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Altered intestinal epithelial homeostasis in mice with intestine-specific deletion of the Krüppel-like factor 4 gene

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ABSTRACT

The zinc finger transcription factor, Krüppel-like factor 4 (KLF4), is expressed in the post-mitotic, differentiated epithelial cells lining the intestinal tract and exhibits a tumor suppressive effect on intestinal tumorigenesis. Here we report a role for KLF4 in maintaining homeostasis of intestinal epithelial cells. Mice with conditional ablation of the *Klf4* gene from the intestinal epithelium were viable. However, both the rates of proliferation and migration of epithelial cells were increased in the small intestine of mutant mice. In addition, the brush-border alkaline phosphatase was reduced as was expression of ephrine-B1 in the small intestine, resulting in mispositioning of Paneth cells to the upper crypt region. In the colon of mutant mice, there was a reduction of the differentiation marker, carbonic anhydrase-1, and failure of differentiation of goblet cells. Mechanistically, deletion of *Klf4* from the intestine resulted in activation of genes in the Wnt pathway and reduction in expression of genes encoding regulators of differentiation. Taken together, these data provide new insights into the function of KLF4 in regulating postnatal proliferation, migration, differentiation, and positioning of intestinal epithelial cells and demonstrate an essential role for KLF4 in maintaining normal intestinal epithelial homeostasis *in vivo*.

Introduction

The mammalian intestinal epithelium is a dynamic system in which cell proliferation, differentiation, migration, and apoptosis are stringently coordinated to achieve homeostasis. The epithelium of the small and large intestine consists of a crypt/villus and crypt/surface epithelium unit, respectively. The bulk of the villus and surface epithelium is composed of differentiated columnar epithelial cells that are divided into absorptive cells (enterocytes) and secretory cells (including goblet, enteroendocrine, and Paneth cells; the last unique to the small intestine). The differentiated epithelial cells are descendants of the crypt progenitor cells, which are themselves derived from the multi-potent stem cells, also located in the crypt compartment (Barker et al., 2008; Scoville et al., 2008).

The zinc finger transcription factor, Krüppel-like factor 4 (KLF4) (Garrett-Sinha et al., 1996; Shields et al., 1996), is normally expressed in the differentiated epithelial cells of the intestine, suggesting that KLF4 may function in the switch from proliferation to differentiation. *In vitro*, KLF4 inhibits cell proliferation by functioning as a cell cycle checkpoint protein (Chen et al., 2001; Shields et al., 1996). *In vivo*, KLF4 exhibits a tumor suppressive effect on intestinal tumorigenesis (Ghaleb et al., 2007). Consistent with this finding, *KLF4* is down-regulated in a variety of human cancers including esophageal, gastric, colorectal, and urinary bladder cancers (Kanai et al., 2006; Ohnishi et al., 2003; Wang et al., 2002; Wei et al., 2005; Zhao et al., 2004). However, KLF4 can promote tumorigenesis in a different context, for example, in the absence of $p21^{CIP1}$ (Rowland et al., 2005; Rowland and Peeper, 2006).

Delineation of the physiologic function of KLF4 in the intestinal epithelium is hampered by the early lethality of mice lacking *Klf4* (Katz et al., 2002; Segre et al., 1999). *Klf4*-null mice die within 1 day after birth and suffer from a loss of barrier function of the epidermis (Segre et al., 1999). Additionally, the colons of the *Klf4*-null mice have a 90% reduction in the number of goblet cells, suggesting that KLF4 plays a crucial role in colonic epithelial cell differentiation *in vivo* (Katz et al., 2002). Mice with conditional deletion of *Klf4* from specific tissues have been described. Targeted deletion of *Klf4* from the stomach and esophagus causes altered differentiation and precancerous changes (Katz et al., 2005; Tetreault et al., 2010). Here, we use the Cre recombinase system under control of the *villin* promoter to drive specific deletion of *Klf4* from the intestinal epithelium. The resultant mutant mice had significantly altered homeostasis that involved proliferation, migration, differentiation, and positioning of intestinal epithelial cells. This study provides the first definitive evidence that KLF4 exerts a crucial function in maintaining intestinal epithelial cell homeostasis *in vivo*.

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Materials and methods

Generation of mice with intestine-specific deletion of the Klf4 gene

C57BL/6 mice carrying floxed *Klf4* gene (*Klf4*$^{fl/fl}$) were previously described (Katz et al., 2002). C57BL/6 mice carrying *Cre* recombinase gene under the regulation of *villin* promoter (*Vil*/*Cre*) were purchased from The Jackson Laboratory in Bar Harbor, ME (Madison et al., 2002). Mice lacking *Klf4* in their intestinal epithelium were generated by mating *Klf4*$^{fl/fl}$ mice with *Vil*/*Cre* mice followed by backcrossing to obtain *Vil*/*Cre*;*Klf4*$^{fl/fl}$ mice (designated *Klf4*$^{\Delta IS}$ for intestine-specific deletion). All protocols involving mouse work have been approved by the Institutional Animal Care and Use Committee of Emory University (protocols #098-2007 and 099-2007).

Histology

The small and large intestines were removed from age-matched littermates of *Klf4* mutant mice (*Klf4*$^{\Delta IS}$) and control (*Klf4*$^{fl/fl}$) mice for histological and immunohistochemical characterization of the intestinal tract. Isolated small and large intestines were flushed with modified Bouin's fixative (50% ethanol, 5% acetic acid, and 10% formaldehyde), and cut open longitudinally for gross examination. The intestines were then rolled into a Swiss-roll, fixed, and embedded in paraffin. Five-μm sections were cut and stained with hematoxylin and eosin (H&E). Age-matched littermate control and mutant mice were examined histologically at ages 3 weeks, 4, 7, and 10 months.

Alcian blue (AB) and periodic acid-Schiff (PAS) staining

Goblet cell staining was carried out as described before (Ghaleb et al., 2008) with slight modifications. For AB staining, sections were deparaffinized in xylene, rehydrated in ethanol and brought to distilled water for 5 min. AB 8GX (Biocare Medical) was applied to the sections for 15 min at RT, followed by a 2-min wash in running tap water, counterstained with Nuclear Fast Red (Biocare Medical), followed by dehydration (twice in 95% EtOH and twice in 100% EtOH) and cover-slipped. For PAS staining, deparaffinized and rehydrated sections were treated with Periodic acid (Biocare Medical) for 5 min at RT. Slides were washed in distilled water then stained with Schiff's reagent (Biocare Medical) for 15 min at RT, followed by a 5-min wash in running tap water. The sections were then counterstained with hematoxylin, washed in running tap water for 2 min, followed by dehydration (twice in 95% EtOH and twice in 100% EtOH) and cover-slipped.

Intestinal alkaline phosphatase staining

Deparaffinized and rehydrated sections were stained for endogenous intestinal alkaline phosphatase activity using Vulcan Fast Red Chromogen kit (Biocare Medical), following manufacturer's recommendations.

