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# Variants in a *cis*-regulatory element of *TBX1* in conotruncal heart defect patients impair GATA6-mediated transactivation

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## Abstract

**Background:** *TBX1* (T-box transcription factor 1) is a major candidate gene that likely contributes to the etiology of velo-cardio-facial syndrome/DiGeorge syndrome (VCFS/DGS). Although the haploinsufficiency of *TBX1* in both mice and humans results in congenital cardiac malformations, little has been elucidated about its upstream regulation. We aimed to explore the transcriptional regulation and dysregulation of *TBX1*.

**Methods:** Different *TBX1* promoter reporters were constructed. Luciferase assays and electrophoretic mobility shift assays (EMSAs) were used to identify a *cis*-regulatory element within the *TBX1* promoter region and its *trans*-acting factor. The expression of proteins was identified by immunohistochemistry and immunofluorescence. Variants in the *cis*-regulatory element were screened in conotruncal defect (CTD) patients. In vitro functional assays were performed to show the effects of the variants found in CTD patients on the transactivation of *TBX1*.

**Results:** We identified a *cis*-regulatory element within intron 1 of *TBX1* that was found to be responsive to GATA6 (GATA binding protein 6), a transcription factor crucial for cardiogenesis. The expression patterns of GATA6 and *TBX1* overlapped in the pharyngeal arches of human embryos. Transfection experiments and EMSA indicated that GATA6 could activate the transcription of *TBX1* by directly binding with its GATA *cis*-regulatory element in vitro. Furthermore, sequencing analyses of 195 sporadic CTD patients without the 22q11.2 deletion or duplication identified 3 variants (NC\_000022.11:g.19756832C>G, NC\_000022.11:g.19756845C>T, and NC\_000022.11:g.19756902G>T) in the non-coding *cis*-regulatory element of *TBX1*. Luciferase assays showed that all 3 variants led to reduced transcription of *TBX1* when incubated with GATA6.

**Conclusions:** Our findings showed that *TBX1* might be a direct transcriptional target of GATA6, and variants in the non-coding *cis*-regulatory element of *TBX1* disrupted GATA6-mediated transactivation.

**Keywords:** *TBX1*, GATA6, *Cis*-regulatory element, Transcription, Pharyngeal arches, Conotruncal defect, Variant

## Background

*TBX1*, encoding a T-box transcription factor, is located within the 1.5 Mb deletion of chromosome 22q11.2 [1–3]. *TBX1* is considered to be a major candidate gene of 22q11.2 deletion syndrome (22q11.2DS) [1–5], and point mutations or indels of *TBX1* were also identified in individuals with 22q11.2DS-like phenotypes [6–11]. *Tbx1* is expressed in the pharyngeal arches and in the cardiac progenitors of the second heart field (SHF) [12–14], which gives rise to the outflow tract (OFT), right

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ventricle, and right and left atria [15–20]. Both the loss-of-function (LOF) and gain-of-function (GOF) of *Tbx1* in mice results in congenital heart malformations, such as tetralogy of Fallot (TOF), double outlet right ventricle (DORV), and ventricular septal defect (VSD) [1, 4, 21–23].

Most research has focused on the *TBX1* downstream transcription factors in cardiovascular development, such as *Isl1*, *Tbx5*, *Pax9*, *Fgf8*, *Fgf10*, *Pitx2* and *Mef2c* [13, 14, 24–29]. Genetic studies indicate that *TBX1* is a dosage-dependent transcriptional regulator [30, 31], but the mechanisms of *TBX1* gene upstream regulation remain largely unexplored. Previous studies have shown that *TBX1* is regulated by sonic hedgehog (Shh) signaling [32, 33], vascular endothelial growth factor (VEGF) [34] and Ripply3 [35] or has a feedback loop with retinoic acid (RA) signaling [36], which are involved in pharyngeal arch development. However, how these signals affect the transcriptional regulation of *TBX1* remains unclear. Only Forkhead proteins have been reported to regulate the transcription of *Tbx1* directly [27, 33, 37], concerning that *Tbx1* is regulated by Shh signaling cascade through a Fox-binding site upstream of *Tbx1* in the pharyngeal endoderm [33]. *Cis*-regulatory elements such as enhancers and promoters are crucial for regulating gene expression [38, 39], and cardiac *cis*-regulatory elements functions are activated by a large number of both commonly expressed and cardiac-specific transcription factors, such as *SNAI3*, *NKX2-5*, *GATA4*, *GATA6*, *SOX9*, and *NFATC1* [40]. Genome-wide association studies (GWAS) suggest that genetic variants associated with the risk of cardiovascular disease are enriched within

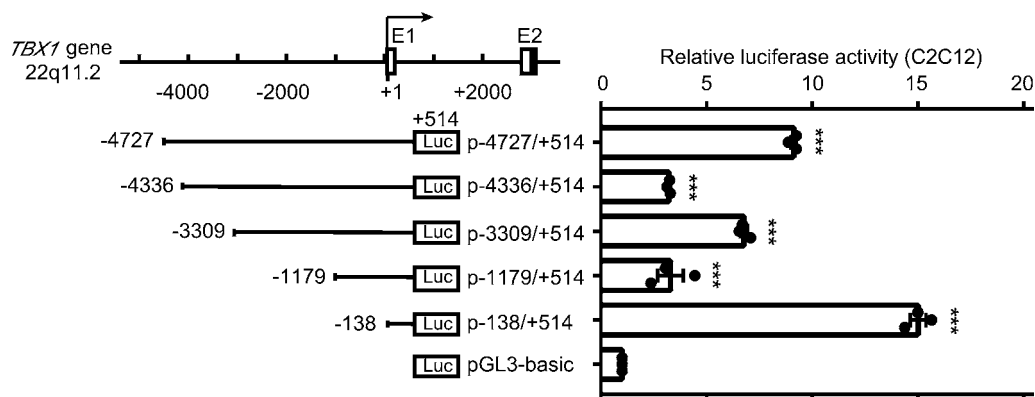
annotated candidate *cis*-regulatory elements (cCREs) [41, 42]). The study of the *cis*-regulatory elements of *TBX1* will help to further understand the role of *TBX1* in the occurrence of CTD.

In this study, we found that *GATA6* could positively activate *TBX1* transcription by directly binding to the *cis*-regulatory element within the *TBX1* promoter region, which contains *GATA* binding sites. We demonstrated that the expression patterns of *TBX1* and *GATA6* overlapped in the pharyngeal arches of human embryos. In addition, we identified 3 rare *TBX1* mutations within the *cis*-regulatory element in sporadic CTD patients without mutations in the coding region of a known CTD pathogenic gene and 22q11.2 deletions. Luciferase assays showed that these variants impaired the *GATA6*-mediated transcriptional activation of *TBX1*. These results provide novel insight into the molecular mechanisms associated with *TBX1* during the pathogenesis of CTD.

