**Foxf Genes Integrate Tbx5 and Hedgehog Pathways in the Second Heart Field for Cardiac Septation**

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**Abstract**

The Second Heart Field (SHF) has been implicated in several forms of congenital heart disease (CHD), including atrioventricular septal defects (AVSDs). Identifying the SHF gene regulatory networks required for atrioventricular septation is therefore an essential goal for understanding the molecular basis of AVSDs. We defined a SHF Hedgehog-dependent gene regulatory network using whole genome transcriptional profiling and GLI-chromatin interaction studies. The Forkhead box transcription factors Foxf1a and Foxf2 were identified as SHF Hedgehog targets. Compound haploinsufficiency for Foxf1a and Foxf2 caused atrioventricular septal defects, demonstrating the biological relevance of this regulatory network. We identified a Foxf1a cis-regulatory element that bound the Hedgehog transcriptional regulators GLI1 and GLI3 and the T-box transcription factor Tbx5 in vivo. GLI1 and Tbx5 synergistically activated transcription from this cis-regulatory element in vitro. This enhancer drove reproducible expression in vivo in the posterior SHF, the only region where GlI1 and Tbx5 expression overlaps. Our findings implicate Foxf genes in atrioventricular septation, describe the molecular underpinnings of the genetic interaction between Hedgehog expression and Tbx5, and establish a molecular model for the selection of the SHF gene regulatory network for cardiac septation.

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**Introduction**

Cardiac septation, the morphogenetic process that transitions the looped heart tube into the multi-chambered heart observed in mammals, is complex and often goes awry in Congenital Heart Disease (CHD). A novel paradigm for the developmental ontogeny of the atrioventricular septum has recently emerged [1–6]. This work describes atrioventricular septation as a process driven by molecular events in second heart field (SHF) cardiac progenitors rather than in the heart itself [1–6]. The identification of extracardiac lineages that generate the atrial and atrioventricular septum implies that the search for gene regulatory networks germane to cardiac septation should occur in SHF cardiac progenitors not in the heart itself.

Hedgehog signaling is an essential developmental pathway conserved from flies to man [7,8]. Mutations in key Hedgehog pathway genes, including ligands such as Sonic hedgehog (Shh; 20423) and downstream signaling cascade member Smo (319757) cause significant cardiac defects including complete atrioventricular septal defects [9,10]. Tissue specific knockout of Hedgehog signaling in the SHF recapitulates atrioventricular septal defects [4,5] and genetic inducible fate mapping showed that the atrial/atrioventricular septum is derived from Hedgehog-receiving SHF cardiac progenitors [5]. These observations laid the groundwork for identifying the Hedgehog-dependent SHF gene regulatory networks essential for atrial septation.

Cardiogenic transcription factor genes Tbx5 (21388), Nkx2.5 (18091) and GATA4 (14463) have been implicated in human atrial septation [11–14]. These transcription factors form a complex and can co-activate gene expression [12,15–17]. Tbx5 has been shown to be required in multiple contexts during cardiac development and adult function in mice. Tbx5 is required in the SHF for atrioventricular septation [6,15], in embryonic cardiomyocytes for proliferation [18], in adult myocardium for contractile function [19], and in the adult cardiac conduction system for cardiac rhythm control [20]. Tbx5 target genes differ significantly between these distinct cellular and temporal contexts [6,21]. Yet the Tbx5-responsive cis-regulatory elements specific to these cellular contexts and the molecular cues that establish context dependent selectivity remain unknown.

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**Author Summary**

Atrioventricular septal defects (AVSDs) are a common severe class of congenital heart defects. Recent work demonstrates that events in the second heart field (SHF) progenitors, rather than in the heart, drive atrioventricular (AV) septation. Our laboratory has shown that both Hedgehog signaling and the T-box transcription factor, Tbx5, are required in the SHF for AV septation. To understand the molecular underpinnings of the AV septation process we investigated SHF Hedgehog-dependent gene regulatory networks. Transcriptional profiling and chromatin interaction assays identified the Forkhead box transcription factors Foxf1a and Foxf2 as SHF Hedgehog targets. Compound haploinsufficiency for Foxf1a and Foxf2 caused AVSDs in mice, demonstrating the biological relevance of this pathway. We identified a cis-regulatory element at Foxf1a that bound Tbx5 and Hedgehog transcriptional regulators GLI1 and GLI3 in vivo. Furthermore, Tbx5 and GLI1 co-activate transcription of the identified cis-regulatory element in vitro. The enhancer is expressed primarily in the pSHF in vivo, where Tbx5 and GLI1 expression overlap. Our findings implicate Foxf1a and Foxf2 in AV septation and establish Tbx5 and Hedgehog signaling upstream of Foxf genes in a gene regulatory network for cardiac septation.

