Supplemental Material

Supplemental Methods

PSC-CM culture and CRISPR-Cas9 genome editing

The H7 pluripotent stem cell (PSCs; WA07, WiCell) were maintained and differentiated into PSC-derived cardiomyocytes (PSC-CMs) according to a previously published protocol[75]. Three transgenic PSC lines were generated using CRISPR-Cas9-mediated genome-editing[76]. A pair of sgRNAs targeting the boundary of a ~200 bp region centered around rs12931021 (sgRNA 1: GCTTCACAAACAGGTTCTGT; sgRNA2: AATAGAGTTCTTTACTCAAG) were used to establish the ∆rs12931021 line. For generating the isogenic line with either AA or CC genotype at rs12931021 (parental PSCs are heterozygous at rs12931021), we employed a single-stranded oligo donor (ssODN) in combination with the sgRNA targeting rs12931021. The sequences of the sgRNAs and the ssODNs are listed as follows: sgRNA_A: CAATGGTGCTGTCTTGCATA, paired with ssODN_C (to generate PSCs with CC genotype): CGGGGTAAGATCTGTCTTGCATATCATGGCGGTCTCCATCCCACTGGAGAATTCAATGGTGCTGTCTTGCCTATGG GCTGTGAAACTTGAACAACCGGATTCTGAGGGTCCAGACGTCTCTGTTTGAGCAACAGCTGCCTTCCTTT. sgRNA_C: CAATGGTGCTGTCTTGCCTA, paired with ssODN_A (to generate PSCs with AA genotype): CGGGGTAAGATCTGTCTTGCATATCATGGCGGTCTCCATCCCACTGGAGAATTCAATGGTGCTGTCTTGCATATGG GCTGTGAAACTTGAACAACCGGATTCTGAGGGTCCAGACGTCTCTGTTTGAGCAACAGCTGCCTTCCTTT. Each sgRNA was cloned into the PX458 vector containing both Cas9 and GFP reporter (Addgene; 48138)[2]. The sgRNA construct (13 μg) and/or ssODN (0.5 nmol) were introduced into PSCs by electroporation with the Neon Transfection System (ThermoFisher Scientific) at 1,050 v, 30 ms and 2 pulses. Positively transfected cells were enriched based on fluorescent signal using a Sony SH800 cell sorter (Sony Biotechnology) followed by single cell seeding and colony expansion. The genotype of each line was confirmed by Sanger sequencing of the target region.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed in PSC-CMs heterozygous at rs12931021 using a previously described protocol[77]. Antibodies against H3K4me1 (Cell Signaling Technology; 5326T, 1:50), H3K27ac (Cell Signaling Technology; 4353S, 1:50) or IgG (Cell Signaling Technology; 2729S, 1:50) were used to pull down the sheared chromatin. The allele-specific qPCR primers for amplifying the rs12931021 region with either A or C allele are listed as follows:

rs12931021A: Forward: TGGCGGGGTAAGATCTGTCTTG; Reverse: GGTTGTTCAAGTTTCACAGCCCATAT

rs12931021C: Forward: TGGCGGGGTAAGATCTGTCTTG; Reverse: GGTTGTTCAAGTTTCACAGCCCATAG

Luciferase assay

The genomic regions flanking the candidate SNPs with both alleles were synthesized by Integrated DNA Technologies and cloned into the pGL4.23 vector (Promega; E8411). These genomic fragments were inserted upstream of a minimal promoter driving a firefly luciferase gene. Lipofectamine LTX (ThermoFisher; 15338100) was used to transfect the luciferase constructs together with pGL4.73 expressing renilla luciferase (Promega; E6911) into PSC-CMs at 80% confluency. Cells were harvested 48 hrs post transfection and the luciferase signal was measured using the Dual-Luciferase Reporter Assay kit (Promega; E1910).

Generation of mice with cardiac-specific Zfhx3 knockout

Male and female homozygous cardiomyocyte-restricted *Zfhx3* knockout mice (α-MHC-*Zfhx3*$^{fl/fl}$, denoted *Zfhx3* KO) were generated using the Cre-loxP system, crossbreeding homozygous floxed *Zfhx3* (*Zfhx3*$^{fl/fl}$) with *Zfhx3* homozygous or heterozygous KO expressing α-myosin heavy chain-cre ((B.6FVB-Tg(Myh6-cre)2182Mds/J, The Jackson Laboratory; Myh6-cre-*Zfhx3*$^{fl/fl}$ or Myh6-cre-*Zfhx3*$^{fl/+}$, respectively). We used

previously published *Zfhx3*$^{fl/fl}$ mice, generated using a targeting construct with inserted loxP sites flanking the seventh and eighth exon of the *Zfhx3* gene so that exons 7 and 8 are deleted in the presence of cre-recombinase[78]. The second mouse line contained an α-myosin heavy chain promoter driving cre-recombinase expression. Homozygous floxed (*Zfhx3*$^{fl/fl}$) mice were used as controls (denoted WT). Both sexes were used for comparison. No animals were excluded from the study. To minimize stress and discomfort during tail snipping and *in vivo* electrophysiology studies, mice were anaesthetized with 5% isoflurane (VetOne, USA) as previously described[79].

