Journal of Cellular Biochemistry 104:1022–1033 (2008)

Characterization of TBX20 in Human Hearts and Its Regulation by TFAP2

Stefanie Hammer,[1] Martje Toenjes,[1] Martin Lange,[1] Jenny J. Fischer,[1] Ilona Dunkel,[1] Siegrun Mebus,[2] Christina H. Grimm,[1] Roland Hetzer,[2] Felix Berger,[3] and Silke Sperling[1]*

[1]Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany
[2]Department of Cardiac Surgery, German Heart Center, Berlin, Germany
[3]Department of Pediatric Cardiology, German Heart Center, Berlin, Germany

Abstract The T-box family of transcription factors has been shown to have major impact on human development and disease. In animal studies Tbx20 is essential for the development of the atrioventricular channel, the outflow tract and valves, suggesting its potential causative role for the development of Tetralogy of Fallot (TOF) in humans. In the presented study, we analyzed *TBX20* in cardiac biopsies derived from patients with TOF, ventricular septal defects (VSDs) and normal hearts. Mutation analysis did not reveal any disease causing sequence variation, however, *TBX20* is significantly upregulated in tissue samples of patients with TOF, but not VSD. In depth analysis of *TBX20* transcripts lead to the identification of two new exons 3′ to the known *TBX20* message resembling the mouse variant *Tbx20a*, as well as an extended 5′UTR. Functional analysis of the human *TBX20* promoter revealed a 100 bp region that contains strong activating elements. Within this core promoter region we recognized functional binding sites for TFAP2 transcription factors and identified TFAP2 as repressors of the *TBX20* gene in vitro and in vivo. Moreover, decreased *TFAP2C* levels in cardiac biopsies of TOF patients underline the biological significance of the pathway described. In summary, we provide first insights into the regulation of TBX20 and show its potential for human congenital heart diseases. J. Cell. Biochem. 104: 1022–1033, 2008.

Key words: T-box; TBX20; TFAP2; congenital heart disease; gene expression

Congenital heart defects (CHD) account for the largest number of birth defects in human, with an incidence of about eight per 1,000 live births. Nearly 30% of major cardiac malformations are associated with additional developmental abnormalities and result from a recognized chromosomal anomaly or occur as part of a syndrome. Major insights into cardiac development and disease have been gained in studies of animal models, such as mice, chicken, and zebrafish, showing that a complex molecular regulatory network is required to initiate and complete the formation of a functional heart [Cripps and Olson, 2002; Brown et al., 2005]. The transcriptional regulation process seems to play one key role in this process (e.g., *Pitx2*, *Isl1*, *Myocardin*, *Hand2*) [Bruneau, 2002], supported also by knowledge gained from mutation reports of patients (e.g., *NKX2-5*, *ZIC3*, *GATA4*, and *CITED2*) [Schott et al., 1998; Garg et al., 2003; Ware et al., 2004; Sperling et al., 2005]. Tetralogy of Fallot (TOF) is a combination of anatomic abnormalities arising mainly from the maldevelopment of the right ventricular outflow tract. Clinically, TOF is characterized by a subaortic ventricular septal defect (VSD), right ventricular infundibular stenosis, aortic valve overriding the right ventricle and right ventricular hypertrophy. As for the overwhelming majority of CHD, the molecular pathology of TOF is so far still poorly understood and major efforts to identify associated molecular factors are currently undertaken.

S. Hammer and M. Toenjes contributed equally to this study.

Grant sponsor: BMBF; Grant number: # 0313349; Grant sponsor: EU FP6 IP Moltools; Grant number: # 503155; Grant sponsor: EU FP6 IP HeartRepair; Grant number: # 018630.

*Correspondence to: Silke Sperling, Max Planck Institute for Molecular Genetics, Ihnestr. 73, 14195 Berlin, Germany. E-mail: sperling@molgen.mpg.de

Received 16 October 2007; Accepted 7 December 2007

DOI 10.1002/jcb.21686

T-box genes represent a family of transcription factors that share a highly conserved DNA-binding region (called T-box) and are suggested to play a crucial role in the development of CHD in human. Several family members show cardiac expression during early embryogenesis, such as *Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx18*, and *Tbx20* [Plageman and Yutzey, 2005; Stennard and Harvey, 2005]. Deletions of *TBX1* have been shown in individuals with DiGeorge syndrome [Yagi et al., 2003] and mutations or haploinsufficiency of *TBX5* are frequent causes of Holt–Oram syndrome associated with atrial septal defects and first or second degree atrioventricular block [Basson et al., 1997; Li et al., 1997]. Together with *Tbx5*, the T-box transcription factor *Tbx20* is one of the first genes expressed in the vertebrate cardiac lineage showing a conserved expression pattern in cardiac structures from *drosophila* to mammals [Meins et al., 2000; Kraus et al., 2001; Plageman and Yutzey, 2005]. During development *Tbx20* expression becomes gradually enriched in the atrioventricular channel, the outflow tract and the developing right ventricle and valves [Iio et al., 2001; Kraus et al., 2001; Stennard et al., 2003; Plageman and Yutzey, 2004; Takeuchi et al., 2005]. It is essential for the correct formation of these structures as reduced *Tbx20* expression results in abnormal heart morphogenesis in zebrafish and mouse models [Szeto et al., 2002; Brown et al., 2005; Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005]. Mechanistically, Tbx20 interacts with major players in the regulation of cardiac development such as Tbx5, Gata4, Gata5, Isl1, and Nkx2-5, acting as a transcriptional repressor of *Tbx2* or activator of *Mef2C* and *Nkx2-5* [Stennard et al., 2003; Brown et al., 2005; Cai et al., 2005; Singh et al., 2005; Shelton and Yutzey, 2007]. Thus Tbx20 has been recognized as a key component of the genetic network controlling regional identity, proliferation and differentiation within the developing heart in a dose-sensitive manner. Phenotypes of mouse embryos with a mild reduction of TBX20 levels show its role in right ventricular growth and outflow tract development [Takeuchi et al., 2005]. Recently, mutations in the T-box DNA binding domain of *TBX20* have been detected in two families with cardiac pathologies including septation defects and cardiomyopathy [Kirk et al., 2007]. The regulation of *TBX20* and its impact as disease gene for TOF in humans, however, has not been investigated to date.

In the study presented, we analyzed the *TBX20* gene in human and show increased *TBX20* expression levels in atrial and ventricular biopsies from TOF patients compared to patients with isolated VSD and normal human heart samples. Further, we characterized the core promoter of *TBX20* and show that TFAP2 transcription factors are direct repressors of *TBX20* in vitro and in vivo. This might represent a regulatory pathway for *TBX20* upregulation in TOF patients as *TFAP2C* expression levels are decreased in respective samples. No sequence mutations could be observed for *TBX20* or the DNA binding domain of *TFAP2C* in analyzed patients.

