Tbx1 is regulated by tissue-specific forkhead proteins through a common Sonic hedgehog-responsive enhancer

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Haploinsufficiency of Tbx1 is likely a major determinant of cardiac and craniofacial birth defects associated with DiGeorge syndrome. Although mice deficient in Tbx1 exhibit pharyngeal and aortic arch defects, the developmental program and mechanisms through which Tbx1 functions are relatively unknown. We identified a single *cis*-element upstream of *Tbx1* that recognized winged helix/forkhead box (Fox)-containing transcription factors and was essential for regulation of *Tbx1* transcription in the pharyngeal endoderm and head mesenchyme. The *Tbx1* regulatory region was responsive to signaling by Sonic hedgehog (Shh) in vivo. We show that Shh is necessary for aortic arch development, similar to Tbx1, and is also required for expression of *Foxa2* and *Foxc2* in the pharyngeal endoderm and head mesenchyme, respectively. Foxa2, Foxc1, or Foxc2 could bind and activate transcription through the critical *cis*-element upstream of *Tbx1*, and Foxc proteins were required, within their expression domains, for *Tbx1* transcription in vivo. We propose that *Tbx1* is a direct transcriptional target of Fox proteins and that Fox proteins may serve an intermediary role in Shh regulation of *Tbx1*.

[*Keywords*: Tbx1; Shh; Fox; DiGeorge syndrome]

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The 22q11.2 deletion syndrome (22q11DS) is the most frequent chromosomal microdeletion syndrome in humans and is typically characterized by a 3-Mb heterozygous deletion, congenital cardiac outflow tract, aortic arch, and craniofacial defects (Scambler 2000; Lindsay 2001; Yamagishi 2002). The embryologic basis for this disease, also known as DiGeorge syndrome, resides in tissues derived from, or cells traversing through, the pharyngeal arches. The pharyngeal arches are bilaterally symmetric structures that arise in a segmental fashion along the anterior–posterior (AP) axis. They are precisely patterned by clustered homeobox (Hox) genes that establish unique identity to each arch based on its AP positioning (for review, see Schilling 1997). Numerous epithelial–mesenchymal interactions are necessary for appropriate segmentation and outgrowth of the pharyngeal arch in a manner similar to the developing limb bud, where Sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Wnts regulate patterning along all three embryonic axes (for review, see Martin 2001).

Each pharyngeal arch consists of an aortic arch artery and a mesodermally derived core, covered externally and internally by ectoderm and endoderm-derived epithelium, respectively (Sadler 2000). The bilaterally symmetric aortic arch arteries undergo extensive remodeling to ultimately form the mature aortic arch and pulmonary artery, whereas the mesodermal core gives rise to facial and neck muscles and the cardiac outflow tract. The endoderm-derived epithelial layer is essential for segmentation of the pharyngeal arches and contributes to craniofacial organs, including the thymus and parathyroid glands. Neural crest cells migrate from the caudal hindbrain to each pharyngeal arch, surrounding the aortic arch arteries and central core of mesodermal cells, with some cells proceeding to populate the cardiac outflow tract (conotruncus). The neural-crest-derived mesenchyme contributes to skeletal structures of the face and is important in patterning the aortic arch and septation of the conotruncus (Kirby and Waldo 1995). Reciprocal

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interactions between the various cell types are essential for proper development of the pharyngeal arch derivatives.

Recent evidence in mice indicates that haploinsufficiency of Tbx1, a T-box transcription factor that is expressed in the developing pharyngeal arches (Chapman et al. 1996; Garg et al. 2001), may be a major contributor to aortic arch defects in the 22q11 deletion phenotype. Mice harboring a hemizygous deletion of part of the mouse equivalent of 22q11.2 have some features of 22q11DS, including aortic arch defects and occasional conotruncal and thymic defects (Lindsay et al. 1999; Merscher et al. 2001). A subset of mice heterozygous for *Tbx1* display defects in aortic arch patterning that are reminiscent of defects seen in 22q11DS (Jerome and Papaionnou 2001; Lindsay et al. 2001; Merscher et al. 2001). Furthermore, aortic arch defects observed in mouse chromosomal deletions syntenic to 22q11 can be rescued by a transgene encompassing *Tbx1* (Lindsay et al. 2001; Merscher et al. 2001). Mice homozygous-null for *Tbx1* display additional features of 22q11DS, including pharyngeal arch segmentation defects, strengthening a role for Tbx1 in this syndrome (Jerome and Papaionnou 2001; Vitelli et al. 2002a).

Tbx1 belongs to a large family of transcription factors that shares an evolutionarily conserved T-box first described in the *brachyury* (T) protein that is necessary for mesoderm formation (Bollag et al. 1994). The T-box is necessary for DNA-binding and can mediate combinatorial interactions with other transcriptional regulators (for review, see Papaionnou and Silver 1998; Smith 1999). *Tbx1* is expressed in discrete domains, including the pharyngeal endoderm and the pharyngeal mesoderm, and broadly in the head mesenchyme (Chapman et al. 1996; Garg et al. 2001). It is notable that *Tbx1* is excluded from the neural-crest-derived mesenchyme of the pharyngeal arches, although signaling from the epithelial and mesodermal layers can affect differentiation of the adjacent neural crest cells. Recent evidence suggests that Tbx1 may regulate fibroblast growth factor 8 (Fgf8) in the endoderm, which may signal to adjacent neural crest cells and regulate aortic arch development (Abu-Issa et al. 2002; Frank et al. 2002; Vitelli et al. 2002b).

We previously suggested that *Tbx1* responds to signals initiated by the secreted morphogen Shh. *Shh* mutant mice die soon after birth with severe craniofacial defects (Chiang et al. 1996; Helms et al. 1997) and fail to maintain *Tbx1* expression (Garg et al. 2001). Consistent with a role for Shh in regulation of *Tbx1*, chick embryos exposed to Shh-soaked beads responded by up-regulating *Tbx1* expression in the pharyngeal arches (Garg et al. 2001). However, the mechanism through which this phenomenon occurred was unknown. Here, we describe a genomic regulatory region that controls the in vivo expression of *Tbx1* and is responsive to Shh signaling. We demonstrate that a single *cis*-element that recognizes winged helix/forkhead box (Fox)-containing transcription factors (Kaufmann and Knochel 1996; Kaestner et al. 2000) is essential for regulation of *Tbx1* transcription in the pharyngeal endoderm and head mesenchyme. Foxa2, required for endoderm development (Ang and Rossant 1994; Weinstein et al. 1994), and Foxc1 or Foxc2, required for head mesenchyme and aortic arch formation (Iida et al. 1997; Winnier et al. 1997, 1999; Kume et al. 1998, 2001), bind and activate Tbx1 transcription in their respective subdomains through this regulatory element. We show that Shh is necessary for aortic arch development, similar to Tbx1, and is also required for expression of *Foxa2* and *Foxc2* in the pharyngeal endoderm and head mesenchyme, respectively. These studies reveal a cascade of events that culminates in transcription of *Tbx1* and establish, to our knowledge, the first known direct target of Foxc proteins during development.

Results

Separable enhancers direct tissue-specific expression of Tbx1

To define the developmental program in which Tbx1 functions, we searched for the *cis*-regulatory elements that control *Tbx1* expression during development. We isolated a mouse bacterial artificial clone (BAC) containing genomic sequence 5′ of *Tbx1* and subcloned a fragment of DNA extending from the 5′-untranslated region (UTR) to –14.3 kb (12.8 kb in length) upstream of a *lacZ* reporter gene (Fig. 1a, construct 1). Mouse embryos harboring this transgene showed X-gal staining in the mesodermal core and endodermal epithelium of pharyngeal arches, as well as in the head mesenchyme surrounding the dorsal aortas (Fig. 1d,e). This pattern accurately recapitulated the expression of endogenous *Tbx1* transcripts (Fig. 1b,c).

