Supplementary Information

Detailed Materials and Methods

Animal experiments
All animal experiments were in accordance to protocols approved by the Institutional Animal Care and Use Committee.

Explant culture
Pregnant females received 10 mg tamoxifen by gavage, 12 hours before sacrifice, to induce recombination in $Wt1^{CreERT2/+}$; $R26^{mTmG}$ embryos. Embryos were collected at E11.5. The head was removed, as well as the caudal trunk right below the liver. The chest was opened to expose the heart and the embryos placed in supine position in single wells of a 24 well dish. One milliliter DMEM medium, supplemented with 20% FCS and antibiotic-antimycotic (Invitrogen). Wnt inhibitor was used at 1 ug/ml recombinant human sFRP1, 1 ug/ml recombinant mouse sFRP2, or 100 ng/ml recombinant mouse DKK1 (all R&D Systems), was added to the embryos. The samples were incubated for 48 hours at 37˚C, 5% CO_2, then fixed in PFA and embedded in OCT.

Tamoxifen administration
Tamoxifen was dissolved 12 mg/ml in sunflower seed oil by sonication. At the timepoints indicated, 0.12 mg tamoxifen per g bodyweight was given to dams by gavage.

Retinoic acid administration
Pregnant females received 2.5 ug/g body weight all trans retinoic acid (ATRA), dissolved in DMSO at 25 g/ml. ATRA was given daily by gavage feeding from E10.5 to E13.5. Embryos were collected at E13.5 and processed for immunohistochemical analysis.

FACS Sorting
Embryos were collected at 13.5 embryonic days. Ventricles were dissected free of the atrium and atrioventricular canal and transferred to HBSS until all littermate samples were collected. Remaining tissue was used for genotyping. Each single heart was dissociated separately in individual round bottom tubes placed in a Thermomixer (Eppendorf) set to 300 rpm, 37°C. We used the collagenase enzyme mix and other buffers provided in the Neomyts isolation kit (Cellutron), in accordance to the provided protocol. In a brief: Buffer D2 plus EC was prepared as suggested, added to the dissected ventricles and replaced every 10 minutes. The buffer containing the released cells was transferred to a polypropylene FACS tube containing 500 µl buffer D3 to inactivate the enzyme. Digest was facilitated by passing the sample each time through a 200 ul tip. After complete dissociation of the sample, we centrifuged the collected cells at 200 g for 5 minutes, resuspended in 200 µl buffer D3 and placed the samples on ice. FACS was performed on a MoFlo cell sorter. Gates were set using a GFP negative sample so that contamination by GFP

negative cells was less than 0.1%. The yield was 2 – 5 * 10^3 cells per heart sample. GFP positive and negative cells were collected in 350 µl RLT buffer (Qiagen) to protect RNA from degradation. Samples were stored at – 80°C.

RNA preparation and amplification

We used the Qiagen RNeasy micro kit according to the manufacturer's protocol to extract total RNA from FAC sorted samples protected in RLT buffer (Qiagen). Genomic DNA was eliminated by on column DNase digest with RNase free DNase (Qiagen) as outlined in the company's protocol. High quality of RNA samples was confirmed on an Agilent Bioanalyzer prior to reverse transcription or RNA amplification.

RNA was amplified using the NuGEN PicoSL WTA System. The manufacturers protocol was strictly followed. Total RNA input of 1 – 4 ng yielded 3 – 4 µg of amplified single stranded cDNA, which was diluted 1:100 before analysis by qPCR.

Quantitative Reverse Transcription PCR

RNA was transcribed with the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) using random and oligo DT primers, in accordance to the product manual. Transcript of 500 ng total RNA input was used for 4 subsequent qPCR reactions.

QPCR was performed on a ABI 7300 real time PCR system with either Sybr green or Taqman qPCR master mixes (ABI). Primers were validated to yield efficient amplification and a single peak in the melting curve, consistent with a single predominant PCR product. The primer sequences are given in Supplementary Table 2. Relative gene expression was calculated with the ΔΔCt method and normalized to control and *Gapdh.*

Histology

Dams were euthanized at the timepoints indicated. Embryos were collected and fixed in 4% PFA for 4 hours at 4°C, followed by a washing step in PBS for 1 hour. For H&E staining, samples were dehydrated in an ethanol series, then xylene, then embedded in paraffin. 5-10 um sections were cut and stained with hematoxylin and eosin.

