CardioGeNet
Definition of a Genetic Network Involved in Congenital Heart Disease

CardioGeNet public website: www.cardiogenet.eu

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The CardioGeNet consortium aimed at the identification of a network of genes controlling the development of the heart, and thus involved in congenital heart disease (CHD), which represents the most common birth developmental defect of the heart.

The entry point for the interrogation and perturbation of cardiogenic gene networks was the transcription factor *Tbx1*, the major gene responsible of 22q11.2 deletion syndrome (22q11DS), or DiGeorge syndrome, the most common genetic cause of CHD after Down syndrome.

The overall goal of the CardioGeNet project was to define gene networks perturbed in models of congenital heart disease and in patients with CHD. The ultimate goal was to deliver to the genetics and clinical communities a catalogue of interacting, validated genes involved in the etiology and pathogenesis of CHD.

The specific objectives were as follows:

1. **Define the role and interactions of the T-box family of transcription factors in the development of the cardiac outflow tract (OFT).**
2. **Define gene networks perturbed in OFT developmental abnormalities.**
3. **Integrate gene expression dynamics, cell lineage distribution and phenotypic data.**

Human birth defects can be caused by chromosome deletions in which patients have only one copy of genes affected by the deletion rather that two. 22q11 deletion syndrome is the most common microdeletion in man; clinically, the diagnoses may be termed DiGeorge or velocardiofacial syndrome. The gene *TBX1* is affected by the majority of these deletions. Tbx1 is vital for many aspects of heart and vessel development and it controls the expression of other genes in a “network”. Partner’s aim was to include other genes within the network, to assess their participation in heart and vessel formation and to investigate whether their eventual role in the malformations produced when one copy of *TBX1* is lost.

Partners have used different methods to identify genes that are regulated by Tbx1 (i.e. genes whose expression is changed by the action of Tbx1 during development). Much of this work has involved the use of mouse models where *TBX1* has been switched off. One step involved the screening of all the genes whose expression changes in the absence of Tbx1. These screening could produce false positive results and therefore each “candidate” Tbx1 target needed to be validated using an independent technique (i.e. polymerase chain reaction). Once identified what look like true positives, i.e. genes whose expression pattern changes when Tbx1 is absent, gene’s expression during mouse

embryo development has been investigated. The data obtained were used to select genes for the next stages of the analysis:

- Is the gene directly regulated by Tbx1?
- In which embryonic tissues is the gene required?
- Does the gene interact with Tbx1 during development of the cardiovascular system?

Tbx1 protein is a transcription factor, it binds to DNA sequences that control primary target gene expression. Of course, the Tbx1 targets may not be directly regulated by the protein, there may be intermediate steps involved. By using computational searches, Partners searched for short DNA sequences that are likely to bind Tbx1, then cloned these sequences and used cell systems to test whether Tbx1 can increase (or decrease) expression of the target. Finally, they attempted to detect binding of Tbx1 protein to the target gene sequences. The one disappointing aspect of the results has been the paucity of direct targets: Partners have been able to detect only one gene, *Smad7*. In order to increase the sensitivity in detecting Tbx1 binding, Partners created a new mouse line which expresses Tbx1 with a tag. This line allows to use antibodies against the tag rather than antibodies against Tbx1 for the work, and antibodies against the tag are generally much better in the following purification steps.

Brief Description of Genes Studied in Greater Detail

Gbx2: transcription factor. *Gbx2* is required in cells in the surface of the embryo for the main vessel, the aorta, to develop properly. Animals which had just one copy of *Gbx2* as well as just one copy of *TBX1* (double heterozygotes) had a much more severe cardiovascular defects versus single mutants.

Hes1: transcription factor active in several regions of the embryo. *Hes1* (actually called her6 in fish) and *TBX1* interact during development of the pharyngeal structures. Extra *Hes1* could partially compensate for the lack of *TBX1*.

Cyp26b1: encodes an enzyme that degrades retinoic acid (vitamin A). Retinoic acid acts as a signal between cells and has already been shown to be important for heart and vessel development. A deficiency of this enzyme, which would result in a local excess of retinoic acid, results in defects of the head and neck vessels. It has been known for several years that exposure of the embryo to excess of retinoic acid can produce malformations similar to 22q11DS.

Sema3c: encodes a protein produced by cells that can either repel or attract the movement of other cells. Tbx1-Sema3c interactions have been investigated. A new mouse strain has been created to allow testing the tissue specific requirements for this protein.

Cxcl12: encodes a protein produced by cells that can attract other cell types through its receptor called Cxcr4. *Cxcl12* mutants have great vessel malformations, this is due to defective Cxcl12 signalling to the cells that constitute the innermost lining of the vessels.

Smad7: encodes a protein that is inside the cell but modulates signalling from the cell surface. Smad7 is required for development of both the heart and the great vessels. An interesting interaction between Smad7 and Tbx1 has been found. Embryos lacking one copy of *TBX1* have fewer vessel defects at birth than they do earlier in development. Thus *TBX1* heterozygotes “recover” from a proportion of the earlier vessel defects. *TBX1* heterozygous animals, which had just one copy of Smad7, did not show this recovery; Smad7 is vital for the remodelling of the early vessel configuration as it matures into the newborn pattern. Tbx1 was discovered to bind DNA at Smad7 directly at the relevant stages of mouse embryo development.

