T-box factors determine cardiac design

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Abstract. The heart of higher vertebrates is a structurally complicated multi-chambered pump that contracts synchronously. For its proper function a number of distinct integrated components have to be generated, including force-generating compartments, unidirectional valves, septa and a system in charge of the initiation and coordinated propagation of the depolarizing impulse over the heart. Not surprisingly, a large number of regulating factors are involved in these processes that act in complex and intertwined pathways to regulate the activity of target genes responsible for morphogenesis and function. The finding that mutations in T-box transcription factor-encoding genes in humans lead to congenital heart defects has focused attention on the importance of this family of regulators in heart development. Functional and genetic analyses in a variety of divergent species has demonstrated the critical roles of multiple T-box factor gene family members, including Tbx1, -2, -3, -5, -18 and -20, in the patterning, recruitment, specification, differentiation and growth processes underlying formation and integration of the heart components. Insight into the roles of T-box factors in these processes will enhance our understanding of heart formation and the underlying molecular regulatory pathways.

Keywords. T-box transcription factor, Tbx, heart, patterning, heart fields, progenitor populations, chamber, conduction system.

Introduction

The heart of vertebrates is a unidirectional muscular pump that propels blood by means of synchronized contractions of two (fish) or four (mammals, birds) chambers, while the one-way valves prevent return flow [1]. In contrast to most other organs, the heart of higher vertebrates must function to supply the embryo and the extra embryonic membranes with blood from the moment it forms, pumping first in a peristaltic wave along the length of the primitive heart tube, before ballooning, looping and transforming into the complex four-chambered heart with its regular paced beat contraction. To accomplish this, the morphogenetic and functional requirements of the heart have been integrated into a perfectly coordinated genetic program underlying heart formation. Several key transcription factors involved in this genetic program have been identified, including Nkx2-5 and members of the Mef, Gata, Hand, Iroquois, Forkhead and T-box families of factors [2, 3]. Although functional requirement of these factors has been demonstrated, their precise functions and interactions during cardiac morphogenesis have yet to be defined satisfactorily. Although a few early studies made mention of T-box factor genes expressed in the heart [4, 5], it was the discovery that mutations in T-box factor TBX5 lead to Holt-Oram syndrome, a disease associated with congenital heart defects of differing severities, that initiated a series of studies to unravel the function of this family of transcription factors in heart development [6, 7]. The T-box family presently counts 17 members in mouse and humans [8]. Members of the Tbx1 subfamily, Tbx1, Tbx18 and Tbx20, and of the Tbx2 subfamily, Tbx2, Tbx3 and Tbx5, have all been found to play specific roles in heart development [8–10].

The embryonic heart is a relatively simple tube emerging from two sheets of cardioblasts derived from visceral mesoderm that fuse at the midline.

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Despite decades of experimental evidence that the myocardial cells of the initial heart tube represent only the precursors of a small part of the mature heart, a tubular heart containing antero-posteriorly aligned segments of precursor cells of all mature structures has been the paradigm for developmental patterning until recently. Two new insights have invoked a shift in conception. First, direct lineage evidence has been provided that non-cardiac progenitor cells peripheral to the initial heart tube make extensive contributions to the heart [11] (Fig. 1). Second, chambers differentiate and expand locally at positions along the heart tube in a non-segmental fashion, a process requiring antero-posterior as well as ventro-dorsal patterning [12] (Fig. 2). T-box factors play very prominent roles in both these processes. In this review, we will provide an update on T-box factor function in heart development, and discuss these functions in the context of novel insights that have significantly increased our appreciation and understanding of heart development.

