

Peer Review File

Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Yena Oh and coworkers performed single cell RNA seq on isolated and purified Cntn2+ (Cntn2-Cre+) derived cardiomyocytes shortly after birth. These cells comprise the components of the conduction system, SAN, AVN, AVB, BB, PF, and probably some additional cardiomyocytes (the boundaries of the CCS components are not sharp, with “transitional cells” expressing only subsets of markers for that component).

Their analysis of the data revealed they successfully isolated and characterized cardiomyocytes of the different CCS components. The authors confirmed many previously identified expression patterns in CCS components and also found novel markers. In addition, using published ATAC-seq and Tbx3 ChIP-seq data sets (of a Tbx3 overexpression model), the authors mapped accessibility and Tbx3 occupancy of selected promoters/genes that correlated with the expected patterns for SAN, AVJ, A and V. They provide some functional data, overexpressing Tbx3 and Irx3 in atrial and ventricular cardiomyocytes, to see if these same selected genes would respond according to expectations.

While the scRNAseq data represents a valuable resource, it largely confirms previously published expression data, adding some novel markers. The bioinformatic analysis does not provide significant novel mechanistic insight. Furthermore, the functional analysis was performed using overexpression of Tbx3 and Irx3 and a very small target gene set as readout. That these factors are able to induce or reduce expression of particular target genes has been well established in knockout and overexpression models of either transcription factor gene. The in vitro experiment performed by authors is largely confirmatory, and does not yield novel mechanistic insight.

The comparison between CCS components is valuable. PF cardiomyocytes (and BB and to some extent AVB cardiomyocytes) share developmental history (lineage) with ventricular cardiomyocytes. It would have been interesting to compare these cells with ventricular cardiomyocytes.

A more extensive comparison of the data with published transcriptomes of CCS components could be presented.

Reviewer #2 (Remarks to the Author):

Comments on submission from Oh et al. 2023 to Nat Comm, entitled “Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity”

In the manuscript entitled “Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity”, the authors provide a description of the transcriptional landscape of the postnatal murine cardiac conduction system, providing particular robustness to the various cell types within the ventricular conduction system (VCS) (His, bundle branches and PFs), not previously captured. The authors go on to demonstrate sufficiency experiments in rat cardiomyocyte culture system to suggest a role for two established TFs (Irx3 and Tbx3) in regulating the expression of certain CCS-related genes. The authors collected the largest amount of CCS cells for analyses to date, however notably the nodal representation was surprisingly limited, with the dataset consisting almost exclusively of VCS conduction cells. Overall, while the work is of value, I believe the authors require significant work to both validate some of their findings as well as to rework the manuscript substantially for clarity as detailed in the major and minor comments below.

Major Comments:

1. The authors should, upfront, provide a clear definition of what they are calling Proximal vs. Distal CCS (page 4, line 26). The terms are not common use and could refer to prox vs. distal VCS versus, as the authors imply (I believe), proximal CCS including nodal tissue SAN, AVN, His bundle and bundle branches (i.e. everything except purkinje fibers – which is not a standard term for CCS classification). If the latter, then ideally their FindClusters single cell data analysis should be performed at a higher resolution to see if the individual clusters emerge at baseline, separating out these different subsets of cell types. This would help avoid confusion and muddled data interpretation by not having the nodal cells and proximal VCS combined as they are, as the authors point out, such distinct cell types.
2. Fig. 2c and S7a: Igfbp7 staining is ambiguous. There appears to be some baseline staining in the interventricular septum and more notably, in/around PFs in the supplement figure. To ensure a direct comparison in staining, the authors should present a single four chamber view with magnification of the His bundle region and Purkinje fiber from the same tissue slice, using the exact same exposure and laser intensity. If a higher mag is required, then the tissue slice can be imaged using tiling. Same for the Scn10a staining Fig 2f and S7b.
3. Fig. 3g, h, k, l in combination with Page 6, lines 30-36: same comment as above. If the authors want to claim protein-level validation of their findings of a “His-specific” (e.g. Pcp4) or “bundle-branch-specific” gene candidate, one would want to see a single staining of a tissue slice (again a standard four chamber view would suffice) that contains both the his bundle and bundle branches showing differential expression. To this point, Pcp4 is in fact expressed in the PFs and not His-specific (as previously established – Kim et al. JCI 2014). The authors, to their credit, do mention this in the discussion however it highlights the overall assertion that to make a claim for CCS subcomponent-specific expression, one would like to see (at least a handful of examples) with

proper validation by immunofluorescence. Given that almost all of their data represents the VCS, I believe the authors should really focus on determining and validating (with either IF vs. in situ hybridization of tissue slices) whether there are any truly significant molecular differences between the His, BBs and PFs.

4. Fig. 3h and Page 6, line 27-28. The authors state: “In the AV node, *Rspo3* expression was enriched, which was validated by immunostaining (Fig. 3f,h).” Enriched, presumably compared to the SAN nodal cells per their single cell analyses, however this is not shown by IF (only an AVN is shown). Does the SAN show relatively reduced *Rspo3* by staining? If so, it should be shown. Further, the outlined putative AVN shows expression of *Rspo3* in the putative AVN cells (labeled by tdTomato) as well as, seemingly, equally, in the surrounding interventricular septum cells. Does the working myocardium show similar levels of *Rspo3* transcript as the staining would suggest? The authors should comment on this specifically.

5. Supp Figure 5b: There is surprisingly low amounts of tdTomato expression in all of the sorted CCS cells, and is almost complete absent from the bottom-most cells of the “Prox CCS cells” cluster. How do the authors explain this? This fact should be highlighted in the text and explained in the discussion. Further, Fig3g would suggest that the tdTomato labeling of CCS cells is incomplete based on their stated outlines for the various CCS structures (i.e. it appears as if many CCS cells are not successfully labeled). The authors should provide a counterstain with endogenous *Cntn2* to demonstrate the degree to which the tdTomato labels each CCS component as well as to comment on whether all cell types are truly represented (e.g. SAN transitional cells).

6. The reviewer would like to see some additional verification that the stated cell annotations in their single-cell data are appropriate. Specifically, the rare cells annotated as “AV node” are *Tbx3*⁺ and negative for *Shox2*. But the authors should comment specifically on other classic AVN markers which should be *Hcn4*⁺/*Kcne1*⁺/*Cx30.2*⁺/*Cx45*⁺/*Cacna2d2*⁺/*Cx40*[−]/*Cx43*[−]/*Nppa*[−]/*Myl2*[−] as well as negative for all distal VCS markers *Etv1*[−]/*Scn5a*[−] (Bhattacharyya et al 2019 Sci Rep; Goodyer et al 2019 Circ Res). Further, for the SAN, the authors note that they did not appreciate *Tbx18* but did the authors not detect other expected markers *Hcn4*⁺/*Isl1*⁺? If not, the authors should clearly state this as a limitation (presumably due to the limited number of captured cells).

7. Section entitled: “Transcriptional diversity of key cardiac functional genes within the CCS”. The authors spend quite a bit of the manuscript detailing differential gene expression for various gene family members, with the many of the findings being previously established. While this is reassuring from a validation perspective, it would be helpful for the authors to clearly distinguish which are novel findings. Further, this reviewer would like the authors to specifically compare their findings to prior scRNAseq studies of the CCS (Goodyer et al. 2019 and (Bhattacharyya et al 2019 Sci Rep; Goodyer et al 2019 Circ Res) to see how they differ. In particular, since the authors found no significant change in differential gene expression between their three postnatal time points it would be interesting, from a developmental standpoint, to see if there were gene expression changes suggestive of maturation (one of their initial stated goals) between the E16.5 embryonic stage and postnatal stages of their data.

8. Page 9, Line 35-37. “On the other hand, *Shox2*, a well-known SA node marker, was not affected by *Tbx3* or *Irx3* in both A-CMs and V-CMs (Fig. S10a), supporting the result that *Shox2* likely acts earlier than *Tbx3* in SA node formation (40).” The authors should not conclude this from their result. They

can only state that overexpression of either Tbx3 and Irx3 were not sufficient to induce Shox2 expression in neonatal atrial or ventricular CMs.

9. Page 9, Lines 43-45: “These data suggest that for SA and AV nodal genes, Tbx3 and Irx3 play antithetical roles in gene expression: Tbx3 as an activator and Irx3 as a repressor.” Again, these claims are overstated as the presented data only represents sufficiency experiments in a very limited setting (AAV overexpression in cultured neonatal rat atrial and ventricular CMs with relatively modest gene expression changes). Further, this reviewer may have missed it but did the authors show to what degree they achieved overexpression of either TF (both by mRNA and protein)?

Minor Comments:

1. Suppl Fig 1: While the Cntn2Cre/+;Rosa26tdTomato^{+/+} is a previously published reporter line, the authors really should provide co-staining for each major CCS component (SAN, AVN, His, PF) with known markers (e.g. endogenous Cntn2, Hcn4, etc.) to confirm both specificity and sensitivity of labelling the postnatal CCS, as opposed to just showing DAPI.
2. Figure 1D: Violin plots lacking gene labels
3. Figure 1F: “Goodyer et al. 2020” likely mis-labelled and should be “Goodyer et al. 2019” instead.
4. Figure 1i: needs to be enlarged and gene names included – consider fewer top genes per cluster to make legible.
5. Page 4, lines 40-43: The authors write, “Of note, the Proximal CCS cluster contained both atrial and ventricular cells, which highly expressed atrial genes (Myl4 and Myl7) and ventricular genes (Myl2 and Myl3), respectively (Fig. S5c,d). This suggested the presence of nodal cells (SA node and AV node) in this cluster and the necessity for sub-clustering analysis.” The sentence is misleading and should be removed as the presence of atrial and ventricular markers would not suggest the presence of SAN and AVN necessarily. The authors later go on to show subcluster analysis but this earlier statement is superfluous and misleading. This comment harkens back to Major comment #1 – where the classification of these cells at the outset is confusing and the initial classification of “Proximal” vs. “Distal” CCS should be avoided altogether.
6. Figure S10 a and d: are mislabeled in the legend. Says Vsnl1 and Gja1 but the actual figures show expression of Shox2 and Ryr2 per the labels. Should correct.
7. Fig. 3g: Shox2 and Vsnl1 are well known markers of the SAN. Staining of the SAN with these markers alone doesn’t add anything re: validation given that they are established markers.
8. Figure 5d: “CC” should be defined in legend.

Recommendation: Conditional accept pending substantial revisions that address major and minor comments.

Reviewer #3 (Remarks to the Author):

The manuscript by Oh et al., describes the transcription landscape and the heterogeneity within the cardiac conduction system (CSS) across early pre-post-natal developmental timepoints, immediately after birth using scRNA-seq. As studies and our understanding of these specialised cardiomyocyte cell-types remains limited, the CCS single cell transcriptomics for post-natal hearts present a good resource to incorporate together with pre-existing studies from those of Goodyer W. Et al., Bhattacharyya S et al., and Liang et al. By analyzing ~7000 CCS cells and identifying 4 distinct clusters which include proliferative CCS, contractile cardiomyocytes, distal CCS and proximal CCS – the latter of which was further subclustered into three categories: nodes (SA and AV), His bundle, bundle branches. The use of a reporter system also helps to comprehensively capture cells of the CSS. Genes pertaining to ion channels, saromeric proteins and transcription factors were studied across the clusters and the transcription factors *Irx3* and *Tbx3* were chosen as candidates for adenovirus over-expression experiments in isolated atrial and ventricular CMs. (ACM, VCM), which the authors used to influence the expression of specific CSS markers and potentially its cell state/identity.

Genes typically expressed in the SA node were markedly increased by *Tbx3* and suppressed by *Irx3*. The roles of the transcription factors were inversed for genes typically enriched in the Purkinje fibres, with *Irx3* activating and *Tbx3* suppressing gene expression. Both *Irx3* and *Tbx3* functioned as activators for genes enriched in the His bundle, and repressors for genes in contractile cardiomyocytes. As said, this paper provides a valuable resource for CCS single-cell transcriptomic data with an interesting angle providing molecular targets that could be important modulators of gene expression across different tissue types.

1. Could the authors justify and clarify the use of the CnTn2 reporter system in text, apart from it being a postnatal CCS reporter, and make comparisons to other conduction reporter systems like those of Gjd3 reporter system for comprehensive isolation of CSS cell types?
2. Figure 1d: Please include the genes on the right y-axis used in the analysis used to identify the conduction and contraction categories.
3. Where possible, it would be helpful to include p-values to test for significance in the violin plots.
4. There is quite a large proportion of proliferative CCS which is interesting, Can figure 1H be augmented with more canonical cell-cycle markers?

5. Please identify the specific GO database used to conduct the pathway analyses and obtain the gene set descriptions (ie. KEGG, GO biological processes)
6. Within Figure 3A, it will be helpful and insightful for the reader for Figure 3D to highlight both the known and novel markers of SA node, AV node, His bundle and bundle branches.
7. Figure 3h: The IF staining for Rspo3 does not appear to be specific to the AV node site, though it definitely appears to be enriched. Could the authors comment on this, as the scRNA analysis shows site specificity?
8. Figure 4c-e: It would be helpful to group/categorize the list of genes in the heatmap to highlight specific genes/groups of genes described in the main manuscript.
9. Figure 4e: Could the reviewers describe how the panel of genes for ion channels, sarcomeric proteins and transcription factors were identified? Ie. Specific databases/papers. Was there a way to determine/confirm transcription factors beyond obtaining DEG's from the transcriptomic analysis? Ie. Packages such as SCENIC to determine regulons.
10. In Figure 5, the authors have overlapped existing Tbx3 ChIP-seq and ATAC-seq datasets, and also highlighted the Irx motifs zoomed into specific locuses. Overall, in relation to the markers identified from the sub clustering of the scRNA-seq data in this study (Figure 3D), what are the number/percentage overlaps with Tbx3 ChIP/ATAC/IRX signals?
11. The results in Figure 6 are interesting, showing cardiomyocyte subtype specific response to Tbx3 and Irx3. But the authors have not included a negative control gene locus to the figure (using neuronal markers or markers of other cell types)?

Minor comments:

Can the authors include in detail of R-packages / Python modules for in silicon analysis of data? For example, details for gene marker identification and clustering/sub-clustering in the methods? e.g using of FindAllMarkers() function for the clustering of cells, and include if any pre-processing steps

With reference to Figure 5, it will be helpful to name the following in legends: BB, PF, VCS and CC.

Oh et al. Response to Reviewer comments

REVIEWER COMMENTS

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Yena Oh and coworkers performed single cell RNA seq on isolated and purified Cntn2+ (Cntn2-Cre+) derived cardiomyocytes shortly after birth. These cells comprise the components of the conduction system, SAN, AVN, AVB, BB, PF, and probably some additional cardiomyocytes (the boundaries of the CCS components are not sharp, with “transitional cells” expressing only subsets of markers for that component).

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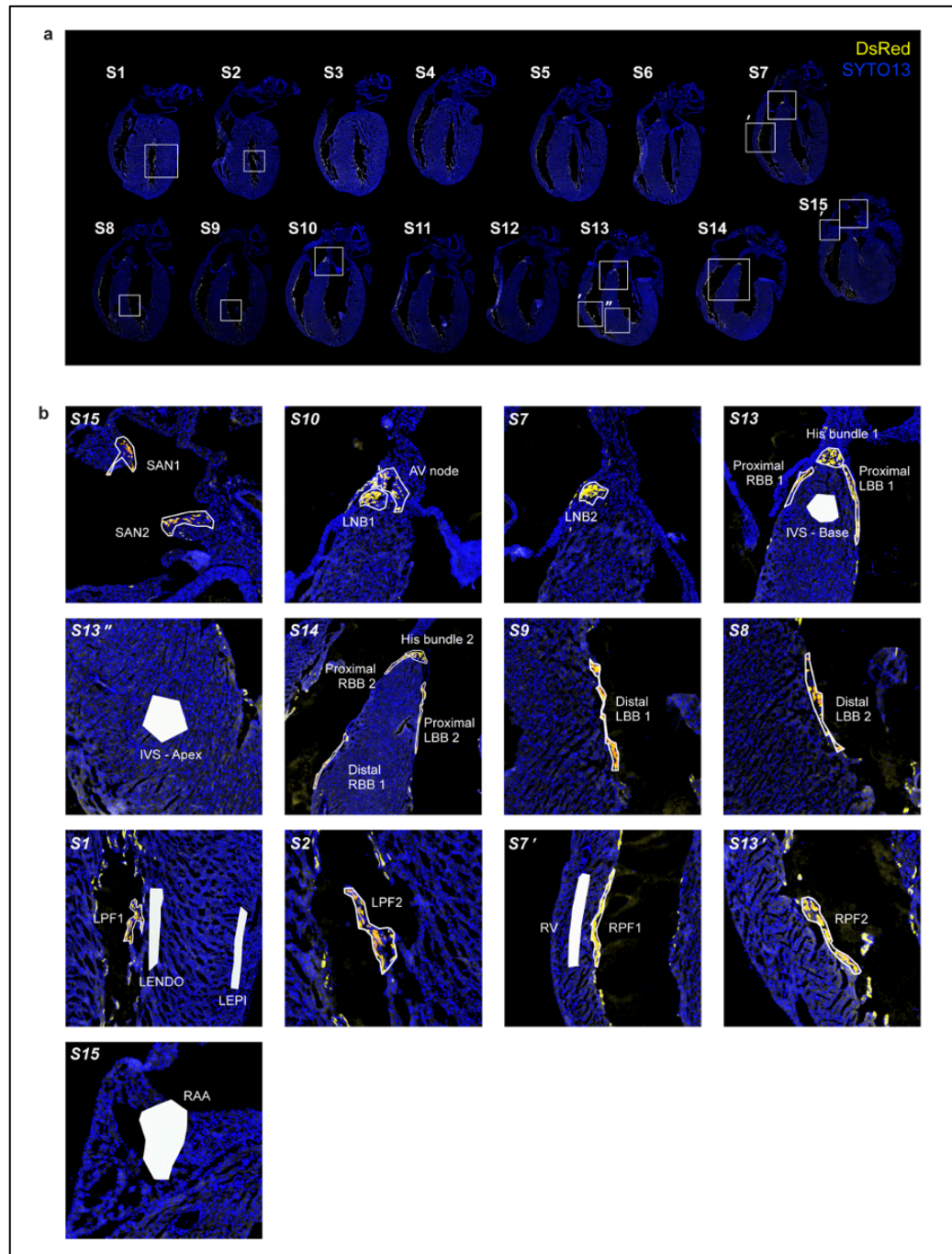
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We very much appreciate the reviewer's critical comments and suggestions. While analyzing large data using recently popular molecular profiling platforms, we have come to truly appreciate the impressive body of work that was done in the field of cardiac conduction system (CCS) many years prior. All of these findings served as an excellent guide for our work, and since our study is built upon bodies of these great pieces of work, our findings certainly confirm some of the previous results, as the reviewer correctly points out. At the same time, we believe our study, now with major improvements, adds more foundational value and another critical layer of insight.

