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A novel interaction between T-box and Sox transcription factors

Submitted in modified form

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Introduction

The T-box genes encode a phylogenetically conserved family of transcription factors that share a common DNA-binding motif known as the T-box domain. They play crucial roles in development where they are implicated in patterning, early cell fate decisions, and many aspects of organogenesis. Mutations of T-box genes have been associated with human disorders such as DiGeorge and Holt-Oram syndromes (Packham and Brook, 2003).

Tbx2 and Tbx3 are closely related paralogues of the T-box family that are expressed in many overlapping areas during development, including the heart, limbs and lungs (Chapman et al., 1996). They act as transcriptional repressors and have at least some target genes in common, including regulators of the cell cycle (Naiche et al., 2005). In addition to their roles during development, Tbx2 and Tbx3 are over-expressed in melanoma, breast, and pancreatic cancers (Jacobs et al., 2000; Prince et al., 2004; Fan et al., 2004). Their role in cancer may be related to their capacity to bypass senescence by repressing expression of p14ARF and P21CIP1 (Brummelkamp et al., 2002; Lingbeek et al., 2002; Yarosh et al., 2008)

During heart development, Tbx3 is required for normal development of the cardiac conduction system and outflow tract (Hoogaars et al., 2007a; Bakker et al., 2008; Mesbah et al., 2008). In the myocardium of the sinus node and the atrioventricular canal Tbx3 represses a chamber myocardium-specific gene program, including the gap junction genes *Gja1* and *Gja5*, encoding connexin 43 (Cx43) and Cx40 respectively, and *natriuretic peptide precursor type A* (*Nppa*), thereby retaining a primitive myocardial phenotype (Christoffels et al., 2000; Christoffels et al., 2004; Mommersteeg et al., 2007). In the absence of Tbx3, chamber specific genes are erroneously expressed in these areas (Hoogaars et al., 2007a). In addition to the essential role of Tbx3 in patterning the myocardium, it has recently become clear that Tbx3 also induces the formation of cushion mesenchyme and epithelial to mesenchymal transformation (Hoogaars et al., unpublished observations). Furthermore, Tbx3 null mice display defects in outflow tract development that are suggestive of roles of Tbx3 in formation of the outflow tract cushions and patterning of the second heart field via signaling between neural crest and second heart field (Bakker et al., 2008; Mesbah et al., 2008). Although these results have provided valuable insights into the roles of Tbx3 during multiple aspects of heart development, many of the underlying molecular mechanisms remain to be elucidated.

Using a yeast 2-hybrid screen, we have identified a novel interaction between Tbx3 and Sox4. With truncation studies we show that the T-box of Tbx3 and the high mobility group

(HMG) box of Sox4 are sufficient to support the interaction. The significance for cardiac development of this interaction is underscored by the co-expression of Sox4 and Tbx3 in the mesenchyme and endocardium of the cardiac cushions, as well as a specific region of conduction system myocardium at the border of the left ventricle and outflow tract. When testing whether the interaction is exclusive for Tbx3, we found that both Tbx2 and Tbx5 can also interact with Sox4. This suggests that there may be a more widespread mechanism of HMG-box - T-box interaction, and that the functionality of these interactions during development is dictated by their spatiotemporal expression patterns. By comparing results from Tbx3 and Sox4 chromatin immunoprecipitation (ChIP) experiments we identified a number of cardiac relevant genes that are identified in both assays and thus are candidate genes to be regulated by an interaction between Tbx3 and Sox4.

Methods

Plasmid constructs

Full length (aa 1-723 / 743) and T-box region (aa 94-300 / 320) of Tbx3 or Tbx3 isoform2 (+ exon 2a) were PCR amplified from human cDNA (NM_005996 / NM_016569) and cloned into pMAL2C (Clontech) to generate MBP fusion constructs. Full length (aa 1-440) and N-terminal fragments (aa1-153, aa1-136, aa1-125) of Sox4 were PCR amplified from mouse cDNA (NM_009238) and cloned into pRP256nb to generate GST fusion constructs, or into pcDNA-myc (full length only) to generate myc-Sox4. Constructs encoding MBP-Tbx2-Tbox, MBP-Tbx5-Tbox, GST-Nkx2.5, HA-Tbx3, myc-Nkx2.5 have been described before (Hoogaars et al., 2007a; Boogerd et al., 2008).

Yeast 2-hybrid screen

The T-box region of mouse Tbx3+2a (aa 94-320, NM_198052) was cloned into pGBKT7 (Clontech) and tested for self-activation by co-transfection to yeast strain AH109 (Clontech) with empty activation domain (AD) plasmid pGADT7 (Clontech). Bait construct was transformed into AH109, which was subsequently mated with yeast strain Y187 that was pretransformed with prey library of mouse embryonic day (E) 11.5 cDNA (Clontech) according to the manufacturer's instructions. Clones were selected on triple-drop-out selection media lacking leucine, tryptophan and histidine in the presence of the galactoside X-α-Gal. Surviving colonies were replated to triple drop out medium and subsequently picked for AD-plasmid rescue and sequencing.

In-vitro protein interactions assay

MBP pulldown assays were performed as described before (Boogerd et al., 2008), using anti-GST (GST-2, Sigma-Aldrich) as primary antibody for western detection.

Immunofluorescence

Experiments in HEK293 cells were performed according to standard protocols. Cells were transfected with 375ng DNA of each plasmid, empty vector was added such that all cells received the same amount of total DNA. Primary antibodies used were rabbit anti-HA (H6908, Sigma-Aldrich), mouse anti-myc (9E10, Santa-Cruz) at 1:250 dilutions, and secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG (Molecular probes), at 1:250 dilutions. TO-PRO3 (Invitrogen) was used for nuclear counterstaining. Immunofluorescent detection of proteins was repeated at least three times, and representative examples were photographed on a Leica DM5500 confocal laser microscope (Leica).