We previously described genetic interactions between Tbx5 and Hedgehog signaling in the SHF for atrioventricular septation in mice [6]. Mice haploinsufficient for both Tbx5 and the obligatory Hedgehog signaling receptor gene Smo express AVSDs more frequently than mice haploinsufficient for either gene alone [6]. Furthermore, constitutive Hedgehog signaling in Tbx5-mutant SHF progenitors can rescue atrioventricular septation [6]. These studies predict that Hedgehog-dependent and Tbx5-dependent gene regulatory networks share vital, yet undescribed overlap in the SHF that is necessary for atrioventricular septation.

In this study we attempted to define Hedgehog-dependent SHF gene regulatory networks and identify the molecular basis of the genetic interaction between Hedgehog signaling and Tbx5. We characterized the Hedgehog-dependent SHF gene regulatory networks by in vivo whole genome transcriptional profiling and GLI-chromatin interaction studies. We found that Foxf1a (15227) and Foxf2 (14238) are downstream of Hedgehog signaling in the SHF. Mice haploinsufficient for both Foxf1a and Foxf2 compound heterozygotes have atrial septal defects, demonstrating the biological relevance of these Hedgehog targets. GLI3T (14634) binding data identified a candidate cis-regulatory element upstream of Foxf1a that contained an adjacent Tbx5 binding site. This enhancer binds to GLI1 (14632), GLI3 and Tbx5 in the SHF in vivo. In vitro and in vivo analysis demonstrated that this cis-regulatory element integrates Hedgehog signaling with Tbx5 activity and provides strong specific activity in the posterior SHF. This work identifies a novel role for Foxf transcription factors at the intersection of Tbx5 and hedgehog signaling in atrioventricular septation and describes a SHF gene regulatory network for cardiac morphogenesis.

**Results**

Transcriptional profiling of the posterior SHF in Shh mutants

Progenitor cells for the atrial and atrioventricular septum require Shh signaling in the posterior SHF (pSHF) between embryonic day 8 and embryonic day 10 (E8–E10) to migrate into the heart to form the atrial septum between E9–E11 [4,5]. To identify the Hedgehog-dependent gene regulatory networks required for this process, we compared transcriptional profiling of the posterior SHF from wild-type and Shh (MGI: 1932461) null embryos at E9.5 to identify differentially expressed transcripts. We isolated the pSHF by microdissection including the dorsal mesenchymal protrusion and closely associated surrounding ventral lateral plate mesenchyme. Our dissection included the attached foregut, but excluded the heart, dorsal lateral plate mesenchyme and neural tube (Figure 1A). RNA was isolated and known Hedgehog-dependent transcripts were evaluated by RT-PCR to verify genotyping prior to whole genome transcriptional profiling. Shh, Ptc1 (192906) and Gli1 all demonstrated significantly reduced expression (p>0.05) in the Shh null samples compared to wild-type micro-dissected samples (Figure 1B). Specifically, Shh was reduced more than 90%, while Ptc1 and Gli1 were each reduced approximately 50%, consistent with significantly reduced Hedgehog signaling in the mutant samples and confirming the genotypic fidelity of the isolated samples.

Transcriptional profiling of pSHF samples was performed on Agilent Mouse Whole Genome Arrays. Using a significance threshold with a multi-test adjusted p-value (Q-value) <0.005 and absolute fold change larger than 2, comparing Shh−/− mutant mouse embryos (n = 4) with wild-type embryos (n = 3) identified a differentially expressed 560-gene signature (Table S1). Gene Ontology (GO) enrichment analysis of differentially expressed genes captured known processes disrupted in Hedgehog pathway mutants, such as pattern specification and organ morphogenesis (Figure 1C) [22]. To further identify the best candidates for an experimental validation, 65 genes were computationally evaluated according to more stringent criteria by three statistical tests (non-parameter Wilcoxon-tested theoretical p<0.15, empirical t-tested FDR<0.1, and absolute fold change>3, Figure S1) on the same data sets. From the Shh down-regulated candidates, we chose 21 targets and validated significant misexpression of 13 by qPCR (p<2e-16, Fisher’s Exact test, FET) (Figure 1D). Eight others did not meet criteria for statistically significant misexpression primarily due to large expression variation, possibly related to the presence of non-SHF tissue isolated by our dissection process.

