

Expanded View Figures

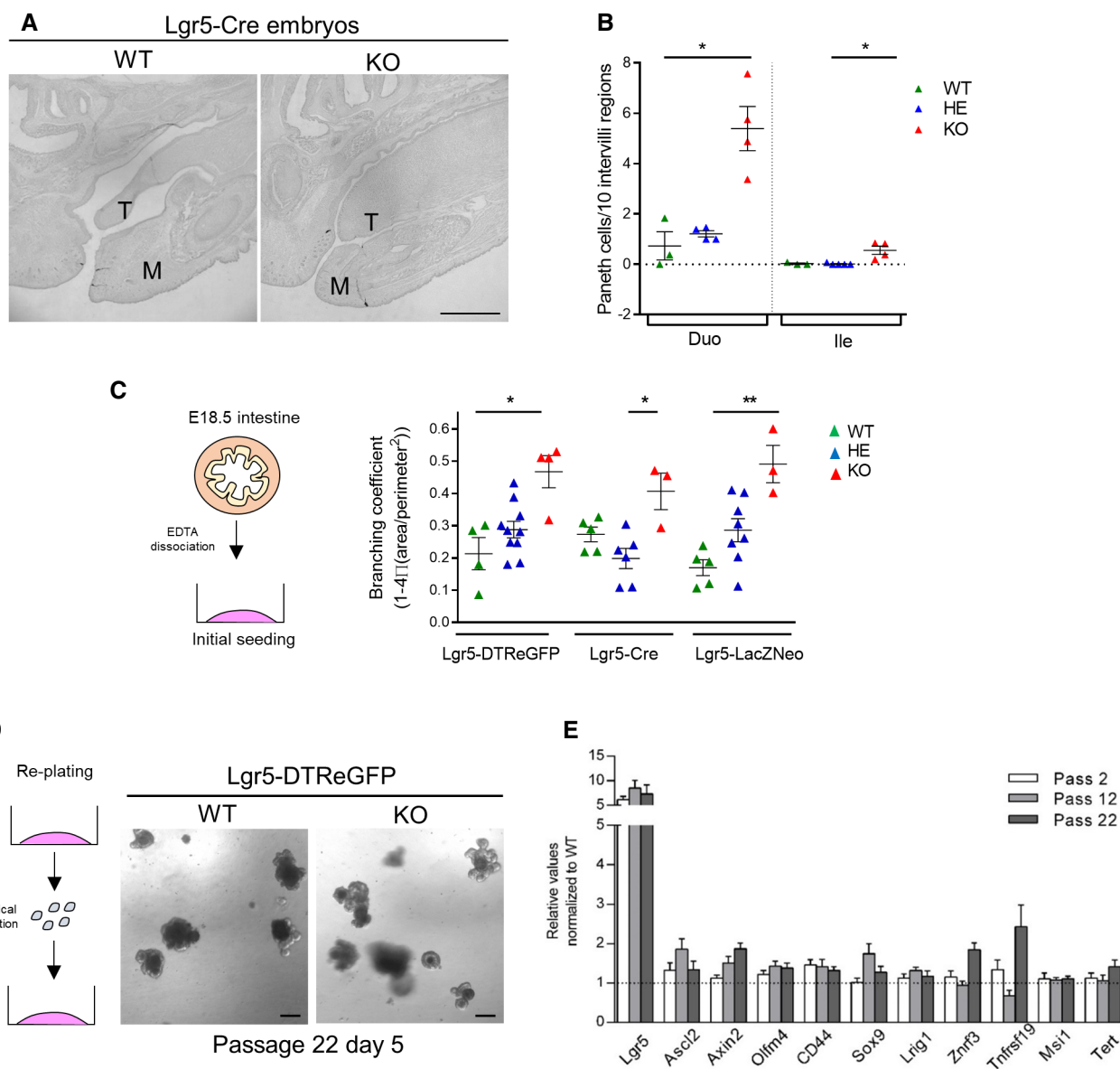


Figure EV1. Lgr5 deficiency induces early Paneth cell differentiation and stem cell expansion in the small intestine at E18.5.