Chd7: encodes a protein that also affects gene expression. However, Chd7 alters the way DNA is packaged in the nucleus, rather than bind DNA directly like Tbx1. Mutations of *CHD7* are a rare cause of DiGeorge syndrome in man; in addition Chd7 interacts with Tbx1. Like Tbx1, Chd7 is required in the surface layer of the embryo for correct formation of the embryo, but is also required in neural crest cells. Partners are now examining whether Chd7 and Tbx1 regulate common genes in a cardiovascular network.

Wnt5a: Encodes an important ligand of the Wnt non-canonical pathway thought to be important for cell polarity and cell migration. Partners found that this gene is a direct target of Tbx1 and that interacts genetically with it during heart development, in particular in the SHF.

Mef2c: encodes a transcription factor required for muscle differentiation. Partners found that Tbx1 can directly repress the expression of this gene by binding to its SHF enhancer. This interaction is likely to be one of the mechanisms by which Tbx1 inhibits premature differentiation of cardiac progenitors.

Fgf8: encodes a ligand of the FGF signaling pathway, known to be a transcriptional target of Tbx1. Partners have demonstrated that forced Fgf8 expression (using a transgenic mouse line) can partially rescue the Tbx1 mutant phenotype.

Vegfr3: encodes a receptor of Vegfc and Vegfd. Partners found that Vegfr3 is a direct target of Tbx1 in endothelial cells, and probably, through this regulation, it is required for lymphatic vessel development.

In conclusion, analysis of global changes in gene expression in *TBX1* embryo mutant embryos has proved a useful method of identifying genes important for cardiovascular morphogenesis and genes that interact in a Tbx1-mediated network in this process. The project has also provided a sound basis for extending the network into genes regulated by the chromatin remodellers CHD7 and Baf60a/Smarcd1.

Definition of molecular mechanisms of Tbx1 function.

Tbx1 is a transcription factor that binds DNA of target genes. Partners have investigated the mechanisms by which it regulates target genes. Searches for protein interactors led for several factors important for gene regulations. One of these is Smarcd1 (a.k.a. Baf60a) a chromatin remodeling factor of the SWI-SNF-like complex. Elimination of Baf60a makes Tbx1 unable to regulate its targets. Another interactor identified by Partners is the histone methyltransferase Setd7. Tbx1 dosage correlates with H3K4me1 enrichment. These findings led partners to propose a model by which Tbx1 binds to its target enhancers of target genes, and recruits chromatin remodeling factors as well as histone modifiers that together increase the likelyhood of the target gene to be transcribed.

Identification of the genome-wide occupation of Tbx1 in Cardiac Progenitors

In contrast to Tbx3, Tbx1 is not expressed in differentiated heart tissue, but in cardiac progenitors. Thus, to investigate its occupation Partners have used an in vitro model of cardiac progenitors differentiation (differentiating P19 cells). ChIP-seq analyses using an antibody against the endogenous Tbx1 protein revealed approximately 2000 peaks of enrichment (after highly stringent data analysis) corresponding to at least 500 genes. ChIP-seq data using an anti H3K4me1 antibody carried out in parallel with the above experiments, revealed that most if not all the regions of Tbx1 enrichment co-localize with regions of H3K4me1 enrichment, a marker of active enhancers. The most represented categories of genes among the targets identified by these experiments were those involved in gene

expression and embryonic development (using Ingenuity ontology), suggesting a broad transcriptional relevance of Tbx1 in cardiac progenitors.

Definition of a T-box gene network regulating heart tube elongation

Partners have explored the genetic interactions between *Tbx1* and two other T-box transcription factor encoding genes, *Tbx2* and *Tbx3,* during formation of the arterial pole of the heart. The experiments were based on the observation that *Tbx2* and *Tbx3* are down-regulated in the pharyngeal region of Tbx1 null embryos at mid-gestation, including cells in the region of the second heart field. Using immunohistochemistry and in situ hybridization coupled with 3D-reconstruction analysis Partners generated detailed expression maps for these three genes at the time of heart tube elongation by addition of progenitor cells from the second heart field. These experiments revealed overlapping expression profiles in different cell types in the pharyngeal region including pharyngeal mesoderm (*Tbx1, Tbx2* and Tbx*3*), neural crest derived mesenchyme (*Tbx2* and *Tbx3*) and pharyngeal endoderm (*Tbx1, Tbx2* and *Tbx3*). Genetic crosses between each pair of T-box genes revealed that the genes play overlapping functions during pharyngeal morphogenesis and heart tube elongation. Removing two of the three genes resulted in very severe heart tube elongation phenotypes and failure of looping and dorsal mesocardial breakdown (Figure 1 A-D). Analysis of the downstream mechanisms, in particular the impact of loss of two of the three *Tbx* genes on intercellular signaling pathways during heart tube elongation, led to the finding that the domains of fibroblast growth factor and bone morphogenetic protein signaling were altered in double mutant embryos as follows. The domain of lateral pro-proliferative FGF signaling was shifted centrally, proximal to the outflow tract, and pro-differentiation BMP signaling in the distal outflow tract region was down-regulated (Figure 1E). These alterations significantly perturb the balance between proliferation and differentiation that is required for coordinated heart tube elongation and pharyngeal morphogenesis. Our results thus define a T-box gene regulatory network operative during early heart development and furthermore identify *TBX2* and *TBX3* as genes that potentially interact with *TBX1* to modify the cardiovascular phenotype of DiGeorge syndrome patients. Partners results were published in early 2012 in *Human Molecular Genetics* (Mesbah et al., 2012). This work has been followed through to reveal a role for *Tbx1* in *Tbx2* positive cells in the dorsal mesenchymal protrusion, derived from the second heart field and critically required for atrial septation. Failure of segregation of progenitor cells adding to the venous and arterial pole of *Tbx1*$^{-/-}$ hearts results in atrioventricular septal defects in a fraction of mutant embryos.

