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Lymphangiocrine signals are required for proper intestinal repair after cytotoxic injury

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Summary

The intestinal epithelium undergoes continuous renewal and has an exceptional capacity to regenerate after injury. Maintenance and proliferation of intestinal stem cells (ISCs) are regulated by their surrounding niche, largely through Wnt signaling. However, it remains unclear which niche cells produce signals during different injury states, and the role of endothelial cells (ECs) as a component of the ISC niche during homeostasis and after injury has been underappreciated. Here, we show that lymphatic endothelial cells (LECs) reside in proximity to crypt epithelial cells and secrete molecules that support epithelial renewal and repair. LECs are an essential source of Wnt signaling in the small intestine, as loss of LEC-derived *Rspo3* leads to a lower number of stem and progenitor cells and hinders recovery after cytotoxic injury. Together, our findings identify LECs as an essential niche component for optimal intestinal recovery after cytotoxic injury.

Graphical Abstract

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Author contributions

B.P., J.R., and O.K. conceived and designed the study. B.P., J.R. and E.A.R. performed all experiments. B.P., J.R. and O.K. interpreted the data and wrote the manuscript. B.P., D.V. and D.B. performed analysis of the single cell sequencing data. All the authors read and provided feedback on the figures and manuscript.

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Declaration of interests

The authors declare no competing interests.



In brief

Palikuqi et al. employ imaging of cleared intestinal tissue, transcriptional analyses and genetic mouse models to investigate the role of lymphatic endothelial cells in the intestinal stem cell niche. The authors demonstrate that lymphatic endothelial cells are an essential source of *Rspo3* for proper intestinal recovery after cytotoxic injury.

Introduction

Enteric blood and lymphatic networks are intimately associated with the small intestinal epithelium, including the ISCs that reside in epithelial invaginations called crypts (Bernier-Latmani et al., 2015; Bernier-Latmani and Petrova, 2017; Beumer and Clevers, 2020; Cifarelli and Eichmann, 2019). Over the last decade, several groups have shown that paracrine factors secreted by endothelial cells lining blood and lymphatic vessels, known as angiocrine factors, are important for stem cell maintenance and regeneration in many tissues (Augustin and Koh, 2017; Rafii et al., 2016). Blood endothelial cells (BECs) play a central role in the regeneration of several organs, such as liver, lung, bone marrow, and thymus (Butler et al., 2010; Ding et al., 2010; Ding et al., 2011; Ding et al., 2012; Hu et al., 2014; Wertheimer et al., 2018). Notably, LECs, through the secretion of the lymphangiocrine factor Reelin (RELN), have been linked to heart regeneration (Liu et al., 2020). In addition, the interaction of stem cells with LECs regulates stem cell cycling in the skin (Gur-Cohen et

al., 2019). However, the role of intestinal ECs in ISC self-renewal and regeneration remains undetermined.

ISCs, which are marked by *Lgr5*, and their transit amplifying descendants self-renew and differentiate along the various intestinal epithelial lineages (Barker et al., 2007; Gehart and Clevers, 2019). Maintenance of ISCs is dependent on the niche, which is comprised of several cell types and is an essential source of Wnt ligands and modulators (McCarthy et al., 2020a; Palikuqi et al., 2021). Among these molecules, R-spondins (RSPOs), which bind to LGR receptors (LGR4 and LGR5 in the intestine), potentiate Wnt signaling (de Lau et al., 2011; Glinka et al., 2011) by stabilizing Frizzled receptors (Hao et al., 2012). RSPOs are essential for the expansion and renewal of ISCs both *in vivo* (Yan et al., 2017) and *in vitro* (Sato et al., 2009). RSPO3 is the predominant RSPO in the small intestine (Ogasawara et al., 2018), and inhibition of RSPO3 along with RSPO2 with a neutralizing antibody negatively affects epithelial regeneration after irradiation (Storm et al., 2015).

Several mesenchymal populations have been reported to be essential sources of Wnt ligands and modulators in the intestine (Degirmenci et al., 2018; Kabiri, 2014; McCarthy et al., 2020b; Stzepourginski et al., 2017; Valenta et al., 2016). For instance, mesenchymal cells marked by *Foxl1*⁺, known as telocytes, express high levels of *Wnt2* and *Wnt5a* (Aoki et al., 2016; Kondo et al., 2019; Shoshkes-Carmel et al., 2018). The inhibition of Wnt ligand secretion by deletion of *Porcupine (Porc)* in *Foxl1*⁺ telocytes results in rapid stem and progenitor cell loss (Shoshkes-Carmel et al., 2018). Another subtype of mesenchymal cells, known as trophocytes and marked by expression of *Grem1* and *Cd81* and low expression of *Pdgfra*, resides in proximity to ISCs and expresses the Wnt-family member *Wnt2b* as well as high levels of all R-spondins. Ablation of the trophocyte mesenchymal population leads to loss of *Lgr5*⁺ cells, while the population of transient amplifying (TA) cells remains stable (McCarthy et al., 2020b). Moreover, deletion of *Rspo3* in *Pdgfra*⁺ cells does not have a major effect on epithelial cells during homeostasis (Greicius et al., 2018), suggesting that this essential signal is produced by several different niche cell types.

LECs express high levels of *Rspo3* (Kalucka et al., 2020; Ogasawara et al., 2018), but it is unknown if LECs are an essential source of Wnt signaling in the small intestine during homeostasis or injury. Additionally, while several lines of evidence suggest that endothelial cells become activated and start proliferating in response to intestinal injury to drive epithelial recovery (Abel et al., 2005; Kinchen et al., 2018; Paris et al., 2001; Rehal et al., 2018), the contribution of endothelial cells and specifically LECs to intestinal maintenance and repair remains an open question. Here, we establish the spatial organization of lymphatic vessels in relation to crypt cells and utilize single cell RNA-sequencing (scRNAseq) to uncover the transcriptional composition of LECs, both during homeostasis and after cytotoxic injury. Using mouse genetics, we show that LEC-secreted *Rspo3* is essential for optimal intestinal repair after cytotoxic injury, positioning LECs as a key component of the intestinal niche and a critical source of Wnt signaling.

Results

Lymphatic endothelial cells reside in proximity to crypt cells

We first set out to delineate the location and molecular composition of LECs in the small intestinal niche. We utilized the CUBIC clearing method (Matsumoto et al., 2019), paired with whole mount imaging, to determine the spatial distribution of LECs in the mouse small intestine during homeostasis and to establish the relationship of LECs to BECs and mesenchymal cells in the niche (Figures 1A-B and S1A-B). VECAD⁺ BECs and PDGFRA⁺ mesenchymal cells surrounded every crypt (Figures 1B and S1A), but lymphatic vessels displayed a unique pattern of organization. We found two distinct sets of lymphatic vessels: large lymphatic vessels that encircled the base of the crypts and gave rise to narrow lymphatic vessels in the villi, known as lacteals (Figures 1A-B and S1A-B). To determine the proximity of LECs to *Lgr5*⁺ stem cells in the crypts, we performed whole mount imaging on cleared small intestinal tissue from *Lgr5*^{GFP-CreER} mice. LECs were in proximity to *Lgr5*⁺ crypt cells at the base of the crypts and at the mid-crypt level (Figures 1C and S1C). More than 80 percent of crypts were in proximity to lymphatics at the base and/or mid-crypt levels, with 36 percent of crypts in proximity to LECs only at the base, 23 percent only at the mid-crypt region, and 26 percent at both (Figure 1D).

