Tbx3 Is Required for Outflow Tract Development

Karim Mesbah, Zachary Harrelson, Magali Théveniau-Ruissy, Virginia E. Papaioannou, Robert G. Kelly

Abstract—Conotruncal and ventricular septal congenital heart anomalies result from defects in formation and division of the embryonic outflow tract. Cardiac remodeling during outflow tract and ventricular septation converts the tubular embryonic heart into a parallel circulatory system with an independent left ventricular outlet and right ventricular inlet. Tbx3 encodes a T-box–containing transcription factor expressed in the developing conduction system of the heart. Mutations in TBX3 cause ulnar–mammary syndrome. Here we show that mice lacking Tbx3 develop severe outflow tract defects, including connection of both the aorta and pulmonary trunk with the right ventricle, in addition to aortic arch artery anomalies and abnormal communication between the right atrium and left ventricle. Alignment defects are preceded by a delay in caudal displacement of the arterial pole of the heart during aortic arch artery formation. Embryonic anterior–posterior patterning and cardiac chamber development are unaffected in Tbx3 mutant embryos. However, the contribution of second heart field derived progenitor cells to the arterial pole of the heart is impaired. Tbx3 is expressed in pharyngeal epithelia and neural crest cells in the pharyngeal region, suggesting an indirect role in second heart field deployment. Loss of Tbx3 affects multiple signaling pathways regulating second heart field proliferation and outflow tract morphogenesis, including fibroblast growth factor signaling, leading to a failure of normal heart tube extension and consequent atrioventricular and ventriculoarterial alignment defects. (Circ Res. 2008;103:743-750.)

Key Words: Tbx3 • outflow tract • congenital heart defect • neural crest

Morphogenesis of the arterial pole of the heart involves formation and subsequent division of the myocardial outflow tract (OFT) to generate the ascending aorta and pulmonary trunk connected to the left and right ventricles, respectively.1 This complex process requires interactions between myocardial, endocardial and neural crest–derived (NC) cells. Defects in OFT morphogenesis in man and mouse models result in congenital heart anomalies, including abnormal ventriculoarterial alignment such as double outlet right ventricle and overriding aorta, associated with ventricular septal defects. The myocardial wall of the OFT is derived from progenitor cells of the second heart field (SHF), a population of pharyngeal mesodermal cells expressing the transcription factors Isl1 and Tbx1.2 Contribution of SHF-derived myocardium to the heart tube is essential for normal elongation of the heart tube and subsequent ventriculoarterial alignment.3 Division of the OFT occurs concomitantly with ventricular septation and is driven by cardiac NC cells.4 Before their entry into the OFT, NC cells are required in the pharyngeal region for normal addition of SHF cells to the distal heart tube.5,5 T-box containing transcription factors control multiple aspects of embryonic development including morphogenesis and patterning of the heart; haploinsufficiency or mutation of a number of Tbx genes in humans and mice result in congenital heart defects (reviewed elsewhere6,7). Tbx2 and Tbx3 are closely related paralogs of the T-box family with many areas of overlapping gene expression during development.8 They act as transcriptional repressors and have at least some target genes in common, including regulators of proliferation and senescence.6 Tbx2-null embryos display cardiac abnormalities, including atrioventricular canal (AVC) anomalies and defects in OFT alignment.9 Tbx3-null embryos die over a range of several days during midgestation with severe but variable yolk sac abnormalities in addition to hindlimb defects and mammary gland aplasia.10 Tbx3 mutations underlie ulnar–mammary syndrome in humans.11 The cause of lethality in Tbx3 mutant embryos is uncertain, although cardiac abnormalities could be a contributing factor. Tbx3 is expressed in the AVC and the sinoatrial and central components of the cardiac conduction system.12 Tbx2 represses the transcriptional program of ventricular and atrial myocardium in the AVC, and gain- and loss-of-function experiments have demonstrated that Tbx3 is required for sinoatrial node identity.9,13–16 In addition, a recent report has described abnormalities in cardiac looping in mice carrying a novel Tbx3 mutation (Tbx3Neo/Neo).17

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Here, we show that Tbx3 is required for arterial pole morphogenesis. In Tbx3/H11002/H11002 embryos elongation of the arterial pole of the heart is perturbed resulting in double outlet right ventricle, where both the aorta and pulmonary trunk are aligned with the right ventricle. Tbx3 is expressed in pharyngeal epithelia and NC cells in the pharyngeal region and loss of Tbx3 function is associated with elevated proliferation and defective deployment of SHF cells. Our data suggest that Tbx3 regulates multiple signaling pathways required for normal SHF development and OFT elongation.