Genotyping transgenic mice

Total DNA was isolated from tail snips using a DNeasy tissue kit (Qiagen; 69504) following manufacturer's instructions. By PCR we assessed the presence of the floxed *Zfhx3* gene (forward primer, 5' TGACTGCGTGGAGAAGCTC 3'; reverse, 5' AAACCAAGACCTGGGGACTC 3'). These primers were used to distinguish the *Zfhx3* wild-type and *Zfhx3* floxed genes by amplification of a 383-bp fragment in the *Zfhx3* WT and a 273-bp fragment in the *Zfhx3* floxed mice. Cre-recombinase primers (forward, 5' ATGACAGACAGATCCCTCCTATCTCC 3'; reverse, 5' CTCATCACTCGTTGCATCATCGAC 3') amplified a 300-bp fragment. The amplification protocol for both sets of primers consisted of 5 min 95°C followed by 38 cycles of 30 sec 95°C, 45 sec 58°C, 60 sec 68°C, and 5 min 68°C.

Immunofluorescence and histology

For immunofluorescence, hearts were embedded in O.C.T. compound (Fisher Scientific; 23-730-625) and snap-frozen in a 2-methylbutane (Millipore Sigma; 277258) bath on dry ice. Fresh frozen sections (10 um thickness) were prepared with a cryostat (Leica CM3050 S) and fixed with 4% formalin for 5 minutes at room temperature. After washing three times in PBS, tissue permeabilization was performed by incubation 0.1% TritonX-100 (Millipore Sigma; X100) in PBS at room temperature. After washing three

times in PBS, blocking was performed though incubation with 3% BSA (Millipore Sigma; A9418) for 1 h in PBS. Samples were then incubated at 4°C overnight in 1% BSA in PBS containing primary antibodies directed against *ZFHX3* (anti-*ZFHX3*, 1:50, Novus Biologicals; AF7384) and Troponin (*anti-TNNI1*, 1:450, Novus Biologicals; NBP1-56641). After washing three times in PBS, samples were incubated with Alexa-Fluor 488- and Alexa-Fluor 594-conjugated secondary antibodies (ThermoFisher Scientific, A11015 and A32740; 1:400) in 1% BSA in PBS for 1.5 hours at room temperature in the dark. After washing three times with 0.2% Tween 20 (Millipore Sigma; P1379) in PBS then washing three times with PBS, tissues were mounted with NucBlue Fixed Cell ReadyProbes Reagent DAPI (ThermoFisher Scientific, R37606) to visualize *Dapi.* All immunostainings were specific, and there was no detectable signal on tissue sections stained by isotype control IgG. Slides were analyzed using a Leica TCS SP8 confocal microscope and LAS X.

For histology, hearts were embedded in O.C.T. compound (Fisher Scientific; 23-730-625) and snap-frozen in a 2-methylbutane (Millipore Sigma; 277258) bath on dry ice. Fresh frozen sections (14 um thickness) were prepared with a cryostat (Leica CM3050 S) and fixed with 10% formalin for 30 minutes at room temperature. After the tissue sections were rinsed in dH2O for 10 minutes, the sections were incubated in Bouin's Fixative solution (Electron Microscopy Sciences; 15990) at room temperature overnight. The slides were washed in tap water to remove yellow color from sections and nuclei were stained with the mixture of Weigert's Iron Hematoxylin Solution A and B (Electron Microscopy Sciences; 26044-05 and 26044-15, respectively) for 5 minutes at room temperature. Masson's Trichrome staining was carried out per the manufacturer's instructions (Sigma-Aldrich; HT15-1KT) using Biebrich Scarlet-Acid Fuchsin, Phosphotungstic/Phosphomolybdic Acid Solution, and Aniline Blue Solution. Slides were scanned by a digital slide scanner, NanoZoomer 2.0-RS (Hamamatsu).

Quantification of fibrosis

Masson's trichrome stained images of hearts were analyzed using the color deconvolution plugin[80] in Fiji (ImageJ 2.0.0-rc-69, NIH). Regions of interest (ROI) were defined from whole heart images. For each ROI, the background was subtracted, and the color deconvolution option was applied on the RGB image. The threshold was adjusted from the Blue frame (color 1) and the percentage of fibrosis was calculated by the ratio between the threshold and the RGB image.

Echocardiography

Echocardiography was performed on conscious mice at 3 months using a GE Vivid 5 with a 15L8 15 MHz linear array transducer at a frame rate of 100 frames/s. Left ventricular chamber dimensions and wall thickness were measured using M-mode recordings in parasternal short axis views at the level of the papillary muscles. Left atrial diameter was measured from the parasternal long-axis view. Left ventricular fractional shortening and ejection fraction were calculated as previously described[81].

