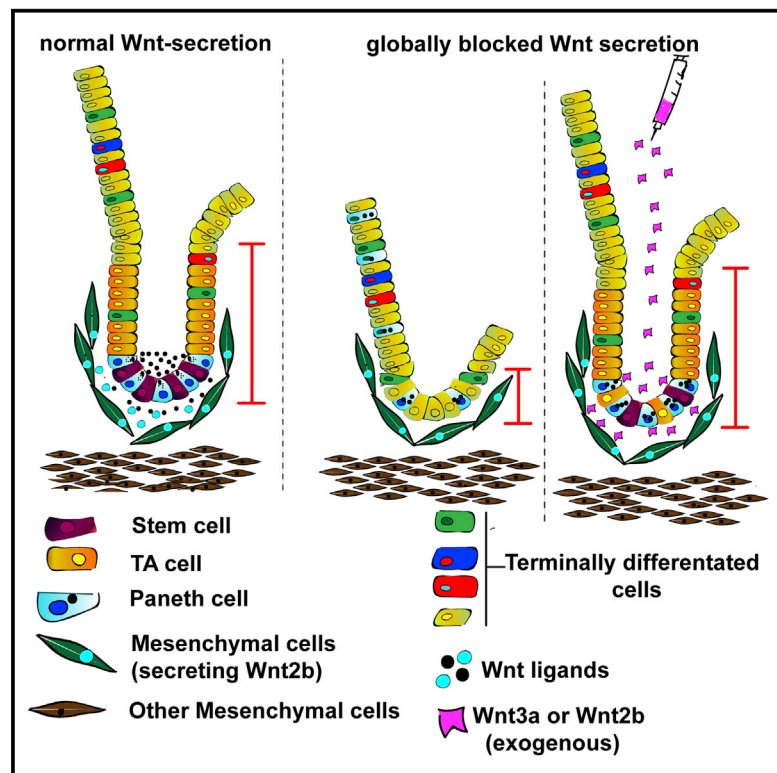


Cell Reports

Wnt Ligands Secreted by Subepithelial Mesenchymal Cells Are Essential for the Survival of Intestinal Stem Cells and Gut Homeostasis

Graphical Abstract



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In Brief

Valenta et al. find that globally blocking Wnt secretion impairs intestinal homeostasis by affecting intestinal epithelial stem cells. Reconstitution of Wnt/ β -catenin signaling by exogenous Wnts preserves stem cells, demonstrating the role for extra-epithelial Wnts, possibly Wnt2b, secreted by mesenchymal cells expressing Gli1, Acta2, or both.

Highlights

- Intestinal epithelial stem cells are sensitive to global attenuation of Wnt secretion
- Extra-epithelial Wnts maintain the renewal of intestinal epithelial stem cells
- Exogenous reconstitution of Wnt/ β -catenin signaling promotes intestinal renewal
- Gli1 and Acta2 mark the majority of Wnt2b⁺ subepithelial mesenchymal cells



Wnt Ligands Secreted by Subepithelial Mesenchymal Cells Are Essential for the Survival of Intestinal Stem Cells and Gut Homeostasis

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SUMMARY

Targeting of Wnt signaling represents a promising anti-cancer therapy. However, the consequences of systemically attenuating the Wnt pathway in an adult organism are unknown. Here, we globally prevent Wnt secretion by genetically ablating *Wntless*. We find that preventing Wnt signaling in the entire body causes mortality due to impaired intestinal homeostasis. This is caused by the loss of intestinal stem cells. Reconstitution of Wnt/ β -catenin signaling via delivery of external Wnt ligands prolongs the survival of intestinal stem cells and reveals the essential role of extra-epithelial Wnt ligands for the renewal of the intestinal epithelium. *Wnt2b* is a key extra-epithelial Wnt ligand capable of promoting Wnt/ β -catenin signaling and intestinal homeostasis. *Wnt2b* is secreted by subepithelial mesenchymal cells that co-express either *Gli1* or *Acta2*. Subepithelial mesenchymal cells expressing high levels of *Wnt2b* are predominantly *Gli1* positive.

INTRODUCTION

Ectopic Wnt signaling has been implicated in the initiation and progression of various cancers and, therefore, represents a promising therapeutic target. However, physiological Wnt signaling is pivotal for the renewal of stem cells in the adult organism (Anastas and Moon, 2013; Clevers et al., 2014). To be able to avoid the potential complications of an anti-Wnt signaling treatment, it is essential to know the impact of globally attenuating pathway activity in an adult organism.

Wnt ligands (Wnts) are secreted glycoproteins that are palmitoylated in the endoplasmic reticulum by the acyltransferase Porcupine (Porcn). *Wntless* (Wls) is a transmembrane protein that is required for the secretion of lipid-modified Wnts (Bänziger

et al., 2006). Wnts elicit various signaling outputs; these are β -catenin dependent (Wnt/ β -catenin) or β -catenin independent (Anastas and Moon, 2013; Valenta et al., 2011). Importantly, although often initiated by mutation(s) downstream of the Wnt-receptor complex, the progression of colon cancer still seems to be augmented by Wnt-ligand-mediated signaling (Voloshanenko et al., 2013). Therefore, blocking Porcn or Wls activity, as non-redundant components required for the secretion of all Wnt ligands, appears as an attractive therapeutic approach in Wnt-activated cancers. Porcn inhibitors are already in clinical development. Surprisingly, there is little information on the effects of globally repressed Wnt-secretion at the level of adult organism.

RESULTS

To better delineate the therapeutic limits of such inhibitors, we sought to determine the consequences of attenuated Wnt secretion in the entire organism using a genetic model. This was achieved by combining a conditional *Wls* allele (*Wls^{flox}*) with an inducible and ubiquitous Cre driver (*Rosa26-Cre^{ERT2}*). Whereas adult heterozygous (*Rosa26-Cre^{ERT2}, Wls^{flox/wt}*) mice were indistinguishable from wild-type animals after induction of Cre activity, the homozygous mutants (*Rosa26-Cre^{ERT2}, Wls^{flox/flox}*, hereinafter referred to *R26-Wls^{CKO}*) died 14 days after induction of recombination. Histopathological analyses of tissues isolated 12 days after induction revealed a strong phenotype in the intestine, whereas other tissues seemed intact. Most affected was the proximal intestine (duodenum), as evidenced by the complete absence of intestinal crypts and aberrant villi morphology (Figure 1A). The impact of blocked Wnt secretion on duodenal renewal—absence of intestinal crypts—was first apparent 10 days after induction (Figure 1A). Stem cells residing in the crypts normally differentiate along the crypt-villi axis via intermediate and highly proliferating transient-amplifying (TA) cells into enterocytes or a secretory lineage as Goblet cells (Barker, 2014; Clevers et al., 2014). Paneth cells residing at the bottom of the crypt also originate from stem cells. When crypts were

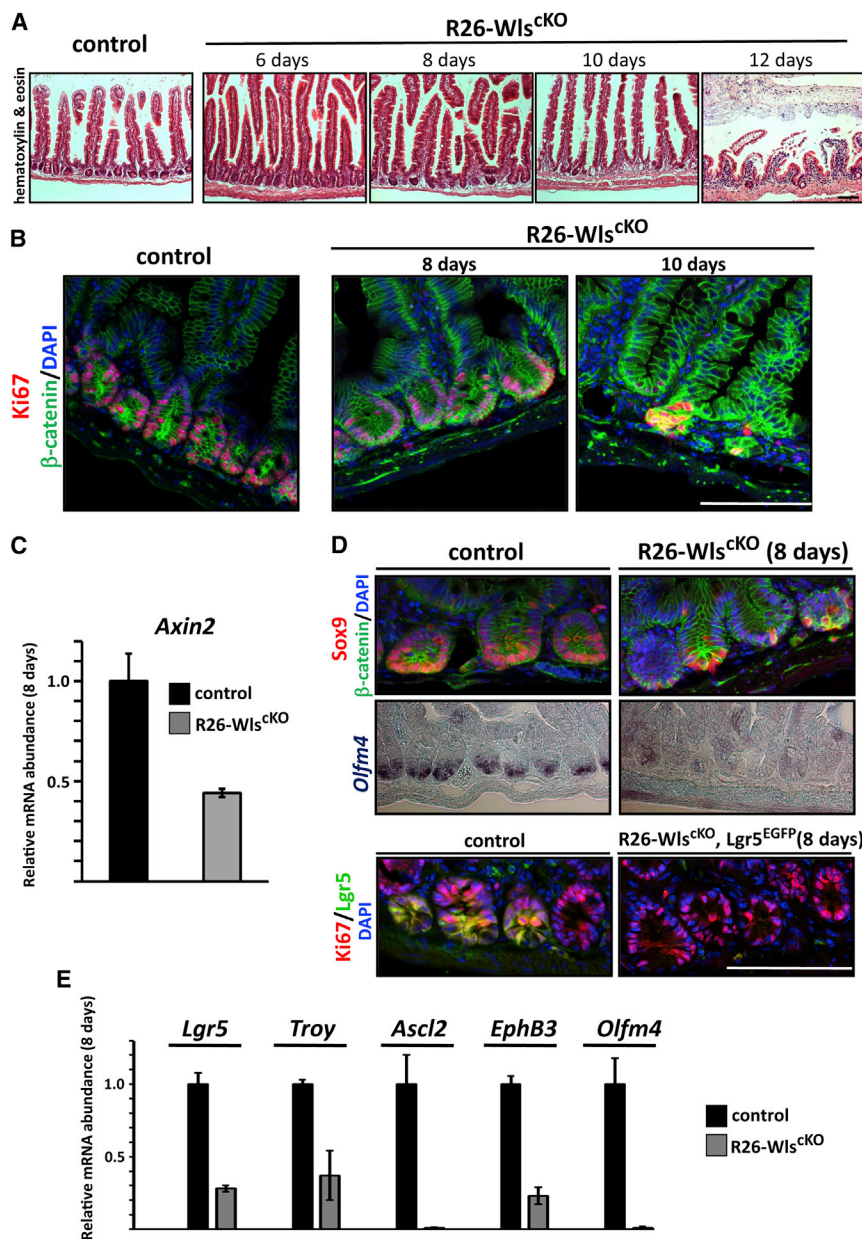


Figure 1. Systemically Blocking the Secretion of Wnt Ligands Affects Intestinal Self-Renewal

(A) Intestinal epithelial crypts are lost from the small intestine 10 days after induction by tamoxifen in R26-Wls^{CKO} animals (H&E staining).

(B) Proliferation activity marked by Ki67 disappears together with intestinal crypts (immunohistochemistry).

(C) Expression of *Axin2* decreases when blocking Wnt secretion (real-time qPCR).