Materials and Methods

Mice

The null alleles Tbx3tm1Pa and Tbx2tm1Pa (hereafter referred to as Tbx3 and Tbx2) were maintained on a mixed genetic background.9,10 Genotyping details are provided in the online data supplement, available at http://circres.ahajournals.org. Cx40eGFP and Mlc1v-nlacZ24 transgenic mice were genotyped as described.18,19 Mouse care and procedures were in accordance with institutional and US and French national guidelines.

Histology and Immunohistochemistry

Details of procedures are provided in the online data supplement. Primary antibody concentrations were: Tbx3 (1/200, Santa Cruz Biotechnology), β-galactosidase (1/300, Cappel), MF20 (1/50, DSHB), Islet-1 (clone 40.2D6; 1/100, DSHB), rat anti-bromodeoxyuridine (BrdU) (1/100, Immunologics), activator protein (AP)-2α (clone 3B5; 1/50, DSHB), and phospho-extracellular signal-regulated kinase (phospho-ERK) (1/100, Cell Signaling).

In Situ Hybridization

Whole-mount in situ hybridization was performed as previously described.19 For each experiment a minimum of 3 embryos of each genotype were scored. Details of riboprobes used are provided in the online data supplement.

Cell Proliferation Analysis

Cell proliferation was evaluated by BrdU incorporation. Pregnant females were injected intraperitoneally on embryonic day (E)9 with 10 μmol/L of BrdU (Sigma) per 100 g of body weight 1.5 hours before embryo harvest. Embryos were sectioned, followed by immunohistochemical detection of BrdU-incorporated cells.

Quantitative RT-PCR

Details of procedures and primer sequences are provided in the online data supplement.

Results

OFT and Atrioventricular Alignment Defects in Tbx3−/− Embryos

Tbx3−/− embryos surviving to E12.5 display a defect in leftward positioning and ventricular alignment of the ascending aorta (Figure 1, Table I). Twelve of 12 embryos scored at E12.5 and E13.5 displayed double outlet right ventricle (DORV) such that the ascending aorta was aligned with the right rather than left ventricle. Ventricular septation, normally complete by E13.5, was incomplete in Tbx3−/− hearts at E13.5 (6 of 6). Two types of DORV were observed in Tbx3−/− embryos. In the first type, the ascending aorta is dextraposed
such that the aorta and pulmonary trunk emerge from the right ventricle in a side-by-side configuration (5 of 12 embryos; Figure 1B). In the second type, the aorta is positioned ventrally, a configuration resembling transposition of the great arteries (7 of 12 embryos; Figure 1C, 1G, and 1J). histological analysis revealed that the pulmonary trunk is contiguous with cushion mesenchyme in the inner curvature of the OFT (Figure 1H and 1K). In addition to defects in alignment of the ascending aorta, aortic arch artery anomalies were observed in Tbx3−/− embryos, including persistence of the right fourth (9 of 12 embryos; Table and Figure 1J) and right sixth (5 of 12; Table and Figure 1K) arch arteries.

Atrioventricular alignment defects were also observed in Tbx3−/− hearts; the right atrium frequently connected with the left rather than right ventricle, a configuration termed double inlet left ventricle (9 of 12 hearts; Figure 1I and 1L). Tbx3−/− hearts, therefore, display defective ventriculoarterial and atrioventricular alignment such that both great arteries emerge from the right ventricle and both atria connect with the left ventricle.

**Tbx3 Is Required for Complete Cardiac Looping**

Cardiac looping is a prerequisite for correct alignment of the future cardiac chambers. Looping is a multistep process involving ventral then rightward looping of the elongating heart tube, followed by displacement of the inflow region of the heart dorsal to the ventricular segment, a process termed convergence.20 We observed a high incidence of Tbx3−/− embryos in which convergence was delayed or defective compared to somite matched Tbx3+/+ and Tbx3+/− littermates at E9.5 (Figure 2A through 2D and supplemental Table I). In normal hearts, convergence positions the atria immediately posterior to the OFT (Figure 2A and 2B). In contrast, in Tbx3−/− embryos anterior displacement of the atria is delayed or defective, resulting in a gap between the OFT and atria (observed in 18 of 25 embryos; Figure 2C and 2D). This was accompanied in several cases by hypoplasia of the right ventricle and OFT of variable severity (Figures 2E and 5C). Four of 25 Tbx3−/− embryos displayed a more severe phenotype in which a distended thin-walled heart tube looped ventrally and rightward looping and convergence were blocked (Figure 2E and 2F). In addition to a defect in looping, we noted an overall developmental delay at this stage: the average somite number was 20.4±3.5 (n=32) for Tbx3+/+, 20.1±4.5 (n=48) for Tbx3+/−, and 16.6±4.4 (n=29) for Tbx3−/− embryos (P<0.001, Student’s t test). Within litters, 27 of 29 (93%) Tbx3−/− embryos were at or below the median somite number for the litter (n=14 litters).

