Genomic mapping of Tbx3 interactions in the heart

Wong, L.Y.E.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 4

Combinatorial ChIP-seqs of Multiple Cardiac Factors Accurately Predicts Cardiac Scn5a/Scn10a Enhancers

L.Y. Elaine Wong and Phil Barnett

Modified version published as:
Genetic Variation in T-box Binding Element Functionally Affects SCN5A/SCN10A Enhancer
Malou van den Boogaard, L.Y. Elaine Wong, Federico Tessadori, Martijn L. Bakker, Lisa K. Dreizehnter, Vincent Wakker, Connie R. Bezzina, Peter A.C. ‘t Hoen, Jeroen Bakkers, Phil Barnett, and Vincent M. Christoffels

Chapter 4

Abstract

Tbx3, belonging to the T-box transcription factor family, plays an important role in development of the cardiac conduction system. It maintains the nodal-like phenotype of the nodal components, the atrioventricular bundle, and the proximal bundle branches by suppressing working myocardial genes and thereby the differentiation of nodal-like myocardium into working myocardium. Except for a few of the working myocardial genes found to be directly repressed by Tbx3, little data exists regarding the direct gene targets of Tbx3. Here, we perform chromatin immunoprecipitation sequencing (ChIP-seq) analysis of Tbx3 in mouse heart, revealing Tbx3-bindings genome-wide. By combining the Tbx3 binding-profile and the expression profile of genes regulated by Tbx3 in the AV canal/node, we find that many of the Tbx3 direct target genes are related to ion channels, including cardiac sodium channel genes Scn5a and Scn10a. During transcriptional regulation, Tbx3 interacts and works synergistically with other cardiac transcription factors like Gata4 and Nkx2-5. The combinatorial approach of Tbx3, Gata4, Nkx2-5 and enhancer-associated co-activator p300 ChIP-seq data sets shows two co-occupied regions in Scn5a and Scn10a, which we demonstrate to be enhancer elements regulated by Tbx3 and Tbx5. Our transgenic mouse enhancer assay shows that the LacZ reporter gene expression-pattern driven by these enhancer elements resemble to Scn5a and Scn10a expression-patterns in the heart, implicating that these enhancer elements are potentially responsible for the expression of Scn5a and Scn10a in the heart.

Introduction

The evolutionarily conserved T-box transcription factor family plays a crucial role in defining gene expression networks in developmental processes and lineage specification \(^1, 2\). T-box genes are expressed in various tissues in both unique and overlapping areas at different developmental stages \(^3\). T-box proteins can bind DNA through their highly conserved \(~180\) amino acids long DNA-binding domain termed the ‘T-box’. For so far tested \textit{in vitro}, T-box factors share a common DNA binding sequence (TCACACCT) termed the T-box binding element (TBE) \(^4-7\). Although it seems that T-box factors bind the same sequence \textit{in vitro}, they differ in their transcriptional properties; e.g. Tbx5 is known to act as an activator while Tbx3 can repress gene transcription \(^8, 9\). Transcriptional functionality of T-box factors is provided by binding of specific gene promoters and enhancer elements, and by the interactions with other transcription factors \(^2, 10-12\). Zinc finger-containing Gata4 and homeobox-containing Nkx2-5 are known to be interacting protein partners of Tbx2,
Tbx3 and Tbx5, and Msx1, Msx2 and Sox4 interact with Tbx3 \(^1,^{13-15}\), synergistically working with the T-box factors to modulate gene expression.

T-box factor Tbx3, belonging to the Tbx2 subfamily, is closely related to Tbx2 in structure and function. Tbx3 is expressed in various tissues during development, including the heart, limbs and lungs \(^2\). It functions as transcriptional repressor, and like other members of the T-box transcription factor family, it is involved in many embryonic developmental processes \(^1,^{2}\). In addition to its role during development, Tbx3 seems to play a role in tumorigenesis; it has been found over-expressed in a number of cancers including lung and breast cancers. Tbx3 contributes to tumorigenesis probably by suppressing the expression of tumor suppressor proteins p14ARF and p21CIP1 and thereby promoting bypass of senescence \(^16-18\).

Tbx3 plays an important role in heart development \(^2,^{19,^{20}}\). Mutations in Tbx3 have been associated with the human genetic disease ulnar-mammary syndrome, which has recently been reported to display cardiac defects as one of the phenotypes \(^21,^{22}\). In the heart throughout embryogenesis, Tbx3 is specifically expressed in the sinusatrial node (SAN), atrioventricular node (AVN), atrioventricular bundle (AVB), and the proximal part of the bundle branches (BBs), which are components of the cardiac conduction system. Tbx3 defines and maintains the nodal like phenotype of these components by repressing the working myocardial genes (such as \(Nppa\) and \(Cx43\)), thereby preventing their differentiation into the working myocardial phenotype \(^23,^{24}\). Tbx3 expression persists in these tissues in the heart into adult life \(^23-26\). Deficiency of Tbx3 in mice resulted in expansion of expression of working myocardial genes into the SAN. Normally, the expression of Tbx3 is absent in the atrial and ventricular working myocardium. Ectopic expression of Tbx3 in the atrial working myocardium of mice led to ectopic pacemaking activity in the atria and arrhythmias, showing the important role of Tbx3 in the regulation of the electrical components of the nodal cells \(^24\). These findings provided insights into the role of Tbx3 in development of the cardiac conduction system. However, the underlying molecular mechanisms need to be further elucidated and little data exists regarding the direct DNA targets of Tbx3.

Chromatin immunoprecipitation coupled to massively parallel sequencing (ChIP-seq) together with microarray analysis can provide insights into direct target genes of a specific protein such as a transcription factor. ChIP-seq analysis has been shown to be an invaluable tool for mapping genome-wide protein-binding sites and epigenetic marks \(^27-30\). Transcription factors typically regulate gene transcription through binding to DNA in a sequence-specific fashion. The genomic binding sites of transcription factors are often cis-acting regulatory DNA elements, elements that are responsible for the spatial and temporal expression patterns of genes \(^31-34\). Such elements, enhancers, have been identified within introns in genes
and in conserved non-coding regions flanking genes, some of which are located at a considerable distance from their target transcription start site (>100 kb).

