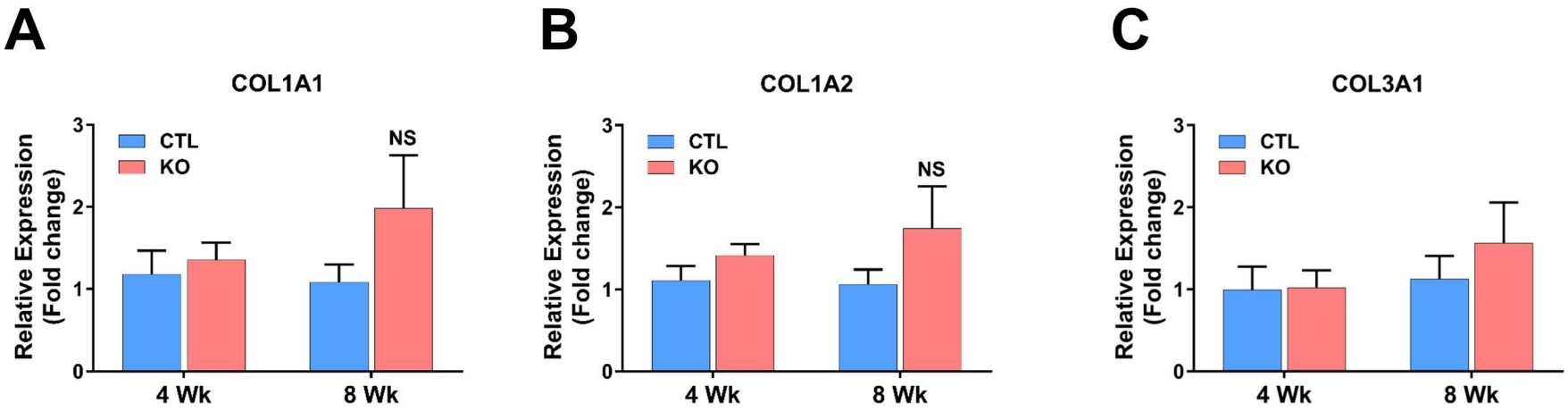
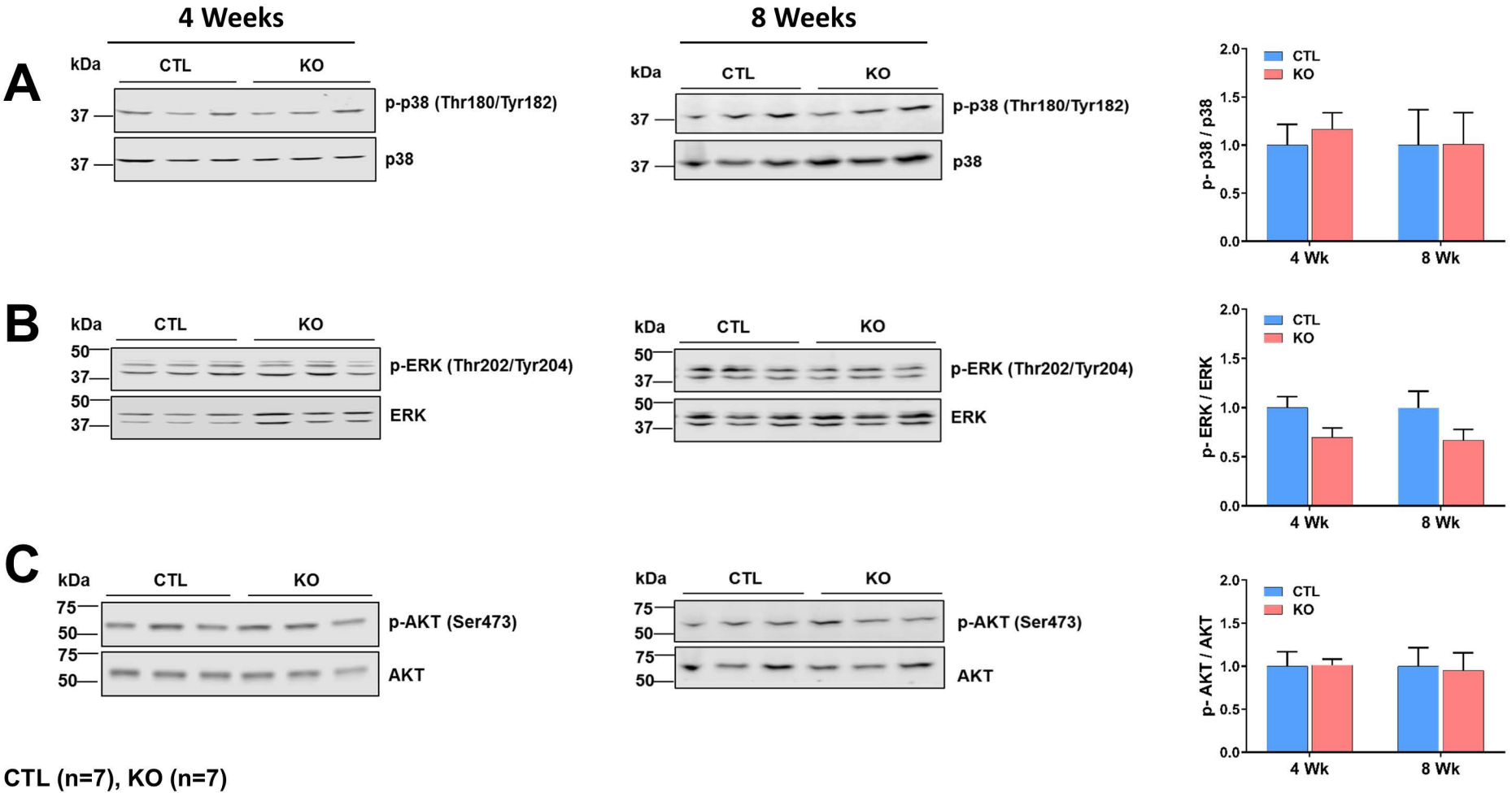
Online Figure 1



