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Supplementary Information for

An evolutionarily conserved *Tbx5-Wnt2/2b* pathway orchestrates cardiopulmonary development

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Supplementary Information Text

Extended Methods.

Xenopus Experiments.

Xenopus explants were microdissected in 1× Modified Barth's saline (MBS) (79) + 50 μg/mL gentamycin sulfate (1676045; MP Biochemicals) and then were cultured in 0.5× MBS + 0.1% fatty acid-free BSA (BP9704; Fisher) + 50 μg/mL gentamycin sulfate. Concentrations of small molecules or reagents were as follows: 20 μM purmorphamine (catalog no. 540220; EMD Millipore/Calbiochem); 1 μM DEX (D4902; Sigma); and 5 μg/mL CHX (C1988; Sigma). For DEX+CHX cotreatment, explants were incubated in CHX alone for 45 min before culture in DEX+CHX for 6 h.

Transcriptional profiling by RNA-seq.

Embryos for RNA-seq were generated by crossing *Tbx5*$^{+/-}$ animals together in timed matings and embryos harvested and micro-dissected as shown in Fig. 1 *A* at E9.5. After genotyping, micro-dissected tissues from four embryos were pooled. Total RNA was extracted from five *Tbx5*$^{+/+}$ and two *Tbx5*$^{-/-}$ pools. 51-bp single-ended sequencing libraries (TruSeq RNA Sample prep kit v2; Part no. RS-122- 2001) were prepared and sequenced using the Illumina HiSeq2500 platform by the Genomics Core Facility at the University of Chicago (Invitrogen, 2013). We focused on the 38-bp reads on the right-side with a general declining pattern of quality scores as expected and ensured a larger than 30 quality score per base. Around 17-26 million RNA-seq reads were generated for each replicate and aligned to the GRCm38/mm10 build of the *Mus musculus* genome using TopHat v2.0.6 (1, 2). Gene-level expression was quantified as read counts per exon using featureCounts in the Bioconductor package RSubRead (3). Reads overlapping exons in annotation build 38.1 of NCBI RefSeq database were included. Counts were converted to log2 counts, fit to a generalized linear model, and normalized to a gene- and sample specific normalization factor generated by the model with the DESeq2 package (4). Data was plotted using the ggplot2 v2.2.1 (5) and scales v0.4.1 (6) packages in R (7). The raw data was deposited in GEO with an accession number GSE75077.

In situ Hybridization.

Mouse embryonic *in situ* hybridization was performed mainly as previously described (8-10). RNA probes were generated using a digoxigenin RNA labeling kit (Roche), and hybridized overnight at 70°C onto bleached (6% Hydrogen Peroxide for 1 hour), proteinase-digested (10μg/ml proteinase K at 37°C for 8-10 minutes), and fixed (0.2% glutaraldehyde/4% PFA, 20 minutes) E9.5 embryos. Following hybridization, embryos were washed 2x30 minutes at 70°C in Solution I (50% formamide, 4X SSC, 1% SDS), 1x10 minutes at 70°C in a 1:1 solution of Solution I and Solution II (500mM NaCl, 10mM Tris 7.5, 0.1% Tween-20), 3x5 minutes Solution II at room temperature, RNase A treated (Sigma) in Solution II (37°C for 1 hour), washed in Solution II 1x5 minutes (room temperature), washed 1x5 minutes in Solution III (50% formamide, 2X SSC, 0.1% Tween-20 at room temperature), washed 2x30 minutes in Solution III at 65°C, and then washed 3x5 minutes in MABTL (100mM Maleic acid, 150mM NaCl, 0.1% Tween-20, and 2mM levamisole, pH 7.5, room temperature). Embryos were blocked for 3 hours in 10% sheep serum, 2% Boehringer Mannheim Blocking Reagent (BMBR) in MABTL. DIG-labeled probes were detected by anti-digoxin-AP Fab fragments (1:4000 dilution, Roche) in MABTL+2% BMBR (4°C overnight), washed 3x5 minutes in MABTL, washed 5-8x60 minutes MABTL, and washed overnight in MABTL (room temperature). Embryos were washed 3x10 minutes in NTMTL (100mM NaCl, 100mM Tris, pH9.5, 50mM MgCl2, 0.1% Tween-20, and 2mM levamisole) and precipitated by BM purple AP substrate (covered, room temperature, Roche). *Wnt2b* and *Nkx2-1* probes were provided by Ed Morrisey (Perelman School of Medicine, University of Pennsylvania). *Shh* probes were provided by Elizabeth Grove (University of

Chicago). The stained section of *Tbx5* come from a series used in a previous publication (11), but the shown sections have not been previously published.

In situ hybridization of *Xenopus* embryos and explants was performed mostly as described (12). Embryos and explants were fixed overnight (12-16 hours) at 4°C in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), washed in MEMFA buffer without the formaldehyde 3x5 minutes, dehydrated directly into 100% ethanol, washed 4-6 times in 100% ethanol, and stored at -20°C. The following minor modifications to the *in situ* protocol were used: proteinase K (ThermoFisher AM2548) on day 1 was used at 2ug/mL for 10 minutes on explants, and at 3.5ug/mL for 10 minutes on whole embryos; the RNAse A step was consisted of 0.5ug/mL for 15 minutes on day 2; and finally the anti-DIG-alkaline phosphatase antibody (Sigma 11093274910) was used at a 1:5000 dilution in MAB buffer (maleic acid buffer, 100 mM Maleic acid, 150 mM NaCl, pH7.5) + 10% heat-inactivated lamb serum (Gibco 16070096) + 2% blocking reagent (Sigma 11096176001) on day2/3. Anti-sense DIG labeled *in situ* probes were generated using linearized plasmid full-length cDNA templates with the 10X DIG RNA labeling mix (Sigma 11277073910) according to manufacturer's instructions.