Cardiac T-box proteins and their partners

T-box factors take on a major role in guiding transcription events during cardiac development, and their combinatorial T-box protein interaction events must be seen as key to determining the individual fate each cell ultimately derives. For example, the combinatorial activities and interactions of Tbx5, Tbx20, Nkx2-5 and Gata4 lead to cardiac chamber differentiation of which a molecular signature is the upregulation and expression of Nppa (Natriuretic Precursor Peptide type A, also known as ANF). Inclusion of Tbx2 and Tbx3 in this network leads to Nppa suppression, notably in the atrioventricular canal, signifying an apparent suppression of differentiation [8, 12]. Attempts then to examine the function of T-box factors at the molecular level often turn to techniques that seek to identify interacting protein partners. Perhaps one of the better-known examples was the use of the yeast two-hybrid system by Hiroi and co-workers [13] in conjunction with the homeodomain-containing transcription factor Nkx2-5. Using Nkx2-5 as bait in a human complementary DNA (cDNA) library screen Tbx5 was identified as interacting partner, the synergistic interaction of which was shown to be a key event in the differentiation of working myocardium [13, 14]. Mutations which interfere with either the capacity of Tbx5 to bind DNA or its ability to interact with Nkx2-5 can cause human Holt-Oram syndrome [15–17]. Similarly, a synergistic interaction between Tbx1 and Nkx2-5 has recently postulated to play a role in another human congenital disease displaying varying heart malformations, DiGeorge syndrome [18]. Although on its own, in vitro, Nkx2-5 appears to function as a moderate transcriptional activator, its ultimate activity as an activator or repressor depends on interaction with the T-box factors present in the same cell [12]. One may then hypothesize that Nkx2-5 acts as a T-box guidance factor instead of a direct transcriptional activator or repressor. Studying congenital defects can itself represent a ‘natural’ experimental setting to discover and elucidate novel protein interactions. One such study involving isolated septal defects leads to the inclusion of GATA4 as a new synergistic interacting partner of Tbx5, an interaction shown to play a role in the expression of Nppa [19]. In our lab we have also demonstrated an in vitro interaction between GATA4...
and Tbx2/3 [unpublished observations], which taken together with the described interaction of Tbx20 with GATA4 [20, 21] suggests that in the case of overlapping expression of T-box proteins, the stoichiometry of T-box proteins and the restricted presence of other positive or negative regulatory factors will ultimately decide timing and direction of cellular fate. In line with this, several other protein interacting partners have been assigned to the Tbx5 list [22–24]. The cardiac-enriched MYST family histone acetyltransferase TIP60 and Tbx5 were observed to be mutual interactive cofactors through the TIP60 zinc finger. In transfection assays, TIP60, Tbx5 and Tbx2 activate an enhancer in the SRF gene required for expression in the developing heart. The zinc-finger-containing protein Sall4, as well as being regulated by Tbx5, takes on a dual role, functioning co-operatively with Tbx5 and Nkx2-5 to upregulate Cx40 (Gja5) and Fgf10 and antagonistically to downregulate certain genes such as Nppa. Notably, the presence of Tbx2/3 can completely block the co-operative activity of Sall4 on Tbx5, though the dependence for this activity on DNA binding or protein-protein interaction was not explored. Further, as is observed for the interaction of Gata4 and Nkx2-5 [13, 19], Sall4 is also unable to associate with the Tbx5 mutants Gly80Arg and
Arg237Trp. This may be indicative of a similar mode of interaction, though one must be careful of over-interpreting the effect of point mutations on interactions since even small perturbations in molecular structure can have significant effects on the overall fold of a protein.

TAZ, a WW domain protein, also represents a recently discovered protein interacting partner of Tbx5 [24]. Postulated to function as a co-activator of Tbx5, TAZ and a related protein, YAP, are hypothesized to play a role in cardiac development and hypertrophy. Although expression patterns of TAZ in the heart are not available, an interesting feature of TAZ is its ability to associate with the histone acetyltransferase proteins p300 and PCAF. Co-transfections of Tbx5, TAZ and p300 or PCAF were shown to upregulate the Nppa promoter in vitro. However, the true in vivo significance of this interaction still needs to be tested. This role of epigenetics in cardiac development can be postulated from the discovery that T-box factors can associate with chromatin-modifying proteins. Chromatin structure and function have been known to have a key role in cell lineage patterning for many years, acetylation or deacetylation, for instance, increasing or decreasing the accessibility of transcription factor binding sites, respectively. In line with this, Tbx5 activity is influenced by the presence of the chromatin remodeling protein BAF60c, shown to potentiate an interaction between Tbx5 and the Baf complex ATPase Brgl [25], thereby assisting in upregulation of cardiac differentiation. Likewise, though functioning in a negative fashion, Tbx2 has been shown to recruit and associate with the histone deacetylase HDAC1 [26].

Recently, the PDZ-LIM domain protein LMP4 was found to interact with Tbx5 and to repress its transcriptional activity [27, 28]. LMP4 is localized in the cytoplasm, associated with the actin cytoskeleton. Interaction between Tbx5 and LMP4, which is dynamic and depends on undefined signals, leads to the localization of Tbx5 to actin filaments. LMP4-mediated regulation of Tbx5 nuclear localization is thought to be involved in the transcriptional regulatory activity of Tbx5.

In a review concerning aspects of T-box proteins in respect to cell fate decision, it is interesting to mention the protein-protein interaction of Tbx20 with a marker of the second heart field, Isl1. This Lim/homeodomain protein has so far only been demonstrated to interact directly with Tbx20 [20]. This interaction, together with a synergistic interaction between Gata4 and Tbx20, plays a role in regulating the expression of Nkx2-5 and Mef2c in the precursors of the outflow tract and right ventricle.

Finally, T-box family members Tbx2 and Tbx5, and Tbx5 and Tbx20, respectively, have also been demonstrated to directly interact with each other [22, 29] and demonstrate a synergistic role during heart development. Although T-box dimerization has often been postulated, chiefly on the basis of the Xenopus T protein (Xbra) homodimer crystal structure and DNA binding characteristics of TBX1 [30, 31], these examples represent the first T-box proteins demonstrated to heterodimerize, forming a synergistically functional complex.

