Corrigendum

Tbx1 expression in pharyngeal epithelia is necessary for pharyngeal arch artery development

Zhen Zhang, Fabiana Cerrato, Huansheng Xu, Francesca Vitelli, Masae Morishima, Joshua Vincentz, Yasuhide Furuta, Lijiang Ma, James F. Martin, Antonio Baldini and Elizabeth Lindsay *Development* **132**, 5307-5315.

An error in Table 1 of this article was not corrected before going to press. Several of the genotypes were listed incorrectly.

The correct table is printed below.

The authors apologise to readers for this mistake.

Table 1. *Tbx1* gene expression and Cre recombination in pharyngeal tissues

Genotype	Fourth PAA hypoplasia	Stage of onset*	Pharyngeal endoderm	Pharyngeal ectoderm	Pharyngeal mesoderm	Neural crest	Vascular endothelium
Tbx1$^{+/-}$	Y (100%)	E8	+	+	+	–	–
Hoxa3$^{Cre/+}$;*Tbx1*$^{flox/+}$	Y (50%)	E8.5	+	+	+	+	+
Foxg1$^{Cre/+}$;*Tbx1*$^{flox/+}$	Y (42%)	E8.5	+	+	+	–	+
TgFgf15Cre;*Tbx1*$^{flox/+}$	Y (20%)	E9.25	+	+	–	–	–
Nkx2.5$^{Cre/+}$;*Tbx1*$^{flox/+}$	N	E8‡	+†	+†	+†	–	+
Mesp1$^{Cre/+}$;*Tbx1*$^{flox/+}$	N	<E8	–	–	+	–	+
Tie2Cre;*Tbx1*$^{flox/+}$	N	N/A	–	–	–	–	+

*Stage of onset of gene expression or Cre recombination in the pharyngeal region.
†Recombination incomplete in these tissues.
‡Moses et al., 2001.
N, no; N/A, not applicable; Y, yes.

Tbx1 expression in pharyngeal epithelia is necessary for pharyngeal arch artery development

Zhen Zhang[1,2], Fabiana Cerrato[1,7], Huansheng Xu[1,2], Francesca Vitelli[1], Masae Morishima[1], Joshua Vincentz[3], Yasuhide Furuta[3], Lijiang Ma[4], James F. Martin[4], Antonio Baldini[1,2,5,6,8] and Elizabeth Lindsay[1,8,*]

[1]Department of Pediatrics (Cardiology), Baylor College of Medicine, Houston, TX 77030, USA
[2]Program in Cardiovascular Sciences, Baylor College of Medicine, Houston, TX 77030, USA
[3]Department of Biochemistry and Molecular Biology, MD Anderson Cancer Center and Program in Genes and Development, Graduate School of Biomedical Sciences, University of Texas, Houston, TX 77030, USA
[4]Alkek Institute of Biosciences and Technology, Texas A and M System Health Science Center, Houston, TX 77030, USA
[5]Center for Cardiovascular Development, Baylor College of Medicine, Houston, TX 77030, USA
[6]Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, TX 77030, USA
[7]CEINGE – Biotecnologie Avanzate S.C. ar. l., via Communale Margherita, 482-80145, Naples, Italy
[8]Division of Cardiology, Second University of Naples, Naples, Italy
*Author for correspondence (e-mail: elindsay@bcm.tmc.edu)

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Summary

During embryonic life, the initially paired pharyngeal arch arteries (PAAs) follow a precisely orchestrated program of persistence and regression that leads to the formation of the mature aortic arch and great vessels. When this program fails, specific cardiovascular defects arise that may be life threatening or mild, according to the identity of the affected artery. Fourth PAA-derived cardiovascular defects occur commonly in DiGeorge syndrome and velocardiofacial syndrome (22q11DS), and in *Tbx1*$^{+/-}$ mice that model the 22q11DS cardiovascular phenotype. *Tbx1* is expressed in pharyngeal mesoderm, endoderm and ectoderm, and, in addition, we show that it is expressed in precursors of the endothelial cells that line the PAAs, thus expanding the number of tissues in which Tbx1 is potentially required for fourth PAA development. In this study, we have used cell fate mapping and tissue-specific gene deletion, driven by six different Cre lines, to explore *Tbx1* gene-dosage requirements in the embryonic pharynx for fourth PAA development. Through this approach, we have resolved the spatial requirements for *Tbx1* in this process, and we show pharyngeal epithelia to be a critical tissue. We also thereby demonstrate conclusively that the role of Tbx1 in fourth PAA development is cell non-autonomous.

Key words: Tbx1, DiGeorge syndrome, 22q11DS, Pharyngeal epithelia, Mouse

Introduction

The mature aortic arch and great vessels derive from the embryonic pharyngeal arch arteries (PAAs) and the aortic sac. The PAAs are components of the pharyngeal apparatus, a vertebrate-specific structure comprising alternating bulges (arches) and indentations (pouches and clefts) that develops sequentially along the body axis in a head-to-tail direction. The pharyngeal arches are overlaid by surface ectoderm and lined by endoderm, which we refer to together as the pharyngeal epithelia. Between the epithelial layers of the pharyngeal apparatus there is mesenchyme of mesodermal origin that is later infiltrated by ectomesenchyme of neural crest origin. The PAAs, which connect the aortic sac with the dorsal aortae, and which pass through the core of the arches, initially form as five pairs of symmetrical vessels. Beginning around embryonic day (E) 11.5 of mouse development, the caudal PAAs and the aortic sac remodel extensively to form the mature asymmetric aortic arch and great vessels. It is unclear how this morphogenic process is regulated, but the increasing number of mouse lines that carry gene mutations that affect PAA development is allowing us to address which genes and tissues are important for this key developmental process.

Tbx1 has been identified as a human disease gene. Mutational analysis has revealed that *TBX1* mutation causes a disease phenotype that is essentially identical to that associated with the relatively common genetic disorder known as 22q11 deletion syndrome (22q11DS) (Yagi et al., 2003), which includes DiGeorge syndrome and velocardiofacial syndrome. 22q11DS is caused by gene haploinsufficiency and it is the most common microdeletion syndrome, occurring in ~1:4000 live births (Botto et al., 2003; McDonald-McGinn et al., 1997; Ryan et al., 1997; Wilson et al., 1994). 22q11DS is caused by an ~3 Mb heterozygous deletion in 22q11.2 that includes *TBX1* and ~40 other genes (http://www.ensembl.org). One of the cardinal features of 22q11DS and of *TBX1* mutation is congenital heart disease, the most common defects being cardiac outflow tract defects and aortic arch abnormalities. In particular, interrupted aortic arch type B (IAA-B) is mainly caused by this genetic defect (Lewin et al., 1997; Rauch et al., 1998), making it one of the most etiologically homogeneous

cardiovascular defects known. Other common cardiovascular defects are aberrant origin of the right subclavian artery (ARSA) and the right aortic arch (RAA). In mice, heterozygous inactivation of *Tbx1* causes the same aortic arch abnormalities as those seen in patients (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001), while *Tbx1* loss of function causes a much more severe phenotype that is only rarely seen in patients. Therefore, the $Tbx1^{+/-}$ mouse is a genetically accurate model of the 22q11DS cardiovascular phenotype and, in particular, of fourth PAA-derived aortic arch abnormalities.

