Tbx20 Interacts With Smads to Confine Tbx2 Expression to the Atrioventricular Canal

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Rationale: T-box transcription factors play critical roles in the coordinated formation of the working chambers and the atrioventricular canal (AVC). Tbx2 patterns embryonic myocardial cells to form the AVC and suppresses their differentiation into chamber myocardium. Tbx20-deficient embryos, which fail to form chambers, ectopically express Tbx2 throughout the entire heart tube, providing a potential mechanism for the function of Tbx20 in chamber differentiation.

Objective: To identify the mechanism of Tbx2 suppression by Tbx20 and to investigate the involvement of Tbx2 in Tbx20-mediated chamber formation.

Methods and Results: We generated Tbx20 and Tbx2 single and double knockout embryos and observed that loss of Tbx2 did not rescue the Tbx20-deficient heart from failure to form chambers. However, Tbx20 is required to suppress Tbx2 in the developing chambers, a prerequisite to localize its strong differentiation-inhibiting activity to the AVC. We identified a bone morphogenetic protein (Bmp)/Smad-dependent Tbx2 enhancer conferring AVC-restricted expression and Tbx20-dependent chamber suppression of Tbx2 in vivo. Unexpectedly, we found in transfection and localization studies in vitro that both Tbx20 and mutant isoforms of Tbx20 unable to bind DNA attenuate Bmp/Smad-dependent activation of Tbx2 by binding Smad1 and Smad5 and sequestering them from Smad4.

Conclusions: Our data suggest that Tbx20 directly interferes with Bmp/Smad signaling to suppress Tbx2 expression in the chambers, thereby confining Tbx2 expression to the prospective AVC region. (Circ Res. 2009;105:442-452.)

Key Words: cardiac ■ T-box factors ■ differentiation ■ interaction ■ repression

The complex multichambered heart of vertebrates arises from a simple, rapidly elongating tubular structure through a coordinated program of cellular differentiation and proliferation and tissue morphogenesis. Highly localized processes of further myocardial differentiation and increased proliferation within the growing heart tube mediate the formation of the atrial and ventricular chambers. Regions separating and bordering the developing chambers, the atrioventricular canal (AVC) and the outflow tract (OFT), retain low proliferation rates and slow impulse conduction and resist differentiation in chamber myocardium.¹

Functional analyses in the mouse revealed that members of the T-box family of transcription factors participate in myocardial patterning and cardiac compartmentalization.²,³ Both Tbx5 and Tbx20 are activated in the early cardiac field by bone morphogenetic protein (Bmp) signaling⁴ and act as transcriptional activators that cooperate with other conserved cardiac transcription factors including Nkx2.5 and Gap43, binding proteins to activate expression of chamber-specific genes such as ANF (Nppa) and connexin40 (Cx40).³⁵⁷ Mice homozygous mutant for Tbx20 establish a heart tube with a primary myocardial phenotype but fail to undergo looping morphogenesis and to initiate chamber formation.⁵⁸⁻¹⁰ Tbx5 acts independently of Tbx20 and maintains posterior domains of the heart.⁷

Tbx2 encodes a transcriptional repressor that suppresses differentiation and the chamber-specific gene program.² Individual loss of Tbx2 results in a locally restricted gain of a chamber myocardial program in the AVC,¹¹¹² whereas forced expression of Tbx2 in the early heart tube leads to an early cardiac developmental arrest and a complete failure of chamber formation.¹³ Tbx2 competes with activating T-box proteins such as Tbx5 and Tbx20 for binding to conserved T-box-binding elements (TBEs) in promoters of chamber specific genes.⁶ Regionally restricted induction of Tbx2 by Bmp signaling in the AVC and OFT¹³,¹⁴ may therefore
underlie the inhibition of the chamber myocardial gene program and maintenance of the primary myocardial phenotype in these areas.

Notably, expression of Tbx2 is precociously activated in the cardiac crescent and heart tube of Tbx20-deficient embryos and may thus be responsible for the observed block in cardiac chamber differentiation in these embryos.\(^8\)\(^-\)\(^10\) This suggests a primary role for Tbx20 in repressing Tbx2 for the progression to a multichambered heart. Here, we present genetic experiments in the mouse that further decipher the molecular pathways that underlie localized formation of the chambers and the AVC.

Methods

Animal care was in accordance with national and institutional guidelines.

