

Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity

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ABSTRACT

The cardiac conduction system (CCS) is a network of specialized cardiomyocytes coordinating electrical impulse propagation for synchronized heart contractions. Although the components of the CCS, including the sinoatrial node, atrioventricular node, *His* bundle, bundle branches, and *Purkinje* fibers, were anatomically discovered more than 100 years ago, their molecular constituents as well as regulators remain not fully understood. Here, we demonstrated the transcriptomic landscape of the postnatal mouse CCS at a single-cell resolution with spatial information. Integration of single-cell and spatial transcriptomics uncovered region-specific markers and zonation patterns of gene expression across the CCS, which were histologically validated. Network inference showed heterogeneous gene regulatory networks across the CCS. Notably, CCS region-specific gene regulation was recapitulated in a dish using neonatal mouse atrial and ventricular myocytes overexpressing CCS-specific transcription factors, *Tbx3* and/or *Irx3*. This finding was supported by ATAC-seq of different CCS regions, *Tbx3* ChIP-seq, and the identification of *Irx* motifs. Overall, this study demonstrates comprehensive molecular profiles within the postnatal CCS and provides evidence of the regulatory mechanisms contributing to its heterogeneity.

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LIST OF ABBREVIATIONS

A

Ad	Adenovirus
Adj. P-val	Adjusted P-value
A-CMs	Atrial cardiomyocytes
AP	Action potential
ATAC-seq	Assay for transposase-accessible chromatin using sequencing
AUC	Area under the curve
AV	Atrioventricular
AVC	Atrioventricular canal
AVJ	Atrioventricular junction
AVN	Atrioventricular node

B

BB	Bundle branch
bpm	Beats per minute

C

cAVN	Compact atrioventricular node
CCS	Cardiac conduction system
ChIP	Chromatin immunoprecipitation
CM	Contractile myocytes
Co-IP	Co-immunoprecipitation

D

DBB	Distal bundle branch
DLBB	Distal left bundle branch
DRBB	Distal right bundle branch
DSP	Digital spatial profiler

E

E	Embryonic day
ECG	Electrocardiogram

F

FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FC	Fold change
FISH	Fluorescence in situ hybridization

G

GFP	Green fluorescence protein
GWAS	Genome-wide association studies

H

HET	Heterozygous
HIS	<i>His</i> bundle

I

INE	Inferior nodal extension
IVS-A	Interventricular septum (Apex)
IVS-B	Interventricular septum (Base)

K

KO	Knock-out
----	-----------

L

LAA	Left atrial appendage
LBB	Left bundle branch
LEND	Left endomyocardium
LEPI	Left epimyocardium
LNB	Lower nodal bundle
LPF	Left <i>Purkinje</i> fibers
LV	Left ventricle

M

MULTI-seq	Multiplexing using lipid-tagged indices for single-cell and single-nucleus RNA sequencing
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P

P	Postnatal day
PBB	Proximal bundle branch
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCW	Post conception weeks
PF	<i>Purkinje</i> fibers
PLBB	Proximal left bundle branch
PRBB	Proximal right bundle branch
Prolif. CCS	Proliferative CCS
P/S	Penicillin/Streptomycin

Q

qPCR Quantitative polymerase chain reaction

R

RA Right atria
RAA Right atrial appendage
RBB Right bundle branch
RFP Red fluorescence protein
ROI Region of interest
RPF Right *Purkinje* fibers
RV Right ventricle

S

SA Sinatrial
SAN Sinoatrial node
SCENIC Single-cell regulatory network inference
scRNA-seq Single-cell RNA-sequencing
SEM Standard error of the mean
ST Spatial transcriptomics

T

tdT Tandem dimer tomato
TF Transcription factor

U

UMAP Uniform manifold approximation and projection
UMI Unique molecular identifiers

V

V-CMs Ventricular cardiomyocytes
VCS Ventricular conduction system

W

WT Wild-type

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SECTION 1 – INTRODUCTION

1.1 The cardiac conduction system (CCS)

1.1.1 The evolution of CCS discovery

In the early 19th to 20th century, cardiac scientists were involved in a heated debate regarding how the heart could generate beats¹. This “Myogenic versus Neurogenic Debate” was between a group of scientists who believed in the ‘*Myogenic theory*’ which posited that the heart’s rhythm is established intrinsically by the heart itself. This theory was supported by histological observation that excised hearts continued to beat. Scientists on the counter side of the argument believed in the ‘*Neurogenic theory*,’ which hypothesized that the heart beats through external stimuli, such as the nervous system. Others also believed that blood is the origin of heartbeats². This controversial debate was eventually settled by a series of discoveries made by several anatomists, who discovered the major components of the cardiac conduction system (CCS): Drs. Arthur Keith and Martin Flack, who discovered the sinoatrial node (SAN) in 1907³; Sunao Tawara, who discovered the atrioventricular node (AVN) in 1906⁴; Wilhelm His Jr., who discovered the *His* bundle (*HIS*) in 1893⁵; R. Retzer who discovered the right bundle branch (RBB) in 1904; K. Braeunig, who discovered the left bundle branch (LBB)^{6,7} and; Johann Evangelist Purkinje, who discovered the *Purkinje* fibers (PF) in 1839⁸. These breakthroughs revolutionized our understanding of one of the heart’s most essential functions – its role as an electrical generator and conductor.

1.1.2 Physiological roles of the CCS

The cardiac conduction system (CCS) in the heart is a small group of specialized cells which are responsible for allowing synchronized heart contractions. For every heartbeat, electrical impulses are initiated by the pacemaker cells in the sinoatrial node (SAN) and

spread throughout the atrial myocardium to reach the atrioventricular node (AVN), which slows down the conduction to ensure sufficient filling of the ventricle. The electrical signal then reaches the fast-conducting network of the ventricular conduction system (VCS), which consists of the *His* bundle, left and right bundle branches and *Purkinje* fibers (**Figure 1.1**).

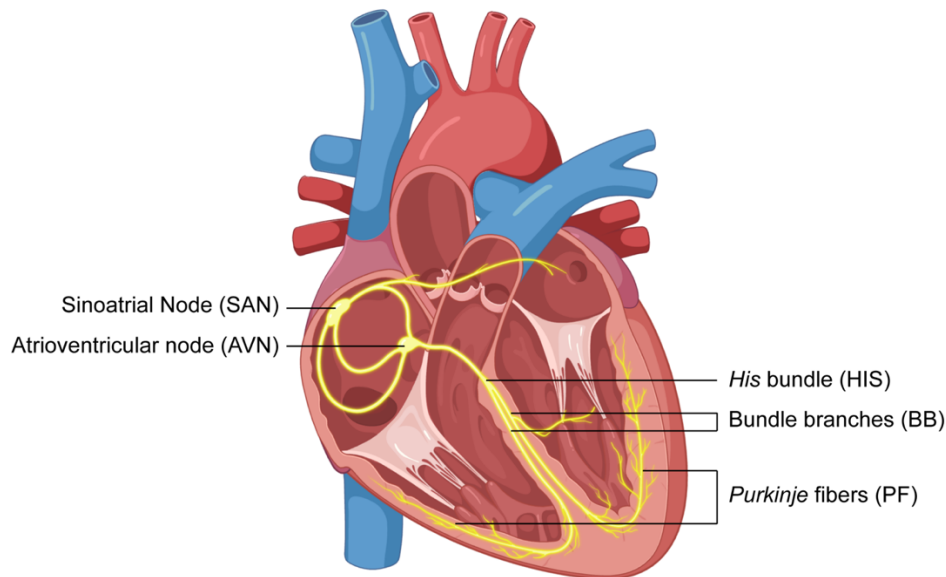


Figure 1.1 The cardiac conduction system (CCS) comprises of the sinoatrial node (SAN), atrioventricular node (AVN), *His* bundle (HIS), bundle branches (BB) and *Purkinje* fibers (PF). Figure generated by BioRender.com

Electrical activities generated by each CCS component can be observed in an electrocardiogram (ECG). The action potential generated by the SAN marks the beginning of the P wave. The delay between atrial and ventricular activation is mediated by the AV node and is represented by the PR interval, the flat segment between the P wave and the QRS complex. The beginning of the QRS complex is driven through the *His* bundle, and the synchronized ventricular depolarization driven by the left and right bundle branches towards the apex of the heart. The *Purkinje* fibers continue this ventricular depolarization, activating both ventricular chambers, represented by the QRS complex.

Each component of the CCS has a specific role in initiating and propagating electrical impulses throughout the heart. To add to the complexity, within each component, there are multiple heterogeneous cell types. Consequently, defects in various regions of the CCS can lead to various types and severities of rhythm disorders, such as heart blocks, ventricular fibrillation, and ventricular tachycardias, potentially leading to sudden cardiac death⁹⁻¹⁴. Therefore, understanding the molecular composition of the CCS is critical, as it will not only provide insight into the precise formation of each component of the CCS, but will also reveal the molecular mechanisms underlying these disorders. This knowledge will be translatable into the discovery of targeted therapies to improve clinical outcomes for patients with conduction disorders.

1.1.3 Characterization of the CCS

The CCS is reported to have distinct morphological and electrophysiological properties compared to the surrounding non-CCS, contractile myocytes (CMs). When the CCS were beginning to be identified and recognized in the late 1800's and early 1900's, three histological criteria were laid out for the distinct of the CCS: (1) The CCS should be histologically discrete; (2) The CCS should be traceable in serial sections of the heart and; (3) The CCS should be insulated by a fibrous sheath apart from the surrounding non-CCS tissue^{15,16}. To this day, these criteria still remain helpful in identifying CCS components or subcomponents that require further examination. Each CCS component is also found to be divided into several other components, based on its morphology, location and physiological properties, which will be discussed in detail in the next sections (**Sections 1.1.3.1-1.1.3.3**).

1.1.3.1 Sinoatrial node (SAN)

The sinoatrial node (SAN) was first anatomically described by Drs. Arthur Keith and Martin Flack in 1907³ as a structure in the right atria proximal to where “the superior vena cava

enters that chamber". The SAN is the primary pacemaker of the heart with the highest natural rate of depolarization, thereby controlling the rate of the heart beats¹⁷. As expected, the SAN cells differ significantly from the surrounding atrial myocytes in their morphological and electrical properties. One of the most distinguishing properties of the SAN from all other cell types in the heart is its strong ability to spontaneously generate action potentials. This automaticity is driven primarily by mechanisms involving the "membrane clock" and "Ca²⁺ clock."

The "membrane clock" is modulated by ion channel activity, particularly the hyperpolarization-activated cyclic nucleotide-gated channels (HCN). These channels are named for their unique property of being activated at hyperpolarized membrane potentials (around -60 mV to -70 mV), unlike most other channels which are activated at more depolarized states. Among the 4 members of the HCN channels (HCN1 - 4), HCN4 is the most prevalent form in the SAN and generates the I_f current, commonly referred to as the 'funny' current¹⁸. During Phase 4 of the pacemaker action potential, HCN4 channels allow a large inward Na⁺ flux and a smaller outward K⁺ flux, creating a net inward current resulting in a slow depolarization (**Figure 1.2**). As depolarization slowly progresses, T-type and L-type Ca²⁺ channels¹⁹ allow a rapid influx of Ca²⁺ ions, leading to the rapid depolarization in Phase 0. Subsequently, outward K⁺ channels restore the membrane potential to a hyperpolarized state during Phase 3, allowing for HCN4 channels to initiate the next pacemaking cycle.

The "Ca²⁺ clock" is governed by intracellular Ca²⁺ cycling. During this process, ryanodine receptors (Ryr2) on the sarcoplasmic reticulum release Ca²⁺ into the cytosol, which in turn activates the sodium-calcium exchanger (NCX), allowing the influx of Na⁺, in exchange for Ca²⁺. The resulting Na⁺ influx further depolarizes the membrane, promoting HCN4 and Ca²⁺ channel activation as part of the membrane clock. Further, Ca²⁺-induced Ca²⁺ release from the sarcoplasmic reticulum contributes to Ca²⁺ transients, which, in

conjunction with L-type Ca^{2+} channels, enhance the sharp upstroke of the action potential. Altogether, this interplay between the membrane clock and Ca^{2+} clock ensures the proper pacemaker activity of the SAN²⁰.

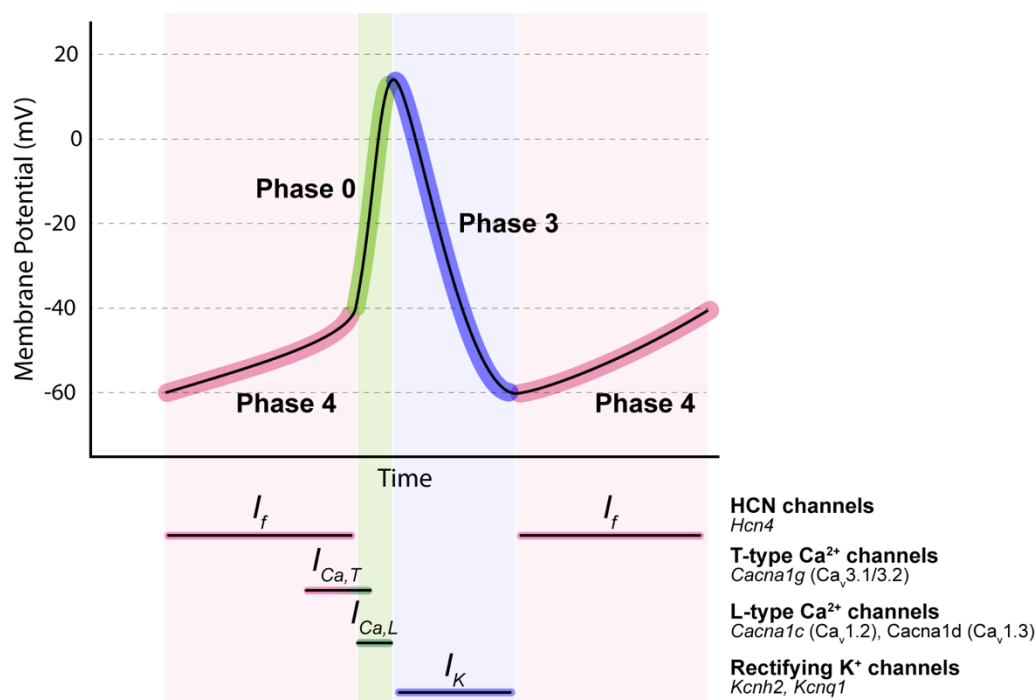


Figure 1.2 Pacemaker action potential properties, with its corresponding representative currents and ion channels. Figure generated by Adobe Illustrator.

The SAN is also structurally isolated from the surrounding atrial myocytes by connective tissue consisting of collagen and fibroblasts, in order to preserve its pacemaking activity and to properly direct the impulse to the rest of the conduction system²¹. Morphologically, when examined under the light microscope, the SAN presents with heavily dense nuclei, compared to atrial myocytes, indicative of its smaller cell size. Indeed, human SAN cells were found to be 5 - 10 μm in diameter, compared to 15 - 20 μm for atrial cells²². Distinct electrophysiological features of the SAN are largely influenced by the unique compositions of gap junctions, which are structures that allow electrical communication between adjacent cells. In mice, the SAN primarily expresses gap junctions with low

conductance, such as connexin 45 (Cx45, encoded by *Gjc1*) and Connexin 30.2 (Cx30.2, encoded by *Gjd3*). These low conductance gap junctions allow synchronizing all excitation frequencies of pacemaker cells to establish a defined and consistent rhythm²³. On the contrary, working myocytes of the atria predominantly express gap junctions with high conductance, such as Connexin 40 (Cx40, encoded by *Gja5*) and Connexin 43 (Cx43, encoded by *Gja1*)²¹, to rapidly distribute the electrical impulse throughout the atrial chamber. As a consequence, the conduction velocity within the SA node (0.03 - 0.05 m/sec) is much lower than the atrial tissue (~1 m/sec)²². SAN cells also express fewer sarcomeric components as well as lower mitochondrial content, compared to atrial myocytes, aligning with their specific role to generate electrical signals rather than to contract²⁴.

The SAN is largely divided into the head and tail regions, where anatomically, the larger head is located proximal to the opening of the superior vena cava, and the smaller tail extends towards the inferior vena cava (**Figure 1.3**)²⁵. Studies using patch clamping to perform electrophysiological assessments have shown unique action potential duration, amplitude as well as upstroke in these populations^{21,26}. While the SAN head is the primary impulse generator, the SAN tail region displayed hybrid electrical properties between the SAN head and atrial myocytes. Particularly, the SAN tail showed a larger AP amplitude and faster AP upstroke compared to the SAN head, but a smaller AP amplitude and slower AP upstroke compared to atrial myocytes²⁶. These suggest a signal-amplifying role of the SAN tail, compared to a more precise impulse-generating role of the SAN head. Interestingly, *ex vivo* optical mapping of rat and human right atrial tissues, have shown that the primary pacemaking site within the SAN shifts depending on the autonomic input. For instance, under sympathetic regulation, pacemaker activity is predominantly driven by the SAN head, while under parasympathetic regulation, the SAN tail dominates²⁷. These unique properties reflect the specialized roles of the SAN head and tail in pacemaking. In addition to the head

and tail of the SAN, recent studies have suggested the presence of transitional nodal cells, which are responsible for mediating signals from the SAN to the atrial myocytes²⁸.

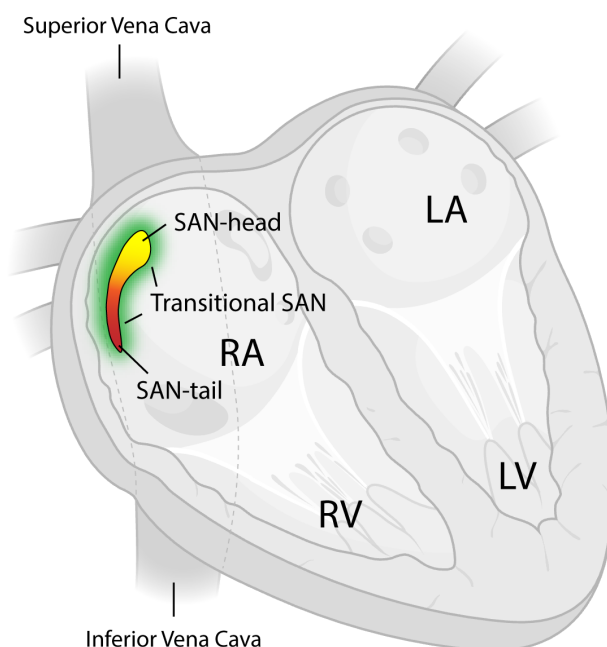


Figure 1.3 Subpopulations of the sinoatrial node (SAN), showing the SAN-head, SAN-tail and transitional SAN. SAN-head, yellow; SAN-tail, red; Transitional SAN, green. SAN, Sinoatrial node; RA, Right atrium; LA, Left atrium; RV, Right ventricle; LV, Left ventricle. Figure generated by BioRender.com and Adobe Illustrator.

1.1.3.2 Atrioventricular node (AVN)

The atrioventricular node (AVN) was first morphologically discovered by Dr. Sunao Tawara in 1906 and was described to appear like a “Knoten” (node) of cells connected to the *His* bundle^{6,7}. Located in the triangle of Koch, at the bottom of the right atria within the junction between the atria and ventricles²⁹, the AVN also possesses a degree of automaticity activity, acting as a secondary pacemaker in case of SAN failure³⁰. As such, the AVN exhibits similar morphological and gap junction characteristics as the SAN, such as lower mitochondrial and sarcomeric protein content³¹ as well as enriched low conductance gap junctions³². Being the only structure that electrically connects the atria and ventricle, the AVN’s predominant role is

to delay the electrical impulse to allow a sufficient amount of time for the ventricles to completely fill before contracting. This delay is represented by the PR interval in an ECG recording, which lasts approximately 30 - 40 ms in C57BL/6J mice^{33,34}, and 150 - 200 ms in humans³⁵.

The mechanism underlying this AVN-mediated delay has been widely speculated upon, with proposed contributions from its complex cellular composition, resulting from molecular profiles (i.e. distinct expressions of gap junctions^{36,37}), electrophysiological profiles (i.e. slow and fast pathway) and possibly its anatomical positioning (i.e. location relative to central fibrous body). Among the various subpopulations of the AVN described, the cAVN and the LNB are the most frequently discussed, though throughout the literature, these terms are used interchangeably or confused with other intermediate structures between the AVN and the HIS. In this dissertation and my study, the term 'LNB' will be used to describe a structure basally located on the plane of atrioventricular insulation or the central fibrous sheath³⁸.

Molecularly, the most extensively studied characterization of the AVN involves gap junction expressions: Cx30.2 (encoded by *Gjd3*, ultra-low conductance), Cx45 (encoded by *Gjc1*, low conductance), Cx43 (encoded by *Gja1*, medium conductance) and Cx40 (encoded by *Gja5*, high conductance). In rabbits³⁹ and humans⁴⁰, the cAVN predominantly expresses Cx45 (and in the case of mice, also Cx30.2⁴¹), while Cx40 and Cx43 are largely absent. Contrary to the cAVN, the LNB co-expresses Cx45 and Cx43⁴², with some studies reporting Cx40 expression⁴¹. This suggests a low coupling of the cells within the cAVN⁴³, which may explain its role in delaying the conduction. In mice, Cx30.2 plays a crucial role in the AVN, as demonstrated by the use of *Gjd3*^{CreEFP} (i.e. Cx30.2-Cre) model, which specifically marks AV nodal cells⁴¹. Cx30.2 is regulated by TFs, *Gata4* and *Tbx5*, which are both essential for AVN development. In mice with heterozygous deletions of *Gata4* or *Tbx5*, Cx30.2 expression was reduced, leading to shortened PR interval⁴⁴, further supporting the

role of the cAVN in sufficient AV delay. Interestingly, Cx40^{-/-} mouse models show a significantly prolonged PR interval, representative of a longer delay. This suggests that Cx40, a fast-conducting gap junction, paradoxically may also play a role in conduction delays⁴⁵. Unfortunately, little is known about other molecular distinctions between AVN structures beyond gap junction proteins. For example, the specific ion channel distributions, the expression of specific transcription factors, or signaling pathways in the regulation of conduction delay properties within the AVN are not fully characterized.

Electrophysiologically, the AVN conducts electrical impulses through two major distinct pathways: the fast pathway and the slow pathway⁴⁶. The fast pathway goes through the cAVN⁴⁷ and has a short conduction delay with long refractory periods, (i.e. requiring a longer time to be re-activated). In contrast, the slow pathway goes through the lower nodal bundle⁴⁷ and has longer conduction delays with short refractory periods (**Figure 1.4**). Normally, electrical signal can be transmitted through both pathways; however, the fast pathway reaches the HIS at a faster rate due to the anatomical distance as well as its high conduction velocity, therefore being the predominant pathway in the AV conduction^{48,49}. This characterization of the AVN, however, is not consistent with the gap junction properties described above, as the cAVN is enriched with low conductance gap junctions, Cx30.2 and Cx45, and lacks fast conductance gap junction, Cx40, which one would suspect will lead to slower conduction velocity. Therefore, this apparent discrepancy necessitates further research linking the molecular and electrophysiological properties of the AVN.

Anatomically, the AVN is encased by a fibrous sheath, called the central fibrous body, before reaching the HIS, though this has yet to be confirmed. As described, the cellular compositions of the AVN have been described by many; however, the anatomical complexity, as well as the inconsistency in labeling its identified subpopulations, has led to ambiguity in understanding the heterogeneity and properties of the AVN. Therefore, it is critical for future research to standardize the naming conventions and perform simultaneous

correlations between the structural and functional characteristics of the subpopulations of the AVN. In this study, two subpopulations will be discussed – the cAVN and the LNB – considering their clear anatomical landmarks (**Figure 1.4**).

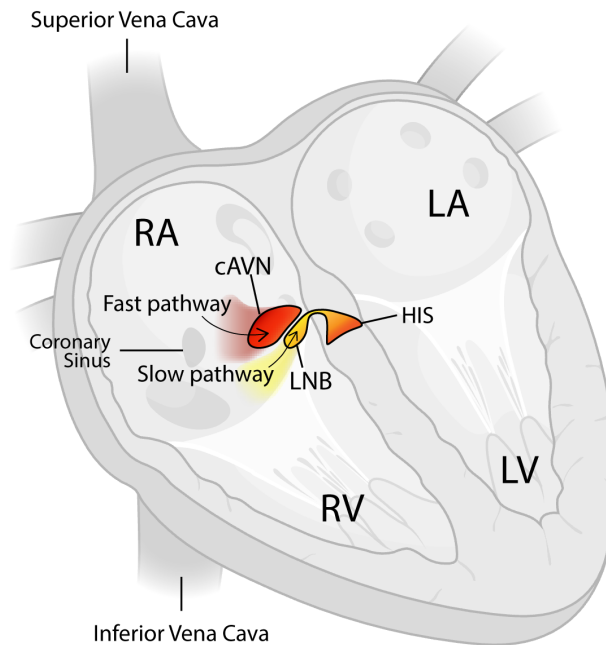


Figure 1.4 Subpopulations of the atrioventricular node (AVN), showing the cAVN and LNB as well as the fast and slow pathway. cAVN, Compact AV node; LNB, Lower nodal bundle; HIS, His bundle; RA, Right atrium; LA, Left atrium; RV, Right ventricle; LV, Left ventricle. Figure generated by BioRender.com and Adobe Illustrator.

1.1.3.3 Ventricular Conduction System (VCS)

Originally discovered by Drs. Wilhelm His Jr.,⁵ Retzer and Braenig, and Johann Evangelist Purkinje⁸, components of the ventricular conduction system (VCS) were described as “muscle bundles which unite the auricular [atrial] and ventricular septal walls,” “subendocardially scattered characteristic muscular bundles,” and “flat, grey gelatinous fibers.” These early observations highlighted the differentiating structures of the VCS from the surrounding cardiac muscle, which, despite comprising only 1% of the ventricular volume, it has a crucial role in heart contractions. The VCS rapidly spreads electrical impulses throughout both ventricles, coordinating ventricular contractions in an apex-to-

basal orientation and maximizing the efficiency of pumping blood⁵⁰. Like other regions of the CCS, the VCS is significantly distinct from the surrounding ventricular myocytes. Having an elongated and rod-shaped morphology⁵¹, the VCS shows less developed contractile machinery and mitochondrial content compared to the surrounding ventricular myocytes⁵², and is also surrounded by a fibrous sheath, similar to the SAN and AVN. The main distinction between VCS and nodal cells (and ventricular myocytes) is the expression of a fast-conducting Connexin 40 (Cx40) in the VCS, which allows rapid propagation of electrical impulses⁵³⁻⁵⁵. Therefore, *Purkinje* fiber cells, the most distal VCS structure, have faster conduction velocities (~2 - 4 m/sec) compared to ventricular myocytes (0.3 - 0.4 m/sec)⁵⁶ as well as the SAN cells.

The VCS is anatomically divided into the *His* bundle (also known as 'bundle of *His*' or 'atrioventricular bundle'; In this thesis, the abbreviation, HIS, will be used), left and right bundle branches (LBB/RBB) and left and right *Purkinje* fibers (LPF/RPF). The HIS is anatomically found deep within a fibrous tissue located at the top of the interventricular septum (IVS). It further bifurcates into the multiple fibers of the LBB and a single fiber of the RBB⁵⁷, all running along the sides of the IVS wrapped around a sheath of collagen⁵⁸. As the BBs reach the apex of the ventricles, they progress into a mesh-like network of multi-stranded LPF and single-stranded RPF¹⁰, which finally transmit the electrical impulses to the adjacent myocytes. Although the primary role of the VCS is to rapidly propagate electrical signals throughout the ventricles, studies have described several electrophysiological differences between VCS components, such as automaticity⁵⁹, conduction velocity⁶⁰, and cell-cell electrical coupling^{61,62}. For instance, in humans, conduction velocity in the HIS is ~1.3 - 1.7 m/sec⁶⁰ compared to ~2 - 4 m/sec for PFs⁵⁶. This difference is likely contributed by the distinct composition of gap junctions: the HIS expresses moderate levels of Cx40, Cx43 and Cx45 while PFs express higher levels of Cx40 and Cx43, with lower levels of Cx45 compared to the HIS⁶³.

Within the VCS, the presence of transitional PF cells, connecting the conduction cells to the surrounding ventricular myocytes, has been described anatomically and electrophysiologically. These transitional cells are functionally relevant, as they are proposed to act as a high-resistance barrier (i.e. lowering the conduction velocity of impulses generated) as well as amplifiers of the current when passing the impulses onto the large mass of myocardium¹⁴. Despite this function, its molecular characterization has remained largely unexplored until a recent publication in E16.5 mouse CCS⁶⁴, which showed expression of PF makers (i.e. *Gja5* (Cx40), *Etv1*, *Cacna2d2* and *Sema3a*) but at lower levels. These cells also did not express a well-known conduction marker, *Hcn4*, while expressing high degrees of a contractile myocyte gap junction, Cx43, suggesting that these transitional cells exhibit more of a hybrid characteristic between conduction cells and the surrounding contractile myocytes. Much more remains to be understood, however, including how their expression profiles transition during both embryonic and postnatal development, and when these cells both start and end to acquire the conduction cell-like properties.

1.2 Diseases of the CCS

1.2.1 Diseases of nodal cells

Abnormalities of signal generators, namely the SAN and AVN, can lead to various types of atrial rhythm disorders. Sick sinus syndrome (SSS) (also known as sinus node dysfunction) is a term used to describe various diseases related to abnormal impulses generated by the nodal cells⁶⁵. These include sinus arrest (pause in normal heart rhythm), sinus bradycardia (decreased resting heart rhythm), sinus tachycardia (increased resting heart rhythm) and tachy-brady syndrome (alternating between sinus bradycardia and tachycardia)⁶⁶. Patients with these abnormal rhythm disorders can present with various degrees and durations of syncope, palpitations or dizziness⁶⁷, and in most cases, its treatment requires electronic

pacemaker placements^{67,68}. Most commonly, nodal dysfunction involves intrinsic remodeling of the nodal cells or the surrounding cells, such as fibrosis⁶⁷, typically concurring with age. However, some cases involve familial inheritance, suggesting that genetic mutations also play a role⁶⁶. Indeed, genetic screening as well as genome-wide association studies (GWAS) have shown mutations and variants of genes associated with nodal disorders, including *SCN5A*⁶⁸⁻⁷¹ (encoding the alpha subunit of a voltage-gated sodium channel, Na_v1.5), *MYH6*^{72,73} (encoding the alpha myosin heavy chain) and *HCN4*⁷⁴ (encoding hyperpolarization-activated cyclic nucleotide-gated channel 4).

Mutations in *SCN5A* are among the most extensively documented, first identified as mutations in its coding region in several independent case studies in the late 1990s⁶⁸⁻⁷¹. To date, over 700 *SCN5A* variants, in both coding and non-coding regions, have been associated with various cardiac disorders⁷⁵. Interestingly, the central nodal cells are devoid of *SCN5A*-encoded Na_v1.5⁷⁶. However, subsequent studies have found that cells in the periphery of the SAN express Na_v1.5⁷⁶, suggesting that patients with *SCN5A* mutations have altered signal transmission from the SAN to the atrial chamber⁷⁷. Mutations in *MYH6* have been identified through GWAS⁷³. In adult humans, *MYH6* is predominantly expressed in the entire atria, and plays a critical role in cardiac development, and more specifically in sarcomere organizations^{78,79} and contractility²⁶. Due to its wide expression pattern as well as its critical role, mutations of *MYH6* are not only limited to rhythm dysfunction, but are also implicated in hypertrophic and dilated cardiomyopathies⁸⁰ and congenital heart defects⁷⁸. Mutations in *HCN4*, a hallmark of SAN's automaticity, have been identified in humans with SSS⁸¹, and in *Hcn4* knock-out mice, *I_f* was reduced by 75%, leading to a decrease in heart rate⁸².

Disorders related to the AVN also exist. One such form is AV blocks, where the electrical signals from the atria to the ventricles are excessively hindered (first-degree AV block), leading to occasionally dropped heartbeats (second-degree AV block) or completely

blocked (third-degree AV block)⁸³. Most cases of AV blocks are acquired (e.g. structural remodeling, age) or arise as a secondary complication of other diseases (e.g. maternal viral infection⁸⁴ or systemic lupus erythematosus⁸⁵). However, there have also been rare reports of inherited AV blocks^{86,87}, although the specific genetic causes were initially unclear. A few studies have identified possible pathogenic variants in genes, such as *LMNA*^{88,89} (encoding lamin a/c), *TTN*⁹⁰ (encoding titin) and *SCN5A*⁹¹; however, these cases often involved complex cardiac defects, such as dilated cardiomyopathy^{89,91,92}. Moreover, *LMNA*, *TTN* and *SCN5A* are expressed across various regions of the heart, not restricted only to the conduction system, making them unsuitable therapeutics targets for treating AV blocks specifically.

One of the most common types of supraventricular tachycardias is atrioventricular nodal re-entrant tachycardia (AVNRT)⁹³. AVNRT occurs when a premature atrial activation reaches the AVN, while the fast pathway is still in a refractory phase from the beat prior. Instead of the normally dominant fast pathway, the impulse travels through the slow pathway of the AVN to the ventricles, and this can retrogradely (i.e. from ventricles to atria) activate the fast pathway. This consequently results in the AVN activating the HIS bundle at a higher rate than the nodal sinus rhythm. This reentry accelerates the heart rate, causing a supraventricular tachycardia with a heart rate over 140 - 280 bpm and a narrower QRS^{94,95}. Structural mechanisms can also occur in the AVN to cause arrhythmias. In the case of atrioventricular re-entrant tachycardia (AVRT), two conduction pathways from the atria to the ventricles exist: the normal AVN and an accessory pathway. The accessory pathway allows signals from the SAN to reach the ventricles without the usual delay imposed by the AVN, leading to early ventricular activation. If this premature activation retrogradely activates the AVN, while the accessory pathway is in a refractory pathway, this can lead to a re-entry circuit, causing tachyarrhythmia⁹⁶. Despite the high risks associated with these

AVN-mediated tachycardias, the molecular characterization of the AVN and, therefore, the underlying mechanisms of these conditions, largely remains unknown.

Genetic screens and GWAS studies have significantly advanced our understanding of mutations and genes linked to rhythm disorders. However, limitations still exist in pinpointing therapeutic candidate genes that are specific to nodal cells. While several mutations and variants have been found to be associated with nodal diseases, many of these genes are not specific to the conduction system, and thus, are difficult to be deemed therapeutic targets. Additionally, while associations between genes and phenotypes have been established, the genes identified within these *loci* are not always causal genes. To address these challenges, molecular profiling of nodal cells can be a valuable tool. By identifying node-specific genes and transcription programs, this method can reveal functionally relevant target genes, which are more suitable candidates for treating nodal conduction diseases.

1.2.2 Diseases of the ventricular conduction system (VCS)

The ventricular conduction system (VCS) plays a crucial role in ensuring that electrical impulses are rapidly and synchronously transmitted throughout the ventricles, leading to coordinated contractions. A defect in the VCS can disrupt this synchronization, causing the ventricles to contract irregularly, which impairs the heart's ability to pump blood effectively and significantly increases the risk of sudden cardiac death⁹⁷. Unlike nodal abnormalities, where compensatory pacemaker mechanisms between the SAN and AVN can often mitigate the impact, VCS defects or ventricular arrhythmias lack such redundancy, making them generally more life-threatening. As such, ventricular arrhythmias account for the highest cause of sudden cardiac death⁹⁸⁻¹⁰⁰.

A range of ventricular arrhythmias can result from abnormal VCS and are commonly noticed by an abnormal duration or peak of the QRS wave. For instance, abnormalities in

the *HIS* can lead to junctional ectopic tachycardia (JET), which is identified by a narrowed QRS wave and a faster ventricular activation rate compared to atrial activation. Though most often arising postoperatively¹⁰¹, JET can be observed in neonates and infants 6 months or younger. In a study involving 26 cases of JET, half were related to familial causes, though no specific causative gene or its etiology has been identified to date^{102,103}.

Among intraventricular blocks, bundle branch blocks (BBBs) are the most common type¹⁰⁴. Resulting from an asynchronous activation of the ventricles driven by a block in either the LBB or RBB is referred to as a left or right bundle branch block (LBBB/RBBB), found in 1 - 2% of the adult human population¹⁰⁵, and becomes more common with age. LBB and RBB exhibit asymmetric anatomy, where the LBB consists of multiple fascicles, while RBB is a single fascicle¹⁰⁶. Consistently, in humans, RBBB is more common than LBBB. Therefore, an error in a single fascicle is more likely to exhibit more severe conduction consequences¹⁰⁷. Specifically, patients with RBBB alone, without other cardiovascular diseases, or even those who were asymptomatic, showed an increased overall risk of mortality and cardiovascular-related mortality¹⁰⁸. Studies have found genetic associations between BBBs and ion channel genes, including *HCN4*, *GJA5* and *SCN5A*. However, these BBBs often co-occur with other conduction defects, such as SSS and atrial fibrillation, making it unclear whether BB disorders are the direct result of these genetic mutations.

In the ventricles, PFs are a major origin of arrhythmias^{11,109-111}, and their dysfunction can affect the systolic function of the heart. PFs have been implicated in the initiation or maintenance of arrhythmias, even in patients with structurally normal hearts¹¹², as well as in those with conditions such as dilated cardiomyopathy¹¹³, myocardial infarction^{109,114}, ischemia¹¹⁵, Brugada syndrome, and Long QT syndrome¹¹⁶. Studies have shown that in domesticated pigs, *Purkinje* fibers exhibit a more disorganized structure, extending not only within the subendomyocardium of the ventricular walls, but also deep into the

midmyocardium. This atypical distribution can serve as an arrhythmogenic substrate, suggesting the importance of properly organized PFs for normal conduction¹¹⁷. Moreover, ion current remodeling, specifically involving Ca^{2+} and K^{+} currents, occurs in PFs of failing hearts^{118,119}. Consistently, inherited ventricular arrhythmias, such as Brugada syndrome and long QT syndromes, are strongly associated with mutations in genes responsible for generating these ion currents, such as *KCNQ1*¹²⁰⁻¹²³, *KCNH2*¹²⁴⁻¹²⁶ and *SCN5A*^{75,127,128}.

While certain molecular mechanisms have been identified and extensively studied (i.e. *SCN5A*, *GJA5*, *HCN4*), mutations in these genes result in a broad array of CCS defects, demonstrating its rather global effect throughout the CCS. However, the diversity of phenotypes suggests that different molecular players may have distinct roles within the various components of the CCS. This highlights the need to explore the unique molecular features of each CCS region, as they may contribute to different types and degrees of conduction dysfunction and arrhythmogenesis.

1.3 Development of the CCS

1.3.1 Overview of CCS development

The cardiovascular system is the first organ system to form and function in all vertebrate embryos¹²⁹, and the CCS forms simultaneously and interdependently with the development of the four heart chambers¹³⁰. First action potential activities were observed as early as E7.5 in mouse embryos, 6 - 8 somite stage in chick embryos^{131,132}, and in rats at a 3 somite stage, even before the first heartbeat is detected¹². At around ~E7.5 - E8 in mouse embryos, and 16 days after fertilization in humans, the heart forms a linear tube from the cardiac crescent¹³³. Subsequently, this primitive heart tube starts to elongate and initiate spontaneous beats, driven by primary myocardial cells with intrinsic automaticity – marking the onset of the heart's electrical activity. By E9.5 - 10.5 in mouse embryos and week 6 - 8

in the human fetus, this tube undergoes looping and trabeculation (i.e. invaginations of the inner ventricular wall) of the prospective ventricles initiate¹³⁴. During this process, blood flow forces and wall sheer stress generated from the peristaltic pumping motion, as well as differential rates of cell proliferation, guide the looping process^{135,136}. Moreover, at this stage, the SAN becomes histologically evident, and a conduction delay around the AV canal is evident. By E12.5, the direction of electrical activation in the ventricles shifts from 'base-to-apex' to 'apex-to-base,' indicating the initiation of VCS development from the trabeculae. By E15.5 in mouse embryos and in week 8 in human fetuses, cardiac morphogenesis is considered complete¹³⁷.

Upon birth, when neonatal and maternal circulations are disconnected, the neonatal heart undergoes intricate anatomical and physiological transitions¹³⁶. Simultaneously the CCS is expected to undergo a postnatal maturation phase, where the embryonically developed CCS is now required to meet the demands of the rapidly growing heart postnatally. During postnatal maturation, parts of the CCS are more distinctively specific and mature through further recruitment as well as the regulation of cell cycle and cell differentiation^{50,138}.

Compared to the embryonic development of the CCS, the processes involved in postnatal maturation of the overall CCS, especially the individual components within the CCS, are studied to a lesser degree, likely due to the difficulty in isolating CCS cells from postnatal stages and the low number of CCS cells in the heart (i.e. ~0.1% of cardiomyocytes). Uncovering these molecular characteristics and differences is crucial to understand how the CCS maintains its identity and function throughout life.

1.3.2 Embryonic development of the CCS

Each component of the CCS develops at distinct spatiotemporal origins during embryonic development. The formation of the SAN begins around E9.5 in mice¹³⁹, which involves a

complex interplay of TFs, such as NK2 Homeobox 5 (*Nkx2-5*)^{26,138}, T-box transcription factor 3 (*Tbx3*)¹⁴⁰⁻¹⁴³, T-box transcription factor 5 (*Tbx5*)^{144,145}, T-box transcription factor 18 (*Tbx18*)^{139,143}, *Islet-1* (*Isl1*)^{146,147} and short stature homeobox 2 (*Shox2*)^{148,149}. Mutant mouse embryos lacking these TFs exhibit malformation of the SAN and/or altered spontaneous activity of the SAN cells. For instance, loss of *Isl1* results in a reduced SAN population and aberrant expression of atrial chamber genes in the SAN, together with sinus arrhythmia¹⁴⁶. Similarly, loss of *Tbx3* leads to the ectopic expression of atrial chamber genes and impaired pacemaker function, while not affecting SAN morphology. Moreover, loss of *Nkx2-5* in the SAN leads to its dysfunction. The development of the SAN also involves further segregation into the SAN head and tail populations, where the SAN head arises specifically from *Tbx18*⁺ progenitor cell populations. These eventually give rise to the molecularly distinct SAN head (*Tbx18*⁺/*Nkx2.5*⁻) and SAN tail (*Tbx18*/*Nkx2.5*⁺)¹⁴³.

The embryonic development of the AVN was studied through functional observations, retrospective clonal analyses, lineage tracing¹⁵⁰, morphometric analyses³⁸, electron microscopy¹⁵¹ and histological assessments¹⁵² to show that the AVN arises from the AV canal (AVC). Particularly, in the early embryonic atrioventricular junction, cardiomyocytes expressing a neural tissue antigen, G1N2⁺, were observed in the expected locations of cells giving rise to the AVN, as well as parts of the VCS¹⁵². Moreover, genetic lineage tracing using *Tbx2*^{Cre} showed that *Tbx2*⁺ cells in the AVC gave rise to the cAVN, but not the LNB, HIS or the BBs¹⁵⁰. It is noteworthy that these cells also give rise to the adjacent working myocardium. Studies have also noted an atrioventricular delay in the embryonic AVC like that from the adult AVN^{153,154}. These observations collectively led to the widely accepted hypothesis that the AVC contains progenitors for the AVN. However, it remains unclear if one distinct population within the AVC is solely responsible for the development of the AVN exclusively.

The VCS originates from the myocardium itself. The developmental origin of the VCS was first documented in chick embryos. Two primary hypotheses were proposed: (1) the '*Ingrowth*' hypothesis: VCS cells form through a gradual recruitment of new myocytes without proliferation; or (2) the '*Outgrowth*' hypothesis: a distinct pool of differentiated conduction cells proliferates independently from working myocytes to form the VCS. The '*Ingrowth*' hypothesis was largely supported by studies in the 1990s using chick embryos, in which retrovirus-based lineage tracing demonstrated that myocytes located adjacent to the coronary arterial vessel were recruited to differentiate into mature PFs^{155,156}. This finding suggested that cardiomyocytes were recruited by factors released from the vasculature, such as endothelin-1 (ET-1), in a paracrine manner to form PF cells. *In vitro* studies further supported this idea, showing that chick myocytes exposed to ET-1 upregulated the expression of Cx40, a molecular marker of PFs¹⁵⁷. Moreover, VCS cells showed limited proliferative capacities, indicated by the same number of VCS cells between E14 and E16 in chick embryos.

In mice, the VCS forms through mechanisms supporting the '*Outgrowth*,' hypothesis. Like avians, the VCS shares a common progenitor with the working myocytes – the trabecular cells. However, genetic labeling using a Cx40-CreERT lineage tracing system showed that the VCS contains a small, predetermined population of cells that proliferate, supporting the outgrowth model rather than recruitment²⁶. In parallel, lineage tracing studies have shown that the proximal part of the VCS (i.e. HIS) differentiated independently of distal parts of the VCS (i.e. PFs)¹⁵⁸. This suggests that different regions of the VCS follow distinct developmental pathways, highlighting the regional specialization in the formation of the VCS.

1.3.3 Postnatal maturation of the CCS

After birth, maturation of the CCS takes place where conductive myocytes largely discontinue proliferation¹⁵⁹, and the fast-conducting identity of the CCS must be maintained after birth. Tamoxifen-inducible Cre animal models for manipulating TFs postnatally, combined with phenotypic and morphological assessments of the conduction system in neonatal or postnatal/adult hearts, have significantly advanced our understanding of CCS maintenance and function. For instance, studies using transgenic mouse models have demonstrated that the disruption of transcription factors, such as *Nkx2.5*^{+/-}, *Etv1*^{-/-} and *Irx3*^{-/-} in adult mice, leads to delayed ventricular activation, QRS complex prolongation in ECGs, and anatomic VCS hypoplasia^{50,138,160, 161}, with reduced expression of Cx40⁵².

In mice lacking other TFs, proximal regions of the postnatal CCS are affected. For instance, conditional deletion of *Tbx3* in adult mice leads to SAN dysfunction and AV block¹⁶². Similarly, mice heterozygous for *Tbx3* display AVN and HIS/BB hypoplasia only after birth¹⁶³. Further supporting these findings, the overexpression of *Tbx3* or the deletion of *Tbx5*, specifically in adult mouse VCS, shifts the fast conduction physiology into a more slowed nodal-like physiology¹⁶⁴.

These findings collectively suggest the importance of not only the development of the CCS, but also the continued maintenance of its specialized identity throughout postnatal and adult stages. However, how these transcription factors are involved specifically during the early CCS maturation stage, as well as whether they form a transcriptional network during this stage is not yet known.

1.3.4 Transcription factors in CCS development

As outlined in the above sections, the development of the CCS requires a sophisticated yet precisely orchestrated series of cellular and molecular events that unfold in a spatiotemporal

manner. These processes are regulated by transcription factors which modulate gene expressions. In the development of the CCS, TFs such as *Shox2*¹⁴⁹, *Isl1*¹⁴⁷, *Nkx2.5*¹⁶⁵, ETS translocation variant 1 (*Etv1*), homeodomain-only protein (*Hopx*)^{166,167}, DNA-binding protein inhibitor ID2 (*Id2*)¹⁴⁵, *Tbx5*¹⁴⁴, *Tbx3*¹⁶⁸ and *Iroquois* homeobox protein 3 (*Irx3*)^{50,53}, have been well described to be involved in the development of specific CCS components. Some of these transcription factors have been investigated, revealing the distinct and overlapping expression patterns within the CCS (**Table 1.1**). For instance, *Tbx3* is expressed in the SAN, AVN, and HIS, and proximal portions of the BBs and; *Irx3* is expressed in the HIS, bundle branches and the PFs.

Transcription Factors	Working Myocytes		Atrial Conduction System		Ventricular Conduction System		
	Atria	Ventricle	SA node	AV node	His bundle	Bundle Branches	Purkinje Fibers
<i>Shox2</i>	✗	✗	✓	✗	✗	✗	✗
<i>Nkx2.5</i>	✓	✓	✓/✗	✓	✓	✓	✓
<i>Tbx5</i>	✓	✓	✓	✓	✓	✓	✓
<i>Etv1</i>	✓	✗	✓	✓	✓	✓	✓
<i>Hop</i>	✓	✗	✓	✓	✓	✓	✗
<i>Id2</i>	✗	✗	?	✓	✓	✓	✗
<i>Tbx3</i>	✗	✗	✓	✓	✓	✓/✗	✗
<i>Irx3</i>	✗	✗	✗	✗	✓	✓	✓

Table 1.1 Unique and overlapping expression patterns of cardiac transcription factors in the CCS and working myocardium.

Interestingly, many of these conduction phenotypes overlap between the mutants. For example, both mutants lacking *Etv1* and *Irx3* exhibit prolonged ventricular activation (increased QRS wave duration), BBBs (notched QRS wave) and hypoplasia of the VCS (reduced PF networks)^{50,53,160}. Additionally, loss of *Tbx3* and *Tbx5* both lead to ventricular septal defects^{140,169}. These overlapping phenotypes between mutants of different

transcription factors suggest possible compensatory or collaborative functions among the TFs.

Despite extensive research on certain well-documented transcription factors, many questions remain unanswered about their precise mechanism of action – whether through repression, activation, or their specific transcriptional targets – as well as the presence of other transcription factors that may be involved in CCS development. Specifically, it is still unknown which additional TFs are expressed in the CCS, the precise timing of their expression during development, and how they differentially regulate the molecular profiles in distinct CCS components. For instance, TFs may be activators or repressors of downstream targets depending on the region of the heart, or they may work with other TFs to fine-tune the molecular properties. Furthermore, it is not yet fully understood how the expression of these TFs changes during embryonic-to-postnatal development, which leaves critical gaps in our knowledge of CCS regulation and heterogeneity. In this thesis, I will be focusing on two CCS-specific (exclusively expressed in conduction cells) TFs – *Tbx3* and *Irx3* – to investigate their cooperative roles in establishing the molecular heterogeneity within the CCS.

1.3.4.1 The role of *T-box (Tbx)* transcription factors in the heart and CCS

T-box (Tbx) transcription factors, named after its large and conserved T-box DNA binding domain, are a family of proteins that are heavily involved in diverse gene expression regulation and tissue differentiation. The 180 - 200 amino acid T-box domain is able to not only recognize specific DNA elements but also serve as an interaction hub for other transcription factors, chromatin remodeling complexes¹⁷⁰ and histone-modifying enzymes¹⁷¹, which all act to regulate gene expression. *Tbx* TFs also consist of a transcriptional activator or a repressor domain or both, highlighting its complex regulatory function¹⁷². *Tbx* TFs are

expressed in specific organs or cell types during its development and are generally required for this process¹⁷³.

8 out of 18 Tbx members (*Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx6*, *Tbx18* and *Tbx20*) are expressed in the developing mammalian heart¹⁷³⁻¹⁷⁵, and some (*Tbx3*^{142,163,176}, *Tbx5*¹⁷⁷⁻¹⁷⁹, *Tbx6*¹⁸⁰, *Tbx18*^{181,182} and *Tbx20*¹⁸³) have been documented for their roles in postnatal or adult heart function. Some of these TFs are closely linked on the same chromosome – *Tbx2* and *Tbx4* on chromosomes 17 (humans) and 11 (mouse) and; *Tbx3* and *Tbx5* on chromosomes 12 (humans) and 5 (mouse)¹⁸⁴. Phylogenetic analyses suggest *Tbx2/3* and *Tbx4/5* form paralogous groups that likely arose from an evolutionary chromosomal duplication event¹⁸⁴. This shared evolutionary origin is reflected in the similar phenotypes observed in mutations or deletions of these, such as *Tbx3* and *Tbx5* in Holt-Oram and Ulnar-Mammary syndromes^{185,186}, which also involve conduction system anomalies. Despite these potential functional similarities, these T-box genes play more complex transcriptional regulatory roles in development, often involving dual roles as both transcriptional activators and repressors¹⁴⁰.

In the heart, *Tbx3* encodes a critical transcription factor implicated in the formation of the CCS. It acts as a molecular switch that generates either SAN cells or working CMs in the atria, specifically by inducing the pacemaker gene program and repressing atrial working myocardium genes (i.e. *Cx43* and *Cx40*)^{141,168,176,187}. Consequently, homozygote *Tbx3* knockout (KO) are embryonically lethal at E14.5. Notably, *Tbx3* is also expressed in the proximal VCS, including the HIS and BBs, where *Cx40* is normally highly expressed¹⁸⁷. However, the exact role of *Tbx3* in the VCS remains elusive due to complex and conflicting results. For example, in mid-gestational embryos (E12.5 - E14.5), similar to the SAN, loss of *Tbx3* leads to increased *Gja5* expression in the proximal VCS¹⁶⁸, whereas, in the adult stage, hypomorphic *Tbx3* mutant heart shows reduced *Cx40* expression in the proximal bundle branches¹⁶². These results suggest a few possibilities: (1) *Tbx3* represses *Cx40*

gene expression in the prenatal developing VCS, while activating Cx40 transcript in the postnatal VCS, similar to *Irx3*'s functions; and (2) *Tbx3* exerts different downstream effects in the atrial vs. ventricular conduction system. Additionally, this leads us to postulate that *Tbx3* forms a complex with other TFs.

1.3.4.2 The role of *Iroquois (Irx)* transcription factors in the heart and CCS

The *Iroquois (Irx)* homeobox transcription factors (TFs) are a family of TFs characterized by a highly conserved 180 bp TALE (three-amino acid loop extension) homeodomain, a DNA binding domain containing repeated 33 - 34 amino acid sequences. Like other TALE homeodomain-containing TFs, such as Mohawk (*Mkx*), *Meis*, 5'-TG-3'-interacting factor (*Tgif*), and Pre-B-cell leukemia (*Pbx*), the *Irx* TFs are essential for various developmental processes, including tissue patterning and cell specification, across different developmental stages^{188,189}. Originally identified in *Drosophila melanogaster*, the three *Irx* TFs – *araucan* (*ara*), *caupolican* (*caup*), and *mirror* (*mirr*) – were named after a bristle pattern phenotype observed in mutants that resembled a traditional hairstyle of the *Iroquois* Native American tribe¹⁹⁰.

So far, six *Irx* genes have been identified in mammals, including mice and humans¹⁹¹, eleven in zebrafish¹⁹², with overlapping yet distinct roles. Within the heart, spatial distributions of these genes were discovered by *in situ* hybridization studies. *Irx1* and *Irx2* are expressed in the interventricular septum including the ventricular conduction system^{189,193}; *Irx4* is exclusively in the ventricular myocardium with a role of establishing ventricular identity through repressing atrial gene expression while activating ventricular gene expression^{193,194} and; *Irx5* is expressed in a transmural gradient within the ventricular wall, where it is highly expressed in the endomyocardium, while minimally in the epimyocardium¹⁹³. *Irx5* is known to repress the ventricular repolarization current by suppressing *I_{to}*-encoding K_v4.2 channel transcription, which exists in an opposite transmural

pattern as *Irx5*¹⁹⁵. It is currently under investigation whether it regulates other transmural gradients as well. *Irx6* expression is known to be comparable to that of *Irx5* during development¹⁹⁶, although at weaker levels. However, the role of *Irx6* in the heart has not been studied (**Figure 1.5**).

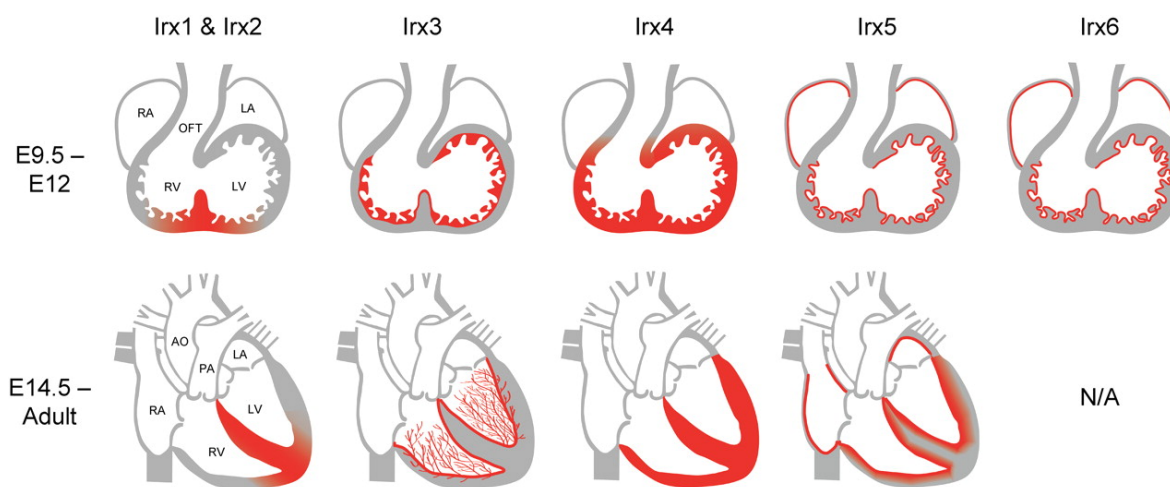


Figure 1.5 *Iroquois (Irx)* homeobox transcription family expression in embryonic and adult mouse hearts. Figure from KH Kim et al. 2012¹⁹⁷.

Out of the six *Irx* family of transcription factors, the expression of *Irx3* is confined to the VCS of the heart. Our previous study has demonstrated that mice lacking *Irx3* exhibit abnormal electrophysiological phenotypes, such as prolonged QRS complex durations and notched R' wave in electrocardiograms (ECGs)⁵³, which indicates delayed ventricular activation and right bundle branch block, respectively. Specifically, *Irx3* regulates the expression of gap junctions in the heart by directly repressing working myocardium-specific connexin 43 (Cx43, encoded by *Gja1*) but indirectly activating VCS-specific Cx40 (encoded by *Gja5*). The role of *IRX3* in the human VCS has also been documented, where loss-of-function mutations in *IRX3* are associated with ventricular arrhythmias in otherwise normal human hearts (i.e. idiopathic)¹⁹⁸. Together, these studies suggest that *Irx3* is a critical TF establishing VCS as well as its proper function of rapid electrical propagation.

We have further found that *Irx3*KO mice show hypoplastic VCS morphology, as fiber densities are reduced in the BBs and PFs, and the right BB is disconnected⁵⁰, suggesting that *Irx3* is critical for the formation of VCS structure. Further supporting this, Neuregulin-1 (*Nrg1*), a key inducer of trabeculation, a process essential for proper VCS formation, was identified to regulate *Irx3* expression¹⁹⁹. Of note, the abnormal electrophysiological and morphological phenotypes in *Irx3*KOs were evident at postnatal day 4 (P4), but not at P0 or embryonic stages, suggesting a possibility that *Irx3*'s regulatory roles are critical during early postnatal VCS development, rather than during embryogenesis. However, the exact role and mechanism of *Irx3* in regulating VCS maturation, specifically during this postnatal stage, have not been well-established and will be addressed in **Section 5.1** as part of Future Directions.

1.4 Recent high-throughput studies on the CCS

As the CCS comprises such a small cardiac population, molecular investigations of the CCS have long been considered difficult. Since the discovery of transcriptomic profiling in single-cells or single-nuclei, our understanding of the CCS has significantly advanced. Goodyer WR. *et al.* (2019), showed the molecular heterogeneity of the micro-dissected conduction system in an E16.5 mouse using scRNA-seq, and specifically examined the molecular profiles within three different 'zones,' such as the SAN, AVN-HIS and BB-PF, while also revealing the gene expression profiles of 'transitional cells' of each zone⁶⁴; Bhattacharyya S, *et al.* (2019) generated and used a *Gjd3*-Cre mouse model to investigate the molecular diversity within the AVN through fluorescence-guided microdissection of the P0 mouse heart⁴¹ and; Liang D. *et al.* (2021) focused on the cellular diversity of the SAN in mammals, including mouse, rabbit and monkey, and have shed light on a novel SAN marker, *Vsnl1*²⁰⁰.

These studies have offered valuable new insights into the cellular diversity within specific regions of the CCS, demonstrating that each CCS component is much more complex in its cellular heterogeneity. However, a comprehensive comparison of all CCS components to each other in an unbiased manner, especially in a postnatal heart, has yet to be conducted. Furthermore, the transcriptional mechanism underlying how these molecular heterogeneities may arise remains unexplored. Additionally, the reliance on microdissection in some of the studies often leads to the underrepresentation of CCS cells, as non-CCS cells (contractile cardiomyocytes or non-cardiomyocytes) are more likely to be captured. While this may enable the comparison between CCS and non-CCS cells, this may lead to statistical bias arising from the large difference in cell numbers.

1.5 Rationale and Objectives

The CCS is believed to be a continuous structure, yet is composed of distinct anatomical regions, each derived from specific spatiotemporal developmental origins. In addition to these developmental distinctions, it is evident that the CCS possesses region-specific electrophysiological properties, such as conduction velocity and action potential profiles, reflecting the specialized functions of each CCS component in precisely regulating the heart rhythm and propagating electrical signals. Nevertheless, the molecular insights into the CCS heterogeneities and how these differences transition from embryonic to postnatal stages, are not fully understood. Therefore, the main objective of my Ph.D. thesis project is to test the hypothesis that ***the CCS is comprised of heterogeneous populations of specialized conduction cells***. Results from this objective are in **Sections 3.1 - 3.4**.

