Online Data Supplement

Voltage-gated sodium channels are required for heart development in zebrafish

Sameer S. Chopra, Dina Myers Stroud, Hiroshi Watanabe, Jeffrey S. Bennett, C. Geoffrey Burns , K. Sam Wells, Tao Yang, Tao P. Zhong, Dan M. Roden

Full Materials and Methods

Zebrafish strains: The zebrafish strains used in this study were raised according to standard procedures. Morphant phenotypes were generated in wild-type AB and TuAB strains as well as in *Tg(cmlc2:GFP)* and *Tg(cmlc2:DsRed2-nuc)* transgenic lines that have been previously described.[1, 2]

Heterologous expression and electrophysiology: Cultured Chinese Hamster Ovary (CHO) cells were transiently transfected with either pBK-CMV-*scn5Laa* or pBK-CMV-*scn5Lab* constructs using FuGENE6 (Roche). Cells were grown for 48 hours after transfection prior to electrophysiologic study. Whole-cell voltage clamp was performed at room temperature with 2-MΩ patch microelectrodes and an Axopatch 200A amplifier. To minimize the capacitive transients, we compensated for approximately 70% to 80% of the cell capacitance and series resistance.[3] Cells exhibiting very large currents (>6nA) were also excluded from further analysis. Cells were also excluded if voltage control was compromised. The extracellular bath solution contained (in mmol/L) NaCl 145, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 1.8, glucose 10, and HEPES 10; the pH was 7.4, adjusted with NaOH. The pipette (intracellular) solution contained (in mmol/L) NaF 10, CsF 110, CsCl 20, EGTA 10, and HEPES 10; the pH was 7.4, adjusted with CsOH. Cells were held at -120 mV, and activating currents were elicited with depolarizing pulses from -100 to +50 mV in 10 mV increments. Specific clamp protocols are indicated as insets within the figures. Data were acquired by pClamp8.0 (Axon Instruments Inc), sampled at 50 kHz, and low-pass filtered at 5 kHz. All currents were normalized to the cell capacitance calculated by Membrane Test (OUT O) in pClamp8.0.

Identification, cloning of *scn5Lab*: At the outset of this work, a putative homolog to mammalian *scn8a* was the only zebrafish voltage-gated sodium channel sequence available in GenBank (NM_131628). To identify additional zebrafish sodium channel genes, BLAST searches of the draft zebrafish genome cDNA database (ensembl Zv2, Zv3; 2003) were performed using full-length cDNA sequences and individual exons of mammalian voltage-gated sodium channel genes. This search yielded >60 incomplete, predicted zebrafish sodium channel cDNA sequences. To identify a putative zebrafish homolog to *SCN5A*, forward and reverse primers were designed against the 15 most conserved predicted sequences and PCR was performed using a zebrafish adult heart cDNA library (Stratagene) as template. Primers directed against only one partial predicted cDNA, whose deduced translation product aligned with 75% identity and 84% similarity to amino acids 1445-2002 of human SCN5A, consistently amplified products of the anticipated size and sequence from the adult zebrafish heart library. To clone the full-length sequence of this gene, reverse primers were generated from the partial 3' clone and used in combination with forward primers directed against vector-specific promoter sites to amplify 3 additional, overlapping sections of the sodium channel cDNA from two

different adult zebrafish cDNA libraries (heart cDNA, total cDNA, Stratagene). 5' and 3' RLM-RACE (Ambion) were then used to extend 5' and 3' untranslated regions (UTRs) and complete the preliminary full-length cDNA sequence. All amplicons were subcloned directly into the pGEM-Teasy vector (Invitrogen), transformed into DH5α competent cells, and sequenced in their entirety in forward and reverse directions. The preliminary, full-length *zscn5a* cDNA sequence was confirmed by using new primer pairs to amplify the entire channel in 6 overlapping sections (A through F) from total embryonic RNA isolated at 2 days post-fertilization (dpf) using one-step Titan RT-PCR (Roche). The PCR primers successfully used to amplify these sections are as follows:

Section	Nest*	Forward (5'-3')	Reverse (5'-3')	Length (bp)
A	Outer	CGAAGTTTCACATCTGTGAA	GATGAAAGTGCTGTTGTTGT	950
A	Inner	TCGGACAGAGGCTCAGGATG	TGTCGGTAAGGTGGTGTTGA	901
B	Outer	AGTACACTTTCACTGGCATT	AGAAGAACTGTGAGCTGTGG	1269
B	Inner	TACACGTTCGAGTCGCTGAT	TCTGAGCACTGGTGCGTCTG	1219
C	Outer	AGACAAACCGGCCAAACTGG	ATCACGCCGGCTGTCCCATT	1557
C	Inner	ACCCCATAGAGGGCATTAAGCAA	CGCTTCAGTTTCAGCATGGC	1486
D	Outer	CATGTGGGATTGTATGGAAG	ATAGATGAAACCCGTCCGGT	1264
D	Inner	ACAGCCGCTCTGCATCCTGGTGTT	CGGCCGAACTTTCCAGCGAA	1209
E	Outer	CTTCATTCTGGAGATGAGCT	ATCATCAATGCAAACAGGAG	1200
E	Inner	AAGTGGATCGCCTACGGTTT	CGGATCACGCGGAAGAGAGT	1100
F	Outer	AGAGCAGAAGAAATATTACAACGC	AACACGTTTCCTCAAGAGAA	1469
F	Inner	CATGAAGAAACTGGGCTCCA	CGTCACAGAAAAGTTTCGCT	1377

*****Although nesting was not frequently necessary, primers were initially designed for this purpose.

The final *zscn5a* cDNA consensus sequence was determined after aligning the forward and reverse sequences of 8-10 clones of each of the 6 overlapping channel sections and was later deposited in GenBank (DQ837300). Subsequently, a detailed phylogenetic analysis of zebrafish sodium channel genes published by others referred to our cloned channel sequence as "*scn5Lab*" for "*scn5a-like gene b".*[4] We have therefore adopted this nomenclature.

Zebrafish sodium channel gene expression screen: Mammalian hearts express multiple sodium channel genes.[5-8] In order to confirm the phylogenetic identify of our clone and identify additional cardiac sodium channel sequences, we developed a screen for all conserved zebrafish voltage-gated sodium channel genes that used *in silico* approaches (updated versions of the draft zebrafish genome sequence, ensembl Zv4, Zv5) in parallel with molecular biology (Online Figure I). This strategy took advantage of the unique genomic structure of cloned mammalian sodium channel genes, which are distinguished by large terminal exons ("TE", >1000 nucleotides) that encode nearly all of domain IV of the protein and preceding exons ("TE-1," exactly 271 nucleotides long in every sodium channel gene) that encode part of the highly-

conserved inactivation gate. These 3' sequences distinguish each individual mammalian sodium channel gene from other isoforms and from closely-related genes in the ion channel superfamily such as L- and T-type voltage-gated calcium channels. BLAST searches of the zebrafish genome using the TE or TE-1 of mammalian sodium channel genes identified 8 non-redundant, predicted zebrafish sodium channel gene sequences. Putative gene identities were assigned based on alignment with the nucleotide and amino acid sequences of equivalent regions of human sodium channel genes and by phylogeny. The identified sequences represented putative *in silico* zebrafish sodium channel "sequence tags." Chromosome assignment was based on the reported map positions of the relevant contigs within the draft genome sequence. *In silico* zebrafish sodium channel sequence tags were validated using RT-PCR and used to screen different adult zebrafish tissues. For RT-PCR, tissues were freshly dissected from wild type adult zebrafish (strain TuAB, 12-16 months old) and flash frozen on dry ice in ethanol. Tissue-specific total RNA was isolated RNA using Trizol (GIBCO), digested with RQ1 DNase (Promega), and purified with the RNeasy Mini Kit (Qiagen). First strand cDNA synthesis was performed using 1µg of RNA from each tissue, random hexamer primers, and Transcriptor reverse transcriptase enzyme (Roche). 2µl of first strand cDNA was used in 25µl PCR reactions with Expand High Fidelity DNA polymerase (Roche). Forward and reverse primers were in different exons (TE, TE-1) to prevent amplification of genomic DNA. Amplicons were analyzed on a 1-2% agarose gel made with 1x Tris-Acetate-EDTA (TAE) buffer. All amplicons were subcloned directly into the pGEM-TEasy vector (Promega) and sequenced in their entirety in both sense and antisense directions. The gene-specific primers used in this screen were as follows:

Gene	Set	Forward (5'-3')	Reverse (5'-3')	Length (bp)
zscn1	1	GAGACAGAGAACATCTTGTATTGG	AGTTTGTTGTTGGCAGGCCT	1133
zscn1	2	TCAGACGAGACAGAGAACAT	TTAACTGTGGTTCCTGGGTT	560
zscn1	3, nest[†]	AAACTTCGTATTCATCGTGGCC	GTTTGCAGATGAAGCCCCTG	1085
zscn2	1	GACAGAGGATATGGACAATA	CCTTCTTAACTGTTTGTCTT	1118
zscn2	2	GACAGAGGATATGGACAATA	AATCTGGATCTTGCTTGTTC	527
zscn3	1	AGTGCTGAGAAAGAATATGTC	TTGAGAAGATGCCTACGGTA	1100
zscn3	2	AGTGCTGAGAAAGAATATGTC	GAGGGCCACTGTTTAGAATA	520
zscn4	1	CAATCACAGGAGACGGAAAA	ATGAAGCCTTTCCGAATCAG	1112
zscn4	2	ATCACAGGAGACGGAAAACA	GGATTTTCTTTAGTTGGCTC	546
zscn5	1	TGCTGAGATAGAGGAAATATTG	CTTCTCCGGTTGGTTTCTTT	1163
zscn5	2	AAGTGCTGAGATAGAGGAAA	GTAGTTGAACCTGGGTTATC	558
zscn6[‡]	1	AATCTGCTCGCATGGAGACC	CCAGGGCGTCTCTTGGTTAA	1160
zscn6	2	ATCCTCAATAACATCAACTTGG	CCACAGTTGCCCTTAGTATT	505
zscn7[‡]	1	CGCCCTCCATCAAACACATC	TTTATCAGCGTCCGGCATGG	1160

zscn7	2	GCCCTCCATCAAACACATCC	GCCAGTGTGGGGAATTTCGG	550
zscn8	1	ATTGAAATGACCCGTGCGCT	CCCATTCTGATGCTTGTTTT	1152
zscn8	2	GATTGAAATGACCCGTGCGC	GCCTACGACCGAACTTCCAG	565
zscn8	3, nest[†]	GTACTGGATCAATGTCATTTTC	TGCGAACTTTCTTCATGCTA	1100

[†]As indicated for *zscn1* and *zscn8*, additional nested primers were designed to facilitate subcloning because of low gene expression levels and/or low PCR amplification efficiency. For other genes, the following nested primer combinations were used as necessary: *zscn2* (1F/1R, 2F/2R); *zscn3* (1F/1R, 2F/2R); *zscn4* (1F/1R, 2F/2R); *zscn5* (1F/1R, 2F/1R); *zscn6* (1F/1R, 2F/2R); *zscn7* (1F/1R, 2F/2R).