Immunohistochemistry (IHC) and immunofluorescence (IF)

Mice were sacrificed by CO_2 asphyxiation prior to IHC and IF examination. The entire small intestine and colon were dissected longitudinally and washed in modified Bouin's fixative (50% EtOH, 5% acetic acid, and 10% formaldehyde). The small intestine was divided into 3 equal segments (proximal, middle, and distal). Both the small and large intestines were cut open along their longitudinal axis, rinsed briefly in phosphate-buffered saline (PBS), and examined under a dissecting microscope. Each segment of the intestine was then rolled in a Swiss-roll to allow for histopathological examination of the entire length of both the small and large intestines. Following the dissecting microscopic examination, intestinal tissues were fixed in 10% formalin in PBS and subsequently embedded in paraffin. Five μm-thick paraffin sections were cut and applied to Superfrost Plus slides (VWR). Some sections were used for standard H&E staining. For IHC, sections were deparaffinized in xylene, incubated in 3% hydrogen peroxide in methanol for 30 min, rehydrated in ethanol gradient, and then treated with 10 mM Na citrate buffer, pH 6.0, at 120 °C for 10 min (except for Muc2 which was for 1 min) in a pressure cooker. For lysozyme staining, antigen retrieval was done by Protienase K (Millipore) digestion (1:10 dilution in PBS, for 15 min at 37 °C). All histological sections were incubated with a blocking buffer (2% non-fat dry milk and 0.01% Tween 20 in PBS) for 1 h at RT. An avidin/biotin blocking kit (Vector Laboratories) was used in conjunction with the blocking buffer according to manufacturer's directions to reduce background and nonspecific secondary antibody binding. Sections were then stained using goat anti-KLF4 (1:300 dilution; R&D), rabbit anti-lysozyme (1:200 dilution; Dako), goat anti-LBP (1:200 dilution; Santa Cruz), rabbit anti-Muc2 (1:500 dilution; Santa Cruz), mouse anti-BrdU (1:50 dilution; BD Pharmingen), rabbit anti-ephrin-B1 (1:500 dilution; Santa Cruz), goat anti-EphB2 and goat ant-EphB3 (1:500 dilution; R&D), rabbit anti-Ki67 (1:800 dilution; Leica Microsystems), rabbit anti-chromogranin A, rabbit anti-cleaved caspase-3 (1:500 dilution; Cell Signal), and rabbit anti-colonic carbonic anhydrase-1 (1:500 dilution; Santa Cruz). Detection of primary antibodies for IHC was carried out using appropriate biotinylated secondary antibodies at 1:500 dilutions for 30 min at 37 °C, and color development was performed using the Vectastain ABC kit (Vector Laboratories). Sections were then counterstained with hematoxylin, dehydrated, and cover-slipped. Detection of primary antibodies for IF was carried out using appropriate AlexaFluor labeled secondary antibodies (Molecular Probes) at 1:500 dilutions in 3% bovine serum albumin (BSA) in PBS for 30 min at 37 °C, counterstained with Hoechst 33258 (2 μg/ml), mounted with Prolong gold (Molecular Probes), and cover-slipped. Images were acquired using an Axioskop 2 plus microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) equipped with an AxioCam MRc5 CCD camera (Carl Zeiss MicroImaging, Thornwood, NY, USA).

5-Bromo-2-deoxyuridine (BrdU) labeling

Mice were injected intraperitoneally (IP) with BrdU (Sigma) at 50 μg/g body weight, then sacrificed at 4 and 24 h post-injection. Following immunostaining for BrdU, the number and position of BrdU-positive cells were counted from at least 30 crypts per mouse per genotype per time point. Statistical significance for number of BrdU positive cells was performed by *t*-test and for cumulative frequency, Kolmogorov–Smirnov (K–S) test.

Ki67 staining and quantification

Following immunostaining for Ki67, the number of Ki67-positive cells was counted from at least 30 crypts per mouse per genotype. Statistical analysis for number of Ki67-positive cells was performed by *t*-test.

Goblet cells counting and size measuring

The number and diameter of Muc2-positive cells in the colon were counted and measured from at least 30 crypts per mouse per genotype. Diameter measurement was done using AxiovisionLE software (Carl Zeiss MicroImaging). Statistical analysis for number and diameter of Muc2-positive cells was performed by *t*-test.

Western blot analysis

Following euthanasia, intestines were removed, flushed once with cold PBS containing protease and phosphatase inhibitors. The

intestines were then cut open longitudinally, and comparable regions from the intestines of both the control and *Klf4*$^{\Delta IS}$ mice were used for protein extraction. Proteins were extracted by scraping the luminal side of the intestine onto a clean glass slide, using another clean glass slide. The scraped tissue was then homogenized in lysis buffer containing 50 mM Tris–HCl (pH 6.8) and 2% sodium dodecyl sulfate (SDS). Insoluble material was removed by centrifugation at 12,000 rpm, and the supernatant was collected for protein quantification and SDS gel electrophoresis. Following protein transfer, the membrane was immunoblotted with the following primary antibodies: rabbit anti-ephrin-B1 (1:500 dilution; Santa Cruz) and mouse monoclonal anti-β actin (1:2,000 dilution; Sigma), overnight at 4 °C. The membrane was incubated for 1 h at RT with appropriate secondary antibodies, and the signal detected by chemiluminscence.

Real-time PCR analysis

Total RNA was extracted from both the small and large intestines of three *Klf4*$^{fl/fl}$ control mice and three *Klf4*$^{\Delta IS}$ mice. In brief, after the mice being euthanized, 2-cm segments from the mid section of the small intestine or the colon were dissected out and quickly flushed with cold PBS containing RNaseOut (1:500; Invitrogen). The segments were then cut open, placed with luminal side facing upward on a clean glass slide on ice, and the epithelium scraped off the segment using a clean glass slide. The scraped epithelium was immediately collected in a tube containing RNA extraction buffer, Trizol (Invitrogen) and extraction proceeded according to manufacturer's recommendations. Equal amounts of extracted RNA from mice per group were pooled for cDNA synthesis. cDNA synthesis was done using RT^2 First Strand kit (SABiosciences) according to manufacturer's recommendation. Real-Time PCR array was done using RT^2 Profiler PCR Array (SABiosciences) for mouse Wnt signaling pathway (cat. #PAMM-043A-2) or ready-made individual primers encoding regulatory genes of differentiation (QuantiTect Primer Assay, Qiagen). Samples were run in triplicates on Real-time thermal cycler Mastercycler® ep *realplex* machine (Eppendorf).

Results

Mice with intestine-specific deletion of Klf4 are viable

To delete *Klf4* from the intestine, we employed *Vil*/*Cre* mice, which carry the *Cre* recombinase directed by 12.4 kb of the mouse *villin* promoter (Madison et al., 2002). *Villin* is normally expressed in the gut epithelium and the proximal tubule of the kidney beginning at embryonic day 11 (Maunoury et al., 1988, 1992). *Vil*/*Cre* mice were crossed with mice carrying floxed alleles of *Klf4* (*Klf4*$^{fl/fl}$) (Katz et al., 2005) and then backcrossed to obtain *Vil*/*Cre*;*Klf4*$^{fl/fl}$ (*Klf4*$^{\Delta IS}$) mice. *Klf4*$^{fl/fl}$ mice without *Cre* served as controls. *Klf4*$^{\Delta IS}$ mice were born in a Mendelian ratio, and at three weeks of age appeared grossly normal without any significant difference in weight from the control *Klf4*$^{fl/fl}$ or wild type mice. Deletion of the *Klf4* gene from the intestine was confirmed by immunohistochemistry. Klf4 staining was seen in the differentiated intestinal epithelial cells of both the small and large intestines of the control mice (Fig. 1A and C), while absent from the epithelia of the small and large intestines of the *Klf4*$^{\Delta IS}$ mice (Fig. 1B and D), confirming Cre-mediated deletion of *Klf4* from the intestinal epithelium.

