A Large Permissive Regulatory Domain Exclusively Controls Tbx3 Expression in the Cardiac Conduction System

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Rationale: The evolutionary conserved Tbx3/Tbx5 gene cluster encodes T-box transcription factors that play crucial roles in the development and homeostasis of the cardiac conduction system in human and mouse. Both genes are expressed in overlapping patterns and function in strictly tissue-specific and dose-dependent manners, yet, their regulation is poorly understood.

Objective: To analyze the mechanism underlying the complex regulation of the Tbx3/Tbx5 cluster.

Methods and Results: By probing the 3-dimensional architecture of the Tbx3/Tbx5 cluster using high-resolution circular chromosome conformation capture sequencing in vivo, we found that its regulatory landscape is in a preformed conformation similar in embryonic heart, limbs, and brain. Tbx3 and its flanking gene desert form a 1 Mbp loop between CCCTC-binding factor (CTCF)-binding sites that is separated from the neighboring Tbx5 loop. However, Ctf1 inactivation did not result in transcriptional regulatory interaction between Tbx3 and Tbx5. Multiple sites within the Tbx3 locus contact the promoter, including sites corresponding to regions known to contain variations in the human genome influencing conduction. We identified an atrioventricular-specific enhancer and a pan-cardiac enhancer that contact the promoter and each other and synergize to activate transcription in the atrioventricular conduction system.

Conclusions: We provide a high-resolution model of the 3-dimensional structure and function of the Tbx3/Tbx5 locus and show that the locus is organized in a preformed, permissive structure. The Tbx3 locus forms a CTCF-independent autonomous regulatory domain with multiple combinatorial regulatory elements that control the precise pattern of Tbx3 in the cardiac conduction system. (Circ Res. 2014;115:432-441.)

Key Words: anatomy & histology ■ heart conduction system

The heart comprises working muscle and an integrated cardiac conduction system (CCS) that initiates and propagates the electric impulse required for the coordinated contraction of the chambers. The development of the CCS is regulated by transcription factors that act in strictly stage, location, and dose-dependent manners.1,2 Disrupted function or expression of these factors may lead to disorders in development or function of the CCS, which are associated with morbidity, heart failure, and sudden death.3 The evolutionary conserved Tbx3/Tbx5 gene cluster encodes T-box transcription factors that play key roles in the development and function of multiple organ systems including heart, limbs, lungs, and liver.4 Haploinsufficiency of Tbx5 causes Holt–Oram syndrome (OMIM [Online Mendelian Inheritance of Man]: 142900), a developmental disorder characterized by hand- and heart defects,5,6 whereas haploinsufficiency of Tbx3 causes the ulnar-mammary syndrome (OMIM: 181450), a developmental disorder characterized by limb, mammary gland, tooth, and genital abnormalities.7 Both genes play crucial roles in the development and patterning of the CCS. In the heart, Tbx5 is expressed in a pattern including the atria and CCS and acts as an activator of genes promoting conduction.8,9 Tbx3 is expressed specifically in the CCS components.

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432
and functions by imposing a pacemaker phenotype on cells within its expression domain. Ectopic expression of Tbx3 in the myocardium induces a pacemaker phenotype. A decrease in Tbx3 below critical levels results in lethal arrhythmias. Therefore, the correct spatiotemporal pattern and dosage of expression of Tbx3 and of Tbx5 are critical for development and functional integration of the CCS.

The regulatory architecture of loci harboring genes encoding transcription factors or other developmental factors often comprises long-range regulatory elements, each responsible for a subset of the temporal, spatial, and quantitative aspects of expression of the gene. Genome-wide association studies suggest that a large number of noncoding variants underlie the increased risk to various common diseases, often by disrupting regulatory elements that control the expression of nearby genes. Genome-wide association studies revealed that common variants in the noncoding regions around Tbx3 and Tbx5 are associated with cardiac impulse conduction and depolarization properties, whereas a congenital heart defect patient has been identified with a homozygous variant in an enhancer regulating Tbx5. These findings further underscore the critical importance of understanding the regulatory system controlling the expression pattern and dosage of Tbx3 and Tbx5.

The 3-dimensional (3D) organization of chromatin has been recognized as an important regulatory layer for gene regulation, setting the stage for physical contact of the control sequences, such as enhancers, and their target promoters. High-resolution circular chromosome conformation capture combined with sequencing (4C-seq) is a recent technology examining the spatial organization of DNA and measuring the contact frequencies of a chosen genomic site, or viewpoint, with the entire genome. We assessed the 3D architecture of the Tbx3/Tbx5 cluster using high-resolution 4C-seq of embryonic day (E) 10.5 mouse hearts. The 4C-seq experiments show results typical for chromosome conformation capture experiments, in that most of the signal is close to the viewpoint, intrachromosomal contacts outnumber interchromosomal contacts, and contacts at distal sites tend to cluster. To assess the conformation of the Tbx3 locus and identify putative regulatory regions that contact Tbx3 and may regulate its expression, the Tbx3 promoter was used as viewpoint. The broad peak around the Tbx3 promoter reflects contact by linear proximity, whereas more distant signal clusters (peaks) represent discernable interactions through looping. The capture profile reveals that the Tbx3 promoter contacts multiple regions upstream of Tbx3, with interactions reaching up to neighboring gene Med13l, located 903 kb upstream (Figure 1A). Analysis of these data reveals relatively strict boundaries of the contact profile, with most contacts being confined to the domain spanning ≈900 kb upstream to 50 kb downstream of Tbx3 (Figure 1A). Interestingly, analysis of chromosomal contacts with TBX3 and Tbx5 as assessed by Hi-C in human fibroblasts reveals, albeit in much lower resolution, a similar pattern with multiple regions in the Tbx3 domain contacting the gene. Again, contacts with the Tbx5 or MED13L domains are almost absent (Online Figure I).

