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Last updated by author(s): *YYYY-MM-DD*

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Isolated P1, P2, and P4 neonatal cardiomyocytes suspended in culture medium (DMEM/F-12 with 10% FBS + 1% P/S) were filtered using a 100 um cell strainer (VWR, 10199-658), then transferred to separate polypropylene tubes (Falcon). Cells were incubated with SytoxTM Blue dead cell stain (Thermofisher, S34857, 1:2000) prior to Fluorescence Activated Cell Sorting (FACS). Gating was done for size and granularity, then for Sytox-based viability, and finally for tdTomato-based identification of conduction cells. Approximately 0.1-0.2% of total viable cardiomyocytes were tdTomato-positive for all P1, P2, and P4 cells (Supplementary Fig. 2). MULTI-seq reagents were generously provided by the developers, and multiplexing of P1, P2, and P4 cells was conducted following the developer's protocols36. In brief, following separate FACS sorting of P1, P2, and P4 CCS cells into culture medium (DMEM/F-12 with 10% FBS + 1% P/S), cells were pelleted and washed using centrifugation (250 x g, 5 min, 4°C) and 1x PBS. Lipid-modified oligonucleotide anchor mix and unique barcodes were added to each sample at final concentrations of 200 nM. Cells were resuspended by gentle pipetting and were incubated at 4°C for 10 min, ensuring to periodically mix by gently tapping. Then, a co-anchor lipid-modified oligonucleotide was added to each sample (final concentration of 200 nM), and cells were incubated at 4°C for 5 min. Cells were then pelleted using centrifugation (250 x g, 5 min, 4°C) and resuspended in PBS containing 1% BSA to quench residual barcodes. P1, P2, and P4 cells were then pooled into a single tube and subjected to viability assessments and cell counting prior to scRNA-seq library generation using the 10x Chromium Single Cell 3' v3 kit (10x Genomics). All gene expression libraries were sequenced with NextSeq500 (Illumina).
Data analysis	All codes used to generate the results and figures in this study are available on Github (https://github.com/hankimlab/2024-Postnatal-Cardiac-Conduction-System). Other codes are available from the authors upon request. Packages used in this study are : R (version 4.2.2) CellRanger (version 7.0.0)

Seurat (version 5.0.1)
pySCENIC (version 0.12.1)
iGraph (version 1.5.1)
FastQC (version 0.11.7)
Bowtie2 (Galaxy Version 2.4.2+galaxy0)
Integrative Genomics Viewer (IGV) genome browser (version 2.16.2)
ggplot2 (version 3.4.4)
ComplexHeatmap (version 2.15.4)
corrplot (version 0.92)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

ScRNA-seq data and spatial transcriptomics data from this study have been deposited into the Gene Expression Omnibus (GEO) database under the accession number GSE231547

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	CCS cells from P1, P2, P4 CCS-reporter hearts (Cntn2-cre;R26tdTomato) were pooled to generate 6,855 cells for scRNA-seq analysis
Data exclusions	Low quality cells were excluded from the analysis. Low quality cells were determined by : 1) <2000 detected genes, 2) >5500 detected genes, 3) % mitochondrial content >25% . Cells enriched with non-cardiomyocyte markers (ex. fibroblasts, smooth muscle cells)
Replication	Duplicate CCS regions were collected for spatial transcriptomics, except the compact AV node as well as non-CCS regions.
Randomization	N/A
Blinding	N/A

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Primary Antibodies used in this study are :
chicken anti-RFP (600-901-379, 1:100)
goat anti-Cntn2 (AF4439, 1:50)
rabbit anti-Gnao1 (Thermo Fisher Scientific, PA5-30044, 1:100)
rabbit anti-Scn10a (Alomone Labs, ASC-016, 1:50)
rabbit anti-Igfbp7 (Abcam, ab74169, 1:50)
rabbit anti-S100a6 (Abcam, ab181975, 1:100)
rabbit anti-Nppa (EMD Millipore, ab5490, 1:250)
mouse anti-Shox2 IgG2a (Santa Cruz Biotechnology, sc-81955, 1:50)
rabbit anti-Vsnl1 (GeneTex, GTX115039, 1:100)
rabbit anti-Ppp1r17 (PA5-61599, 1:200)

