Islet1 is a direct transcriptional target of the homeodomain transcription factor Shox2 and rescues the Shox2-mediated bradycardia

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Abstract The heart’s rhythm is initiated and regulated by a group of specialized cells in the sinoatrial node (SAN), the primary pacemaker of the heart. Abnormalities in the development of the SAN can result in irregular heart rates (arrhythmias). Although several of the critical genes important for SAN formation have been identified, our understanding of the transcriptional network controlling SAN development remains at a relatively early stage. The homeodomain transcription factor Shox2 is involved in the specification and patterning of the SAN. While the Shox2 knockout in mice results in embryonic lethality due to severe cardiac defects including improper SAN development, Shox2 knockdown in zebrafish causes a reduced heart rate (bradycardia). In order to gain deeper insight into molecular pathways involving Shox2, we compared gene expression levels in right atria of wildtype and Shox2−/− hearts using microarray experiments and identified the LIM homeodomain transcription factor Islet1 (Isl1) as one of its putative target genes. The downregulation of Isl1 expression in Shox2−/− hearts was confirmed and the affected region narrowed down to the SAN by whole-mount in situ hybridization. Using luciferase reporter assays and EMSA studies, we identified two specific SHOX2 binding sites within intron 2 of the ISL1 locus. We also provide functional evidence for Isl1 as a transcriptional target of Shox2 by rescuing the Shox2-mediated bradycardia phenotype with Isl1 using zebrafish as a model system. Our findings demonstrate a novel epistatic relationship between Shox2 and Isl1 in the heart with important developmental consequences for SAN formation and heart beat.

Keywords Arrhythmia · Gene regulation · Islet1 · Shox2 · Sinoatrial node · Transcription factors

Introduction

During embryogenesis most cells have to become specialized into one of many various cell types that build a whole organism. These patterning and differentiation processes are ensured by the temporal and spatial activation of different transcription factors [50]. Homeobox genes, for example, regulate numerous processes during embryogenesis including heart development. Complex transcriptional regulation networks are important in many aspects of heart cell lineage development and morphogenesis [46].

The cardiac conduction system consists of electrically coupled cardiomyocytes that are responsible for impulse propagation and essential for the rhythmic and coordinated contraction of the heart. A specialized group of cells in the sinoatrial node (SAN) located at the junction of the
superior caval vein and right atrium generates the electric impulse to activate the atrial myocardium (also termed primary pacemaker). Together with specialized cells in the atrioventricular node (termed secondary pacemaker) and conduction fibers in the interventricular septum, they form the cardiac conduction system [7, 19]. Abnormalities in the cardiac conduction pathway are responsible for various forms of arrhythmias [41, 44, 58]. Sinus node dysfunction, for example, is associated with sinus bradycardia, atrial tachyarrhythmias, sinus pause or arrest and sinoatrial exit block [10]. Although the morphology, cellular components and electrophysiological properties of the SAN are rather well described [34, 38], knowledge of pathways and transcriptional regulation of the SAN formation are poorly understood. Unraveling the pathways that control pacemaker development and function is of crucial relevance to our understanding of pathologies associated with cardiac conduction.

So far, several transcription factors of the homeobox- and T-box gene families have been shown to be involved in cardiac conduction system specification, patterning, maturation, and function [17, 37]. Among these genes is the paired-related homeodomain transcription factor Shox2, which is known to play a crucial role in early cardiac formation, particularly in SAN development and specification [2, 11, 12, 30, 42]. Shox2 expression can first be detected in the posterior region of the primitive heart tube at murine embryonic stage E8.5 and as development progresses, Shox2 is specifically expressed in the sinus venosus myocardium comprising the SAN and the venous valves [2, 12]. These Shox2 expressing domains develop from myocardium that is added to the venous pole of the developing heart and contributes to the second heart field lineage [5, 52]. Newly recruited myocardium that is added to the arterial pole is divided into the anterior or secondary heart field, while the myocardium added to the venous pole is referred to as the posterior heart field [15]. Loss of function studies revealed an essential role for Shox2 prevents the SAN from atrialization by repressing Nkx2.5 expression [11, 12]. Recently, a further link between Tbx5, Shox2 and Bmp4 could be established in the developing heart, demonstrating that Tbx5 signaling in the cardiac pacemaker region controls the expression of Shox2, which in turn regulates Bmp4 in a direct manner [42]. It has also been reported that Pitx2c represents an important susceptibility gene for atrial arrhythmias by suppressing left-sided sinus node formation via direct repression of Shox2 [25, 53]. Taken together, these findings illustrate the importance of Shox2 function in SAN development and highlight the need for further elucidation of the molecular networks involved.

