Supplementary Methods

Tamoxifen-induced gene knockout

Foxa2$^{Cre/Es1}$ pregnant females were treated with a single interaperitoneal injection of tamoxifen (100 mg/kg; Sigma, T5648) dissolved in corn oil at E6.5-7 as described previously (Park et al., 2008).

In vivo ChIP assays

Whole heart was extracted from wild-type E10.5 embryos and chopped into small pieces using two razor blades; 1 ml nuclei extraction buffer [0.5% Triton X-100, 10 mM Tris-HCl (pH 7.5), 3 mM $CaCl_2$, 0.25 M sucrose, protease inhibitor (one tablet/10 ml), 1 mM DTT, 0.2 mM PMSF] was added and then the tissue was transferred to 1.7 ml tubes, 25 µl 37% formaldehyde was added, and rotated at room temperature for 15 min followed by addition of 100 µl 1 M Tris-HCl (pH 9.5) and rotation for another 5 min. The sediment was centrifuged at 2000 ***g*** at 4° C for 2 min and the pellet transferred into 1 ml nuclei extraction buffer and homogenized with 5-10 strokes. The pellet was then filtered through a Falcon 100 µm cell strainer and recentrifuged. 200-300 µl SDS lysis buffer was added to the resuspended pellet. Sonication, immunoprecipitation and elution were carried out using a CHIP Assay Kit (Upstate).

ChiP assay primers:

SITE 1-F: GCA TTA CAA AAG AAT CCT TCA
SITE 1-R: TAA ACC ACT ACC AGG GAA AA

SITE 2-F: GTG CTT TTC AAT GAG ACA GG
SITE 2-R: CAA GCC TCA AAG CAT TTT TA

SITE 3-F: GTG CTC ATT TCC CTG AAC T
SITE 3-R: CAG CTT TTT GTT CCT TCT TG

SITE 4-F: TGG ATC AAG AAC CTT AGC TG
SITE 4-R: CTT GAG CTG GAT AGG ATG AG

SITE 5-F: CCA TTC CTG TTC TCA TCC TA
SITE 5-R: GAA TAT CGG CAT CAT CAA CT

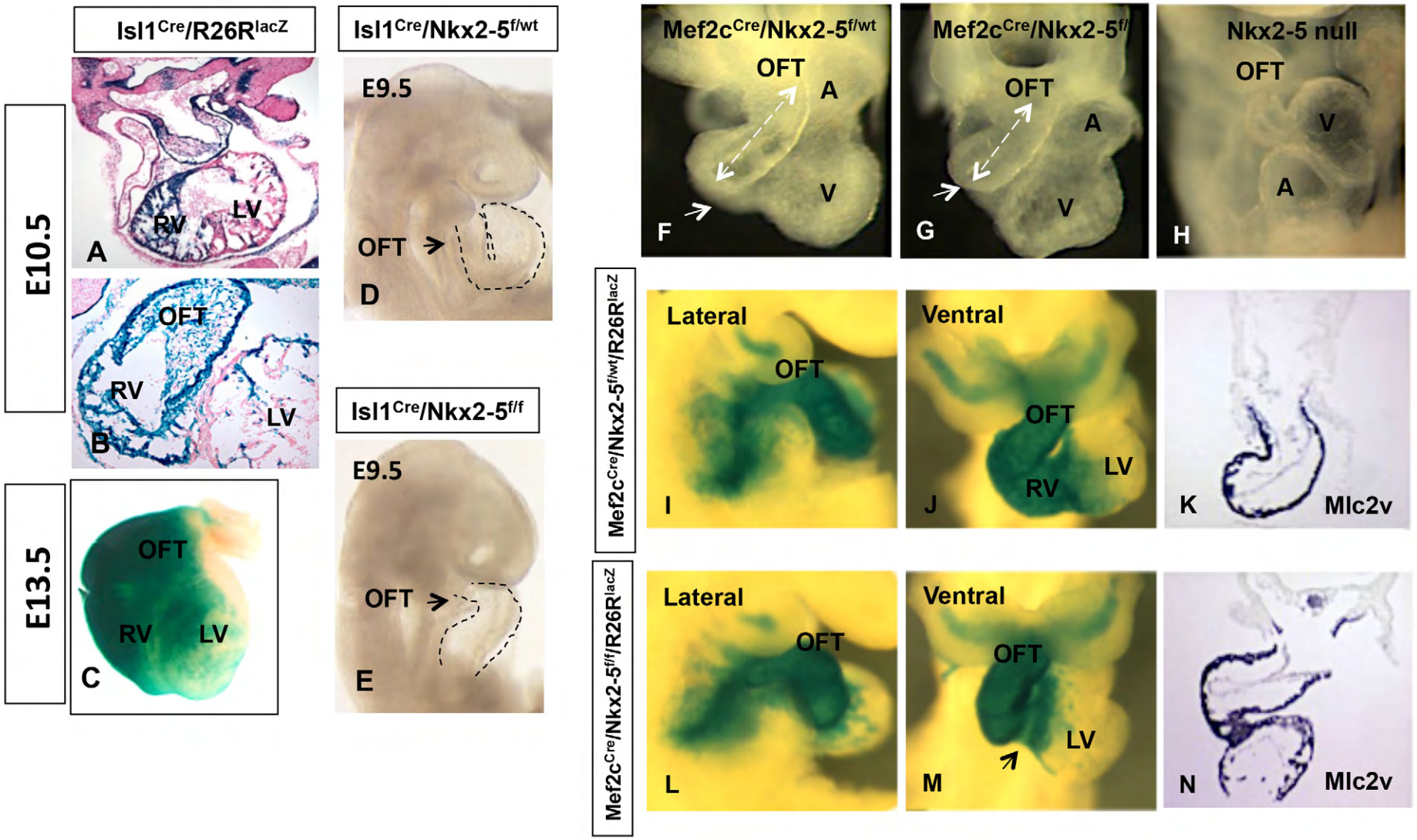
SITE 6-F: CCC ATA ACT TCA TAG AGA AGG A
SITE 6-R: ATG GAG TCT AAC CAC CCT TT

Promoter cloning and luciferase reporter assays

The 3 kb genomic DNA fragment containing the putative Nkx2-5 binding site upstream of the *Rspo3* (ENSMUSG00000019880) start codon, and 290 bp (forward 5′-AGATGCCCACCCAATTCCAA-3′ and reverse 5′-GGTGTCCGTCATTGTGCTCA-3′) and

