Regulation of the diversification of the nodal and chamber myocardium

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Interplay between Tbx20, Tbx2/Tbx3 and Bmp/Smad-signaling controls compartmentalization of the developing heart tube into working chambers and the atrioventricular canal

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Abstract

The generation of efficient unidirectional blood flows in the mammalian heart relies on the functional compartmentalization into chambers separated by valves and septa. Here, we report on a genetic network involving the T-box transcription factor genes Tbx20, Tbx2 and Tbx3 and Bmp/Smad-signaling that coordinates the formation of the working chambers and the atrioventricular (AV) canal, from which the AV conduction system and the mesenchymal cushions, primordia of valves and septa, arise during cardiac development. We show that Tbx2 and its close relative Tbx3 are redundantly required and individually sufficient to specify AV myocardium, induce formation of the AV cushions and suppress chamber differentiation. Tbx20 is required for chamber formation independently from Tbx2, but suppresses Tbx2 in the developing chambers, thereby localizing its activity to the AV canal. We identified a Bmp/Smad-dependent enhancer conferring AV canal restricted expression and Tbx20-dependent chamber suppression of Tbx2 in vivo. Unexpectedly, Tbx20 does not repress expression of Tbx2 and other genes by binding to DNA-elements, but attenuates Bmp/Smad-dependent activation by binding Smad1 and 5 and sequestering them from Smad4. Our findings suggest that opposing regulation of Bmp-signaling by Tbx20 and Tbx2 may underlie specification of the chambers and the AV canal, respectively.
Introduction

The complex multi-chambered heart of vertebrates arises from a simple tubular structure through a coordinated program of cellular differentiation and proliferation, and tissue morphogenesis. Elongation of this simple tube is supported by recruitment of precursor cells and differentiation into cardiomyocytes at the two poles. Highly localized processes of further myocardial differentiation and increased proliferation within the growing heart tube mediate the local out-bulging of the atrial and ventricular chambers. Regions separating and bordering the developing chambers retain low proliferation rates and slow impulse conduction and resist differentiation in a working type of myocardium, resulting in the generation of primitive morphological constrictions, the AV canal (AVC) and outflow tract, and a delay in AV conduction (Moorman and Christoffels 2003). The primary AV myocardium, from which the definitive AV node derives, induces the overlying endocardium to undergo an epithelial-mesenchymal transition (EMT) and to invade the cardiac jelly, an extracellular matrix that is deposited by the primary myocardium. These mesenchymal cushions are subsequently remodeled into thin valve leaflets and components of the septa (Person et al. 2005) that ensure structural and functional compartmentalization of the heart.

Formation of the initial heart tube from the cardiac crescent and further elongation of the tube from mesodermal progenitor pools, requires Bmp-signaling, which induces the differentiation into cardiomyocytes (Klaus et al. 2007; Yang et al. 2006). Bmp-signaling regulates expression of an evolutionary conserved network of transcription factor genes that drives the development of chambers and the differentiation of working myocardium therein (Klaus et al. 2007; Prall et al. 2007). Ablation of any member of the network, including Nkx2-5, Gata and Mef2 factors, and the T-box transcription factors Tbx5 and Tbx20 causes major heart defects and early developmental arrest. Mice homozygous mutant for Nkx2-5 and Tbx20 establish a heart tube with a primary myocardial phenotype but fail to undergo looping morphogenesis and to initiate chamber formation (Cai et al. 2005; Singh et al. 2005; Stennard et al. 2005; Takeuchi et al. 2005; Prall et al. 2007). Tbx5 acts independently of Tbx20 and maintains posterior domains of the heart (Bruneau et al. 2001). Both Tbx20 and Tbx5 synergize on the biochemical level with other members of the conserved network, Nkx2-5 and Gata4, to activate expression of chamber specific genes such as Nppa and Cx40 (for a review see (Hoogaars et al. 2007a)).

Formation of the AVC again relies on Bmp-signaling (Sugi et al. 2004; Ma et al. 2005) and is elaborated and stabilized by other pathways (Rutenberg et al. 2006; Kokubo et al. 2007). Transcriptional repressor Tbx2 acts downstream of Bmp-signaling (Yamada et al. 2000) and regionally inhibits a chamber myocardial gene program in the AV canal by competing with activating T-box proteins such as Tbx5 for binding to conserved T-box binding elements (TBE)s in promoters of chamber specific genes (Habets et al. 2002; Christoffels et al. 2004b; Harrelson et al. 2004; Hoogaars et al. 2004; Chi et al. 2008). Tbx3 is genetically and functionally related to Tbx2, and suppresses chamber differentiation of the
sinus node, the AV bundle and bundle branches (Hoogaars et al. 2007b; Mommersteeg et al. 2007; Bakker et al. 2008). Tbx2 and Tbx3 expression overlaps in the AV canal, suggesting that functional redundancy has prevented a full appreciation of their role in the development of this tissue to date (Ribeiro et al. 2007; Bakker et al. 2008; Mesbah et al. 2008).

Although important signaling and transcriptional modules involved in the establishment of the cardiac components have been identified (Olson 2006; Dunwoodie 2007), the genetic hierarchies and the spatial and temporal interplay of these pathways have remained elusive. How for example Bmp-signaling, after inducing cardiogenesis, is redeployed to locally activate Tbx2 expression and AV canal formation has remained unclear, as has the role of core cardiac transcription factors in orchestrating these morphogenetic processes. The observation that Tbx2 is ectopically up-regulated in the entire embryonic heart tube of Tbx20-deficient embryos, possibly causing the block in chamber differentiation in Tbx20-deficient hearts, has suggested that Tbx20 is required to repress Tbx2 for the progression to a multi-chambered heart (Cai et al. 2005; Singh et al. 2005; Stennard et al. 2005). Here, we present genetic experiments in the mouse that further decipher the molecular pathways that underlie localized formation of the chambers and the AV canal. We show that Tbx20 plays a dual role in compartmentalization of the heart. It stimulates chamber differentiation independently from Tbx2 and simultaneously suppresses Bmp/Smad-dependent activation of Tbx2 in the heart tube by a previously unrecognized DNA binding-independent mechanism, thereby restricting its expression to the prospective AVC region. Tbx2, together with Tbx3, subsequently impose the AV canal phenotype on this region of the heart tube. Together, our data provide insight into the hierarchical mechanisms underlying the spatial delimitation of a broadly active signaling pathway and its redeployment to compartmentalize the heart.

**Materials and methods**

**Mice and genotyping**

Mice carrying a null allele of Tbx20 (Tbx20\textsuperscript{tm1Akis}, synonyms: Tbx20\textsuperscript{−/−}, Tbx20\textsuperscript{laZ}) (Singh et al. 2005), Tbx2 (Tbx2\textsuperscript{tm1.1(cre)Ymc}, synonyms: Tbx2\textsuperscript{−/−}, Tbx2\textsuperscript{Cre}) and/or Tbx3 (Tbx3\textsuperscript{tm1.1(cre)Ymc}, synonyms: Tbx3\textsuperscript{−/−}, Tbx3\textsuperscript{Cre}) (Hoogaars et al. 2007b) were maintained on an outbred (NMRI) background. The Tbx2\textsuperscript{Cre} transgenic line, which harbors a Cre gene at the translation start site and from which the Pgk-neo cassette was removed, is a null allele that will be described elsewhere. For the generation of mutant embryos, heterozygous mice were intercrossed. For the generation of double mutant embryos, double heterozygous mice were intercrossed. For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos were harvested in PBS, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at –20°C before further use. Wild-type littermates were used as controls. CAG-CAT-TBX3 (CT) and Nppa-Cre4 (Cre4) transgenic mice have
been described (Hoogaars et al. 2007b). Double-transgenic mice conditionally expressing TBX3 in the atria were generated by crossing CT mice with Cre4 mice. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (Details are available on request).

An expanded Materials and Methods section is available in the online data supplement and included at the end of this chapter.