To localize the regulatory elements responsible for embryonic expression of *Tbx1*, we constructed a series of deletions of the 12.8-kb genomic fragment and analyzed their ability to direct *lacZ* expression from a heterologous promoter (*hsp68*) in F_0 transgenic mice at embryonic day 9.5 (E9.5). Comparison of sequence upstream of mouse and human *TBX1* within a 6.4-kb fragment (construct 3) that was sufficient for appropriate expression (Fig. 1f,g) revealed sequence conservation in four regions spanning 1.5 kb (–12.8 to –14.3 kb). This 1.5-kb fragment (construct 4) directed *lacZ* expression in pharyngeal arch endoderm and head mesenchyme, but not in pharyngeal mesoderm (Fig. 1h,i). Further deletions revealed that the critical *cis*-element(s) necessary for *Tbx1* expression in the pharyngeal endoderm and head mesenchyme lay between –14.3 and –13.2 kb (Fig. 1j,k).

Enhancer-dependent temporospatial expression of Tbx1

The temporospatial expression pattern of *Tbx1* during early embryogenesis was determined using stable transgenic mouse lines bearing *lacZ* under control of the 12.8-kb (construct 1) or 1.1-kb (construct 6) upstream fragments described above. At E7.5, the 12.8-kb transgene directed *lacZ* expression in the paraxial mesodermal

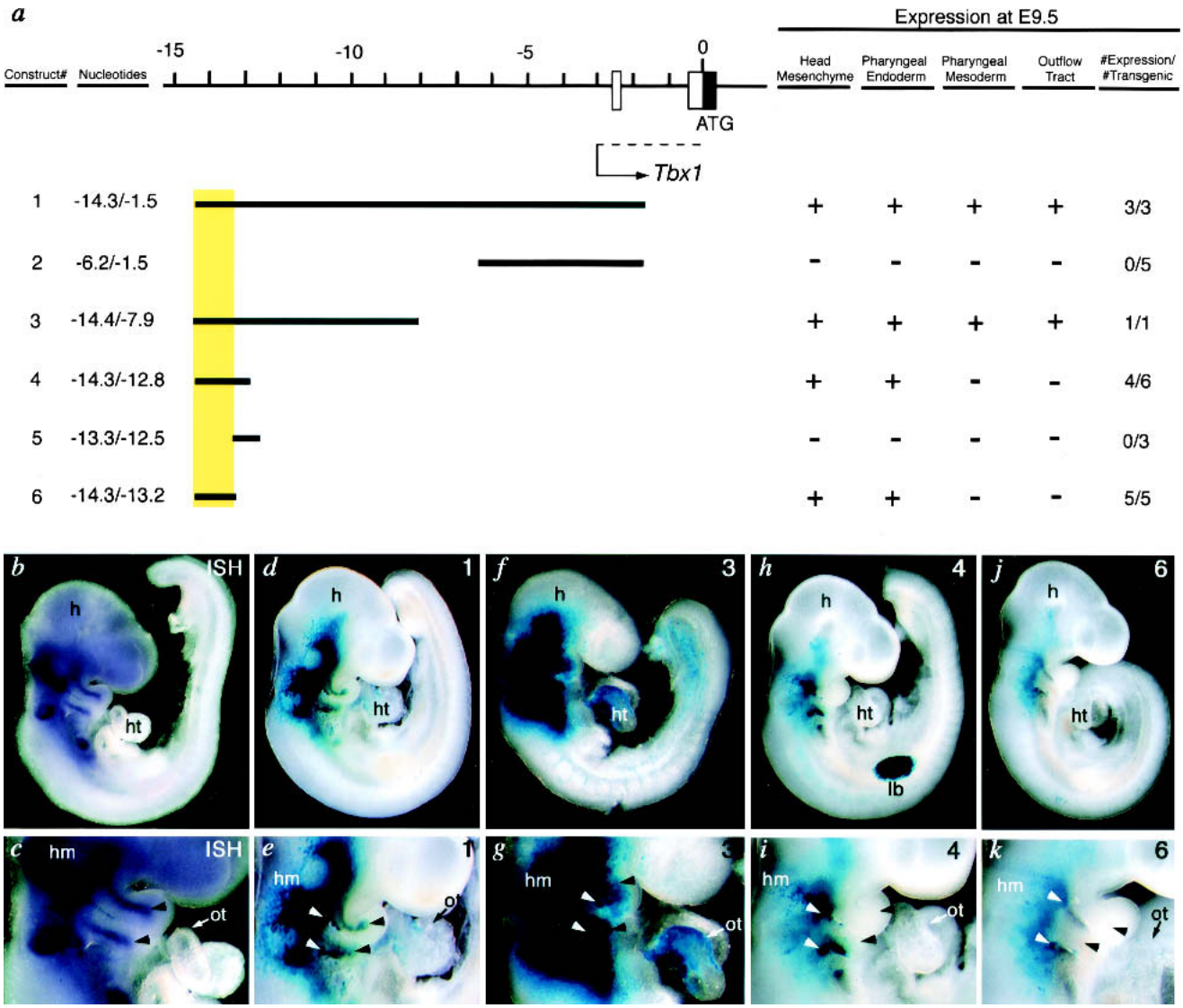

Figure 1. *Tbx1* expression is controlled by separable pharyngeal endoderm and mesoderm regulatory regions in transgenic mice. (*a*) Genomic organization of the 5′ mouse *Tbx1* locus and flanking region. Boxes indicate exons, and translation start site (ATG) is designated as nucleotide number zero. Construct number is indicated on the *left*, and the corresponding expression pattern of *lacZ* at E9.5 is summarized on the *right*. The far-right column indicates the fraction of F_0 transgenic embryos with *Tbx1*-like *lacZ* expression/ *lacZ* gene positive embryos. (*b*,*c*) Endogenous expression of *Tbx1* transcripts is shown by whole-mount RNA in situ hybridization in E9.5 mouse embryos. (*d*–*k*) Right lateral views of representative embryos obtained with each construct (indicated in *upper-right* corner of each panel). (*c*,*e*,*g*,*i*,*k*) Higher magnification of the pharyngeal arch of embryos in *upper* panels. Expression of *lacZ* in embryos with construct 1 (*d*,*e*) recapitulated endogenous *Tbx1* expression (*b*,*c*). *LacZ* expression in the head mesenchyme (hm) and pharyngeal endoderm (white arrowheads) was detectable in each embryo with construct 1 (*e*), 3 (*g*), 4 (*i*), and 6 (*k*), whereas that in the pharyngeal mesoderm (black arrowheads) and cardiac outflow tract (ot) was only in embryos with construct 1 (*e*) or 3 (*g*). *lacZ* expression in limb buds (lb) in panel *h* represents ectopic expression. h, head; ht, heart.

cells that give rise to cranial mesenchyme, but not in the lateral plate mesoderm or cardiac crescent that forms the heart tube (Fig. 2a,f). Slightly later (E8.5), *lacZ* expression was observed in a segment of the cranial mesoderm including the first pharyngeal arch mesoderm (Fig. 2b,g). At E9.5, the transgene directed high levels of *lacZ* expression in the mesoderm-derived head mesenchyme and the mesodermal core of the pharyngeal arches (Fig. 2c,h–j). *LacZ* expression in pharyngeal endoderm became detectable at this stage and was pronounced in the pharyngeal pouches. Expression was not detectable in pharyngeal arch mesenchyme derived from neural crest cells, consistent with the endogenous *Tbx1* expression previously shown by RNA in situ hybridization. At E10.5, *lacZ* expression was maintained in the head mesenchyme, pharyngeal mesoderm, and endoderm, and began to extend to the precursors of the vertebral column (Fig. 2d). Although expression of *Tbx1* in the developing