MATERIALS AND METHODS

Patient Samples

All cardiac samples were obtained from the German Heart Center during cardiac surgery with ethical approval by the Institutional Review Committee and informed consent of the patients or parents. Biopsies were taken from the right ventricle and atrium of patients with TOF as well as age and sex matched samples from individuals with VSD from the same tissue region. Samples of all four heart chambers were obtained from normal human hearts.

RNA and DNA Isolation and Quantitative Real-Time PCR

Total RNA and genomic DNA of all cardiac tissues were extracted using TRIzol (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. Five micrograms of total RNA was reverse transcribed and real-time PCR carried out using SYBR Green PCR master mix (ABgene, Epsorn, UK) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with primers for *TBX5*, *TBX20* isoforms as well as *TFAP2* genes. The housekeeping genes *ABL*, *B2M*, and *HPRT* were used for normalization as described [Vandesompele et al., 2002].

Mutation Analysis

Genomic DNA extracted from patient heart biopsies was amplified using the GenomiPhi-Kit (Amersham Biosciences, Piscataway, NJ).

All exons and the 700 bp promoter region of *TBX20* as well as exons 4 and 5 of *TFAP2C* were amplified by PCR using Hotstar *Taq* polymerase (Qiagen, Hilden, Germany). Sequences of the primers utilized in this study are available upon request. PCR fragments were sequenced by the Services in Molecular Biology Company (Berlin, Germany).

Plasmid Constructs

Human *TBX20* promoter-luciferase plasmids were generated by cloning the 1,540 bp fragment of the human *TBX20* 5′flanking region between −1,546 and −7 bp relative to the initiation codon into *Kpn*I/*Nhe*I sites of the luciferase reporter gene plasmid *pGL3basic* (Promega, Mannheim, Germany). The resulting full-length promoter–reporter plasmid was denoted as *−1,546-TBX20-Luc*. Sequential deletion constructs were created as indicated in Figure 3. Expression vectors for SP1, TFAP2A, TFAP2B, TFAP2C, and E2F1 were described previously and generously donated by Guntram Suske, Helen Hurst, Ronald J. Weigel, and Joseph R. Nevins [Hagen et al., 1994; Schwarz et al., 1995; Bosher et al., 1996; Bamforth et al., 2001].

Cell Culture, Transfection, and Luciferase Assay

The human cell lines HEK293 and HepG2 as well as C2C12 mouse myoblasts were maintained in DMEM +10% FBS. HL1 mouse cardiomyocytes were obtained from William C. Claycomb and cultured as described [Claycomb et al., 1998]. Cells were transfected using Transfast (Promega) or Dreamfect (Oz Biosciences, Marseille, France) according to manufacturers' instructions. Reporter gene assays for luciferase activity were performed as described previously [Sperling et al., 2005].

5′UTR Mapping

The investigation of the *TBX20* 5′UTR was carried out by PCR using cDNA derived from HEK293 cells. The reverse primer was located in exon 2 (+168 to +188 bp relative to the A of the ATG initiation codon) and a panel of forward primers upstream of the translation start site as indicated in Figure 3b.

Electromobility Shift Assay

Nuclear extracts were prepared from HEK293 cells after transfection with TFAP2C expression plasmid or empty vector. Double-stranded oligonucleotides containing the putative TFAP2 binding sites within the *TBX20* promoter were generated by annealing complementary single-stranded oligonucleotides (cgcccggcccgcggccccgccccccggcggcggaatca) and subsequently end-labeled with digoxygenin-11-ddUTP using the DIG Gel Shift Kit 2nd Generation (Roche Diagnostics, Mannheim, Germany). For binding reactions, 3 μg of nuclear extract and 0.8 ng labeled oligonucleotides were incubated, for competition experiments a 100-fold excess of unlabeled competitor DNAs was added to the mixture. After the binding reaction, samples were subjected to electrophoresis on a 6% TBE DNA Retardation Gel (Novex, Invitrogen) and visualized by autoradiography.

Chromatin-Immunoprecipitation (ChIP)

ChIP experiments were performed on duplicate sets of HL1 cells essentially as described previously [Horak et al., 2002]. Modifications of the assay protocol were as follows: cells were cross-linked for 10 min at 37°C and samples sonified using a Branson 250 Sonifier with 12 pulses at power-setting of 6% and 100% duty-cycle for 30 s and 2 min on ice between pulses. Immunoprecipitation was carried out with magnetic protein A/G beads (Invitrogen) and TFAP2 antibody (#sc-8977, Santa Cruz Biotechnology, Inc., CA) at 5 μg/ml concentration. Enrichment of TFAP2 target sequences over input was quantified by real-time PCR as described above.

RESULTS

Human *TBX20* Splice Variants and Their Expression in Normal Human Hearts

To characterize the human *TBX20* gene in more detail we generated alignments of known murine *Tbx20* transcripts with the human genome. This analysis suggested the potential presence of further *TBX20* splice variants in addition to the annotated human transcript harboring six exons (NM_020417). RT-PCR performed on cDNA from HEK293 cells as well as human myocardium showed expression of exons 7 and 8, homologous to the mouse *Tbx20α* splice variant. We cloned the full-length human *TBX20A* transcript, submitted to Genbank (accession number NM_001077653; Fig. 1a,b).

This novel human isoform contains a region of 150 amino acids C-terminal to the T-box, which

