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Supplemental Information

ADAR2 increases in exercised heart and protects against myocardial infarction and doxorubicin-induced cardiotoxicity

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Supplementary Figures and legends:

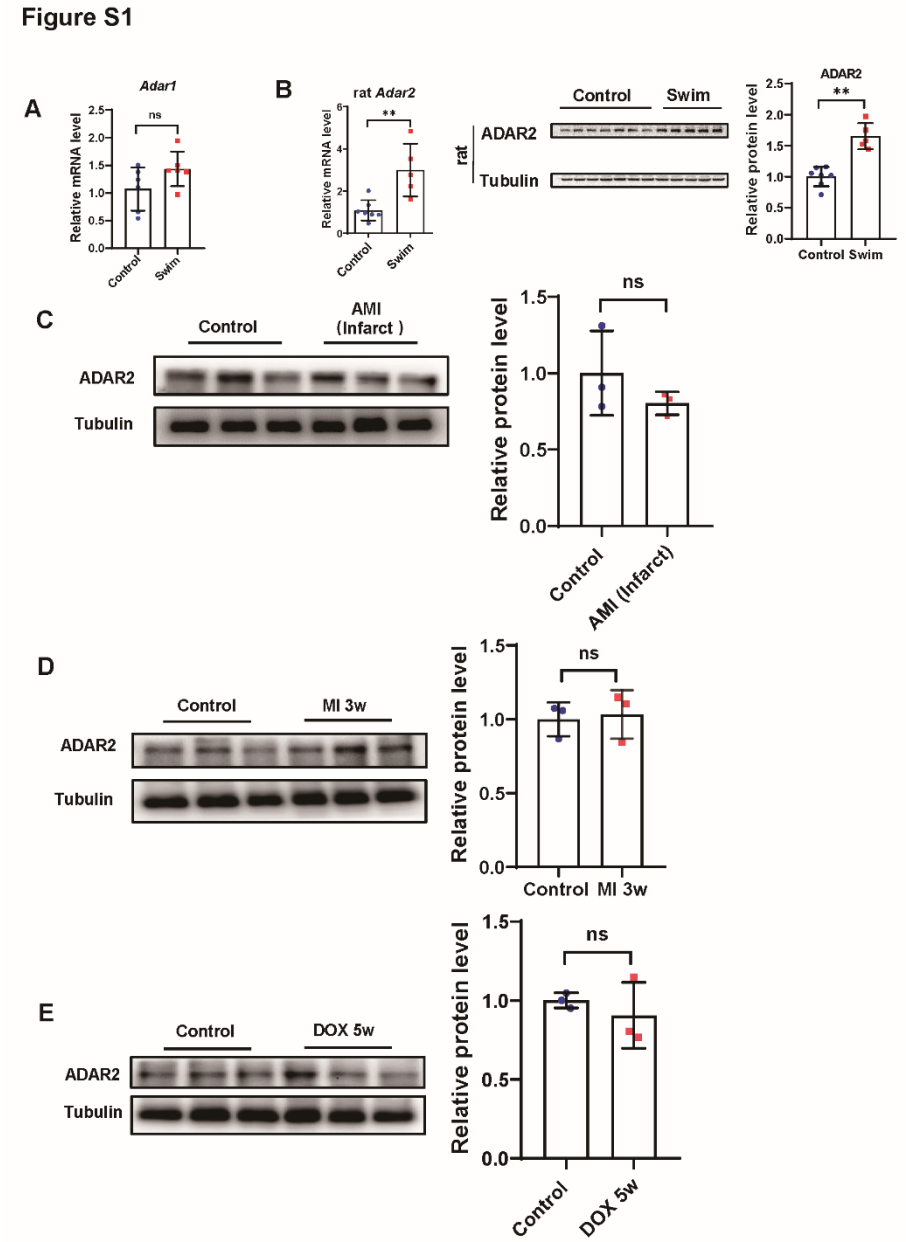

Figure S1. The expression of ADARs in swimming trained hearts. A, ADAR1 expression is unchanged in swimming trained mice (n=6). **B,** Quantitative real-time PCR and representative western blot showing expression change of ADAR2 in swimming trained hearts vs control in rat (n=7:5, **p<0.01). **C,** Representative western blot showing expression of ADAR2 with or without acute myocardial infarction (AMI) surgery (n=3). **D,** Representative western blot showing expression of ADAR2 with or without MI 3w remodeling (n=3). **E,** Representative western blot showing expression of ADAR2 with doxorubicin or saline treatment for 5 weeks (n=3). ns, non-statistically significant.

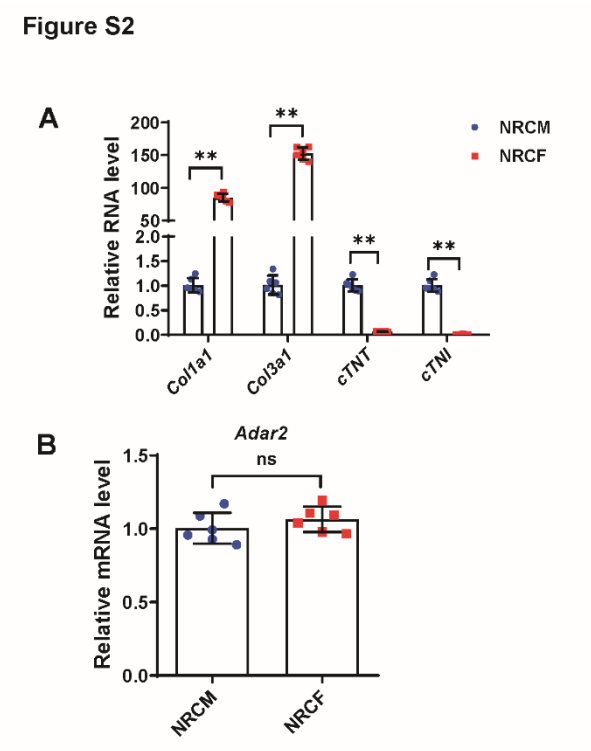

Figure S2. ADAR2 distribute evenly in cardiomyocyte and cardiac fibroblast. A, Quantitative real-time PCR analyzing the fibrotic markers (*Col1a1* and *Col3a1*) and myocyte markers (*cTNT* and *cTNI*) (n=6, **p<0.01). **B,** Quantitative real-time PCR showing the expression of ADAR2 in NRCM and NRCF (n=6). ns, non-statistically significant.

