SUPPLEMENTAL INFORMATION INVENTORY

Figure S1, related to Figures 1 and 2: *Rspo3* is not expressed by cardiac neural crest or epicardial derived cells in the heart and *Rspo3* is highly expressed in regions with secondary heart field progenitors at E9.5.

Figure S2, related to Figures 1 and 2: The $Myh6^{MerCreMer}$ line shows poor recombination in the heart when induced at E11.5 and variable recombination when induced at E8.5, coronary stem proliferation is decreased in MYHR3 mutants and deletion of *Rspo3* with the $CAGGCreER^{TM}$ line is very efficient.

Figure S3, related to Figure 3*: Lgr4* Is expressed in the myocardium and endothelial cells of hearts during embryonic development and adulthood.

Figure S4, Related to Figure 4: *Lgr4* coding sequence carrying a 5 base pair deletion produces a truncated protein.

Figure S5 related to Figure 4: Less severe *Lgr4* mutants do not display noticeable edema or cardiac defects and *Lgr4* mutants display decreased intestinal crypt cell proliferation.

Figure S6, Related to Figure 4: *Lgr4* mutant kidneys display dilated tubules and cyst formation.

Antibodies table: List of antibodies used in this study.

Primers table: Complete list of primers used for qPCR analysis in this study.

Supplemental Methods: Description of supplementary materials and methods not included in the main text.