ChIP data-analysis

Conditional Tbx3 over-expressing and cardiac specific tamoxifen inducible Cre mice have been described before (Sohal et al., 2001; Hoogaars et al., 2007a). Mice hearts were isolated 1 week after intra-peritoneal injections of tamoxifen, and Tbx3 over-expression was confirmed by qRT-PCR, *in-situ* hybridization and immunohistochemistry (not shown). ChIP was performed on mouse hearts using anti-Tbx3 (E-20, Santa-Cruz). Isolated DNA fragments were analyzed using high-throughput sequencing (Data will be published elsewhere). Sox4 ChIP data were obtained from NCBI gene expression omnibus (accession: GSE11874; (Scharer et al., 2009). Annotated genes co-occuring in both assays were selected for further analysis. Gene ontology (GO) terms were obtained from Mouse Genome Informatics (MGI) and statistical overrepresentation of these was determined using Ontologizer software (http://compbio.charite.de/index.php/ontologizer2.html; (Robinson et al., 2004).

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from transfected H10 cells using the Nucleopsin RNA II kit (Bioké) according to the manufacturer's instructions. cDNA was reverse-transcribed from 1 µg of total RNA using SuperScript II (Invitrogen). Expression of different genes was assayed using the LighCycler480 real time pcr system (Roche) and subsequently analyzed using LinReg software (Ruijter et al., 2009). HPRT expression served as internal control.

Results

Tbx3 interacts with Sox4

Tbx3 has been shown to play crucial roles during development of the cardiac conduction system and the cushions of the atrioventricular canal and outflow tract. To gain further insight into the molecular mechanisms by which Tbx3 exerts these functions, we performed a yeast 2-hybrid screen with Tbx3 as bait. From an initial screen of $>1 * 10^6$ colonies, 12

surviving clones revealed a GAL4 fusion to a peptide (>40 aa) in a reading frame coding for a BLASTP genome identifiable sequence. Two of these clones encoded an N-terminal fragment of Sox4, a high mobility group (HMG) domain containing transcription factor that has been previously shown to be essential for normal outflow tract development and atrioventricular valve formation (Schilham et al., 1996; Ya et al., 1998; Goldsworthy et al., 2008). The fragment encoded amino acids 3-153 of mouse Sox4, which contains the entire HMG domain. No other functional domains have been identified within this part of the protein and a database search for conserved domains using the NCBI CDD search option revealed no other conserved domains in this fragment (Figure 1a; (Hur et al., 2004; Dy et al., 2008; Marchler-Bauer et al., 2009)). The interaction could be confirmed using a direct yeast 2-hybrid assay (data not shown).

Tbx3 and Sox4 interact via their DNA-binding domains

To validate and further investigate the interaction between Tbx3 and Sox4, we performed *in-vitro* binding assays using bacterially expressed Tbx3 fused to MBP and Sox4 fused to GST. Both full length Sox4 and the N-terminal fragment that was identified in the screen, are able to interact with MBP-Tbx3, but not with MBP alone (Figure 1b). We next tested whether binding of Tbx3 to Sox4 is unique among T-box proteins, or whether the closely related T-box proteins Tbx2 and Tbx5 can also bind to Sox4. In this *in-vitro* binding assay (Figure 1b) we found that the T-box of Tbx2 and Tbx5 are able to bind the N-terminal Sox4 fragment as well, suggesting promiscuity of binding between Sox4 and T-box proteins.

Multiple bands were observed in the binding between full length Sox4 and Tbx3 (Figure 1b), which are likely to represent carboxy-terminal specific protein degradation by *Escherichia coli* endoproteases. Strikingly, the size of the smallest degradation product that is still retained by Tbx3 equals the size of the N-terminal fragment that was picked up in the 2-hybrid screen. Smaller protein fragments therefore are not retained by Tbx3, indicating that further shortening of Sox4 would disrupt the interaction domain. To test this hypothesis, we compared binding of three N-terminal fragments. Stepwise truncation of Sox4 showed that the shortest construct that still binds Tbx3 contains the full HMG domain and its C-terminal tail, whereas the construct that lacks the C-terminal tail does not bind anymore.

In summary, our *in-vitro* binding assays show a strong interaction between Tbx3 and Sox4, which is mediated by their conserved DNA binding regions; the T-box and the HMG-domain.

