Transcription Factor Tbx3 Is Required for the Specification of the Atrioventricular Conduction System

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Abstract—The cardiac conduction system consists of distinctive heart muscle cells that initiate and propagate the electric impulse required for coordinated contraction. The conduction system expresses the transcriptional repressor Tbx3, which is required for vertebrate development and controls the formation of the sinus node. In humans, mutations in Tbx3 cause ulnar–mammary syndrome. Here, we investigated the role of Tbx3 in the molecular specification of the atrioventricular conduction system. Expression analysis revealed early delineation of the atrioventricular bundle and proximal bundle branches by Tbx3 expression in human, mouse, and chicken. Tbx3-deficient mice, which die between embryonic day 12.5 and 15.5, ectopically expressed genes for connexin (Cx)43, atrial natriuretic factor (Nppa), Tbx18, and Tbx20 in the atrioventricular bundle and proximal bundle branches. Cx40 was precociously upregulated in the atrioventricular bundle of Tbx3 mutants. Moreover, the atrioventricular bundle and branches failed to exit the cell cycle in Tbx3 mutant embryos. Finally, Tbx3-deficient embryos developed outflow tract malformations and ventricular septal defects. These data reveal that Tbx3 is required for the molecular specification of the atrioventricular bundle and bundle branches and for the development of the ventricular septum and outflow tract. Our data suggest a mechanism in which Tbx3 represses differentiation into ventricular working myocardium, thereby imposing the conduction system phenotype on cells within its expression domain. (Circ Res. 2008;102:1340-1349.)

Key Words: Tbx3 ■ conduction system ■ atrioventricular bundle ■ bundle branches ■ development

The cardiac conduction system is responsible for the initiation, coordination, and propagation of the electric impulse. The impulse is initiated in the sinus node, delayed in the atrioventricular (AV) node, and then rapidly propagated by the AV bundle (bundle of His), bundle branches, and the peripheral conduction system to activate the ventricular working myocardium from apex to base. Disorders of the conduction system, including sinus node dysfunction and AV block, occur commonly and may cause life-threatening arrhythmias requiring pacemaker implantation or treatment with antiarrhythmic medication. Congenital heart defects and pacemaker implantation are common and may result in further developmental defects.1–3 Since the anatomic identification of the conduction system components 100 years ago, knowledge regarding their development, morphology, and physiological function has increased steadily.4–10 However, insight into the molecular and genetic underpinnings of the specification and formation of the conduction system is very limited. Human and mouse studies have identified cardiac homeobox factor Nkx2-5, T-box transcription factor Tbx5, Id2, and bone morphogenetic protein signaling as important molecular components for the formation of the AV conduction system11–15 and Tbx3 as a critical factor in the formation of the sinus node.14

Tbx3 is a T-box transcription factor involved in developmental patterning, regulation of proliferation, senescence, cell cycle exit, and apoptosis.16,17 Mutations in Tbx3 cause ulnar–mammary syndrome (UMS) in humans, a congenital disorder associated with defects in limbs, mammary glands, teeth and, occasionally, the heart.18,19 In the heart, Tbx3 is significantly expressed in the conduction system.20 Recently, we have found that Tbx3 acts as a molecular switch that determines whether embryonic cardiac cells differentiate into pacemaker cells or working myocardium.14 Here, we investigated the role of Tbx3 in the formation of the AV conduction system. We found that Tbx3 marks the AV bundle and bundle branches before other functionally important genes and markers and that Tbx3 is required for the specification of these conduction system components by protecting them from obtaining the ventricular working myocardium phenotype.

Materials and Methods

Mice

The Tbx3<sup>−/−</sup> allele has been previously described.14 For age determination of the embryos, couples were put together overnight when the female was in estrus. The next day, the female was inspected for a vaginal plug and the animals were separated. Noon was considered embryonic day (E)0.5. Genomic DNA prepared from amnion or tail
biopsies was used for genotyping by PCR, using primers specific for \textit{Cre} and the wild-type allele. Animal care was in accordance with national and institutional guidelines.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

**Tbx3 Delineates the Developing and Mature AV Bundle and Bundle Branches**

To explore the possible role of Tbx3 in the development of the AV bundle and branches, we investigated its expression pattern in these components. In situ hybridization was performed on consecutive sections using probes against Tbx3 and Cx43, a gene expressed in the working myocardium but excluded from sinus node and AV conduction system components during normal cardiac development.\textsuperscript{20–25} Tbx3 expression was confined to the crest of the ventricular septum from E10.5 to adulthood, demarcating the nascent AV bundle. From E12.5 onward, Tbx3 expression was additionally observed in the proximal bundle branches, where it was expressed in a diminishing gradient toward the apex. In the myocardium of the AV bundle and AV canal, the Tbx3 pattern was strictly complementary to that of Cx43 (Figure 1).