Below are key points summarizing of the significant improvements made during this revision.

1. The major accomplishment that we have made to improve our manuscript during this revision was conducting spatial transcriptomics in the cardiac conduction system (CCS) fluorescent reporter mice. Specifically, we utilized NanoString GeoMX Digital Spatial Profiler in heart sections of postnatal day 4 (P4) CCS-reporter mice ($Cntn2^{Cre/+}; Rosa26^{tdTomato/+}$) and captured all CCS components, including sinoatrial node (SAN), compact atrioventricular node (cAVN), lower nodal bundle (LNB), *His* bundle (HIS), proximal bundle branch (PBB), distal bundle branch (DBB) and *Purkinje* fiber (PF) (Fig. 1b, d, e, and Supplementary Fig. 6, shown below).



a

scRNA-seq

Scaled Expression

2
0
-2

Spatial transcriptomics

Scaled Expression

2
0
-1

SA node

Shox2 *Gnao1* *Cdh12* *Nid2* *Bmp2* *Tbx3*

Compact AV node

Lin7a *Rims1* *Rspo3* *Gcgr* *Scars3* *Ednrb*
Lpl1 *Prdm6* *Hamp* *Shox2* *Fla2r1* *Rd3*
Hs3a3a1 *Shc2* *Gup1* *Boc* *Nid2* *Spon1*
Bmp2 *Casq1* *Pipr5* *Znf1* *Cacna1* *Srp5*
Rgs12 *Gde1* *Bambi* *Adk* *Gor1*

Lower nodal bundle

Hs3a3a1 *Lap1* *Ppp1r17* *Dok7* *Pirt* *Col4a4*
Cdo1 *Robo1*

His bundle

Tubb4a *Pcp4* *Cdh6* *Cyp4b1* *Scube2* *Grih1*
Lg3 *Cpne5* *Gprc22* *Fgf9*

Proximal bundle branch

Igfbp7 *Axes1*

Distal bundle branch

Gal *Npsa* *Mbp* *Capn6* *Pam* *Col13a1*
Slc16a2 *Gn5111* *Pde10a* *Co5a1* *Ecm1*
Cited1 *Gpr103* *Arhgap28* *Sbk2* *Mest* *Hsf17b11*
Prrg *Thbs4* *Unc5b* *B2m* *Calr* *Hsp90b1*
Ccdc167 *Cybb* *Alpbap11* *Pin*

Purkinje fibers

Ogn *Corin* *Abhd13* *Mpped2* *Max* *Sh3a19*
Myh4

Proliferative CCS

Klf2c *Deafc1a* *Pik1* *Xkr5* *Ccnb1* *Hst1h1b*
Hmng *Ccnw2* *Klf4* *Cdca8* *Sapo2* *Cfcd2*
Hmnr *Pfk* *Cdca5* *Bub1* *Cdca3* *Cep55*
Sxa1 *Shc25* *Cdc20* *Pct1* *Cdc25c* *Mxd3*
Chap21 *Nuf2* *Aurkb* *Birc5* *Oligap5* *Racgap1*

SAN1 SAN2 GAVN1 GAVN2 LNB1 LNB2 HIS1 HIS2 PLBB1 PLBB2 PRBB1 PRBB2 DLBB1 DLBB2 DRBB1 DRBB2 LFP1 LFP2 RFP1 RFP2

b

Enriched in 'Nodal cells' (SA node, Compact AV node)

Enriched in 'LNB & Proximal VCS' (Lower nodal bundle, His bundle, Proximal bundle branch)

Enriched in 'Distal VCS' (Distal bundle branch, Purkinje fibers)

scRNA-seq

SA node

Compact AV node

Lower nodal bundle

His bundle

Proximal BB

Distal BB

Purkinje fibers

Proliferative CCS

Contractile myocytes

Shox2, Ggrr, Rld3, Nid2, Bmp2, Csrp2, Tbx3, Enpp, Ccna1d, Gsk1, Ctsp1, Akt, Hssc1a1, Ppp117, S100a6, Lrr2, Col4a4, Col4a3, Cdh6, Robo1, Susd2, Cyp4b1, Scube2, Mga1, Ache, Grn1, Hmg13, Ndn, Alpl1, Alcam, Nsp2, Myk4, Sema3a, Sbk2, Mipn2, Slit2, Inx2, Mgp, Irf1, Pdzrn3, Tsc22d3, Gja5

Percent Expressed

0, 20, 40, 60, 80, 100

Average Expression

1.5, 1.0, 0.5, 0

Spatial transcriptomics

SA node

Compact AV node

Lower nodal bundle

His bundle

Proximal RBB

Proximal LBB

Distal LBB

Distal RBB

Left purkinje fibers

Right purkinje fibers

RAV

L-ENDO

IVS-BASE

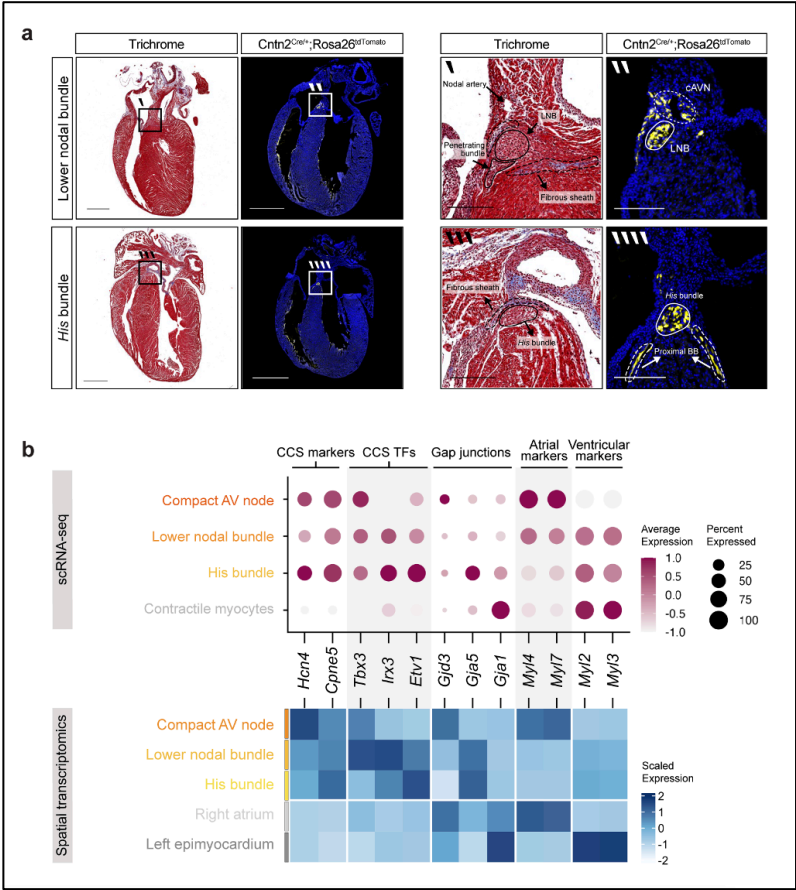
IVS-APEX

RV

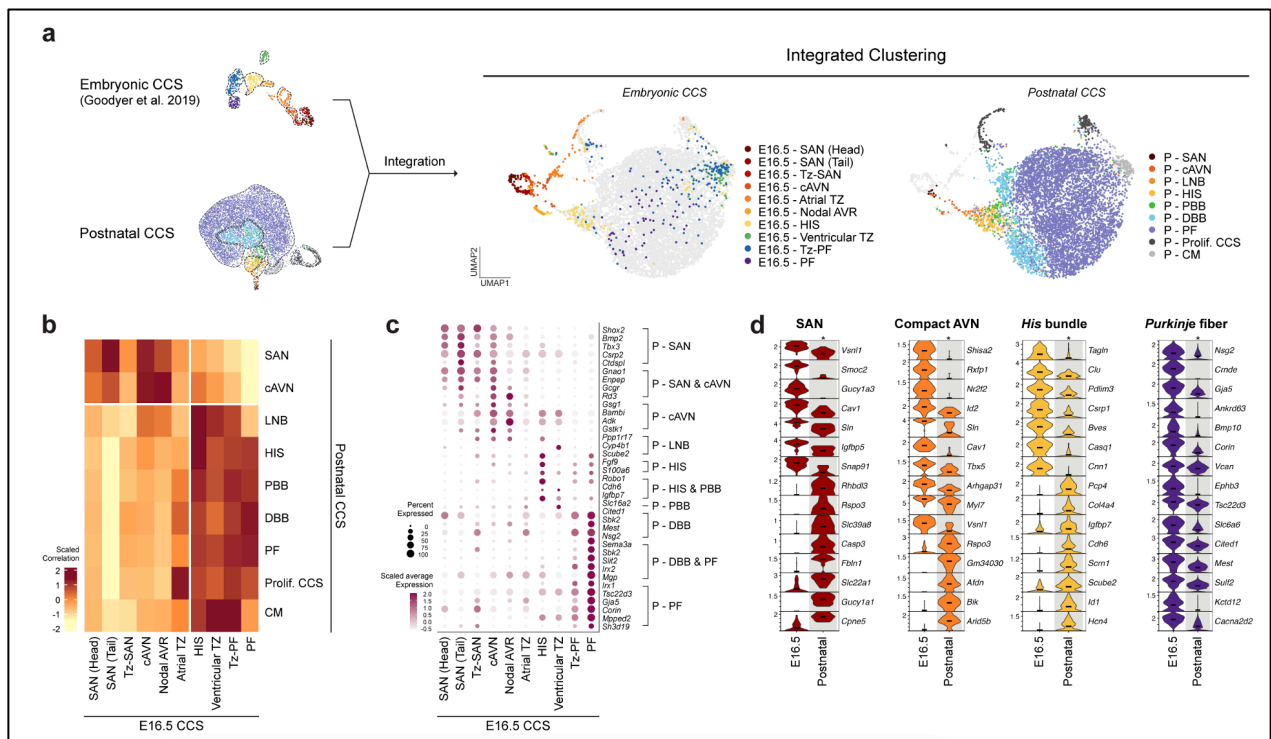
Scaled Expression

2, 1, 0, -1, -2

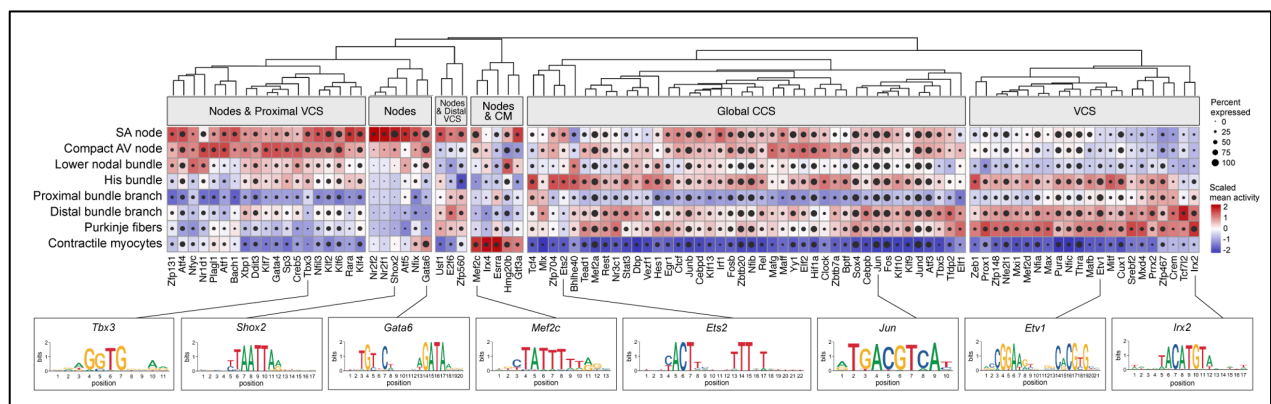
2. An additional finding that was added to the revised manuscript is the transcriptional identification of the lower nodal bundle (LNB) (Supplementary Fig. 7, shown below). The AVN is anatomically subdivided into compact AVN (cAVN) and LNB as they are situated adjacent to each other within the atrioventricular junction (Supplementary Fig. 7a). However, their molecular identities were not fully understood. Owing to spatial transcriptomics, we were able to capture both cAVN and LNB regions located in the same histological section and profiled the gene expression in these AVN components (Fig. 1d and 2a). Despite the anatomical proximity between the cAVN and LNB, a direct comparison between cAVN, LNB and HIS revealed that the LNB is molecularly more similar to HIS than to cAVN. This reinforces previous studies demonstrating that LNB is structurally continuous with HIS and is derived from a ventricular lineage (Supplementary Fig. 7b).



3. The Reviewer suggested 'more extensive comparison of the data with published transcriptomes of CCS components'. We thank you for this valuable comment. In the revised manuscript, we have integrated embryonic (E16.5) CCS data (Goodyer, 2019) with our scRNA-seq data to examine the molecular similarities and differences between embryonic and postnatal CCS. Notably, we found that new CCS markers that we have identified in the postnatal CCS show very good similarities in the embryonic CCS. This suggests that CCS specification may largely occur embryonically. Meanwhile, we have also identified molecular differences between two temporal stages. In particular, we found downregulation of *Corin*, *Bmp10*, *Tagln* and *Cnn1*, which are known to be expressed in the developing heart, but repressed in the mature heart. This data provides evidence of prenatal to postnatal CCS maturation. This result is shown in Fig. 4 (shown below) of the revised manuscript.



4. The Reviewer commented that “*The bioinformatic analysis does not provide significant novel mechanistic insight*”. To address this comment and to demonstrate improved mechanistic insight into how the molecular patterning across the CCS is transcriptionally established, we conducted network inference analysis using SCENIC (Single-Cell Regulatory Network Inference and Clustering). We identified CCS region-specific regulons (transcription factors and their target genes) that may contribute to the establishment of heterogeneous gene expressions within the CCS. Examples of identified transcription factors include well known CCS TFs, such as *Shox2*, *Gata6*, *Tbx3*, *Tbx5*, and *Etv1* as well as less explored TFs, including *Nr2f1*, *Atf5*, and *Bach1*. Through SCENIC analysis, we have also found that these transcription factors may regulate some of the novel markers we have identified. Together, this analysis provides novel insights into the gene regulatory networks underlying CCS heterogeneity. This result is shown in Fig. 5a (shown below) of the revised manuscript.



5. We further extended our study investigating the role of *Tbx3* and *Irx3* in the establishment of CCS heterogeneity. Specifically, we tested the effects of *Tbx3* and *Irx3* overexpression in known and

novel markers of each CCS component: *Vsnl1* and *Shox2* for SAN, *Prdm6* for cAVN, *Igfbp7*, *S100a6* and *Pcp4* for proximal VCS, *Slit2*, *Nppa*, *Mpped2*, *Scn10a* for distal VCS, and *Gja1* and *Ryr2* for contractile myocytes. We therefore developed an *in vitro* model to study the molecular mechanism underlying the CCS regional heterogeneity. Our data clearly showed that *Tbx3* and/or *Irx3* regulates gene expression individually, cooperatively or antagonistically. More importantly, through this method, we showed that *Tbx3*'s and *Irx3*'s transcriptional regulation is distinct in different heart chambers (Ventricle vs. Atria). This finding was further supported by examining the epigenetic landscape of these newly identified CCS markers, by incorporating ATAC-seq data of the SAN and atrioventricular junction as well as *Tbx3* ChIP-seq data and *Irx* motifs. Therefore, we respectfully disagree with the comment that "*The in vitro experiment performed by authors is largely confirmatory, and does not yield novel mechanistic insight*". While we acknowledge that prior work tested *Gja5*, *Nppa*, *Gja1* and *Ryr2* in loss- or gain-of-function mouse models of *Tbx3* or *Irx3*, none have investigated how these transcription factors regulate the expressions of downstream targets in a chamber-specific manner. Our findings suggest that *Tbx3* and *Irx3* control target gene expression distinctly in the ventricles and atria, thereby contributing to the establishment of the molecular heterogeneities within the CCS. To address this comment, however, we have further underscored the aspect of "chamber-specific transcriptional regulation of *Tbx3* and *Irx3*" in both the results and discussion sections of our manuscript, in order to clearly elucidate our novel findings. This result is shown in Fig. 6 and Supplementary Fig. 25 of the revised manuscript.

Together, we truly appreciate the reviewer again for the valuable comments, which played a crucial role in improving the manuscript.

Reviewer #2 (Remarks to the Author):

Comments on submission from Oh et al. 2023 to Nat Comm, entitled "Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity"

In the manuscript entitled "Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity", the authors provide a description of the transcriptional landscape of the postnatal murine cardiac conduction system, providing particular robustness to the various cell types within the ventricular conduction system (VCS) (His, bundle branches and PFs), not previously captured. The authors go on to demonstrate sufficiency experiments in rat cardiomyocyte culture system to suggest a role for two established TFs (*Irx3* and *Tbx3*) in regulating the expression of certain CCS-related genes. The authors collected the largest amount of CCS cells for analyses to date, however notably the nodal representation was surprisingly limited, with the dataset consisting almost exclusively of VCS conduction cells. Overall, while the work is of value, I believe the authors require significant work to both validate some of their findings as well as to rework the manuscript substantially for clarity as detailed in the major and minor comments below.