[‡]*zscn6* = *scn5Laa* and *zscn7* = *scn5Lab*.

Identification of *scn5Laa*: Our screen for conserved sodium channel genes identified 2 genes homologous to mammalian *scn5a* (Online Figure I), one of which we previously cloned (*scn5Lab*) after detecting its expression in the adult zebrafish heart. Although we did not detect expression of the second *scn5a* homolog (*scn5Laa*) in adult zebrafish cardiac tissue, its expression was demonstrated in the embryonic myocardium by RT-PCR.[9] We confirmed these findings using similar approaches (RT-PCR with *scn5Laa*-specific primers and cardiac RNA template isolated from embryos at 54 and 76hpf) and thus incorporated this gene into our study. We also independently confirmed the full-length sequence of *scn5Laa* by amplifying this gene from total zebrafish embryonic RNA in several overlapping sections using the following primer sets:

Section	**Forward (5'-3')**	**Reverse (5'-3')**	**Length (bp)**
A-1	CTTTGAGTAACTTCTAGTACAGG	GATGGTAAAGGCTTTCCCAG	1184
A-2	ACACAAGCTTTGACACTTTT	TAACGTGTTGAGAACTATGC	1100
A-rc*	GCCGATCAGGATGAAAAGAT	CGTGTTGAGAACTATGCAGA	2175
B	TCAGCTTCTTCAGTGACGCC	TCCTCAAGAAATTCCACGTC	1150
B	ATGTTTTTCCAGAAACCGAG	CCGATGAACAAATTCAGGGT	1150
B-rc*	TGGAAGAGGCCCGTCAGAAGT	CGGGAATCAACGGCGGCATA	2065
C	CAATAGGACAGGATTCATCC	TCGCAAAACTGGAGCTACAC	1100
C	GTTGGAATAACCTGCTGGAT	CACATCCAAATGTGGTTAAA	962
C-rc*	GGACTCGGATACCTCTCACT	CCTAAAGAAAGGTCTCTTCG	1825

*A-rc, B-rc, and C-rc are nested primers that were used to recombine smaller sections and amplify large enough quantities of DNA for routine subcloning and sequencing. For example, A-rc primers successfully amplified the A-1/A-2 recombination product when A-1 and A-2 were incorporated as templates in a single reaction mix.

Amplification, subcloning, and sequencing were performed as described above for *scn5Lab*.

Gene Expression: Total RNA was isolated and purified from whole embryos at the specified timepoints using the TRIzol reagent (Invitrogen). First strand cDNA synthesis was performed using 3-4µg of total RNA, random hexamer primers, and Transcriptor reverse transcriptase enzyme (Roche). 2µl of first strand cDNA was used in 25µl PCR reactions with Expand High Fidelity DNA polymerase (Roche). Amplicons were analyzed on a 1-2% agarose gel made with 1x Tris-Acetate-EDTA (TAE) buffer.

***In situ* hybridization**: Whole zebrafish embryos were fixed in 4% paraformaldehyde in PBS overnight at 4ºC. *In situ* hybridization was performed under standard conditions using digoxigenin-labeled probes as previously reported.[10] Antisense probes against the following zebrafish genes were used: *nkx2.5* (NM_131421), *gata4* (NM_131236), *gata5* (NM_131235), *dHand* (NM_131626), *cmlc2* (NM_131692), *vmhc* (AF114427), and *amhc* (NM_198823). For *scn5Laa* and *scn5Lab*, probes were obtained from the Ribera laboratory[9] and extended hybridization times were used to enhance signal. Wash times were prolonged prior to staining to preserve specificity. Embryonic hearts were microdissected at the relevant stages to confirm expression via RT-PCR (brain samples were not analyzed). Images of stained embryos were captured using either a Zeiss Axioplan 2 or Leica dissecting microscope equipped with an Axiocam CCD camera.

Design and use of antisense morpholino oligonucleotides: Morpholinos were designed to inhibit mRNA translation or perturb pre-mRNA splicing. The sequences of morpholino oligonucleotides used in this study appear in Online Table I. Optimal splice site targets were identified by examining pre-mRNA sequences for exons/introns predicted to induce translation frameshift and premature protein termination when skipped/retained. The specificity of all morpholino oligonucleotides targeting *scn5Laa* and *scn5Lab* was confirmed by alignment of the relevant sequences and quantification of the number of mismatches at the homologous gene (Online Figure V, Online Table I). Morpholino target specificity was also investigated by BLAST queries of the zebrafish genome. 5-mispair control morpholinos were used as an additional test of the specificity of active morpholinos, and the Gene Tools "standard control" morpholino was used as an injection control for morpholinos lacking corresponding 5-mispair controls. Identical stock and working concentrations of both active and control morpholinos were prepared in Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES) or water. Solutions were injected into 1-2 cell stage wild-type zebrafish embryos at a consistent volume calculated from the direct measurement of the injectate diameter under a stereomicroscope. The efficacy of splice-blocking morpholinos was confirmed by RT-PCR amplification using primers flanking the target splice junction (Online Figure VI). Primers used for *scn5Lab* splice morpholinos included: E6I6 MO (fwd) 5'-TTCCAGAACCGCAGAACTTT-3' and (rev) 5'-GAGGCCCAGCTTCTGTTATT-3' (product = 668bp) and E25I25 (fwd) 5'-GTGAACCGGACGGGTTTCATCTATAATTCC-3' and (rev) 5'-AACAGGAGCGTCCGGATGCCTTTA-3' (product = 845bp). Total RNA from morphant, control MO injected, and WT embryos was extracted using the Trizol reagent (Invitrogen). 4µg of total RNA was used for first strand cDNA synthesis with the Transcriptor reverse transcriptase enzyme (Roche), and 2µl of cDNA was subsequently used as template for PCR (25µl reaction volume) using Expand Hi-Fidelity polymerase (Roche).

Cell death assay: Acridine orange staining was used to visualize cell death in live embryos. Embryos at the desired stage were incubated for 30 minutes in 10µg/mL acridine orange (Sigma) made from a 100-fold dilution of 1mg/mL stock solution in E3 embryo medium. Acridine orange-labeled embryos were visualized with fluorescence microscopy.

Confocal imaging and quantification of cardiomyocyte number: For high-resolution confocal imaging, tricaine-anesthetized embryos were stabilized ventral side down with 1% low melt agarose (BioRad) in a glass-bottom culture dish (MatTek). Images of the hearts of live *Tg(cmlc2:GFP)* embryos were captured using a Zeiss LSM 510 Confocal Microscope System equipped with either 20x or 40x (oil immersion) objective lenses. Three dimensional projections of image z-stacks were rendered in LSM Browser software (Zeiss). For ascertainment of total cardiomyocyte number, *Tg(cmlc2:DsRed2-nuc)* embryos were either gently flat-mounted between two coverslips (embryos at 2dpf) or whole-mounted in 1% agarose in a glass-bottom culture dish (embryos at 3dpf) as described above. Images captured in the z-dimension extended from the ventral to the dorsal aspect of each heart. Total embryonic cell number was quantified using the Pointpicker function of Image J, which permits a single user-defined mark to appear through multiple images in a z-stack. This allowed the tracking of a single cell in subsequent z sections without double counting.

Quantitative PCR: Transcript levels of zebrafish *nkx2.5* (NM_131421), *gata5* (NM_131235), and *β-actin* (NM_131031) were measured using SYBR green dye (Applied Biosystems) and the following primers: *nkx2.5* (fwd) 5'-CTTCAGTGCTTCAGGCTTTTACGCG-3' and (rev) 5'-GCTCCGCATCATCCAGCTTCAGATC-3' (cDNA product = 155bp, genomic DNA product = 1333bp); *gata5* (fwd) 5'-CCGGAGATGGCAACCTCATGGA-3' and (rev) 5'-ATTGAGCCGCAGTTCACACACTCG-3' (cDNA product = 155bp, genomic DNA product = 2069bp); *β-actin* (fwd) 5'-CTCCCCTTGTTCACAATAACCTACTAATACACAGC-3' and (rev) 5'-TTCTGTCCCATGCCAACCATCACTC-3' (cDNA product = 185bp, genomic DNA product = 1552bp). Data were collected on an ABI 7900HT real-time PCR instrument, quantified by the standard curve method, and normalized to zebrafish β-actin levels. No RT and water templates did not yield signal. Real-time PCR primers for SYBR green assays spanned introns, amplified single bands of the appropriate size as visualized by gel electrophoresis, and did not form primer dimers as analyzed by the first derivative of the product melting curve. Standard curves were generated from serial dilutions of plasmids containing each of the respective targets.

Action potential recordings: The intact, beating heart was exposed in 1 year old TuAB strain zebrafish anesthetized on ice and fixed into a tissue chamber perfused with 95% O_2/5% CO_2 and Tyrode's solution (120mM NaCl, 4mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 20mM $NaHCO_3$, 1.2mM NaH_2PO_4, 10mM glucose, 5mM sucrose, pH 7.35 with NaOH) warmed to 37 degrees C. Action potentials were recorded from ventricular muscle using a conventional 3M KCl-filling microelectrode technique. Data were acquired using AcqKnowledge 3.7.3 software (Biopac System, Santa Barbara, CA). Ventricular action potentials were recorded prior to and after perfusion with 50nM TTX prepared in Tyrode's solution.

Drug and toxin studies: The drugs/toxins, doses, and delivery methods used in this study are summarized in both Table 1 and Online Table III. The sodium channel modulators tetrodotoxin (TTX), flecainide, lidocaine, anemone toxin II (ATX II), and veratridine, the L- and T-type calcium channel blocker mibefradil, and the potassium channel blocker E-4031 were all obtained from Sigma-Aldrich. The L-type calcium channel blocker nisoldipine was obtained from Bayer. ATX II working solutions were prepared from 100μm stock solution in water. Veratridine working solutions were prepared from 10mM stock solution in ethanol. TTX working solutions were prepared from 3.13mM (1mg/mL) stock solutions in water. Since TTX is soluble in a weak acid buffer, TTX powder was supplied with 1mg of tetrodotoxin and approximately 5mg of citrate buffer, pH 4.8, in the same vial. When obtained without citrate, TTX powder was dissolved in

dilute citrate buffer, pH 5.0. Nisoldipine working solutions were prepared from 1mM stock solution in ethanol, and mibefradil working solutions were prepared from 1mM stock solution in water. Flecainide acetate and lidocaine hydrochloride working solutions were prepared from 10mM and 5mM stock solutions in water, respectively. E-4031 working solutions were prepared from 10mM stock solution in water. Working solutions were prepared in embryo medium (for bath exposure experiments) or in water or a physiological buffer such as Tyrode's solution (for pericardial microinjections).

