Expanded Materials & Methods

Cell culture and genetic modification

Authentic cell lines, HT1080 cells, HEK293, and HEK293T cells, were obtained from the American Type Culture Collection. Neonatal mouse cardiomyocytes from 1-2 day-old mice were isolated and cultured on a culture dish coated with 1 μg/mL of fibronectin [45]. Adult mouse cardiomyocytes were isolated from animals at 15 weeks of age using a standard perfusion protocol [46], plated on laminin (10 μg/mL) coated culture dishes and incubated in the presence of insulin (1 μg/mL). This insulin concentration was chosen as a standard dose for cultured cardiomyocytes [47]. Adult mouse cardiac fibroblasts were isolated from the heart as described [48]. Monocytes were extracted from adult mouse splenocytes using EasySep Mouse Monocyte Isolation Kit (Stemcell Technologies). Cells were maintained at 37°C in Dulbecco's modified Eagle medium containing penicillin/streptomycin and 10% fetal bovine serum. For overexpression experiments, the human alpha arrestins were subcloned into pCDH-CMV-MCS-EF1-GFP-T2A-Puro with a Strep/FLAG (SF) tag (System Biosciences) or pEGFP-C3 plasmid (Addgene). For genetic knockdown, small interfering RNA (siRNA) of human ARRDC4 (Thermo Fisher Scientific, Catalog# 4427037, siRNA ID s40820) or a non-targeting negative control siRNA (Thermo Fisher Scientific, Catalog# 4390843) was used. Cells were transfected using PureFection transfection reagent (System Biosciences). For adenoviral overexpression, recombinant adenoviral constructs expressing mouse *Arrdc4* in tandem with mCherry were generated using the pAV[Exp]-CMV vector system. Cells were infected with an adenoviral gene vector at a multiplicity of infection of 20 for 18 hr. In all experiments, the identical construct expressing empty vector (EV) or the non-targeting siRNA control was used as control. For hypoxic experiments, cells were incubated at 1% O_2 balanced with 94% N_2 and 5% CO_2 at 37°C in a hypoxic chamber (Coy Laboratory Products Inc.). Dyngo, a dynamin inhibitor, was obtained from Abcam. N-acetylglucosamine (GlcNac), uridine diphosphate-GlcNac (UDP-GlcNac), and

lactic acid were purchased from Sigma. A cell-permeable clathrin inhibitor Pitstop1 (2-(4-Aminobenzyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinoline-5-sulfonic acid sodium salt) and its negative control (2-(4-Aminobenzyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinoline-6-sulfonic acid potassium salt) were obtained from Abcam.

Gene and protein expression analyses

Gene expression was analyzed by real-time quantitative PCR as described [12] using Taqman primers (Thermo Fisher Scientific) for human *ARRDC4* (Assay ID: Hs00411771_m1), mouse *Arrdc4* (Mm00508442_m1), human *XBP1* (Hs00231936_m1), mouse *Xbp1* (Mm00457357_m1), human *ATF4* (Hs00909569_g1), mouse *Atf4* (Mm00515324_m1), human *ATF6* (Hs00232586_m1), mouse *Atf6* (Mm01295317_m1), human *CHOP* (Hs00358796_g1), mouse *Chop* (Mm00492097_m1), mouse *Bnp* (Mm01255770_g1), mouse *Col1a1* (Mm00801666_g1) and *Col3a1* (Mm00802331_m1). Relative amounts of mRNA were normalized with human *GAPDH* (Hs02786624_g1) or mouse *Gapdh* (Mm99999915_g1). Thermocycler conditions used in amplification are enzyme activation at 95 °C for 20 sec (1 cycle), denaturation at 95 °C for 1 sec, and annealing/extension at 60 °C for 20 sec (40 cycles) using QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The $\Delta\Delta$Ct method was used to calculate the relative gene expression of samples.