We thank the Reviewer for the very positive comments with enthusiasm. We especially appreciate the comments recognizing the strength of our manuscript while providing critical suggestions. We have revised our manuscript extensively by performing additional experiments and analyses, which we

believe fully address the Reviewers' concerns and suggestions, thereby significantly improving the manuscript.

In particular, to facilitate and complement our findings from scRNA-seq, we have advanced our study by performing spatial transcriptomic (ST) analysis in P4 *Cntn2^{Cre/+};Rosa26^{tdTomato/+}* mouse heart. By integrating scRNA-seq and ST datasets, we identified CCS component and regional markers with confidence. In addition, as suggested by the Reviewer, we conducted additional bioinformatic analysis. Particularly by comparing our postnatal CCS data with published embryonic CCS scRNA-seq data, we have demonstrated the similarities and differences between prenatal and postnatal CCS, reflecting the molecular transition during the maturation process. In addition, based on other Reviewers' comment, we performed network inference analysis using SCENIC, which provided insights into the regulatory mechanism underlying the transcriptional heterogeneity within the CCS.

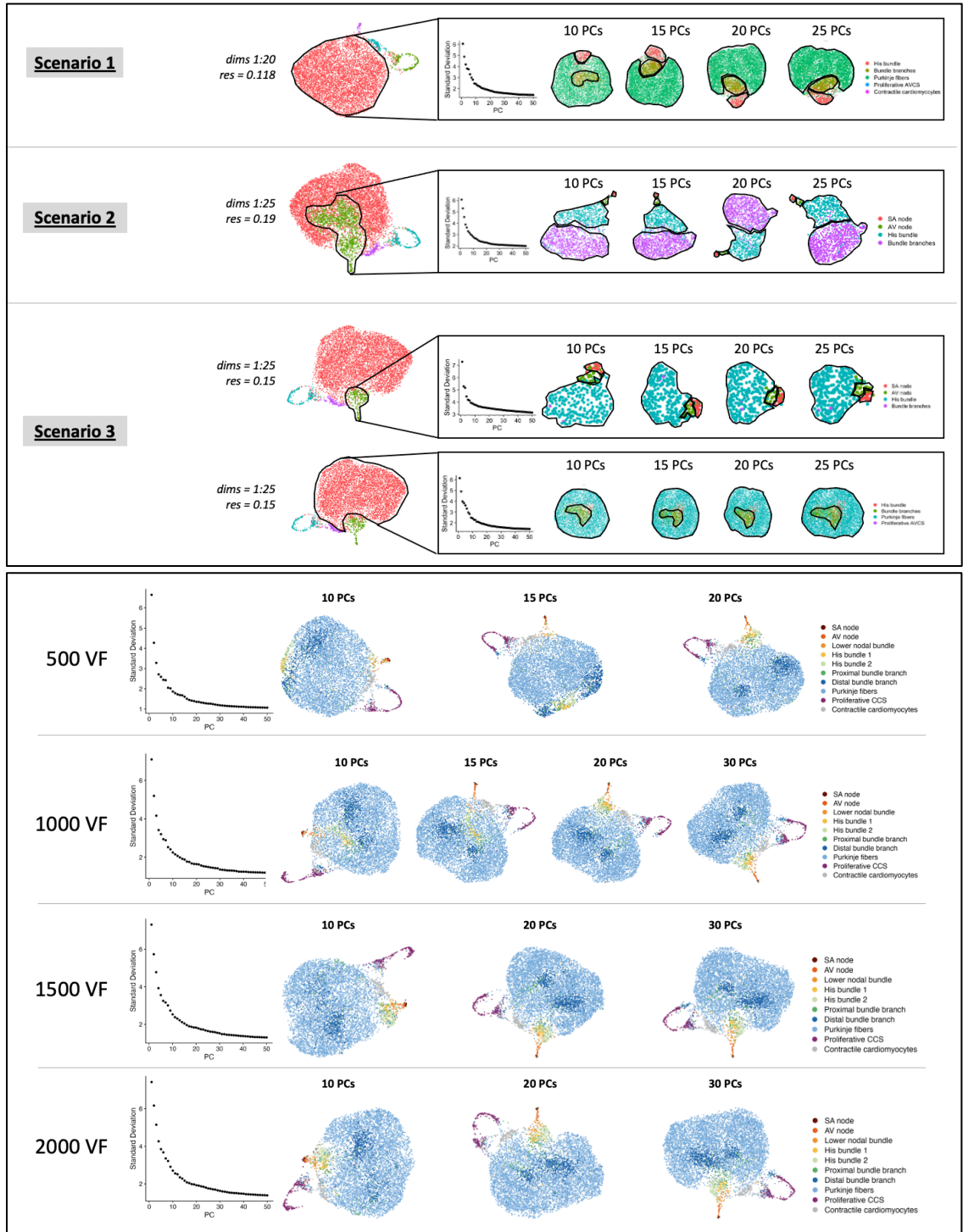
To address the Reviewer's specific comments, we provide our responses to each point below.

Major Comments:

1. The authors should, upfront, provide a clear definition of what they are calling Proximal vs. Distal CCS (page 4, line 26). The terms are not common use and could refer to prox vs. distal VCS versus, as the authors imply (I believe), proximal CCS including nodal tissue SAN, AVN, His bundle and bundle branches (i.e. everything except purkinje fibers – which is not a standard term for CCS classification). If the latter, then ideally their FindClusters single cell data analysis should be performed at a higher resolution to see if the individual clusters emerge at baseline, separating out these different subsets of cell types. This would help avoid confusion and muddled data interpretation by not having the nodal cells and proximal VCS combined as they are, as the authors point out, such distinct cell types.

Thank you for the valuable comment. We understand the confusion involved in the proximal and distal CCS classifications, as these are not commonly used terms as the reviewer points out. In the prior version of the manuscript, we structured the figures so that it demonstrates the order of our clustering analysis, which caused difficulty in data interpretation. As per the Reviewer's suggestions, in the revised manuscript, we do not use 'proximal/distal CCS', but instead use the more specific CCS regional names, or "proximal/distal VCS," as commonly used in literature¹⁻⁴. We also clearly define these terms as "proximal VCS regions (HIS and PBB); and distal VCS components (DBB and PF)."

In terms of finding optimal clusters, we agree that it would be ideal to see all CCS subpopulation clusters emerge at baseline using higher resolutions. However, after performing clustering using several combinations of parameters (variable features = 500, 1000, 1500, 2000; dimensions = 10, 20, 25, 30; resolutions = 0 - 1 at increments of even smaller than 0.05), we found that it was not possible to generate the clusters all at once using the same *FindClusters* and *FindNeighbours* clustering parameters. (Figures shown below for the Reviewer to show different clustering methods)



However, this is not unexpected, as CCS components exhibit different degrees of similarities and differences with zoned and gradient expression patterns, which implies that clustering will not happen all at once. Therefore, we performed a series of sub-clustering analysis (Supplementary Fig. 5) to reliably achieve the clusters that aligned with anticipated cell populations based on established marker genes, like we did in the previous version of the manuscript. In the revised manuscript, we show upfront the re-constructed UMAP of the global CCS, containing information on all subclusters (Fig. 1c, shown below) while providing detailed information in Supplementary Fig. 5. We are quite confident with our update clusters, especially with the addition of our spatial transcriptomics data, which shows accurate regional information.

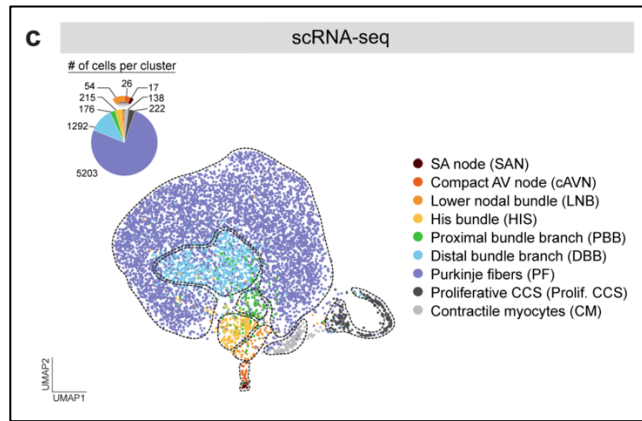
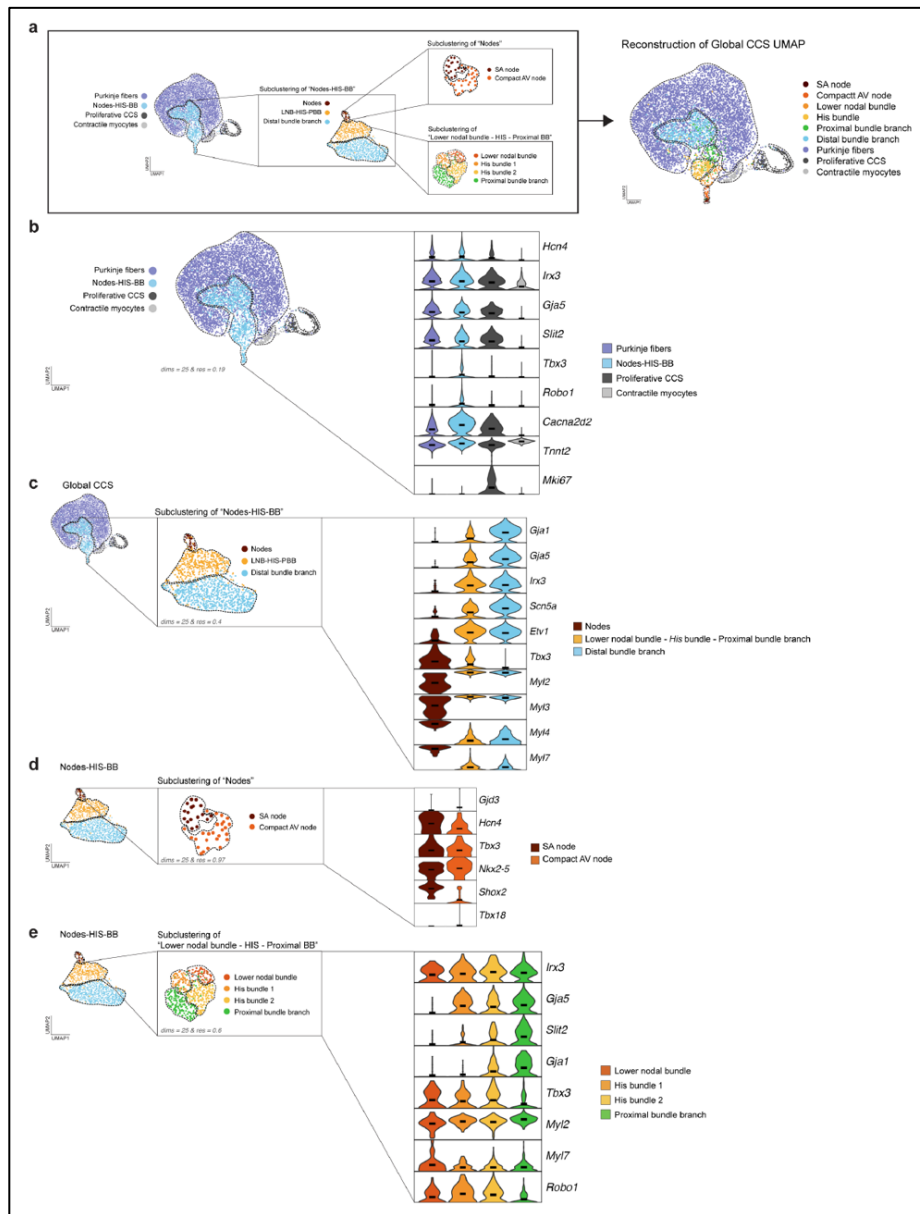


Fig. 1c: CCS scRNA-seq UMAP



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

2. Fig. 2c and S7a: Igfbp7 staining is ambiguous. There appears to be some baseline staining in the interventricular septum and more notably, in/around PFs in the supplement figure. To ensure a direct comparison in staining, the authors should present a single four chamber view with magnification of the His bundle region and Purkinje fiber from the same tissue slice, using the exact same exposure and laser intensity. If a higher mag is required, then the tissue slice can be imaged using tiling. Same for the Scn10a staining Fig 2f and S7b.

Thank you for the important comment. For all our imaging experiments, we do agree that it would be ideal to take images of all CCS components in one single four chamber view section. As shown in our image of P4 Cntn2-Cre;R26tdT sections used for spatial transcriptomic analysis (Supplementary Fig. 6), since CCS components are located in different anatomical positions (i.e. AVN in posterior vs. HIS in anterior), it is not technically feasible to capture all CCS component in a single section. Thus, we had done our best to capture all CCS components by performing staining of one target on multiple sections from the same heart at the same time in order the best capture all parts of the CCS. As staining was done simultaneously, the same staining parameters (antibody concentration, incubation time) and image acquisition settings (laser intensity and exposure time) were used. Therefore, all the images of a single target were taken in technically identical conditions.

This also applies to the expression data of Igfbp7, which was identified as the proximal bundle branch (PBB) marker in the revised manuscript. To show specificity of Igfbp7, we included data from the *His* bundle and bundle branches (Fig. 3e) as well as left and right *Purkinje* fibers (Supplementary Fig. 17) and found that Igfbp7 is enriched in the proximal VCS, but not in PF, suggesting Igfbp7 could serve as a proximal VCS marker. On the other hand, we also noticed a very mild Igfbp7 expression outside the LV near the Cntn2-positive PF (Supplementary Fig. 17a). Since Igfbp7 expression in failing heart and regulates pathological cardiac remodeling, it is possible that Igfbp7 is expressed in non-CCS regions. This was added to 'Discussion' section.

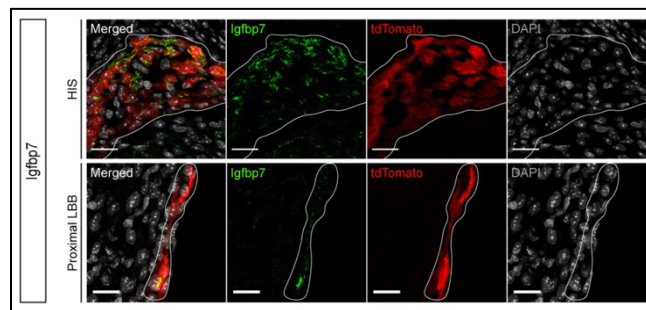
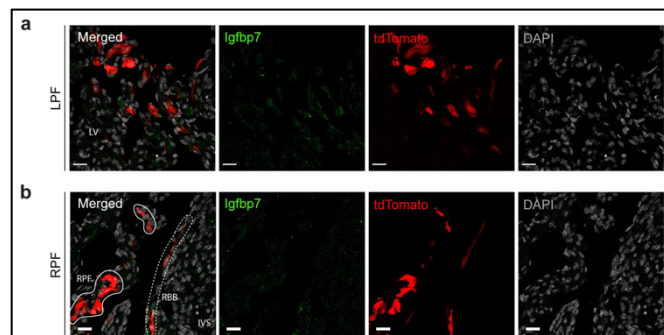


Fig. 3e: Igfbp7 expression in the HIS and PLBB.



Supplementary Fig. 17: Igfbp7 expression in LPF and RPF.

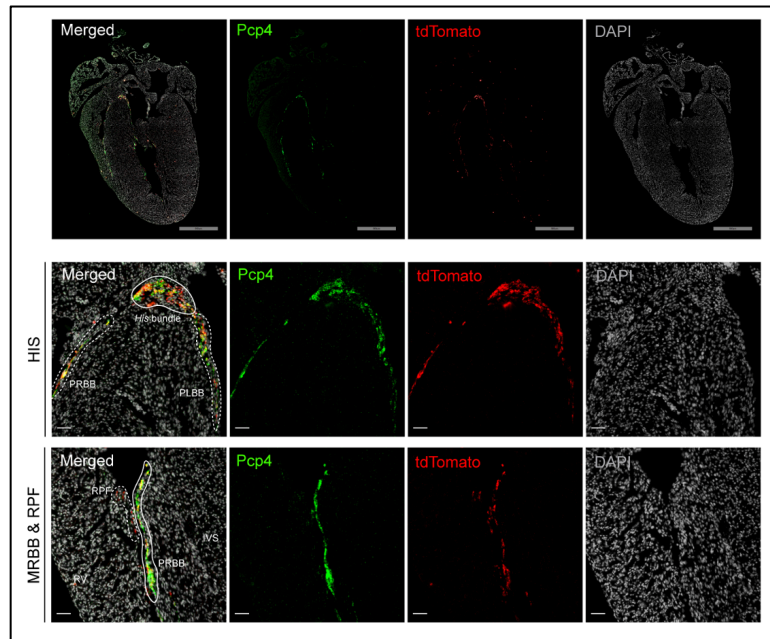
As the Reviewer commented, the above-mentioned process was identically done for *Scn10a* as well as *Mpped2*, which were identified to be enriched in the distal VCS (DBB and PF) (Fig. 3g and Supplementary Fig. 20 and 21). We showed images of *Scn10a* and *Mpped2* expression in different CCS regions (His bundle, bundle branches and Purkinje fibers) to support our findings of their enrichment in the distal VCS. For all new genes (e.g. *Gnao1*, *Prdm6*, *Ppp1r17*, *Mpped2*) tested for the revised manuscript, as per the Reviewer's suggestion, we also performed fluorescent scanning of the slide. Since we also observed slightly broader expression of *Mpped2* and *Scn10a* than tdTomato+ VCS cells, we described these findings in the revised manuscript that "*Mpped2* and *Nav1.8* exhibited a slightly broader expression pattern showing enrichment along the endomyocardium of the ventricular chambers, suggesting its potential role within and around the VCS."

Lastly, we would like to emphasize that all CCS genes identified from our scRNA-seq and ST analyses result from the comparative analyses where we compared each region to the non-CCS contractile myocytes region, suggesting their enrichment in the CCS region. An additional validation of our analysis is demonstrated by the similar expression patterns of our newly identified CCS genes (e.g. *Igfbp7*, *S100a6*, *Mpped2*, *Mpg*) in the corresponding regions of the embryonic CCS (Fig. 4C).

