Developmental Origin, Growth, and Three-Dimensional Architecture of the Atrioventricular Conduction Axis of the Mouse Heart


Rationale: The clinically important atrioventricular conduction axis is structurally complex and heterogeneous, and its molecular composition and developmental origin are uncertain.

Objective: To assess the molecular composition and 3D architecture of the atrioventricular conduction axis in the postnatal mouse heart and to define the developmental origin of its component parts.

Methods and Results: We generated an interactive 3D model of the atrioventricular junctions in the mouse heart using the patterns of expression of Tbx3, Hcn4, Cx40, Cx43, Cx45, and Nav1.5, which are important for conduction system function. We found extensive figure-of-eight rings of nodal and transitional cells around the mitral and tricuspid junctions and in the base of the atrial septum. The rings included the compact node and nodal extensions. We then used genetic lineage labeling tools (Tbx2+/Cre, Mef2c-AHF-Cre, Tbx18+/Cre), along with morphometric analyses, to assess the developmental origin of the specific components of the axis. The majority of the atrial components, including the atrioventricular rings and compact node, are derived from the embryonic atrioventricular canal. The atrioventricular bundle, including the lower cells of the atrioventricular node, in contrast, is derived from the ventricular myocardium. No contributions to the conduction system myocardium were identified from the sinus venosus, the epicardium, or the dorsal mesenchymal protrusion.

Conclusions: The atrioventricular conduction axis comprises multiple domains with distinctive molecular signatures. The atrial part proliferates from the embryonic atrioventricular canal, along with myocytes derived from the developing atrial septum. The atrioventricular bundle and lower nodal cells are derived from ventricular myocardium. (Circ Res. 2010;107:728-736.)

Key Words: atrioventricular canal ■ atrioventricular node ■ three-dimensional reconstruction ■ lineage analysis ■ heart development ■ transgenic mice

The atria and ventricles are separated by the connective tissues of the atrioventricular junction that insulate the atrial and ventricular muscle masses. A small part of the musculature, however, the atrioventricular conduction axis, crosses the plane of insulation, thus allowing conduction of the impulse generated by the sinus node to the ventricles. The axis has atrial parts, including the atrioventricular node and atrioventricular ring bundles, and ventricular parts, the atrioventricular bundle and the bundle branches.1-2 The atrioventricular node delays the electric impulse, thus permitting the ventricles to fill before ventricular contraction. The node can also function as a subsidiary pacemaker. Several arrhythmias, such as atrioventricular block and reentrant tachycardia, have their anatomic substrates within the axis,2-5 which is complex and heterogeneous in terms of its morphology.2-6

Insight into the mechanisms of the arrhythmias can be provided by an understanding of development. Of the transcription factors implicated in the regulation of the developmental process, transcription factor Tbx3 is expressed specifically in the central conduction system, thus providing a key marker with which to delineate these tissues throughout development and in the adult.7-9 The origin and lineages of the atrioventricular junctions, however, have still to be clarified. It is currently thought that the atrial components are derived from the embryonic atrioventricular canal, whereas the atrioventricular bundle developed from the interventricular ring. In this model, the

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atrioventricular node develops at the caudal crossing point of the atrioventricular and interventricular rings. Others have indicated that the rate of proliferation of the proposed precursors is insufficient to generate all the cells of the adult axis, which, hence, requires recruitment of adjacent nonaxis myocardium. It has also been suggested that the sinus horns, atrial septal myocytes, and the atrioventricular bundle could contribute to the atrial part of the axis.

Although the mouse is the most widely used mammal for experimental analysis, the structure and composition of the atrioventricular junctions, including the conduction axis, remain undefined. This perhaps reflects the difficulty in defining morphologically the components of the axis as it develops. With this in mind, we have assessed the molecular composition and structure of the mouse atrioventricular junctions and analyzed the patterns of expression of key functional genes in the adult heart using 3D reconstructions. We then determined the developmental origin of the identified components of the conduction axis.

**Methods**

Experimental procedures for in situ hybridization, immunohistochemistry, 3D reconstruction, and statistical analyses are provided in the Online Data Supplement, available at http://circres.ahajournals.org. Animal care was in accordance with national and institutional guidelines.