Here, we performed ChIP-seqs of three major cardiac transcription factors Tbx3, Gata4 and Nkx2-5. Overlaps of the results of these ChIP-seqs revealed co-occupation in a significant number of genes involved in cardiac conduction including cardiac sodium channel genes Scn5a and Scn10a. These genes were used as model genes to prove our hypothesis that the combined ChIP-seqs of cardiac transcription factors could reveal the locations of in vivo cardiac enhancers. Two sequences in Scn5a and Scn10a were co-occupied by multiple factors, and in vivo and in vitro enhancer assays demonstrated active enhancing activities exerted by these sequences. Mutation and EMSA analysis showed that T-box binding site present in these two enhancer sequences was responsible for binding and regulation of Tbx3. All together, we identified cardiac specific Scn5a and Scn10a enhancers modulated by a network of cardiac regulators involving Tbx3.

**Figure 1** Tbx3 ChIP-seq validation.

(A) Tbx3 binding-regions (peak 1 and 2) and non binding-regions (i en ii) in Scn5a found in Tbx3 ChIP-seq of adult mouse heart with ectopic TBX3 expression are validated for Tbx3 bindings by ChIP-PCR. Hprt was used as non-binding control gene. (B) Significant Tbx3 binding peaks are present in a number of previously published T-box binding genes, including Nppa, Cx43, Cx30.2 and Myh6.
Figure 2  In vivo ChIP-seq analysis in adult mouse heart (A) Alignment of ChIP-seq datasets from mouse heart (Tbx3, red; Gata4, blue; Nkx2-5, green; p300, brown) described in this manuscript in the vicinity of Hopx, a gene involved in conduction system function. (B) Overlap of all ChIP-seq binding regions with genes in refGene plus their promoters, defined here as the 1 kb upstream region of those genes. Values are percent of all peaks. (C) Results obtained from the MEME motif discovery analysis. Binding motifs are in good agreement with those recently published for the HL-1 based ChIP-seq and JASPAR motifs. (D) Venn diagram showing the number of overlapping binding regions between heart-derived ChIP-seq datasets of Tbx3, Nkx2-5 and Gata4. (E) Molecular function GO-term analysis of genes containing an intragenic overlapping Tbx3/Nkx2-5/Gata4-ChIP-seq peak region.

### Table: Molecular function GO-term analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Count</th>
<th>%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeleton organization</td>
<td>8</td>
<td>8.9</td>
<td>1.4E-4</td>
</tr>
<tr>
<td>Actin cytoskeleton organization</td>
<td>5</td>
<td>5.6</td>
<td>1.0E-2</td>
</tr>
<tr>
<td>Actin filament-based process</td>
<td>5</td>
<td>5.6</td>
<td>1.3E-2</td>
</tr>
<tr>
<td>Heart development</td>
<td>5</td>
<td>5.6</td>
<td>2.8E-2</td>
</tr>
<tr>
<td>Actin filament organization</td>
<td>3</td>
<td>3.3</td>
<td>3.4E-2</td>
</tr>
<tr>
<td>Muscle contraction</td>
<td>3</td>
<td>3.3</td>
<td>3.8E-2</td>
</tr>
<tr>
<td>Intracellular signalling cascade</td>
<td>3</td>
<td>3.3</td>
<td>4.4E-2</td>
</tr>
<tr>
<td>Muscle system process</td>
<td>10</td>
<td>11.1</td>
<td>4.7E-2</td>
</tr>
</tbody>
</table>
Results

Validation of Tbx3 ChIP-seq data for T-box bindings
Chromatin-immunoprecipitation coupled to massively parallel sequencing (ChIP-seq) of Tbx3 was applied to determine genome-wide binding-sites of Tbx3 in adult mouse heart. Tbx3 is only expressed in a small fraction (the conduction system) of the total adult heart (Horsthuis et al. 2009) and its amount is too low for efficient ChIP-seq. Therefore, for the Tbx3 ChIP-seq analysis, we used adult mouse heart with ectopic Tbx3 expression in cardiomyocytes. This mouse heart contained a Cre-inducible TBX3 expression cassette (CT3) and a cardiomyocyte-specific tamoxifen inducible Cre expression cassette (Myh6-mER-Cre-mER) (mouse model was described previously 23). Mouse heart that only possessed the Myh6-mER-Cre-mER cassette served as ChIP control.

To validate the results of the Tbx3 ChIP-seq, a number of regions with or without Tbx3 binding-peaks were tested for Tbx3 bindings by ChIP-PCR using adult mouse heart with ectopic TBX3 expression (Fig. 1a). Hprt was used as non-binding control gene. The ChIP-PCR confirmed that regions with ChIP-seq peaks represented indeed Tbx3-bindings and regions without the peaks represented non-binding. Furthermore, we checked occupancy of a number of genes previously published as T-box binding targets. Numerous known T-box binding genes were identified as significant Tbx3-binding regions in our Tbx3 ChIP-seq, including natriuretic precursor peptide A (Nppa) 4, 40, connexin 43 (Cx43, also known as Gja1) 24, connexin 30.2 (Cx30.2, also known as Gjd3) 11 and alpha-cardiac myosin heavy polypeptide 6 (Myh6) 41 (Fig. 1b). JASPAR database (MA0009.1 T binding-site with score threshold 70%) was used for the search of consensus T-box binding sites within the Tbx3-binding peaks in these genes. The T-box binding site found within the Tbx3 binding-peak in the Nppa promoter (Fig. 1b) matched a published T-box binding site responsible for Tbx5 activation of Nppa 4. Taken together, the Tbx3 binding-peaks found in our Tbx3 ChIP-seq could be considered as solid Tbx3 binding-sites.