Protein expression was analyzed by Western blotting. After adjusting protein concentrations, each sample was separated by electrophoresis on NuPAGE Bis-Tris gels (Thermo Fisher Scientific). Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific) was used to extract membrane fractions of proteins. Immunoblotting was carried out using the primary antibodies against ARRDC4 (Thermo Fisher Scientific, Catalog# PA5-38852), ATF4 (Cell Signaling Technology, Catalog# 11815), CHOP (Cell Signaling Technology, Catalog# 2895), GLUT1 (Abcam, Catalog# ab115730), pan-cadherin (Abcam, Catalog# ab51034), mCherry (Abcam, Catalog# ab167453), GAPDH (Cell Signaling Technology,

Catalog# 8884), and HRP conjugated anti-rabbit (Bio-rad, Catalog# 1721019) or anti-mouse (Bio-rad, Catalog# 1721011) IgG as a secondary antibody. We also performed immunostaining with an isotype control antibody (rabbit IgG, Abcam, Catalog# ab172730) or the secondary antibody alone to distinguish non-specific background signals. Enhanced chemiluminescent (ECL) reagents were used to detect horseradish peroxidase on immunoblots exposed to X-ray film in a darkroom. The size of proteins on SDS-PAGE gels was determined by a pre-stained protein standard (Thermo Fisher Scientific, Catalog# LC5925).

Pulldown assay

HEK293T cells were transfected with human *ARRDC4*, its truncated or mutated form in pCDH-CMV-MCS-EF1-GFP-T2A-Puro with a Strep/FLAG (SF) tag. Human GLUT1 (*SLC2A1*) cDNA was subcloned into the same plasmid with a hemagglutinin (HA) tag and co-transfected with *ARRDC4*. Cells were lysed, and a pulldown assay was performed with magnetic Strep-Tactin beads (IBA Lifesciences) as previously described [41, 49]. Western blot analyses of pulled-down proteins were performed with anti-FLAG (Abcam, Catalog# ab1162 or MBL International, Catalog# M185-7) and anti-HA (Abcam, Catalog# ab9110 or MBL International, Catalog# M180-7) antibodies.

Assays for cellular glucose metabolism

We generated a pCDH-EF1-MCS1-puro plasmid encoding mCherry-conjugated human GLUT1 with an HA epitope tag in the first exo-facial loop (55-64 amino acid). Cell surface GLUT1 was detected by anti-HA (Abcam, Catalog# ab9110 or MBL International, Catalog# M180-7) and Alexa Fluor 405-conjugated secondary (Abcam, Catalog# ab175660) antibodies in non-permeabilized cells. Total expression of exogenous GLUT1 was estimated by fluorescence levels of mCherry. Fluorescence emitted by Alexa Fluor 405 or mCherry was quantified by SpectraMax i3x microplate reader (Molecular Devices), and the ratio between cell surface over

total GLUT1 was calculated. Localization of GLUT1-mCherry or ARRDC4-GFP was monitored by confocal imaging with a Zeiss LSM 800 confocal microscope.

For glucose uptake assay, cells were incubated with 100 μM 2-deoxy-D-glucose and 0.3 μM 2-[^{3}H]deoxy-D-glucose (1 mCi/ml, PerkinElmer Life Science) for 30 min as described [49]. The radioactivity in cell lysates was measured by liquid scintillation counting with an LS6500 scintillation counter (Beckman Coulter). The values were divided by the total protein content of the cell lysates to yield uptake per weight of total protein and normalized to the level of control. Tissue levels of glycogen were measured by a glycogen assay kit (Sigma). Cellular amounts of ATP were analyzed by CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Assays for ER stress, oxidative stress, and cell death

A cellular response for endoplasmic reticulum (ER) stress was quantified using ERAI ER Stress Detector system (Cosmo Bio). Briefly, cells were transfected with a plasmid containing a reporter gene in which luciferase was fused with the 3' exon side of the intron region of *XBP1*. In this system, ER stress splices the *XBP1* intron sequence, and the luciferase-fused XBP1s (activated XBP1) increases luciferase activities. Cellular response to oxidative stress was evaluated by NRF2 activation with OKD Oxidative Stress Detector system (Cosmo Bio). In this assay, cells were transfected with a plasmid containing a reporter gene in which luciferase was fused with the NRF2 ubiquitination domain. Oxidative stress stabilizes the luciferase-fusion protein and increases luciferase activities. For ER stress and oxidative stress analyses, luciferase activities were measured by ONE-Glo™ Luciferase Assay System (Promega). Glutathione level was quantified by EarlyTox Glutathione Assay Kit (Molecular Devices).

