

Supplemental Material

Supplemental Methods

Magnetic resonance imaging (MRI)

The mouse was positioned in a custom-made cradle where anesthesia was induced with 4-5% isoflurane in a medical air/oxygen mixture, and maintained with 1-2% isoflurane. Breathing rate was monitored and gas adjusted to maintain a rate of 30-60 breaths per minute. Core body temperature (monitored via rectal probe) was maintained at 35 °C with the animal on a heated circulating-water pad or via a warm air blower. MRI images were scanned by a 7 Tesla Bruker Avance MRI scanner (Ettlingen, Germany). The sequence parameters were TE (echo time) = 10.3 ms with a 4 echo train, FOV (Field of View) = 6 x 4 cm or 4 x 4 cm, slice thickness = 1 mm, matrix = 256 x 256, and NA (number of average) = 4. To reduce motion artifacts, monitored breathing was used to gate acquisition of lines of k-space between mouse breaths. Breathing, maintained at 45 breaths per minute, allowed acquisition of 3-4 lines of k-space at each break. First image was comprised of 16 coronal slices and second 40 axial slices.

Two-dimensional gel electrophoresis

Protein samples were pre-labeled prior to two-dimensional electrophoresis with CyDye DIGE fluors (GE Healthcare) and “spot-pick” gels were stained with Sypro Ruby (Bio-Rad) following manufacturer’s instructions. Briefly, heart lysates (50 µg) from *ARHI*^{-/-} mice were incubated (2 h, 30 °C) with recombinant mouse ARH1 (rmARH1, 30 µg) or PBS, followed by labeling with Cy5 (400 pmol, red) or Cy3 (400 pmol, green), respectively. After quenching reactions with 1 µl of 10 mM lysine, rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% ampholytes, and 13 mM DTT) was added to samples. For spot picking, heart lysates of *ARHI*^{-/-} mice (500 µg) were stained with Sypro Ruby. Samples were loaded on an immobilized pH gradient strip (24 cm; pH 3-10 NL, GE Healthcare) for isoelectric focusing: 30 V, 10-12 h; 250 V, 250 Vh; 500 V, 500 Vh; 1000 V, 1000 Vh; a gradient to 8000 V, 66667 Vh (Ettan IPGphor II, GE Healthcare). Each strip was equilibrated for 15 min in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) with 0.5% DTT followed by a second 15-min equilibration with 4.5% iodoacetamide and brief rinsing in SDS-PAGE buffer (25 mM Tris, 192 mM

glycine, 0.2% SDS, pH 8.3) before application to 10-15% SDS-polyacrylamide gels and sealing with 0.5% agarose containing bromophenol blue. Electrophoresis in SDS-PAGE buffer at 30 V, 30 min, followed by 110 V, 15 h was performed in an Ettan DALT-12 tank (GE Healthcare).

Image acquisition spot picking and identification

Gels for spot picking were fixed (3 h) in 500 ml of 30% methanol, 7.5% glacial acetic acid then stained with SYPRO Ruby Protein Gel Stain (Bio-Rad) according to manufacturer's instructions. Gels with CyDye-labeled samples and SYPRO Ruby-stained gels were scanned on a Typhoon 9400 variable mode imager (GE Healthcare) at a resolution of 100 μ m. Image analysis was performed using the cross-stain analysis function with Progenesis Discovery software (Nonlinear Dynamics). For protein identifications from 2D gels, protein spots were excised from SYPRO Ruby gels using the Ettan Spot Handling Workstation (GE Healthcare). Gel plugs were digested overnight with trypsin (Promega) and desalted with C18 ZipTips (Millipore) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (LTQ-FTMS mass spectrometer, Thermo Fisher Scientific).

2DE-Western blotting analysis

Pelleted proteins for 2D gels were washed twice with cold-acetone twice and dissolved in 50 μ l of lysis buffer (15 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS). Each sample treated with ARH1 or hydroxylamine was subjected to 2DE as described above and transferred to PVDF membranes. The PVDF membranes were incubated with anti-TRIM72 antibodies (Supplemental Figure 1), and membranes stained with GelCode Blue Stain Reagent (PIERCE) for comparison of different TRIM72 blots.