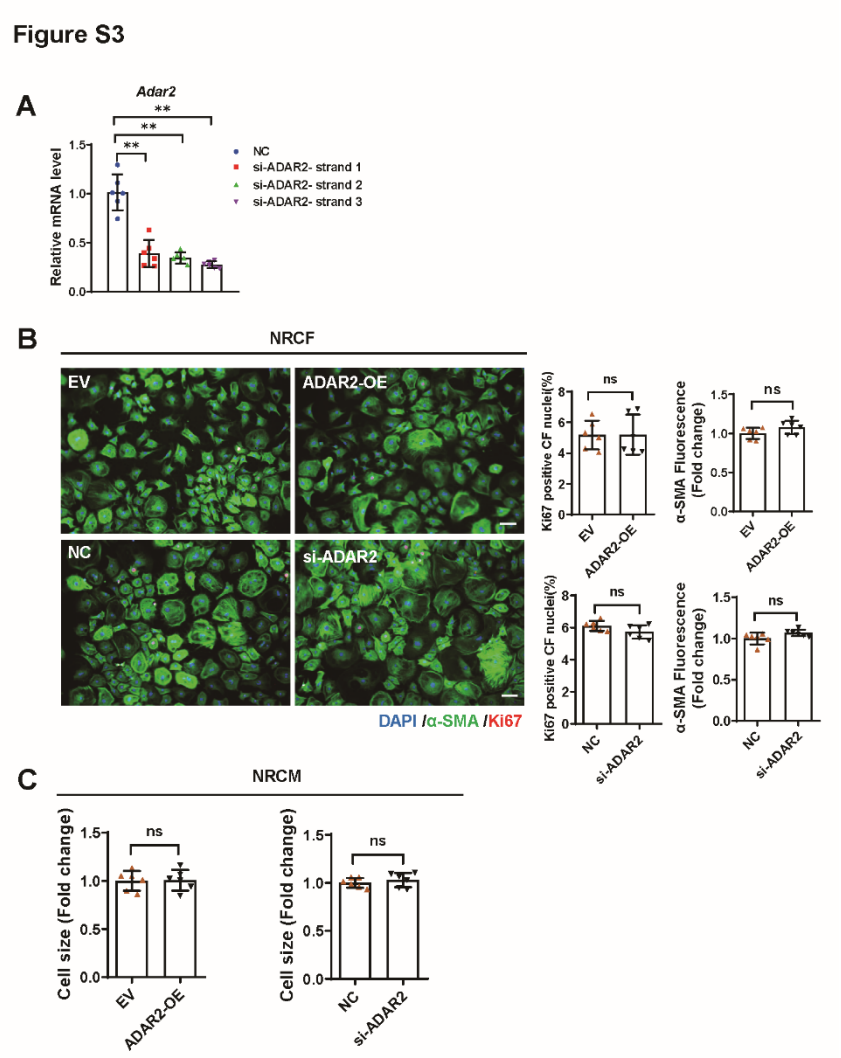

Figure S3. ADAR2 has no effect on cardiac fibroblast and cardiomyocyte hypertrophy. A, Quantitative real-time PCR testing expression change of ADAR2 after siRNA treatment (n=6, **p<0.01). **B,** Ki67 immunofluorescent staining of NRCF after ADAR2 overexpression or knockdown (n=6), scale bar, 50μm. **C,** Cell size measurement of NRCM after ADAR2 overexpression or knockdown (n=6). ns, non-statistically significant.

Figure S4

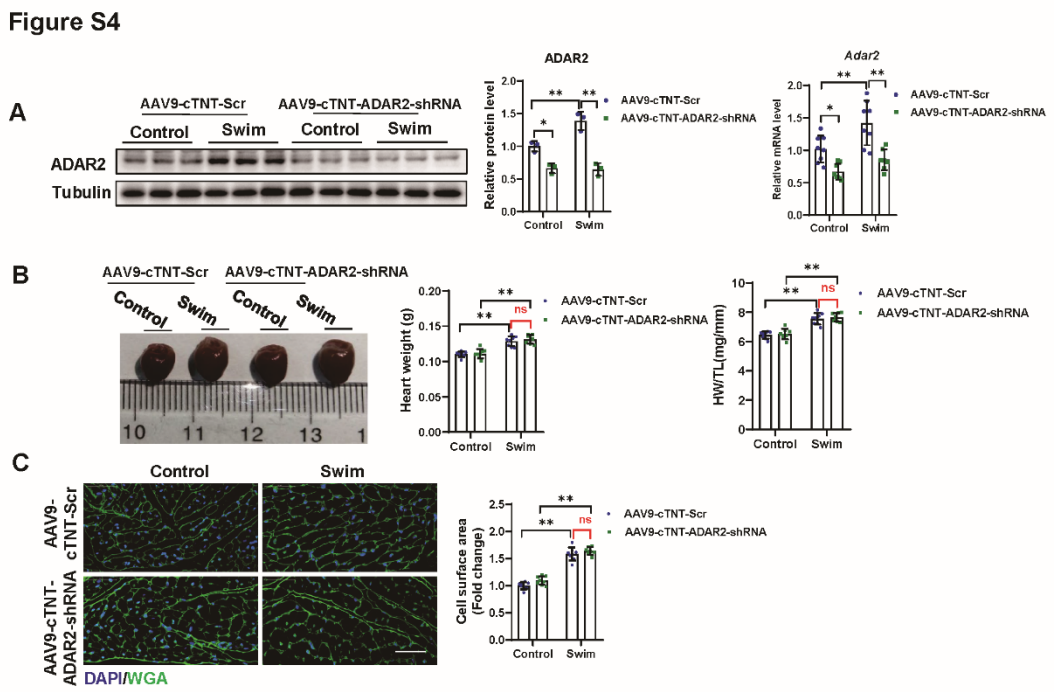

Figure S4. ADAR2 is not necessary for exercise-induced physiological cardiac hypertrophy *in vivo*. A, Quantitative real-time PCR (n=8:8:8:7) and representative western blot (n=3) showing cardiac-specific knockdown of ADAR2 in mice tail-vein injected with AAV9-cTNT-ADAR2-shRNA, and mice injected with empty AAV9-cTNT-scramble with or without swimming training (*p<0.05, **p<0.01). **B,** Heart weight and heart weight/tibia length (HW/TL) of ADAR2 cardiomyocyte-specific knockdown mice with or without swimming training (n=8:8:8:7, **p<0.01). **C,** WGA staining (n=8:8:8:7) showing cell size in ADAR2 cardiac-specific knockdown mice with or without swimming training (**p<0.01), scale bar, 50μm. ns, non-statistically significant.