The number of dead cells was counted by trypan blue (Thermo Fisher Scientific) uptake using a hemocytometer and light microscopy [50]. Cellular LDH release was measured by CytoTOX-One homogeneous membrane integrity assay (Promega). Types of cell death were

determined by Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell) and Apoptosis/Necrosis Assay Kit (Abcam). Caspase-3/7 activities were measured using EarlyTox Caspase 3/7 R110 Assay Kit (Molecular Devices).

Generation of *Arrdc4*- KO mice

We generated a novel *Arrdc4*-KO mouse model on a C57BL/6 background by CRISPR/Cas9 genome-editing technology. Two pairs of gRNA were designed to target the wild-type *Arrdc4* gene exons 1-8 (NM_ 001042592.2) on mouse chromosome 7. The gRNA target sequences were gRNA1 (forward) 5'-CGGGGTTACGAAATCCTTTTGGG-3' and gRNA2 (reverse) 5'-ACTAGGCAACCAACTCATCGTGG-3' with the protospacer adjacent motif (PAM) shown underlined. The gRNAs and Cas9 mRNA were injected into fertilized mouse eggs to generate targeted knockout offspring. F0 founder mice were identified by PCR of tail-extracted DNA to verify proper targeting to the *Arrdc4* locus and bred to wild-type mice to test germline transmission. F1 animals were further crossed with wild-type C57BL/6 mice (Taconic) to generate heterozygous mice, which were intercrossed to generate homozygous *Arrdc4*-KO mice and wild-type littermate control mice. PCR primers for the KO allele were Primers F (forward) 5'-AAGTATGCGTTTGGAAGGAGTTAGGG-3', R1 (reverse) 5'-CAAGTGGAGACCTCCCAGAGAATGTAG-3', and R2 (reverse) 5'-TTGTACTAGAACATGAACTTGTGGCTGC-3'. The successful deletion of exons 1-8 in F1 animals was confirmed by DNA sequence analysis.

Next-generation sequencing and blood chemistry

Complementary DNA libraries were constructed using NEBNext Ultra II RNA Library Prep kit (New England Biolabs). The libraries were sequenced with Illumina HiSeq 2x150bp configuration. 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 on ENSEMBL using STAR ultrafast universal RNA-seq aligner.

Circulating serum levels of glucose, insulin, a lipid profile, and renal and hepatic function panels were analyzed. After a fasting period of 6 hr, blood was obtained from mice by cardiocentesis under inhaled anesthesia (1-2% isoflurane) and centrifuged (2,300 × g, 10 min) in serum separator gel tubes (IDEXX BioAnalyttics). Blood chemistry was analyzed with Beckman Coulter analyzers. For glucose tolerance testing, whole blood was collected from the tail vein to measure blood sugar levels with a glucometer (Bayer).

Coronary artery ligation, electrocardiogram, and echocardiogram

Mice were housed in a well-ventilated and light-proof cabinet and kept under regular light/dark cycles (12 hr light: 12 hr dark) at room temperature. Animals had free access to a standard chow diet and water. All procedures involving animal use and surgeries were approved by the Institutional Animal Care and Use Committee. Both genders were included in this study, and sex was used as a biological variable. Experimental myocardial infarction (MI) was produced in 12-week-old mice by permanent ligation of the left anterior descending coronary artery with an 8-0 polyvinylidene fluorides suture of a knot as described [12]. Sham groups had the same procedure without the suture around the coronary artery. During the MI surgeries, electrocardiograms (EKG) were recorded using Small Animal Physiology Monitoring System 75-1500 (Harvard Apparatus). Echocardiography was performed without anesthesia to measure left ventricular (LV) parameters using a GE Vivid 7 Dimension machine and a GE i13L 10-14 MHz transducer. LV end-diastolic and end-systolic volumes were estimated from the Teichholz formula as described previously [51]. The inclusion criteria included age, gender, and the genotype (homozygous or wild-type only) of animals. The exclusion criteria were that if an animal showed significant distress, it would have been humanely euthanized.

A priori sample power calculation was performed using G*Power 3.1.9.2. Detailed parameters and predicted values are listed in Major Resources Table. Based on our previous MI study [12], we estimated at least N=20 and N=50 per group to generate valid scientific conclusions for infarct size and survival rate post-MI, respectively. The animals were ear-tagged upon weaning and randomly selected for the procedure using Microsoft Excel software. The procedure was performed by an investigator who used the ear-tag number as identification and was blinded to the genotype of animals.