Purification of recombinant mouse TRIM72, ARH1, ART1 and ART5

Mouse TRIM72 (NM 001079932) protein was expressed in E. coli BL21 (DE3) (Invitrogen) using pGEX-6P-1 vector GST Gene Fusion System (GE Healthcare). GST tag was cleaved by PreScission

protease according to manufacturer's instructions (GE Health). ARH1 protein synthesized in *E. coli* BL-21 was purified as described (1). For preparation of mouse ART1, rat mammary adenocarcinoma cells (ATCC) transfected with plasmid containing the mouse ART1 gene were grown in Eagle's MEM with 10% FBS and 0.5 mg/mL Geneticin (G-418, Sigma). Proteins released from the cells by phosphatidylinositol-specific phospholipase C (PI-PLC, Sigma) were collected for ADP-ribosyltransferase assays where the transfer of ADP-ribose from NAD to agmatine was quantified as described (2). Mouse lymphocyte ART5 cDNA was subcloned into a pFLAG-MAC expression vector (Sigma), which was transfected into *E. coli* BL-21 (DE3) competent cells (Novagen). ART5 with FLAG tag was purified on anti-FLAG M2 affinity gels (Sigma) according to the manufacturer's instructions.

Design of shRNA-resistant cDNA

To overexpress TRIM72-GFP and TRIM72 (R207K, R260K)-GFP in TRIM72 shRNA cells, shRNA-resistant cDNA was prepared with 13 different silent mutations placed into sites corresponding to pEGFP-N1-TRIM72 and pEGFP-N1-TRIM72 (R207K, R260K) vectors. The original sequence of TRIM72 5'-GGCGTCCTGGCTTTCTATGATGCGAGCAAC-3' was replaced with 5'-GGAGTGTTAGCATT**TT**ACGACGCAT**CAA**AAT-3', where the nucleotide substitutions are indicated by bold letters. The QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) was used for performing single and multiple mutagenesis. The mutations were verified by sequencing the cDNA (Macrogen, Rockville, MD).

Subcellular fractionation

After harvest, WT mouse heart was frozen in liquid nitrogen and ground with a mortar. The tissue powder was dissolved with ice-cold hypotonic buffer (20 mM Tris-HCl, 20 mM NaCl, pH 7.4) with protease inhibitor cocktail (complete EDTA-free, Roche) and mechanically lysed with a Dounce homogenizer. After centrifuging the heart lysates at 10,000 $\times g$ for 10 min at 4 °C, the supernatant containing plasma membrane and cytosol was transferred into clean tubes. Repeated centrifugation at 100,000 $\times g$ for 60 min

at 4 °C was performed to separate plasma membrane and cytosol. The pellet of plasma membrane was dissolved in the same volume of cytosol fraction.

Cell lines

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco or Atlanta), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. The gene-specific knockdowns in C2C12 cells (ATCC, CRL-1772) stably expressing short-hairpin RNA (shRNA) vectors (Origene) targeting mouse ARH1 (5'-TTGTGAAGGAGAATCTTCAACACTGGTCC-3'), ART1 (5'-TGAAGATTCCCTGCTATGATGTCTCTCCTA-3') and TRIM72 (5'-TAGAGCAACTGAGGCAGATGGAGAAGGTG-3') were generated by using Lipofectamin 2000 (Invitrogen), followed by selection with 3.0 µg/ml puromycin (InvivoGen); the cells were transferred to antibiotic-free medium 1 day before experiments. Cells transiently transfected with pEGFP-N1, pEGFP-N1-TRIM72, and pEGFP-N1-mutant TRIM72 (R207K, R260K) Vectors (Clontech) were cultured in DMEM with 10% FBS and 200 µg/ml G418. DAPI staining showed no bacterial contamination in the C2C12 cells transformed with ARH1 shRNA, ART1 shRNA, TRIM72 shRNA, double knockdown for ART1 and ARH1 shRNA, or control scrambled shRNA (Supplemental Figure 5).