Results

**Tbx20 is required for chamber formation, whereas Tbx2 stimulates cardiac jelly formation**

Previous analysis has shown that ectopic expression of *Tbx2* in the simple heart tube leads to arrest of cardiogenesis (Christoffels et al. 2004b). To test the hypothesis that ectopic *Tbx2* expression is responsible for the observed block in chamber differentiation in *Tbx20*-deficient hearts (Cai et al. 2005; Singh et al. 2005; Stennard et al. 2005), we generated embryos with combined deficiencies of *Tbx2* and *Tbx20* by interbreeding double heterozygous animals. Similar to *Tbx20* single mutants, *Tbx20lacZ/lacZ;Tbx2Cre/Cre* embryos were severely growth retarded and died at E10.5 due to hemodynamic failure (data not shown). At E9.5, when cardiac chambers were clearly delineated in wild-type embryos, *Tbx20* single mutant and *Tbx20/Tbx2* double mutant embryos featured a simple heart tube (Fig. 1A-C). On histological stainings of sagittal sections both myocardium and endocardium appeared homogenously thin throughout the *Tbx20lacZ/lacZ;Tbx2Cre/Cre* heart tube that sometimes appeared dilated. This is in contrast to *Tbx20lacZ/lacZ* hearts, where a thick layer of cardiac jelly filled the space between myocardium and endocardium. Cardiac jelly production was not associated with endocardial EMT and cushion formation in the *Tbx20lacZ/lacZ* linear heart tube as revealed by histological inspection as well as expression analysis of cushion markers *Sox9* (Akiyama et al. 2004) and *Msx2* (Abdelwahid et al. 2001) (Fig. 1D-F and data not shown). Molecular analysis using markers with differential expression along the linear heart tube (*Tbx5, Myh7*) (Bruneau et al. 1999; Christoffels et al. 2000) confirmed that anterior-posterior patterning occurred normally in *Tbx20lacZ/lacZ;Tbx2Cre/Cre* hearts (Fig. 1G-L). Chamber myocardium, however, was not formed, as shown by absence of expression of *Nppa (Anf)* and *Cited1* (Fig. 1M-R). *Bmp2* that is expressed in the primary myocardium of the AV canal in the wild-type (Ma et al. 2005), was found throughout the tubular heart of single and double mutant embryos (Fig. 1S-U). In summary, loss of *Tbx2* does not rescue the *Tbx20*-deficient heart from developmental arrest at the linear heart tube stage, but results in loss of cardiac jelly formation. These data show that *Tbx20* is a critical factor in cardiac chamber development, and that *Tbx2* is sufficient to induce the first step in cushion formation, the production of a thick layer of cardiac jelly in the developing heart.
Figure 1. Loss of Tbx2 does not rescue cardiac arrest of Tbx20-deficient mice. Comparative analysis of wild-type, Tbx20^-/- and Tbx20^-/-Tbx2^-/- embryos for cardiac morphology, histology and molecular marker expression at E9.5. (A-C) Left lateral views of whole E9.5 embryos reveal growth retardation and linear heart tube phenotype in Tbx20/Tbx2 double mutants similar to Tbx20-deficient embryos. Boxes mark the heart regions to be magnified in the following images. (D-F) Histological analysis of sagittal sections by hematoxylin and eosin (H&E) staining uncovers loss of cardiac jelly and cushion tissue (asterisk) in the Tbx20/Tbx2-deficient linear heart tube that appears as a slightly inflated sac. (G-U) In situ hybridization analysis of marker gene expression in whole embryos with probes as indicated. avc, atrioventricular canal; ec, endocardium; la, left atrium; lv, left ventricle; mc, myocardium; oft, outflow tract; pv, primitive ventricle; rv, right ventricle.
**Tbx2 and Tbx3 are cooperatively required for myocardial patterning and formation of cushion mesenchyme in the AV canal**

Previous analyses indicated that Tbx2 is necessary and sufficient to suppress chamber gene expression in the AV canal (Christoffels et al. 2004b; Harrelson et al. 2004). However, in our Tbx2 loss-of-function mouse AV canal formation at E9.5 was grossly normal, and Nppa and other chamber markers were not ectopically expressed in the AV canal (Fig. 2F,J,N).

**Figure 2.** Combined loss of Tbx2 and Tbx3 abrogates cushion formation and myocardial patterning in the atrioventricular canal. Comparative analysis of wild-type, Tbx2<sup>−/−</sup>, Tbx2<sup>−/−</sup>Tbx3<sup>−/−</sup> and Tbx3<sup>−/−</sup> embryos for cardiac morphology, histology and molecular marker expression at E9.5. (A-D) Left lateral views of whole E9.5 embryos reveal growth retardation and dilated avc phenotype in Tbx2/Tbx3 double mutant embryos. Boxes mark the heart regions to be magnified in the following images. (E-H) Histological analysis of sagittal sections through the left atrium (la), atrioventricular canal (avc) and left ventricle (lv) by hematoxylin and eosin (H&E) staining uncovers loss of cardiac jelly and cushion tissue (asterisk) in the avc of Tbx2/Tbx3-deficient hearts. (I-X) In situ hybridization analysis of marker gene expression in sagittal sections through the avc with probes as indicated. Abbreviations are as in Fig.1.
However, co-expression with the close family member $Tbx3$ in the AVC myocardium argues that functional redundancy precludes full appreciation of $Tbx2/Tbx3$ requirement in this region. We therefore aimed to generate mice double mutant for $Tbx2$ and $Tbx3$. Mice double heterozygous for $Tbx2$ and $Tbx3$ null alleles are afflicted with craniofacial defects that lead to early postnatal lethality (unpubl. observ.). Breeding onto an NMRI wild-type background partially rescued this phenotype so that double heterozygous mice could be interbred. Since pregnancies were badly maintained in these mothers, we obtained only a total of four viable $Tbx2/Tbx3$ double homozygous embryos from few litters at E9.5. These embryos appeared slightly retarded in their development, most likely due to arising hemodynamic failure. Morphologically, the constriction between the left ventricle and the atrium in the wild-type was largely absent (Fig. 2C). Histologically, the AV canal appeared as an inflated tube with little investment of cardiac jelly, and, in contrast to the other genotypes, complete absence of cushion tissue (Fig. 2G). Markers of chamber myocardium ($Nppa$, $Gja5$ ($Cx40$)) (Christoffels et al. 2004a) were expanded into this region, whereas markers for primary myocardium of the AV canal, $Bmp2$ (Ma et al. 2005) and $Cre$ from the mutant alleles reflecting endogenous $Tbx2$ and $Tbx3$ expression, were present but reduced in their expression levels and spatial extent (Fig. 2I-X). A similar phenotype was observed in compound mutants with loss of three functional alleles of $Tbx2$ and $Tbx3$ (Suppl. Fig. 1), whereas $Tbx2$ and $Tbx3$ single mutants showed normal cushion formation and proper chamber gene repression in the AV canal (Fig. 2). Hence, $Tbx2$ and $Tbx3$ are required in a redundant fashion to maintain, expand and differentiate the primary myocardium of the AV canal, and to induce the formation of cushion tissue from the endocardium in this region.

**$Tbx3$ regulates the AV myocardial gene program**

The difficulty in obtaining a sufficiently high number of $Tbx2^{Cre/Cre};Tbx3^{Cre/Cre}$ embryos for a more detailed analysis of the molecular consequences of combined loss of $Tbx2$ and $Tbx3$, prompted us to use a complimentary gain-of-function approach to further elucidate the role of these factors in AV canal development. We used ectopic expression of $Tbx3$ in the atrial myocardium that we have previously shown to be powerful in achieving molecular insight into cardiac $Tbx3$ function (Hoogaars et al. 2007b). $Nppa-Cre4$ ($Cre4$) mice were crossed with $CT$ mice to obtain permanent activation of $Tbx3$ in atrial chamber myocardium from E10.5 onwards in double transgenic $Cre4;CT$ mice (Hoogaars et al. 2007b). Micro-array analysis comparing the transcriptome of atria of adult $Cre4;CT$ and $Cre4$ males confirmed earlier findings of strong reduction in expression of the known chamber genes $Nppa$, $Smpx$ (Chisel), $Scn5a$ ($Nav1.5$), $Kcnj3$ ($Kir3.1$), $Gja1$ ($Cx43$) and $Gja5$ ($Cx40$) in $Tbx3$-expressing atria of $Cre4;CT$ mice (Hoogaars et al. 2007b), and additionally revealed down-regulation of $Ckm$, $Nppb$, $Bmp10$, $Ednra$ and $Aldh1b1$ (Suppl. Table 1). qRT-PCR and in situ hybridization analysis confirmed normal chamber restricted expression and $Tbx3$-mediated atrial repression of $Ckm$ (Wessels et al. 1990), $Nppb$ (Houweling et al. 2005), $Aldh1b1$ and $Ednra$ (Clouthier et
Figure 3. Myocardial Tbx3 expression induces endocardial mesenchyme formation and nodal gene expression. Sections of E17.5 atria of CT and Cre4;CT mice were probed for expression of indicated genes. Note the reduced complexity and smoothness of the pectinated muscle structure in atria of Cre4;CT fetuses. Black arrow head indicates sinus node (san), white arrow head the myocardium in which Cacna2d2, transgenic TBX3 and Bmp2 expression was seen to be induced in Cre4;CT atria. Red arrow heads depict the thick endocardial mesenchymal layer that forms in Cre4;CT atria, that is devoid of myocardial gene expression (cTnI), but expresses Fbln2 and Tgfβ2. Black bar, 100µm.

