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Krüppel-like Factor 5 is Important for Maintenance of Crypt Architecture and Barrier Function in Mouse Intestine

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Abstract

Background & Aims—Krüppel-like factor 5 (KLF5) is transcription factor that is expressed by dividing epithelial cells of the intestinal epithelium. KLF5 promotes proliferation in vitro and in vivo and is induced by mitogens and various stress stimuli. To study the role of KLF5 in intestinal epithelial homeostasis, we examined the phenotype of mice with conditional deletion of *Klf5* in the gut.

Methods—Mice were generated with intestinal-specific deletion of *Klf5* (Vil-Cre;*Klf5*^{fl/fl}).

Morphological changes in the small intestine and colon were examined by immunohistochemistry, immunoblotting, and real-time PCR.

Results—*Klf5* mutant mice were born at a normal Mendelian ratio but had high mortality compared to controls. Complete deletion of *Klf5* from the intestinal mucosa resulted in neonatal lethality that corresponded with an absence of epithelial proliferation. Variegated intestinal-specific deletion of *Klf5* in adult mice resulted in morphological changes that included a regenerative phenotype, impaired barrier function, and inflammation. Adult mutant mice exhibited defects in epithelial differentiation and migration. These changes were associated with reduced

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Authors' Contributions:

BBM designed and performed experiments, analyzed data and drafted the manuscript

SSK conducted mouse experiments and immunohistochemical staining

KY conducted immunohistochemical staining and quantitative RT-PCR experiments

AMG provided technical expertise for tissue staining

NT developed the mouse model used in experiments

MI developed the mouse model used in experiments

AN provided histological analysis, advised on study design

RN provided the mouse model for these studies and contributed to study design

VWY advised on experimental design, analyzed data and edited the manuscript

All authors read and approved the final manuscript.

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expression of Cdx 1, Cdx2, and Eph and ephrin signaling proteins. Concomitantly, Wnt signaling to β -catenin was reduced. Proliferation in regenerative crypts was associated with increased expression of the progenitor cell marker Sox9.

Conclusions—Deletion of *Klf5* in the gut epithelium of mice demonstrated that KLF5 maintains epithelial proliferation, differentiation, and cell positioning along the crypt radial axis. Morphological changes that occur with deletion of *Klf5* are associated with disruption of canonical Wnt signaling and increased expression of Sox9.

Keywords

intestinal homeostasis; gastrointestinal development; genetics; GI tract

The mammalian intestinal epithelium is a continuously renewing system in which several biological processes including stem cell self-renewal, proliferation, terminal differentiation, migration, and apoptosis are carefully choreographed in order to maintain tissue homeostasis. The epithelium of the small intestine is composed of a crypt and a villus compartment. Similarly, the colon consists of a crypt and a surface epithelium compartment. The bulk of the villus and surface epithelium of the small and large intestine, respectively, is composed of differentiated columnar epithelial cells that are divided into two main classes: absorptive and secretory. These cells are descendants of the multipotent stem cells residing in the base of the crypt and are no longer dividing. In contrast, crypt stem cells are capable of self-renewal and asymmetric division. They give rise to the proliferative progenitor cells, also residing in the crypt compartment, which then become fully differentiated daughter cells as they migrate along the crypt-villus axis of the intestine ^{1, 2}.

Recent studies have illustrated the importance of several signaling pathways including the Wnt, Notch, Hedgehog, and bone morphogenetic protein pathways in controlling proliferation of intestinal stem cells and progenitor cells and their subsequent differentiation into different lineages of mature, differentiated epithelial cells ³⁻⁵. Among these, the Wnt pathway is the best characterized. Wnt genes, of which there are 19 in humans, encode secreted glycoproteins ⁶. Upon binding to cell surface receptors such as Frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP), Wnt elicits a signaling cascade that culminates in the formation of nuclear β -catenin/T cell factor (TCF) complexes, which then drive cell proliferation ⁷⁻⁹. It is well established that there is a Wnt gradient along the crypt-to-villus or crypt-to-surface epithelium axis of the small and large intestine, respectively, which provides important signals for proliferation of stem cells and progenitor cells in the crypt region ^{2, 3}. Additionally, Wnt signaling participates in intestinal epithelial cell maturation through regulating expression of the *caudal* related homologue Cdx1, which, along with Cdx2, promotes development of a mature intestinal phenotype ^{10, 11}. Wnt signaling also controls proper positioning of differentiated Paneth cells and enterocytes along the crypt-villus axis by reciprocally regulating expression of the EphB2/EphB3 receptors and their ligand ephrin B1 ¹²⁻¹⁴.

The Krüppel-like factor 5 (KLF5) gene is one of over 20 mammalian genes which exhibit homology to the *Drosophila* Krüppel gene that is essential for embryogenesis ¹⁵⁻¹⁸. In the intestine, expression of KLF5 is enriched in the proliferating crypt epithelial cells ^{19, 20}. Consistent with its cellular localization, KLF5 has been associated with, and in several instances, regulates, cellular proliferation in both physiological and pathophysiological conditions ²¹⁻²⁵. Until recently, a definitive assessment of KLF5 function in the intestinal epithelium has not been possible, as homozygous deletion of the *Klf5* gene is embryonic lethal ²⁶. Here, we use the Cre-LoxP system to generate mice with conditional deletion of the *Klf5* gene to investigate the physiological function of KLF5 in maintaining intestinal epithelial homeostasis.

