

# Unraveling the genetic and developmental mysteries of 22q11 deletion syndrome

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**Birth defects occur in nearly 5% of all live births and are the major cause of infant mortality and morbidity. Despite the recent progress in molecular and developmental biology, the underlying genetic etiology of most congenital anomalies remains unknown. Heterozygous deletion of the 22q11.2 locus results in the most common human genetic deletion syndrome, known as DiGeorge syndrome, and has served as an entry to understanding the basis for numerous congenital heart and craniofacial anomalies, among many other defects. Extensive human genetic analyses, mouse modeling and studies of developmental molecular cascades involved in 22q11 deletion syndrome are revealing complex networks of signaling and transcriptional events that are essential for normal embryonic development. Armed with this knowledge, we can now begin to consider the multiple genetic 'hits' that might contribute to developmental anomalies, some of which could provide targets for *in utero* prevention of birth defects.**

Monoallelic microdeletion of chromosome 22q11.2 (del22q11) occurs with an incidence of 1:4000 live births and is the most common human genetic deletion syndrome (reviewed in Refs [1,2]). Recognition that DiGeorge syndrome (DGS), velo-cardio-facial syndrome (VCFS) and conotruncal anomaly face syndrome (CAFS) have overlapping clinical presentations and share similar deletions of 22q11.2 revealed a common etiology of these clinical entities, together known as 22q11 deletion syndrome (22q11DS). Over the past decade, the combined efforts of genetic and developmental biology approaches have begun to clarify the molecular mechanisms that contribute to the spectrum of birth defects found in 22q11DS. As is often the case, there is hope that the lessons learned from this syndrome will reveal the basis for normal and abnormal development of the affected structures, broadening the relevance of these studies to isolated, nonsyndromic defects in children. Recent progress in understanding the molecular cascades contributing to features of 22q11DS has led to much excitement that we might soon be able to understand the genetic

susceptibilities that predispose individuals to a variety of birth defects. Here, we review the recent advances that have begun to reveal the human genetic, developmental biology and molecular biology underpinnings of this common syndrome.

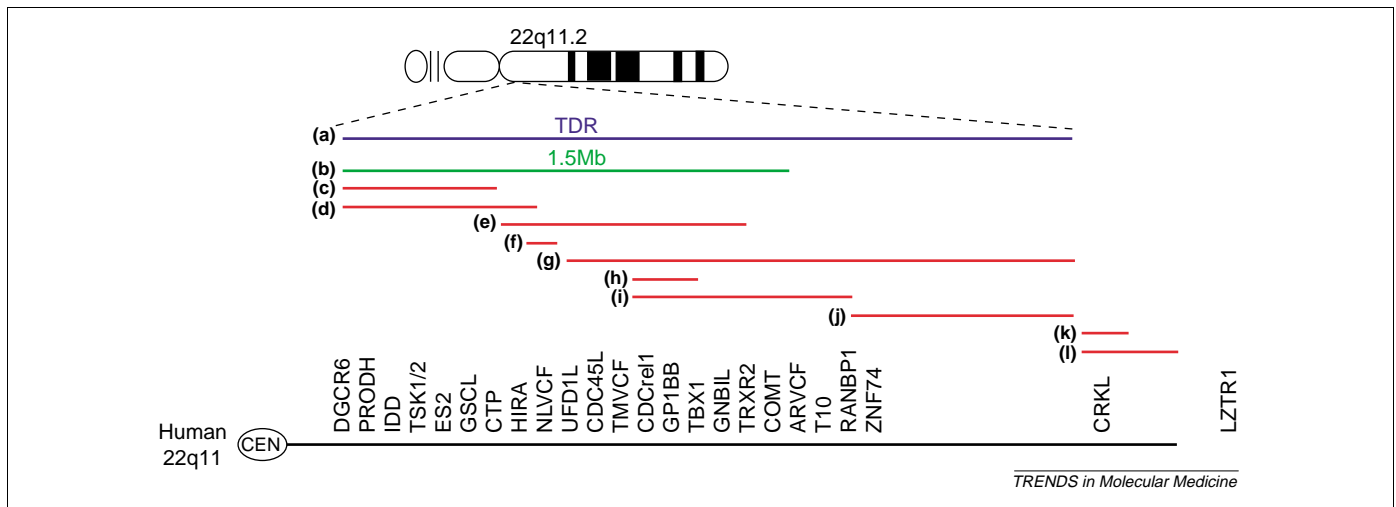
## Clinical features of 22q11DS

The clinical findings associated with del22q11 are highly variable. Approximately 75% of patients with 22q11DS are born with congenital heart defects (CHD), mainly of the cardiac outflow tract and aortic arch. Other common features of 22q11DS include a characteristic facial appearance, immunodeficiency from thymic hypoplasia, velopharyngeal dysfunction with or without cleft palate, hypocalcemia as a result of hypoparathyroidism, developmental and behavioral problems, and psychiatric disorders in adulthood.

