Cell Reports, Volume *22*

Supplemental Information

Epithelial WNT Ligands Are Essential Drivers of Intestinal Stem Cell Activation

Winnie Y. Zou, Sarah E. Blutt, Xi-Lei Zeng, Min-Shan Chen, Yuan-Hung Lo, David Castillo-Azofeifa, Ophir D. Klein, Noah F. Shroyer, Mark Donowitz, and Mary K. Estes

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Flow cytometry. Crypts isolated from previous steps were made into single cell suspensions by incubating with 0.8mg/mL dispase solution at 37°C for 10min. Solution was vortexed every 2min to ensure single cell dissociation. 1mL Fetal Bovine Serum (FBS) was added to stop the enzymatic digestion and 50uL 10mg/µL DNAse was added to prevent cell clumps. Single cells were filtered through a 40µm cell strainer (BD Falcon) and then spun down at 1000g, 4°C for 5 min. 10% FBS in HBSS was used to wash cells before proceeding to antibody staining. Please refer to **Table S1** for a list of antibodies and respective dilutions. n in all experiments refer to separate crypt preps from different animals in each group. Discrimination of each cell population via flow cytometry is shown in **Figure S3**. Dead cells and doublets were excluded using side and forward scatters. At least 30,000 live, epithelial cells were collected using the $EpCam^{APC}$/DAPI gate for analysis. Lgr5-GFP^{high} population was analyzed using previously published gating schemes (Sato et al., 2009). All data were collected using BD LSRFortessa™ and analyzed on FlowJo software. Flow cytometry data were uploaded to Flow Repository with IDs: FR-FCM-ZYF8 and FR-FCM-ZYF9.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Crypts were isolated from control- or RV-infected mice small intestine. Total RNA was extracted from isolated crypts using the Zymo Research Directo-zol™ RNA kit, following the manufacture's protocol. The amount and purity of RNA were determined using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, TA). Reverse transcription-quantitative PCR (RT-qPCR) was performed on a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA), using One-Step RT-qPCR ToughMix with the ROX reference dye according to the manufacturer's protocol (Quanta Biosciences, Gaithersburg,

MD). TaqMan primer-probe mixes were obtained from Molecular Probes (Eugene, OR), with the assay identification numbers listed in **Table S2**. Expression levels were normalized to *Gapdh* levels and analyzed with StepOne v2.1 software (Applied Biosystems) by using the $2^{-\Delta\Delta CT}$ method as previously described (Schmittgen and Livak, 2008). n in all experiments refer to biological replicates, with two technical RT-qPCR replicates for each biological replicate. Dots represent relative gene expression of a single animal. Bars represent means ± SD of respective groups. Statistical analyses were performed using Student's t-test. $^{*}p < 0.05$.

Tissue staining. Intestinal tissues were fixed in 4% paraformaldehyde in PBS at 4°C overnight, transferred to 70% ethanol, paraffin-embedded, and sectioned at 5 µm thickness. Paraffin-embedded sections were deparaffinized and rehydrated before staining. Antigen retrieval was performed using sodium citrate buffer (10 mM sodium citrate pH 6.0).

For immunofluorescence, the sections were blocked using BioGenex Power Block™ Universal Block Reagent at room temperature (RT) for 5 min and then incubated with primary antibody at 4°C overnight. Slides were washed three times with PBST (0.05% Tween 20 in PBS) at RT for 5 min and incubated with secondary antibody at RT for 1 h. Slides were then washed three times again with PBST and mounted by VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories). For immunohistochemistry, endogenous peroxidase were quenched by 3% H_2O_2 in methanol for 20 min at RT. The sections were then blocked by Avidin/Biotin and animal serum for 15 min each at RT. Then, primary antibody were incubated at 4°C overnight. Slides were washed by PBST at RT for 5 min three times and incubated with secondary antibody at RT for 0.5

h. Slides were then washed three times again by PBST and visualized via ABC and DAB reagents (Vector Laboratories). A hematoxylin counterstain was used before dehydration and mounting using Permount Mounting Medium (Fisher Scientific). For EdU detection, the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (ThermoFisher Scientific) were used according to the manufacture's protocols.