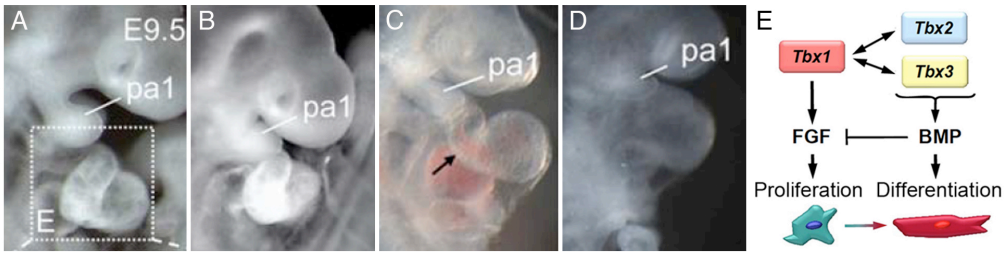

Figure 1. **E9.5 embryos comparing wildtype (A), Tbx1$^{-/-}$ (B), Tbx3$^{-/-}$ (c) and Tbx1$^{-/-}$;Tbx3$^{-/-}$ (D).** Note the severely impaired heart tube in the double mutant embryo. Pa1, first pharyngeal arch. A T-box regulatory network operates upstream of intercellular signaling events controlling progressive differentiation of arterial pole progenitor cells (E).

Identification and characterization of a conduction system defect in *Tbx1* null hearts

T-box genes play an important role in development of the cardiac conduction system that ensures cardiac rhythm. In the course of analysis of *Tbx1*$^{-/-}$ hearts Partners observed, using a *Cx40-GFP* reporter line that permits visualization of the ventricular conduction system, that there is a

morphological discontinuity between the atrioventricular bundle and right ventricular Purkinje fiber system in the region of the right bundle branch. Optical mapping of the electrical activity in *Tbx1*$^{-/-}$ fetal hearts confirmed that instead of a normal bi-ventricular breakthrough, only the left ventricular apex showed a breakthrough revealing right bundle branch block (Figure 2A), a defect seen in human patients. Comparison with different mouse models with outflow tract defects and analysis of earlier timepoints has revealed that this defect emerges during the fetal period, is specific to the type of ventricular septal defect observed in *Tbx1*$^{-/-}$ hearts, is associated with abnormal morphogenesis of the right ventricular papillary muscle and is not associated with altered cell death, proliferation or gene expression in the early ventricular conduction system. Genetic tracing experiments are underway to further probe the origin of this anomaly.

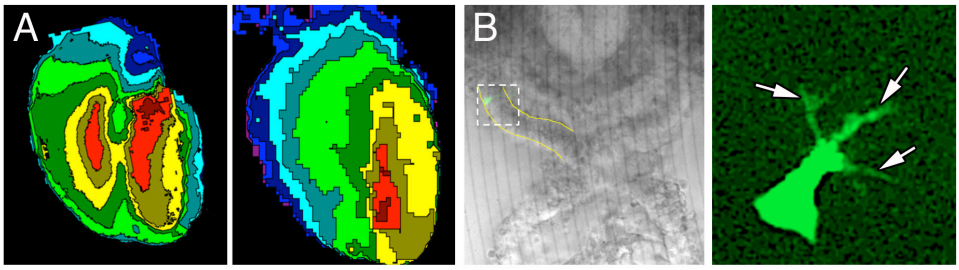

Figure 2**. Optical mapping data for a wildtype heart (A, left) and Tbx1**$^{-/-}$ **heart (A, right) at E17.5, showing activation only at the apex of the left ventricle in the mutant heart.** GFP expression in a cell in the dorsal pericardial wall after electroporation (B, left); high magnification view of the boxed region showing dynamic filopodia-like protrusions (arrows) on the basal side of an electroporated cell (B, right).

Tbx1 and Tbx2 function in outflow tract development and conduction

The right ventricular outflow tract (RVOT) of the heart, affected in *Tbx1*$^{-/-}$ embryos and hypoplastic in tetralogy of Fallot patients, is associated with life threatening arrhythmias including arrhythmogenic right ventricular dysplasia/cardiomyopathy and Brugada syndrome. The RVOT is derived from the embryonic outflow tract in which chamber differentiation is postponed, possibly by the action of Tbx2. Aspects of the embryonic phenotype of the outflow tract are maintained in the fetal and adult RVOT and contribute to conduction slowing and arrhythmogenesis especially after sodium current reduction. Using optical mapping Partners found that the conduction velocity was lower in the RVOT than in the right ventricular free wall. The fetal RVOT displayed an embryonic outflow tract gene expression profile compatible with slow conductivity. Unexpectedly, a difference in conduction velocity in the RVOT between *Tbx1* mutants and controls and between *Tbx2* mutants and controls was not observed. However, in hearts of *Scn5a*$^{1798insD/+}$ mice (which have reduced sodium channel function), conduction was slower in the RVOT than in the right ventricular wall. Furthermore, low expression of several genes (Cx43, Scn5a, both involved in conduction) was maintained in the RVOT. Partners conclude that aspects of the slowly conducting embryonic phenotype are maintained in the fetal and adult RVOT, and are unmasked when cardiac sodium channel function is reduced.