We used a stage 11 lizard, staged according to Sanger et al. (13). *In situ* hybridization for *Tbx5* was performed as previously described (14). The sections of the American alligator and chicken come from stained section series used in previous publications (15, 16), but the shown sections have not been published before.

3D Reconstruction.

Reconstructions of embryonic lung histology were performed using AMIRA (version 5.3.2) on a Linux platform. Manual review of each image for a stack was performed and corrections were made when necessary. LabelFields for mesenchymal and endodermal tissue were generated from the same tissue using separate CastField and LabelVoxel modules. SurfaceGen modules were used to generate surfaces from these LabelFields. Tissue models for the two different tissues were initially aligned using the Landmark (2sets) module. A minimum of three landmarks were used to align the separate models. Final alignments were fine-tuned manually using the Transform editor.

Chromatin Immunoprecipitation and Analysis.

To prepare chromatin extract, micro-dissected tissue (Fig. 1 *A*) from E9.5 CD-1 mouse embryos (2x from 50 embryo pools each) obtained from Charles River or pelleted IMR90 cells (4x from 5 million cells each) were cross-linked in PBS containing 1% formaldehyde at 25°C for 10 minutes. The reaction was quenched by 125 mM glycine. The cross-linked tissues were incubated in LB1 (50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% Glycerol; 0.5% NP-40; 0.25% Triton X-100) with protease and phosphatase inhibitors on ice for 10 minutes. The tissues were then sonicated in LB3 (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 0.1% sodium deoxycholate; 0.5% N-lauroyl sarcosine; 1% Triton X-100) with protease and phosphatase inhibitors. Chromatin extract was then cleared by centrifugation at 14,000g, 4°C for 10 minutes.

For immunoprecipitation, the chromatin extract was incubated with 5ug of the anti-TBX5 antibody (Santa Cruz Biotechnology sc-17866; Lot #G1516), 1µg of anti-H3K4me3 (Wako Chemicals #305-34819; Lot #14004), or 1µg of anti-H3K4me1 (Abcam ab8895; Lot #GR257926-1) at 4°C for >12 hours in a total volume of 200 µL. The immune-complexes were captured by Protein G-conjugated magnetic beads (Life Technologies, 1003D), washed in sequence by LB3, LB3 with 1 M NaCl, LB3 with 0.5 M NaCl, LB3, and TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The captured chromatin was eluted in ChIP Elution Buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1% SDS; 250 mM NaCl) at 65°C. After RNase and proteinase K treatment and reverse cross-linking, DNA were purified.

High-throughput sequencing libraries from ChIP and input DNA were prepared using NEBNext Ultra DNA Library Prep Kit (New England Biolabs, E7370S). During library preparation, adaptor-ligated DNA fragments of 200-650 bp in size were selected before PCR amplification using Sera-Mag magnetic beads (GE, 6515-2105-050-250). DNA libraries were sequenced using Illumina Hi-seq instruments (single-end 50 base) by the Genomics Core Facility at the University of Chicago.

Raw sequencing reads were aligned to the mm10 genome using Bowtie2 (17) and SAMtools (18, 19) requiring a minimum mapping quality of 10 (-q 10). Pooled peak calling was performed using default settings of MACS2 callpeak (20, 21) with a q-value set to 0.05 and tag size set to 6 (-q 0.05 -s 6). A fold-enrichment track was generated using MACS2 using the bdgcmp function (-m FE) for visualization on the genome browser. Peaks overlapping with ENCODE blacklist sites (22) were removed. TBX5 peaks were annotated in the genome browser using the R (7) package ChIPseeker (23) and TxDb.Mmusculus.UCSC.mm10.knownGene (24) with the promoter defined as within 2kb of the TSS. Density plots, heatmaps, and peak overlaps were generated using the R packages ChIPpeakAnno (25, 26), GenomicRanges (27), trackViewer (28), rtracklayer (29), RColorBrewer (30), and org.Mm.eg.db (31). Correlation analysis between tissues was performed using multiBigwigSummary and plotCorrelation functions of deepTools (32). The raw data was deposited in GEO with an accession number GSE119885.

ATAC-seq and Analysis.

ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) was performed as previously described (33). Nuclei were isolated from micro-dissected tissue (Fig. 1 *A*) from E9.5 CD-1 mouse embryos (2x from 5 embryo pools each) from Charles River according to published protocols and transposition reaction performed by published methods (33). Libraries were amplified and normalized with the Illumina Nextera DNA Library prep kit (FC-121–1031) according to the manufacturer's protocols. Libraries were quantitated using the Agilent Bio-analyzer, pooled in equimolar amounts, and sequenced with 50-bp single-end reads on the Illumina HiSeq following the manufacturer's protocols through the Genomics Core Facility at the University of Chicago.

Sequencing reads were aligned to the mm10 genome using Bowtie2 (17) and SAMtools (18, 19) in a similar manner to the ChIP-seq. Peak calling was performed using MACS2 callpeak (20, 21) using the settings --nomodel --shift -100 --extsize 200 -q 0.05. A fold-enrichment track was generated using MACS2 using the bdgcmp function (-m FE) for visualization on the genome browser. The raw data was deposited in GEO with an accession number GSE119885.

***Tbx5*OE-mESC Generation, CRISPR, and In Vitro Differentiation.**

The inducible *Tbx5*OE-mESC line was generated as previously published (34). *Tbx5* cDNA, without the stop codon, was Gateway cloned into the p2Lox plasmid, which was transfected into the A2Lox.cre mESC, which contains a doxycycline-inducible loxP-flanked cre-recombinase upstream of the *Hprt* locus. Using cre-mediated cassette exchange, the cre locus was replaced with the *Tbx5* cDNA, generating the doxycycline-inducible *Tbx5*OE-mESC. Multiple clones were generated and characterized for growth and differentiation. The best clone was expanded and frozen down for subsequent experimentation.

