Human Molecular Genetics, 2010, Vol. 19, No. 23 **4625–4633**
doi:10.1093/hmg/ddq393
Advance Access published on September 21, 2010

Shox2 mediates Tbx5 activity by regulating Bmp4 in the pacemaker region of the developing heart

Sandra Puskaric[1], Stefanie Schmitteckert[2], Alessandro D. Mori[3], Anne Glaser[1], Katja U. Schneider[1], Benoit G. Bruneau[3], Rüdiger J. Blaschke[1], Herbert Steinbeisser[2,*] and Gudrun Rappold[1,*]

[1]Department of Human Molecular Genetics, [2]Division of Developmental Genetics, Institute of Human Genetics, University of Heidelberg, INF 366, 69120 Heidelberg, Germany and [3]Department of Pediatrics and Cardiovascular Research Institute, Gladstone Institute for Cardiovascular Disease, University of California, 1650 Owens Street, San Francisco, CA 94158, USA

Received September 3, 2010; Revised and Accepted September 8, 2010

Heart formation requires a highly balanced network of transcriptional activation of genes. The homeodomain transcription factor, Shox2, is essential for the formation of the sinoatrial valves and for the development of the pacemaking system. The elucidation of molecular mechanisms underlying the development of pacemaker tissue has gained clinical interest as defects in its patterning can be related to atrial arrhythmias. We have analyzed putative targets of Shox2 and identified the *Bmp4* gene as a direct target. Shox2 interacts directly with the *Bmp4* promoter in chromatin immunoprecipitation assays and activates transcription in luciferase-reporter assays. In addition, ectopic expression of *Shox2* in *Xenopus* embryos stimulates transcription of the *Bmp4* gene, and silencing of *Shox2* in cardiomyocytes leads to a reduction in the expression of *Bmp4*. In *Tbx5*$^{del/+}$ mice, a model for Holt-Oram syndrome, and *Shox2*$^{-/-}$ mice, we show that the T-box transcription factor Tbx5 is a regulator of *Shox2* expression in the inflow tract and that *Bmp4* is regulated by Shox2 in this compartment of the embryonic heart. In addition, we could show that Tbx5 acts cooperatively with Nkx2.5 to regulate the expression of *Shox2* and *Bmp4*. This work establishes a link between *Tbx5*, *Shox2* and *Bmp4* in the pacemaker region of the developing heart and thus contributes to the unraveling of the intricate interplay between the heart-specific transcriptional machinery and developmental signaling pathways.

INTRODUCTION

Heart formation requires the coordinated recruitment of various transcription factors including members of the T-box and homeobox-containing gene families (1). Different family members have been implicated in vertebrate heart tissue patterning and differentiation. In early stages of development, the heart is a slow conducting linear tube. During embryogenesis, the primitive tube develops into a synchronous and regular beating heart structure initiated by a small group of specialized cells forming the pacemaker region or sinoatrial node (SAN). Cells of the SAN locate at the junction of the right atrial wall and the superior caval vein and are spontaneously active (2,3). Yet, the exact molecular mechanisms underlying pacemaker potentials and its defects leading to arrhythmias have not been fully elucidated.

One key regulator of pacemaker differentiation is the homeobox transcription factor, *Shox2*. This gene has a highly restricted expression pattern in the sinus venosus myocardium comprising the SAN region (4,5). Knock-down and knock-out mouse models of *Shox2* have demonstrated a crucial role of this gene in the development of heart and limb (4–7). Loss of Shox2 leads to embryonic lethality owing to heart defects occurring between E11.5 and E17.5 (4,7). Furthermore, *Shox2* deficiency has been shown to lead to impaired pacemaking function in embryonic Zebrafish

*To whom correspondence should be addressed. Tel: +49 6221565050; Fax: +49 6221565155; Email: herbert.steinbeisser@med.uni-heidelberg.de (H.S.); Tel: +49 6221565059; Fax: +49 6221565155; Email: gudrun.rappold@med.uni-heidelberg.de (G.R)

hearts and isolated hearts of *Shox2* mutant mice have shown a slower heart beat rate (4,5). Very recently, *Shox2* has been shown to be a direct target of *Pitx2c*, which is known to prevent susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification (8). These findings have revealed a critical role for *Shox2* in heart development and pacemaker function, but the detailed molecular mechanism of *Shox2* function remains unclear. To investigate the transcriptional regulation of *Shox2* and to identify putative target genes, we have used *Xenopus* and mouse as model systems. We provide genetic evidence for an epistatic relationship between *Tbx5*, *Shox2* and *Bmp4* in a very distinct region of the developing heart.

RESULTS

Shox2 regulates the expression of *Bmp4*

As a first step towards a functional analysis of *Shox2*, we cloned the full-length coding region of the *Xenopus tropicalis* orthologous gene. Sequence comparison between human and *Xenopus Shox2* revealed an overall homology of 80% at the DNA and 85% at the amino acid level. Crucial functional features are conserved between the human and *Xenopus* Shox2 proteins including an identical OAR- (orthopedia, aristaless and rx homeoproteins) and homeodomain (Supplementary Material, Fig. S1). *Shox2*-specific transcripts can be first detected in stage 23 embryos and the expression level increases until stage 45 (Supplementary Material, Fig. S2A). Whole-mount *in situ* hybridization and following transverse sections of the heart anlage showed that *Shox2* expression is restricted to the posterior domain of the myocardium (Supplementary Material, Fig. S2B–H), which continues to cover the dorsolateral aspect of the endocardium, anterior to its bifurcation in the sinus venosus. Endogenous *Shox2* transcripts are not found in gastrula and early neurula *Xenopus* embryos, but when *Shox2* mRNA was provided experimentally before gastrulation, defects were observed in embryonic patterning. Synthetic *Shox2* mRNA was injected into each blastomere of 4-cell stage embryos. Subsequently, they were cultured until stage 36 and the dorso-anterior index (DAI) was determined. All embryos injected either with human or *Xenopus Shox2* mRNA were ventro-posteriorized with DAI scores ranging from 2 to 4 (Fig. 1A–D, Supplementary Material, Table S1). The ventralized phenotype obtained by *Shox2* injection was dose-dependent. Embryos injected with 0.5 ng of *Shox2* mRNA exhibit reduced eyes and foreheads (Fig. 1A and C), whereas those that received higher doses (1 ng) were acephalic (Fig. 1B and D). These results indicate that ectopic *Shox2* suppresses dorso-anterior structures and promotes ventro-posterior development. The ventralized phenotype observed in *Xenopus* embryos after injection of *Shox2* RNA could be owing to the inhibition of early Wnt signaling, which also results in the loss of dorsal structures. Therefore, we analyzed the expression of the Wnt targets *siamois* (*sia*) and *nodal-related-3* (*Xnr3*) at early gastrula stage after *Shox2* injection. Whole-mount *in situ* hybridization for *Xnr3* and quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) for *sia* and *Xnr3* demonstrated that expression of these Wnt targets was unaffected in *Shox2*-injected embryos (data not shown). This shows that ectopic Shox2 does not inhibit early Wnt-signaling.

