Murine T-box transcription factor Tbx20 acts as a repressor during heart development, and is essential for adult heart integrity, function and adaptation

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Summary

The genetic hierarchies guiding lineage specification and morphogenesis of the mammalian embryonic heart are poorly understood. We now show by gene targeting that murine T-box transcription factor Tbx20 plays a central role in these pathways, and has important activities in both cardiac development and adult function. Loss of Tbx20 results in death of embryos at mid-gestation with grossly abnormal heart morphogenesis. Underlying these disturbances was a severely compromised cardiac transcriptional program, defects in the molecular pre-pattern, reduced expansion of cardiac progenitors and a block to chamber differentiation. Notably, Tbx20-null embryos showed ectopic activation of Tbx2 across the whole heart myogenic field. Tbx2 encodes a transcriptional repressor normally expressed in non-chamber myocardium, and in the atrioventricular canal it has been proposed to inhibit chamber-specific gene expression through competition with positive factor Tbx5. Our data demonstrate a repressive activity for Tbx20 and place it upstream of Tbx2 in the cardiac genetic program. Thus, hierarchical, repressive interactions between Tbx20 and other T-box genes and factors underlie the primary lineage split into chamber and non-chamber myocardium in the forming heart, an early event upon which all subsequent morphogenesis depends. Additional roles for Tbx20 in adult heart integrity and contractile function were revealed by in-vivo cardiac functional analysis of Tbx20 heterozygous mutant mice. These data suggest that mutations in human cardiac transcription factor genes, possibly including TBX20, underlie both congenital heart disease and adult cardiomyopathies.

Key words: T-box, Tbx20, Heart, Nkx2-5, Tbx2, Chamber myocardium, Dilated cardiomyopathy, mice

Introduction

Cardiac development in mammals is guided by an ancient and conserved genetic program (Cripps and Olson, 2002; Harvey, 2002; Zaffran and Frasch, 2002). However, how the cardiac program unfolds, the specific relationships between patterning events and the transcription factor hierarchy, and how cardiomyocyte function impacts on heart form remain poorly understood.

Of 18 T-box factor genes identified in mammals, at least six of them (Tbx1/2/3/5/18/20) are expressed in the developing heart (Plageman and Yutzey, 2004). T-box proteins are characterized by the presence of a sequence-specific DNA-binding domain called the T-box (Smith, 1999). During embryogenesis, T-box genes are expressed in restricted and sometimes overlapping domains throughout gastrulation and/or organogenesis, and in some cases roles in controlling cell fate and migration have been demonstrated (Chapman and Papaioannou, 1998; Naiche and Papaioannou, 2003; Russ et al., 2000). T-box factors can act up- or downstream of signaling factors of the TGF-β (Suzuki et al., 2004), fibroblast growth factor (Brown et al., 2004; Hu et al., 2004; Sakiyama et al., 2003; Yamagishi et al., 2003), sonic hedgehog (Suzuki et al., 2004; Yamagishi et al., 2003) and wingless-related (Takeuchi et al., 2003) superfamilies.

Haploinsufficiencies for several human T-box genes have been linked to congenital anomaly syndromes (Bongers et al., 2004; Packham and Brook, 2003). Two of these involve cardiac malformations. Di George syndrome, also occurring as part of chromosome 22q11 deletion syndrome, is characterized by dysmorphogenesis of the cardiac outflow tract (OFT), as well as thymic, splenic and craniofacial abnormalities (Yamagishi and Srivastava, 2003). Holt Oram
syndrome is characterized by congenital abnormalities of the upper limbs and heart, the latter involving atrial and ventricular septal defects, tetralogy of Fallot and atrioventricular conduction block (Gruber and Epstein, 2004). Targeted mutation of causative genes in mice has reproduced many aspects of the human cardiac disease phenotypes, thus providing valuable models for understanding underlying mechanisms (Bruneau et al., 2001; Lindsay et al., 2001; Merscher et al., 2001; Yamagishi and Srivastava, 2003).

Tbx20 is an ancient member of the T-box gene subfamily related to Tbx1 (Plageman and Yutzey, 2005). The Drosophila gene is expressed in early cardioblasts of the dorsal vessel of the fly, a primitive heart-like organ (Griffin et al., 2000). During fish development, Tbx20 (left) is expressed in cardiac progenitors, developing heart and endothelial cells of the dorsal aorta (Ahn et al., 2000; Griffin et al., 2000), and Tbx20 orthologs with analogous expression patterns have since been identified from frog, chicken, mice and humans (Carson et al., 2000; Horb and Thomsen, 1999; Kraus et al., 2001; Meins et al., 2000; Stennard et al., 2003). During mouse development, Tbx20 is expressed in the cardiac crescent prior to heart tube formation, then in myocardium and endocardium of the looping heart (Kraus et al., 2001; Stennard et al., 2003).

As for other T-box factors (Bruneau et al., 2001; Casey et al., 1999; Habets et al., 2002; Harrelson et al., 2004; He et al., 1999; Hoogaars et al., 2004; Hsueh et al., 2000; Kispert et al., 1995; Lamolet et al., 2001; Paxton et al., 2002; Stennard et al., 2003; Tada and Smith, 2000), Tbx20 can regulate transcription of target genes positively and negatively, depending on the particular isoform expressed and, potentially, cellular context (Plageman and Yutzey, 2004; Stennard et al., 2003).

The T-box DNA-binding domain of Tbx20 can associate specifically, albeit weakly, with the consensus DNA half site sequence defined for the brachyury (T) protein, the founding member of the T-box family, and can interact in solution and function synergistically with homeodomain factor Nkx2-5 and zinc finger factor Gata4 (Stennard et al., 2003).