In mice, *Tbx1* is required for fourth PAA formation and growth. We have shown that 100% of $Tbx1^{+/-}$ embryos have hypoplastic fourth PAAs at E10.5, whereas, at term, 30-50% have fourth PAA-derived cardiovascular defects (specifically, IAA-B, RAA and ARSA), according to whether the left, right, or both fourth PAAs are affected (Lindsay and Baldini, 2001). The penetrance of these defects in E10.5 and term embryos varies with genetic background (Lindsay and Baldini, 2001) (this study). Although it is not usually possible to ascertain the embryonic origin of these cardiovascular abnormalities in humans, it is likely that they have the same embryological basis as in mice.

The *Tbx1* expression domains of mid-gestation mouse embryos have been described (Chapman et al., 1996; Garg et al., 2001; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Vitelli et al., 2002a). Briefly, they comprise pharyngeal endoderm, mesoderm and ectoderm, mesenchyme of arches III and IV, sclerotome, otocyst and head mesenchyme. *Tbx1* is not expressed in neural crest cells that infiltrate the pharyngeal arches and cardiac outflow tract. When the fourth PAA forms at around E9.75, *Tbx1* is most highly expressed in the endoderm of the fourth arch and pouch. *Fgf8* is also expressed in pharyngeal endoderm at this time, and we have hypothesized that Tbx1 may regulate fourth PAA development by activating signals from pharyngeal endoderm, perhaps via Fgf8, that are directed towards the underlying mesenchyme surrounding the PAAs (Vitelli et al., 2002b). In support of this hypothesis, we have shown that *Tbx1* and *Fgf8* interact genetically in fourth PAA development (Vitelli et al., 2002b), and a more recent study has shown that *Fgf8* expression is required in surface ectoderm for fourth PAA development (Macatee et al., 2003). However, *Tbx1* is expressed in many tissues potentially involved in the development of these arteries.

To understand the developmental and genetic mechanisms governing the formation, growth and remodeling of the fourth PAAs, it is necessary to understand the role of the individual tissues. This can be approached using tissue-specific, gene-dosage reduction. To this end, we have used a panel of Cre drivers (Table 1) that induce recombination in different tissues of the pharyngeal apparatus to delete one copy of the *Tbx1* gene. Results clearly indicate that the critical tissue for early fourth PAA development is the pharyngeal epithelia. We believe that the use of multiple Cre drivers, with unique but partially overlapping patterns of Cre recombination, strengthens the major conclusions of our study because it permits the confirmation of results obtained with individual Cre drivers, which are likely to have some inherent variability due to minor differences in the onset and extent of recombination.

Materials and methods

Mouse mutants and breeding

The following mouse lines have been described: R26R (Soriano, 1999), Tie2-Cre (Kisanuki et al., 2001), $Tbx1^{+/-}$ (Lindsay et al., 2001), $Tbx1^{flox/+}$ and $Tbx1^{mcm/+}$ (Xu et al., 2004), $Nkx2.5^{Cre/+}$ (Moses et al., 2001), $Foxg1^{Cre/+}$ (Hebert and McConnell, 2000), $Mesp1^{Cre/+}$ (Saga et al., 1996), $Hoxa3^{Cre/+}$ (Macatee et al., 2003). Mice were genotyped by PCR as described in the original reports. The *Tbx1* conditional (floxed) allele is functionally normal, and $Tbx1^{flox/flox}$ mice are healthy and fertile (Xu et al., 2004). $Tbx1^{flox/-}$ embryos have fourth PAA hypoplasia at a similar penetrance to $Tbx1^{+/-}$ embryos (data not shown). In this study, all of the 100 $Tbx1^{flox/+}$ embryos analyzed had normal fourth PAAs. In order to induce nuclear translocation of the MerCreMer fusion protein (Verrou et al., 1999) encoded by the $Tbx1^{mcm}$ allele for cell fate-mapping experiments, pregnant female mice were given a single injection of Tamoxifen (75 mg/kg body weight) on gestation day 7.5, and embryos were collected at E10.5. *Tbx1* expression, as tested by a *lacZ* knock-in reporter allele, is not affected by Tamoxifen treatment (Xu et al., 2004). To visualize Cre recombination, Cre-driver mice were bred with R26R reporter mice and their offspring analyzed for β-gal activity.

Generation of new mouse lines

We generated two stable transgenic Fgf15-Cre lines. For the first line, we cloned an Hsp68 minimal promoter and Cre recombinase downstream of a 4.2-kb *Xho*I-*Bam*HI fragment from the upstream promoter region of *Fgf15* (J.V. and Y.F., unpublished). This fragment, in combination with the Hsp68 minimal promoter and a *lacZ* reporter, drives expression specifically in pharyngeal ectoderm and endoderm (not shown). To analyze Fgf15-Cre-induced recombination, we bred a single founder (TgFgf15HspCre) with R26R reporter mice and analyzed embryos with the genotype TgFgf15HspCre; R26R by X-gal staining. For the second transgenic line, we used the endogenous Fgf15 promoter instead of Hsp68. This promoter fragment extends from base pair –520 to –80 (considering the first base of the *Fgf15* trascription start codon (ATG) as base pair 1). The promoter fragment, in combination with Cre recombinase was cloned downstream of a 4-kb *Xho*I-*Nhe*I fragment from the upstream promoter region of *Fgf15* (J.V. and Y.F., unpublished). Cre-induced recombination from this second transgenic line was analyzed by breeding a single founder (TgFgf15Cre) with R26R reporter mice and analyzing embryos with the genotype TgFgf15Cre; R26R by X-gal staining. To generate *Tbx1* conditional mutants, we bred TgFgf15HspCre or TgFgf15Cre mice with $Tbx1^{flox/flox}$ mice and analyzed the phenotype of embryos with the genotypes TgFgf15HspCre; $Tbx1^{flox/+}$ or TgFgf15Cre; $Tbx1^{flox/+}$, respectively.

X-gal staining, histology and RNA in situ hybridization

To visualize β-gal activity, paraformaldehyde-fixed embryos were stained using X-gal substrate, according to standard procedures. Stained embryos were photographed as whole-mount specimens and then embedded in paraffin wax and cut into 10 μm histological sections. Sections were counterstained with Nuclear Fast Red. RNA in situ hybridization experiments were performed on 10 μm embryo sections, according to a published protocol (Albrecht et al., 1997). Labeled sense and antisense probes were prepared by reverse transcription of DNA clones in the presence of ^{35}S-UTP (MP Biomedical).