Additional Tbx3 expression was also observed in the cushion mesenchyme and outside the heart (eg, body wall, lung), where it was coexpressed with Cx43 (Figure 1 and Figure I in the online data supplement). Localized expression of Tbx3 could be traced back to the myocytes in between the just emerging ventricular chambers of the E9.5 heart, at the position where the ventricular septum will develop (Figure 1A). The presence of Tbx3 protein in the crest of the mouse ventricular septum (Figure 1J) was detected by immunohistochemistry, which is evolutionarily conserved in human (Figure 1H and 1I) and chicken (data not shown).

**Gene Expression in the Developing AV Bundle and Bundle Branches**

We next assessed the expression pattern of Cx43 and Nppa (atrial natriuretic factor), which mark the developing working myocardium and are specifically absent from the developing and adult nodal (sinus node, AV canal/node) conduction system components.\textsuperscript{10} These markers were found to be absent from the developing and mature AV bundle (Figure 1 and supplemental Figures II and III), consistent with the notion that the developing AV bundle shares its expression profile

**Figure 1.** Tbx3 expression delineates the forming AV bundle and bundle branches from early stages onwards. A through G, Section in situ hybridizations of mouse ventricular septa. For each stage, serial sections were stained for Tbx3 and Cx43, respectively. Probes and developmental stages are indicated in the panels. The square bracket in A demarcates the interventricular area in the embryonic ventricle with increased Tbx3 expression. Black arrowheads in B through G indicate the Tbx3-expressing AV bundle and bundle branches. The square brackets in D through G depict the region of graded Tbx3 and Cx43 expression. H through J, Fluorescent immunohistochemistry sections stained for Tbx3. H, Twelve-week human fetus. I, Enlargement of H. J, E12.5 mouse embryo. The white arrowheads indicate Tbx3 protein detection in the AV bundle and branches. Scale bar=100 μm. avb indicates AV bundle; rv/lv, right/left ventricle; lbb, left bundle branch; vs, ventricular septum.
and phenotype with the nodal conduction system components. Nppa, however, was expressed in more distal parts of the bundle branches (supplemental Figure IIE). Two other members of the T-box transcription factor family, Tbx18 and Tbx20, were found to be expressed in the working myocardium of the ventricular septum, but their expression was selectively absent from the AV bundle (supplemental Figure IIB and III). The gap junction protein connexin (Cx)40, another marker of developing working myocardium,22,23 is responsible for the fast impulse propagation between myocardial cells of the AV bundle and branches. Cx40 is not expressed in the Tbx3-positive developing AV bundle until E14.5 but is expressed in the bundle branches (Figure 2F).23 However, unlike the other markers, Cx40 is progressively expressed in more proximal parts of the AV bundle during its further maturation (Figure 2I), until the entire AV bundle expresses Cx40 around birth.21,25,26 The AV canal/AV node never initiated expression of Cx40. We conclude that the developing AV bundle and the nodal components of the conduction system share a similar (Tbx3-positive, Cx40/Cx43/Nppa-negative) expression profile, which distinguishes them from the working myocardium.

We next compared expression of Tbx3 with expression of transcription factors Tbx5 and Nkx2-5, which are involved in patterning and specification of the AV bundle and bundle branches.11,12,27 In the developing heart tube, Tbx5 is expressed in a gradient toward the outflow tract,28,29 and, after E11.5, Tbx5 becomes confined to the left ventricle, the trabecular component of the right ventricle, and the crest of the ventricular septum (Figure 2B, 2E, and 2H).28 Nkx2-5 was reported to be expressed at higher levels in the ventricular conduction system compared with the working myocardium.12,30 At E11.5, Nkx2-5 was homogeneously expressed in the ventricular myocardium (not shown), but from E12.5 onward, its mRNA (Figure 2C) and protein (Figure 5I) were slightly enriched in the crest of the septum. Taken together, expression of Nkx2-5 and Tbx5 is initially broad but becomes significantly higher in the developing AV bundle several days later than Tbx3.