Materials and Methods

Generation of mice with Intestinal-specific deletion of *Klf5*

C57BL/6 mice carrying *Klf5* alleles flanked by loxP sites were described previously²⁷. C57BL/6 mice carrying the Cre recombinase gene under regulation of the *villin* promoter (*Vil-Cre* mice)²⁸ were obtained from Jackson Laboratories. *Klf5^{fl/fl}* mice were crossed with *Vil-Cre* mice to generate *Vil-Cre;Klf5^{fl/+}* progeny. These mice were backcrossed to *Klf5^{fl/fl}* mice to produce *Vil-Cre;Klf5^{fl/fl}* offspring (designated *Klf5^{Δ/Δ}*). The Animal Care Committee of Emory University approved all procedures performed, and handling of the mice was in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States Public Health Service.

In vivo Permeability Assay

Gut permeability assays were performed to determine barrier function in control and mutant mice at 6 weeks of age. Mice were administered FITC dextran by gavage (60 mg/100 g body weight, FD-4, M 4000, Sigma Aldrich, St. Louis, MO) to determine the amount of dextran crossing the gut epithelial barrier and entering the bloodstream. Blood serum was collected 4 hours post-treatment. Levels of FITC dextran in the blood serum were measured by fluorescence intensity of the samples (excitation, 492, nm; emission, 525 nm; Cytofluor 2300). Results were normalized to the amount of serum protein assayed.

Myeloperoxidase (MPO) Activity

Neutrophil infiltration into tissue was quantified by measuring MPO enzyme activity (a marker for neutrophils). Briefly, colon tissues were washed to remove luminal contents and homogenized with a Tissuemizer (Fisher Scientific, Hampton, NH) in 0.5% hexadecyltrimethyl ammonium bromide (Sigma Aldrich) in 50 mM PBS, pH 6.0. Samples were subjected to three rounds of freeze/thaw and homogenized in the Bullet Blender with .1 mm zirconium silicate beads (Next Advance, Averill Park, NY). MPO activity was assayed from cleared supernatants by adding 1 mg/ml of dianisidine dihydrochloride (Sigma Aldrich) and $5 \times 10^{-4}\%$ H_2O_2 (Sigma Aldrich) and measuring the change in optical density at 450 nm. Activity was normalized to total protein assayed.

Immunohistochemistry

Tissue isolated from the colon and small intestine (duodenum, jejunum, ileum) of 8-week-old mice were extracted, cut longitudinally and formed into Swiss rolls. Samples were prepared and subjected to immunohistochemical staining as described previously²⁹. Immunofluorescence staining was performed by blocking with 3% BSA/PBS/Tween20 solution, incubating with primary antibody overnight at 4°C and incubation with Alexa fluor 488 or Alexa fluor 568 secondary antibodies (Invitrogen, Carlsbad, CA). Images were taken using Zeiss Axioskop2 plus microscope (Carl Zeiss Microimaging, Inc.). Areas of *Klf5* deletion were verified in stained sections by co-staining with *Klf5* antibody or by comparing with serial sections stained for *Klf5*.

Alcian blue/Periodic acid Schiff (PAS) staining

Alcian blue/PAS staining of paraffin-embedded sections to identify goblet cells was carried out using the Alcian Blue and PAS Staining Kit (Biocare Medical, Concord, CA) per manufacturer's instructions.

Western Blot Analysis

Protein lysates were prepared from distal ileum or proximal colon where deletion of *Klf5* was most complete. Epithelial tissues were scraped into cell extraction buffer (Invitrogen)

supplemented with Complete Protease Inhibitor Cocktail tablets (Roche, Indianapolis, IN). Samples were homogenized briefly with a TissueMizer (Fisher Scientific) and further homogenized with 0.1 mm zirconium silicate beads in the Bullet Blender (Next Advance). Lysates were prepared in a 1:1 ratio with 2X Laemmli Buffer, subjected to polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were blocked for 30 minutes in 5% non-fat dried milk in Tween/Tris-buffered salt solution (20mmol/L Tris-HCl, 3mmol/L KCl, 137 mmol/L NaCl, 0.1% Tween20, pH 7.5) and hybridized overnight at 4°C with primary antibodies.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA from scraped colonic musosa of 3 control and 3 *Klf5* mutant mice was prepared using Trizol reagent (Invitrogen) followed by DNase treatment and RNA cleanup with RNeasy columns (Qiagen, Valencia, CA). First strand synthesis of cDNA was performed with the RT² First Strand Kit per manufacturers' instructions (SA Biosciences, Frederick, MD). For analysis of Wnt pathway genes, PCR reactions were set up using the Wnt Signaling Pathway PCR Array plates (SA Biosciences) and analyzed with the manufacturer's software. Reactions were run on the Eppendorf Realplex PCR machine. Information on primers is included in Supplementary Methods.