The aim of our current study was to identify Shox2 target genes during heart development. We used expression analysis to compare the transcriptomes in right atria of wildtype and Shox2$^{-/-}$ mouse hearts, and Islet1 (Isl1) was identified as a direct transcriptional target. We have examined putative regulatory elements in the human ISL1 gene and show a pivotal role for sequences within intron 2 in activating transcription. Furthermore, a functional link between Shox2 and Isl1 in vivo was demonstrated using zebrafish as a model by rescuing the Shox2-mediated bradycardia via ectopic expression of Isl1.

Methods

Animals and tissue samples

Shox2$^{-/-}$ mice were generated and genotyped as previously described [2]. For maximal reproduction, we crossed our mice into the CD-1 outbred strain. To improve specificity of the microarray analysis, we dissected the right atria of E11.5 hearts that comprise the Shox2 expressing sinus venosus myocardium and venous valves. For total RNA isolation by TRIzol® (Invitrogen), samples from embryonic right atria (E11.5) of the same genotype (wildtype and Shox2$^{-/-}$) were pooled. For whole-mount in situ hybridization, complete hearts were dissected from E11.5 and E12.5 embryos and fixed overnight in 4 % paraformaldehyde at 4 °C.

Microarray analysis

Gene expression profiling was performed using the GeneChip Mouse Gene 1.0 ST array from Affymetrix (Santa Clara, CA) according to the manufacturer’s protocol. For each genotype (wildtype and Shox2$^{-/-}$), RNA from 6 right atria from embryos of 2 independent pregnancies was pooled. Purity and quality of isolated RNA were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies,
Santa Clara, CA) and showed a RIN >9. 200 ng RNA were used for production of end-labeled biotinylated ssDNA and subsequently hybridized to the arrays. Statistical comparisons of chip data based on ANOVA were performed using the software package JMP Genomics, version 4.0 from SAS (SAS Institute, Cary, NC, USA). Briefly, values of perfect-matches were log transformed, quantile normalized and fitted with log-linear models, with probe ID and genotype considered to be constant. Custom CDF version 14 with Entrez gene-based gene definitions (http://brainarray.mblni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp) different from the original mbni.med.umich.edu/Brainarray/Database/CustomCDF/Entrez gene-based gene definitions (http://brainarray.mblni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp) were used to annotate the arrays. The microarray data were deposited in the NCBI GEO database with accession number GSE39924.

Quantitative RT-PCR

cDNA was synthesized using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green ROX dye (Thermo Scientific). Primer sets used are presented in Supplemental Material (Table S1).

Generation of plasmid constructs

Cloning of the murine Isl1 in situ probe, the zebrafish shox2 and isl1 expression constructs, the human SHOX2a expression construct and generation of the ISL1 luciferase reporter constructs are described online in the Supplemental Material.

In situ hybridization

Shox2−/− mice have been described previously [2]. Whole-mount in situ hybridization on embryonic mouse hearts was performed as reported [16]. Specific digoxigenin- or fluorescein-labeled RNA probes were as follows: Isl1 (see Supplemental Material) and Hcn4 (kindly provided by B. Bruneau, Gladstone Institute of Cardiovascular Disease, USA).