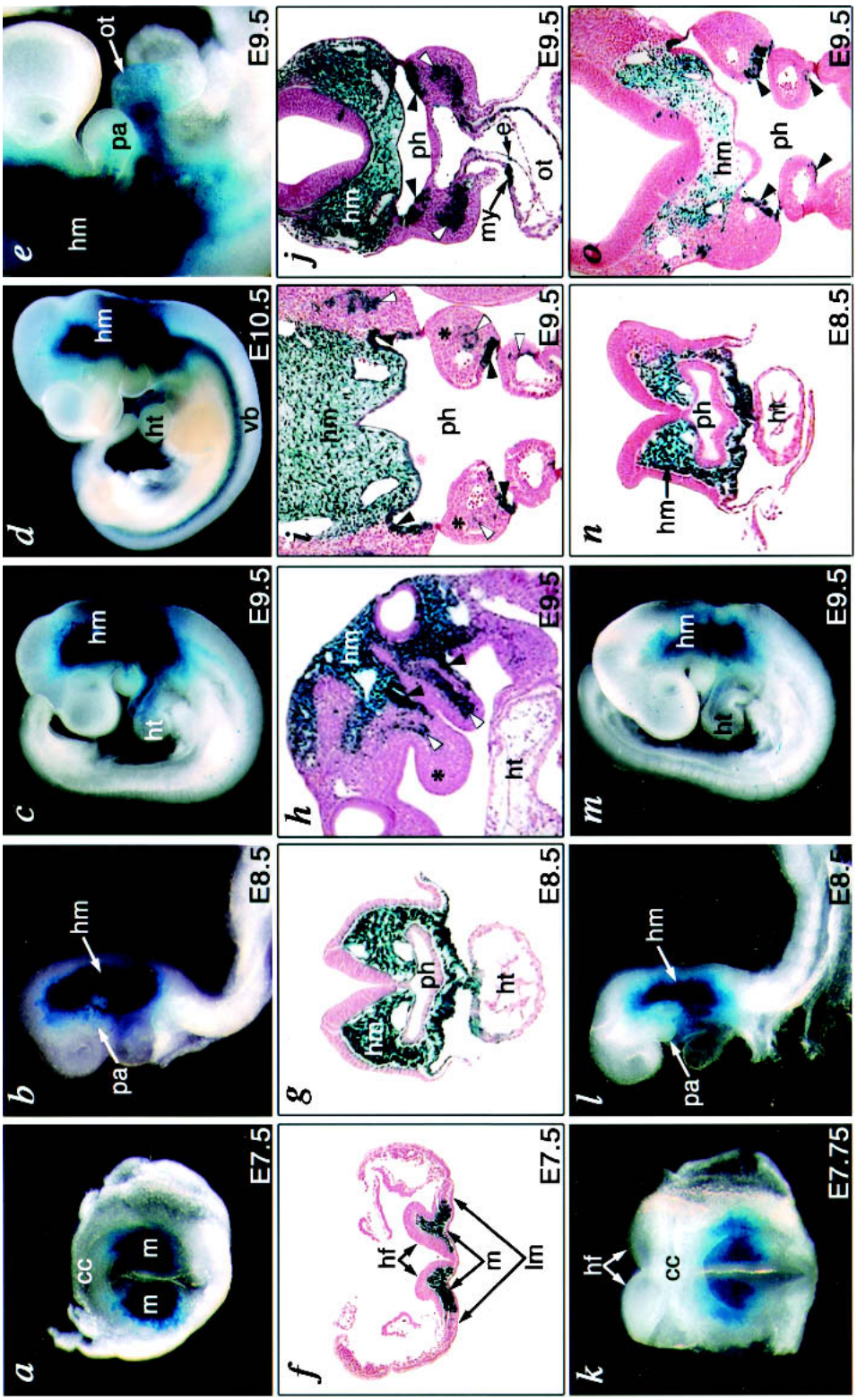

Figure 2. Developmental regulation of *Tbx1* expression by tissue-specific enhancers. Embryos from a stable transgenic line harboring the 12.8-kb fragment (construct 1) were analyzed at various times during mouse embryogenesis. (*a–d*) Whole-mount photographs of embryos from E7.5 to E10.5. (*e*) Right lateral view of embryo focusing on the pharyngeal arch and heart at E9.5. (*f–i*) Transverse (*f,g,i*), sagittal (*h*), and frontal (*i*) sections counterstained by Nuclear Fast Red from E7.5 to E9.5. (*a,f*) *lacZ* was expressed in mesoderm cells (m) that give rise to cranial mesenchyme, but not in the cardiac crescent (cc) or lateral plate mesoderm (lm) at E7.5. (*b,g*) Expression of *lacZ* was detectable in the head mesenchyme (hm) and pharyngeal arch (pa) at E8.5. (*c,h,i,j*) *lacZ* expression was detectable in head mesenchyme, pharyngeal arch mesoderm (white arrowheads), and endoderm (black arrowheads), but not in neural-crest-derived mesenchyme (asterisks) at E9.5. (*d*) Expression of *lacZ* extended to the primordia of vertebral bodies (vb). (*e,i*) *lacZ* was expressed in both myocardial (my) and endocardial (e) layers of the cardiac outflow tract (ot) at E9.5. (*k–m*) Whole-mount photographs of embryos from a stable transgenic line harboring the 1.1-kb fragment (construct 6) at E7.75 to E9.5. (*n,o*) Transverse (*n*) or frontal (*o*) sections of *l* or *m*, respectively, counterstained by Nuclear Fast Red. (*k*) *lacZ* expression in mesoderm cells was observed similar to panel *a* at E7.75. (*l,n*) *lacZ* was detectable in head mesoderm as in *b* and *g*, but excluded from the pharyngeal arch at E8.5. (*m,o*) Expression of *lacZ* in head mesenchyme and pharyngeal endoderm was detectable similar to *c*, *i*, and *j*, but expression in pharyngeal mesoderm was absent. hf, head fold; ht, heart; ph, pharynx.

heart had not been detectable by mRNA expression analyses, a recent study using mice harboring the *lacZ* gene targeted into the *Tbx1* locus demonstrated *Tbx1* expression in the cardiac outflow tract between E9.5 and E12.5 (Vitelli et al. 2002a,b). *LacZ* expression under control of the 12.8-kb fragment was detectable in the myocardial and endocardial layer of the outflow tract between E8.5 and E10.5 (Fig. 2e,j), indicating that *Tbx1* may be expressed in the recently identified cells of the anterior heart tube that are derived from pharyngeal mesoderm (Kelly et al. 2001; Mjaatvedt et al. 2001; Waldo et al. 2001).

A stable transgenic mouse line harboring the 1.1-kb enhancer (construct 6) showed *lacZ* expression in the head mesenchyme from E8.0 through E10.5, although it was relatively weaker than that of transgenic mice with the 12.8-kb fragment (Fig. 2k–o). Differences in expression level could be secondary to positional effects of transgene insertion or other elements that further enhance expression. The 1.1-kb enhancer also directed *lacZ* expression in the pharyngeal arch endoderm from E9.5, similar to the 12.8-kb fragment, but excluded pharyngeal arch mesoderm and cardiac outflow tract expression (Fig. 2l,m,o), indicating that separable enhancers direct distinct domains of *Tbx1* expression.

Tbx1 *enhancer is responsive to Shh signaling*

We have previously reported regulation of *Tbx1* by Shh in mouse and chick embryos (Garg et al. 2001). *Shh*-null mice die soon after birth and have numerous craniofacial defects (Chiang et al. 1996; Helms et al. 1997) that are distinct but overlapping with defects in *Tbx1* mutants. To determine whether the identified enhancer of *Tbx1* was dependent on Shh signaling, we examined activity of the *Tbx1–lacZ* transgene in the *Shh*-null background. At E9.25, *lacZ* expression was relatively normal in the pharyngeal arches and head mesenchyme of *Shh*-null mutant embryos compared with wild-type embryos (Fig. 3a,b). By E9.5, *lacZ* expression was detectable but down-regulated in pharyngeal arches and the head mesenchyme of *Shh* mutant embryos (Fig. 3c–f). Only 1 d later (E10.5), *lacZ* expression was nearly undetectable in *Shh* mutant embryos, consistent with our previous results from *Tbx1* mRNA in situ hybridization (Garg et al. 2001). These results suggest that the *Tbx1* enhancer responds to Shh signaling and promotes maintenance, rather than induction, of *Tbx1* expression in a temporo–spatial fashion.