In the CCS, key cardiac TFs exhibit both unique and overlapping expression patterns, suggesting distinct regulatory mechanisms that govern different regions of the CCS. These TFs are essential for defining the specific identities and functions of the CCS

components, as evidenced by the phenotypes observed in mutants. Interestingly, genetic and functional studies in mice lacking one or more of these TFs frequently lead to similar developmental abnormalities, suggesting that TFs form a collaborative network to regulate CCS formation and function. Of the TFs found to be present or involved in CCS development, some exhibit highly region-specific expression patterns. In particular, *Tbx3* is expressed in the SAN and AVN as well as in the proximal regions of the VCS (i.e., His bundle and proximal bundle branches), while *Irx3* has been demonstrated to be expressed throughout the VCS, but not in nodal regions. Although *Tbx3* and *Irx3* have distinct expression patterns, they share common downstream targets, such as Cx40 and Cx43, which are essential gap junctions regulating electrical conduction. Moreover, *Tbx3* and *Irx3* are key players involved in the regulation of early postnatal CCS development, shown through the abnormal phenotypes after birth in mutants. Despite these insights on each of these TFs, our fundamental and biological understanding of the ***transcriptional regulatory mechanism of Tbx3 and Irx3 and its regional implications establishing molecular heterogeneities*** within the CCS is limited. The results are in **Sections 3.5** and **3.6**. The AIMS for my project are as follows:

AIM 1: To construct a high-resolution molecular and cellular map of the postnatal CCS
(Results are in **Sections 3.1 - 3.3**)

AIM 2: To investigate the molecular changes that occur in the CCS during embryonic-to-postnatal development (Results are in **Section 3.4**)

AIM 3: To understand the regulatory role of *Tbx3* and *Irx3* in the establishment of regional molecular identities of the CCS (Results are in **Sections 3.5 - 3.6**)

Results from all aims are included in the manuscript (**Section 8.1**).

SECTION 2 – METHODS

2.1 Mice

All experimental protocols were approved by the Animal Care Committees of the University of Ottawa (HI-2950 and 3060), in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). All mice had free access to standard chow (2019 Teklad Global Diet; Envigo) and water and were housed in a controlled environment with a 12 hr light/12 hr dark cycle, ambient temperature of 22 - 24 °C and humidity levels of 30 - 60%. Mice were humanely euthanized via CO₂ inhalation and/or cervical dislocation based on CCAC guidelines on the euthanasia of animals used in science.

For single-cell RNA-sequencing (scRNA-seq), spatial transcriptomics (ST), and histological validation experiments, homozygous *Cntn2*^{tm1.1(cre/EGFP)*N*mun} (*Cntn2*^{Cre}) and homozygous *Rosa26*^{tdTomato} mice²⁰¹, which were maintained on a CD-1 background, were crossed to generate CCS-fluorescent reporter mice (*Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+}, hereafter CCS-tdT) (**Figure 2.1**).

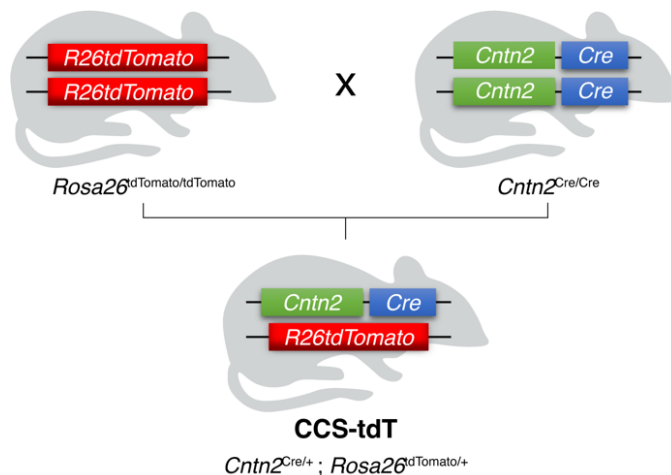


Figure 2.1 Schematic of CCS reporter transgenic mouse generation. *Rosa26*^{tdTomato/tdTomato} was crossed with *Cntn2*^{Cre/Cre} to generate the CCS reporter transgenic mouse (*Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+}) used in the study.

To achieve the maximum number of age-matched pups simultaneously, 8 - 15 weeks old female mice were housed together without exposure to male mice for 2 - 3 weeks prior to mating to synchronize their estrous cycles. Following the mating setup, vaginal plugs were monitored daily. Neonatal mice on postnatal day 1, 2, and 4 (P0, P1, and P4) were euthanized by cervical dislocation immediately before heart collection for cardiomyocyte isolation, ST or histological validation. A total of 103 CCS-tdT mouse pups were used for scRNA-seq. For *in vitro* adenovirus-based overexpression studies, 2 to 3 P0 neonatal litters from CD-1 male and female breeders were used to isolate atrial and ventricular cardiomyocytes. Both males and females were included in all experiments, and sex differences were not considered.

2.2 Neonatal mouse cardiomyocyte isolation

The atria and ventricles of P1 - P4 neonatal mice were collected separately in sterile ice-cold 1x phosphate-buffered saline (PBS). The ventricles were cut into 3 - 4 pieces for efficient digestion. Atrial and ventricular cardiomyocytes were separately placed into 50 mL Falcon tubes containing filtered Hanks' Balanced Salt Solution (1x HBSS, Thermofisher) with 0.0125% Trypsin-EDTA (Thermofisher) and 1% penicillin/streptomycin (P/S; Thermofisher). Cells were subjected to overnight trypsin digestion at 4°C while gently rotating. Then, the trypsin solution was aspirated and discarded. 5 mL of filtered and warm (37°C) L-15 + 0.075% Collagenase II/Dispase (Roche, 10269638001) + 1% P/S solution was added to the 50 mL falcon tube containing cardiomyocytes, and a fine magnetic stir bar was placed. The falcon tubes were placed in a tube rack, which was placed above the stir plate. After gentle stirring of the solution and cells for 5 min, the supernatant containing single cardiomyocytes was collected into 5 mL of cold fetal bovine serum (FBS) to prevent cells from over-digestion. An additional 5 mL of collagenase digestion solution was added to

the cells, and this digestion-collection step was repeated until sufficient cells were collected. After the final collection, debris was removed using a 100 μ m cell strainer (VWR International). Cells were then pelleted at 200 g for 5 min. Purified cells were resuspended and plated in a culture medium containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Thermofisher) with 10% FBS + 1% P/S. Cells were pre-plated for 1 hr in the incubator to allow separation of fibroblasts from cardiomyocytes. Cardiomyocytes were then collected for either FACS or plated in new 12- or 24-well plates for adenovirus-mediated overexpression studies (**Figure 2.2**). Detailed cardiomyocyte isolation methods are included in the appendix as a manuscript in preparation (**Section 8.2**).

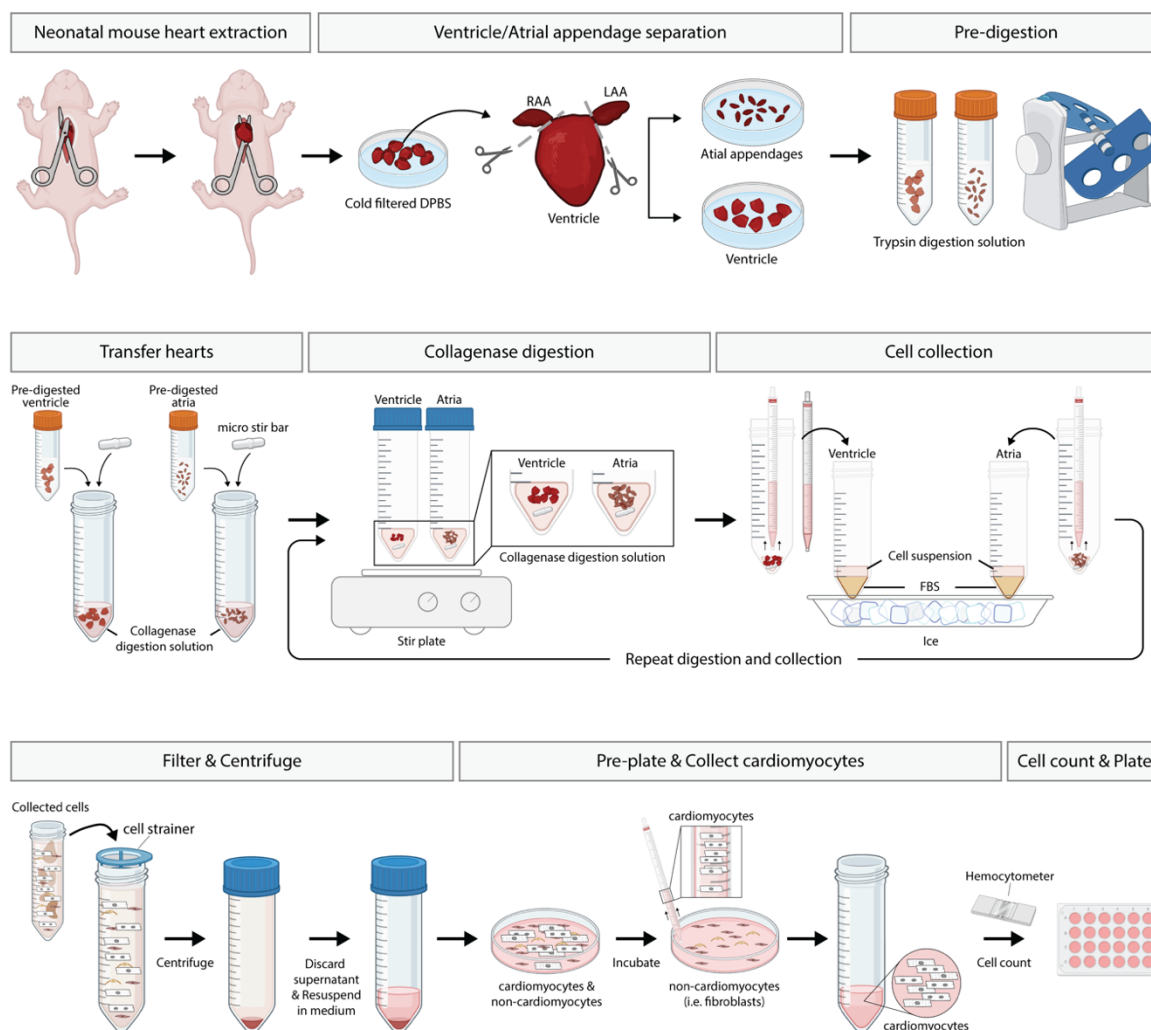


Figure 2.2 Experimental workflow of neonatal mouse atrial and ventricular cardiomyocyte isolation.

2.3 Single-cell RNA sequencing (scRNA-seq) with multiplexed CCS cells

Isolated P1, P2, and P4 neonatal cardiomyocytes suspended in culture medium (DMEM/F-12 with 10% FBS + 1% P/S) were filtered using a 100 μ m cell strainer (VWR, 10199-658), then transferred to separate polypropylene tubes (Falcon). Cells were incubated with SytoxTM Blue dead cell stain (ThermoFisher, S34857, 1:2000) prior to Fluorescence Activated Cell Sorting (FACS) using the BeckmanCoulter MoFlo XDP instrument (Flow Cytometry and Cell Sorting Facility, The Ottawa Hospital Research Institute). Cells were gated based on forward scatter (FSC) to capture non-debris based on cell size, side scatter (SSC) to capture singlets and exclude doublets or multiplets based on cell complexity and Sytox staining to distinguish live (Sytox⁻) from dead (Sytox⁺) cells. Finally, native tdTomato fluorescence was used to differentiate between CCS (tdTomato⁺) and non-CCS (tdTomato⁻) cells.

Multiplexing reagents using lipid-tagged indices for single-cell and single-nucleus RNA sequencing (MULTI-seq) were generously provided by the developers, and multiplexing of P1, P2, and P4 cells was conducted following the developer's protocols²⁰². In brief, following separate FACS sorting of P1, P2, and P4 CCS cells into the culture medium (DMEM/F-12 with 10% FBS + 1% P/S), cells were pelleted and washed using centrifugation (250 g, 5 min, 4°C) and 1x PBS. Lipid-modified oligonucleotide anchor mix and unique barcodes were added to each sample at final concentrations of 200 nM. Cells were resuspended by gentle pipetting and were incubated at 4°C for 10 min, ensuring to periodically mix by gently tapping. Then, a co-anchor lipid-modified oligonucleotide was added to each sample (final concentration of 200 nM), and cells were incubated at 4°C for 5 min. Cells were then pelleted using centrifugation (250 g, 5 min, 4°C) and resuspended in PBS containing 1% BSA to quench residual barcodes. P1, P2, and P4 cells were then pooled into a single tube and subjected to viability assessments and cell counting prior to

scRNA-seq library generation using the 10x Chromium Single Cell 3' v3 kit (10x Genomics). All gene expression libraries were sequenced with NextSeq500 (Illumina) at the StemCore at the Ottawa Hospital Research Institute.

2.4 Adenovirus-mediated overexpression of Tbx3 and Irx3

24 hrs following the isolation and plating of atrial and ventricular cardiomyocytes, debris and dead cells were washed with pre-warmed PBS. Following washing, pre-warmed fresh culture medium (DMEM/F-12 + 10% FBS + 1% P/S) containing adenoviruses (Ad) expressing green fluorescent protein (GFP), Irx3 or Tbx3 or both Irx3 and Tbx3 were provided to the cells at a multiplicity of infection of 10 virus particles per cell. Adenovirus was washed after 7-12 hrs, and after culturing for 48 hrs, virus infection was confirmed through fluorescence – GFP for Ad-GFP, GFP for Ad-Irx3, red fluorescent protein (RFP) for Ad-Tbx3 – prior to collection for RNA extraction. All adenovirus was generated and generously provided by Dr. Chi-Chung Hui (The Hospital for Sick Children, Toronto) and Dr. Peter H. Backx (University of Toronto, currently York University).

2.5 scRNA-seq data analysis

2.5.1 scRNA-seq analysis of postnatal CCS

The generated fastq files were processed with CellRanger (version 7). Reads were aligned to the mm10-3.0.0 reference transcriptome annotation file. Sequences and annotations for tdTomato and Cre were manually added to the fasta and gtf files (**Table 2.1**), respectively, using the function *cellranger mkref*.

Gene	Sequence
<i>Tdtomato</i>	ATGGTGAGCAAGGGCGAGGAGGTCATCAAAGAGTTCATGCGCTTCAAGGTGCGCA TGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCC GCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCC TGCCCTTCGCCTGGGACATCCTGTCCCCCAGTTCATGTACGGCTCCAAGGCGTA CGTGAAGCACCCCGCCGACATCCCCGATTACAAGAAGCTGTCCTTCCCCGAGGGC TTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGTCTGGTGACCGTGACCC AGGACTCCTCCCTGCAGGACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCAC CAACTTCCCCCCCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGC CTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAAGGGCGAGATCCACCA GGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGACCATCTAC ATGGCCAAGAAGCCCGTGCAACTGCCCGGCTACTACTACGTGGACACCAAGCTGG ACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAGCGCTCCGA GGGCCGCCACCACCTGTTCTGGGGCATGGCACCGGCAGCACCGGCAGCGGCAG CTCCGGCACCGCCTCCTCCGAGGACAACAACATGGCCGTCATCAAAGAGTTCATG CGCTTCAAGGTGCGCATGGAGGGCTCCATGAACGGCCACGAGTTCGAGATCGAG GGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGT GACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCAGTTCATG TACGGCTCCAAGGCGTACGTGAAGCACCCCGCGACATCCCGATTACAAGAAGC TGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCG GTCTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCACGCTGATCTACAA GGTGAAGATGCGCGGCACCAACTTCCCCCCCCGACGGCCCCGTAATGCAGAAGAA GACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCT GAAGGGCGAGATCCACCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGT GGAGTTCAAGACCATCTACATGGCCAAGAAGCCCGTGCAACTGCCCGGCTACTAC TACGTGGACACCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTGG AACAGTACGAGCGCTCCGAGGGCCGCCACCACCTGTTCTGTACGGCATGGACGA GCTGTACAAGTAA
<i>Cre</i>	ATGGCCAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTGATGCAAC GAGTGATGAGGTTGCAAGAACCTGATGGACATGTTTCAGGGATCGCCAGGCGTTT TCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCTGGGCGGCATGGT GCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTGCGGATTAT CTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAACTATCCAGCAACATTTGGG CCAGCTAAACATGCTTCATCGTCCGTCCGGGTGCCACGACCAAGTGACAGCAAT GCTGTTTCACTGGTTATGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAAC GTGCAAAACAGGCTCTAGCGTTTCAACGCACTGATTTTCGACCAGGTTTGGTCACTC ATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTCTGGGGATTGC TTATAACACCCTGTTACGTATAGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTC ACGTAAGTACGGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTA GCACCGCAGGTGTAGAGAAGGCACTTAGCCTGGGGGTAATAACTGGTCGAGCG ATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGG TCAGAAAAAATGGTGTGCGCGGCCATCTGCCACCAGCCAGCTATCAACTCGCGC CCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAGGATGACT CTGGTCAGAGATACCTGGCCTGGTCTGGACACAGTGCCCGTGTGCGAGCCGCGC GAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGGTGGCTG GACCAATGTAAATATTGTCATGAACTATATCCGTAACTGGATAGTGAACAGGGG CAATGGTGCGCCTGCTGGAAGATGGCGATTAG

Table 2.1 Reference sequences for *Tdtomato* and *Cre*. Sequences of *Tdtomato* and *Cre* used to manually add to FASTA reference transcriptome.

A total of 10,407 cells were sequenced, with a mean of 35,041 reads per cell and a median of 3,055 genes detected per cell. Demultiplexing was performed using Python

according to the unique MULTI-seq barcodes given to each P1, P2, and P4 sample prior to pooling.

Downstream bioinformatic analysis was performed on R (version 4.2.2) using the 'Seurat' R package (version 5.0.1)²⁰³. Briefly, pre-processing of the data was performed following quality assessment to include cells with >2,000 and <5,500 detected genes, and percentages of mitochondrial genes of >5% and <25% (**Figure 3.5**). Additional biological filtering was applied based on markers of common cell types such as cardiomyocytes, fibroblasts and smooth muscle cells known to exist in the heart. Non-cardiomyocytes were removed from downstream analysis (**Section 3.1.3, Figure 3.6**). Following pre-processing of the data, feature counts were normalized by log-transformation using the function *NormalizeData*. The top 5,000 features were identified using "vst" as the selection method using *FindVariableFeatures*. Normalized counts for these variable genes were then scaled using *ScaleData* with regression of mitochondrial gene counts and the number of unique molecular identifiers (UMIs). Linear dimensional reduction was performed using principal component analysis (PCA) using *RunPCA*. For analysis using all cells, including non-cardiomyocytes (**Figure 3.6**), CCS cells (**Figure 3.7b**), 'Nodes-HIS-BB' (**Figure 3.7c**), 'Nodes' (**Figure 3.7d**), and the 'LNB-HIS-PBB' (**Figure 3.7e**), the first 25 principal components were used for Uniform Manifold Approximation and Projection (UMAP) embedding using *RunUMAP*. Optimal clusters were identified by *FindNeighbors* and *FindClusters* using resolutions of 0.25, 0.19, 0.4, 0.97 and 0.6, respectively, determined by the examination of known marker expression patterns. Heatmaps were generated using the 'ComplexHeatmap' R package (version 2.15.4).

2.5.2 E16.5 CCS UMAP construction

E16.5 mouse CCS data (GSE132658) published by Goodyer WR. *et al.* 2019, *Circulation research*⁶⁴ was obtained and analyzed according to the author's described methods as well

as using markers demonstrated in the study. Specifically, the study micro-dissected tissues from 3 distinct zones: (1) Zone I: SAN; (2) Zone 2: AVN/HIS; and (3) Zone III: PF. Clustering of all cells from each zone was performed using the following criteria Zone I (dimensions = 1:10, resolution = 0.7), Zone II (dimensions = 1:10, resolution = 0.45) and Zone III (dimensions = 1:10, resolution = 0.77) in order to reproduce the published t-SNE (t-distributed stochastic neighbor embedding) plots. From the t-SNE plots of each zone, conduction cells were isolated using a set of cardiomyocyte and conduction markers. Specifically, SAN cells were isolated from Zone I using *Actn2*, *Hcn4*, *Isl1*, *Shox2* and *Tbx3*; AVN/HIS cells were isolated from Zone II using *Actn2*, *Hcn4*, *Cacna2d2* and *Cacna1g* and; PF cells were isolated from Zone III using *Actn2*, *Gja5*, *Scn5a*, *Irx3* and *Cacna2d2*. These conduction cells were combined and re-clustered on a UMAP using dimensions = 1:30 and resolution = 2, which gave rise to the most appropriate identification of the specific CCS subcomponents.

2.5.3 Integration of embryonic and postnatal CCS scRNA-seq data

Integration of E16.5 and postnatal CCS was performed using Seurat's Integration pipeline. Briefly, anchors between the datasets were found using *FindIntegrationAnchors* followed by data integration using *IntegrateData*. For correlation between E16.5 and postnatal CCS components, averaged expressions of genes for each CCS component given by *AverageExpression* were used. Correlation analysis was then performed and visualized using the 'corrplot' R package (version 0.92). Direct comparison was performed on integrated data using *FindMarkers*.

2.6 Gene regulatory network inference

Gene regulatory network inference was performed using 'pySCENIC' (version 0.12.1) following the SCENIC (single-cell regulatory network inference and clustering) pipeline^{204,205}.

The count matrix was provided as input after filtering out genes with zero expression.

Repressive regulons were excluded, and putative regulons lacking expression of the TF in at least 40% of any region were filtered out, resulting in 97 retained regulons. Regulon activity was quantified in individual cells using AUCell and averaged across each region.

To construct regional gene regulatory networks, regulons were scored based on regional specificity²⁰⁶. The top 5 regulons specific to each region were retained after filtering for 40% expression of the TF in the respective region. Target genes differentially expressed in each region (adj. $P < 0.05$) were retained. The resulting regional networks with top 5 regulons were then visualized using the Fruchterman-Reingold layout ('iGraph', version 1.5.1), with node colours corresponding to the classification of the gene (TFs and CCS marker genes).

2.7 Spatial transcriptomics and data analysis

Spatial Transcriptomic (ST) analysis was performed in frozen tissue sections of P4 CCS-tdT mouse heart using the NanoString GeoMx Digital Spatial Profiler (DSP)^{207,208} in collaboration with the Advanced Genomics Core at the University of Michigan. Slides for ST were prepared with fixed cryopreserved hearts (See **Section 2.9.1**). Briefly, following trimming of excess Tissue-Tek® O.C.T compound (Sakura, #4683) and cryosectioning, heart sections were mounted on a slide within a required scan area with at least 2 mm spacing between sections. Frequent examination for endogenous tdTomato in the CCS was performed to ensure the capture of all CCS components on a single slide (**Figure 2.3**).

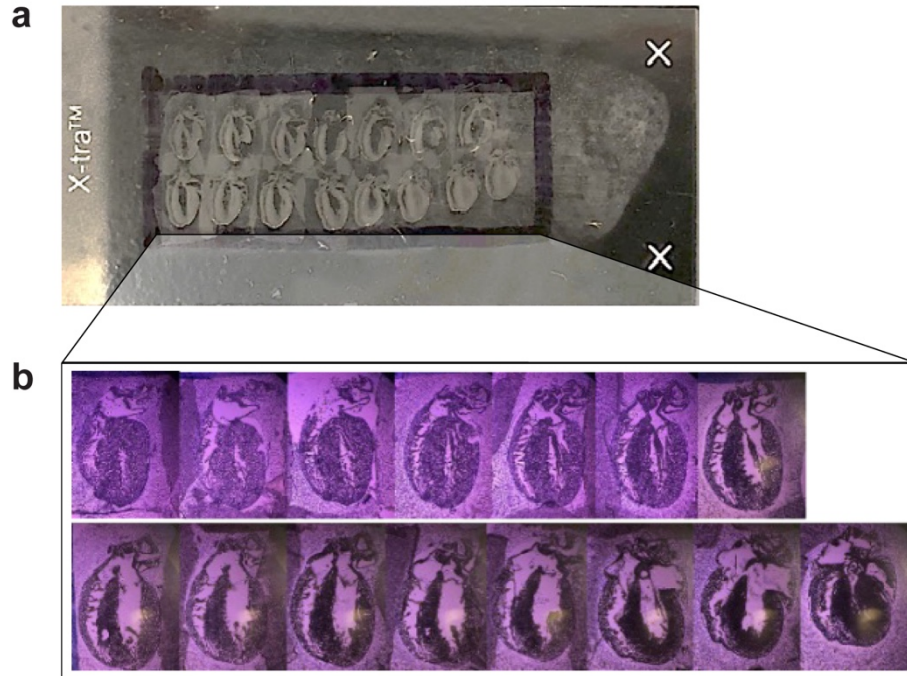


Figure 2.3 Slide image of CCS-tdT sections used for GeoMx Digital Spatial Profiler (DSP) spatial transcriptomics. (a) Image of slide with heart sections and (b) zoomed in bright-view images of the sections. Fluorescence images are shown in Figure 3.10.

The sections were subjected to *in situ* hybridization with the GeoMx Probe Mix for NGS readout (Mouse Whole Transcriptome Atlas RNA v1.0). CCS structure was visualized with anti-DsRed primary antibody (Takara, #632496, 1:200) and anti-rabbit AlexaFluor™ 594 secondary antibody (ThermoFisher Scientific, A11012, 1:500). SYTO13 nuclear staining was performed for visualization of tissue morphology and cell counting. Regions of interest (ROI) were chosen using the fluorescent segmentation function on the DSP. Two ROIs were selected for each CCS region, including the SAN, LNB, HIS, PLBB/PRBB, DLBB/DRBB, LPF/RPF. One ROI was collected for the compact AV node (cAVN). Non-CCS regions adjacent to each CCS region were also selected, including right atrial appendage (RAA), left endomyocardium (LEND), left epimyocardium (LEPI), basal interventricular septum (IVS-B), apical interventricular septum (IVS-A), and right ventricle (RV). Following ROI selection, DNA oligos were cleaved from RNAScope probes by directing UV light at each ROI using the digital micromirror device. Cleaved oligos were then collected into the wells of a 96-well

collection plate, which were subjected to library preparation and next-generation sequencing. Sequencing depth was estimated by multiplying the total area of all the ROIs (μm^2) and sequencing of the pooled library was performed on NovaSeq 6000 (Illumina).

Raw FASTQ files were processed using NanoString's Automated Data Processing Pipeline, which includes adapter trimming and aligning stitched paired-end reads to the barcodes in the reference assay before the removal of PCR duplicates based on the UMI of each read. The resulting data collection container (DCC) files for each ROI were then processed with the 'GeoMxTools' package (version 3.0.1) using the corresponding NanoString DSP configuration file and the instrument-generated worksheet with ROI identifiers. Initial quality control filtering and third quartile (Q3) normalization were performed with minimum average negative control measurements for ROI retention and minimum counts above background for target retention. The resulting filtered and Q3 normalized data were analyzed for differential expression analysis using an unpaired t-test. A gene was considered CCS-specific as well as significantly differentially expressed, if it satisfied both of the following conditions: 1) $|\text{Fold change}| > 1.5$, $P < 0.05$ in the region of interest compared to all other CCS components, and 2) $|\text{Fold change}| > 1.5$ in the region of interest compared to its respective non-CCS components. For SAN and cAVN, its non-CCS control was RAA; for LNB, HIS, PLBB and PRBB, its control was IVS-B; for DLBB and DRBB, its control was IVS-A; for LPF, its control was LENDO and; for RPF, its control was RV.

2.8 Assay for Transposase-Accessible Chromatin with sequencing (ATAC-seq) and Chromatin Immunoprecipitation sequencing (ChIP-seq) analyses

For chromatin accessibility and binding analyses, raw data was downloaded in fastq format using the *fastq-dump* function in the SRA toolkit. Fastq files were then uploaded onto the UseGalaxy server²⁰⁹. Quality control assessments were performed using FastQC (version

0.11.7)²¹⁰. Reads were then aligned to the mm9 mouse reference genome using Bowtie2 (Galaxy Version 2.4.2+galaxy0) with the ‘--very-sensitive’ parameter applied for optimal mapping²¹¹. Mitochondrial genome reads and low mapping quality (mapQuality <30) were considered uninformative reads and filtered out using the *Filter BAM datasets on a variety of attributes* function (Galaxy Version 2.4.1)²¹². Duplicate reads were then removed using ‘Picard MarkDuplicates’ (Galaxy Version 2.18.2.2) (broadinstitute.github.io/picard). *MACS2 callpeak* function (Galaxy Version 2.1.1.20160309.6) was used to call peaks with the following parameters: ‘Single-end BED’, ‘--nomodel’, ‘--shift -100’, ‘--extend 200’, and FDR < 0.05²¹³. The following datasets were analysed: E13.5 Tbx3+ AVJ ATAC-seq ([GSE121464](#))¹⁶³, adult ventricle ATAC-seq ([GSE110209](#))²¹⁴, P0 sinoatrial pacemaker ATAC-seq ([GSE148515](#))¹⁴⁷, P0 right atrial cardiomyocyte ATAC-seq ([GSE148515](#)), and adult heart Tbx3 ChIP-seq ([GSE35151](#))²¹⁵. Moreover, two *Iroquois (Irx)* motifs, ACATGT (Motif ID: UP00223_2)²¹⁶ and ACAAnnTGT²¹⁷ were used for predicting genome-wide motif occurrences. Each motif sequence was uploaded onto FIMO (Find Individual Motif Occurrences), part of the MEME software package (tools.genouest.org/tools/meme/), and motifs were scanned in the UCSC mm9 mouse genome. Only motif occurrences with *P* values $\leq 1.0 \times 10^{-4}$ were considered significant, which are automatically calculated by FIMO based on a log-likelihood ratio score conversion²¹⁸.

Data visualization was performed as previously described²¹⁹. Briefly, peaks were visualized in bigWig format by converting MACS2 Bedgraph output to bigWig via the *Wig/BedGraph-to-bigWig* function (Galaxy version 1.1.1) and uploading it onto the Integrative Genomics Viewer (IGV) genome browser (version 2.16.2). For datasets where peaks were readily available in bigWig format, peak files were directly downloaded from the NCBI Gene Expression Omnibus (GEO) and uploaded onto IGV. For datasets with multiple replicates, a mean bigWig file was generated using the deepTools2 ‘bigwigCompare’ function (Galaxy version 3.5.1.0.0)²²⁰.

2.9 Histological assessments

2.9.1 Immunofluorescence staining

Freshly isolated neonatal hearts were fixed in a methanol-free 4% paraformaldehyde solution (Thermo Scientific™ Pierce™, #28906) in PBS overnight at 4°C. Hearts were washed twice in PBS for 30 mins each, then incubated in filtered 30% sucrose in PBS overnight at 4°C. Fixed cryopreserved hearts were embedded in Tissue-Tek® O.C.T compound (Sakura, #4683) and immediately frozen on powdered dry ice. Embedded hearts were sectioned using a cryostat at 8 µm thickness and stored at -20°C until use.

For immunofluorescence staining, heart sections were air-dried for 30 min at room temperature before incubating in PBS for 5 min three times to wash. Heart sections were permeabilized in 0.1% Triton X-100 in PBS for 15 min. Blocking was done using the appropriate host serum in 0.3% triton X-100 in PBS for 1hr at room temperature. Following blocking, the following primary antibodies were used: chicken anti-RFP (600-901-379, 1:100), goat anti-Cntn2 (AF5549, 1:50), rabbit anti-Gnao1 (Thermo Fisher Scientific, PA5-30044, 1:100), rabbit anti-Scn10a (Alomone Labs, ASC-016, 1:50), rabbit anti-Igfbp7 (Abcam, ab74169, 1:50), rabbit anti-S100a6 (Abcam, ab181975, 1:100), rabbit anti-Nppa (EMD Millipore, ab5490, 1:250), mouse anti-Shox2 IgG2a (Santa Cruz Biotechnology, sc-81955, 1:50) or rabbit anti-Vsnl1 (GeneTex, GTX115039, 1:100), rabbit anti-Ppp1r17 (PA-61599, 1:200). Following primary antibody incubation overnight at 4°C, sections were washed three times for 5 mins with PBS. Sections were then incubated with secondary antibodies: goat anti-chicken IgY Alexa Fluor™ 568 (Thermo Fisher Scientific, A11041, 1:200), donkey anti-chicken IgY Alexa Fluor™ 568 (Thermo Fisher Scientific, A78950, 1:200), donkey anti-rabbit Alexa Fluor™ 647 (Thermo Fisher Scientific, 711-605-1523, 1:200), goat anti-rabbit Alexa Fluor™ 488 (Thermo Fisher Scientific, A-11008, 1:500), goat anti-mouse IgG2a Alexa Fluor™ 488 (Thermo Fisher Scientific, A21131, 1:500), donkey

anti-rabbit Alexa Fluor® 657 (Jackson ImmunoResearch Laboratories Inc., 711-605-152, 1:200), goat anti-mouse IgG2b Alexa Fluor™ 488 (Thermo Fisher Scientific, A21141, 1:500) for 1 hr at room temperature in the dark, followed by three times of 5 min PBS wash. Autofluorescence of the heart was minimized using the Vector® TrueVIEW Autofluorescence quenching kit (Vector laboratories, SP-8400) according to the manufacturer's protocol. Mounting medium with DAPI (Abcam, ab104139) was used for nuclei staining. Airyscan confocal imaging was performed using LSM 880 confocal microscope (Zeiss) or AxioObserver 7 microscope (Zeiss), and processing was performed using ZEN microscopy software with Airyscan processing and orthogonal projection. Whole tissue fluorescence imaging to examine Cntn2-Cre mediated recombination and tdTomato expression was performed using the Zeiss SteREO Discovery V8 stereomicroscope. Fluorescence scanning was performed using Aperio VERSA 8 Scanner (Leica Biosystems).

2.9.2 Fluorescence *In-Situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was performed using the RNAscope Multiplex Fluorescent Reagent Kits v2 (Advanced Cell Diagnostics, ACD) as per the manufacturer's protocol. In brief, frozen tissue sections (8 µm thickness) of P4 CCS-tdT mouse heart were baked at 60°C for 30 min, followed by post-fixation in 4% PFA for 30 min at 4°C. After target retrieval for 5 min and Protease Plus incubation for 15 min, the sections were incubated for 2 hours at 40°C with the following probes: *Prdm6* (mouse, Cat #456891), *Mpped2* (mouse, ACD, #848371), *Pcp4* (mouse, ACD, #402311) and *tdTomato* (mouse, ACD, #317041-C2). Opal 690 Reagent Pack (Akoya Biosciences, #FP1497001KT, 1:200) and Opal 570 Reagent Pack (SKU FP1488001KT, 1:200) were used to visualize the FISH signal. Fluorescence microscopy was performed using the LSM 880 confocal microscope (Zeiss) or AxioObserver 7 microscope (Zeiss), followed by image processing with ZEN microscopy software.

2.9.3 Trichrome staining

To examine the cellular morphology and fibrous insulation in the atrioventricular region of the cardiac conduction system (i.e., cAVN, LNB and HIS), P4 mouse hearts were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μ m thickness. Fibrosis was visualized by Masson's trichrome staining, and images were captured using the Aperio VERSA 8 Scanner (Leica Biosystems).

2.10 Gene expression assessments

2.10.1 RNA isolation and cDNA synthesis

Cultured cardiomyocytes were lysed in Trizol™ Reagent (Invitrogen™) through vigorous pipetting. For every 1 mL of Trizol™, 0.2 mL of chloroform (C607-4, Fisher Chemical) was added to the cell lysate, followed by vigorous shaking for 15 sec and incubation at room temperature for 5 min. Centrifugation was done at 12,000 *g* for 15 min at 4°C to allow separation of the aqueous layer collection. Using the aqueous layer, RNA was purified with PureLink™ RNA Mini Kit (Invitrogen™) according to the manufacturer's protocol.

Nanodrop2000 spectrophotometer (Thermo Fisher Scientific) was used for spectrophotometric quality and quantity assessments. cDNA was synthesized from purified RNA through reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) following the manufacturer's instructions, and to inhibit RNA degradation, RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen™) was used.

2.10.2 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative Polymerase Chain Reaction (qPCR) was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™) with QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). Relative fold change and normalization of genes of interest were

performed using the $2^{-\Delta\Delta CT}$ method, with TATA-box binding protein (*Tbp*) serving as the housekeeping gene after ensuring its stable expression across all conditions. Primer sequences used are listed below (**Table 2.3**).

Gene	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')
<i>Gja1</i>	TCATTAAGTGAAAGAGAGGTGCCC	TGGAGTAGGCTTGGACCTTGTC
<i>Gja5</i>	AAGCAGAAGGCTCGGCCTC	GGAAGCTCCAGTCACCCATCTT
<i>Mppd2</i>	ATGGCACATGGGATTCTTCT	AGGCATCTGGATACCATCTGTT
<i>MyI2</i>	ATCGACAAGAATGACCTAAGGGA	ATTTTTCAGTTCACTCGTCCT
<i>MyI3</i>	TGCCTCCAAGATTAAGATCGAGT	CTCTGCCTGGGTAGGATTCTG
<i>MyI4</i>	AAGAAACCCGAGCCTAAGAAGG	TGGGTCAAAGGCAGAGTCCT
<i>MyI7</i>	GGCACAACGTGGCTCTTCTAA	TGCAGATGATCCCATCCCTGT
<i>Nppa</i>	ATCTGCCCTCTTGAAAAGCA	ACACACCACAAGGGCTTAGG
<i>Pcp4</i>	AGCTGTGGCCATTGAGTCTC	GTGTCTTCAGGTGGACCAGG
<i>Prdm6</i>	AGCGCATGGAAATCATCCCG	CACTCCCCGTTACGGTTGT
<i>Ryr2</i>	GCGAGCTGGCTACTATGACC	CGTTGCTAATGCTCACGAAA
<i>S100a6</i>	TTCTCGTGGCCATCTTCCAC	GCCAATGGTGAGCTCCTTCT
<i>Scn10a-short</i>	AGCAACCCATTTCCGTCG	ATGTACAGGCTCTCCTCCCAAT
<i>Shox2</i>	GGAAGAACTTACGGCGTTTCG	ACGTGATGGCCTCCTTCTTC
<i>Slit2</i>	CCATGTAAAAATGATGGCACCTG	ATCACAGTCCTGACCCTTGAA
<i>Tbp</i>	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
<i>Vsnl1</i>	ACAGCGGGTGGACAAGATTT	GGAAGGGTCGCTTTTGCAG

Table 2.2 Sequences of primers used for qPCR

SECTION 3 – RESULTS

3.1 Single-cell RNA-sequencing and spatial transcriptomic analyses capture molecular profiles of the postnatal mouse cardiac conduction system

3.1.1 *Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+} heart marks all the components of the postnatal CCS

To understand the cellular and molecular constituents of the postnatal CCS, we simultaneously performed single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics (ST) using the CCS reporter mouse model, *Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+} (hereafter CCS-tdT)²⁰¹ (Figure 3.1).

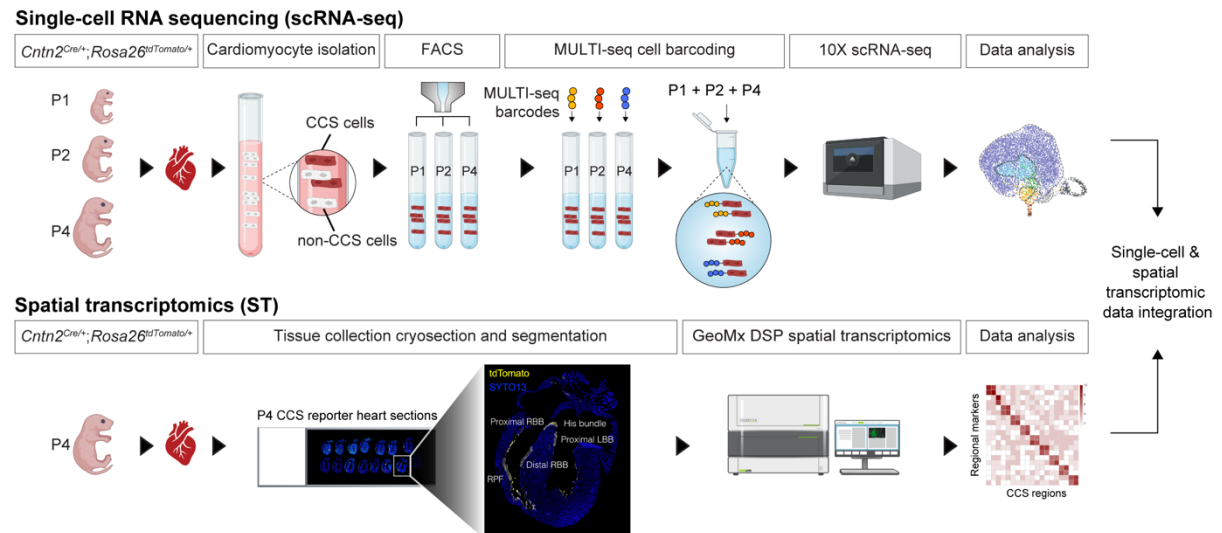


Figure 3.1 Schematic of the experimental workflow. (a) Schematic overview of scRNA-seq experiment for the CCS profiling. Neonatal mouse cardiomyocytes were isolated from postnatal day 1, 2 and 4 (P1, P2, and P4) CCS-reporter mouse hearts, and tdTomato-positive CCS cells were purified using fluorescence-activated cell sorting (FACS). P1, P2 and P4 cells were barcoded separately using MULTI-seq, followed by 10x Genomics scRNA-seq. **(b)** Schematic overview of spatial transcriptomics of the CCS using NanoString GeoMx Digital Spatial Profiler (DSP). Cryosections of P4 CCS-reporter mouse hearts were collected on a slide, followed by the selection of regions of interest using segmentation and sequencing. Results from scRNA-seq and ST were integrated for analysis.

Consistent with the previous studies that utilized Contactin-2 (*Cntn2*)-driven reporters and *Cntn2*-targeting antibody to visualize the entire CCS system in mice^{51,160,221}, epifluorescence imaging in P4 and P42 CCS-tdT hearts showed labelling of all CCS components at the expected locations (**Figure 3.2**), suggesting efficient Cre-mediated recombination of the *Rosa26* locus. However, the intensity of the tdTomato signal was stronger in the P42 CCS, especially in the SAN, suggesting possible higher Cre recombination with age or greater *Cntn2* expression in more mature CCS.

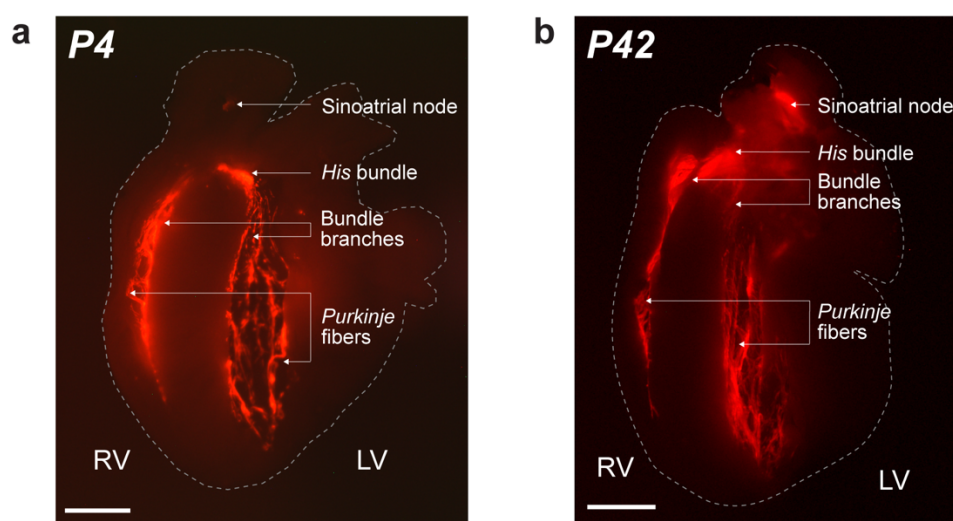


Figure 3.2 Representative epifluorescence image of (a) P4 and (b) P42 CCS-reporter mouse (*Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+}) hearts showing tdTomato marking the sinoatrial node, *His* bundle, bundle branches and *Purkinje* fibers. LV, Left ventricle; RV, Right ventricle. Scale bar = 1mm

To more accurately validate whether the CCS-tdT captures all CCS components, immunostaining was performed using *Cntn2* antibody to capture endogenous *Cntn2* protein expression. As expected, we observed specific co-expression of tdTomato and endogenous *Cntn2* in all components of the CCS – SAN, HIS, LBB, RBB, LPF and RPF (**Figure 3.3**). Additional expression of *Cntn2* in the compact AVN and LNB is demonstrated in **Figure 3.10**.

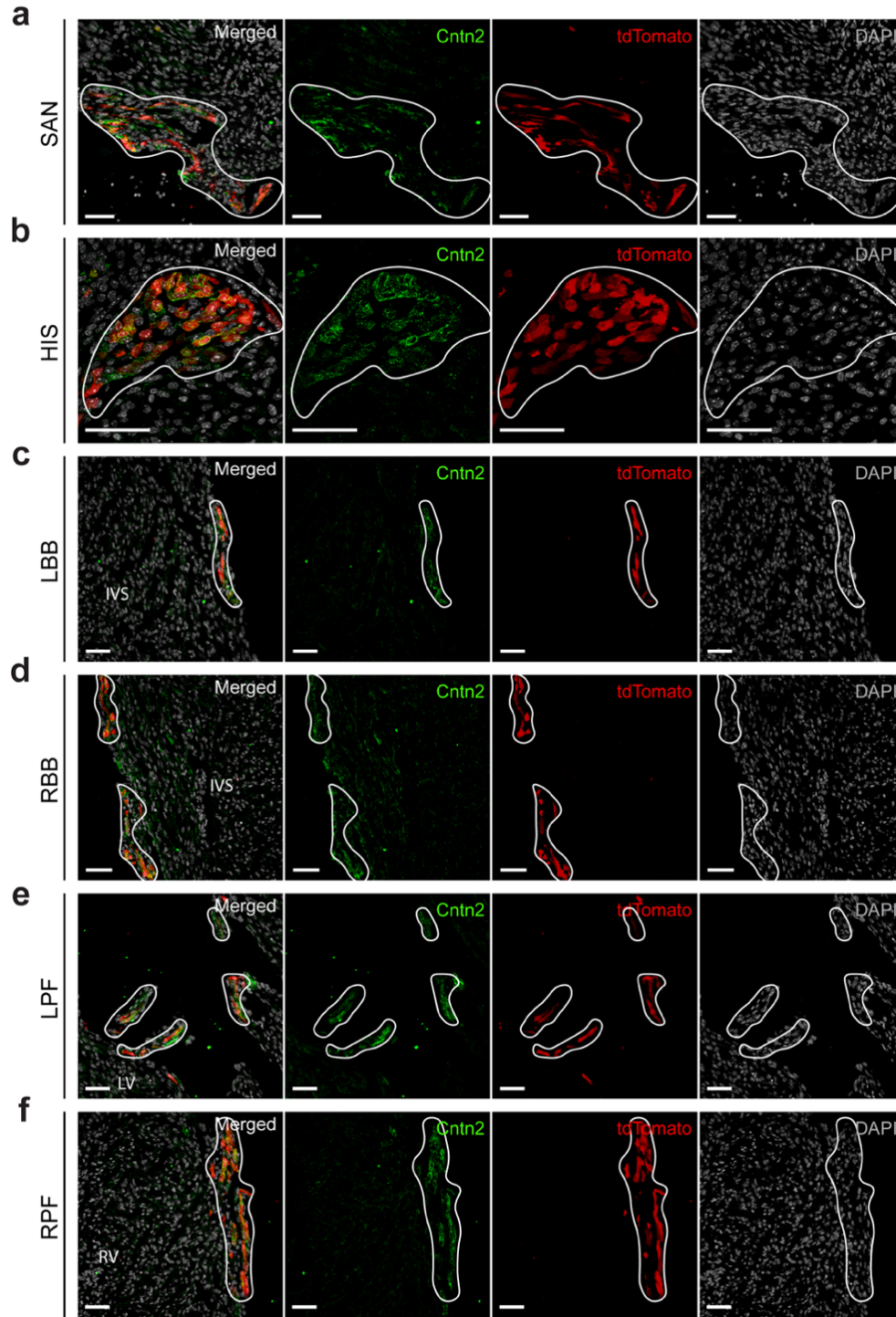


Figure 3.3 P4 *Cntn2*^{Cre/+};*Rosa26*^{tdTomato/+} heart marks all the components of the postnatal CCS. (a-f) Confocal microscope images of the CCS components of the CCS-tdT mouse heart, including (a) SAN, (b) HIS, (c) LBB, (d) RBB, (e) LPF and (f) RPF. tdTomato-marked CCS components (red) were stained with antibodies targeting endogenous *Cntn2* (green). SAN, sinoatrial node; HIS, *His* bundle; LBB, left bundle branch; RBB, right bundle branch; LPF, left *Purkinje* fiber; RPF, right *Purkinje* fiber. Scale bar = 50 μ m.

3.1.2 Fluorescence-activated cell sorting (FACS) of P1, P2 and P4 CCS cells

For scRNA-seq, cardiomyocytes were isolated from 31, 39 and 21 CCS-tdT mouse hearts at postnatal day 1, 2, and 4 (P1, P2, and P4), respectively. Subsequently, CCS cells were isolated using fluorescence-activated cell sorting (FACS), resulting in a purified CCS cell population making up 0.1 - 0.2% of the total cardiomyocyte population at all stages (**Figure 3.4**). These isolated CCS cells were then subjected to multiplexed scRNA-seq (MULTI-seq)²⁰²

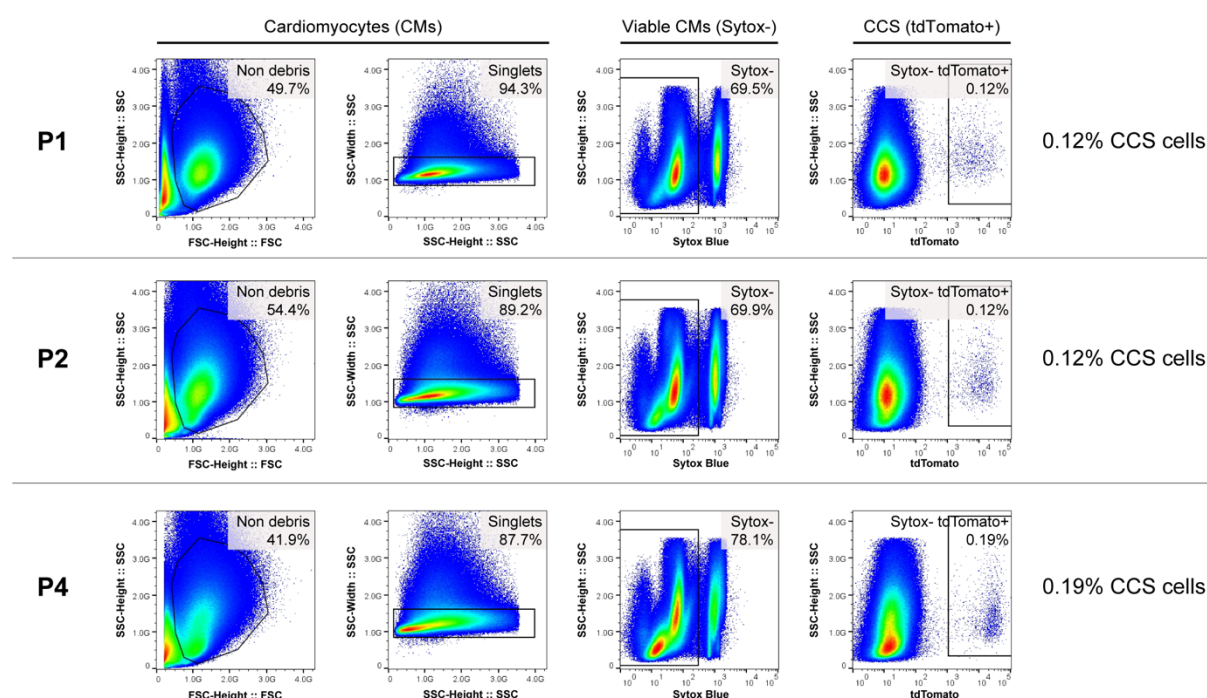


Figure 3.4 Fluorescence-activated cell sorting (FACS) of P1, P2 and P4 CCS cells. Gating was performed for size and granularity, then for Sytox-based cell viability, and finally for tdTomato-based identification of the CCS cells. Isolated neonatal mouse cardiomyocytes from P1, P2 and P4 consist of comparable percentages of CCS cells (0.1 - 0.2%) of viable cardiomyocytes.

3.1.3 Pre-processing of scRNA-seq data allows capture of pure cardiomyocytes

From a total of 10,407 cells, pre-processing of scRNA-seq was performed using mitochondrial gene percentage, number of genes per cell and number of UMIs (number of transcripts per cell). This led to the removal of 3,480 low-quality cells, leaving 6,927 cells

with an average of 3,393 identified genes, 13.3% mitochondrial genes, and 14,392 UMIs per cell for from downstream analysis (**Figure 3.5**).

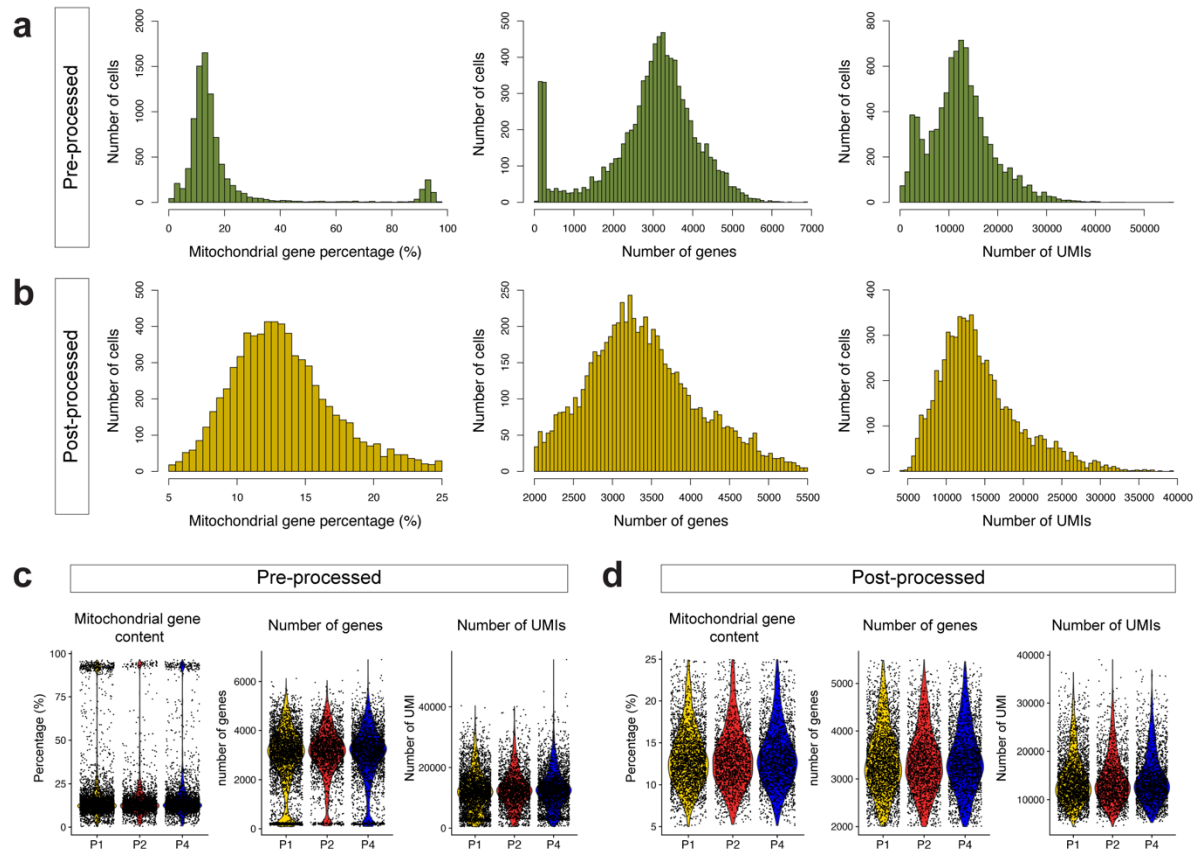


Figure 3.5 Quality assessment of single-cell RNA-sequencing (scRNA-seq). (a-b) Histograms of mitochondrial content (%), number of detected genes, and number of unique molecular identifiers (UMIs) of (a) pre-processed and (b) post-processed scRNA-seq data. (c-d) Violin plots showing percentages of mitochondrial content (%), number of detected genes, and number of UMIs per cell from (c) pre-processed and (d) post-processed scRNA-seq data in P1, P2 and P4 cells. Each dot represents a single cell.

Further biological assessment was performed to identify and exclude non-cardiomyocytes. Of the 6,927 high-quality cells, 72 cells (1.03% of all cells) highly expressed markers of fibroblasts (*Postn*, *Col1a1*, *Col1a2* and *Col13a1*) or smooth muscle cells (*Cald1*, *Emilin1*, *Hexim1*, *Tagln* and *Fabp4*) (**Figure 3.6**). These non-cardiomyocytes were removed leaving a total of 6,855 cardiomyocytes highly expressing cardiomyocyte

genes (*Myh6*, *Myh7*, *Tnnt2*, *Tnni1* and *Ryr2*) for scRNA-seq analysis, suggesting successful enrichment of pure cardiomyocytes.

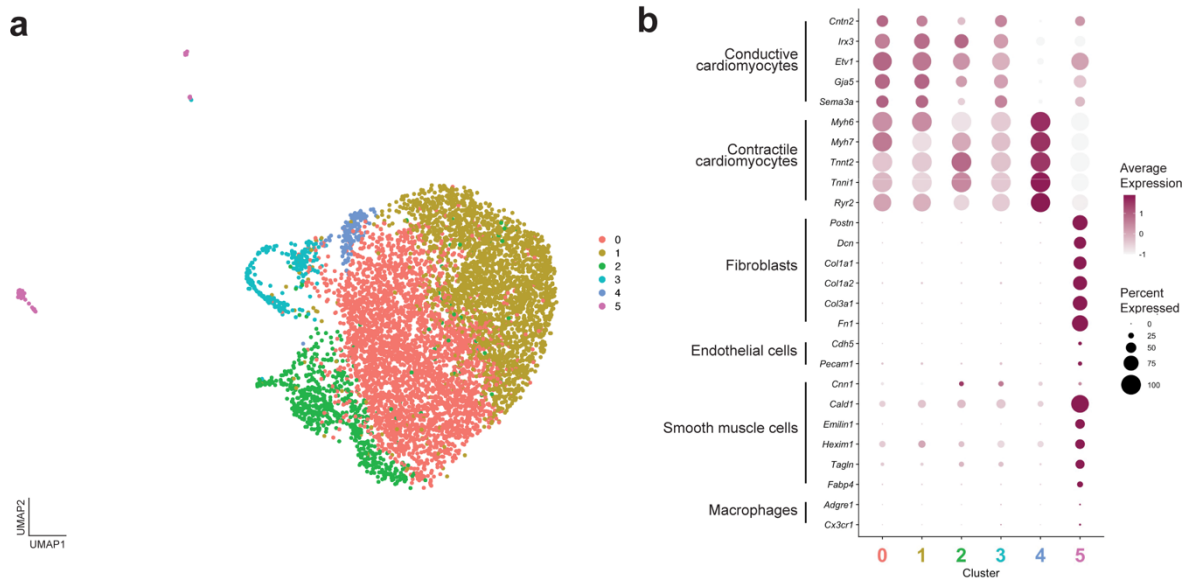


Figure 3.6 Identification of cardiomyocyte and non-cardiomyocyte populations. (a) UMAP clustering of all sequenced cells. **(b)** Dot plot showing enriched cardiac genes (*Myh6*, *Myh7*, *Tnnt2*, *Tnni1*, *Ryr2*) in clusters 0-4 and fibroblast/smooth muscle cell markers in cluster 5. Cells belonging to cluster 5 were removed for downstream CCS analysis.

3.1.4 Subclustering analysis of scRNA-seq data identifies components of the CCS

In the pure cardiomyocyte population, we performed a series of sub-clustering analysis through unsupervised dimensional reduction analysis using uniform manifold approximation and projection (UMAP)²²². Four unique clusters of cells – ‘Purkinje fibers’, ‘Nodes-HIS-PBB’, ‘Proliferative CCS’, and ‘Contractile myocytes’ – were initially identified through a thorough investigation of known cell-type and regional marker genes (**Figure 3.7**). In particular, the three CCS clusters displayed high expression of well-established conduction system markers (*Hcn4*, *Irx3*, *Gja5* and *Cacna2d2*^{145,223}), while exhibiting lower expression of contraction-related genes (*Tnnt2*, *Tnni* and *Mb*) (**Figure 3.7b** and **Figure 3.12a**). In contrast, the Contractile cardiomyocytes cluster (138 of 6,855 cells, ~2%) expressed minimal levels

of conduction genes, but higher levels of contraction genes. This suggested that most of the cells sequenced were conductive CCS cells.

Next, the three CCS clusters were further identified by examining established regional markers of the CCS. For example, while pan-CCS markers (*Hcn4*, *Cpne5* and *Slit2*) were highly expressed in all CCS clusters, markers of the proximal CCS (*Tbx3*, *Robo1*^{224,225} and *Cacna2d2*^{64,226}), were enriched mostly in the 'Nodes-HIS-BB' cluster, and PF markers (*Irx3*, *Etv1*, *Sema3a*) was most highly expressed in the 'Purkinje fibers'. Additionally, the 'Proliferative CCS' cluster showed enrichment of genes related to the G2/M phase of the cell cycle (*Ube2c* and *Birc5*), indicating the presence of committed CCS cells undergoing proliferation after birth (**Figure 3.7b** and **Figure 3.12a**).