Antibodies

See Supplementary Methods for a list of antibodies and dilutions used for Western blotting and immunohistochemistry.

Statistics

Numerical values in the figures are expressed as the mean $\pm$ S.E. Sample sizes are indicated in the figure legends. *P* values comparing the data for two groups of mice were calculated using a 2-tailed *t* test. Differences were considered to be significant if the *P* value was less than 0.05.

Results

To examine the consequences of intestinal-specific deletion of the *Klf5* gene, we generated *Vil-Cre;Klf5^{fl/fl}* mice (designated *Klf5 Δ^S* for “intestinal-specific deletion”) by crossing mice carrying floxed alleles of the *Klf5* gene (*Klf5^{fl/fl}*) with mice expressing the Cre recombinase under the control of a 12.4-kb regulatory region of the murine *villin* gene, followed by backcrossing to obtain the mutant mice. *Klf5 Δ^S* mice were born at a normal Mendelian ratio, but approximately two-thirds of these mice died within two days after birth. The remainder survived up to 8 weeks before dying or being sacrificed due to significant weight loss and rectal prolapse. Deletion of *Klf5* in intestinal tissues was confirmed by immunohistochemical staining of *Klf5*. In mutant mice that died shortly after birth, *Klf5* was deleted completely in the small intestine and colon (Figure 1A, panels a-d). Intestinal villi were sparse in newborn mutant mice compared to control mice, with the villus tips having a bulbous rather than tapered shape. In the colon, tissue architecture was distorted, with no identifiable crypt structures. To examine the effect of intestinal-specific deletion of *Klf5* on epithelial proliferation in the neonatal gut, tissues from control and *Klf5 Δ^S* mice were stained for expression of the proliferation marker, Ki67 (Figure 1A, panels e-h). Whereas proliferating cells were abundant in the crypts of control mice, they were virtually absent in corresponding areas in the mutant mice in both the small and large intestine.

In examining *Klf5* expression in surviving mice at 8 weeks of age, small and large intestines from *Klf5^{fl/fl}* control mice and *Klf5* mutant mice were prepared as Swiss rolls and stained for *Klf5* (Figure2A). Adult control mice showed *Klf5* localized to epithelial cells in

proliferating regions of intestinal crypts. In contrast, adult *Klf5*^{Δ/Δ} mice exhibited patchy expression of *Klf5* in segments of the small and large intestine, indicating incomplete deletion of the *Klf5* gene from intestinal stem cells. *Klf5* was deleted in approximately 50% of small intestinal tissues and in 80% of colon tissues (Figure 2B).

We next examined morphology of the small and large intestines in control and surviving *Klf5* mutant mice. *Klf5* expression in areas of representative histology was confirmed by staining serial sections with *Klf5* antibody (Figure 2C). Hematoxylin and eosin (H&E) staining indicated that normal crypt architecture was disrupted throughout the gut of the mutant mice. In the small bowel, villi were shortened and crypts were elongated, with some areas exhibiting erosion at the villus tips. In the colon, crypts were severely distorted, with dilated glands and reduced numbers of goblet cells. Somewhat surprisingly, mutant mice displayed active proliferation, with increased crypt lengths and crypt densities in the small intestine and increased crypt fission and proliferation in the colon (Supplementary Figure 1). These results were in contrast to those in newborn mice which showed absence of epithelial proliferation. Altered morphologies in the adult *Klf5*^{Δ/Δ} mice are consistent with a regenerative phenotype.

The average lifespan of *Klf5*^{Δ/Δ} mice with variegated *Klf5* expression was approximately 8 weeks, with the mice succumbing to severe wasting or being sacrificed due to rectal prolapse (Figure 3 A and B). Histological analysis of mutant mice with rectal prolapse revealed cryptitis, with infiltration of neutrophils into the epithelium and lamina propria of the small and large intestines, disruption of crypt architecture in the small intestine, and the presence of neutrophil exudate in the glands of the colon (Figure 3C). Neutrophil infiltration was also examined in *Klf5* mutant mice by myeloperoxidase (MPO) activity, a lysosomal enzyme abundant in neutrophils (Figure 3D). MPO activity was significantly higher in *Klf5* mutant mice than in control mice at 6 weeks of age, prior to the development of rectal prolapse. To determine whether inflammation was associated with epithelial barrier defects, barrier function was measured in control and mutant mice at 6 weeks of age by the FITC dextran method (Figure 3E). Results indicated a significantly increased permeability across the intestinal epithelium of *Klf5*^{Δ/Δ} mice, corresponding to increased MPO activity.

Accompanying the perturbed crypt architecture and barrier defects in *Klf5* mutant mice were alterations in intestinal epithelial differentiation, with aberrant localization of differentiated cells. In control mice, immunohistochemical staining for carbonic anhydrase-I (CA-I), a colonic marker for epithelial differentiation³⁰, showed CA-I restricted to epithelial cells in the upper third of the colonic crypt (Figure 4A). However, in *Klf5* mutant mice, CA-I expression was seen throughout the epithelial mucosa. Disruption of normal patterns of terminal differentiation was associated with reduced mRNA expression of key regulators of differentiation in the small and large intestine, *Cdx1* and *Cdx2*¹⁰ (Figure 4B).

