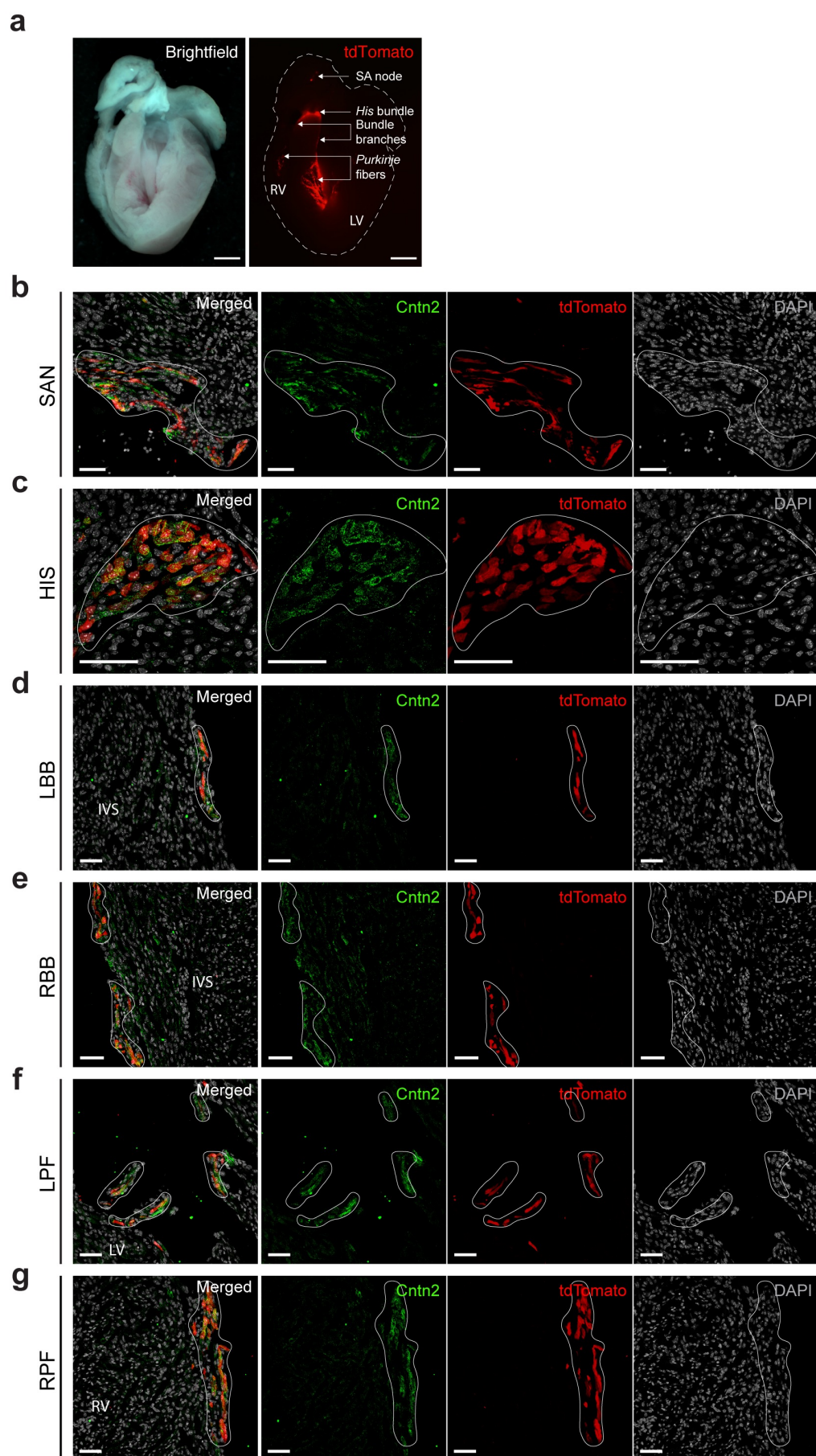
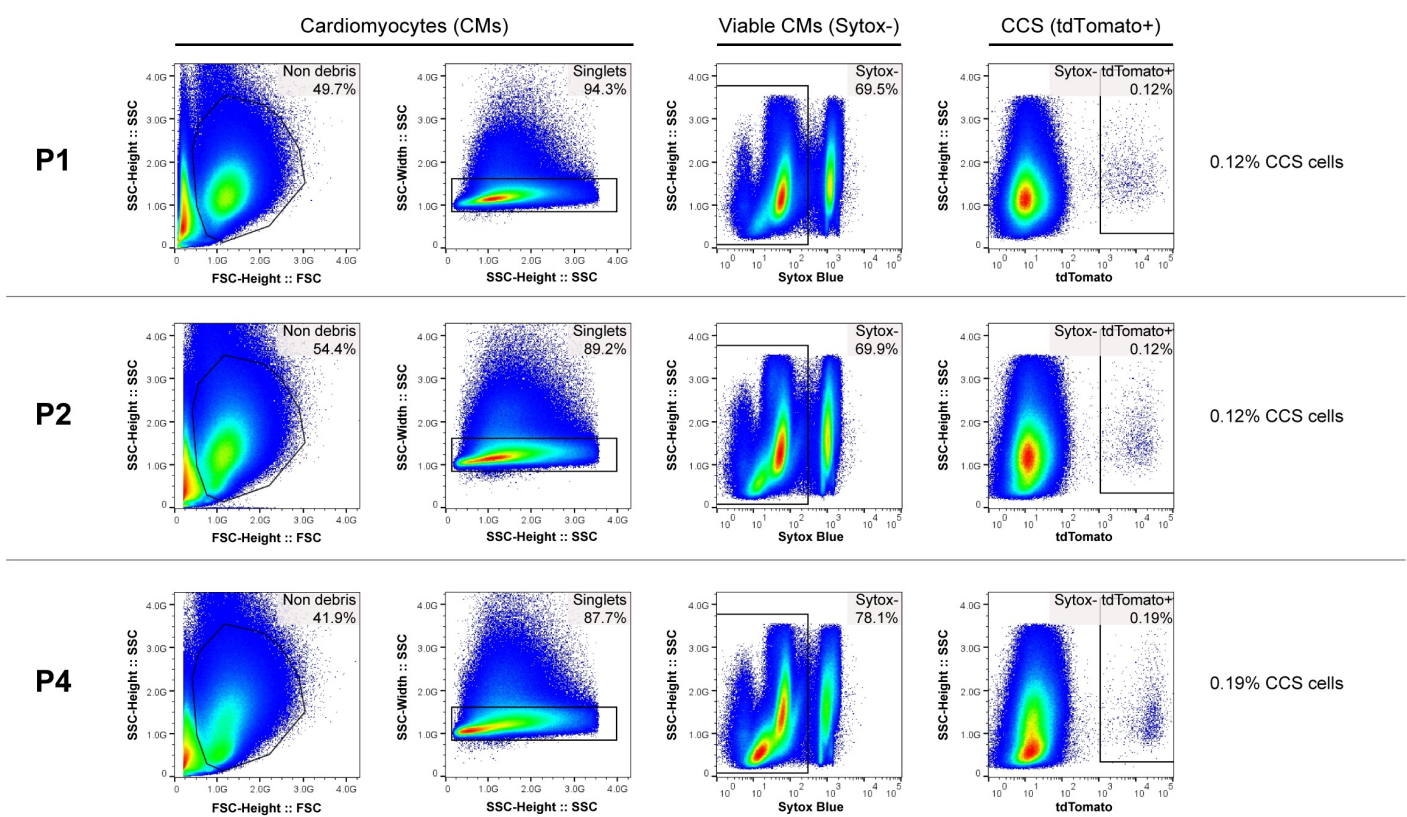


Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity

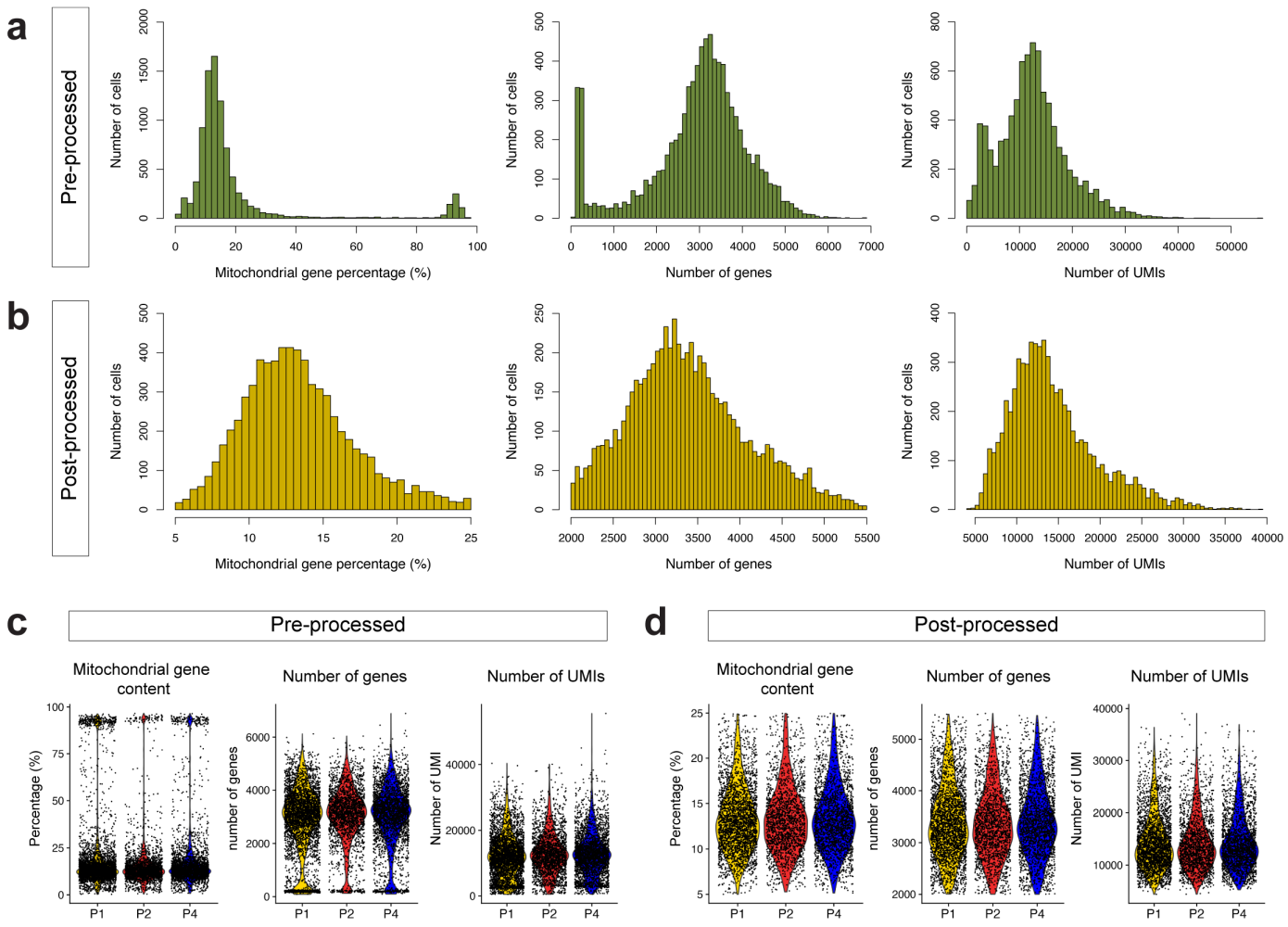


Supplementary Fig. 1. *Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+} heart marks all the components of the postnatal CCS.