Magnetic resonance imaging (MRI)

MRI was carried out on 3 and 9-month-old WT and *Zfhx3* KO mice. Cine images of short and long axis views of left and right ventricles and atria were obtained using a 4.7 Tesla horizontal bore Pharmascan (Bruker) and a custom-built mouse cardiac coil (RAPID Biomedical). Images were analyzed using the software Segment (Medviso).

Electrophysiology studies

Electrophysiology studies were performed in 3-month-old mice as previously described[81]. Following induction of general anesthesia, the right internal jugular vein was exposed by surgical cut down. A 1.1 French octapolar catheter (Millar Instruments) was introduced into the vein and positioned in the right

atrium and right ventricle. Electrophysiology studies were performed using a previously described protocol to measure function of the sinus node, AV node and conduction properties of atrial and ventricular tissue[81]. Burst pacing, single, double, and triple extra stimuli at two different drives were performed to assess for inducibility of atrial arrhythmias. Mice that exhibited AA/AF evoked by burst pacing at least 2 times with loss of P waves, irregular R-R intervals, and durations of at least 1 sec were considered AF-positive. The incidence of inducible AF was calculated as the percentage of the AF-positive mice divided by the total number of mice studied.

Optical mapping

Isolation and perfusion of mice hearts was performed as previously described[81]. Briefly, mice were deeply anaesthetized, and hearts were removed and perfused by aortic cannulation. A Langendorff preparation was used to maintain perfusion of cannulated hearts with a modified Krebs-Henseleit solution (in mM, 24.9 NaHCO3, 1.2 KH2PO4, 11.1 Dextrose, 1.2 MgSO4, 4.7 KCl, 118.0 NaCl, 1.0 CaCl2; pH 7.35) at a constant pressure, pH and temperature. Heart motion was arrested with blebbistatin (5 mM; Selleck Chemicals; S7099) and stained with voltage sensitive dye (di-4-ANEPPs, 2.5 mM; ThermoFisher Scientific; D1199). Pacing was performed at a 120 ms cycle length at twice the capture threshold (1 ms square wave stimuli). A Mercury Vapor Short Arc lamp was used to excite fluorescence which was collected by a CCD-camera. Programmed electrical stimulation (PES) was performed using epicardial stimulation electrodes to assess inducibility of atrial and ventricular arrhythmias. Refractory periods were identified by delivering a pacing train of 8 beats at a fixed cycle length followed by an extrastimulus at progressively shorter coupling intervals. Arrhythmia induction was performed by delivery of burst pacing for 3 and 6 seconds at progressively shorter cycle lengths, starting at 50ms and decrementing by 5ms per maneuver down to bursts at 10ms. To measure conduction velocity, we used a custom MATLAB algorithm[82] that calculated the conduction velocity as the vector normal to the

wavefront at all points within the activation map. For analysis, conduction velocity vectors were selected from the same area in the posterior right atrium and left atrium, and these values were averaged to give the conduction velocity from each chamber.

Primary atrial cardiomyocyte isolation

Mice were anaesthetized with isoflurane, and hearts were removed rapidly and retrograde perfused with arrest solution containing Krebs-Henseleit (KH) Solution, KCl (1 mg/ml), Dextrose (1 mg/ml) and Heparin (50 U/ml). KH solution consists of NaHCO3 (24.9 mM), KH2PO4 (1.2 mM), MgSO4 (1.2 mM), KCl (4.7 mM), and NaCl (118 mM). The aorta was cannulated and attached to a Langendorff apparatus (Radnoti). The heart was perfused with KH solution containing 1 mM calcium for 3 minutes, followed by calcium-free KH solution for 3 minutes and 0.2 mM calcium KH solution containing collagenase (0.3 mg/ml). After 56 minutes of enzyme digestion the left atrial appendage was dissected and stored in Kraftbrühe (KB) buffer: KCl (25 mM), KH2PO4 (10 mM), Glucose (20 mM), DL aspatic acid potassium salt (10 mM), bovine albumin (0.1%), L-Glutamic acid potassium salt (100 mM), MgSO4 (2 mM), Taurine (20 mM), EGTA (0.5 mM), Creatine (5 mM), HEPES (5 mM), pH 7.2. Single cardiomyocytes were obtained by gently triturating the tissue using a polished glass pipette. After isolation of cardiomyocytes calcium concentration was slowly increased over 60 minutes to a final concentration of 1.2 mM calcium.

Calcium Transients

Intracellular calcium [Ca^{2+}]i transients were studied with intracellular calcium indicator Fluo-3 acetoxymethyl ester (Fluo-3 AM) at room temperature. Murine atrial cardiomyocytes were loaded with ~4.4 µmol/L Fluo-3 AM (Invitrogen; F1241) in Tyrode's solution in the presence of 0.02% Pluronic F-127 for 10 minutes at room temperature, followed by a 30 minute washout of Fluo-3 AM for its deesterification. Fluo-3 fluorescence was obtained with a FITC filter set (excitation: HQ480, mirror:

Q505LP, emission: HQ535/50 m; Chroma) and an X-Cite exacte mercury arc lamp (Luman Dynamics) with a 50% output for illumination. Fluorescent images were recorded with a Nikon Eclipse Ti-U inverted microscope (Nikon Instruments Inc.), a NeuroCCDSM camera (RedShirtImaging), and the Neuroplex software (RedShirt Imaging). Calcium transients were analyzed with Neuroplex and Clampfit 9.2 (Molecular Devices Inc).

