**Tbx1** mutation causes multiple cardiovascular defects and disrupts neural crest and cranial nerve migratory pathways

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**INTRODUCTION**

DiGeorge syndrome (DGS) (1) is characterized by aortic arch patterning defects, conotruncal heart defects, thymus and parathyroid aplasia/hypoplasia, and craniofacial anomalies. This phenotypic complex has suggested a role for pharyngeal arch/pouch maldevelopment in the pathogenesis of DGS, because the organs affected are developmentally related to the pharyngeal apparatus (reviewed in 2). The pharyngeal apparatus is composed of derivatives of all three embryonic germ layers, patterned by the anteroposterior growth and dorsolateral outpouching of the pharyngeal endoderm. Meso- dermal and ectomesenchymal (neural-crest-derived) cells colonize the region between the pouches to form the pharyngeal arches, from which connective tissue and nerves of the face and neck develop. Arteries form within these arches, and subsequently undergo extensive remodeling that ensures proper outflow connection between the heart and systemic and pulmonary circulation.

DGS is mostly caused by a heterozygous chromosomal deletion of chromosome 22q11.2 (referred to as del22q11). del22q11 is estimated to be the most common chromosomal deletion associated with birth defects in humans (3). Unveiling the genetic basis of DGS is important for understanding the disease pathogenesis and the developmental genetics of the pharyngeal apparatus. To this end, we have generated a mouse model of DGS by deleting part of the murine region on chromosome 16 that is homologous to del22q11. Heterozygously deleted mice, referred to as Df1/+ mice, present with cardiovascular defects (4), mild thymic and parathyroid abnormalities (5), and behavioral abnormalities (6) characteristic of the human syndrome. The haploinsufficient gene, within Df1, responsible for the cardiovascular abnormalities is Tbx1 (7–9). Homozygous mutation of Tbx1 causes severe developmental defects of the pharyngeal arches and pouches (7,8). However, a detailed analysis of the effects of Tbx1 deficiency on the cardiovascular system has not been reported, and the role of neural crest cells (NCCs), postulated to be of pathogenetic importance in DGS, has not been assessed in Tbx1 mutants. Here we show that, consistent with the patients' phenotype, the cardiovascular phenotype in Tbx1 mutants is multifaceted and includes a substantial intracardiac phenotype.

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Potentially related to these phenotypic abnormalities is the discovery of previously unreported Tbx1 expression domains in the outflow tract. We show that Tbx1 is unlikely to have a function in NCCs, but the paths of the caudal streams of NCCs, and of the cranial nerves that are derived from these cells, are profoundly affected, probably secondary to the lack of the caudal arches and pouches.

RESULTS

Tbx1 is required for patterning and growth of the pharyngeal endoderm

To correlate Tbx1 gene expression and the mutant phenotype, we have used a mutant Tbx1 allele carrying a knocked-in nuclear-lacZ reporter gene. The generation and establishment of mice with this allele, named Tbx1tm1Bld has been described elsewhere (8), but its suitability as a Tbx1 expression reporter had not been tested. To establish whether the β-galactosidase (β-gal) activity recapitulates the expression of the wild-type (WT) Tbx1 allele, we compared β-gal activity (detected by X-gal staining) and Tbx1 expression (detected by RNA in situ hybridization) in Tbx1wt/wt embryos, hereinafter referred to as Tbx1+/++. Results did not identify any significant differences between the two patterns (Fig. 1A and B) indicating that, in these mutants, β-gal activity recapitulates Tbx1 gene expression.