3. Fig. 3g, h, k, l in combination with Page 6, lines 30-36: same comment as above. If the authors want to claim protein-level validation of their findings of a "His-specific" (e.g. *Pcp4*) or "bundle-branch-specific" gene candidate, one would want to see a single staining of a tissue slice (again a standard four chamber view would suffice) that contains both the his bundle and bundle branches showing differential expression. To this point, *Pcp4* is in fact expressed in the PFs and not His-specific (as previously established – Kim et al. JCI 2014). The authors, to their credit, do mention this in the discussion however it highlights the overall assertion that to make a claim for CCS subcomponent-specific expression, one would like to see (at least a handful of examples) with proper validation by immunofluorescence. Given that almost all of their data represents the VCS, I believe the authors should really focus on determining and validating (with either IF vs. in situ hybridization of tissue slices) whether there are any truly significant molecular differences between the His, BBs and PFs.

Thank you for the important comment about *Pcp4*. Our intention in the previous version of the manuscript was that *Pcp4* is enriched, not specific, in the *His* bundle, compared to other parts of the CCS. However, as you rightly pointed out, we do agree that our previous Discussion section may have inadvertently conveyed the wrong impression that *Pcp4* expression is exclusive to the *His* bundle.

During the revision, we also checked *Pcp4* expression in our spatial transcriptomic data. Indeed, as you can see in Fig. 2a, our integrated transcriptomic data consistently identified *Pcp4* as His bundle-enriched gene, further validating our findings. Due to a potential discrepancy with the previous literature (Kim et al., JCI 2014), we performed RNAScope and examined *Pcp4* expression in the *Cntn2-Cre*;R26tdTomato postnatal mouse heart. We have confirmed that *Pcp4* is expressed throughout the VCS from the *His* bundle, bundle branches and *Purkinje* fibers, however the level of expression was in the order of His bundle > bundle branches > PF (Supplementary Fig. 18, partially shown below), aligning with our integrated transcriptomic analysis results.



Supplementary Fig. 18: Pcp4 expression in the CCS

To address these, we have changed the text in the result section describing the analysis as follows :
 (Page 6) “The HIS showed enrichment of *Pcp4*, *Cdh6* and *Cyp4b1*. Although our analysis showed that these genes are most highly expressed in the *His* bundle, *Pcp4* is known to be expressed throughout the VCS⁵⁷ with critical roles in cardiac electrophysiology, and *Cdh6* is known to be enriched in the CCS compared to non-CCS cardiomyocytes⁵⁸. This suggests that there may be regional heterogeneity in the expression levels of these genes within the CCS.”

Also, we have described the histological validation of *Pcp4* as follows:

(Page 7) “We also found that an established VCS marker⁸⁰, *Pcp4*, was indeed expressed throughout the VCS, but showed stronger expression in the HIS and BB than in the PF, confirming our scRNA-seq and ST data (**Supplementary Fig. 18**).”

4. Fig. 3h and Page 6, line 27-28. The authors state: “In the AV node, *Rspo3* expression was enriched, which was validated by immunostaining (Fig. 3f,h).” Enriched, presumably compared to the SAN nodal cells per their single cell analyses, however this is not shown by IF (only an AVN is shown). Does the SAN show relatively reduced *Rspo3* by staining? If so, it should be shown. Further, the outlined putative AVN shows expression of *Rspo3* in the putative AVN cells (labeled by tdTomato) as well as, seemingly, equally, in the surrounding interventricular septum cells. Does the working myocardium show similar levels of *Rspo3* transcript as the staining would suggest? The authors should comment on this specifically.

Thank you for the comment on this important validation. Indeed, Reviewer #3 pointed out the same issue. We examined our image data and agreed that *Rspo3* expression is broader than simply compact AVN, suggesting *Rspo3* may be an AV junctional marker, rather than cAVN-specific marker. We also encountered technical challenges due to unstable antibody. Thus, we tested another cAVN marker, *Prdm6*, by performing validation experiments using RNAScope. As shown in Fig. 3b, *Prdm6* expression was found in cAVN and AV nodal ring regions, but not in LNB region or other CCS or non-CCS regions (Supplementary Fig. 14). Notably, *PRDM6* has been shown as a potential gene regulator

in human nodal cells (Kanemaru et al., Nature 2023), suggesting that it may be an evolutionarily conserved cAVN regulator.

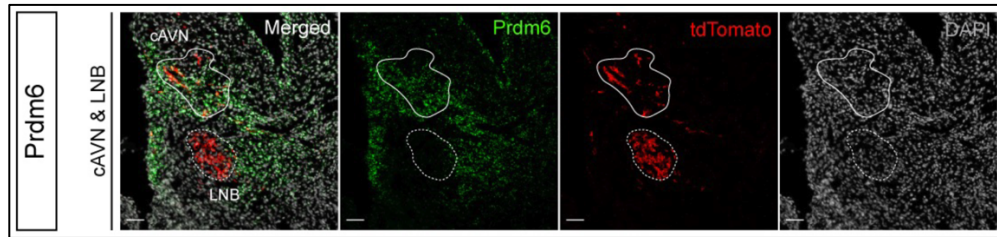


Fig. 3b: *Prdm6* expression in the cAVN, not LNB.

In the revised manuscript, we have written as follows:

(Page 7) “We performed FISH to examine cAVN-enriched *Prdm6*, showing that it was primarily expressed in cAVN and AV ring, but not in the LNB and other CCS or non-CCS regions (**Fig. 3b** and **Supplementary Fig. 14**).”

5. Supp Figure 5b: There is surprisingly low amounts of tdTomato expression in all of the sorted CCS cells, and is almost complete absent from the bottom-most cells of the “Prox CCS cells” cluster. How do the authors explain this? This fact should be highlighted in the text and explained in the discussion. Further, Fig3g would suggest that the tdTomato labeling of CCS cells is incomplete based on their stated outlines for the various CCS structures (i.e. it appears as if many CCS cells are not successfully labeled). The authors should provide a counterstain with endogenous *Cntn2* to demonstrate the degree to which the tdTomato labels each CCS component as well as to comment on whether all cell types are truly represented (e.g. SAN transitional cells).

Thank you for this insightful comment. We acknowledge that the expression of tdTomato in the FeaturePlot in the previous manuscript (previous sFig. 5b) appeared to be low. This issue regarding the low transcript levels aligned specifically to tdTomato sequence has previously been reported, where it has been demonstrated that the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), located in the downstream of the tdTomato sequence in the targeting vector, represses the mRNA readthrough of the tdTomato sequence⁷. For this reason, studies often either represent tdTomato signal with WPRE expression instead⁸ or include the WPRE sequence in the reference genome⁹. The Rosa26tdTomato model that we utilized in our study, B6.Cg-Gt(*ROSA*)26Sor^{tm14(CAG-tdTomato)Hze}/J (Jackson 997014), indeed contains the WPRE element in the targeting vector (Rosa-CAG-LSL-tdTomato-WPRE). In our study, a customized reference genome was constructed by the additions of Cre and tdTomato sequences, but not the WPRE sequence. We suspect this may be the reason for the low transcript levels of tdTomato. However, we did validate that the sequencing reads were specifically aligned to the added tdTomato gene, as shown in the figure below.

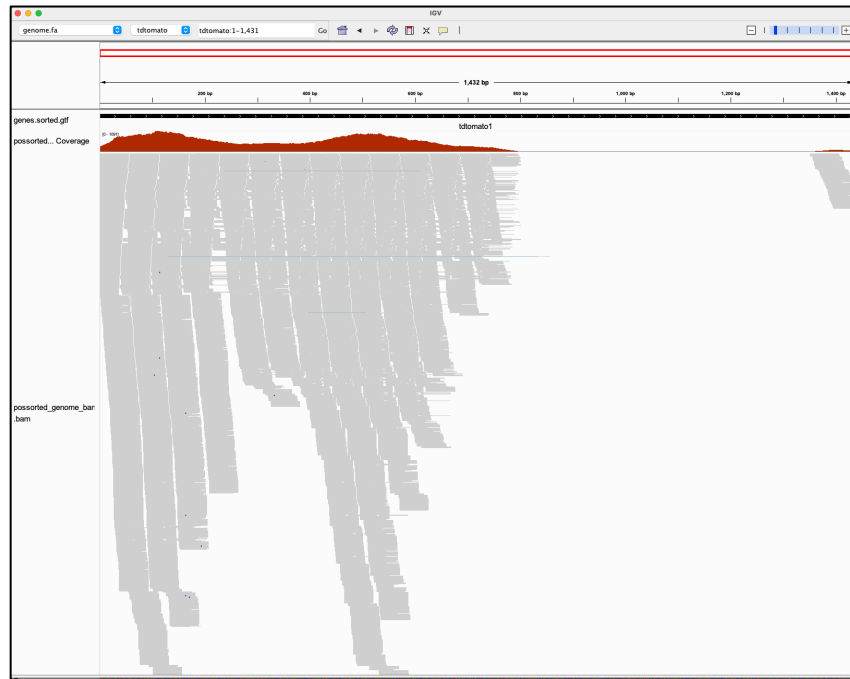
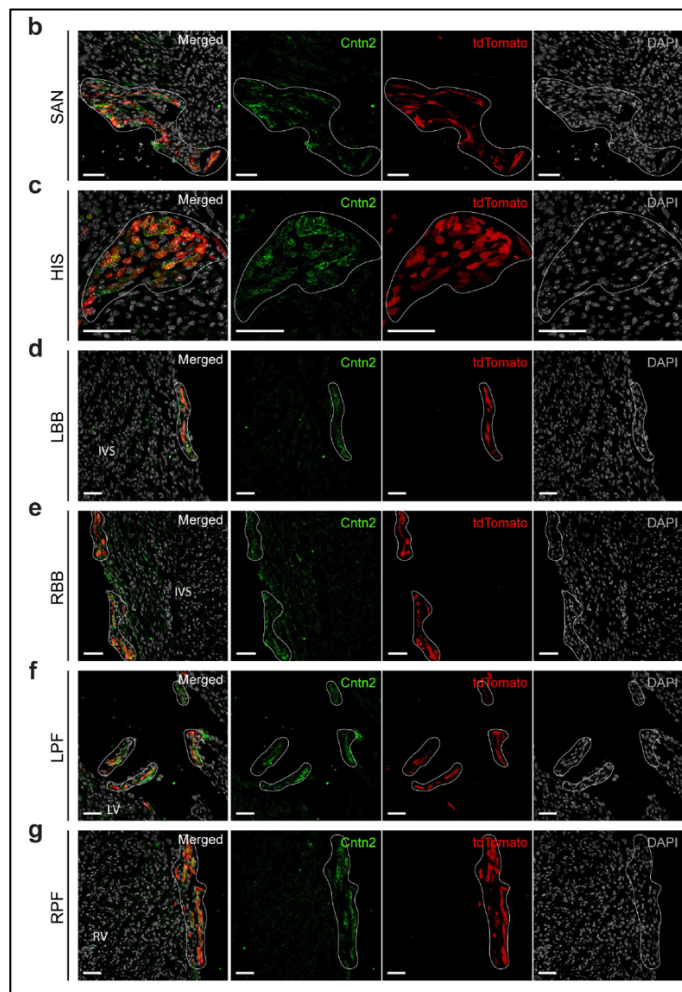


Figure for the Reviewer: Sequence alignment of tdTomato.

We also believe that transcript levels often do not linearly correlate with its protein levels, and this is indirectly demonstrated by strong tdTomato signal in the epifluorescence image of the CCS heart, as well as our FACS results, which show clear separation between tdTomato- and tdTomato+ cells. Unfortunately, as we aimed to input a maximum number of cells for scRNA-seq, we do not have a fluorescence image of FACS-sorted tdTomato+ cells.

A recent study [Goodyer et al., JCI, 2022] used an engineered antibody targeting Cntn2 to visualize the entire CCS in mice, strongly suggesting that Cntn2 can be an excellent target for marking all the CCS components from SAN to PF. Therefore, as Reviewer #2 (and Reviewer #3) suggested, we conducted co-immunostaining using endogenous Cntn2 antibody, showing that tdTomato signaling from Cntn2-Cre driven labeling were fully co-labelled with endogenous Cntn2 throughout the CCS (Supplementary Fig. 1). In addition, we repeated immunostaining for a well-established SAN-specific maker, Shox2, and found that tdTomato signal in the SAN almost fully overlaps with Shox2 staining suggesting Cntn2-Cre/Rosa26-tdT reliably marks SAN (Supplementary Fig. 12). We hope these new data address the Reviewer's concern.



Supplementary Fig. 1: *Cntn2*Cre/+;*Rosa26*tdTomato/+ heart mark all the components of the postnatal CCS

We also wrote in the revised manuscript:

(Page 4) “Consistent with the previous studies that utilized Contactin-2 (*Cntn2*)-driven reporters and *Cntn2*-targeting antibody to visualize the entire CCS system in mice^{21,33-35}, all CCS components were reliably marked in the postnatal CCS-tdT mouse heart, which was validated by endogenous *Cntn2* co-immunostaining (**Fig. 1a** and **Supplementary Fig. 1**).”

6. The reviewer would like to see some additional verification that the stated cell annotations in their single-cell data are appropriate. Specifically, the rare cells annotated as “AV node” are *Tbx3*+ and negative for *Shox2*. But the authors should comment specifically on other classic AVN markers which should be *Hcn4*+/*Kcne1*+/*Cx30.2*+/*Cx45*+/*Cacna2d2*+/*Cx40*-/*Cx43*-/*Nppa*-/*Myl2*- as well as negative for all distal VCS markers *Etv1*-/*Scn5a*- (Bhattacharyya et al 2019 Sci Rep; Goodyer et al 2019 Circ Res). Further, for the SAN, the authors note that they did not appreciate *Tbx18* but did the authors not detect other expected markers *Hcn4*+/*Isl1*+? If not, the authors should clearly state this as a limitation (presumably due to the limited number of captured cells).

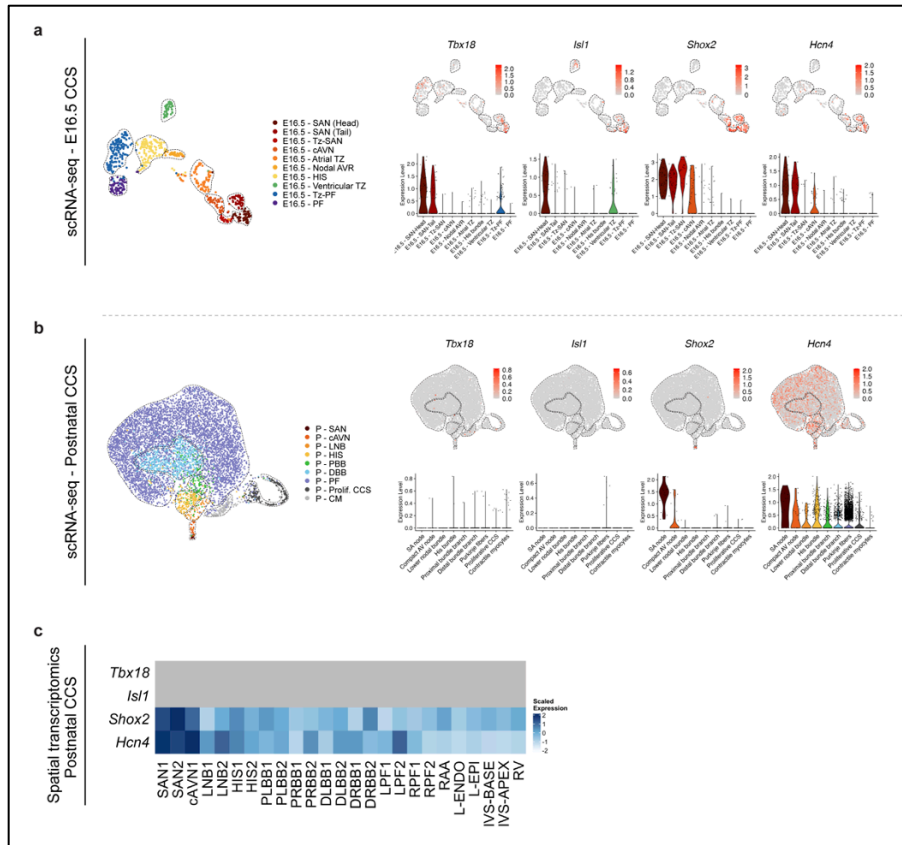
Thank you for your important comments. Since in the revised manuscript, we utilized both scRNA-seq and spatial transcriptomic data and tried to ensure if all CCS components including SAN and compact AVN (cAVN) were accurately annotated. As shown in Fig. 1e, f, we examined the expression patterns

of known pan-CCS and regional markers, such as *Hcn4*, *Cpne5*, *Cacna2d2*, *Gja5*, *Shox2*, *Tbx3*, *Irx3*, *Slit2*, and *Sema3a*, in the scRNA-seq and ST datasets, demonstrating clear annotations of CCS components which corroborate with established markers. Additionally, we directly compared cAVN with lower nodal bundle (LNB) and His bundle (HIS), based on *Tbx3*, *Irx3*, *Etv1*, *Gjd3* (*Cx30.2*), *Gja5*, and *Gja1*. Consequently, we annotated CCS components as follows:

- SAN (*MyI2^{low}/MyI3^{low}/MyI7^{high}/MyI4^{high}/Hcn4^{high}/Tbx3^{high}/Nkx2.5⁺/Shox2^{high}/Irx3⁺/Robo1⁻*);
- cAVN (*MyI2^{mid}/MyI3^{mid}/MyI7^{high}/MyI4^{high}/Hcn4^{high}/Tbx3^{high}/Nkx2.5⁺/Robo1^{low}/Shox2^{low}*);
- LNB (*MyI4^{low}/MyI7^{low}/MyI2^{high}/MyI3^{high}/Hcn4⁺/Gja5^{low}/Gja1⁺/Irx3⁺/Tbx3⁺*);
- HIS and PBB (*MyI2⁺/MyI3⁺/MyI4⁺/MyI7⁺/Irx3⁺/Tbx3⁺/Robo1⁺*);
- DBB and PF (*MyI2⁺/MyI3⁺/MyI4⁺/MyI7⁺/Irx3⁺/Gja5⁺/Gja1⁺/Slit2⁺/Sema3a⁺*);
- VCS (*MyI4⁺/MyI7⁺/MyI2⁺/MyI3⁺/Irx3⁺/Gja5⁺/Sema3a⁺/Slit2⁺*);
- Non-CCS cardiomyocytes (*Hcn4⁻/Cpne5⁻/Cacna2d2⁻/Gja5⁻/Sema3a⁻/Tnnt2^{high}/Tnni1^{high}/Mb^{high}*)

This annotation information has been added to the revised manuscript. Together, we believe that annotated CCS regions align very well with known literature.

