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Shared molecular programs govern the formation of heart and head during mammalian embryogenesis. Development of both structures is disrupted in human chromosomal microdeletion of 22q11.2 (del22q11), which causes DiGeorge syndrome (DGS) and velocardio-facial syndrome (VCFS). Here, we have identified a genetic pathway involving the Six1/Eya1 transcription complex that regulates cardiovascular and craniofacial development. We demonstrate that murine mutation of both *Six1* and *Eya1* recapitulated most features of human del22q11 syndromes, including craniofacial, cardiac outflow tract, and aortic arch malformations. The mutant phenotypes were attributable in part to a reduction of fibroblast growth factor 8 (*Fgf8*), which was shown to be a direct downstream effector of Six1 and Eya1. Furthermore, we showed that *Six1* and *Eya1* genetically interacted with *Fgf8* and the critical del22q11 gene T-box transcription factor 1 (*Tbx1*) in mice. Together, these findings reveal a *Tbx1-Six1/Eya1-Fgf8* genetic pathway that is crucial for mammalian cardiocraniofacial morphogenesis and provide insights into the pathogenesis of human del22q11 syndromes.

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A Tbx1-Six1/Eya1-Fgf8 genetic pathway controls mammalian cardiovascular and craniofacial morphogenesis

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Shared molecular programs govern the formation of heart and head during mammalian embryogenesis. Development of both structures is disrupted in human chromosomal microdeletion of 22q11.2 (del22q11), which causes DiGeorge syndrome (DGS) and velo-cardio-facial syndrome (VCFS). Here, we have identified a genetic pathway involving the Six1/Eya1 transcription complex that regulates cardiovascular and craniofacial development. We demonstrate that murine mutation of both Six1 and Eya1 recapitulated most features of human del22q11 syndromes, including craniofacial, cardiac outflow tract, and aortic arch malformations. The mutant phenotypes were attributable in part to a reduction of fibroblast growth factor 8 (Fgf8), which was shown to be a direct downstream effector of Six1 and Eya1. Furthermore, we showed that Six1 and Eya1 genetically interacted with Fgf8 and the critical del22q11 gene T-box transcription factor 1 (Tbx1) in mice. Together, these findings reveal a Tbx1-Six1/Eya1-Fgf8 genetic pathway that is crucial for mammalian cardiocraniofacial morphogenesis and provide insights into the pathogenesis of human del22q11 syndromes.

Introduction
Progenitors of the cardiac and craniofacial skeletal muscles arise from subdomains of a contiguous field of cephalic mesoderm: the ventrolateral primary heart field (PHF) and the dorsomedial cranial paraxial mesoderm (CPM), respectively. A distinct population of mesodermal progenitors located in between the PHF and CPM, than 30% of del22q11 patients have renal defects; however, the thymus and parathyroid glands (9, 10). Additionally, more and than 30% of del22q11 patients have renal defects; however, the underlying etiology remains to be elucidated. T-box transcription factor 1 (TBX1) is located in the 22q11.2 critical region, and TBX1 haploinsufficiency is a major contributor to human del22q11 phenotypes and to murine models of the syndrome (11, 12). Mouse Tbx1 is expressed in SHF cardiac progenitors and is required for their proliferation as well as for cranial skeletal muscle development (13–17). Similar to human hemizygous del22q11 phenotypes, mouse Tbx1 haploinsufficiency causes aortic arch defects, whereas Tbx1-null mutants exhibit more severe cardiac and craniofacial anomalies (18–20).

Although human fibroblast growth factor 8 (FGF8) does not map to the 22q11.2 region, FGF8 and FGF signaling pathways are critical for cardiovascular development and have been directly implicated in the etiology of del22q11 syndromes (12, 21). Autocrine function of murine Fgf8 controls proliferation and survival of SHF cardiac progenitors (22, 23), whereas paracrine Fgf8 signals from the pharyngeal ectoderm and endoderm epithelia control formation and remodeling of both craniofacial structures and aortic arch arteries and cardiac OFT (13, 24–26). Double-heterozygous Tbx1+/− Fgf8+/− mutant embryos exhibit increased incidence of pharyngeal arch (PA) artery remodeling anomalies (27, 28), demonstrating a genetic link between Tbx1 and Fgf8 during vascular morphogenesis. Fgf8 activity has to be precisely controlled, as too much or too little is detrimental to cardiovascular development (29). For example, mutations of components of the Notch signaling pathway cause congenital heart defects in humans (30), which depends in part on modulation of Fgf8 expression in the SHF (31). Fgf8 hypomorphic mice with reduced Fgf8 expression display a range of pharyngeal and cardiovascular phenotypes (32). Forced expression of Fgf8 in a Tbx1-null background could not fully rescue the cardiac defects of Tbx1-null mutants and even increased the incidence of cardiovascular defects (28, 33). Despite the critical role of Fgf8, little is known about the direct transcriptional mechanism responsible for its gene regulation during cardiovascular development (14, 27, 34).

