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Supplemental Information

Dynamic Cellular Integration Drives Functional Assembly of the Heart's Pacemaker Complex

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Supplemental Experimental Procedures:

METHOD DETAILS

Live imaging of voltage sensitive dyes: Hearts were isolated in pre-warmed sterile Tryode's solution [137mM NaCl, 2.7 mM KCL, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 0.2 mM Ha2HPO4, 5.5mM D-glucose, 15mM Hepes, pH 7.4], and allowed to recover for 20-30 min. Hearts were then transferred into staining solution (Tyrodes Solution, 10mM Hepes, 12mM NaHCO3, 10 μM Di-4-ANEPPs [Invitrogen], and 10 μM Cytochalasin-D [Tocris Biosciences], pH 7.4) saturated with 95% O2 and 5% CO2, for 10-30 min. Both the imaging chamber and staining solution were maintained at 37.5 ° C +/- 0.5 ° C throughout the imaging process using a Warner CL-100 in-line media temperature control unit and a BioOptecs Delta T Stage heater. Live imaging was conducted at 2000fps using a 14 bit, 100x100 pixel, CMOS Camera (MiCAM Ultima, SciMedia), mounted on a vertical THT Microscope (SciMedia) illuminated with a Moritex 150w Halogen light source.

Cardiac slices were prepared by embedding freshly isolated embryonic hearts in 3.5% low melting temperature agarose. To ensure that the pacemaker region was captured in the slices to be imaged, the top 100-200um of the atria were removed via sectioning with a Lecia VT 1000 vibrating blade microtome and the next 400 um segment was cut and prepared for imaging. These slices were imaged at 1000fps using the same protocol described above.

3D reconstructions: Chick and mouse tissues was fixed in 4% PFA for 30 min and then washed three times in standard PBS. To preserve morphology and limit possible tissue deformation, no clearing or dehydration steps were performed. Fixed tissue was then embedded in 4% low melting agarose and 100um thick sections were cut through the transverse axis of the heart using a vibrating blade microtome. Sections were blocked for two hours in PBS, 1% BSA, 0.1% triton X 100, before being transferred into primary antibodies overnight at 4 ° C. Antibodies against MF20 (diluted 1:500), Vim H5 (diluted 1:100), TPN1 CH1 (diluted 1:50) were purchased from Developmental Studies Hybridoma Bank (University of Iowa), while antibodies against HCN4 (diluted 1:1000) were purchased from Abcam. Sections were then washed four times for 40 min in PBS and incubated with subtype specific alexa conjugated secondary antibodies (Life Technologies) overnight at 4 ° C. Following this, slices were washed four times for 40 min in PBS and counterstained with Dapi.

Equipment and Settings: Thick sections of cardiac tissue were imaged on a two-photon upright LSM 7 MP laser-scanning microscope (Carl Zeiss MicroImaging) using a Plan-Apochromat water-immersion 20x object (1.0 DIC M27 70mm). The imaging platform was controlled using ZEN software (Carl Zeiss MicroImaging). 8 bit scans were acquired using Zeiss LSM NDD detectors at an x,y resolution of 2048x2048 pixels. Each tissue section was imaged first with a digital zoom factor of 1 spanning 80um through the z axis, and then again at a digital zoom factor of 5 spanning 30um through the Z axis. Fluorophores were simultaneously excited using two laser lines set at 920nm and 780nm respectively.

Standard Histology: 10 um paraffin sections were used for standard histological studies. Immunofluorescence was performed using previously reported protocols (Bressan et al., 2014). MF20, and HCN4 were used as described above, Col3 3b2 (Developmental Studies Hybridoma Bank) and WT1 (Abcam) were applied at 1:500 and 1:200 respectively.