Results

Tbx1 is expressed in the surface ectoderm overlying the nascent caudal arches

Ectodermal expression of *Tbx1* has been noted (Vitelli et al., 2002a) but not previously characterized. We have analyzed *Tbx1* expression in early pharyngeal development using a *lacZ*

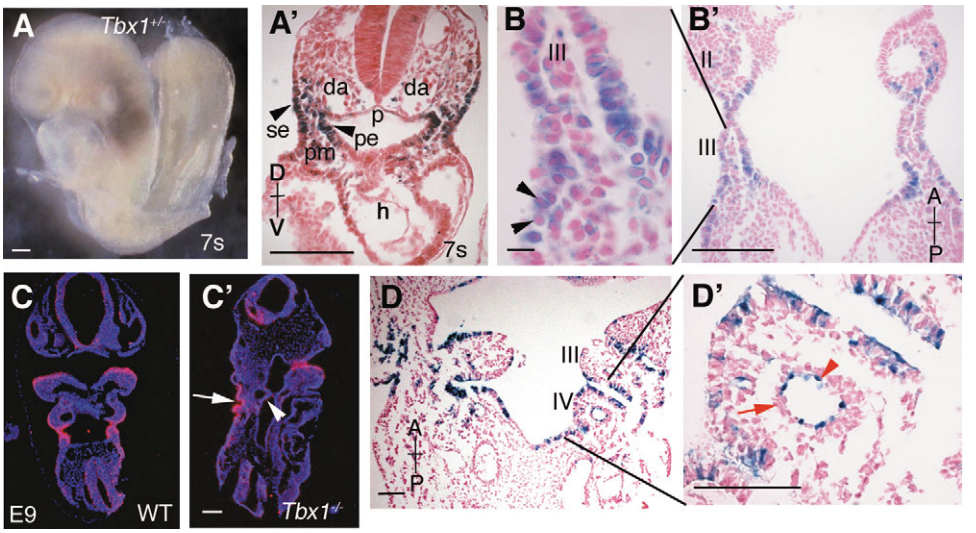

Fig. 1. *Tbx1* expression in the pharyngeal region. (A,A′) X-gal staining pattern generated by a *Tbx1-lacZ* knock-in allele (*Tbx1*$^{+/–}$) at E8 (A), and in a transverse section through the pharynx of the same embryo (A′). D, dorsal; V, ventral; da, dorsal aorta; se, surface ectoderm; pe, pharyngeal endoderm; pm, pharyngeal mesoderm; p, pharynx; h, heart. (B,B′) *Tbx1* expression in surface ectoderm overlying the presumptive caudal arches (arrowheads) at E9. A, anterior; P, posterior; II, III, second and third pharyngeal arch. (C,C′) Radioactive in situ hybridization with an *Fgf8* antisense probe on coronal sections of wild-type (C) and *Tbx1*$^{–/–}$ (C′) embryos at E9. There is a loss of *Tbx1* expression in the pharyngeal endoderm (arrowhead) but not in the surface ectoderm (arrow) of *Tbx1*$^{–/–}$ mutants. (D,D′) Fate map of *Tbx1*-expressing cells in the fourth pharyngeal arch: X-gal staining pattern in coronal sections of a *Tbx1*$^{mcm/+}$;R26R embryo at E10.5. Red arrowhead in D′ indicates X-gal staining in endothelial cells lining the fourth pharyngeal arch artery (PAA); no staining is observed in the surrounding vascular smooth muscle (red arrow). A, anterior; P, posterior; III, IV, third and fourth pharyngeal arch. Scale bars: 100 μm in A,A′,B′,C-D′; 10 μm in B.

knock-in reporter (Lindsay et al., 2001). Expression in the pharyngeal region was first seen at embryonic stage E8 in the surface ectoderm overlying the pharynx, in the pharyngeal endoderm and mesoderm (Fig. 1A,A′, Table 1), and in the head mesenchyme (not shown). At E9, expression in the surface ectoderm extended over the presumptive caudal arches (Fig. 1B,B′), whereas after E9.5, ectodermal expression was no longer detectable. Thus, *Tbx1* expression in surface ectoderm precedes the formation of the fourth PAA at ~E9.75, suggesting that it may contribute to this process. Hence, both endoderm and ectoderm express *Tbx1* in a region relevant to fourth PAA formation. *Fgf8* is also expressed in surface ectoderm at E9 and its expression in that tissue is required for fourth PAA development (Macatee et al., 2003). Thus, it is possible that the reported genetic interaction between *Tbx1* and *Fgf8* (Vitelli et al., 2002b) operates through transcriptional regulation of the *Fgf8* gene by Tbx1 in ectoderm. We analyzed *Fgf8* expression in *Tbx1*$^{–/–}$ embryos between E9 and E10 and found it to be robustly expressed in surface ectoderm (Fig. 1C,C′), although it was not expressed in the pharyngeal endoderm of these mutants. Thus, it is unlikely that Tbx1 regulates *Fgf8* expression in pharyngeal ectoderm.

Tbx1 is expressed but not required in progenitors of fourth PAA endothelial cells

The complete lack of caudal (third, fourth and sixth) PAAs in *Tbx1*$^{–/–}$ mutants indicates that *Tbx1* is required for PAA formation. *Tbx1* is not expressed in the structural components of these vessels. However, *Tbx1*-expressing endothelial cells are present at the junction of the fourth PAAs with the dorsal aorta (Vitelli et al., 2002a). To establish whether the endothelial lining of the fourth PAA derives from *Tbx1*-expressing cells, we analyzed their fate using a Tamoxifen-inducible *Tbx1*-Cre mouse (*Tbx1*^{mcm}) in breedings with R26R reporter mice. The fate of *Tbx1*-expressing cells was analyzed in *Tbx1*$^{mcm/+}$; R26R embryos at E10.5, where Tamoxifen had been administered to the pregnant mother on gestation day 7.5. We found that *Tbx1*-expressing cells do indeed contribute to the endothelial lining of the fourth PAA, but not to the vascular smooth muscle (Fig. 1D,D′).

