SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

miR1-1 knockout (miR1-1$^{-/-}$:miR1-2$^{+/+}$) and miR1-2 knockout (miR1-1$^{+/+}$:miR1-2$^{-/-}$) mice[10] were generously shared by Dr. Deepak Srivastava at the Gladstone Institutes. Upon receival, these transgenic mice were crossbred back with C57BL/6N (Charles river) to generate miR1-deficient offspring with various genotypes, which were then intercrossed to generate 75%-miR1-knockdown (75%KD, miR1-1$^{+/-}$:miR1-2$^{-/-}$ or miR1-1$^{-/-}$:miR1-2$^{+/-}$) and wildtype (WT) siblings' offspring. All mice were housed following institutional guidelines at the Ohio State University (OSU); experiments were performed according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) under the protocol 2019A00000085. Both female and male mice with matched gender and ages were used for molecular and biochemistry studies, cell isolation, and functional examinations of animal hearts/cardiomyocytes. Researchers were blinded to groups when conducting experiments and data analyses.

Echocardiography and electrocardiogram

Echocardiography was performed by using a Vevo 3100 system (FUJIFILM VisualSonics) with a MX550S transducer as described previously.[48] Briefly, 75%KD and WT Mice (3-6 months) were anesthetized with 2.5% isoflurane in Oxygen. Anesthesia was maintained by administration of oxygen and ~1.25% isoflurane on a heated pad during the whole imaging procedure. Animal heart rate (HR) was monitored throughout the imaging procedure; recordings taken at a HR less than 400bpm were excluded from further analysis. Parasternal long-axis images, inclusive of two-dimensional loops and freeze-frame end-diastolic images, were collected to determine the left ventricular internal diameter (LVID), posterior wall thickness (LVPW), and interventricular septal thickness (LVS) in both systole (s) and diastole (d). Parasternal short-axis images at the level of the papillary muscles, inclusive of M-mode imaging, were obtained to determine the LV anterior wall dimension, LCPW and LVID in both systole and diastole. Fractional shortening (FS%) and ejection fraction (EF%) were calculated from the analyses of both parasternal long-axis two-dimensional real-time 3-beat loops and M-mode measurements.

Non-invasive surface electrocardiogram (ECG) recording was performed on anesthetized mice. ECG electrodes were inserted under the skin of three paws of mice for a 2-minute continuous recording. Animals were administrated epinephrine (2mg/kg) via intraperitoneal injection, followed by a 20-minute surface ECG recording to assess the risk of arrhythmogenesis. ECG data were subsequently analyzed using LabChart software.

In vivo administration of miR mimics through intravenous tail vein injections

Conscious littermate mice were placed in a restrainer tube. miR1 mimics or control mimics (0.67 nmol/per animal) were encapsulated with Polyethylenimine (PEI, Sigma-Aldrich) in 100 µL Opti-MEM™ I media (ThermoFisher Scientific) and delivered into animals via intravenous tail vein injection by using a BD insulin syringe (1/2cc) with ultra-fine 28G needle. miR administrations were conducted bi-weekly; the function of the heart was evaluated by echocardiography.

Optical mapping of the *ex vivo* heart

Optical mapping of the *ex vivo* heart was performed as described previously.[47] Briefly, mice were injected with heparin (500 units per mouse) and euthanized with Ketamine (100mg/Kg)/Xylazine (10mg/kg) mixture. Hearts were excised and cannulated for Langendorff perfusion with O_2-bubbled Tyrode solution (140 mM NaCl, 4.5 mM KCl, 1.25 mM $CaCl_2$, 0.7 mM $MgCl_2$, 10 mM HEPES, and 5.5 mM dextrose, adjust pH to 7.4 with NaOH) and placed in a heated custom-built Plexiglas chamber. Blebbistatin (6 μM) was added to the perfusate to eliminate motion artifacts during optical recordings. Hearts were perfused with a bolus of the voltage-sensitive dye 4-(2-(6-(Dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)pyridinium (di-4-ANEPPS, 10 μM, Sigma-Aldrich) and optical action potentials were recorded simultaneously from the anterior epicardial surface of the ventricles (10x10mm mapping field). Steady-state ventricular epicardial pacing was performed at a basic cycle length of 130ms. Arrhythmia inducibility was determined by programmed electrical stimulation with up to 3 premature stimuli (S1-S4 pacing protocol). Activation times were assigned for each action potential by identifying the greatest positive change in fluorescence during the upstroke of the action potential. Local conduction velocity at each recording site was calculated during steady-state pacing and determined by averaging 10-15 sites corresponding to longitudinal and transverse fiber orientation. Ventricular tachycardia (VT) was defined as lasting >3 beats.