Macroscopically, both the small and large intestines of the *Klf4*$^{\Delta IS}$ mice appeared normal. At 3 weeks of age, histology of the small intestinal epithelium of *Klf4*$^{\Delta IS}$ mice appeared normal (Fig. 1F) as compared to controls (Fig. 1E). In contrast, the colonic epithelium of the *Klf4*$^{\Delta IS}$ mice had a distorted architecture with increased glandular formation and reduced numbers of goblet cells (compare Fig. 1G and H). No evidence of hypertrophy or hyperplasia was observed in either the small or large intestine of the *Klf4*$^{\Delta IS}$ mice up to 10 months of age.

Intestinal deletion of Klf4 increases rates of proliferation and migration of epithelial cells along the crypt–villus axis

Since KLF4 negatively regulates cellular proliferation, we examined the effect of *Klf4* deletion on the rate of proliferation of intestinal epithelial cells *in vivo*. At 3 weeks of age, mice were pulse-labeled in the S-phase with bromodeoxyuridine (BrdU) and sacrificed at 4 and 24 h later for immunohistochemical examination. The number of BrdU-positive cells was higher in the small intestine of *Klf4*$^{\Delta IS}$ mice compared to the control littermates at both 4 h ($p<0.001$) and 24 h ($p<0.001$) (Fig. 2A–F and H). The rate of migration of epithelial cells as defined by the cumulative frequency of BrdU-positive cells along the crypt–villus axis was also higher in the small intestine of *Klf4*$^{\Delta IS}$ mice compared to the control littermates at 4 h ($p<0.05$) and 24 h ($p<0.001$) (Fig. 2G). Furthermore, there was a significant difference ($p<0.05$) in the number of cells positive for the proliferation marker, Ki67, in the crypts between control and *Klf4*$^{\Delta IS}$ mice (Fig. 2I–K). These results suggest that the number of progenitor cells or stem cells is increased by *Klf4* deletion, leading to an increase in the number of cells incorporating BrdU.

In contrast to the small intestine, there was no difference in the number of BrdU-positive cells in the colon of *Klf4*$^{\Delta IS}$ mice compared to control mice (Suppl. Figs. S1A–C). We also stained the small and large intestines of the mutant and control mice for cleaved caspase-3 but found no difference in the rate of apoptosis between the two groups (data not shown). These results indicate that both the rates of proliferation and migration along the crypt–villus axis are increased when *Klf4* is deleted from the small intestine.

Intestinal deletion of Klf4 results in altered goblet cell maturation and differentiation

The effect of intestinal *Klf4* deletion on cellular differentiation was then investigated. Differentiation of intestinal epithelial cells into goblet cells was examined by staining for acidic, neutral, and Muc2 mucins using Alcian blue (AB), Periodic-acid Schiff (PAS), and anti-Muc2 stains, respectively. Compared to the controls, *Klf4*$^{\Delta IS}$ mice showed a reduction in acidic mucin expression, as demonstrated by AB staining, in the small intestine but no change in neutral mucin or Muc2 mucin expression (Fig. 3A–F). In contrast, the colon of *Klf4*$^{\Delta IS}$ mice showed an overall altered goblet cell maturation and differentiation as indicated by the reduction in staining for all three mucins (Fig. 3G–L), as well as a reduction in the number and size of goblet cells, when compared to the controls (Suppl. Figs. S2A and B). These results support the previous finding that Klf4 is required for terminal differentiation of goblet cells in the colon of one day-old *Klf4*-null mice (Katz et al., 2002).

Deletion of Klf4 alters terminal differentiation of intestinal enterocytes and colonocytes

The intestinal alkaline phosphatase (ALP) gene is only expressed in differentiated enterocytes of the small intestine and has been shown to be an *in vitro* target gene of KLF4 (Hinnebusch et al., 2004). To investigate the effect of intestinal *Klf4* deletion on the terminal differentiation of enterocytes, we stained the small intestine for ALP. Compared to the controls, the small intestine of *Klf4*$^{\Delta IS}$ mice showed a reduction in ALP staining, indicating altered differentiation of intestinal enterocytes (Fig. 4A and B). In the colon, the effect of *Klf4* deletion on the terminal differentiation of colonocytes was determined by staining for carbonic anhydrase-1 (CA1). CA1 is expressed in the colonic epithelium but not the small intestine (Parkkila et al., 1994; Sowden et al., 1993). The staining of CA1 in the colon of *Klf4*$^{\Delta IS}$ mice varied from weak to absent (Fig. 4D), while it was present throughout the colon in the upper one-third of the colonic epithelium of control mice (Fig. 4C). These results indicate that KLF4 is required for terminal differentiation of both small intestinal enterocytes and colonocytes.

