Published in final edited form as:

Biochem Biophys Res Commun. 2019 November 19; 519(4): 754–760. doi:10.1016/j.bbrc.2019.09.044.

Epithelial Wntless is dispensable for intestinal tumorigenesis in mouse models

Ganglong Gao^{1,3,4,7}, Gaigai Wei^{2,7}, Shijie Liu^{2,7}, Jiwei Chen^{2,7}, Zhiyang Zeng², Xinyan Zhang², Fangrui Chen², Lingang Zhuo², Wei Hsu⁵, Dali Li², Mingyao Liu^{2,6,*}, Xueli Zhang^{1,3,*}

¹Department of General Surgery, Affiliated Fengxian Hospital, Southern Medical University (Shanghai Fengxian Central Hospital), Shanghai, 201499, China.

²Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China

³The Third School of Clinical Medicine, Southern Medical University, Guangzhou, 510500, China

⁴Department of Biliary-Pancreatic Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiaotong University, 1630, Dongfang road, Pudong district, Shanghai, 200127, China

⁵Center for Oral Biology, University of Rochester Medical Center, Rochester, NY, USA.

⁶Department of Molecular and Cellular Medicine, The Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, TX 77030, USA

⁷These authors contributed equally to this work

Abstract

Wnt signaling is essential for the maintenance of adult stem cells and its aberrant activation is a stimulator of carcinogenesis. The transmembrane protein, Wntless, is an essential Wnt signaling component through regulating the secretion of Wnt ligands. Here, we generated a mouse model with specific Wntless knockout in intestinal epithelium to study its function in the intestinal epithelium. Wntless knockout exhibits no obvious defects in mice but significantly disrupted proliferation and differentiation of small intestinal organoids. We also discovered that these deficiencies could be partially rescued by Wnt3a supplement but not Wnt9b. To further investigate the role of Wntless in tumorigenesis, APC-deficient spontaneous intestinal tumors and chemical induced colorectal cancer mouse models were employed. To our surprise, intestinal epithelium-specific knockout of Wntless did not cause significant differences in tumor number and size. In summary, our data demonstrated that epithelial Wntless was required for the growth and differentiation of small intestinal organoids but not in live animals, suggesting the other tissues, such as mesenchymal tissue, play critical role for Wnt secretion in both intestinal homeostasis as well as tumorigenesis.

*To whom correspondence should be addressed: Dr. Mingyao Liu, 500 Dongchuan Road School of life sciences Room 501, Shanghai, China, 200241; myliu@bio.ecnu.edu.cn; Dr. Xueli Zhang, 6600 NanFeng Road, Shanghai, 201499, China. lejing1996@aliyun.com;.

Conflict of interest statement

The authors declare no competing financial interests.

Keywords

Wntless; intestinal epithelium; small intestinal organoids; colorectal cancer

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1. Introduction

The secretion of Wnt ligands is reported to mediate cell-cell communication in tissue homeostasis and tumorigenesis[1]. Dysregulation in Wnt signaling is associated with various human diseases, including developmental defects and cancers[2]. Wntless, also known as Gpr177, is a conserved transmembrane protein and a component required for Wnt ligand secretion in mammalian cells [3]. In the endoplasmic reticulum (ER), Wntless binds to palmitoleated Wnt ligands and transports them to the plasma membrane where they are released [3]. Then Wntless is taken up by clathrin-mediated endocytosis [4,5], and recycled to the Golgi and ER to replay the role of Wnt-transporter [3]. Depletion of Wntless in animal models can cause developmental abnormalities which is similar to the phenotype caused by Wnt loss-of-function [6].

