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Haploinsufficiency of Krüppel-Like Factor 4 Promotes Adenomatous Polyposis Coli–Dependent Intestinal Tumorigenesis

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Abstract

The zinc finger transcription factor Krüppel-like factor 4 (KLF4) is frequently down-regulated in colorectal cancer. Previous studies showed that the expression of *KLF4* was activated by the colorectal cancer tumor suppressor adeno-matous polyposis coli (APC) and that KLF4 repressed the Wnt/β-catenin pathway. Here, we examined whether KLF4 plays a role in modulating intestinal tumorigenesis by comparing the tumor burdens in mice heterozygous for the Apc^{Min} allele ($Apc^{Min/+}$) and those heterozygous for both the Apc^{Min} and *Klf4* alleles ($Klf4^{+/-}/Apc^{Min/+}$). Between 10 and 20 weeks of age, $Klf4^{+/-}/Apc^{Min/+}$ mice developed, on average, 59% more intestinal adenomas than $Apc^{Min/+}$ mice ($P < 0.0001$). Immunohistochemical staining showed that Klf4 protein levels were lower in the normal-appearing intestinal tissues of $Klf4^{+/-}/Apc^{Min/+}$ mice compared with wild-type, $Klf4^{+/-}$, or $Apc^{Min/+}$ mice. In contrast, the levels of β-catenin and cyclin D1 were higher in the normal-appearing intestinal tissues of $Klf4^{+/-}/Apc^{Min/+}$ mice compared with the other three genotypes. Klf4 levels were further decreased in adenomas from both $Apc^{Min/+}$ and $Klf4^{+/-}/Apc^{Min/+}$ mice compared with their corresponding normal-appearing tissues. Reverse transcription-PCR showed an inverse correlation between adenoma size and *Klf4* mRNA levels in both $Klf4^{+/-}/Apc^{Min/+}$ and $Apc^{Min/+}$ mice. There was also a progressive loss of heterozygosity of the wild-type *Apc* allele in adenomas with increasing size from $Klf4^{+/-}/Apc^{Min/+}$ and $Apc^{Min/+}$ mice. Results from this study show that KLF4 plays an important role in promoting the development of intestinal adenomas in the presence of Apc^{Min} mutation.

Introduction

Colorectal cancer is a major cause of cancer mortality in the United States. More than 80% of colorectal cancers contain mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene (1). APC is present in the normal intestinal mucosa with an increasing gradient

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of expression in mature epithelial cells located in the upper crypt region (2). Overexpression of *APC* leads to cell cycle arrest at the G_1-S and G_2-M boundaries (3–6). In addition, APC antagonizes the pro-proliferative Wnt pathway by negatively regulating the steady-state level of intracellular β-catenin (7–9). When APC is inactivated by mutation, Wnt signaling is unimpeded, resulting in the nuclear accumulation of β-catenin and subsequent activation of downstream target genes such as *cyclin D1* and *c-MYC* that promote cell proliferation (10, 11).

The nuclear transcription factor Krüppel-like factor 4 (KLF4; also known as gut-enriched Krüppel-like factor or GKLF), is a member of the C_2H_2-zinc finger–containing proteins exhibiting homology to the *Drosophila melanogaster* segmentation gene product, Krüppel (12–16). KLF4 is highly expressed in the terminally differentiated, postmitotic intestinal epithelial cells and is an inhibitor of cell proliferation (17,18). We previously showed that KLF4 was transcrip-tionally activated by p53 following DNA damage (19) and caused cell cycle arrest at both the G_1-S and G_2-M boundaries (20,21). These results indicate that KLF4 is an important factor in mediating the checkpoint functions of p53 following DNA damage. In the intestine, the *KLF4* promoter is regulated by APC in a CDX2-dependent manner; CDX2 is an intestine-specific transcription factor that controls intestinal development (22). Conversely, KLF4 has been shown to regulate colonic cell growth by inhibiting β-catenin activity (23,24). Accordingly, studies have shown a potentially causal relationship between KLF4 and several kinds of human cancers. For example, the expression of *KLF4* is often reduced in tumors of the gastrointestinal tract (25–29). In addition, loss of heterozygosity (LOH) and promoter hypermethylation are thought to be possible reasons for the reduced expression of *KLF4* in a subset of colorectal cancers (25). However, whether KLF4 plays an *in vivo* role in the development of intestinal tumors has not been established.

The $Apc^{Min/+}$ mice are an excellent model for studying intestinal tumorigenesis (30). The mutant mice, multiple intestinal neoplasia (Min; ref. 30), carry a truncating mutation at codon 850 of the murine *Apc* gene (31). In a C57BL/6J background, $Apc^{Min/+}$ mice develop, on average, 30 adenomatous polyps in the intestines, with a predominant distribution in the small intestine (30). All intestinal adenomas are established by 100 days of age or sooner and new tumors do not arise continuously over the remaining life span of the animals (32). In the current study, we investigated the *in vivo* role of KLF4 in intestinal tumorigenesis in the setting of the Apc^{Min} mutation.

Materials and Methods

Mice

Founder C57BL/6J male mice heterozygous for the Apc^{Min} allele ($Apc^{Min/+}$) were purchased from The Jackson Laboratory. Founder C57BL/6J male mice heterozygous for the *Klf4* allele ($Klf4^{+/-}$) were previously established (33). A colony of each genotype was established by mating the founder male with wild-type C57BL/6J female mice. Allele-specific PCR assays were used to identify the Apc^{Min} (34) and the *Klf4* (33) mutations. $Apc^{Min/+}$ males were subsequently mated with $Klf4^{+/-}$ females to obtain mice wild-type for both alleles, heterozygous for the *Klf4* allele ($Klf4^{+/-}$), heterozygous for the Apc^{Min} allele ($Apc^{Min/+}$), or heterozygous for both the *Klf4* and Apc^{Min} alleles ($Klf4^{+/-}/Apc^{Min/+}$).