In order to generate the W2E mutants, we transfected the mESC with pSpCas9(BB)-2-Puro (PX459) plasmid vectors containing one of four guides (TACTGGTAACGGCCCTCCCT, ACTGGTAACGGCCCTCCCTC, CCTAAGATGCAGGCGTGCGC, and TCCTAAGATGCAGGCGTGCG) designed to generate an approximately 4.6kbp deletion of W2E1 and W2E2 without disrupting the last exon of *St7* or the predicted splice acceptor. Following clone selection and expansion, two homozygous deletion clones (W2E mutants) and two wildtype clones (W2E controls) were successfully generated and evaluated.

Stem cell differentiation to the cardiac lineage was based on the original protocol out of the lab of Gordon Keller (35) with some modifications. In brief, cells were treated with 1.5ng/ml BMP4 (R&D), 8ng/ml Activin A (R&D), 6ng/ml bFGF (R&D), and 200ug/ml Transferin (Sigma) on day 2 of the differentiation, and treated with 20ng/ml PDGFAA (eBioscience) on day 3. Live cells, positive for PDGFRα (Invitrogen) and Flk-1 (BioLegend), were sorted at day 3.75. Following cell sorting on day 4, cells were cultured in StemPro (LifeTechnologies) with 2mMol Glutamax, 1mM ascorbic acid, 1ug/ml SB431542 and 20mg/ml Normocine (Invitrogen).

Tbx5 overexpression dosage was determined through a doxycycline dose responsive curve using the following concentrations: 0, 5, 10, 25, 50, 100, 250, 500ng/mL. For all mESC experiments utilizing overexpression, *Tbx5*OE-mESC cells were treated with doxycycline (Sigma D9891) at the cardiac progenitor-like stage (day 6) then harvested for RNA 24 hours later. For CRISPR cell line evaluation, a dose of 100ng/mL was used. RNA isolation was performed with the NucleoSpin RNA kit (Macherey-Nagel) and cDNA was generated using qScript cDNA Supermix (Quantabio).

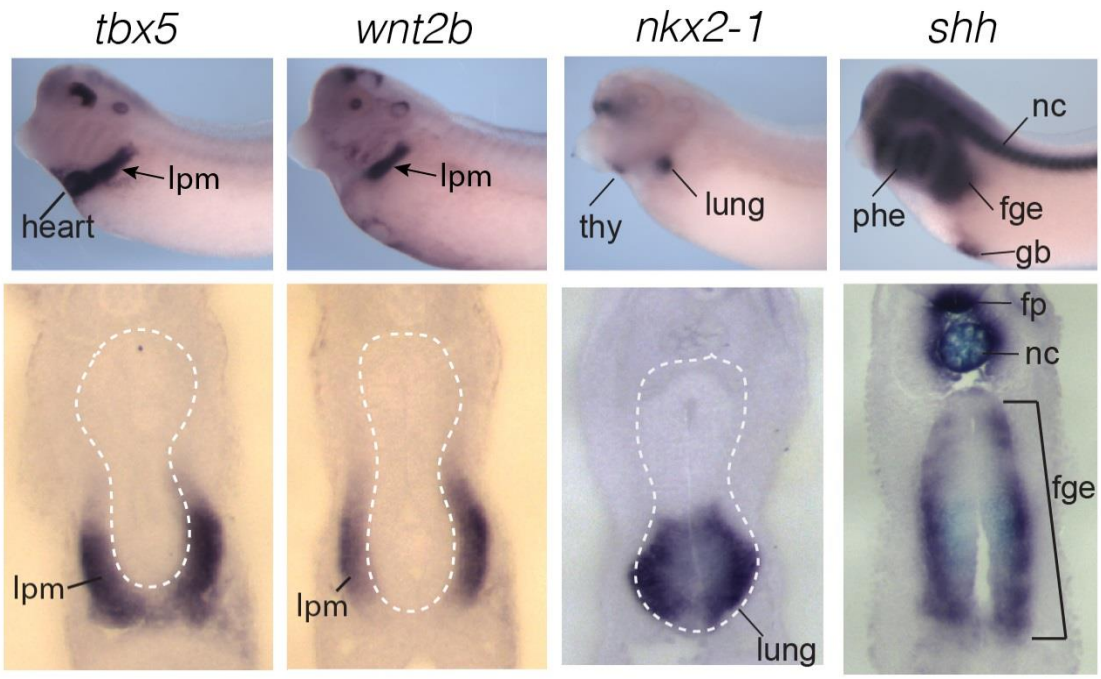


Fig. S1. *Xenopus* whole mount *in situ* hybridization. RNA *in situ* hybridization was performed in NF35 *Xenopus* embryos for *tbx5*, *wnt2b*, *nkx2-1*, and *shh* (whole mount side-view on top, transverse cross-section on bottom). lpm = lateral plate mesoderm, thy = thyroid, nc = notochord, phe = pharyngeal endoderm, fge = foregut endoderm, gb = gall bladder, fp = floor plate.