22q11DS has attracted attention as a model for investigating the genetic and developmental basis for many birth defects because of the high incidence and association with numerous clinical features. In particular, del22q11 is the most common cause of several CHDs, including (1) tetralogy of Fallot, characterized by malalignment of the major vessels with the ventricular chambers, (2) interruption of the aortic arch type B, which results from maldevelopment of the left fourth pharyngeal arch artery, and (3) persistent truncus arteriosus (PTA), which results from failure of cardiac outflow tract (conotruncus) septation into the aorta and pulmonary artery. Although CHDs are the major cause of mortality in 22q11DS, survivors have an exceptionally high incidence of schizophrenia and obsessive-compulsive disorder (OCD), making del22q11 the most frequent genetic cause of such psychiatric diagnoses.

## Molecular genetics of 22q11DS

Despite heterogeneous clinical presentations, remarkably homogenous deletions in the 22q11.2 region are present in patients with 22q11DS. Approximately 90% of patients have a typical deleted region (TDR) of ~3 Mb, which encompasses an estimated 30 genes, whereas ~8% of patients have a smaller deletion of ~1.5 Mb, which contains 24 genes (reviewed in Refs [2,3]). Recent molecular studies have described low copy repeat



**Fig. 1.** Deletions of 22q11 associated with the DiGeorge phenotype. The positions of reported microdeletions in the 22q11 locus: (a) typical deleted region (TDR) of 3 Mb; (b) 1.5 Mb deletion; (c–l) atypical deletions. The organization of candidate genes in the 22q11 locus is shown.

sequences in the 22q11 region (LCR22), which flank the 3.0 Mb and the 1.5 Mb deletion region. Unequal recombination events between flanking LCRs can explain the uniform recurrence of deletions of uniform size [4]. A small number of atypical, non-overlapping deletions have also been reported, all with similar phenotypes, suggesting that deletions might have long-range chromosomal effects.

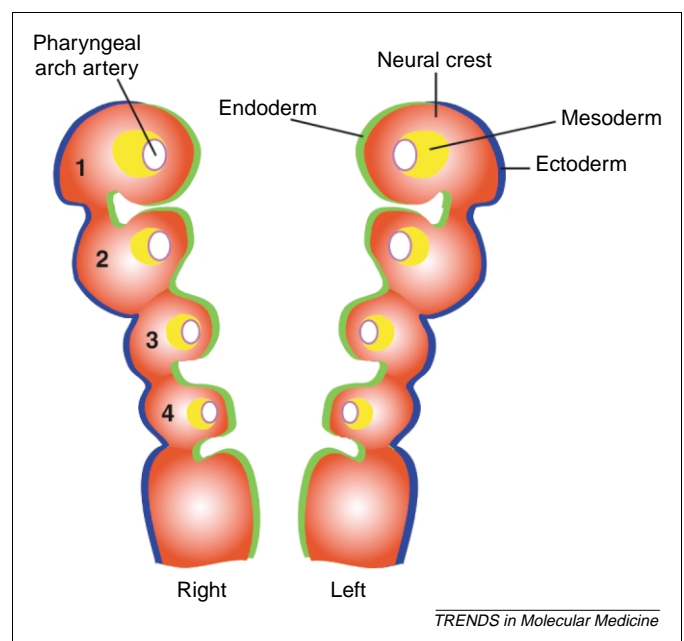
The basis of phenotypic variability in 22q11DS remains unknown. There is poor genotype–phenotype correlation [5–7], with phenotypic variability typical even in familial cases with identical deletions, including monozygotic twins [8,9] (Fig. 1). Proposed explanations for the phenotypic diversity include allelic variability of a genetic modifier(s), variable penetrance or variable expressivity caused by environmental factors or stochastic events during fetal development.