Please see **Table S1** for a list of antibodies and respective dilutions. Immunofluorescent confocal images were captured using a Nikon A1-Rs confocal laser scanning microscope. Light microscopy images were captured using a Biotek Cytation 5 Imager and Plate Reader or a Nikon Ci-L Upright Microscope. All image analyses were performed using the Integrated Microscopy Core at Baylor College of Medicine.

RNA *in situ* hybridization and RNAScope®

Detection of *Axin2* mRNA by *in situ* hybridization was performed as described (Gregorieff and Clevers, 2010) on tissue fixed by immersion in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 minutes and embedded in Tissue-Tek O.C.T. Compound. Digoxigenin (DIG)-labeled *Axin2* antisense RNA probe was generated by *in vitro* transcription using NotI and SP6 polymerase (plasmid provided by I. Thesleff). *Wnt3* was detected on paraffin-embedded intestinal slides using RNAScope® probe 312241 following manufacturer's protocols.

SUPPLEMENTAL REFERENCES

GREGORIEFF, A. & CLEVERS, H. 2010. In situ hybridization to identify gut stem cells. *Current protocols in stem cell biology*, 2F. 1.1-2F. 1.11.

SATO, T., VRIES, R. G., SNIPPERT, H. J., VAN DE WETERING, M., BARKER, N., STANGE, D. E., VAN ES, J. H., ABO, A., KUJALA, P., PETERS, P. J. & CLEVERS, H. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature,* 459**,** 262-5.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc,* 3**,** 1101-8.

Figure S1.