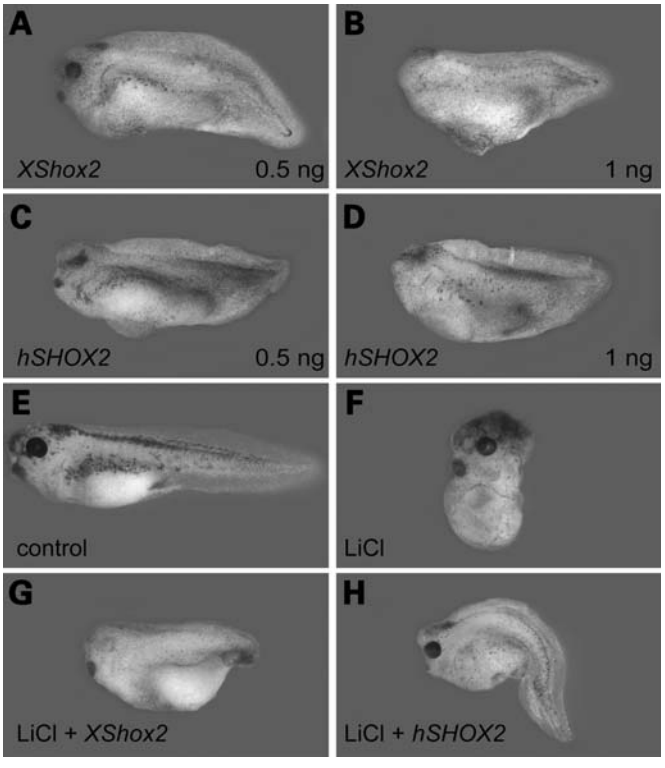


Figure 1. Ectopic *Shox2* expression induces a ventralizing effect during early *Xenopus* development and rescues embryos partially dorsalized by LiCl. Lateral view of stage 36 embryos, radially injected at 4-cell stage with 0.5 ng (**A**, **C**) and 1 ng (**B**, **D**) *Xenopus tropicalis Shox2* RNA (A, B) and human *SHOX2a* RNA (C, D). *Shox2*-injected embryos show a dose-dependent ventralizing effect. Uninjected stage 36 control embryo (**E**) and control embryo treated with 120 mM LiCl (**F**). Ventral injection of 1 ng *Shox2* RNA rescues embryos partially dorsalized by LiCl (**G**, **H**).

Bmp4 mediates dorsal–ventral patterning in *Xenopus* embryos by inhibiting differentiation of dorsal mesoderm and neural tissue. Since the phenotype in *Shox2*-injected embryos strongly resembled the effect of early ectopic *Bmp4* expression in *Xenopus*, we speculated that *Shox2* might induce *Bmp4* transcription. We therefore inhibited zygotic transcription of *Bmp4* by lithium chloride (LiCl) treatment. LiCl acts through inhibition of the glycogen synthase kinase-3β (GSK-3β), which allows activation of the Wnt/β-catenin signaling pathway and leads to dorsalization of the mesoderm (9). Induction of early Wnt signaling dorsalizes the mesoderm and the entire mesoderm acquires properties of the Spemann Organizer (dorsal mesoderm). In this situation, no *Bmp4* transcripts are detected in the mesoderm. Thus, LiCl treatment inhibits ventro-posterior structures, resulting in a dorso-anteriorized phenotype with DAI scores >5 (Fig. 1F, Supplementary Material, Table S1). Ectopic expression of *Shox2* antagonizes the dorsalized LiCl-phenotype and partially rescues posterior structures, suggesting an upregulation of *Bmp4* by *Shox2* (Fig. 1G and H, Supplementary Material, Table S1). To validate this hypothesis, we performed whole-mount *in situ* hybridization on *Shox2*-injected, LiCl-treated embryos. *Bmp4*-specific

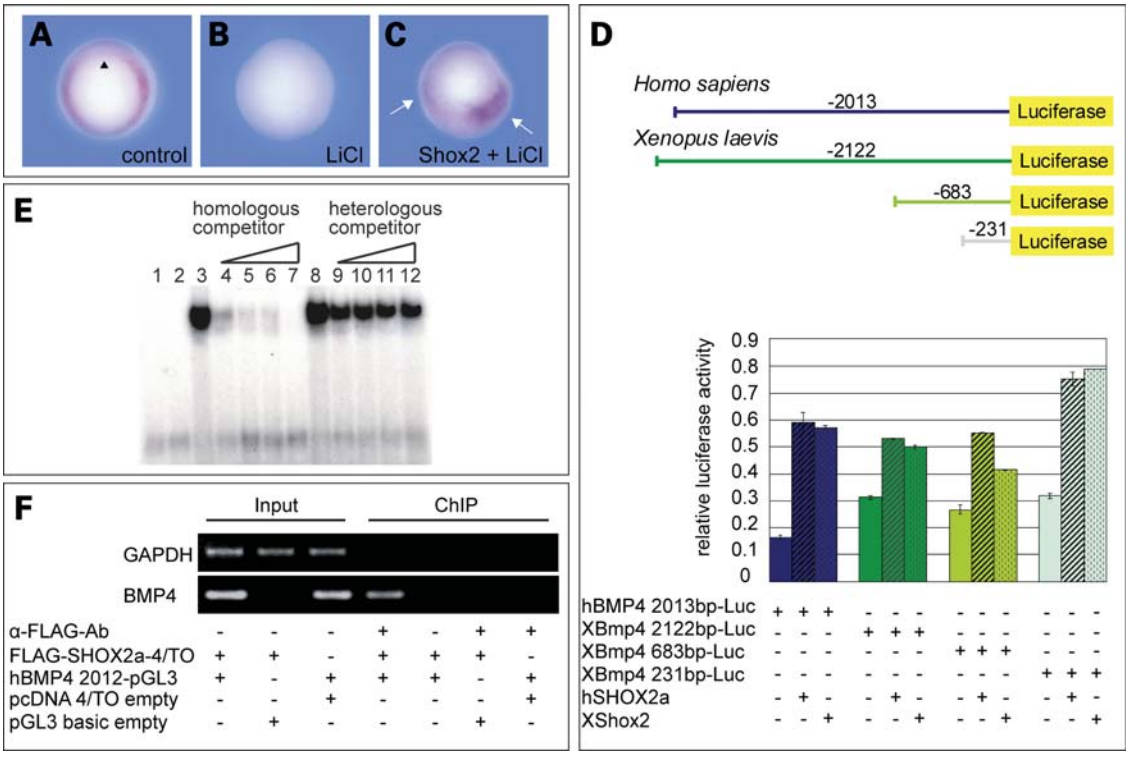
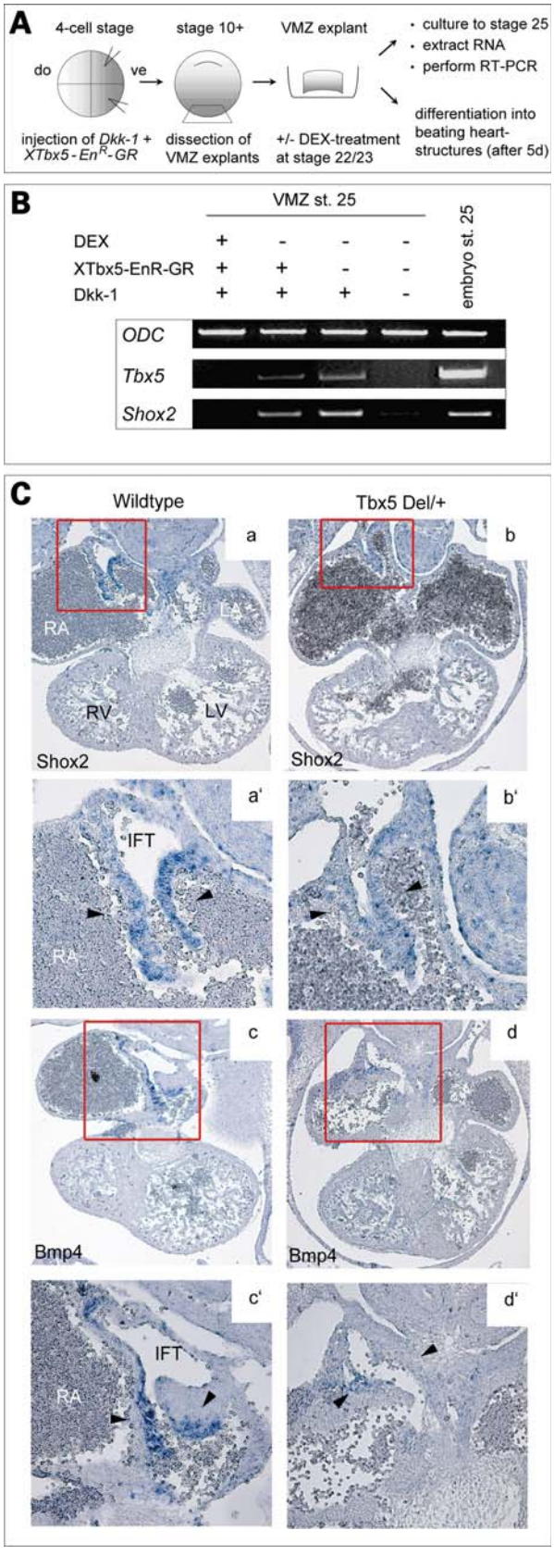