**Results**

**An Interactive Three-Dimensional Model of the Adult Mouse Atrioventricular Junctions**

We used the patterns of expression of key genes to create an interactive 3D reconstruction of the adult atrioventricular junctions. Hcn4 is involved in the spontaneous depolarization, or automaticity, of cardiomyocytes. It is expressed in all components of the mouse atrioventricular conduction axis. The gap junction subunit connexin (Cx)40 forms high-conductance gap junctions and is expressed in the atrial working myocardium and the atrioventricular bundle and branches but not in the atrial part of the axis. Cx43 is the major gap-junction subunit that is expressed in the working myocardium. In rodents, it is not expressed anywhere in the atrioventricular conduction axis. The low conductance subunit Cx45 is enriched in the atrioventricular canal. The major cardiac sodium channel Nav1.5 is expressed in working myocardium, but within the axis is confined to the atrioventricular bundle and its branches. Cx45 is expressed in all components of the conduction axis. In slow-conducting atrial components, it is involved in the repression of Cx40, Cx43, and Nav1.5. Cardiomyocytes were detected by their expression of cardiac troponin (cTnI). We also reconstructed the location of all cTnI-negative cells (connective tissue and fibroblasts), along with extracellular material. Taken together, the patterns of these genes allowed mapping of the components of the atrioventricular axis based on their molecular signatures, independent of their morphological features and eventual locations. The interactive 3D reconstruction can be found as Online Figure I. It supports Figure 1, as well as Online Figures II and III. We used general vertebrate terms of anatomic location, specifically cranial, caudal, ventral, dorsal, left, and right, to describe the location of the cardiac structures relative to the murine body axes, hoping to avoid confusion with terms for description of locations in the human.

The atrial working myocardium expressed Cx40, Cx43, Cx45, and Nav1.5 but not Tbx3 and Hcn4. Adjacent to the connective tissues of the insulating atrioventricular plane (Figure 1A), we observed a ring of myocardium positive for Tbx3, Hcn4, and Cx45 at a lower level, but negative for Cx40, Cx43, and Nav1.5. We nominated this myocardium as the nodal atrioventricular ring and colored it yellow. It formed a figure-of-eight structure around the mitral and tricuspid junctions (Figure 1B; Online Figures II and III), incorporating also the base of the atrial septum. This domain was less extensive around the mitral than the tricuspid orifice (Online Figure III). Between this nodal ring and the atrial working myocardium, we observed an additional figure-of-eight-like domain of cardiomyocytes negative for Tbx3, Hcn4, and Cx40, which expressed Cx43 and Nav1.5 in a gradient toward the atrial myocardium (Figure 1B and 1C; Online Figures II and III). We called this domain the transitional atrioventricular ring and colored it red.

Beneath the nodal ring, at its caudal (inferior) side, we noted a domain with high Hcn4 and Cx45 expression, which we colored green. This corresponds to the compact atrioventricular node and its inferior nodal extension (Figure 1B and 1C; Online Figures II and III). This domain was partly ensheathed by connective tissue. The connective tissue between the compact node and the nodal atrioventricular ring, however, was very thin and discontinuous (Online Figure IV). We observed, nonetheless, 2 major sites of direct connection between the node and the rings: a small site at the end of the inferior nodal extension, which corresponds to the slow nodal pathway; and a large site toward the left atrioventricular ring, which was in continuity with the myocytes of the atrial septum. Within the basal part of the atrioventricular node, we observed a domain that was negative for Cx43, but positive for Cx40 and Nav1.5. This domain extended through the major atrioventricular insulating fibrous tissue to become continuous with the atrioventricular bundle. It corresponds to the so-called lower nodal cells (Figure 1B and 1C; Online Figures II and III). When traced cranially, the atrioventricular bundle continued to encircle the base of the aorta, whereas when traced apically, it gave rise to the bundle branches.

**Lineage Contributions to the Atrial Part of the Conduction Axis**

Several tissues have been suggested to contribute to the atrial part of the conduction axis during its development, including the atrioventricular canal, the myocardium of the sinus horns, the atrial septum, and the atrioventricular bundle. Genetic
lineage analysis using specific Cre-drivers (cGATA6-enhancer-Cre or Tbx2\textsuperscript{Cre}) revealed that the atrioventricular canal contributes to the atrial components of the conduction axis.\textsuperscript{12,13} The contributions to the atrioventricular bundle and lower nodal cells, however, were not properly assessed in these studies, because these structures were not appropriately identified. Furthermore, in case of the cGATA6-enhancer-Cre study, the expression of Cre in the atrioventricular bundle was not excluded. We examined postnatal day (P)14 Tbx2\textsuperscript{2+}Z/EG mice to determine the contributions of the descendants of the Tbx2\textsuperscript{+} cells of the atrioventricular canal, the atrial septum, and the dorsal mesenchymal protrusion to the postnatal conduction axis (Figure 2 and Online Figure VII). The atrioventricular bundle and the lower nodal cells were discriminated by expression of Cx40. At P14, the Tbx2\textsuperscript{2+} lineage was found in all atrial Hcn4\textsuperscript{+} Tbx3\textsuperscript{+} cells, indicating that the atrioventricular canal, along with other atrial structures, may contribute to the atrial components of the axis. In contrast, recombination was virtually excluded from the Cx40\textsuperscript{+} domain, revealing that the atrioventricular bundle and the lower nodal cells did not receive cellular contributions from the Tbx2\textsuperscript{2+} myocardium of the atrioventricular canal and atrium (Figure 2).