The function of T-box-factors in the progenitor populations of the heart

During its development, the embryonic heart tube increases in length severalfold. Paradoxically, the tube itself shows limited proliferation, indicating that the increase must be attributable to recruitment of precursors to the myocardial phenotype [32]. Current estimates in mouse suggest that only the precursors of the future left ventricle, and perhaps part of the future atrioventricular canal and atria, are derived from the first lineage of myocardial cells that comprise the embryonic heart tube (Fig. 1). The remainder of the compartments, the outflow tract, right ventricle and atria, are derived from undifferentiated precursor cells present outside the heart tube. This population has been referred to as the second lineage or second heart field and contains subpopulations referred to as secondary heart field and anterior heart field [3, 11]. At 7.5 days of mouse development, this second field is present medially to the cardiac crescent that forms the heart tube (Fig. 1). During and after folding of the embryo, the second field becomes positioned in the pharyngeal mesoderm, the pericardial mesothelium and mesoderm cranially, dorsal and caudal to the tubular heart. This precursor field progressively provides contributions to both poles and to the dorsal wall of the heart tube via the dorsal mesocardium until this structure is disrupted later during development. It is currently unclear whether the fields are discrete entities, or just subpopulations of one large heart precursor field. However, several transcriptional regulators, including Tbx1, have been identified that are selectively expressed in, or are selectively required for a cellular subset of the second heart field. Recent evidence from our laboratory indicates the presence of yet another precursor population with a molecular signature distinct from that of the first and second heart fields (see below). Therefore, a picture emerges of one large precursor pool for the heart comprising subpopulations, such as the first and second heart fields, each with distinctive gene programs, and
providing spatially and temporally segregated contributions to the heart tube.

**Tbx1 and the second heart field**

DiGeorge syndrome, also known as del22q11 syndrome based on a 1.5–3 MB genomic deletion at the 22q11.2 region seen in many patients [33, 34], is a common congenital disorder characterized by a variety of abnormalities, including absence or hypoplasia of the thymus, cleft palate, facial dysmorphism and cardiovascular anomalies such as aortic arch malformation (specifically hypoplasia of 4th pharyngeal arch artery), outflow tract defects and ventricular septal defects. Tbx1-deficient mice phenocopy important aspects of DiGeorge Syndrome, including outflow tract abnormalities [35–37]. Therefore, Tbx1 has a specific function in outflow tract morphogenesis in the developing heart.

Expression of Tbx1 can be detected in pharyngeal endoderm, the mesodermal core of the pharyngeal arches and the second heart field [38–42]. Genetic lineage studies have demonstrated that Tbx1-positive cells in the second heart field provide extensive contributions between E8.5 and E9.5 to the outflow tract myocardium, endocardium and mesenchymal cushions [42–44]. A limited contribution can also be seen in the atria [44], suggesting that Tbx1 is also expressed in the larger second heart field. Tracing of the Tbx1-positive lineage revealed diminished contribution of these cells to the outflow tract in a Tbx1-deficient background. Furthermore, conditional deletion of Tbx1 within the second heart field results in reduced cell proliferation, which may underlie the reduced contributions of the second heart field to the outflow tract in Tbx1-deficient mice [42]. Elegant time-course deletion experiments have further demonstrated that Tbx1 is required for outflow tract development between E8.5/9 and E9.5, coinciding with the secondary heart field progenitors contributing to the outflow tract [42].

Cardiac defects seen in Tbx1-deficient mice are similar to those observed in Fgf8 hypomorphs [45, 46], suggesting that both may act along a common pathway. Fgf8, Fgf10 and Tbx1 are all co-expressed in the second heart field [40–42]. Tbx1 null mice show reduced Fgf8 expression in the outflow tract and reduced expression of both Fgf8 and Fgf10 in the pharyngeal mesoderm [40–42]. Conversely, overexpression of Tbx1 in the myocardium appears to induce Fgf8 and Fgf10 expression, and increase outflow tract myocardium [40]. Furthermore, Tbx1 was found to regulate transcriptional activity of Fgf8 and Fgf10 through association with conserved T-box binding sites in their promoter sequences [40, 42]. Consistent with these findings, conditional deletion of Fgf8 in the Tbx1-positive region resulted in outflow tract defects, suggesting a genetic interaction [43]. Although Fgf8 signaling is indeed crucial for normal development of second heart field progenitors and outflow tract septation, as two recent studies demonstrated [47, 48], another recent study showed that forced expression of Fgf8 in Tbx1 null mice or conditional deletion of Fgf8 in the Tbx1-expressing region did not rescue or change the Tbx1 outflow tract phenotype. This indicates that Tbx1 and Fgf8 act independently in outflow tract development [49].

Pitx2c-deficient mice have outflow tract defects, and recently Pitx2c was identified as a putative target of Tbx1 in a subpopulation of the second heart field [18]. In Tbx1 null mice the expression of Pitx2 is down-regulated in this subpopulation, along with the outflow tract and splanchnic mesoderm. The phenotype of Tbx1+/− Pitx2+− heterozygous mice appeared more severe when compared with single heterozygous mutant mice of either gene, suggesting a genetic interaction. Moreover, Tbx1 binds to a critical Pitx2 enhancer and synergistically induces transcriptional activity of this enhancer together with Nkx2-5, further indicating that Tbx1 and Nkx2-5 act in the same pathway for outflow tract morphogenesis [18].

