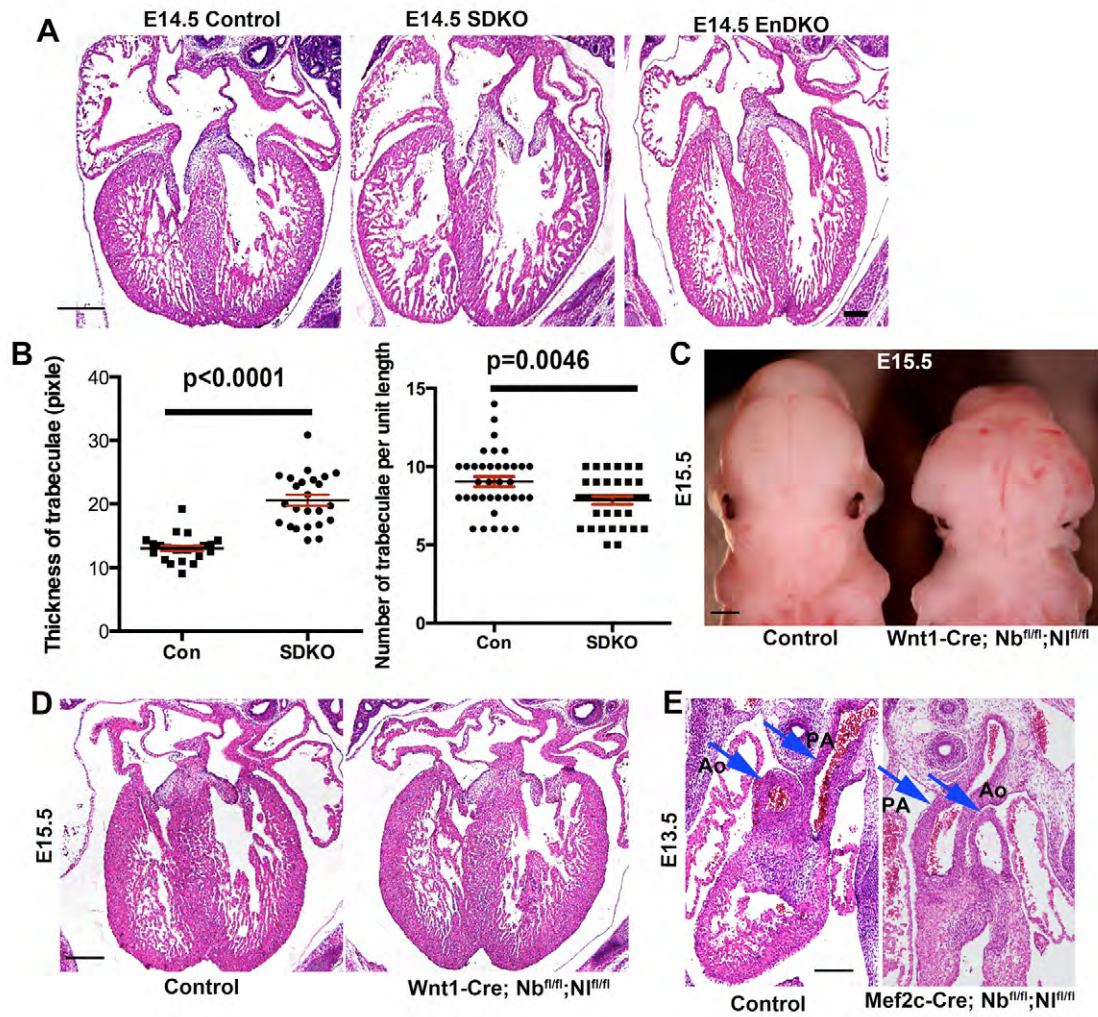


**Figure S1. Expression patterns of *Nkx2.5*<sup>Cre/+</sup> and phenotype of MDKO.**

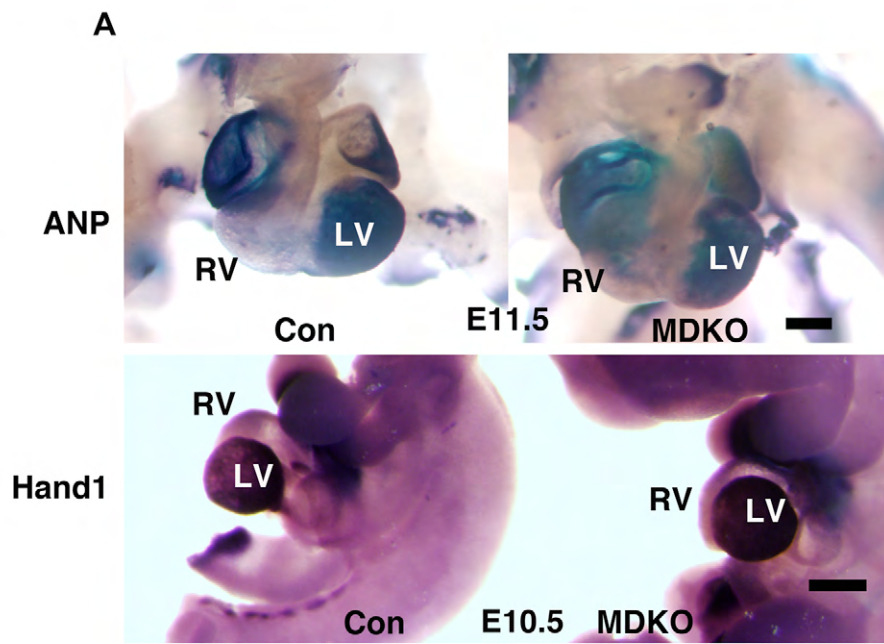
(A-C) *Nkx2.5*<sup>Cre/+</sup> was active in cardiomyocytes and endocardial cells of the IFT and OFT, but in very few endocardial cells of ventricles, indicated by green arrows. Sections through the whole heart of E9.5 (A+B) and E10.5 (C) embryos were examined (n=3 for each age). The section in (C) is from a 3D imaged E10.5 whole heart and all of its sections are shown in Movie 1. (D) Heterozygotes heart did not display obvious cardiac defects. (E&F) MDKO embryos displayed edema at E13.5. (G) OFT of MDKO hearts displayed abnormal alignment with ventricles at E11.5. Dotted line delineates the OFT curve. (H) MDKO displayed alignment defect, as both aorta and pulmonary arteries extend from right ventricles. Scale bar in A-C, D, G and H are 20  $\mu$ m, in E&F are 300 $\mu$ m.