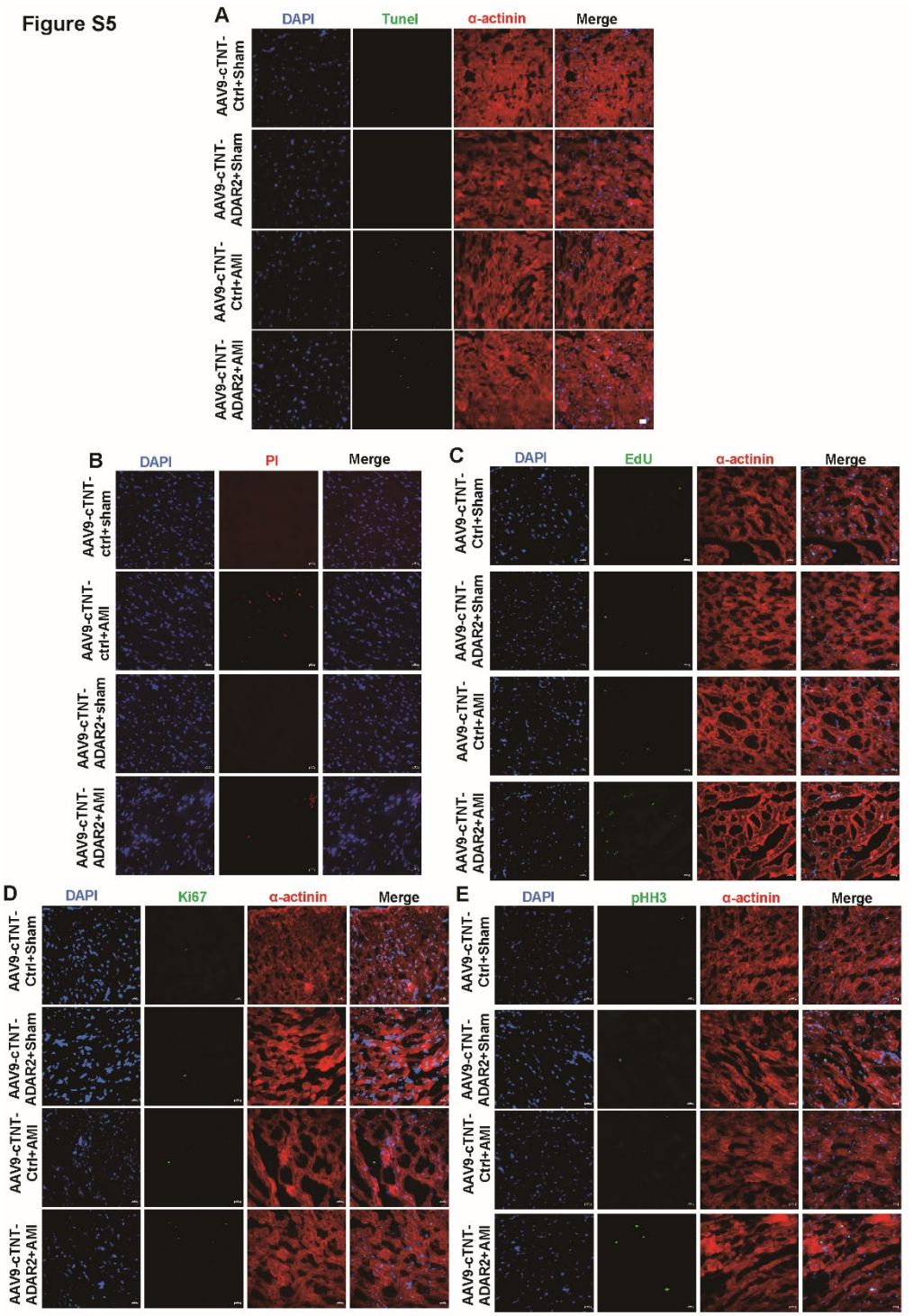

Figure S5. Single channel images of immunofluorescence staining. A, Single channel images of Tunel staining with or without acute myocardial infarction (AMI) surgery about figure 2D. **B,** Single channel images of PI staining with or without AMI surgery about figure 2G. **C,** Single channel images of EdU staining with or without AMI surgery about figure 2H. **D,** Single channel images of Ki67 staining with or without AMI surgery about figure 2I. **E,** Single channel images of pHH3 staining with or without AMI surgery about figure 2I. Ctrl, Control.

Figure S6

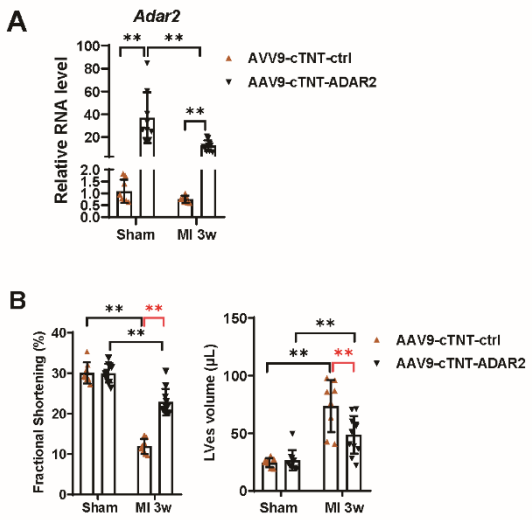

Figure S6. Cardiac-specific overexpression of ADAR2 improves cardiac function 3 weeks post-MI. A, Quantitative real-time PCR showing expression change of ADAR2 in hearts in AAV9-cTNT-ADAR2 or AAV9-cTNT-ctrl treated mice (n=8:9:7:10, **p<0.01). **B**, Echocardiography of mice treated with AAV9-cTNT-ADAR2 with or without MI surgery (n=8:10:8:11). LVes;volume, left ventricular end-systolic volume.