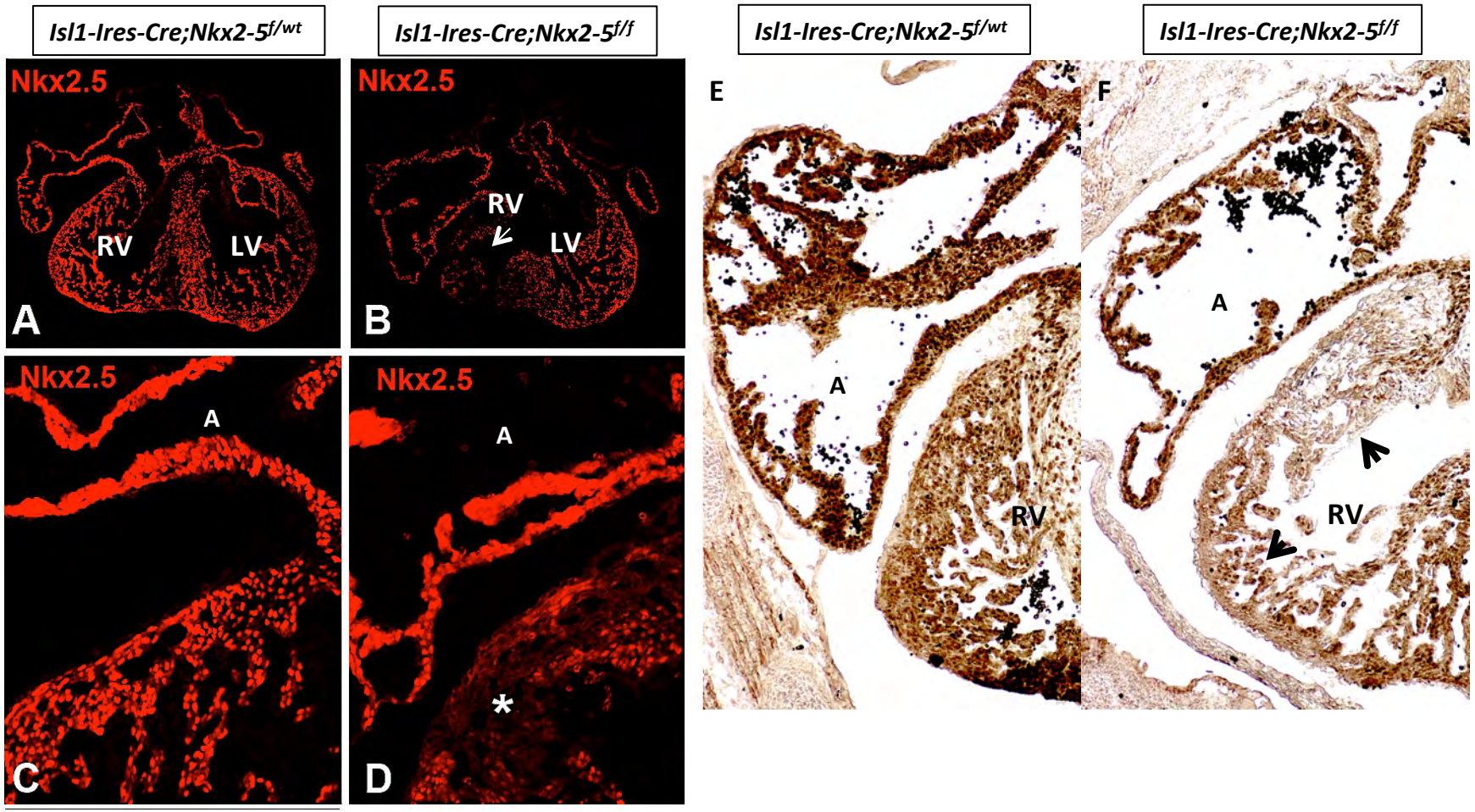
460 bp (forward 5′-AGATGCCCACCCAATTCCAA-3′ and reverse 5′-TGAATGGTGCTTGTTCCTTG-3′) fragments were amplified with Phusion high-fidelity DNA polymerase (New England BioLabs, F-530S/L) and cloned into the pGL3-basic vector (Promega, E1751).

For the luciferase transfection assay, we transfected 293T cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Unless otherwise indicated, 100 ng reporter and 100 ng of each plasmid were used; 10 ng CMV-*Renilla* luciferase was used as an internal control to normalize for variations in transfection efficiency. Luciferase assays were performed using a Dual Luciferase Reporter Assay System (Promega), and reported values are the mean ± s.e.m. of three assays performed in duplicate.