Immunohistochemistry

E11.5 embryos were fixed in 4 % paraformaldehyde at 4 °C overnight, embedded in OCT and transversely cryosectioned (10 μm). Tbx18 and Hcn4 stainings were enhanced using the ABC (Avidin–Biotin-Complex) reagent from Vector Laboratories (VECTASTAIN Elite Peroxidase ABC-Kit) and the TSA (Tyramide Signal Amplification) system (NEL741, Perkin Elmer) according to the manufacturers’ instructions. For double staining (Isl1/Tbx18 or Isl1/Hcn4), the respective fluorescent secondary antibody was added either during the biotinylated antibody incubation or in a second step afterwards. The following primary antibodies were used: mouse monoclonal antibody against Isl1 (1:100; 39.4D5, Developmental Studies Hybridoma Bank), goat polyclonal antibody against Tbx18 (1:250; C-20, Santa Cruz) and rabbit polyclonal antibody against Hcn4 (1:500; APC-052, Alomone). Alexa Fluor 568 rabbit anti-mouse (1:800; A-11061, Invitrogen), biotinylated horse anti-goat (1:500; BA-9500, Vector Laboratories) and biotinylated goat anti-rabbit (1:500; 550338, BD Pharmingen) were used as secondary antibodies. Nuclei were counterstained with Hoechst (Invitrogen). Imaging was carried out on a Nikon 90i upright epi-fluorescence microscope with a Nikon DS-Qi1 mc camera.

Cell culture, transfection and luciferase assay

HEK 293 cells were cultured at 37 °C in DMEM medium containing high glucose, supplemented with 10 % fetal calf serum and antibiotics. For luciferase assay analysis, the cells were cotransfected in triplicate with different constructs using polyethylenimine (Sigma-Aldrich). 24 h after transfection, luciferase activity was determined and normalized to Renilla luciferase activity with a dual luciferase transfection, luciferase assay constructs using polyethylenimine (Sigma-Aldrich). 24 h after transfection, luciferase activity was determined and normalized to Renilla luciferase activity with a dual luciferase assay kit (Promega). Experiments were repeated at least three times in triplicate with consistent results and representative data are shown.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using standard protocols. For the binding reaction, 32P-labeled, double stranded ISL1 oligonucleotides (Oligo1 (+2364/+2418), Oligo2 (+2404/+2458), Oligo3 (+2447/+2502), Oligo4 (+2496/2561); Supplemental Material Table S1), were used together with purified, bacterially expressed recombinant GST-SHOX2 protein [3].

Zebrafish embryos and microinjections

Care and breeding of zebrafish, Danio rerio, were as described previously [55]. For all plasmid and morpholino injection procedures, the TE4/6 wildtype strain was used. Morpholino-modified antisense oligonucleotides (MO; Gene Tools) were directed against the translational start site (shox2-MO [5′-ACGCTGTAGTCTTCCATCAGTGC-3′]) of zebrafish shox2, the splice donor site of exon 2 (isl1-MO [5′-TTAACACGATCGTACCTGACATGTC-3′]) of zebrafish isl1 and the translational start site (isl1-MO2 [5′-GATCCCCCATGTCCTCCATGTCAGAAG-3′]) of zebrafish isl1. A shox2-MO directed against the splice donor site of intron 3 causing the identical bradycardia phenotype has
been described previously [2]. shox2 and isl1 antisense oligonucleotides or a standard control oligonucleotide (MO-control), diluted in 0.2 mol/liter KCl, were microinjected into one-cell-stage zebrafish embryos [20]. For rescue experiments 0.32 ng of pDestTo2CG2-shox2 or pDestTo2CG2-isl1 was microinjected into one-cell-stage embryos directly after injection of 2.8 ng of shox2-MO.

For histology, embryos were fixed in 4 % paraformaldehyde and embedded in JB-4 (Polysciences, Inc). Then, 5-µm sections were cut, dried, and stained with hematoxylin and eosin [43].

