	24.8±0.7	25.7±1.1	25.9±0.7
TL(mm)	18.0±0.2	17.9±0.3	18.3±0.2	18.1±0.2
LV (mg)	88.3±2.7	126.7±8.7[A]	94.7±4.8	158.5±6.2[A,B]
RV (mg)	18.6±1.4	22.3±1.7	20.7±2.0	23.9±2.0
LV weight/BW (mg/g)	3.47±0.11	5.08±0.27[A]	3.69±0.11	6.13±0.19 [A,B]
Lung weight/BW (mg/g)	5.24±0.15	6.30±0.87	5.15±0.17	6.86±0.69
Liver weight/BW (mg/g)	45.0±4.5	40.8±1.9	44.9±2.6	42.6±1.0
LV weight/TL (mg/mm)	4.99±0.15	7.05±0.41[A]	5.19±0.25	8.73±0.27 [A,B]
Lung weight/TL (mg/mm)	7.31±0.09	8.70±1.17	7.23±0.34	9.79±1.05
Liver weight/TL (mg/mm)	62.7±5.8	56.4±2.8	63.5±5.6	60.8±2.4

Data are mean ± SEM. [A]$P < 0.01$ compared with same genotype sham mice. [B]$P < 0.05$ compared with WT 2 weeks after TAC.

Online Table 2

Postmortem pathologic measurements in control and *Murf1*$^{-/-}$ mice 4 weeks after TAC

	WT		***Murf1*$^{-/-}$**	
	Sham	**TAC**	**Sham**	**TAC**
N	7	5	5	5
BW (g)	26.4±1.1	28.1±1.1	25.8±1.3	29.8±0.6
TL(mm)	18.0±0.2	17.6±0.2	18.3±0.1	17.4±0.2
LV (mg)	88.3±2.7	155.0±12.7[A]	90.5±4.4	205.0±11.1[A,C]
RV (mg)	18.6±1.4	21.4±1.7 [A]	20.0±1.8	31.0±3.4 [A]
LV weight/BW (mg/g)	3.58±0.15	5.50±0.30 [A]	3.52±0.10	6.89±0.40 [A,C]
Lung weight/BW (mg/g)	5.24±0.15	6.48±0.49 [A]	4.96±0.21	9.55±1.49 [A]
Liver weight/BW (mg/g)	45.0±4.5	39.3±1.8	41.0±0.9	45.5±1.5
LV weight/TL (mg/mm)	4.99±0.15	8.88±0.64 [A]	5.11±0.30	11.9±0.63 [A,C]
Lung weight/TL (mg/mm)	7.31±0.09	10.4±0.51 [A]	7.21±0.50	16.6±2.70 [A,C]
Liver weight/TL (mg/mm)	62.7±5.8	63.5±4.2	59.7±4.2	78.9±3.4

Data are mean ± SEM. [A]$P < 0.01$ compared with same genotype sham mice. [C]$P < 0.01$ compared with WT 4 weeks after TAC.

Online Table 3

Echocardiographic analyses in control and *Murf1*$^{-/-}$ mice 2 weeks after TAC

	WT		***Murf1*$^{-/-}$**	
	Sham	**TAC**	**Sham**	**TAC**
N	7	6	6	8
DSEPWT (mm)	0.69±0.02	0.97±0.04^{B}	0.75±0.03	1.17±0.08^{B,C}
LVEDD (mm)	4.13±0.13	3.99±0.12	4.15±0.12	4.10±0.11
DPWT (mm)	0.64±0.03	0.92±0.04^{B}	0.72±0.03	1.11±0.06^{B,C}
LVESD (mm)	2.97±0.11	2.88±0.11	2.98±0.11	3.13±0.13
EF (%)	62.9±1.8	57±3	63.06±2.02	52.6±2.3^{A}
FS (%)	28.2±1.1	27.8±1.0	28.4±1.1	21.8±1.9^{A}
HR (bpm)	412±19	411±13	406±13	395±10

Data are mean ± SEM. $^{A}P < 0.05$, $^{B}P < 0.01$ compared with same genotype sham mice. $^{C}P < 0.01$ compared with WT 2 weeks after TAC.

DSEPWT: diastolic septal wall thickness; LVEDD: left ventricular end-diastolic dimension; DPWT: diastolic posterior wall thickness; LVESD: left ventricular end-systolic dimension; EF: ejection fraction; FS: fractional shortening; HR: heart rate.

