SUPPLEMENTAL MATERIAL

Expanded Methods

Animals

Gata6 conditional mutant mice (*Gata6$^{f/f}$*), in which exon 4 encoding the carboxyl-terminal, zinc-finger domain flanked by loxP (floxed) sites have been previously described[1]. *Mlc2v-Cre* mice, with Cre-recombinase genetically engineered to replace the first exon in one allele of the myosin light chain 2v (*Mlc2v*) locus[2], were intercrossed with *Gata6* floxed mice to generate mice harboring cardiomyocyte-restricted deletion of the carboxyl zinc-finger domain of *Gata6* (*Mlc2vCre-Gata6$^{f/f}$*). To obtain staged embryos, male and female mice were placed together overnight in breeding cages when the female was in estrus. The next day the female was inspected for a vaginal plug and if a plug was present the mice were separated. Noon was considered embryonic day E0.5. Mice were bred to the F6 generation or greater on a pure C57BL/6 background. All protocols conformed to the guidelines established by the Association for the Assessment and Accreditation of Laboratory Animal Care and approved by the University of Pennsylvania Animal Care and Use Committees. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

In situ hybridization

In-situ hybridization was performed on murine embryos recovered from pregnant mice euthanized by carbon dioxide inhalation followed by cervical dislocation. as previously described[1]. The embryos were removed from the uterus, trimmed, washed (2X in PBS) and fixed overnight in 2% paraformaldehyde. They were then dehydrated through an ethanol series followed by xylene, embedded in paraffin and sectioned (4-6 µm). The sections were then deparaffinized, rehydrated, treated with Proteinase K and incubated overnight with digoxigenin–labeled riboprobes. Probes complementary to *Gata6 exon4* and *Id2* have been previously described[1, 3]. An in situ probe to detect RNA sequences encoded by the mouse *Tbx3* gene was generated by subcloning a 695-bp fragment from basepair +1047 through +1741 of the mous *Tbx3* cDNA into the pGEM-T vector and using SP6 polymerase to *in vitro* transcribe an antisense cRNA probe. The DNA fragment corresponding to the 5′ and 3′ ends of the mouse *Tbx3* cDNA was generated by PCR from cDNA obtained by reverse transcription of total RNA using the SuperScript One-Cycle cDNA kit (Life Technologies, Grand Island, NY). The primer pairs used were 5’-TGCCGAGCTCCTACTGAAACCGA-3’ and 5’-

TTCGACTAGTCCTTCCTGACTTCG-3’ and the resulting PCR generated DNA fragment was cloned into the *SacI* and *SpeI* sites of the pGEM-T vector (Promega, Corp., Madison, WI). After washing, the slides were incubated overnight with anti-digoxigenin antibody (1:1500) to mark transcriptional expression, and then visualized under epifluorescence and dark-field microscopy on a Nikon E600 microscope.

Immunoblot analysis

Heart lysates were obtained from age-matched, adult littermate mice and immunoblot analysis performed as previously described[4]. The superior aspect of the interventricular septum containing the AV node from adult *Mcl2vCre-Gata6*$^{f/f}$ and *Gata6*$^{f/f}$ mice were dissected and homogenized in buffer containing 1% Igepal surfactant, 0.5% deoxycholate sodium, 2% SDS, 5 mM EDTA (pH=7.4) and 1% protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Samples were cleared of debris by centrifugation at 5000 g for 10 min. and the protein concentration was determined using a BCA protein assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). Protein samples were run on a 4-12% gradient pre-cast SDS-PAGE gel (Life Technologies) and after electrophoresis were transferred to PVDF membranes (Millipore Inc., Billerica, MA). Membranes were incubated with primary antibodies in 5% milk and PBS overnight at 4°C and then washed for 5 min. 4 times with PBS-T solution. The following primary antibodies were used for immunoblot analysis at a concentration of 1:1000 (unless otherwise specified) and consisted of: goat polyclonal anti-GATA6 antibody (AF1700, R&D Systems Inc., Minneapolis, MN); rabbit polyclonal anti-ID2 antibody (sc-489, Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit polyclonal anti-HCN4 antibody (AB5808, Millipore); rabbit polyclonal anti-α-MHC antibody (sc-32732, Santa Cruz); rabbit polyclonal anti-α-cardiac actin antibody (P68032, Millipore); rabbit polyclonal anti-connexin40 antibody (cs-20466, Santa Cruz Biotechnology Inc.) and mouse monoclonal anti-GAPDH antibody at 1 μg/ml (MAB374, Millipore). Membranes were then incubated with secondary antibody (Western Breeze, Life Technologies) for 45 min. at room temperature and subsequently washed in PBS-T for 5 min. 4 times. Results were visualized by enhanced chemiluminescence using a commercially available kit (Western Breeze, Life Technologies) and data were recorded on BioMax-MR film (Kodak, Rochester, NY). Densitometry was determined using the Image-J software package (NIH).

Histology and immunohistochemistry

Detailed protocols used for histological and immunohistochemical staining have been previously described[4, 5]. For immunohistochemical analysis, mice were injected with heparin (100 units IP), followed by euthanasia with pentobarbital overdose (120 mg/kg IP) 5 minutes later and the hearts were rapidly excised through a sternotomy while embryos were harvested from the uterus. The hearts and embryos were then rinsed of blood in ice-cold phosphate-buffered saline (PBS) and either fixed in 2% paraformalin in PBS for 30 min. or equilibrated in 30% sucrose/PBS and embedded for cryosectioning in OCT medium (Sakura Fine Tek, Torrance, CA). After sectioning, and just prior to staining, frozen hearts were fixed with a 0.5% glutaraldehyde/PBS solution for 10 min. at room temperature. Fixed hearts and embryos were dehydrated and embedded in paraffin wax according to standard procedures. Sections were cut and mounted on slides from fixed tissues (4-6 μm) or frozen hearts (8-12 μm). Fixed sectioned were then dewaxed in xylene, rehydrated through an ethanol series and then heated in 1X Antigen Unmasking Solution (Vector Laboratories) in a 350W microwave oven for 10 min. to expose the epitope. Both frozen and fixed sections were washed in PBS before blocking with 5% skim milk in PBS for 30 minutes. Fixed sections were incubated overnight at 4ºC with either a goat polyclonal anti-GATA6 antibody (1:20, AF1700, R&D Systems); rabbit polyclonal anti-GFP antibody (1:50, AB6673, AbCam), rabbit polyclonal anti-ID2 antibody (1:10, sc-489, Santa Cruz); goat polyclonal anti-TBX3 antibody (1:50, sc-17871, Santa Cruz) or a rabbit polyclonal anti-PCNA (1:50 sc-9857-R, Santa Cruz) diluted with 5% skim milk in PBS. Frozen sections were incubated overnight at 4ºC with either a rabbit polyclonal anti-HCN4 antibody (1:50, PCA-052, Alomone Labs), rabbit polyclonal anti-connexin40 antibody (1:50, cs-20466, Santa Cruz) or goat polyclonal anti-TBX3 antibody, also diluted with 5% skim milk in PBS. After washing in PBS sections were incubated with Alexa488- or Texas Red-conjugated rabbit anti-mouse, goat anti-rabbit, donkey anti-goat (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or AMCA streptavidin-conjugated goat anti-rabbit secondary antibodies (Vector Laboratories) at a 1:500 dilution for 1 hour at room temperature. Sections were then washed in PBS and mounted for analysis using either a Nikon i80 microscope equipped with a digital camera and fluorescence capabilities or a Leica TCS SP8 confocal microscope.

For wheat germ agglutinin staining, deparaffinized slides were incubated for 1 hour at room temperature with primary antibody against WGA conjugated to Texas Red (10 μg/ml, Invitrogen) in PBS. The slides were then rinsed 3 times in PBS, mounted in Vectashield with DAPI (Vector Labs) and imaged by fluorescence microscopy using a Nikon i80 microscope. For

each section we captured 10-20 images at 20X magnification and cell size was calculated using ImageJ software.

Transthoracic echocardiography

Protocols for murine transthoracic echocardiography have been previously described[5, 6]. Briefly, five *Mlc2vCre-Gata6*$^{f/f}$ and five *Gata6*$^{f/f}$ mice were anesthetized with 0.75-1% isoflourane plus 100% oxygen while supine on a heated platform. An additional infrared heating lamp was used to maintain body temperature at 37±0.5°C throughout the procedure. An ECG signal was obtained from the electrode pads on the heated platform, and chest hairs removed with a chemical depilator (Nair) to minimize ultrasound attenuation. Transthoracic echocardiography was performed using a Vevo 770 VisualSonic machine (Toronto, Canada) equipped with a linear 30-MHz probe (RMV 707B). Two-dimensional images were recorded in the parasternal long- and short-axis projections to guide M-mode recordings obtained at the mid-ventricular level in both views. Digital images were obtained at a frame rate of 180 images/sec. Thickness of the ventricular (LV) interventricular septum (IVS) and posterior wall (PW) were measured and the average value from both views reported. We computed LV fractional shortening, LVFS=(LVIDd-LVIDs)/LVIDd), and LV ejection fraction, $LVEF=(LVIDd^3-LVIDs^3)/LVIDd^3$, from the M-mode measurements.