CTL (n=8), KO (n=11)

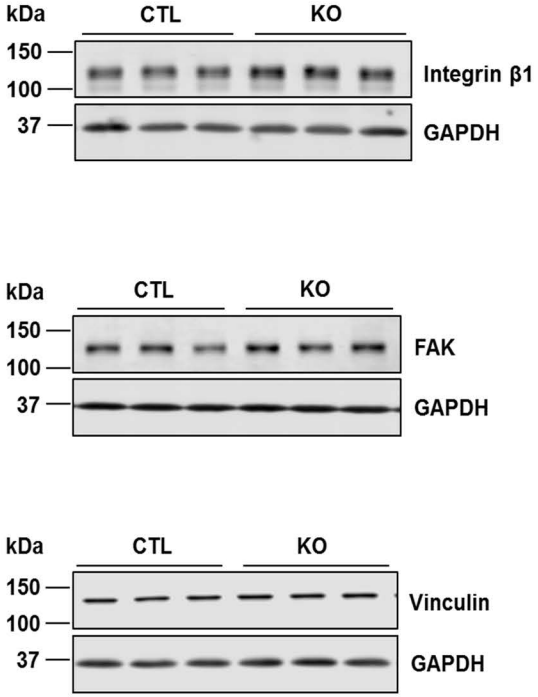
Online Figure 2

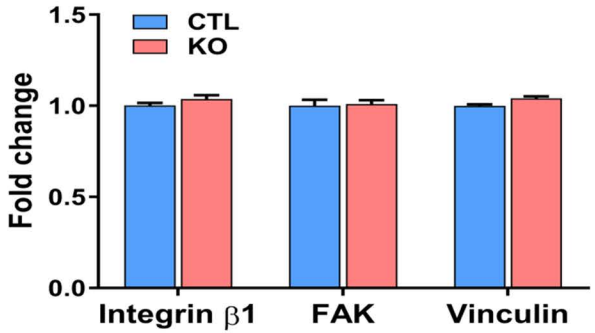


CTL (n=7), KO (n=7)