(D) Expression of the stem cell and early progenitor marker Sox9 is changed. It localizes to the bottom of the crypts in R26-Wls^{CKO} animals (immunohistochemistry). The stem cell marker *Olfm4* is lost 8 days after the induction (RNA in situ hybridization). Similarly, the lack of *Lgr5* staining indicated that stem cells disappeared after 8 days, when there are still proliferating cells within crypts (immunohistochemistry: anti-GFP staining of *Lgr5*^{EGFP} allele; proliferation: Ki67 staining).

(E) Relative expression of the intestinal epithelial stem cell markers (*Ascl2*, *Lgr5*, *Olfm4*, and *Troy*) and early progenitors (*EphB3*) in duodenum of R26-Wls^{CKO} animals 6 days after induction (real-time qPCR).

R26-Wls^{CKO} indicates *Rosa26-Cre*^{ERT2}, *Wntless*^{flax/flax}. Immunohistochemistry: DAPI marks nuclei, and β-catenin denotes epithelial cells. Scale bars, 100 μm. Real-time qPCR: y axes show normalized relative mRNA abundance, control levels were set to 1. Error bars indicate SD.

of *Lgr5*, *Troy*, *Ascl2*, and *Olfm4*) was observed in R26-Wls^{CKO} already 8 days after Cre induction (Figures 1D and 1E), when the morphology and proliferation activity of the epithelium still appeared as normal (Figure 1D). An altered expression pattern of Sox9, a stem cell and early progenitor marker, was also observed in R26-Wls^{CKO} 8 days after induction (Figure 1D). The expression of key stem cell regulators (*Lgr5* and *Troy*) is controlled by Wnt/β-catenin signaling (Barker et al., 2007; Clevers et al., 2014; Fafilek et al.,

2013). Their reduced expression suggested that Wnt/β-catenin-dependent transcription was abrogated in R26-Wls^{CKO} crypts; consistent with this, expression of the Wnt/β-catenin target gene *Axin2* was also reduced (Figure 1C).

To further probe the mechanism underlying the observed defects, we compared the consequences of a systemic loss of Wnt secretion to a universal block of β-catenin signaling outputs. Eliminating β-catenin in the entire adult mouse by combining *Rosa26-Cre*^{ERT2} with a β-catenin conditional allele (R26-β-catenin^{CKO}) led to a phenotype similar to that of R26-Wls^{CKO} animals (Figure S1D) and resembled that observed when β-catenin was selectively eliminated in the intestinal epithelium (Figure S1E) (Fevr et al., 2007). However, the effect was already evident 4 days after the induction, compared with

lost 10 days after Cre induction, Paneth cells, marked by expression of lysozyme, were no longer present at their proper position. Instead, lysozyme-positive cells were ectopically situated in villi (Figure S1A). Morphologically, these cells resemble Goblet cells, suggesting that the secretory lineage underwent improper terminal differentiation. In contrast to Paneth cells, other cell types (e.g., enterocytes) did not exhibit any apparent alteration (Figure S1A). The high proliferation of TA cells was also lost within the intestinal crypts (Figure 1B) at 10 days. In contrast to the duodenum, the morphology of the colon did not seem to be affected in the R26-Wls^{CKO} animals; however, similar to the duodenum, colonic crypts exhibit strongly reduced proliferation activity (Figure S1B). Crypt loss was preceded by the disappearance of stem cells: a reduction of stem cell traits (expression

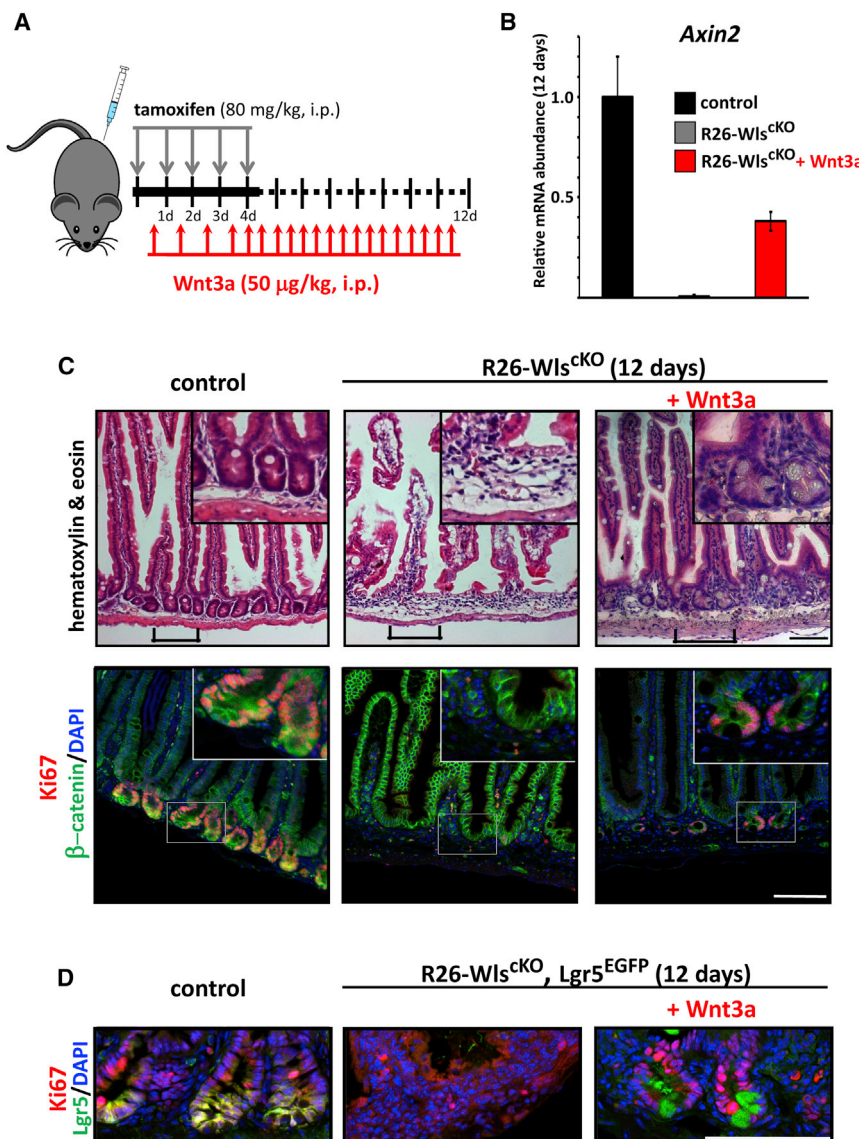


Figure 2. Extra-epithelial Wnt ligands Are Essential for the Maintenance of Intestinal Homeostasis

(A) Scheme of external Wnt3a application regimen. i.p., intraperitoneal; d, days.

(B) Injected Wnt3a partially restores intestinal expression of *Axin2*, indicating restoration of active Wnt/ β -catenin signaling (real-time qPCR).

(C) Restored intestinal morphology (upper panels) and proliferation determined by Ki67 (lower panels) in R26-Wls^{CKO} animals receiving external Wnt3a (H&E staining and immunohistochemistry).

(D) Stem cells marked by Lgr5 survive longer in the intestinal epithelium when Wnt/ β -catenin signaling was restored by external Wnt3a. Prolonged renewal of intestinal stem cells is associated with active proliferation as assayed by Ki67 expression. At the same time, intestinal crypts, including stem cells, are completely absent in R26-Wls^{CKO} animals (immunohistochemistry: anti-GFP staining of Lgr5^{EGFP}).

R26-Wls^{CKO} indicates *Rosa26-Cre^{ERT2}, Wntless^{flx/flx}*. Immunohistochemistry: DAPI marks nuclei, and β -catenin denotes epithelial cells. Scale bars, 100 μ m. Real-time qPCR: y axes show normalized relative mRNA abundance; control levels were set to 1. Error bars indicate SD.

the direct Wnt-pathway target *Axin2* (Figure S2B). The effects of attenuated Wnt/ β -catenin transcription on the expression of the markers of intestinal epithelial stem cells (IESCs) were apparent 1 day after induction (Figure S2B).

In sum, IESCs are sensitive to perturbations of Wnt/ β -catenin signaling and disappear first when Wnt/ β -catenin signaling is attenuated either by blocking the transcriptional outputs of β -catenin or preventing the secretion of Wnt ligands.

Renewal of IESCs depends on Wnt/ β -catenin, and many IESC markers are direct targets of this pathway (Barker et al., 2007; Clevers et al., 2014; Schuijers et al., 2014). Our results confirm that secreted Wnt ligands are essential for maintaining intestinal homeostasis via renewal of the stem cell pool. In the intestine, Wnt ligands are secreted from the epithelium by Paneth cells and possibly also by extra-epithelial cells (Durand et al., 2012; Farin et al., 2012; Sato et al., 2011). When we prevented Wnt secretion only from the intestinal epithelium using *villinCre^{ERT2}*, animals (*villin-Wls^{CKO}*) lived normally (Figure S2C), consistent with earlier work on epithelial Porcn or Wnt3 (Farin et al., 2012; Kabiri et al., 2014; San Roman et al., 2014). However, it was not possible to establish Villin-Wls^{CKO} intestinal organoid cultures, as the organoids died within 1 week if cultured under standard conditions. Organoids of this genotype could, however, be fully rescued by the addition of Wnt3a ligand or partially rescued by a GSK3 inhibitor resulting in stabilizing β -catenin (Figure S2D). These observations indicate that, although Wnt ligands secreted by Paneth cells are essential when they

10 days in the case of R26-Wls^{CKO}. This difference in timing is likely due to the perdurance of Wls protein, which was still detectable after 10 days, although the transcript was no longer detectable after 6 days (Figure S1C). A prolonged half-life of Wls protein may result from its recycling via the retromer complex (Belenkaya et al., 2008; de Groot et al., 2013; Port et al., 2008). To specifically probe the contribution of the transcriptional output of canonical Wnt signaling, we used the β -catenin-dm allele (Valenta et al., 2011). β -catenin-dm is functional at the adherens junctions but its signaling function is completely abrogated. Intestinal epithelia expressing only β -catenin-dm (*villin- β -catenin^{dm}*) stopped proliferating 2 days after loss of the floxed wild-type allele (Figure S2A). The loss of cellular proliferation was preceded, as in R26-Wls^{CKO}, by the loss of stem cells, as revealed by the absence of the stem cell markers *Lgr5*, *Troy*, and *Ascl2* (Figure S2B). In *villin- β -catenin^{dm}* crypts, the loss of stem cell traits was also accompanied with the reduced expression of