Secondary Antibodies used in this study are:
goat anti-chicken IgY Alexa FluorTM 568 (Thermo Fisher Scientific, A11041, 1:200)
donkey anti-chicken IgY Alexa FluorTM 568 (Thermo Fisher Scientific, A78950, 1:200)
goat anti-rabbit Alexa FluorTM 488 (Thermo Fisher Scientific, A-11008, 1:500)
goat anti-mouse IgG2a Alexa FluorTM 488 (Thermo Fisher Scientific, A21131, 1:500)
donkey anti-rabbit Alexa FluorTM 647 (Jackson ImmunoResearch Laboratories Inc., 711-605-152, 1:200)
goat anti-mouse IgG2b Alexa FluorTM 488 (Thermo Fisher Scientific, A21141, 1:500)

Validation

chicken anti-RFP (600-901-379) - https://www.rockland.com/categories/primary-antibodies/rfp-antibody-600-901-379/
goat anti-Cntn2 (AF4439) - https://www.rndsystems.com/products/human-mouse-rat-contactin-2-tag1-antibody_af4439
rabbit anti-Gnao1 (PA5-30044) - https://www.thermofisher.com/antibody/product/GNAO1-Antibody-Polyclonal/PA5-30044
rabbit anti-Scn10a (ASC-016) - https://www.alomone.com/p/anti-nav1-8-2/ASC-016
rabbit anti-Igfbp7 (ab74169) - https://www.abcam.com/products/primary-antibodies/igfbp7-antibody-ab74169.html
rabbit anti-S100a6 (ab181975) - https://www.abcam.com/products/primary-antibodies/s100-alpha-6pra-antibody-epr13084-69-ab181975.html
rabbit anti-Nppa (ab5490) - https://www.emdmillipore.com/US/en/product/Anti-Atrial-Natriuretic-Peptide-Antibody,MM_NF-AB5490
mouse anti-Shox2 IgG2a (sc-81955) - https://www.scbt.com/p/shox2-antibody-jk-6e
rabbit anti-Vsnl1 (GTX115039) - https://www.genetex.com/Product/Detail/Visinin-like-1-antibody/GTX115039
rabbit anti-Ppp1r17 (PA5-61599) - https://www.thermofisher.com/antibody/product/PPP1R17-Antibody-Polyclonal/PA5-61599
goat anti-chicken IgY Alexa FluorTM 568 (A11041) - https://www.thermofisher.com/antibody/product/Goat-anti-Chicken-IgY-H-L-Secondary-Antibody-Polyclonal/A-11041
donkey anti-chicken IgY Alexa FluorTM 568 (A78950) - https://www.thermofisher.com/antibody/product/Donkey-anti-Chicken-IgY-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A78950
donkey anti-rabbit Alexa FluorTM 647 (711-605-152) - https://www.jacksonimmuno.com/catalog/products/711-605-152
goat anti-rabbit Alexa FluorTM 488 (A-11008) - https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11008
goat anti-mouse IgG2a Alexa FluorTM 488 (A21131) - https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG2a-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21131
goat anti-mouse IgG2b Alexa FluorTM 488 (A21141) - https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG2b-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21141
Chicken anti-RFP (600-901-379) - https://www.rockland.com/categories/primary-antibodies/rfp-antibody-600-901-379/
Goat anti-Cntn2 (AF4439) - https://www.rndsystems.com/products/human-mouse-rat-contactin-2-tag1-antibody_af4439
Rabbit anti-Gnao1 (PA5-30044) - https://www.thermofisher.com/antibody/product/GNAO1-Antibody-Polyclonal/PA5-30044
Rabbit anti-Scn10a (ASC-016) - https://www.alomone.com/p/anti-nav1-8-2/ASC-016
Rabbit anti-Igfbp7 (ab74169) - https://www.abcam.com/products/primary-antibodies/igfbp7-antibody-ab74169.html
Rabbit anti-S100a6 (ab181975) - https://www.abcam.com/products/primary-antibodies/s100-alpha-6pra-antibody-epr13084-69-ab181975.html
Rabbit anti-Nppa (ab5490) - https://www.emdmillipore.com/US/en/product/Anti-Atrial-Natriuretic-Peptide-Antibody,MM_NF-AB5490
Mouse anti-Shox2 IgG2a (sc-81955) - https://www.scbt.com/p/shox2-antibody-jk-6e
Rabbit anti-Vsnl1 (GTX115039) - https://www.genetex.com/Product/Detail/Visinin-like-1-antibody/GTX115039
Rabbit anti-Ppp1r17 (PA5-61599) - https://www.thermofisher.com/antibody/product/PPP1R17-Antibody-Polyclonal/PA5-61599