In situ hybridization: Whole mount and section *in situ* hybridization were performed using to published protocols (Bressan et al., 2013; Ishii et al., 2010a). Probes used for detecting *Hcn4*, *Tbx3*, *Tbx18*, and *Bmp2* have been previously reported (Ishii et al., 2010a; 2010b; Bressan et al, 2013). The following primers were used for additional probe generation:

Snai2-F TGGAGCAGACAATGCGATAG, Snai2-R CTGTTGCAGTGAGGACAGGA;
Rhob-F TCCTCATGTGCTTCTCAGTG, Rhob-R GTCTCAGCAGCTTCTTGTTG;
Lsp1-F CACAGGAGAACCACTATGAC, Lsp1-R GGAAGTCATGGCATTGAGGA;
Shox2-F AAGGAGCTCATCACCTACCG, Shox2-R ATGCTCGAGTTCTTGCTGGT;
Tmem46-F CCTCATTGTCGGCTCCGTCT, Tmem-R CTCACAAGCACAGTGATCTG;
Mmd1-F GCAATACTGAAGGACTTGAG, Mmd1-R CATCTATGCTACAACCCTTG,
Anxa2-F GTACAAGAGCTACAGCCCAT, Anxa2-R TGGCAGAACCAAGCAGAGC;
Gpx3-F GTACCTCGAACTGAATGCAC, Gpx3-R TGGAGATGTCATCCTCTGCT;
Fgfrl1-F GCTACAGTTTTCGCAGTGCT, Fgfrl1-R TGGGATCCGGAGTTCCTTCA;
Sfrp1-F CACTGCTTGCTTTACCACCA, Sfrp1-R GCTTCTACAGCTTCGGATGG;
Sfrp2-F TCGACGACCTGGACGAGATC, Sfrp2-R CAGATAAGAGTCACATTTGC,
Cxcr7-F CGCTTCACATCACTCAGTGT, Cxcr7-R GTCATCCTCTTACCAAGGAT,
Col3a1-F AAGCTGATGAGCTCTGTGGA, Col3a1-R CCACTGACTTGACCAATGGA;
Ncs1-F AAGCCTGAAGTTGTGGAGGA, Ncs1-R TCTTCTCCGGTGTGTTTTCC;
Sik1-F CACGACTTCTGTTGGTGTCA, Sik1-R GCCATCTCCATGTCCTCTG,
Col18a1-F TTGGAGATGCTGCGCAGTGT, Col18a1-R ACCACATGTGGTCAACATGC.

RNA isolation and RNAseq: Embryos were incubated to HH stage 18-19 and cardiac tissue was isolated. Fragments of the ventral right inflow (pacemaker region), left component of the common atria, and apex of the common ventricle were manually dissected and pooled (n = 50 per group). RNA was extracted using standard phenol-chloroform techniques yielding approximately 2mg of total RNA per sample group. RNAseq was performed as described previously (Christodoulou et al., 2011; DeLaughter et al., 2013).

Chimeric embryo generation: Donor embryos (quail or GFP expressing chick) were incubated to HH stage 17-18. PEs were isolated form these embryos using previously described techniques (Ishii et al., 2010a) and stored in room temperature Tyrodes solution. Host chick embryos were incubated to HH stage 17-18 and isotopic, isochonic transplantations were performed. Briefly, a window was cut in the top of the host embryo's eggshell and the viteline membrane was opened to expose the embryo. 100uL of 10% india ink was injected into the yolk below the embryo to improve contrast. A small incision was made through the pericardium to expose the heart and DiI (Life Technologies) was used to tag the region of the right inflow tract that contributes to the mature SAN (Bressan et al., 2013). The native PE was then excised using sharpened fine forceps (Dumont #5SF Forceps). The donor PE was placed in the pericardial space and a strip of eggshell membrane was used to wedge the donor PE into the position that the native PE had been excised from. These embryos were allowed to recover for 30 min at 37° C and then the strip of eggshell membrane was removed. The egg was then sealed and re-incubated for 72 hours and the embryos were prepared for imaging. A total of 7 quail-chick chimeras and 5 GFP chimeras were analyzed. Quail derived PE cells were traced using an antibody against the quail specific antigen QCPN (Developmental Studies Hybridoma Bank, University of Iowa).

PE ablations: PE ablations were performed as described above for host embryo preparation. Due to variation in PE size and outgrowth, the PE of some embryos had already made contact with the inner curvature of the heart by stage 17-18. In these cases, the PE was removed from the inflow region of the heart, and a strip of eggshell membrane was placed between the inflow and inner curvature to prevent PE/epicardial cells located on the heart from potentially being transferred back onto the inflow. During control (Sham)-operations the heart was accessed as described above but only a 50-100 um segment of the most distal portion of the PE was removed leaving the majority of the PE intact.