Regarding the comment about the absence of *Tbx18* in SAN, we revisited our postnatal CCS scRNA-seq data and ST data and also checked the comparative analysis with E16.5 CCS data. We do think that this Reviewer's comment is very important because our correlation analysis showed that our postnatal SAN is correlated more strongly with SAN (tail) than with SAN (head) (Fig 4b). As described in the previous paper (Goodyer et al. 2019 Circ Res), *Tbx18* and *Isl1* were expressed in SAN of the E16.5 heart, whereas they were not detected in both our scRNA and ST results (figure is shown below for the Reviewer).



Considering that *Tbx18* and *Isl1* are involved in early SAN development and their expression diminishes in the adult heart¹⁰, their absence in our data might indicate pre-to-postnatal maturation. However, we do agree with the Reviewer that there might be a possibility that the entire SAN was not captured due to the relatively lower number of nodal cells in our scRNA-seq data compared to VCS cells. To address this, we have added the following sentences in the Discussion.

(Page 13), "Meanwhile, the correlation analysis between E16.5 and our postnatal CCS data showed that the postnatal SAN cells were more strongly correlated with E16.5 SAN (tail) than with SAN (head). We also observed that *Tbx18*, which marks SAN (head)¹⁰⁵ as well as *Isl1*¹⁰⁶ were undetected in the postnatal SAN region of our scRNA-seq and ST data (data not shown). Considering that *Tbx18* and *Isl1* are involved in early SAN development and their expression diminishes in the adult heart¹⁰⁶, their absence in our data might indicate pre-to-postnatal maturation. However, it may also be attributed to the relatively lower number of nodal cells in our scRNA-seq data compared to VCS cells. Therefore, while the tdTomato signal in the postnatal CCS-tdT mouse heart overlapped with *Shox2* and *Vsnl1* expression in the SAN (**Supplementary Fig. 12**), further investigation to ascertain whether entire SAN regions are captured by *Cntn2* could elucidate this discrepancy."

7. Section entitled: "Transcriptional diversity of key cardiac functional genes within the CCS". The authors spend quite a bit of the manuscript detailing differential gene expression for various gene family members, with the many of the findings being previously established. While this is reassuring from a validation perspective, it would be helpful for the authors to clearly distinguish which are novel findings. Further, this reviewer would like the authors to specifically compare their findings to prior scRNAseq studies of the CCS (Goodyer et al. 2019 and (Bhattacharyya et al 2019 Sci Rep; Goodyer et al 2019 Circ Res) to see how they differ. In particular, since the authors found no significant change in differential gene expression between their three postnatal time points it would be interesting, from a developmental standpoint, to see if there were gene expression changes suggestive of maturation (one of their initial stated goals) between the E16.5 embryonic stage and postnatal stages of their data.

Thank you for the constructive comments. We agree that the novel aspects of our work were not adequately emphasized, as the previous version of the manuscript placed a rather heavy focus on previously demonstrated work, particularly regarding the profiling data of ion channels, sarcomeric proteins and transcription factors (previously Fig. 4). In the current version of the manuscript, we made several revisions. (1) Profiling of ion channels and sarcomeric genes was performed using integrated scRNA-seq and ST data, thus generating a more confident novel result. (2) We removed this figure from the main figures and moved them to Supplementary Figs. 10 and 11. (3) We made this section of the manuscript more concise. In particular, in addition to the validation of a few known electrophysiological and sarcomeric genes, we highlighted novel genes that we believe are worth future functional exploration, such as *Kcnj12*, *Gria3*, *Grik5* and *Ank3*. They were found to have associations with normal or pathological cardiac conduction although its role in the CCS are not well understood. To address the Reviewer's comment regarding emphasis on novel findings, we have made the following change:

8. Page 9, Line 35-37. “On the other hand, *Shox2*, a well-known SA node marker, was not affected by *Tbx3* or *Irx3* in both A-CMs and V-CMs (Fig. S10a), supporting the result that *Shox2* likely acts earlier than *Tbx3* in SA node formation (40).” The authors should not conclude this from their result. They can only state that overexpression of either *Tbx3* and *Irx3* were not sufficient to induce *Shox2* expression in neonatal atrial or ventricular CMs.

We concur with the point the Reviewer mentioned. We revised the sentence and toned down the interpretation as follows:

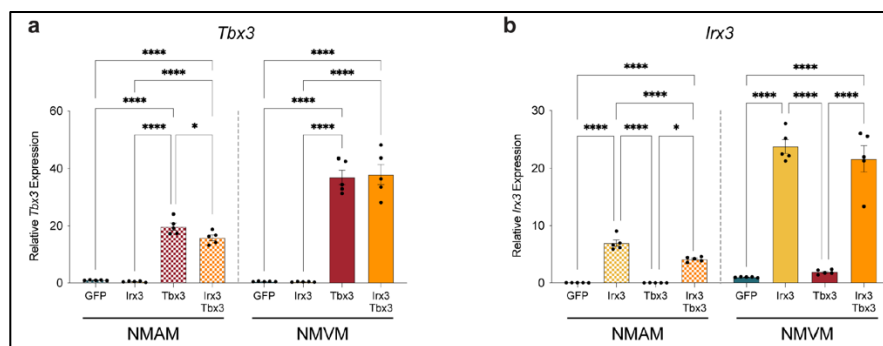
(Page 10) “On the other hand, another SAN marker, *Shox2* was not affected by either *Tbx3* or *Irx3* in both A-CMs and V-CMs (**Supplementary Fig. 25a**), suggesting their insufficiency in regulating *Shox2* expression in neonatal cardiomyocytes. This aligns with the previous data and supports the notion that *Shox2* potentially acts earlier than *Tbx3* in SAN development³⁹.”

9. Page 9, Lines 43-45: “These data suggest that for SA and AV nodal genes, *Tbx3* and *Irx3* play antithetical roles in gene expression: *Tbx3* as an activator and *Irx3* as a repressor.” Again, these claims are overstated as the presented data only represents sufficiency experiments in a very limited setting (AAV overexpression in cultured neonatal rat atrial and ventricular CMs with relatively modest gene expression changes). Further, this reviewer may have missed it but did the authors show to what degree they achieved overexpression of either TF (both by mRNA and protein)?

Thank you for the comment. Although we do agree the sentence that we wrote may read overstating, our findings on the effect of *Tbx3* and *Irx3* on nodal genes, such as *Vsnl1*, *Prdm6* and *Rspo3* suggest this antithetical relationship. We toned down the sentence in the revised manuscript as follows:

(Page 12) “Together, these data suggest that *Tbx3* and *Irx3* can play antithetical roles in gene expression: *Tbx3* as an activator and *Irx3* as a repressor of these tested nodal genes.”

In addition, we have included data for *Tbx3* and *Irx3* mRNA expression levels after adenovirus overexpression in neonatal mouse atrial and ventricular myocytes, to ensure their overexpression. This data is shown in the Supplementary Fig. 24.



Supplementary Fig. 24. *Tbx3* and *Irx3* mRNA expression in NMAMs and NMVMs overexpressing *Tbx3* and *Irx3*.

Minor Comments:

1. Suppl Fig 1: While the *Cntn2Cre/+; Rosa26tdTomato^{+/+}* is a previously published reporter line, the authors really should provide co-staining for each major CCS component (SAN, AVN, His, PF) with

known markers (e.g. endogenous *Cntn2*, *Hcn4*, etc.) to confirm both specificity and sensitivity of labelling the postnatal CCS, as opposed to just showing DAPI.

Thank you for the valuable comment. Indeed, a recent study (Goodyer et al., JCI, 2022) used an engineered antibody targeting *Cntn2* to visualize the entire CCS in mice, strongly suggesting that *Cntn2* can be an excellent target for marking all the CCS components from SAN to PF. As suggested, we performed immunostaining for endogenous *Cntn2* in *Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+} mouse heart and showed that endogenous *Cntn2* and *Cntn2*-cre driven tdTomato overlapped and reliably marked all the CCS (Supplementary Fig. 1). To address this point, we wrote in the revised manuscript as follows:

“Consistent with the previous studies that utilized Contactin-2 (*Cntn2*)-driven reporters and *Cntn2*-targeting antibody to visualize the entire CCS system in mice¹⁴⁻¹⁷, all CCS components were reliably marked in the postnatal CCS-tdT mouse heart, which was validated by endogenous *Cntn2* co-immunostaining (**Fig. 1a** and **Supplementary Fig. 1**).”

2. Figure 1D: Violin plots lacking gene labels

This is our oversight. Revised from previous Fig. 1d, violin plots have now been expanded with additional known CCS markers in newly annotated CCS components. This is shown in Fig. 1e in the revised manuscript.

3. Figure 1F: “Goodyer et al. 2020” likely mis-labelled and should be “Goodyer et al. 2019” instead.

Our apologies for the mislabelling. This information has been corrected in the revised manuscript. E16.5 CCS data is now shown in Fig. 4a.

4. Figure 1i: needs to be enlarged and gene names included – consider fewer top genes per cluster to make legible.

The heatmap of CCS scRNA-seq data (previously Fig. 1i) has now moved to Fig. 2 in the revised manuscript with updated annotation of CCS components. We now have updated heatmaps showing integration of scRNAseq and spatial transcriptomic data for a more reliable identification of novel regional markers. We have also included the list of common genes for each region, as suggested.

5. Page 4, lines 40-43: The authors write, “Of note, the Proximal CCS cluster contained both atrial and ventricular cells, which highly expressed atrial genes (*Myl4* and *Myl7*) and ventricular genes (*Myl2* and *Myl3*), respectively (Fig. S5c,d). This suggested the presence of nodal cells (SA node and AV node) in this cluster and the necessity for sub-clustering analysis.” The sentence is misleading and should be removed as the presence of atrial and ventricular markers would not suggest the presence of SAN and AVN necessarily. The authors later go on to show subcluster analysis but this earlier statement is superfluous and misleading. This comment harkens back to Major comment #1 – where the classification of these cells at the outset is confusing and the initial classification of “Proximal” vs. “Distal” CCS should be avoided altogether.

Thank you for the comments. We understand the possibility that this information could be misleading because these are not the only markers that we used to identify nodal cells. The fact that SAN and

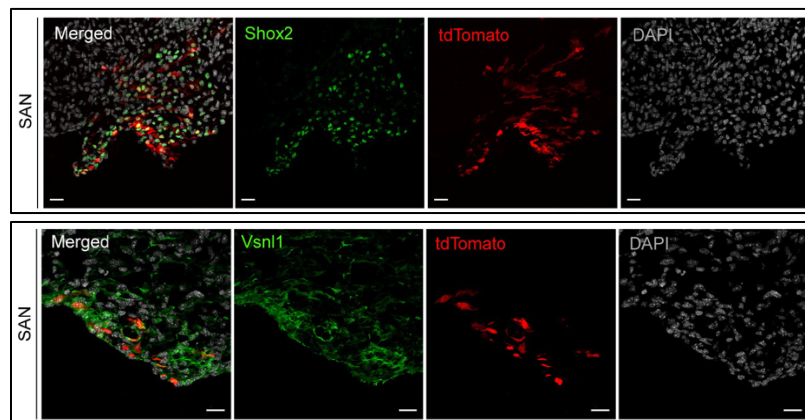
AVN express atrial light chain (Myl4 and Myl7) while VCS expresses ventricular light chain (Myl2 and Myl3) helps as an index to identify nodal cells and VCS in combination with other CCS markers (i.e. Shox2, Tbx3 for nodal cells and Irx3, Etv1 and Gja5 for VCS). Therefore, in the revised manuscript, we have removed this explanation. As described above, we have also removed the terms, 'Proximal CCS' and 'Distal CCS' to describe components within the global CCS in the revised manuscript.

6. Figure S10 a and d: are mislabeled in the legend. Says Vsnl1 and Gja1 but the actual figures show expression of Shox2 and Ryr2 per the labels. Should correct.

We apologize for the mistake. We have fixed the errors in the legend. The qPCR data for Shox2 and Ryr2 is now shown in Supplementary Fig. 25.

7. Fig. 3g: Shox2 and Vsnl1 are well known markers of the SAN. Staining of the SAN with these markers alone doesn't add anything re: validation given that they are established markers.

Thank you for the comment. To provide context, Vsnl1 was the most unique SAN marker we had identified and validated before Vsnl1 in the SAN was described in Liang et al, Nat Commun, 2021. We understand that Vsnl1 is no longer considered novel in our study, so in order to appreciate the published work on Vsnl1, in the revised manuscript, we used Shox2 and Vsnl1 as established positive markers for SAN and showed that they overlapped with tdTomato in the SAN, but not other regions. These data are shown in the Supplementary Fig. 12.



Supplementary Fig. 12: Immunofluorescence staining of Shox2 and Vsnl1 in a P4 CCS reporter heart allows identification of the SAN

In addition, we performed immunostaining for an SAN-enriched marker, Gnao1. We found that Gnao1 is expressed in the SAN as well as the cAVN and atrioventricular ring, but not in the LNB. However, its expression was not specific to nodal cells, as it was observed in the right atrial appendage, although not in the left atrial appendage as well as other CCS regions. This data is shown in Fig.3a (shown below) and Supplementary Fig. 13 and is described in the text as follows:

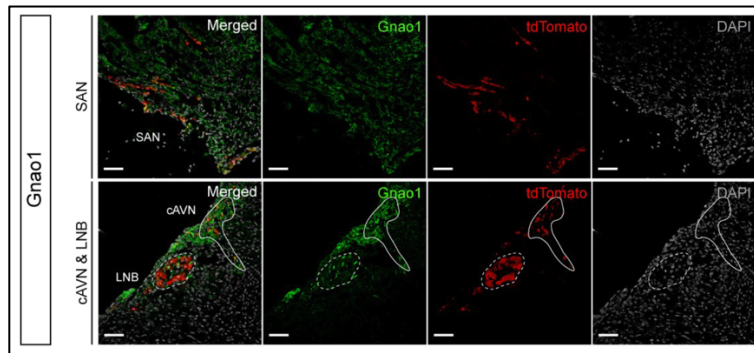


Fig. 3a: Gnao1 expression in SAN and cAVN

In the revised manuscript, we have written as follows:

(Page 7) “An SAN-enriched marker, Gnao1 was expressed in tdTomato-positive pacemaker cells (**Fig. 3a**), where Shox2 and another recently discovered SAN marker, Vsnl1³¹, were expressed (**Supplementary Fig. 12**). Gnao1 expression was also detected in the cAVN and atrioventricular (AV) ring, but not in LNB. However, its expression was not specific to nodal cells, as it was observed in the right atrial appendage, although not in the left atrial appendage as well as other CCS regions (**Supplementary Fig. 13**).”

8. Figure 5d: “CC” should be defined in legend.

Thank you. We have defined all the acronyms in the legends.

Recommendation: Conditional accept pending substantial revisions that address major and minor comments.

Reviewer #3 (Remarks to the Author):

The manuscript by Oh et al., describes the transcription landscape and the heterogeneity within the cardiac conduction system (CSS) across early pre-post-natal developmental timepoints, immediately after birth using scRNA-seq. As studies and our understanding of these specialised cardiomyocyte cell-types remains limited, the CCS single cell transcriptomics for post-natal hearts present a good resource to incorporate together with pre-existing studies from those of Goodyer W. Et al., Bhattacharyya S et al., and Liang et al. By analyzing ~7000 CCS cells and identifying 4 distinct clusters which include proliferative CCS, contractile cardiomyocytes, distal CCS and proximal CCS – the latter of which was further subclustered into three categories: nodes (SA and AV), His bundle, bundle branches. The use of a reporter system also helps to comprehensively capture cells of the CSS. Genes pertaining to ion channels, saromeric proteins and transcription factors were studied across the clusters and the transcription factors *Irx3* and *Tbx3* were chosen as candidates for adenovirus over-expression experiments in isolated atrial and ventricular CMs. (ACM, VCM), which the authors used to influence the expression of specific CSS markers and potentially its cell state/identity.

Genes typically expressed in the SA node were markedly increased by *Tbx3* and suppressed by *Irx3*. The roles of the transcription factors were inversed for genes typically enriched in the Purkinje fibres,

with *Irx3* activating and *Tbx3* suppressing gene expression. Both *Irx3* and *Tbx3* functioned as activators for genes enriched in the His bundle, and repressors for genes in contractile cardiomyocytes. As said, this paper provides a valuable resource for CCS single-cell transcriptomic data with an interesting angle providing molecular targets that could be important modulators of gene expression across different tissue types.

We thank the Reviewer for the very positive comments. The specific comments from the Reviewer truly helped improving our manuscript. As summarized in the responses to two other Reviewers above, we have revised our manuscript significantly. First, we performed new spatial transcriptomics experiment to examine the CCS components and identify novel genes with confidence. Second, we conducted additional bioinformatic analyses to address the Reviewer's concerns and suggestions. Please see the point-by-point responses below.