**Figure S2. Functions of NFPs in different cardiac cell types.**

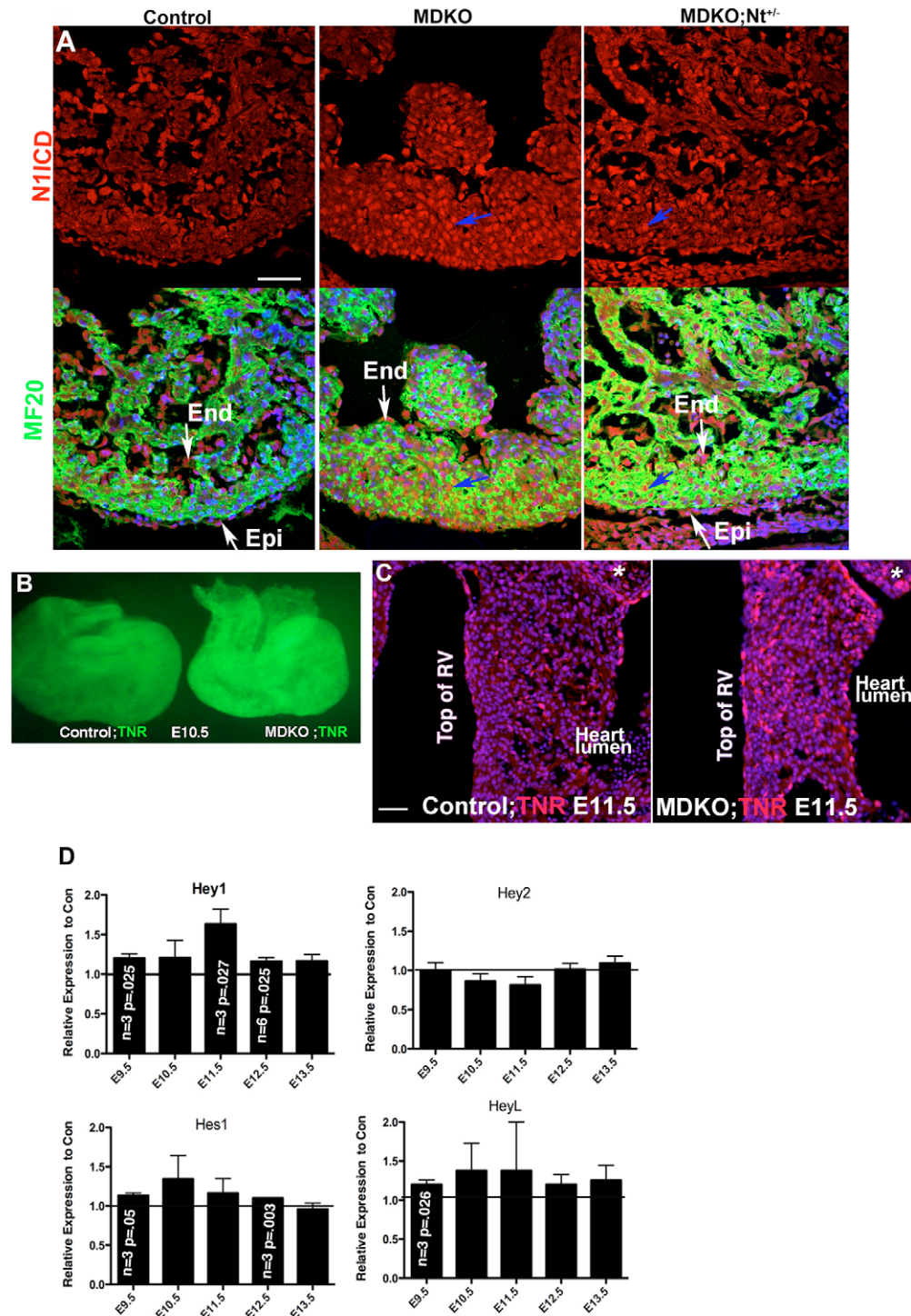
(A) *SM22-Cre<sup>Tg/0</sup>; Numb<sup>fl/fl</sup>; Numbl<sup>fl/fl</sup>* (SDKO) displayed trabeculation defects, showing thicker trabeculae and slight fewer number of trabeculae per unit length. The endocardial cell double knockout (EnDKO) (*Tie2-Cre<sup>Tg/0</sup>; Numb<sup>fl/fl</sup>; Numbl<sup>fl/fl</sup>*) displayed normal trabeculation and morphogenesis. (B) Shows the quantification