Isolated perfused heart experiments

Isolated perfused heart experiments were performed in the Langendorff mode as described [12, 52]. Briefly, the heart was perfused with a constant pressure of 80 mmHg with modified Krebs-Henseleit (KH) buffer (in mM): 118 NaCl, 25 $NaHCO_3$, 4.5 KCl, 2.5 $CaCl_2$, 1.7 $MgSO_4$, 1.2 KH_2PO_4, and 5.6 glucose, equilibrated with 95% O_2-5% CO_2 (pH 7.4). After an equilibration period, the heart was subjected to hypoxia with the buffer equilibrated with 95% N_2-5% CO_2 or global ischemia-reperfusion injuries. Isovolumic ventricular function was quantified by a water-filled balloon inserted into the LV and analyzed by a data-acquisition system (PowerLab, AD Instruments).

To measure myocardial glucose uptake, the heart was perfused with the KH buffer containing 83 nM 2-[^{3}H]deoxy-D-glucose (1 mCi/ml, PerkinElmer Life Science) for 20 min and then switched to the KH buffer without the isotope for another 5 min to wash out the radioactive tracer. Exogenous oxidation rates of fatty acid were determined by measuring the rates of ^3H_2O production from 0.15 nM [^{3}H]-palmitate (1 mCi/ml, PerkinElmer Life Science) as described [53]. At the end of the experiments, hearts were homogenized. The radioactivity was measured by liquid scintillation counting with an LS6500 scintillation counter (Beckman Coulter) and normalized by the total protein content of the tissue.

Histopathological examination

After fixation with 4% paraformaldehyde (PFA) and permeabilization with 0.1% Triton X-100, cells were incubated in a blocking solution (10% normal goat serum in PBS) for 1 hr, stained with an anti-GLUT1 antibody (Abcam, Catalog# ab115730, 1:400 dilution) for overnight, washed with PBS over three times, and stained with the secondary antibody conjugated with Alexa flour 488 (Abcam, Catalog# ab150077, 1:600) for 1 hr. The mounting medium (Electron Microscopy Sciences, Catalog# 17966) was used for fixed-cell imaging. Clathrin and its adaptor protein AP-2 were detected by an anti-clathrin (Cell Signaling, Catalog# 4796, 1:400) and AP2M1 antibodies (Abcam, Catalog# ab 233712, 1:400), followed by the secondary antibody conjugated with aminomethyl coumarin acetate (AMCA) (Jackson ImmunoResearch Laboratories, Catalog#711-155-152 or 715-155-151, 1:600). CellBrite Blue (biotium) was used for cell membrane staining. Concanavalin A conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, Catalog# C11252, 1:400) was used to detect *N*-linked protein glycosylation by immunocytochemistry.

The harvested heart was paraffin-embedded, sectioned at 10 μm, and stained with picrosirius red (to evaluate collagen deposition and myocyte cross-sectional area) as previously described [54]. Triple staining with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling), an anti-sarcomeric alpha-actinin antibody (Abcam, Catalog# ab68167, 1:400), and DAPI was performed using In Situ Cell Death Detection Kit (Sigma) according to the instruction manual, and apoptotic cell death was detected using quantified by fluorescence microscopy. To assess the tissue levels of reactive oxygen species (ROS), heart tissues were embedded in the Tissue-Tek OCT compound (Electron Microscopy Science) and snap-frozen in dry ice. Cryosections were stained with the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Thermo Fisher Scientific) as described [55]. Staining, scanning, and quantitation were performed with a minimum of 5 sections for each animal. Image analyses

were performed by ImageJ. We selected representative images that were most similar to all the other images in each study set.

To measure LV infarct size, the heart was perfused with 1% triphenyl tetrazolium chloride solution. To calculate the area at risk, the heart was perfused with 0.3% phthalocyanine blue. Non-jeopardized, ischemic but viable, and necrotic LV tissues were identified by blue, red, and white coloration, respectively. Planimetry image analysis was used with ImageJ (NIH Image) to quantitate the infarct size and the area at risk.

***Artificial intelligence*-guided identification of protein-protein interaction**

The structure of GLUT1 was based on the Protein Data Bank (PDB ID: 4PYP). The structure of ARRDC4 was predicted by the AlphaFold Protein Structure Database (Q8NCT1). The possible protein-protein docking model was constructed using an algorithm on the HDOCK server. Based on the previous finding that TXNIP interacts with intracellular loops of GLUT1 in a 1:1 ratio [14], we used the dataset from intracellular loops in GLUT1 as potential docking sites with ARRDC4. The protein-protein interface was analyzed by PyMOL software to calculate the parameters of hydrogen-bond geometry.