**Tbx3 Is Required for Septation and Outflow Tract Formation**

To unravel the role of Tbx3 in the formation of the AV conduction system, Tbx3-deficient embryos14 were analyzed. Consistent with the defects seen in UMS patients (heterozygous for a mutation in Tbx3) and previously reported Tbx3-deficient mice,18,31 homozygous Tbx3-deficient (Tbx3^Cre/Cre) embryos have limb defects and fail to form mammary glands. The embryos die between E12.5 and E15.5. At E12.5, Mendelian ratios were as expected (wild type/heterozygous/homozygous: 24/57/27). However, at E13.5, we found 34 wild-type embryos and only 15 homozygous mutants, which is 44% of the expected fraction. At E14.5, we found 49 wild-types and 4 homozygous mutants (8% of expected fraction). No homozygous Tbx3-deficient embryos were found at later stages.

A consistent finding in mutant hearts was a double outlet right ventricle. The great arteries lie side by side with the aorta positioned to the right of the pulmonary trunk (Figure 3). Three-dimensional reconstruction demonstrates that the aorta does not originate from the left ventricle but from the right ventricle, whereas the pulmonary trunk is positioned normally (Figure 3C and 3D). This congenital cardiac malformation was observed in 24 of 27 mutant embryos, compared with 2 of 31 wild-types (P<0.001; supplemental Figure 1VA). A severe delay in closure of the interventricular foramen was observed. In wild-type embryos, the septum was closed in 75% of embryos at E13.5 and 100% at E14.5, compared with 12.5% at E13.5 and 0% at E14.5 in mutant embryos (P=0.012 (E13.5) and 0.014 (E14.5); supplemental Figure IVB). We observed a short and blunted ventricular septum in multiple sections of mutants. Measurements revealed that the length of the ventricular septum is 19% (P=0.012) smaller in mutants than in wild types, whereas there is no significant difference in the width of the heart (4% difference, P=0.31).

**Loss of AV Bundle Specification in Tbx3 Mutants**

To investigate the role of Tbx3 in AV conduction system development, hearts of Tbx3 homozygous mutants and wild-type littermates were analyzed for gene expression, proliferation, and apoptosis. Sections of Tbx3 mutant hearts were stained for Cre expression, revealing the Tbx3 expression domain in the absence of Tbx3 protein. Expression of Cre was observed in the expected region in the crest of the ventricular septum of Tbx3 mutants, indicating that these cells have received the instructions to initiate Tbx3 expression and that Tbx3 protein is not required for Tbx3 gene activity. In these mutants, both the distal (cranial) and proximal (caudal) Cre-expressing AV bundle domain showed coexpression of Cx43 (Figure 4A through 4H). Three-dimensional reconstruction of the Tbx3/Cre-expressing domain revealed mutually exclusive expression of Tbx3 and Cx43 in wild-type embryos but extensive overlap in expression of Cre and Cx43 in the dorsal AV canal (presumptive AV node), AV bundle, and proximal branches in mutants (Figure 4I and 4J). No ectopic expression of Cx43 was observed in other parts of the AV canal. Moreover, the reconstructions show that the overall morphology of the AV canal of mutants was not affected (Figure 4I; compare Cre-expressing AV domain in mutants with Tbx3-expressing AV domain in wild types).

Consistent with the ectopic expression of Cx43, also Nppa, Tbx18, and Tbx20 were ectopically expressed in the Cre-expressing domain (Figure 5). Moreover, Cx40 was precociously upregulated in the AV bundle (Figure 5G and 5J). The enrichment of Nkx2-5 protein in the crest of the septum was largely lost in mutants (Figure 5I and 5L). Expression of Id2 and Tbx5 was similar in wild-type and mutant hearts, indicating that they do not depend on Tbx3 (supplemental Figure V).

The components of the conduction system proliferate slowly.12–34 In mouse, it was shown that the AV bundle exits the cell cycle at approximately E12.5.12 Based on proliferating cell nuclear antigen staining, we observed cell cycle exit as early as E11.5 (not shown). We then investigated the role of Tbx3 in regulation of proliferation and cell cycle exit, using 5-bromodeoxyuridine (BrdUrD) incorporation as a measure for proliferation rate. Adjacent sections stained for
BrdUrd incorporation and for Tbx3 expression revealed that the Tbx3-expressing domain contains few BrdUrd positive cells (Figure 6A). In mutants, however, the number of BrdUrd-positive cells in the crest of the ventricular septum was similar to that of the working myocardium in the remainder of the septum (Figure 6B). The same results were obtained using proliferating cell nuclear antigen staining (not shown). Quantitative analysis of local proliferation rates in the ventricular septum demonstrated relatively slow proliferation in the crest of wild-type AV bundles, whereas proliferation in the AV bundles of mutants was similar to the remainder of the septum (Figure 6C and 6D). We conclude