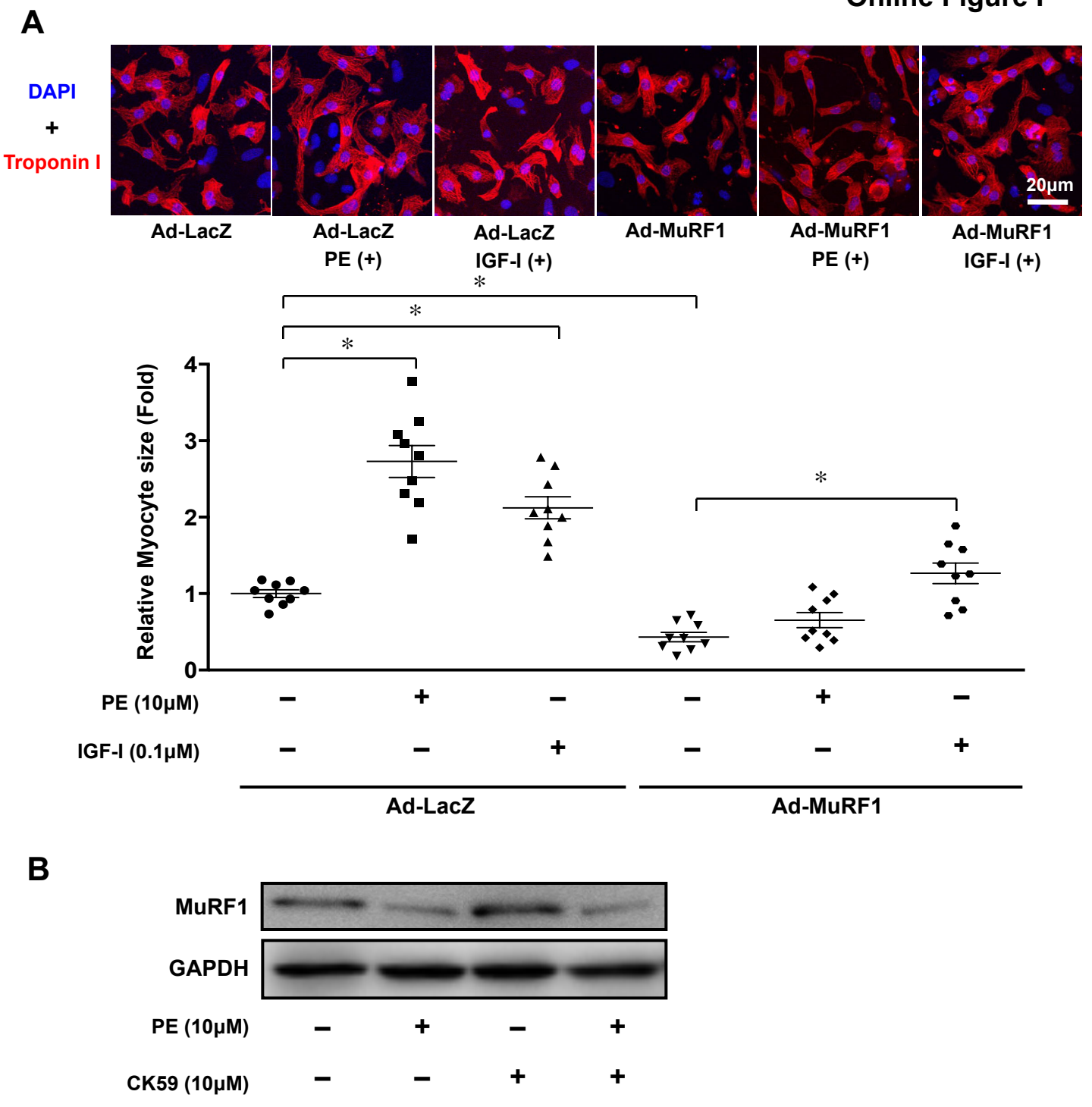
Online Table 4

Echocardiographic analyses in control and *Murf1*$^{-/-}$ mice 4 weeks after TAC

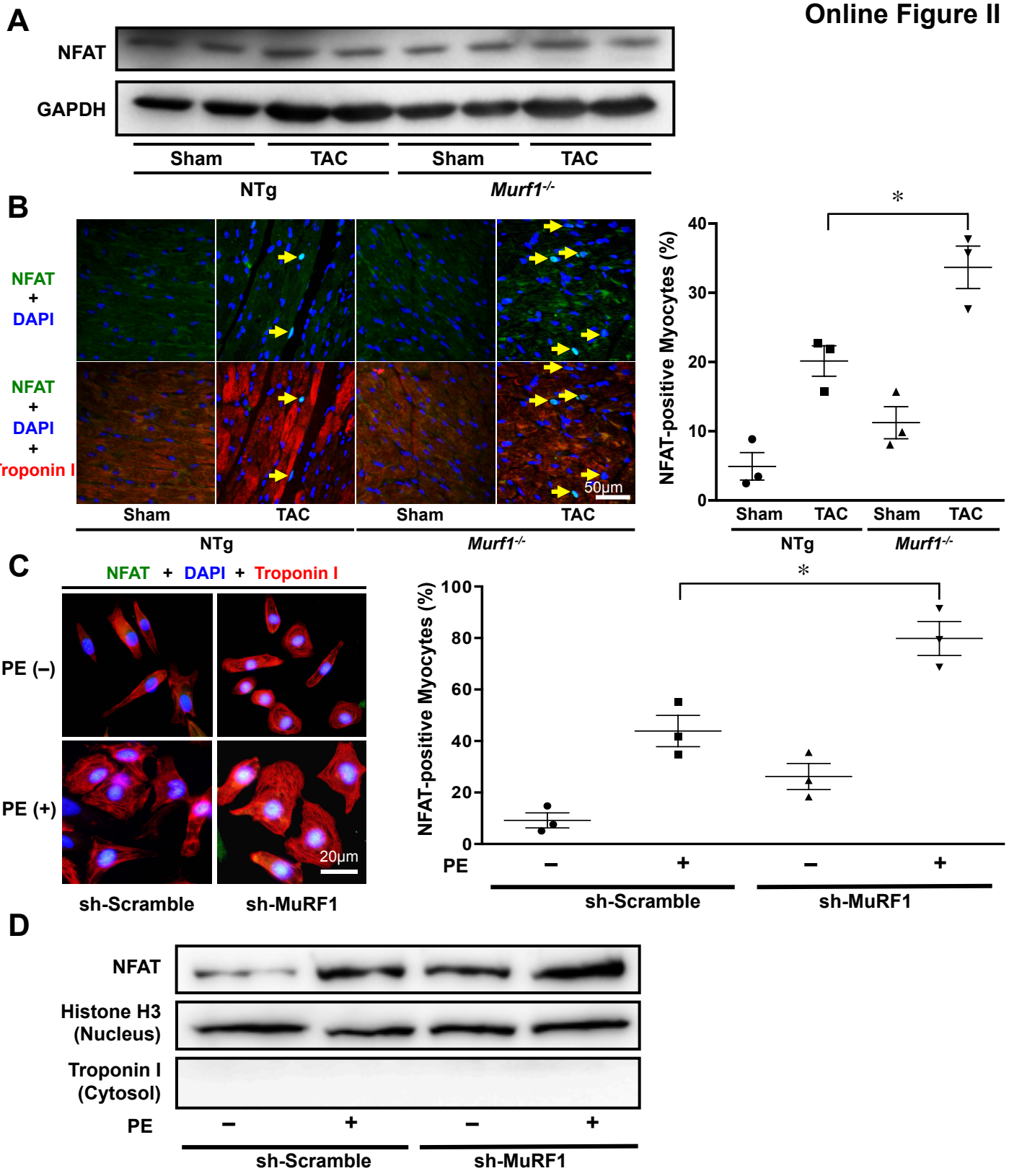
	WT		***Murf1*$^{-/-}$**	
	Sham	**TAC**	**Sham**	**TAC**
N	7	5	5	5
DSEPWT (mm)	0.69±0.02	1.23±0.06^{A}	0.74±0.04	1.25±0.05^{A}
LVEDD (mm)	4.13±0.13	3.95±0.12	4.02±0.16	4.16±0.15
DPWT (mm)	0.64±0.03	1.15±0.08^{A}	0.72±0.04	1.20±0.07^{A}
LVESD (mm)	2.97±0.11	2.89±0.11	2.81±0.14	3.21±0.07
EF (%)	62.87±1.76	50.51±2.66	65.70±1.49	40.25±2.36 ^{A,B}
FS (%)	28.23±1.12	26.83±1.47	30.04±1.00	16.67±1.68 ^{A,B}
HR (bpm)	412±19	405±14	413±15	432±18

Data are mean ± SEM. $^{A}P < 0.01$ compared with sham mice of the same genotype. $^{B}P < 0.05$ compared with WT 4 weeks after TAC.

DSEPWT: diastolic septal wall thickness; LVEDD: left ventricular end-diastolic dimension; DPWT: diastolic posterior wall thickness; LVESD: left ventricular end-systolic dimension; EF: ejection fraction; FS: fractional shortening; HR: heart rate.



Online Figure I. A, *Upper:* Representative images of neonatal rat cultured cardiomyocytes treated with PE or IGF-I and the indicated adenoviruses. The cells were observed under