For immunofluorescence and immunohistochemical staining, fixed embryos were incubated in 30% sucrose overnight at 4°C, equilibrated in OCT for one hour and then frozen in 2-butylbutane cooled in dry ice. Cryopreserved tissue was cut with a cryotome in 5 – 10 um thick sections, air dried for 30 minutes, and stored at -20°C until further use. For staining, we washed and permeabilzed frozen sections in PBS + 0.1% Triton-X100 (PBST) for 10 minutes and proceeded with staining according to the application.

Sections derived from $R26^{mTmG}$ mice were stained for the specific antigen and subsequently visualized by a far red fluorophor. For applications requiring immunostaining in the green or red channels fluorescent proteins were bleached by incubation with 3% H2O2 in methanol on a fluorescent light box for four hours to overnight at 4°C. Effective bleaching was confirmed under a fluorescent microscope before staining was carried out.

Immunofluorescent (IF) staining

Permeabilzed sections were blocked for non-specific antibody binding with PBST + 5% normal donkey serum (PBSST), 1 hour at room temperature. Primary antibodies and the working concentrations are indicated in Supplementary Table 1. Primary antibodies were incubated with tissue sections overnight at 4°C. Sections were washed 3 times 10 minutes prior to incubation with secondary antibody. All secondary antibodies conjugated to Alexa fluorophores (488, 555, 647) were derived in donkey (Invitrogen) and applied 1:250 or 1:1000 (total Ctnnb1) in PBSST, 2 hours at room temperature. Sections were washed 3 times 10 minutes, counterstained with DAPI (Invitrogen) and mounted in Vectashield (Vector Labs).

Staining for non-phosphorylated CTNNB1 (Millipore) was performed using the MOM kit (Vector Labs) according to the manufacturer's instructions. Slides were washed 3 times 10 minutes, counterstained with DAPI and mounted with Vectashield (IF) or directly mounted in glycerol (IHC).

Immunohistochemistry (IHC) and signal amplification

In case an enzymatic detection system was used, endogenous enzyme activity was quenched with 3% H2O2 in PBS, 5 minutes at room temperature, prior to the blocking step. Primary antibody incubation was performed as described above followed by application of SuperPicture (Invitrogen) secondary antibodies, according to manufacturers recommendation. Signal was developed using the DAB kit (Vector Labs), following the kit instructions, or the TSA kit (Perkin Elmer) at a substrate concentration of 1:200, 5 minutes at room temperature.

Supplementary Tables

Supplementary Figures

Suppl. Table 1. Primers used in this study.

gene	forward	reverse
Wt1	*ATCCGCAACCAAGGATACAG*	*GGTCCTCGTGTTTGAAGGAA*
Aldh1a2	*GGCACTGTGTGGATCAACTG*	*TCACTTCTGTGTACGCCTGC*
Ctnnb1	*CGACTAAGCAGGAAGGGATG*	*TGGCACACCATCATCTTGTT*
Lef1	*Mm00550265_m1 ABI predesigned Taqman primers/probe*	
sFrp1	*CCGTGTGTCCTCCATGCGACA*	*TGGGCCCCAGCTTCAAGGGT*
sFrp2	*CCGGTCATGTCCGCCTTCGG*	*TGCAGGCTTCACACACCTTGGG*
Axin2	*GCGTGGCCAGTCAGCAGAGG*	*CCTGGAGCGCGTGGACACTT*
CyclinD1	*GCAAGGCCTGAACCTGGGCA*	*CGACGTTCTGCTGGGCCTGG*
CyclinD2	*GAACCTGGCCGCAGTCACCC*	*CGACGGCGGGTACATGGCAA*
Wnt5a	*AATTCCTCGGCCGCCTTCGC*	*GCGGTCCCCAAAGCCACTCC*
Snail	*CGTGTGTGGAGTTCACCTTC*	*GGAGAGAGTCCCAGATGAGG*
Slug	*CACATTCGAACCCACACATT*	*TATTGCAGTGAGGGCAAGAG*
Gapdh	*ACAACTTTGGCATTGTGGAA*	*GATGCAGGGATGATGTTCTG*