The intestinal epithelium renews itself every 3 to 5 days[7]. The transit-amplifying (TA) cells, which are generated from the $Lgr5^+$ crypt base columnar (CBC) stem cells, can differentiate into all types of intestinal epithelial cells, including enterocytes, goblet, enteroendocrine, and Paneth cells[7]. Numerous studies have demonstrated that Wnt signaling is vital for the intestinal epithelial homeostasis [8]. Intestinal crypts can form organoids when cultured in a medium containing recombinant EGF, Noggin, and R-spondin (ENR) [9], and maintaining the ability of self-renewal and differentiation. In the organoid model, Wnt ligands secreted from epithelial and non-epithelial cells are found to function as stem cell growth factors and contribute to the Wnt-dependent stem cell niche[10,11]

Aberrant activation of the Wnt signaling is also reported to be one of the major causes of colorectal tumor [12] [13]. These findings suggest that regulators of the Wnt signaling pathway could be potential therapeutic targets. Impairment of Wnt secretion by Wntless knockdown in colon cancer cell lines is known to inhibit cancer cell proliferation *in vitro* and *in vivo* [14]. However, the function of Wnt ligands secreted from the intestinal epithelial cells in colorectal carcinogenesis are not well studied especially in spontaneous colon cancer animal models which is more relevant to human disease.

Here, we generated an intestinal epithelium specific Wntless knockout mouse model to investigate the function of epithelial Wntless in intestinal homeostasis and tumorigenesis. We found that deletion of Wntless in the intestinal epithelium inhibited small intestinal organoid growth. The small intestinal organoids with Wntless knockout tended to differentiate into goblet cells. Wnt3a supplement could rescue the growth inhibition and differentiation of the small intestinal organoids. However, specific deficiency of Wntless in the intestinal epithelium did not cause significant differences in tumor number and size in both spontaneous intestinal tumor and colitis-associated cancer (CAC) mouse models. In summary, we report that although Wntless plays important roles in small intestinal organoid formation, in the intestinal epithelium, Wntless was not sufficient for regulating intestinal tumorigenesis *in vivo*.

2. Materials and Methods

2.1. Mice and tumor induction

Wntless^{loxp/+} were a gift from Dr, Wei Hsu. Villin-Cre mice were obtained from the National Resource Center of Mutant Mice (NRCMM). All experiments conformed to the regulations drafted by the Association for the Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by the East China Normal University Centre for Animal Research.

For the CAC tumor model, Six to eight-week-old mice were treated as described previously [15].

For the spontaneous tumor model, We generate the control group mice Villin-cre;Wntless^{loxp/+};Apc^{loxp/+}, and the experimental group mice Villin-cre;Wntless^{loxp/loxp};Apc^{loxp/+}. When the mice were 4 months old, Tumor counts and size estimations were performed, and the samples were fixed in 4% paraformaldehyde at 4°C overnight and paraffin-embedded.

2.2. Small intestine crypts isolation, culture and treatment

The small intestine crypts isolation was performed as described [16]. The following proteins were used for rescue experiments: Wnt3a (100 ng/ml; R&D), Wnt9b (100 ng/ml; MedChemExpress). Stocks were dissolved in sterile PBS and concentrations were kept uniform across all wells during the experiments.

2.3. *In situ* organoid immunofluorescence

Organoids were fixed in plates (4% paraformaldehyde, 4 hours at 4°C), then, permeabilized in 0.2% Triton X-100/PBS, blocked with 2% goat serum for 20 minutes, and incubated with primary antibody overnight at 4°C. After that, fluorescence was detected using the second Alexa Fluor 488 antibodies with DAPI counterstaining. The antibodies used were as follows: Ki67 (1:2000; NeoMarker), Lysozyme (1:2000; Dako) and Muc2 (1:50; SantaCruz).

2.4. mRNA level gene expression analysis

The intestinal organoid or whole intestinal tumor tissue RNA was obtained by homogenization in TRIzol reagent (Takara), followed by RNA precipitation in isopropanol, resuspension in DEPC-treated water. cDNA was synthesized with the Superscript RNase H2 Reverse Transcriptase Kit (Takara). Real-time PCR was performed using the SYBR Green PCR kit (Takara) on Mx3005P thermal cycler.