Figure 2. *Shox2* regulates the expression of *Bmp4*. (**A**) *Bmp4* expression in uninjected embryos at stage 10.5 ($n = 35$). Black arrowhead indicates the dorsal lip. (**B**) *Bmp4* expression is reduced or absent in 87% of LiCl-treated embryos ($n = 31$). (**C**) Spots of *Bmp4* expression in 77% of previously *Shox2*-injected, LiCl-treated embryos ($n = 30$). *Shox2* RNA was injected diagonally into two blastomers of a 4-cell stage embryo. *Bmp4* staining in two areas of the embryo (marked by white arrows) indicates an upregulation of *Bmp4* expression corresponding to the *Shox2* injection. (**D**) *Shox2* increases the activity of the human and *Xenopus laevis Bmp4* promoter upon co-transfection of *Shox2* expression plasmids (1 μg) and the indicated *Bmp4* reporter-constructs (1 μg) into Cos-7 cells. Data using HEK-293 cells are not shown. (**E**) Electrophoretic mobility shift assay of the GST–SHOX2 fusion protein on a conserved promoter oligonucleotide sequence (*BMP4*). An excess of non-radioactively labelled oligonucleotides (homologous competitor) reduces the DNA-binding ability of SHOX2, whereas excess of a random oligonucleotide sequence (heterologous competitor) does not affect DNA-binding. 1, free oligonucleotide; 2, GST alone; 3, GST–SHOX2; 4–7, 10-fold, 50-fold, 75-fold and 150-fold molar excess of homologous competitor; 8, GST–SHOX2; 9–12, 10-fold, 50-fold, 75-fold and 150-fold molar excess of heterologous competitor. (**F**) Chromatin immunoprecipitation (ChIP) assay. HEK-293 cells were co-transfected with the FLAG-SHOX2a expression or empty control vector and with either the hBMP4 2013-Luc reporter or empty control vector as indicated. Formaldehyde-crosslinked DNA was immunoprecipitated using an anti-FLAG antibody (α-FLAG-Ab) or no antibody, as a negative control. Precipitated DNA fragments (ChIP) and DNA from lysate before immunoprecipitation (Input) were subjected to PCR using primer sets amplifying the putative SHOX2-binding element in the *BMP4* gene (lower panel) or *GAPDH* (upper panel) as a control.

mRNA was almost undetectable in LiCl-treated embryos (Fig. 2B). In contrast, *Shox2*-injected, LiCl-treated embryos clearly showed an upregulation of *Bmp4* expression in those cells derived from the *Shox2*-injected blastomeres (Fig. 2C). Since Shox2 acts as a transcription factor, a direct regulation of the *Bmp4* promoter was possible. Comparative analysis of genomic *Bmp4* sequences has indicated that exons 3, 4 and 5 of the human *BMP4* gene correspond to exons 1, 2 and 3 of the orthologous *Xenopus* gene (Supplementary Material, Fig. S3). To identify specific DNA regulatory elements within the *Bmp4* gene that are essential for the transactivation by *Shox2*, we used human and the corresponding *Xenopus Bmp4* promoter-constructs and tested their activities by transient transfections of Cos-7 and HEK-293 cells in dual-luciferase-reporter assays. Co-transfections of the different *Bmp4* reporter-constructs together with the expression plasmids for either human or *X. tropicalis Shox2* led to an approximately 3-fold upregulation in luciferase activity, compared to cells transfected only with the corresponding *Bmp4* reporter-constructs (Fig. 2D). By transfection of serial gene deletion-constructs, we have narrowed down the *Shox2*-responsive element of the *Bmp4* promoter to an interval of 231 bp upstream of the mRNA start site. Within this 231 bp fragment, a 34 bp sequence motif, highly conserved between human, mouse and *Xenopus* was identified (Supplementary Material, Fig. S4). Electrophoretic mobility shift assay (EMSA) competitive experiments showed that a SHOX2–GST fusion protein could bind to this 34 bp *BMP4*-sequence motif with high affinity and was competed away by an excess of the unlabeled *BMP4* oligonucleotide (Fig. 2E). A non-specific random oligonucleotide sequence did not affect the binding of the SHOX2–GST to the *BMP4* oligonucleotide.