We reported previously that the evolutionarily conserved homeodomain transcription factor Six1 and its canonical coactivator Eya1 synergistically control organ-specific progenitor cell proliferation and survival (35). Deletion of either Six1 or Eya1 phe-
nocopies human branchio-oto-renal (BOR) syndrome, which is characterized by congenital preauricular pits, branchial fistulas, hearing loss, and renal agenesis (35–37). Human EYA1 mutations are also found in patients with congenital cardiac abnormalities (38, 39). Mutation of the EYA1 homolog EYA4 causes dilated cardiomyopathy (40). Therefore, the Six1/Eya1 complex may have yet unidentified roles during cardiovascular development.

Here, we demonstrate that double-null Six1−/−Eya1−/− mutant mice exhibited phenotypes similar to Tbx1 and Fgf8 mouse mutants and phenocopied human del22q11 syndromes, including the major craniofacial and cardiovascular phenotypes. We provide genetic and molecular evidence indicating that a Tbx1-Six1/Eya1-Fgf8 pathway is critical for morphogenesis of cardiocraniofacial structures and propose that mutations of SIX1 and EYA1 may contribute to the pathogenesis of both del22q11-like and BOR syndromes in humans.

Results

Six1 and Eya1 regulate cardiovascular and craniofacial morphogenesis. To determine a potential role of Six1 and Eya1 in cardiovascular development, we analyzed the cardiac phenotype of embryos with null mutation of 1 or both copies of Six1 and Eya1. While Six1−/−Eya1−/− embryos had normal morphology of the aorta and pulmonary arteries, further loss of function of either Six1 or Eya1 caused a spectrum of abnormalities of great artery patterning (Figure 1 and Table 1) consistent with abnormal development of the PA arteries and characteristic of del22q11 syndromes (9, 10). For instance, 78% of Eya1−/− mutants had aortic arch defects (n = 13), including 54% with interrupted aortic arch type B (IAA-B), in which the transverse arch is disrupted between the left common carotid and left subclavian arteries. Other vascular anomalies found in Eya1−/− mutants included retroesophageal right subclavian artery (23%) and vascular ring (16%). Six1−/−Eya1−/− mutant embryos had a higher incidence and greater severity of cardiovascular phenotypes (Table 1). Instead of the normal OFT pattern, in which the aorta and pulmonary arteries connect to the LV and RV, respectively, 100% of Six1−/−Eya1−/− embryos exhibited OFT defects (n = 7). We found 14% had double-outlet RV (DORV), in which both great vessels arise from the RV, and egress of blood from the LV is via a ventricular septal defect (Figure 1I). Such defects were present in 100% of compound mutants. Approximately 70% of Six1−/−Eya1−/− mutants had a single, unseptated outflow vessel, and the outflow valve contained 3 or 4 leaflets (Figure 1J). These SHF-related defects are found in the human del22q11 patient population: persistent truncus arteriosus (PTA; a single unseptated OFT, gross morphology of the outflow valves in a Tbx1-Six1/Eya1−/− mutant (I and J) compared with Six1−/−Eya1−/− control (G and H). Ao, aortic artery; BCA, brachiocephalic artery; dA, descending aorta; DLCC, duplicated left common carotid artery; IAA-B, left common carotid artery; LCC, left subclavian artery; p, pulmonary artery; RCC, right common carotid artery; RRSOA, retroesophageal right subclavian artery; RSC, right subclavian artery; V, ventricle; VR, vascular ring. Asterisks denote interrupted aortic arches. Original magnification, ×80 (G and I); ×80 (H and J).

Figure 1

Six1−/−Eya1−/− mutants phenocopy features of human del22q11 syndromes. (A–F) Gross morphological defects of the OFT and great arteries of newborn mutants (B–F) versus WT control (A). (G–J) H&E histological staining of sectioned hearts revealed VSD and dysmorphology of the outflow valves in a Tbx1-Six1/Eya1−/− mutant (I and J) compared with Six1−/−Eya1−/− control (G and H).
branchiomeric skeletal muscles (6, 7), we examined all cranial skeletal muscles using an antibody marker, MF20 (myosin heavy chain–specific antibody required for both cardiovascular and craniofacial development.