### Developmental biology of 22q11DS

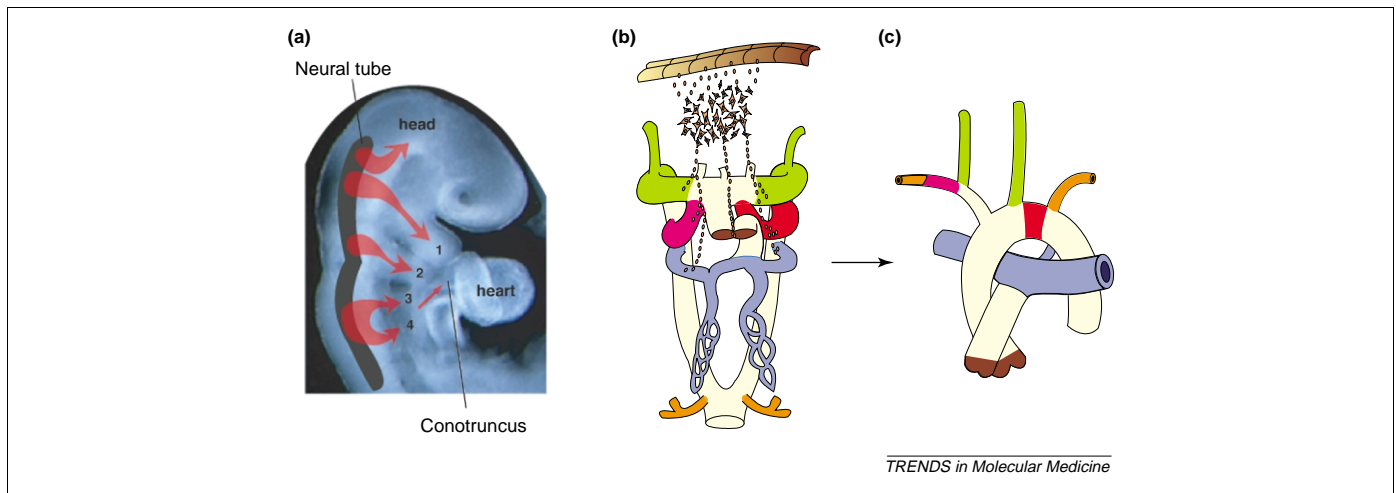
The structures primarily affected in patients with 22q11DS are derivatives of the embryonic pharyngeal arches and pouches, suggesting that 22q11DS is a developmental field defect of the pharyngeal apparatus. Pharyngeal arches are bilaterally symmetric structures that develop in a segmental fashion along the anterior–posterior axis and are composed of several different embryonic cell types (reviewed in Ref. [10]). Each arch has an outer covering of ectoderm, an inner covering of endoderm and a central core of mesoderm adjacent to a pharyngeal arch artery (Fig. 2). Cephalic neural crest cells migrate from the neural folds in the caudal hindbrain to each pharyngeal arch, where they form the mesenchyme that surrounds the pharyngeal arch arteries and the mesodermal core. The mesoderm gives rise to facial and neck muscles, as well as the cardiac outflow tract. The neural-crest-derived mesenchyme contributes to skeletal structures of the face as well as to cardiovascular structures. Pharyngeal pouches, formed by the endoderm-derived epithelial layer, provide precursors to craniofacial organs, including the thymus and parathyroid glands, whereas pharyngeal clefts, formed by the ectoderm-derived

epithelial layer, are precursors for the epidermis and the sensory neurons of the arch-associated ganglia.

Neural crest cells that migrate into third and fourth pharyngeal arches and the cardiac outflow tract (conotruncus) are known as cardiac neural crest cells (reviewed in [11]). They contribute to remodeling of the six pairs of bilaterally symmetric pharyngeal arch arteries that eventually results in formation of the ascending aorta, proximal subclavian, carotid and pulmonary arteries. Cardiac neural crest cells are also necessary for septation of the truncus arteriosus into the aorta and pulmonary artery, as well as formation of part of the ventricular septum (Fig. 3). Disruption of pathways implicated in neural crest development in mouse, such as ones dependent on *Pax3* and *endothelin-1*, can result in malformations reminiscent of 22q11DS [12–14].



**Fig. 2.** Pharyngeal arch development. Pharyngeal arches from embryonic day 10.0 mouse embryo in frontal cross-section. Endodermal and ectodermal epithelial linings are indicated in green and blue, respectively. Mesodermal cells are shown in yellow and neural-crest-derived mesenchyme is in red.