To determine whether heterozygous loss of *Tbx1* in endothelial precursors causes fourth PAA abnormalities, we bred Tie2-Cre or *Mesp1*$^{Cre/+}$ mice, both of which express Cre in endothelial precursors (Kisanuki et al., 2001; Saga et al., 1996), with *Tbx1*$^{flox/flox}$ mice and analyzed the fourth PAAs of

Table 1. *Tbx1* gene expression and Cre recombination in pharyngeal tissues

Genotype	Fourth PAA hypoplasia	Stage of onset*	Pharyngeal endoderm	Pharyngeal ectoderm	Pharyngeal mesoderm	Neural crest	Vascular endothelium
Tbx1$^{+/–}$	Y (100%)	E8	+	+	+	–	–
Hoxa3$^{Cre/+}$;*Tbx1*$^{+/–}$	Y (50%)	E8.5	+	+	+	+	+
Foxg1$^{Cre/+}$;*Tbx1*$^{+/–}$	Y (42%)	E8.5	+	+	+	–	+
TgFgf15Cre;*Tbx1*$^{+/–}$	Y (20%)	E9.25	+	+	–	–	–
Nkx2.5$^{Cre/+}$;*Tbx1*$^{+/–}$	N	E8‡	+†	+†	+†	–	+
Mesp1$^{Cre/+}$;*Tbx1*$^{+/–}$	N	<E8	–	–	+	–	+
Tie2Cre; *Tbx1*$^{+/–}$	N	N/A	–	–	–	–	+

*Stage of onset of gene expression or Cre recombination in the pharyngeal region.
†Recombination incomplete in these tissues.
‡Moses et al., 2001.
N, no; N/A, not applicable; Y, yes.

conditional mutants at E10.5 by intracardiac ink injection. No fourth PAA abnormalities were seen in Tie2-Cre; *Tbx1*$^{flox/+}$ embryos (data not shown), or *Mesp1*$^{Cre/+}$; *Tbx1*$^{flox/+}$ embryos (reported fully below), indicating that loss of *Tbx1* in endothelial precursors does not cause the *Tbx1*$^{+/-}$ haploinsufficiency phenotype.

Mesodermal expression of *Tbx1* is not required for fourth PAA development

Nkx2.5^{cre}-induced recombination occurs in pharyngeal mesoderm, specifically in the core mesoderm of arches I-III (Fig. 2B, part a), the cardiac outflow tract and the secondary heart field (Fig. 2B, part b), as well as in pharyngeal endoderm (Fig. 2B, part c, Fig. 3B′) and ectoderm (Fig. 3B′). *Tbx1* is also expressed in these tissues (Fig. 2A). However, there is little overlap between *Tbx1* expression and *Nkx2.5*^{cre}-induced recombination in the pharyngeal endoderm at E10.5 (compare Fig. 2A part c with 2B part c). Specifically, *Nkx2.5*^{cre} recombination is most prominent in the floor of the pharynx, whereas *Tbx1* expression is most prominent in the lateral pharynx and developing pouches. Furthermore, in earlier embryos (E9), both *Tbx1* expression and *Nkx2.5*^{Cre}-induced recombination in the mesoderm is patchy (compare Fig. 3A′ with 3B′). Xu et al. showed that *Nkx2.5*^{Cre}; *Tbx1*$^{flox/-}$ embryos have the same outflow tract phenotype as *Tbx1*$^{-/-}$ embryos do, but that the aortic arch phenotype is much milder (Xu et al., 2004). In that study, the effect of deleting one copy of *Tbx1* in the *Nkx2.5* expression domain on fourth PAA formation was