- A Sagittal sections of craniofacial region showing the presence of an ankyloglossia in Lgr5-null Cre embryos at E18.5 as compared to the WT littermate. The tongue (T) and mandible (M) are indicated.
- B Paneth cell quantification as number of cells per 10 intervilli regions on Lgr5-Cre duodenum (Duo) and ileum (Ile). Each symbol indicates the value for a given embryo.
- C Quantification showing organoid complexity ex vivo measured by the branching coefficient for wild-type (WT), heterozygous (HE), and KO organoids from 3 different mouse strains: Lgr5-DTRReGFP, Lgr5-Cre, and Lgr5-LacZNeo at day 6 of culture upon initial seeding. Each symbol indicates the mean value for a given embryo.
- D Representative pictures of WT and KO Lgr5-DTRReGFP organoids at day 5 of passage 22.
- E Gene expression analysis by qRT-PCR of the indicated stem cell markers in Lgr5-DTRReGFP WT and Lgr5 KO organoid cultures, each originating from a given embryo ($n = 7$ WT and 9 KO at passage 2 and passage 12, $n = 2$ WT and 5 KO at passage 22). Values are normalized to the WT at passage 2.

Data information: Scale bars, 1 mm (A) and 50 μ m (D). Data are represented as means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$ by Kruskal–Wallis test followed by Dunn's multiple comparison test (B, C).