of the thickness of trabeculae and the number of trabeculae per unit length in control and SDKO hearts at E14.5. Error bars indicate s.d. ( $n=5$ ). (C&D) *Wnt1-Cre* mediated NFPs knockout displayed a cranial facial defect, but no cardiac morphogenesis defect. (E) *Mef2c-Cre*-mediated NFPs knockout displayed an OFT alignment defect. Scale bars in A, C, D are 100  $\mu$ m.





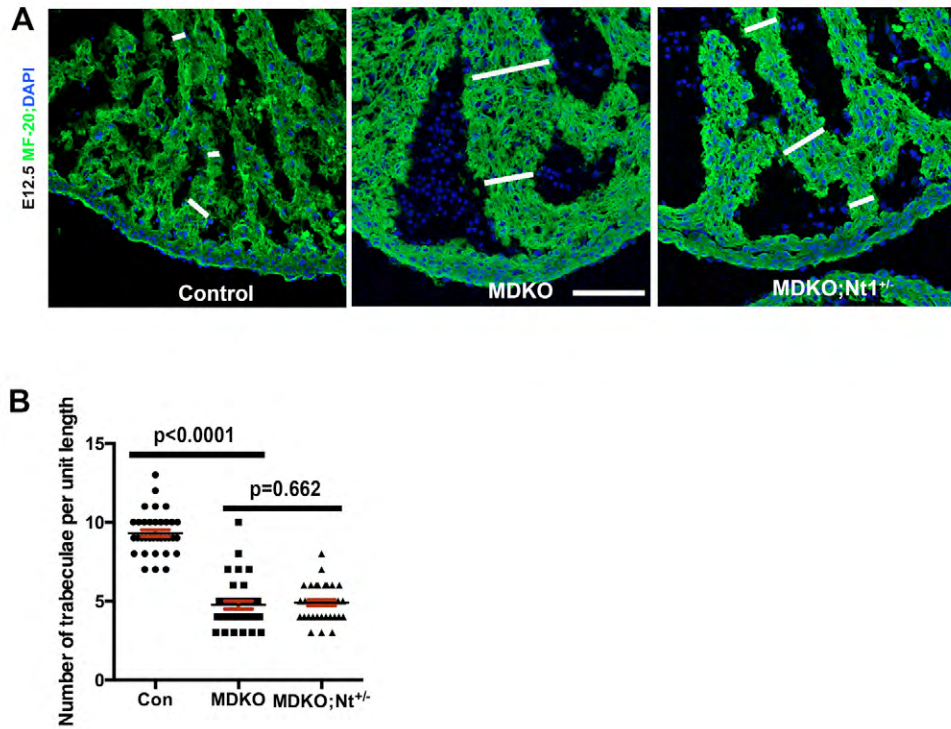
**Figure S3. MDKO hearts did not display chamber specification defects.**