The 'Nodes-HIS-BB' cluster contained both atrial and ventricular cells, which highly expressed atrial chamber/conduction genes (*Myl4*⁺/*Myl7*⁺/*Tbx3*⁺) and ventricular conduction/chamber genes (*Myl2*⁺/*Myl3*⁺/*Gja5*⁺/*Irx3*⁺/*Scn5a*⁺/*Etv1*⁺), respectively (**Figure 3.7c** and **Figure 3.12a**). This allowed the distinction of the atrial 'Nodes' cluster from the ventricular 'LNB-HIS-PBB' cluster. The 'Nodes' cluster was further divided into the SAN (*Tbx3*⁺/*Shox2*⁺/*Gjd3*⁻) and cAVN (*Tbx3*⁺/*Shox2*/*Gjd3*⁺) (**Figure 3.7d** and **Figure 3.12a**). Within the 'LNB-HIS-PBB,' the LNB was identified by *Irx3*⁺/*Tbx3*⁺/*Gja5*⁻ and the HIS by *Irx3*⁺/*Tbx3*⁺/*Gja5*⁺. We found two populations of HIS, HIS1 (*Slit2*^{low}/*Gja1*⁻) and HIS2 (*Slit2*^{high}/*Gja1*⁺) (**Figure 3.7e**). For downstream analysis, the two HIS populations were combined.

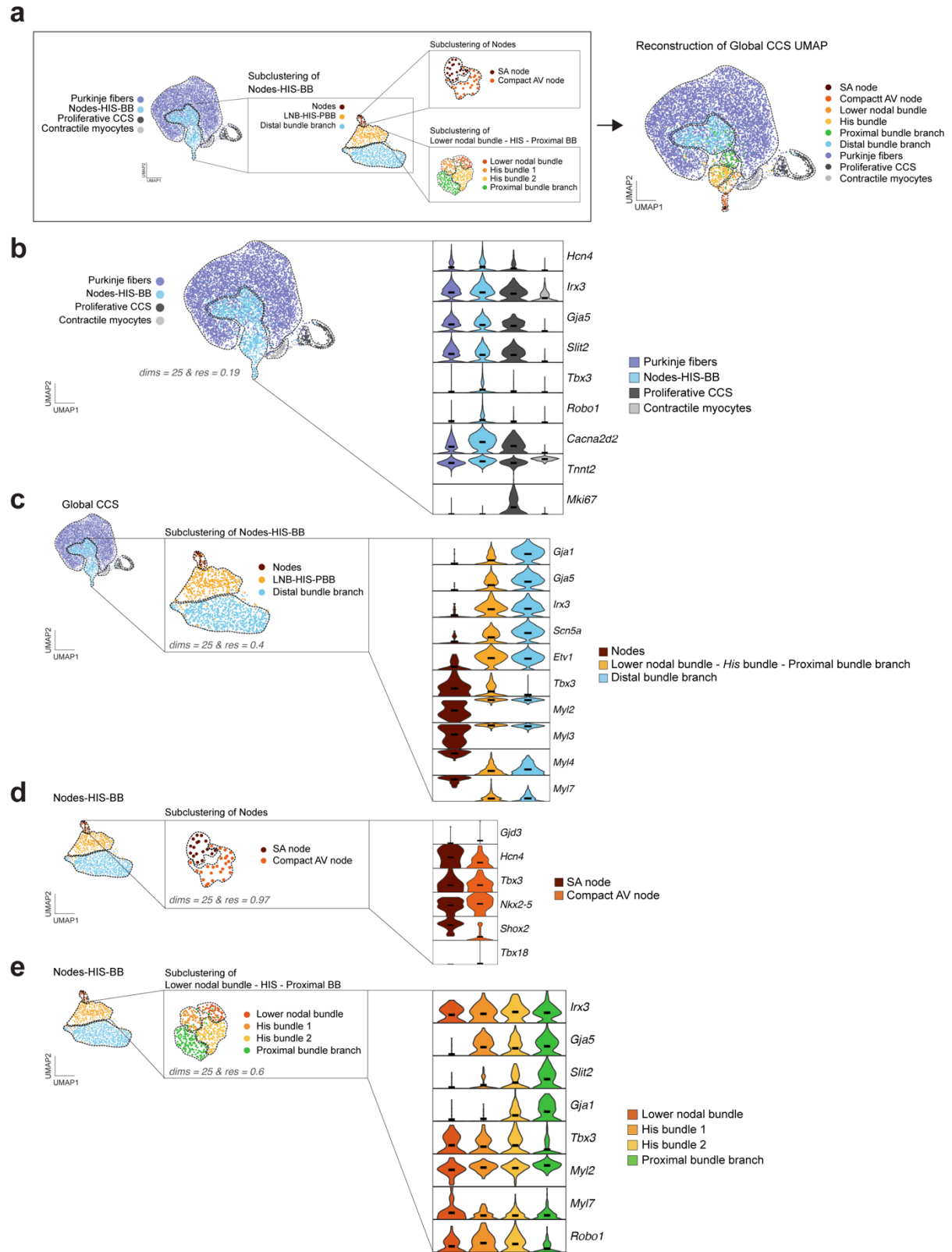


Figure 3.7 Identification of CCS components in scRNA-seq data through subclustering analysis. (a) Overall schematic showing the process of subclustering analyses and reconstruction of the global CCS UMAP. (b) Subclustering analysis of the global CCS identifies *Purkinje* fibers

(*Hcn4*⁺/*Tbx3*⁺/*Robo1*⁺), Nodes-HIS-BB (*Tbx3*⁺/*Cacna2d2*⁺/*Robo1*⁺), Proliferative CCS (*Hcn4*⁺/*Mki67*⁺) and Contractile myocytes (*Hcn4*⁺/*Tnnt2*^{high}). (c) Subclustering analysis of the Nodes-HIS-BB identifies the Nodes (*Hcn4*⁺/*Myl4*⁺/*Myl7*⁺/*Myl2*⁺/*Myl3*⁺/*Irx3*⁺/*Tbx3*⁺), LNB-HIS-PBB (*Gja1*^{low}/*Gja5*^{low}/*Scn5a*^{low}) and Distal Bundle Branch (*Gja1*^{high}/*Gja5*^{high}/*Scn5a*^{high}). (d) Subclustering analysis of the Nodes identifies the SAN (*Hcn4*^{high}/*Shox2*⁺) and Compact AV node (*Gjd3*⁺/*Shox2*⁺). (e) Subclustering analysis of the LNB-HIS-PBB identifies the Lower nodal bundle (*Irx3*⁺/*Gja5*⁺), His bundle 1 (*Irx3*⁺/*Gja5*⁺/*Slit2*^{low}/*Gja1*⁺/*Tbx3*⁺), His bundle 2 (*Irx3*⁺/*Gja5*⁺/*Slit2*^{high}/*Gja1*⁺/*Tbx3*⁺), and Proximal bundle branch (*Irx3*⁺/*Gja5*⁺/*Slit2*⁺/*Gja1*⁺/*Tbx3*⁺). His bundle 1 and 2 were combined for downstream analysis. LNB, lower nodal bundle; HIS, His bundle; BB, bundle branches; CCS, cardiac conduction system; dims, dimension; res, resolution.

Altogether, the identified subpopulations reconstructed in the UMAP of all sequenced cardiomyocytes, leading to the identification of CCS and 1 non-CCS populations - sinoatrial node (SAN), compact atrioventricular node (cAVN), lower nodal bundle (LNB), His bundle (HIS), proximal bundle branch (PBB) and distal bundle branch (DBB), Purkinje fibers (PF), proliferative CCS (Prolif. CCS) and contractile myocytes (CM) (**Figure 3.8**).

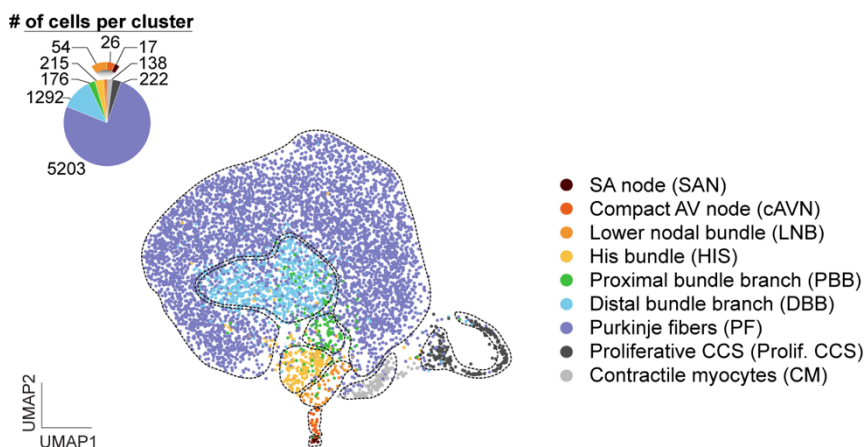


Figure 3.8 Clustering of scRNA-seq of CCS cells. Uniform Manifold Approximation and Projection (UMAP) of scRNA-seq showing 8 CCS and 1 non-CCS clusters. A pie chart (top, left) shows the number of cells in each cluster.

3.1.5 CCS components are enriched with neuronal pathways

Next, to associate differentially expressed genes of each CCS component with their functional implications, all statistically significant (adj. $P < 0.05$, $\text{Log}_2\text{FC} > 0.20$) genes enriched in each cluster compared to the rest were identified and subjected to gene

ontology analysis (**Figure 3.9**). Most CCS components showed enrichment of gene sets linked to neurons (*e.g.* neuronal cell body, neuron projection, presynaptic membrane and axon) as well as cardiomyocytes (*e.g.* actin cytoskeleton, muscle contraction, Z disc, striated muscle contraction, sarcomere organization). This corroborates previous findings, as genes enriched in the CCS are often associated with pathways linked to neuronal development²²⁶. Among CCS components, the LNB showed unique enrichment gene sets linked to structural components, such as focal adhesion, cell surface and sarcolemma, which reflects its unique structural properties connecting the atrial and ventricular chambers within the AVJ. The Prolif. CCS cluster displayed enrichment of nucleus- and cell cycle-related gene sets (*e.g.*, cell division, chromosome, cell cycle (mitosis) and nucleus). As anticipated, the CMs cluster was enriched with mitochondrion, the citric acid cycle/respiratory electron transport, oxidative phosphorylation, thermogenesis and metabolism. Collectively, our scRNA-seq analysis identified and annotated gross postnatal populations of the conduction system using established regional markers and previously observed functional characteristics.

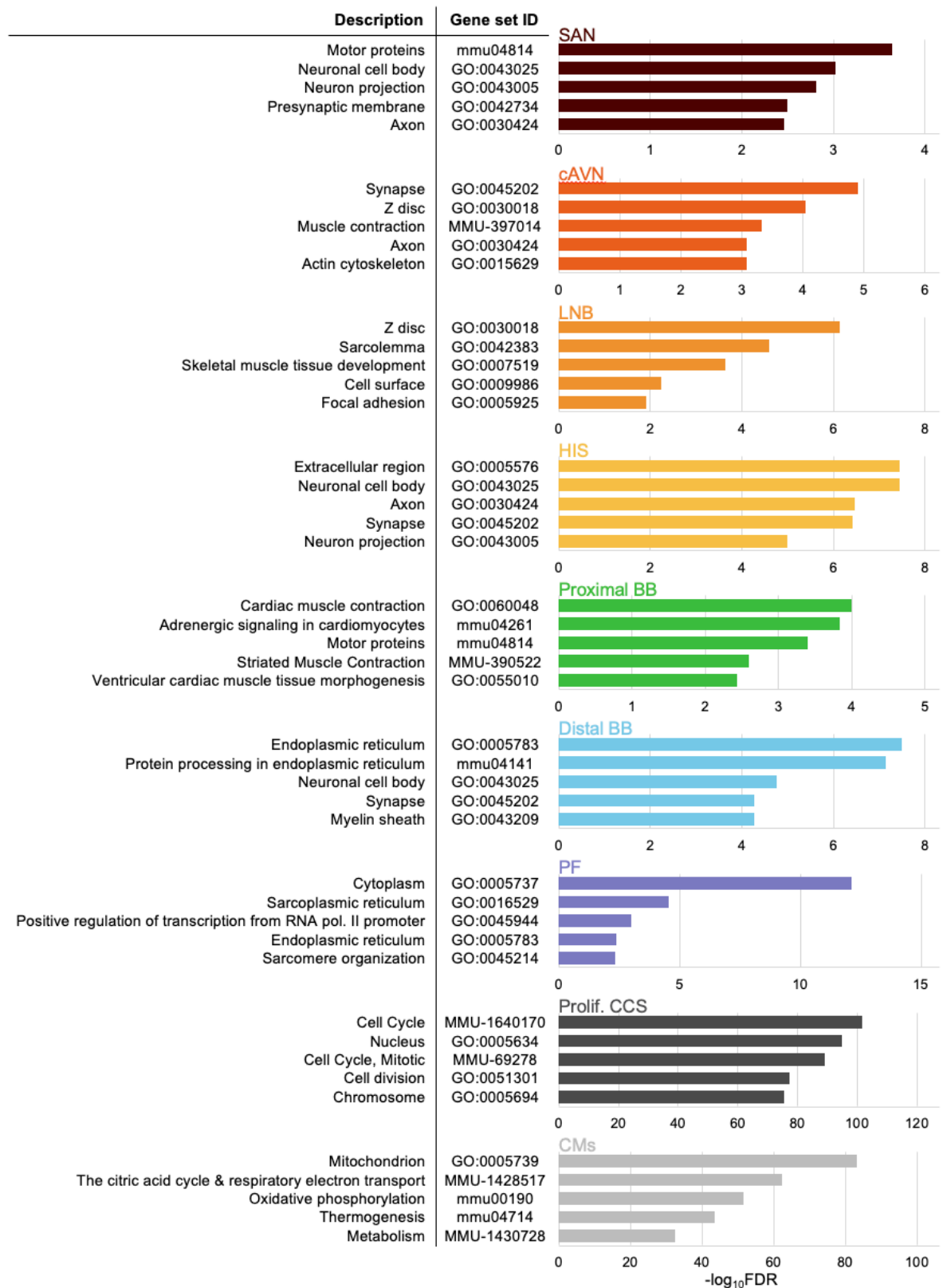


Figure 3.9 Gene ontology analysis of CCS clusters from scRNA-seq.

3.1.6 Spatial transcriptomics of postnatal CCS

In parallel, ST was performed on cryosections of the P4 CCS-tdT mouse heart using the GeoMx Digital Spatial Profiler (**Figure 3.1**). A segmentation approach allowed the precise capture of all tdTomato-positive cells in the CCS regions, such as SAN, cAVN, LNB, HIS, left and right proximal bundle branches (PLBB/PRBB), left and right distal bundle branches (DLBB/DRBB), left and right *Purkinje* fibers (LPF/RPF) (**Figure 3.10**). Of note, our ST analysis included two sub-compartments of the AVN^{36,38,46}, cAVN and LNB, which are anatomically situated adjacent to each other within the atrioventricular junction (**Figure 3.13**). As described previously^{36,38,46}, trichrome staining of the atrioventricular region showed that the LNB was surrounded by insulating fibrous tissue and basally located on the plane of atrioventricular insulation, in comparison to the apically located HIS. Additionally, non-CCS regions adjacent to the selected CCS components, including the right atrial appendage (RAA), basal and apical interventricular septum (IVS-B, IVS-A), left ventricular endomyocardium (LEND), left ventricular epimyocardium (LEPI), and right ventricle (RV) were collected for comparison (**Figure 3.10**).

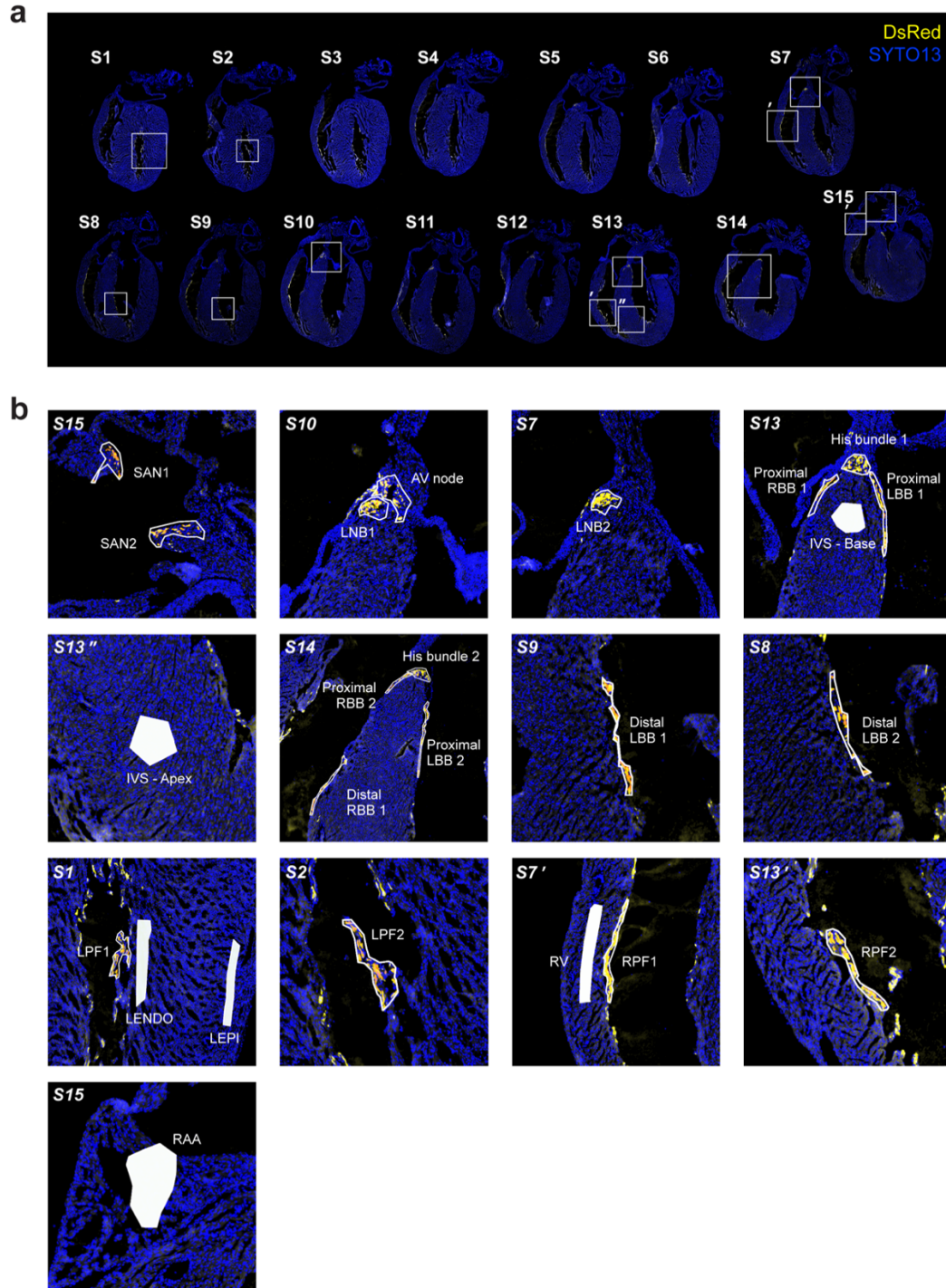


Figure 3.10 CCS regions selected for spatial transcriptomics. (a) A scanned fluorescent image of the single slide containing continuous cryosections of P4 CCS-tdT reporter hearts, encompassing all CCS components. Section 1 (S1) through 15 (S15) were collected from the same heart, ranging from posterior (S1) to anterior (S15) regions. tdTomato was stained with anti-DsRed antibody to amplify the signal for capture (yellow) and nuclei with SYTO13 (blue). **(b)** Zoomed images of CCS regions captured by segmentation for spatial transcriptomics data of the study. SAN, sinoatrial node; LNB, lower nodal bundle; LBB/RBB, left/right bundle branch; IVS, interventricular septum; LPF/RPF, left/right *Purkinje* fiber; RAA, right atrial appendage; RV, right ventricle; LENDO, left ventricular endomyocardium; LEPI, left ventricular epimyocardium.

3.1.7 Principal component analysis of CCS components profiled by spatial transcriptomics

Among the 12,638 transcripts detected in ST, differentially expressed genes (4,708 transcripts) showing $|FC| > 3$ and $P < 0.05$ in any possible comparison of two CCS components, were used to perform principal component analysis (PCA). PCA of ST data demonstrated that CCS components exhibited a globally linear clustering pattern, reflecting the sequence of cardiac electrical activation, from impulse generation in SAN to propagation through the VCS. 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) (Figure 3.11).

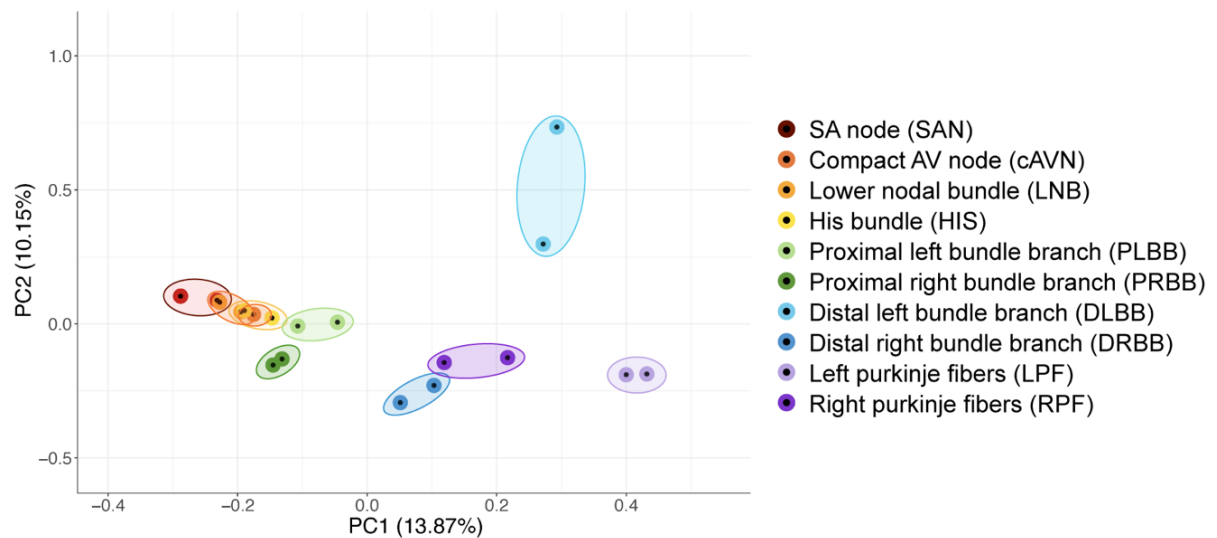


Figure 3.11 Principal component analysis (PCA) of the CCS and non-CCS regions profiled by spatial transcriptomics.

3.1.8 Expression profiles of established CCS and non-CCS marker genes

In both scRNA-seq and ST analyses, all CCS regions highly expressed known pan-CCS markers (*Hcn4*, *Cpne5* and *Cacna2d2*)^{64,145,223,226}, whereas the non-CCS regions, such as the ‘Contractile myocytes’ cluster in scRNA-seq, and LENDO, LEPI, IVS-B, IVS-A and RV in ST, expressed higher levels of contraction genes (*Tnnt2*, *Tnni1*, *Mb*) with low expression of conduction genes (**Figure 3.12**). Also, both scRNA-seq and ST results corroborate the expression patterns of the known regional CCS markers, including *Shox2* in the SAN^{148,227}; *Tbx3* in the SAN, AVN, and HIS^{168,187}; *Irx3* and *Gja5* in the VCS^{50,53}; *Robo1* in the proximal VCS^{224,225}; *Slit2* and *Sema3a* in the distal VCS^{64,224}; *Gja1* in distal VCS and CM²²⁸; as well as atrial (*Myl4*, *Myl7*) and ventricular (*Myl2*, *Myl3*) sarcomere genes. Based on these observations, we characterized the CCS components as follows: SAN

(*Myl2*^{low}/*Myl3*^{low}/*Myl4*^{high}/*Myl7*^{high}/*Hcn4*^{high}/*Tbx3*^{high}/*Nkx2.5*⁺/*Shox2*^{high}/*Irx3*/*Robo1*⁻); cAVN(*Myl2*^{mid}/*Myl3*^{mid}/*Myl4*^{high}/*Myl7*^{high}/*Hcn4*^{high}/*Tbx3*^{high}/*Nkx2.5*⁺/*Robo1*^{low}/*Shox2*^{low}); LNB (*Myl2*^{high}/*Myl3*^{high}/*Myl4*^{low}/*Myl7*^{low}/*Hcn4*⁺/*Gja5*^{low}/*Gja1*/*Irx3*⁺/*Tbx3*⁺); HIS and PBB (*Myl2*⁺/*Myl3*⁺/*Myl4*⁻/*Myl7*⁻/*Irx3*⁺/*Tbx3*⁺/*Robo1*⁺); DBB and PF (*Myl2*⁺/*Myl3*⁺/*Myl4*⁻/*Myl7*⁻/*Irx3*⁺/*Gja5*⁺/*Gja1*⁺/*Slit2*⁺/*Sema3a*⁺); VCS (*Myl2*⁺/*Myl3*⁺/*Myl4*⁻/*Myl7*⁻/*Irx3*⁺/*Gja5*⁺/*Sema3a*⁺/*Slit2*⁺) and non-CCS cardiomyocytes (*Hcn4*/*Cpne5*/*Cacna2d2*/*Gja5*⁻/*Sema3a*⁻/*Tnnt2*^{high}/*Tnni1*^{high}/*Mb*^{high}).

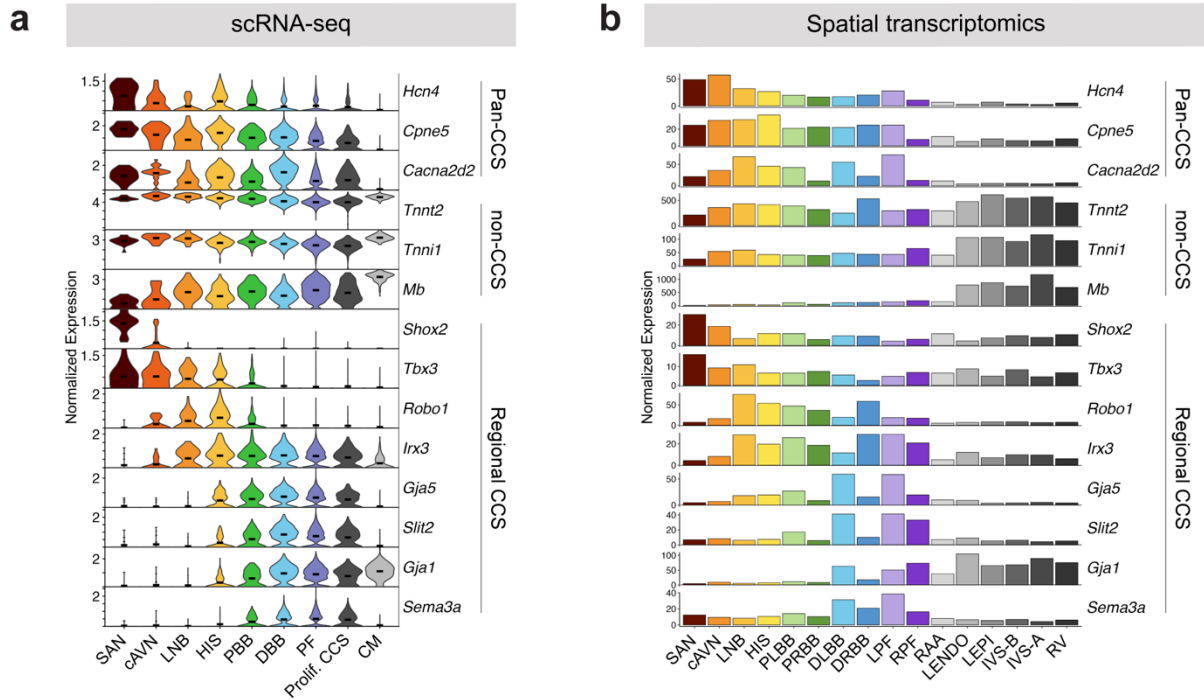


Figure 3.12 Expression profiles of established CCS and non-CCS marker genes in each cluster of (a) scRNA-seq and (b) spatial transcriptomics data. SAN, sinoatrial node; cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, *His* bundle; PBB, proximal bundle branch; PLBB, proximal left bundle branch; PRBB, proximal right bundle branch; DBB, distal bundle branch; DLBB, distal left bundle branch; DRBB, distal right bundle branch; PF, *Purkinje* fibers; RPF, right *Purkinje* fibers; LRF, left *Purkinje* fibers; RAA, right atrial appendage; LENDO, left ventricular endo-myocardium; LEPI, left ventricular epi-myocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

3.1.9 Correlation analysis between CCS components from scRNA-seq and spatial transcriptomics

Next, I conducted a comparison between the transcriptomic profiles of corresponding regions detected by both technologies (**Figure 3.13**). Correlation analysis showed strong concordances between scRNA-seq and ST datasets within nodal cells (SAN and cAVN); LNB and proximal VCS (HIS and PBB); and distal VCS (DBB and PF) components. Also, all ventricular non-CCS components in ST (LEND, LEPI, IVS-B, IVS-A and RV) were highly correlated to the CM cluster in scRNA-seq, while the RAA in ST showed high similarity to the SAN and cAVN in scRNA-seq. Collectively, these results suggest that our scRNA-seq and ST methods credibly captured all CCS and non-CCS regions.

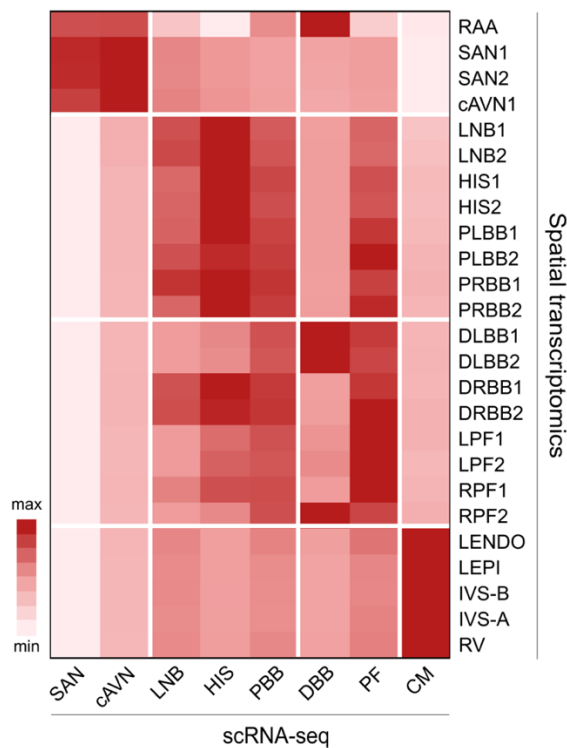
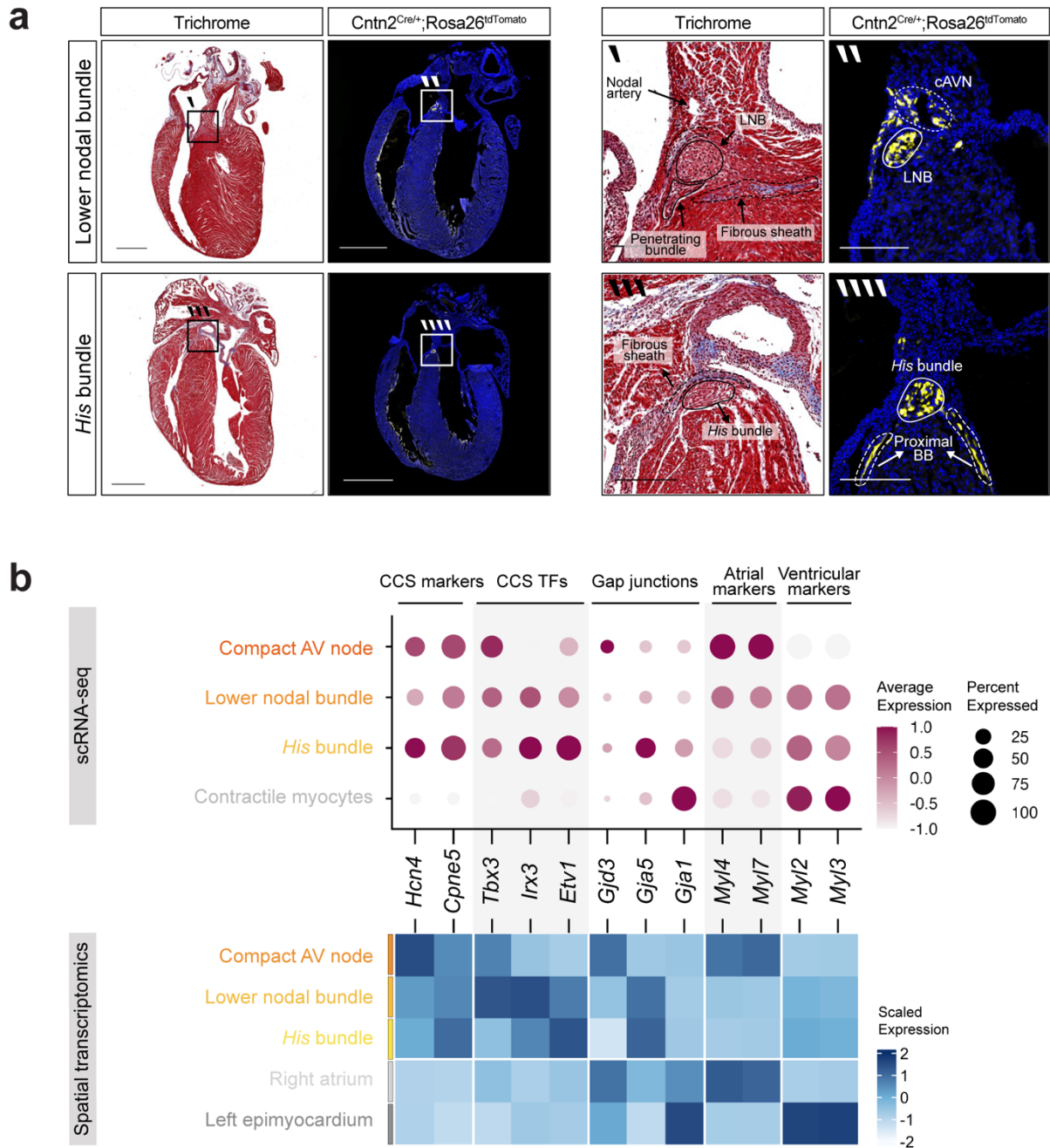


Figure 3.13 Correlation analysis of CCS components between scRNA-seq and spatial transcriptomics data. SAN, sinoatrial node; cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, *His* bundle; PBB, proximal bundle branch; PLBB, proximal left bundle branch; PRBB, proximal right bundle branch; DBB, distal bundle branch; DLBB, distal left bundle branch; DRBB, distal right bundle branch; PF, *Purkinje* fibers; RPF, right *Purkinje* fibers; LPF, left *Purkinje* fibers; RAA, right atrial appendage; LENDO, left ventricular endo-myocardium; LEPI, left ventricular epi-myocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

3.1.10 Histological and molecular identification of the lower nodal bundle, compact AV node and *His* bundle

Among the components of the CCS, the identification of the LNB remains ambiguous, possibly due to its anatomical proximity to the cAVN. To ensure the correct collection of the LNB for spatial transcriptomics, we examined their histological features through Masson's trichrome staining. Consistent with the literature²²⁹, the LNB was encased in a fibrous sheath while the cAVN was penetrated by the suspected atrioventricular node artery (**Figure 3.14a**). The HIS was situated adjacent to a longitudinally oriented collagen structure²³⁰. Despite the anatomical proximity between the cAVN and LNB, a direct

molecular comparison between the cAVN, LNB and HIS revealed that the LNB expressed ventricular genes (*MyI2*, *MyI3*) and VCS markers (*Irx3*, *Etv1*, *Gja5*), which are characteristics more similar to HIS than to cAVN (**Figure 3.14b**). This is consistent with previous studies demonstrating that the LNB is structurally continuous with the HIS and is derived from a ventricular lineage^{36,38,41,46}. Altogether, this confirms the correct capture of the cAVN, LNB and HIS.



3.1.11 Molecular profiling of the proliferative CCS

Our scRNA-seq showed a cluster of cells (222 out of 6,855 cells, 3.24%) annotated as 'Proliferative CCS' (Prolif. CCS), as these cells expressed CCS genes (*Cntn2*, *Etv1*, *Irx3*, *Gja5*) and were also enriched in genes involved in G1/S phase and G2/M phase (**Figure 3.15a**). Further subclustering of the Prolif. CCS cluster led to 3 clusters (**Figure 3.15b**), each of which contained comparable proportions of P1, P2 and P4 cells (**Figure 3.15c-d**). Each subcluster also contained a combination of G1, G2/M and S phase cells (**Figure 3.15e-f**) and was enriched with G1/S transition genes (*Mcm4* and *Mcm5*), G2/M genes (*Fam83d* and *Cdc25b*), and G2/M - G1/S transition genes, such as (*Cdkn3* and *Ccnb2*) (**Figure 3.15g-i**). This suggests the presence of committed conduction cells undergoing proliferation in early postnatal heart.

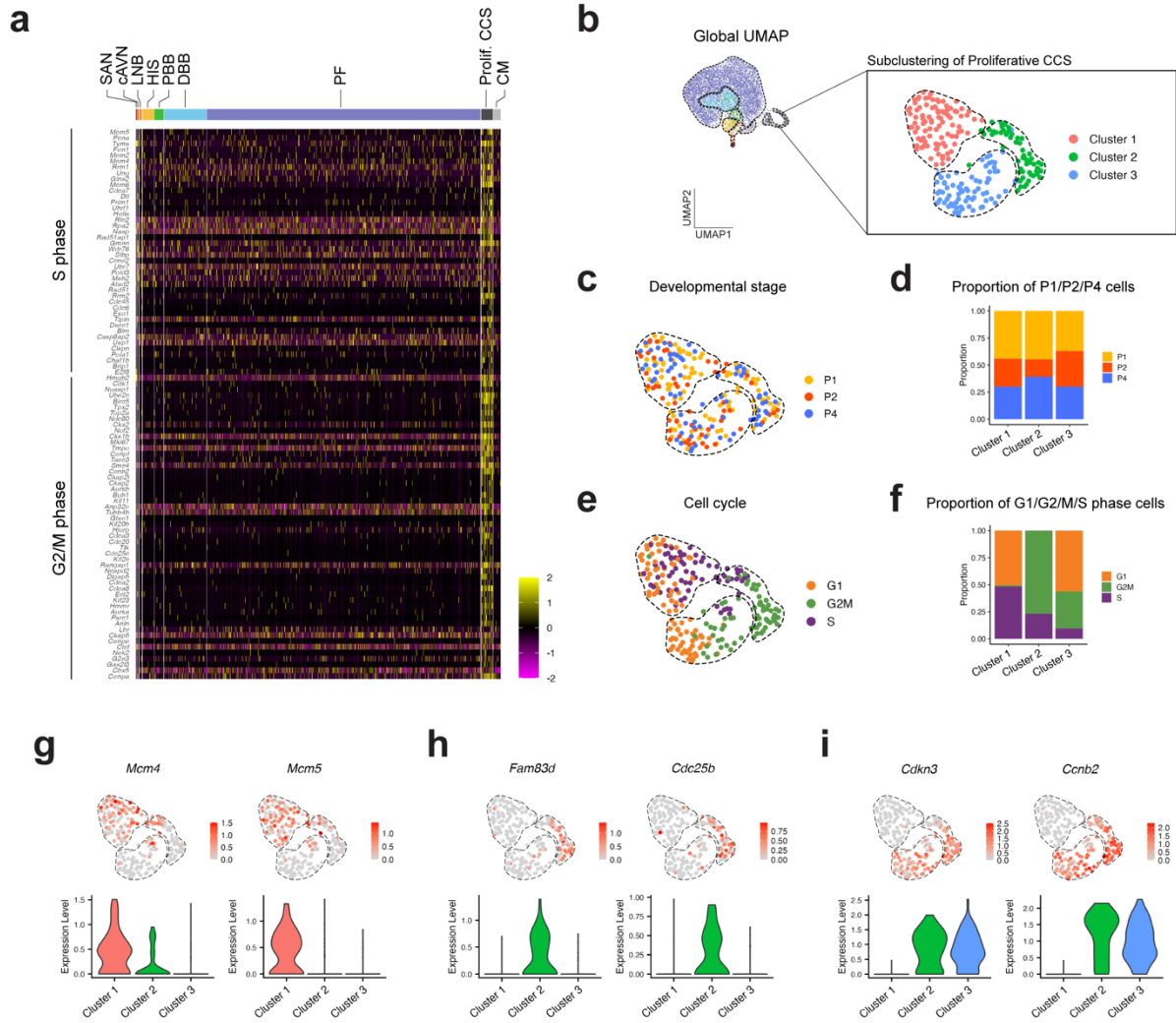


Figure 3.15 Postnatal CCS contains proliferative CCS (Prolif. CCS) cells. (a) Heatmap showing specific enrichment of S phase and G2/M phase cell cycle genes in the Proliferative CCS cluster. (b) Subclustering analysis of the 'Proliferative CCS' cluster leads to three subclusters. (c-d) Each subcluster contains a comparable number of P1, P2 and P4 cells. (e-f) Each subcluster contains a combination of cells with G1, G2/M, and S phase scores. (g) Cluster 1 is enriched with G1/S transition genes, *Mcm4* and *Mcm5*. (h) Cluster 2 with G2/M phase markers, *Fam83d* and *Cdc25b*. (i) Cluster 3 with *Cdkn3* and *Ccnb2*.

3.1.12 Comparison between CCS of postnatal day 1, 2 and 4

Studies have shown unique CCS responses to mutated genes at different postnatal stages. For instance, mice lacking *Ir33*⁵⁰, *Tbx3*¹⁶³ or *Nkx2-5*¹⁶¹ show abnormal phenotypes at early postnatal stages, but not in P0, suggesting there may be dynamic transcriptomic changes that occur postnatally. Therefore, leveraging our P1, P2 and P4 MULTI-seq data, we examined these transcriptomic changes during early postnatal development in the CCS.

Unlike our expectation, P1, P2 and P4 cells were found homogeneously distributed in the compiled UMAP (**Figure 3.16a, c**). Moreover, no differences in the proportion of P1, P2 and P4 cells per cluster, or in clusters per development stage, were observed (**Figure 3.16b, d**).

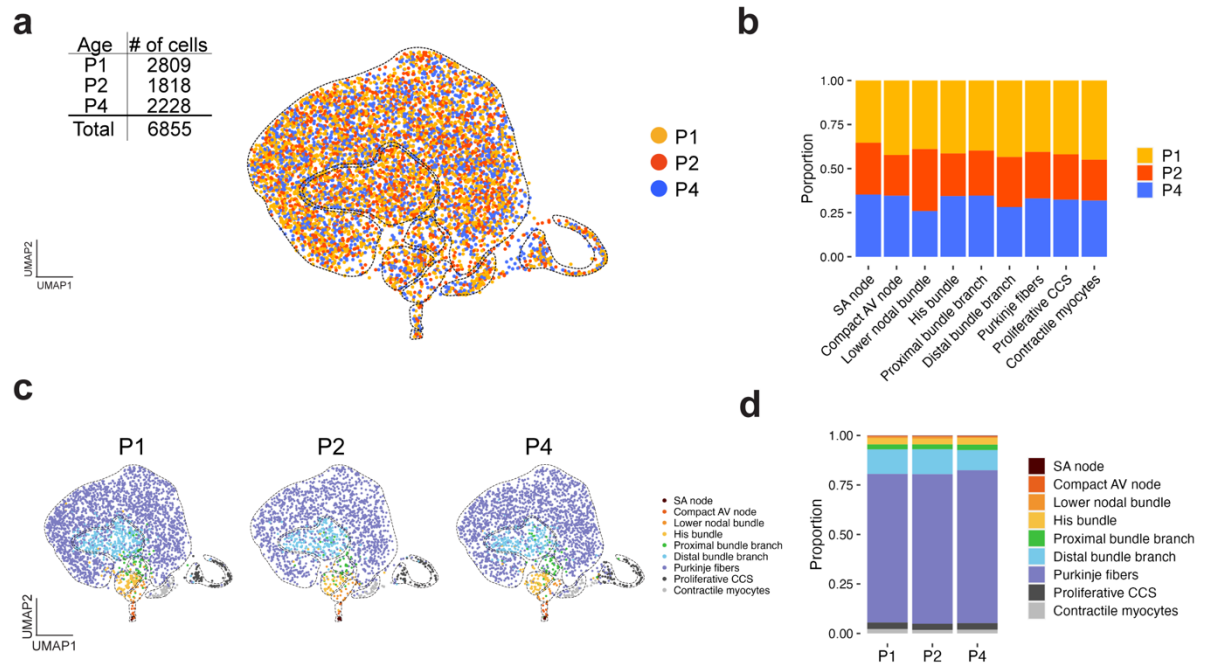


Figure 3.16 CCS cells undergo minimal transcriptomic changes during early postnatal development. **a** UMAP of CCS cells labelled by developmental stages (P1, P2 and P4) and the number of P1, P2 and P4 CCS cells sequenced. **b** Stacked bar graphs showing that all CCS components contain comparable proportions of P1, P2 and P4 cells. **c** Individual UMAPs for P1, P2 and P4 CCS cells. **d** Stacked bar graphs showing different developmental stages contain comparable proportions of each CCS cluster. **e-f** Expression levels of (**e**) conductive genes (*Cntn2*, *Cpne5*, *Cacna2d2*) and (**f**) cardiac maturation markers (*Myh6*, *Myh7*, mitochondrial content) are comparable in P1, P2 and P4 in all clusters.

Furthermore, no significant changes in gene expression were found in all clusters, including the commonly known molecular signatures for conduction (*Cpne5*, *Hcn4* and *Cacna2d2*), cardiomyocyte maturation (a transition in myosin heavy chain isoforms from *Myh6* to *Myh7*) and an increase in mitochondrial gene content (**Figure 3.17**).

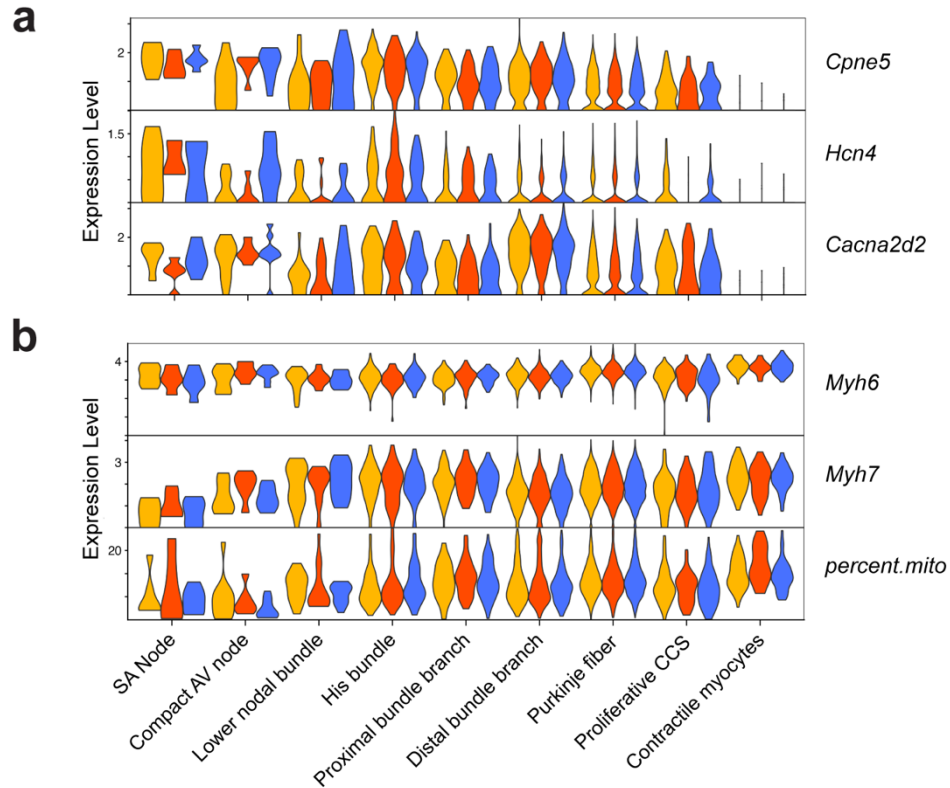


Figure 3.17 Expression levels of (a) conduction genes (*Cntn2*, *Cpne5*, *Cacna2d2*) and (b) cardiac maturation markers (*Myh6*, *Myh7*, mitochondrial content) are comparable in P1, P2 and P4 in all clusters.

3.2 Transcriptional profiling of the postnatal CCS regional components

3.2.1 CCS-specific enriched genes for each CCS component

Next, I sought to identify regional CCS markers by integrating scRNA-seq and ST data.

First, enriched genes of each CCS region were identified from scRNA-seq and ST datasets separately, by comparing each CCS region to other CCS and non-CCS clusters/regions ($|\text{FC}| > 1.5$, $P < 0.05$) (**Figure 3.18**). Subsequently, genes that were identified as common between scRNA-seq and ST were considered specific regional markers with high confidence.

In the SAN, as expected, *Shox2*^{148,227} and *Tbx3*^{168,187}, well-known transcription factors in SAN development, were observed. In addition, we identified other potential markers, including *Gnao1*, which encodes G protein subunit alpha O1 and has been found to be involved in heart development²³¹, 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^{232,233}, as well as *Cacna1d*, which encodes Ca_v1.3 channel known to regulate AVN automaticity²³⁴. Newly identified markers of the cAVN included *Prdm6*, which encodes PR/SET domain 6 and has been shown to be expressed in the developing cardiovascular²³⁵⁻²³⁷ and nervous systems²³⁸. Interestingly, a genetic variant of *Prdm6* has been reported to be associated with heart rate recovery during exercise²³⁹. 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 stages²⁴⁰. Like many other cardiac conduction genes, *Ppp1r17* is also expressed in neurons²⁴¹, 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 VCS²⁴² with critical roles in cardiac electrophysiology, and *Cdh6* is known to be enriched in the CCS compared to non-CCS cardiomyocytes²⁴³. 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^{244,245}, is known to be expressed in embryonic trabecular cardiomyocytes^{246,247}, 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 ventricles²³³, although its functional involvement in the CCS has not yet been described.

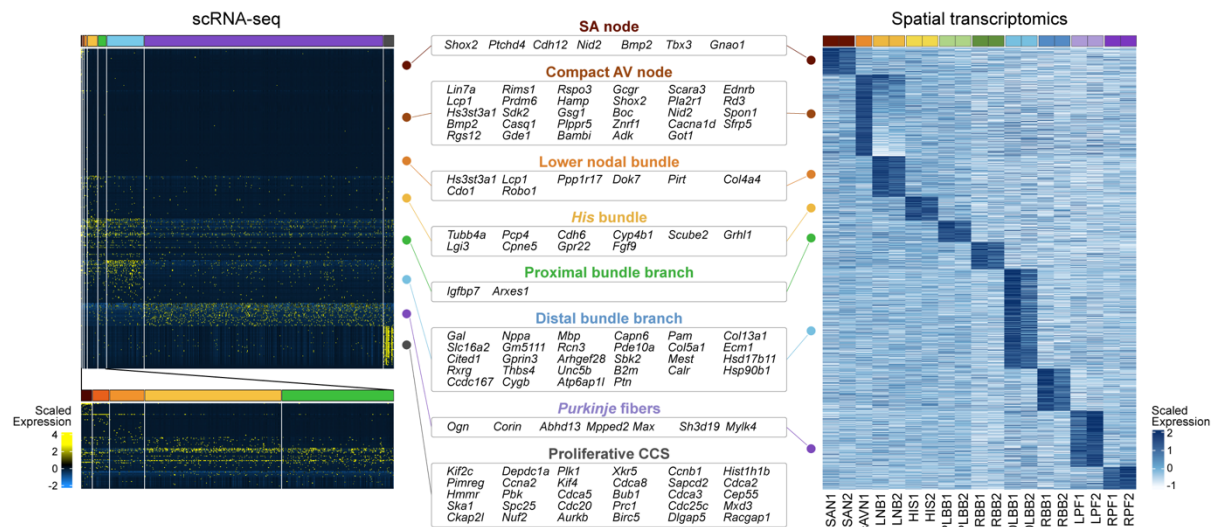


Figure 3.18 Integration of scRNA-seq and spatial transcriptomic data of the CCS. CCS-specific enriched genes for each CCS component identified by both scRNA-seq and spatial transcriptomics. SAN, sinoatrial node; cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, *His* bundle; PBB, proximal bundle branch; PLBB, proximal left bundle branch; PRBB, proximal right bundle branch; DBB, distal bundle branch; DLBB, distal left bundle branch; DRBB, distal right bundle branch; PF, *Purkinje* fibers; RPF, right *Purkinje* fibers; LPF, left *Purkinje* fibers; RAA, right atrial appendage; LENDO, left ventricular endo-myocardium; LEPI, left ventricular epi-myocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

3.2.2 CCS-specific enriched genes for zones within the CCS

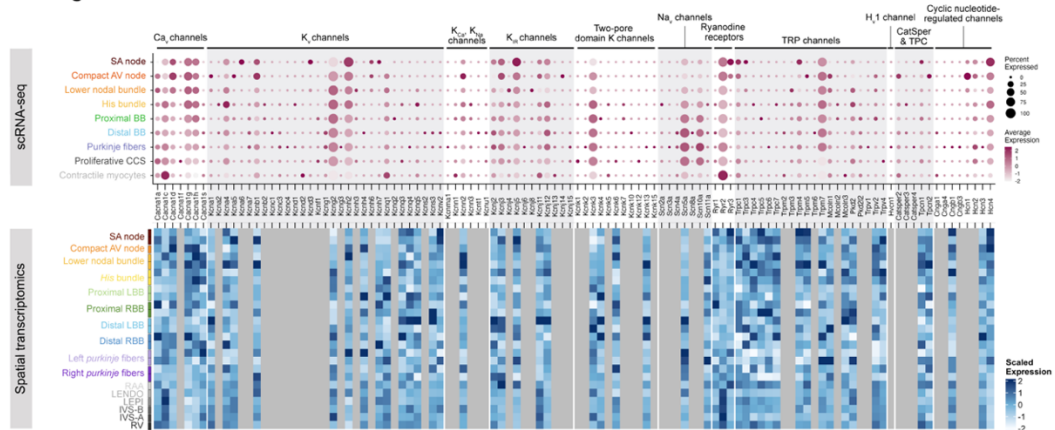
The CCS is an anatomically continuous structure with a highly specialized function of generating and propagating electrical signals. Thus, while the integrated analytic approach above effectively uncovers the enrichment of gene expression in each individual CCS component, it may not be effective in capturing the gradual changes in gene expression along the CCS, in which case, genes would be enriched in more than one defined component of the CCS. Therefore, according to the PCA (**Figure 3.11**) and the correlation

bundle & Proximal VCS (*His* bundle, Proximal bundle branch), and Distal VCS (Distal bundle branch, *Purkinje* fibers). LBB, left bundle branch; RBB, right bundle branch; RAA, right atrial appendage; LENDO, left ventricular endo-myocardium; LEPI, left ventricular epi-myocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle; VCS, ventricular conduction system.

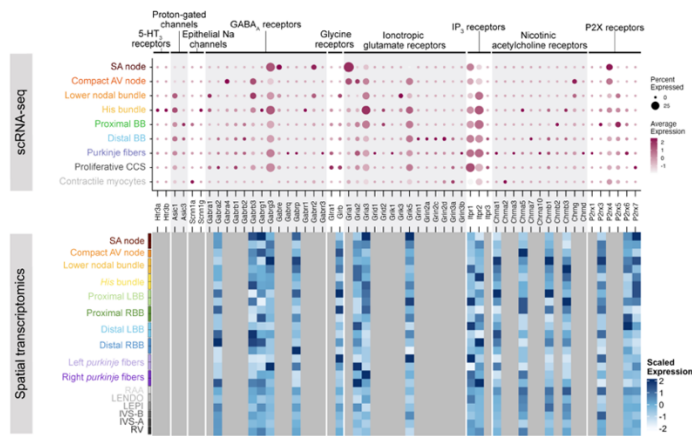
3.2.3 Ion channel profiling of the CCS

To further gain molecular insights into the distinctive physiological and electrophysiological properties of each CCS component, including automaticity and conduction velocity, we profiled the expression patterns of genes functionally associated with ion channels, receptors and gap junctions and using our scRNA-seq and ST datasets (**Figure 3.20**). For example, *Kcnj3* and *Kcnj5*, which form the acetylcholine-activated potassium channel (I_{KACH}) *Kir3.1* and *Kir3.4*, respectively, and are associated with bradyarrhythmia and sporadic atrial fibrillation²⁵⁸, as well as *Cacna1g* and *Cacna1h*, which encode $Ca_v3.1$ and $Ca_v3.2$, respectively, known to contribute to pacemaker activity²⁵⁹, were highly expressed in the CCS with strong enrichment in SAN and cAVN. Also, consistent with a previous study²⁶⁰, *Kcnk3*, encoding TASK-1 channel, showed enrichment in the VCS. It was notable that *Kcnj12*, encoding *Kir2.2* responsible for cardiac inwardly rectifying potassium current (I_{K1}), *Gria3* encoding glutamate ionotropic receptor, *Grik5* encoding glutamate ionotropic receptor kainite, and *Ank3*, encoding a cytoskeletal element ankyrin-G that regulates cardiac excitability²⁶¹, were found to be enriched in the CCS compared to contractile myocytes. However, their functional implications in the CCS are not well understood.

a Voltage Gated Ion Channels



b Ligand Gated Ion Channels



c Other Ion Channels

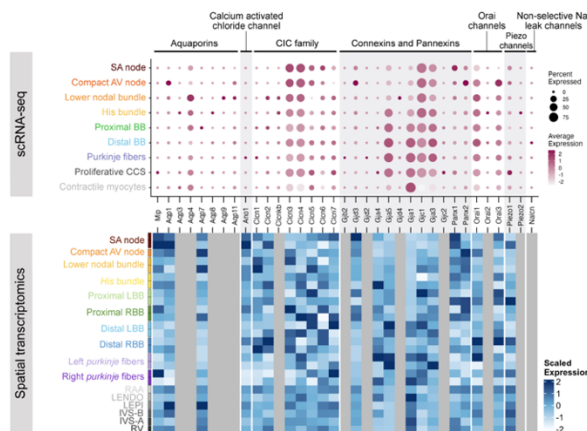


Figure 3.20 Ion channel profiling within the CCS using scRNA-seq and spatial transcriptomics. Expression patterns of genes annotated with (a) voltage-gated ion channels, (b) ligand-gated ion channels, and (c) other ion channels. Gene lists obtained from IUPHAR/BPS Guide to PHARMACOLOGY. LBB/RBB, left/right bundle branch; RAA, right atrial appendage; LENDO, left ventricular endomyocardium; LEPI, left ventricular epimyocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

We have also found several CCS genes identified from scRNA-seq that were not detected in ST, likely due to the limitation of the probe-based approach used in ST. Among those genes, we noted strong and CCS-specific expression of *Scn10a* (Nav1.8), known to modulate *Scn5a*-encoded Nav1.5 currents²⁶², in the PF. In line with a recent study showing that only a short form of *Scn10a* mRNA is expressed in the VCS²⁶², we observed a predominant expression of the short *Scn10a* transcript in the postnatal CCS. Mapping sequenced reads to the *Scn10a* gene in our scRNA-seq data revealed higher sequencing coverage in the last seven exons of the gene (exons 22 to 28), compared to exons 1 to 21 (**Figure 3.21**), further supporting the dominant expression of the short *Scn10a* transcript.

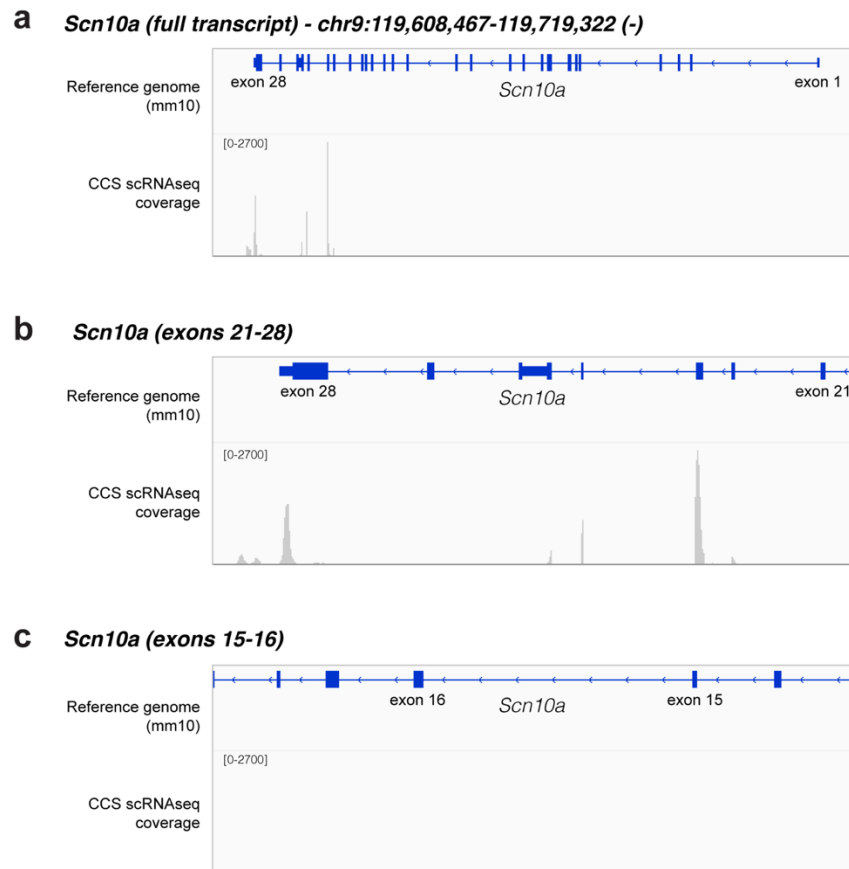
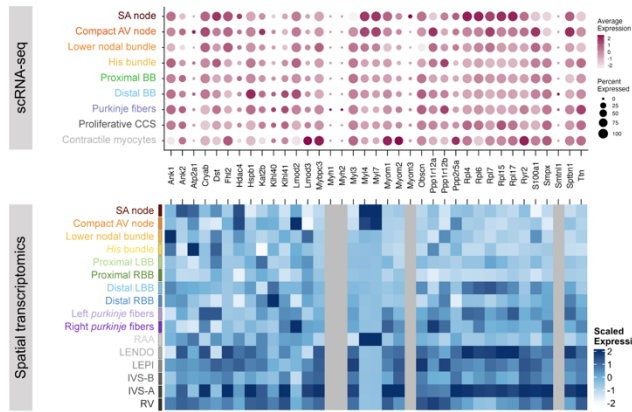


Figure 3.21 scRNA-seq of mouse postnatal CCS captures predominantly the short form of *Scn10a*. Alignment of scRNA-seq reads to (a) full, (b) short form and (c) long form of *Scn10a* transcript, shows high coverage of regions spanning exons 21-28 of *Scn10a* (short form of *Scn10a* transcript) than the rest of the transcript. mm10 was used as the mouse reference genome. Integrative genomics Viewer (IGV, v2.17.4) was used for reads alignment.