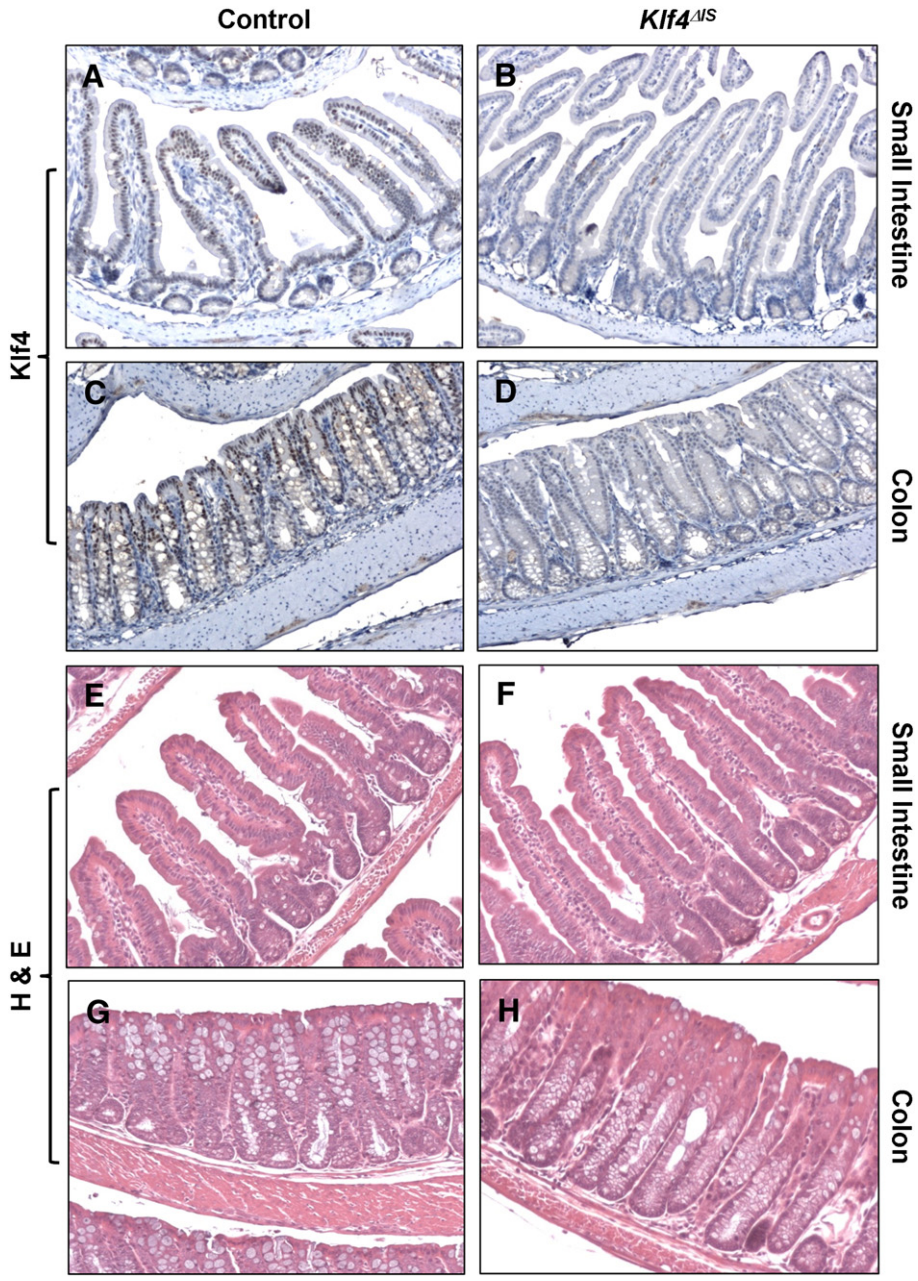

Fig. 1. Deletion of Klf4 from the intestine *Klf4*$^{\Delta IS}$ mice. The small intestine and colon of control *Klf4*$^{fl/fl}$ mice (A and C) and *Vil/Cre*;*Klf4*$^{fl/fl}$ (*Klf4*$^{\Delta IS}$) mice (B and D) were immunostained with anti-Klf4 followed by secondary antibodies as described in Materials and methods. Sections were also stained with H & E (E–H).

Intestinal Klf4 deletion causes mislocalization of Paneth cells and decreased ephrin-B1 expression

The effect of *Klf4* deletion of enteroendocrine and Paneth cell differentiation was also investigated. As reported before (Katz et al., 2002), we found no difference in enteroendocrine cells differentiation between the intestines of control and *Klf4*$^{\Delta IS}$ mice (data not shown). However, compared to the controls, *Klf4*$^{\Delta IS}$ mice had mislocalized Paneth cells in the small intestine as demonstrated by the displacement of the cells normally residing at the bottom of the crypts when stained for Paneth cell markers lysozyme (Fig. 4E–H) and lipopolysaccharide-binding protein (LBP) (Suppl. Figs. S3A–D).

Ephrin-B/EphB signaling has been shown to regulate Paneth cell positioning in the intestine. In both *EphB3*-null mice (Batlle et al., 2002) and mice with conditional *ephrin-B1* deletion (Cortina et al., 2007), Paneth cells are no longer restricted to the crypt base. To determine whether mispositioning of Paneth cells in the *Klf4*$^{\Delta IS}$ mice was due to alterations in ephrin-B/EphB signaling, we stained the intestine for ephrin-B1, EphB2, and EphB3. Compared to the controls, ephrin-B1 staining in the small intestine was significantly reduced in *Klf4*$^{\Delta IS}$ mice (Fig. 5A, D, G, and J); and while there was no change in EphB2 staining between the two groups (Fig. 5B and E), there was a relative increase in EphB3 staining in *Klf4*$^{\Delta IS}$ mice (Fig. 5H and K). The effect *Klf4* deletion on ephrin-B1 was confirmed by Western blot analysis, which showed an absence of ephrin-B1 expression from the small intestine of *Klf4*$^{\Delta IS}$ mice (Fig. 5M).

Intestinal Klf4 deletion leads to perturbation of proliferation and differentiation pathways

Since deletion of *Klf4* from the small intestine results in increased epithelial proliferation, we examined the consequence of *Klf4* deletion on expression of components of the Wnt pathway using a real-time PCR array. As shown in Fig. 6 and Suppl. Table S1, there was an overall

