Haploinsufﬁciency of Krüppel-Like Factor 5 Rescues the Tumor-Initiating Effect of the ApcMin Mutation in the Intestine

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Abstract

Inactivation of the tumor suppressor adenomatous polyposis coli, with the resultant activation of β-catenin, is the initiating event in the development of a majority of colorectal cancers. Krüppel-like factor 5 (KLF5), a proliferative transcription factor, is highly expressed in the proliferating intestinal crypt epithelial cells. To determine whether KLF5 contributes to intestinal adenoma formation, we examined tumor burdens in ApcMin/+ mice and ApcMin/+/Klf5−/− mice. Compared with ApcMin/+ mice, ApcMin/+/Klf5−/− mice had a 96% reduction in the number of intestinal adenomas. Reduced tumorigenicity in the ApcMin/+/Klf5−/− mice correlated with reduced levels and nuclear localization of β-catenin as well as reduced expression of two β-catenin targets, cyclin D1 and c-Myc. In vitro studies revealed a physical interaction between KLF5 and β-catenin that enhanced the nuclear localization and transcriptional activity of β-catenin. Thus, KLF5 is necessary for the tumor-initiating activity of β-catenin during intestinal adenoma formation in ApcMin/+ mice, and reduced expression of KLF5 offsets the tumor-initiating activity of the ApcMin mutation by reducing the nuclear localization and activity of β-catenin. [Cancer Res 2009;69(10):4125–33]

Introduction

Homeostasis of intestinal epithelial cells requires stringent regulation of proliferation, differentiation, migration, and apoptosis to ensure continued tissue renewal. Intestinal tumors occur when these processes become dysregulated, resulting in hyperproliferation at the cost of differentiation (1). A gene proved critical in the initiation of colorectal cancer (CRC) is adenomatous polyposis coli (APC). Germline loss-of-function mutations in APC were identiﬁed as the genetic basis for familial adenomatous polyposis (2). Subsequently, APC has been shown to be inactivated in >80% of sporadic CRC (2). The association between loss of APC function and CRC formation has been conﬁrmed in experimental mouse models, with the most widely used model being the ApcMin/+ mouse (Min for multiple intestinal neoplasia; refs. 3, 4). Mice heterozygous for the ApcMin mutation exhibit the intestinal manifestations observed in familial adenomatous polyposis patients—they develop numerous adenomas but predominantly in the small intestine rather than the colon. One of the primary contributions of APC to intestinal tumorigenesis is its regulation of β-catenin (5). β-catenin is the key mediator of Wnt signaling, which is critical for maintaining proliferation of the intestinal crypt epithelium (6). In the absence of Wnt signaling, APC allows for destabilization of β-catenin through phosphorylation events (7), and the loss of APC results in accumulation and constitutive activation of β-catenin (8). Mutations that affect stabilization of β-catenin have been identiﬁed in CRC cell lines and tumors (9); thus, it seems that any mutation event that constitutively activates Wnt signaling via β-catenin may lead to tumor initiation in the gut (10).

Krüppel-like factor 5 (KLF5) is a zinc-finger transcription factor that is highly expressed in proliferating epithelial cells of the intestinal crypts. KLF5 belongs to the KLF family of transcription factors that regulate diverse cellular functions, including proliferation, differentiation, apoptosis, and embryogenesis (11, 12). KLF5 has been shown to drive proliferation in cultured cells and in mouse intestinal tissues (13–15). In addition, KLF5 has been reported to be a target of Wnt signaling (16). Because KLF5 is growth promoting and has been implicated as an oncogene in various types of solid tumors (17–20), we sought to determine whether KLF5 contributes to the tumorigenic effects of the ApcMin mutation by investigating mice that are heterozygous for mutations in each gene.

Materials and Methods

Mice. Founder C57BL/6J male mice heterozygous for the ApcMin allele (ApcMin/+ ) were purchased from the Jackson Laboratory. Founder C57BL/6J mice heterozygous for the Klf5 alleles (Klf5−/+ ) were previously developed (15). ApcMin−/+ males were mated with Klf5−/+ females to obtain mice wild-type (WT) for both alleles, mice heterozygous for either the Klf5−/− allele (Klf5−/+ ) or the ApcMin−/− allele (ApcMin−/+ ), or mice heterozygous for both the ApcMin−/− and Klf5−/− alleles (ApcMin−/+ , Klf5−/−).

Tumor assessment. Mice were sacriﬁced at age 16 to 18 wk or at age 28 wk by CO2 asphyxiation. The entire small intestine was dissected longitudinally. Intestinal tissues were examined under a dissecting microscope in a blinded fashion for the presence of adenomas. Intestinal adenomas were counted and grouped by size as follows: <1, 1 to 2, 2 to 3, and ≥3 mm.

Cell culture. Cell culture medium and fetal bovine serum (FBS) were purchased from Invitrogen. COS-1 cells were acquired from American Type Culture Collection and maintained in DMEM and 10% FBS with 100 U/mL penicillin and 100 μg/mL streptomycin.

Plasmid constructs and antibodies. Information regarding plasmids and antibodies used can be found in Supplementary Materials and Methods.

Immunohistochemistry. Immunohistochemical staining using a chromogenic substrate was performed as described previously (19). For immunofluorescence staining, tissue sections were deparaffinized, rehydrated, subjected to antigen retrieval using Rodent Decloaker (Biocare Medical), and incubated with primary antibody overnight at 4°C. Slides were then incubated with Alexa Fluor secondary antibodies (Invitrogen).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and counterstained with Hoechst 33258 to visualize nuclei. Fluorescent staining was viewed with a Zeiss Axioskop 2 plus microscope or a Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging, Inc.). Images were analyzed for intensities of nuclear KLF5 staining using Metamorph Imaging software (Molecular Devices).