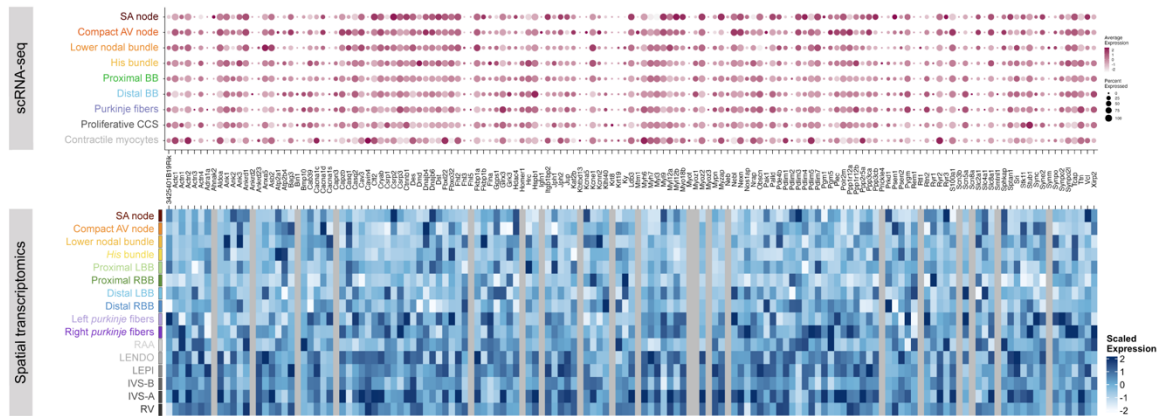
3.2.4 Sarcomeric gene profiling of the CCS

In addition to ion channel genes, the expression of sarcomeric genes within the CCS was examined (**Figure 3.22**). As expected, well-known sarcomere genes, such as *Ttn*, *Myh6*, *Mybpc3*, *Actc1*, *Myl12a*, *Myom1*, *Ryr2*, *Tnni1*, *Tnni3*, *Actn2*, and *Tnnc1*, showed the highest expression in contractile cardiomyocytes, although were still abundantly expressed in all CCS components. Ventricular genes, *Myl3* and *Myh7*, were enriched in the VCS components as well as the contractile cardiomyocytes, while atrial genes, *Myl4*, *Myl7* and *Myl9*, were enriched in the SA and AV nodes, aligning with their different developmental origins and functional implications of CCS components. Among CCS-specific genes (*i.e.*, those with low expression in contractile cells), *Synpo2* (Synaptopodin 2 localized in z-disk structure) and *Fbxo32* (F-box protein 32, cardiac ubiquitin ligase) were expressed globally in the CCS; *Actn1* (alpha-actinin 1) and *Tpm2* (Tropomyosin 2) were enriched in the SA node; *Casq1* (Calsequestrin-1, associated with heart rate regulation and arrhythmia)²⁶³ and *Sphkap* (SPHK1 interactor, AKAP domain containing) were enriched in the AV node; *Myoz1* (Myozenin 1), *Jph1* (Junctophilin 1), *Capn3* (Calpain 3), *Grk3* G protein-coupled receptor kinase-3), *Atp2b4* (ATPase plasma membrane Ca²⁺ transporting 4), and *Ptroxd1* (Oxidoreductase) were enriched in VCS components. *Neb* (Nebulin), *Mypn* (Myopalladin) and *Klhl40* (kelch-like 40) were found to be enriched in the *Purkinje* fibers and bundle branches. Since some ion channel genes and most of these sarcomeric genes have not been linked to a conduction defect, it is important to determine whether they play a functionally significant role in the CCS.

a A band (GO:0031672)



b I band (GO:0031674)



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

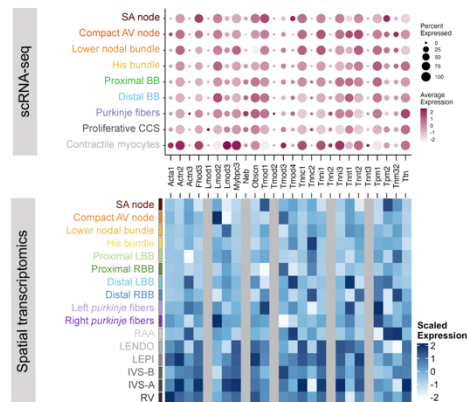


Figure 3.22 Sarcomeric gene profiling within the CCS using scRNA-seq and spatial transcriptomics. Expression patterns of genes annotated with (a) A band (GO:0031672), (b) I band (GO:0031674), and (c) striated muscle thin (GO:0005863) and thick filament (GO:0005865). LBB/RBB, left/right bundle branch; RAA, right atrial appendage; LENDO, left ventricular endomyocardium; LEPI, left ventricular epimyocardium; IVS-B, interventricular septum (Base); IVS-A, interventricular septum (Apex); RV, right ventricle.

3.3 Histological assessments of marker genes validate molecular heterogeneity of the CCS

We next validated the identified CCS markers by performing immunostaining and fluorescence *in situ* hybridization (FISH) in the P4 CCS-tdT mouse heart.

3.3.1 Histological identification of the SAN using Shox2 and Vsnl1

The SAN was identified using a well-established SAN marker, *Shox2* (**Figure 3.23**), as well as a recently discovered marker, *Vsnl1*²⁰⁰ (**Figure 3.24**), both of which were also identified in our integrated analysis.

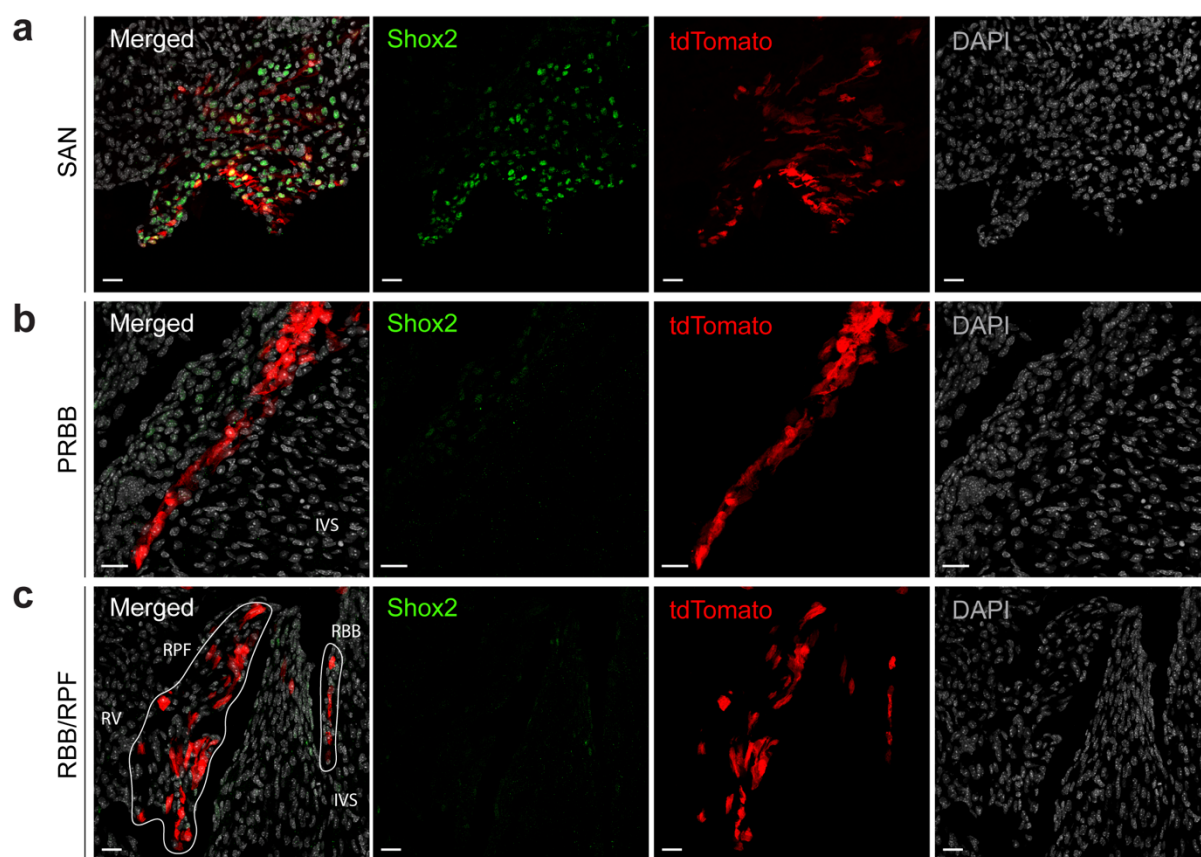


Figure 3.23 Immunofluorescence staining of *Shox2* in a P4 CCS reporter heart allows identification of the SAN. *Shox2* is expressed in the (a) SAN, but not in other CCS components, such as (b) proximal RBB and (c) RBB/RPF. SAN, sinoatrial node; PLBB/PRBB, proximal left/right bundle branch; RBB, right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 20 μ m.

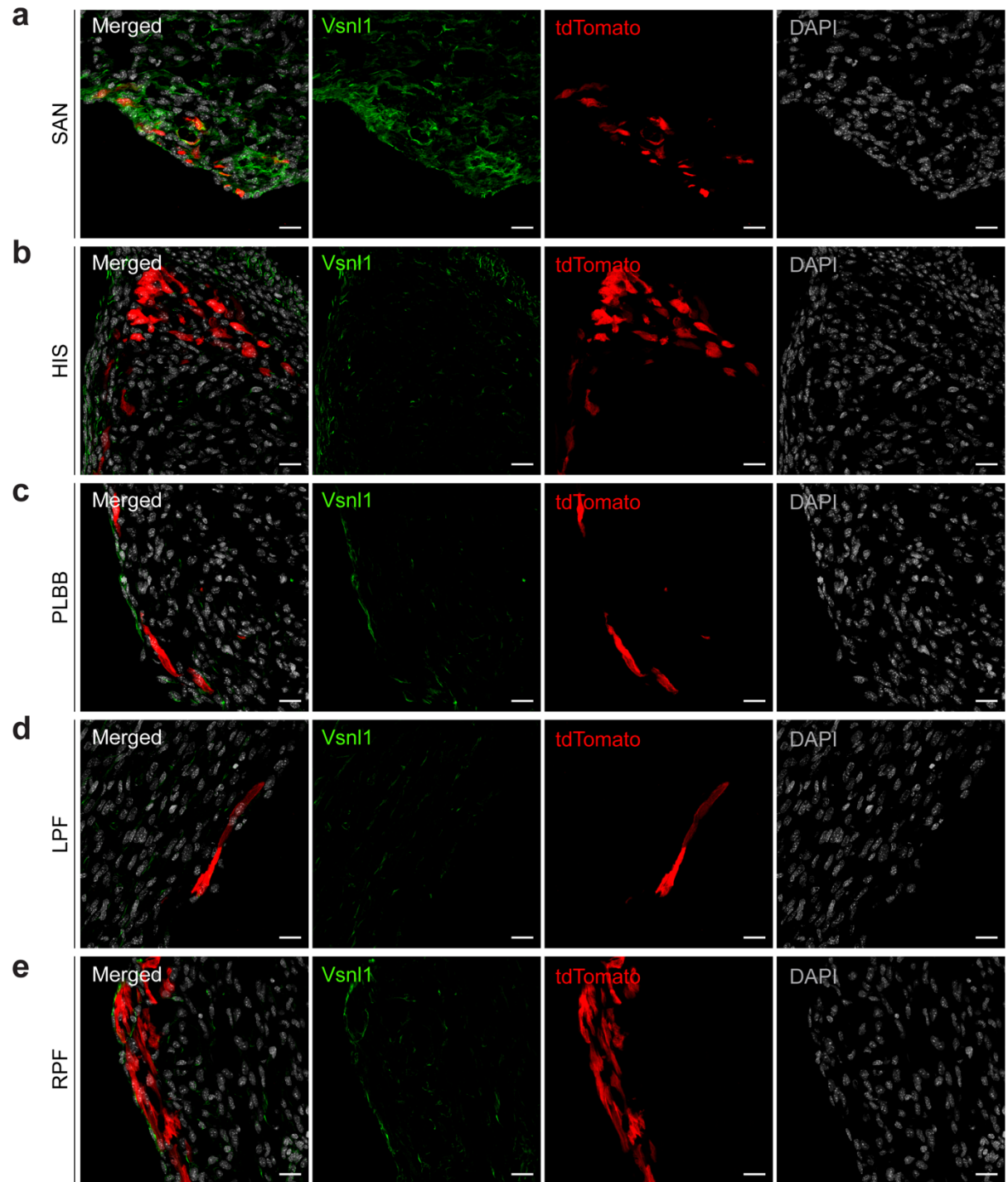


Figure 3.24 Immunofluorescence staining of Vsnl1 in a P4 CCS reporter heart allows identification of the SAN. Vsnl1 is expressed in the (a) SAN, but not in other CCS components, such as (b) HIS, (c) proximal LBB, (d) LPF and (e) RPF. SAN, sinoatrial node; PLBB/PRBB, proximal left/right bundle branch; RBB, right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 20 μ m.

3.3.2 Immunofluorescence staining of Gnao1

Consistent with scRNA-seq and spatial transcriptomic analyses, an SAN-enriched marker, Gnao1, was expressed in tdTomato-positive pacemaker cells (**Figure 3.25a, b**). Gnao1 expression was also detected in the cAVN and atrioventricular (AV) ring, but not in the LNB, HIS, PLBB, LPF or RPF (**Figure 3.25c-g**). However, its expression was not specific to only nodal cells, as it was observed globally in the right atrial appendage (**Figure 3.25a, b**).

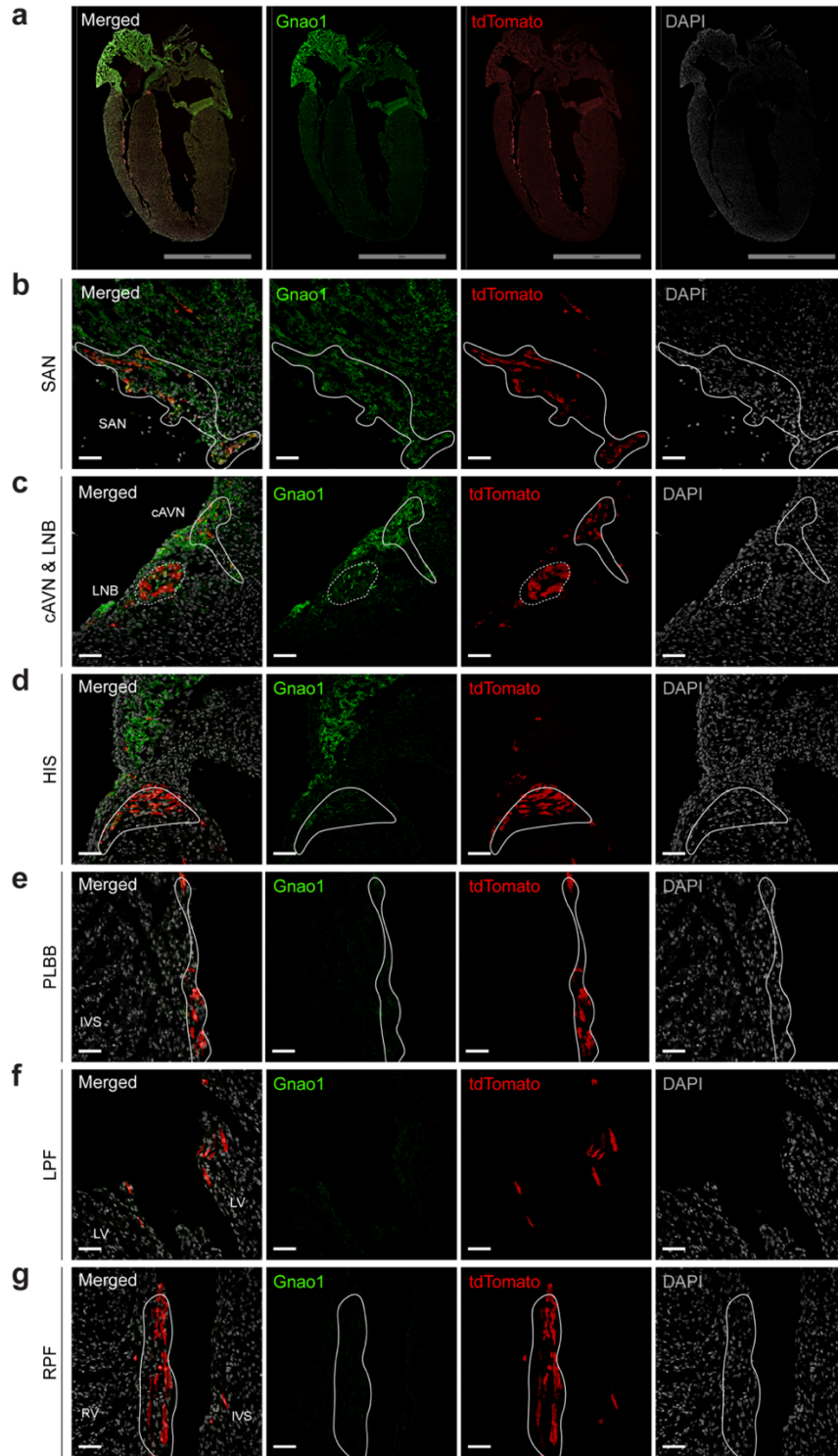


Figure 3.25 Immunofluorescence staining of Gnao1 in a P4 CCS reporter heart. (a) Fluorescence scanning of the stained whole heart. Scale bar = 2 mm. **(b-g)** Gnao1 is expressed in the SAN and the cAVN but not the **(c)** LNB, **(d)** HIS, **(e)** proximal LBB, **(f)** LPF or the **(g)** RPF. cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, *His* bundle; PLBB, proximal left bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 50 μ m.

3.3.3 Fluorescence *in situ* hybridization of *Prdm6*

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 (**Figure 3.26**).

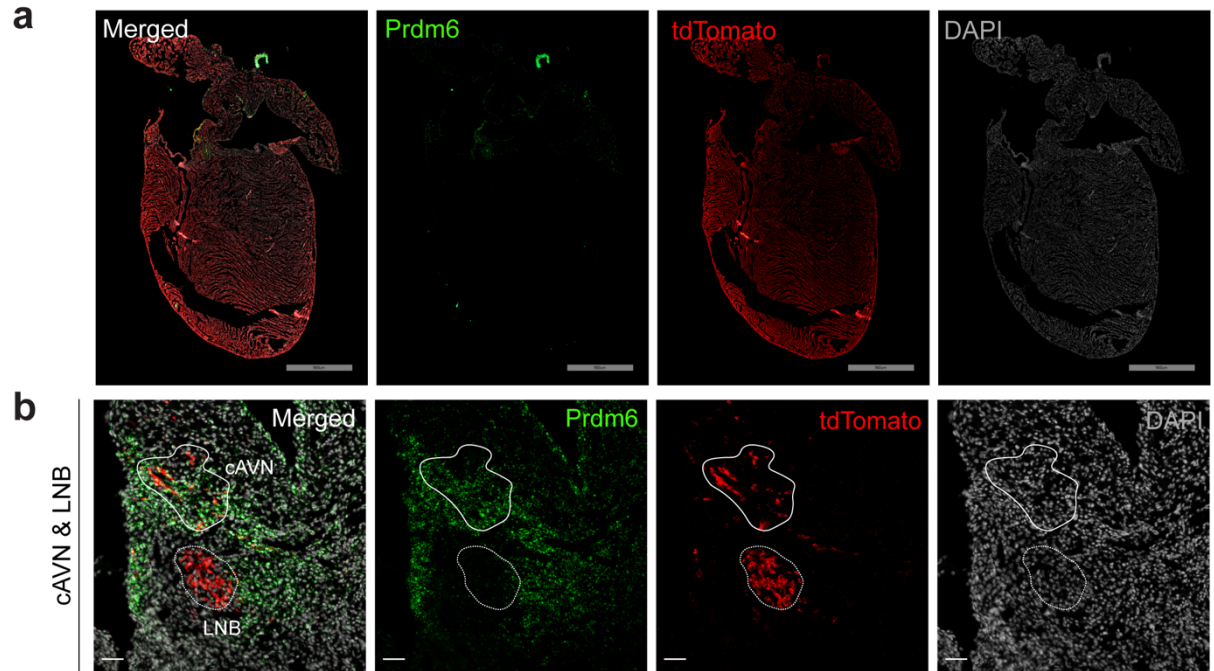


Figure 3.26 Fluorescence *in situ* hybridization (FISH) staining of *Prdm6* in a P4 CCS reporter heart. **(a)** Fluorescence scanning of the stained whole heart. Scale bar = 2 mm. **(b)** *Prdm6* is expressed in the compact AV node, but not in the lower nodal bundle and other CCS components. cAVN, compact atrioventricular node; LNB, lower nodal bundle. Scale bar = 900 μ m.

3.3.4 Immunofluorescence staining of Ppp1r17

In agreement with our integrated scRNA-seq and ST data, Ppp1r17 was expressed in the LNB and HIS, but not in cAVN (**Figure 3.27a-d**), supporting our finding that LNB is molecularly more similar to HIS than to cAVN. Ppp1r17 was not expressed in the VCS, including the distal BB or the PF (**Figure 3.27e-f**).

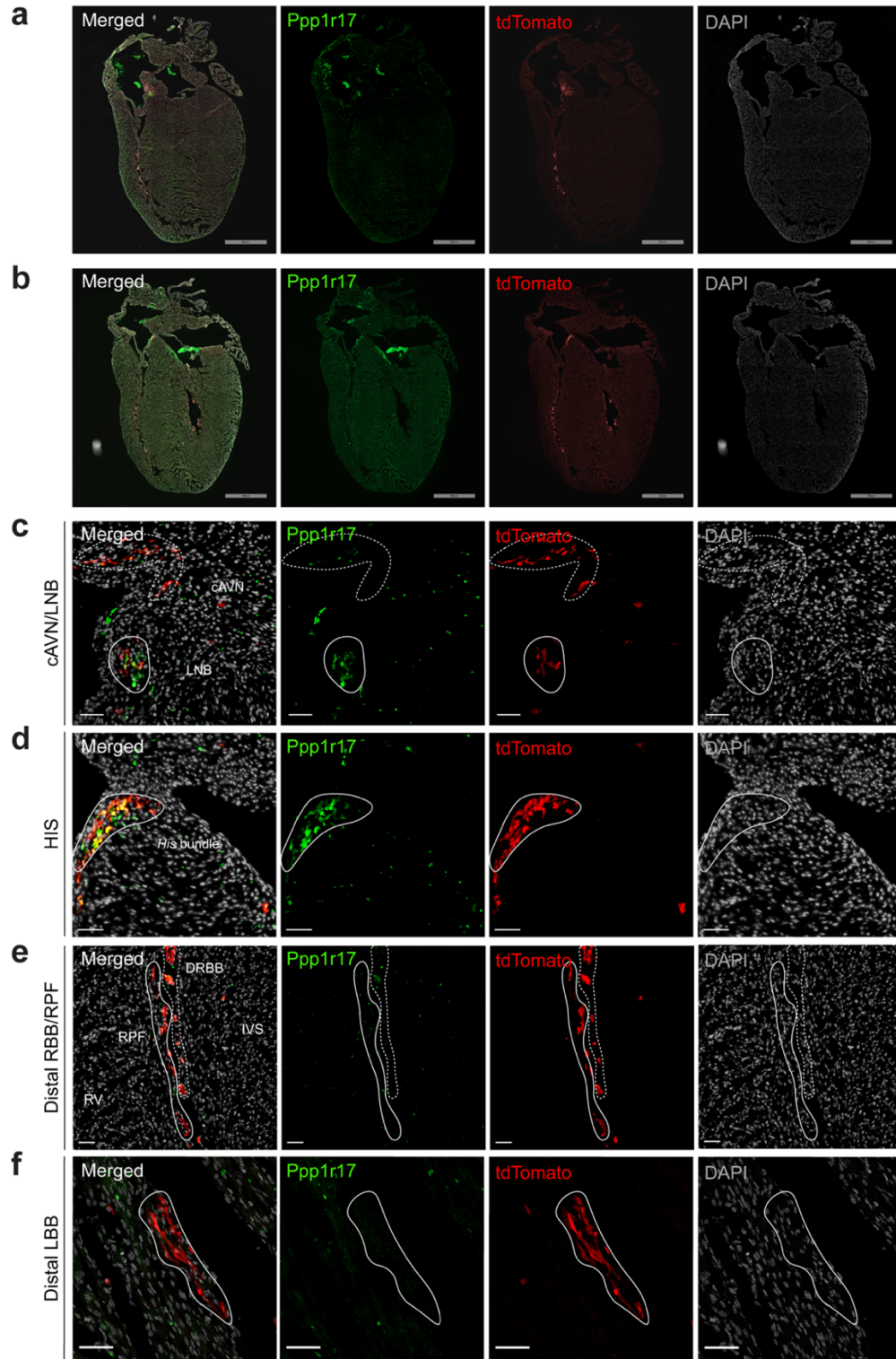


Figure 3.27 Immunofluorescence staining of Ppp1r17 in a P4 CCS reporter heart. (a-b) Fluorescence scanning of the stained whole heart section with (a) LNB and cAVN, and (b) HIS. Scale bar = 900 μ m. (c-d) Ppp1r17 is expressed in the (c) cAVN and (d) HIS, but not in the (e) distal RBB/RPF or the (f) distal LBB. LBB/RBB, left/right bundle branch; RPF, right Purkinje fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 50 μ m.

3.3.5 Immunofluorescence staining of S100a6

We also examined proximal VCS-enriched markers. Consistent with scRNA-seq data, S100a6 was detected in the HIS, but not in any other CCS regions, including the LBB, RBB, LPF and RPF (**Figure 3.28**). However, its expression in the HIS only partially overlapped with tdTomato-positive cells and was also found in tdTomato-negative cells around the HIS and in the IVS. Previous studies have reported S100a6 expression in other cell types, including neurons²⁶⁴, fibroblasts and epithelial cells²⁶⁵, suggesting that the role of S100a6 may not be restricted to the CCS.

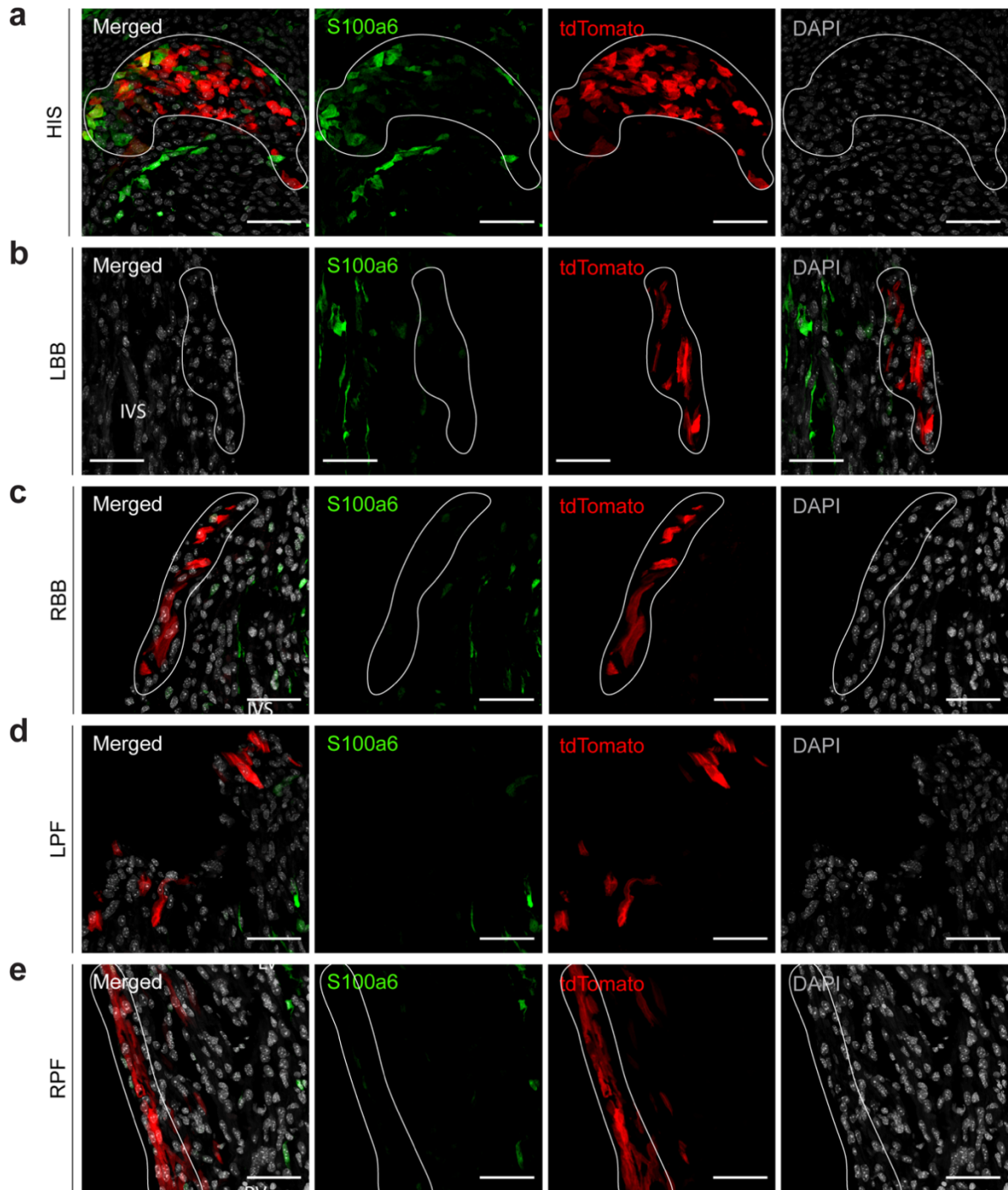


Figure 3.28 Immunofluorescence staining of S100a6 in a P4 CCS reporter heart shows expression of S100a6 in the (a) HIS, but not in the (b) LBB, (c) RBB, (d) LPF or the (e) RPF. LBB/RBB, left/right bundle branch; HIS, *His* bundle; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; LV/RV, left/right ventricle. Scale bar = 50 μ m.

3.3.6 Immunofluorescence staining of Igfbp7

Igfbp7, identified to be enriched in PBB in our integrated analysis, displayed strong expression in both HIS and PBB, but not in the PF (**Figure 3.29**). This suggests that Igfbp7 could serve as a proximal VCS marker.

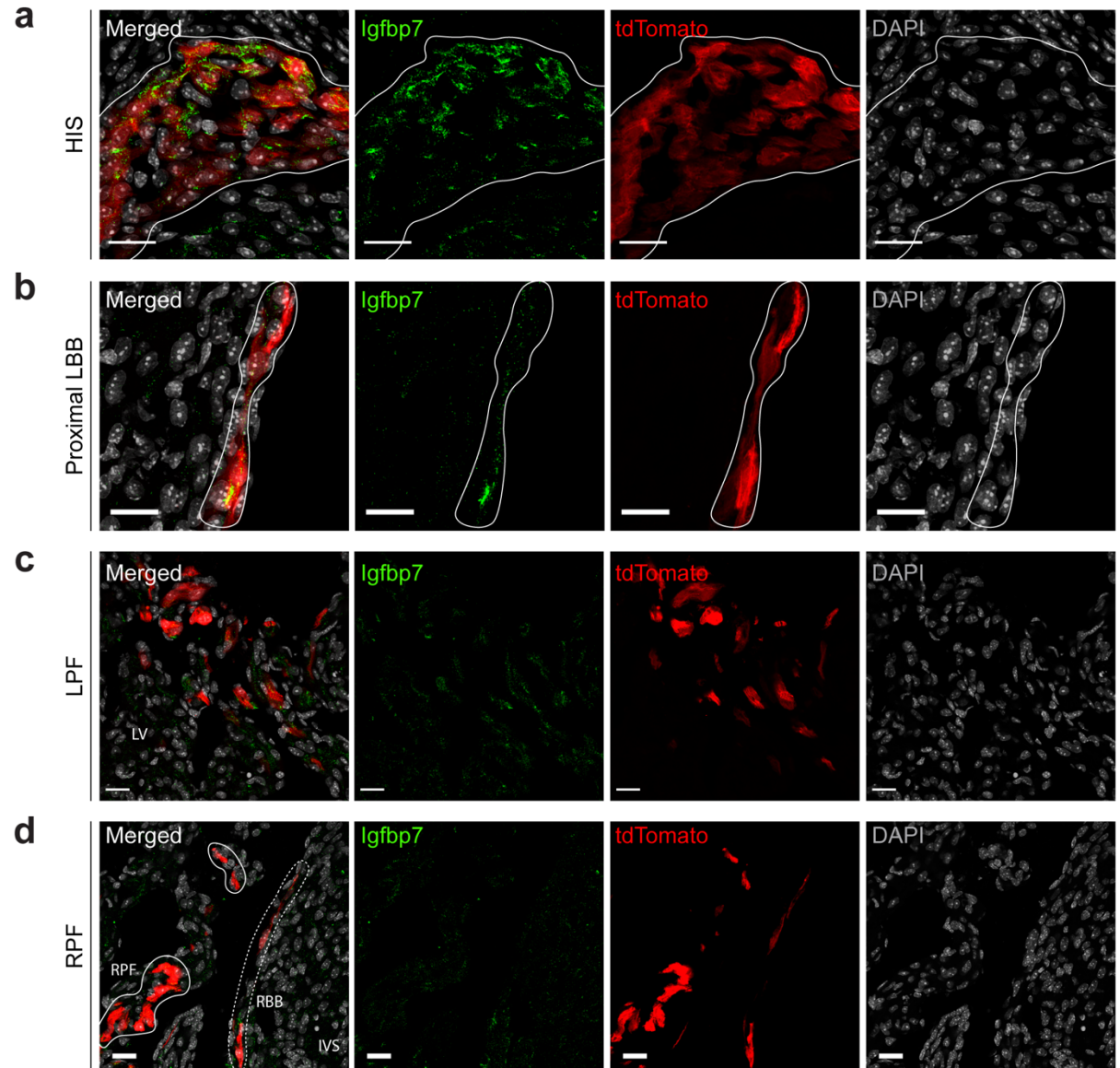


Figure 3.29 Immunofluorescence staining of Igfbp7 in a P4 CCS reporter heart shows expression of Igfbp7 in the (a) HIS and (b) Proximal LBB, but not in the (c) LPF and (d) RPF. HIS, *His* bundle; RBB, right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; LV, left ventricle. Scale bar = 20 μ m.

3.3.7 Fluorescence *in situ* hybridization of *Pcp4*

Confirming our scRNA-seq and ST data, FISH staining showed that an established VCS marker, *Pcp4*²⁴², was indeed expressed specifically and throughout the VCS, but showed stronger expression in the proximal VCS (i.e. HIS and BB) than in the distal VCS (i.e. PF) (Figure 3.30).

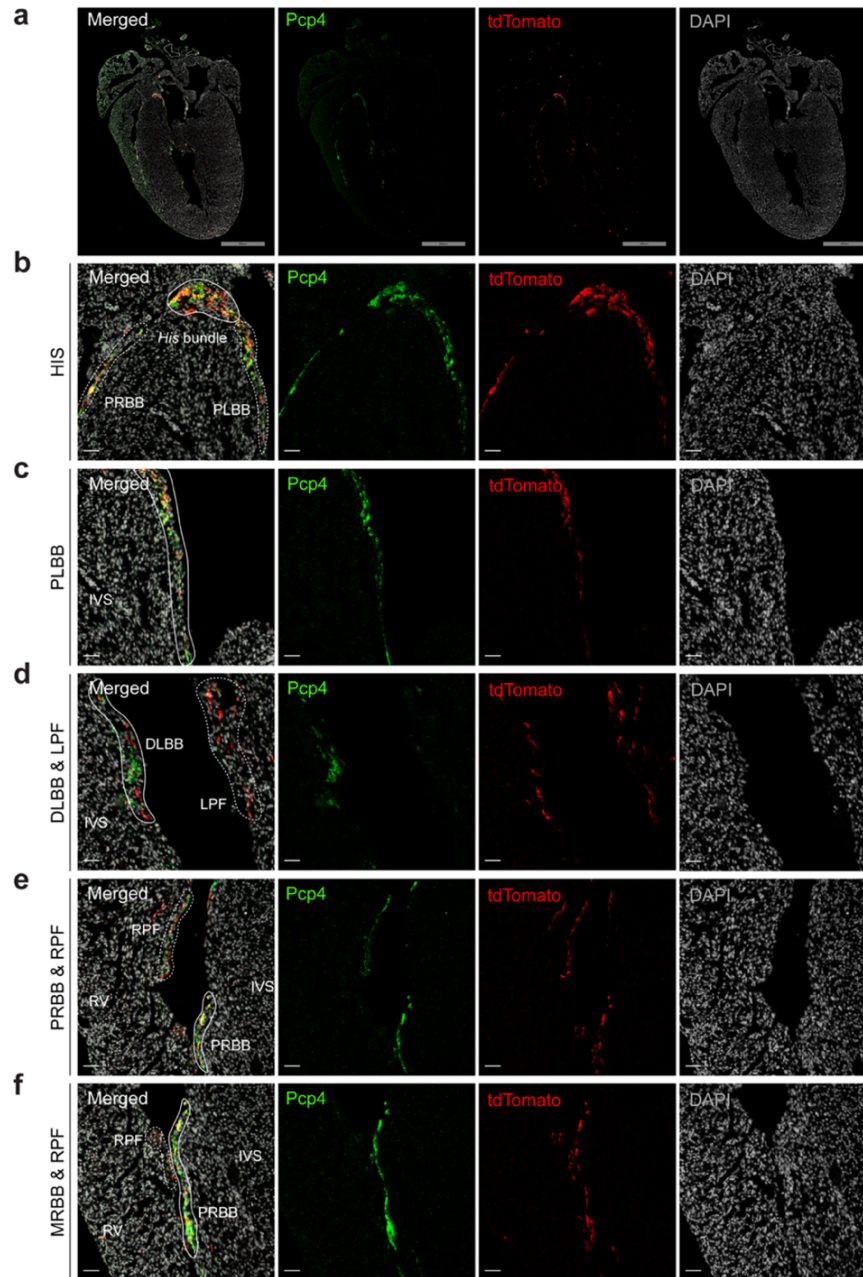


Figure 3.30 Fluorescence *in situ* hybridization (FISH) staining of *Pcp4* in a P4 CCS reporter heart. (a) Fluorescence scanning of the stained whole heart. Scale bar = 900 μ m. (b-f) *Pcp4* is

expressed specifically in the CSS, including (b) HIS, (c) proximal LBB, (d) distal LBB/LPF, (e) proximal RBB/RPF or the (f) mid-RBB/RPF. HIS, *His* bundle; PLBB/PRBB, proximal left/right bundle branch; DLBB, distal left bundle branch; MRBB, mid right bundle branch; LPF/RPF, left/right *Purkinje* fiber; IVS, interventricular septum; RV, right ventricle. Scale bar = 50 μ m.

3.3.8 Immunofluorescence staining of *Nppa*

In agreement with our integrated analysis, the expression of *Nppa*-encoded atrial natriuretic peptide (ANP), a well-established atrial chamber gene (**Figure 3.31a**), was primarily found in the distal VCS, including DLBB but not in other CCS proximal components (**Figure 3.31b**).

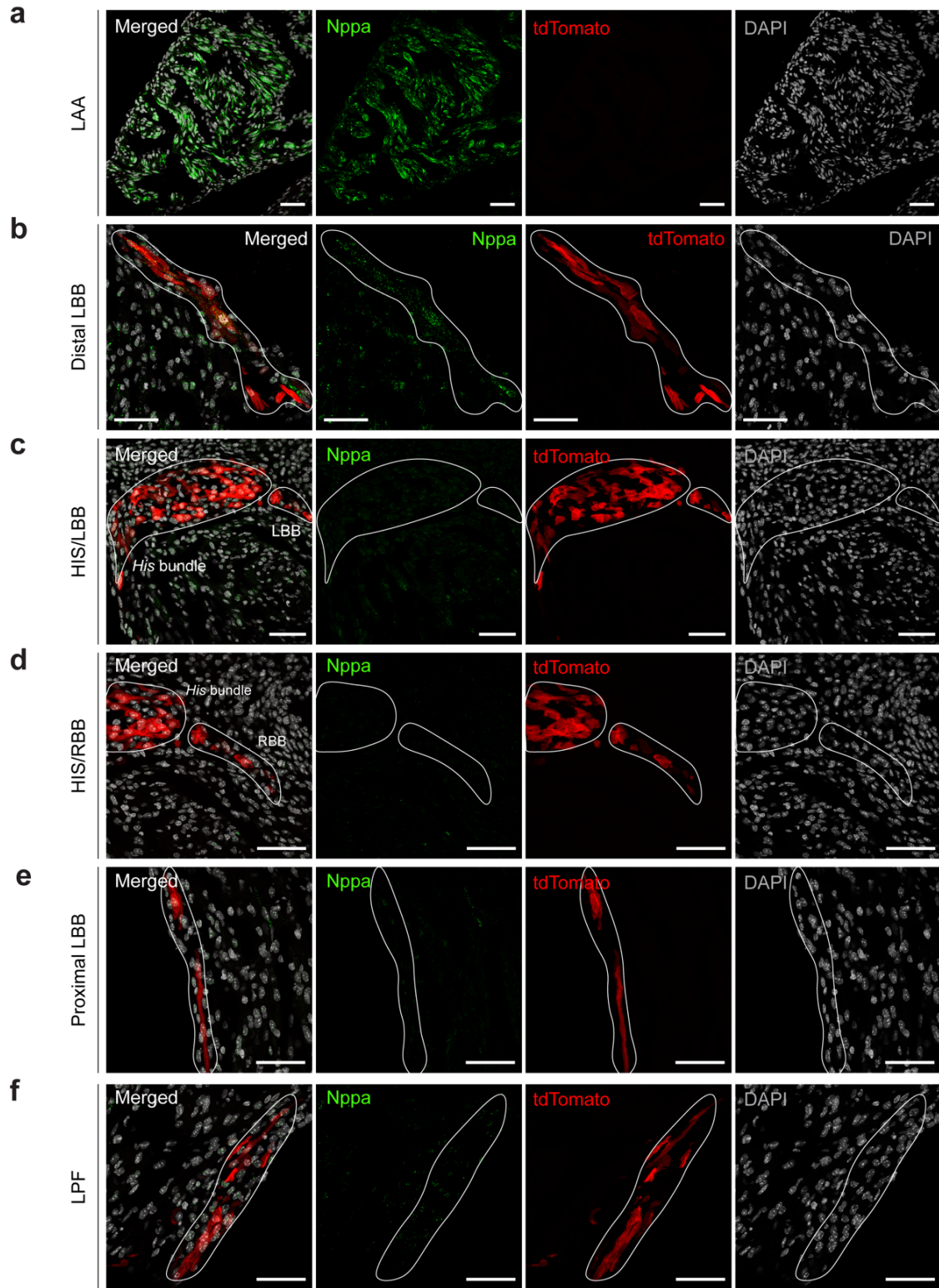


Figure 3.31 Immunofluorescence staining of Nppa-encoded atrial natriuretic peptide (ANP) in a P4 CCS reporter heart. ANP is primarily expressed in the (a) Nppa in the DBB and (b) LAA, but not in the (c) HIS/LBB, (d) HIS/RBB, (e) Proximal LBB or (f) LPF. LAA, left atrial appendage; HIS, His bundle; LBB/RBB, left/right bundle branch; LPF, left Purkinje fiber. Scale bar = 50 μ m.

3.3.9 Immunofluorescence staining of Scn10a

To confirm *Scn10a* enrichment in the PF, as observed in our scRNA-seq data further validated through read alignment to the reference genome (**Figure 3.21**), we performed immunofluorescence staining for Nav1.8 encoded by *Scn10a*, which showed its higher expression in the PF, compared to the HIS (**Figure 3.32**). *Scn10a* was also expressed in the cardiomyocytes proximal to the PFs, resembling a transmural gradient.

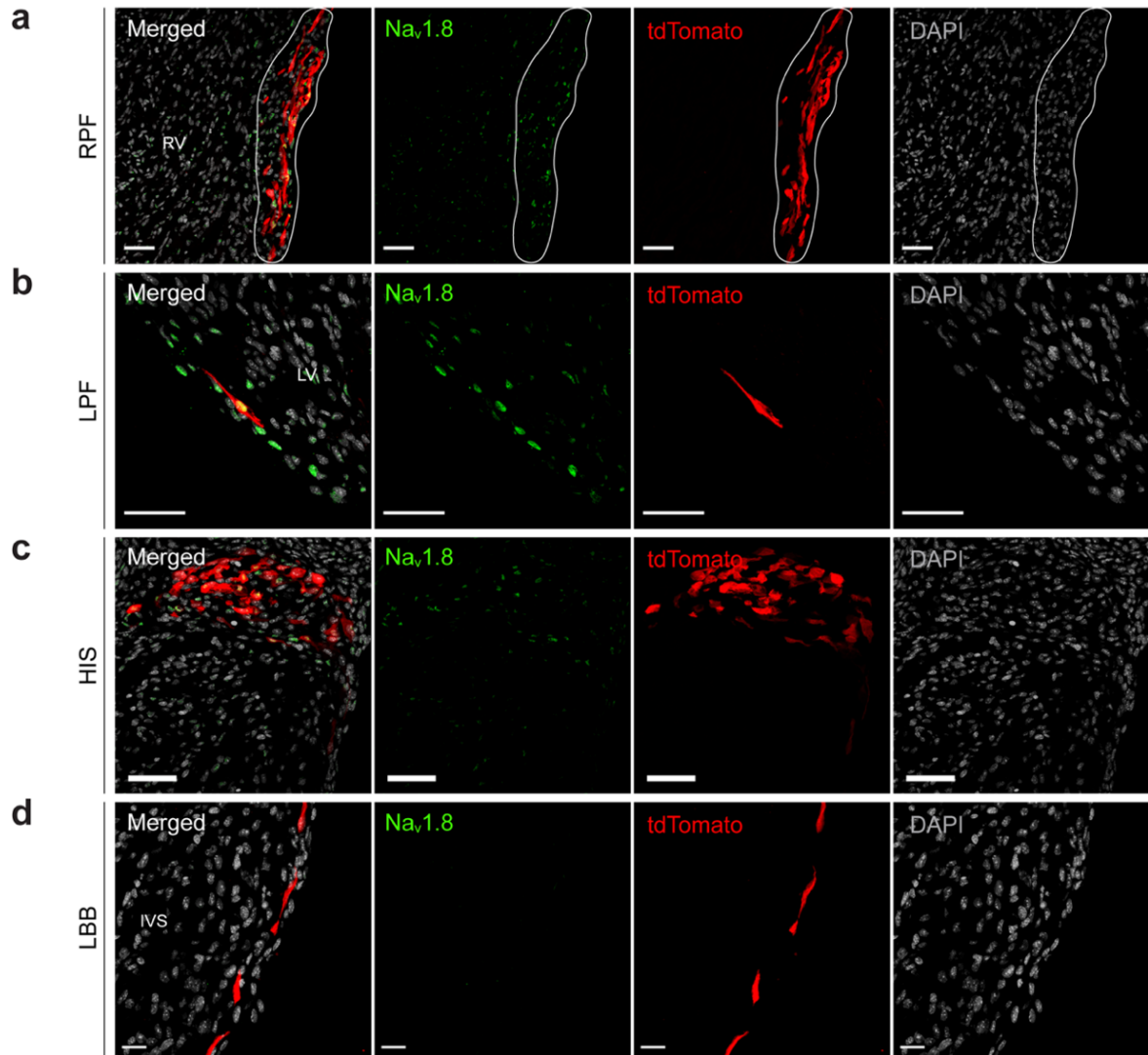


Figure 3.32 Immunofluorescence staining of *Scn10a*-encoded Nav1.8 in a P4 CCS reporter heart. Nav1.8, encoded by *Scn10a*, is enriched in the (a-b) distal VCS (RPF, LPF), but not in (c-d) proximal VCS (HIS, LBB). LPF/RPF, left/right Purkinje fibers; HIS, His bundle; LBB, left bundle branch; LV/RV, left/right ventricle; VCS, ventricular conduction system. Scale bar: (a-c) 50 μ m and (d) 20 μ m.

3.3.10 Fluorescence *in situ* hybridization of *Mpped2*

A newly identified PF-enriched gene, *Mpped2*, was strongly expressed throughout the VCS, albeit with a lower level of expression in the HIS and PBB (**Figure 3.33**). Like Na_v1.8, *Mpped2* also exhibited a slightly broader expression pattern, especially in the endomyocardium of the ventricular chambers, suggesting its roles within and around the VCS.

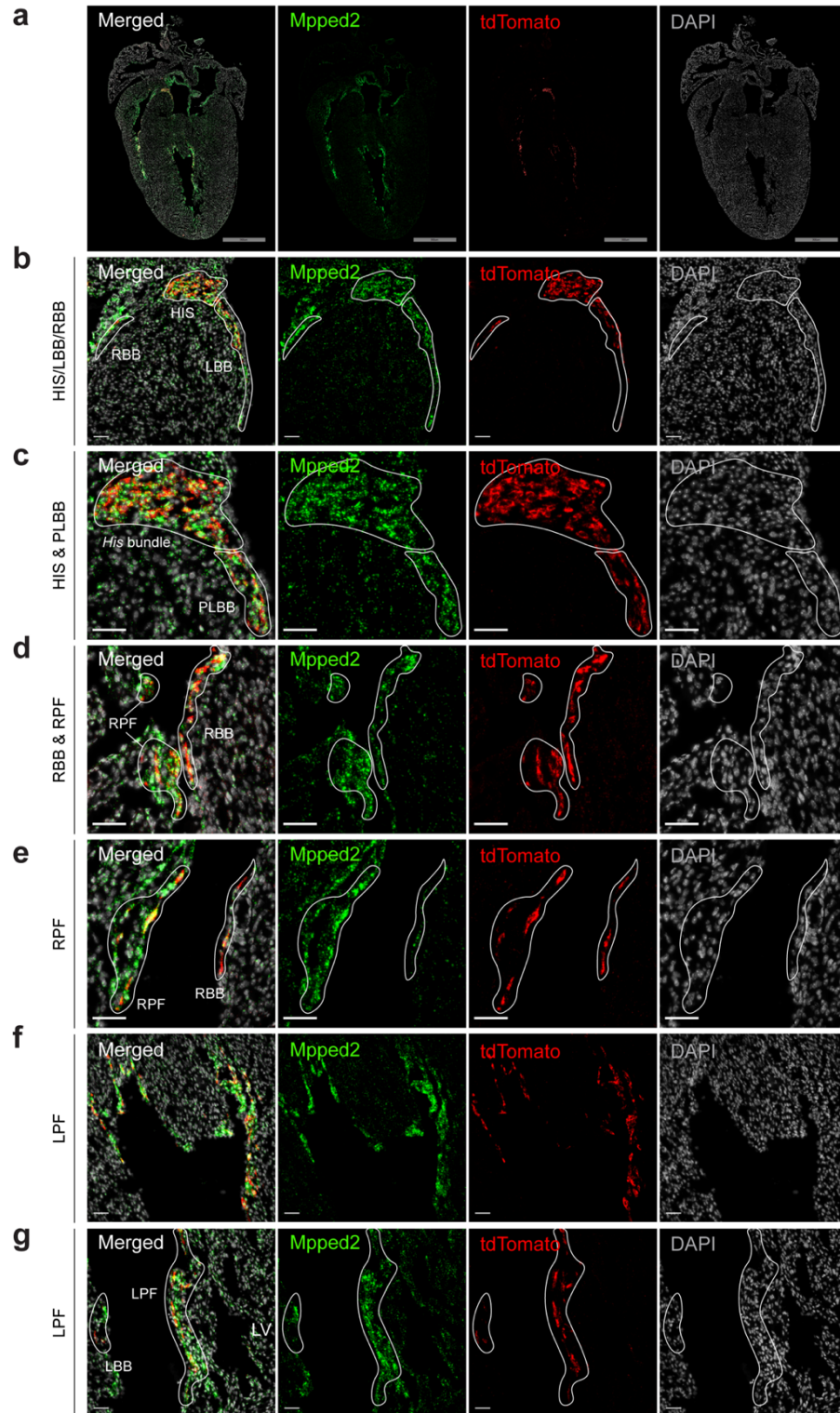


Figure 3.33 Fluorescence *in situ* hybridization (FISH) staining of *Mpped2* in a P4 CCS reporter heart. (a) Fluorescence scanning of the whole heart showing enrichment of *Mpped2* in the CCS and valves. Scale bar = 900 μm . (b-g) Representative images showing *Mpped2* expression in the (b-c) HIS bundle and PLBB, Scale bar = 20 μm , (d-e) RBB & RPF, scale bar = 20 μm and (f-g) LPF, scale bar = 50 μm . HIS, *His* bundle; PLBB, Proximal left bundle branch; RBB, Right bundle branch; LPF/RPF, Left/Right *Purkinje* fibers.

Collectively, the simultaneous examination of both scRNA-seq and ST followed by histological validation demonstrates that the CCS consists of both specific and gradational molecular profiles.

3.4 Comparison of molecular profiles of CCS in embryonic and early postnatal stages

3.4.1 Integration of E16.5 and postnatal CCS scRNA-seq data

The CCS forms through a tightly coordinated process of cell differentiation and specification during embryonic development, and maturation during early postnatal development^{50,163}. As a recent study has characterized the embryonic (E16.5) CCS through scRNA-seq⁶⁴, we sought to understand the transcriptional changes of the major CCS components during late embryonic and postnatal development. Embryonic CCS cells were first extracted from the original scRNA-seq datasets ([GSE132658](#)) based on cellular identification profiles⁶⁴, and then were integrated with our postnatal CCS scRNA-seq data with minimization of potential batch effects. This approach enabled a direct comparison of the molecular profiles of CCS components between embryonic and early postnatal stages.

Unbiased clustering of integrated cells displayed aligned patterns between individual CCS components from E16.5 and postnatal stages. For instance, the SAN, cAVN, HIS and PF cells of E16.5 and postnatal stages were well overlapped in the UMAP. Additionally, embryonic transitional cells, such as E16.5-Ventricular TZ and E16.5-TZ-PF, were aligned with the P-CM cluster, emphasizing their hybrid molecular profile comprising conduction and contractile cell identities (**Figure 3.34**).

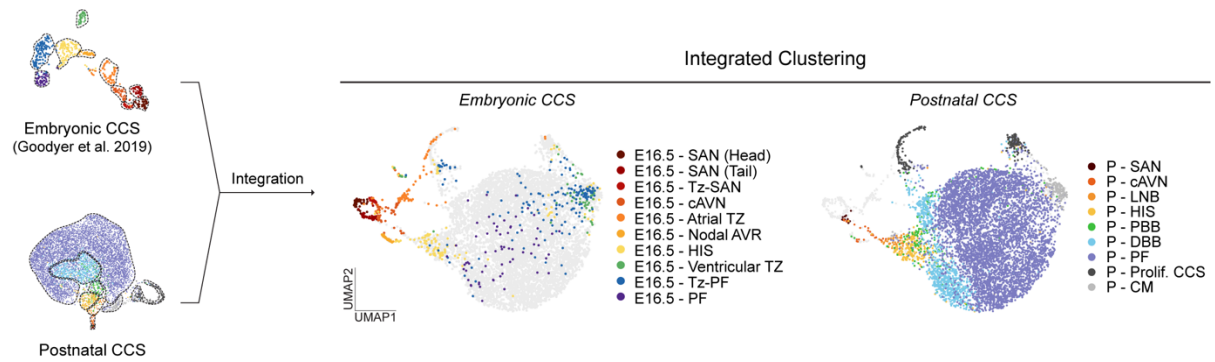


Figure 3.34 Data integration of embryonic and postnatal CCS. Integration of embryonic day 16.5 (E16.5) and postnatal (P1-P4) CCS scRNA-seq datasets shows a close overlay of the CCS components from different developmental stages. P: postnatal.

3.4.2 Correlation analysis between E16.5 and postnatal CCS components

Consistently, correlation analysis between E16.5 and postnatal CCS components demonstrated high concordances between the respective CCS regions, with significant clustering of CCS regions within each cardiac chamber (*i.e.*, the atria and the ventricles) (Figure 3.35).

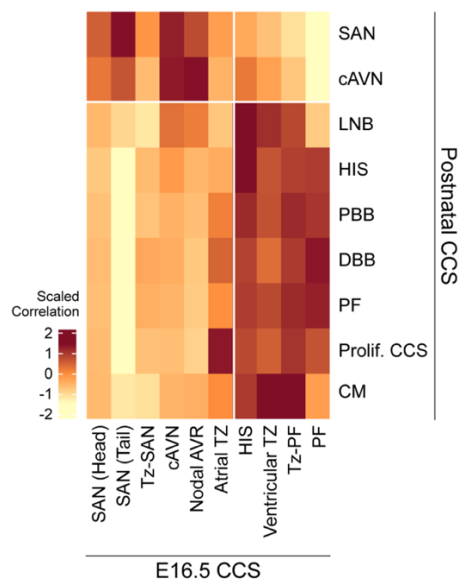


Figure 3.35 Molecular correlation between embryonic and postnatal CCS. Pearson correlation analysis between embryonic and postnatal CCS components exhibits a high degree of concordance between respective groups. SAN, sinoatrial node; cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, *His* bundle; PBB, proximal bundle branch; DBB, distal bundle branch; PF, *Purkinje* fibers; Prolif. CCS, proliferative CCS; CM, contractile myocytes; Tz-SAN, transitional SAN; AVR, AV ring; Atrial TZ, atrial transitional zone, Ventricular TZ, ventricular transitional cells; Tz-PF, transitional PF.

3.4.3 Molecular similarities between E16.5 and postnatal CCS components

We further examined whether the CCS marker genes identified in the postnatal CCS displayed similar expression patterns in the E16.5 CCS (**Figure 3.36**). Notably, SAN-enriched genes (*Gnao1*, *Nid2*, *Bmp2*), cAVN-enriched genes (*Bambi*, *Gsg1*, *Rd3*, *Prdm6*), nodal cells-enriched genes (*Gcgr*, *Csrp2*, *Enpep*, *Ctdspl*), HIS/proximal VCS-enriched genes (*S100a6*, *Robo1*, *Scube2*, *Cdh6*), and PF/distal VCS-enriched genes (*Mpped2*, *Corin*, *Nsg2*, *Sema3a*, *Sbk2*, *Tsc22d3*, *Irxa* and *Irxb*) were consistently observed in the corresponding regions of the E16.5 CCS. This finding not only affirms the specificity of the newly identified CCS markers, but also indicates a high degree of transcriptomic consistency between these developmental stages, suggesting early establishment of molecular heterogeneity within the CCS.

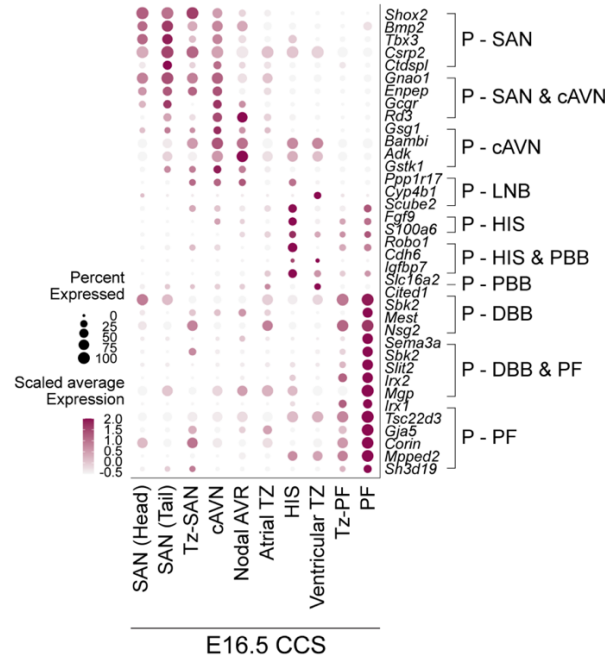


Figure 3.36 Molecular similarities between embryonic and postnatal CCS. Expression patterns of the identified CCS component and regional markers in E16.5 CCS clusters, showing molecular similarities between embryonic and postnatal CCS. SAN, sinoatrial node; cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, His bundle; PBB, proximal bundle branch; DBB, distal bundle branch; PF, Purkinje fibers; Prolif. CCS, proliferative CCS; CM, contractile myocytes; Tz-SAN, transitional SAN; AVR, AV ring; Atrial TZ, atrial transitional zone, Ventricular TZ, ventricular transitional cells; Tz-PF, transitional PF.