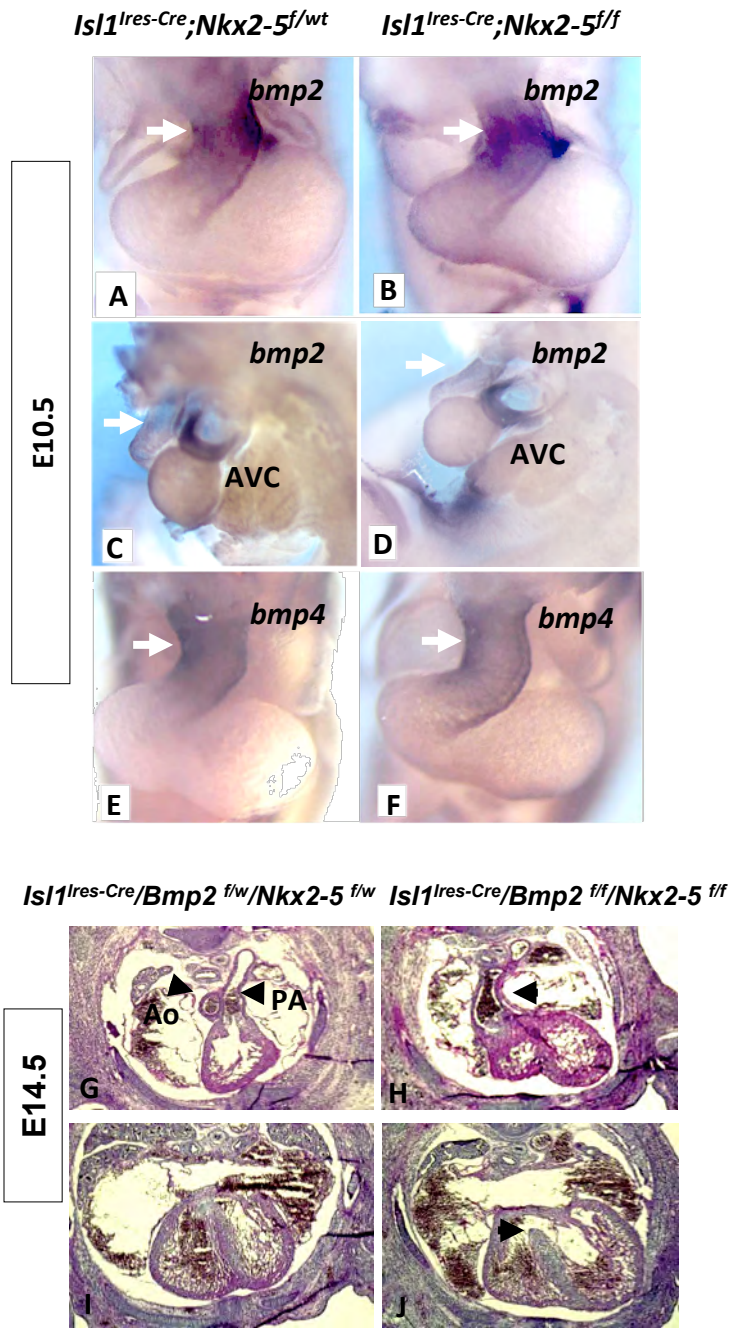
Suppl. Fig. 1. Conditional mutants of Nkx2-5 by Isl1-Cre and Mef2c-Cre.

(A-C) Lineage analysis of Isl1Cre (Yang et al., 2006) line crossed with R26R-lacZ reporter line showing efficient recombination in descendants of SHF that give rise to RV and OFT, and a large number of cells in the LV at E10.5 (A,B) and E13.5 (C). (D,E) Comparison of heterozygous ($Isl1^{Cre}/Nkx2\text{-}5^{f/w}$) and mutant ($Isl1^{Cre}/Nkx2\text{-}5^{f/f}$) embryos at E9.5, showing poorly developed RV and OFT. (F-H) Comparisons of heart development in the conditional heterozygote (F), conditional mutant (G), and the conventional Nkx2-5 mutant (H) at E9.5. Compared to the conventional Nkx2-5 mutants, the OFT is better developed in the conditional mutants. (I,J,L,M) Whole-mount X-gal analysis of the heterozygote ($Mef2cCre/Nkx2\text{-}5^{f/wt}$) and mutant ($Mef2cCre/Nkx2\text{-}5^{f/f}$) hearts shown in R26R-lacZ background at E9.5. The right ventricle is virtually absent in the Nkx2-5 mutant embryos, while OFT myocardium is still present. (K,N) In situ hybridization using Mlc2v probe showing properly differentiated myocardium in the conditional Nkx2-5 mutants.