Previously, we observed that nodal conduction system genes Hcn1, Hcn4, Cx30.2, Cav3.1 and Lbh are induced in Cre4;CT mice (Hoogaars et al. 2007b), indicating that Tbx3 stimulates the nodal gene program. Micro-array analysis and subsequent validation by qRT-
PCR and/or in situ hybridization revealed induction of additional genes in Cre4;CT atria including Cx45, Itpr1, Slco3A1, Id2, Cacna2d2, and Hnt (Fig. 4). These genes are enriched in the conduction system components, including the AV node, and associated with (Slco3A1, Cacna2d2, Hnt) or involved in the formation (Id2) or function (Cx45, Itpr1) of the conduction system (Gorza et al. 1993; Coppen et al. 1999; Kreuzberg et al. 2005; Mery et al. 2005; Moskowitz et al. 2007) (Fig. 3, 4A,B). Together, these data indicate that Tbx3 is able to suppress a large number of chamber genes that are reduced in the AV canal and conduction system, and to induce AV canal-enriched genes implicated in conduction system specification or function.

**Tbx3 induces sub-endocardial mesenchyme formation and Bmp-signaling**

Histological analysis of Cre4;CT atria and controls at E17.5 revealed that the pectinated muscles, trabecule-like structures in the atrial appendages, were abnormally thick, smooth and uncomplicated and that a thick sub-endocardial layer of cells had formed (Fig. 3). This tissue expressed mesenchymal marker genes including Acta2/αSMA, Fbln2, Cspg2/Versican and Lumican (Fig. 3, 4A, Suppl. Table 1) that are associated with AV cushions. In addition, micro-array analysis and in situ hybridization revealed strong activation of components of the Tgfß-, Bmp-, Fgf- and Wnt-signaling pathways that have been functionally implicated with AV cushion and valve formation (Armstrong and Bischoff 2004; Person et al. 2005). Notably, we detected induction of Bmp2 expression in the myocardium, and of Bmp4 in endocardial layers of Cre4;CT mice (Fig. 3, 4A). Since Bmp2 expression in the AV canal myocardium is both required and sufficient to induce cushion formation (Yamagishi et al. 1999; Sugi et al. 2004; Ma et al. 2005), Bmp2 is likely to be a pivotal downstream mediator of cushion induction by Tbx3. Tgfß2, a Bmp2 target in this tissue that is required for cushion formation (Camenisch et al. 2002; Mercado-Pimentel and Runyan 2007) was upregulated in the endocardium (Fig. 3, 4A). In addition, qRT-PCR and in situ hybridization analyses confirmed expression of genes in the endocardial mesenchymal layer of Cre4;CT mice (Twist1, Msx1, Meox1, Sox9, Id3 and Smad6) (Fig. 4, Suppl. Fig. 2 and 3, Suppl. Table 1), whose expression and functional relevance in EMT and cushion formation in the AV canal have been reported (Galvin et al. 2000; Akiyama et al. 2004; Ma et al. 2005; Lincoln et al. 2006; Chen et al. 2008; Shelton and Yutzey 2008). Furthermore, expression of Fgfr2, and of Wnt antagonists Frzb, sFRP2 and Nkd2 were up-regulated in atria of Cre4;CT mice, compatible with the known role for Fgf- and Wnt-signaling in cushion and valve formation (Gitler et al. 2003; Hurlstone et al. 2003; Sugi et al. 2003; Person et al. 2005) (Suppl. Fig. 2, 3). Pkd2 is normally expressed in the developing valves and was induced in atrial mesenchyme of Cre4;CT mice. (Fig. 4A). In human and mouse, mutations of Pkd2 result in valve abnormalities (Wu et al. 2000; Stypmann et al. 2007). Finally, we identified additional 47 induced genes in the micro-array data whose specific expression in the fetal AV valvular mesenchyme was reported by Genepaint (http://www.genepaint.org/) (Suppl. Table 2).
In summary, these data show that ectopic myocardial expression of Tbx3 is sufficient to induce the mesenchymal transition of endocardial cells and the formation of AV cushion tissues. Hence, Tbx3 collectively regulates the genetic circuits of signaling pathways and transcriptional activities mediating these processes.

**Figure 4.** Tbx3 suppresses chamber-specific genes and induces genes involved in cardiac cushion formation and conduction system function. (A) qRT-PCR analysis of left atria of Cre4-CT double transgenic mice compared to CT control mice. Expression levels in CT atria were set to 1. Expression of chamber-specific genes was reduced, whereas genes associated with conduction system were induced (nodal/channel). Genes encoding signaling proteins and transcription factors involved in cardiac cushion formation and valve development were induced in atria of Cre4-CT mice. Error bars represent SD (n=4 per group). *P<0.05. (B) Cross-sections of E12.5 mouse hearts showing expression of genes up-regulated in Cre4;CT atria (micro-array) in the developing AV canal and AV cushions and valves.
Regulation of cardiac Tbx2 expression by Bmp/Smad-signaling and Tbx20

To gain insight into the molecular pathways that confine Tbx2 expression to the developing AVC, we functionally examined the Tbx2 regulatory region. We first tested a 6 kbp genomic fragment previously shown to mediate AVC expression (Kokubo et al. 2007), and found that Eyfp reporter gene expression driven by this fragment in transgenic animals recapitulated endogenous cardiac expression of Tbx2 in the AV canal and outflow tract in E9.5 to E11.5 wild-type embryos. Since this fragment also conferred ectopic expression in Tbx20-deficient hearts (Fig. 5A-G), all elements required for control of cardiac Tbx2 expression reside within this genomic DNA fragment that contains several phylogenetically conserved regions (Fig. 5A, Suppl. Fig. 4). Deletion analysis of this fragment showed that the previously identified TBE recognized by Tbx20 (Cai et al. 2005) is neither required for AV canal and outflow tract-specific expression nor Tbx20-mediated repression. We identified a 0.9 kbp genomic fragment located 2.3 kbp upstream of the transcriptional start site, that in combination with a minimal promoter piece of 0.6 kbp (-1.5mTbx2) recapitulated cardiac expression of Tbx2 in wild-type and up-regulation in Tbx20-deficient embryos faithfully (Fig. 5H-J, Suppl. Fig. 5). Recent reports have pinpointed the relevance of conserved Foxn and additional TBE sites in the zebrafish tbx2b promoter for activation of the gene in the AV canal (Chi et al. 2008). Moreover, β-catenin has been shown to regulate Tbx3, the T-box factor most closely related to Tbx2, in cancer cells by direct binding to a Lef/Tcf site (Renard et al. 2007). A 1.5 kbp genomic fragment deprived of these conserved sites still drove reporter gene expression to the AV canal and outflow tract, negating a role for T-box factors, Lef/Tcf proteins and Fox transcription factors in regulating cardiac Tbx2 expression on the DNA level in the mouse (Fig. 5A, Suppl. Fig. 5). However, this -1.3mTbx2 genomic fragment contained a large number of putative Smad binding sites (SBE), supporting a role of Bmp-signaling in the regulation of cardiac Tbx2 expression (Suppl. Fig. 4). Taken together, this analysis indicates that a small SBE-containing enhancer in Tbx2 is sufficient to drive AV canal expression in vivo.