Tissue harvesting and tumor assessment

At 10, 16, or 20 weeks of age, wild-type, $Klf4^{+/-}$, $Apc^{Min/+}$, and $Klf4^{+/-}/Apc^{Min/+}$ mice were sacrificed by CO_2 asphyxiation. The entire small intestine and colon were dissected longitudinally and washed in PBS. The intestines were examined under a dissecting microscope for the presence of adenomas. The number and size of adenomas in both the small and large

intestines were recorded. Adenomas identified in the small and large intestines were grouped by size (<1, 1–2, 2–3, and >3 mm and ≤2 and >2 mm, respectively).

Immunohistochemistry

Intestinal tissues for immunohistochemistry were fixed in 10% formalin in PBS and subsequently embedded in paraffin. Five-micrometer-thick paraffin sections were cut and applied to Superfrost Plus slides (VWR). Sections were deparaffinized in xylene, rehydrated in ethanol, and then treated with 10 mmol/L of sodium citrate buffer (pH 6.0), at 120°C for 10 min in a pressure cooker. The histologic sections were incubated with a blocking buffer (10% nonfat dry milk, 0.01% Tween 20, and 10% normal horse serum in PBS) for 1 h at room temperature. An avidin/biotin blocking kit (Vector Laboratories) was used in conjunction with the blocking buffer according to the manufacturer's directions to reduce background and nonspecific secondary antibody binding. Sections were then stained for Klf4 (goat anti-GKLF, T-16; Santa Cruz Biotechnology), β-catenin (monoclonal anti–β-catenin; Zymed/Invitrogen), or cyclin D1 (rabbit anti–cyclin D1; Santa Cruz Biotechnology). The Klf4, β-catenin, and cyclin D1 antibodies were used at dilutions of 1:50, 1:1,000, and 1:100, respectively, in the blocking buffer for 1 h at room temperature. Detection of primary antibodies and color development were done using the Dako (k6090) kit (Dako North America, Inc.). Sections were then counterstained with hematoxylin, dehydrated, and coverslipped. Images were acquired using an Axioskop 2 plus microscope (Zeiss) equipped with an AxioCam MRc5 CCD camera (Zeiss).

Reverse transcription-PCR

Adenomas and adjacent normal-appearing intestinal tissues from $Apc^{Min/+}$ and $Klf4^{+/-}/Apc^{Min/+}$ mice were retrieved under a dissecting microscope. Adenomas from six 16- to 20-week-old mice of each genotype were pooled by size (<1, 1–3, and >3 mm). Total RNA from the adenomas was then extracted using RNeasy Micro Kit (QIAGEN). Two micrograms of RNA per sample was used for cDNA synthesis using SuperScript III First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) kit (Invitrogen). *Klf4* cDNA amplification was carried out using primers F-5′-TGATGGGCAAGTTTGTGCTGAAGG-3′ and R-5′-ACAGTGGT-AAGGTTTCTCGCCTGT-3′ that amplified the wild-type allele. PCR reactions were carried out for 25 cycles (95°C for 30 s, 56°C for 30 s, 68°C for 45 s) in a buffer of 50 μmol/L of each deoxynucleotide triphosphate, 1.25 units of Taq polymerase, 1× Taq buffer containing 1.5 mmol/L of $MgSO_4$. The wild-type allele produced a band of 632 bp. As a control, RT-PCR for β-actin was carried out using the primers described previously (35). Band intensities were quantified using the Scion Image software.[5]

Measurement of LOH of the *Apc*$^+$ locus

To reduce contamination of adenomatous tissues with the surrounding normal tissues, adenomas versus normal-appearing tissues were dissected using laser capture microscopy (LCM). Intestines were rinsed thrice with PBS without being cut open, and quickly embedded in Tissue-Tek optimal cutting temperature compound (VWR), and then snap-frozen in liquid nitrogen. Eight-micrometer-thick sections were cut using a Leica CM1510-3 cryostat (Leica Microsystems) and applied to Superfrost Plus slides. Sections were counterstained for visualization using HistoGene LCM Frozen Section Staining Kit (Arcturus) following the manufacturer's protocol. Cells from normal tissue and adenomas (<1, 1–3, and >3 mm in size) were dissected using a PixCell IIe microscope (Arcturus) and captured on CapSure Macro LCM Caps LCM 0211 (Arcturus). DNA was extracted from the captured cells using QIAmp DNA Micro Kit (QIAGEN) following the manufacturer's protocol. PCR for the *Apc* locus and *Hin*dIII digestion was carried out as described previously (36). Ten microliters of each *Hin*dIII

[5]http://www.scioncorp.com/

digestion reaction was electrophoresed through a 2.5% agarose gel. Bands were quantified using Scion Image software. Determination of LOH was carried out as described previously (36).

Results

The *in vivo* role of Klf4 in the development of intestinal adenomas

Mice null for *Klf4* (*Klf4*$^{-/-}$) are not suitable for the study of intestinal tumorigenesis because they die shortly after birth (33,37). We therefore used an alternative approach to investigate the role of KLF4 in intestinal adenoma formation in the setting of the well-established model of intestinal tumorigenesis, the *Apc*^{Min} mouse (30). We first compared the number and size of the adenomas that developed in *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice between 10 and 20 weeks of age. Neither wild-type nor *Klf4*$^{+/-}$ mice developed adenomas in this period. *Apc*$^{Min/+}$ mice developed, on average, 18.4 ± 6.8 adenomas per mouse (n = 40) in the small intestine and less than one adenoma per mouse in the large intestine (Fig. 1*A* and *B*, respectively). The average number of adenomas in the small intestine of *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice was 28.9 ± 7.8 adenomas per mouse (N = 40; Fig. 1*A*), 57% more than *Apc*$^{Min/+}$ mice ($P = 1.3 \times 10^{-7}$ by paired two-tailed t test). In the large intestine, the average number of adenomas in *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice trended higher than *Apc*$^{Min/+}$ mice, although it did not reach statistical significance (P = 0.053; Fig. 1B). Combining the tumor burden in the small and large intestines, the average number of adenomas per mouse was 18.7 ± 6.9 and 29.7 ± 7.7 for *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice, respectively, with a highly statistically difference of 59% ($P = 4 \times 10^{-8}$ by paired two-tailed t test; Fig. 1*C*).

