T-box transcription factors and their roles in regulatory hierarchies in the developing heart

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Summary

T-box transcription factors are important players in the molecular circuitry that generates lineage diversity and form in the developing embryo. At least seven family members are expressed in the developing mammalian heart, and the human T-box genes *TBX1* and *TBX5* are mutated in cardiac congenital anomaly syndromes. Here, we review T-box gene function during mammalian heart development in the light of new insights into heart morphogenesis. We see for the first time how hierarchies of transcriptional activation and repression involving multiple T-box factors play out in three-dimensional space to establish the cardiac progenitors fields, to define their subservient lineages, and to generate heart form and function.

Introduction

The mammalian heart is a mechanical pump that can be viewed developmentally as a highly modified muscular vessel. The atria are collecting chambers and the ventricles pumping chambers. Valves separate chambers and guard the arteries to prevent reflux, and the conduction system coordinates the timing and efficiency of chamber filling and ejection. One of the remarkable features of the mammalian heart is that it begins to function from virtually the moment it comes into being as a simple muscular tube, before the development of the valves and specialized conduction components. Henceforth, the functional development of the heart accompanies its morphological development, and we are now beginning to realize that, to a large (but unknown) extent, function dictates form. The formation and maturation of chambers and valves, in particular, are highly dependent on the forces associated with contraction and blood flow (Bartman et al., 2004; Hove et al., 2003), and on other epigenetic influences such as hypoxia (Sugishita et al., 2004). In this fashion, the efficiencies implicit in adult heart form (Kilner et al., 2000) are actually molded in the embryo by function itself. The molecular details of the interplay between patterning and functional feedback are largely unknown, and this additional dimension to developmental regulation brings with it numerous challenges as we attempt to unravel regulatory circuits.

We have been aided in this endeavour by the discovery a decade ago that transcription factor pathways guiding cardiogenesis have been conserved in evolution (Bodmer and Venkatesh, 1998; Harvey, 1996). A core transcriptional network that involves homeodomain factors, zinc finger factors of the GATA family, Mef2 factors of the MADS Box family and, indeed, T-box factors, appears to guide cardiac specification and differentiation from *Drosophila* to man, and many of the details of molecular mechanisms can now be studied using the powerful genetics of *Drosophila* (Cripps and Olson, 2002).

Nonetheless, mammalian heart development involves many morphological innovations that are not evident in the simple muscular hearts of flies (Harvey, 2002). In evolutionary terms, these modifications are relatively recent, and it may be partly for this reason that they appear vulnerable to genetic perturbation. Indeed, almost one live-born human baby in 100 has some form of structural anomaly of the heart (Hoffman and Kaplan, 2002), many requiring surgical intervention. The evident connections between cardiac development and human congenital heart disease (CHD), and our increasing ability to accurately measure heart function in animal models, even in utero (Zhou et al., 2002), ensures that the heart will continue to be a prominent platform for understanding the origins, the development and the evolution of organ systems.

Development proceeds via a series of transcriptional and post-transcriptional switches that build regional complexity and functional diversity. Many transcriptional processes in development and organogenesis involve members of the ancient family of T-box transcription factors, named after its founding member, the T protein, now known as Brachyury (see Box 1). This review examines recent new insights into heart morphogenesis and the involvement of T-box genes in these processes. In particular, we explore the roles of T-box genes in defining cardiac progenitor populations, and in the hierarchical transcription factor pathways that specify cardiac chambers and the conduction system.

Heart morphogenesis: new concepts

The basic morphological steps of mammalian heart development are well described (see Box 2). We focus here on two recent insights that have significantly clarified and extended our view of heart morphogenesis, and that have a key relevance to T-box gene function.

The secondary heart field

The discovery that there is selective deployment of two distinct progenitor pools to the forming heart represents a major advance in how we view cardiac morphogenesis, and has many implications for the evolution and patterning of cardiac structures.

The early linear heart tube encompasses progenitors for parts of the ventricles, with other compartments formed by the addition of cells to its cranial and caudal poles (De la Cruz et al., 1977; Noden, 1991; Redkar et al., 2001). We now know that this growth process is based on a series of progressive lineage restrictions. An elegant fate-mapping technique termed 'retrospective lineage analysis' has shown that the whole heart is likely to be derived from a single cardiac progenitor pool (Meilhac et al., 2004). However, an early lineage restriction, occurring prior to heart formation, creates two distinct pools that show profoundly different behaviors (Fig. 1).

The 'first lineage' (Kelly, 2005; Meilhac et al., 2004) comes to occupy what we have classically regarded as the cardiac crescent (see Fig. 1D). This lineage undergoes early differentiation, as judged by the expression of myofilament gene markers at the cardiac crescent stage (Franco et al., 1998), and is subsequently used to build the initial linear heart tube, which (in the mouse) is composed largely of precursors of the left ventricle (LV), with the inflow region, including the atrioventricular (AV) canal and parts of the atria, added progressively. The first lineage contributes minimally, if at all, to the right ventricle (RV) and outflow tract (OFT).

A 'second lineage' [referred to at later stages as the secondary heart field or anterior heart field (Kelly, 2005)] occupies a position caudally and medially to the first lineage at the cardiac crescent stage, and its differentiation and deployment to the heart is delayed. On the basis of evidence from a number of sources, including gene expression patterns

(see Fig. 1A-C) and lineage tracings using DiI injection, retroviral tagging and Cre-Lox methods, it is now clear that the second lineage is deployed exclusively to build the RV and OFT, including its myocardial, smooth muscle and endothelial investments, as well as parts of the atria (Cai et al., 2003; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). It
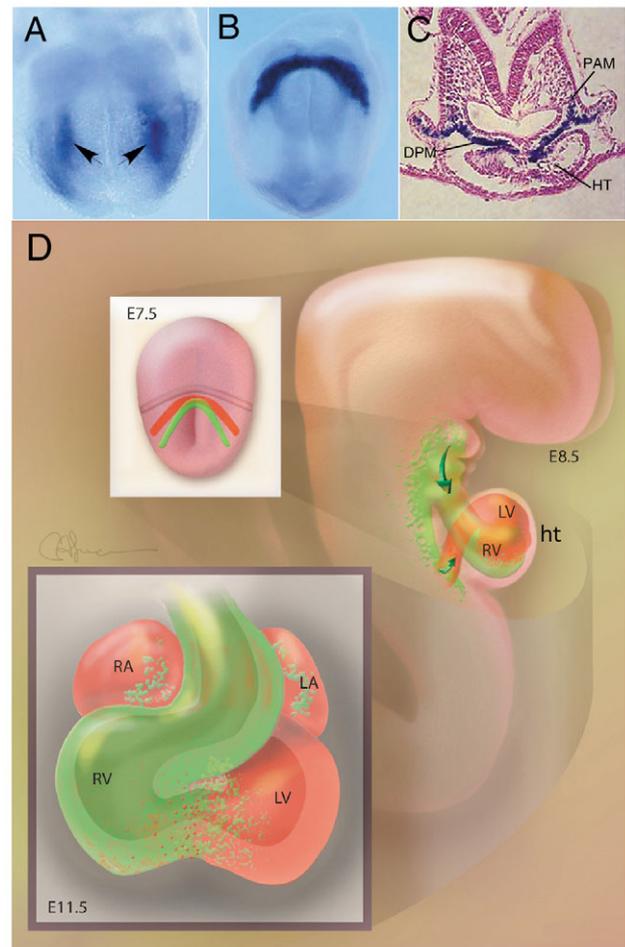