Morpholino oligonucleotide knockdown of Tbx20 in fish produces small and dysmorphic hearts showing upregulation of Tbx5, ectopic expression of blood markers caudally and an abnormally patterned aorta (Szeto et al., 2002). Tbx20 downregulation in frogs leads to absent (Horb and Thomsen, 1999) or dysmorphic (Brown et al., 2005) hearts. Frog Tbx20 physically interacts with Tbx5 and cardiac defects in embryos are more frequent and severe if both proteins are concomitantly inhibited (Brown et al., 2005). Enforced expression experiments in frog embryos show that mouse Tbx20 can induce mesodermal and endodermal cell fates and their coordinated cell migration (Stennard et al., 2003).

We report here the loss-of-function phenotype of murine Tbx20. Tbx20 null embryos showed grossly abnormal cardiac development and arrested yolk sac vascular remodeling. Our analysis has highlighted a role for Tbx20 as a transcriptional repressor during the primary lineage split in myocardium into chamber and non-chamber fates. Furthermore, hierarchical interaction between different T-box genes was revealed as a central element of early heart patterning and morphogenesis. Tbx20 also acts in adult heart function and homeostasis, with implications for human cardiomyopathies.

**Materials and methods**

**Expression analysis**

In-situ hybridization and histochemical methods were as described (Biben and Harvey, 1997; Wang et al., 2000). Rat anti-Pecam (DAKO) and rabbit anti-phosphohistone H3 (Upstate) antibodies were used at 1:200, and secondary antibodies at 1:250, with the Elite ABC kit (Vector Laboratories) and DAB substrate (Sigma). Apoptotic cells were detected using DeadEnd™ Fluorometric TUNEL System (Promega). Total RNA was isolated from hearts using Trizol (Invitrogen) and subjected to Northern analysis as described (Fatkin et al., 2000). RT-PCR using 1 μg of total RNA treated with RQ1 DNase (Promega) and 35 cycles of PCR (Rotor-Gene3000, Corbett Technologies) was as described (Stennard et al., 2003).

**Embryo culture**

Explants from C57BL/6J E8.5 embryos were cultured in DMEM media containing 0.5% (v/v) heat-inactivated fetal bovine serum, 10 mmol/l glutamine, 100 units/ml penicillin and streptomycin (GibcoBRL) in 1% agarose-coated wells for 24 hours with or without recombinant human Heregulin β2 (10–9 mol/l) (Fiddes et al., 1995).

**Gene targeted mice**

Tbx20lacZ/+ mice were generated by Ozgene Pty Ltd (Perth, Australia). Nkx2.5lacZ mice were generated using a vector similar to one described (Biben et al., 2000), in which a lacZ cassette was inserted in frame into exon 1 (M.S., C.B. and R.P.H., unpublished).

**Transthoracic echocardiography**

Two-dimensional echocardiographic images were obtained using a Sonos 5500 ultrasonograph with 12 MHz probe (Philips Medical Systems) as described (Fatkin et al., 2000). Statistical significance of data was determined by ANOVA and Student’s t-test.

**Results**

**Generation of Tbx20 mutant mice**

To target the Tbx20 gene by homologous recombination, we engineered a conditional vector (Fig. 1A) in which a Cre recombinase recognition site (loxP) was inserted just 5′ of the methionine initiation codon in exon I, and a cassette consisting of a loxP-flanked neomycin resistance gene (pgkneo) followed by a nuclear-localizing β-galactosidase gene (lacZ) was incorporated into intron 3 (Fig. 1A). Founders were crossed with transgenic mice expressing Cre recombinase in the germline (Schwenk et al., 1995). Cre deletion of floxed Tbx20 sequences led to excision of genomic regions encoding the methionine initiation codon, first 181 amino acids of the Tbx20 protein and the pgkneo cassette (Fig. 1B-E). Deletion also brought the lacZ reporter directly adjacent to Tbx20 cis-regulatory elements.

In Tbx20lacZ/+ embryos at embryonic day (E) 7.5, lacZ expression was robust in the cardiac crescent, as well as amniotic and yolk sac mesoderm (Fig. 1F), confirming published in-situ hybridization patterns (Carson et al., 2000; Kraus et al., 2001; Stennard et al., 2003). Patterns at E7.5-10.5 in heart, yolk sac, brain, spinal chord, eye and allantois also confirmed published studies and revealed additional sites of expression in pharyngeal endoderm, endothelium of the dorsal aorta and associated sympathetic ganglia, vitelline and placental vessels, and adrenal medulla (Fig. 1F-I, Fig. 2E-G and data not shown).
Abnormal cardiac morphogenesis in Tbx20lacZ/lacZ embryos

Homozygous Tbx20lacZ/lacZ embryos appeared normal until E7.5 but subsequently showed severe cardiac dysmorphogenesis and arrested yolk sac vascular remodeling, and all died around E10.5. Heart tube formation was retarded and abnormal from the outset. The primary ventricular chamber was small, looping was blocked and, notably, there was a significant delay in closure of the foregut pocket at caudal levels. From E8.0-9.5, a distinct additional compartment in the outflow region became progressively obvious in mutant hearts, resulting by E9.5 in a heart tube with two small chamber-like swellings separated by a circumferential sulcus (Fig. 2A-D). Apart from occasional inward myocardial protrusions, these chambers did not form trabeculae and no endocardial cushions were evident in the outflow or atrioventricular regions (Fig. 2E-J and data not shown). LacZ expression was largely as expected in mutant hearts, although there was ectopic activation in dorsal mesocardial tissue in the sinusatrial region (Fig. 2G,J).