Identification of the genome-wide occupation of TBX3, NKX2-5 and GATA4 in the heart *in vivo*

To identify direct targets of Tbox and other transcription factors in the PA and OFT, Partners tried to perform ChIP-seq (chromatin immunoprecipitation-massive parallel sequencing) on embryonic material. However, this turned out to be technically not feasible. Therefore, partners assessed the genome-wide occupation of transcription factors Tbx3, Nkx2-5 and Gata4 and of enhancer-associated co-activator p300 in the mouse heart, uncovering cardiac enhancers throughout the genome. Many of the

enhancers co-localize with ion channel genes repressed by Tbx3, including the clustered sodium channel genes *Scn5a*, essential for cardiac function, and *Scn10a.* Two enhancers in the *Scn5a/Scn10a* locus have been identified; they are regulated by Tbx3 and its family member and activator Tbx5, and are functionally conserved in humans. Partners provide evidence that a common single-nucleotide polymorphism in the *SCN10A* enhancer, associated with alterations in cardiac conduction patterns in humans, disrupts Tbx3/5 binding and reduces the cardiac activity of the enhancer in vivo. Thus, the identification of key regulatory elements for cardiac conduction helps to explain how genetic variants in non-coding regulatory DNA sequences influence the regulation of cardiac conduction and the predisposition for cardiac arrhythmias (van den Boogaard M et al. 2012).

Identification of the regulatory DNA regions of Tbx3 involved in cardiac and PA expression

Tbx3 is expressed in the cardiac neural crest in the PA and OFT, and in the second heart field, foregut, valve mesenchyme and conduction system. Tbx3 function is crucial for OFT morphogenesis. Identification of the factors that regulate its expression may reveal upstream regulatory mechanisms for OFT development. Partners have used ChIP-seq data to identify enhancers of Tbx3, and tested these enhancers in transient transgenic embryos. The activity of an enhancer (eA) 20 kbp upstream of the transcription start site recapitulates key aspects of this pattern (Figure 3). An enhancer (eB) further upstream acts as a booster for this enhancer. The human orthologous enhancer regions were found to be functionally conserved. For robust characterization of the physical organization around selected promoters and other functional elements, Partners optimized chromosome conformation capture combined with high-throughput sequencing. Partners defined the physical interactions with the Tbx3 promoter and enhancers in the embryonic mouse heart (Figure 3). These data confirmed interactions between the promoter and the enhancers in vivo, and revealed many more sequences within the Tbx3 locus that interact with the promoter. Thus, two key regulatory sequences that control expression of Tbx3 in the heart and PA have been identified and characterized; moreover, the 3D organization of the Tbx3 locus and the sequences that interact with the promoter of Tbx3 have been defined.

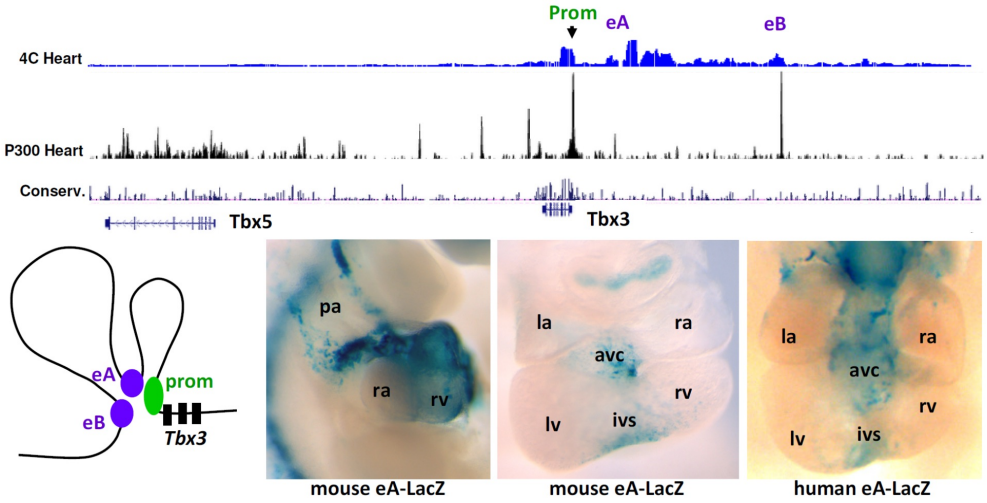

Figure 3. **Browser views of 4C-seq data from embryonic heart tissue, p300 binding profile in heart tissue and conservation of the Tbx3 locus.** Using the promoter of Tbx3 as viewpoint, the interactions with enhancers eA and eB are clearly visible. A model for the interactions of the promoter (green) and enhancers (purple) is shown in the lower left panel. Transgenic embryos in which lacZ reporter activity is driven by mouse or human enhancer A are shown in the lower right panels. pa, pharyngeal arches; ra, la, right- left atrium; rv, lv, right- left ventricle; avc, atrioventricular canal; ivs interventricular septum.