Regulation of Tbx1 in the second heart field subpopulation has been shown to depend upon the presence of two enhancers that contain conserved binding sites for forkhead transcription factors [40, 44, 50]. The forkhead transcription factors Foxa2, Foxc1 and Foxc2 are able to bind to these sites and to induce transcriptional activity of these Tbx1 enhancers. Furthermore, Foxa2 and Tbx1 co-localize in the second heart field, and Foxa2 has been demonstrated to be a target of Tbx1 [40, 44]. Taken together, these data suggest that Foxa2 and Tbx1 act in a common pathway in the second heart field critical for normal outflow tract development.

**Roles for T-box factors in recruitment and elongation of the heart tube**

Tbx20, of which several splice variants exist, is expressed in the first heart field, in a subset of second heart field progenitors, and in the endocardium and derived mesenchyme of the atrioventricular and outflow tract cushions [20, 21, 51] (Table 1). After looping, the expression of Tbx20 decreases in chamber myocardium compared with atrioventricular and cardiac outflow regions [20, 21]. A recent series of papers showed that mice deficient for Tbx20 die early during development, displaying severely underdevel-
oped, short heart tubes [20, 52–54]. Failure in the deployment or recruitment of the second heart field is likely to be a contributing factor to this phenomenon, as elongation of the heart tube is primarily achieved by recruitment of the extra-cardiac second heart field progenitors rather than by proliferation. Defects in either a cell-autonomous or a non-cell-autonomous process may underlie this problem, though the expression pattern of \( Tbx20 \) is compatible with both.

In \( Tbx20 \) null and RNA interference (RNAi) knock-down embryos the expression of \( Nkx2-5 \) and \( Mef2c \), both required for anterior heart field formation, was found to be moderately to severely downregulated in two studies [20, 54]. RNAi knock-down of \( Tbx20 \) in mice results in hypoplasia of the outflow tract and right ventricle [20], derivatives of the anterior heart field. Furthermore, \( Tbx20 \) was found to synergize with \( Islet1 \) and \( Gata4 \) to activate a \( Mef2c \) anterior heart field enhancer and a \( Nkx2-5 \) cardiac enhancer. Together, these data indicate that \( Tbx20 \) is directly involved in controlling the anterior heart field. Although downregulation of \( Nkx2-5 \) and \( Mef2c \)

### Table 1. Cardiac expression patterns of T-box factors during recruitment and chamber formation (E9.5) and during septation (E12.5).

| E9.5          | Tbx1 | Tbx2 | Tbx3 | Tbx5 | Tbx18 | Tbx20 | Cx40 | Nppa |
|---------------|------|------|------|------|-------|-------|------|------|
| 2nd HF, posterior | –    | +    | +    | –    | –     | –     | –    | –    |
| Caudal progenitors | –    | –    | –    | –    | ++    | –     | –    | –    |
| Inflow tract | –    | +/–  | +    | ++   | –     | +     | –    | –    |
| Atria | –    | –    | –    | +    | +     | +     | +    | +    |
| AVC myocard. | –    | +    | +    | –    | +     | +     | +    | +    |
| AVC mesench. | –    | +    | +    | –    | +     | +     | +    | +    |
| Left ventricle | –    | –    | –    | +    | –     | +     | +    | +    |
| Right ventricle | –    | –    | –    | –    | –     | +     | +    | –    |
| OFT myocard. | –    | +    | –    | –    | –     | +     | –    | –    |
| OFT mesench. | –    | +    | +/–  | –    | +     | +     | –    | –    |
| 2nd HF, anterior | +    | +    | +    | –    | –     | +     | –    | –    |
| Pro epicardium | –    | –    | –    | +    | +     | +     | +/–  | –    |

| E12.5         |      |      |      |      |       |       |      |      |
|---------------|------|------|------|------|-------|-------|------|------|
| Sinus horns | –    | –    | –    | +    | +     | –     | –    | –    |
| Atria | –    | –    | –    | –    | +     | +     | +    | +    |
| Mediastinal myocard. | –    | –    | –    | +    | –     | +     | +    | –    |
| Pulm. vein myocard. | –    | –    | –    | +    | –     | +     | +    | +    |
| Pulm. vein mesench. | –    | –    | –    | +    | –     | +     | –    | –    |
| Sinoatrial node | –    | –    | +    | +    | +     | +/–   | –    | –    |
| Venous valves | –    | –    | +/–  | +    | +     | –     | +    | +    |
| Primary atrial septum | –    | –    | –    | +    | –     | +     | +    | +    |
| AV canal myocard. | –    | +    | +    | –    | +     | –     | –    | –    |
| AVC mesench. | –    | +/2  | +/2  | +/3  | ++    | –     | –    | –    |
| Left ventricle | –    | –    | –    | +/4  | +/5   | +/6   | +/4  | +/4  |
| Interventr. septum | –    | –    | –    | +/7  | +     | +     | +/–  | +    |
| AV bundle | –    | –    | –    | +    | –     | –     | –    | –    |
| Right ventricle | –    | –    | –    | +/8  | –     | +/6   | +/4  | +/4  |
| OFT myocard. | –    | +    | –    | –    | –     | +     | –    | –    |
| OFT mesench. | –    | +/–  | +/–  | –    | +     | +     | –    | –    |
| Intraper. art. trunk | –    | +    | –    | –    | +     | –     | –    | –    |
| Epicardium | –    | –    | –    | –    | –     | +     | –    | –    |