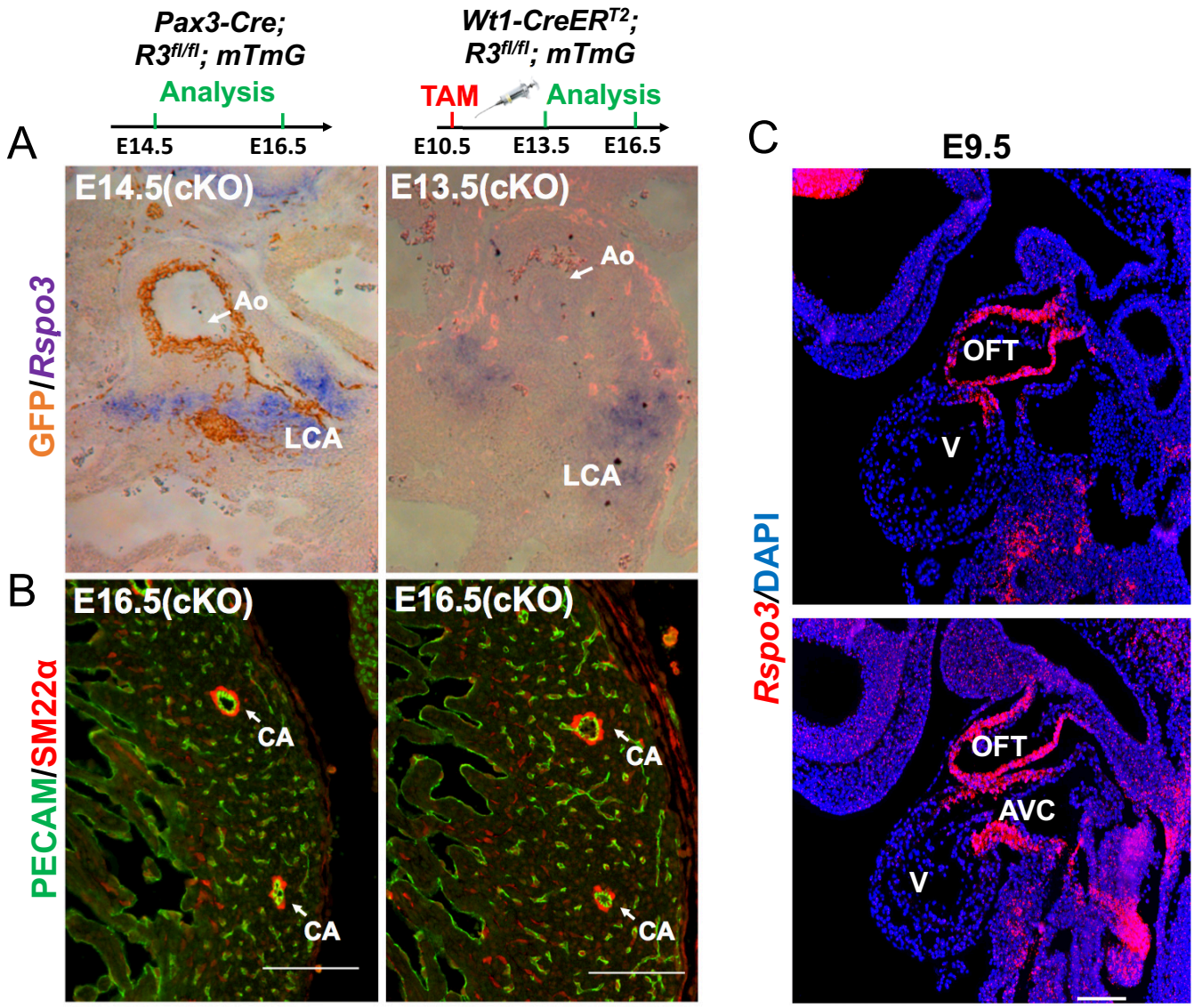

Fig. S1. *Rspo3* is not expressed by cardiac neural crest or epicardial derived cells in the heart and *Rspo3* is highly expressed in regions with secondary heart field progenitors at E9.5. (A) The *Pax3Cre* and *WT1CreER*^{T2} lines were crossed with the *mTmG* reporter allele to analyze their recombination rates in upper sections of the heart, where *Rspo3* is normally expressed. GFP immunostaining overlayed on *in situ* hybridization analysis of *Rspo3* mRNA levels demonstrates minimal co-expression of *Rspo3* with the cardiac neural crest or epicardial derived cells of the heart around the left coronary artery stem. (B) Coronary artery formation is not affected when *Rspo3* is deleted by the *Pax3Cre* and *WT1CreER*^{T2} lines, as demonstrated by PECAM/SM22α staining in the left ventricles of mutant hearts. (C) *RNAScope* analysis of *Rspo3* mRNA levels at E9.5 reveals high *Rspo3* expression in the outflow tract (OFT), the atrioventricular canal (AVC) and the ventricle (V). All scale bars: 100 μM.