Figure S7

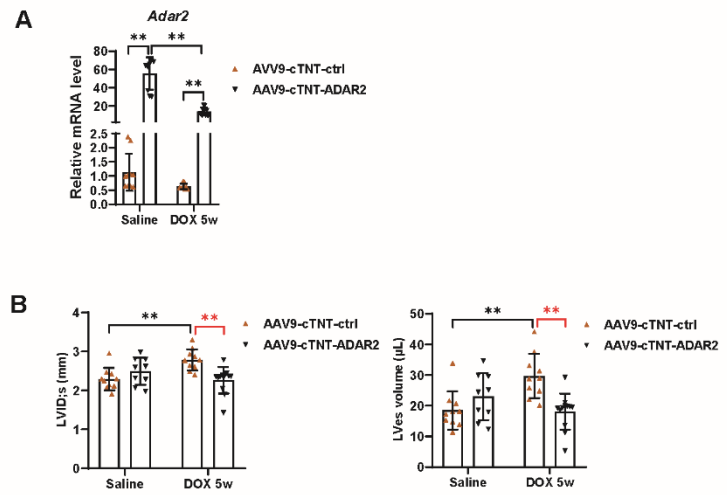

Figure S7. ADAR2 overexpression alleviates doxorubicin-induced cardiotoxicity. **A,** Quantitative real-time PCR showing expression change of ADAR2 in hearts in AAV9-cTNT-ADAR2 or AAV9-cTNT-ctrl treated mice with or without doxorubicin (DOX) treatment (n=10:9:10:10, **p<0.01). **B**, Echocardiography of mice treated with AAV9-cTNT-ADAR2 followed by doxorubicin or saline treatment (n=10:9:10:12). LVID;s, Left ventricular internal diameters of systole. LVes;volume, left ventricular end-systolic volume.

Figure S8

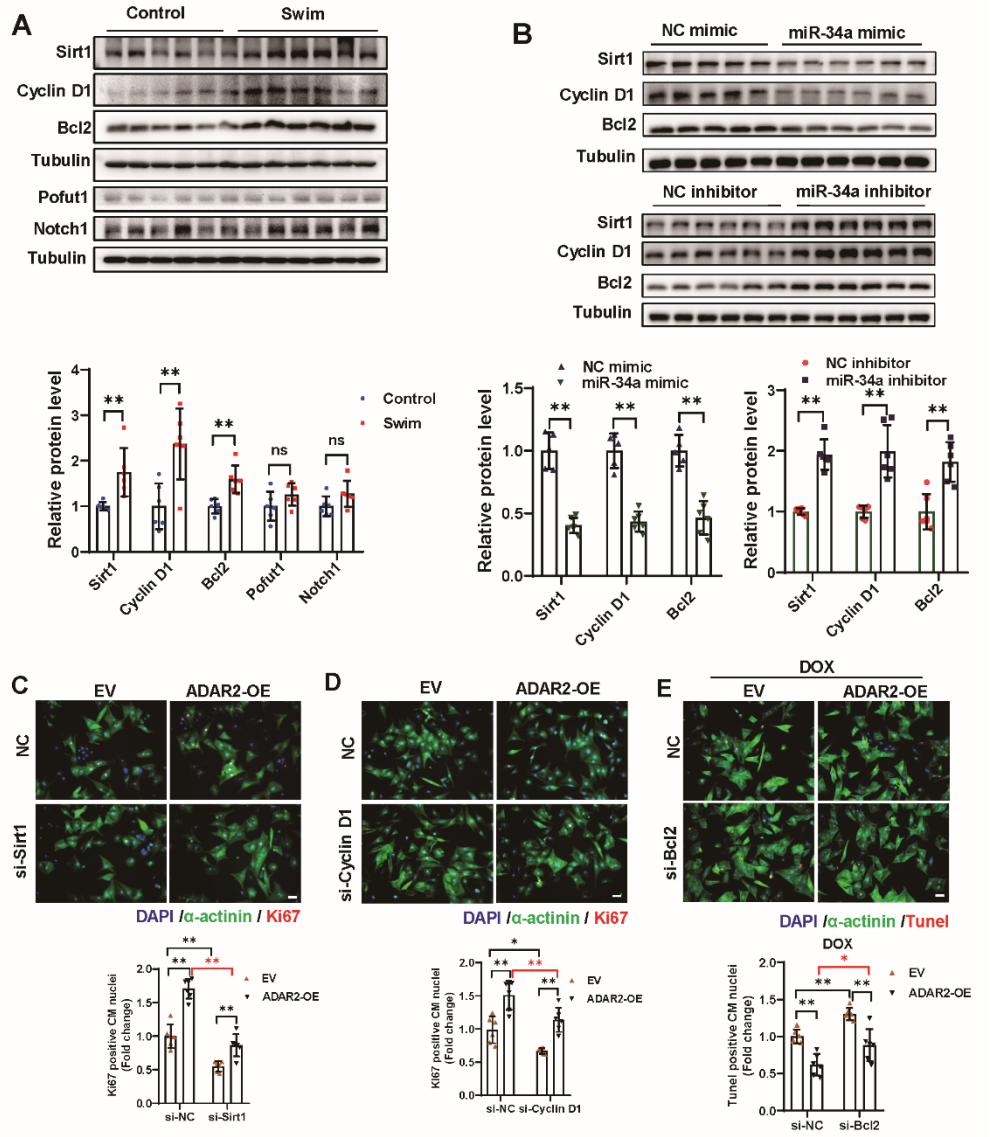

Figure S8. The role of Sirt1, Cyclin D1, and Bcl2 in NRCMs. A, Representative western blot showing expression change of Notch1, Pofut1, Sirt1, Cyclin D1, and Bcl2 in swimming trained hearts vs control in mice hearts (n=6, **p<0.01). **B**, Representative western blot showing expression change of Sirt1, Cyclin D1, and Bcl2 in NRCMs after up or down-regulation of miR-34a by miR-34a mimic (n=5:6) or miR-34a inhibitor respectively (n=6, **p<0.01). **C-D**, Ki67 immunofluorescent staining of NRCM after ADAR2 overexpression and knockdown of either Sirt1 or Cyclin D1 (n=6, *p<0.05, **p<0.01), scale bar, 50μm. **E**, Tunel staining analyzing doxorubicin (DOX)-induced NRCMs apoptosis after ADAR2 overexpression and knockdown of Bcl2 (n=6, *p<0.05, **p<0.01), scale bar, 50μm. ns, non-statistically significant.