The Mef2c-AHF-Cre line has been used to label the anterior second heart field, which provides the precursors of the outflow tract and the right ventricle, as well as the dorsal mesenchymal protrusion, or vestibular spine.\textsuperscript{30,31} At embry-
Tbx2\(^{+/Cre,Z/EG}\)

A

B

C

D

Figure 2. Tbx2\(^{+}\) embryonic atrioventricular (AV) canal myocardium does not contribute to the AV bundle. In situ hybridization analyses of serial sections of P14 Tbx2\(^{+/Cre,Z/EG}\) mice. Tbx3 and Hcn4 delineate the central conduction system. Cx40 demarcates the AV bundle. Egfp represents the Tbx2\(^{+}\) lineage. A and B, The Cx40\(^{+}\) parts of the AV conduction system are not derived from the Tbx2\(^{+}\) cells. C and D, Only at the caudal most part is there overlap between AV canal-derived cells and Cx40\(^{+}\) population. avb indicates atrioventricular bundle; cavn, compact atrioventricular node; la, left atrium; ra, right atrium; rv, right ventricle.

The Expression Domain of the Developing Atrioventricular Conduction Axis

Tbx3 is expressed early in development in a pattern suggested to delineate the precursors of the adult axis.\(^7\) The expression pattern of a BacTbx3Egfp construct contains regulatory sequences that directs expression of Egfp to a subdomain of Tbx3\(^{+}\) cell population in the heart.\(^{31}\) Therefore, at P14, we compared the expression of Egfp to that of Tbx3, Cx40, and Hcn4, thus establishing the relation between these markers in the conduction axis. Expression of Egfp was limited to a subdomain positive for both Tbx3 and Hcn4, which included the compact atrioventricular node and its inferior extension, the nodal atrioventricular junctional ring myocardium encircling the tricuspid valvar orifice, and the cranial crossing point of the atrioventricular and interventricular rings, known as the retroaortic node. No expression was found in the Tbx3\(^{+}\) atrial septal component of the ring encircling the mitral valve, nor the Cx40\(^{+}\) atrioventricular bundle (Figure 5A and B; Online Figure V). We reconstructed hearts from embryos at E10.5 and E17.5. At both stages, Tbx3 was expressed in all components of the developing atrioventricular conduction system, and Egfp in a subdomain which corresponded to the putative precursors of the atrioventricular node and the other components positive for Egfp at P14, but which excluded the Tbx3\(^{+}\) atrial roof at E10.5, and the Tbx3\(^{+}\) atrial septal component later in development (Figure 5A and B; Online Figures IV [A and B] and V). These spatiotem-
poral patterns of gene expression suggest that, throughout development, *Tbx3* marks the entire atrioventricular conduction axis, whereas a pool of *Egfp* myocytes within this domain specified at an early stage forms specific components of the axis, including the compact atrioventricular node.

**Proliferation of the Atrioventricular Canal Myocardium**

Previous studies have been interpreted to suggest that the rate of proliferation of the myocytes within the embryonic atrioventricular conduction axis is insufficient to form all the

![Figure 3. In situ hybridization analyses of serial sections of E14.5 (A) and P14 (B) *Mef2c-AHF-Cre;Z/EG* mice. A, The *Mef2c-AHF-Cre* transgene is active in the ventricle, including the atrioventricular (AV) bundle, but not in the cells derived from the dorsal mesenchymal protrusion, which, nevertheless, are recombined because their progenitors have expressed Cre. This transgene can be used to assess the contribution of these cells to the AV conduction system. B, *Tbx3* represents the central conduction system. The ventricular myocardium, AV bundle, and the dorsal mesenchymal protrusion do not contribute to the Cx40-negative myocardium of the AV conduction system. asnc indicates atrial septal nodal cells; avb, atrioventricular bundle; cavn, compact atrioventricular node; dmp-d, dorsal mesenchymal protrusion-derived; la, left atrium; ls, left sinus horn; lv, left ventricle; pv, pulmonary vein; ra, right atrium; rv, right ventricle; ravr, right atrioventricular ring.](Image)