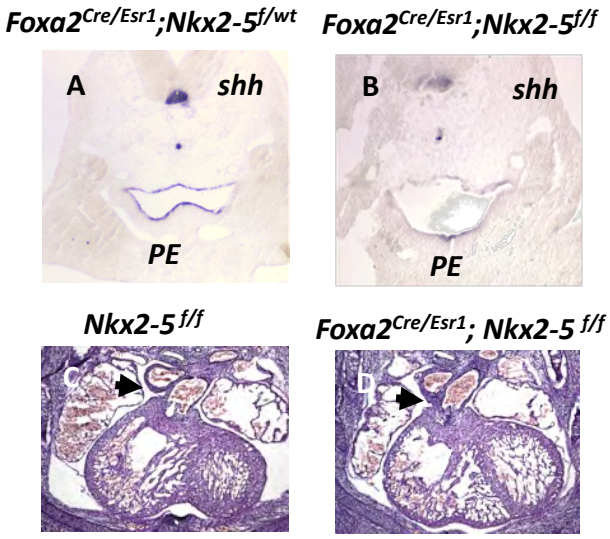


Suppl. Fig. 2. Efficiency of Isl1-Ires-Cre recombination in the Nkx2-5 mutants.

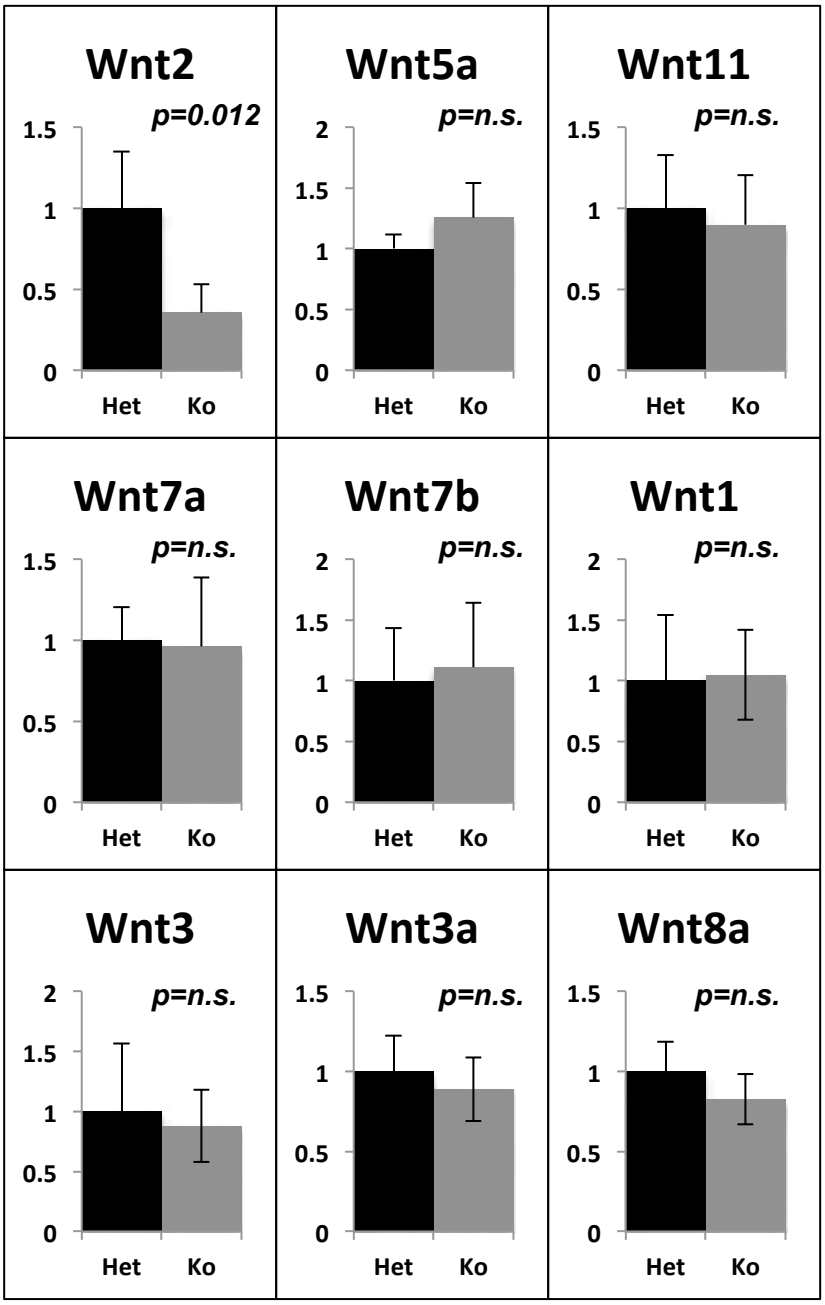
(A-D) Immunofluorescence stain using Nkx2-5 antibody shows prominent loss of Nkx2- 5 in the RV of the mutants but not in the LV. (E,F) Immunohistochemical staining for Nkx2-5 showing marked loss of Nkx2-5 in the RV and OFT. NKx2-5-positive nuclei are uniformly present in the heterozygous RV.