Isolation of adult ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from the left ventricle of age-matched (3-5 months) adult wild-type and 75%KD mice by using an enzymatic dispersion technique as described previously.[49] The Tyrode's solution (130 mM NaCl, 5.4 mM KCl, 3.5 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 20 mM taurine, 10 mM glucose, 5 mM HEPES, adjust pH to 7.35 with NaOH) was used as the basic solution with modification at different steps of cell isolation. Briefly, hearts were cannulated for Langendorff perfusion (at 37°C) with calcium-free Tyrode's solution containing 0.1mM EGTA for 2.5 minutes, then with enzyme solution (1mg/ml Type II collagenase [Worthington] with 0.05 mM $CaCl_2$) for 13 minutes (recycling the solution). The left ventricle tissue was washed and trimmed with high potassium solution (110 mM potassium L-glutamate, 25 mM KCl, 2 mM $MgSO_4 \cdot 7H_2O$, 10 mM KH_2PO_4, 20 mM taurine, 5 mM creatine, 0.5 mM EGTA, 5 mM HEPES, 20 mM glucose, adjust pH to 7.35 with KOH). Gentle trituration was performed and the resultant cell suspension was filtered by using a 100-μm cell strainer (Corning) to remove undissociated tissues. Final cells were collected by centrifuge, re-suspended, and stored in Medium 199 for whole-cell patch clamp recording on the same day at 35℃.

Electrophysiology of ventricular cardiomyocytes

Action potentials (APs) were recorded in individual ventricular cardiomyocytes of WT and 75%KD mice by ruptured-patch whole cell current clamp. Briefly, isolated myocytes were bathed in a chamber continuously perfused with Tyrode's solution (134 mM NaCl, 4.5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM Dextrose, 5 mM HEPES, adjust pH to 7.35 with NaOH). Patch pipettes were pulled from borosilicate capillary glass using a Sutter micropipette puller P-97 and lightly fire-polished to resistance 0.9-1.5 MΩ when filled with electrode solution (120 mM K-aspartic acid, 20 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM K_2ATP, 11 mM EGTA, 5 mM HEPES, pH 7.3). Action potentials were elicited in current clamp mode using a supra-threshold depolarizing current pulse (5ms) at 1Hz. The membrane potential data were adjusted by subtracting the junction potential of 14.3mV. Command and data acquisition were operated with an Axopatch 200B patch clamp amplifier controlled by a personal computer using a Digidata 1200 acquisition board driven by pCLAMP 9.0 software (Axon Instruments, Foster City, CA).

I_{to}, and $I_{Ca,L}$ currents were recorded by ruptured-patch whole cell voltage clamp and normalized with cell capacitance. Cell capacitance and series resistance were compensated electronically at ~80%. To measure I_{to}, cells were placed in the same Tyrode's solution as for AP recordings, and microelectrodes were filled with a solution (pH 7.3) containing 120 mM aspartic acid, 20 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, 10 mM NaCl, 5 mM EGTA, 0.3 mM Na-GTP, 14 mM Phosphocreatine, 4 mM K-ATP, 2 mM Creatine phosphokinase. I_{to} amplitude was measured as the difference between peak current and steady-state current during a 400-ms voltage step ranging from –30 to +80 mV from a holding potential of –40 mV. To measure $I_{Ca,L}$, isolated cells were placed in a solution (pH 7.3) consisting of 137 mM NaCl, 5.4 mM CsCl, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES. Microelectrodes were filled with a solution (pH 7.3) containing 130 mM CsMES, 20 mM TEA Cl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, 0.3 mM TRIS GTP, 14 mM Phosphocreatine, 4 mM Mg ATP, 2 mM Creatine phosphokinase. $I_{Ca,L}$ were elicited from a holding potential of -40 mV with depolarizing voltage pulses from -30 mV to 60 mV for 300 ms.

Na^+ currents (I_{Na}) were recorded by ruptured-patch whole cell voltage clamp at room temperature and normalized with cell capacitance. Microelectrodes were filled with a solution (pH7.3) containing CsF 120 mM, $MgCl_2$ 2 mM, HEPES 10 mM, and EGTA 11 mM. Isolated myocytes were placed in the solution (pH 7.3) containing NaCl 25 mM, N-methyl D-glucamine 115 mM, CsCl 5 mM, $MgCl_2$ 1 mM, $NiCl_2$ 1 mM, glucose 10 mM, and HEPES 10 mM. I_{Na} were elicited from a holding potential of -80 mV with depolarizing voltage pulses from -60 mV to 45 mV for 50 ms with an increment of 5 mV.

Ca^{2+} transients and sarcomere shortening

To measure intracellular Ca^{2+} transients, isolated ventricular cardiomyocytes were loaded with Fura-2-AM (1 μg/ml) for 20 min. Cells settled on the glass bottom of a flow chamber and perfused with Tyrode's buffer (4 mM KCl, 131 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, and 10 mM glucose, pH 7.4) at 1 ml/min at ~35°C. Fura-2 fluorescence (340/380nm) and sarcomere shortening were measured by using an IonOptix system. Cells were field-stimulated at 1 Hz, 3 Hz or 5 Hz. Data were analyzed by the IonOptix software. Caffeine (10 mM) was used to study Ca^{2+} stock of sarcoplasmic reticulum (SR).

Transmission Electron Microscopy

Ultrastructure of WT and 75%KD cardiomyocytes were studied by electron microscopy in the Campus Imaging Facility (CMIF) at the OSU. 75%KD or WT heart tissues were dissected and fixed in 2.5% glutaraldehyde (product #18426, Ted Pella Inc., Redding, CA) in 0.1 M phosphate buffer. Samples were post-fixed with 1% osmium tetroxide (product #18456, Ted Pella Inc.) and then en bloc stained with 1% aqueous uranyl acetate (product #19481, Ted Pella Inc.), dehydrated in a graded series of ethanol, and embedded in Eponate 12 epoxy resin (product #18012, Ted Pella Inc.). Ultrathin sections were cut with a Leica EM UC7 ultramicrotome (Leica microsystems Inc., Deerfield, IL). Images were acquired with an FEI Technai G2 Spirit BioTwin transmission electron microscope (Thermo Fisher Scientific, Waltham, MA) operating at 80kV, and a Macrofire (Optronics, Inc., Chelmsford, MA) digital camera and AMT image capture software.

