Wnt ligands secreted by subepithelial mesenchymal cells are essential for the survival of intestinal stem cells and gut homeostasis

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Abstract: 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/-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/-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.

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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

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.
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 endoplasmatic 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 (Wlsfloxflo) with an inducible and ubiquitous Cre driver (Rosa26-CreERT2), Whereas adult heterozygous (Rosa26-CreERT2, Wlsfloxflo) mice were indistinguishable from wild-type animals after induction of Cre activity, the homozygous mutants (Rosa26-CreERT2, Wlsfloxflox) hereinafter referred to R26-WlsKO) 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...
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 S1 A). 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 S1 A). The high proliferation of TA cells was also lost within the intestinal crypts (Figure 1 B) 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 S1 B). Crypt loss was preceded by the disappearance of stem cells: a reduction of stem cell traits (expression of Lgr5, Troy, Ascl2, and Olfm4) was observed in R26-Wls cKO already 8 days after Cre induction (Figures 1 D and 1E), when the morphology and proliferation activity of the epithelium still appeared as normal (Figure 1 D). An altered expression pattern of Sox9, a stem cell and early progenitor marker, was also observed in R26-Wls cKO 8 days after induction (real-time qPCR). The expression of key stem cells 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 1 C).

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-CreERT2 with a β-catenin conditional allele (R26-β-catenincKO) led to a phenotype similar to that of R26-Wls cKO animals (Figure S1 D) and resembled that observed when β-catenin was selectively eliminated in the intestinal epithelium (Figure S1 E) (Fevr et al., 2007). However, the effect was already evident 4 days after the induction, compared with
10 days in the case of R26-Wls\textsuperscript{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 \(\beta\)-catenin\textsuperscript{dm} allele (Valenta et al., 2011). \(\beta\)-catenin\textsuperscript{dm} is functional at the adherens junctions but its signaling function is completely abrogated. Intestinal epithelia expressing only \(\beta\)-catenin\textsuperscript{dm} (villin-\(\beta\)-catenin\textsuperscript{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\textsuperscript{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-\(\beta\)-catenin\textsuperscript{dm} crypts, the loss of stem cell traits was also accompanied with the reduced expression of the direct Wnt-pathway target Axin2 (Figure S2B). The effects of attenuated Wnt/\(\beta\)-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/\(\beta\)-catenin signaling and disappear first when Wnt/\(\beta\)-catenin signaling is attenuated either by blocking the transcriptional outputs of \(\beta\)-catenin or preventing the secretion of Wnt ligands. Renewal of IESCs depends on Wnt/\(\beta\)-catenin, and many IESCs 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\textsuperscript{ERT2}, animals (villin-Wls\textsuperscript{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\textsuperscript{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 \(\beta\)-catenin (Figure S2D). These observations indicate that, although Wnt ligands secreted by Paneth cells are essential when they

**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/\(\beta\)-catenin signaling (real-time qPCR).

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

(D) Stem cells marked by Lgr5 survive longer in the intestinal epithelium when Wnt/\(\beta\)-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\textsuperscript{cKO} animals (immunohistochemistry: anti-GFP staining of Lgr5EGFP).

R26-Wls\textsuperscript{cKO} indicates Rosa26-Cre\textsuperscript{ERT2}, Wntless\textsuperscript{flox/flox}. Immunohistochemistry: DAPI marks nuclei, and \(\beta\)-catenin denotes epithelial cells. Scale bars, 100 \(\mu\)m. Real-time qPCR: y axes show normalized relative mRNA abundance; control levels were set to 1. Error bars indicate SD.
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-WlscKO animals could be prevented by the addition of exogenous Wnt3a protein (Figure 2A). Indeed, intraperitoneal injection of Wnt3a was able to restore Wnt/b-catenin signaling in the proximal intestine (Figure 2B). Restoration of b-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 b-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/b-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-Wlscko animals. As with Wnt3a treatment, Wnt2b-treated organoids grew as spheroids (basically as closed crypts) and did not promote growth. If Villin-Wlscko 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-Wlscko animals. Injection of Wnt2b could partially...
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 (αSMA), 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 (αSMA) negative (Figures S3A and S3B). Nevertheless, 65% of the Wnt2b-positive cells and 55% of the Wnt5a-expressing ones are myofibroblasts

epithelium, some of them in close proximity to Lgr5+ IESCs (Figure 4A; Figures S3A and S3B). Hence, Wnt2b represents a driver of 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 pericytic subepithelial cells. We focused on the transcription factors Fox1 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. Fox1 was found to be expressed by subepithelial mesenchymal cells, but only 21% of the
Wnt2b-positive (further as Wnt2b⁺) cells co-express Fox1 (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 (αSMA). 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 (αSMA) 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 (αSMA). 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; Fev 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 (Gregoireff 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-WlsKO; 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 (villinCreERT2) and in myofibroblasts (Myh11CreERT2) 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 (αSMA). 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) in Lgr5⁺ IESCs and triggers the Wnt/β-catenin signal essential 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**

**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 Vaudois, Switzerland.

To completely 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 (Brau et al., 2001), Lgr5-EGFP-IRESCreERT2 (Barker et al., 2007), β-cateninmin (Valenta et al., 2011), Rosa26-CreERT2 (Ventera et al., 2007), and villin-CreERT2 (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 µg/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 Grazc et al. (2012), were lysed in TRI-Reagent (Sigma). RNA isolation, cDNA synthesis, and Real-Time qPCR 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 ΔΔCT 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 Gregoreff and Clevers (2010). Protein extraction from proximal intestine and immunoblot were performed according to Schwitalla et al. (2013).

Intestinal Organoids
Intestinal organoids were generated from villin-Wls<sup>lox/lox</sup> 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 μM 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, Fox1, and Postn by utilizing the Stellaria 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.cellrep.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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