MATERIALS AND METHODS

Gene targeting and generation of ZO-1 cardiac-myocyte specific knockout mice and animal use

Genomic DNA was isolated from R1 ES cells and used to create a *ZO-1*-targeting construct **(Online Figure IA)**. The construct was produced using a pBluescript II KS+ vector backbone (Stratagene, La Jolla, CA). The 5′ arm of the targeting vector consisted of a 2.3-kb *Not* I–*Sal* I fragment fused with an FRT-Neo-FRT cassette, followed by the first loxP site positioned upstream of ZO-1 exon 3. The 3′ arm of the targeting vector was a 4.0-kb *Xho* I–*Bgl* II fragment located downstream of a second loxP site. The targeting construct sequence and arrangement was verified by sequencing. The vector was linearized with *Not* I before electroporation into R1 ES cells at the Transgenic / Knockout Core Facility of the University of California, San Diego.

Following electroporation and antibiotic selection, genomic DNA was extracted from G418-resistant ES cell clones as previously described[28]. ES cell DNA was digested with *Hind* III and analyzed by Southern blot analyses. For probing the Southern blot, a 265-bp fragment was generated by PCR from genomic mouse DNA with *ZO-1* specific primers (forward, 5'TCCCCAGCCTCCTGCACAAA3';
reverse, 5'CCCTGCACCTTGGCAACTTA3'). The PCR product was radiolabeled using α-[^{32}P]-dCTP random priming (Invitrogen, Carlsbad, CA). Autoradiographs demonstrated the wild-type allele (9.1-kb) and in appropriately targeted clones, a 6.6-kb band representing the mutant allele **(Online Figure IB)**. Four clones of six hundred G418-resistant ES clonal isolates were proven to have had appropriate homologous recombination by Southern blot analysis.

Subsequently, two independent recombinant ES clones were microinjected into blastocysts from C57/Bl6 mice at the UCSD Transgenic / Knockout Core. Male chimeras were first bred with female Black Swiss mice to generate germ line-transmitted heterozygous mice. These genetically targeted mice were then crossed with FLPe mice from Jackson Laboratory[62] to delete the neomycin selection cassette and to generate ZO-1 – floxed (loxp) mice (ZO-1$^{f/+}$). ZO-1$^{f/+}$ were then crossed with αMHC-nuclear-Cre mice (as used by use previously)[36], to generate mice (ZO-1$^{f/+,\ Cre}$) which had the ZO-1 allele excised specifically in cardiac myocytes. ZO-1$^{f/+,\ Cre}$ mice were then used to generate ZO-1$^{f/+,\ Cre}$ (used as control mice throughout the study and termed CTL) and ultimately ZO-1$^{f/f,\ Cre}$ (termed ZO-1cKO) which were both used for subsequent experiments.

Offspring were genotyped by PCR analysis using mouse tail DNA and genotype primers (forward, GCTTTTGAGGGCCTTTCACAGGAGAGTC;
reverse, GCAGGTCCTTCACAAGAGCATTTAGCAG).

In order to investigate the morphological changes in the conduction system, ZO-1cKO and CTL mice were also crossed with HCN4-GFP-ERT2 mice[29] to generate ZO-1$^{f/f,Cre,\ GFP}$ (ZO-1 cKO-G) mice and CTL-GFP mice ZO-1$^{f/+,Cre,\ GFP}$ (CTL-G) as outlined in the main manuscript

Initial experiments were performed on a subset of animals and found no variance between sexes. Still, male mice were used in a majority of studies and unless specified in the figure legends, male mice were used. If both sexes were used, details are provided in the figure legends. Within specific genotypes, animals or samples from them, were randomly assigned to experiments. Studies were performed in a blinded manner.

Histology, transmission electron and immunomicroscopy

For standard histology, three pairs of ZO-1cKO and CTL mice at 6m were anesthetized by ketamine and xylazine and perfused with Relaxing buffer (77mM NaCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4 and 62.7mM KCl) for 5 min, followed by fixation with 4% paraformaldehyde (PFA) in phosphate buffer for 5 min. Hearts were then dehydrated and paraffin embedded. Sections (10uM) were analyzed for standard histology by hematoxylin and eosin or Masson's trichrome staining[63]. Images were obtained by standard microscopy NanoZoomer and analyzed using NanoZoomer Digital Pathology (NDP, Hamamatsu Corporation, San Jose, CA).

For electron microscopy (EM) three pairs of ZO-1cKO and CTL mice (6m) were anesthetized as above, and perfused with Tyrode's buffer for 5 min, followed by fixation with 2% glutaraldehyde and 2% PFA in phosphate buffer. Hearts were then immersed in the fixative for 24 hrs. Following rinses by 0.15mM sodium cacodylate buffer (pH7.4), tissues were post-fixed by 2% aqueous osmium tetroxide and 0.8% potassium ferrocyanide in 0.15mM sodium cacodylate buffer for 2h, washed in ddH2O twice, incubated in 2% uranyl acetate (UA) overnight, and then dehydrated in a graded alcohol series (50, 80, 90, 100) and acetone. Subsequently tissue was infiltrated with a 1:1 mixture of acetone and araldite resin (Electron Microscopy Science, Hatfield, PA) for 2h placed into 100% araldite resin overnight, and then embedded in fresh araldite resin. Thick sections (0.5μm) were cut with glass knives and stained with toluidine blue prior to initial light microscopic examination. Then from these thin sections (60-90 nm) were cut with a diamond knife and stained with uranyl acetate and statured lead citrate, before examination in a JEOL 1200EX microscope (JEOL, Peabody, MA). The negative films were scanned with an Epson 4990 scanner (Epson, Long Beach, CA). The images were analyzed by Photoshop CS or image J (NIH, Bethesda, MD).

For immunomicroscopic analysis of fluorescent reporter expressing tissue, mice were anesthetized and perfused with relaxing buffer, as above, and then perfusion fixed with 4% paraformaldehyde (PFA) in phosphate buffer for 5 min. Hearts were then dissected, fixed with 4% PFA in PBS solution overnight at 4°C and placed on silicon coated petri dishes for imaging. Bright field and fluorescence GFP images were acquired with an Olympus SZX12 Macro-View microscope and merged light and fluorescent fields by Abode photoshop CS5.

For immunomicroscopy of tissue sections, mice were anesthetized and perfused with Relaxing buffer for 5 min. Hearts were then excised and collected immediately. Hearts were then flash frozen in isopentane pre-cooled in liquid nitrogen and stored at -80°C until

further processed. Cryostat sections (8 μm) were cut and fixed with acetone at -20°C for 10 min. Specimen were washed in PBS twice, permeabilized in PBST once and incubated in blocking buffer including 5% BSA, 5% normal donkey serum in PBST. And then incubated with specific antibodies: primary rat anti-ZO-1 Clone R40.76 (1:50, MATB11, Millipore-Sigma, Burlington, MA), ZO-2 (1:25, Abgent, San Diego, CA), desmoplakin (1:200, AbD Serotec, Hercules, CA), goat anti-mouse PECAM/CD31 (1:100, AF3628, R & D Systems, Minneapolis, MN), Connexin 43 (1:500, 71-0700, Invitrogen, Carlsbad, CA), β-catenin (1:100, Abcam, Cambridge, MA), Y-catenin (1:50, ThermoFisher, Carlsbad, CA), N-cad (1:100, BD Biosciences, San Diego, CA), contactin-2 (1:50, AF439, R & D Systems, Minneapolis, MN). Cx45 antibody was provided kindly by Dr. Ulrike Janssen-Bienhold (University of Oldenburg, Germany) and staining protocol followed reference[52, 64]. Nav1.5 antibody was kindly provided by Dr. Robert Gourdie (Virginia Tech Carilion Research Institute) and was verified previously in work from his laboratory[65]. CAR antibody was used previously by our group and published[24].

Sections were then washed in PBST 3 times, for 10 min each, and incubated with secondary antibodies: donkey anti-Rabbit Alex-488 (1:100, 711-545-152, Jackson Lab), anti-Rabbit Alex-647 (1:200, 711-605-152, Jackson Lab), cy3 donkey anti-Rat (1:200, 712-165-153, Jackson Lab) cy5 donkey anti-Mouse (1:200, 715-175-151, Jackson Lab), cy3 donkey anti-goat (1:200, 705-165-147). DAPI (10ug/ml, D1306, ThermoFisher, Carlsbad, CA) was used to label nuclei, as indicated in figures. Finally, sections were washed in PBST 3 times and mounted under coverslips with Fluoromount-G™ (00-4958-02, eBioscience, San Diego, CA). Controls for all immunostaining experiments were performed by using either IgG or secondary antibody alone, without use of primary antibodies. Images was obtained with Olympus FV1000 (Waltham, MA) in the microscope core of UCSD and analyzed by FV10-ASW viewer.