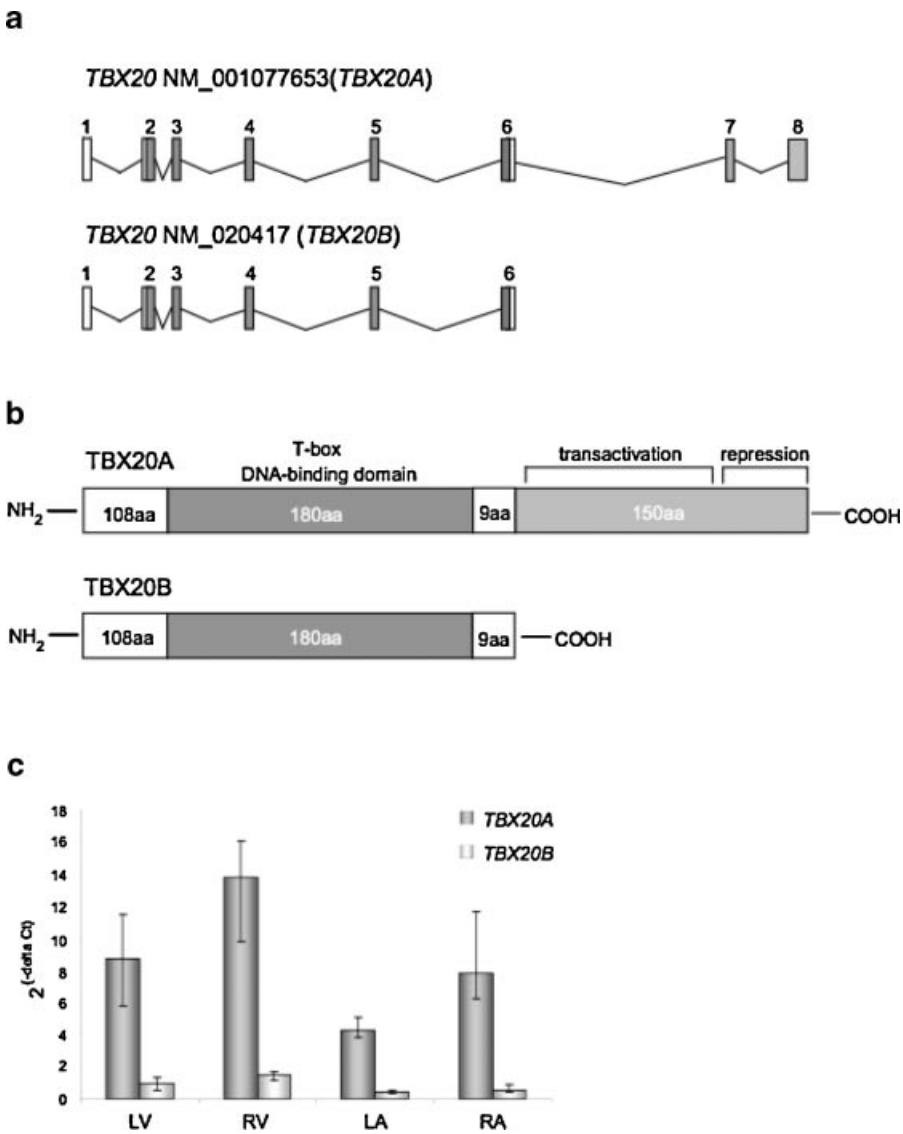

Fig. 1. Structure and expression of human TBX20 isoforms. **a**: Intron/exon structure of human *TBX20* transcript variant A isolated from HEK293 total cDNA compared to known *TBX20B*. Exons are represented as boxes and the position of the 180aa T-box domain is shown in dark gray. Novel exons are depicted in light gray. **b**: Schematic representation (not to scale) of the TBX20 isoforms. Note that variant TBX20A contains an extension harboring transactivation and transrepression domains. **c**: Real-time PCR analysis of *TBX20A* and *TBX20B* splice variants in cDNA derived from normal human heart tissues (n = 4) of left atrium (LA), right atrium (RA), left ventricle (LV), and right ventricle (RV). Results represent median expression levels with 25% and 75% quantile; assays were performed in triplicates.

is predicted to carry strong transactivation and transrepression domains in mice [Stennard et al., 2003]. The corresponding murine *Tbx20a* transcript has been shown to be the most abundant splice variant of *Tbx20* in mouse. In accordance with this quantitative real-time PCR analysis of cDNA derived from normal human heart samples showed a much stronger expression of the *TBX20A* isoform compared to the previously described splice variant in human, which is designated *TBX20B* in the paper presented. Expression profiles of the *TBX20A* and *TBX20B* transcripts were similar in cDNAs from all four chambers of the human heart (Fig. 1c).

Mutational Analysis of *TBX20* in Patients With TOF

To analyze genomic alterations of *TBX20* potentially causative for CHD in human, we screened 23 patients with TOF by sequencing

TABLE I. Mutation Analysis of the *TBX20* Gene in Patients With TOF

dbSNP	Position	Nucleotide variation	Amino acid variation	Mut chr	Total chr	Mut allele freq	Mut allele freq dbSNP
	5′UTR	c.−186T > C		13	46	0.283	
rs336283	Exon 1	c.39T > C	p.Ser13Ser	33	46	0.717	0.735
rs17675148	Intron 3	c.545 + 13A > G		9	38	0.237	0.263

Systematic nomenclature for SNPs (www.hgvs.org) based on GenBank NM_001077653 (*TBX20A* cDNA) and counting +1 as A of the initiation codon. Mut, mutant; chr, chromosome; freq, frequency.

all *TBX20* exons including their flanking intronic regions and 700 bp 5′ of the translation start site, a region potentially containing regulatory elements for *TBX20*. The results from this mutation screen are presented in Table I. We detected two previously known sequence variations showing the same distribution as in the normal population (NCBI dbSNP) and one additional nucleotide variation 5′ to the start codon. Further sequence variations, which are also currently associated with *TBX20* in dbSNP, resulted from amplification of the *TBX20* pseudogene on chromosome 12 that comprises exons 5–8 of *TBX20* on chromosome 7. However, analysis of cDNA demonstrated that the pseudogene is not transcribed suggesting its functional silence and cDNA analysis of *TBX20* showed the absence of the proposed alterations. Homology studies revealed that the mouse genome lacks a *Tbx20* pseudogene.

Increased Cardiac *TBX20* Expression Levels in Patients With TOF

In addition to mutations potentially causing deficient transcription factor activity the regulatory network during cardiac development has been shown to be dependent on the amount of transcription factors present in the corresponding tissue [Cai et al., 2005; Singh et al., 2005; Takeuchi et al., 2005]. Therefore we questioned whether the T-box genes *TBX5* and *TBX20* would be deregulated in biopsies of 13 patients with TOF whose genomic DNA was included in the mutation analysis. A group of 8 samples of normal human hearts served as control and 12 age matched biopsies from patients with isolated VSD. Quantitative real-time PCR displayed a significant upregulation of *TBX20* in TOF samples compared to normal human hearts and VSD samples ($P < 0.005$; Fig. 2a). In contrast, expression levels of *TBX5* were not significantly altered in either group of individuals (Fig. 2b). Next, we analyzed the expression of the different *TBX20* splice variants in representative atrial and ventricular samples of TOF patients compared to normal human hearts. In these samples both *TBX20* isoforms were found to be upregulated compared to normal human hearts ($P < 0.05$ and $P < 0.005$; Fig. 2c). Again, *TBX5* levels did not differ between the groups (data not shown).