Results in Supplementary Fig. S1 compare the number of adenomas per mouse between *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice in each of the three age groups—10, 16, and 20 weeks. Consistent with a previous report (32), *Apc*$^{Min/+}$ mice developed adenomas early in life but tumor numbers did not increase over the life span of the animals (Supplementary Fig. S1*A*, *C*, and *E*). *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice developed significantly more small intestinal adenomas in as early as 10 weeks (Supplementary Fig. S1*A*) and this increase was maintained up to 20 weeks (Supplementary Fig. S1*C* and *E*). Adenomas in the large intestine developed later in life in both groups. There was a trend for the *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice to have more colonic adenomas than the *Apc*$^{Min/+}$ mice, although it did not reach statistical significance (Supplementary Fig. S1*D* and *F*).

We also examined whether there were any differences in the size of adenomas between the small and large intestines of *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. As shown in Supplementary Fig. S2*A*, the adenomas which developed in the small intestines of both *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice at 10 weeks were mostly small (<1 and 1–2 mm). By week 16, there was a shift in the size of adenomas to larger ones in both *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice, and this trend continued at 20 weeks (Supplementary Fig. S2*A*). Although the *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice had higher numbers of adenomas in each of the size categories than the *Apc*$^{Min/+}$ mice at any given age, there was no significant difference in the distribution of sizes between the two groups of mice. There was also no significant difference in the size distribution of adenomas formed in the large intestines of *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice (Supplementary Fig. S2*B*).

Immunohistochemical characterization of the normal-appearing small intestines and adenomas from *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice

To investigate the mechanisms by which the haploinsufficiency of *Klf4* enhances intestinal adenoma formation in the *Apc*^{Min} background, we examined the levels of Klf4, β-catenin, and cyclin D1 by immunohistochemistry in the normal-appearing intestinal tissues obtained from

age-matched wild-type, *Klf4*$^{+/-}$, *Apc*$^{Min/+}$, and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice and in adenomas derived from mice with the latter two genotypes. As seen in Fig. 2, Klf4 was present primarily in epithelial cells lining the villi of the small intestine of wild-type mice (Fig. 2*A*). Consistent with previous findings (17,24), Klf4 is absent from the proliferating crypt cell compartment in the wild-type intestine. As expected from the reduced gene dosage, *Klf4*$^{+/-}$ mice had less expression of Klf4 than wild-type mice (Fig. 2*B*). Also, compared with the *Apc*$^{Min/+}$ mice (Fig. 2*C*), immunostaining for Klf4 was significantly decreased in the normal-appearing small intestine of *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice (Fig. 2*D*).

KLF4 has been shown to repress Wnt/β-catenin activity in colonic epithelial cells (24). We therefore determined the levels of β-catenin in the normal-appearing intestines of mice with the four different genotypes. Figure 3 shows that β-catenin is present in the basolateral membranes of epithelial cells lining the villi and crypts of the intestines from wild-type and Klf4$^{+/-}$ mice (Fig. 3*A* and *B*, respectively). The staining of β-catenin is increased in the *Apc*$^{Min/+}$ mice and further increased in the *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice (Fig. 3*C* and *D*, respectively). Notably, the staining of β-catenin seems higher in the crypts than in the villi in both *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice with a shift in the staining pattern from membranous in the villi to cytoplasmic in the crypts. The increase in β-catenin in the *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice was reflected by a corresponding increase in the staining of cyclin D1, a transcriptional target of β-catenin (ref. 10; compare Fig. 4*C* and *D* with Fig. 4*A* and *B*). Importantly, although the staining of cyclin D1 was primarily present in the crypt cells of wild-type (Fig. 4*A*) and *Klf4*$^{+/-}$ mice (Fig. 4*B*), the staining patterns in the *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice seems to have extended into the villus cells (Fig. 4*C* and *D*, respectively).

Figure 5 shows immunostaining for Klf4, β-catenin, and cyclin D1 in the adenomas derived from *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. Klf4 levels were decreased in intestinal adenomas from both *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice (Fig. 5*A*) compared with their respective normal-appearing tissues (Fig. 2*C* and *D*, respectively). Reflecting this reduction, the levels of β-catenin (Fig. 5*B*) and cyclin D1 (Fig. 5*C*) were elevated in adenomas derived from both *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. Moreover, the pattern of β-catenin staining in tumor cells from *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice now becomes cytoplasmic/nuclear (Fig. 5B).