Supplementary Table 2. Antibodies used in this study.

antibody	company	catalog #	dilution	amplification*	notes
Wt1	Calbiochem	CA1026	1:100	multi-HRP	antigen retrieval
β-catenin	Cell Signaling	9562	1:500	no	
β-catenin (ph-S657)	Cell Signaling	4176	1:500	no	
β-catenin (anti-ABC)	Millipore	05-665	1:500	no	Mouse on mouse kit (Vector)
Snail	Cell Signaling	C15D3		no	
Slug	Cell Signaling	C19G7		no	
Aldh1a2	Sigma	HPA010022		multi-HRP	
TNNT2	Thermo Scientific	MS-295-P1			
phospho-Histone H3	Upstate	06-570		no	
CD-31 (Pecam)	BD Pharmingen	553371	1:250	ABC	
GFP-Alexa488	Invitrogen	A21311	1:100	no	
E-cadherin	Cell Signaling	3195		no	

*Amplification was performed using multimerized HRP or the Vector ABC kit, and detected with tyramide-conjugated fluorophore.

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Aldh1a2	*GGCACTGTGTGGATCAACTG*	*TCACTTCTGTGTACGCCTGC*
Ctnnb1	*CGACTAAGCAGGAAGGGATG*	*TGGCACACCATCATCTTGTT*
Lef1	*Mm00550265_m1 ABI predesigned Taqman primers/probe*	
sFrp1	*CCGTGTGTCCTCCATGCGACA*	*TGGGCCCCAGCTTCAAGGGT*
sFrp2	*CCGGTCATGTCCGCCTTCGG*	*TGCAGGCTTCACACACCTTGGG*
Axin2	*GCGTGGCCAGTCAGCAGAGG*	*CCTGGAGCGCGTGGACACTT*
CyclinD1	*GCAAGGCCTGAACCTGGGCA*	*CGACGTTCTGCTGGGCCTGG*
CyclinD2	*GAACCTGGCCGCAGTCACCC*	*CGACGGCGGGTACATGGCAA*
Wnt5a	*AATTCCTCGGCCGCCTTCGC*	*GCGGTCCCCAAAGCCACTCC*
Snail	*CGTGTGTGGAGTTCACCTTC*	*GGAGAGAGTCCCAGATGAGG*
Slug	*CACATTCGAACCCACACATT*	*TATTGCAGTGAGGGCAAGAG*
Gapdh	*ACAACTTTGGCATTGTGGAA*	*GATGCAGGGATGATGTTCTG*