The contact profile of the promoter of the adjacent Tbx5 gene reveals an independent, neighboring, regulatory domain with boundaries near Tbx3 and the neighboring Rbm19 gene, located ≈230 kb downstream of Tbx5 (Figure 1B). The 2 domains hardly overlap, indicating that sites in the Tbx3 domain do not contact the Tbx5 domain and vice versa. Med13l is isolated from these regulatory landscapes, as revealed by the contact profile of the Med13l promoter (Online Figure I). In contrast, the contact profile of Rbm19, downstream of Tbx5, partially overlaps with that of Tbx5 but not with the Tbx3 regulatory landscape (data not shown). This demonstrates that Tbx3 forms a large independent regulatory domain not used by the neighboring genes Tbx5, Med13l, or Rbm19. The strict boundaries of the contact profiles correspond with the location of CTCF-binding sites in heart and other tissues (Figure 1D; Online Figure IIA). CTCF is involved in chromatin looping and in insulation of loci. We studied the interaction profile of one of the CTCF-binding sites in between Tbx3 and Tbx5. This site physically interacts with distal CTCF-binding site-containing regions located 974 kb up- and 358 kb downstream, forming chromatin loops that demarcate the Tbx3 and Tbx5 regulatory landscapes, respectively (Figure 1C). These observations suggest that CTCF may mediate the physical separation of the regulatory domains of Tbx3 and Tbx5 (Figure 1E), preventing enhancer sharing between these genes. To test this possibility, we analyzed the expression of Tbx3 and Tbx5 in

### Methods

An expanded methods section is provided in the Online Data Supplement. Fore- and midbrain, limbs and hearts were dissected from E10.5 embryos and processed to obtain single-cell samples for circular chromosome conformation capture (4C), which was performed as described. Bacterial artificial chromosomes (BACs) were modified as described. Enhancer fragments were amplified from genomic DNA and cloned in the Hsp68-LacZ reporter vector. Embryos were isolated for F0 screening, fixed in 4% paraformaldehyde in PBS, and processed for in situ hybridization and immunohistochemistry. Animal work was performed in accordance with national and international guidelines and approved by the Animal Experiments Committee of the Academic Medical Center.
embryos in which Ctf was inactivated specifically in the cardiomyocytes. However, neither the level nor the pattern of Tbx3 and Tbx5 was affected, indicating that CTCF is not required to prevent the separation of the regulatory sequences of Tbx3 and Tbx5 (Online Figure IIB and IIC).

Active enhancers are marked by acetylation of histone H3 on lysine K27 (H3K27ac).29 Chromatin immunoprecipitation (ChIP) sequencing with an antibody against H3K27ac in both embryonic chamber and atrioventricular canal (AVC) myocardial cells10 revealed that the region flanking Tbx3 is extensively acetylated on H3K27 in AVC cells, corresponding to the AVC-specific expression of Tbx3 (Online Figure III). In contrast, the region flanking Tbx5 is acetylated in both chamber myocardium and AVC, corresponding to its expression in both compartments. The boundary between the complementary H3K27ac signatures colocalizes roughly with the boundary of the contact profiles for Tbx3 and Tbx5. Taken together, our data indicate that the Tbx3 locus forms an independent regulatory domain which is physically separated from that of Tbx5 and that this domain contains multiple regions that contact the Tbx3 gene.

Green Fluorescent Protein-Modified BACs Recapitulate Atrioventricular Conduction System Expression of Tbx3

Although 4C-seq physically wire gene promoters to potential enhancer sequences, physical contact does not necessarily reflect functional interaction.31 To identify regulatory elements within the Tbx3 locus, we generated transgenic mice with modified BACs. BAC RP23-143N21 contains genomic sequences from −73 kb to +147 kb relative to the transcription start site of Tbx3, BAC RP23-366H17 contains sequences from −82 kb to +78 kb and BAC RP24-89K7 contains sequences from −149 to +66 kb. To investigate whether regulatory elements required for cardiac Tbx3 expression are located within the regions covered by these BACs, we modified them by inserting a green fluorescent protein (GFP)-encoding reporter gene at the translation start site of Tbx3 (143N21-GFP, 366H17-GFP, 89K7-GFP; Figure 2A), also resulting in the inactivation of Tbx3 within the BAC, negating any potential gain of Tbx3 function in transgenic mice. Analysis of the GFP expression pattern in F0 embryos of embryonic day (E) 11.5 revealed that all 3 modified BACs are able to recapitulate many aspects of the extracardiac