Fig. 1. First and second heart lineages. (A) The second lineage of cardiac progenitor cells is highlighted in a cardiac crescent-stage mouse embryo (E7.5) by the expression of a β-galactosidase (*lacZ*) transgene under the transcriptional control of the *Fgf10* locus (arrows). (B) Expression of α-cardiac actin mRNA in differentiating cardiomyocytes of the cardiac crescent (first lineage) in a similarly staged mouse embryo. Comparison with staining in A demonstrates that the second lineage progenitors lie medial and caudal to the first lineage progenitors of the crescent. (C) Transverse section through an E8.5 mouse embryo, showing expression of the *Fgf10-lacZ* transgene in dorsal pericardial mesoderm (DPM) in continuity with myocardium of the heart tube (HT), and in pharyngeal arch mesoderm (PAM). (D) Illustration of the position of the first (red) and second (green) heart precursor lineages at E7.5 (small inset), and during deployment to the heart tube at E8.5 (main figure) in the mouse embryo. Arrows indicate deployment at both poles of the heart. Large inset shows the contribution of the first and second heart lineages to the E11.5 heart. Figures reprinted, with permission, from Kelly and Harvey (Kelly and Harvey, 2004), and Parmacek and Epstein (Parmacek and Epstein, 2005), Copyright 2005 with permission from Elsevier. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

Box 1. T-box genes: an historical perspective

The story of the T-box genes began in Paris at the Pasteur laboratory in the 1920s with the Russian scientist Nadine Dobrovolskaïa-Zavadskaïa, who embarked on a pioneering screen for X-ray-induced developmental mouse mutants. Her isolation of a mouse strain with a short tail, caused by a semi-dominant heterozygous mutation in a locus she called *T*, represented one of the first successful mammalian genetic screens, and provided one of the earliest links between gene activity and cell behaviour during embryogenesis (for a review, see Korzh and Grunwald, 2001). The mid-gestational death of homozygous *T* embryos, with perturbed development of the posterior mesoderm and notochord, demonstrated an essential requirement for *T* during gastrulation, and led to the earliest insights into the inductive influences of notochord on neural tube and somite development (Chelsey, 1935). Over 60 years later *T*, now also known as brachyury, meaning 'short tail' in Greek, was cloned in one of the earliest positional cloning efforts in the mouse (Herrmann et al., 1990). At the time, lack of homology in the *T*-gene product to any previously characterized protein gave no clues as to its biochemical role until, in 1993, it was revealed to be a novel sequence-specific DNA-binding protein (Kispert and Herrmann, 1993). Crystallographic determination of the structure of the DNA-binding domain, now called the T-box, revealed a new way through which proteins recognize DNA (Muller and Herrmann, 1997).

contributes only a small number of cells to the LV, at least initially (Brown et al., 2004). At the time of its deployment to the poles of the growing heart, beginning around E8.5 in the mouse (Zaffran et al., 2004), the secondary heart field appears to encompass cells positioned dorsally and anterior to the linear heart tube. These cells, collectively termed pharyngeal mesoderm (Zaffran et al., 2004), equate to the dorsal mesocardium and the dorsal pericardial mesoderm (see Box 2), as well as to head mesenchyme occupying the future posterior pharyngeal arches. There may be subdivisions of the second lineage (Kelly, 2005), although this is still a matter of debate (Abu-Issa et al., 2004). Nevertheless, cells of the secondary heart field are beautifully highlighted by the expression of a number of genes and transgenes, and their deployment to the heart can be followed using tagging methods (Cai et al., 2003;

Dodou et al., 2004; Hu et al., 2004; Kelly et al., 2001; Stanley et al., 2001; Xu et al., 2004) (Fig. 1A-C).

The lineage split that gives rise to the first and second cardiac precursor pools may stem from temporal, spatial or determinative constraints earlier in development. Evidence for the latter is based on the apparently restricted early expression patterns of the transcription factor genes *Isl1* and *Tbx1* in the secondary heart field (Brown et al., 2004; Cai et al., 2003).

Cardiac chamber formation

The origin of chamber myocardium in the forming heart tube has been a much-debated topic. Recent analyses have now given us a clearer perspective of this issue, which is so important for our understanding of CHD.

During the incorporation of the first and second lineages into

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Box 2. Morphological development of the mammalian heart
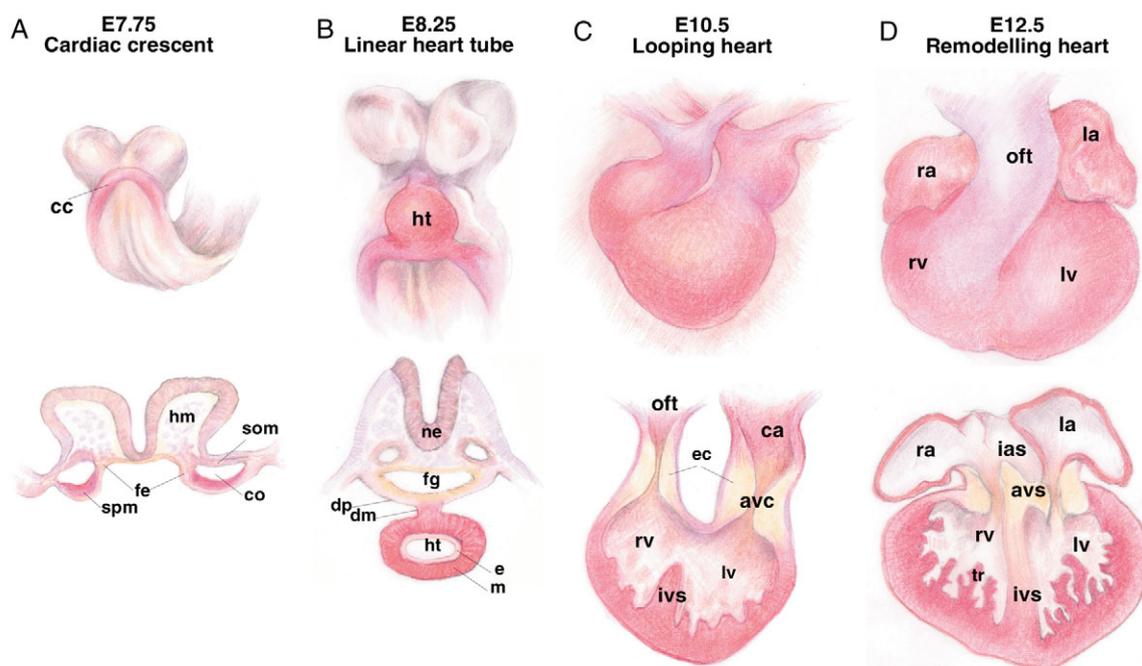
At gastrulation, cardiac progenitor cells enter the mesodermal layer then migrate laterally, merging across the anterior midline to form the 'cardiac crescent' (A). (In the accompanying figure, the whole embryo or isolated heart is shown at the top with a representative informative section shown beneath.) At E7.75, a coelum separates the heart tube progenitors (splanchnic mesoderm) from those that line the peri-cardial cavity (somatic mesoderm). Myocardial and endocardial cells become evident now, and both express cardiac-restricted transcription factors (Franco et al., 1998; Harvey, 2002). Heart progenitors move ventrally, then, due to growth of the head folds, caudally, to form a muscular vessel within the ventral trunk (B). The myocardium initially remains open dorsally, in continuity with pericardial mesoderm. This opening then converges to form the dorsal mesocardium, a transient attachment to the ventral foregut that eventually resolves, leaving the heart tethered only at its poles. From E8.5-E10.5, the heart tube elongates within the pericardial cavity and loops into a rightward spiral (C) under the influence

of the embryonic left/right axial pathway (Levin, 2005). Many changes occur during cardiac looping (C,D): the future cardiac chambers become evident as swellings; rib-like myocardial protrusions called trabeculae form; endocardial cushions, precursors of the cardiac valves and parts of the septa, form in the AVC and OFT; and muscular septa arise within the primitive ventricles and atria. Further remodelling occurs in subsequent phases. [Figure adapted with permission from Harvey (Harvey, 2002), Copyright 2002 Macmillan Magazines Limited.] avc, atrioventricular canal; avs, atrioventricular septum; ca, common atrium; cc, cardiac crescent; co, coelum; dm, dorsal mesocardium; dp, dorsal pericardium; e, endocardium; fg, foregut; hm, head mesoderm; ias, interatrial septum; ivs, inter-ventricular septum; la, left atrium; lv, left ventricle; m, myocardium; ne, neural epithelium; oft, outflow tract; fe, foregut endoderm; pm, pharyngeal mesoderm; ra, right atrium; rv, right ventricle; som, somatic mesoderm; spm, splanchnic mesoderm; tr, trabeculae.

the heart, chamber myocardium arises in descendents of both lineages (Christoffels et al., 2004a; Christoffels et al., 2000). Previously, emphasis was placed on the segmental arrangement of chamber primordia in the forming heart. However, molecular markers, along with anatomical and electrophysiological observations, now show that chambers are specified in discreet zones along the outer curvature of the heart tube: the ventricles at the original ventral surface of the heart tube, and atria located more caudally in dorsolateral zones (red and blue regions, respectively, in Fig. 2B) (Christoffels et al., 2004a; Christoffels et al., 2000). Several gene markers highlight the forming chambers – among them *Nppa*, encoding atrial natriuretic factor (ANF; Fig. 2E-G), and *Chisel/Smpx* and *Gja5*, which encode cytoskeletal and connexin proteins, respectively. Chamber myocardium is unlikely to arise from a distinct lineage; rather, it may be induced within primary myocardium in response to positional cues. Compared with primary myocardium, chamber myocardium acquires a more differentiated state that is adapted for a lifetime of mechanical work.