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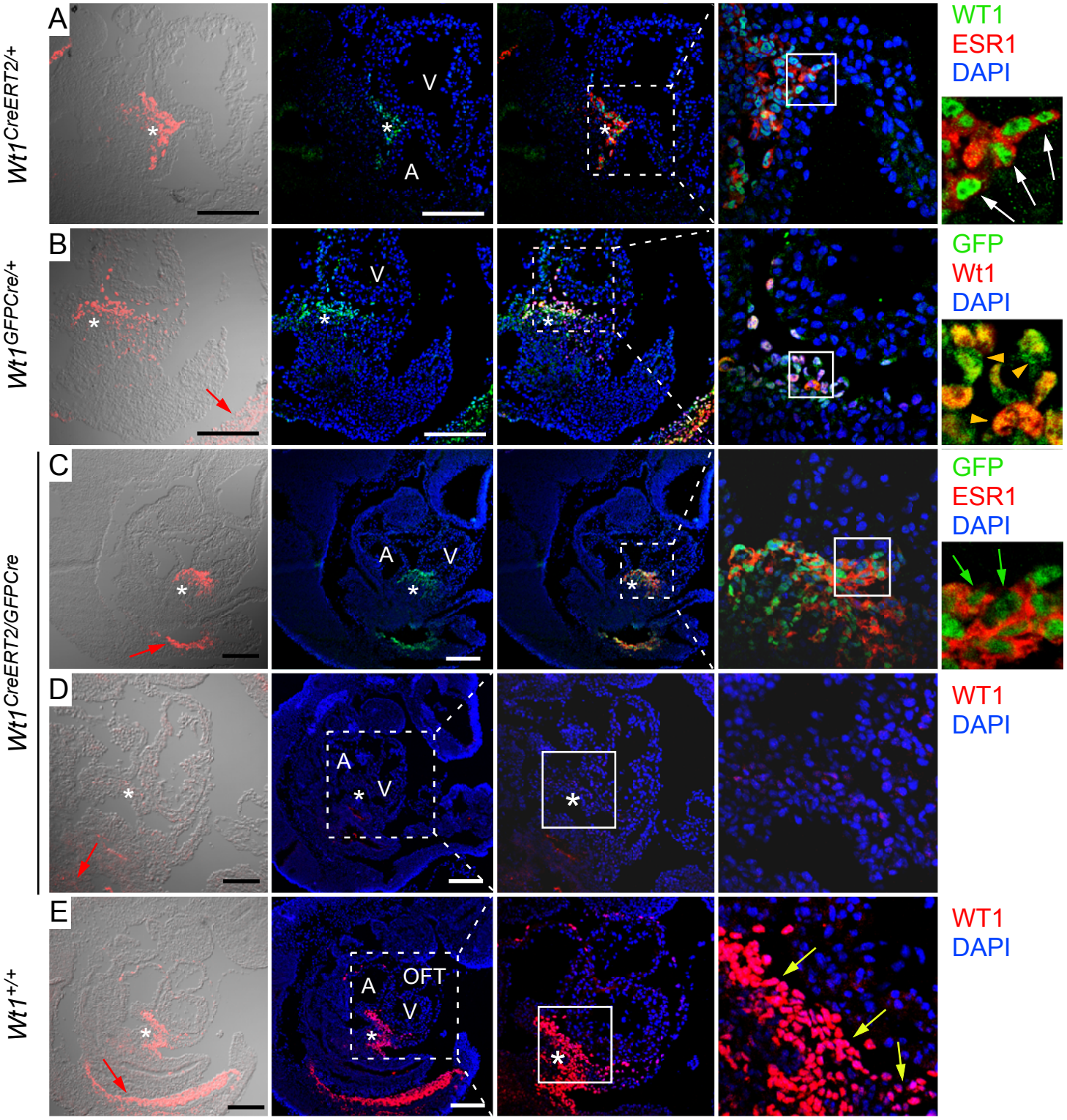
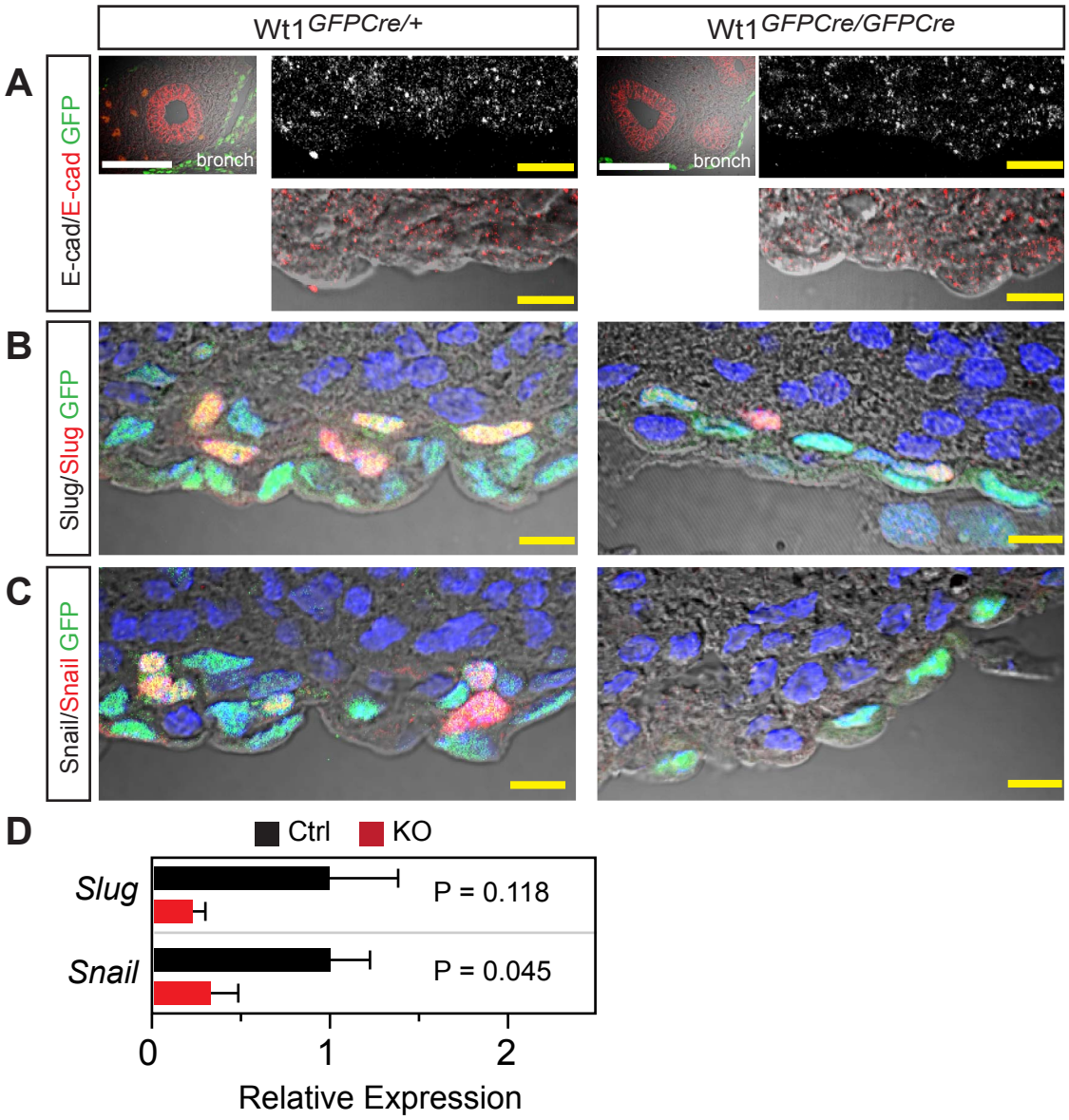
β-catenin (anti-ABC)	Millipore	05-665	1:500	no	Mouse on mouse kit (Vector)
Snail	Cell Signaling	C15D3		no	
Slug	Cell Signaling	C19G7		no	
Aldh1a2	Sigma	HPA010022		multi-HRP	
TNNT2	Thermo Scientific	MS-295-P1			
phospho-Histone H3	Upstate	06-570		no	
CD-31 (Pecam)	BD Pharmingen	553371	1:250	ABC	
GFP-Alexa488	Invitrogen	A21311	1:100	no	
E-cadherin	Cell Signaling	3195		no	

