# Expression of R-spondin1 in *Apc*$^{Min/+}$ Mice Reduces Growth of Intestinal Adenomas by Altering Wnt and TGFB Signaling

## Short title: RSPO1 suppresses intestinal adenomas in mice

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This work was funded by The Academy of Finland (Centre of Excellence Program 2014–2019 grant 273817, Translational Cancer Biology 307366 and the iCAN Flagship 2019-2022: grant 320185), Cancer Foundation Finland, Sigrid Juselius Foundation, Hospital District of Helsinki and Uusimaa, Helsinki Institute of Life Sciences (HiLIFE) and Biocenter Finland (all to K.A.), The Finnish Medical Foundation, Biomedicum Helsinki Foundation (both to M.L.) and Cancer Foundation Finland (to S.H.), and Cancer Research UK (grant A17196, to D.F. and O.J.S.). T.T. is a Josie Robertson Scholar at the Sloan Kettering Institute and is supported by NIH (grant R00CA187317) and the American Association for Cancer Research (grant 18-20-01-TAMM).

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Abbreviations used in this paper: AAV, adeno-associated viral vector; APC, adenomatous polyposis coli; CRC, colorectal cancer; EdU, 5-ethynyl-2-deoxyuridine; FAP, familial adenomatous polyposis; KEGG, Kyoto encyclopedia of genes and genomes; LEF, lymphoid enhancer-binding factor, LGR, Leucine-rich repeat-containing G-protein coupled receptor; Min, multiple intestinal neoplasia; PROX1, prospero homeobox protein 1; RSPO, R-spondin; scRNAseq, single cell RNA-sequencing; TAM, tamoxifen; TCF, T-cell factor; TA, transit amplifying; TGFB, transforming growth factor beta; TGFBR, TGFB receptor; TGFBRi, TGFBR kinase inhibitor; UMAP, uniform manifold approximation and projection; WT, wild type

Transcription profiling data (single cell RNA sequencing) can be accessed in Gene Expression Omnibus with accession number GSE146139 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146139)

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The authors declare no potential conflicts of interest.

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M.L., S.H., and K.A. designed the experiments, M.L., S.H., J.H., S.K., A.A., D.F, and P.K. performed the experiments, M.L. and S.H. processed the experimental data and performed the analysis, V-M.L. designed and S.K. cloned the AAV constructions, A.R. analyzed the tumor histology, O.R. provided RSPO1–Fc ORF for AAV construction, K.W. and T.T. performed staining of the liver sections, M.H. and O.J.S. performed RNAscope staining, M.L. and K.A. wrote the manuscript and designed the figures, M.L., S.H., J.H., T.T., and O.J.S. discussed the results and provided critical feedback on the manuscript. K.A. supervised the work.

## Abstract

**Background & Aims:** Mutations in genes in the Wnt and beta catenin signaling pathway contribute to development of colorectal carcinomas. R-spondin (RSPO) proteins are secreted proteins that increase Wnt signaling in intestinal stem cells. Alterations in *RSPO* genes were identified in human colorectal tumors. We studied the effects of expressing RSPO1 in $Apc^{Min/+}$ mice.

**Methods:** $Apc^{Min/+}$ mice were given intraperitoneal injection of adeno-associated viral vector encoding an RSPO1–Fc fusion protein or a control vector. Intestinal crypts were isolated from $Apc^{Min/+}$ mice and cultured as organoids, which were incubated with or without RSPO1–Fc and an inhibitor of transforming growth factor beta receptor (TGFBR). Livers were collected from mice and analyzed by immunoblots and immunohistochemistry. Organoids and adenomas were analyzed by quantitative reverse-transcription PCR and single-cell 3′RNA sequencing.

**Results:** Although the RSPO1–Fc vector increased proliferation of intestinal epithelial cells, $Apc^{Min/+}$ mice injected with the vector encoding RSPO1–Fc developed fewer and smaller intestinal tumors and had significantly longer survival times than mice injected with the control vector. also developed fewer and smaller intestinal tumors and had significantly longer survival times. Adenomas of $Apc^{Min/+}$ mice injected with the RSPO1–Fc vector had a rapid increase in expression of genes regulated by the Wnt pathway and apoptosis, followed by reduced expression of mRNAs and proteins regulated by the Wnt pathway, reduced cell proliferation, and less crypt branching than adenomas of mice given the control vector. Addition of RSPO1 to organoids

derived from *Apc*$^{Min/+}$ adenomas decreased frequency of formed organoids and expression of genes regulated by Wnt, but increased phosphorylation of SMAD2 and transcription of genes regulated by SMAD. Addition of the TGFBR inhibitor to organoids incubated with RSPO1–Fc restored organoid formation and expression of genes regulated by Wnt.

**Conclusions:** Expression of RSPO1 in *Apc*$^{Min/+}$ mice increases apoptosis and reduces proliferation and Wnt signaling in adenoma cells, resulting in development of fewer and smaller intestinal tumors and longer survival times of mice. Addition of RSPO1 to organoids derived from adenomas inhibits their growth and promotes proliferation of intestinal stem cells that retain the APC protein; these effects are reversed by TGFB inhibitors. Strategies to increase expression of RSPO1 might be developed for treatment of intestinal adenomas.

**Keywords:** CRC, familial adenomatous polyposis, PROX1, LGR5, KRAS

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality in the Western world. Despite recent advances in the treatment of CRC, one-third of patients succumb to metastatic CRC.[1] The majority of CRC cases are caused by aberrant activation of the Wnt/beta-catenin signaling pathway, commonly due to loss-of-function mutations of the adenomatous polyposis coli (*APC*) tumor suppressor gene. Hereditary forms of *APC* mutations in humans lead to familial adenomatous polyposis (FAP), which is a dominantly inherited autosomal disorder, causing multiple polyps in the colon and small intestine and an extremely high risk of CRC development.[2] After their diagnosis, FAP patients are commonly treated with total colectomy plus ileorectal anastomosis or proctocolectomy with ileal pouch–anal anastomosis[3], leading to decreased quality of life. Inactivation of APC inhibits the proteasomal degradation of beta-catenin, leading to accumulation of beta-catenin and its translocation into the nucleus, where it binds to T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors and activates target genes that stimulate cell-cycle progression and tumorigenesis.[4] During intestinal tumorigenesis, the beta-catenin/TCF/LEF complex activates transcription of the prospero homeobox 1 (*PROX1*) gene, which drives dysplasia and an invasive phenotype.[5] Furthermore, PROX1 promotes proliferation and tumor-initiating properties of cells expressing the intestinal stem cell marker leucine-rich repeat-containing G-protein coupled receptor 5 (*LGR5*).[6]

