## SUPPLEMENTAL MATERIALS

## METHODS

### Western blot analysis

Total protein extracts were isolated from adult hearts with 250mM sucrose buffer. Antibodies against NCX1 (Swant, 1:2000), CaMKIId (BD Biosciences, 1:1000), CypD (Mitosciences, 1:2000), BIM (Cell Signaling, 1:1000), GAPDH (Millipore, 1:10,000), VDAC1 (Abcam, 1:1000), and α-tubulin (Sigma; T9026, 1:1,000) were used. Quantification of Western blot was performed using ImageJ software.

### Qualitative RT PCR to assess *Dnm3os* splicing

The primer sets used include:

Set 1: For 5'-GCCACGTCAAGACTGGAAAT,

Set 1: Rev 5' -AAGTGCGTTTGCATTGTCTG

Set 2: For 5'-GCAGGCCAGGGGAATAGCGC,

Set 2: Rev 5'-TGGTGCCTCGCCTGTTTCACC

Set 3: For 5'- CTTGACTAACGCGTCACGAA,

Set 3: Rev 5'- TGATGGAGTTGAGGTTGCAG

Set 4: For 5'- TGGTGTCCTGCAGATGATGT,

Set 4: Rev 5'- TGTGCAGTGCCTAGAGATGG

Set 5: For 5'- TGCACACACCTTTGCTGCCTCC,

Set 5: Rev 5'-AGGCCTGTGGGCAAGGCAGT

**Generation of miR-214 KO mice**

Our targeting strategy introduced a neomycin resistance cassette and loxP sites flanking the genomic region encompassing the 106bp pre-miR (Supplemental Figure 1A). To generate the miR-214 targeting vector, a 4.2 kb fragment (5' arm) extending upstream of the miR-214 coding region was digested with SacII and NotI and ligated into the pGKneoF2L2dta targeting plasmid upstream of the loxP sites and the Frt-flanked neomycin cassette. The KO arm (671 bp) was generated by digestion with Sma and ligated between the proximal loxP and Frt sites. A 1.6 kb fragment (3' arm) was digested with NheI and SalI and ligated into the vector between the loxP site and Dta negative selection cassette. Targeted ES-cells carrying the disrupted allele were identified by Southern blot analysis and PCR. miR-214 targeted ES clones were used for blastocyst injection. The resulting chimeric mice were bred to C57BL/6 mice to obtain germline transmission of the mutant allele (conditional) and CAG-Cre mice to directly obtain the global miR-214 KO.

**Histology and immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5-μm intervals. Hematoxylin and eosin and Masson's trichrome stains were performed using standard procedures.

For wheat germ agglutinin staining, deparaffinized slides were incubated for 1 hour at room temperature with primary antibody against WGA conjugated to FITC (50 μg/ml, Invitrogen) in PBS. Slides were washed 3 times in PBS,

mounted in Vectashield with DAPI (Vector Labs) and imaged by fluorescence microscopy. For each section, 10-20 images were captured using 20x magnification and cell size calculated using ImageJ software.

TUNEL staining was performed using the In Situ Cell Death Detection kit (Roche) according to the manufacturers instructions. Hoechst staining was performed to visualize nuclei and the percentage of TUNEL positive nuclei was quantified using ImageJ software. For double staining, the TUNEL protocol was preceded by immunofluorescence with a mouse monoclonal Desmin antibody (Dako, 1:100) and Alexa555-conjugated anti-mouse secondary (Invitrogen, 1:500).

**Transthoracic echocardiography**

Cardiac function and heart dimensions were evaluated by two-dimensional echocardiography using a Visual Sonics Vevo 2100 Ultrasound (Visual Sonics, Canada) on conscious mice. M-mode tracings were used to measure anterior and posterior wall thicknesses at end diastole and end systole. Left ventricular (LV) internal diameter (LVID) was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). A single observer blinded to mouse genotypes performed echocardiography and data analysis. FS was calculated according to the following formula: $FS(\%) = [(LVIDd - LVIDs)/LVIDd] \times 100$. EF% was calculated by: EF= EDV-ESV/EDV (ESV= end systolic volume, EDV=end diastolic volume).