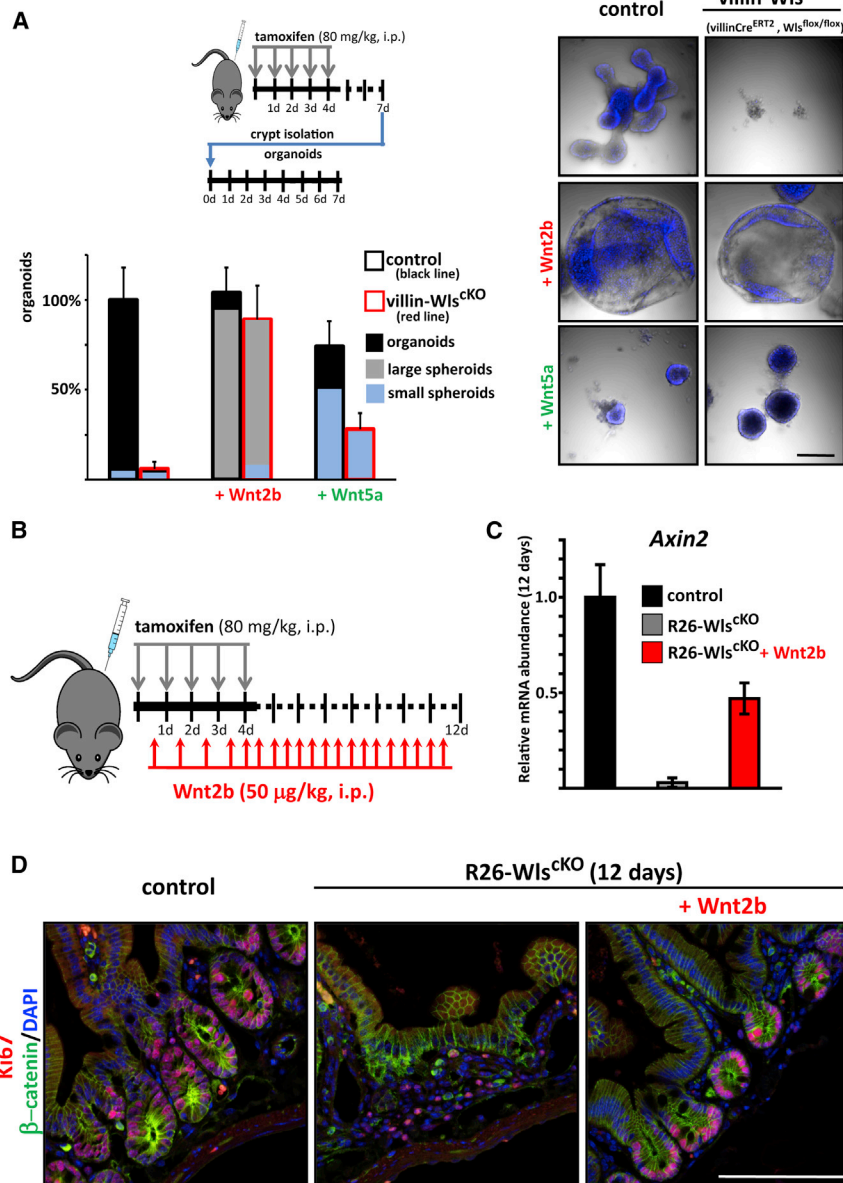


Figure 3. Wnt2b Ligand Secreted by Extra-epithelial Cells Drives β -Catenin Signaling Outputs and Is Essential for the Maintenance of Intestinal Homeostasis

(A) (Upper left) Scheme depicting how the intestinal organoids were generated. Intestinal crypts were isolated from villin-*Wls*^{CKO} (or control) animals and cultivated in Matrigel as intestinal organoids with standard cultivation medium (SCM) or SCM containing either 100 ng/ml Wnt2b or 100 ng/ml Wnt5a. (Right) Whereas organoids from control animals grew normally, it was not possible to establish growing organoids from villin-*Wls*^{CKO} crypts. Addition of Wnt2b changed the morphology of control organoids to large spheroids (compare with finger-like protrusions in SCM). External Wnt2b enables organoids from villin-*Wls*^{CKO} crypts to be established. Wnt5a promoted survival of villin-*Wls*^{CKO} crypts but did not promote growth. Organoid representing typical shape of organoids (apparent in more than 75% of the organoids) is shown. DAPI (blue) counterstains the nuclei. (Bottom left) The fraction of living organoids after 7 days (d) in culture is shown; the proportion of various shapes of organoids is indicated. Crypts were seeded at the same initial density. Number of control organoids growing in SCM is set as 100%, each column summarizes data from two independent experiments (three replicates each). Error bars indicate SD. Scale bar, 100 μ m. i.p., intraperitoneal.

(B) Scheme of external Wnt2b application regimen. (C) Injected Wnt2b partially restores intestinal expression of *Axin2* indicating active Wnt/ β -catenin signaling. For real-time qRT-PCR, y axes show normalized relative mRNA abundance; control levels were set to 1. Error bars indicate SD.

(D) Externally delivered Wnt2b preserves intestinal morphology and proliferation determined by Ki67 in R26-*Wls*^{CKO} animals. R26-*Wls*^{CKO} indicates *Rosa26-Cre*^{ERT2} *Wntless*^{flx/flx}. Immunohistochemistry: DAPI marks nuclei, and β -catenin denotes epithelial cells. Scale bar, 100 μ m.

represent the only source (in organoids), they are dispensable in vivo due to Wnts supplied by extra-epithelial cells. To test this notion further, we asked whether the loss of IESCs in R26-*Wls*^{CKO} animals could be prevented by the addition of exogenous Wnt3a protein (Figure 2A). Indeed, intraperitoneal injection of Wnt3a was able to restore Wnt/ β -catenin signaling in the proximal intestine (Figure 2B). Restoration of β -catenin-dependent signaling outputs preserved intestinal crypts up to 12 days after induction of *Wls* loss; they were usually lost by this time. These rescued crypts were proliferatively active (Figures 2C and 2D). Reconstituted β -catenin signaling also promoted the renewal and survival of IESCs (Figure 2D).

Since Wnt3a is not expressed in the small intestine (Klostermeier et al., 2011), we sought to determine which extra-epithelial

Wnts might be responsible for the maintenance of IESC renewal. Wnt2b and Wnt5a are highly expressed outside of the intestinal epithelium (Klostermeier et al., 2011; Farin et al., 2012). Importantly, Wnt2b was shown to be a potent activator of Wnt/ β -catenin signaling and able to compensate for the loss of epithelial Wnt3 in intestinal organoids (Goss et al., 2009; Farin et al., 2012). Consistent with these results, recombinant Wnt2b could restore the growth of organoids derived from Villin-*Wls*^{CKO} intestinal crypts. As with Wnt3a treatment, Wnt2b-treated organoids grew as spheroids (Figure 3A). In contrast to Wnt2b, the other highly expressed Wnt, Wnt5a, only allowed Villin-*Wls*^{CKO} crypts to survive as small spheroids (basically as closed crypts) and did not promote their growth. If Villin-*Wls*^{CKO} organoids were passaged further, only those treated with Wnt2b could self-renew, whereas Wnt5a-treated ones died (data not shown). The in vitro potency of Wnt2b prompted us to test its effect in R26-*Wls*^{CKO} animals. Injection of Wnt2b could partially

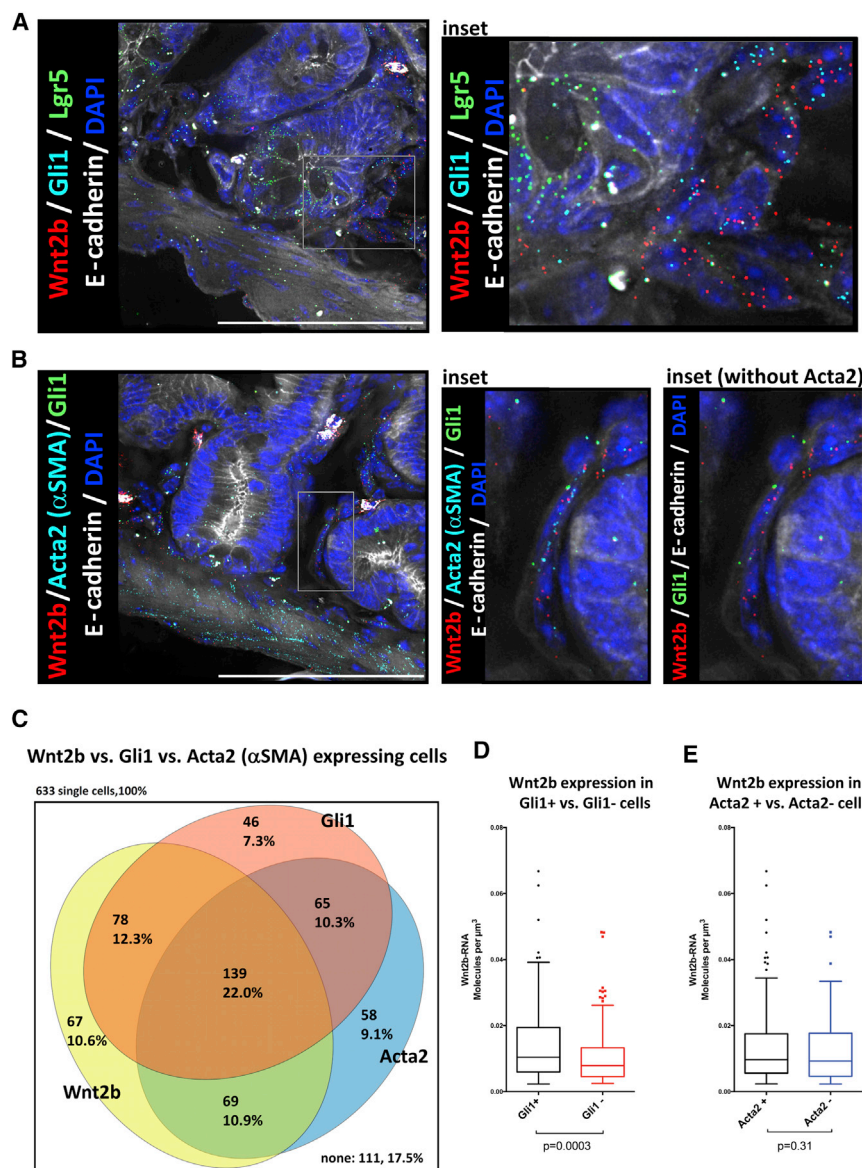


Figure 4. Subepithelial Mesenchymal Cells Expressing High Levels of *Wnt2b* Are Predominantly *Gli1* Positive and the Expression of Either *Gli1* or *Acta2* Marks the Majority of *Wnt2b*-Secreting Cells

(A) Murine duodenum tissue sections were hybridized with smFISH probe libraries for *Wnt2b* (red dots), *Lgr5* (green dots), and *Gli1* (cyan dots) single-mRNA molecules. Nuclei were stained with DAPI (blue), and E-cadherin protein was stained with a FITC-coupled antibody (gray) to visualize cell membranes of epithelial cells. Paneth cell granules exhibit non-specific fluorescence appearing in multiple channels. Scale bar, 100 μ m.