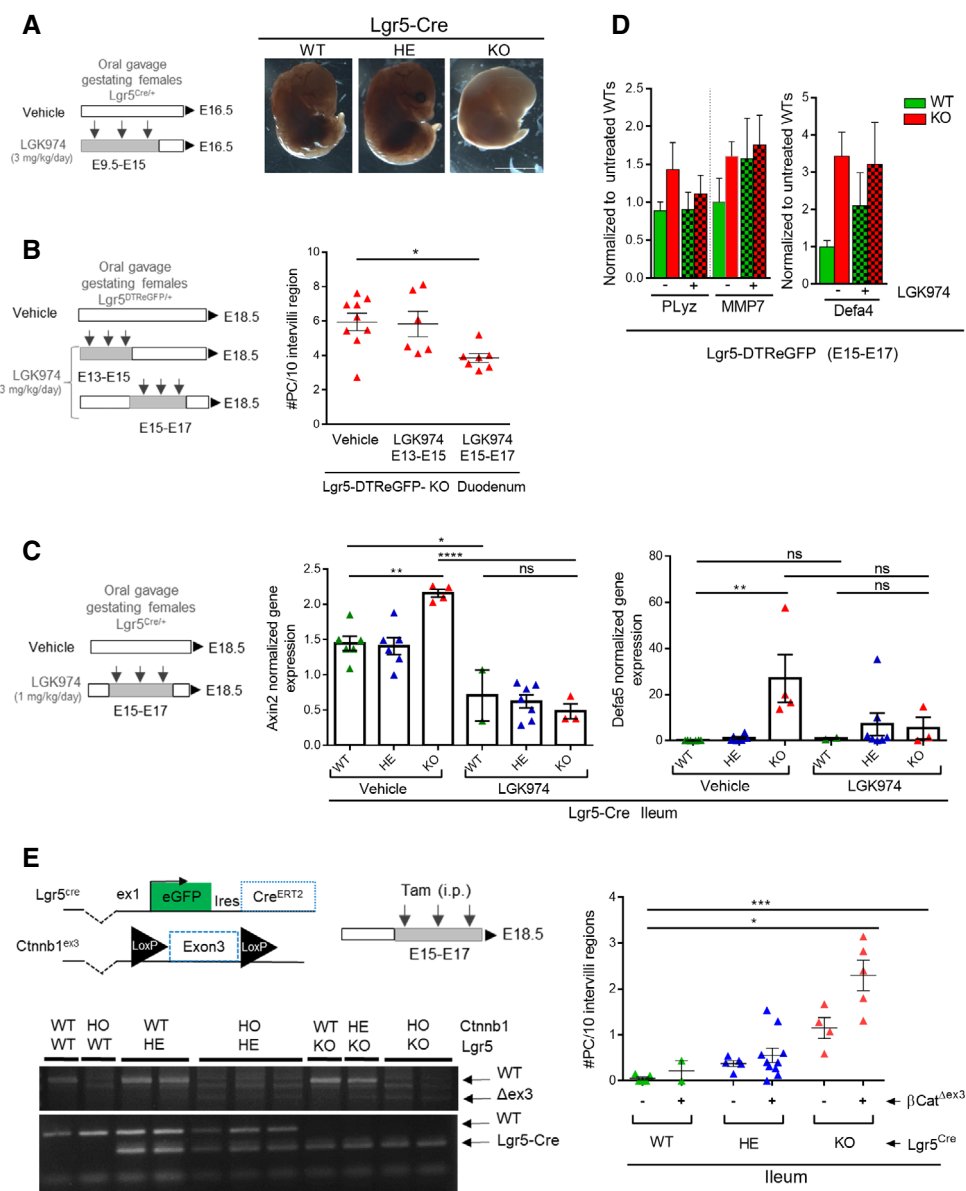


Figure EV2. In utero inhibition of Wnt activity counteracts early Paneth cell differentiation induced by *Lgr5* deficiency.

- A Gestating *Lgr5^{Cre}* heterozygous females were vehicle- or LGK974-treated by oral gavage between E9.5 and E15 at the indicated dose. Global morphological analysis of whole embryos was done at E16.5.
- B Gestating *Lgr5^{DTReGFP}* heterozygous females were vehicle- or LGK974-treated by oral gavage between E13-E15 or E15-E17 at the indicated dose. Duodenum of treated *Lgr5^{DTReGFP}* KO embryos was analyzed at E18.5 for Paneth cell differentiation. Each symbol indicates the value for a given embryo.
- C Gestating *Lgr5^{Cre}* heterozygous females were vehicle- or LGK974-treated by oral gavage between E15 and E17 at the indicated dose. Gene expression analysis of stem cell (*Axin2*) and Paneth differentiation (*Defa5*) markers was performed at E18.5 in ileums of *Lgr5^{Cre}* embryos by qRT-PCR. Each symbol indicates the value for a given embryo.
- D Gene expression analysis of Paneth differentiation markers was performed at E18.5 in ileums of *Lgr5^{DTReGFP}* embryos by qRT-PCR (vehicle-treated: 7 WT and 7 KO; LGK974-treated: 3 WT and 6 KO).
- E Gestating females were intra-peritoneally injected with tamoxifen between E15 and E17. Left panel: Recombination of floxed b-catenin exon 3 (Δ ex3) was verified by PCR on ileums of embryos. Genotypes of embryos for b-catenin (*Ctnnb1*) and *Lgr5* loci are indicated. Right panel: Ileums of embryos were analyzed at E18.5 for Paneth cell differentiation by Lendrum staining. Each symbol indicates the value for a given embryo.

Data information: Scale bar, 500 μ m. Data are represented as means $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 by Kruskal–Wallis test followed by Dunn's multiple comparison test (B, E) and two-way ANOVA followed by Tukey's multiple comparison test (C, D).