Combinatorial ChIP-seq analysis of Tbx3, Nkx2-5 and Gata4
We also established the occupancy of endogenous Nkx2-5 and Gata4, two cardiac transcription factors that cooperate with T-box factors. It has been shown that occupancy by the enhancer-associated protein p300 can predict the location of heart enhancers 42. Therefore, we also include the previously published occupancy profile of p300 42 (Fig. 2a) in the assessment of enhancer locations. We first assessed the quality of our ChIP-seq experiments by determining the overlap in peaks between the Tbx3 ChIP-seq dataset and the control ChIP-seq dataset. These datasets possessed a total of 25446 and 1089 peaks, respectively, with an
Combinatorial ChIP-seqs Accurately Predicts Cardiac Scn5a/Scn10a Enhancers

overlap of 131. The overlapping peaks were subtracted from the main Tbx3 dataset as likely false positive peaks, remaining 25315 as Tbx3 binding-peaks (Fig. 2b). Next, we determined the number of peaks for both the Nkx2-5 and the Gata4 ChIP-seq datasets (Fig. 2b). The resulting datasets were used to analyze the distribution of factor binding regions across the genome. For this analysis, a promoter region of 1kb upstream of all genes was used to define the number of peaks associated with a promoter. The distribution of the remaining peaks was then assessed in terms of intragenic or intergenic. This analysis revealed that the majority of peaks in all 3 data sets were positioned within a gene sequence (exons and introns), with a significant enrichment (p<0.001) of peaks associated with a promoter region.

To investigate whether the ChIP peaks could be directly correlated to DNA binding elements for Tbx3, Nkx2-5 and Gata4, we performed de novo motif discovery using MEME. Using 600 random peaks of each transcription factor, MEME yielded motifs (Fig. 2c) in good agreement with the motifs derived from ChIP-seq data of biotinylated Tbx5, Nkx2-5 and Gata4 expressed in HL-1 cells.

### GOTERM: Molecular Function

<table>
<thead>
<tr>
<th>Genes</th>
<th>Count</th>
<th>%</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>metal ion binding</td>
<td>67</td>
<td>26.9</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>ion binding</td>
<td>68</td>
<td>27.3</td>
<td>1.10E-03</td>
</tr>
<tr>
<td>cation binding</td>
<td>67</td>
<td>26.9</td>
<td>1.30E-03</td>
</tr>
<tr>
<td>gated channel activity</td>
<td>11</td>
<td>4.4</td>
<td>2.50E-03</td>
</tr>
<tr>
<td>cation channel activity</td>
<td>7</td>
<td>2.8</td>
<td>2.80E-03</td>
</tr>
<tr>
<td>calmodulin binding</td>
<td>3</td>
<td>1.2</td>
<td>3.00E-03</td>
</tr>
<tr>
<td>NAD(P)+-protein-arginine ADP-ribosyltransferase activity</td>
<td>10</td>
<td>4</td>
<td>3.50E-03</td>
</tr>
<tr>
<td>cation channel activity</td>
<td>12</td>
<td>4.8</td>
<td>5.40E-03</td>
</tr>
<tr>
<td>channel activity</td>
<td>12</td>
<td>4.8</td>
<td>5.40E-03</td>
</tr>
<tr>
<td>passive transmembrane transporter activity</td>
<td>12</td>
<td>4.8</td>
<td>5.40E-03</td>
</tr>
<tr>
<td>voltage-gated ion channel activity</td>
<td>8</td>
<td>3.2</td>
<td>6.60E-03</td>
</tr>
<tr>
<td>voltage-gated channel activity</td>
<td>8</td>
<td>3.2</td>
<td>6.60E-03</td>
</tr>
<tr>
<td>metal ion transmembrane transporter activity</td>
<td>10</td>
<td>4</td>
<td>9.50E-03</td>
</tr>
<tr>
<td>ion channel activity</td>
<td>11</td>
<td>4.4</td>
<td>1.10E-02</td>
</tr>
<tr>
<td>substrate specific channel activity</td>
<td>11</td>
<td>4.4</td>
<td>1.30E-02</td>
</tr>
<tr>
<td>alkali metal ion binding</td>
<td>8</td>
<td>3.2</td>
<td>1.40E-02</td>
</tr>
</tbody>
</table>

**Figure 3** Tbx3 direct target genes are related to ion channel genes. (A) Molecular function Gene Ontology (GO) analysis using DAVID on genes significantly bound by Tbx3, repressed by ectopic Tbx3 expression and reduced in the AV canal/node. (B) Quantitative RT-PCR analysis shows reduced expression of 10 representative ion channel genes in mouse hearts with ectopic Tbx3 expression (black bars) compared to the control hearts (light grey bars). These genes are significantly reduced in the AV canal (p < 0.05) and contain ChIP-seq peaks for Tbx3, Gata4 and Nkx2-5.
Figure 4 Scn5a and Scn10a are down-regulated by Tbx3. (A) Relative mRNA expression levels of Scn5a and Scn10a in right and left atrium, right and left ventricle, brain, lung and spine of E14.5 mice. Both genes have high expression in the heart chambers compared to the other tested tissues. (B) In situ hybridization of Scn5a and Scn10a in the E17.5 sinoatrial node region showing complementary patterns to Tbx3 (upper panels). In situ hybridization performed in E14.5 wild-type mouse hearts showing overlap of Scn5a and Scn10a expression. SAN, sinoatrial node; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; IVS, interventricular septum. (C) Quantitative RT-PCR analysis showing significantly (p < 0.05) reduced relative mRNA levels of Scn5a and Scn10a in mouse hearts with ectopic Tbx3 expression (black bars) compared to control hearts (light grey bars), and the relative mRNA levels of neighboring genes Scn11a and Endogl1 are not affected by enhanced Tbx3 expression. (D) Overview of ChIP-seq datasets of Tbx3, Nkx2-5, Gata4 performed in adult mouse hearts and p300 performed in E11.5 embryonic mouse hearts (Blow et al.) showing the binding-peaks of these factors within the Scn5a/Scn10a locus. Tbx3 (red), Gata4 (blue), Nkx2-5 (green) and p300 (brown). (E) Tbx3 ChIP-PCR (left panel) and Tbx3 ChIP-qPCR (right panel) of E10.5 embryonic mouse hearts showing bindings of TBE2 and TBE9 with endogenous Tbx3. (F) Luciferase reporter assay showing enhancer activity in vitro. Enhancers cloned upstream of pGL2 and a minimal promoter were transfected into H10 cells with (light grey bars) or without (black bars) Nkx2-5 and Gata4. Fragments TBE1, TBE2, TBE6, TBE7 and TBE8 respond to addition of Gata4 and Nkx2-5. Fragments TBE4 and TBE9 show strong constitutive activity. The table below shows the genomic locations and sizes of the fragments tested in the luciferase assay. (G) Luciferase reporter assay showing the response of TBE1, TBE2 and TBE9 on the T-box factors. TBE1 and TBE2 are induced by Tbx5 and repressed by Tbx2 and Tbx3. TBE9 is repressed by Tbx2 and Tbx3 but not induced by Tbx5.
Combinatorial ChIP-seqs Accurately Predicts Cardiac *Scn5a/Scn10a* Enhancers