Figure S9

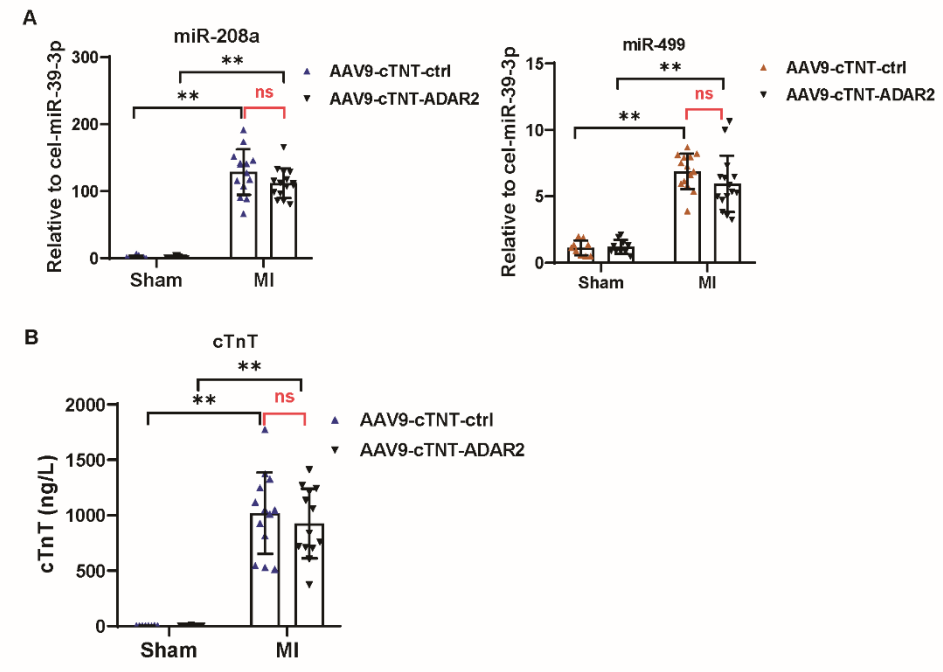

Figure S9. MI surgery homogeneity in mice plasma was evaluated by plasma miR-208a, miR-499 and high sensitive cTnT. A, Mice plasma miR-208a and miR-499 level at six hours after MI surgery (n=9:10:14:15, **p<0.01). **B,** Mice plasma cardiac Troponin T (cTnT) level at six hours after MI surgery (n=8:9:13:13, **p<0.01). ns, non-statistically significant.