As indicated in Table 1 and Online Table III, the pH of working solutions of compounds that are weak bases (e.g. lidocaine) were adjusted with 1N NaOH to values that were close to or above the pKA to facilitate absorption. Embryo medium with pH values up to 10.0 were observed not to affect development or embryonic heart function. Control solutions contained no active drug but were equivalent in pH and contained equivalent amounts of solvent (dilute citrate buffer, ethanol) as the active drug solution. Similarly, no developmental or functional differences were observed when embryos were exposed to dilute citrate buffer solvent or dilute ethanol compared to embryo medium (for bath exposure) or water (for microinjection).

For experiments designed to test whether modulators of sodium channel function could affect cardiac lineage specification or differentiation, *Tg(cmlc2:GFP)* embryos were reared in or injected with solutions of ion channel-modulating drugs as presented in Table I. Embryos were then fixed at the 6-8 somite stage in 4% paraformaldehyde overnight and processed for *in situ* hybridization with antisense probes directed against gene *nkx2.5* or allowed to develop until 26-28 hours post-fertilization (hpf) when the developing heart tube was examined by fluorescence microscopy. Embryos were also commonly left in drug solution through 60hpf or longer to evaluate the functional and morphological consequences of ion channel blockade at later stages. To determine whether L-type calcium channel blockade could influence cardiac morphogenesis or cell number, blastula-stage embryos were placed in 10μM nisoldipine solution and reared in darkness at 28.5ºC until the desired timepoints. *Tg(cmlc2:GFP)* embryos were used to examine cardiac morphogenesis at the 30 somite stage, at 60hpf and at 78hpf. To quantify cell number, *Tg(cmlc2:DsRed2-nuc)* embryos were reared to 78hpf in nisoldipine and then analyzed by confocal microscopy. The hearts of embryos reared in 10μM nisoldipine solution never contracted at any stage of development. Although nisoldipine-treated embryos grew relatively normally through 80hpf, they displayed reduced head size and died prior to 96hpf.

For experiments designed to investigate the role of sodium channels in embryonic heart rhythm (Online Table III), TTX, ATX II, nisoldipine or control solutions were pressure injected into the trunk circulation (26-28hpf), sinus venosus (48-54hpf), or directly into the pericardial space (72-78hpf and all later stages). Live embryos were first anesthetized with the minimum dose of tricaine that permitted stabilization of embryos on their lateral aspect in methylcellulose but caused no detectable effects on heart rate, rhythm or contractility. Injections were performed with sharp-tipped, borosilicate micropipettes inserted directly from the caudal aspect into the sinus venous or the pericardial space.

Image processing and statistics: To improve clarity, photographs of embryos were adjusted for brightness and/or contrast in Adobe Photoshop and/or Microsoft Powerpoint. Identical adjustments were made for active treatment groups and controls. Statistical analyses were performed as indicated in the text and figure legends.

References

1. Burns CG, Milan DJ, Grande EJ, Rottbauer W, Macrae CA, Fishman MC. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nat Chem Biol*. 2005;1:263-4.

2. Mably JD, Mohideen MA, Burns CG, Chen JN, Fishman MC. heart of glass regulates the concentric growth of the heart in zebrafish. *Curr Biol*. 2003;13:2138-47.

3. Darbar D, Yang T, Churchwell K, Wilde AA, Roden DM. Unmasking of brugada syndrome by lithium. *Circulation*. 2005;112:1527-31.

4. Novak AE, Jost MC, Lu Y, Taylor AD, Zakon HH, Ribera AB. Gene duplications and evolution of vertebrate voltage-gated sodium channels. *J Mol Evol*. 2006;63:208-21.

5. Maier SK, Westenbroek RE, McCormick KA, Curtis R, Scheuer T, Catterall WA. Distinct subcellular localization of different sodium channel alpha and beta subunits in single ventricular myocytes from mouse heart. *Circulation*. 2004;109:1421-7.

6. Maier SK, Westenbroek RE, Yamanushi TT, Dobrzynski H, Boyett MR, Catterall WA, Scheuer T. An unexpected requirement for brain-type sodium channels for control of heart rate in the mouse sinoatrial node. *Proc Natl Acad Sci U S A*. 2003;100:3507-12.

7. Zimmer T, Bollensdorff C, Haufe V, Birch-Hirschfeld E, Benndorf K. Mouse heart Na+ channels: primary structure and function of two isoforms and alternatively spliced variants. *Am J Physiol Heart Circ Physiol*. 2002;282:H1007-H1017.

8. Maier SK, Westenbroek RE, Schenkman KA, Feigl EO, Scheuer T, Catterall WA. An unexpected role for brain-type sodium channels in coupling of cell surface depolarization to contraction in the heart. *Proc Natl Acad Sci U S A*. 2002;99:4073-8.

9. Novak AE, Taylor AD, Pineda RH, Lasda EL, Wright MA, Ribera AB. Embryonic and larval expression of zebrafish voltage-gated sodium channel alpha-subunit genes. *Dev Dyn*. 2006;235:1962-73.

10. Jia H, King IN, Chopra SS, Wan H, Ni TT, Jiang C, Guan X, Wells S, Srivastava D, Zhong TP. Vertebrate heart growth is regulated by functional antagonism between Gridlock and Gata5. *Proc Natl Acad Sci U S A*. 2007;104:14008-13.

Online Table I: Morpholino antisense oligonucleotides used in this study.

Target Gene	Morpholino name	Morpholino type	Target location	Sequence (5'-3')	Mismatch nucleotides - *scn5Laa*	Mismatch nucleotides - *scn5Lab*	Dose injected (ng)
scn5Laa	AA_MO1	translation blocking	ATG translation initiation site	CCGCTGGTAAGAGCAT GGTCGCCAT	0	6	6
scn5Laa	AA_MIS1	5-mismatch control	ATG translation initiation site	CC**c**CTG**c**TAAGA**c**CAT G**c**TCGC**g**AT	5	11	6
scn5Laa	AA_MO2	translation blocking	5' untranslated sequence	CTTTTCATCCTGATCG GCCTGTACT	0	11	5
scn5Lab	AB_MO1	splice blocking	exon 6-intron 6 splice donor site	AGCGGCCTCTCACCT GGGATGACTG	8	0	4
scn5Lab	AB_MIS1	5-mismatch control	exon 6-intron 6 splice donor site	A**c**CG**c**CCTCT**g**ACCTG **c**GATGA**g**TG	12	5	4
scn5Lab	AB_MO2	splice blocking	exon 25-intron 25 splice donor site	AGTTTGTGCTGACCGG TGGTCTGGG	10	0	1.5
zerg	zerg_MO	translation blocking	5' untranslated sequence	GACATGTCCGCGGCG CACGGGCAT	n/a	n/a	up to 14.5
p53	p53_MO	translation blocking	ATG translation initiation site	GCGCCATTGCTTTGCA AGAATTG	n/a	n/a	2-6
control	STD	standard control morpholino	n/a	CCTCTTACCTCAGTTA CAATTTATA	25	25	2-8

Online Table II: Summary of antisense-induced cardiac developmental defects observed at 60-72 h.p.f. (%, n)*.

Group	Target Gene(s)	Morpholino(s)	Chamber Morphogenesis	Looping	Bradycardia	Circulation
1	-	-	0.4% (232)	1.3% (232)	1.0% (209)	3.8% (132)
2	-	standard control	2.9% (138)	7.2% (138)	5.7% (87)	9.0% (145)
3	*scn5Laa*	AA_MO1	84.7% (215)	92.6% (215)	95.6% (113)	n/a
4	*scn5Laa, p53*	AA_MO1, p53_MO	94.2% (294)	96.0% (294)	96.0% (294)	71.0% (255)
5	*scn5Laa*	AA_MIS1	1.1% (184)	3.3% (184)	2.2% (91)	0% (41)
6	*scn5Laa, p53*	AA_MIS1, p53_MO	0.6% (170)	1.8% (170)	1.8% (170)	0.0% (145)
7	*scn5Laa, p53*	AA_MO2, p53_MO	57.7% (430)	80.9% (430)	70.0% (430)	66.7% (204)
8	*scn5Lab, p53*	AB_MO1, p53_MO	83.0% (112)	90.2% (112)	90.2% (112)	55.0% (109)
9	*scn5Lab*	AB_MIS1	0% (69)	0% (69)	0% (69)	0% (69)
10	*scn5Lab, p53*	AB_MIS1, p53_MO	7.7% (221)	7.7% (221)	7.7% (221)	0.0% (143)
11	*scn5Lab*	AB_MO2	78.0% (123)	97.6% (123)	98.1% (108)	93.9% (246)
12	*scn5Lab, p53*	AB_MO2, p53_MO	88.0% (150)	96.0% (150)	96.0% (150)	90.1% (121)
13	*scn5Laa, scn5Lab, p53*	AA_MO1, AB_MO2, p53_MO	92.9% (255)	94.5% (255)	92.9% (255)	92.4% (144)
14	*scn5Laa, scn5Lab, p53*	AA_MIS1, AB_MIS1, p53_MO	0.0% (51)	0.0% (51)	0.0% (51)	0.0% (51)
15	*scn5Laa, scn5Lab, p53*	AA_MIS1, STD_MO, p53_MO	2.5% (157)	36.3% (157)[†]	2.5% (157)	2.5% (157)
16	*zerg*	zerg_MO	4.8% (146)	8.9% (146)	100% (141)[‡]	16.1% (31)

* percent shown is percentage of embryos displaying an abnormal phenotype from the total number (n) of embryos injected shown below in parentheses. Defects in chamber morphogenesis indicate abnormalities in shape and/or size of the atrium and ventricle. Looping defects referred to aberrant positioning of the atrium relative to the ventricle. Percent of bradycardic embryos was qualitatively determined by individual comparison to wild-type embryos. Lack of circulation was directly observed due to the transparent nature of the embryo.

† Unlike the permanent looping *defect* observed in group 13 (active treatment), approximately 1/3 embryos in group 15 (controls) exhibited a looping *delay*. In embryos with looping delay, looping was normal relative to controls by 84-90hpf. In embryos scored as having looping defects, no normal looping pattern was ever observed.