Electrophysiology studies

Surface ECG and invasive mouse electrophysiology studies were performed as previously described[4, 7]. Surface ECG recordings and complete in vivo electrophysiological studies (EPS) were obtained from twenty-one *Mlc2vCre-Gata6*$^{f/f}$ mice (aged 50-58 weeks) and twenty-one age-matched *Gata6*$^{f/f}$ littermates. Each mouse was anesthetized with 1.0-1.5% isoflourane plus 100% oxygen, and multi-lead ECGs were obtained using 26-gauge subcutaneous electrodes. Core body temperature was maintained with an infrared heating lamp at 33-34°C and monitored with a rectal probe. A jugular vein cutdown was performed and an octapolar 1.0-French electrode catheter (EP800, Millar Instruments Inc. Houston, TX) placed in the right atrium and ventricle under electrogram guidance to confirm catheter position. A programmed digital stimulator (DTU-215A, Bloom Associates Ltd., Reading, PA) was used to deliver electrical impulses at approximately twice the diastolic threshold. Surface ECG and intracardiac electrograms were simultaneously displayed on a multichannel oscilloscope recorder (Bard Electrophysiology, Inc. Lowell, MA) at a digitization rate of 2 kHz and stored on optical media for offline analysis. ECG channels were filtered from 0.5-250 Hz and intracardiac electrograms

were filtered from 5-400 Hz. ECG intervals were measured by two independent observers blinded to the animal's genotype. We considered an arrhythmic episode to be the induction of three or more consecutive ectopic beats following the last extrastimuli of a drive train.

In vivo electrophysiology stimulation protocol

The *in vivo* study began by determining atrial and ventricular capture thresholds. We start with a capture output of 0.1 mA at 1.0 ms (with good catheter position capture threshold should be 0.1 mA or less) and then place the output at 0.2 mA or twice the diastolic threshold if capture threshold is between 0.1 - 0.2 mA. If capture threshold is above 0.2 mA, then the catheter position is readjusted until the capture threshold is acceptable. Sinus Node Recovery Times were determined by delivering atrial pacing at drive cycle lengths of 120 and 100 ms for 15 seconds each, and then measuring the recovery time to the first atrial electrogram from the last S1 of the train. AV Nodal Conduction was determined by delivering atrial pacing starting at 150 ms and decrement by 10 ms down to the AV Wenckebach cycle length. Then we increase the atrial pacing cycle length by 5 ms to see if there is either 1:1 conduction or Wenckebach. We continue delivering atrial burst pacing and decrement by 10 ms down to 2:1 conduction and then increase the atrial pacing cycle length by 5 ms to see if there is still 2:1 conduction or Wenckebach. AV Node Effective Refractory Period was determining by delivering a drive cycle of 120 ms with an S2 coupled at 115 ms and we then brought in S2 by 5 ms until there was no longer conduction to the ventricle. Atrial Effective Refractory Period was determined by beginning at a drive cycle of 120 ms with an S2 coupled at 70 ms and bringing in S2 by 10 ms until there was loss of atrial capture. We then brought out S2 by 5 ms until atrial capture returns. This procedure was repeated with a drive cycle 100 ms beginning with an S2 coupled at 70 ms. *Atrial Double Extrastimuli* were delivered to determine atrial arrhythmia inducibility with a train of 8 S1s at a cycle length of 120 ms, an S2 coupled at 70 ms and an S3 coupled at 70 ms. We then brought in S3 by 10 ms until it was just below AERP and then brought in S2 by 10 ms until it was below AERP. *Atrial Triple Extrastimuli* were also delivered to determine atrial arrhythmia inducibility with a train of 8 S1s at a cycle length of 120 ms with an S2 coupled at 70 ms, an S3 coupled at 70 ms and an S4 also coupled at 70 ms. We brought in S4 by 10 ms until it was just below AERP and then repeated the protocol as above with atrial doubles. Atrial Burst Pacing, to determine atrial arrhythmia inducibility, was delivered as a train of 28 S1s at a cycle length of 50 ms followed by four extrastimuli with a coupling interval of 30 ms for about 20 seconds with a pause of one second in between trains. We then repeated this protocol with a train of 48 S1s for 20 more seconds followed by 20 seconds more with a train of 8 S1s. Retrograde AV Conduction

was determined by delivering ventricular pacing at a cycle length of 120 ms and incrementing the pacing cycle length by 10 ms steps until there is 1:1 VA conduction or the paced cycle length exceeded the sinus cycle length. Right Ventricular Effective Refractory Period was determined by delivering a train of 8 S1s at a cycle length of 120 ms with an S2 coupled at 70 ms and bringing in S2 by 10 ms until S2 failed to capture, and then bringing out S2 by 5 ms to find the refractory period. The protocol was repeated at a drive cycle of 120 ms and then 100 ms coupled with an S2 at 70 ms. *Ventricular Double Extrastimuli* were delivered as a train of 8 S1s at a cycle length of 120 ms with an S2 coupled at 70 ms and an S3 coupled at 70 ms. We brought in S3 by 10 ms decrements until it was refractory or down to 50 ms, then we brought in S2 by 10 ms and brought out S3 by 10 ms and continued this stepwise process until S2 was refractory. *Ventricular Triple Extrastimuli* were also delivered as a train of 8 S1s with the same drive cycle as used for the ventricular double extrastimuli with an S2, S3 and S4 coupled at 70 ms each. We repeated the protocol as above for ventricular double extrastimuli starting with S4 and brought in each extrastimuli until it was refractory or down to 50 ms in the same stepwise process until S2 was refractory. Ventricular Burst Pacing to determine ventricular arrhythmia inducibility was delivered as a train of 28 S1s at a cycle length of 50 ms followed by four extrastimuli with a coupling interval of 30 ms for about 20 seconds with a pause of one second in between trains. We then repeated the protocol with a train of 48 S1s for 20 more seconds followed by 20 seconds more with a train of 8 S1s.

Ambulatory ECG recordings

Ambulatory ECG recordings were obtained by aseptic, subcutaneous implantation of a 1.1-g wireless radiofrequency telemetry device (ETA-F10; Data Sciences International, St. Paul, MN) configured to record a signal analogous to ECG lead I as previously described[17]. Following a 7 day recovery period, continuous ECG recordings were obtained for at least 24 hours from eight *Mlc2vCre-Gata6$^{F/F}$* and eight *Gata6$^{F/F}$* mice in separate cages overlying a receiver. ECG intervals were measured by two independent observers blinded to the genotypes using digital calipers in the LabChart 5.0 analysis suite (ADInstruments, Inc., Colorado Springs, CO).

CCS volume calculations

Volumetric determination of the proximal CCS was performed as previously described[4]. To determine acetylcholine esterase (AchE) activity in the proximal CCS of five *Mlc2vCre-Gata6$^{f/f}$* and five *Gata6$^{f/f}$* mice (aged 40-46 weeks), the animals were injected with heparin (100 units IP), followed by euthanasia with pentobarbital overdose (120 mg/kg IP) 5 minutes later. The