Supplementary Figure 1

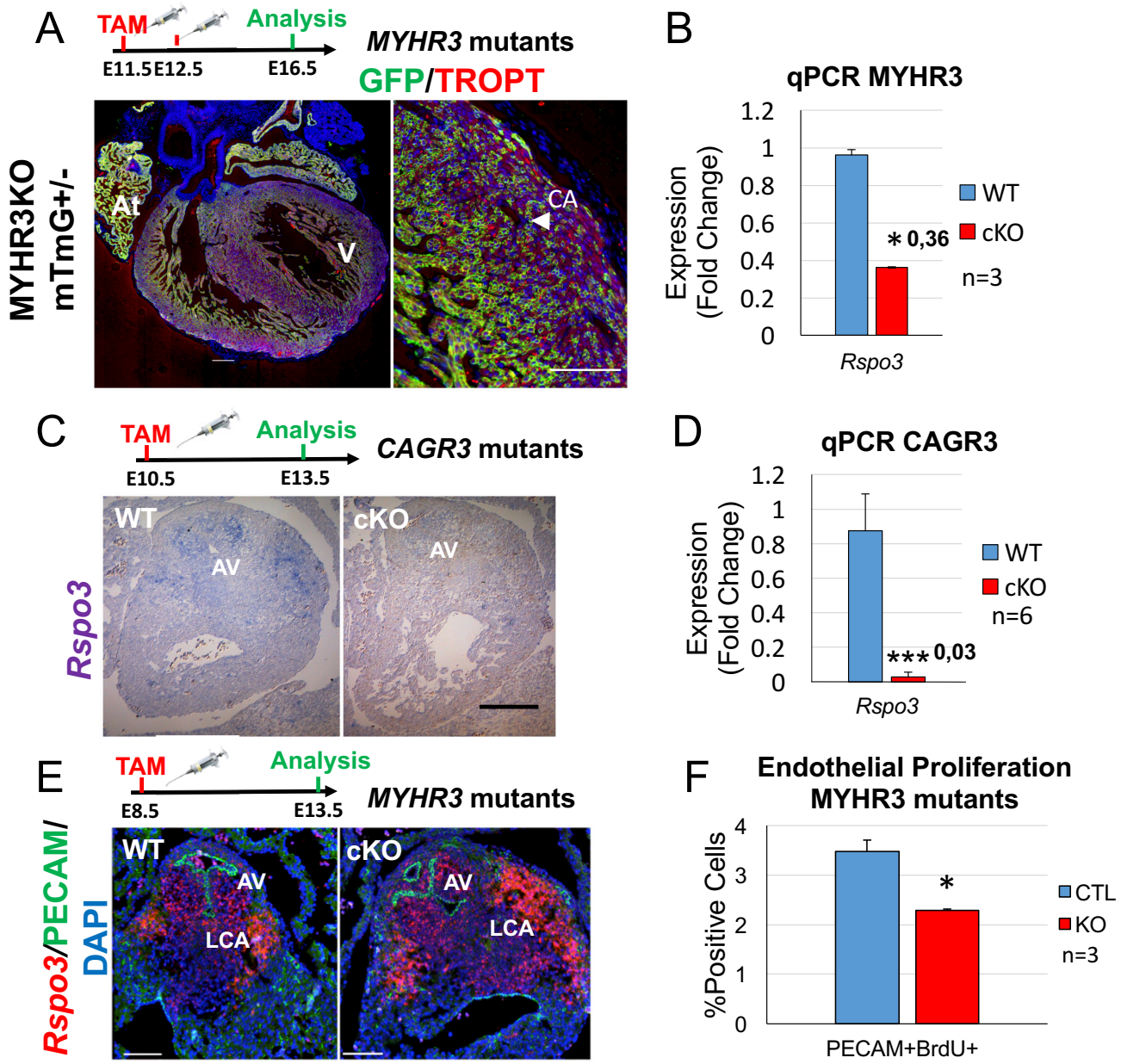

Fig. S2. The *Myh6*^{MerCreMer} line shows poor recombination in the heart when induced at E11.5 and variable recombination when deleted at E8.5, coronary stem proliferation is decreased in MYHR3 mutants and deletion of *Rspo3* with the *CAGGCreER*^{TM} line is very efficient. (A) *Rspo3* deletion at E11.5 followed by an extra pulse at E12.5 with the *Myh6*^{MerCreMer} line crossed with the *mTmG* reporter shows efficient recombination in the atria, but poor recombination in the compact myocardium of the ventricles as demonstrated by GFP and TroponinT (TROPT) co-immunostaining (3 hearts analyzed). (B) qPCR analysis of RNA extracted from the upper halves of MYHR3 mutants reveals only a 60% reduction in *Rspo3* expression levels. Data are expressed as fold change vs. controls and columns are means ± SEM. (C) The *CAGGCreER*^{TM} line (CAGR3 mutants) is very efficient as demonstrated by *in situ* hybridization analysis with an *Rspo3* probe. (D) qPCR analysis of RNA extracted from the upper halves of hearts demonstrates a 97% reduction in *Rspo3* expression in CAGR3 mutants. (E) Deletion of *Rspo3* with the *Myh6*^{MerCreMer} line at E8.5 leads to variable recombination rates with some mutants demonstrating persistence of high levels of *Rspo3* around the coronary stems. *Rspo3* mRNA levels were analyzed by RNAScope. (F) Quantification of endothelial proliferation around the developing left coronary artery demonstrates a significant reduction in MYHR3 mutants when compared to controls (3 mutants, 2 litters). Columns are means ± SEM. Data are expressed as fold change vs. controls and columns are means ± SEM. Scale bars: mosaics 200 µM, close ups 100 µM. For all statistical analyses the paired student t-test was used assuming unequal variances, *p<0.05, ***p<0.001. AV=aortic valve, LCA=left coronary artery, At=atrium, V=ventricle, CA=coronary artery.