**Electron microscopy**

Adult (8-10 week old) miR-214 KO males and control littermate mice were anesthetized and transcardially perfused with 0.1 M phosphate buffer (pH 7.3), followed by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer. Hearts were removed, and left ventricles were sliced into 1-$mm^3$ cubes and fixed overnight at 4°C. Samples were processed by the University of Texas Southwestern Medical Center Electron Microscopy Core facility. Sections were imaged on Jeol 1200 EX TEM at the indicated magnification. Mitochondrial volume was measured from EM images using ImageJ software.

**Microarray analysis**

Samples were labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). Briefly, cDNA was column purified following synthesis according to the manufacturer's protocol and then used for in vitro transcription to generate cRNA. After purification, cRNA quality was checked by NanoDrop before proceeding with hybridization. Samples were hybridized on chips overnight at 58$^{o}$C with rocking, washed, blocked, and then stained with StrepAvidin-Cy3. After washing, slides were dried and scanned using the Direct-Hybridization program on the BeadArray Reader. Fold change was calculated based on signal intensity ratios between miR-214 KO and WT samples. Genes that were 1.5 fold or greater up-regulated were examined and those listed in Table 2 were confirmed by qPCR.

**Cell culture, transfection, and luciferase assays**

miR-214 expression plasmid was transfected into HEK 293T or COS cells. Two different portions of the *Ncx1* 3' UTR were cloned into the pmiR-report vector (Ambion). The construct containing site 3 encompasses the region from nucleotide 73 to 1095 while the region from nucleotide 1439 to 15376 contained sites 1 and 2. Full length *CamkIId* and *Ppif* 3' UTRs were also cloned into pmiR-report. Mutations in the 3′ UTRs were generated using the QuickChange Mutagenesis kit (Stratagene) to alter nucleotides 2-4 of the targeting sequence.

**Neonatal rat cardiomyocyte culture**

Neonatal rat cardiomyocytes were prepared by dissociation of 1- to 2-day-old Sprague-Dawley rats with the Isolation System for Neonatal Rat/Mouse Cardiomyocytes (Cellutron) according to the manufacturer's instructions. After isolation, cardiomyocytes were maintained in Dulbecco's modified Eagle's medium (DMEM)/199 medium (4:1) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. 15-nucleotide LNA-modified antimiRs (100nM) were transfected into cardiomyocytes with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and cultured for 48-72 hours before harvesting cells for RNA or performing in vitro IR assays.

**Mouse model of MI and IR**

MI and IR: 8-12 week old miR-214 KO male mice or WT controls were anesthetized with 2.4% isoflurane and placed in a supine position on a heating

pad (37°C). Animals were intubated with a 19G stump needle and ventilated with room air, using a MiniVent mouse ventilator (Hugo Sachs Elektronik; stroke volume, 250 ul, respiratory rate, 210 breaths per minute). Via left thoracotomy between the fourth and fifth ribs, the LAD was visualized under a microscope and ligated by using a 6–0 prolene suture. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Leica) by discoloration of the occluded distal myocardium. For IR, the ligation was released after 45 minutes of ischemia and tissue allowed to re-perfuse as confirmed by visual inspection. Sham operated animals underwent the same procedure without occlusion of the LAD.

**Adult mouse cardiomyocyte isolation**

Cardiomyocytes were isolated from adult mouse hearts as previously described (1). In brief, after retrograde perfusion with the $Ca^{2+}$-free Krebs-Ringer buffer and following perfusion with collagenase solution (Collagenase II, 8 mg/mL), the left ventricle was removed and minced. After gentle trituration , cells were kept in KB solution at room temperature. Extracellular $Ca^{2+}$ was added to KB solution in increments to 1.2 mM. Only rod-shaped, clearly striated cardiomyocytes that were $Ca^{2+}$ tolerant were used within 6 hours at room temperature in all the experiments.