‡ The predominant phenotype following *zerg* knockdown was bradycardia with 2:1, 3:1 or complete atrioventricular conduction block.

Online Table III: Effects of acute exposure to pharmacological modulators of ion channel function at different developmental stages.

stage	heart morphology	agent	target	delivery	[drug]	n	rhythm phenotypes
Day 1 (26-32 h.p.f.)	simple beating heart tube	tetrodotoxin (TTX)	VGSC[1]	embryo medium[2]	150µm	51	▪ HR[3], pre: 80.5±10.6 ▪ HR, 30' post: 84.1±10.5
					500µm (pH 8.0)	25	▪ HR, pre: 96.4±9.6 ▪ HR, 60' post: 89.3±7.0
		dilute citrate buffer[4]	n/a	embryo medium	n/a	38	▪ HR, pre: 90.0±9.8 ▪ HR, 30' post: 87.1±8.3
		flecainide	VGSC	embryo medium	150µm	56	▪ HR, pre: 95.9±6.3 ▪ HR, 30' post: 94.2±7.7
		veratridine	VGSC	embryo medium	150µm	59	▪ HR, pre: 99.4±6.2 ▪ HR, 30' post: 102.7±6.1
		anemone toxin II (ATX II)	VGSC	embryo medium	20µm	29	▪ HR, pre: 88.9±10.8 ▪ HR, 30' post: 86.9±14.9
		nisoldipine	LTCC[1]	embryo medium	50µm	53	▪ HR, pre: 70.1±8.2 ▪ HR, 30' post: 0±0 (asystole)
32-36 h.p.f.	simple beating heart tube	tetrodotoxin	VGSC	embryo medium	500µm (pH 8.5)	18	▪ HR, pre:115.3±9.0 ▪ HR, 30' post:126.2±8.8
		dilute citrate buffer[4]	n/a	embryo medium	pH 8.5	17	▪ HR, pre:117.1±7.1 ▪ HR, 30' post:116.1±5.4
		flecainide	VGSC	embryo medium	500µm (pH 8.5)	15	▪ HR, pre:121.2±12.9 ▪ HR, 30' post:125.5±8.1
Day 2 (54-60 h.p.f.)	two-chambered heart	TTX	VGSC	embryo medium	500µm (pH 9.8)	49[5]	▪ EM, HR 15' post: 115.3±12.3 ▪ TTX, HR 15' post: 112.2±12.5 ▪ EM, HR 30' post: 117.5±10.1 ▪ TTX, HR 30' post: 111.2±13.5
		nisoldipine	LTCC	embryo medium	10µm (pH10)	15	▪ HR, pre: 188±14.5 ▪ HR, 5' post: 113.5±11.4 ▪ HR, 15' post: 0±0 (asystole)
		E-4031	ERG[1]	embryo medium	250µm (pH 7.5)	15	▪ HR, pre: 173±12 ▪ HR, 5' post: 4/15 with 2:1 AV block, 3/15 with silent ventricle. ▪ HR, 15' post: 8/15 with 2:1 AV block, 6/15 with silent ventricle, atrial rate = 143±19.
		TTX	VGSC	microinjection in sinus venosus[6]	150µm	10	▪ 2' post-injection: 3/10 embryos with bradycardia, 7/10 unperturbed. ▪ No abnormalities in 7/7 control-injected embryos.[7]
		ATX II	VGSC	microinjection in sinus venosus	100µm	22	▪ 2' post-injection: 11/22 embryos with bradycardia, 7/22 with 2:1 AV block, 1/22 with irregular ventricular rate. ▪ 6/22 embryos with hypercontracted ventricle.

		nisoldipine	LTCC	microinjection in sinus venosus	100µm	10	▪ 5’ post-injection: 7/10 in asystole, profound bradycardia in 3/10. ▪ 15’ post-injection: 5/10 in asystole, bradycardia in 5/10.
		nisoldipine	LTCC	microinjection in sinus venosus	150µm	9	▪ 2’ post-injection: 6/9 embryos with silent atria, ventricular twitching, 3/9 embryos with bradycardia.
Day 3 (78-84 h.p.f.)	two-chambered heart	TTX	VGSC	embryo medium	500µm (pH 9.8)	53[8]	▪ EM, HR, 15’ post: 138±11.3 ▪ TTX, HR, 15’ post: 129.4±14.0 ▪ EM, HR, 30’ post: 152.9±9 ▪ TTX, HR, 30’ post: 132.8±11.1
				microinjection in pericardium	150µm	5	▪ 2’ post-injection: 5/5 with bradycardia.
					100µm	12	▪ 2’ post-injection: 5/12 embryos with bradycardia. ▪ No change in heart rate in 30 control-injected embryos.
		ATX II	VGSC	microinjection in pericardium	100µm	6	2’ post-injection: 3/6 embryos with tonically contracted ventricle. ▪ 2/6 with complete AV block with hypercontracted ventricle. ▪ 1/6 with 2:1 AV block with hypercontracted ventricle. ▪ Atrial rhythm, contractility unaffected in 6/6 embryos.
Day 4 (102-108 h.p.f.)	two-chambered heart	TTX	VGSC	microinjection in pericardium	100µm	7	2’ post-injection: ▪ 3/7 embryos with AV block, irregular ventricular response rate. Arrhythmias durable for at least 30”. ▪ 2/7 embryos with bradycardia. ▪ No rate/rhythm defects in 5/5 control-injected embryos.
		ATX II	VGSC	microinjection in pericardium	100µm	10	2’ post-injection: ▪ 4/10 embryos with complete heart block, 3/10 embryos with 2:1 AV block. ▪ 1mM TTX injection rescues abnormal rhythm in 4/4 ATX II-injected embryos. ▪ 9/10 embryos with hypercontracted ventricle.
Day 7 (174-180 h.p.f.)	two-chambered heart	TTX	VGSC	microinjection in pericardium	100µm	32	2’ post-injection: ▪ 28/32 embryos with AV conduction block, irregular ventricular response rate, sinus pauses. ▪ No rate/rhythm defects in 3/3 control-injected embryos.

Notes:

1. VGSC = voltage-gated sodium channel; LTCC = L-type calcium channel; ERG = ether-a-go-go related gene (potassium channel); AV = atrioventricular.
2. To account for poor absorption of drug, TTX (highly charged molecule) and ATX II (peptide toxin) were also pressure injected directly into the trunk circulation of day 1 embryos. Shortly after acute

delivery into the circulation, 150μM TTX completely inhibited normal spontaneous movements of the embryos and their response to mechanical stimulation (Online Video V). Conversely, injection of 20μM ATX II promptly resulted in hyperkinetic movements of the embryo for approximately 15-20 seconds, followed by tonic contraction and then complete relaxation (Online Video VI). After this initial response, hyperkinetic movements were readily elicited by gentle stimulation. Despite these dramatic effects on sodium channel related physiological responses, no effect was observed on the beating of the day 1 heart tube.

3. HR = heart rate and is reported as mean ± standard deviation.
4. Control for TTX. TTX stock solution is prepared in a dilute citrate buffer, pH 5.0.
5. n = 49 control embryos evaluated at 15 and 30 minutes, 49 TTX-treated embryos evaluated at 15 minutes, and 46 TTX-treated embryos evaluated at 30 minutes.
6. The sinus venosus was injected with 3-4nL of drug solution.
7. Injections of vehicle (dilute citrate, Tyrode's solution, or water) at different stages did not affect heart function. Water pH = 8.5 injected into the pericardium resulted in full recovery of cardiac function by 1 minute.
8. n=49 control embryos evaluated at 15 minutes, 48 control embryos evaluated at 30 minutes, 53 TTX-treated embryos evaluated at 15 minutes and 54 TTX-treated embryos evaluated at 30 minutes.

Online Video Legends

Online Video I

Lateral view of the beating heart of a wild-type *Tg(cmlc2:GFP)* embryo at 100 hours post-fertilization (hpf). The atrium is on top and the ventricle is on the bottom (Quicktime; 9.9 MB).

Online Video II

Lateral view of the beating heart of the same wild-type *Tg(cmlc2:GFP)* embryo at 100hpf after microinjection of 500μM TTX directly into the pericardial sac. The atrium is on top and the ventricle is on the bottom. Injection of TTX but not vehicle induced atrioventricular conduction block (Quicktime; 9.6 MB).

Online Video III

Ventral view of the beating heart of a wild-type *Tg(cmlc2:GFP)* embryo at 82hpf. The atrium is on the left and the ventricle is on the right (Quicktime; 9.5 MB).

Online Video IV

Ventral view of the beating heart of the same wild-type *Tg(cmlc2:GFP)* embryo at 82hpf after microinjection of 100μM ATX II directly into the pericardial sac. The atrium is on the left and the ventricle is on the right. Injection of ATX II but not vehicle induced tonic ventricular contraction. Other embryos displayed atrioventricular conduction block (Quicktime; 9.9 MB).

Online Video V

A group of embryos at 32hpf following microinjection of 2 large boluses of 1mM TTX (sodium channel blocker) into the yoke sac shortly after fertilization (1-2 cell stage). Embryos are completely paralyzed and show no escape response upon probe stimulation. However, their hearts have developed normally and are beating at a rate indistinguishable from vehicle-injected clutchmates. (Quicktime; 10.0 MB).