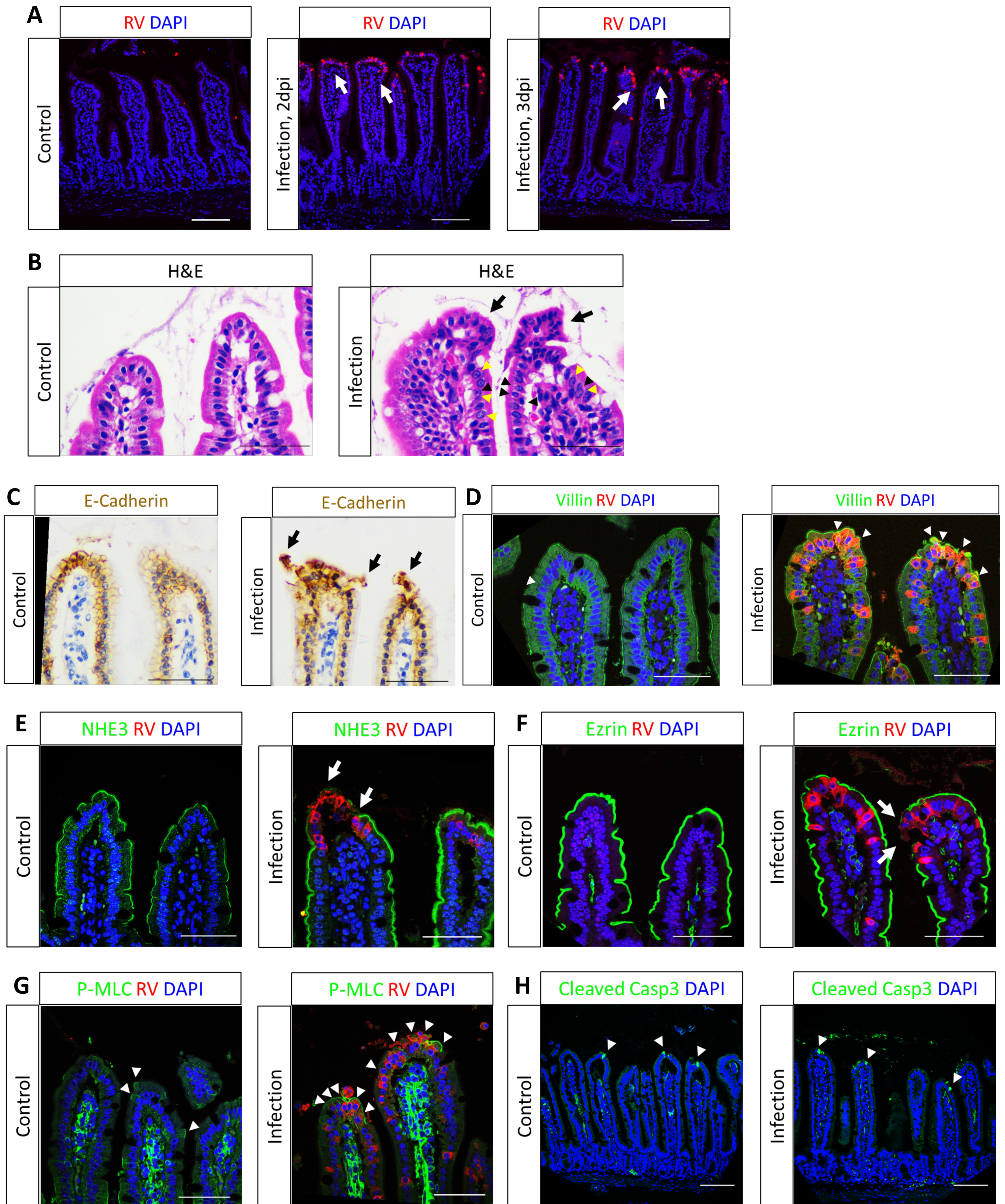

Figure S1. Related to Figure 1. **RV infection causes localized damage at tips of villi. (A)** Representative confocal images of control- and RV-infected mouse epithelium at 2dpi and 3dpi. RV-infected cells are detected using a laboratory-generated, polyclonal guinea pig anti-RV antibody that detects viral structural proteins. Infected villi are noted by white arrows. **(B)** Representative H&E images of control- vs. RV-infected villi at 4dpi. Black arrows denote altered shedding at the tip of the villus. Black arrowheads denote nuclei mis-positioned either towards the apical or the basolateral surface of the cell. Yellow arrowheads denote distorted and enlarged nuclei with hypodense hematoxylin stain. **(C)** Representative immunohistochemistry staining of E-Cadherin staining in control- and RV-infected animals at 4dpi. E-Cadherin protein is mis-localized to the cytoplasm in RV-infected villus tip cells. Black arrows point to shedding cells with fragmented nuclei, indicating necrosis. **(D)** Representative confocal images of villin and RV staining in control- and RV-infected animals at 4dpi. White arrowheads denote irregular villin staining in infected villus tips with intensified villin stain on the apical cell surface and its mis-localization to the cytoplasm. **(E)** Representative confocal images of sodium–hydrogen antiporter 3 (NHE3) and RV staining in control- and RV-infected animals at 4dpi. NHE3 protein is lost in parts of RV-infected villi (white arrowheads). **(F)** Representative confocal images of ezrin and RV staining in control- and RV-infected animals at 4dpi. Ezrin protein is lost in parts of RV-infected cells (white arrowheads). **(G)** Representative confocal images of phosphorylated myosin light chain (P-MLC), a marker of anoikis, and RV staining in control- and RV-infected animals at 4dpi. P-MLC-labeled cells are increased in number following RV infection (white arrowheads). **(H)** Representative confocal images of cleaved caspase 3 (cleaved casp3), a marker of apoptosis, in control- and RV-infected animals at 4dpi. Cleaved casp3-labeled cells remained stable following RV infection (white arrowheads). Scale bars: 100 µm in A and H, 50 µm in B-G.

Figure S2.