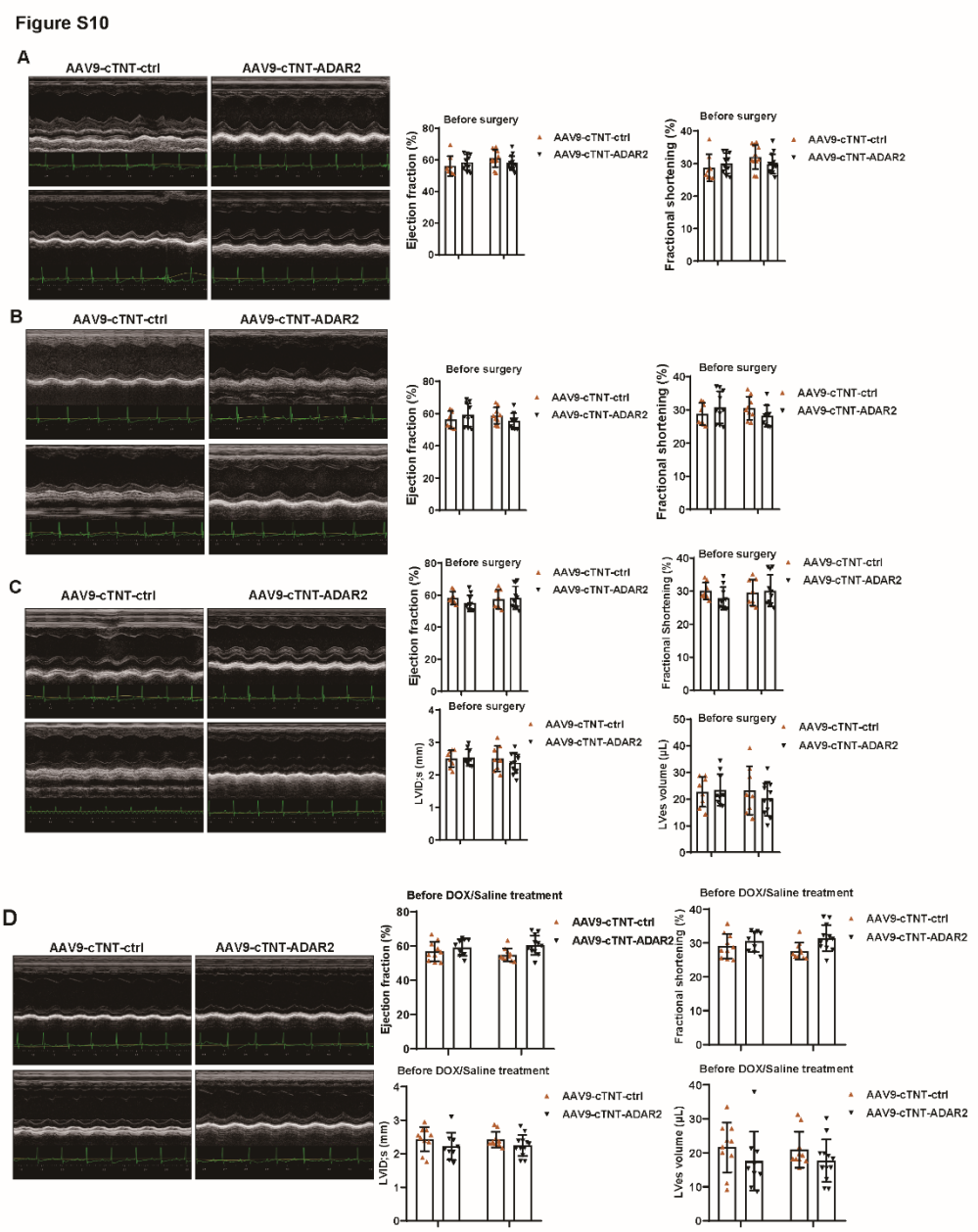

Figure S10. The mice baseline cardiac function before MI surgery (for AMI, MI remodeling), and doxorubicin treatment used in this study. A, The mice baseline cardiac function before AMI surgery for apoptosis and proliferation detection assays (n=8:10:11:14). **B**, The mice baseline cardiac function before AMI surgery for necrosis detection assays (n=8:10:9:9). **C**, The mice baseline cardiac function before MI surgery for MI remodeling (MI 3w) (n=8:10:8:11). **D**, The mice baseline cardiac function before doxorubicin or saline treatment for doxorubicin-induced cardiotoxicity detection (n=10:9:10:12). LVID;s, Left ventricular internal diameters of systole. LVes;volume, left ventricular end-systolic volume. DOX, doxorubicin.

Supplementary Tables:

Table S1

The primer sequences used for quantitative PCR were as follows:

gene	Forward	Reverse
m-*GAPDH*	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
m-*18S*	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCAC
m-*ADAR1*	TGAGCATAGCAAGTGGAGATACC	GCCGCCCTTTGAGAAACTCT
m-*ADAR2*	GTTTCGACAGGGACGAAGTGT	TGGCGTCATACCCTCTAGCA
m-*Col1a1*	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
m-*Col3a1*	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
r-*GAPDH*	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
r-*18S*	ATTCGAACGTCTGCCCTATCAA	CGGGAGTGGGTAATTTGCG
r-*ADAR2*	TGATAGACATCCGAATCGCAAAG	TAGATGGGCTCCACGAAAATG
r-*Col1a1*	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG
r-*Col3a1*	TGCCATTGCTGGAGTTGGA	GAAGACATGATCTCCTCAGTGTTGA
r-*cTNT*	TCGACCACCTGAATGAAGACC	TTCCTGCAGGTCGAACTTCTC
r-*cTNI*	GACGTGGAAGCAAAAGTCACC	GAGAGTGGGCCGCTTAAACTT
m-*Ctgf*	CTTCTGCAGACTGGAGAAGC	CAGCCAGAAAGCTCAAACTTG

Supplementary materials and methods:

Cardiomyocyte isolation, culture

Neonatal rat cardiomyocyte (NRCMs) was isolated from 0 to 1-day-old SD rats as described[33]. Briefly, the cells were purified using percoll gradient centrifugation (GE Healthcare). Isolated NRCMs were seeded in appropriate plates and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum and 10% horse serum. After serum starvation treatment, all transfection of cardiomyocyte was performed using Lipofectamine 3000 with containing indicated constructs. Briefly, Opti-MEM I reduced serum medium (Thermo, #31985088) was used as medium to mix Lipofectamine 3000 and plasmid or siRNA, and serum free DMEM was added to the mixture after 15 min. Then the solution was exposed to cardiomyocytes at a concentration of 2μg/ml for plasmid and 75nM for siRNA. Eight hours later, the solution was removed and replaced with DMEM for further experiment.

Cardiac fibroblasts isolation, culture, and Ki67 staining

Cardiac fibroblasts were isolated from 0 to 1-day-old SD rats. NRCFs were isolated and cultured as previously reported[22]. Cardiac fibroblasts at passage 2 were used in this study. All transfection of Cardiac fibroblasts was performed using Lipofectamine 3000 (Invitrogen) with containing indicated constructs. NRCFs were incubated with anti-α-SMA (1:200, Sigma, C6198) and Ki67 antibody (1: 200, Abcam, ab16667). The following secondary antibodies were used: Fluorescein (FITC) AffiniPure Goat Anti-Mouse IgG (H+L) (1: 200, Jackson), Cy3 AffiniPure Goat Anti-Rabbit IgG (H+L) (1: 200, Jackson). DAPI (Beyotime) was used for nuclear counterstaining and the number of Ki67-positive nuclei was counted. Inverted Fluorescence Microscope (Carl Zeiss, Thuringia, Germany) was used to capture images.

Plasmid and siRNAs

For ratADAR2 overexpression, the coding sequence of rat ADAR2 was obtained from NCBI. Gene fragment was generated by PCR amplification and cloned into FUGW and verified by Sanger sequence. PCR primers were used as follows:

ratADAR2 Forward Primer: 5'-
gggctgcaggtcgactctagaGGATCCatgGATATAGAAGACGAAGAGAAT-3'
ratADAR2 Reverse Primer: 5'-
tgctccatgtttttctaggtCTCGAGttaGGGAGTGAAGGAGAACTGGTCCTGCT-3'
ratADAR2-N Forward Primer:5'-
gctgcaggtcgactctagaGgccaccatgGATATAGAAGACGAAGAGAATATGAGT-3'
ratADAR2-N Reverse Primer: 5'-
gataagcttgatatcgaattTTATATCACCTTGGCATCTTTGACATCT-3'

SiRNAs were purchased by RiboBio (Guangzhou, China). Target sequences of siRNAs were used as follows: si-rat-Adar2_001, 5'-TCAGGTTTCTATACGCACA-3'; si-rat-Adar2_002, 5'-CATCCGAATCGCAAAGCAA-3'; si-rat-Adar2_003, 5'-GGTCATCAATGCCACAACA-3'; si-rat-Sirt1, 5'-GCCACCAACACCTCTTCAT-3'; si-rat-Bcl2, 5'-GGGAGATCGTGATGAAGTA-3'; si-rat-Cyclin D1, 5'-CAAGCAGATCATCCGCAAA-3'; si-rat-C/EBPβ, 5'-GTTTCGAGCATTAAAGTGA-3'.