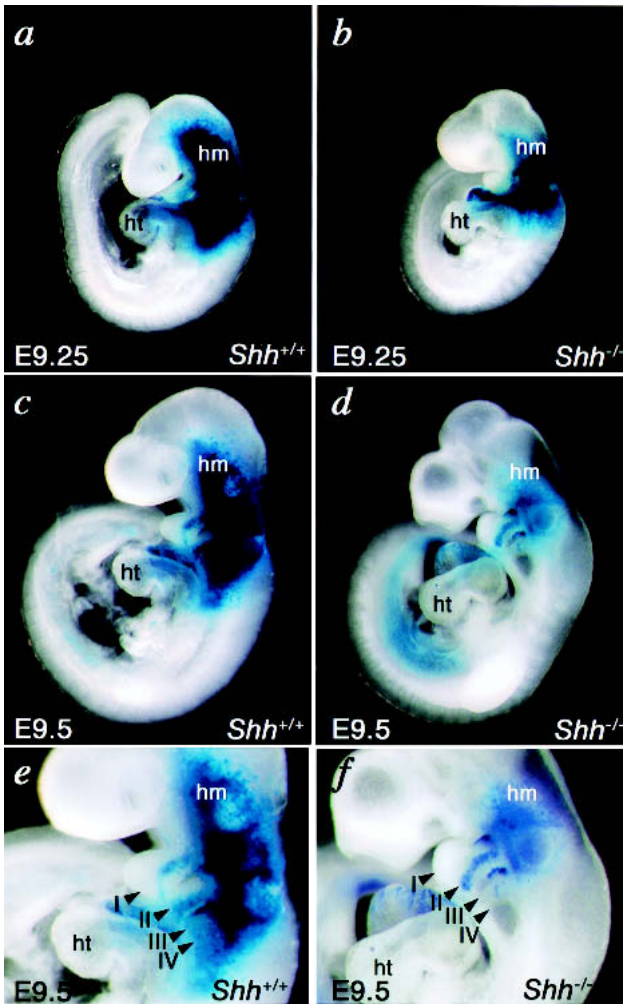

Figure 3. The *Tbx1* enhancer is dependent on Shh signaling in a temporo–spatial fashion. (*a*,*b*) X-gal staining of wild-type (*Shh*$^{+/+}$) and *Shh* mutant (*Shh*$^{-/-}$) embryos harboring the 12.8-kb *Tbx1–lacZ* transgene at E9.25 showed relatively normal *lacZ* expression in the pharyngeal arches and head mesenchyme (hm) of *Shh* mutant embryos compared with wild-type embryos. (*c*,*d*) *LacZ* expression in wild-type and *Shh* mutant embryos at E9.5. (*e*,*f*) Higher magnification photographs of *c* and *d* focusing on pharyngeal arches show down-regulation of *lacZ* expression in the head mesenchyme and first (I), third (III), and fourth (IV) pharyngeal arches of *Shh* mutant embryos compared with wild-type embryos. *lacZ* expression in the second pharyngeal arch (II) was also down-regulated, but expression was still detected in the mesoderm of the second arch of *Shh* mutant embryos. ht, heart.

A forkhead-transcription-factor-binding element is necessary for Tbx1 *transcription in pharyngeal endoderm and head mesenchyme*

We inspected the 1.1-kb enhancer for binding sites of transcription factors using the MatInspector V.2.2 based on TRANSFAC 4.0 (Quandt et al. 1995). We did not identify a consensus binding site for Gli proteins that might mediate transcriptional activities directly downstream of Shh signaling (Sasaki et al. 1997), suggesting that the regulation of the *Tbx1* enhancer by Shh may be through an intermediate step. Alignment of the 1.1-kb mouse *Tbx1* enhancer with the corresponding human *TBX1* upstream sequence revealed conservation of several potential transcription-factor-binding sites. In particular, a consensus binding site for the winged helix/forkhead transcription factor family (GCCTGTTTGTTTT) was completely conserved across species (Fig. 4a). Members of the winged helix/forkhead class of transcription fac-

