SUPPLEMENTAL MATERIAL

Supplemental Mehtods

Mice

All experimental procedures were performed according to the guidelines established by Chiba University for experiments in animals and all protocols were approved by our institutional review board. We generated transgenic mice (mActin-Tg) that expressed a mutated cardiac α-actin (R312H) with HA tag in the heart. This mutation has been reported in patients with DCM.[1] Wild-type cDNA for murine cardiac α-actin was obtained by reverse transcription-polymerase chain reaction (PCR) using total RNA isolated from murine cardiac ventricle as template. The wild-type cDNA was subcloned into pGEM T-easy vector (Promega, Madison, WI) and sequenced. Mutated cardiac α-actin (R312H) was created by substituting histidine for arginine using site-directed mutagenesis kit (STRATAGENE, La Jolla, CA). A HA tag was inserted at the amino terminus just distal to the translational start site of the mutated cardiac α-actin cDNA. The mutated cDNA was subcloned into murine α-myosin heavy chain

(MHC) promoter-containing expression vector.[2] The linearized DNA was injected pronuclei of eggs from C57BL/6 mice, and the eggs were transferred into the oviducts of pseudopregnant ICR mice. The transgene was identified by PCR with transgene-specific primers and by Southern blot analyses. Founder mice were bred with C57BL/6 wild-type mice. We also generated transgenic mice that expressed mutated cardiac α-actin (A331P) with HA tag in the heart. This mutation has been reported in patients with hypertrophic cardiomyopathy.[3] Generation and genotyping of transgenic mice with cardiac-restricted overexpression of human Bcl-2, AC3-I or nuclear factor of activated T cells (NFAT)-luciferase have been previously described.[4-6] Heterozygous p53 deficient mice were purchased from the Jackson Laboratory (Maine, ME).[7] The strain of mActin-Tg, AC3-I transgenic and p53 deficient mice was C57BL/6 background, and the strain of Bcl-2 transgenic and NFAT-luciferase reporter mice was mix-background between FVBN and C57BL/6. The wild-type littermates were served as controls for all studies. To inhibit activation of CaMKII, KN-93 (10μmol/kg/day, D. Western Therapeutics Institute, Nagoya, Japan) was continuously administered by osmotic minipump (DURECT, Cupertino, CA) beginning at 2 months of age until 5

months of age. KN-92 (10μmol/kg/day, D. Western Therapeutics Institute) was used as a control. Bisoprolol (100mg/kg/day, Mitsubishi Tanabe Pharma Corp., Osaka, Japan) was continuously administered from 2 months of age to 5 months of age.

Echocardiography, Blood Pressure Measurements and Electrocardiography

Cardiac function was examined by echocardiogram (Vevo 660, VISUAL SONICS, Ontario, Canada) provided with a 25-MHz imaging transducer. Blood pressure was measured with an indirect tail-cuff method (BP-98A, Softron, Tokyo, Japan). Surface electrocardiography (ECG) was recorded from subcutaneous needle electrodes attached to each limb using the Powerlab acquisition system (AD Instruments, New South Wales, Australia). All recordings were performed on conscious mice.

Histology

Hearts fixed in 10% formalin were embedded in paraffin, sectioned at 4μm thickness, and stained with Masson trichrome. For electron microscopic analyses, hearts were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde, postfixed in 1%

osmium tetroxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. For detection of apoptotic cardiomyocytes, we performed TUNEL staining (Takara, Shiga, Japan) along with immunostaining for dystrophin (Novocastra Laboratories, Newcastle, UK). We counted the numbers of TUNEL/ dystrophin-double positive cardiomyocytes and hematoxylin-stained nuclei in a whole section of each samples. For immunohistological analyses, 6μm cryostat sections of fresh-frozen hearts were prepared. Anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-α actinin (Sigma, Saint Louis, MO) primary antibodies were applied overnight at 4°C. Alexa Fluor 488-or 555-conjugated secondary antibodies were applied to visualize expression of specific proteins (Invitrogen, Carlsbad, CA). Nuclear staining was performed with TOPRO-3 (Invitrogen). Confocal images were acquired at room temperature using a microscope (Radiance 2000; Bio-Rad Laboratories, Hercules, CA) and Laser Sharp 2000 confocal software (Bio-Rad Laboratories).