3D reconstruction of key marker genes during normal development (E8.5-11.5).

Partners generated a series of 3D reconstructions in which the mRNA expression patterns of 13 genes (Tbx5, Pitx2c, Tbx2, Tbx3, Isl1, Tbx18, Hey2, Lbh, Nppa, Cx40, Mlc2a, Mlc2v and Bmp10) were visualized at embryonic day E9.5, E11.5 and E13.5. The expression patterns contained in the individual gene expression reconstructions were mapped using an elastic image registration algorithm age matched to reference models. Hierarchical clustering was used to identify regions that have similar expression profiles. The clustering procedure was applied to several combinations of genes and stages with increasing complexity. The regions found by the cluster analyses not only show classical anatomical compartments but also show regions which are not directly evident from anatomical landmarks. The great advantage of this genetic annotation approach is that it is fully independent of prior recognition of anatomic boundaries. The protein expression patterns of Nkx2.5 and Ki67 were also collected and will be provided with the clusters. The inclusion of the proliferation marker Ki67 allows the linkage between gene programs and the proliferative fraction.

3D reconstruction of gene expression patterns in mutant embryos.

The expression of *Tbx1*, *Tbx2* and *Tbx3* in *Tbx1* mutants and controls has been analyzed, and 3D pdf files of these patterns have been generated. Expression patterns in the pharyngeal ectoderm, foregut endoderm, pharyngeal mesoderm including the second heart field, outflow tract/whole heart, and neural crest were separately defined. This work has shown that Tbx1 is part of a T-box gene regulatory network including Tbx2 and Tbx3 required for pharyngeal morphogenesis and heart tube elongation (Mesbah, Rana et al. 2012). The results have shown that the dorsal mesenchymal protrusion (DMP), a second heart derivative that plays a critical role in formation of the primary atrial septum, is abnormal in $Tbx1^{-/-}$ embryos. An early defect in dorsal mesocardial development and DMP morphogenesis is associated with a failure of segregation of second heart field progenitor cells to the arterial and venous poles of the heart and misrouting of Fgf10 enhancer trap transgene positive cells which normally give rise to subpulmonary myocardium, to the venous pole. These conclusions are supported by detailed 3D reconstructions of gene expression and proliferation patterns, genetic tracing experiments and DiI labeling in cultured wildtype and $Tbx1^{-/-}$ mouse embryos, revealing a temporal requirement for Tbx1 from the 7 somite stage. Of particular interest is the observation that abnormal early development of the DMP in $Tbx1^{-/-}$ embryos leads to atrioventricular septal defects in a significant fraction of mutant embryos at fetal stages, a previously unnoted aspect of the Tbx1 null phenotype that reveals the importance of Tbx1 regulated progenitor cell segregation to the poles of the elongating heart tube.

Assessment of PAA and OFT development during human embryonic development

Congenital aortic arch malformations can cause serious cardiovascular and respiratory problems. 22q11.2 deletion syndrome (22q11.2DS) is the most common human microdeletion disorder and increases the risk for aortic arch anomalies. In order to test whether current insights from animal studies also are applicable to the human situation, Partners studied patterns of gene expression of 22q11.2DS candidate gene *TBX1* and of other transcription factors and developmental regulators relevant during PAA development and remodeling in human embryos. Based on the (interactive) 3D reconstructions that were generated and on reported congenital anomalies of the pulmonary trunk and tributaries, we postulate that the pulmonary arteries originate from the aortic sac, rather than from the sixth pharyngeal arch arteries. Similar to mouse, *TBX1* gene is expressed in pharyngeal mesenchyme and epithelia. The endothelium of the pharyngeal arch arteries is largely negative for *TBX1* and family member *TBX2* but expresses neural crest marker AP2a, which gradually decreases with ongoing development of vascular smooth muscle. At early stages, the pharyngeal arch arteries, aortic sac, and the dorsal aortae in particular were largely negative for proliferation marker Ki67, potentially an important parameter during aortic arch system remodeling. Together, the data obtained support current animal-to-human

extrapolations and future genetic and molecular analyses using animal models of congenital heart disease (M. Sameer Rana et al., 2013, in press.).

A novel *Tbx1* mutant mouse line.

Using ENU mutagenesis, a novel mutation which models DiGeorge syndrome has been identified. Using MRI, features of cleft palate, absent cochlea, absent thymus and cardiac malformations, including interrupted aortic arch, vascular ring, right sided aortic arch, common arterial trunk and ventricular septal defects have been identified. The mutation was genetically mapped to *Tbx1* gene and characterized as a G/A transition at the end of Tbx1 exon 3, which introduces a R160Q and alters mRNA splicing. Partners showed that the mutation (called George):

a) is a strong hypomorphic allele of *Tbx1* as it fails to complement a null allele and the mutation results in markedly reduced levels of TBX1 protein.
b) the 34 amino acid deletion does not affect the transactivation capacity of Tbx1 in reporter gene assays.
c) Tbx1 interacts with transcription factors RBBP5, GATA4, JMJD6 and SMARCAD1. Of particular interest, GATA4 and JMJD6 are known to be involved in heart development.
d) exon 3 deletion results in impaired Tbx1 homodimerisation.