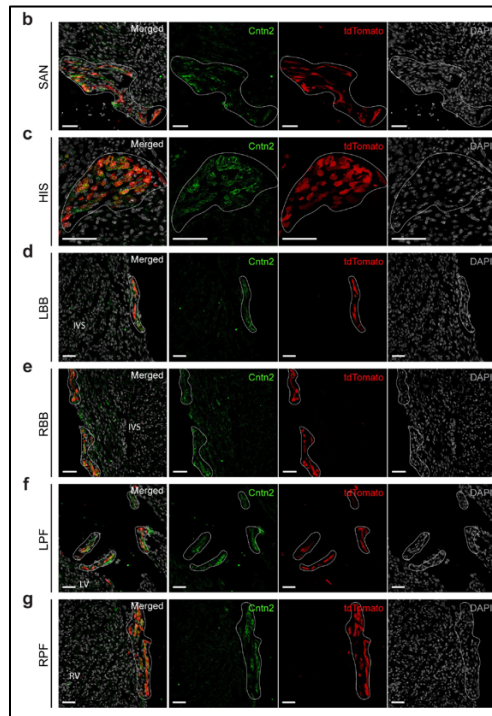
1. Could the authors justify and clarify the use of the CnTn2 reporter system in text, apart from it being a postnatal CCS reporter, and make comparisons to other conduction reporter systems like those of Gjd3 reporter system for comprehensive isolation of CSS cell types?

Thank you for the valuable comment. We have received a similar comment from Reviewer #2. To address this comment, we have revised the text to emphasize the Cntn2 reporter system and its validity. In the 'Results' and 'Discussion' sections, we have written as follows:

(Page 4) "Consistent with the previous studies that utilized Contactin-2 (*Cntn2*)-driven reporters and Cntn2-targeting antibody to visualize the entire CCS system in mice^{21,33-35}, all CCS components were reliably marked in the postnatal CCS-tdT mouse heart, which was validated by endogenous Cntn2 co-immunostaining (**Fig. 1a** and **Supplementary Fig. 1**)."

(Page 12) "Among several Cre lines targeting the CCS, including Hcn4CreERT2⁹⁷, Cx40-CreERT2⁹⁸, Gjd3-CreEGFP³⁰ and Sema3a-CreERT2⁶⁷, the *Cntn2*^{Cre/+} model was chosen as it could mark all components of the early postnatal CCS in mice without tamoxifen induction, consistent with *Cntn2*-driven reporters and Cntn2-targeting antibody^{21,33-35}. To our knowledge, this study has incorporated the highest number of purified entire mouse CCS cells to conduct a single-cell transcriptomic investigation

In addition, we conducted co-immunostaining using endogenous Cntn2 antibody, showing that tdTomato signaling from Cntn2-Cre driven labeling was fully co-labelled with endogenous Cntn2 throughout the CCS (Supplementary Figure 1), further addressing why Cntn2-cre is an appropriate CCS reporter model:



Supplementary Fig. 1: *Cntn2*Cre/+;*Rosa26*tdTomato/+ heart mark all the CCS components

2. Figure 1d: Please include the genes on the right y-axis used in the analysis used to identify the conduction and contraction categories.

We are sorry for the oversight. As we responded to Reviewer #2 above, previous Fig. 1d, violin plots have been expanded with additional CCS markers in newly annotated CCS components, which is now shown in Fig. 1e in the revised manuscript.

3. Where possible, it would be helpful to include p-values to test for significance in the violin plots.

Due to new spatial transcriptomic data and its integration with scRNA-seq, most of the violin plots that we showed in the previous manuscript are now removed. In Fig. 4d where we compared E16.5 and postnatal CCS, we used violin plots and added asterisk to indicate significance. In addition, we attached the entire list of differentially expressed genes between E16.5 and postnatal CCS which also contains P-values as Supplementary Table. 3.

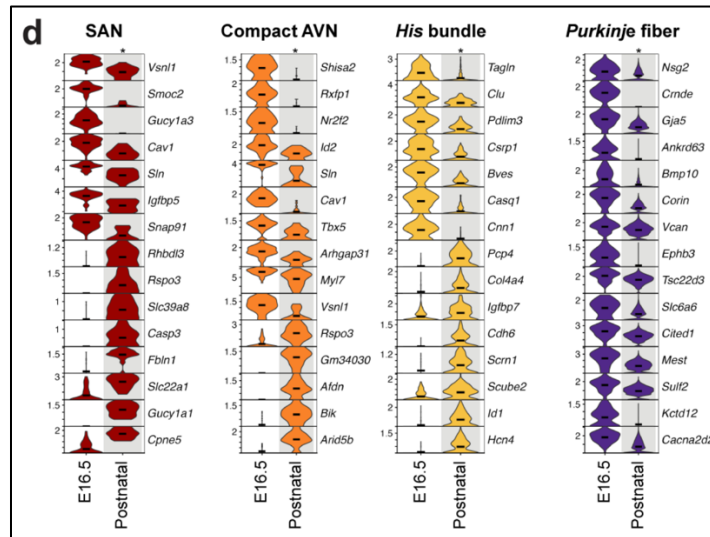
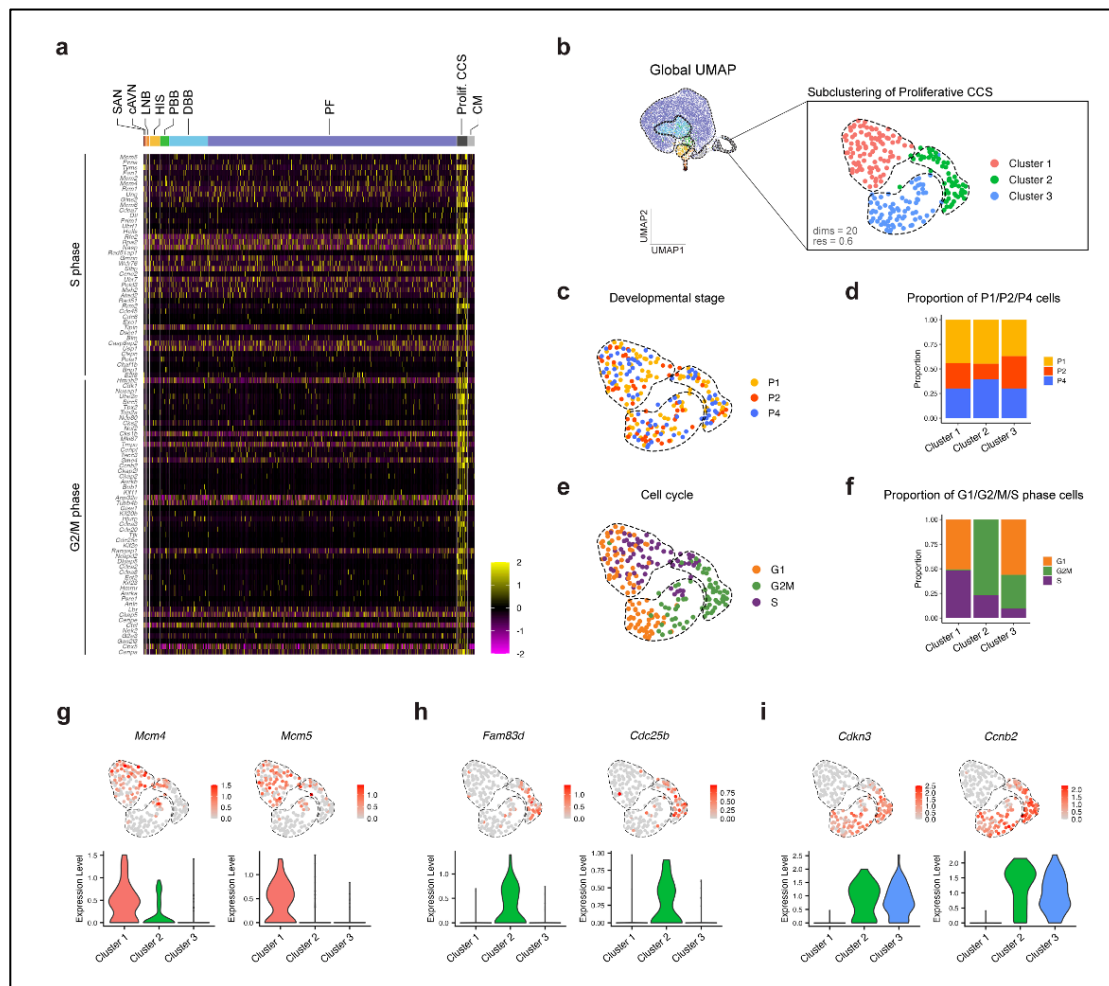


Fig. 4d: Pairwise differential gene expression analysis between embryonic and postnatal CCS

4. There is quite a large proportion of proliferative CCS which is interesting, Can figure 1H be augmented with more canonical cell-cycle markers?

Thank you for the comment. We also found it very intriguing that 3.24% of CCS cells (222 out of 6855 cells) are proliferating. As suggested, we performed additional analysis on these cells (Supplementary Fig. 8). We examined the expression patterns of all characterized S phase and G2/M phase genes⁹, and found specific enrichment of both of these genes in the Proliferative CCS cluster. Sub-clustering analysis of the Proliferative CCS cluster generated three subclusters, which were not based on the developmental stage of the CCS (P1/P2/P4). Consistently, the proportion of P1/P2/P4 cells in each cluster was relatively comparable. We observed a cluster enriched with G1/S transition genes (*Mcm4*, *Mcm5*), another enriched with G2/M phase genes (*Fam83d*, *Cdc25b*) and another with enriched with both G1/S and G2/M genes (*Cdkn3* and *Ccnb2*, respectively). Together, this data shows that a significant number of cells remain actively engaged in the cell cycle and proliferating in an early postnatal CCS. In the revised manuscript, these results are shown in Supplementary Fig. 8 (shown below) with the following text:

(Page 5) “Our scRNA-seq showed a cluster of cells (222 out of 6855 cells, 3.24%) annotated as ‘proliferating CCS’, because these cells expressed CCS genes (*Cntn2*, *Etv1*, *Irx3*, *Gja5*) as well as cell cycle markers of G1/S phase (*Mcm4*, *Mcm5*) and G2/M phase (*Fam83d*, *Cdc25b*) (**Supplementary Fig. 8**). This suggests the presence of committed conduction cells undergoing proliferation in the early postnatal heart.”



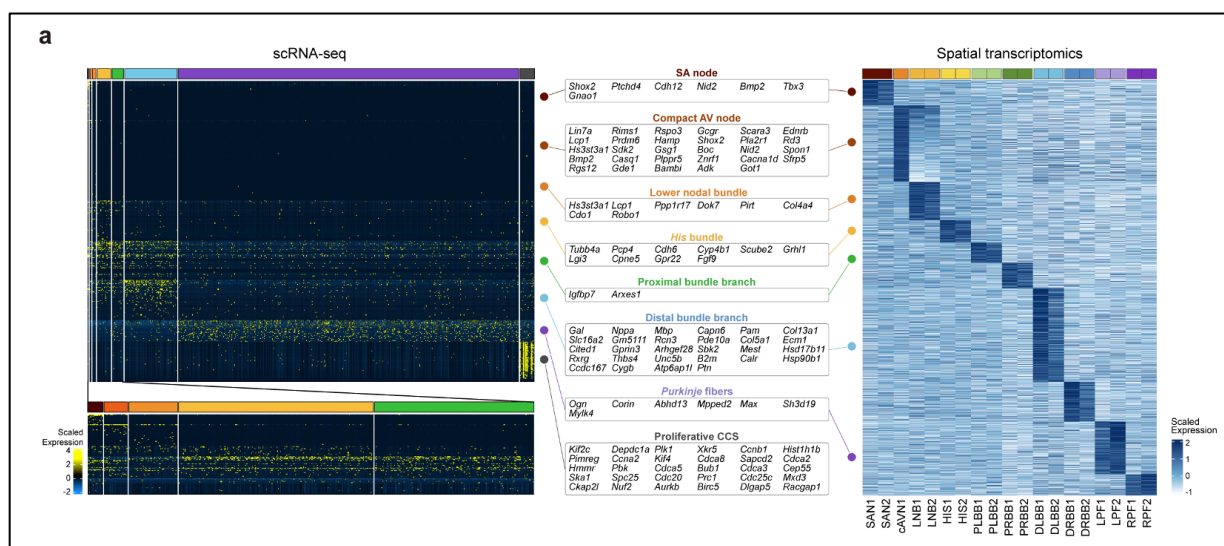
Supplementary Fig. 8. Postnatal CCS contains a cluster of Proliferative CCS cells.

5. Please identify the specific GO database used to conduct the pathway analyses and obtain the gene set descriptions (ie. KEGG, GO biological processes)

Thank you for the comment. Previous Fig. 1J showing gene ontology analysis has been omitted in the revised manuscript.

6. Within Figure 3A, it will be helpful and insightful for the reader for Figure 3D to highlight both the known and novel markers of SA node, AV node, His bundle and bundle branches.

In the revised manuscript, we performed a new spatial transcriptomic analysis and integrated it with scRNA-seq data to identify CCS-specific genes with high confidence. This led us to confirm the previously known CCS markers and also identify novel marker genes. The list is now shorter enhancing the readability of the figure (Fig 2a, shown below).



In the revised manuscript, we have highlighted known and novel markers of each CCS component as well as CCS regions. We have revised the text as follows:

(Page 6) “In the SAN, as expected, *Shox2*^{39,40} and *Tbx3*^{41,42}, well-known transcription factors in SAN development, were observed. In addition, we identified other potential novel markers, including *Gnao1*, which encodes G protein subunit alpha O1 and has been found to be involved in heart development⁴⁶, as well as *Ptchd4*, *Cdh12*, and *Nid2*. The cAVN showed enrichment of known genes, such as *Rspo3* and *Bmp2*, both of which are expressed in the atrioventricular canal of the developing mouse heart (E10.5) from which the AVN originates^{47,48}, as well as *Cacna1d*, which encodes Ca_v1.3 channel known to regulate AVN automaticity⁴⁹. Newly identified markers of the cAVN included *Prdm6*, which encodes PR/SET domain 6 and has been shown to be expressed in the developing cardiovascular⁵⁰⁻⁵² and nervous system⁵³. Interestingly, a genetic variant of *Prdm6* has been reported to be associated with heart rate recovery during exercise⁵⁴. The LNB showed enrichment of genes, including *Ppp1r17*, *Hs3st3a1*, and *Lcp1*. A previous study has shown that *Ppp1r17*, encoding protein phosphatase 1 regulatory subunit 17, is expressed in the interventricular septum during embryonic heart development, and is mostly lost in later developmental stages⁵⁵. Like many other cardiac conduction genes, *Ppp1r17* is also expressed in neurons⁵⁶, yet its expression and function in the CCS are unknown. The HIS showed enrichment of *Pcp4*, *Cdh6* and *Cyp4b1*. Although our analysis showed that these genes are most highly expressed in the *His* bundle, *Pcp4* is known to be expressed throughout the VCS⁵⁷ with critical roles in cardiac electrophysiology, and *Cdh6* is known to be enriched in the CCS compared to non-CCS cardiomyocytes⁵⁸. This suggests that there may be regional heterogeneity in the expression levels of these genes within the CCS. The PBB highly expressed *Igfbp7* and *Arxes1*, while the DBB showed high expression of genes, such as *Nppa*, *Gal*, *Ecm1*, *Cygb*, *Slc16a2*, *Pde10a* and *Mbp*. *Nppa*, encoding atrial natriuretic peptide^{59,60}, is known to be expressed in embryonic trabecular cardiomyocytes^{61,62}, and its expression in the ventricle is strongly reduced after birth. However, we observed low but specific expression of *Nppa* in the DBB, suggesting a possibility of persisting *Nppa* expression in the distal VCS postnatally. The PF showed enrichments of genes, including *Mpped2*, *Corin*, and *Mylk4*. In particular, *Mpped2*, encoding Metallophosphodiesterase domain-

containing protein 2, has previously been reported to be expressed in neurons and embryonic ventricles⁴⁸, although its functional involvement in the CCS has not been described.

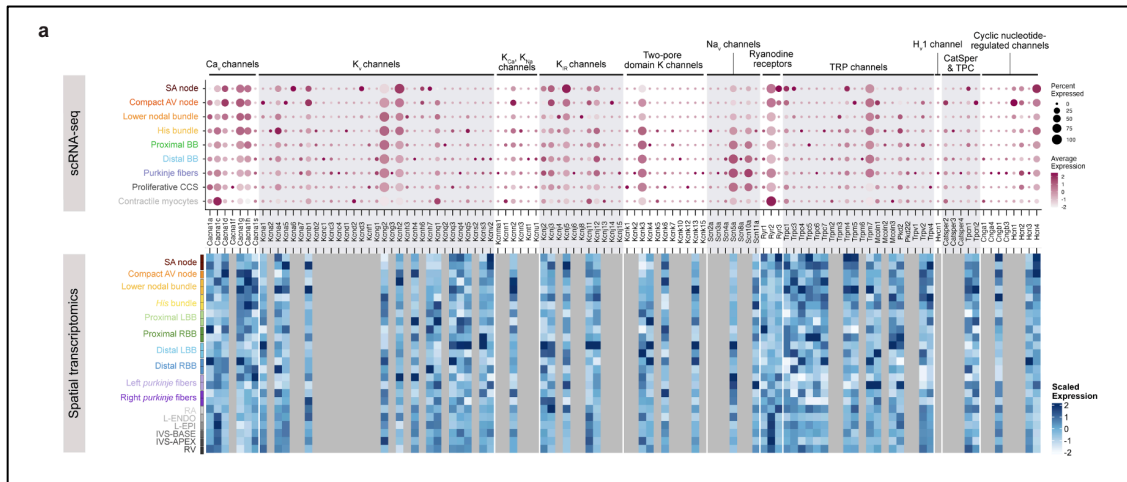
7. Figure 3h: The IF staining for Rspo3 does not appear to be specific to the AV node site, though it definitely appears to be enriched. Could the authors comment on this, as the scRNA analysis shows site specificity?