3.4.4 Molecular differences between E16.5 and Postnatal CCS components

Subsequently, we conducted pairwise differential gene expression analysis to identify the molecular differences between E16.5 and postnatal CCS components ($|FC| > 1.5$; $P < 0.05$; minimum % of expressing cells in either compared group $> 50\%$). We noted significant differences in the expression of known CCS marker genes, including higher *Cpne5* and lower *Igfbp5*, *Sln*, and *Vsnl1* expression in the postnatal SAN (**Figure 3.37a**). Also, lower expression levels of *Id2*, *Sln*, *Cav1*, and *Vsnl1*, along with higher *Rspo3* levels, were observed in postnatal cAVN, compared to the embryonic cAVN (**Figure 3.37b**). Compared to the embryonic HIS, the postnatal HIS exhibited higher levels of *Pcp4*, *Hcn4*, and *Igfbp7*, yet lower levels of *Tagln* and *Cnn1*, encoding Transgelin (also known as SM22 α) and Calponin 1, respectively, both of which are smooth muscle cell markers²⁶⁶. Given that *Cnn1* is expressed in the embryonic mouse heart (E13.5) but becomes restricted to smooth muscle cells in adult mouse tissues²⁶⁷, this reduction may reflect postnatal CCS maturation (**Figure 3.37c**). Similarly, the postnatal PF showed significant decreases in *Bmp10*²⁶⁸ and *Corin*²⁶⁹, genes that are expressed in the ventricular trabecular myocardium during development (**Figure 3.37d**).

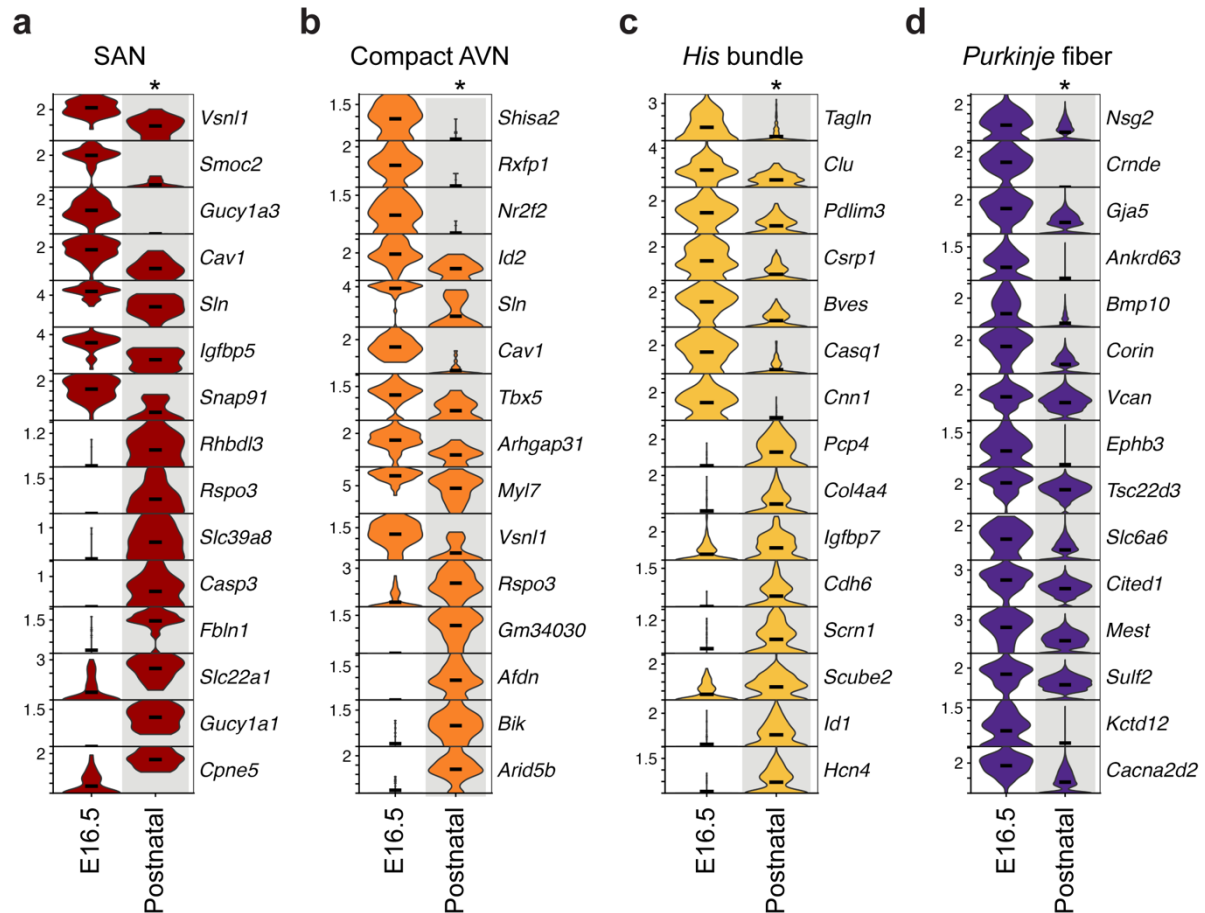


Figure 3.37 Molecular differences between embryonic and postnatal CCS. Pairwise differential gene expression analysis between embryonic and postnatal CCS components, including the **(a)** SAN, **(b)** Compact AVN, **(c)** His bundle, **(d)** Purkinje fiber using the Wilcoxon Rank Sum Test. *, adjusted P -value < 0.05. SAN, sinoatrial node; cAVN, compact atrioventricular node; LNB, lower nodal bundle; HIS, His bundle; PBB, proximal bundle branch; DBB, distal bundle branch; PF, Purkinje fibers; Prolif. CCS, proliferative CCS; CM, contractile myocytes; Tz-SAN, transitional SAN; AVR, AV ring; Atrial TZ, atrial transitional zone, Ventricular TZ, ventricular transitional cells; Tz-PF, transitional PF.

These observations collectively suggest that despite the high similarity in the overall molecular profiles of CCS components between E16.5 and early postnatal stages (P1 - P4), there are discernible molecular changes that indicate the dynamic maturation process in the CCS during late embryonic and early postnatal development.

3.5 Heterogeneous transcriptional regulation of the CCS

3.5.1 Enriched regulons in CCS components and gene regulatory networks

To gain mechanistic insights into how distinct molecular patterning across the CCS is transcriptionally established, we performed network inference using SCENIC (Single-Cell Regulatory Network Inference and Clustering)²⁰⁴, which identifies regulons by predicting the transcription factors (TFs) and their candidate target genes based on co-expression and motif enrichment analysis. TF activity in each individual cell was calculated using AUCell and subjected to unbiased clustering. This analysis revealed TFs that potentially contributed to the establishment of the regional differences within the CCS (**Figure 3.38**).

Transcriptional activities by Tbx3, Klf7, Creb5, Rara and Sp3 were enriched in the nodes and proximal VCS; Shox2, Nr2f1, Nr2f2 and Gata6 in the nodes only; E2f6 in the nodes and distal VCS; as well as Mef2c and Gtf3a in the nodes and contractile cardiomyocytes. In addition, enrichments of Tcf4, Tbx5, Cebpb, Cebpd, Jun, Ets2, Fos, and Irf1 activities were observed across all CCS components, except contractile myocytes, whereas Irx2, Tcf7l2, Etv1, Srebf2, and Max displayed VCS-specific enrichment. These findings corroborated a large body of literature highlighting the regulatory functions of Shox2 and Gata6 in the SAN²⁷⁰, Tbx3 in the SAN/cAVN/HIS^{162,163,168,271}, and Tbx5^{145,164,168} and Etv1^{160,272} in the VCS.

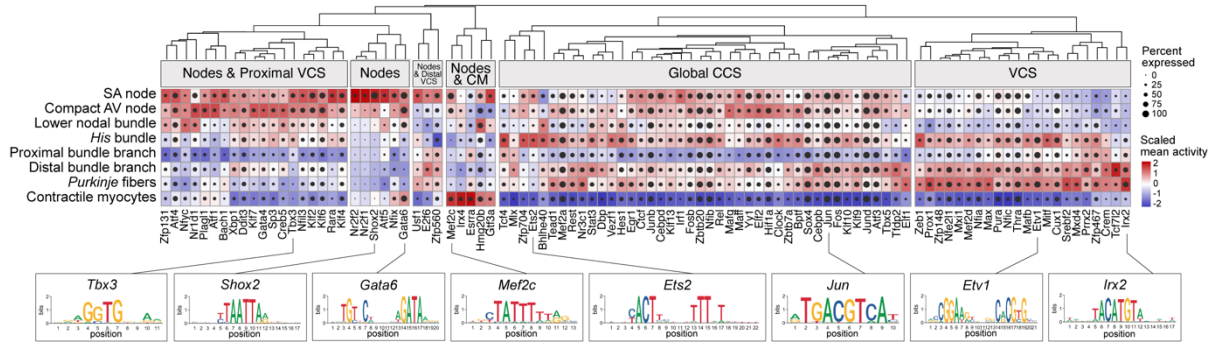


Figure 3.38 Heterogeneous transcriptional regulation of the CCS. (Top) Integrated heatmap/dotplot of CCS regulons, as inferred by the SCENIC algorithm. Heatmap colour represents regulon activities for each CCS component based on the area under the curve (AUC) scores. Dot size represents transcription factor expression in each CCS component. **(Bottom)** Motifs of representative transcription factors in the CCS regions.

Gene regulatory networks of the top 5 regulons in the SAN, cAVN, HIS and PF were also constructed, elucidating the regulation of the regional marker genes, such as *Gnao1*, *Gcgr*, *Bmp2* and *Vsn11* in the SAN (**Figure 3.39a**); *Prdm6*, *Rspo3*, and *Gde1* in the cAVN (**Figure 3.39b**); *Igf1bp7*, *Cpne5*, *Scube2*, and *Cdh6* in the HIS (**Figure 3.39c**) as well as; *Mpped2*, *Slit2*, *Scn10a*, and *Tsc22d3* in the PF (**Figure 3.39d**).

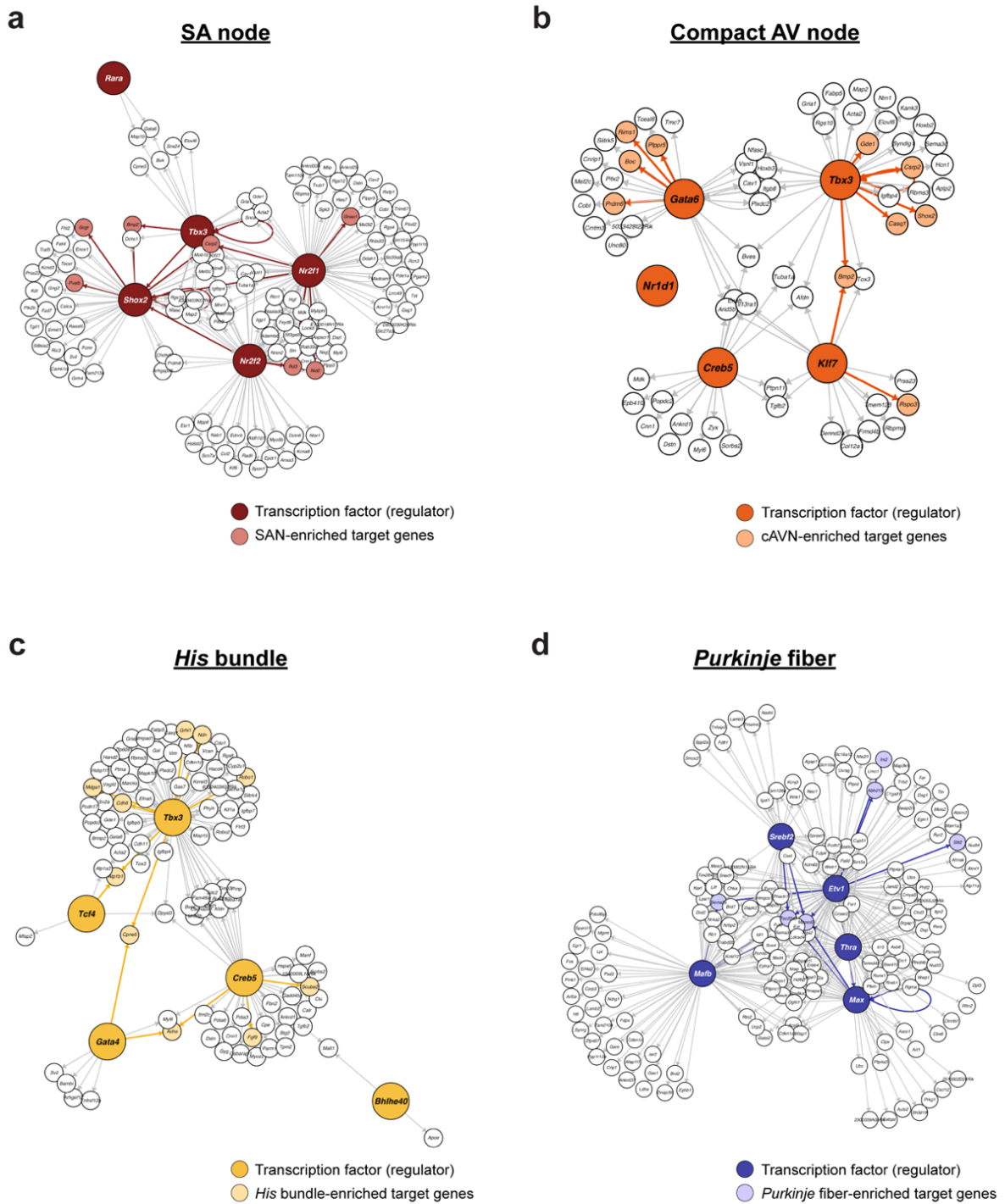


Figure 3.39 Gene regulatory networks of CCS components. Gene regulatory networks featuring the top 5 regulons, enriched transcription factors (darker colours) and their target genes (lighter colours), in **(a)** SA node, **(b)** compact AV node, **(c)** *His* bundle and **(d)** *Purkinje* fiber, are presented.

3.5.2 Unique and overlapping expression patterns of *Tbx3* and *Irx3* in the CCS

To deepen our understanding of transcriptional regulation of CCS heterogeneity, we focused on two TFs, *Tbx3* and *Irx3*, known for their critical roles in CCS development and functions^{50,53,141,163,168} (**Figure 3.40a**). Our scRNA-seq and ST analyses showed that *Tbx3* and *Irx3* exhibited distinct and overlapping expression patterns within the CCS: *Tbx3* alone in the atrial components of the CCS (SAN and cAVN); both *Irx3* and *Tbx3* in the proximal VCS (LNB, HIS, PBB); and *Irx3* alone in the distal VCS (DBB and PF) (**Figure 3.40b, c**). Although *Irx3* was not identified from SCENIC analysis, we observed strong VCS-enrichment of the *Irx2* regulon, another member of the *Irx* TF family sharing the same *Irx* motif^{216,217} (**Figure 3.38**). Given that *Irx3*, not *Irx2*, is well described to be essential for VCS development and function^{50,53,255}, we hypothesized that individual and cooperative transcriptional regulation by *Tbx3* and *Irx3* contributed to the molecular heterogeneity of the CCS.

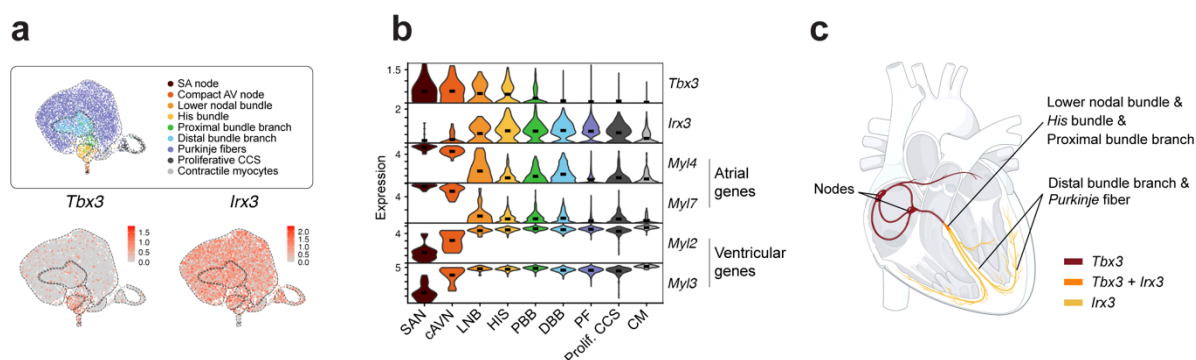


Figure 3.40 Heterogeneous transcriptional regulation of the CCS by *Tbx3* and *Irx3*. (a) *Tbx3* and *Irx3* show unique and overlapping expression patterns exclusively within the CCS. (b) SAN and cAVN show enrichment of *Tbx3* with atrial genes (*Myh4* and *Myh7*); LNB, HIS and PBB show enrichment of both *Tbx3* and *Irx3* with ventricular genes (*Myh2* and *Myh3*); and DBB and PF show enrichment of *Irx3* with ventricular genes. (c) Schematic showing unique and overlapping expression patterns of *Tbx3* and *Irx3* throughout the CCS. Created with BioRender.com.

To test this possibility, we utilized publicly available datasets of the assay for transposase-accessible chromatin using sequencing (ATAC-seq) in different CCS regions:

P0 mouse SAN¹⁴⁷ and E13.5 Tbx3-positive atrioventricular junction (AVJ encompassing both AVN and HIS)¹⁶³, along with their respective non-CCS counterparts: P0 right atria (RA) and mouse ventricle²¹⁴. Additionally, we used Tbx3 ChIP-seq data performed in adult mouse cardiomyocytes²¹⁵, and searched for two Irx TF binding motifs: 'ACAnnTGT'²¹⁷ and 'ACATGT'²¹⁶. 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.

Overall, 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 TFs across the CCS.

3.5.3 Chromatin accessibilities, Tbx3 binding and Irx3 motifs of identified CCS marker genes

We closely examined the epigenomic landscape for several new and established CCS marker genes. SAN markers, *Shox2* and *Vsnl1*, as well as a cAVN marker, *Prdm6* displayed higher ATAC-seq peaks in the SAN and AVJ compared to the atria or ventricle, respectively. This suggests a spatial specificity in their genomic accessibility. Tbx3-binding peaks and Irx

motif sequences were also found in *Vsnl1* and *Prdm6*, indicating potential regulation by Tbx3 and Irx TFs (**Figure 3.41**).

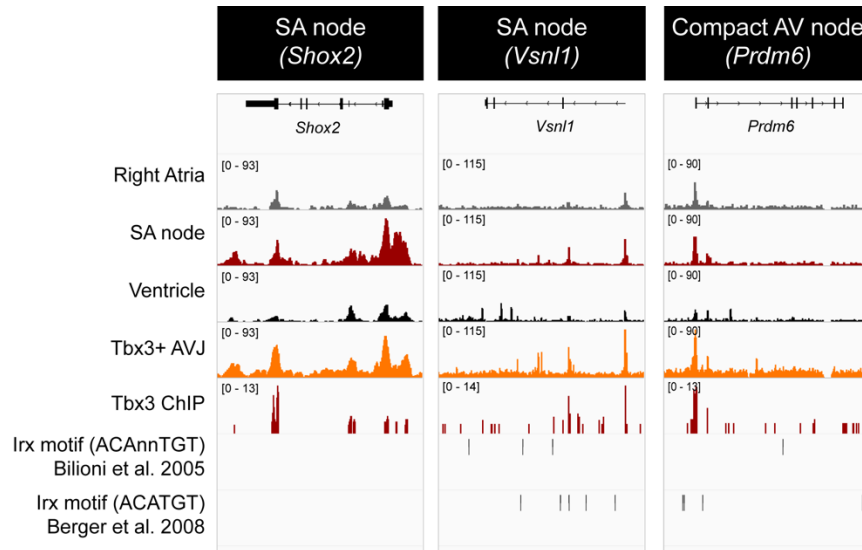


Figure 3.41 Heterogeneous transcriptional regulation of nodal markers by *Tbx3* and *Irx3*. Genome browser views of chromatin accessibilities in the right atria, SA node, ventricle, and *Tbx3*⁺ atrioventricular junction (AVJ) as well as Tbx3 ChIP-seq, and the presence of *Iroquois* (*Irx*) transcription factor motifs for markers of nodal cells.

In accordance with its enriched expression in the proximal VCS, *S100a6* exhibited higher chromatin accessibility peaks in Tbx3⁺ AVJ, compared to the ventricle. This aligned with the Tbx3 ChIP-seq peak, but not with Irx motifs, suggesting a possible direct transcriptional regulation by Tbx3, but not Irx TFs. Interestingly, genes enriched in the distal VCS where *Tbx3* expression is absent, such as *Nppa*, *Gja5*, *Mpped2*, and *Slit2*, still displayed open chromatin regions in the Tbx3⁺ AVJ. They also showed Tbx3 ChIP-seq peaks and Irx motifs. On the other hand, another distal VCS gene, *Scn10a*, showed no apparent differences in ATAC-seq peaks between the Tbx3⁺ AVJ and the ventricle, although it did exhibit Tbx3 ChIP-seq peaks and Irx motifs (**Figure 3.42**).

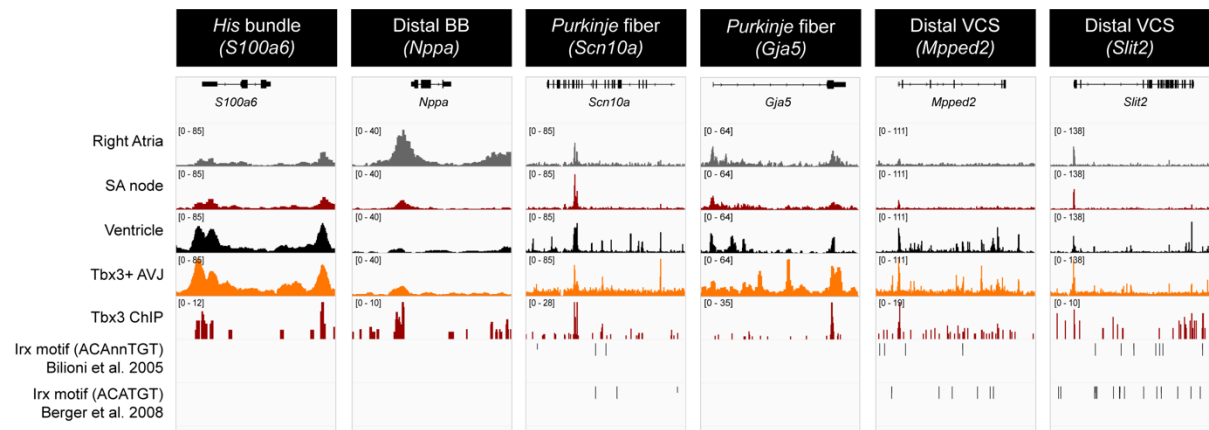


Figure 3.42 Heterogeneous transcriptional regulation of VCS markers by *Tbx3* and *Ir*3. Genome browser views of chromatin accessibilities in the right atria, SA node, ventricle, and *Tbx3*⁺ atrioventricular junction (AVJ) as well as *Tbx3* ChIP-seq, and the presence of *Iroquois* (*Ir*) transcription factor motifs for markers of VCS components.

Lastly, consistent with their high expression in contractile myocytes, *Gja1* and *Ryr2* showed greater chromatin accessibility in the ventricle compared to AVJ, atria, and SAN, alongside the presence of *Ir*x motifs and *Tbx3* binding peaks. Collectively, these findings suggest a complex and region-specific potential transcriptional regulation of CCS regional marker genes by *Tbx3* and/or *Ir*x3.

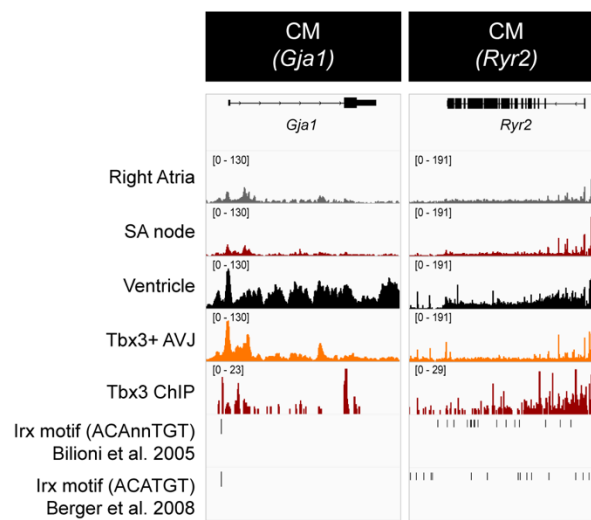


Figure 3.43 Heterogeneous transcriptional regulation of contractile myocyte markers by *Tbx3* and *Ir*3. Genome browser views of chromatin accessibilities in the right atria, SA node, ventricle, and *Tbx3*⁺ atrioventricular junction (AVJ) as well as *Tbx3* ChIP-seq, and the presence of *Iroquois* (*Ir*) transcription factor motifs for markers of contractile myocytes.

3.6 *Tbx3* and *Irx3* contribute to the establishment of CCS heterogeneity

3.6.1 *In vitro* model of CCS regional heterogeneity

To further investigate the individual and combined actions of *Tbx3* and *Irx3* on CCS gene regulation, we established an *in vitro* model that simulated various CCS regions by introducing *Tbx3* and/or *Irx3* in neonatal mouse atrial and ventricular cardiomyocytes (A-CMs and V-CMs, respectively) using adenovirus-mediated overexpression (**Figure 3.44**).

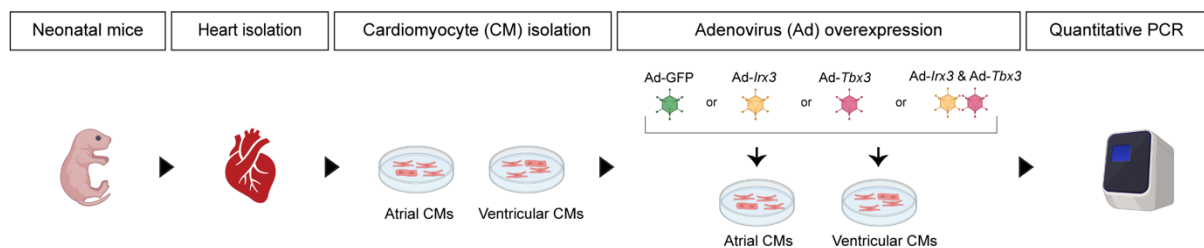


Figure 3.44 Schematic of the experimental workflow to model the CCS regional heterogeneity *in vitro*. Neonatal mouse atrial and ventricular cardiomyocytes were infected with Ad-GFP, Ad-Irx3, Ad-Tbx3 or both Ad-Irx3 and Ad-Tbx3.

Here, *Tbx3* overexpression in A-CMs modelled the nodes (SAN and cAVN); *Irx3* and *Tbx3* overexpression in V-CMs, the proximal VCS (HIS and PBB); and *Irx3* overexpression in V-CMs, the distal VCS (DBB and PF) (**Figure 3.45**).

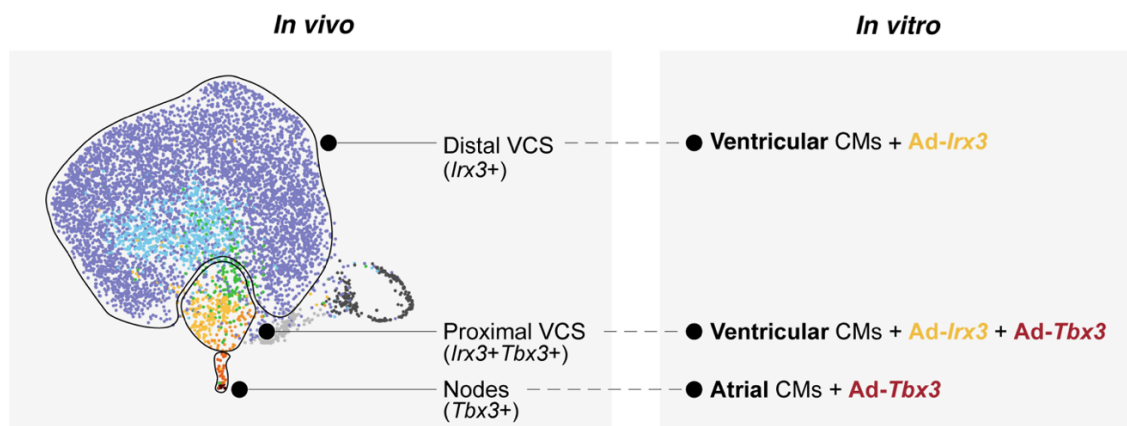


Figure 3.45 Schematic of the *in vitro* model of the CCS regions. Nodes were simulated by *Tbx3* overexpression in atrial cardiomyocytes (CMs); the Proximal VCS by *Tbx3* and *Irx3* overexpression in ventricular CMs; and the Distal VCS by *Irx3* overexpression in ventricular CMs.

The regional properties of isolated A-CMs and V-CMs were validated by the significantly high atrial gene expression (e.g., *MyI4* and *MyI7*) in A-CMs and ventricular gene expression (e.g., *MyI2* and *MyI3*) in V-CMs (Figure 3.46).

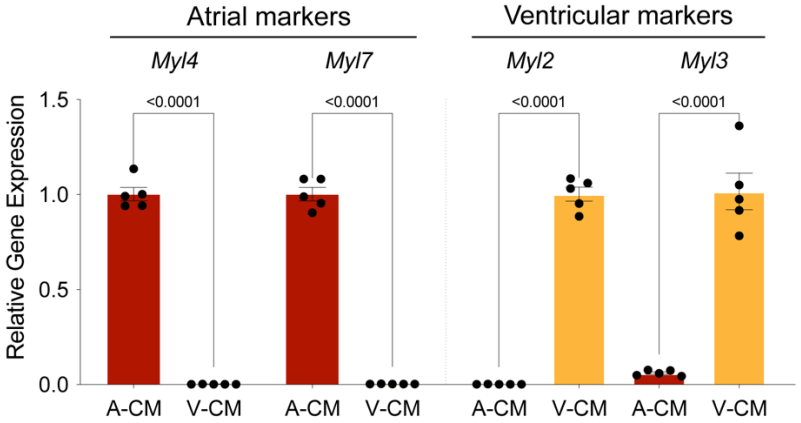


Figure 3.46 Gene expression of atrial (*MyI4* and *MyI7*) and ventricular (*MyI2* and *MyI3*) genes in cultured atrial and ventricular cardiomyocytes. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

Successful overexpression of *Tbx3* and *Irx3* was also confirmed by high *Tbx3* expression in cells infected with Ad-*Tbx3* or Ad-*Irx3*+*Tbx3*, and high *Irx3* expression in cells infected with Ad-*Irx3* or Ad-*Irx3*+*Tbx3*, respectively (Figure 3.47). Subsequently, we assessed how *Tbx3* and/or *Irx3* influence the expression of CCS regional markers.

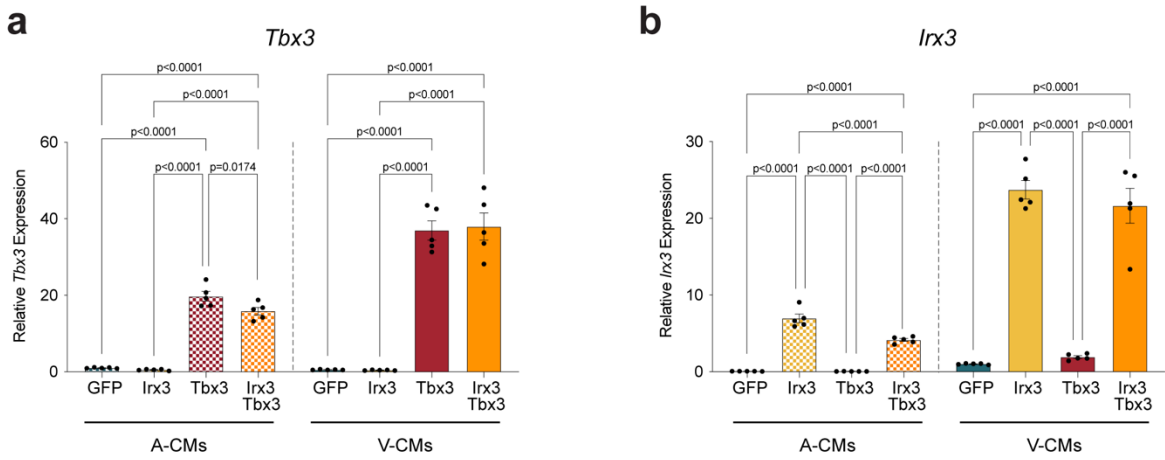


Figure 3.47 Gene expression of (a) *Tbx3* and (b) *Irx3* in cultured atrial and ventricular cardiomyocytes. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

3.6.2 Transcriptional regulation of CCS marker genes by *Tbx3* and/or *Irx3*

3.6.2.1 Transcriptional regulation of nodal genes by *Tbx3* and/or *Irx3*

We found that *Vsnl1*, exclusively expressed in the *Tbx3*⁺ SAN, was markedly increased by *Tbx3* overexpression in A-CMs (**Figure 3.48**). Interestingly, the addition of *Irx3*, as well as *Irx3* overexpression alone in A-CMs, suppressed *Vsnl1* expression, suggesting the antagonistic actions of *Irx3* against *Tbx3* outside nodal cells. In V-CMs, *Vsnl1* was unaffected by *Tbx3* or *Irx3* overexpression, suggesting that *Vsnl1* expression in the SAN is predominantly activated by *Tbx3* in an atria-specific manner.

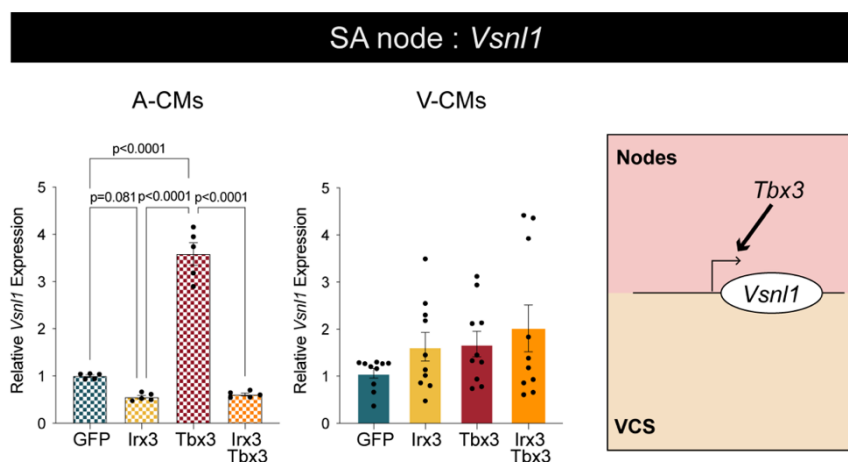


Figure 3.48 Region-specific transcriptional regulation of a SA node marker, *Vsnl1*, by *Tbx3* and *Irx3*. *n* = 5 per group for A-CMs, and *n* = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

On the other hand, another SAN marker, *Shox2* was not affected by either *Tbx3* or *Irx3* overexpression in both A-CMs and V-CMs (**Figure 3.49**), 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 development¹⁴⁸.

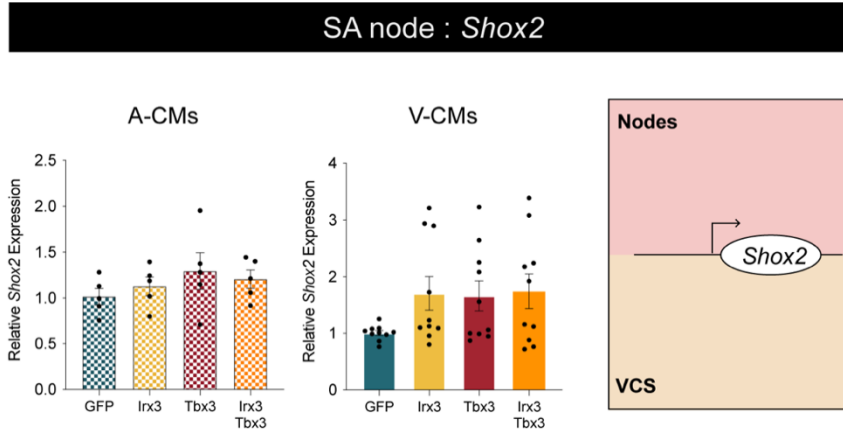


Figure 3.49 Region-specific transcriptional regulation of a SA node marker, *Shox2*, by *Tbx3* and *Irx3*. n = 5 per group for A-CMs, and n = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

Similarly, cAVN genes, *Prdm6* and *Rspo3* were unaffected by *Tbx3* and/or *Irx3* in A-CMs (**Figures 3.50 and 3.51**). However, in V-CMs, *Prdm6* and *Rspo3* were significantly repressed by *Irx3* overexpression, supporting their absence in the *Irx3*⁺ VCS. This observation suggests that their expression in cAVN might be primarily driven by other transcription factors. 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.

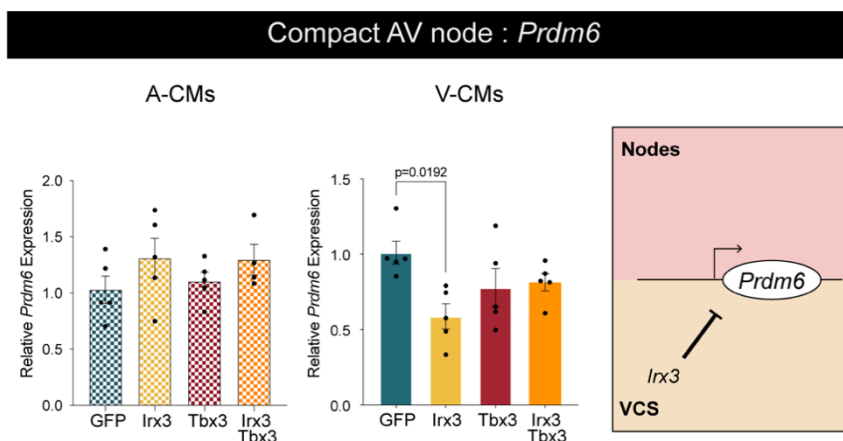


Figure 3.50 Region-specific transcriptional regulation of a compact AV node marker, *Prdm6*, by *Tbx3* and *Irx3*. n = 5 per group for A-CMs, and n = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

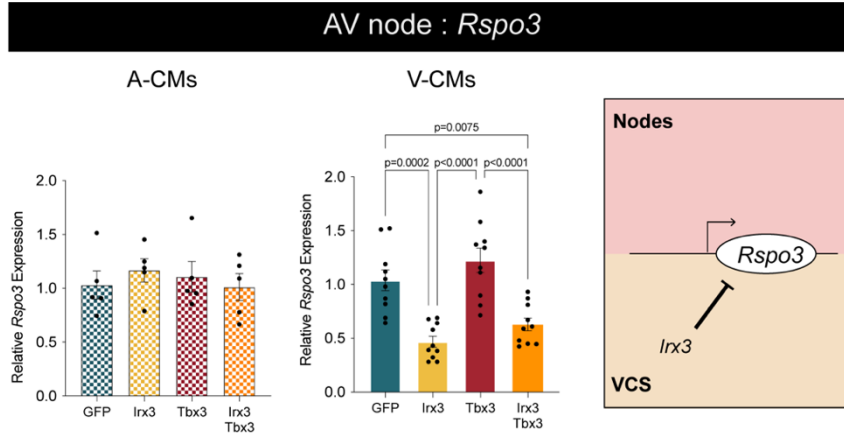


Figure 3.51 Region-specific transcriptional regulation of a compact AV node marker, *Rspo3*, by *Tbx3* and *Irx3*. $n = 5$ per group for A-CMs, and $n = 10$ for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

3.6.2.2 Transcriptional regulation of proximal VCS genes by *Tbx3* and/or *Irx3*

For genes enriched in the *Tbx3*⁺/*Irx3*⁺ proximal VCS, both *Tbx3* and *Irx3* appeared to function primarily as activators. For example, *S100a6* was upregulated by *Tbx3* and *Irx3* together in the V-CMs (**Figure 3.52**). Interestingly, this upregulation was largely driven by *Tbx3*, in agreement with the enriched *Tbx3*-binding peaks, while *Irx3* overexpression alone in V-CMs had no effect on its expression, consistent with the lack of *Irx* motifs in the *S100a6* gene (**Figure 3.42**).

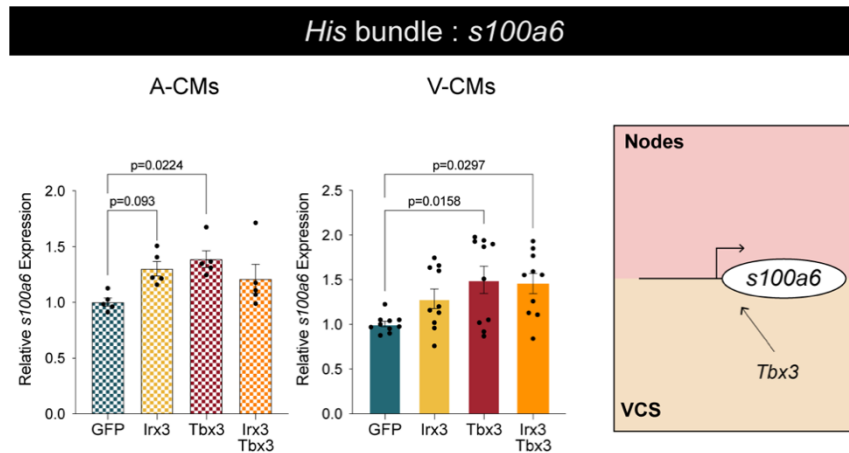


Figure 3.52 Region-specific transcriptional regulation of a *His* bundle marker, *s100a6*, by *Tbx3* and *Irx3*. $n = 5$ per group for A-CMs, and $n = 10$ for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

Additionally, *Pcp4* was significantly increased upon overexpression of both *Tbx3* and *Irx3* in A-CMs and V-CMs (**Figure 3.53**), supporting the cooperative activator properties of *Irx3* and *Tbx3* of the identified proximal VCS markers.

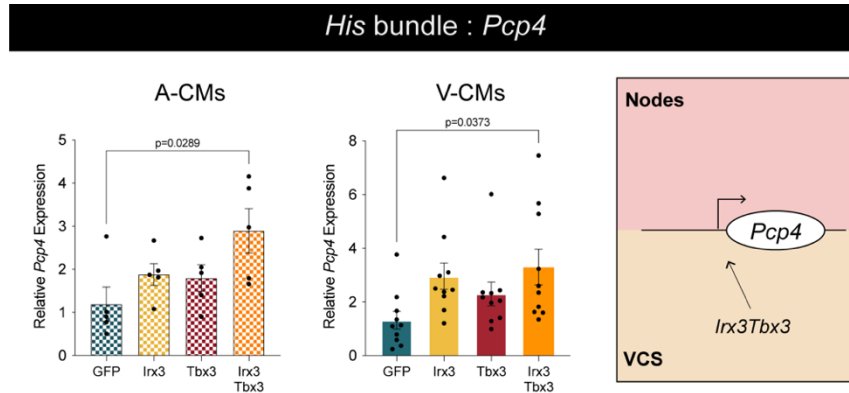


Figure 3.53 Region-specific transcriptional regulation of a *His* bundle marker, *Pcp4*, by *Tbx3* and *Irx3*. *n* = 5 per group for A-CMs, and *n* = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

For the markers identified in the *Tbx3*⁺/*Irx3*⁺ distal VCS, we observed an inverse regulatory relationship between *Tbx3* and *Irx3* compared to nodal genes: *Tbx3* as a repressor and *Irx3* as an activator. For example, *Irx3* overexpression significantly upregulated *Nppa*, *Scn10a*, and *Gja5* in both A-CMs and V-CMs, supporting their enrichments in the distal VCS (**Figure 3.54**).

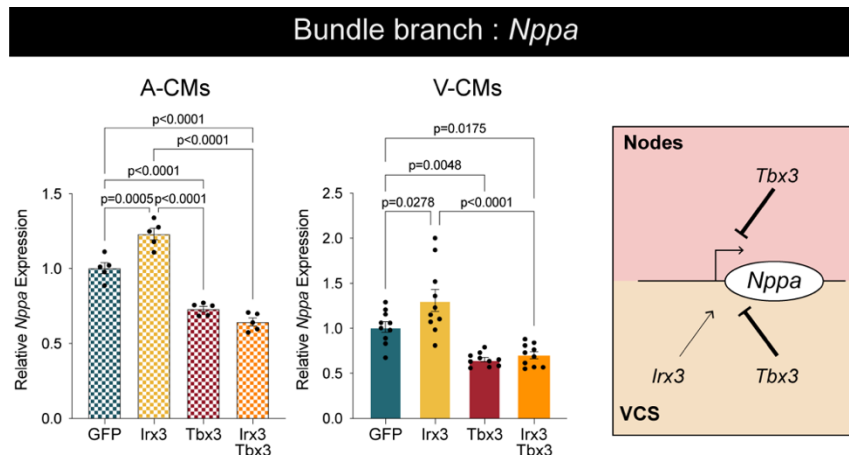


Figure 3.54 Region-specific transcriptional regulation of bundle branch marker, *Nppa*, by *Tbx3* and *Irx3*. *n* = 5 per group for A-CMs, and *n* = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

3.6.2.3 Transcriptional regulation of distal VCS genes by *Tbx3* and/or *Irx3*

For the markers identified in the *Tbx3*⁻/*Irx3*⁺ distal VCS, we observed an inverse regulatory relationship between *Tbx3* and *Irx3* compared to nodal genes: *Tbx3* as a repressor and *Irx3* as an activator. For example, *Irx3* overexpression significantly upregulated *Nppa*, *Scn10a*, and *Gja5* in both A-CMs and V-CMs, supporting their enrichments in the distal VCS (**Figures 3.55 - 3.58**). Importantly, when *Irx3* and *Tbx3* were overexpressed together, the activating role of *Irx3* on *Nppa*, *Scn10a*, and *Gja5* was largely abolished or reduced, demonstrating the dominant repressive role of *Tbx3* on these genes. These findings corresponded with *Tbx3*-binding peaks in *Nppa*, *Scn10a*, and *Gja5* (**Figure 3.42**), and explained their lower expressions in the *His* bundle. Notably, while *Tbx3* overexpression alone significantly repressed *Gja5* expression in A-CMs, it mildly increased *Gja5* levels in the V-CMs, suggesting that *Tbx3*'s function on *Gja5* depends on the chamber identity. Similarly, *Tbx3* and *Irx3* activated *Mpped2* and *Slit2* expression in the V-CMs, but had no impact on their expression in the A-CMs, suggesting chamber-specific actions of *Tbx3* and *Irx3* on these genes (**Figures 3.55 - 3.58**).

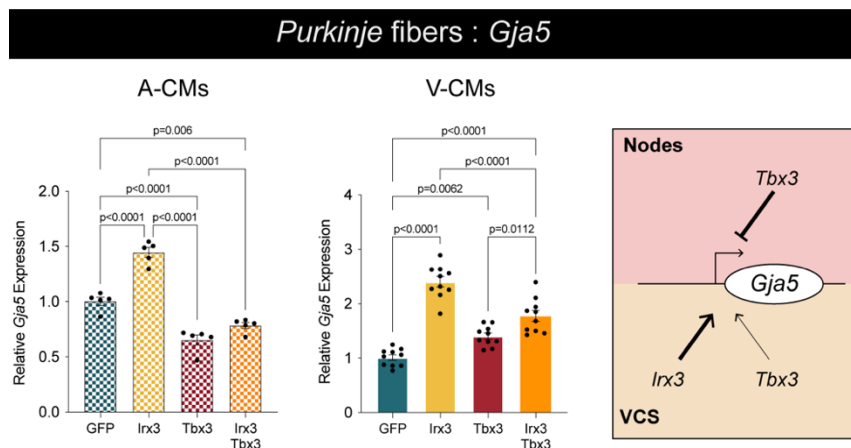


Figure 3.55 Region-specific transcriptional regulation of a distal VCS marker, *Gja5*, by *Tbx3* and *Irx3*. *n* = 5 per group for A-CMs, and *n* = 10 for V-CMs. Data are means ± SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

Purkinje fibers : *Scn10a*

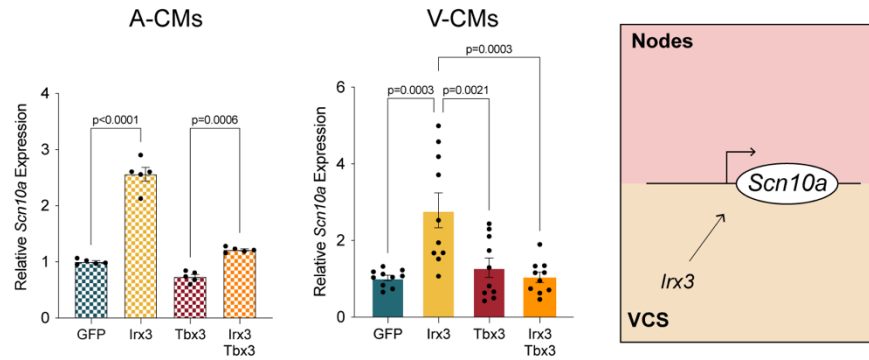


Figure 3.56 Region-specific transcriptional regulation of a distal VCS marker, *Scn10a*, by *Tbx3* and *Irx3*. n = 5 per group for A-CMs, and n = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

Distal VCS : *Mpped2*

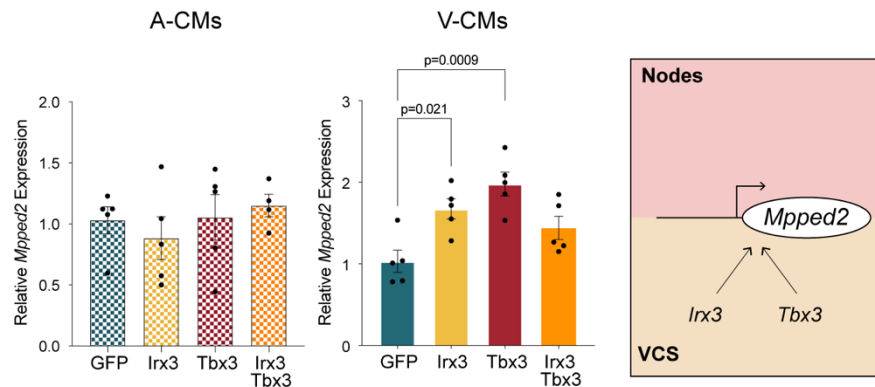


Figure 3.57 Region-specific transcriptional regulation of a distal VCS marker, *Mpped2*, by *Tbx3* and *Irx3*. n = 5 per group for A-CMs, and n = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

Distal VCS : *Slit2*

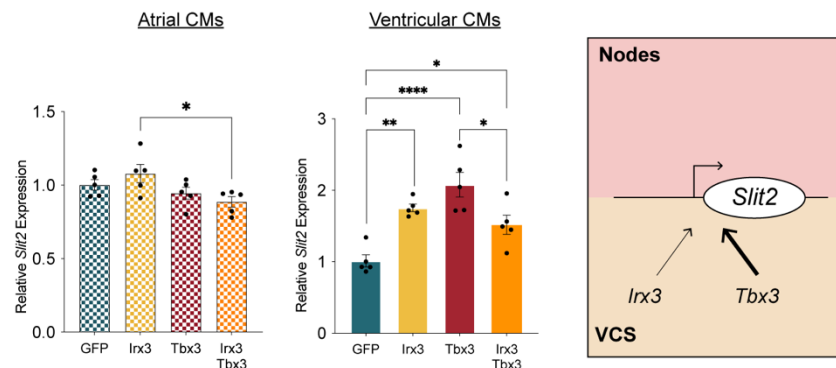


Figure 3.58 Region-specific transcriptional regulation of a distal VCS marker, *Slit2*, by *Tbx3* and *Irx3*. n = 5 per group for A-CMs, and n = 10 for V-CMs. Data are means $\pm$ SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

3.6.2.4 Transcriptional regulation of contractile myocyte genes by *Tbx3* and/or *Irx3*

Lastly, for genes enriched in the *Tbx3*⁻/*Irx3*⁻ contractile myocytes, *Tbx3* and *Irx3* mainly play a repressive role. For instance, *Gja1* and *Ryr2* were significantly downregulated by *Tbx3* and *Irx3* overexpression together in V-CMs (**Figures 3.59 and 3.60**), but not by *Tbx3* or *Irx3* alone. This demonstrates their synergistic repressive role in V-CMs and supports the absence of *Gja1* in the *His* bundle, but not in the *Purkinje* fibers. On the contrary, in A-CMs, *Tbx3* overexpression alone dominantly repressed the expression of both *Gja1* and *Ryr2*, corroborating with the detection of *Tbx3*-binding peaks (**Figure 3.43**) and previous reports on reduced *Gja1* and *Ryr2* expression in the SAN^{273,274}.

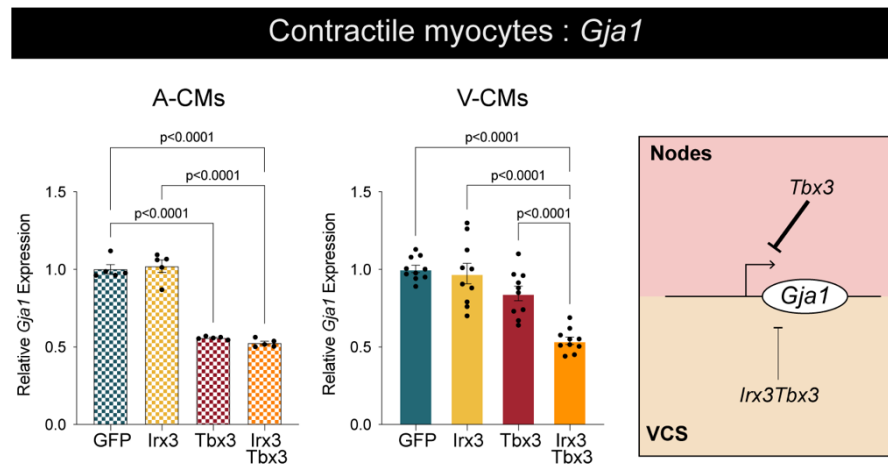


Figure 3.59 Region-specific transcriptional regulation of a contractile myocyte marker, *Gja1*, by *Tbx3* and *Irx3*. n = 5 per group for A-CMs, and n = 10 for V-CMs. Data are means ± SEM. One-way ANOVA and Tukey post hoc test. A-CM: atrial cardiomyocytes; V-CM: ventricular cardiomyocytes.

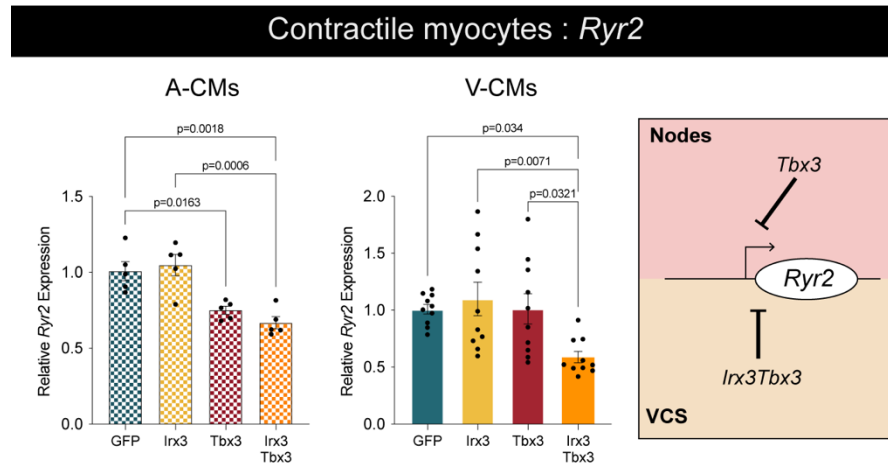


Figure 3.60 Region-specific transcriptional regulation of a contractile myocyte marker, *Ryr2*, by *Tbx3* and *lrx3*. *n* = 5 per group for A-CMs, and *n* = 10 for V-CMs. Data are means ± SEM. One-way ANOVA and Tukey post hoc test. A-CM, atrial cardiomyocytes; V-CM, ventricular cardiomyocytes.

Together, our *in vitro* study using primary atrial and ventricular cardiomyocytes successfully recapitulated the unique expression patterns of several CCS regional marker genes in a dish. Furthermore, gene expression analysis integrated with ATAC-seq, *Tbx3* ChIP-seq and *lrx* motif data suggests that the transcriptional regulatory program mediated by *lrx3* and *Tbx3* is dependent on CCS regions as well as heart chamber identity, thereby contributing to the molecular heterogeneity within the CCS.

SECTION 4 – DISCUSSION

4.1 Summary of findings

4.1.1 Molecular profiling of the global CCS

In this study, I conducted a comprehensive molecular and spatial profiling of the transcriptional landscape of postnatal mouse CCS cells at a single-cell resolution. My findings demonstrate how CCS components, from the SAN to PF, are molecularly continuous yet also distinct from each other. As noted in previous studies⁶⁴, major limitations of investigating the CCS include the low number of CCS cells in the heart (~0.1 - 0.2% of the entire cardiomyocyte population) and difficulties in isolating viable postnatal CCS cells. For our scRNA-seq analysis, I utilized approximately 7,000 CCS cells that were isolated from postnatal *Cntn2*^{Cre/+}; *Rosa26*^{tdTomato/+} mouse hearts and purified using FACS. Among several Cre lines targeting the CCS, including *Hcn4*-CreERT2²⁷⁵, *Cx40*-CreERT2²⁷⁶, *Gjd3*-CreEGFP⁴¹ and *Sema3a*-CreERT2²⁵², 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^{51,160,221}. To my knowledge, this study has incorporated the highest number of purified entire mouse CCS cells to conduct a single-cell transcriptomic investigation to date.

Transcriptional profiling of the CCS using scRNA-seq was further facilitated by integration with spatial transcriptomics. Among various spatial transcriptomic technologies available, we chose NanoString GeoMx Digital Spatial Profiler (DSP) for its several advantages. First, given that all CCS components cannot be fully captured in one or two histological sections, the GeoMX DSP offered a larger slide scan area that accommodated over 10 sections of the P4 heart encompassing all CCS regions. Second, its segmentation tool enabled precise capture of tdTomato-positive CCS cells only, allowing transcriptomic

assessments without the need for a cell-type deconvolution method. Lastly, it provided the whole transcriptome profiling. As a result, we were able to reliably acquire transcriptomic profiles of each CCS component.

My study identified several marker genes of each CCS component, while also consolidating recently recognized CCS markers. In addition, zonation patterns of genes were identified within the CCS: specifically, nodes consisting of the SAN and cAVN, proximal VCS consisting of the LNB, HIS and PBB, and distal VCS consisting of the DBB and PF. These marker genes include *Gnao1* and *Vsnl1* in SAN, *Prdm6* in cAVN, *Ppp1r17* in LNB, *S100a6* and *Igfbp7* in proximal VCS, and *Nppa*, *Scn10a* and *Mpped2* in distal VCS. A recent study has shown that *GNAO1* was found to be highly enriched in the human SAN, while *PRDM6* was predicted as a potential TF in human nodal cells²⁷⁷. This suggests possible evolutionarily conserved roles of these genes in the CCS. While previous studies have demonstrated that *Igfbp7* regulates pathological cardiac remodeling²⁷⁸ and that *S100a6*, a Ca^{2+} binding protein, modulates adverse ventricular remodeling²⁷⁹, it has not been tested whether they play physiological roles in the CCS. Together, it remains for future research to elucidate the functional implications of these CCS marker genes in both healthy and diseased hearts. It is important to highlight that the spatial information from ST significantly enhanced our ability to identify all CCS components in the scRNA-seq data. 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 may arise from varying numbers of cells in CCS components. Collectively, this integrative approach, supported by histological validations, enabled the discovery of CCS marker genes with greater reliability.

4.1.2 Molecular identification of the LNB and its functional relevance

Noteworthy among my findings, specifically solidified by our ST analysis, is the molecular identification and comparison of CCS components within the atrioventricular junction: two sub-compartments of AVN^{36,38,46}, the cAVN and LNB, as well as the HIS. Despite their anatomical proximity, my results indicate that the LNB shares more molecular similarities with the HIS than with the cAVN (**Figure 3.14b**). For instance, like the HIS, the LNB expressed both *Tbx3* and *Irx3*. This corroborates with the previous studies, demonstrating that LNB is developmentally originated from the ventricular myocardium, whereas cAVN is derived from Tbx2⁺ atrioventricular canal^{36,38,41,46}. Interestingly, the LNB expressed both atrial chamber genes (*MyI4*, *MyI7*) and ventricular chamber genes (*MyI2*, *MyI3*), suggesting a hybrid atrial and ventricular characteristic. While this unique expression profile as well as its origin suggests that the LNB may serve as a transitional structure bridging atrial and ventricular chambers, facilitating coordinated conduction across the chambers.