**D**

```
<table>
<thead>
<tr>
<th>Fragment</th>
<th>Genomic location</th>
<th>Size fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE1</td>
<td>119543388-119543582</td>
<td>215</td>
</tr>
<tr>
<td>TBE2</td>
<td>119543272-119541627</td>
<td>356</td>
</tr>
<tr>
<td>TBE3</td>
<td>119479888-119480145</td>
<td>258</td>
</tr>
<tr>
<td>TBE4</td>
<td>119472726-119473099</td>
<td>374</td>
</tr>
<tr>
<td>TBE5</td>
<td>119406718-119406984</td>
<td>246</td>
</tr>
<tr>
<td>TBE6</td>
<td>119398457-119398800</td>
<td>344</td>
</tr>
<tr>
<td>TBE7</td>
<td>119387350-119387991</td>
<td>619</td>
</tr>
<tr>
<td>TBE8</td>
<td>119385814-119384133</td>
<td>319</td>
</tr>
<tr>
<td>TBE9</td>
<td>119376706-119379271</td>
<td>572</td>
</tr>
</tbody>
</table>
```
We found near-perfect matches for the Gata4 motif in the JASPAR and TRANSFAC database. Interestingly, the Nkx2-5 ChIP-seq motif showed a closer resemblance to the Nkx3-2 consensus site found in the JASPAR database, compared to the Nkx2-5 consensus site. The ChIP-seq motif for Tbx3 resembles the motif for T in the JASPAR database and for Tbx5 in the TRANSFAC database.

Tbx3, Nkx2-5 and Gata4 are known to interact with each other in the regulation of transcription. Based on a recent ChIP-seq analysis of biotinylated Tbx5, Nkx2-5 and Gata4 in HL-1 cells in which it was demonstrated that overlapping peak regions often represented bona fide regulatory elements, we assessed the intersections of our in vivo Tbx3, Nkx2-5 and Gata4 datasets (Fig. 2d). Indeed, the intragenic portion of the 185 overlapping peak regions revealed significant enrichment of GO-terms for heart and muscle system development (Fig. 2e).

Combinatorial approach of Tbx3 binding-profile and profile of genes regulated by Tbx3 reveals that majority of Tbx3 direct target genes are related to ion channels

We previously identified sets of genes differentially expressed between the AV canal/node and the working myocardium. Tbx3 is important for the maintenance of the nodal like phenotype of the SAN, AVN, AVB and the proximal part of the BBs by repressing working myocardial genes in these regions. Therefore, the set of genes expressed at a lower level in the AV canal/node compared to the working myocardium may include genes repressed by Tbx3. Further, previously we also performed microarrays in mouse hearts with ectopic TBX3 expression in the myocardium (the same mouse model used for the Tbx3 ChIP-seq), which provided expression profile of genes regulated by Tbx3 (data available online as GSE31969 in GEO Accession View). The gene-set that was expressed at a lower level in hearts with ectopic TBX3 expression compared to the control hearts was assigned to be putative direct targets of Tbx3. In terms of identifying direct target genes of Tbx3, we combined these two gene-sets with peak regions of Tbx3 ChIP-seq. Only ChIP-seq peaks in or within 1 kb up-stream or down-stream of a gene were assigned to that gene. We identified 182 genes significantly bound by Tbx3, repressed by ectopic Tbx3 expression and reduced in the AV canal/node, meaning that these genes were probably directly repressed by Tbx3 in the AV canal/node. Molecular function Gene Ontology (GO) analysis using DAVID revealed that these genes were enriched for the terms related to ion channels like ion binding and gated channel activity (Fig. 3a). By using quantitative RT-PCR, we tested a number of ion channel genes that had Tbx3 binding-peaks and were reduced in the AV canal/node on their response to Tbx3. All tested ion channel genes were reduced in mouse hearts with ectopic Tbx3 expression compared to the control.
Combinatorial ChIP-seqs Accurately Predicts Cardiac Scn5a/Scn10a Enhancers

hearts, confirming that these ion channel genes might be directly down-regulated by Tbx3 (Fig. 3b).

**Tbx3 down-regulates Scn5a and Scn10a**

*Scn5a*, which encodes the main voltage-gated sodium channel Nav1.5 in the heart, was one of the ion channel genes revealed by the combinatorial approach of gene data sets described above that were down-regulated by Tbx3 in the AV canal/node. Mutations in SCN5A have been associated with multiple clinical arrhythmia syndromes including long QT syndrome and Brugada syndrome, showing the importance of Scn5a in cardiac conduction. In addition to Scn5a, Scn10a was also found to be down-regulated by Tbx3 in our approach. It encodes the Nav1.8 sodium channel that belongs to the same gene family as Scn5a. SCN10A has only recently been revealed to have a role in cardiac conduction in genome-wide association studies (GWAS). SCN10A is located upstream next to SCN5A on human chromosome 3. Both SCN5A and SCN10A are highly conserved in man and mice. Quantitative RT-PCR and *in situ* hybridization showed that the expression patterns of Scn5a and Scn10a were similar during the development of the mouse heart (Fig. 4a and 4b). The mRNA expression levels of Scn5a and Scn10a were high in the heart chambers compared to tissues of the brain, lung and spine (Fig. 4a), although Scn5a expression was stronger than Scn10a expression (Fig. 4a and 4b). Scn5a and Scn10a expression were present in the crest of the interventricular septum and the ventricular trabeculated subendocardium (Fig. 4b, bottom panels) and were low in the SAN and AV canal/node, regions where Tbx3 is expressed (Fig. 4b). Quantitative RT-PCR analysis showed that Scn5a and Scn10a expression were reduced significantly (p<0.05) by 60% and 80% in the hearts with ectopic Tbx3 expression compared to the control hearts, respectively (Fig. 4c). Scn11a and Endogl1, which are located up-stream of Scn10a and down-stream of Scn5a, respectively, did not respond to Tbx3 (Fig. 4c), indicating that the Tbx3-regulation of this locus is confined to Scn5a and Scn10a. Taken together, Scn5a and Scn10a are down-regulated by Tbx3 in the AV canal/node and this regulation might be in a coordinated fashion.