Statistical analysis

We performed *in vitro* experiments with 3-5 independent cell preparations with 2-3 technical replicates of each sample. We averaged technical replicates for each biological replicate. The sample size (N) represents biological replicates.

Data are presented as means±SEM. Statistical analysis was performed by GraphPad Prism (GraphPad software). Comparisons between groups were tested by Student's unpaired two-tail *t*-test. Comparisons among groups were tested by one-way or two-way ANOVA with Bonferroni's or Tukey's test for post-hoc analysis. Multiple testing corrections were performed using Bonferroni correction and the Benjamini-Hochberg-Yekutieli procedure to confirm no

occurrence of false positives. Shapiro-Wilk test was used to determine if a dataset was modeled by a normal distribution. When the P-value was less than the alpha level (0.05), a non-parametric alternative (Mann-Whitney *U* test or Kruskal-Wallis test followed by post-hoc Dunn's test) was performed. When the sample size (N) was smaller than 6, a non-parametric test was used. For the data not normally distributed, values of median and quartiles are listed in Table 1 or Online Dataset 2. Survival rates among groups were compared using Kaplan-Meier estimation and log-rank test. A P-value less than 0.05 was considered significant.

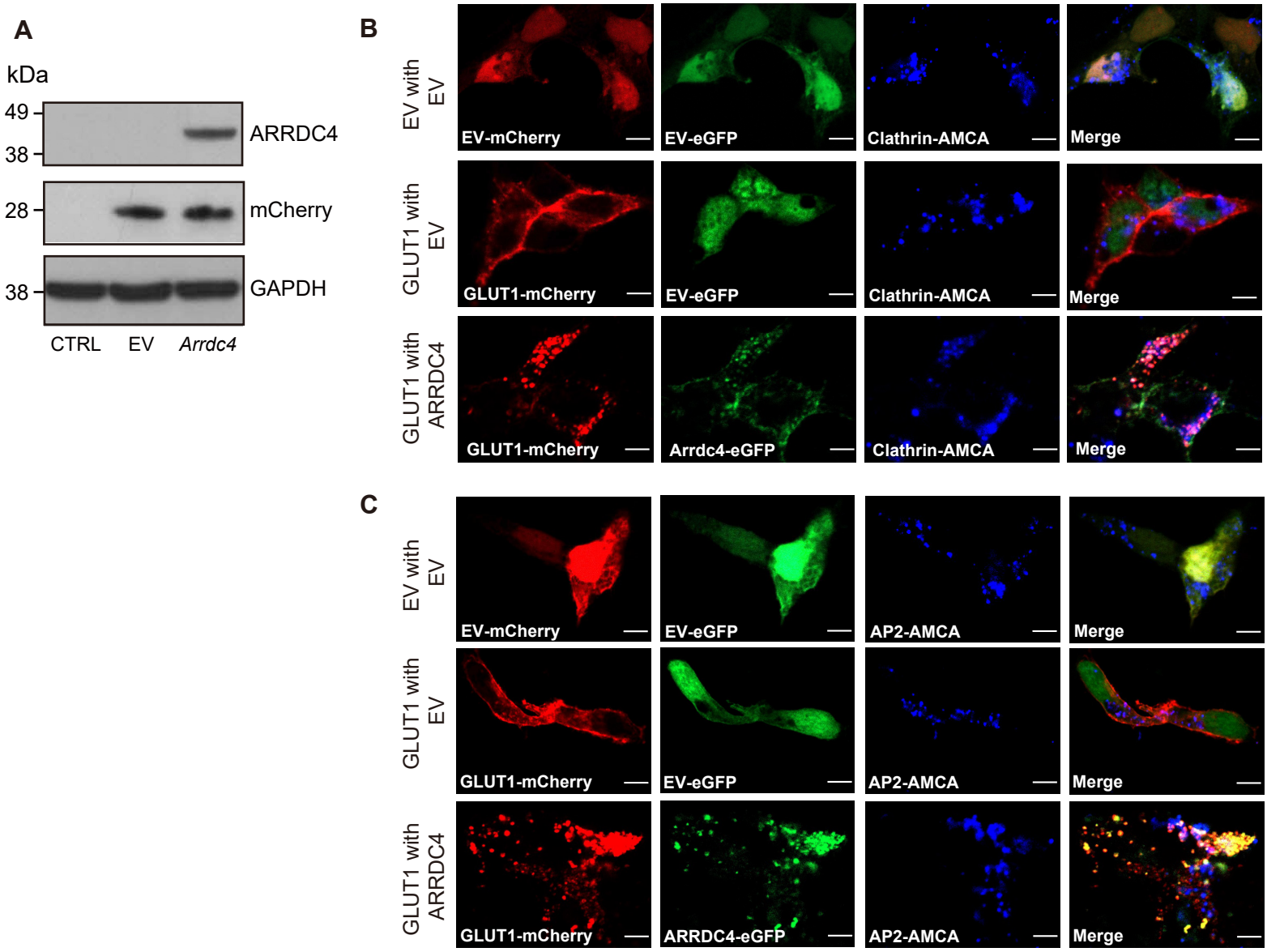

Figure S1. A. HT1080 cells were infected with an adenoviral gene vector of *Arrdc4* wild-type (WT) or empty vector (EV) in tandem with mCherry as a transfection marker. Control (CTRL) is cells without adenoviral transduction.Expression of the *Arrdc4* construct was verified by Western analysis of cell lysates with anti-ARRDC4 and mCherry antibodies.
B and **C**. ARRDC4-eGFP fusion or EV-eGFP was co-transfected with GLUT1-mCherry or EV-mCherry fusion construct in HEK293T cells. After fixation and permeabilization, cells were stained with an anti-clathrin (B) or anti-AP-2 (C) primary antibody followed by the secondary antibody conjugated with a blue fluorescent dye, aminomethyl coumarin acetate (AMCA). Confocal images show that clathrin and its adaptor protein AP-2 were co-localized with GLUT1 upon internalization by ARRDC4, as shown in the merged images. Scale bars, 5 μm.