Online Figure 3

A: Integrin complex proteins

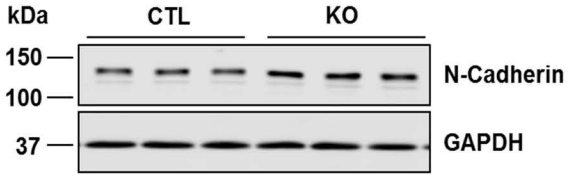


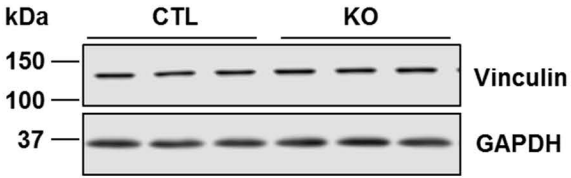


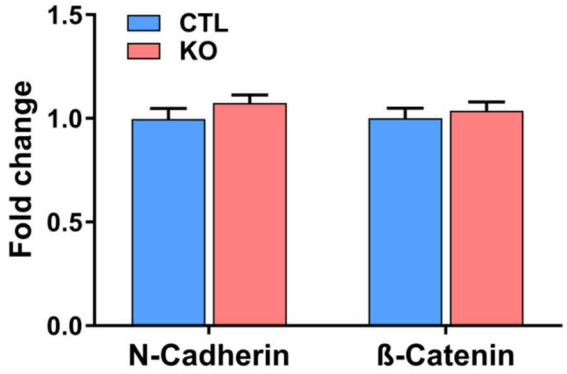
CTL (n=7), KO (n=7)

Online Figure 3

B: Adherens junction proteins



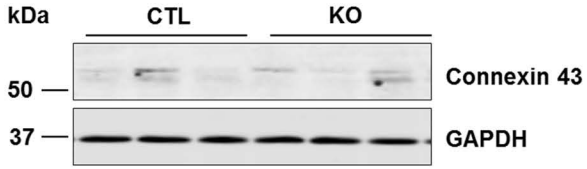


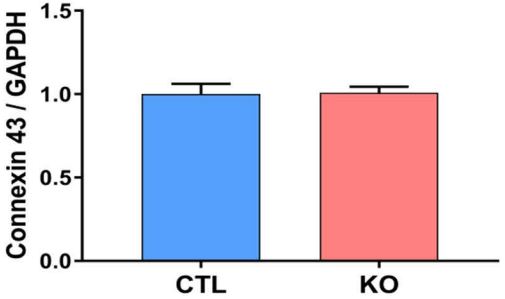


CTL (n=7), KO (n=7)

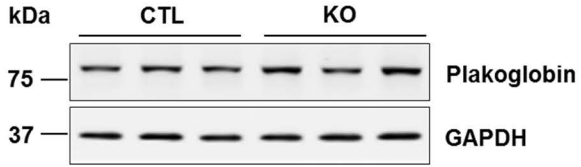
Online Figure 3

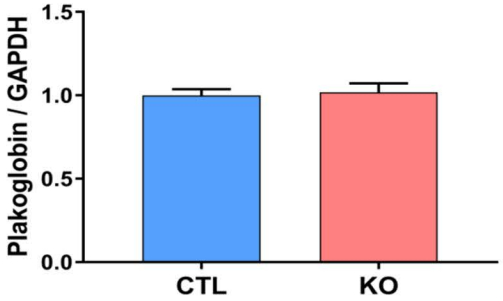
C: Gap Junction protein





D: Desmosome protein





CTL (n=7), KO (n=7)

ONLINE FIGURE LEGENDS

Online Figure 1. Analysis of cardiac fibrosis markers by qPCR method

At the end of each time point qPCR analysis was performed to examine the mRNA levels of **A**, Collagen Type I Alpha 1 Chain (COL1A1). **B,** Collagen Type I Alpha 2 Chain (COL1A2). **C**, Collagen Type III Alpha 1 Chain (COL3A1).