Figure 1. Separation of the Tbx3 and Tbx5 regulatory domains. A–D, Contact profiles of the Tbx3 and Tbx5 loci as determined by circular chromosome conformation capture (4C) reveal genomic regions that physically contact the Tbx3 promoter in embryonic heart cells. The upper track shows the UCSC (The University of California, Santa Cruz) genome browser traces of a running window approach for viewpoints of the Tbx3 promoter (A), Tbx5 promoter (B), and CTCF site (C and D). The second track shows the respective normalized contact intensities (gray dots) and their truncated mean trends (black line) for all viewpoints in and around Tbx3 and Tbx5. Means are computed for 5 kb windows, and the gray band displays the 20% to 80% percentiles for these windows. The scaling-plot below depicts data points across differently scaled window sizes (from 2 kb [top row] to 50 kb [bottom]). The data points and the colors are scaled to the maximum mean value of a 5 kb window size.25 Local changes in color codes indicate regions enriched for captured sequences, which correspond to the promoter–enhancer contacts described. The contact profile of Tbx5 (B) reveals a complementary pattern to that of Tbx3 (A), with hardly any overlap in contacting regions. C, The contact profile of the CTCF-binding site in between Tbx3 and Tbx5 (marked by arrow in D) as determined by 4C reveals interaction with neighboring CTCF-binding site islands (marked by asterisks). Distribution of CTCF binding sites as determined by chromatin immunoprecipitation sequencing (ChIP-seq) in heart cells.20 CTCF-binding sites are located in between Tbx3 and Tbx5 and near flanking genes Med13l and Rbm19, colocalizing with the boundaries in contact profiles of Tbx3 and Tbx5 promoters. Arrow marks CTCF site used for 4C. E, Schematic overview of the Tbx3 and Tbx5 loci, depicting the physical separation of the Tbx3 and Tbx5 regulatory domains.
Tbx3 expression pattern. GFP expression was found in the retina, limb buds, mammary glands, and pharyngeal mesenchyme (described earlier for 366H17-GFP),32 corresponding to the endogenous Tbx3 expression pattern (Figure 2B). In situ hybridization revealed that 143N21-GFP and 366H17-GFP recapitulate Tbx3 expression in the foregut endoderm and ventral body wall (Figure 2C). Cardiac GFP expression was detected in the AVC myocardium but in a domain smaller than that of Tbx3 itself. The GFP expression domain included ventral, right-sided, and dorsal aspects (primordial atrioventricular node) of the AVC. The Tbx3-positive atrioventricular bundle primordium (interventricular ring) did not express GFP, indicating that the region from −82 kb to +147 kb of Tbx3 lacks DNA sequences for Tbx3 activation in the complete CCS. GFP expression was also observed in Tbx3-positive cardiac neural crest cells distal from the outflow tract and the outflow tract cushions (neural crest-derived cells; Online Figure IV). 89K7-GFP, spanning an additional 60 kb region upstream of the other BACs, drove GFP expression in the entire AVC, recapitulating the pattern of Tbx3. Furthermore, GFP expression was observed in the interventricular ring, indicating that the region −149 kb to −82 kb upstream of Tbx3 harbors regulatory elements required for Tbx3 expression in the prospective atrioventricular bundle. These data reveal that the genomic region of −82 kb to +147 kb of Tbx3 harbors regulatory elements that drive atrioventricular activity of Tbx3, whereas the region from −149 kb to −82 kb of Tbx3 harbors additional regulatory sequences driving the full AVC and bundle expression pattern of Tbx3. We observed no GFP expression in the sinoatrial node, indicating that the regulatory elements driving Tbx3 expression in this region are absent from the regions spanned by the tested BACs.

**Two Putative Enhancers Physically Contact the Tbx3 Promoter**

To identify potential enhancers within the Tbx3 BACs conferring tissue-specific expression, we analyzed the contact profile of the promoter by 4C in embryonic heart, brain, and limb cells. Contact profiles of all 3 cell types revealed a highly similar pattern with multiple sites contacting the Tbx3 promoter (Figure 3A). Two regions showed strong interaction with the promoter, one ≈25 kb upstream of the promoter and present in all 3 modified BACs and one at ≈90 kb upstream of the promoter and present in only BAC 89K7-GFP. To identify putative enhancers within the contact regions, we evaluated ChIP-sequencing datasets25,33–35 of the genome-wide occupancy profiles of cardiac transcription factors Gata4, Nkx2-5, Tbx5, Tbx3, the enhancer-associated histone acetyltransferase p300, RNA polymerase II (Pol2), and histone modifications H3K27ac and monomethylation of histone H3 on lysine K4 (H3K4me1; Figure 3B). Based on these occupancy profiles, 2 candidate regions were identified upstream of Tbx3. Putative enhancer A (eA)
is located 19 kb upstream of \(Tbx3\) within the proximal contact region and is occupied by Tbx5, Gata4, and Nkx2-5 and marked by H3K4me1. Putative enhancer B (eB), which shows occupancy by all factors except Pol2, is located 95 kb upstream of \(Tbx3\) within the distal contact region that is present only in BAC 89K7 (Figure 3B and 3C; Online Figure V). Tbx3, H3K27ac, p300, and Pol2 occupancy of eA and Pol2 occupancy of eB were not called by MACS (Model-based Analysis of ChIP-Seq). However, inspection of the raw data tracks revealed distinguishable peaks for all these factors on both sites (Online Figure VIA). The weak signal might be explained by the fact that the ChIP-sequencing datasets are derived from whole hearts, whereas the CCS in which \(Tbx3\) is active comprises only a small fraction of the total cardiac cell population. The association of both enhancers with the enhancer-associated histone modifications H3K27ac and H3K4me1 was confirmed by ChIP-quantitative polymerase chain reaction. Both putative enhancers showed enrichment of H3K27ac and H3K4me1 occupancy over a negative control region, revealing their possible role as transcriptional enhancers (Online Figure VIB).

Figure 3. Identification of 2 putative enhancers contacting \(Tbx3\). A, Contact profile in heart with \(Tbx3\) promoter as viewpoint as assessed by circular chromosome conformation capture (4C) reveals multiple sites contacting \(Tbx3\). Y axis depicts contact tag count. B, Occupancy profiles of the \(Tbx3\) locus of cardiac transcription factors Tbx5, Tbx3, Gata4, and Nkx2; histone modifications H3K4me1 and H3K27ac, and enhancer-associated proteins p300 and Pol2 and as assessed by chromatin immunoprecipitation sequencing (ChIP-seq). Peaks were called using the MACS (Model-based Analysis of ChIP-Seq) peak calling algorithm. C, Two potential enhancers are identified which are present in all 3 modified bacterial artificial chromosomes (BACs; enhancer A [eA]) or exclusively in 89K7-GFP (enhancer B [eB]).
We used putative eA and eB as viewpoints in 4C-seq analysis and found that eA strongly interacts with both eB and the Tbx3 promoter. The same holds for eB, albeit to a much lesser extent (Figure 4; Online Figure VII). These findings reveal that both enhancers and the Tbx3 promoter are spatially clustered together. Comparison of contact profiles from heart, limb, and brain indicated that the chromosomal conformation of the Tbx3 locus is not tissue specific because the contact profiles for all 3 cell types with different viewpoints reveal a striking similarity (Figure 4). Only minor differences in contact profiles are observed between heart and limb. In brain, slightly more appreciable differences are observed in the contact profiles. The region upstream of Tbx3, encompassing both eA and eB, shows more enhancer–promoter contacts in heart compared with brain. Furthermore, the Tbx3 promoter contacts Tbx5 and several flanking intergenic regions in brain, exceeding the boundaries marked by CTCF-binding sites, and vice versa (Figure 4B). The separation between both domains is, therefore, less strict in embryonic brain.