Thank you for the comment on this important validation. Indeed, the Reviewer #2 pointed out the same issue, and we addressed this comment as follows:

“Thank you for the comment on this important validation. Indeed, Reviewer #3 pointed out the same issue. We examined our image data and agreed that Rspo3 expression is broader than simply compact AVN, suggesting Rspo3 may be an AV junctional marker, rather than cAVN-specific marker. We also encountered technical challenges due to unstable antibody. Thus, we sought another cAVN marker, Prdm6, and performed validation experiments using RNAScope. As shown in Fig. 3b, Prdm6 expression was found in cAVN and AV nodal ring regions, but not in the LNB region or other CCS or non-CCS regions (Supplementary Fig. 14). Notably, PRDM6 has been shown as a potential gene regulator in human nodal cells (Kanemaru et al., Nature 2023), suggesting that it may be an evolutionarily conserved cAVN regulator.”

8. Figure 4c-e: It would be helpful to group/categorize the list of genes in the heatmap to highlight specific genes/groups of genes described in the main manuscript.

Thank you for the helpful comments. We agree that the profiling data of ion channels, sarcomeric proteins and transcription factors that we showed in previous Fig. 4 was difficult to follow. We have updated this data by showing profiling of ion channels and sarcomeric proteins from the integrated scRNA-seq/spatial transcriptomic analysis. To make the combined dotplot-heatmaps clearer and more legible, we categorized the ion channel genes into voltage-gated ion channels, ligand-gated ion channels, and other ion channels based on classifications on IUPHAR-DB (the International Union of Basic and Clinical Pharmacology Database). In addition, we categorized sarcomeric genes into A band, I band, and Striated muscle thin & thick filament according to gene ontology classifications, GO0031672, GO0031674, GO0005863 & GO0005865, respectively. These profiling results are now shown in the Supplementary Figs. 10 (shown below as an example) and 11 in the revised manuscript, and we anticipate the updated dotplot-heatmaps to help readers find information more easily. This information for analysis method is also included in the Methods section of the revised manuscript.



9. Figure 4e: Could the reviewers describe how the panel of genes for ion channels, sarcomeric proteins and transcription factors were identified? Ie. Specific databases/papers. Was there a way to determine/confirm transcription factors beyond obtaining DEG's from the transcriptomic analysis? Ie. Packages such as SCENIC to determine regulons.

Thank you. As for the first comment, please refer to the above response for information on the database used for ion channels and sarcomeric gene profiling.

We thank you for the Reviewer's insightful comment and suggestion. We agree that simple DEG analysis to identify meaningful transcription factors (as we showed in previous Fig. 4c) was insufficient to provide mechanistic insights into transcriptional regulation of the CCS. Following your suggestion, we performed SCENIC to identify regulons and examine transcription factor activity in each cell in separate CCS regions (Fig. 5a in the revised manuscript, shown below). SCENIC analysis mechanistically identified different TFs exerting their actions in different components/zones within the CCS. Using these results, we constructed gene regulatory networks in SAN, AVN, HIS and PF, inferring major transcriptional regulators and their target genes within each CCS region (Supplementary Fig. 22, partly shown below). More importantly, SCENIC analysis uncovered Tbx3 and Irx TFs, suggesting their implications regulating the transcriptional heterogeneity of the CCS. We believe this analysis greatly improved the reliability as well as the depth of the study, so we once again thank the Reviewer for the excellent suggestion.

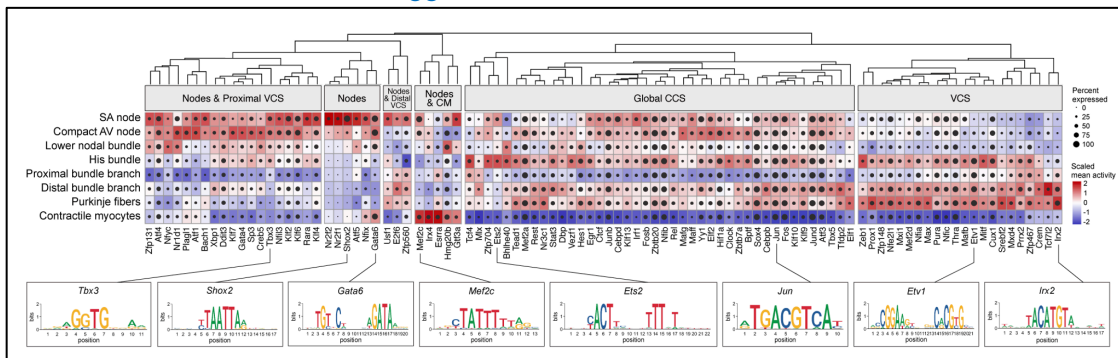
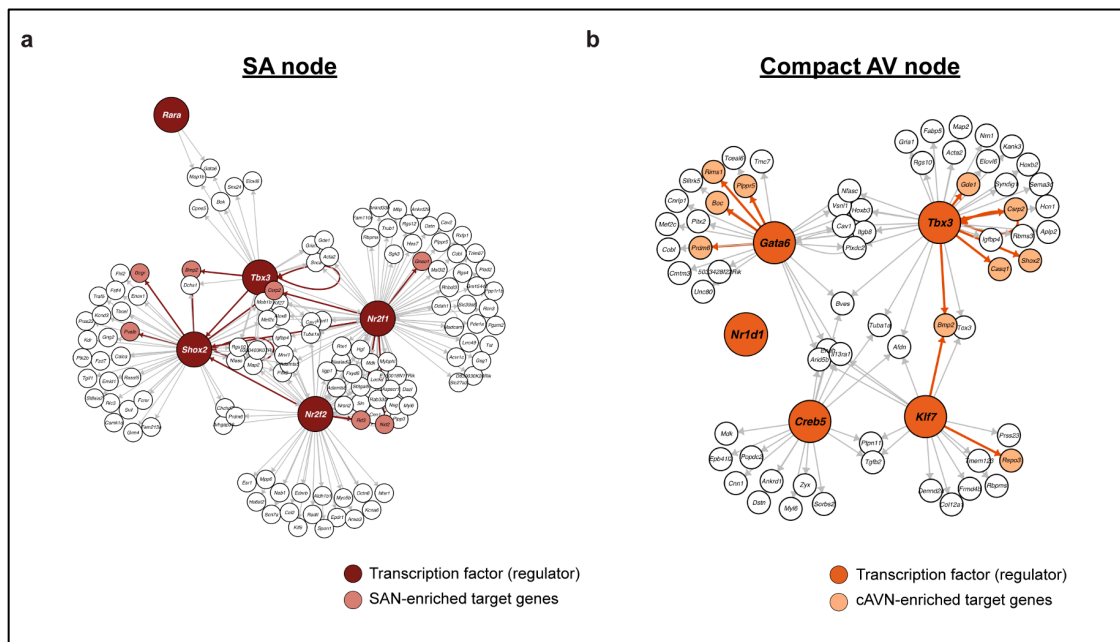


Fig. 5a: Integrated heatmap/dotplot of CCS regulons, as inferred by SCENIC algorithm. Heatmap colour represents regulon activities for each CCS component based on the area under curve (AUC) scores.



Supplementary Fig. 22. Gene regulatory networks of CCS components

10. In Figure 5, the authors have overlapped existing Tbx3 ChIP-seq and ATAC-seq datasets, and also highlighted the *Irx* motifs zoomed into specific locuses. Overall, in relation to the markers identified from the sub clustering of the scRNA-seq data in this study (Figure 3D), what are the number/percentage overlaps with Tbx3 ChIP/ATAC/*IRX* signals?

Thank you for another important and insightful comment. To address this question, we examined how many of the CCS component and regional markers (i.e. nodal markers, proximal VCS markers, and distal VCS markers) that we identified from scRNA-seq/ST analyses show chromatin accessibilities in CCS and non-CCS regions, Tbx3-binding, and the presence of *Irx* TF-binding motifs. Specifically, we found that the nodal markers showed greater chromatin accessibilities in SAN ATAC-seq data, compared to VCS genes. The nodal genes and proximal VCS genes exhibited greater open chromatin peaks in AVJ ATAC-seq data, than distal VCS genes. Also, Tbx3-ChIP-seq peaks were found more often in the nodal genes than the distal VCS genes. Lastly, *Irx* motif were observed more often in the distal VCS, compared to nodal genes. Therefore, the results from this analysis are aligned very well with other expression data, further strengthening our conclusion. We have included this finding in the text of the revised manuscript as follows:

(Page 9) “By intersecting these datasets, we examined chromatin accessibilities in CCS and non-CCS regions, Tbx3-binding, and the presence of *Irx* TF-binding motifs for the marker genes that we identified in this study. 45.9% (17/37) of the nodal markers showed SAN ATAC-seq peaks, which contrasts with 16.1% (5/31) of the proximal VCS genes and 11.4% (5/44) of the distal VCS genes, corroborating our findings. Similarly, 89.2% (33/37) of the nodal markers and 80.6% (25/31) of the proximal VCS markers showed chromatin accessibilities in AVJ, compared to 70.5% (31/44) of the distal VCS markers. Furthermore, 79.5% (89/112) of the CCS markers appeared to be potentially regulated by Tbx3 or *Irx* TFs. Specifically, Tbx3-ChIP-seq peaks were present in 70.3% (26/37) of the nodal genes, with a mildly lower incidence of 61.4% (27/44) in the distal VCS genes. In contrast, *Irx* motifs were found in 37.8% (14/37) of the nodal genes, while a greater prevalence of 61.4% (27/44) was noted in the distal VCS

genes. These observations align with the expression patterns of the CCS markers and suggest the individual and cooperative regulatory role of Tbx3 and Irx TF across the CCS.”

11. The results in Figure 6 are interesting, showing cardiomyocyte subtype specific response to Tbx3 and Irx3. But the authors have not included a negative control gene locus to the figure (using neuronal markers or markers of other cell types)?

Thank you for the positive comments on our data using Tbx3/Irx3 overexpression in vitro model. To address the Reviewer’s comments, we thought about potential negative control genes, but found difficulty to chose one because Tbx3 and Irx3 are expressed in many different organs and tissues, including brain, liver etc. At the same time, we believed that our best negative control gene was indeed housekeeping gene (Tbp, TATA box binding protein) that we used for relative comparison analysis. Certainly, we found very stable gene expression (cT values) of Tbp, functioning as a negative control (Reference figure shown below for the Reviewer). In addition, we believe that our data showing clearly different regulatory responses by Tbx3 and Irx3 in atrial and/or ventricular cardiomyocytes function as internal negative control. For example, Mpped2 and Slit2 were regulated by Tbx3 and Irx3 in the ventricular cardiomyocytes, but not in atrial cardiomyocytes. I hope this addresses the Reviewer’s concern.

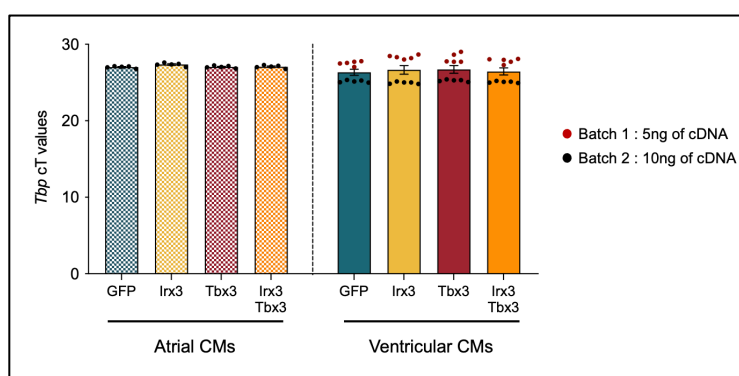
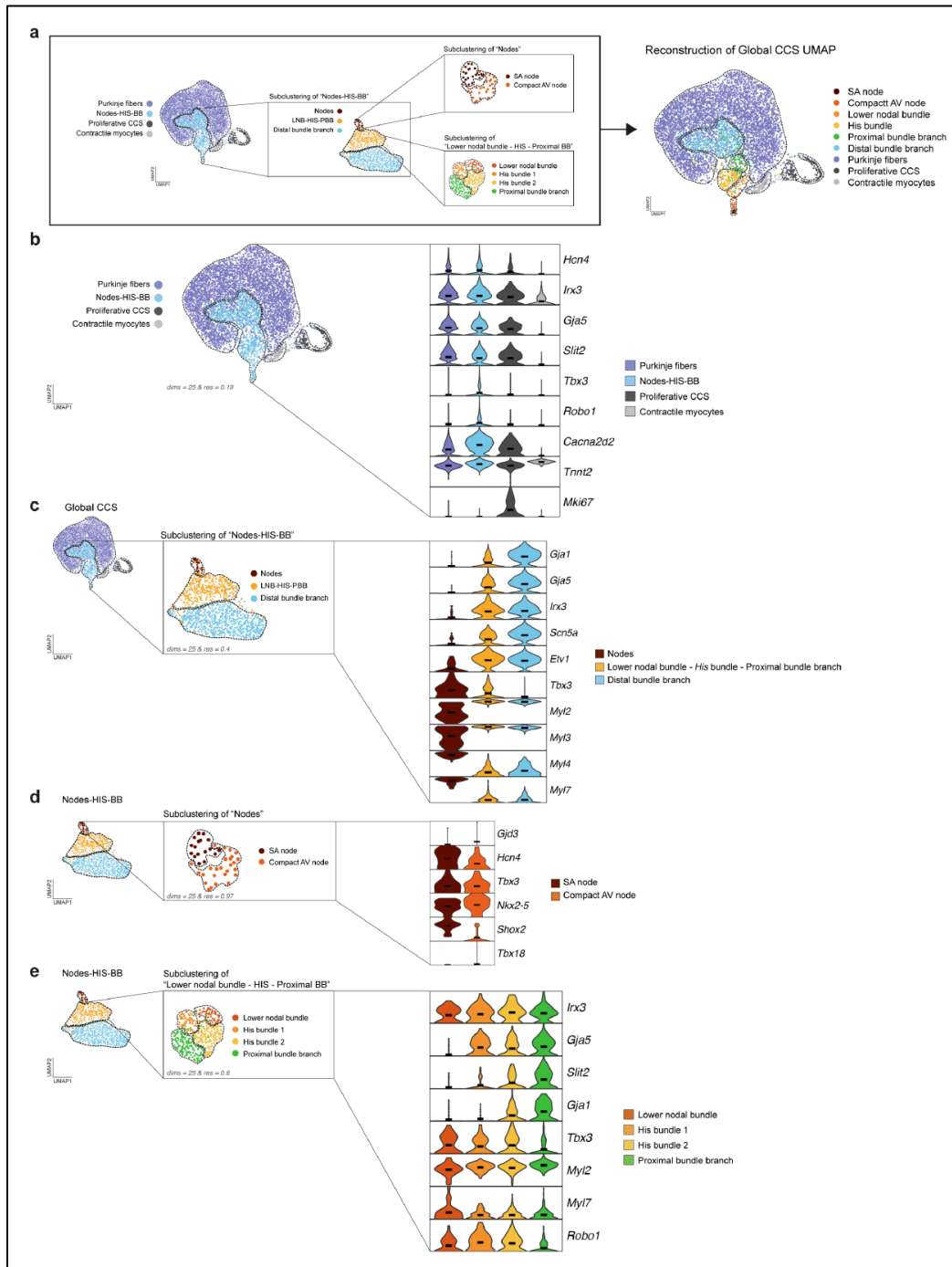


Figure. Tbp expression in atrial and ventricular CMs upon Irx3 and Tbx3 overexpression

Minor comments:

Can the authors include in detail of R-packages / Python modules for in silicon analysis of data? For example, details for gene marker identification and clustering/sub-clustering in the methods? e.g using of FindAllMarkers() function for the clustering of cells, and include if any pre-processing steps

Thank you for the comment. We have expanded our methods section of the revised manuscript to include information on the R/Python packages used, as well as the functions and parameters that were different from the default settings. We have also included values for the parameters (dimensions used, resolution for cluster identification) in the figure showing detailed subclustering analysis (Supplementary Fig. 5). We have uploaded codes used for figure generation as well as the following bioinformatic analysis: (1) scRNA-seq postnatal CCS data, (2) integration of embryonic (E16.5, Goodyer et al. 2019) and postnatal CCS data, and (3) SCENIC analysis.



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

With reference to Figure 5, it will be helpful to name the following in legends: BB, PF, VCS and CC.

Thank you. We have included the full names of the acronyms in our revised figures.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have added spatial transcriptomics data (GeoMX DSP) and integrated these with their scRNAseq data. In addition, they compared their scRNAseq data with those of Goodyer 2019 (an in our experience excellent data set). With this, the revised manuscript has gained depth and value.

Some concerns remain regarding the balance between the data regarding CCS components provided and the attention they receive in the manuscript. If I am not mistaken, the authors provide scRNAseq data of 17 SAN cells and 26 cAVN cells, vs 1200 DBB and >5000 PF cells? If this is correct, then the analysis of the nodal cells is underpowered and can easily be overinterpreted, whereas the VCS cell analysis is well powered, allowing interpretation with greater confidence. Perhaps the authors can comment on this and also take that into account in their manuscript.

The Adv-mediated overexpression of TFs in atrial or ventricular cells is in my opinion not as informative as the authors suggest. 1) using such a delivery system, these very dose-sensitive TFs will be expressed at high levels, which is outside the physiologically relevant range, and 2) the cells do not represent CCS cells, the added TFs thus acting on non-CCS gene regulatory networks. In addition, the cause of the small effect sizes is not clear (e.g. Nppa is expected to be much more than 2 fold down-regulated by Tbx3).

Reviewer #2 (Remarks to the Author):

Comments on re-submission from Oh et al. 2024 to Nat Comm, entitled “Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity”

In summary, the authors provide a description of the transcriptional landscape of the postnatal murine cardiac conduction system, providing particular robustness to the various cell types within the ventricular conduction system (VCS) (His, bundle branches and PFs), not previously captured. In their resubmission, the authors did a robust job at addressing reviewer comments and suggestions, dramatically improving both the novelty and quality of the overall manuscript. Specifically, the authors went on to combine both spatial transcriptomics as well as additional bioinformatic analyses including the incorporation and contrasting with embryonic datasets (Goodyer et al. 2019) to illustrate maturational changes at the transcriptomic level throughout the VCS components, as well as network inference analysis using SCENIC to shed new light on the

potential transcriptional regulators underpinning these differences. I believe that the authors' characterization of the individual subcomponents of the VCS using both single cell and spatial analyses provides a level of detail not previously achieved and backed up with validation assays, be it IF or FISH (Fig 3 and Supp Figs 13-21). These data are of value to the field as they improve our basic understanding of this complex, important and heterogeneous cell population, in particular as we move towards trying to generate individual CCS components in vitro. Overall, the authors did a comprehensive job in their resubmission. Well done.