Surface electrocardiography, conscious telemetry, transverse aortic constriction, echocardiography and intracardiac electrophysiology

For all of these studies mice were lightly anesthetized with 1% isoflurane and maintained on 100% supplemental oxygen throughout the study.

For surface electrocardiography (ECG) studies needle electrodes (30 gauge) were inserted subcutaneously into the limbs and chest. ECG signals were recorded using LabChart software and Bio Amplifier (AD Instruments, Colorado Springs, CO) with bandpass filtered between 0.3 and 1000 Hz. and analyzed using LabChart software. Conscious ECG telemetry was performed by using an implantable wireless transmitter/receiver system (Ponemah, Data Science International, New Brighton, MN) as performed[26].

Left ventricular pressure overload was produced in mice by performing transverse aortic constriction (TAC) as described previously[30]. Briefly, we monitored the function of heart by transthoracic echocardiography (Echo) each week after the surgery. At 4 weeks

following surgery, the pressure gradient generated by aortic banding was measured by introducing high-fidelity pressure transducers into the left and right common carotids. Only mice showing an adequate pressure gradient (>40 mmHg) were included in the analysis.

Echo analyses were performed using a VEVO 2100 ultrasound machine with a 45-MHz transducer (FUJIFILM Visualsonics Inc. Toronto, ON, Canada), M-mode echocardiograms were performed and measured as described previously[66].

For intracardiac electrophysiological recordings, an octopolar electrode (EPA-800, Millar, Houston, Texas) was inserted through the right jugular vein, and advanced into the right atrium (RA) and subsequently into the right ventricle (RV). Intracardiac electrograms were sampled at 4.0 kHz and filtered at 0.3to 1,000 Hz and recorded by use of Acknowledge software (LabChart Systems) described, as above. Studies were initiated by testing 'capture threshold' to determine minimum pulse amplitude (mA) to induce pacing and then baseline recordings were obtained. To evaluate atrioventricular (A-V) conduction properties: Sinus nodal recovery time (SNRT), Wenckebach (Mobitz type I), 2:1 AV block (Mobitz type 2), atrial effective refractory periods (AERP) and AV-nodal refractory period (AVERP), were evaluated. Atrial fibrillation was induced also using 40, 20, 10, 5, 2ms pulses stimulation. CTL mice analysis was able to be completed through the entire testing protocol described above, but since ZO-1cKO could not transmit atrial paced beats through to ventricle, the analysis of all these parameters could not be measured.

Optical mapping studies

For optical mapping studies, hearts were excised from 6m ZO-1 cKO and CTL mice (n=4 each) after mice were anesthetized with 2.5% isoflurane and after receiving 100% supplemental oxygen. The hearts were hung in a custom optical chamber and Langendorff perfused with warmed (37°C) modified Krebs Henseleit solution at a constant pressure (70 mmHg) as previously described [24]. After equilibration, the heart was perfused with the voltage-sensitive fluorescent dye, di-4-ANEPPS, and the excitation-contraction uncoupler Blebbistatin (10 mM) was added to the perfusate to reduce motion artifact. To optically record the epicardial membrane potential, the fluorophore was excited by a 470 nm LED array (LEDtronics, Torrance, CA). Fluorescence emission was gathered by a custom-built tandem lens optical system (1x magnification Plano APO objectives; Leica, Wetzlar, Germany), which passed images through a >610 nm long-pass optical filter (Omega Optical Brattleboro, VT), and then focused them onto a high-speed CMOS camera (MiCAM Ultima L, SciMedia, Costa Mesa, CA). Imaging was acquired on the 1 cm x 1 cm sensor at 1000 Hz with 0.1 mm x 0.1 mm pixel resolution. Atrial and ventricular pacing was performed using a Teflon coated platinum electrode. The 2-ms rectangular constant-current pacing pulse was generated by a digital stimulator (DS8000 stimulator and SLS100 isolator, World Precision Instruments, Sarasota, FL). Data analysis was performed in MATLAB (Natick, MA) as previously described [31]. Atrial and ventricular activation times were obtained from optical voltage signals recorded by

the anterior and posterior cameras. Calculation of paced and intrinsic conduction velocity was described as in Bayly et al [67]

Immunoblot analysis

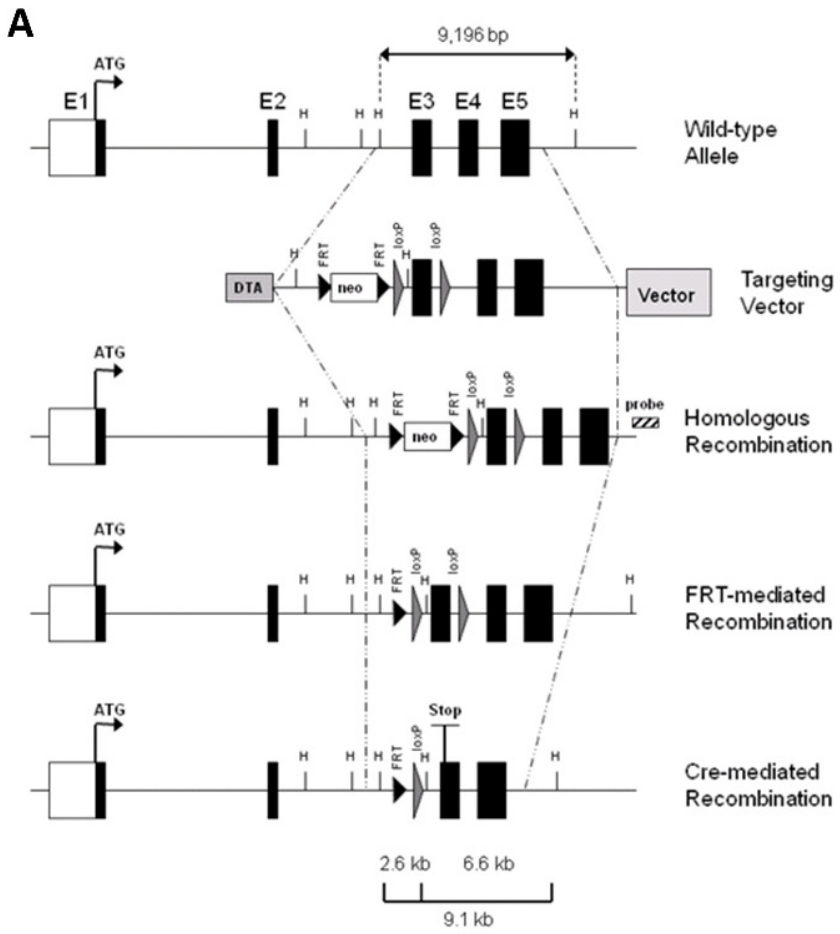
To evaluate ZO-1 and intercalated disc (ICD) protein expression level, protein lysates from 12-week-old mice (n = 6) were subjected to immunoblot analysis using an ECL detection system as described previously[68]. Primary antibodies used were rat anti-ZO-1 Clone R40.76 (1:1000, MATB11, Millipore-Sigma, Burlington, MA), desmoplakin (1:1000, 2722-5204, AbD Serotec, Hercules, CA), Connexin 43 (1:5000, 71-0700, Invitrogen, Carlsbad, CA), p-Connexin 43 (1:1000, 3511, Cell Signaling Technology, Danvers, MA) Vinculin (1;10000, V9131, Sigma, Burlington, MA), N-cadherin (1:1000, MNCD2, University of Iowa Hybridoma Bank, Iowa City, Iowa), CAR (1:1000, SC-15405, Santa Cruz Biotechnology Inc., Santa Cruz, CA), β-catenin (1:1000, AB16051, Abcam, Cambridge, MA), Y-catenin (1:1000, SC-7900, Santa Cruz Biotechnology Inc., Santa Cruz, CA), ZO-2 (1;1000, ABO11279, Abgent, San Diego, CA), Connexin 30.2 (1:1000, 40-7400, Invitrogen, Carlsbad, CA), Connexin 40 (1:1000, 36-5000, Invitrogen, Carlsbad, CA), Connexin 45 (1:1000, 41-4700, Invitrogen, Carlsbad, CA), GAPDH (1:10000, MAB374, Millipore-Sigma, Burlington, MA). Secondary antibodies were donkey anti-Mouse (615-035-214), donkey anti-rabbit (711-035-152), donkey anti-rat (712-035-153), donkey anti-goat (705-035-003) from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, Protein expression levels were evaluated with Image Lab software (Bio Rad laboratories, Hercules, CA) based on the signal intensity of each protein using GAPDH as an internal control for protein loading.

Statistical analysis

Tests for statistical significance were computed using the Prism, version 7.04, software (GraphPad Software, San Diego, CA) with a probability value of <0.05 considered significant. Sample sizes were based on a previous studies of similar types that we performed and published[24, 36, 69]. Formal sample size and power calculations were not conducted. Each data set, irrespective of the sample size, was tested for normality typically using the Shapiro-Wilk test. Thereafter, a parametric test was chosen as appropriate. Statistical analyses were performed for most studies with 2-tailed Student t test (parametric unpaired, 2 group of analysis) with Welch's correction. For multiple comparisons, a two-way ANOVA followed by Sidak's test was used as indicated. All data are represented as mean ± SD. A value of P (or corrected P in case of multiple groups) <0.05 was considered to be statistically significant.