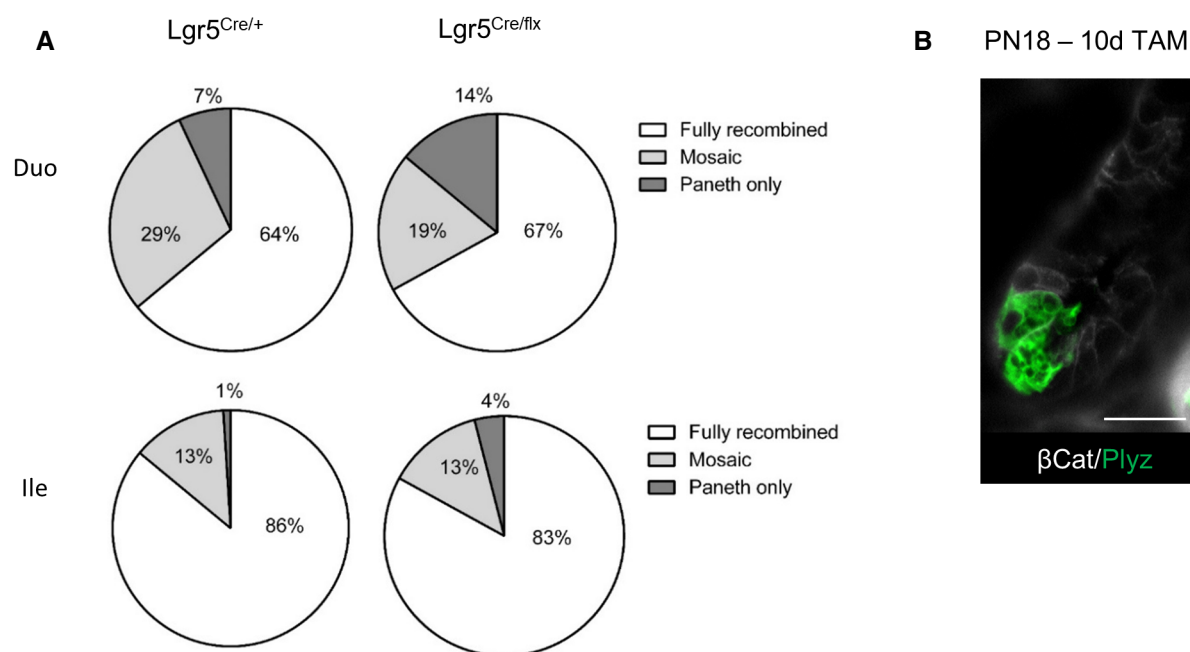


Figure EV3. Postnatal Lgr5 ablation in ISCs alters stem cell fate toward the Paneth cell lineage.

- A** Fate of RFP⁺-traced clones in control (Lgr5^{Cre/+}) and cKO (Lgr5^{Cre/fix}) at postnatal day 18 (PN18) after 10 days of chase. The proportion of fully recombined and mosaic RFP⁺ crypt/villus units as well as RFP⁺ cells only labeling Paneth cells is indicated for the duodenum and ileum of controls and cKOs ($n = 5$ for each genotype).
- B** Representative immunofluorescence picture showing Paneth cells (Plyz marker) in co-staining with the epithelial cell membrane marker b-catenin in a control (Lgr5^{Cre/+}) at PN18 in ileum 10 days after tamoxifen injection. Scale bar, 50 μ m.

