SUPPLEMENTAL MATERIAL

Supplemental Methods

pRIP3 immunoprecipitation and western blot. Left ventricular tissue samples (~60 mg) from hearts (N = 6/group) collected at the end of the DCD protocol were homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM glycerophosphate, 5 mM dithiothreitol (DTT), 15% Roche cocktail protease inhibitors; pH 7.4). Samples were centrifuged at 10,000 x *g* for 5 min at 4°C and the supernatant collected. The protein concentration of each lysate was measured using a Bradford assay kit (Pierce Biotechnology, Rockford, IL), with bovine serum albumin used as standard. In order to detect and enrich for pRIP3, immunoprecipitations (IP) were performed on equal amounts of total protein from each sample. Lysate samples from individual hearts were centrifuged for 10 min at 10,000 x *g* (4°C) and diluted with lysis buffer without DTT to make a final volume of 0.5 mL containing 1 mg of total protein (1 μg/μL). Pre-clearing of the samples was performed by adding 60 μL of 50% Protein A agarose beads to 1 mL of diluted lysate from above. Samples were tumbled for 30 min at 4°C in a tube rotator, prior to being centrifuged for 5 min at 1,000 x *g* (4°C), and supernatants were collected for immunoprecipitation. The cleared lysates were divided into two 400-μL aliquots and 1:100 specific primary antibody added to form the Ag/Ab complex (pRIP3 Ser227, Cell Signalling Technology, USA). 50 μl of 50% Protein A beads were added followed by gentle rocking for 2 h at 4°C. Following centrifugation at 4°C for 30 s at 10,000 x *g*, the supernatant (containing unbound proteins) was discarded. The remaining pellet was washed five times with 500 μL of ice-cold lysis buffer. For each wash, the beads were re-suspended by inversion and centrifuged for 30 s at 1,000 x *g* at 4°C, prior to the supernatant being aspirated and discarded. The final pellet was resuspended with 50 μL of 2 x Laemmli sample loading buffer without β-mercaptoethanol. The samples were mixed vigorously and heat denatured for 5 min at 95°C. Following a 30 s centrifugation at 10,000 x *g*, the supernatants were transferred to new vials and β-mercaptoethanol was added. The prepared samples were electrophoretically separated on 4–20% graduated precast gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, membranes were incubated overnight at 4°C with primary antibody (pRIP3 Ser227, Cell Signalling Technology, USA). Membranes were incubated with secondary anti-rabbit (1:2000 in 5% non-fat dry milk + TBS-

T) or anti-mouse (1:5000 in 1% BSA + TBS-T) IgG conjugated to horseradish peroxidase (GE healthcare, Ryadalmere, Australia) for 1 h at 25°C. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Life Technologies, Scoresby, VIC, Australia), digitized, and quantified using Image J software (Version 1.52a, National Institute of Health, USA). Within each blot, the signal intensity from each lane was normalized to the Celsior-only group average from the same membrane. Blots were repeated to confirm results. All antibodies, unless otherwise stated, were sourced from Cell Signalling Technology (Beverly, MA).

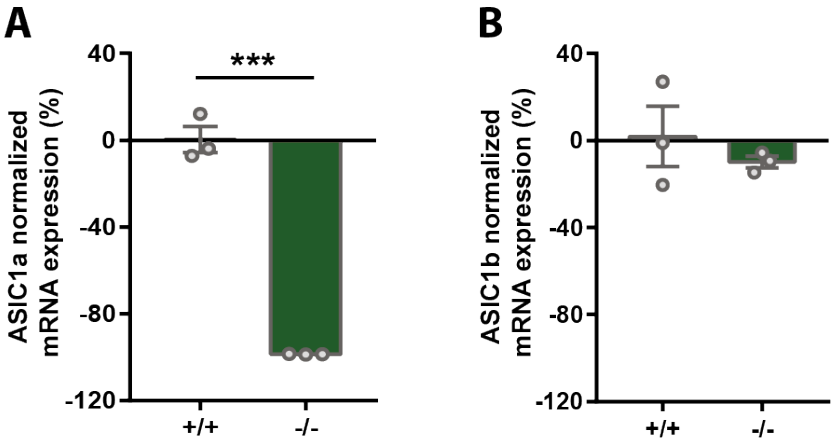
Flow cytometry. To assess the cardiomyocyte purity of differentiated cell populations, cells were fixed with 4% paraformaldehyde (Sigma Aldrich), permeabilized in 0.75% saponin (Sigma Aldrich), and labelled with Phycoerythrin (PE)-conjugated sarcomeric α-actinin (SA) antibody (Miltenyi Biotec Australia Pty) or PE-conjugated mouse isotype (IgG) control (Miltenyi Biotec Australia Pty). Stained samples were analysed on a FACS CANTO II (Becton Dickinson) machine with FACSDiva software (BD Biosciences). Data were analysed was using FlowJo software and cardiac populations were determined with population gating from isotype controls.