ONLINE FIGURES

Online Figure I



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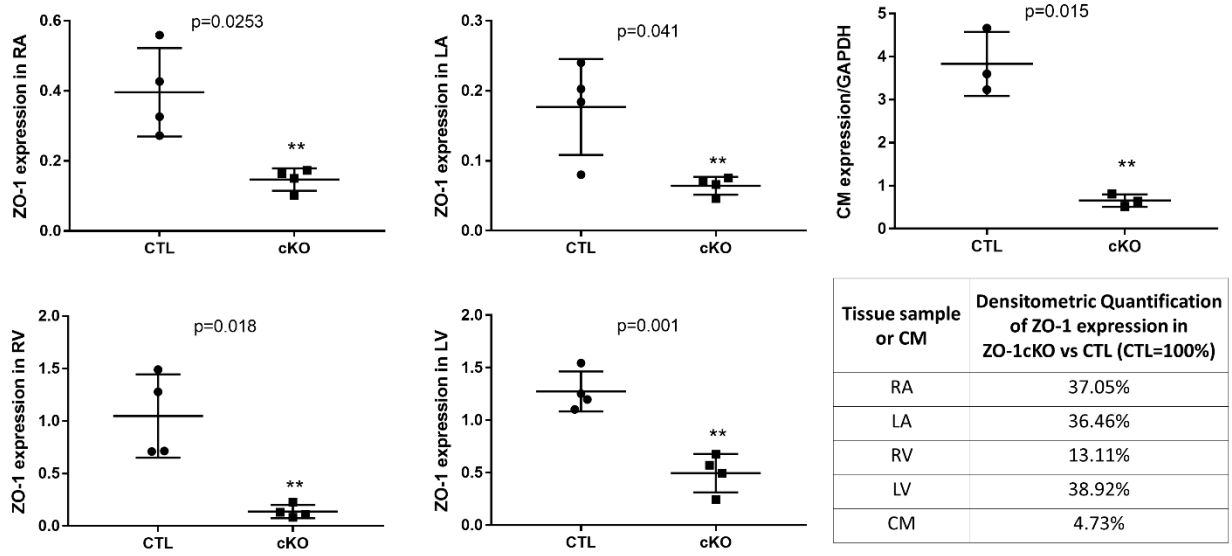
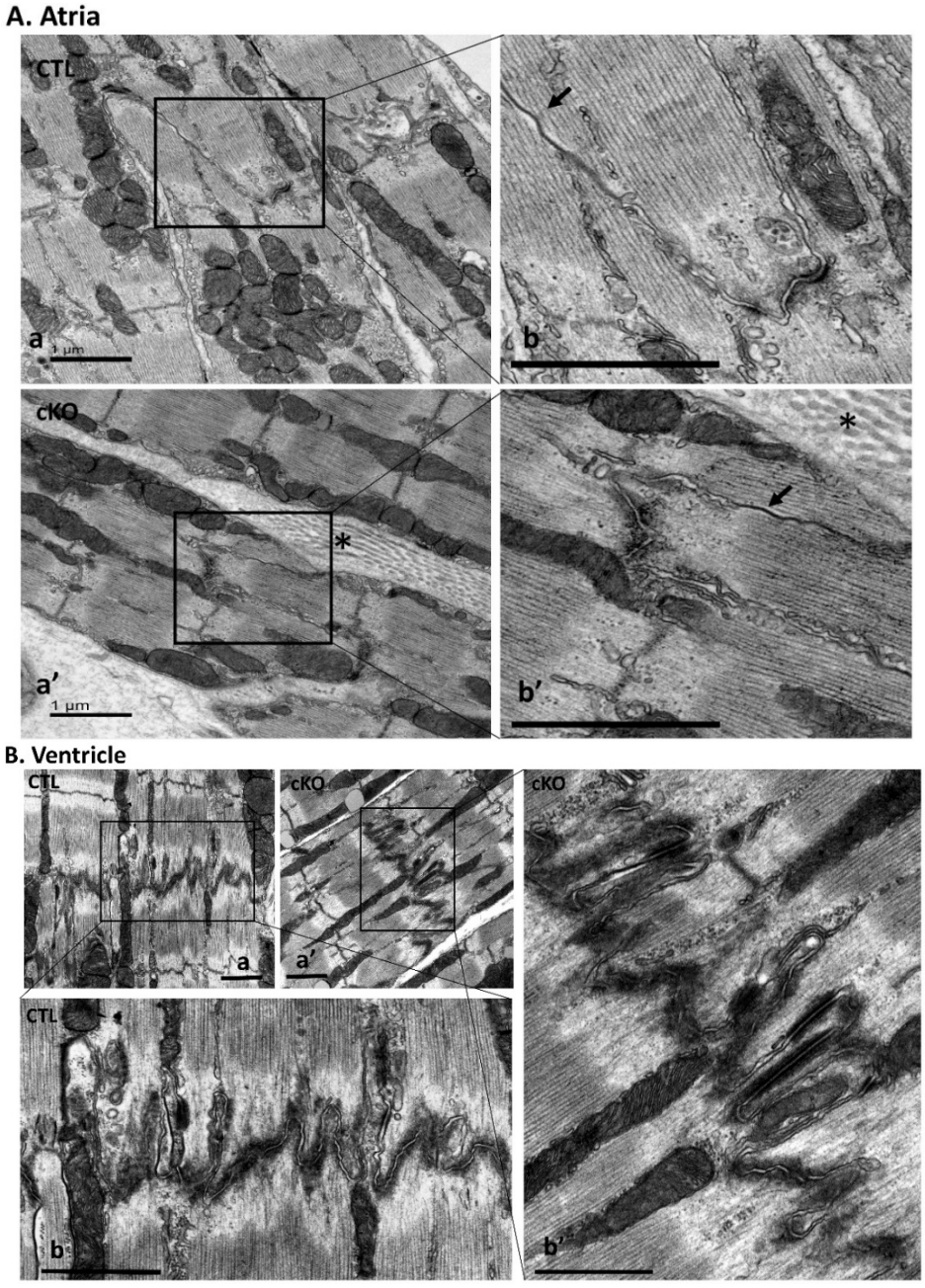


Tissue sample or CM	Densitometric Quantification of ZO-1 expression in ZO-1cKO vs CTL (CTL=100%)
RA	37.05%
LA	36.46%
RV	13.11%
LV	38.92%
CM	4.73%

Online Figure I. Targeting strategy of the ZO-1 gene.

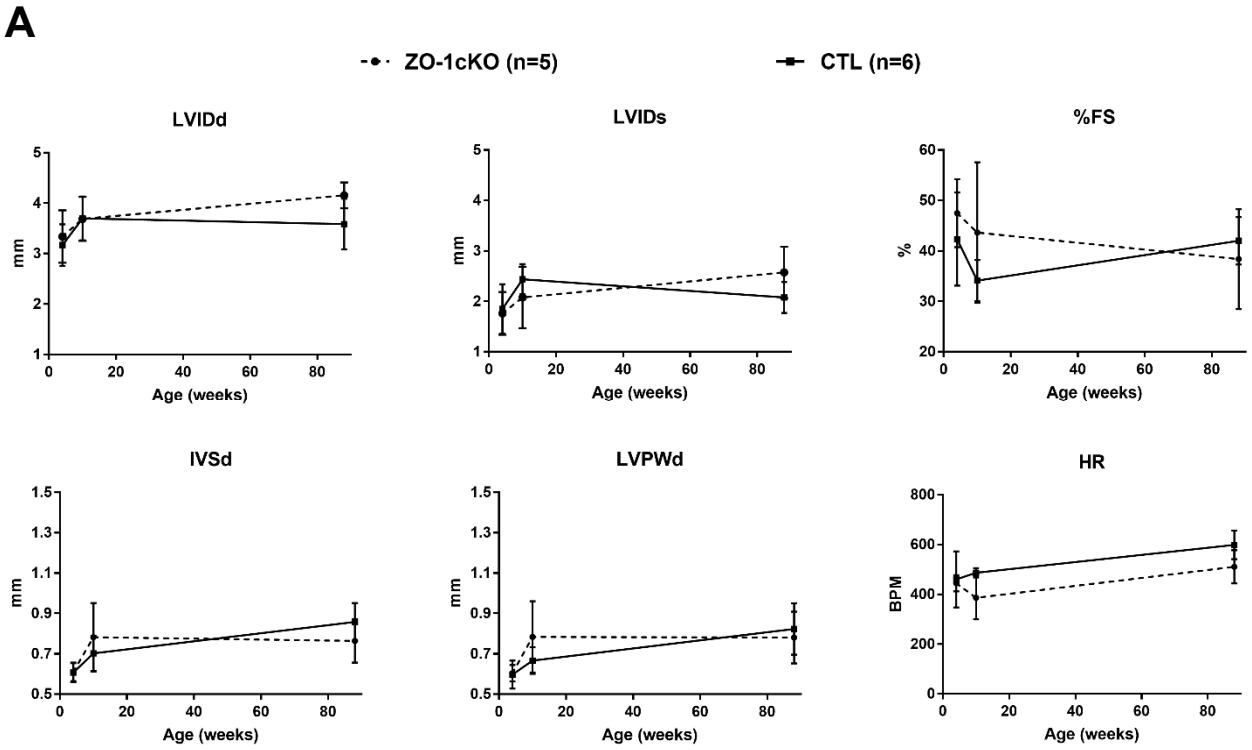
(**A**) **Targeting strategy for deletion of the *ZO-1* gene.** Exon 3 of the murine *ZO-1* gene was flanked by loxP sites, and a neomycin (Neo) resistance cassette was inserted upstream, flanked by FRT sites. H, Hind III.
(**B**) **Southern blot confirmation** of the wildtype (WT) allele at 9.1kb and presence of the appropriately targeted mutant allele at 6.6kb (f/+). Details as described in methods, above.
(**C) Densitometric quantification of ZO-1 expression in ZO-1cKO vs CTL**, from Western blots of protein lysates from tissue of specific chambers (n=4 for CTL and ZO-1cKO), and also isolated CMs (CTL - n=4, ZO-1cKO - n=5). CTL = 100%). Normality was assessed with the Shapiro-Wilk statistical test. Data are represented as mean ± SD. Unpaired two-tailed Student's *t*-test with Welch correction was used to assess the p values by groups.