(B) smFISH of duodenum probed for *Wnt2b* (red dots), *Gli1* (green dots), and *Acta2* (α SMA) (cyan dots) single-mRNA molecules. The shape of the epithelial cells is visualized by E-cadherin staining; DAPI marks nuclei (similarly as in A). Scale bar, 100 μ m.

(C) Venn diagram showing overlap of *Wnt2b*, *Gli1*, and *Acta2* (α SMA). Single-crypt-associated stromal cells were stratified into groups of expressing (>0 molecules per cubic micron) or non-expressing cells (0 molecules per cubic micron) for each RNA of interest according to smFISH analyses. The probability of random co-expression was assessed with the hypergeometric enrichment test.

(D and E) High expression levels of *Wnt2b* correlate with *Gli1* positivity but not with expression of *Acta2* (α SMA). *Wnt2b*-expressing subepithelial cells were divided into two groups based on their expression of *Gli1* (*Gli1*⁺ versus *Gli1*⁻) (D) or *Acta2* (α SMA; *Acta2*⁺ versus *Acta2*⁻) (E). Cells expressing high levels of *Wnt2b* (more *Wnt2b*-RNA molecules per cubic micron) are *Gli1* positive. No significant difference based on *Acta2* (α SMA) expression was identified. The y axes show numbers of *Wnt2b*-RNA molecules per cubic micron. Data shown as Tukey-style boxplots.

activate Wnt/ β -catenin signaling within the intestine as determined by expression of the target gene *Axin2* (Figures 3B and 3C). Addition of *Wnt2b* preserved both the morphology and the proliferation activity of intestinal crypts (Figure 3D). Animals can live at least 5 days longer than the lethality point upon *Wnt2b* administration.

As a next step, we determined which cells secrete *Wnt2b* (and *Wnt5a*) using single-molecule-RNA FISH (smFISH). We simultaneously probed tissue sections for *Wnt2b* (or *Wnt5a*) and *Acta2* (α SMA), a marker for intestinal myofibroblasts. Intestinal myofibroblasts are potential source of Wnts (Lahar et al., 2011; Ong et al., 2014; Powell et al., 2011). Although myofibroblasts seemed to express *Wnt2b* and *Wnt5a*, both *Wnt2b* and *Wnt5a* are also strongly expressed by cells that are *Acta2* (α SMA) negative (Figures S3A and S3B). Nevertheless, 65% of the *Wnt2b*-positive cells and 55% of the *Wnt5a*-expressing ones are myofibroblasts

(Figure S3C). However, there is no correlation between *Wnt2b* and *Acta2* expression (Figure S3D). *Wnt2b*-expressing cells are E-cadherin negative (i.e., non-epithelial) and localized adjacent to the intestinal epithelium, some of them in close proximity to *Lgr5*⁺ IESCs (Figure 4A; Figures S3A and S3B). Hence, *Wnt2b* represents a driver of Wnt/ β -catenin signaling and is expressed by subepithelial mesenchymal cells that are not exclusively myofibroblasts. To determine the identity of these cells more precisely, we performed smFISH against genes expressed in specific patterns in pericryptic subepithelial cells. We focused on the transcription factors *Foxl1* and *Gli1* and matrix protein Periostin (*Postn*); all are expressed in the mesenchymal cells adjacent to the crypts (Varnat et al., 2010; Büller et al., 2015; Sackett et al., 2007; Malanchi et al., 2011). With the designed probe set, we were not able to detect any subepithelial *Postn* expression. Although consistent with reports that *Postn* expression in the duodenum is low or undetectable (Klostermeier et al., 2011), we cannot exclude an effect of the probe design. *Foxl1* was found to be expressed by subepithelial mesenchymal cells, but only 21% of the

Wnt2b-positive (further as Wnt2b⁺) cells co-express *Foxl1* (Figures S4A and S4B). *Gli1* is expressed broadly by subepithelial mesenchymal cells; 65% of Wnt2b⁺ cells are *Gli1* positive (Figures 4A–4C). This situation resembles the co-expression of *Wnt2b* and *Acta2* (α SMA). However, cells that express high levels of *Wnt2b* also co-express *Gli1* significantly more often (Figure 4C); there is no such relationship between Wnt2b⁺ cells and *Acta2* (α SMA) expression (Figure 4E). Wnt2b⁺/*Gli1*⁺ cells expressing high levels of *Wnt2b* are often adjacent to *Lgr5*⁺ IESCs (Figure 4A). Hence, *Gli1* serves as a marker of cells with high levels of *Wnt2b*. Importantly, the majority (81%) of Wnt2b-secreting subepithelial mesenchymal cells co-express either *Gli1* or *Acta2* (α SMA). The Wnt2b produced by this subpopulation of cells is likely the Wnt source that compensates for the loss of Wnt production in the intestinal epithelia.

DISCUSSION

The role of Wnt/ β -catenin signaling for the maintenance of the intestinal epithelium has been recognized more than 2 decades ago (Korinek et al., 1998; Fevr et al., 2007; Barker, 2014; Clevers et al., 2014). As we show here, this is the most essential role of the Wnt pathway at the organismal level. Whereas the outputs of the receptor complex and their consequences for intestinal renewal have been described in detail, the intricate universe of Wnt ligands triggering various downstream actions is only partially understood. Currently, the source—and relevance—of Wnt ligands for the maintenance of intestinal homeostasis is under dispute (Farin et al., 2012; Durand et al., 2012; Kabiri et al., 2014; San Roman et al., 2014).

First indications pointed toward Paneth cells secreting Wnt3, Wnt6, and Wnt9b as providing a niche for IESCs (Sato et al., 2011). While this may be the case under normal conditions, the intestine can also renew itself in the absence of Paneth cells (and thus without Wnt3, Wnt6, and Wnt9b), suggesting that extra-epithelial Wnts can play a role (Durand et al., 2012; Kim et al., 2012). We show here that blocking Wnt secretion in the intestinal epithelium does not influence intestinal homeostasis and renewal of IESCs. Similar observations were reported using *Porcn* knockout animals (Kabiri et al., 2014; San Roman et al., 2014). However, a complete block of Wnt secretion severely affects the renewal of IESCs by attenuating β -catenin signaling outputs.

Extra-epithelial mesenchymal cells secrete various Wnt ligands with possibly divergent outputs (Gregorieff et al., 2005; Klostermeier et al., 2011; Farin et al., 2012). We show that, in vitro and in vivo, Wnt2b is a driver of β -catenin signaling outputs and is capable of sustaining the self-renewal of intestinal crypts. Importantly, Wnt2b is the only extra-epithelial Wnt ligand that rescues the growth of organoids, which either lack epithelial Wnt3 (Farin et al., 2012) or cannot secrete any Wnt (Villin-Wls^{CKO}; Figure 3A). Wnt2b is secreted by subepithelial mesenchymal cells, including a subpopulation that does not constitute myofibroblasts and that so far has not successfully been targeted. Blocking Wnt secretion in vivo using conditional alleles of either *Wls* or *Porcn* in combination with Cre drivers that are active in the intestinal epithelium (*villinCre^{ERT2}*) and in myofibroblasts (*Myh11Cre^{ERT2}*) does not affect IESC renewal (data not shown; San Roman et al., 2014).

Here, we show that cells expressing high levels of *Wnt2b* are predominantly *Gli1* positive. Moreover, the majority of Wnt2b⁺ subepithelial mesenchymal cells co-express either *Gli1* or *Acta2* (α SMA). It remains unclear whether Wnt2b⁺ cells serve only as a backup or safeguard for the situation when secretion from epithelial cells is impaired (Figure S2C; Durand et al., 2012; Kim et al., 2012) or whether they are also important for normal intestinal homeostasis. Support for serving primarily as a backup comes from the observation that mice lacking Wnt2b can live normally; the intestine is functional (Goss et al., 2009). Since Wnt2b was shown to have a similar affinity to the Fzd7 receptor as Wnt3 secreted by the epithelium, extra-epithelial Wnt2b likely binds to this key receptor (and LRP-co-receptors) for the maintenance of epithelial homeostasis (Flanagan et al., 2015). Importantly, Wnt2b⁺/*Gli1*⁺ cells with high Wnt2b levels are in close proximity to *Lgr5*⁺ IESCs, reducing the distance between Wnt-secreted and Wnt-receiving cells to the minimum. Such short-range signaling was recently shown to be important for epithelially secreted Wnt3 (Farin et al., 2016).

In sum, we show that a systemic block of Wnt secretion in the adult mouse results in lethality caused by aberrant intestinal renewal due to the loss of IESCs. IESCs require Wnt ligands to maintain the activity of the canonical Wnt/ β -catenin pathway. Whereas the lack of Wnt secretion from Paneth cells can be compensated by extra-epithelial sources, a complete block of Wnt secretion can only be rescued by delivering external Wnt ligands. The high sensitivity of IESCs to Wnt pathway perturbations will have to be taken into account for any anti-cancer therapy based on Wnt secretion inhibitors.

EXPERIMENTAL PROCEDURES

For a detailed description see the [Supplemental Experimental Procedures](#).

Mouse Experiments

Mouse experiments were performed in accordance with Swiss guidelines and approved by the Veterinarian Office of Kanton Zürich, Switzerland, and the Veterinarian Office of Kanton Vaud, Switzerland.

To conditionally eliminate the Wntless allele, a conditional *Wls* strain was generated (Gay et al., 2015).

The following mouse strains were used within the study: conditional β -catenin allele (Brault et al., 2001), *Lgr5-EGFP-IRES-Cre^{ERT2}* (Barker et al., 2007), β -catenin^{dm} and β -catenin^{KO} (Valenta et al., 2011), *Rosa26-Cre^{ERT2}* (Ventura et al., 2007), and *villin-Cre^{ERT2}* (el Marjou et al., 2004).

To induce Cre-mediated recombination, tamoxifen (Sigma) was injected (80 mg/kg) intraperitoneally for 5 consecutive days. External mouse Wnt3a (Abcam) or mouse Wnt2b (R&D Systems) was injected intraperitoneally (50 μ g/kg) twice a day, starting 12 hr after the first tamoxifen injection.