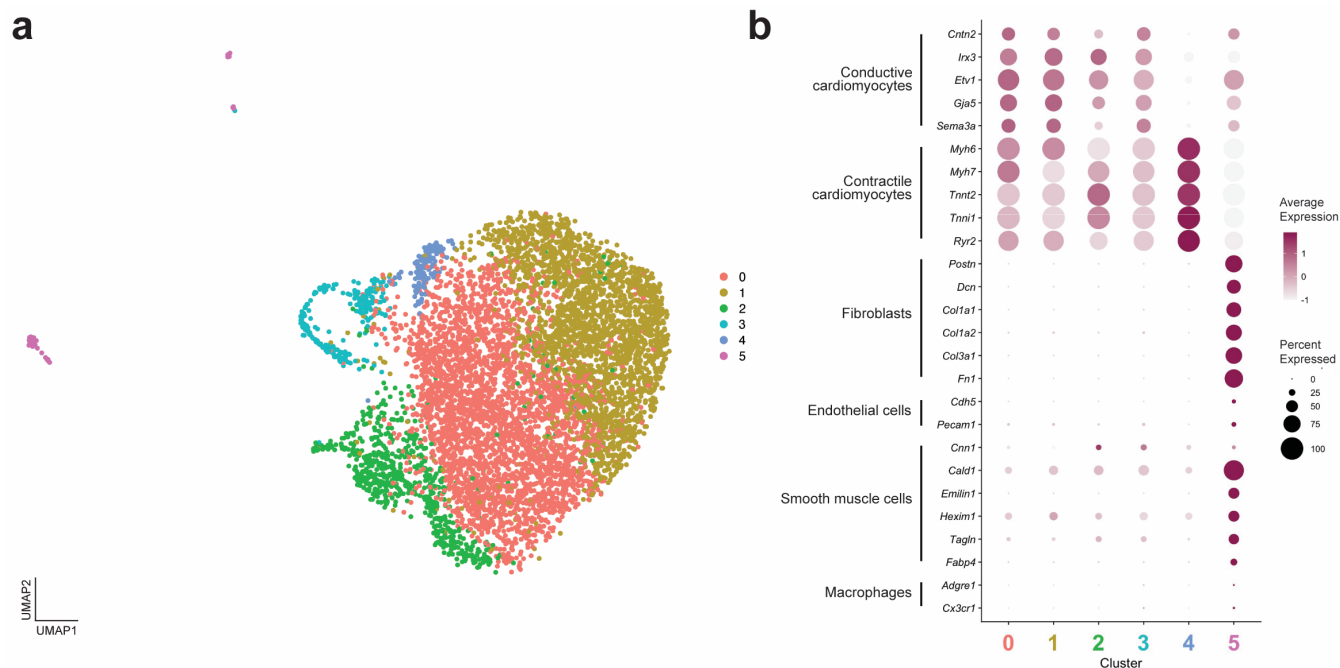
a Representative brightfield and fluorescent images of the CCS-tdT reporter (*Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+}) mouse heart at postnatal day 4 (P4). Scale bar = 1 mm. LV/RV, Left/Right ventricle. **b-g** Confocal microscope images of the CCS components of the CCS-tdT mouse heart, including (**b**) SAN, (**c**) HIS, (**d**) LBB, (**e**) RBB, (**f**) LPF and (**g**) RPF. tdTomato-marked CCS components (red) were stained with antibodies targeting endogenous Cntn2 (green). Scale bar = 50 μm. SAN, sinoatrial node; HIS, His bundle; LBB, left bundle branch; RBB, right bundle branch; LPF, left Purkinje fiber; RPF, right Purkinje fiber.



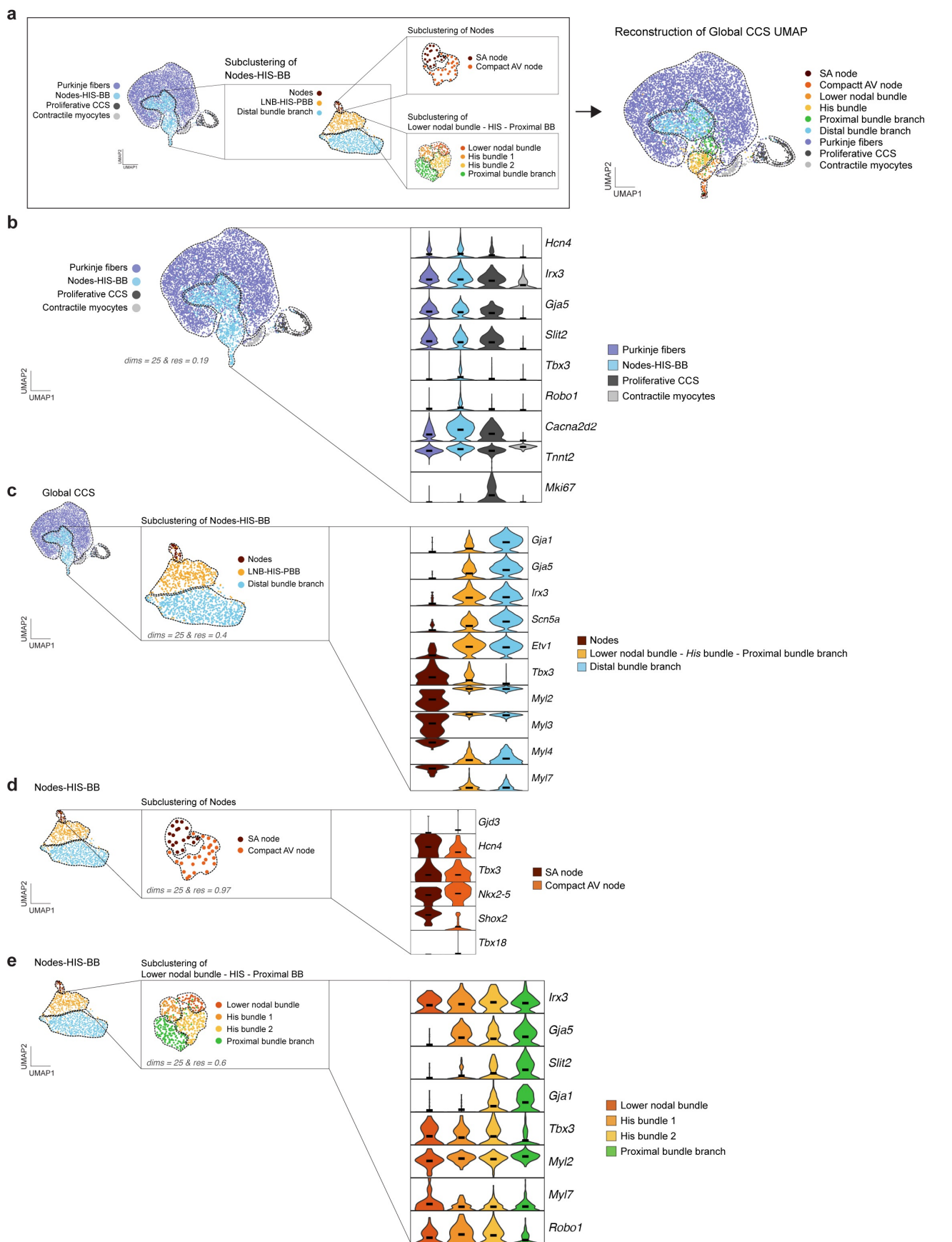
Supplementary Fig. 2. Fluorescence-activated cell sorting (FACS) of P1, P2 and P4 CCS cells. Gating was performed for size and granularity, then for Sytox-based cell viability, and finally for tdTomato-based identification of the CCS cells. Isolated neonatal mouse cardiomyocytes from P1, P2 and P4 consist of comparable percentages of CCS cells (0.1 - 0.2%) of viable cardiomyocytes.



Supplementary Fig. 3. Quality assessment of single-cell RNA-sequencing. **a-b** Histograms of mitochondrial content (%), number of detected genes, and number of unique molecular identifiers (UMIs) of **(a)** pre-processed and **(b)** post-processed scRNA-seq data. **c-d** Violin plots showing percentages of mitochondrial content (%), number of detected genes, and number of UMIs per cell from **(c)** pre-processed and **(d)** post-processed scRNA-seq data in P1, P2 and P4 cells. Each dot represents a single cell.

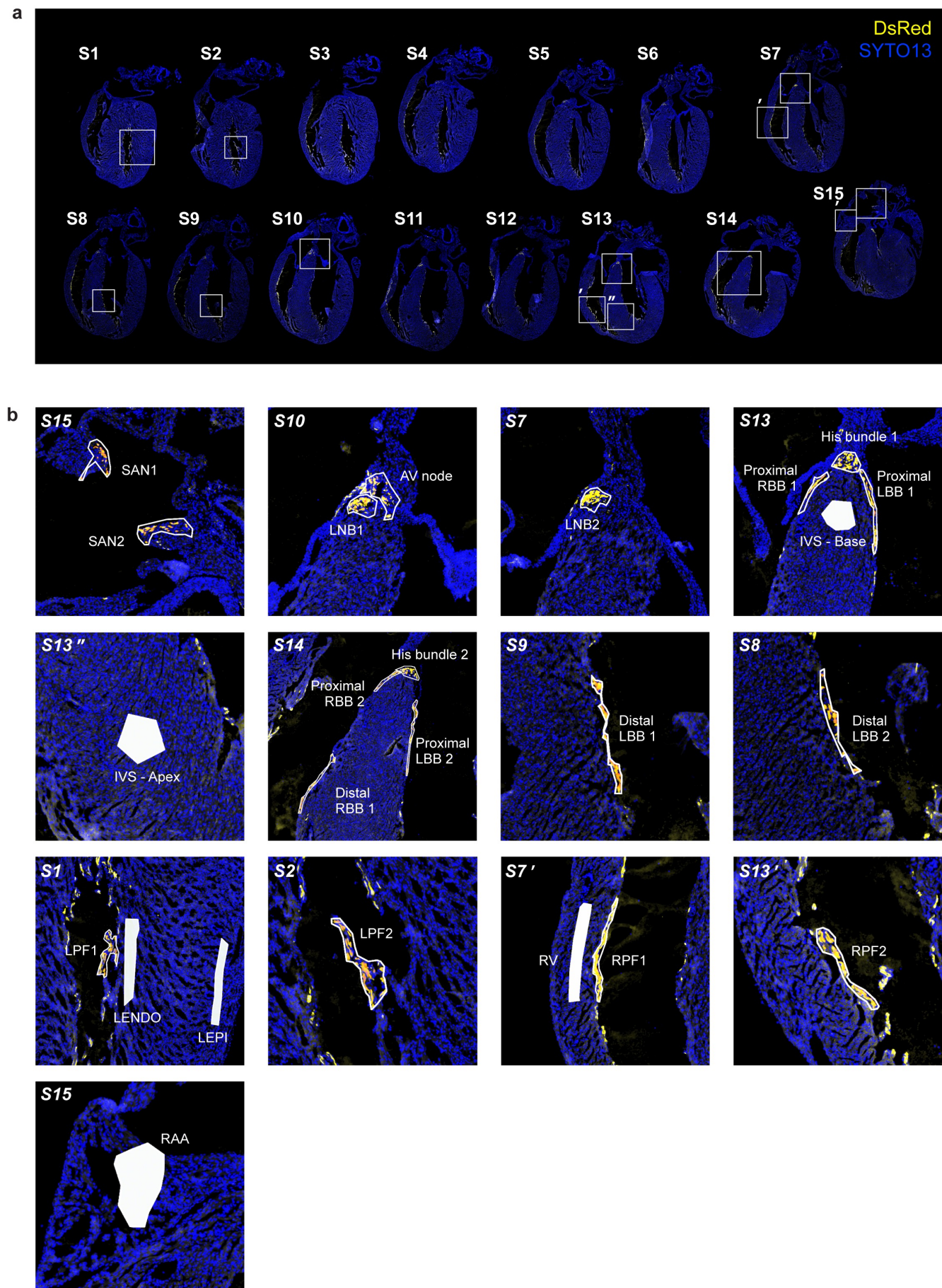


Supplementary Fig. 4. Identification of cardiomyocyte and non-cardiomyocyte populations. **a** UMAP clustering of all sequenced cells. **b** Dot plot showing enriched cardiac genes (*Myh6*, *Myh7*, *Tnnt2*, *Tnni1*, *Ryr2*) in clusters 0-4 and fibroblast/smooth muscle cell markers in cluster 5. Cells belonging to cluster 5 were removed for downstream cardiac conduction system (CCS) analysis.