a fluorescent microscope. *Lower:* Relative cell size of myocytes treated with the indicated adenoviruses in the presence or absence of PE or IGF-I for 48 hours was examined * $P<0.05$ ($N=9$). **B,** Cultured cardiomyocytes were treated with CK59, a CaMKII inhibitor, and then harvested after 48 hr. Immunoblot analysis was performed using anti-MuRF1 antibody.



Online Figure II. A, Expression of NFAT in mouse hearts in response to TAC (4 weeks) was determined by immunoblotting. **B,** *Left:* Immunohistochemistry of NFAT. Arrows indicate nuclear localization of NFAT. *Right:* NFAT-positive myocytes were counted. * $P<0.05$ ($N=3$). **C,** *Left:* The extent of nuclear expression of NFAT was determined by triple immunostaining with anti-NFAT antibody (green), anti-troponin I antibody (red) and DAPI (blue). *Right:* Nuclear NFAT-positive myocytes were counted. * $P<0.05$ ($N=3$). **D,** Cytosolic and nuclear fractions were prepared from Ad-shScramble- and Ad-shMuRF1-transduced cardiomyocytes. Immunoblot analyses were conducted with NFAT antibody. The purity of the nuclear fraction was confirmed by the lack of Troponin I. Representative immunoblot images are shown.