Convergence occurs concomitantly with caudal displacement of the OFT in the pharyngeal region as bilateral arch arteries form sequentially in an anterior to posterior progression.20 The tubular heart is initially connected to the first arch arteries, subsequently to the first and second and then to the third, fourth and sixth arch arteries, during which process the connection with the first and second arch arteries is lost. We scored arch artery connections in Tbx3−/− embryos at E10.5 using a Cx40-eGFP allele expressed in arterial endothelial cells.18 A delay in caudal displacement of the OFT was observed such that whereas in Tbx3+/+ and Tbx3+/− embryos

**Table. Incidence of Heart Defects in Tbx3−/− embryos at E12.5 and E13.5**

<table>
<thead>
<tr>
<th>Defect</th>
<th>Tbx3−/−</th>
</tr>
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<tbody>
<tr>
<td>Double outlet right ventricle</td>
<td>12/12</td>
</tr>
<tr>
<td>Double outlet right ventricle with transposition</td>
<td>7/12</td>
</tr>
<tr>
<td>Persistent right fourth pharyngeal arch artery</td>
<td>9/12</td>
</tr>
<tr>
<td>Persistent right sixth pharyngeal arch artery</td>
<td>5/12</td>
</tr>
</tbody>
</table>

Data are from 6 E12.5 and 6 E13.5 Tbx3−/− embryos; no abnormalities were observed in Tbx3+/- and Tbx3+/+ litters.
the OFT was connected to the third and fourth arch arteries and the connection with the second arch artery no longer existed (Figure 2G and 2H), the OFT of somite-matched mutant embryos maintained a connection with the second arch artery (Figure 2I and 2J). This phenotype was observed in 9 of 10 mutant embryos (Figure 2K). Subsequently, posterior arch arteries are present in Tbx3−/− embryos at E11.5 and E12.5 (Figure 1 and data not shown).

Molecular Patterning of Tbx3−/− Hearts

The above phenotypic analysis suggests that OFT defects in Tbx3−/− embryos arise as a result of incomplete looping and a delay in caudal displacement of the OFT. The anterior limit of 2 genes expressed at defined levels along the embryonic anterior-posterior axis, Hoxb1, and Radh2 was unaltered in Tbx3−/− embryos, suggesting that overall anterior-posterior patterning of the pharyngeal region is not perturbed (online data supplement, Figure IA through ID). Nkx2-5 and Tbx5 are expressed normally in Tbx3−/− hearts at E9.5 (supplemental Figure IE through IL), suggesting that Tbx3 is not required for global cardiac patterning.

Tbx2 is closely related to Tbx3 and is required to repress the expression of the chamber-specific genes Csl, Cx40, and Nppa in AVC myocardium at E9.5.9,13,14 As Tbx3 is also expressed in AVC myocardium,8,12 patterning of the prospective chambers and AVC was analyzed in Tbx3−/− mutant embryos. Expression of Tbx2 in the OFT and AVC is maintained in Tbx3−/− hearts (Figure 3A through 3D). Similarly, Tbx3 expression is normal in Tbx2−/− hearts at E9.5, suggesting that the AVC expression domains of Tbx2 and Tbx3 are not interdependent.9 Expression of Csl, Nppa, and Cx40 is not expanded in the AVC of Tbx3−/− embryos (Figure 3E through 3J), suggesting that AVC specification proceeds normally in the absence of Tbx3. Precocious expression of Nppa was observed in atrial myocardium of somite matched Tbx3−/− embryos at E9.5 (Figure 3G and 3H); however, no differences were observed at E10 (data not shown). As Tbx2 and Tbx3 interact genetically during mammary gland development,21 we explored a potential interaction of Tbx2 and Tbx3 during cardiac development. Nppa, Csl, and Cx40 transcripts did not accumulate in AVC myocardium of Tbx2+/−;Tbx3+/− double heterozygous mutant embryos at E9.5 (Figure 3K and 3L; data not shown).