Tbx1 homozygous mutants (Df1/Tbx1– or Tbx1–/–) present with hypoplasia of the pharynx, which has a simple tube-like appearance and lacks the characteristic segmented pattern (7,8) (Fig. 1C and D); pouches 2–4 and arches 3–6 are not recognizable. At E8.0, the earliest stage tested, Tbx1 is expressed in the pharyngeal endoderm and in the mesodermal core of the 1st pharyngeal arch. At E9.5, the strongest expression is observed in the endoderm lining the 3rd pouch and 3rd arch (Fig. 2A), which are the most posterior pharyngeal segments at this stage. At E10.5, when the pharynx has grown to include the 4th arch, 4th pouch, and 6th arch, Tbx1 in the endoderm is again expressed most strongly in the posterior segments (Fig. 2B). In addition to this anteroposterior 'gradient' of expression, we observed higher expression towards the dorsolateral folds of the pharynx. At E11.5, endodermal expression persists almost exclusively in the 4th pouch (Fig. 2C). Hence, Tbx1 is expressed following anterior-to-posterior and medial-to-lateral gradients. These are also the directions of growth of the pharynx, suggesting that Tbx1 may be involved in the growth process. To test whether cell proliferation is affected, we calculated the mitotic index in the

![Figure 1](https://example.com/figure1.png)

Figure 1. (A, B) Tbx1-lacZ recapitulates the wild-type Tbx1 expression pattern. Sagittal sections of E10.5 Tbx1+/– embryos; dorsal is right, cranial is up. (A) RNA in situ hybridization using a Tbx1 antisense riboprobe. (B) X-gal staining. 3p, 3rd pouch; 4p, 4th pouch. DAo, dorsal aorta; arrowheads point to the expression domain in the core mesenchyme of the 1st and 2nd arches (I, II, arches); O, otocyst; H, heart. (C, D) Tbx1 homozygous mutants have a hypoplastic pharynx. Coronal sections of E9.5 X-gal stained embryos; cranial is up. (C) Tbx1+/–. (D) Df1/Tbx1–. The pharynx of the homozygous mutant is severely hypoplastic and lacks the characteristic segmented arrangement. I–IV: pharyngeal arches. 2a, 3a, pharyngeal arch arteries; DAo, dorsal aorta; fg, foregut; ph, pharynx.

![Figure 2](https://example.com/figure2.png)

Figure 2. There is a gradient of Tbx1 expression in the pharyngeal endoderm. X-gal stained embryos at E9.5 (A), E10.5 (B) and E11.5 (C). Sagittal (A) and coronal (C) sections through the pharynx (ph) demonstrate that at each stage, the most caudal portion of the pharyngeal endoderm shows the strongest Tbx1 expression. At E9.5 (A), the 3rd pouch (3p) and endoderm of the 3rd arch and arch artery (3a) are the most posterior structures and are highly Tbx1-positive. (B) By E10.5, the 4th arch has developed, and the strongest Tbx1 expression shifts to the 4th pouch (4p). This dorsal view of a cleared embryo shows the anteroposterior, mediolateral gradient of expression. The drawing adjacent to panel (B) is an outline of the pharynx; A, anterior; P, posterior; L, left; R, right; o, otocyst. (C) By E11.5, the 6th arch arteries (6a) have formed, and endodermal expression is strongest in the 4th pouch; note that the right 4th arch artery is hypoplastic (4a). In (A), cranial is up, dorsal is left; in (D), cranial is up.
pharyngeal endoderm and in the mesenchyme of pharyngeal arches 1 and 2 of Tbx1⁺/−/C0 and Df1/Tbx1/C0 embryos.

In the endoderm, the mitotic index was 42% (n = 1084) in the heterozygous, and 39% (n = 1065) in the homozygous mutant. The mitotic index of the mesenchyme was 53% (n = 1082) in heterozygous mutants and 51% (n = 1103) in homozygous mutants. These differences were not statistically significant.

With the exception of the central, mesodermal core of the 1st, 2nd and 3rd arches, no Tbx1 expression was detected in the pharyngeal arch mesenchyme. To establish whether pharyngeal hypoplasia in homozygous mutants could be due to increased cell death, we performed the TUNEL assay, but no difference could be found at the stage tested (E10.0, not shown).

Tbx1 is required for normal neural crest cell distribution and normal patterning of the peripheral nervous system in the pharyngeal apparatus.