RNA sequencing and RNA Sequencing Analysis

Total RNA was extracted from murine cardiac tissue using the Direct-zol RNA Miniprep kit (Zymo Research, R2051). RNA-seq libraries were generated with an mRNA enrichment protocol (KAPA mRNA HyperPrep, 08098115702). All RNA-seq libraries were sequenced on an Illumina NovaSeq. Sequenced reads were aligned to the mouse genome (mm10) using STAR (2.7.9a)[83]. Differential expression analysis was performed with DESeq2 (1.30.1)[84]. Pathway enrichment analysis was carried out with Metascape[85-89].

Motif enrichment analysis

We generated a consensus ZFHX3 motif according to Parson's et al[90]. Briefly, we extracted the well-known *ZFHX3* motifs from Mouse, Human, and Rhesus and then generated a consensus sequence using a mixture model by multiple EM (expectation maximization) for motif elicitation (MEME) using default parameters (version 5.4.1)[91]. We next extracted the nucleotide probability matrix to generate a motif file for calculating instances of the *ZFHX3* motif within human atrial snATAC peaks with Homer (version 4.11; annotatePeaks.pl function, default parameters)[92]. All atrial human genome browser tracks were obtained via http://cepigenomics.org/CARE_portal/[93]. For gene enrichment analysis we utilized Metascape (www.metascape.org)[94]. All gene enrichment procedures within metascape were performed with default statistical parameters (minimum overlap > 3 genes, p-value < 0.01, minimum enrichment >

1.5). The publicly available human snATAC-seq data was obtained from the NCBI with accession number GSE165839[93].

