Supplementary Figure 1: GATA6 human mutations associated with Atrial Fibrillation. ZF: zinc finger. NLS: nuclear localization signal.
Supplementary Figure 2: Expression of GATA6 in Atria and ventricles of GATA6 mice. (A) and (B) Western blot analysis and quantification of GATA6 expression in adult hearts showing its decreased expression in Gata6<sup>−/−</sup> mice when compared to their control littermates. (C) Masson trichrome staining on adult transverse heart from Gata6<sup>+/+</sup> and Gata6<sup>−/−</sup> mice showing dilated right atria in Gata6<sup>−/−</sup>. RA: right atria. LA: left atria. Scale bar: 2000 μm. (D) and (E) Masson trichrome staining and LV thickness measurement on adult frontal heart from Gata6<sup>+/+</sup> and Gata6<sup>−/−</sup> mice showing dilated left ventricle with thinner LV wall in Gata6<sup>−/−</sup>. RV: right ventricle. LV: left ventricle. Scale bar: 2000 μm.
Supplementary Materials and Methods

**Animals and Histology.** Mouse handling and experimentation were performed in accordance with the guidelines of the Canadian Council on Animal Care and the Guide of the Care and Use of Laboratory Animals of the US National Institutes of Health. Experiments were approved by the Animal Care Committee of the University of Ottawa (protocol number BMI-1973). Gata6 heterozygous (Gata6<sup>+/−</sup>, C57BL/6) mice have been previously described<sup>1</sup>. Cell-specific knockout mice were obtained by crossing the Gata6<sup>+/−</sup> line with the respective cre lines<sup>1,2</sup>. Embryos and adult heart tissues were fixed with 4% paraformaldehyde in PBS, paraffin embedded, sectioned at 4-μm intervals, and processed. Masson Trichrome staining was performed by the histology service of the University of Ottawa. The opening of the aortic valve on the left ventricle was used as a reference to stain SAN in all animals ensuring the same plane in the heart.

**Electrophysiology.** Surface ECG recording was done using EMKA technologies platform. PR interval, QRS duration, QT Interval, RR Interval were analyzed using the IOX 2.4.2.6 software and reported. The QT intervals were corrected (QTc) for the heart rate using the standard formula for mice (QTc=QT/(RR/100)<sup>1/2</sup>)<sup>3</sup>.

**Telemetry analysis (Arrhythmias at baseline).** Implantable radio frequency transmitter (Data Services International, DSI’s PhysioTel® ETA-F10) with subcutaneous (SC) leads was placed subcutaneously along the lateral flank in 280 days old mice. The negative lead was fed SC to the right pectoral muscle and the positive lead was fed SC to the left caudal rib region. Precision and proper placement of the ECG leads were crucial to allow recording of the ECG waveforms over a long period of time and with high signal-to-noise tracings. Data acquisition started at 1 week post-surgery and was acquired 20 seconds from every 1 hour for 72 hours. Analysis was performed using Ponomah Physiology Platform Software (Version 5.2 SP7, DSI).

**Ex vivo arrhythmia induction.** Mouse hearts were collected and perfused on a Langendorff system as was previously described<sup>4</sup>. A standard programmed electrical stimulation (PES) was performed using an electrical stimulator (IonOptix, Myopacer EP, USA) in hearts perfused with Tyrode solution containing 100 nM isoproterenol. A train of 10 consecutive stimuli (S1, 5V, 1ms pulse width, and 100 ms interval) was delivered and followed by an extra stimulus (S2, 5V, 1ms pulse width) starting at a coupling interval of 80 ms and by 2 ms decrements until reaching the effective refractory period (ERP). If ventricular tachycardia or fibrillation were not induced, a second extra stimulus (S3) was introduced from 80 ms at progressively decremental coupling intervals until ERP was reached. A third extra stimulus (S4) was introduced from an 80 ms coupling interval until ERP was reached finally. If the mouse failed to develop ventricular arrhythmias with 3 extra stimuli (S1-S2-S3-S4), it is deemed that arrhythmias are non-inducible in this animal.