(A) Expression of *ANP* and *Hand1* in MDKO hearts at E10.5 and E11.5 hearts appears normal based on In situ hybridization. Scale bar in A is 200  $\mu$ m.



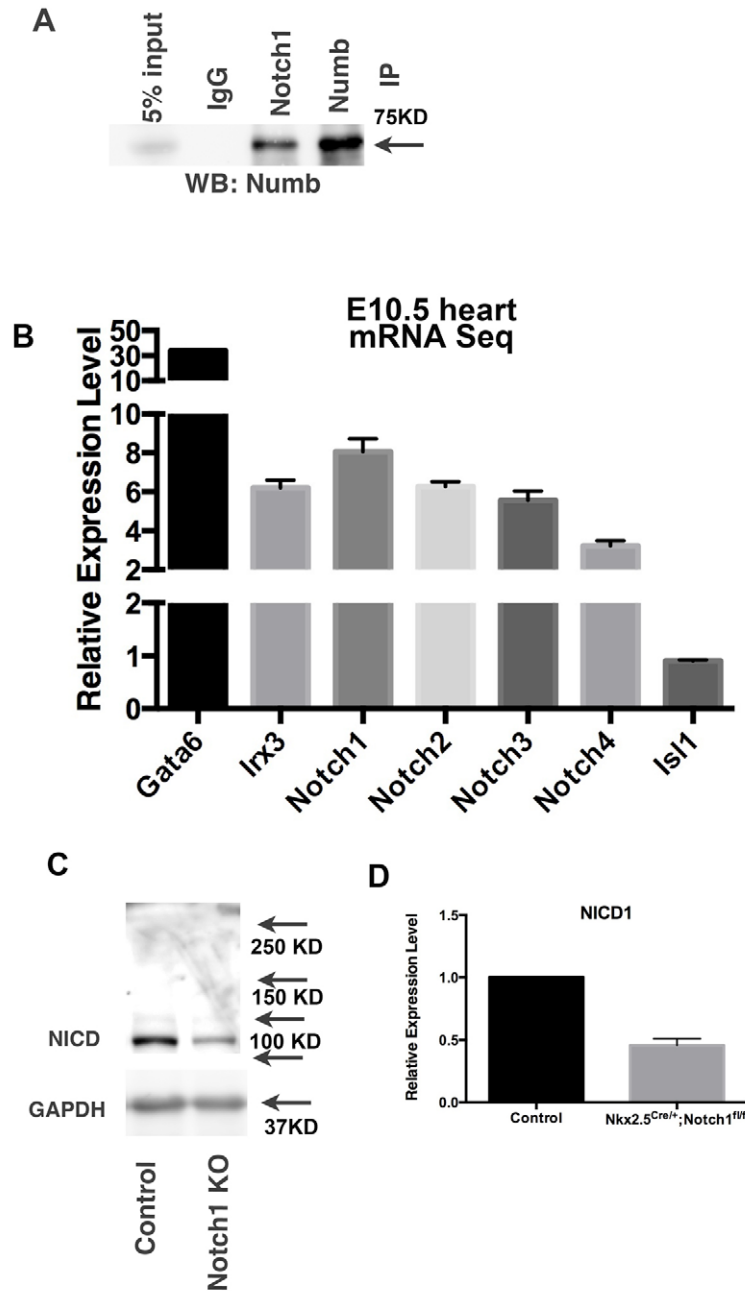
**Figure S4. MDKO displayed CBF1 dependent and independent Notch signaling activation.**