Together with its homologs LGR4 and LGR6, LGR5 functions as a receptor for ligands of the R-spondin (RSPO) family ligands that function as essential Wnt signal enhancers in multiple adult stem cell compartments, including the intestine.[7-12] However, the role of RSPO/LGR5 signaling in intestinal tumorigenesis is controversial. Some studies suggest that RSPO/LGR5 suppresses intestinal tumorigenesis[13,14], whereas others have reported that LGR5 promotes tumor

progression[15-17]. Interestingly, recurrent and mutually exclusive *RSPO2* or *RSPO3* gene fusions without concomitant *APC* or *CTNNB1* mutations occur in ~10% of colon tumors.[18] Given the function of RSPO ligands in amplifying Wnt signaling in normal tissues, the RSPO fusion proteins are assumed to drive Wnt signaling and tumorigenesis in a Wnt-dependent manner.[18,19] However, frequent occurrence of *RSPO2* promoter hypermethylation that reduces RSPO2 expression in human CRC suggested that RSPO2 functions as a tumor suppressor in CRCs driven by *APC* or *CTNNB1* mutations.[13]

Loss-of-function mutations of the transforming growth factor beta/SMAD (TGFB/SMAD) signaling pathway accumulate during the malignant conversion step in CRC, indicating that the TGFB pathway has a tumor suppressor function in human CRC.[20-23] In mouse models, TGFB exerts its tumor-suppressive functions by inhibiting the transition of premalignant cells to a more malignant phenotype and by limiting the dedifferentiation of cancer cells[20]; loss of TGFB signaling activity induces formation of more aggressive tumors in *Apc;Kras* mutant mice.[24] Mutations inactivate the TGFB signaling pathway in 30–40 % of colon cancers, and reduced TGFB pathway activity is associated with metastatic properties of the intestinal tumors.[25] TGFB ligands bind to type II TGFB receptors (TGFBR2), leading to their heterodimerization with the type I receptor (TGFBR1), which phosphorylates the SMAD2/3 transcription factors.[20] These form a complex with SMAD4, translocate into the nucleus and activate TGFB target gene expression.[20] A recent study showed that the binding of exogenous RSPO1 to LGR5 could directly activate TGFB signaling cooperatively with TGFBR2 in cultured CRC cells, enhancing TGFB-mediated growth inhibition and apoptosis.[14] However, the effects of TGFB have been reported to turn from tumor

suppressive to pro-metastatic as cancers advance, and high stromal TGFB activity has been shown to promote metastasis.[20]

We sought to compare the roles of exogenous Wnt signals and tumor cell-autonomous beta-catenin activation as drivers of PROX1 expression during intestinal cancer progression. To elucidate the function of RSPO1 in PROX1 regulation and in intestinal tumorigenesis, we utilized adeno-associated virus vectors (AAV) to induce systemic expression of RSPO1 in *Apc*-deficient $Apc^{Min/+}$ mice at a stage when these mice harbored aberrant crypt foci or adenomas in their intestine. Surprisingly, we found that in contrast to augmenting Wnt function in the normal intestine, RSPO1 induced tumor cell apoptosis and decreased Wnt/beta-catenin signaling and proliferation of *Apc* mutant adenoma cells via a TGFB pathway-mediated mechanism, leading to regression of the majority of the tumors.

## Materials and methods

### *In vivo* experiments

The National Animal Experiment Board at the Provincial State Office of Southern Finland approved all animal experiments (ESAVI/6306/04.10.07/2016). Mice were housed in individually ventilated cages with enrichment materials, following the guidelines and recommendations of the Federation of European Laboratory Animal Science Association. The *in vivo* experiments were performed with $Apc^{+/+}$ and $Apc^{Min/+}$ mice in the C57BL/6 background (Jackson Laboratories).

For *Prox1* lineage tracing, $Apc^{Min/+}$*;* $Rosa26^{LSL\text{-}TdTomato}$ mice (Jackson Laboratories) were crossed with *Prox1-*$Cre^{ERT2}$ mice.[26] To activate *Prox1* lineage tracing, a single 2 mg dose of tamoxifen

(T5648, Sigma) dissolved in 100 μL of corn oil (8001-30-7, Sigma) was administered by oral gavage.

To label proliferating cells, mice were given a single intraperitoneal (i.p.) injection of 1 μg of EdU diluted in 100 μL of 0.9% saline 4 hours before euthanasia.

For survival analysis of the mice, they were closely monitored and weighed every 3 days during the experiment. Euthanasia was performed upon >15% loss of body weight or detection of melena.

All *in vivo* experiments were repeated at least three times, and 10-12 mice were used for each experiment, with approximately equal numbers of male and female mice of the same age in each experimental group.

Additional materials and methods are described in the Supplemental Material.

## Results

**Systemic expression of RSPO1–Fc suppresses Wnt/beta-catenin signaling and proliferation of adenoma cells in *Apc* mutant mice.** Intraperitoneal administration of RSPO1 is known to potentiate Wnt/beta-catenin signaling in LGR5-expressing intestinal stem cells.[8] To determine whether PROX1 expression in the intestinal epithelium or adenomas is regulated by exogenous stimulation of the Wnt signaling pathway, we constructed an AAV vector encoding a dimeric human RSPO1–Fc fusion protein (AAV-RSPO1–Fc) (**Supplementary Figure 1*A***). Fc–region was included in the protein in order to enhance its solubility and to enable detection of expressed protein in mouse serum samples. To test the vector *in vivo*, we injected $10^{12}$ AAV particles encoding

RSPO1–Fc or empty vector (Ctrl), intraperitoneally into wild-type (WT) mice. Expression of the RSPO1–Fc protein was confirmed in the liver by immunostaining for human Fc (**Supplementary Figure 1*B***), and in mouse serum samples by Western blot analysis at the indicated time points (**Supplementary Figure 1*C***). As expected, we detected an increase in small intestinal crypt depth and villus length already after 2 days of RSPO1–Fc expression and up to 10 weeks thereafter (**Supplementary Figure 1*D*-*E***), whereas the AAV-Ctrl did not produce an effect. Given that the AAV9 vector transduces hepatocytes with high efficiency, we confirmed the expression of the transgene and its biological activity by staining liver sections for human Fc and glutamine synthetase, a marker of metabolic zonation induced by Wnt signaling activity[27], respectively (**Supplementary Figure 1*F***).