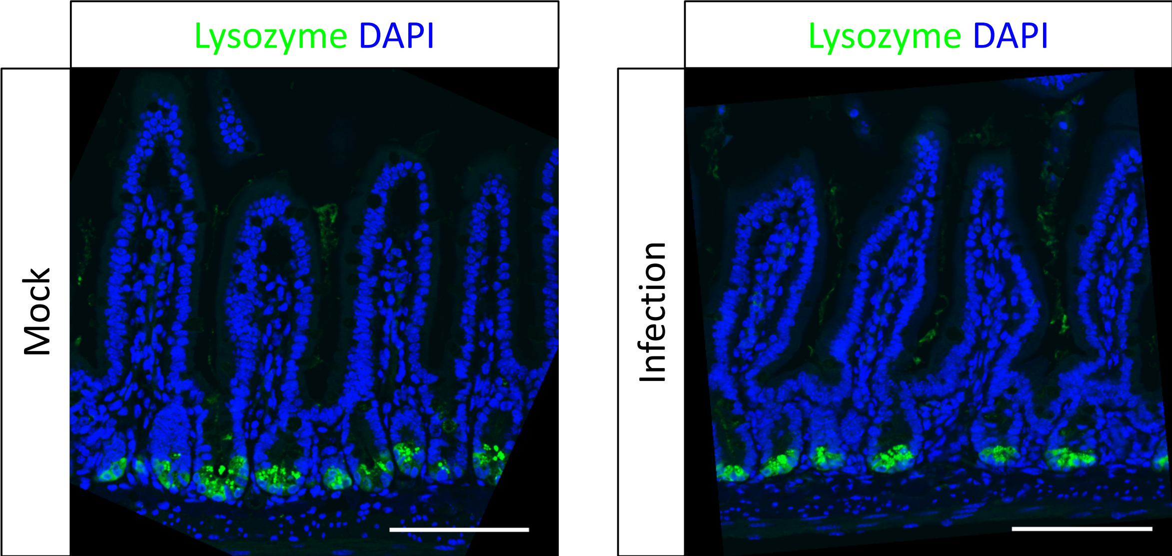

Figure S2. Related to Figure 1. **Paneth cells are intact in RV-infected animals.** Representative confocal images of lysozyme staining in control- and RV-infected animals at 4dpi. Scale bars: 100 µm.

Figure S3.