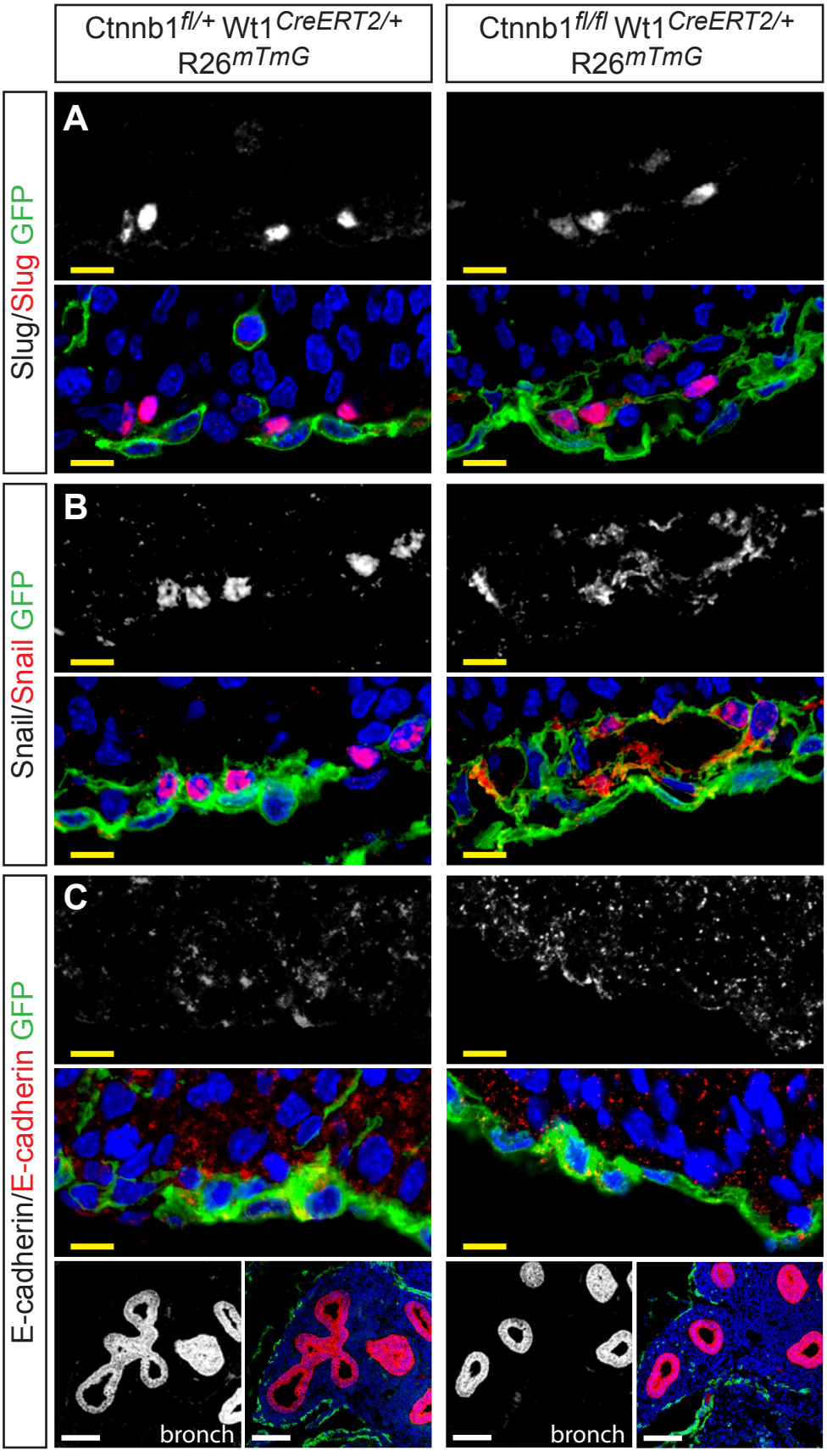
*Amplification was performed using multimerized HRP or the Vector ABC kit, and detected with tyramide-conjugated fluorophore.