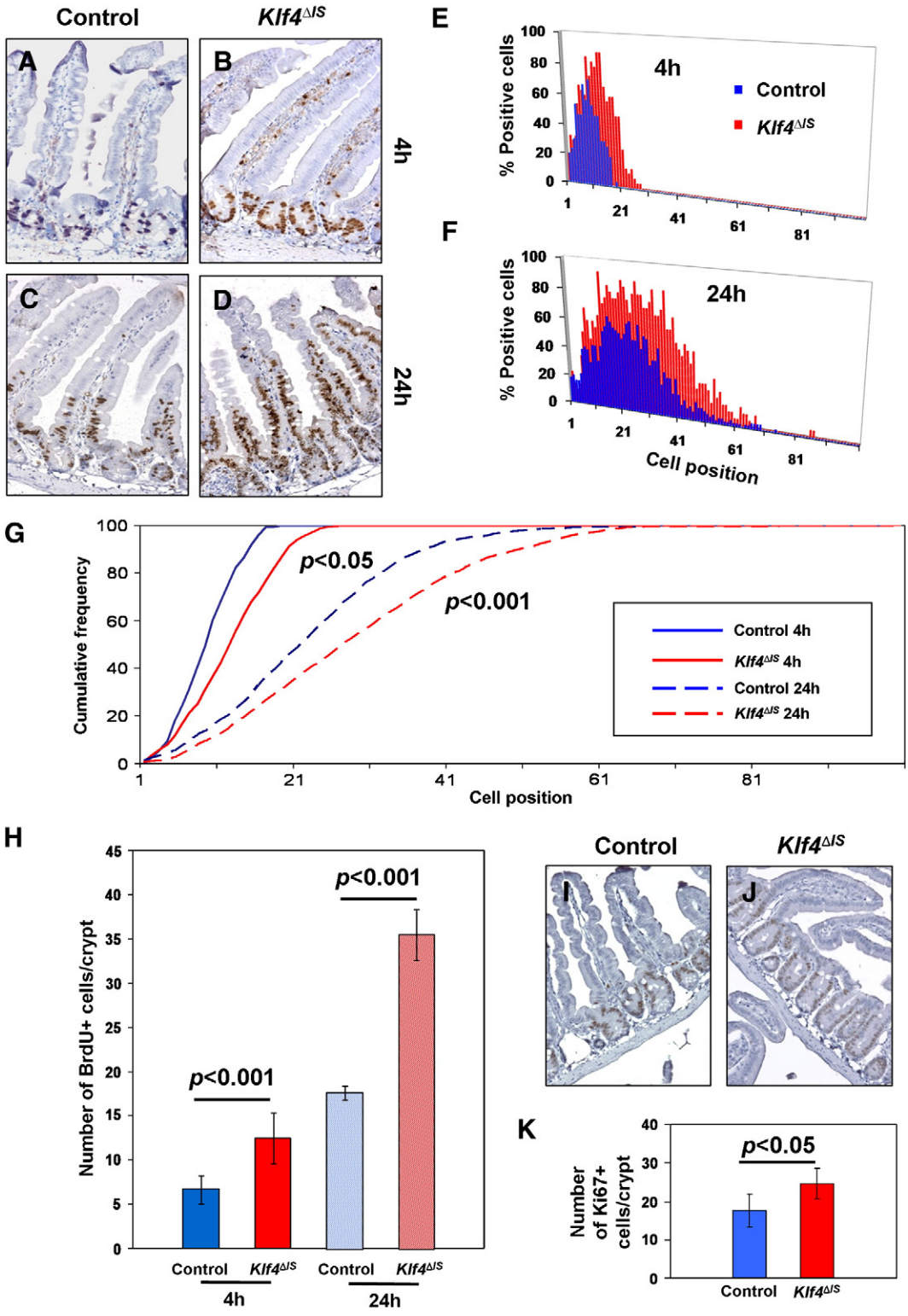

Fig. 2. Intestinal deletion of Klf4 increases the rate of proliferation and migration of small intestinal epithelial cells. Control (*Klf4*$^{fl/fl}$) or *Klf4*$^{\Delta IS}$ mice were injected with BrdU intraperitoneally and euthanized 4 or 24 h later. Intestinal sections were immunostained for BrdU as described in Materials and methods. Shown are representative sections of intestines from control (A and C) and *Klf4*$^{\Delta IS}$ (B and D) mice at 4 and 24 h, respectively. (E and F) are the percentages of BrdU-positive cells according to their cell positions in the crypts (position 1 is at the very bottom of the crypt) of control and *Klf4*$^{\Delta IS}$ mice at 4 and 24 h. At least 30 crypts were examined per mouse per genotype per time point. (G) Cumulative frequency of BrdU-positive cells of control and *Klf4*$^{\Delta IS}$ mice along the crypt–villus axis. The rates of migration between control and *Klf4*$^{\Delta IS}$ mice were significantly different at both 4 h ($p<0.05$) and 24 h ($p<0.001$). (H) The total number of BrdU-positive cells per crypt were compared between control and *Klf4*$^{\Delta IS}$ mice at 4 and 24 h. At least 30 crypts were examined per mouse per genotype per time point. (I and J) Representative patterns of immunostaining for Ki67 in the small intestine of control and *Klf4*$^{\Delta IS}$ mice. (K) The number of Ki67-positive cells per crypt in the small intestine of control and *Klf4*$^{\Delta IS}$ mice. At least 30 crypts were examined per mouse per genotype.

increase in the transcript levels of the genes in the Wnt pathway, including β-catenin (Ctnnb1) and c-Myc, in the small intestine of *Klf4*$^{\Delta IS}$ mice compared to controls. This is consistent with the observed increase in cellular proliferation in the small intestinal epithelium in the absence of *Klf4*. The result also suggests that Klf4 is a negative regulator of Wnt signaling *in vivo*. Because deletion of *Klf4* appears to

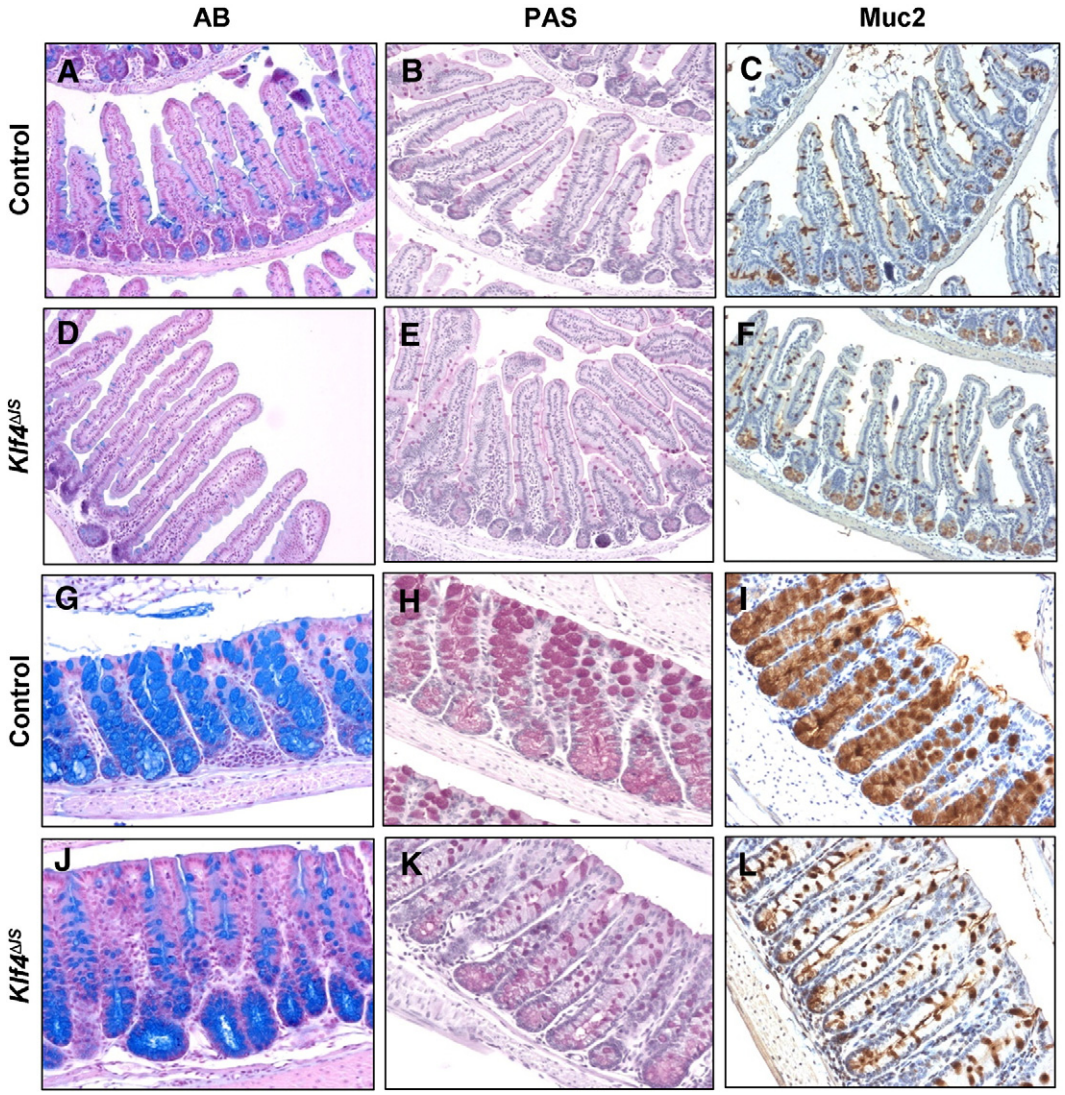

Fig. 3. Intestinal deletion of Klf4 perturbs terminal differentiation and maturation of goblet cells. Histochemical staining for acidic mucin by Alcian blue (AB) (A, D, G, and J), neutral mucin by Periodic acid-Schiff's (PAS) (B, E, H, and K), and immunohistochemical staining for Muc2 mucin (C, F, I, and L) were conducted in the small (A–F) and large (G–L) intestines of control (A–C; G–I) and $Klf4^{\Delta IS}$ (D–F; J–L) mice.

perturb differentiation more in the colon than the small intestine, we compared the transcript levels of a panel of genes involved in regulation of differentiation between $Klf4^{\Delta IS}$ and control mice. As seen in Fig. 7, there was a general decrease in the expression levels of these genes in the colon of $Klf4^{\Delta IS}$ mice compared to controls. This result suggests that Klf4 is involved in global regulation of colonocyte differentiation.

Discussion

Much has been learned in the recent past about the mechanisms by which differentiation and development of the intestinal tract are regulated (Barker et al., 2008; de Santa Barbara et al., 2003; Hauck et al., 2005; Jedlicka and Gutierrez-Hartmann, 2008; Scoville et al., 2008). It is clear from these studies that several signaling pathways including Wnt, Notch, hedgehog, and bone morphogenetic protein (BMP), play critical roles in the regulatory process. The roles of individual regulatory proteins in the epithelium in controlling proliferation, differentiation, and migration of intestinal epithelial cells, however, are still not well defined. Using targeted deletion of *Klf4* from the intestinal epithelium, we demonstrate for the first time that the zinc finger transcription factor, KLF4, has a crucial role in regulating intestinal epithelial cell homeostasis *in vivo*.