**Fig. 3.** Neural crest in cardiac and pharyngeal arch development. (a) Neural crest cells migrate from the neural folds and populate the pharyngeal arches (1–4) (red arrows). Some mesenchymal cells continue to migrate into the conotruncus, surround the aortic arch arteries, and contribute to their remodeling. (b) Mesenchymal cells originating from the neural folds contribute to the bilaterally symmetrical third (green), fourth (pink and red) and sixth (purple) aortic arch arteries as they arise from the heart (removed in this cartoon). (c) The origins of the mature aortic arch and pulmonary artery, color-coded as in (b). Orange segments in (b) and (c) represent the seventh intersegmental arteries and their derivatives.

### Mouse models for 22q11DS implicate *Tbx1* in aortic arch anomalies

Approximately 30 genes reside in the crucial 22q11.2 locus; however, direct sequencing has failed to detect mutations in any candidate genes in patients with the DGS or VCFS phenotype who lack a cytogenetic aberration [2,3]. *HIRA*, *UFD1L* and *CRKL* have been suggested as candidate genes but are not haploinsufficient in mice, although they might be important as modifier genes and could contribute to disease in humans [15–24].

Recent genome manipulation in mice has provided more-definitive insight into the role of genes in the 22q11 locus [3]. Elegant efforts by several groups to model del22q11 in mice by creating orthologous chromosomal deletions were successful in reproducing at least the aortic arch defects of 22q11DS [22,25]. Subsequent complementation experiments suggested that disruption of the T-box-containing transcription factor, *Tbx1*, was responsible for the aortic arch phenotype. Heterozygosity of *Tbx1* alone also caused aortic arch defects [23,25], whereas homozygosity of *Tbx1* resulted in most of the cardiac and pharyngeal arch defects observed in the 22q11DS, including conotruncal defects, abnormal facial features, cleft palate and hypoplasia of the thymus and parathyroid glands [26].

### *Tbx1* expression in pharyngeal arches

*Tbx1* belongs to a family of proteins containing a T-box necessary for DNA binding and protein–protein interactions. In mouse and chick embryos, *Tbx1* is preferentially expressed in the pharyngeal arches, the ventral half of the otic vesicle, and in the head [23,25–28]. Within the pharyngeal arch region, *Tbx1* is expressed in the pharyngeal mesoderm, pharyngeal endoderm and head mesenchyme. Surprisingly, *Tbx1* transcripts were not detectable in the neural-crest-derived mesenchyme of the pharyngeal arches nor in primary culture of chick neural crest cells [28]. These results suggest that defects of

neural-crest-derived tissues in 22q11DS could occur in a non-cell-autonomous fashion.

Numerous recent studies have demonstrated that reciprocal epithelial–mesenchymal signaling is essential for proper development of the pharyngeal arches, and that primary impairment of epithelial endoderm might secondarily affect migration or differentiation of neural crest cells during pharyngeal arch development [14,29–31]. Importantly, mutations of genes expressed in either pharyngeal neural crest or non-neural crest cells can result in similar aortic arch defects in mice. Many studies have thus focused on the signals that mediate communication between the neural crest, endodermal and mesodermal cells in the pharyngeal arches to uncover the tissue interactions underlying 22q11DS.

### *Tbx1* in pharyngeal endoderm and aortic arch patterning

Recently, three independent studies reported a possible genetic link between fibroblast growth factor 8 (FGF-8), expressed in the pharyngeal endoderm, and the 22q11DS phenotype [32–34]. In chick and zebrafish, *Fgf8* is important for craniofacial [35,36] and cardiovascular development [37,38]. Analyses of mouse mutants hypomorphic for *Fgf8* demonstrated the array of cardiovascular and craniofacial phenotypes seen in 22q11DS, possibly from a neural crest survival defect [32,33]. Interestingly, *Fgf8* is downregulated in the pharyngeal endoderm of *Tbx1* mutants, and mice trans-heterozygous for both *Tbx1* and *Fgf8* have a higher penetrance of aortic arch defects than *Tbx1* heterozygotes [34,39,40]. These observations support a model in which *Tbx1* somehow regulates *Fgf8* in the pharyngeal endoderm, which in turn signals to adjacent neural crest cells and promotes aortic arch patterning through epithelial–mesenchymal interaction.