Figure 2. Spatial and temporal expression pattern of genes expressed in the developing AV bundle and bundle branches. Section in situ hybridizations of prenatal mouse ventricular septa. For each stage, adjacent sections were used. Probes and developmental stages are indicated in A through I. Black arrowheads indicate the AV bundle. White arrowheads indicate staining in the bundle branches. Scale bar=100 μm.
that Tbx3 is required for the low proliferation rate in the developing AV bundle.

To assess whether the loss of Tbx3 affects programmed cell death in the AV bundle, we analyzed apoptosis in wild-type and mutant hearts using cleaved caspase-3 staining and TUNEL assays. In the ventricular septum, very low rates of apoptosis were detected, which were not different between wild types and mutants (supplemental Figure VI).

![Image](https://example.com/image.png)

**Figure 3. Ventricular septum and outflow tract defects in Tbx3 mutant hearts.** A, C, and E, Sections of an E13.5 wild-type (WT) fetus. B, D, and F, Tbx3-deficient littermate. A, B, E, and F, Representative sections of the outflow tract (A and B) and AV canal region (E and F). Black arrow in F indicates interventricular communication/ventricular septal defect. C and D, Three-dimensional reconstructions showing the blood-filled lumen. Yellow circle indicates the abnormal origin of the aorta from the right ventricle in the mutant (D). Scale bar=100 μm. ao, aorta; pt, pulmonary trunk; ra/la, right/left atrium.

No Change in AV Conduction Time in Tbx3 Mutants

To investigate whether deregulation of gene expression in the AV canal and AV bundle caused alterations in impulse propagation, the activation pattern and AV conduction time were assessed in wild-type and Tbx3 mutant embryos (E12.5) by optical mapping. The activation pattern and interval between excitation of the atrium and excitation of the ventricle were not different (Figure 7). Next, we performed surface ECG on adult Tbx3+/Cre heterozygous mutants and wild-type littermates to evaluate whether a decrease in Tbx3 affects cardiac conduction. No difference was found in heart rate, PQ, QRS, QT, or QTc duration (wild type/Tbx3+/Cre; n=5/4; supplemental Figure VII). Morphological analysis revealed the absence of outflow tract malformations and absence of ventricular septal defects. Immunohistochemical analysis revealed an intact AV node (Hcn4+/Cx40−), AV bundle, and branches (Hcn4+/Cx40−) in Tbx3+/Cre mice (n=6; supplemental Figure VII).

Discussion

Our study shows that Tbx3 is required for the specification of the AV bundle and proximal bundle branches and for the development of the ventricular septum and outflow tract. Mutations in Tbx3 cause UMS in human, which includes hypoplasia of the mammary and other apocrine glands, malformations of the limbs (ulnar side), teeth, and genitalia. Interestingly, ventricular septal defects and pulmonary stenosis were observed in one UMS family, suggesting that this phenotype may be part of this syndrome, albeit with low penetrance, indicating that the occurrence depends on other mutations or genetic background. Conduction system defects have not been reported in UMS patients, and our findings indicate that heterozygous Tbx3 mutant mice do not have obvious structural heart defects or conduction system defects. These observations indicate that Tbx3 levels in heterozygous mutants are sufficient for the formation of the conduction system, ventricular septum, and outflow tract. Nevertheless, a large screen among UMS patients may reveal such abnormalities in the patient population. More importantly, the role of Tbx3 identified here and in previous studies render Tbx3 an important candidate as modifier gene in congenital or acquired conduction system disease (sinus node dysfunction, AV block) or congenital defects of the ventricular septum and outflow tract.1–3

Tbx3 Specifies the AV Bundle and Prevents Its Differentiation Into Working Myocardium