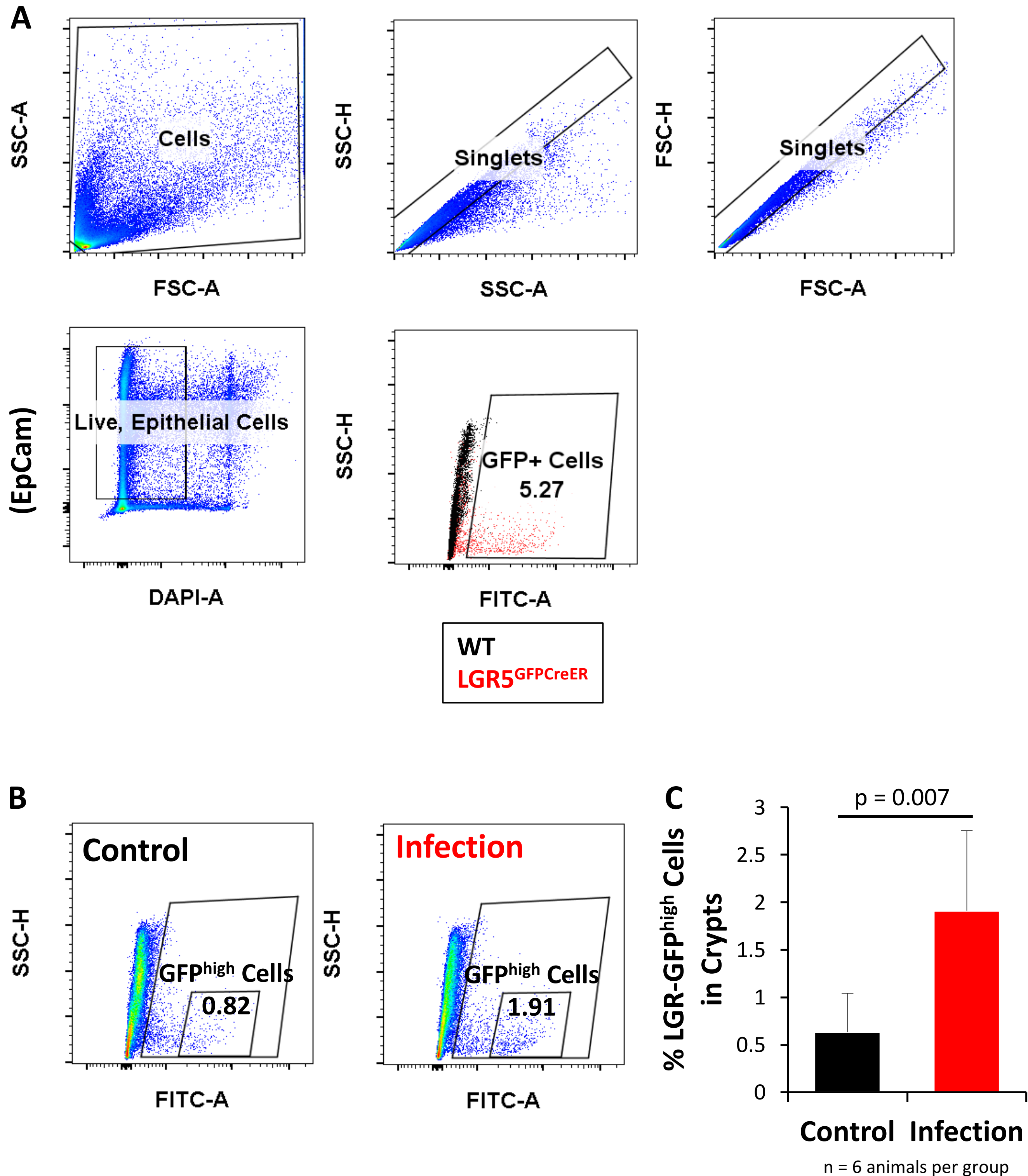

Figure S3. Related to Figure 3. **LGR5-GFP^{high} cells are increased in number following RV infection. (A)** Representative flow gating scheme for isolating viable (DAPI^{neg}), single intestinal epithelial cells (EpCAM^{pos}), then *Lgr5^{GFPCreERT}* population. All flow analysis were performed using crypt-enriched epithelial preparation. **(B)** Representative flow cytometry analysis of LGR5-GFP^{high} cell population in control- and RV-infected *LGR5^{GFPCreERT}* mice. **(C)** Quantification of LGR5-GFP^{high} cell population in control- and RV-infected animals (n= 6 mice per group with at least two independent experiments).

Figure S4.