Tbx3 Controls OFT Elongation

In addition to AVC myocardium, Tbx3 transcripts are observed in the pharyngeal region at E9.5 (Figure 4A and 4B).8,12 Analysis of Tbx3 protein distribution revealed that the caudal pharyngeal region expression domain includes ectoderm, pericardium, ventral pharyngeal endoderm, and mesenchymal cells (Figure 4C through 4F). Tbx3 distribution was compared to that of nuclear localized β-galactosidase in embryos carrying the Mlc1v-nlacZ-24 transgene, expressed in pharyngeal mesoderm and AVC myocardium as a result of integration upstream of the gene encoding Fibroblast growth factor 10.19 Within the heart, Tbx3-positive nuclei are observed in AVC myocardium whereas β-galactosidase positive nuclei are found in myocardium of the right ventricle and OFT. A reciprocal distribution of Tbx3 and β-galactosidase-positive nuclei was observed in pharyngeal mesenchyme (Figure 4D through 4F), such that most cells expressed one or the other epitope but not both. These results suggest that Tbx3 is expressed in NC cells adjacent to the SHF. Immunohistochemistry with AP-2α revealed that Tbx3 and AP-2α-positive nuclei colocalize in mesenchymal cells in the caudal pharynx, confirming that these cells are NC-derived (Figure 4G through 4I). The contribution of NC cells to the OFT was investigated in Tbx3−/− hearts. Crabp1-expressing and AP-2α-positive NC cells were observed in the pharyngeal region of Tbx3−/− embryos (supplemental Figure II2A through IID). Subsequently, PlexinA2-expressing cells were observed in the distal OFT cushions of Tbx3−/− embryos in a pattern similar to that in control hearts, revealing that NC cells colonize the Tbx3−/− OFT (supplemental Figure IIE through IIH).

The OFT defects observed in Tbx3−/− embryos suggest that Tbx3 might act indirectly on SHF deployment. Expression of the Mlc1v-nlacZ-24 transgene in SHF cells in the dorsal pericardial wall was observed in Tbx3+/− and Tbx3−/− em-
bryos (Figure 5B and 5D), even in embryos with severe OFT and right ventricular hypoplasia (Figure 5C and 5D). Isl1 transcripts accumulate normally in the SHF of Tbx3−/− embryos (Figure 5E and 5F) and Isl1 protein was observed in pharyngeal mesoderm and the distal OFT of Tbx3−/− embryos; however, less Isl1-positive cells were observed in the OFT of hypoplastic hearts (Figure 5G and 5H). Quantitative analysis at E10.5 demonstrated a significant reduction in OFT length in Tbx3−/− compared to Tbx3+/+ (P<0.05) and Tbx3+/− (P<0.01) embryos; in contrast, mutant OFTs were significantly broader than in control littermates (supplemental Figure III). These data reveal a failure of normal OFT elongation and morphogenesis in the absence of Tbx3.

Signaling molecules required for OFT elongation were evaluated in Tbx3−/− embryos. Fgf8 transcripts are normally detectable in the SHF and OFT and were slightly elevated in the distal OFT of Tbx3−/− embryos (Figure 6A and 6B). Bmp4 is expressed in the distal OFT and has been implicated in recruitment of SHF cells to the arterial pole of the heart. A slight reduction in Bmp4 transcript levels was observed in the OFT of Tbx3−/− embryos (Figure 6C and 6D). Quantitative RT-PCR revealed a significant increase in Fgf8 (1.5-fold) and Fgf10 (2-fold) transcript levels in the distal OFT and ventral pharynx of Tbx3−/− embryos; in contrast, Bmp4 transcript levels were reduced (0.6-fold; supplemental Figure IV). Downstream mediators of fibroblast growth factor (FGF) signaling were evaluated. Pea3 transcript levels were slightly elevated in the caudal pharynx and an increase in phospho-ERK was observed in SHF cells in ventral pharyngeal mesoderm of Tbx3−/− embryos, consistent with elevated FGF signaling (Figure 6E through 6H). Shh in ventral pharyngeal endoderm regulates addition of SHF cells to the heart tube25, a reduction in Shh expression dorsal to the heart was observed in Tbx3−/− embryos (supplemental Figure VA and VB). Ptx2 is required in the SHF for normal OFT development.26 Whereas Ptx2 transcripts are maintained in the first arch and in pharyngeal mesoderm, expression in the OFT was reduced in Tbx3−/− embryos (supplemental Figure VC and VD). Expression of Wnt11, which functions downstream of Ptx2 in OFT development,27 was slightly reduced in Tbx3−/− embryos (supplemental Figure VE and VF); TGFβ2, downstream of Wnt11,27 was also reduced in the OFT and pharyngeal region of Tbx3−/− embryos (supplemental Figure VG and VH). Loss of Tbx3 is thus associated with altered gene expression affecting multiple signaling pathways implicated in SHF and OFT development.