Df1 mutant embryos interaction with high-fat diet

Partners have shown that high-fat maternal diet affects the phenotype of *Cited2* mutant embryos by increasing the penetrance of left-right patterning defects. Partner's aim is to determine if high-fat diet would affect the penetrance of cardiac and outflow tract abnormalities in a mouse model of DiGeorge Syndrome (Df1), where the cardiac defects are related to abnormal neural crest cell development.

Partners imaged and analysed 300 embryos using MRI in an experiment where wild-type females were put on high-fat or control diet for 8 weeks before being mated with DF1/+ males. The results showed that the penetrance of cardiac defects in Df1/+ embryos is not significantly different between the two diet groups. Moreover, no difference in the severity of cardiac defects was observed between the two diet groups, suggesting that the left-right patterning network is more sensitive to maternal high-fat diet than neural crest cell development pathways.

***Hes1* mutant embryos**

Hes1 is a target of Tbx1. Partners imaged and analyzed 135 embryos that showed partially penetrant range of 22q11DS-like defects including pharyngeal arch artery (PAA), outflow tract, craniofacial and thymic abnormalities (van Bueren et al., 2010)

***Tbx1-Sprouty* genetic interactions**

Partners imaged 110 embryos and showed that:

a) *Sprouty* genes, which encode feedback antagonists of receptor tyrosine kinase (RTK) signaling, phenocopy many defects associated with DiGeorge syndrome;
b) *Tbx1* heterozygosity significantly exacerbated the severity of all these defects.

In conclusion, Tbx1 functions as an essential component of a buffering mechanism which protects the embryo against perturbations in RTK signaling that may lead to developmental defects characteristic of DGS/VCFS. (Simrick et al., 2012).

Phenotypic Characterization and setup of in vitro assays to study the function of CHD genes in endothelial cells

Using various cell based assays, including endothelial tube formation assay, Partners are investigating the function of congenital heart disease (CHD) genes in endothelial cells, in particular their ability to form angiogenic tubulular networks. Partners have characterized the cellular phenotypes resulting from siRNA mediated CHD gene knock-down and inhibition using chemical compounds. This will enable to screen for genetic interactors in cell-based assays using siRNA or chemichal libraries and in the further development of endothelial cell-based models and high-throughput screening systems which could potentially be used as a surrogate for animal models of CHD.

a) Using the endothelial tube formation assay to investigate the function of congenital heart disease (CHD) genes in endothelial cells and a panel of genes that are known to affect cardiovascular development when conditionally deleted in the endothelium, which Partners are using as positive controls, they have established and characterized a range of possible phenotypes which will serve as baseline for later experiments and screens.
b) Knockdown of the several of our positive control genes severely impairs tubulogenesis on the matrigel assay, including CXCR7, EFNB2, FOXP1, KLF2, MAPK7, PTPN11, TMEM100, and ZPFM1. Milder effects were seen in other genes such as CITED2 and INPP5a.
c) Partners have explored the phenotypes caused by inhibition of the BMP, Tgf-b, Hh, Notch, retinoic and Wnt pathways using chemical inhibitors, as these pathways are necessary for normal endothelial tube formation. Using chemical inhibitors, Partners found that inhibition of TGF-b, BMP and Notch signalling pathways affected tube formation, with inhibition of the BMP and Notch pathways inhibiting tube formation and inhibition of the TGF-b pathway increasing the overall tube thickness. Inhibition of the WNT and Retinoic Acid signalling pathways did not have an effect on this assay.
d) Using the cell migration assay, Partners found that RNAi mediated knockdown in endothelial cells of various genes, including TBX1, reduces cell migration.

Tbx1 is required in endothelial cells for lymphatic development and regulates Vegfr3

Partners have found that *Tbx1*-/- embryos have a profoundly defective lymphatic system. Using endothelial-specific deletion of Tbx1, it was determined that it is required in these cells for lymphatic development. In particular, Vegfr3, essential for lymphatic vessel development and maintenance, is regulated by Tbx1. It was also found an enhancer that responds to Tbx1 *in vitro* and *in vivo*. ChIP analyses determined that Tbx1 occupies this enhancer in endothelial cells. Thus it was concluded that *Vegfr3* is a direct target of Tbx1 in lymphatic vessels.

Investigation of genetic interactions between *Tbx1*, *Sema3c* and *Hes1*

Sema3c and *Hes1* were identified as TBX1 target genes and as endogenous genes adjacent to two transgenes expressed in subdomains of the outflow tract through integration site position effects. *Sema3c*, encoding the Semaphorin3c ligand, is expressed in subpulmonary myocardium, a region of the heart severely reduced in $Tbx1^{-/-}$ embryos. *Sema3c* is known to regulate cardiac neural crest cell entry into the outflow tract. Partners crossed *Tbx1* and *Sema3c* heterozygous mutant animals in order to score the incidence of cardiovascular anomalies in compound mutant embryos. Double heterozygous mice were obtained at the expected frequency and no alteration in the incidence or severity of arch artery defects was observed at E10.5 or E17.5. $Tbx1^{+/-}$;$Sema3c^{-/-}$ embryos resulting from interbreeding