The levels of *Klf4* mRNA in *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mice decrease as tumor sizes increase

To verify the reduction in Klf4 levels observed by immunohistochemical staining of *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mouse intestines, we isolated tumors of different sizes (<1, 1–3, and >3 mm) from *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mouse intestines by microdissection. mRNA was purified from these tumors and semiquantitative RT-PCR was conducted using mouse Klf4-specific primers. As shown in Supplementary Fig. S3, the level of *Klf4* mRNA in the normal-appearing intestinal tissues of *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice was significantly lower than that of *Apc*$^{Min/+}$ mice (Supplementary Fig. S3B). The *Klf4* mRNA levels further decreased in adenomas from both *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mice as the size of the adenoma increases compared with their respective normal tissues. Lastly, there was a greater reduction in *Klf4* mRNA levels in adenomas 1 to 3 mm and >3 mm in size isolated from *Klf4*$^{+/-}$/Apc$^{Min/+}$ mice as compared with those from *Apc*$^{Min/+}$ mice. Supplementary Fig. S3*A* shows a representative RT-PCR result for Klf4 and β-actin. Supplementary Fig. S3*B* is a densito-metric analysis of the amplified Klf4 bands after normalization to β-actin.

Evidence for progressive LOH in adenomas from *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice

We further investigated whether there were any differences in the extent of loss of the wild-type *Apc* allele (LOH) between intestinal adenomas derived from *Apc*$^{Min/+}$ and *Klf4*$^{+/3}$/*Apc*$^{Min/+}$ mice. As seen in Fig. 6, there was a progressive increase in LOH of the *Apc* allele

that correlated with the increase in tumor size in both $Apc^{Min/+}$ and $Klf4^{+/3}/Apc^{Min/+}$ mice. The rates of LOH were similar in smaller tumors between the two groups. However, adenomas >3 mm in size showed a higher level of LOH in $Klf4^{+/3}/Apc^{Min/+}$ mice as compared with $Apc^{Min/+}$ mice. Figure 6A shows a representative PCR result for LOH of *Apc*. Figure 6*B* is a densitometric analysis of the relative band intensity ratio of the amplified wild-type and mutated *Apc* bands are shown in Fig. 6*A*.

Discussion

Previous studies indicate that KLF4 inhibits cell proliferation by activating crucial checkpoints in the cell cycle (18,20,21,38–40). These findings led to the suggestion that KLF4 may function as a tumor suppressor. Indeed, expression of *KLF4* was found to be significantly reduced in intestinal adenomas from $Apc^{Min/+}$ mice and patients with familial adenomatous polyposis when compared with their respective matched normal-appearing tissues (41). Similarly, the levels of *KLF4* mRNA were reduced in a panel of human colorectal cancers when compared with their matched control tissues (25). Moreover, there is evidence for LOH of the *KLF4* locus as well as promoter hypermethylation in a subset of colorectal cancer specimens (25). Conversely, reexpression of *KLF4* in a colorectal cancer cell line resulted in reduced tumorigenecity in a xenotransplantation model (42). Results from the current study provide the first *in vivo* evidence that haploinsufficiency of *Klf4* promotes intestinal tumorigenesis in the setting of the Apc^{Min} mutation, thus further solidifying the concept that KLF4 exerts a tumor-suppressive function in the gastrointestinal system.

The Apc^{Min} mouse, which carries a germ line nonsense mutation in the murine *Apc* gene (31), has served as a remarkably useful model for the study of intestinal tumorigenesis. Similar to the Apc^{Min} model, familial adenomatous polyposis is a human disorder in which germ line mutations of the *APC* gene lead to polyposis early in life (1,43). A notable difference between the human syndrome and the mouse model is the much higher prevalence of adenomas in the small intestine than in the colon in $Apc^{Min/+}$ mice (32), whereas it is the reverse in humans. Another unique nature of the Apc^{Min} mouse model is that all adenomas were established by 100 days of age or sooner; no new tumors were formed over the remaining life span of the animals (32), a finding confirmed by the current study. Our study also shows that $Klf4^{+/-}/Apc^{Min/+}$ mice developed significantly more intestinal adenomas than $Apc^{Min/+}$ mice in as early as 10 weeks of age (Supplementary Fig. S1). This increase was maintained later in life, at 16 and 20 weeks. However, the size distribution of the adenomas in the two groups of mice were comparable at all time points (Supplementary Fig. S2), suggesting that the rates of tumor growth in both $Apc^{Min/+}$ and $Klf4^{+/-}/Apc^{Min/+}$ mice were similar. In the colon, although there was no statistically significant difference in the number of tumors between the two groups, the $Klf4^{+/-}/Apc^{Min/+}$ mice tended to have more tumors than the $Apc^{Min/+}$ mice at 16 and 20 weeks (Supplementary Fig. S2). Analysis of intestinal tumor multiplicity in the $Apc^{Min/+}$ mice suggests that tumor number does not significantly increase over time (32,44). The fact that $Klf4^{+/-}/Apc^{Min/+}$ mice maintained a higher number of intestinal adenomas, but a similar size distribution when compared with $Apc^{Min/+}$ mice throughout the course of the study, suggests that haploinsufficiency of *Klf4* contributes to the initiation but not the subsequent progression of adenomas.

The results of this study show that the levels of Klf4 protein and mRNA were reduced in the normal-appearing small intestinal tissues from $Klf4^{+/-}/Apc^{Min/+}$ mice as compared with $Apc^{Min/+}$ mice (Fig. 2; Supplementary Fig. S3). In addition, the level of *Klf4* mRNA decreased progressively as the size of the adenomas increased in both $Apc^{Min/+}$ and $Klf4^{+/-}/Apc^{Min/+}$ mice (Supplementary Fig. S3). The reduced level of Klf4 in the normal-appearing small intestinal tissues of the $Klf4^{+/-}/Apc^{Min/+}$ mice, as compared with the $Apc^{Min/+}$ mice, may be a contributing factor to the early formation of intestinal adenomas in mice that are heterozygous

for the *Klf4* and Apc^{Min} alleles. Reduced expression or loss of *KLF4* has been reported in gastrointestinal tumors and other types of solid tumors (25–29,45,46). Our findings are not only consistent with these reports, but provide direct evidence to show an *in vivo* effect of *KLF4* reduction in promoting intestinal tumorigenesis.