Masson's Trichrome staining

To study the fibrosis of the heart, 75%KD, and WT adult heart tissue were fixed in 4% paraformaldehyde, embedded in paraffin, and used for a standard Masson's trichrome staining. Briefly, paraffin-embedded heart tissue was stained with hematoxylin for the nuclei, ponceau red liquid dye acid

complex for all the acidic tissues, and then incubated with the phosphomolybdic acid solution. Lastly, the tissue was stained with aniline blue liquid with 1% acetic acid added for collagen and photographed with a microscope.

Whole transcriptomic and proteomic assays

Total RNA was extracted from isolated cardiomyocytes of 75%KD or WT mice by using RNeasy Kits (Qiagen). Library preparation was performed with ribosomal RNA-deletion method and sequencing was achieved on HiSeq (Illumina), with 2 × 150 bp paired-end reads. Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Mus musculus GRCm38 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. Unique gene hit counts were calculated by using Feature-Counts from the Subread package v.1.5.2. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Comparison of gene expression between 75%KD and WT was performed by using DESeq2. The Wald test was used to generate p-values and log2 (fold changes). Genes with an adjusted p-value < 0.05 and absolute fold change > 1.5 were called as differentially expressed genes for each comparison.

For proteomic assay, 75%KD or WT ventricular tissues were homogenized/lysed, digested with sequencing grade trypsin and quantified using Pierce Quantitative Colorimetric Peptide Assay kit (Thermo Fisher Scientific). Peptides (25 μg) were taken from each sample and labeled with tandem mass tags (TMT10plex, Thermo Fisher Scientific). Labeled samples were fractionated using a high pH reversed phase HPLC, followed by LCMS analysis with ThermoScientific Fusion Lumos mass spectrometry system. The data were searched against the mouse UniProtKB databases with the program Proteome Discoverer 2.1 and samples were analyzed utilizing an LC gradient from 2 to 80% acetonitrile in 2hrs. Two different filters were used to identify differentially expressed proteins those display a fold change greater to 1.2 with a p-value less than 0.05.

For the analyses of transcriptomic and proteomic data, the biological processes associated with these genes were determined by using CluegO[50] plugged-in Cytoscape network analysis tool[51] ($p< 0.05$). GSEA was performed using the GSEA software (v.4.2.3, UC San Diego and Broad Institute)[52,53] on normalized reads ranked based on log2 (fold change). Gene sets were extracted from the available collection in the Molecular Signatures Database (MSigDB)[52,54,55]. Protein-protein association network was generated with String database[56] and visualized within Cytoscape. Only proteins physically or functionally interacting with miR1 target proteins were reserved for the gene network.

Western blotting

75%KD or WT hearts were perfused with Ca^{2+}-free Tyrode Buffer, minced into small tissue pieces, washed twice with ice-cold PBS, homogenized with a tissue tearor, and used for protein extraction with RIPA buffer (Thermo Fisher Scientific). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Primary antibodies including anti-Kir2.1 (Alomone Lab), and anti-GAPDH (Sigma) were used.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from isolated adult ventricular cardiomyocytes using TRIzol RNA isolation reagents buffer (Thermo Fisher Scientific) according to the manufacturer's instructions. High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA synthesis of mRNA; qPCR of

mRNA was performed by using the SsoFast-EvaGreen Supermix with Low ROX (Bio-Rad). TaqMan™ MicroRNA Reverse Transcription Kit (Life Technologies) was used for converting miRNA to cDNA; qPCR of miRNA was performed using the TaqMan advanced miRNA assay (Applied Biosystems) on thermocycler ABI Prism 7500 fast (Applied Biosystems).