## Results

### Identification of a novel *cis*-regulatory element in human *TBX1* adjacent to the 5' flanking region

To identify potential *cis*-regulatory elements controlling *TBX1* transcription, a series of truncated human *TBX1* 5'-flanking regions were cloned into luciferase reporter vectors (Fig. 1). The subsequent dual-luciferase reporter analysis of these fragments showed that p-138/+514, which is located on chromosome 22: 19756565–19757216, significantly increased transcriptional activity by nearly 15-fold compared with the control vector in the C2C12 cell line (Fig. 1); and similar results were obtained in the NIH/3T3 cell line (Additional file 1: Fig. S1). This



**Fig. 1** Deletion analysis identifies a 0.65-kb region around the *TBX1* TSS essential for transcriptional activity in the C2C12 cell line. (Upper left), genomic organization of the 5' flanking region of human *TBX1*. The boxes show exons: blank box, non-coding exon; dark box, coding exon; E1, exon 1; E2, exon 2. Numbering shows the position relative to the TSS of the *TBX1* gene at g.19756703 (+1) of NC\_000022.11. (Bottom left), schematic representation of 5' truncated luciferase constructs. (Right), deletion analysis of the 5' human *TBX1* flanking region. Relative luciferase activity of different 5'-serially deleted *TBX1* reporter constructs transfected in C2C12 cells, which were normalized to Renilla and represented as the fold increase when compared to the pGL3-basic vector. Data are shown as mean  $\pm$  SEM, statistical significance was calculated by one-way ANOVA with Dunnett's post hoc test, n = 3 independent experiments, \*\*\* $p < 0.001$  vs pGL3-basic

suggests that the *TBX1*-138/+514 region around the *TBX1* transcription start site (TSS) may be a *cis*-regulatory element, resulting in increased reporter expression.

We performed predictive analysis of cCREs around the *TBX1* TSS (Additional file 1: Fig. S2) with SCREEN (<http://screen.encodeproject.org>) [38], which is a web interface for searching and visualizing the registry of cCREs derived from ENCODE (Encyclopedia of DNA Elements) data (<https://www.encodeproject.org/>) [38, 43]. There is a cCRE (ENCODE Accession: EH38E2149905) possessing high DNase signals and high H3K4me3 signals, which is annotated as a proximal promoter-like signature (PLS). This cCRE-PLS genome browser was obtained from the UCSC Genome Bioinformatics Site (<http://genome.cse.ucsc.edu/>) [44], located on chromosome 22: 19756591–19756937 (Additional file 1: Fig. S2), and the *TBX1*-112/+235 region relative to the TSS, which is contained in the *TBX1*-138/+514 region.

#### **GATA6 significantly improved the promoter activity of *TBX1* by binding with a *cis*-regulatory element within the *TBX1* promoter**

Cardiac *cis*-regulatory elements play important roles during transcriptional regulation by recruiting cardiac-specific transcription factors [40]. Therefore, to identify specific transcription factor binding sites within *TBX1*-138/+514, a non-exhaustive search for predicted candidate transcription factor binding sites was performed using JASPAR (<http://jaspar.genereg.net/>) [45], and the results showed that this region contained six GATA motifs, three NKX2-5 motifs and one serine response factor (SRF) motif (Fig. 2a). GATA4 and GATA6, two highly conserved transcription factors, are primarily expressed in the myocardium [46, 47], and are capable of binding to the same predicted GATA-binding sites [48–50]. We cotransfected the p-138/+514 reporter gene with individual expression vectors containing GATA4, GATA6, NKX2-5 or SRF in the C2C12 and NIH/3T3 cell lines (Fig. 2b). The results showed that GATA6 significantly increased luciferase activity by nearly sixfold compared with the control pcDNA3.1 vector, NKX2-5 slightly increased luciferase activity by only twofold, and GATA4 and SRF had no effects on luciferase activity (Fig. 2b).

Since *TBX1*-967/+185 [51] and *TBX1*-577/+439 [52] were used as promoters in previous literature, we used *TBX1*-1179/+514 as the *TBX1* promoter to test whether *TBX1*-1179/+514 could be activated by GATA6 as *TBX1*-138/+514. Similar results showing that GATA6 significantly increased *TBX1* promoter activity in the C2C12 and NIH/3T3 cell lines were acquired (Fig. 2c). Moreover, we compared the activation effects of GATA6 on *TBX1*-1179/-1 and *TBX1*-1179/+514. The GATA6 expression vector and the corresponding reporter genes

p-1179/-1 or p-1179/+514 were cotransfected into the C2C12 (Fig. 2d) and NIH/3T3 cell lines (Fig. 2e). We found that the overexpression of GATA6 significantly increased the luciferase activity of p-1179/+514 compared with that of the control pcDNA3.1 vector but had almost no effect on p-1179/-1 (Fig. 2d, e), which further suggests that GATA6 regulates *TBX1* promoter activity by binding with the *TBX1*+1/+514.

#### **Endogenous overlapping expression of GATA6 and *TBX1* in the pharyngeal arches of human embryos**

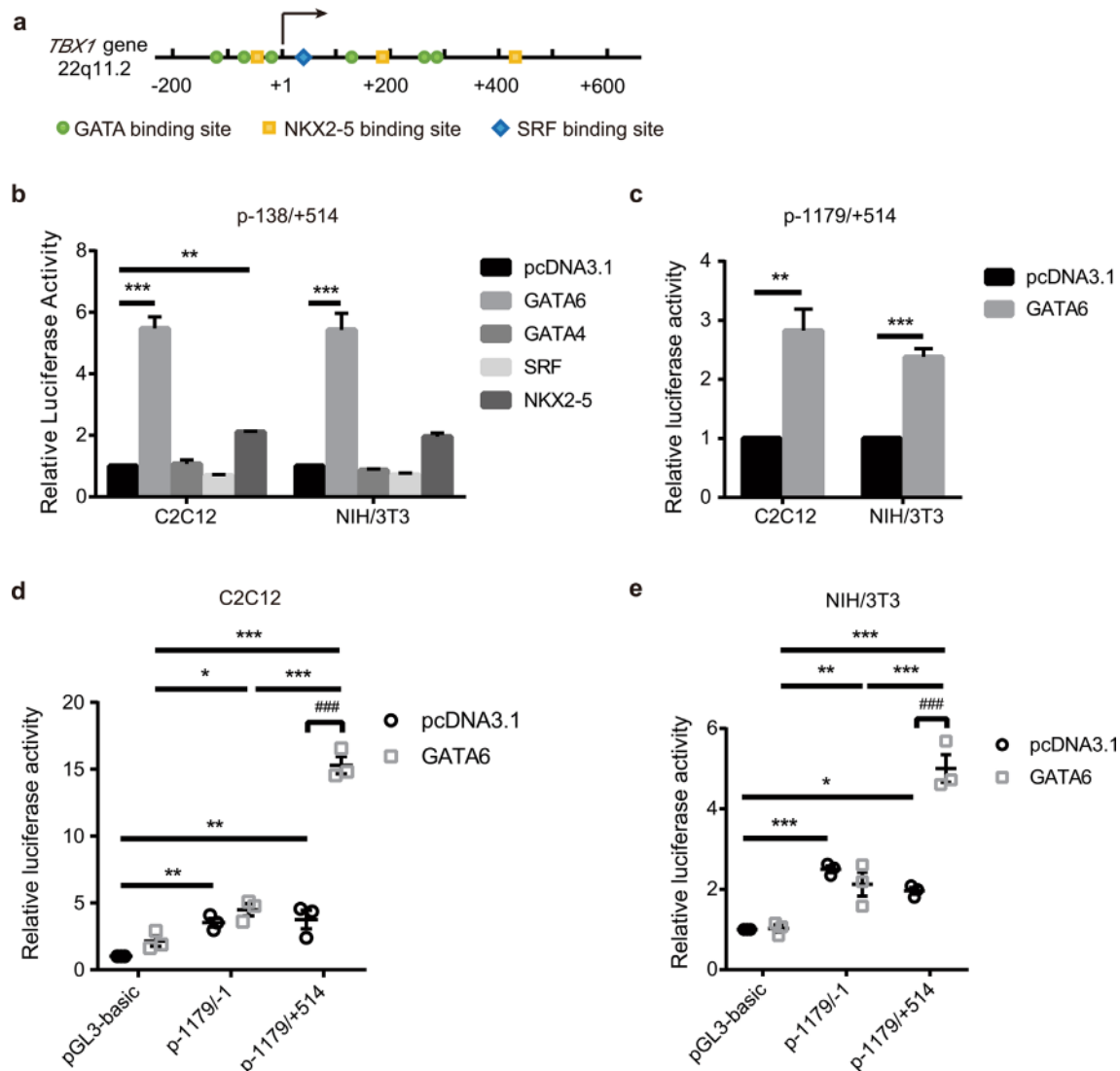
We performed immunohistochemistry (IHC) analyses for *TBX1* and GATA6 in human Carnegie Stage 13 (CS13) embryos. As previously reported, *TBX1* was predominantly expressed in the pharyngeal arches, whereas GATA6 was widely expressed in the pharyngeal arches, along with all atrial and ventricular chambers (Fig. 3a). To further determine the distinct but overlapping expression patterns of *TBX1* and GATA6, we performed double-immunofluorescence staining. The examination of confocal projections revealed that *TBX1* and GATA6 expression primarily overlapped in pharyngeal arches, which is a transient vertebrate-specific complex that participates in OFT development 27 (Fig. 3b).