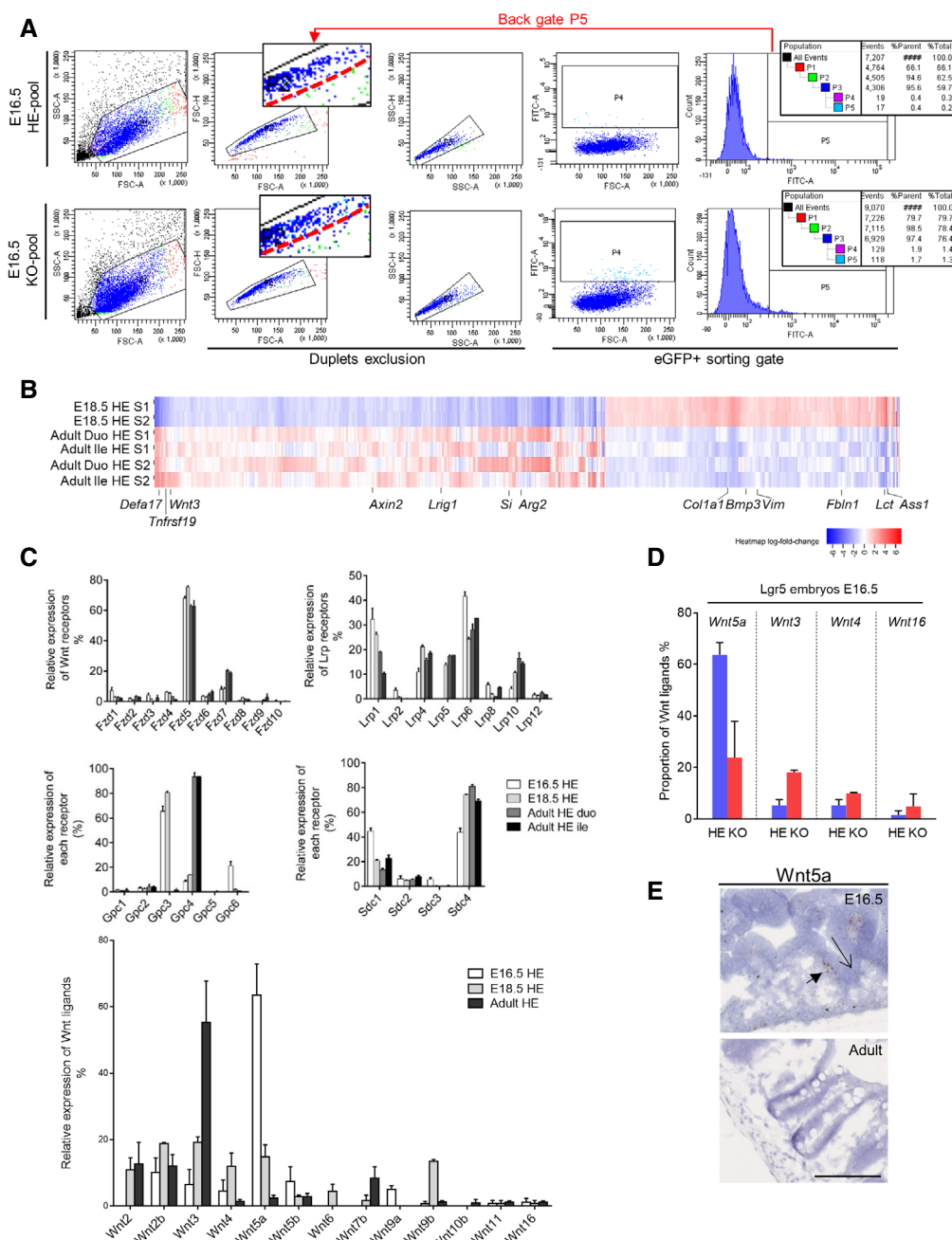


Figure EV4. Transcriptome analysis of Lgr5 ISC precursors in adults.

- A Representative FACS plots showing gating strategy used for sorting experiments (Lgr5-DTReGFP E16.5). Back gate for eGFP+ sorted cells (visualized as cyan dots) shows high stringency for duplets exclusion (depicted by a red dashed line).
- B ISC (eGFP^{+/ve}) cells from Lgr5-DTReGFP embryonic E18.5 and adult stages were sorted by FACS and subjected to RNAseq analysis. Heatmap of differentially regulated genes (fold change $\times 1.5$, FDR 0.1) in E18.5 HE as compared to adult ISCs from duodenum (Duo) and ileum (Ile). Selected genes are evidenced. Highlighted genes: Wnt-related genes (Wnt3, Axin2), differentiation markers (Defa17, Si), extracellular matrix-related genes (Col1a1, Vim), and metabolic-related genes (Lct, Ass1).
- C Graphs showing relative expression levels of the Wnt ligands and Wnt receptors/co-receptors (Fzd, Lrp, Gpc, Sdc) at different developmental stages E16.5, E18.5, and adult HEs based on the RNAseq data.
- D Graph showing relative proportion of the main Wnt ligands expressed in Lgr5 KOs and HEs at E16.5.
- E Expression of the Wnt ligand Wnt5a in normal embryonic and adult small intestines by RNA scope. The arrows show Wnt5a-expressing cells localized in stroma and epithelium in E16.5 embryos.

Data information: Scale bar, 100 μ m. Biological replicates for RNAseq experiments on ISC (B, C, D): Lgr5-DTReGFP E16.5 independent embryonic pools ($n = 2$ HE, 2 KO); Lgr5-DTReGFP E18.5 individual embryos ($n = 2$ HE depicted as S1 and S2 in panel B), and Lgr5-DTReGFP adult individual animals ($n = 2$ HE depicted as S1 and S2 in panel B).