To further analyze SHF development in Tbx3−/− embryos, we evaluated differentiation, cell death, and proliferation within pharyngeal mesoderm at E9.5. We found no evidence of precocious or delayed myocardial differentiation. Normal accumulation of sarcomeric myosin heavy chain was observed in the distal OFT of Tbx3−/− embryos and not in adjacent splanchic mesodermal cells (Figure 7A through 7C). No significant differences in cell survival between Tbx3+/− and Tbx3−/− embryos were observed in pharyngeal mesoderm using caspase3 immunohistochemistry (data not shown). However, a significant increase in proliferation of pharyngeal mesoderm was observed in Tbx3−/− embryos (Figure 7D through 7F). Using a BrdU incorporation assay in

Figure 4. Tbx3 expression in the pharyngeal region. A and B, Tbx3 in situ hybridization at E9.5. Tbx3 is expressed in the first branchial arch (arrowhead), caudal pharynx (arrow), and AVC. C through F, Immunohistochemistry with anti-Tbx3 (green) and anti–β-galactosidase (red) antibodies in a transverse section through the caudal pharynx and heart of an E9.5 embryo carrying the Mic1v-nlacZ-24 transgene. Tbx3 protein is observed in the AVC, ectoderm (arrowhead), pericardium (arrow), pharyngeal endoderm, and NC cells (box in C); β-galactosidase is observed in right ventricular myocardium and the dorsal pericardial wall. D through F, High magnification of the boxed region in C showing the reciprocal distribution of Tbx3 and β-galactosidase in caudal pharyngeal mesenchyme. Tbx3 is observed in pharyngeal endoderm (arrowhead in D) and NC-derived mesenchyme (arrow). β-Galactosidase is observed in pharyngeal mesoderm (arrowheads in E) including the SHF (arrow). G through I, Immunohistochemistry with anti–Tbx3 (red) and anti–AP-2 (green) antibodies. AP-2α colocalizes with Tbx3 in pharyngeal ectoderm and NC-derived mesenchyme (arrows). Ph indicates pharynx. Scale bars: 100 μm (C); 50 μm (D through I).

Figure 5. SHF and OFT defects in Tbx3 mutant embryos. A through D, X-Gal–stained E9.5 Mic1v-nlacZ-24 transgenic embryos in right lateral (A and C) and ventral views with the heart removed (B and D). SHF cells in the dorsal pericardial wall are indicated by white arrowheads. Note the hypoplastic OFT and right ventricle in the embryo in C (arrowhead) and reduced transgene expression in the second arch. E and F, Isl1 transcripts accumulate normally in the pharyngeal region of Tbx3−/− embryos. G and H, Isl1 protein is observed in pharyngeal mesoderm and OFT myocardium of Tbx3−/− and Tbx3+/− hearts. Scale bar: 100 μm.
Mlc1v-nlacZ-24 transgenic embryos, we observed a 20% increase in the percentage of BrdU-positive lacZ-positive SHF cells in Tbx3<sup>+/−</sup> versus Tbx3<sup>+</sup> embryos versus (Figure 7F, based on counts from 3 Tbx3<sup>+/−</sup> and 3 Tbx3<sup>+</sup> embryos; P<0.001, Student’s t test). In contrast, no difference was detected in BrdU incorporation in adjacent β-galactosidase negative nuclei (Figure 7F).

**Discussion**

The coordinated development of different cell types is critical for arterial pole morphogenesis. In particular, interactions between SHF and NC cells orchestrate OFT elongation and septation. Our results demonstrate that between SHF and NC cells orchestrate OFT elongation and for arterial pole morphogenesis. In particular, interactions

The lack of survival of Neo/Neo mutant mouse embryos to fetal stages, and also to aberration of SHF deployment. Furthermore, we propose that this regulation is indirect and may be mediated by NC cells or pharyngeal endoderm. A subset of Tbx3<sup>+/−</sup> embryos exhibit general growth failure and have severely affected hearts where both rightward looping and convergence are blocked, suggesting the existence of an earlier, potentially distinct, role for Tbx3 in heart tube formation. Arch artery anomalies are observed in a subset of Tbx3<sup>+/−</sup> embryos at E12.5, revealing a later role for Tbx3 in asymmetrical arch artery remodeling. The lack of survival of Tbx3<sup>+/−</sup> embryos to fetal stages