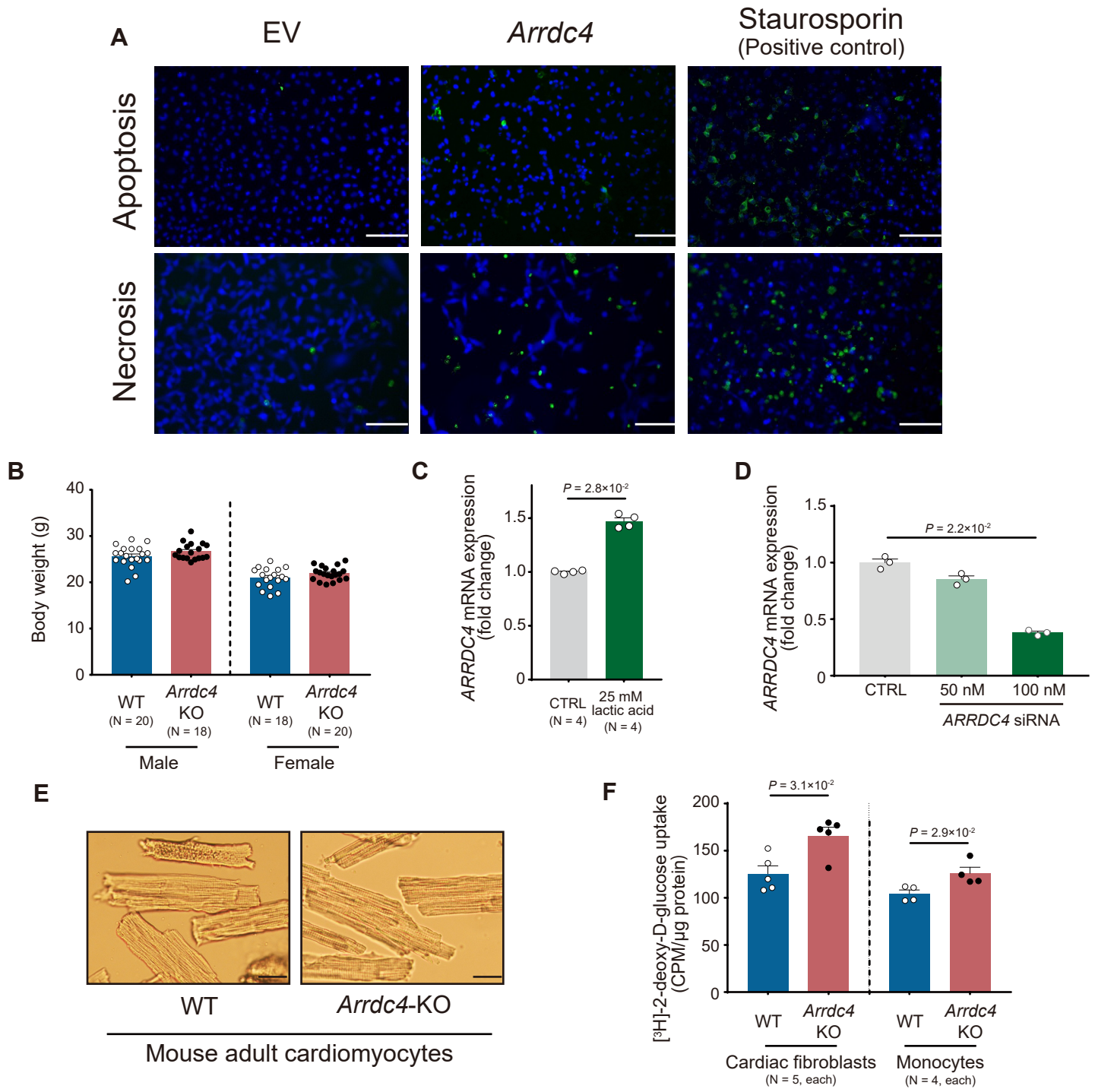

Figure S2. A. HT1080 cells were infected with an adenoviral gene vector of *Arrdc4* or empty vector (EV). The number of apoptotic (green-stained FITC-Annexin V) or necrotic (green-stained Nuclear Green DCS1) cells was counted over that of healthy (blue-stained CytoCalcein Violet 450) cells. Staurosporine (2 μM, 2 hr) was used for a positive control. Scale bars, 100 μm. **B**. *Arrdc4*-KO and wild-type (WT) littermates, aged 13 weeks old, had no differences in body weight (*t*-test). **C**. The mRNA expression was quantified by quantitative PCR. Incubation with lactic acid (25 mM, 3 hr) in HT1080 cells (pH=7.0) up-regulated the gene expression of *ARRDC4* (Mann-Whitney *U* test). **D**. Quantitative PCR confirmed successful small interfering RNA (siRNA)-mediated knockdown of *ARRDC4* mRNA in HT1080 cells in a dose-dependent manner (50 and 100 nM) compared with siRNA control (CTRL, 100 nM) (Kruskal-Wallis post-hoc Dunn's test). **E**. Representative images of cultured cardiomyocytes derived from an adult wild-type (WT) or *Arrdc4*-knockout (KO) mouse. Bright-field microscopic images. Scale bars, 10 μm. **F**. 