(A) Control hearts showed NICD1 positive staining in the endocardial cells indicated by white arrows, while MDKO and MDKO; *Nt*<sup>-/-</sup> hearts additionally displayed NICD1 staining in cardiomyocytes as indicated by blue arrows. (B) Whole heart imaging showed that Notch signaling was up-regulated in MDKO based on the GFP staining of MDKO; TNR heart. (C) MDKO hearts display more TNR positive cells (GFP+) compared to control, but not all cells are TNR positive. (D) Expression of *Hey1* and *Hes1* in MDKO is 1.1 to 1.7-fold higher than control based on Q-PCR at certain ages. Error bars indicate s.d.; *n* values are shown within each bar.



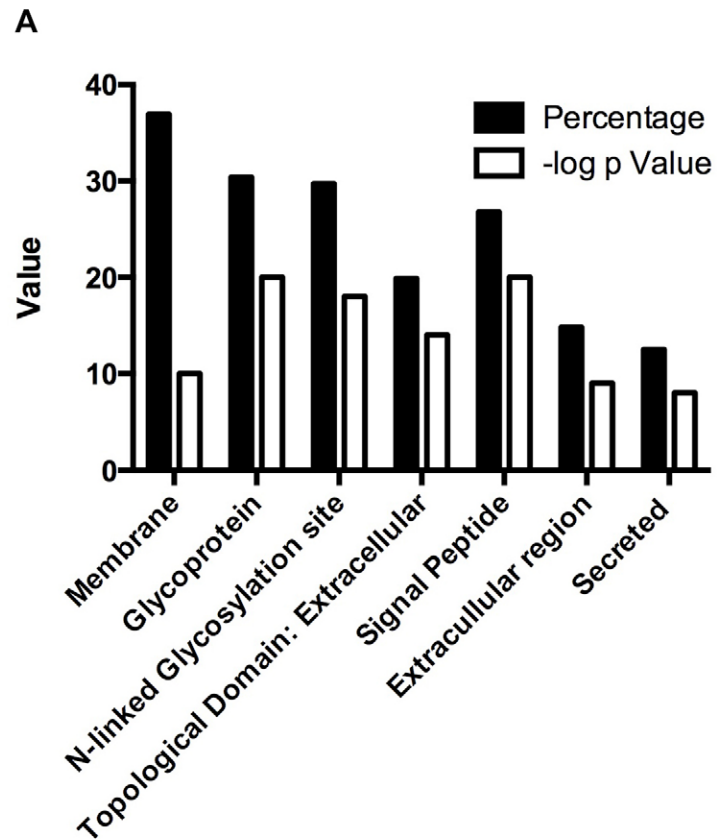
**Figure S5. Notch1 suppression decreased the thickness of trabeculae, but not the number of trabeculae per unit length in MDKO.**

(A) *MDKO; Nt1<sup>-/-</sup>* displayed less thick trabecula compared to MDKO. (B) Notch1 suppression in MDKO did not increase the number of trabeculae per unit length. Error bars indicate s.d. ( $n=5$ ). Scale bar in A is 100  $\mu$ m.



**Figure S6. Notch expression in the embryonic heart.**

(A) Immunoprecipitation shows that both Numb and Notch1 can pull down Numb from lysate of the heart, in which NFP in endocardial/endothelial cells were deleted via *Nfatc1-Cre*. This indicates that Numb and *Notch1* in cardiomyocytes can bind each other. (B) shows the relative expression of *Gata6*, *Irx3*, *Isl1* and all four Notch receptors based on mRNA deep sequencing using E10.5 hearts. (C&D) *Nkx2.5<sup>Cre/+</sup>*-mediated-*Notch1* deletion reduced the NICD1 to 46% of the control hearts based on Western blot using lysates of E12.5 ventricles. This indicates that the Notch1 is expressed in cardiomyocytes, considering that *Nkx2.5<sup>Cre/+</sup>* is active in very few endocardial cells in the ventricles.