**Identification of Hedgehog signaling direct targets in the SHF**

To define loci directly downstream of Hedgehog signaling, we analyzed genome-wide chromosomal binding locations of the Hedgehog transcriptional regulator Gli3 in the embryonic SHF by chromatin immunoprecipitation with deep sequencing (ChIP-seq). We performed ChIP using a Cre-inducible flag-tagged Gli3T expression line (RosaGli3TFlag/Cre MGE: 3828280) [23] combined with the SHF Cre driver Mef2c-AHF-Cre [24] (MGE: 3639735). The SHF tissue from 50 Mef2c-AHF-Cre/+; RosaGli3TFlag/+ embryos was micro-dissected and immunoprecipitated using an anti-FlagM2 antibody (Sigma). To verify enrichment of Gli3T bound sequences by immunoprecipitation prior to sequencing, we tested a previously identified Gli3T peak upstream of Ptc1 (Chromosome 13, nucleotides 63577408–63579384, mm9), a known Gli3T-bound cis-regulatory element in the limb [23]. This sequence was 13.7-fold enriched in the SHF IP fraction by ChIP-PCR. We proceeded to sequence the IP library and apply Model-based Analysis for ChiP-seq (MACS) [25]. We identified 1316 Gli3T-bound peaks by comparing 68 million sequence tags in IP to 21 million sequence tags in input (tag size = 36 bps, effective genome size = 2e+9, band width = 200, 2<model fold<200, and p-value cutoff = 1e-05) [25]. From these peaks, we analyzed the
indicates p

Shh

heart (IV). Microdissected tissue was kept as anterior SHF (Va), posterior tube was removed (III). SHF tissue was bisected and separated from the isolated (I). Thoracic tissues including the heart were removed from the transcriptional profile analysis of SHF tissue from wild-type and profiling (C) Gene Ontology biological processes (GOBPs) enriched in using RT-qPCR (relative quantitation, RQ). * indicates p

identified in the transcriptional profile were verified as Shh-dependent mutant embryos identifies developmental terms. (D) 13 genes

Figure 1. Transcriptional profiling of SHF from shh−/− embryos. (A) Microdissection for isolation of SHF tissues. E9.5 embryos were isolated (I). Thoracic tissues including the heart were removed from head and tail, kept for genotyping or non-cardiac controls (II). Neural tube was removed (III). SHF tissue was bisected and separated from the heart (IV). Microdissected tissue was kept as anterior SHF (Va), posterior SHF (Vb) or heart (Vc). (B) RT-PCR demonstrates decreased expression of Shh, Gli1 and Ptc1 in shh mutant SHF tissues isolated for transcriptional profiling (C) Gene Ontology biological processes (GOBPs) enriched in the transcriptional profile analysis of SHF tissue from wild-type and Shh mutant embryos identifies developmental terms. (D) 13 genes identified in the transcriptional profile were verified as Shh-dependent using RT-qPCR (relative quantitation, RQ). * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. doi:10.1371/journal.pgen.1004604.g001
distribution of the signal around the peak center and identified a typical distribution, confirming successful sequencing (Figure 2A). The predominant GLI3T peak location from the binding data was typical distribution, confirming successful sequencing (Figure 2A). The predominant GLI3T peak location from the binding data was
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derived from GLI3T-bound and SHh-dependent genes was significant among approximately 22,000 mouse genes (FET p<0.01, Figure 2B).

To define the direct Hedgehog-dependent SHF gene regulatory networks, we intersected the SHF Shh-dependent transcriptional profile signature with the SHF GLI3T chromatin contact results to define candidate Hedgehog-dependent Gli-target genes. This dataset intersection comprised 119 peaks annotated to 112 genes (Figure 2B, Table S3). The enrichment between GLI3T-bound and Shh-dependent genes was significant among ~22k mouse genes (FET p = 0.003, odds ration = 1.4, Figure 2B). The 119 Shh-dependent GLI3T-bound sites contained significant enrichment of the de novo and known Gli3-binding motif, as derived by ChiP-Chip (CGTGGGTGGTCC) [23] and by computational implication (TRANSFAC database; Figure 2C, bottom panel) [30,31] at a high degree of significance (p≤1e-10; Figures 2C, top panel).

A significant enrichment of transcription factors was observed in SHF Hedgehog target genes. Transcription factor activity and DNA binding were the two most significant gene-sets over-represented among the 112 Shh-dependent Gli3-bound genes. We directly analyzed our gene set for overrepresentation of transcription factors by searching TRANSFAC version 2013.1 [31] and identified 26 TFs among the 112 unique genes with significant GLI3T-bound peaks (Figure 2D, Table S4), representing a significant enrichment (p = 0.0001, odds ratio = 2.7, Fishers exact test). Specifically, Shh transcriptional profiling and GLI3T chromatin interaction data both identified an enrichment of FOX gene family members, encoding Forkhead transcription factors, identifying FOX genes as potential SHF Hedgehog targets (Figure S2). The set of 112 Shh-dependent Gli3T-bound genes included four Fox transcription factors, Fox31 (64290), Fox1 (17300), Fox1 (15229) and Fox1, representing a significant enrichment (Figure 2E, p = 0.0001, odds ratio = 18.4).