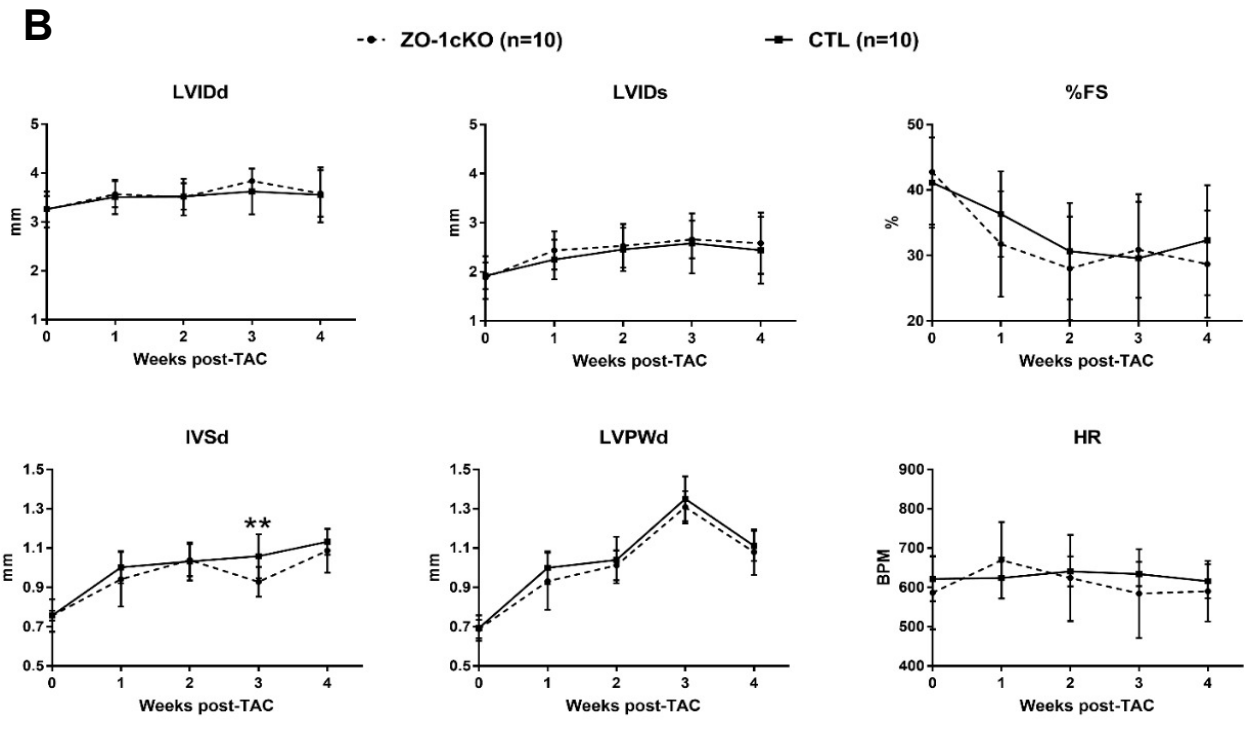
Online Figure II

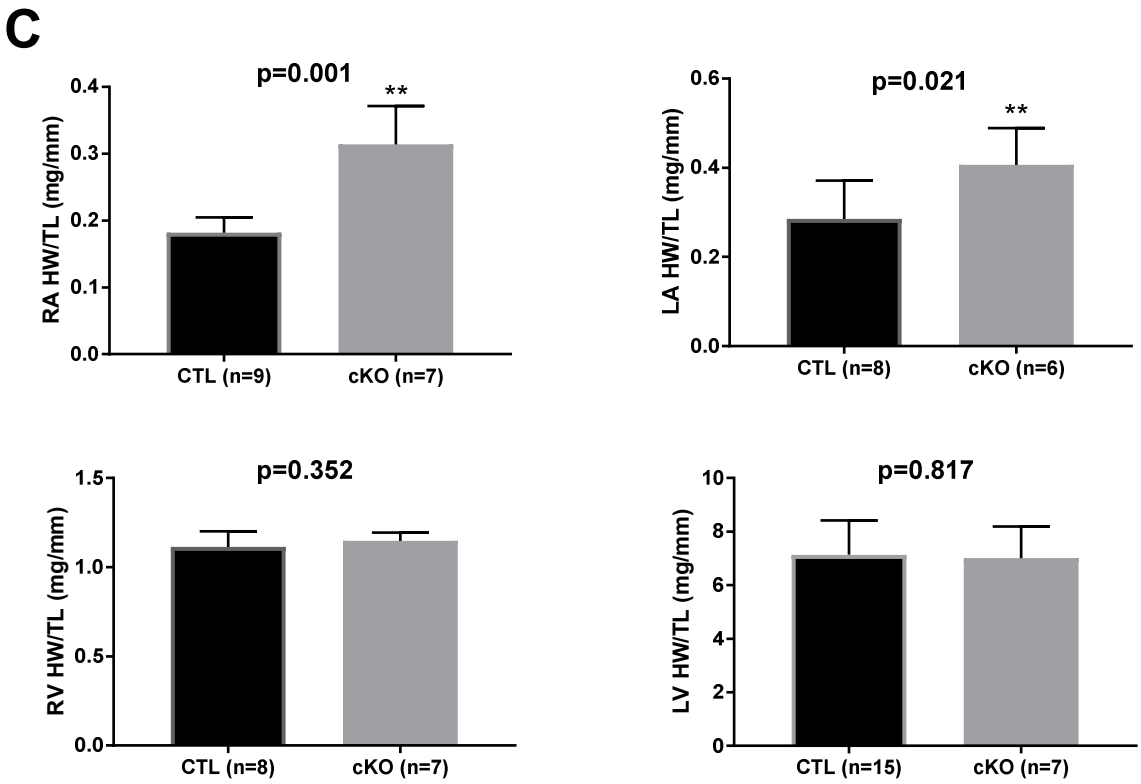


Online Figure II. Transmission electron microscopy (TEM) of right atria (RA) and left ventricle (LV) tissue.
(A) Right Atria - Interstitial matrix was noted to be increased between atrial myocytes of the cKO (indicated by *) that was not seen in CTL specimens. There were no obvious structural differences of GAP junctions (indicated by arrows) in atria between the groups. Boxed areas in a and a' are shown as enlarged micrographs in b and b', respectively. Bar = 1um. **(B) Left Ventricle -** There were no abnormalities in the intercalated disc in ventricular tissue of ZO-1cKO vs CTL. Boxed areas in a and b are shown as enlarged micrographs in a' and b', respectively. Bars = 500nm.

Online Figure III







Online Figure III. Investigation of cardiac function by echocardiography at baseline and up to 4w after hemodynamic loading produced by transverse aortic constriction (TAC).