Immunoprecipitation (IP)

Mouse heart was ground by mortar in liquid nitrogen and dissolved in Dulbecco's phosphate-buffered saline (DPBS) (21-030-CV, Corning). Heart lysate was fractionated by centrifugation at 700 xg for 2 min. The resulting supernatant was collected and 500 µg of total extract for each sample were pre-cleared with 50 µl of protein G-Sepharose beads (Dynabeads, Invitrogen) for 30 min on ice, mixing every 10 min. After removal of the Dynabeads using a magnet (Invitrogen), lysates were incubated with 5 µg of anti-TRIM72, anti-ARH1, anti-ART1 or anti-caveolin-3 (A-3, Santa Cruz) antibodies for 30 min on ice. As control, 500 µg of heart lysate were incubated with 5 µg of normal mouse IgG or normal rabbit IgG. Resulting complexes were collected with protein G-Sepharose beads during a 1.5-h incubation, followed by three washes with 20 mM Tris-HCl, pH 7.4.

Blue native PAGE and Western blotting analysis

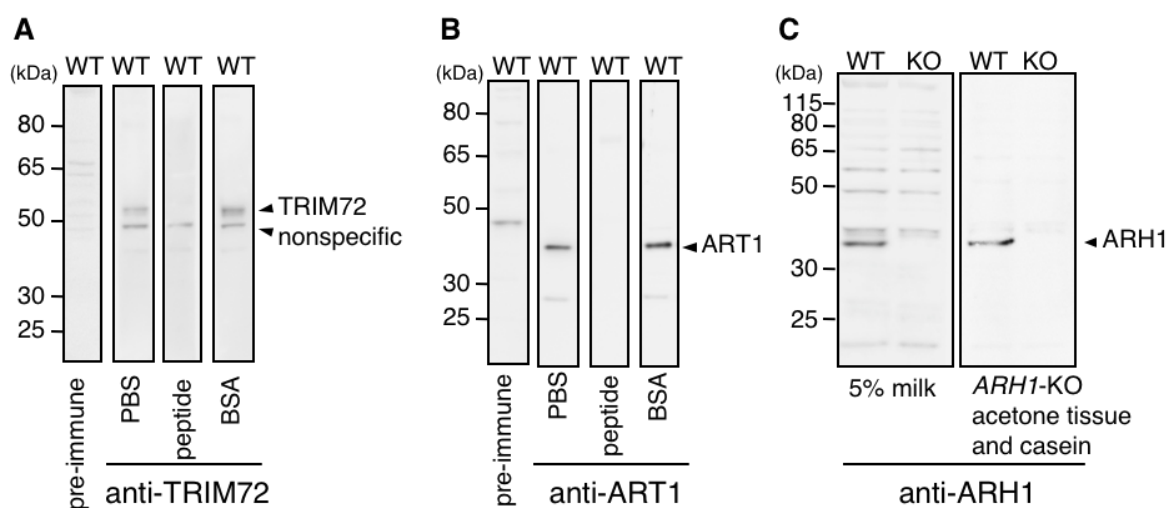
Wild-type mouse heart was minced by hand with blade into approximately 1-mm³ pieces. The minced tissue was placed in a Dounce homogenizer and homogenized with 30 strokes in ice cold DPBS with protease inhibitors. The tissue lysate was centrifuged at 700 xg for 10 min at 4 °C. The supernatant (50 µg) was dissolved with digitonin and NP-40 with final concentrations of 2% and 0.5%, respectively, Native PAGE sample buffer was added followed by 30-minuts incubation on ice. After gently pipetting, samples were spun at 13,000 xg for 10 min at 4 °C. The supernatant was added Coomassie G-250 (final concentration 0.625%). Samples were applied to a native PAGE 3-12% bis-tris gradient gel (Thermo Fisher Scientific). Blue native PAGE was performed according to company's instructions with the following modification. After 30-minuts electrophoresis at 150 V, dark blue cathode buffer was replaced with light blue cathode buffer, and continued electrophoresis for 120 min at 200 V. Immediately after finishing electrophoreses, blue native PAGE gel was incubated with 2x transfer buffer for 10 min at room temperature to remove excess Coomassie G-250. Proteins were transferred to a PVDF membrane, and Western blots were performed with primary antibody and IRDye 800CW anti-rabbit IgG (LI-COR) as the secondary antibody. Blots were visualized by Odyssey imaging system (LI-COR).