#### **GATA6 directly binds with the *TBX1*-138/+514 *cis*-regulatory element within the *TBX1* promoter**

To investigate whether GATA6 directly binds to the *TBX1*-138/+514 *cis*-regulatory element within the *TBX1* promoter, EMSA was performed using GATA6 protein and a biotin-labeled *TBX1*+115/+302 probe containing three candidate GATA binding sites. An in vitro-translated GATA6 protein was verified by immunoblotting (Additional file 1: Fig. S3). The EMSA results showed that the GATA6 protein and the biotin-labeled probe *TBX1*+115/+302 formed a protein-DNA supershift, which could be inhibited by the addition of a cold competition probe or an antibody targeting GATA6 (Fig. 4). Together, these results indicated that the human GATA6 protein was able to directly bind to the *TBX1*-138/+514 *cis*-regulatory element within the *TBX1* promoter.

#### **Variants within the *TBX1 cis*-regulatory element in CTD patients impair activation of the *TBX1* promoter by GATA6**

In an effort to explore whether *TBX1 cis*-regulatory element variants are related to CTD, we screened the *TBX1*-138/+514 sequence in 195 patients with CTD. The proportions of CTD variations in these patients were as follows: 43.59% TOF, 23.08% DORV, 14.36% pulmonary atresia with ventricular septal defect (PA/VSD), 15.38% transposition of the great arteries (TGA), 2.56% interrupted aortic arch (IAA) and 1.03% persistent truncus arteriosus (PTA) (Table 1). None of the patients or control



**Fig. 2** GATA6 transcriptional regulation of *TBX1* promoter. **a** Diagrammatic representation of the *TBX1 cis*-regulatory element showing putative potential GATA, NKX2-5, and SRF binding sites. **b** The influence of transcription factors on the p-138/+ 514 reporter gene in the C2C12 and NIH/3T3 cell lines. Luciferase analysis after cotransfection with luciferase reporter constructs containing the *TBX1 cis*-regulatory element *TBX1*-138/+ 514 and the expression vector of GATA6, GATA4, NKX2-5, SRF or empty vector (pcDNA3.1). The results were normalized to Renilla and presented as the fold induction over the pcDNA3.1 vector. Data are shown as mean  $\pm$  SEM, significance was calculated by one-way ANOVA with Dunnett’s multiple comparisons test,  $n = 3$  independent experiments,  $**p < 0.01$  vs pcDNA3.1,  $***p < 0.001$  vs pcDNA3.1 in each cell line. **c** The influence of GATA6 on the *TBX1* promoter reporter gene p-1179/+ 514 in the C2C12 and NIH/3T3 cell lines. Luciferase analysis after cotransfection with luciferase reporter p-1179/+ 514 and GATA6 expression vector or empty vector (pcDNA3.1). The results were normalized to Renilla and presented as the fold induction over the pcDNA3.1 vector. Data are shown as mean  $\pm$  SEM, two-tailed unpaired *t* test was used for statistical calculation,  $n = 3$  independent experiments,  $**p < 0.01$  vs pcDNA3.1,  $***p < 0.001$  vs pcDNA3.1 in each cell line. **d** and **e** The effects of the transcription factor GATA6 on the *TBX1* promoter reporter gene in C2C12 and NIH/3T3 cell lines. Luciferase analysis after cotransfection of luciferase reporter and GATA6 expression construct or empty vector (pcDNA3.1). The results were normalized to Renilla and presented as the fold induction over basal reporter (pGL3-basic + pcDNA3.1). Data are shown as mean  $\pm$  SEM,  $n = 3$  independent experiments. Significance was calculated by two-way ANOVA with Sidak’s post hoc test,  $###p < 0.001$ . Significance was calculated by two-way ANOVA with Tukey’s multiple comparisons test,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$

individuals presented copy number variants of 22q11.2 or any other chromosomal region (data not shown) [53]. We identified three heterozygous sequence variations

in three patients (NC\_000022.11:g.19756832C>G, NC\_000022.11:g.19756845C>T and NC\_000022.11:g.19756902G>T), and all of the results were verified by

(See figure on next page.)