To further examine the SHOX2 binding to the *BMP4* promoter *in vivo*, we performed a Chromatin immunoprecipitation (ChIP) assay (10). An anti-FLAG antibody was used to immunoprecipitate chromatin from HEK-293 cells transiently co-transfected with the FLAG-tagged SHOX2a expression plasmid and the hBMP4 2013-Luc reporter-construct containing the putative SHOX2-binding site of the *BMP4* promoter. The binding of SHOX2 to the *BMP4* promoter was detected by PCR analysis using specific primers spanning the putative SHOX2-binding site within the *BMP4* promoter. We obtained



PCR products in the sample that was immunoprecipitated with the anti-FLAG antibody as well as in the input DNA, whereas the negative controls (no antibody control, transfections with empty vectors) failed to yield a product (Fig. 2F). Identical results were also obtained using *Xenopus* FLAG-tagged Shox2 and XBmp4 231 bp-Luc-constructs (data not shown). Taken together, these results indicate that SHOX2 binds specifically to a 34 bp conserved DNA interval in the *Bmp4* promoter *in vitro* and *in vivo*.

Tbx5 acts upstream of *Shox2* and *Bmp4* in the inflow tract of the developing heart

Mutations in the T-box transcription factor gene *TBX5* cause heart and limb malformations in the Holt-Oram syndrome (11,12). Because of a common heart and limb defect pattern between *Tbx5* (13) and *Shox2*, we speculated on a possible epistatic relationship between *Tbx5*, *Shox2* and *Bmp4* in the developing heart. We have carried out two independent sets of experiments to assess this hypothesis.

To study *Tbx5*-dependent *Shox2* expression, we used experimentally induced heart structures in *Xenopus*. Injection of the *Wnt* antagonist *Dickkopf-1* (*Dkk-1*) into *Xenopus* embryos can induce heart differentiation in explants of ventral marginal zone (VMZ) mesoderm (14). '*In vitro* hearts' only form when Wnt is inhibited. Thus, we injected *Dkk-1* mRNA together with an inducible *Tbx5* Engrailed-repressor glucocorticoid-receptor fusion protein (*XTbx5-En^R-GR*), which blocks endogenous Tbx5 activity, into ventral blastomeres of early *Xenopus* embryos. *XTbx5-En^R-GR* was activated by dexamethasone (DEX) treatment in *Dkk-1*-injected VMZ explants at later embryonic stage 22/23 to avoid complete loss of heart tissue. In such samples, the heart marker *Troponin* is expressed, indicating that heart tissue is present under these experimental conditions (data not shown). Remarkably, RT–PCR analysis of the explants at stage 25 revealed that *XTbx5-En^R-GR* completely blocks *Shox2* expression in *Dkk-1*-injected VMZ explants (Fig. 3A and B). These results indicate that Tbx5 function is critical for the expression of *Shox2* in cardiac tissues.

Figure 3. *Shox2* mediates *Tbx5* expression to *Bmp4* signaling in the developing heart. (**A**) Scheme of the ventral marginal zone (VMZ) experiment. *Xenopus* embryos were injected (vegetal dorsal) with 1 ng *Dkk-1* mRNA and 0.2 ng of *XTbx5 En^R-GR* DNA into two blastomeres at the 4-cell stage and VMZ explants were dissected at early gastrula stage. Explants were cultured until stage 22/23 and then treated with 0.5 μM dexamethasone (DEX)-solution to activate the injected repressor-construct. RNA was extracted at stage 25 and analysed by RT–RCR for the presence of *XTbx5* and *XShox2*. (**B**) RT–PCR shows that *Dkk-1* induces *Tbx5* and *Shox2* expression in VMZ explants. *XTbx5 En^R-GR* abolishes *Dkk-1* induced *Tbx5* and *Shox2* expression after DEX-treatment. *Ornithine decarboxylase* was used as a loading control. (**C**) Section *in situ* hybridization on E11.5 wild-type and *Tbx5$^{del/+}$* mouse hearts. Murine *Shox2* is strongly expressed in the inflow tract of the developing heart in wild-type embryos (a), which is markedly decreased in *Tbx5$^{del/+}$* embryos (b). Murine *Bmp4* expression overlaps with the *Shox2* expression domain in wild-type hearts (c) and is almost absent in *Tbx5$^{del/+}$* embryos (d). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

