SUPPLEMENTAL MATERIAL

Materials and Methods

All animal care and procedures were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee of the University of California, Davis. Animal use was in accordance with National Institutes of Health guidelines. De-identified human ventricular specimens were obtained from a commercial source, in accordance with the approved UC Davis Institutional Review Board (IRB) protocol.

We used 129S6/SvEv wild type (WT) and *Slc26a6*$^{-/-}$ mice previously generated and reported[1] (a kind gift from by Dr. Peter S. Aronson, Yale University) in our study. All experiments described in the study were conducted in a blinded fashion. Specifically, the investigators who performed the physiological recordings, cell/tissue dissection, and staining had no knowledge of the genotypes of the animals.

Cardiac tissue preparation and cardiomyocyte isolation

Mice were anesthesized by intraperitoneal injection of 80 mg/kg of ketamine and 5 mg/kg of xylazine and sacrificed by exsanguination with rapid heart excision. Isolation of mouse cardiomyocytes followed the conventional enzymatic dissociation methods, as we have previously described[2]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except specifically indicated.

Genotyping, western blot, and histological analyses

Histological analyses of cardiac tissue were performed as we have previously described[3]. Briefly, excised hearts were retrogradely perfused with phosphate-buffered solution to remove blood. Fixed hearts were embedded in paraffin, and serial cardiac sections of 5 μm in thickness were taken along the transverse axis and stained with wheat germ agglutinin and Picrosirius Red

to assess for collagen content. Western blot analyses were performed using isolated ventricular myocytes from WT and *Slc26a6*$^{-/-}$ mice following the standard protocols by using mouse monoclonal anti-Slc26a6 antibody (Santa Cruz Biotechnology, Dallas, TX). Genotyping analyses were performed using RT-PCR of genomic DNA from wild-type (WT), *Slc26a6*$^{+/-}$, and *Slc26a6*$^{-/-}$ mice (**Online Supplemental Figure 1**). Three sets of primers were used with 2 forward (F) primers for WT or mutant (KO) alleles and one reverse (R) primer for both WT and mutant alleles. The primers used are as follow:

Slc26a6-WT-F, CAAAGCCCTGGCTTCAGGGTGAATGATCTAG;

Slc26a6-WT-KO-R, GAAGAGGCCGACCAGGAAGCTGAGTGTGTAG;

Slc26a6-KO-F, CTTCCATTGCTCAGCGGTGCTGTCCATCTG.

Electrocardiographic Recordings

ECG recordings were performed using Bioamplifier (BMA 831, CWE, Inc., Ardmore, PA), as we have previously described.[4] Briefly, mice were placed on a temperature-controlled warming blanket at 37 ºC. Four consecutive two-minute epochs of ECG data were recorded. Signals were low-pass filtered at 0.2 kHz and digitized using Digidata 1200 (Molecular Devices LLC., Sunnyvale, CA). A total of 100 beats were analyzed from each animal in a blinded fashion. The rate-corrected QT interval (QT_c) was calculated using modified Bazett's formula as reported by Mitchell et al for mouse models, and QT_c interval was defined as (QT interval (in ms)/$(RR/100)^{1/2}$).[5]

Analysis of cardiac function by echocardiography

Echocardiograms using M-mode and two-dimensional (2D) measurements to assess systolic function were performed in conscious animals using Vevo 2100 (FUJIFILM VisualSonics, Toronto, ON, Canada), as we have previously described[4]. The measurements

represented the average of six selected cardiac cycles from at least two separate scans performed in blinded fashion with papillary muscles used as a point of reference for consistency in the level of the scan. End diastole was defined as the maximal left ventricular (LV) diastolic dimension and end systole was defined as the peak of posterior wall motion. Fractional shortening (FS), a surrogate of systolic function, was calculated from LV dimensions as follows: FS = ((EDD-ESD)/EDD) x100%, where EDD and ESD are LV end diastolic and end systolic dimension, respectively.