Antibodies

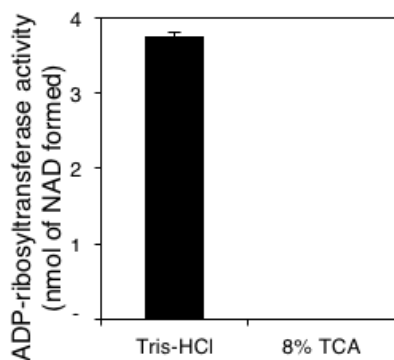
Monoclonal anti-myogenin (MAB3876), anti-β-actin (A1978) and anti-GFP (G1544) antibodies were from Sigma-Aldrich. Normal rabbit IgG and mouse IgG were from R&D Systems. Anti-TRIM72 and anti-ART1 antibodies were raised against mouse-specific peptides, CARLKTQLPQQKMQLQEA and CANSPLHKEFNAAVREA, respectively, by 21st Century Biochemicals, Marlboro, MA (Supplemental Figure 1, A and B). Rabbit polyclonal anti-ARH1 antibodies were raised against full-length recombinant mouse ARH1 by Cocalico Biologicals, Inc, Stevens, PA (Supplemental Figure 1C) (1). Anti-ART1 (H110) and anti-caveolin-3 (A-3) antibodies were from Santa Cruz Biotechnology.

Supplemental References

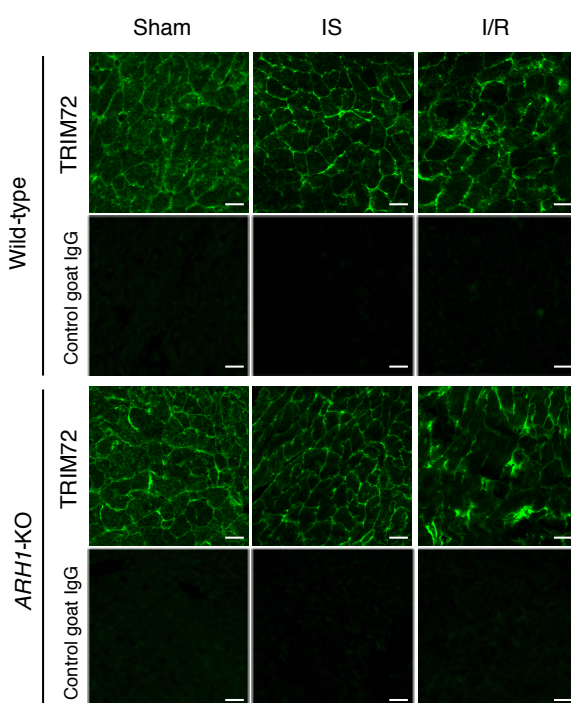
1. Kato J, Zhu J, Liu C, and Moss J. Enhanced sensitivity to cholera toxin in ADP-ribosylarginine hydrolase-deficient mice. *Mol Cell Biol.* 2007;27(15):5534-43.
2. Stevens LA, Levine RL, Gochuico BR, and Moss J. ADP-ribosylation of human defensin HNP-1 results in the replacement of the modified arginine with the noncoded amino acid ornithine. *Proc Natl Acad Sci U S A.* 2009;106(47):19796-800.