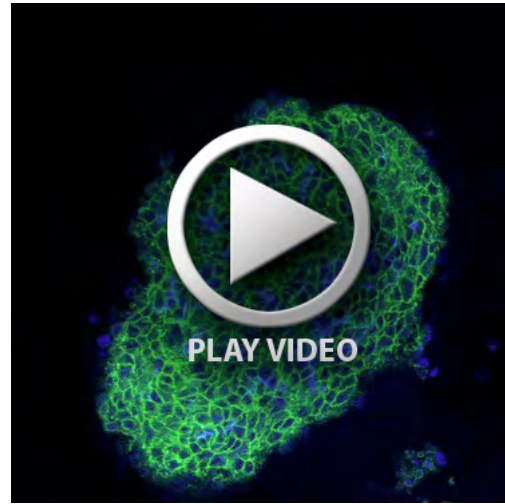
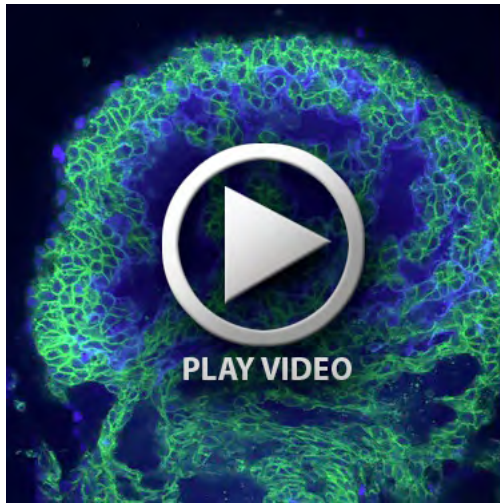
**Figure S7. NFPs are involved in multiple signaling pathways during cardiac development.**

(A) The percentage of each category of genes that are dysregulated in the MDKO and their  $-\log(p)$  values are shown. This indicates that NFPs regulate multiple signaling pathways during cardiac morphogenesis.



**Movie 1.** E10.5 *Nkx2.5<sup>Cre/+</sup>; mTmG* whole hearts were stained with PECAM (blue) and 3D imaged. The movie shows a Z stack of an E10.5 heart with 3  $\mu\text{m}$  per section. This movie indicates that very few endocardial cells of the ventricles are Cre active.





**Movies 2 and 3.** E10.5 *Nkx2.5<sup>Cre/+</sup>; mTmG* and *MDKO; mTmG* whole hearts were stained with PECAM (blue) and 3D imaged. The movie shows a Z stack of an E10.5 heart with 3  $\mu$ m per section. The movies indicate that the trabecula of MDKO is thicker than that of the control.



**Movies 4 and 5.** *Mef2c-Cre*-mediated-NFP deletion failed to form Dorsal Mesenchymal Protrusion (DMP) and displayed AVSD. E13.5 *Mef2c-Cre; mTmG* control and knockout hearts were stained with PECAM (Blue) and then 3D imaged. Movies show the Z stack of *Mef2c-Cre* labeled control (Movie 2) and NFPs-null cells (Movie 3) around the DMP region with 3  $\mu$ m per section. One section of Movie 4/5 is shown in Fig. 3C/D. The genotypes are *Mef2c-Cre; Numb<sup>fl/+</sup>; Numb<sup>fl/+</sup>; mTmG* for Movie 4 and *Mef2c-Cre; Numb<sup>fl/fl</sup>; Numb<sup>fl/fl</sup>; mTmG* for Movie 5

**Table S1. The results of mRNA deep sequencing.** RNA-Seq data of Con vs MDKO at E10.5 including all genes with p-value  $\leq 0.05$  are listed (15273 genes in total). This experiment was repeated three times and the data of the average of the three were shown. The ratio of MDKO:WT with p-value is presented.

[Download Table S1](#)

**Table S2. The results of mRNA deep sequencing.** Genes whose transcriptional levels were significantly changed are listed. Genes highlighted in red are downregulated, whereas genes highlighted in blue are upregulated.