Hemodynamic monitoring

Mice were anesthetized by intraperitoneal injection of 80 mg/kg of ketamine and 5 mg/kg of xylazine and maintained at 37 ºC. The arterial catheter was inserted retrogradely into the left ventricle *via* carotid artery. The recording of pressure and volume was performed by using Millar Pressure-Volume System MPVS-300 (Millar, Inc., Houston, TX), Power Lab, and Lab Chart 6.0 software (AD Instruments, Colorado Springs, CO). The pressure and volume were calibrated before recordings. The volume calibration used fresh heparinized 37 ^{o}C mouse blood and a cuvette (P/N 910-1049, Millar, Inc.). To change the preload, a gentle and quick abdominal compression was applied to occlude inferior vena cava.

Patch-clamp recordings

Whole-cell current and AP recordings were performed using an Axopatch 200A amplifier, Digidata 1440 digitizer, and pClamp10 software (Molecular Devices, LLC., Sunnyvale, CA). All experiments were performed using 3 M KCl agar bridges.

For current recordings, the clamped and suspended whole-cell was switched between two capillary tubes dispensing control solution and test solution, respectively, at a potential of 0 mV as we have previously described[2]. The fast solution exchange was controlled by SF-77 solution

exchanger (Warner Instruments, LLC., Hamden, CT) and pClamp10 software. The sequential exposure to the control and test solution induces the exchanger currents. The currents were digitally filtered at 1 kHz and digitized at 2 kHz. Borosilicate glass electrodes were pulled with P-97 micropipette puller (Sutter Instruments, Novato, CA). The resistance of the electrodes was 1.5-3.0 MΩ when filled with the pipette solution. For the outward current recording, the pipette solution contained (in mM): 140 NaCl, 10 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 1 EGTA, and pH 7.4. The bath control solution contained (in mM): 139 Na-glutamate, 1 NaCl, 10 HEPES, 1 EGTA, and pH 7.4; 1 mM Na oxalate was added to the bath control solution to make the Cl^-/oxalate test solution. The test solution for Cl^-/HCO_3^- exchange contained (in mM): 24 $NaHCO_3$, 116 Na Glutamate, 1 EGTA, 1 NaCl, gassed with 5% CO_2 and 95% O_2 during usage.

For the inward current recording, the pipette solution contained (in mM): 24 $NaHCO_3$, 116 Na Glutamate, 1 EGTA, 1.4 NaCl, gassed with 5% CO_2 and 95% O_2. The bath control solution is the same as pipette solution. The bath test solution with 140 mM Cl^- contained (in mM): 140 NaCl, 10 HEPEs, 1 EGTA, and pH 7.4; the bath test solution with 14 mM Cl^- contained (in mM): 14 NaCl, 126 Na Glutamate, 10 HEPEs, 1 EGTA, and pH 7.4. Current recordings were performed at the room temperature.

APs were recorded at 36 ^oC by perforated patch-clamp techniques using a temperature controller TC-344C (Warner Instruments, Hamden, CT). The signals were filtered at 1 kHz and digitized at a sampling frequency of 5 kHz. The patch-pipettes were backfilled with amphotericin (200 μg/ml). The pipette solution contained (in mM): 120 K-glutamate, 25 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, with pH 7.4. The external solution contained (in mM): 120 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, 24 $NaHCO_3$, gassed with 5% CO_2 and 95% O_2

during usage. Data analysis was performed using Clampfit 10 software (Molecular Devices) and Origin 6.1 software (OriginLab Corp., Northampton, MA).