The role for *Tbx1* in pharyngeal endoderm development is conserved across species. The zebrafish mutant *van gogh* is characterized by failure of pharyngeal endoderm segmentation and is caused by a mutation in the fish ortholog of *Tbx1* (T. Piotrowski, pers. commun.). Although

many of the downstream genes affected by disruption of *Tbx1* differ between mouse and fish, the overall similarity in pharyngeal arch phenotype is striking.

### **Tbx1 in cardiac outflow tract development**

During heart development, there is a common outflow tract (truncus arteriosus) that is invaded by neural crest cells, resulting in septation into separate aortic and pulmonary vessels. Homozygous mutation of *Tbx1* causes failure of outflow septation, leading to a PTA at term [26,39], suggesting a crucial role of *Tbx1* in outflow tract development.

The cardiac outflow tract originates from a pool of mesodermal cells distinct from those that form the cardiac chambers. A population of myocardial precursor cells in chick and mouse embryos originates in the pharyngeal mesoderm anterior to the early heart tube, and is known as the secondary or anterior heart-forming field (AHF) [41–43]. Migration of cells from the AHF into the outflow region of the mouse heart occurs at embryonic day 8.25–10.5 and gives rise to myocardium of the outflow tract. *Fgf10* is expressed in cells of the AHF as well as in the mesodermal core of the pharyngeal arches and might function in regulating this event [43]. Interestingly, the expression of *Tbx1* represented by *lacZ* inserted in the *Tbx1* locus [39] or by *lacZ* under control of the upstream *Tbx1* regulatory region [44] overlaps that of *Fgf10* in the pharyngeal mesoderm and AHF, suggesting that *Tbx1* is expressed in cells derived from the AHF during early outflow tract development.

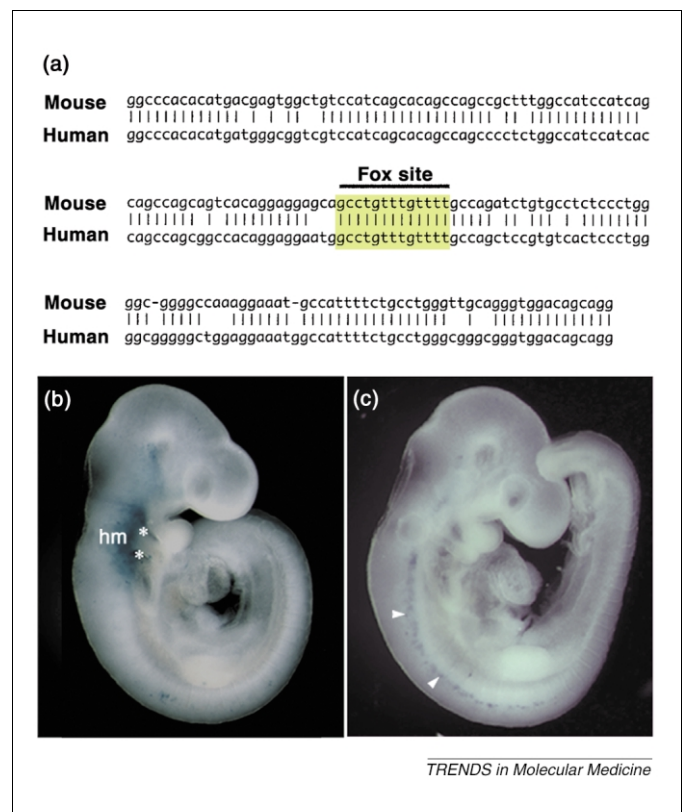
It is tempting to speculate that *Tbx1* and *Fgf10* function in a common pathway during development of the outflow tract by regulating the adjacent neural crest that is necessary for septation. The expression of *Fgf10* is down-regulated in the pharyngeal mesoderm of *Tbx1* mutants, consistent with this hypothesis [39,40]. Evidence for a primary *Tbx1*-dependent defect in the conotruncus comes from the reduced conotruncal diameter as early as embryonic day 9.5 in *Tbx1* mutants, before neural crest cell migration [39]. We have recently established mouse mutants hypomorphic for *Tbx1*, which display only a subset of the defects observed in *Tbx1*-null mice. Specifically, conotruncal and third and fourth pharyngeal arch defects, including PTA and hypoplasia of thymus, were observed, but not first and second pharyngeal arch defects, such as mandibular hypoplasia and cleft palate (T. Hu *et al.*, unpublished). These observations suggest that the cardiac outflow tract is more susceptible to reduction of *Tbx1* gene dosage than is craniofacial development. This is consistent with human disease, in which 75% of patients with 22q11DS have cardiac defects but only 10–30% suffer from overt clefts of the hard palate.