2.5. Statistical analysis

Statistical analyses were performed using the GraphPad Prism software. $P < 0.05$ was considered to be statistically significant. Unless indicated in the figure legends, all the experiments were performed at least three times with similar results. Results are displayed as mean $\pm$ standard error of the means (SEM).

3. Results

3.1. Intestinal epithelium-specific deletion of Wntless impairs cell proliferation and differentiation in organoids culture but not in live animals

To generate intestinal epithelium-specific Wntless knockout mouse model, we crossed Villin-cre mice with our previously reported Wntless^{loxP/loxP} (hereafter referred to as CTR) mice [6] to generate Villin-cre; Wntless^{loxP/loxP} (hereafter referred to as Wntless^{E-Del}) mice. Deletion of Wntless in intestinal epithelial cells did not show any obvious defects in mice through 6 months of observation, even challenged by dextran sulfate sodium salt (DSS) to induce acute inflammatory bowel disease (data not shown). Since the resource of Wnt ligands is not limited to epithelial cells, we leveraged organoid culture system to exclude the influence of non-epithelial cells. We isolated small intestinal crypts from both CTR and Wntless^{E-Del} adult mice for organoid culture in ENR (EGF, Noggin and R-spondin) medium. After 24-hour cultivation, organoids from the CTR crypts began to change from round to bud-shaped (Figure 1A, left), while the Wntless^{E-Del} crypts maintained the round shape (Figure 1A, right). The size of the round Wntless^{E-Del} organoids remained unchanged, indicating no significant increase in the total cell number in crypts (Figure 1A, right). To investigate the proliferation and differentiation of the organoids, we performed immunofluorescence staining with the organoids after 4 days of cultivation. Consistent with our previous report [16], positive Ki67 staining was observed in the budding region of the CTR organoids, while there were almost no Ki67-positive staining in the Wntless^{E-Del} organoids (Figure 1B, top). Additionally, the number of lysozyme-positive Paneth cells was significantly decreased in the Wntless^{E-Del} organoids (Figure 1B, middle). Interestingly, the number of Muc2-positive goblet cells was increased in the Wntless^{E-Del} organoids (Figure 1B, bottom). These results were confirmed by qPCR. The expression of the stem cell markers (Lgr5 and Olfr4), TA cell markers (Wdr43), Paneth cell markers (Lysozyme), and target genes of the Wnt signaling pathway (C-myc, Cyclind1, Axin2, and Ctnnb1) were decreased in the Wntless^{E-Del} organoids (Figure 1C). Further, the expression of the goblet cell marker, Muc2, increased in the Wntless^{E-Del} organoids (Figure 1C). It was reported that intestinal stem cells ultimately differentiate into goblet cells when the Notch signaling pathway is inhibited [17]. We then checked the expression level of genes related to the Notch signaling pathway and found those genes (Dll1, Notch1, Jag1, Hes1, and Atoh1) were repressed in Wntless deleted small intestinal organoids (Figure 1D). These data suggest that Wntless is critical for cell proliferation and differentiation in organoid culture mainly through modulation of Wnt signaling.

3.2. Wnt3a supplement could partially rescue cell proliferation and differentiation in Wntless^{E-Del} organoids

Both epithelial and non-epithelial Wnt ligands are reported to be involved in small intestinal epithelial homeostasis [11]. We assume that the defect of Wntless^{E-Del} cells observed in organoid culture is due to Wnt ligand deficiency. The Wntless^{E-Del} intestinal epithelium organoid culture system provided us a tool for studying the function of a single Wnt family member. Due to technical limitations, we only used Wnt3a and Wnt9b. When supplied with Wnt3a, the Wntless^{E-Del} crypts either budded or grew larger (Figure 2A, middle). However, adding Wnt9b (Figure 2A, right) resulted in no differences compared with the Wntless^{E-Del}