Fox1a and Fox2 are downstream of Shh in the SHF

We investigated the hypothesis that Fox1a and Fox2 expression was downstream of Hedgehog signaling in cardiac development. Shh-dependent expression of both genes in the SHF was confirmed by qPCR: Fox1a expression was reduced by 50% (p = 0.05) and Fox2 was reduced by 80% in the SHF of Shh−/− versus wild-type controls (p = 0.01) (Figure 1D). In-situ hybridization to evaluate the patterning of expression showed that Fox1a and Fox2 were both expressed in the posterior SHF, but not in the heart, in wild-type embryos at E9.5, with Fox1a expression extending more ventrally than Fox2 to include the DMP (Figures 3A, A, E, E’). Mesenchymal expression of both Fox1a and Fox2 demonstrated a severe decrement in shh−/− mutant embryos (Figures 3B, B’, F, F’).

In a search for common targets between Tbx5 and Hedgehog signaling in the SHF, we tested whether Fox1a and/or Fox2 SHF expression was Tbx5-dependent. We performed in situ hybridization for Fox1a and Fox2 in Tbx5+/− heterozygous mutant embryos (MGI: 2387580), which demonstrate 40% penetrance of AVSDs [13]. Fox1a but not Fox2 expression demonstrated significant reduction in Tbx5 heterozygotes at E9.5. In Tbx5+/− embryos, Fox1a expression was specifically decreased in the posterior SHF (Figure 3C, C’, D, D’, arrow) in the area of under control of GLI-mediated cis-regulatory elements, given that enhancers often reside thousands of base pairs away from their target of regulation and act independently of their orientation [26,27]. We therefore annotated GLI3T-bound regions to all transcription start sites within 100 kbp and to the nearest TSS if it resided outside the 100 kbp window [28,29]. This consideration resulted in mapping the 1316 peaks to 3296 neighbor genes (Table S2). The enrichment between GLI3T-bound and Shh-dependent genes was significant among approximately 22,000 mouse genes (FET p<0.01, Figure 2B).
expression overlap with Tbx5 expression [6]. In regions where Foxf1a expression does not overlap with Tbx5 expression, such as the anterior SHF, Foxf1a expression appeared normal (Figure 3D, D9). Foxf2 expression in Tbx5+/2 embryos appeared unaltered compared to wild-type embryos (Figure 3G, G9, H9, H9). Taken together, this analysis demonstrates that posterior SHF Foxf1a expression was Shh- and Tbx5-dependent whereas Foxf2 expression was Shh-dependent alone.

Foxf1a and Foxf2 are required for atrioventricular septation

We hypothesized that Foxf1a and Foxf2 were required in a dosage sensitive manner for atrioventricular septation. We analyzed the cardiac anatomy of embryos from an intercross between Foxf1a+/2 and Foxf2+/2 at E14.5, when cardiac septation is normally complete. Foxf1a+/2; Foxf2+/2 double-heterozygote embryos all exhibited atrioventricular septal defects (Figure 4D, D9 arrow; p = 0.03). Primum-type atrial septal defects, characterized by absence of the dorsal mesenchymal protrusion, were observed in each case (Figure 4D, D9). Additionally, Foxf1a+/2; Foxf2+/2 double-heterozygotes displayed larger than normal mesenchymal caps covering the primary atrial septum (Figure 4D, D9 arrow), an observation in keeping with the known redundant requirement for Foxf1a and Foxf2 in limiting mesenchymal growth in other contexts [32]. Atrial septal defects were never observed in Foxf1a+/2 or Foxf2+/2 single-heterozygotes or wildtype control littermate embryos (Figure 4B, B9). We concluded that Foxf1a and Foxf2 are redundantly required for atrioventricular septation.