Figure S1

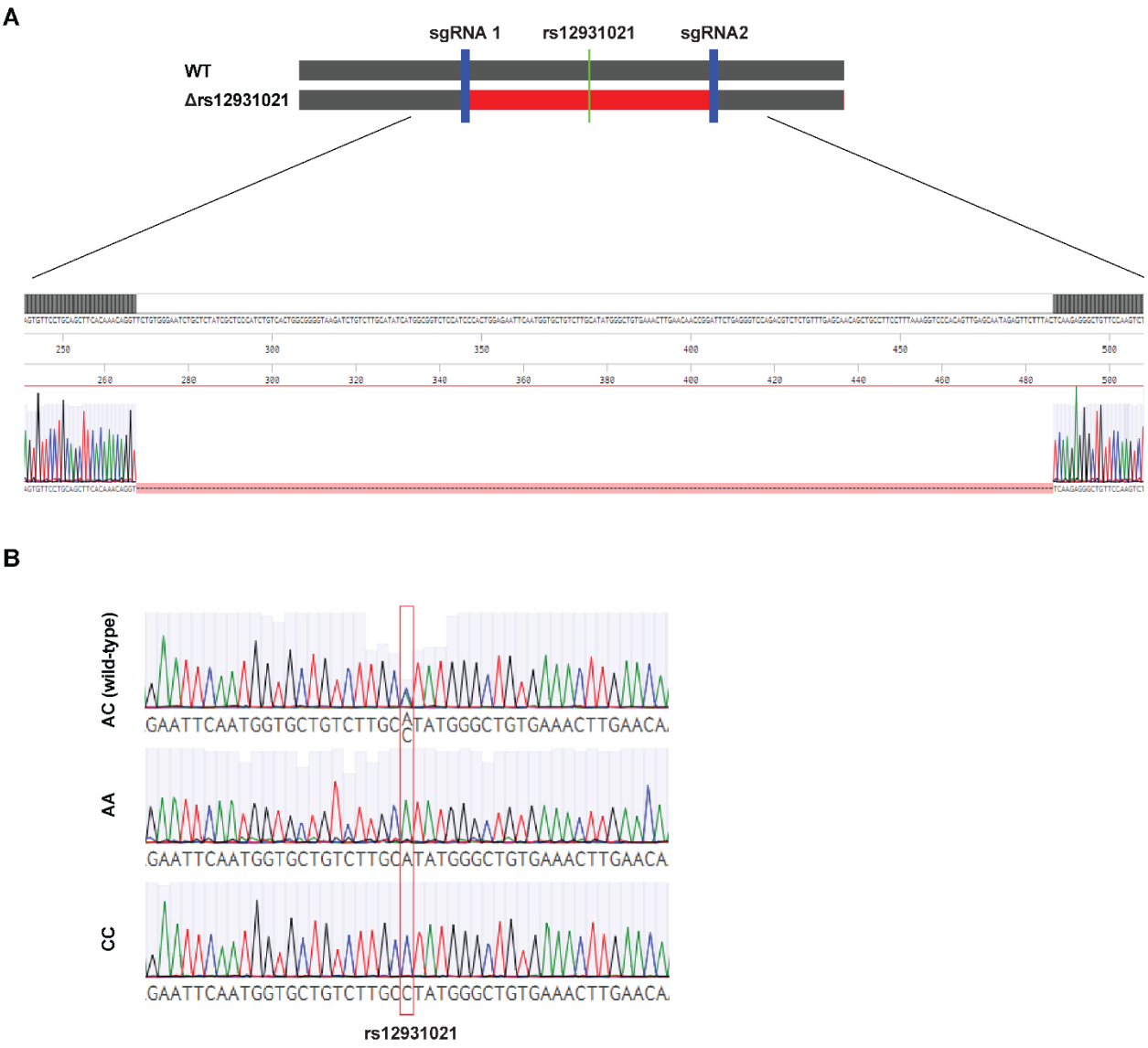

Figure S1. Sanger sequencing of the rs12931021 region in WT and genome-edited PSCs. Representative chromatograms showing the deletion around rs12931021 in the Δrs12931021 line (**A**) and the base pair change in the PSCs carrying AA or AC at rs12931021 (**B**). rs12931021 is highlighted in red box. The sequencing primers are CAGTGTGGCCAAGAAAGCTACC (forward) and TGTAGCGAGAAGACTGAGTCGC (reverse).

Figure S2

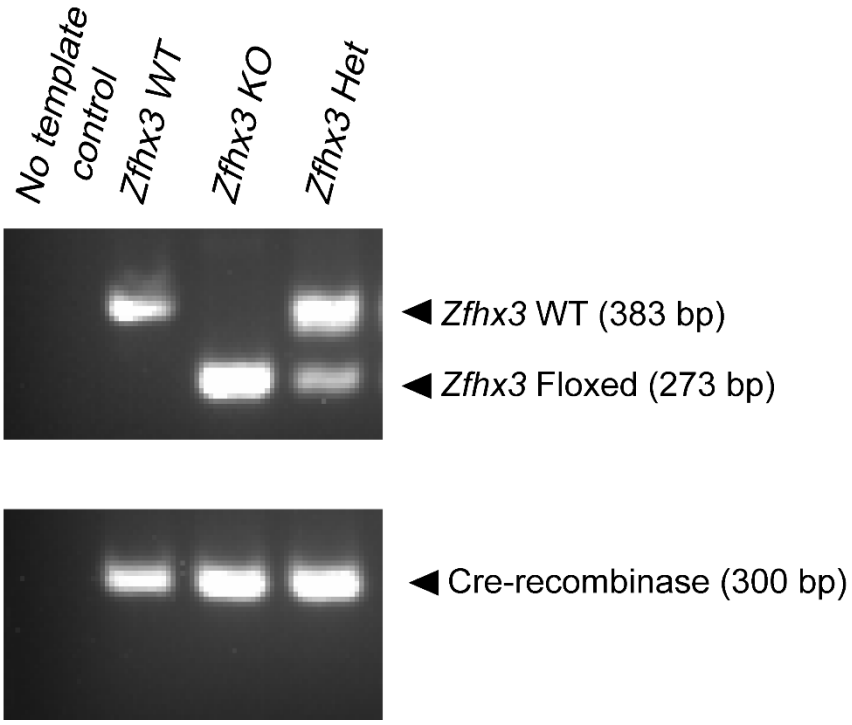

Figure S2. **Generation of cardiac-specific *Zfhx3* Het and KO.** Representative gel electrophoresis showing results of genotyping PCR from tails of *Zfhx3* WT, Het, and KO mice and no template control. The alleles are indicated.