For immunoblotting, monoclonal mouse IgG1 anti-rat islet1 antibody (Developmental Studies Hybridoma Bank, DSHB, University of Iowa) and polyclonal anti pan-cadherin (Abcam) were used. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. Blots were probed with the mentioned antibodies, signals were detected by chemiluminescence (monoclonal anti-rabbit-Hrp and anti-mouse-Hrp) and imaging was performed with Image Quant LAS4000mini (GE Healthcare). Quantification of the immunoblot was performed using the Image Quant TL software.

Whole-mount antisense RNA in situ hybridization was carried out as described [21] using a digoxigenin-labeled antisense probe for zebrafish shox2 and islet1. For immunostaining, zebrafish embryos were fixed in Dent’s fixative and a monoclonal anti-acetylated tubulin antibody (Sigma) was used [40].

Statistical analysis

Data are presented as mean ± SEM from at least three independent experiments. Comparisons between experimental groups were performed using paired t test or 1-way ANOVA. Differences were considered significant if they showed a value of \( P < 0.05 \).

Results

Shox2 has regulatory effects on Isl1 expression in the developing sinoatrial node

To identify novel transcriptional targets of Shox2, we performed microarray analysis and compared gene expression levels in right atria of wildtype and Shox2\(^{-/-}\) hearts. Only right atria comprising the Shox2 expressing sinus venosus myocardium and venous valves were dissected from murine E11.5 hearts to increase the specificity of the array analysis. Genes were considered to be up- or downregulated if they showed log2 ratios of \( \geq 0.35 \) or \( \leq -0.4 \) with a significance of \( P \leq 0.0001 \). Using these selection criteria, we identified 321 genes with significantly altered gene expression (80 up- and 241 downregulated genes). As expected, Shox2 was among the highest negatively regulated genes. Classification of the 321 genes into gene families using the Gene Set Enrichment Analysis (GSEA) revealed that the highest proportion of regulated genes belongs to the group of transcription factors. Among these, the LIM homeodomain transcription factor Isl1 showed a reduced expression in right atria of Shox2\(^{-/-}\) embryos.

To verify Isl1 as a putative target gene, we carried out qRT-PCR analyses using right atrial tissue samples of wildtype and Shox2 knockout embryos and confirmed the downregulation of Isl1 expression in a second independent pool of embryonic heart tissue (Fig. 1A). To further investigate Isl1 as a putative target gene of Shox2, we compared the spatial expression of both genes and found that Shox2 and Isl1 expression overlap in embryonic mouse hearts. Isl1 expression was detected in the SAN of wildtype hearts, which corresponds to the area where Shox2 normally is expressed (Fig. 1B). Whole-mount in situ hybridization on isolated E11.5 wildtype and knockout hearts demonstrated that Isl1 transcripts are completely absent in the SAN region of Shox2 mutants (Fig. 1C b’), while normal expression was observed in the outflow tract (Fig. 1C b). The lack of Isl1 expression in the SAN, but unaffected Isl1 expression in the outflow tract of Shox2-null mice could be also confirmed at embryonic stage E12.5 (Fig. S1A). In situ analyses revealed a complete loss of Isl1 expression in the right atrium (SAN) of knockout hearts, suggesting that the residual Isl1 expression detected in Shox2 knockout tissue by qRT-PCR and microarray experiments is likely due to some remaining outflow tract tissue following microdissection. Quantitative expression analysis using outflow tract tissue samples showed high and consistent Isl1 expression levels in both wildtype and Shox2 knockout embryos (Fig. S1B).