The regions of the heart tube that do not give rise to atrial or ventricular chambers retain the embryonic, less differentiated myogenic phenotype of the primary heart tube. These regions, shown in grey in Fig. 2B, include the OFT, the inner curvature of the ventricular loop, the AV canal and the inflow complex, including the original body of the common atrium. The characteristics that distinguish these regions from working chamber myocardium include a distinct gene expression signature, reduced cellular proliferation rate, lower conduction velocity and the ability to spontaneously depolarize. One of the principal molecular markers of non-chamber myocardium is the T-box gene *Tbx2* (Fig. 2H-J, see below).

The non-chamber myocardium serves several crucial roles in heart development. The ability of its myocytes to spontaneously depolarize is greatest in the caudal region of the heart tube. This creates a dominant pacemaker-like activity in the caudal heart that initiates the slow anterior-ward propagation of action potentials and peristaltic contraction waves. Rapid conduction through chamber myocardium is acquired as chambers mature. Non-chamber myocardium also provides the signals that induce the formation of prominent endocardial cushions from endothelium, the precursor structures of valves and septa (see Box 2). Importantly, non-chamber myocardium develops into the more specialized elements of the central conduction system, including the sinuatrial (SA) node, the AV node and the Bundle of His (see Box 3) (Christoffels et al., 2004a). Thus, the specification of chamber and non-chamber myocardium in the forming heart is one of the crucial early lineage decisions upon which much of the subsequent heart development is based.
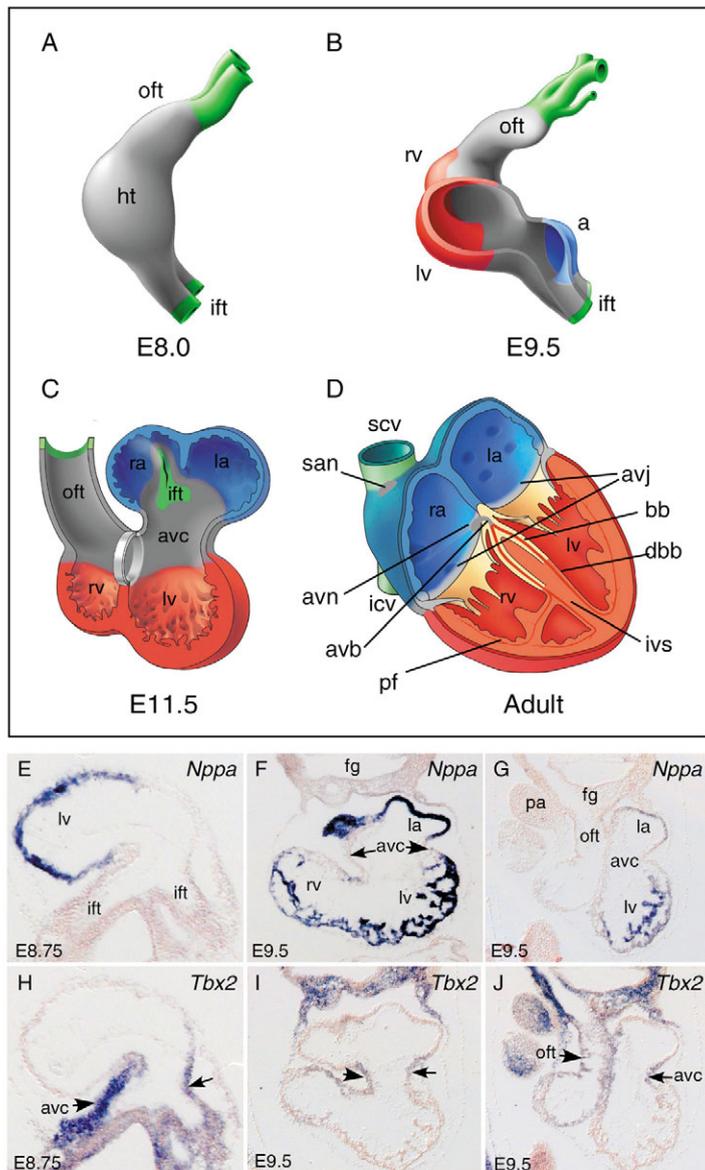

Fig. 2. Chamber and non-chamber myocardium in the forming heart. (A-D) Illustrations of the developing mouse heart at E8.0 (A), E9.5 (B) and E11.5 (C), showing non-chamber (primary) myocardium (grey), ventricular chamber myocardium (red), atrial chamber myocardium (blue), and the inflow and outflow vessels (green). Note that chamber myocardium forms at the outer curvature of the looping heart tube. Components in the forming heart (A-C) can be traced to the adult heart (D). Primary myocardium forms the elements of the central conduction system (see Box 3), including the sinuatrial node (san), atrioventricular node (avn), atrioventricular bundle (avb; bundle of His) and bundle branches (bb), as well as insulating tissue of the atrioventricular junction (avj). Distal bundle branches (dbb) and Purkinje fibres (pf) form within the interventricular septum (IVS) and ventricular chambers, respectively. Yellow indicates the derivatives of the AV canal, including insulating elements formed from myocardium and valve leaflets formed from endocardial cushions. Modified from Christoffels et al. (Christoffels et al., 2004a), Copyright 2004 with permission from Elsevier. (E-J) In situ hybridization analysis of sections of E8.75-E9.5 mouse embryos showing mutually exclusive expression patterns of the heart chamber marker *Nppa* (E-G) and *Tbx2*, a marker of non-chamber myocardium (H-J, arrows). Reproduced with permission from Habets et al. (Habets et al., 2002). a, atrium; avc, atrioventricular canal; ev, embryonic ventricle; fg, foregut; icv, inferior caval vein; ift, inflow tract; la, left atrium; lv, left ventricle; oft, outflow tract; oftc, outflow tract cushion; pa, pharyngeal arch; ra, right atrium; rv, right ventricle; scv, superior caval vein.

T-box genes

T-box proteins are characterized by the presence of a highly conserved 180-amino acid, sequence-specific, DNA-binding domain termed the T-box. The T-box transcription factor family, of which there are 18 members in mice, is divided into five subfamilies (Naiche et al., 2005). Although the crystal structure of Brachyury shows that it can form a dimer on a palindromic DNA-binding site (Muller and Herrmann, 1997), studies of the binding sites of Brachyury and other T-box factors in genuine target promoters show that these proteins bind as monomers to one half of the palindromic site demonstrated for Brachyury (Naiche et al., 2005). T-box proteins function as transcriptional activators, repressors, or both, depending on the cellular context (Naiche et al., 2005), and can interact with other transcription factors (Bruneau et al., 2001; Garg et al., 2003; Habets et al., 2002; Hiroi et al., 2001; Krause et al., 2004; Lamolet et al., 2001; Maira et al., 2003; Stennard et al., 2003), as well as with transcriptional co-activators and co-repressors (Barron et al., 2005; Maira et al., 2003; Vance et al., 2005), nucleosome assembly proteins (Wang et al., 2004) and chromatin-modifying proteins (Lickert et al., 2004).