Tbx1$^{+/-}$;*Sema3c*$^{+/-}$ mice were also scored for arch artery anomalies at E10.5. A significantly elevated incidence of the most severe class of defects (bilateral non-patency of the 4th aortic arch) was observed in *Tbx1*$^{+/-}$ embryos in the absence of both *Sema3c* alleles. Loss of *Sema3c* alone does not result in defects in arch artery formation, although later remodeling of aortic arch arteries is *Sema3c*-dependent. The results suggest that haploinsufficiency for *Tbx1* reveals a cryptic role for Sema3c in arch artery development, suggesting that suggest that Sema3c may function in arch artery development together with a TBX1 target gene. Comparison of *Sema3c* and *Tbx1* expression reveals overlapping sites in pharyngeal ectoderm and mesoderm.

The transcription factor and progenitor cell marker *Hes1* is expressed in pharyngeal mesoderm at the time of heart tube elongation, including both cells of the second heart field and adjacent pharyngeal endoderm. Outflow tract defects are observed in 30% of *Hes1*$^{-/-}$ embryos in a CD1 outbred background, showing a rightward displaced ascending aorta overriding a ventricular septal defect. These defects are associated with a shorter, straighter outflow tract at E10.5 and decreased proliferation in the dorsal pericardial wall of *Hes1*$^{-/-}$ embryos at E8.5 together with defects in pharyngeal arch artery development. While founding no evidence for genetic interaction between *Tbx1* and *Hes1,* Partners used conditional mutagenesis to determine the critical cell type in which Hes1 function is required for heart tube elongation. Inactivation of a conditional *Hes1* allele using a *Mesp1 Cre* driver expressed in anterior mesoderm, including second heart field cardiac progenitor cells, resulted in a low frequency of conotruncal defects (overriding aorta, double outlet right ventricle and ventricular septal defects) in 2/21 embryos. This incidence of 10% is comparable to the penetrance of outflow tract defects (2/23) observed in *Hes1*$^{-/-}$ embryos on the MF1/C57Bl/6 background but less that that observed on the CD1 background. These embryos also display, on average, a significantly shorter outflow tract with a larger angle between the proximal and distal regions compared to wildtype embryos. *Hes1* is thus directly required in mesoderm for optimal heart tube elongation.

Genetic interaction between Wnt5a and Tbx1

Wnt5a encodes a ligand of the non canonical Wnt pathway. Wnt5a is expressed in the SHF, and is required for cardiac outflow tract development. Using genetic crosses, Partners demonstrated that *Wnt5a* and *Tbx1* interact genetically. Indeed, *Tbx1* mutation enhances significantly the phenotype of *Wnt5a* mutants and viceversa. *Wnt5a* harbors two *Tbx1*-responding enhancers that are occupied by the Tbx1 protein.

Establishment of a system for dynamic imaging of living cells in the second heart field and identification of basal filopodia-like structures

Partners have investigated the dynamic nature of second heart field development in wildtype and *Tbx1*$^{-/-}$ embryos. Using a slice culture technique coupled with fluorescent reporter genes and time-lapse confocal microscopy, Partners set up a technique to study living cells in the second heart field, allowing to make conclusions about cell movement in the dorsal pericardial wall compared to cell movement in neural crest populations and to identify highly motile filopodia-like structures on the basal side of cells in the dorsal pericardial wall that extend towards the overlying ventral endoderm. These basal protrusions are enriched in filamentous actin and can also be visualized after electroporation of GFP encoding reporter genes into the dorsal pericardial wall prior to slice culture. Analysis of *Tbx1*$^{-/-}$ embryos after electroporation reveals that cells in the dorsal pericardial wall maintain their apical/basal polarity but are more rounded with less and shorter filopodial-like extensions. This *ex vivo* system provides a means to further investigate the role of *Tbx1* in the second heart field and to test the effects of inhibitors and agonists of different signaling pathways on cell micro-behaviour during heart tube elongation.

Analysis of T-box transcription networks in congenital heart disease

A large number of known cardiac transcription factors are still expressed in adult myocardium and moreover are reactivated through so far unclear embryological reprogramming of disease myocardium. This provides the opportunity to study the regulatory relationships of transcription factors and target genes based on gene expression profile disturbances in cardiac tissue from malformed and diseased hearts. However, the relevance of identified relationships for cardiogenesis need to be proven in model systems.

Partners focused on two main tasks; (A) Analysing in the mouse model transcription networks driven by the interplay of DNA-binding transcription factors, histone modifications and microRNAs, and (B) Studying gene expression in human malformed and normal hearts and correlating obtained data to knowledge gained in mouse models.