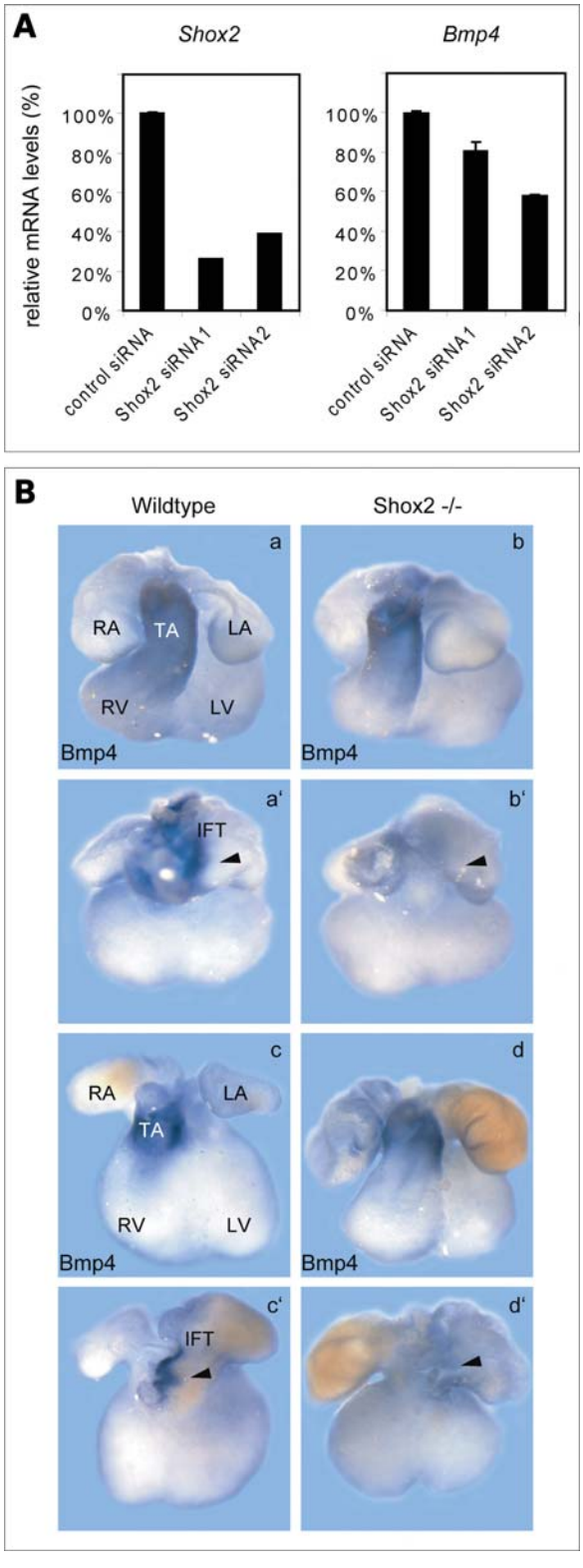


Figure 4. *Shox2* deficiency impairs *Bmp4* expression. (**A**) H10 cells were transfected with two different *Shox2* siRNAs (*Shox2* siRNA1 and *Shox2* siRNA2) in parallel with a control siRNA. Expression levels of *Shox2* (left panel) and *Bmp4* (right panel) were assessed 24 h after transfection by qRT–PCR analysis. siRNA-mediated knock-down of *Shox2* results in 19–42% reduction of *Bmp4* mRNA levels. All results were normalized to *Hprt1* (*hypoxanthine phosphoribosyltransferase 1*) mRNA values. (**B**) Whole-mount *in situ* hybridization on E11.5 (a, b) and E12.5 (c, d) wild-type and $Shox2^{-/-}$ mouse hearts using a *Bmp4* RNA probe. Both, ventral (a–d) and dorsal (a′–d′) views are shown. Murine *Bmp4* is strongly expressed in the truncus arteriosus (TA) (a, c) and in the inflow tract (a′, c′) of the developing heart in wild-type embryos. In the $Shox2^{-/-}$ mouse hearts, *Bmp4* expression is still present in the truncus arteriosus (b, d) but completely absent in the IFT (b′, d′), where *Shox2* and *Bmp4* expression domains overlap in the wild-type heart. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

Using mouse as a model, we found that expression of *Shox2* and *Bmp4* overlaps in the inflow tract (IFT) of the developing heart (Fig. 3Ca and c). This is consistent with the data published by Norden *et al*. (15). To assess the relationship between *Tbx5*, *Shox2* and *Bmp4*, we have analyzed mice with a heterozygous deletion of the *Tbx5* gene ($Tbx5^{del/+}$). Homozygous $Tbx5^{del/del}$ mice do not survive past E10.5 (13). We investigated whether expression of *Shox2* or *Bmp4* in the IFT of the right atrium relies on *Tbx5* function in a dose-dependent manner. *In situ* hybridization on histological sections of embryonic hearts demonstrated reduced *Shox2* expression in the IFT of the $Tbx5^{del/+}$ mutant mice at E11.5 (Fig. 3Ca and b). Similarly, *Bmp4* expression, which overlaps with the *Shox2* expression domain, is strongly reduced and almost absent in this distinct heart region of $Tbx5^{del/+}$ embryos (Fig. 3Cc and d). This is consistent with data from the microarray experiments, confirmed by quantitative RT–PCR, which suggested *Shox2* as one of the roughly 100 putatively downregulated genes in *Tbx5*-deficient mouse hearts (16). Other genes important for normal development and function of the conduction system, like *α1D L-type calcium channel*, *Cx43* and *Tbx3*, were either normally expressed, or in the case of *Tbx3*, downregulated but still expressed in $Tbx5^{del/+}$ hearts (13,16). Hence, we can state that the strongly reduced expression of *Shox2* and *Bmp4* in $Tbx5^{del/+}$ hearts is not owing to incorrect formation of the IFT.

We next performed gene-specific silencing of *Shox2* expression in neonatal rat cardiomyocytes by RNAi. H10 cells were transiently transfected with two different siRNAs directed against the *Shox2* cDNA with a knock-down efficiency of 74% and 61% (Fig. 4A). We could also show that *Shox2* silencing leads to a reduction in *Bmp4* expression (Fig. 4A).

We also carried out whole-mount *in situ* hybridization on $Shox2^{-/-}$ embryonic mouse hearts (4) to confirm that *Bmp4* expression depends on *Shox2* function in the IFT of the developing heart. These stainings revealed that *Bmp4* is completely absent in the IFT of *Shox2*-deficient mice, whereas a strong expression in the truncus arteriosus is still detectable (Fig. 4B). *Shox2* and *Bmp4* stained $Shox2^{-/-}$ hearts, as well as histological sections of $Shox2^{-/-}$ embryos, clearly showed that the IFT in *Shox2*-deficient mice develops properly, even if the venous valve formation is abnormal (Fig. 5). We also carried out *in situ* hybridization using *Tbx3* and *Hcn4* as markers for the pacemaker region and can show that *Tbx3* and *Hcn4* are downregulated but still present in $Shox2^{-/-}$ hearts (data not shown). These data are consistent with a recent study investigating the role of *Shox2* by expression analysis of different key regulators in SAN development (5). Thus, we demonstrate that the absence of *Bmp4* expression in the IFT of $Shox2^{-/-}$ hearts is not owing to incorrect formation of this tissue. Taken together, these data

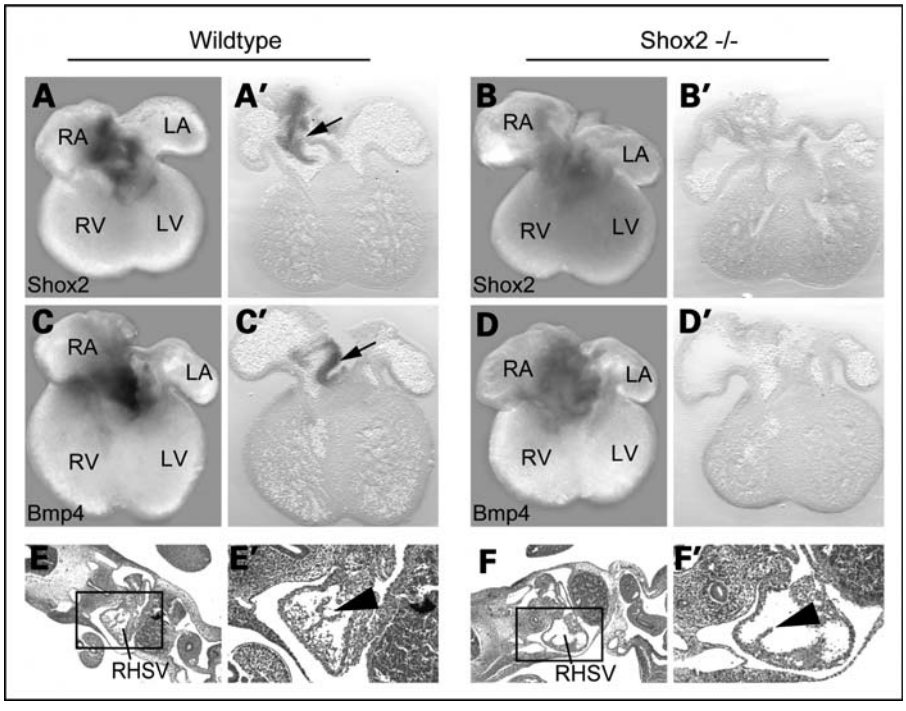


Figure 5. *Bmp4* expression is absent in the inflow tract (IFT) of $Shox2^{-/-}$ embryos. Whole-mount *in situ* hybridization with corresponding sections (50 μm) on E12.5 wild-type and $Shox2^{-/-}$ mouse hearts using *Shox2* (**A**, **B**) and *Bmp4* (**C**, **D**) RNA probes. Murine *Shox2* (A, **A**′) and *Bmp4* (C, **C**′) are expressed in the IFT of wild-type hearts (indicated by black arrows), but are completely absent in the IFT of $Shox2^{-/-}$ hearts (B, **B**′, D, **D**′). Note that IFT tissue is still present in $Shox2^{-/-}$ hearts (B′, D′). Histological sections of wild-type (**E**, **E**′) and $Shox2^{-/-}$ (**F**, **F**′) embryos at E10.5. Squares in E and F show the right horn of the sinus venosus that is magnified in E′ and F′. *Shox2*-deficient embryos develop IFT tissue (F, F′), even if the venous valve formation is abnormal (black arrowhead in F′). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; RHSV, right horn of the sinus venosus.

strongly suggest that *Tbx5* acts upstream of *Shox2*, which, in turn, activates *Bmp4* in the IFT of the heart.