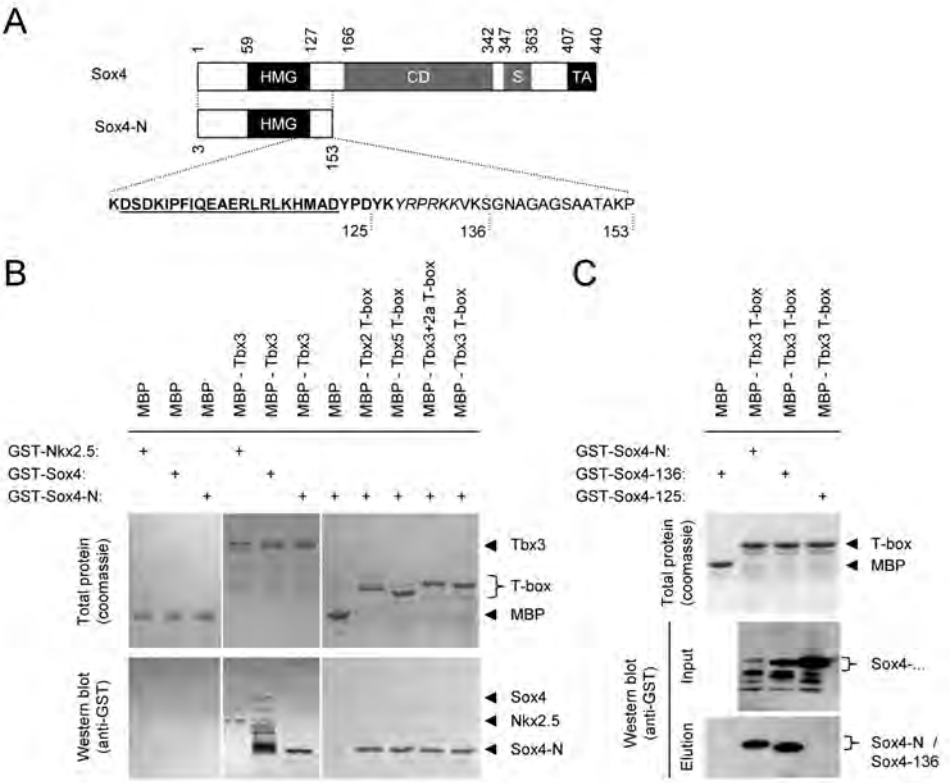


Figure 1. The T-box of Tbx3 interacts with the HMG domain of Sox4. a) Diagram showing full length Sox4 with conserved domains, and the clone that was identified in our screen (Sox4-N). The sequence of a fragment containing the 3rd α-helix (underlined) of the HMG box (bold) and its c-terminal tail (italics) is shown, with the positions of truncated constructs (125, 136) b) MBP pulldown assays showing that GST tagged Nkx2.5, Sox4 and Sox4-N bind to MBP-Tbx3 (middle panel) but not MBP alone (left). The T-box of Tbx3, but also Tbx2, Tbx3+2a and Tbx5 bind to the HMG domain of Sox4 (right). c) Mapping of the interaction domain of Sox4 showing that the construct that misses the C-terminal tail (Sox4-125) does not ineract with the T-box, whereas longer constructs do. Abbreviations: CD, central domain; S, serine rich region; TA, transactivation domain.

Tbx3 and Sox4 interact in a mammalian cellular context

To address whether the interaction between Tbx3 and Sox4 can also occur *in-vivo*, we analyzed the sub-cellular distribution of HA-tagged Tbx3 by immunofluorescence in HEK293 cells. When transfected to HEK cells, both Tbx3 isoforms (Tbx3, Tbx3+2a) are localized primarily in the cytoplasm, although some nuclear localization can be detected (Figure 2a and data not shown). However, upon co-expression of Sox4 or Nkx2.5 as a positive control, Tbx3 was detected nearly exclusively in nuclei, strongly suggesting that Sox4 and Tbx3 dimerize and are translocated to the nucleus together. The absence of nuclear recruitment upon co-expression of unrelated nuclear proteins confirmed the specificity of the interaction.