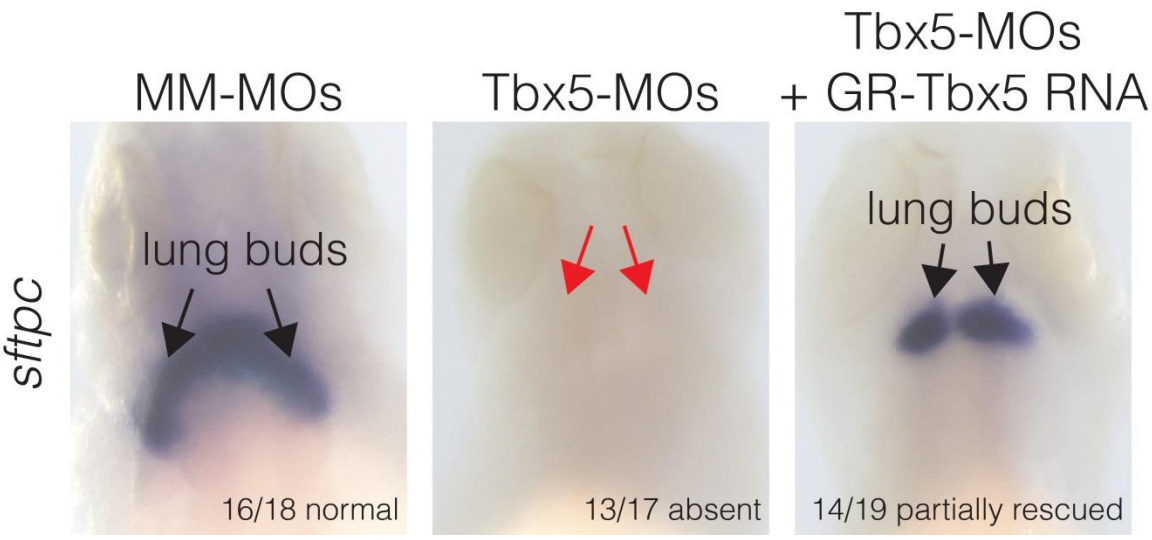


Fig. S2. Expression of *sftpc* is *tbx5*-dependent in *Xenopus*. RNA *in situ* hybridization was performed for the pulmonary epithelium marker *sftpc* in NF42 *Xenopus* embryos injected with mismatched morpholinos (MM-MOs), Tbx5-MOs, or Tbx5-MOs and GR-Tbx5 RNA. Black arrows indicate the branching lung structures as indicated by *sftpc* expression, while the red indicates absence.

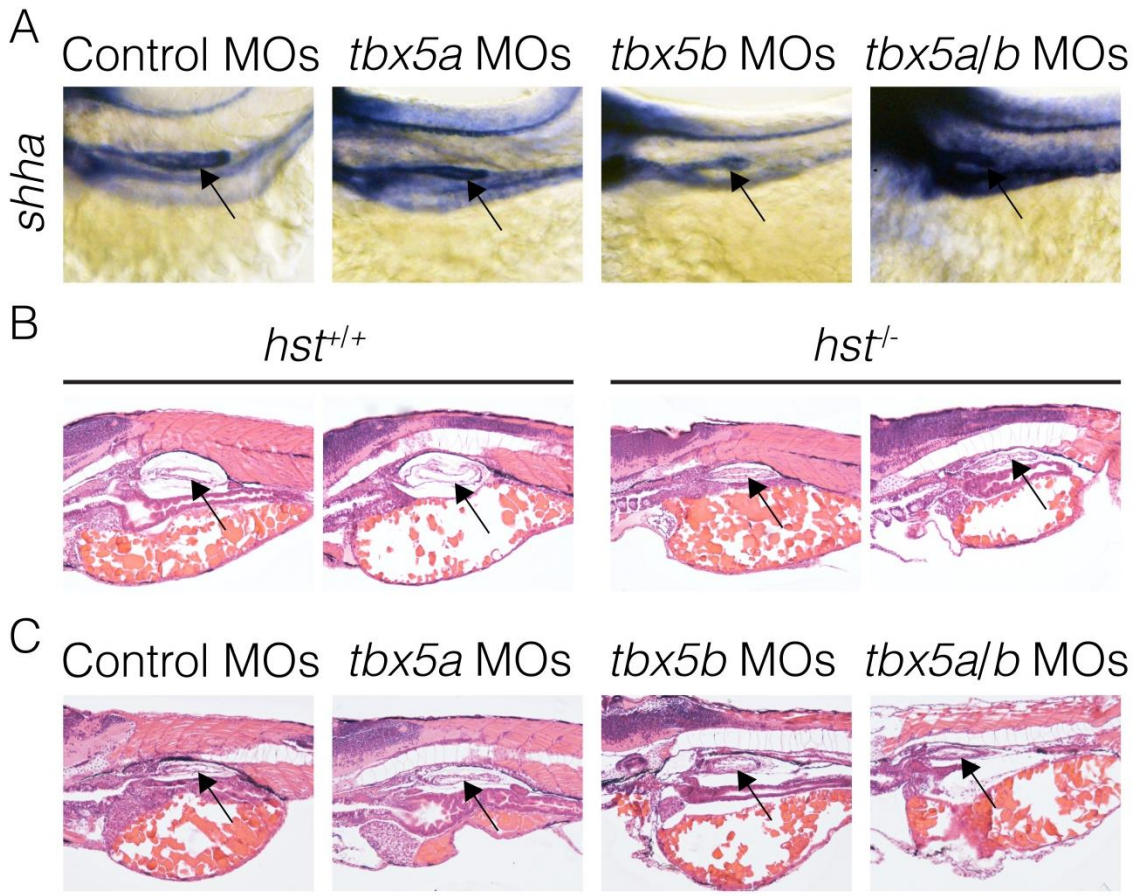


Fig. S3. Initiation of the swim bladder occurs independent of *tbx5a/b* in zebrafish. A. RNA *in situ* hybridization was performed for *shha* in 72hpf zebrafish embryos injected with controls, *tbx5a*, *tbx5b*, or *tbx5a* and *tbx5b* morpholinos (MOs). B. Zebrafish embryos were collected at 96hpf and histologically sectioned and stained with H&E comparing *heartstrings* (*hst*) controls and homozygous mutants. C. Similarly, zebrafish embryos were collected at 96hpf and histologically sectioned and stained with H&E comparing control, *tbx5a*, *tbx5b*, and *tbx5a* and *tbx5b* MOs. In all images, the black arrows indicate the forming swim bladder.

