SUPPLEMENTARY FIGURES

Figure S1.

(A) Luciferase reporter assay indicating β-catenin mediated transcription in HEK cells upon transient *Rspo3* transfection (left panel) and upon stimulation with conditioned medium of L-cells stably expressing *Rspo3* (right panel). Both approaches show that the used *Rspo3* coding sequence provides RSPO3 protein that is capable to enhance Wnt/β-catenin signaling slightly on its own, and strongly in synergy with Wnt3a. Graphs show averages and standard deviations of three independent experiments. (B) Schematic representation of the generation of the *Rspo3*^{inv} construct. Shortly, the *Rspo3* coding sequence was inserted in antisense orientation between two sets of *lox* sites, 3' to the CAGGS promoter in a *Rosa26* gene targeting cassette. For further details please note the description in the supplementary materials and methods. The obtained *Rspo3*^{inv} construct provides transgenic *Rspo3* expression upon Cre recombinase activity. (C) Schematic representation of the *Rspo3*^{inv} construct, either or not converted to the sense orientation. Black arrow heads indicate the location and orientation of the primer sequences used for genotyping and validation of sense-oriented *Rspo3* expression. Primer set 1/2 can only amplify a product when the *Rspo3* gene has been switched into sense orientation and set 2/3 detects the presence of the construct (D) RT-PCR confirming sense-oriented *Rspo3* mRNA expression in double transgenic mice exclusively, and throughout the entire intestinal tract, i.e. the duodenum, jejunum, ileum, colon and rectum. Intermediate products (upper bands) and the final, locked product (lower bands) are shown, and were sequence verified. Low levels of transgenic *Rspo3* were expressed in Lgr5;Rspo3 mice injected with vehicle only, indicating mild leakage activity of the Cre^{ERT2} recombinase in the absence of tamoxifen. (E) Immunohistochemical RSPO3 staining in the small intestine of a tamoxifen-injected, double transgenic Lgr5;Rspo3 mouse, demonstrating RSPO3 protein expression. Staining was observed in double transgenic mice only.

Figure S2. (A) H&E stainings of cecum and most proximal colon of Rspo3 single transgenic and Lgr5;Rspo3 double transgenic mice illustrating the hyperplastic phenotype in this region of the intestinal tract. The lower part of each panel represents an enlargement of the boxed regions. (B) Tumor incidence in Lgr5;Rspo3 mice (n=15) injected with tamoxifen at day 25 and maintained 1-2 months prior to analysis, lines indicating the median. Tumor incidence does not differ significantly from that of animals injected at 2 months of age (as depicted in Figure 2F). Ad: adenoma; AdCa: adenocarcinoma.

Figure S3. (A) Immunohistochemical β-catenin staining in an intestinal tumor of an *Apc*^{Min} mouse (FVB), showing clear detection of nuclear β-catenin in a prominent part of the lesion. (B) Quantification of BrDU-retaining cells (LRC) in the jejunum, being lysozyme-negative or lysozyme-positive (1 week after BrDU), showing averages and standard deviations over three independent experiments. * $p<0.05$ Student t-test