**Fig. 3** Histological staining analysis of *TBX1* and *GATA6* in human Carnegie Stage 13 (CS13) embryos. **a** Immunohistochemical staining for *TBX1* and *GATA6* in human CS13 embryos. Upper panel, *TBX1* expression localized in pharyngeal arches (arrowheads); lower panel, *GATA6* was expressed at pharyngeal arches (arrowheads) along with all atrial and ventricular chambers. Representative sagittal sections are shown (n = 3 embryos): dorsal is right; cranial is up. pa, pharyngeal arch; A, atrium; V, ventricle. Left panel, scale bars = 500  $\mu$ m; middle panel, scale bars = 100  $\mu$ m; right panel, scale bars = 50  $\mu$ m. **b** Double immunofluorescence staining for *TBX1* (red) and *GATA6* (green) in human CS13 embryos. White arrows denote that both *TBX1* and *GATA6* are expressed in pharyngeal arches. Representative sagittal sections are shown (n = 3 embryos). pa, pharyngeal arch. Upper panel, scale bars = 100  $\mu$ m; lower panel, scale bars = 50  $\mu$ m

Sanger sequencing (Fig. 5a). The clinical information and *TBX1*-138/+514 sequence mutations in patients with CTD are shown in Table 2. These three patients had PA, TOF and TGA respectively, combined with other congenital heart diseases (CHDs) such as patent ductus arteriosus (PDA), VSD and pulmonary stenosis (PS). The identified variant (NC\_000022.11:g.19756832C>G) is located within the GATA binding site, whereas the other two variants (NC\_000022.11:g.19756845C>T; NC\_000022.11:g.19756902G>T) are located between the GATA binding sites (Fig. 5b). None of the three variants were found in the 145 ethnically matched healthy controls, but two variants (NC\_000022.11:g.19756845C>T and NC\_000022.11:g.19756902G>T) were reported in the 1000 Genomes database and gnomAD database (Table 2). Several known CHD pathogenic genes (*GATA4*, *GATA6*, *GATA5*, *HAND2*, *FOG2*, *NKX2-5*, *MEF2C*, *TBX1*, *TBX5*, *SOX7*, *PITX2*, and *MESPI*) were also screened in these three patients. There were no non-synonymous variants identified in these known CHD pathogenic genes, including *TBX1*.

We constructed vectors containing each mutation individually using the template *TBX1* promoter p-1179/+514, named M-130, M-143, and M-200. The wild-type (WT) or mutant *TBX1* promoter reporter gene were transfected alone into the C2C12 cell line or cotransfected with the *GATA6* expression vectors to test whether these variants had any effects on *TBX1* transcriptional activation. The luciferase activity measurements showed that the mutation vector alone had no effect on *TBX1* promoter activity itself, but when transfected with *GATA6*, *TBX1* promoter activity decreased significantly compared with the WT (Fig. 5c). Taken together, these results revealed that these variants within the *TBX1* *cis*-regulatory element inhibited *GATA6*-mediated transactivation of *TBX1*.

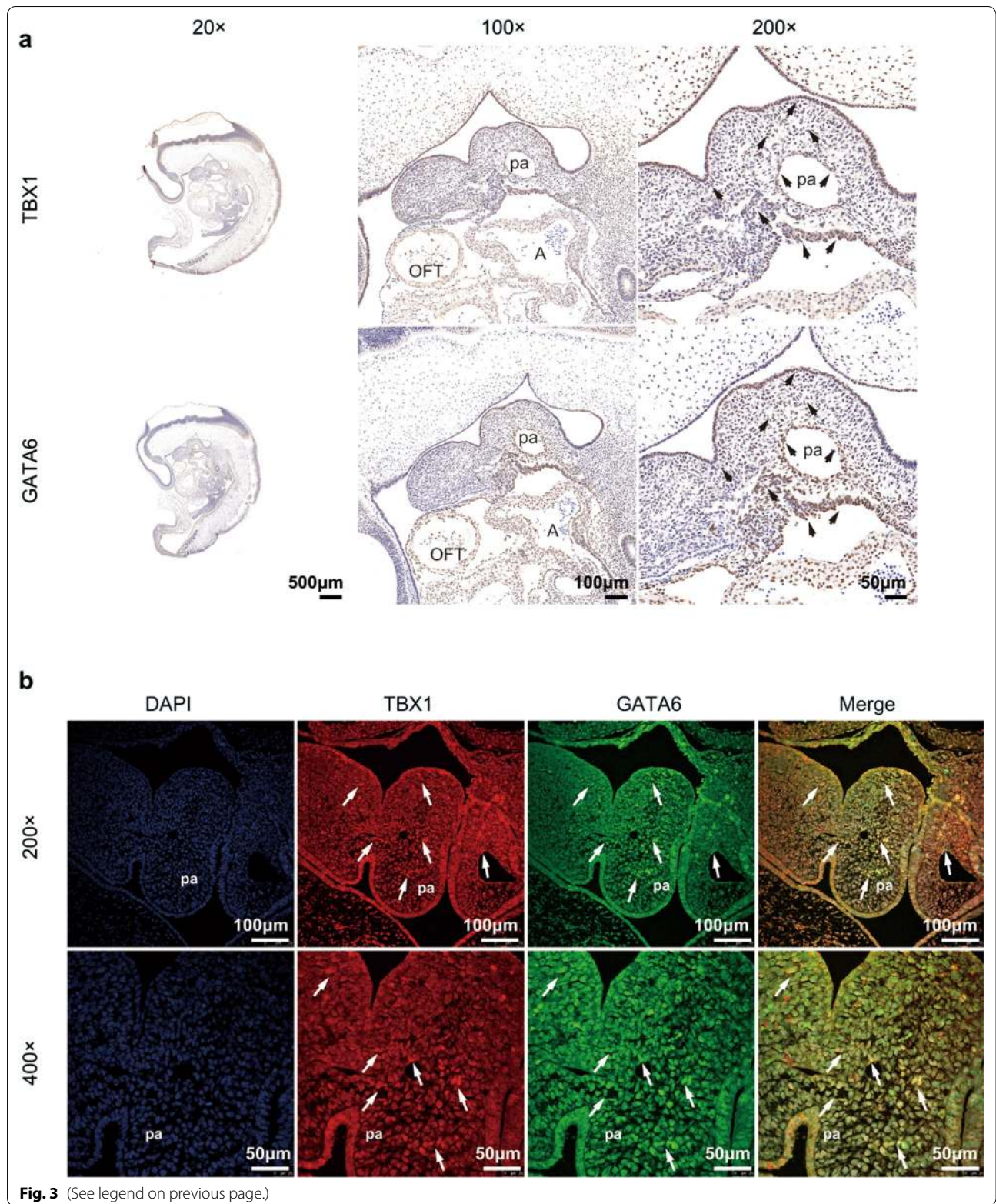
## Discussion

Our results identified a novel non-coding *cis*-regulatory element within the *TBX1* proximal promoter region, which is capable of binding with *GATA6*; and variants in the *cis*-element in CTD patients disrupt *GATA6*-mediated *TBX1* transactivation. CTD, the common type of cyanotic cardiac disease, is usually defined as