**3D reconstruction.** Serial sections for the SAN from E14.5 embryos were used for the reconstruction using the FreeD software (version 1.14) according to the developer’s instructions<sup>5</sup>. The 3D reconstruction involved analysis of 90 sections per animal with minimum 4 embryos per genotype. Sections were registered before the SAN tracing for each of the serial sections so that the tracing does not interfere with the alignment. Following the SAN tracing, images were stacked to obtain the 3D reconstructions. To avoid bias, sinus node structural definition was performed by 2 different investigators.

**Immunohistochemistry and Immunofluorescence.** Immunohistochemical stainings were performed as previously described<sup>6</sup>. The dotted lines around the SAN in sections stained with non-SAN specific markers were drawn based on the corresponding HCN4-stain consecutive section. The GATA6 and TBX5 antibodies<sup>7,8</sup> were used at a dilution of 1/2000 and 1/2500 respectively. Experiments were carried out using GATA4 (C20) goat polyclonal IgG (SC-1237X; dilution 1/600), TBX3 E-20 (SC-31656; dilution 1/750), HCN4 (Alomone (APC-052, dilution 1/1000), ANP (Peninsula T-4014; RGG-9103, dilution 1/1000), NKX2.5 (ab35842; dilution 1/100) and ISL1 (ab20670; 1/500). The biotinylated anti-Goat IgG was from Vector Laboratories (BA5000). The anti-Rabbit and anti-Mouse antibodies were from Jackson (Cederlane) (711-065-152 and 715-065-151 respectively). Streptavidin-HRP conjugate was from Perkin Elmer (NEL 750000 1EA). Immunofluorescence was carried out using anti-HCN4 (ABCAM, ab32675,
**Chromatin Immunoprecipitation (ChiP).** ChiP was completed using a modification of a previously described protocol. AD293 cells were transected with 20 μg total DNA (TBX3 promoter-pGL3, GATA6-pCGN and/or relevant empty vectors) using calcium phosphate as previously described. 48 hours post-transfection, cells were fixed directly in media with formaldehyde buffer (1% formaldehyde, 10 mM NaCl, 100 mM EDTA pH 8, 50 mM EGTA, 5 mM Hepes pH 8) for 30 minutes with agitation at room temperature, quenched with 125 mM glycine for 5 minutes and washed twice with ice cold 1x PBS. Cells were then incubated in Lysis Buffer 1 (50 mM Hepes-KOH pH 7.4, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.75% Nonidet P-40) for 10 minutes at 4°C, centrifuged at 1000 x g for 5 minutes at 4°C, resuspended in Lysis Buffer 2 (200 mM NaCl, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 10 mM Tris-HCl pH 8) for 10 minutes at RT and centrifuged again under the same conditions. The resulting nuclear pellet was resuspended in Lysis Buffer 3 (1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 10 mM Tris-HCl pH 8, 0.5% (w/v) Sodium Sarcosyl) and sonicated using a Covaris S220 Ultrasonicator (Peak Power of 140, Duty Factor of 5, 200 cycles/burst, 5 minutes) to achieve fragments of between 200 and 600 bp as observed on a 2% agarose gel. 50 μg of fragmented chromatin was then precleared with MagnaChIP Protein A magnetic beads (EMD-Millipore, 16-661) for 1 hour with agitation at 4°C. The sample was then divided into equal fractions for immunoprecipitation with IgG (Cell Signaling, 2729) or GATA6 antibody (Cell Signaling, 5851), with 10% having been reserved for the input. Immunoprecipitated chromatin was washed 3 times for 5 minutes at RT. Both samples and inputs were then reverse crosslinked overnight at 65°C, incubated with 50 ng/ul RNase A (Sigma, R4642) at 37°C for 30 minutes and centrifuged again under the same conditions. The resulting DNA was purified by fractionation with Phenol:Chloroform:Isomyl Alcohol (Sigma-Aldrich P2069) and resuspended in 50 ul of water and analysed by qPCR at an annealing temperature of 60°C. Amplification of a chromosome 20 gene desert was used as negative control. The sequences of the primers used are the following:

- **Negative control:** chromosome 20 gene desert taken from ChiP-seq data from GSE77360-5′-CATACGGATGGCCTGAATG-3′ and 5′-CTGACATTGCCTCCCCAATC-3′
- **GATA site 1 on TbX3 promoter:** 5′-CTCCCTCCGCCACTCTGTG-3′ and 5′-GGTGCCAGCACAGGGTAAGGTAAG-3′
- **GATA sites 2/3 on TbX3 promoter:** 5′-TTCCGCTGTGGTCTTTCGAGCCTCC-3′ and 5′-CGGCCATATCTCAGCACACAC-3′
- **GATA site 4 on TbX3 promoter:** 5′-GACTGAGGCATTCCAGACGTG-3′ and 5′-GTCGCTCTTAGGGTGGTTATC-3′

**Western Blot and Immunofluorescent Electrophoretic Mobility Shift Assays (EMSA).** Western blot was performed as previously described. Primary antibodies anti-HA (SANTA CRUZ, SC-7392) and anti-Lamin B (SANTA CRUZ, SC-6217) were used. Secondary donkey anti-mouse (JACKSON CEDERLANE, 715-035-151) and donkey anti-goat (JACKSON CEDERLANE, 705-035-147) antibodies were used at 1/2000 dilution. Immunofluorescent gel shift was performed on 20-ng of nuclear protein extracts from AD293. The binding reaction (15 μl) was carried out at RT for 20 min in binding buffer (final concentration 170mM KCl, 125mM HEPES, 25mM MgCl2, 50% Glycerol, 5 mM dithiothreitol). An excess of unlabeled GATA sites oligonucleotide from TBX3 promoter (Site 1, 2 and 4) were added to the binding reaction mixture and incubated for 20 min before the addition of labeled probe. An excess of ANF double-stranded DNA probe (Eurofins- 60 fmole) was then added and incubated for 15 min at room temperature. Complexes were resolved on a 5% native acrylamide gel run for 1 h and then visualized.

**Quantitative real-time PCR.** For analysis of gene expression changes in Gata6 mutant mice and control, total RNA was isolated from snap-frozen hearts with TRIzol reagent (Life technologies, 15596018) using...
FastPrep beads (MP-Bio, 6913-100); cDNAs were generated using the Omniscript RT kit (Qiagen, 205113). Oligonucleotide sequences (Figure 2E) are the following:

| Gene   | Forward primer          | Reverse Primer          |
|--------|-------------------------|-------------------------|
| NaV1.5 | ATGCCCTGAAGATCCAGATG    | TGGAGGAGATGGAAGAGCTG    |
| KIR3.1 | TGAACAGTTGAGTTGTCG      | ATTTTCTGCAAGTCTGATGG    |
| KV4.2  | TCGGGAGAAATATCGTCAG     | ACAAGAGGAGGCCACAG       |
| CAV1.3 | CAAGAAGGAAAGAGCAAGG     | GCAAGTCAGGATATAGCCCC    |
| SCL8A1 | CGTCCTGCTTTGACTACGTG    | GGCCTTGACTGACTCTTG      |
| CX43   | CTCTCTCTGGGTACAAGCTG    | AGTGCCTGATCCACAGATAG    |
| CX45   | CTCTAATCGATGCTAAAGA     | CTCTGGCTTCTGGGCTATA     |
| NKX2.5 | GTGGGTCTCAATGCGCTATGG   | TCATCGCCCTTCTCTAAGG     |
| NPPA   | GTAGGATTGACAGGATTGG     | TGATAGATGAAAGGCAGGA     |
| NRG1   | CCTGTAACCCCTCAAGATAC    | CGCTTCCATAAATTCAATCCC   |
| EDN1   | GTTTTCCCTAGGCTGTCTGC    | TTGCATGGACTTTGGAGTTTC   |
| EDNRA  | GTCTTGAACTCCTGTGCTCCTC  | GATCCGGATTCCTTTAAGACTCG |
| EDNRB  | CAATCGCTCTGTATTGGTGAG   | CGTGATCGTTGGCCTTTGAA    |

**Luciferase assay.** Transfections were carried out as previously described\(^\text{11}\). Appropriate amounts of empty DNA vector were added to maintain a constant total amount of DNA. TBX3-Luc reporter construct was a kind gift from Dr. Sharon Prince\(^\text{12}\).

**Statistics.** Unless otherwise indicated, Student’s 2-tailed t-test was used. Data are presented as mean ± SEM. P values < 0.05 were considered as an index of statistical significance. For statistical analysis of phenotype-genotype association (PAC), the Fisher exact test (2×2 contingency table) was used.

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