Minor Comment:

P. 7 lines 253-254: "We performed FISH to examine cAVN-enriched Prdm6, showing that it was primarily expressed in cAVN and AV ring, but not in the LNB and other CCS or non-CCS regions". Prdm6 seems to be clearly expressed in surrounding non-CCS tissue. The authors should amend their statement to reflect this.

Reviewer #3 (Remarks to the Author):

The authors have revised and improved the manuscript with added spatial transcriptomics data and additional in-silico analysis. The authors have also expanded with integrating embryonic CCS data from Goodyer et al., 2019.

Below are just some additional comments for clarification:

1. In Figure 1D, it seems that the PCA cluster for RAA, LENDO, LEPI, IVS-B, IVS-A, and RV is omitted. It may be missed out inadvertently.
2. Sub-cluster for Compact AV node for one of the replicate seem to be missing, though there are two replicates in the PCA.

Oh et al. Response to Reviewer comments

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have added spatial transcriptomics data (GeoMX DSP) and integrated these with their scRNAseq data. In addition, they compared their scRNAseq data with those of Goodyer 2019 (an in our experience excellent data set). With this, the revised manuscript has gained depth and value.

Some concerns remain regarding the balance between the data regarding CCS components provided and the attention they receive in the manuscript. If I am not mistaken, the authors provide scRNAseq data of 17 SAN cells and 26 cAVN cells, vs 1200 DBB and >5000 PF cells? If this is correct, then the analysis of the nodal cells is underpowered and can easily be overinterpreted, whereas the VCS cell analysis is well powered, allowing interpretation with greater confidence. Perhaps the authors can comment on this and also take that into account in their manuscript.

First and foremost, we are truly thankful to the Reviewer for recognizing the value of the added work, which includes the integration of spatial transcriptomics as well as the comparison between P0 and E16.5 CCS datasets. We are confident that our data provides valuable information, especially to those studying the cardiac conduction system.

We also fully agree and understand the Reviewer's concern about a potential over- and/or under-estimation of differentially expressed genes due to the differing cell numbers per cluster. This is a common issue of scRNA-seq analysis that is also under active discussion. Aware of these potential issues arising from varying numbers of cells in CCS components, we spent a significant amount of time ensuring the validity of our data using various verification methods. Because of this concern, we decided to perform spatial transcriptomics during the previous revision. We believe that integrating scRNA-seq with spatial transcriptomics has significantly helped to overcome this issue, therefore allowing for the discovery of novel CCS marker genes with greater reliability. We further validated our findings histologically using immunofluorescent and fluorescent in situ hybridization methods.

As suggested by the Reviewer, we have discussed this potential issue of scRNA-seq data and addressed how this limitation was mitigated by spatial transcriptomics in the revised manuscript as follows:

(Page 12) "Furthermore, although GeoMX DSP presents technical limitations due to its probe-based transcript counting method, ST data compensated potential statistical biases in our scRNA-seq data that arose from varying numbers of cells in CCS components. Collectively, this integrative approach, supported by histological validations, enabled the discovery of novel CCS marker genes with greater reliability."

The Adv-mediated overexpression of TFs in atrial or ventricular cells is in my opinion not as informative as the authors suggest. 1) using such a delivery system, these very dose-sensitive TFs will be expressed at high levels, which is outside the physiologically relevant range, and 2) the cells do not represent CCS cells, the added TFs thus acting on non-CCS gene regulatory networks. In addition, the cause of the small effect sizes is not clear (e.g. *Nppa* is expected to be much more than 2 fold down-regulated by *Tbx3*).

Thank you for the comments; we appreciate your valid concern.

The primary objective of this experiment using adenovirus-mediated overexpression was to understand the combinatorial role of *Tbx3* and *Irx3* in the regulation of CCS genes as an *in vitro* model to mimic CCS heterogeneity. Strongly supported by accessibility peaks (ATAC-seq) from different CCS components (i.e. SAN, *Tbx3*+ atrioventricular junction), *Tbx3*-binding (TBX3 ChIP-seq) and *Irx* motifs, our experiment indeed demonstrated that the expression patterns of several known and newly identified CCS regional markers were regulated through the cooperative or antagonistic actions of *Tbx3* and *Irx3*. Previous studies have highlighted the individual roles of *Tbx3* (Bakker et al., *Cardiovasc Res*, 2012; Lu et al, *Can J Cardiol*, 2020) and *Irx3* (Zhang et al, *PNAS* 2011; Kim et al, *Sci Rep*, 2016) in gene regulation. However, we would like to emphasize that our study is the first to reveal the joint actions of *Tbx3* and *Irx3* in the regulation of CCS genes in a chamber-specific manner. Notable examples include the activation of *Vsnl1*, a recently identified SAN marker, exclusively in atrial cardiomyocytes, not in ventricular cardiomyocytes. Conversely, distal VCS genes, such as *Mpped2*, and *Slit2*, were increased by *Tbx3* and *Irx3* in ventricular cardiomyocytes, not in atrial cardiomyocytes. Furthermore, the well-known *Tbx3* target, *Gja5*, was repressed by *Tbx3* in atrial cardiomyocytes, but was mildly activated by *Tbx3* in ventricular cardiomyocytes. *Irx3*-mediated increase of *Gja5* expression was also diminished by *Tbx3* overexpression. These data suggest that *Tbx3* and *Irx3* can play distinct functional roles in gene regulation in the neonatal mouse atrial and ventricular cardiomyocytes. Therefore, we believe our data provides proof-of-principle evidence of their contributions to CCS regional heterogeneity in chamber-specific manners.

Nevertheless, we do agree with the Reviewer's concerns about potential limitations related to adenovirus-mediated overexpression of TFs, which exceed physiological levels. In addition, the relatively moderate effects of *Tbx3* and *Irx3* on gene regulation may be attributed to the maturity of neonatal mouse cardiomyocytes. As our comparative analysis of postnatal CCS with E16.5 CCS data suggests, CCS heterogeneity may be established as early as the E16.5 heart stage. Therefore, utilizing immature cardiomyocytes from embryonic hearts could potentially enhance the responsiveness to TF overexpression. As noted by another Reviewer, our results could inform future efforts to generate individual CCS components *in vitro*, such as with iPSC-derived cardiomyocytes.

To address the Reviewer's concerns and outline potential limitations as well as possible future direction, we have revised the discussion in the manuscript as follows:

(Page 13) "The *in vitro* model we utilized has potential limitations due to adenovirus-mediated high TF expression, which exceeds physiological levels, and the relatively moderate effects of *Tbx3* and *Irx3* overexpression on gene regulation, which can be attributed to the maturity of

neonatal mouse cardiomyocytes. Therefore, further investigation is warranted to elucidate the precise regulatory mechanisms by which the gene dosage balance between *Tbx3* and *Irx3* positively and negatively fine-tunes gene expression in a developmentally more potent system.”

Reviewer #2 (Remarks to the Author):

Comments on re-submission from Oh et al. 2024 to Nat Comm, entitled “Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity”

In summary, the authors provide a description of the transcriptional landscape of the postnatal murine cardiac conduction system, providing particular robustness to the various cell types within the ventricular conduction system (VCS) (His, bundle branches and PFs), not previously captured. In their resubmission, the authors did a robust job at addressing reviewer comments and suggestions, dramatically improving both the novelty and quality of the overall manuscript. Specifically, the authors went on to combine both spatial transcriptomics as well as additional bioinformatic analyses including the incorporation and contrasting with embryonic datasets (Goodyer et al. 2019) to illustrate maturational changes at the transcriptomic level throughout the VCS components, as well as network inference analysis using SCENIC to shed new light on the potential transcriptional regulators underpinning these differences. I believe that the authors’ characterization of the individual subcomponents of the VCS using both single cell and spatial analyses provides a level of detail not previously achieved and backed up with validation assays, be it IF or FISH (Fig 3 and Supp Figs 13-21). These data are of value to the field as they improve our basic understanding of this complex, important and heterogeneous cell population, in particular as we move towards trying to generate individual CCS components in vitro. Overall, the authors did a comprehensive job in their resubmission. Well done.

We truly appreciate the Reviewer for recognizing our efforts to address the Reviewers’ comments during the last revision. The constructive comments by the Reviewer played a pivotal role in enhancing the overall coherence as well as the depth of our study. In particular, the incorporation of the suggested analyses (i.e. network inference, comparison between embryonic and postnatal CCS) showed that there is high consistency between the distinct methods of analyses. Moreover, a more thorough histological assessment of each marker gene consolidated our findings with greater confidence. We are grateful that the novelty of our added work is convincingly recognized by the Reviewers. Once again, we would like to sincerely thank the Reviewer for the insightful feedback as well as the encouraging words.

Minor Comment:

P. 7 lines 253-254: “We performed FISH to examine cAVN-enriched *Prdm6*, showing that it was primarily expressed in cAVN and AV ring, but not in the LNB and other CCS or non-CCS regions”. *Prdm6* seems to be clearly expressed in surrounding non-CCS tissue. The authors should amend their statement to reflect this.

Thank you for the comment. The Reviewer is correct that *Prdm6* expression was observed in the surrounding non-CCS cells, especially within the atrioventricular junction, but not in the LNB and ventricular regions. To address this comment and more accurately reflect its expression pattern, we have changed the text in the revised manuscript as follows:

(Page 7) We performed FISH to examine cAVN-enriched *Prdm6*, showing that it was primarily expressed in the cAVN and AV ring, as well as in surrounding non-CCS within the atrioventricular junction, but not in the LNB and other CCS regions (**Fig. 3b** and **Supplementary Fig. 14**).

Reviewer #3 (Remarks to the Author):

The authors have revised and improved the manuscript with added spatial transcriptomics data and additional in-silico analysis. The authors have also expanded with integrating embryonic CCS data from Goodyer et al., 2019. Below are just some additional comments for clarification:

1. In Figure 1D, it seems that the PCA cluster for RAA, LENDO, LEPI, IVS-B, IVS-A, and RV is omitted. It may be missed out inadvertently.

Thank you for the comment. Although we had initially performed PCA using both CCS and non-CCS components, as we placed a major visual focus on CCS components and the way they cluster with each other, we did not include non-CCS regional components in the submitted manuscript. However, as the Reviewer pointed out, we realize that this is not consistent with figures showing our scRNA-seq analysis (**Fig. 1c, 1e**) as well as spatial transcriptomics analysis (**Fig. 1f**).

In the revised manuscript, we have updated the PCA to include both CCS and non-CCS components (**Fig. 1d**). As shown in the PCA below, and consistent with our correlation analysis (**Fig. 1g**), there are high proximities between components of the same chamber. For example, the right atria (RAA) clusters closely with the SAN, AVN and LNB; the right ventricle (RV), basal interventricular septum (IVS-B), and left epimyocardium (LEPI) cluster with the proximal VCS components (HIS and PLBB, PRBB) and; the apical interventricular septum (IVS-A) and the left endomyocardium (LENDO) cluster with distal VCS components (DLBB, LPF). This further supports our analysis of spatial transcriptomic data, in which enriched genes of each CCS region were identified through comparing genes in each region to its respective non-CCS components. (i.e. SAN vs RAA, PLBB vs IVS-B, DLBB vs IVS-A, LPF vs LENDO, RPF vs RV). Moreover, it is interesting to note that, like the CCS components, the non-CCS components also exhibited a linear pattern in our PCA, closely reflecting the sequence of activation (from RAA to IVSB to IVSA to LENDO).

Following the comment by the Reviewer, **Fig. 1d** and its figure legend have been updated to include both CCS and non-CCS components:

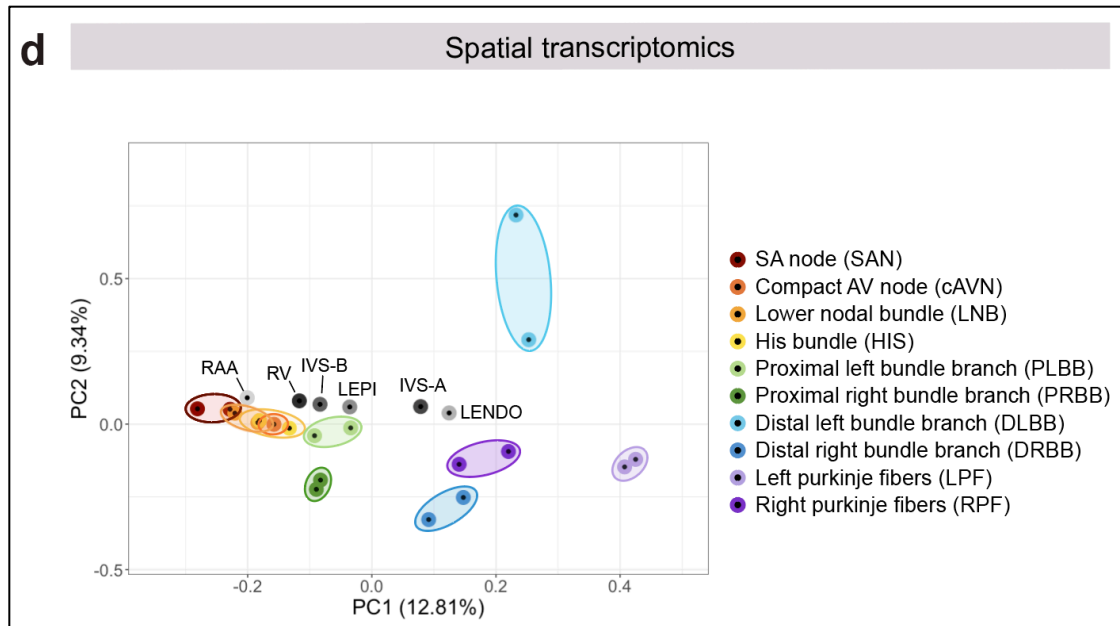


Fig. 1d. Principal component analysis (PCA) of the CCS and non-CCS regions profiled by spatial transcriptomics (ST).

The text has also been updated to include both CCS and non-CCS:

(Page 5) “Moreover, these CCS components and their non-CCS counterparts were closely positioned. For instance, the RAA was grouped with the SAN, cAVN and LNB; IVS-B was grouped with the proximal VCS components (HIS, PLBB, and PRBB); and the IVS-A and the LENDO were grouped with distal VCS components (DLBB/DRBB and LPF/RPF).”

2. Sub-cluster for Compact AV node for one of the replicate seem to be missing, though there are two replicates in the PCA.

Thank you for the comment. Unfortunately, when we performed spatial transcriptomics, we were able to identify and capture only 1 region of the cAVN in our sections. We also recognize the potential difficulty in identifying the different components in our PCA due to the similar colours between the cAVN and LNB, as well as the partial overlap between these points. However, with the updated PCA, we believe the components are now better distinguishable. For clarity, the figure below shows the PCA with all labels.

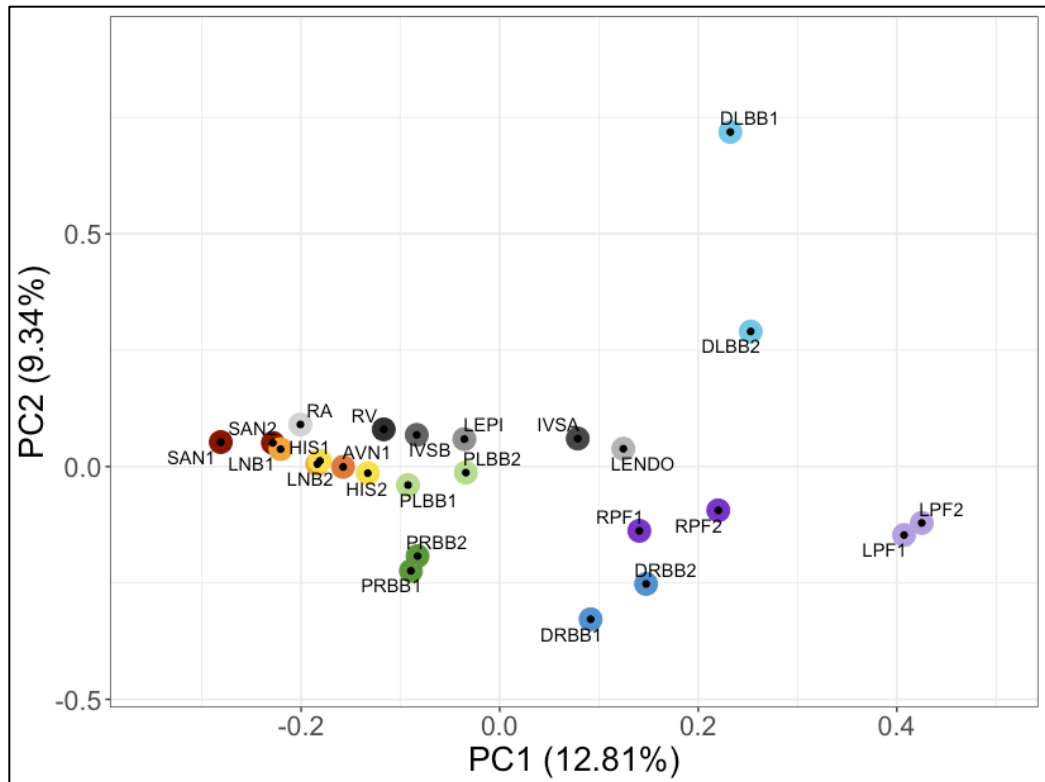


Figure. Principal component analysis (PCA) of the CCS and non-CCS regions profiled by spatial transcriptomics (ST) with all labels.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments satisfactorily. This will be a valuable contribution to the field.

Reviewer #3 (Remarks to the Author):

No further comments. All my points have been addressed.