These data have been extracted from [20, 21, 38, 39, 41, 51, 61–63, 74, 107] and our own observations. AV, atrioventricular; Intraper. art. trunk, intrapericardial arterial trunk (non-myocardial); OFT, outflow tract; Pulm., pulmonary. 1, only in small dorsal part; 2, lumen to myocardium gradient; 3, endocardium; 4, higher in trabecular myocardium; 5, restricted to compact myocardium; 6, higher in compact myocardium; 7, left ventricular component is positive; 8, restricted to trabecular myocardium; 9, very weak expression; 10, high in epicardium of AV groove.
expression in Tbx20 mutants was not always found [53], ectopic upregulation of Tbx2 was consistently observed [52–54]. Our lab previously showed that β-myosin heavy chain promoter-driven expression of Tbx2 in the heart tube prior to chamber formation caused a complete block of chamber differentiation (see below) and failure to elongate the heart tube. The cardiac phenotype of these embryos is very similar to that of Tbx20 mutant mice, strongly suggesting that de-repression of Tbx2 can largely account for the phenotype in Tbx20 mutants. If we assume that defective recruitment of second heart field progenitors underlies the phenotypes of Tbx2 overexpression and Tbx20 null mice, the question is how excess Tbx2 in myocardium can interfere with recruitment from, or deployment of the extra-cardiac progenitors. The answer to this remains elusive, but leads one to speculate that the myocardium itself controls progenitor recruitment. Soluble factors, such as members of the bone morphogenetic protein (BMP) or fibroblast growth factor (FGF) families, are involved in differentiation of myocardium and in regulation of the anterior heart field [55]. Several studies have shown that BMP and FGF factors are regulated by T-box factors in several tissues [41, 56–58]. Changes in T-box gene expression in the heart tube may affect the production of these soluble factors, leading to defective intercellular signaling underlying progenitor proliferation or recruitment and differentiation.

Tbx5 mutant heart tubes are similarly short and hypoplastic. They also display a caudal to cranial gradient in the severity of the defect and a failure of looping [59]. Once again, a defect in the process of elongation by recruitment of progenitors that reside in the dorso-caudal region of the second heart field to the caudal end of the heart tube cannot be excluded as an underlying cause of the mutant phenotype. Tbx5 is also expressed in the dorso-caudal domain of the second heart field (Table 1, Fig. 3). Therefore, one cannot discriminate between direct and indirect (non-cell-autonomous) functions of Tbx5 in recruitment of progenitors to the heart tube. The same holds for mice deficient in Tbx2 or Tbx3. Both display outflow tract defects [60] [Robert Kelly and V. Papaioannou, personal communication], and both are expressed in a subpopulation of the second heart field [60–62] (Table 1, Fig. 3).

**Tbx18 and the caudal heart field**

During folding of the embryo, Tbx18 is expressed in a small subpopulation of cells ventral to the developing heart tube (Fig. 1, Table 1). This region is spatially associated with the precursors of the forming septum transversum, and gives rise to the pro-epicardium and the mesenchyme that borders the myocardial inflow tract of the heart [63]. Tbx18 is required for maintaining antero-posterior polarity in somites [64]. Tbx18-deficient mice die shortly after birth as a result
of severe skeletal malformations. The absence of any noticeable defects in the highly Tbx18-positive (pro) epicardial cells and their derivatives, including coronary arteries, is unexpected. Nevertheless, Tbx18-deficient mice do develop heart defects. The sinus horns are the myocardial parts of the common cardinal veins upstream of the venous valves. Recent mouse lineage and expression analysis experiments from our lab have demonstrated that sinus horn myocardium forms after E9.5 by recruitment of Nkx2-5-negative, but Tbx18-positive, mesenchymal precursors located in the periphery of the inflow tract [65]. This reciprocal pattern was found to be conserved in the chick. Normally, the common cardinal veins are released from the mesenchymal pericardial wall into the pericardium, and the wall of the released vein subsequently differentiates into myocardium. Both processes fail in mice that lack Tbx18. As a consequence, the right and left superior caval veins run through the pleuro-pericardial membrane, where they eventually become myocardialized. Intriguingly, the expression pattern of Tbx18 in the sinus horns of mouse and chicken is conserved in zebra fish [66], supporting the view that the sinus venosus of fish is the evolutionary equivalent of the sinus horns. 

Issl expression was found to be largely excluded from the Tbx18-positive precursor population. The fact that the first and second heart fields express Issl and Nkx2-5, but not Tbx18, defines the sinus horn precursor population as a genetically distinct field in its own right. Furthermore, the second heart field is positioned medially to the cardiac crescent before folding of the embryo (Fig. 1). In contrast, the septum transversum progenitors, with which the Tbx18-expressing precursors are associated, are localized laterally and cranially to the cardiac crescent (Fig. 1). The sinus horn precursors are thus spatially separated from the second heart field progenitors. Of interest, the two fields meet at the inflow tract (Fig. 1), suggesting that sinus horn myocardium recruited first directly adjacent to the atria, such as the sinoatrial node, may receive contributions from both populations.