qRT-PCR. To assess mRNA transcript levels in hiPSC-CM populations, total RNA was extracted using a RNeasy Mini Kit (QIAGEN). Superscript III First Strand Synthesis (ThermoFisher) was used to generate cDNA and qRT-PCR was performed on ViiA 7 Real-Time PCR Machine (Applied Biosystems) with SYBR Green PCR Master Mix (ThermoFisher). Transcript copy numbers were calculated using 2-ΔΔCt method with normalization to *HPRT1* housekeeping gene. Primers used in this study were *ASIC1* forward 5'-GGATGGAGGTCTACCCTGGA-3' and reverse 5'-GACCTCAGCTTCTGCCTGTCA-3' and *HPRT1* forward 5'-TGACACTGGCAAAACAATGCA-3' and reverse 5'GGTCCTTTTCACCAGCAAGCT-3'.

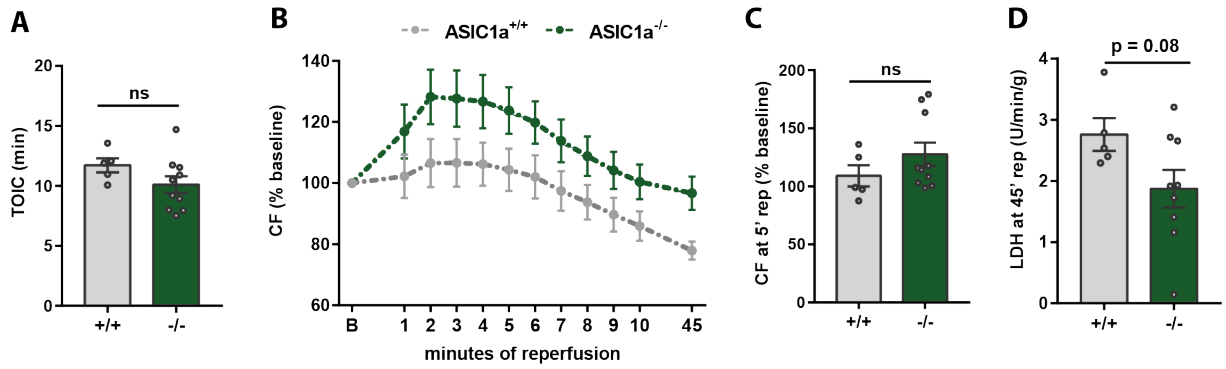
Immunohistochemisty/TUNEL stain. After overnight treatment at low pH, replated hiPSC-CMs were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and incubated for 1 h at room temperature in blocking solution (PBS containing 2% heat-inactivated sheep serum (HISS) and 0.05% Triton X-100). Cells were incubated overnight at 4°C with primary antibody followed by incubation with secondary antibody for 1 h at room temperature. Nuclei were

counterstained with 1 µg/mL DAPI. The samples were washed with PBS, then TUNEL stain (Abcam) was performed as per manufacturer protocol. Stained samples were imaged within one week. All antibody dilutions were prepared in blocking solution. The following antibodies were used: mouse anti-α-actinin (Clone EA-53, Sigma Aldrich, 1:100) and donkey anti-mouse AlexaFluor 647 (ThermoFisher, 1:100). High-resolution images were obtained using a Zeiss LSM710 AiryScan confocal microscope with 20x 0.8 NA or 40x 1.3 NA Plan Apochromat objectives running Zeiss Zen Black. All image processing and quantitation was performed in FIJI (*1*).