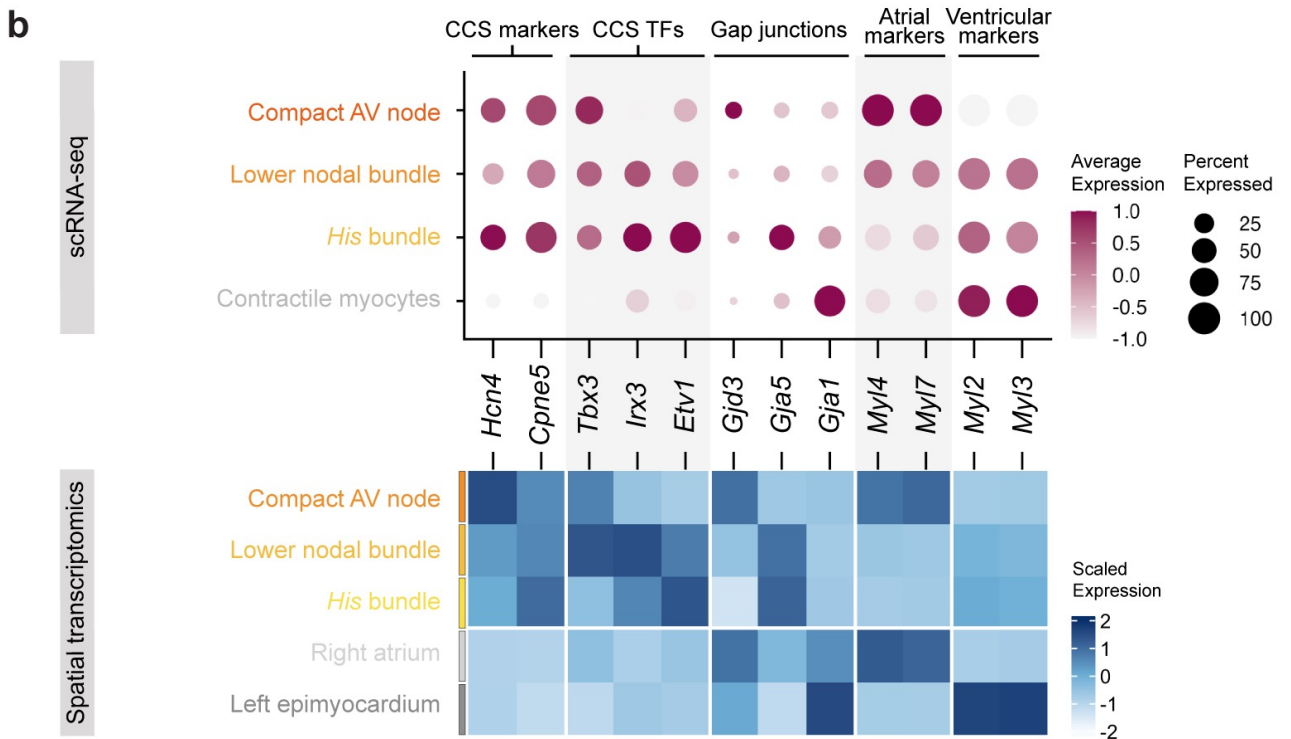
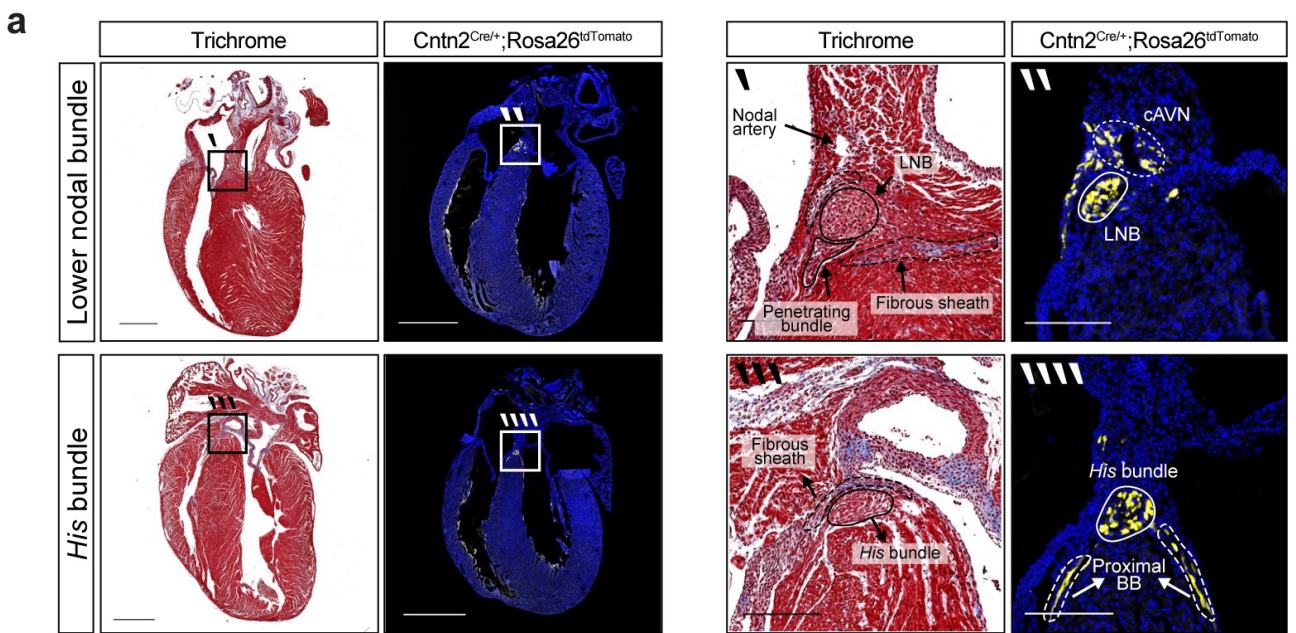


Supplementary Fig. 5. Identification of CCS components in scRNA-seq data through a series of subclustering analyses.

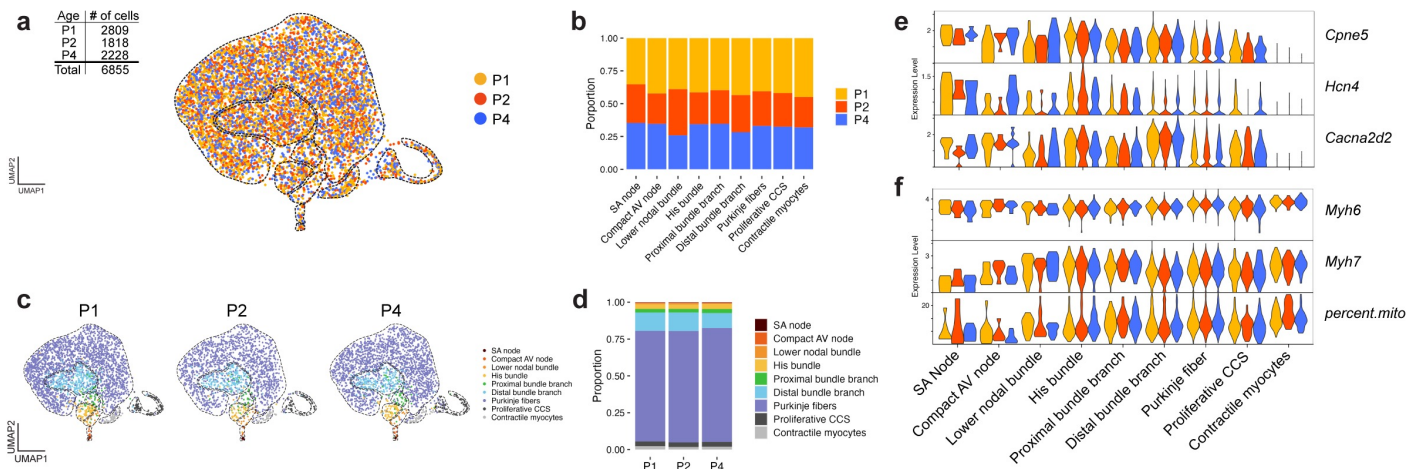
a Overall schematic showing the process of subclustering analyses and reconstruction of the global CCS UMAP. **b** Subclustering analysis of the global CCS identifies *Purkinje* fibers (*Hcn4*⁺/*Tbx3*⁺/*Robo1*⁺), *Nodes-HIS-BB* (*Tbx3*⁺/*Cacna2d2*⁺/*Robo1*⁺), *Proliferative CCS* (*Hcn4*⁺/*Mki67*⁺) and *Contractile myocytes* (*Hcn4*⁺/*Tnnt2*^{high}). **c** Subclustering analysis of the *Nodes-HIS-BB* identifies the *Nodes* (*Hcn4*⁺/*Myl4*⁺/*Myl7*⁺/*Myl2*⁺/*Myl3*⁺/*Irx3*⁺/*Tbx3*⁺), *LNB-HIS-PBB* (*Gja1*^{low}/*Gja5*^{low}/*Scn5a*^{low}) and *Distal Bundle Branch* (*Gja1*^{high}/*Gja5*^{high}/*Scn5a*^{high}). **d** Subclustering analysis of the *Nodes* identifies the *SA node* (*Hcn4*^{high}/*Shox2*⁺) and *Compact AV node* (*Gjd3*⁺/*Shox2*⁺). **e** Subclustering analysis of the *LNB-HIS-PBB* identifies the *Lower nodal bundle* (*Irx3*⁺/*Gja5*⁺), *His bundle 1* (*Irx3*⁺/*Gja5*⁺/*Slit2*^{low}/*Gja1*⁺/*Tbx3*⁺), *His bundle 2* (*Irx3*⁺/*Gja5*⁺/*Slit2*^{high}/*Gja1*⁺/*Tbx3*⁺), and *Proximal bundle branch* (*Irx3*⁺/*Gja5*⁺/*Slit2*^{high}/*Gja1*⁺/*Tbx3*⁺). *His* bundle 1 and 2 were combined for downstream analysis. LNB, lower nodal bundle; HIS, *His* bundle; BB, bundle branches; CCS, cardiac conduction system.



Supplementary Fig. 6. CCS regions selected for spatial transcriptomics. **a** A scanned fluorescent image of the single slide containing continuous cryosections of P4 CCS-tdT reporter hearts, encompassing all CCS components. Section 1 (S1) through 15 (S15) were collected from the same heart, ranging from posterior (S1) to anterior (S15) regions. tdTomato was stained with anti-DsRed antibody to amplify the signal for capture (yellow) and nuclei with SYTO13 (blue). **b** Zoomed images of CCS regions captured by segmentation for spatial transcriptomics data of the study. SAN, sinoatrial node; LNB, lower nodal bundle; LBB/RBB, left/right bundle branch; IVS, interventricular septum; LPF/RPF, left/right Purkinje fiber; RAA, right atrial appendage; RV, right ventricle; LENDO, left ventricular endomyocardium; LEPI, left ventricular epimyocardium.

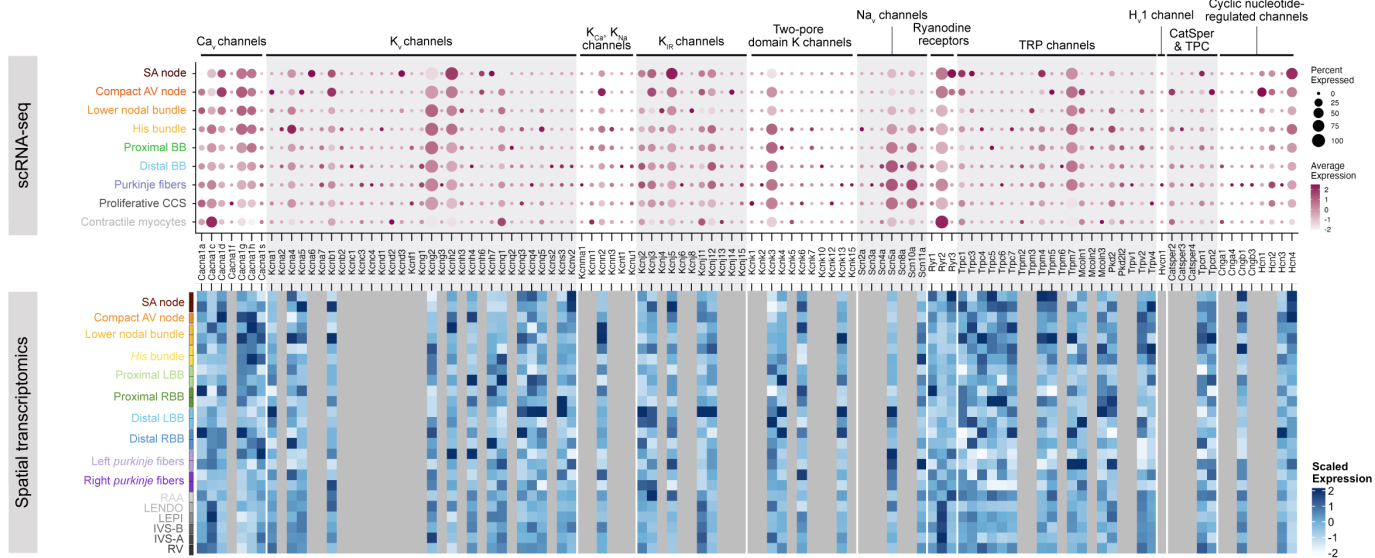


Supplementary Fig. 7. Distinct histological and molecular characteristics of the compact AV node (cAVN), lower nodal bundle (LNB) and His bundle (HIS). **a** Trichrome staining and fluorescence imaging of the lower nodal bundle (LNB) of AV node and His bundle show their distinct anatomical and histological landmarks. LNB is found above an atrioventricular fibrous sheath, while the HIS is found below. The LNB is linked to a structure called the “penetrating bundle,” which eventually becomes the HIS. Scale bar = 200 μ m. **b** Comparison of the transcriptomes of the compact AVN, LNB and HIS using scRNA-seq and spatial transcriptomics shows that all three structures are *Hcn4*⁺/*Cpne5*⁺ conduction cells with distinct molecular profiles: cAVN (*Hcn4*⁺/*Cpne5*⁺/*Tbx3*⁺/*Irx3*⁺/*Etv1*⁺/*Gjd3*⁺/*Gja5*⁺/*Gja1*⁺/*Myl4*⁺/*Myl7*⁺/*Myl2*⁺/*Myl3*⁺), LNB (*Hcn4*⁺/*Cpne5*⁺/*Tbx3*⁺/*Irx3*⁺/*Etv1*⁺/*Gjd3*⁺/*Gja5*⁺/*Gja1*⁺/*Myl4*⁺/*Myl7*⁺/*Myl2*⁺/*Myl3*⁺) and HIS (*Hcn4*⁺/*Cpne5*⁺/*Tbx3*⁺/*Irx3*⁺/*Etv1*^{high}/*Gjd3*⁺/*Gja5*^{high}/*Gja1*⁺/*Myl4*⁺/*Myl7*⁺/*Myl2*⁺/*Myl3*⁺).