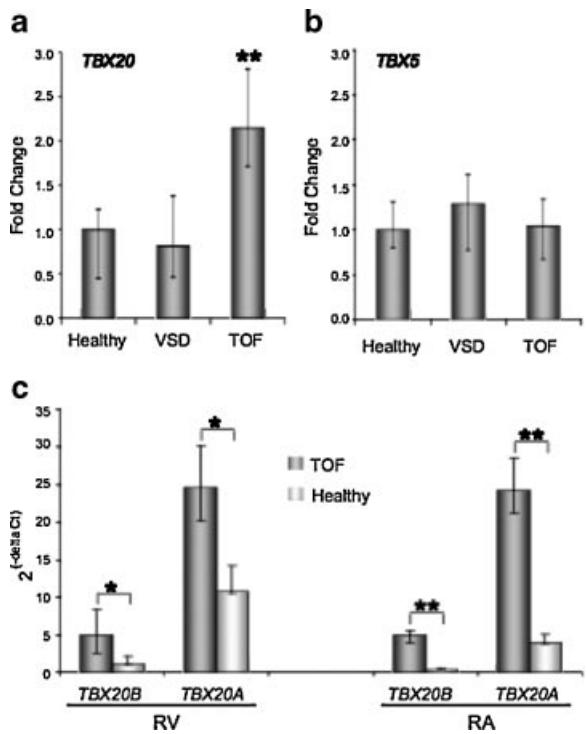

Fig. 2. Overexpression of *TBX20* variants in cardiac samples of patients with TOF. **a**: *TBX20* (both splice forms) and (**b**) *TBX5* mRNA expression levels in right ventricular biopsies of patients with Tetralogy of Fallot (TOF; n = 13), isolated ventricular septal defects (VSDs; n = 12) and normal human hearts (healthy; n = 6) were quantified by real-time PCR. **c**: Expression of *TBX20* splice variants in right ventricular (RV) and right atrial (RA) samples of patients with TOF (n = 4) compared to normal human hearts (n = 4) as determined by real-time PCR. Results represent median expression levels with 25% and 75% quantile. * Indicates statistical significance according to Wilcoxon testing. (*) $P < 0.05$; (**) $P < 0.005$.

Identification of the *TBX20* Core Promoter and 5′UTR

To elucidate the regulatory region of the human *TBX20* gene we cloned a fragment comprising nucleotides −1,546 and −7 relative to the translation start site counting the A of the initiation codon as +1. This region was able to drive expression of a luciferase gene when cloned in a corresponding vector about 30-fold higher compared to the activity of the empty vector after transfection in HEK293 cells (Fig. 3a). To define the minimal promoter region of *TBX20* we generated a series of truncated constructs and characterized the basal activity in HEK293 cells. As shown in Figure 3a, a region between −629 and −527 bp relative to the translational start site is responsible for the major increase in promoter activity, as between −629 and −527 bp the transcriptional activity of the construct decreased sequentially by about five- to sixfold. Similar results were obtained in HepG2 cells as well as C2C12 mouse myoblasts (data not shown), suggesting that major regulatory elements of the *TBX20* gene are located in a region between −629 and −527 bp 5′ of the ATG initiation codon. We therefore suggest that this region represents the *TBX20* core promoter serving as recognition site for the basal transcription apparatus which is typically a 100 bp region flanking the transcriptional start site (TSS). Moreover, our data show that all constructs with inserts containing less than −527 bp exhibit only minor transcriptional activity. This 527 bp region is homologous to the murine *Tbx20* 5′UTR and using primer walking analysis we could also annotate it as the 527 bp long 5′UTR in the human *TBX20* transcripts (Fig. 3b). This TSS maps well with the one proposed by prediction programs (Dragon GSF1.0, Eponine, Mc Promoter, NNPP2.1, Promoter Scan, TSSG and TSSW).

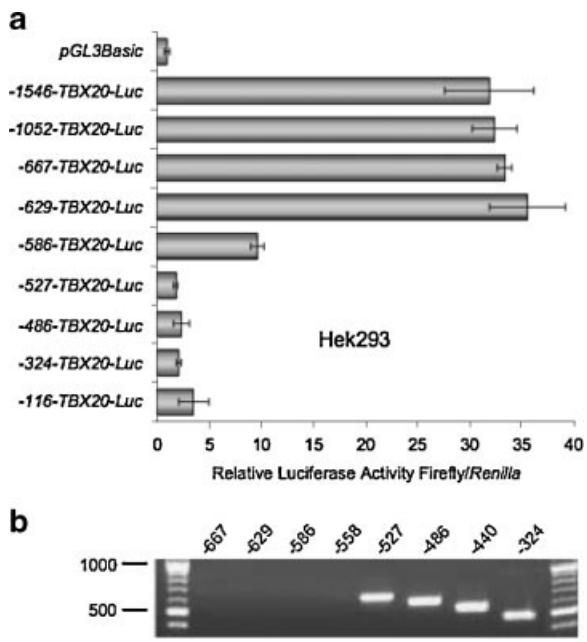

Fig. 3. Identification of the *TBX20* core promoter and 5′UTR. **a**: Luciferase activity assays in HEK293 cells transfected with different *TBX20* promoter constructs. The fragments between −1,546, −1,052, −667, −629, −586, −527, −486, −324, and −116 to −7 relative to the A of the initiation codon of the human *TBX20* gene (NM_001077653) were PCR amplified and cloned into *pGL3basic*. Firefly luciferase activity of the resulting plasmids was normalized to *Renilla* luciferase activity to account for differences in transfection efficiency. The mean luciferase activity of transient transformants is presented as fold change compared to basal activity of the *pGL3basic* vector from one representative experiment performed in triplicates, error bars represent standard deviations. The assays were repeated at least three times independently. **b**: Mapping of the transcriptional start site of human *TBX20* by RT-PCR analysis of cDNA from HEK293 cells with forward primers upstream of the translation start site as indicated.

TFAP2 Isoforms Dose Dependently Downregulate the *TBX20* Promoter

Promoter analysis using TRANSFAC [Matys et al., 2003] revealed that the region identified as the *TBX20* core promoter harbors several GC-boxes that represent potential binding sites for the transcription factors SP1, E2F, and the TFAP2 family (Fig. 4a). Cotransfection of corresponding expression constructs in HEK293 cells with the −667 to −7 bp *TBX20* promoter construct in the presence of empty vector or TFAP2 expression plasmids revealed that TFAP2A, TFAP2B, and TFAP2C significantly downregulate *TBX20* promoter activity by about threefold. In contrast cotransfection with expression constructs for transcription factors SP1 and E2F had no effect on the luciferase level (Fig. 4b). The repressive effects of all three TFAP2 isoforms showed dose-dependency (Fig. 4c and data not shown).