For whole-cell Ca^{2+} current recording, the pipette solution contained (in mM): 100 CsOH, 100 aspartic acid, 20 CsCl, 1 $MgCl_2$, 2 ATP, 0.5 GTP, 10 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA; Life Technologies), and 5 HEPES, pH 7.4 with CsOH. The external solution contained (in mM): 140 *N*-methylglucamine (NMG), 5 CsCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 2 4-AP, 10 glucose, and 10 HEPES, pH 7.4 with HCl. For whole-cell total K^+ current recordings, the pipette solution contained (in mM): 144 potassium gluconate, 1.15 $MgCl_2$, 5 EGTA, 10 HEPES, 3.85 $CaCl_2$, pH 7.25 with KOH. The external solution contained (in mM): 140 NMG, 4 KCl, 1$MgCl_2$, 5 glucose, 10 HEPES, pH 7.4 with HCl. The recordings were performed at the room temperature.

Measurement of sarcomere shortening and Ca^{2+} transient (CaT)

The IonOptix sarcomere detection (IonOptix LLC., Westwood, MA) and fast Fourier transform (FFT) method[6], rather than Fluo-4 confocal imaging edge detection, were used to measure single cardiomyocyte contraction because the latter may lose precision if the cell's edges move out of the focal plane during contraction. Contraction was measured using a high-speed camera (MyoCam-S, 240 to 1000 frames/s) to record sarcomere movement. The sarcomere pattern was used to calculate the sarcomere length using an FFT algorithm. The fractional shortening was calculated as the percentage change in sarcomere length during contraction. Simultaneous Ca^{2+} transients (CaT) were recorded using Fura-2 dual-wavelength ratiometric method, which is more precise than using the Fluo-4 single-wavelength method. Additional analyses included assessment of sarcoplasmic reticulum (SR) Ca^{2+} load as we described before[6].

Cardiomyocytes loaded with Fura-2 were seeded in a recording chamber with pacing electrodes connected to a stimulator. Cells were paced at 0.5 Hz and continuously perfused during recording. The recording solution contained (in mM): 145 NaCl, 4 KCl, 1 $CaCl_2$, 0.33 NaH_2PO_4, 1 $MgCl_2$, 10 Glucose, 10 HEPES, and pH 7.4. In a separate set of experiments, HCO_3^- was used as a buffer, and the recording solution contained (in mM): 120 NaCl, 4 KCl, 1 $CaCl_2$, 0.33 NaH_2PO_4, 1 $MgCl_2$, 10 Glucose, 24 $NaHCO_3$, gassed by 5% CO_2 and 95% O_2. To reduce the pH_i, but keep the pH_o steady, sodium acetate was applied to clamp the pH_i[7, 8]. Sodium acetate was used to replace an equal concentration of NaCl and 5 μM 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) was added to inhibit the Na^+/H^+ exchanger for acetate application.

Intracellular pH (pH_i) measurement

The pH_i of cardiomyocytes was measured using carboxy-SNARF-1 fluorescent pH indicator. Isolated cardiomyocytes were loaded by 10 μM SNARF-1 AM in Tyrode's solution containing (mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, 10 HEPES, and pH 7.4. The loaded cells were perfused continuously using a self-made fast perfusion system. Cells were excited at 488 nm and the emission was measured simultaneously at 580 and 640 nm, with band pass emission filters by confocal microscope (LSM 700, Carl Zeiss, Oberkochen, Germany). The measurement was performed using Na^+-free solutions either buffered by HEPES or HCO_3^-. HEPES-buffered solution contained (in mM): 144 NMG-Cl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, 10 HEPES, and pH 7.4; HCO_3^--buffered solution contained (in mM): 120 NMG-Cl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, 24 Choline-HCO_3, gassed by 5% CO_2 and 95% O_2. Sodium acetate was used to clamp pH_i, and 5 μM 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) was added to inhibit the Na^+/H^+ exchanger for acetate application.

The SNARF emission ratio (F_{580}/F_{640}) was converted to a pH_i value using standard calibration[9-11]. The calibration was performed by measuring the SNARF emission ratios when the cells were perfused by calibration solutions with five different pH values. The calibration solutions contained (in mM): 140 KCl, 1 $MgCl_2$, 20 HEPES (or MES at pH 5.5), with pH 5.5, 6.5, 7.0, 7.5, 8.0. 10 μM negericin (a K^+/H^+ antiporter ionophore) was added to the calibration solution before use.