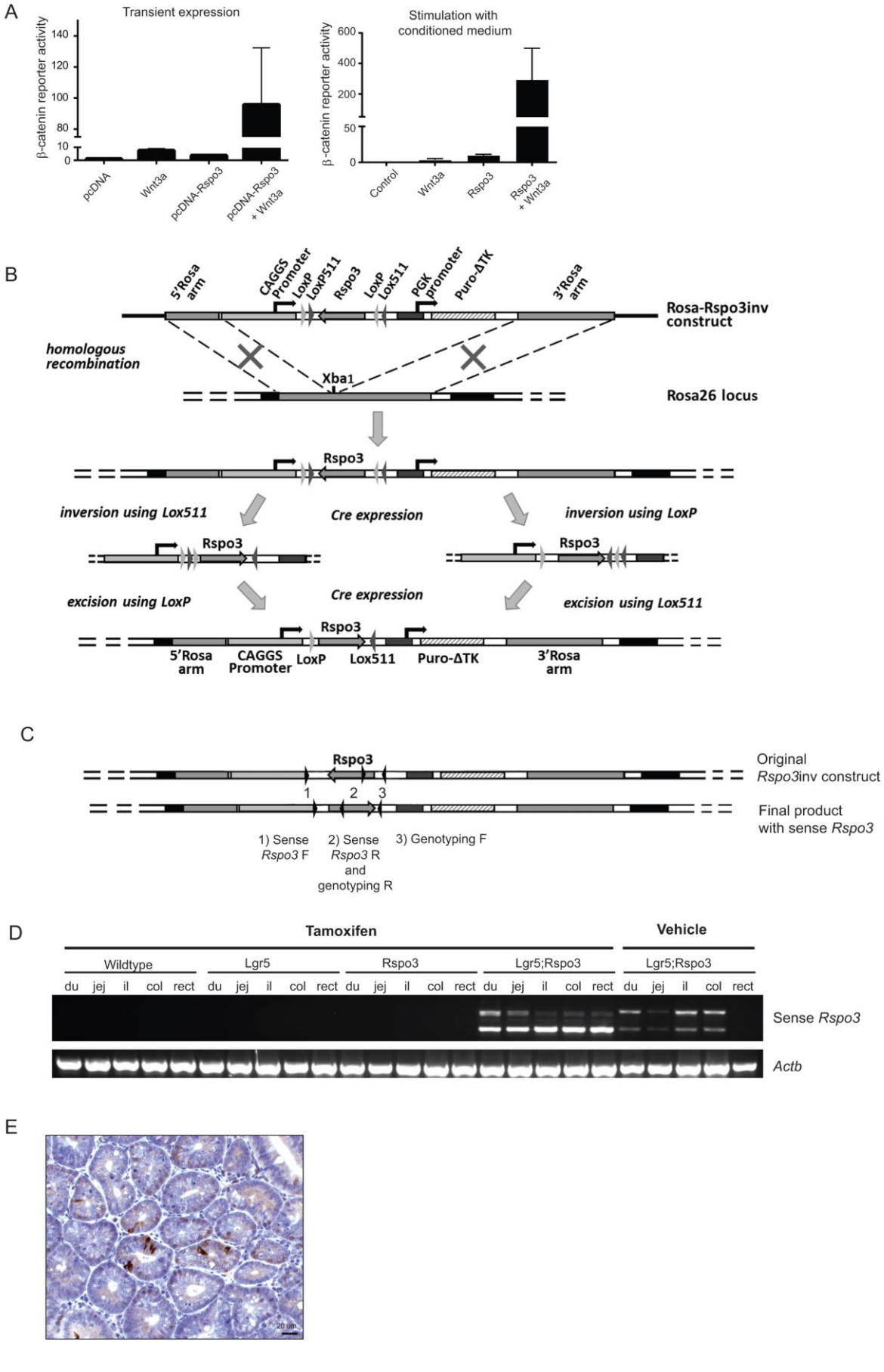
Figure S4. Diagram of unsupervised clustering based upon RNA sequencing of jejunum tissues of Lgr5, Rspo3 and Lgr5;Rspo3 mice. Lgr5;Rspo3 double transgenic animals cluster together and separately from the single transgenic controls, whereas Lgr5 and Rspo3 single transgenic controls do not cluster together. Scale bar indicates the relative phylogenetic distance.

Figure S5. (A) Overview of the individual genes being upregulated per signaling pathway upon *Rspo3* expression, ordered to fold change highest-to-lowest. (B) Most significantly downregulated molecular and cellular functions and (C) signaling pathways upon *Rspo3* expression. Grey points indicate gene ratios per pathway. (D) Overview of the individual genes being downregulated per signaling pathway upon *Rspo3* expression, ordered to fold change highest-to-lowest. All conducted using Ingenuity Pathway Analysis.