Roles for T-box factors in chamber development and conduction system formation

Some time around E10, shortly after looping, the cardiac blueprint has been set and development is well under way. While the embryonic heart tube enlarges by recruiting precursor cells at the poles and via the dorsal mesocardium, a secondary process of chamber differentiation is being initiated. At E8–8.5, the expression of marker genes for ventricular and atrial chamber myocardium, including Nppa, Chisel (Smpx) and Cx40, can already be observed at the ventral side of the heart tube [12] (Fig. 2). This region will expand (‘balloon’) to form the embryonic left ventricle at the outer curvature [67]. Somewhat later, right-ventricular and atrial expression of the chamber markers is observed at discrete sites of the outer curvature, these being the regions which will expand to form the respective chamber compartments. Importantly, the sinus venosus, atrioventricular canal, inner curvature and the outflow tract will not initiate expression of chamber markers and will not expand. These structures initially retain the original embryonic phenotype. The sinus venosus and atrioventricular canal presumably will give rise to the nodal components of the conduction system [1] (Fig. 2). The compartments do not arise from specific heart fields, but rather to a varying degree obtain contributions from the first and second heart fields, although the initially formed left-ventricular compartment receives the greatest contribution from the first heart field [55, 68] (Fig. 1). Therefore, local cues rather than distinct lineages probably regulate the sites of chamber differentiation. Taking into account the positioning of the differentiating chambers, it is likely that antero-posterior and dorso-ventral patterning underlies chamber formation or the repression thereof (Fig. 2, 4).

The non-chamber myocardium of the atrioventricular canal, inner curvatures and outflow tract provide signals to the underlying endocardium to form cushions [69], from which subsequently the valves and major parts of the septa will be formed. Furthermore, the atrioventricular canal retains its slow conducting properties, and will serve to delay the propagation of the impulse from atria to ventricles. The accordingly acquired configuration of slow conducting and contracting primary myocardium and fast conducting and contracting atrial and ventricular chambers, with dominant pacemaker activity always found at the caudal venous end, is sufficient to obtain a synchronously contracting heart with a functional conduction system [1]. Obviously, many more morphogenetic steps will still have to be taken to generate septa, valves, and a mature nodal and ventricular conduction system before the heart truly reaches its mature form (Fig. 2).

Tbx5 and establishing the antero-posterior pattern

T-box factors play critical roles in the establishment of the cardiac blueprint. The presence of antero-posterior, or crano-caudal, patterning in the heart tube is a well-established phenomenon, believed to guide the formation of distinct components along the antero-posterior axis. Retinoic acid plays a determining role
in antero-posterior patterning, as it provides caudal cardiac progenitors with positional information, thus invoking the sinusatrial identity and further development of these precursors [70, 71]. Tbx5 is expressed in a caudal-high antero-posterior gradient in the heart tube, a gradient regulated by retinoic acid [71, 72]. Tbx5 deficiency results in cardiac developmental arrest, the formed but unlooped heart tube being characterized by a hypoplastic caudal end, indicating that Tbx5 plays a pivotal role in development (recruitment or expansion) of the sinusatrial precursor population. Forced expression of Tbx5 in the entire heart causes an arrest in heart development and loss of Mlc2v expression, an anterior marker gene not normally expressed in the sinusatrial region [72]. Furthermore, expression of myosin heavy chain 6, a gene important for development of the sinoatrial region, has been shown to be regulated by Tbx5 in vitro [73]. Thus, Tbx5 may represent a patterning factor linking positional information provided by retinoic acid and development of the sinusatrial region of the heart.

Formation of the interventricular septum is initiated as early as E9.5–10, concomitant with differentiation and expansion of the left and right ventricles. Since the left and right ventricles are specified along the antero-posterior axis, the interventricular septum can be regarded as an antero-posterior boundary structure between these two ventricles. Normally, the left ventricle expresses more Tbx5 than the right ventricle.

Figure 4. Role of T-box factors in early heart development. (a) Schematic representation of an E9.5–10.5 heart showing T-box patterning in the different emerging structures. Tbx2 and Tbx3 exert their function in the non-chamber myocardium, Tbx1 in the outflow tract and Tbx18 in the sinus horns. Yellow bars indicate expression patterns of Tbx5, Tbx20 and Nkx2-5. Tbx5 is required for antero-posterior patterning and, along with Tbx20 and Nkx2-5, for chamber differentiation. Note the absence of Nkx2-5 expression from the sinus horns. (b) Working model of a T-box factor regulatory network for chamber formation. Tbx2 and Tbx3 act as repressors of chamber differentiation in primary myocardium where they compete with Tbx5, while BMP signaling stimulates Tbx2/3 (and Tbx20) expression in the primary myocardium. Tbx20 represses Tbx2 expression in chamber myocardium and regulates proliferation. Tbx5 acts as a positive regulator of chamber genes and proliferation, thus stimulating chamber differentiation.
T-box factors control chamber position and node retention

Tbx5 and Nkx2-5 mutant embryos fail to develop chambers [59, 76, 77]. Both factors control growth and are critical to the activation of chamber-specific genes, including Cx40 and Nppa [13, 59, 78, 79]. These findings have been fundamental to our insights into the molecular programs that drive chamber differentiation. However, the highly localized differentiation of the chambers cannot be explained by the broad expression patterns of these factors in chamber and primary myocardium. Moreover, both Tbx5 and Nkx2-5 are involved in the formation of, again very localized, atrioventricular derived components of the conduction system [80–82].