To study the effect of RSPO1 in intestinal adenomas, we injected RSPO1–Fc or Ctrl AAV into 17-week-old *Apc*$^{Min/+}$ mice, followed by euthanasia and analysis of the intestines 4 days thereafter (**Supplementary Figure 2*A***). We observed an increase in the diameter of the macroscopic adenomas but no significant difference in their numbers in RSPO1–Fc injected mice (**Supplementary Figure 2*B*-*D***). Strikingly, however, in the immunofluorescence analysis, the relative PROX1 and nuclear beta-catenin-positive (beta-catenin+) adenoma areas appeared to be decreased in the tumors (**Supplementary Figure 2*E, H***), suggesting that RSPO1–Fc suppresses Wnt signaling. Furthermore, we detected a remarkable reduction in the expression of mRNA of *Notum*, a gene regulated by Wnt, in the adenomas from AAV-RSPO1–Fc injected mice (**Supplementary Figure 2*F***). Interestingly, RSPO1–Fc decreased the number of proliferating adenoma cells (**Supplementary Figure 2*G, I***), indicating that RSPO1–Fc suppresses the growth of beta-catenin+ adenoma cells.

To confirm that Wnt signaling activity was indeed reduced within the adenomas of mice given AAV-RSPO1–Fc, we dissociated the tumors into single cells and subjected them to single-cell RNA sequencing (scRNAseq). The subspace among the cells was aligned, followed by nonlinear dimensionality reduction using uniform manifold approximation and projection (UMAP). By using the hierarchical clustering of the scRNAseq data in UMAP, we identified 11 epithelial cell clusters (**Figure 1*A, B***). The results indicated that genes regulated by Wnt/beta-catenin pathway were downregulated by the RSPO1–Fc expression, as indicated by the decreased number of cells in the Wnt-high cell clusters expressing *Prox1*, *Nkd1*, *Notum*, *Lef1*, *Lgr5*, and *Tcf4* (**Figure 1*B,* Supplementary figure 2*J***). The downregulation of Wnt pathway activity was also consistent with KEGG pathway enrichment analysis of the data (**Supplementary Figure 2*K***).

**Prolonged systemic expression of RSPO1–Fc reduces tumor number and dysplasia.** To better assess the impact of systemic RSPO1 expression on the growth of of the adenomas, we next injected 12-week-old $Apc^{Min/+}$ mice with a single dose of AAV-RSPO1–Fc or AAV-Ctrl and quantified tumor diameter and frequency at 1, 4, and 6 weeks thereafter (**Figure 1*C***). Consistent with our previous findings, we found that the diameters of the macroscopic small intestinal tumors were increased during the first week of systemic RSPO1–Fc expression, whereas the total tumor burden was not affected (**Figure 1*D***). Strikingly, however, considerably fewer tumors were found after 4 and 6 weeks in the RSPO1–Fc injected mice compared with the Ctrl group, and the intestinal tumor burden was decreased in the 4- and 6-week groups (**Figure 1*D***). These results indicated that after the first week of RSPO1–Fc expression, the tumors underwent significant regression in the $Apc^{Min/+}$ mice.

Next, we analyzed the effect of systemic RSPO1–Fc expression on Wnt signaling activity in the intestinal tumors. Based on immunofluorescence, the areas positive for nuclear beta-catenin in the RSPO1–Fc expressing mice were located further from the crypts, towards the lumen, when compared to the Ctrl mice (**Figure 1*E***). Moreover, the PROX1 and nuclear beta-catenin+ adenoma areas were much smaller in intestines of AAV-RSPO1–Fc expressing mice than the AAV-Ctrl mice, and only a few such areas remained 4 and 6 weeks after AAV-RSPO1–Fc injection; yet, the relative PROX1+ area within the remaining adenomas remained similar as in the Ctrl group (**Figure 1*E, F***). A remarkable decrease in epithelial cell proliferation in the nuclear beta-catenin+ adenoma areas was observed by EdU labeling 1, 4, and 6 weeks after RSPO1–Fc injection (**Figure 1*E, G***), whereas the proliferation rate of the nuclear beta-catenin-negative normal intestinal epithelial cells was increased (**Figure 1*E, H***). These results also indicated that the initial increase in diameter of the macroscopic adenomas was due to the increased proliferation of the adjacent normal epithelium.

To assess whether the RSPO1–Fc expression affects the histological properties of the tumors, epithelial dysplasia was evaluated by a gastrointestinal pathologist in blind-coded hematoxylin & eosin stained tumor sections (**Figure 2*A*)**. All specimens in the Ctrl group were diagnosed as broad-based tubular adenomas with mild dysplasia. Their crypts were often branched, and they contained reduced interglandular stroma. Cell nuclei were elongated, relatively well polarized, hyperchromatic, and had inconspicuous nucleoli. One week after AAV injection, the tumors in the RSPO1–Fc expressing mice presented less dysplasia and crypt branching than the Ctrl tumors. After 4 and 6 weeks of RSPO1–Fc expression, all specimens were diagnosed as hyperplastic polyps that contained elongated and straight glandular structures. Their ovoid nuclei abutted the epithelial

basement membrane, lacked atypia and showed only mild serration. Thus, systemic expression of RSPO1–Fc gradually decreased dysplasia in adenomas of the *Apc*$^{Min/+}$ mice.

To exclude the possible involvement of the Fc region in the effects of the AAV-RSPO1–Fc vector, we next used an AAV vector to express a monomeric RSPO1–FLAG protein (**Supplementary Figure 3*A***). We injected the vector into 15-week-old *Apc*$^{Min/+}$ mice, which were analyzed 1 and 4 weeks thereafter. As with the dimeric RSPO1–Fc protein, the monomeric RSPO1–FLAG protein also decreased the nuclear beta-catenin+ adenoma area and increased proliferation of nuclear beta-catenin-negative cells (**Supplementary Figure 3*C*** and data not shown). Similar results were obtained with the vector encoding mouse RSPO1–Fc (**Supplementary Figure 3*A*** and data not shown). However, the effects of RSPO1–FLAG on tumor number and burden did not reach statistical significance (**Supplementary Figure 3*B***), suggesting that the dimeric form was more effective in reducing adenoma growth.