Although the specific function of most T-box genes is largely unknown, they clearly act in a combinatorial and/or hierarchical fashion within the progenitor fields that shape the developing embryo, including those of the early mesoderm (Suzuki et al., 2004), limbs (Messenger et al., 2005) and heart (see below). They can also determine distinct morphogenetic behaviors (Bruce et al., 2005; Kwan and Kirschner, 2003; Russ et al., 2000; Yamomoto et al., 1998). T-box genes can be rapidly induced by growth factors, often in a dose-responsive manner (O'Reilly et al., 1995). Indeed, development appears to be exquisitely sensitive to the level of T-box gene expression (Hatcher and Basson, 2001), with several human congenital anomaly syndromes being linked to T-box gene haploinsufficiency (reviewed by Packham and Brook, 2003). Mice bearing mutations in these genes show many aspects of the corresponding human diseases, providing valuable models for the further dissection of disease mechanisms (Naiche et al., 2005).

At least seven members of the T-box gene family are expressed in the embryonic heart in humans and vertebrate models, namely *Tbx1-5*, *Tbx18* and *Tbx20*, and these genes show overlapping expression patterns in the first and second heart precursor lineages, the myocardium, the endocardium and valves, the conduction system and the epicardium (reviewed by Plageman and Yutzey, 2004). Table 1 documents these genes and their known loss-of-function phenotypes.

T-box genes and heart progenitor specification in *Drosophila*

An analysis of the *Drosophila Dorsocross* complex (*Doc*), which contains three linked T-box genes related to mammalian *Tbx4/Tbx5/Tbx6*, indicates that these genes function in association with the Nkx2-5-related homeodomain factor 'tinman' and the Gata4-related factor 'pannier' at the very heart of cardiac specification (Reim and Frasch, 2005). Indeed, *Doc* triple mutants have no heart. Other *Drosophila* T-box genes, *midline* and *H15*, related to mammalian *Tbx20*, may also participate in cardiac specification, but their main roles occur later in determining cardioblast differentiation, polarity and patterning (Miskolczi-McCallum et al., 2005; Qian et al., 2005; Reim et al., 2005). An essential feature of regulation in the *Drosophila* heart is the interaction between different T-box genes. Midline acts in a pathway that represses *Doc* in a subset of cardioblasts, and this is crucial for establishing the morphological and functional characteristics of the distinct classes of muscle cells, one functioning in the propulsion of haemolymph, and another acting as valves (Reim et al., 2005). The early, instructive and hierarchical roles for T-box genes in fly heart development appear to be conserved, at least in outline, in the mammalian heart, as described below.

Tbx1 regulation and function in the secondary heart field

It is likely, as in *Drosophila*, that *TBX5*, the gene mutated in Holt-Oram syndrome, is involved in cardiac specification in mammals. However, a muscular heart tube is able to form in *Tbx5* null mice, albeit one that is highly truncated, demonstrating that the proposed early function is not absolutely essential (Bruneau et al., 2001). *Tbx5* is also involved in heart tube morphogenesis and chamber formation, as discussed in detail in sections below. More extensive information on the role of T-box genes in cardiac progenitor populations has come from analysis of *TBX1*, which is also involved in human CHD. In this section, we describe the role

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Box 3. The cardiac conduction system

The cardiac conduction system (CCS) is composed of several distinct but integrated components (reviewed by Christoffels et al., 2004a). The SA node, located in the right atrium, generates the pacemaker impulse that sets the beat rate. Impulses from the SA node stimulate atrial contraction and are transmitted to the AV node, which acts as the electrical bridge between the atria and ventricles. The AV node is composed of slow-conducting fibres, imposing a delay in transmission of the pacemaker impulse, allowing atrial contraction to be completed before ventricular filling. Impulses from the AV node are transmitted to the ventricular (peripheral) conduction system. This occurs through special muscle fibres of the AV bundle (Bundle of His) that bifurcate into left and right distal bundle branches before leading into the ventricular Purkinje system. Purkinje fibres are electrically coupled to muscle cells via gap junctions and initiate an apex-to-base contraction of the ventricles.

A functional CCS arises in the tubular heart before specialized components become evident anatomically (Christoffels et al., 2004a; Moorman et al., 2004). Indeed, several transgenic markers highlight regions in the developing heart tube that will form the CCS (reviewed by Myers and Fishman, 2003). Caudal myocytes are intrinsically inclined to depolarize and therefore act as pacemakers, propagating impulses from inflow to outflow. Gradients of morphogens and transcription factors probably control this activity (Moorman et al., 2004). The electrical pattern, initially producing a peristaltic contraction wave, changes when chamber myocardium is formed. Caudal pacemaker activity, slow conduction through non-chamber myocardium and rapid conduction through chamber myocardium represent the essential features of the CCS. The SA and AV nodes arise from non-chamber myocardium of the slow-conducting inflow tract and AV canal, respectively. The ventricular conduction system is proposed to originate from the trabecular component of the ventricles, including the inter-ventricular septum.

Table 1. Embryonic expression and mutant phenotypes of mouse cardiac T-box genes

Gene	Main expression sites during embryogenesis	Mouse knockout phenotype	Mouse heterozygous phenotype	Associated human disease
Tbx1	E8 pharyngeal endoderm, mesodermal core of first pharyngeal arch; E9.5-E12.5 endoderm lining posterior pharyngeal pouches, head mesoderm ventral to hindbrain including secondary heart lineage mesoderm, sclerotome (Chapman et al., 1996; Lindsay et al., 2001; Vitelli et al., 2002a; Xu et al., 2004).	Perinatal lethality. Severely disrupted pharyngeal arch arteries. Abnormal growth and septation of the outflow tract (OT), interventricular septation and conotruncal alignment defects. Hypoplastic thymus and parathyroid glands, abnormal facial structures and vertebrae, cleft palate (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Vitelli et al., 2002a).	Viable and fertile. Abnormal development of fourth pharyngeal arch artery, aorta and pulmonary artery (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001).	DiGeorge and chromosome 22q11 deletion syndromes (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001).
Tbx2	E8.5 allantois; E8.75-E9.5 non-chamber myocardium; E9.5 optic and otic vesicles, naso-facial mesenchyme, limbs, lungs, genitalia (Chapman et al., 1996; Christoffels et al., 2004b; Gibson-Brown, 1996; Gibson-Brown et al., 1998a; Gibson-Brown et al., 1998b; Mahlapuu et al., 2001).	Embryonic lethality by 14.5 dpc, abnormal atrioventricular canal and OT septation, facial dysmorphogenesis, hypoplastic pharyngeal arches, abnormal eye development (Harrelson et al., 2004).	Apparently normal (Harrelson et al., 2004).	None identified.
Tbx3	E8.5 non-chamber myocardium, specifically sinuatrial region, AV canal and interventricular ring; eventually delineates the cardiac central conduction system (de Lange et al., 2004; Hoogaars et al., 2004).	Embryonic lethality by E16.5 (50% mortality by E11.5). Yolk sac, fore- and hindlimb defects, lack of mammary glands, small liver (Davenport et al., 2003).	Viable and fertile. Minor morphological difference in external genitalia (Davenport et al., 2003).	Ulnar mammary syndrome (Bamshad et al., 1997).
Tbx4	Hindlimb, proctodeum, mandibular and lung mesenchyme, atrium and body wall (Chapman et al., 1996).	Embryonic lethality by E10.5. Lack of chorioallantoic fusion and hindlimb defects (Naiche and Papaioannou, 2003; Rodriguez-Esteban et al., 1999; Takeuchi et al., 2003a).	Apparently normal (Naiche and Papaioannou, 2003).	Small patella syndrome (Bongers et al., 2004).
Tbx5	Cardiac crescent, heart tube (graded, highest caudally), sinus venosus, common atrium, left ventricle (LV), trabeculae of the right ventricle (RV), forelimb, eye (Bruneau et al., 1999).	Embryonic lethality by E10.5. Failure of cardiac looping, hypoplasia of sinuatria and LV, forelimb defects (Bruneau et al., 2001).	Perinatal lethality in 40% of pups. Upper limb, conduction, atrial and ventricular septal defects (Bruneau et al., 2001).	Holt Oram syndrome (Basson et al., 1997; Li et al., 1997).
Tbx18	Splanchnic mesoderm, septum transversum, proepicardial organ, epicardium (Bussen et al., 2004).	Perinatal lethality likely due to respiratory failure, severe skeletal malformation of vertebral column and thoracic cavity (Bussen et al., 2004).	Apparently normal (Bussen et al., 2004).	None identified.
Tbx20	Allantois, lateral plate mesoderm, cardiac crescent, heart tube (graded, highest caudally and in outflow region), endocardial cushions, hindbrain, eye (Carson et al., 2000; Kraus et al., 2001; Meins et al., 2000; Stennard et al., 2003).	Embryonic lethality by E11.5. Abnormal development of yolk sac vasculature. Failed cardiac looping, hypoplastic heart, lack of cardiac chamber differentiation (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005).	Viable and fertile. Mild dilated cardiomyopathy of LV (Stennard et al., 2005).	None identified.

of *TBX1* in CHD, and its regulation and function in the secondary heart field.