**eA and eB Are Required for CCS Expression of Tbx3**

To investigate whether putative enhancers eA and eB are involved in the regulation of Tbx3 expression, fragments containing the enhancers were deleted from 89K7-GFP to construct ΔeAeB-89K7-GFP. Inspection of GFP expression in E10.5 F0 ΔeAeB-89K7-GFP embryos revealed that extracardiac Tbx3 expression was largely unaffected by deletion of both fragments (Figure 5). GFP expression was detected in the limb buds, eye placode, pharyngeal arches, and cardiac neural crest cells in a pattern that is similar to 89K7-GFP, indicating that Tbx3 expression in these regions is not mediated by eA and eB. However, GFP expression in the heart is completely abolished in ΔeAeB-89K7-GFP embryos. Immunohistochemistry revealed that GFP expression in the AVC, atrioventricular bundle, foregut endoderm, and ventral body wall is absent in ΔeAeB-89K7-GFP embryos (Figure 5). These findings reveal that putative enhancers eA and eB are required for CCS expression of Tbx3.

**Evolutionary Conserved eA and eB Are Sufficient to Drive Atrioventricular Tbx3 Expression**

To elucidate whether both enhancer fragments on their own are sufficient to drive atrioventricular Tbx3 expression, we isolated fragments of ≈2 kb spanning eA or eB, cloned them in a LacZ-reporter vector with a minimal promoter (eA-LacZ and eB-LacZ, respectively), and assessed their in vivo activity. F0 screening of transient transgenic eA-LacZ E10.5 embryos revealed that eA is sufficient to drive LacZ expression in the AVC, strong in the ventral, right, and dorsal aspects, weak at the left side (Figure 6A). This cardiac expression pattern corresponds to the GFP expression as observed in BACs 143N21-GFP and 366H17-GFP, in which eA but not eB is located. Consistent LacZ expression was also observed in the pharyngeal arches, whereas ventral body wall expression was not detected in any of the embryos.

eB-LacZ embryos showed a variable expression pattern. Two embryos showed cardiac expression in only few spots in the heart not correlating with the pattern of endogenous Tbx3 expression, whereas 2 other embryos showed cardiac expression throughout the entire heart (Figure 6B). This indicates that on its own eB is cardiac specific, is weak and influenced by repressive effects of the genomic region at the site of its integration, and, within the heart, lacks spatial information. Interestingly, when coupled to eA and tested in transient transgenic mouse embryos (eAeB-LacZ), robust expression was observed throughout the entire AVC and prospective atrioventricular bundle (interventricular ring; Figure 6C). Strong ventral body wall expression was also observed in eAeB-LacZ embryos, in contrast to either eA-LacZ or eB-LacZ. This expression pattern in eAeB-LacZ embryos is highly similar to the GFP expression we observed in 89K7-GFP embryos, corresponding to the presence of both eA and eB in BAC 89K7. These data imply that eA and eB do not act as independent modules, but act in synergy, whereby eB enhances the activity of eA and complements the pattern of eA in the atrioventricular conduction system precursors (Figure 6G).

To investigate whether the mechanism of Tbx3 regulation in the atrioventricular conduction system is evolutionary conserved, we tested the human and chicken orthologues of eA in vivo. Human eA-LacZ showed an expression pattern comparable to its murine counterpart, with expression in the major portion of the AVC, mainly in the ventral, right, and dorsal side (Figure 6D). Weak activity was also observed in the prospective atrioventricular bundle. Chicken eA-LacZ shows similar atrioventricular ring expression; however, expression in the prospective atrioventricular bundle was not detected in any of the embryos (Figure 6E). The chicken eA-LacZ construct also showed clear expression around the fore limbs, possibly reflecting Tbx3 expression in the zone of polarizing activity implicated in limb bud development, which was not detected in the human or murine ortholog of eA.

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**Figure 5.** Enhancer A (eA) and enhancer B (eB) are required for atrioventricular conduction system expression of Tbx3. 89K7-GFP recapitulates (extra)cardiac expression pattern of Tbx3. Deletion of both eA and eB from 89K7-GFP (ΔeAeB-89K7-GFP) leads to complete loss of cardiac GFP expression, whereas extracardiac expression is unaffected. Rectangles depict regions shown in greater magnification below. Circle depicts loss of GFP expression in the heart. Arrows depict atrioventricular canal, and arrowheads depict interventricular region. ia indicates left atrium; lv, left ventricle; ra, right atrium; and rv, right ventricle.
 interspecies consistency in AVC activity of eA indicates that aspects of the Tbx3-mediated formation of the CCS are evolutionary conserved.

**Tbx3 Regulation in the AVC by eA Is Mediated by Bone Morphogenetic Protein Signaling**

We recently found that several atrioventricular canal-specific enhancers are GATA-binding site dependent and are synergistically activated by Gata4 and Smads (downstream bone morphogenetic protein [BMP]-signaling effectors), which recruit histone acetyltransferases such as p300.30,36 Because the enhancers here are occupied by Gata4 and p300, we hypothesized that they may be activated by Gata4 and BMP signaling. In transient transfection assays, we found that eA and eB are not activated by Gata4 or BMP effectors alone. Cotransfection with Gata4 and BMP effectors, however, resulted in a synergistic induction of eA (Figure 6F).