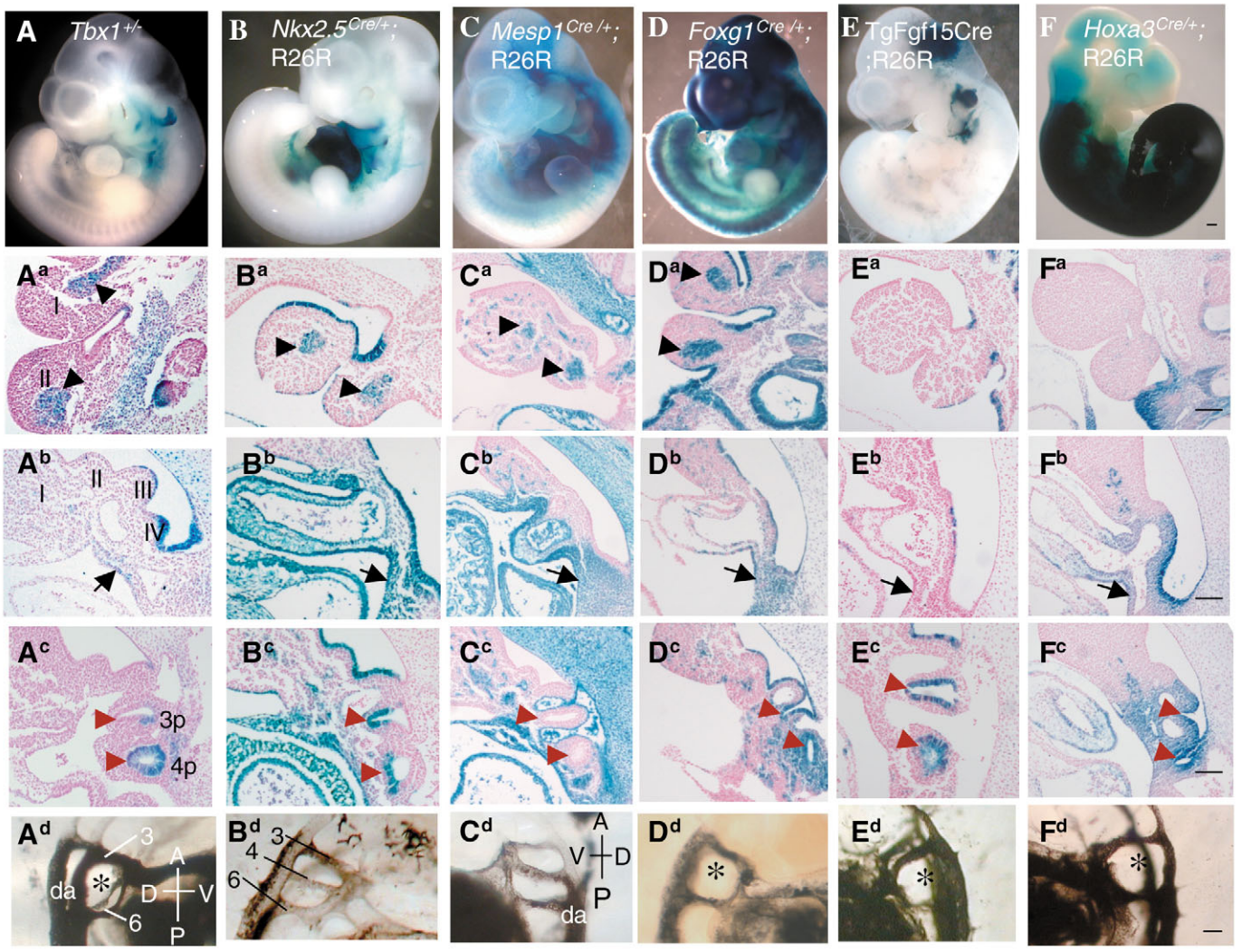

Fig. 2. *Tbx1-lacZ* expression and Cre-induced recombination in the pharyngeal region of embryos at E10.5. (A) *Tbx1-lacZ* expression in a whole-mount *Tbx1*$^{+/-}$ embryo (A), and on sagittal sections of the same embryo (Aa-Ad) showing expression in the core arch mesoderm of pharyngeal arches I and II (arrowheads in Aa), in the pharyngeal mesoderm (secondary heart field, arrow in Ab) and in the third and fourth pharyngeal pouches (red arrowheads in Ac). The absent fourth PAA (asterisk in Ad, Dd, Ed, Fd) at E10.5 is revealed by intracardiac ink injection. I-IV, pharyngeal arches I-IV; 3p, 4p, third and fourth pharyngeal pouches; da, dorsal aorta; 3, 6, third and sixth pharyngeal arch artery; A, anterior; P, posterior; D, dorsal; V, ventral. (B-F) X-gal-stained wholemounts and sagittal sections of Cre-driver; R26R embryos. (B) *Nkx2.5*$^{Cre/+}$; R26R; (C) *Mesp1*^{Cre}; R26R (D) *Foxg1*$^{Cre/+}$; R26R; (E) TgFgf15Cre; R26R; (F) *Hoxa3*$^{Cre/+}$; R26R. Sagittal sections in B-F are similar to those in A, and arrows and arrowheads show the same tissues and structures. In all histological sections, cranial is up and dorsal is right. In the lower panel of ink injected embryos, Ad, Bd and Dd are oriented as indicated in Ad; Cd, Ed and Fd are in the opposite orientation, as indicated in Cd. Scale bars: 100 μm.

not tested. To do this, we analyzed conditional mutant embryos (*Nkx2.5*$^{Cre/+}$; *Tbx1*$^{flox/+}$) at E10.5 by intracardiac ink injection at E10.5. None of the twelve conditional mutants had fourth PAA abnormalities (Fig. 2B, part d), suggesting that *Nkx2.5*^{cre}-induced recombination does not occur in the cells that require *Tbx1* for fourth PAA formation and growth. In order to achieve more extensive recombination in pharyngeal mesoderm, we used an alternative mesoderm Cre driver.

Mesp1^{Cre} induces recombination in mesoderm-derived tissues (Saga et al., 1996), but rigorously spares pharyngeal epithelia. In gastrulating embryos, *Mesp1* is expressed by all mesodermal cells as they exit the primitive streak between E6.5 and E7. It is rapidly downregulated after E7.0-E7.5 (Saga et al., 1996; Saga et al., 1999). As a result, *Mesp1*^{Cre} induces extensive recombination in a sub-population of mesoderm-derived tissues from the cardiac crescent stage. Pertinent to this study, this includes core arch mesoderm (Fig. 2C, part a; Fig. 3C′), pharyngeal mesoderm (Fig. 2C, part b; Fig. 3C′, Fig. 4A′) and vascular endothelium (Fig. 2C, part c; Fig. 3C′, Fig. 4A′). Caudal axial mesoderm is spared (Fig. 3C), because precursors of this tissue do not express *Mesp1*. We bred *Mesp1*$^{Cre/+}$ mutants with *Tbx1*$^{flox/flox}$ mutants and analyzed the phenotype of conditional (*Mesp1*$^{Cre/+}$; *Tbx1*$^{flox/+}$) mutants at E10.5 by intracardiac ink injection. None of the 19 embryos tested had fourth PAA defects (Fig. 2C, part d). Overall, the data obtained with *Nkx2.5*^{Cre} and *Mesp1*^{Cre} suggest that the role of Tbx1 in fourth PAA development is not mediated by mesodermal expression.

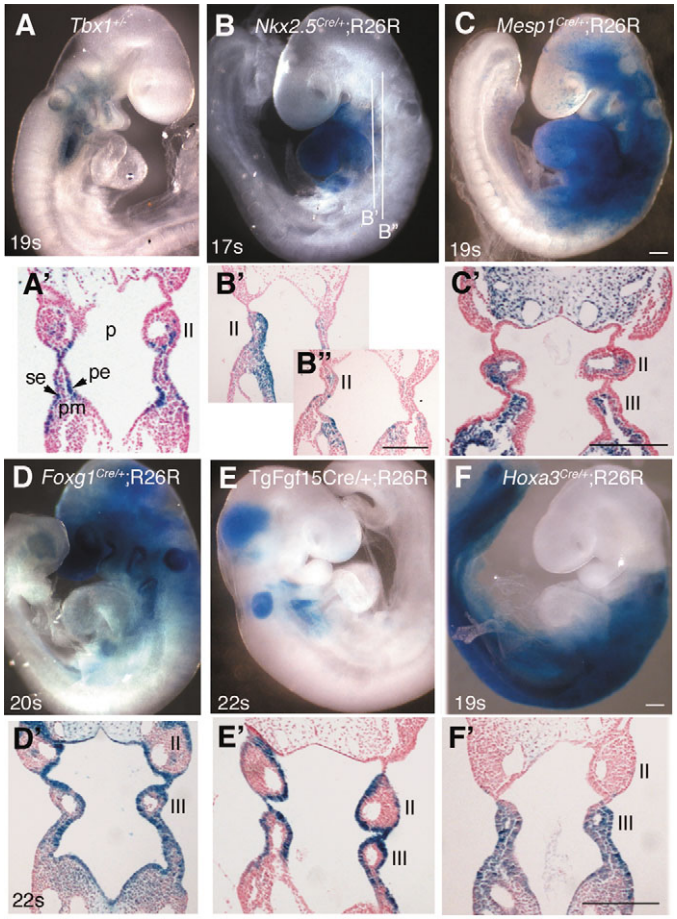

Fig. 3. *Tbx1* gene expression and Cre recombination in embryos at E9. X-gal-stained wholemount embryos (A-F) and coronal sections through the pharynx of the same embryos (A′-F′). (A,A′) *Tbx1*$^{+/-}$; (B-B′′) *Nkx2.5*$^{Cre/+}$; R26R; (C,C′) *Mesp1*$^{Cre/+}$; R26R; (D,D′) *Foxg1*$^{Cre/+}$; R26R; (E,E′) TgFgf15Cre; R26R (E9.5 embryo); (F,F′) *Hoxa3*$^{Cre/+}$; R26R. Position of the sections in B′ and B′′ are indicated in B. se, surface ectoderm; pm, pharyngeal mesoderm; pe, pharyngeal endoderm; p, pharynx; II, III, second and third pharyngeal arches. Scale bars: 200 μm.