of the genetic program required for formation of both cardiac and genetic programs of the foregut endoderm. We posit that the combination of Six1 and Eya1 function in all these cells is required for normal cardiovascular and craniofacial morphogenesis. Six1 and Eya1 are required for cell survival and proliferation. We have shown previously that the Six1/Eya1 transcription complex is essential for renal progenitor cell survival and proliferation at E11.5 and E12.5 (35, 36, 52). Thus, we analyzed early-stage (E9.5 and E10.5) Six1<sup>−/−</sup> Eya1<sup>−/−</sup> mutants to determine whether a similar mechanism contributes to cardiovascular morphogenesis (Figure 3). We detected an approximately 50% reduction in phospho–histone H3–labeled (pH3-labeled) cells in the PAs and OFT of whole-mount and sectioned Six1<sup>−/−*/Eya1<sup>−/−</sup></sub> specimens (Figure 3 and data not shown). Cardiac progenitors in the SHF/SpM had an approximately 48% reduction in pH3 labeling, and those in the foregut endoderm had an approximately 40% reduction (Figure 3, A–D). More apoptotic cells were detected in the SHF pharyngeal endoderm and endoderm of the Six1/Eya1 mutants relative to controls, based on both LysoTracker and TUNEL staining (Figure 3, E–L, and data not shown). Thus, reduced proliferation and survival of the SHF and the pharyngeal endoderm and endoderm epithelia in Six1<sup>−/−*/Eya1<sup>−/−</sup></sub> mutants likely contributes to the observed cardiovascular phenotypes, as has been reported in Tbx1 and Fgfbp mouse mutants with similar phenotypes.

### Table 1

Six1 and Eya1 synergistically regulate cardiovascular development

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| Six1<sup>−/−</sup> Eya1<sup>−/−</sup> | 7 | 100%<sup>B</sup> | 29%<sup>B</sup> | 43%<sup>B</sup> | 29%<sup>B</sup> | 29%<sup>B</sup> | 0% | 14%<sup>B</sup> | 14%<sup>A</sup> | 71%<sup>B,C</sup>

DLCC, duplicated left common carotid artery; RAA, right-sided aortic arch; RPA, right-sided pulmonary artery; RRSA, retroesophageal right subclavian artery; VR, vascular ring. Statistical analyses were done by Fisher exact test. *P < 0.05 versus Six1<sup>−/−</sup>Eya1<sup>−/−</sup> control. **P < 0.05 versus Six1<sup>−/−</sup>Eya1<sup>−/−</sup> control.

Six1<sup>−/−</sup> progenitors contribute to multiple cardiac lineages. To determine whether Six1<sup>−/−</sup> progenitors contribute to mature cardiovascular tissues, we used double-heterozygous Six1<sup>Gtv/hpAP</sup>R26R<sup>lacZ</sup> and Six1<sup>Gtv/hpAP</sup>R26R<sup>R26R</sup> embryos, in which Six1<sup>−/−</sup> progenitors and their progeny are permanently labeled with membrane-localized eGFP and β-gal reporters, respectively (Figure 2 and Supplemental Figure 4). eGFP<sup>+</sup> cells were detected in the inflow tract and OFT of the looped heart at E9.0 (Figure 2, G–I), consistent with Six1 expression in the SHF. This was further supported by colocalization of eGFP with the cardiac progenitor marker Isl1 (4, 48). Consistently, Six1<sup>−/−</sup> progenitors differentiated into multiple cell types, including ventricular cardiomyocytes and smooth muscle cells of the proximal aortic and pulmonary arteries, based on coimmunostaining with the cardiac-specific TNNT2 and ACTN2 and the smooth muscle–specific SM22α and CNN1 antibodies (49), respectively (Figure 2, J–N). In addition, eGFP colocalized with WT1 and RALDH2 (Figure 2O and Supplemental Figure 4, C–J), which suggests that Six1 is expressed in epicardial progenitors (49–51). Thus, in addition to the previous reports of Six1 expression in the pharyngeal endoderm and endoderm, and the cranial skeletal muscle progenitors (35, 36), our detailed expression and fate-mapping analyses demonstrated that Six1 was also expressed in cardiac progenitors in the SHF and proepicardium during cardiovascular development (Figure 2O).
We also observed a substantial increase in apoptosis in the pharyngeal mesenchyme cells derived from either mesoderm or neural crests (Figure 3). Defects of a specialized population of cardiac neural crest cells (CNCs) result in cardiovascular phenotypes similar to those found in the Six1/Eya1 mutants (53–55). To analyze CNCs specifically, we examined the expression patterns of transcription factor AP-2α (AP2) and plexin A2 (Plxna2) (Supplemental Figure 5). These genes were detected in migrating CNCs in the mutant pharynx (AP2) and postmigratory CNCs in the mutant OFT (Plxna2) (Supplemental Figure 5). AP2 expression in the ectoderm was unaffected (Supplemental Figure 5H), but staining in the caudal pharynx was decreased (Supplemental Figure 5, D and G, arrow). Consistent with a potential increase of CNC apoptosis (Figure 3I), the intensity of Plxna2 staining in the prongs of CNCs invading the OFT appeared to be decreased (Supplemental Figure 5B), suggestive of fewer invading CNCs in the mutants. Together, these findings suggest that Six1 and Eya1 are required for CNC survival and that decreased CNC number and/or CNC dysfunction in the mutants may also contribute to the hypoplastic OFT cushions and septation failure.