Lymphatic endothelial cells express high levels of *Rspo3*

Next, we set out to establish the molecular composition of LECs in the small intestine by performing scRNAseq. BECs and LECs were enriched by fluorescence-activated cell sorting (FACS) from the small intestine of wild type mice and identified as CDH5⁺, PODOPLANIN (PDPN)^{Neg} and CDH5⁺, PDPN⁺ cells, respectively (Figures 2A-B). In all, 6000 BECs and 2000 LECs were captured by scRNAseq with BECs clustering in 13 groups and LECs clustering in 2 groups (Figure 2C). LECs could be distinguished from BECs by the high expression *Lyve1* and *Pdpn* (Figure 2D). Clustering was similar amongst 4 different mice in 2 separate experiments (Figure S2D). Notably, LECs uniquely expressed high levels of *Aqp1*, *Cyp4b1*, *Il33* and *Mmrn1* genes compared to BECs (Figure S1E). The transcription factor *Maf* was also selectively expressed in LECs (Figure S1E). *Reelin* (RELN), which is expressed in the heart by LECs and has been found to be essential for heart regeneration, was also highly expressed by LECs but not BECs in the small intestine (Figure S1E).

As Wnt signaling is essential for ISC maintenance and proliferation, we mined the scRNAseq data for the expression of Wnt-family members. As previously reported (Kalucka et al., 2020; Ogasawara et al., 2018), LECs in the small intestine expressed high levels of *Rspo3* (Figures 2E and S1F). Additionally, LECs expressed high levels of *Wntless* (*Wls*, essential for the secretion of Wnt ligands) and the canonical Wnt-family member *Wnt2* (Figures 2E and S1F). Interestingly, *Rspo3* and *Wnt2* were uniquely expressed by LECs, whereas *Wls* was also expressed by BECs (Figures 2E and S1F). *Rspo3* expression by LECs in proximity to epithelial crypt cells was confirmed by *in situ* hybridization (RNAscope) (Figure 2F); no other Wnt-family members were expressed by LECs during homeostasis.

Loss of lymphatic *Rspo3* leads to impaired intestinal recovery after cytotoxic injury

As *Rspo3* is important for the proliferation of stem and progenitor cells in the crypt, we next utilized a genetic model to specifically delete *Rspo3* in LECs. We bred mice carrying the lymphatic driver *Prox1^{CreER}* with *Rspo3^{fl/fl}* mice to generate *Prox1^{CreERT2};Rspo3^{fl/fl}* mice (Figure 3A). Deletion of *Rspo3* in adult LECs did not significantly alter crypts in the small intestine during homeostasis. Control and knock-out mice had similar levels of EdU incorporation, crypt number and OLFM4 expression after tamoxifen induction (Figures 3B-E). Thus, during homeostasis, Wnt signaling from other, redundant niche components, such as PDGFRA⁺ cells, is sufficient for ISC maintenance.

We then assessed whether lymphatic *Rspo3* is essential for intestinal recovery after injury, when crypt epithelial cells must undergo high proliferation. To test this hypothesis, we utilized 5-fluorouracil (5FU), a chemotherapeutic that selectively kills proliferating cells. Control and *Rspo3* knock-out mice underwent sublethal 5FU injury and were sacrificed at day 3 after the last 5FU injection (Figures 3F and S2A-B). Sustained deletion of *Rspo3* after injury was confirmed by qPCR (Figure 3G). After 5FU injury, control crypt epithelium exhibited high levels of proliferation as measured by EdU incorporation, but knock-out mice had significantly decreased proliferation (Figures 3H-I). The total number of regenerating crypts was significantly reduced in knock-out mice (Figure 3J). Additionally, we quantified the size of crypts after 5FU and determined that, in knock-out mice, crypts in proximity to LECs were decreased in size more than those not close to LECs (Figure 3K). The total levels of *Lgr5* mRNA and OLFM4 protein were also decreased in knock-out crypt cells (Figures 3L-O). Lastly, we asked whether loss of *Rspo3* resulted in structural changes in LECs after 5FU injury. LEC density and percent of crypts found in proximity to LECs were similar in control and knock-out mice, suggesting that changes in crypt cells after 5FU were due to lymphangiogenic signaling rather than to loss of LEC structures (Figures S2B-D).

5FU treatment induces substantial damage to proliferating cells, potentially affecting both epithelial and niche cells. We therefore investigated whether a similar requirement for LEC-derived *Rspo3* exists when *Lgr5⁺* cells alone are ablated. We bred *Prox1^{CreERT2};Rspo3^{fl/fl};Lgr5^{DTR}* mice, in which tamoxifen injection leads to deletion of *Rspo3* in LECs, and Diphtheria Toxin (DT) (Tian et al., 2011) injection results in ablation of *Lgr5⁺* cells. Tamoxifen induction was followed by *Lgr5⁺* cell ablation for 3 consecutive days (Figure S2E). Although there was a slight decrease in levels of proliferation as measured by EdU incorporation, the effect was less pronounced compared with that observed after cytotoxic injury (Figures S2F-J). Thus, LEC-derived *Rspo3* is essential for adequate intestinal recovery when there is a requirement for a marked increase in proliferation of epithelial cells and when the niche becomes activated during injury.

Loss of lymphatic *Rspo3* results in lower TA and *Sca1^{high}* cells after cytotoxic injury

Next, we set out to further uncover molecular changes in *Prox1^{CreERT2};Rspo3^{fl/fl}* knock-out mice after 5FU injury. At day 3 after the final 5FU injection, we isolated the following from both control and knock-out mice: crypt epithelial cells as EPCAM⁺, CD44⁺; LECs as CD31⁺, PDPN⁺; BECs as CD31⁺, PDPN^{neg}; and other stromal cells as EPCAM^{neg}, CD31^{neg}. We then performed scRNAseq on these cells (Figures 4A and S3A). Analysis

included cells from two biological replicates with a total of 20,803 cells for control mice and 9,153 for knock-out mice, with cells grouping in 29 clusters (Figures 4B and S3B). Clustering patterns were similar among the two experiments (Figure S3B). Crypt epithelial cells were identified as *Epcam*⁺, *Cd44*⁺, and LECs were identified by the pan-endothelial markers *Cd31*, *Cdh5* or *Kdr* and expression of *Lyve1* or *Pdpr* (Figures S3C-D). We also identified clusters of BECs as positive for *Cd31*, *Cdh5* or *Kdr* and negative for *Lyve1* and *Pdpr*, mesenchymal cells as *Pdgfra*⁺, and myofibroblasts as *Myh11*⁺ (Figures S3C-D). Within the *Pdgfra*⁺ mesenchymal populations, we identified telocytes as *Foxl1*⁺, *Pdgfra*^{high} and trophocytes as *Grem1*⁺, *Cd81*⁺/*Cd81*^{neg}, *Pdgfra*^{low}, amongst others (Figures S4F-G).

To compare the effect of 5FU treatment on crypt epithelial cells in control and knock-out mice, we isolated *Epcam*⁺, *Cd44*⁺ cells from the merged cell population and re-performed cluster analysis (Figure 4C). We identified 9 clusters in *Epcam*⁺, *Cd44*⁺ epithelial cells: stem cells (*Olfm4*⁺, *Lgr5*⁺), cycling transit amplifying cells (TA) (*Mki67*⁺), secretory progenitors (*Atoh1*⁺), secretory cells (including Paneth cells as *Lyz1*⁺ and goblet cells as *Muc2*⁺), enteroendocrine cells (*ChgA*⁺, *ChgB*⁺) and mature enterocytes (*Alpi*⁺) (Figures 4C and S3E). We also identified 3 other clusters of crypt epithelial cells. One was a *Sca1*^{high} (*Ly6a*) cluster; *Sca1* has been identified as a marker of dedifferentiation and of the transition to a fetal-like state in the small intestine after injury (Nusse et al., 2018). We also identified 2 clusters with elevated expression of markers such as *Cd74* and *Gpx2* (activated epithelium 1 and 2), which have been associated with activated epithelium in bacterial and parasitic infections (Haber et al., 2017) (Figures 4C and S3E). We determined differential cell composition and gene expression between control and knock-out samples for each cluster. Knock-out crypt epithelial cells had a lower expression of *Agr2*, *Ang4*, *Muc2*, *Ly6a* (*Sca1*) and *Il33*, amongst others (Figure 4D). Control crypt epithelial cells had a higher proportion of TA and *Sca1*^{high} cells (Figure S3F). Lower numbers of TA and *Sca1*^{high} cells in mutant mice than in control were accompanied by a higher proportion of *Olfm4*⁺, *Lgr5*⁺ cells compared to other crypt epithelial cells (Figure S3F). We confirmed decreased SCA-1 expression in knock-out crypt epithelial cells compared to control cells via flow-cytometry (Figure 4E-G).