Previous studies indicate that KLF4 is mainly expressed in the post-mitotic, differentiated epithelial cells in the small intestine and colon (McConnell et al., 2007; Shields et al., 1996). Consistent with this observation, expression of KLF4 is associated with growth arrest *in vitro* (Shields et al., 1996) and forced expression of KLF4 leads to cell cycle arrest (Chen et al., 2001; Shields et al., 1996). Results of the current study showing that conditional deletion of *Klf4* from the small intestinal epithelium causes an increased rate of proliferation (Fig. 2) further substantiate the inhibitory function of KLF4 on cell proliferation. These results suggest that one of KLF4's physiologic functions in the intestinal tract is to regulate proliferation of stem cells or progenitor cells and that its deletion results in an increase in the rate of the division of such cells. This increase could also explain the observed increase in migration of epithelial cells along the crypt–villus axis. In contrast, these changes were not observed in the colonic epithelial cells, suggesting that additional mechanisms may guard against increased proliferation of colonic epithelial cells. The increased proliferation of small intestinal epithelial cells following loss of *Klf4* is similar to that observed in mice with conditional deletion of *Klf4* from the esophagus and stomach (Katz et al., 2005; Tetreault et al., 2010).

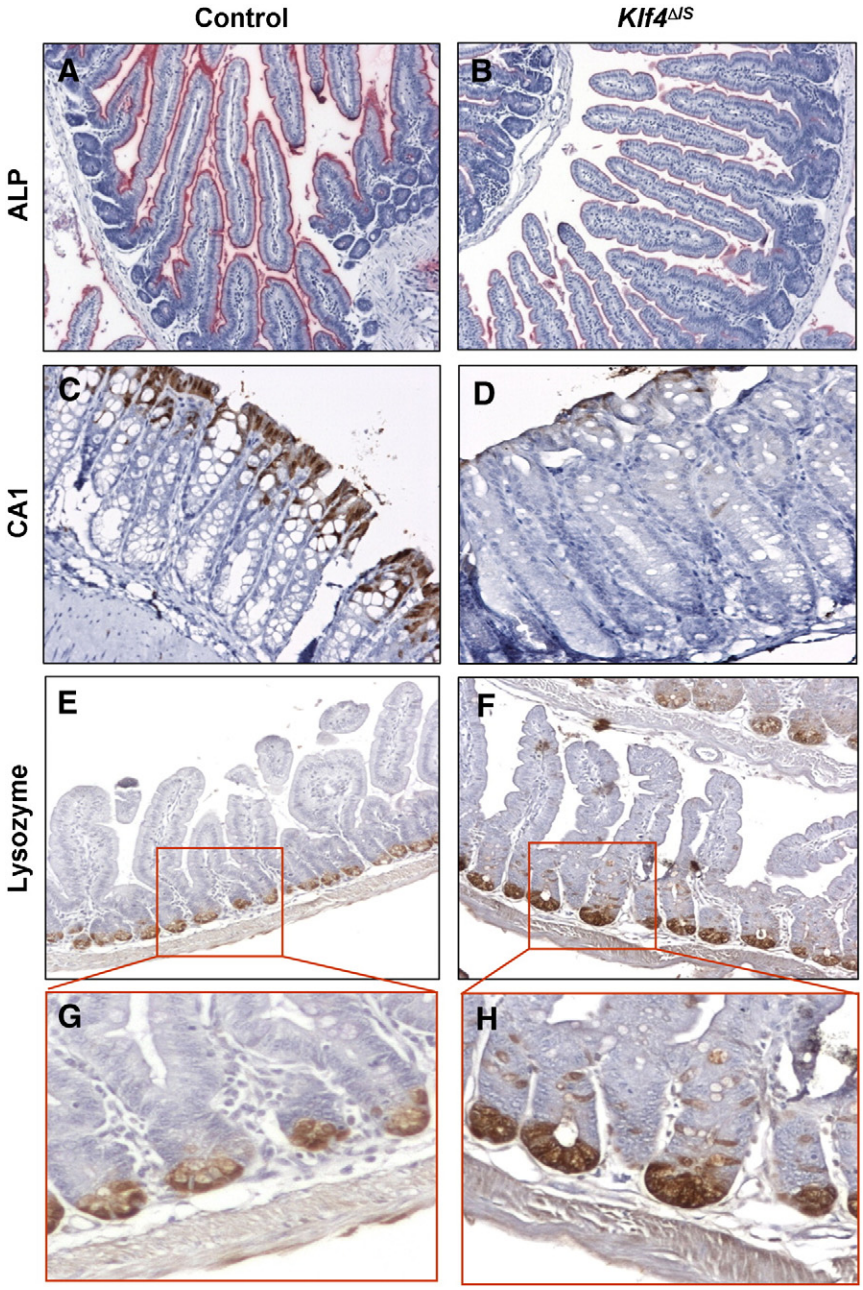

Fig. 4. Intestinal Klf4 deletion results in altered terminal differentiation of enterocytes and colonocytes and mispositioning of Paneth cells. (A and B) Alkaline phosphatase (ALP) staining of small intestinal enterocytes in control and *Klf4*$^{\Delta IS}$ mice. (C and D) Carbonic anhydrase-1 (CA1) staining of colonocytes in control and *Klf4*$^{\Delta IS}$ mice. (E and F) Lysozme staining for Paneth cells in the small intestines of control and *Klf4*$^{\Delta IS}$ mice. (G and H) are enlargement of the inserts in panels E and F.

Homeostasis in epithelial tissues is balanced between proliferation and apoptosis (Guasch et al., 2007; Squier and Kremer, 2001; Walker and Stappenbeck, 2008). This is demonstrated by the finding that conditional deletion of the tumor suppressor gene, *Apc*, from the small intestine led to an increase in both proliferation and apoptosis (Sansom et al., 2004). In contrast, loss of *Klf4* did not result in an increased rate of apoptosis in the intestine. We also did not observe any intestinal epithelial hyperplasia as a consequence of *Klf4* deletion, as might be expected from the combination of increased proliferation and unchanged apoptotic rates. This could potentially be explained by the increased migratory rate, which may be accompanied by increased shedding of epithelial cells into the lumen. It is worth noting that mice with intestine-specific deletion of *Apc* was accompanied by abrogated epithelial cell migration along the crypt–villus axis, which led to the retention of *Apc* mutant cells, allowing them to acquire additional genetic changes and produce daughter cells that exhibit properties of premalignant cells (Sansom et al., 2004). In this regard, the increased migration of epithelial cells in the intestine of *Klf4* mutant mice may offset the acquisition of any genetically altered premalignant cells by increasing their migration and eventual shedding in the lumen. This may also explain the lack of any intestinal neoplasia from the intestine of *Klf4* mutant mice up to 10 months of life. A similar finding of increased proliferation with unchanged apoptosis has been observed in the intestine of mice with conditional deletion of the transcription factor, Math1 (Shroyer et al., 2007).