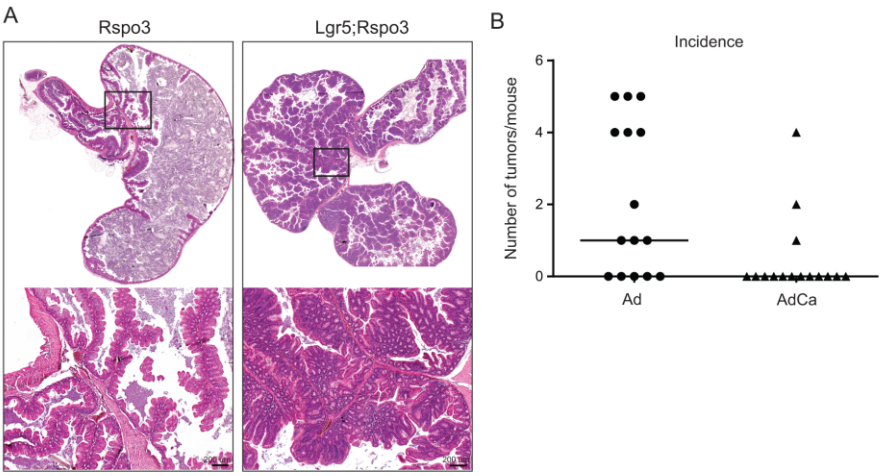
Figure S6. Pictures of additional time points showing Lgr5;Rspo3 double transgenic organoids, (A) 4 and (B) 7 days following seeding, further exemplifying the phenotype of *Rspo3*-expressing organoids. Both at 4 and 7 days, the developing crypts appear aberrant, with enlarged, open lumen.

Figure S7. Kaplan-Meier survival curves of mice expressing transgenic *Rspo3* and/or the mutant *Kras* allele. Although co-expression of mutant *Kras* with *Rspo3* shows a trend to a shorter survival, log-rank test (Mantel-Cox) does not indicate a significant difference in survival ($p=0.07$).

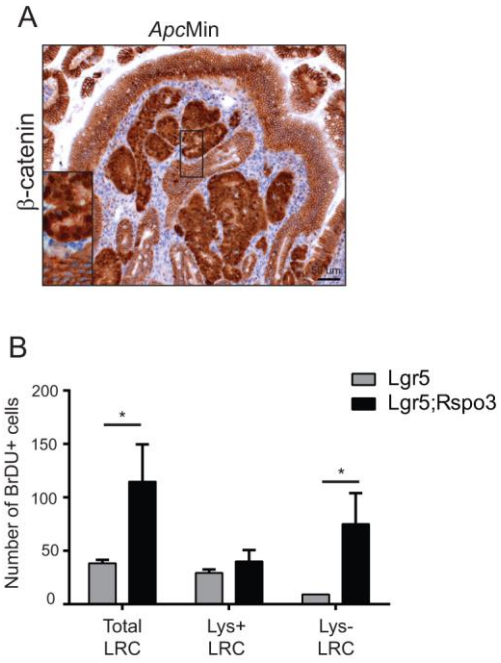
Supplementary Figure 1



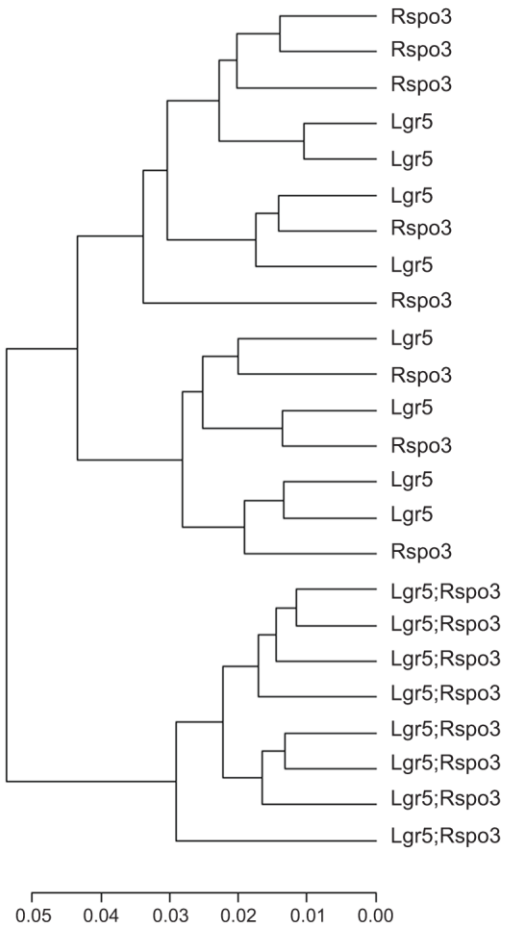
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