group (Figure 2A, left). We statistically analyzed the organoid survival rate and the number of buds. Almost all Wntless^{E-Del} organoids with the Wnt3a supplement survived and grew normally in the 4 days of cultivation, while half of the Wntless^{E-Del} organoids with or without the Wnt9b supplement died (Figure 2B). Wnt3a supplementation led to the budding of some Wntless^{E-Del} organoids, while Wnt9b did not (Figure 2C). These data showed that the Wnt3a supplement could partially rescue the proliferation of Wntless^{E-Del} organoids, suggesting Wnt3a is at least one of the critical Wnt ligand to maintain intestinal epithelial cell activity.

Next, Wnt3a supplementation promoted budding and proliferation of many Wntless^{E-Del} organoids (Figure 2D, top right). Paneth cell marker lysozyme staining showed that the Wnt3a-treated Wntless^{E-Del} organoids differentiated into Paneth cells in the sprout bud region (Figure 2D, top right). Accordingly, the Wntless^{E-Del} organoids with Wnt3a treatment had less goblet cells (Figure 2D, top right) compared to the Wntless^{E-Del} organoids. Wnt9b-treated Wntless^{E-Del} organoids (Figure 2D, bottom) showed no significant difference compared with the Wntless^{E-Del} group (Figure 2D, top left). Quantitative PCR analysis showed that compared with the Wntless^{E-Del} organoids, with or without Wnt9b treatment, Wntless^{E-Del} organoids with Wnt3a supplement indeed had higher expression levels of the stem cell markers (Lgr5 and Olfm4), TA cell marker (Wdr43), and genes related to the activated Wnt and Notch signaling pathways (C-myc, Cyclind1, Ephb2, Ascl2, Notch1, and Hes1) (Figure 2E).

3.3. Epithelium Wntless deficiency did not reduce tumorigenesis in spontaneous intestinal tumor models

As Wnt signaling is also strongly related to intestinal tumorigenesis, we next investigated whether Wnt ligand secretion in epithelial cells played critical role in colorectal cancer. We used Apc^{loxP/+} mouse model which could develop tumors resembling human sporadic adenomas [18]. We obtain control group mice, Villin-Cre; Apc^{loxP/+}, and the experimental group mice, Villin-Cre; Wntless^{loxP/loxP}; Apc^{loxP/+} mice. In these models, the adenomas occurred mainly in the small intestine. Tumor number and size were analyzed at the age of 4 months. We found no significant differences in tumor number between the CTR (29.33 ± 14.09 adenomas per mouse) and the Wntless^{E-Del} (33.5 ± 11.68) groups (Figure 3A). The Ki67-positive cell numbers were also comparable between both groups (Figure 3B). These data suggest that intestinal epithelium-specific Wntless knockout did not significantly change the phenotype in APC-deficient spontaneous intestinal tumor mouse model.

To comprehensively address the role of Wntless in intestinal tumor, we also used a CAC mouse model. Eight-week old CTR and Wntless^{E-Del} mice were injected with the procarcinogen azoxymethane (AOM; 12.5mg/kg), followed by three cycles of DSS administration (Figure 4A). All the mice treated with AOM and DSS developed tumors located in the colon, where the most severe pathological changes happened during DSS colitis [19]. Deletion of Wntless in the intestinal epithelium had almost no effect on tumor incidence and size 100 days post-treatment (Figure 4B). Consistently, no significant differences in the mRNA levels of the key factors in the Wnt signaling pathway (Cyclind1, Ephb2, PCNA, Bak and IL6), were detected in tumor samples (Figure 4C). H&E staining

also revealed no significant changes in histology (Figure 4D). Immunohistochemical staining of Ki67 showed the cell proliferation was not significantly different in the Wntless^{E-Del} group compared to the control (Figure 4D). Collectively, these data indicate that Wntless in epithelial cells is not sufficient for preventing tumor development and growth in both spontaneous intestinal tumor and induced colorectal cancer models, suggesting Wnt ligands secretion from other cell types, such as GLI1-expressing mesenchymal cells[20] and subepithelial telocytes[8] in intestine is more critical.