2-[3H]deoxy-D-glucose uptake was measured in cardiac fibroblasts or inflammatory monocytes isolated from the spleen of mice (Mann-Whitney *U* test).

Table S1. Predicted docking sites between ARRDC4 and GLUT1

ARRDC4		GLUT1		Distance (Å)	Side chain interaction
Residue number	Residue	Residue number	Residue		
80	Ala	158	Thr	2.3	−
81	Ala	329	Gln	2.5	−
82	Leu	154	Gly	3.1	−
243	Lys	153	Arg	2.6	+
244	Thr	150	Thr	3.2	−
244	Thr	153	Arg	3	−
244	Thr	247	Glu	2.3	+
284	Cys	250	Gln	3.4	−
285	Cys	397	Gln	2.8	−
290	Asp	249	Arg	2	+
308	Glu	242	Gln	3	+
308	Glu	242	Gln	2.8	+

ARRDC4 protein structure was predicted based on AlphaFold Protein Structure Database (Q8NCT1). Structural Protein Data Bank (PBL) file was used for GLUT1 (4PYP). Side chain interaction (+) indicates that the side chain of amino acid residues in ARRDC4 forms a hydrogen bond with GLUT1, while (-) indicates that the main chain in ARRDC4 forms a hydrogen bond with GLUT1. Residues shown in red were mutated to Alanine.