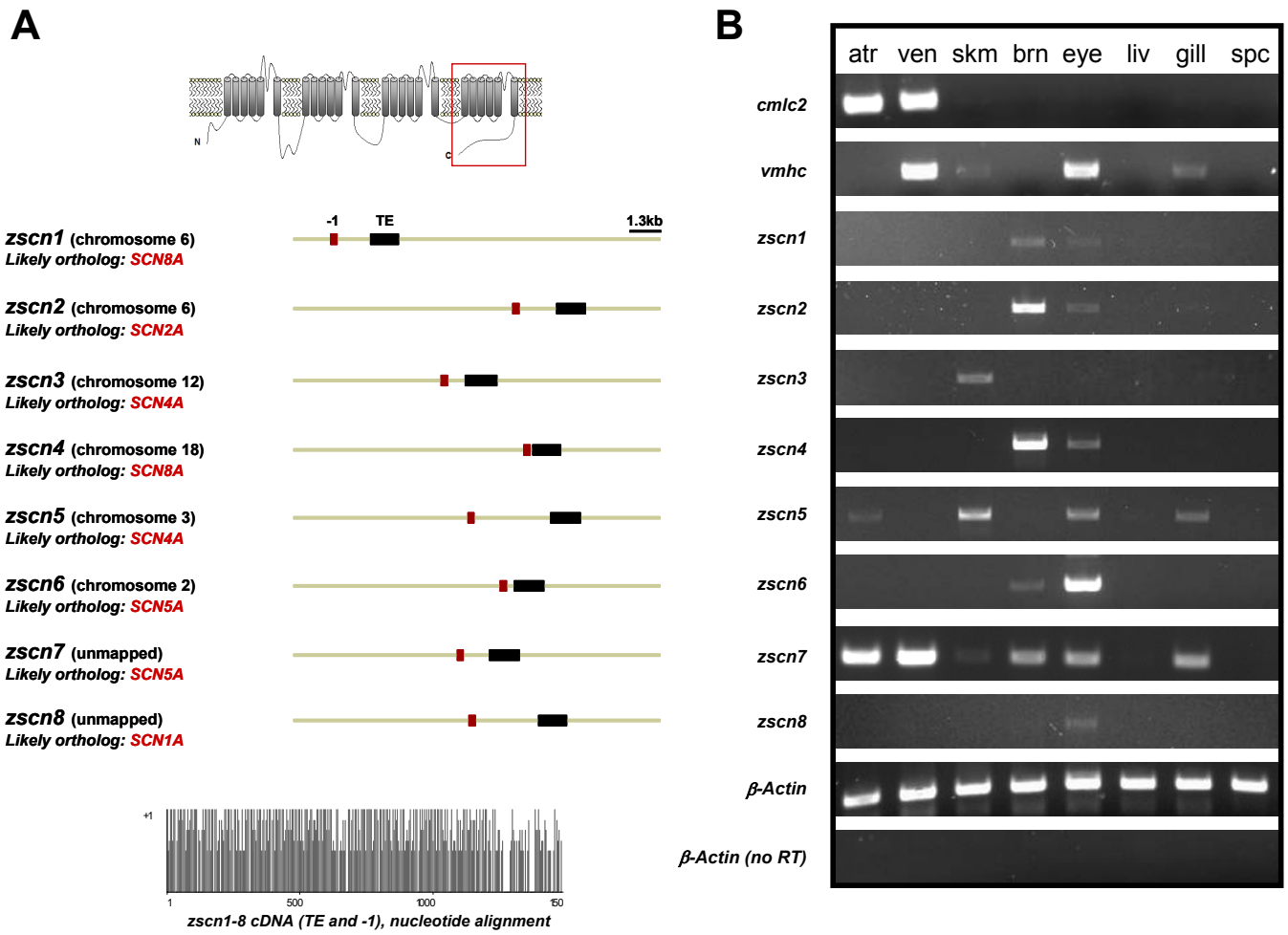
Online Video VI

A group of embryos at 32hpf following microinjection of 2 large boluses of 1mM veratridine (sodium channel activator) into the yoke sac shortly after fertilization (1-2 cell stage). Probe stimulation induces tonic contraction and hyperexcitability rather than the typical escape response. However, the hearts of these embryos have developed normally and are beating at a rate indistinguishable from vehicle-injected clutchmates. Similar results were obtained following treatment with ATX II. (Quicktime; 9.8 MB).

Online Figures I-VIII

Voltage-gated sodium channels are required for heart development in zebrafish

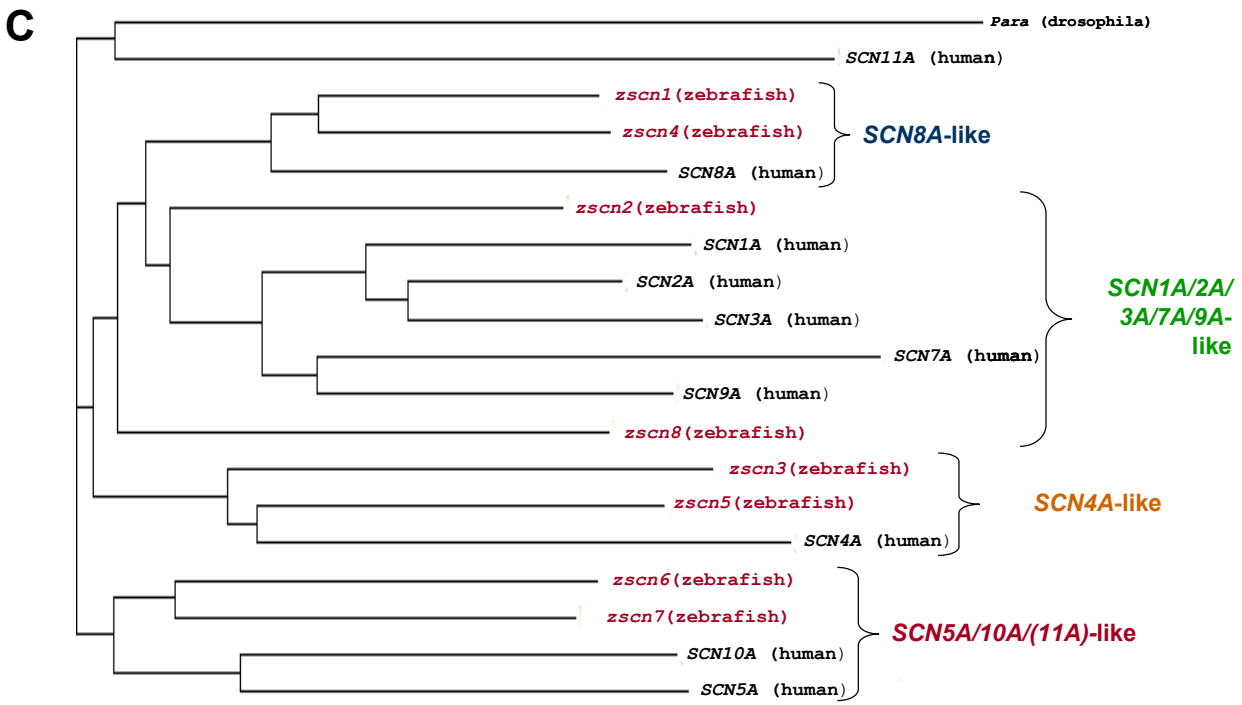
Sameer S. Chopra, Dina Myers Stroud, Hiroshi Watanabe, Jeffrey S. Bennett, C. Geoffrey Burns, K. Sam Wells, Tao Yang, Tao P. Zhong, Dan M. Roden



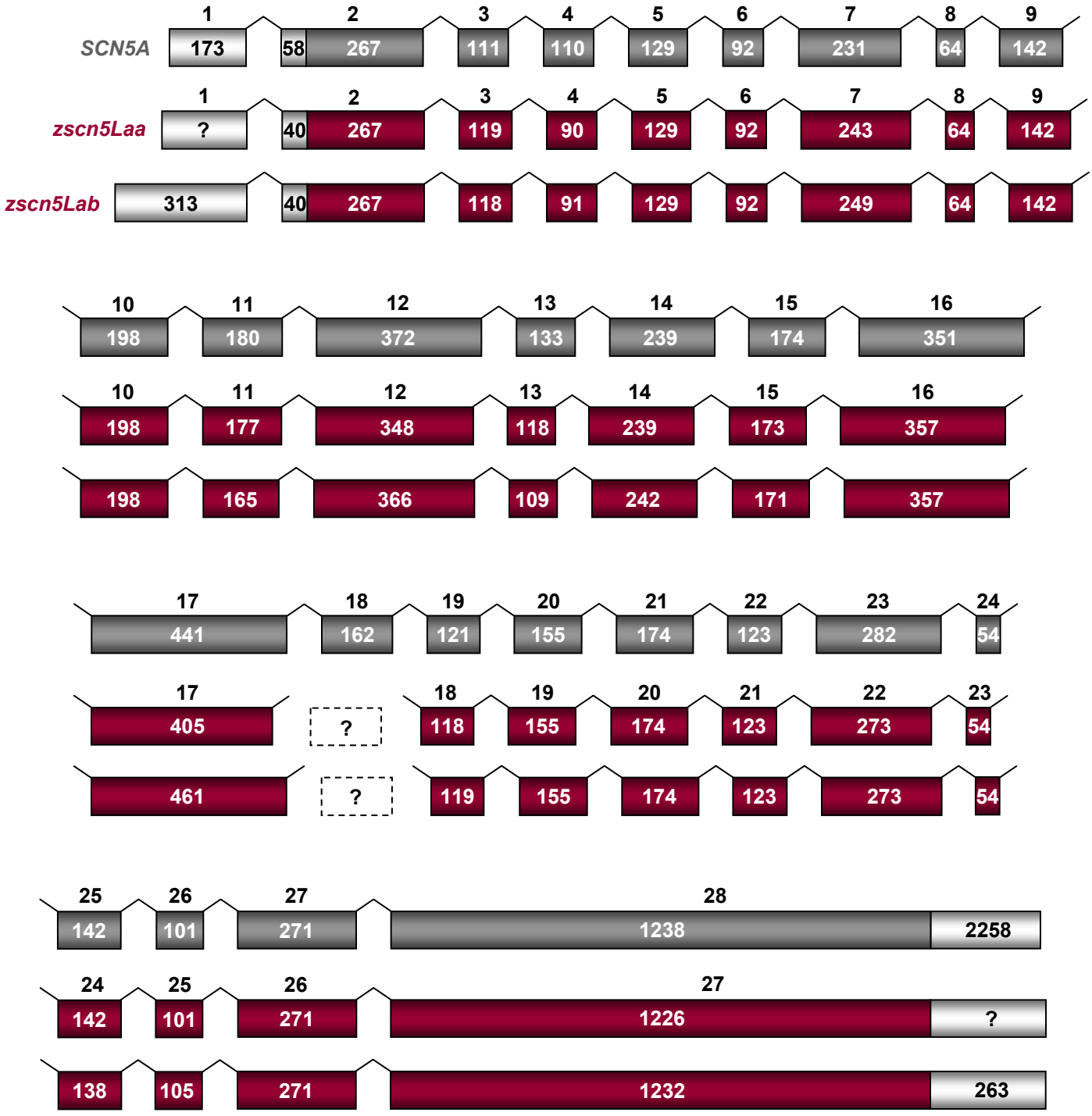
Online Figure I. Identification of cardiac-type sodium channels in zebrafish.

(Note: The Zebrafish *scn5a* homologs *zscn6* and *zscn5* were renamed *scn5Laa* and *scn5Lab*, respectively).