4. Discussion

The Wnt signaling pathway is essential for the stemness of stem cells, proliferation of the TA progenitor cells in intestinal crypts.[21]. Wntless, which is required for the transportation of all Wnt ligands is discovered to be essential for planarian regeneration and angiogenesis [22,23]. Previous studies have reported that Wnt ligands could be secreted from Paneth cells [11,24], and extra-epithelial Wnt-producing niche in the small intestine, e.g., subepithelial mesenchyme and stroma cells [10,25]. In this study, we investigated the function of the Wntless in intestinal epithelial cell homeostasis and tumorigenesis.

We used Villin-Cre to specifically delete Wntless in mouse intestinal epithelium. To exclude the influence of Wnts secreted from the mesenchyme, we established Wntless^{E-Del} mice small intestinal organoids. This organoid culture system provides an accessible model for investigating the single molecular function of Wnt ligands and molecular interactions in the intestinal stem cell-niche. In ENR culture medium, normal small intestinal cells are able to secrete Wnt ligands to support organoid homeostasis. Knockout of Wntless in epithelial cells lead to loss of the Wnt ligands secretion in organoid culture system and caused growth inhibition and impaired cell differentiation. However, this phenotype is not observed in live mice. It is because that the mesenchymal cells are able to secrete abundant Wnt ligands to maintain epithelial homeostasis. Our observation suggests that Wnt ligands secreted from epithelial cells is functional but is not essential for homeostasis. Moreover, we found that the growth inhibition of small intestinal organoids induced by Wntless deletion could be partially rescued by supplementation with Wnt3a. Although Wnt9b is also expressed in the intestinal epithelium and the stromal cells, its addition had no effect on Wntless^{E-Del} small intestinal organoid growth, suggesting Wnt3a is a critical factor to activate Wnt signaling for the maintenance of epithelial cell activity.

The expression levels of Wntless and some Wnt ligands were reported to be upregulated in colon cancer samples [14]. Our mouse models will be useful for investigating whether Wnt ligands secreted by the intestinal epithelium are required for intestinal tumorigenesis. The tumor cells regulate their survival and growth by autocrine and paracrine ways [13]. Stroma cells from the tumor microenvironment regulate tumor cell growth by influencing metabolism, tumor immunity and other signaling processes. Wnt ligands expressed both in the intestinal epithelium and the stromal cells. Thus in our tumor mouse models, consistent with other in vivo studies [26,27], Wnt secreted from extra-epithelial cells could maintain the Wnt pathway. The experiment regarding the function of Wnt ligands in tumorigenesis using cancer cell lines which were composed of single type of cells, might not mimic the actual situation in vivo. Indeed, our animal experiment results differed from previous studies

conducted using cell lines [14]. We found that Wnt ligands secretion in the intestinal epithelium was not required for intestinal adenoma formation and growth. The main difference of animal model and cell culture system is in animals numerous cell types and systems work together to maintain the biological process while cell culture system is better to understand molecular mechanisms. In colon cancer cell lines or organoid culture system, epithelial-specific knockout of Wntless could significantly defect Wnt secretion in epithelial cells and inhibit tumor cell proliferation or organoid formation. It is because in this system the source of Wnt ligands are from epithelial cell themselves. However, in mouse models, epithelial-specific knockout of Wntless could only impair epithelial Wnt secretion. While Wnt secreted from extra-epithelial cells was sufficient to activate the Wnt pathway in epithelial cells. Thus, epithelial knockout of Wntless had different phenotypes between cell lines and mouse models.