The human *APC* gene was mutated in a majority of colorectal cancer (1). Loss of the normal *APC* allele occurred in up to 70% of colorectal adenomas (47–49) and 80% of colorectal cancer cell lines (2). These studies also indicate that one mechanism of adenoma formation is the inactivation of both alleles of the *APC* tumor suppressor gene. Consistent with this, quantitative site-specific PCR assays showed extensive loss of the wild-type *Apc* allele in adenomas derived from $Apc^{Min/+}$ mice (36). Using the same assay, we were able to show that LOH of the *Apc* allele occurred not only in the normal-appearing intestines of $Apc^{Min/+}$ mice but also in those of $Klf4^{+/-}$/ApcMin/+ mice (Fig. 6). Moreover, the LOH increased progressively as tumor size increased in both groups of mice (Fig. 6). It is of interest to note that in the largest adenomas (>3 mm), the LOH of *Apc* was greater in $Klf4^{+/-}/Apc^{Min/+}$ than in $Apc^{Min/+}$ mice (Fig. 6). This finding raises the possibility that reduced KLF4 levels may help facilitate LOH of *APC*.

The results of our study support a role for KLF4 in suppressing intestinal tumorigenesis by modulating β-catenin and cyclin D1 activity. KLF4 has been shown to regulate the Wnt/β-catenin pathway by inhibiting the expression of β-catenin (23). In addition, KLF4 binds the transcriptional activation domain of β-catenin and inhibits β-catenin–mediated transcription (24). Both of these findings were confirmed by the *in vivo* data in the current study showing the significant increase in the levels of β-catenin in the normal-appearing small intestinal tissues in the $Klf4^{+/-}/Apc^{Min/+}$ mice. Moreover, β-catenin staining in the normal-appearing intestine of $Klf4^{+/-}/Apc^{Min/+}$ mice changes from a membranous pattern in the villi to a cytoplasmic pattern in the crypts (Fig. 3). This is further changed to a cytoplasmic/nuclear distribution in the adenomas (Fig. 4). A previous study showed that both the cytoplasmic and cytoplasmic/nuclear patterns of β-catenin staining were associated with increased proliferative index (50). The different staining patterns of β-catenin in the $Klf4^{+/-}$/*ApcMin*/+ mice may thus represent different mechanisms of cyclin D1 induction. In cells with membranous β-catenin, Klf4 may directly repress cyclin D1 promoter; and the decrease in Klf4 releases cyclin D1 from repression. In contrast, in cells with cytoplasmic and cytoplasmic/nuclear distribution of β-catenin, cyclin D1 may be directly induced by β-catenin.

It has also been previously shown that the expression of *KLF4* was dependent on APC (22). Our observations that Klf4 mRNA and protein levels were decreased in the normal-appearing intestinal tissues of $Klf4^{+/-}/Apc^{Min/+}$ mice compared with wild-type, $Klf4^{+/-}$, and $Apc^{Min/+}$ mice also supports the notion that Klf4 is regulated by Apc. Moreover, the cellular localization of KLF4 and APC in the intestinal epithelium was similar to each other (2,17). Taken together, the findings of the previous studies and the current study suggest a synergistic mechanism by which KLF4 and APC may function to regulate intestinal epithelial cell proliferation.

The increase in adenoma formation in $Klf4^{+/-}/Apc^{Min/+}$ mice may thus be viewed in the context of crosstalk between KLF4 and the Wnt/β-catenin pathway. The decrease in the wild-type Apc level by LOH in the $Klf4^{+/-}/Apc^{Min/+}$ mice leads to a decrease in Klf4 to a level below that of $Apc^{Min/+}$ mice. This decrease is accentuated by having only one wild-type *Klf4* allele in the $Klf4^{+/-}/Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice. As KLF4 has been shown to inhibit β-catenin–mediated gene expression (23,24), the simultaneous reduction in Klf4 and Apc could exacerbate the dysregulation of β-catenin in the intestinal epithelium, leading to an increased rate of proliferation, a greater chance for early LOH, and earlier initiation of tumorigenesis. We also speculate that mutations in the *APC* gene, along with haploinsufficiency of the *KLF4* gene in the intestine, would result in the constitutive activation of other β-catenin target genes that promote cell proliferation, and eventually, transformation.