A. *In silico* approaches using 3' mammalian sodium channel sequences (terminal exon, terminal exon -1) and the draft zebrafish genome sequence (ensembl Zv4, Zv5) identified 8 unique zebrafish voltage-gated sodium channel genes (see: Online Methods). Putative gene identities were assigned based on alignment with the nucleotide and amino acid sequences of equivalent regions of human sodium channel

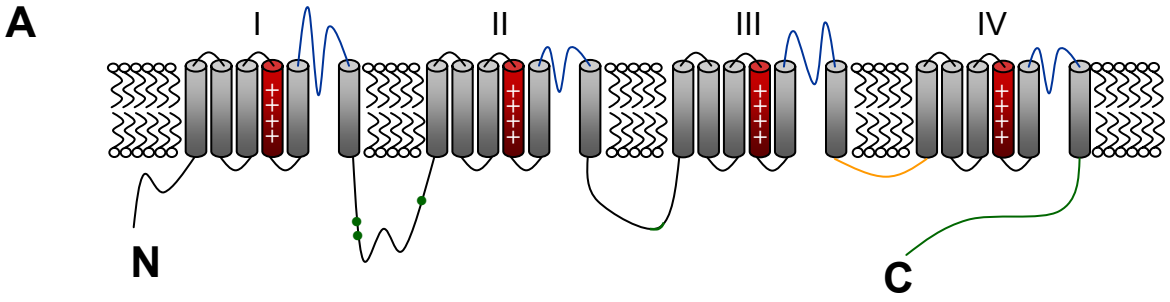


genes and by phylogeny (panel C). The identified non-redundant, 3' sequences represented putative *in silico* zebrafish sodium channel "sequence tags." **B.** *In silico* zebrafish sodium channel sequence tags were validated by using RT-PCR and to screen different adult zebrafish tissues. Amplicon specificity was confirmed by direct sequencing. Atr = atrium; ven = ventricle; skm = skeletal muscle; brn = brain; eye = eye and optic nerve; liv = liver; gill; spc = spinal cord. Despite two putative *scn5a*-like genes in the zebrafish genome (*zscn6*, *zscn7*), only one Na_v1 gene was found to be highly-expressed in the adult zebrafish atrium and ventricle (*zscn7*). Expression of *zscn5* was also detected in the atrium, but at very low levels compared to its expression in skeletal muscle. **C.** Neighbor-joining phylogenetic tree based on the alignment of zebrafish *in silico* sequence tags with equivalent regions of mammalian sodium channel genes. Zebrafish Na_v1 channel sequences appear in red. The *Para* sodium channel gene sequence from *Drosophila* was included as an outgroup. *Zscn6* and *zscn7* gene sequences are most closely related to the human sodium channel genes *SCN5A* and *SCN10A*. A detailed phylogenetic analysis performed by the AB Ribera lab using full-length zebrafish sodium channel clones (Online Materials and Methods Referefence #4) led to our renaming of *zscn6* as ***scn5Laa*** and *zscn7* as ***scn5Lab***.



Online Figure II. Genomic organization of *scn5a*-like genes in humans and zebrafish.

The genomic structures of zebrafish *scn5Laa* and *scn5Lab* were solved by comparing cloned cDNA sequences with contigs of genomic DNA derived from the draft zebrafish genome sequence (ensembl). The genomic structures of coding regions of zebrafish *scn5a*-like genes are entirely conserved with human *SCN5A* with the exception of a single exon (18) that encodes for part of the cytoplasmic domain II-III linker. Despite several attempts, 5' and 3' RACE PCR were unable to fully resolve the extended 5' and 3' regions of *scn5Laa*.



Alignment
$Na_V1.5$ (human *SCN5A*)
$Na_V1.5La$ (zebrafish *scn5Laa*)
$Na_V1.5Lb$ (zebrafish *scn5Lab*)

VOLTAGE SENSORS

S4 (DI)	S4 (DII)	S4 (DIII)	S4 (DIV)
ALRTFRVLRALKTISVISGL	VLRSFRLLRVFKLAKSWPTL	IKSLRTLRALRPLRALSRF	RVIRLARIGRILRLIRGAKGIR
ALRTFRVLRALKTISVIPGL	VLRSFRLLRVFKLAKSWPTL	IKSLRTLRALRPLRALSRF	RVIRLARIGRVLRLIRAAKGIR
ALRTFRVLRALKTISVIPGL	VLRSFRLLRVFKLAKSWPTL	IKSLRTLRALRPLRALSRF	RVIRLARIGRILRLIRGAKGIR

PORE LOOPS (partial, region surrounding selectivity filter)

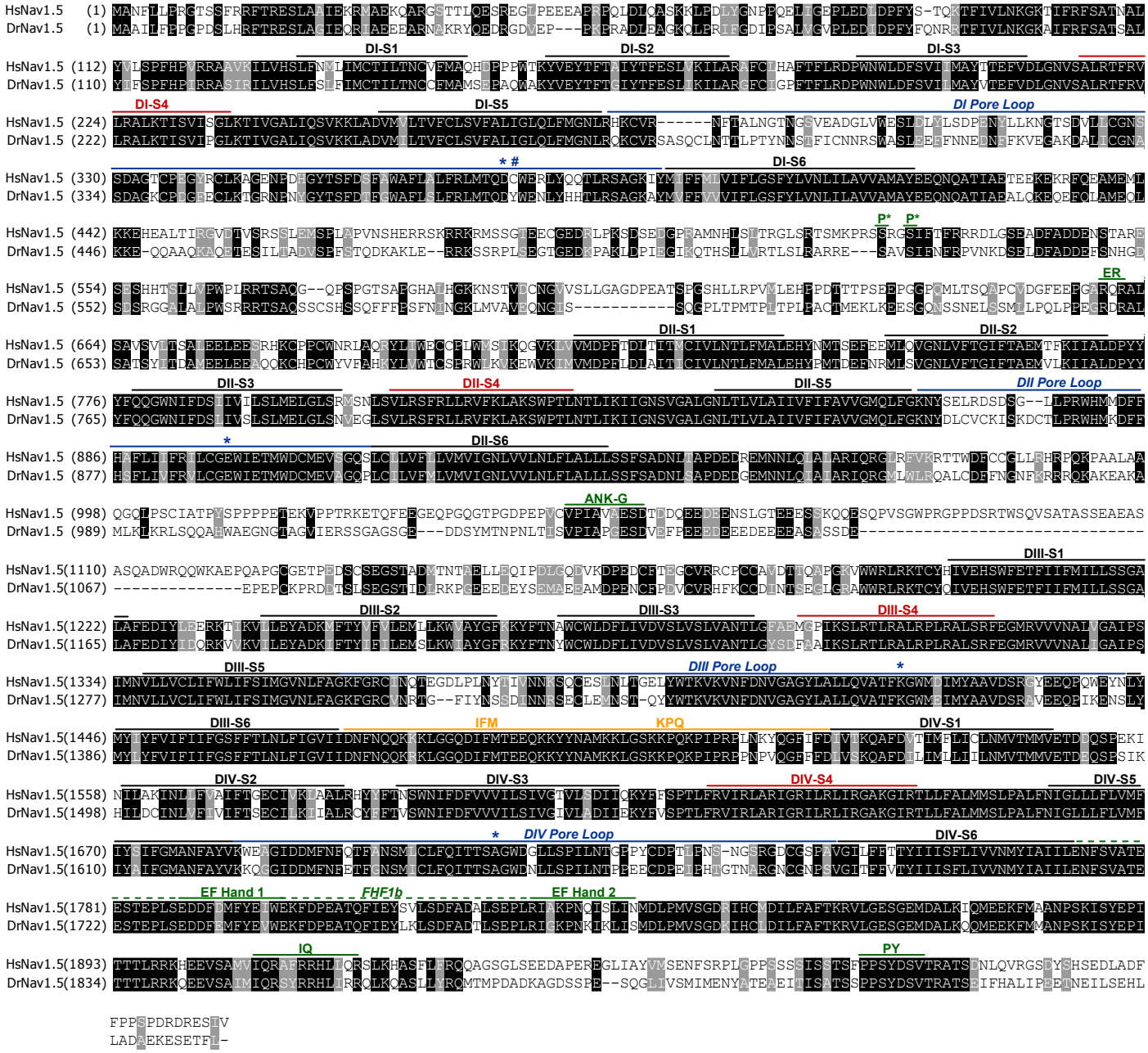
S5-6 (DI)	S5-6 (DII)	S5-6 (DIII)	S5-6 (DIV)
FLALFRLMTQDCWERLYQQTL	FLIIFRILCGEWIETMWDCME	YLALLQVATFKGWMDIMYAAV	MLCLFQITTSAGWDGLLSPIL
FLGLFRLMTQDYWESLYHQTL	FLIVFRVLCGEWIETMWDCME	YLSLLQVATFKGWMEIMYAAV	MICLFQITTSAGWNNLLDPIL
FLSLFRLMTQDYWENLYHHTL	FLIVFRVLCGEWIETMWDCME	YLALLQVATFKGWMEIMYAAV	MICLFQITTSAGWDNLLSPIL
*	*	*	*

INACTIVATION GATE (DIII-IV LINKER)

DNFNQQKKKLGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPLNKYQGFIFD
DNFNQQKRMIGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPMNILQGFFFD
DNFNQQKRKLGGQDIFMTEEQKKYYNAMKKLGSKKPQKPIPRPPNPVQGFFFD