**Intracellular calcium ( [Ca2+]i ) measurements**

Fura-2 AM was used to detect intracellular $Ca^{2+}$concentration ([Ca2+]i) as described before (2). Cardiomyocytes were loaded with 2.5 μM Fura-2 AM, washed, and placed on laminin-coated coverslips (Deckgläser, Germany), which were inserted into the bottom of a perfusion chamber connected with Myopace Cell Stimulator (Ionoptix Inc, MA). The cells were perfused with regular (1.8 mM $Ca^{2+}$) or high $Ca^{2+}$ (5 mM $Ca^{2+}$) extracellular buffer. Fura-2 fluorescence was recorded with a dual-excitation fluorescence photo multiplier system (PTI, NJ) with an Olympus IX-70 inverted microscope, a Fluor 40x oil objective and a CCD camera. Cells were exposed to light emitted by a 75W lamp and passed through either a 340 or a 380 filter, while being stimulated to contract at 0.5 Hz. Changes in [Ca2+]i were monitored by Fura-2 excitation at 340 and 380 nm and emission at 510 nm. Data are expressed as the 340/380 ratio following subtraction of background fluorescence. $Ca^{2+}$ transient amplitude was calculated from the difference of between systolic and diastolic [$Ca^{2+}$]i level (corrected F340/380 ratio). The time course of the fluorescence signal decay (t: the duration where $Ca^{2+}$ transient decays 67% from the peak level) was calculated to assess intracellular $Ca^{2+}$clearance rate.

**Mitochondrial respiratory activity and superoxide production**

Mitochondrial electron transport activity and superoxide production were assessed as previously described (3). Snap-frozen WT or miR-214 KO hearts were homogenized by Polytron homogenizer in 5.0 mL of ice-cold buffer

containing 10 mM MOPS and 1.0 mM EDTA, pH 7.4. The homogenate was then spun at 500g for 5 min at 4°C, the supernatant collected and passed through cheese cloth, and spun again for 10 min at 10000g. The resulting mitoplast pellet was resuspended in 10 mM MOPS buffer pH 7.4 and protein concentration determined by the BCA method (Pierce) using BSA as a standard. All assays were performed in 25 mM KCl, 10 mM MOPS at pH 7.4 (25°C) with 25 mg/mL frozen-thawed mitoplast. NADH oxidase activity, an assessment of overall electron transport through complexes I, III, and IV, was measured spectrophotometrically as the rotenone-sensitive rate of NADH oxidation (e340=6200 M-1cm-1) following addition of 0.2 mM NADH. The NADH-supported rate of superoxide production was measured by monitoring the superoxide-dependent, SOD-sensitive, oxidation of hydroethidine to the fluorescent product 2-hydroxyethidium. The fluorescent signal changes were recorded (excitation, 480nm; emission, 567nm) over time utilizing 10 mM hydroethidine and 0.5 mM NADH.