In conclusion, we show for the first time that reduced *Klf4* expression *in vivo* plays a role in the onset of intestinal adenoma formation in $Apc^{Min/+}$ mice. Our findings suggest that genetic events which inactivate *KLF4* may promote tumorigenesis in human colorectal cancers, particularly in those with mutations in the *APC* gene. Examination of human colorectal cancer tissues for evidence of *KLF4* mutations may further help to substantiate the role of KLF4 in colorectal carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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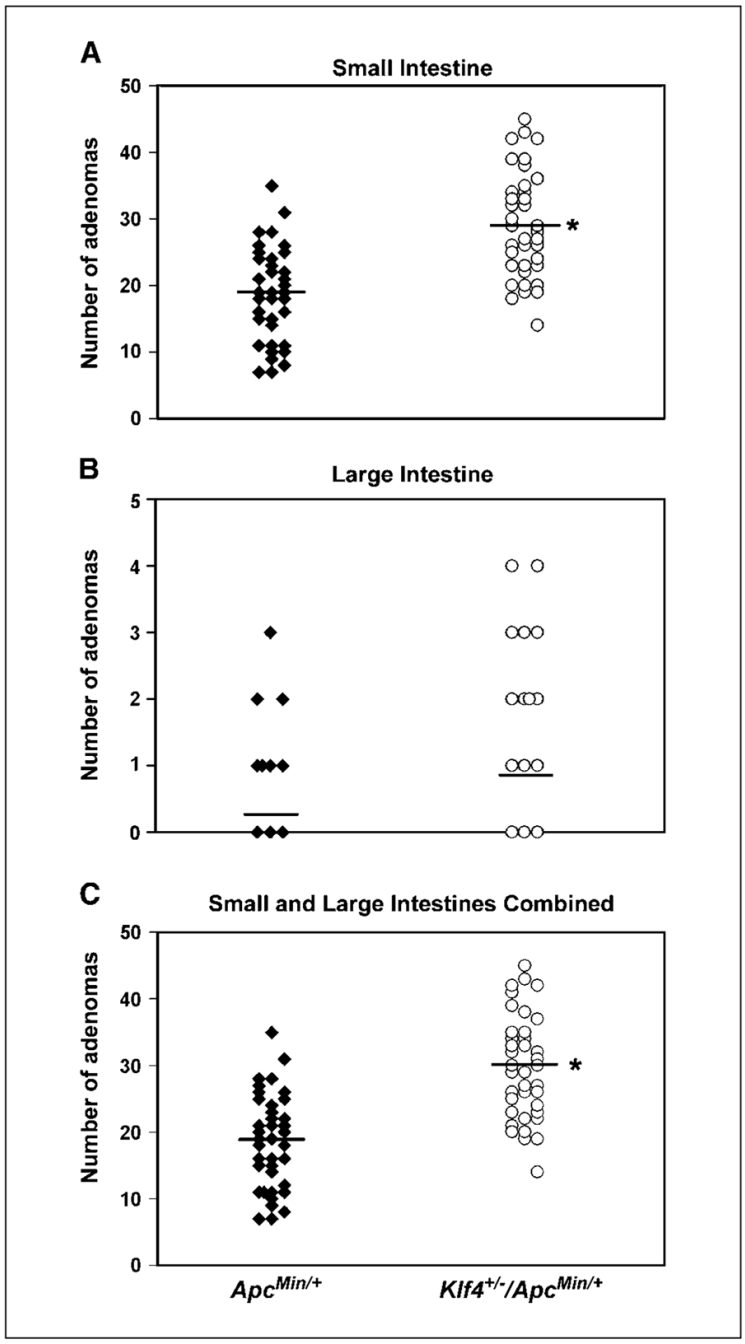

Figure 1.

Klf4$^{+/-}$/*Apc*$^{Min/+}$ mice develop more intestinal adenomas than *Apc*$^{Min/+}$ mice. *Klf4*$^{+/-}$ mice were bred with *Apc*$^{Min/+}$ mice. Mice were sacrificed at 10, 16, and 20 weeks and the number of adenomas of the three age groups combined was counted and compared between the two genotypes. *A*, comparison of the number of adenomas per mouse in the small intestine between *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. *B*, comparison of the number of adenomas per mouse in the large intestine between *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. *C*, comparison of the total number of adenomas per mouse in both the small and large intestines between *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. *Points*, number of adenomas per mouse; *, $P < 0.0001$ by paired two-tailed *t* test between the two genotypes.

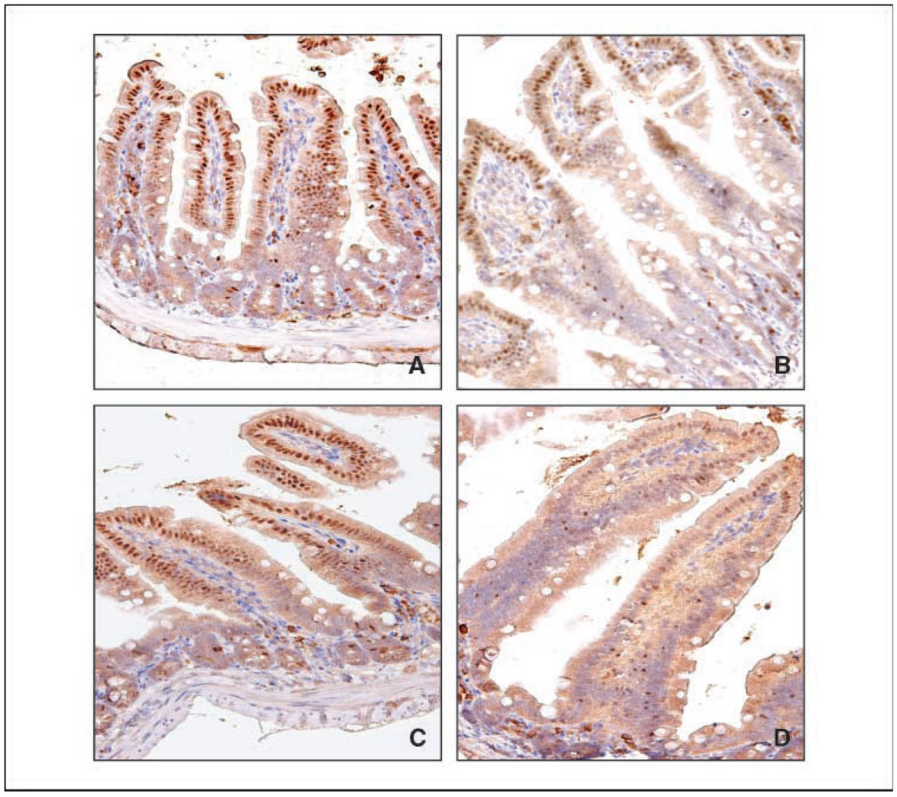

Figure 2.
Immunohistochemical localization of Klf4 in normal-appearing small intestinal tissues.
Normal-appearing small intestinal tissues obtained from age-matched wild-type (*A*), *Klf4*$^{+/-}$ (*B*), *Apc*$^{Min/+}$ (*C*), and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ (*D*) mice were stained with a Klf4 antibody.