Epithelial cell lineages also appeared to be altered in the *Klf5*^{Δ/Δ} mice. Numbers of goblet cells were reduced in the colon of mutant mice as determined by Alcian blue/Periodic acid-Schiff staining (Figure 4C). A reduction in goblet cell number was accompanied by a reciprocal increase in the number of enteroendocrine cells, as indicated by chromogranin A staining (Figure 4D). In the small intestine, no significant changes in epithelial cell lineage decisions were noted in mutant mice compared to control mice. Numbers of goblet cells and enteroendocrine cells as a percentage of total epithelial cells were comparable between the two groups (data not shown).

Paneth cell numbers and distribution in the small intestine of mutant and control mice were examined by staining for the Paneth cell marker, lysozyme³¹. While numbers of Paneth cells per crypt were similar in control and mutant mice, the localization of Paneth cells

within the crypts was altered in *Klf5* mutant mice. In control mice, Paneth cells were anchored at the very base of the crypts (Figure 5A, panel *a*). However, in mutant mice, some of the lysozyme-positive cells were no longer retained at the crypt base and could be found at higher positions in the crypts (Figure 5A, panels *b,c*). Mispositioning of cells was also observed for actively proliferating epithelial cells in *Klf5* mutant mice. Whereas cells stained positive for the proliferation marker, Ki67, were localized to the base of the crypts in the small and large intestines of control mice (Figure 5B, panels *d,g*), Ki67-positive proliferating cells were intermingled with non-proliferating cells throughout the intestinal crypts of mutant mice (Figure 5B, panels *e,f, h* and *i*). These results suggest that one of the physiological functions of KLF5 is to control cell migration and position along the crypt/villus axis.

The scattered distribution of Paneth cells and Ki67-positive cells in the intestines of *Klf5* mutant mice is reminiscent of the phenotype observed in mice deficient for the EphB2/EphB3 receptors^{12,13}. These receptors are normally expressed in the intestinal crypts. Together with their ligand, ephrin B1 which is expressed in the villi, EphB2/EphB3 regulate positioning of cells in the intestinal epithelium^{12, 14}. We therefore performed immunohistochemical staining and Western blot analysis for EphB2, EphB3 and ephrin B1 in intestinal tissues from *Klf5* mutant and control mice. Deletion of *Klf5* in regions of immunohistochemical staining was verified by co-staining for *Klf5* or staining serial sections for *Klf5* (Supplementary Figure 2). In examining expression of EphB2 and EphB3 in the colon, both receptors were confined to cells at the base of the crypts in control mice (Figure 6A). In the mutant mice, however, EphB2 exhibited a more diffuse staining throughout the crypt, with expression levels only slightly reduced compared to those in control mice (Figure 6A and B). In contrast, EphB3 expression was not detectable in the colon of mutant mice by Western blot or immunohistochemistry. Likewise, ephrin B1, which is normally expressed in cells in the upper region of the colonic epithelium, was undetectable in mutant mice. Similar although less dramatic changes in EphB3 and ephrin B1 expression were seen in the small intestine (Supplementary Figure 3). Thus, KLF5 regulates cell positioning in the gut epithelium by controlling expression of EphB2/EphB3 receptors and the ephrin B1 ligand.

KLF5 has been reported to promote β -catenin activity through its effects on β -catenin nuclear localization²⁹. Since differentiation and cell positioning are regulated by Wnt/ β -catenin signaling in the intestine, we investigated whether deletion of *Klf5* affects Wnt pathway activation. Immunohistochemical staining of β -catenin revealed reduced nuclear localization at the base of colonic crypts in *Klf5* mutant mice compared to controls (Figure 7A). *Klf5* expression in regions stained for β -catenin was verified by immunohistochemical staining of *Klf5* in serial sections (Supplementary Figure 4.) Levels of β -catenin in nuclear lysates from mucosal scrapings were also reduced in *Klf5* ^{Δ /S} mice (Figure 7B). Using a quantitative PCR array for analysis of Wnt pathway genes, numerous Wnt-related genes were found to be downregulated in colonic tissues of *Klf5* ^{Δ /S} mice (Figure 7C). Genes with reduced expression included direct targets of Wnt signaling (*Ccnd1*, *Fgf4*, *Jun*, *Sox17*) as well as genes encoding receptors and ligands for Wnt signaling (*Dvl2*, *Fzd4*, *Fzd7*, *Lrp5*, *Wnt2*, *Wnt4*) and proteins involved in Wnt activation (*Lef1* and *Pygo1*). Thus, deletion of *Klf5* results in reduced activation of β -catenin and inhibition of Wnt signaling in the intestine.