Western Blot Analysis

Whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose transfer membrane (Whatman, Dassel, Germany). Western blot analyses were performed with antibodies against Ca^{2+}/calmodulin-dependent kinase IIδ (CaMKIIδ), Bax, HA, phospho- phospholamban (PLB) (Thr17), GAPDH (Santa Cruz Biotechnology), p53, caspase-12, phospho-CaMKII (Cell Signaling Technology, Danvers, MA), phospho-PLB (Ser16), PLB (Upstate Biotechnology, Temecula, CA), myc (Invitrogen), talin, or actin (Sigma). Hybridizing bands were visualized using an ECL detection kit (GE Healthcare, Buckinghamshire, UK).

Luciferase assay

Left ventricles were homogenized in luciferase assay buffer, and the homogenates were assayed for luciferease activity as described previously.[5]

Force measurements

A small fiber was dissected from the skinned left ventricular papillary muscle of

wild-type littermates and mActin-Tg mice, and isometric force was measured as described previously.[8]

RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was isolated from the heart with RNAZol-B (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. cDNA synthesis of 1μg of RNA was carried out by using QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). Quantitative real time (RT)-PCR was performed by using the LightCycler with Taqman Universal Probe Library and the Light Cycler Master (Roche, Basel, Switzerland).

Calcium Transient

The hearts were quickly excised and perfused by Langendorff's method at 37°C for 5 min with a calcium (Ca^{2+})-free solution containing 126 mM NaCl, 22 mM dextrose, 5 mM $MgCl_2$, 4.4 mM KCl, 20 mM taurine, 5 mM creatine, 5 mM Na-pyruvate, 1 mM NaH_2PO_4, 24 mM HEPES, pH 7.4. Then they were digested for 15 min by perfusion

with an enzyme solution containing 1.0 mg/ml collagenase type 2 (Worthington Biochemical, Lakewood, NJ), 0.1 mg/ml protease (Sigma), and 0.1 mM $CaCl_2$ added to the Ca^{2+}-free solution. Finally, the enzyme was washed out for 5 min by perfusion with a solution containing 0.1 mM $CaCl_2$ added to the Ca^{2+}-free solution. Ventricles were then cut out, minced and shaken gently at 37°C for 10 min. The dissociated cardiomyocytes were filtered and kept at room temperature in a solution containing 0.5 mM $CaCl_2$ added to the Ca^{2+}-free solution. Cardiomyocytes were loaded with 30 μM fluo-3 AM (Invitrogen) for 30 min. We placed them in a laminin-coated chamber perfused with a bath solution containing 138 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 4.4 mM KCl, 11 mM Dextrose, 24 mM HEPES, pH 7.4. We left them in the chamber for 10 min to allow them to attach to the bottom and permit deesterification of fluo-3 AM. We obtained fluorescent images with a Leica TCL SL laser-scanning confocal system with a DM IRE2 inverted microscope and a 63× oil immersion objective lens. Intracellular fluo-3 was excited at 488 nm with a krypton/ argon laser and the spectral component of the emission between 500 and 650 nm was measured with a photomultiplier tube. Cardiomyocytes were placed with their long axis within ± 10

degrees along the longitudinal axis of the imaging window. All images were acquired in XT mode with 0.2 μm and 2.5 ms per pixel resolution and with 512 × 512 pixels in size. We stimulated cardiomyocytes with a pair of platinum electrodes with 4-ms pulses at 2× threshold voltage at 0.5 Hz. After conditioning stimulation for more than 2 min, we recorded 10 synchronized images with stimulation (images of stimulation). We stopped stimulation and one minute later we recorded consecutive 10 images without stimulation (images at rest). All the experiments were performed at room temperature.

Cell Culture.

Cardiomyocytes were prepared from ventricles of one-day-old Wistar rats and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 ºC in a mixture of 95% air and 5% CO_2. Cardiomyocytes were transfected with an expression plasmid encoding Myc-tagged constitutively active form of CaMKIIδ[9] using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and were cultured for 5 days. Cardiomyocytes were treated with MG132 (100μM, Calbiochem,

La Jolla, CA) and were incubated for 24 hours before harvest.