Cardiac gene expression in Tbx20 null embryos

The early transcription factor program was significantly compromised in Tbx20lacZ/lacZ hearts. Expression of T-box factor gene Tbx5 was reduced at E8.5, although the caudal-high, graded pattern seen in normal hearts was preserved (Bruneau et al., 1999) (Fig. 3A). Expression had recovered somewhat by E9.5 (Fig. 3B), suggesting delayed activation. The cranial limit of Tbx5 expression at E9.5 is normally at the level of the interventricular sulcus (Bruneau et al., 1999). In mutants, it was at the sulcus between the inflow and outflow chamber-like swellings (Fig. 3B), suggesting that these swellings represent precursors of the normal systemic (left) ventricle and pulmonary (right) ventricle/OFT, respectively. Consistent with this model, Hey1, encoding a basic helix-loop-helix factor acting downstream of Notch signaling and
expressed predominantly in endocardium of the OFT in normal E9.5 hearts (Iso et al., 2003), was expressed only in the outflow ventricle-like chamber in mutants (Fig. 3C).

Expression levels of the genes for homeodomain factor Nkx2-5, zinc finger factor Gata4 and MADS domain factor Mef2c were also significantly reduced in Tbx20lacZ/lacZ hearts at both E8.5 and 9.5 (Fig. 3D-F and data not shown). At E8.0, Gata4 expression was undetectable in the cranial portion of the heart progenitor field corresponding to the primary myogenic lineage, suggesting delayed activation. However, Gata4 was already activated at this time in medial and caudal portions of the heart progenitor region (Fig. 3E), potentially occupied by a distinct cardiac progenitor population termed the secondary heart field (SHF) (Cai et al., 2003; Meilhac et al., 2004). Tbx20 mRNA and Tbx20-lacZ were not substantially expressed in SHF cells dorsal to the heart once the heart tube had formed (Stennard et al., 2003) (Fig. 2E-G). SHF cells are deployed to the heart after formation of the primary heart tube and form the pulmonary ventricle and OFT, with significant contributions also to the atria. Other markers of the SHF, including Isl1 and Fgf8 (Cai et al., 2003), were expressed approximately normally in mutants at E8.5. Foxh1, encoding a potential upstream regulator of Mef2c in SHF (data not shown) derivatives (von Both et al., 2004) was also expressed normally (Fig. 3G). However, expression of Tnc, encoding the matrix protein tenascin C, while normal in mutant SHF cells dorsal and caudal to the forming heart at E8.5, failed to be maintained in the mutant outflow ventricle-like chamber (Fig. 3H).

As noted above, the outflow chamber in mutant hearts probably corresponds to precursors of the pulmonary ventricle and outflow tract of normal embryos, which are derived from the anterior SHF. To assess this further, we constructed transgenic mice bearing the human placental alkaline phosphatase gene (hPLAP) driven by an enhancer of the Mef2c gene, previously shown to accurately mark cells of the anterior SHF and their pulmonary ventricle and outflow derivatives (Dodou et al., 2004) (Fig. 3I). Tbx20lacZ/lacZ embryos carrying the transgene showed strong hPLAP staining in the outflow chamber extending...
caudally to the sulcus at E9.0, strongly supporting our hypothesis that this chamber is SHF-derived. Weaker staining was also seen in the caudal ventricle-like chamber, suggesting a contribution to this chamber from the anterior SHF. A minority of anterior SHF cells do contribute to the left ventricle in wild-type embryos (Cai et al., 2003), although the Mef2c enhancer used in these experiments is normally downregulated in those cells (D.J.M. and B.L.B., unpublished).

Excessive cell death was not detected in mutant myocardium or endocardium (data not shown). We therefore measured the mitotic index (proportion of cells expressing phosphohistone H3) in three zones of E9.0 hearts corresponding in wild-type embryos to the sinuatrium, systemic ventricle and pulmonary ventricle/OFT. The myocardium of the outflow ventricle-like chamber in mutants (n=2) had a mitotic index 6 to 7-fold less than the equivalent region in controls (n=2; P<0.0001, chi-squared test) (see Table S1 in the supplementary material). The index in the inflow ventricle-like chamber was also reduced, although less so (2.2 and 3.3-fold; P=0.056 and 0.008), while indices in the sinuatrium and head mesoderm were normal.

**Expanded cardiac pre-pattern in Tbx20 and Tbx20/Nkx2-5 homozygotes**

The *Myl2* gene, which encodes myosin light chain 2v, is expressed in ventricles and the atrioventricular canal (AVC), but not atria, betraying a molecular pre-pattern in the forming heart (Fig. 4A). How this pre-pattern is established is unknown, although maximal *Myl2* expression requires the homeodomain factor Nkx2-5 (Lyons et al., 1995). *Myl2* was expressed in *Tbx20*ΔΔ*lacZ* hearts at a level diminished compared with wild-type controls at E9.25, but nonetheless considerably higher than seen in single *Nkx2-5GFP/GFP* embryos, which lack Nkx2-5 function (Biben et al., 2000) (Fig. 4A). Expression encroached partially into the outflow ventricle-like chamber of *Tbx20* mutants (Fig. 4B), consistent with the notion discussed above that this chamber is SHF-derived and composed of progenitors that would normally form the pulmonary ventricle and OFT.