Loss of lymphatic *Rspo3* leads to a decrease in mucus-secreting cells

Secretory progenitors and secretory lineage cells were also decreased in knock-out mice. Crypt epithelial cells displayed a decrease in genes involved in mucus production such as *Agr2* and *Muc2* (Figure 4D). *Agr2* is important for mucus secretion and processing, and its loss has been associated with mucus barrier dysfunction and colitis (Zheng et al., 2006). We confirmed by immunofluorescence a decrease in both MUC2 and AGR2 in crypt epithelial cells in mutant mice (Figures 4H-K). Notably, these changes in secretory cells resulted in lower mucus levels in knock-out mice compared to control mice after 5FU injury (Figure 4M).

Knock-out LECs upregulate an endothelial to mesenchymal transition program after 5FU injury

We next asked what molecular changes occurred in LECs after 5FU injury in knock-out mice compared to control mice. We isolated all endothelial cells from the scRNAseq

data and performed clustering analysis. Endothelial cells grouped into 11 distinct clusters (Figures S4A-B). Amongst those, LECs were identified by the expression of *Lyve1* and included 2 clusters: cluster 0 and 3 (Figures S4A-B). In order to better understand these transcriptional changes in LECs, we performed differential expression analysis of all LECs in control versus knock-out mice. Interestingly, genes related to endothelial to mesenchymal transition such as *Prss23* (Bayoumi et al., 2017), *Tgfb1* and *Tgfb2* (Maleszewska et al., 2013; Yoshimatsu et al., 2020), and genes shown to have anti-lymphangiogenic effects such as *Cavin1* (Yang et al., 2020), were expressed at significantly higher levels in *Rspo3* knock-out LECs (Figures S4C-D). Cluster 3, which consists largely of knock-out LECs, was especially enriched in these genes. We confirmed these patterns of changes in LECs by performing qPCR analysis on FACS-isolated LECs (Figure S4E). Lastly, we examined the mesenchymal cell populations and observed that a new *Rspo3^{neg}* myofibroblast population was present in knock-out mice, but not in control mice (Figure S4F-G).

In sum, loss of *Rspo3* from LECs leads to reduced crypt proliferation after cytotoxic injury, which is reflected in a lower number of TA and *Sca^{high}* crypt epithelial cells. *Rspo3* loss in LECs also causes a decrease in mucus-secreting cells and a reduction in mucus production after 5FU injury.

Discussion

The cellular components that constitute the ISC niche are currently the subject of intensive investigation, but the involvement of endothelial cells has been understudied. We report that LECs are physically part of the niche, with large vessels located just under the crypts and in proximity to ISCs, and with smaller lacteal vessels ascending alongside the crypt to the top of villi. Together, over 80% of crypts are in proximity to lymphatic vessels. Analysis of our scRNAseq data revealed that LECs secrete several components of the Wnt pathway, such as *Rspo3*, *Wls* and *Wnt2*. A parallel study utilizing spatial transcriptomics demonstrated that *Rspo3* and *Wnt2* are highly expressed in LECs in the crypt region, compared to lacteals in the villus region (Niec et al, 2022). Deleting *Rspo3* specifically in LECs demonstrated that LEC-secreted *Rspo3* is essential for the proper regeneration of the intestinal epithelium after injury caused by 5FU treatment. *Rspo3* knock-out mice displayed a decrease in proliferative cells in the crypt and lower levels of SCA1+ cells after 5FU injury. Additionally, loss of *Rspo3* in LECs resulted in a marked decrease in mucus-producing cells and in levels of mucus after 5FU injury; this could potentially result in increased vulnerability of crypt epithelial cells to pathogens.

Rspo3 produced by LECs supports regeneration of the small intestine after 5FU-induced injury, but it is dispensable during homeostasis. This indicates that *Rspo3* is produced by several sources in the ISC niche during homeostasis. Consistent with this notion, other studies have shown that deletion of *Rspo3* in *Pdgfra⁺* fibroblasts (Goto et al., 2022; Greicius et al., 2018) or in myofibroblasts (Harnack et al., 2019) alone, or generalized inhibition of RSPO3 by a neutralizing antibody (Storm et al., 2015) has no obvious effect on epithelial homeostasis. Additionally, loss of LEC-derived *Rspo3* seems to have a negligible effect on intestinal repair after ablation of *Lgr5⁺* cells. We report that after a perturbation that is addressed through high levels of proliferation, such as 5FU treatment, *Rspo3* secreted

by LECs is essential for the timely repair of epithelial cells. In addition, LECs themselves appear relatively stable to injury and are relatively unaffected by deleterious conditions, such as 5FU (this paper) or irradiation (Goto et al., 2022).

The 5FU injury triggers a massive proliferative and damage response, as seen by the high number of EdU⁺ cells, and this is not seen when *Lgr5*⁺ cells alone are ablated. The number of proliferative cells per crypt stays about the same as during homeostasis after ablation of *Lgr5*⁺ cells, but it more than doubles after 5FU injury. The massive proliferation in the crypts after 5FU requires all sources of *Rspo3* available including that coming from LECs. We propose that there is a hierarchy of lymphatic *Rspo3* need that increases from homeostasis through ablation of *Lgr5*⁺ cells to 5FU injury. It therefore appears that intestinal maintenance and regeneration rely on distinct niche sources of *Rspo3*, presumably depending on the level of damage and the proliferation required to achieve repair. This also suggests that the intestinal niche should not be seen as static under all conditions, but rather that different niche sources come together depending on need and injury to facilitate proper regeneration.

Angiocrine factors support stem cells and regenerative processes of several tissues. This has led to an appreciation that ECs are more than just the plumbing that carries blood and oxygen, but rather that they provide essential molecular signaling for maintaining tissue function. Our findings demonstrate that lymphangiocrine-derived factors in the intestine are critical for intestinal regeneration after cytotoxic injury. This suggests that LECs could be an important source of secreted factors in other tissues as well. The finding of a supportive role for LECs to ISC and regenerative cells is also relevant to diseases in the gut. In colorectal cancer (CRC), lymphangiogenesis is one cause of the development of metastasis (Li et al., 2011; Tacconi et al., 2015). Our finding that after 5FU treatment LEC-secreted *Rspo3* contributes to ISC maintenance suggests that LECs could also be involved in the promotion of cancer. Furthermore, maintenance of the lymphatic vessel density of LECs after 5FU treatment raises questions about the potential involvement of LECs in cancer cell survival and escape from chemotherapy.

Limitations of the study

In our work, we determined that lymphatic derived *Rspo3* contributes to intestinal regeneration after 5FU injury. However, we did not observe a phenotype after conditional deletion of *Rspo3* from the lymphatics during homeostasis. In a complementary study to ours, Goto et. al explored all sources of *Rspo3* in the intestine. They found that when *Rspo3* is deleted from both mesenchymal and lymphatic sources, ISC maintenance at homeostasis was affected (Goto et al., 2022). We also determined that LEC derived *Rspo3* is dispensable for intestinal recovery after ablation of *Lgr5*⁺ cells, but it is possible that we could have identified a phenotype if we looked at shorter or longer time points after ablation of *Lgr5*⁺ cells.

STAR METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ophir Klein (Ophir.klein@ucsf.edu).

Materials availability—All the materials will be available upon request to the lead contact under material transfer agreement with UCSF.