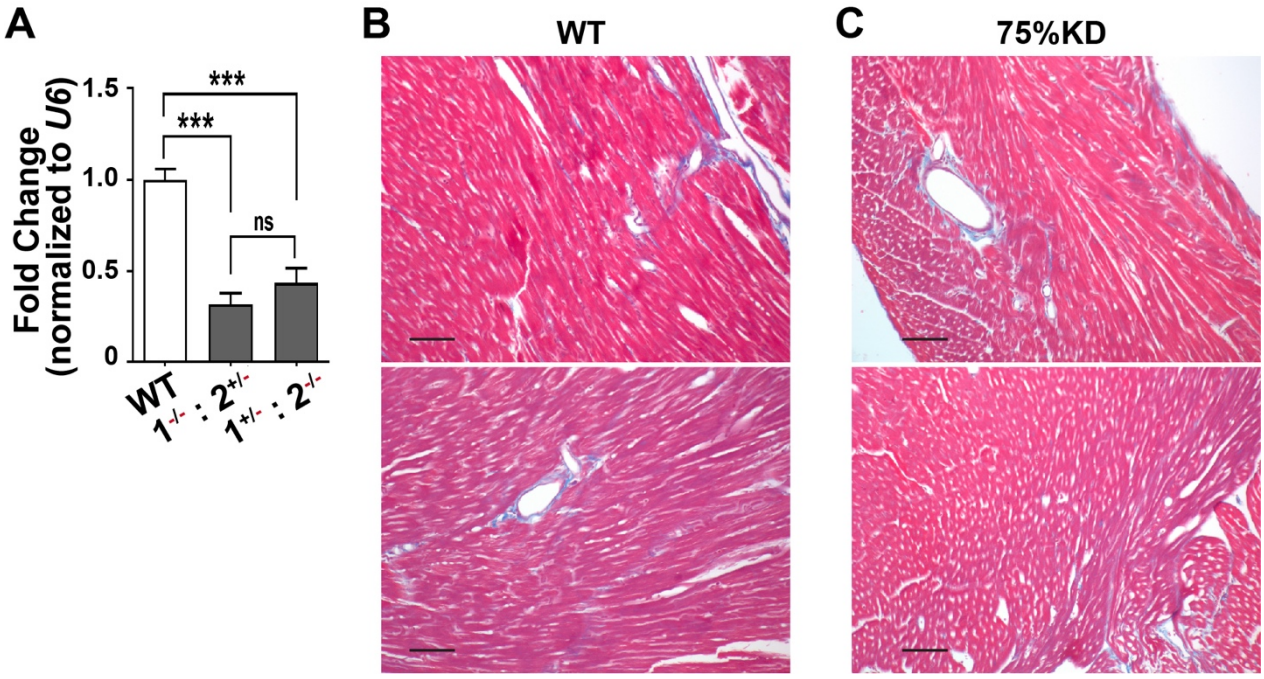
Quantification and statistical analysis

All biochemistry experiments have been replicated at least three times independently. Data are expressed as mean±S.E.M, with n indicating the number of distinct biological samples. Statistical significance of differences in means was estimated by one-way ANOVA and subsequent Student's t test, Log-rank (Mantel-Cox) test, or 1-sided Fisher exact test by using GraphPad Prism version 9.4.1 for Windows (GraphPad Software, www.graphpad.com). $p<0.05$ indicates a statistical significance.

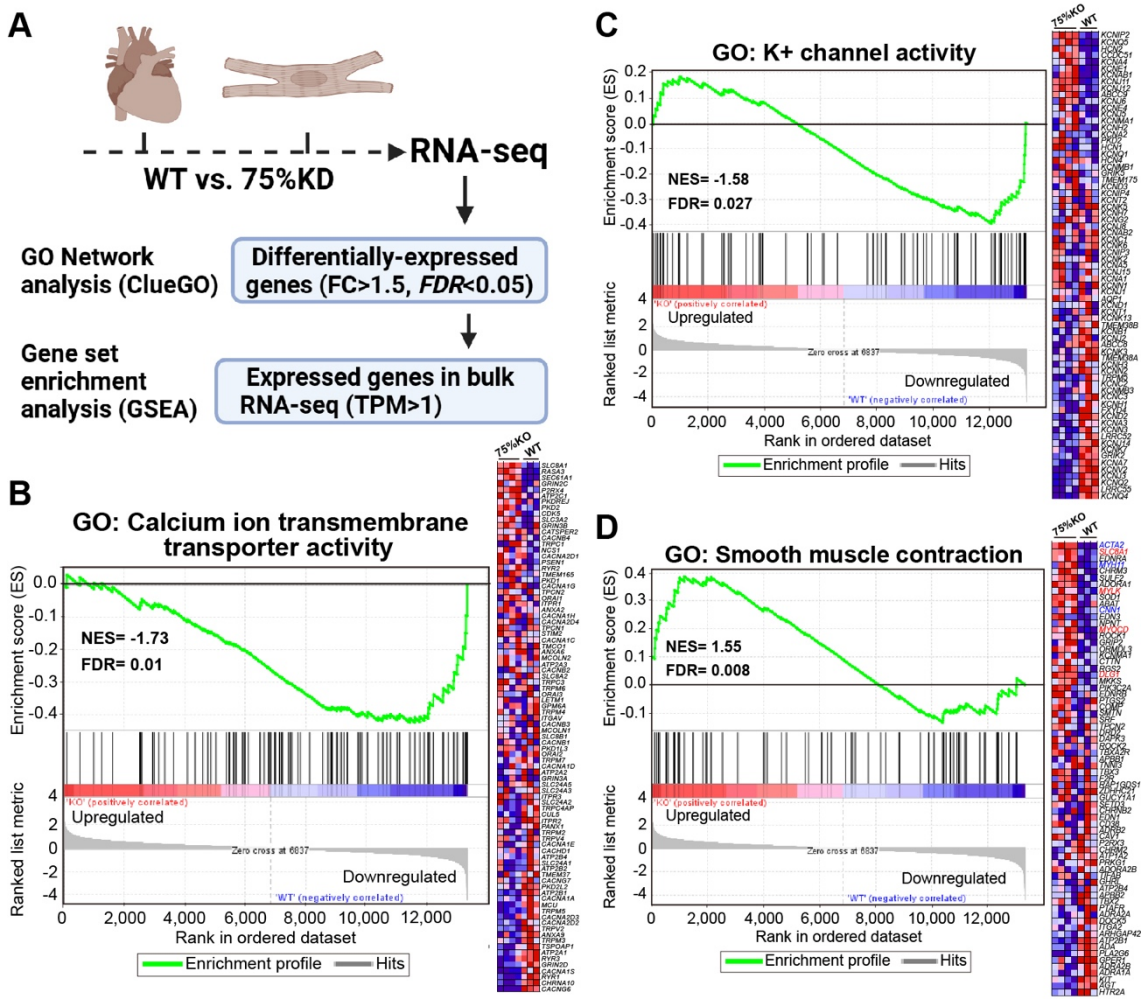
Data availability

The raw and processed RNA/Protein-sequencing data generated in this study will be deposited in the NCBI GEO database upon publication. The data that support the findings of this study are available from the corresponding author upon reasonable request.