Cardiomyocytes of the conduction system, including the AV bundle and branches, are less well differentiated than working myocardium cells. They have a poorly developed sarcomere apparatus, display high automaticity, and proliferate slowly, in these respects resembling embryonic myocytes. This suggests that conduction system cells have been limited in their differentiation into working myocardial cells. We hypothesize that Tbx3 is required to prevent differentiation of the AV conduction system into working myocardium. Nppa, Cx43, and Cx40 are markers for chamber myocardium (ie, precursor of working and Purkinje fiber myocardium). In Tbx3-deficient embryos, these markers were ectopically expressed in the AV bundle. Two novel negative markers for the AV bundle, Tbx18 and Tbx20, were also ectopically expressed in the AV bundle of Tbx3 mutants. Furthermore, AV bundles in Tbx3-deficient embryos completely failed to exit the cell cycle and acquired or maintained proliferation at the same rate as the surrounding working myocardium. Previous studies further support the hypothesized function of
Figure 4. Ectopic expression of Cx43 in the AV conduction system. A through H, Section in situ hybridizations of wild-type and Tbx3 mutant (Tbx3<sup>C<sub>Cre</sub>C<sub>Cre</sub></sup>) ventricular septa. Probes and genotypes are indicated. Caudal sections are close to the presumptive AV node. Cre staining (C and G) reveals the activity of the Tbx3 gene in the absence of Tbx3 protein. I and J, Three-dimensional reconstructions of the Tbx3 (or Cre)-positive and Cx43-negative myocardial area. I, Cranial (4-chamber) view of the transparent lumen of an E12.5 heart in which the Tbx3<sup>−/−</sup>, Cx43<sup>−/−</sup> myocardial AV region has been made visible. J, The isolated myocardial area of the AV region shown from ventral (top), caudal (middle), and right side (bottom). Color code is depicted below the reconstructions. Scale bar=100 μm. lbb/rbb, left/right bundle branch; cran., cranial; caud., caudal; D, dorsal; V, ventral; R, right; L, left; lavrb/ravrb, left/right AV ring bundle; avn, AV node.
Tbx3 in the specification of the AV conduction system. Tbx3 has been shown to function as a repressor of gene expression and is able to suppress myogenic differentiation in C2C12 myoblasts. Furthermore, ectopic expression of Tbx3 in the entire embryonic heart causes arrest of heart growth and chamber differentiation. Tbx3 is able to suppress a large panel of atrial working myocardial genes and Tbx3 directly interacts with the Cx43 regulatory DNA to suppress its activity in vivo. Tbx3 may also stimulate the conduction system phenotype as it was found to induce pacemaker-specific genes. Together, the data suggest a mechanism in which Tbx3 represses differentiation into ventricular working myocardium.

**Figure 5.** Ectopic expression of ventricular markers in the AV conduction system. A through H, J, and K, Section in situ hybridizations of wild-type and Tbx3 mutant ventricular septa. Probes and genotypes are indicated. Black arrowheads indicate the Tbx3 (or Cre)-positive AV bundle region. I and L, Fluorescent immunohistochemistry sections. Black arrowheads indicate the Tbx3 (or Cre)-positive AV bundle region. The difference in expression level of Nkx2-5 between the crest and compact myocardium of the ventricular septum is largely lost (white arrowheads). Scale bar=100 μm.

**Figure 6.** Tbx3 is required to suppress proliferation in the AV bundle. A and B, Fluorescent immunohistochemistry sections of E12.5 mouse hearts stained as indicated. White arrowheads in A indicate the Tbx3-expressing AV bundle, where BrdUrd incorporation is not observed in wild-type embryos. B, Myocardium is visualized using antibody against cardiac troponin I (cTnl). White arrowheads indicate the AV bundle, which is free of BrdUrd incorporation in wild-type, but not in mutant, embryos. C, Two-dimensional visualization of the BrdUrd-labeling index in the ventricular septum. The arrowheads indicate the AV bundle. Color code for BrdUrd incorporation is depicted. D, Quantification of fraction of BrdUrd-positive nuclei in the base of the septum and the AV bundle. *P<0.05. Scale bar=100 μm.
myocardium, imposing the conduction system phenotype on cells within its expression domain.

Mechanism of Specification and Formation of the Conduction System

Transcription factors Tbx5 and Nkx2-5 have important roles in the formation of the conduction system.11,12,27 Mutations in these genes cause congenital heart malformations and conduction system defects, including AV block, in human and mouse. Mice haploinsufficient for both Tbx5 and Nkx2-5 fail to establish an AV bundle, as monitored by the loss of minK-lacZ expression, fail to acquire slow proliferation in the crest of the septum, and develop functional conduction block in postnatal animals.12 However, both Tbx5 and Nkx2-5 stimulate working myocardial gene expression (Cx40, Cx43, Nppa), and both factors are required for chamber (working myocardial) differentiation.41,42 Moreover, Tbx5 is required for the progression of the cell cycle.43 These functions are not compatible with a direct role in the suppression of working myocardial gene expression, differentiation, and proliferation in the AV bundle. Tbx5-Nkx2-5 double heterozygous mice fail to activate transcriptional repressor Id2 in the crest of the septum, which may be responsible for the observed repression of differentiation and gene expression,12 a possible role that needs to be addressed.