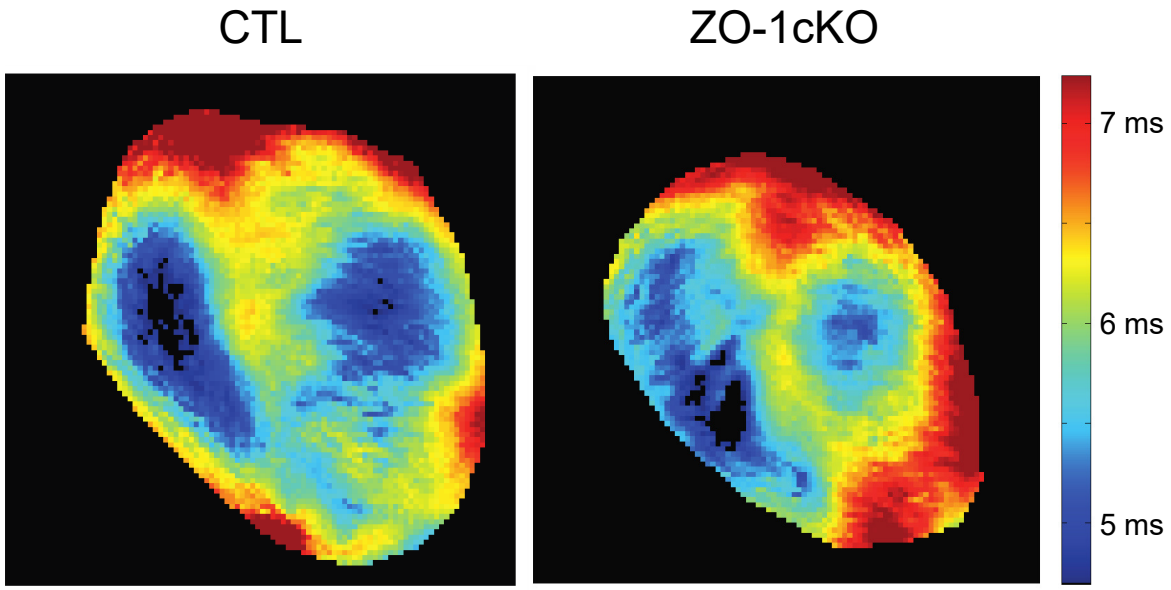
(**A**) **Basal evaluation –** Echocardiography was performed in basal mice at ages 4, 10 and 88 weeks. ZO-1cKO (n=5; M=4, F=1) showed no significant changes in echocardiographic parameters, except for reduced HR at 88w, vs. CTL (n=6; M=2, F=4). No differences were seen at earlier timepoints. Statistical analysis was performed by 2-way ANOVA, followed by Sidak's test for multiple comparisons and with $P \leqslant 0.05$ regarded as significant.

(**B**) **Hemodynamic Loading** – **Baseline echocardiography was performed, followed by transverse aortic constriction (TAC) for 4w.** Mice at 11-12w were challenged with TAC or Sham (control) surgery. No significant differences were seen between groups basally, as we showed in the aging study, in Panel A, or when mice were evaluated at weekly time points up to 4w post-TAC, except for a single variance of the IVSd at the 3-week time point. Statistical analysis was performed by 2-way ANOVA, followed Sidak's test for multiple comparisons and with $P \leqslant 0.05$ regarded as significant indicated by **. (n=10, each group.)

(C) Normalized weights of cardiac chambers in CTL vs ZO-1cKO obtained at termination of TAC study. Dissected and blotted tissue chamber weights were normalized to tibia length and show that RA and LA normalized weights are increased in cKO mice vs CTL, as they were in the basal state. Normality was assessed with the Shapiro-Wilk statistical test. Data were represented as mean ± SD. Unpaired two-tailed

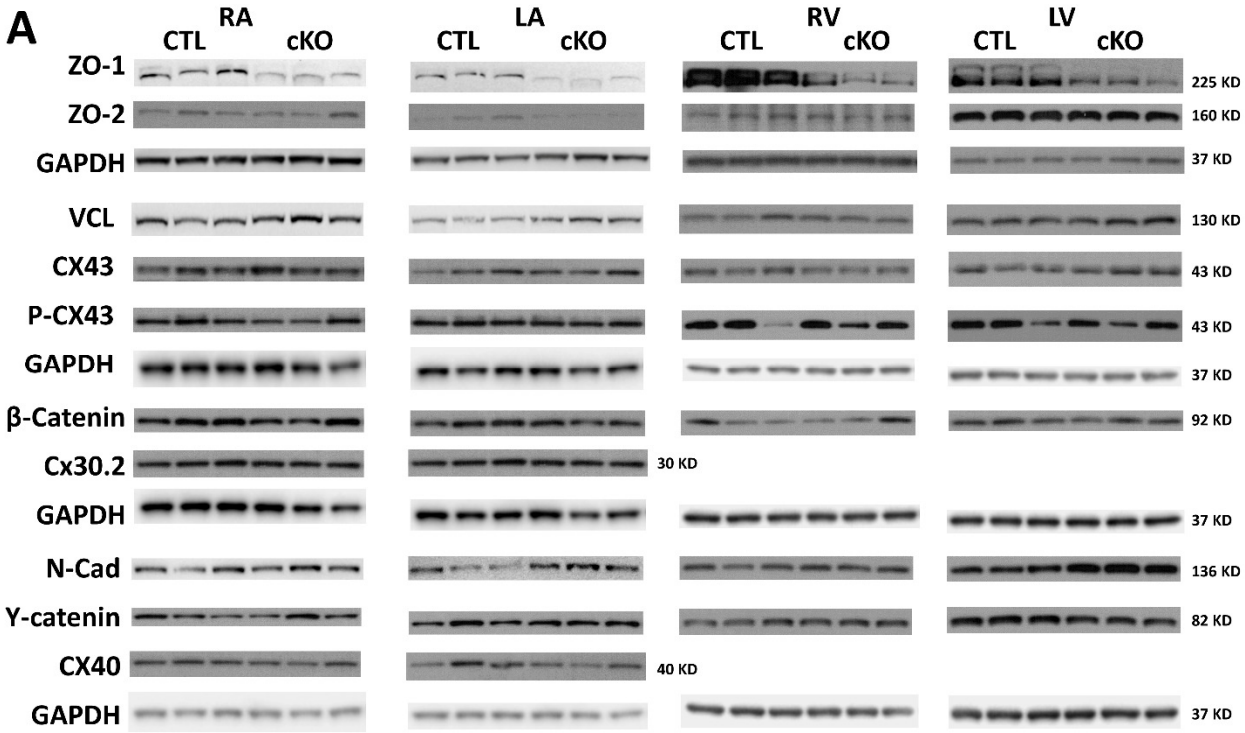
Student's *t*-test with Welch correction was used to assess the p value by groups. **p<0.05; Data = mean +/- SD. (LVIDd and LVIDs=LV internal diameter in end-diastole and end-systole, respectively. %FS=% fractional shortening. IVSd and LVPWd = interventricular septal dimension in end-diastole and LV posterior wall dimension in end-diastole, respectively. HR=heart rate.) Numbers of mice in each group are shown in figure.