QUANTIFICATION AND STATISTICAL ANALYSIS

Live imaging of voltage sensitive dyes: Quantification of PC-to-atria conduction time was conducted by subtracting the time of pacemaker activation (dF/dT max at the electrical impulse initiation site) from the atrial activation time (dF/dT max of a region of the atria approximately 250 um from the pacemaker site along the direct conduction route. At E5 (HH stage 28) and E6 (HH stage 30), the dF/dT max of the electrical impulse initiation site was subtracted from the dF/dT max at the first point of atrial activation as the entire conduction route between the two regions could not always be captured in a single field of view. A total of 45 hearts were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) and imaged for this analysis. Quantification was performed using SciMedia BV-Ana analysis software.

3D reconstruction measurements: 3D reconstructions and volume rendering were performed using ImageJ v1.48 (National Institutes of Health) or Imaris Scientific 3D/4D Image Processing & Analysis Software (Bitplane). Wall thickness was defined as the diameter of a circle bound by the epicardial and endocardial surfaces of the pacemaker region (n=3 embryos per biological group). Cellular density was calculated by counting the number of MF20 positive nuclei in a 50 um x 50um x 30 um volume of the pacemaker region (n=3 embryos per biological group). Fiber alignment was determined by tracing MF20 positive fibers (defined as three consecutive interconnected cells) through a 30 um thick 3D reconstruction (n=3 embryos per biological group). The center of mass for the Dapi signal was used as an anchor point for the location of the three cells. Lines connecting these points were then drawn using Imaris and the angle across the central nucleus was used as a measure of the fiber's alignment.

RNA isolation and RNAseq: Genes were defined as differentially expressed if they displayed a normalized read count of > 2 per/million reads, and a fold of >,< 2 when compared to both atrial and ventricular reads. Gene ontology analysis was preformed using the Gene Ontology Consortium Panther Overrepresentation Test (release 20160321). Go terms that met the following criteria are reported: terms were not present in genes sets enriched in either the ventricular or atria RNA pools, > 10 genes were associated with the GO term, p-value > 0.05.

Chimeric embryo generation: A total of 7 quail-chick chimeras and 5 GFP chimeras were analyzed. Quail derived PE cells were traced using an antibody against the quail specific antigen QCPN (Developmental Studies Hybridoma Bank, University of Iowa).

PE ablations: For this study, 36 sham-operation and 64 PE-ablations were performed. For viability analysis 15 sham and 26 PE-ablated embryos were examined at E4, 8 sham and 10 PE-ablated embryos were examined at E5, and 13 sham and 42 PE-ablated embryos were allowed to develop until E6. Sham-operated embryos were divided for general histology (n=8), 3D reconstructions (n=3), or were prepared for live imaging of voltage sensitive dyes (n=6, cardiac slices). Among the PE-ablated embryos, 12 hearts were not analyzed due to the obvious morphological defects noted in Sup Figure 3H. 14 hearts were prepared for general histology, 3 were used for 3D reconstructions, and 4 were sectioned and used for living imaging of voltage sensitive dyes. As the magnitude of

effect on morphology and conduction characteristics could not be predicted prior to data analysis, no statistical metric was used to prejudge the number of PE ablation surgeries required for appropriate statistical power prior to data collection.