DiGeorge syndrome, velo-cardio-facial syndrome and conotruncal anomaly face syndrome are three human congenital anomaly syndromes that share certain clinical features, including cardiovascular malformations (OMIM numbers 18840, 192430, 217095). They also share the same 1.5 Mb or ~3 Mb monoallelic microdeletion of chromosome 22q11.2, and are now collectively termed the 22q11 deletion syndrome (22q11DS) (for a review, see Yamagishi and Srivastava, 2003). This is the most common genetic deletion syndrome in humans and, as such, its underlying mechanism has attracted intense scrutiny. Through heroic chromosomal engineering feats in transgenic and knockout mice, the T-box gene *TBX1* has emerged as the leading candidate for disease causation (reviewed by Lindsay, 2001), and, indeed, *TBX1* mutations have recently been found in patients who have 22q11DS-like symptoms but not the deletion (Yagi et al.,

2003). The clinical features of 22q11DS include severe cardiac OFT and aortic arch anomalies, as well as other structural malformations of the pharyngeal complex and behavioural problems. Cardiovascular defects include tetralogy of Fallot, caused by mal-partitioning of the OFT vessels, persistent truncus arteriosus, in which the OFT is not septated, and interruption of the left fourth aortic arch artery. These abnormalities are similar to those caused by the ablation of cranial neural crest (CNC) cells, and the syndrome has long been regarded as a 'neurocristopathy' (reviewed by Kirby and Waldo, 1995). *Tbx1* heterozygous mice show defects of aortic arch artery four, whereas *Tbx1* null mice develop most of the clinical features of 22q11DS, albeit at the severe end of the spectrum, causing perinatal death (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001).

Tbx1 is a transcriptional activator (Altaliotis et al., 2005; Hu et al., 2004) and, in mouse, the gene is expressed in head mesoderm, from as early as E7.5, and in pharyngeal endoderm

(Chapman et al., 1996; Yamagishi et al., 2003). Cre-based lineage tracking shows that the *Tbx1* mesodermal domain includes cells of the secondary heart field (Xu et al., 2004). The finding that *Tbx1* is not expressed in neural crest cells revealed, contrary to expectation, that defects of the pharyngeal complex in 22q11DS secondarily lead to failure of the cellular mechanisms that support CNC development. *Tbx1* expression in head mesoderm and endoderm is maintained by the signaling morphogen sonic hedgehog (Shh), which is secreted from pharyngeal ectoderm (Hu et al., 2004; Yamagishi et al., 2003). Shh induces expression of the forkhead-class transcription factors Foxc1 and Foxc2 in mesoderm, and Foxa2 in endoderm, and these factors act directly on *Tbx1*. In mesoderm, the expression of *Foxc1* and *Foxc2* are themselves dependent on Tbx1, showing that a positive-feedback loop involving Fox proteins maintains mesodermal *Tbx1* expression (Hu et al., 2004). Retinoic acid signaling represses *Tbx1* expression in the caudal pharynx (Roberts et al., 2005).

Fibroblast growth factors (Fgfs) 8 and 10 are key downstream effectors of Tbx1 in the pharyngeal region. *Fgf8* and *Fgf10* are both expressed in pharyngeal tissues in patterns that overlap that of *Tbx1* (Kelly et al., 2001; Vitelli et al., 2002b) (Fig. 1A,C), and both Fgf genes are downregulated in mesoderm of *Tbx1* null and hypomorphic embryos (Hu et al., 2004; Vitelli et al., 2002b). Furthermore, embryos hypomorphic or conditionally deleted for *Fgf8* display the same cardiac and pharyngeal abnormalities seen in *Tbx1* null mice and 22q11DS (Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003; Trumpp et al., 1999). Genetic tests have confirmed that *Tbx1* and *Fgf8* act in the same pathway (Vitelli et al., 2002b).

The Shh→Foxc1/c2/a2→Tbx1→Fgf8/10 pathway has multiple roles in the pharyngeal region: it maintains cell proliferation and differentiation in mesenchyme, and is important for patterning the endoderm, as well as for the survival, differentiation and migration of neural crest cells (Abu-Issa et al., 2002; Frank et al., 2002; Hu et al., 2004; Macatee et al., 2003; Xu et al., 2004). The variable penetrance of 22q11DS phenotypes is modelled in mutant mice carrying various combinations of null, hypomorphic or conditionally deleted alleles (Abu-Issa et al., 2002; Hu et al., 2004; Xu et al., 2004). This probably reflects the multiplicity and complexity of the developmental processes involved, and in particular their common dependence upon secreted factors, such as Fgf8 and Fgf10, that are expressed in multiple tissues. Interestingly, the infiltration of neural crest into the pharyngeal region is necessary for the normal deployment of myocardium to the OFT, highlighting the inter-dependence of tissue development in this zone (Waldo et al., 2005).

In the secondary heart field, Tbx1 functions in both growth and differentiation. *Fgf8* and *Fgf10* are expressed in secondary heart field mesoderm and associated endoderm, as well as weakly in the OFT (Hu et al., 2004; Kelly et al., 2001). The OFTs of hearts mutant for *Tbx1* or hypomorphic for *Fgf8* are truncated, due to the reduced deployment of *Tbx1*-positive cells (Xu et al., 2004). For *Tbx1*, this effect is cell non-autonomous, confirming the involvement of secreted factors in cell behaviour. These data are consistent with a role for the Tbx1→Fgf8/Fgf10 pathway in driving proliferation in secondary heart field mesoderm, contributing to OFT growth. BMP proteins are also induced in secondary heart field cells

proximal to the inflow and outflow poles of the heart (Cai et al., 2003; Waldo et al., 2001). BMPs can induce cardiomyogenic differentiation in collaboration with Fgf8 (Alsan and Schultheiss, 2002) and can moderate the proliferation of secondary heart field mesoderm (Waldo et al., 2001). Therefore, a delicate balance between the levels of Fgf and BMP factors appears essential for secondary heart field development.

T-box genes in chamber development

Mutations in human *TBX5* cause the rare autosomal-dominant Holt-Oram syndrome, which is characterized by forelimb and cardiac congenital abnormalities, the latter including atrial and ventricular septal defects, tetralogy of Fallot, hypoplastic left heart, and conduction anomalies (OMIM number 142900) (Basson et al., 1997; Li et al., 1997). Similar defects are found in *Tbx5* heterozygous mice (Bruneau et al., 2001).