**Figure 6.** Analysis of signaling pathways in Tbx3 mutant embryos at E9.5. A and B, In situ hybridization showing elevated levels of Fgf8 transcript accumulation in the OFT of Tbx3<sup>+/−</sup> (B) relative to Tbx3<sup>+/+</sup> (A) embryos (arrowheads). C and D, Bmp4 transcripts are reduced in the OFT of Tbx3<sup>+/−</sup> (D) relative to Tbx3<sup>+/+</sup> (C) hearts (arrowheads). E and F, Pea3 transcripts are slightly upregulated in the caudal pharynx of Tbx3<sup>+/−</sup> embryos (F, arrowhead). G and H, Fluorescent immunohistochemistry showing elevated levels of nuclear phospho-ERK (green) in Isl1-positive ventral pharyngeal mesoderm (red) of Tbx3<sup>+/−</sup> (H, arrowhead) compared to Tbx3<sup>+/+</sup> embryos (G). Scale bar (G and H): 100 μm.

**Figure 7.** Properties of the SHF in Tbx3 mutant embryos. A through C, Immunohistochemistry with MF20 anti–sarcomeric myosin and anti–β-galactosidase antibodies in paraffin sections of E9.5 Tbx3<sup>+/−</sup> (A) and Tbx3<sup>+/−</sup> (B and C) embryos carrying the Mlc1v-nlacZ-24 transgene. A through C, No differentiating cardiomyocytes are observed in mesodermal adjacent to the OFT (arrow) or in the dorsal pericardial wall (arrowhead) of Tbx3<sup>+/−</sup> or Tbx3<sup>++</sup> embryos. Immunohistochemistry with anti–BrdU and anti–β-galactosidase antibodies in paraffin sections of E9.5 Tbx3<sup>+/−</sup> (D) and Tbx3<sup>+/−</sup> (E) embryos carrying the Mlc1v-nlacZ-24 transgene and treated with BrdU in utero. F, Histogram showing the percentage of β-galactosidase–positive and –negative nuclei that are BrdU-positive. There is a 20% increase in BrdU-positive β-galactosidase–positive nuclei in Tbx3<sup>+/−</sup> compared to Tbx3<sup>++</sup> embryos (P<0.001, Student’s t test). Nuclei are labeled with Hoechst (blue). Scale bars: 100 μm (A through C); 50 μm (D and E).
precludes investigation of whether this phenotype is linked to the earlier developmental delay.

The addition of SHF cells to the arterial pole of the heart is coordinated by signals from adjacent cell types, including pharyngeal endoderm and NC cells. The signals and upstream regulators mediating such effects, however, largely remain to be identified. Tbx3 is expressed in pharyngeal endoderm and NC cells in the pharyngeal region rather than in the SHF itself. Impaired NC function in the absence of Tbx3 could result in defective proliferation and deployment of the SHF. In the chick, NC in the pharyngeal region is required for normal SHF development in addition to OFT septation. NC ablation results in myocardial hypoplasia and alignment defects associated with a reduced contribution of SHF cells to the elongating heart tube. GDF signaling is elevated in the pharynx of NC ablated embryos, leading to hyperproliferation and defective differentiation of the SHF. Reduction in GDF signaling has been shown to partially rescue the effects of NC ablation on the SHF. Precise levels of FGF signaling are critical for SHF deployment as pharmacological or genetic changes. Although not part of the classically defined ulnar–mammary syndrome phenotype, congenital heart defects have been reported in ulnar–mammary patients; our results identify Tbx3 as a candidate gene for human congenital heart defects affecting the arterial pole of the heart.

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Disclosures

None.

References


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Expanded Materials and Methods

Mouse genotyping

*Tbx3*\(^{\text{+/−}}\) mice were mated to produce embryos of the required genotypes. Embryos were dissected from timed pregnancies in phosphate buffered saline (PBS) with 4% bovine serum albumen. Somites were counted for developmental staging and a sample of the yolk sac was taken for PCR genotyping using the following primers.

- **Tbx3**: the primers 5'-GGC CTC AAG TAG CTT GGA A-3', 5'-AGG CCA ACA Aaa GAG CAG A-3', and 5'-CTA AGC CTG ATG G TG TGA G-3' result in a 350bp wild type band and a 500bp mutant band.
- **Tbx2**: the primers 5'-CCA GCC AGG GAA CAT AAT GAG G-3', 5'-CTG TCC CCT GGC ATT TCT GG-3', and 5'-CCT GCA GGA ATT CCT CGA CC-3' result in a 180bp wild type band and an 88bp mutant band.