![Figure 4. The sinus horn myocardium and epicardium do not contribute cells to the atrioventricular (AV) node and junction. In situ hybridization analyses of serial sections of *Tbx18*+/Cre;R26R<sup>LacZ</sup> embryos (A) and E17.5 (B). A, At E10.5, Cre expression recapitulates that of *Tbx18* in the sinus horn myocardium (red arrowhead) and in the epicardium. No expression is seen in the AV canal myocardium (black arrowhead) nor in the venous valve (blue arrowhead). Recombination (*LacZ<sup>+</sup>* ) is found in all *Tbx18*<sup>+</sup> areas and in the venous valve (A, blue arrowhead). B, At E17.5, a sharp border is found between the sinus horn myocardium and the AV canal (black arrowhead). Note the absence of recombination in the AV node in contrast to the robust recombination of the surrounding connective tissue. avc indicates atrioventricular canal; cavn, compact atrioventricular node; ct, connective tissue; lv, left ventricle; ra, right atrium; rv, right ventricle; sh, sinus horn; pv, pulmonary vein.](Image)
myocytes of the mature conduction axis, implying the need for recruitment of myocytes from adjacent working myocardium.\textsuperscript{15,16} Therefore, we determined the number of cells in the Tbx3\textsuperscript{+} atrioventricular canal and the BacTbx3Egfp subdomain and then used a bromodeoxyuridine-incorporation assay to assess whether these populations proliferated sufficiently to form the myocytes of the definitive atrioventricular conduction axis. The number of Tbx3\textsuperscript{+} and of Egfp\textsuperscript{+} cells was similar at subsequent stages of development and was increased at P14 (Figure 5D). We found that, after 1 hour of exposure to bromodeoxyuridine, 10\% to 15\% of the Tbx3\textsuperscript{+} and of the Egfp\textsuperscript{+} myocardium was labeled (Figure 5C). With the calculated cell cycle length (see the Online Data Supplement), we determined the expected number of cells after 48 hours, which was at least 2 times higher than observed (not shown). This indicated that the embryonic populations proliferated sufficiently to supply all myocytes within the Tbx3\textsuperscript{+} and Egfp\textsuperscript{+} parts of the postnatal atrioventricular conduction axis.

**Discussion**

**Location and Extent of the Mouse Atrioventricular Conduction System Based on Gene Expression Patterns**

The atrioventricular conduction axis is a frequent source of arrhythmias. Insight into the mechanisms underlying those arrhythmias can be provided by an understanding of the development and structure of the atrioventricular conduction axis. We have now created a 3D reconstruction of the mouse atrioventricular conduction system, mapping the patterns of expression of a panel of key functional genes, so as to provide insight into the structure and identity of its component parts. The 3D model is provided in an interactive format to permit independent interpretation (see Online Figure I). The domains identified show marked similarities to those seen in humans, rats, and rabbits.\textsuperscript{3,4,6,28} In the hearts of both man and mouse, the compact atrioventricular node is recognized as a group of specialized cardiomyocytes set against the central fibrous body. At the atrial side, the nodal cells extend caudally into the tricuspid and mitral rings and make contact with the atrial working myocardium through transitional cells in the atrial septum and caudal atrial wall. The compact node and lower nodal cells are continuous with the atrioventricular bundle, the transition from node to bundle, as first established by Tawara,\textsuperscript{1} being the point at which the atrioventricular conduction axis crosses the plane of atrioventricular insulation.