Identification of the source of Wnt secretion is a critical question in development biology and cancer science. Here we show that specific ablation of Wntless in intestinal epithelial cells impaired small intestinal organoids growth and differentiation but no defect was observed in homeostasis, tissue repair and tumorigenesis in animal, suggesting mesenchymal cells in the intestine play critical roles in Wnt ligands secretion and maintenance of stem cell niche.

Acknowledgments

Funding

This work was supported by grants from the National Natural Science Foundation of China (no. 81874140) and China Postdoctoral Science Foundation (no. 11301-41211C-18018).

References

- [1]. Gao X, Hannoush RN, Single-cell imaging of Wnt palmitoylation by the acyltransferase porcupine, *Nat Chem Biol* 10 (2014) 61–68. [PubMed: 24292069]
- [2]. Clevers H, Wnt/beta-catenin signaling in development and disease, *Cell* 127 (2006) 469–480. [PubMed: 17081971]
- [3]. Banziger C, Soldini D, Schutt C, Zipperlen P, Hausmann G, Basler K, Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells, *Cell* 125 (2006) 509–522. [PubMed: 16678095]
- [4]. Belenkaya TY, Wu Y, Tang X, Zhou B, Cheng L, Sharma YV, Yan D, Selva EM, Lin X, The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network, *Dev Cell* 14 (2008) 120–131. [PubMed: 18160348]
- [5]. Yang PT, Lorenowicz MJ, Silhankova M, Coudreuse DY, Betist MC, Korswagen HC, Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells, *Dev Cell* 14 (2008) 140–147. [PubMed: 18160347]
- [6]. Fu J, Jiang M, Mirando AJ, Yu HM, Hsu W, Reciprocal regulation of Wnt and Gpr177/mouse Wntless is required for embryonic axis formation, *Proc Natl Acad Sci U S A* 106 (2009) 18598–18603. [PubMed: 19841259]
- [7]. Clevers H, The intestinal crypt, a prototype stem cell compartment, *Cell* 154 (2013) 274–284. [PubMed: 23870119]
- [8]. Shoshkes-Carmel M, Wang YJ, Wangenstein KJ, Toth B, Kondo A, Massasa EE, Itzkovitz S, Kaestner KH, Subepithelial telocytes are an important source of Wnts that supports intestinal crypts, *Nature* 557 (2018) 242–246. [PubMed: 29720649]