Supplemental Figure 1. Specificity of anti-TRIM72, anti-ART1 and anti-ARH1 antibodies. (A and B) Four lanes contain four samples from the same wild-type (WT) mouse heart lysate (50 μ g) separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes, which were divided into 4 strips. These were reacted with affinity-purified anti-TRIM72 or anti-ART1 antibodies (each 5 μ g) mixed with 20 μ g of peptide CARLKTQLPQQKMQLQEA or CANSPLHKEFNAAVREA, respectively, or 20 μ g of BSA in PBS for 30 min at room temperature. Membranes were blocked with 5% skim milk (Bio-Rad) in TBST for 1 h at room temperature. Antibody mixtures and pre-immune serum were diluted to working concentrations of 1:1000 for Western blotting. Primary antibodies were incubated overnight at 4 $^{\circ}$ C, followed by 3 washes with TBST for 10 min each, then incubated with secondary rabbit IgG antibody for 1 h at room temperature. The ECL system was used for detection of TRIM72 (**A**) and ART1 (**B**). (**A**) Lower band is nonspecific reactivity with anti-TRIM72 antibodies. (**C**) WT and *ARH1*-KO (KO) mouse heart lysate (50 μ g) separated by SDS-PAGE and transferred to nitrocellulose membranes, which were divided into 2 strips. Membranes were blocked with 5% skim milk or 0.2% casein (I-Block, Thermo Fisher) in TBST for 1 h at room temperature. Affinity-purified anti-ARH1 antibody (10 μ g) was incubated with/without *ARH1*-KO mouse heart acetone tissue powder in TBST (0.1 ml) for 1 h at room temperature. Primary antibodies were diluted to working concentrations of 1:1000 and incubated with membranes for 2 h at room temperature, followed by 3 washes with TBST for 10 min each, then incubated with secondary rabbit IgG antibody for 1 h at room temperature. The ECL system was used for detection of ARH1 protein. Primary antibody incubated with *ARH1*-KO mouse heart acetone tissue powder was used for Western blotting analysis in Supplemental Figure 8. Images representative of three experiments are shown.