DISCUSSION

Shox2 encodes a homeodomain transcription factor that among other features plays a critical role in heart development (4,5,17). The aim of our study was to understand the molecular mechanisms underlying *Shox2* expression in the developing heart. Our data show that the transformation growth factor-β, Bmp4, is a direct target of Shox2. In contrast to the repressive effect of Shox2 on *Bmp4* expression during limb development (7), we could show that in the heart, Shox2 activates *Bmp4.* Several lines of evidence support this conclusion. We demonstrate that ectopic expression of *Shox2* in *Xenopus* embryos stimulates transcription of the *Bmp4* gene and silencing of *Shox2* in cardiomyocytes leads to a reduction in *Bmp4* expression. In addition, Shox2 activates *Bmp4* transcription in luciferase-reporter assays. We also show by EMSA and ChIP assays that Shox2 binds specifically to a 34 bp AT-rich sequence within the *Bmp4* promoter region. *Bmp4* thus represents the first gene identified as a direct Shox2 target. In loss-of-function experiments using *Xenopus* embryos, as well as $Tbx5^{del/+}$ and $Shox2^{-/-}$ mice, we show that the T-box transcription factor Tbx5 is required for *Shox2* expression in the IFT and that *Bmp4* is regulated by *Shox2* in this compartment of the embryonic heart.

Bmp4 is known to exert numerous developmental functions including different roles in cardiogenesis. Homozygosity for a null mutation, for example, leads to severe looping defects and embryonic death at E9.5 or earlier (18,19). The analysis of a hypomorphic allele has demonstrated additional later functions in atrioventricular septation, valvulogenesis and formation of the outflow tract (20,21). Both *Shox2* and *Bmp4* are expressed already strongly at E9.5, prior to the disturbed development in the *Shox2* mutant. At this time, incorporation of the sinus venosus into the right atrium takes place and at the venous pole new myocardium is still added to the sinus venosus (22). Most probably, both processes are disturbed and contribute to the abnormalities seen in $Shox2^{-/-}$ embryos. Abnormal *Bmp4* expression has also been reported to result in abnormal myofibrillogenesis of the precardiac mesoderm (23), which also might contribute to embryonic heart failure in *Shox2*-deficient embryos. The essential cardiogenic functions previously attributed to *Bmp4* pertain to the atrioventricular and semilunar valves that, in contrast to the venous valves of the IFT, develop from endocardial cushions. Our analyses suggest a link between *Bmp4* expression and early sinoatrial development and venous valve formation.

It was also reported that expression of *Bmp4* is dysregulated in the developing heart of the zebrafish *heartstrings* (*hst*) mutant with *Tbx5* deficiency (24). Although this *hst* mutation does not perturb atrioventricular patterning as described for other species with *Tbx5* mutations, it impairs pacemaking functions, similar to that observed in *Shox2* knock-down

experiments with morpholino-modified antisense oligonucleotides (4,24). These data suggest that the *hst* mutation affecting the cardiac conduction system may also regulate the expression of downstream genes such as, for example, *Shox2* and *Bmp4*. Overall, our data indicate that in specific structures and during different time points, *Bmp4* also can act downstream of *Tbx5*.

Tbx5 overexpression experiments in *Xenopus* animal cap (AC) and dorsal marginal zone (DMZ) explants do not provide support for the regulation of *Shox2* by *Tbx5* alone (data not shown), suggesting that *Tbx5* affects *Shox2* expression also through other factors not present in the AC and DMZ explants. Previous studies have demonstrated that Tbx5 directly associates with co-factors, such as the homeodomain transcription factor Nkx2.5 and the zinc finger factor Gata4 to induce cardiac-specific genes like *Nppa* and *Gja5* (13,25,26). Moreover, it has been shown that co-activators such as *Baf60c* or *TAZ* and *YAP* are important for the control of Tbx5-dependent transcription in cardiac development (27,28). Very recent work has also demonstrated that *Baf60c* and *Gata4* cooperate with Tbx5 to set-up a cardiac gene expression program, including *Nkx2.5*, which transforms non-cardiac embryonic mesoderm into beating heart cells (29). It cannot be excluded that *Shox2* may play a role in these processes too.

An involvement of further co-factors in cardiac development in the described epistatic relationship between *Tbx5*, *Shox2* and *Bmp4* can be assumed. In contrast to the highly restricted expression pattern of *Shox2* in the sinus venosus myocardium, *Tbx5* and *Bmp4* are widely expressed during embryonic heart development (4,17,30,31). Owing to the fact that the overlapping expression domains of these three genes are confined to a restricted region of the IFT of the heart, it is likely that additional as yet unknown factors may contribute to control the transcriptional readout in other regions of the developing heart. To find out if heart-specific co-factors of Tbx5, such as Nkx2.5 contribute to the regulation of *Shox2*, we analyzed the *SHOX2* promoter for TBX5 and NKX2.5 binding sites, and three putative TBX5 and three NKX2.5 binding sites upstream of the *SHOX2* start codon could be identified. In luciferase-reporter assays using a *SHOX2* reporter-construct containing all putative binding sites, we could demonstrate that TBX5 acts cooperatively with NKX2.5 to activate *SHOX2* expression. In contrast, TBX5 alone is not able to activate the *SHOX2* promoter. The same effect could be shown by using the *BMP4* reporter-construct (Supplementary Material, Fig. S5). Our data indicate that Nkx2.5 acts upstream of Shox2. The findings of Espinoza-Lewis *et al*. (5) have shown that Shox2 can also inhibit the expression of *Nkx2.5*, arguing for a regulatory feedback-loop between Shox2 and Nkx2.5. These novel findings support the idea that a cardiac-specific molecular environment is necessary for Tbx5 to regulate Shox2.

In summary, we have demonstrated a previously undescribed connection of *Tbx5*, *Shox2* and *Bmp4* in a distinct subregion of the developing heart. Our results suggest that *Tbx5* signaling in the IFT of the heart directly or indirectly controls the expression of the transcription factor *Shox2*, which regulates *Bmp4* in a direct manner. Taken into account the crucial role of Shox2 in pacemaking function, an investigation in patients with atrial arrhythmia of unknown molecular cause may be of interest. Indeed, the recent genetic linkage of Tbx5 and Nkx2.5 to atrial fibrillation suggests that this pathway may be of relevance in the general population (32,33).