Despite the differences in apoptosis and migration of intestinal epithelial cells, *Klf4* mutant mice shared several features with those with intestine-specific deletion of *Apc*, aside from the increased epithelial cell proliferation. For example, *Apc* mutant mice had

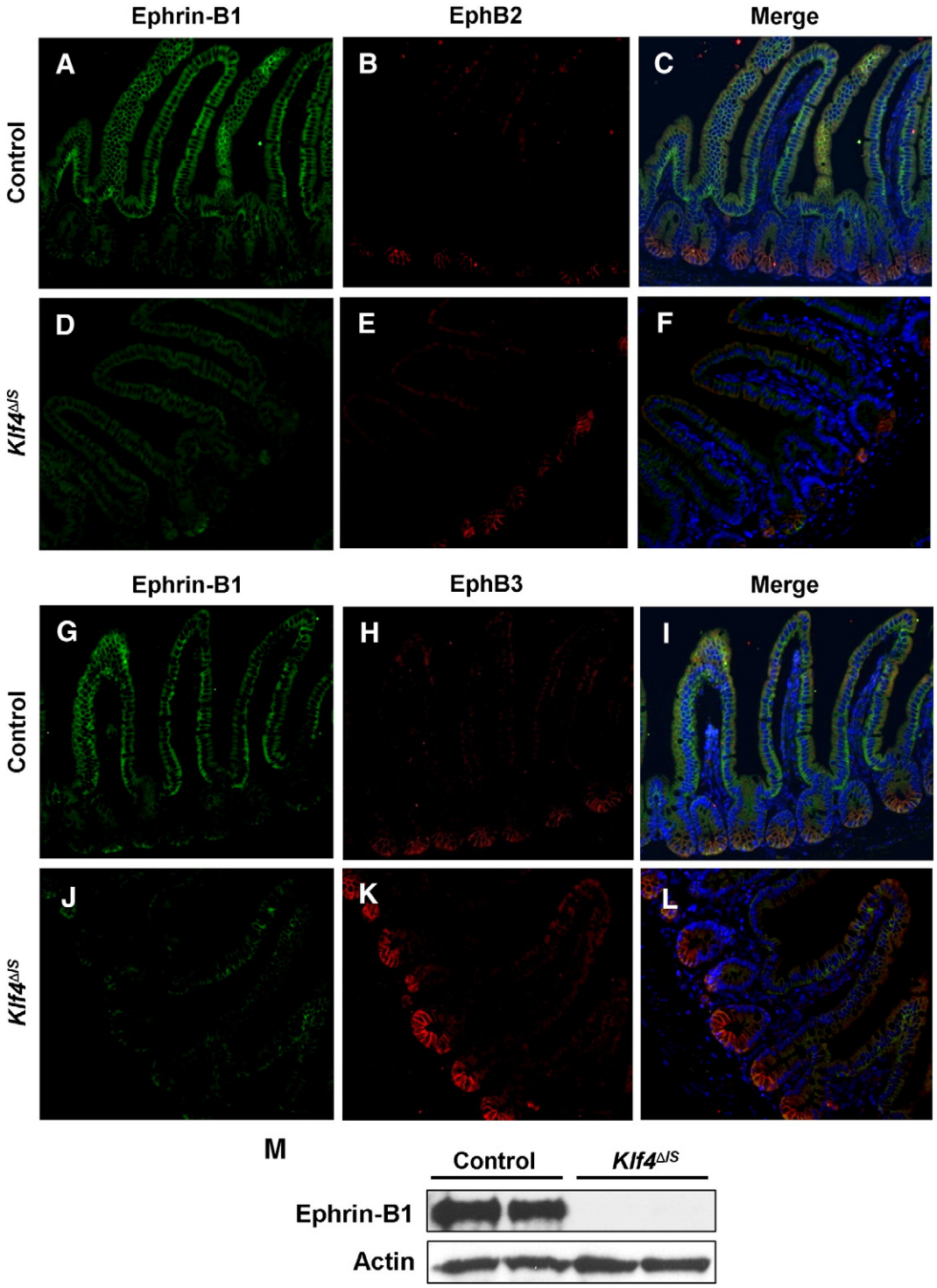

Fig. 5. Intestinal deletion of Klf4 causes a decrease in ephrin-B1 expression in the small intestine. Immunofluorescence staining for ephrin-B1 (A, D, G, and J), EphB2 (B and E), and EphB3 (H and K) were performed on sections of small intestines from control (A, B, G, and H) and $Klf4^{\Delta IS}$ (D, E, J, and K) mice. Panels C, F, I, and L are merged images. (M) Western blot analysis of ephrin-B1 and actin in small intestines of control and $Klf4^{\Delta IS}$ mice. Shown are representative results of two individual mice from each group.

reduced staining for alkaline phosphatase in the brush border, reduced goblet cells, and mispositioned Paneth cells in their small intestine (Sansom et al., 2004). The last observation is particularly intriguing as the mispositioning of Paneth cells in the *Apc* mutant mice is a consequence of perturbed ephrin-B/EphB signaling system (Sansom et al., 2004). In the intestine of *Apc* mutant mice, both EphB2 and EphB3 are up-regulated while ephrin-B2 is down-regulated. This is similar to our findings of up-regulation of EphB3 and down-regulation of ephrin-B1 in the intestine lacking *Klf4* (Fig. 5). A similar phenotype of mispositioned Paneth cells has also been described in the intestine of mice null for *EphB3* (Batlle et al., 2002) or with conditional deletion of *ephrin-B1* (Cortina et al., 2007). These studies underscore the importance of the ephrin-B/EphB repulsion system in determining cell positioning. Significantly, consistent with previous reports that KLF4 is a downstream mediator of APC (Dang et al., 2001; Stone et al., 2002; Zhang et al., 2006), the phenotypic overlaps between *Klf4* and *Apc* mutant mouse intestines lend further support to this notion. To this end, KLF4, ephrin-B1, intestinal alkaline

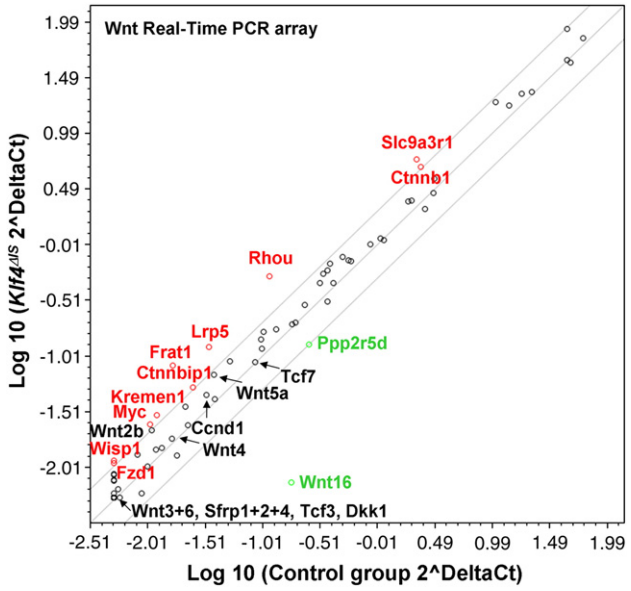

Fig. 6. Comparison of expression of Wnt pathway genes in the small intestines of *Klf4*$^{\Delta IS}$ and control mice. Shown is a scatter plot of expression levels of Wnt pathway genes in the intestines of *Klf4* mutant and control mice as determined by real-time PCR analysis. The analysis was conducted with pooled RNA specimens from three mice for each genotype. Transcripts with two or more fold-increase in the *Klf4* mutant mice compared to the controls are labeled in red and those with two or more fold-decrease in the *Klf4* mutant mice are labeled in green. Detailed results are shown in Suppl. Table S1.

phosphatase, Muc2, and p21^{CIP1} are among the highest up-regulated markers of intestinal differentiation in colorectal cancer cells with abrogated Wnt signaling (which functions in opposition to APC) (van de Wetering et al., 2002). Previous studies have already established that KLF4 directly regulates intestinal alkaline phosphatase and p21^{CIP1} expression (Chen et al., 2001, 2003; Hinnebusch et al., 2004; Zhang et al., 2000). The results of the current study suggest that Klf4 is also responsible for the expression of ephrin-B1 in the small intestinal epithelium (Fig. 5) and possibly Muc2 and CA-1 in the colonic epithelium (Figs. 3 and 4). In addition, results of our recent microarray analysis showed that transcripts for ephrin-B1, B2, and B4 were among those down-regulated in mouse embryonic fibroblasts null for *Klf4* (Hagos et al., 2011), suggesting that Klf4 regulates expression of the ephrin-B family of ligands, perhaps at the transcriptional level.