Mice carrying a null allele of Tbx20 (Tbx20\(^{m1AKis}\); aliases: Tbx20\(^{-}\), Tbx20\(^{m1AJ\alpha}\) and Tbx2 (Tbx2\(^{m1ICre/VNeo}\); aliases: Tbx2\(^{-}\), Tbx2\(^{m1B}\))\(^{11}\) were maintained on an outbred (NMRI) background.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

A Minimal Regulatory Region for Restriction of Tbx2 Expression to the AVC

Precocious expression of Tbx2 in the cardiac crescent of Tbx20-deficient embryos suggested a primary role for Tbx20 in restricting Tbx2 expression and AVC formation.\(^8\)\(^-\)\(^10\) To gain insight into the molecular mechanism operated by Tbx20 to confine Tbx2 expression to the developing AVC, we functionally examined the Tbx2 regulatory region (Figure 1). We first tested a 6-kbp genomic fragment previously shown to mediate AVC expression\(^15\) and found that enhanced yellow fluorescent protein (Eyfp) reporter gene expression driven by this fragment in transgenic animals (Tbx2\(^{-}/\Phi\) +0.316/Eyfp) recapitulated endogenous cardiac expression of Tbx2 in the AVC and OFT (Figure 1A and 1B). This fragment also conferred ectopic expression in Tbx20-deficient hearts (Figure 1C). Deletion analysis showed that a 0.9-kbp fragment, located 2.3 kbp upstream of the transcriptional start site, in combination with a minimal promoter piece of 0.6 kbp (Tbx2\(^{-}/\Phi\) −3.2/−2.3, −0.314/+0.316/Eyfp) recapitulated cardiac expression of Tbx2 in wild-type and upregulation in Tbx20-deficient embryos faithfully (Figure 1A through 1C). The previously identified TBE recognized by Tbx20\(^{10}\) is absent from this construct and, therefore, is neither required for AVC and OFT-specific expression nor Tbx20-mediated repression. 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 AVC.\(^{16}\) Moreover, β-catenin could regulate Tbx2 by direct binding to a Lef/Tcf site. Genomic Tbx2 fragments deprived of these deeply conserved sites (Tbx2\(^{-}/\Phi\) −3.2/−2.3,0.063/+0.316/Eyfp and Tbx2[LEFmut, −0.314/0.316/Eyfp, respectively) still drove reporter gene expression to the AVC and OFT, making it unlikely that T-box factors, Lef/Tcf proteins, or Fox transcription factors regulate this Tbx2 genomic fragment (Figure 1A and 1B; Online Figure I). Further truncation of the 0.9-kbp region revealed that a 380-bp Tbx2 subfragment (−2.7/−2.3) in conjunction with a minimal promoter (−0.063/+0.316) in the Tbx2(−2.7/−2.3, −0.063/ +0.316)Eyfp construct is sufficient to completely recapitulate expression of Tbx2 in the AVC and OFT (Figure 1A and 1B).

The 380-bp Tbx2 genomic fragment (−2.7/−2.3) contains a large number of putative Smad-binding sites (SBEs),\(^{17}\) supporting a role of Bmp/Smad signaling in the regulation of cardiac Tbx2 expression (Figure 1D). Taken together, this analysis indicates that a small SBE-containing enhancer in Tbx2 is sufficient to drive AVC expression in vivo.

Regulation of Cardiac Tbx2 Expression by Bmp/Smad Signaling and Tbx20

In in vitro reporter assays, we observed a strong activation of the Tbx2 upstream regulatory region (Tbx2\(^{−}/\Phi\) −5.6/ +0.316/Luc) on coexpression of Smad5 and constitutively active Bmp receptor Alk3 (Alk3CA)\(^{18}\) and a reduction to basal activity in the presence of increasing concentrations of Tbx20 (Figure 2A and 2B). Deletion analysis of the Tbx2 promoter fragment revealed the absolute requirement of an 0.9-kbp Nhe1/Alf1I fragment (Tbx2\(^{−}/\Phi\) −3.2/−2.3) for Bmp/Smad-dependent activation of the promoter (Figure 2A; Online Figure II). This fragment on its own mediated only weak activation, whereas inclusion of the adjacent 5′ region in the construct Tbx2\(^{−}/\Phi\) −3.9/−2.3, −0.314/+0.316/Luc gave strong Bmp/Smad-dependent activation (Figure 2A; Online Figure II). We identified several SBEs in the phylogenetically conserved 3′ region of the Tbx2\(^{−}/\Phi\) −3.9/−3.2) fragment (Online Figure I). We assume that these sites normally cooperate with SBEs in the Tbx2\(^{−}/\Phi\) −3.2/−2.3) fragment. Sufficiency of the Tbx2\(^{−}/\Phi\) −3.2/−2.3) fragment to confer AVC/OFT expression of Eyfp in vivo (Figure 1A and 1B) may thus result from multimerization of this fragment by tandem integration of the reporter construct in the genome. In support of this hypothesis, its trimerization in the construct Tbx2\(^{−}/\Phi\) −3.2/−2.3)x3, −0.314/+0.316/Luc strongly increased the Bmp/Smad-dependent activation (Figure 2A; Online Figure II, H).