In search of a possible mechanism for chamber-specific expression of Nppa, we demonstrated that both a single TBE (T-box binding site) and adjacent NKE (Nkx2-5 binding site) present in the Nppa promoter are required for repression of Nppa in the atrioventricular canal [83] and outflow tract [84]. Tbx2 and Tbx3 were found to interact with the TBE, to repress Nppa through this site and to effectively compete with Tbx5 in the trans-activation of the Nppa and Cx40 promoter [61, 62, 83]. Expression of Tbx2 and Tbx3 is confined to primary (non-chamber) myocardium, remarkably mutually exclusive of Nppa, Cx40, Cx43, Chisel and other chamber-specific genes [60–62, 83]. These findings seem to dictate a model in which chamber formation (atria, left and right ventricle) and differentiation is driven by broadly expressed factors. An additional layer of spatially restricted repressors inhibits this process in regions where chambers do not develop, i.e. the inflow tract, atrioventricular canal, inner curvatures and outflow tract [12] (Fig. 4). Tbx2 gain and loss of function experiments have demonstrated that Tbx2 is indeed able and required to inhibit chamber formation and expression of chamber marker genes [60, 61]. Tbx3 is expressed in a subdomain of the Tbx2 domain, and whereas it is able to block chamber formation when expressed ectopically, its deficiency does not lead to obvious defects in atrioventricular canal patterning, indicating functional redundancy with Tbx2 [our unpublished observations].

How do Tbx2 and Tbx3 exert their functions? Both factors act as repressors of transcription and share DNA binding properties and target genes [31, 85–88]. They effectively compete with Tbx5, a transcriptional activator, for TBE-binding and for Nkx2-5, a cardiac accessory factor, thus repressing chamber-specific genes and chamber differentiation [61, 62, 83]. The finding that Tbx3 inhibits myogenic differentiation [89] is compatible with the assumed roles of these T-box factors in inhibiting differentiation of chamber muscle. A conspicuous property of primary myocardium is that it retains its low proliferation rate while the chambers are rapidly proliferating and expanding. Both Tbx2 and Tbx3 appear able to bypass senescence, and are reported to be amplified and overexpressed in various cancers [28, 89–93]. They directly suppress the tumor suppressor/cell-cycle inhibitors p19ARF (Arf) and p21Cip1 (Cdkn1a) [28, 89–91, 94], the latter by recruiting HDAC1 to the initiator of the p21 promoter [26]. Moreover, Tbx2 is tightly regulated during the cell cycle, with highest expression levels during late S-phase and G2 [95]. These properties would seem to support a role for Tbx2 and Tbx3 in regulating proliferation in the primary myocardium, but fall short of explaining why their functions seem opposing in myocardium as compared with other systems. Moreover, p21, p19ARF/p16INK4a and p15INK4b are not elevated in Tbx2-deficient embryos, and mutation of p53, upregulated by p19 suppression, did not rescue the Tbx2 mutant phenotype [60]. However, the lack of response may be due to compensating factors participating in this pathway, such as Tbx3 that is co-expressed in the heart [60].

Nmyc1 (N-myc) is required for early myocardial proliferation [96, 97]. Its transcripts are enriched in the compact, fast-proliferating layer of the chambers, in a pattern complementary to that of Tbx2 [52, 96]. Evans and co-workers [52] found that Tbx2 directly represses Nmyc1 and cyclin A2 (Cena2), a feature...
Tbx5 haploinsufficient for atrioventricular conduction system, we may begin to of Tbx2/3 into the Tbx5-Nkx2-5 pathway in the expression is not specific to these areas. By integration conduction system development [80, 81], while their rent efforts to address this issue are ongoing, and hint that the sinoatrial node develops from primary myocardium at the junction between the right sinus horn and the right atrium, whereas the atrioventricular node develops from the atrioventricular canal. The node precursors consequently express Tbx2 and Tbx3. During development, Tbx2 becomes down-regulated, whereas Tbx3 expression is maintained specifically in the nodes, and is the only known transcription factor to be so expressed [61, 62]. As mature nodes display many features that resemble primary myocardium in the embryo, it is attractive to hypothesize that their formation is derived by Tbx2 and Tbx3 maintaining the primary phenotype. Current efforts to address this issue are ongoing, and hint at an even more active role of Tbx3 in node formation [our unpublished results]. As discussed above, both Tbx5 and Nkx2-5 are required for atrioventricular conduction system development [80, 81], while their expression is not specific to these areas. By integration of Tbx2/3 into the Tbx5-Nkx2-5 pathway in the atrioventricular conduction system, we may begin to explain some of the highly localized defects in mice haploinsufficient for Tbx5 or Nkx2-5.