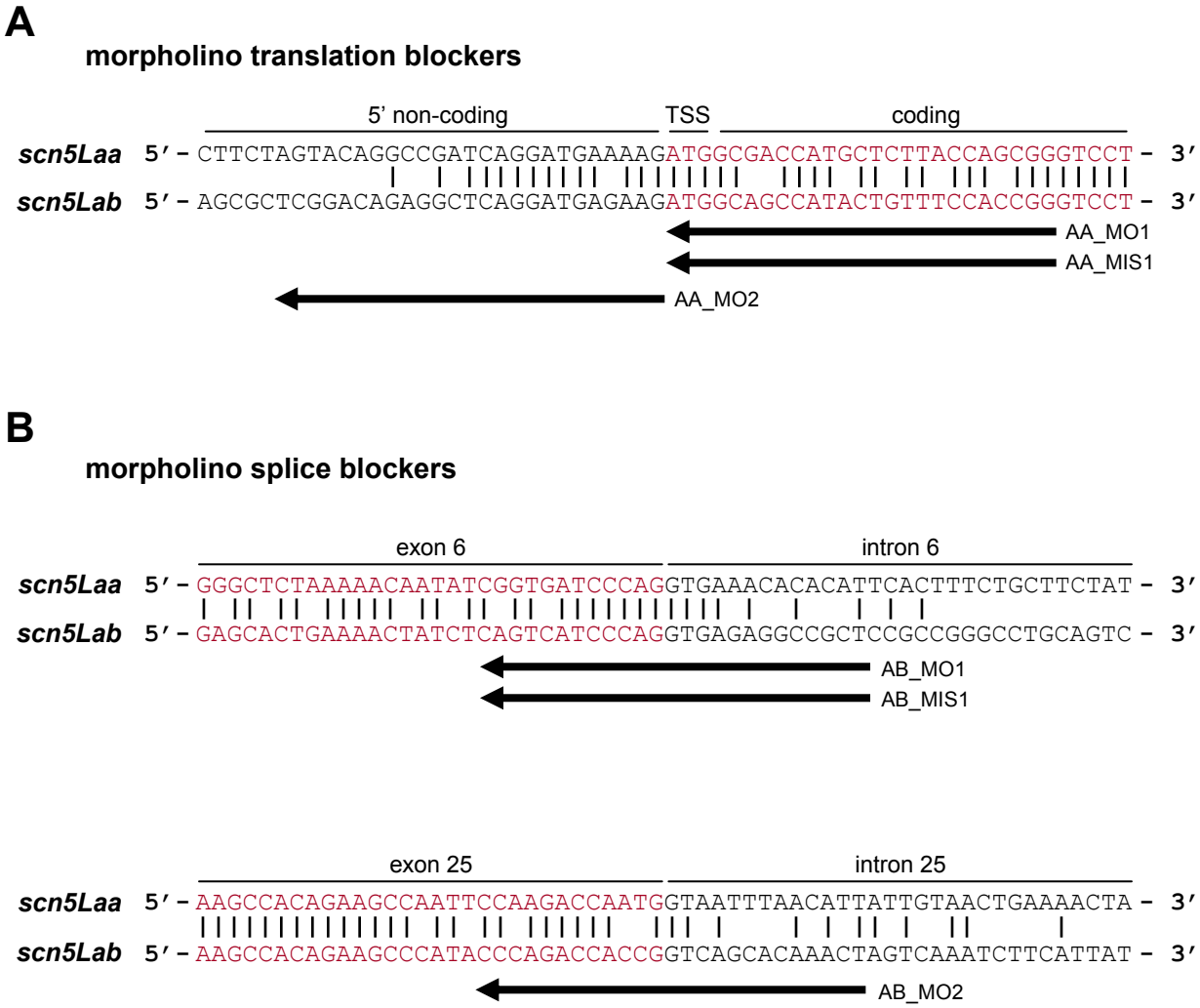
C-TERMINUS (proximal)

ENFSVATEESTEPLSEDDFDMFYEIWEKFDPEATQFIEYSVLSDFADALSEPLRIAKPNQISLINMDLPMVSGDRIHCMDILFAFTKR
ENFSVATEESTEPLSEDDFEMFYEVWEKFDVEATHFIEYAKLSNFADSLSEPLRIAKPNKIKLIHMDLPMVNGDRIHCLDILFAFTKR
ENFSVATEESTEPLSEDDFEMFYEVWEKFDPEATQFIEYLKLSDFADTLSEPLRIGKPNKIKLISMDLPMVSGDKIHCLDILFAFTKR



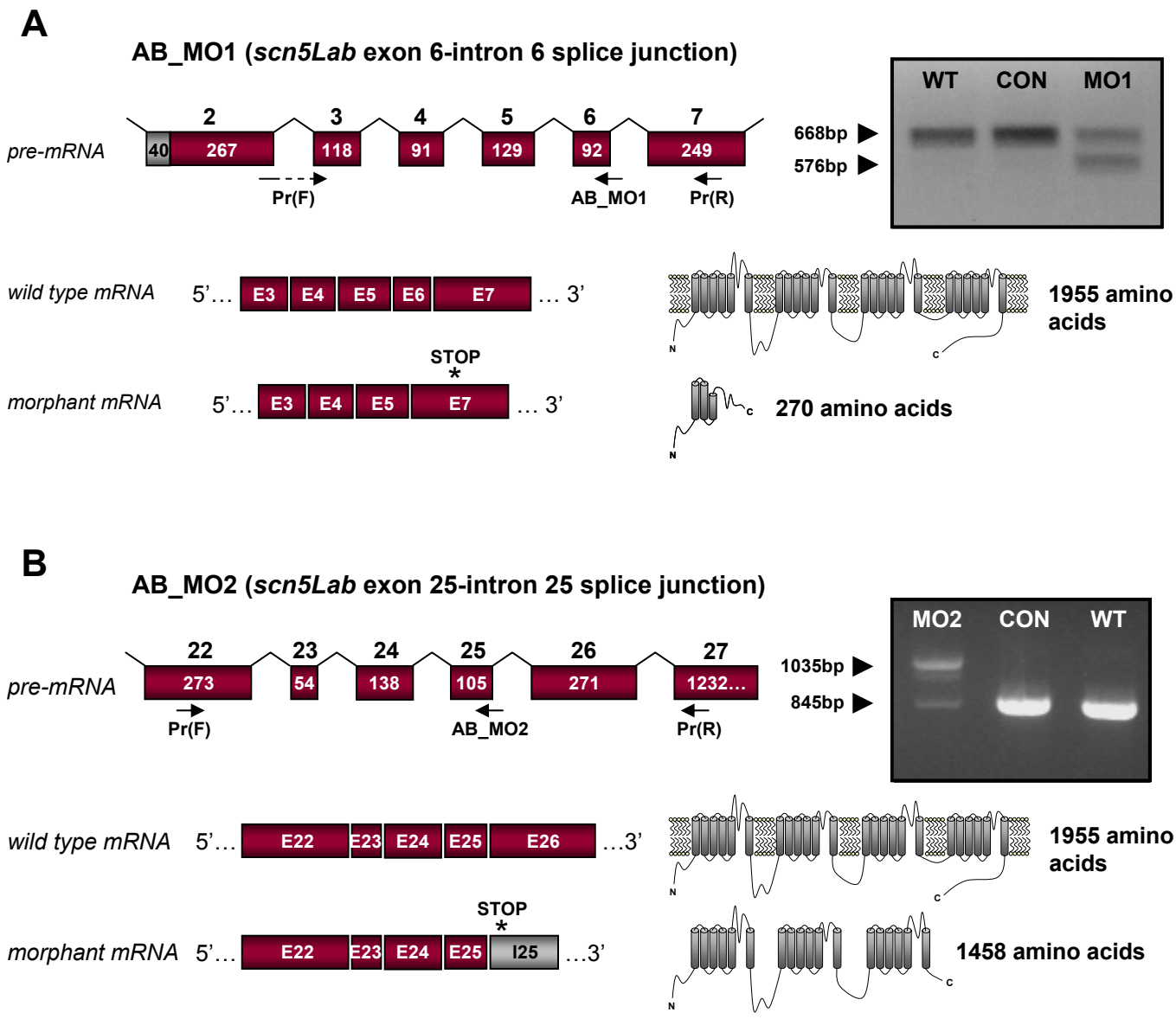
Online Figure III. Zebrafish $Na_v1.5La$ (*scn5Laa*) and $Na_v1.5Lb$ (*scn5Lab*) are conserved voltage-gated sodium channels.

A. Schematic of human $Na_v1.5$ with important functional domains highlighted in color: voltage "sensors" = S4 membrane-spanning segments of domains I-IV; pore loops = S5-6 linkers of domains I-IV, with * = D-E-K-A residues that constitute the ion selectivity filter; inactivation gate = intracellular DIII-IV linker; sites of predicted protein-protein interaction/Ca2+ regulation including the proximal C-terminus. Amino acid alignment of important functional domains of human $Na_v1.5$ (*SCN5A*), zebrafish $Na_v1.5La$ (*scn5Laa*), and zebrafish $Na_v1.5Lb$ (*scn5Lab*) are shown below. Black and grey highlighting denote identity and similarity, respectively. **B.** Annotated alignment of the full-length human $Na_v1.5$ ("HsNa$_v$1.5") and zebrafish $Na_v1.5Lb$ ("DrNa$_v$1.5") amino acid sequences. Color coding as in **A**, with the addition of: # = single cysteine residue in $Na_v1.5$ that confers TTX resistance; P* = PKA phosphorylation site; ER = endoplasmic reticulum retention motif; ANK-G = ankyrin-G interaction motif; EF hand = Ca+ binding motif; *FHF1b* = fibroblast growth factor homologous factor 1b interacting domain; IQ = calmodulin binding domain; PY = interaction motif for Nedd4-like E3 ubiquitin-protein ligases.



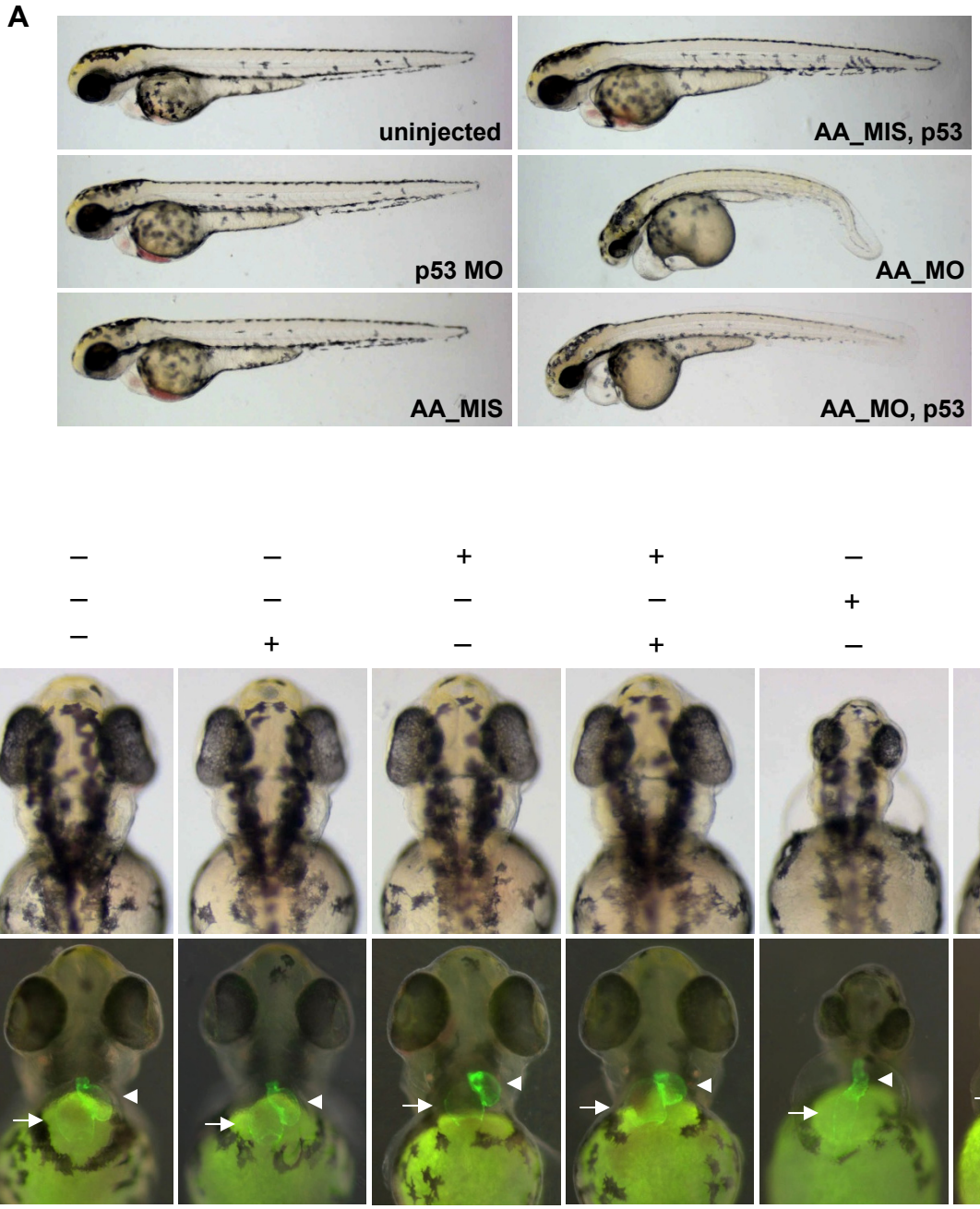
Online Figure IV. Target and sequence specificity of morpholino oligonucleotides directed against *scn5Laa* and *scn5Lab*.