Immunofluorescent staining for NRCMs

NRCMs were fixed, permeabilized, and blocked at room temperature, then incubated with primary antibodies at 4°C overnight. For Ki67 staining, NRCMs were incubated with anti-α-actinin antibody (1: 200, Sigma, A7811) and Ki67 antibody (1: 200, Abcam, ab16667). For EdU staining, EdU assays were performed using the Click-iT® EdU Alexa Fluor®594 Imaging Kit (Life Technologies) and Click-iT® EDU Alexa Fluor®488 Imaging Kit (Life Technologies) according to the manufacturer's instructions. For NRCMs, the following secondary antibodies were used: Fluorescein (FITC) AffiniPure Goat Anti-Mouse IgG (H+L) (1: 200, Jackson), Cy3 AffiniPure Goat Anti-Mouse IgG (H+L) (1: 200, Jackson), Cy3 AffiniPure Goat Anti-Rabbit IgG (H+L) (1: 200, Jackson). Nuclei were counterstained with DAPI (Beyotime) and the number of Ki67- or EdU-positive α-actinin-labeled cardiomyocyte were calculated to determine NRCMs proliferation. To evaluate the cardiomyocyte area, Image J was used to determine α-actinin-labeled NRCMs area. Inverted Fluorescence Microscope (Carl Zeiss, Thuringia, Germany) was used to capture images.

In Vitro doxorubicin-induced apoptosis model and Tunel staining

NRCMs were treated with doxorubicin (0.3 μM) for 24 hours. NRCMs were incubated with anti-α-actinin antibody (1: 200, Sigma, A7811) at 4°C overnight. Next day, NRCMs were incubated with secondary antibody Cy3 AffiniPure Goat Anti-Mouse IgG (H+L) (1: 200, Jackson). To detect apoptosis, Tunel assays was performed using the Tunel Staining Kit (Roche) according to the manufacturer's instructions. Nuclei were counterstained with DAPI and the number of Tunel-positive NRCMs nuclei was counted. The images were captured by Inverted Fluorescence Microscope (Carl Zeiss, Thuringia, Germany).

TA cloning and sequencing analysis

RT-PCR products from NRCMs treated with ADAR2 OE or empty vector (EV) were recovered by TIANquick Midi Purification Kit (TIANGEN BIOTECH (BEIJING) CO., LTD) and cloned into pLB vector (TIANGEN BIOTECH (BEIJING) CO., LTD) by TA cloning. Sequencing analysis was performed on positive clones containing inserts to detect nucleotide changes, and the sequencing results were compared to the sequences of EV infected control samples. The sequencing primer is: 5'-CGACT CACTATAGGGAGAGCGGC-3'.

High-resolution melting (HRM) analysis

Primer 3 Input was used to design primers for miR-34a. The primer sequence was pri-miR-34a-F: 5'-ATGGGGAGGCACTGACGTA-3', pri-miR-34a-R: 5'-CAACGTG CAGCACTTCTAGGG-3', and the length of the amplified fragment was 268bp, which included the entire pre-miR-34a, and extended at least 50bp to both ends. The PCR amplification and HRM analysis were performed on LightCyclerH 480 (Roche Diagnostics Applied Science) using LightCycler® 480 High Resolution Melting Dye (Roche Molecular Systems, Inc.), and the results were analyzed using LightCycler® 480 Gene Scanning Software.

Western blot

NRCMs or cardiac tissues were lysed using the protein extraction kit (KGI Biotech, China, KGP2100). The protein sample (30 μg) was loaded onto the sodium dodecyl

sulfate polyacrylamide gel (Bio-Rad, USA). Membranes were immunoblotted overnight on a rocking platform at 4 ℃ with the following antibodies incubated: ADAR2 (Absin Bioscience lnc, China, abs133529, 1: 1000), Bax (Proteintech, USA, 50599-2-Ig, 1: 1000), Bcl2 (Proteintech, USA, 12789-I-AP, 1: 1000), Caspase-3 (CST, USA, 9662, 1: 1000), Cleaved-Caspase 3 (CST, USA, 9661, 1: 1000), Sirt1 (Proteintech, USA,13161-1-AP,1:1000), Cyclin D1 (CST, USA, 2922, 1: 1000), Tubulin (CST, USA, 2146, 1: 5000), and GAPDH (CST, USA, 5174, 1: 5000) . Next day, membranes were incubated with the secondary antibody (CST, USA, 7074 to anti-rabbit, 7076 to anti-mouse, 1: 5000). Tubulin were used as the loading control. All proteins were detected by SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher, USA) and visualized using ChemiDoc Imaging Systems (Bio-Rad, USA). Band intensity was calculated by Image J.

AAV9-cTNT-ADAR2/ADAR2-shRNA cloning and virus packaging

AAV9-cTNT-ADAR2 / ADAR2-shRNA cloning and virus packaging were completed by Shanghai Hanbio Biotechnology Co.,Ltd according to standard procedures. For all constructs, AAV9 vectors carrying the cTnT promoter were used to generate cardiomyocyte-specific overexpression or knockdown of ADAR2. For AAV9-ADAR2, the coding sequence of ADAR2 was synthesized and directly inserted into the pAAV-cTNT-MCS-CMV-Zsgreen vector. For AAV9-ADAR2-shRNA, the shRNA sequence of the control virus was as follows:

Forward sequence: 5’-
GATCCGTTCTCCGAACGTGTCACGTAATTCAAGAGATTACGTGACACGTTC
GGAGAATTTTTTC-3’.

Reverse sequence: 5’-
AATTGAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGAATTACGTGACA
CGTTCGGAGAACG-3’.

The shRNA sequence of the ADAR2 was as follows:

Forward sequence: 5’-
AATTCGCAGCTCAAGTGGAGATGTCAGCCTATTCAAGAGATAGGCTGACAT
CTCCACTTGAGCTGTTTTTTG-3’.