Removal of previously identified TBEs\(^{10}\) did not affect the repression activity of Tbx20 on the promoter (Figure 2A; Online Figure II, A). 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 anymore (Online Figure III). Unexpectedly, these mutant Tbx20 pro-
Figure 1. In vivo 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 locations of conserved TBE, Foxn, and LEF sites are indicated. Deletion constructs of the 6-kbp Tbx2 genomic fragment Tbx2[−5.6/+0.316] used in vivo to drive Eyfp reporter expression. Colors refer to the constructs that are used in Figures B and C to show Eyfp activity. 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. expr./TG). B, Comparative analysis of Tbx2 mRNA expression (first row) and Eyfp activity from the indicated reporter constructs of the Tbx2 genomic region in transgenic embryos (subsequent rows). C, Comparative analysis of Eyfp activity of the 6-kbp Tbx2 regulatory region (Tbx2[−5.6/+0.316]Eyfp) and the Tbx2[−3.2/−2.3, −0.314/+0.316]Eyfp deletion construct in wild-type and in Tbx2-deficient embryos. Arrows point to the simple heart tube in Tbx2-deficient embryos. D, Nucleotide sequence of the 380-bp Tbx2 genomic fragment (Tbx2[−2.7/−2.3]) sufficient to recapitulate endogenous Tbx2 expression in the heart. * indicates nucleotides conserved between mouse and human. Consensus putative SBEs have been highlighted in gray. The Lef site has been boxed and highlighted in yellow. The putative TBE, overlapping SBEs, and the Lef site are underlined. Nucleotides changed to inactivate these sites have been indicated. avc indicates atrioventricular canal; fl, fore limb bud; la, left atrium; lv, left ventricle; oft, outflow tract; op, optic placode; ot, otic placode; ra, right atrium; rv, right ventricle.
proteins still repressed transactivation of the Tbx2(−5.6/+0.316) fragment by Bmp/Smad signaling both in the presence of transfected Smad5 (Figure 2B) and in the absence of transfected, ie, in the presence of endogenous Smad5 only (Figure 2C). All deletion constructs of this Tbx2 genomic fragment that were activated by Bmp/Smad signaling to variable degree were repressed by the DNA binding–deficient Tbx20 protein, whereas this Tbx20mut protein had no effect on the constructs that were not activated by Bmp/Smad signaling. Thus, Smad activation is required to observe the DNA-binding independent Tbx20 effect (Online Figure II). Repression achieved by the DNA binding–deficient mutant of Tbx20 was lower than with the wild-type protein suggesting the coexistence of DNA binding–dependent and –independent mechanisms of repression for Tbx20 protein in the regulation of Tbx2 in this assay (Figure 2B and 2C). Together, the in vivo and in vitro analyses of the Tbx2 promoter argue that temporal and spatial confinement of cardiac Tbx2

Figure 2. In vitro reporter assays to detect transcriptional activation of a luciferase reporter from Tbx2 genomic fragments. A, Plasmids encoding constitutively active Bmp receptor Alk3, full-length Smad5 protein, full-length Tbx20 protein, and the luciferase reporter construct were cotransfected in NIH3T3 cells, and luciferase activity was determined and normalized as fold over the reporter alone. Strength (++++, ++, + or –) of activation by Bmp/Smad signaling, and the presence (+) or absence (−) of repression by Tbx20 is listed for the constructs tested. B and C, Luciferase reporter assay for the 6-kbp Tbx2 genomic fragment (Tbx2[−5.6/+0.316]). Numbers indicate micrograms of plasmids for the reporter (Tbx2[−5.6/+0.316]Luc), and the expression plasmids for Alk3CA, Tbx20 and the non–DNA-binding Tbx20 protein (Tbx20mut) in the presence (B) or absence of Smad5 plasmid (C) cotransfected into NIH3T3 cells.
expression is supported by Tbx20-dependent repression of Bmp/Smad-mediated transcriptional activation.