Epithelial recombination is necessary and sufficient to cause fourth PAA hypoplasia

Foxg1^{Cre} induces recombination in pharyngeal epithelia (Fig. 2D, part c; Fig. 3D′) and in pharyngeal mesoderm, including the core arch mesoderm (Fig. 2D, part a; Fig. 3D′) and secondary heart field (Fig. 2D, part b). We bred *Foxg1*$^{cre/+}$ mutants with *Tbx1*$^{flox/flox}$ mutants and analyzed the phenotype of conditional (*Foxg1*$^{Cre/+}$; *Tbx1*$^{flox/+}$) mutants by ink injection at E10.5. Forty-two percent of *Foxg1*^{Cre} conditional mutant embryos had hypoplasia of one or both fourth PAAs (Fig. 2D, part d; n=21). Thus, *Tbx1* gene-dosage reduction in *Foxg1*^{Cre}-expressing cells, or daughter cells, is sufficient to cause fourth PAA hypoplasia.

To investigate the cause of reduced penetrance of the fourth PAA phenotype, we analyzed Cre-induced recombination at earlier embryonic stages. Recombination was first detectable in pharyngeal endoderm, and to a lesser extent in surface ectoderm, at E8.5 (Fig. 4B′, Table 1). At this stage and at E9 (Fig. 3D′), recombination was patchy. Therefore, reduced penetrance of the fourth PAA phenotype in *Foxg1*^{Cre} conditional mutants may be due to the late activation of Cre-induced recombination of the *Tbx1*-floxed allele in some cells. As with *Nkx2.5*^{Cre} and *Mesp1*^{Cre}, *Foxg1*^{Cre} induces recombination in mesodermally derived tissues of the pharyngeal apparatus, but, unlike the former Cre drivers, *Foxg1*^{Cre} also induces extensive recombination in pharyngeal endoderm and surface ectoderm, indicating that it is the recombination in these latter tissues that causes the fourth PAA hypoplasia in conditional mutants.

To dissect further the tissue requirement of Tbx1 in fourth PAA development, we generated two transgenic Fgf15Cre driver lines that are specific for pharyngeal epithelia. For the first line, we used a 4.2-kb enhancer fragment located ~4 kb upstream from the transcription start site of the *Fgf15* gene that contains putative tissue-specific regulatory elements (J.V. and Y.F., unpublished). This fragment, in combination with an Hsp68 minimal promoter can drive the expression of a *lacZ* reporter in pharyngeal ectoderm and endoderm in transgenic embryos (not shown). We used this enhancer fragment and an Hsp68 promoter in combination with Cre recombinase to generate a stable transgenic line. A second stable transgenic line was generated using a closely related enhancer fragment (~4 kb *Xho*I-*Nhe*I) in combination with the endogenous *Fgf15* promoter and Cre recombinase. Cre-induced recombination was analyzed by crossing transgenic founders with R26R mice. A similar pattern of pharyngeal epithelial-specific Cre-induced recombination was seen from founders of both transgenic lines, although, from the TgFgf15HspCre founder, we observed some embryos (~20%) with variable levels of ectopic Cre-induced recombination. From the TgFgf15Cre founder, no ectopic recombination was seen in any of the 23 TgFgf15Cre;

R26R embryos analyzed. All illustrations are from this latter transgenic line. Cre-induced recombination was first seen in pharyngeal endoderm at E9 (Fig. 4C′, Table 1), in a more anterior position to the *Tbx1* endodermal expression that is observed at a similar stage (compare Fig. 3A′ with Fig. 4C′). From E9.25 (Fig. 3E), Cre-induced recombination encompassed all surface ectoderm and pharyngeal endoderm (Fig. 2E, part c; Fig. 3E′), between and including arch I and arch IV. As no recombination was seen in the core arch mesoderm (Fig. 2E, part a) or arch mesenchyme (Fig. 2E, part c; Fig. 3E′), we conclude that recombination is epithelial specific.

In order to evaluate whether *Tbx1* dosage reduction specifically in pharyngeal endoderm and ectoderm was sufficient to recapitulate the $Tbx1^{+/-}$ haploinsufficiency phenotype, we bred founders from both transgenic lines with $Tbx1^{flox/flox}$ mice and analyzed the phenotype of conditional mutants (TgFgf15HspCre; $Tbx1^{flox/+}$ or TgFgf15Cre; $Tbx1^{flox/+}$) at E10.5 by intracardiac ink injection. Fifty percent of TgFgf15HspCre conditional mutants had hypoplasia of one or both fourth PAAs (n=14, not shown). From founder TgFgf15Cre, which gave no embryos with ectopic recombination, we obtained similar results, although at a lower penetrance (20%, n=34, Fig. 2E, part d). Together, these data indicate that *Tbx1* dosage reduction in pharyngeal epithelia is sufficient to cause fourth PAA hypoplasia.

The TgFgf15Cre lines were maintained on an FVB/C57BL/6 background, whereas the other Cre lines and *Tbx1* mutants were on a mixed C57BL/6;129SvEv background. To test whether the FVB/C57BL/6 background may affect penetrance of the haploinsufficiency phenotype, we crossed the TgFgf15Cre founder with $Tbx1^{+/-}$ mice and analyzed embryos at E10.5 by ink injection. Sixty percent of $Tbx1^{+/-}$ embryos from this breeding had fourth PAA hypoplasia (n=7) compared with 100% on the mixed C57BL/6;129SvEv background (Lindsay and Baldini, 2001). This suggests that the low penetrance of fourth PAA hypoplasia observed in TgFgf15Cre conditional mutants may be due, at least in part, to a protective effect of the FVB background. Weak recombination, partially overlapping expression or delayed Cre activation may also contribute to reduced penetrance.