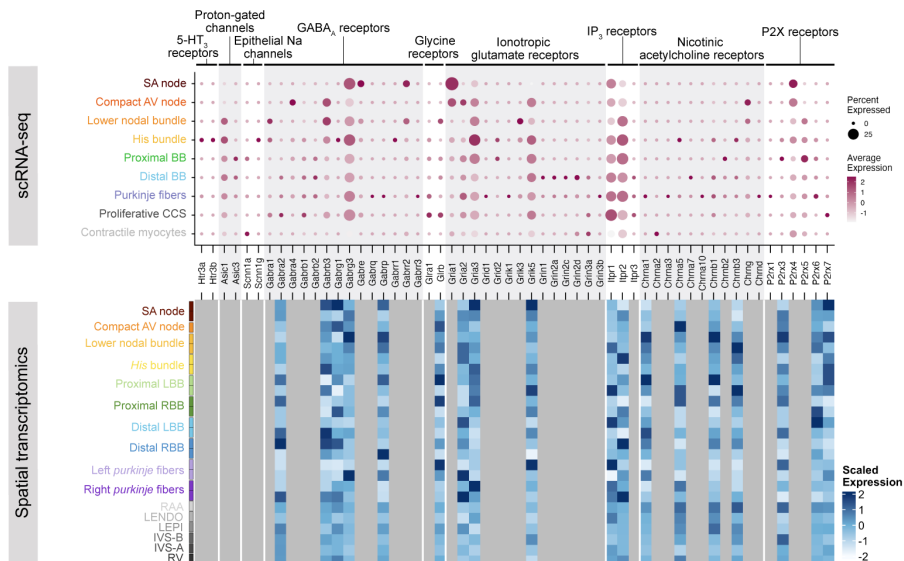


Supplementary Fig. 9. CCS cells undergo minimal transcriptomic changes during early postnatal development. **a** UMAP of CCS cells labelled by developmental stages (P1, P2 and P4) and the number of P1, P2 and P4 CCS cells sequenced. **b** Stacked bar graphs showing that all CCS components contain comparable proportions of P1, P2 and P4 cells. **c** Individual UMAPs for P1, P2 and P4 CCS cells. **d** Stacked bar graphs showing different developmental stages contain comparable proportions of each CCS cluster. **e-f** Expression levels of **(e)** conductive genes (*Cntn2*, *Cpne5*, *Cacna2d2*) and **(f)** cardiac maturation markers (*Myh6*, *Myh7*, mitochondrial content) are comparable in P1, P2 and P4 in all clusters.

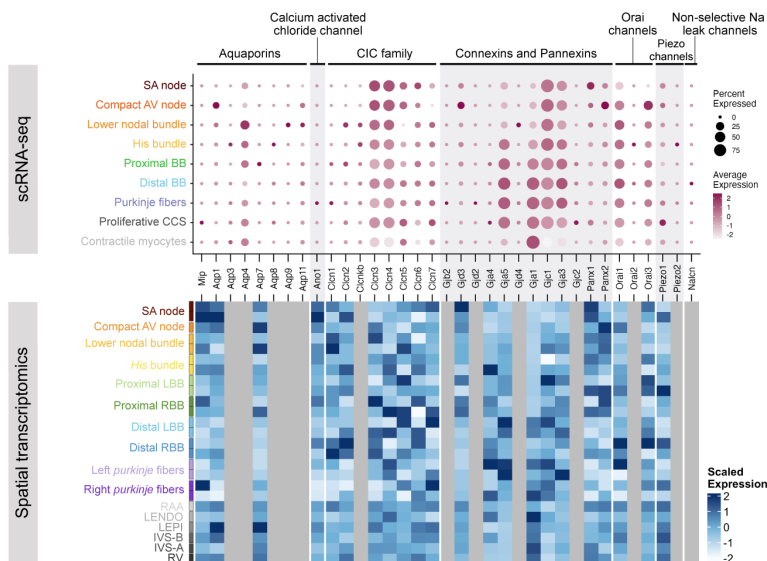
a



b

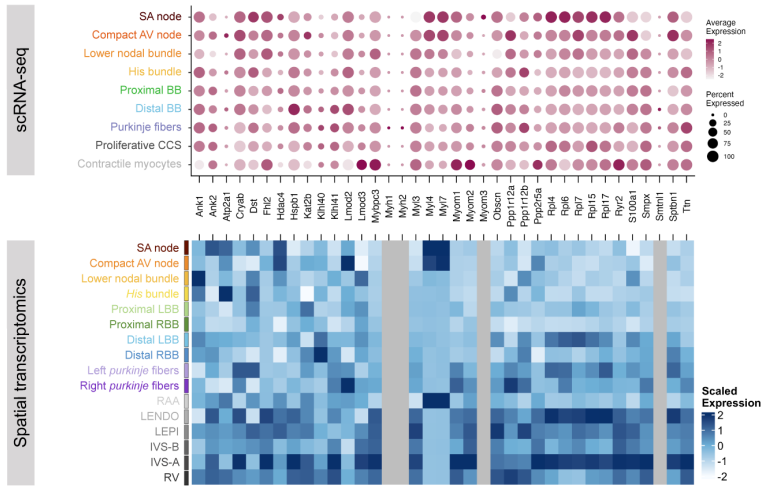


c

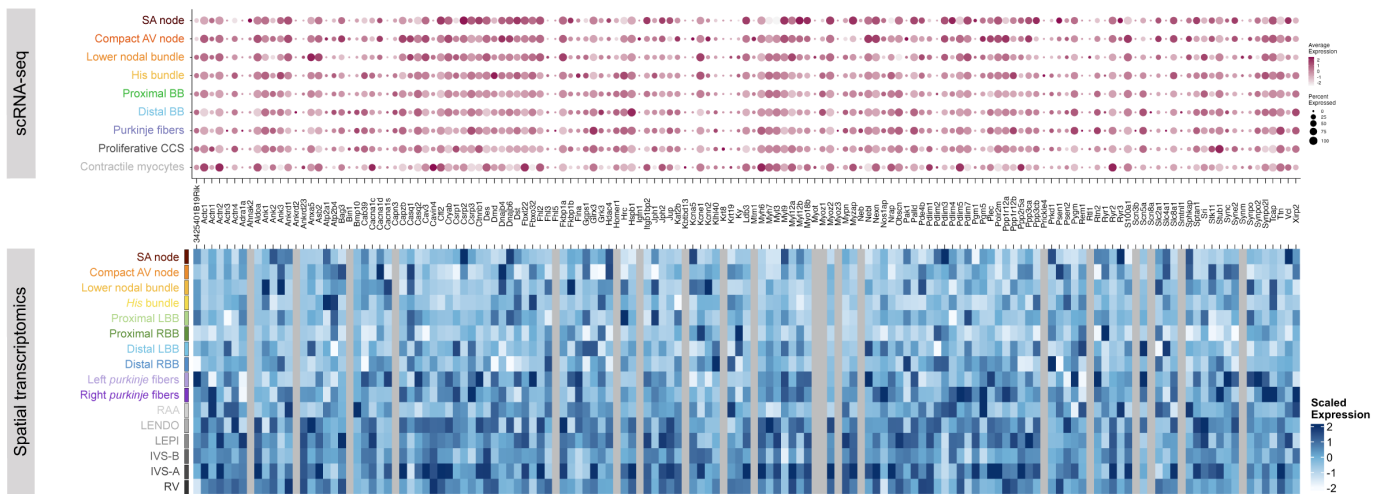


Supplementary Fig. 10. Ion channel profiling within the CCS using scRNA-seq and spatial transcriptomics. Expression patterns of genes annotated with (a) voltage-gated ion channels, (b) ligand-gated ion channels, and (c) other ion channels. Gene lists obtained from IUPHAR/BPS Guide to PHARMACOLOGY. LBB/RBB, left/right bundle branch; RAA, right atrial appendage; LENDO, left ventricular endomyocardium; LEPI, left ventricular epimyocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

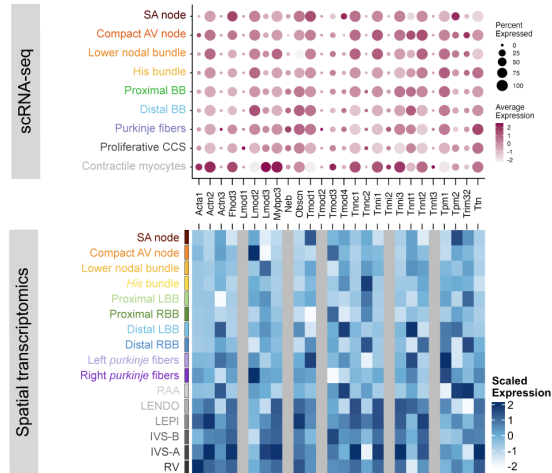
a A band (GO:0031672)



b I band (GO:0031674)

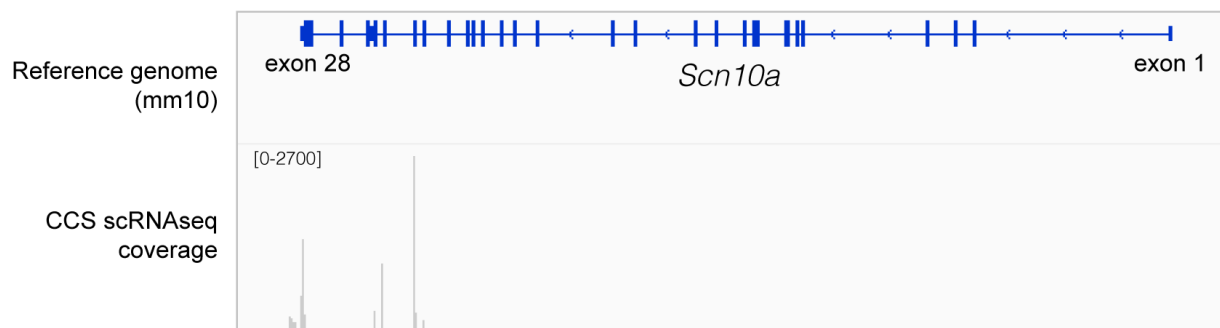


c Striated muscle thin & thick filament (GO:0005863 & GO:0005865)