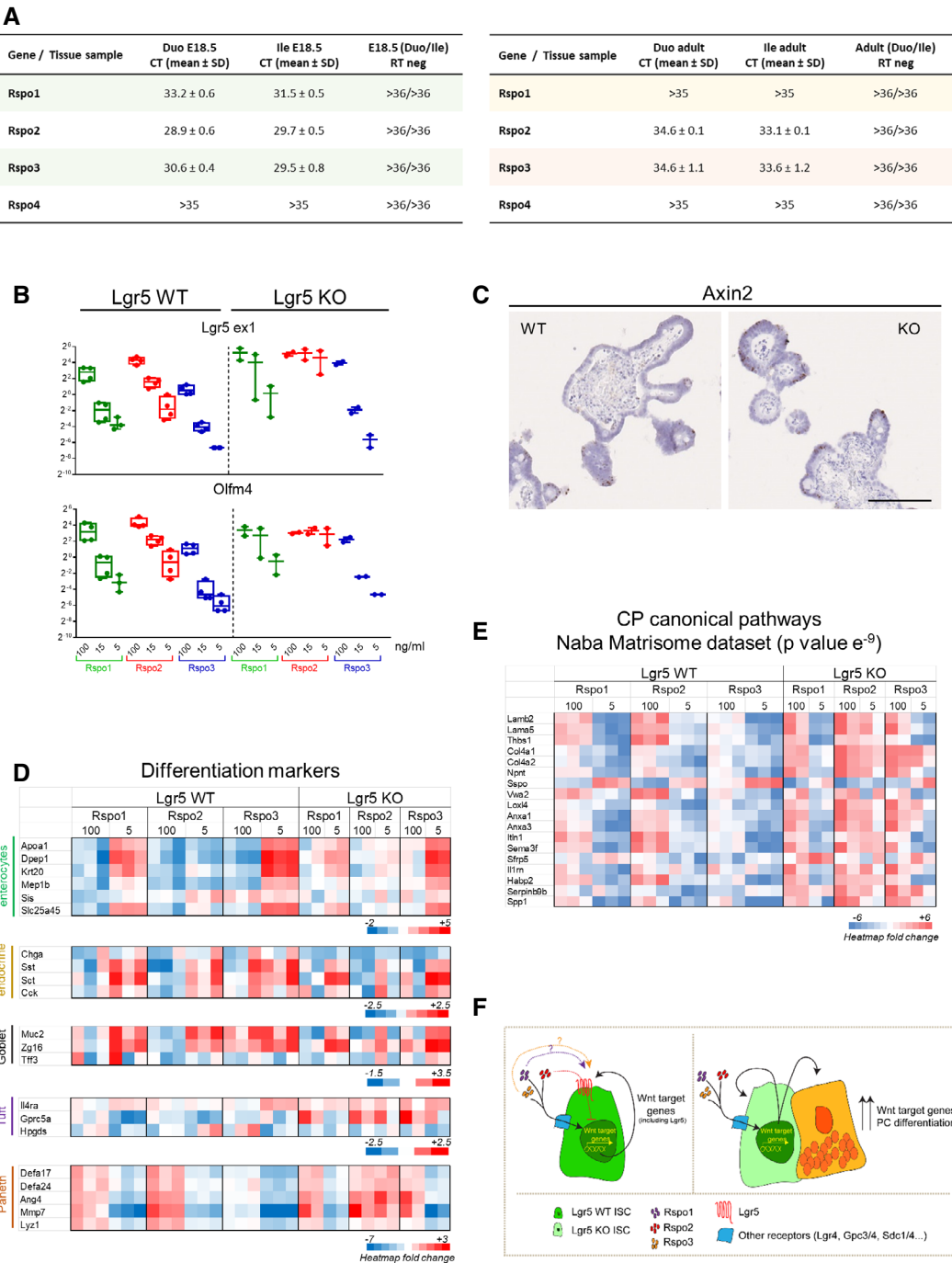


Figure EV5. Rspodin 2/Lgr5 interaction regulates stem cell fate in organoids.

- A Expression levels of Rspodin ligands during intestinal maturation. Tables showing Ct values for *Rspo1*, *Rspo2*, *Rspo3*, and *Rspo4* genes expression levels analyzed by qRT-PCR on duodenum (Duo) and ileum (Ile) from E18.5 embryos ($n = 11$ samples originating from individual embryos) and adult animals ($n = 3$ samples originating from individual animals).
- B Gene expression analysis by qRT-PCR of stem cell markers in Lgr5-DTReGFP WT and KO organoids exposed to 100, 15, or 5 ng/ml Rspodins after 5 days of culture. Each symbol indicates the value for an organoid culture originated from a given embryo.
- C Expression of the Wnt target gene *Axin2* by RNAscope in WT and KO organoids cultured in the presence of Rspodin 2 at 5 ng/ml. Scale bars: 100 μ m.
- D Heatmap showing the impact of Rspodin type and concentration on relative cell lineage differentiation (enterocyte, enteroendocrine, goblet, tuft, and Paneth cell lineages) in Lgr5-DTReGFP WT and KO organoids analyzed by RNAseq ($n = 3$ WT and 2 KO organoid culture samples, each originating from a given embryo).
- E Heatmap showing the impact of Rspodin type and concentration on matrisome components gene expression (list from the Naba Matrisome dataset) in Lgr5-DTReGFP WT and KO organoids analyzed by RNAseq ($n = 3$ WT and 2 organoid culture samples, each originating from a given embryo).
- F Scheme describing current hypothesis/model for Rspodins action on Wnt signaling regulation in ISC through Lgr5 and alternative receptors.