- [9]. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H, Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche, *Nature* 459 (2009) 262–265. [PubMed: 19329995]
- [10]. Kabiri Z, Greicius G, Madan B, Biechele S, Zhong Z, Zaribafzadeh H, Edison, Aliyev J, Wu Y, Bunte R, Williams BO, Rossant J, Virshup DM, Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts, *Development* 141 (2014) 2206–2215. [PubMed: 24821987]
- [11]. Farin HF, Van Es JH, Clevers H, Redundant Sources of Wnt Regulate Intestinal Stem Cells and Promote Formation of Paneth Cells, *Gastroenterology* 143 (2012) 1518–+. [PubMed: 22922422]
- [12]. Huels DJ, Bruens L, Hodder MC, Cammareri P, Campbell AD, Ridgway RA, Gay DM, Solar-Aboud M, Faller WJ, Nixon C, Zeiger LB, McLaughlin ME, Morrissey E, Winton DJ, Snippert HJ, van Rheeën J, Sansom OJ, Wnt ligands influence tumour initiation by controlling the number of intestinal stem cells, *Nat Commun* 9 (2018) 1132. [PubMed: 29556067]
- [13]. Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP, Wnt activity defines colon cancer stem cells and is regulated by the microenvironment, *Nat Cell Biol* 12 (2010) 468–476. [PubMed: 20418870]
- [14]. Voloshanenko O, Erdmann G, Dubash TD, Augustin I, Metzger M, Moffa G, Hundsruker C, Kerr G, Sandmann T, Anchang B, Demir K, Boehm C, Leible S, Ball CR, Glimm H, Spang R, Boutros M, Wnt secretion is required to maintain high levels of Wnt activity in colon cancer cells, *Nat Commun* 4 (2013) 2610. [PubMed: 24162018]
- [15]. Eckmann L, Nebelsiek T, Fingerle AA, Dann SM, Mages J, Lang R, Robine S, Kagnoff MF, Schmid RM, Karin M, Arkan MC, Greten FR, Opposing functions of IKK β during acute and chronic intestinal inflammation, *Proc Natl Acad Sci U S A* 105 (2008) 15058–15063. [PubMed: 18815378]
- [16]. Zhang X, Liu S, Wang Y, Hu H, Li L, Wu Y, Cao D, Cai Y, Zhang J, Zhang X, Interleukin22 regulates the homeostasis of the intestinal epithelium during inflammation, *Int J Mol Med* 43 (2019) 1657–1668. [PubMed: 30816423]
- [17]. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H, Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells, *Nature* 435 (2005) 959–963. [PubMed: 15959515]
- [18]. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF, Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene, *Science* 256 (1992) 668–670. [PubMed: 1350108]
- [19]. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R, A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice, *Gastroenterology* 98 (1990) 694–702. [PubMed: 1688816]
- [20]. Degirmenci B, Valenta T, Dimitrova S, Hausmann G, Basler K, GLI1-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells, *Nature* 558 (2018) 449–453. [PubMed: 29875413]
- [21]. Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, Danenberg E, Clarke AR, Sansom OJ, Clevers H, Crypt stem cells as the cells-of-origin of intestinal cancer, *Nature* 457 (2009) 608–U119. [PubMed: 19092804]
- [22]. Adell T, Salo E, Boutros M, Bartscherer K, Smed-Evi/Wntless is required for beta-catenin-dependent and-independent processes during planarian regeneration, *Development* 136 (2009) 905–910. [PubMed: 19211673]
- [23]. Stefater JA 3rd, Lewkowich I, Rao S, Mariggi G, Carpenter AC, Burr AR, Fan J, Ajima R, Molkentin JD, Williams BO, Wills-Karp M, Pollard JW, Yamaguchi T, Ferrara N, Gerhardt H, Lang RA, Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in myeloid cells, *Nature* 474 (2011) 511–515. [PubMed: 21623369]
- [24]. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H, Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts, *Nature* 469 (2011) 415–418. [PubMed: 21113151]

- [25]. Valenta T, Degirmenci B, Moor AE, Herr P, Zimmerli D, Moor MB, Hausmann G, Cantu C, Aguet M, Basler K, Wnt Ligands Secreted by Subepithelial Mesenchymal Cells Are Essential for the Survival of Intestinal Stem Cells and Gut Homeostasis, *Cell Rep* 15 (2016) 911–918. [PubMed: 27117411]
- [26]. Durand A, Donahue B, Peignon G, Letourneur F, Cagnard N, Slomianny C, Perret C, Shroyer NF, Romagnolo B, Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1), *Proc Natl Acad Sci U S A* 109 (2012) 8965–8970. [PubMed: 22586121]
- [27]. Kim TH, Escudero S, Shivdasani RA, Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells, *Proc Natl Acad Sci U S A* 109 (2012) 3932–3937. [PubMed: 22355124]

Figure 1. Wntless was important for adult mice small intestinal organoid proliferation and differentiation

(A) Organoid morphological images from control (A, left) and Wntless^{E-Del} (A, right) mice small intestinal crypts cultured in ENR medium.

(B) Whole mount IF for detecting cell proliferation and differentiation after 4 days of organoid cultivation. Reduced Ki67 positive cells; Fluorescence inverted microscopic images of Ki67 immunostaining (green), Nuclei stained with DAPI (blue) (B, top). Lysozyme staining showing a reduced number of Paneth cells after 4-day Wntless^{E-Del} organoid cultivation compared to the CTR organoids (B, middle). Goblet cell marker, Muc2, immunostaining showing Wntless^{E-Del} mice small intestinal organoid differentiation into more goblet cells than CTR (B, bottom).