Fgf8 expression depends on Six1 and Eya1 in vivo. To identify Six1/Eya1-dependent genetic pathways that regulate cardiovascular morphogenesis, we examined expression of a panel of molecular markers known to be important in SHF survival and function, including Tbx1, Isl1, Fgf8, and Fgf10 (Figure 4 and Supplemental Figures 6–8). Of these genes, Fgf8 exhibited a drastic reduction in expression throughout the PAs and the SHF at E9.5 in Six1−/−Eya1−/− mutants; expression levels of Tbx1 and Isl1 were unaltered despite the severe hypoplastic caudal arch defect (Figure 4, A–D and Supplemental Figures 6–8). Consistent with the gross cardiovascular phenotypes (Figure 1), we noted that Fgf8 expression in the caudal pharyngeal region and the SHF was more sensitive to the loss of Eya1 than of Six1, and the reverse was true in the first arch (Figure 4, B and C). Similar to Six1 and Eya1, Fgf8 is required for SHF progenitor cell proliferation and survival (22, 23, 25, 26), and Fgf8 mouse mutants display phenotypic features of del22q11 syndrome (32, 56). In contrast to Tbx1 mutants, in which both Fgf8 and Fgf10 are reduced, expression of Fgf10 in Six1−/−Eya1−/− mutants was slightly increased (Supplemental Figure 7). Baldini and col-
leagues have demonstrated that Tbx1 can directly regulate Fgf10 in vitro (16), but it is not yet clear whether Fgf8 is also a direct target of Tbx1 in some tissues (14). Our results suggest an alternate mechanism, in which Tbx1 may regulate Fgf8 expression through the Six1/Eya1 transcription complex.

The Six1/Eya1 transcription complex directly controls Fgf8 expression in vivo. To test whether Six1 and Eya1 directly control Fgf8 gene expression, we first questioned whether they are expressed in the same cells. We used double-heterozygous Six1<sup>hpAP/Cre/+</sup> Fgf8/eGFP<sup>+</sup> embryos to address this question. Expression of eGFP in these embryos depends on the endogenous promoter activity of both Fgf8 and Six1 and therefore can be used as a sensitive indicator of the same cell lineages (25). Six1<sup>hpAP/Cre/+</sup> Fgf8/eGFP<sup>+</sup> embryos displayed strong Fgf8/eGFP expression in the pharyngeal region and the cardiac OFT (Figure 4E), which is similar to previously reported Fgf8/eGFP<sup>+</sup> lines (32). These Fgf8/eGFP<sup>+</sup> lineages included Nkx2.5<sup>+</sup> cells in the bulbus cordis and the conotruncus (which contribute to the RV and OFT, respectively; Figure 4, F and G) and Isl1<sup>+</sup> cells in the pharyngeal endoderm and ectoderm and the SHF/SpM (Figure 4, H and I). Furthermore, Fgf8/eGFP colocalized with Six1/Cre immunoreactivity in the SHF/SpM and pharyngeal endoderm (Figure 4, J and K), which suggests that Six1 and Fgf8 are coexpressed in multiple cell lineages.

To examine whether Six1/Eya1 can directly regulate Fgf8 expression, we analyzed a genomic fragment containing an Fgf8 enhancer sufficient to drive transgene reporter expression in pharyngeal ectoderm and endoderm and in cardiac OFT (14). The proximal Fgf8 promoter has 3 conserved regions in mice, rats, cows, and primates, and we identified 4 putative Six1 binding sites distributed among those 3 regions (Figure 4L). The second and third sites were clustered in the second conserved region (~130 bp). A luciferase reporter driven by a 2.4-kb mouse Fgf8 enhancer fragment had negligible activity after transient transfection in HEK293 cells. Cotransfection of either Six1 or Eya1 resulted in a 3-fold increase in reporter activity, and there was synergistic enhancement of the reporter activity to approximately 13-fold when both factors were present (Figure 4M). Because truncation analyses indicated that the second conserved region (Fgf8-d) had relatively higher activity than the first and third regions (Figure 4N), we focused on the 2 putative Six1-binding sites within the second conserved region. Mutation of these 2 sites significantly decreased reporter activity (Figure 4O). To confirm that Six1 binds directly to the endogenous Fgf8 enhancer, we purified Six1-associat chromatin from E9.5 mouse embryonic PAs and cardiac tissues. Real-time quantitative PCR analyses indicated that binding of Six1 to the endogenous Fgf8 enhancer was enriched more than 4-fold relative to the coding region of Fgf8 and IgG control (Figure 4P).