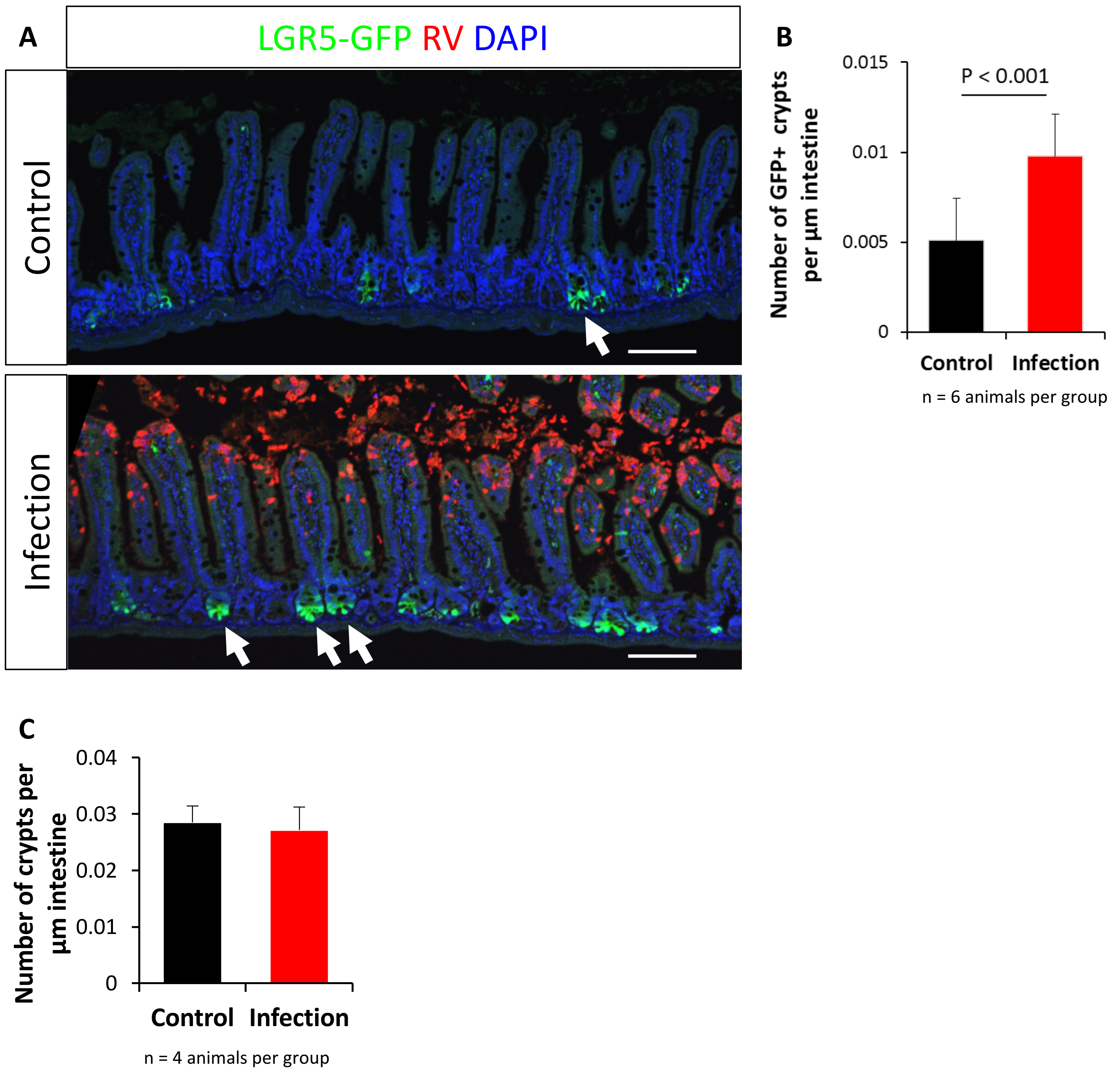

Figure S4. Related to Figure 3. **RV infection resulted in increased number of LGR5+ crypts. (A)** Representative immunofluorescent image of LGR5+ crypts in control- and RV-infected mice. Scale bar: 100 µm. **(B)** Quantification of LGR5+ crypts per µm of intestine in control- and RV-infected animals (n= 6 mice per group with at least two independent experiments). **(C)** Quantification of number of crypts per µm of intestine in control- and RV-infected animals (n= 4 mice per group with at least two independent experiments).

Figure S5.

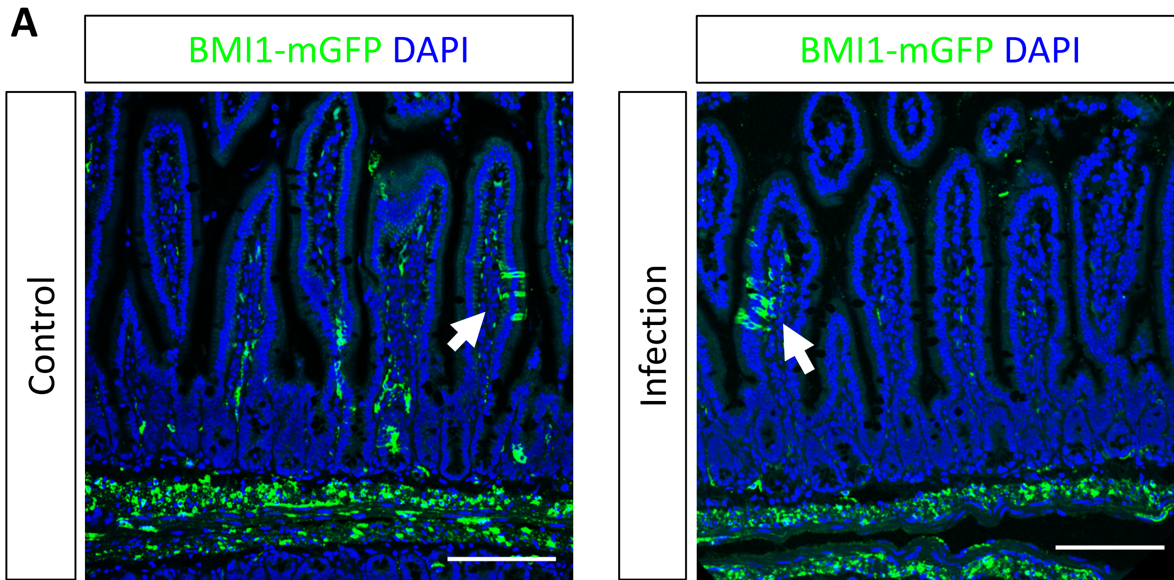

Figure S5. Related to Figure 3. **BMI1 lineage is not changed following RV infection.** Representative confocal images of *Bmi1*^{Cre};R26^{mTmG} mice 7 days after tamoxifen injection and RV infection. BMI1 lineage remained similar in control- and RV-infected animals (white arrows). Scale bar: 100 µm.