Given that the LNB is characterized as a component of the AVN, one might expect that it contributes to the conduction delay, the main function of the AVN. However, the LNB is known to be positioned anatomically along the slow pathway (**Figure 1.4**), which typically does not transmit impulses directly to the HIS bundle under normal conditions. Moreover, we observed through serial histological sections that the LNB is physically connected to the HIS, which shows a discrepancy between its anatomy and its documented electrophysiological properties. These seemingly paradoxical and ambiguous properties of the LNB raise intriguing questions regarding its role in signal conduction under normal physiological conditions. In pathological conditions, such as atrioventricular nodal reentrant tachyarrhythmias (AVNRT), its ablation is the most common treatment of choice¹⁸¹, reinforcing its importance. Additionally, inconsistencies in terminology used within the literature, where similar structures within the AVJ are variably referred to as cAVN, LNB or

INE, emphasize the need for standardizing the nomenclature to clarify these distinctions and consolidate critical data to properly understand its function and structure.

4.1.3 Embryonic to early postnatal developmental transitions of the CCS

We found high molecular similarities between E16.5 and postnatal stages within the same CCS regions, suggesting that cell specifications within the CCS, particularly for the SAN, cAVN and proximal VCS, may largely occur by E16.5. On the other hand, higher expression of early-development genes, such as *Tagln*, *Cnn1*²⁶⁷, *Bmp10*²⁶⁸ and *Corin*²⁶⁹, was noted in the E16.5 CCS, compared to the postnatal CCS, indicating CCS maturation during pre-to-postnatal development. *Corin* is a notable gene that encodes a transmembrane cardiac serine protease, which activates *Nppa*-encoded ANP by cleaving pro-ANP²⁸⁰. Since ANP signaling is implicated in embryonic VCS development²⁸¹, our findings raise a possibility that *Corin*-ANP signaling may be crucial for CCS development and maturation. Additionally, our observation that more than 3% of early postnatal CCS cells are still proliferating, highlights the ongoing progress of postnatal CCS maturation. Further studies are needed to examine the molecular profiles of fully mature adult CCS cells. As conducted in a series of postnatal cardiomyocytes (from P0 - P28)²⁸², it would also be informative to examine the trajectory of CCS postnatal maturation and identify a mediator in this developmental process.

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)¹⁴³ as well as *Isl1*¹⁴⁶ were undetected in the postnatal SAN region of our scRNA-seq and ST data. Considering that *Tbx18* and *Isl1* are involved in early SAN development and their expression diminishes in the adult heart¹⁴⁶, 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.

4.2 Distinct and overlapping transcriptional regulation of the CCS by Tbx3 and Irx3

In the CCS, which is essentially a continuous structure, molecular heterogeneities govern distinct functional properties of each CCS component, ensuring harmonious contraction and relaxation of the heart. While the roles of individual transcription factors in the development and function of CCS components have been extensively studied²⁸³⁻²⁸⁵, our understanding of how these transcription factors cooperate across different regions of the CCS is comparatively limited. In our study, we utilized SCENIC analysis to infer gene regulatory networks within the CCS. We found that the CCS was controlled by well-established TFs such as Shox2, Gata6²⁷⁰, Tbx3^{162,163,168,271}, Tbx5^{145,164,168}, and Etv1^{160,272} as well as by less explored TFs, including Nr2f1, Atf5, and Bach1. It is also notable that CCS-specific regulons were largely clustered within the nodes only, nodes & proximal VCS, and the VCS, suggesting the zonation patterns of gene regulatory networks within the CCS. As expected, Tbx3 regulon activity was enriched in the nodes as well as in proximal VCS. Although the regulon activity specifically for Irx3 was not identified, the activity of Irx2, which shares the same binding motif as Irx3, was found enriched in the VCS, leading us to further investigate the co-regulatory roles of Tbx3 and Irx3.

I utilized an *in vitro* model with primary atrial and ventricular cardiomyocytes overexpressing *Irx3* and *Tbx3*, along with ATAC-seq, ChIP-seq and motif analyses to provide proof-of-principle evidence of their contributions to the establishment of molecular heterogeneities within the CCS. Specifically, my findings suggest that Tbx3 and Irx3 play individual, cooperative, or antagonistic actions in a chamber-specific and CCS component-specific manner. Notable examples include the activation of *Vsnl1*, a recently identified SAN

marker, exclusively in A-CMs, not in V-CMs. Conversely, distal VCS genes, such as *Mpped2* and *Slit2*, were increased by *Tbx3* and *Irx3* in V-CMs but not in A-CMs. Furthermore, the well-known *Tbx3* target, *Gja5*, was repressed by *Tbx3* in A-CMs, but was mildly activated by *Tbx3* in V-CMs. *Irx3*-mediated increase of *Gja5* expression was also diminished by *Tbx3* overexpression in the A-CMs. These data suggest that *Tbx3* and *Irx3* can play distinct functional roles in gene regulation in the neonatal mouse atrial and ventricular cardiomyocytes.

In conclusion, this study generates a transcriptomic resource of the postnatal murine CCS and provides functional evidence of how molecular heterogeneities across the CCS can be transcriptionally established. These findings help advance our understanding of the molecular identities within the CCS beyond its anatomical structures and, therefore, have implications for therapeutic approaches against conduction system defects.

4.3 Limitations

4.3.1 Capture of the CCS

In this study, the capture of CCS cells for both scRNA-seq and spatial transcriptomics was achieved using *Cntn2*-mediated recombination of tdTomato. This enabled precise isolation and enrichment of a purified population of CCS cells, while minimizing contamination from non-CCS cells. Although tdTomato signal in the postnatal mouse heart well-overlapped with endogenous *Cntn2* expression, my analysis suggests a possibility that *Cntn2* does not mark the entire CCS, such as subpopulations of the SAN – SAN-head and SAN-tail. In addition, transitional cells, bridging the conduction system and the surrounding myocardium⁶⁴, appear to be unmarked by *Cntn2*. However, I did observe a few genes with expression patterns resembling the anticipated positions of transitional cells. Among these, *Mpped2* and *Scn10a* expressions were slightly broader than the tdTomato-positive PFs in our fluorescence *in situ* hybridization (FISH) and immunofluorescence validations. Several *SCN10A* variants have

been described to be associated with conduction disorders in humans²⁸⁶. *Mpped2* expression was found to be ventricular-specific in early developing hearts of both mice (E10.5)²³³ and zebrafish (2 - 4 cell stage)²⁸⁷, suggesting its chamber-specific role. This raises the possibility that *Mpped2* and *Scn10a* could be expressed in transitional cells within the mature CCS, even if transitional cells were not fully captured by our *Cntn2*-Cre model. The absence of transitional cells in our study may be accounted by several biological or technical reasons.

Biologically, it is possible that *Cntn2* does not label transitional cells at all. Since *Cntn2* is a marker specific to the “postnatal” CCS, it is unclear whether the transitional cells identified in the E16.5 dataset will ever express *Cntn2*, even postnatally. Goodyer WR. *et al.* (2019) indeed has shown that these transitional cells, such as transitional-SAN and transitional-PF, express low or absent levels of the well-established CCS marker, *Hcn4*. A similar pattern may apply to *Cntn2*. In a scenario where *Cntn2* does label transitional cells, it is possible that four postnatal days is insufficient for recombination to fully occur in these cells. This would suggest that recombination efficiency might differ across different regions of the CCS. Supporting this, my epifluorescence imaging of the CCS-tdT (*Cntn2*-Cre;*Rosa26*^{tdTomato}) heart showed notably weaker tdTomato signal in the P4 SAN, compared to P42 SAN (**Figure 3.2**), which may also hold true for transitional cells.

Technically, it is also possible that the subtle differences in the molecular profiles between CCS cells and transitional cells were not detected bioinformatically. To test this, I have performed extensive clustering on the global CCS (**Figure 4.1**), as well as in subsets of the CCS (**Figure 4.2**) using several combinations of bioinformatical parameters (variable features = 500, 1,000, 1,500, 2,000; dimensions = 10, 20, 25, 30; resolutions between 0 and 1, at increments of less than 0.05).

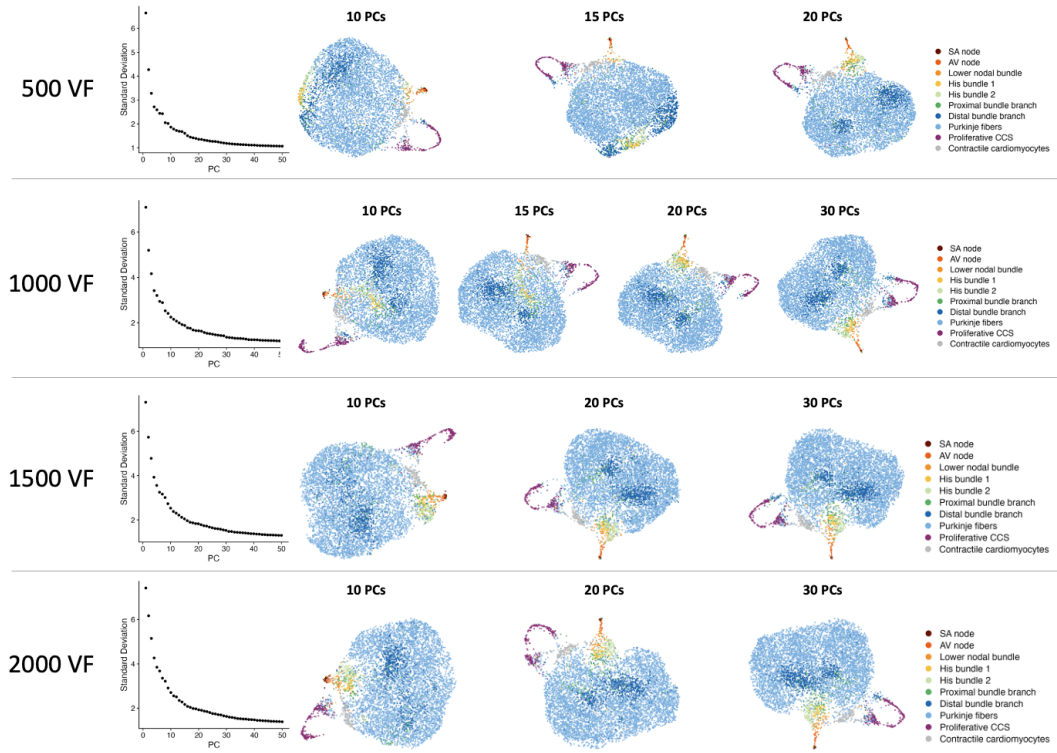


Figure 4.1 Examples of clustering of the global CCS using number of variable features (VF) and number of principal components (PC).

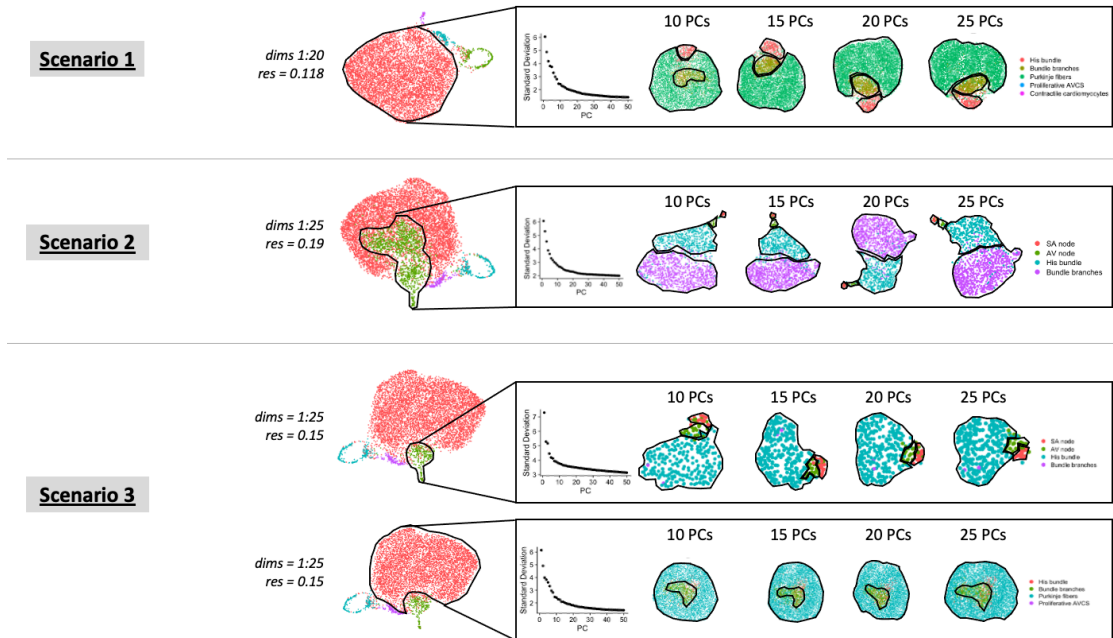


Figure 4.2 Examples of clustering of CCS subpopulations using various number of dimensions (dims), resolution (res) and number of principal components (PCs).

In addition, in our dataset, I was unable to identify known subpopulations of the SAN, specifically the SAN-head. SAN-head is characterized by *Shox2*⁺/*Nkx2.5*⁻; while SAN-tail by *Shox2*⁺/*Nkx2.5*⁺¹⁴⁸. However, nearly all the SAN cells in our data expressed high levels of *Nkx2.5*, suggesting that the cells labelled as “SAN” are likely SAN-tail cells. The differential expression of *Nkx2.5* within the SAN is noteworthy, as it is perhaps the most well-characterized cardiac markers described. The absence of *Nkx2.5* in SAN-head cells highlights their distinct molecular identity. Studies have shown that the SAN-head and SAN-tail originate from distinct developmental progenitors¹⁴³, which explains their divergent expression of these general cardiac markers. This may also be the case for a mature CCS marker, *Cntn2*, which in turn could account for why *Cntn2* was unable to label all SAN populations.

4.3.2 Histological validation of CCS markers using antibodies

In this study, markers identified through bioinformatic analysis of integrated scRNA-seq and spatial transcriptomics were validated histologically. Protein expression was confirmed through immunofluorescence and mRNA expression was validated through FISH, in cases where IF was not successful. While these methods are the most standard approach for validating computational findings, we faced several challenges, especially in finding functional antibodies. All antibodies used were tested using two proximal sections of the heart ~6 µm apart – one stained using both primary and secondary antibodies, and the other a negative control only without the primary antibody. Antibodies were considered ineffective if no noticeable difference was observed throughout the whole heart between the negative and positively stained sections and, therefore, were not pursued. In total, 21 antibodies were tested, with 9 effective and validatory and 12 ineffective or non-supporting antibodies. Details of these antibodies are shown in **Table 4.1**.

Target Protein	Catalogue #	Description of immunofluorescence results
Cdh6	HPA007047	Not specific to CCS
Gcgr	26784-1-AP	Not specific to CCS
Igfbp4	NBP1-80549	Not specific to CCS
Irf1	8478S	Antibody did not work, observed native autofluorescence from tissue only
Irs2	#4502 (CST)	Not specific to CCS
Jun	#9165 (CST)	Not specific to CCS
Lcp1	13025-1-AP	Not specific to CCS
Lyz2	MA5-32154	Not specific to CCS
Magp-1/MFAP2	AF4977-SP	Not specific to CCS
Nsg2	PA563263	Not specific to CCS
Psd3	NBP2-55678	Antibody did not work, observed native autofluorescence from tissue only, FISH attempted using designed probes, but was unable to capture the specific variant
Sbk2	HPA030631	Not specific to CCS

Table 4.1 List of all tested antibodies for histological validation of CCS markers.

The high number of ineffective antibodies may be attributed to the fact that many of the markers identified in this study were novel, with limited prior knowledge about their expression patterns or functions in the CCS or even in the whole heart. Moreover, not all antibodies were suitable for histological assessments, but rather for other applications, such as western blot, FACS, or immunohistochemistry (IHC). There were antibodies which showed that the tested proteins were not CCS- or cardiomyocyte-specific. This is not unexpected, as our scRNA-seq and spatial transcriptomics data, were highly specific to the CCS only, leading to identifications of CCS marker genes, which are also expressed in other cardiac cell types (i.e. fibroblasts, endothelial cells, or macrophages). Additionally, different fixatives have been suggested for different tissues. One notable study explored techniques to improve histological staining methods, including immunofluorescence and immunohistochemistry, in neuronal tissues²⁸⁸. Their findings demonstrated that glyoxal-based fixation significantly improved immunoreactivity and antibody penetration compared to the commonly used 4% paraformaldehyde (PFA), which was also utilized in my study.

Given that the CCS shares molecular similarities with neurons⁵¹, alternative fixation methods could potentially increase the success rate of antibody staining. In addition, mRNA and protein often do not always display a linear relationship due to processes involved in post-transcription, post-translation, as well as technical limitations in mRNA and/or protein assessments²⁸⁹. In a study examining the relationship between mRNA and protein levels in undisturbed, or 'steady state' mammalian cells, it was found that mRNA variance accounted for 56 - 84% of the protein variance, emphasizing the more complex nature of mRNA-to-protein transitions. While our study sought to improve data reliability by incorporating spatial transcriptomics as an additional layer of high-throughput data, these findings highlight the necessity of thorough validation of high-throughput studies.

4.3.3 *In vitro* model of the CCS

This study utilizes an *in vitro* model to mimic different regions of the CCS, where neonatal mouse atrial and ventricular cardiomyocytes (A-CMs and V-CMs) were isolated and were overexpressed with CCS-specific transcription factors, *Tbx3* and/or *Irx3*. Specifically, A-CMs and V-CMs from neonatal mouse hearts were isolated, cultured, and infected with adenoviruses overexpressing either GFP, *Tbx3* and/or *Irx3*. While this model successfully recapitulated some of the regional heterogeneities that were identified through our scRNA-seq and spatial transcriptomics, there are a few limitations. First, the introduction of high levels of TFs may not be an accurate reflection of physiological conditions. Given that transcription factors are responsible for controlling gene regulatory networks, the method of their introduction, as well as their precise levels or timings of transcription factors can alter cell homeostasis, disease status or even the identity or fate of cells²⁹⁰. In our model, the overexpression of transcription factors was performed *in vitro*, which inevitably introduces an artificial environment compared to the physiological *in vivo* conditions. Furthermore, the degree of adenovirus-mediated overexpression led to expression levels much above the

physiological norms, potentially introducing secondary effects unrelated to Tbx3 and/or Irx3. Another limitation of this model is that A-CMs and V-CMs used contain not only CCS cells but also contractile cells from the atria and ventricle, respectively; therefore, the effects of Tbx3 and/or Irx3 may not accurately show transcriptional effects specific to the CCS only. Moreover, transcription factors were introduced to the cells specifically at early postnatal stages, suggesting that the responses we observed may be unique to this developmental period. As a result, the transcriptional regulation by Tbx3 and/or Irx3 that we present may not hold true if these TFs were introduced to cells during embryonic stages or fully developed adult stages, where their effects could differ. I anticipate that the overexpression of TFs during developmental stages more accurately represents the physiological processes, potentially better capturing their roles in their transcriptional roles and establishing the molecular heterogeneity in the CCS. Given these limitations, further investigation is warranted to elucidate the precise regulatory mechanisms by which the gene dosage balance between Tbx3 and Irx3 fine-tunes gene expression in a developmentally more potent system.

Nevertheless, in order to overcome these challenges, we have performed additional analysis using datasets published by several independent studies, such as the accessibility peaks (ATAC-seq) from different CCS components (i.e. SAN, Tbx3⁺ atrioventricular junction), Tbx3-binding (TBX3 ChIP-seq) and *Irx* motifs. By investigating the combination of these datasets, we have 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. Therefore, despite the limitations of the *in vitro* model used in the study, I believe this study, together with simultaneous examination of several published works, provides proof-of-principle evidence of their contributions to CCS regional heterogeneity in chamber-specific manners.

4.3.4 Cooperative actions of other CCS transcription factors

Our work highlights the critical transcriptional role of two CCS-specific TFs, Tbx3 and Irx3. Each of these TFs is well-recognized as a critical regulator of CCS development as well as function^{50,53,141,143,163,176,187,193}. However, the proper development of the CCS is a complex process that requires the coordinated action of many TFs working in a time- and region-specific manner.

Even for Tbx3 and Irx3, evidence strongly suggests that their roles are not independent; rather, they exhibit significant co-regulatory functions. Irx3, for instance, demonstrate bidirectional transcriptional roles as activators or repressors⁵³. This dual functionality, especially its repressive role, may involve its interactions with an unidentified repressor, suggesting that Irx3's regulatory function is likely more complex and involves a cooperative network with other TFs. Studies have also shown that Irx3 interacts with key cardiac TFs, Tbx5 and Nkx2.5, to directly regulate its downstream target, *Gja1*⁵³. Additionally, members of the *Irx* family are also able to compensate for each other's roles in other physiological systems. For instance, in a single KO of *Irx* TFs, the phenotypes were shown to be relatively mild, but in combined KO models, more severe defects are observed, such as *Irx3* and *Irx5* KOs, in bone and limb development²⁹¹⁻²⁹⁵. Similarly, previous studies on Tbx3 have also shown its co-regulatory functions in the CCS through interaction with other TFs. Among *Tbx* members, gene dosage between Tbx3 and Tbx5 has been shown to be crucial for determining whether the CCS adopts a slow-conducting nodal-like identity, or a fast-conducting VCS-like identity, respectively¹⁶². Mechanistically, this is driven by Tbx5 activating Tbx3 expression, and Tbx3 counteracting Tbx5's activation of genes by Tbx5, such as *Cx40* and *Nppa*. This emphasizes the tightly regulated interaction among the *Tbx* TF family members. Furthermore, Tbx3 interacts with the same TFs as Irx3, such as Tbx5 and Nkx2.5, in regulating its downstream target, *Gja5*^{50,141}.

Collectively, these findings suggest that *Tbx3* and *Irx3* are not simply working independently in their regulatory actions. Rather, they are part of a larger transcription network that involves a complex interplay of TFs, potentially the TFs introduced in the Introduction (**Section 1.3.4**) as well as our results (**Section 3.6**), working in concert to orchestrate the molecular heterogeneity of the CCS. In order to understand this, more mechanistic studies are needed. For instance, direct binding sites for *Irx* TFs, as well as other co-regulatory TFs, remain to be identified. It is also unknown whether the novel CCS markers identified in our study are direct or indirect targets of *Irx3* and/or *Tbx3*. Thus, more mechanistic studies on *Tbx3* and *Irx3*, as well as other TFs, in the context of chamber-specific or CCS region-specific gene regulation would significantly enhance our understanding of transcriptional regulatory networks in the CCS.

4.3.5 Postnatal maturation of the CCS

The completion of CCS maturation occurs postnatally. Since deficiencies in *Nkx2-5*, *Tbx3*, and *Irx3* lead to CCS hypoplasia in the postnatal mouse (as early as P4), but not during embryonic development^{50,162,163}, it was unexpected that no significant differences in the transcriptomic profiles between P1 and P4 were observed in our scRNA-seq results (**Figure 3.17**). This observation led us to consider a few possibilities: (a) the temporal duration between P1 and P4 may be too narrow to capture the transcriptional differences; (b) postnatal CCS development may be primarily regulated and established by the embryonic transcriptional program; and/or (c) the subtle molecular differences may have been concealed during cell isolation.

It is possible that major changes in postnatal maturation occur at later postnatal stages, such as P14 or even much later, as suggested by several studies investigating the maturation of general cardiomyocytes. The possibility that postnatal CCS heterogeneity is largely already determined embryonically was tested by directly comparing our postnatal

CCS scRNA-seq data with embryonic CCS data⁶⁴, which indeed showed high overall similarities. However, there were certainly evidence of maturity differences, suggesting that while the CCS may be embryonically pre-established, there is still postnatal maturation taking place. Consistently, studies have shown evidence for transcriptional transitions peaking between P8 - 15²⁹⁶. Another group using bulk RNA-seq of P1, P7, P21 and P56 primary mouse cardiomyocytes observed almost identical transcriptomic profiles (~89% similarity) between P1 and P7, and a much distinct profile at P21²⁹⁷, suggesting a transcriptomic switch occurs after P7. Similarly, studies using various neonatal mouse models of cardiac injury (e.g. apical resection, myocardial infarction or cryoinjury) have shown that the regenerative capacity of cardiomyocytes is largely diminished at P7, consistently indicating a transition from an immature proliferative state to a more mature differentiated state²⁹⁸⁻³⁰⁰. Like general cardiomyocytes, CCS cells also undergo postnatal maturation at later time points, as evidenced by the delayed phenotypes observed in mice lacking CCS- or cardiac-specific TFs, such as Tbx3¹⁶³ or Tbx5¹⁴⁴, which manifest only at later developmental stages, such as P21. Interestingly, in a study using genetic tracing of trabecular derivatives (Cx40^{CreERT2}) in a model of neonatal myocardial infarction, PFs were found to have limited regenerative capacities, but they were found to contribute to the regenerated myocardium upon myocardial infarction³⁰¹, suggesting that the postnatal maturation process of contractile cardiomyocytes and CCS cells may be interconnected or interdependent. However, the exact timing of their postnatal plasticity, as well as its distinct or overlapping molecular mechanisms, need further clarification.

Another possibility that we observed no molecular differences between P1, P2 and P4 CCS cells is that any variation may not have reached statistical significance, especially given such purified population of cells. There may also be technical reasons involved during digestion and isolation of cells, where cells at all stages may have exhibited similar stress response, therefore, changing their genetic program and masking the physiological

difference between the three stages. Due to technical challenges in isolating healthy primary cardiomyocytes required for scRNA-seq, P4 was the latest stage for maximizing both cell yield and quality. However, with the development of single-nucleus RNA sequencing (snRNA-seq), which allows the use of frozen samples instead of viable cells, or large-particle FACS (LP-FACS), which enables the sorting of larger cardiomyocytes³⁰², it would be interesting to study the cellular populations at later postnatal stages. Future studies using this approach would provide valuable insights into the transcriptomic changes that occur within a wider postnatal window, such as P7 or P14.

4.3.6 CCS in normal and diseased human hearts

While our study shows strong evidence of the molecular heterogeneity within the postnatal mouse CCS and its high overall similarity to embryonic mouse CCS, whether or not these molecular characteristics are conserved in humans remains unclear. The investigation of the human CCS has been a great challenge, particularly due to the limited availability of high-throughput data of human heart samples. Since CCS cells make up only a small fraction of the heart, they are often undetected or underrepresented in these studies. Moreover, to comprehensively examine different components of the CCS, samples from specific anatomical regions of the hearts must be collected. For example, PFs from the ventricular free wall; the HIS from the top of the IVS; and the SAN from the RAA. An important piece of work that remains to be thoroughly investigated is the molecular remodeling that occurs under stress or in disease conditions. This is particularly important as remodeling of the CCS is frequently observed in arrhythmic pathologies (**Section 1.2**). For example, the HIS-PF system was found to play a significant pathogenic role in both triggering and maintaining ventricular arrhythmias, the leading cause of cardiac mortality^{14,303}. Also, a wide range of genetic variants in PF-enriched genes, such as *SCN5A*^{68,69,71,75,91}, *SCN10A*^{286,304-306}, *IRX3*^{198,292,307} and *GJA5*^{308,309}, demonstrate strong associations with cardiac conduction

diseases, underscoring how altered molecular compositions in the PFs can directly relate to conduction diseases.

To address this gap in our knowledge, we first sought to investigate the CCS heterogeneity in normal humans and further compare its molecular characteristics to those of mice. Recently, two snRNA-seq studies in humans were published – one from a fetus heart of 9-16 post-conception weeks³¹⁰ and another from 25 - 75 year old adult hearts²⁷⁷. We computationally isolated CCS cells and performed comparative analysis with our data on the postnatal mouse CCS and embryonic E16.5 mouse⁶⁴, in order to investigate developmentally- and species-conserved molecular profiles of the CCS. We have further explored possible disease associations based on conserved and unique CCS marker genes. Preliminary works of this analysis are shown in **Section 5.2**.

SECTION 5 – FUTURE DIRECTIONS

5.1 The role of postnatal *Irx3* in VCS maturation

Previous works on *Irx3* in the VCS showed that *Irx3*KO mice exhibit morphological and electrophysiological defects with hypoplastic and reduced fiber densities, as well as right bundle branch block (RBBB)⁵⁰. These defects were observed at P4, but not P0, suggesting that *Irx3* plays a specific role during early postnatal development. Interestingly, despite these findings, our MULTI-seq experiment in the CCS showed no significant difference in its molecular profiles at P1, P2 and P4. However, the functional implication of *Irx3* during postnatal VCS development and maturation remains to be elucidated. To investigate the role of postnatal *Irx3* during VCS establishment, early maturation and maintenance, postnatal VCS-specific *Irx3* KO (*Irx3*^{VCS-KO}) mice were generated by crossing *Cntn2*^{3'UTR-IRES-Cre-EGFP}, a postnatal VCS-specific Cre, with *Irx3*^{fllox} mouse and *Rosa26*^{tdTomato} mouse lines (Figure 5.1).

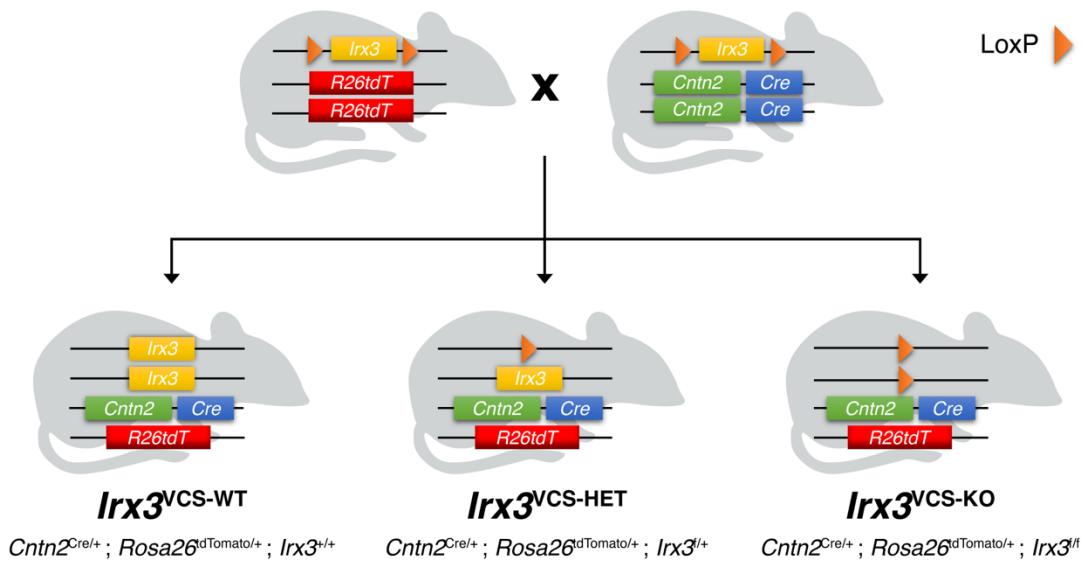


Figure 5.1 Breeding schematic of postnatal VCS-specific *Irx3* wildtype (WT), heterozygous (HET) and knock-out (KO) reporter mouse

5.1.1 The role of postnatal *Irx3* in VCS electrophysiology

For all electrophysiological assessments, the control group consisted of *Cntn2*^{+/+};*Irx3*^{fl/+}, *Cntn2*^{+/+};*Irx3*^{fl/fl} and *Cntn2*^{Cre/+};*Irx3*^{+/+}, as there was no significant difference in the phenotypes between these genotypes (data not shown). sECG data showed that 8-week-old *Irx3*^{VCS-KO} mice have a nearly significant prolongation of ventricular activation duration (indicated by increased QRS intervals) compared to control mice (p=0.0513) (**Figure 5.2**), while other electrophysiological parameters, such as RR interval and QT interval, showed no significant difference (data not shown). Although not significant, there was a trend towards QRS prolongation with the loss of each *Irx3* allele, suggesting a possible gene dosage effect of *Irx3* in VCS function. This mild phenotype, however, was only evident in 8-week-old adult mice, but not in postnatal day 4 (P4) or 3-week-old *Irx3*^{VCS-KO}. Also, there was no evident bundle branch block (indicated by notched R' waves) in *Irx3*^{VCS-KO} hearts at any age. This suggests that postnatal *Irx3* may be dispensable for VCS establishment or early VCS maturation, but rather is more important for VCS maintenance.

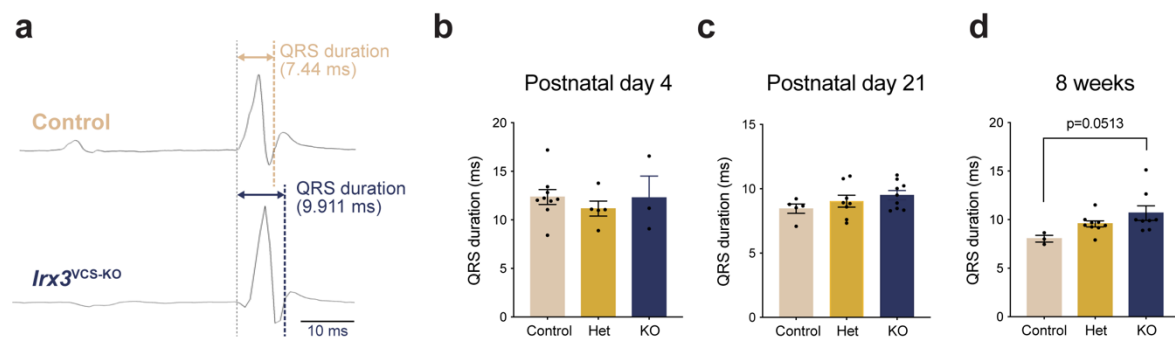


Figure 5.2 Electrophysiological assessment of *Irx3*^{VCS-KO} mice at different postnatal development stages. (a) Representative surface ECG traces of 8-week-old control (top) and *Irx3*^{VCS-KO} (bottom) mice. (b-d) QRS duration in control, HET(*Irx3*^{VCS-Het}) and KO (*Irx3*^{VCS-KO}) mice at (b) postnatal day 4 (P4), (c) P21, and (d) 8 weeks. *P-value determined by one-way ANOVA

5.1.2 The role of postnatal *Irx3* in VCS morphology

VCS morphology assessments showed that VCS structure does not contribute to the mild phenotype observed in *Irx3*^{VCS-KO}. VCS morphology in *Irx3*^{VCS-KO} was comparable to that

from *Irx3*^{WT} adult hearts from published results⁵³. There was no noticeable difference in the gross VCS structure, thickness or density between *Irx3*^{VCS-WT}, *Irx3*^{VCS-Het} and *Irx3*^{VCS-KO} mice (Figure 5.3).

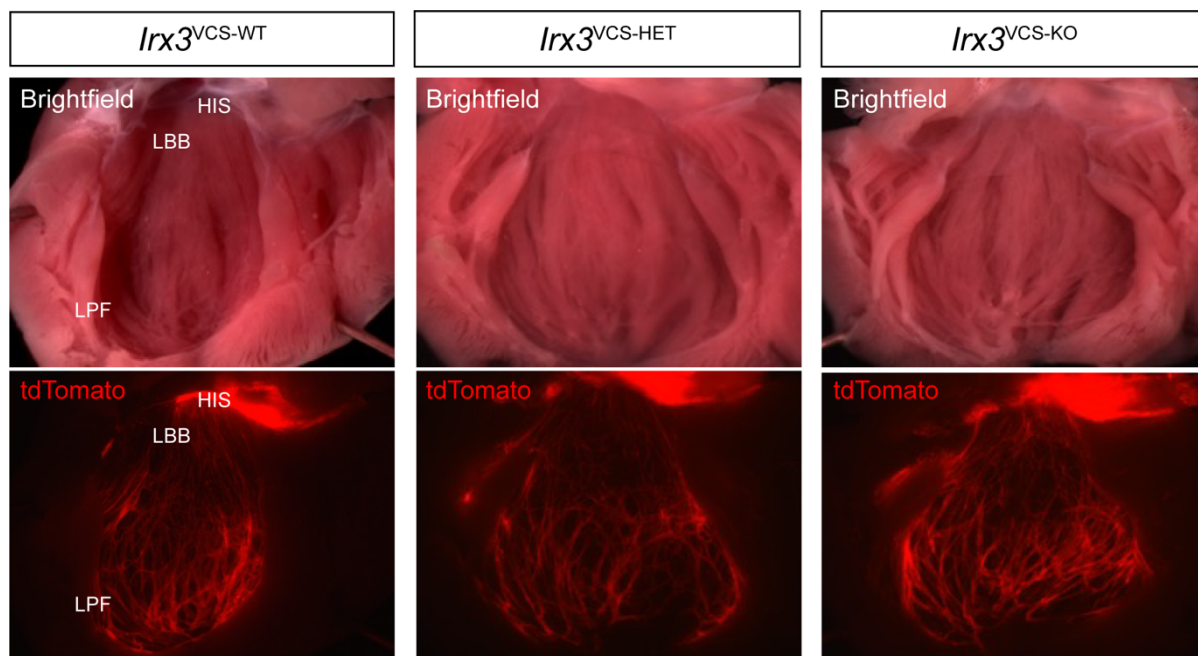


Figure 5.3 Epifluorescence imaging suggests *Irx3*^{VCS-KO} hearts have grossly normal VCS morphology. VCS structure in *Irx3*^{VCS-WT}, *Irx3*^{VCS-HET} and *Irx3*^{VCS-KO} hearts. HIS, His bundle; LBB, Left bundle branch; LPF, Left Purkinje fiber.

5.1.3 Summary of preliminary findings and discussion

No noticeable morphological and electrophysiological differences were observed between controls and *Irx3*^{VCS-KO} hearts. This result is unexpected, considering our previous results from whole-body *Irx3* KO mice (*Irx3*^{LacZ/LacZ}). Compared to postnatal VCS-specific *Irx3* KO mice (i.e. *Irx3*^{VCS-KO}), whole-body *Irx3* KO mice (i.e. *Irx3*^{LacZ/LacZ}) displayed a much more severe electrophysiological phenotype with a more prolonged QRS (10.7 ms in *Irx3*^{VCS-KO} vs. 12.2 ms in *Irx3*^{LacZ/LacZ})^{50,53} with prominent notched R' waves, indicative of prolonged ventricular activation and a block in conduction in the bundle branches, respectively. This suggests that even though it appears that postnatal *Irx3* is important for VCS maintenance,

embryonic *Irx3* expression is crucial for the overall process of early postnatal VCS maturation as well as maintenance.

Unlike my initial hypothesis, and as described in the results section above, loss of postnatal *Irx3* in the VCS did not lead to significant changes in ventricular activation (QRS interval), or bundle branch blocks (R' wave notches). It was only seen in 8-week adult mice that QRS intervals reached a nearly significant and mild prolongation in the QRS interval compared to controls. Moreover, in *Irx3*^{VCS-KO} hearts, the VCS morphology had no observable structural abnormalities, contrary to what was found in whole-body *Irx3*KO hearts, where fiber density as well as CX40-derived fluorescence intensity in the VCS were significantly reduced. This suggests that the mild prolongation in the QRS interval in postnatal VCS-*Irx3* KO mice is not a consequence of abnormal VCS structure.

It is possible that there are other mechanisms that may explain such mild phenotype. Since *Irx3* is able to transcriptionally regulate the expression of Cx40 and Cx43, loss of *Irx3* may alter the expression level or pattern of these gap junctions, leading to delayed electrical propagation, resulting in prolonged QRS durations. It should also be noted that the loss of *Irx3* has not been fully validated in the *Irx3*^{VCS-KO} to validate if *Irx3* expression is truly diminished in *Irx3*^{VCS-KO} compared to *Irx3*^{VCS-WT}. This will confirm the deletion efficiency in these mice. Nevertheless, published data using the *Cntn2*-Cre mouse line, in addition to my epifluorescence imaging of the tdTomato-positive VCS, demonstrates a reliable degree of recombination throughout the VCS. Therefore, the minimal phenotype in the *Irx3*^{VCS-KO} is surprising. While *Irx3* deletion should be confirmed, the results so far suggest that postnatal VCS development is largely contributed by embryonic *Irx3* expression and function. To test this, other CCS-specific Cre mouse lines should be considered to examine the role of embryonic *Irx3*. For instance, the *Hcn4*-CreERT2 mouse line generated by Liang *et al.* 2014²⁷⁵ is commercially available (The Jackson Laboratory), in which *Hcn4* is a general CCS marker that is expressed during embryonic VCS development. It is expressed in both

the atrial and ventricular conduction system (SA node, AV node, *His*-bundle, Bundle branches and *Purkinje* fibers). Since *Irx3* is only expressed in the VCS, using this global CCS Cre mouse line will still be helpful in explaining the role of *Irx3* in the VCS. Additionally, given that *Hcn4*-CreERT2 mice allow tamoxifen-inducible Cre recombination, we can ablate *Irx3* at desired stages (i.e. embryonic, early postnatal, adult). Similarly, *Kcne1*-Cre (mink-CreERT2) developed by Arnolds *et al.* 2011³¹¹ can be considered.

5.2 CCS heterogeneity in humans and mice

While understanding the molecular properties of the conduction system in mice has now been conducted through a few recent studies, conducting a global investigation into the molecular heterogeneity within human CCS has not been done, as outlined in **Section 4.2.6**. Therefore, using publications that have performed single-cell or single-nuclei RNA-sequencing in human hearts, we were able to isolate the conduction cells that were captured in these studies.

We bioinformatically identified and isolated CCS cells from E16.5 mouse embryonic hearts⁶⁴, P1 - P4 mouse (data presented in current dissertation)³¹², 9 - 15 post-conception weeks human fetus hearts³¹⁰ and 20 - 75 year old human hearts²⁷⁷. Using each of the datasets, the CCS components were compiled, and UMAPs of each were generated using marker identifications published in these studies, and the CCS clusters were identified (**Figure 5.4**). Interestingly, in UMAPs of mouse embryos and human fetuses, CCS components of the atria, such as the SAN and compact AV node, were clearly separated from the VCS components, such as the HIS and PFs (**Figure 5.4a, c**). On the other hand, all CCS components of postnatal mouse and human adult were more integrated to each other (**Figure 5.4b, d**). This could be due to much more distinctive transcriptomic profiles between impulse generators (i.e. nodes) and propagators (i.e. VCS). Alternatively, this could

simply be due to the disproportionate numbers between atrial and ventricular conduction cells in more developed mouse and human CCS. Of note, recent studies have suggested that the distances between clusters in UMAP do not necessarily signify the degree of differences³¹³.

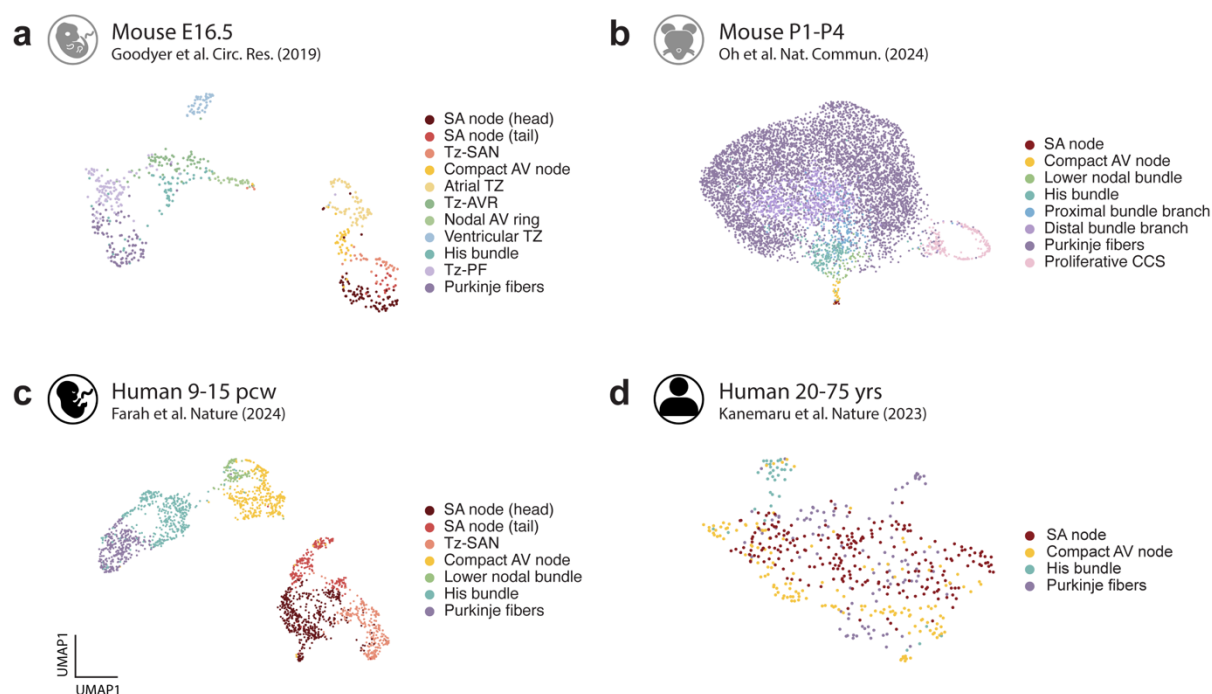


Figure 5.4 UMAP constructions of CCS cells from various developmental stages of mouse and human hearts: (a) E16.5 mouse, (b) P1-P4 mouse, (c) 9-15 post conception weeks (pcw) human fetus and; (d) 20-75 year old human hearts. ATZ, Atrial transitional zone; cAVN, Compact atrioventricular node; DBB, distal bundle branch; HIS, His bundle; LNB, Lower nodal bundle; nAVR, Nodal atrioventricular ring; PF, Purkinje fibers; SAN, Sinoatrial node; SAN-H, Sinoatrial node (head); SAN-T, Sinoatrial node (tail); Tz-AVR, Transitional atrioventricular ring; Tz-SAN; Transitional sinoatrial node; VTZ, Ventricular transitional zone

To investigate the molecular similarities between all components from all datasets, an unbiased correlation analysis was performed (**Figure 5.5**). The resulting dendrogram showed an overall high proximity between the nodal cells from all datasets and between the VCS from all datasets, showing a high degree of species/developmental stage conservation of molecular profiles. Notably, the atrial transitional zone (ATZ) from the embryonic mouse dataset was clustered among the VCS components; and the HIS from human adults as well as LNB from human fetuses were clustered among the atrial conduction components.

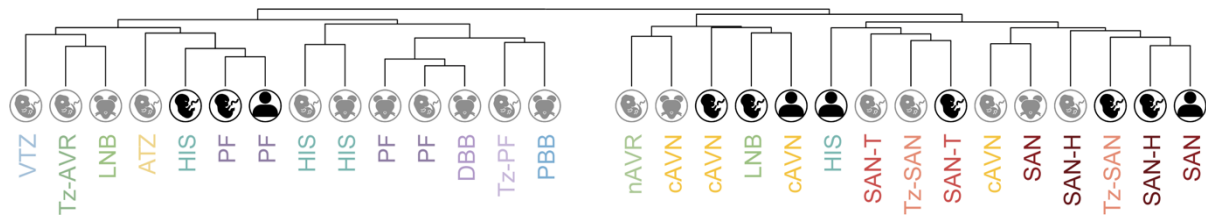


Figure 5.5 Dendrogram demonstrating molecular correlation among developing and adult mouse and human CCS components. ATZ, Atrial transitional zone; cAVN, Compact atrioventricular node; DBB, distal bundle branch; HIS, His bundle; LNB, Lower nodal bundle; nAVR, Nodal atrioventricular ring; PF, Purkinje fibers; SAN, Sinoatrial node; SAN-H, Sinoatrial node (head); SAN-T, Sinoatrial node (tail); Tz-AVR, Transitional atrioventricular ring; Tz-SAN, Transitional sinoatrial node; VTZ, Ventricular transitional zone

Further work performing comparative analysis between the described datasets is currently ongoing. Specifically, molecular profiles of the CCS between different developmental stages and between mouse and humans are under investigation, as well as its possible transcriptional regulation underlying the heterogeneity at each stage and species. We further identify potential therapeutic targets through genome-wide association analysis, linking mutations in our newly identified CCS markers to reported cardiac conduction diseases.

This study is the first to directly compare all components of the CCS in developing and postnatal/adult mice and humans using high-throughput approaches. Through this comprehensive comparison, this study will highlight species- and developmental stage-specific elements within the CCS. This study will benefit researchers and clinicians with foundational knowledge into the differences and similarities in the CCS across species and developmental stages, as well as potential transcriptional mechanisms that establish these profiles. Furthermore, pinpointing genetic variations associated with cardiac conduction defects will identify the most relevant genes for the development of targeted therapies. This will additionally help prioritize critical genes for in-depth investigation through mouse models, which will significantly enhance the translational potential of basic research in the field of CCS.

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SECTION 7 – CONTRIBUTIONS OF COLLABORATORS

10X Genomics scRNA-sequencing was performed by Katayoun Sheikholeslami (StemCore Laboratories, The Ottawa Hospital Research Institute) and fluorescence-activated cell sorting (FACS) was performed by Fernando Ortiz (Flow Cytometry and Cell Sorting Facility, The Ottawa Hospital Research Institute)

NanoString GeoMX Digital Spatial Profiler (Spatial transcriptomics) was performed by the Epigenomics core (University of Michigan). Initial staining of spatial slides with DsRED and CYTO13 was performed by the Advanced Genomics Core (University of Michigan). Basic quality assessment of each sample was performed by Dana King from the Bioinformatics core (University of Michigan).

Sectioning of hearts for several histological experiments as well as spatial transcriptomics was performed by Rimshah Abid; FISH staining was performed by Termeh Aslani; SCENIC analysis was performed by Marwan Bakr; ATAC-seq, ChIP-seq and motif analysis were performed by Saif Dababneh.

SECTION 8 – APPENDIX

8.1 PUBLICATION : OH *ET AL.* NATURE COMMUNICATIONS. 2024

Transcriptional regulation of the postnatal cardiac conduction system heterogeneity

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The cardiac conduction system (CCS) is a network of specialized cardiomyocytes that coordinates electrical impulse generation and propagation for synchronized heart contractions. Although the components of the CCS, including the sinoatrial node, atrioventricular node, *His* bundle, bundle branches, and *Purkinje* fibers, were anatomically discovered more than 100 years ago, their molecular constituents and regulatory mechanisms remain incompletely understood. Here, we demonstrate the transcriptomic landscape of the postnatal mouse CCS at a single-cell resolution with spatial information. Integration of single-cell and spatial transcriptomics uncover region-specific markers and zonation patterns of expression. Network inference shows heterogeneous gene regulatory networks across the CCS. Notably, region-specific gene regulation is recapitulated in vitro using neonatal mouse atrial and ventricular myocytes overexpressing CCS-specific transcription factors, *Tbx3* and/or *Irx3*. This finding is supported by ATAC-seq of different CCS regions, *Tbx3* ChIP-seq, and *Irx* motifs. Overall, this study provides comprehensive molecular profiles of the postnatal CCS and elucidates gene regulatory mechanisms contributing to its heterogeneity.

For every heartbeat, electrical impulses are initiated by the pacemaker cells in the sinoatrial node (SAN) and spread throughout the atrial myocardium to reach the atrioventricular node (AVN) and the fast-conducting network of the ventricular conduction system (VCS), including the *His* bundle, bundle branches and *Purkinje* fibers. Disturbances in the CCS can manifest in various types of cardiac rhythm abnormalities, such as heart blocks, ventricular fibrillation, and ventricular tachycardias, potentially leading to sudden cardiac death^{1–6}.

Following the anatomical discoveries of the SAN, AVN, the *His* bundle, bundle branches, and the *Purkinje* fibers more than 100 years ago by Arthur Keith and Martin Flack⁷, Wilhelm His Jr⁸, Sunao Tawara⁹, and Johann Evangelist Purkinje¹⁰, respectively, electrophysiological and morphological differences between CCS components, such as automaticity¹¹, conduction velocity¹², and cell-cell electrical coupling^{13,14}, have been demonstrated. In addition, the developmental processes of the CCS and its origin have been studied using genetic model organisms^{15–17}. These studies have revealed specific expression

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patterns and/or functional implications of individual key cardiac transcription factors within the CCS. For instance, T-box 3 transcription factor 3 (*Tbx3*) is found in the SAN and AVN, as well as the proximal part of the VCS¹⁸. On the other hand, *Iroquois* homeobox 3 (*Irx3*) is expressed exclusively in the VCS, while being absent in the SAN and AVN^{19,20}. *Etv1*, a member of the E-twenty-six (ETS) transcription factor family, is also expressed in the VCS as well as atrial cardiomyocytes at a high level but not in the SAN and AVN²¹. Inhibitor of DNA binding 2 (*Id2*), an inhibitor of the helix-loop-helix transcription factors, is expressed in all components of the CCS, except the SAN^{22,23}. Interestingly, loss of many of these CCS transcription factors (e.g., *Tbx3*, *Irx3*, *Etv1*, and *Nkx2-5*) often results in similar developmental and functional phenotypes, such as CCS hypoplasia and conduction defects, only after birth^{20,24,25}. Collectively, these studies suggest that the CCS is developmentally dynamic through the pre-to-postnatal stages, potentially arising through complex interactions among transcription factors. Nevertheless, due to the low number of CCS cells and technical difficulties in isolating healthy CCS cells from the postnatal heart, our understanding of the molecular heterogeneity of the CCS and its regulatory mechanisms at a cellular level has been comparatively limited. In particular, molecular diversities within the AVN, which is anatomically subdivided into the compact AVN and lower nodal bundle (LNB, also known as lower nodal cells)^{26–28}, as well as those within the VCS, consisting of morphologically distinct structures like the *His* bundle, left and right bundle branches, and *Purkinje* fibers, are not yet fully understood.

Leveraging the recent advancements in single-cell RNA-sequencing (scRNA-seq) techniques, a few studies have highlighted the cellular heterogeneity within the CCS. Goodyer et al. have demonstrated the molecular heterogeneity of different components of the CCS using micro-dissected embryonic day 16.5 (E16.5) mouse hearts²⁹. Bhattacharyya et al. have shown heterogeneity in the atrioventricular region using fluorescence-guided micro-dissection in *Gjd3-CreEGFP; Rosa26^{tdTomato}* mice at postnatal day 0 (PO)³⁰. Additionally, Liang et al. have reported the cellular diversity within the micro-dissected SAN of adult mouse hearts³¹. While these studies elaborately demonstrate distinct CCS cell populations and their unique expression profiles, further research on the transcriptomic landscape across the postnatal CCS and comparative profiling of different CCS components would enhance our understanding of the molecular, anatomical, and functional diversities within the CCS. In addition, the mechanisms governing the establishment of these CCS heterogeneities and the potential molecular transitions during pre-to-postnatal developmental stages remain to be elucidated.

Here, we utilize the integration of scRNA-seq and spatial transcriptomic analyses in a postnatal CCS-reporter mouse model (*Cntn2^{Cre/+}; Rosa26^{tdTomato/+}*) to provide a comprehensive view of the cellular heterogeneity within the postnatal CCS at a high-resolution with spatial information. Our study identifies CCS regional markers with unique and overlapping patterns and compares the expression of functional genes (i.e., ion channels and sarcomeres) within the CCS. By integrating with embryonic CCS scRNA-seq data²⁹, we examine the molecular similarities and differences between prenatal and postnatal stages of the CCS. Furthermore, we provide insights into the gene regulatory mechanisms of the CCS and further investigate regulatory relationships between two CCS-specific transcription factors, *Tbx3* and *Irx3*. This involves functional validation of CCS marker regulation using isolated neonatal mouse atrial and ventricular myocytes overexpressing *Tbx3* and/or *Irx3*. Our analysis explores region-specific transcriptional regulation that contributes to the heterogeneity within the CCS.

Results

Single-cell and spatial transcriptomic analyses of the CCS

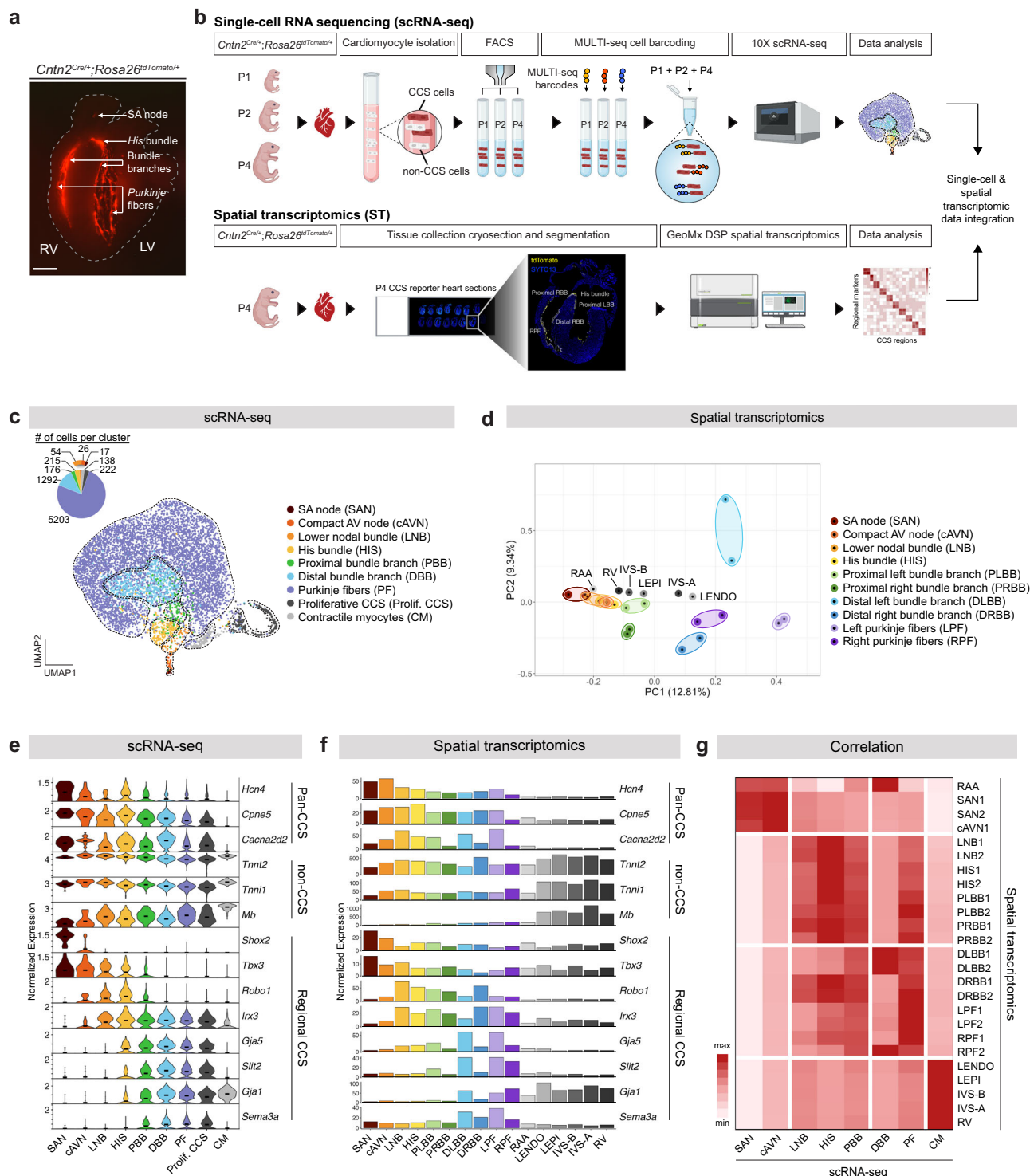
To understand the cellular and molecular constituents of the postnatal CCS, we simultaneously performed single-cell RNA sequencing

(scRNA-seq) and spatial transcriptomics (ST) using the CCS reporter mouse model, *Cntn2^{Cre/+}; Rosa26^{tdTomato/+}* (CCS-tdT)³² (Fig. 1a). Consistent with the previous studies that utilized Contactin-2 (*Cntn2*)-driven reporters and *Cntn2*-targeting antibody to visualize the entire CCS system in mice^{21,33,34}, 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).

For scRNA-seq, cardiomyocytes were isolated from CCS-tdT mouse hearts at postnatal day 1, 2, and 4 (P1, P2, and P4). Subsequently, fluorescence-activated cell sorting (FACS)-purified CCS cells, which made up 0.1 – 0.2% of the total cardiomyocyte population (Supplementary Fig. 2), were subjected to multiplexed scRNA-seq (MULTI-seq)³⁵ (Fig. 1b, Top). After pre-processing which excluded low-quality cells (Supplementary Fig. 3) and non-cardiomyocytes (1.03% of all cells) (Supplementary Fig. 4), a total of 6855 cardiomyocytes expressing an average number of 3388 genes per cell were identified. Through a series of unsupervised dimensional reduction analyses using uniform manifold approximation and projection (UMAP) and based on the expression of known CCS marker genes (see below), we identified 8 CCS and 1 non-CCS populations, including the sinoatrial node (SAN), compact atrioventricular node (cAVN), lower nodal bundle (LNB), *His* bundle (HIS), proximal bundle branch (PBB) and distal bundle branch (DBB), *Purkinje* fibers (PF), proliferative CCS (Prolif. CCS) and contractile myocytes (CM) (Fig. 1c and Supplementary Fig. 5).