**Lineage tracing reveals that RSPO1 provides a competitive growth advantage to the normal epithelium.** To establish whether the outgrowth of normal epithelium displaces the adenoma cells, we used 16-week old *Apc*$^{Min/+}$ mice harboring *Rosa26*$^{LSL-tdTomato}$*; Prox1-Cre*$^{ERT2}$ cell lineage tracer alleles (**Figure 2*B***). We gave the mice tamoxifen by oral gavage, followed by AAV-RSPO1–Fc or AAV-Ctrl 2 days later, and analyzed the mice 7 days thereafter. We found less tdTomato expressing cells in the intestinal epithelium of the RSPO1–Fc-expressing mice than in the Ctrl mice (**Figure 2*C***). The tdTomato+ adenoma areas in mice injected with AAV-RSPO1–Fc were translocated to more luminal parts of the villi than in Ctrl mice, with an intervening layer of proliferating normal epithelial cells (**Figure 2*C***). To confirm the *Apc* status of the remaining nuclear beta-catenin negative cells, we stained the sections with an antibody against the APC C-

terminus, which, together with the all the Axin and beta-catenin binding regions of APC, deleted by the *Apc*$^{Min/+}$ mutation. As expected, the antibody stained only the apical borders of the polarized epithelial cells[28] negative for nuclear beta-catenin but did not stain the beta-catenin-positive adenoma areas (**Supplementary Figure 4*A, B***). In contrast, staining with an antibody against the N-terminal region of APC showed that the truncated mutant APC$^{Min}$ protein was expressed also in the adenomas (**Supplementary Figure 4*A, C***). These results indicated that although small APC deficient tumor cell areas were still found after *in vivo* RSPO1 exposure, the systemic expression of RSPO1–Fc suppresses their growth, as shown by the EdU staining, and also the growth of the clonal progeny of PROX1 positive (Wnt high) adenoma cells. The simultaneously increased RSPO1 stimulated growth of the adjacent *Apc* wild-type epithelium then gradually displaces the remaining adenoma cells, pushing them towards the lumen.

**Long-term systemic expression of AAV-RSPO1–Fc suppresses intestinal tumorigenesis and improves survival of the *Apc*$^{Min/+}$ mice.** To assess the significance of the effect of long-term RSPO1–Fc expression on the *Apc*$^{Min/+}$ mice, we next injected AAV-RSPO1–Fc into 8-week-old mice, which had developed few or no macroscopic tumors at this timepoint (**Figure 3*A***). Upon analysis 10 weeks after RSPO1–Fc injection, the number of tumors in the RSPO1–Fc expressing mice was reduced when compared to the Ctrl mice, and the remaining tumor cells were negative for nuclear beta-catenin and PROX1 (**Figure 3*B*-*E***).

In order to further evaluate if AAV-RSPO1–Fc suppresses the formation of mouse intestinal tumors, we used the tamoxifen-inducible *Apc*$^{fl/fl}$*; Villin-Cre*$^{ERT}$ mouse model, in which rapid tumorigenesis occurs because of complete loss of both *Apc* alleles in all villin-positive intestinal epithelial cells. The mice were injected with RSPO1–Fc and Ctrl vectors, followed by tamoxifen 3

days later to initiate intestinal tumorigenesis (**Supplementary Figure 5*A***). When analyzed 5 days after the tamoxifen injection, the intestinal epithelial cells in the RSPO1–Fc expressing mice showed less PROX1 expression, fewer proliferating (EdU+) cells and more normal tissue architecture than the Ctrl mice (**Supplementary Figure 5*B***), indicating downregulation of Wnt-signaling activity.

To determine whether RSPO1–Fc has an effect on the survival of the $Apc^{Min/+}$ mice, we repeated the experiment in three cohorts of 8-week-old $Apc^{Min/+}$ mice and closely monitored their well-being until they fulfilled humane criteria for euthanasia. RSPO1–Fc expression improved the survival of the $Apc^{Min/+}$ mice by approximately 10 weeks (±SD) (**Figure 3*F***). Furthermore, mice that received RSPO1–Fc had fewer tumors in the small intestine and had a lower overall tumor burden than the Ctrl mice (**Figure 3*G, H***). However, although the RSPO1–Fc mice survived longer than the $Apc^{Min/+}$ mice, they eventually fulfilled the termination criteria due to weight loss or rectal bleeding caused by colonic tumors that were not affected by the RSPO1–Fc expression (**Supplementary Figure 6*A, B***).

**RSPO1–Fc suppresses Wnt signaling and activates the TGFB /SMAD pathway in adenoma cells.** To explore the mechanism of how RSPO1–Fc suppresses Wnt signaling in the intestinal adenoma cells, we investigated the effect of RSPO1–Fc on organoids derived from $Apc^{Min/+}$ mice. A recent study suggested that exogenous RSPO1 binding to LGR5 can directly activate TGFB signaling cooperatively with the TGFBR2 to inhibit cell growth and to induce apoptosis in at least some cultured CRC cell lines.[14] We thus sought to investigate whether decreased growth of the adenoma cells is due to the activation of the TGFB/SMAD signaling pathway. Organoid cultures

obtained from *Apc*$^{Min/+}$ intestinal adenomas were cultured in the presence of RSPO1–Fc with or without the TGFB receptor kinase inhibitor SB-431542 (TGFBRi), and analyzed on days 1, 3, and 6 (**Figure 4*A***). The addition of RSPO1–Fc to the cultures decreased the frequency of organoids in comparison with control cultures when analyzed at 3 days, but this phenotype was rescued by the TGFBRi (**Figure 4*B, C***). The remaining organoids continued to proliferate as indicated by the EdU staining, suggesting that they are resistant to inhibition by the TGFB pathway (**Figure 4*D***). Similar results were obtained by analysis of 4-OH-TAM induced organoids from *Apc*$^{fl/fl}$; *Lgr5-eGFP-IRES-Cre*$^{ERT}$ mice (**Supplementary Figure 7**). RSPO1–Fc led to the suppression of mRNAs of *Prox1* and *Notum,* genes regulated by Wnt pathway (**Figure 4*E***). Surprisingly, however, *Lgr5* and *Myc* mRNAs were upregulated in the organoids that remained after 6 days of addition of RSPO1–Fc as analyzed by qPCR (**Figure 4*E***). This is most likely due to residual epithelial cells that lack the *Apc* mutation that remained in the cultures, although we cannot exclude the possibility that the elevated LGR5 expression level provides a survival advantage for cultured *Apc* mutant cells, as LGR5 expression has been reported to correlate with metastatic properties and poor prognosis in CRC.[16] Immunostaining of the *Apc*$^{Min/+}$ organoids 3 days after RSPO1–Fc addition confirmed the decreased expression of PROX1, which was rescued in the presence of TGFBRi (**Figure 4*D***). These data suggest that RSPO1-mediated suppression of the Wnt pathway is mediated by activation of the TGFB/SMAD pathway.

To confirm the involvement of the TGFB/SMAD pathway, we performed Western blotting analysis of the organoids cultured with RSPO1–Fc. We observed that RSPO1–Fc stimulated the phosphorylation of SMAD2 (**Figure 4*F***), indicating activation of the TGFB/SMAD pathway. Furthermore, scRNAseq of the *Apc*$^{Min/+}$ organoids cultured with RSPO1–Fc for 24h showed

increased expression of several genes regulated by TGFB/SMAD pathway, such as *Cdkn2b*, *Hes1*, *Tgfbr2*, *Smad4*, *Gadd45a*, *Tgfbr1*, *Ep300*, and *Furin* (**Figure 4*G***), suggesting that TGFB/SMAD pathway activation is enhanced soon after the addition of RSPO1–Fc. These results indicated that RSPO1–Fc suppresses the growth of *Apc* mutant organoids by suppressing the Wnt pathway and activating the TGFB/SMAD pathway, which leads to cell death.