**Tbx20 Inhibits Transcriptional Activation of Bmp/Smad-Dependent Promoters in a DNA Binding–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 Mxx2 and Id1 promoters known to be activated by Smad binding in transactivation experiments in NIH3T3 cells (Figure 3A through 3C).19,20 Both Tbx20 wild-type and Tbx20 DNA-binding mutant proteins repressed Bmp/Smad-mediated activation of the Mxx2 and Id1 promoter in a dose-dependent manner. 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 that the wild-type protein in addition regulates transcription directly. Interestingly, the DNA-binding deficient mutant of Tbx20 exerted very weak repression of Bmp/Smad-mediated activation of the Id1 promoter in the absence of exogenous Smad5 (Figure 3B and 3C), suggesting a role as direct DNA-dependent transcriptional repressor of Tbx20 in this context. Wild-type Tbx20 protein acted as a DNA-dependent transcriptional activator of the Nppa promoter that did not respond to Bmp/Smad signaling. The Tbx20 mutant protein did not show a transactivation in this context (Figure 3D).

Introduction of a potent transcriptional activator into eukaryotic cells can suppress the transcription of a cointroduced target gene most likely by titration of 1 or more general transcription factors that might be in limiting supply. Because very high doses of Tbx20 protein activated the Nppa promoter to the same degree as lower doses (Figure 3E), we deem it unlikely that the repressive effect of Tbx20 on the Bmp/Smad-dependent promoters that use the same pGL3-based reporter plasmids can be explained by such a general squelching mechanism. Together, these experiments suggest that Tbx20 can regulate transcription of target genes in multiple ways: as an inhibitor of Bmp/Smad-mediated transactivation (Tbx2), as a transcriptional activator of Bmp/Smad-independent promoters (Nppa), and most likely also as a direct transcriptional repressor.

**Tbx20 Binds to Regulatory Smad Proteins**

DNA binding–independent inhibition of Bmp/Smad-mediated transactivation by Tbx20 may rely on physical interaction and/or functional interference with the transcriptional activators Smad5 or Smad1. In vitro pull-down assays with glutathione S-transferase (GST)–Tbx20 fusion proteins showed that Tbx20 directly binds to regulatory Smad5 and the closely related Smad1 proteins but not to co-Smad4 and the inhibitory Smads, Smad6, and Smad7 (Figure 4A). 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 (Figure 4A). Tbx20 binding to Smad1 and Smad5 also occurred in a cellular context as shown by cotransfection/coprecipitation experiments in HeLa cells (Figure 4B and 4C). Tbx20 bound to the phosphorylated form of Smad5 (Figure 4D). Because of the lack of a specific antibody against unphosphorylated Smad1/5/8 we cannot exclude that Tbx20 binds to this form of the protein as well. However, cotransfection experiments of Smad5/Smad1 and Tbx20 expression constructs and detection by immunofluorescence confirmed that Tbx20 predominantly localized to the nucleus and did not mediate cytoplasmic–nuclear shuttling of the unphosphorylated form of Smad5 and Smad1 (Figure 4E and 4F). Together, these data suggest that Tbx20 binds to phospho-Smad1/5 in the nuclear compartment. Smad5 also bound to Tbx2 and Tbx5 in a cellular system, suggesting a more general nature of interaction of regulatory Smad proteins with T-box transcription factors (Online Figure IV). However, Tbx-Smad interaction may not necessarily influence Smad-dependent transcriptional activation because we did not detect an effect of Tbx5 expression on Bmp/Smad-dependent activation of the Tbx2 promoter in reporter assays in vitro (data not shown).

**Inhibition of Smad1/5-Smad4 Complex Formation by Tbx20**

Because Smad4 is a necessary cofactor for nuclear translocation and transcriptional activation by Smad1/Smad5, we investigated whether Tbx20 binding to Smad1/Smad5 competes with Smad1/5-Smad4 complex formation. These experiments revealed a decrease of coimmunoprecipitated Smad4.HA protein in the presence of increasing amounts of Tbx20 (Figure 5A). In an alternative assay, we transfected expression constructs for Myc-tagged Smad1 and hemagglutinin (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 complete release of Smad4 from the complex (Figure 5B). In a reverse experiment, we observed that addition of in vitro translated Smad4 protein to resuspended immunocomplexes resulted in release of Smad5 from the complex (Figure 5C). Finally, we eased the suppressive effect of DNA binding–deficient Tbx20 protein on Bmp/Smad-mediated activation of the Tbx2 regulatory fragment by increasing the concentration of Smad4 in the cellular system (Figure 5D). 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 Is Required for Chamber Formation Independently From Tbx2**