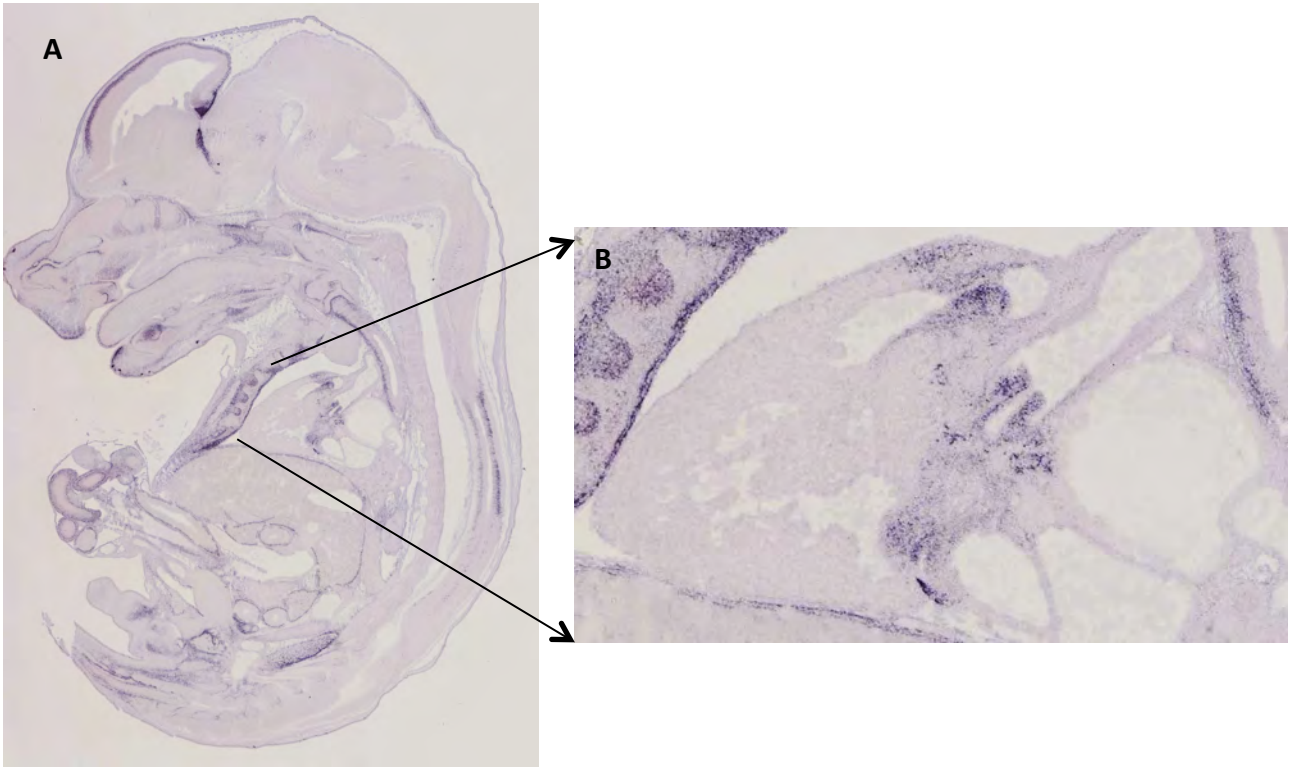
Suppl. Fig.3. Loss of Bmp2 does not rescue conditional Nkx2-5 mutants.
In situ hybridization for Bmp2 (A-D) and Bmp4 (E,F) showing comparable expression in the control and mutant OFTs. (G-J) H&E staining of E14.5 Bmp2 and compound heterozygous (G,I) and compound mutants (H,J) sections showing the presence of PTA and VSD in the compound mutants of Nkx2-5 and Bmp2.