hearts were then excised through a sternotomy. The hearts were rinsed of blood in ice-cold phosphate-buffered saline (PBS), snap frozen in liquid nitrogen and embedded unfixed in paraffin. Sections (4-6 μm) were cut and mounted on slides from the frozen hearts and fixed in 5% glutaraldehyde for 15 min. and then rinsed in PBS x 3. Images were obtained following staining for acetylcholine esterase activity as previously described[8]. Sections from adult hearts were incubated with fresh acetylcholine esterase stain (5 mM NaOAc, pH=5; 1 mM glycine; 0.2 mM $CuSO_4$; 1.15 mg/ml acetylthiocholine iodide - Sigma A5751) overnight at room temperature. Following incubation, the sections were rinsed with distilled water and counterstained with hematoxylin for 1 min. Stained sections were dehydrated with ethanol. To assess the extent of HCN4-positive staining in the proximal CCS of adult hearts, the hearts were excised, fixed, embedded and sectioned as described above for immunohistochemical staining. Sections were then deparrafinized and rehydrated as described above, blocked with 3% hydrogen peroxide in PBS and then rinsed in PBS-T with 10% goat serum. The sections were then incubated with a rabbit polyclonal anti-HCN4 antibody (1:50, PCA-052, Alomone Labs). After staining the sections with the primary antibody, the slides were rinsed in PBS-T x 6 and then stained with a biotinylated goat anti-rabbit IgG antibody (1:200; BA-1000, Vector Labs) at room temperature for 1 hour. The slides were then incubated in ABC solution for 45-60 min. following the manufacturer's directions (VECTASTAIN ABC Kit, Rabbit IgG, PK-4001, Vector Labs) The slides were rinsed in PBS-T x 4 and the color allowed to develop. Slides were then counterstained with hematoxylin for 1 min. To estimate the proximal CCS volume we obtained digital photomicrographs of all histologic sections through the proximal CCS from five hearts of each genotype using a Nikon i80 microscope (Nikon Inc., Melville, NY) equipped with a Nikon DXM1200 digital camera. The structural regions of the CCS were defined according to the method of Rentschler et al.[9] where stained sections above the bifurcation of the bundle branches, encompassed by atrial tissues, were taken as the AV node and stained tissues distal to the AV node, but proximal to the division of the bundle branches, were taken as the His-bundle. Measurement of the area of interest (the AV node and His-bundle) was performed using the ImageJ software suite (NIH). The sum of the areas of interest multiplied by the section thickness (5 μm assumed average thickness) was used to compute CCS volume.

To assess bundle branch size we excised the hearts from four adult *HopXLacZ::Gata6$^{f/f}$* and four adult *HopXLacZ::Mlc2vCre-Gata6$^{f/f}$* (aged 40-46 weeks) as described above for calculating proximal CCS volume. These hearts were pre-fixed in 2% paraformaldehyde for 2 hours, rinsed in PBS x 5 and then both free ventricular walls were removed and the hearts were incubated in X-gal staining solution (2mM $MgCl_2$, 5mM K-Ferricyamide, 5mM K-Ferrocyamide,

0.1% deoxycholate, 1 X PBS, 0.01% NP40, and 0.1% X-gal) overnight at room temperature. The hearts were then washed with PBS, fixed in 4% paraformaldehyde overnight and then rinsed with PBS X 5. They were then dehydrated through an ethanol series. Sections (8-10 μm) were cut transversely from the superior 2/3 of the intraventricular septum starting at just below the level of the AV annulus, mounted on slides and counterstained with hemoxylin for 1 min. To estimate the area of both bundle branches we obtained digital photomicrographs of 10 histologic sections through three different regions of the intraventricular septum that were defined as: Level 1 (2 mm below the level of the fibrous AV annulus); Level 2 (2 mm below the last section analyzed from Level 1) and Level 3 (2 mm below the last section analyzed from Level 2). Measurement of the area of interest (the right and left bundle branches) was performed using the ImageJ software suite (NIH).

Plasmids and transient co-transfection analyses

The pcDNA3–GATA6 plasmid encoding the full-length mouse *Gata6* cDNA, the pcDNA3–GATA6–Δexon4 plasmid containing GATA-6 lacking amino acids encoded by exon 4 and the pcDNA3-GATA4 plasmid encoding the full-length mouse *Gata4* cDNA have been previously described[1]. The *Id2*-LUC luciferase reporter plasmid contains a 5.2-kb genomic fragment from the mouse *inhibitor of DNA 2* gene consisting of the promoter sequence from basepair –5992 through –732 cloned upstream of firefly luciferase (Figure 8C). The construct was generated using the bacterial artificial chromosome (BAC) recombineering method[10]. Briefly, the promoter fragment corresponding to the 5′ and 3′ ends of the mouse *inhibitor of DNA 2* genomic fragment were generated by PCR from BAC *CH29-585M17* (CHORI) containing the gene using primer pairs 5'-CGGGGTACCAAGGAGCTAAGGTCGGA-ATTGTGG-3' and 5'-CCGCTCGAGAAAGGAGGTCACTGTCTCATCGCA-3', respectively, cloned into the *KpnI* and *XhoI* sites of the pGL3-promoter vector (Promega Corp.). Similarly, a 5.1-kb promoter fragment of the mouse *sodium-calcium exchanger 1* gene containing the sequence from basepair -4795 through +359 was also generated by PCR from BAC CH29-557N07 (CHORI). The primer pairs used were 5'-CGGGGTACCTGAGCAGCCATCTTGCATGTGTG-3' and 5'-CCGCTCGAGAA-GTAAGCAAGCCTTCCCAGACCT-3', and this promoter fragment was then cloned into the *KpnI* and *XhoI* sites of the pGL3-promoter vector (Promega Corp.). The *Id2-LUC* and *Ncx1-LUC* plasmids were transformed into JM109 bacteria and transformants were screened for ampicillin resistance. HL-1 immortalized murine cardiomyocytes (1×10^5) were co-transfected with 100 ng of either the Id2*-LUC* or the *Ncx1-LUC* reporter plasmid; with 0.125-0.5 μg of either pcDNA3–GATA6, pcDNA3–GATA6–Δexon4 or pcDNA3-GATA4 and 100 ng of the pRL-CMV reference

plasmid (Promega Corp.) using the FuGENE 6 system (Roche Applied Science). Luciferase activity was measured and normalized for transfection efficiency using the Dual Luciferase Assay System (Promega Corp.). Data are reported as the mean normalized to relative light units (fold activation) ± one standard deviation.

Myocyte Isolation and Size Quantification

Ventricular myocytes were isolated from adult *Mcl2vCre-Gata6*$^{f/f}$ and *Gata6*$^{f/f}$ mice as previously described[11]. Briefly, excised hearts were mounted on a Langendorf apparatus and perfused with Ca^{2+}-free Tyrode's solution for 6 minutes at 3.0-3.5 mL/min. at a temperature of 36°-37°C. This was followed by 12-15 minutes of perfusion with Ca^{2+}-free Tyrode's solution containing: 0.35 mg/ml collagenase B & 0.25 mg/ml collagenase D (Roche Diagnostics, Inc., Indianapolis, IN) plus 0.05 mg/ml protease (Type XIV, Sigma-Aldrich Co., St. Louis, MO). Myocyte length and area were assessed from digital images taken at 200X on a Nikon i80 (Nikon Instruments Inc, Melville, NY) microscope equipped with a CCD camera using the Elements D imaging suite (Nikon, Inc.).

Quantitative RT-PCR

Quantitative RT-PCR was performed using SYBR Green according to the manufacturer's protocol. Total RNA was isolated from the crest of the intraventricular septum using a commercial kit (RNeasy, Qiagen, Valencia, CA). PCR was preformed with 4 µl of reverse transcribed cDNA reaction mixture, 500 nM of specific forward and reverse primers and 1X SYBR Green PCR Master Mix (Life Tecnologies, Carlsbad, CA). Quantification of the reaction product was performed using the MJ Research DNA Engine Opticon 2 real time detection system. PCR cycle conditions were 95°C for 10 min., followed by 40 cycles of denaturation at 95°C for 15 sec. and annealing and extension at 60°C for 1 min. All reactions were performed in triplicate with and without RT as controls. Cycle threshold *C(t)* values were converted to relative gene expression levels using the $2^{-\Delta\Delta C(t)}$ method. Primer pair sequences and expected product sizes are listed in Supplemental Table 7.

Chromatin Immunoprecipitation

For these assays we used the Chromatin Immunoprecipitation (ChIP) Assay Kit (17-925, Millipore) and followed the manufacturer's protocol as provided. Briefly, whole hearts were quickly excised from euthanized wild-type mice and Gata6 mice with deletion of the carboxyl zinc-finger domain and homogenized in a dounce homogenizer in 1 ml of cold phosphate-

buffered saline (PBS) with protease inhibitor cocktail. Histones were cross-linked to the DNA by adding formaldehyde to a final concentration of 1% and incubating for 15 min. at room temperature. Cross-linking was stopped by directly adding 1.25 M glycine and gentle rotation on an automated rotator for 5 min. The homogenate was then washed 2X with PBS plus protease inhibitors and resuspended in lysis buffer (50 mM Tris-Cl [pH 8.1], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) containing protease inhibitor cocktail. The samples were then sonicated to obtain chromatin fragments between 200 to 500 bp. Sonicated samples were resuspended in ChIP dilution buffer (0.01% SDS,1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1,167 mM NaCl,) and rotated overnight at 4°C with goat anti-GATA6 antibodies (sc-7244X, Santa Cruz) or goat IgG (negative control, sc-34665, Santa Cruz). The antibody/histone complexes were collected by adding protein A agarose beads with salmon sperm DNA (50% slurry) and rotating the samples for one hour at 4°C. The agarose beads were pelleted by gentle centrifugation (1000 rpm at 4°C for 1 min.), and the pellet was washed 1X with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1,150 mM NaCl). The pellet was then washed 1X with high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) followed by a wash with LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and then washed 2X with TE buffer. The histone complexes were eluted from the antibody by adding freshly prepared elution buffer (1% SDS, 100 mM $NaHCO_3$) and the DNA was reverse cross-linked by adding 5 M NaCl and incubating for 4 to 10 hours at 65°C. DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation and the pellet was resuspended in ddH_2O and quantified by SYBR Green (Life Technologies). Four nanograms of DNA was used per qPCR reaction using the primer sequences listed in Supplemental Table 8.