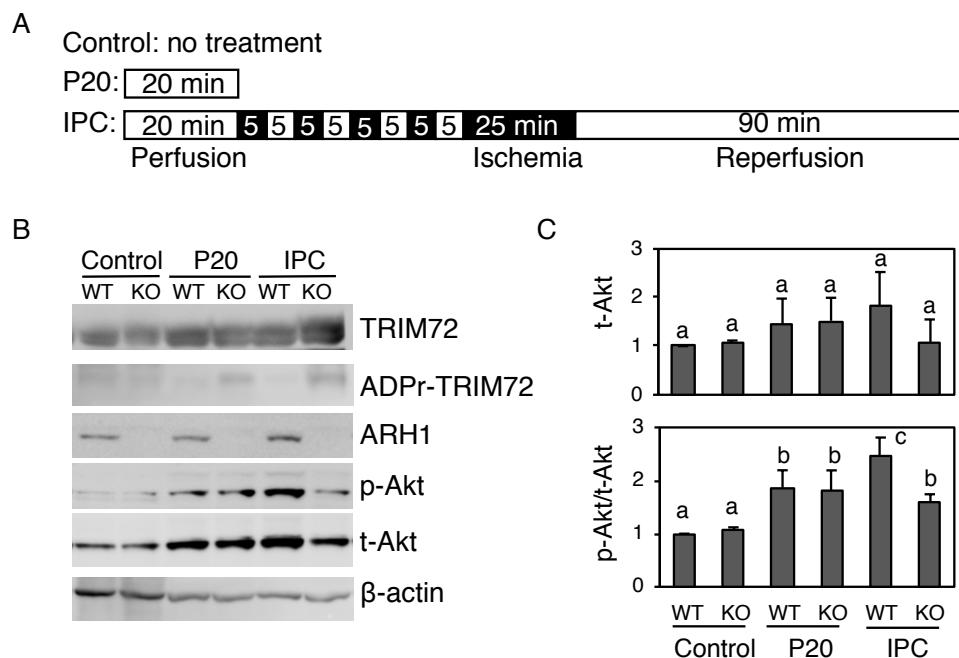


Supplemental Figure 2. ADP-ribosyltransferases inactivated by trichloroacetic acid (TCA). Mouse hearts were extracted, immediately frozen in liquid nitrogen and ground in a mortar. After evaporation of liquid nitrogen, 8% ice-cold TCA or Tris-HCl was added immediately. TCA-precipitated proteins were collected by centrifugation (20,000 xg, 30 min) and solubilized in 4 ml of ice-cold Tris-HCl buffer (pH adjusted to 7.4 with 2N NaOH). Mouse heart lysates with or without TCA treatment (100 μ g/150 μ l) were incubated (16 h, 4 $^{\circ}$ C) with 0.1 mM [adenine- 14 C] NAD in Tris-HCl buffer, pH 7.4, with 0.1 mM β -NAD and 20 mM agmatine before application to AG1-X2 (Bio-Rad) columns and elution of [14 C] ADP-ribose- β -agmatine for quantification of 14 C in a scintillation counter. Data are means \pm SD. $n = 4$ in each group.

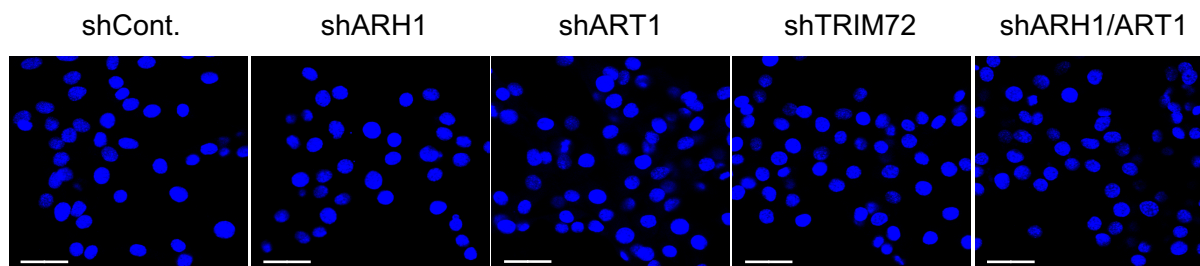


Supplemental Figure 3. Ischemia-reperfusion-induced disruption of TRIM72 localization in *ARH1*-KO mouse. Cross-sections of wild-type and *ARH1*-KO mouse hearts after sham operation ($n = 4$), ischemia (IS)($n = 4$) and ischemia-reperfusion (I/R)($n = 4$) in vivo were stained with antibody against