RNA Isolation, cDNA Synthesis, and Real-Time qPCR

Intestine or intestinal epithelial cells, isolated as described by Gracz et al. (2012), were lysed in TRI-Reagent (Sigma). RNA isolation, cDNA synthesis, and qRT-PCR were performed as described previously (Valenta et al., 2011). For qRT-PCR, samples were measured in triplicates, and average cycle threshold values were quantified relative to three reference genes (β -actin, GAPDH, and SDHA) using the $\Delta\Delta$ CT method.

Histology, Immunohistochemistry, In Situ Hybridization, and Immunoblot

Standard immunohistochemical protocols for optimal-cutting-temperature (OCT)-frozen sections or formalin-fixed paraffin-embedded (FFPE) sections

were performed. RNA in situ hybridization was performed as described by Gregorieff and Clevers (2010).

Protein extraction from proximal intestine and immunoblot were performed according to Schwitala et al. (2013).

Intestinal Organoids

Intestinal organoids were generated from villin-Wls^{CKO} or control animals 7 days after the first tamoxifen application and cultured as previously described (Sato et al., 2011; Sato and Clevers, 2013). To activate the Wnt/ β -catenin pathway, mWnt3a (Abcam) or mWnt2b (R&D Systems) (both 100 ng/ml) or 7.5 μ M CHIR99021 (Abcam) was added. Wnt5a (R&D Systems) was used at 100 ng/ml.

smFISH Analyses

Murine duodenum tissues were processed and used for smFISH staining as previously described (Raj et al., 2008; Itzkovitz et al., 2011; Bahar Halpern et al., 2015), including smFISH probes for Lgr5-RNA. Custom probes were designed against Wnt2b, Wnt5a, Acta2, Gli1, Foxl1, and Postn by utilizing the Stellaris FISH Probe Designer (Biosearch Technologies). See the Supplemental Experimental Procedures for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.03.088>.

AUTHOR CONTRIBUTIONS

T.V. and K.B. proposed and designed the research. T.V. and B.D. designed and performed the experiments and analyzed the data. A.E.M. performed smFISH experiments and analyzed the data. A.E.M. and M.B.M. did histopathological analysis. P.H. generated the conditional Wls allele. D.Z. assisted with doing experiments. D.Z., C.C., G.H. and M.A. critically discussed the data. T.V. and K.B. wrote and prepared the manuscript. B.D., A.E.M. and G.H. assisted with manuscript preparation. K.B. financed the research.

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Cell Reports, Volume 15

Supplemental Information

Wnt Ligands Secreted by Subepithelial

Mesenchymal Cells Are Essential for the Survival

of Intestinal Stem Cells and Gut Homeostasis

Tomas Valenta, Bahar Degirmenci, Andreas E. Moor, Patrick Herr, Dario Zimmerli, Matthias B. Moor, George Hausmann, Claudio Cantù, Michel Aguet, and Konrad Basler

SUPPLEMENTAL INFORMATION

Summary

A) Supplemental Figures

Figure S1, Related to Figure 1. *Effects of blocked Wnt secretion on the terminally differentiated intestinal epithelial cells and the role of repressed β -catenin outputs for the intestinal morphology.*

Figure S2, Related to Figure 2. *Transcription-specific outputs of β -catenin are required to maintain intestinal homeostasis. Extra-epithelial Wnt ligands promote the growth of the intestinal epithelium in vivo and ex vivo (intestinal organoids).*

Figure S3, Related to Figure 4. *Wnt2b and Wnt5a are expressed by subepithelial mesenchymal cells that are not exclusively myofibroblasts.*

Figure S4, Related to Figure 4. *Wnt2b expressing cells only partially overlap with Foxl1 pericryptic mesenchymal cells.*

B) Extended Experimental Procedures

Figure S1, Related to Figure 1

Effects of blocked Wnt secretion on the terminally differentiated intestinal epithelial cells and the role of repressed β -catenin outputs for the intestinal morphology.

A) Whereas distribution of enterocytes (alkaline phosphatase) or Goblet cells (Muc2) is unaffected, Paneth cells (Lysozyme) display an irregular pattern in villi and are lost together with crypts.

(immunohistochemistry; DAPI marks nuclei, β -catenin denotes epithelial cells)

B) The morphology of R26-Wls^{CKO} colonic crypts seems not to be altered 12 days after tamoxifen application (hematoxylin & eosin staining), but these crypts show decreased proliferation activity as determined by Ki67 expression (immunohistochemistry; DAPI marks nuclei, β -catenin denotes epithelial cells). The average number of Ki67⁺ cells per colonic crypt is indicated in a graph.

C) (upper panel) Relative expression of the *Wls* mRNA in duodenum of R26-Wls^{CKO} animals (real-time qRT PCR, y-axes show normalized relative mRNA levels; control was set to 1. Error bars show standard deviations. nd=not determined).

(lower panel) Protein levels of Wls extracted from duodenum of R26-Wls^{CKO}. Actin is used as the loading control. Days after induction are indicated.

D) Proper intestinal morphology is disrupted and intestinal crypts are lost in R26- β -catenin^{CKO} (i.e. *Rosa26-Cre^{ERT2}, β -catenin^{fllox/fllox}*) animals 4 days after the induction of the recombination by tamoxifen (H&E staining).

E) Complete epithelial loss of β -catenin (*villin- β -catenin^{CKO}* animals) has a similar impact on intestinal homeostasis as blocking solely the transcriptional outputs of β -catenin (*villin- β -catenin^{dm}* animals). To induce the loss of conditional allele *villin-Cre^{ERT2}* driver active only within intestinal epithelium was used. Both phenotypes recapitulate the one observed in R26- β -catenin^{CKO}. These results indicate that cells residing within intestinal epithelial crypts critically require Wnt/ β -catenin signaling and receiving Wnt ligands initiating this pathway (H&E staining).

(Scale bars indicate 100 μ m)

Figure S1

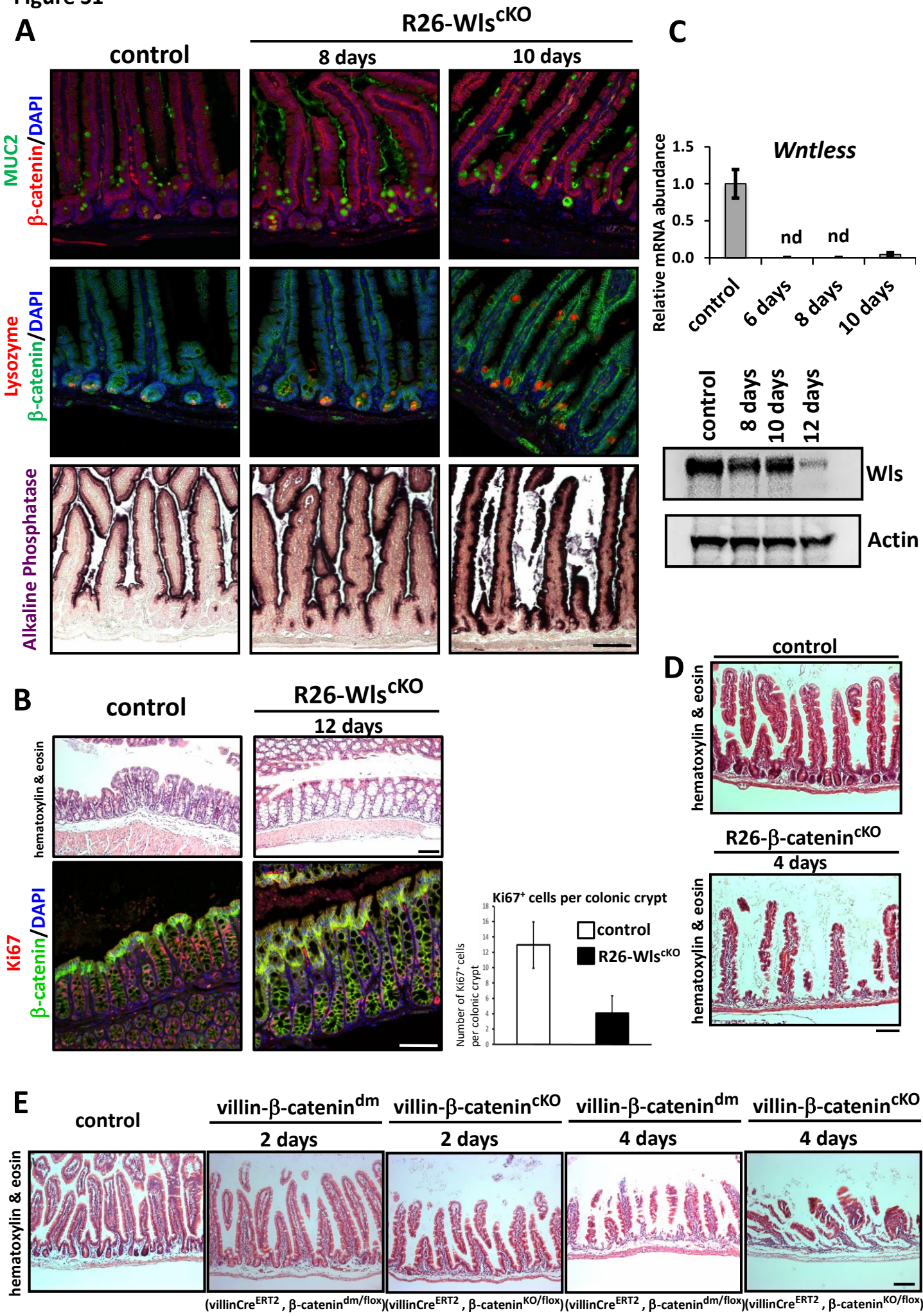


Figure S2, Related to Figure 2

Transcription-specific outputs of β -catenin are required to maintain intestinal homeostasis. Extra-epithelial Wnt ligands promote the growth of the intestinal epithelium in vivo and ex vivo (intestinal organoids).

A) Proliferation activity marked by Ki67 is lost already 2 days after the induction in villin- β -catenin^{dm} animals. (immunohistochemistry; DAPI marks nuclei, β -catenin denotes epithelial cells).

B) Relative expression of *Axin2* and the intestinal epithelial stem cell markers (*Ascl2*, *Lgr5*, *Troy*) in intestinal epithelium isolated from villin- β -catenin^{dm} animals. Time after tamoxifen injection is indicated (real-time qRT PCR: y-axes show normalized relative mRNA abundance, control levels were set to 1. Error bars show standard deviations).