To verify the activation of TGFB signaling in the adenomas *in vivo*, we stained the tumors from mice after 4 days of AAV-RSPO1–Fc expression for the TGFB target pSMAD3 and the downstream cell cycle kinase inhibitor p21 (**Figure 5*A*-*C***). Increased phosphorylation of SMAD3 and increased expression of p21 were detected in the tumors from RSPO1–Fc expressing mice (**Figure 5*B, C***). Moreover, we observed increased expression of genes regulated by TGFB/SMAD, such as *Tgfbr2*, *Smad4*, *Cdkn1b*, *Myc*, *Bach1*, and *Ep300*, in the scRNAseq analysis of the tumors from mice 4 days after AAV-RSPO1–Fc injection (**Figure 5*D***). KEGG pathway enrichment analysis of these tumors indicated upregulation of the SMAD2/3 nuclear pathway after 4 days of RSPO1–Fc expression (**Figure 5*E***), confirming that the growth-suppressive effect of systemic RSPO1–Fc expression is associated with an enhancement of the TGFB/SMAD signaling *in vivo*.

**RSPO1–Fc induces an early wave of apoptosis in *Apc*$^{Min/+}$ adenomas.** Given that the effect of systemic RSPO1–Fc expression on intestinal tumors was already obvious 4 days after gene delivery, we tested whether RSPO1–Fc induces apoptosis specifically in beta-catenin+ adenoma cells. We injected 17-week-old *Apc*$^{Min/+}$ mice with AAV-RSPO1–Fc or AAV-Ctrl and analyzed them 1, 2, 3, and 4 days thereafter (**Figure 6*A***). AAV-RSPO1 caused a significant increase in apoptosis in the beta-catenin positive adenoma areas already after 1 day of RSPO1–Fc expression

(**Figure 6*B, C***). The apoptosis rate was decreased on day 2 and even further on day 3, yet remaining higher than in the AAV-Ctrl tumors (**Figure 6*B, C***). Furthermore, in scRNAseq analysis, the expression levels of apoptosis-related genes, such as *Trp53*, *Cdkn1c*, *Bcl2l11*, *Bax*, and *Pycard*, were enriched in AAV-RSPO1–Fc vs. AAV-Ctrl injected tumors on day 4 after the RSPO1–Fc injection (**Figure 6*D***). KEGG pathway enrichment analysis indicated that several apoptosis-related pathways (apoptosis pathway, apoptotic execution phase pathway, and p53 activity regulation pathway), were upregulated in tumors from AAV-RSPO1–Fc expressing mice (**Figure 5*E***). Moreover, concomitant administration of the TGFBRi in combination with AAV-RSPO1–Fc, restored the apoptosis rate back to the level observed in adenomas from AAV-Ctrl injected mice (**Figure 6*B, C***). Interestingly, the scRNA analysis indicated that the expression of several genes regulated by Wnt also increased simultaneously with the increase in apoptosis 1 day after the AAV-RSPO1–Fc injection, but the Wnt signaling activity subsequently began to decrease already on day 2 after the AAV-RSPO1–Fc injection (**Supplementary Figure 8**). These findings suggest that the combination of rapidly increased Wnt signaling and TGFB/SMAD pathway activation leads to apoptosis and eventual loss of $Apc^{Min/+}$ adenomas.

**Oncogenic mutant *Kras* protects tumor cells from the growth-suppressing effect of systemic RSPO1–Fc expression.** We previously showed that oncogenic mutant *Kras* protects *Apc*-mutant organoids from TGFB–induced apoptosis.[23] In a majority of advanced CRCs, many of the TGFB signals that promote tumor progression are mediated via stromal cells.[20] Thus, additional genetic insults that are acquired during tumor progression, such as KRAS-activating and TGFB pathway-inactivating mutations, may protect CRC cells from the tumor-suppressive effects of RSPO1. In order to examine whether RSPO1-mediated tumor suppression could be abrogated by an oncogenic

*Kras* mutation in the adenoma cells, we next injected *Apc*$^{fl/fl}$*; Villin-Cre*$^{ERT}$ (*VApc*) and *Apc*$^{fl/fl}$*; Kras*$^{G12D/+}$*; Villin-Cre*$^{ERT}$ (*VApcK*) mice with either RSPO1–Fc or Ctrl AAVs, induced Cre activation with tamoxifen by oral gavage during the 2 subsequent days and analyzed the mice 4 days later (**Supplementary Figure 9*A***). Consistent with our previous findings, we also found increased staining for phosphor-SMAD3 and p21 in the intestines from *VApc* mice, but not from *VApcK* mice injected with AAV- RSPO1–Fc (**Supplementary Figure 9*B, C***). Thus, KRAS abolishes the adenoma-suppressing effect of RSPO1.

## Discussion

Our results indicate that systemic delivery of soluble RSPO1 protein extends the survival of *Apc*$^{Min/+}$ mice. The combination of increased growth of the normal intestinal epithelium and inhibition of the adenomatous growth resulted in decreased number and luminal displacement of the adenomas, eventually leading to the regression of most of the tumors and substantial improvement in the survival of the *Apc*$^{Min/+}$ mice. The tumor-suppressive effect of RSPO1 was also reproduced in intestinal organoid cultures from *Apc* mutant mice, and the phenotype was rescued *in vivo* and *ex vivo* by inhibition of the TGFB/SMAD pathway.

In the *Apc*$^{Min/+}$ mouse model, the mice develop multiple intestinal adenomas and a smaller number of colonic tumors, whereas in humans, most of the intestinal tumors develop in the colon and rectum, with only a small proportion in the small intestine.[29] In our experiments, some of the AAV-RSPO1–Fc injected mice met the termination criteria prematurely because of colonic tumors that did not respond to RSPO1–Fc expression. The cellular mechanisms that are responsible for such

difference in the RSPO1 response between small intestinal and colonic tumors remain to be investigated.