Supplemental Table 1. Summary of Echocardiography in Cardiac α-Actin A331P Mutant Transgenic Mice

	2 months		5 months		10 months	
	WT	A331P	WT	A331P	WT	A331P
n	6	5	5	10	8	6
LVDd (mm)	3.28±0.08	3.39±0.18	3.40±0.10	3.32±0.11	3.72±0.23	3.63±0.18
LVDs (mm)	1.87±0.22	1.68±0.16	1.58±0.03	1.56±0.14	1.81±0.21	1.88±0.20
Wall thickness (mm)	0.68±0.07	0.66±0.04	0.72±0.10	0.71±0.05	0.88±0.05	0.86±0.07
FS (%)	48.35±3.33	50.70±3.16	53.47±1.75	54.05±2.91	50.11±2.35	47.15±2.66

Data are shown the mean±SE. Multiple group comparison was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic

dimension; FS, fractional shortening; WT, wild-type littermates; A331P, cardiac α-actin A331P mutant transgenic mice.

Supplemental Table 2. Summary of Echocardiography, Blood Pressure, and ECG Date

	2 months		5 months		10 months	
	WT	Tg	WT	Tg	WT	Tg
n	14	7	7	5	5	5
BW (g)	24.37±0.63	23.59±0.52	27.80±0.14	30.23±1.20	34.83±2.36	31.43±0.80
HW (mg)	117.1±4.9	113.6±3.1	128.4±3.6	150.0±9.5	150.7±6.0	172.7±24.0*
HW/ BW (mg/g)	4.80±0.13	4.81±0.06	4.66±0.17	4.98±0.36	4.43±0.33	5.46±0.67*†
Echo, n	11	12	10	13	6	10

LVDd (mm)	2.95±0.10	3.21±0.14	3.41±0.10	3.84±0.07*	3.89±0.14	4.50±0.25*
LVDs (mm)	1.67±0.07	2.07±0.12	1.98±0.09	2.76±0.11*	2.44±0.14	3.68±0.32*
Wall thickness (mm)	0.62±0.02	0.58±0.02	0.79±0.03	0.71±0.03	0.88±0.04	0.71±0.04*
FS (%)	43.01±1.29	35.88±1.47*	42.06±1.26	28.26±1.86*†	37.57±1.79	19.70±3.07*†‡
Blood Pressure, n	10	11	10	8	6	6
HR (bpm)	587±25	568±22	602±19	631±9	617±12	710±9*†
SBP (mmHg)	104±4	101±3	106±4	100±3	99±2	96±3
DBP (mmHg)	75±4	71±2	63±3	67±3	66±4	66±2

ECG, n	6	7	7	7	6	8
HR (bpm)	654±20	718±11	589±23	639±17	637±17	709±13*
P interval (msec)	18.30±0.64	17.83±0.88	20.67±0.82	19.42±0.35	18.92±0.49	18.86±0.62
PR interval (msec)	41.40±3.02	34.77±1.10	48.33±2.56	40.50±1.94	41.33±2.19	36.07±2.14
QRS interval (msec)	13.44±0.81	14.80±0.91	13.75±1.66	16.17±1.01	16.50±1.48	15.79±1.24
R amplitude (mV)	1.17±0.09	1.01±0.11	1.10±0.16	0.66±0.03*†	0.99±0.06	0.65±0.03*†

Data are shown the mean±SE. Multiple group comparison was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. BW, body weight; HW, heart weight; HW/ BW, HW to BW ratio; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; FS, fractional shortening; HR, heart rate; SBP systolic blood pressure; DBP, diastolic blood pressure; WT, wild-type littermates; Tg, mActin-Tg. * $P< 0.05$ versus WT at same age.

† *P<0.05* versus Tg at 2 months of age. ‡ *P<0.05* versus Tg at 5 months of age.