### **Molecular regulation of *Tbx1***

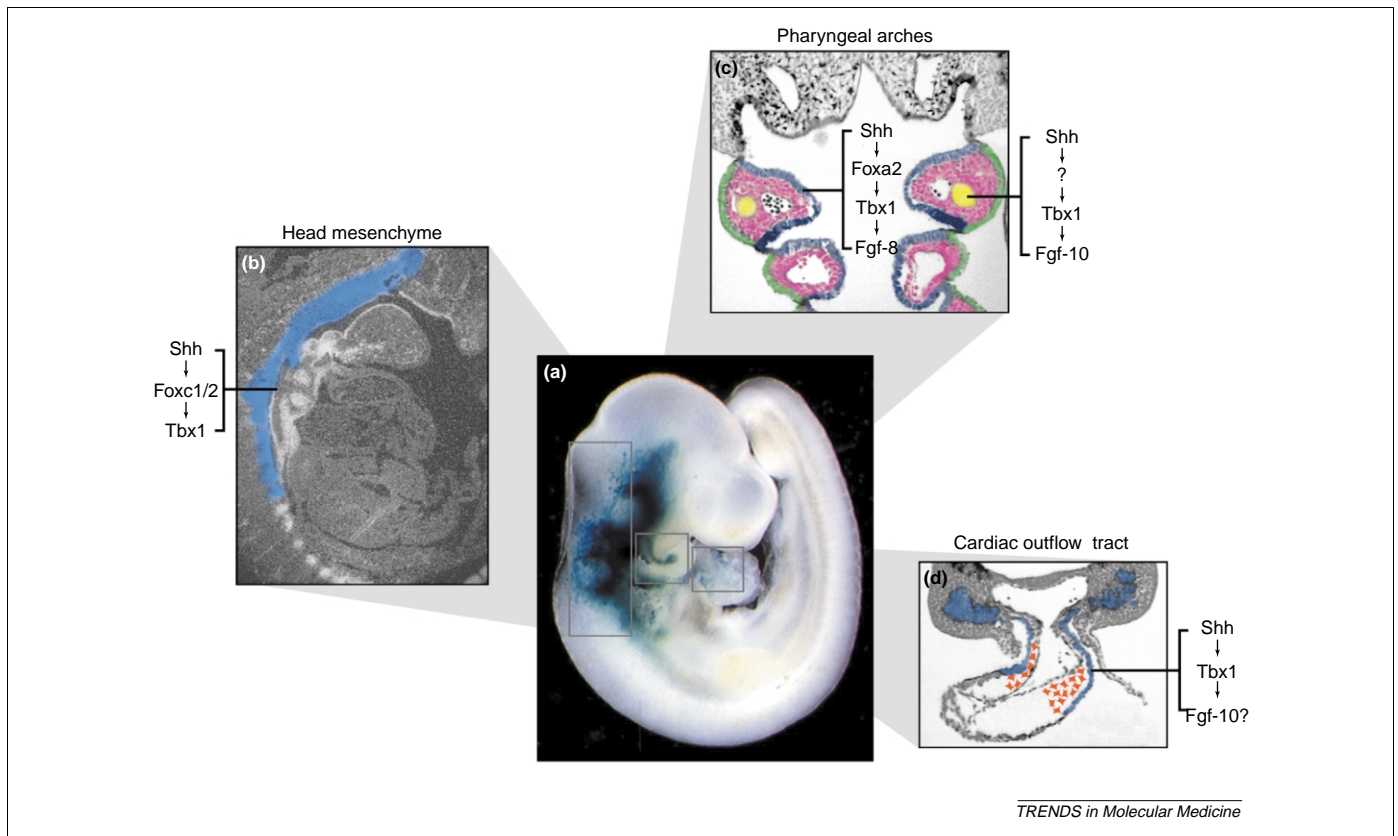
Because the *Tbx1* dose is particularly important for developmental events, alterations in *Tbx1* gene regulation could contribute to disease. We have therefore investigated a signaling cascade involving sonic hedgehog (Shh) that culminates in *Tbx1* transcription [28,44]. Shh, a vertebrate ortholog of the *Drosophila* segment polarity gene *Hedgehog*, is essential for normal development of many