To identify mechanisms promoting colonic epithelial proliferation in regions with no *Klf5* expression and reduced Wnt signaling, several candidate pathways and molecules were examined. Canonical TCF/ β -catenin targets, cyclin D1 and c-Myc, were reduced or unchanged, respectively, in areas of *Klf5* deletion (Supplementary Figure 5). Notch signaling was likewise modestly reduced in colonic tissues of *Klf5* mutant mice

(Supplementary Figure 6). However, expression of Sry (sex determining region of Y)-box 9 (Sox9), a transcription factor that participates in decisions of self-renewal and differentiation^{32, 33}, was increased approximately four-fold in colonic tissues of *Klf5* mutant mice (Figure 7D and E). Staining of Sox9 in *Klf5* mutant mice revealed high levels throughout the regenerative glands. Thus, Sox9 may mediate aspects of the regenerative phenotype in intestinal tissues of *Klf5*^{Δ/Δ} mice.

Discussion

The results presented here demonstrate that *Klf5* plays a critical role in homeostasis of the intestinal epithelium, with loss of *Klf5* affecting mucosal crypt architecture and barrier function. Deletion of *Klf5* from the gut epithelium culminates in a regenerative phenotype that is characteristic of wound repair. This phenotype is likely a compensatory response to changes in the mucosal epithelium that are associated with reduced Wnt signaling. Interestingly, changes in tissue architecture with deletion of *Klf5* are more severe in the colon than in the small intestine. This may be due to a more complete deletion of *Klf5* in colonic tissues (Figure 2B) or to *Klf5* playing a more significant role in maintenance of epithelial homeostasis in the colon.

Our results indicate that *Klf5* is not essential to maintain intestinal epithelial proliferation in adult mice, in contrast to its requirement for proliferation in newborn mice. This finding may be attributable to regenerative processes in adult intestinal tissues that are independent of canonical Wnt signaling. In adult *Klf5* mutant mice, epithelial cell proliferation was observed in regenerative crypts where *KLF5* is deleted and Wnt signaling is reduced. Concomitant with these changes was increased expression of the progenitor cell marker, Sox9. Sox9 is linked to progenitor status in adult liver, exocrine pancreas and intestine, and is proposed to maintain the precursor cell population during physiological cell replacement and/or regeneration after injury³⁴. Thus, Sox9 may promote proliferation in *Klf5* mutant mice by maintaining epithelial cells in a progenitor state.

In regard to changes in tissue morphology, we show a direct effect of *KLF5* deletion on epithelial differentiation within the gut epithelium. Defects in terminal differentiation along the crypt-luminal axis are apparent in the colon of *Klf5* mutant mice stained with the differentiation marker, carbonic anhydrase-I. Normal expression of CA-I is limited to colonocytes in the upper one third of the crypt³⁰; however, in *KLF5*^{Δ/Δ} mice, CA-I-expressing cells are scattered throughout the crypt, indicating that terminal differentiation is no longer restricted to the luminal surface. A similar effect is seen with the differentiation marker alkaline phosphatase in mice with transgenic intestinal-specific expression of the Wnt inhibitor *Dkk1*³⁵. Perturbations in terminal differentiation in *Klf5* mutant mice were associated with reduced expression of the homeobox transcription factors, *Cdx1* and *Cdx2*. These factors are reported to stimulate differentiation and inhibit proliferation of intestinal epithelial cells^{10, 11}. *Cdx1* is a transcriptional target of TCF4/β-catenin³⁶, while *Cdx2* is repressed by Sox9³⁷. In accordance with reports on regulation of *Cdx1* and *Cdx2*, reduced expression of these factors in *Klf5* mutant mice correlates with impaired Wnt signaling and increased Sox9 expression. In examining epithelial lineage decisions, *Klf5*^{Δ/Δ} mice exhibit changes in the prevalence of secretory cells, with fewer goblet cells and increased numbers of enteroendocrine cells in the colon compared to controls. These effects are similar to those seen with deletion of the transcriptional repressor, *Gfi1*, which drives the selection of goblet and Paneth cells over enteroendocrine progenitors³⁸.

Defects in epithelial migration in *Klf5* mutant mice are reminiscent of mice with disrupted Eph/ephrin signaling, correlating with altered expression of Eph/ephrin proteins in the mutant mice. In the intestine of *EphB3*-null mice, localization of Paneth cells is no longer

restricted to the crypt base; rather, Paneth cells migrate upwards and are found dispersed throughout the epithelium¹². Mislocalization of Paneth cells is also observed in mice with intestinal specific deletion of ephrin B1³⁹. Other architectural changes in *Klf5*^{Δ/Δ} mice are similar to those reported in *EphB2*^{-/-}; *EphB3*^{-/-} double knockout mice including intermingling of proliferative and differentiated cells¹². Future studies will examine whether genes encoding *EphB3* or *ephrinB1* are direct transcriptional targets of KLF5.