Together, these findings strongly suggest that the Six1/Eya1 transcription complex directly regulates Fgf8 gene expression in vivo. To further establish the functional relationship in vivo between Six1 and Fgf8, we analyzed compound Six1<sup>Cre/hpAP/+</sup> Fgf8<sup>−/−</sup> mutants (ref. 32 and Supplemental Figure 9). In these mutants, a single allele of Fgf8 is inactivated specifically in Six1-expressing cells. Six1<sup>−/−</sup> and Six1<sup>Cre/hpAP/+</sup> Fgf8<sup>−/−</sup> mutants had only rare great vessel defects (Table 2); however, 83% of Six1<sup>Cre/hpAP/+</sup> Fgf8<sup>−/−</sup> mutants exhibited IAA-B or cervical aortic arch phenotypes. This functional interaction strongly suggests that Six1 and Fgf8 are in the same genetic pathway and synergistically regulate cardiovascular morphogenesis.

Decreased expression of Fgf8 likely contributes to the observed del22q11-like phenotypes in Six1<sup>−/−</sup> Eya1<sup>−/−</sup> mutants.

Exogenous Fgf8 rescues endothelial-to-mesenchymal transformation defects. Fgf8 signaling is critical for endothelial-to-mesenchymal transformation (EMT) in the proximal OFT cushions and for

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CNC migration and invasion of both proximal and distal OFT cushions (26, 31). Consistently, we found that Six1–/–Eya1–/– mutants had severely hypoplastic proximal and distal OFT cushions compared with WT littermate controls (Figure 5, A–H).

Using an established OFT explant culture assay (26, 31, 57), we found that both Six1+/–Eya1–/– and Six1–/–Eya1–/– mutants had a dramatic reduction in the number of mesenchymal cells that invaded the gel and migrated away from the beating myocardial explants (Figure 5, I–P). These results suggest that the Six1/Eya1 complex is required indirectly through Fgf8 for EMT of OFT endothelial cells during cushion remodeling.

To determine whether Fgf8 is sufficient to rescue EMT in Six1–/–Eya1–/– mutant phenotypes, we treated WT and mutant explants with recombinant Fgf8 isoform b protein (rFgf8b) or BSA control.
Six1 and Fgf8 are in the same genetic pathway

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RRSA, retroesophageal right subclavian artery; VR, vascular ring. Statistical analyses were done by Fisher exact test. *p < 0.05. No significant difference.

and assayed EMT by counting the total number of mesenchymal cells that invaded the gel as well as the distance migrated from the explant after invasion. Whereas very few mesenchymal cells were present in untreated compound mutant explants and the few present had not migrated far (Figure 5J), rFgf8b treatment almost fully rescued EMT and migration defects of Six1+/−; Eya1−/− mutants (Figure 5, I–P). The Six1+/−; Eya1−/− mutant phenotypes were also rescued, but to a lesser extent, indicating, not surprisingly, that additional factors downstream of Six1/Eya1 contribute to defects in the Six1+/−; Eya1−/− mutant.

Tbx1 is a genetic upstream regulator of Six1 and Eya1. Although human SIX1 and EYA1 do not map to chromosome 22q11.2, the shared morphological defects and molecular phenotype of decreased Fgf8 expression in Six1/Eya1 and Tbx1 mutant mouse models suggests that human SIX1 and EYA1 may be genetic modifiers of the del22q11 syndrome. To test this possibility using mouse models, we examined the functional relationship between Six1 or Eya1 and Tbx1 by analyzing compound mutants of these genes in a C57BL6/CD-1 mixed genetic background (Figure 6, A–D, and Table 3). Whereas 25% of Tbx1−/− mutants (n = 12) had cardiovascular defects at E17.5, none of the Eya1−/− mutants did (n > 10). Penetrance of these defects increased to 73% in Eya1−/−; Tbx1−/− mutants (n = 15). Remarkably, although approximately 80% of Eya1−/− mutants had cardiovascular phenotypes (n = 13), none of them had the severe defect of a single outflow vessel seen in Tbx1−/− mutants, in contrast to 100% of Eya1−/−; Tbx1−/− mutants (n = 5; Figure 6D and Table 3). Similar to Eya1/Tbx1 compound mutants, 63% of Six1+/−; Tbx1−/− mutants (n = 8) had cardiovascular phenotypes, in contrast to less than 5% of Six1−/− mutants (n = 20) and 33% of Tbx1−/− mutants (n = 9). Together, these findings demonstrated that Six1, Eya1, and Tbx1 are in a common genetic pathway controlling cardiovascular and craniofacial development.