Statistical analysis

Continuous variables, such as ECG intervals and cardiac conduction properties were compared using 2-tailed, unpaired Student's *t*-test. Data sets with smaller sample sizes ($n \leq 5$) were compared using the Wilcoxon rank sum test. All values are reported as the mean ± 1 standard deviation, unless otherwise noted. A probability value < 0.05 was considered significant.

Supplemental References

1. Lepore JJ, Mericko PA, Cheng L, Lu MM, Morrisey EE, Parmacek MS. GATA-6 regulates semaphorin 3C and is required in cardiac neural crest for cardiovascular morphogenesis. *J Clin Invest.* 2006;116:929-939.
2. Chen J, Kubalak SW, Chien KR. Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development.* 1998;125:1943-1949.
3. Moskowitz IP, Kim JB, Moore ML, Wolf CM, Peterson MA, Shendure J, Nobrega MA, Yokota Y, Berul C, Izumo S, Seidman JG, Seidman CE. A molecular pathway including Id2, Tbx5, and Nkx2-5 required for cardiac conduction system development. *Cell.* 2007;129:1365-1376.
4. Ismat FA, Zhang M, Kook H, Huang B, Zhou R, Ferrari VA, Epstein JA, Patel VV. Homeobox protein Hop functions in the adult cardiac conduction system. *Circ Res.* 2005;96:898-903.
5. Liu F, Levin MD, Petrenko NB, Luu MM, Wang T, Yuan LJ, Stout AL, Epstein JA, Patel VV. Histone-deacetylase inhibition reverses atrial arrhythmia inducibility and fibrosis in cardiac hypertrophy independent of angiotensin. *J Mol Cell Cardiol.* 2008;45:715-723.
6. Yuan L, Wang T, Liu F, Cohen ED, Patel VV. An evaluation of transmitral and pulmonary venous Doppler indices for assessing murine left ventricular diastolic function. *J Am Soc Echocardiogr.* 2010;23:887-897.
7. Patel VV, Arad M, Moskowitz IP, Maguire CT, Branco D, Seidman JG, Seidman CE, Berul CI. Electrophysiologic characterization and postnatal development of ventricular pre-excitation in a mouse model of cardiac hypertrophy and Wolff-Parkinson-White syndrome. *J Am Coll Cardiol.* 2003;42:942-951.
8. El-Badawi A, Schenk EA. Histochemical methods for separate, consecutive and simultaneous demonstration of acetylcholinesterase and norepinephrine in cryostat sections. *J Histochem Cytochem.* 1967;15:580-588.
9. Rentschler S, Vaidya DM, Tamaddon H, Degenhardt K, Sassoon D, Morley GE, Jalife J, GI. F. Visualization and functional characterization of the developing murine cardiac conduction system. *Development.* 2001;128:1785-1792.
10. Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res.* 2003;13:476-484.

11. Liu F, Levin MD, Petrenko NB, Lu MM, Wang T, Yuan LJ, Stout AL, Epstein JA, Patel VV. Histone-deacetylase inhibition reverses atrial arrhythmia inducibility and fibrosis in cardiac hypertrophy independent of angiotensin. *J Mol Cell Cardiol.* 2008;45:715-723.

ADDITIONAL TABLES

Supplemental Table 1. Morphometric and Transthoracic Echocardiograpic Data

	***Gata6*$^{f/f}$ (n=5)**	***Mlc2vCre-Gata6*$^{f/f}$ (n=5)**
Heart weight (mg)	158 ± 11.4	153 ± 10.8
Body weight (g)	26.8 ± 2.2	26.4 ± 2.0
HW/BW (mg/g)	5.90 ± 0.57	5.80 ± 0.50
LV mass (mg)	90 ± 7.8	88 ± 6.6
LV mass/BW (mg/g)	3.36 ± 0.26	3.33 ± 0.22
LVEF (%)	53.9 ± 6.2	50.1 ± 4.1
LV Fractional Shortening (%)	27.5 ± 3.8	25.0 ± 2.4
IVS Thickness (mm)	0.75 ± 0.07	0.77 ± 0.06
PW Thickness (mm)	0.69 ± 0.06	0.67 ± 0.08
LVID in systole (mm)	3.11 ± 0.27	2.95 ± 0.22
LVID in diastole (mm)	3.72 ± 0.30	3.56 ± 0.35

Mean values ± SD. *$p<0.05$ for *Gata6*$^{f/f}$ versus *Mlc2vCre-Gata6*$^{f/f}$ mice. HW = Heart weight; BW = Body weight; LV = Left Ventricular; LVEF = LV Ejection Fraction; IVS = Intraventricular Septum; PW = Posterior LV Wall.

Supplemental Table 2. Surface ECG Data Summary

			Conscious Telemetry ECG	
	Gata6$^{f/f}$ (n=21)	*Cre-Gata6*$^{f/f}$ (n=21)	*Gata6*$^{f/f}$ (n=8)	*Cre-Gata6*$^{f/f}$ (n=8)
Age (days)	341 ± 12.4	345 ± 13.0	268 ± 7.6	274 ± 6.9
Weight (gm)	26.9 ± 2.8	25.3 ± 2.5	26.4 ± 2.2	25.8 ± 1.9
SCL (ms)	137 ± 32.4	133 ± 31.0	108 ± 21.4	111 ± 19.5
HR (bpm)	438 ± 44.9	451 ± 47.7	556 ± 33.1	541 ± 27.2
PR (ms)	47.6 ± 3.7	58.8 ± 3.4*	35.4 ± 3.3	40.0 ± 2.7*
P-wave (ms)	24.7 ± 3.0	25.1 ± 3.2	17.6 ± 1.2	18.2 ± 1.0
QRS (ms)	10.4 ± 1.3	10.6 ± 1.4	10.4 ± 1.1	10.7 ± 0.9
QT (ms)	38.3 ± 4.2	37.8 ± 3.6	29.2 ± 3.1	28.9 ± 2.9
QTm (ms)	29.0 ± 2.1	28.4 ± 2.6	29.0 ± 2.0	28.7 ± 1.8

*$p<0.05$ for *Gata6*$^{f/f}$ versus *Cre-Gata6*$^{f/f}$ mice. *Cre-Gata6*$^{f/f}$ = *Mlc2vCre-Gata6*$^{f/f}$. SCL = Sinus cycle length; HR = Heart rate; PR = PR-interval duration; QRS = QRS-complex width; QT = QT-interval duration; QT_m = Corrected QT-interval duration.

Supplemental Table 3. Invasive EP Data Summary

	***Gata6*$^{f/f}$ (n=21)**	***Cre-Gata6*$^{f/f}$ (n=21)**
AH (ms)	28.1 ± 2.9	37.2 ± 3.1*
H_d (ms)	4.0 ± 1.0	4.2 ± 1.1
HV (ms)	11.9 ± 1.2	12.1 ± 1.6
AVI (ms)	41.0 ± 2.8	48.3 ± 4.2*
$SNRT_{120}$ (ms)	219 ± 68.1	212 ± 66.5
$SNRT_{100}$ (ms)	247 ± 69.8	253 ± 74.0
$AVERP_{120}$ (ms)	61.5 ± 6.0	70.2 ± 5.7
AVWBCL (ms)	94.6 ± 4.7	106 ± 5.0*
AVN 2:1	75.7 ± 6.6	87.2 ± 6.8*
$AERP_{120}$ (ms)	40.9 ± 7.5	41.2 ± 7.2
$AERP_{100}$ (ms)	43.2 ± 8.0	45.4 ± 7.8
$VERP_{120}$ (ms)	41.0 ± 12.2	51.7 ± 9.6
$VERP_{100}$ (ms)	44.3 ± 10.1	52.4 ± 9.8
Duration AT (ms)	165 ± 41.0	182 ± 45.1
AT CL (ms)	38.9 ± 8.0	39.7 ± 10.5
AT/Mouse	0.5	0.5
Duration VT (ms)	235 ± 28.4	248 ± 30.2
VT CL (ms)	58.7 ± 7.4	59..2 ± 8.0
VT/Mouse	0.25	0.25

Symbols are the same as described above for Supplemental Table 2. AH = AtrioHisian interval; H_d = His-duration; HV= Hisioventricular interval; AVI = Atrioventricular interval; $SNRT_{120}$ = Sinus node recovery time at drive train of 120 ms; $SNRT_{100}$ = Sinus node recovery time at drive train of 100 ms; $AERP_{120}$ = Atrial ERP at drive train of 120 ms; $AERP_{100}$ = Atrial ERP at drive train of

100 ms; $AVERP_{120}$ = Atrioventricular ERP, drive train 120 ms; AVWBCL = AV Wenckebach block cycle length; AV 2:1 = AV 2:1 block cycle length; VAWBCL = Ventriculoatrial Wenckebach block cycle length; $VERP_{120}$ = Ventricular ERP at drive train of 120 ms; $VERP_{100}$ = Ventricular ERP at drive train of 100 ms.