Our analysis reveals several previously unappreciated features. Within the myocardium of the atrioventricular junction, we identified dual molecular domains, both having the shape of a figure-of-eight. The first domain, the nodal atrioventricular ring myocardium, is directly adjacent to the plane of atrioventricular insulation, and has a slow-conducting nodal signature. It includes the lower rim of the atrial septum, the rings encircling the valves, the retroaortic root branch, and the retroaortic node.\textsuperscript{21,28,34} This domain was adjacent to a second figure-of-eight–like domain in which we did not detect nodal markers or the activity pattern of BacTbx3Egfp based on the expression pattern of Tbx3 and BacTbx3Egfp at E10.5, E17.5, and P14 show a similar shape of the Egfp\textsuperscript{+} subpopulation throughout development. C and D, Using a bromodeoxyuridine-incorporation assay, we determined the proliferation rate within the Tbx3\textsuperscript{+} and Egfp\textsuperscript{+} AV canal (n=1 per stage), and we were able to determine that the proliferation rate within the AV canal is sufficient to form all cells of the consecutive stage. Scale bar, 100 \mu m. avb indicates atrioventricular bundle; avc, atrioventricular canal; cavn, compact atrioventricular node; ine, inferior nodal extension; lavc, left atrioventricular canal; lavr, left atrioventricular ring; rarb, retro-aortic root branch; ravc, right atrioventricular canal; ravr, right atrioventricular ring.
tissue remains to be established. Nevertheless, the location of the right myocardial connection (inferior nodal extension) indicates it is the equivalent of the slow nodal pathway observed in other species (Figure 1).2–5

We also observed an atrial septal component of the conduction axis that connects the caudal and cranial parts of the atrioventricular rings (Figures 1 and 6; and Online Figures I and II). These myocytes are insulated from the atrioventricular bundle (Figure 6). They have not received much attention in literature, and their origin and functional significance is unclear.

Developmental Origin of the Components of the Atrioventricular Conduction Axis

Several features remained to be clarified concerning the developmental origin of the atrioventricular conduction axis. For example, is the embryonic atrioventricular canal the major source of the atrial part of the conduction axis,10,11,13 or are major contributions made by adjacent tissues, such as the atrial chamber myocardium, the left sinus horn, the dorsal mesenchymal protrusion, or the atrioventricular bundle?15,17–20 What are the lineage origins of the atrial and ventricular parts of the axis, respectively?19,20 By our use of genetic lineage tracing, we have excluded the notion that contributions to the atrial part of the axis are made from the sinus horns or the epicardium (Tbx18+/−), from the ventricular myocardium, including the atrioventricular bundle, or from the dorsal mesenchymal protrusion (Mef2c-AHF-Cre−/− derived) with the atrioventricular bundle. The Tbx18− lineage formed the sinus horn myocardium and contributed to the connective tissues but did not contribute to the AV conduction axis.

Figure 6. Two-dimensional representation depicting the expression domains and lineages of the atrioventricular (AV) conduction axis. The Cx40-negative AV nodal cells are derived from the embryonic Tbx2+ population, which did not contribute to the Cx40+ AV bundle. Part of the Cx40+ cells located within the atrial tissues are referred to as the lower nodal cells. These cells share their expression signature and lineage origin (Mef2c-AHF-Cre−/− derived) with the atrioventricular bundle. The Tbx18− lineage formed the sinus horn myocardium and contributed to the connective tissues but did not contribute to the AV conduction axis.

![Figure 6](attachment:Figure_6.png)

Figure 7. Model to show that the embryonic atrioventricular (AV) canal, together with the embryonic interventricular ring and Tbx3+ atrial septum, represents the putative precursors for the adult AV conduction tissues. The AV canal myocardium contributes to the atrial parts of the AV conduction axis except for the lower nodal cells. The myocardium of the embryonic interventricular ring forms the lower nodal cells, the AV bundle, and the septal branch. We propose that the atrial septal nodal cells are derived from the Tbx3+ atrial septum.

![Figure 7](attachment:Figure_7.png)
The origin of the atrial septal component of the conduction axis is unclear. Its position and its Bactbx3Egfp negativity suggest it is not derived from the embryonic atrioventricular canal. The finding that the myocytes are Tbx2+ derived in this case is not informative, because in addition to the atrioventricular canal, several other tissues in the atrium were found to initiate expression of Tbx2 (Cre) (Online Figure VII). These myocytes are probably not derived from the endocardium of the atrioventricular cushions, nor from the mesenchymal cap of the primary atrial septum, as neither population muscularizes. The dorsal mesenchymal protrusion, in contrast, is known to muscularize and is a possible contributor to the nodal myocytes of the lower atrial rim. This nodal component, however, was not labeled by the Mef2C-AHF-Cre lineage tool. Provided that all cells in the dorsal mesenchymal protrusion express Cre, this indicates that this structure does not contribute myocytes to the atrioventricular node nor the atrial septal nodal component. Then, the only remaining sources for these nodal cells are the myocardium of the atrial roof lining the dorsal mesenchymal protrusion, the right and left pulmonary ridges, or the myoccardium of the developing primary atrial septum cranial to the endothelial-derived cap. These cells express Tbx3 from early stages onward, suggesting that they have a nodal gene program from the outset (Online Figure VI). We propose that the atrial septum nodal cells are derived from the half-ring of Tbx3+ myocytes in the atrial roof at the site of development of the primary atrial septum (Figure 7).