Figure S6.

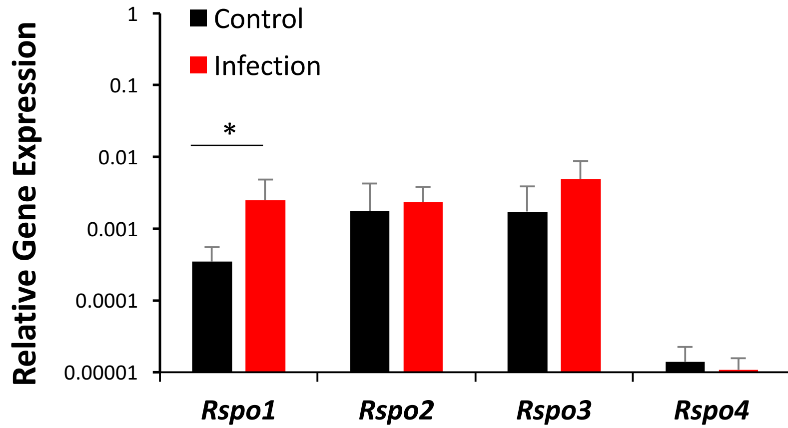

Figure S6. Related to Figure 4. **Upregulation of *R-Spondin 1* following RV infection.** RT-qPCR results showed expression of the *R-Spondin* family genes in isolated mesenchyme of WT mice following RV infection. Transcripts were normalized to *Gapdh* and relative gene expression was obtained using the $2^{-\Delta\Delta Ct}$ method (n = 3 mice per group in at least 2 independent experiments). Dots represent relative gene expression of one animal. Bars represent means ± standard deviation of respective groups. Statistical analyses were performed using Student's t-test. *$p < 0.05$.

Figure S7.