Figure S3

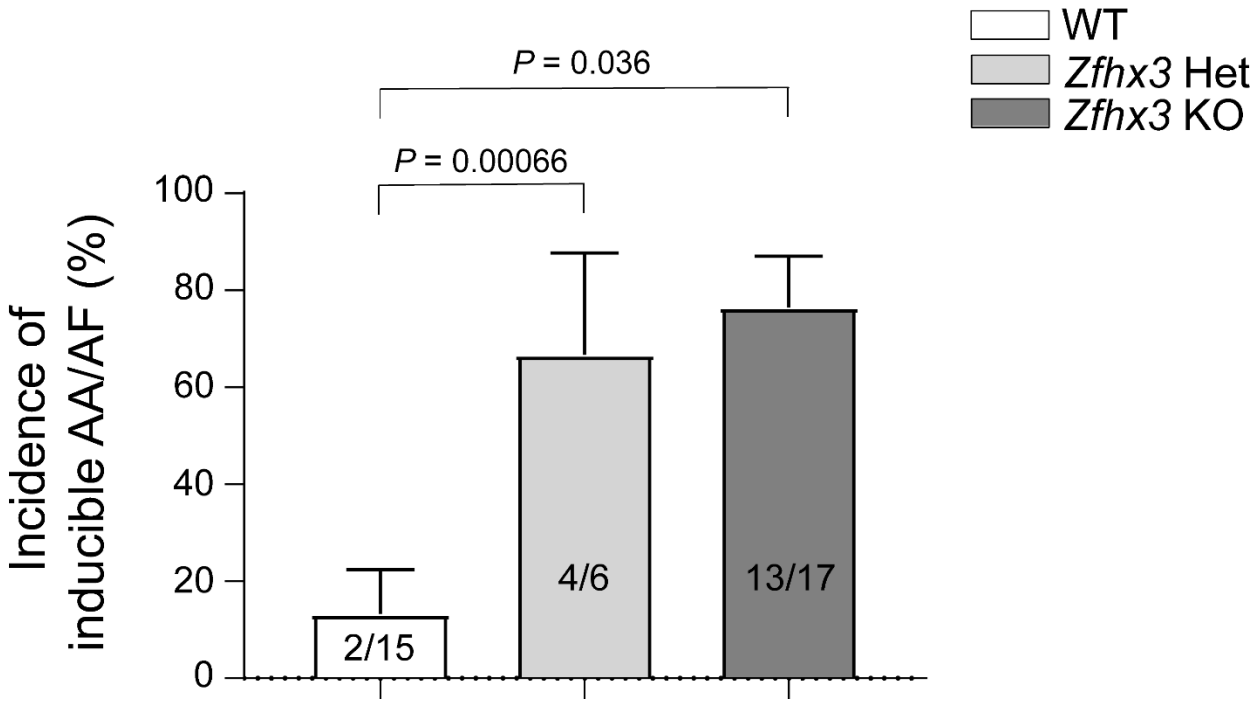

Figure S3. **Incidence of inducible AA/AF.** Percentage of mice with inducible AA/AF lasting at least 15 sec (n = 2/15 WT, 4/6 *Zfhx3* Het, and 13/17 *Zfhx3* KO mice). AA indicates atrial arrhythmia; AF, atrial fibrillation. Data are mean ± s.e.m. *P* values indicated. Significance was determined using a one-way ANOVA with Tukey's multiple comparisons test.