Data availability—All scRNAseq data have been deposited on the Gene Expression Omnibus (GEO) and can be viewed under accession number GSE198469. This manuscript did not generate any new code. Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse strains—All mouse experiments were conducted under the IACUC protocol AN176864 at the University of California San Francisco. All mice (male and female) were used at 8-12 weeks of age at the beginning of each experiment. *Rspo3^{fl/fl}* mice were a gift of Christof Niehrs (Scholz et al., 2016), *Prox1^{CreERT2}* mice were purchased from Jackson labs (022075), *Lgr5^{DTR/GFP}* were originally derived at Genentech and are a kind gift of Fred de Sauvage (Tian et al., 2011), *Lgr5^{GFP-IRES-CreERT2}* were purchased from Jackson labs (008875; (Barker et al., 2007)). For knock-out induction, mice were gavaged with tamoxifen in corn oil (200 mg/kg) 6 times, 2 x 3 days interspersed by 3 days of rest. For EdU proliferation experiments, mice were injected with EdU (5mg/mL) 2 hours before sacrifice. For injury experiments, mice were injected for 3 consecutive days with either 5FU at 75 mg/kg in 0.9% saline or Diphtheria toxin (DT) (50 µg/kg). For vessel visualization, mice were injected with an antibody against VECAD (25 µg/mouse in 100 µl of PBS) conjugated to Alexa-647 8 min prior to sacrifice.

METHOD DETAILS

Tissue collection and preparation for microscopy—For all microscopy experiments, the jejunum was collected and fixed in 4% PFA for 24h. For sections, tissues were incubated in 30% sucrose for 24h at 4°C and then embedded in OCT before being sectioned to a thickness of 8 µm. For whole mount, fixed tissues were cleared for 3 to 6 days in CUBIC-L solution (10% N-butyldiethanolamine, 10% Triton X-100 (Matsumoto et al., 2019)).

Immunofluorescence, RNAscope, EdU labeling on sections and Whole mount staining—Frozen sections were baked at 60°C for 30min, unmasked for 30min, blocked (5% NDS, 1X Animal Free blocking, 0.3% Triton X-100) for 1h and incubated overnight with primary antibodies at 4°C (table). Then, sections were incubated with secondary antibodies for 1h at RT (table).

RNAscope was done according to the manufacturer's protocol. Briefly, the sections were post-fixed in 4% PFA, incubated with hydrogen peroxide to inhibit endogenous peroxidase

before performing target retrieval. Subsequently, the tissues were incubated with specific RNA probes (table), followed by revelation and amplification steps.

For EdU labeling, after the baking, slides were permeabilized for 5min in 1% Triton X-100 and EdU staining performed according to manufacturer protocol (table).

For whole mount staining, 1 cm tissue pieces were blocked for 1 day, incubated with primary and then secondary antibodies for 2 days and finally post-fixed for 1 day in 4% PFA (Bernier-Latmani, 2016). Each step was done at 4°C.

For all staining and labeling, tissues were counterstained with DAPI and mounted in Prolong Gold. The images were taken using a Zeiss Apotome 910 system.

Quantification of staining—All image quantifications were done using ImageJ software. The proliferative cell number was quantified by counting the number of EdU⁺ cells per crypt and calculating the mean for each mouse (~30 crypts per mouse). MUC2⁺ and AGR2⁺ cell numbers were quantified by counting positive cells in the crypts except Paneth cells present at the bottom (~30 crypts per mouse). For quantification of MUC2 intensity, crypts were traced and manually segmented. Yen thresholding followed by particle analysis was then performed on the segmented image and the mean grey value was measured for each region of interest (~40 crypts/mouse). The crypt number was calculated by counting OLFM4⁺ crypts in 20 to 30 mm of tissue length. The OLFM4 intensity was analyzed by calculating the mean of the staining in a defined area corresponding to the crypt (~50 crypts per mouse). *Lgr5* RNAscope was quantified by calculating the mean of positive dots in each crypt (~20 crypts per mouse). For the quantification of crypt number in close proximity to lymphatic vessels, crypts were assigned to different categories: 1) crypt base: at least one cell of the crypt base is above a lymphatic vessel, 2) mid-crypt: at least one side of the mid-crypt is beside of a lymphatic vessel, 3) mid-crypt only, 4) crypt base only, 5) both crypt base and mid-crypt, 6) either crypt base or mid-crypt and 7) not in proximity: neither crypt-base nor mid-crypt (~200 crypts per mouse). For the quantification of lymphatic vessel density, after Z-projection of all optical sections, the area fraction of the LYVE-1 signal (LYVE-1 positive area on total area) was quantified for each mouse. For crypt size calculation, in whole mount staining, crypts were first classified as in proximity or not with lymphatics as previously, then transverse optical sections at the mid crypt position were chosen to quantify the area of the width using ImageJ (~200 crypts per mouse).

Tissue preparation and digestion—Small intestines from adult mice were collected and cut into small pieces (0.5 cm) and put in 30 mL of dissociation solution (RPMI + 1% Glutamax, 1% non-essential amino acids, 2.5% HEPES, 1% Pen-strep, 5mM EDTA, 3% FBS and 1 μM DTT) in a rotator at 37°C for 30 min. Tissues were washed twice in HBSS, spun down and further minced using scissors. The minced tissue was then digested in a rotator for 30 min at 37°C in 4 ml of digestion solution (Collagenase A (2.5 mg/ml), Dispase II (1 U/ml) and DNase (30 μg/ml) in HBSS with 1% Glutamax, 1% non-essential amino acids and 2.5% HEPES). The cells were strained (100 μM strainer) and the left-over tissue digested for another 30 min in more 4 ml of digestion solution. Both fractions were stained with antibodies and Aria II with a 100-micron nozzle utilized to perform the sort.

For sequencing experiments during homeostasis: Small intestines were digested and prepared as described above. Data are from two biological replicates. Each biological replicates contains cells from one adult male and one adult female mouse. Lymphatic endothelial cells were sorted as CD31⁺, PDPN⁺, EPCAM^{neg}, CD45^{neg} and blood endothelial cells as CD31⁺, PDPN^{neg}, EPCAM^{neg} and CD45^{neg}. 6000 BECs and 2000 LECs were sequenced in total.

For 5FU experiments: At day 3 after the last 5FU injection, small intestines were digested and prepared as described above. Data are from two biological replicates. Each biological replicate contains cells from one adult male and one adult female mouse. Crypt epithelial cells were sorted as EPCAM⁺, CD44⁺, CD45^{neg}, CD31^{neg}, lymphatic endothelial cells were sorted as CD31⁺, PDPN⁺, EPCAM^{neg}, CD45^{neg}, blood endothelial cells as CD31⁺, PDPN^{neg}, EPCAM^{neg}, CD45^{neg}, epithelial cells as EPCAM⁺, CD45^{neg}, CD31^{neg} and stroma cells EPCAM^{neg}, CD45^{neg}, CD31^{neg}. After sorting, the different populations were mixed and loaded into 10x Chromium.

For SCA-1 validation experiments: At day 3 after the last 5FU injection, small intestines were digested and prepared as described above. Data are from 7 biological replicates. SCA-1⁺ cells were gated on the EPCAM⁺, CD44⁺, CD45^{neg}, CD31^{neg} population.

scRNAseq analysis—The single-cell suspension was loaded onto the 10X Chromium Single Cell instrument (10X Genomics). Single-cell barcoding, and cDNA libraries were constructed using the 10X Chromium single-cell 3' Library Kit according to the manufacturer's protocol. Libraries were sequenced on an Illumina NovaSeq 6000 and the sequence output processed with Cell Ranger 5.0.1 to produce filtered feature-barcode count matrices (<http://10xgenomics.com>).

Single-cell analyses were performed using the Seurat package in R (v.4.1.0) (Butler et al., 2018). Count matrices filtered to retain only genes expressed in three or more cells in a sample, were used for further analysis. Cells with high mitochondrial gene percentage or in the 1st or 99th percentile of transcript counts were also removed. Initially, all sequenced cell types were analyzed together. Data were processed following standard practices, including log-normalization of UMI counts, and regression of mitochondrial gene expression. Principal component analysis was subsequently performed on the most variable genes. Cluster identification (at a resolution of 0.5) and UMAP visualization was performed on the top 20 principal components. Differential gene expression for gene-marker discovery across the clusters was performed using the Wilcoxon rank-sum test.