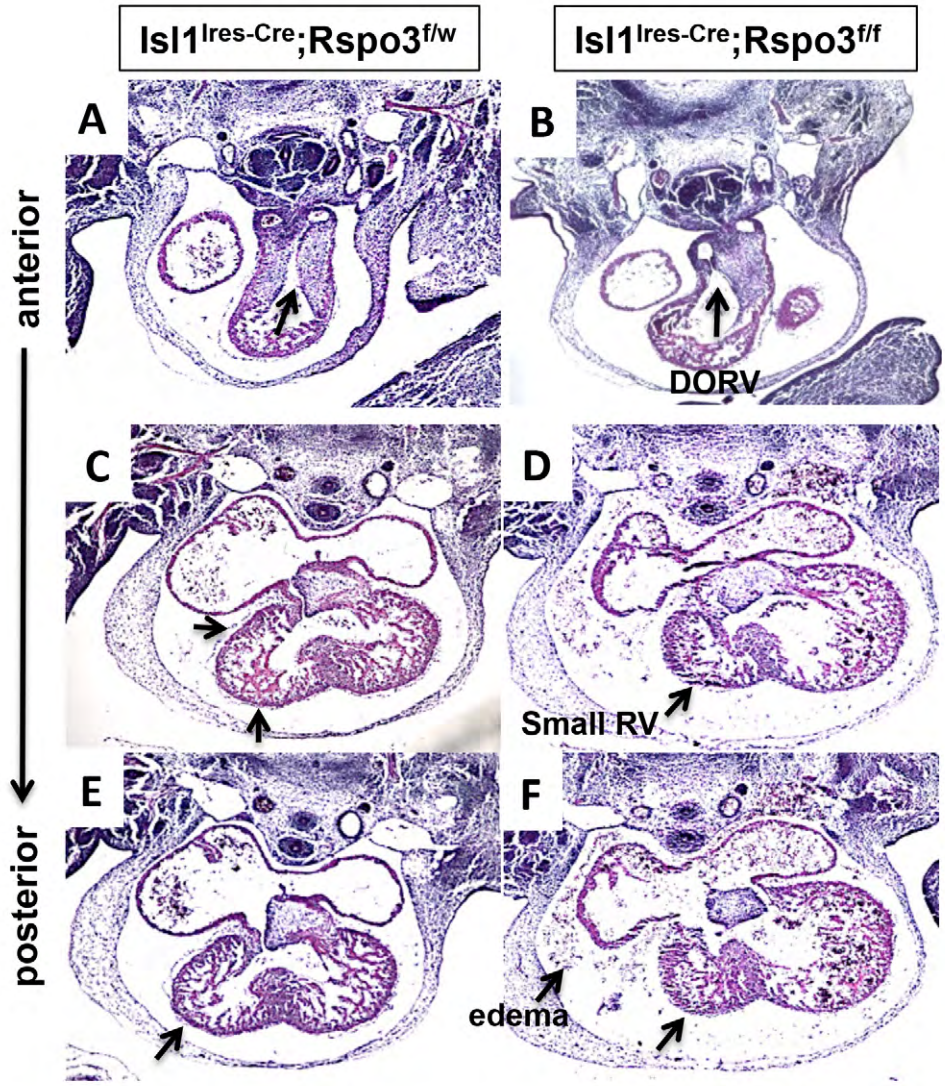
Suppl. Fig. 4. Loss of Nkx2-5 in the pharyngeal endoderm does not affect OFT septation.
(A,B) In situ hybridization showing expression of Shh in the endoderm-specific conditional Nkx2-5 mutants. (C,D) Endoderm-specific ablation of Nkx2-5 using $Foxa2^{Cre/Esr1}$ does not result in OFT septation or alignment defects.