Reduced expression of Eph/ephrin proteins and Cdx1 in *Klf5*^{Δ/Δ} mice indicates that Wnt signaling is inhibited in the mutant mice. Absence of epithelial proliferation in the intestines of *Klf5*^{Δ/Δ} newborn mice (Figure 1B) phenocopies genetic models for disruption of Wnt signaling that include deletion of Tcf4 and transgenic expression of the Wnt inhibitor Dkk-1^{35, 40-42}. In these models, the neonatal epithelium was composed entirely of differentiated, non-dividing villus cells, resulting in loss of intestinal stem cell compartments and neonatal lethality. In surviving *Klf5*^{Δ/Δ} mice, we observe downregulation of numerous Wnt target genes, corresponding to reduced expression of active β-catenin at the base of the crypts (Figure 7). Previous evidence from our laboratory demonstrates that changes in expression of *Klf5* have a significant impact on β-catenin localization and activity²⁹. Haploinsufficiency of *Klf5* in the *Apc*^{Min/+} mouse rescues the tumor-initiating effects of the *Apc*^{Min} mutation by inhibiting β-catenin nuclear accumulation. Furthermore, downregulation of KLF5 in colon cancer cell lines inhibits nuclear localization of β-catenin and reduces expression of TCF/β-catenin target genes²⁹. Inhibition of tumor formation by heterozygous deletion of *Klf5* is also reported in mice with combined *KRas* and *Apc*^{Min} mutations, indicating a critical role for KLF5 in proliferative and transforming events associated with intestinal tumorigenesis⁴³.

Homeostasis of the intestinal epithelium is dependent on tight regulation of pathways that regulate cell proliferation, differentiation and migration. An unexpected finding of our work is the necessity of intestinal-specific expression of KLF5 for viability. By targeting expression of *Klf5*, we have identified a novel effector of Wnt signaling that is critical for maintenance of proper crypt organization and structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. *Science*. 2005; 307:1904–9. [PubMed: 15790842]
2. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol*. 2009; 71:241–60. [PubMed: 18808327]
3. Crosnier C, Stamataki D, Lewis J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet*. 2006; 7:349–59. [PubMed: 16619050]
4. Fre S, Pallavi SK, Huyghe M, Lae M, Janssen KP, Robine S, Artavanis-Tsakonas S, Louvard D. Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. *Proc Natl Acad Sci U S A*. 2009; 106:6309–14. [PubMed: 19251639]
5. Ishizuya-Oka A, Hasebe T. Sonic hedgehog and bone morphogenetic protein-4 signaling pathway involved in epithelial cell renewal along the radial axis of the intestine. *Digestion*. 2008; 77 1:42–7. [PubMed: 18204261]

6. Gregorieff A, Pinto D, Begthel H, Destree O, Kielman M, Clevers H. Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology*. 2005; 129:626–38. [PubMed: 16083717]
7. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature*. 2005; 434:843–50. [PubMed: 15829953]
8. Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature*. 1996; 382:225–30. [PubMed: 8717036]
9. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. LDL-receptor-related proteins in Wnt signal transduction. *Nature*. 2000; 407:530–5. [PubMed: 11029007]
10. Guo RJ, Suh ER, Lynch JP. The role of Cdx proteins in intestinal development and cancer. *Cancer Biol Ther*. 2004; 3:593–601. [PubMed: 15136761]
11. Crissey MA, Guo RJ, Funakoshi S, Kong J, Liu J, Lynch JP. Cdx2 levels modulate intestinal epithelium maturity and Paneth cell development. *Gastroenterology*. 2011; 140:517–528 e8. [PubMed: 21081128]
12. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell*. 2002; 111:251–63. [PubMed: 12408869]
13. Holmberg J, Genander M, Halford MM, Anneren C, Sondell M, Chumley MJ, Silvary RE, Henkemeyer M, Frisen J. EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. *Cell*. 2006; 125:1151–63. [PubMed: 16777604]
14. Merlos-Suarez A, Batlle E. Eph-ephrin signalling in adult tissues and cancer. *Curr Opin Cell Biol*. 2008; 20:194–200. [PubMed: 18353626]
15. Black AR, Black JD, Azizkhan-Clifford J. Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol*. 2001; 188:143–60. [PubMed: 11424081]
16. Dang DT, Pevsner J, Yang VW. The biology of the mammalian Kruppel-like family of transcription factors. *Int J Biochem Cell Biol*. 2000; 32:1103–21. [PubMed: 11137451]
17. Kaczynski J, Cook T, Urrutia R. Sp1- and Kruppel-like transcription factors. *Genome Biol*. 2003; 4:206. [PubMed: 12620113]
18. Pearson R, Fleetwood J, Eaton S, Crossley M, Bao S. Kruppel-like transcription factors: a functional family. *Int J Biochem Cell Biol*. 2008; 40:1996–2001. [PubMed: 17904406]
19. Konkright MD, Wani MA, Anderson KP, Lingrel JB. A gene encoding an intestinal-enriched member of the Kruppel-like factor family expressed in intestinal epithelial cells. *Nucleic Acids Res*. 1999; 27:1263–70. [PubMed: 9973612]
20. McConnell BB, Ghaleb AM, Nandan MO, Yang VW. The diverse functions of Kruppel-like factors 4 and 5 in epithelial biology and pathobiology. *Bioessays*. 2007; 29:549–57. [PubMed: 17508399]
21. Sun R, Chen X, Yang VW. Intestinal-enriched Kruppel-like factor (Kruppel-like factor 5) is a positive regulator of cellular proliferation. *J Biol Chem*. 2001; 276:6897–900. [PubMed: 11152667]
22. Chanchevalap S, Nandan MO, Merlin D, Yang VW. All-trans retinoic acid inhibits proliferation of intestinal epithelial cells by inhibiting expression of the gene encoding Kruppel-like factor 5. *FEBS Lett*. 2004; 578:99–105. [PubMed: 15581624]
23. Nandan MO, Chanchevalap S, Dalton WB, Yang VW. Kruppel-like factor 5 promotes mitosis by activating the cyclin B1/Cdc2 complex during oncogenic Ras-mediated transformation. *FEBS Lett*. 2005; 579:4757–62. [PubMed: 16102754]
24. McConnell BB, Klapproth JM, Sasaki M, Nandan MO, Yang VW. Kruppel-like factor 5 mediates transmissible murine colonic hyperplasia caused by *Citrobacter rodentium* infection. *Gastroenterology*. 2008; 134:1007–16. [PubMed: 18395082]