Although there is no significant overlap between Tbx1 expression pattern and cardiac neural crest cells (NCC) in the pharyngeal apparatus, the anatomical defects in homozygous mutants predict abnormal NCC distribution. Crabp1, a marker of migrating NCCs, shows that NCCs in homozygous mutants are initially organized in apparently normal migratory streams after exiting the hindbrain. However, the Crabp1 expression pattern is abnormal in the pharyngeal arch region, with absence of the caudal expression domain (Fig. 3A and B) and ectopic expression (arrows in Fig. 3B). In situ hybridization on tissue sections confirmed the abnormal distribution of NCCs presumably destined to the caudal arches, which are absent in the homozygous mutant (not shown). At E10, the circumpharyngeal stream migrates caudal to the 4th arch (IV, white arrowhead) (C). The mandibular branch of the trigeminal nerve (Vm in G, white arrow in H) is misdirected caudally in the homozygous mutant and fuses with branches of the facial (VII) nerve. In this mutant, the glossopharyngeal nerve (IX) is hypoplastic and its distal ganglia (IXd in G) are abnormally fused to the distal ganglia of the vagus nerve (X, compare G and H); axonal projections show defasciculation and disarray (bracket in H); XI, accessory nerve.
1, 2, 3 and 4, respectively. To observe the fate of cranial nerves in homozygous mutants, we immunostained embryos to detect neurofilament-M. Results reveal a number of abnormalities. The mandibular branch of the trigeminal (V) nerve is abnormally directed caudally and fuses with the facial (VII) nerve (compare Fig. 3G and H). The fibers of the glossopharyngeal (IX) nerve are either hypoplastic or bundled to the fibers of the vagus (X) nerve, and the axonal projections show defasciculation and disarray (bracket in Fig. 3H). Terminal projections of the vagus and the accessory (XI) nerves are misdirected rostrally (Fig. 3H). Hence, cranial nerves are formed but their migration paths are abnormal. These results indicate that NCCs are able to migrate and differentiate, but they appear to lack appropriate directional cues. Peripheral nervous system abnormalities are not limited to NCC-derived structures. Indeed, the distal ganglia of the IX and X, which derive from the ectodermal placodes, are abnormally fused (Fig. 3H, red arrow), consistent with an extensive disorganization of the pharyngeal apparatus in Tbx1 homozygous mutants.

Tbx1 is required for formation and early remodeling of the pharyngeal arch arteries

It has been noted that in homozygous mutant embryos (Tbx1−/− or Df1/Tbx1−) pharyngeal arch arteries (PAAs) 3, 4, and 6 do not form (7,8), while in heterozygous mutants (Df1/+ or Tbx1+/−), the 4th PAA is hypoplastic in its early stages of development (8,10). To gain insight as to how Tbx1 expression relates to early 4th PAA remodeling, we examined Tbx1+/− embryos stained with β-gal at E9.75 and at E10.5. At E9.75, the nascent 4th PAA is nestled between the surface ectoderm and the pharyngeal endoderm; Tbx1 is expressed in the pharyngeal endoderm of the developing 4th pouch and, to a lesser extent, on the surface ectoderm and in the 4th arch mesoderm (Fig. 4A and B). At this stage, no phenotypic difference could be detected between WT and mutant embryos, consistent with previously reported data obtained with the Df1/+ mutant (10). At E10.5, when the 4th PAAs are hypoplastic in heterozygous mutants, Tbx1 expression in the pharyngeal region is restricted to the endoderm (Fig. 4C). The same expression pattern is observed in Dp1/Tbx1− embryos (which carry two functional copies of the Tbx1 gene), but in these animals, the size of the 4th PAA is normal (Fig. 4D). These data suggest that the role of Tbx1 in early remodeling of this artery is likely to operate through expression in endodermal cells. However, we also considered an alternative hypothesis, since we detected a few Tbx1-positive cells in the vessel wall near the confluence of the 4th artery into the dorsal aorta (Fig. 4E and F). Because Tbx1 is expressed in a subpopulation of vascular smooth muscle (vsm) cells in the
Tbx1 is required for growth and septation of the conotruncus