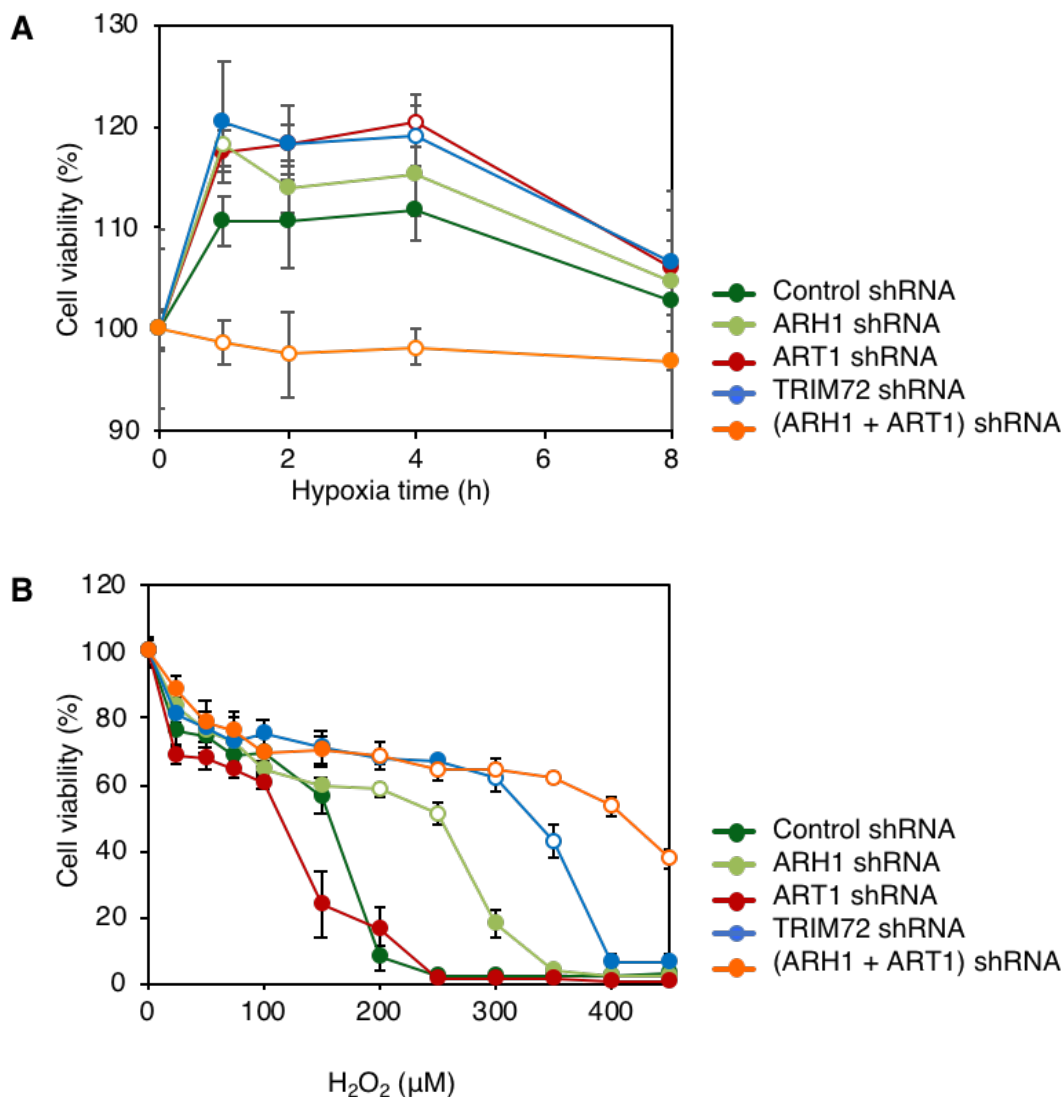
TRIM72 (SAB2501571, SIGMA) conjugated with Alexa fluor 488. Representative immunofluorescence images show localization of TRIM72 in the left ventricle (scale bars, 20 μ m).



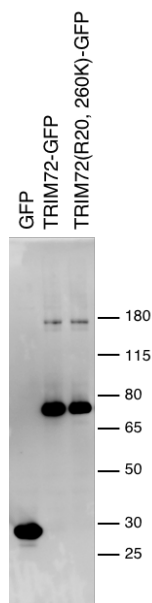
Supplemental Figure 4. Phosphorylation of Akt in Langendorff perfused mouse heart is not affected by ischemic preconditioning (IPC). (A) After IPC as described in Experimental Procedures, mouse hearts were immediately frozen in liquid nitrogen as a control. (B and C) Western blot images and densitometric quantification of total Akt (t-Akt) and the ratio of p-Akt to t-Akt (p-Akt/t-Akt). Phosphorylation of Akt (p-Akt) was similar in WT and *ARH1*-KO (KO) mouse hearts perfused for 20 min (P20) and control, however, p-Akt increased during IPC in WT heart, but decreased in *ARH1*-KO mouse heart. Representative images of three experiments are shown. Data are means \pm SEM. Comparison was analyzed by two-way ANOVA, followed by Tukey's multiple comparison test.



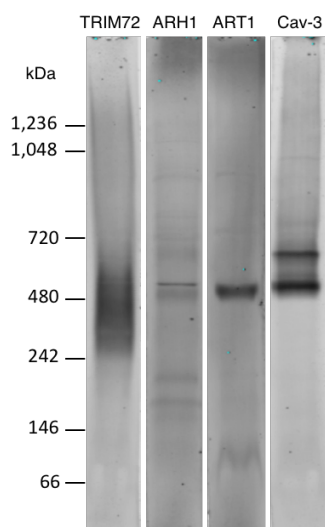
Supplemental Figure 5. DAPI staining images of cells transformed with ARH1 shRNA (shARH1), ART1 shRNA (shART1), TRIM72 shRNA (shTRIM72), or double knockdown for shART1 and shARH1 (shART1/ARH1), or control scrambled shRNA (shCont.). Nuclei were stained with Vectashield mounting medium with DAPI. Images were taken by 510 Meta (zeiss). Scale bar, 40 μ m.