[Download Table S2](#)



**Table S3. Primers used for Q-PCR.** Both *Hey1* and *Isl1* used two sets of primers.

Gene	Forward	Reverse
<i>Nkx2.5</i>	GACGTAGCCTGGTGTCTCG	GTGTGGAATCCGTCGAAAGT
<i>Hey2</i>	GTGGGGAGCGAGAACAATTA	GTTGTCGGTGAATTGGACCT
<i>Hey1</i>	ACGACATCGTCCCAGGTTTTG	GGTGATCCACAGTCATCTGCAAG
<i>Hey1</i>	CATGAAGAGAGCTCACCCAGA	CGCCGAACTCAAGTTTCC
<i>Hes1</i>	ACACCGGACAAACCAAAGAC	CGCCTCTTCTCCATGATAGG
<i>HeyL</i>	CTGAATTGCGACGATTGGT	GCAAGACCTCAGCTTTCTCC
<i>Hes5</i>	CCAAGGAGAAAAACCGACTG	TGCTCTATGCTGCTGTTGATG
cyclophilin	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT
<i>Jag1</i>	GAGGCGTCTCTGAAAAACA	ACCCAAGCCACTGTTAAGACA
<i>Wnt2</i>	CCTGATGAACCTTCACAACAAC	TCTTGTTTCAAGAAGCGCTTTAC
<i>Tanni2</i>	CAGGATGGGAGATGAGGAGA	TCTGGAGCATCACACTCTTCAG
<i>Gata4</i>	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC
<i>Gata5</i>	CCTTCGACAGCAGCATCC	TCCTCCAAGAAGTCAGGTACG
<i>Gata6</i>	GGTCTCTACAGCAAGATGAATGG	TGGCACAGGACAGTCCAAG
<i>Isl1</i>	AGCAACCCAACGACAAAAC	CCATCATGTCTCTCCGGACT
<i>Isl1</i>	CCACAAGCAGCCGGAGAAGAC	GAGGGTTGGCGGCATAGCAG
<i>Tbx2</i>	GAACGGCCGTCGGGAGAAAAG	TGGGGGAGGGCGGTGGTT
<i>Tbx1</i>	TTTGTGCCCGTAGATGACAA	CTCGGCCAGGTGTAGCAG
<i>Tbx3</i>	TTGCAAAGGGTTTTTCGAGAC	TGCAGTGTGAGCTGCTTTCT
<i>Tbx5</i>	CGAAGTGGGCACAGAGATG	CACCTTCACTTTGTAAGTAGGAAACA
<i>Hand1</i>	CAAGCGGAAAAGGGAGTTG	GTGCGCCCTTAATCCTCTT
<i>Hand2</i>	GGAGAAGAGGAAGAAAGAGCTGA	ATGAGGCCCTACTGCTTGAG
<i>Mef2c</i>	TCTGCCCTCAGTCAGTTGG	CGTGGTGTGTTGTGGGTATC
<i>Myh7</i>	CGCATCAAGGAGCTCACC	CTGCAGCCGCAGTAGGTT
<i>Myh6</i>	CGCATCAAGGAGCTCACC	CCTGCAGCCGCATTAAGT
<i>Vcam1</i>	TGGTGAAATGGAATCTGAACC	CCCAGATGGTGGTTTCCTT
<i>Numb</i>	CCACATCAGTGGCAGACAGA	TTCTACGTGGCCGAGGTACT
cyclin D1	TCTTTCCAGAGTCATCAAGTGTG	GACTCCAGAAGGGCTTCAATC
<i>Numb1</i>	CTGCAGCTTCCCTGTTAGGT	TCTTCACAAACGTGCATTCC
<i>Bmp10</i>	TGGTGAGGGATAGACACATTG	CGGAGCTTCAAGAACGAAGA
<i>Irx5</i>	ACAGAAGCCCAGGACAAG	AAAATCCGAGTCGCTGAGG
<i>Irx3</i>	AAAAGTTACTCAAGACAGCTTTCCA	CGATTTAAAAATGGTTGAAAAGTTAAG
<i>Hop</i>	ACCACGCTGTGCCTCATC	GCGCTGCTTAAACCATTTCT

<i>p57</i>	CAGGACGAGAATCAAGAGCA	GCTTGGCGAAGAAGTCGT
<i>ANF</i>	CACAGATCTGATGGATTTCAAGA	CCTCATCTTCTACCGGCATC
<i>Irx4</i>	AGGGCTATGGCAACTACGTG	CTTGGACTCGAAGCTGTTCA
<i>FoxH1</i>	TTCCTCTAATCGGTGCTTCC	AGCAGGAATCAGGCTCACAT
<i>Cxcl12</i>	CTGTGCCCTTCAGATTGTTG	TAATTTCGGGTCAATGCACA
<i>Nrg1</i>	GTTAGGAAACGACAGTGCCTCT	TTCAGTTGAGGCTGACATGC