We switched to in vitro reporter assays in NIH3T3 cells to further investigate the precise role of Tbx20 in conjunction with Bmp/Smad-signaling in cardiac Tbx2 expression (Fig. 5K,L). We used expression constructs for full-length mouse proteins for Smad5, the constitutively active Bmp receptor Alk3 (Alk3CA) (Wessely et al. 2001) and HA-tagged Tbx20, and a reporter plasmid containing the luciferase reporter gene downstream of the 6 kbp Tbx2 genomic fragment in these transactivation assays. We observed a strong activation of reporter gene activity upon co-expression of Smad5 and Alk3CA and a reduction to basal activity in the presence of increasing concentrations of Tbx20 (Fig. 5L). Deletion analysis of the Tbx2 promoter fragment revealed the requirement of a 0.9 kbp Nhel/AflIII fragment for Bmp/Smad-dependent activation of the promoter (Fig. 5K, Suppl. Fig. 6). Since this fragment was found to be sufficient to confer AV canal/outflow tract expression of Eyfp in vivo, Bmp/Smad-signaling is likely to activate cardiac Tbx2 expression in vivo. Removal of previously identified TBEs (Cai et al. 2005) did not affect the repression activity of Tbx20 on...
the promoter (Fig. 5K, Suppl. Fig. 6). This may suggest the presence of cryptic DNA binding sites for Tbx20. Alternatively, repression by Tbx20 may not be mediated by DNA binding but by protein interaction. To test the latter, we constructed point mutants of Tbx20 that do not exert specific DNA binding any more (Suppl. Fig. 7B,C). Unexpectedly, these mutant Tbx20 proteins still repressed transactivation of the 6 kbp Tbx2 fragment by Bmp/Smad- signaling

Figure 5. Bmp/Smad-signaling controls cardiac expression of Tbx2. In vivo and in vitro reporter analyses of the control of cardiac Tbx2 expression. (A) Phylogenetic analysis of a 6 kbp genomic region upstream and around the Tbx2 transcription start site for conserved sequences. The location of conserved T-box binding elements (TBE) and Foxn sites is marked by boxes. Deletion constructs of the 6 kbp Tbx2 genomic fragment used in vivo to drive Eyfp reporter expression. Presence (+) and absence (-) of Eyfp expression from these deletion constructs in the atrium (A), atrioventricular canal (AVC), ventricle (V) and outflow tract (OFT) in transgenic mouse embryos. Numbers indicate cardiac expression of Eyfp and the number of transgenic embryos analyzed (Card. expr/tg) and extracardiac expression domains detected in the same embryos (Extra card. exp/tg). A 0.9 kbp NheI/AflII fragment from the Tbx2 locus contains elements sufficient and required to drive reporter gene activity in the regions of primary myocardium. (B-J) Comparative analysis of Tbx2 mRNA expression (B-D), Eyfp activity from the 6.0 kbp Tbx2 genomic region in transgenic embryos (E-G), and Eyfp activity from the 1.5 kbp Tbx2 genomic region in transgenic embryos (H-J) in E9.5 wild-type embryos (B,E,H, arrows point to the AVC), E10.5 hearts (C,F,I), and E9.5 Tbx20-deficient embryos (D,G), arrows point to mutant linear heart tube). (K) In vitro reporter assays to detect transcriptional activation of a luciferase reporter from Tbx2 genomic fragments. Plasmids encoding constitutively active Bmp receptor Alk3, full length Smad5 protein, full length Tbx20 protein and the luciferase reporter construct were co-transfected in NIH3T3 cells and luciferase activity determined and normalized as fold over the reporter alone. Presence (+) and absence of activation (-) by Bmp/Smad-signaling and repression by Tbx20 is listed for the constructs tested. (L), Luciferase reporter assay for the 6 kbp Tbx2 genomic fragment. Numbers indicate µg of plasmids for the reporter -6.0mTbx2-luc, and the expression plasmids for Alk3CA, Smad5, Tbx20 and the non-DNA binding Tbx20 protein (Tbx20 mut) co-transfected into NIH3T3 cells. Reporter activation upon co-transfection of expression constructs for Alk3CA and Smad5 is inhibited by co-expression of Tbx20, independent of its ability to bind to DNA.
both in presence and absence of transfected Smad5 in co-transfection experiments (Fig. 5L). Repression achieved by the DNA-binding deficient mutant of Tbx20 was lower than with the wild-type protein suggesting the co-existence of DNA-binding dependent and independent mechanisms for Tbx20 protein in the regulation of Tbx2 (Fig. 5L). Together, our analyses of the Tbx2 promoter argue that temporal and spatial confinement of cardiac Tbx2 expression is achieved by Tbx20-dependent repression of Bmp/Smad-mediated transcriptional activation.

Figure 6. Tbx20 inhibits Bmp/Smad-mediated transcriptional activation. (A-D) Luciferase reporter assays on a 0.7 kbp Msx2 genomic fragment (A), a 91 bp array of Smad bindings sites derived from the Id1 promoter (B,C), and the 0.7 kbp Nppa promotor fragment (D). Numbers indicate µg of plasmids for the luciferase reporter plasmids, and the expression plasmids for Alk3CA, Smad5, Tbx20 and the non-DNA binding Tbx20 proteins Tbx20mut1 (L126R), Tbx20mut2 (L127R) and Tbx20mut (LL126,127RR) co-transfected into NIH3T3 cells. Wild-type as well as the DNA-binding dead mutant Tbx20 protein inhibit dose-dependently activation by Bmp/Smad whereas the Tbx20 protein activates the Bmp/Smad-independent Nppa promoter.

**Tbx20 inhibits transcriptional activation of Bmp/Smad-dependent promoters in a DNA-independent manner**

We wondered whether the repressive effect of Tbx20 on Bmp/Smad-dependent transcriptional activation might be of a more general nature. We tested minimal fragments of Msx2 and Id1 promoters known to be activated by Smad binding in transactivation experiments in NIH3T3 cells (Brugger et al. 2004; Monteiro et al. 2004) (Fig. 6A-C). Both Tbx20 wild-type and Tbx20 DNA-binding mutant proteins repressed Bmp/Smad-mediated activation of Msx2 and Id1 promoter in a dose-dependent manner. Repression was independent from the addition of
exogenous Smad5 since it was also observed when cells were only transfected with constructs for Alk3CA. As in the case of the Tbx2 promoter, repression by the DNA binding-deficient mutant form of Tbx20 did not reach the level of the wild-type protein suggesting participation of direct transcriptional activities of Tbx20 wild-type protein in these assays. Wild-type Tbx20 protein acted as a DNA-dependent transcriptional activator on the Bmp/Smad-independent Nppa promoter (Habets et al. 2002) while the Tbx20-mutant protein did not show a transcriptional effect in this context (Fig. 6D). Hence, Tbx20 acts as a transcriptional activator of Bmp/Smad-independent promoters but as an inhibitor of Bmp/Smad-mediated transactivation.

**DNA binding-independent inhibition of Bmp/Smad-mediated transactivation by Tbx20**

**Figure 7.** Tbx20 inhibits Bmp/Smad-signaling in a DNA-independent manner by binding to activating Smad1 and Smad5 proteins. (A) Pull-down of in vitro synthesized Flag-tagged Smad5, Myc-tagged Smad1 and HA-tagged Smad4, Smad6, and Smad7 proteins by GST fusion proteins bound to GSH-agarose beads and subsequent detection by anti-Flag, anti-Myc and anti-HA immunohistochemistry on Western blot. All GST fusion proteins harboring a T-domain retain Smad5 and Smad1 proteins on the column. (B) Co-immunoprecipitation of Myc-tagged Tbx20 and Flag-tagged Smad5 from HeLa cells co-transfected with expression constructs for the two proteins in presence and absence of an expression construct for constitutively active Bmp receptor Alk3 (Alk3CA). Arrow indicates the presence of Smad5 protein after IP with anti-Myc antibody and subsequent detection with anti-Flag antibody. The lower band in the Western blot represents the Ig heavy chain. (C) Co-immunoprecipitation of Flag-tagged Smad5, HA-tagged Smad4, and increasing amounts of Tbx20.HA in the presence of AlkCA from HeLa cells. Subsequent immunohistochemistry for different epitopes on Western blot detects competition of Smad4 binding to Smad5 by Tbx20. (D) Co-immunoprecipitation of Myc-tagged Smad1 and HA-tagged Smad4 from HeLa cells co-transfected with expression constructs for the two proteins, and subsequent addition of in vitro translated Tbx20 protein to Protein A bound complexes. Anti-HA immunohistochemistry on Western blot detects competition of Smad4 binding to Smad1 by Tbx20.
may rely on physical interaction and/or functional interference with the transcriptional activators Smad5 or Smad1. In vitro pull-down assays with GST-Tbx20 fusion proteins showed that Tbx20 directly binds to the activating Smad5 and the closely related Smad1, but not to the regulatory Smad4 and the inhibitory Smads, Smad6 and Smad7 (Fig. 7A, Suppl. Fig. 7A). Binding was mediated by the T-box of Tbx20 as shown by GST pull-down assays with fusion constructs of GST and various Tbx20 protein fragments (Fig. 7A). Tbx20 binding to Smad5 also occurred in a cellular context as shown by co-transfection/co-precipitation experiments in HeLa cells (Fig. 7B). Since Smad4 is a necessary co-factor for nuclear translocation and transcriptional activation by Smad1/Smad5 we investigated whether Tbx20 binding to Smad1/Smad5 competes with Smad1/5-Smad4 complex formation. We transfected expression constructs for Alk3CA, Smad4.HA, Smad5-Flag and increasing amounts of HA-tagged Tbx20 into HeLa cells and precipitated Smad5/Smad4/Tbx20 complexes with anti-Flag antibodies. Detection of HA-epitopes on Western Blots revealed a decrease of co-immunoprecipitated Smad4.HA protein in the presence of increasing amounts of Tbx20 (Fig. 7C).