Morpholino antisense oligonucleotides were designed to specifically target either *scn5Laa* or *scn5Lab*. Gene sequences are presented in 5'-3' orientation for both *scn5Laa* and *scn5Lab*, with coding sequence highlighted in red and non-coding sequence in black. Vertical lines mark sequence identity. **A.** Translation-blocking morpholino oligonucleotides were directed against the translation start site or 5' UTR of *scn5Laa.* **B.** Splice-blocking morpholino oligonucleotides were directed against the exon 6-intron 6 and exon 25-intron 25 splice donor sites of *scn5Lab*. The specificity of each morpholino (number of mismatches against *scn5Laa* or *scn5Lab*) is indicated in Online Table 1.



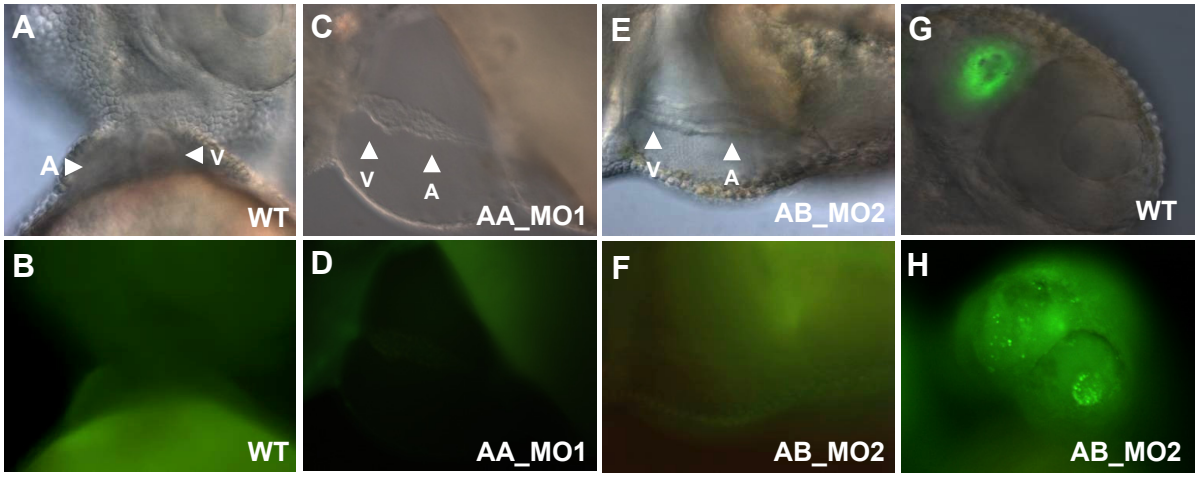
Online Figure V. Validation of splice morpholinos (72-78hpf).

A. A morpholino oligonucleotide directed against the exon 6-intron 6 splice donor site of *scn5Lab* (AB_MO1) resulted in skipping of exon 6 and a shift of translation frame, introducing a premature stop codon in exon 7. The translation product of the morphant RNA is predicted to be 270 amino acids in length. **B.** A morpholino oligonucleotide directed against the exon 25-intron 25 splice donor site of *scn5Lab* resulted in retention of intron 25, introducing a premature stop codon in the mRNA. The morphant RNA translation product is predicted to be 1458 amino acids long and lacks all of domain 4 of the channel protein. Pr(F) and Pr(R) indicate the location of PCR primers used to amplify across the targeted splice junctions.



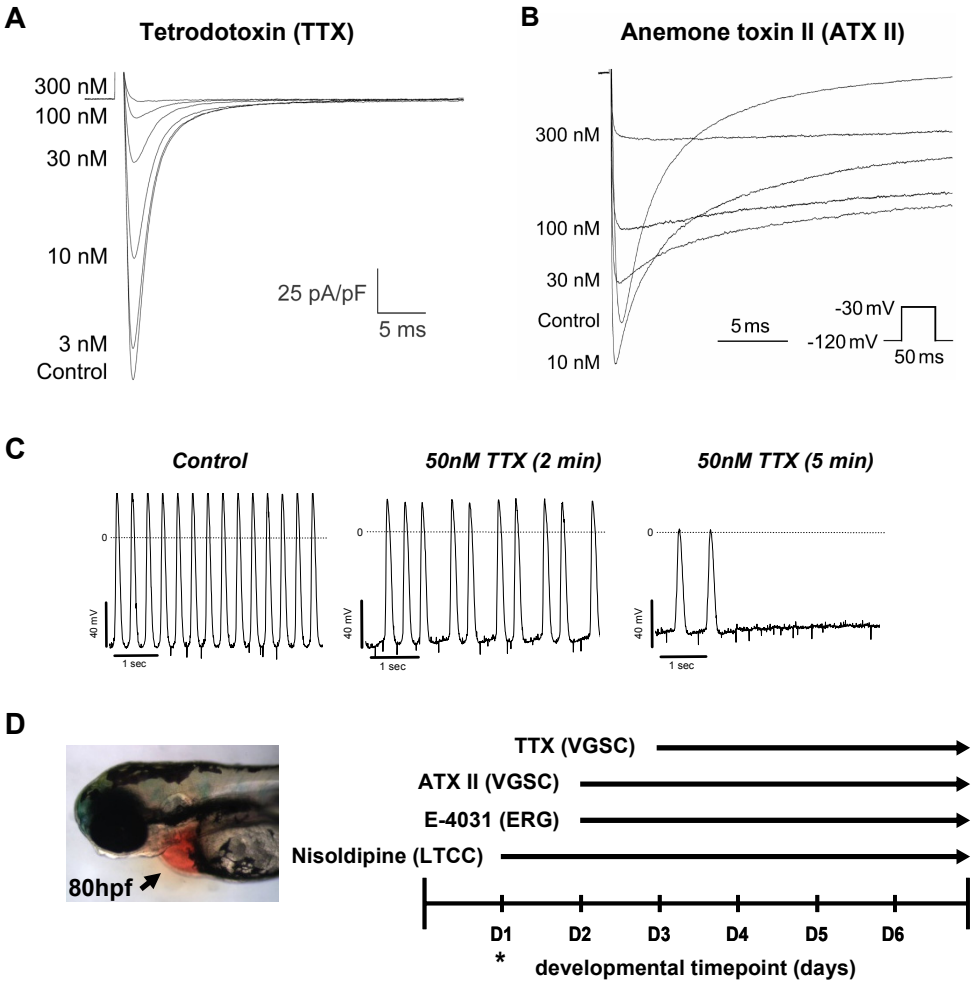
Online Figure VI: The cardiac phenotype of sodium channel morphant zebrafish is not attributable to apoptosis and/or morpholino toxicity.

A, B. Knockdown of p53 partially rescues the body and head phenotypes of sodium channel knockdown but does not ameliorate defects in heart development. Arrow = atrium, arrowhead = ventricle. Shown are morpholinos against *scn5Laa*, with and without p53 morpholino. P53 morpholino alone did not produce any phenotype. Similar results were observed for *scn5Lab*.



Online Figure VII. Absence of apoptotic cells in the dysmorphic, hypoplastic sodium channel morphant heart at 60 h.p.f.

Control and morphant embryos were stained with 5μg/ml of acridine orange for 30 minutes at room temperature and then examined for apoptotic cells using fluorescence microscopy. N = 35 in each group. **A-F**. Treatment groups are as marked. A, atrium and V, ventricle. **G, H.** Non-cardiac tissues served as positive controls for the acridine orange stain. For example, staining was consistently observed in the olfactory epithelium of wild type embryos and in the forebrain and eye of morphant embryos. The p53 morpholino was not used in these experiments.



Online Figure VIII: *In vitro* and *in vivo* activity of the sodium channel toxins tetrodotoxin (TTX) and anemone toxin II (ATX II) in zebrafish.

A-B. Heterologous expression of the full-length zebrafish *scn5Lab* cDNA in CHO cells demonstrates conserved sodium channel pharmacology. The sodium channel encoded by *scn5Lab* is highly sensitive to nanomolar concentrations of both tetrodotoxin (TTX) and anemone toxin II (ATX II). While TTX blocks inward sodium current, ATX II interferes with channel inactivation resulting in persistent sodium current. **C.** Low doses of the sodium channel pore blocker tetrodotoxin (TTX) (50nM) inhibited zebrafish adult ventricular action potentials and silenced the heart. **D.** TTX and ATX II were delivered directly to the embryonic heart via pericardial microinjection (arrow, phenol red dye) at different developmental timepoints. Nisoldipine (calcium channel blocker) and E-4031 (potassium channel blocker) were used as positive controls at early stages. Black horizontal line indicates the timepoint at which the compound has an observed effect on heart rhythm. Similar to findings in other model systems, sodium current (I_{Na}) is not required for early heart function. An asterisk marks the timepoint of heartbeat initiation (D1, Day 1, 22-24hpf). The sodium channel activator ATX II elicited arrhythmias such as 2:1 AV block as early as day 2 (54-60hpf) while TTX produced bradycardia starting on day 3 (78-84hpf) and AV conduction block starting on day 4 (102-108hpf). See Online Table III, Online Video Legend, and Online Videos.