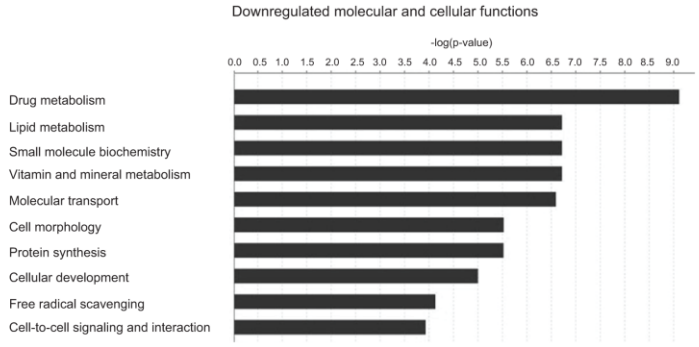
Supplementary Figure 5

A

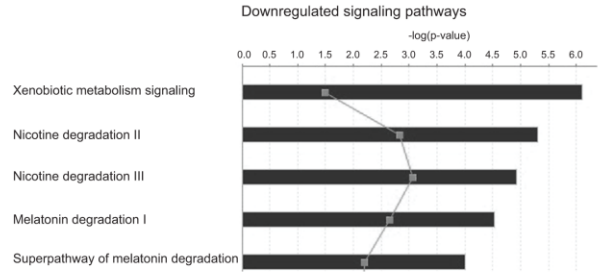
Upregulated

Signaling pathway	Gene ratio	Genes
Atherosclerosis signaling	8/125	Pla2g5, Pla2g2a, Pla2g2f, Pla2g4c, Alox15, Pla2g12a, Lyz, Tnfrsf12a
Wnt/β-catenin signaling	9/170	Lef1, Mmp7, Axin2, Fzd9, Wnt3, Sox9, Bcl9, Myc, Cd44
Eicosanoid signaling	6/82	Pla2g5, Pla2g2a, Pla2g2f, Pla2g4c, Alox15, Pla2g12a
MIF-mediated glucocorticoid regulation	4/35	Pla2g5, Pla2g2f, Pla2g4c, Pla2g12a
Axonal guidance signaling	13/440	Myl7, Mmp7, Fzd9, Wnt3, Ephb3, Sema3b, Tubb2b, Dpysl5, Plxnb1, Sema3c, Epha4, Plxna3, Ephb2