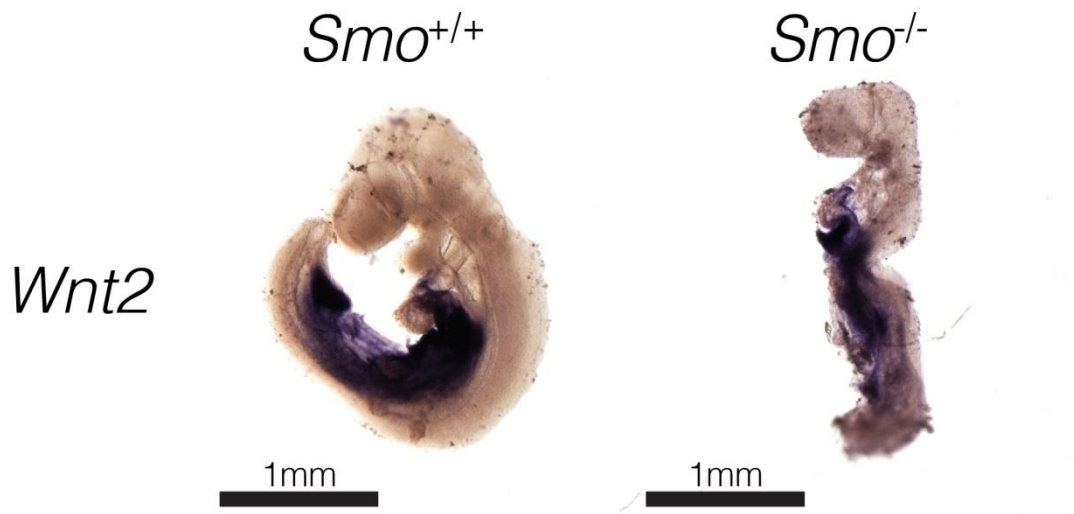


Fig. S4. *Wnt2* expression is independent of Hedgehog signaling. Whole mount *in situ* hybridization for *Wnt2* was performed on E9.5 *Smo*$^{+/+}$ and *Smo*$^{-/-}$ embryos.