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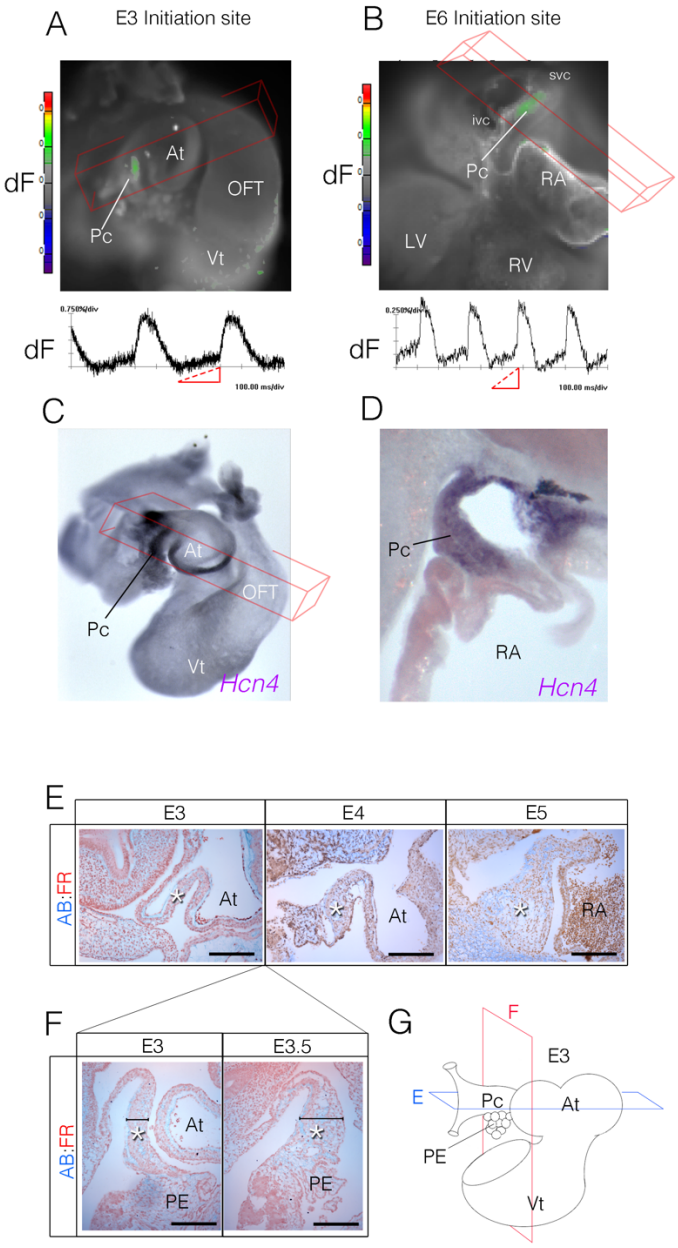