Since Tbx1 expression in the pharyngeal epithelia appeared to be unaffected in Six1+/−; Eya1−/− mutants (Supplemental Figures 7 and 8), we next examined the possibility that Six1 and Eya1 function down-stream of Tbx1. Both Six1 and Eya1 were downregulated in the presumptive caudal arch tissue (PA3–PA6 region) of Tbx1 mutants (Figure 6, F and I). It is not clear whether the observed reduction of Six1 and Eya1 expression in Tbx1 mutants is attributable to arch hypoplasia and failed segmentation (i.e., fewer cells to express the genes), or rather reflects a direct effect of Tbx1 loss of function on expression of these genes (i.e., less expression in affected cells).

We reasoned that if Six1 and Eya1 are genetically downstream of Tbx1, increased levels of Tbx1 should result in their upregulation. We therefore examined transgenic mice that overexpress human TBX1
using a BAC (20). As expected, 54% of the transgenic mice had vascular defects involving the aortic arch and/or great vessels in the face of relatively normal morphology of the pharyngeal apparatus (20, 58, 59). We noted enhanced expression of both Six1 and Eya1 in the PA3–PA6 region (Figure 6, G and J). Six1, but not Eya1, was also upregulated in pharyngeal mesoderm (Figure 6G, arrows), including primordium of the lateral rectus eye muscle and the first branchial arch core mesoderm. Eya1 expression in the pharyngeal epithelia, particularly in the pharyngeal clefts, was increased in Tbx1Bac/+ transgenic mice (Figure 6J, arrowheads). Together, these loss-of-function and gain-of-function analyses revealed a functional relationship in which Tbx1 is genetically upstream of Six1 and Eya1 during cardio-craniofacial morphogenesis. It remains to be determined whether Tbx1 directly regulates Six1 and Eya1 gene expression in vivo.

Discussion

Here, we uncovered what we believe to be a novel function for the Six1/Eya1 transcription complex in cardiovascular development and delineated a Tbx1-Six1/Eya1-Fgf8 regulatory cascade that controls morphogenesis of mammalian heart and face. Our findings suggest that dysregulation of this molecular pathway is likely to be a critical mechanism underlying the pathogenesis of del22q11-like syndromes in humans.

The present study provides evidence that Six1 is transiently expressed in cardiac progenitors in the SHF and proepicardium and contributes directly to cardiovascular development. The restricted distribution pattern of Six1 progeny, which is reminiscent of the SHF in chicks (3), suggests that Six1 is expressed in a subpopulation of the SHF lineages marked by Isl1 or Tbx1 (4, 60). It is also possible, however, that the lineage tracing experiments may be limited by inefficient recombination rate as a result of weak enhancer activity of the endogenous Six1 locus in these cells. We showed that Six1 and Eya1 were broadly expressed in the pharyngeal ectoderm, endoderm, and mesoderm (including SHF). Consequently, deletion of both genes resulted in downregulation of Fgf8 in all 3 cell layers, all of which are likely to contribute to the observed morphological defects of cardio-craniofacial structures (21, 24, 25). Future conditional knockout experiments will be needed to define the tissue-specific roles of Six1/Eya1.

Six1−/−Eya1−/− mutants had a single unseptated OFT in addition to other typical vascular defects seen in del22q11 human patients (Figure 1). It has been proposed that the lesion diagnosed as PTA in some cases of human del22q11 and animal models of this syndrome may in fact be a severe form of pulmonary atresia in which the entire RV outflow fails to form, resulting in a single outflow vessel that is the aorta (43, 61). However, at this point, the morphological and molecular criteria for this important distinction remain to be established. Based on the variable and abnormal outflow valve morphology and the size and location of the outflow vessel in Six1−/−Eya1−/− mutants, we think the defect is likely PTA, but recognize the possibility that the spectrum of defects in these mutants may include pulmonary atresia.