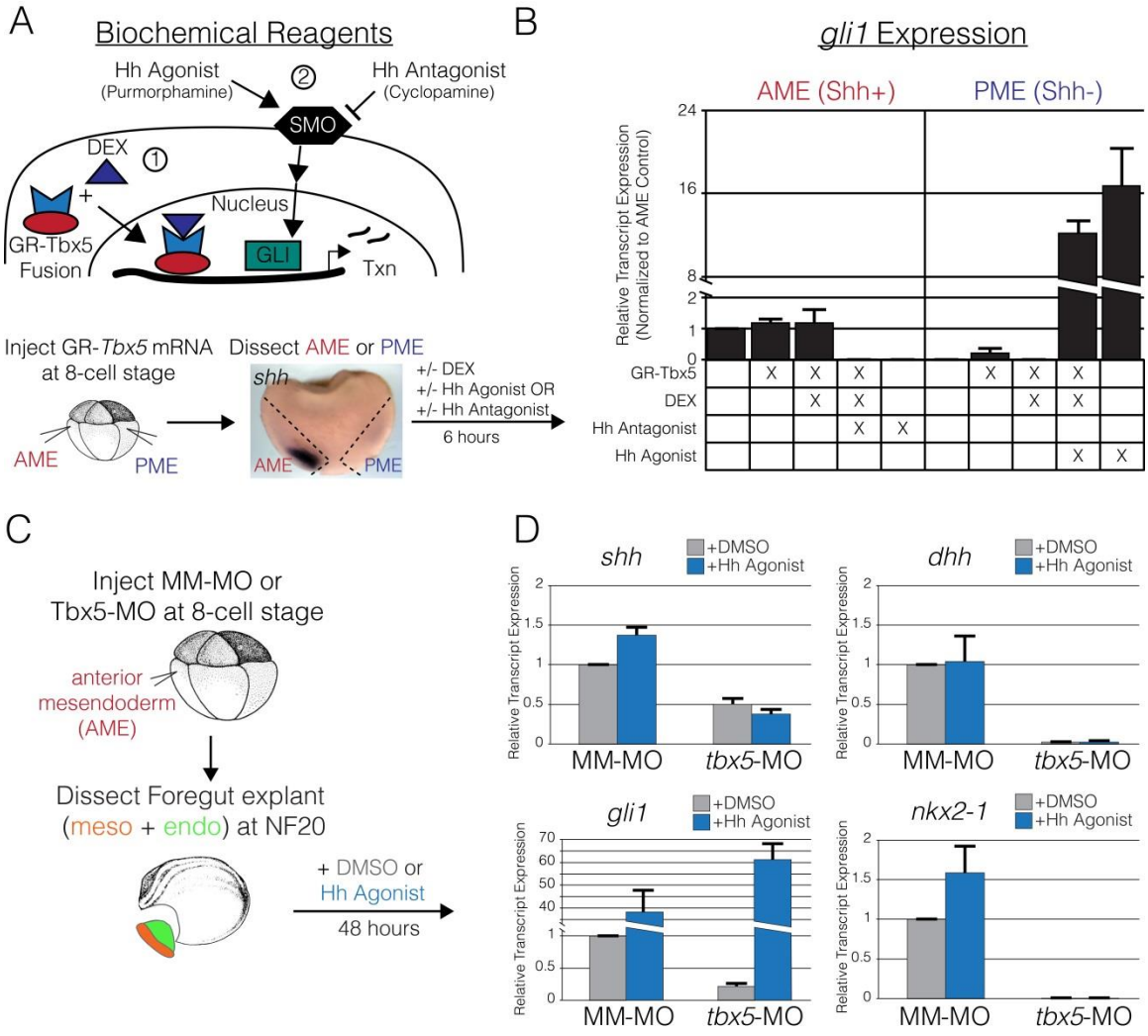


Fig. S5. Utilizing Xenopus system to understand the genetic interaction between Tbx5 and Shh signaling. A. To study the interaction of Tbx5 and Hedgehog signaling, we utilized: (1) a dexamethasone (DEX) inducible glucocorticoid receptor-Tbx5 (GR-Tbx5) fusion protein, and (2) pharmacological agonists (purmorphamine) and antagonists (cyclopamine) of SMO to activate or repress Hh signaling similar to Fig. 4 (top). Experimental details for testing Hedgehog response in anterior mesendoderm (AME, red, Shh+) and posterior mesendoderm (PME, blue, Shh-) tissues measured by *gli1* expression (bottom). Dissected AME or PME tissue was cultured for 6 hours in combinations of dexamethasone (DEX, translocates GR-Tbx5 fusion protein to the nucleus), purmorphamine (Hh agonist), and cyclopamine (Hh antagonist). B. Expression of *gli1* examined by qRT-PCR in culture conditions as indicated by the checked boxes. All conditions were normalized to the AME controls (far left). C. Experimental plan for investigating markers of lung development in embryos injected with mistmatched morpholinos (MM-MOs) or Tbx5-MOs. Embryos were cultured in DMSO (grey) or Hh agonist (purmorphamine, blue) for 48 hours. D. qRT-PCR of *shh*, *dhh*, *gli1*, and *nkx2-1* from Tbx5-morphant or control embryonic explants treated with DMSO or Hh agonist.

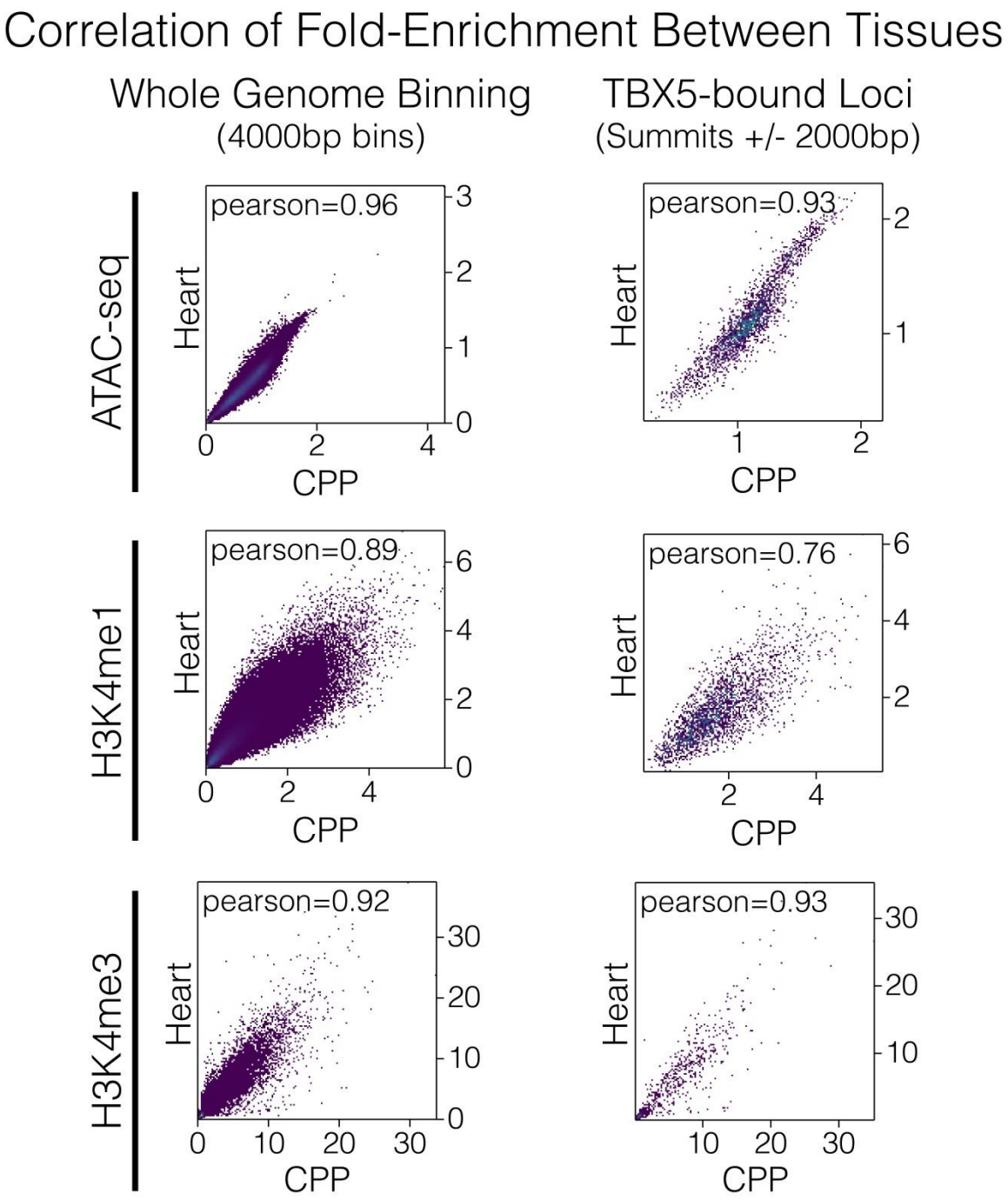


Fig. S6. Genomic correlation between tissues. Fold-enrichment signals from the CPP micro-dissected tissue were plotted against fold-enrichment signals from the heart for each ATAC-seq, H3K4me1 ChIP-seq, and H3K4me3 ChIP-seq. On the left, the signal was calculated from 4000bp bins of the whole genome, while on the right, signal was determined from the 2000bp window to either side of a TBX5 ChIP-seq summit.