Supplemental Figure 6. Effects of down-regulation of ARH1, ART1, TRIM72 gene expression in C2C12 myocytes after hypoxia/re-oxygenation injury and H₂O₂-induced oxidative stress. After a 7-days incubation, cells (2×10^4) were seeded on 96-well plats and incubated in DMEM with 10% FBS for 24–32 h before the cells were exposed to hypoxia and H₂O₂. (A) Hypoxia/re-oxygenation injury were performed with C2C12 cells transformed with control scrambled shRNA, ARH1 shRNA, ART1 shRNA, TRIM72 shRNA or double knockdown for shART1 and shARH1. The cells were exposed to hypoxia condition at 5% CO₂ and 2.5% O₂ for indicated time followed by 24-hour re-oxygenation at 5% CO₂ and 18% O₂ before subjected to cell viability assay. (B) Cells were exposed to H₂O₂ (Sigma) for 24 h at indicated concentrations before assessment of cell viability. The cell viability was measured by cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Japan). Data are means \pm SEM. $n = 6$. Open symbols are values significantly different ($p < 0.05$) from those of control shRNA cells by two-way ANOVA, followed by Tukey's multiple comparisons test.



Supplemental Figure 7. TRIM72 oligomerization in C2C12 myocytes. The C2C12 myoblasts were overexpressed with GFP, wild-type TRIM72, and double mutant TRIM72 (R207K, R260K)-GFP. The C2C12 cells were cultured until differentiated and lysed for non-reducing SDS-PAGE followed by Western blot analysis with anti-GFP antibody. Data shown are representative of three experiments.



Supplemental Figure 8. TRIM72 complexes are heterogeneous. As described in Supplemental Methods, wild-type mouse heart lysate (50 μ g) was applied to a native PAGE 3-12% bis-tris gradient gel. Proteins were separated by blue native PAGE. After finishing electrophoresis, proteins were transferred to a PVDF membrane, and Western blots were performed with anti-TRIM72, ARH1, ART1 and caveolin-3 (Cav-3) antibodies. IRDye 800CW anti-rabbit IgG (LI-COR) was used as the second antibody. Blots were visualized by an Odyssey imaging system (LI-COR). Representative images of three experiments are shown.

Movie 1. Echocardiography (Echo) B-mode imaging of 8-month WT mouse shows normal cardiac motion (related to Figure 1).

Movie 2. Echo B-mode imaging of 8-month *ARH1*-KO mouse shows reduced cardiac function (related to Figure 1).

Movie 3. MRI of 8-month WT mouse shows normal cardiac motion (related to Figure 1).

Movie 4. Reduced cardiac function in 8-month *ARH1*-KO mouse was observed on MRI (related to Figure 1).

Movie 5. TRIM72-GFP localization after laser-induced damage in C2C12 cells stably expressing control shRNA (related to Figure 5 B and C).

Movie 6. TRIM72-GFP localization after laser-induced damage in C2C12 cells stably expressing ARH1 (related to Figure 5 B and C).

Movie 7. TRIM72-GFP localization after laser-induced damage in C2C12 cells stably expressing TRIM72 shRNA (related to Figure 5 B and C).

Movie 8. TRIM72 (R207K, R260K)-GFP localization after laser-induced damage in C2C12 cells stably expressing TRIM72 shRNA (related to Figure 5 B and C).

Movie 9. TRIM72-GFP localization after laser-induced damage in C2C12 cells stably expressing ART1 shRNA (related to Figure 5 B and C).

Movie10. TRIM72-GFP localization after laser-induced damage in C2C12 cells stably expressing ARH1shRNA and ART1 shRNA (related to Figure 5 B and C).