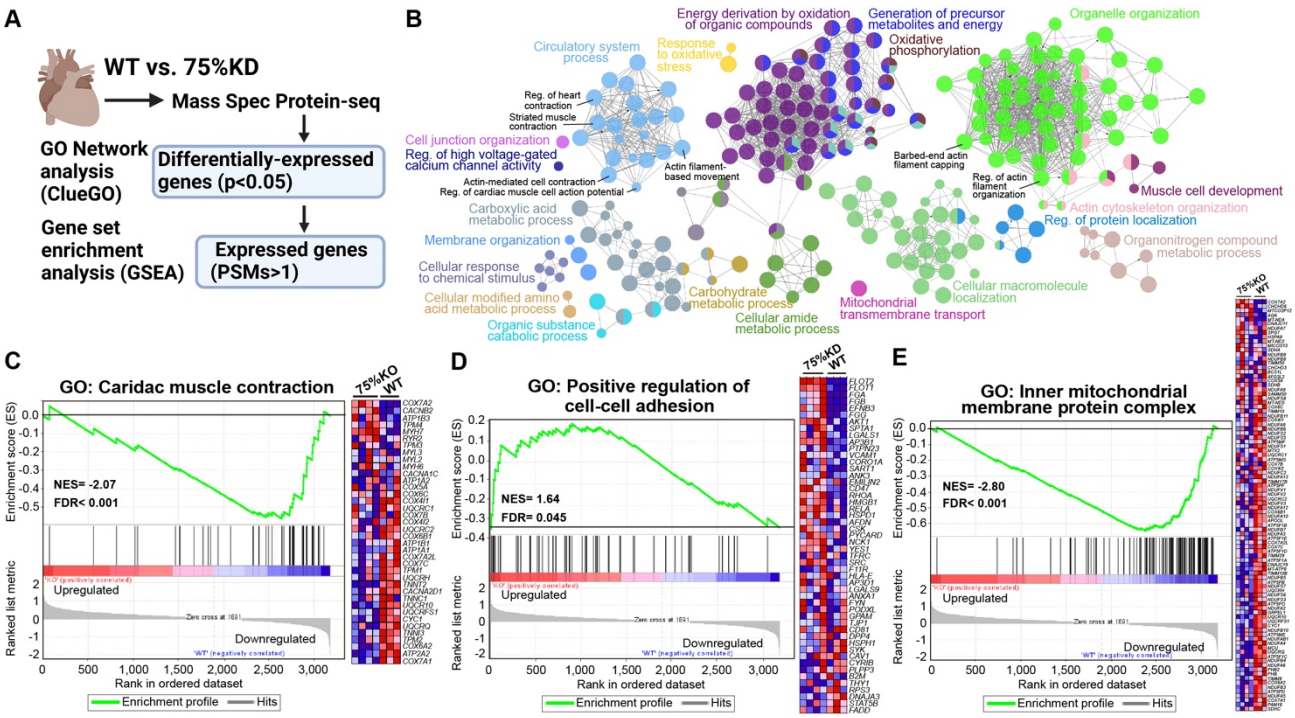
Supplemental Figures and Figure Legends



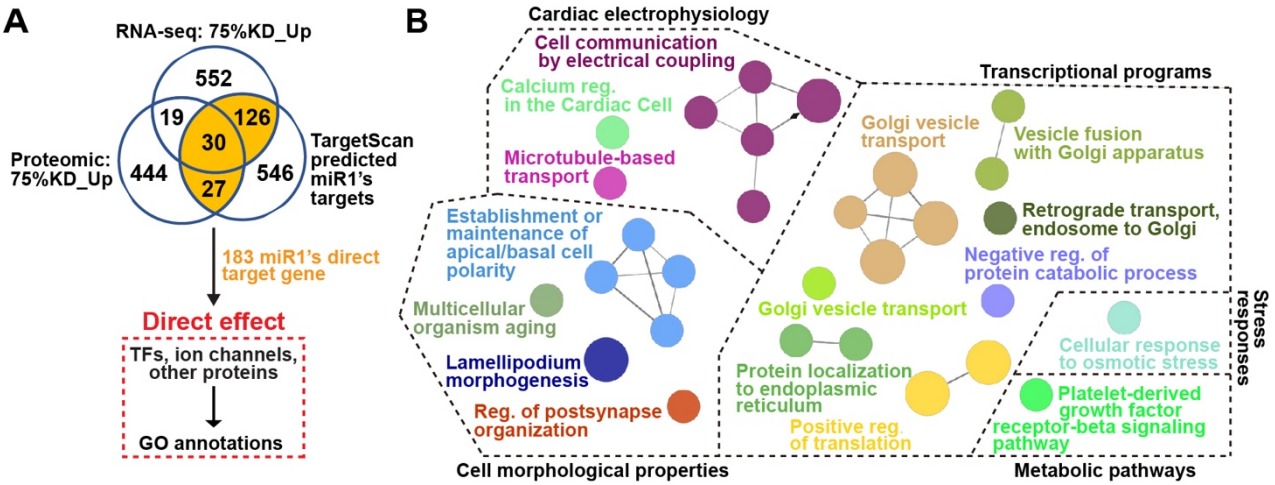
S-Fig I. (**A**) qPCR analysis showing lower expression level of miR in the heart of adult 75%KD mice ($1^{+/-}$:$2^{-/-}$ or $1^{+/-}$:$2^{-/-}$ genotype, n=5 for each group, P<0.001) compared to that in WT mice (n=7). (**B-C**) Masson's Trichrome staining showing comparable levels of fibrotic tissues between WT (B, n=3) and 75%KD hearts (C, n=3). Bars indicate 100 μM.