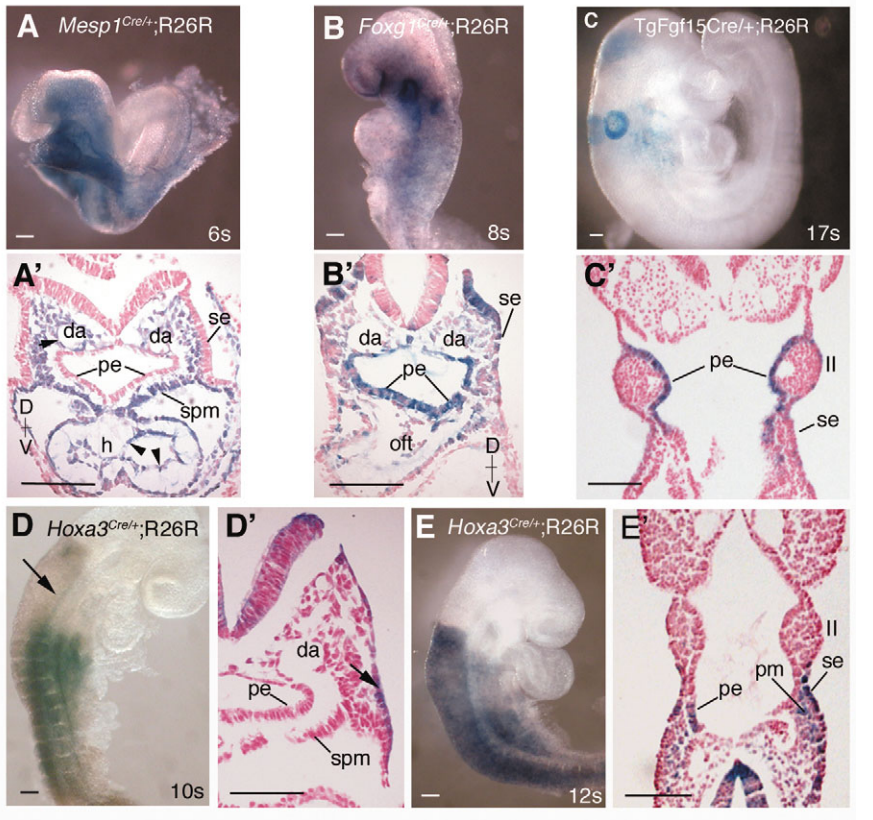

Fig. 4. Early Cre-induced recombination from Cre drivers $Mesp1^{Cre}$ (A,A′), $Foxg1^{Cre}$ (B,B′), TgFgf15Cre (C,C′) and $Hoxa3^{Cre}$ (D-E′). A′,B′ and D′ are transverse sections through the pharynx, D, dorsal; V, ventral. C′ and E′ are coronal sections. (A,A′) $Mesp1^{Cre}$ induces recombination in all mesoderm-derived tissues, including vascular endothelia (arrowheads in A′). da, dorsal aorta; pe, pharyngeal endoderm; se, surface ectoderm; spm, splanchnic mesoderm; h, heart; oft, outflow tract. (B,B′) $Foxg1^{Cre}$ induces patchy recombination in pharyngeal ectoderm, endoderm and mesoderm from E8.5. oft, outflow tract. (C,C′) TgFgf15Cre recombination begins in pharyngeal endoderm (pe) at E9. $Hoxa3^{Cre}$ recombination in the pharyngeal region begins at E8.25 in surface ectoderm (D,D′, arrows), and extends into pharyngeal endoderm and mesoderm by E8.5 (E,E′). Scale bars: 100 μm.

Tbx1 expression in non-epithelial tissues does not contribute to early fourth PAA development

Reduced penetrance of fourth PAA hypoplasia in TgFgf15Cre and $Foxg1^{Cre}$ conditional mutants may occur because Tbx1 is required before these Cre alleles are fully activated (after E9), or because *Tbx1* expression in non-epithelial tissues, although insufficient to ensure normal fourth PAA formation, has an additive effect. To begin to address these possibilities, we used the $Hoxa3^{Cre}$ driver, which induces recombination in all pharyngeal tissues caudal to pharyngeal arch II. $Hoxa3^{Cre}$-induced recombination begins in the caudal part of embryos before E8 (not shown). At E8.25, recombination extends into the pharyngeal region, where it is weak and confined to the surface ectoderm (Fig. 4D,D′). By E8.5, recombination extends into the pharyngeal mesoderm and endoderm (Fig. 4E,E′), and from late E9, it includes all of the pharyngeal tissues (Fig. 3F,F′, Fig. 2F). We bred $Hoxa3^{cre/+}$ mutants with $Tbx1^{flox/flox}$ mutants and analyzed the phenotype of conditional ($Hoxa3^{Cre/+}$; $Tbx1^{flox/+}$) mutants by intracardiac ink injection at E10.5. Fifty percent of conditional mutants had fourth PAA hypoplasia (n=17, Fig. 2F, part d). Thus, even though $Hoxa3^{Cre}$ recombines in all pharyngeal tissues, the penetrance of fourth PAA hypoplasia was similar to that obtained with other Cre drivers that recombine in the pharyngeal epithelia ($Foxg1^{Cre}$ and TgFgf15Cre). This suggests that *Tbx1* expression in non-epithelial tissues does not have an additive effect on fourth PAA growth at this stage. Our results also suggest that the requirement for *Tbx1*

expression begins before *Hoxa3*^{Cre} recombination in pharyngeal tissues occurs.

Discussion

Development of the fourth PAAs appears to be particularly susceptible to insult during embryonic life, and a variety of teratogens, blood flow perturbations and gene mutations result in characteristic cardiovascular defects – specifically, interrupted aortic arch type B, and aberrant origin of the right subclavian artery and right aortic arch. In mice, loss of function of several genes causes these defects, including *Pitx2c* (Liu et al., 2002), *Crkl* (Guris et al., 2001), *Foxc1* (Kume et al., 2000; Kume et al., 2001; Winnier et al., 1999), *Foxc2* (Iida et al., 1997; Winnier et al., 1999), *Tgfβ2* (Molin et al., 2002), *Vegf*^{164} (Stalmans et al., 2003) and genes of the endothelin pathway, *Et-1* (Edn1 – Mouse Genome Informatics), *Ece1 and Et*$_A$ (*Ednra* – Mouse Genome Informatics) (Clouthier et al., 1998). *Fgf8* dosage reduction also causes the same defects (Abu-Issa et al., 2002; Frank et al., 2002). However, to our knowledge, *Tbx1* is the only gene identified for which heterozygous loss of function is sufficient to cause these cardiovascular defects (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001).

The recent finding of *TBX1* mutations in patients with a classical clinical presentation but without a 22q11.2 deletion strongly supports the extensive evidence gathered from mouse studies that *Tbx1* is the major gene involved in 22q11DS. In humans, the most common genetic cause of IAA-B is 22q11DS and *Tbx1* mutation causes similar defects. Thus, *Tbx1* gene dosage is critical for fourth PAA development, making the *Tbx1*$^{+/-}$ mouse an excellent model in which to study the molecular basis of aortic arch artery abnormalities. In order to understand the mechanism by which *Tbx1* dosage reduction affects fourth PAA development it is important to determine where Tbx1 is required for this function. It is likely that fourth PAA development requires the interaction of different tissues. We have previously hypothesized that Tbx1 in pharyngeal endoderm may provide molecular instructions for the formation, growth and remodeling of the PAAs. However, *Tbx1* is expressed in many tissues that could contribute to fourth PAA development, including, as we have shown here, pharyngeal ectoderm and precursors of fourth PAA endothelial cells. We therefore considered the Cre-driver strategy to be an effective way to address systematically the tissue requirement for Tbx1 in fourth PAA development. Furthermore, by using multiple Cre drivers with different but partially overlapping patterns of recombination, we have been able to reaffirm results obtained with individual Cre drivers. Thus, we have found fourth PAA defects in embryos where *Tbx1* expression was conditionally reduced via three Cre drivers, all of which are robustly expressed in pharyngeal epithelia (TgFgf15Cre, *Foxg1*^{Cre}, *Hoxa3*^{Cre}), whereas we did not find these abnormalities with Cre drivers that were not expressed in pharyngeal epithelia (*Mesp1*^{Cre}), or where it was only partially expressed (*Nkx2.5*^{Cre}). The most compelling data were obtained with the TgFgf15Cre driver, which expresses exclusively in pharyngeal epithelia and thereby demonstrates conclusively that *Tbx1* is required in this tissue for early fourth PAA development. We thereby also demonstrate for the first time that *Tbx1* function in fourth PAA development is cell non-autonomous. We exclude that Tbx1 in mesoderm contributes to early phases of fourth PAA development because of the early expression of the mesoderm-specific Cre driver *Mesp1*^{Cre}.