TFAP2-Response Elements Drive Promoter Activity In Vitro and In Vivo

To investigate the impact of putative TFAP2 binding sites on promoter regulation we transfected HEK293 cells with different *TBX20* promoter constructs in the presence or absence

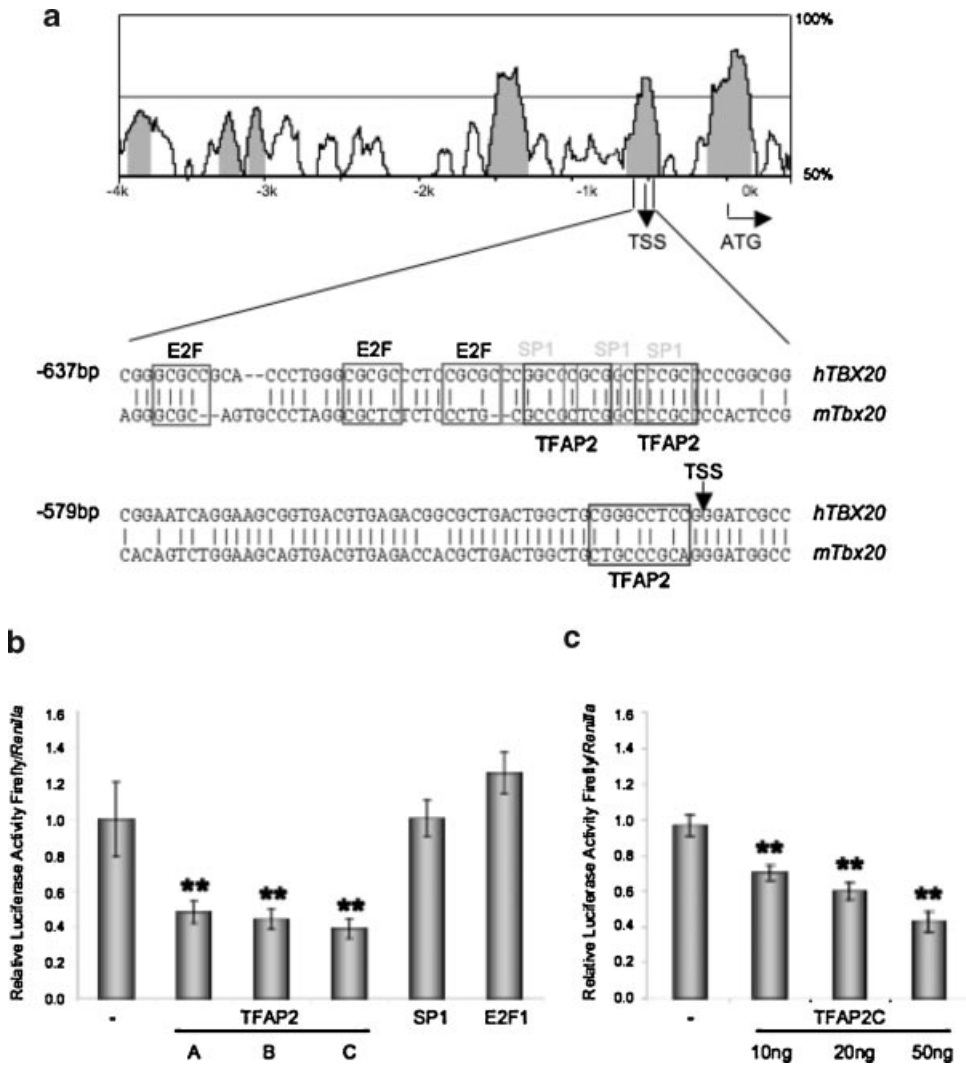

Fig. 4. Transcription factor binding sites and regulation of the *TBX20* core promoter. **a**: Alignment of the human and mouse *TBX20* 5′flanking sequence generated by mVISTA (http://genome.lbl.gov/vista/index.shtml) and potential transcription factor binding sites identified by TRANSFAC [Matys et al., 2003]. Predicted transcription factor binding sites for E2F, SP1, and TFAP2 in the putative core promoter are boxed. **b**,**c**: Regulation of the *TBX20* promoter by various transcription factors. HEK293 cells were transfected with expression vectors for the transcription factors as indicated or corresponding empty vectors. Normalized mean luciferase activity is shown compared to unstimulated activity of the −667 to −7 bp construct set as one.

of TFAP2 expression plasmids. TFAP2 isoforms repressed transcriptional activity of the −667 to −7 bp and −629 to −7 bp promoter constructs by two- to threefold, in contrast no effects could be observed when cotransfecting TFAP2A or TFAP2C to the −586 to −7 bp and −527 to −7 bp promoter construct (Fig. 5a and data not shown). These results suggest the functionality of a repressive TFAP2 binding site between 629 and 586 bp upstream of the *TBX20* initiation codon. To test whether TFAP2 binds to those sites in vitro, we performed gel shift assays of nuclear extracts from HEK293 cells transfected with TFAP2 expression plasmids and oligonucleotides representing the potential binding sites between −629 and −586 bp of the *TBX20* promoter. Figure 5b shows binding with nuclear extracts from TFAP2 transfected cells, whereas there is no signal in the non-transfected cells. In the presence of a 100-fold molar excess of competitor oligonucleotides, complexes of the labeled DNA fragments with TFAP2 were abolished. ChIP experiments in cultured HL1 cells showed about 20-fold enrichment of the corresponding *TBX20* core promoter in samples after precipitation of cross-linked chromatin with TFAP2 antibodies compared to unrelated promoter regions (Fig. 5c). Thus, TFAP2 binds

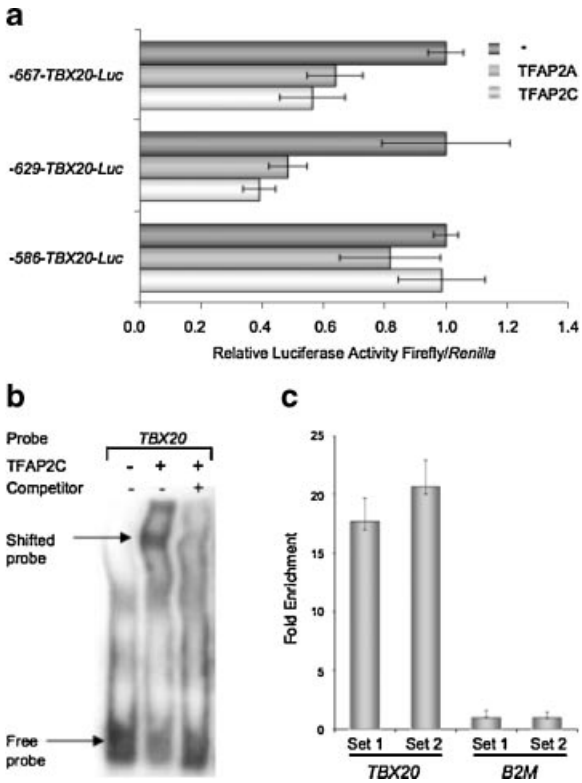

Fig. 5. Identification of functional TFAP2 binding sites in the *TBX20* core promoter. **a**: Effects of serial deletions of the region harboring TFAP2 binding sites on promoter regulation by TFAP2C. HEK293 cells were transfected with different *TBX20* promoter constructs as indicated in the presence or absence of TFAP2A and TFAP2C expression vectors. Normalized mean luciferase activities are shown with the luciferase activity of the corresponding unstimulated promoter constructs set to one. **b**: Electrophoretic mobility shift assay with nuclear extracts from HEK293 cells transfected with TFAP2 expression constructs or empty vector and end-labeled oligonucleotide probes containing potential TFAP2 binding sites in the presence or absence of a 100-fold excess of unlabeled oligonucleotides. **c**: Chromatin-immunoprecipitation analysis of HL1 cell extracts immunoprecipitated with TFAP2 antibody in replicates (set 1, set 2). Bound DNA was detected using real-time PCR analysis targeting the *TBX20* core promoter primers and an unrelated negative control (*B2M*).