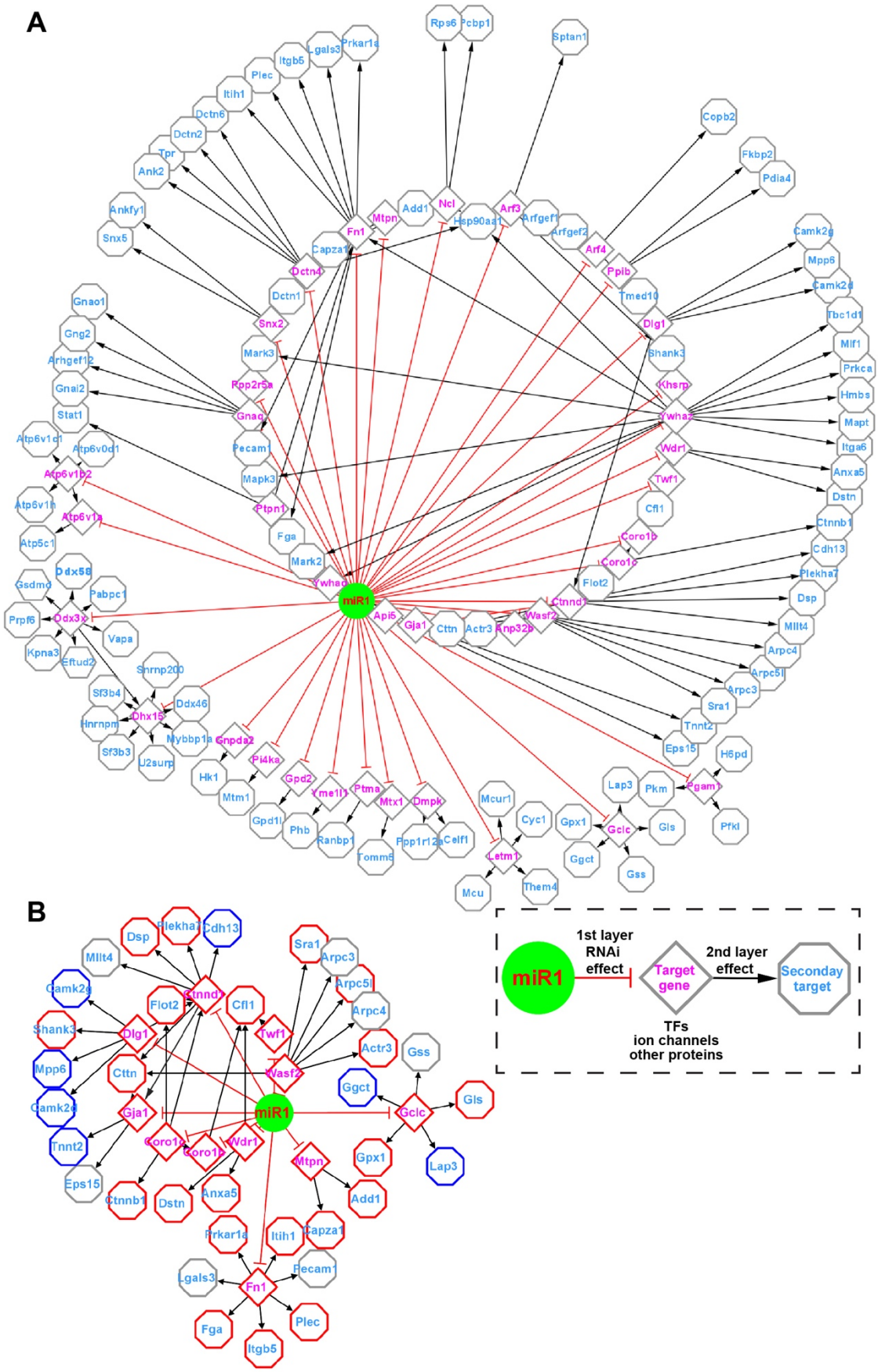
S-Fig II. Gene set enrichment analysis (GSEA) of transcriptome comparison between 75%KD and WT myocytes. (**A**) Experimental scheme of RNA sequencing for GO term identification and downstream gene set enrichment analysis. (**B-D**) Testing of the regulation of specific GO terms, including voltage-gated calcium channel activity (**B**), potassium channel activity (**C**), and smooth muscle contraction (**D**), along with heatmaps depicting the expression of individual component genes from low (blue) to high (red). miR1 target genes associated with smooth muscle cells (SMCs) are highlighted in red, while other SMC cell-fate regulators are highlighted in blue.