Molecular cloning from human cardiac tissues

De-identified human ventricular specimens were obtained from a commercial source (T Cubed), in accordance with the approved UC Davis Institutional Review Board (IRB) protocol. Total RNA and mRNA were extracted as previously described[2]. The cDNA was synthesized using oligo-dT primer. For amplification of full length coding sequence of human cardiac SLC26A6, two different forward primers were designed corresponding to the genomic DNA (gDNA) sequence in chromosome 3 (NC_000003.12), one on 5'UTR of exon 1 and the other on the intron sequence between exon 1 and exon 2, based on the exon structures of human SLC26A6 variant 1 and 4 (NM_001040454.1 and NM_022911.2) to verify isoforms having distinct 5'UTR and/or 5' end of CDS (**Figure 7A**) (For-long primer; 5'-CCTGGGGGAGCTGTTTGAA, For-short primer 5'-GGCTCTGTGGTCCCAAGTA, Rev primer: 5' GGGGGTTTCATGAGGGTGAC). The PCR products were then cloned into pIRES-DsRed plasmid vector and the full-length sequence was confirmed by automated sequencing.

Heterologous expression in Chinese hamster ovary (CHO) cells

Human cardiac SLC26A6 isoforms were expressed in CHO cells following the protocol we used previously[2]. CHO cells were cultured in F-12 media with 10% fetal bovine serum at 37 °C with 5% CO_2. Human cardiac SlC26A6 clones were transiently transfected into cells using

Lipofectamine 2000 (ThermoFisher Scientific) following the manufacturer's instructions. The cells were plated on glass coverslips for patch clamp recordings 2 to 3 days after transfection.

Statistical analyses

Data are presented as mean ± S.E.M. Statistical comparisons were analyzed by student's t-test. Statistical significance was considered to be achieved when $p<0.05$.