**Co-occupancy by multiple cardiac transcription regulators reveals candidates for Scn5a and Scn10a enhancers**

Scn5a and Scn10a were used as model genes to prove our hypothesis that the combined ChIP-seqs of cardiac factors Tbx3, Nkx2-5, Gata4 and p300 could reveal the locations of *in vivo* Tbx3 regulated cardiac enhancers. From the overlapping data we observed that, within and near Scn5a and Scn10a, the binding-sites of these factors aligned in three regions, which we labeled TBE1, TBE2 and TBE9 (Fig. 4d). TBE1 and TBE2 were located close to each other in the non-coding
Figure 5 Reporter activity of mouse Scn5a and Scn10a enhancers. (A) Overview of the genomic positions of the putative Scn10a enhancer TBE1-2 and Scn5a enhancer TBE9. Magnifications of these enhancer regions depicting the presence of bindings of Tbx3 (red), Gata4 (blue), Nkx2-5 (green) and p300 (brown). (B) Lateral views of whole embryos and magnified dorsal view of hearts containing TBE1-2-LacZ or TBE9-LacZ reporter construct. Both TBE1-2 and TBE9 enhancers show LacZ expression in the interventricular septum, atria and ventricles. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; IVS, interventricular septum.
Figure 6 Regulatory T-box binding sites in Scn5a and Scn10a enhancers. (A) Overview depicting the position of the generated 3 bp mutations labeled as TBE2m and TBE9m (marked in red) in the T-box binding sites (in frame) present in TBE2 and TBE9. (B) Luciferase reporter assay in Cos-7 cells shows that the mutations TBE2m and TBE9m generated in TBE2 and TBE9 lead to less effect of Tbx3 on the enhancing activities. (C) Electrophoretic mobility shift assay (EMSA) showing relative oligonucleotide binding of MBO fused Tbx3 (T-box3) compared to MBP only. MBP-Tbx3 fusion associates well with an Nppa probe. MBP-Tbx3 shows lower level of association with a probe containing the TBE2m or TBE9m mutation compared to a probe containing the wild-type (wt) TBE2 or TBE9 enhancer sequence.
region of Scn10a. TBE9 was located in the intergenic region down-stream of Scn5a. TBE2 and TBE9 were found to interact with endogenous Tbx3 in embryonic (E10.5) mouse hearts by using Tbx3 ChIP-PCR and ChIP-qPCR (Fig. 4e). We tested TBE1, TBE2 and TBE9, together with six other regions with Tbx3 binding-peaks within or near Scn5a and Scn10a (TBE3 to TBE8), for their ability to induce expression of a luciferase reporter gene in H10 cells (Fig. 4f). The previous findings that the region flanking TBE4 acted as Scn5a promoter was consistent with our observation that the TBE4 containing construct could up-regulate luciferase when compared to the empty vector possessing the minimal promoter alone. When Gata4 and Nkx2-5 were added in the luciferase reporter assay, TBE1 and TBE2 showed higher induction of luciferase expression than the other tested fragments (Fig. 4f). Further, TBE1 and TBE2 were stimulated by Tbx5 and repressed by Tbx2 and Tbx3 (Fig. 4g). TBE9 showed constitutive activity (Fig. 4f) and was repressed by Tbx2 and Tbx3, but not induced by Tbx5 (Fig. 4g).

We postulated that Tbx3 is responsible for the down-regulation of Scn5a and Scn10a and this regulation probably occurs in the Tbx3-positive SAN and AVN. TBE1, TBE2 and TBE9 seemed to be functional binding-sites of cardiac transcriptional regulators Tbx3, Gata4, Nkx2-5 and p300, and therefore could be potential enhancers. Since these fragments are located near Scn5a and within Scn10a, it was assumable that they are Scn5a and Scn10a enhancers. We continued with TBE1, TBE2 and TBE9 fragments for further analysis.

TBE1-2 and TBE9 function as enhancers in vivo
Considering TBE1, TBE2 and TBE9 sites as the potential enhancer candidates for Scn5a and Scn10a (Fig. 5a), we validated their enhancing activity in a transgenic mouse enhancer assay. Since TBE1 and TBE2 were located adjacent to each other, we tested them as one fragment (labeled as TBE1-2). Fragments containing the mouse sequence of TBE1-2 (chr9:119,541,166-119,543,699) and TBE9 (chr9:119,378,500-119,379,597) (Fig. 5a) were cloned into an enhancer LacZ-reporter construct and the LacZ-staining pattern was analyzed in the transgenic mice. E14.5 mice containing the TBE1-2 construct showed reproducible LacZ-staining in the atrium and interventricular septum (Fig. 5b), which were areas where also expression of endogenous Scn10a was found (Fig. 4b). Transverse sections through hearts of E10.5 embryos containing TBE9 construct showed that LacZ-staining was present in the atrium, interventricular septum and the ventricular trabeculated subendocardium (Fig. 5b), a pattern that was also found in E14.5 transgenic hearts (Fig. 5b) and comparable to endogenous Scn5a expression pattern (Fig. 4b).

TBE1-2 as well as TBE9 fragment could drive reporter gene expression in the embryonic hearts, demonstrating their enhancing ability in the heart. The
Combinatorial ChIP-seqs Accurately Predicts Cardiac *Scn5a/Scn10a* Enhancers

reporter gene expression pattern was very similar to the expression pattern of *Scn10a* and *Scn5a*, supporting the hypothesis that TBE1-2 and TBE9 are active *Scn10a* and *Scn5a* enhancer elements, respectively.