Our data have revealed that Tbx3 is required to specify the AV bundle and proximal branches and to suppress ventricular working myocardial genes and proliferation in this cell population. Moreover, Id2 expression was not affected in Tbx3-deficient embryos, indicating that Id2 is not sufficient to fulfill the tasks of suppressing differentiation, proliferation, and gene expression. Therefore, we hypothesize that Tbx3 is a main determinant in AV bundle formation.

In the heart, both Tbx5 and Tbx3 physically interact with Nkx2-5 to activate or repress target genes (P. Barnett, unpublished data, 2007).42,44 A transcriptional regulatory
network is active in which Tbx3 expression is induced by Tbx5 and suppressed by Nkx2-5, possibly to maintain and balance expression of Tbx3 in the conduction system.\textsuperscript{13,45} Tbx3 (repressor), in turn, competes with Tbx5 (activator) for the regulation of working myocardial genes Cx40 and Nppa, differentiation, and proliferation in a dose-dependent manner (Figure 8).\textsuperscript{20} We hypothesize that Tbx5 and Nkx2-5 specify the AV conduction system largely through the regulation of Tbx3 gene activity and interactions with Tbx3 protein. The broad expression pattern of Tbx5 and other known regulatory factors in the early developing heart indicates that a yet undefined factor is required to confine Tbx3 expression to the early developing conduction system.

Conduction System Function Is Not Affected in Tbx3 Mutants

In mutants, ectopic induction was observed of Cx40 and Cx43 in the AV bundle and of Cx43 in the dorsal AV canal, where the AV node develops. These high conductance gap junctions are required for rapid impulse propagation.\textsuperscript{46} However, we did not observe changes in the AV conduction velocity in homozygous mutant embryos. The presence of a slow conducting AV canal in the absence of Tbx3 strongly suggests additional Tbx3-independent mechanisms for slow conduction. In the AV canal AV Tbx3 may be redundant, because Tbx2, which is functionally closely related to Tbx3, is robustly expressed in the AV canal during development. Another mechanism may be the presence in the AV canal of the very low conductance gap junction Cx30.2 that is required for conduction slowing in the adult AV node.\textsuperscript{46}

The ventricular activation pattern was also normal in E12.5 mutant embryos, indicating that the AV bundle is not yet required for fast propagation of the action potential from the AV node to the ventricular myocardium at this developmental stage. This is consistent with the notion that the AV bundle does not express Cx40 before E14.5 (Figure 2). Moreover, at this developmental stage, the Cx40/Cx43-positive trabecular myocardium is directly connected to the dorsal AV canal (future AV node).\textsuperscript{6} This connection may underlie the normal activation pattern observed at this stage\textsuperscript{26} (Figure 7A) in the absence of a specified AV bundle.

Because of lethality before E14.5, homozygous mutants could not be analyzed at later stages, when the AV bundle has become the only electric connection between the AV node and ventricle. ECG recordings revealed the absence of conduction disturbances in heterozygous mutants. The AV node and AV bundle appeared to be intact. These observations indicate that the decreased Tbx3 dose in heterozygous mutants is sufficient for the formation of the conduction system.

Tbx3 Deficiency and Outflow Tract Malformations

Double outlet right ventricle and ventricular septal defects were observed in nearly all mutant embryos. We believe that these malformations are not related to the conduction system phenotype but represent a separate function of Tbx3 in cardiac morphogenesis. Tbx3 is expressed in pharyngeal mesenchyme and cardiac neural crest cells that populate the outflow tract (supplemental Figure IVD) and are involved in its rotation and septation.\textsuperscript{47} We hypothesize that Tbx3 is involved in a crucial aspect of the regulation of these cells, but the precise role of Tbx3 in outflow tract morphogenesis remains to be elucidated.

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Disclosures

None.

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Expanded Materials and Methods

BrdU assay

Pregnant females were injected with 50 mg of 5’-bromo-2’-deoxyUridine (BrdU) / kg bodyweight (Sigma B5002). After 1 hour of BrdU exposure the mice were stupefied with carbon dioxide and killed by cervical dislocation. The embryos were isolated on ice cold PBS to stop the BrdU incorporation, and further processed for immunohistochemistry.