As we have emphasized, it was Tawara, in his original description of the atrioventricular conduction axis, who divided the axis into atrial and ventricular segments using an anatomic criterion, setting the border at its site of penetration into the central fibrous body, and noting a coincident cellular change at that site in the sheep heart. In the rabbit heart, cells with electrophysiological features of the atrioventricular bundle are known to extend into the atrial segment, and have been called the lower nodal cells. Our lineage analysis now shows that this lower nodal cells are derived from the half-ring of Tbx3+ myocytes in the atrial roof at the site of development of the primary atrial septum (Figure 7).

An Embryonic Blueprint for the Mature Atrioventricular Conduction Axis

Based on our correlations, we postulate that the atrioventricular conduction axis is already represented at the earliest stages of development, being made up of the atrioventricular canal ring, along with an interventricular half-ring. It proliferates sufficiently to form the adult components. The atrioventricular canal forms the nodal and transitional atrioventricular myocardial rings, including the compact atrioventricular node with its inferior nodal extension, the retroaortic root branch, and the retroaortic node. The interventricular myocardial ring forms the lower nodal cells, the atrioventricular bundle, and the septal branch. The nodal and transitional atrioventricular cells in the lower rim of the atrial septum are possibly derived from the half-ring of Tbx3+ myocytes in the atrial roof at the site of development of the primary atrial septum (Figure 7 and Online Figure VI).

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Disclosures

None.

References

We have generated a portable and interactive 3D model of the structure of the atrioventricular node. The atrioventricular conduction system electrically connects the atria and ventricles and delays the electrical impulse, permitting the ventricles to fill before their contraction. Defects in the development of this system contribute to cardiac arrhythmias, yet little is known about the developmental origin and formation of this system. We have defined the different component parts and the 3D structure of the mouse atrioventricular conduction system. We found extensive rings of nodal and transitional cells around the atrioventricular valves of the adult heart, which include the compact nodal and transitional cell components.

The atrioventricular bundle and lower nodal cells are derived from the embryonic ventricular myocardium. Detailed understanding of the composition of the atrioventricular conduction system and the process of its formation is an important issue given that arrhythmias are a major cause of morbidity and mortality and can arise from defects in embryonic development. The mouse and human conduction system appeared to be highly similar, indicating that the mouse is a powerful model to study errors in human conduction system development and disease.
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Detailed Methods

Transgenic mice

$Tbx2^\text{Cre}$, $Mef2c$-$AHF$-$\text{Cre}$ and $Tbx18^\text{Cre}$ transgenic mice have been described are previously.\textsuperscript{1-3} $Tbx2^\text{Cre}$ labels the atrioventricular canal, base of the ventricles, outflow tract, atrial septum and dorsal mesenchymal protrusion. The $Mef2c$-$AHF$-$\text{Cre}$ line labels the anterior, or second, heart field, the outflow tract, the right ventricle, the interventricular septum, and the dorsal mesenchymal protrusion. $Tbx18^\text{Cre}$ labels the epicardium, sinus horn myocardium, myocytes and other cell types in the compact left ventricular wall and interventricular septum, and endocardium-derived cells. The different $\text{Cre}$ transgenic lines were crossed with the reporter strains $Z/EG$\textsuperscript{4} or $\text{R}26\text{R}^{\text{LacZ}}$,\textsuperscript{5} to obtain the lineage. $Tbx3\text{BacEgfp}$ was previously described.\textsuperscript{6} All mice were bred on FVB background except for the $Tbx18^\text{Cre}$ animals, which were bred on NMRI background. Animal care was in accordance with national and institutional guidelines.

BrdU assay

Pregnant females were injected intraperitoneally with 50 mg of 5'-bromo-2'-deoxyuridine (BrdU) / kg bodyweight (Sigma B5002) dissolved in 0.9% NaCl. After 1 hour of BrdU exposure the mice were killed by cervical dislocation after CO2 1 l/min anesthesia. The embryos were isolated on ice-cold PBS and further processed for immunohistochemistry (see below).