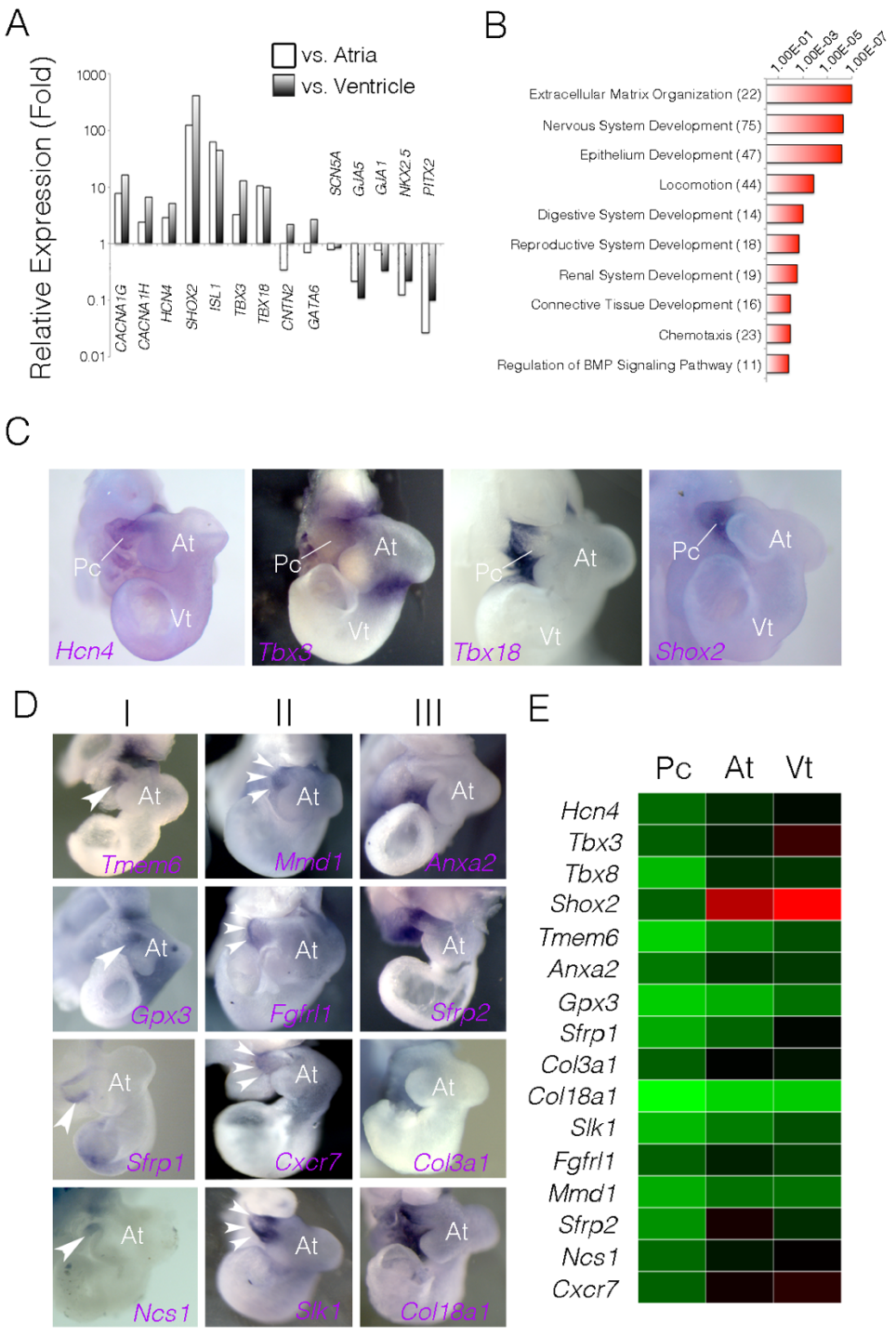
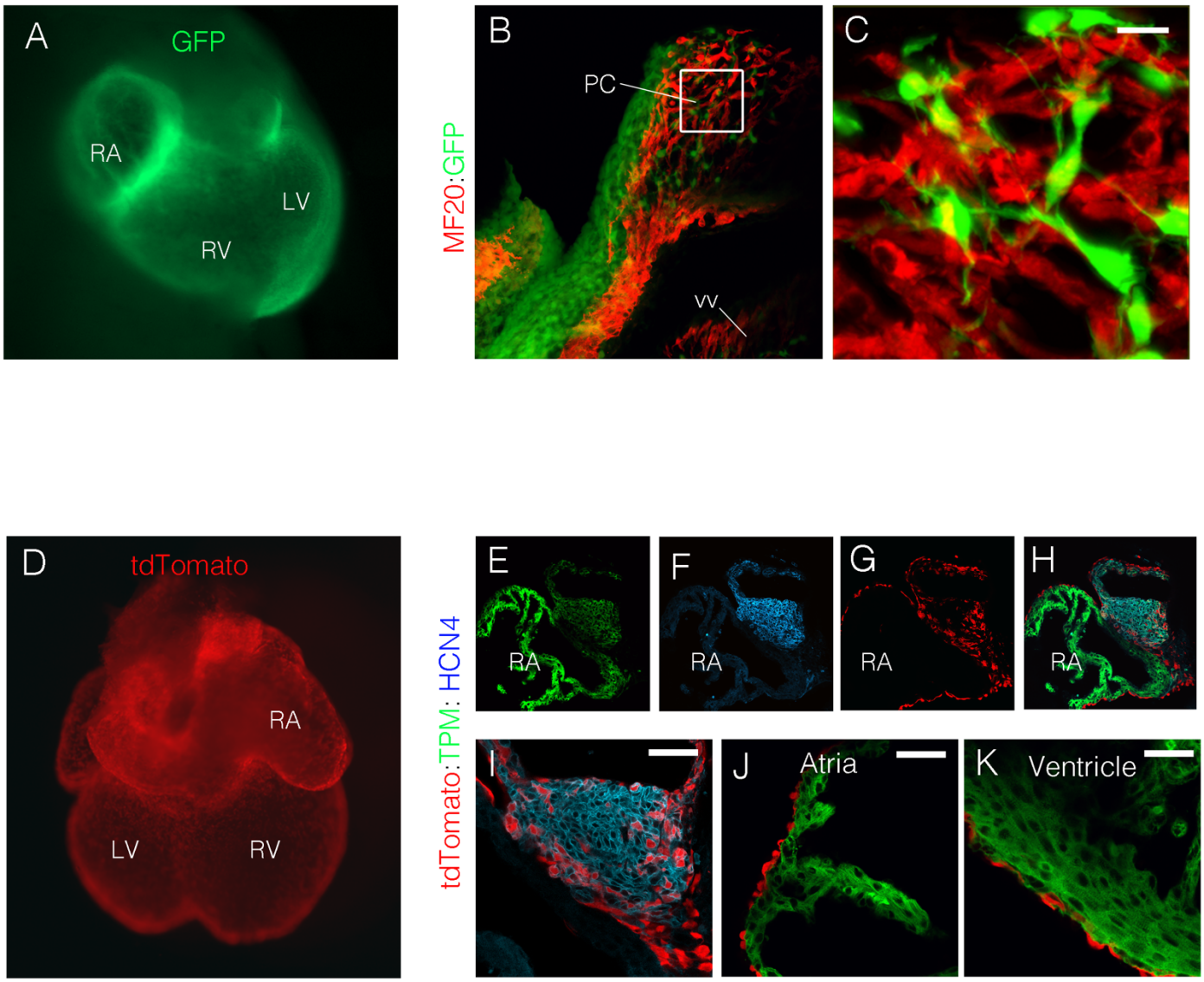
Supplemental Figures:



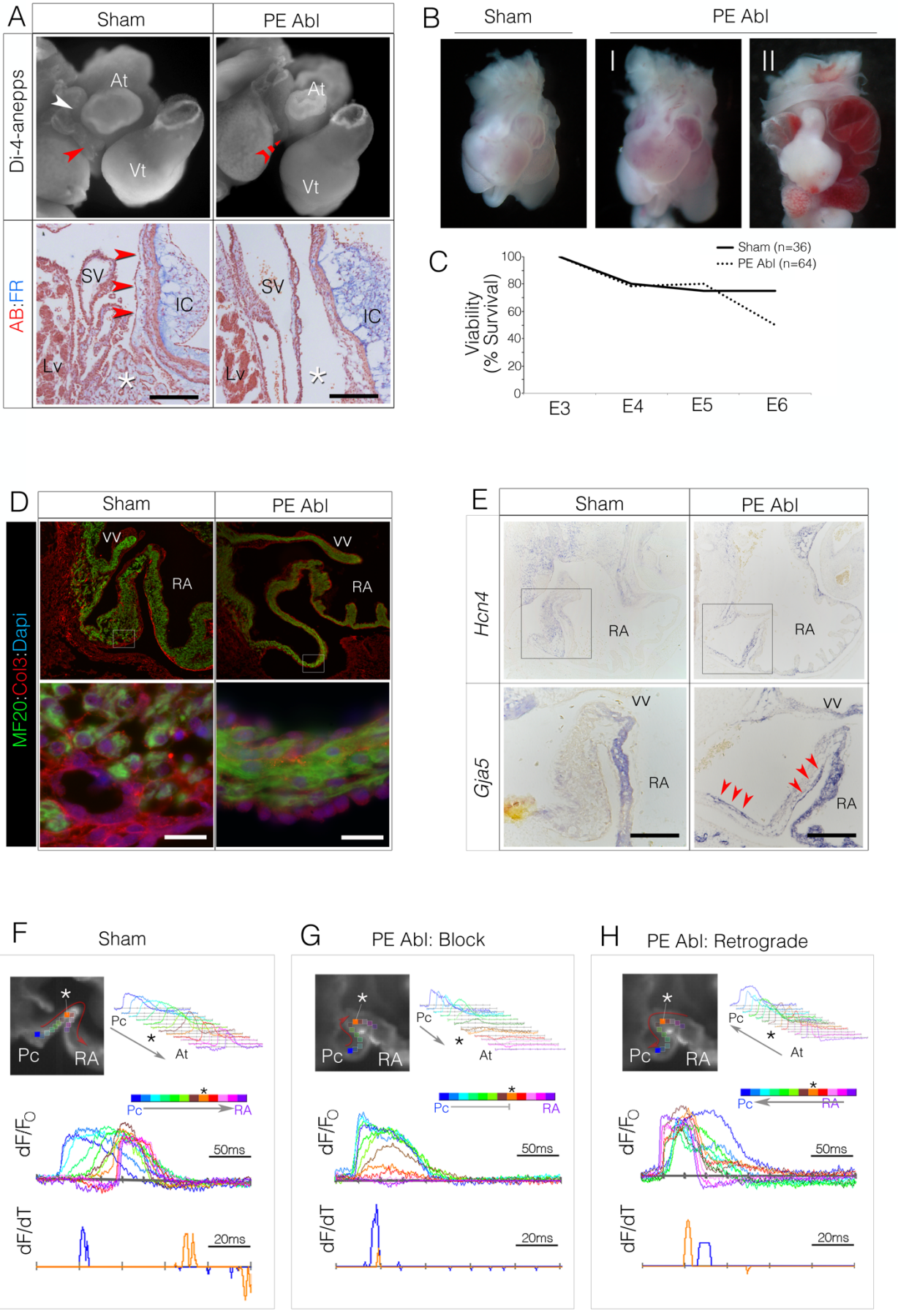
Sup Figure 1: Developmental progression of cardiac pacemaker region physiology and anatomy, related to Figures 1,3,4. **A)** Optically mapped E3 heart imaged from the right lateral view. Initial activation (change in voltage-sensitive dye fluorescence – dF, green signal) is detected along the ventral surface of the right inflow. Boxed region denotes position of thick section used for 3D reconstruction (Figure 1). Trace of membrane potential change (change in voltage sensitive dye fluorescence – dF) from the initiation site in (A). Note slow diastolic depolarization (red dashed line), between consecutive depolarizations. **B)** Optically mapped E6 heart imaged from the dorsal-inferior view. Initial activation (change in voltage-sensitive dye fluorescence – dF, green signal) is detected within the inflow myocardium between the inferior *vena cava* and superior *vena cava*. As in (A), trace of membrane potential shows slow diastolic depolarization. **C)** Whole mount *in situ* hybridization of optically mapped heart from (A) using probe for *Hcn4*. Hcn4 corresponds to the action potential initiation site from (A). **D)** *In situ* hybridization for *Hcn4* in a thick section cut through the pacemaker region of an E6 heart (this section plane is also used in Figure 1 I-M, Figure 3 D, Figure 4 A, B,I: see methods). **E)** Time series of SAN morphogenesis spanning E3 to E6. Transverse sections stained with nuclear fast red and alcian blue demonstrate that the pacemaker region (white asterisks) progressively thickens between E3-E5. **F)** Sagittal sections trough the pacemaker region at E3 and E3.5. Sections demonstrate that initial remodeling (wall thickening indicated by brackets) is detectable as early as E3.5. **G)** Diagram depicting the section plans for (E) and (F). Trace of membrane potential change (change in voltage-sensitive dye fluorescence – dF) from the initiation site in (D). At-Atria, VT-ventricle, Pc-pacemaker, OFT-out flow tract, –RA-right atria, RV-right ventricle, LV-left ventricle, ivc-inferior vena cava, svc-superior vena cava. Scale bars – 100um.