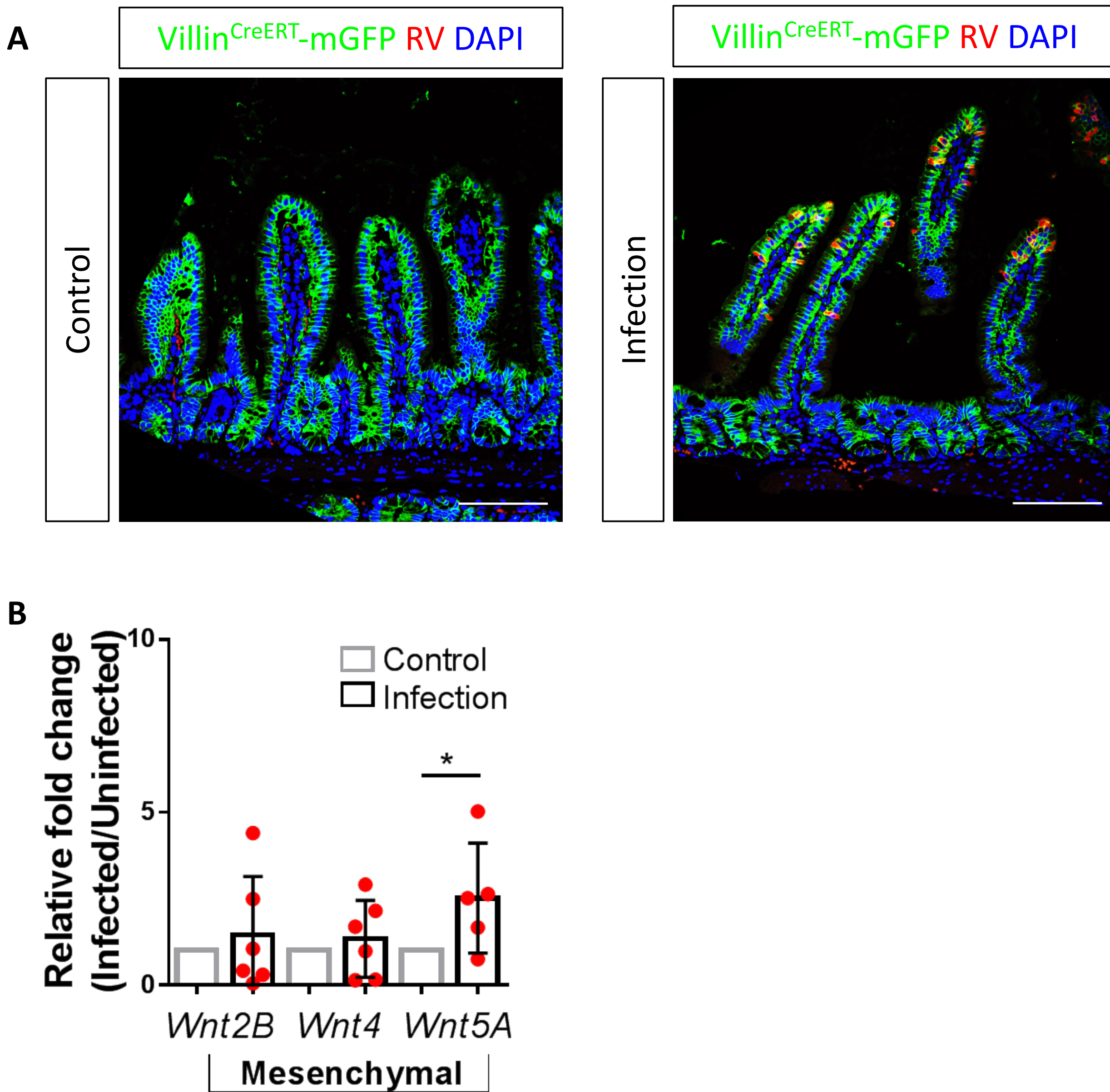

Figure S7. Related to Figure 5. **Epithelial WLS conditional KO results in mesenchymal *Wnt5A* upregulation following infection. (A)** Representative confocal images of $Villin^{CreERT};WLS^{f/f};R26^{mTmG}$ (WLS KO) mouse following tamoxifen injection in control and RV infection. mGFP+ cells represent epithelial cells with *Wntless* gene knockout. RV infected cells (red) visualized by guinea pig polyclonal serum that detected viral structural proteins. Scar bar: 100 µm. (B) RT-qPCR results showed expression of the mesenchymal *Wnt* family genes in isolated mesenchyme from WLS KO mice following RV infection. Transcripts were normalized to *Gapdh* and relative gene expression was obtained using the $2^{-\Delta\Delta Ct}$ method (n = 6 mice per group). Dots represent relative gene expression of one animal. Bars represent means ± standard deviation of respective groups. Statistical analyses were performed using Student's t-test. *$p < 0.05$.

Table S1. Related to Material and Methods. **Antibody information and dilutions for flow cytometry and immunofluorescence.**

Antibody	Host	Company and Catalogue no.	Dilution
EpCam/CD326	Rat	BioLegend 118213	1:200
GFP	Goat	Abcam Ab6662	1:500
Lysozyme	Rabbit	ThermoFisher 18-0039	1:1000
OLFM4	Rabbit	Cell signaling 39141	1:200
PCNA	Mouse	Cell Signaling 2586	1:500
RV (GP511)	Guinea pig	Laboratory Generated	1:200
β-catenin	Rabbit	Abcam ab32572	1:500
CC44v6	Rat	eBiosciences BMS145	1:1000
E-Cadherin	Mouse	BD 610181	1:1000
Villin	Goat	Santa Cruz sc-7672	1:500
NHE3	Rabbit	Novus NBP1-82574	1:200
Ezrin	Rabbit	Abcam ab76247	1:200
P-MLC	Rabbit	Cell Signaling 3671S	1:50
Clev. Casp. 3	Rabbit	Cell Signaling 9551	1:200

Table S2. Related to Material and Methods. **TaqMan probes used for RT-qPCR gene expression analysis.**

Gene name	Probe ID
Ascl2	Mm01268891_g1
Axin2	Mm00443610_m1
Bmi1	Mm03053308_g1
Ccnd1	Mm00432359_m1
Cd44	Mm01277161_m1
Dclk1	Mm00444950_m1
Gapdh	Mm99999915_g1
EphB2	Mm01181021_m1
Hopx	Mm00558630_m1
Ki67	Mm01278617_m1
Lgr5	Mm00438890_m1
Lrig1	Mm00456116_m1
Myc	Mm00487804_m1
Olfm4	Mm01320260_m1
Pcna	Mm00448100_g1
Sox9	Mm00448840_m1
Tert	Mm00436931_m1