Online Figure 2. CM-Specific SMAD4 Deletion Does Not Affect MAPK or AKT Signaling Pathways

Western blot analysis of MAPK and AKT phosphorylation in CTL and SMAD4 KO hearts harvested at 4 weeks post-TAM treatment. Representative western blots and quantification **A,** p38 **B,** ERK1/2 **C,** AKT.

Online Figure 3. Effect of CM-Specific SMAD4 Deletion on Expression of Costameres and Intercalated Disk Proteins

Proteins were harvested from CTL and SMAD4 KO hearts at 4 weeks post-TAM treatment and western blot analysis was performed. Representative western blots and quantification **A,** Integrin complex proteins **B,** Adherens junction proteins **C,** Gap Junction protein **D,** Desmosome protein. FAK=focal adhesion kinase.

ONLINE APPENDIX

Mice

To achieve conditional deletion of SMAD4 specifically in CM, $SMAD4^{fl/fl}$ mice (Stock No: 017462, The Jackson Laboratory) were crossed with mice carrying the tamoxifen (TAM) inducible α-MHC promoter driven Mer-Cre-Mer transgene (Myh6-$Cre^{+/+}$) (Stock No: 005657, The Jackson Laboratory). Also, Myh6-$Cre^{+/+}$ transgenic mice were crossed with C57BL/6 mice (Stock No: 000664, The Jackson Laboratory) to generate Myh6-$Cre^{+/–}$. The $SMAD4^{fl/fl}$/$Cre^{+/–}$/TAM mice were the conditional knockouts (KO), whereas littermates $SMAD4^{fl/fl}$/$Cre^{-/-}$/TAM represented controls (CTL). Myh6-$Cre^{+/–}$/TAM mice were used as no-flox Cre controls (MCM). The Institutional Animal Care and Use Committee of Vanderbilt University Medical Center approved all animal procedures and treatments used in this study (protocol # M1700133-00).

Echocardiography

Transthoracic echocardiography was performed as described previously (1). In brief, mice were anesthetized by inhalation of isoflurane (1-1.5%). M-mode interrogation was performed with a MS400:18–38 MHz transducer (VisualSonics) in the parasternal short-axis view. Left ventricular internal dimension in diastole (LVID; d), left ventricular internal dimension in systole (LVID; s), left ventricular posterior wall thickness in diastole (LVPW; d), left ventricular posterior wall thickness in systole (LVPW; s), left ventricle ejection fraction (LVEF), left ventricle fractional shortening (LVFS), intraventricular septum dimension in systole (IVS; s), intraventricular septum dimension in diastole (IVS; d), heart rate (HR) values were obtained by analyzing data using the Vevo 2100 program.

Electrocardiography (ECG)

Mice were anesthetized by inhalation of isoflurane (1-1.5%) and ECG leads were recorded with surface electrodes (ADInstruments). The mean values for each parameter were obtained by analyzing data using LabChart software (ADInstruments). Corrected QT (QT_C) intervals were analyzed for an evaluation independent of heart rate. QTc was calculated with mean values and the Bazett's Formula (QTc = QT Interval/√ (RR interval).

Histology

Heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm thickness. Sections were stained with Hematoxylin-Eosin (HT1079, Sigma-Aldrich) or Masson Trichrome (HT15, Sigma-Aldrich) as per manufacturer's instruction. The images of LV region were captured using Zeiss AxioPlan2 microscope with AxioVision software. The quantification of LV fibrosis and cross-sectional area of cardiomyocytes (CSA) were determined with ImageJ software. At least 200-300 cardiomyocytes per heart (n=5 hearts per group) were taken for CSA measurement.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed using In Situ Cell Death Detection Kit, TMR red (12156792910, Sigma-Aldrich) as per manufacturer's instructions. Briefly, the sections were deparaffinized and rehydrated using xylene and ethanol gradings, and permeabilized using Proteinase K solution. Sections were incubated with the reaction mixture containing TdT and TMR red labeled dUTP for 1 h at 37 °C. Stained sections were mounted in mounting medium and images were captured with a confocal microscope (Olympus FV1000). TUNEL-positive and 4′,6-diamidino-2-

phenylindole (DAPI) stained nuclei were counted using the NIH ImageJ software. At least 500 nuclei were counted in each field.