In situ hybridization and immunohistochemistry

The non radioactive in situ hybridization method and probes were described previously. The probe for Id2 was kindly provided by Dr. Ivan Moskowitz (Institute of Molecular Pediatric Sciences, University of Chicago). Embryos were fixed in 4% formaldehyde, embedded in paraplast and sectioned at 7 μm for immunohistochemistry and at 12 μm for in situ hybridization. Immunohistochemistry of adult tissue was performed on cryosections. The primary antibodies used were: Nkx2-5 rabbit polyclonal (1:100; Santa Cruz), Tbx3 goat polyclonal (1:300; Santa Cruz), BrdU rat polyclonal (1:600; AbD serotec), PCNA mouse monoclonal (1:200; Santa Cruz biotechnology), Cx40 mouse monoclonal (1:100; USBio), Hcn4 rabbit polyclonal (1:250; Chemicin), and cTnI rabbit polyclonal (1:100; Hytest Ltd). For Nkx2-5 and Tbx3 a Tyramide signal amplification kit was used (Perkin Elmer LAS, Inc). The secondary antibodies used were Alexa 647 goat-anti-rabbit, goat-anti-mouse and goat-anti-rat, Alexa 568 goat-anti-rabbit and goat-anti-mouse antibodies (1:250; Molecular Probes), biotinylated goat-anti-rabbit (1:200; DAKO) and biotinylated Donkey-anti-goat antibody (1:100; Jackson immunotech). Nuclei were stained using Sytox Green nucleic acid
stain (1:30,000; Molecular Probes). All expression patterns were confirmed in at least 3 independent embryos per genotype.

**Apoptosis assay**

Apoptotic cells were identified using the above described procedure for immunohistochemistry with a primary antibody against cleaved caspase 3 (rabbit polyclonal, 1:250; Cell Signaling Technology). Terminal Transferase dUTP Nick End Labeling (TUNEL) assay was performed according to the manufacturer’s instructions (Boehringer-Mannheim). The DNA nicks were detected by a peroxidase-coupled monoclonal antibody against flourescein (converter-POD, 1:15, Boehringer-Mannheim). The antibody was demonstrated with diaminobenzidine and H$_2$O$_2$.

**Reconstruction and quantification**

The 3D reconstruction procedure has been previously described. For the quantification of proliferation and 2D visualization of the BrdU labeling index, sections were stained for BrdU, Sytox green and a myocardial marker. Data from 6 serial sections were projected onto one section to allow reliable counting and visualization of local BrdU labeling indices. For regional quantification, 100-150 cTnI-positive myocardial cells were marked (using a “cTnI-mask”) in the crest (AV bundle domain) and in the compact myocardium in the base of the septum (the region around “vs” in panels H and I in revised Fig. 6). This was performed for at least three representative sections of the ventricular septum per embryo, n=3 embryos per genotype. Then, the BrdU staining was visualized to count the number of marked cells that were proliferating. Proliferation rates were calculated by dividing the BrdU-positive cTnI-positive cells by the total number of cTnI-positive cells.
**Optical mapping**

After removal of the embryos from the uterus hearts were isolated. Each heart was incubated for 5 minutes with Tyrode’s solution containing 5 μM Di-4 Anneps at 37 °C, and placed on an inverted microscope set up for recording optical signals. Excitation light was provided by a 5 Watt power led (filtered 510 +/- 20 nm). Emitted fluorescence (filtered >610nm) was transmitted through a tandem lens system on a photo diode array (16x16 elements) connected to a data-acquisition system (biosemi Mark-6). Optical action potentials were analyzed with custom software.

**ECG**

Three unipolar electrocardiograms were recorded for a period of 5 minutes during 1.5 % isoflurane anesthesia. Signals were averaged after which RR, PQ, QRS, QT and QTc were calculated.

**Statistics**

Statistical analysis was performed using a two-sample two tailed Student t-test. Genotype and phenotype frequencies were tested with a Chi-square test.