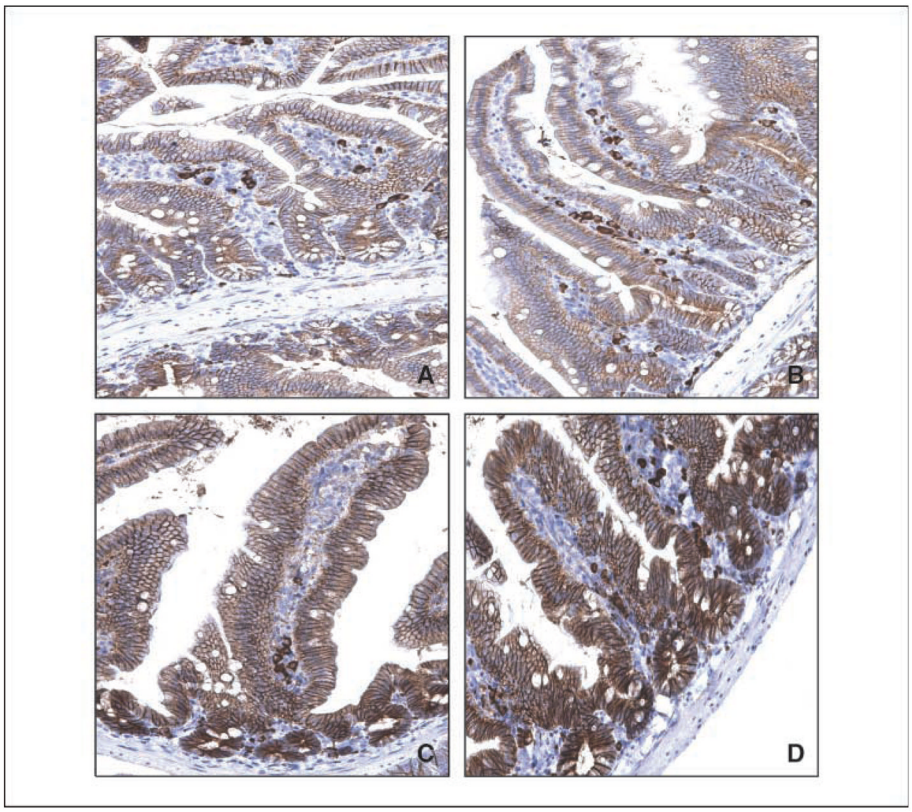

Figure 3.
Immunostaining of β-catenin in normal-appearing small intestinal tissues. Normal-appearing small intestinal tissues obtained from age-matched wild-type (*A*), *Klf4*$^{+/-}$ (*B*), *Apc*$^{Min/+}$ (*C*), and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ (*D*) mice were stained with a β-catenin antibody.

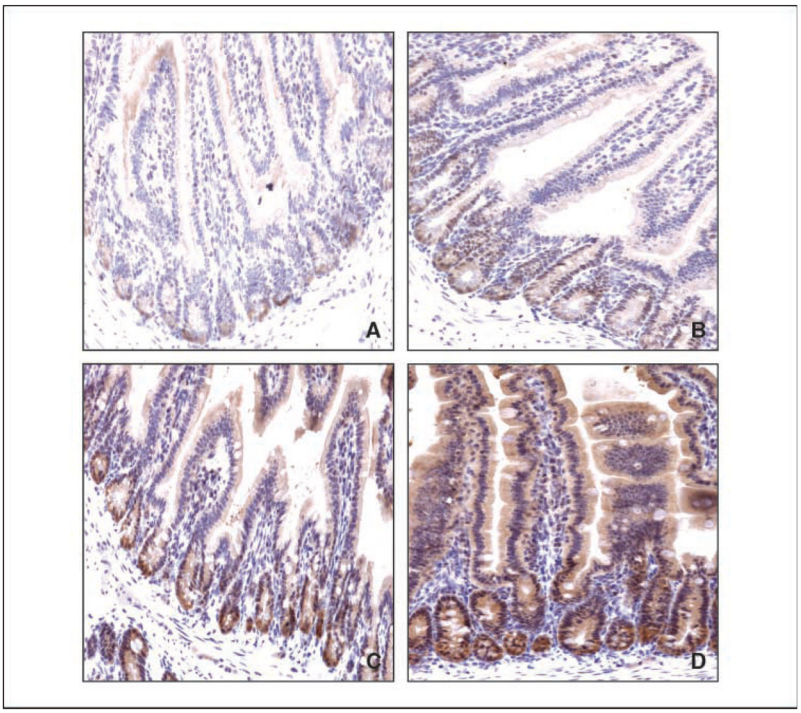

Figure 4.
Immunostaining of cyclin D1 in normal-appearing small intestinal tissues. Normal-appearing small intestinal tissues obtained from age-matched wild-type (*A*), *Klf4*$^{+/-}$ (*B*), *Apc*$^{Min/+}$ (*C*), and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ (*D*) mice were stained with a cyclin D1 antibody.