B



C

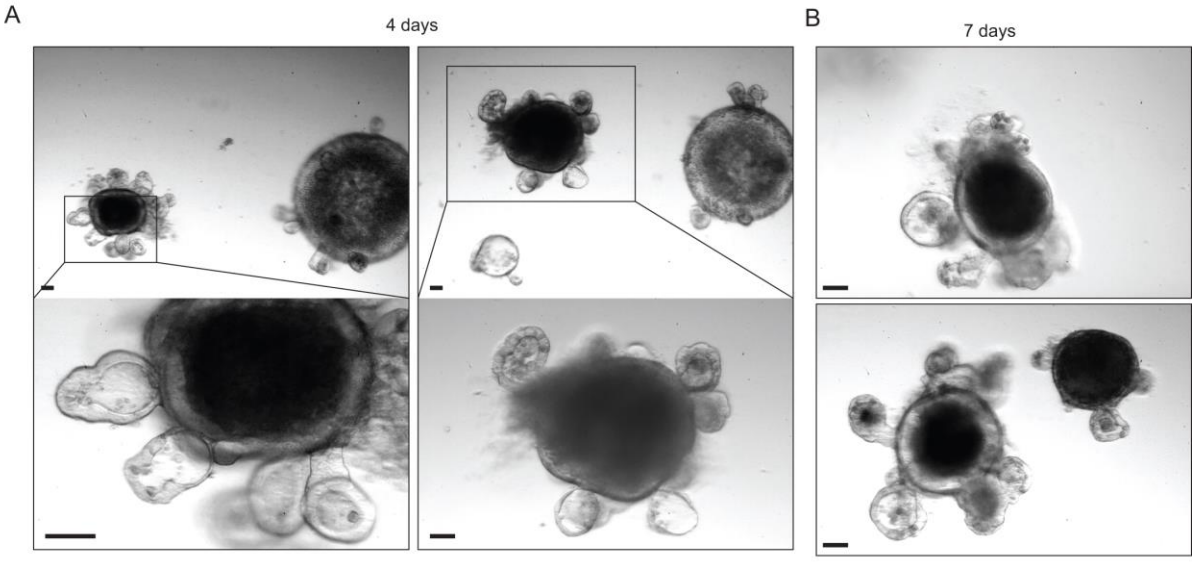


D

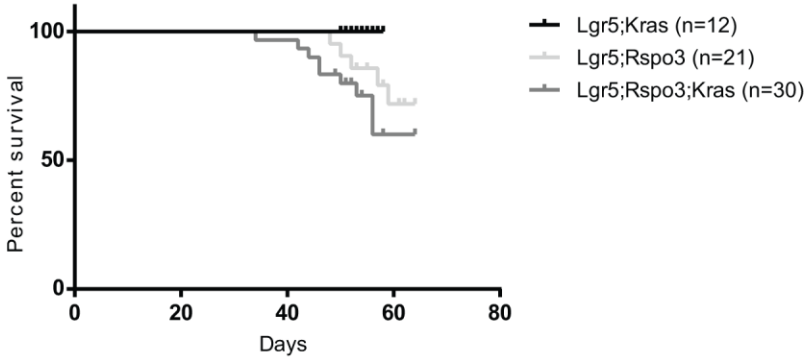
Downregulated

Signaling pathway	Gene ratio	Genes
Xenobiotic metabolism signaling	16/276	Cyp3a5, Cyp2c9, Ugt2b28, Ugt2b7, Aldh1a1, Ahrr, Cyp2b6, Scand1, Ppm1j, Aldh1a3, Chst10, Ugt1a7, Prkcq, Fmo2, Gstk1, Gsta3
Nicotine degradation II	8/73	Cyp3a5, Cyp2c9, Ugt2b28, Ugt2b7, Cyp2b6, Ugt1a7, Fmo2, Cyp4b1
Nicotine degradation III	7/59	Cyp3a5, Cyp2c9, Ugt2b28, Ugt2b7, Cyp2b6, Ugt1a7, Cyp4b1
Melatonin degradation I	7/68	Cyp3a5, Cyp2c9, Ugt2b28, Ugt2b7, Cyp2b6, Ugt1a7, Cyp4b1
Superpathway of melatonin degradation	7/82	Cyp3a5, Cyp2c9, Ugt2b28, Ugt2b7, Cyp2b6, Ugt1a7, Cyp4b1