In an alternative assay, we transfected expression constructs for Myc-tagged Smad1 and HA-tagged Smad4 into HeLa cells and precipitated Smad1/Smad4 complexes by anti-Myc antibodies. Addition of in vitro translated Tbx20 protein to resuspended immunocomplexes resulted in a complete release of Smad4 from the complex (Fig. 7D). Thus, Tbx20 effectively competes with Smad4 for binding to Smad1/Smad5 explaining the DNA-independent inhibition of Bmp/Smad-mediated activation of target promoters including Tbx2.
**Tbx20 restricts Tbx2 to the prospective AV canal by attenuating Bmp/Smad-signaling**

Expression analyses, embryological manipulation in the chick and genetic ablation experiments in the mouse have revealed a range of Bmp-dependent processes during amniote heart development (van Wijk et al. 2007). Bmp-signaling is broadly activated in the precardiac mesoderm and the cardiac crescent and is required for differentiation of progenitors of the first and second heart field into cardiomyocytes (Monteiro et al. 2004; Klaus et al. 2007). Ablation of Bmpr1a in early mesoderm resulted in a complete failure to establish cardiomyocytes expressing conserved core cardiogenic factors including Tbx5, Tbx20, Nkx2.5 and Gata family members, implicating them as downstream mediators of early Bmp-signaling. After cardiac specification, Bmp-signaling is redeployed for specification of the AV canal. Conditional deletion of Bmp2 showed that Bmp-signaling is required to establish an AV myocardium, to enhance formation of the cardiac jelly and to induce endocardial EMT (Ma et al. 2005). Bmp2 regulates expression of the transcriptional repressor Tbx2 in the AV canal: Tbx2 expression is lost in Bmp2 mutants, and Tbx2 is induced by Bmp2-loaded beads in chick epiblast cultures (Yamada et al. 2000; Ma et al. 2005). Our analysis of the regulatory region of the Tbx2 gene identified a genomic fragment that is sufficient to direct AV canal expression in vivo. This fragment is rich in Smad-binding sites and responsive to Bmp/Smad-signaling in vitro, strongly arguing that Tbx2 is a direct target gene of this pathway in the heart.

Analysis of the regulatory region of tbx2b in the zebrafish heart has identified Foxn4 and Tbx5 as activators of tbx2b expression (Chi et al. 2008). Deletion of (conserved) TBE and Foxn4 sites in the Tbx2 upstream region of the mouse showed that these sites are not relevant for cardiac Tbx2 expression, suggesting that AV canal restriction of Tbx2 might have been achieved by different molecular pathways in vertebrate evolution.

Expression of Tbx2 in the early heart tube prevents chamber formation (Christoffels et al. 2004b), illuminating the necessity to prevent premature activation of Tbx2 by the first wave of Bmp/Smad-signaling in the cardiac crescent, the heart tube and the prospective chambers. In Tbx20-mutant hearts, Tbx2 is induced in the developing cardiac crescent and throughout the linear heart tube (Singh et al. 2005), demonstrating that temporal and spatial restriction of Tbx2 to the developing AV canal is not achieved by positive regulatory inputs, but by Tbx20-mediated inhibition of broad activation in regions outside the AV canal. Absence of TBE sites from a minimal Tbx2 promotor fragment that is sufficient to recapitulate cardiac Tbx2 expression in wild-type and Tbx20-mutant embryos strongly argues against Tbx20 acting as a transcriptional repressor in this context, as indicated by ChIP experiments (Cai et al. 2005).

To our surprise, we discovered that Tbx20 binds to activating Smad1 and 5 and sequesters them from binding to the common Smad4, abolishing the formation of transcriptionally active Smad1/5,Smad4 complexes. Hence, transcriptional modulation of target gene expression by T-box transcription factors may not only rely on the presence of
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TBE sites that feature a particular orientation and spacing. Direct binding and sequestration of transcriptional regulators by the conserved T-domain suggests another level of complexity of transcriptional regulation and establishes the T-box as a versatile interface both for DNA and protein interaction.

Tbx20-mediated sequestration of activating Smads may be one of several mechanisms that synergize to shut off Bmp/Smad-signaling after cardiac specification of the lateral plate mesoderm. In Nkx2.5-mutant hearts, Bmp/Smad-signaling is dramatically augmented and expanded suggesting that Nkx2.5 represses Bmp/Smad-signaling (Prall et al. 2007). Smad6 that is expressed in the cardiac crescent stably binds to activated type I receptors and competes with regulatory Smad4 for receptor activation (Imamura et al. 1997). Moreover, similar and likely in addition to Tbx20, Smad6 specifically competes with Smad4 for binding to receptor-activated Smad1, yielding an inactive Smad1/Smad6 complex (Hata et al. 1998). Since Tbx20, Nkx2-5 and Smad6 are targets of Bmp-signaling in the cardiac crescent, they may be part of a concerted negative feed-back loop.

Since Tbx2 is known from other systems like the limb to respond to high levels of Bmp only (Suzuki et al. 2004; Behesti et al. 2006; Yang et al. 2006), all these mechanisms may collectively dampen Bmp/Smad-signaling to a level insufficient to activate Tbx2. Intriguingly, we noted that ectopic expression of Tbx2 and Tbx3 resulted in increased expression of Bmp2, Bmp4 and Smad-target genes (e.g. Id). Thus Tbx2/Tbx3 may activate a feed-forward loop of Bmp-signaling that further increases Tbx2/Tbx3 expression, and thereby development of the AV canal.

Tbx20 and Tbx2/3 regulate chamber versus AV canal development

Previous analyses by a number of research groups revealed the crucial role of Tbx20 in cardiac chamber formation in vertebrates (Cai et al. 2005; Singh et al. 2005; Stennard et al. 2005; Takeuchi et al. 2005). Tbx2 expression, normally restricted to the AV canal (Habets et al. 2002) was expanded into the entire heart tube of Tbx20-deficient embryos arguing that chambers have been lost at the expense of an AV canal. Since ectopic expression of Tbx2 in the pre-chamber heart tube resulted in a similar phenotypic outcome (Christoffels et al. 2004b), it was hypothesized that de-repression of Tbx2 fully explains the cardiac phenotype in Tbx20-deficient embryos. Our analysis of Tbx20/Tbx2 double mutant embryos revealed that loss of Tbx2 does not rescue the early cardiac arrest, identifying Tbx20 as a positive factor for chamber formation independent from Tbx2. However, given the dominant chamber differentiation-blocking capacity of Tbx2 (Christoffels et al. 2004b), the ectopic expression of Tbx2 in Tbx20 mutants is likely to add to its cardiac phenotype. This is further supported by biochemical studies that show that the transcriptional repressor Tbx2 can compete with transcriptional activators of the T-box family including Tbx5 and Tbx20 for binding to conserved DNA-elements such as TBE sites in the Nppa promoter (Habets et al. 2002). Thus, Tbx20 has a dual role in cardiac development, acting to stimulate formation of chambers and to confine repressor Tbx2 to the AV canal.
Tbx2 and Tbx3, a pair of evolutionary closely related T-box proteins, share identical biochemical properties (reviewed in (Naiche et al. 2005). Co-expression in the AV canal argues for a role of these genes in regionalization of the simple heart tube and establishment of the AV canal phenotype. In gain-of-function scenarios both Tbx2 and Tbx3 were able to prevent chamber differentiation (Christoffels et al. 2004b; Mommersteeg et al. 2007) and to suppress a broad spectrum of working myocardium associated genes, including sarcomere components and mitochondrial genes. Ectopic expression of Tbx3 in atrial myocardium led to induction of a set of genes associated with the AV canal / conduction system in the atrial working myocardium. Individual loss of function of either Tbx2 or Tbx3 did not have a major impact on AV canal development (Harrelson et al. 2004; Ribeiro et al. 2007; Bakker et al. 2008; Mesbah et al. 2008). However, Tbx2/3 double mutant embryos largely failed to establish a morphological AV canal indicating that the two genes act redundantly in this process, possibly augmented by other factors including Id genes that suppress chamber differentiation (Moskowitz et al. 2007). Together, these data indicate that Tbx2 and Tbx3 coordinately regulate the AV gene program by suppressing working myocardial and by activating AV-specific features.