We found that RSPO1–Fc leads to enhanced TGFB/SMAD pathway-mediated growth suppression specifically in the adenomas, but not in the adjacent WT intestinal epithelium. This is consistent with our previous work showing that the *Apc* mutation increases intestinal epithelial cell sensitivity to the proapoptotic effects of TGFB/SMAD signaling.[23] Our previous studies showed that TGFB-induced apoptosis in *Apc* mutant organoids, including the LGR5+ adenoma stem cells, was mediated by upregulation of the BH3-only proapoptotic protein Bcl-2-like protein 11 (BIM).[23] In the present study, we found rapid upregulation of several genes regulated by Wnt after RSPO1–Fc expression. According to the "just-right Wnt" hypothesis[30], this by itself may make the adenoma cells more susceptible to apoptosis. However, after a few days, apoptosis and the expression of several genes regulated by Wnt and cell proliferation in the adenomas was decreased, explaining the further suppression of tumor growth.

AAV-RSPO1–Fc injection increased expression of *Tgfbr2* and *Smad4*, phosphorylation of SMAD2 and SMAD3, and their downstream targets, BIM and the cell-cycle inhibitor p21 in the adenomas. Furthermore, our experiments with the TGFB pathway inhibitor indicated that the TGFB pathway contributed to induction of apoptosis specifically in the beta-catenin positive adenoma cells. This is consistent with the previously described mechanism in which RSPO1 receptor LGR5 abnormally activates TGFBR2 in CRC cells and leads to TGFB/SMAD-mediated growth inhibition and apoptosis.[14] The differential sensitivity to TGFB gives the healthy intestinal epithelial cells a growth advantage over the adenoma cells, eventually leading to the extrusion of the adenomatous tissue. The RSPO1-TGFB mechanism also explains why the deletion of the RSPO1 target *LGR5*

in a human CRC cell line increased liver and lung metastasis of tumor fragments transplanted into the caecal subserosa in mice.[14] When considering these results, it should also be noted that the effect of the TGFB/SMAD pathway turns from tumor suppressive to pro-metastatic in more progressed tumors via activation of stromal paracrine signals.[20]

In the normal intestine, RSPOs act as agonists of the Wnt signaling pathway, stimulating crypt cell proliferation by stabilizing beta-catenin.[8-12] RSPOs have been reported to augment Wnt signaling activity by binding to LGR4, LGR5, and LGR6, and promoting their interaction with transmembrane E3 ligases RING finger protein 43/zinc and RING finger 3 (RNF43/ZNRF3), which are encoded by genes regulated by Wnt, forming a negative Wnt feedback loop.[31] Interestingly, a mild reduction of Wnt ligand secretion by a porcupine inhibitor accelerated fixation of Apc-deficient cells within the crypt leading to accelerated tumorigenesis.[32] In agreement with this, RSPO1 stimulated Wnt signaling in our experiments led to loss of the competitive growth advantage of $Apc^{-/-}$ vs. WT epithelial cells.

RSPO2 and RSPO3 gene fusions found in CRCs are mutually exclusive with *APC* and *CTNNB1* mutations.[18,19] As such, it has been speculated that the former activate the Wnt pathway and stimulate intestinal tumorigenesis, although it is uncertain whether RSPO rearrangements alone can induce intestinal tumorigenesis.[18,19] Here we find that systemic RSPO1–Fc expression suppresses the growth of $Apc^{Min/+}$ adenomas, suggesting that at least in the early phases of CRC development, RSPOs could be utilized therapeutically. Our findings are consistent with the report showing that the related RSPO2 can act as a tumor suppressor in human CRCs, where promoter hypermethylation followed by RSPO2 downregulation correlated with tumor cell differentiation, tumor size, and metastasis.[13] In several CRC cell lines, RSPO2 induced an LGR5-dependent

feedback loop inhibiting Wnt signaling, thus exerting a net growth-suppressive effect on the CRC cells. However, RSPO2 has also non-redundant functions with the other RSPO members, especially during embryogenesis.[33] Further studies should show if the effect of systemic RSPO2 or RSPO3 expression differs from that of RSPO1.

LGR5 homologs are facultative components of the Wnt signaling pathway, mediating Wnt signal enhancement by the RSPOs, as evidenced by abrogation of RSPO1 responses following knockdown of *LGR5*.[13,14] Both LGR4 and LGR5 are co-expressed in stem cells in the intestine, colon, stomach, and hair follicles.[7,34,35] In human CRC cell lines, LGR5 expression is highly variable, whereas LGR4 is strongly expressed and LGR6 is almost undetectable.[14] When LGR5 expression was knocked down in two colon carcinoma cell lines, LGR4 expression was not affected, suggesting that LGR4 does not compensate for LGR5 loss.[14] Hence, the effect of RSPOs on LGR4-mediated Wnt signaling in colon cancer remains to be determined.

The TGFB pathway has been implicated in the control of epithelial regeneration in the intestine. A recent study discovered that expression of Wnt5a, a non-canonical Wnt ligand capable of activating other Wnt responses besides beta-catenin, is upregulated during crypt regeneration after injury. Wnt5a inhibits intestinal epithelial stem cell proliferation by activation of TGFB signaling via the Wnt5a receptor Ror2 and TGFB receptor I kinase activity.[36] However, unlike RSPO1, Wnt5a does not activate TGFB signaling in colon cancer cells, and Wnt and RSPO ligands are functionally non-equivalent.[14,37]

In conclusion, we show that RSPO1 plays a role in intestinal tumorigenesis, yet this role appears to be opposite to the one previously considered. Systemic expression of RSPO1–Fc activates a

negative feedback loop by a rapid enhancement of Wnt-signaling pathway that leads to apoptosis of the adenoma cells due to the recruitment of the TGFB/SMAD pathway via LGR5/TGFBRII heterodimer, and thereafter mainly by suppression of the growth of the remaining adenoma cells. In contrast, RSPO1–Fc increases the proliferation of the neighboring normal intestinal stem cells. This intriguing difference in sensitivity and qualitative response could provide a novel mechanism for inhibition of tumor growth or even the eradication of early-stage tumors. The remarkable increase in survival of the $Apc^{Min/+}$ mice obtained by systemic expression of a growth factor is unprecedented and highly significant. For a possible translation of these results, the RSPO1 delivery would need to be controlled and better targeted to the intestinal epithelium. One could also envision future use of RSPO1 together with targeted repair of *APC* or *CTNNB1* mutations in e.g. FAP patients to secure selection of the patient's successfully repaired intestinal epithelial cells in organoid cultures, before cell transplantation back into the intestine. Further developments along these lines might eventually benefit FAP patients, for whom the only currently available treatment is subtotal prophylactic colectomy, leading to a significantly reduced quality of life.