Supplementary Figure 6



Supplementary Figure 7



SUPPLEMENTARY MATERIALS AND METHODS

Generation of the *Rspo3*^{inv} mouse

The *Rspo3* coding sequence was cloned from cDNA obtained from a Balb/c mouse [1] and sequence verified. The biological activity of the cloned *Rspo3* gene was confirmed in a β-catenin luciferase reporter assay (Figure S1A). Subsequently, the *Rspo3* coding sequence was inserted in antisense orientation in the Xba1 restriction site of a derivative of the pR26-MCS13-puro Rosa26 gene targeting cassette.[2] The original cassette contained the CAGGS promoter, the BGH poly A site and a PGK-Puromycin selection cassette flanked at the 5' and 3' ends by two adjacent long sequences from the Gt(Rosa)26Sor locus. The plasmid was further modified by Dr. Antony Uren, Netherlands Cancer Institute, by adding two pairs of non-homologues lox sites, LoxP and Lox511, in a head to head orientation, 3' to the CAGGS promoter and 5' of the poly A site. In addition, the delta thymidine kinase gene, for negative selection in eukaryotic cells and Frt sites to excise the selection markers were introduced. Upon insertion of *Rspo3* in the plasmid, this was denoted pRosa26-CAGGS-loxP-lox511-Rspo3inv-loxP-lox511-polyA-FRT-PGK-PuroTK- FRT- ROSA26 in concordance with the order of the genetic elements or shortly, ROSA-Rspo3inv (Figure S1B). The construct was verified by restriction enzyme analysis and sequencing. When this construct is introduced in cells that express Cre, either of the homologous Lox pairs will recombine stochastically as depicted in Figure S1B, resulting in inversion of the intervening sequence and thus sense orientation of the *Rspo3* coding sequence. This recombination event will first lead to intermediate products containing direct repeats of either two homologous Lox sites with an intervening non-homologous Lox site up- or downstream of *Rspo3*. Upon continuing Cre activity, both homologous Lox sites will recombine excising one of the homologous and the non-homologous Lox site, leaving the *Rspo3* gene flanked by two non-homologous Lox sites locked in the sense orientation. These consecutive recombination events bring *Rspo3* under control of the upstream ubiquitous CAGGS promoter while transcription terminates at the BGH-polyadenylation sequence.

To generate a transgenic mouse strain that can conditionally express *Rspo3* in various tissues, purified ROSA-Rspo3inv construct was linearized and introduced into strain 129 derived IB10 E14ES cells by electroporation. Cells were cloned by limiting dilution in 96 well plates on mouse embryonic fibroblast feeder cells and wells with single puromycin resistant clones were selected and expanded. DNA was subjected to *KpnI* and *HpaI* digestion followed by Southern blotting. The blots were probed with a radiolabeled probe representing a sequence downstream of the 3'Rosa26 arm present in the construct but within the *KpnI* fragment containing most of the insert. More than half of the puromycin resistant clones gave a band of the predicted size indicating that in these clones the insert is present in one of the Rosa26 alleles. To assure that the constructs were not inserted as a concatemer in the Rosa locus, we also hybridized the blots with an internal (puromycin) probe, which labels DNA fragments at the same position as the 3'Rosa probe. No additional bands were observed in the clones with the correct insert in the Rosa locus. Two clones were selected and karyotyping using Giemsa staining did not show any gross chromosomal abnormalities. Cells of both *Rspo3*^{inv} ES clones were injected into 129/Ola derived

blastocysts, obtaining two male chimeric mice. Backcrossing these mice to the 129/Ola strain resulted in germ line transmission. Subsequently, the offspring of these mice were further backcrossed to 129/Ola mice to maintain two founder lines. Subsequent backcrosses for >9 generations of one of the founder lines to the FVB/NA strain established FVB.129P2-Gt(Rosa)26Sor$^{tm6(CAG-Rspo3)Nki}$/A (MGI:5697338, abbreviated to *Rspo3*^{inv}) strain. The transgenic strain is maintained heterozygous by continuous backcrossing to FVB/NA. Genotyping was performed using forward primer CGCGATTAAATCGATCCCG and reverse primer CCTATCTGCTTCATGCCAATCC (indicated in Figure S1C).

Generation of a *Rspo3*-expressing cell line

Mouse L cells were infected with a LZRS-Zeo retroviral vector containing the *Rspo3* coding sequence,[1] followed by Zeocin-mediated selection (400ug/ml) of transfected cells. Expression of *Rspo3* mRNA was verified by PCR.

β-catenin reporter assay

β-catenin reporter assay was performed in HEK293T cells using either Top-Flash/Fop-Flash (Figure S1 left panel), or Wnt- and mutant responsive element-luciferase reporters (Figure S1 right panel).[3] TK-Renilla was always co-transfected as a control for transfection efficiency. RSPO3 was provided by pcDNA-*Rspo3* transfection or stimulation with conditioned medium derived from stably expressing L-Rspo3 cells, either or not co-stimulated with Wnt3a conditioned medium. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega). Assays were performed three times in duplicate.