**Identification of regulatory T-box binding sites in TBE2 and TBE9**

The putative T-box binding sites in TBE2 and TBE9 were predicted by JASPAR database (MA0009.1 T binding-site with score threshold 70%) (Fig. 6a). We next tested whether the T-box binding site present in TBE2 and TBE9 was responsible for the regulation by Tbx3. Mutations were introduced in the putative T-box binding site in TBE2, labeled as TBE2m, and in TBE9, labeled as TBE9m (Fig. 6a, mutation sites labeled in red). The effect of the mutations on the enhancing activity was tested by a luciferase reporter assay in Cos-7 cells. Upon co-transfection of Nkx2-5 and Gata4, wild-type TBE2 and TBE9 exhibited increased enhancing effect on the transcription of the luciferase reporter gene compared to no addition of Nkx2-5/Gata4 (Fig. 6b). Addition of Tbx3 to the Nkx2-5/Gata4-complex led to a lowered enhancing activity, indicating the repressive role of Tbx3 in this transcriptional regulation-complex. Tbx3 failed to repress the Nkx2-5/Gata4-induced enhancing activity in the TBE2m reporter construct and showed less repression of the induced activity in the TBE9m compared to TBE9 reporter construct. TBE2m and TBE9m mutations resulted in reduced Tbx3 binding demonstrated by electrophoretic mobility shift assay (EMSA) (Fig. 6c), indicating that these T-box binding-sites in TBE2 and TBE9 might be important for the Tbx3-modulation.

**Discussion**

Tbx3 ChIP-seq reveals Tbx3-bindings at thousands of novel locations throughout the genome, and confirming several known T-box binding locations. The combinatorial approach of the Tbx3 binding-profile and expression profile of genes regulated by Tbx3 in the AV canal/node reveals that many of the Tbx3 direct target genes are related to ion channels including *Scn5a* and *Scn10a*. Co-occupancy by cardiac regulators Tbx3, Gata4, Nkx2-5 and p300 accurately predicts candidates for *Scn5a* and *Scn10a* regulatory elements, which we validated by *in vitro* and *in vivo* enhancer assays. In our *in vivo* transgenic mouse enhancer assay, the expression pattern of LacZ reflects the active area of the tested enhancer. We observe that TBE1-2 and TBE9 both can drive LacZ reporter gene expression in the interventricular septum, the structure that will give rise to the AVB and BB. The LacZ expression-patterns resemble to the expression-pattern of *Scn10a* and *Scn5a*, respectively, strongly indicating that TBE1-2 and TBE9 fragments are *Scn10a* and *Scn5a* enhancers. All together, we have identified *Scn5a* and *Scn10a*
enhancers that are responsible for the expression of these genes in the heart and a transcriptional regulation-complex involving Tbx3 that modulates these enhancers.

Tbx3 probably competes with Tbx5 in the regulation of Scn5a and Scn10a

Low expression of Scn5a and Scn10a has been observed in the SAN and AVN, area where high expression of Tbx3 has been found\(^57\) (Fig. 4b). The expression of Scn5a/10a and Tbx3 is not mutually exclusive everywhere; they overlap in the AVB and BB\(^23,57\) (Fig. 4b). Thereby, the repression of Scn5a/10a by Tbx3 seems not to occur in the AVB and BB but only in the nodal tissues of the conduction system. This suggests that the regulation of Scn5a/10a in the AVB and BB might depend on factors other than Tbx3, and Tbx5 might be such a factor. Tbx5 and Tbx3 have been shown \textit{in vitro} to recognize a similar T-box binding element.\(^58,59\) Recently published data of Tbx5 ChIP-seq performed in HL-1 cells shows that Tbx5 binding-pattern is comparable to our Tbx3 binding-pattern in Scn5a and Scn10a including TBE1-2 and TBE9 region\(^33\). Tbx5 and Tbx3 are co-expressed the AVB and BB\(^23\). Further, Tbx5 (activator) and Tbx3 (repressor) protein share Nkx2-5 and Gata4 protein as physical interacting partners to regulate transcription of target genes\(^15,24\). Taken together, it is possible that Tbx5 and Tbx3 are competitors of each other for binding of DNA and co-factors in the AVB and BB. The mechanism for this competitive process has not been clarified yet, but stoichiometry may be involved (see Chapter 3). The level of Tbx5 might be higher than Tbx3 in the AVB and BB and thereby enforcing Tbx5 to activate Scn5a and Scn10a. However, TBE9 does not respond to the induction of Tbx5 shown in our \textit{in vitro} luciferase reporter assay. This indicates that in the AVB and BB, Tbx5 might need other (unknown) co-factors to activate Scn5a. In addition, TBE1 and TBE2 do react on Tbx5 induction in our \textit{in vitro} luciferase reporter assay. Thus, Tbx5 seems to at least activate TBE1-2 that leads to Scn10a expression in the AVB and BB. It is noteworthy to point out that due to technical reason, the lengths of the enhancer fragments used in the \textit{in vitro} reporter assay were shorter than those used in the \textit{in vivo} reporter assay. Therefore, results from the \textit{in vitro} reporter assay might not fully reflect the effects of the full length functional enhancers. Clearly, the molecular mechanisms of these enhancers need to be investigated properly in order to understand their functions. In the SAN and AVN region, where the expression of Tbx5 and Tbx3 overlap\(^26\), Tbx3 appears to `win' the competitions in Scn5a and Scn10a regulation since these genes are not activated by Tbx5 but are repressed by Tbx3 most likely by modulating enhancers TBE1-2 and TBE9.

Taken together, it seems that Tbx3 and Tbx5 can both regulate Scn5a and Scn10a by binding the same enhancer regions, and the regulation by which one of
these T-box factors might depend on their expression level and the presence of cofactors, it might thereby be tissue-specific.

**Human genetic variants associated with PR- and QRS-interval are located in TBE1-2**

The alignment of the human DNA sequence with the mouse TBE1-2 sequence tested in the transgenic mouse enhancer assay shows that two SNPs in SCN10A (rs6795970 and rs6801957) associated with the PR- and QRS-interval identified in different genome-wide association (GWAS) studies 51-54 are located within the TBE1-2 sequence. The PR-interval is an electrocardiographic measure reflecting the time required for an electrical signal to travel from the SAN through the AVN to the ventricles, and the QRS-interval reflects the ventricular activation. Our study suggests a mechanism how genetic variants in TBE1-2 region in SCN10A could cause differences in the ECG-parameters. We identify TBE1-2 as an enhancer region for possibly Scn10a in mouse, meaning that this region might be of importance in the regulation of the expression of this gene. We hypothesize that the genetic variants in TBE1-2 region could possibly influence the binding of Tbx3 (or other factors) to Scn10a and thereby affecting the level of Scn10a expression, causing difference in conduction velocity and thereby the ECG-parameters. However, further experiments are needed in order to demonstrate the exact function of this enhancer region and whether it indeed can drive the Scn10a promoter. Our study is following up on this line to address these issues. Since Scn5a and Scn10a play important roles in the generation of cardiac arrhythmias, identifying the function of genetic variations in these genes provides insights in predicting disease trait susceptibility.