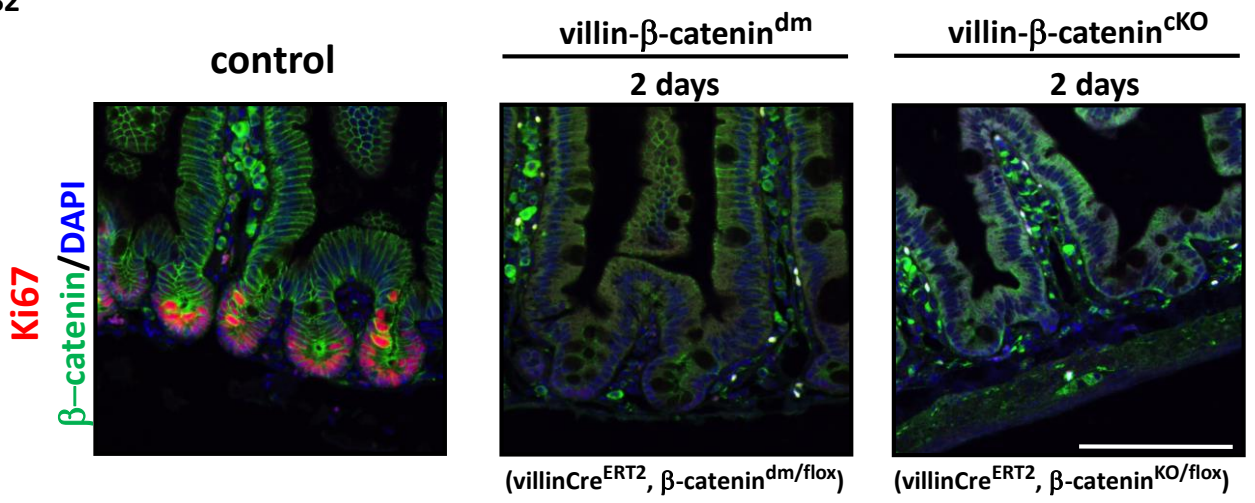
C) Blocking Wnt secretion from intestinal epithelial cells using *villin-Cre^{ERT2}* driver (villin-Wls^{ckO} animals) does not affect intestinal morphology and proliferation activity within intestinal crypts detected by Ki67 staining (immunohistochemistry; DAPI marks nuclei, β -catenin denotes epithelial cells).

D) (Upper left) Scheme depicting how the intestinal organoids were generated. Intestinal crypts were isolated from villin-Wls^{ckO} (or control) animals and cultivated in matrigel as intestinal organoids with standard cultivation medium (SCM) or SCM containing either 100 ng/ml Wnt3a or 7.5 μ M CHIR99021 (inhibitor of GSK3). (right panel) Whereas organoids from control animals grew normally, it was not possible to establish growing organoids from villin-Wls^{ckO} crypts. Addition of Wnt3a changed the morphology of control organoids to spheroids (compare with finger-like protrusions in SCM). External Wnt3a enables organoids from villin-Wls^{ckO} crypts to be established. CHIR99021 (stabilizing β -catenin) only partially restored the growth of villin-Wls^{ckO} organoids. Organoid representing typical shape of organoids (apparent in more than 75% of organoid) is shown. DAPI (blue) counterstains the nuclei. (bottom left) The fraction of living organoids after 7 days in culture is shown in graph. Crypts were seeded at the same initial density. For each cultivation parallel control organoids are set as 100%, each column summarizes data from two independent experiment (each 3 replicates), error bars show standard deviations.

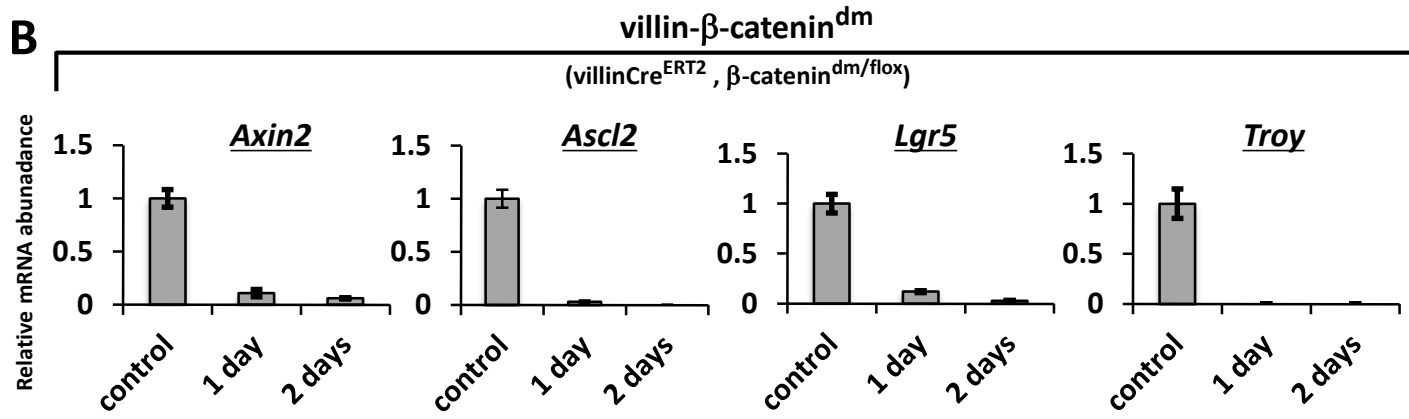
(Scale bars indicate 100 μ m)

Figure S2

A



B



C



D

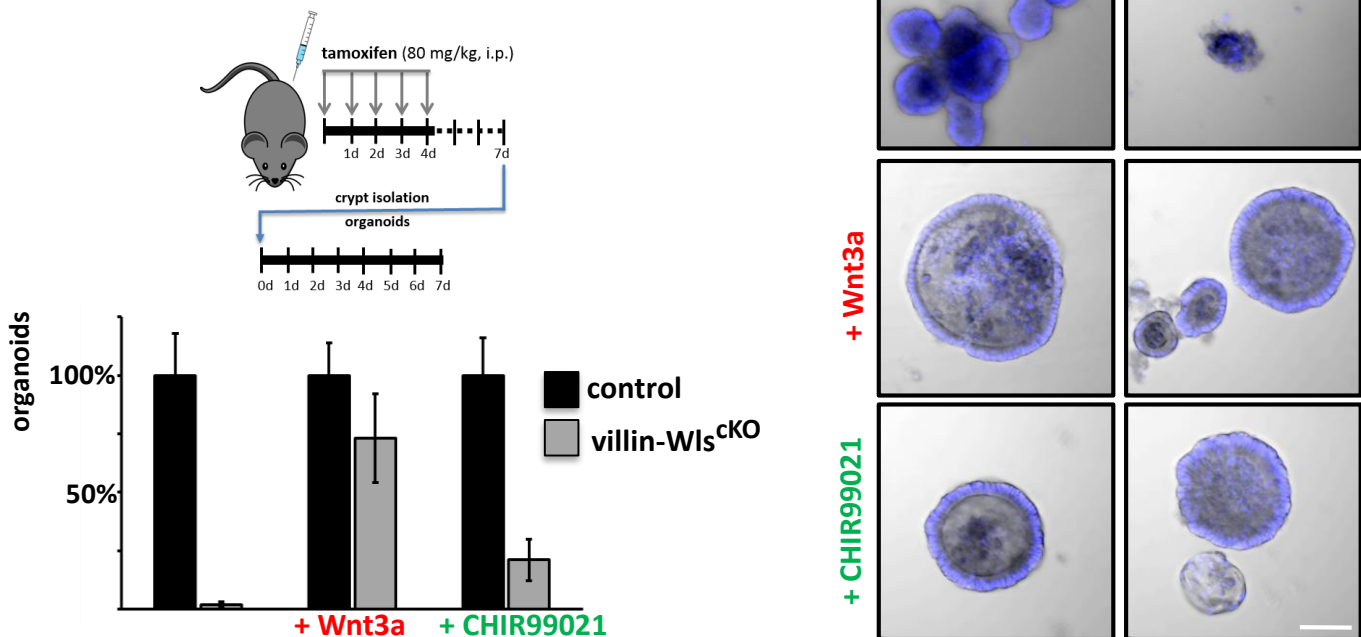


Figure S3, Related to Figure 4

Wnt2b and Wnt5a are expressed by subepithelial mesenchymal cells that are not exclusively myofibroblasts.

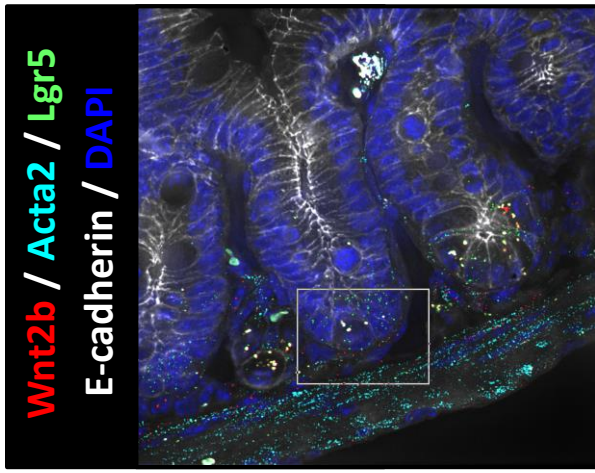
A and B) Murine duodenum tissue sections were hybridized with smFISH probe libraries for *Wnt2b* (red dots, panel A) or *Wnt5a* (red dots, panel B), *Lgr5* (green dots) and *Acta2* (α SMA) (cyan dots) single mRNA molecules. Cell nuclei were stained with DAPI (blue), E-cadherin protein was stained with a FITC-coupled antibody (grey) to visualize cell membranes of epithelial cells. Paneth cell granules exhibit non-specific fluorescence appearing in multiple channels. Scale bar: 100 μ m.

C) Venn diagram showing overlap of *Wnt2b* and *Acta2* (α SMA) (left panel) or *Wnt5a* and *Acta2* (α SMA) expression (right panel). Single crypt-associated stromal cells were stratified into groups of expressing (>0 molecules per μm^3) or non-expressing cells (0 molecules per μm^3) for each RNA of interest according to smFISH analyses. The probability of random co-expression was assessed with the hypergeometric enrichment test.

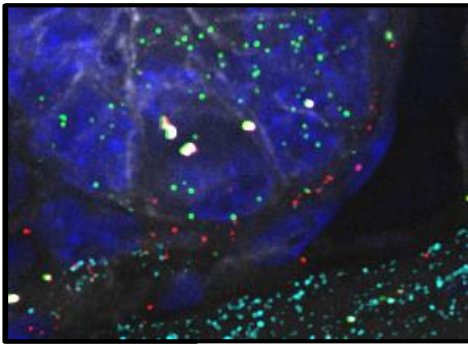
D) Correlation of RNA abundance in single crypt-associated stromal cell by smFISH. *Wnt2b* plot: n=488, *Wnt5a* plot: n=392. Cells without any detectable expression of either RNA were excluded from the analyses.