Sup Figure 2: RNAseq validation reveals complex expression patterns relative to the newly active pacemaker cells, related to Figure 2. A) RNAseq fold difference of known positive and negative makers of pacemaker/conduction system cells. **B)** P-values of Gene Ontology (GO) terms uniquely associated the pacemaker region. E3 whole mount *in situ* hybridization for previously reported pacemaker (PC) marker genes. **C)** Whole mount in situ hybridization for previously reported PC enriched transcripts. The outflow tract of these E3 hearts have been removed to more easily view the pacemaker region. **D)** E3 whole mount *in situ* hybridization of a subset of genes upregulated based on RNAseq. Expression patterns are organized into three columns: I. Genes expressed in a patch on the ventral right inflow corresponding to the pacemaker electrical impulse initiation site (white arrowhead), II. Genes expressed broadly across the entire right inflow including the pacemaker region (white arrowheads), but absent from the adjacent atria, III. Genes expressed surrounding the patch of cells from column I including expression in the PE. **D)** Heat map indicating RNAseq expression levels (Log_2 counts) of genes from (C,D). At-atria, Vt-ventricle.



Sup Figure 3: PE cell tracing studies demonstrate integration into the forming SAN, related to Figure 3. **A)** Example of a heart in which a GFP-positive PE was implanted at E3 and development was allowed to proceed to E6. GFP positive cells cover the ventricle and atria. **B)** Volumetric 3D reconstruction of an 80 um thick segment of the pacemaker region (PC) in a GFP PE chimera. Note GFP-positive cells (green) are seen interdigitating with the pacemaker muscle (red). Boxed region indicates area in **C)** which is presented in primary Figure 3 F-H. Scale bar – 5um. **D)** E13.5 heart from inducible TFC21 merCREmer x Lox-Stop-Lox tdTomato lineage tracing. E-H) Thick section cut through the atria and SAN head of heart from (D), stained with HCN4 (blue) and Tropomyosin (green). **I)** Higher magnification image of SAN from (E-H). Scale bare – 20um. **J,K)** Higher magnification images of atria and ventricle from (D). Note TCF21 labeled cells have integrated into the HCN4 positive myocardium of the SAN, but not the atrial or ventricular myocardium (n=3). Scale bar – 20um.