Cell count and proliferation

The $Tbx3^+$ population and the Egfp$^+$ population where both identified on sections and separately counted manually for all nuclei and the BrdU$^+$ nuclei within that population. The labeling index was calculated by dividing the BrdU$^+$ nuclei by all nuclei. Based on the labeling index we could calculate the cell cycle length based on the following formula: $F_B=\frac{T_S}{T_G+1/T_G}$, with $T_S$ and $T_G$ representing the length of the S phase and the cell cycle, respectively. The length of the S-phase was set on 4 hours. Based on the cell cycle length we calculated the expected number of cells from E10.5 to E12.5 and from E12.5 to E14.5 for both domains with the following equation: $N_{\text{Exp}} = N_0 * 2^{\frac{T_G}{T}}$ in which $N_0$ is the number of cells at either E10.5 or E12.5 and T is 48 hours.

Immunohistochemistry and in situ hybridization

Probes and methodology of the non-radioactive in situ hybridization analysis and immunohistochemistry were described previously.\textsuperscript{1,8} Embryos and adult hearts were fixed in 4% formaldehyde (ISH and IHC), embedded in paraplast and sectioned at 8 µm for immunohistochemistry and in situ hybridization. Alternatively, the embryos and hearts were frozen for cryosection (IHC). For IHC, the primary antibodies used were: BrdU rat polyclonal (1:600; AbD serotec), cTnI rabbit polyclonal (1:250; Hytest Ltd), Tbx3-goat polyclonal (1:250; Santa Cruz), Egfp rabbit polyclonal (1:250; Santa Cruz), Cx40 goat polyclonal (1:250; Santa Cruz), Cx40 mouse monoclonal (1:250; BD transduction), Cx43 mouse monoclonal (1:250 BD transduction), Cx45 rabbit polyclonal (1:250; Chemicon), Cx30.2 rabbit polyclonal (1:200 kindly provided by Dr. Klaus Willecke, Bonn, Germany), Nav1.5 rabbit polyclonal (1:250; Alamone Labs), Hcn4 rabbit poly clonal (1:250; Chemicon). The secondary antibodies used were Alexa 680 (1:250; Molecular Probes), Alexa 647 (1:250; Molecular Probes), Alexa 568 (1:250; Molecular Probes) Alexa 488 (1:250; Molecular Probes) with the epitope appropriate to visualize the primary antibody. Nuclei were stained using Dapi stain (1:40,000; Molecular Probes).

3D-reconstruction protocol

7, 8 and 10 µm serial sections were stained by in situ hybridization or immunohistochemistry, and 3D reconstructions were performed as described previously using Amira 5.2 software.\textsuperscript{9} The expression patterns of the different proteins were independently confirmed in samples of at least two additional hearts. The interactive 3D pdf was created using Adobe Acrobat Pro Extended ® version 9.3. The 3D
pdf can be viewed with the freeware version: Adobe Reader® (version 9.3 or higher) with Javascript® enabled. Detailed methods used to generate the 3D pdf will be described elsewhere.
Online Figure Legends

Online Figure I. To view Online Figure I, please scroll to the end of the manuscript PDF or download the separate online supplement - Interactive 3D PDF file.

Interactive 3D reconstruction of the AV junction in a adult mouse heart. This pdf document enables one to examine the 3D reconstruction of the AV conduction system in 3 dimensions with the ability to visualize the annotated domains individually. This pdf should be viewed in Adobe Reader® version 9.3 or higher with Javascript® enabled. The left column contains buttons to switch structures on or off and to make them transparent. To move the reconstruction, left-click on the reconstruction itself.

Rotate: push and hold the left mouse-button and move.
Translate: push and hold the left and right mouse-button and move.
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Online Figure II. (A) The atrioventricular (AV) conduction axis as shown in Figure 1, now viewed from the ventricular side. From left to right the distinct layers of the AV junction peeled off. The AV junction, compact AV node and proximal part of the AV bundle are shielded from the ventricular myocardium by connective tissue. The AV bundle travels along the crest of the ventricular septum approaching anteriorly the AV ring via the septal branch, without making physical contact due to the annulus fibrosus. (B, C) Immunohistochemical sections showing the expression patterns of Hcn4, Cx40, Cx43, Cx45, Nav1.5 and cTnl in the AV node (B) and AV bundle (C) region. Importantly, the Hcn4 pattern differs between hearts depending on the applied level of detection. In the compact AV node Hcn4 is always visible due to relative high expression levels. In the remainder of the AV junction, Hcn4 is only detectable when a high detection level is applied. For an example of detection of low levels of Hcn4, see Online Figure III. Dashed lines indicate level of sectioning. lavc, left atrioventricular canal; ravc, right atrioventricular canal; ao, aorta; lavr, left atrioventricular ring; ravr, right atrioventricular ring; tavr, transitional atrioventricular ring; navr, nodal atrioventricular ring; asnc, atrial septum nodal cell; rarb, retro aortic root branch; sb, septal branch; cavn, compact atrioventricular node; avb, atrioventricular bundle; ine, inferior nodal extension; inc, lower nodal cells; cs, coronary sinus; ra, right atrium; as, atrial septum, astc, atrial septum transitional cells; vs, ventricular septum; lv, left ventricle; rv, right ventricle; la, left atrium.