Supplementary Figure 2

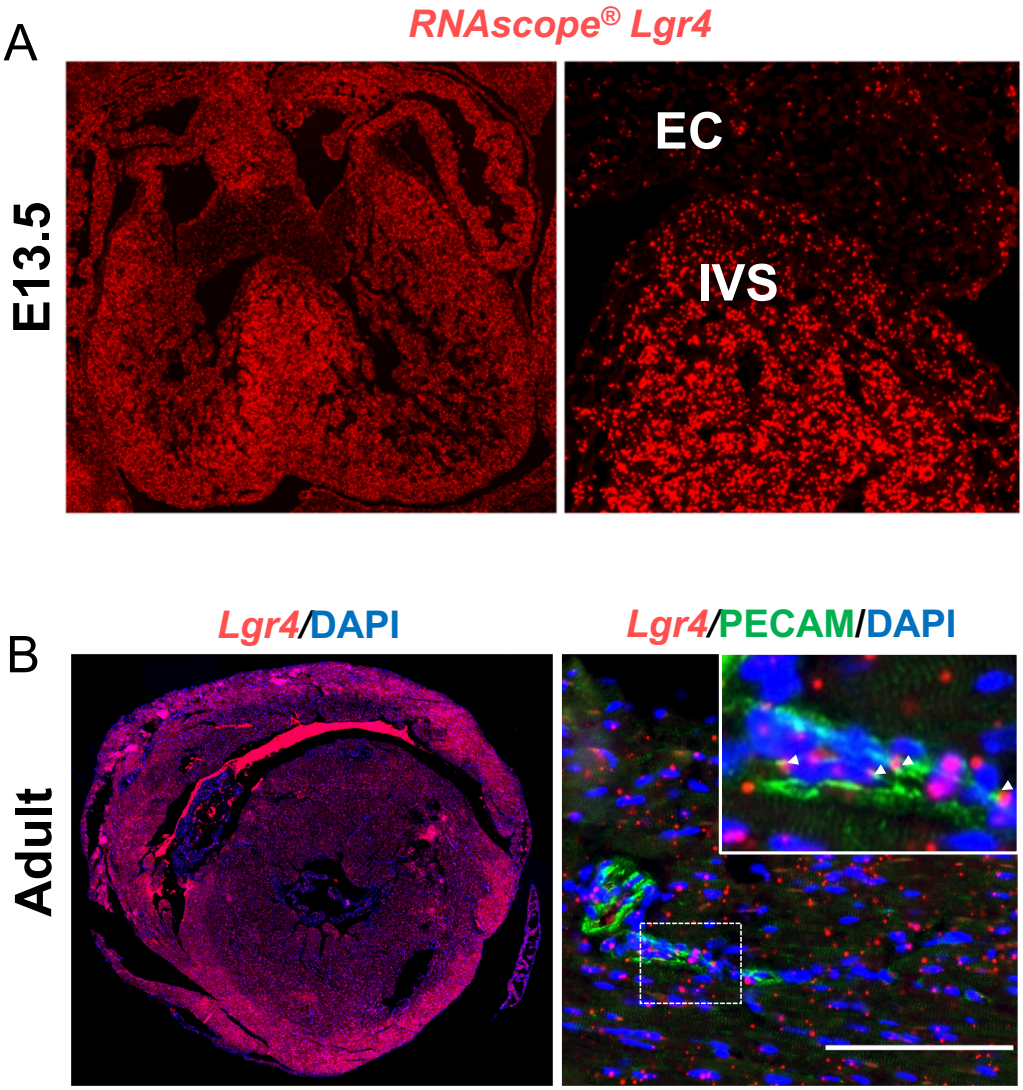

Fig. S3. *Lgr4* Is expressed in the myocardium and endothelial cells of hearts during embryonic development and adulthood (A) *RNAScope* analysis reveals *Lgr4* is specifically expressed in the myocardium of the interventricular septum. The endocardial cushions do not express *Lgr4* and serve as a negative control that demonstrate the specificity of the *Lgr4* probe and *RNAScope* analysis. (B) *RNAScope* analysis of adult hearts reveals *Lgr4* is expressed throughout the entire heart. Closer inspection reveals *Lgr4* is expressed by endothelial cells (PECAM-positive, white arrowheads, insets) in the adult heart (Scale bar 100 μM). EC=endocardial cushion, IVS=interventricular septum.