Transfection of 2 subfragments of eA revealed that the regulatory information of this enhancer is harbored within eA2. Consistently, this subfragment also drives atrioventricular canal expression in transgenic embryos (not shown). The eB enhancer fragment was not strongly activated. Together these data are consistent with the observed activity patterns of the 2 enhancer fragments in vivo, in which eA provides atrioventricular canal specificity, whereas eB is weak and does not show specificity within the heart.

**Discussion**

The T-box transcription factors Tbx5 and Tbx3 are crucial for the development and function of the heart and its electric components. In this study, we provided a high-resolution model of the 3D conformation of the evolutionary conserved Tbx3/Tbx5 genomic locus using high-resolution 4C-seq in vivo. We found that Tbx3 and Tbx5 are physically organized into separate loci and that the genomic organization is independent of tissue type. We identified 2 enhancers within the Tbx3 domain that contact each other and the Tbx3 promoter and that synergize to drive Tbx3 expression in the atrioventricular conduction system.

Tbx3 and Tbx5 form an evolutionary conserved cluster of developmental transcription factor genes originating from a primitive T-box gene.37 Studies on the transcriptional regulation of clustered developmental genes, like the Irx and Hox clusters, revealed that regulatory DNA elements are not uniquely associated with single promoters.38,39 Instead, genes within these clusters extensively share enhancers, coordinating the regulation of their expression pattern. It has been proposed that enhancer sharing explains the conservation of the genomic organization of such clusters during evolution.38,39 Our data indicate that, contrary to the Irx and Hox gene clusters, the Tbx3/Tbx5 cluster is divided into 2 separate regulatory domains. Interdomain contacts seem almost absent in all tissue types we tested, indicating that Tbx3 and Tbx5 are regulated independently. This also implies that genomic variation affecting regulatory elements of either one of the genes does not directly influence the expression pattern and level of the other.

The strict boundaries between the Tbx3 and Tbx5 domains are marked by CTCF-binding sites. CTCF-bound sites can function as boundary regions, interacting with each other to physically segregate the chromosome into topologically associated domains within which enhancer–promoter contacts have been shown to be particularly frequent.37,40,41 CTCF-binding sites have been suggested to isolate genes or gene clusters from neighboring transcriptional interference. However, in cardiac-specific Ctf4 mutants, we did not observe
any ectopic expression of Tbx3 in the larger Tbx5 expression domain or any change in the level of expression of either gene. These data indicate that although CTCF-binding marks regions involved in looping and locus separation, CTCF itself is not required to transcriptionally insulate the loci from neighboring regulatory activity. Consistently, inactivation of Ctcf in erythroid cells or limb buds did not lead to ectopic activation of genes adjacent to the β-globin locus or Hoxd locus, respectively.20,42

Tissue-specific gene expression is mediated by specific interactions of the promoter with distal tissue-specific enhancers, and as such a tissue-specific 3D chromosomal conformation is expected for different cell types. Contrary to this, however, our data indicate that the chromosomal architecture of the Tbx3/Tbx5 locus is strikingly similar between different tissue types. Contact profiles in heart and limb cells reveal a fixed chromosomal architecture with multiple sites contacting the Tbx3 promoter, including eA and eB. In brain cells, the contact profile seems somewhat different, displaying slightly more Tbx3-Tbx5 interdomain contacts than other tissues tested (Figure 4B and 4C). Tbx3 is expressed in the developing heart and limbs, whereas its expression in the developing brain is limited or absent. The observed variation in the topology of the Tbx3 locus might thus correspond to its inactivity in this tissue. We propose that the genomic contacts of eA, eB, and the Tbx3 promoter are mostly tissue independent and organized in a preformed, permissive chromatin structure, in which functional enhancer–promoter interactions are facilitated by the rigid, cell-type invariant structural enhancer contacts.19 The enhancer regions and the promoter are in close proximity irrespective of tissue type, which thus facilitates CCS-specific transcription factor complexes in the activation of Tbx3 transcription in the CCS. In other cell types, the CCS-specific enhancers are not activated but remain in close proximity to the promoter.

Analysis of the chromosomal interactions in human fibroblasts assessed by Hi-C reveals a similar pattern as observed in mouse. Multiple regions upstream of Tbx3 contact the gene and these regions do not overlap with regions contacting Tbx5 or MED13L (Online Figure 1). This indicates that the genomic organization of the Tbx3/Tbx5 locus is not only conserved between tissues but also between mammalian species, which reveals a robust conservation of the topological Tbx3/Tbx5 domains. Although CTCF appeared to be not required for the insulation of the Tbx3 regulatory domain, it has been well described as a factor frequently present at the boundaries of topologically associated domains. The highly similar occupancy profiles of CTCF between different tissue types (Online Figure II A) therefore further indicates that the 3D organization of the Tbx3 locus is largely conserved and cell type independent.

Genome-wide association studies have identified common genetic variants in the human TBX3/TBX5 locus that influence CCS function.16,17 Tbx3 and Tbx5 are required for functional development, maturation, and homeostasis of the CCS in a highly dosage-sensitive manner, indicating that minor changes in regulatory sequences could potentially have consequences for CCS function and homeostasis.12,43 Based on the contact profile of the Tbx3 and Tbx5 promoters and their separate regulatory domains, we propose that all the regulatory elements mediating Tbx3 or Tbx5 expression, respectively, are confined within their respective domains marked by CTCF-binding sites. This indicates that the functional effect of genetic variation is limited to either the Tbx3 or Tbx5 domain, respectively, and within those domains to the regions contacting the respective promoters. Our data, therefore, facilitates the assignment of function to genomic variants identified in genome-wide association studies.