Prior to E11.5, there is a common outflow tract, the truncus arteriosus, which connects the heart to the systemic circulation. Subsequently, the outflow undergoes septation to separate aortico (systemic) and pulmonary flows. Formally, the septum is divided in three components (from distal to proximal, relative to the heart): (a) the aortico-pulmonary (AP) septum, (b) the truncal septum and (c) the conal septum (intracardiac), which is also important for the closure of the interventricular foramen. Tbx1 homozygous mutation causes disruption of all three components, ultimately leading to truncus arteriosus communis (TAC) in all homozygous mutant embryos examined at term (n = 9) (Fig. 5A and B), while the semilunar valve leaflets (which divide the truncal from the conal region) form normally (Fig. 5C). The AP septum agenesis is caused by the severe abnormalities of the pharyngeal region and the aortic sac, from which the AP septum originates (Fig. 5D and E). In homozygous mutants, the conotruncal conduit is considerably reduced in diameter as early as E9.5 (Fig. 5H and I). X-gal staining revealed for the first time a Tbx1 expression domain in the muscular wall of the outflow of both heterozygous and homozygous mutants (Fig. 5J and K), these X-gal-positive cells become more abundant later in development (E11.5, Fig. 5L, N, P, R). A niter expression domain is also found in the outflow myocardium near the inner curvature of the heart from E10.5 through at least E12.5 (arrowheads in Fig. 5F and U). Immunostaining confirmed that these X-gal-positive cells are muscle cells, since they are α-smooth muscle actin (α-sm) antibody (Fig. 4G–J). Hence, it is unlikely that Tbx1 has a cell-autonomous role in building the structure of the vessel.

DISCUSSION

While the evidence that mutation of TBX1 alone can cause DGS is not yet available, mouse studies provide compelling evidence that Tbx1 mutations can cause severe and gene-dosage-dependent pharyngeal and pouch abnormalities that are strikingly similar to the DGS phenotype (7–9). These studies, however, have left a number of questions open. Expression studies of Tbx1 using conventional in situ hybridization (7–9,13,14) have not reported expression in the conotruncus, begging the question of whether Tbx1 is a good candidate for the non-aortic arch cardiovascular defects commonly observed in patients, and whether the truncus arteriosus observed in Tbx1+/− mutants (7) may in fact be secondary to the severe disruption of the pharyngeal arch system. A potentially related question is what is the role of neural crest cells in the pathogenesis of the Tbx1 mutant phenotype. To begin addressing these questions, we initiated the study presented here. Our results show for the first time that Tbx1 gene expression is consistent not only with a role in arch artery formation and early remodeling, but also with a role in the development of the cardiac outflow tract. Tbx1 has two expression domains in the conotruncus: an early expression domain in the muscular wall of the outflow tract and a later expression domain in endocardial and subendothelial cells. The conotruncal muscle cells derive from a region of the splanchnic mesoderm underlying the caudal pharynx (15–17). We have shown here that Tbx1 is not required for muscle cell differentiation. However, it is tempting to speculate that the early conotruncal hypoplasia in Tbx1-deficient embryos, at a stage preceding NCC migration, may be due to a reduction in the number of the abovementioned mesodermal precursor cells. The expression of Tbx1 in endocardial and subendothelial cells of the conotruncus could be related to the septation defects in homozygous mutants, although it is also possible that the reduction of NCCs in the truncal swellings, which are hypoplastic, could contribute to the pathogenesis of these defects. As a consequence of the loss of septation throughout the outflow tract, Tbx1 deficiency causes truncus arteriosus and...
a large perimembranous/infundibular VSD. The truncus communicates with the right ventricle only, implying a defective alignment of the conal septum with the truncal septum, potentially due to failed rotation of the outflow tract. Interestingly, perimembranous VSD was also detected in the haploinsufficient mouse model Df1+/− (4) and is commonly seen in DGS patients. The conal alignment defect observed in homozygous mutants is developmentally related to the overriding of the aorta seen in some Df1+/− mutants (4) and in DGS patients. The fact that related defects are observed in the haploinsufficiency model indicates that these defects are not secondary to the severe anatomical disruption of the pharyngeal apparatus in homozygous mutants. The expression of Tbx1 in a subpopulation of vsm cells of the inner carotids is