Supplemental Table 4. Transcript Expression in the Mature Proximal CCS

Transcript	ΔC(t) *Cre-Gata6*$^{f/f}$/ *Gapdh*	ΔC(t) *Gata6*$^{f/f}$ /*Gapdh*	*Cre-Gata6*$^{f/f}$/*Gata6*$^{f/f}$ Fold Change	samples/ group
Gata6	5.38±0.57	8.96±0.82*	0.08	6
Hcn4	4.21±0.29	6.40±0.33*	0.22	5
Tbx3	4.62±0.31	6.31±0.36*	0.31	5
Id2	7.34±0.19	8.53±0.21*	0.44	5
Ncx1	4.13±0.16	5.42±0.18*	0.41	5
Sema3c	6.08±0.37	6.38±0.40	0.62	5
Scn5a	4.19±0.29	4.42±0.34	0.85	5
Nkx2.5	4.93±0.32	5.2±0.37	0.85	6
Gata4	5.29±0.94	5.53±0.95	0.85	5
Gjd3	4.85±0.36	5.02±0.51	0.89	6
Gja5	7.97±0.42	8.16±0.68	0.88	6
Gja1	1.92±0.67	2.33±0.69	0.75	5
Gja7	5.31±0.48	6.26±0.39*	0.52	6
Bcl2	7.97±0.9	8.6±0.19	0.65	4
Kcn1	14.04±2.59	14.2±1.02	0.88	4
Kv4.2	6.8±0.74	7.7±0.49	0.52	4
Carp	3.28±0.53	4.40±0.44	0.61	6

*$p<0.05$ for *Gata6*$^{f/f}$ versus *Cre-Gata6*$^{f/f}$; *Cre-Gata6*$^{f/f}$ = *Mlc2vCre-Gata6*$^{f/f}$.

Supplemental Table 5. Proximal CCS Volume Measurements in Mature Mice

AchE	*Gata6*$^{f/f}$ (n=5)	*Cre-Gata6*$^{f/f}$ (n=5)
Heart weight (mg)	156±17.2	152±15.4
Heart weight/Body weight (mg/g)	5.9±0.7	5.8±0.6
CCS vol. (x10^6 μm^3)	7.7±1.4	4.3±1.6*
CCS vol./heart wt. (x10^3 μm^3/mg)	49.4±0.7	28.3±1.0*
HCN4	*Gata6*$^{f/f}$ (n=4)	*Cre-Gata6*$^{f/f}$ (n=4)
Heart weight (mg)	158±18.6	154±17.9
Heart weight/Body weight (mg/g)	5.9±0.6	5.8±0.8
CCS vol. (x10^3 μm^3)	2880±87.8	550±40.7*
CCS vol./heart wt. (10^3 μm^3/mg)	18.2±0.9	3.6±0.6*

**p<0.05 for* Cre-Gata6$^{f/f}$ *versus* Gata6$^{f/f}$ *mice.* Cre-Gata6$^{f/f}$ = Mlc2vCre-Gata6$^{f/f}$*.*

Supplemental Table 6. Candidate Transcripts with Known GATA Binding Sites

Transcript	Transcript Description
Id2	Inhibitor of DNA binding 2
Ncx1	Sodium-calcium exchanger 1
Nkx2.5	Homeobox protein Nkx-2.5 (Homeobox protein NK-2 homolog E)
Sema3c	Semaphorin, subfamily 3, member C
Scn5a	sodium channel, voltage-gated, type V, alpha subunit
Gata4	GATA-binding factor 4
Gjd3	Gap junction protein, delta 3
Gja5	Gap junction protein, alpha 5
Gja1	Gap junction protein, alpha 1
Bcl2	B-cell leukemia/lymphoma 2
Kcn1	Potassium voltage-gated channel, subfamily E, member 1
Kv4.2	Potassium voltage-gated channel, subfamily D, member 2
Carp	Ankyrin repeat domain-containing protein 1

Supplemental Table 7. Real-Time PCR Primer Sequences

Transcript	Primer Sequence	Product Size (bp)
Gapdh	5'-gcatggactgtggtcatgag-3' 5'-ccatcaccatcttccaggag-3'	134
Gata6	5'-tcattacctgtgcaatgcatgcgg-3' 5'-acgccataaggtagtggttgtggt-3'	148
Tbx3	5'-agccaacgatatcctgaaactg-3' 5'-gtgtctcgaaaaccctttgc-3'	122
Hcn4	5'-gattatccacccctacagtgac-3' 5'-accacattgaagacgatccag-3'	109
Id2	5'-tgagacccaccctgaacacggacat-3' 5'-ctatcattcgacataagctcagaaggg-3'	80
Sema3c	5'-aagggatcggcagtgtgtgtgtat-3' 5'-aacgtcatctgggaagtccttggt-3'	135
Ncx1	5'-tggagagctcgaattccagaacga-3' 5'-tggtcagtggctgcttgtcatcat-3'	88
Nkx2.5	5'-aagtgctctcctgctttccca-3' 5'-tttgtccagctccactgccttct-3'	132
Scn5a	5'-cctcatcttctggctcatcttc-3' 5'-gactcacactcgctcttgtt-3'	135
Gata4	5'-tcacaagatgaacggcatcaaccg-3' 5'-tcacaagatgaacggcatcaaccg-3'	102
Gjd3	5'-atcatgctgatcttccgcatcctg-3' 5'-gcatggagtagatgacgaaca-3'	114
Gja5	5'-ttcatattccgcatgctggtgctg-3' 5'-ttggtcgtagcagacattcccaca-3'	117
Gja1	5'-tcatcttcatgctggtggtgtcct-3' 5'-tggtgaggagcagccattgaagta-3'	124
Gja7	5'-tttccctgcttgtatgtctcc-3' 5'-atagcgtctcacaaccatctg-3'	112
Bcl2	5'- ttgtggccttctttgagttcggtg-3' 5'-aatcaaacagaggtcgcatgctgg-3'	105
Kcn1	5'-ctgaagttcctccagcaactgact-3' 5'-aaactctgggtgcctggtcct-3'	93
Kv4.2	5'-cttcagcaagcaagttcaccagca-3' 5'-tgaagttcgacacgatcacaggca-3'	113