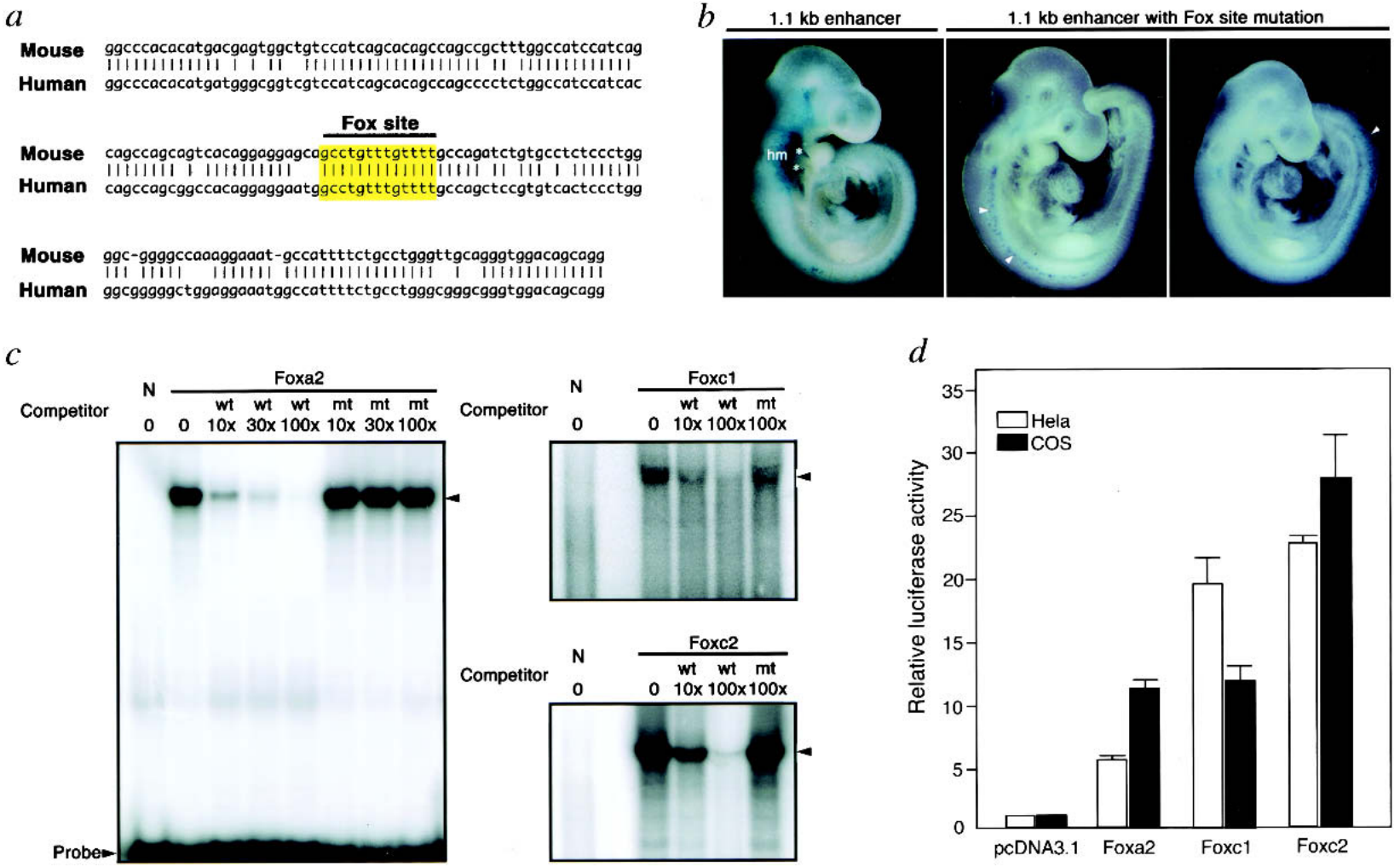

Figure 4. A consensus Fox-binding site is essential for regulation of *Tbx1*. (*a*) One-hundred percent cross-species conservation of the consensus Fox-binding site at −13,435/−13,423 bp in the 1.1-kb enhancer of *Tbx1* is indicated. (*b*) Mutation of Fox site abolished head mesenchyme (hm) and pharyngeal endoderm (asterisks) enhancer activity in 6/6 *lacZ*-positive embryos. Ectopic *lacZ* staining (arrowheads) was observed in some embryos. (*c*) Electrophoretic gel mobility shift assays (EMSA) revealed DNA–protein complexes of in vitro translated Foxa2, Foxc1, or Foxc2 with the Fox *cis*-element in the *Tbx1* enhancer. The interaction was competed specifically by excess unlabeled wild-type (wt) oligonucleotide, but not by a mutant (mt) oligonucleotide. Fold-excess of competitor DNA compared with labeled probe is shown. No DNA–protein complex (in lane *N*) was observed with lysate alone. (*d*) Luciferase expression under control of the *Tbx1* genomic DNA fragment shown in panel *a* after cotransfection of luciferase reporter and Foxa2, Foxc1, or Foxc2 expression construct or empty vector (pcDNA3.1). Results are presented as relative luciferase activity, corrected for transfection efficiency. The control (pcDNA3.1) transfection value is set at 1.

tors, also known as Fox proteins (Kaestner et al. 2000), serve as critical regulators during embryogenesis, tumorigenesis, and in maintenance of differentiation (Kaufmann and Knochel 1996). To determine if the Fox-binding site was required for *Tbx1* expression in vivo, we mutated this site in the context of the 1.1-kb enhancer and generated transgenic embryos. *LacZ* expression in pharyngeal endoderm and head mesenchyme was abolished in 6/6 embryos harboring the mutant transgene, although ectopic expression of *lacZ* was observed in some embryos (Fig. 4b). This result identified *Tbx1* as a direct transcriptional target of Fox transcription factors during development in vivo.

Tissue-specific forkhead proteins bind and activate Tbx1 *transcription through a common* cis*-acting element*

Of the numerous Fox factors, Foxc1 and Foxc2 (formerly Mf1 and Mfh1, respectively) are closely related Fox proteins with virtually identical DNA-binding domains that are expressed in the mesoderm-derived head mesenchyme and play redundant roles in cardiovascular development (Iida et al. 1997; Winnier et al. 1997, 1999; Kume et al. 1998, 2001). Embryos lacking either *Foxc1* or *Foxc2*, and most compound heterozygotes, die perinatally from aortic arch defects, including interruption of the aortic arch (IAA; Winnier et al. 1999; Kume et al. 2001), reminiscent of mice heterozygous for a *Tbx1* mutation (Jerome and Papaionnou 2001; Lindsay et al. 2001; Merscher et al. 2001). In contrast, Foxa proteins, also known as hepatocyte nuclear factors (HNF)-3, are expressed in the pharyngeal endoderm (Kaufmann and Knochel 1996), and one member, Foxa2 (HNF-3β), is essential for endoderm development (Ang and Rossant 1994; Weinstein et al. 1994). Based on these data, we hypothesized that Foxa2 might regulate expression of *Tbx1* in the pharyngeal endoderm, whereas Foxc1 and Foxc2 may regulate expression of *Tbx1* in the head mesenchyme through a common *cis*-regulatory element.

To determine if the conserved Fox-binding site was capable of binding Foxa2, we performed electrophoretic mobility shift assays (EMSA) with ^{32}P-labeled oligonucleotides containing the *cis*-element. Foxa2 protein specifically shifted this element, and DNA binding was effectively competed by an excess of unlabeled cognate competitor but not by a mutated oligonucleotide, suggesting that Foxa2 specifically interacts with the Fox consensus site in the *Tbx1* enhancer (Fig. 4c). Similar studies using Foxc1 or Foxc2 protein also demonstrated interaction with this site with varying affinities (Fig. 4c).

To test whether Fox proteins could not only bind, but also activate transcription through this site, we transiently transfected HeLa cells with a luciferase reporter cloned downstream of the Fox-binding element (FBE) in the 1.1-kb *Tbx1* enhancer. Transfection with expression vectors of Foxa2, Foxc1, or Foxc2 activated this reporter by ~5-, ~20-, or ~25-fold, respectively (Fig. 4d). Similar results were obtained in COS-7 cells (Fig. 4d), suggesting that Foxa2, Foxc1, and Foxc2 can bind and activate transcription through the consensus Fox *cis*-element upstream of *Tbx1* in vitro.

Foxc1 and Foxc2 are required for normal Tbx1 *expression in vivo*

Unlike *Foxa2* mutants, *Foxc1*-null or *Foxc2*-null embryos survive until E18.5 (Iida et al. 1997; Winnier et al. 1997; Kume et al. 1998), allowing us to test whether these factors are involved in regulation of *Tbx1* in vivo. The expression of *Tbx1* was partially down-regulated in either *Foxc1*-null or *Foxc2*-null embryos at E9.5–E10.5 (Fig. 5a–d), suggesting that Foxc1 and Foxc2 may regulate *Tbx1* in a dose-dependent and redundant fashion. Comparison of the domains of *Foxc1* expression, represented by *lacZ*-positive cells (red), and *Tbx1* expression (white) in wild-type or *Foxc1* mutants revealed that *Tbx1* expression in *Foxc1* mutants was only affected in areas in which *Foxc1* and *Tbx1* were coexpressed, consistent with Foxc regulation of *Tbx1* (Fig. 5e–g). It is worth noting that cell numbers in *Foxc* mutants are comparable to wild type as no defect in cell death or proliferation has been found (Winnier et al. 1999; T. Kume, unpubl.). Mice homozygous-null for both *Foxc1* and *Foxc2* die at E9.5–E10.5 from abnormal development of the vasculature and pharyngeal arches (Kume et al. 2001), precluding formal examination of *Tbx1* expression in *Foxc1*/*Foxc2* double homozygous mutants.

Foxa2 is dependent on Shh signaling in the pharyngeal endoderm

Previous studies demonstrated that the expression of *Foxa2* was down-regulated in the neural tube floor plate of *Shh*-null mice from E8.5, suggesting that Shh signaling may be required for maintenance, but not induction, of *Foxa2* expression (Chiang et al. 1996; Kaufmann and Knochel 1996; Sasaki et al. 1997). Identification of a Gli-dependent floor plate enhancer upstream of *Foxa2* further established *Foxa2* as a direct transcriptional target of Shh signaling (Sasaki et al. 1997). We specifically examined expression of *Foxa2* in *Shh*-null pharyngeal endoderm and found that *Foxa2* was dependent on Shh signaling in this domain as well as in the neural tube floor plate (Fig. 6a,b). The early demise of *Foxa2* mutants prevented formal examination of *Tbx1* expression in pharyngeal arches of *Foxa2* mutants. The down-regulation of *Foxa2* in *Shh* mutant endoderm is consistent with Foxa2 involvement in the maintenance of endodermal *Tbx1* expression by Shh.