However, while the caudal boundary of *Myl2* expression was within the AVC in normal embryos, it was inappropriately positioned within the sinuatrial region of *Tbx20*ΔΔ*lacZ* hearts (Fig. 4B), clearly evident in sections (Fig. 4C). This was even more pronounced in doubly homozygous *Tbx20*ΔΔ*lacZ*/Nkx2-5GFP/GFP embryos, in which *Myl2* expression extended throughout the sinus venosus (Fig. 4B). The hearts of these embryos appeared to show a combination of the abnormalities seen in single homozygotes. However, the fact that the doubly homozygous embryos ‘reactivate’ *Myl2* to levels well above those seen in single *Nkx2-5GFP/GFP* homozygotes (Fig. 4A) strongly implicates transcriptional repression at or downstream of *Tbx20* in the genetic circuitry controlling *Myl2* (see Discussion). To investigate the apparent expansion of the *Myl2* expression domain further, we compared expression of *Myl2* and its relative *Myl7* (encoding myosin light chain 2a) at earlier stages. Despite the obvious delay in heart tube formation in mutants, dimensions of the cardiac myogenic field, as highlighted by *Myl7*, appeared comparable to those of controls at E8.5 (Fig. 4D,E). *Myl2* expression was highly regional within the *Myl7* domain. However, expression in mutants, unlike that in wild types, extended into the apparent sinuatrial region. Furthermore, before
Spatial specification of the atrial domain appeared nonetheless normal in mutants. Although diminished, a morphological sinuatrium and AVC had formed by E9.5 (Fig. 2C,D). Furthermore, the regional expression of Hey1 was spatially correct in the outflow domain (see above) and sinuatrial region, albeit downregulated significantly in this latter domain (Fig. 4G). Expression of Aldh1a2, which overlaps the sinuatrial region of normal hearts and is essential for atrial specification (Niederreither et al., 2001), was also normal in Tbx20lacZ/lacZ embryos (Fig. 4H).

Chamber formation in Tbx20 mutants

The myogenic layer of the early heart tube undergoes an initial regional specialization to form working myocardium of the ventricles and atrial appendages (Christoffels et al., 2000), an event that depends on transcription factors Nkx2-5 (Palmer et al., 2001), Tbx5 (Brunneau et al., 2001) and Foxh1 (von Both et al., 2004). Expression levels of Nppa and Smox, markers of chamber myocardium (Christoffels et al., 2000; Palmer et al., 2001), were severely reduced in mutant hearts at E8.5 and 9.5 (Fig. 4I-J), demonstrating lack of chamber differentiation. Hand1, expressed predominantly in the forming left ventricle, was also dramatically downregulated (Fig. 4K).

In the looping heart, non-chamber myocardium retains the slow conduction features evident in the primary heart tube and is destined to form elements of the central conduction system (Christoffels et al., 2004a). Tbx2, encoding another member of the T-box family, is expressed in non-chamber myocardium, most prominently in the AVC (Habets et al., 2002), where it has been proposed to repress formation of chamber myocardium (Christoffels et al., 2004b; Harrelson et al., 2004). By contrast to the highly regional expression of Tbx2 in the forming AVC in normal embryos at E8.5, Tbx2 was strikingly upregulated and ectopically expressed throughout the entire Tbx20lacZ/lacZ mutant heart (Fig. 4L). The pattern extended considerably more caudally in lateral plate mesoderm than in wild-type embryos, identical to the patterns of Myl7 and Tbx20-lacZ at this stage (Fig. 4E and data not shown). We examined Tbx2 expression in Nkx2-5GFP/GFP embryos, in which formation of chamber myocardium is also blocked (Palmer et al., 2001). Tbx2 was expressed in the normal pattern in these embryos, although slightly diminished in level (Fig. 4L), suggesting that upregulation of Tbx2 in all or most myogenic progenitor cells in Tbx20lacZ/lacZ embryos is not a default state arising from loss of chamber myocardium (see Discussion), and that Tbx20, directly or indirectly, represses Tbx2 and plays a major role in its regional expression. As expected, Tbx20lacZ/Nkx2-5GFP/GFP doubly homozygous embryos also showed upregulation of Tbx2 across the heart (Fig. 4L).

Bmp2 is expressed in myocardium of the AVC and OFT in the looping heart and has been proposed to positively regulate Tbx2 and establish its regional pattern (Yamada et al., 2000). We therefore assessed expression of Bmp2 and Bmp4 in the early looping hearts of E8.0 embryos. Bmp2 expression was in fact severely downregulated in Tbx20lacZ/lacZ hearts at this stage (Fig. 4M), demonstrating that Tbx2 upregulation in Tbx20lacZ/lacZ hearts occurs independently of Bmp2. The pattern of Bmp4 expression was normal (data not shown).

Tbx20 is repressed by neuregulin 1

The data above show that Tbx20 is required for chamber differentiation, although it is unclear whether this is direct or indirect. For example, loss of chamber myocardium could result from the depressed Nkx2-5 expression (Palmer et al., 2001) or, importantly, ectopic activation of Tbx2, a repressor of chamber-specific gene expression (Christoffels et al., 2004b). Paradoxically, Tbx20 may itself be a chamber repressor – the long Tbx20a isoform, which carries strong transcriptional activation and repression domains in its C-terminal region (Stennard et al., 2003), suggesting that it is non-essential for the later stages of chamber differentiation. To clarify this issue, we asked whether Tbx20 expression increased or decreased after treatment of myocardium in situ with a pro-chamber stimulus. Neuregulin 1 (Nrg1), a member of the epidermal growth factor family of signaling ligands, is expressed in the endocardium of the early heart tube (Garratt et al., 2003) and, along with its co-receptors ErbB2 and 4, expressed in myocardium, is essential for formation of trabeculae, a morphological feature of chamber myocardium. Excess Nrg1 induces trabecular overgrowth in vivo.
and enhances myofibrillogenesis in vitro (Hertig et al., 1999).