Figure 6

Eya1 functionally interacts with Tbx1 in vivo. (A–D) Gross views of cardiovascular structures of newborn Eya1/Tbx1 compound mutants. Asterisk in C denotes interrupted aortic arches. (E–J) Whole-mount RNA in situ hybridization revealed altered Six1 and Eya1 expression in Tbx1−/− (F and I) and Tbx1Bac/+ gain-of-function mutants (G and J). Tbx1−/− mutants had hypoplastic and unsegmented caudal PAs (F and I, double asterisks). Brackets in G and J indicate increased expression of Six1 and Eya1 in the Tbx1 gain-of-function mutants. Arrows in G denote Six1 upregulation. Arrowheads in J indicate enhanced Eya1 expression. CA, cervical aortic arch; LR, lateral rectus muscle; md, mandible; mx, maxillary; np, nasal placode; O, otic vesicle; Tr, trachea. Original magnification, ×63 (E–J).
To our knowledge, no direct in vivo transcriptional regulator of Fgf8 has previously been described. Loss of Tbx1 causes a significant reduction of both Fgf8 and Fgf10, which may have redundant roles during cardiovascular development (12, 14, 16, 21, 27, 62). Fgf10 in the SHF, but not Fgf8, is a direct downstream target of Tbx1 (1, 14, 16). Hu et al. demonstrated that Tbx1 is required for an orthologous region upstream of the human FGF8 locus to enhance reporter activity in the OFT of transgenic mice; expression of the enhancer in the PAs (including SHF) was not affected by loss of Tbx1 (14). Furthermore, mutation of 3 putative Tbx1 binding sites found in the FGF8 enhancer did not affect its activity in vivo. Thus, it is unclear whether Tbx1 directly activates Fgf8 in vivo. We found that Tbx1 expression was unaltered in the Six1−/−Eya1−/− mutants (Supplemental Figures 6–8), despite the severe morphological cardiac arch defects. Both Six1 and Eya1 were reduced in Tbx1−/− loss-of-function mutants and enhanced in the Tbx1fl/fl gain-of-function transgenics (Figure 6). Tbx1 may therefore be genetic upstream of Six1 and Eya1. Furthermore, we demonstrated that Six1 and Fgf8 were coexpressed in the pharyngeal ectoderm and endoderm and in some cardiac progenitors in the SHF. Fgf8, but not Fgf10, was severely reduced in all these cells in the Six1−/−Eya1−/− mutants (Figure 4 and Supplemental Figure 7). We showed that not only did Six1/Eya1 compound mutants share cardiovascular and craniofacial features with Fgf8 mutants, they synergistically interacted to regulate pharyngeal artery patterning, which strongly suggests that they are in the same genetic pathway. Taken together, our results indicate that the Six1/Eya1 transcription complex directly controls Fgf8 expression through an evolutionarily conserved enhancer element (Figure 4). Future studies will determine whether Tbx1 is a direct regulator of Six1 and Eya1.

Recent studies in Drosophila melanogaster demonstrate that the fly eya1 ortholog is a direct downstream target of tinman. Nkx2.5, the mammalian ortholog of tinman, is required for both PHF and SHF development (63, 64). In Drosophila, eya1 and tinman are initially coexpressed throughout the mesoderm, after which their localization becomes mutually exclusive, with eya restricted to ventral mesoderm and tinman present only dorsally. tinman is also required for Drosophila expression of six4 and differentiation of muscle along the dorsoventral axis (65). In addition, zebrafish six1 and eya1 were reported to function downstream of tbx1 during craniofacial myogenesis (66). Thus, an evolutionarily conserved network consisting of Tbx1, Nkx2.5, Six1, and Eya1 may be critical for specification and differentiation of cranial skeletal and cardiac muscle.

Haploinsufficiency of SIX1 and EYA1 in humans causes BOR syndrome (35, 36), which often exhibits kidney and ear anomalies but does not routinely feature cardiovascular defects. It is important to note that frequent and severe cardiovascular defects were found only in Six1−/−Eya1−/− mutant mice (Table 1). Renal defects are seen in nearly one-third of del22q11 patients (9, 10, 41, 42), but Tbx1-null mutants do not exhibit any apparent renal defects (18–20). Thus, TBX1 haploinsufficiency is unlikely to be the genetic basis for the renal defects in affected human del22q11 patients. In contrast, Six1 and Eya1 were critical regulators of kidney development (Supplemental Table 1 and refs. 35, 52). We hypothesize that mutations of human SIX1 and EYA1 likely contribute to the pathogenesis of del22q11 syndrome and that the pathogenesis of human BOR and del22q11-like syndromes may be linked.