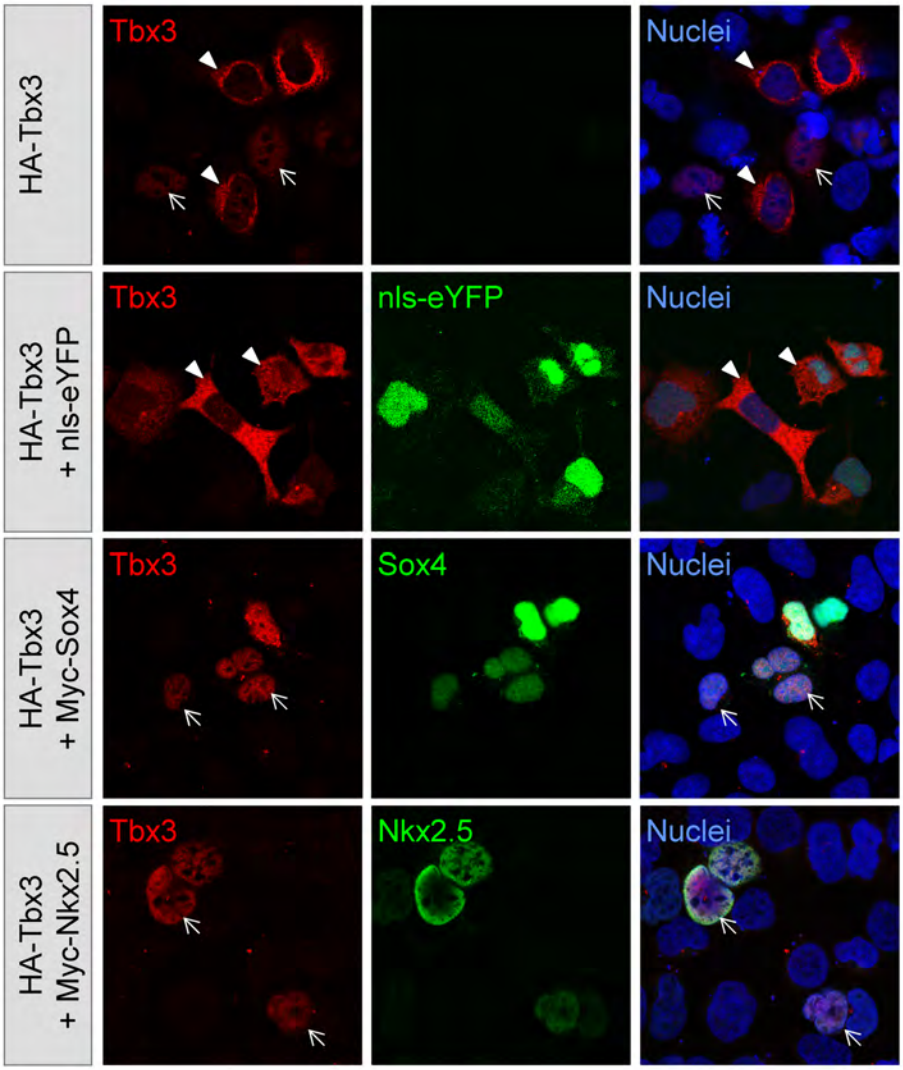


Figure 2. Tbx3 and Sox4 interact in Hek293 cells. Cells were transfected with expression constructs for HA-tagged Tbx3, in the presence or absence of nls-eYFP, Sox4 or Nkx2.5 (myc-tagged). Cytoplasmic Tbx3 is efficiently relocalized to the nucleus upon co-expression of Sox4 and Nkx2.5, whereas co-expression of the unrelated eYFP protein does not influence subcellular localization of Tbx3.

Tbx3 and Sox4 are co-expressed during heart development

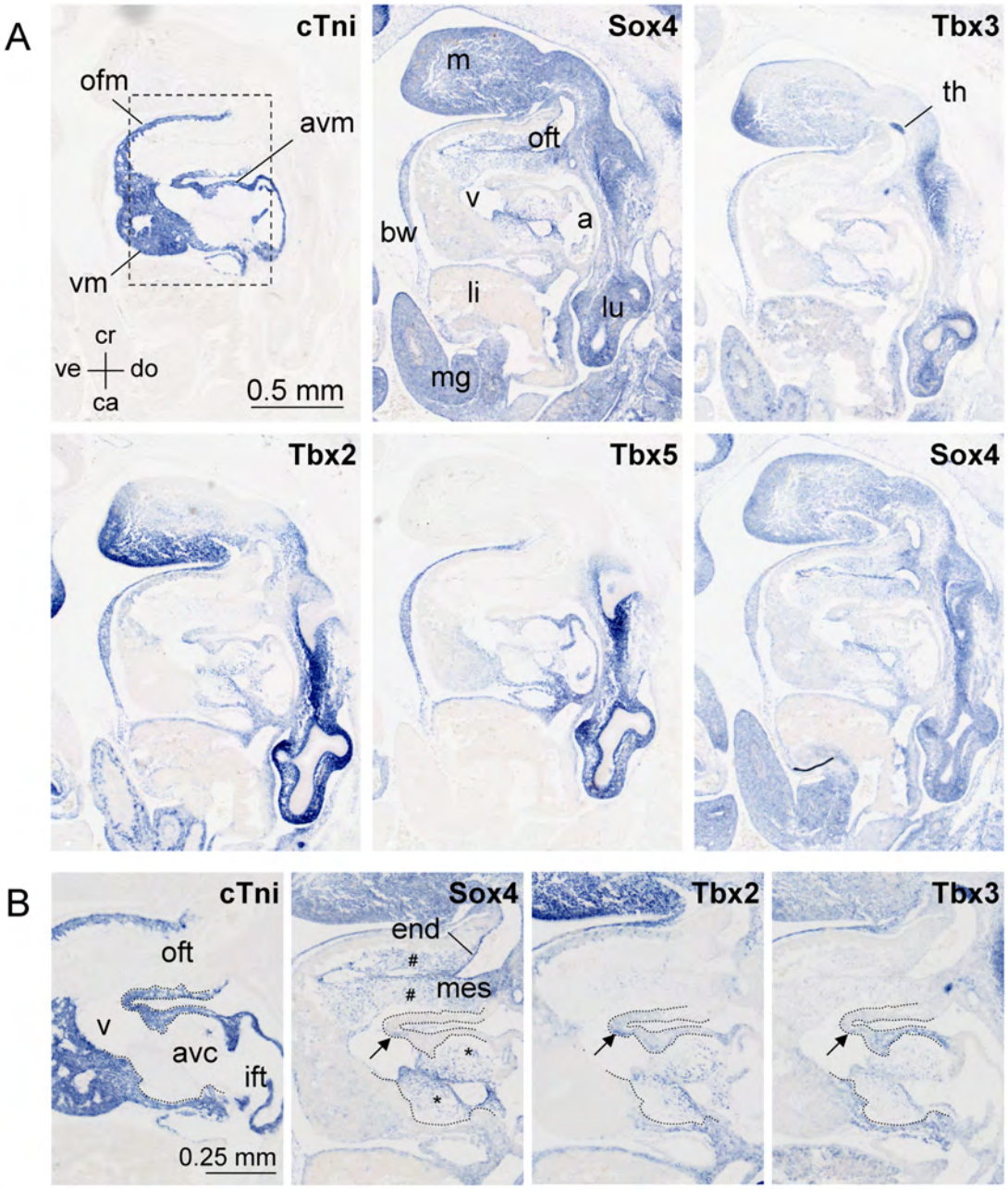
The observation that Tbx3 and Sox4 interact *in-vitro* and in mammalian cells raises the question whether these proteins might also interact during development. To determine in which tissues such a molecular interaction may occur, we compared the expression patterns of *Sox4* and *Tbx3* and the two closely related *Tbx2* and *Tbx5* genes using *in-situ* hybridization analysis of E11.5 wild-type mouse embryos. *Sox4* is coexpressed with *Tbx2*,

Tbx3 and *Tbx5* in the thoracic body wall, maxillary component of the first branchial arch, the developing lungs, and the midgut (Figure 3; Chapman et al., 1996; Hoogaars et al., 2004). In the heart, *Sox4* expression in the endocardium and mesenchyme of the cardiac cushions overlaps with *Tbx2* and *Tbx3*. These regions are subject to extensive remodeling and differentiation processes, in which a protein-protein interaction between Sox and Tbx may play a role. We also detect Sox4 expression in the ventral aspect of the interventricular ring, a subpopulation of primitive myocardium at the border of the left ventricle and outflow tract (Figure 3b, 4; Wessels et al., 1992). While at E11.5 Tbx3 is expressed throughout the interventricular ring myocardium, Sox4 expression seems to co-localize with the part of the ring that will cease to express Tbx3 during later development and will not differentiate into conduction system myocardium(Moorman et al., 1997; Hoogaars et al., 2004).