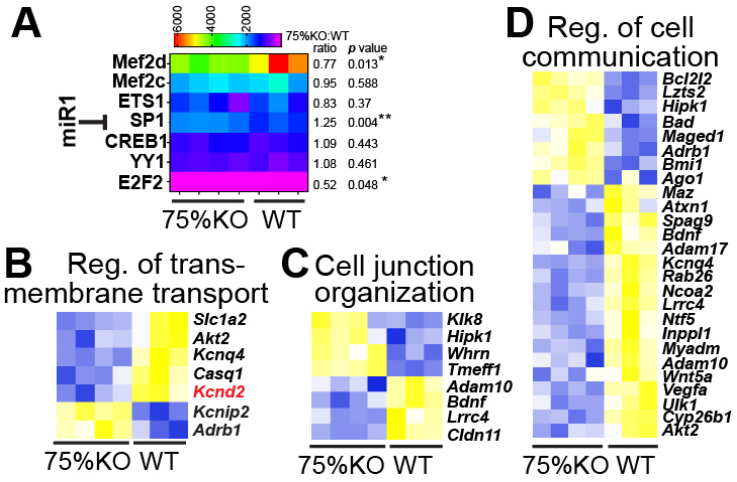
S-Fig III. Proteomic profiling reveals dysregulated biological process networks upon miR1 deficiency. (**A**) Experimental scheme of mass spectrometry for GO term identification and downstream gene set enrichment analysis. (**B**) ClueGO analysis of differentially expressed proteins in 75%KD and WT hearts, highlighting broad categories of biological processes that were altered due to miR1 deficiency. Each network node represents a specific biological process, and network edges illustrate relationships between different terms. Nodes of the same color belong to functionally similar terms or pathways. Node size indicates the significance of term enrichment (P<0.0005 to <0.05). (**C-E**) GSEA of proteomic assays revealed negative regulation of the GO term cardiac muscle contraction (C), inner mitochondrial membrane protein complex (D), and positive regulation of cell-cell adhesion (E) in 75%KD hearts. NES determines negative or positive enrichments in 75%KD hearts. An *FDR* < 0.05 indictes a significant enrichment. Heatmaps represent the expression levels of individual genes in different terms, ranging from low (blue) to high (red).



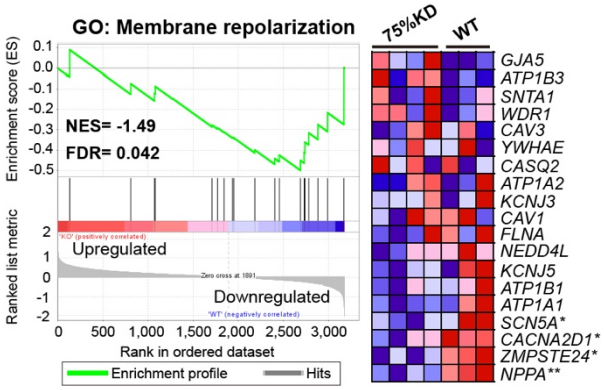
S-Fig IV. Pathway analysis of miR1 direct target genes dysregulated in 75%KD hearts. **(A)** Venn diagram depicting the overlap among predicted miR1's target genes by TargetScan and the upregulated genes in 75%KD hearts identified through RNA sequencing and proteomic assays. (**B**) ClueGO annotation of 183 miR1 direct target genes that displayed upregulation at either transcriptional or protein levels in 75%KD hearts. Each node in the network represents a specific biological process, and edges indicate the relationships of terms. Nodes of the same color indicate functionally similar terms. Node size represents the significance of term enrichment (P<0.0005 to <0.05).


A
B
miR1
1st layer
RNAi
effect
Target
gene
2nd layer
effect
Seconday
target
TFs
ion channels
other proteins

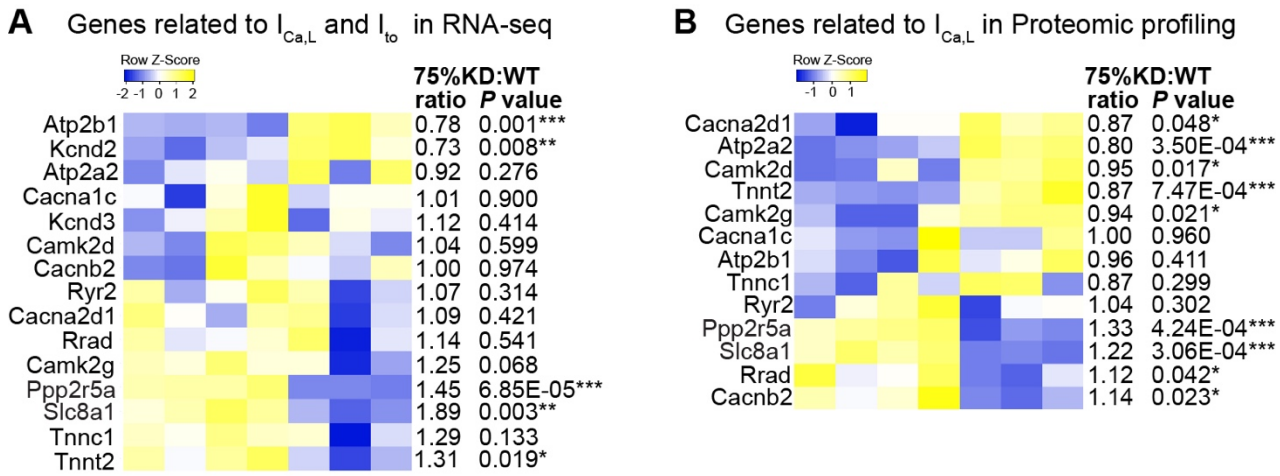
S-Fig V. miR1 broadly targets regulatory molecular networks through protein-protein associations. (**A**) Molecular networks of miR1 targets, which exhibited upregulated upon miR1-deficiency, were generated using the String database and visualized within Cytoscape. Each node represents a distinct protein; red edges indicate 1st-layer effect of miR1's direct target (highlighted in pink), while black edge represents 2nd effects through protein-protein association, as shown in the dashed line box. (**B**) An enlarged view of miR1-regulated gene networks related to cell adhesion, communication, and the actin cytoskeleton. Genes outlined in red were upregulated, whereas genes outlined in blue were downregulated in 75%KD hearts.