We explanted the cardiac region of wild-type E8.5 embryos and cultured them with and without Nrg1 (1 nmol/l) in low serum (0.5%) medium for 24 hours. Overall, cardiac-specific gene expression was reduced in cultured explants, for some genes dramatically (Fig. 5 and data not shown), a possible consequence of cardiac unloading. However, expression of the chamber markers \textit{Nppa} and \textit{Cited1} in explants was restored to approximately normal levels and the correct pattern by Nrg1. The pan-myocardial marker \textit{Actc1} (encoding α-cardiac actin) was also slightly increased (Fig. 5A,B,D). Notably, however, \textit{Tbx20} expression was significantly repressed by Nrg1 in a dose-dependent manner, and remaining expression was mostly in endocardium (Fig. 5C and data not shown). These findings support the expression data suggesting a non-essential role for \textit{Tbx20} in the later stages of chamber differentiation. The simplest interpretation is that chamber loss in \textit{Tbx20lacZ/lacZ} hearts is indirect, although an early direct role for \textit{Tbx20} in setting up the chamber program cannot be excluded. Additionally, Nrg1 may be the agent that actively represses \textit{Tbx20} during formation of chamber myocardium in vivo.

**Arrested vascular development in \textit{Tbx20} mutant yolk sacs**

Yolk sac vasculature remodeling was also defective in \textit{Tbx20lacZ/lacZ} embryos. An initial vascular plexus formed, but remodeling into a mature vascular bed did not occur, as highlighted by staining for \textit{lacZ} and vascular markers (Fig. 6A-D,G,H). \textit{Tbx20lacZ} was expressed only in the mesodermal layer of the yolk sac, then later in all vascular derivatives (Fig. 1F; Fig. 6C,E,F). Elevated apoptosis was detected by TUNEL assay specifically in the mesodermal layer at E9.5, and ultrastructural studies showed occasional perforation of the endothelial layer (red arrowheads) in the mutant. (M) RT-PCR analysis of E9.0 yolk sacs (n=3) for markers of hemangioblast specification (\textit{Tal1}), angioblast specification (\textit{Kdr}) and remodeling (\textit{Angpt1, Tek}) and vessel maturation (\textit{Acta2, Gja5}). Scale bar: 5 µm. e, endoderm; ec, endothelial; wt, wild type.

**Fig. 6.** Arrested development of yolk sac vasculature in \textit{Tbx20} null embryos. (A,B) \textit{LacZ} expression in yolk sac flat mounts showing lack of mature vessels in \textit{Tbx20lacZ/lacZ} embryos, as seen in wild type. (C,D) Sections of E9.5 \textit{Tbx20lacZ/+/} or \textit{Tbx20lacZ/lacZ} yolk sacs showing expression of \textit{lacZ} in the mesodermal layer, but not in blood cells. (E,F) \textit{Tbx20} is expressed in yolk sac mesoderm and vessel derivatives of \textit{Tbx20lacZ/lacZ} embryos from E11.5-14.5. Note that expression was never observed in the endodermal layer or hematopoietic cells. (G,H) Whole-mount immunohistochemical detection of \textit{Pecam1}, a marker of mature endothelial cells at E9.5. (IJ) TUNEL showing increased apoptosis in the mesodermal layer of E9.5 mutant yolk sacs compared with wild type. (KL) Transmission electron microscopy of E9.5 yolk sacs showing an example of perforation of the endothelial layer (red arrowheads) in the mutant. (M) RT-PCR analysis of E9.0 yolk sacs (n=3) for markers of hemangioblast specification (\textit{Tal1}), angioblast specification (\textit{Kdr}) and remodeling (\textit{Angpt1, Tek}) and vessel maturation (\textit{Acta2, Gja5}). Scale bar: 5 µm.

**Tbx20 and Nkx2-5 genetically interact in vivo**

\textit{Tbx20} protein associates directly with the homeodomain factor Nkx2-5 and zinc finger factors Gata4 and 5, and these factors can function synergistically to regulate promoters of cardiac genes in vitro (Stennard et al., 2003). To test for a genetic interaction between \textit{Tbx20} and \textit{Nkx2-5} that would support the idea of their function in common pathways, we inter-crossed \textit{Tbx20lacZ/+/} and \textit{Nkx2-5GFP/+/} mice. A proportion of \textit{Tbx20lacZ+/Nkx2-5GFP+/} compound heterozygotes survived to adulthood and were apparently healthy and fertile, although substantially lower numbers than the expected 25% were found at weaning (10%; n=18/184; \textit{P}=0.0001), suggesting partial embryonic or perinatal lethality due to structural or functional
malformations. Single heterozygotes were represented normally.

Humans with NKK2.5 mutations show secundum atrial septal defect (ASD) at high penetrance (Schott et al., 1998). Nkk2-5 heterozygous mutant mice also show ASD but only rarely (1% on C57BL/6 background), although they do manifest a spectrum of less severe atrial septal abnormalities including shortened septum primum, patent foramen ovale and atrial septal aneurysm (Biben et al., 2000). They may therefore be sensitized to mutation or downregulation of other genes involved in atrial septation. Tbx20 may be one such gene, since it is expressed in the inter-atrial septum primum (Fig. 1G). Indeed, anatomical dissection revealed frank ASD in 16% (n=4/24) of Tbx20lacZ/+\textsuperscript{2} and Nkk2-5GFP/+\textsuperscript{2} mice, while none were found in Tbx20lacZ/+\textsuperscript{2} and Nkk2-5GFP/+\textsuperscript{2} mice (P<0.05) (Table 1).