Identification of enhancers driving CCS-specific Tbx3 expression is crucial if one is to fully understand the mechanisms underlying CCS development and function. We report the identification of 2 enhancers within the Tbx3 domain that synergistically drive the atrioventricular conduction system expression of Tbx3. Major components of the Tbx3+ atrioventricular conduction system are the slow-conducting atrioventricular node and rings and the fast-conducting atrioventricular bundle and bundle branches. These components derive from the embryonic atrioventricular canal and crest of the interventricular septum, respectively, which are interconnected throughout development.44 The atrioventricular canal component is evolutionarily conserved in vertebrates.45 Our data reveal that the regulatory sequences regulating Tbx3 in these respective components are separated in the genome and suggest that eA is responsible for driving Tbx3 expression in the conserved AVC component through a BMP signaling–mediated mechanism. Although on its own weak, eB seems to synergize with eA to drive robust Tbx3 expression in the AVC and atrioventricular bundle precursor region in the interventricular septum. We speculate that eB may have been recruited during the evolution of the atrioventricular bundle, a structure, as yet, only found in birds and mammals. These elements may come together to form a single regulatory unit, leading to an expression pattern that is wider than the sum of each isolated element alone.46,47 The contribution of presumably weak elements such as eB could, therefore, be to provide robustness to the complex regulatory structure.

Taken together, our data show that the Tbx3 locus forms a conserved, independent regulatory unit physically separated from neighboring loci. The chromosomal topology of this unit is in a preformed and permissive organization, which is tissue independent and evolutionary conserved. Multiple sites within this unit synergize to control the precise expression of Tbx3 in the CCS. This study provides insight into the tight relationship between chromatin topology and the strict regulation of the evolutionary conserved Tbx3/Tbx5 gene cluster.

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Disclosures

None.

References

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Novelty and Significance

**What Is Known?**

- The T-box transcription factors Tbx3 and Tbx5 play crucial roles in the development and function of the cardiac conduction system.
- Tissue-specific gene expression is mediated by genomic regulatory sequences that act in a time-, tissue- and dose-dependent manner.
- Common genetic variants in noncoding sequences close to Tbx3 and Tbx5 are associated with cardiac conduction system parameters.

**What New Information Does This Article Contribute?**

- The Tbx3/5 genomic cluster is organized in a preformed 3-dimensional structure, independent of tissue type.
- The Tbx3 genomic locus is physically separated from that of Tbx5, indicating both genes do not share regulatory sequences to drive their transcription.
- We identified 2 genomic enhancers that are required and sufficient to drive Tbx3 expression in the atrioventricular conduction system.

Tbx3 and Tbx5 are crucial for the development and function of the cardiac conduction system. Recent studies have identified common variations in the human genome close to Tbx3 and Tbx5 to be associated with cardiac impulse conduction. We show here that the Tbx3/5 genomic locus is organized in a preformed 3-dimensional conformation, independent of tissue type. The Tbx3 domain is physically separated from the neighboring Tbx5 domain, indicating both genes do not share regulatory sequences to drive their transcription. This suggests that the functional effect of genetic variation in either one of the domains can be exclusively attributed to the respective gene, which facilitates the assignment of function to genomic variations associated with cardiac conduction. Furthermore, we identified 2 transcriptional enhancers that are sufficient and required to drive Tbx3 expression in the atrioventricular conduction system. Together, these findings provide insight into the close relationship between chromatin conformation and the complex regulation of the Tbx3/5 gene cluster.
A Large Permissive Regulatory Domain Exclusively Controls Tbx3 Expression in the Cardiac Conduction System
Jan Hendrik van Weerd, Ileana Badi, Malou van den Boogaard, Sonia Stefanovic, Harmen J.G. van de Werken, Melisa Gomez-Velazquez, Claudio Badia-Careaga, Miguel Manzanares, Wouter de Laat, Phil Barnett and Vincent M. Christoffels

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Circular chromosome conformation capture

4C was performed as previously described. In short, fore- and midbrain, limbs and heart were dissected from E10.5 embryos and processed as described to obtain single-cell samples. Approximately $10^7$ cells were used for fixation and lysis, and cross-linked chromatin was digested with *DpnII* (New England Biolabs, cat.no. R0543M). Samples were treated with T4 DNA ligase (Roche, cat.no. 10799009001) to obtain large DNA circular molecules, encompassing multiple restriction fragments held together by cross-links. After cross-link removal by heating, DNA was digested with *Csp6I* (Fermentas, cat.no. ER0211). Final ligation of restriction fragments yields circular DNA templates for 4C PCR amplification. For all experiments, 200 ng of the resulting 4C template was used for the subsequent PCR reaction, of which 16 (total: 3.2 μg of 4C template) were pooled and purified for next-generation sequencing. The PCR products were purified using two columns per sample of the High Pure PCR Product Purification Kit (Roche cat. no. 11732676001). Inverse PCR primers specific for fragment of interest (viewpoint) are listed in Table S1. Primers were designed and data analysis for mapping and normalizing 4C-seq data was performed as described in ref. In short, the rich fragment pool (around 6-8 fragment ends per 1kb) was used to generate statistically robust semiquantitative contact maps in the 10kb to 1Mb region surrounding the viewpoint. Moreover, the high-resolution restriction site grid was used to quantify contact intensities in genomic windows varying in size from a few kilobases to tens of kilobases. Chromosomal contacts in human fibroblasts were assessed using Hi-C data generated by Jin *et al.*