Figure S4

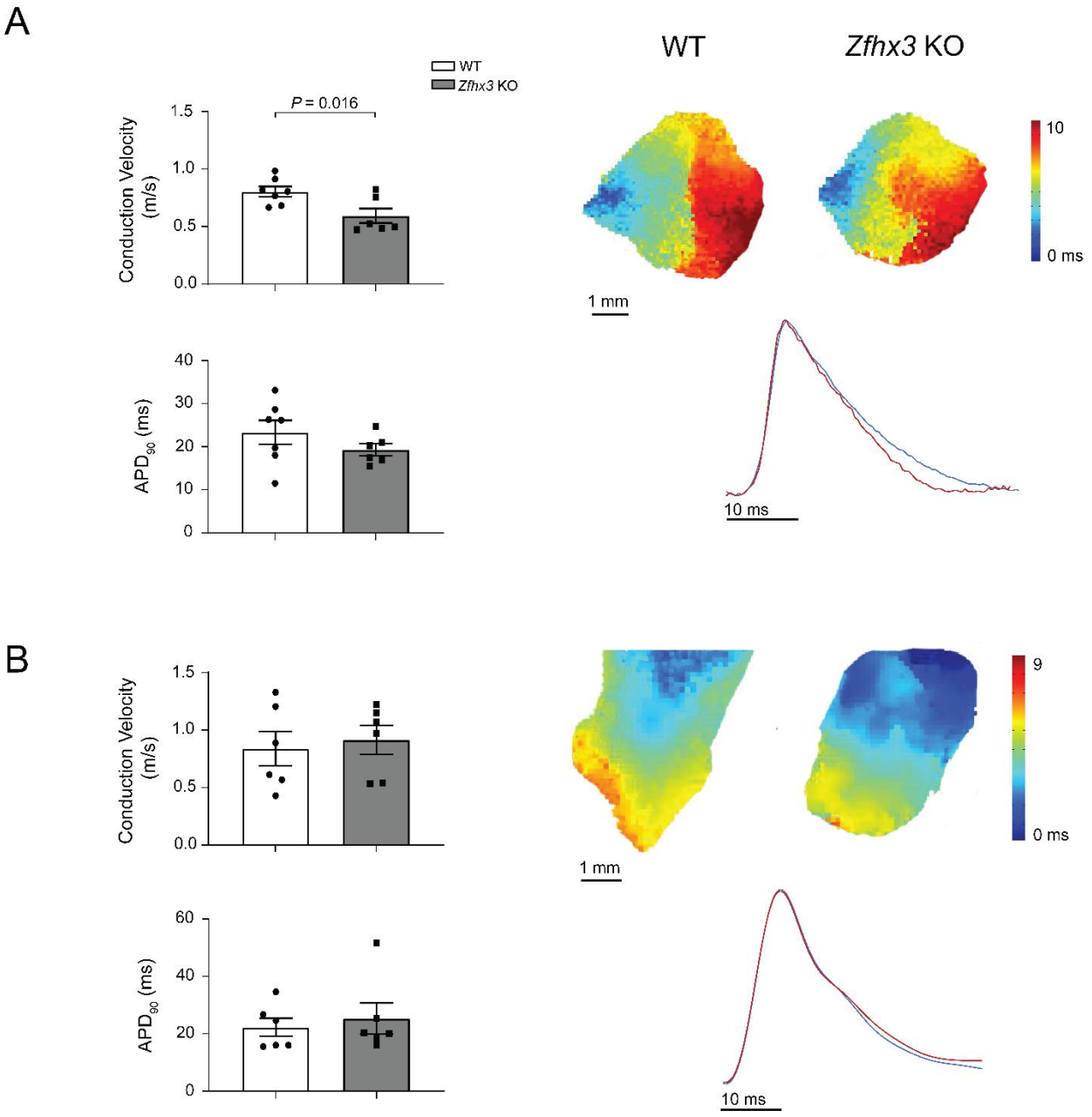

Figure S4. **Optical mapping in *Zfhx3* KO hearts at 3-months-old. A.** Quantitative comparison (left) of right atrial conduction velocity (top) and APD_{90} (bottom) between WT and *Zfhx3* KO mice; activation maps (right) recorded during right atrial pacing with a drive cycle length of 120 ms from WT (top left) and *Zfhx3* KO mice (top right) Representative right atrial optical action potentials (bottom). n = 7 WT for CV; 6 WT for APD_{90} and 6 *Zfhx3* KO mice. **B**. Quantitative comparison (left) of left atrial conduction velocity (top) and APD_{90} (bottom) between WT and *Zfhx3* KO mice; activation maps (right) recorded

during left atrial pacing with a drive cycle length of 120 ms from WT (top left) and *Zfhx3* KO mice (top right) Representative left atrial optical action potentials (bottom). n = 6 per genotype. Data are mean ± s.e.m. *P* values are indicated. Groups were compared using unpaired *t* tests.

Figure S5.

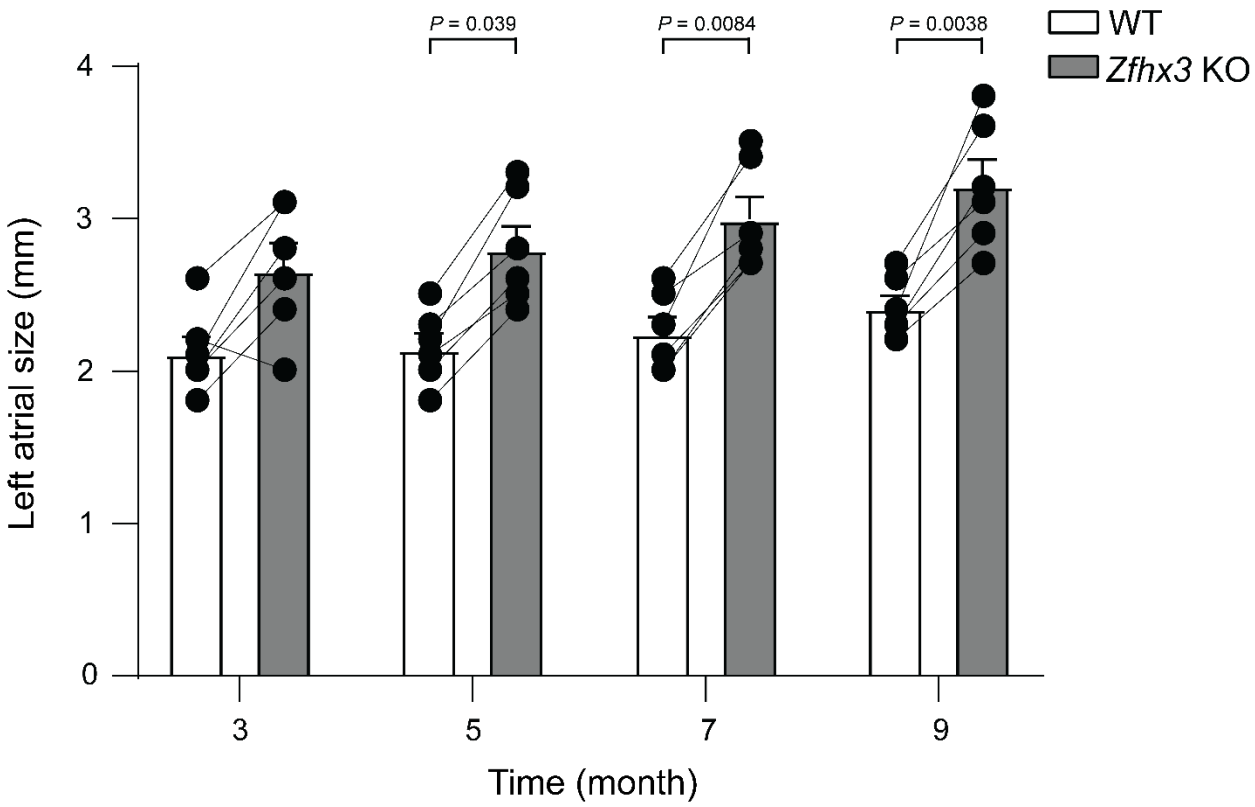

Figure S5. Left atrial size over time. Serial in vivo MRI-acquired images in WT and *Zfhx3* KO mice at 3, 5, 7, and 9 months of age. n = 6 per genotype. Data are mean ± s.e.m. *P* values are indicated. Groups were compared using 2way ANOVA with Sidak's multiple comparisons test.