Histology and immunochemistry

Embryos were collected and whole embryos or trunk regions were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 10mm sections were stained with haemotoxilin and eosin. Immunohistochemistry was performed on hydrated 10mm paraffin-embedded sections treated for 15min with antigen unmasking solution (Vector). Primary antibodies were incubated overnight. Primary antibody concentrations were: *Tbx3* (1/200, Santa Cruz Biotechnology), β-galactosidase (1/300, Cappel), MF20 (1/50, DSHB), Islet-1 (clone 40.2D6 : 1/100, DSHB), rat anti-BrdU (1/100, Immunologics), AP-2α (1/50, clone 3B5 DSHB), phospho-ERK (1/100, phospho-p44/42 MAPK (Thr202/Tyr204) (20G11) Cell
Signaling). Secondary antibody concentrations were: anti-goat biotin (1/500, Jackson), anti-mouse Cy3 (1/500, Jackson) and anti-rabbit Cy3 (1/500, Jackson). Protein was revealed using the Renaissance TSA Fluorescence System (Perkin Elmer), according to the manufacturer's instructions, counterstained with Hoechst and observed using an ApoTome microscope (Zeiss). For each experiment a minimum of 3 embryos of each genotype was scored. X-gal staining was carried out as described in 1, embryos were collected and fixed for 10 min in 4% paraformaldehyde, extensively washed in 1x PBS and stained for 5 hours at 37°C in a solution containing 4mg/ml of X-gal. After staining, the samples were washed in PBS, post-fixed and observed under a Zeiss Lumar stereomicroscope.

In situ hybridization

The following riboprobes were used: Tbx2, Tbx3, Tbx5 (Chapman et al. 1); Crabp1, Nppa, Csl, Cx40 (Harrelson et al. 2); Fgf8 (Kelly et al. 3); PlexinA2 (Mesbah et al. 4); Nkx2.5 (Lints et al. 5); Raldh2, Hoxb1 (kindly provided by S. Zaffran, IBDML, Ryckebusch et al. 6); Pea3 (kindly provided by Francoise Helmbacher, IBDML, Helmbacher et al. 7), Wnt11 (Zhou et al. 8); Isl1 (Pfaff et al. 9); Pitx2 and TGFβ2 probes were synthesized using T7 RNA polymerase from DNA fragments generated by the following PCR primers: Pitx2 5'- AGG GAC ACA TGT AAC TCG A -3'; 5'-GTA ATA CGA CTC ACT ATA GGG ACA TTC TGC TGC CAA GC-3'; TGFβ2 5'- ATC GTC CGC TTT GAT GTC TC -3'; 5'- GTA ATA CGA CTC ACT ATA GGG TTC GAT CTT GGG CGT ATT TC -3'.

Quantitative RT-PCR
The ventral pharyngeal region and outflow tract were dissected from E9.5 embryos in cold 1X PBS. Total RNA was isolated using PureLink Micro-to-midi total RNA purification System with Trizol (Invitrogen). First strand cDNA was synthesized using SuperScript III First-strand synthesis superMix for qRT-PCR (Invitrogen) following manufacture’s instructions. qRT–PCR analysis was performed using the following primers and SYBR greenER qPCR SuperMix (Invitrogen) and a Bio-Rad iCycler. Each experiment was performed in triplicate from a pool of seven samples of each genotype and normalized to Hprt.

Quantitative RT-PCR primers:
- Fgf8 F 5’-TGG AAG CAG AGT CCG AGT TC-3’; Fgf8 R 5’-TGT GAA TAC GCA GTC CTT GC-3’; Fgf10 F 5’-GCC ACC AAC TGC TCT TCT TC-3’; Fgf10 R 5’-CTG ACC TTG CCG TTC TTC TC-3’; Bmp4 F 5’-TCG TTA CCT CAA GGG AGT GG-3’; Bmp4 R 5’-ATG CTT GGG ACT ACG TTT GG-3’; Hprt F 5’-CTG GTG AAA AGG ACC TCT CG-3’; Hprt R 5’-TGG CAA CAT CAA CAG GAC TC-3’; Tbx3 E1 F 5’-ACA AGC GGG GTA CAG AGA TG-3’; Tbx3 E3 5’-ACG GCG ATG AAT TCT GTT TC-3’.