**References**

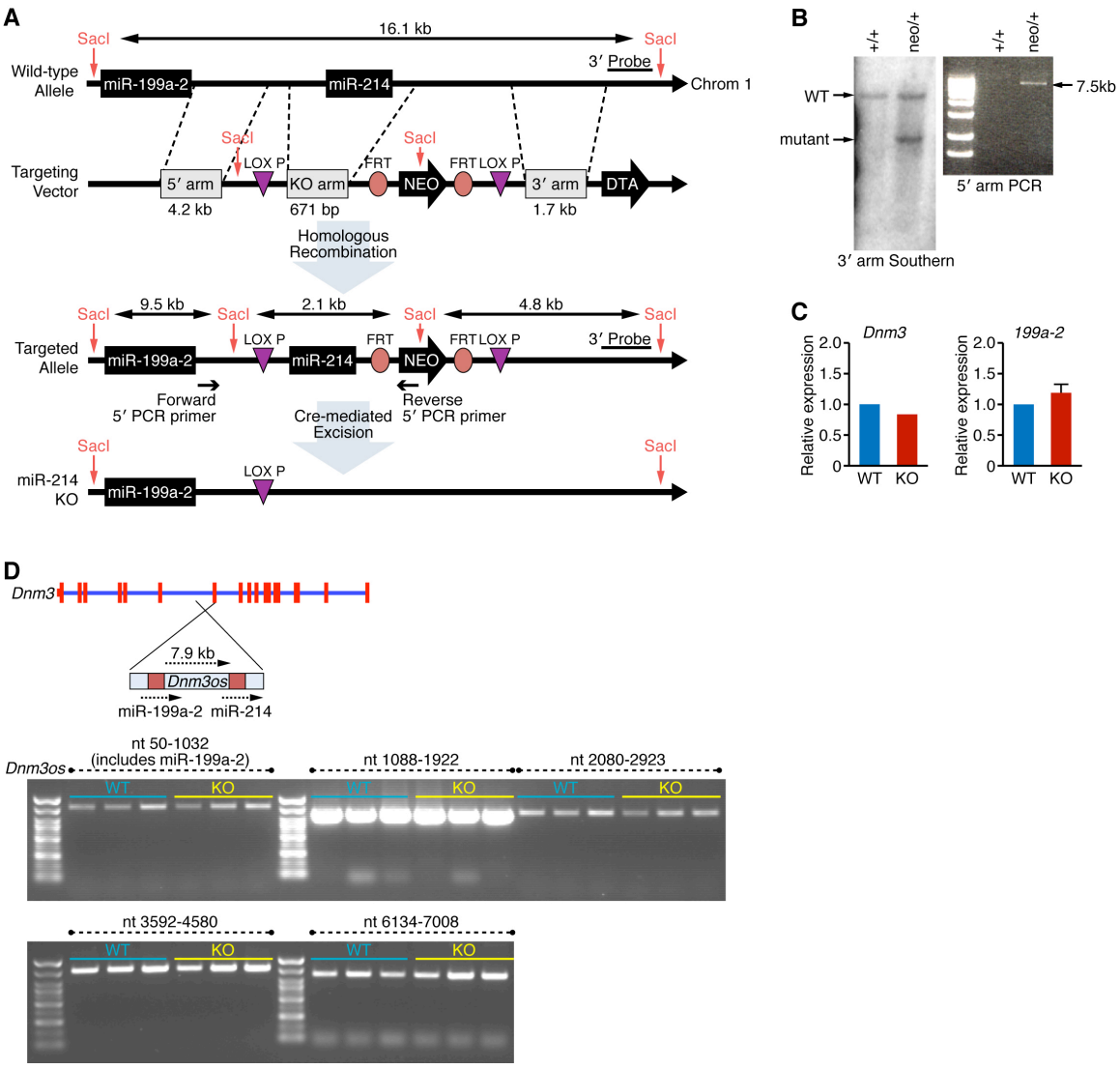

1. Tandan, S., et al. Physical and functional interaction between calcineurin and the cardiac L-type Ca2+ channel. *Circ. res*. 2009;105(1):51-60.
2. Luo, X., Shin, D.M., Wang, X., Konieczny, S.F., and Muallem, S. Aberrant localization of intracellular organelles, Ca2+ signaling, and exocytosis in Mist1 null mice. *J. Biol. Chem*. 2005;280(13):12668-12675.
3. Matsuzaki, S., Szweda, L.I., and Humphries, K.M. Mitochondrial superoxide production and respiratory activity: biphasic response to ischemic duration. *Arch. Biochem. Biophys*. 2009;484(1):87-93.

**Supplemental Table S1.** Numbers of offspring of the indicated genotypes resulting from crosses between miR-214 heterozygous male and females. Percent expected and observed are shown.

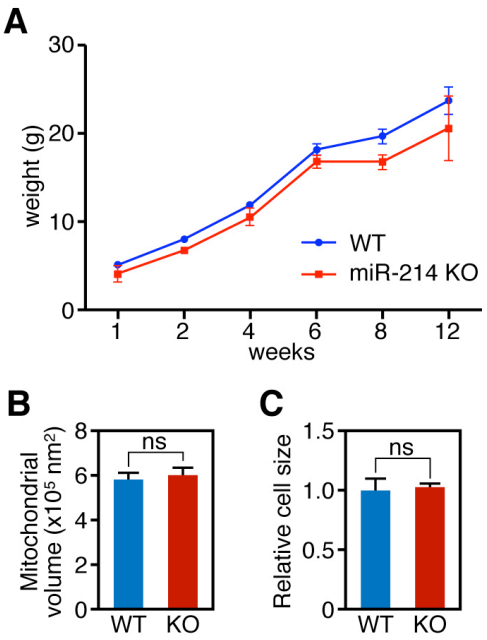
| Total # mice | +/+ | +/- | -/- |
|---|---|---|---|
| 255 | Observed 64 (25%)<br>Expected 64 (25%) | Observed 129 (51%)<br>Expected 128 (50%) | Observed 62 (24%)<br>Expected 64 (25%) |