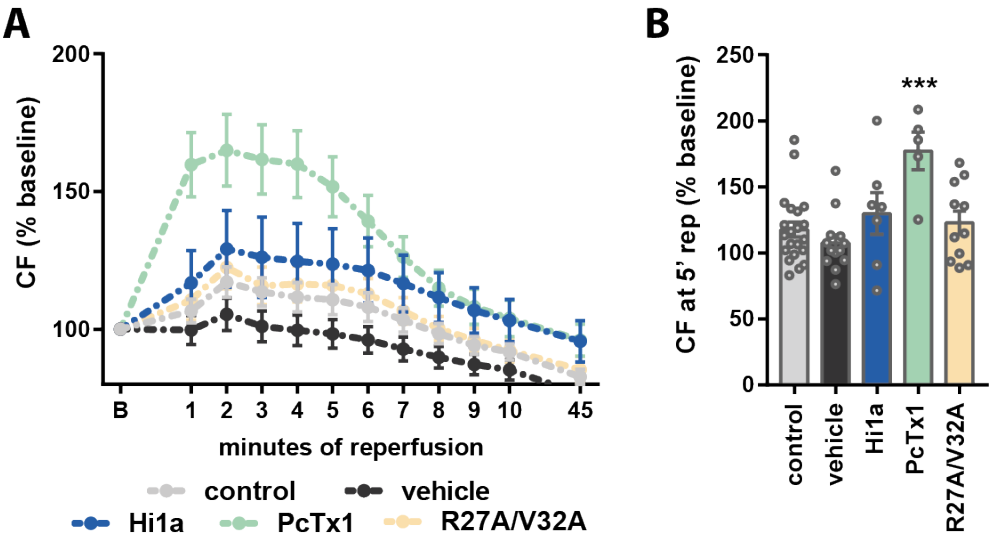
Supplemental Figures



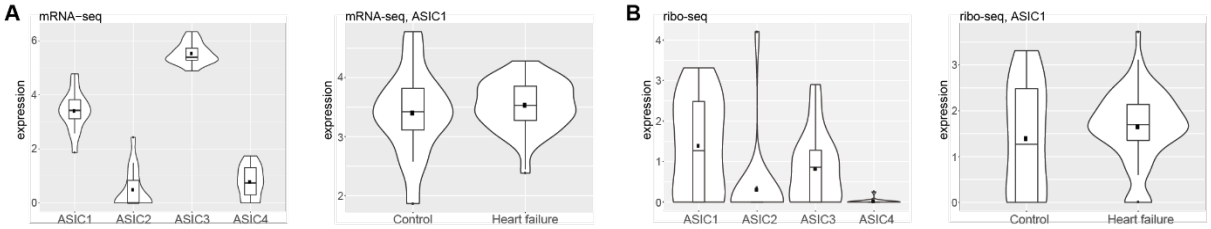
Supplemental Fig. S1. Verification of the loss of ASIC1a in knockout mouse strain. **(A-B)** Normalized mRNA expression levels of **(A)** ASIC1a ($p<0.0001$, WT 0.3 ± 6.0%, ASIC1a$^{-/-}$ –98.7 ± 0.7%) and **(B)** ASIC1b ($p = 45$, WT 1.9 ± 13.8%, ASIC1a$^{-/-}$ –9.9 ± 2.6%) from brain samples from WT (black, $n = 3$) and ASIC1a$^{-/-}$ mice (green, $n = 3$). Statistical significance was determined with two-tailed unpaired Student's *t*-test (***$p<0.001$). Data are presented as mean ± SEM.