Mouse *Tbx5* is expressed in the cardiac crescent, indicating its involvement in the earliest stages of cardiac induction, then in a graded fashion along the heart tube with the highest levels in the sinuatrial region (Bruneau et al., 1999). The graded pattern is established by retinoic acid signaling, which is known to be essential for the formation of the sinuatrium (Liberatore et al., 2000; Niederreither et al., 2001). Expression of *Tbx5* remains high in the caudal heart during subsequent development, but also occurs at lower levels in the LV, left half of the interventricular septum and trabeculae of the RV (Bruneau et al., 1999). The graded pattern of *Tbx5* expression across the heart appears to play an instructive role in determining the molecular identity and morphogenesis of chambers. For example, enforced expression of *Tbx5* relatively evenly throughout the heart tube leads to what is likely to be a 'caudalization' of more anterior regions: the upregulation in the RV of markers that are typically high in the LV; loss of the inter-ventricular septum and trabeculae; and downregulation of a ventricle marker in the LV (Liberatore et al., 2000; Takeuchi et al., 2003b). Tbx5 overexpression inhibits myocyte proliferation (Hatcher et al., 2001), which could also be interpreted in this light. In *Tbx5* null embryos, the left ventricular and sinuatrial regions are severely hypoplastic (Fig. 3C), and numerous chamber markers, including *Nppa*, are not expressed (Bruneau et al., 2001).

Known target genes of Tbx5 are few. However, Tbx5 associates directly with other conserved cardiac transcription factors, including the homeodomain factor Nkx2-5 and the zinc finger factor Gata4 (Bruneau et al., 2001; Garg et al., 2003; Hiroi et al., 2001), as well as with the transcriptional co-activators Tip60, a histone acetyltransferase (Barron et al., 2005) and Baf60c, a component of the Swi/Snf-like BAF chromatin remodeling complex (Lickert et al., 2004). CHD-causing mutations in TBX5 or in its partner factors are known to disrupt some of these interactions (Bruneau et al., 2001; Fan et al., 2003; Garg et al., 2003; Hiroi et al., 2001). Acting in synergy with Nkx2-5 and Gata4, Tbx5 can stimulate transcription from the promoters of chamber-specific genes such as *Nppa* and *Gja5* (which encodes connexin 40) in vitro (Bruneau et al., 2001; Habets et al., 2002; Hiroi et al., 2001; Lickert et al., 2004).

We can conclude that Tbx5 is a positive transcriptional driver of cardiac specification, as well as of chamber morphogenesis and differentiation, in the developing

mammalian heart. Other cardiac transcription factors, including Nkx2-5, are also essential for chamber differentiation (Lyons et al., 1995; Palmer et al., 2001; Tanaka et al., 1999). However, the documented features of *Tbx5* and *Nkx2-5* fall well short of explaining the spatial specificity of chamber development. For example, *Tbx5* and *Nkx2-5* are not expressed in a chamber-specific manner, and, paradoxically, both factors are essential for the differentiation of the central conduction system, a derivative of non-chamber myocardium (Jay et al., 2004; Moskowitz et al., 2004; Pashmforoush et al.,

Fig. 3. T-box gene mutant phenotypes in mice. (A,B) Scanning electron micrographs showing hearts from wild-type (wt; A) and *Tbx20*$^{-/-}$ (B) E9.0 embryos (scale bars indicate relative size), showing hypoplasia, lack of looping and abnormal chamber development in the mutant. (C) In situ hybridization showing expression of cardiac marker *Nkx2-5* in wild-type and *Tbx5*$^{-/-}$ E8.5 embryos, demonstrating a severe reduction of the LV and SA region in the mutant. (D-J) Comparison of *Tbx20*$^{-/-}$ embryos with wild-type siblings at ~E8.5, showing upregulation of *Tbx2* (D), reduced proliferation (immunostaining for phosphohistone H3; arrows indicate the heart tube; E-F) and downregulation of cell-cycle gene *Nmyc1* (G-J) in mutants. G,H are at the level of the OFT, I,J are at the level of the AVC. Figures reproduced from Bruneau et al., Cai et al. and Stennard et al. (Bruneau et al., 2001; Cai et al., 2005; Stennard et al., 2003), with permission from Elsevier and Company of Biologists Limited. avc, atrioventricular canal; iv, inflow ventricle-like chamber; lv, left ventricle; oft, outflow tract; ov, outflow ventricle-like chamber; rv, right ventricle; sa, sinuatrium.

2004). These factors are likely to act as 'selector genes', defining an organ-specific context for many cardiac processes (Barolo and Posakony, 2002). To understand spatial specificity in heart development, other players are required, and the dynamics of signal-dependent transcriptional changes need to be defined.

Tbx2/Tbx3 and spatial definition of cardiac chambers

The inclusion of Tbx2 and its close relative Tbx3 in the cardiac regulatory network has given us a way to visualize at least some of the spatial aspects of cardiac chamber formation. Tbx2 and Tbx3 are transcriptional repressors (Carreira et al., 1998; Habets et al., 2002; Hoogaars et al., 2004; Jacobs et al., 2000; Sinha et al., 2000), and Tbx2 can bind the co-repressor Hdac1, a histone deacetylase (Vance et al., 2005). In the developing heart, Tbx2 and Tbx3 are expressed in non-chamber myocardium, mutually exclusively of markers of chamber myocardium such as *Nppa* (Habets et al., 2002; Hoogaars et al., 2004) (Fig. 2E-J). In vitro they act as potent repressors of the promoters of *Nppa* and *Gja5*, which are normally activated in chamber myocardium by Tbx5 and Nkx2-5 (Habets et al., 2002; Hoogaars et al., 2004). Indeed, Tbx2 and Nkx2-5 form a repressive ternary complex on *Nppa* promoter DNA, and mutagenesis has shown that this complex formation depends on a T-box binding element (TBE) and, to a lesser extent, on a Nkx2-5-binding element (NKE). Indeed, by transgenic analysis, the TBE and NKE have been shown to be crucial for *Nppa* repression in non-chamber myocardium of the AV canal, with the mutation of either element causing promiscuous transgene expression in that region (Habets et al., 2002). An attractive model is that Tbx2 competes away the positive chamber factor Tbx5 from the TBE/NKE element of the *Nppa* gene in non-chamber myocardium, forming a repressive complex with Nkx2-5 (Fig. 4). In support of this model, enforced expression of *Tbx2* (or *Tbx3*) throughout the developing heart tube completely blocks chamber formation (Christoffels et al., 2004b; Hoogaars et al., 2004), and, in *Tbx2* knockout mice, several chamber markers are activated inappropriately across the AV canal, effectively merging the LV with the atria (Harrelson et al., 2004).

Tbx2 controls regional proliferation through Nmyc1

The data above show that *Tbx2* and *Tbx3* participate in setting or reinforcing the boundaries between atrial and ventricular chambers, but do they guide morphogenesis directly? In analyzing *Tbx20* knockout mice (see below), Evans and colleagues revealed a direct role for Tbx2 in repressing the expression of *Nmyc1* in non-chamber myocardium (Cai et al., 2005). *Nmyc1* encodes a basic helix-loop-helix leucine zipper transcription factor related to *Myc* (previously known as *c-myc*) that heterodimerizes with its partner Max. Myc proteins are thought to 'tune' the expression of many genes involved in metabolism (protein synthesis and/or cell growth), apoptosis and the cell cycle (Hipfner and Cohen, 2004). Studies in the developing lung and brain show that Nmyc1 drives proliferation in their precursor populations (Kenney et al., 2003; Okubo et al., 2005).