**Supplemental Table S2.** Illumina array performed on miR-214 KO hearts and WT hearts at P14 and 8 weeks of age. RNA from 3 different mice was pooled for each sample. Shown are top most changed genes that relate to calcium handling or energy metabolism.

| Gene | p14 hearts | Adult heart | Function |
|---|---|---|---|
| Cox7a2l | 14x down | 17.7x up | Regulatory subunit of cytochrome c oxidase |
| Ndufb10 | 14x down | 3.2x up | Elec. transfer from NADH to the respiratory chain |
| Mylpf | 3.4x up | 3x up | Fast skeletal myosin light chain |
| Pdk4 | 1.7x up | 4.6x up | Sensor/modulator of glucose homeostasis |
| Tnnc2,Tnni2, Tpm2 | 3-10x up | 5.5x up | Fast skeletal troponins; Ca-dependent muscle contractility |
| Angptl4 | 4x up | 2x up | Inhibitor of lipoprotein lipase |
| Atp2a1 | 8x up | 9x up | Calcium ATPase pump; muscular contraction |
| Pvalb | 10x up | -- | Calcium shuttling protein; high on fast muscle |
| Calr | 2x up | -- | ER Ca-storage protein; protein folding |
| Mt1a | 2x up | 2.5x up | Inhibits ischemia/reperfusion induced injury |
| Ucp3 | 3x up | 2x up | Protects mitochondria against lipid-induced oxidative stress |




**Supplemental Figure 1.** miR-214 genetic deletion.
**(A)** Strategy for the conditional targeting of miR-214. The targeting vector and targeted allele are shown.
**(B)** Mutant miR-214 targeting of ES cells demonstrated by Southern blot. The WT targeted and mutant bands are shown. Genomic DNA was also subjected to PCR with primers for the 5' arm as depicted in (A). Genotypes of mice are shown on top.
**(C)** qPCR specific for pre-miR 199a-2 and for *Dnm3* performed using RNA from miR-214 WT and KO hearts. For each analysis, 2-3 mice of each genotype were tested per litter and three different litters were analyzed.
**(D)** RNA samples from WT and miR-214 KO littermates were subjected to RT-PCR and amplification using primers spanning the entire length of the *Dnm3os* transcript. Samples from WT and KO mice were run side by side for each primer set to detect any splicing differences between genotypes. Numbers above indicate the nucleotides of *Dnm3os* amplified for each primer set. n=3 for each group.