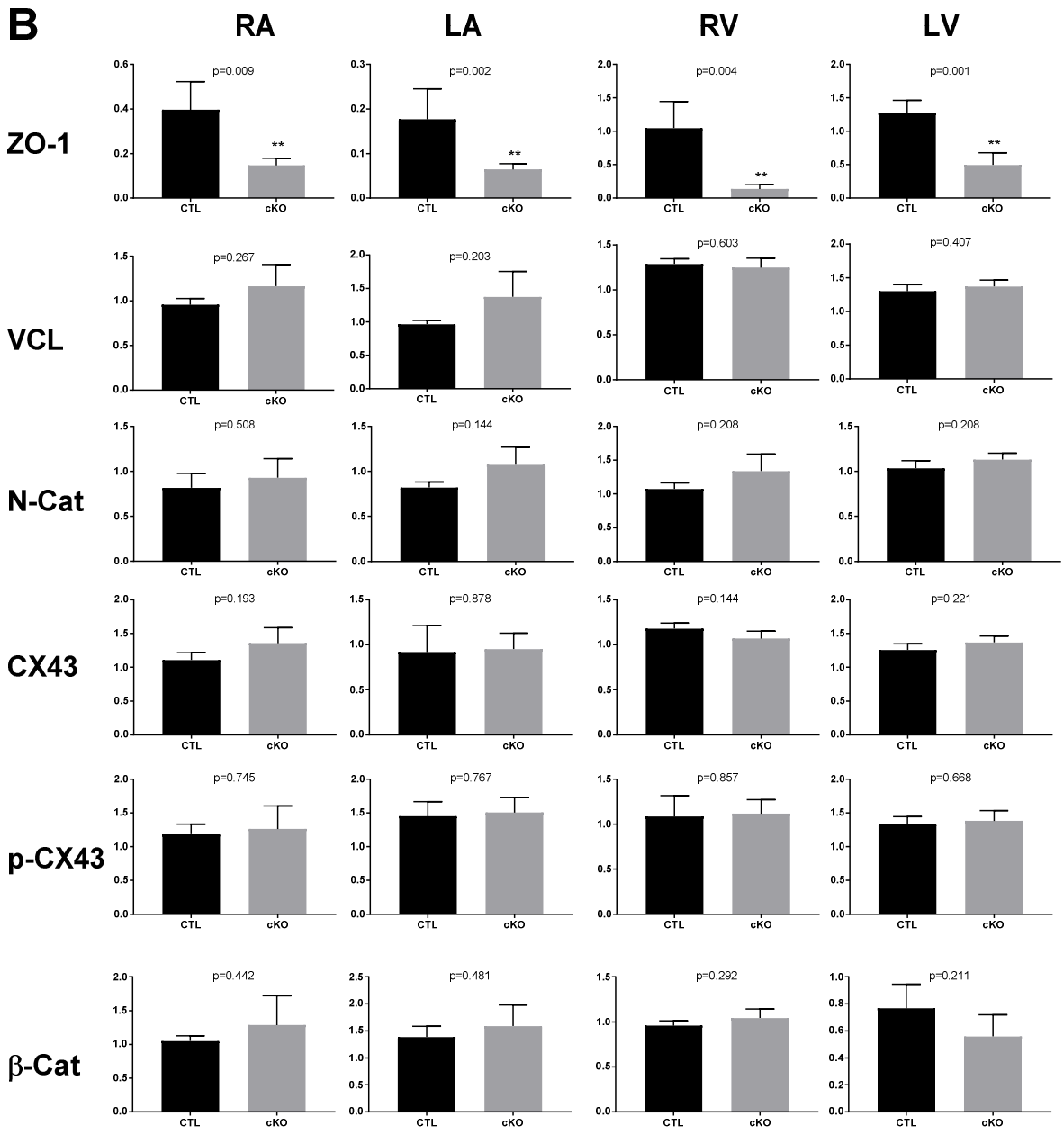
Online Figure IV

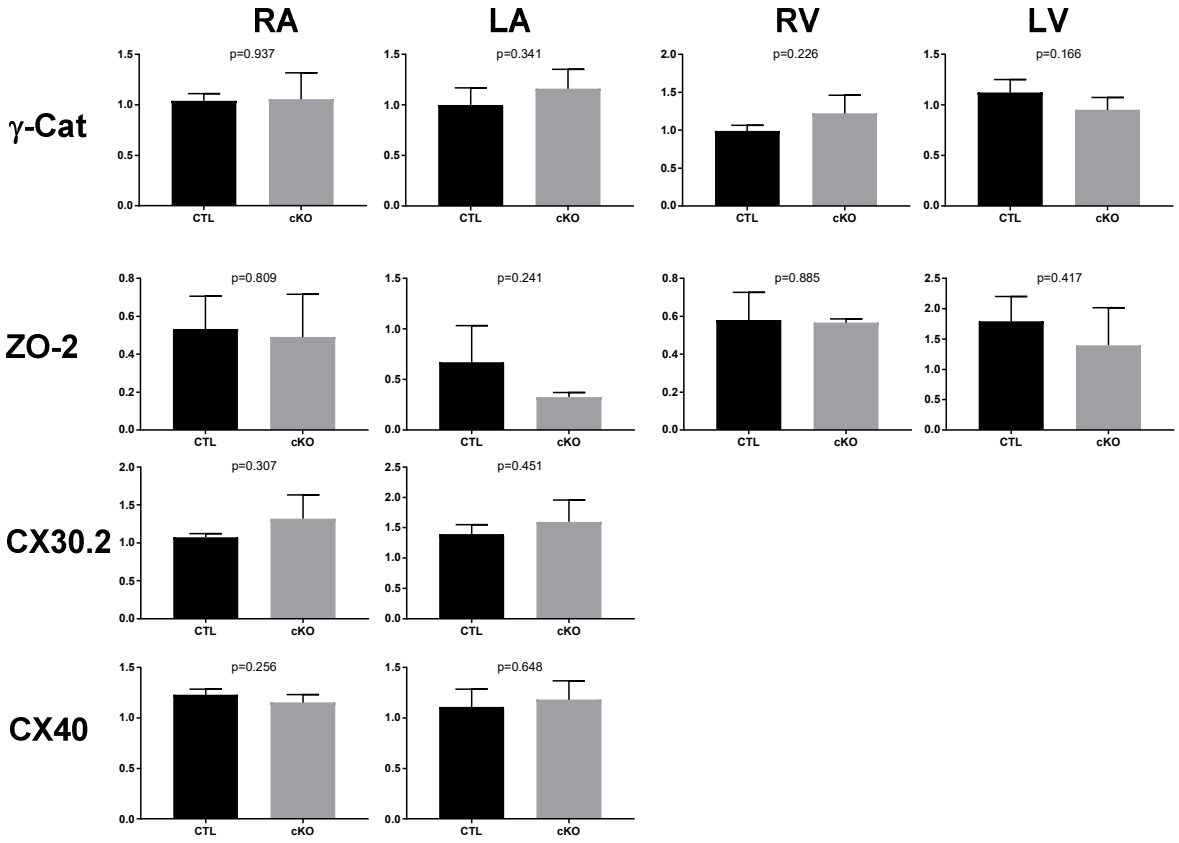


Online Figure IV. Optical mapping studies show normal Purkinje activation in ZO-1cKO mice. Activation maps of the anterior epicardium in representative CTL and ZO-1cKO mice show a similar pattern of two distinct breakthrough locations due to electrical propagation through the Purkinje system. (n=4 examined for each group.)

Online Figure V




B
RA
LA
RV
LV
ZO-1
VCL
N-Cat
CX43
p-CX43
β-Cat
p=0.009
p=0.002
p=0.004
p=0.001
p=0.267
p=0.203
p=0.603
p=0.407
p=0.508
p=0.144
p=0.208
p=0.208
p=0.193
p=0.878
p=0.144
p=0.221
p=0.745
p=0.767
p=0.857
p=0.668
p=0.442
p=0.481
p=0.292
p=0.211
**
CTL
cKO

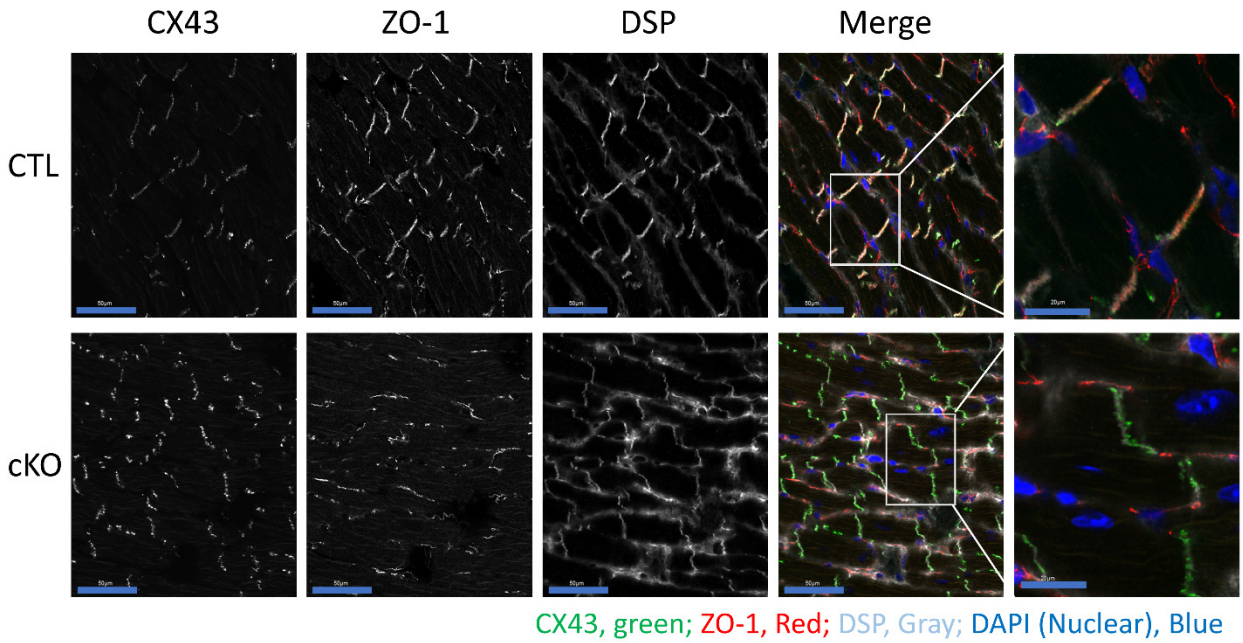


Online Figure V. Western blot analysis.

(A) - ZO-1 and a series of ZO-1 associated proteins were evaluated by Western blotting of whole protein lysates obtained from various cardiac chambers, as shown.