Nmyc1 is expressed in the heart tube, with transcripts being enriched in chamber myocardium in a complementary pattern to that of Tbx2 (Cai et al., 2005; Moens et al., 1993) (Fig.
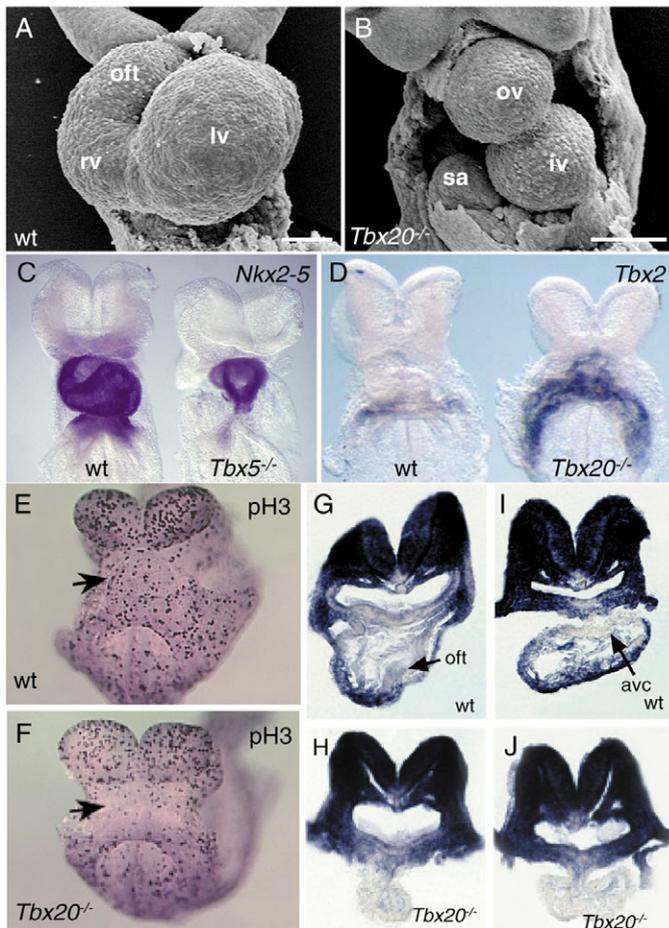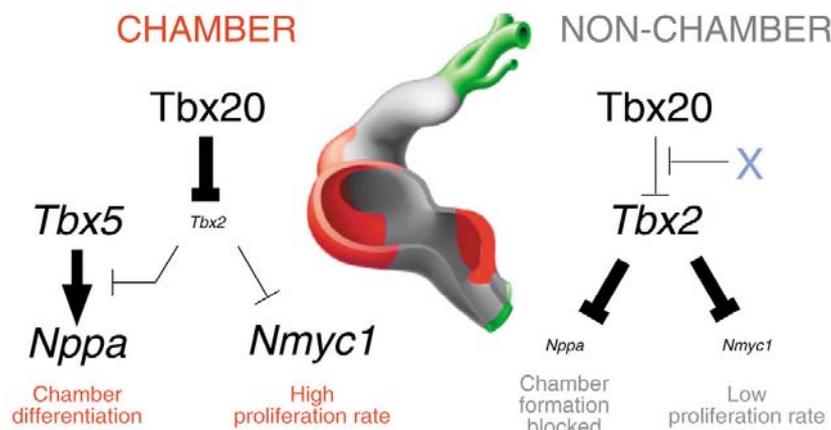
Fig. 4. Model of T-box transcription factor regulation of cardiac chamber development. Chamber myocardium is shown in red and non-chamber (primary) myocardium is shown in grey. Interactions between T-box genes in each of these compartments are indicated, with the relative expression levels of genes, as determined by positive and repressive interactions, reflected in the size of the lettering. Factor X (blue) is proposed to activate *Tbx2* in non-chamber myocardium through repression of the *Tbx2* inhibitory activity of Tbx20.

3G,I). With further development, expression becomes restricted to the so-called 'compact myocardium', corresponding to the outer cellular layers of the ventricles that expand massively through proliferation. *Nmyc1* is in fact essential for early chamber growth and expansion of the compact layer, as shown by the analysis of knockout and hypomorphic embryos (Charron et al., 1992; Moens et al., 1993; Sawai et al., 1993).

Tbx2 binds directly to TBEs in the proximal promoter of the *Nmyc1* gene, and Tbx2 can repress *Nmyc1* reporter constructs in vitro (Cai et al., 2005). Although mechanistic details are lacking, the Tbx2-Nmyc1 inhibitory pathway ensures that non-chamber myocardium and its conduction system descendents are kept in a relatively mitotically inactive state. Chamber myocardium, which does not express *Tbx2*, retains high levels of *Nmyc1*, shows a high proliferative rate and 'balloons out' from primary myocardium, generating the characteristic form of the looping heart (Christoffels et al., 2000) (Fig. 2B,C).

It should be noted that Tbx3 has anti-apoptotic activity in bladder cells (Ito et al., 2005), and that Tbx2 and Tbx3 are amplified or overexpressed in pancreatic, breast and skin tumours. These T-box genes are suspected to override senescence by directly repressing the cell-cycle inhibitor $p21^{Cip1}$ gene (Vance et al., 2005). The specific roles for Tbx2 and Tbx3 in facilitating proliferation in other systems contrast with those described in the heart, in which Tbx2 inhibits proliferation via the repression of *Nmyc1*. The roles of T-box genes in cell-cycle control may therefore vary in different tissues. A common theme, nonetheless, is that Tbx2 and Tbx3 mediate the transcriptional repression of cell cycle-related genes.

Hierarchies of repression: Tbx20 negatively regulates *Tbx2*

A yet higher tier of T-box regulation in the heart has recently come to light. Four papers this year reported the genetic or RNAi-mediated knockout/knockdown of *Tbx20* in mice (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005), and knockdowns of *Tbx20* orthologues in fish and frogs have also been reported (Brown et al., 2005; Szeto et al., 2002). *Tbx20* is an ancient member of the T-box family and belongs to the same sub-family as *Tbx1* (Plageman and Yutzey, 2004). In the mouse, it is expressed in the cardiac crescent, with some possible overlap with cells of the secondary heart

field (Kraus et al., 2001; Takeuchi et al., 2005). In the heart tube, *Tbx20* is expressed across the myocardium and also strongly in endothelial cells associated with endocardial cushions (Stennard et al., 2003; Stennard et al., 2005; Takeuchi et al., 2005). With further development, expression is turned down in chamber myocardium, but remains high in endocardial cushions (Stennard et al., 2005). Tbx20 carries strong transcriptional repression and activation domains, and interacts directly with other cardiac transcription factors, including Tbx5, Nkx2-5, Gata4/5 and Isl1 (Brown et al., 2005; Stennard et al., 2003; Takeuchi et al., 2005).

The consequences of Tbx20 loss in the mouse are catastrophic for heart development (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). The mutant heart tube is small, does not loop, and the deployment of progenitor cells is impaired (Fig. 3A,B). Whilst some details of characterization differ between groups, several clear findings emerge. The expression of the transcription factors genes *Nkx2-5*, *Gata4* and *Mef2c*, and the cardiac inducers *Bmp2/Bmp5*, is downregulated or delayed, suggesting some positive, albeit redundant, involvement of *Tbx20* in inducing and supporting the core cardiac transcription factor network, as has been suggested for *Tbx20* orthologues in *Drosophila* (Miskolczi-McCallum et al., 2005; Qian et al., 2005). This is consistent with the ability of Tbx20 to activate cardiac enhancers of *Nkx2-5*, *Mef2c* and other heart regulatory genes in vitro (Takeuchi et al., 2005). Small ventricle-like chambers are formed in null mutants, and these appear to represent precursor pools for the left and right ventricles, indicating that both first and second heart lineages contribute to the mutant heart tube. However, chamber myocardium does not differentiate, as seen by the lack of chamber-restricted markers *Nppa*, *Smpx*, *Cited1* and *Hand1*.

A key finding is that *Tbx2* expression is dramatically upregulated in probably all myocardial progenitor cells in *Tbx20* knockout embryos (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005) (Fig. 3D). As discussed, Tbx2 is a transcriptional repressor that normally inhibits cell proliferation and chamber differentiation in non-chamber myocardium. *Tbx2* overexpression might therefore explain both the complete block of chamber differentiation, and the severely hypoplastic state of myocardium in *Tbx20* mutant hearts. Hypoplasia is evident after staining in wholemount or on sections for phospho-histone H3 (Fig. 3E,F). *Nmyc1*

expression, normally high in chamber myocardium, is virtually eliminated in the mutant hearts (Fig. 3G-J).

An important mechanistic detail is whether repression of *Tbx2* by Tbx20 is direct or indirect. A pair of TBEs was found ~680 bp upstream of the *Tbx2* transcriptional start site, and ChIP analysis shows that these are occupied by Tbx20 (Cai et al., 2005). Tbx20 can repress this portion of the Tbx2 promoter in vitro in a TBE-dependent manner. We can conclude that Tbx20 represses *Tbx2* directly, and one of its principal roles is to keep Tbx2, a repressor of chamber differentiation, off in the developing chamber myocardium (Fig. 4). This seems to fit the concept of 'default repression', a feature of virtually all conserved signal-responsive transcriptional pathways acting in development (Barolo and Posakony, 2002). In essence, genes that are required to affect an important developmental switch need to be actively repressed so that other positive drivers of their transcription, particularly 'selector genes', do not cause cryptic activation. When activated by an external signal, the repressor is displaced or converted to an activator.