to these regulatory elements in the *TBX20* promoter in cardiac cells in vivo.

Decreased Expression Levels of *TFAP2C* in Biopsies of Patients With TOF

To strengthen the biological relevance of TFAP2 regulation of *TBX20* we assessed mRNA levels of *TFAP2* genes in human heart samples. *TFAP2A* and *C* mRNA was present in atrial and ventricular samples, while *TFAP2B* mRNA was not detectable by real-time PCR. Interestingly, we found that *TFAP2C* was significantly downregulated in tissue samples of patients with TOF compared to normal human hearts ($P < 0.005$; Fig. 6a) and patients with VSD ($P < 0.05$), providing a possible explanation for the overexpression of *TBX20*. In contrast, expression levels of *TFAP2A* were unchanged (Fig. 6b). Mutation analysis did not show any structural alterations of the TFAP2C DNA binding domain (data not shown) suggesting that again deregulation rather than mutation is more likely to be responsible for *TBX20* overexpression in TOF patients.

DISCUSSION

Splice Variants and Sequence Variations of *TBX20* in Human

The T-box genes *TBX5* and *TBX1* have long been known as disease genes for human CHD. In addition to these two family members, *TBX20* represents a key regulator of embryogenesis and particularly early cardiac development. Lack of Tbx20 leads to various cardiac malformations in animal models such as outflow tract defects and malformed valves and a disturbed expression pattern of a number of other key cardiac transcription factors [Brown et al., 2005; Cai et al., 2005; Singh et al., 2005; Takeuchi et al., 2005]. Moreover, in a recent study mutations in the T-box DNA binding domain of *TBX20* were linked to cardiomyopathy and cardiac septation defects in human

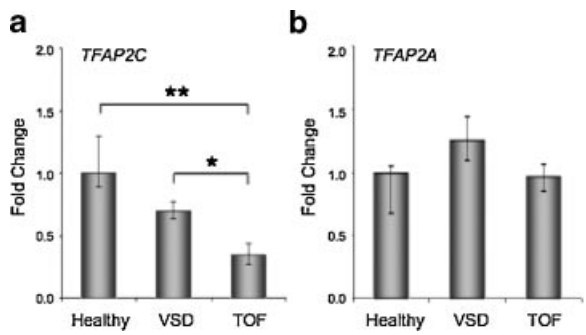

Fig. 6. Decreased expression levels of *TFAP2C* in cardiac samples of patients with TOF. **a**: *TFAP2C* and (**b**) *TFAP2A* mRNA expression levels in right ventricular biopsies of patients with Tetralogy of Fallot (TOF; n = 13), isolated ventricular septal defects (VSDs; n = 12) and normal human hearts (healthy; n = 6) were quantified by real-time PCR. Results represent median expression levels with 25% and 75% quantile. Results are shown in relation to the expression levels of the healthy human heart samples. * Indicates statistical significance according to Wilcoxon testing. (*) $P < 0.05$; (**) $P < 0.005$.

[Kirk et al., 2007]. However, the presence of different *TBX20* transcripts in human as well as sequence variations and expression levels of *TBX20* in patients with TOF have not been investigated before. Here, we discovered the presence of a human *TBX20A* splice variant homologous to the murine *Tbx20a* transcript representing the major transcript in both species. The newly discovered human TBX20A comprises C-terminal to the T-box the transactivation and transrepression domains, which are potentially of major impact for the transcriptional activity of TBX20. In contrast to the preferential expression of *Tbx20* transcripts in distinct cardiac regions during cardiac development in mouse, the human *TBX20* splice variants are equally expressed in human left and right atrial and ventricular samples of normal adult hearts.

The cardiac malformations observed in mouse models lacking Tbx20 proposed a potential primarily causative impact of TBX20 on the development of TOF in human. However, in 23 patients studied we could not identify any amino acid changing mutation. This suggests that mutations of TBX20 are not common in humans live births or they may be associated with other CHD not studied [Kirk et al., 2007]. Two sequence variations present in the dbSNP database (www.hgvs.org) could be confirmed at equal frequencies compared to the normal population. One novel nucleotide exchange was discovered within the 5′UTR. Interestingly in contrast to mouse, the human genome harbors a *TBX20* pseudogene on chromosome 12 including exons 5–8 of the *TBX20* transcript. This has to be considered when genotyping *TBX20* DNA as many *TBX20* sequence variations listed in dbSNP arise from the non-transcribed pseudogene.

Expression of *TBX20* in Human Right Ventricular Samples

Various results from mouse studies have revealed the impact of Tbx20 as a key regulator of transcriptional networks in cardiac development. Thereby, the level of transcription factors plays an important role and is tightly regulated. However, the expression levels of transcription factors in human heart development and malformed hearts are still largely unknown. A previous study on gene expression in malformed human hearts [Kaynak et al., 2003] demonstrated disease specific molecular portraits, with a higher number of genes being upregulated in TOF patients compared to individuals with VSD. This analysis, however, did not include all transcription factors known to play a role in cardiac development. Here we determined the expression levels of the T-box transcription factors *TBX20* and *TBX5* using quantitative real-time PCR in cDNAs derived from human heart tissue samples showing elevated expression of *TBX20* in patients with TOF. In contrast, levels of *TBX5* were not altered in either of the groups.

In depth analysis of *TBX20* transcripts in human revealed that both human isoforms, namely *TBX20A* and *TBX20B* are overexpressed in patients with TOF. This upregulation could be detected in atrial and ventricular samples pointing to a general deregulation in TOF rather than adaptation processes related to cardiac pressure overload and altered hemodynamic features in the ventricle. Thus, the altered expression level of *TBX20* may have a potential impact on the development of TOF in human and we further investigated the upstream regulatory cascade of *TBX20*.