Previous analysis has shown that ectopic expression of Tbx2 in the early heart tube leads to arrest of cardiogenesis and chamber differentiation.11 To test the hypothesis that ectopic Tbx2 expression is responsible for this observed arrest in Tbx2-deficient hearts,8-10 we generated embryos with combined deficiencies of Tbx2 and Tbx20 by interbreeding double heterozygous animals. Similar to Tbx20 single mutants, Tbx20<sup>Tbx2</sup> homozygous embryos were severely growth retarded and died at embryonic day (E)10.5 because of hemodynamic failure (data not shown). At E9.5, Tbx20 mutant embryos featured a straight and short tubular heart (Figure 6A through 6D). In Tbx20/Tbx2 double mutants, the heart tube appeared morphologically more varied from being straight...
and short like in the Tbx20 single mutants to being more extended and inflated with some rightwards looping. On histologically stained sagittal sections, both myocardium and endocardium appeared homogenously thin throughout the Tbx20\textsuperscript{lacZ/lacZ; Tbx2\textsuperscript{Cre/Cre}} heart tube. This is in contrast to Tbx20\textsuperscript{lacZ/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 Tbx20\textsuperscript{lacZ/lacZ} linear heart tube as revealed by histological inspection (Figure 6E through 6H) as well as expression analysis of the cushion marker Sox9 (Figure 6I through 6L). This suggests that ectopic Tbx2 mediates the induction of cardiac jelly formation in Tbx20-deficient hearts.

Molecular analysis using markers with differential expression along the linear heart tube (Tbx5, Myh7) confirmed that anterior-posterior patterning occurred normally in Tbx20\textsuperscript{lacZ/lacZ; Tbx2\textsuperscript{Cre/Cre}} hearts (Figure 6M through 6T). Chamber myocardium, however, was not formed, as shown by absence of expression of Nppa (Anf) and Cited1 (Figure 6U through 6B’). Bmp2 is

Figure 3. Tbx20 inhibits Bmp/Smad-mediated transcriptional activation. A through E, 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 and C), and the 0.7-kbp Nppa promoter fragment (D and E). Numbers indicate micrograms 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) cotransfected into NIH3T3 cells.
expressed in the primary myocardium of the AVC in the wild type. In the tubular hearts of single and double mutant embryos, Bmp2 expression is restricted to the posterior part of the primitive ventricle suggesting that a molecular AVC domain has been established in these embryos (Figure 6C through 6F). In summary, loss of Tbx2 does not rescue the Tbx20-deficient heart from developmental arrest at the linear heart tube stage, defining a role of Tbx20 as regulator of
cardiac chamber formation independent from its repression of Tbx2.

**Discussion**

Our genetic and biochemical analyses suggest that Tbx20 function may couple and localize cardiac compartmentalization into chambers and AVC by suppressing the Bmp/Smad-signaling pathway and Tbx2 activation in the entire heart tube (Figure 7). The future AVC, however, expresses Bmp2 at levels sufficient to overcome the suppressive function of Tbx20 to activate Tbx2.

**Tbx20 Restricts Tbx2 to the Prospective AVC 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. Bmp signaling is broadly activated in the precardiac mesoderm and the cardiac crescent and is required for myocardial differentiation of progenitor cells in the first heart field and for proliferation and recruitment of second heart field cells to the forming OFT. 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 AVC. Conditional deletion of Bmp2 showed that Bmp signaling is required to establish an AV myocardium and to induce endocardial EMT. Furthermore, Bmp2 regulates expression of the transcriptional repressor Tbx2 in the AVC. Our analysis of the regulatory region of the Tbx2 gene identified a genomic fragment that is sufficient to direct AVC expression in vivo. This fragment is rich in SBEs and responsive to Bmp/Smad signaling in vitro, strongly arguing that Tbx2 is a direct target gene of this pathway in the heart.