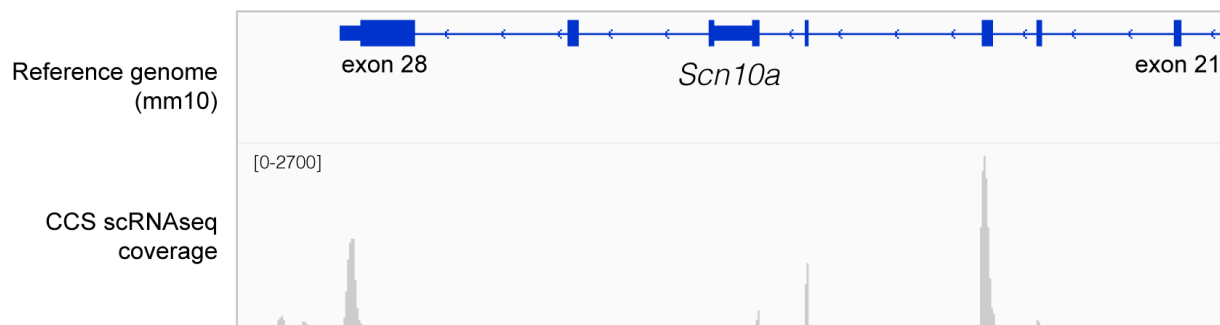


Supplementary Fig. 11. Sarcomeric gene profiling within the CCS using scRNA-seq and spatial transcriptomics. Expression patterns of genes annotated with (a) A band (GO:0031672), (b) I band (GO:0031674), and (c) striated muscle thin (GO:0005863) and thick filament (GO:0005865). LBB/RBB, left/right bundle branch; RAA, right atrial appendage; LENDO, left ventricular endomyocardium; LEPI, left ventricular epimyocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

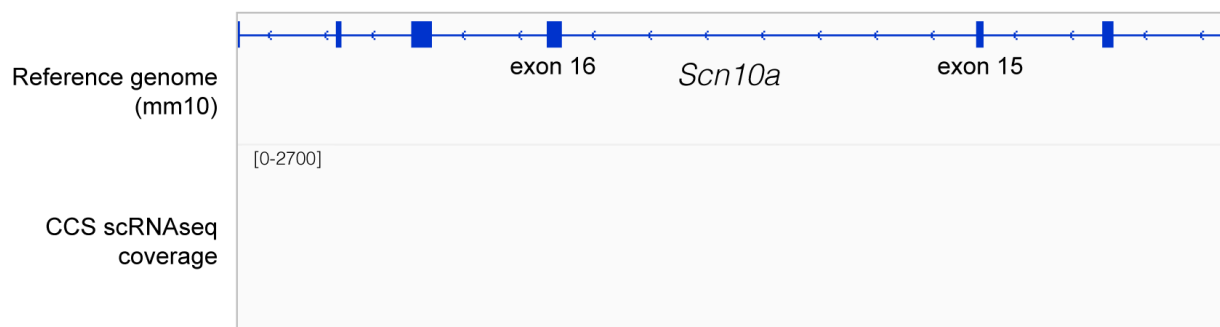
a *Scn10a* (full transcript) - chr9:119,608,467-119,719,322 (-)



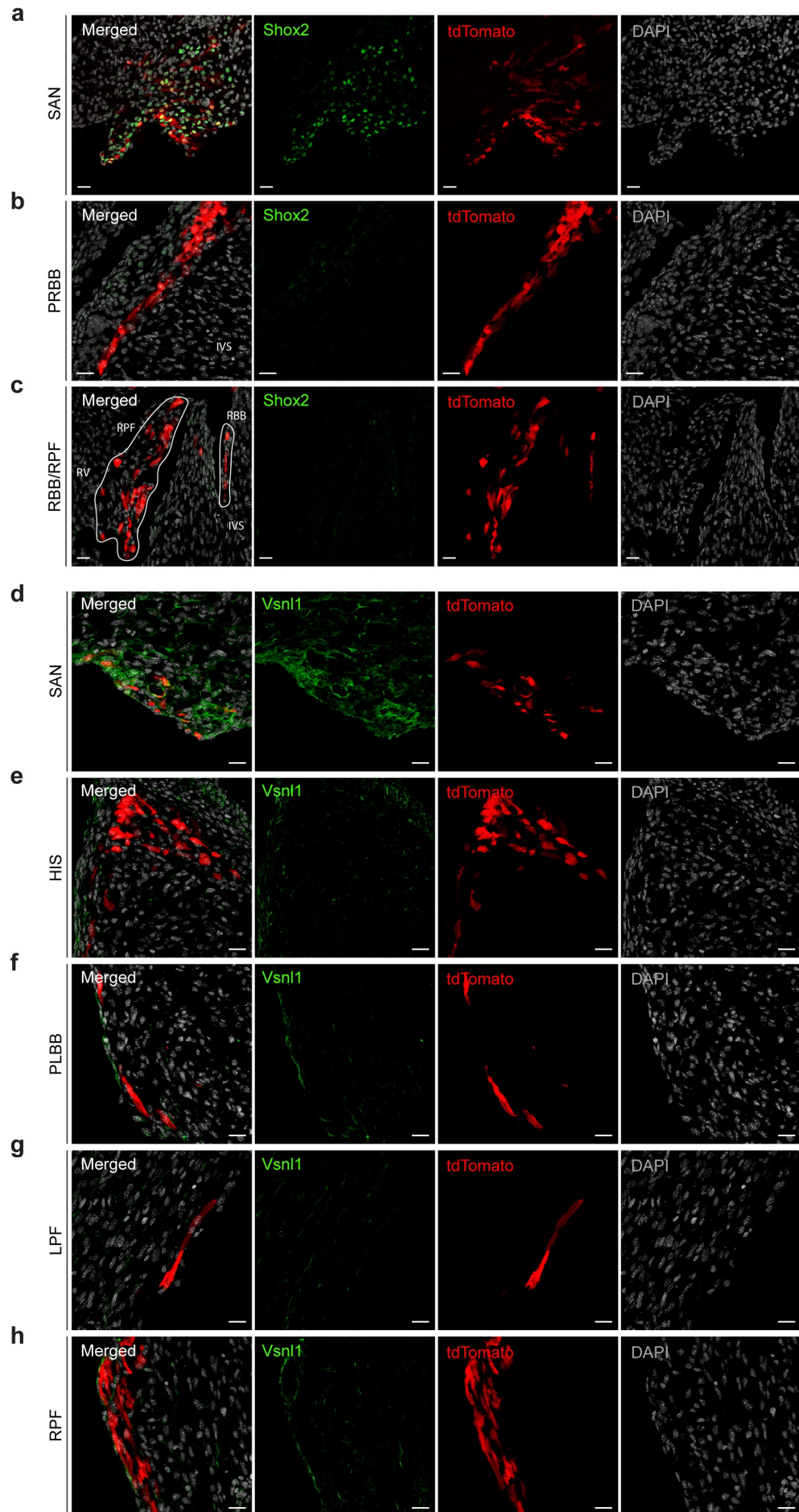
b *Scn10a* (exons 21-28)



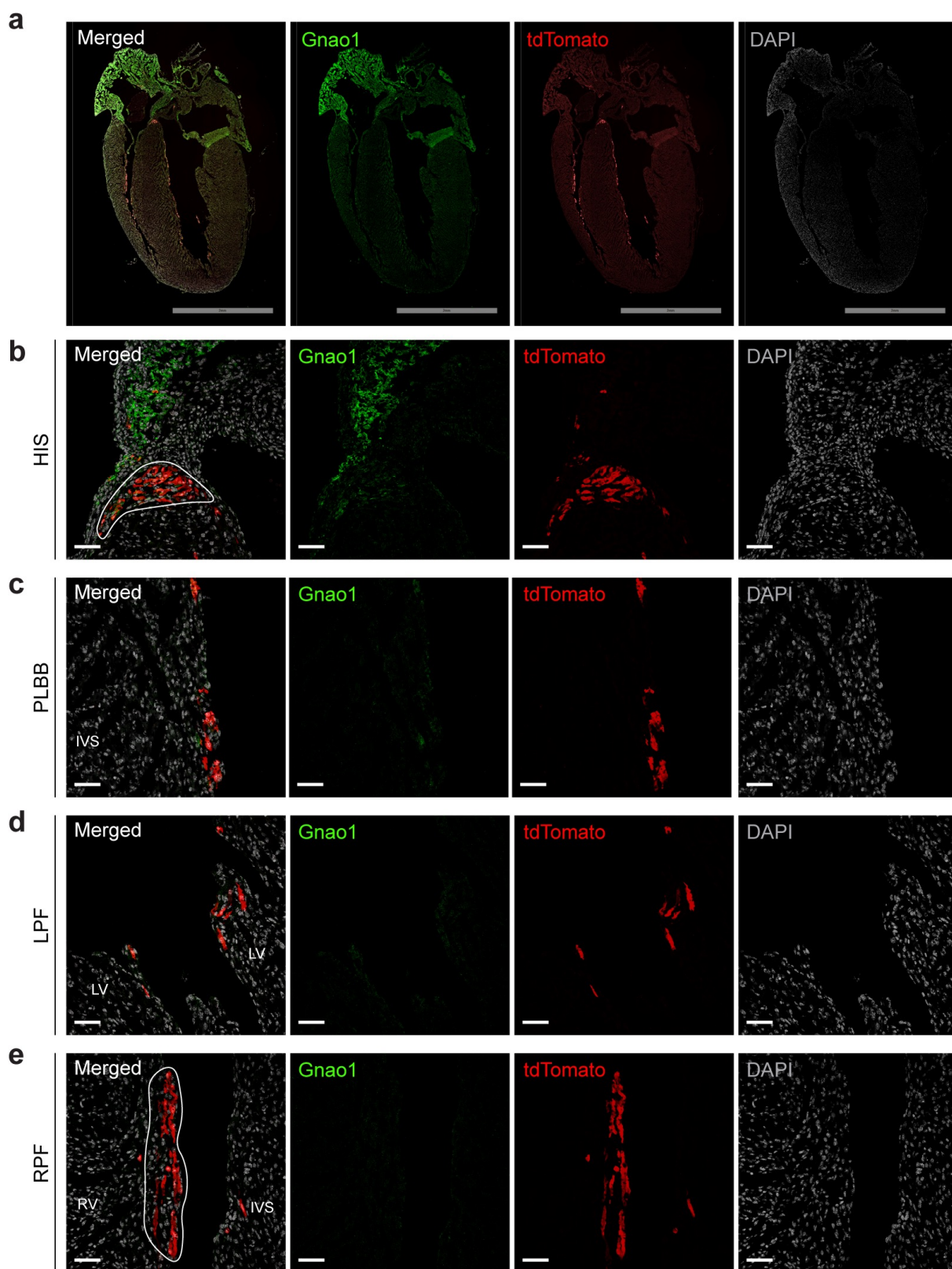
c *Scn10a* (exons 15-16)



Supplementary Fig. 12. scRNA-seq of mouse postnatal CCS captures predominantly the short form of *Scn10a*. Alignment of scRNA-seq reads to (a) full, (b) short form and (c) long form of *Scn10a* transcript, shows high coverage of regions spanning exons 21-28 of *Scn10a* (short form of *Scn10a* transcript) than the rest of the transcript. mm10 was used as the mouse reference genome. Integrative genomics Viewer (IGV, v2.17.4) was used for reads alignment

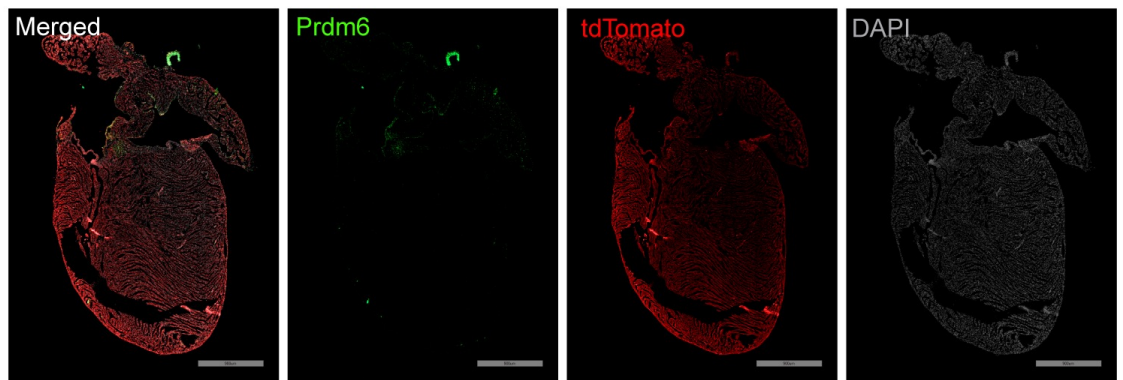


Supplementary Fig. 13. Immunofluorescence staining of Shox2 and Vsn1 in a P4 CCS reporter heart allows identification of the SAN. Shox2 is expressed in the (a) SAN, but not in other CCS components, such as (b) proximal RBB and (c) RBB/RPF. Vsn1 is expressed in the (d) SAN, but not in other CCS components, such as (e) HIS, (f) proximal LBB, (g) LPF and (h) RPF. SAN, sinoatrial node; PLBB/PRBB, proximal left/right bundle branch; RBB, right bundle branch; LPF/RPF, left/right Purkinje fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 20 μm .