Supplementary Figure 3

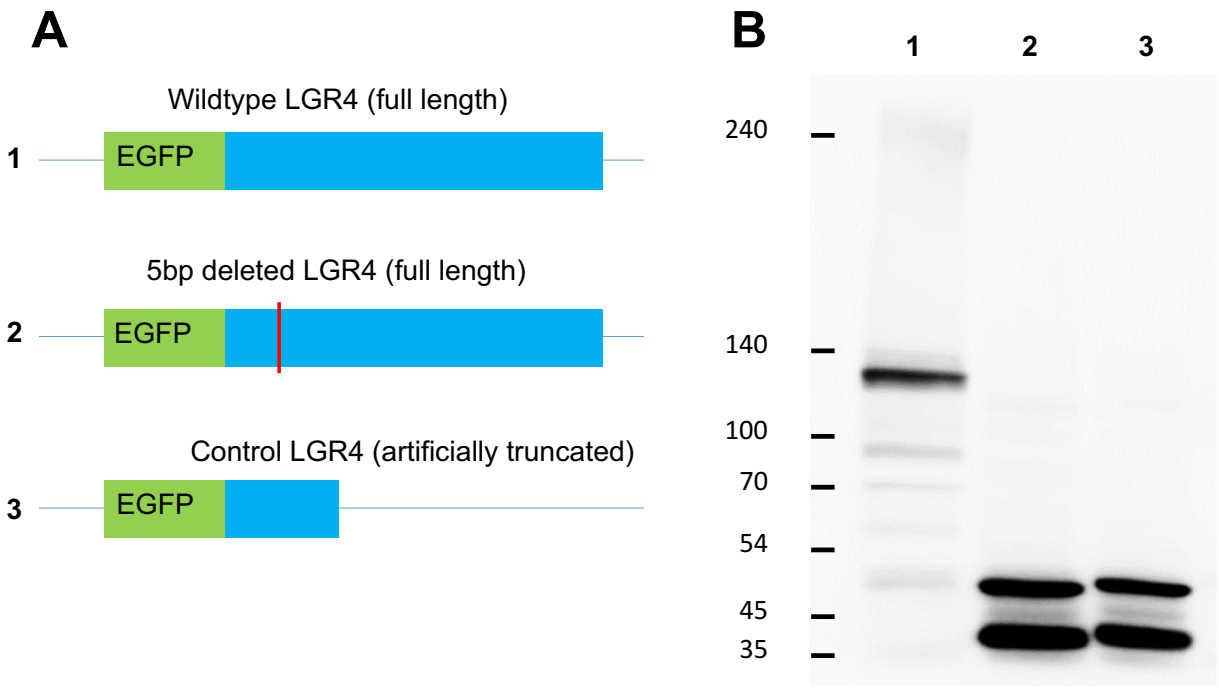

Fig. S4. LGR4 coding sequence carrying a 5 base pair-deletion produces a truncated protein. (A) Schematic representation of LGR4 expression constructs. Full length coding sequence was amplified from RNA isolated from wildtype or KO (5bp deleted) heart tissue and inserted, in frame with EGFP, into a pCDNA3 expression vector. A truncated LGR4 expression construct with a stop codon at the predicted truncation site was artificially generated by PCR as a control. **(B)** Western blot analysis on HEK129T cells transfected with wildtype, full length EGFP-LGR4 coding sequence (lane 1), EGFP-LGR4 sequence harboring the 5bp deletion (lane 2) and artificially truncated EGFP-LGR4 coding sequence (lane 3).The plasmid expressing the full-length coding sequence derived from LGR4 mutant embryos (lane 2) is unable to produce a full length protein compared to the wild type sequence. The size of the truncated protein is comparable with the expected size of the artificially shortened coding sequence.