References
2. Harrelson Z, Kelly RG, Goldin SN, Gibson-Brown JJ, Bollag RJ, Silver LM, Papaioannou VE. Tbx2 is essential for patterning the atrioventricular canal and


Supplementary Table 1. Incidence of cardiac defects observed in *Tbx3* mutant embryos at E9.5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Convergence defect (%)</th>
<th>Hypoplasia OFT and RV (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Severe delay (%)</th>
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<td><em>Tbx3&lt;sup&gt;+/+&lt;/sup&gt;</em></td>
<td>0 / 20</td>
<td>1 / 20 (5)</td>
<td>0/20</td>
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<tr>
<td><em>Tbx3&lt;sup&gt;+/-&lt;/sup&gt;</em></td>
<td>2 / 35 (3)</td>
<td>2 / 35 (3)</td>
<td>0/35</td>
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<tr>
<td><em>Tbx3&lt;sup&gt;-/-&lt;/sup&gt;</em></td>
<td>18 / 25 (72)</td>
<td>13 / 25 (52)</td>
<td>4/25 (16)</td>
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<sup>a</sup> OFT, Outflow tract; RV, right ventricle.
Supplementary Figure Legends

Supplementary Figure 1. Molecular patterning in Tbx3 mutant embryos. Whole-mount views of E9.5 Tbx3+/− or Tbx3+/+ (A, C, E, F, I, J) and Tbx3−/− (B, D, G, H, K, L) embryos after in situ hybridization. A-D, E, G, I, K, left lateral views; F, H, right lateral views; J, L, ventral views. A-D, in situ hybridization for Hoxb1 (A, B) and Raldh2 (C, D) transcripts shows that a similar anterior expression border in Tbx3+/− and Tbx3−/− embryos (arrowheads). (E-H) Nkx2.5 transcripts accumulate indistinguishably in Tbx3+/− (E, F) and Tbx3−/− (G, H) hearts. (I-L) Tbx5 is expressed equivalently in the left ventricle and atria, but not the outflow tract (arrows) of Tbx3+/+ (I, J) and Tbx3−/− (K, L) hearts.

Supplementary Figure 2. Neural crest development in Tbx3 mutant embryos. (A, B) Wholemount embryos after in situ hybridization with a Crabp1 riboprobe showing normal NC cell migration in Tbx3+/− (A) and Tbx3−/− (B) embryos at E9.5. (C, D) Paraffin sections after fluorescent immunohistochemistry showing AP-2α positive cells in pharyngeal ectoderm and NC derived mesenchyme of Tbx3+/− (C) and Tbx3−/− (D) embryos. Wholemount hearts (E, F) and cryosections (G, H) after in situ hybridization with a PlexinA2 riboprobe showing NC derived cells migrating into the outflow tract of E11.5 Tbx3−/− embryos (F, H). Scale bars (C, D): 50μm; (G, H): 200μm.

Supplementary Figure 3. Outflow tract development in Tbx3 mutant embryos at E10.5. (A-D) Ventral views of Tbx3+/− (A) and Tbx3−/− (B-D) embryos carrying the Mlc1v-nlacZ-24 transgene stained with X-gal showing transgene expression in the
future right ventricle and OFT. (E) Histogram showing quantitative analysis of outflow tract length and diameter (plus SEM) in Tbx3<sup>+/+</sup> (n=2), Tbx3<sup>+/−</sup> (n=3) and Tbx3<sup>−/−</sup> (n=4) embryos at E10.5 using WCIF ImageJ, including the embryos in panels A-D.

Supplementary Figure 4. Quantitative RT-PCR analysis of gene expression in Tbx3 mutant embryos. (A) Histogram showing relative normalized transcript levels (plus SEM) for Bmp4, Fgf8 and Fgf10 in microdissected E9.5 outflow tract and pharyngeal tissue from Tbx3<sup>+/−</sup> and Tbx3<sup>−/−</sup> embryos. (B) Histogram showing endogenous Tbx3 transcript levels in microdissected E9.5 outflow tract and pharyngeal tissue from Tbx3<sup>+/+</sup>, Tbx3<sup>+/−</sup> and Tbx3<sup>−/−</sup> embryos.

Supplementary Figure 5. Analysis of signaling pathways in Tbx3 mutant embryos at E9.5. (A B) In situ hybridization showing that Shh transcript accumulation in the ventral pharynx is reduced in Tbx3<sup>−/−</sup> embryos (B, arrowhead). (C, D) Pitx2 transcripts are observed in the first arch and caudal pharyngeal region (arrow) of Tbx3<sup>−/−</sup> embryos but are reduced in OFT myocardium (arrowhead) relative to Tbx3<sup>+/−</sup> embryos. (E, F) Wnt11 transcript levels are slightly reduced in Tbx3<sup>−/−</sup> hearts (F). (G, H) TGFβ2 expression is reduced in the OFT (arrows) and pharyngeal mesoderm (arrowheads) of Tbx3<sup>−/−</sup> embryos (H).
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A

Relative normalized mRNA level

B

Relative normalized mRNA level

- Bmp4
- Fgf8
- Fgf10

- Tbx3^{+/+}
- Tbx3^{+/-}
- Tbx3^{-/-}