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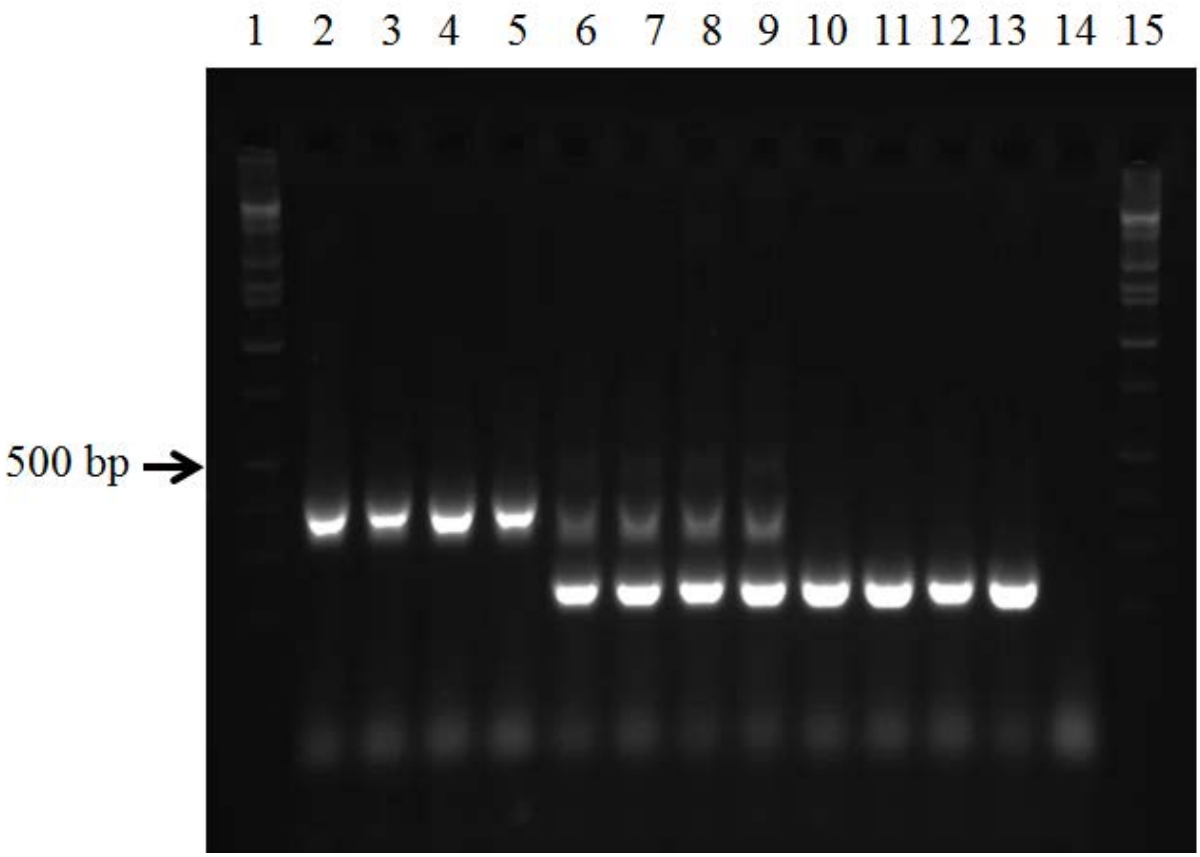
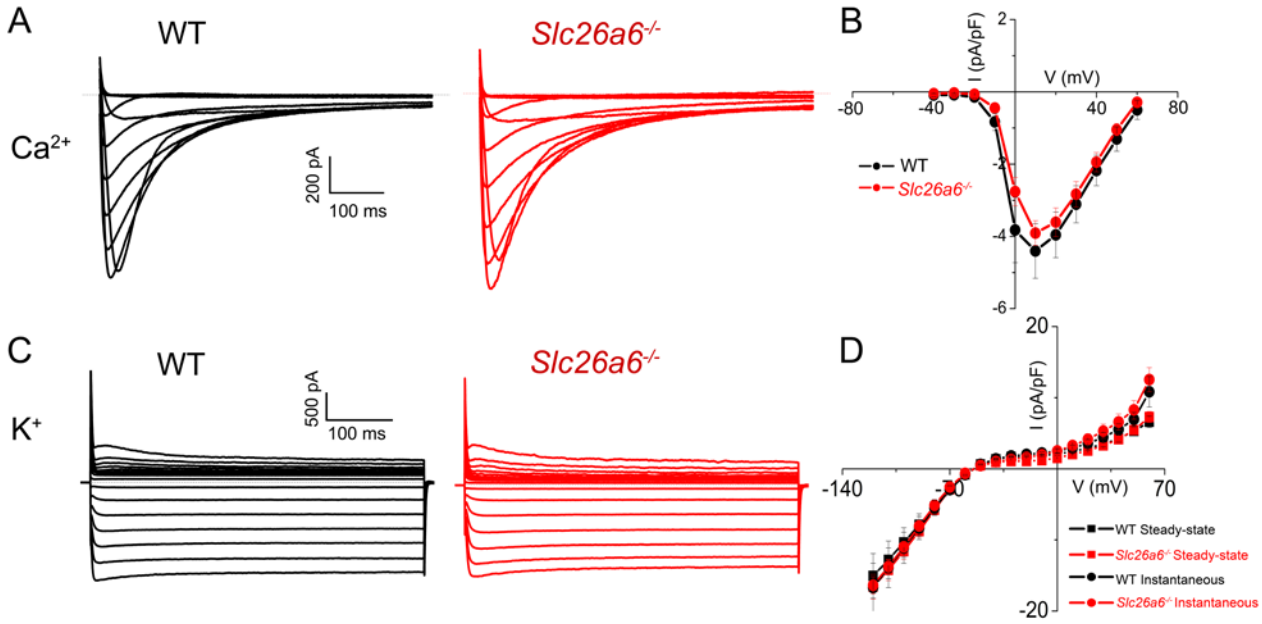