Table S1. *ZFHX3* candidate SNPs

Candidate SNP	r^2 with rs2106261	*P* value	Odds ratio	Directionality
rs12596810	0.6672	2.62e-40	1.15	Increased risk
rs7192350	0.7815	3.08e-59	0.865	Decreased risk
rs12931021	0.7988	3.06e-60	0.864	Decreased risk
rs11643592	0.7704	3.11e-53	0.872	Decreased risk
rs1548373	0.5459	1.56e-49	1.12	Increased risk
rs5817827	0.6384	NA	NA	NA

The *P* value and odds ratio of each candidate SNP for AF association from the 2018 AF HRC GWAS in the Cardiovascular Disease Knowledge Portal (http://www.broadcvdi.org/). The r^2 values for assessing linkage disequilibrium with sentinel SNP rs2106261 in European ancestry populations were obtained from LDlink (https://ldlink.nci.nih.gov/).

Table S2. Cardiac morphology and function of 3-month-old WT, *Zfhx3* Het, and *Zfhx3* KO mice assessed by echocardiography.

Echocardiogram Parameters	WT	*Zfhx3 Het*	*Zfhx3* KO
LVIDs (mm)	2.85 ± 0.12	2.87 ± 0.20	2.93 ± 0.17
LVIDd (mm)	3.97 ± 0.06	4.00 ± 0.19	3.97 ± 0.13
IVSs (mm)	1.04 ± 0.05	1.02 ± 0.08	1.09 ± 0.08
IVSd (mm)	0.68 ± 0.04	0.72 ± 0.04	0.73 ± 0.04
PWs (mm)	0.92 ± 0.07	1.05 ± 0.10	0.91 ± 0.5
PWd (mm)	0.64 ± 0.04	0.67 ± 0.03	0.72 ± 0.05
Fractional shortening (%)	28.3 ± 2.19	28.6 ± 2.47	26.4 ± 2.30
LA Diameter (mm)	1.76 ± 0.04	1.69 ± 0.06	1.73 ± 0.04

n = 7 WT, 6 *Zfhx3* Het, and 8 *Zfhx3* KO mice. Data are mean ± s.e.m. Groups were compared using ordinary one-way ANOVA with Tukey's multiple comparisons test. LVIDs, left ventricular internal diameter end systole; LVIDd, left ventricular internal diameter end diastole; IVSs, interventricular septum thickness end systole; IVSd, interventricular septum thickness end diastole; PWs, posterior wall thickness end systole; PWd, posterior wall thickness end diastole; LA, left atrium.

Table S3. Baseline intracardiac conduction intervals in 3-month-old mice.

Interval (ms)	WT	*Zfhx3* Het	*Zfhx3* KO
PR interval	49.6 ± 2.78	45.3 ± 5.36	51.1 ± 3.64
QRS duration	21.5 ± 0.65	25.5 ± 5.32	21.9 ± 1.09
$SNRT_{120ms}$	95.3 ± 5.84	90.7 ± 6.94	94.4 ± 5.66
$SNRT_{100ms}$	117.1 ± 5.09	130.2 ± 11.7	119.7 ± 5.06
AV Wenckebach	90.5 ± 3.46	82.5 ± 5.06	89.5 ± 2.56
2:1 AV block	64.9 ± 1.88	61.8 ± 2.77	65.5 ± 1.70
$AVERP_{120ms}$	48.8 ± 1.47	44.8 ± 2.70	50.6 ± 2.61
$AVERP_{100ms}$	53.5 ± 2.28	48.2 ± 3.27	53.7 ± 2.60
$AERP_{120ms}$	29.5 ± 1.11	33.2 ± 3.22	32.2 ± 1.15
$AERP_{100ms}$	29.5 ± 1.52	35.0 ± 3.47	31.6 ± 1.31
1:1 VA conduction	94.6 ± 6.26	99.0 ± 7.13	93.9 ± 3.78
$VERP_{120ms}$	37.0 ± 2.68	38.0 ± 2.86	38.6 ± 2.63
$VERP_{100ms}$	38.4 ± 3.14	40.7 ± 3.51	40.2 ± 2.38

n = 13 WT, 6 *Zfhx3* Het, and 17 *Zfhx3* KO mice. Data are mean ± s.e.m. Groups were compared using Oneway ANOVA with Tukey's multiple comparisons test. SNRT, sinus node recovery time; AV, atrioventricular; AVERP, atrioventricular effective refractory period; AERP, atrial effective refractory period; VA, ventriculoatrial; VERP, ventricular effective refractory period.