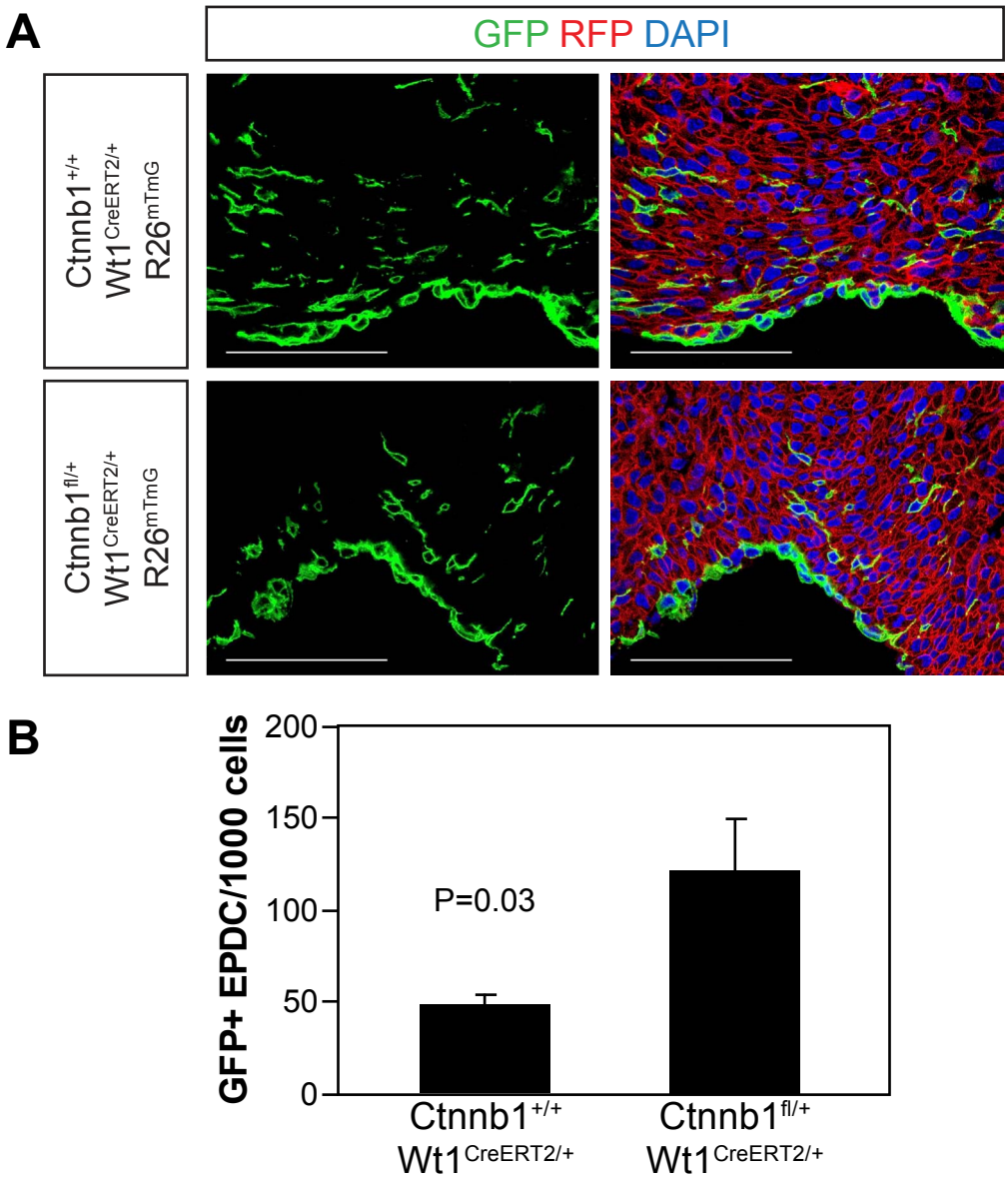
Suppl Fig. 1. $Wt1^{GFPCre}$ and $Wt1^{CreERT2}$ alleles are WT1 protein null. Sagittal sections of E9.5 embryos stained for WT1, GFP, and estrogen receptor 1 (ESR1). Asterisks (*) indicate proepicardium (PE), and red arrows indicate urogenital ridge (UR). **A**. In $Wt1^{CreERT2/+}$ embryos, estrogen receptor 1 (ESR1, red) was co-expressed in PE and UR cells with Wt1 (green). **B.** In $Wt1^{GFPCre/+}$ embryos, GFP-Cre fusion protein (green) was co-expressed in cells with WT1 protein (red) in PE and UR. **C-D.** In $Wt1^{CreERT2/GFPCre}$ embryos, GFPCre fusion protein (green) was co-expressed in cells with ESR1. These cells were negative for WT1 protein, demonstrating that both *GFPCre* and *CreERT2* are WT1 protein null. **E**. WT1 was readily detected in $Wt1^{+/+}$ wildtype embryos in PE and UR. Bar = 200 μm. A, atrium; V, ventricle; OFT, outflow tract. Each figure is a representative of 3 embryos in each genotype.