Supplementary Figure 1. Photomicrograph showing genotype analyses using RT-PCR for wild-type (WT), *Slc26a6*$^{+/-}$ and *Slc26a6*$^{-/-}$ mice. Lanes 1 and 15 are ladders (DNA MARKER, HI - LO 50 - 10,000 bp Marker, BIONEXUS). Lanes 2-5, 6-9, and 10-13 are WT, *Slc26a6*$^{+/-}$ and *Slc26a6*$^{-/-}$, respectively. Lane 14 is no template control. The expected sizes for the 2 bands are 250 and 400 bp for the WT and mutant alleles, respectively. Three sets of primers were used with 2 forward (F) primers for WT or mutant (KO) alleles and one reverse (R) primer for both WT and mutant alleles. The primers used are as follow:

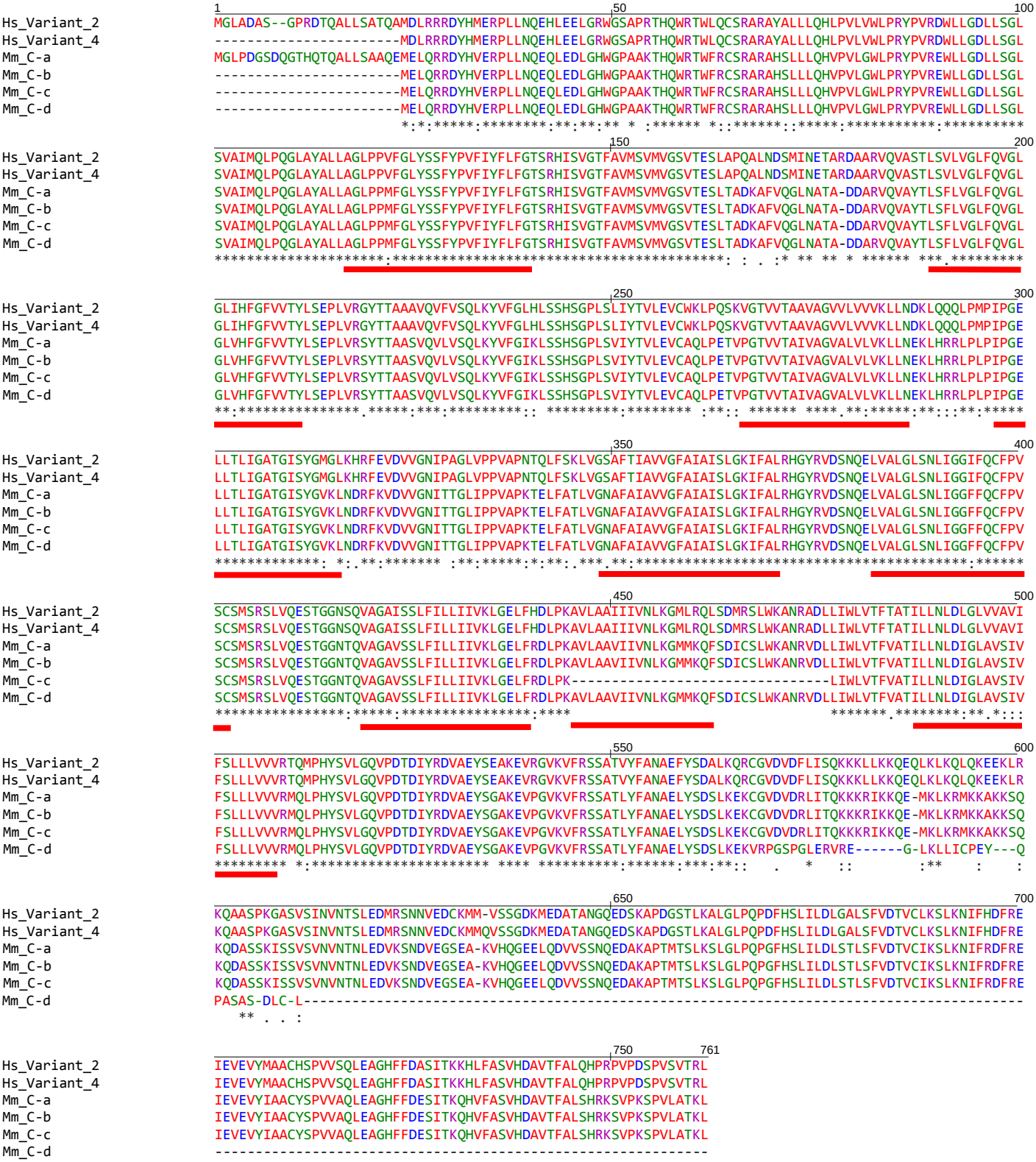
Slc26a6-WT-F, CAAAGCCCTGGCTTCAGGGTGAATGATCTAG;