Shh is required for Foxc2 *expression and aortic arch development*

Based on our observation that Foxc proteins directly regulate the Shh-responsive *Tbx1* enhancer, we asked whether Foxc1 or Foxc2 might serve as a transcriptional link between Shh signaling and *Tbx1* expression. To test this idea, we examined whether *Foxc1* or *Foxc2* expres-

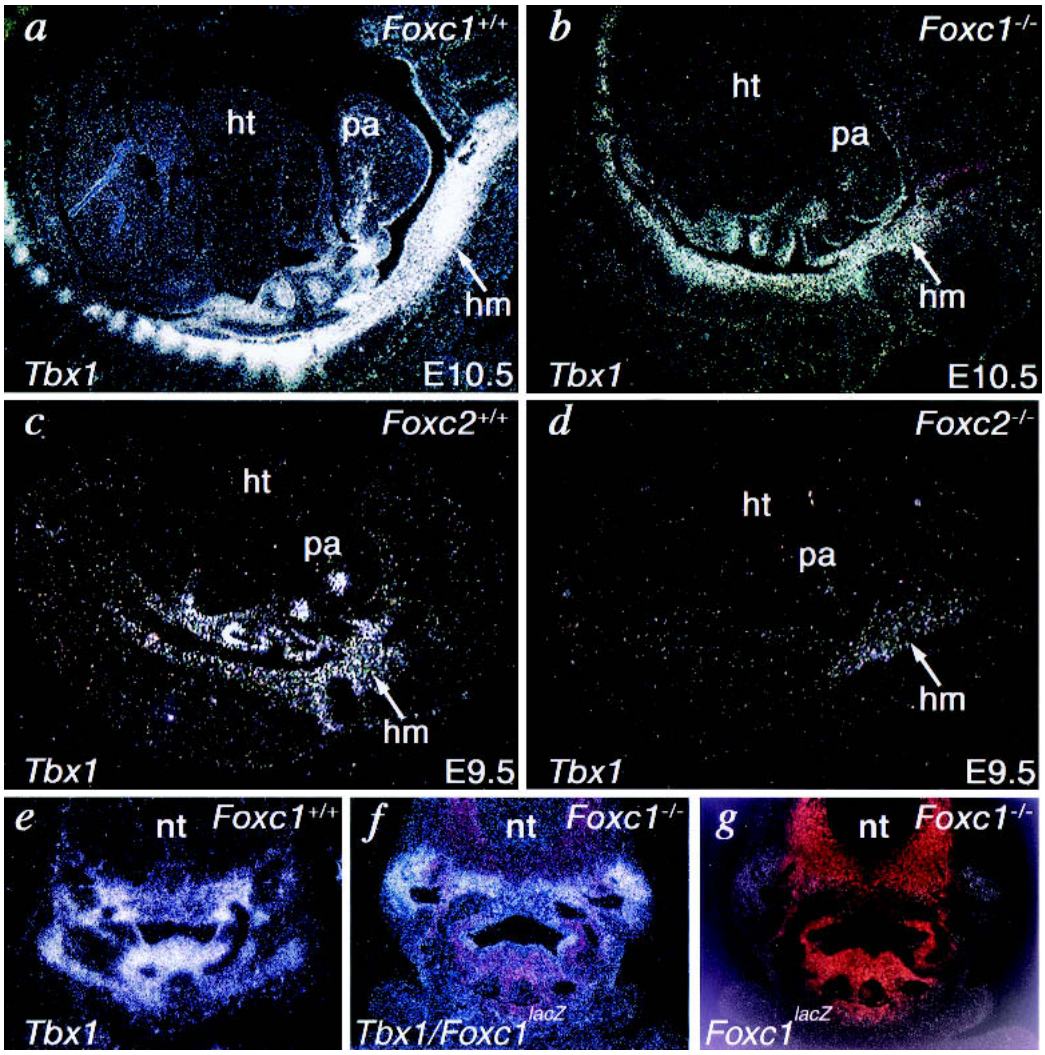

Figure 5. Foxc proteins regulate *Tbx1* expression in vivo. (*a–d*) Radioactive in situ hybridization on sagittal sections of E9.5–E10.5 mouse embryos showed that *Tbx1* was expressed, but partially down-regulated in the head mesenchyme (hm) of *Foxc1* or *Foxc2* mutant (*Foxc1*$^{-/-}$ or *Foxc2*$^{-/-}$) embryos compared with wild-type (*Foxc1*$^{+/+}$ or *Foxc2*$^{+/+}$) embryos. (*e–g*) Transverse section of wild-type or *Foxc1* mutants at the pharyngeal arch level. *Tbx1* expression (white) in *Foxc1*-null embryo was down-regulated (*f*) compared with wild type (*e*) in the subdomain corresponding to *Foxc1* expression, as marked by *lacZ* expression under control of the endogenous *Foxc1* promoter (red in *g*). *Tbx1* expression and *lacZ* expression are overlaid in *f*. ht, heart; pa, pharyngeal arch; nt, neural tube.

sion was dependent on Shh signaling. *Foxc2* expression was undetectable, whereas *Foxc1* expression was detectable, although somewhat diminished, in the pharyngeal arches of *Shh* mutants at E9.5 (Fig. 6c–f). Because fewer cells are present in the *Shh* mutant pharyngeal arch, the apparent *Foxc1* down-regulation may be secondary to alterations in cell number; however, the complete absence of *Foxc2* expression suggests a requirement of Shh signaling for *Foxc2* activation.

Because our data supported a model in which Fox proteins mediate a Shh signaling cascade resulting in maintenance of *Tbx1* expression, we hypothesized that *Shh* mutants might have aortic arch defects similar to *Tbx1* mutants. To better detail the developing aortic arch arteries, we crossed mice containing a *lacZ* reporter downstream of the endothelial-specific *Tie2* promoter (Schlaeger et al. 1997) into the *Shh*-null background. At E9.5, the first, second, and third arch arteries were apparent in *Shh* mutant and wild-type embryos (Fig. 6g,h), although the first arch artery was smaller in the absence of Shh. The first and second arch arteries normally begin to regress by E10.5, at which time the fourth and sixth arch arteries are visible (Fig. 6i). In contrast, embryos lacking Shh had only a third aortic arch artery at this stage with no evidence of a fourth or sixth arch artery on the left or right side (Fig. 6j). This finding was confirmed by injection of India ink into the cardiovascular system to delineate the aortic arch, which also indicated the isolated presence of the third arch artery (Fig. 6k,l). *Tbx1* heterozygotes have a diminutive or absent fourth arch artery at this stage, consistent with a common role for Shh and Tbx1 in aortic arch patterning and development.