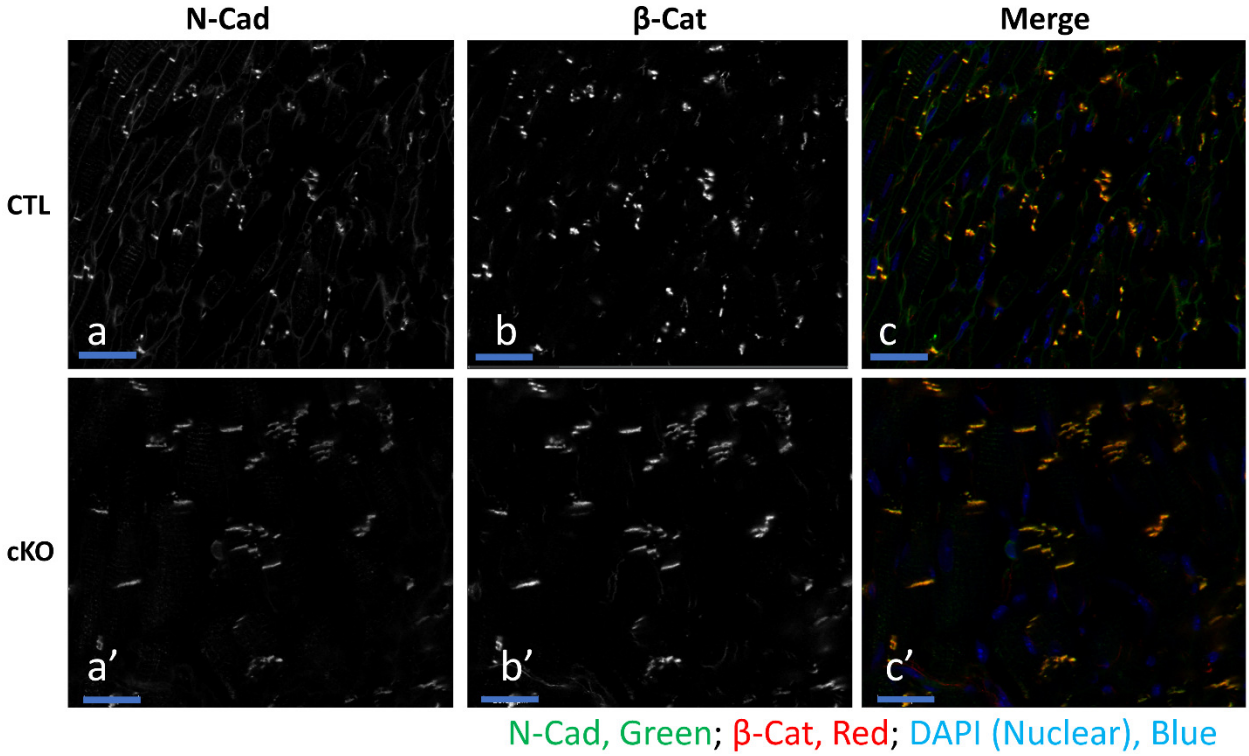
(B) - Densitometric evaluation of each protein, normalized to GAPDH as a loading control, was then obtained for the samples. Only ZO-1 was reduced in all four chambers and the Coxsackie-adenovirus receptor (CAR) was reduced in the atria of the ZO-1cKO compared to CTL. However, there was not a significant difference in expression of any of the other proteins analyzed. . Normality was assessed with the Shapiro-Wilk statistical test. Data were represented as mean ± SD. Unpaired two-tailed Student's *t*-test with Welch correction was used to assess the p value by groups. (**P < 0.05, n=3 for each sample. Vin, Vinculin; N-Cad, N- Cadherin; CAR, Coxsackie Adenovirus Receptor; CX, connexin; CAT, catenin) (RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

Online Figure VI



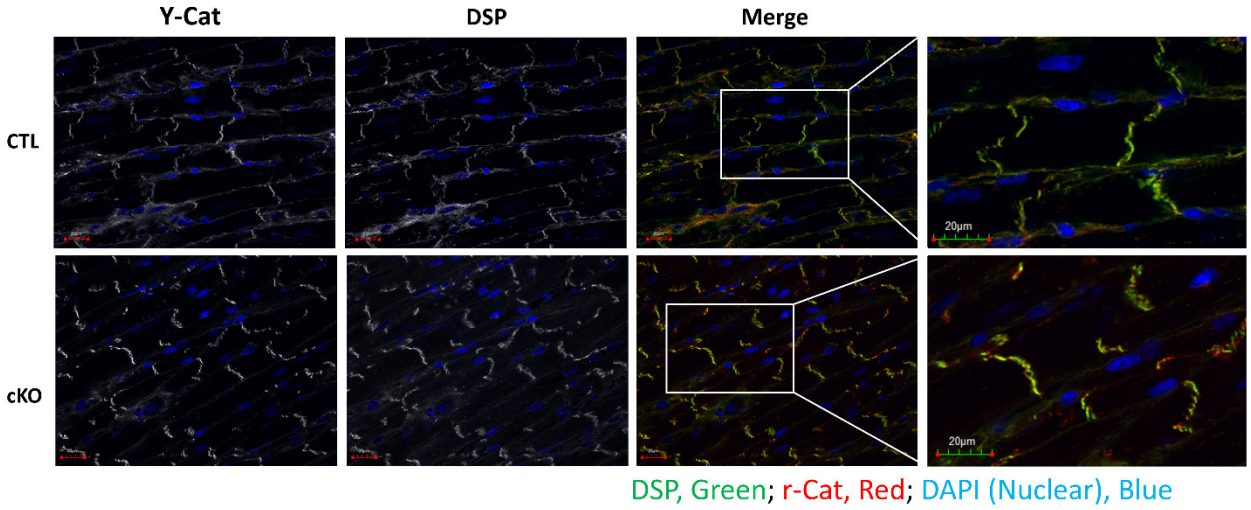
Online Figure VI. Immunostaining of LV tissues from CTL and ZO-1cKO heart show no significant changes in Cx43 staining pattern in ZO-1cKO vs CTL. Connexin 43 expression was preserved with no significant visual change in intercalated disks in ventricular tissue of ZO-1cKO vs. CTL. (In 'Merge' figure panel: CX43=Green, ZO-1=Red, DSP=Gray, Nuclei DAPI=Blue, Bar = 50um in low magnification and Bar = 20um in high magnification)

Online Figure VII



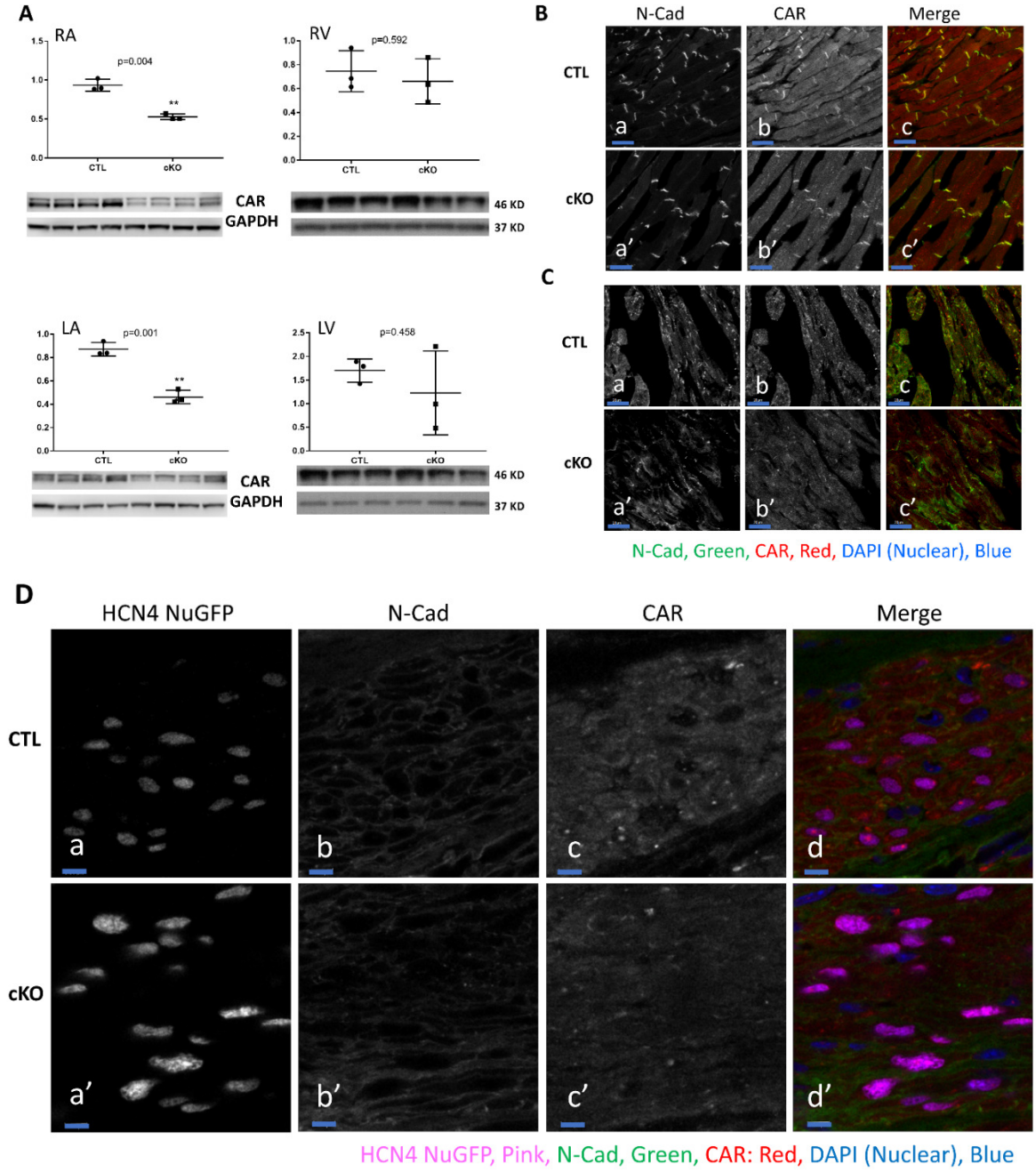
Online Figure VII. Immunostaining of LV tissues from CTL and ZO-1cKO heart show no significant changes in N-cadherin and β-catenin staining pattern in ZO-1cKO vs CTL. A similar expression pattern of ZO-1 associated proteins N-cadherin and β-catenin is seen in the ventricular tissue evaluated from ZO-1cKO and CTL mouse groups. (In 'Merge' figure panel: N-Cad=Green, β-Cat=Red, Nuclei DAPI=Blue, Bar 20uM).