organs, including the limb, lung, nervous system and cardiovascular system (reviewed in [45]). Shh is expressed in numerous tissues, including the pharyngeal arch epithelium, and *Shh*-mutant mice die soon after birth with severe craniofacial defects [46,47]. In the absence of Shh, *Tbx1* expression in the pharyngeal arches is not maintained; conversely, chick embryos exposed to Shh beads respond by upregulating *Tbx1* expression in the pharyngeal arches [28]. Recently, we identified a genomic regulatory region that controls the *in vivo* expression of *Tbx1* and responds to Shh signaling in promoting maintenance, rather than induction, of *Tbx1* expression in a tempero-spatial fashion [44]. Detailed analysis of *Shh* mutant embryos demonstrated similar types of aortic arch patterning defects compared with *Tbx1* mutant embryos, consistent with the notion that Shh and *Tbx1* function in a common molecular pathway for aortic arch development [44]. The pharyngeal arch defects in *Shh* mutants are not as severe as *Tbx1* mutants, consistent with our observations that other signals are also involved in *Tbx1* regulation.

A single *cis*-element upstream of *Tbx1* that recognizes winged helix/forkhead box (Fox)-containing transcription factors [48,49] is essential for regulation of a *lacZ* reporter in the pharyngeal endoderm and head mesenchyme domains of *Tbx1* expression [44] (Fig. 4). *Foxa2*, required for endoderm development [50,51], and *Foxc1* or *Foxc2*,



**Fig. 4.** A winged helix/forkhead box (Fox)-binding *cis*-element is essential for endoderm and head mesenchyme regulation of *Tbx1*. (a) The 200 bp genomic sequence conserved between human and mouse with the Fox-binding element (FBE) highlighted in yellow. (b) *LacZ* expression directed by the 200 bp regulatory sequence in transgenic mice recapitulates endogenous *Tbx1* expression in the head mesenchyme (HM) and pharyngeal endoderm (\*) of embryonic day 9.5 embryos. (c) Mutation of the FBE abolishes *lacZ* expression in the HM and pharyngeal endoderm of transgenic mice. White arrowheads indicate ectopic expression of *lacZ*.



**Fig. 5.** The involvement of *Tbx1* in cardiac and pharyngeal arch development. (a) Whole-embryo *Tbx1* expression visualized in lateral view. (b–d) Sagittal, coronal and transverse sections of the head mesenchyme, pharyngeal arches and cardiac outflow tract, respectively, are color enhanced to indicate *Tbx1* expression domains (blue or yellow) in head mesenchyme, pharyngeal endoderm and mesoderm, and cardiac outflow tract. Pharyngeal arch and cardiac mesenchymal cells are shown in pink and red, respectively. (b) The sagittal section includes head mesenchyme expression and also pharyngeal arches, which are not highlighted. Sonic hedgehog (Shh) regulates winged helix/forkhead box c (*Foxc*) proteins, which directly control transcription of *Tbx1* in the head mesenchyme. (c) The coronal section of the pharyngeal endoderm indicates that Shh regulates *Foxa2* in the endoderm, which then directly activates *Tbx1* expression (blue epithelium). *Tbx1* is necessary for fibroblast growth factor 8 (*Fgf-8*) secretion from the pharyngeal endoderm into the adjacent neural-crest-derived mesenchyme (pink), where its signal is necessary for proper neural crest differentiation. Pharyngeal ectoderm is indicated in green. Similar *Tbx1*-dependent signals might emanate from the pharyngeal mesoderm (yellow circles), cells of which contribute to the cardiac outflow myocardium. These signals might function non-cell-autonomously on the adjacent neural-crest-derived outflow mesenchyme, similar to the interactions between the pharyngeal endoderm and neural crest. (d) The transverse section of the cardiac outflow tract demonstrates *Tbx1* expression in the myocardium of the outflow tract and pharyngeal mesoderm. Signals from the myocardium, regulated by Shh, *Tbx1* and possibly *Fgf-10*, might modulate adjacent mesenchymal cells (red).

required for head mesenchyme and aortic arch formation [52–56], bind and activate transcription in their respective subdomains through this regulatory element. We therefore 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 (Fig. 4).