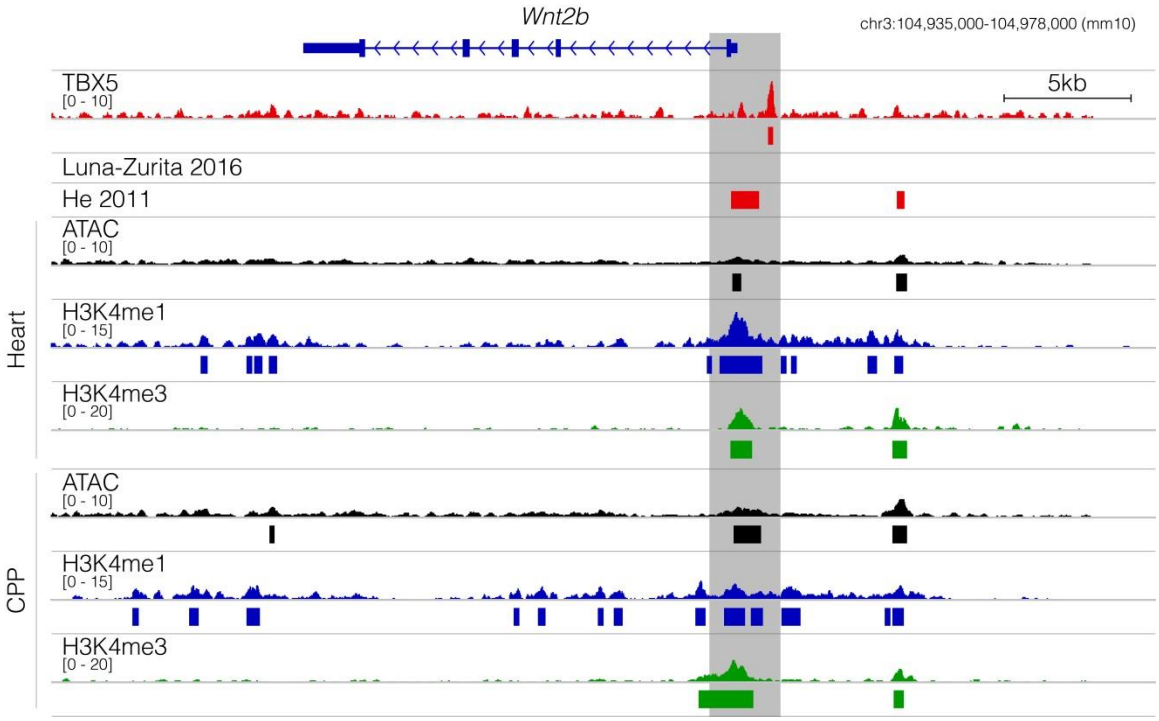
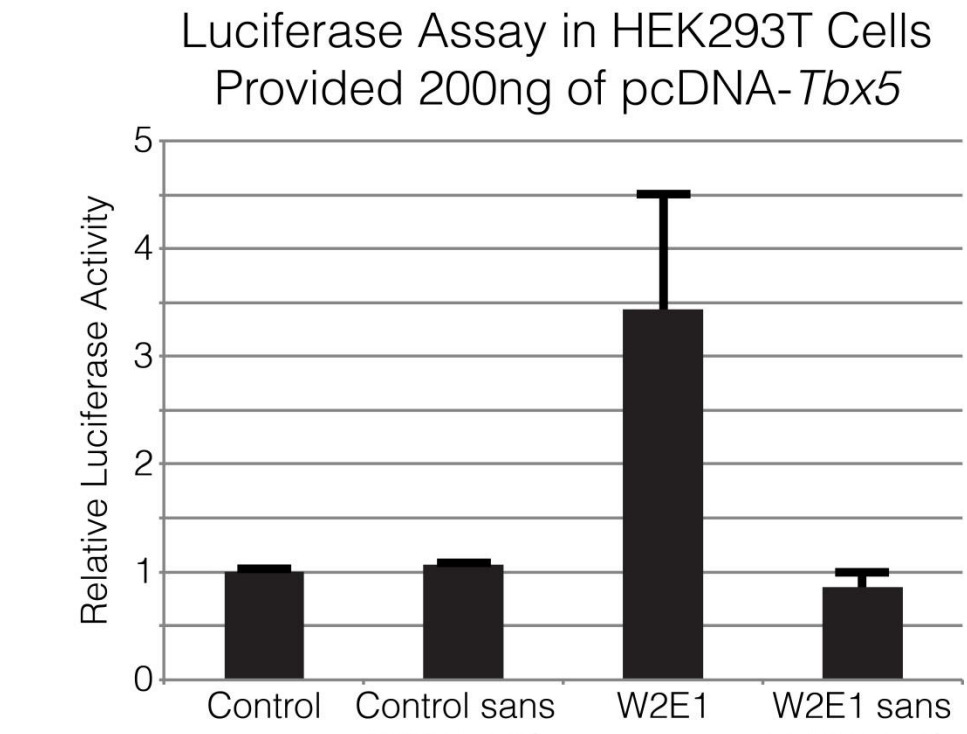