A cis-regulatory element at Foxf1a binds TBX5, GLI1, and GLI3 in vivo

We hypothesized that Foxf1a may represent a direct downstream target of Hedgehog signaling and/or Tbx5 in the SHF. We identified Foxf1a as a candidate direct target based on unbiased interrogation of GLI3T and Tbx5 transcription factor chromatin interaction and transcriptional profiling data sets. We intersected our SHF GLI3T ChIP data set (Figure 2B) with a published
TBX5 ChIP-seq data set generated from HL-1 cardiomyocytes [33] to define regions with potential co-occupancy of both transcription factors. The intersection of the ChIP-seq datasets identified a single overlapping interaction peak for Gli3T (in the SHF (Figure 2B)) and TBX5 (in HL-1 cardiomyocytes) [33] located approximately 90 kb upstream of the Foxf1a transcription start site (Figure 5A and Figure S3). The Foxf1a transcriptional start site is the closest protein-coding gene to the described peak. The transcriptional start site for a non-coding RNA is located approximately 1.3 kbp upstream of Foxf1a, oriented in the opposite direction [34]. Closer interrogation of the sequence underlying the interaction domains revealed a conserved canonical T-box binding site (AGGTGTGG; chr 8, nucleotides 123,517,714–721, NCBI137/mm9 assembly) and a conserved canonical Gli binding site (GGACCACCCAGC; chr 8, nucleotides 123,517,754–762, NCBI137/mm9 assembly) within 30 base pairs of one another (Figure 5A). We evaluated the sequence information content for these sites from our SHF Gli3 ChIP-seq experiment and found close agreement with published binding sites for Gli3 [23,33]. This chromatin interaction data in combination with the Tbx5 and Hedgehog signaling-dependent Foxf1a SHF expression (Figure 3) identified this conserved region (mouse chromosome 8, nucleotides 123,517,714–762) as a candidate Foxf1a cis-regulatory element.

We evaluated the binding of TBX5 and the Hedgehog transcriptional regulators GLI1 and GLI3 to the candidate cis-regulatory element at Foxf1a in vivo in the SHF. We evaluated TBX5 binding in vivo by performing ChIP using an anti-TBX5 antibody on the micro-dissected wildtype SHF at E10.5 and observed 35-fold enrichment of the cis-regulatory element in the TBX5-immunoprecipitation fraction compared to the input fraction by qPCR (Figure 5B). We evaluated GLI1 and GLI3T binding in vivo by performing ChIP on the micro-dissected SHF of mice carrying either a Cre-activated flag-tagged Gli3 (Rosa-Gli3TFlag c/c) or Gli1 (RosaGli1Flag c/c MGI: 4460761) allele (RosaGli1Flag c/c MGI: 4460761) in concert with the Nkx2.5-Cre (MGI 2654594), broadly expressed cardiac tissues and progenitors. We performed ChIP using an anti-flag antibody on the SHF from R26R-Gli3-flag Nkx2.5-Cre/+ or R26R-Gli1-flag Nkx2.5-Cre/+ embryos at E10.5 and observed, respectively, 6.8-fold and 7.1-fold enrichment of the Foxf1a cis-regulatory element in the GLI1- and GLI3T-overexpressing embryos over the input control by qPCR (Figure 5B). We also evaluated two genomic loci between our identified binding site and the Foxf1a transcription start site to determine whether nonspe-
A cis-regulatory element at *Foxf1a* integrates Tbx5 and Hedgehog activity

We hypothesized that the conserved, adjacent, and functional *in vivo* Gli and Tbx5 binding sites may integrate Tbx5 and Hedgehog activity as a component of a downstream gene regulatory network. We evaluated the activity of Tbx5 and GLI1 on the candidate *Foxf1a* enhancer *in vitro*. The conserved element was cloned into a pGL4.23 vector containing a minimal promoter driving luciferase as a transcriptional readout and was transfected into HEK293T cells along with expression vectors for Gli1 and/or Tbx5. Co-transfection with the expression vector for Gli1, a Hedgehog-responsive transcriptional activator, provided a 91.9-fold induction of luciferase activity (p = 0.0017). Co-transfection with the expression vector for Tbx5 alone provided a 3.9-fold increase of luciferase activity (p = 0.039). Co-transfection with both Gli1 and Tbx5 expression constructs provided a 171.6-fold induction of luciferase activity (p = 0.0017). Interestingly, transcriptional activation by Tbx5-mutant construct profoundly diminished transcriptional activation by GLI1 alone (p = 0.001) (Figure 5C). Interestingly, transcriptional activation by Tbx5 and GLI1 on the GLI-mutant enhancer construct was only modestly abrogated luciferase compared to the activity of GLI1 and Tbx5 on the wild-type enhancer (p = 0.003).

**SHF-specific in vivo expression of the cis-regulatory element at *Foxf1a***

We hypothesized that the cis-regulatory element at *Foxf1a* may integrate Hedgehog signaling and Tbx5 activity as a SHF-specific enhancer *in vivo*. We cloned the Foxf1a genomic region into an *Hsp68-LacZ* expression construct, whose minimal promoter affords no intrinsic *in vivo* expression activity [35]. We evaluated the enhancer activity of the *Foxf1a* genomic fragment by evaluating *LacZ* expression in transient transgenic mouse embryos at E9.5. The posterior SHF demonstrated strong *lacZ* expression and was the only anatomic region demonstrating consistent and robust expression in the 8 transgenic embryos genetically positive for *Foxf1a* genomic activity [6], including the early dorsal mesenchymal protrusion and surrounding mesenchyme of the posterior SHF (less frequent and intense expression was also observed in other anatomic locations that receive Hedgehog signaling outside of the Tbx5 expression domain, including the anterior SHF (5/8), anterior lateral plate mesoderm (5/8) and somites (2/8) (Figure 5D). These observations, in concert with the *in vitro* analysis suggested that Hedgehog and Tbx5 act synergistically to provide strong reproducible transcriptional activation of this enhancer in the posterior SHF.