Tbx2/Tbx3 double mutant embryos failed to establish AV swellings (cushions), which are the precursors of the valves, and contribute to the septal structures and to the fibrous insulation. The Tbx20 mutants formed cardiac jelly in the entire tube, whereas Tbx2/Tbx20 double mutants did not, suggesting that Tbx2 triggers cardiac jelly formation. These data also suggest that Tbx20 that has been implicated in later aspects of cushion development (Shelton and Yutzey 2007) is not required for the initial steps of cushion formation. Furthermore, we observed a striking mesenchymal layer in the atria of mice ectopically expressing Tbx3 in the atria. Together, these observations strongly suggest that Tbx2 and Tbx3 in the AV canal are required and individually sufficient to initiate cushion formation and EMT. The mechanism of cushion formation has been extensively studied, and important roles for ligands and receptors of the Tgfß-superfamily have been exposed (Bartram et al. 2001; Camenisch et al. 2002; Sugi et al. 2004; Mercado-Pimentel and Runyan 2007). Bmp2 expression in the AV canal myocardium is both required and sufficient to induce cushion formation (Sugi et al. 2004; Ma et al. 2005). Bmp2 was selectively upregulated in the Tbx3-expressing atrial myocardium, and is likely to be a pivotal downstream mediator of cushion induction by Tbx3. This is further confirmed by enhanced expression in the ectopic mesenchyme of Tgfß2 and other Bmp2 target genes that are required for cushion formation (Bartram et al. 2001; Camenisch et al. 2002; Sugi et al. 2004; Mercado-Pimentel and Runyan 2007). However, alternative possibilities could be considered, because Bmp2 is not absent from Tbx2/Tbx3 double mutant hearts that fail to initiate cushion formation. One possibility is that in addition to its role of directly signaling to the endocardium through receptor Bmpr1a, Bmp2 may act through Tbx2 to activate another intercellular signaling pathway, such as the Notch pathway also implicated in cushion formation (Timmerman et al. 2004).

Together, our loss- and gain-of-function analyses suggest that Tbx2/Tbx3 and Tbx20 antagonistically regulate regionalization of the heart. Tbx20 promotes chamber formation
whereas Tbx2/Tbx3 directs the AV canal phenotype in the growing heart tube. Chamber and AV canal formation may be coupled and localized by the antagonistic regulation of Bmp/Smad-signaling pathway by these T-box transcription factors.

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References


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Supplementary data

Supplementary materials and methods

Histological analysis
Embryos were embedded in paraffin wax and sectioned to 10 µm. For histological analyses sections were stained with Hematoxylin and Eosin.

In situ hybridization analysis
In situ hybridization analyses on whole embryos and on paraffine sections using digoxigenin-labeled antisense riboprobes were performed according to standard protocols (Wilkinson and Nieto 1993; Moorman et al. 2001). Details of probes are available upon request. Stained whole mounts were transferred into 80% glycerol prior to documentation on a Leica M420 microscope with a Fujix digital camera HC-300Z. Sections of in situ hybridizations were photographed using a Leica DM5000 microscope with a Leica DFC300FX digital camera. All images were processed in Adobe Photoshop CS.

Microarray analysis
Left atria of six Cre4-CT mice and six control (Cre4) mice (male, 6 weeks) were dissected and snap frozen in liquid nitrogen. Total RNA was isolated and purified using single prep nucleospin columns according to the manufacturer’s instructions (Macherey-Nagel). RNA quality was checked using a bioanalyzer (Agilent Technologies). 250 ng of total RNA was used for biotin-16-UTP cRNA labeling and amplification using the Illumina RNA amplification kit (Ambion Inc., Austin, Txs). Labeled RNA was hybridized to Illumina MouseRef-6 BeadChip following the manufacturer’s instructions (Illumina Inc., San Diego, CA). The arrays were scanned using an Illumina Bead array reader confocal scanner. Beadstudio software was used to assess the individual array quality. Unprocessed intensity values were averaged per bead type, exported from beadstudio and subsequently normalized using VSN in R (Huber et al. 2002). Genes were tested for significant differential expression using the empirical Bayes moderated t-statistics test in the R-Limma package (Smyth 2004) at a 5% Benjamini-Hochberg false discovery rate (Reiner et al. 2003). We found that the expression of 737 transcripts was significantly reduced in atria of Cre4-CT mice, whereas 809 transcripts were significantly induced (threshold: P-value<0.05). A comprehensive analysis of this data set will be presented elsewhere.

Quantitative Real-Time PCR analysis
Quantitative real time PCR analysis was performed as described before (Hoogaars et al. 2007). In short, total RNA was isolated from left atrial appendices of 4 week old adult mice using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen). cDNA was reverse transcribed from 300 ng total RNA using the Superscript II system (Invitrogen) and expression of different genes was assayed with quantitative real time-PCR using the Roche 480 Lightcycler. Relative start concentration \(N_{(0)}\) was calculated using the following equation: \(N_{(0)}=10^{\log(threshold)-Ct \text{ (mean Eff)}}\). Values were normalized to Gapdh expression levels. Primers sequences can be provided upon request.
Mutagenesis

For construction of a DNA-binding deficient mutant form of Tbx20 conserved amino acid residues in the DNA-binding region were mutated based on reports for the Tbx2 protein (Habets et al. 2002; Lingbeek et al. 2002). Primer sets used for making the DNA binding dead mutant were:

ATCACCAAGTCTGGCGAGAGGATGTTCCCCACCATCC converting arginine 126 to glutamic acid,
ATCACCAAGTCTGGCGAGGGAGATGTTCCCCACCATCC converting arginine 127 to glutamic acid and
ATCACCAAGTCTGGCGAGGAGATGTTCCCCACCATCC converting arginines 126 and 127 to glutamic acid residues. pcDNA3.1Tbx20.HA was used as a template for the amplification. Either of the three primers was used for the amplification of the entire plasmid using PfuTurbo DNA polymerase (Stratagene QuickChange XL Site-Directed kit manual). Positive clones were sequenced to confirm the mutation, and protein synthesis was analyzed in transfected Hela cells to check efficiency of expression. Details on all other constructs upon request.

In vitro transcription/in vitro translation

Coding regions of mouse Tbx20, Smad4, Smad6 and Smad7 were amplified by PCR from the respective cDNAs and inserted in pSP64 modified to contain 5'-ß-globin leader and 3'-ß-globin trailer sequences as C-terminal fusion proteins with Myc or HA-epitope tag (Kispert and Herrmann 1993). SP6-coupled in vitro transcription/translation kit (TNT, Promega) was used for synthesis of the proteins in wheat germ lysate.

Expression in cell lines

For cytomegalovirus promoter/enhancer-driven expression of Tbx20, Smad1 and Smad5 proteins in cells, the globin leader/cDNA/globin trailer cassette of pSP64 was shuttled into HindIII and EcoRI sites of pcDNA3 (Invitrogen). Constructs were transfected in HeLa cells using the calcium phosphate method and in NIH3T3 cells employing Fugene reagent (Roche).

In vitro reporter assays

Luciferase reporter assays were used to determine transactivation properties of Tbx20 on various promoter fragments. Promotor fragments cloned in pGL2 or pGL3-luciferase constructs (Promega) were the 6 kbp genomic Tbx2 fragment and deletions thereof (pGL3basic.Tbx2-Luc), a minimal Bmp-responsive Msx2 promoter element (pGL2basic.Msx2-Luc) (Brugger et al. 2004) a short 91 bp Bmp-response element derived from the Id1 promoter (pGL3.BRE2-Luc) (Monteiro et al. 2004) and a 0.7 kbp Nppa genomic fragment (pGL3basic.Nppa(0.7)-Luc) (Habets et al. 2002). Reporter constructs were co-transfected with expression constructs for HA-tagged mouse Tbx20 (pcDNA3.Tbx20.HA), HA-tagged mouse DNA-binding-deficient form of Tbx20 (pcDNA3.Tbx20.HAmut) alone or in the presence of Flag-tagged mouse Smad5 (pcDNA3.Smad5.Flag) and constitutively active Bmp-receptor Alk3 (Alk3CA) (pCS2.BmpR1a.CA)(Wessely et al. 2001). Constructs were transfected into NIH3T3 cells (6×10^5 cells per well of 6-well plates) with the Fugene HD
transfection reagent (Roche). 40 ng of pCMV.ßGal vector were co-transfected to normalize the transfection efficiency by colorimetric determination of X-Gal turnover in the ß-Gal assay. After 48 h of further culture, cell lysates were prepared and the luciferase and ß-galactosidase activities were measured. All transfections were performed in duplicates and experiments were repeated at least three times. After normalization, the mean luciferase activities and standard deviations were plotted as “fold activation” when compared with the empty expression plasmid.