Supplemental Table 3. Ca^{2+} Release

	No Spark	Ca^{2+} sparks	Ca^{2+} waves
WT	28	8	0
Tg	15	12	3

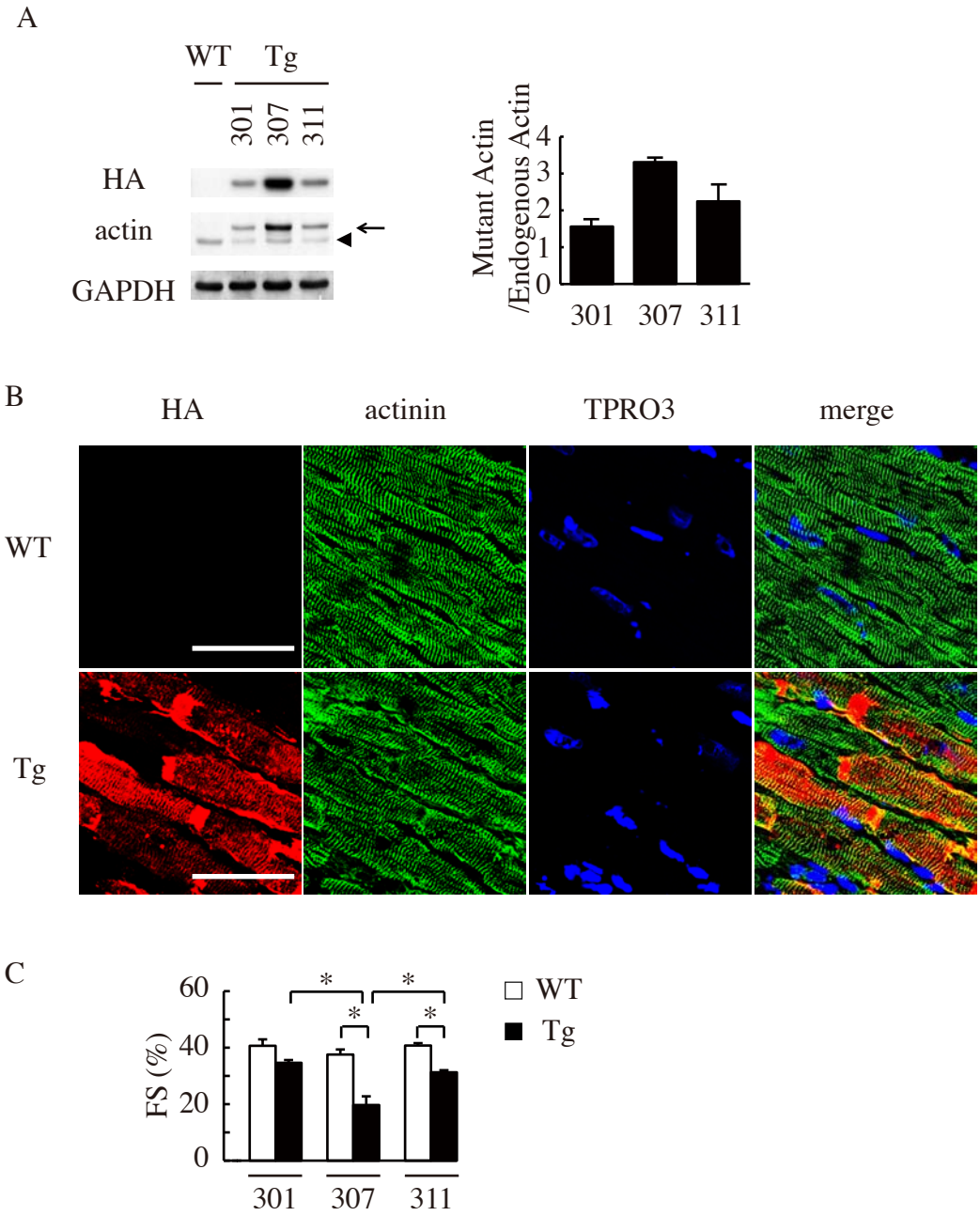
Statistical analysis was performed by Chi-square test. WT, wild-type littermates; Tg, mActin-Tg. $P<0.05$, WT versus Tg.

Supplemental Table 4. Raw Data

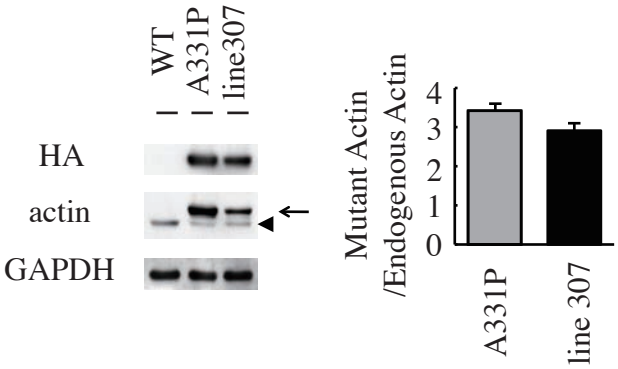
WT (RLU/μg)	4863 ± 860 (4113, 7321, 4645, 3374)
Tg (RLU/μg)	13860 ± 922 (11478, 15837, 13541, 14583)

The mean ± SEM (raw data) of The NFAT-luciferase reporter activity in the hearts at 5 months of age. Statistical analysis was performed by Student's t-test test. WT, wild-type littermates; Tg, mActin-Tg. $P<0.05$, WT mice versus Tg mice.