A
Sham
PE Abl
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At
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IC
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B
Sham
PE Abl
I
II
C
Sham (n=36)
PE Abl (n=64)
Viability (% Survival)
100
80
60
40
20
0
E3
E4
E5
E6
D
Sham
PE Abl
MF20:Col3:Dapi
VV
RA
E
Sham
PE Abl
Hcn4
Gja5
RA
VV
F
Sham
G
PE Abl: Block
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PE Abl: Retrograde
Pc
RA
At
dF/F0
dF/dT
50ms
20ms

Sup Figure 4: PE ablation disrupts structural, molecular, and function patterning of the forming SAN, related to Figure 4. A) Comparison between sham and PE-ablated embryos 24 hrs. after surgery. Hearts were stained with Di-4-anepps to provide better contrast. The PE (red arrowhead) is located directly adjacent to the pacemaker region (white arrowhead) in Sham embryos, but is missing following ablation (dashed red arrowhead). Transverse sections through the atrioventricular junction (stained with alcian blue and nuclear fast red) demonstrate that the PE is visible (white asterisks) in the space between the sinus venosus (SV) and the atrioventricular myocardium of Sham embryos, but is not detectable following PE-ablation. Scale bars – 100um. **B)** Examples of hearts isolated from sham or PE-ablated embryos following incubation to E6. Two major classes of hearts were observed in PE ablated embryos: "I" hearts with grossly normal anatomy, and "II" hearts that displayed enlarged atria, smaller ventricles, and underdeveloped outflow tracts. Only category "I" hearts were used for PC structural and functional analysis (see Supplemental Experimental Procedures). **C)** Survival rates of sham-operated and PE-ablated embryos between E3 and E6. **D)** Comparison of Collagen 3 (COL3-red) deposition in sham vs PE-ablated pacemaker regions. Note, in sham embryos Col3 is localized between and surrounding small clusters of pacemaker myocardium, whereas only small puncta of Col3 are present in the PE-ablated embryos (n=3). Scale bars – 10um. **E)** Comparison of the low resistance gap junction *Gja5* expression in sham vs PE-ablated embryos. In sham embryos, *Gja5* is not detected above background levels in the *Hcn4* positive SAN myocardium. In contrast strands of *Gja5* positive cells are seen running through the *Hcn4* positive region of PE-ablated embryos (red arrowheads) (n=3). Scale bars – 100um. **F)** Conduction characteristics in sham-operated cardiac slice preparation. Cropped image of the pacemaker region from Figure 4 I demonstrates direction of propagation (red arrow). Colored boxes denote the region from which wave traces are presented. Orange box (asterisks) is the approximate anatomical junction between PC region and atria. Analysis of dV/dT (indicating the point of maximal action potential up stroke velocity) at the impulse initiation site (Blue) and the PC-atria boundary (orange) demonstrates the extent of conduction delay across the pacemker region. **G)** As for (F), in a PE-ablated embryo displaying SAN conduction block. Impulse amplitude dramatically decreases as current flows across the pacemaker region towards the atria, and there is a lack of temporal separation in the dV/dT between initiation site (Blue) and PC-Atria boundary (orange). **H)** Example of retrograde (Atria-to-PC) conduction the PE-ablated embryo from (G). There is no drop in impulse amplitude in the retrograde direction and, consistent with (G), retrograde propagation displays a dramatically decrease temporal separation between the dV/dTs at the PC-atrial boundary (orange) and the central PC region (Blue) indicative of rapid current flow. IC – inferior cushion, RA – right atria, vv – venous valve.