Cardiomyocyte isolation and measurements of sarcomere shortening

Adult mouse cardiomyocytes were isolated as described previously (2). For recordings, myocytes were placed in a chamber on the stage of an inverted microscope and perfused with a normal physiological Tyrode's solution containing (in mmol/L): 150 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 10 glucose, 2 Na-pyruvate, 1 $CaCl_2$ and 5 HEPES, pH 7.4. Myocytes were paced at 1 Hz with a MyoPacer field stimulator (IonOptix). Adult CM contractility measurements were made using sarcomere length (SarcLen) parameters and processed with IonWizard 6.0 software. The following parameters were analyzed: sarcomere peak shortening normalized to resting sarcomere length (%PS), maximal shortening velocity (-dL/dT) and re-lengthening velocity (+dL/dT).

Western blotting

Western blotting was performed to evaluate protein levels. LV tissues were dissected from mouse heart and homogenized with cell lysis buffer (9803S, Cell Signaling Technology) having 50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1mM EDTA, 0.25% sodium deoxycholate, 1% NP-40 and freshly supplemented Protease Inhibitor Cocktail (P8340, Sigma-Aldrich) and Phosphatase Inhibitor Cocktail (P0044, Sigma-Aldrich). Protein concentration was determined with Bio-Rad Protein Assay Dye Reagent Concentrate (500-0006, Bio-Rad). The equal amount of proteins were denatured in SDS–PAGE sample buffer, resolved by SDS–PAGE and transferred to Immobilon-P PVDF Membrane (IPVH00010, EMD Millipore). The membranes were blocked in Odyssey blocking buffer (927-40000, LI-COR Biosciences) for 1h at RT. Primary antibody incubations were performed at different dilutions as described in antibody list (Online Table 2).

All incubations for primary antibodies were done for overnight at 4°C and followed by incubation with secondary antibodies described in antibody list (Online Table 3) for 1h at RT. Proteins were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensity was quantified by NIH Image J software.

RNA deep sequencing

Total RNA was extracted from ventricles of SMAD4 KO and littermate controls (n=4/group) using RNeasy Mini kit (74104, Qiagen) as per the manufacturer's instructions. RNA integrity was confirmed using Agilent Bioanalyzer. RNA sequencing (RNA-Seq) was performed by the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core. RNA libraries were constructed using the Illumina TruSeq Stranded Total RNA kit. Libraries were sequenced using Illumina HiSeq 3000 on paired-end-75 flow cell runs at ~35M PF reads per sample**.** Raw reads (fastq files) were uploaded to the Partek Flow server and pre-alignment quality assessment performed. Mean base-call quality scores were above Phred-like values of 36 in all positions of all samples, and no hard trimming of the reads was necessary. Sequences were aligned to the mm10 assembly of the mouse genome using STAR 2.5.3a and resulting summary of reads quantified at the gene level to Ensemble transcripts 83 using Partek's expectation maximization (E/M) annotation model. Gene counts were normalized to total read count per sample and then log-transformed (with an offset of 0.0001).

To identify differentially expressed genes, statistical analysis was performed using Partek's Gene Specific Analysis (GSA) multimodel estimation algorithm, which identifies the statistical model that is the best for each gene among all the selected models (lognormal, Poisson, etc.), and then uses that best model to calculate p-value and fold change. Hierarchical clustering was performed

on normalized and log transformed counts using Partek Genomics Suite 6.6. Statistical analyses (including correction for multiple hypothesis testing) for identification of overrepresented ontologies, functions, and pathways were performed using DAVID (https://david.ncifcrf.gov), a freely available online-based functional analysis tool to identify relevant gene sets.