Potential downstream targets of Tbx3 - Sox4 interaction

For many transcription factors, including T-box proteins, target promoter specificity may be achieved through interaction with other proteins (Black and Olson, 1998; Bruneau et al., 2001; Hiroi et al., 2001; Habets et al., 2002). To identify genes that are potentially regulated by simultaneous binding of Sox4 and Tbx3 to their promoters, we compared data from ChIP experiments. A recently published Sox4 ChIP-chip experiment in prostate cancer cells was compared with our own Tbx3 ChIP-seq data that was obtained by performing ChIP on mouse hearts over-expressing Tbx3 throughout the myocardium upon tamoxifen injections (Tbx3 ChIP results to be published elsewhere; Scharer et al., 2009). From this experiment we selected peaks with high significance ($p<0.0001$, 1917 peaks) corresponding to 1649 genes. 204 of these genes were also identified in the Sox4 ChIP. These genes have thus been shown to be bound by both Tbx3 and Sox4, and their expression may therefore be regulated by Tbx3 and Sox4 via a synergetic or antagonistic mechanism. Gene-ontology categories that were overrepresented in this list include “muscle system processes”, “communication and signaling” and “developmentally regulated processes”, which are in agreement with documented roles for Tbx3 during heart development (Table 1). To address whether Tbx3 and Sox4 interact in the regulation of these genes, we performed qRT-PCR on transfected H10 cells for all genes underlined in Table 1. Although measurements within cDNA samples were highly reproducible, results between duplo transfections varied. Further investigation therefore requires an optimized method, which is currently being tested.

Figure 3 (next page). Saggital sections of E11.5 mouse embryos showing colocalization of Sox4 with T-box factors at multiple sites. a) Consecutive sections of mouse embryo showing colocalization of Sox4 with Tbx2 and Tbx3 in maxillary component of the first branchial arch (m), and the midgut (mg) and with Tbx2, Tbx3 and Tbx5 in the developing heart, lungs and body wall. cTni marks all myocardium. b) Expression of Sox4 in the heart is localized in the endocardium and mesenchyme of the atrioventricular (*) outflow tract cushions (#), sites of abundant Tbx2 and Tbx3 expression. Tbx2 and Tbx3 are also expressed in the atrioventricular myocardium



underlying the cushions, a region that does not express Sox4. dotted lines mark contours of the myocardium. Abbreviations: a, atrial lumen; v, ventricular lumen; oft, outflow tract; ift, inflow tract; end, endocardium; mes, cushion mesenchyme; cr, cranial; ca, caudal; ve, ventral; do, dorsal.

When also taking into consideration available microarray data, we found that 67 out of 204 genes are differentially regulated upon over-expression of Tbx3 or Sox4 (Figure 5; Hoogaars et al., 2007a; Scharer et al., 2009). To select for cardiac relevant genes we focused on the Tbx3 array experiment and show that 25% of the genes with promoter occupancy by Tbx3 and Sox4 can also be regulated by Tbx3. This group of genes includes known target genes like *Gja1*, *Cacna2d2*, *KcnJ3* and *Tpm1* (Hoogaars et al., 2007a). These genes thus represent the most likely candidates for our future studies on the interaction between Tbx3 and Sox4.

Discussion

Members of the T-box and Sox families of transcriptional regulators control a diverse array of processes during vertebrate embryonic development. In this study, we present evidence that Tbx3 and Sox4 interact via their DNA binding domains, both *in-vitro* and in mammalian cells. Comparative expression analysis showed that this interaction may be functional at multiple sites during development, including the cardiac valves and conduction system.

Tbx3 interacts with Sox4

The interaction studies presented here show that the DNA binding domains of Tbx3 and Sox4 interact. Since the interaction occurs through highly conserved domains, it is expected that other members of the T-box family will also interact with Sox4. Indeed, the related proteins Tbx2 and Tbx5 also bind Sox4. The lack of specificity is also evident for Nkx2.5 and Gata4, which partner-up with multiple T-box genes (reviewed in (Boogerd et al., 2009)). The functionality of these interactions is likely dictated by the timing and (co-) localization of expression and the relative expression levels of the different T-box factors.

Similarly, the high degree of sequence conservation between HMG domains suggests that other members of the Sox family may also interact with Tbx3 (Kamachi et al., 2001). For instance, the very early expression of Tbx3 in the inner cell mass of the blastocyst, where related T-box factors are not yet expressed, coincides with Sox2 expression, thus representing an example of a potentially interesting interaction that might be worth to further investigate (Chapman et al., 1996; Avilion et al., 2003). On the other hand, the interaction between the HMG domain of Sox4 and the ubiquitin conjugating enzyme Ubc9 is specific for Sox4 and not shared with other Sox proteins, the specificity of which, although less likely, may also count for the interaction between Tbx3 and Sox4 (Pan et al., 2006).