**Methods**

**ChIP sequencing from adult mouse heart**

Previously described CAG-CAT-TBX3 (CTBX3) heterozygous mice 24 were crossed with Myh6MCM mice 60. Tamoxifen (Sigma T5648) was injected intraperitoneally (100 mg per mouse per day) at four day intervals in adult males. Hearts were removed on day 5, prior to the onset of heart dysfunction (the phenotype will be described elsewhere). qRT PCR was performed to determine the efficiency of recombination. For Nkx2-5, Gata4 and p300 ChIPs, hearts were isolated from adult FVB males. Tissues were cross-linked in 1% (p300) or 2% (Tbx3, Gata4, Nkx2-5) formaldehyde for 30 minutes at room temperature. Cross-linking was quenched by addition of 0.125M Glycine. Tissues were dissociated by IKA Ultra Turrax T5 FU, pelleted and resuspended in cold Lysis Buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA (pH 8.0), 1% SDS, 1X Protease Inhibitor Cocktail
(Roche)). By use of a tight glass dounce homogenizer nuclei were obtained. Cross-linked nuclei were sonicated under conditions established to yield an average fragment size of ~300bp. All antibodies were purchased from Santa Cruz (Tbx3 (A-20, sc-17871, lot#J0306), Tbx5 (C-20, sc-17866, lot#D1708), Nkx2-5 (N-19, sc-8697, lot#A1109, #J2010), Gata4 (C-20, sc-1237, lot#F3010) and p300 (N-15, sc-583, lot#K3009). Immunoprecipitation, washing, elution and reverse cross-linking were performed as previously described \(^\text{13}\). Quality of the ChIP was assessed with primers on locations of known cardiac enhancers. Material was used for library preparation with standard NEBNext library kit protocol for Illumina sequencing (New England BioLabs, #E6000S/L, #E6040S/L).

ChIP-qPCR was performed on a Roche LightCycler 480 System using Sybr Green detection. Triplicate biological repeats were measured each in duplo. Fold enrichment indicates the ratio of ChIPed DNA to a negative control region in the \textit{Hprt} locus, normalized for input DNA.

Primers used for the ChIP-qPCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scn10a TBE2 (mouse)</td>
<td>F: TGACATTCCTCACACTGAAGGG</td>
<td>R: CCACTGTAAGAAACTCAAGGCT</td>
</tr>
<tr>
<td>Scn5a TBE9 (mouse)</td>
<td>F: TTTGCAGAGGAGGCATGGTG</td>
<td>R: TCCTCCCTGCAGAAGGGGCCT</td>
</tr>
<tr>
<td>Hprt (mouse)</td>
<td>F: GTCAACGGGGGACATAAAAAG</td>
<td>R: CATTTGGCTATAAAGTCCAGG</td>
</tr>
</tbody>
</table>

**Processing of ChIP-sequencing data**

Sequencing results were run through the standard Illumina GAPipeline (v1.3 for GAII runs) to convert images to reads of 36bp in length (unaligned sequences produced by the Illumina Genome Analyzer) and edit for quality (FIRECREST, Bustard and GERALD). Reads were then processed and aligned to the mouse reference genome (NCBI Build 37, mm9) as previously described, allowing a maximum of two mismatches and retaining only sequences with unique alignments \(^6\). Peaks were extracted and defined from the wiggle files containing the coverage per basepair using an in-house algorithm that returns peak start and stop coordinates in a BED file format.

Gene sets were extracted from UCSC refGene for the mouse using the Galaxy software interface. Promoter regions were defined as 1 kb regions directly upstream of all genes. Overlaps between gene regions and peak regions were identified using Galaxy. For determining the intersection between gene arrays and Tbx3 ChIP-seq peak regions, significant up or down regulated genes from the various microarray datasets were assigned coordinates using the Biomart Central Portal. All chromosomal coordinates were subsequently extended both up- and down-stream by 1 kb. Coordinates were then intersected using the Galaxy software.
Combinatorial ChIP-seqs Accurately Predicts Cardiac Scn5a/Scn10a Enhancers

interface. Two control gene sets were generated, both for the induced Tbx3 array analysis and the AVC/working myocardium array analysis, using 6 sets of equivalent numbers of unique genes, randomly extracted from the non-significant genes from the AVC versus working myocardium microarray study. These control gene sets were further treated in exactly the same way as the test gene sets. Statistical analysis of the significance of enrichment between intersects and controls was carried out using a Z-test, which uses the normal approximation of the binomial distribution to compare proportions \(62, 63\).

Proteins assigned to overlaps were extracted using Galaxy and further analyzed using the DAVID bioinformatics resource GO-gene ontology analysis software \(47, 64\). Unique gene names were called using chromosome locations via the BioMart Central Portal. Motif analysis was performed with MEME \(43\) using 600 random peaks with an average length of ~165bp per total dataset. For scanning ChIP-seq peaks with known TF motifs, position weight matrices were obtained from JASPAR \(44, 45\) and TRANSFAC (v11.3) \(46\) database.

**Transgenic mouse enhancer assay**
Enhancer candidate regions of 1-2.5 kb in the Scn5a-Scn10a locus were cloned into the Hsp68-LacZ reporter vector as previously described \(65\). DNA was injected into the pronucleus of 0.5-day-old fertilized FVB/N eggs, which were subsequently transferred into the oviducts of CD-1 pseudo-pregnant foster females (Cyagen Inc.). At least 200 injections were performed per construct. Embryos were harvested, stained with X-gal to detect LacZ activity, and yolk sacs were processed for PCR genotyping.