Subsequently, for 5FU experiments the following analyses were performed. 1) For crypt epithelial cells: *Epcam*⁺, *Cd44*⁺ cells were identified as having expression of *Epcam* >1, *Cd44* >1 and expression of *Cdh5*, *Cd31*, *Kdr* and *Pdgfra* <1. *Myh11*⁺ cells were also excluded. 2) ECs were isolated as *Cdh5*, *Cd31*, *Kdr* >1 and *Epcam*, *Cdh1*, *Pdgfra* <1. EC clusters that expressed *Lyve1* were identified as LECs. 3) Mesenchymal cells were isolated as *Pdgfra* >1, *Cdh5*, *Kdr*, *Cd31*, *Epcam*, *Cdh1* <1. For each cell type, filtering, clustering, and differential gene expression testing was done as described above. For pathway analysis, WEB-GESTALT (WEB-based Gene SeT Analysis Toolkit) (Liao et al., 2019) was utilized,

where genes that had at least a 0.5 Log₂ upregulation in control LEC vs knock-out LEC or genes that had at least a 0.5 Log₂ upregulation in knock-out LECs vs. control LECs were utilized for over-representation analysis. False discovery rate (FDR)-adjusted *P* values of less than 0.05 were considered statistically significant.

Mucin staining—For mucin staining, the jejunum (middle third of the small intestine) was isolated 3 days after the last 5FU injection. Fragments of 1.5 cm in length containing fecal pellets were then selected and placed directly into methacarn fixative (60% methanol, 30% chloroform, 10% acetic acid). Fragments were fixed for 3 days rocking at room temperature. Following fixation, fragments were washed twice in methanol for 20 minutes each wash, twice in absolute ethanol for 20 minutes each, and twice in xylene for 10 minutes each. The tissue was then embedded in paraffin blocks following standard procedures and cut into 4, 7, and 14 μm sections. Sections were stained with 20 μg/ml UEA1-FITC.

mRNA extraction and RT-qPCR analysis—Tissues were collected and LECs were sorted as described above. 20,000 LECs were then resuspended in lysis buffer from the PicoPure™ RNA Isolation Kit. mRNA was extracted following the manufacturer's protocol and resuspended in 13.2μL of elution water. mRNA was then used for reverse transcription using the High-Capacity cDNA Reverse Transcription Kit. The resulting cDNA was diluted (4X) before proceeding to qPCR using TaKaRa Taq DNA Polymerase mix. Relative gene expression was calculated by using Ct method and normalized to *β2m* expression. Results are displayed as mean +/- s.e.m., Mann Whitney U-test, two-sided.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were assessed and analyzed using appropriate statistical methods. Mann Whitney U-test was utilized unless otherwise noted. GraphPad Prism v.9 was used for all statistical analysis, unless otherwise indicated. No statistical methods were used to determine sample size. Unless otherwise stated, the experiments were not randomized. The investigators were blinded during 5FU and DT experiment quantifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Intestinal crypts are in proximity to lymphatics, which express *Rspo3*.
- Intestinal recovery is impaired in lymphatic *Rspo3* knock-out mice after 5FU injury.
- Lymphatic *Rspo3* knock-out mice have decreased progenitor cells after 5FU injury.
- Lymphatic *Rspo3* knock-out mice have reduced mucus production after 5FU injury.

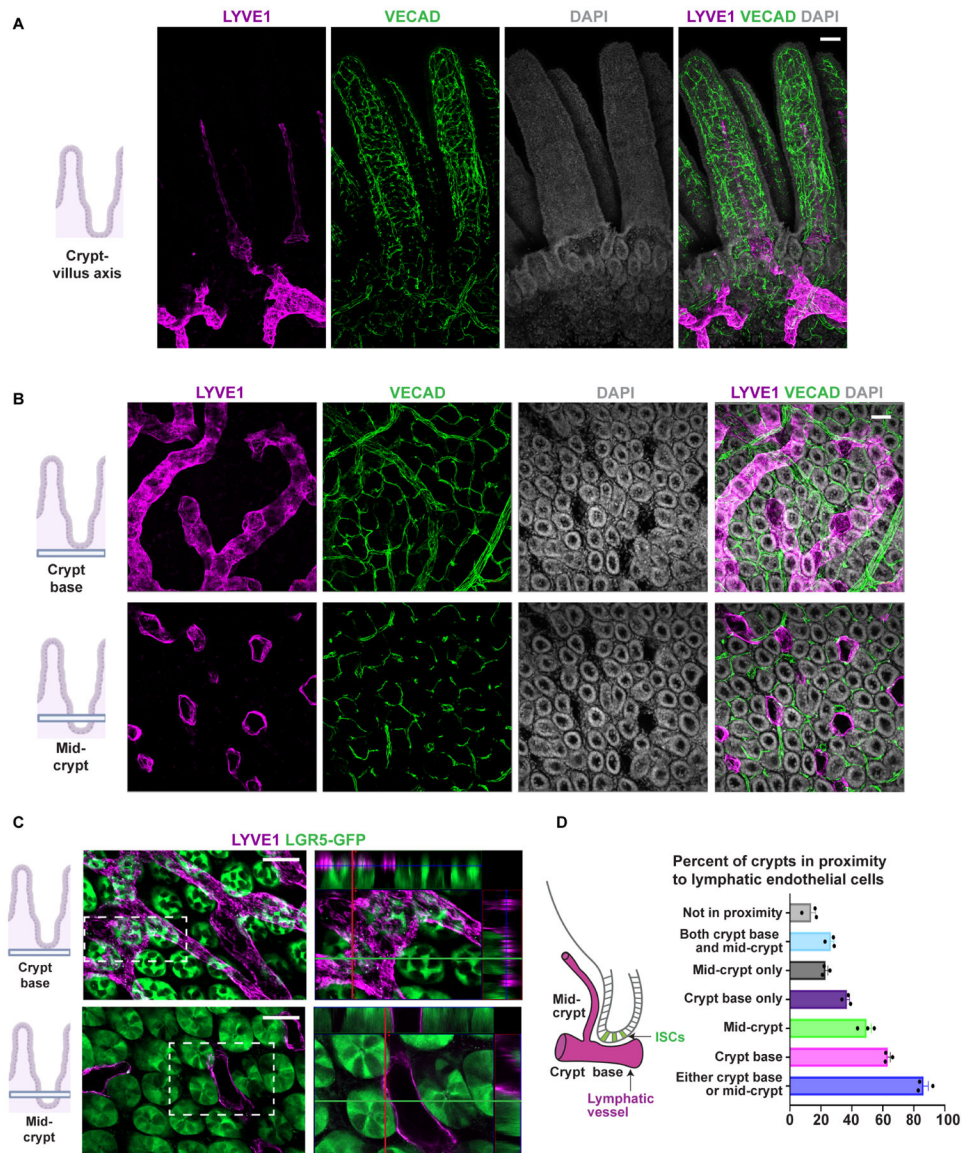


Figure 1. LECs reside in proximity to crypt cells.

A) Sagittal view of whole mount imaging of CUBIC cleared tissue from adult wild type mouse small intestine stained for LYVE1 and VECAD showing the localization of lymphatic and blood vessels within the crypt-villus axis in the small intestine. **B-C)** Transverse view of LYVE1 and VECAD showing **(B)** the organization of LECs and BECs at both the crypt base region and mid-crypt region in adult wild type mice, as well as, **(C)** the LECs proximity to *Lgr5*⁺ ISCs in adult *Lgr5*^{GFP-Cre} mice. Insets represent orthogonal projections of the whole mounts depicting adjacency and at times contact of LECs to *Lgr5*⁺ ISCs. **D)** Left: schematic representation of position of lymphatic vessels in relation to crypts. Right: quantification of percentage of crypts in vicinity to LECs at the base of the crypts, at mid-crypt level or not in proximity to any LECs (n=3 mice). Scale bars = 50 μ m. See also Figure S1.