Supplementary Figure 4

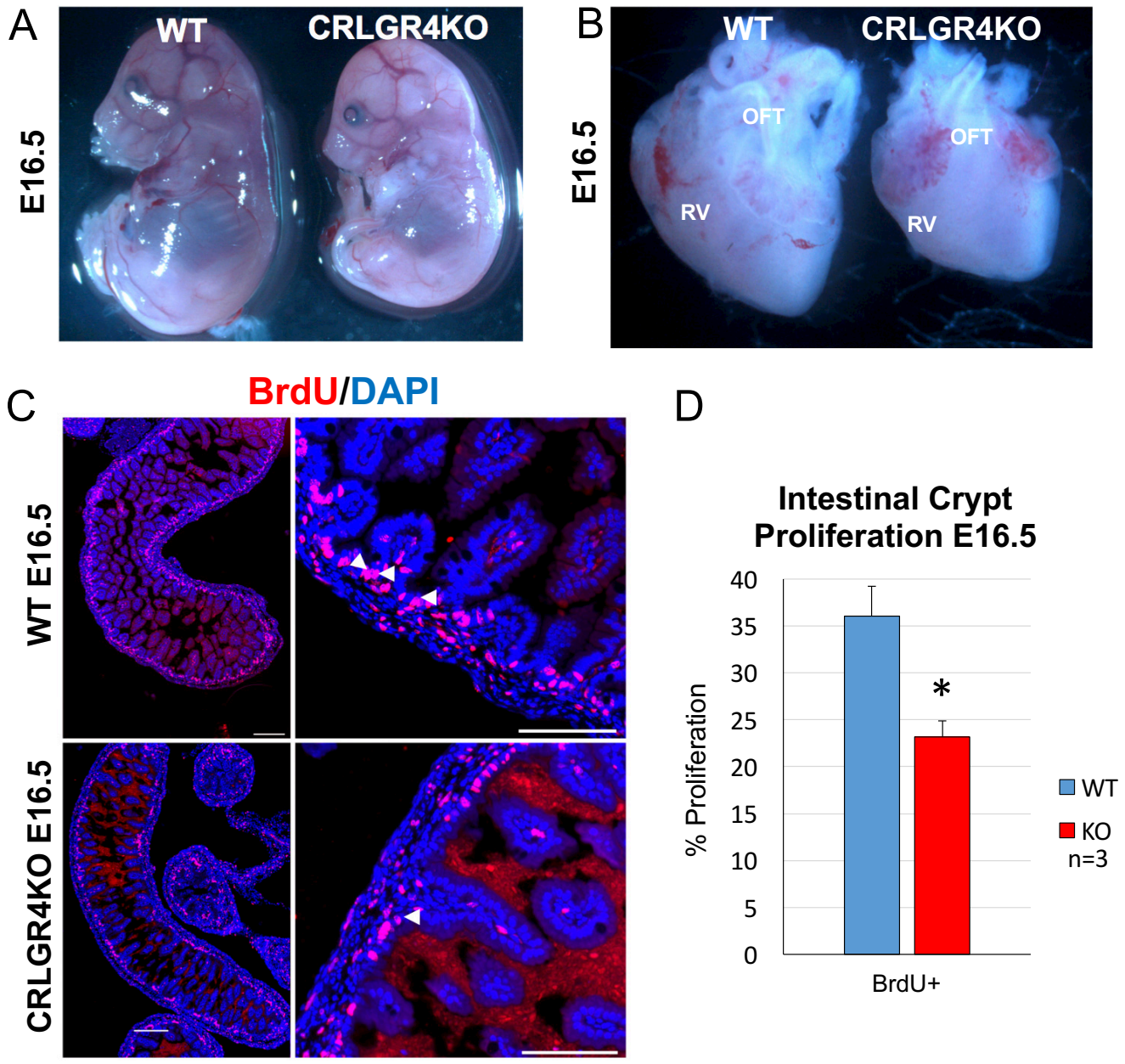

Fig. S5. Less severe *Lgr4* mutants do not display noticeable edema or cardiac defects and *Lgr4* mutants display decreased intestinal crypt cell proliferation (A) Analysis of CRLGR4 mutants reveals that many mutants are smaller, but do not display noticeable edema or haemorrhages. (B) Less severely affected CRLGR4 mutant hearts are smaller, but appear to have normal morphology. OFT=outflow tract, RV=right ventricle. (C) Immunostaining with an anti-BrdU antibody demonstrates decreased proliferation (white arrowheads) in the intestinal crypts of CRLGR4 mutant intestines at E16.5. (D) Quantification of intestinal crypt cell proliferation demonstrates a significant decrease in CRLGR4 mutants when compared to controls. Data are expressed as fold change vs. controls and columns are means ± SEM. For statistical analysis the paired student test was used assuming unequal variance, *p<0.05. Scale bars: mosaics 200 μM, close ups 100 μM.