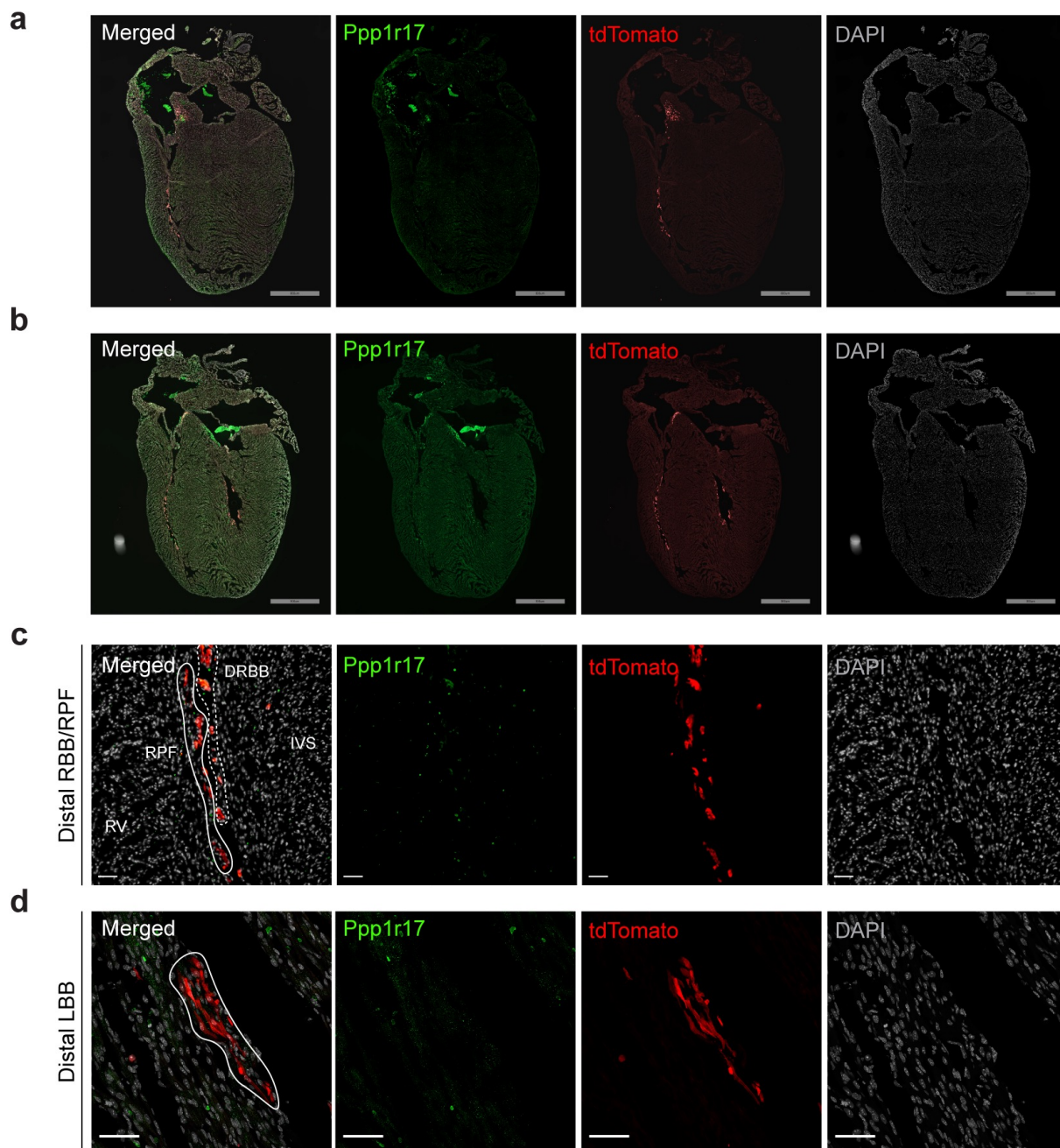


Supplementary Fig. 14. Immunofluorescence staining of Gnao1 in a P4 CCS reporter heart. **a** Fluorescence scanning of the stained whole heart. Scale bar = 2 mm. **b-e** Gnao1 is expressed in the SAN but not the **(b)** HIS, **(c)** proximal LBB, **(d)** LPF or the **(e)** RPF. cAVN, compact atrioventricular node; HIS, *His* bundle; PLBB, proximal left bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 50 μ m.

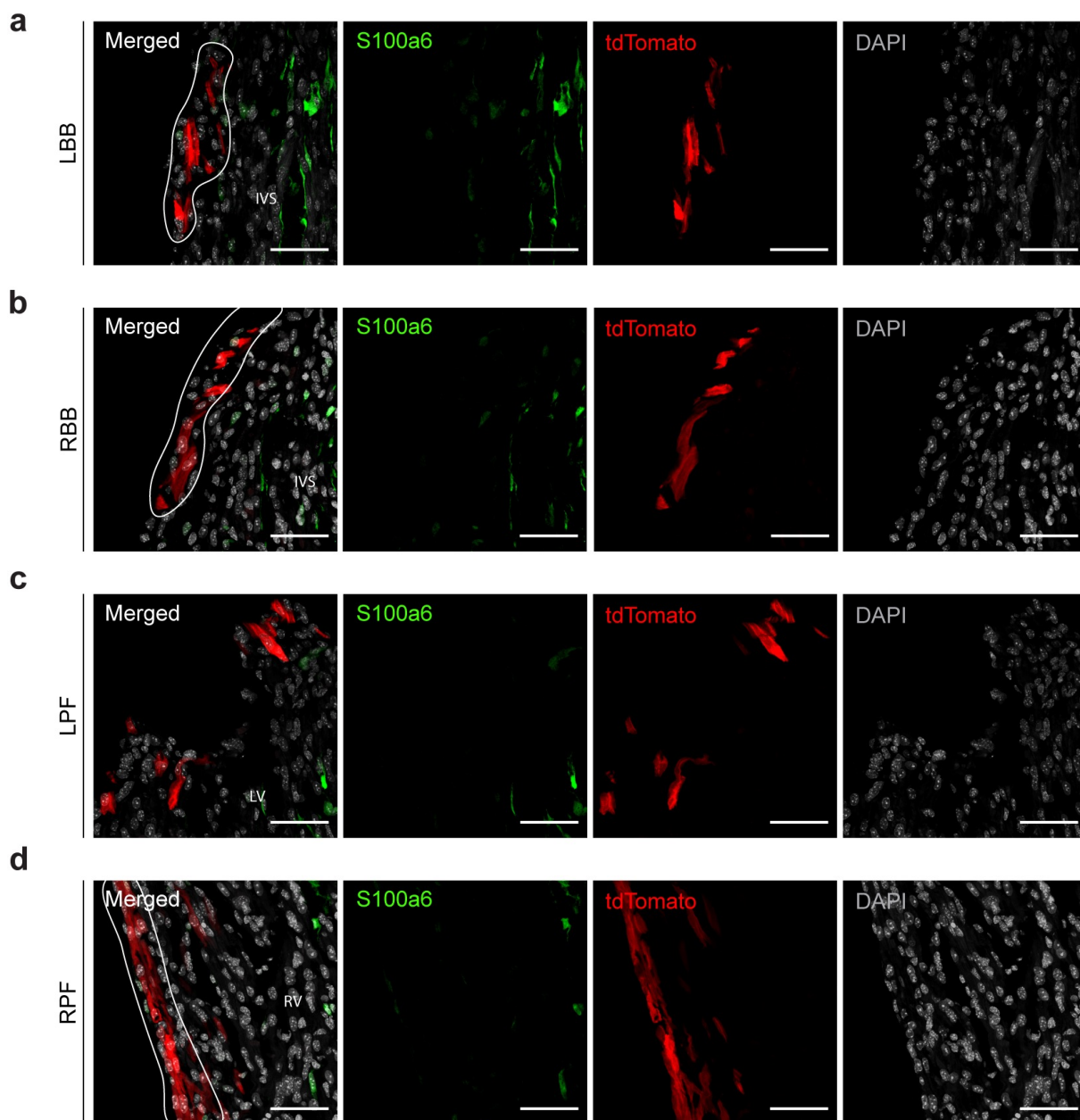
a



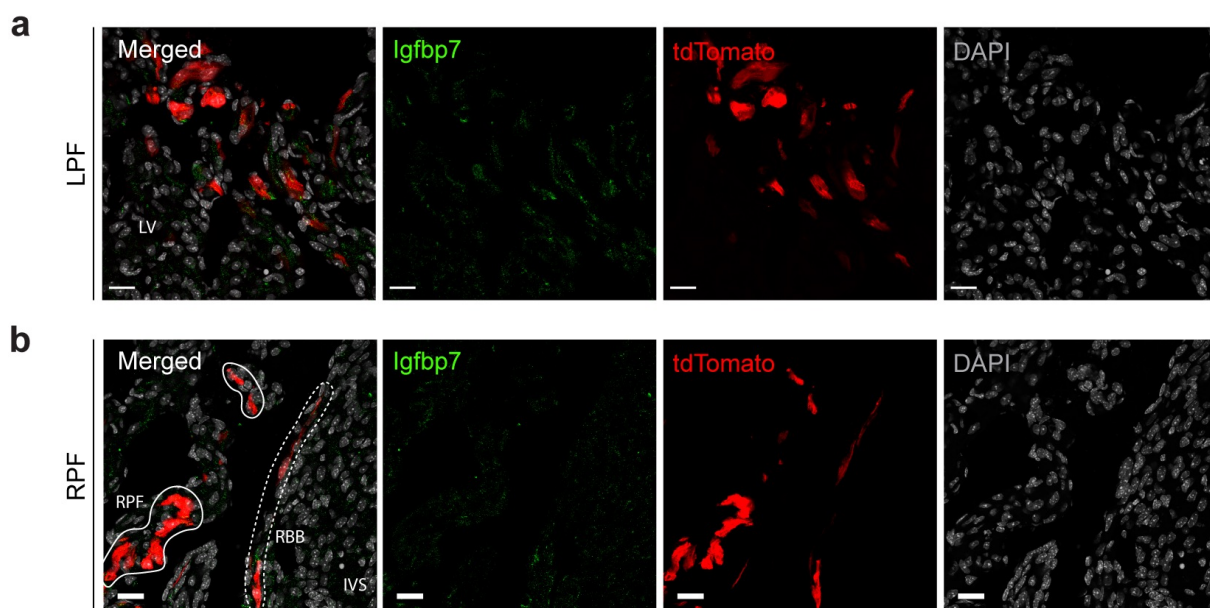
Supplementary Fig. 15. Fluorescence *in situ* hybridization (FISH) staining of *Prdm6* in a P4 CCS reporter heart shows specific expression of *Prdm6* in the compact AV node, but not in the lower nodal bundle and other CCS components. The zoomed-in figure is shown in Fig. 3b. Scale bar = 900 μ m.