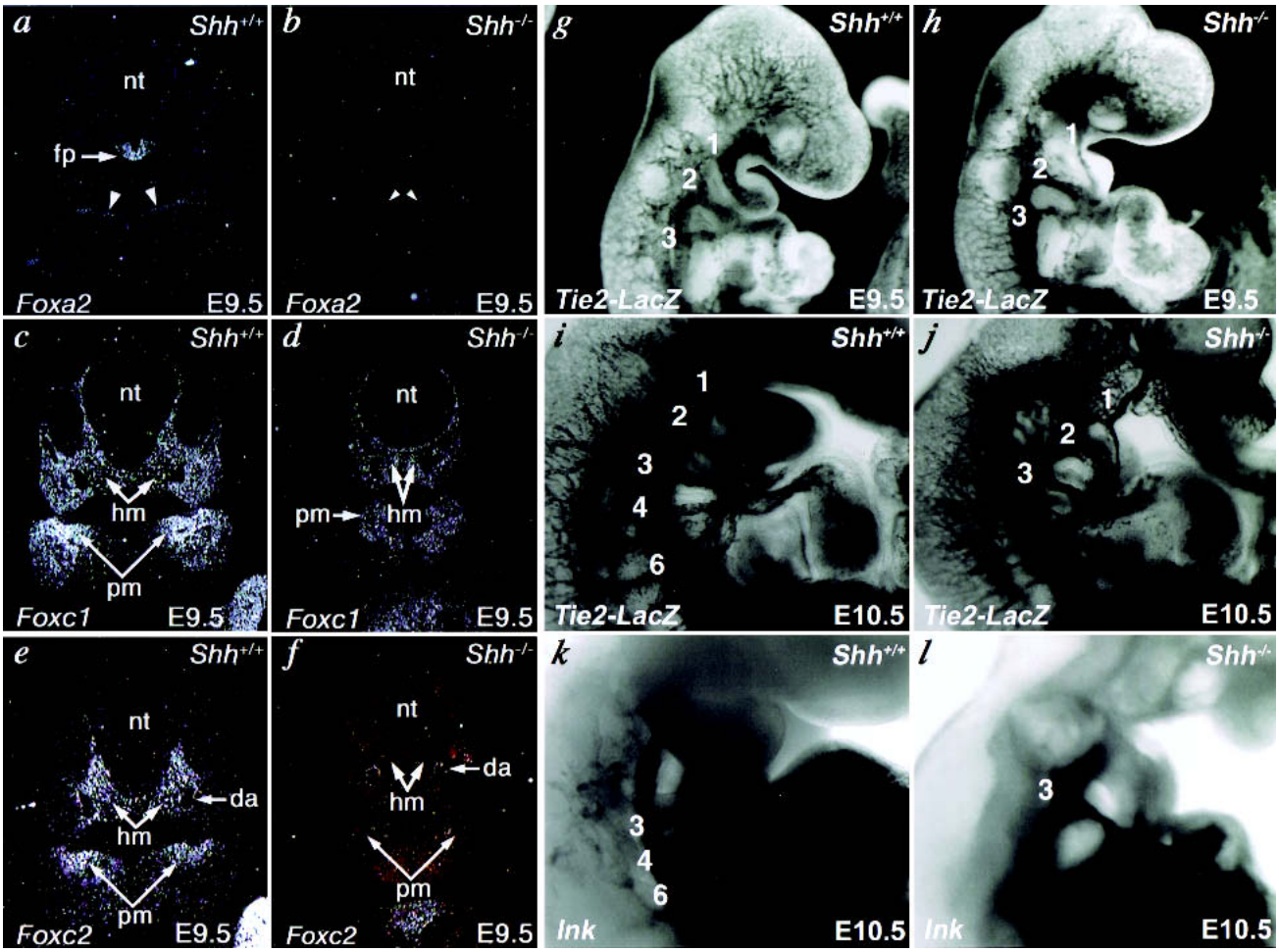

Figure 6. Shh regulates Fox proteins and aortic arch development. (*a*,*b*) Radioactive in situ hybridization on transverse sections of E9.5 mouse embryos shows that *Foxa2* is down-regulated in floor plate (fp) of neural tube (nt) and pharyngeal endoderm (arrowheads) of $Shh^{-/-}$ embryos compared with wild-type ($Shh^{+/+}$) embryos. (*c*,*d*) *Foxc1* was expressed in the head mesenchyme (hm) and pharyngeal arch mesenchyme (pm) of *Shh* mutant embryos at relatively low levels compared with wild-type embryos. (*e*,*f*) *Foxc2* was undetectable in the head mesenchyme and pharyngeal arch mesenchyme of *Shh* mutant embryos compared with wild-type embryos. *Foxc2* expression in dorsal aortae (da) was normal in *Shh* mutant embryos. *a*, *c*, and *e* are serial sections from a wild-type embryo, and *b*, *d*, and *f* are serial sections from an *Shh* mutant embryo. (*g–l*), Aortic arch defects in *Shh* mutants. *lacZ* driven by the *Tie2* promoter marked the vasculature and revealed a small first aortic arch artery at E9.5 (*h*) and absence of the fourth and sixth arch arteries at E10.5 (*j*) compared with wild type (*g*,*i*). India ink injection demonstrated similar absence of the fourth and sixth arch arteries. Arch arteries are indicated numerically. Right lateral views are shown with close-ups of the pharyngeal arch region in *i–l*.

Discussion

The pharyngeal arches are highly susceptible to perturbation as reflected by the numerous congenital syndromes affecting their derivatives. The close relationship of human cardiac malformations with pharyngeal arch derivatives reflects, in part, their common ontogeny. The signaling cascade involving Shh and Fox proteins that culminates on the regulatory apparatus of *Tbx1*, as revealed here (Fig. 7), provides an understanding of the events involved in development of the pharyngeal and aortic arch derivatives and some features of 22q11DS.

Transcriptional regulation of Tbx1 *by Fox proteins*

We found that a single Fox-binding element is essential for expression of *Tbx1* in the head mesenchyme and in the pharyngeal endoderm. We propose that a common Fox-binding element can interpret transcriptional messages from Foxa or Foxc proteins to activate *Tbx1* transcription in either the endoderm or head mesenchyme, respectively. Although Foxa and Foxc proteins have somewhat divergent forkhead DNA-binding domains and likely have varying affinities for the conserved Fox-binding site, it is interesting that a single *cis*-element translates information for both spatial domains of *Tbx1* expression. It is possible that relative affinities for the binding element dictate onset of gene expression in the mesenchyme or endoderm, analogous to recent evidence in *Caenorhabditis elegans*, suggesting that variable affinity of the Foxa2 ortholog PHA-4 for distinct forkhead regulatory elements predicts activation of target genes (Gaudet and Mango 2002). Although the endoderm and

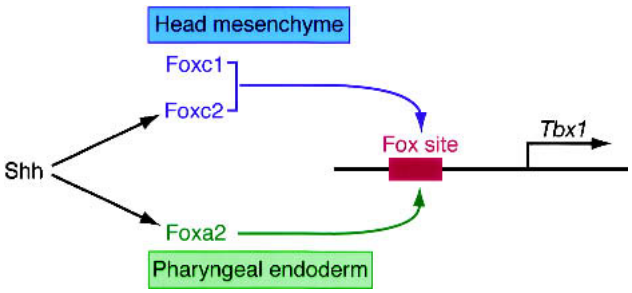

Figure 7. A proposed model for molecular regulation of *Tbx1*. Our data support a model in which *Tbx1* is directly regulated by Foxc1 and Foxc2 in the head mesenchyme, and Foxa2 in the pharyngeal endoderm, through a common Fox-binding site upstream of *Tbx1*. Shh signaling functions to maintain the expression of *Foxa2* and *Foxc2*, which subsequently activate *Tbx1* expression.

mesenchyme enhancers are not separable, the region regulating mesoderm expression of *Tbx1* appears to be under separate transcriptional regulation. Foxa proteins are inherently capable of initiating chromatin relaxation events (Cirillo et al. 2002) in collaboration with the zinc-finger transcription factor GATA4, suggesting a general mechanism for transcriptional activation by Fox proteins. It will be interesting to determine whether Foxa2 similarly cooperates with GATA3 to regulate *Tbx1*, as GATA3 is coexpressed in the pharyngeal endoderm (Lim et al. 2000) and is linked to a second DiGeorge syndrome locus on 10p (Gottlieb et al. 1998).

Foxc1 and Foxc2 are redundant transcription factors that are expressed in the head mesenchyme and mesenchyme surrounding the aortic arch arteries. The observation that mice lacking any two of the four alleles of *Foxc1* or *Foxc2* display aortic arch malformations (Winnier et al. 1999; Kume et al. 2001) strikingly similar to those of *Tbx1* heterozygotes (Jerome and Papaionnou 2001; Lindsay et al. 2001; Merscher et al. 2001), including interruption of the aortic arch, is consistent with our data that Foxc proteins and Tbx1 function in a common program. To our knowledge, *Tbx1* is the first direct target gene identified for Foxc1 or Foxc2 during development, and the down-regulation of *Tbx1* in the mesenchyme of $Foxc1^{-/-}$ or $Foxc2^{-/-}$ embryos by at least 50% provides a potential mechanism for the aortic arch defects observed in *Foxc* mutants.

Fox proteins as mediators of Shh signaling

Our in vitro and in vivo studies demonstrate that one mechanism by which Shh signaling may be linked to *Tbx1* transcription is through members of the Fox family of transcription factors. Shh signals through the transmembrane receptor Patched, which results in transcriptional activation of target genes by the Gli family of DNA-binding proteins (for review, see McMahon 2000). Shh is a direct upstream regulator of a Gli-binding site 5′ of *Foxa2* that is necessary for *Foxa2* expression in the floor plate of the notochord (Sasaki et al. 1997). Shh is also required for *Foxa2* expression in the pharyngeal endoderm, as shown here, supporting an intermediary role for Foxa2 in *Tbx1* regulation. The ability of Foxa2 to bind and activate transcription through the *Tbx1* enhancer is consistent with a role for Foxa2 in linking Shh signaling to *Tbx1* transcription in the endoderm.