BAC modification and generation of transgenic mice

BACs RP24-89K7 and RP23-143N21 were obtained from C57BL/6J mouse BAC libraries (CHORI, BACPAC Resources) and modified to generate 89K7-GFP and 143N21-GFP. A GFP reporter gene cassette was inserted in the translational start site of Tbx3 according to the two-step BAC modification protocol as described in ref. 3. Generation of the 366H17-GFP mouse line was described previously. To obtain ΔeAeB 89K7-GFP, eA and eB enhancer sequences were sequentially deleted from GFP-89K7 using the abovementioned protocol with shuttle vectors in which both fragments were absent. Modified BAC DNA was purified with the NucleoBond PC 20 kit (Macherey-Nagel) according to manufacturer's instructions and injected into mouse pronuclei of FVB/N mice. Microinjected zygotes were implanted into pseudo-pregnant females (E0.5) and transgenic embryos were isolated in PBS at the indicated day, visualized by fluorescence microscopy, then fixed in 4% paraformaldehyde in PBS for 4 h and processed for *in situ* hybridization or immunohistochemistry. At least two independent transgenic lines per BAC construct were analysed to define the GFP expression pattern and to account
for variation in expression pattern due to position effect. The sequences of primers used for cloning and genotyping are available upon request. All animal work was approved by the Animal Experimental Committee of the Academic Medical Center, University of Amsterdam, and carried out in compliance with Dutch government guidelines.

**Ctcf conditional knockout**
The *Ctcf* floxed allele and *Nkx2.5-Cre* line were previously described.5,6 Mice were bred in the core animal facility in the Centro Nacional de Investigaciones Cardiovasculares in accordance with national and European legislation. Three pools of 2 to 4 wildtype and *Ctcf* KO hearts were used for qRT-PCR. 500 ng of total RNA was reverse transcribed using random primers (Applied Biosystems). Expression changes were normalized to actin. PCR was performed in an AB7900_384 (Applied Biosystems) using Sybrgreen (Applied Biosystems) as reporter. qRT-PCR primers are available upon request. Homo- or heterozygous mice for the *Ctcf*-floxed allele that did not carry the *Nkx2.5-Cre* were used as controls.

**In-situ hybridisation**
After fixation in 4% PFA in PBS, embryos were embedded in paraplast and sectioned at 10 µm. Non-radioactive in situ hybridization on sections was performed as previously described.7,8 Probes for Tbx3 and GFP have been described in ref. 4 Sections were observed with a Zeiss Axiophot microscope and photographed with a Leica DFC320 Digital Camera.

**Immunohistochemistry**
After fixation, embryos were embedded in paraplast and sectioned at 8-10 µm for immunohistochemistry. Anti-Tbx3 (E-20, sc-31656) goat polyclonal (1:200; Santa Cruz Biotechnology Inc.), anti-GFP (ab5450) goat polyclonal (1:200; Abcam) primary antibodies and Alexa Fluor 488 donkey anti-goat IgG, Fluor 568 donkey anti-goat IgG or Fluor 680 donkey anti-goat IgG (1:250; Invitrogen) secondary antibodies were used. Stained sections were photographed with a fluorescence microscope Leica DM6000.

**ChIP-sequencing**
ChIP-sequencing data sets for Nkx2-5, Gata4, Tbx5 and Tbx3 were described in refs. 9,10. ChIP-seq datasets for p300, Pol2, CTCF and DNase hypersensitivity sites were generated by the ENCODE consortium.11 ChIP-sequencing datasets for H3K27ac are described in ref. 12. Peaks were called using the peak caller MACS, using corresponding input control tissue, and by an in-house peak calling algorithm dubbed Occupeak (de Boer, van Duijvenboden et al., PLoS-One, in press).
Identification of consensus binding sites

Consensus binding sites were identified using JASPAR\textsuperscript{13} and TRANSFAC\textsuperscript{14} software. Sequences were tested for enrichment of binding sites for cardiac transcription factors Nkx2-5, Gata4, T-box factors and Bmp signaling effector Smads. Relative profile score threshold for JASPAR was set to 85%. TRANSFAC parameters were set at the default setting for vertebrate sequences and the minFP setting to minimize false positive scores.

Chromatin immunoprecipitation

E14.5 embryos were dissected, and the hearts were isolated and collected in cold PBS. ChIP was performed using the True MicroChIP kit (Diagenode) according to the manufacturer’s protocol. The antibodies used for ChIP were anti-H3K27ac (0.5μg, Abcam ab4729) and anti H3K4me1 (0.5μg, Abcam ab8895). Quantitative PCR was performed on a Roche LightCycler 480 System using Sybr Green detection. Fold enrichment indicates the ratio of ChIPed DNA to a negative control region, normalized for input DNA. Primer sequences are provided in Table S2.

In vivo enhancer screening assay

Mouse eB (chr5:120,024,484-120,025,600; NCBI37/mm9), 3.4kb-eB (chr5:120,024,225-120,027,612), eA (chr5:120,100,494-120,102,560), eAb (chr5:120,101,525-120,102,566), human eA (chr12:115,140,877-115,142,783; NCBI36/hg18) and chicken eA (chr15:12,509,382-12,511,893; WUGSC2.1/galGal3) enhancer sequences were PCR-amplified and cloned in the HindIII restriction site of the Hsp68-LacZ reporter vector.\textsuperscript{15} eA was subcloned into the SmaI site of eB-Hsp68-LacZ vector to obtain the eAeB-Hsp68-LacZ construct. After vector backbone excision, linearized fragments were injected into the pronucleus of 0.5-day-old fertilized FVB/N eggs, which were transferred into the oviducts of CD-1 pseudopregnant foster females (Cyagen Biosciences). Embryos were harvested and stained with X-gal to detect LacZ activity.