Supplemental Figure 1



Supplemental Figure 2



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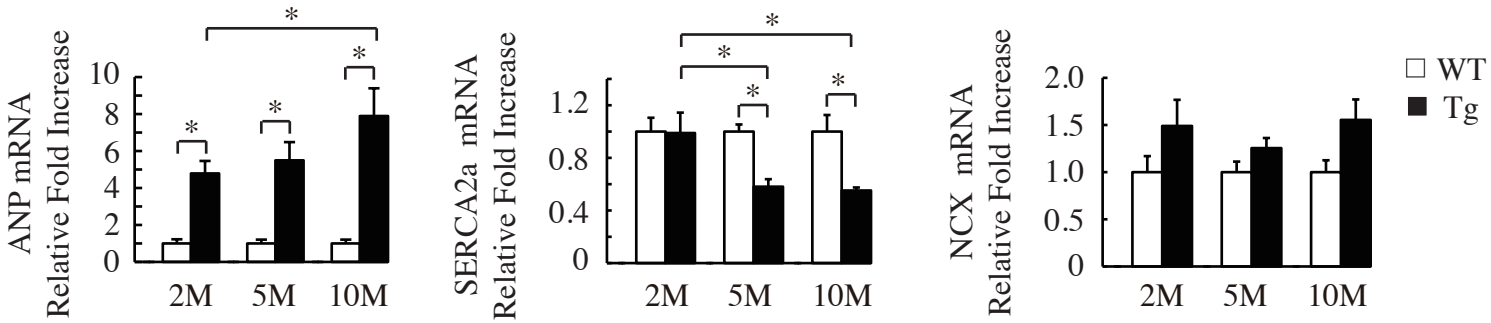

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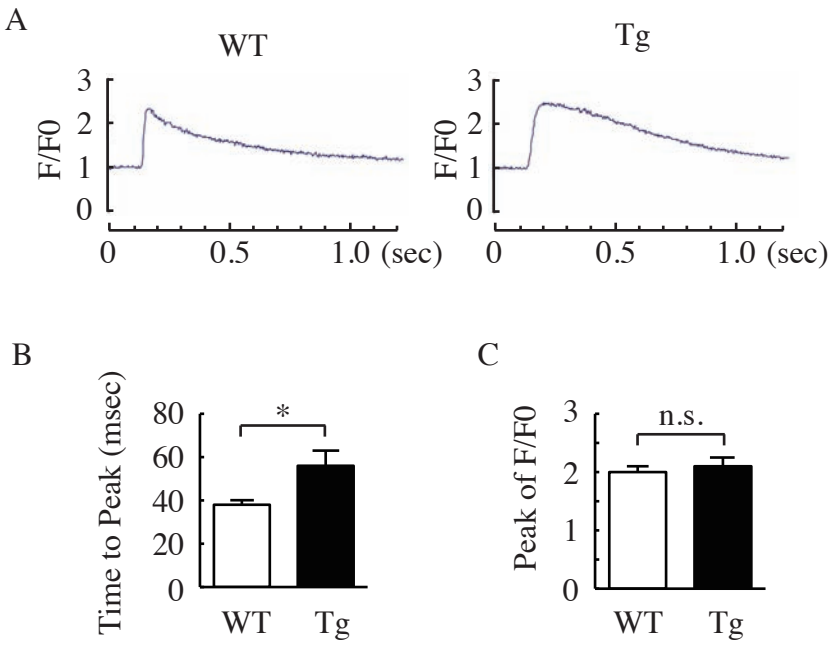