Online Figure VIII



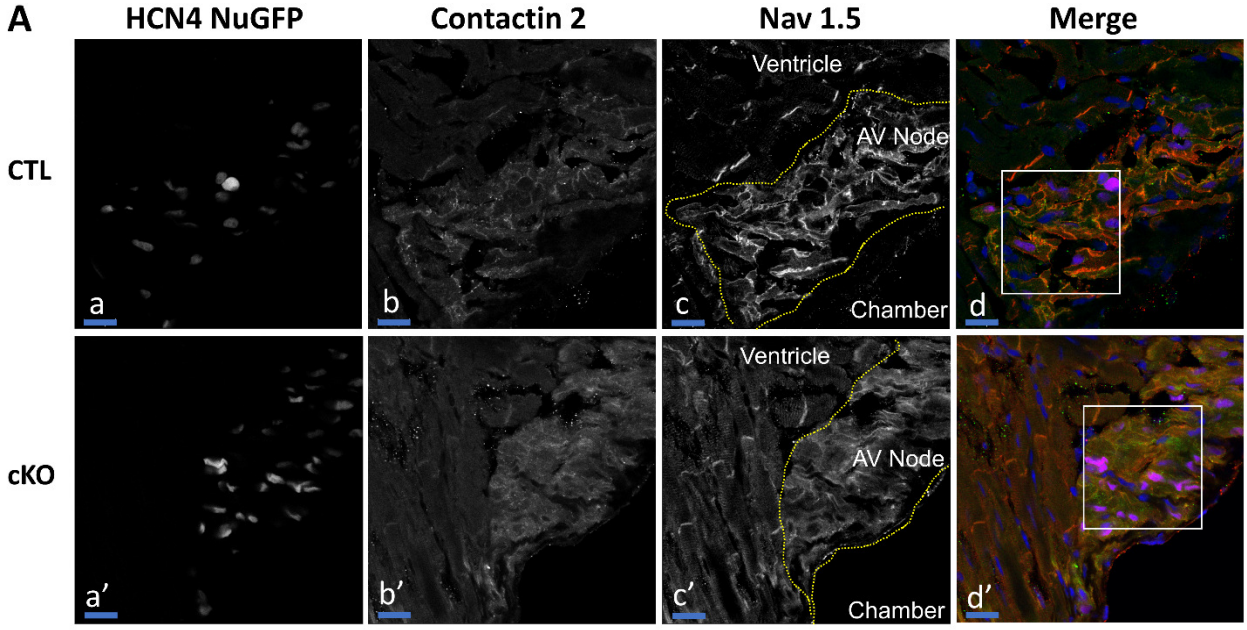
Online Figure VIII. Immunostaining of LV tissues from CTL and ZO-1cKO heart show no significant changes in Y-catenin and desmoplakin staining pattern in ZO-1cKO vs CTL. Similar expression patterns of ZO-1 associated proteins Y-catenin and desmoplakin (DSP) is detected in ventricular tissue evaluated from ZO-1cKO and CTL groups. (In 'Merge' figure panels (both original and magnified version from boxed area) : Nuclear DSP=Green, Y-Cat=Red, Nuclei DAPI=Blue, Bar 20uM in a-c and a'-c', 5uM in d and d').

Online Figure IX



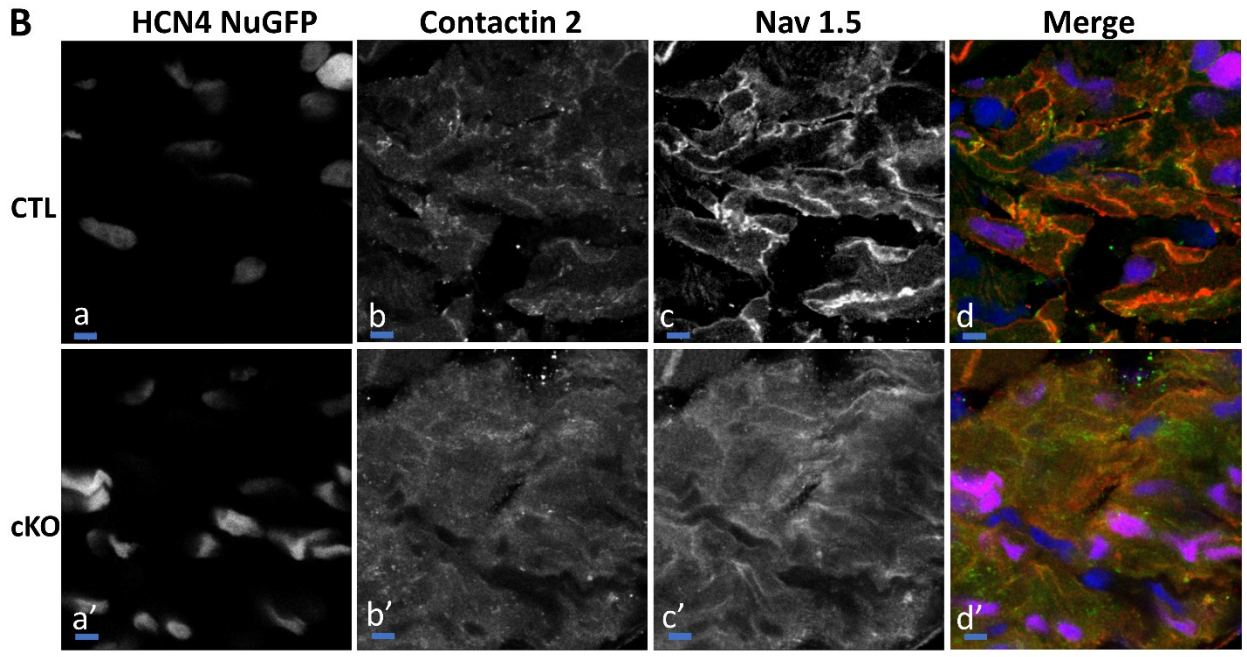
Online Figure IX, CAR expression detected in the different chambers and AV Node. (A, B, C) CAR protein was reduced in atrial specimens from cKO vs. CTL detected by Western blot and immunostaining, but not in ventricular tissue or cells. Normality was evaluated using the Shapiro-Wilk statistical test. Data were represented as mean ± SD. Unpaired two-tailed Student's *t*-test with Welch correction was used to assess the p value by groups. (n=3 in each group) (**D**) No significant qualitative difference in CAR expression was seen in AV nodal tissue marked by HCN4 NuGFP staining. (In 'Merge' figure panel: Nuclear HCN4-GFP=pink, N-Cad=Green, CAR=Red, Nuclei DAPI=Blue, Bar 20uM in B and C, 5uM in D).

Online Figure X



HCN4 GFP, purke; Contactin 2, green; Nav 1.5, Red; DAPI (Nuclear), Blue; Bar = 20uM

NB: Box in panel A, d and d' are enlarged and shown in Panel B.



HCN4 GFP, purke; Contactin2, green; Nav 1.5, Red; DAPI (Nuclear), Blue; Bar = 5uM

Online Figure X, Expression of Nav 1.5 was altered in ZO-1cKO samples compared to CTL. Loss of ZO-1 from AV nodal cells caused qualitative alteration in the expression of Nav1.5. **Panels in (A) are shown enlarged in (B).** AV node was marked by HCN4 NuGFP and also contactin-2. In 'Merge' figure panels (Ad, Ad' and Bd, Bd') HCN4-nuGFP = pink, Contactin-2 = Green, Nav1.5 = Red, Nuclei are marked by DAPI = Blue.