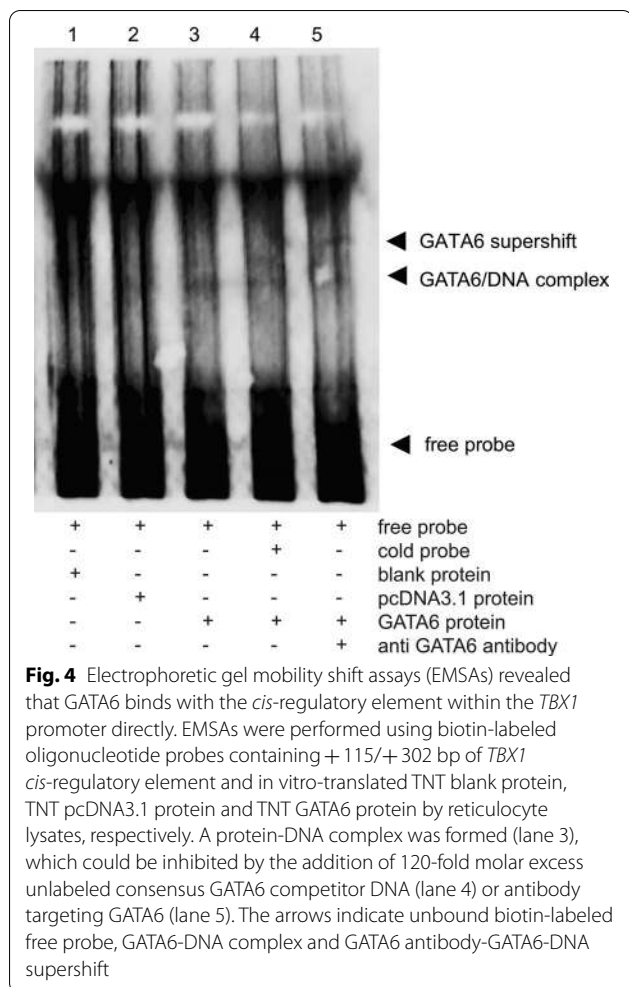
malformations of the OFT, and *TBX1* participates in OFT development. The findings not only help to deepen the understanding of the molecular mechanism of *TBX1* transcription, but also provide ideas for the pathogenesis of CTD.

*TBX1*, the major candidate gene for 22q11.2DS, is a dosage-sensitive transcription factor contributing to the formation of the OFT during cardiogenesis [15, 31]. Understanding the mechanisms of *TBX1* upstream transcriptional regulation may uncover novel determinants of CTD. Transcriptional regulation is a significant mechanism that controls the spatial and temporal expression of genes during cardiac development, and non-coding *cis*-regulatory elements are critical participants in the transcriptional regulation process [54]. A series of truncated portions of the human *TBX1* flanking region were used to identify transcriptional *cis*-regulatory elements associated with *TBX1*. *TBX1*-138/+514, a 0.65 kb fragment around the *TBX1* TSS, was identified as a *cis*-regulatory element. As the *TBX1*-138/+514 region consists of a cCRE-PLS (ENCODE Accession: EH38E2149905, also named *TBX1*-112/+235), combined with previous reports [51, 52], this suggests that *TBX1*-138/+514 may be a *cis*-regulatory element within the *TBX1* promoter.

*Cis*-regulatory elements can alter transcriptional activity by recruiting DNA-binding transcription factors [40], and we identified potential transcription factor binding sites within the *TBX1*-138/+514 sequence, including GATA/NKX2-5/SRF motifs. Our results revealed a clear requirement for *GATA6* to activate the *TBX1*-138/+514 *cis*-regulatory element within the *TBX1* promoter (*TBX1*-1179/+514). *GATA6* belongs to the GATA family of zinc finger transcription factors that consists of six known family members (*GATA1-6*) [55]. *GATA6* is capable of binding the GATA consensus motif (A/T)GATA(A/G) through zinc finger domains [48–50], which are essential *cis*-elements in the promoter or enhancer regions of a variety of cardiac-specific genes [49, 56]. Accumulating research underscores the important roles played by GATA binding motifs in the promoter regions of several myocardial genes, such as *wnt2* [57] and *Bmp-4* [48], which were both identified as direct downstream targets for *Gata6*. Previously, no evidence of a genetic interaction between *GATA6* and *TBX1* has been reported. Using an



**Fig. 3** (See legend on previous page.)



**Table 1** Frequency and spectrum of CTD patients

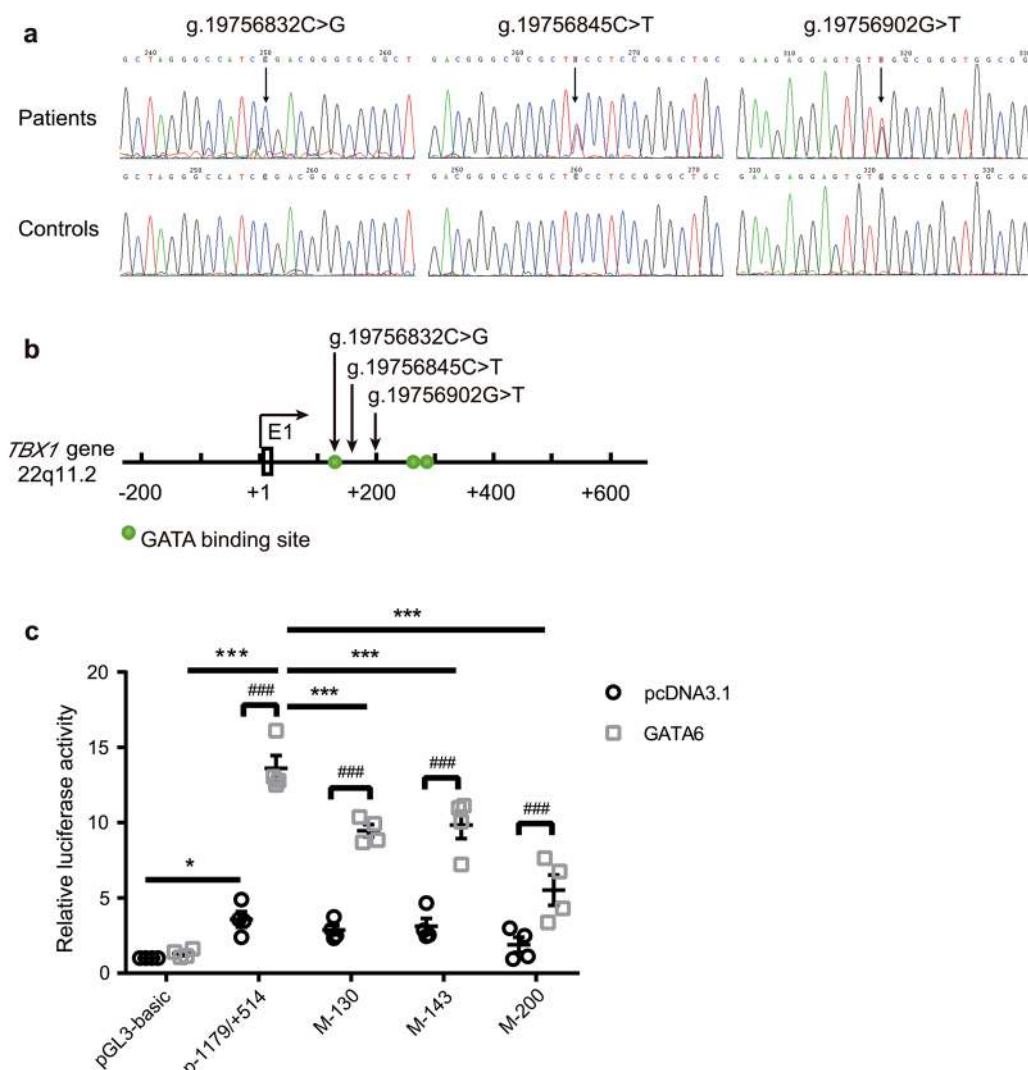
Cardiac defects	Number	Percentage
TOF	85	43.59
DORV	45	23.08
PA/VSD	28	14.36
TGA	30	15.38
IAA	5	2.56
PTA	2	1.03
Total	195	100