Supplemental Table 8. ChIP Real-Time PCR Primer Sequences

Primer Pairs	Primer Sequences	Genomic Bases Primers Span*	GATA Consensus	Product Size (bp)
Id2-F1/R1	5'- accacaacaacactcggataag-3' 5'-cctaaaaccccaagggtgagg -3'	-4001 to -3981 -3917 to -3898	-3978 to -3983	103
Id2-F2/R2	5'-ttggcatctggttcctaaaag-3' 5'-tcacctagagtccagaaacttg-3'	-3787 to -3767 -3705 to -3684	-3751 to -3756	103
Id2-F3/R3	5'-cagaaacacatgcacacaaag-3' 5'-cgactcgcctctgttattaag-3'	-1622 to -1602 -1540 to -1520	-1561 to -1566	102
Id2-F4/R4	5'-gcatctgaattcccttctgagc-3' 5'-attaccgaaacgcgcacac-3'	+636 to +657 +722 to +740	+668 to +673	104
Ncx1-F1/R1	5'-caacctaagcttatcctagt-3' 5'-cagtgagataatctaagaccac-3'	-4166 to -4147 -4086 to -4065	-4115 to -4120	101
Ncx1-F2/R2	5'-agagtgaacctgtcttaccc-3' 5'-tttccatgatgactcagacaac-3'	-3182 to -3163 -3099 to -3078	-3097 to -3102	104
Ncx1-F3/R3	5'-agttctgactgtgacctcttc-3' 5'-acctggaatttgcgctaaatg-3'	-1307 to -1287 -1220 to -1200	-1271 to -1276 -1037 to -1042	107
Ncx1-F4/R4	5'-aactgccaacatatcttcacc-3' 5'-aggagtggcgattctatctg-3'	-1116 to -1096 -1019 to -999	-526 to -531	97
Ncx1-F5/R5	5'-agcttgaagatttctctgttcc-3' 5'-aagagccacatcttgaaactc-3'	-609 to -588 -485 to -466	-526 to -531	144
Ncx1-F6/R6	5'-actttgcgtacatcaaagagc-3' 5'-gcactccctcatgattcctaac-3'	+2035 to +2055 +2121 to +2141	+2098 to +2103	106
Id2-FC/RC	5'-aagtgacttggttgggaagggg-3' 5'-gtgcgactcacatactcgtacc-3'	+2813 to +2834 +2894 to +2915	N/A	102
Ncx1-FC/RC	5'-gtgactttggctatagacagc-3' 5'-aaagcttcctgtgtcaac-3'	+3379 to +3399 -3463 to +3481	N/A	102

*Relative to translational start codon.

Supplemental Table 9. GATA Consensus Sequence ChIP Expression: Wild-type Hearts

Primer Pairs	ΔC(t) Gata6 IgG/ IgG	Gata6 IgG/IgG Fold Change	samples/ group
Id2-F1/R1	0.50±0.29	1.41	6
Id2-F2/F2	0.60±0.37	1.51	6
Id2-F3/R3	5.28±0.72*	38.9	6
Id2-F4/R4	5.10±0.67*	34.3	6
Id2-FC/RC	0.21±0.15	1.16	6
Ncx1-F1/R1	2.99±0.55*	7.94	6
Ncx1-F2/R2	4.68±0.72*	25.6	6
Ncx1-F3/R3	7.11±0.95*	138	6
Ncx1-F4/R4	4.11±0.86*	17.3	6
Ncx1-F5/R5	0.89±0.45	1.85	6
Ncx1-F6/R6	0.76±0.42	1.69	6
Ncx1-FC/RC	0.45±0.27	1.37	6

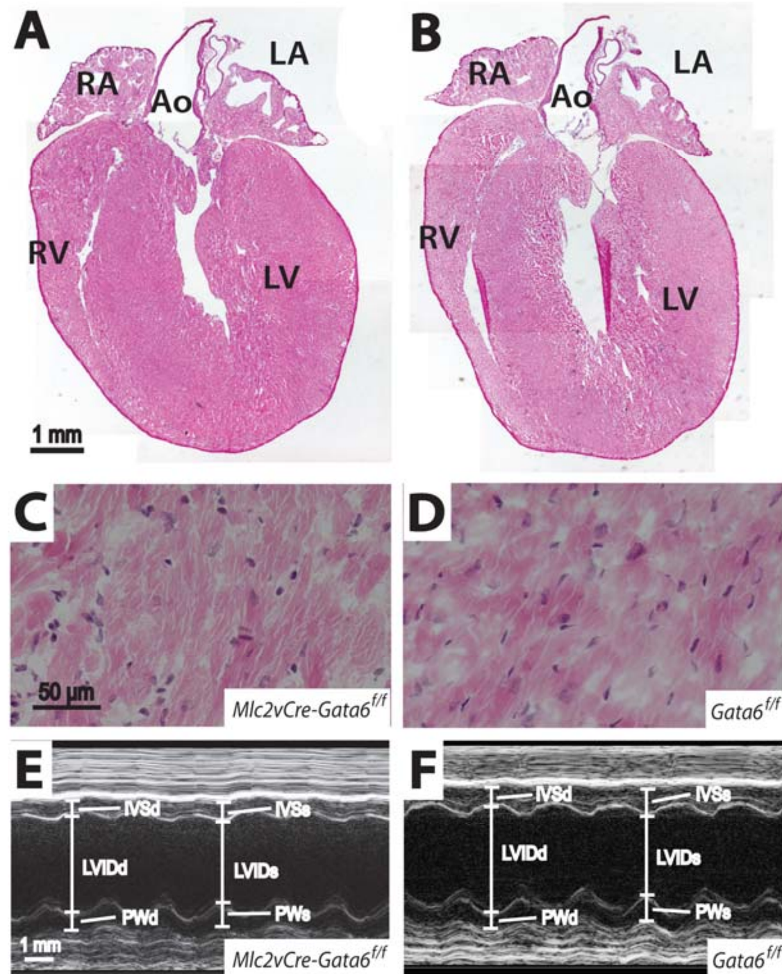
*p<0.05 compared to corresponding Id2 or Ncx1 control segment (FC/RC).

Supplemental Table 10. GATA Consensus Sequence ChIP Expression: Gata6 Mutant Hearts

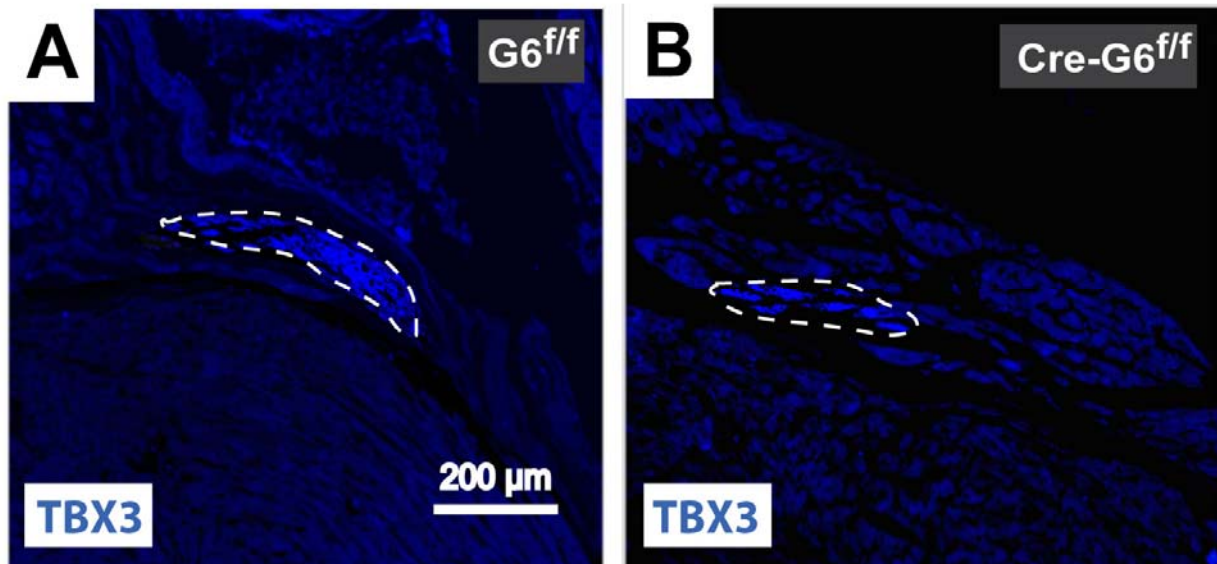
Primer Pairs	ΔC(t) Gata6 IgG/ IgG	Gata6 IgG/IgG Fold Change	samples/ group
Id2-F1/R1	0.40±0.24	1.32	5
Id2-F2/F2	0.46±0.20	1.38	5
Id2-F3/R3	0.79±0.54	1.73	5
Id2-F4/R4	0.61±0.49	1.53	5
Id2-FC/RC	0.51±0.22	1.42	5
Ncx1-F1/R1	0.54±0.31	1.45	5
Ncx1-F2/R2	0.66±0.40	1.58	5
Ncx1-F3/R3	1.17±0.64	2.25	5
Ncx1-F4/R4	0.70±0.59	1.62	5
Ncx1-F5/R5	0.90±0.38	1.87	5
Ncx1-F6/R6	0.73±0.35	1.66	5
Ncx1-FC/RC	0.59±0.29	1.51	5

*$p<0.05$ compared to corresponding Id2 or Ncx1 control segment (FC/RC).