In parallel, ST was performed on cryosections of the P4 CCS-tdT mouse heart using the GeoMx Digital Spatial Profiler (Fig. 1b, Bottom). A segmentation approach allowed the precise capture of all tdTomato-positive cells in the CCS regions, such as SAN, cAVN, LNB, HIS, left and right proximal bundle branches (PLBB/PRBB), left and right distal bundle branches (DLBB/DRBB), left and right *Purkinje* fibers (LPF/RPF) (Supplementary Fig. 6; see the “Methods” section). Of note, our ST analysis included two sub-compartments of the AVN^{26–28}, cAVN and LNB, which are anatomically situated adjacent to each other within the atrioventricular junction (Supplementary Fig. 6b and 7a). As described previously^{26–28}, trichrome staining of the atrioventricular region showed that the LNB was surrounded by insulating fibrous tissue and basally located on the plane of atrioventricular insulation, in comparison to the apically located HIS (Supplementary Fig. 7a). Additionally, non-CCS regions adjacent to the selected CCS components, including the right atrial appendage (RAA), basal and apical interventricular septum (IVS-B, IVS-A), left ventricular endomyocardium (LENDO), left ventricular epimyocardium (LEPI), and right ventricle (RV) were collected for comparison. Among the 12,638 transcripts detected in ST, differentially expressed genes (4708 transcripts) showing $|FC| > 3$ and $P < 0.05$ in any possible comparison of two CCS components, were used to perform principal component analysis (PCA). PCA of ST data demonstrated that CCS components exhibited a globally linear clustering pattern reflecting the sequence of cardiac electrical activation, from impulse generation in SAN to propagation through the VCS (Fig. 1d). 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).

In both scRNA-seq and ST analyses, all CCS regions highly expressed known pan-CCS markers (*Hcn4*, *Cpne5* and *Cacna2d2*)^{22,29,36,37}, whereas the non-CCS regions, such as the ‘Contractile myocytes’ cluster in scRNA-seq, and LENDO, LEPI, IVS-B, IVS-A and RV in ST, expressed higher levels of contraction genes (*Tnnt2*, *Tnni1*, *Mb*) with low expression of conduction genes (Fig. 1e, f). Also, both scRNA-seq and ST results corroborate the expression patterns of the known regional CCS markers, including *Shox2* in the SAN^{38,39}, *Tbx3* in the SAN, AVN, and HIS^{40,41}, *Irx3* and *Gja5* in the VCS^{19,20}, *Robo1* in the proximal VCS^{42,43}, *Slit2* and *Sema3a* in the distal VCS^{29,42}, *Gja1* in distal VCS and CM⁴⁴; as well as atrial (*Myl4*, *Myl7*) and ventricular (*Myl2*, *Myl3*)



sarcomere genes. Based on these observations, we characterized the CCS components as follows: SAN ($MyL2^{low}/MyL3^{low}/MyL4^{high}/MyL7^{high}/Hcn4^{high}/Tbx3^{high}/Nkx2.5^{+}/Shox2^{high}/Irx3^{+}/Robo1^{-}$); cAVN ($MyL2^{mid}/MyL3^{mid}/MyL4^{high}/MyL7^{high}/Hcn4^{high}/Tbx3^{high}/Nkx2.5^{+}/Robo1^{low}/Shox2^{low}$); LNB ($MyL2^{high}/MyL3^{high}/MyL4^{low}/MyL7^{low}/Hcn4^{+}/Gja5^{low}/Gja1/Irx3^{+}/Tbx3^{+}$); HIS and PBB ($MyL2^{+}/MyL3^{+}/MyL4^{+}/MyL7/Irx3^{+}/Tbx3^{+}/Robo1^{+}$); DBB and PF ($MyL2^{+}/MyL3^{+}/MyL4^{+}/MyL7/Irx3^{+}/Gja5^{+}/Gja1/Slit2/Sema3a^{+}$); VCS ($MyL2^{+}/MyL3^{+}/MyL4^{+}/MyL7/Irx3^{+}/Gja5^{+}/Sema3a^{+}/Slit2^{+}$) and non-CCS cardiomyocytes ($Hcn4/Cpn5/Cacna2d2/Gja5/Sema3a/Tnni2^{high}/Tnni1^{high}/Mb^{high}$). Notably, despite the anatomical proximity between the cAVN and LNB, a direct comparison between cAVN, LNB

and HIS revealed that the LNB expressed ventricular genes ($MyL2$, $MyL3$) and VCS markers ($Irx3$, $Etv1$, $Gja5$), which were characteristics more similar to HIS than to cAVN (Supplementary Fig. 7b). This is consistent with previous studies demonstrating that the LNB is structurally continuous with the HIS and is derived from a ventricular lineage^{26–28,30}.

Our scRNA-seq showed a cluster of cells (222 out of 6855 cells, 3.24%) annotated as 'Proliferative CCS', as 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. Additionally, we

Fig. 1 | Single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics (ST) of postnatal mouse cardiac conduction system (CCS). **a** Representative epi-fluorescence image of a P4 CCS-reporter mouse (*Cntn2*^{Cre/+}; *Rosa26*^{tdTomato/+}) heart showing tdTomato-marked sinoatrial node, *His* bundle, bundle branches and *Purkinje* fibers. Scale bar = 1 mm. Imaging was performed on more than five hearts. **b** (top) Schematic overview of scRNA-seq experiment for the CCS profiling. Neonatal mouse cardiomyocytes were isolated from postnatal days 1, 2, and 4 (P1, P2, and P4) CCS-reporter mouse hearts, and tdTomato-positive CCS cells were purified using fluorescence-activated cell sorting (FACS). P1, P2, and P4 cells were barcoded separately using MULTI-seq, followed by 10x Genomics scRNA-seq. (bottom) Schematic overview of spatial transcriptomics of the CCS using GeoMx Digital Spatial Profiler (DSP). Cryosections of P4 CCS-reporter mouse hearts were collected on a slide, followed by the selection of regions of interest using segmentation and sequencing. Results from scRNA-seq and ST were integrated for analysis. Created with BioRender.com. **c** Uniform Manifold Approximation and Projection

(UMAP) of scRNA-seq showing 8 CCS and 1 non-CCS clusters. A pie chart (top, left) shows the number of cells in each cluster. **d** Principal component analysis (PCA) of the CCS and non-CCS regions profiled by spatial transcriptomics (ST). **e** Expression profiles of the established CCS and non-CCS marker genes in each cluster of scRNA-seq data. **f** Expression profiles of the established CCS and non-CCS marker genes in each collected region of ST data. **g** Correlation analysis of CCS components between scRNA-seq and ST data. SAN sinoatrial node, cAVN compact atrioventricular node, LNB lower nodal bundle, HIS *His* bundle, PBB proximal bundle branch, PLBB proximal left bundle branch, PRBB proximal right bundle branch, DBB distal bundle branch, DLBB distal left bundle branch, DRBB distal right bundle branch, PF *Purkinje* fibers, RPF right *Purkinje* fibers, LPF left *Purkinje* fibers, RAA right atrial appendage, LENDO left ventricular endo-myocardium, LEPI left ventricular epi-myocardium, IVS-B interventricular septum (Base), IVS-A interventricular septum (Apex), RV right ventricle.

compared P1, P2, and P4 MULTI-seq data to examine potential transcriptomic changes during early postnatal development in the CCS. Unlike our expectation, no differences in the proportion of P1, P2, and P4 cells per cluster, or in clusters per development stage were observed (Supplementary Fig. 9). Furthermore, no significant changes in gene expression were found, including the commonly known molecular signatures for cardiomyocyte maturation, such as a transition in myosin heavy chain isoforms from *Myh6* to *Myh7* and an increase in mitochondrial gene content.

Next, we conducted a comparison between the transcriptomic profiles of corresponding regions detected by both technologies (Fig. 1g). Correlation analysis showed strong concordances between scRNA-seq and ST datasets within nodal cells (SAN and cAVN); LNB and proximal VCS (HIS and PBB), and distal VCS (DBB and PF) components. Also, all ventricular non-CCS components in ST (LENDO, LEPI, IVS-B, IVS-A and RV) were highly correlated to the CM cluster in scRNA-seq, while the RAA in ST showed high similarity to the SAN and cAVN in scRNA-seq. Collectively, these results suggest that our scRNA-seq and ST methods credibly captured all CCS and non-CCS regions.

Transcriptional profiling of the postnatal CCS components

Next, we sought to identify regional CCS markers by integrating scRNA-seq and ST data. First, enriched genes of each CCS region were identified from scRNA-seq and ST datasets separately, by comparing each CCS region to other CCS and non-CCS clusters/regions ($|FC| > 1.5$, $P < 0.05$) (Fig. 2a and Supplementary Data 1 and 2). Subsequently, genes that were identified as common between scRNA-seq and ST were considered as specific regional markers with high confidence.

In the SAN, as expected, *Shox2*^{38,39} and *Tbx3*^{40,41}, well-known transcription factors in SAN development, were observed. In addition, we identified other potential markers, including *Gnao1*, which encodes G protein subunit alpha O1 and has been found to be involved in heart development⁴⁵, 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^{46,47}, as well as *Cacna1d*, which encodes Ca_v1.3 channel known to regulate AVN automaticity⁴⁸. Newly identified markers of the cAVN included *Prdm6*, which encodes PR/SET domain 6 and has been shown to be expressed in the developing cardiovascular^{49–51} and nervous system⁵². Interestingly, a genetic variant of *Prdm6* has been reported to be associated with heart rate recovery during exercise⁵³. 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 stages⁵⁴. Like many other cardiac conduction genes, *Ppp1r17* is also expressed in neurons⁵⁵, 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 VCS⁵⁶ with critical roles in cardiac electrophysiology, and *Cdh6* is known to be enriched in the CCS compared to non-CCS cardiomyocytes⁵⁷. This suggests that there may be regional heterogeneity in the expression levels of these genes within the CCS. The PBB highly expressed *Igf1bp7* and *Arxex1*, while the DBB showed high expression of genes, such as *Nppa*, *Gal*, *Ecm1*, *Cygb*, *Slc16a2*, *Pde10a*, and *Mbp*. *Nppa*, encoding atrial natriuretic peptide^{58,59}, is known to be expressed in embryonic trabecular cardiomyocytes^{60,61}, 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 *Mppd2*, *Corin*, and *Mylk4*. In particular, *Mppd2*, encoding Metallophosphodiesterase domain-containing protein 2, has previously been reported to be expressed in neurons and embryonic ventricles⁴⁷, although its functional involvement in the CCS has not yet been described.

The CCS is an anatomically continuous structure with a highly specialized function of generating and propagating electrical signals. Thus, while the integrated analytic approach above effectively uncovers the enrichment of gene expression in each individual CCS component, it may not be effective in capturing the gradual changes in gene expression along the CCS, in which case, genes would be enriched in more than one defined component of the CCS. Therefore, according to the PCA (Fig. 1d) and the correlation heatmap (Fig. 1g), we divided CCS components into three zones: 'Nodal cells' (SAN, cAVN), 'LNB & Proximal VCS' (HIS, PBB) and 'Distal VCS' (DBB, PF), and identified markers enriched in each zone by integrating scRNA-seq and ST datasets (Fig. 2b). 'Nodal cells' showed enrichment of genes, including *Gcgr*, which encodes glucagon receptor⁶² with roles in heart rate regulation⁶³, *Ctdspl*, which encodes CTD Small Phosphatase Like, associated with atrial fibrillation through miR-26a-1⁶⁴, *Enpep*, and *Bmp2*. The 'Proximal VCS' zone showed high expression patterns of *S100a6* encoding a Ca²⁺ binding protein Calcyclin⁶⁵, *Col4a4*, *Alcam*, *Cdh6*, and *Atp1b2*. Also, in addition to the established PF markers, *Sema3a*⁶⁶ and *Gja5*⁶⁷, the 'Distal VCS' zone showed enrichment of *Pdzrn3*, involved in heart maturation⁶⁸, *Irx1* and *Irx2*⁶⁹ encoding *Iroquois* homeobox (*Irx*) transcription factors, *Mgp* encoding Matrix Gla protein, *Nsg2*, and *Sbk2*. Interestingly, several PF and distal VCS genes, *Mylk4*, *Corin*, *Sbk2*, and *Mgp*, are also known to be expressed in the atria^{70,71}. Together, these results demonstrate the zoned and gradient expression patterns of CCS marker genes, which reflect its highly specialized and integrated conductive structure and functions.

To further gain molecular insights into the distinctive physiological and electrophysiological properties of each CCS component, including automaticity and conduction velocity, we profiled the expression patterns of genes functionally associated with ion channels, receptors, gap junctions, and sarcomeres using our scRNA-seq and ST

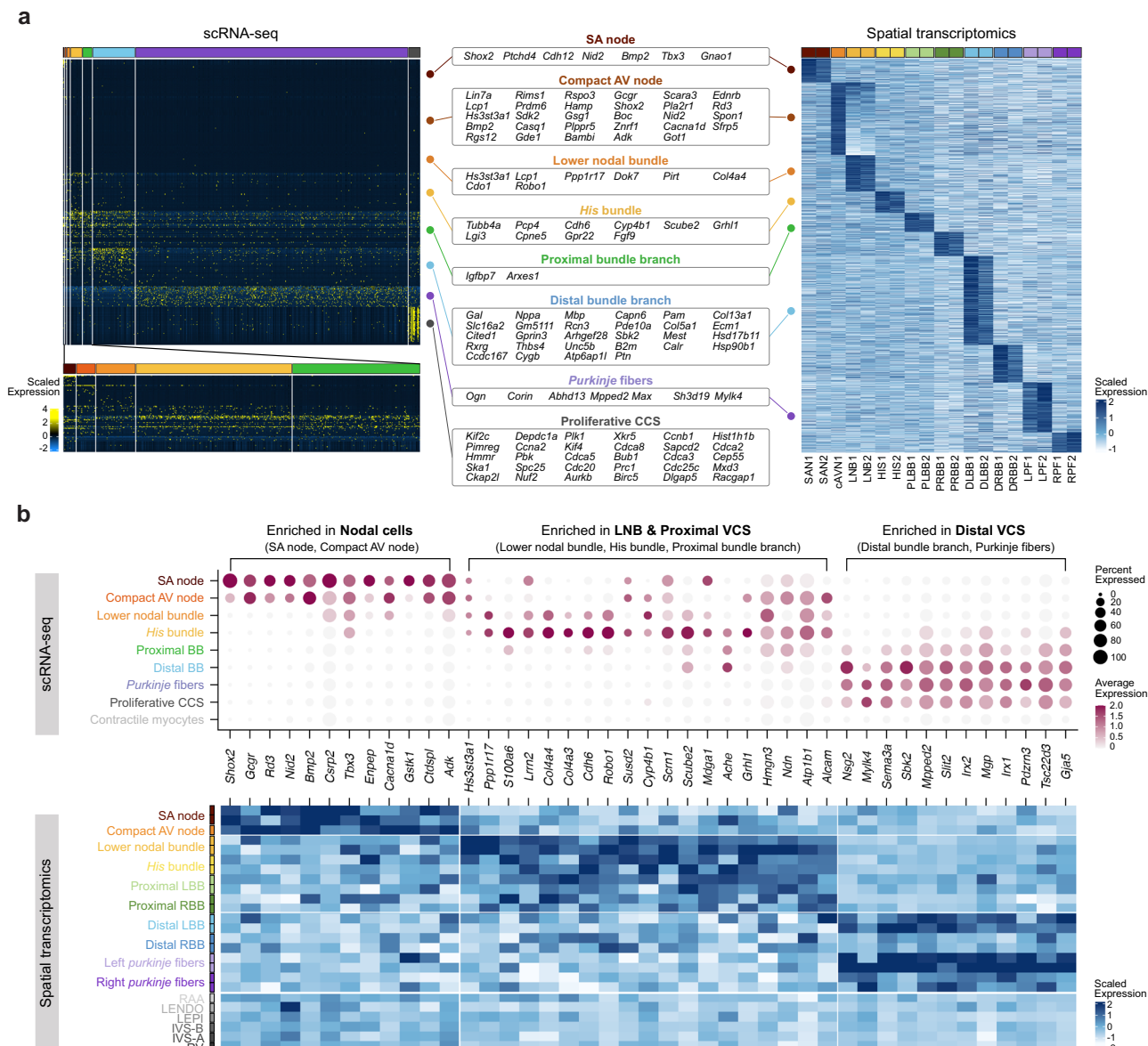


Fig. 2 | Integration of scRNA-seq and spatial transcriptomic data of the CCS. **a** CCS-specific enriched genes for each CCS component identified by both scRNA-seq and spatial transcriptomics. **b** Molecular gradients within the CCS components showing zoned enrichment of genes in Nodal cells (SA node, Compact AV node), Lower nodal bundle & Proximal VCS (*His* bundle, Proximal bundle branch), and

Distal VCS (Distal bundle branch, *Purkinje* fibers). LBB left bundle branch, RBB right bundle branch, RAA right atrial appendage, LENDO left ventricular endo-myocardium, LEPI left ventricular epi-myocardium, IVS-B interventricular septum (Base), IVS-A interventricular septum (Apex), RV right ventricle, VCS ventricular conduction system.

datasets (Supplementary Figs. 10 and 11). For example, *Kcnj3* and *Kcnj5*, which form the acetylcholine-activated potassium channel (I_{KACH}) Kir3.1 and Kir3.4, respectively, and are associated with bradyarrhythmia and sporadic atrial fibrillation⁷², as well as *Cacna1g* and *Cacna1h*, which encode $Ca_v3.1$ and $Ca_v3.2$, respectively, known to contribute to pacemaker activity⁷³, were highly expressed in the CCS with strong enrichment in SAN and cAVN. Also, consistent with a previous study⁷⁴, *Kcnk3*, encoding TASK-1 channel, showed enrichment in the VCS. It was notable that *Kcnj12*, encoding Kir2.2 responsible for cardiac inwardly rectifying potassium current (I_{K1}), *Gria3* encoding glutamate ionotropic receptor, *Grik5* encoding glutamate ionotropic receptor kainite, and *Ank3*, encoding a cytoskeletal element ankyrin-G that regulates cardiac excitability⁷⁵, were found to be enriched in the CCS compared to contractile myocytes. However, their functional implications in the CCS are not well understood. On the other hand, we

found several CCS genes identified from scRNA-seq, which were not detected in ST, likely due to the limitation of the probe-based approach used in ST. Among those genes, we noted strong and CCS-specific expression of *Scn10a* ($Na_v1.8$), known to modulate *Scn5a*-encoded $Na_v1.5$ currents⁷⁶, in the PF. In line with a recent study showing that only a short form of *Scn10a* mRNA is expressed in the VCS⁷⁶, we observed a predominant expression of the short *Scn10a* transcript in the postnatal CCS. Mapping sequenced reads to the *Scn10a* gene in our scRNA-seq data revealed higher sequencing coverage in the last seven exons of the gene (exons 22 to 28), compared to exons 1 to 21 (Supplementary Fig. 12).

Next, we validated the identified CCS markers by performing immunostaining and fluorescence in situ hybridization (FISH) in the P4 CCS-tdT mouse heart. An SAN-enriched marker, *Gnao1* was expressed in tdTomato-positive pacemaker cells (Fig. 3a), where *Shox2* and

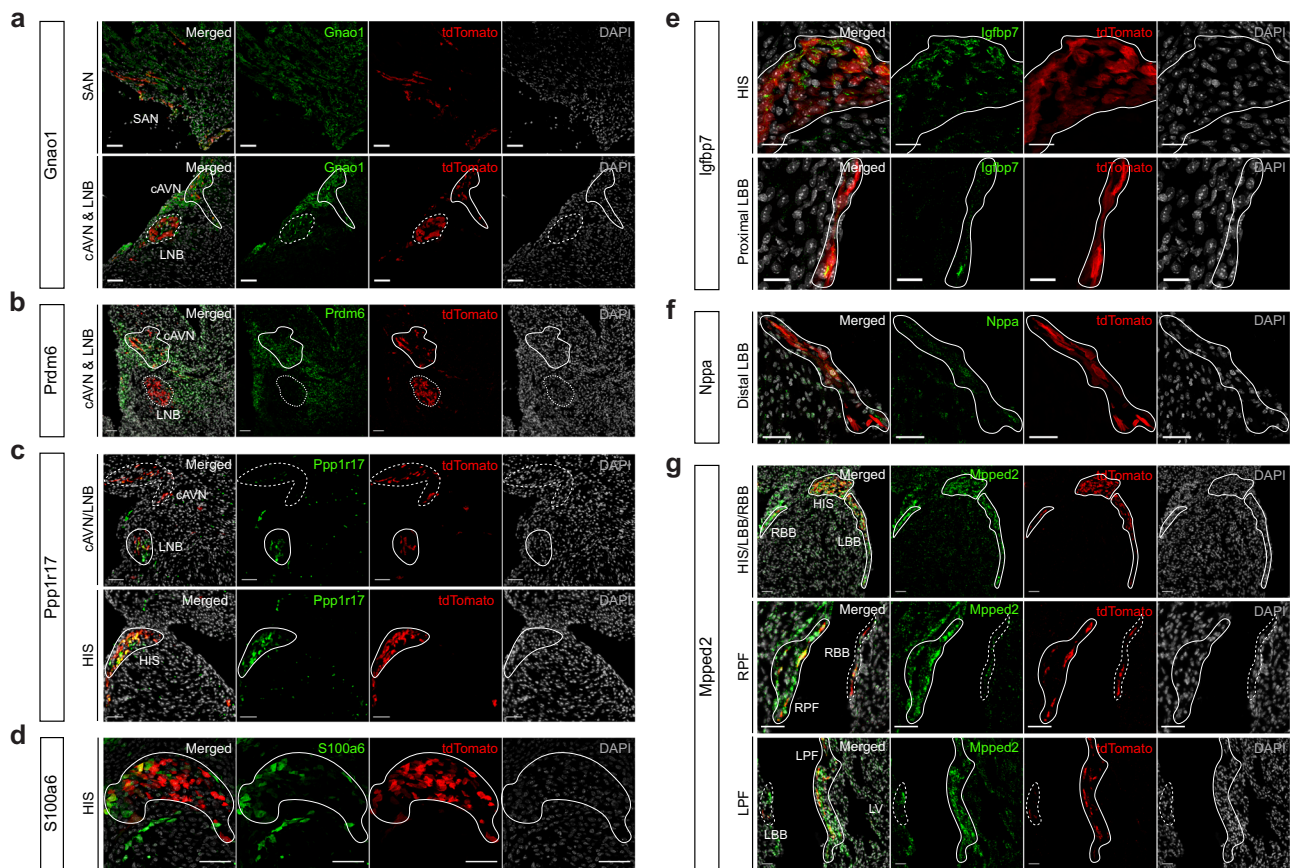


Fig. 3 | Histological validation of CCS component and regional markers.

Immunofluorescence (IF) and fluorescence in situ hybridization (FISH) staining were performed in P4 *Cntn2*^{Cre/+}; *Rosa26*^{tdTomato/+} heart sections. **a** *Gnao1* IF staining in tdTomato-positive SAN and cAVN, but not LNB; **b** *Prdm6* FISH staining in the cAVN, but not LNB; **c** *Ppp1r17* IF staining in the LNB and HIS; **d** *S100a6* IF staining in the HIS; **e** *Igfbp7* IF staining in the HIS and proximal LBB; **f** *Nppa* IF staining in the DBB;

g *Mpped2* FISH staining in the VCS. scale bars = 50 μm. The figures shown are representative of at least three independently repeated IF experiments or two FISH experiments. SAN sinoatrial node, cAVN compact atrioventricular node, LNB lower nodal bundle, HIS His bundle, LBB left bundle branch, RBB right bundle branch, LPF left Purkinje fibers, RPF right Purkinje fibers.

another recently discovered SAN marker, *Vsnl1*³¹, were expressed (Supplementary Fig. 13). *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 (Supplementary Fig. 14). 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. 15). On the other hand, *Ppp1r17* was expressed in the LNB and HIS, but not in cAVN (Fig. 3c and Supplementary Fig. 16), supporting our finding that LNB is molecularly more similar to HIS than to cAVN. We also examined proximal VCS-enriched markers. Consistent with scRNA-seq data, *S100a6* was detected in the HIS, but not in any other CCS regions (Fig. 3d and Supplementary Fig. 17). However, its expression only partially overlapped with tdTomato-positive cells in the HIS and was also found in tdTomato-negative cells around the HIS and in the IVS. Previous studies have reported *S100a6* expression in other cell types, including neurons⁷⁷, fibroblasts and epithelial cells⁷⁸, suggesting that the role of *S100a6* may not be restricted to the CCS. *Igfbp7*, identified to be enriched in PBB in our analysis, displayed strong expression in both HIS and PBB, but not in PF (Fig. 3e and Supplementary Fig. 18). This suggests that *Igfbp7* could serve as a proximal VCS marker. FISH staining showed that an established VCS marker⁷⁹, *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. 19). In addition, the expression of *Nppa*-encoded atrial natriuretic peptide (ANP) was primarily found in the distal VCS, including DLBB (Fig. 3f and Supplementary Fig. 20). *Scn10a*-encoded *Nav1.8* was expressed highly in the PF, compared to the HIS (Supplementary Fig. 21). Moreover, another PF-enriched gene, *Mpped2*, was strongly and specifically expressed throughout the VCS, albeit with a lower level of expression in the HIS and PBB (Fig. 3g and Supplementary Fig. 22). Interestingly, *Mpped2* and *Nav1.8* exhibited a slightly broader expression pattern showing enrichment along the endomyocardium of the ventricular chambers, suggesting their potential roles within and around the VCS. Collectively, the simultaneous examination of both scRNA-seq and ST followed by histological validation demonstrates that the CCS consists of both specific and gradational molecular profiles.

Transcriptomic comparison of the embryonic and postnatal CCS

The CCS forms through a tightly coordinated process of cell differentiation and specification during embryonic development, and maturation during early postnatal development^{20,25}. As a recent study has characterized the embryonic (E16.5) CCS through scRNA-seq²⁹, we sought to understand the transcriptional changes of the major CCS components during late embryonic and postnatal development. Embryonic CCS cells were first extracted from the original scRNA-seq datasets (GSE132658 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132658>]) based on cellular identification profiles²⁹, and

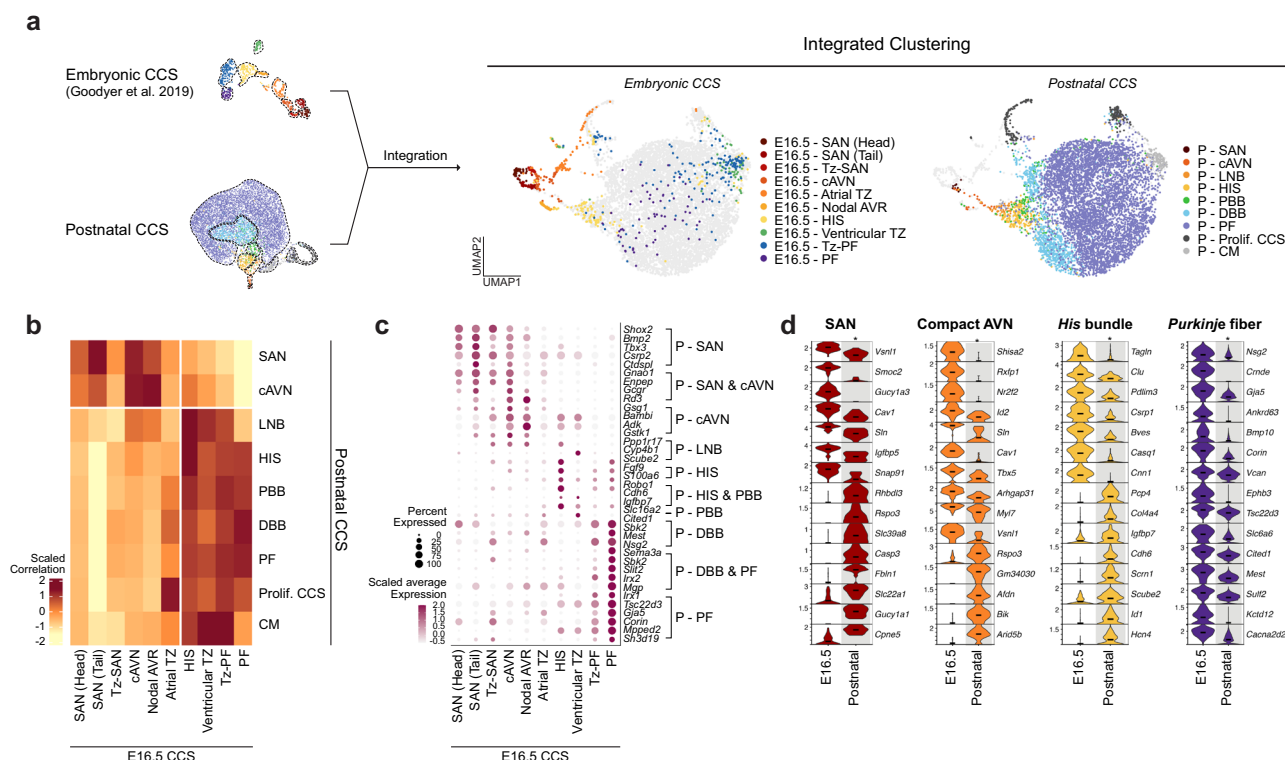


Fig. 4 | Molecular comparison between embryonic and postnatal CCS.

a Integration of embryonic day 16.5 (E16.5) and postnatal (P1-P4) CCS scRNA-seq datasets shows a close overlay of the CCS components from different developmental stages. P: postnatal. **b** Correlation analysis between embryonic and postnatal CCS components exhibits a high degree of concordance between respective groups. **c** Expression patterns of the identified CCS component and regional markers in E16.5 CCS clusters, showing molecular similarities between embryonic and postnatal CCS. **d** Pairwise differential gene expression analysis between

embryonic and postnatal CCS components, using the Wilcoxon Rank-Sum Test.

*, adjusted P -value < 0.05 . Source data are provided in Supplementary Data 3. SAN sinoatrial node, cAVN compact atrioventricular node, LNB lower nodal bundle, HIS His bundle, PBB proximal bundle branch, DBB distal bundle branch, PF Purkinje fibers, Prolif. CCS proliferative CCS, CM contractile myocytes, Tz-SAN transitional SAN, AVR AV ring, Atrial TZ atrial transitional zone, Ventricular TZ ventricular transitional cells, Tz-PF transitional PF.

then were integrated with our postnatal CCS scRNA-seq data with minimization of potential batch effects (Fig. 4a). This approach enabled a direct comparison of the molecular profiles of CCS components between embryonic and early postnatal stages.

Unbiased clustering of integrated cells displayed aligned patterns between individual CCS components from E16.5 and postnatal stages. For instance, the SAN, cAVN, HIS and PF cells of E16.5 and postnatal stages were well overlapped in the UMAP (Fig. 4a). Consistently, correlation analysis between E16.5 and postnatal CCS components demonstrated high concordances between the respective CCS regions, with significant clustering of CCS regions within each cardiac chamber (*i.e.*, the atria and the ventricles) (Fig. 4b). We further examined whether the CCS marker genes identified in the postnatal CCS displayed similar expression patterns in the E16.5 CCS (Fig. 4c). Notably, SAN-enriched genes (*Gnao1*, *Nid2*, *Bmp2*), cAVN-enriched genes (*Bambi*, *Gsg1*, *Rd3*, *Prdm6*), nodal cells-enriched genes (*Gcgr*, *Csrp2*, *Enpep*, *Ctdspl*), HIS/proximal VCS-enriched genes (*S100a6*, *Robo1*, *Scube2*, *Cdh6*), and PF/distal VCS-enriched genes (*Mppd2*, *Corin*, *Nsg2*, *Sema3a*, *Sbk2*, *Tsc22d3*, *Irx1* and *Irx2*) were consistently observed in the corresponding regions of the E16.5 CCS. This finding not only affirms the specificity of the newly identified CCS markers, but also indicates a high degree of transcriptomic consistency between these developmental stages, suggesting early establishment of molecular heterogeneity within the CCS.

Subsequently, we conducted pairwise differential gene expression analysis to identify the molecular differences between E16.5 and postnatal CCS components (IFC > 1.5 ; $P < 0.05$; minimum % of expressing cells in either compared group $> 50\%$) (Fig. 4d and

Supplementary Data 3). We noted significant differences in the expression of known CCS marker genes, including higher *Cpne5* and lower *Igf1bp5*, *Sln*, and *Vsnl1* expression in the postnatal SAN. Also, lower expression levels of *Id2*, *Sln*, *Cav1*, and *Vsnl1*, along with higher *Rspo3* levels, were observed in postnatal cAVN, compared to the embryonic cAVN. Compared to the embryonic HIS, the postnatal HIS exhibited higher levels of *Pcp4*, *Hcn4*, and *Igf1bp7*, yet lower levels of *Tagln* and *Cnn1*, encoding Transgelin (also known as SM22 α) and Calponin 1, respectively, both of which are smooth muscle cell markers⁸⁰. Given that *Cnn1* is expressed in the embryonic mouse heart (E13.5) but becomes restricted to smooth muscle cells in adult mouse tissues⁸¹, this reduction may reflect postnatal CCS maturation. Similarly, the postnatal PF showed significant decreases in *Bmp10*⁸² and *Corin*⁸³, genes that are expressed in the ventricular trabecular myocardium during development.

These observations collectively suggest that despite the high similarity in the overall molecular profiles of CCS components between E16.5 and early postnatal stages (P1-P4), there are discernible molecular changes that indicate the dynamic maturation process in the CCS during late embryonic and early postnatal development.

Heterogeneous transcriptional regulation of the CCS

To gain mechanistic insights into how distinct molecular patterning across the CCS is transcriptionally established, we performed network inference using SCENIC (Single-Cell Regulatory Network Inference and Clustering)⁸⁴, which identifies regulons by predicting the transcription factors (TFs) and their candidate target genes based on co-expression and motif enrichment analysis. TF activity in each individual cell was

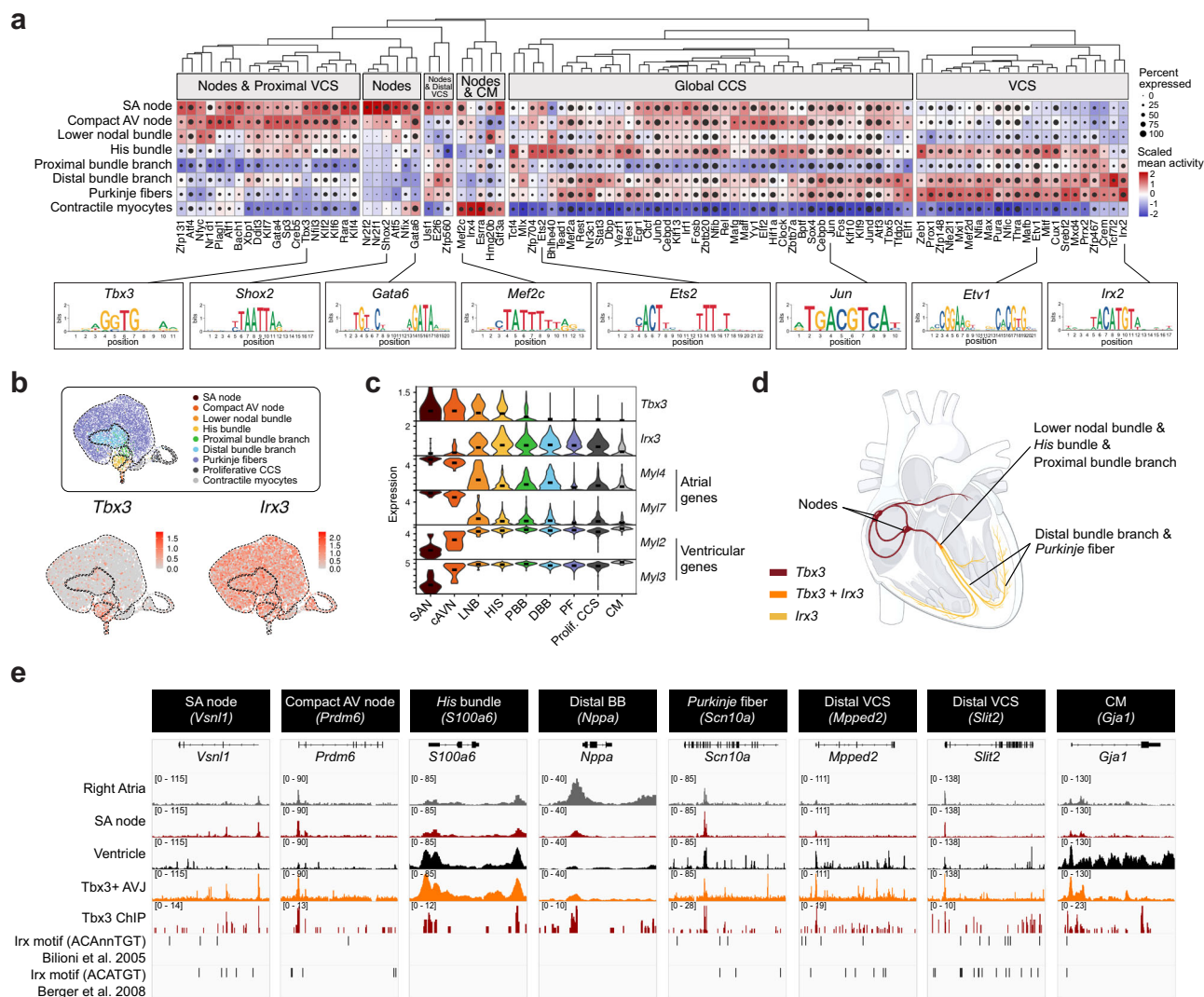


Fig. 5 | Heterogeneous transcriptional regulation of the CCS. a (Top) Integrated heatmap/dotplot of CCS regulons, as inferred by the SCENIC algorithm. The heatmap color represents regular activities for each CCS component based on the area under the curve (AUC) scores. Dot size represents transcription factor expression in each CCS component. (Bottom) Motifs of representative transcription factors in the CCS regions. **b** *Tbx3* and *Irx3* show unique and overlapping expression patterns exclusively within the CCS. **c** SAN and cAVN show enrichment of *Tbx3* with atrial genes (*Myf4* and *Myf7*); LNB, HIS, and PBB show enrichment of both *Tbx3* and *Irx3* with ventricular genes (*Myf2* and *Myf3*); and DBB and PF show enrichment of *Irx3* with ventricular genes. **d** Schematic showing unique and

overlapping expression patterns of *Tbx3* and *Irx3* throughout the CCS. Created with BioRender.com. **e** Genome browser views of chromatin accessibilities in the right atria, SA node, ventricle, and *Tbx3*-positive atrioventricular junction (*Tbx3*⁺ AVJ) as well as *Tbx3* ChIP-seq, and the presence of *Iroquois* homeobox (*Irx*) transcription factor motifs, for the CCS component and regional marker genes. SAN sinoatrial node, cAVN compact atrioventricular node, LNB lower nodal bundle, HIS *His* bundle, PBB proximal bundle branch, DBB distal bundle branch, PF *Purkinje* fibers, Prolif. CCS proliferative CCS, CM contractile myocyte, VCS ventricular conduction system.

calculated using AUCcell and subjected to unbiased clustering. This analysis revealed TFs that potentially contributed to the establishment of the regional differences within the CCS (Fig. 5a and Supplementary Data 4). Transcriptional activities by *Tbx3*, *Klf7*, *Creb5*, *Rara*, and *Sp3* were enriched in the nodes and proximal VCS; *Shox2*, *Nr2f1*, *Nr2f2*, and *Gata6* in the nodes only; *E2f6* in the nodes and distal VCS; as well as *Mef2c* and *Gtf3a* in the nodes and contractile cardiomyocytes. In addition, enrichments of *Tcf4*, *Tbx5*, *Cebpb*, *Cebpd*, *Jun*, *Ets2*, *Fos*, and *Irf1* activities were observed across all CCS components, except contractile myocytes, whereas *Irx2*, *Tcf7l2*, *Etv1*, *Srebf2*, and *Max* displayed VCS-specific enrichment. These findings corroborated a large body of the literature highlighting the regulatory functions of *Shox2* and *Gata6* in the SAN⁸⁵, *Tbx3* in the SAN/cAVN/HIS^{24,25,41,86}, and *Tbx5*^{22,41,87} and *Etv1*^{21,88} in the VCS. Gene regulatory networks of SAN, cAVN, HIS and PF were also constructed, elucidating the regulation of the regional

marker genes, such as *Gnao1*, *Gcgr*, *Bmp2* and *Vsn1l* in the SAN; *Prdm6*, *Rspo3*, and *Gde1* in the cAVN; *Igf1p7*, *Cpne5*, *Scube2*, and *Cdh6* in the HIS; as well as *Mpped2*, *Slit2*, *Scn10a*, and *Tsc22d3* in the PF (Supplementary Fig. 23).

To deepen our understanding of transcriptional regulation of CCS heterogeneity, we focused on two TFs, *Tbx3* and *Irx3*, known for their critical roles in CCS development and functions^{18–20,25,41} (Fig. 5b). Our scRNA-seq and ST analyses showed that *Tbx3* and *Irx3* exhibited distinct and overlapping expression patterns within the CCS: *Tbx3* alone in the atrial components of the CCS (SAN and cAVN); both *Irx3* and *Tbx3* in the proximal VCS (LNB, HIS, PBB); and *Irx3* alone in the distal VCS (DBB and PF) (Fig. 5c, d). Although *Irx3* was not identified from SCENIC analysis, we observed strong VCS-enrichment of the *Irx2* regulon, another member of the *Irx* TF family sharing the same *Irx* motif^{89,90}. Given that *Irx3*, not *Irx2*, is well described to be essential for

VCS development and function^{19,20,69}, we hypothesized that individual and cooperative transcriptional regulation by Tbx3 and Irx3 contributed to the molecular heterogeneity of the CCS.

To test this possibility, we utilized publicly available datasets of the assay for transposase-accessible chromatin using sequencing (ATAC-seq) in different CCS regions: P0 mouse SAN⁹¹ and E13.5 Tbx3-positive atrioventricular junction (AVJ) encompassing both AVN and HIS²⁵, along with their respective non-CCS counterparts: P0 right atria (RA) and mouse ventricle⁹². Additionally, we used Tbx3 ChIP-seq data performed in adult mouse cardiomyocytes⁹³, and searched for two Irx TF binding motifs: 'ACAnnTGT'⁸⁹ and 'ACATGT'⁹⁰ (Fig. 5e). 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 TFs across the CCS.

Next, we closely examined the epigenomic landscape for several new and established CCS marker genes (Fig. 5e and Supplementary Fig. 24). SAN markers, *Shox2* and *Vsnl1*, as well as a cAVN marker, *Prdm6* displayed higher ATAC-seq peaks in the SAN and AVJ compared to the atria or ventricle, respectively. This suggests a spatial specificity in their genomic accessibility. Tbx3-binding peaks and Irx motif sequences were also found in *Vsnl1* and *Prdm6*, indicating potential regulation by Tbx3 and Irx TFs. In accordance with its enriched expression in the proximal VCS, *S100a6* exhibited higher chromatin accessibility peaks in Tbx3⁺ AVJ, compared to the ventricle. This aligned with the Tbx3 ChIP-seq peak, but not with Irx motifs, suggesting a possible direct transcriptional regulation by Tbx3, but not Irx TFs. Interestingly, genes enriched in the distal VCS where *Tbx3* expression is absent, such as *Nppa*, *Gja5*, *Mppd2*, and *Slit2*, still displayed open chromatin regions in the Tbx3⁺ AVJ. They also showed Tbx3 ChIP-seq peaks and Irx motifs. On the other hand, another distal VCS gene, *Scn10a*, showed no apparent differences in ATAC-seq peaks between the Tbx3⁺ AVJ and the ventricle, although it did exhibit Tbx3 ChIP-seq peaks and Irx motifs. Lastly, consistent with their high expression in contractile myocytes, *Gja1* and *Ryr2* showed greater chromatin accessibility in the ventricle compared to AVJ, atria, and SAN, alongside the presence of Irx motifs and Tbx3 binding peaks. Collectively, these findings suggest a complex and region-specific potential transcriptional regulation of CCS regional marker genes by Tbx3 and/or Irx3.

Tbx3 and Irx3 contribute to CCS heterogeneity

To further investigate the individual and combined actions of Tbx3 and Irx3 on CCS gene regulation, we established an in vitro model that simulated various CCS regions by introducing Tbx3 and/or Irx3 in neonatal mouse atrial and ventricular cardiomyocytes (A-CMs and V-CMs, respectively) using adenovirus-mediated overexpression (Fig. 6a and Supplementary Fig. 25). Here, Tbx3 overexpression in A-CMs modeled the nodes (SAN and cAVN); Irx3 and Tbx3 overexpression in V-CMs, the proximal VCS (HIS and PBB); and Irx3 overexpression in V-CMs, the distal VCS (DBB and PF) (Fig. 6b). The regional properties of isolated A-CMs and V-CMs were validated by the significantly high atrial gene expression (e.g., *Myl4* and *Myl7*) in A-CMs and ventricular

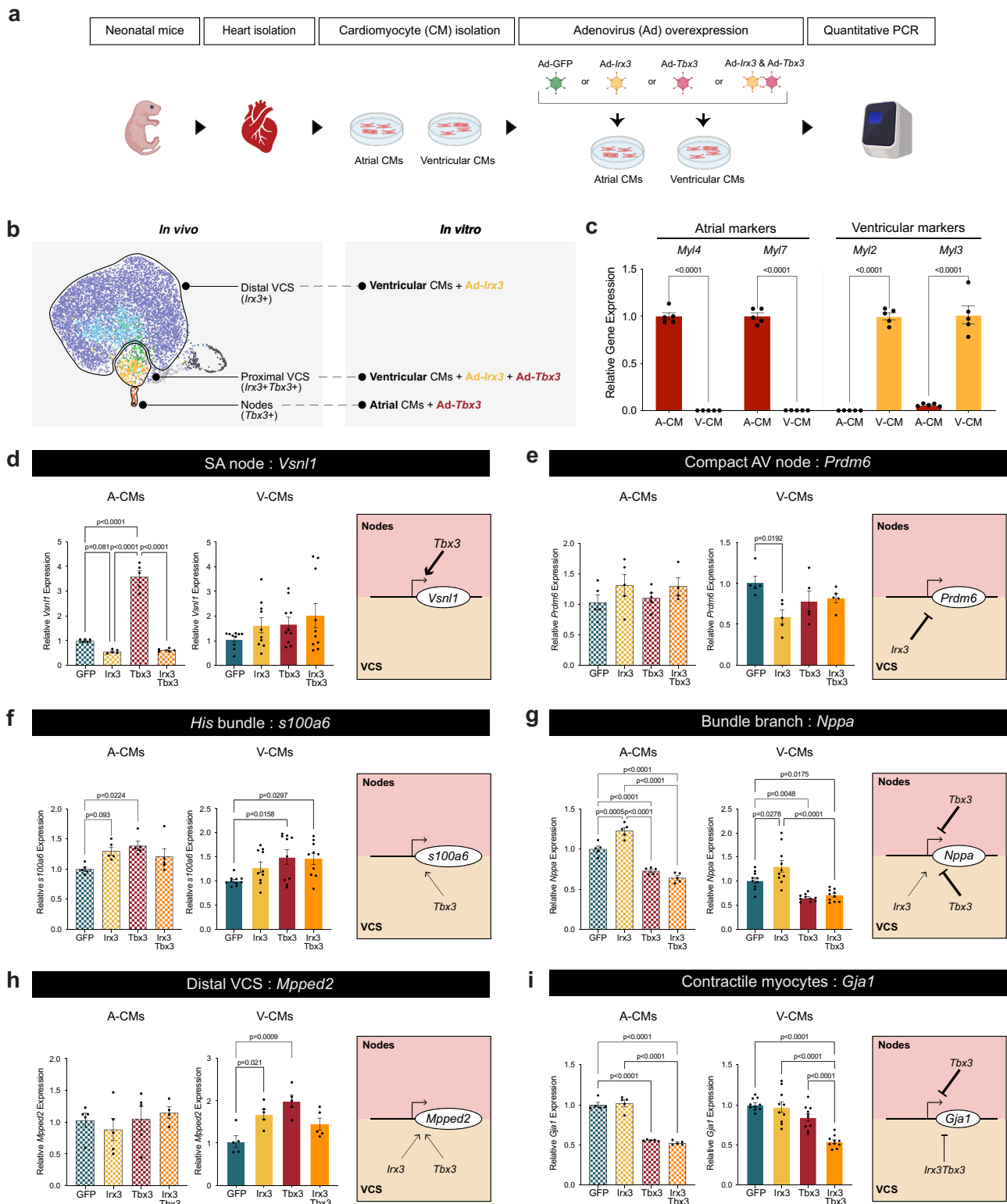
gene expression (e.g., *Myl2* and *Myl3*) in V-CMs (Fig. 6c). Subsequently, we assessed how Tbx3 and/or Irx3 influence the expression of CCS regional markers.

We found that *Vsnl1*, exclusively expressed in the Tbx3⁺ SAN, was markedly increased by Tbx3 overexpression in A-CMs (Fig. 6d). Interestingly, the addition of Irx3 as well as Irx3 overexpression alone in A-CMs suppressed *Vsnl1* expression, suggesting the antagonistic actions of Irx3 against Tbx3 outside nodal cells. In V-CMs, *Vsnl1* was unaffected by Tbx3 or Irx3 overexpression, suggesting that *Vsnl1* expression in the SAN is predominantly activated by Tbx3 in an atria-specific manner. On the other hand, another SAN marker, *Shox2* was not affected by either Tbx3 or Irx3 overexpression in both A-CMs and V-CMs (Supplementary Fig. 26a), 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 development³⁸. Similarly, cAVN genes, *Prdm6* and *Rspo3* were unaffected by Tbx3 and/or Irx3 in A-CMs (Fig. 6e and Supplementary Fig. 26b). However, in V-CMs, *Prdm6* and *Rspo3* were significantly repressed by Irx3 overexpression, supporting their absent expression in the Irx3⁺ VCS. This observation suggests that their expression in cAVN might be primarily driven by other transcription factors. Together, these data suggest that Tbx3 and Irx3 can play antithetical roles in gene expression: Tbx3 is an activator and Irx3 is a repressor of these tested nodal genes.

For genes enriched in the Tbx3⁺/Irx3⁺ proximal VCS, both Tbx3 and Irx3 appeared to function primarily as activators. For example, *S100a6* was upregulated by Tbx3 and Irx3 together in the V-CMs (Fig. 6f). Interestingly, this upregulation was largely driven by Tbx3, in agreement with the enriched Tbx3-binding peaks, while Irx3 overexpression alone in V-CMs had no effect on its expression, consistent with the lack of Irx motifs in the *S100a6* gene (Fig. 5d). Additionally, *Pcp4* was also significantly increased upon overexpression of both Tbx3 and Irx3 in A-CMs and V-CMs (Supplementary Fig. 26c), supporting the cooperative activator properties of Irx3 and Tbx3 of the identified proximal VCS markers.

For the markers identified in the Tbx3⁻/Irx3⁺ distal VCS, we observed an inverse regulatory relationship between Tbx3 and Irx3 compared to nodal genes: Tbx3 as a repressor and Irx3 as an activator. For example, Irx3 overexpression significantly upregulated *Nppa*, *Scn10a*, and *Gja5* in both A-CMs and V-CMs, supporting their enrichments in the distal VCS (Fig. 6g and Supplementary Fig. 26d, e). Importantly, when Irx3 and Tbx3 were overexpressed together, the activating role of Irx3 on *Nppa*, *Scn10a*, and *Gja5* was largely abolished or reduced, demonstrating the dominant repressive role of Tbx3 on these genes. These findings corresponded with Tbx3-binding peaks in *Nppa*, *Scn10a*, and *Gja5* (Fig. 5d), and explained their lower expressions in the His bundle. Notably, while *Tbx3* overexpression alone significantly repressed *Gja5* expression in A-CMs, it mildly increased *Gja5* levels in the V-CMs, suggesting that Tbx3's function on *Gja5* depends on the chamber identity. Similarly, Tbx3 and Irx3 activated *Mppd2* and *Slit2* expression in the V-CMs, but had no impact on their expression in the A-CMs, suggesting chamber-specific actions of Tbx3 and Irx3 on these genes (Fig. 6h and Supplementary Fig. 26f).

Lastly, for genes enriched in the Tbx3⁻/Irx3⁻ contractile myocytes, Tbx3 and Irx3 mainly play a repressive role. For instance, *Gja1* and *Ryr2* were significantly downregulated by Tbx3 and Irx3 overexpression together in V-CMs (Fig. 6i and Supplementary Fig. 26f), but not by Tbx3 or Irx3 alone. This demonstrates their synergistic repressive role in V-CMs and supports the absence of *Gja1* in the His bundle, but not in the Purkinje fibers. On the contrary, in A-CMs, Tbx3 overexpression alone dominantly repressed the expression of both *Gja1* and *Ryr2*, corroborating with the detection of Tbx3-binding peaks (Fig. 5d) and previous reports on reduced *Gja1* and *Ryr2* expression in the SAN^{94,95}.



Together, our *in vitro* study using primary atrial and ventricular cardiomyocytes successfully recapitulated the unique expression patterns of several CCS regional marker genes in a dish. Furthermore, gene expression analysis integrated with ATAC-seq, ChIP-seq, and Irx motif data suggests that the transcriptional regulatory program mediated by Irx3 and Tbx3 is dependent on CCS regions as well as heart chamber identity, thereby contributing to the molecular heterogeneity within the CCS.

Discussion

In this study, we conducted a comprehensive molecular and spatial profiling of the transcriptional landscape of postnatal mouse CCS cells at a single-cell resolution. Our findings demonstrate how CCS components, from the SAN to PF, are molecularly continuous yet also distinct from each other. As noted in previous studies²⁹, major limitations of investigating the CCS include the low number of CCS cells in the heart (~0.1–0.2% of the entire cardiomyocyte population) and

Fig. 6 | Region-specific transcriptional regulation of CCS genes by Tbx3 and Irx3. **a** Schematic of the experimental workflow to model the CCS regional heterogeneity in vitro. Neonatal mouse atrial cardiomyocytes (A-CMs) and ventricular cardiomyocytes (V-CMs) were infected with Ad-GFP, Ad-Irx3, Ad-Tbx3 or both Ad-Irx3 and Ad-Tbx3. Created with BioRender.com. **b** Schematic of the in vitro model of the CCS regions. Nodes were simulated by Tbx3 overexpression in A-CMs; the Proximal VCS by Tbx3 and Irx3 overexpression in V-CMs; and the Distal VCS by Irx3 overexpression in V-CMs. **c** Gene expression of atrial (*Myl4* and *Myl7*) and ventricular (*Myl2* and *Myl3*) genes in cultured A-CMs and V-CMs. $n = 5$ per group. Data are presented as the mean $\pm$ SEM. P -values were determined by unpaired two-tailed Student's t -test. **d–i** Gene expression levels of the CCS component and

regional markers in A-CMs and V-CMs upon Tbx3 and/or Irx3 overexpression. Checkered bar graphs represent A-CMs and solid bar graphs represent V-CMs. The schematic of the transcriptional regulatory model in each gene is displayed. Tested genes include **(d)** SA node marker, *Vsnl1*; **(e)** AV node marker, *Prdm6*; **(f)** His bundle marker, *S100a6*; **(g)** distal bundle branch marker, *Nppa*; **(h)** distal VCS marker, *Mpped2*; and **(i)** contractile myocyte marker, *Gja1*. $n = 5$ per group for A-CMs, and $n = 5$ (*Prdm6*, *Mpped2*) or 10 (*Vsnl1*, *S100a6*, *Nppa*, *Gja1*) for V-CMs. Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test. Source data are provided as a Source Data file. VCS ventricular conduction system, A-CMs atrial cardiomyocytes, V-CMs ventricular cardiomyocytes.

difficulties in isolating viable postnatal CCS cells. For our scRNA-seq analysis, we utilized approximately 7000 CCS cells that were isolated from postnatal *Cntn2*^{Cre/+}; *Rosa26*^{tdTomato/+} mouse hearts and purified using FACS. Among several Cre lines targeting the CCS, including *Hcn4*-CreERT2⁹⁶, *Cx40*-CreERT2⁹⁷, *Gjd3*-CreEGFP³⁰ and *Sema3a*-CreERT2⁶⁶, 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^{21,33,34}. To our knowledge, this study has incorporated the highest number of purified entire mouse CCS cells to conduct a single-cell transcriptomic investigation to date.

Transcriptional profiling of the CCS using scRNA-seq was further facilitated by integration with spatial transcriptomics. Among various spatial transcriptomic technologies available, we chose NanoString GeoMx Digital Spatial Profiler (DSP) for its several advantages. First, given that all CCS components cannot be fully captured in one or two histological sections, the GeoMX DSP offered a larger slide scan area that accommodated over 10 sections of the P4 heart encompassing all CCS regions. Second, its segmentation tool enabled precise capture of tdTomato-positive CCS cells only, allowing transcriptomic assessments without the need for a cell-type deconvolution method. Lastly, it provided the whole transcriptome profiling. As a result, we were able to reliably acquire transcriptomic profiles of each CCS component. Noteworthy among our findings from ST is the molecular identification and comparison of CCS components within the atrioventricular junction: two sub-compartments of AVN^{26–28}, the cAVN and LNB, as well as the HIS. Despite their anatomical proximity, our results indicate that the LNB shares more molecular similarities with the HIS than with the cAVN (Supplementary Fig. 6 and 7). This corroborates with the previous studies, demonstrating that LNB is developmentally originated from the ventricular myocardium, whereas cAVN is derived from Tbx2⁺ atrioventricular canal^{26–28,30}. It is, therefore, important to highlight that the spatial information from ST significantly enhanced our ability to identify all CCS components in the scRNA-seq data. 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 may arise from varying numbers of cells in CCS components. Collectively, this integrative approach, supported by histological validations, enabled the discovery of CCS marker genes with greater reliability.

Our study identified several marker genes of each CCS component, while also consolidating recently recognized CCS markers. In addition, zonation patterns of genes were identified within the CCS: specifically, nodes consisting of the SAN and cAVN, proximal VCS consisting of the LNB, HIS, and PBB, and distal VCS consisting of the DBB and PF. These marker genes include *Gnao1* and *Vsnl1* in SAN, *Prdm6* in cAVN, *Ppp1r17* in LNB, *S100a6* and *Igfbp7* in proximal VCS, and *Nppa*, *Scn10a* and *Mpped2* in distal VCS. A recent study has shown that *GNAO1* was found to be highly enriched in the human SAN, while *PRDM6* was predicted as a potential TF in human nodal cells⁹⁸. This suggests possible evolutionarily conserved roles of these genes in the CCS. While previous studies have demonstrated that *Igfbp7* regulates

pathological cardiac remodeling⁹⁹ and that *S100a6*, a Ca²⁺ binding protein, modulates adverse ventricular remodeling¹⁰⁰, it has not been tested whether they play physiological roles in the CCS. Together, it remains for future research to elucidate the functional implications of these CCS marker genes in both healthy and diseased hearts.

The completion of CCS development occurs postnatally. Since deficiencies in *Nkx2-5*, *Tbx3*, and *Irx3* lead to CCS hypoplasia in the postnatal mouse (as early as P4), but not during embryonic development^{20,24,25}, it was unexpected that no significant differences in gene expression between P1 and P4 were observed in our scRNA-seq results (Supplementary Fig. 9). This observation led us to consider a few possibilities: (a) the temporal duration between P1 and P4 may be too narrow to capture the transcriptional differences; (b) the major changes in postnatal maturation may occur at a later stage, such as P14, as shown previously¹⁰¹; and/or (c) postnatal CCS development may be primarily regulated and established by the embryonic transcriptional program. These possibilities were partially tested by directly comparing our postnatal CCS scRNA-seq data with embryonic CCS data²⁹. We found high molecular similarities between E16.5 and postnatal stages within the same CCS regions, suggesting that cell specifications within the CCS, particularly for the SAN, cAVN and proximal VCS, may largely occur by E16.5. On the other hand, higher expression of early-development genes, such as *Tagln*, *Cnn1*⁸¹, *Bmp10*⁸³, and *Corin*⁸³, was noted in the E16.5 CCS, compared to the postnatal CCS, indicating CCS maturation during pre-to-postnatal development. *Corin* is a notable gene that encodes a transmembrane cardiac serine protease, which activates *Nppa*-encoded ANP by cleaving pro-ANP¹⁰². Since ANP signaling is implicated in embryonic VCS development¹⁰³, our findings raise a possibility that *Corin*-ANP signaling may be crucial for CCS development and maturation. Additionally, our observation that more than 3% of early postnatal CCS cells are still proliferating, highlights the ongoing progress of postnatal CCS maturation. Further studies are needed to examine the molecular profiles of fully mature adult CCS cells. As conducted in a series of postnatal cardiomyocytes (from P0–P28)¹⁰¹, it would also be informative to examine the trajectory of CCS postnatal maturation and identify a mediator in this developmental process. 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)¹⁰⁴ as well as *Isl1*¹⁰⁵ were undetected in the postnatal SAN region of our scRNA-seq and ST data. Considering that *Tbx18* and *Isl1* are involved in early SAN development and their expression diminishes in the adult heart¹⁰⁵, 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. 13), further investigation to ascertain whether entire SAN regions are captured by *Cntn2* could elucidate this discrepancy.