Supplementary Figure 5

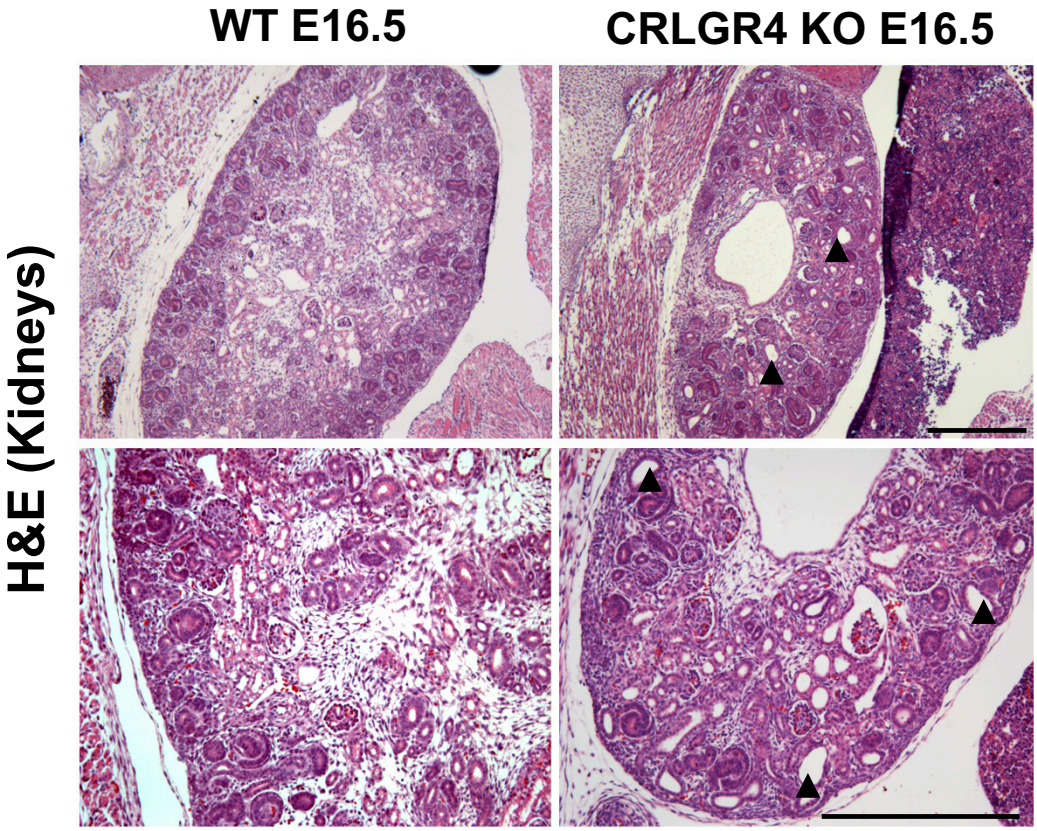

Fig. S6. *Lgr4* mutant kidneys display dilated tubules and cyst formation. H & E staining of kidney sections demonstrates the presence of various cysts and dilated tubules (black arrowheads) in CRLGR4 mutants. Scale bars 200 μM.

Supplementary Figure 6

SUPPLEMENTAL TABLES:

Antibodies table, related to Materials and Methods: List of Antibodies used in this study

Protein	**Host**	**Type**	**Dilution**	**Secondary**	**Manufacturer**
PECAM	Goat	polyclonal	1:200	AlexaFluor 647	Santa Cruz
CONNEXIN40	Rabbit	polyclonal	1:400	AlexaFluor 555	Alpha Diagnostic International
SM22α	Rabbit	polyclonal	1:400	AlexaFluor 555	Abcam
GFP	Goat	polyclonal	1:400	AlexaFluor 647	Abcam
TROPT	mouse	monoclonal	1:200	AlexaFluor 555	Thermofisher
MF20	mouse	monoclonal	1:20	Donkey anti-mouse-HRP	DSHB
BrdU	mouse	monoclonal	1:250	AlexaFluor 555	BD Bioscience
GFP	Goat	polyclonal	1:2000	Rabbit anti-Goat-HRP	Abcam

Primers table, related to Materials and Methods: List of primers used for qPCR analysis in this study

Name	Sequence	Direction	Usage
Rspo3 right	CAGCCATTGTAATCTGAACACG	antisense	qPCR
Rspo3 left	TCATTTTGAACTTTATGGAATACATTG	sense	qPCR
Lgr4 right	CAAGGCTTTTGGGTGGATAA	antisense	qPCR
Lgr4 left	CAGTTACCAGAAGATGCATTTAAGAA	sense	qPCR
Lgr5 right	CAGCCAGCTACCAAATAGGTG	antisense	qPCR
Lgr5 left	CTTCACTCGGTGCAGTGCT	sense	qPCR
Lgr6 right	AGAGGTGGTTCCCTGAGAG	antisense	qPCR
Lgr6 left	AGCTTCAGCCGGGTCTCT	sense	qPCR
Sox17 right	CCACCACCTCGCCTTTCAC	antisense	qPCR
Sox17 left	GATGCGGGATACGCCAGTG	sense	qPCR
Pecam right	CTTCATCCACCGGGGCTATC	antisense	qPCR
Pecam left	CTGCCAGTCCGAAAATGGAAC	sense	qPCR
TropT right	CTCTCTCCATCGGGGATCTT	antisense	qPCR
TropT left	GGTCCAGTAGAGGACACCAAA	sense	qPCR
Nkx2-5 right	GTGTGGAATCCGTCGAAAGT	antisense	qPCR
Nkx2-5 left	GACGTAGCCTGGTGTCTCG	sense	qPCR
Vegfr2 right	CAAAGCATTGCCCATTCGAT	antisense	qPCR
Vegfr2 left	GCCCTGCTGTGGTCTCACTAC	sense	qPCR
Islet1 right	GTCTTCTCGGGCTGTTTGTG	antisense	qPCR
Islet1 left	CCCTCTCAGTCCCTTGCAT	sense	qPCR
Axin2 right	CAGTTTCTTTGGCTCTTTGTGA	antisense	qPCR
Axin2 left	AGGAACCACTCGGCTGCT	sense	qPCR