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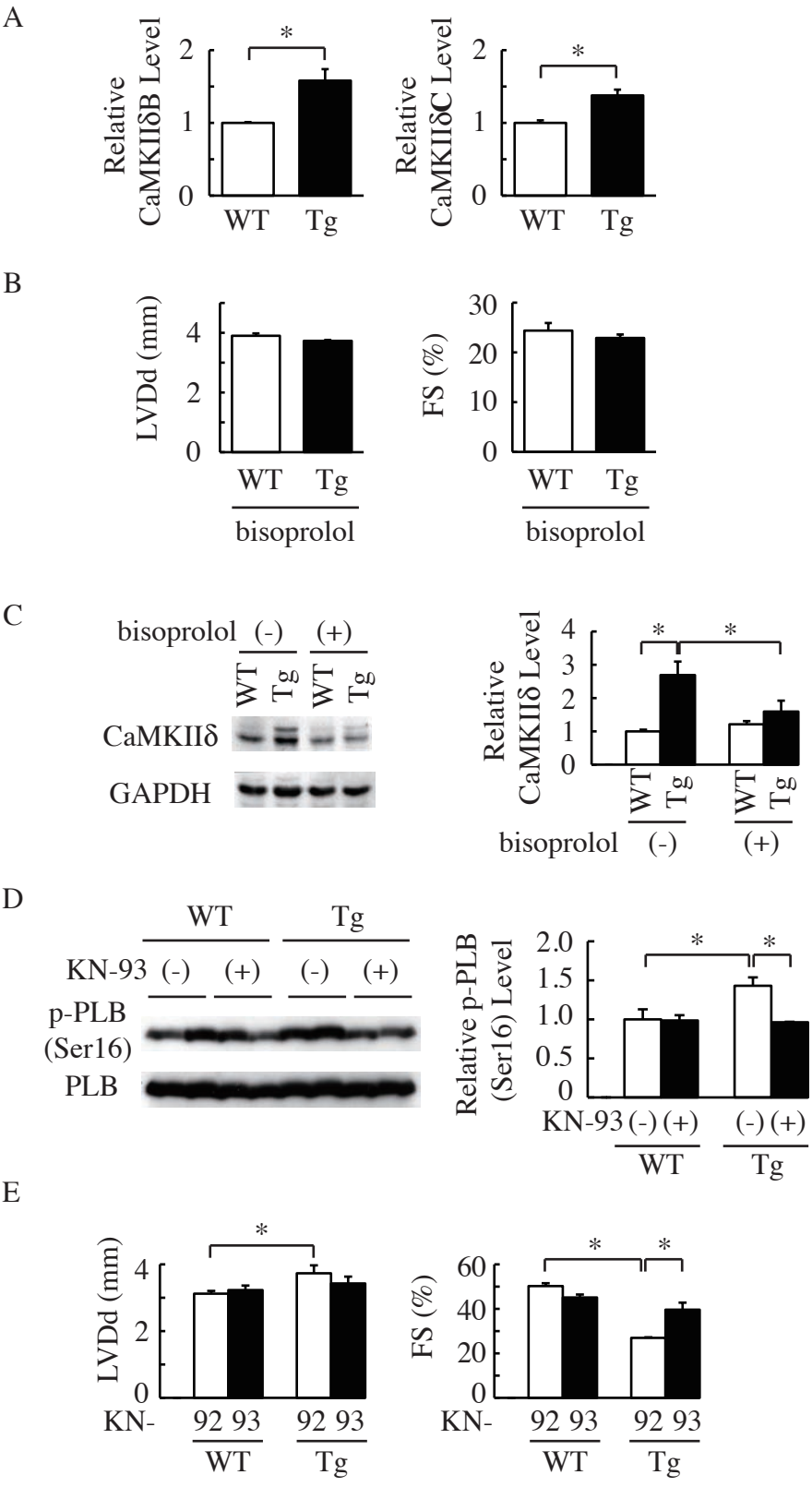
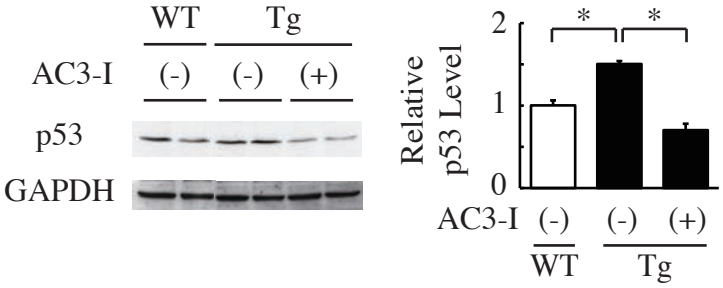


Supplemental Figure 6

A



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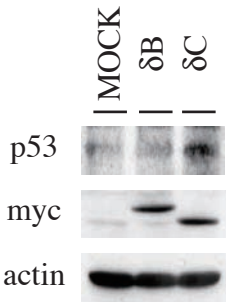