*Foxc1* and *Foxc2* are redundant transcription factors that are expressed in the head mesenchyme and in the 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 [55,56] strikingly similar to *Tbx1* heterozygotes is consistent with our data that *Foxc* proteins and *Tbx1* function in a common program. In addition, ~15% of patients with *FOXC2* mutations, a cause for lymphedema–distichiasis syndrome, have conotruncal cardiac defects [57].

*Tbx1* is the first direct target gene identified for *Foxc1* or *Foxc2* during development, and the downregulation of *Tbx1* in the mesenchyme of *Foxc1*<sup>-/-</sup> or *Foxc2*<sup>-/-</sup> embryos provides a potential mechanism for the aortic arch defects observed in *Foxc* mutants. Moreover, Shh is necessary for expression of *Foxa2* and *Foxc2* in the

pharyngeal endoderm and head mesenchyme, respectively, suggesting an intermediary role for *Foxa2* and *Foxc2* in Shh regulation of *Tbx1* [44]. Interestingly, disruption of the signaling pathway mediated by an isoform of vascular endothelial growth factor (*Vegf*)<sub>164</sub> affects *Tbx1* expression and also results in a 22q11DS phenotype in mice [58]. Because Shh is a regulator of *Vegf* in endothelial cells, it will be interesting to determine if Shh signals via *Vegf* to regulate *Tbx1* in the pharyngeal arches or cardiac outflow tract [59]. Together, these studies have revealed a cascade of events that culminates in transcription of *Tbx1* and provides an understanding of the events involved in development of the pharyngeal and aortic arch derivatives and some features of 22q11DS (Fig. 5).

#### Unresolved questions

It is a significant finding that *TBX1* haploinsufficiency is likely to be the major determinant of aortic arch defects in patients with 22q11DS. Although mutational analysis of the coding region of *TBX1* in over 200 non-deleted patients failed to identify obvious disease-causing *TBX1* gene mutations [23,60], a few amino acid variations were recently found in highly selected phenotypes (R. Matsuoka, pers. commun.). The functional significance of these

mutations remains to be described but provide hope for definitive evidence implicating TBX1 in 22q11DS.

Although TBX1 is presently the strongest candidate for many features of 22q11DS, animal models for 22q11DS suggest that haploinsufficiency of *TBX1* cannot solely account for the entire clinical presentation of 22q11DS [23,25,26]. It remains possible that the cumulative effect of several genes within the 22q11 region results in the full del22q11 phenotype. These include HIRA and UFD1L, which are expressed in the pharyngeal arches and are essential for survival, and CRKL, which causes a 22q11DS phenotype in homozygous-null mice. In addition, *COMT* and *PRODH*, which encode catechol-O-methyltransferase and proline dehydrogenase, respectively, might be involved in the behavioral and psychiatric phenotype of 22q11DS [61,62]. *Comt* mutant mice display impairment in emotional behavior, whereas *Prodh* mutant mice have a deficit in sensorimotor gating, a neural-filtering process that allows attention to be focused on a given stimulus. Defects in this process are fundamental to the pathogenesis of schizophrenia, OCD and other psychiatric disorders common to 22q11DS, and are phenocopied in mice with a large deletion of the 22q11 locus [63].

The contribution of modifier genes and other 22q11 genes, as well as the genetic interaction between these genes and *Tbx1*, remain to be studied. Genes such as *Shh*, *Vegf*, *Fgf8* and *Foxc2* might modify the phenotype because they function in a common pathway with *Tbx1* in cardiovascular and pharyngeal arch development.

### Concluding remarks

In the coming years, the molecular and developmental basis of 22q11DS will become clearer by identification of TBX1 target genes, elucidation of molecular pathways controlling *TBX1* expression, and understanding the combinatorial interactions through which TBX1 regulates transcription. Although the advances described here indicate that the Shh–Fox–Tbx1–Fgf signaling pathway is likely to play a crucial role in 22q11DS, whether *Tbx1* directly or indirectly regulates *Fgf8* or *10* remains to be studied. The *cis* and *trans* regulators of *Tbx1* in pharyngeal mesoderm and AHF remain unknown and might reveal important information regarding conotruncal defects in 22q11DS. A multifaceted approach combining information from biochemical studies, animal models and human genetic studies will develop a deeper understanding of 22q11DS and establish the foundation for future preventive and therapeutic intervention of multiple human birth defects.

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