**References**


Supplementary Figure 1.
Identity of Tbx3 expressing cells. In situ hybridization sections of E12.5 (A-D) and E11.5 (E-H) wild-type mouse hearts. Probes have been indicated. B, D and F, H are enlargements of A, C and E, G respectively. A, C, black arrows indicate regions outside the heart where Tbx3 and Cx43 are co-expressed. B, D, black arrowheads indicate myocardium (venous valve, AVC and AV bundle), where Tbx3 and Cx43 are strictly complementary expressed. Yellow arrowhead indicates the endocardium where Tbx3 and Cx43 are co-expressed. The brackets illustrate the gradients of Tbx3 and Cx43 within the bundle branches. E-H show that the areas of complementary expression of Tbx3 and Cx43 are myocardial (black arrowheads). These panels also show that Tbx3 is not only expressed in myocardium but also in the mesenchyme of the cushions. Scale bar = 100 µm. r/l v, right/left ventricle; bwm, body wall mesenchyme; lb, lung bud; AV-c, AV cushion; bb, bundle branch; r/l a, right/left atrium; ep, epicardium; mes, mesenchyme; myo, myocardium.
Supplementary Figure 2.
A through G, *Tbx3* delineates the forming AV bundle and bundle branches from early stages onwards. Section in situ hybridizations of mouse ventricular septa. For each stage, adjacent sections were used. Probes and developmental stages are indicated in the panels. Black arrowheads in panels indicate the *Tbx3* expressing AV bundle and bundle branches. Scale bar = 100 μm. avb, atrioventricular bundle; vs, ventricular septum; lbb, left bundle branch.
Supplementary Figure 3.
Spatial and temporal expression pattern of genes expressed in the developing AV bundle and bundle branches. Section in situ hybridizations of pre-natal mouse ventricular septa. For each stage, adjacent sections were used. Probes and developmental stages are indicated. Black arrowheads indicate the AV bundle. The white arrowhead indicates staining in the bundle branches. Scale bar = 100 μm. avb, atrioventricular bundle; r/l v, right/left ventricle; lbb, left bundle branch; vs, ventricular septum.
Supplementary Figure 4.

A, table summarizing the numbers of double outlet right ventricle (DORV) observed in wild-type (WT) and Tbx3-deficient (KO) mice at different stages of development. B, Summary of the number of open (gray) and closed (black) interventricular foramen at different stages of development of wild-type (WT) and Tbx3-deficient (KO) mice. Numbers of animals per group are depicted inside the bars. *, p<0.05. C, bargraph summarizing the length of the ventricular septum at different stages of development. A delay in growth of nearly one day can be observed. D, E, in situ hybridization sections of E9.5 mouse embryo sections cranial of the heart in the outflow tract and pharyngeal arch region. Arrowheads indicate expression of Tbx3 and Tbx2 in the outflow tract and the pharyngeal mesenchyme and neural crest cells. Scale bar = 100 μm. pa, pharyngeal arch; fg, fore gut; nt, neural tube; oft, outflow tract; la, left atrium.
**Supplementary Figure 5.**
Expression patterns of *Tbx5* and *Id2* are not altered in Tbx3-deficient embryos. A-F section in situ hybridizations of wild-type and *Tbx3* mutant ventricular septa. Probes and genotypes are indicated in the panels. Black arrowheads indicate the *Tbx3* (or Cre) positive AV bundle region. Scale bar = 100 µm. avb, atrioventricular bundle; lbb, left bundle branch; vs, ventricular septum.
Supplementary Figure 6.

Apoptosis is not increased in the ventricular septum of Tbx3-deficient embryos. A-C, fluorescent immunohistochemistry sections of E12.5 mouse hearts stained for cleaved caspase 3. No apoptosis was observed in septa of wild-type or mutant embryos. C, representative dorsal ganglion with cleaved caspase 3 positive apoptotic cells as a positive control. D, E, TUNEL assay. Representative sections show apoptotic cells in the ventricular septum of wild-type (D) and mutant (E) E12.5 embryos. The dorsal ganglion served as a positive control (F). Scale bar = 100 µm. vs, ventricular septum; gangl., dorsal ganglion.
Supplementary Figure 7.

Structure and function of the AV conduction system in heterozygous mutants (Tbx3\(^{+/\text{Cre}}\)). Panels A, C and B, D, show adjacent sections of the AV bundle-AV node transition region in adult wild-type (A, C) and heterozygous mutants (B, D), respectively. The AV node is identified by presence of Hcn4 and absence of Cx40. The AV bundle is characterized by the presence of both Hcn4 and Cx40. E, bar graph showing RR, PR, QRS, QT and QTc duration in adult wild-type and heterozygous mutant (Tbx3\(^{+/\text{Cre}}\)) mice. Scale bar = 100 \(\mu\)m. avb, atrioventricular bundle; avn, atrioventricular node; vs, ventricular septum.