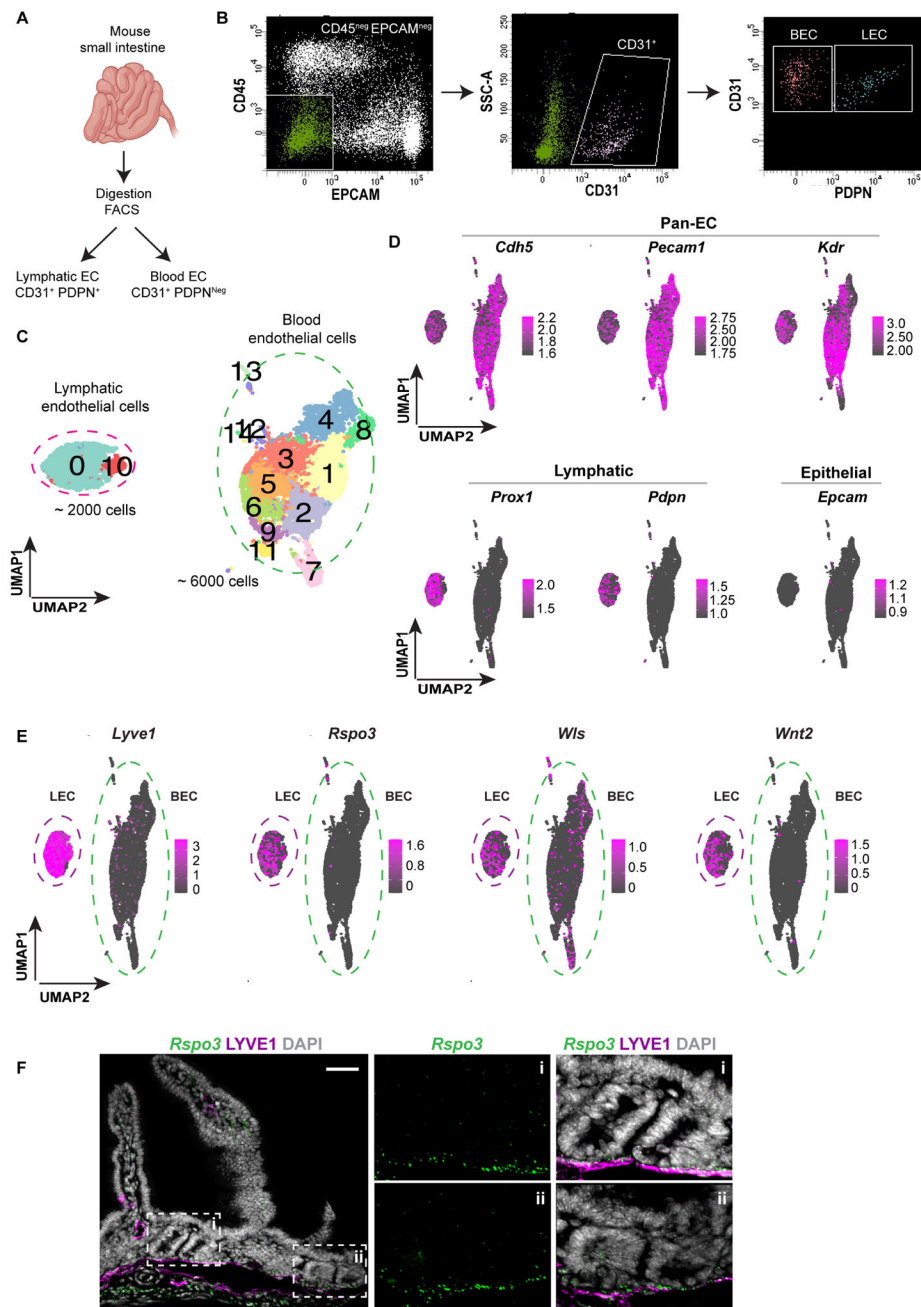


Figure 2. LEC scRNAseq data reveal expression of *Rspo3* and other Wnt signaling molecules. **A)** Small intestines from 4 wild-type mice in 2 independent experiments were digested, and LECs and BECs were isolated by sorting CD31⁺, PDPN⁺ and CD31⁺, PDPN^{Neg} cells, respectively. **B)** Gating strategy to isolate LECs and BECs. First, we selected CD45^{Neg} and EPCAM^{Neg} cells to exclude immune and epithelial cells, then CD31⁺ endothelial cells were isolated, and finally LECs and BECs were distinguished as PDPN⁺ and PDPN^{Neg}, respectively. **C)** UMAP (Uniform Manifold Approximation and Projection) plot of all ECs showing that LECs group in 2 clusters (cluster 0 and 10) and BECs group in 13 different clusters. **D)** UMAP plots of the expression of markers for pan-endothelial, lymphatic, and

epithelial cells validating the identity of LECs and BECs. **E)** UMAP plots of *Lyve1*, *Rspo3*, *Wls* and *Wnt2* expressions inside EC clusters showing that LECs in the small intestine express high levels of *Rspo3* and *Wls* and *Wnt2*. **F)** *Rspo3* *In situ* hybridization (RNAscope) coupled with LYVE1 immunofluorescence of adult mouse small intestine demonstrating expression of *Rspo3* mRNA in LECs that are in proximity to the crypt region. Scale bar = 50 μm . See also Figure S1.

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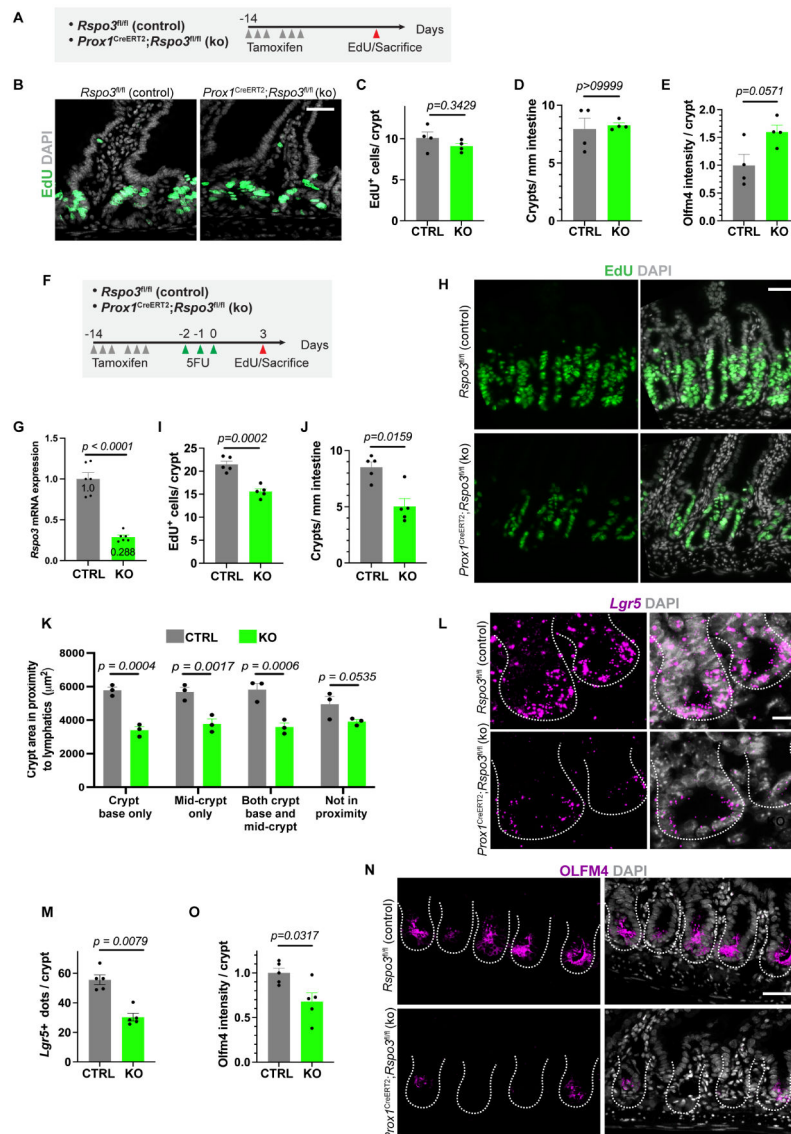


Figure 3. Lymphatic $Rspo3$ is essential for intestinal recovery after 5FU injury.