We show that the C-terminal part of the HMG domain is essential for the interaction between Tbx3 and Sox4. Protein-protein interactions of Sox2, Sox8 and Sox10 with other transcription factors were also shown to be mediated by the C-terminal part of the HMG domain, which includes helix 3 and the C-terminal tail region (Kamachi et al., 2001;

Remenyi et al., 2003; Wissmuller et al., 2006)). Since these regions are not involved directly in establishing DNA contacts, they are still available for interactions with other proteins while Sox proteins are bound to the DNA (Werner et al., 1995; Remenyi et al., 2003). The C-terminal tail region is unstructured in solution and therefore is a good candidate for these interactions. However, the possibility that Tbx3 and Sox4 interact independent of DNA binding must also be considered. This was shown to be the mechanism of action for the interaction between Sox17, β-catenin and LEF/TCF (Sinner et al., 2007). Thus, the domains through which Tbx3 and Sox4 interact are also bound by numerous other transcriptional modulators and these interactions can occur while bound to the DNA.

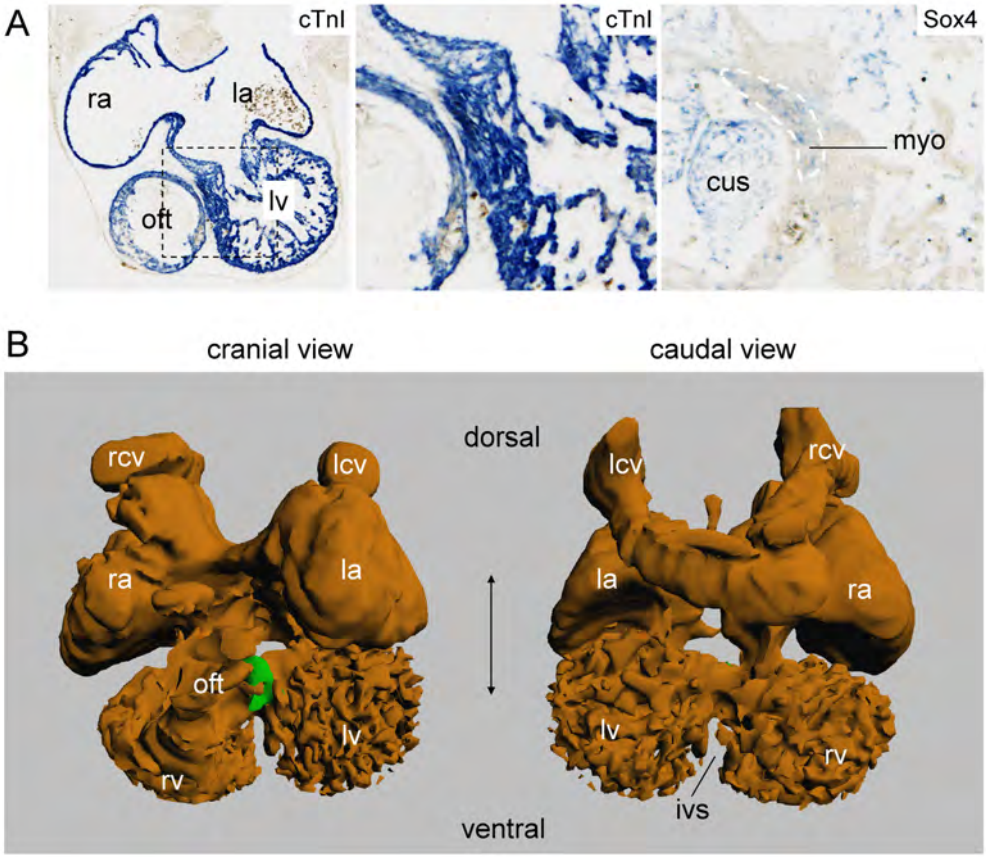


Figure 4. Sox4 is expressed in the ventral aspect of the interventricular ring. a) *In situ* hybridization on E11.5 section, showing Sox4 expression in the myocardium at the border of the left ventricle and outflow tract. b) Projection of Sox4 expression domain (green) on the lumen of an E11.5 mouse heart (orange). In cranial view (left) the domain overlays the connection between left ventricle and outflow tract, whereas the interventricular septum does not express Sox4 (caudal view, right). Abbreviations: la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle; oft, outflow tract; ivs, interventricular septum; rcv, right caval vein; lcv, left caval vein.

The finding that Tbx3 was primarily localized in the cytoplasm in Hek239 cells was contrary to our expectation, and not in accordance with a previous report (Carlson et al., 2001). Artificially high expression levels of Tbx3 may prevent its efficient import into the nucleus in the present study. Cytoplasmic localization has also been observed in malignant cells in breast cancer tissues that are associated with high Tbx3 levels (Lomnytska et al., 2006) (Yarosh et al., 2008). On the other hand, Tbx3 can be actively translocated to the cytoplasm by the nuclear export machinery (Kulisz and Simon, 2008). The shift in the balance between import and export by co-expression of Sox4 with Tbx3 reflects either the nuclear recruitment of Tbx3, or its retention inside the nucleus and thereby prevention of nuclear export. The observation that Tbx3 is specifically translocated to the nucleus in the presence of Sox4 is a strong indication of a direct interaction between Sox4 and Tbx3 in mammalian cells, and may be of mechanistic significance in terms of normal Tbx3 function.