TOF, tetralogy of Fallot; DORV, double outlet right ventricle; PA/VSD, pulmonary atresia with ventricular septal defect; TGA, transposition of the great arteries; IAA, interrupted aortic arch; PTA, persistent truncus arteriosus

EMSA, we showed that GATA6 could directly activate *TBX1* transcription by binding with the *cis*-regulatory element *TBX1*-138/+514. Our results also showed that *TBX1* and GATA6, which are two key cardiac regulatory factors, showed an overlapping distribution in the

pharyngeal arches of human embryos. Previous reports showed that *Semaphorin 3c* (*Sema3c*) transcript was downregulated in the OFT of *Tbx1*<sup>-/-</sup> mouse embryos [17], and SEMA3C was regulated directly by GATA6 in the developing OFT [58, 59], suggesting that GATA6 may share a common molecular pathway with *TBX1*, or at least in part during OFT development [5, 59]. Our findings provide evidence of a molecular link between GATA6 and *TBX1*, confirming the above hypothesis. The conditional inactivation of *Gata6* in cardiac neural crest cells results in failed OFT septation [58], and CHD patients with GATA6 mutations have OFT malformations [59–61], demonstrating a role for GATA6 in CTD.

*TBX1* is critical for normal pharyngeal arch development, and both reductions and increases in *TBX1* levels increase the risk of pharyngeal arch birth defects. Any allelic *TBX1* variations that cause *TBX1* levels to fall below a threshold for proper pharyngeal arch development increase the risk of birth defects [34]. Although *TBX1* is the causative gene of 22q11.2, only a few mutations in the coding region of *TBX1* were identified in patients with the 22q11.2 syndrome phenotype without detectable deletion of 22q11.2, and it is possible that mutations in the *cis*-regulatory region of *TBX1* also play a role [9, 30, 37]. The identification of a novel *TBX1* *cis*-regulatory element provided an opportunity to examine whether mutations within the *cis*-regulatory element occurred in patients with the 22q11DS phenotype but no chromosomal deletion. In our study, three mutations within the *TBX1* *cis*-regulatory element in CTD patients were consistently associated with significantly reduced transcriptional activation by GATA6. The mutation NC\_000022.11:g.19756832C>G is located within the GATA binding site and is therefore expected to impact the GATA6-binding ability. The other two variants NC\_000022.11:g.19756845C>T and NC\_000022.11:g.19756902G>T identified in this study were located adjacent to GATA binding sites, which may influence the biological function of the *TBX1* gene through other mechanisms. Therefore, further study is warranted. There are also reports of *TBX1* *cis*-regulatory elements being involved in cardiac development [32, 33, 51, 62], such as a mutation of the Fox-binding site upstream of *Tbx1* abolishing *Tbx1* expression in the pharyngeal endoderm [32, 33] and heterozygous variants NC\_000022.11:g.19756055C>T and NC\_000022.11:g.19756212A>C upstream of the *TBX1* TSS in VSD patients significantly decreasing the activity of the *TBX1* gene promoter [51]. In addition, studies have reported that variants in the regulatory region of other OFT development-related genes are also associated with CTD, such as *WNT5A* [63].



**Fig. 5** Mutation studies on the *cis*-regulatory element of the *TBX1* promoter associated with CTD. **a** Chromatograms of sequence variants within the *TBX1* *cis*-regulatory element. (Top) shows the heterozygous mutation of patients, (Bottom) shows healthy controls, and all the variants are marked with arrows. **b** Schematic representation of the identified *TBX1* *cis*-regulatory element variants in CTD patients. The transcription start site is at the position of g.19756703 (+1) in the first non-coding exon (E1). The numbers represent the genomic DNA sequences of the *TBX1* gene (NC\_000022.11). **c** The effects of GATA6 on the *TBX1* mutation luciferase reporter genes M-130, M-143, and M-200. The WT or mutant *TBX1* promoter reporter gene were transfected alone into the C2C12 cell line or cotransfected with the GATA6 expression vectors. The luciferase activities were normalized to Renilla and presented as the fold increase relative to the activity of the reporter in the presence of an empty expression plasmid (pGL3-basic + pcDNA3.1). Patient variants are associated with a significant transcription activity reduction by GATA6. Data shown are mean  $\pm$  SEM, n = 4 independent experiments. Significance was calculated by two-way ANOVA with Sidak's post hoc test, ###  $p < 0.001$ . Significance was calculated by two-way ANOVA with Dunnett's multiple comparisons test, \*  $p < 0.05$  vs p-1179/+514, \*\*\*  $p < 0.001$  vs p-1179/+514

Although almost all 22q11.2DS patients are hemizygous for the deletion, highly variable genotypes and phenotypes are commonly observed [13, 64]; thus, it has been hypothesized that genes located outside the deleted region may serve as potential modifiers of 22q11.2DS [13, 65, 66]. Our findings uncover a novel mechanism of the GATA6 transcription factor in the regulation of the *TBX1* gene by a *cis*-regulatory element, and variants

in the *cis*-regulatory element in CTD patients impair *TBX1* promoter activity by GATA6, thus providing a basis for the above hypothesis. In addition, with imaging in assessing the phenotype of CTD, computed tomography angiography (CTA) and contrast magnetic resonance angiography (MRA) precisely detect associated aortic, pulmonary venous and coronary anomalies, and other CHDs [67, 68], which will contribute to a better



**Table 2** Clinical information and mutations of *TBX1* cis-regulatory element in patients with CTD

Patient no	Gender	Age	Cardiac phenotype	Variant (NC_000022.11)	Location	Relative position*	1000 Genomes allele frequency	Gnomad allele frequency
91-PF	F	22 months	PA/VSD/PDA	g.19756832C>G	intron1	130 bp	–	–
193-PF	M	26 months	TOF	g.19756845C>T	intron 1	143 bp	0.0006000	0.00009866
40-PF	M	16 months	TGA/VSD/PS	g.19756902G>T	intron1	200 bp	0.0002000	0.00001317

F, female; M, male; PA, pulmonary atresia; VSD, ventricular septal defect; PDA, patent ductus arteriosus; TOF, tetralogy of Fallot; TGA, transposition of the great arteries; PS: pulmonary stenosis

\*Variants were located relative to the transcription start site of *TBX1* gene at g.19756703 (+1) of NC\_000022.11

estimation of the variants and to a clear genotype–phenotype correlation.

## Conclusions

In conclusion, our data provide the first evidence of a genetic link between *TBX1* and GATA6 signaling. A GATA6-binding cis-regulatory element within the *TBX1* promoter was identified, and variants in the cis-regulatory element impaired GATA6-mediated *TBX1* transactivation. However, our study is preliminary. We do not have sufficient evidence to show that *TBX1* expression is directly dependent on the GATA6-binding cis-regulatory element during cardiovascular development in vivo, and the mutations in the *TBX1* cis-regulatory element reported in this paper are causal for CTD. This is the limitation of our study. Further functional assays for the GATA6-TBX1 pathway in CTD patients need to be performed. Our study can be used as the first evidence for future research on the pathogenic non-coding variants in cis-regulatory elements in CTD. This is hoped to be further confirmed in other populations.