T-box factors and transcriptional networks for septation and conduction system function

A strict dosage level of Tbx5 is required for septum formation and conduction system maturation [81]. A large fraction of individuals with Holt-Oram syndrome have heart defects, including atrial and/or (muscular) ventricular septal defects, and they are at risk for progressive atrioventricular block and atrial fibrillation [15, 98]. The dose-dependent regulation of target genes [99] and the requirement of Tbx5 for cell-cycle progression [78] probably represent the underlying cause of these defects. Not surprisingly, perhaps, proteins working together to coordinate a process often derive a similar spectrum of phenotypes when mutated. Mutations in the Tbx5 interacting partners Nkx2-5, Gata4 and Sall4, like Tbx5 itself, are known to cause septum defects and, in the case of Nkx2-5, atrioventricular conduction defects [19, 23, 100]. Tbx20 can interact with all of these factors, and is required for septation [20, 21, 54]. Careful analysis of Tbx20 heterozygous mutant mice revealed atrial septal defects that were more frequent and severe than in Nkx2-5+/– Tbx20+/– double mutant mice [54]. Tbx3 is also part of this network. Mutations in TBX3 cause ulnar-mammary syndrome characterized by defects in breast development, apocrine gland, limb and genital formation [101], and, indeed, low penetrance ventricular septal defects and pulmonary stenosis [102]. These genetic and functional data indicate that septation of the atria and ventricles is governed by a tightly regulated and often spatially constrained network of interacting transcription factors [103]. The sensitivity of this network and the many manifestations of septum defects seen in mutants possibly reflects the complexity of formation of these structures, which involves complex signaling and coordinated directional growth and apoptosis of the cushion mesenchyme and myocardial muscle. The challenge will be to elucidate the requirements, functions and interaction of the transcription factors in the distinct tissues during septation.

A regulatory T-box factor network

Recent studies of the regulation of T-box genes and the identification of some of their target genes have provided a picture, albeit far from complete, of the regulatory network that controls the temporally and spatially resolved formation of the heart components (Fig. 4). Whereas Tbx2 and Tbx3 suppress chamber-specific genes, their own regulation is controlled by Bmp-Smad signaling. Bmp2 is expressed in the atrioventricular canal from its earliest stages of formation onward, and is required for Tbx2 expression [69, 104]. Furthermore, Bmp2-soaked beads induce Tbx2 and Tbx3 expression [104]. Conditional inactivation of the type 1 Bmp receptor (Bmpr1a/Alk3) in the heart leads to reduced Tbx2 and Tbx3 levels [105]. Evidence has been put forward which suggests that the Tbx3 promoter is directly regulated by Bmp Smads that interact with a consensus Smad binding element 1.3 kbp upstream of the transcription start site [105]. Since our results seem to demonstrate that a 6 kbp upstream promoter fragment of Tbx3 is not sufficient to drive cardiac expression in transgenic mice [our unpublished observations], the contribution of this pathway to Tbx3 regulation in vivo remains to be verified. Whereas BMP2 induces Tbx2 and Tbx3 in the atrioventricular canal, the expression profile of
revealed that Tbx3 [104, 106]. Furthermore, a Tbx20 expression in the undifferentiated pre-cardiac mesoderm in chick [106] and inactivation of type I Bmp receptor in cardiac progenitor cells results in a modest downregulation of Tbx20. However, again inconsistently, inactivation of Bmp2 in the heart progenitors does not lead to reduced Tbx20 expression [69]. Tbx3 is not de-repressed in Tbx20 mutants, demonstrating that despite their similarity in structure and function, Tbx2 and Tbx3 are regulated by distinct inputs. Similarly, applied BMP2 induces Tbx2, Tbx3 and Tbx20 but not Tbx5 [104, 106]. Furthermore, a recent genome-wide analysis of Tbx5 target genes revealed that Tbx3, but not Tbx2, is positively regulated by Tbx5 [99].

Together, these findings provide a first glimpse into the complicated and multi-layered T-box factor network that controls critical steps in the localized formation of the components of the heart.

**Concluding remarks**

T-box transcription factors have multiple and diverse roles in gene regulation and heart development. They are critical to patterning, localized proliferation, differentiation, chamber formation, development of conduction system components, septation and valvulogenesis. Studying their functions has been rewarding, bringing our understanding of heart development to a higher level. The challenge will be to understand precisely how T-box factors regulate the different aspects of heart morphogenesis. Current evidence shows that T-box factors act in genetic cross-regulatory networks, that they act together with a variety of other proteins, that they have overlapping as well as unique functions, and that their functions depend on tissue-specific context and stage of development. To study these functions, new animal models that allow cell type- and stage-specific activation or inactivation of multiple T-box factor genes are being constructed and analyzed, and large-scale screens for T-box factor target genes and interaction partners in different animal models and tissues, are being performed. These studies will reveal novel and important details of known molecular pathways controlling specific aspects of heart morphogenesis, which will bring us closer to understanding congenital heart defects.

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