To validate that the loss of Isl1 expression in the SAN of Shox2 knockout hearts is not due to incorrect formation of SAN tissue, we examined the expression of SAN markers in wildtype and knockout hearts using in situ hybridization and immunohistochemistry. While Tbx18 is mainly expressed in the SAN head, Hcn4 is a very prominent marker of the complete developing SAN [45, 56]. Double in situ hybridization experiments using an Isl1 antisense probe together with Hcn4 showed coexpression in the SAN of E12.5 and E13.5 wildtype hearts (Fig. 2A a, a’, c, c’). In Shox2\(^{-/-}\) mouse hearts Isl1 expression is completely absent in this region, while Hcn4 transcripts are still detectable (Fig. 2A b, b’, d, d’). Double immunostainings revealed that Hcn4 and Tbx18 colocalize with Isl1 in the SAN of E11.5 wildtype embryos (Fig. 2B a, g). While both markers could be detected in the SAN of wildtype and knockout hearts (Fig. 2B c, f, i, l), Isl1 staining is not
detectable in this region in Shox2−/− embryos (Fig. 2B b, k). Together, these stainings confirm the loss of Isl1 expression in the SAN of Shox2 mutants on transcript and protein level at different embryonic stages, while the presence of Hcn4 and Tbx18 confirm that the SAN is formed. The loss of Isl1 expression is thereby very likely linked to Shox2 deficiency and does not constitute an indirect effect due to disrupted development of SAN tissue in knockout embryos.

SHOX2 binds to regulatory elements in an intronic region of the ISL1 gene

To further study the transcriptional control mechanism of ISL1 by SHOX2, we investigated specific binding sites within the ISL1 locus. As a first step, we performed multispecies conservation analysis for the human ISL1 locus to identify evolutionary conserved regions. Conservation of DNA sequences provides a route for identifying functional elements in genomes [32]. Sequence comparison of human, frog, chicken, and mouse Isl1 revealed several highly conserved regions (Fig. S2A). Using luciferase assays, we functionally characterized these highly conserved elements. The reporter constructs used are depicted as black boxes below the conservation plot in Supplemental Material Fig. S2A and are referred to as ISL1-reporter-1 to ISL1-reporter-5. ISL1-reporter-3 and -4 include regulatory elements previously identified downstream of the translational stop codon [22, 23], while ISL1-reporter-1 contains a recently published minimal promoter of the Isl1 gene [31]. Cotransfections of the different ISL1 reporter constructs along with a SHOX2a expression plasmid revealed a strong increase in luciferase activity only for ISL1-reporter-2, compared to the basal level of the respective reporter alone (Fig. S2B). By transfection of serial gene deletion constructs (genomic locations of the constructs are depicted in Fig. 3A), we confined the SHOX2 transcriptional regulatory element to a 184-bp region located in the second intron of ISL1, corresponding to ISL1-reporter-2del 3 (Fig. 3A, B). To identify the precise SHOX2 binding site, EMSA was performed using four different oligonucleotide probes (Oligo1–Oligo4; Fig. S3A) that cover the respective intronic ISL1 sequence. We obtained a shift with both Oligo1 (strong binding) and 3 (weaker binding), but not with Oligo2 and 4 (Fig. 3C, Fig. S3B). Competitive EMSAs demonstrated that only high excess (150-fold) of unlabeled Oligo3 (SpC3) competitively affects binding of Oligo1 to SHOX2 (Fig. S4A, left panel), while already a low molar excess (50-fold) of unlabeled Oligo1 (SpC1) competitively inhibits binding of labeled Oligo3 to SHOX2 (Fig. S4A, right panel). Our luciferase assays also show that the sequence encompassing Oligo1 alone is not sufficient to activate ISL1 by SHOX2 (as this region was also partly included in the ISL1-reporter-2del 2 construct which did not show an increase in luciferase activity upon cotransfection with SHOX2) (Fig. 3B), suggesting that both sequences (encompassing Oligo1 and Oligo3) are necessary to activate the ISL1 gene by SHOX2. Oligo1 and Oligo3 both include AATT-palindromic sequences, which were
mutated to CCGG (Oligo1 mut and Oligo3 mut) to confirm sequence specific binding. No shift was obtained using the mutated Oligos, confirming that the AATT motif is essential for SHOX2 binding (Fig. 3C). In addition, competition assays revealed that an excess of unlabeled wildtype Oligos (SpC1 and SpC3) reduce the DNA binding ability of SHOX2, whereas excess of mutant Oligos (MuC1 and MuC3) do not affect the binding (Fig. S4B).
Bradycardia caused by shox2 deficiency depends on isl1 expression