A) Tamoxifen induced $Prox1^{CreERT2};Rspo3^{fl/fl}$ knock-out mice and control $Rspo3^{fl/fl}$ were sacrificed 14 days after beginning of tamoxifen induction and after a 2-hour EdU pulse. **B)** Representative images of EdU staining from control and knock-out mice at homeostasis (n=4 mice). Scale bar = 50 μm . **C)** Quantification of EdU⁺ cells per crypt at homeostasis for control (CTRL) and knock-out (KO) mice (n=4 mice). **D)** Quantification of total crypts per millimeter (mm) at homeostasis for control (CTRL) and knock-out (KO) mice (n=4 mice). **E)** Quantification of OLFM4 staining intensity for control (CTRL) and knock-out (KO) mice at homeostasis (n=4 mice). **F)** After tamoxifen treatment, control (CTRL) and $Rspo3$ knock-out (KO) mice were treated for 3 days with 5FU at 75 mg/kg. Recovery was assessed at day 3 after injury. Mice were injected with EdU 2 hours before sacrifice. **G)** Deletion efficiency as quantified by $Rspo3$ mRNA levels analyzed by RT-qPCR in sorted LECs at day 3 after the end of 5FU treatment (n=6 mice). **H)** Representative images of EdU staining in the crypts of control and $Rspo3$ knock-out mice at day 3 and **I)** quantification of EdU⁺

cells per crypt at day 3 (n=5 mice). Scale bar = 50 μm . **J**) Quantification of total crypts per millimeter (mm) at day 3 after 5FU treatment for control (CTRL) and knock-out (KO) mice (n=5 mice). **K**) Quantification of crypt size (area in μm^2 of crypt section) in relation to their position to lymphatic vessels in control and *Rspo3* knock-out mice at day 3 after 5FU (n=3 mice). **L**) Representative images of *Lgr5*⁺ *in situ* hybridization (RNAscope) for control and knock-out mice at day 3 after 5FU and **M**) quantification of *Lgr5* transcripts per crypt (n=5 mice). Scale bar = 20 μm . **N**) OLFM4 staining in control and knock mice at day 3 after 5FU (n=5 mice). Scale bar = 50 μm . **O**) Quantification of OLFM4 intensity staining per crypt at day 3 after 5FU (n=5 mice). Bar graph data are mean \pm s.e.m., Mann Whitney U-test, two-sided. See also Figure S2.

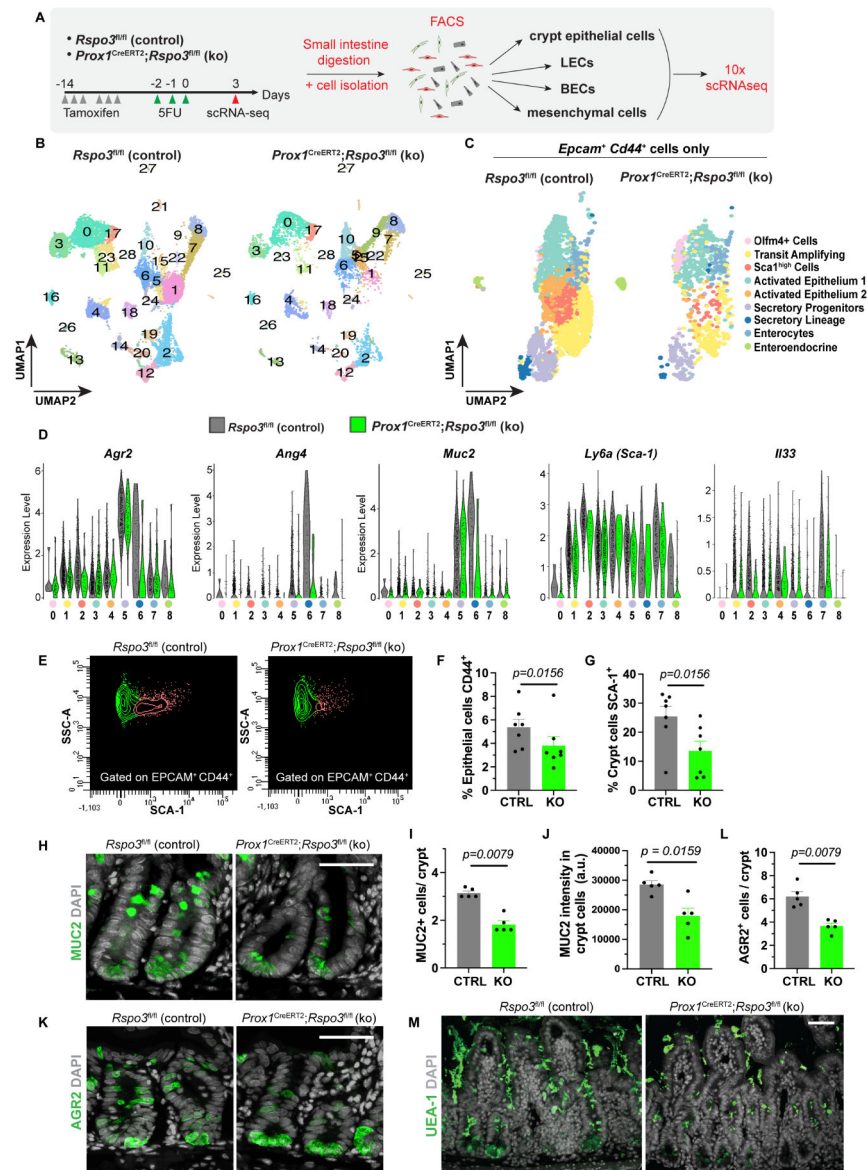


Figure 4. Loss of LEC *Rspo3* results in decreased numbers of TA, SCA1^{high} and mucus secreting cells.

A) Schema of scRNAseq experiment. Tamoxifen treated control and knock-out mice were administered 5FU on 3 consecutive days. Crypt epithelial cells, LECs, BECs and other stromal cells were enriched by FACS, and the samples submitted for 10X scRNAseq analysis. Data are from 2 independent biological experiments. Each biological experiment included 2 control and 2 knock-out mice. **B)** The sequenced cells group in 29 clusters as depicted in UMAP plots. **C)** Crypt epithelial cells were identified as *Epcam*⁺, *Cd44*⁺ cells and re-clustered. Crypt epithelial cells group in 9 distinct clusters as shown in UMAP plots. **D)** Violin plot of differentially expressed genes (DEGs) in crypt epithelial cells from control (grey) versus knock-out (green) mice. **E)** Flow cytometry analysis of SCA-1 negative (green) and positive (red) cells, gated on crypt epithelial cells (EPCAM⁺ and CD44⁺), in control and knock-out mice at day 3 after 5FU (n=7 mice). **F-G)** Proportion of epithelial