25. Nandan MO, McConnell BB, Ghaleb AM, Bialkowska AB, Sheng H, Shao J, Babbitt BA, Robine S, Yang VW. Kruppel-like factor 5 mediates cellular transformation during oncogenic KRAS-induced intestinal tumorigenesis. *Gastroenterology*. 2008; 134:120–30. [PubMed: 18054006]
26. Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R. Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med*. 2002; 8:856–63. [PubMed: 12101409]
27. Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, Shindo T, Sano M, Otsu K, Snider P, Conway SJ, Nagai R. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. *J Clin Invest*. 2010; 120:254–65. [PubMed: 20038803]
28. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem*. 2002; 277:33275–83. [PubMed: 12065599]
29. McConnell BB, Bialkowska AB, Nandan MO, Ghaleb AM, Gordon FJ, Yang VW. Haploinsufficiency of Kruppel-like factor 5 rescues the tumor-initiating effect of the Apc(Min) mutation in the intestine. *Cancer Res*. 2009; 69:4125–33. [PubMed: 19435907]
30. Bekku S, Mochizuki H, Takayama E, Shinomiya N, Fukamachi H, Ichinose M, Tadakuma T, Yamamoto T. Carbonic anhydrase I and II as a differentiation marker of human and rat colonic enterocytes. *Res Exp Med (Berl)*. 1998; 198:175–85. [PubMed: 9879596]
31. Bry L, Falk P, Huttner K, Ouellette A, Midtvedt T, Gordon JI. Paneth cell differentiation in the developing intestine of normal and transgenic mice. *Proc Natl Acad Sci U S A*. 1994; 91:10335–9. [PubMed: 7937951]
32. Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, Bibeau F, Scherer G, Joubert D, Hollande F, Blache P, Jay P. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J Cell Biol*. 2007; 178:635–48. [PubMed: 17698607]
33. Formeister EJ, Sionas AL, Lorange DK, Barkley CL, Lee GH, Magness ST. Distinct SOX9 levels differentially mark stem/progenitor populations and enteroendocrine cells of the small intestine epithelium. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:G1108–18. [PubMed: 19228882]
34. Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T, Uemoto S. Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. *Nat Genet*. 2011; 43:34–41. [PubMed: 21113154]
35. Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev*. 2003; 17:1709–13. [PubMed: 12865297]
36. Lickert H, Domon C, Huls G, Wehrle C, Duluc I, Clevers H, Meyer BI, Freund JN, Kemler R. Wnt/(beta)-catenin signaling regulates the expression of the homeobox gene Cdx1 in embryonic intestine. *Development*. 2000; 127:3805–13. [PubMed: 10934025]
37. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H, Jay P. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol*. 2004; 166:37–47. [PubMed: 15240568]
38. Shroyer NF, Wallis D, Venken KJ, Bellen HJ, Zoghbi HY. Gfi1 functions downstream of Math1 to control intestinal secretory cell subtype allocation and differentiation. *Genes Dev*. 2005; 19:2412–7. [PubMed: 16230531]
39. Cortina C, Palomo-Ponce S, Iglesias M, Fernandez-Masip JL, Vivancos A, Whissell G, Huma M, Peiro N, Gallego L, Jonkheer S, Davy A, Lloreta J, Sancho E, Batlle E. EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. *Nat Genet*. 2007; 39:1376–83. [PubMed: 17906625]
40. Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet*. 1998; 19:379–83. [PubMed: 9697701]

41. Ireland H, Kemp R, Houghton C, Howard L, Clarke AR, Sansom OJ, Winton DJ. Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. *Gastroenterology*. 2004; 126:1236–46. [PubMed: 15131783]
42. Kuhnert F, Davis CR, Wang HT, Chu P, Lee M, Yuan J, Nusse R, Kuo CJ. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A*. 2004; 101:266–71. [PubMed: 14695885]
43. Nandan MO, Ghaleb AM, McConnell BB, Patel NV, Robine S, Yang VW. Kruppel-like factor 5 is a crucial mediator of intestinal tumorigenesis in mice harboring combined ApcMin and KRASV12 mutations. *Mol Cancer*. 2010; 9:63. [PubMed: 20298593]