## Materials and methods

### Human samples

Human embryonic samples were obtained at Department of Obstetrics and Gynecology of Xinhua Hospital according to the procedure we previously reported [69]. In brief, the pregnant women who could not continue pregnancy for certain reasons, and elective medical termination of pregnancy were performed under the Maternity Protection Law of China. All the donors signed the informed consents from Xinhua Hospital, Affiliated to Shanghai Jiao Tong University School of Medicine. The study was approved by the Medical Ethics Committee of Xinhua Hospital (no. XHEC-C-2012-018). The aborted embryos were isolated with an Olympus stereomicroscope and categorized in accordance with the Carnegie stage (CS). The well-preserved specimens evaluated as normal, were fixed in 4% paraformaldehyde (PFA) overnight,

embedded in paraffin sagittally, and then sectioned at the thickness of 4  $\mu$ m.

Blood samples from 195 sporadic CTD patients (75 female, 120 male; median age 2.3 years) of Han ethnicity, which included 85 TOF, 45 DORV, 28 PA/VSD, 30 TGA, 5 IAA and 2 PTA patients, were collected in Shanghai Children's Medical Center (SCMC) from November 2011 to January 2014 (Table 1) [53]. Clinical records like echocardiography, computed tomography angiography and magnetic resonance angiography [67, 68] were reviewed by pediatric cardiologists before recruitment, and patients who had extracardiac anomalies were excluded. None of which harbored 22q11.2 deletion or duplication tested by CNVplex [53]. A total of 145 healthy control individuals (57 female, 88 male, median age 6.43 years) were ethnically gender-matched and recruited from the same geographical area. DNA was extracted from the whole blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Duesseldorf, Germany). The human experimentations were approved by the Medical Ethics Committee of Xinhua Hospital (no. XHEC-C-2012-018) and Shanghai Children's Medical Center (no. SCMC-201015). Fully written informed consent was obtained from all participants or their guardians. All procedures were in accordance with the ethical standards of the institutional and national research committees and the Declaration of Helsinki.

### Plasmid construction and site-directed mutagenesis

5'-nested polymerase chain reaction (PCR) primers (–4727/+514 F, –4336/+514 F, –3309/+514 F, –1179/+514 F, –138/+514 F, –1179/–1 F, Additional file 2: Table S1) and 3' PCR primers (common 3' primer +514 R, –1179/–1 R, Additional file 2: Table S1) were used to amplify serial deletion fragments of the 5' end region of *TBX1* by the template *TBX1* (NC\_000022.11). Numbering shows the position relative to the TSS of the *TBX1* gene at g.19756703 (+1) of NC\_000022.11. Then, these fragments were subcloned into the *KpnI* and *XhoI* sites of the pGL3-basic

vector (Promega, Madison, Wisconsin, USA) with T4 DNA ligase to construct a series of truncated vectors such as p-4727/+514, p-4336/+514, p-3309/+514, p-1179/+514, p-138/+514, and p-1179/-1. All of the vectors were verified by Sanger sequencing and extracted by an Endo-free Plasmid Mini Kit (OMEGA, USA).

The p-1179/+514 vector was used as templates for all mutagenesis experiments. The mutant plasmids M-130, M-143 and M-200 were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The mutant primers are shown in Additional file 2: Table S1.

The wild-type GATA6 expression plasmid, pcDNA3.1(+)-GATA6 was kindly gifted by Professor Hiroyuki Yamagishi [59]. The GATA4, NKX2-5 and SRF wild-type plasmid was generated by subcloning the corresponding cDNA amplified from the vector pCMV6-Entry-GATA4/NKX2-5/SRF separately purchased from Origene (Rockville, USA) into the expression vector pcDNA3.1(+).

#### Bioinformatics analysis of *TBX1-138+514 cis-regulatory element*

Predictive analysis of cCREs around the *TBX1* TSS were performed with the SCREEN (<http://screen.encodeproject.org>) [38, 43]. The cCRE-PLS genome browser were obtained from the UCSC Genome Bioinformatics Site (<http://genome.cse.ucsc.edu/>) [44]. A non-exhaustive search for potential transcription factor binding sites of *TBX1-138/+514* fragment using the JASPAR (<http://jaspar.genereg.net/>) [45].

#### Cell culture and transfection

C2C12 (mouse myoblast) and NIH/3T3 (mouse embryo fibroblast) cells were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured at 37°C in a 5% CO<sub>2</sub> humidified incubator in DMEM (Gibco, Portland, OR, USA) supplemented with 10% fetal bovine serum (Gibco, Portland, OR, USA).

For luciferase reporter assay, cells were transfected with firefly luciferase reporter plasmid and Renilla luciferase expression plasmid pRL-TK (Promega, Madison, Wisconsin, USA) as an internal control per well using Eugene HD. The firefly and Renilla luciferase activities were measured 40 h after transfection by the Dual Luciferase reporter assay system (Promega, Madison, Wisconsin, USA). The transcriptional activities were represented as ratios of firefly luciferase activities over Renilla luciferase activities.

#### Electrophoretic mobility shift assays (EMSA)

Plasmids pcDNA-3.1 and pcDNA3.1-GATA6 were used for in vitro protein synthesis with the TNT<sup>®</sup>T7 Quick Coupled transcription/translation system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The oligonucleotide probes (+115/+302 bp) acquired from the *TBX1 cis-regulatory element* were synthesized by PCR using 5'-end biotin-labeled primers (GATA-biotin +115/+302, Additional file 2: Table S1). Unlabeled consensus oligonucleotides were used as competitors (GATA cons, Additional file 2: Table S1) [70]. The EMSA was performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific, USA) according to the manufacturers' protocol. Briefly, for the binding reactions with volume 20 µl, 4µl TNT protein sample were incubated with 500 fmol of 5'-labeled biotin probes in the following buffer: 2 µl of 10× binding buffer, 1 µl of 50% Glycerol, 1 µl of 1% NP-40, 1 µl of 1 M KCl, 1 µl of 100 mM MgCl<sub>2</sub>, 1 µl of 200 mM EDTA and 1 µl Salmon DNA. All reactions were incubated at room temperature for 20 min. For competitor lanes, a 120-fold excess of competitor oligonucleotides was added and incubated at room temperature for 10 min before the addition of biotin-labeled probe. For antibody lanes, assays were conducted in a similar manner with the following exceptions: addition of ~5 µg of antibodies against GATA6 (sc-9055 X, Santa Cruz Biotechnology Inc., USA) and incubation at room temperature for 20 min before the addition of the biotin-labeled DNA probe. The products were then separated by 6.5% nondenaturing polyacrylamide gels in 0.5× TBE. The DNA-protein complex was electrotransferred to a positively charged of nylon membrane (Pierce), and the signal was detected by the chemiluminescent nucleic acid detection.