In vitro reporter assay

eA (chr5:120,100,494-120,102,560), eA1 (chr5:120,100,494-120,101,620), eA2 (chr5:120,101,526-120,102,560), eB (chr5:120,024,484-120,025,600) and eAeB were cloned in the pGL2-SV40 luciferase construct containing a minimal promoter (Promega). (Tbx2)4 construct was previously described.\textsuperscript{12} The constructs were transfected in Cos-7 cells using PEI as transfection reagent (DNA:PEI ratio 1:4). Constructs were co-transfected with expression constructs for Gata4, Smad1, Smad4 (all cloned in pcDNA3.1) and constitutively active Bmp-receptor Alk3 (Alk3CA; pCS2.BmpR1a.CA). Cell extracts and luciferase assays were performed according to the manufacturer’s protocol (Promega). Mean luciferase activities and standard deviations (s.d.) were
plotted as fold activation compared to the empty pGL2-SV40 expression plasmid. Transfections were carried out in triplo.
Online Figure II

A

Heart

Cerebellum

Limb

500kb

Med13l  Tbx3  Tbx5  Rbm19

B

WT  Ctcf -/-

Tbx3

ra  la  avc  rv  lv

Tbx5

C

mRNA Relative Expression

**

Ctcf  Tbx3  Tbx5

wt  Ctcf -/-
Consensus binding sites for **Gata4**, **Nkx2-5**, **Tbx3/5** and **Smad** identified by JASPAR\textsuperscript{13} and TRANSFAC\textsuperscript{14}.
SUPPLEMENTAL FIGURE LEGENDS

Online Figure I. Conservation of topological Tbx3/Tbx5 domains
Contact profiles with Med13l, Tbx3 and Tbx5 promoters as viewpoints in mouse heart (upper tracks, 4C data) and human fibroblasts (lower tracks, Hi-C data generated by Jin et al.2). Arrows depict point of view for each track. Similar to the mouse genome, contacts with TBX3 in human are restricted to the upstream domain and do not overlap with regions contacting MED13L (upstream) and TBX5 (downstream).

Online Figure II. cKO of CTCF in cardiomyocytes does not result in altered gene expression
(A) UCSC browser views of ChIP-seq data (ENCODE consortium11) depicting occupancy of CTCF in heart, cerebellum and limb (E14.5). Lower traces depict peaks called by MACS, indicating the high similarity of occupancy profiles between the three tissue types. (B) In-situ hybridisation on sections through WT and Ctf-/- embryos at E10.5 reveal no misexpression of Tbx3 and Tbx5 in the heart. (C) qPCR validation of CTCF, Tbx3 and Tbx5 mRNA levels. CTCF mRNA levels are significantly reduced in Ctf-/- embryos, whereas Tbx3 and Tbx5 levels were unaffected.

Online Figure III. Cell-type specific H3K27 acetylation associates with gene activity
Occupancy profiles of H3K27ac in the Tbx3 and Tbx5 locus as assessed by ChIP-seq in AVC and chamber myocardium. Y-axis in upper tracks depicts normalized tag count. Lower tracks depict peaks called by our in-house peakcalling algorithm called OccuPeak (de Boer, van Duijvenboden et al., PLoS-One, in press).

Online Figure IV. GFP-modified BACs recapitulate Tbx3 expression in neural crest-derived cell
In-situ hybridisation and immunohistochemistry on sections through 89K7-GFP, 143N21-GFP and 366H17-GFP (not shown) embryos at E11.5 reveal GFP expression in cardiac neural crest cells flanking the outflow tract (arrows) and the outflow tract cushions (neural crest-derived cells; asterisks).

Online Figure V. Overview of transcription factor binding sites in eA and eB
Enrichment of binding sites for cardiac transcription factors Nkx2-5, Gata4, T-box factors and Bmp signalling effector Smads as revealed by JASPAR13 and TRANSFAC14 software.

Online Figure VI. Transcription factor occupancy and histone modifications of eA and eB
(A) UCSC browser view of ChIP-sequencing datasets, revealing the occupancy of eA and eB by Tbx3, p300, Pol2 and H3K27ac. Y-axis depict normalized tag count. (B) ChIP-qPCR confirming eA and eB
are marked by H3K4me1 and H3K27ac. Y-axis depicts fold activation over a negative control region. Bars represent mean±s.d. Experiments are performed in duplo.

**Online Figure VII. Physical interactions of Tbx3, eA and eB**

Contact profiles of with Tbx3 promoter, eA and eB as viewpoints (arrows) reveal both enhancers and the Tbx3 promoter physically contact each other (asterisks). The legend for the color-coded scaling plots can be found in the legend of Figure 1.
<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>CTTACACGACGCTCTTCCGATCTGAATGCTCTGATTC</td>
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<td>CTTACACGACGCTCTTCCGATCTGAATGCTCTGATTC</td>
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<tr>
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<tr>
<td>eB_F</td>
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<td>TCAGAAAAAAGAAGGAGGAGGAGGAGGAGGAGGAGG</td>
</tr>
</tbody>
</table>
Online Table II. qPCR primer sequences

| Primer |
|------------------|--------------------------|
| Tbx3_Promoter_F  | CAGCAGCTCGACCTGTGAAAA   |
| Tbx3_Promoter_R  | ATTGGCTCTTTTGAGGCTTTTC  |
| Tbx3_eA_F        | CAGCTGAGGCCTTCAGGAT     |
| Tbx3_eA_R        | GGTGTGAAACATCCCTTCTGA   |
| Tbx3_eB_F        | TCCCCGAAGCTGACCTTTCTGA  |
| Tbx3_eB_R        | GTGTGTGCTGGCTTTCTTG     |
| negative control region 1_F | TCCAGACACACTGAACAGCAC |
| negative control region 1_R | TCCCAGATCAAGTCTCAC     |
| negative control region 2_F | CCAGACACACTGACAGCAC   |
| negative control region 2_R | TGCAACAGTGTCTGGAATG    |

* (chr4: 147397477 – 147397580)
** (chr4: 147397478 – 147397598)
References