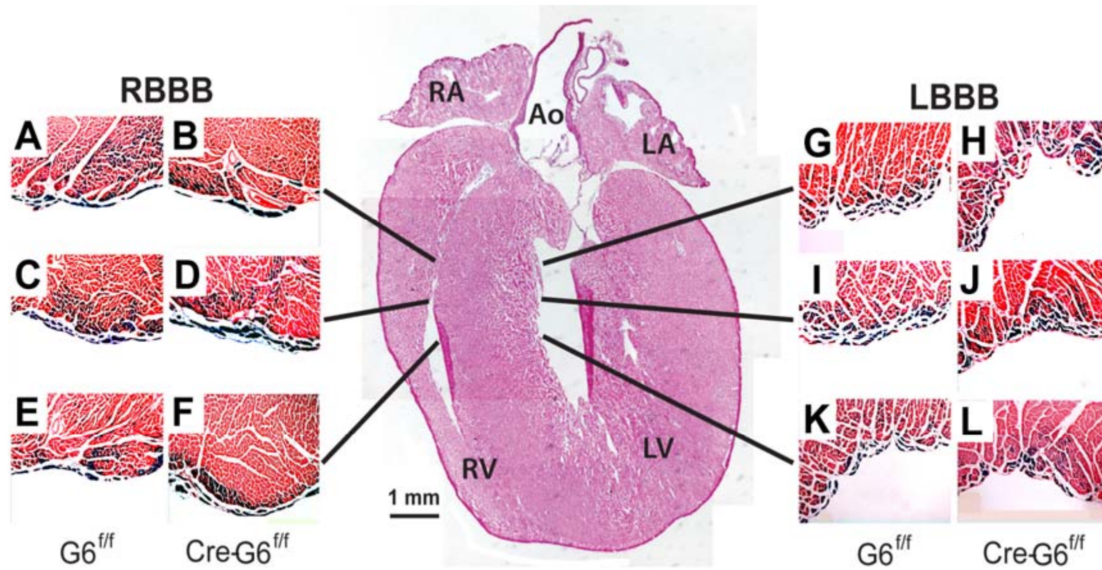
ADDITIONAL FIGURES

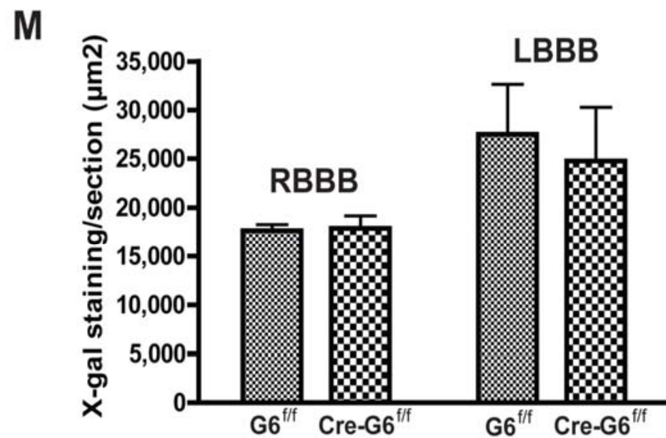


Supplemental Figure 1. Myocardial-restricted deletion of the carboxyl zinc-finger domain of *Gata6* does not affect ventricular structure or function. (A, B) Montage of a representative heart from an *Mlc2vCre-Gata6*$^{f/f}$ mouse (A) stained with H&E reveals no gross anatomic abnormalities compared to a heart from a *Gata6*$^{f/f}$ mouse without Cre (B). (C, D) Higher magnification of the left ventricular free wall (400X) from H&E stained sections. Note lack of *Gata6* without a carboxyl zinc-finger does not significantly alter myofibril structure or induce interstitial fibrosis. (E, F) M-mode echocardiography of the left ventricle from an *Mlc2vCre-Gata6*$^{f/f}$ mouse (E) and a *Gata6*$^{f/f}$ mouse with Cre (F) demonstrates normal chamber size and function with normal wall thickness in the *Gata6* mutant hearts. Ao=aorta; RA=right atrium; RV=right ventricle; LA=left atrium; LV=left ventricle; IVS=interventricular septum.

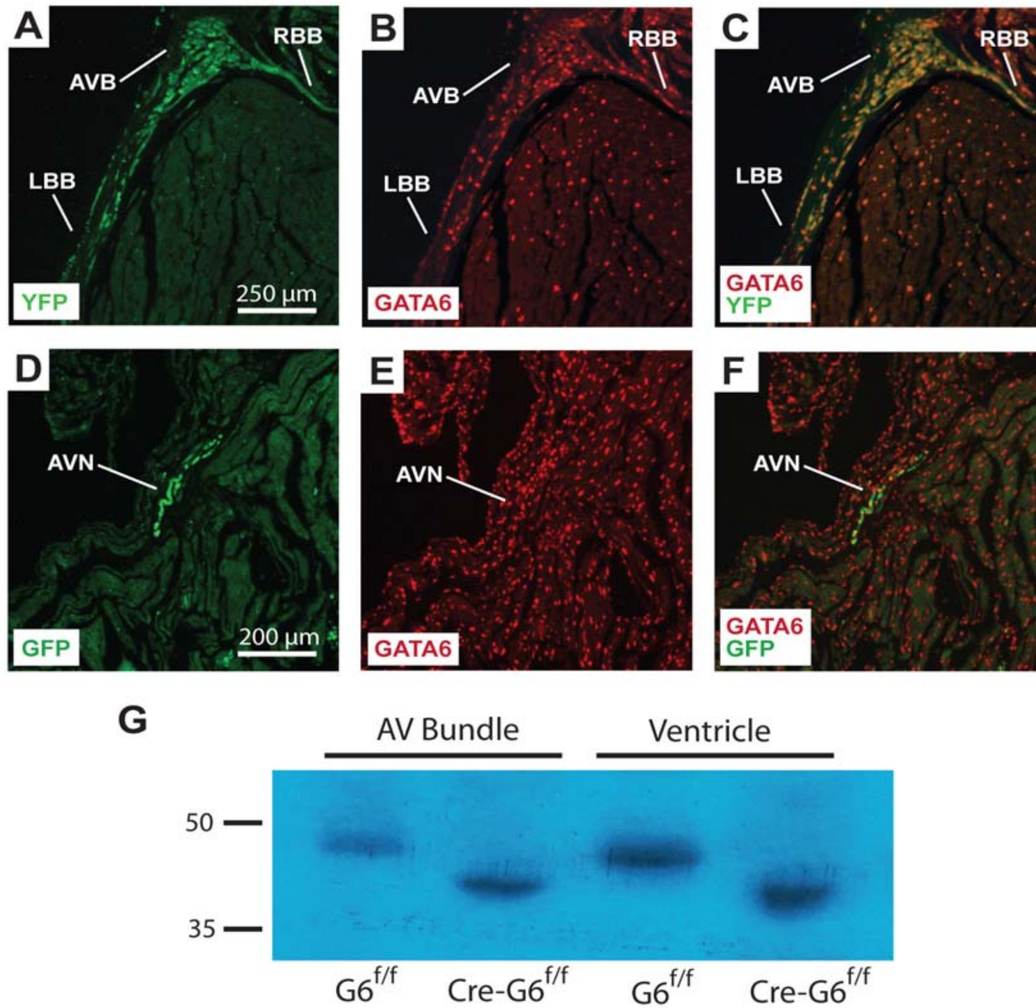


Supplemental Figure 2. Deletion of the carboxyl-zinc finger of *Gata6* reduces TBX3 expression in the AV node. Immunohistochemical staining of frontal sections from mature murine hearts against TBX3 (blue) with *Gata6* in control hearts (A), and the absence of the carboxyl zinc-finger of *Gata6* in mutant hearts (B), shows TBX3 expression is reduced in the AV node in the absence of the carboxyl zinc-finger of *Gata6*. The dotted white lines show the region of TBX3+ staining in both hearts. The scale bar applies to both panels.