Expression of Tbx2 in the early heart tube prevents chamber formation, 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 (Figure 7). In Tbx20 mutant hearts, Tbx2 is induced in the developing cardiac crescent and throughout the linear heart tube, demonstrating that temporal and spatial restriction of Tbx2 to the developing AVC is not achieved by positive regulatory inputs, but by Tbx20-mediated inhibition of broad activation in regions outside the AVC. Absence of TBE sites from a minimal genomic Tbx2 regulatory region, 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 previously by chromatin immuno-
It is formally possible that Tbx20 acts indirectly as a DNA-dependent transcriptional repressor of an inducer of Tbx2, including Bmp2. However, confinement and low-level expression of Bmp2 in the posterior region of the primitive heart tube in Tbx20-deficient embryos does not support this model. Alternatively, Tbx20 may transcriptionally activate a repressor of Tbx2 expression outside the AVC, compatible with its known biochemical function as a transactivator as shown for the Nppa promoter. We unexpectedly found that Tbx20 binds to the phosphorylated forms of Smad1 and -5 in the nucleus and sequesters them from binding to the co-Smad4, abolishing the formation of transcriptionally active P-Smad1/5-Smad4 complexes. Hence, transcriptional modulation of target gene expression by T-box transcription factors may not only rely on the presence of TBE sites. 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. Indeed, previous evidence has suggested that the T-box represents a common interface for...
binding to numerous classes of DNA-binding domains.\textsuperscript{2,3} Notably, binding of T-box proteins to Smad proteins as shown in this study is not without precedence. In \textit{Xenopus}, Brachyury, the prototypical member of the T-box family, interacts with Smad1 to activate expression of the transcriptional repressor \textit{Xom}. In this case, Smad binding is conferred by an N-terminal region of XBra and not the T-box.\textsuperscript{3} In addition, phosphorylated Smad1 interacts with Tpit in the corticotroph ACT-20 cell line to prevent the transcriptional activation of the \textit{POMC} gene by a Tpit/Pitx1 complex, suggesting a third mode of target gene regulation by T-box/Smad interaction.\textsuperscript{24} Finally, and similar to the findings of this study, Tbx1 binding to Smad1 was found to interfere with Smad1-Smad4 complex formation and Smad signaling.\textsuperscript{25}

Thus, Tbx-Smad interaction seems to be widespread but may have different context-dependent functional implications on gene regulation. Tbx20-mediated sequestration of regulatory Smads may be one of several mechanisms that synergize to shut off Bmp/Smad signaling after cardiac specification of the lateral plate mesoderm. In \textit{Nkx2.5} mutant hearts, Bmp/Smad signaling is dramatically augmented and expanded, suggesting that Nkx2.5 represses Bmp/Smad signaling.\textsuperscript{26} Smad6, which is expressed in the cardiac crescent, stably binds to activated type I receptors and competes with co-Smad4 for receptor activation.\textsuperscript{27} 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.\textsuperscript{28} Because Tbx20, Nkx2.5, and Smad6 are targets of Bmp signaling in the cardiac crescent, they may be part of a concerted feed-back inhibition loop.

**Tbx20 and Tbx2 Regulate Chamber Versus AVC Development**

Previous analyses by a number of research groups revealed the crucial role of Tbx20 in cardiac chamber formation in vertebrates.\textsuperscript{5,8–10} Tbx2 expression, normally restricted to the AVC,\textsuperscript{4,9} was expanded into the entire heart tube of Tbx20-deficient embryos arguing that chambers have been lost at the expense of an AVC. Because ectopic expression of Tbx2 in the prechamber heart tube also inhibited chamber formation, similar to the phenotype in Tbx20 mutants,\textsuperscript{13} it was hypothesized that derepression 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 failure to form chambers. We assume that Tbx20 similar to Tbx5 promotes chamber formation as a DNA-dependent transcriptional activator of chamber-specific genes such as \textit{Nppa}. Ectopic expression of the strong transcriptional repressor Tbx2 in a wild-type embryo is likely to block this process by its ability to compete with Tbx20 (and Tbx5) binding to conserved T-binding sites in promoters of these genes and prevent their activation. Hence, ectopic expression of Tbx2 in the Tbx20 mutant heart does not further enhance the loss of Tbx20 phenotype. On the other hand, Tbx2 may require Tbx20 to suppress chamber differentiation, providing an alternative explanation for the failure to rescue the Tbx20 mutant phenotype in Tbx2/Tbx20 double mutants.

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**Disclosures**

None.

**References**


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Legends for Online Figures

**Online Figure I.** Sequence analysis of the 6 kbp promoter region of Tbx2 identifies conserved elements.

**Online Figure II.** 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, Tbx20 DNA-binding deficient mutant (Tbx20mut) 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 Tbx2(-5.6/+0.316DTBE)Luc (A), Tbx2(-3.9/+0.316)Luc (B), Tbx2(-3.2/0.316)Luc (C), Tbx2(-2.3/+0.316) (D) and Tbx2(-3.9/-2.3,-0.314/+0.316)Luc (E), Tbx2(-5.6/-3.2,-2.3/+0.316)Luc (F), Tbx2(-3.2/-2.3,-0.314/+0.316)Luc (G) and Tbx2((-3.2/-2.3)x3,-0.314/+0.316)Luc (H) and the expression plasmids for Alk3CA, Smad5, Tbx20 and Tbx20mut co-transfected into NIH3T3 cells.
**Online Figure III.** 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).