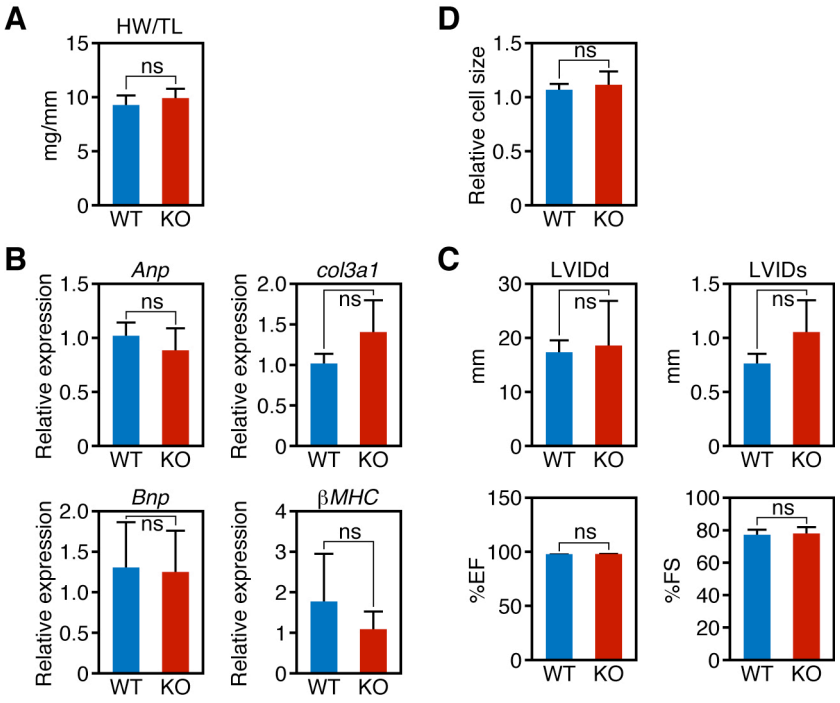



**Supplemental Figure 2.** Body weight, mitochondrial volume and cardiomyocyte size in miR-214 KO mice.

**(A)** Body weight of miR-214 KO mice and their WT littermates measured starting from 1 week to 12 weeks of age. While KO mice had modestly lower body weight at most time-points examined, the difference was not statistically significant.

**(B)** Electron microscopy images from miR-214 heterozygotes and miR-214 KO mice at 8 weeks of age were analyzed for mitochondrial volume by measuring the area (representative images Fig. 2C). At least 125 mitochondria were examined from each genotype and results are expressed relative to WT average volume.

**(C)** Wheat germ agglutinin staining was performed on sections from 4-month-old WT or miR-214 KO mouse hearts to assess cardiomyocyte size (expressed relative to WT). 8-10 40x fields were analyzed from each mouse (n=3). Data are mean values ± S.E.M., ns=not significant.



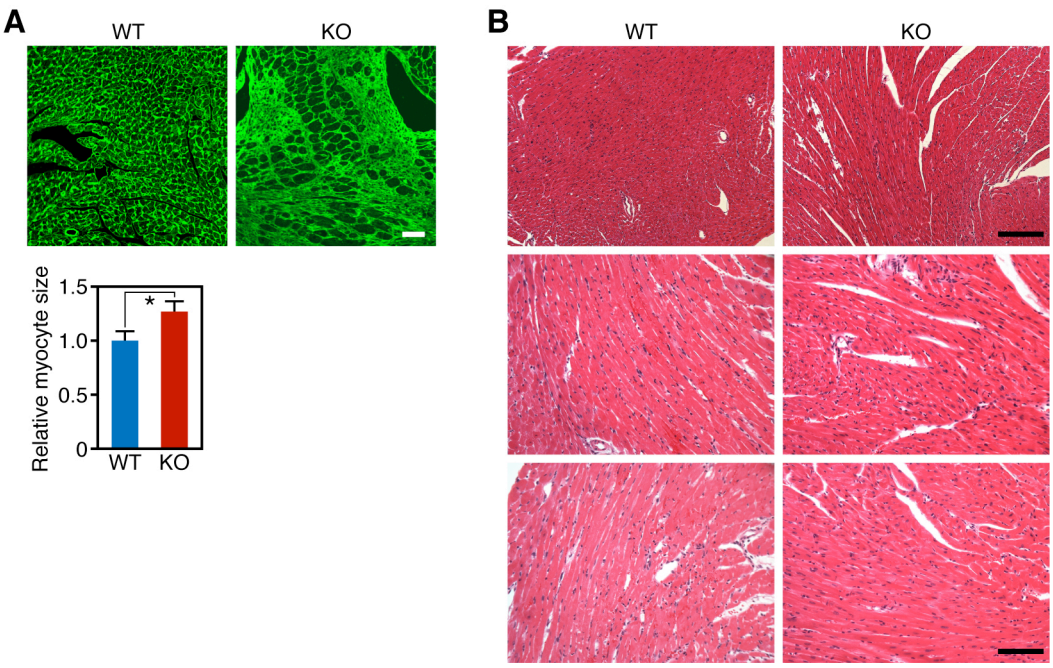

**Supplemental Figure 3.** Cardiac structure and function in miR-214 KO mice upon aging.

**(A)** Heart weight, tibia length (HW/TL) ratios shown for 10-12 month old mice. Mean values ± S.E.M. with n=3. ns=not significant.