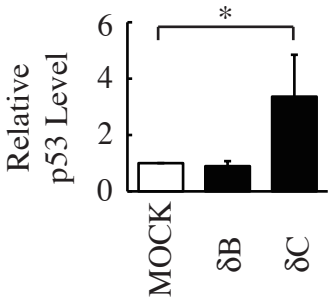

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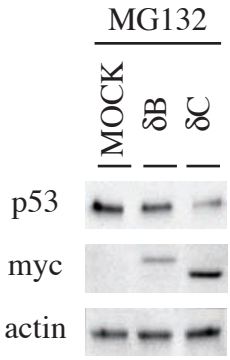

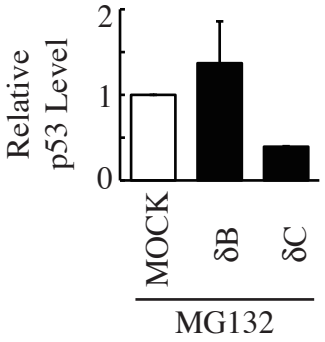

Supplemental Figure Legends

Figure 1 Mutated cardiac α-actin R312H transgenic mice. A, Western blot analyses in the hearts of wild-type littermates (WT) or three independent founders of mActin-Tg (Tg) mice. Arrow and arrowhead indicate exogenous and endogenous cardiac α-actin, respectively. The graph indicates quantitative analyses of the ratio of the mutant actin to endogenous actin proteins. n=3 in each group. B, Immunohistochemistry for HA (red) or actinin (green) in the hearts of WT and mActin-Tg (line 307) mice. Nuclei were stained with TPRO3 (blue). Scale bar, 50μm. C, Echocardiographic analyses at 10 months of age. *P< 0.05. n=4 in each group.

Figure 2 Mutated cardiac α-actin A331P transgenic mice. Western blot analyses in the hearts of wild-type littermates (WT), mutated cardiac α-actin A331P transgenic (A331P) or line 307 of mActin-Tg (line 307) mice. Arrow and arrowhead indicate exogenous and endogenous cardiac α-actin, respectively. The graph indicates quantitative analyses of the ratio of the mutant actin to endogenous actin proteins. n=3 in each group.

Figure 3 The gene expression levels in mActin-Tg mice. The expression levels of ANP, SERCA2a and Na^+/ Ca^{2+} exchanger (NCX) in hearts of wild-type littermates (WT) or mActin-Tg (Tg) mice were quantified by RT-PCR analyses, normalized against GAPDH mRNA expression. * *P<0.05*. n=4 in each group.

Figure 4 Ca^{2+} transient in mActin-Tg mice. A, Ca^{2+} transients induced by electrical stimulation at 0.5 Hz in left ventricular cardiomyocytes loaded with fluo-3 AM at 10 months of age. WT, wild-type littermates. Tg, mActin-Tg mice. B, Time to peak amplitude of Ca^{2+} transient. *$P< 0.05$. C, The ratio of peak to basal amplitude of Ca^{2+} transient. n.s., not significant. B, C, WT, n=36; Tg, n=30.

Figure 5 The role of CaMKIIδ in mActin Tg mice. A, Relative protein levels of CaMKIIδB or CaMKIIδC. * $P< 0.05$. n=4 in each group. WT, wild-type littermates. Tg, mActin-Tg mice. B, Cardiac Function is improved by bisoprolol in mActin-Tg mice. Echocardiographic analyses at 5 months of age. LVDd, left

ventricular end-diastolic dimension. FS, fractional shortening. n=5 in each group. C, Western blot analyses in the hearts at 5 months of age. The graph indicates relative protein levels of CaMKIIδ. *P< 0.05. n=4 in each group. D, Western blot analyses in the hearts at 5 months of age. The graph indicates relative protein levels of phosphorylated PLB (Ser16). *P< 0.05. n=4 in each group. E, Cardiac Function is improved by KN-93 not KN-92 in mActin-Tg mice. Echocardiographic analyses at 5 months of age. *P< 0.05. WT, n=4; Tg, n=5.

Figure 6 CaMKII increases expression level of p53. A, Western blot analyses in the hearts at 5 months of age. The graph indicates relative protein levels of p53. * P< 0.05. n=4 in each group. WT, wild-type littermates. Tg, mActin-Tg mice. B, Western blot analyses in cardiomyocytes transfected with caCaMKIIδB (δB) or caCaMKIIδC (δC). The graph indicates relative protein levels of p53. * P< 0.05. n=4 in each group. C, Western blot analyses in cardiomyocytes with treatment of MG132. The graph indicates relative protein levels of p53. * P< 0.05. n=4 in each group.

Supplemental References

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