**Online Figure IV.** Expression constructs for Flag-tagged Smad5 and HA-tagged Tbx2 and Tbx5, respectively, were co-transfected in HeLa cells. Complexes were immunoprecipitated with anti-HA antibody and bound Smad5 was detected on Western blot by Anti-Flag immunohistochemistry. Smad5 binding was weak to Tbx2 and strong to Tbx5.

**Expanded Materials and Methods**

**Embryos**

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.

**Genotyping**

Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR. AKO0427 sense primer (5’-AGGATCTCCTGTCATCCTCACCTTGCTCCTG) and AKO1013 antisense primer (5’-CGCAAGTATAAAATGCGGTTCTGACC) amplified a 1000 bp fragment of the mutant Tbx20lacZ allele, sense primer AKO01012 (5’-CCCAGGAGAGAGGCAGCAGAAC) and antisense primer AKO1013 amplified a 608 bp fragment of the Tbx20 wild-type allele. In addition, a PCR protocol was established to speed up genotyping of Tbx2 mutant embryos and mice. Primers AKO01154 (sense, 5’-AAGCTAAAAATCAGCAATCAGAGG) and AKO01157 (antisense, 5’-AAGGCATCGCCGGAACGTCG) amplified a 450-bp fragment from the wild-type allele, and primers AKO01154 and AKO01047 (5’-GGAAATGGTCTTCGCCAGACC) amplified a 1000-bp fragment from the Tbx2Cre mutant allele.

**Histological analysis**

Embryos were embedded in paraffin wax and sectioned to 5 µm. For histological analyses sections were stained with Hematoxylin and Eosin.

**In situ hybridization analysis**

In situ hybridization analyses on whole embryos and on 10 µm paraffine sections using digoxigenin-labeled antisense riboprobes were performed according to standard protocols.\(^1\,^2\) Details of probes used 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.

**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.\(^3\),\(^4\) 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. The reaction setup, PCR amplification and transformation were done according to the 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 Tbx2, Tbx5, 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. 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 Tbx2, Tbx5, 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 promotor fragments. Promotor fragments cloned in *pGL2* or *pGL3-luciferase* constructs (Promega) were the 6 kbp genomic *Tbx2* fragment *pGL2.Tbx2(-5.6/+0.316)Luc* and deletions thereof (details on cloning upon request), a minimal Bmp-responsive *Msx2* promotor element (*pGL2basic.Msx2-Luc*), a short 91 bp Bmp-response element derived from the *Id1* promotor (*pGL3.BRE2-Luc*), and a 0.7 kbp *Nppa* genomic fragment (*pGL3basic.Nppa(0.7)-Luc*). 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*). Constructs were transfected into NIH3T3 cells (6×10^5 cells per well of 6-well plates) with the Fugene HD transfection reagent (Roche), according to the manufacturer’s instructions. 40 ng of *pCMV.βGal* vector were co-transfected to normalize the transfection efficiency by colorimetric determination of X-Gal turnover.
in the β-Gal assays. After 48 h of further culture, cell lysates were prepared and the luciferase and β-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 Tbx2, Tbx5 and Tbx20 to Smad5 by co-immunoprecipitation experiments, Hela cells were either transfected with expression constructs for HA-tagged mouse Tbx2 (pcDNA3.Tbx2.HA), Tbx5 (pcDNA3.Tbx20.HA), 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). 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 debri was precipitated by centrifugation for 20 min at 4 ºC. 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 debri 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 no, 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 the supernatant 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 HA-tagged mouse Tbx20 (pcDNA3.Tbx20.HA) and Flag-tagged mouse Smad5 (pcDNA3.Smad5.Flag). Transfections and immunoprecipitations with anti-HA antibody were performed as above. Beads with complexes of Smad5.Flag and Tbx20.HA bound to anti-HA antibody were resuspended in 300 µl Nonidet P-40 buffer, and no, 10 and 50 µl of *in vitro* translated Smad4 protein was added. After 2 h incubation at 4°C, beads were precipitated and washed. Proteins were released by boiling in SDS-buffer, and the supernatant separated by SDS-PAGE. After Western blotting, Flag-tagged Smad5 protein was detected by anti-Flag immunohistochemistry. We additionally determined competition of Tbx20 and Smad4 for binding to Smad5 in co-immunoprecipitation experiments, by transfecting HeLa cells 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. 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. The probe for Tbx20 binding was generated by annealing the two oligonucleotides BS.dirF, 5'-GATCCGGAGGTGTGAAGGTGTGAAAGGA-3'; and BS.dirR, 5'-GATCTCCTTTCACACCTTCACCTCCG-3'. Protein for the binding assay was prepared using TNT SP6 High-Yield protein expression system (Promega).