Goat anti-chicken IgY Alexa FluorTM 568 (A11041) - https://www.thermofisher.com/antibody/product/Goat-anti-Chicken-IgY-H-L-Secondary-Antibody-Polyclonal/A-11041
Donkey anti-chicken IgY Alexa FluorTM 568 (A78950) - https://www.thermofisher.com/antibody/product/Donkey-anti-Chicken-IgY-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A78950
Donkey anti-rabbit Alexa FluorTM 647 (711-605-152) - https://www.jacksonimmuno.com/catalog/products/711-605-152
Goat anti-rabbit Alexa FluorTM 488 (A-11008) - https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11008
Goat anti-mouse IgG2a Alexa FluorTM 488 (A21131) - https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG2a-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21131
Goat anti-mouse IgG2b Alexa FluorTM 488 (A21141) - https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG2b-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21141

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	Early postnatal mice (1 to 4 days old) were used for experiments. Cntn2tm1.1(cre/EGFP)Nmun, B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J and CD1 mice were used for the study
Wild animals	N/A
Reporting on sex	Both males and females were used for the study.
Field-collected samples	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	N/A
Novel plant genotypes	N/A
Authentication	N/A

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	The atria and ventricles of neonatal mice were collected separately in sterile ice-cold 1x phosphate- buffered saline (PBS). The ventricles were cut into 3-4 pieces for efficient digestion. Atrial and ventricular cardiomyocytes were separately placed into 50 ml Falcon tubes containing filtered Hanks' Balanced Salt Solution (1x HBSS, Thermofisher) with 0.0125% Trypsin-EDTA (Thermofisher) and 1% penicillin/streptomycin (P/S; Thermofisher). Cells were subjected to overnight trypsin digestion at 4°C while gently rotating. Then, the trypsin solution was aspirated and discarded. 5mL of filtered and warm (37°C) L-15 + 0.075% Collagenase II/Dispase (Roche, 10269638001) + 1% P/S solution was added to the 50 mL falcon tube containing cardiomyocytes, and a fine magnetic stir bar was placed. The falcon tubes were placed in a tube rack, which was placed above the stir plate. After gentle stirring of the solution and cells for 5 min, the supernatant containing single cardiomyocytes was collected into 5 mL of cold fetal bovine serum (FBS) to prevent cells from over-digestion. An additional 5 mL of collagenase digestion solution was added to the cells, and this digestion-collection step was repeated until sufficient cells were collected. After the final collection, debris was removed using a 100 mm cell strainer (VWR International). Cells were then pelleted at 1,000 rpm for 5 min. Purified cells were resuspended and plated in culture medium

	containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Thermofisher) with 10% FBS + 1% P/S. Pre-plating was done for 1 hr in the incubator to allow separation of fibroblasts from cardiomyocytes. Cardiomyocytes were then collected for either FACS Isolated P1, P2, and P4 neonatal cardiomyocytes suspended in culture medium (DMEM/F-12 with 10% FBS + 1% P/S) were filtered using a 100 µm cell strainer (VWR, 10199-658), then transferred to separate polypropylene tubes (Falcon). Cells were incubated with SytoxTM Blue dead cell stain (Thermofisher, S34857, 1:2000) prior to Fluorescence Activated Cell Sorting (FACS).
Instrument	MoFlo XDP
Software	FlowJo v. 10 (BD)
Cell population abundance	40-55% of cells were Non-debris cells, of which ~90% were singlets. Among these cells, 69-79% were viable (Sytox-) and approximately 0.1-0.2% of total viable cardiomyocytes were tdTomato-positive. (Supplementary Figure 2)
Gating strategy	Gating was performed for size and granularity, then for Sytox-based cell viability, and finally for tdTomato-based identification of the CCS cells. Isolated neonatal mouse cardiomyocytes from P1, P2 and P4 consist of comparable percentages of CCS cells (0.1 - 0.2% of viable cardiomyocytes).

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.