**Quantitative expression analysis**
Total RNA was isolated from left atrial appendices of adult mice with induced TBX3 expression using the RNeasy Mini Kit according to manufacturer’s protocol (Qiagen). For expression analysis at embryonic and adult stages, different tissues (brain, lung, spine, atria and ventricles) from wildtype animals were isolated in equal proportions. Total RNA from these tissues was isolated with the TRIzol kit according to manufacturer’s protocol (Invitrogen). cDNA was reverse transcribed from 300ng total RNA using the Superscript II system (Invitrogen). Expression of different genes was assayed with quantitative real-time PCR using the Roche LightCycler 480 system. Relative start concentration (N(0)) was calculated as previously described \(66\). Values were normalized to Hprt expression levels.
Primers used for the qRT-PCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (F)</th>
<th>Reverse (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scn5a</td>
<td>F: GGGACCTCATTGCTACATGA</td>
<td>R: GCACCTGGGAGGTTATCAG</td>
</tr>
<tr>
<td>Scn10a</td>
<td>F: CTAGTCTGTGGTTTCTCCTGGA</td>
<td>R: GCGAAGCAGCAGTGGAGAATG</td>
</tr>
<tr>
<td>Kcnq1</td>
<td>F: GAGAACAAGGTGAACAAACTG</td>
<td>R: CTGGCTACAAACTTGTGCCTATG</td>
</tr>
<tr>
<td>Kcnk3</td>
<td>F: CTCTCTCTACCTGCGCATCA</td>
<td>R: GAAGGTTGTGATGCGTTC</td>
</tr>
<tr>
<td>Kcnq3</td>
<td>F: CCACCATAAGAACCATAAG</td>
<td>R: GTCTTCTGTGCTACGAGG</td>
</tr>
<tr>
<td>Kcnj1</td>
<td>F: GGACCTCGCTTTCTCAGGT</td>
<td>R: GCATCACTAAACTGCTGGA</td>
</tr>
<tr>
<td>Kcnj2</td>
<td>F: AGAGGCAGTGTGCAAGAACT</td>
<td>R: GTGGTCTGTGAGGTGTGG</td>
</tr>
<tr>
<td>Kcnj3</td>
<td>F: CCACCCATTCTCTGTGCTGTC</td>
<td>R: GAACCAATCAGTGCCCTAA</td>
</tr>
<tr>
<td>Kcnd3</td>
<td>F: GGTCTCATTGGAACACAGG</td>
<td>R: GGTTGCATGGAACTGGG</td>
</tr>
<tr>
<td>Kcnv2</td>
<td>F: CTTACCAGCATCCTCCATG</td>
<td>R: GCATCCATTGAGAATAATGCC</td>
</tr>
<tr>
<td>Pkp4</td>
<td>F: GCCACATTTGGAAGTGCAT</td>
<td>R: CAGAGTTGTGTTGGTCAG</td>
</tr>
<tr>
<td>Hprt</td>
<td>F: CCATTCCTATGACTGTAGAT</td>
<td>R: CAATCAAGACGTTCCTCTCCAG</td>
</tr>
</tbody>
</table>

Luciferase assays

COS7 and H10 cells, grown in 12-well plates in DMEM supplemented with 10% FCS (Gibco-BRL) and glutamine, were transfected using polyethylenimine 25 kDa (PEI, Brunschwick) at a 1:3 ratio (DNA:PEI). Reporter construct was generated by ligating putative enhancer regions to pGL2basic+minimal promoter (control reporter). Standard transfections used 1.4 μg of reporter (or control reporter) vector co-transfected with 3 ng phRG-TK Renilla vector (Promega) as normalization control. pcDNA3 constructs expressing Gata4, Nkx2-5, Tbx2, Tbx3 and Tbx5 were co-transfected as appropriate. Transfections were carried out at least three times and measured in duplo. Luciferase measurements were performed using a Promega Turner Biosystems Modulus Multimode Reader luminometer. All data was statistically validated using an ANOVA two-way test for all combinations.
Combinatorial ChIP-seqs Accurately Predicts Cardiac Scn5a/Scn10a Enhancers

**In situ hybridization**
Non-radioactive section *in situ* hybridization was performed on 12 μm serial section as described previously 67. *In vitro* transcribed RNA probes complementary to Scn5a and Tbx3 are described 26, 57. Scn10a probe construct was generated by PCR amplification of a 946 bp fragment with Scn10a specific primers (Forward: GCCCTCTTAGAATCCCCAAC, Reverse: GCAAACCCTATTAGCAGTGC), which was subsequently cloned into pBluescript SK+ vector.

**Electrophoretic Mobility Shift Assay**
Non-radioactive electromobility shift assay was performed using bacterially expressed MBP-Tbx3 (Tbox) or MBP only (control) as previously described 14. Oligonucleotide probes used are:

TBE2-F      TTTAAGGCCTTTAACTTGACACCTCGCCGCCCAAGTGCAG
TBE2-R      TTTCTGCACTTTGAGGCAGGTGCTCGCAAGTGAAGCCTTT
TBE2M-F     TTTAAGGCCTTTAACTTGAGGGCTCGCCGCCCAAGTGCAG
TBE2M-R     TTTCTGCACTTTGAGGCAGGTGCTCGCAAGTGAAGCCTTT
TBE9-F      TTTGGGCCTCTGAGGAGGTGTGAATGGGAGGAGCCGA
TBE9-R      TTTTCACCTCCTCGAGTGTGAAGGGAGGAGGCCGA
TBE9M-F     TTTGGGCCTCTGAGGAGGCCGAATGGGAGGAGGCCGA
TBE9M-R     TTTTCACCTCCTCGAGTGTGAAGGGAGGAGGCCGA

**Animal work**
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal work was approved by the Animal Experimental Committee of the Academic Medical Center, Amsterdam, and carried out in compliance with the Dutch government guidelines.

**Acknowledgement**
We thank Jan Ruijter for help with the statistical analysis, and Carol Verhoek-Pocock, Corrie de Gier-de Vries and Mojtaba Amini for technical assistance.
References

(20) Stennard FA, Harvey RP. T-box transcription factors and their roles in regulatory hierarchies in the developing heart. Dev 2005 November;132(22):4897-910.
Combinatorial ChIP-seqs Accurately Predicts Cardiac Scn5a/Scn10a Enhancers


Combinatorial ChIP-seqs Accurately Predicts Cardiac Scn5a/Scn10a Enhancers