Figure S3

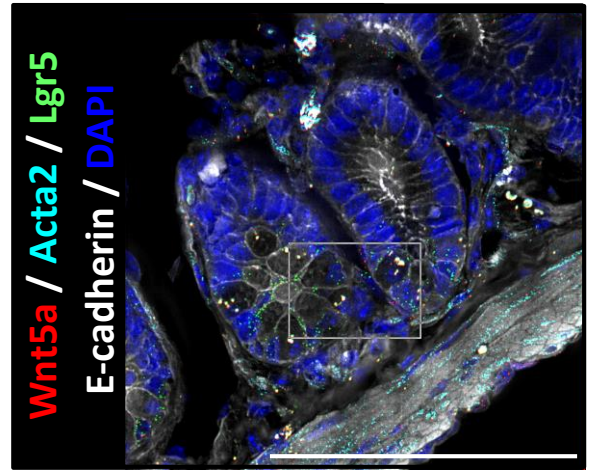
A



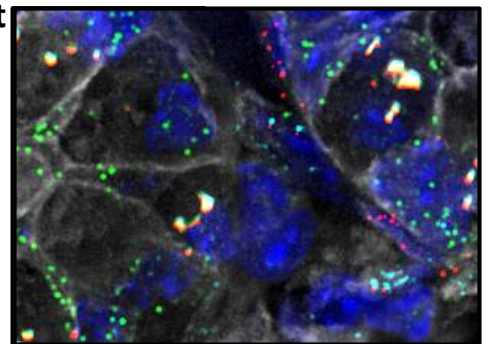
inset



B



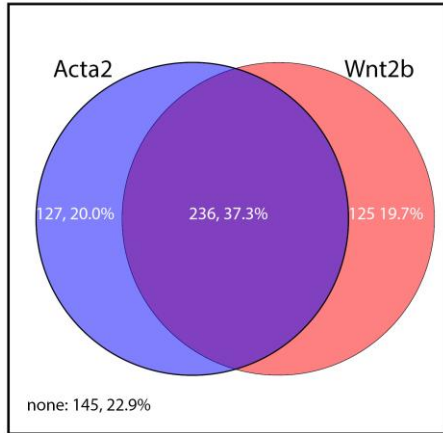
inset



C

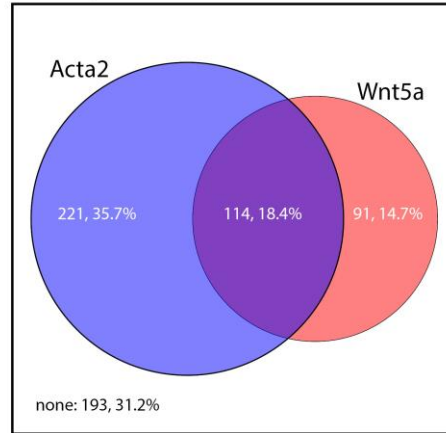
Wnt2b vs. Acta2 (α SMA) expressing cells

633 single cells, 100%



Wnt5a vs. Acta2 (α SMA) expressing cells

619 single cells, 100%



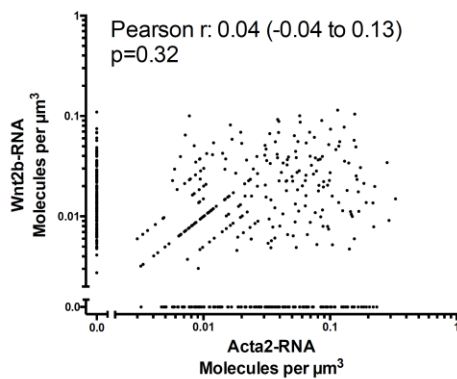
Enrichment (hypergeometric test):

$p=8.35e-7$

$p=0.27$

D

Acta2 (α SMA) vs. Wnt2b



Acta2 (α SMA) vs. Wnt5a

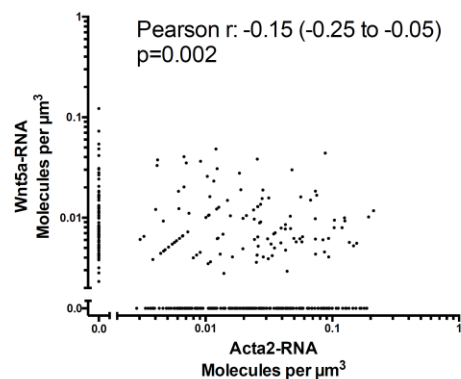


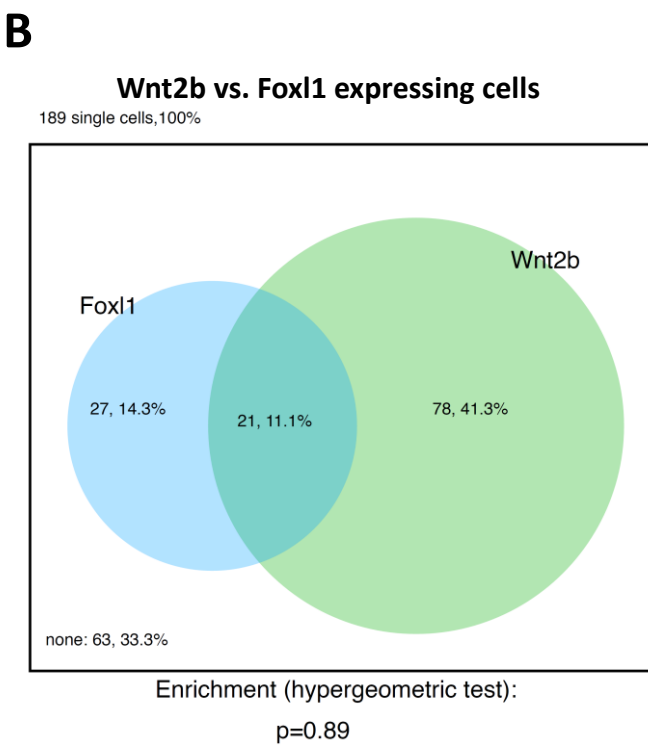
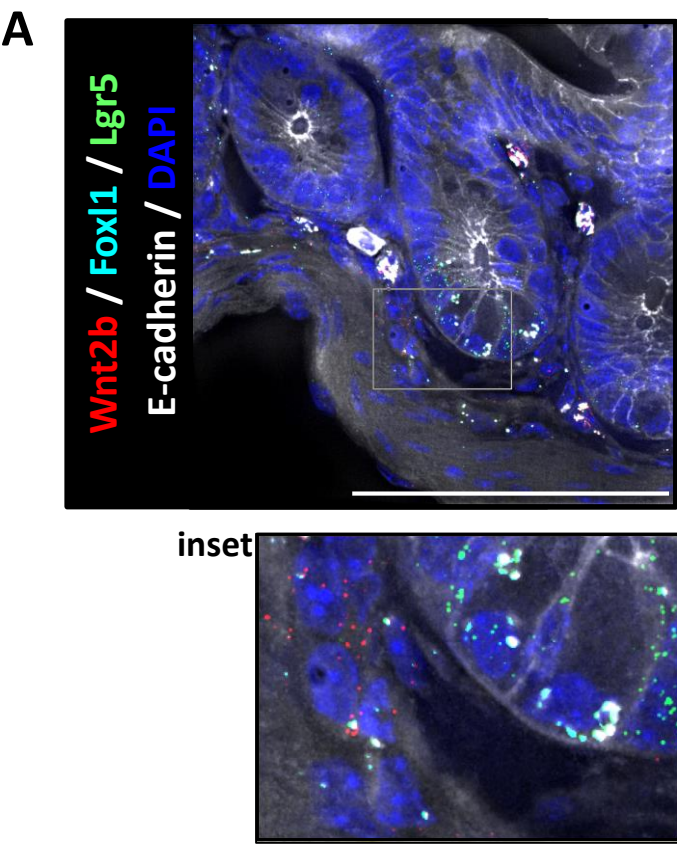
Figure S4, Related to Figure 4

***Wnt2b* expressing cells only partially overlap with *Foxl1* subepithelial mesenchymal cells.**

A) Murine duodenum tissue sections were hybridized with smFISH probe libraries for *Wnt2b* (red dots) *Lgr5* (green dots) and *Foxl1* (cyan dots) single mRNA molecules. Cell nuclei were stained with DAPI (blue), E-cadherin protein was stained with a FITC-coupled antibody (grey) to visualize cell membranes of epithelial cells. Paneth cell granules exhibit non-specific fluorescence appearing in multiple channels. Scale bar: 100 μm .

B) Venn diagram showing overlap of *Wnt2b* and *Foxl1* expression. Single crypt-associated stromal cells were stratified into groups of expressing (>0 molecules per μm^3) or non-expressing cells (0 molecules per μm^3) for each RNA of interest according to smFISH analyses. The probability of random co-expression was assessed with the hypergeometric enrichment test.

Figure S4



Extended Experimental Procedures

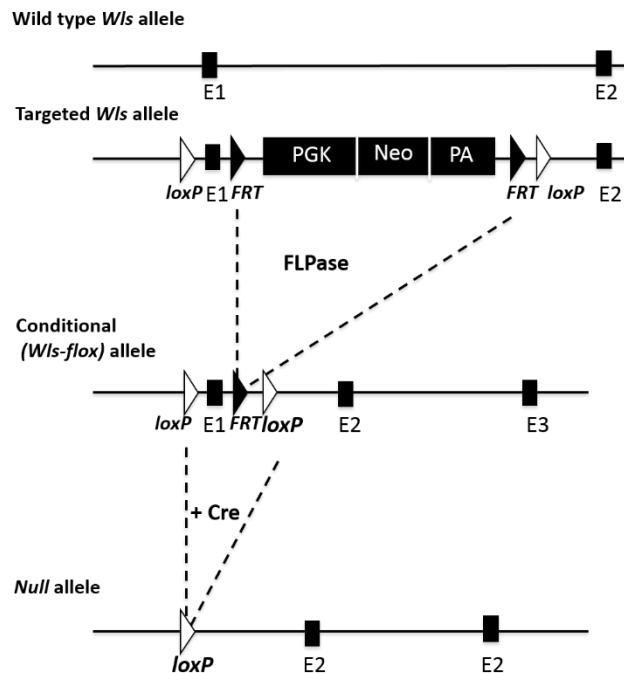
Mouse experiments

Mouse experiments were performed in accordance with Swiss guidelines and approved by the Veterinarian Office of Kanton Zürich, Switzerland and Veterinarian Office of Kanton Vaud, Switzerland. To conditionally eliminate *Wntless* allele conditional *Wls* strain was generated (by Ozgene, Perth, Australia). In this strain first exon of *Wls* containing also translation start is flanked by two loxP sites (as shown in scheme of the locus below). Cre-mediated recombination results in absence of functional RNA and protein. This strain was already used previously (Gay et al., 2015). For genotyping standard PCR was used. Primers recognizing conditional allele (forward primer: 5'- CCC CCT TTC CCT CTC GGT TCC-3', reverse primer: 5'-GGC GGC ATG GAA GCC AAG GGC-3') give 267bp product for wt *Wls* allele and 353bp product for conditional allele.