(C) Quantitative real-time PCR analysis of the relative expression level of intestinal epithelial cell markers and Wnt/ β -catenin target genes in small intestinal organoids of CTR and Wntless^{E-Del} at day 4.

(D) Quantitative real-time PCR analysis of the Notch signaling pathway in small intestinal organoids of CTR and Wntless^{E-Del} at day 4.

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Figure 2. Wnt3a supplements partially restored the proliferation and differentiation abilities in Wntless^{E-Del} small intestinal organoids

(A) Wntless^{E-Del} small intestinal organoids were cultured 5 days in ENR medium; images were taken at different time points (A, left). Images from the Wntless^{E-Del} mice small intestinal organoids, which budded again when Wnt3a was added to the ENR medium (A, middle). Image showing the Wntless^{E-Del} mice small intestinal organoids, which still could not bud when Wnt9b was added to the ENR medium (A, right).

(B and C) Quantitation of Wntless^{E-Del} and Wntless^{E-Del}+Wnt3a and Wntless^{E-Del}+Wnt9b organoid numbers (B) (the crypts plated at day 0 were defined as 100) and the average number of buds per organoid (C) in the 4-day culture (n=5 mice). Results shown as mean ± SEM. (*P<0.05; **P<0.01; ***P<0.001)

(D) Ki67, Lysozyme and Muc2 staining indicated Wntless^{E-Del} small intestinal organoids cultured in the ENR culture medium for 4 days lost their proliferation ability and differentiation was abnormal. Reduced Paneth cell and increased goblet cells numbers were

observed (D, top left). Wntless^{E-Del} small intestinal organoids growth in the ENR medium with Wnt3a was partially rescued. Some organoids budded again; the Ki67 and lysozyme positive cells distributed among the budded zone and goblet cell marker, Muc2, were reduced (D, top right). When Wnt9b was added to the ENR culture medium, the Wntless^{E-Del} organoid growth state remained the same as the Wntless^{E-Del} organoid growth rate (D, bottom).

(E) Quantitative real-time PCR analysis of the relative expression level of intestinal epithelial cell markers, Wnt/ β -catenin target genes and Notch signaling pathway target genes from organoids of Wntless^{E-Del} and Wntless^{E-Del} cultured with Wnt3a and Wnt9b at day 4 (n=5 mice). (Data are mean $\pm$ SEM, *P<0.05; **P<0.01; ***P<0.001).

Figure 3. Intestinal epithelium Wntless knockout did not inhibit tumorigenesis in Apc mutant adenoma mouse model

(A) CTR mice (n=12) displayed a mean of 29.33 ± 14.09 adenomas per mouse. Wntless^{E-Del} mice developed 33.5 ± 11.68 adenomas per mouse (n=12). Adenomas of CTR and Wntless^{E-Del} mice had a similar size distribution and the mean number of adenomas increased in mutant mice, but not significantly.

(B) Immunostaining of Ki67 in the small intestinal tumor from CTR mice and Wntless^{E-Del} mice at 4 months.

Figure 4. Intestinal epithelium Wntless knockout did not inhibit tumorigenesis in the CAC model
(A) Schematic representation of the CAC model.

(B) Tumor incidence and mean tumor size in CTR (n=14) and Wntless^{E-Del} mice (n=15) after completion of the CAC challenge at day 100.

(C) Relative expression levels of the Wnt signaling pathway, tumor cell proliferation apoptosis and inflammation-related cytokine mRNAs isolated from the tumors in CTR and Wntless^{E-Del} mice at day 100 of the CAC model (Data are mean $\pm$ SEM. n=7 mice).

(D) Representative H&E-stained sections and immunohistochemical analysis of Ki67 of colon tumor from CTR mice and Wntless^{E-Del} mice at day 100 of the CAC challenge.