In non-chamber myocardium, the expression of *Tbx20* and *Tbx2* overlap. Thus, the inhibitory role of Tbx20 on *Tbx2*, clearly evident in chambers, must itself be somehow inhibited in non-chamber myocardium. We ascribe this function to factor X in Fig. 4, and because Bmp2 and Bmp4 are expressed in non-chamber myocardium of the looping heart, and because they have been implicated in the induction of *Tbx2* in vitro (Yamada et al., 2000), they are good candidates for this role. Factor X is presumably a signal that converges on Tbx20 to release the default repression of *Tbx2*.

Other repressive roles for Tbx20 are also evident in heart development. Expression of *Myl2*, a ventricle region marker, is downregulated in *Nkx2-5* mutant embryos, but was 're-expressed' in doubly homozygous *Nkx2-5/Tbx20* mutants, inextricably implicating Tbx20 as a repressor in the pathway leading to *Myl2* expression (Stennard et al., 2005).

Role of Tbx20 in heart disease

Heterozygous *Tbx20* mutant mice appear to be healthy and are fertile. However, several findings suggest that we should be looking for the involvement of *TBX20* in human CHD and in adult cardiac pathologies. It is well known that mutations in the cardiac transcription factors TBX5, NKX2.5 and GATA4 cause a range of CHDs, including atrial septal defect (ASD) (Prall et al., 2002). TBX20 associates directly with all of these factors (Brown et al., 2005; Stennard et al., 2003). As in *Nkx2-5* heterozygous mice (Biben et al., 2000), there is an increased prevalence of mild atrial septal anomalies, including atrial septal aneurysm (ASA) and patent foramen ovale (PFO), in *Tbx20* heterozygous mice (Stennard et al., 2005). Our previous work has suggested that ASD, ASA and PFO are degrees of the same anatomical and pathological continuum (Biben et al., 2000), raising the possibility that the *Tbx20* heterozygotes are actually genetically sensitized to ASD. Indeed, 16% of doubly heterozygous *Nkx2-5/Tbx20* mutants show fully frank ASD (Stennard et al., 2005). Echocardiography on these mice has also shown that they have dilated cardiomyopathy, with reduced ejection fraction, increased end-systolic LV wall dimensions, and occasional gross dilation of the RV with
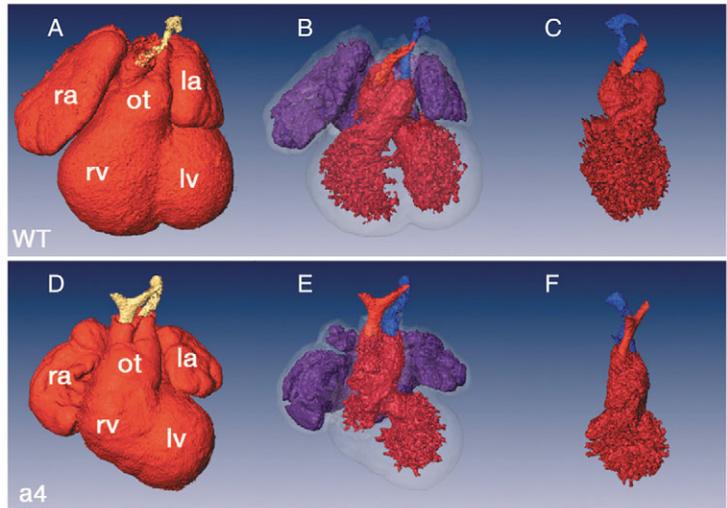

Fig. 5. Cardiac defects resulting from partial RNAi knockdown of *Tbx20*. Rendered optical projection tomography (OPT) images of hearts from wild-type (WT; A-C) and RNAi knockdown (a4; D-F) E12.5 mouse embryos. Embryonic stem cells from which knockdown embryos were derived had a 65% reduction in *Tbx20* mRNA levels and a 40% reduction in protein levels compared with wild type. (A,D) Surface rendered view. (B,C,E,F) Space-filling images of chamber lumina showing atria in purple (removed in C,F), ventricles in dark red, pulmonary artery in blue and aorta in red. Note the hypoplastic right ventricle and the lack of outflow vessel spiraling in the knockdown heart. Reproduced from Takeuchi et al. (Takeuchi et al., 2005), with permission from Company of Biologists Limited. la, left atrium; lv, left ventricle; ot, outflow tract; ra, right atrium; rv, right ventricle.

myocyte disarray (Stennard et al., 2005). These features are similar to those seen during heart failure in humans. Intriguingly, no cardiomyocyte hypertrophy was seen in these mice at an anatomical or molecular level, suggesting that hypertrophy, which is normally seen as a compensatory response to cardiac pathology, is blocked if *Tbx20* levels are compromised (Stennard et al., 2005). Other developmental transcription factors, including Gata4 and Mef2c, are directly involved in the hypertrophic response (Liang and Molkentin, 2002), and Tbx20 may be an important partner for these factors.

The elegant transgenic RNAi knockdown approach to assessing *Tbx20* function, which was taken by Bruneau and colleagues, allows hypomorphic phenotypes to be examined (Takeuchi et al., 2005). Foetuses with 65% knockdown of *Tbx20* showed normal heart patterning but severely compromised RV and OFT development, including hypoplastic RV, double outlet RV, persistent truncus arteriosis, and defects in outflow and tricuspid valve formation (Fig. 5). These phenotypes most certainly relate to the high level of *Tbx20* expression in endocardial cushion and OFT, and potentially to a partial manifestation of the developmental phenotypes discussed above. Thus, dominant *TBX20* mutations in humans might be expected to preferentially affect the RV and OFT.

Perspectives

There is much more to learn about cardiac development and its

relationship to CHD. Understanding T-box gene function in both first and second heart lineages, and during chamber formation, is clearly at the centre of this endeavour. Our view of T-box factor involvement in cardiac chamber formation (Fig. 4) deliberately places Tbx2 in a nodal position. This is because it is a key regulator of the chamber/non-chamber lineage separation upon which all subsequent cardiac morphogenesis depends. But why has this important lineage decision in cardiac morphogenesis utilized repression of a repressor, or, if Factor X is invoked, repression of a repressor of a repressor? Superficially, this seems terribly inefficient, so why has it evolved? The answer to this may lie in how functional efficiencies in the hearts of mammals and other species were achieved through the innovation of valves, septa and specialized conduction components. It is likely that the most expedient evolutionary strategy for crafting these parts was to modify lineages already existing in the simplest and most ancient of hearts, namely myocytes and endothelial cells. We propose that an essential developmental and evolutionary theme in the chamber/non-chamber lineage split is modulation of the extent of myogenesis driven by the ancient conserved core transcriptional pathway. In chambers, ancestral myogenesis is 'enhanced', and the neuregulin pathway, essential for full chamber differentiation, is likely to be involved in this process (Burden and Yarden, 1997). In non-chamber myocardium, myogenesis is 'repressed', allowing the specialization of other components, such as conduction cells, which essentially retain the electrical but not contractile properties of muscle. Tbx2 is the nodal element that 'de-tunes' myogenesis in non-chamber myocardium. It is interesting that Tbx2 and Tbx3 are also expressed in the second heart lineage mesoderm, so we can anticipate interesting, perhaps repressive, interactions with Tbx1 function in this region.

Applied to mammalian heart development, this viewpoint would posit that neither chamber nor non-chamber myocardium is the default evolutionary state. Importantly, the model also allows us to see how function begets form in heart development (see Introduction). Pathways responsive to biomechanical stress or other cues associated with function would somehow exaggerate chamber growth and differentiation (Sedmera et al., 2000). The scheme shown in Fig. 4 will therefore serve as a template for expanding our understanding, not only of chamber formation per se, but of the many other inputs affecting developmental regulation in this important organ.

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