In vivo reporter assays

The Tbx2(-5.6/+0.314)Eyfp construct for generating transgenic Tbx2 promoter-reporter lines (Figure 1A) 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), removing the CMV promoter. The Tbx2(-3.2/+0.316)Eyfp and Tbx2(-2.3/+0.316)Eyfp constructs were
generated by truncation of the Tbx2(-5.6/+0.314)Eyfp construct using the restriction sites NheI and AflII, respectively (Figure 1A). To generate the Tbx2(-3.2/-2.3,-0.314/+0.316)Eyfp and Tbx2(-3.2/-2.3,-0.063/+0.316)Eyfp constructs, the Tbx2(-3.2/+0.316)Eyfp construct was restricted with AflII and BglII, after which a fragment was cloned in between with an artificial AflII site at -314 bp and -63 bp, respectively. Within the Tbx2(-3.2/-2.3,-0.314/+0.316)Eyfp construct, a conserved LEF1/TCF binding site CTTTGTT at -2620 bp was mutated into CcgCGcgGT to generate the Tbx2(LEFmut,-0.314/+0.316)Eyfp construct. Further details on these and other constructs upon request. Vector sequences were removed and constructs were injected into pronuclei of zygotes of FVB mice.

References


Putative binding sites in the 6 kbp upstream regulatory region of *Tbx2*.

Reference sequence NCBI July 2007: >ref|NT_096135.5|Mt11_95772_37:51144025-51167297 Mus musculus chromosome 11 genomic contig, strain C57BL/6J.

*Tbx2* genomic fragment in construct 6-Eyfp

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. 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. The enhancer fragment of 929 bp present in the *Tbx2*(−3.2/−2.3, −0.314/+0.316)Eyfp constructs and *Tbx2*(LEFmut, −0.314/+0.316)Eyfp construct (see Figure 1) reaches from the unique restriction site NheI at −3233 to the unique restriction site AflII at −2305 bp (underlined). A 735 bp sequence from PstI at −3962 to NheI at −3233 (underlined) upstream of this enhancer, present in the *Tbx2*(−3.9/−2.3, −0.314/+0.316)Luc construct (see Figure 2), is activated by Smad in cell culture but is not required in vivo. The PstI-AflII fragment, sequences highly conserved across multiple species have been marked gray. The first and last nucleotides of the core enhancer (Figure 1D) are marked red.
GGAGTGCCGGTGGAGGCCAGCTGGTTGACGGAAGACAGCGCCGGCTCCCAGCAGGAGGAAAG
GGCGCCCTCTCCTGCAAG

ACCCACAATTTGCTCAAAAGCTTGAATCAACAGGAGGAGGCCTGGCTGTATTAGCGCAAGGATC

Putative binding sites indicated in literature
TBE at -426: GTGTCAATGTTGCA

Tbx5 BE at -173: AAGGTGTCGAGA (anti-sense)
Foxn binding site at -84: TTACCCTTT (anti-sense)

Putative LEF1 and T-box binding sites in the enhancer
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 it has been mutated to: GCCGCGC (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: TGTTGTGGA. It partially overlaps with the LEF1 binding site, and has been inactivated by the mutation of the LEF1 binding site (GCCGCGC).

Consensus Smad Binding Elements (SBEs)
Based on Jonk LJC 1998. J Biol Chem 273:21145-52; Shi Y 1998. Cell 94:585-594; Zawel L 1998. Mol Cell 1:611-617; Massagué J 2000. EMBO J 19:1745-1754; Korchynskyi & ten Dijke 2002. J Biol Chem 277:4883) the following Smad binding elements were assigned: GTCTG, GTCTT, GGGCC. All sites have been indicated in the regulatory sequence above. In addition, GCCG, GCAT, CAGC and CGCC have been implicated in BMP-mediated activation. These sites have been indicated in the 386 bp enhancer (Figure 1D).
Non-consensus SMAD-binding sequences
5'-GTGGAGCCTCGGGCCGTGGCGCACGGCGAGGCG-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.
Online Figure III

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Image showing Smad5.Flag.