Fig. S7. *Wnt2b* genomic locus. Genome browser view of *Wnt2b* and surrounding locus (chr3:104,935,000-104,978,000) with TBX5 ChIP-seq signals from the E9.5 heart and literature (42, 43), H3K4me1 and H3K4me3 ChIP-seq signals from the heart and CPP micro-dissected tissues, and ATAC-seq from the heart and CPP micro-dissected tissues. Significant peak calls below fold-enrichment signal tracks.



p-value:	Control sans TBX5 Motifs	W2E1	W2E1 sans TBX5 Motifs
Relative to Control	0.524	0.0309	0.308
Relative to W2E1			0.0252

Fig. S8. Putative T-box motifs of W2E1 are required for TBX5-dependent activation in HEK293T cells. Luciferase assay examining TBX5 activation of the random sequence control, W2E1, and mutant variations of each lacking the canonical T-box motif. Data was normalized to the control vector.

Dataset S1 (separate file)

List of all significantly dysregulated genes (FDR < 0.05, FC ≥ 1.5 or FC ≤ 1/1.5) identified by RNA-seq in the micro-dissected CPPs from *Tbx5*$^{+/+}$ and *Tbx5*$^{-/-}$ embryos at E9.5.

Dataset S2 (separate file)

List of all 3883 peaks called by MACS2 for the TBX5 ChIP-seq, including positional information (mm10), fold-change over input, significance, and fold enrichment.

Dataset S3 (separate file)

List of all 162 genes that were both significantly down and the closest gene to a TBX5 ChIP-seq peak. Some of these dysregulated genes were associated with more than one TBX5 ChIP-seq peak.

References

1. Kim D, *et al.* (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14(4):R36.
2. Trapnell C, Pachter L, & Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25(9):1105-1111.
3. Liao Y, Smyth GK, & Shi W (2013) The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 41(10):e108.
4. Love MI, Huber W, & Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15(12):550.
5. Wickham H (2009) *ggplot2: Elegant Graphics for Data Analysis* (Springer-Verlag New York).
6. Wickham H (2017) scales: Scale Functions for Visualization), R package version 0.5.0.
7. R Core Team (2016) R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing).
8. Hoffmann AD, *et al.* (2014) Foxf genes integrate tbx5 and hedgehog pathways in the second heart field for cardiac septation. *PLoS Genet* 10(10):e1004604.
9. Wilkinson DG & Nieto MA (1993) Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol* 225:361-373.
10. Takada S, *et al.* (1994) Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes Dev* 8(2):174-189.
11. Xie L, *et al.* (2012) Tbx5-hedgehog molecular networks are essential in the second heart field for atrial septation. *Dev Cell* 23(2):280-291.
12. Sive HL, Grainger RM, & Harland RM (2000) *Early development of Xenopus laevis : a laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) pp ix, 338 p.
13. Sanger TJ, Losos JB, & Gibson-Brown JJ (2008) A developmental staging series for the lizard genus Anolis: a new system for the integration of evolution, development, and ecology. *J Morphol* 269(2):129-137.
14. Jensen B, *et al.* (2012) Identifying the evolutionary building blocks of the cardiac conduction system. *PLoS One* 7(9):e44231.
15. Jensen B, *et al.* (2018) Specialized impulse conduction pathway in the alligator heart. *Elife* 7.
16. Poelmann RE, *et al.* (2014) Evolution and development of ventricular septation in the amniote heart. *PLoS One* 9(9):e106569.
17. Langmead B & Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9(4):357-359.
18. Li H, *et al.* (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25(16):2078-2079.
19. Li H (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* 27(21):2987-2993.
20. Zhang Y, *et al.* (2008) Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 9(9):R137.
21. Feng J, Liu T, Qin B, Zhang Y, & Liu XS (2012) Identifying ChIP-seq enrichment using MACS. *Nat Protoc* 7(9):1728-1740.
22. Encode Project Consortium (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* 489(7414):57-74.
23. Yu G, Wang LG, & He QY (2015) ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* 31(14):2382-2383.

24. Bioconductor Core Team and Bioconductor Package Maintainer (2016) TxDb.Mmusculus.UCSC.mm10.knownGene: Annotation package for TxDb object(s)), R package version 3.4.0.
25. Zhu LJ, *et al.* (2010) ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq and ChIP-chip data. *BMC Bioinformatics* 11:237.
26. Zhu LJ (2013) Integrative analysis of ChIP-chip and ChIP-seq dataset. *Methods Mol Biol* 1067:105-124.
27. Lawrence M, *et al.* (2013) Software for computing and annotating genomic ranges. *PLoS Comput Biol* 9(8):e1003118.
28. Ou J, Wang Y-X, & Zhu LJ (2016) trackViewer: A bioconductor package with minimalist design for drawing elegant tracks or lollipop plot), R package version 1.10.2.
29. Lawrence M, Gentleman R, & Carey V (2009) rtracklayer: an R package for interfacing with genome browsers. *Bioinformatics* 25(14):1841-1842.
30. Neuwirth E (2014) RColorBrewer: ColorBrewer Palettes), 1.1-2.
31. Carlson M (2016) org.Mm.eg.db: Genome wide annotation for Mouse), 3.4.0.
32. Ramirez F, *et al.* (2016) deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* 44(W1):W160-165.
33. Buenrostro JD, Wu B, Chang HY, & Greenleaf WJ (2015) ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr Protoc Mol Biol* 109:21 29 21-29.
34. Iacovino M, *et al.* (2011) Inducible cassette exchange: a rapid and efficient system enabling conditional gene expression in embryonic stem and primary cells. *Stem Cells* 29(10):1580-1588.
35. Kattman SJ, *et al.* (2011) Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 8(2):228-240.