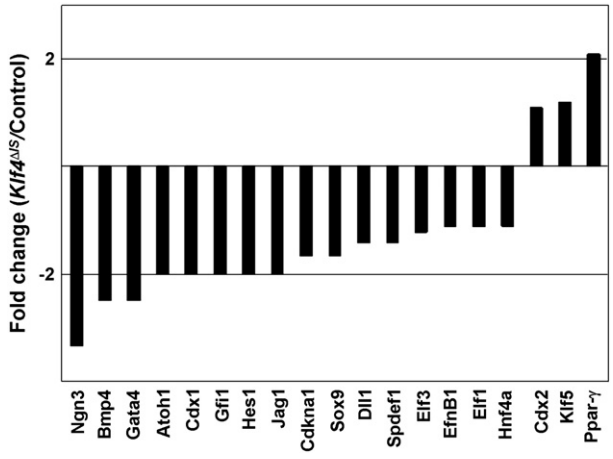

Fig. 7. Comparison of expression of genes involved in regulating differentiation in the colon of *Klf4*$^{\Delta IS}$ and control mice. Real-time PCR analysis of transcript levels in pooled RNA specimens of colonic epithelial cells from three mutant or control mice was conducted on a panel of genes that are involved in regulating epithelial differentiation. Shown are the fold-changes in transcript levels between *Klf4*$^{\Delta IS}$ and control mice.

Finally, the observation that *Klf4* deletion leads to a general up-regulation of genes in the Wnt pathway (Fig. 6 and Suppl. Table S1) is highly suggestive that Klf4 is involved in the network of Wnt signaling.

Results of our study also suggest that intestine-specific *Klf4* deletion alters differentiation and maturation of goblet cells, albeit to a different extent between the small intestine and colon (Fig. 3). In the small bowel of *Klf4* mutant mice, there is a reduction in staining for acidic mucin (as demonstrated by the Alcian blue stain) but no change in staining for neutral mucin (as demonstrated by the PAS stain) and the goblet cell-specific marker, Muc2. As acidic mucin is considered the more mature form of mucin produced by goblet cells (Fontaine et al., 1998), these findings suggest that Klf4 is required for maturation of goblet cells in the small intestine. In the colon, Klf4 is required not only for the maturation but differentiation of goblet cells as demonstrated by the significant reduction in the number and size of goblet cells, as well as in staining for Muc2, and both acidic and neural mucins in the colon of *Klf4*$^{\Delta IS}$ mice compared to controls (Fig. 3 and Suppl. Fig. S2). These results are similar to those observed in the colon of 1-day-old newborn mice lacking *Klf4* (*Klf4*$^{-/-}$) (Katz et al., 2002). Combining the results of these studies, it is apparent that Klf4 functions as a colonic goblet cell-differentiation factor *in vivo*.

Perturbations in goblet cell homeostasis in the intestine have been described in several other mouse models in which specific genes were deleted from the intestine. Mice with intestine-specific ablation of *Math1* (*Atoh1*) lost cells of the secretory lineage from their intestine including goblet, Paneth, and enteroendocrine cells (Shroyer et al., 2007). The transcription repressor, Gfi1, functions downstream of Math1 in intestinal secretory lineage differentiation-*Gfi1*-null mice lacked Paneth cells, had fewer goblet cells, and supernumerary enteroendocrine cells (Shroyer et al., 2005). Another transcription factor, Spdef, when over-expressed in the intestine, caused the expansion of goblet cells and a corresponding reduction in Paneth and enteroendocrine cells (Noah et al., 2010). It is of interest to note that the transcript levels of several factors involved in goblet cell differentiation including Atoh1, Gfi1, Spdef1 and Elf3, are lower in the colonic epithelial cells from *Klf4* mutant mice than controls (Fig. 7). In addition, *Klf4* deletion results in the down-regulation of several other genes known to regulate epithelial differentiation such as Ngn3, Bmp4, Gata4, Sox9 and Hnf4a (Fig. 7). These results suggest that Klf4 has a global function in regulating epithelial differentiation, which may be linked to its ability to regulate epithelial proliferation.

The down-regulation of expression of some components of the Notch pathway such as Hes1, Jag1 and Dll1 (Fig. 7), is somewhat unexpected since Notch signaling has been shown to inhibit differentiation of the secretory lineage including goblet cells (Jensen et al., 2000; van Es et al., 2005). Notch regulates terminal differentiation by Hes1-dependent repression of Math1, which is required for commitment to the secretory cell lineage in the mouse intestine (Yang et al., 2001). Notch also inhibits expression of KLF4 in colonic epithelial cells and in the mouse intestine (Ghaleb et al., 2008; Zheng et al., 2009). It is possible that KLF4 is involved in a feedback loop by positively regulating Notch signaling, thus explaining the reduction in transcript levels of the Notch pathway components in the colon of *Klf4* mutant mice (Fig. 7). However, the concomitant reduction in the transcript levels of Atoh1, which is required for the differentiation effects of Notch inhibitors (Kazanjian et al., 2010), in *Klf4* mutant mice may account for the lack of goblet cell differentiation in the colon.

In contrast to the previous finding that haploinsufficiency of *Klf4* results in increased intestinal tumor formation in the *Apc*^{Min} mouse background (Ghaleb et al., 2007), *Klf4*$^{\Delta IS}$ mice remain tumor-free up to 10 months of age despite evidence for increased intestinal epithelial proliferation. These results suggest that *Klf4* deletion alone is not sufficient to initiate tumor formation in the mouse, and that it may require a "second hit" in order to do so. We can also conclude that perturbed maturation and differentiation of goblet cells in the colon of

Klf4$^{\Delta IS}$ mice, which also occurs in *Klf4*$^{-/-}$ newborn mice, does not play a role in the premature death of *Klf4*-null mice (Katz et al., 2002; Segre et al., 1999). The additional phenotypic changes such as increased proliferation and migration of epithelial cells observed in the *Klf4*$^{\Delta IS}$ mice but not in *Klf4*$^{-/-}$ mice likely reflect a true physiologic function of Klf4 in the intestine since the former survived to adulthood. Additionally, ablation of goblet cells from the colon results in a reduction in susceptibility to chemical-induced colonic injury (Itoh et al., 1999). On the other hand, chronic inflammation such as ulcerative colitis is characterized by goblet cell depletion (Podolsky, 1997; Tytgat et al., 1996a,b; Van Klinken et al., 1999) and loss of *Muc2* in mice leads to the development of spontaneous colitis and rectal tumors on a permissive genetic background (Chutkan, 2001; Van der Sluis et al., 2006; Velcich et al., 2002). The physiologic role of KLF4 in mediating the epithelial response to injury therefore remains to be determined.

Conclusion

The results of our study provide new insights into the *in vivo* function of KLF4 in the postnatal proliferation, migration, differentiation, and positioning of intestinal epithelial cells, and highlight an essential role for KLF4 in maintaining normal intestinal epithelial cell homeostasis.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.11.001.

Conflict of interest

The authors declare that they have no conflict of interest.

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