### Table 1. Atrial septal dysmorphogenesis in adult mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Normal (%)</th>
<th>PFO (%)</th>
<th>ASA (%)</th>
<th>PFO + ASA (%)</th>
<th>ASD (%)</th>
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<td>21</td>
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<td>3 (14.3)</td>
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<td>4 (20)</td>
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<tr>
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<td>12 (50)</td>
<td>1 (4.2)</td>
<td>9 (37.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tbx20lacZ/+\textsuperscript{2}/Nkk2-5GFP/+\textsuperscript{2}</td>
<td>24</td>
<td>3 (12.5)</td>
<td>4 (16.6)</td>
<td>1 (4.2)</td>
<td>12 (50)*</td>
<td>4 (16.7)</td>
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</tbody>
</table>

PFO, patent foramen ovale; ASA, atrial septal aneurysm; ASD, atrial septal defect.
*Increase compared with PFO+ASA in Nkk2-5GFP/+\textsuperscript{2} not significant.
†P<0.05 compared with all other genotypes.

In-vivo cardiac function in Tbx20 and Nkk2-5 heterozygous mice

Both Tbx20 and Nkk2-5 were expressed in adult atrial and ventricular myocardium and interventricular septum, as judged by lacZ staining of hearts from Tbx20lacZ/+\textsuperscript{2} and Nkk2-5lacZ/+\textsuperscript{2} knock-in mice (Fig. 1A; see Materials and methods). We therefore examined cardiac chamber function and morphology in Tbx20 and Nkk2-5 single and compound heterozygous mutant embryos. Echocardiographic analysis was performed on a cohort of 28 aged-matched male mice of wild type, and single or compound heterozygous genotypes at 3-4 months of age (Table 2). Body weight and heart rate did not differ between genotypes. However, the left ventricular (LV) diastolic dimension was mildly, although significantly, increased in the Tbx20lacZ/+\textsuperscript{2} and Tbx20lacZ/+\textsuperscript{2}/Nkk2-5 lacZ/+\textsuperscript{2} genotypes (P=0.027; ANOVA), while wall thickness was decreased by 35 and 40%, respectively (P<0.001). Cardiac contractile function was also compromised, as seen by the increase in LV systolic dimension by 42 and 47% (P<0.001), and decrease in fractional shortening by 21 and 24% (P<0.001). These findings are indicative of the onset of dilated cardiomyopathy (DCM). LV parameters in Nkk2-5GFP/+\textsuperscript{2} mice were also abnormal, although less so. It is noteworthy that for all LV parameters measured there was no apparent interaction between Tbx20 and Nkk2-5 alleles. By contrast, an increase in left atrial dimensions, evident in all mutant genotypes, was significantly more severe in Tbx20lacZ/+\textsuperscript{2}/Nkk2-5GFP/+\textsuperscript{2} mice (P<0.001).

Based on the above findings, we examined gross cardiac morphology and gene expression in a subset of mice of each genotype selected randomly from the echocardiography cohort. After arrest in diastole, overall cardiac morphology and degree of trabeculation appeared within the normal range in single heterozygotes, despite their functional deficit. However, in three of four Tbx20lacZ/+\textsuperscript{2}/Nkk2-5GFP/+\textsuperscript{2} mice examined, the right ventricle was misshapen and/or enlarged without increased wall thickness, and this appeared unrelated to the
presence of ASD (Fig. 7B). Histology on transverse sections from one of the three such affected hearts showed pronounced myocyte disarray and patches of fibrosis specifically in RV myocardium (Fig. 7C-F), although examination of additional mice showed that gross myocyte disarray was not a general feature of the doubly heterozygous hearts showing RV dilation. Despite reduced contractile function, compensatory myocardial hypertrophy was not evident in any of the mutant hearts. Specifically, there was no change in heart weights or heart weight/body weight ratios (Table 2), and no overt signs of myofiber hypertrophy. Moreover, while northern analysis showed upregulation of Nppa, a general marker of myocardial stress, in Tbx20lacZ/lacZ hearts, multiple markers of cardiac hypertrophy were either normally expressed or diminished in mutant genotypes (Fig. 7G). Nppa was significantly downregulated in Nkx2-5GFP/+ and Tbx20lacZ/lacZ+Nkx2-5GFP/+ hearts, probably because Nkx2-5 plays a direct role in Nppa transcription (Durocher et al., 1996).

**Discussion**

Nearly 1% of live born humans have some form of structural malformation of the heart. Recent data suggest that a few percent of these are caused by mutations in cardiac transcription factors acting in development, including T-box factors Tbx5 and Tbx1 (Garg et al., 2003; Prall et al., 2002; Yagi et al., 2003). In this paper, we address the function of cardiac T-box factor Tbx20. Our data demonstrate a central role for Tbx20 in patterning and chamber formation in the embryonic heart via positive and negative influences on other T-box factors that induce and support differentiation (and migration) of the cardiomyocyte lineage (Nascone and Mercola, 1996).