X-gal staining

Jejunum tissues were fixed in 2% PFA/ 0.25% glutaraldehyde/ 0.01% NP40 in PBS for 1 hr at RT, followed by washing with PBS and incubating 30 min at RT with equilibration buffer 2mM $MgCl_2$/ 0.02% NP40/ 0.01% sodium deoxycholate in PBS. LacZ substrate consisting of 5mM $K_3Fe(CN)_6$/ 5mM $K_4Fe(CN)_6 \bullet 3H_2O$/ 2mM $MgCl_2$/ 0,02% NP40/ 0,1% sodium deoxycholate/ 1mg/ml X-gal in PBS was incubated overnight at RT. Tissues were washed in PBS and fixed in 4% PFA overnight at 4°C. Finally, tissues were paraffin-embedded and nuclear fast red staining was performed according to routine protocols.

1 Theodorou V, Kimm MA, Boer M, et al. MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nature genetics* 2007;39:759-69.

2 Vooijs M, Jonkers J, Berns A. A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. *EMBO reports* 2001;2:292-7.

3 van Veelen W, Le NH, Helvensteijn W, et al. beta-catenin tyrosine 654 phosphorylation increases Wnt signalling and intestinal tumorigenesis. *Gut* 2011;60:1204-12.

Supplementary Tables

Table I Immunohistochemistry

Single protein stainings

Primary antibody	Company	Dilution	Retrieval	Secondary (all DakoCytomation)
Rabbit-a-Rspo3	ProteinTech	1:100	Citrate	EnVision Goat-a-rabbit-HRP
Rabbit-a-GFP	Abcam	1:1000	Tris/EDTA	EnVision Goat-a-rabbit-HRP
Rabbit-a-Lysozyme	DakoCytomation	1:2000	Citrate	EnVision Goat-a-rabbit-HRP
Rabbit-a-Ki67	Monosan	1:600	Tris/EDTA	EnVision Goat-a-rabbit-HRP
Mouse-a-β-catenin	BD Bioscience	1:200	Tris/EDTA	EnVision Goat-a-mouse-HRP
Rabbit-a-Sox9	Millipore	1:3500	Tris/EDTA	Goat-a-rabbit-biotin, Streptavidin/HRP
Mouse-a-BrDU	DakoCytomation	1:100	Citrate	Goat-a-mouse-biotin, Streptavidin/HRP

Visualization all with DAB (Sigma Aldrich)

Double staining BrDU-Lysozyme

Primary antibodies	Company	Dilution	Retrieval	Secondary
Mouse-a-BrDU	DakoCytomation	1:100	Citrate	Goat-a-mouse-biotin (DakoCytomation), Streptavidin-AP (Invitrogen), AP Visualization Blue (Vectorlabs)
Rabbit-a-Lysozyme	DakoCytomation	1:2000	Citrate	EnVision Goat-a-rabbit-HRP (DakoCytomation), HRP Visualization Red (Vectorlabs)

Table II

RT-PCR

Sense Rspo3 F	5’ TGGGCAACGTGCTGGTTATT 3’
Sense Rspo3 R	5’ CCTATCTGCTTCATGCCAATCC 3’
Actb F	5’ TGAGACCTTCAACACCCCAG 3’
Actb R	5’ GAGCCAGAGCAGTAATCTCC 3’

qPCR

Actb F	5’ GGCTGTATTCCCCTCCATCG 3’
Actb R	5’ CCAGTTGGTAACAATGCCATGT 3’
Axin2 F	5’ GCTCCAGAAGATCACAAAG 3’
Axin2 R	5’ CTTCAGCATCCTCCTGTAT3’
Sox9 F	5’ AGTACCCGCATCTGCACAAC 3’
Sox 9 R	5’ ACGAAGGGTCTCTTCTCGCT 3’
Lgr5 F	5’ GGACCAGATGCGATACCGC 3’
Lgr5 R	5’ CAGAGGCGATGTAGGAGACTG 3’
Rnf43 F	5’ CTGGCTATACCAGCATCGGACT 3’
Rnf43 R	5’ ATGCTGGCGAATGAGGTGGAGT 3’
Cd44 F	5’ CGGAACCACAGCCTCCTTTCAA 3’
Cd44 R	5’ TGCCATCCGTTCTGAAACCACG 3’