Slc26a6-WT-KO-R, GAAGAGGCCGACCAGGAAGCTGAGTGTGTAG;

and *Slc26a6*-KO-F, CTTCCATTGCTCAGCGGTGCTGTCCATCTG.



Supplementary Figure 2. Ca^{2+} currents and total K^+ currents recorded from WT and *Slc26a6*$^{-/-}$ ventricular myocytes. A. Representative Ca^{2+} current traces. Ca^{2+} currents were elicited from a holding potential of −55 mV using a family of test pulses stepping from −40 to +60 mV with a 10-mV increment and 500 ms in duration. The interpulse interval was 5 s. The dotted line represents zero current. **B**. Current-voltage (I-V) relationship of Ca^{2+} currents from WT and *Slc26a6*$^{-/-}$ ventricular myocytes (n=7 for WT, and n=6 for *Slc26a6*$^{-/-}$). **C**. Representative total K^+ current traces. K^+ currents were elicited from a holding potential of −55 mV using a family of test pulses stepping from +60 to -120 mV with a 10-mV decrement and 500 ms in duration. The interpulse interval was 3 s. The dotted line represents zero current. **D**. Steady-state and instantaneous I-V relationship of total K^+ currents from WT and *Slc26a6*$^{-/-}$ ventricular myocytes (n=6 for WT, and n=5 for *Slc26a6*$^{-/-}$). The steady-state currents were measured at the end of the test pulse, and the instantaneous currents were measured as the peak currents at the beginning of the test pulse.


Hs_Variant_2
Hs_Variant_4
Mm_C-a
Mm_C-b
Mm_C-c
Mm_C-d

Supplementary Figure 3. Molecular identification and sequence analysis of human cardiac Slc26a6 isoforms. Sequence analysis and alignments of the two human cardiac Slc26a6 isoforms (Hs_Variant_2 and Hs_Variant_4), the same sequence as the previously published variant 2 (NM_134263.2) and variant 4 (NM_001040454.1) from human. The amino acid sequence were aligned to our previously published four mouse cardiac isoforms Mm_C-a, Mm_C-b, Mm_C-c, and Mm_C-d, respectively. Mm_C-a has a 23 a.a. extended NH_2-terminus. Mm_C-c lacks in-frame exon 12 resulting in protein that is 32 a.a. shorter than Mm_C-b. Mm_C-d used an alternate splice site, which caused frame-shift and early termination. Residues that are identical among all of the clones are marked by an asterisk, while a colon marks the highly conservative and a period the weakly conservative substitutions, respectively, and no mark represents nonconservative substitution. Amino acid numbers for the full-length coding sequences are given on the right. The predicted transmembrane domains were labelled by the red bar under the sequence.