**Immunoprecipitation**

To determine binding of Tbx20 to Smad5 by co-immunoprecipitation experiments, Hela cells were either transfected with expression constructs for HA-tagged mouse Tbx20 (pcDNA3.Tbx20.HA) alone or in the presence of Flag-tagged mouse Smad5 (pcDNA3.Smad5.Flag) and constitutively active Bmp-receptor Alk3 (Alk3CA) (pcS2.BmpR1a.CA) (Wessely et al. 2001). Transfections were performed using the calcium phosphate method in 10-cm dishes at 50-60% confluency. After 48 h, cells were lysed in 1000 µl of Nonidet P-40 buffer, cellular debris was precipitated by centrifugation for 20 min at 4°C. The supernatant was split for immunoprecipitation with anti-HA and anti-Flag antibody (2.5 µg). After 1h of incubation, 25 µl of protein G sepharose beads (Amersham Biosciences) were added for 2h at 4 ºC followed by the precipitation and washing of beads. Beads were boiled in SDS-loading buffer and eluted proteins analyzed by Western blot.

To determine competition of Tbx20 and Smad4 for binding to Smad1 in co-immunoprecipitation experiments, Hela cells were transfected with expression constructs for HA-tagged mouse Smad4 (pcDNA3.Smad4.HA) and myc-tagged mouse Smad1 (pcDNA3.Smad1.Myc). Transfections were performed by the calcium phosphate method in 10-cm dishes at 30% confluency. After 48h, cells were lysed in 500 µl of Nonidet P-40 buffer, cellular debris was precipitated by centrifugation for 15 min at 4°C. The supernatant was split in three aliquots for immunoprecipitation with anti-Myc antibody (2.5 µg). After 2 h of incubation, 30 µl of proteinA agarose beads (Amersham Biosciences) were added for 1 h at 4°C followed by the precipitation and washing of beads. Beads were resuspended in 300 µl Nonidet P-40 buffer, and 0, 10 and 50 µl of in vitro translated Tbx20 protein was added. After 2 h incubation at 4°C, beads were precipitated and washed. Proteins were released by boiling in SDS-buffer, and separated by SDS-PAGE. After Western blotting, Myc-tagged proteins (Smad1.Myc, Tbx20.Myc) were detected by anti-Myc, while Smad4.HA was detected by anti HA-immunohistochemistry.

To determine competition of Tbx20 and Smad4 for binding to Smad5 in co-immunoprecipitation experiments, HeLa cells were transfected with expression constructs for Alk3CA, HA-tagged mouse Smad4 (pcDNA3.Smad4.HA), Myc-tagged mouse Smad5 (pcDNA3.Smad5.Myc) using the calcium phosphate method as described. Specific proteins were detected with immunohistochemistry on Western blots as described.

**GST pull down**

For GST pull-down experiments, GST or GST fusion proteins with five different deletion domains of Tbx20 were expressed in E. coli strain BL21 and bound in the presence of DNaseI to glutathione sepharose 4B beads (Amersham Biosciences) as described (Leger et al. 1995). An aliquot of the washed and equilibrated beads, now carrying GST or GST fusion protein, was incubated with one-tenth of an extract of HeLa cells transfected with pcDNA3.1 expression constructs for Smad5.Flag or Smad1.Myc protein (from 10cm plates), or with an
aliquot of in vitro translated protein of Smad4.HA, Smad6.HA and Smad7.HA in interaction buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 10 mM KCl, 5mM MgCl₂, 0.5 mM EDTA, 5% glycerol, 0.05% Triton X-100, and 1 mM DTT). After extensive washing, the proteins were eluted and analyzed by SDS-PAGE and Western blot.

**Electrophoretic mobility shift assay**

EMSA was done as previously described (Farin et al. 2007). The probe for Tbx20 binding was generated by annealing the two oligonucleotides BS.dirF, 5'–GATCCGGAGGTGAAGGTGTGAAAGGA-3'; and BS.dirR, 5'–GATCTCCTTTCACACCTCCTCCTCCG-3'. Protein for the binding assay was prepared using TNT SP6 High-Yield protein expression system (Promega) (Farin et al. 2007).

**In vivo reporter assays**

The -6mTbx2-Eyfp construct for generating transgenic Tbx2 promoter-reporter lines (Fig. 5A) was generated by inserting a 6 kbp Tbx2-promoter fragment (from -5.557 bp to +310 bp relative to the human TBX2 transcription start site) into expression vector pCS2, upstream of Eyfp (Venus) (Nagai et al. 2002), removing the CMV promoter. The -3.6mTbx2-Eyfp and -2.7mTbx2-Eyfp constructs were generated by truncation of the -6mTbx2-Eyfp construct using the restriction sites NheI and AflIII, respectively (Fig. 5A). To generate the -1.5mTbx2-Eyfp and -1.3mTbx2-Eyfp constructs, the -3.6mTbx2-Eyfp construct was restricted with AflIII and BglIII, after which a fragment was cloned in between with an artificial AflII site at -314 bp and -63 bp, respectively. Within the -1.5mTbx2-Eyfp construct, a conserved LEF1/Tgf binding site CTTTGTT (Arce et al. 2006) at -2620 bp was mutated into CcgcGcgGT to generate the -1.5(LEFmut)mTbx2-Eyfp construct. Vector sequences were removed and constructs were injected into pronuclei of zygotes of FVB mice.
Supplementary Figure 1. Analysis of AVC formation in an allelic series of Tbx2 and Tbx3 embryos by cardiac morphology, histology and expression of the chamber marker Nppa at E9.5; genotypes are as indicated. (A-F) Left lateral views of whole embryos. Boxes mark the heart regions to be magnified in the following figures (G-L). (M-R) Histological analysis of sagittal sections through the left atrium (la), atrioventricular canal (avc) and left ventricle (lv) by hematoxylin and eosin staining (H&E). (S-D') in situ hybridization analysis of Nppa expression in whole hearts in dorsal views (S-X) and in left lateral views (Y-D'). Embryos with a loss of three functional alleles of Tbx2 and Tbx3 exhibit expansion of chamber myocardium in the AVC, and lack of AVC cushion formation, while single mutants appear phenotypically normal in this respect.
Supplementary Figure 2. Tbx3 induces expression of genes involved in cardiac cushion formation and valve development. Serial sections of E17.5 hearts of CT and Cre4;CT mice, showing induced myocardial expression of transgenic TBX3, reduced myocardial expression of Smpx and Bmp10, induced myocardial expression of Cacna2d2 and Hcn4, and induced mesenchymal expression of Odd1, Id3, Meox1 (also myocardial), Frodo (also myocardial), Bmp6, Smad6 and Collagen III. ra, right atrium; san, sinoatrial node. Black bar, 100 μm.
Supplementary Figure 3. Cross-sections of E12.5 mouse hearts showing expression of genes up-regulated in Cre4;CT atria (micro-array) in the developing AVC and AV cushions and valves. In situ hybridization shows expression of Tbx3 in AVC myocardium (green arrow heads) and endocardial cushions (asterisk). Aldh1b1 expression is absent from the AVC, whereas Hnt, Cacna2d2 and Hcn4 are expressed in the AVC and sinus node. We observed broad expression of Sox9, Mdk, Fbln2, Ttxnc5, Lum and Lef1 in the AV cushions (black asterisk) and the valve leaflets, whereas expression of Meox1, Id3, Nkd2, Fgfr2 and Frzb was more restricted.
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Supplementary Figure 4. Sequence analysis of the 6 kbp promoter region of Tbx2 identifies conserved elements.