Regulation of the *TBX20* Gene

So far biochemical and animal studies have investigated the regulation of potential target genes of Tbx20 and its interactions with other cardiac transcription factors. The regulation of the *Tbx20* gene itself, however, is largely unknown to date. The only described signaling molecule upstream of *Tbx20* is Bmp2, as cultured chicken embryo explants display overexpression of *Tbx20* in its presence [Plageman and Yutzey, 2004]. Here, we were able to identify a fragment between −629 and −527 bp upstream of the translation start site of *TBX20* that is responsible for 95% of the transcriptional activity resulting from the *TBX20* locus. In accordance to this, we discovered an extended 5′UTR for the *TBX20* transcripts of 527 bp. Therefore the mapped transcriptionally active region is about 100 bp upstream of the TSS and represents the *TBX20* core promoter. Its sequence is highly conserved between mice and human and contains a GC rich region, harboring potential binding sites for the transcription factors TFAP2 and SP1 as well as E2F. We show that all three isoforms of TFAP2, namely TFAP2A, TFAP2B, and TFAP2C repress the *TBX20* promoter by two- to threefold, whereas SP1 and E2F do not alter *TBX20* promoter

activity. In addition, TFAP2 transcription factors are able to bind to the *TBX20* promoter in vitro and in vivo.

Members of the TFAP2 family share a homologous C-terminal helix-span-helix domain responsible for dimerization and DNA-binding and a proline-glutamine rich transactivation domain at the N-terminus [Eckert et al., 2005]. Interestingly, the three TFAP2 family members shown to regulate *TBX20* are expressed in the neural crest during development [Chazaud et al., 1996; Moser et al., 1997]. This region contributes to cardiogenesis as progenitor cells from the cardiac neural crest migrate into the developing heart and participate in septation and outflow tract morphogenesis [Harvey, 2002]. Moreover, TFAP2A and TFAP2B have been associated with CHD. Knock-in mice with functionally deficient *Tfap2a* display cardiac malformations in addition to failing neural tube closure and craniofacial defects [Brewer et al., 2002]. The observed cardiac malformations include a panel of defects associated with perturbed outflow tract formation such as double outlet right ventricle, persistant truncus arteriosus, TOF and severe pulmonary stenosis. In contrast, mutations of *TFAP2B* leading to haploinsufficiency or a dominant negative form of the TFAP2B protein have been associated with Char syndrome in humans, characterized by persistant ductus arteriosus, facial dysmorphism and skeletal abnormalities of the hand [Satoda et al., 2000]. These findings illustrate the role of the *TFAP2* gene family in cardiac morphogenesis, mainly outflow tract formation and cardiac septation, by controlling cell proliferation and terminal differentiation [Eckert et al., 2005; Hutson and Kirby, 2007].

The TFAP2C family member so far has not been implicated in CHD, however, recent studies in zebrafish embryos showed redundant activities of Tfap2a and Tfap2c in neural crest development [Li and Cornell, 2007]. Results presented in this study suggest that overexpression of *TBX20* in TOF patients may result from lack of repression by TFAP2C. Whereas mutational analysis did not show any structural alterations of the TFAP2C DNA binding domain or its cofactor CITED2, a known causative factor for CHD [Schott et al., 1998; Garg et al., 2003; Ware et al., 2004; Sperling et al., 2005], gene expression analysis demonstrated downregulation of *TFAP2C* mRNA in cardiac biopsies from TOF patients.

To summarize, the present study reveals that mutations in TBX20 and the DNA binding domain of TFAP2C are unlikely to be a major cause of TOF or VSD in human. In contrast, we show that TBX20, a key transcription factor for chamber specific cell differentiation, is overexpressed in TOF patients. Our expression profiling and functional analysis support a role of TFAP2C as a direct transcriptional regulator of *TBX20* which adds another piece to the transcriptional network important for cardiac development. Animal studies, however, have not yet addressed the consequences of *TBX20* gain of function. These experiments will demonstrate whether elevated levels of *TBX20* alone can mirror the cardiac malformations seen in TOF patients and explain how the cardiac transcriptional network is influenced by *TBX20* overexpression.

ACKNOWLEDGMENTS

We are deeply grateful to the patients and families for their cooperation. This work has been supported by the European Community's Sixth Framework Programme contract ('Heart-Repair') LSHM-CT-2005-018630.

REFERENCES

Bamforth SD, Braganca J, Eloranta JJ, Murdoch JN, Marques FI, Kranc KR, Farza H, Henderson DJ, Hurst HC, Bhattacharya S. 2001. Cardiac malformations, adrenal agenesis, neural crest defects and exencephaly in mice lacking Cited2, a new Tfap2 co-activator. Nat Genet 29:469–474.

Basson CT, Bachinsky DR, Lin RC, Levi T, Elkins JA, Soults J, Grayzel D, Kroumpouzou E, Traill TA, Leblanc-Straceski J, Renault B, Kucherlapati R, Seidman JG, Seidman CE. 1997. Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. Nat Genet 15:30–35.

Bosher JM, Totty NF, Hsuan JJ, Williams T, Hurst HC. 1996. A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. Oncogene 13:1701–1707.

Brewer S, Jiang X, Donaldson S, Williams T, Sucov HM. 2002. Requirement for AP-2alpha in cardiac outflow tract morphogenesis. Mech Dev 110:139–149.

Brown DD, Martz SN, Binder O, Goetz SC, Price BM, Smith JC, Conlon FL. 2005. Tbx5 and Tbx20 act synergistically to control vertebrate heart morphogenesis. Development 132:553–563.

Bruneau BG. 2002. Transcriptional regulation of vertebrate cardiac morphogenesis. Circ Res 90:509–519.

Cai CL, Zhou W, Yang L, Bu L, Qyang Y, Zhang X, Li X, Rosenfeld MG, Chen J, Evans S. 2005. T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis. Development 132: 2475–2487.

Chazaud C, Oulad-Abdelghani M, Bouillet P, Decimo D, Chambon P, Dolle P. 1996. AP-2.2, a novel gene related to AP-2, is expressed in the forebrain, limbs and face during mouse embryogenesis. Mech Dev 54:83–94.

Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ, Jr. 1998. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci USA 95:2979–2984.

Cripps RM, Olson EN. 2002. Control of cardiac development by an evolutionarily conserved transcriptional network. Dev Biol 246:14–28.

Eckert D, Buhl S, Weber S, Jager R, Schorle H. 2005. The AP-2 family of transcription factors. Genome Biol 6:246.

Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, Rothrock CR, Eapen RS, Hirayama-Yamada K, Joo K, Matsuoka R, Cohen JC, Srivastava D. 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424:443–447.

Hagen G, Muller S, Beato M, Suske G. 1994. Sp1-mediated transcriptional activation is repressed by Sp3. EMBO J 13:3843–3851.