MATERIALS AND METHODS

Generation of plasmid-constructs

Cloning of the *Xenopus Shox2* cDNA, generation of *Xenopus Shox2*, human *SHOX2* and NKX2.5 expression-constructs, and *BMP4* and *SHOX2* promoter-constructs are described in the Supplementary Material.

Egg and embryo manipulations

Ovulation, *in vitro* fertilization, embryo culture and manipulation were performed as previously described (34). LiCl treatment was carried out as described (35).

mRNA synthesis

Capped mRNAs were synthesized from linearized plasmids using mMessage mMachine Kit (Ambion). pCS2$^+$-*SHOX2a* was linearized with *ApaI*, pCS2$^+$-Xtropicalis *Shox2* and pCS2$^+$-*Dkk-1* were linearized with *NotI*. mRNAs were synthesized using SP6 RNA polymerase.

Semi-quantitative and quantitative RT–PCR

For embryos, total RNA was prepared with Trizol® reagent (Invitrogen) and for transfected H10 cells with the RNeasy®-Mini Kit (Qiagen) with subsequent DNase I treatment. cDNA was synthesized using the SuperScript™ First-Strand Synthesis System for RT–PCR (Invitrogen). Semi-quantitative PCR was performed using standard conditions. Quantitative RT–PCR (qRT–PCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green ROX dye (Thermo Scientific). Primer sets used are presented in Supplementary Material, Table S2.

Xenopus in situ hybridization

Whole-mount *in situ* hybridization and antisense probe preparation was carried out as described (36). Digoxigenin-labelled antisense RNA was synthesized from the plasmids pTOPO-X.trop-*Shox2* (linearized with *Hin*dIII) and pBSK/B12-*Bmp4* (35) (linearized with *Eco*RI) using T7 RNA polymerase.

Cell culture, transfection and luciferase assay

Cos-7 and HEK-293 cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM) medium containing high glucose, supplemented with 10% fetal calf serum and antibiotics. Neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40T antigen (H10 cells), were cultured at 33°C in DMEM medium (see above). For luciferase assay analysis, the cells were co-transfected in triplicates with different constructs using polyethylenimine (Sigma-Aldrich).

Forty-eight hours after transfection, luciferase activity was determined and normalized to Renilla luciferase activity with a dual-luciferase assay kit (Promega). Experiments were repeated at least three times in triplicates with consistent results and representative data were shown. Knock-down experiments were performed using Stealth RNAi Duplex (Invitrogen), directed against the *Shox2* cDNA (*Shox2* siRNA1: 5′-GGACCAATTTCACCCTGGAACAACT-3′, *Shox2* siRNA2: 5′-GCAAGGACTCCAGCATCGCCGATCT-3′). H10 cells were transiently transfected with 100 pmol siRNA in 12-well plates using Lipofectamine 2000 (Invitrogen) and the relative knock-down efficiency was determined by qRT–PCR 24 h after transfection. Stealth RNAi negative control GC high was used as a control siRNA. Representative data from three independent experiments with consistent results are shown.

Electrophoretic mobility shift assay

EMSA was performed using standard protocols. For the binding reaction, ^{32}P-labeled, double-stranded *BMP4* oligonucleotides (Supplementary Material, Table S2) were used together with purified, bacterially expressed recombinant GST–SHOX2 protein.

Chromatin Immunoprecipitation assay

HEK-293 cells were transiently co-transfected with hBMP4 2013-Luc reporter or empty control vector and with either the FLAG-SHOX2a expression vector or empty control vector using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were cultured for 48 h and the ChIP assay carried out as described by Abcam protocols using 10 μg of anti-FLAG antibody (M2, Sigma-Aldrich). PCR analysis of immunoprecipitated DNA and input DNA was performed under standard conditions using primer sets amplifying the putative SHOX2-binding element in the *BMP4* gene (−1241 to −1082 bp region) or *GAPDH* as control (Supplementary Material, Table S2).

Mouse whole-mount and section *in situ* hybridization

Shox2$^{-/-}$ and *Tbx5*$^{del/+}$ mice were described previously (4,13). Whole-mount *in situ* hybridization was performed as previously reported. Digoxigenin-labelled antisense RNA was synthesized from the plasmids pCR-S-Og12 (Shox2) (17) (linearized with *Sac*I) and pSP72-Bmp4 (linearized with *Acc*I) using T7 RNA polymerase. Section *in situ* hybridization was performed on 10 μm paraffin sections using standard protocols. The murine *Shox2* antisense probe was made from a cDNA clone ordered from Open Biosystems (Huntsville, USA; Cat no. EMM1032-595060) using T7 polymerase after linearizing with *Eco*RI. The *Bmp4* antisense probe was synthesized from pSP72-Bmp4 (see above).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

This study is dedicated to Rüdiger Blaschke. We thank A. Gittenberger-de Groot for reading the manuscript, A. Schweikert for assistance on sectioning, G. Thomsen for providing the *X. laevis Tbx5*-construct, W. Knöchel for the *X. laevis Bmp4*-promoter plasmids, C. Niehrs for the *Dkk-1* plasmid, H.-J. Hippe for the H10 cell line and B. Hogan for the murine *Bmp4 in situ* probe.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft grant no. BL 372/4-2. Funding to pay the Open Access Charge was provided by the Medical Faculty Heidelberg.