Figure 5. Tbx1 mutation and conotruncal development. (A) Normal aortic arch pattern at E18.5. (B) A Df1/Tbx1−/− littermate shows truncus arteriosus communis (TAC) and aberrant (retroesophageal) right subclavian artery (rsa); Ao, aorta; P, pulmonary trunk; t, trachea; rcc, lcc, right or left common carotid; Isa, left subclavian artery; pa, pulmonary arteries. (C) Top view of a homozygous mutant heart after dissection of the truncus arteriosus to show the four leaflets of the semilunar valve (arrowheads). (D, E) Sagittal sections through the aortic sac (AS) of Tbx1+/− (D) and Df1/Tbx1−/− (E) at E10.5. Homozygous mutants lack the mesenchyme dorsal to the AS, from which the A/P septum arises: I–III, pharyngeal arches; ‘II’, hypoplastic 2nd arch; DAo, dorsal aorta; H, heart; 6a, 6th arch artery. (F) High-power view of the boxed region in (D) showing Tbx1-positive cells near the inner curvature of the heart (arrowhead). (G) Immunohistochemistry of an analogous section with α-smooth muscle actin shows that the region is populated by muscle cells (arrowhead). (H–S) Coronal sections through the outflow tract (OFT) of heterozygous (H, J, L, M, P, Q) or homozygous (I, N, O, R, S) mutants at E9.5 (H–K) and E11.5 (L–S) stained with X-gal. The size of the outflow of the homozygous mutant is reduced (I) with respect to the heterozygote (H); ph, pharynx; o, otocyst. (J, K) Enlargement of the boxed regions in (H) and (I), showing lacZ-positive cells in the wall of the outflow. (L–P) This cell population is more abundant at E11.5, (P) is an enlargement of the boxed area in (L). Immunostaining with α-smooth muscle actin shows that the lacZ-positive cells are α-smooth muscle actin-positive (Q–S); arrows and outlines show single cells stained with both X-gal and α-smooth muscle actin antibody. Note the hypoplasia of the truncal swelling (ts) in (N, O) compared with (L, M). For (H–T), cranial is up, (T, U) Tbx1 expression in endothelial and subendothelial cells (ec) in the outflow of heterozygous embryos at E11.5 (T). At E12.5 (U), these cells are found along the entire length of the pulmonary artery (pa) and aorta (Ao). The arrowhead indicates a group of X-gal-positive cells in the conal myocardium. Sagittal section, dorsal is up and cranial is left; Ao, aortic valve, atr, atrium. (V) Lateral view of a homozygous E18.5 heart in which the free wall of the right ventricle (rv) has been removed to show a large ventricular septal defect (VSD, arrow) and the origin of the truncus arteriosus from the right ventricle. The arrowhead indicates the path of the outflow tract; lv, left ventricle. (W, X) Sagittal sections at the level of the valves of the outflow tract, in heterozygous (W) and homozygous (X) embryos at E12.5. (W) Tbx1 is expressed in the septum (sept) separating right and left outlets; the physiological VSD is shown. (X) In a homozygous littermate, the corresponding region appears as a linear tube, with Tbx1 expression on the wall of the outlet leading to the truncus arteriosus communis (TAC). atr, atrium; For (V, X), cranial is up, and dorsal is right.
intriguing because del22q11 patients often present with abnormalities (mainly tortuosity and malpositioning) of inner carotid arteries (18). However, we have not observed such abnormalities in Tbx1 mouse mutants (heterozygous or homozygous), suggesting that in humans TBX1 plays a more critical role in carotid morphology, or that this particular phenotype is caused by deletion of another gene.