Reverse sequence: 5’-
GATCCAAAAAACAGCTCAAGTGGAGATGTCAGCCTATCTCTTGAATAGGCT

GACATCTCCACTTGAGCTGCG-3'.

Luciferase reporter assays

The functional domain in promoter region of ADAR2 was amplified from genomic DNA of mouse heart (Forward primer, 5'-TCGAGTTTGGGTCATGCACGGAGAA-3', Reverse primer, 5'-AGCTTCAAGCTGCACGTGGCAAAAG-3'). The reported construct was generated by linking the PCR product to HindIII (New England Biolabs, #R3104) -linearized PGL3-basic vector using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech, C113-01). HEK293T was transfected with 2μg C/EBPβ overexpression plasmid with Sinofection in 12-well plates for 24 h; and then co-transfected for 24 h. Dual-luciferase reporter assay kit (Promega) was applied to analyzed the activation of firefly and Renilla luciferase according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay

About 1×10^7 neonatal mouse cardiomyocytes (NMCM) were obtained by centrifuging after cross-linking with 37% formaldehyde and 1.5M glycine. Lysis buffer together with 100×Protease inhibitor (Thermo) were used to resuspend NMCMs, and after 15 minutes of incubation on ice, the supernatant was removed and the nucleus components were collected. Nucleus components were suspended in nuclei lysis buffer with protease inhibitors and allowed incubation on ice for 30 minutes. NMCM nuclei were completely lysed after 10 sets of 20-sec interval pulses using an ultrasonicator (Scientz) with 60% ultrasonic power. The DNA fragment obtained should be optimized at 250bp~500bp and the supernatant was collected. Dilution Buffer was added to the supernatant to reach 1 mL final volume. 1% supernatant was taken as input and store at 4℃ after adding 5μL PI. The remaining supernatant was divided into two tubes to incubate IgG antibody (Sigma, #SAB3700848) and C/EBPβ antibody (Abcam, #ab32358) respectively at 4℃ overnight. The supernatant incubated with the antibodies were then transferred into new tubes containing pre-balanced G-sepharose beads and allowed a 90 min incubation at 4℃. After that, the supernatant was removed and beads

were obtained by centrifugation. IP elution buffer was added to the beads, and the unlocked crosslinking was performed overnight at 65℃. Finally, DNA was extracted for qPCR quantification. The primer sequences were as follows: Forward primer, 5'-TCCTCCCTCCAGCTTCTTTG-3', Reverse primer, 5'-CAAGCACTCAGAGTTCCCCT-3'.

Propidium iodide staining

Treated mice was injected with 20 mg/kg propidium iodide (PI, Sigma) intraperitoneally to label necrotic cells 1 h before experiment termination. Heart samples were harvested and snap-frozen in liquid nitrogen. Tissue blocks were cut into 5μm frozen sections and counterstained with DAPI. Quantification of necrotic cells was performed by accessing at least 3 sections for each sample. Images were captured by fluorescent microscope (Zeiss, Oberkochen, Germany). The percentage of necrotic cell death was calculated by counting the total number of PI-stained nuclei divided by total DAPI-positive nuclei.

Lactate dehydrogenase assay

At least 500μL of fresh blood was collected in anticoagulant centrifuge tube containing heparin to determine the total lactate dehydrogenase (LDH) released immediately after the mice were terminated. After centrifugation at 3000rpm for 10 minutes, Toxicology Assay Kit (ROCHE#11644793001) was used to determine the LDH concentration in 100μL serum. Briefly, standard solutions with gradient concentrations were prepared by mixing 100μL LDH standard and 100μL reaction buffer. 100μL of sample mixtures and standard solutions were transferred to 96-well plate and allowed to incubate at 25°C for 30 minutes. After incubation, 50μL stop solution was added to each well, the absorbance at 492nm and 655nm was detected using a microplate reader (Molecular Devices). The LDH concentration in the samples is calculated according to the absolute absorbance that is the absorbance at 492nm minus the absorbance at 655nm.

Immunofluorescent staining and EdU staining

50mg/kg 5-ethynyl-2-deoxyuridine (EdU) was intraperitoneally injected twice before sample harvesting. The frozen sections of mouse heart tissue blocks were fixed with 4% paraformaldehyde (PFA) for 15 minutes and permeabilized with 0.5% Triton in PBS for 20 minutes. After blocked with 4% bovine serum album (BSA) for 1 hour, the sections were incubated with primary antibodies overnight at 4℃: phpspho-HistoneH3 (pHH3, 1:100, PA5-17869, Invitrogen), Ki67 (1:200, ab16667, Abcam) and α-actinin (1:200, A7811, Sigma). Afterwards, secondary antibodies were applied for 2 hours at room temperature: Cy3 AffiniPure Donkey Anti-Mouse IgG (H+L) (715-165-151, Jackson), Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG (H+L) (115-545-003, Jackson), Alexa Fluor 488 AffiniPure Goat Anti-Rabbit IgG (H+L) (715-165-151, Jackson). EdU staining was carried out according to manufacturer's instruction of Cell-LightTM Apollo 567 Stain Kit (C10371-1, Ribobio). Nuclei were counterstained with DAPI. Images were captured by fluorescent microscope (Zeiss, Oberkochen, Germany). Images were analyzed with Image J; pHH3, Ki67, and EdU positive cardiomyocytes percentage in tissues was calculated, respectively.

Quantification of plasma miRNAs and high sensitive cTnT

To determine the plasma level of miR-208a and miR-499, total miRNAs were isolated from plasma samples using the mirVana™ miRNA isolation kit (Life Technologies) in accordance with the manufacturer's instructions. Mature miRNAs were converted to cDNA with iScript cDNA Kits (Bio-Rad) and miRNA reverse transcription primer set (Ribobio) and the miRNA expression levels were quantified by SYBR green–based qPCR using LightCycler 480 real-time PCR detection system (Roche). Normalization was performed by spike-in cel-miR-39 exogenously added to each sample.

Plasma high sensitive cTnT concentrations were measured using commercially available electrochemiluminescence immunoassays on an Elecsys 601 analyzer (Elecsys Troponin T hs STAT, cGmbH, Mannheim, Germany).