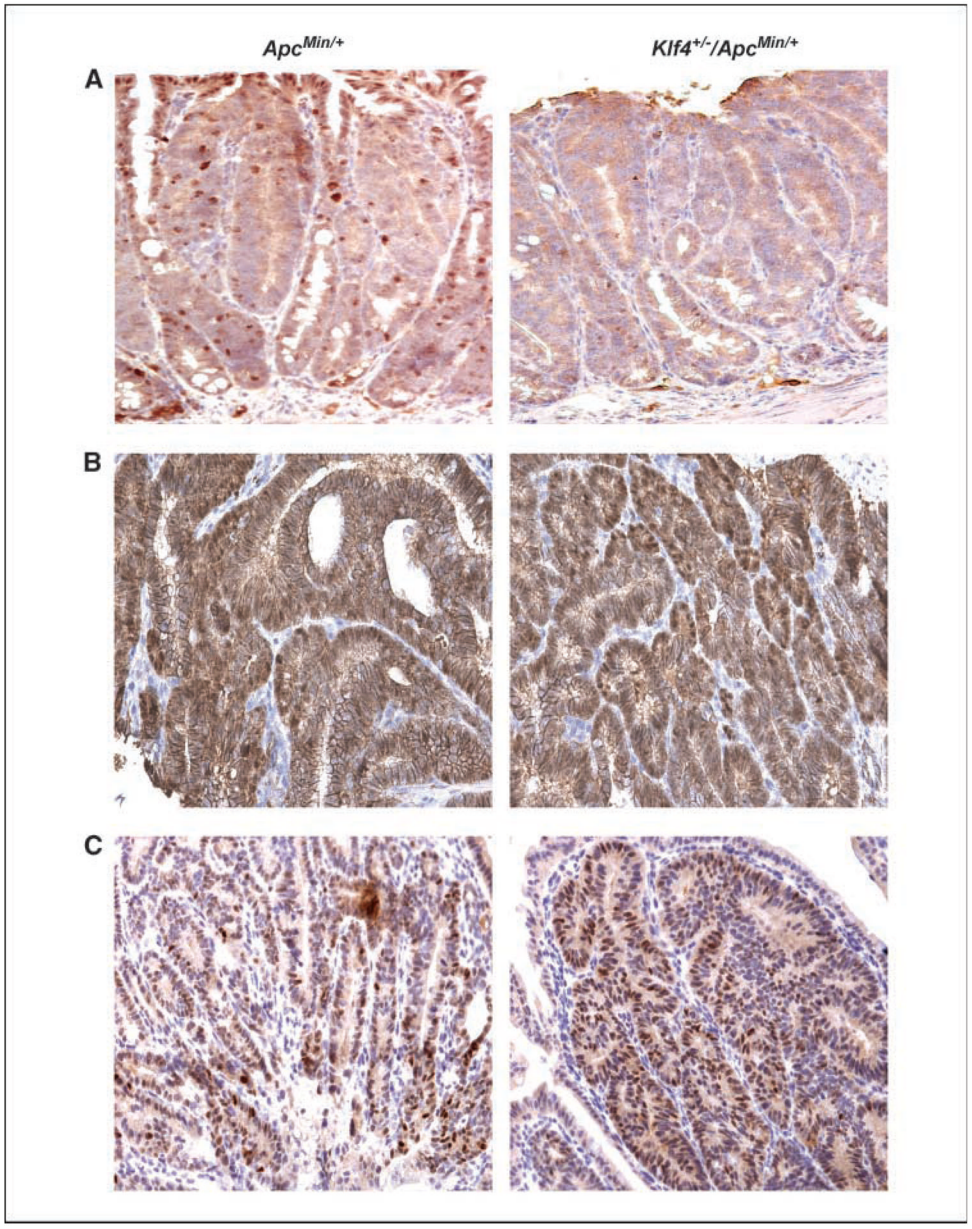

Figure 5.
Immunohistochemical characterization of small intestinal adenomas from *Apc*$^{Min/+}$ and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice. Adenomas from small intestines of *Apc*$^{Min/+}$ mice and *Klf4*$^{+/-}$/*Apc*$^{Min/+}$ mice were stained with antibodies against Klf4 (*A*), β-catenin (*B*), and cyclin D1 (*C*).

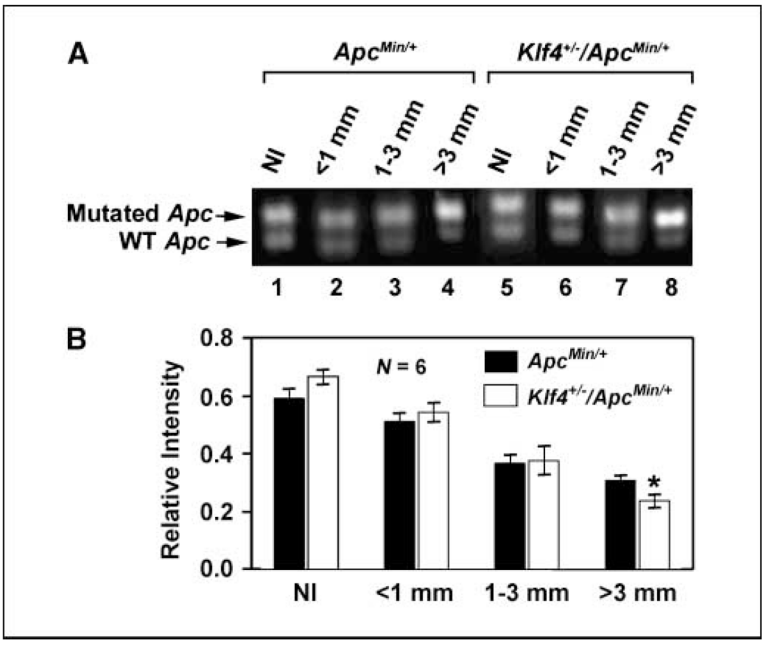

Figure 6.

LOH of *Apc* increases as adenoma size increases. *A*, adenomas of various sizes and neighboring normal tissue were microdissected out from intestines of $Apc^{Min/+}$ and $Klf4^{+/-}/Apc^{Min/+}$ mice. DNA was extracted and the extent of LOH was analyzed by semiquantitative PCR. Adenomas sizes were <1 mm (*lanes 2* and 6), 1 to 3 mm (*lanes 3* and 7), and >3 mm (*lanes 4* and 8). Normal-appearing intestinal tissues (*Nl*) were also included in the analysis (*lanes 1* and 5). *B*, densitometric analysis of the amplified *Apc* bands in (*A*).