Reference sequence NCBI July 2007: >ref|NT_096135.5|Mm11_95772_37:51144025-51167297 Mus musculus chromosome 11 genomic contig, strain C57BL/6J.

Positions of restriction sites and putative binding sites refer to the middle of a site.

-6mTbx2 promoter
The first nucleotide (G) and last (A) are depicted in green. Total length is 5867bp, from -5557 to +310 relative to the transcription start site.

5’-GGGATGTTAGAAGCTCTGATAGGACGACCCTGGCCAGCTTTAAGTGGGCAAAAAACAGATGTCGAGCTCTGATACCTGGACCTTCTCTGCAGCCTTTTCAATCAGAGCACTACCTGTGTTTCTGGGAGAAGAGGCGGAAGGCTCTTACTGCCACTAACCCTAGAGGGGCCAGCTTCTTATCCTCTACAGATAACATCCGAAACAACCTCAGACCTCCTCCCCCCCCCTTAACCCCTAGACCGCATCAGATACCATCACTCAGAAAGCCATCAGAACATGAACTAATTGTGACTGGGCCAGGTGACCTTGAGGAAGGCAAGTGACCTCCTACTTCTCACAGCACTACCTACTTACGGGGCTCCCTTTCAATCTGGGAGGAAGAGGCTGCTGGGAACTGGCCACTCATAAGGTATGGCACTGTGCTGGGAGTGAATCAGTAAAAAGCCCAATGGTCCAAGAGCAAAAATCACACAAAACA

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

CAGAC

LEF1 / TBE

GAGCTTTGTTGTT

GTGGGA

GCCTCGGCCGCCT

GGCGCC

A

GGCG

CCCTCGTCCGTGCCCTCCCCCTCCAGCCCCG

GGCGGCCCGCGAGGCGCCCCCCGGGGCCTCTTAAAGACACGCACACTCTGCCCAGGGGACTCCCCCAG

GGGCGCCTGTTCCTGGGAAGGGGAGATGAGTGGGGGTAGACCCAGGGGGGGTAGGGCG AAGCAGGAGTTGCGAGCCGCAAGGCGCCGCTCGAGAGGCTGCTTAGGCCCCCCAGCACCAGTTTTCGATGCTACAAGGATATTCTAAGAAAATTTTGTTGGGGATGGGTCGATAAAGAATCTTAAGAGCGGCTAAAGCAGGTCATCCAGGTGACTACGTGATGGGGGTGGGGGGGTGGCTGGAGCTGTTGGTGGAAACCCAACCTGCCCTGAAAACGTCTCTGACCAGTCGCAGGTCCTTAGCTCTATAGGTGGGATTCAGATCCCCTAATGTAGCCACTGGGC
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GACAGCTGCGGATTTCTGGGACCAATAACTCTCAAGCTGCTCTCAATCTCTCCAGGCACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTGCAAACGCAGAG
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ACCTCAGATGCTTACAGCATCAGGGGACACTGAGGTCAA

**Transcription start site +1 = CAGAGATCA**, conserved across species. Translation start site at +316 (ATG), conserved across species. The 5' untranslated region has been underlined. Transcription and translation start site of mouse have been inaccurately assigned in the NCBI database.

0.9 kb NheI-AflII enhancer
This fragment of 929 bp reaches from the unique restriction site NheI at -3233 to the unique restriction site AflII at -2305 bp. Its sequence has been marked in light grey.

-1.5 kb construct: promoter starts at -314
It contains the 0.9 kb NheI-AflII enhancer, which has been cloned upstream of a -0.6 kb promoter fragment from -314 to +310. The 5' nucleotide of the proximal promoter has been marked black.

-1.3 kb construct: promoter starts at -63
It contains the 0.9 kb NheI-AflII enhancer, which has been cloned upstream of a -0.4 kb proximal promoter fragment from -63 to +310. The 5' nucleotide of the proximal promoter has been marked light grey.

**Putative binding sites previous literature on Tbx2 promoter:**

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Tbx5 BE at -173: AAGGTGTGGAA (anti-sense)
Foxn binding site at -84: TTTACGCTTT (anti-sense)

Novel localized putative LEF1 and T-box binding sites present study
1) LEF1 binding site at -2620: 5'-GCTTTGTT-3' (reviewed in Arce L 2006. Oncogene 25:7492-7504), found to be conserved between human and mouse using Transfac matrices. To inactivate the binding site in the context of the -1.5(LEFmut)mTbx2-Eyfp construct it has been mutated into: GCcgcGcg (Giese K 1992. Cell 69:185-195).

2) Non-consensus putative Tbx5 site (for consensus sites see Sinha S 2000. Gene 258:15-29; Farin HF 2007. J Biol Chem 282:25748-59), localized using rVISTA and Transfac matrices, at -2615: 5'-TGTTGTGGA-3'. It partially overlaps with the LEF1 binding site. Within the -1.5(LEFmut)mTbx2-Eyfp construct the site has been inactivated by mutation to: 5'-cGcgGTGGA-3'.

Novel localized putative SMAD binding sites present study

Consensus Smad Binding Elements (SBEs)
5'-GTCTG-3' or 5'-CAGAC-3' was the original consensus binding sequence; 5'-GTCTG-3' or 5'-CAGAC-3' was indicated by Jonk LJC 1998. J Biol Chem 273:21145-52. However, also 5'-GTCTG-3' or 5'-AGAC-3' has been published to be sufficient (Shi Y 1998. Cell 94:585-594; Zawel L 1998. Mol Cell 1:611-617; reviewed in Massagué J 2000. EMBO J 19:1745-1754). All sites have been indicated in the regulatory sequence.

GC-rich palindromes shown to be important for SMAD activation
5'-GCCGCC-3' (Korchynskyi O and ten Dijke P 2002. J Biol Chem 277:4883-4891)

Non-consensus SMAD-binding sequences
5'-GTGGAGCCTCGGGCCGGCGCTCTGCCCAGGCCGCCGCCGC-3': Sequence at -2608, found to contain several SMAD binding elements which are conserved between mouse-human-chicken, using rVISTA and Transfac matrices. The sequence has been underlined.
Supplementary Figure 5. Identification of cardiac regulatory elements by deletion analysis of the Tbx2 promoter in transgenic embryos. RankVISTA alignment of human against mouse Tbx2 regulatory sequences show conserved regions. Conserved T-box binding elements (TBE), LEF and Foxn sites are marked by boxes. Top panels show pictures of the Tbx2 expression pattern in E10.5 hearts visualized by whole mount in situ hybridization. Below these are fluorescence pictures of hearts of representative embryos with constructs depicted in the right panel. White arrowheads depict the atrioventricular canal (AVC), grey arrowheads the outflow tract (OFT). Grey lines indicate Tbx2 regulatory sequences, boxes represent exons, green boxes reporter gene Egfp. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.
Supplementary Figure 6. Identification of regions of the Tbx2 promoter conferring transcriptional activation by Bmp/Smad-signaling and repression by Tbx20 in transactivation experiments in vitro by deletion analysis. Plasmids encoding constitutively active Bmp receptor Alk3 (Alk3CA), full length Smad5 protein, full length Tbx20 protein and the luciferase reporter constructs were co-transfected in NIH3T3 cells and luciferase activity determined and normalized as fold activation over the reporter alone. Numbers indicate µg of plasmids for the reporter -6.0Tbx2-TBE-luc (A), -5.0mTbx2-luc (B), -3.6mTbx2-luc (C), -2.7mTbx2-luc (D) and -6.0mTbx2-0.9-luc (E), and the expression plasmids for Alk3CA, Smad5 and Tbx20 co-transfected into NIH3T3 cells.
Supplementary Figure 7. Biochemical analyses of Tbx20 proteins. (A) Coomassie staining of Tbx20-GST fusion proteins shows that all proteins are expressed in and purified equally well from bacteria. N refers to the N-terminal protein domain, T to the T-box region and C to the C-terminal protein domain. (B) Western blot analysis of the HA-tag of different Tbx20 proteins in transfected HeLa cells shows that the wild-type (wt), and the non-DNA binding Tbx20mut1 (L126R), Tbx20mut2 (L127R) and Tbx20mut (LL126,127RR) forms are expressed equally well. (C) Electrophoretic mobility shift assay of wild-type and mutant Tbx20 proteins confirms that amino acid changes L126R and L127R abolish binding of the Tbx20 protein to the DNA probe. Note the absence of the supershift band (arrow) in the mutant situations (Tbx20.HAmut1, mut2, mut).
References supplementary data