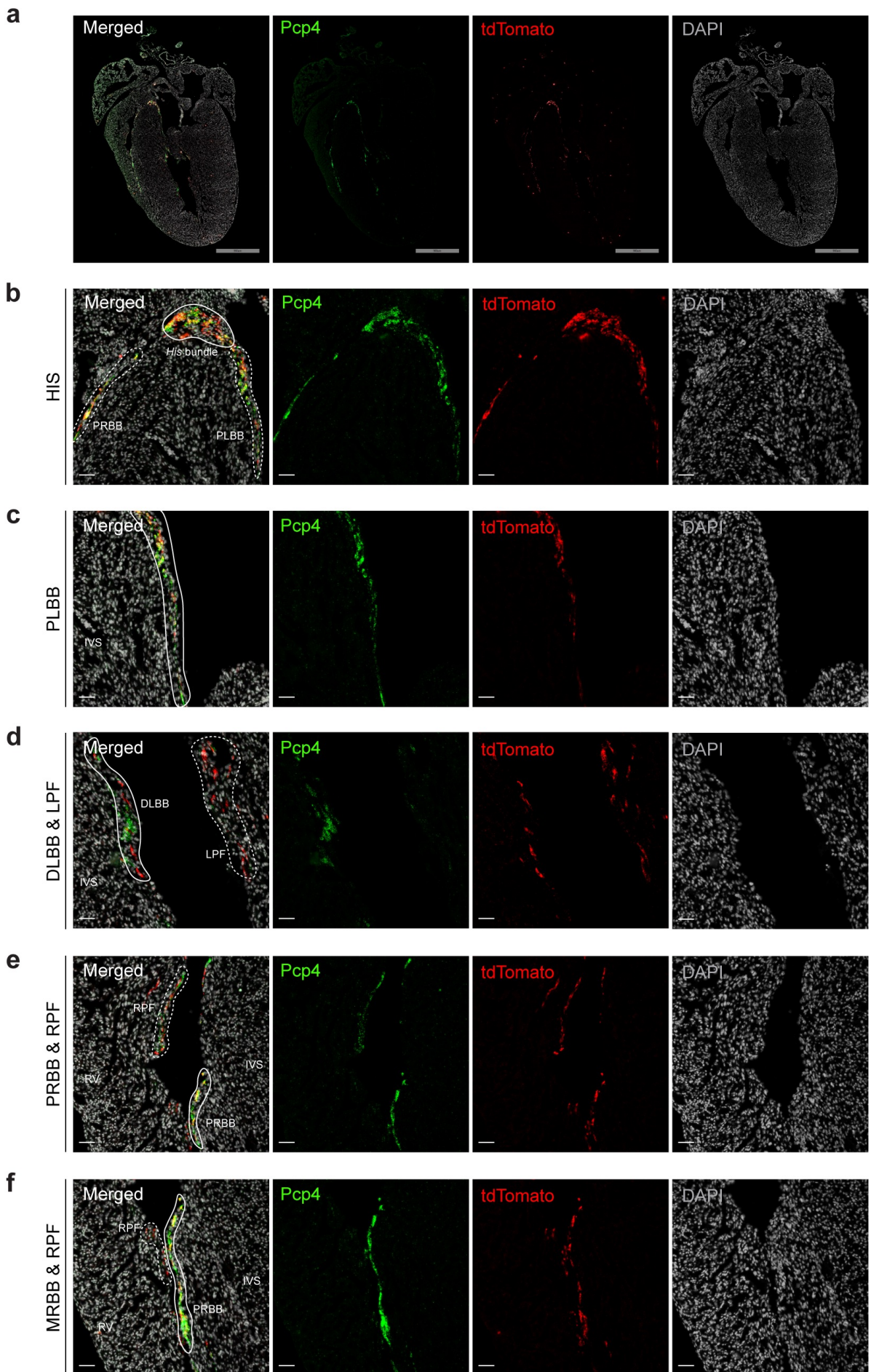
Supplementary Fig. 16. Immunofluorescence staining of Ppp1r17 in a P4 CCS reporter heart. **a-b** Fluorescence scanning of the stained whole heart section with **(a)** LNB and cAVN, and **(b)** HIS. Scale bar = 900 μm . **c-d** Ppp1r17 is not expressed in the **(c)** distal RBB/RPF or the **(d)** distal LBB. LBB/RBB, left/right bundle branch; RPF, right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 50 μm .



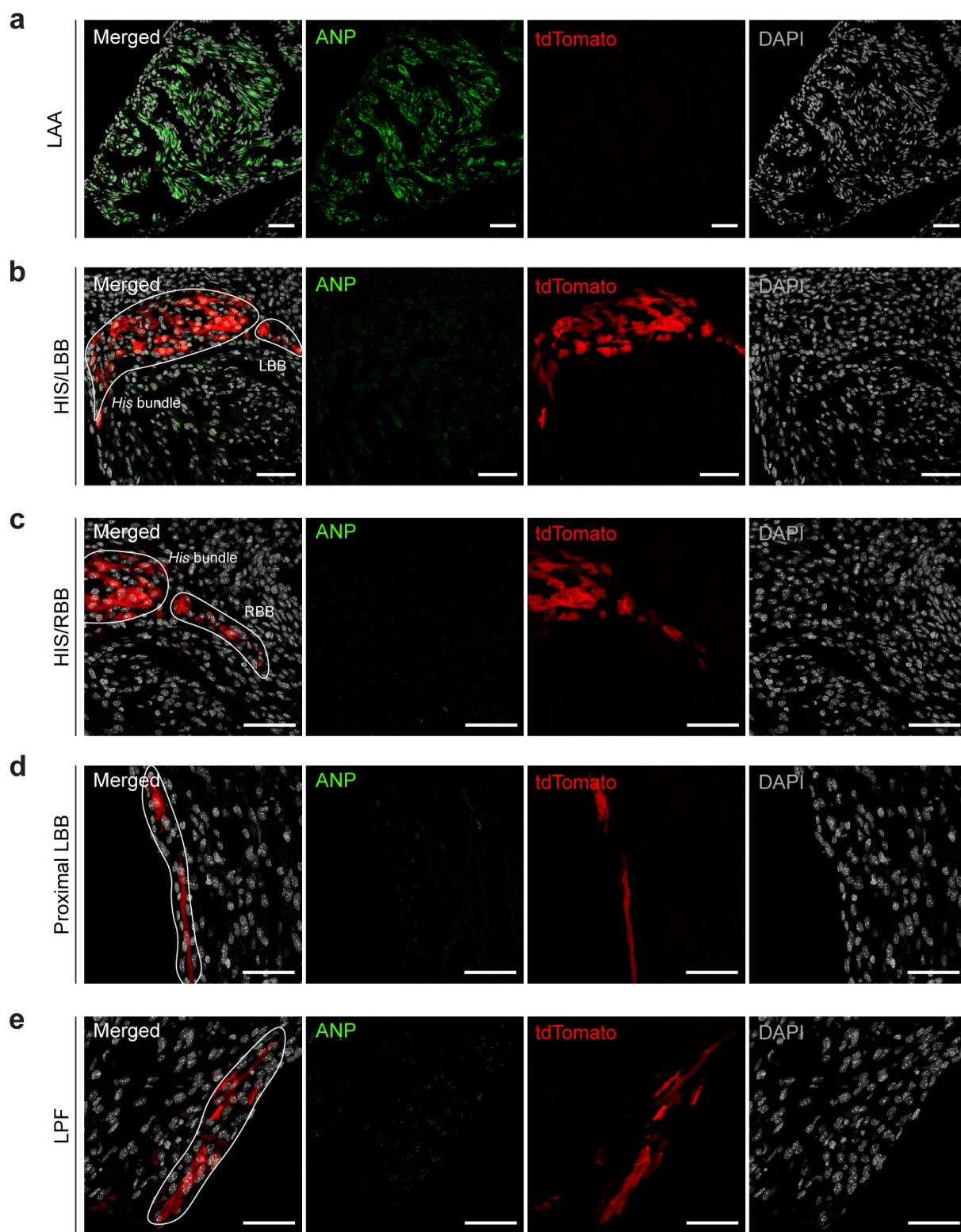
Supplementary Fig. 17. Immunofluorescence staining of S100a6 in a P4 CCS reporter heart shows absence of S100a6 in the (a) LBB, (b) RBB, (c) LPF or the (d) RPF. LBB/RBB, left/right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; LV/RV, left/right ventricle. Scale bar = 50 μ m.



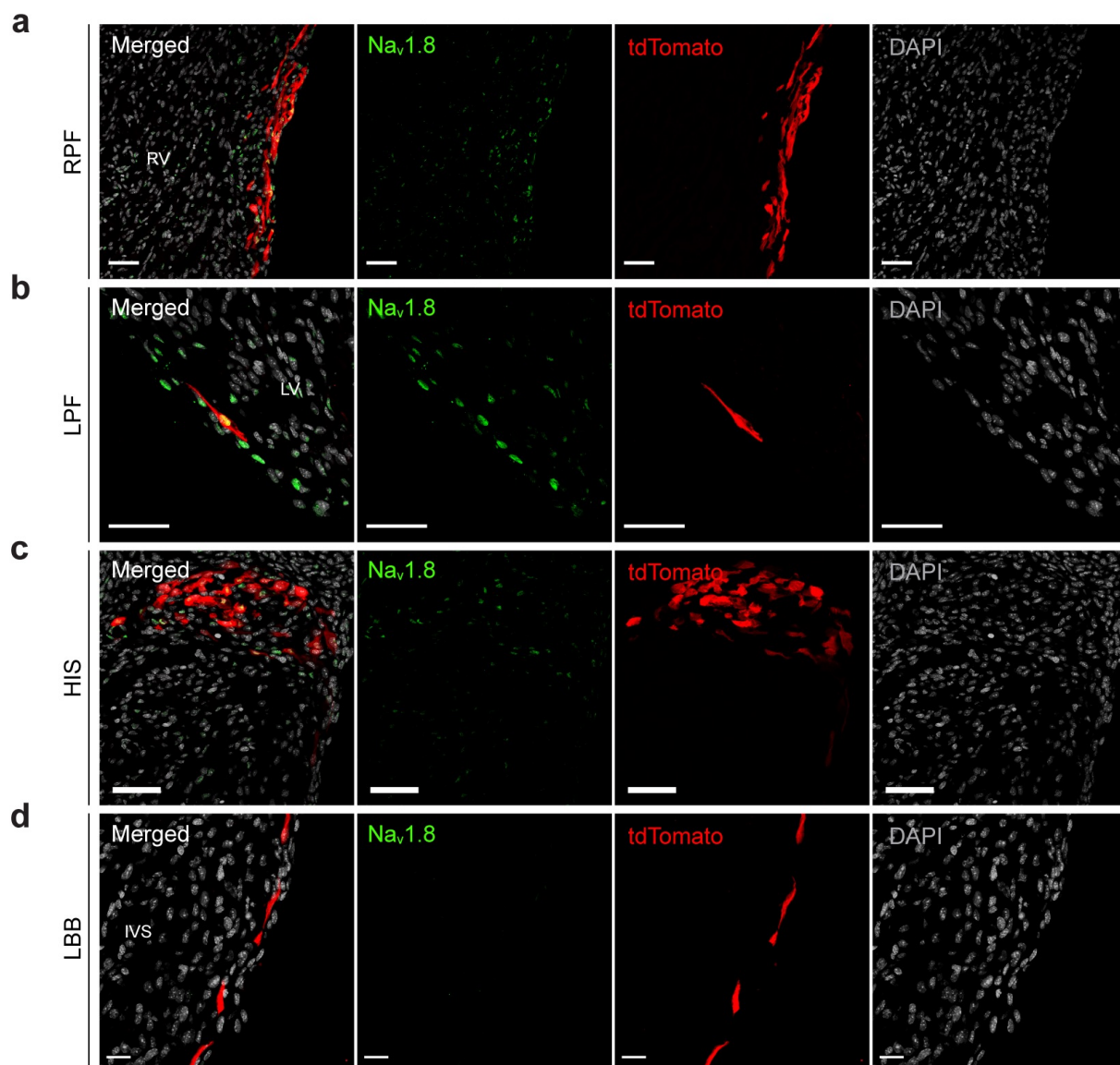
Supplementary Fig. 18. Immunofluorescence staining of Igfbp7 in a P4 CCS-tdT reporter heart shows absence of Igfbp7 in the (a) LPF and (b) RPF. RBB, right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; LV, left ventricle. Scale bar = 20 μ m.