DiGeorge syndrome is a classical pharyngeal arch/pouch disorder (2). Whether or not NCCs play a pathogenic role has been the subject of speculation, but there are no experimental data to answer this question conclusively. Data from genetically modified mice do not support a NCC migration defect, in either the heterozygous multigene deletion mutant (10) or in the homozygous Tbx1 mutant, as shown here. However, we show that the distribution of neural-crest-derived cells, as detected by migratory, postmigratory, and differentiation markers, is profoundly disrupted in homozygous mutants. This disruption is also reflected in abnormalities of the peripheral nervous system, particularly of cranial nerves IX and X, which may be profoundly disrupted in homozygous mutants. This disruption is seen in the context of a general disruption of the pharyngeal arch and pouch derivatives. The latter role may be the basis for a wide range of birth defects. These include craniofacial abnormalities, palatal clefts, thymic and parathyroid defects, and some of the most common cardiovascular defects.

**MATERIALS AND METHODS**

Mouse mutants and breeding

Heterozygous Tbx1 mice carrying the lacZ knock-in allele Tbx1tm1Bal (here referred to as Tbx1+/−) were obtained as described previously (8). Mutants were maintained and analyzed on a C57BL/6 × 129SvEvBrd (129S5) mixed genetic background and were crossed to each other and to mice carrying a chromosomal deletion or duplication that includes the Tbx1 locus (Df1/+ and Dp1/+, respectively (4)) or wild-type mice of the same genetic background. Embryos were collected at various time points, considering the day of observation of a vaginal plug to be embryonic day (E) 0.5. Embryo stage was also confirmed by somite count. Mice and embryos were genotyped by PCR of DNA extracted from tail biopsies or yolk sacs, respectively, using previously published PCR primer pairs (8). Because to date we could not identify phenotypic differences between Tbx1−/− and Df1/Tbx1−/− animals, we selected the latter genotype for this work because it carries a single copy of the lacZ reporter gene and is hence more directly comparable with Tbx1+/− animals. The term ‘homozygous Tbx1 mutant’ throughout this paper refers to the genotype Df1/Tbx1−/−, unless otherwise specified. At least three embryos were analyzed per time point per genotype and per phenotyping procedure.

β-Galactosidase detection, histology and immunohistochemistry

β-Gal activity was detected in paraformaldehyde-fixed embryos using the X-gal substrate, according to standard procedures. Stained embryos were photographed as whole mounts and then embedded in paraffin and cut into 10 μm histological sections. Sections were counterstained with Nuclear Fast Red. Immunohistochemistry using an anti-α-smooth muscle actin (sma) monoclonal antibody (Clone 1A4, Sigma) was used to identify muscle cells. We used the monoclonal antibody 2H3 (Developmental Studies Hybridoma Bank) to detect neurofilament-M (165 kDa). To assay cell proliferation, pregnant females were injected with 5 mg/100 g body weight of bromodeoxyuridine (BrDU) and sacrificed 1 h after injection to harvest embryos. Embryos were fixed in ethanol, embedded in paraffin and cut into 7 μm sections. BrDU incorporation was detected on histological sections using an anti-BrDU monoclonal antibody (Clone # 85-2C8, Novacstra). Apoptosis was assayed by TUNEL analysis using a commercial kit (Roche).

In situ hybridization

Radioactive or non-radioactive in situ hybridization experiments were performed on sectioned or whole-mouse embryos, respectively, using a published protocol (24). Sense and antisense riboprobes were prepared by reverse transcription of DNA probes and labeled by incorporation of digoxigenin-conjugated UTP (Roche) or 35S-UTP (ICN). A Tbx1 probe (13) was obtained from Dr V. Papaloannou. Crabp1 transcripts were detected using a probe described previously (25). Dix2 transcripts were detected using the Expressed Sequence Tag clone BG228249.
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