In the CCS, which is essentially a continuous structure, molecular heterogeneities govern distinct functional properties of each CCS

component, ensuring harmonious contraction and relaxation of the heart. While the roles of individual transcription factors in the development and function of CCS components have been extensively studied^{15–17}, our understanding of how these transcription factors cooperate across different regions of the CCS is comparatively limited. Our SCENIC analysis inferred gene regulatory networks within the CCS, controlled by established TFs such as *Shox2*, *Gata6*⁸⁵, *Tbx3*^{24,25,41,86}, *Tbx5*^{22,41,87}, and *Etv1*^{21,88} as well as by less explored TFs, including *Nr2f1*, *Atf5*, and *Bach1*. It is also notable that CCS-specific regulons were largely clustered within the nodes only, nodes & proximal VCS, and the VCS, suggesting the zonation patterns of gene regulatory networks within the CCS. This was further tested functionally by examining the roles of two CCS-specific TFs, *Tbx3*, and *Irx3*^{18–20,25,41}. Our in vitro model using primary atrial and ventricular cardiomyocytes overexpressing *Irx3* and *Tbx3*, along with ATAC-seq, ChIP-seq, and motif analyses, provides proof-of-principle evidence of their contributions to the establishment of molecular heterogeneities within the CCS. Specifically, our findings suggest that *Tbx3* and *Irx3* play individual, cooperative, or antagonistic actions in a chamber-specific, and CCS component-specific manner. 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. We have previously demonstrated that *Irx3* can promote transcription as a repressor (possibly through an unidentified repressor)¹⁹, suggesting a potential co-regulatory function of *Irx3*. Also, direct binding sites for *Irx* TFs remain to be identified. Thus, identifying the co-regulator(s) of *Tbx3* and *Irx3* in the context of chamber-specific or CCS region-specific gene regulation would significantly enhance our understanding of transcriptional regulatory networks in developing hearts. Indeed, our results are well aligned with a study demonstrating that the gene dosage balance between *Tbx5* and *Tbx3* orchestrates VCS-specific gene expression profiles and function⁸⁷. Specifically, the VCS-specific removal of *Tbx5* was sufficient to alter gene expression profiles of the VCS towards those of nodal cells by elevating *Tbx3* expression. Since *Irx3* physically interacts with *Tbx5*²⁰, this suggests a potential gene regulatory network among *Tbx5*, *Tbx3*, and *Irx3* in establishing CCS heterogeneities.

In conclusion, our study generates a transcriptomic resource of the postnatal murine CCS and provides functional evidence of how molecular heterogeneities across the CCS can be transcriptionally established. These findings help advance our understanding of the molecular identities within the CCS beyond its anatomical structures, and therefore have implications for therapeutic approaches against conduction system defects.

Methods

Mice

All experimental protocols were approved by the Animal Care Committees of the University of Ottawa (HI-2950 and 3060), in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). All mice had free access to standard chow (2019 Teklad Global Diet; Envigo) and water and were housed in a controlled environment with a 12 h light/12 h dark cycle, ambient temperature of 22–24 °C and humidity levels of 30–60%. Mice were humanely euthanized via CO₂ inhalation and/or cervical dislocation based on CCAC guidelines on euthanasia of animals used in science.

For scRNA-seq, ST, and histological validation experiments, homozygous *Cntn2*^{tm1.1(ccre/EGFP)Nmn} (*Cntn2*^{Cre}) and homozygous *Rosa26*^{tdTomato} mice³², which were maintained on a CD-1 background,

were crossed to generate CCS-fluorescent reporter mice (*Cntn2*^{Cre/+}; *Rosa26*^{tdTomato/+}, CCS-tdT). To achieve the maximum number of age-matched pups simultaneously, 8–15-week-old female mice were housed together without exposure to male mice for 2–3 weeks prior to mating to synchronize their estrous cycles. Following the mating setup, vaginal plugs were monitored daily. Neonatal mice on postnatal days 1, 2, and 4 were euthanized by cervical dislocation immediately before heart collection for cardiomyocyte isolation, ST or histological validation. A total of 103 CCS-tdT pups were used for scRNA-seq. For in vitro adenovirus-based overexpression studies, 2–3 P0 neonatal litters from CD-1 male and female breeders were used to isolate atrial and ventricular cardiomyocytes. Both males and females were included in all experiments, and sex differences were not considered.

Neonatal mouse cardiomyocyte isolation

The atria and ventricles of neonatal mice were collected separately in sterile ice-cold 1x phosphate-buffered saline (PBS). The ventricles were cut into 3–4 pieces for efficient digestion. Atrial and ventricular cardiomyocytes were separately placed into 50 mL centrifuge tubes containing filtered Hanks' Balanced Salt Solution (1x HBSS, ThermoFisher) with 0.0125% Trypsin-EDTA (ThermoFisher) and 1% penicillin/streptomycin (P/S; ThermoFisher). Cells were subjected to overnight trypsin digestion at 4 °C while gently rotating. Then, the trypsin solution was aspirated and discarded. 5 mL of filtered and warm (37 °C) L-15 + 0.075% Collagenase II/Dispase (Roche, 10269638001) + 1% P/S solution was added to the 50 mL centrifuge tube containing cardiomyocytes, and a fine magnetic stir bar was placed. The centrifuge tubes were placed in a tube rack, which was placed above the stir plate. After gentle stirring of the solution and cells for 5 min, the supernatant containing single cardiomyocytes was collected into 5 mL of cold fetal bovine serum (FBS) to prevent cells from over-digestion. An additional 5 mL of collagenase digestion solution was added to the cells, and this digestion-collection step was repeated until sufficient cells were collected. After the final collection, debris was removed using a 100 µm cell strainer (VWR International). Cells were then pelleted at 200 *g* for 5 min. Purified cells were resuspended and plated in a culture medium containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, ThermoFisher) with 10% FBS + 1% P/S. Pre-plating was done for 1 hr in the incubator to allow separation of fibroblasts from cardiomyocytes. Cardiomyocytes were then collected for either FACS or plated in new 12- or 24-well plates for adenovirus-mediated overexpression studies.

FACS, multiplexing of cells and single-cell RNA sequencing

Isolated P1, P2, and P4 neonatal cardiomyocytes suspended in culture medium (DMEM/F-12 with 10% FBS + 1% P/S) were filtered using a 100 µm cell strainer (VWR, 10199-658), then transferred to separate polypropylene tubes (Falcon). Cells were incubated with SytoxTM Blue dead cell stain (ThermoFisher, S34857, 1:2000) prior to Fluorescence Activated Cell Sorting (FACS). Gating was done for size and granularity, then for Sytox-based viability, and finally for tdTomato-based identification of conduction cells. Approximately 0.1–0.2% of total viable cardiomyocytes were tdTomato-positive for all P1, P2, and P4 cells (Supplementary Fig. 2).

MULTI-seq reagents were generously provided by the developers, and multiplexing of P1, P2, and P4 cells was conducted following the developer's protocols³⁵. In brief, following separate FACS sorting of P1, P2, and P4 CCS cells into culture medium (DMEM/F-12 with 10% FBS + 1% P/S), cells were pelleted and washed using centrifugation (250 *g*, 5 min, 4 °C) and 1x PBS. Lipid-modified oligonucleotide anchor mix and unique barcodes were added to each sample at final concentrations of 200 nM. Cells were resuspended by gentle pipetting and were incubated at 4 °C for 10 min, ensuring to periodically mix by gently tapping. Then, a co-anchor lipid-modified oligonucleotide was added to each sample (final concentration of 200 nM), and cells were

incubated at 4 °C for 5 min. Cells were then pelleted using centrifugation (250 g, 5 min, 4 °C) and resuspended in PBS containing 1% BSA to quench residual barcodes. P1, P2, and P4 cells were then pooled into a single tube and subjected to viability assessments and cell counting prior to scRNA-seq library generation using the 10x Chromium Single Cell 3' v3 kit (10x Genomics). All gene expression libraries were sequenced with NextSeq500 (Illumina).

scRNA-seq data analysis

The generated fastq files were processed with Cell Ranger (version 7). Reads were aligned to the mm10-3.0.0 reference transcriptome annotation file. Sequences and annotations for tdTomato and Cre were manually added to the fasta and gtf files (Supplementary Data 5), respectively, using the function *cellranger mkref*. A total of 10,407 cells were sequenced, with a mean of 35,041 reads per cell and a median of 3055 genes detected per cell. Demultiplexing was performed using Python according to the unique MULTI-seq barcodes given to each P1, P2, and P4 sample prior to pooling.

Downstream bioinformatic analysis was performed on R (version 4.2.2) using the 'Seurat' R package (version 5.0.1)¹⁰⁶. Briefly, pre-processing of the data was performed following quality assessment to include cells with >2000 and <5500 detected genes, and percentages of mitochondrial genes of >5% and <25% (Supplementary Fig. 3). Additional biological filtering was applied based on markers of common cell types known to exist in the heart. For instance, 1.03% of all cells (72 out of 6927 cells) that lacked expression of cardiomyocyte genes (*Myh6*, *Myh7*, *Tnnt2*, *Tnni1*, *Ryr2*) but expressed markers of fibroblasts (*Postn*, *Col1a1*, *Col1a2*, *Col3a1*) or smooth muscle cells (*Cald1*, *Emilin1*, *Hexim1*, *Tagln*, and *Fabp4*), were excluded from downstream analysis (Supplementary Fig. 4). Following pre-processing of the data, feature counts were normalized by log-transformation using the function *NormalizeData*. The top 5000 features were identified using 'vst' as the selection method using *FindVariableFeatures*. Normalized counts for these variable genes were then scaled using *ScaleData* with regression of mitochondrial gene counts and the number of unique molecular identifiers (UMIs). Linear dimensional reduction was performed using principal component analysis (PCA) using *RunPCA*. For Uniform Manifold Approximation and Projection (UMAP) embedding using *RunUMAP* analysis using all cells, including non-cardiomyocytes (Supplementary Fig. 4), CCS cells (Supplementary Fig. 5b), 'Nodes-HIS-BB' (Supplementary Fig. 5c), 'Nodes' (Supplementary Fig. 5d), the first 25 principal dimensions were used. Optimal clusters were identified by *FindNeighbors* and *FindClusters* using resolutions of 0.25, 0.19, 0.4, and 0.97, respectively, determined by the examination of known marker expression patterns. For the 'LNB-HIS-PBB' (Supplementary Fig. 5e), the first 10 principal components were used with a clustering resolution of 0.6. For 'Proliferative CCS' (Supplementary Fig. 8), 20 principal components were used with a clustering resolution of 0.6. Heatmaps were generated using the 'ComplexHeatmap' R package (version 2.15.4).

E16.5 CCS data (GSE132658)²⁹ was analyzed according to the author's described methods as well as using markers demonstrated in the study. Integration of E16.5 and Postnatal CCS was performed using Seurat's Integration pipeline. Briefly, anchors between the datasets were found using *FindIntegrationAnchors* followed by data integration using *IntegrateData*. For correlation between E16.5 and postnatal CCS components, averaged expressions of genes for each CCS component given by *AverageExpression* were used. Correlation analysis was then performed and visualized using the 'corrplot' R package (version 0.92). Direct comparison was performed on integrated data using *FindMarkers*.

Visualization of sequencing reads alignment to *Scn10a* was generated using the Integrative Genomics Viewer (IGV) (version 2.17.4), with the reference genome mm10 (Supplementary Fig. 12).

Gene regulatory network inference

Gene regulatory network inference was performed using 'pySCENIC' (version 0.12.1) following the SCENIC (single-cell regulatory network inference and clustering) pipeline^{84,107}. The count matrix was provided as input after filtering out genes with zero expression. Repressive regulons were excluded, and putative regulons lacking expression of the TF in at least 40% of any region were filtered out, resulting in 97 retained regulons. Regulon activity was quantified in individual cells using AUCell and averaged across each region.

To construct regional gene regulatory networks, regulons were scored based on regional specificity¹⁰⁸. The top 5 regulons specific to each region were retained after filtering for 40% expression of the TF in the respective region. Target genes differentially expressed in each region (adj. $P < 0.05$) were retained. The resulting regional networks with top 5 regulons were then visualized using the Fruchterman-Reingold layout ('iGraph', version 1.5.1), with node colors corresponding to the classification of the gene (transcription factors and CCS marker genes).

Spatial transcriptomics and data analysis

Spatial Transcriptomic (ST) analysis was performed in frozen tissue sections of P4 CCS-tdT mouse heart using the NanoString GeoMx Digital Spatial Profiler (DSP)^{109,110} in collaboration with the Advanced Genomics Core at the University of Michigan. The sections were subjected to in situ hybridization with the GeoMx Probe Mix for NGS readout (Mouse Whole Transcriptome Atlas RNA v1.0). CCS structure was visualized with anti-DsRed primary antibody (Takara, #632496, 1:200) and anti-rabbit AlexaFluor™ 594 secondary antibody (ThermoFisher Scientific, A11012, 1:500). SYTO13 nuclear staining was performed for visualization of tissue morphology and cell counting. Regions of interest (ROI) were chosen using the fluorescent segmentation function on the DSP. Two ROIs were selected for each CCS region, including the SA node (SAN), lower nodal bundle (LNB), His bundle (HIS), proximal left and right bundle branch (PLBB/PRBB), distal left and right bundle branch (DLBB/DRBB), and left and right Purkinje fibers (LPF/RPF). One ROI was collected for the compact AV node (cAVN). Non-CCS regions adjacent to each CCS region were also selected, including right atrial appendage (RAA), left endomyocardium (LEND), left epimyocardium (LEPI), basal interventricular septum (IVS-B), apical interventricular septum (IVS-A), and right ventricle (RV). Following ROI selection, DNA oligos were cleaved from RNAScope probes by directing UV light at each ROI using the digital micromirror device. Cleaved oligos were then collected into the wells of a 96-well collection plate, which were subjected to library preparation and next-generation sequencing. Sequencing depth was estimated by multiplying the total area of all the ROIs (μm^2) and sequencing of the pooled library was performed on NovaSeq 6000 (Illumina).

Raw FASTQ files were processed using NanoString's Automated Data Processing Pipeline, which includes adapter trimming and aligning stitched paired-end reads to the barcodes in the reference assay before the removal of PCR duplicates based on the UMI of each read. The resulting data collection container (DCC) files for each ROI were then processed with the 'GeoMxTools' package (version 3.0.1) using the corresponding NanoString DSP configuration file and the instrument-generated worksheet with ROI identifiers. Initial quality control filtering and third quartile (Q3) normalization were performed with minimum average negative control measurements for ROI retention and minimum counts above background for target retention. The resulting filtered and Q3 normalized data were analyzed for differential expression analysis using an unpaired t-test. A gene was considered CCS-specific as well as significantly differentially expressed (Fig. 2a, b) if it satisfied both of the following conditions: 1) |Fold change| > 1.5, $P < 0.05$ in the region of interest compared to all other CCS components, and; 2) |Fold change| > 1.5, in the region of interest compared to its respective non-CCS components. For SAN and cAVN,

its non-CCS control was RAA; for LNB, HIS, PLBB, and PRBB, its control was IVS-B; for DLBB and DRBB, its control was IVS-A; for LPF its control was LENDO and; for RPF, its control was RV.

ATAC-seq and ChIP-Seq analyses

For chromatin accessibility and binding analyses, raw data was downloaded in fastq format using the *fastq-dump* function in the SRA toolkit. Fastq files were then uploaded onto the UseGalaxy server¹¹¹. Quality control assessments were performed using FastQC (version 0.11.7)¹¹². Reads were then aligned to the mm9 mouse reference genome using Bowtie2 (Galaxy Version 2.4.2+galaxy0) with the ‘-very-sensitive’ parameter applied for optimal mapping¹¹³. Mitochondrial genome reads and low mapping quality (mapQuality <30) were considered uninformative reads and filtered out using the *Filter BAM datasets on a variety of attributes* function (Galaxy Version 2.4.1)¹¹⁴. Duplicate reads were then removed using ‘Picard MarkDuplicates’ (Galaxy Version 2.18.2.2) (broadinstitute.github.io/picard). *MACS2 callpeak* function (Galaxy Version 2.1.1.20160309.6) was used to call peaks with the following parameters: ‘Single-end BED’, ‘-nomodel’, ‘-shift -100’, ‘-extend 200’, and FDR < 0.05¹¹⁵. The following datasets were analysed: E13.5 Tbx3+ AVJ ATAC-seq (GSE121464 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121464>])²⁵, adult ventricle ATAC-seq (GSE110209 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110209>])⁹², PO sinoatrial pacemaker ATAC-seq (GSE148515 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148515>])⁹¹, PO right atrial cardiomyocyte ATAC-seq (GSE148515 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148515>])⁹¹, and adult heart Tbx3 ChIP-seq (GSE35151 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35151>])⁹³. Moreover, two *Iroquois* (*Irx*) motifs, ACATGT (Motif ID: UP00223_2)⁹⁰ and ACAnnTGT⁸⁹ were used for predicting genome-wide motif occurrences. Each motif sequence was uploaded onto FIMO (Find Individual Motif Occurrences), part of the MEME software package (tools.genouest.org/tools/meme/), and motifs were scanned in the UCSC mm9 mouse genome. Only motif occurrences with *P* values $\leq 1.0 \times 10^{-4}$ were considered significant, which are automatically calculated by FIMO based on a log-likelihood ratio score conversion¹¹⁶.

Peaks were visualized in bigWig format by converting MACS2 Bedgraph output to bigWig via the *Wig/BedGraph-to-bigWig* function (Galaxy version 1.1.1) and uploading it onto the Integrative Genomics Viewer (IGV) genome browser (version 2.16.2)¹¹⁷. For datasets where peaks were readily available in bigWig format, peak files were directly downloaded from the NCBI Gene Expression Omnibus (GEO) and uploaded onto IGV. For datasets with multiple replicates, a mean bigWig file was generated using the deepTools2 ‘bigwigCompare’ function (Galaxy version 3.5.1.0.0)¹¹⁸.

Immunofluorescence staining

Freshly isolated neonatal hearts were fixed in a methanol-free 4% paraformaldehyde solution (Thermo Scientific™ Pierce™, #28906) in PBS overnight at 4 °C. Hearts were washed twice in PBS for 30 mins each, then were incubated in filtered 30% sucrose in PBS overnight at 4 °C. Fixed cryopreserved hearts were embedded in Tissue-Tek® O.C.T compound (Sakura, #4683) and immediately frozen on powdered dry ice. Embedded hearts were sectioned using a cryostat at 8 µm thickness and stored at -20 °C until use.

For immunofluorescence staining, heart sections were air-dried for 30 min at room temperature, then washed three times in PBS for 5 min each. The sections were permeabilized with 0.1% Triton X-100 in PBS for 15 min. Blocking was done using the appropriate host serum in 0.3% triton X-100 in PBS for 1 hr at room temperature. The sections were then incubated overnight at 4 °C with the following primary antibodies: chicken anti-RFP (Rockland Immunochemicals, 600-901-379, lot #42649, 1:100), goat anti-Cntn2 (R&D Systems, AF4439, lot #CDSO0219101, 1:50), rabbit anti-Gnao1 (Thermo Fisher Scientific,

PA5-30044, lot #YH4027897A, 1:100), rabbit anti-Scn10a (Alomone Labs, ASC-016, lot #, ASC016AN2550, 1:50), rabbit anti-Igfbp7 (Abcam, ab74169, lot #GR242021-1, 1:50), rabbit anti-S100a6 (Abcam, ab181975, lot #GR3438351-1, 1:100), rabbit anti-Nppa (EMD Millipore, ab5490, lot #Q3015715, 1:250), mouse anti-Shox2 IgG2a (Santa Cruz Biotechnology, sc-81955, lot #B2822, 1:50) or rabbit anti-Vsn1 (GeneTex, GTX115039, lot #40247, 1:100), rabbit anti-Ppp1r17 (Thermo Fisher Scientific, PA5-61599, lot #YH4028316B, 1:200). The following day, sections were washed three times with PBS for 5 min each, and then incubated with appropriate secondary antibodies: goat anti-chicken IgY Alexa Fluor™ 568 (Thermo Fisher Scientific, A11041, lot #2482953, 1:200), donkey anti-chicken IgY Alexa Fluor™ 568 (Thermo Fisher Scientific, A78950, lot # 2622381, 1:200), goat anti-rabbit Alexa Fluor™ 488 (Thermo Fisher Scientific, A-11008, lot #2420730, 1:500), goat anti-mouse IgG2a Alexa Fluor™ 488 (Thermo Fisher Scientific, A21131, lot #2420706, 1:500), donkey anti-rabbit Alexa Fluor® 647 (Jackson ImmunoResearch Laboratories Inc., 711-605-152, 1:200), goat anti-mouse IgG2b Alexa Fluor™ 488 (Thermo Fisher Scientific, A21141, lot #2432059, 1:500) for 1 hr at room temperature in the dark. Finally, the sections were subjected to the Vector® TrueVIEW Autofluorescence quenching kit (Vector laboratories, SP-8400) to minimize autofluorescence of the heart. A mounting medium with DAPI (Abcam, ab104139) was used for nuclei staining. Imaging was performed using LSM 880 confocal microscope with Airyscan (Zeiss) or AxioObserver 7 microscope (Zeiss), and processing was performed using ZEN microscopy software with Airyscan processing and orthogonal projection. Whole tissue fluorescence imaging to examine *Cntn2*^{Cre}-mediated tdTomato expression of the CCS was performed using the Zeiss Stereo Discovery V8 stereomicroscope. Fluorescence slide scanning was performed using Aperio VERSA 8 Scanner (Leica Biosystems).

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed using the RNAscope Multiplex Fluorescent Reagent Kits v2 (Advanced Cell Diagnostics, ACD) as per the manufacturer's protocol. In brief, frozen tissue sections (8 µm thickness) of P4 CCS-tdT mouse heart were baked at 60 °C for 30 min, followed by post-fixation in 4% PFA for 30 min at 4 °C. After target retrieval for 5 min and Protease Plus incubation for 15 min, the sections were incubated for 2 hrs at 40 °C with the following probes: *Prdm6* (mouse, Cat #456891), *Mpped2* (mouse, ACD, #848371), *Pcp4* (mouse, ACD, #402311) and tdTomato (mouse, ACD, #317041-C2). Opal 690 Reagent Pack (Akoya Biosciences, #FP1497001KT, 1:200) and Opal 570 Reagent Pack (SKU FP1488001KT, 1:200) were used to visualize the FISH signal. Fluorescence microscopy was performed using LSM 880 confocal microscope (Zeiss) or AxioObserver 7 microscope (Zeiss), followed by image processing with ZEN microscopy software.

Trichrome staining

To examine the cellular morphology and fibrous insulation in the atrioventricular region of the cardiac conduction system (*i.e.*, cAVN, LNB and HIS), P4 mouse hearts were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 µm thickness. Fibrosis was visualized by Masson's trichrome staining, and images were captured using the Aperio VERSA 8 Scanner (Leica Biosystems).

Adenovirus-mediated overexpression

24 hrs following the isolation and plating of atrial and ventricular cardiomyocytes, debris and dead cells were washed with pre-warmed PBS. After washing, pre-warmed fresh culture medium (DMEM/F-12 + 10% FBS + 1% P/S) containing adenoviruses expressing GFP, *Irx3*, *Tbx3*, or both *Irx3* and *Tbx3* was added to the cells at a multiplicity of infection of 10 virus particles per cell. Adenovirus was washed off after 7-12 hrs, and the cells were cultured for an additional 48 hrs before being collected for RNA extraction.

Quantitative PCR

Cultured cardiomyocytes were lysed in TrizolTM Reagent (InvitrogenTM) through vigorous pipetting. For every 1 mL of TrizolTM, 0.2 mL of chloroform (C607-4, Fisher Chemical) was added to the cell lysate, followed by vigorous shaking for 15 sec and incubation at room temperature for 5 min. Centrifugation was done at 12,000 g for 15 min at 4 °C to allow separation of the aqueous layer collection. Using the aqueous layer, RNA was purified with PureLinkTM RNA Mini Kit (InvitrogenTM) according to the manufacturer's protocol. Nanodrop2000 spectrophotometer (Thermo Fisher Scientific) was used for spectrophotometric quality and quantity assessments. cDNA was synthesized from purified RNA through reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM) following the manufacturer's instructions, and to inhibit RNA degradation, RNaseOUTTM Recombinant Ribonuclease Inhibitor (InvitrogenTM) was used. Quantitative PCR (qPCR) was performed using PowerUpTM SYBRTM Green Master Mix (Applied BiosystemsTM) with QuantStudioTM 5 Real-Time PCR System (Applied Biosystems). Relative fold change and normalization of genes of interest were performed using the $2^{-\Delta\Delta CT}$ method, with *Tbp* serving as the housekeeping gene after ensuring its stable expression across all conditions. Primer sequences used are listed in the supplemental data (Supplementary Data 6).

Statistical analysis and reproducibility

Fluorescence stereomicroscope imaging of the CCS-tdT heart (Fig. 1a) was performed on more than five hearts. All immunofluorescence experiments (Fig. 3a, c–f and Supplementary Figs. 12, 13, 15–17, 19, and 20) were independently performed at least two times with varying antibody concentrations. All fluorescence in situ hybridization (FISH) experiments (Supplementary Fig. 14, 18, 21) were conducted at least twice. Comparison between the embryonic (E14.5) and postnatal CCS data (Fig. 4d) was conducted using the Wilcoxon Rank Sum Test. Data are provided in Supplementary Data 3. Statistical analyses of qPCR experiments (Fig. 6 and Supplementary Figs. 24 and 25) were performed using GraphPad Prism 10 (GraphPad Software). Results are reported as mean $\pm$ standard error of the mean (SEM). Statistical differences between the means of two or more groups were tested using unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. *P*-values less than 0.05 were considered statistically significant. The number of samples (*n*) is stated in figure legends. No statistical method was used to pre-determine the sample size.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The scRNA-seq data and spatial transcriptomics data generated in this study have been deposited in the Gene Expression Omnibus (GEO) database under accession code, [GSE231547](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE231547). Source data for Fig. 6 and Supplementary Fig. 25 and 26 are provided with this paper. Source data are provided with this paper.

Code availability

All codes used to generate the results and figures in this study are available in Zenodo (<https://doi.org/10.5281/zenodo.12520577>).

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Author contributions

K-H.K. and Y.O. conceived the project and designed experiments. Y.O., R.A., T.A., and K-H.K. performed experiments. Y.O., S.D., M.B., and K-H.K.

conducted bioinformatic analysis. Y.O., R.A., M.B., and K-H.K. prepared the figures. D.P.C., B.V., N.V.M., and C.-C.H. provided reagents. D.P.C. and J.G.P. provided technical support. K-H.K. directed the project. Y.O. and K-H.K. drafted, edited, and revised the manuscript with input from all authors. All authors, Y.O., R.A., S.D., M.B., T.A., D.P.C., B.V., J.G.P., N.V.M., C.-C.H., and K-H.K. read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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8.2 MANUSCRIPT IN PREPARATION

Isolation of neonatal mouse atrial and ventricular myocytes

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Abstract

Neonatal rodent cardiomyocytes serve as a valuable *in vitro* model for investigating cellular and molecular mechanisms of the heart, circumventing the technical challenges associated with isolating and maintaining adult cardiomyocytes. Importantly, studying atrial and ventricular cardiomyocytes separately is crucial due to their distinct functional and genetic properties. Here, we present a protocol for the simultaneous isolation and culture of neonatal mouse atrial and ventricular cardiomyocytes. This protocol includes the dissection of P0 - P1 neonatal mouse hearts, separation of atrial appendages and ventricles, and sequential digestion with trypsin and collagenase. The chamber-specific identities of isolated cardiomyocytes were validated by gene expression analyses of atrial and ventricular markers. The resulting cardiomyocyte populations demonstrate high viability and maintain chamber-specific characteristics, rendering them suitable for a wide array of experimental applications under physiological and pathological conditions. Possible applications include physiological assessments (e.g., calcium transient analysis, patch clamp), morphological assessments, biochemical assays, and drug screening.

Introduction

Cardiac *in vitro* models are continuously evolving to more accurately recapitulate cardiovascular function and facilitate the manipulation of molecular processes and cellular behaviors, such as by altering genetic components or environmental conditions (e.g., metabolic energy substrates and hypoxia). Current models, ranging from two-dimensional (extracellular matrix-patterned cell culture) to three-dimensional (organoids, engineered heart tissue) tissue constructs are able to mimic some of the heart's biological and mechanical properties, including anisotropic tissue structure, extracellular matrix network, and vascularization¹. The cellular components of these models, particularly cardiomyocytes, which are responsible for the heart's contractility and constitute the largest portion of its volume, provide valuable insights into fundamental cardiac functions and mechanisms. Common cardiomyocyte models include human and rodent-derived immortalized cell lines (e.g., AC16 for ventricular², HL-1 for atrial³), human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) including those derived from embryonic stem cells (hESCs-CMs) and induced pluripotent stems (hiPSC-CMs), and primary rodent cardiomyocytes. The selection for the appropriate model depends on factors such as translatability, cellular maturation, production time, cost-effectiveness, genetic manipulation potential, and the ability to distinctly represent atrial and ventricular cardiomyocyte populations.

Studying atrial and ventricular cardiomyocytes separately is crucial due to their distinct functions, structures, and genetic profiles. Immortalized cell lines allow for independent study of distinct atrial or ventricular populations, but often exhibit diverging phenotypes to primary cells due to the induced genetic mutations required for immortalization. While hPSCs can differentiate into atrial or ventricular-like cells⁴⁻⁶, the process can be complex and often yields heterogeneous populations. Rodent primary cardiomyocyte isolation protocols have been optimized over the past 50 years, but the isolation of atria and ventricular cells while achieving high yield and cell viability remains challenging. Protocols for isolating atria and ventricles from adult rat and mouse hearts have

been reported^{7,8}, but adult rodent cardiomyocytes are less viable⁹ and likely to lose their phenotype^{10,11} *in vitro*. Neonatal rodent cardiomyocytes, despite their immature phenotype characterized by different metabolism¹² and excitation-contraction coupling processes¹³⁻¹⁵, are easier to maintain and offer a more cost-effective alternative for studying cardiac physiology and pathology, providing mechanistic insights despite phenotypic and genetic differences.

Here, we present our protocol for simultaneously isolating and culturing neonatal mouse atrial and ventricular cardiomyocytes. The major procedures of this protocol involve microdissection of P0-P4 neonatal mouse hearts, separation of atrial and ventricular appendages, overnight pre-digestion, and subsequent collagenase digestion to release cardiomyocytes. The isolated cells are then purified by pre-plating, counted, and plated in a culture dish. We validate the chamber-specific identity of the isolated cardiomyocytes by gene expression analyses (**Figure 1**) and immunofluorescence staining of atrial and ventricular markers (**Figure 2**). This protocol provides a reliable method for obtaining distinct atrial and ventricular cardiomyocyte populations, facilitating downstream experiments to elucidate chamber-specific cardiac biology and pathophysiology.

Materials

All solutions must be sterile and freshly prepared. All equipment must be sterilized before use.

Dissection and pre-digestion

1. 1× Dulbecco's phosphate-buffered saline (DPBS). Store at 4°C before use.
2. Pre-digestion solution: Hank's balanced salt solution (HBSS) with 0.0125% Trypsin-EDTA and 1% Penicillin/Streptomycin (P/S) (see **Note 1**).
3. 35 mm petri dishes
4. 70% (v/v) ethanol
5. Micro-dissection tools (Dumont No.5 forceps, scissors, micro-scissors).
6. 50 mL tube rotator

Collagenase digestion and cell collection

1. Collagenase digestion solution: L-15 medium (Leibovitz) with 0.075% Collagenase II/Dispase and 1% P/S. Incubate at 37 °C before use (see **Note 2**).
2. Fetal bovine serum (FBS): Store at 4C before use (see **Note 3**).
3. Cell culture medium: Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12) with L-Glutamine, 2.438g/L sodium bicarbonate and phenol red, 10% FBS, 1% P/S. Incubate at 37°C before use.
4. Mini stir bar (see **Note 4**)
5. Magnetic stirrer
6. Ice bucket
7. Serological pipettes (5 mL, 10 mL, 25 mL)
8. 100 µm cell strainer
9. Two timers

Pre-plating and cell culture

1. Trypan blue stain
2. Cell culture medium: Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12) with L-Glutamine, 2.438 g/L sodium bicarbonate and phenol red, 10% FBS, 1% P/S. Incubate at 37 °C before use.
3. Cell counter or Hemocytometer
4. 35 mm or 100 mm culture dishes (see **Note 5**)
5. Incubator (37 °C, 5% CO₂, 95% humidity)

Product Name	Catalog #
DPBS, no calcium, no magnesium	14190144 (Thermo Fisher Scientific)
HBSS, no calcium, no magnesium, no phenol red	14175-095 (Thermo Fisher Scientific)
Trypsin-EDTA (0.5%), no phenol red	15400054 (Thermo Fisher Scientific)
Penicillin-Streptomycin (10,000 U/mL)	15140122 (Thermo Fisher Scientific)
Leibovitz's L-15 Medium, no phenol red	21083027 (Thermo Fisher Scientific)
Collagenase/Dispase	11097113001 (Roche)
DMEM/F12	11320033 (Thermo Fisher Scientific)
Trypan Blue Solution, 0.4%	15250061 (Thermo Fisher Scientific)
Nunc™ Cell-Culture Treated Multidishes	142475 (Thermo Fisher Scientific)
35 mm x 10 mm Easy Grip Style Cell Culture Dish	353801 (Corning)
100mm x 20 mm TC-Treated Culture Dishes	430293 (Corning)

Table 1. Details of materials used

Methods

Dissection and tissue collection

1. Under the biological safety cabinet, prepare 25 mL of pre-digestion solution in a 50 mL conical tube (see **Note 1**).
2. Prepare a minimum of four 35mm culture dishes, each containing approximately 2 mL of ice-cold DPBS.
3. Transfer one DPBS-filled dish and the neonatal mice, to the chemical fumehood.
4. Prepare a container with 70% (v/v) ethanol for surface sterilization.
5. Immerse the neonatal mouse in 70% (v/v) ethanol for surface sterilization.
6. Position the mouse dorsal side up and perform cervical dislocation by applying upward pressure to the posterior cranium while stabilizing the body.
7. Reposition the mouse dorsal side up. Using fine forceps, retract the forelimbs to expose the thoracic cavity.
8. Make an incision along the right lateral side of the sternum and carefully remove the heart.
9. Transfer the collected heart to the first DPBS-filled dish.
10. Repeat the procedure for each neonatal mouse (steps 4-9).
11. After all hearts are collected, pull or cut away any non-cardiac tissue if present, and transfer hearts to the second dish containing fresh cold DPBS to remove blood.
12. Micro-dissect the atria from the ventricles. Move all atria to the third dish, and ventricles to the last dish.

13. Make 5-6 cuts in each ventricle while keeping the tissue intact (see **Note 6**).
14. Using a 10 mL serological pipette, carefully collect the atria and ventricles from each petri dish and transfer them to separate 50 mL conical tubes containing ~2 mL of the pre-digestion solution (see **Note 7**).
15. Place the tubes into a rotator in the 4°C cold room and rotate for 14 - 16 hours at approximately 12 RPM (see **Note 8**).

Collagenase digestion

Perform the following steps for both the atria and ventricle unless otherwise stated:

1. Move the pre-digestion tubes to the biological safety cabinet.
2. Allow tissue to settle at the bottom of the tube, and carefully remove pre-digestion Trypsin solution using a 25 mL pipette. Avoid disrupting tissue.
3. Add 3 mL of pre-warmed (37°C) collagenase digestion solution each to the atrial and ventricular tissue tubes (see **Note 3**).
4. Add a sterile micro stir bar to the tube and place the tubes in a tube rack above the magnetic stirrer. Set the stirrer to 50-100 RPM.
5. Stir the ventricular tissue for 10 mins and the atrial tissue for 5 mins at room temperature (RT).
6. Using a 10 mL pipette, gently aspirate and release the tissue up and down the pipette once and wait briefly for 5 -10 seconds to allow the tissue to settle to the bottom of the tube. Discard the supernatant.
7. Add 3 mL of warm (37°C) collagenase digestion solution to the tissue.
8. Stir the ventricular tissue for 15 mins and the atrial tissue for 5 mins at RT.
9. Using a 10 mL pipette, gently aspirate and release the tissue up and down the pipette 3 – 4 times and wait briefly for a few seconds to allow the tissue settle to the bottom of the tube.
10. Carefully collect the supernatant containing isolated cells and add into a 50 mL tube containing 5 mL of ice-cold FBS (see **Note 10**). Place the tube on ice.
11. Repeat steps 7-10, collecting the digested solution in alternating FBS tubes (see **Note 11**).

Pre-plating and cardiomyocyte collection

Perform the following steps for both the atria and ventricle unless otherwise stated:

1. Strain the collected cells (in FBS) into new 50 mL conical tubes through a 100 µm cell strainer using a 25 mL serological pipette.
2. Balance and centrifuge the FBS tubes at 200 x g for 5 min at RT (see **Note 12**).
3. Gently pour out supernatant into a waste bottle by tilting the 50 mL tube (see **Note 13**).
4. Add 2 mL of media (DMEM/F12 + 10% FBS + 1% P/S) and thoroughly but gently resuspend the pellet using a P1000 pipette (see **Note 14**). Avoid making bubbles.
5. Prepare two 100 mm culture dishes (see **Note 5**). Add 8 mL of media to each empty culture dish. Gently resuspend cells again and add 2 mL of cells from each tube into each culture dish (see **Note 15**).

Cardiomyocyte isolation and culture

1. Pre-plate cells for 1 hr in the incubator (37 °C, 5% CO₂).
2. Remove the plates from the incubator, and gently pipette supernatant (containing unattached CMs) up and down to collect weakly attached CMs. Collect CM-containing supernatant using a 10 mL pipette into a new 50 mL falcon tube (see **Note 16**). Avoid making bubbles.
3. Count cells using a hemocytometer and Trypan blue stain (see **Note 17**).
4. Plate the cardiomyocytes in a culture plate at a density of 0.5×10^6 cells/well in a 24 well plate (see **Notes 14-15**). Adjust the final volume in each well to ~1 mL (see **Note 18**).
5. Incubate overnight (37 °C, 5% CO₂).
6. Assess cell viability and morphology the following day.
7. Wash the cells with 2 mL of pre-warmed (37 °C) culture medium.
8. If cells exhibit suboptimal health, replace the medium and continue culture for an additional 24 hours. Otherwise, proceed with experimental protocols.

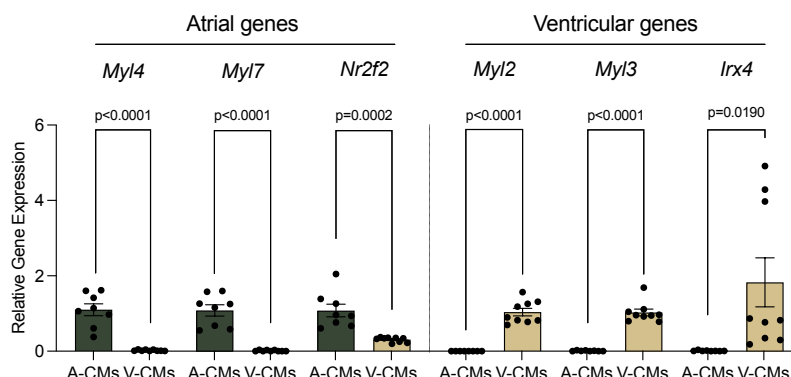


Figure 1 Quantitative PCR shows significant enrichment of atrial (*MyI4*, *MyI7*, *Nr2f2*) and ventricular (*MyI2*, *MyI3* and *Irx4*) gene expression in isolated A-CMs and V-CMs, respectively. n=8 for A-CMs and n=9 for V-CMs. Data are presented as mean $\pm$ standard error of mean (SEM). Unpaired student's t-test. A-CMs, atrial cardiomyocytes; V-CMs, ventricular cardiomyocytes.

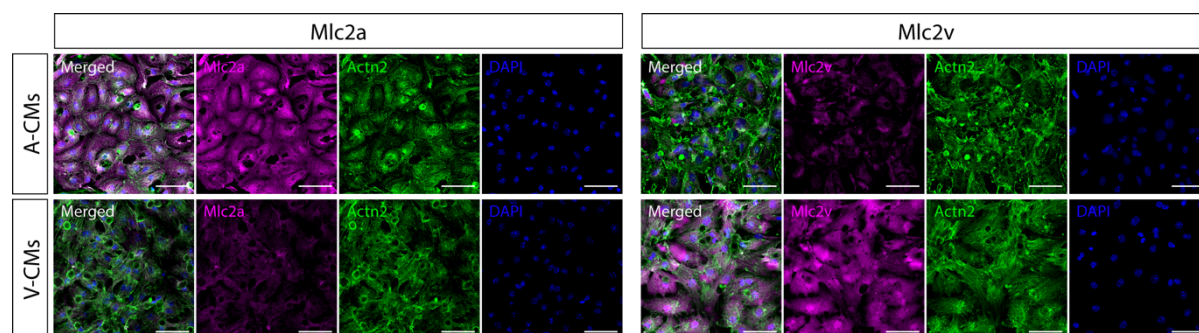


Figure 2 Immunofluorescence stainings of Mlc2a (encoded by an atrial gene, *MyI7*) and Mlc2v (encoded by a ventricular gene, *MyI2*) in A-CMs and V-CMs show enriched protein expression of Mlc2a and Mlc2v in isolated A-CMs and V-CMs, respectively. A-CMs, atrial cardiomyocytes; V-CMs, ventricular cardiomyocytes. Scale bar = 50 μ M.

Notes

1. Prepare 25 mL of pre-digestion solution for each tube with approximately 40 atria or 20 ventricles per tube.
2. More than 10 rounds of collagenase digestion are performed to ensure complete digestion.
3. FBS inhibits the enzymatic activity of collagenase, therefore preventing excessive digestion of collected cells.
4. Add 70% EtOH to a 30 mm petri dish and place the stir bar in the dish using forceps. After swirling around the stir bar, take it out and place it on the petri dish lid and let it air dry.
5. Cutting the ventricles open helps efficient digestion by increasing surface area exposed to digestion enzyme. However, it is preferred that the ventricles stay intact even after cutting as over-cutting the ventricles may lead to over digestion. Make a transverse cut at least once so that blood inside the chambers can be washed out and tissue can be efficiently digested.
6. The tissue will be highly adhesive, so pipetting up the tissues excessively can lead to the hearts attaching to the inner walls of the serological pipette, and will lead to tissue loss. Ensure the hearts stay near the bottom of the pipette.
7. Ensure proper balance of the laboratory rotator to maintain consistent agitation during overnight incubation.
8. The hearts will have clustered together. Do not touch the hearts, they have been digested overnight, so cells will fall off.
9. Complete tissue digestion is characterized by loss of structural integrity and a viscous consistency. Adjust collagenase digestion time and increase collection frequency for highly digested tissues to prevent over-digestion.
10. Equilibrate FBS volumes between tubes if necessary. Post-centrifugation, cells will form a dark pink pellet at the tube bottom.
11. Do not tilt the falcon tube too slowly as cells can drain out.
12. Ensure thorough resuspension of the cell pellet to generate a homogeneous single-cell suspension. This will be crucial for uniform plating and to prevent cellular aggregation.
13. Adjust culture vessel dimensions, media volume, and cell density based on the total cell yield and experimental requirements. For ventricular cardiomyocytes, 100 mm culture dishes with 10 mL media are typically sufficient. For less abundant atrial cardiomyocytes, use smaller vessels (e.g., 30 mm dishes with 2 mL media) to optimize cell concentration. Consider downstream applications (e.g., imaging, pharmacological treatments) when determining final plating conditions.
14. Check under microscope to ensure even plating. Cells will initially appear round and clear when first plated.
15. Fibroblasts are highly adhesive so they will stick to the plate during pre-plating. Cardiomyocytes remain in suspension or weakly adherent, allowing for the selective removal of fibroblasts¹⁶.
16. Efficient digestion leads to $\sim 0.5 \times 10^6$ cells per neonatal mouse heart.

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8.3 CURRICULUM VITAE

Yena Oh

EDUCATION

Ph.D. in Cellular and Molecular Medicine (expected February 2025) University of Ottawa, Ottawa, ON, Canada <i>Supervisor: Kyoung-Han Kim, PhD</i>	Jan 2019 – Present
M.Sc in Physiology , Specialization in Cardiovascular Sciences University of Toronto, Toronto, ON, Canada <i>Supervisor: Peter H. Backx, PhD & DVM</i>	2014 – 2017
B.Sc in Human Biology (Health & Disease) and Cell Systems Biology University of Toronto, Toronto, ON, Canada <i>Graduated with High Distinction, Dean's List</i>	2009 – 2014

DISTINCTION

• Trainee of the Year. University of Ottawa Heart Institute	May 14, 2024
• Paul Dudley White International Scholar (Best abstract in Canada). 2023 Basic Cardiovascular Sciences Scientific Sessions, American Heart Association	Jul 31, 2023
• Award of Excellence – PhD Graduate. Faculty of Medicine Awards in Education Ceremony (Awarded to a PhD student demonstrating academic excellence through overall average and research capabilities)	Dec 1, 2022
• Paul Dudley White International Scholar (Best abstract in Canada). 2022 Basic Cardiovascular Sciences Scientific Sessions, American Heart Association	Jul 24, 2022
• Gordon K. Moe Young Investigator Winner. The 26 th Annual Upstate New York Cardiac Electrophysiology Society Conference, Utica, New York, USA	Oct 28, 2016
• Gold Medal. Cayley Canadian Mathematics Competition, University of Waterloo	2006

SCHOLARSHIPS

• Korean-Canadian Science Scholarship Foundation Award – Young Professional Forum (awarded a fully funded conference trip, including flight, accommodation and conference attendance)	Jul 2024
• Canadian Institutes of Health Research (CIHR) Travel Award (1,500 CAD)	Oct 2023
• Canadian Institutes of Health Research (CIHR) Travel Award (1,500 CAD) - <i>declined</i>	Feb 2023
• University of Ottawa Heart Institute Travel Award (1,000 CAD)	May 2022
• CIHR Doctoral Award – Frederick Banting and Charles Best Canada Graduate Scholarship (105,000 CAD)	2021-2024
• University of Ottawa Admission Scholarship – Doctorate (36,000 CAD)	2019-2023
• University of Ottawa Heart Institute Endowed Graduate Scholarship (40,000 CAD)	2019-2021
• Queen Elizabeth II Graduate Scholarship in Science and Technology Award (QEII-GSST) (\$15,000 CAD)	2019-2020
• University of Ottawa Excellence Scholarship (10,000 CAD)	2019-2020
• Queen Elizabeth II/Heart & Stroke Foundation of Ontario Graduate Scholarships in Science and Technology Award (15,000 CAD)	2016-2017
• University of Toronto Fellowship – Physiology (10,000 CAD)	2014-2015

PRESENTATION AWARDS

Oral Presentation Awards

- **2nd place - Fame Lab Presentation.** Young Professional Forum (World Congress of Korean Scientists & Engineers, Seoul, South Korea) Jul 10, 2024
- **Trainee Oral Presentation Award – PhD category.** Academy of Cardiovascular Research Excellence, Ottawa, Canada Jun 30, 2023
- **2nd place - Korean Cardiovascular Society Best Abstract Presentation.** The 4th Asian Cardiovascular Symposium at Basic Cardiovascular Sciences Scientific Sessions, Chicago, USA (150 CAD) Jul 24, 2022
- **Innovation Award.** 34th Annual Ottawa Cardiovascular Research Day 2022, Ottawa, Canada (1,000 CAD) May 27, 2022
- **Trainee Research Award – Basic Science, Runner Up Award.** Canadian Cardiovascular Congress, Virtual (250 CAD) Oct 22, 2021
- **Honourable Mention for Best Research Seminar Presentation.** Cellular and Molecular Medicine Seminar Day, University of Ottawa, Ottawa, Canada (50 CAD) Feb 25, 2020
- **Best Talk Cardiovascular Biology and Neuroscience.** 1st Annual uOttawa Faculty of Medicine Research Day, Ottawa, Canada (500 CAD) Sep 25, 2019
- **Dr. Yves Marcel Award for Best Basic Science Oral Presentation.** Ottawa Cardiovascular Research Day 2019, Ottawa, Canada (200 CAD) May 13, 2019
- **1st place in Online Research & Leadership Competition.** Canada-Korea Conference on Science and Technology, Ottawa, Canada Aug 8, 2016

Poster Presentation Awards

- **Best Basic Science Poster.** The 35th Ottawa Cardiovascular Research Day, Ottawa, Canada (150 CAD) May 16, 2023
- **2022 Margaret Moffat Award for Best Poster in Biomedical Sciences.** The 41st North American Meeting of the International Society of Heart Research, Winnipeg, Canada (250 USD) Sep 8, 2022
- **Honourable Mention award.** Canadian Student Health Research Forum, Winnipeg, Canada Jun 15, 2022
- **Best Poster Award.** 1st China-Canada-USA Pharmacology/Physiology Conference, University of Toronto, Toronto, Canada Jun 30, 2017
- **Gordon K. Moe Young Investigator Winner.** The 26th Annual Upstate New York Cardiac Electrophysiology Society Conference, Utica, New York, USA (1,000 USD) Oct 28, 2016
- **Young Generation Best Poster Award.** Canada-Korea Conference on Science and Technology, Ottawa, Canada (150 CAD) Aug 8, 2016
- **Best Poster Award.** 7th Annual Muscle Health Awareness Day. York University, Toronto, Canada (150 CAD) May 27, 2016
- **1st Place in the Cardiovascular Session.** Frontiers in Physiology, University of Toronto, Toronto, Canada (150 CAD) May 11, 2016
- **3rd Place.** HSRLCE Michael J. Sole Annual Cardiovascular Scientific Day, Toronto, Canada (Top 3 selected for oral presentation, 250 CAD) Apr 4, 2016

PUBLICATIONS

Peer-Reviewed Publications

- **Oh Y.** Abid R, Dababneh S, Bakr M, Aslani T, Cook DP, Vanderhyden BC, Park JG, Munshi NV, Hui CC, Kim KH. *Transcriptional regulation of the postnatal cardiac conduction system heterogeneity.* *Nature Communications.* 2024 Aug 2;15(1):6550. (PMID: 39095365)
- Zhang L, Smyth D, Al-Khalaf M, Blet A, Du Q, Bernick J, Gong M, Chi X, **Oh Y**, Roba-Oshin M, Coletta E, Feletou M, Gramolini AO, Kim KH, Coutinho T, Jannuzzi JL, Tyl B, Ziegler A, Liu PP. *Insulin-like*

growth factor-binding protein-7 (IGFBP7) links senescence to heart failure. Nature Cardiovascular Research. 2022 Dec 22; 1(12):1195-1214. (PMID: 36088651)

- Kim KH, **Oh Y**, Liu J, Dababneh S, Xia Y, Kim RY, Kim DK, Ban K, Husain M, Hui CC, Backx PH. *Irx5 and transient outward K⁺ currents contribute to transmural contractile heterogeneities in the mouse ventricle.* Am J Physiol Heart Circ Physiol. 2022 May 1;322(5):H725-H741. (PMID: 35245131)
- Asif S*, Kim RY*, Fatica T, Sim J, Zhao X, **Oh Y**, Cheung AC, Mulvihill EE, Kim KH. *Hmgcs2-mediated ketogenesis modulates high fat diet-induced hepatosteatosis.* Molecular Metabolism. 2022 Apr 12; 101494. (PMID: 35421611)
- **Oh Y**, Yang S, Liu X, Jana S, Izaddoustdar F, Gao X, Debi R, Kim DK, Kim KH, Yang P, Kassiri Z, Lakin R, Backx PH. *Transcriptomic bioinformatic analyses of atria uncover involvement of pathways related to strain and post-translational modification of collagen in increased atrial fibrillation vulnerability in intensely exercised mice.* Frontiers in Physiology. 2020 Dec 23;11:605671. (PMID: 33424629)
- Lin HB, Naito K, **Oh Y**, Farber G, Kanaan G, Valaperti A, Dawood F, Zhang L, Li GH, Smyth D, Moon M, Liu Y, Liang W, Rotstein B, Philpott DJ, Kim KH, Harper M-E, Liu PP. *Innate Immune Nod1/RIP2 Signaling Is Essential for Cardiac Hypertrophy but Requires Mitochondrial Antiviral Signaling Protein for Signal Transductions and Energy Balance.* Circulation. 2020 Dec 8;142(23):2240-58. (PMID: 33070627)
- Kim RY, Lee JH., **Oh Y**, Sung HK, Kim KH. *Assessment of the metabolic effects of isocaloric 2:1 intermittent fasting in mice.* J Vis Exp. 2019 Nov 27;(153). (PMID: 31840661)
- Panama BK, Korogyi AS, Aschar-Sobbi R, **Oh Y**, Gray B, Gang H, Brown, JH, Kirshenbaum LA and & Backx PH (2016). *Reductions in the cardiac transient outward K⁺ current I_{to} caused by chronic β -adrenergic receptor stimulation are partly rescued by inhibition of nuclear factor kappaB.* J Biol Chem. 2016 Feb 19;291(8):4156-65 (PMID: 26742842)
- Sun H, **Oh Y**, Yan M, Yu BO (2015). *3D Is It a Better Choice for All, or for Some?* J Cardiovasc Electrophysiol. 2015 Oct;26(10):E9. (PMID: 25952964)
- Sun H, **Oh Y**, Ding M, & Yang P (2015). *More caution required for iatrogenic short QT: additional concerns on QTc shortened by testosterone treatment.* Pacing Clin Electrophysiol. 2015 Aug;38(8):1021. (PMID: 25973953)
- Tian J, Ding M, **Oh Y**, & Yan M (2016). *SA-VA Difference with Extended Utility—More to Consider.* Pacing Clin Electrophysiol. 2016 Jan;39(1):107. (PMID: 26481580)

Manuscripts in Preparation

- Bakr M, Dababneh S, Tibbits GF, **Oh Y***, Kim KH*. *Comparative analysis of the mouse and human cardiac conduction system heterogeneity* (*, co-corresponding authors)
- Liu WF*, **Oh Y***, Abid R*, Yin WC, Kim RY, Zhou YQ, Zhang X, Mo R, Puvindran V, Srianjan S, van Eede M, Henkelman M, Bruneau BG, CC Hui, Kim KH. *The combinatorial role of Iroquois Homeobox Genes 3 and 4 in the compaction of the ventricular myocardium.* (*, equal contribution)
- Dababneh S*, **Oh Y***, Pan J*, Oudit GY, Backx PH, Kim KH. *IRX5 mediates ventricular transmural heterogeneity of the healthy and diseased heart.* (*, equal contribution)
- Choe MS, **Oh Y**, Hahn N, Vijitha Puvindran, Hui CC, Son JE, Kim KH. *Repressor form of Irx3 enhances adipose thermogenesis through beige adipocyte differentiation.*

Book Chapters

- **Oh Y***, Pan J*, Kim KH. *Isolation and Culture of Neonatal Mouse Atrial and Ventricular Myocytes.* *Mouse Embryogenesis Methods and Protocols*, Springer Nature (*, equal contribution) (Submitted)
- Abid R*, **Oh Y***, Kim KH. *Visualization of embryonic great vessels using plastic resin injection.* *Mouse Embryogenesis Methods and Protocols*, Springer Nature (*, equal contribution) (Submitted)

SELECTED OR INVITED CONFERENCE TALKS

- *Transcriptional Regulation of the Postnatal Cardiac Conduction System Heterogeneity.* **Ottawa Cardiovascular Research Day**, Ottawa, ON, Canada May 14, 2024
- *Transcriptional Heterogeneity of the Postnatal Cardiac Conduction system.* **AHA Basic Cardiovascular Science Scientific Sessions (BCVS)**, Boston, MS, USA Jul 31, 2023

- *Transcriptomic Landscape of the Postnatal Cardiac Conduction System CCS.* Feb 14, 2023
Keystone Symposium - Heart Development and Disease: From Genes to Cures,
Santa Fe, NM, USA
- *Transcriptional Blueprint of the Postnatal Atrioventricular Conduction system.* Sep 7, 2022
International Society for Heart Research, North American Section, Winnipeg, MB,
Canada
- *Transcriptomic Landscape of the Postnatal Atrioventricular Conduction system.* Jul 24, 2022
**AHA Basic Cardiovascular Science Scientific Session – Asian Cardiovascular
Symposium (BCVS-ACS),** Chicago, IL, USA
- *Transcriptomic heterogeneity of the postnatal atrioventricular conduction system at a
single-cell resolution.* Oct 21, 2021
Canadian Cardiovascular Congress (CCC) (Virtual)
- *Research in Bioinformatics.* Feb 26, 2021
Career Mentorship Seminar hosted by The Association
of Korean-Canadian Scientists and Engineers from University of Ottawa, Queen's
University and McMaster University (Virtual)
- *The Role of *Irx5* in Establishing Ventricular Transmural Gradients.* Sep 25, 2019
1st Annual Faculty of Medicine Research Day, University of Ottawa, Ottawa, Canada
- *Molecular Mechanisms Driving Ventricular Transmural Heterogeneity by *IRX5*.* May 13, 2019
Ottawa Cardiovascular Research Day. Ottawa, ON, Canada
- *Exercise-Induced Differential Atrial and Ventricular Remodeling.* Aug 8, 2016
**Canada-Korea Conference on Science and Technology 2016. Online Research & Leadership
Competition.** Ottawa, ON, Canada

ACADEMIC/WORK EXPERIENCES

- Doctor of Philosophy (Ph.D.) Candidate** Jan 2019
University of Ottawa, Department of Cellular and Molecular Medicine (Supervisor: Dr. Kyoung-Han Kim) – Jun 2024
- Ph.D. thesis: Investigated transcriptional regulation of the postnatal cardiac conduction system heterogeneity (**Oh Y et al. *Nature communications*. 2024**)
 - Led projects investigating the individual and overlapping roles of *Iroquois (Irx)* transcription factors in the heart
 - Investigated molecular heterogeneity within the cardiac conduction system, *Irx3*-mediated transcriptional regulation in the ventricular conduction system and *Irx5*-based transmural gradient establishment using mouse genetics, cardiac physiological analysis, *in vitro* studies and bioinformatics analysis
 - Utilized high-throughput data/database (i.e. microarray, bulk RNA-seq, scRNA-seq, snRNA-seq, multi-seq (scRNA-seq & scATAC-seq), GTEx human consortium) to perform a wide variety of bioinformatics analysis, including differential gene expression, motif, pathway and correlation analysis.
- Laboratory technician** Jan 2018
University of Ottawa Heart Institute (Supervisor: Dr. Kyoung-Han Kim) – Dec 2018
- Led a project and co-supervised an undergraduate student examining the role of *Irx5* in the establishment of transmural gradient property of the heart.
 - Co-authored in Kim et al. *AJP-Heart and Circulatory Physiology*, 2022
 - Review article currently in progress
 - Maintained mouse colony (Ear notching, genotyping, weaning)
- Master of Science (M.Sc.)** Sept 2014
University of Toronto, Department of Physiology - Cardiovascular Sciences (Supervisor: Dr. Peter Backx) – Sept 2017
- M.Sc. thesis: Investigated differential mouse atrial/ventricular remodeling in response to swim exercise using molecular techniques, bulk RNA-seq and bioinformatic analysis (**Oh Y et al. *Frontiers in Physiology*. 2020**)
 - Co-authorship for a project investigating the role of NFκB in the reduction of cardiac transient outward K⁺ currents (*I_{to}*), in response to chronic β-adrenergic receptor stimulation (Panama et al., *J Bio Chem*, 2016)

- Lab volunteer** Sept 2013
– May 2014
University of Toronto, Department of Physiology - Cardiovascular Sciences (Supervisor: Dr. Peter Backx)
- Conducted daily swimming exercise experiment in mice to examine TNF α 's role in exercise-induced atrial arrhythmias. Acknowledged in Aschar-Sobbi et al., *Nature Communications*, 2015
- Medical & health project intern - 'Heart Surgery Project' assistant** May 2012
– Jan 2013
Development for Freedom International, Port-au-Prince, Haiti (Supervisor: Ms. Helen Kim)
- Assisted in the "Heart Surgery Project" to send 8 Haitian children with cardiovascular defects to Korea University Anam Hospital for surgeries.
 - This project involved transporting patients via the South Korean Army military aircraft, with permission from the United Nations Stabilization Mission in Haiti (MINUSTAH)
 - Organized regular meetings and took part in discussions with Haitian government officials and Haitian local NGOs with goals to receive national support for the project
- Research assistant** Jun 2011
– Sept 2011
Toronto Western Hospital, Toronto, ON, Canada (Supervisor: Dr. Robert Lam)
- Generated data to develop a clinical tool for physicians to better interact with elderly patients by conducting questionnaires and interviewing 20 family physicians in the Greater Toronto Area (GTA) who have referred one or more of their patients to the Seniors Wellness Clinic. Acknowledged in Lam et al., *Can Geriatr J*, 2013
- Clinical support volunteer** Dec 2010
– Sept 2011
Family Health Clinic, Toronto Western Hospital, University Health Network, Toronto, ON, Canada
- Interacted and communicated closely with the medical staff to provide optimal service for patients: escorted patients to physicians, restocked medical supplies in inpatient units, performed basic clinical assessments of patients (i.e. height and weight) prior to medical examination

RESEARCH TECHNIQUES

Bioinformatics-related techniques

Quality control, processing, bioinformatic & statistical analysis and visualization of high-throughput data (Microarray, Bulk RNA-seq, scRNA-seq, multi-seq (scRNA-seq & scATAC-seq), human Genotype-Tissue Expression (GTEx) database)

Molecular and biochemical techniques

DNA/RNA isolation, PCR, quantitative PCR, Western blot, Immunofluorescence, Immunohistochemistry, Confocal microscopy

In vivo mouse work

Mouse handling & colony management, Surface electrocardiograms (sECGs), Tissue dissection

In vitro work

Neonatal mouse cardiomyocyte/fibroblast isolation, *in vitro* adenovirus infection

ACADEMIC EXTRACURRICULAR EXPERIENCES

Work-In-Progress (WIP) Coordinator

University of Ottawa Heart Institute, Trainee Committee

Apr 2019
– Jun 2020

- Scheduled, organized and moderated trainee Work in Progress (WIP) rounds every week with more than 60 trainee presenters and >200 audience per week.
- Organized the trainee committee-organized 'Summer Student Research Day' and established evaluation template, incorporating a standardized presentation grading criteria and judging scheme to encourage fair competition and recognition of students' works

President of DFI Youth, International Programs & Operations Coordinator

Development for Freedom International (DFI), Toronto, ON

Jan 2013
– 2016

- Organized the "DFI Youth" group to engage youths in reaching out to support those who are underprivileged or in need, and held weekly meetings.
- Held fundraising events ("Destination Haiti" and "DFI Busking"), where Haitian souvenir products produced by Haitian artists trained in DFI were sold