**Discussion**

Identification of the gene regulatory networks required for atrioventricular septation will be the basis for a mechanistic
understanding of AVSDs, a common severe form of CHD. We investigated Hedgehog-dependent networks and implicated Foxf genes for the first time in vertebrate heart development. We examined the overlap between Hedgehog pathways and Tbx5, both known to be integral to the pSHF for atrioventricular septation. Using transcription factor-chromatin interaction data, we identified a cis-regulatory element at Foxf1a that bound both Tbx5 and the Hedgehog pathway transcriptional activator GLI1. In vitro analysis of Tbx5 and GLI activity on the cis-regulatory element at Foxf1a proved predictive of in vivo biology: this enhancer exhibited strong transcriptional activation only in the pSHF region where Tbx5 expression and Hedgehog signaling intersect. This region is the location of atrial septum progenitors [5], where both Hedgehog signaling and Tbx5 are required for atrioventricular septation (Figure 6) [4–6]. These observations provide molecular detail for the genetic interaction between Tbx5 and Hedgehog signaling in atrioventricular septation.
Foxf transcription factors are required in the SHF for atrial septation

Our observations identified a requirement for the Forkhead-box transcription factors Foxf1a and Foxf2 in heart development. Compound haploinsufficiency for both Foxf1a and Foxf2 caused an atrial septal defect of the primum type, an atrioventricular septal defect characterized by absence of the septum primum. Foxf1a and Foxf2 were expressed selectively in the SHF, not in the heart (Figure 3). The requirement for Foxf genes in atrioventricular septation (Figure 4) provided further support for a model of atrioventricular septation driven by molecular events in SHF cardiac progenitors as opposed to in the heart itself. We found that Foxf1a and Foxf2 are required downstream of Hedgehog signaling in atrioventricular septation, adding cardiac development to the previously described Hedgehog-dependent role for Foxf genes in murine gut development [32,36–37]. Atrioventricular septal defects are also observed in Shh-null mutant embryos [10]. Because Foxf1a and Foxf2 expression were each decreased in the SHF by more than 50% in shh−/− null embryos (Figure 1D), Foxf1a−/+; Foxf2−/+ double heterozygote embryos provided a reasonable developmental facsimile of their diminished expression levels in shh−/− embryos. The observation that Foxf1a and Foxf2 compound haploinsufficiency resulted in AVSDs is therefore consistent with the supposition that Foxf1a and Foxf2 are essential components of the Hedgehog-dependent SHF gene regulatory network.

Foxf genes have also been previously implicated in cardiac specification in the ascidian Ciona intestinalis [38,39]. In ascidians, the single Foxf orthologue lies at the center of a pathway regulating numerous migration-related cellular processes, such as polarity, migration and membrane protrusion in trunk ventral cardiac progenitor cells [38,39]. Ciona trunk ventral cells with disrupted Foxf activity fail to migrate properly, but still differentiate into cardiac tissue at an improper location. Interestingly, removing Hedgehog signaling from the mouse SHF causes a migration failure of SHF progenitors [4,5]. Like the Ciona trunk cells without Foxf, SHF cells without Hedgehog responsiveness differentiate into cardiomyocytes, but their altered migration causes AVSDs [5]. Future efforts will determine whether the requirement for Foxf genes in cardiac progenitor migration is a conserved feature of mammalian cardiac development.

A SHF cis-regulatory element integrates Tbx5 and Hedgehog pathways: Building a gene regulatory network for cardiac septation

Genetic interaction and rescue experiments investigating the requirement for Hedgehog signaling and Tbx5 in atrioventricular septation were consistent with Tbx5 acting either in parallel or upstream of Hedgehog signaling in atrioventricular septation [6]. Our interrogation of these pathways on a cis-regulatory element at Foxf1a provides molecular detail for their interaction. We observed that Tbx5 and GLI1, the Hedgehog-dependent transcriptional activator, synergistically activated the cis-regulatory element in vitro (Figure 5C) predicting strong activation of expression in areas of overlap between Tbx5 expression and Hedgehog signaling. This prediction held in vivo, where transcriptional activity of the enhancer was strong and reproducible only in the posterior SHF region, where Tbx5 expression and Hedgehog signaling overlap (Figures 5D and 6). This work is consistent and a model describing a SHF-specific gene regulatory network driven by GLI1 and Tbx5 and essential for atrioventricular septation (Figure 6). This model provides specific predictions for the logic underlying enhancer choice in the SHF with ramifications for understanding the molecular and biochemical basis of atrioventricular septation and clinical AVSDs.