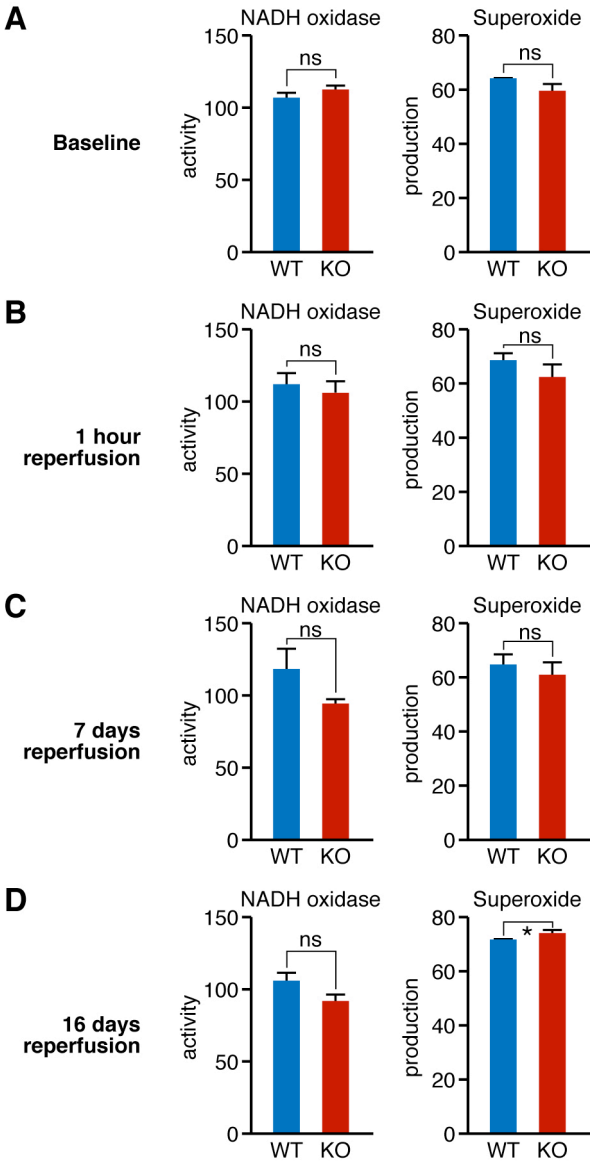
**(B)** qPCR expression analysis of cardiac stress response genes in miR-214 KO hearts relative to WT in 8-10 month old mice. n=3 per group. Data shown are the fold induction of gene expression normalized with 18S and expressed as mean ± S.E.M. ns = not significant.

**(C)** Cardiac function in WT and miR-214 KO mice at 10-12 months of age. Quantification of left ventricular internal diameter in systole or diastole (LVIDs or LVIDd), fractional shortening (FS) and ejection fraction (EF) is shown. Mean ± S.E.M. with n=3 per group. ns = not significant

**(D)** Wheat germ agglutinin staining to assess cardiomyocyte size on sections from 10-12-month-old WT or miR-214 KO mouse (expressed relative to WT). 8-10 40x fields were analyzed from each mouse (n=3). Data are mean values ± S.E.M., ns=not significant.