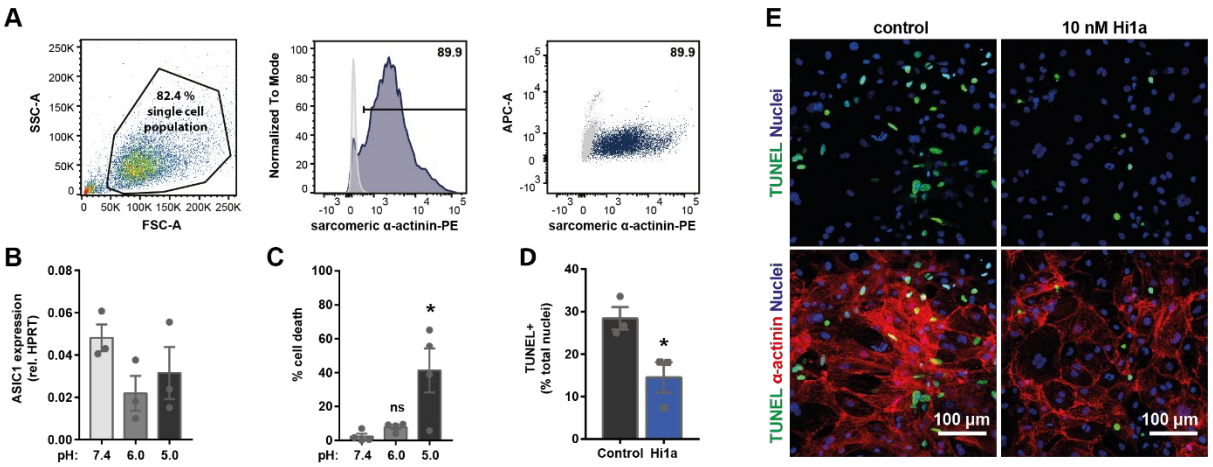
Supplemental Fig. S2. *Ex vivo* IRI in Langendorff-perfused ASIC1a KO mouse hearts compared to wild-type. **(A-D)** Hearts from ASIC1a KO (ASIC1a$^{-/-}$, n = 10, dark gray) and WT (ASIC1a$^{+/+}$, n = 5, light grey) mice were subjected to 25 min of global ischemia followed by 45 min of reperfusion. **(A)** Time to onset of ischemic contracture (TOIC, p = 0.162). **(B)** Coronary flow (CF) at baseline (B, pre-ischemia), during the first 10 min of reperfusion, and at the end of the 45 min reperfusion period. **(C)** CF at 5 min reperfusion (p = 0.26). **(D)** Cell death after 45 min of reperfusion (units of LDH normalized to reperfusion flow rate and heart weight, U/min/g, p = 0.08). For all parameters, baseline values were obtained immediately prior to ischemia, and all data are expressed as mean ± SEM. Statistical significance was evaluated with two-tailed unpaired Student's *t*-test (*p < 0.05).



Supplemental Fig. S3. ASIC1a inhibitors protect mouse hearts from *ex vivo* IRI. **(A-B)** Additional analysis of the experiment described in Figure 2. **(A)** CF plotted versus time (min) at baseline (B, pre-ischemia), during the first 10 min of reperfusion, and at the end reperfusion (45 min). **(B)** CF at 5 min reperfusion. Statistical significance was determined using one-way ANOVA with multiple comparisons (***p < 0.001). Data are presented as mean ± SEM (*n* > 5/group).



Supplemental Fig. S4. ASIC expression in adult human heart. Analysis of published **(A)** transcriptomic (mRNA-seq) and **(B)** translatomic (ribo-seq) data from the left ventricles of control (n = 15) and heart failure (dilated cardiomyopathy) (n = 65) patients (*2*). Data are presented as box blots where the middle box represents interquartile range (IQR), the middle line in the box is median (50th percentile of the data set), and points outside the plot are outliers.



Supplemental Fig. S5. Generation of hiPSC-CMs and treatment at low pH. **(A)** Flow cytometry analysis of differentiated hiPSC-CMs prior to replating. Single cell population from SSC-A (side scatter) versus FSC-A (forward scatter) plot (left) was analysed for the percentage of cells that stained positive for sarcomeric α-actinin (PE-gated population) shown as a histogram (middle) and a scatter plot against an unstained fluorophore (right). Isotype-stained sample (grey) was used to create PE+ gate to analyse sarcomeric α-actinin stained sample (blue). **(B-C)** Replated hiPSC-CMs treated overnight in HBSS at pH 7.4, 6.0, or 5.0 and analysed for **(B)** mRNA expression (qRT-PCR) of ASIC1 and **(C)** cell death (LDH). **(D-E)** Replated hiPSC-CMs treated overnight in HBSS at pH 5.0 and evaluated for cell death. **(D)** Quantification of cell death (TUNEL-positive nuclei normalized to total nuclei) following **(E)** immunohistochemistry for TUNEL (green) and α-actinin (red) with nuclei counterstained with DAPI. Top panel: TUNEL and DAPI merged image. Bottom panel: TUNEL, α-actinin, and DAPI merged image. All data are expressed as mean ± SEM (n = 3 biological replicates, 2–3 technical replicates each). Statistical significance was determined with one-way ANOVA (LDH results, panel c) or with a two-tailed unpaired student's t-test (TUNEL quantification, panel d) (*$p < 0.05$).

1. J. Schindelin *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).
2. S. van Heesch *et al.*, The Translational Landscape of the Human Heart. *Cell* **178**, 242-260.e229 (2019).