Materials and Methods

Ethics statement

Mouse experiments were completed according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the University of Chicago, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Mouse lines and handling

The Shh−/− line was obtained from the Jackson laboratory. The Tbx5+/− mice have been previously reported [15]. Foxf1a+/− and Foxf2+/− mice were generated in the Kalinichenko lab (Cincinnati Children's Hospital Medical Center) by breeding Foxf1-xlfl/fl and Foxf2-xlfl/fl mice with EIIA-Cre transgenic mice (Jackson Lab). Mef2c-AHF-Cre [24], ROSA26-Gli1 [40] and ROSA26-Gli3T [23] were reported previously.

Dissection techniques

For ChIP, transcriptional profiles and in-situ hybridizations, embryos were dissected in nuclease-free PBS on ice. For SHF microdissection procedures, head tissues anterior to the heart were removed, as were tail tissues posterior to the heart. Portions of these tissues were retained for genotyping if necessary. Neural tube tissues were also removed. The SHF mesenchyme was bisected into anterior and posterior portions when necessary, and then removed from the cardiac tissue (Figure 1A).
Transcriptional profiling

Shh<sup>−/+</sup> and Shh<sup>−/−</sup> embryos were dissected as described above at E9.5. SHF tissues from these embryos were pooled to isolate sufficient amount of RNA for synthesis of labeled cRNA. Transcriptional profiles were performed using Agilent Mouse Whole Genome Arrays mgug4122a.

ChIP

Microdissected SHF tissues were grouped into pools of approximately 50. Tissues were briefly fixed in 1.8% formaldehyde, then washed and homogenized. Sonication was performed with a Misonix 4000 sonicator until the sheared chromatin was approximately 100–300 bp in length. Input control samples were reserved prior to overnight immunoprecipitation with the appropriate antibody bound to magnetic Dynabeads (Invitrogen). Beads were precipitated and washed, the chromatin was eluted, de-crosslinked and purified using a PCR cleanup kit (Qiagen). To determine fold enrichment, qPCR was performed using input controls compared with DNA bound to immunoprecipitated proteins, using primers specific to the site of interest as well as primers to two sites not expected to be enriched.

Gli3-bind peaks and the enriched motifs derived from ChIP-seq

**RNA extraction and ChIP-seq.** To prepare the ChIP-seq library, chromatin was fixed and sonicated to 300–500 bp fragments, then was immunoprecipitated, eluted, de-crosslinked and column-purified before submission for sequencing. High-throughput sequencing was performed on Illumina Genome Analyzer following the manufacturer’s protocols. The raw data was deposited in GEO with an accession number GSE44755.

**Binding peaks.** Gli3T ChIP-Seq immunoprecipitated product (IP) was compared to the input from SHF tissue dissected from mouse embryos at E9.5. The raw IP sequence reads were first trimmed 8 bps on the left end for two reasons: 1) they showed unexpected low quality and 2) they were 8 bps longer than the input reads. Then we used Bowtie aligner software to map ChIP-seq and control sequencing reads to the mouse genome reference build mm9. The genome-wide locations of Gli3-binding peaks were identified using a model-based analysis of ChIP-seq (MACS) algorithm version 2.

**Motif identification.** Motifs were identified using HOMER software by the default parameters. Visualization was conducted using R and a local mirror of the UCSC Genome Browser with customized data.

**Candidate Gli3-targtes annotation.** The Gli3-bound sites were first annotated to the mouse mm9 assembly genome by HOMER software to the nearest transcription start sites (TSSs). Additional genes were included following analyses that localized them to the same chromosome within 100 kb distance to any Gli3T-bound peaks. These genes were annotated using Bioconductor packages biomaRt version 2.14.0 and ChIPpeakAnno version 2.6.0.