RNA extraction and quantitative PCR analysis

Total RNA was extracted from heart tissue using the RNeasy Mini Kit (74104, Qiagen) according to manufacturer's protocol. cDNA was synthesized using the iScript cDNA synthesis kit (170-8891, Bio-Rad) following manufacturer's instructions. Gene expression was analyzed by quantitative PCR (qPCR) using the TaqMan Gene Expression Master Mix (4369016, Applied Biosystems) and TaqMan gene expression assays (Applied Biosystems) on a Bio-Rad CFX96 Real-Time PCR Detection machine. Details of TaqMan gene expression assays used in this study is proved as Online Table 4. Relative gene expression was determined by using the comparative C_T method ($2^{-\Delta\Delta C_T}$) and was represented as fold change. Briefly, the first ΔC_T is the difference in threshold cycle between the target and reference genes: $\Delta C_T = C_T$ (a target gene X) − C_T (18S rRNA) while $\Delta\Delta C_T$ is the difference in ΔC_T as described in the above formula between the CTL and KO group, which is = ΔC_T (KO target gene X) − ΔC_T (CTL target gene X). Fold change is calculated using $2^{-\Delta\Delta C_T}$ equation (3).

References:

1. Lal H, Zhou J, Ahmad F et al. Glycogen synthase kinase-3alpha limits ischemic injury, cardiac rupture, post-myocardial infarction remodeling and death. Circulation 2012;125:65-75.

2. O'Connell TD, Rodrigo MC, Simpson PC. Isolation and culture of adult mouse cardiac myocytes. Methods in molecular biology 2007;357:271-96.

3. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101-8.

Online Table 1: Echocardiographic measurements

	LVID, d (mm)	LVID, s (mm)	LVPW, d (mm)	LVPW, s (mm)	IVS, d (mm)	IVS, s (mm)	LVEF (%)	LVFS (%)	HR (bpm)
t=Baseline									
CTL	4.05±0.18	2.69±0.16	0.83±0.04	1.13±0.07	0.80±0.02	1.09±0.03	55.29±4.03	28.59±2.68	444±16
MCM	3.75±0.12	2.99±0.13	0.83±0.05	1.08±0.03	0.88±0.06	1.25±0.05	51.67±1.19	26.08±0.76	442±6
KO	3.81±0.11	2.73±0.12	0.77±0.03	1.10±0.05	0.80±0.02	1.07±0.02	55.47±1.93	28.45±1.23	455±20
t= 4 weeks									
CTL	4.04±0.17	3.07±0.12	0.95±0.08	1.12±0.07	0.91±0.03	1.23±0.05	51.08±1.77	25.80±1.07	404±15
MCM	4.13±0.15	3.14±0.14	1.04±0.05	1.19±0.07	0.88±0.03	1.17±0.05	45.43±2.17	22.34±1.26	470±22
KO	5.06±0.10****	2.73±0.12****	0.82±0.05*	0.92±0.05	0.80±0.02	1.02±0.03*	29.25±1.82**	13.82±0.93*	381±3
t= 8 weeks									
CTL	3.79±0.11	3.10±0.12	0.84±0.04	1.09±0.06	0.80±0.04	1.26±0.03	55.17±2.82	28.89±1.97	499±20
MCM	4.35±0.11	3.04±0.13	0.88±0.02	1.06±0.04	0.93±0.02	1.17±0.05	41.29±3.44$^{\#}$	19.94±1.92$^{\#\#}$	406±15
KO	5.09±0.11***	4.54±0.15****	0.80±0.03	0.84±0.04	0.73±0.02**	1.02±0.03****	23.37±3.38**	10.93±1.69*	491±24

Echocardiographic measurements. (CTL: n=13, MCM: n=7, KO: n=11) Data are mean ± SEM.

LVID, d= left ventricular internal dimension in diastole, ****P<0.0001 vs. MCM, ***P=0.0008 vs. MCM.; LVID, s= left ventricular internal dimension in systole, ****P<0.0001 vs. MCM.; LVPW, d=left ventricular posterior wall thickness in diastole, *P=0.0265 vs. MCM; LVPW, s=left ventricular posterior wall thickness in systole; IVS, d=intraventricular septum dimension in diastole, **P=0.0027 vs. MCM; IVS, s=intraventricular septum dimension in systole, ****P<0.0001 vs. MCM, *P=0.0466 vs. MCM; LVEF=left ventricle ejection fraction, **P=0.0035 vs. MCM (t=4 Wk), **P=0.0014 vs. MCM, #P=0.0161 vs. CTL (t=8 Wk); LVFS=left ventricle fractional shortening, *P=0.0220 vs. MCM (t=4 Wk), *P=0.0184 vs. MCM & #P=0.0096 vs. CTL (t=8 Wk); HR=heart rate; bpm=beats/min; Wk=Week