Knock-out genotype by PCR determination is as follows: forward primer: 5'- 'CCT CTT TCC TAG CGT TCA AAT GGC-3', reverse primer: 5'- GCC AAG GGC TTA AAG GTT GAG ACC-3') giving 1400 bp product for wild type allele and 380 for deleted (KO) allele.

Scheme of *Wls*-conditional allele

(E1 and E2 denote first two exons of *Wls* locus)



Conditional β -catenin animals (Brault et al., 2001) were purchased from Jackson laboratories (Bar Harbor, Maine, USA). Deleted β -catenin^{KO} allele was generated previously using CMV-Cre driver (Valenta et al., 2011). β -catenin^{dm} knock-in allele lacking the ability to promote transcriptional outputs but fully functional in adherens junctions was published previously (Valentat et al., 2011).

To achieve conditional deletion of *Wls* (*Wls*^{cKO}) or β -catenin (β -catenin^{cKO}) the relevant homozygous conditional strain was combined with either *Rosa26-Cre*^{ERT2} driver, which is active in all tissues (Ventura et al., 2007), or *villin-Cre*^{ERT2}, active only in intestinal epithelium (elMarjou et al., 2004). *Rosa26-Cre*^{ERT2} was purchased from Jackson laboratories (Bar Harbor, Maine, USA), *villin-Cre*^{ERT2} was kindly provided by Sylvie Robine. To overcome early embryonic lethality of β -catenin^{dm} allele this allele was combined with the conditional β -catenin allele and inducible Cre driver (either *Rosa26-Cre*^{ERT2} or *villin-Cre*^{ERT2}) as

described in Valenta et al., 2011. To induce Cre-mediated recombination tamoxifen (Sigma) was injected (80 mg/kg) intraperitoneally for 5 consecutive days. The day of the first injection is counted as day 0. In the experiments using β -catenin^{eKO} or β -catenin^{dm} tamoxifen was injected once (tissues collected 24 hour after injection at day 1), two times (tissues collected 48 hour after first injection at day 2), or four times (tissues collected 96 hour after injection at day 4).

To monitor *Lgr5* expression *in vivo*, the knock-in allele *Lgr5-EGFP-IRES-Cre^{ERT2}* (Barker et al., 2007; obtained from Jackson laboratories (Bar Harbor, Maine, USA) was used in combination with *Rosa26-Cre^{ERT2}* and our conditional *Wls* allele.

External active-mouse Wnt3a (Abcam) or mouse Wnt2b (R&D Systems) dissolved in PBS was injected intraperitoneally (minimally 50 μ g/kg, based on the activity of the ligand) twice a day (12 hours intervals) starting 12 hours after first tamoxifen injection. The last Wnt3a or Wnt2b injection was done 6 hours before sacrificing the animals and collecting the tissues. Wnt/ β -catening inducing activity of the particular batch of the recombinant Wnt proteins was assessed using HEK293 STF cells (ATCC CRL-3249) and the injected amount per one injection was adjusted accordingly.

RNA isolation, cDNA synthesis, Quantitative real-time PCR

Freshly isolated intestine was lysed in Tri-Reagent (Sigma) and total RNA was isolated as described by manufacturer's instructions. In the experiments where intestinal epithelial cells were used, they were isolated as described in Gracz et al., 2012. For cDNA synthesis 1 μ g of total RNA was used and cDNA was synthesized by Transcription High Fidelity cDNA Synthesis kit (Roche). Quantitative real-time PCR reactions were performed in three independent biological replicates each with technical triplicate using the Applied Biosystems SYBR Green Kit and monitored by the ABI Prism 7900HT sytem (Applied Biosystem). Representative results are shown at Figures. Specificity of amplification products was verified using melting temperature analysis Primers were designed with ProbeFinder (Roche) software and obtained from Microsynth (Switzerland).

Primer sequences are listed in table below. For qRT-PCR, samples were measured in triplicates and average cycle threshold values were quantified relative to three reference genes (*β -actin*, *GAPDH*, *SDHA*) and using the $\Delta\Delta$ CT method (Valenta et al., 2011).

Primers for real-time qRT-PCR

Gene/mRNA	Forward (5'-3')	Reverse (5'-3')
<i>Ascl2</i>	TGGCACGCCGCAATG	CCTGGAAGCCCAAGTTTACCA
<i>Axin2</i>	GGGGGAAAACACAGCTTACA	ACTGGGTCGGTCTCTTTGAA
<i>Beta-actin</i>	GATCTGGCACCACCTTCT	GGGGTGTGAAGGTCTCAAA
<i>EphB3</i>	AAGAGACTCTCATGGACACGAAAT	ACTTCCCGCCGCCAGATG
<i>GAPDH</i>	AACCTTGGCATTGTGGAAGG	ATCCACAGTCTTCTGGGTGG
<i>Lgr5</i>	CTCCACACTTCGGACTCAACAG	AACCAAGCTAAATGCACCGAAT
<i>Olfm4</i>	GCCACTTTCCAATTTTAC	GAGCCTCTTCTCATAAC
<i>Succinate dehydrogenase A(SDHA)</i>	AAGGCAAATGCTGGAGAAGA	TGGTTCTGCATCGACTTCTG
<i>Troy(Tnfrsf19)</i>	GCTCAGGATGCTCAAAGGAC	CCAGACACCAAGACTGCTCA
<i>Wls</i>	CCCAGCCATGAGCAAAGT	GCATGAGGAAGTGAACCTGA

Histology, Immunohistochemistry, In Situ Hybridisation

Freshly isolated tissues were fixed in 4% paraformaldehyde, dehydrated and mounted in paraffin using standard protocols. Material for frozen sections was processed and embedded in OCT (Tissue-Tek; Sakura

Finetek USA) according to standard protocol. H&E and enzymatic alkaline phosphatase staining was carried out according to standard protocols. Standard immunohistochemical protocols were performed with following antibodies: rabbit anti-Mucin2 (1:100; Santa Cruz sc-15334), mouse anti- β -catenin (1:100; BD Transduction Laboratories 610154), rabbit anti-Ki67 (1:100; Abcam ab15580), rabbit anti-Lysozyme (1:100; Dako A0099), chicken-anti GFP (1:100, Aves; 1:100 Rockland Immunochemicals). Secondary antibodies were anti-rabbit, anti-mouse or anti-chicken antibodies conjugated with Alexa (A488, A594 or A647) diluted 1:500 from Life Technologies. Image was taken using Leica LSM 710 confocal microscope, processed equally using ImageJ (FIJI) software.

RNA in situ hybridization was performed as described in Gregorieff and Clevers, 2010. Vector for the generation of *mOlfm4* was kindly provided by Joerg Huelsken.

Immunoblot

Protein extraction from proximal intestine and immunoblot were performed according to Schwitalla et al., 2013. To detect Wls anti-Wls antibody (Merck Millipore, MABS87) was used, anti-Actin (Sigma, A 3853) serves to check the uniformity of loading of the lysates. Chemiluminescence was detected by Fusion SL VILBER LOURMAT.

Intestinal organoids

Intestinal crypts were isolated as described by Sato et al., 2011 from villin-Wls^{CKO} or control animals injected 5x with tamoxifen 7 days after first tamoxifen application. The culture of small intestinal crypts has been previously described (Sato et al., 2011). Briefly, isolated crypts were and purified, embedded in 50 μ l matrigel drops (Corning, 356231) and overlaid with 700 μ l organoid medium (Advanced DMEM/F12 (Life Technologies, 11320-082), 2mM Glutamax (Life Technologies, 35050-061), 10mM HEPES buffer (Sigma, 83264-100ML-F), 0.5U/ml Penicillin/Streptomycin (Life Technologies, 15070-063), N2 (Life Technologies, 17502-048), B27 (Life Technologies, 12587-010) and growth factors: 50ng/ml mEGF (Life Technologies, PMG8041), 100ng/ml mNoggin (Sigma), 500ng/ml hRSPO1 (Sigma). Crypts were seeded at same density (100 crypts per one matrigel drop) Organoids were grown minimally for 7 days, medium was change after 3 days. To externally activate Wnt/ β -catenin pathway active-Wnt3a (Abcam) or mWnt2b (R&D Systems) (both 100 ng/ml) or 7.5 μ M CHIR99021 (Abcam) was added. Wnt5a (R&D Systems) was used at 100 ng/ml. After 7 days viable organoids were counted using Trypan Blue. For nuclei staining organoids were fixed with 4% paraformaldehyde, and stained with DAPI in PBT.

smFISH analyses

Collected murine duodenum tissues were flushed with PBS, fixed in 4% formaldehyde (FA) in PBS for 3 hours and subsequently agitated in 30% sucrose, 4% FA in PBS overnight at 4°C. Fixed tissues were embedded in OCT (Tissue-Tek), 8 μ m sections were used for smFISH staining. SmFISH probes for *Lgr5*-RNA were previously described (Itzkovitz et al., 2011), custom probes were designed against *Wnt2b*, *Wnt5a*, *Acta2*, *Gli1*, *Foxl1* and *Postn* by utilizing the Stellaris FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA). The duodenum sections were hybridized with smFISH probe sets according to a previously published protocol (Itzkovitz et al., 2011). DAPI (Sigma, D9542) and an E-Cadherin-FITC antibody (BD, 612131) were utilized as nuclear and cell-membrane counterstains, respectively. SmFISH imaging was performed on a Nikon-Ti-E inverted fluorescence microscope with a 100 \times oil-immersion objective and a Photometrics Pixis 1024 CCD camera using MetaMorph software as previously described (Itzkovitz et al., 2011, Bahar Halpern et al., 2015). Single cell segmentation and automatic transcript quantification were performed in a custom Matlab program (Itzkovitz et al., 2011; MATLAB Release 2012a, The MathWorks Inc., USA). For illustrative purposes, the smFISH RNA channels of the images (ref) were filtered with a three-dimensional Laplacian of Gaussian filter (Raj et al., 2008), the counterstain channels DAPI and E-Cadherin-FITC were 3D-deconvoluted with Autoquant X3

(www.mediacy.com), z-stack maximal projections of each deconvoluted counterstain and each filtered RNA channel were merged in Fiji. The hypergeometric distribution was calculated in Matlab by making use of the `hygecdf`-function, single cell expression correlations were calculated in Prism (Version 6, GraphPad Software).