Suppl. Fig .5. Quantitative RT-PCR analysis of selective Wnt genes in the Nkx2-5 mutants.
RNA isolated from SHF (RV/OFT) of Nkx2-5 mutants and controls at E10.5 was subjected to qPCR analysis. Only Wnt2 showing a modest decrease in the conditional Nkx2-5 mutants, but examination of other Wnt ligands showed either undetectable or insignificant changes as analyzed by qPCR.



Suppl. Fig. 6. Expression of Rspo3 in the E14.5 mouse embryo.
(A,B) In situ hybridization showing Rspo3 expression in the heart becomes restricted to the valvular apparatus and myocardium of the inflow and the outflow tract (adapted from: eurexpress.org).



Suppl. Fig. 7. Conditional loss of Rspo3 by Isl1-Ires-Cre results in SHF defects.
(A-F) Inactivation of Rspo3 results in embryonic (E13.5) heart failure with resultant pericardial edema, DORV (B), and a significantly smaller RV (D,F).

Table S1. Primers used for RT-PCR and qPCR reactions

Gene name(s)	Forward primer	Reverse primer
Actb	GCTGTATTCCCCTCCATC	GGGTGTTGAAGGTCTCAAA
Angiopoietin 1	AGCTGACAGATGTTGAGACC	TGATGAATGTCTGACGAGAA
Bmp2	GAGATGAGTGGGAAAACG	GCAGTAAAGGCATGATAGC
Bmp4	AAGTTTCCCACCGTGTCATT	CCGAGCCAACACTGTGAGGAGT
Fgf10	ACATTGTGCCTCAGCCTTTC	TTCCATTCAATGCCACATACAT
Gapdh	CATGGCCTTCCGTGTTCCTA	CCTGCTTCACCACCTTCTTGAT
Hod	TGGAGTACAACTTCAACAAGG	TGTGTTAAATAGCAGGACAGC
Pdgfra	AGATAGCTTCATGAGCCGAC	GGAACAGGGTCAATGTCTGG
Rspo3	GTGTTCAGATTACAATGGCT	CCCTTTTGAAGCCACATGTTT
Sema3C	CCACCCGAAGGACATCATG	GGCAACTGATTCCTCCTGTTTC
Sfrp2	CAGCCCGACTTCTCCTAC	TGGATGGTCTCATCTAGGTC
Sostdc1	TGGATTGGAGGAGGCTATGGAA	ACTTGCAGGCAGTGACTACTGT
Tbx3	CCCGAAGAAGAGGTGGAGGACG	GATGGAGACAGCAGGAGAGGAT
Wnt1	CTACTGGCACTGACCGCTCT	GAATCCGTCAACAGGTTCGT
Wnt11	GGGCCAAGTTTTCCGATGCT	TTCGTGGCTGACAGGTAGCG
Wnt2	CACCCTGGACAGAGATCACA	ACAACGCCAGCTGAAGAGAT
Wnt5a	TCAGGACCACATGCAGTA	CTCATGGCGTTCACCACC
Wnt7a	TGGATGCCCGGGAGATC	CCGACCCGCCTCGTTATT
Wnt7b	TTCTGGAGGACCGCATGAA	GGTCCAGCAAGTTTTGGTGGTA
Wnt8a	AACGGTGGAATTGTCCTGAG	GGTGACTGCGTACATGATGG