Table 2. In vivo structural and functional analysis of Tbx20lacZ/+ and Nkx2-5GFP/+ single and compound heterozygous adult hearts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type (n=8)</th>
<th>Tbx20lacZ (n=7)</th>
<th>Nkx2-5GFP/+ (n=6)</th>
<th>Tbx20lacZ/Nkx2-5GFP/+ (n=7)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>28.9±2.4</td>
<td>28.0±1.4</td>
<td>28.1±3.0</td>
<td>27.3±1.4</td>
<td>NS</td>
</tr>
<tr>
<td>HW (g)</td>
<td>0.16±0.03</td>
<td>0.15±0.02</td>
<td>0.15±0.03</td>
<td>0.16±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>HW/BW</td>
<td>5.39±0.93</td>
<td>5.52±0.81</td>
<td>5.29±0.27</td>
<td>6.07±0.93</td>
<td>NS</td>
</tr>
<tr>
<td>LVWT (mm)</td>
<td>0.63±0.08</td>
<td>0.41±0.07</td>
<td>0.55±0.07</td>
<td>0.38±0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVDD (mm)</td>
<td>3.43±0.08</td>
<td>3.68±0.15</td>
<td>3.57±0.26</td>
<td>3.67±0.18</td>
<td>0.027</td>
</tr>
<tr>
<td>LVSD (mm)</td>
<td>1.35±0.05</td>
<td>1.92±0.31</td>
<td>1.71±0.38</td>
<td>1.98±0.36</td>
<td>0.001</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>61±1</td>
<td>48±6</td>
<td>53±8</td>
<td>46±8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>1.61±0.04</td>
<td>1.87±0.10</td>
<td>1.74±0.11</td>
<td>2.10±0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>62±42</td>
<td>64±22</td>
<td>658±30</td>
<td>60±79</td>
<td>NS</td>
</tr>
</tbody>
</table>

*P value determined by ANOVA.
†Mice aged 12-18 weeks.
‡P<0.05 compared with wild type.
§P<0.05 Tbx20lacZ/+ compared with Nkx2-5GFP/+.
¶P<0.05 Tbx20lacZ/+ compared with Tbx20lacZ/Nkx2-5GFP/+.
**P<0.05 Nkx2-5GFP/+ compared with Tbx20lacZ/Nkx2-5GFP/+.

BW, body weight; HW, heart weight; LV, left ventricle; WT, wall thickness; DD, end-diastolic diameter; SD, end-systolic diameter; FS, fractional shortening (determined as LVDD-LVSD/LVDD); LAD, left atrial diameter; HR, heart rate; NS, not significant.

Our morphological findings are generally consistent with the consequences of Tbx20 morpholino knockdown experiments in fish and frogs, which showed small, unlooped and dysmorphic hearts with poor chamber discrimination (Brown et al., 2005; Szeto et al., 2002).

While the cardiac progenitor field appeared to form normally in Tbx20 mutants, there was initially a profound delay in incorporation of progenitors into the forming primary heart tube. We have previously shown that injection of Tbx20 mRNA into Xenopus embryos induces mesodermal and endodermal cell fates and their coordinated migration via cell non-autonomous mechanisms (Stennard et al., 2003), and numerous other T-box factors have been implicated in control of cell migration at gastrulation (Russ et al., 2000; Tada and Smith, 2000; Yamamoto et al., 1998).

While Tbx20 could control cell migration of cardiac progenitors directly, the effects may in part be indirect due to blocked or delayed differentiation of myocardium or endocardium. Indeed, in Tbx20lacZ/+ mutants, the early cardiac regulatory program involving transcription factors Nkx2-5, Gata4 and Mef2c and cardiac inducing factor Bmp2 was significantly compromised. Furthermore, expression of Tbx20-lacZ in foregut flags the possibility that Tbx20 could also regulate differentiation of endoderm, a well-known source of factors that induce and support differentiation (and migration) of the cardiomyocyte lineage (Nascone and Mercola, 1996).

Development of SHF derivatives in Tbx20lacZ/+ mutants was also defective. Abnormal deployment or differentiation of SHF cells is likely to underlie conotruncal and other congenital heart defects in humans, with haploinsufficiency for the T-box factor gene, TBX1, expressed in SHF cells and associated endoderm, thought to be the major determinant of a spectrum of heart as well as branchial region defects associated with chromosome 22q11 deletion syndrome (Yagi et al., 2003). The outflow ventricle-like chamber in Tbx20lacZ/+ hearts is likely to be derived from the SHF, and this is supported by strong expression of the Mef2c-SHF-hPLAP transgene in this chamber. However, cell proliferation in the outflow chamber was severely compromised and the structure remained bulbous...
and did not undergo elongation or looping as in normal hearts. Furthermore, expression of Tnc, encoding a matrix protein with broad regulatory functions on cell adhesion, migration and proliferation through interactions with other matrix components and cell surface receptors (Jones and Jones, 2000), was not maintained in the outflow region. Thus, Tbx20 is essential for cell proliferation and gene expression in SHF cells once they enter the outflow region of the heart, effects that may be mediated by alterations to the extracellular matrix. This would affect clonal growth patterns in the forming heart that support correct cardiac looping and outflow tract morphogenesis (Meilhac et al., 2004). It is noteworthy that other T-box genes have been implicated in control of cell proliferation (Hatcher et al., 2001; Xu et al., 2004).

Chamber formation and transcriptional repression in the developing heart

Chamber muscle becomes evident early in heart development from the regional expression of several genes, most notably Nppa and Smpx, and formation of trabeculae (Christoffels et al., 2000; Palmer et al., 2001). Transcription factors Tbx5, Nkx2-5 and Foxh1 are essential for its specification (Bruneau et al., 2000; Lyons et al., 1995; von Both et al., 2004), and Tbx5 and Nkx2-5 directly regulate chamber-specific genes in vitro (Bruneau et al., 2001; Stennard et al., 2003). In Tbx20lacZ/+ hearts, expression of chamber-specific markers was severely downregulated, indicating that they do not differentiate chamber muscle. The Nrg1 pathway, which is necessary (but not sufficient) for chamber differentiation, suppressed Tbx20 expression in situ, suggesting that loss of chamber myocardium in Tbx20lacZ/− hearts is indirect, probably a consequence of ectopic activation of Tbx2. It is still feasible, however, that Tbx20 plays a direct positive role at the earliest stages of chamber formation.