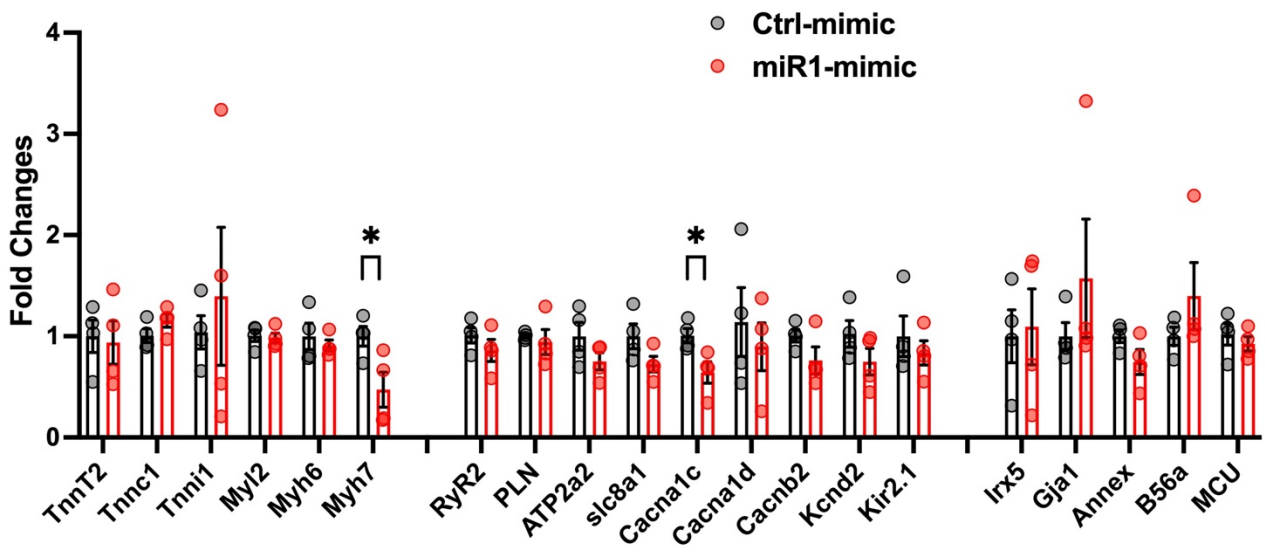
S-Fig VI. Transcription factors function as important mediators of miR1-initiated regulatory effects. (**A**) DESeq2-normalized counts of miR1-targeted transcription factors in RNA-seq. (**B-D**) Numerous transcripts, which contain at least one SP1 binding site and are regulated by SP1, were significantly changed in 75%KD cardiomyocytes. Those SP1-targeted transcripts were associated with biological processes related to the regulation of transmembrane transport (B), cell junction organization (C), and cell communication (D). The heatmaps depict the expression levels of individual genes, ranging from low (blue) to high (yellow). Notably, *Kcnd2*, highlighted in red, is a miR1 direct target gene.



S-Fig VII. The gene ontology term "membrane repolarization" was negatively enriched in 75%KD cardiomyocytes. GSEA was conducted by comparing the transcriptomes of 75%KD and WT groups. The mormalized enrichment score (NES) indicated a negative enrichment in 75%KD cardiomyocytes. A significant enrichment was determined by a false discovery rate (*FDR*) of less than 0.05. The heatmap illustrates the expression level of individual genes associated with the "membrane repolarization" term, ranging from low (blue) to high (red).



S-Fig VIII. Summarized expression of Ca^{2+} handling proteins at the mRNA (**A**) and protein (**B**) levels in 75%KD and WT cardiomyocytes. The heatmaps provide an overview of the expression levels of individual genes involved in Ca^{2+} handling in 75%KD and WT hearts. The color gradient ranges from low (blue) to high (yellow), representing varying expression levels.



S-Fig IX. qPCR analysis comparing the expression of cardiac genes, including myofilament component genes and Ca^{2+} handling genes, in the hearts treated with control (Ctrl) or miR1 mimics. Gene expressions were first normalized to the expression of *Gapdh* housekeeping gene and then further normalized to the average mean of the Ctrl group. *P<0.05 versus Ctrl group. The statistical significance of differences was assessed using unpaired two-tailed Student *t*-tests.