Online Table 2: Primary antibodies and dilutions used for Western blot analysis

S.No.	Antibody	Vendor	Catalog No.	Dilution
1	SMAD4	abcam	ab40759	1:1000
2	p-ERK1/2 (Thr202/Tyr204)	Cell Signaling Technology	4370S	1:1000
3	ERK1/2	Santa Cruz Biotechnology	sc-93	1:1000
4	p-AKT (Ser473)	Cell Signaling Technology	4060	1:1000
5	AKT	Cell Signaling Technology	9272	1:1000
6	p-p38 (Thr180/Tyr182)	Cell Signaling Technology	9211s	1:1000
7	p38	Santa Cruz Biotechnology	sc-535	1:1000
8	p-Phospholamban(p-PLN) (Thr17)	Badrilla	A010-13	1:5000
9	Phospholamban	Cell Signaling Technology	14562	1:1000
10	SERCA2a	Cell Signaling Technology	9580	1:1000
11	p-Troponin I (p-TnI) (Ser23/24)	Cell Signaling Technology	4004	1:1000
12	Troponin I	Cell Signaling Technology	4002	1:1000
13	Integrin β1	abcam	179471	1:1000

Online Table 2 continued: Primary antibodies and dilutions used for Western blot analysis

S.No.	Antibody	Vendor	Catalog No.	Dilution
14	FAK	Cell Signaling Technology	3285	1:1000
15	Vinculin	Sigma	V9131	1:200
16	Connexin 43	Thermo Fisher Scientific	13-8300	1:500
17	β-Catenin	Cell Signaling Technology	8814	1:1000
18	N-Cadherin	BD Transduction Laboratories	610920	1:250
19	γ -Catenin (plakoglobin)	BD Transduction Laboratories	610253	1:8000
20	GAPDH	Fitzgerald	10R-G109a	1:10000
21	cMyBP-C	Ref (1)		1:2500

Online Table 3: Secondary antibodies and dilutions used for Western blot analysis

S.No.	Antibody	Vendor	Catalog No.	Dilution
1	IRDye 680LT Goat anti-Mouse IgG	LI-COR Biosciences	926-68020	1:3000
2	IRDye 680LT Goat anti-Rabbit IgG	LI-COR Biosciences	926-68021	1:3000
3	IRDye® 800CW Goat anti-Mouse IgG	LI-COR Biosciences	925-32210	1:3000
4	IRDye® 800CW Goat anti-Rabbit	LI-COR Biosciences	925-32211	1:3000

Online Table 4: TaqMan gene expression assays and control used for qPCR analysis

S.No.	Gene	Assay ID	Catalog No.	Vendor
1	Col1a1	Mm00801666_g1	4331182	Applied Biosystems
2	Col1a2	Mm00483888_m1	4331182	Applied Biosystems
3	Col3a1	Mm00802300_m1	4331182	Applied Biosystems
4	Kcna2	Mm00434584_s1	4331182	Applied Biosystems
5	Kcnd2	Mm01161732_m1	4331182	Applied Biosystems
6	Kcne1	Mm04207514_m1	4331182	Applied Biosystems
7	Kcnv2	Mm00807577_m1	4331182	Applied Biosystems
8	Scn4b	Mm01175562_m1	4331182	Applied Biosystems
9	Cacng6	Mm01325846_m1	4331182	Applied Biosystems
10	Clcn1	Mm00658624_m1	4331182	Applied Biosystems
11	Eukaryotic 18S rRNA Endogenous Control		4319413E	Applied Biosystems

Reference:

1. Copeland O, Sadayappan S, Messer AE, Steinen GJ, van der Velden J, Marston SB. Analysis of cardiac myosin binding protein-C phosphorylation in human heart muscle. J Mol Cell Cardiol 2010;49:1003-11.