**Acknowledgments**

We thank Dr. Hans Clevers and Dr. Gao Yajing for scientific advice, Dr. Shentong Fang, Dr. Saara Ollila, Dr. Pekka Katajisto, Simon Anderson, and Jennifer Paech for help with the experiments, and María Arrano de Kivikko, Tanja Laakkonen, Tapio Tainola, Katja Salo, David He, Vilja Jokinen, Laura Amoedo Lopez, Tony Helenius, and Jefim Brodkin for technical assistance. We also thank the Biomedicum Imaging Unit for microscopy services, Laboratory Animal Center of the University of Helsinki for technical assistance, Single Cell Analytics core facility at the Technology Centre of Institute for Molecular Medicine Finland (FIMM), HiLIFE, University of

418 Helsinki supported by Biocenter Finland, for providing services and Genome Biology Unit
419 supported by HiLIFE, the Faculty of Medicine, University of Helsinki, and Biocenter Finland, for
420 providing slide scanning services.

## References


1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA: a cancer journal for clinicians. 2018;68:7-30.

2. Vasen HF, Moslein G, Alonso A, et al. Guidelines for the clinical management of familial adenomatous polyposis (FAP). Gut 2008;57:704-713.

3. Gregorieff A, Clevers H. Wnt signaling in the intestinal epithelium: From endoderm to cancer. Genes Dev. 2005;19:877-90.

4. Petrova TV, Nykänen A, Norrmén C, et al. Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. Cancer Cell. 2008;13:407-19.

5. Wiener Z, Högström J, Hyvönen V, et al. Prox1 promotes expansion of the colorectal cancer stem cell population to fuel tumor growth and ischemia resistance. Cell Rep. 2014;8:1943-56.

6. Barker N, Van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449:1003.

7. Kim KA, Kakitani M, Zhao J, et al. Medicine: Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science. 2005;309:1256-9.

8. Binnerts ME, Kim KA, Bright JM, et al. R-Spondin1 regulates wnt signaling by inhibiting internalization of LRP6. Proc Natl Acad Sci U S A. 2007;104:14700-5.

9. Kim KA, Wagle M, Tran K, et al. R-spondin family members regulate the wnt pathway by a common mechanism. Mol Biol Cell. 2008;19:2588-96.

10. de Lau W, Barker N, Low TY, et al. Lgr5 homologues associate with wnt receptors and mediate R-spondin signalling. Nature. 2011;476:293.

11. Storm EE, Durinck S, e Melo, Felipe de Sousa, et al. Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. Nature 2016;529:97-100.

12. Wu C, Qiu S, Lu L, et al. RSPO2–LGR5 signaling has tumour-suppressive activity in colorectal cancer. Nature communications. 2014;5:3149.

13. Zhou X, Geng L, Wang D, et al. R-Spondin1/LGR5 activates TGFB signaling and suppresses colon cancer metastasis. Cancer Res. 2017;77:6589-602.

14. Barker N, Ridgway RA, Van Es JH, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature. 2009;457:608-11.

15. Tsuji S, Kawasaki Y, Furukawa S, et al. The miR-363-GATA6-Lgr5 pathway is critical for colorectal tumourigenesis. Nature communications. 2014;5:3150

16. Baker A, Graham TA, Elia G, et al. Characterization of LGR5 stem cells in colorectal adenomas and carcinomas. Scientific reports. 2015;5:8654.

17. Seshagiri S, Stawiski EW, Durinck S, et al. Recurrent R-spondin fusions in colon cancer. Nature. 2012;488:660.

18. Han T, Schatoff EM, Murphy C, et al. R-spondin chromosome rearrangements drive wnt-dependent tumour initiation and maintenance in the intestine. Nature communications. 2017;8:15945.

19. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. Immunity. 2019;50:924-40.

20. Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev. 2005;19:2783-810.

21. Wang J, Yang L, Yang J, et al. Transforming growth factor beta induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells. Cancer Res. 2008;68:3152-60.

22. Wiener Z, Band AM, Kallio P, et al. Oncogenic mutations in intestinal adenomas regulate bim-mediated apoptosis induced by TGF-B. Proc Natl Acad Sci U S A. 2014;111:E2229-36.

23. Cammareri P, Vincent DF, Hodder MC, et al. TGFβ pathway limits dedifferentiation following WNT and MAPK pathway activation to suppress intestinal tumourigenesis. Cell Death Differ. 2017;24:1681.

24. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor B receptor type II in microsatellite stable colon cancers. Cancer Res. 1999;59:320-4.

25. Harvey NL, Srinivasan RS, Dillard ME, et al. Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. Nat Genet. 2005;37:1072-81.

26. Benhamouche S, Decaens T, Godard C, et al. Apc tumor suppressor gene is the "zonation-keeper" of mouse liver. Developmental cell. 2006;10:759-70.

27. Reinacher-Schick A, Gumbiner BM. Apical membrane localization of the adenomatous polyposis coli tumor suppressor protein and subcellular distribution of the beta-catenin destruction complex in polarized epithelial cells. J Cell Biol 2001;152:491-502.

28. Jackstadt R and Sansom O. Mouse models of intestinal cancer. J Pathol. 2016;238:141–15.

29. Fodde R, Tomlinson I. Nuclear β-catenin expression and Wnt signalling: in defence of the dogma. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland 2010;221:239-241.

30. de Lau W, Peng WC, Gros P, et al. The R-spondin/Lgr5/Rnf43 module: Regulator of wnt signal strength. Genes Dev. 2014;28:305-16.

31. Huels D, Bruens L, Hodder M, et al. Wnt ligands influence tumour initiation by controlling the number of intestinal stem cells. Nature communications 2018;9:1-10.

32. Nam J, Park E, Turcotte TJ, et al. Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. Dev Biol. 2007;311:124-35.

33. Jaks V, Barker N, Kasper M, et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet. 2008;40:1291.

34. Barker N, Huch M, Kujala P, et al. Lgr5 ve stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell stem cell. 2010;6:25-36.

35. Yan KS, Janda CY, Chang J, et al. Non-equivalence of Wnt and R-spondin ligands during Lgr5 intestinal stem-cell self-renewal. Nature 2017;545:238-242.