To join human and mouse network information, Partners generated advanced datasets for mouse transcription networks, focusing attention to the interaction between different molecular levels, involving epigenetic, transcriptional and post-transcriptional mechanisms. The majority of previous studies investigated each of these levels individually, and little was known about their interdependency. Partners identified Nk2.5, Mef2a, Gata4 and Srf as direct regulators of Tbx20 (Figure 4). Partners performed a systems biology study integrating mRNA profiles with DNA–binding events of these cardiac transcription factors, activating histone modifications (H3ac, H4ac, H3K4me2, and H3K4me3), and microRNA profiles obtained in wild-type and RNAi–mediated knockdown. Finally, Partners confirmed conclusions primarily obtained in cardiomyocyte cell culture in a time-course of cardiac maturation in mouse around birth. Partners provide insights into the combinatorial regulation by cardiac transcription factors and show that they can partially compensate each other's function. Genes regulated by multiple transcription factors are less likely differentially expressed in RNAi knockdown of one respective factor. In addition to the analysis of the individual transcription factors, Partners found that histone 3 acetylation correlates with Srf- and Gata4- dependent gene expression and is complementarily reduced in cardiac Srf knockdown (Figure 5). Further, Partners found that altered microRNA expression in Srf knockdown potentially explains up to 45% of indirect mRNA targets. Considering all three levels of regulation, Partners present an Srf-centered transcription network providing on a single-gene level insights into the regulatory circuits establishing respective mRNA profiles.

In summary, Partners show the combinatorial contribution of four DNA–binding transcription factors in regulating the cardiac transcriptome and provide evidence that histone modifications and microRNAs modulate their functional consequence. This opens a new perspective to understand heart development and the complexity of cardiovascular disorders and is incorporated in our further work to elucidate the genetic background of human cardiac diseases.

In addition, gene expression profiles of 30 human heart samples using next-generation sequencing technology (Illumina Genome Analyzer) have been analysed. The myocardial samples were obtained from patients with Tetralogy of Fallot, a frequent congenital malformation in human with known involvement of T-box factors. The obtained profiles underwent an advanced computational analysis including statistical assessment and pathway analysis. The data have been associated to gene expression information obtained in mouse embryonic mouse hearts. Moreover, expression profiles were studied in context to genetic variations found in respective individuals by targeted re-sequencing of ~1,000 genes. The project results are currently finalized in a related manuscript.

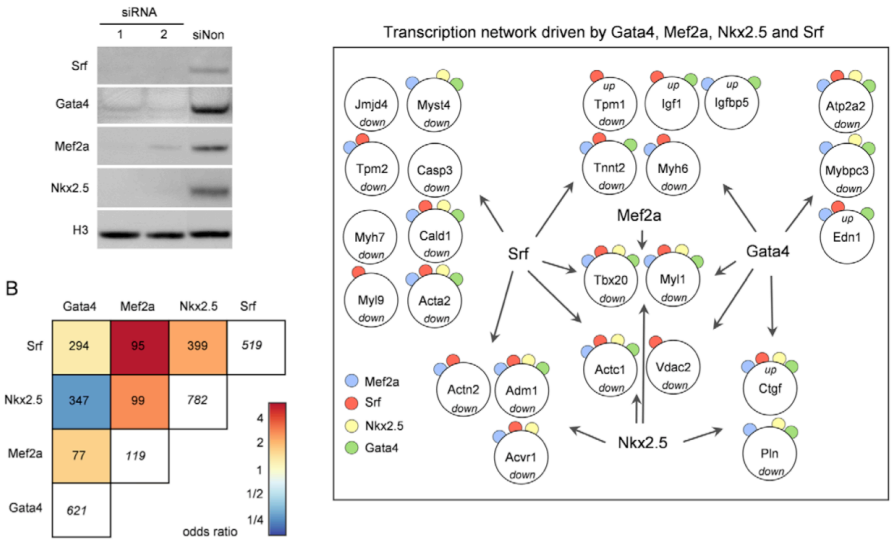

Figure 4. **RNAi–induced knockdown of Gata4, Mef2a, Nkx2.5, and Srf results in reduced expression of Tbx20 and other genes.** Transcription factor network showing a selection of cardiac relevant genes bound in ChIP-chip and/or ChIP-seq, and significantly differentially expressed in RNAi knockdown experiments of the respective factor. Up- and downregulation of genes is depicted and occurrence of ChIP binding marked by color-coded circles.

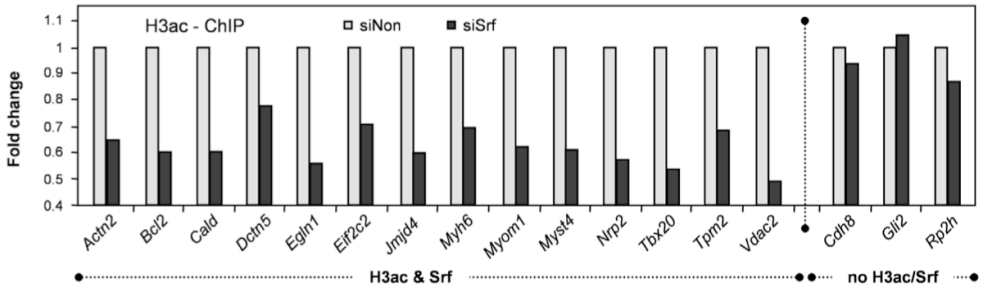

Figure 5. **Srf knockdown in HL-1 cells leads to complementary alterations in H3ac marks at Srf binding sites including Tbx20.** H3ac-ChIP enrichments after Srf knockdown (siSrf) compared to control siRNA (siNon) were measured with qPCR for two groups of promoter regions (H3ac & Srf binding and no H3ac/Srf). The H3ac enrichment was normalized to Input and IgG controls. Fold changes show significant decrease in H3ac enrichment after Srf knockdown.