The down-regulation of *Foxc2* in *Shh* mutants is consistent with Foxc2 functioning as an intermediary between Shh signaling and *Tbx1* regulation in the mesenchyme. The remaining *Foxc1* expression in *Shh* mutants may be insufficient to activate *Tbx1* fully, or Shh may be affecting Foxc1 activity through regulation of other cofactors. In either case, the evidence provided here of aortic arch defects in *Shh* mutants indicates that this molecular cascade is necessary for proper aortic arch patterning, although it remains to be determined whether Shh directly or indirectly controls *Foxc2* expression. In addition, it remains possible that Shh regulates other pathways that also contribute to development of the aortic arch arteries.

Implications for the 22q11.2 deletion syndrome

Despite the relatively uniform microdeletions in patients with 22q11DS, a highly variable phenotype is commonly observed, possibly as a result of mutations or polymorphisms in functionally related genes. Because Shh and Fox proteins function in a common pathway with Tbx1, it will be interesting to determine if such upstream regulators serve as genetic modifiers of the 22q11DS phenotype. *FOXC2* in particular may be such a modifier, as it directly regulates *TBX1* and is responsible for a human syndrome that can be associated with cardiovascular defects, although typically in the setting of lymphedema and distichiasis (double-rowed eyebrows; Fang et al. 2000). The identification of a critical *Tbx1* *cis*-regulatory element provides an opportunity to search for mutations of the Fox-binding element in patients with cardiac and craniofacial anomalies similar to those observed in patients with the 22q11 deletion. This will be particularly important as no mutations in the coding region of *Tbx1* have been found in >200 patients with the DiGeorge phenotype. Mutation analyses of the *cis*- and *trans*-regulators of *Tbx1* in patients with cranial and cardiac defects should ultimately reveal their significance in human disease.

Materials and methods

Generation of transgenic mice

We cloned a 5′ upstream genomic sequence of *Tbx1* by screening a 129SV mouse genomic library of bacterial artificial clones (BAC; RPCI genomic BAC library from BACPAC Resources, Children's Hospital Oakland Research Institute). Different fragments of the *Tbx1* upstream region were cloned into the *hsp68lacZ* reporter construct (Kothary et al. 1989). DNA for pronuclear injection was gel-purified and eluted using a QIAquick gel extraction kit (QIAGEN). Fertilized eggs from B6C3F1 female mice were collected for pronuclear injection. Injected

eggs were implanted into ICR female mice, foster mothers were killed at E9.5, and F_0 embryos were collected and stained for β-galactosidase (β-gal) activity as described previously (Yamagishi et al. 2000). Stable transgenic lines were obtained with a 12.8-kb fragment (construct 1) and a 1.1-kb fragment (construct 6), and F_1 embryos were collected from E7.5 to E10.5 and also stained for β-gal activity. For histological analyses, embryos were dehydrated, embedded in paraffin, sectioned at 7 μm, rehydrated, and stained with Nuclear Fast Red (Moller and Moller 1994). Transgenes were detected by polymerase chain reaction (PCR) or Southern analysis for the *lacZ* gene in yolk sac DNA for embryos or tail DNA for 2-week-old pups. Primer sequences are available on request.

Mouse mutants

Mice with mutations in *Shh* (Chiang et al. 1996), *Foxc1* (Kume et al. 1998), and *Foxc2* (Winnier et al. 1997) were described previously. *Shh* mutant mice were used for intercrosses with *Tbx1–lacZ* or *Tie2–lacZ* (Schlaeger et al. 1997) transgenic mice.

Site-directed mutagenesis

The conserved Fox-binding site at –13,435 bp (TGTTTGT) was mutated to an *Not*I site in the context of the 1.1-kb fragment (–14,329/–13,230 bp). The 1.1-kb fragment was subcloned into plasmid pBluescript (Stratagene), and PCR-based mutagenesis on this plasmid was performed using *pfu* DNA polymerase and a QuikChange site-directed mutagenesis kit (Stratagene). The primers used were 5′-GGAGGAGCAGCCGCGGCCGCTT GCCAGATCTGTGC-3′ and 5′-GCACAGATCTGGCAAGCG GCCGCGGCTGCTCCTCC-3′. Restriction mapping and sequence analysis confirmed the presence of the mutation, and the insert was excised and cloned into the *hsp68lacZ* reporter plasmid.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides corresponding to the conserved Fox-binding site in the *Tbx1* enhancer were synthesized (Integrated DNA Technologies) as follows: 5′-GGAGGAGCAGCCTGTTTGTT TTGCCAGATCTGTGC-3′, 5′-GCACAGATCTGGCAAAAC AAACAGGCTGCTCCTCC-3′. The primer pair for site-directed mutagenesis was used for mutant oligonucleotides. Oligonucleotide pairs were resuspended, boiled for 10 min, and annealed slowly, cooling to room temperature. Annealed oligonucleotides were radiolabeled with [α-^{32}P]dCTP using a Klenow fragment of DNA polymerase and purified using Sephadex G-25 spin columns (Roche). Foxa2, Foxc1, and Foxc2 proteins were translated in vitro with the TNT T7-coupled reticulocyte lysate system (Promega), using full-length cDNA for Foxa2, Foxc1, and Foxc2 cloned into a pcDNA3.1 plasmid (Invitrogen) as DNA template. DNA-binding assays were performed as described previously (Scott et al. 1994). Unlabeled wild-type or mutant competitor oligonucleotide was added to reactions at the indicated concentrations. DNA–protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5× TBE. Gels were exposed to Phosphor screen and read in PhosphorImager (Molecular Dynamics).

Transfection and luciferase assay

A nearly 200-bp DNA sequence (–13,546/–13,348 bp) containing the conserved Fox-binding site in the *Tbx1* enhancer was amplified by PCR using primers 5′-CCCAAGCTTCCTGTGT GTCTGAGCTGGC-3′ and 5′-CGGGATCCTGCTGTCCACC CTGCAACCC-3′, and cloned into the pGL3 basic luciferase vector (Promega). Plasmid transfection was performed in 6-well plates using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. HeLa cells or COS-7 cells were transfected with 0.2 μg of luciferase reporter construct and various expression constructs, including 0.25 μg of pcDNA3.1 plasmids (as control), pcDNA3.1-Foxa2, pcDNA3.1-Foxc1, or pcDNA3.1-Foxc2. Then 0.1 μg of a RSV-*lacZ* expression construct was cotransfected to correct for transfection efficiency. Luciferase and *lacZ* activities in the cell extract were assayed 36–48 h after transfection, using the Luciferase Assay System (Promega) and Lucy2 luminometer (Rosys Anthos). All experiments were repeated at least twice, and representative results are shown.

In situ hybridization

^{35}S-labeled antisense riboprobe was synthesized with T3 or SP6 RNA polymerase (MAXIscript in vitro transcription kit, Ambion) from Foxa2, Foxc1, Foxc2, or Tbx1 cDNA. Hybridization was performed on paraffin-embedded sections of E9.5–E10.5 wild type, *Shh* mutant, *Foxc1* mutant, or *Foxc2* mutant mouse embryos as described previously (Yamagishi et al. 2001). Sources of DNAs for making probes were as follows: Foxc1 and Foxc2 (Kume et al. 2001) and Tbx1 (Garg et al. 2001). Partial cDNA of Foxa2 was PCR-amplified using primer pair 5′-ATGC TGGGAGCCGTGAAGAT-3′ and 5′-CCAGTGACCAGAGC TAGCAG-3′ with full-length cDNA of Foxa2 [gift from K. Kaestner (University of Pennsylvania, Philadelphia)] as a template, and cloned into the pCRII-TOPO (Invitrogen) for generation of the probe.

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