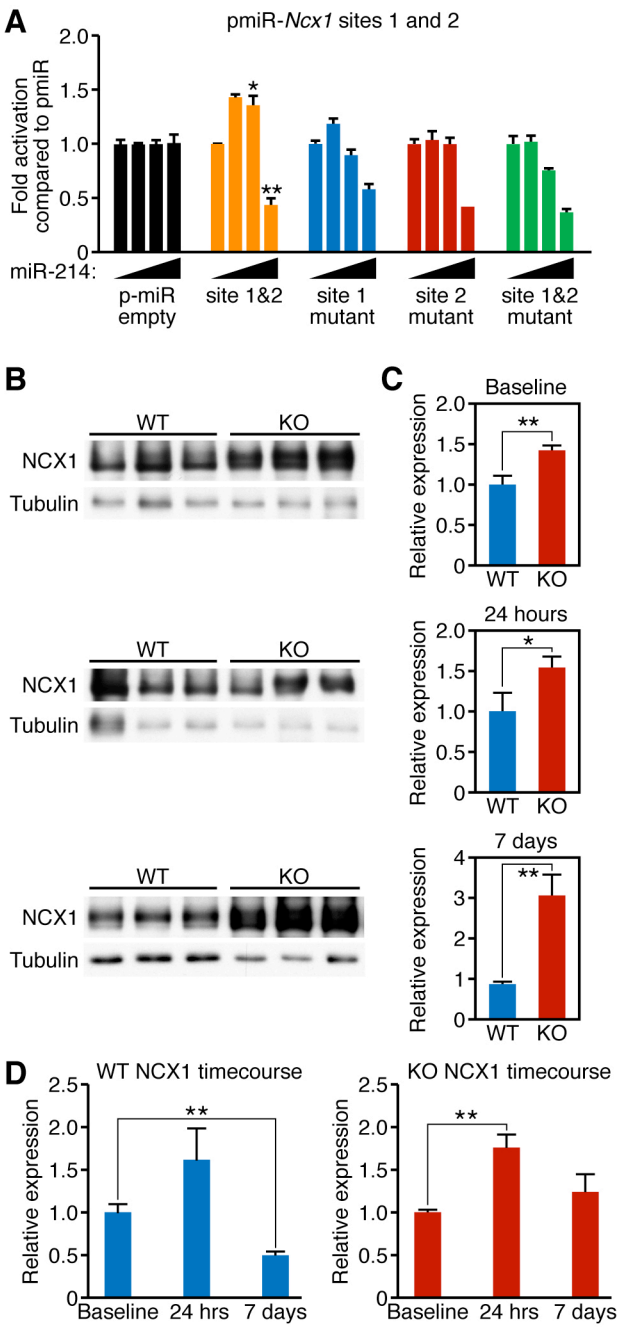



**Supplemental Figure 4.** Cardiomyocyte size and inflammation following IR.
**(A)** Representative images and quantification of wheat germ agglutinin stained heart sections from WT and miR-214 KO mice subjected to IR (7 days reperfusion). Cardiomyocyte size is expressed relative to WT. Mean value ± S.E.M. with n=3. *p=0.03
**(B)** Degree of inflammatory cell infiltration examined in transverse sections of miR-214 KO hearts and WT littermates at 24 hours of reperfusion stained with H&E. Representative images at 10x (top panels) and 20x (middle and bottom panels) are shown. Inflammatory cells were noted in both groups by nuclear morphology. Scale bars: 200μM (top panel) and 100μM (middle and bottom). n=3 mice per group.




**Supplemental Figure 5.** Mitochondrial function and superoxide production is normal in miR-214 KO hearts.

Enzymatic assays were performed to assess mitochondrial respiratory function and the rate of superoxide production in WT or miR-214 KO hearts at baseline (**A**), or after IR at 1 hour (**B**), 7 days (C) or 16 days (**D**) of reperfusion. NADH activity represents the time dependent, rotenone-sensitive, oxidation of NADH measured at 340 nm. Superoxide production represents the time dependent, SOD-sensitive, increase in hydroethidine oxidation. Mean values ± S.E.M. for all measurements. n= 3-5 mice per group. *p<0.05, ns=not significant




**Supplemental Figure 6.** Regulation of NCX1 and miR-214.
**(A)** pmiR luciferase assays for predicted miR-214 binding sites 1 and 2 in the 3' UTR of *Ncx1*. Luciferase assays were performed with the 3' UTR for the target gene *Ncx1* in HEK 293 cells. Luciferase activity for the constructs containing site 1and 2 and mutants of one or both of these sites are shown. Triangle below indicates increasing concentrations of transfected miR-214 expression plasmid (0, 50ng, 100ng, 200ng). Expression is normalized to β-galactosidase activity and relative to the activation level of the empty pmiR vector. Luciferase assays are performed in triplicate and are representative of 2-3 independent

experiments. Data are mean values ± S.E.M. *p<0.05; **p<0.005
**(B)** Side-by-side immunoblot analysis for NCX1 in heart lysates collected from miR-214 or WT littermates at baseline, and following IR (24 hours and 7 days of reperfusion). Tubulin is used as a loading control.
**(C)** Quantification of NCX1 levels in miR-214 KO hearts relative to WT levels at the same time-point.
**(D)** Quantification of NCX1 protein in WT mice or miR-214 KO mice relative to the same genotype at baseline to examine how NCX1 is regulated within each genotype following IR. Levels are normalized to Tubulin and expressed relative to baseline levels of the respective genotype. Data are representative of 2 independent experiments. Mean values± S.E.M. with n=3. *p<0.05; **p<0.01