Online Figure III. (A) Representative immunohistochemical sections showing the expression patterns of Hcn4, Cx40, Cx43, Nav1.5 and cTnl in the left AV ring, right AV ring and atrial septum. The yellow and orange arrowheads indicate the distinct domains within the AV junction corresponding to the colors used in (B). The AV ring is broader on the right compared to the left side, as indicated by the Hcn4 staining. (B) Schematic representation of the right AV junction. ra, right atrium; la, left atrium; rv, right ventricle; lv, left ventricle; vs, ventricular septum; ao, aorta; mv, mitral valve.

Online Figure IV. In situ hybridization of serial sections of a 14 days old mouse revealing the myocardium (cTnl). The sections go from caudal (A) to cranial (F). (A) Caudal section that shows the right atrioventricular (AV) ring connecting the inferior nodal extension. (B) The inferior nodal extension detaches from the right AV ring and connective tissue is found in between (red arrow), although, some small strands still communicate at different sites (green arrow). Notice that the sinus coronarius drains towards the AV canal at this point. (C) The compact AV nodal region is lined by connective tissue (red arrows) which in turn is lined by a small myocardial layer that belongs to the right AV canal myocardium (yellow arrows). Here, the compact AV nodal region does therefore not communicate directly with the right nor left AV ring myocardium. (D) After passing the sinus coronarius the AV nodal area is still covered by connective tissue that separates it from the right AV ring, however, it now directly communicates with the left AV ring and the atrial septum myocardium. (E) The lower nodal cells and the compact AV node are separated from the right AV ring by connective tissue (black arrows). (F) The central fibrous body is now clearly visible (black star). Notice the very small band of connective tissue (black arrows) that separates the AV bundle from the right AV canal myocardium (yellow arrows).

Online Figure V. Representative sections of the 3D-reconstruction of the E10.5 (A) and E17.5 (B) AV junction myocardium. Dashed lines indicate level of sectioning. ra, right atrium; la, left atrium; rv,
right ventricle; lv, left ventricle; oft, outflow tract; rarb, retro-aortic root branch; pv, pulmonary vein; ravr, right atrioventricular ring; asnc, atrial septum nodal cells; avb, atrioventricular bundle.

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**Online Figure VII.** *In situ* hybridization of serial sections of a wildtype heart of E13.5. *Tbx2* is expressed in the atrial septum myocardium at this stage (black arrows). Therefore, contributions of the AV canal, atrial septum or dorsal mesenchymal protrusion cannot be distinguished to the mature AV conduction axis. ra, right atrium; la, left atrium; rv, right ventricle; lv, left ventricle.
References


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**Preferred settings for Adobe Reader® 9.3**

Open *Edit → Preferences* to ensure the following:

1) In *3D & Multimedia* : under *3D Tool Options*
   - for “Open Model Tree on 3D Activation” choose “Use Annotation’s Settings”
   - for “Default Toolbar State” choose “Use Annotation’s Settings”
   - disable "Show 3D orientation axis"
   under *Auto-Degrade Options*
   - for the "Optimization Scheme for Low Frame Rate" select "None".
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   - enable “Enable Acrobat JavaScript”

**How to use this PDF file**

The following page contains on the left the preset view buttons to enable the user to (re)set the 3D model into the orientation shown on the particular button.

Below the buttons for preset views there is a list of the structures ("lumen", etc) with three buttons per structure to permit to:

To further interact with the 3D model:

- **rotate**: click and hold the left mouse button and move the mouse
- **zoom**: click and hold the right mouse button and move the mouse
- **translate**: click and hold the left+right mouse buttons and move the mouse

**Abbreviations**

- ca = caudal
- cr = cranial
- v = ventral
- d = dorsal
- r = right
- l = left
- INE = inferior nodal extension
- LNC = lower nodal cells

Visit [http://www.hfrc.nl](http://www.hfrc.nl) for more information.
3D-pdf

The atrioventricular conduction system of the adult mouse heart

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