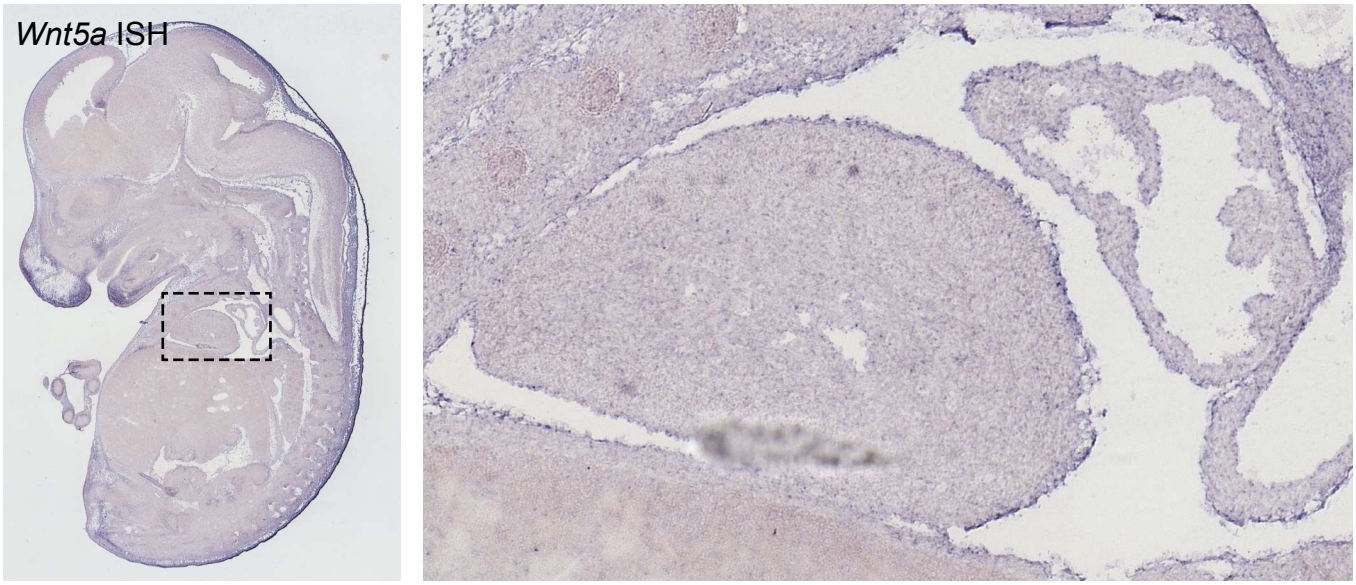
Supplemental Figure 2. Expression of Slug, Snail, and E-cadherin in control and Wt1^{KO} heart. Cryosections of E13.5 heart were immunostained for E-cadherin (A), Slug (B), or Snail (C). GFP was visualized by immunostaining. **A**. E-cadherin was not detected in epicardium but was robustly detected in bronchial epithelium (bronch). **B-C**. Snail and Slug were expressed in subepicardial mesenchymal cells, which were diminished in Wt1^{KO} compared to control. **D**. qRTPCR revealed reduced expression of Slug and Snail in GFP^{+} cells. E-cadherin was undetectable in either group. n=4-5 per group. Scale bars : 100 μm (white), 10 μm (yellow).