A key finding of this work is that Tbx2 was ectopically expressed in all or most committed myocyte progenitors in Tbx20 mutant hearts. Tbx2 is expressed normally in non-chamber myocardium, and in the AVC it is thought to compete with Tbx5 for interaction with Nkx2-5 on the cis-regulatory elements of chamber-specific genes, thus inhibiting their expression (Habets et al., 2002). The global expression of Tbx2 in mutant hearts could merely reflect the loss of chamber myocardium and expansion of non-chamber myocardium. However, two facts argue against this possibility. First, Tbx2 was markedly upregulated (3-fold by RT-PCR quantitation) as well as ectopically expressed. Second, Tbx2 was expressed normally in the hearts of Nkx2-5GFP/− embryos, in which chamber differentiation is also blocked at the level of a controlling transcription factor. We conclude that Tbx20 directly or indirectly represses Tbx2 in myocardium, and that Tbx20 plays a defining role in specification of chamber and non-chamber myocardium, a lineage digression in the early heart upon which all subsequent morphogenesis depends.

Our data suggest a model in which chamber formation in the heart involves ‘default repression’, a feature of virtually all well-studied, conserved, signal-induced transcriptional regulatory systems acting in development (Barolo and Posakony, 2002). Default repression occurs when a developmental process is actively repressed in the absence of its inducing signal to prevent cryptic activation by other positive factors involved in specificity. Thus, specification of the Tbx2 pattern in the AVC and other regions of non-chamber myocardium must involve regional and presumably signal-dependent inhibition of the repressive role of Tbx20 on Tbx2 expression, a possible role for Bmps (Yamada et al., 2000).

A repressive role for Tbx20 was also evident in regulation of the Myl2 cardiac pre-pattern. Myl2 is expressed at only very low levels in hearts of Nkx2-5 null embryos (Biben et al., 2000; Lyons et al., 1995), yet was ‘reactivated’ in Tbx20lacZ/+Nkx2-5GFP/− embryos, which lack both Nkx2-5 and Tbx20 function. This finding inextricably implicates transcriptional repression involving Tbx20 in the regulation of Myl2. The Myl2 pattern was also broader relative to morphological landmarks in Tbx20lacZ/− and Tbx20lacZ/Nkx2-5GFP/GFP hearts. Consistent with these findings, expanded expression of the cmlc2 gene (an Myl2 ortholog) into the atria was noted after morpholino knockdown of zebrafish Tbx20, and the ventricle-specific gene vmhc was also activated in this region (Szeto et al., 2002). These patterns probably reflect loss of repressive roles for Tbx20, and it is noteworthy that expansion of the expression domains of developmental genes, as seen here, is also one hallmark of loss of default repression (Barolo and Posakony, 2002). Our data suggest multiple repressive functions for Tbx20 in the core cardiac regulatory program. We envisage a genetic circuitry for heart development based on interactions between multiple cardiac T-box factors and their co-factors, involving overlapping steps of repression and de-repression.

A role for cardiac transcription factors in adult heart pathology

Our studies have also revealed a key role for Tbx20 in adult cardiac function. Tbx20 haploinsufficiency led to LV dilation, decreased wall thickness and contractile dysfunction, indicative of DCM. Gross dilation was also seen in the RV in some Tbx20lacZ/+Nkx2-5GFP/− mice. In the atrial compartment, ASD was evident in 16% of Tbx20lacZ/+Nkx2-5GFP/− mice, and there was left atrial dilation in all mutant genotypes analyzed, although most severely in the Tbx20lacZ/+Nkx2-5GFP/− mice. The specific roles for Tbx20 in adult cardiac structure and function remain to be determined. ASD is developmental in origin and our data highlight Tbx20 as a candidate ASD gene in humans. In relation to ventricular defects, we found no deficit in expression of developmental genes such as Tbx5, Nkx2-5, Smpx and Gja1 in Tbx20lacZ/+ mice (data not shown). Nevertheless, it will be important to explore the timing of onset of LV DCM to determine whether it is also developmental in origin or reflects specific adult functions for Tbx20. In humans, a large number of disease genes for familial DCM have been identified, including those for sarcomeric, cytoskeletal, nuclear and calcium handling proteins (Fatkin and Graham, 2002). However, known disease genes account for only a small proportion of all familial cases. Mutations in cardiac transcription factor genes may prove to be another cause of DCM in humans.

The onset of LV dilation and contractile dysfunction in Tbx20lacZ/+ mice in the absence of hypertrophy, fingers Tbx20 as an essential gene in the adult cardiac adaptive response. In most models of adult cardiomyopathy, hypertrophy is a component of the pathophysiological response, although it can be bypassed if structural proteins, potential sensors of biomechanical stress, are absent (Brancaccio et al., 2003; Knoll...
et al., 2002). Recent work highlighting the developmental transcription factor Gata4 as a convergence point for cardiac hypertrophy pathways (Liang and Molkentin, 2002), has supported the long-held view that developmental pathways are reactivated in hypertrophy, although mechanistic understanding is still limited and distinctions between adaptive and pathological hypertrophy are unclear (Fatkin and Graham, 2002). Further analysis of the Tbx20 model should advance our understanding of the important link between development and adaptive responses in the adult organ.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/10/2451/DC1

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