36. Miyoshi H, Ajima R, Luo CT, et al. Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. Science. 2012;338:108-13.

## Figure legends

**Figure 1. AAV-RSPO1–Fc suppresses growth and Wnt/beta-catenin signaling activity in intestinal adenomas of *Apc*$^{Min/+}$ mice.** (*A*). Uniform manifold approximation and projection (UMAP) visualization of cluster annotation based on the expression of known cell type markers in intestinal adenomas of AAV-RSPO1–Fc or AAV-Ctrl injected *Apc*$^{Min/+}$ mice based on scRNA-seq. Individual dots correspond to single cells. (*B*). UMAPs representing Wnt-high adenoma cell clusters based on scRNA-seq analysis of tumor samples from AAV-transduced *Apc*$^{Min/+}$ mice. (*C*). Schematic of the experiment. 12-week-old *Apc*$^{Min/+}$ mice were injected with AAV-RSPO1–Fc or AAV-Ctrl ($10^{12}$ vp) and analyzed 1, 4, and 6 weeks thereafter. (*D*). Comparison of tumor number and small intestinal tumor burden in 15 mice per group. (*E*). Immunofluorescent staining of PROX1, beta-catenin, EdU, and Dapi in tumor sections. White dashed line indicates nuclear beta-catenin+ adenoma area. (*F*). Quantification of PROX1+ nuclear beta-catenin+ adenoma area. (*G*). Quantification of EdU+ nuclear beta-catenin+ adenoma area %. (*H*). Quantification of EdU+ area % within the nuclear beta-catenin-negative area. Scale bars: 100 μm, data is presented as mean+SD, n=15+15+15, Student's unpaired *t* test, **P*<0.05, ***P*<0.01.

**Figure 2. RSPO1–Fc induces displacement of adenomas by wild-type intestinal epithelium.** (*A*). Representative hematoxylin & eosin staining of adenomas from AAV-Ctrl and AAV-RSPO1-injected mice 1, 4, and 6 weeks after the AAV injection. (*B*). Schematic of the experiment. 16-week-old *Apc*$^{Min/+}$*; Rosa26*$^{LSL}$*–tdTomato; Prox1-Cre*$^{ERT2}$ mice received tamoxifen by oral gavage to induce tdTomato lineage tracing in PROX1+ adenoma cells. Two days thereafter, AAV-RSPO1–Fc or AAV-Ctrl ($10^{12}$ vp) was injected and the mice were analyzed 7 days later. (*C*). Immunofluorescent staining for PROX1 lineage traced cells (tdTomato), EdU, and Dapi in tumor sections. n=15 per group, Scale bars: 100 μm.

**Figure 3. RSPO1–Fc decreases the number of intestinal adenomas and improves the survival of *Apc*$^{Min/+}$ mice.** (*A*). Schematic of the experiment. 8-week-old *Apc*$^{Min/+}$ mice received one injection of the AAVs and were terminated 10 weeks thereafter. (*B-D*). Comparison of number of remaining tumors and small intestinal tumor burden in 15 mice per group. Black arrowheads in 3*B* indicate intestinal adenomas. (*E*). Staining for PROX1, EdU, beta-catenin, Dapi, and hematoxylin & eosin in intestinal sections. Scale bars: 100 μm. (*F*). Survival of the AAV injected *Apc*$^{Min/+}$ mice by age. (*G*, *H*). Comparison of number of remaining tumors and small intestinal tumor burden of the *Apc*$^{Min/+}$ mice at termination in 20 mice per group. Data is presented as mean+SD, Student's unpaired *t* test, ***P*<0.01, ****P*<0.005.

**Figure 4. RSPO1–Fc suppresses the canonical Wnt/beta-catenin pathway in intestinal organoids from *Apc*$^{Min/+}$ mice.** (*A*). Schematic of the experiment. Intestinal crypts from *Apc*$^{Min/+}$ mice were isolated and cultured in Matrigel to form organoids. RSPO1–Fc fusion protein was added with or without TGFBRi, and the organoids were analyzed 1 and 3 days thereafter. (*B*).

Bright field images of organoids cultured with RSPO1–Fc fusion protein ± TGFBRi for 3 days. (*C*). *Apc*$^{Min/+}$ organoids were plated in equal numbers, and frequency and average diameter of the formed organoids were analyzed after 3 days of culture with RSPO1–Fc fusion protein ± TGFBRi. (*D*). Staining for EdU, PROX1, and Dapi in organoids 3 days after RSPO1–Fc addition. Quantification of PROX1+ area is shown in red in the images. (*E*). Expression of genes regulated by Wnt/beta-catenin pathway in organoids on day 3 (RT-qPCR). (*F*). Western blot analysis of SMAD2 phosphorylation in the intestinal organoids 1 day after RSPO1–Fc addition. (*G*). Violin plots representing expression of mRNA of genes regulated by TGFB/SMAD pathway (*Cdkn2b*, *Hes1*, *Tgfbr2*, *Smad4*, *Gadd45a*, *Tgfbr1*, *Ep300*, and *Furin*) based on scRNA-seq analysis of organoids cultured with RSPO1–Fc for 1 day. Scale bars: 100 μm, data is presented as mean+SD, Student's unpaired *t* test, **P*<0.05, ***P*<0.01, ****P*<0.005.

**Figure 5. RSPO1–Fc activates the TGFB/SMAD pathway in *Apc*$^{Min/+}$ mouse adenomas.** (*A*). Schematic of the experiment. 15-week-old *Apc*$^{Min/+}$ mice received one injection of RSPO1–Fc or Ctrl AAV-vector ($10^{12}$ vp) and were terminated 4 days thereafter, n=15+15. (*B, C*). Staining for phosphorylated SMAD3 and p21 in tumor sections 4 days after injection of the AAVs. Scale bars: 100 μm. (*D*). UMAPs representing expression of mRNA of genes regulated by TGFB/SMAD pathway (*Tgfbr2*, *Smad4*, *Cdkn1b*, *Myc*, *Bach1*, and *Ep300*), based on scRNA-seq analysis of tumor samples from AAV-transduced *Apc*$^{Min/+}$ mice. (*E*). Bar plots of P-values of the upregulated pathways based on KEGG pathway enrichment analysis in the tumor samples.

**Figure 6. AAV-RSPO1–Fc induces apoptosis in *Apc*$^{Min/+}$ adenomas, but TGFBRi rescues the phenotype.** (*A*). Schematic of the experiment. 17-week-old *Apc*$^{Min/+}$ mice received one injection of RSPO1–Fc or Ctrl-AAV vector ($10^{12}$ vp) and were terminated 1, 2, and 3 days thereafter. In addition, the mice received TGFBRi or vehicle on days 0, 1, and 2. (*B*). Staining for cleaved caspase-3 and Dapi in the tumor sections 1, 2, and 3 days after the AAV-RSPO1–Fc or AAV-Ctrl injection ± TGFBRi. Scale bar: 100 μm. (*C*). Quantification of cleaved caspase-3 area % in nuclear beta-catenin-positive and negative adenoma areas. (*C*). Violin plots representing expression of mRNA of genes involved in apoptosis (*Trp53, Cdkn1c*, *Bcl2l11, Bax*, *Bad*, and *Pycard*) based on scRNA-seq analysis of tumor samples from AAV-transduced *Apc*$^{Min/+}$ mice. Data is presented as mean+SD, n=10 per group, Ordinary one-way ANOVA, multiple comparisons, **P*<0.05, ***P*<0.01, ****P*<0.005.