#### Immunohistochemistry

Paraffin-embedded embryo sections underwent deparaffinization, hydration, and antigen retrieval with citrate buffer (Servicebio technology, China). Then quenched endogenous peroxidase activity with 3% H<sub>2</sub>O<sub>2</sub>, blocked in 3% BSA for 1 h at room temperature, followed by incubating with rabbit polyclonal antibody against *TBX1* (Abcam Inc., ab18530, Cambridge, UK) or *GATA6* (Santa Cruz Biotechnology Inc., sc-9055, USA) overnight at 4°C. Next, the secondary antibody, HRP-labelled anti-rabbit immunoglobulin (Gene Tech, Shanghai, China) was applied for 45 min at room temperature. Immunoreactions were visualized by diaminobenzidine substrate (DAB), and nuclei were stained with hematoxylin. Finally, sections were rinsed with water, dehydrated, cleared and mounted with neutral balsam. Immunohistochemical

images were photographed with microscope slide scanner Panoramic MIDI (3D HISTECH).

### Immunofluorescence

For immunofluorescence staining, the sections were deparaffinized, rehydrated, antigen repair with citrate buffer (Servicebio technology, China), washed in PBS briefly and blocked with 3% BSA for 1 h at room temperature. Primary antibodies rabbit anti-TBX1 (Abcam Inc., ab18530, Cambridge, UK), goat anti-GATA6 (Santa Cruz Biotechnology Inc., sc-7244, USA) diluted in 1% BSA and incubated at 4°C overnight. Then sections were washed in 1 × PBS and stained with fluorescent secondary antibodies, Cy3-conjugated donkey anti-rabbit IgG (ServiceBio, GB21403, China) and FITC-conjugated donkey anti-goat IgG (ServiceBio, GB22404, China) for 50 min at room temperature, and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were collected using Leica confocal microscope.

### Western blot analysis

Cells were lysed by RIPA buffer, mixed with SDS loading buffer, and denatured at 100 °C for 10 min. Proteins were separated by 10% SDS-PAGE gels (Beyotime Biotech Inc., Shanghai, China) and transferred electrophoretically onto polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). Then the membranes were blocked with 5% skim milk for 2 h at room temperature, then incubated overnight at 4 °C with anti-GATA6 (Sigma-Aldrich, G2298) and anti-GAPDH (Sigma-Aldrich, G9545) antibodies. After the membranes were rinsed, they were incubated with the HRP-conjugated secondary antibody for 2 h at room temperature. Detection was performed by an enhanced chemiluminescence western blot detection system (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

### Gene sequencing and mutation characterization

Mutation screening analysis of the *TBX1*-138/+514 *cis*-regulatory element sequence was performed in patients and control individuals by Sanger sequencing. The referential genomic DNA sequence of *TBX1* was derived from a nucleotide (NC\_000022.11). Mutations in *TBX1*-138/+514 with a prevalence of under 0.1% in control populations (under 0.1% in both the 145 healthy controls and public variant databases, including 1000 Genomes and gnomAD) were regarded as candidate variants. The variants were validated by Sanger sequencing, and the primers used in the present study are summarized in Additional file 2: Table S1.

To validate the potential function of identified *TBX1*-138/+514 *cis*-regulatory element variants, the coding

sequences and splicing sites of these known CHD pathogenic genes (*GATA4*, *GATA6*, *GATA5*, *HAND2*, *FOG2*, *NKX2-5*, *MEF2C*, *TBX1*, *TBX5*, *SOX7*, *PITX2*, *MESPI*) were also screened in CTD patients and controls using the EasyTarget® amplification kit (Genesky Biotechnologies Inc, Shanghai, China). Nonsynonymous mutations in the coding and splicing regions with a prevalence of under 0.1% in control populations (under 0.1% in both the 145 healthy controls and public variant databases, including 1000 Genomes, ExAC and gnomAD) were regarded as CHD pathogenic gene mutations.

### Statistical analysis

Data were analyzed by PRISM 6.0 (GraphPad Software Inc., La Jolla, CA, USA) software and were presented as the mean ± standard error of the mean (S.E.M.). The experiments were repeated at least three times independently. Two-tailed unpaired t-test was used to determine significant differences between two groups. One-way ANOVA with Dunnett's post hoc test was used to analyze differences among three or more groups. Differences between two independent variables were analyzed by two-way ANOVA with Sidak's multiple comparisons test, Tukey's multiple comparisons test or Dunnett's multiple comparisons test.  $P < 0.05$  were considered statistically significant.

### Abbreviations

TBX1: T-box transcription factor 1; VCFS: Velo-cardio-facial syndrome; DGS: DiGeorge syndrome; EMSAs: Electrophoretic mobility shift assays; CTD: Conotruncal defect; GATA6: GATA binding protein 6; 22q11.2DS: 22Q11.2 deletion syndrome; SHF: Second heart field; OFT: Outflow tract; LOF: Loss-of-function; GOF: Gain-of-function; TOF: Tetralogy of Fallot; DORV: Double outlet right ventricle; VSD: Ventricular septal defect; Shh: Sonic hedgehog; VEGF: Vascular endothelial growth factor; RA: Retinoic acid; GWAS: Genome-wide association studies; cCREs: Candidate *cis*-regulatory elements; TSS: Transcription start site; ENCODE: Encyclopedia of DNA Elements; PLS: Promoter-like signature; SRF: Serine response factor; IHC: Immunohistochemistry; CS13: Carnegie stage 13; PA: Pulmonary atresia; TGA: Transposition of the great arteries; IAA: Interrupted aortic arch; PTA: Persistent truncus arteriosus; CHDs: Congenital heart diseases; PDA: Patent ductus arteriosus; PS: Pulmonary stenosis; WT: Wild-type; Sema3c: Semaphorin 3c; CTA: Computed tomography angiography; MRA: Magnetic resonance angiography; PFA: Paraformaldehyde; PCR: Polymerase chain reaction; DAB: Diaminobenzidine substrate; DAPI: 4',6-Diamidino-2-phenylindole.

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13023-021-01981-4>.

**Additional file 1. Fig. S1** Deletion analysis identifies a 0.65-kb region around the *TBX1* TSS essential for transcriptional activity in the NIH/3T3 cell line. **Fig. S2** UCSC Genome Browser views of cCREs around the *TBX1* TSS and the underlying DNase and ChIP data (hg38 human genomes). **Fig. S3** Verification of in vitro-translated GATA6 by reticulocyte lysates.

**Additional file 2. Table S1** Primers used for PCR, EMSA and Site-directed mutagenesis.

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### Authors' contributions

YX and RX designed the research. XJ, TL and SL performed experiments. QF and FL collected the blood samples of patient samples. XJ and TL analyzed data. XJ wrote the manuscript. YX, RX, SC and KS contributed to the revision of the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Declarations

#### Ethics approval and consent to participate

All assessments about human samples were in accordance with the ethical standards of the institutional and national research committees and the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of Xinhua Hospital (no. XHEC-C-2012-018) and Shanghai Children's Medical Center (no. SCMC-201015). Fully written informed consent was obtained from all participants (or their parents if children were too young to consent by themselves).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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