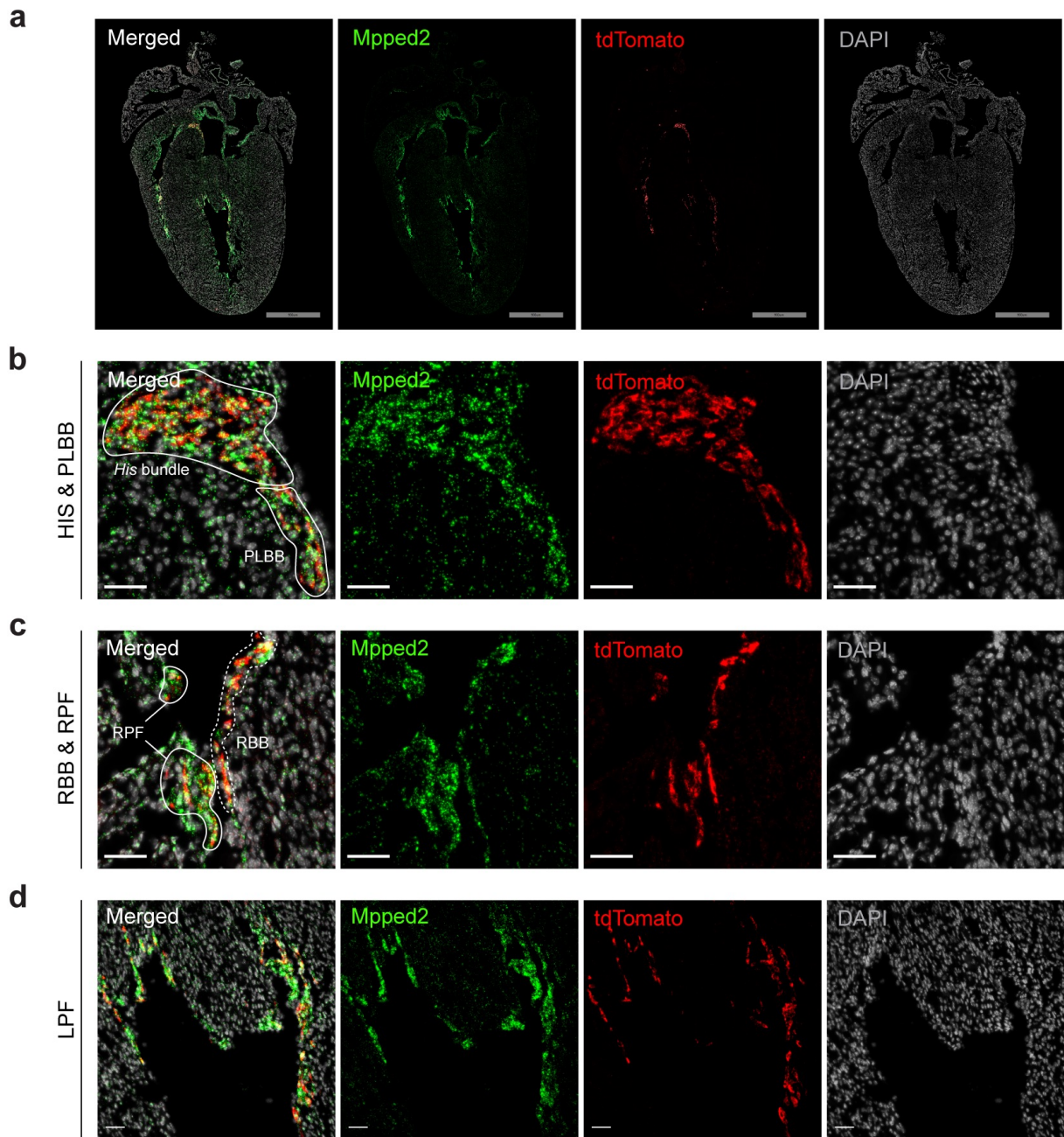
Supplementary Fig. 19. Fluorescence *in situ* hybridization (FISH) staining of Pcp4 in a P4 CCS reporter heart. **a** Fluorescence scanning of the stained whole heart. Scale bar = 900 μ m. **b-f** Pcp4 is expressed specifically in the CSS, including **(b)** HIS, **(c)** proximal LBB, **(d)** distal LBB/LPF, **(e)** proximal RBB/RPF or the **(f)** mid-RBB/RPF. HIS, *His* bundle; PLBB/PRBB, proximal left/right bundle branch; DLBB, distal left bundle branch; MRBB, mid right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 50 μ m.



Supplementary Fig. 20. Immunofluorescence staining of Nppa-encoded atrial natriuretic peptide (ANP) in a P4 CCS reporter heart. ANP is primarily expressed in the (a) LAA, but not in the (b) HIS/LBB, (c) HIS/RBB, (d) Proximal LBB or (e) LPF. LAA, left atrial appendage; HIS, *His* bundle; LBB/RBB, left/right bundle branch; LPF, left *Purkinje* fiber. Scale bar = 50 μ m.

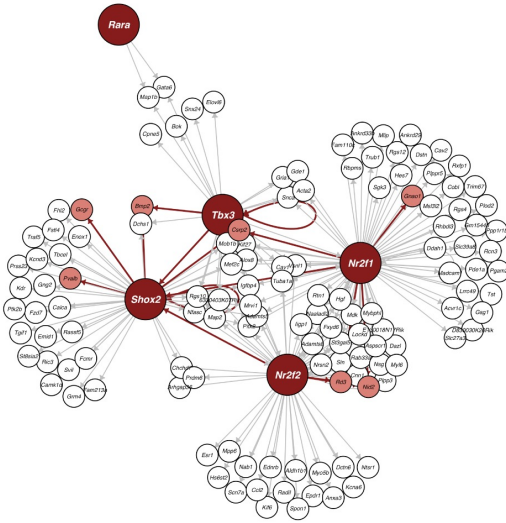


Supplementary Fig. 21. Immunofluorescence staining of *Scn10a*-encoded Nav1.8 in a P4 CCS reporter heart. Nav1.8, encoded by *Scn10a*, is enriched in the (a-b) distal VCS (RPF, LPF), but not in (c-d) proximal VCS (HIS, LBB). LPF/RPF, left/right Purkinje fibers; HIS, His bundle; LBB, left bundle branch; LV/RV, left/right ventricle; VCS, ventricular conduction system. Scale bar: (a-c) 50 μ m and (d) 20 μ m.



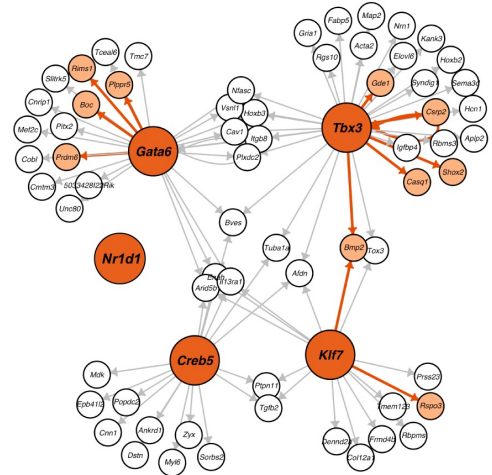
Supplementary Fig. 22. Fluorescence *in situ* hybridization (FISH) staining of *Mpped2* in a P4 CCS reporter heart. **a** Fluorescence scanning of the whole heart showing enrichment of *Mpped2* in the CCS and valves. Scale bar = 900 μm . **b-d** Representative images showing *Mpped2* expression in the **(b)** HIS bundle and PLBB, Scale bar = 20 μm , **(c)** RBB & RPF, scale bar = 20 μm and **(d)** LPF, scale bar = 50 μm . HIS, *His* bundle; PLBB, proximal left bundle branch; RBB, right bundle branch; LPF/RPF, left/right *Purkinje* fibers.

a

SA node

● Transcription factor (regulator)
● SAN-enriched target genes

b

Compact AV node

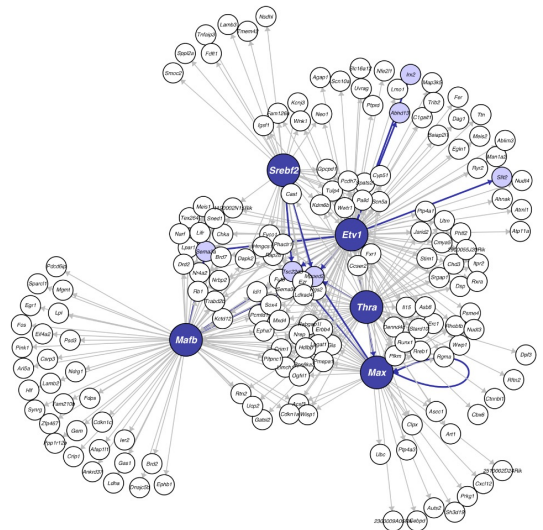
● Transcription factor (regulator)
● cAVN-enriched target genes

c

His bundle

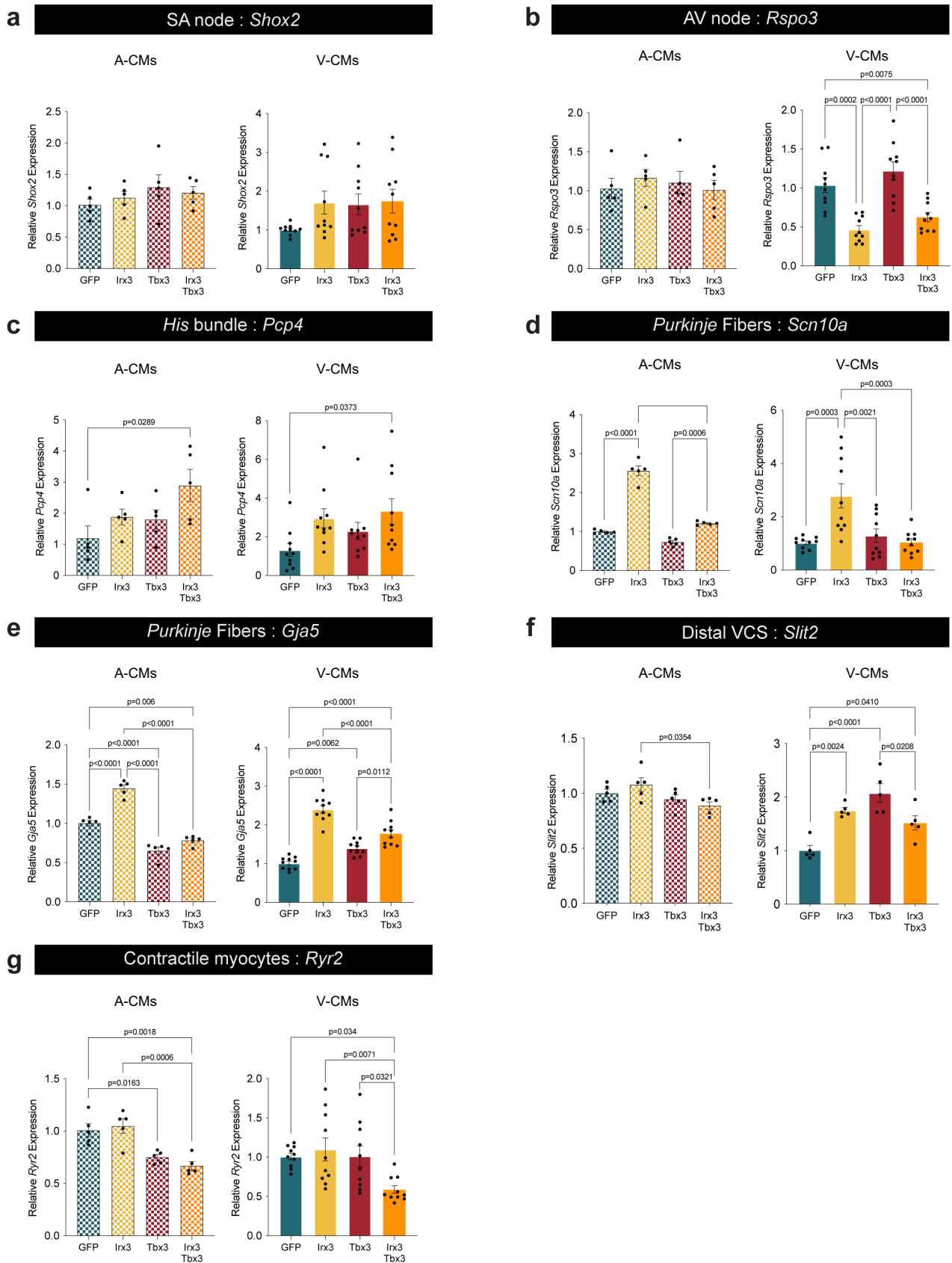
● Transcription factor (regulator)
● His bundle-enriched target genes

d

Purkinje fiber

● Transcription factor (regulator)
● Purkinje fiber-enriched target genes

Supplementary Fig. 23. Gene regulatory networks of CCS components. Gene regulatory networks featuring the top 5 regulons, enriched transcription factors (darker colours) and their target genes (lighter colours), in (a) SA node, (b) compact AV node, (c) His bundle and (d) Purkinje fiber, are presented.



Supplementary Fig. 26. Region-specific transcriptional regulation of CCS genes by *Tbx3* and *Irx3*. Gene expression levels of the CCS component and regional markers in neonatal mouse atrial cardiomyocytes (A-CMs) and ventricular cardiomyocytes (V-CMs) upon *Tbx3* and/or *Irx3* overexpression. Tested genes include (a) SA node marker, *Shox2*; (b) AV node marker, *Rspo3*; (c) His bundle marker, *Pcp4*; (d-e) Purkinje fiber markers, (d) *Scn10a* and (e) *Gja5*; (f) Distal VCS marker, *Slit2*, and (g) contractile myocyte marker, *Gja1*. Checkered boxes represent A-CMs, and solid bars represent V-CMs. $n = 5$ per group for A-CMs, and $n = 5$ or 10 for V-CMs. Data are presented as mean $\pm$ SEM. One-way ANOVA and Tukey's multiple comparisons test. Source data are provided as a Source Data file. VCS, ventricular conduction system; A-CMs, atrial cardiomyocytes; V-CMs, ventricular cardiomyocytes