cells that are CD44⁺ (**F**) and crypt epithelial cells that are SCA-1⁺ (**G**) quantified by flow cytometry in control (CTRL) and *Rspo3* knock-out (KO) mice at day 3 after 5FU (n=7 mice). **H-J** Representative images (**H**) and quantification (**I**) of MUC2⁺ cells and (**J**) quantification of MUC2 staining intensity in the crypts of control (CTRL) and *Rspo3* knock-out (KO) mice at day 3 after 5FU (n=5 mice). **K-L** Representative images (**K**) and quantification (**L**) of AGR2⁺ cells in crypts of control (CTRL) and *Rspo3* knock-out (KO) mice at day 3 after 5FU (n=5 mice). **M** Representative images of mucus (UEA-1) staining in control and *Rspo3* knock-out mice at day 3 after 5FU (n=4 mice). Bar graph data are mean \pm s.e.m., Wilcoxon paired t-test two-sided **F-G**, Mann Whitney U-test, two-sided **I, J, L**. See also Figures S3-4.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-LYVE1 (clone ALY7)	eBioscience	Cat# 14-0443-82, RRID:AB_1633414
Rabbit polyclonal anti-LYVE1	AngioBio	Cat# 11-034, RRID:AB_2813732
Goat polyclonal anti-PDGFR α	R&D systems	Cat# AF1062, RRID:AB_2236897
Rabbit monoclonal anti-OLFM4 (clone D6Y5A)	Cell signaling technology	Cat# 39141, RRID:AB_2650511
Rabbit polyclonal anti-MUC2	Novus biologicals	Cat# NBP1-31231, RRID:AB_10003763
Rabbit polyclonal anti-AGR2	Origene	Cat# TA336715
Donkey anti-Goat IgG Secondary Antibody (Alexa Fluor Plus 488)	Thermo Fischer Scientific	Cat# A32814, RRID:AB_2762838
Donkey Anti-Rat IgG Secondary Antibody (Alexa Fluor 647)	Jackson ImmunoResearch Labs	Cat# 712-605-153, RRID:AB_2340694
Donkey Anti-Rat IgG Secondary Antibody (Alexa Fluor 488)	Thermo Fischer Scientific	Cat# A-21208, RRID:AB_2535794
Donkey anti-Goat IgG Secondary Antibody (Alexa Fluor Plus 647)	Thermo Fischer Scientific	Cat# A32849, RRID:AB_2762840
Donkey Anti-Rabbit IgG Secondary Antibody (Alexa Fluor 555)	Thermo Fischer Scientific	Cat# A32794, RRID:AB_2762834
Goat Anti-Rabbit IgG Secondary Antibody (Alexa Fluor 488)	Thermo Fischer Scientific	Cat# A-11008, RRID:AB_143165
Goat Anti-Rabbit IgG Secondary Antibody (Alexa Fluor 568)	Invitrogen	Cat# A-11011, RRID:AB_143157
Alexa Fluor 647 anti-mouse CD326 (Ep-CAM) antibody	BioLegend	Cat# 118212, RRID:AB_1134101
Alexa Fluor 488 anti-mouse CD31 antibody	BioLegend	Cat# 102414, RRID:AB_493408
APC anti-mouse CD326 (Ep-CAM) antibody	BioLegend	Cat# 118214, RRID:AB_1134102
PE/Cyanine7 anti-mouse Podoplanin antibody	BioLegend	Cat# 127412, RRID:AB_10613648
PE-Cyanine7 anti-mouse LYVE1 Monoclonal Antibody (ALY7)	Thermo Fisher Scientific	Cat# 25-0443-82, RRID:AB_2802237
PE/Cyanine7 anti-mouse/human CD44 antibody	BioLegend	Cat# 103030, RRID:AB_830787
Brilliant Violet 421 anti-mouse CD45 antibody	BioLegend	Cat# 103134, RRID:AB_2562559
PE anti-mouse CD45 antibody	BioLegend	Cat# 103106, RRID:AB_312971
Alexa Fluor 647 anti-mouse CD144 (VE-cadherin) antibody	BioLegend	Cat# 138006, RRID:AB_10569114
APC anti-mouse Ly-6A/E (Sca-1) Monoclonal Antibody (D7)	eBioscience	Cat# 17-5981-82, RRID:AB_469487
Chemicals, peptides, and recombinant proteins		
ProLong Gold Antifade Mounting Solution	Thermo Fischer Scientific	Cat# P36930

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tamoxifen	Sigma-Aldrich	Cat# T5648-5G
Corn Oil	Sigma-Aldrich	Cat# C8267
Mac's SmartStrainers	Miltenyi Biotec	Cat# 130-110-916
Triton X-100	Sigma-Aldrich	Cat# T8787
Normal Donkey Serum	Jackson ImmunoResearch	Cat# 017-000-121
Animal-free Blocker	Vector Laboratories	Cat# sp-5030
PFA	Fischer Scientific	Cat# 04042
Sucrose	VWR	Cat# JT4072-7
Tissue-Tek O.C.T. Compound	Sakura	Cat# 4583
N-butyldiethanolamine	TCI Chemicals	Cat# TCI-B0725
Antigen Unmasking Solution, Citrate-Based	VWR	Cat# 101098-054
DAPI	Sigma-Aldrich	Cat# D9564
RPMI-1640 Medium	Sigma-Aldrich	Cat# R7509
Glutamax	Thermo Fisher Scientific	Cat# 35050061
MEM Non Essential Amino Acids Solution (100X)	Thermo Scientific	Cat# 11140050
HEPES	Thermo Fisher Scientific	Cat# 15630080
Penicillin Streptomycin	Thermo Fisher Scientific	Cat# 15140122
EDTA	Sigma-Aldrich	Cat# E5134
DTT	Sigma-Aldrich	Cat# 10197777001
HBSS	Thermo Scientific	Cat# 14175095
Collagenase A	Sigma-Aldrich	Cat# 10103586001
Dispase II	Sigma-Aldrich	Cat# 4942078001
DNAse	Worthington Biochemical	Cat# LS002007
iTaq™ Universal SYBR® Green Supermix	Biorad	Cat# 1725124
Lectin from <i>Ulex europaeus</i> (FITC conjugated)	Sigma-Aldrich	Cat# L9006
Critical commercial assays		
RNAscope Multiplex Fluorescent V2 Assay	ACD	Cat# 323100
Click-iT™ Plus Edu Alexa Fluor™ 647 Imaging Kit	Thermo Fisher Scientific	Cat# C10640
PicoPure™ RNA Isolation Kit	Thermo Scientific	Cat# KIT0204
High-Capacity cDNA Reverse Transcription Kit	Thermo Scientific	Cat# 4368814
Deposited data		
All scRNAseq data has been deposited on GEO	This paper	GSE198469
Experimental models: Organisms/strains		
Mouse strain: <i>Rspo3^{fl/fl}</i>		Christof Niehrs (Institute of Molecular Biology)
Mouse strain: <i>Prox1^{CreERT2}</i>	The Jackson Laboratory	JAX: 022075
Mouse strain: <i>Lgr5^{DTR GFP}</i>	Genentech (Dr. Frederic de Sauvage)	
Mouse strain: <i>Lgr5^{GFP-IRES-CreERT2}</i>	The Jackson Laboratory	JAX: 008875
Oligonucleotides		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNAscope Probe- Mm-Lgr5	ACD	Cat# 312171
RNAscope Probe- Mm-Rsp03	ACD	Cat# 402011
Rsp03 primers Fw: GACTCGCCTAACACCTACATAC RV GACATTAGGATGCATTCTTCGC	This paper	
Apoa1 primers Fw: CGCTAATGTGTATGTGGATGCG Rv: CCCAATCTGTTCTTTCTCCAGG	This paper	
Cavin1 primers Fw: CCAGATCCAGCTGACCCAAG Rv: TTCTTTATCTGGCCGGCCTG	This paper	
Prss23 primers Fw: CCGCACAGAGACTCGGGT Rv: CCAGTGCAGCCAGTAGACAA	This paper	
Tgfb1 primers Fw: TACAACAGCACCCGCGAC Rv: ACAGCAATGGGGTTTCGG	This paper	
Tgfb2 primers Fw: AGGGATCTGGATGGAAATGGA Rv: CCAATGTAATAGAGAATGGTCAGTGG	This paper	
Vim primers Fw: CGTTTCCAAGCCTGACCTCA Rv: GGCATCCACTTCACAGGTGA	This paper	
Software and algorithms		
GraphPad Prism 9		https://www.graphpad.com
ImageJ (Fiji)	https://imagej.net/software/fiji/	v2.1.0/1.53c; RRID:SCR_002285
Adobe Illustrator	https://www.adobe.com	v25.4
R	https://www.r-project.org/	v.4.0.3
BD FACS Diva	https://www.bdbiosciences.com/	v.8.0.1
ZenBlue	https://www.zeiss.com/	v.3.1
WEB-GESTALT (WEB-based GENE SeT Analysis Toolkit)	https://www.webgestalt.org/	
Seurat		v.4.1.0
Cell Ranger	https://10xgenomics.com	v.5.0.1
Biorender	https://biorender.com	