SUPPLEMENTAL MATERIALS AND METHODS:

RT-qPCR

RNA was extracted from E10.5 or E13.5 hearts using TRIzol® reagent (Invitrogen), following the manufacturer's instructions. Reverse transcription was performed using the M-MLV reverse transcriptase in combination with oligo (dT) primers (Invitrogen). The cDNA was utilized as a template for quantitative PCR analysis with the SybrGREEN® Master Kit (Roche) and a Light Cycler 1.5® (Roche). The expression levels were normalized to *Gapdh*. For each litter or experiment ddCt values were normalized to one control dCt rather than the mean of control delta Cts. Primers (see primer table) were designed on the Universal Probe Library website (Roche).

Endothelial/arterial proliferation quantification for MYHR3 and CRLGR4 E13.5 hearts

Quantification of BrdU-positive endothelial cells was performed by co-immunostaining with anti-BrdU (BD Bioscience) and anti-PECAM antibodies as described in the main experimental procedures. PECAM/BrdU-positive cells were counted on at least 10 consecutive sections in the area immediately surrounding the developing left coronary artery. Three independent embryos per genotype from one dissection were analyzed for the CRLGR4 quantification and three independent embryos from three different dissections were analyzed for the MYHR3 quantification. PECAM staining was quantified by calculating the amount of pixels in the ventricular coronary vessels using ImageJ software.

BrdU quantification for CRLGR4 E10.5 hearts

Quantification of BrdU-positive cells was performed by immunostaining with an anti-BrdU antibody. Results were obtained after scoring the percentage of BrdU positive nuclei/total nuclei in 10 matched sections per embryo. Entire hearts sections that normally would have high levels of *Rspo3* expression were counted. Three independent E10.5 WT embryos were compared with three independent mutants (2 litters).

BrdU quantification for intestines

Detection of BrdU-positive cells was performed by immunostaining with an anti-BrdU antibody. Results were obtained after scoring the percentage of BrdU positive nuclei/total nuclei in four different matched areas of four histological sections per embryo. Three independent E16.5 WT embryos were compared with three independent mutants from the same litter.

Coronary Vessel Quantification

Coronary arteries were defined as Connexin40/PECAM-positive intramyocardial vessels. For the MYHR3-null E16.5 hearts the coronary arteries were counted for the right and left ventricles as well as for the interventricular septum. Six sections were analyzed per embryo and a total of 3 embryos from two different litters were analyzed per genotype. For the CRLGR4 all of the 18 mutant hearts from 8 different dissections were analyzed. Only 6 of these hearts displayed significant differences in coronary artery development. Coronary artery counts for these 6 mutant hearts along with their counterpart controls were performed in the same manner as in the MYHR3 hearts.

CRLGR4 mutant protein analysis in HEK293T cells

LGR4 coding sequences were obtained amplifying the whole LGR4 sequences from wildtype and mutant cDNA. The fragments were cloned, in frame with EGFP, into pCDNA3.1 plasmid using the NEBuilder® HiFi DNA Assembly master mix (NEB, ref: E2621). The artificially truncated coding sequence was obtained by amplifying the wildtype allele up to the presumptive stop codon generated by the 5bp deletion (the primers are available upon request). HEK293T cells were transfected with X-tremeGENE™ HP DNA Transfection Reagent (Roche, ref: 06366236001) according to the manufacturer instructions. The cells were lysed in RIPA buffer 48 hours after transfection. To avoid the aggregation of LGR receptors upon boiling, cell lysates were incubated with 1x Laemmli loading buffer 1 hour at 37°C before loading on a western blot gel. After transfer, the membrane was blocked 1 hour at RT in PBS- tween 0.1%- milk 5% and incubated with anti-GFP antibody (Abcam, ref: ab5450, dilution 1:2000) O/N at 4°C rocking. The protein was visualized using secondary rabbit anti-goat HRP conjugated antibody (SantaCruz, ref: sc-2768, dilution 1:5000).