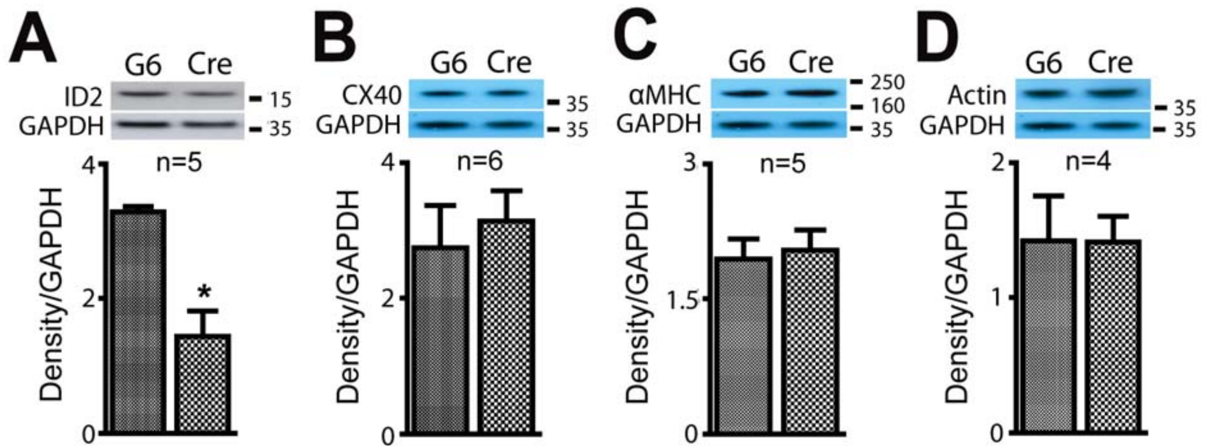




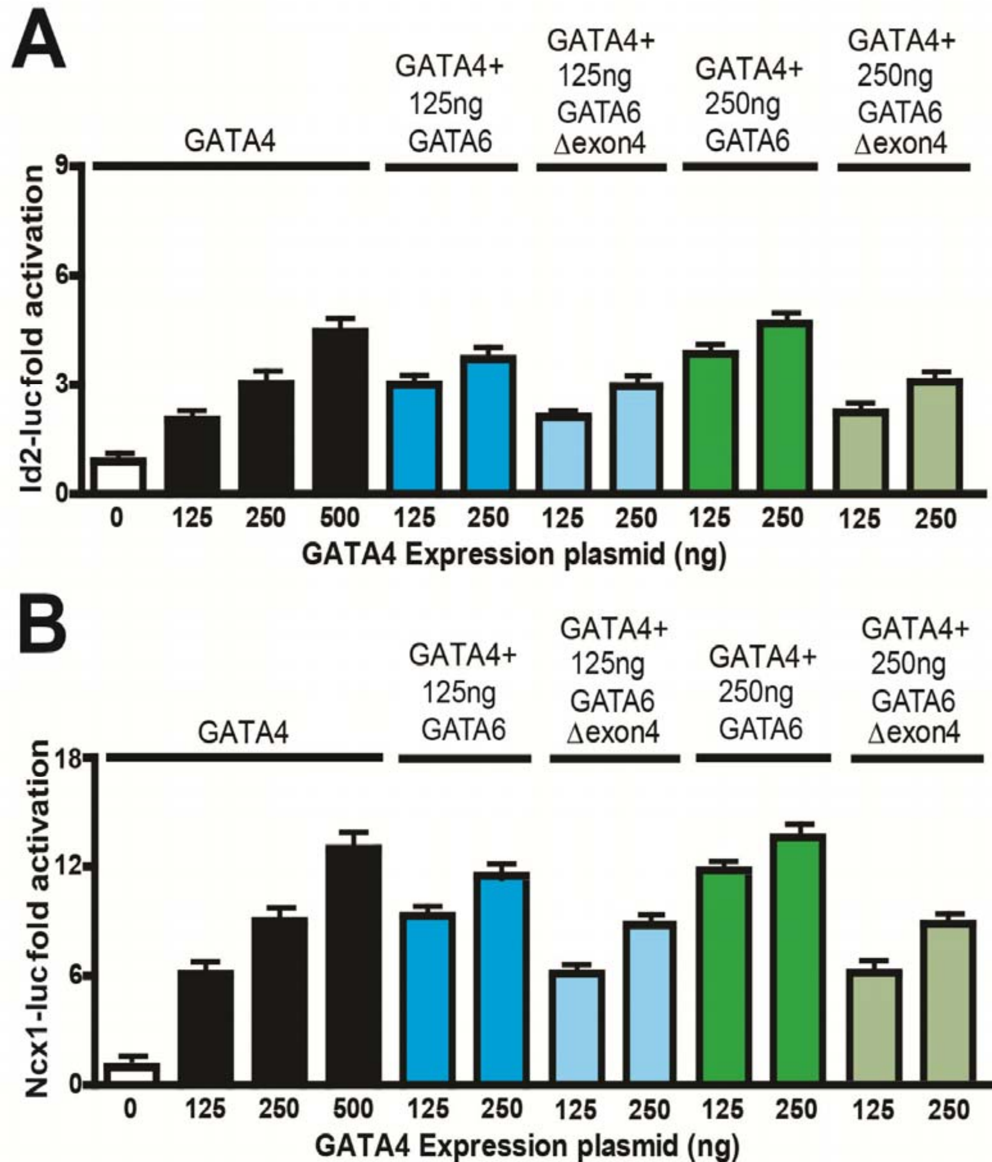
Supplemental Figure 3. Myocardial-restricted deletion of the carboxyl zinc-finger of *Gata6* does not affect the size of the bundle branches. (A-F) Representative images of the right bundle branch following X-gal staining from a *HopXLacz::Gata6*$^{f/f}$ mouse (A, C, E) and a *HopXLacz::Mlc2vCre-Gata6*$^{f/f}$ mouse (B, D, F). (G-L) Representative images of the left bundle branch following X-gal staining from a *HopXLacz::Gata6*$^{f/f}$ mouse (G, I, K) and *HopXLacz::Mlc2vCre-Gata6*$^{f/f}$ mice (H, J, L). The black lines point to the approximate anatomic regions that the sections correspond to in the intact heart. (M) Quantification of the area of the right and left bundle branches, averaged from 10 sections at three separate levels (30 sections total) from three different hearts of each genotype. Ao=aorta; RA=right atrium; RV=right ventricle; LA=left atrium; LV=left ventricle; IVS=interventricular septum. RBB=right bundle branch; LBB=left bundle branch; G6$^{f/f}$=*Gata6*$^{f/f}$; Cre-G6$^{f/f}$=*Mlc2vCre-Gata6*$^{f/f}$; Scale bar=250 µm. *p<0.01.



Supplemental Figure 4. GATA6 expression in the murine CCS. (A-C) Shown is dual-immunohistochemical staining against (A) YFP, (B) GATA6 and (C) overlay in a mature, tamoxifen-induced *MinKCreERT2-R26RYFP* mouse heart which shows GATA6 expression in the AV bundle and bundle branches. (D-F) Similarly, dual immunohistochemical staining of a mature *Tbx3-GFP* BAC transgenic mouse heart against (D) GFP, (E) GATA6 and (F) overlay shows GATA6 is expressed in the AV node. (G) Representative immunoblot with an antibody that recognizes the C-terminus of GATA6 shows the full-length 46-kD GATA6 protein is present in lysates isolated from the crest of the intraventricular septum that contains the AV node, and in lysates from the left ventricle (Ventricle) of *Gata6*$^{f/f}$ hearts. However, the 41-kD truncated GATA6 protein is present in the corresponding lysates isolated from *Mlc2vCre-GATA6*$^{f/f}$ hearts that have recombined to delete exon 4 of the *Gata6* gene. AVN=atrioventricular node; AVB=atrioventricular bundle; LBB=left bundle branch; RBB=right bundle branch.



Supplemental Figure 5. Western blot analysis of selected proteins from the crest of the intraventricular septum of *Gata6* mutant mice. Representative immunoblots and densitometric analysis are shown for (A) ID2. (B) connexin40 (Cx40), (C) α-myosin heavy chain (αMHC) and (D) cardiac actin. All blots were normalized to GAPDH expression and show the mean value ± 1 standard deviation. The number of blots analyzed is shown above the bar graphs with the genotypes of the GATA6 mutants abbreviated as: $G6^{f/f}$=$Gata6^{f/f}$; Cre-$G6^{f/f}$=$Mlc2vCre\text{-}Gata6^{f/f}$. *p-value<0.01.



Supplemental Figure 6. Co-expression of GATA4 and GATA6 on target reporters. (A) Id2-LUC reporter activation by GATA4 and GATA6. HL-1 cells were transiently transfected with Id2-LUC and 125–500 ng of expression plasmid encoding wild-type GATA4 plus wild-type GATA6 or GATA6 lacking exon 4 (Gata6Δexon4). The reporter is appropriately activated by wild-type GATA4 plus GATA6, but adding GATA6Δexon4 does not change Id2-LUC expression over that induced by wild-type GATA4. (B). Ncx1-LUC reporter activation by GATA4 and GATA6. HL-1 cells were similarly transiently transfected with Ncx1-LUC and 125–500 ng of expression plasmid encoding wild-type GATA6 plus wild-type GATA6 or GATA6Δexon4. The Ncx1-LUC reporter is also appropriately activated by wild-type GATA4 plus GATA6, but again adding GATA6Δexon4 does not change Ncx1-LUC expression over that induced by wild-type GATA4.