Supplemental Figure 3. Expression of Slug, Snail, and E-cadherin in epicardial β-catenin loss of function. Cryosections of E13.5 heart were immunostained for Slug (A), Snail (B), or E-cadherin (C). E-cadherin was not detected in epicardium but was robustly detected in bronchial epithelium (bronch). Scale bars: 100 μm (white), 10 μm (yellow).



Supplementary Figure 4. von Gise et al.
E13.5 Ctnnb1$^{+/+}$ Wt1$^{CreERT2/+}$ and Ctnnb1$^{fl/+}$ Wt1$^{CreERT2/+}$ hearts, treated with Tam at E10.5, were analyzed for GFP expression from the Cre-activated Rosa26^{mTmG} reporter. **A.** Representative cryosections, showing endogenous GFP fluorescence marking Cre-recombined cells, and endogenous RFP fluorescence, marking cells without Cre recombination. **B.** Quantitation of EMT frequency as GFP^{+} cells per 1000 cells within myocardium. An average of 2150 cells were counted in each of 3 hearts per group. The frequency of GFP^{+} cells was lower in all double heterozygous hearts compared to Wt1 heterozygous hearts. n=5 per group.



Supplementary Fig. 5. *Wnt5a* in situ hybridization demonstrated epicardial expression. Images are from www.genepaint.org.