To functionally assess whether Shox2 and Isl1 act in the same genetic pathway, we injected zebrafish embryos with morpholinos (MO). When injecting either 2.8 ng of shox2-MO or isl1-MO, 82.0 ± 2.6 % (n = 175; 2 independent experiments) and 79.1 ± 0.3 % (n = 182; 2 independent experiments) of injected embryos, respectively, displayed pericardial edema (Fig. 4A asterisk) and a severely reduced heart rate (Fig. 4B). Embryonic heart morphogenesis proceeds normally in shox2-MO and isl1-MO injected zebrafish. By 72-h post fertilization (hpf), morphant hearts consist of two heart chambers, atrium and ventricle, with properly developed myocardial and endocardial cell layers. The similarity of phenotypes between shox2 and isl1 deficiency in the fish raised the possibility that a close functional interaction between these two genes exists. To address whether the observed cardiac dysfunction caused by shox2 deficiency could depend on isl1 function, we examined the ability of isl1 to compensate the morpholino-mediated loss of shox2 function by transient rescue experiments. DNA constructs expressing isl1 and shox2 under the control of the cardiac myosin light chain 2 (cmle2) promoter [18, 33], which drives myocardial-specific expression were used for the rescue experiment. The cmle2 promoter also drove expression of GFP, which allowed monitoring of cardiomyocyte-specific expression (Fig. S5A). We coinjected 0.32 ng of the isl1-expression construct along with 2.8 ng shox2-MO, which was sufficient to induce the bradycardia phenotype and subsequently measured the heart rate of GFP positive embryos after 72 hpf. Myocardial-specific expression of isl1 could significantly rescue the reduced heart rate in shox2 morphant embryos. As a control, injection of the isl1- or shox2-expression construct alone did not cause any changes in embryonic heart rate (Fig. 4C). Histological sections of shox2-MO injected hearts rescued by isl1 overexpression show normal development of the heart without formation of pericardial edema (Fig. 4D), compared to those of shox2-MO injected embryos alone (Fig. 4A). In addition, we could show a downregulation of isl1 protein levels in embryos injected with shox2-MO (Fig. S5B). Both proteins, shox2 and isl1, are highly expressed during neural development and isl1 plays an important role during differentiation of neurons in the brain. To exclude that shox2 deficiency also affects differentiation of isl1 positive neurons, we analyzed the isl1 expression after shox2 knockdown by whole-mount in situ hybridization. Isl1 expression in the brains of shox2 morphants is indistinguishable from wildtype embryos (Fig. S6A and B). On the other hand, isl1 deficient embryos show a normal expression of shox2 in the head region (Fig. S6C and D). Furthermore, we performed immunostaining using acetylated Tubulin as
neuronal marker (Fig. S6E and F). While the heart rate was reduced after shox2-MO injection, no alteration could be detected in neural development. Together, these results indicate an epistatic relationship and a direct functional link between shox2 and isl1 in the pacemaking system of zebrafish embryos.

**Discussion**

During embryogenesis, the proper development of the cardiac pacemaker is essential for a coordinated heart beat. Defects in the transcriptional network controlling SAN development may cause dysfunction of the primary pacemaker resulting in severe arrhythmias. The elucidation of the molecular mechanisms and key regulators involved in these developmental processes is therefore crucial for our understanding of arrhythmia-related diseases.

The LIM homeodomain transcription factor Isl1 is a prominent marker of second heart field derivatives comprising the right ventricle, outflow tract, inflow tract with the SAN and parts of the atria and plays a crucial role in cardiogenesis [6, 49, 51]. SAN development is initiated in the presence of Isl1 expressing second heart field-derived cells [35, 48, 51]. Homozygous loss of Isl1 results in embryonic lethality between E10.5 and E11.0 due to severe cardiac abnormalities. Besides heart looping defects, Isl1−/− embryos show hypoplasia or complete absence of the right ventricle, the outflow tract and parts of the atria including the inflow tract [6]. Early in embryogenesis, Isl1 positive cells, which exhibit multipotent properties [4, 36], contribute to both the arterial (outflow tract) and the venous...
pole (SAN) of the primary heart tube. Once these progenitor cells differentiate into cardiomyocytes, Isl1 expression becomes downregulated and almost extinguished from E12.5 to E18.5 [27]. Small numbers of Isl1 positive cells can still be detected in the adult hearts, including the SAN [14, 24, 54].