### Table 3

Eya1 functionally interacts with Tbx1 in vivo

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Abnormal</th>
<th>IAA-B</th>
<th>RRSA</th>
<th>VR</th>
<th>CA</th>
<th>PTA</th>
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<tbody>
<tr>
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<td>0%</td>
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<td>0%</td>
<td>0%</td>
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<td>17%</td>
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<td>8%</td>
<td>0%</td>
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<td>26.7%</td>
<td>46.7%</td>
<td>6.7%</td>
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<td>23%</td>
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</tr>
<tr>
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<td>0%</td>
<td>40%</td>
<td>0%</td>
<td>0%</td>
<td>100%a</td>
</tr>
</tbody>
</table>

CA, cervical aortic arch; RRSA, retroesophageal right subclavian artery; VR, vascular ring. Statistical analyses were done by Fisher exact test. *P < 0.05.

### Methods

**Knockout mice and histological analyses.** SIX1 for haplo was generated using the same targeting strategy as previously described (35). SIX1/EYA1 compound mutants were generated by crossing SIX1−/− and EYA1−/− mice (36), and all mutants were confirmed by PCR. Fgfbcre/+; Fgf8cre/+; R26RlacZ, and R26RlacZCre reporter mice were reported previously (20, 25, 32, 67). All mice were maintained on C57BL6, 129sv, or CD-1 mixed genetic backgrounds. Heads, hearts, and aortic arch arteries were dissected, embedded in OCT, sectioned, and stained with H&E using standard procedures. All animal studies were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at the Children’s Hospital Boston.

**Gene expression analyses.** Whole-mount RNA in situ hybridization with digoxigenin-UTP-labeled riboprobes and specific gene probes was performed as previously described (35, 67–69). For quantitative PCR analyses, relative gene expression levels were normalized to a GAPDH internal control from the E9.5 pharynx and heart, analyzed using the SYBR Green method on an ABI-7500 detector (Applied Biosystems).

**Immunohistochemistry and TUNEL assay.** Staged embryos were dissected, fixed in 4% paraformaldehyde for 1–2 hours at 4°C, and then sectioned at 8–10 μm. Anti-PECAM1 (BD Biosciences), cardiac Troponin T (Thermo scientific), WT1, NKX2-5, GATA4 (Santa Cruz Biotech), ISLET1 (DSHB), Eya1 (Eya1 mutant mice and histological analyses), and Fgf10 (Rockland) were all incubated at 1:100 in blocking solution, except for anti-β3 (Upstate), which was incubated at 1:200. TUNEL assay was performed on cryostat sections according to the manufacturer’s instructions (Roche). The number of pH3+ or TUNEL+ cells was averaged from 3 sections per embryo. In total, 3 embryos per genotype were quantified.

**Whole-mount and section β-galactosidase and hpAP staining.** As described previously (35, 68), we detected β-galactosidase activity and hpAP activity in whole-mount embryos using X-gal and BM purple substrate, respectively.

**In vivo ChIP.** ChIP was performed as described previously (67, 70). PAs and cardiovascular tissues from 40 E9.5 embryos were used for each ChIP experiment. Normal serum (IgG) and nonspecific Fgf8 intragenic region were used as negative controls. Anti-Six1 antibody (Sigma-Aldrich) specific oligos for real-time PCR reactions were as follows: Fgfb promoter (con2) forward, 5′-CTGTGAGGGTGTTGGGGACACGTG-3′; Fgfb promoter (con2) reverse, 5′-CGAGTGGTTGATCCCCGGGCTC-3′; Fgfb negative control (exon5) forward, 5′-ACCGGGGCTGCAAGCCAGCAG-3′; Fgfb negative control (exon5) reverse, 5′-CGGCTCTGCTGTTGGTTGGTG-3′. 3 independent ChIPs with triplicate real-time PCR reactions were performed per experiment.

**Luciferase reporter assays.** HEK293 cells were transfected with the designated plasmids (100 ng/plasmid/well, 24-well plate) using Lipofectamine 2000 (Invitrogen). Transfected cells were collected and lysed 48 hours after transfection with 50 mM Tris-MES, pH 7.8; 1 mM DTT; and 0.1% Triton X-100. The resulting cell lysates were measured using a Sirius Single Tube Luminometer (Berthold Detection Systems) according to the manufacturer’s instructions. Results represent 3 independent assays, each having 3 replicates.
OFT explant culture. The OFT explants were cultured as previously described (26, 31, 57). OFTs of E9.5 embryos were microdissected and divided into halves along the longitudinal axis from proximal to distal. For the rescue experiments, 50 ng/ml mouse Fgf8b (R&D Systems) or vehicle solution (0.1% mg/ml BSA in PBS) was added. Cell migration distance from the edge of the explant was measured at 8 different locations every 45° and then averaged. The number of migrating cells was normalized to the explant area. Data represent mean ± SEM (n = 3). Phenotypic data were analyzed with Fisher exact test. P values less than 0.05 were considered statistically significant.

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