Shh-dependent transcriptomic alteration derived from microarray

**Data pre-process.** Expression of the pooled RNAs (8 Shh<sup>−/−</sup> pools and 7 Shh<sup>−/+</sup> pools) were extracted by Feature Extraction Software (v. 10.3.1.1) available from Agilent, using the default variables. Outlier features on the arrays were flagged by the Bioconductor [41] package Agi4x44PreProcess and were excluded. Raw feature intensities (the meanSignals) were background corrected, variance stabilizing normalization (VSN) normalized and log2 transformed [42]. As has been noted in previous studies, non-expressed probes are merely background noise and thus no longer track the expression of genes, lower expressed probes were filtered to increase true positive on the array [43]. As a result, 11,469 probes (encoding 8,867 out of 29,674 genes on the array) were retained for signature identification that met the following three criteria: 1) Met a minimum criterion of signal quality flagged by Agilent Feature Extraction; 2) Cross-sample expression fell into the top half of the inter-quartile range (IQR); 3) Presented known genes as annotated by Bioconductor package mgug4122a.db version 2.6.3. The raw data was deposited in GEO with an accession number GSE44754.

**Data analysis.** To identify Shh-dependent gene signature, the whole mouse genome gene-expression was compared between Shh mutant samples and wide-type controls, using R and Bioconductor package samr (unpaired two-class t-statistic, 100 permutations) [44]. The resultant p-values of all genes with at least 2-fold changes (computed by samr) were corrected for multiple testing using the Q-value adjustment. Differentially expressed genes were identified that had a Q-value less than 0.5%. The hypergeometric test for all Gene Ontology biological processes was performed using Bioconductor package GOStats.

**Tbx5-dependent microarray transcriptomic data analysis**

**Data pre-process.** Analysis performed was identical to that used to identify Shh-dependent transcriptomic alterations. Briefly, VSN [42] was used to do between arrays normalization, and the resultant expression measurements were log2 transformed. The raw data was deposited in GEO with an accession number GSE43599. A Q-value <0.05 indicated significantly differentially expression.

**Candidate Tbx5 and Gli3 co-targets.** Tbx5-bound peaks derived from ChIP-seq were recently published using HL-1 cells [33]. These Tbx5 binding sites were annotated by HOMER software to the nearest transcription start sites (TSSs) in the mouse mm9 reference genome. As T-box motif is significantly enriched among the Tbx5-bound regions, we further searched for candidate Tbx5 and Gli3 co-targets, using HOMER software (Figure 5A).

We checked the intersection of transcriptomic altered genes dependent on either Tbx5 or Shh that intersected with Tbx5 and Gli3 bound genes. The list of resultant genes includes those with Shh- or Tbx5-dependent expression on the microarray experiments and being located within 100 kb distance to a Gli3-bound region derived from the ChIP-seq experiment. These genes were further annotated using Bioconductor package biomaRt version 2.14.0.

**Data accession**

ChIP-seq and microarray data were deposited in the Gene Expression Omnibus (GEO) database with a super accession number GSE44756.

**In-situ hybridization**

**In-situ hybridization** was performed as in Moorman et al. [45] with slight modifications. Specifically, after post-hybridization washes with 50% formamide/2X SSC, specimens were incubated for 30 minutes at 37 degrees C in 20 ug/ml RNase A to remove unbound probe and reduce nonspecific staining. All in-situ hybridization experiments were performed on a minimum of three control and three experimental embryos.

**Luciferase assays**

Expression vectors for Gli1 and Gli3T were obtained from the Vokes lab. Tbx5 was cloned into the pCDNA 3.1 expression vector version 2.14.0, and the reporter was transfected into HEK-293 cells using the transfection agent Lipofectamine 2000.
construct [20] Foxf1a fragment was cloned into the pGLA:23 vector (Promega). Expression and reporter vectors were transfected into HEK293T cells using FuGENE (Promega). Cells were cultured for 48 hours after transfection, then lysed and assayed using the Dual-Luciferase Reporter Assay system (Promega).

**Transient transgenics**

The Foxf1a enhancer and minimal promoter used in the luciferase assays were subcloned from the pENTR vector into the Hsp68-LacZ vector [35] using the Gateway system (Invitrogen). The resulting construct was digested with Nod enzyme to remove the pBlueScript backbone, gel-purified, injected into fertilized mouse eggs at the University of Chicago Transgenics Core Facility and implanted into female mice. Embryos were harvested at E9.5 and stained as described previously [5].

**Supporting Information**

**Figure S1** Optimized shh-dependent candidates for in vitro validation. Panel A) 65 genes generated from all three statistical profiles between the identified among the identified. Panel B) Hierarchical classification of samples based on the expression of these 65 genes splits Shh mutants from wild types. (TIF)

**Figure S2** Known mouse Forkhead-box genes are enriched among the identified shh-dependent and Gli3T-bound genes. (TIF)

**Figure S3** Browser views of the actual sequence mapping profiles between the identified Gli3-bound site and Foxf1a TSS. The two top panels show the estimated enriched peaks and the density measurements respectively. The two bottom panels are the genome (mm9) coordinates and the RefGenes. (TIF)

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