Shared or overlapping functions during development

From our co-expression studies and previous reports we can conclude that Tbx3 and Sox4 are co-expressed in a number of tissues during development. Tbx2, Tbx3 and Sox4 are co-expressed in the outflow tract of the developing heart, which is remarkable, especially when considering that Tbx2, Tbx3 and Sox4 null mice develop comparable outflow tract defects (Schilham et al., 1996; Harrelson et al., 2004; Mesbah et al., 2008). The source of the various defects that occur in these knock-out mice may vary, though. Sox4 is not expressed in the myocardium of the outflow tract, therefore the outflow tract septation and alignment defects in Sox4 null mice are likely to find their origin in the endocardium-derived cushion mesenchyme or the neural crest cells, or communication between these (Ya et al., 1998). The outflow tract septation and alignment defects observed in Tbx2 and Tbx3 null mice may be endocardium or neural crest derived as well, and thus be indicative of a disturbed interaction with Sox4. However, the more broad expression of Tbx2, and to a lesser extent Tbx3, in the myocardium of the outflow tract, in which Sox4 is not expressed, may also contribute to the observed defects in outflow tract patterning in these null mice.

Recently, it was shown that a point-mutation in Sox4 resulting in a functional null allele, causes dysplastic atrioventricular valves in addition to the previously described outflow tract defects (Goldsworthy et al., 2008). Valvulogenesis starts with the formation of the atrioventricular cushions, which are sites of abundant Sox4 as well as Tbx2 and Tbx3 expression. Over-expression studies have shown potential roles for Tbx2 and Tbx3 in atrioventricular cushion development, which are supported by the fact that in addition to the outflow defects observed in the single nulls Tbx2-Tbx3 compound mutants do not develop atrioventricular cushions (Singh et al., 2005; Hoogaars et al., 2007a; VC, personal

communication). Therefore, Sox4 acts in the same processes in atrioventricular cushion development with Tbx2 and Tbx3, which potentially involves a cooperative interaction between these proteins.

We also show an additional, previously unrecognized domain of Sox4 expression in the myocardium at the border of the left ventricle and the outflow tract, which is the ventral aspect of the interventricular ring (Moorman et al., 1997). In contrast to the dorsal part, which will ultimately form the atrioventricular node and bundle, the ventral region will contribute to the septal branch and the retroaortic root bundle, part of which will loose its Tbx3 expression during later development and disappear (Wessels et al., 1992; Hoogaars et al., 2004; Yanni et al., 2009). The highly localized Sox4 expression is suggestive of a role in the specification of this subcomponent of the interventricular ring. Considering that this part will not contribute to the conduction system, Sox4 may be involved in down-regulation of Tbx3 at later stages. On the other hand, the disappearance of this part of the ring during later development may be caused by apoptosis, which would fit with a previously documented role for Sox4 in the apoptotic pathway (van den Hoff et al., 2000; Hur et al., 2004). Further insight into the function of Sox4 in this area could be obtained using myocardial specific ablation of Sox4 (Agah et al., 1997; Penzo-Mendez et al., 2007).

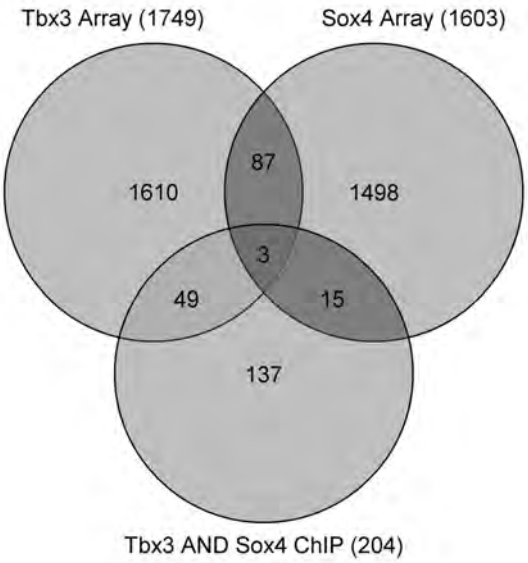


Figure 5. Comparison of ChIP and microarray results. Venn diagram showing numbers of genes that are identified in both the Tbx3 and Sox4 ChIP experiments (bottom circle, 204), and whether these have also been identified as target genes in Tbx3 (left) or Sox4 (right) over-expression micro-array experiments. 67 out of 204 genes also occur in the arrays, of which 3 were found in all four experiments.

Defining downstream targets

To gain insight into the processes that may be regulated by these factors, we defined target genes shared by Tbx3 and Sox4, the expression of which may thus be influenced by an interaction between the two. Comparison of Tbx3 and Sox4 ChIP experiments shows that at least 200 genes are in common. By including microarray datasets we have obtained a list of genes of which we know that their promoters are occupied by Tbx3 and Sox4 *in-vivo*, and these genes are differentially regulated in the myocardium upon overexpression of Tbx3. Within this group of genes we identify several known Tbx3 target genes including *Gja1*, *Cacna2d2*, *KcnJ3* and *Tpm1* (Hoogaars et al., 2007a). These observations indicate that by comparing datasets from different *in-vivo* ChIP and expression arrays we have obtained a relevant list with candidate genes that will be subject of future investigations to further explore the functionality of the interaction between Tbx3 and Sox4.

Unfortunately, a transfection based assay to study differential gene regulation by Tbx3 and Sox4 in H10 cells was unsuccessful. The lack of reproducible results in this assay may be explained by the fact that H10 cells are transformed with an SV40 temperature-sensitive antigen (Jahn et al., 1996). While proliferating when cultured at 33°C, cells differentiate to adapt a more cardiomyocyte like phenotype at 37°C. Possibly, leakyness of this system might explain the variation between duplo transfections. Testing the method on other, more stable, cell-lines in an attempt to try to identify co-regulated genes is currently in progress.