REFERENCES

1. Bruneau, B.G. (2008) The developmental genetics of congenital heart disease. *Nature*, **451**, 943–948.
2. Boyett, M.R., Honjo, H. and Kodama, I. (2000) The sinoatrial node, a heterogeneous pacemaker structure. *Cardiovasc. Res.*, **47**, 658–687.
3. Christoffels, V.M., Smits, G.J., Kispert, A. and Moorman, A.F. (2010) Development of the pacemaker tissues of the heart. *Circ. Res.*, **106**, 240–254.
4. Blaschke, R.J., Hahurij, N.D., Kuijper, S., Just, S., Wisse, L.J., Deissler, K., Maxelon, T., Anastassiadis, K., Spitzer, J., Hardt, S.E. *et al.* (2007) Targeted mutation reveals essential functions of the homeodomain transcription factor Shox2 in sinoatrial and pacemaking development. *Circulation*, **115**, 1830–1838.
5. Espinoza-Lewis, R.A., Yu, L., He, F., Liu, H., Tang, R., Shi, J., Sun, X., Martin, J.F., Wang, D., Yang, J. *et al.* (2009) Shox2 is essential for the differentiation of cardiac pacemaker cells by repressing Nkx2–5. *Dev. Biol.*, **327**, 376–385.
6. Cobb, J., Dierich, A., Huss-Garcia, Y. and Duboule, D. (2006) A mouse model for human short-stature syndromes identifies Shox2 as an upstream regulator of Runx2 during long-bone development. *Proc. Natl Acad. Sci. USA*, **103**, 4511–4515.
7. Yu, L., Liu, H., Yan, M., Yang, J., Long, F., Muneoka, K. and Chen, Y. (2007) Shox2 is required for chondrocyte proliferation and maturation in proximal limb skeleton. *Dev. Biol.*, **306**, 549–559.
8. Wang, J., Klysik, E., Sood, S., Johnson, R.L., Wehrens, X.H. and Martin, J.F. (2010) Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification. *Proc. Natl Acad. Sci. USA*, **107**, 9753–9758.
9. Klein, P.S. and Melton, D.A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl Acad. Sci. USA*, **93**, 8455–8459.
10. Wells, J. and Farnham, P.J. (2002) Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods*, **26**, 48–56.
11. Basson, C.T., Bachinsky, D.R., Lin, R.C., Levi, T., Elkins, J.A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T.A., Leblanc-Straceski, J. *et al.* (1997) Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat. Genet.*, **15**, 30–35.
12. Li, Q.Y., Newbury-Ecob, R.A., Terrett, J.A., Wilson, D.I., Curtis, A.R., Yi, C.H., Gebuhr, T., Bullen, P.J., Robson, S.C., Strachan, T. *et al.* (1997) Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat. Genet.*, **15**, 21–29.
13. Bruneau, B.G., Nemer, G., Schmitt, J.P., Charron, F., Robitaille, L., Caron, S., Conner, D.A., Gessler, M., Nemer, M., Seidman, C.E. *et al.* (2001) A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell*, **106**, 709–721.
14. Schneider, V.A. and Mercola, M. (2001) Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. *Genes Dev.*, **15**, 304–315.

15. Norden, J., Grieskamp, T., Lausch, E., van Wijk, B., van den Hoff, M.J., Englert, C., Petry, M., Mommersteeg, M.T., Christoffels, V.M., and Niederreither, K. *et al.* (2010) Wt1 and retinoic acid signaling in the subcoelomic mesenchyme control the development of the pleuropericardial membranes and the sinus horns. *Circ. Res.*, **106**, 1212–1220.
16. Mori, A.D., Zhu, Y., Vahora, I., Nieman, B., Koshiba-Takeuchi, K., Davidson, L., Pizard, A., Seidman, J.G., Seidman, C.E., Chen, X.J. *et al.* (2006) Tbx5-dependent rheostatic control of cardiac gene expression and morphogenesis. *Dev. Biol.*, **297**, 566–586.
17. Blaschke, R.J., Monaghan, A.P., Schiller, S., Schechinger, B., Rao, E., Padilla-Nash, H., Ried, T. and Rappold, G.A. (1998) SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. *Proc. Natl Acad. Sci. USA*, **95**, 2406–2411.
18. Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P. and Hogan, B.L. (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.*, **13**, 424–436.
19. Fujiwara, T., Dehart, D.B., Sulik, K.K. and Hogan, B.L. (2002) Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. *Development*, **129**, 4685–4696.
20. Jiao, K., Kulessa, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H.S. and Hogan, B.L. (2003) An essential role of Bmp4 in the atrioventricular septation of the mouse heart. *Genes Dev.*, **17**, 2362–2367.
21. Liu, W., Selever, J., Wang, D., Lu, M.F., Moses, K.A., Schwartz, R.J. and Martin, J.F. (2004) Bmp4 signaling is required for outflow-tract septation and branchial-arch artery remodeling. *Proc. Natl Acad. Sci. USA*, **101**, 4489–4494.
22. De la Cruz, M.V., Sánchez-Gómez, G. and Palomino, M.A. (1989) The primitive cardiac regions in the straight tube heart (Stage 9) and their anatomical expression in the mature heart: an experimental study in the chick embryo. *J. Anat.*, **165**, 121–131.
23. Nakajima, Y., Yamagishi, T., Ando, K. and Nakamura, H. (2002) Significance of bone morphogenetic protein-4 function in the initial myofibrillogenesis of chick cardiogenesis. *Dev. Biol.*, **245**, 291–303.
24. Garrity, D.M., Childs, S. and Fishman, M.C. (2002) The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development*, **129**, 4635–4645.
25. Hiroi, Y., Kudoh, S., Monzen, K., Ikeda, Y., Yazaki, Y., Nagai, R. and Komuro, I. (2001) Tbx5 associates with Nkx2–5 and synergistically promotes cardiomyocyte differentiation. *Nat. Genet.*, **28**, 276–280.
26. Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K. *et al.* (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature*, **424**, 443–447.
27. Murakami, M., Nakagawa, M., Olson, E.N. and Nakagawa, O. (2005) A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. *Proc. Natl Acad. Sci. USA*, **102**, 18034–18039.
28. Lickert, H., Takeuchi, J.K., Von Both, I., Walls, J.R., McAuliffe, F., Adamson, S.L., Henkelman, R.M., Wrana, J.L., Rossant, J. and Bruneau, B.G. (2004) Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. *Nature*, **432**, 107–112.
29. Takeuchi, J.K. and Bruneau, B.G. (2009) Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature*, **459**, 708–711.
30. Abdelwahid, E., Rice, D., Pelliniemi, L.J. and Jokinen, E. (2001) Overlapping and differential localization of Bmp-2, Bmp-4, Msx-2 and apoptosis in the endocardial cushion and adjacent tissues of the developing mouse heart. *Cell Tissue Res.*, **305**, 67–78.
31. Chapman, D.L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S.I., Gibson-Brown, J.J., Cebra-Thomas, J., Bollag, R.J., Silver, L.M. and Papaioannou, V.E. (1996) Expression of the T-box family genes, Tbx1–Tbx5, during early mouse development. *Dev. Dyn.*, **206**, 379–390.
32. Holm, H., Gudbjartsson, D.F., Arnar, D.O., Thorleifsson, G., Thorgeirsson, G., Stefansdottir, H., Gudjonsson, S.A., Jonasdottir, A., Mathiesen, E.B., Njolstad, I. *et al.* (2010) Several common variants modulate heart rate, PR interval and QRS duration. *Nat. Genet.*, **42**, 117–122.
33. Pfeufer, A., van Noord, C., Marciante, K.D., Arking, D.E., Larson, M.G., Smith, A.V., Tarasov, K.V., Muller, M., Sotoodehnia, N., Sinner, M.F. *et al.* (2010) Genome-wide association study of PR interval. *Nat. Genet.*, **42**, 153–159.
34. Swain, R.K., Katoh, M., Medina, A. and Steinbeisser, H. (2005) *Xenopus* frizzled-4S, a splicing variant of Xfz4 is a context-dependent activator and inhibitor of Wnt/beta-catenin signaling. *Cell Commun. Signal.*, **3**, 12.
35. Fainsod, A., Steinbeisser, H. and De Robertis, E.M. (1994) On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J.*, **13**, 5015–5025.
36. Harland, R.M. (1991) *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol.*, **36**, 685–695.