Harvey RP. 2002. Patterning the vertebrate heart. Nat Rev Genet 3:544–556.

Horak CE, Mahajan MC, Luscombe NM, Gerstein M, Weissman SM, Snyder M. 2002. GATA-1 binding sites mapped in the beta-globin locus by using mammalian chIp-chip analysis. Proc Natl Acad Sci USA 99:2924–2929.

Hutson MR, Kirby ML. 2007. Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. Semin Cell Dev Biol 18: 101–110.

Iio A, Koide M, Hidaka K, Morisaki T. 2001. Expression pattern of novel chick T-box gene, Tbx20. Dev Genes Evol 211:559–562.

Kaynak B, von Heydebreck A, Mebus S, Seelow D, Hennig S, Vogel J, Sperling HP, Pregla R, Alexi-Meskishvili V, Hetzer R, Lange PE, Vingron M, Lehrach H, Sperling S. 2003. Genome-wide array analysis of normal and malformed human hearts. Circulation 107:2467–2474.

Kirk EP, Sunde M, Costa MW, Rankin SA, Wolstein O, Castro ML, Butler TL, Hyun C, Guo G, Otway R, Mackay JP, Waddell LB, Cole AD, Hayward C, Keogh A, Macdonald P, Griffiths L, Fatkin D, Sholler GF, Zorn AM, Feneley MP, Winlaw DS, Harvey RP. 2007. Mutations in cardiac T-box factor gene TBX20 are associated with diverse cardiac pathologies, including defects of septation and valvulogenesis and cardiomyopathy. Am J Hum Genet 81:280–291.

Kraus F, Haenig B, Kispert A. 2001. Cloning and expression analysis of the mouse T-box gene tbx20. Mech Dev 100:87–91.

Li W, Cornell RA. 2007. Redundant activities of Tfap2a and Tfap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos. Dev Biol 304:338–354.

Li QY, Newbury-Ecob RA, Terrett JA, Wilson DI, Curtis AR, Yi CH, Gebuhr T, Bullen PJ, Robson SC, Strachan T, Bonnet D, Lyonnet S, Young ID, Raeburn JA, Buckler AJ, Law DJ, Brook JD. 1997. Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. Nat Genet 15:21–29.

Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R, Hornischer K, Karas D, Kel AE, Kel-Margoulis OV, Kloos DU, Land S, Lewicki-Potapov B, Michael H, Munch R, Reuter I, Rotert S, Saxel H, Scheer M, Thiele S, Wingender E. 2003. TRANSFAC: Transcriptional regulation, from patterns to profiles. Nucleic Acids Res 31: 374–378.

Meins M, Henderson DJ, Bhattacharya SS, Sowden JC. 2000. Characterization of the human TBX20 gene, a new member of the T-Box gene family closely related to the Drosophila H15 gene. Genomics 67:317–332.

Moser M, Ruschoff J, Buettner R. 1997. Comparative analysis of AP-2 alpha and AP-2 beta gene expression during murine embryogenesis. Dev Dyn 208:115–124.

Plageman TF, Jr., Yutzey KE. 2004. Differential expression and function of Tbx5 and Tbx20 in cardiac development. J Biol Chem 279:19026–19034.

Plageman TF, Jr., Yutzey KE. 2005. T-box genes and heart development: Putting the "T" in heart. Dev Dyn 232: 11–20.

Satoda M, Zhao F, Diaz GA, Burn J, Goodship J, Davidson HR, Pierpont ME, Gelb BD. 2000. Mutations in TFAP2B cause Char syndrome, a familial form of patent ductus arteriosus. Nat Genet 25:42–46.

Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE, Seidman JG. 1998. Congenital heart disease caused by mutations in the transcription factor NK X2-5. Science 281:108–111.

Schwarz JK, Bassing CH, Kovesdi I, Datto MB, Blazing M, George S, Wang XF, Nevins JR. 1995. Expression of the E2F1 transcription factor overcomes type beta transforming growth factor-mediated growth suppression. Proc Natl Acad Sci USA 92:483–487.

Shelton EL, Yutzey KE. 2007. Tbx20 regulation of endocardial cushion cell proliferation and extracellular matrix gene expression. Dev Biol 302:376–388.

Singh MK, Christoffels VM, Dias JM, Trowe MO, Petry M, Schuster-Gossler K, Burger A, Ericson J, Kispert A. 2005. Tbx20 is essential for cardiac chamber differentiation and repression of Tbx2. Development 132: 2697–2707.

Sperling S, Grimm CH, Dunkel I, Mebus S, Sperling HP, Ebner A, Galli R, Lehrach H, Fusch C, Berger F, Hammer S. 2005. Identification and functional analysis of CITED2 mutations in patients with congenital heart defects. Hum Mutat 26:575–582.

Stennard FA, Harvey RP. 2005. T-box transcription factors and their roles in regulatory hierarchies in the developing heart. Development 132:4897–4910.

Stennard FA, Costa MW, Elliott DA, Rankin S, Haast SJ, Lai D, McDonald LP, Niederreither K, Dolle P, Bruneau BG, Zorn AM, Harvey RP. 2003. Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. Dev Biol 262:206–224.

Stennard FA, Costa MW, Lai D, Biben C, Furtado MB, Solloway MJ, McCulley DJ, Leimena C, Preis JI, Dunwoodie SL, Elliott DE, Prall OW, Black BL, Fatkin D, Harvey RP. 2005. Murine T-box transcription factor Tbx20 acts as a repressor during heart development, and is essential for adult heart integrity, function and adaptation. Development 132:2451–2462.

Szeto DP, Griffin KJ, Kimelman D. 2002. HrT is required for cardiovascular development in zebrafish. Development 129:5093–5101.

Takeuchi JK, Mileikovskaia M, Koshiba-Takeuchi K, Heidt AB, Mori AD, Arruda EP, Gertsenstein M, Georges R, Davidson L, Mo R, Hui CC, Henkelman RM, Nemer M, Black BL, Nagy A, Bruneau BG. 2005. Tbx20 dose-dependently regulates transcription factor networks required for mouse heart and motoneuron development. Development 132:2463–2474.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

Ware SM, Peng J, Zhu L, Fernbach S, Colicos S, Casey B, Towbin J, Belmont JW. 2004. Identification and functional analysis of ZIC3 mutations in heterotaxy and related congenital heart defects. Am J Hum Genet 74: 93–105.

Yagi H, Furutani Y, Hamada H, Sasaki T, Asakawa S, Minoshima S, Ichida F, Joo K, Kimura M, Imamura S, Kamatani N, Momma K, Takao A, Nakazawa M, Shimizu N, Matsuoka R. 2003. Role of TBX1 in human del22q11.2 syndrome. Lancet 362:1366–1373.