An alternative approach to identify potentially shared target genes might be to test for promoter co-occupancy by sequential ChIP, preferably using endogenous proteins in a relevant cellular context (Wang et al., 2006a; Medeiros et al., 2009). However, one has to bear in mind that these approaches are technically challenging, especially when considering that the number of cells co-expressing Tbx3 and Sox4 are relatively small. It would be worth considering culturing cushion mesenchyme of the outflow tract to expand the number of cells. These approaches may contribute to the elucidation of the functional role of the interaction between Sox4 and Tbx3.

Table 1. Overrepresented GO-terms in the group of genes that are identified in both Sox4 ChIP and the Tbx3 ChIP. Underlined genes were selected for qRT-PCR experiments.

Group ID	Description	p-value	Genes
Muscle system			
GO:0003012	muscle system process	0.00003	Actn2, Camk2d, Edn1, Myom2, Tpm1, Ttn
Response to stimulus			
GO:0051789	response to protein stimulus	0.0006	Bcl2l1, Eif2ak3, Hsp90aa1, Klf10, Nfe2l2, Nr3c1
GO:0042221	response to chemical stimulus	0.027	Anxa5, Bcl2l1, Cuta, Edn1, Eif2ak3, Hsp90aa1, Klf10, Nfe2l2, Nr3c1, Ppp1r15b, Ptpn1, Rhoq, Sort1
Cell communication			
GO:0060024	rhythmic synaptic transmission	0.003	Cacna2d2, Edn1
GO:0010646	regulation of cell communication	0.096	Agap1, Arhgef7, Bcr, Bmper, Cacna2d2, Edn1, Eif2ak3, Git1, Ncam1, Rgs3
Intracellular signaling			
GO:0007242	Intracellular signaling cascade	0.004	Adcy5, Agap1, Arhgef7, Bcr, Calcoco1, Edn1, Eif2ak3, Git1, Mknk2, Nbn, Ncam1, Nfe2l2, Nr3c1, Pdk1, Ppp1r15b, Rab5a, Rhobtb1, Rhoq, Rps6ka2
GO:0006984	ER-nuclear signaling pathway	0.007	Eif2ak3, Nfe2l2, Ppp1r15b
GO:0007219	Notch signaling pathway	0.046	Rbm15, Spen
GO:0016055	Wnt receptor signaling pathway	0.049	Calcoco1, Celsr2, Mitf
GO:0007229	integrin-mediated signaling pathway	0.074	Adamts2, Itga9
Developmental processes			
GO:0048468	cell development	0.012	Ank3, B3gnt2, B4galt1, Bcl2l1, Cacna2d2, Edn1, Eif2ak3, Gja1, Helt, Mdga1, Nav1, Prdm16, Rps6ka2, Sort1, Tacc2, Ttn
GO:0009888	tissue development	0.015	Adamts2, B4galt1, Cacna2d2, Celsr2, Edn1, Eif2ak3, Gja1, Klf10, Lmo4, Ninj1, Nr3c1, Sort1, Tpm1, Ttn
GO:0007507	heart development	0.018	Adamts1, Crkl, Edn1, Gja1, Rbm15, Tpm1, Ttn
GO:0035295	tube development	0.055	Adamts2, B4galt1, Edn1, Gja1, Lmo4, Nr3c1, Rbm15
Transport			
GO:0051179	localization	0.010	Agap1, Ank3, B3gnt2, B4galt1, Bicd2, Cacna2d2, Camk2d, Cd47, Edn1, Eif2ak3, Gja1, Igf2r, Kcnab1, Kcnd2, Kcnd3, Kcnh2, Kcnj3, Ldlrap1, Mdga1, Nav1, Ndufv1, Osbp, Rab5a, Rhoq, Rin1, Sh3bp4, Sh3glb1, Slc1a4, Slc25a36, Slc28a1, Slc4a4, Slc7a14, Sort1, Stam2, Tacc2, Unc50, Uqcrq, Vamp3, Vps37b, Xpo5, Yes1
GO:0017156	calcium ion-dependent exocytosis	0.021	B4galt1, Vamp3
GO:0008643	carbohydrate transport	0.070	Edn1, Sort1, Yes1
GO:0006812	cation transport	0.081	Cacna2d2, Camk2d, Kcnab1, Kcnd2, Kcnd3, Kcnh2, Kcnj3, Slc4a4
Other GO-terms			
GO:0030029	actin filament-based process	0.018	Abl1, Actr3, Arpc2, Daam1, Rhoq, Ttn
GO:0007155	cell adhesion	0.025	Abl1, B4galt1, Cd47, Celsr2, Ctnna3, Dsg2, Itga9, Lamc1, Ncam1, Ninj1, Pard3, Zyx
GO:0006355	regulation of transcription, DNA-dependent	0.027	Atf3, Bcl9l, Calcoco1, Camta1, Celsr2, Dedd2, Hdac4, Helt, Irf2bp2, Kcnh2, Klf10, Lass2, Lmo4, Mitf, Nfe2l2, Nr1d1, Nr3c1, Pcgf5, Phf17, Rbm15, Rnf141, Rybp, Spen, Tsc22d3, Whsc1
GO:0010608	posttranscriptional regulation of gene expression	0.035	Eif2ak3, Mapkapk2, Mknk2, Ppp1r15b, Zfp36l2
GO:0006793	phosphorus metabolic process	0.036	Abl1, Bcr, Camk2d, Cdk4, Edn1, Eif2ak3, Grk5, Grk6, Mapkapk2, Mark2, Mknk2, Myo3b, Pdk1, Ppap2a, Ptpn1, Rps6ka2, Ttn, Yes1
GO:0016265	death	0.045	B4galt1, Bcl2l1, Bok, Dedd2, Eif2ak3, Fadd, Mitf, Nr3c1, Phf17, Rybp, Sh3glb1, Traf5, Tsc22d3, Unc5b