The Cre driver strategy used here has also given us an insight into the time requirement for Tbx1 for fourth PAA development. Specifically, we have shown that *Tbx1* is expressed in surface ectoderm and pharyngeal endoderm from E8.25 (7 somites), whereas the earliest time in which a uniform Cre-induced recombination occurred in both pharyngeal epithelia (via the *Hoxa3*^{Cre} driver) was at E9 (12-19 somites). As the fourth PAA defects resulting from *Hoxa3*^{Cre}-induced deletion of *Tbx1* occurred at a reduced penetrance (50%), this suggests that there is a critical requirement for *Tbx1* between E8 and E9. This requirement may continue after E9. Such an early time requirement for *Tbx1* was unexpected, as the fourth PAAs do not develop until 18-24 hours later at E9.75, and it may indicate that Tbx1 is involved in patterning the caudal arches.

The only known transcriptional target of *Tbx1* that could potentially mediate a cell non-autonomous function is *Fgf10* (Xu et al., 2004). We have proposed that *Fgf10* may interact with *Tbx1* in the secondary heart field to regulate the proliferation of myocyte precursors fated to the outflow tract (Xu et al., 2004). However, a similar interaction cannot be invoked for the regulation of fourth PAA development because *Fgf10* is not expressed in pharyngeal endoderm or ectoderm at the relevant developmental stage. Three other genes have been proposed to interact with *Tbx1* – *Vegf*, chordin (*Chrd*) and *Fgf8* – all of which are co-expressed with *Tbx1* in pharyngeal endoderm.

Vegf has been proposed to be an upstream regulator of *Tbx1* in PAA development (Stalmans et al., 2003). However, at E10.5, *Vegf* mutants were reported to have an enlargement of the right dorsal aorta and a local narrowing of an otherwise apparently well-grown fourth and sixth PAA. This phenotype is quite different to that seen in *Tbx1*$^{+/-}$ mutants, which is characterized by overall hypoplasia of the fourth PAAs, which is often severe, but does not extend to the sixth PAAs. Therefore, *Vegf* is likely to exert its effect on PAA development via a different mechanism.

Chrd-null mice have a phenotype that is strikingly similar to that of *Tbx1*$^{-/-}$ mutants, including similar fourth PAA-derived cardiovascular defects and cardiac outflow tract defects (Bachiller et al., 2003). *Tbx1* expression was reported to be reduced in *Chrd*$^{-/-}$ embryos at E9, suggesting that the gene may lie upstream of *Tbx1* in the regulation of pharyngeal development. However, neither the development of the fourth PAA in *Chrd*$^{-/-}$ embryos at mid-gestation nor the phenotype of *Chrd*$^{+/-}$ mice have been reported, so we do not know by what mechanism mutation of *Chrd* affects fourth PAA development.

Fgf8 is strongly expressed in the pharyngeal epithelia of mid-gestation embryos (Fig. 1C), and we have demonstrated that the two genes interact genetically in fourth PAA development (Vitelli et al., 2002b). Recently, a *Tbx1*-responsive enhancer has been identified in the 5′ region of the *Fgf8* gene (Hu et al., 2004), but *Fgf8* has not yet been demonstrated to be a direct transcriptional target of *Tbx1*. Currently, *Fgf8* is the only gene other than *Tbx1* for which a tissue-specific requirement in fourth PAA development has been demonstrated (Macatee et al., 2003), intriguingly in pharyngeal ectoderm. Therefore, our finding that *Tbx1* is also

expressed in pharyngeal ectoderm, albeit transiently, raises the question as to whether the two genes may interact in pharyngeal ectoderm to regulate fourth PAA development. However, several lines of evidence from this study and from the study of Macatee et al. suggest that *Tbx1* and *Fgf8* operate through different mechanisms in ectoderm. In the study of Macatee et al., conditional mutagenesis was used to ablate *Fgf8* specifically in pharyngeal ectoderm, which resulted in a range of fourth PAA-derived cardiovascular defects in conditional mutants (Macatee et al., 2003). However, ectodermal expression of *Fgf8* is robust in $Tbx1^{-/-}$ mutants, therefore it is unlikely that Tbx1 regulates *Fgf8* expression in the ectoderm. In addition, extensive apoptosis of neural crest cells was observed in arch IV of *Fgf8* conditional mutants, which could account for the fourth PAA growth failure, as suggested by the authors, but we have not detected increased apoptosis in arch IV of $Tbx1^{+/-}$ mutants (Vitelli et al., 2002b). We do not yet know the effect of endoderm-specific deletion of *Fgf8* on fourth PAA development, thus it is still possible that *Fgf8* and *Tbx1* interact in the endoderm and affect fourth PAA development through a mechanism other than cell death.

The $Tbx1^{-/-}$ phenotype demonstrates that the gene is required for formation of the PAAs, thus it is reasonable to think that fourth PAA hypoplasia in $Tbx^{+/-}$ embryos is a milder consequence of a dosage-sensitive role of *Tbx1* during the formation of the arteries. There is a precedent for endodermal induction of vessel formation from classical embryology and anatomical studies, which showed that the earliest intraembryonic vessels arise close to endoderm. More recent studies, performed with the aid of endothelial-specific molecular markers, have confirmed the overall requirement of endoderm for early vasculogenesis, and several endodermally expressed genes have been shown to be involved in this process in various species, specifically, *Fgf2* (Riese et al., 1995), $Vegf_{122}$ (Cleaver and Krieg, 1998), *Gdf6* (Hall et al., 2002), *Ihh* (Byrd et al., 2002; Dyer et al., 2001), *Shh* (Vokes et al., 2004) and *Hhex* (Hallaq et al., 2004). However, it is less clear whether endodermal induction of vessel formation continues into later stages of mouse embryogenesis.

We propose a model whereby Tbx1 regulates fourth PAA formation by activating signals from the pharyngeal endoderm, via Fgf8 and/or other extracellular signaling systems. These signals are directed either towards the mesenchyme surrounding the nascent vessels, or towards the dorsal aortae, from which the PAAs may sprout and which at the time of fourth PAA formation are in close contact with pharyngeal endoderm (Fig. 1A′).

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