Abbreviations

Fz	Frizzled
LRP	lipoprotein receptor-related protein
KLF5	Krüppel-like factor 5
MPO	myeloperoxidase
PAS	Periodic acid-Schiff
Sox9	Sry (sex determining region of Y)-box 9
TCF	T cell factor

Figure 1. Deletion of *Klf5* from the intestinal epithelium of moribund newborn mice. Immunohistochemical staining of Klf5 (A) and Ki67 (B) was performed on tissues from the small intestine and colon of control (*Klf5*^{fl/fl}) and *Klf5*^{Δ/Δ} (Vil-Cre;*Klf5*^{fl/fl}) newborn mice one day after birth.

Figure 2. Variegated deletion of *Klf5* and evidence of a regenerative phenotype in *Klf5* mutant mice. (A) Immunohistochemical staining of *Klf5* in Swiss rolls from the small intestine and colon of mice at 8 weeks of age. (Red arrows: *Klf5*-positive crypts; black arrows: *Klf5*-negative crypts). (B) Average percent deletion of *Klf5* in Swiss rolls of duodenum, jejunum, ileum and colon stained for *Klf5* protein, n=5. (C) Histology in areas of *Klf5* deletion. Serial sections from the small intestine and colon of adult control and *Klf5^{Δ/S}* mice were stained with *Klf5* antibody or H&E.

Figure 3. Wasting and enterocolitis in *Klf5*^{Δ/Δ} mice. (A) Comparison of size difference of control and *Klf5*^{Δ/Δ} littermates at 8 weeks of age. (B) Rectal prolapse is frequently observed in *Klf5*^{Δ/Δ} mice by approximately 8 weeks of age. (C) H&E staining indicates infiltration of immune cells and disruption of normal crypt architecture in the small intestine and colon of *Klf5*^{Δ/Δ} mice with rectal prolapse. (D) Measurement of neutrophil infiltration by myeloperoxidase assay using colon tissue from control and *Klf5*^{Δ/Δ} mice at 6 weeks of age (**P* < 0.001, n=7). (E) Measurement of gut permeability of mice at 6 weeks of age with the FITC dextran permeability assay (***P* = 0.05, n=7).

Figure 4. Loss of a restricted differentiation zone and changes in epithelial cell lineage with deletion of *Klf5*. Paraffin-embedded colon sections from adult control and *Klf5* mutant mice were stained with various epithelial markers to identify changes in differentiation. (A) Immunohistochemical staining with the colonic differentiation marker, carbonic anhydrase-I. (B) Quantitative real time polymerase chain reaction (qRT-PCR) to measure relative mRNA expression of Cdx1 and Cdx2 in colon tissues from control and mutant mice (* $P < 0.001$, ** $P = 0.004$, $n=3$). (C) Alcian blue/Periodic acid-Schiff (PAS) staining to identify goblet cells and immunohistochemical staining of chromogranin A as a marker for enteroendocrine cells.

Figure 5. Impaired migration of epithelial cells in *Klf5* mutant mice. Tissues from adult control and *Klf5*^{Δ/Δ} mice were examined for alterations in epithelial cell migration. (A) Immunohistochemical staining of the small intestine with lysozyme as a Paneth cell-specific marker. Right panel shows a higher magnification of *Klf5*^{Δ/Δ} staining. The red arrowhead indicates a Paneth cell that has migrated from the crypt base. (B) Immunohistochemical staining with the proliferation marker, Ki67, in the small intestine and colon. Red dotted lines indicate the normal boundaries between proliferative and differentiated compartments. Right panels are higher magnification images of adjacent panels. Green arrowheads indicate Ki67-positive proliferating cells. Red arrowheads indicate Ki67-negative non-cycling cells.

Figure 6. Disrupted expression of Eph/ephrin proteins in *Klf5^{ΔIS}* mice. (A) Immunofluorescence staining of EphB2, EphB3 and ephrin B1 in colon tissues from control and *Klf5* mutant mice. White arrows indicate expected localization of the Eph/ephrin proteins. (B) Western blot analysis of Eph/ephrin levels in colon lysates from control and *Klf5* mutant mice, n=3.

Figure 7. Impaired Wnt signaling and induction of Sox9 in colonic tissues of *Klf5^{ΔIS}* mice. (A) Colonic tissues of control and mutant mice were stained by immunohistochemistry to examine localization of β-catenin. Red arrows indicate the presence or absence of nuclear localization of β-catenin in control and mutant mice, respectively. (B) Nuclear lysates prepared from colons of 3 control and 3 mutant mice were pooled and subjected to Western blotting to determine levels of nuclear-localized β-catenin, n=3. Tata-binding protein (TBP) is shown as a nuclear protein loading control. (C) Wnt pathway PCR array analysis was conducted on RNA samples from control and *Klf5^{ΔIS}* mice to identify differentially

expressed mRNAs. RNA samples were pooled for analysis, n=3. Fold changes in parentheses indicate a fold decrease.