Although signaling pathways up- and downstream of Isl1 have been investigated in the past [1, 6, 8, 26, 39], little is known about direct regulators controlling the transcriptional activity of this gene in cardiac cells. The only proteins known to regulate Isl1 expression so far via direct binding are β-Catenin [28], Forkhead- and Gata4 transcription factors [22, 23] and the POU homeodomain transcription factor Oct1 [31]. While the binding sites for these regulators reside in promoter and enhancer regions up- or downstream of the gene, we could identify a regulatory element within an intronic region of ISL1.

In the present study, a connection between the homeodomain transcription factor Shox2 and Isl1 was revealed during cardiac development. We could show that Shox2 is essential for the expression of Isl1 in the SAN. Based on luciferase reporter studies and EMSA, our data also demonstrated that SHOX2 directly regulates ISL1 expression and that this regulation is conducted via the binding to an intronic regulatory element of ISL1. To study the transcriptional regulation of isl1 by shox2 in vivo, zebrafish was employed as a model. We have shown previously that shox2 loss of function in zebrafish embryos results in severe cardiac dysfunction with sinus bradycardia and intermittent sinus exit block after 72 h of development [2]. Similar data have also been published for Isl1 [9] and we could confirm these findings experimentally. Furthermore, it has been shown that both genes are expressed at the venous pole of the atrium in zebrafish embryos (48–72 hpf) [2, 57]. In the present study, we demonstrate a functional link between shox2 and isl1 in zebrafish embryos by partially rescuing the shox2-mediated bradycardia via ectopic expression of isl1. Previously, two separate phases of cardiomyocyte differentiation have been shown to exist in zebrafish. The first process, which regulates the differentiation of the venous pole comprising the sinus venosus requires Isl1 expression, while in a second FgF8-dependent process new cells are added to the arterial pole of the heart [9]. Interestingly, in both Isl1 mutant fish and Shox2 knockout mice, Bmp4 expression is lost in the inflow tract (sinus venosus), but not the outflow tract of the developing heart [9, 42]. In addition, deficiency of both Isl1 and Shox2 in fish, as well as Shox2 deficiency in the mouse results in a bradycardia phenotype [2, 9]. The similarity of the phenotypes observed in Isl1 and Shox2 mutants point to a genetic link between these two genes in the development or function of the cardiac pacemaker. Taken together, our findings revealed an interesting interplay between Shox2 and Isl1 in the SAN, which contributes to a novel transcriptional regulation in the cardiac pacemaker system (Fig. 5).

Based on the established functional data, SHOX2 as well as SHOX2 targets represent excellent candidates for human arrhythmias. While genetic variation in ISL1 is associated with congenital heart disease and cardiomyopathies [13, 47], SHOX2 has not been linked to any human phenotype yet. In a SHOX/Shox2 replacement mouse model, it was recently shown that a hypomorphic SHOX allele is not able to completely rescue the shox2-/- cardiac phenotype. Although the mice survive, they still show arrhythmias, suggesting that SHOX2 haploinsufficiency may lead to arrhythmia in humans [29]. Thus, it will be interesting to see if both SHOX2 and ISL1 are involved in human heart disease, especially arrhythmias.

A possible connection between Isl1 expressing second heart field-derived cells and the formation of the cardiac conduction system has been previously proposed [35, 48, 54]. As Shox2 directly regulates Isl1 expression specifically in the embryonic SAN, our data show for the first time a direct functional consequence for Isl1 in the pacemaking system of the developing heart. Taken together, our data add another piece to the puzzle of regulatory transcriptional networks controlling the development of the pacemaker.

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Conflict of interest  None.

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