**Immunocytochemistry.** Cells were grown in 4-well chamber slides cells, fixed in 4% ultrapure formaldehyde (Polysciences), and blocked in 3% bovine serum. Cells were stained overnight at 4°C with primary antibodies followed by staining with Alexa Fluor secondary antibodies (Invitrogen).

**Reverse transcription-quantitative PCR.** Total RNA was isolated from mouse distal jejunum, which was inverted and scraped to isolate mucosal tissue. First-strand cDNA was prepared using the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen). Primers used are listed in Supplementary Materials and Methods. PCR reactions were amplified and detected with the iCycler iQ real-time PCR Detection System (Bio-Rad Laboratories), using iQ SYBR Green Supermix (Bio-Rad Laboratories). Relative fold changes were calculated based on the comparative CT (ΔΔCt) method (21).

**Luciferase reporter assays.** COS-1 cells at 80% to 90% confluence were transfected with pCI-neo, pCI-neo-β-catenin-S33Y-FLAG, pCI-neo-KLF5, or in combination using Lipofectamine 2000 (Invitrogen). Cells were additionally transfected with firefly luciferase reporter constructs: pGL2, pTOPFLASH or pA3-cyclin D1, and a Renilla luciferase reporter to normalize for transfection efficiencies. Luciferase activities were determined at 24 h posttransfection using the Dual Luciferase Assay System kit (Promega).

For small interfering RNA (siRNA) experiments, validated Stealth Select siRNAs for mouse KLF5 (1126 or 1127) were obtained from Invitrogen. HCT 116 or DLD-1 cells were transfected using siRNAMax (Invitrogen) with either nonspecific siRNA or siRNAs specific for KLF5, 1126, or 1127 (Invitrogen) and the firefly luciferase reporter, pTOPFLASH. Cells were assayed for luciferase activity 48 h after transfection.

**Western blot analysis.** Equal amounts of protein lysates were subjected to standard Western blotting procedures. Lysates from COS-1 cells were prepared using 1 X Cell Extraction Buffer (Biosource/Invitrogen). Whole cell lysates from mouse jejunum were prepared from 1-cm pieces of small intestine isolated 10 cm proximal to the cecum using Cell Disruption Buffer (Applied Biosystems/Ambion). Before lysates preparation, tissues were screened for absence of polyps.

**Northern blot analysis.** Total RNA was prepared from cells using Trizol reagent (Invitrogen), per manufacturer’s instructions. Northern blots were carried out as previously described (13).

**Coimmunoprecipitation.** In COS-1 cells, nuclear extracts were prepared using the Nuclear Complex Co-IP kit (Active Motif) and immunoprecipitations were performed according to the manufacturer’s protocol. For immunoprecipitation from DLD-1 lysates, cells were pretreated with 2 mmol/L dithiobis[succinimidylpropionate] to cross-link interacting proteins (22) before lystate preparation.

**Subcellular fractionation.** Fractionation of crude cell lysates was carried out as described previously (23).

**Statistical analysis.** Numerical values in text and figures are expressed as the mean ± SE. Data were evaluated for normality and homogeneity of variance by Kolmogorov-Smirnov and Levene’s tests, respectively. When assumptions of normality and equal variance were met, multigroup data were analyzed statistically by ANOVA with multiple pair-wise comparisons between means performed using Tukey’s tests. Data failing

Figure 1. Characterization of tumor incidence and tumor burden in the ApcMin/+ and ApcMin+Klf5+/- mice. Mice at age 16 to 18 wk or age 28 wk were examined macroscopically for adenomas in the small intestine. A, graph of percentage of mice with small intestinal adenomas at age 16 to 18 wk (n = 22). B, scatter plot of the number of adenomas per mouse in the small intestine; ▲, ApcMin+; ◆, ApcMin+Klf5−/−. Black horizontal lines, average number of adenomas for each genotype (*, P < 0.001; 16–18 wk, n = 22; 28 wk, n = 11; Wilcoxon-Mann-Whitney test). C, graph of total numbers of adenomas from mice screened in B, separated by size of adenomas.
to meet the assumptions required for analysis by parametric statistical tests were evaluated using distribution-free methods. Differences between means were considered to be statistically significant when \( P \) value are <0.05. Probability values of greater than \( P \) values of 0.001 are reported as exact \( P \) values.

Results

Haploinsufficiency of \textit{Klf5} inhibits intestinal adenoma formation in \textit{Apc\textsuperscript{Min/\textast}} mice. To investigate the role of KLF5 in the well-established \textit{Apc\textsuperscript{Min/\textast}} mouse model of intestinal tumorigenesis, we crossed \textit{Apc\textsuperscript{Min/\textast}} mice with \textit{Klf5\textsuperscript{+/\textast}} mice, and compared adenoma formation between \textit{Apc\textsuperscript{Min/\textast}} mice and \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} double heterozygous mice. Mice were screened at age 16 to 18 weeks when intestinal adenomas were clearly visible or at age 28 weeks, nearing the end of the approximate life span of the \textit{Apc\textsuperscript{Min/\textast}} mice. As seen in Fig. 1A, at age 16 to 18 weeks, only 41\% of \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice developed adenomas in the small intestine (\( n = 22 \)), compared with 100\% of \textit{Apc\textsuperscript{Min/\textast}} mice (\( n = 22 \)). Furthermore, the number of adenomas per mouse was reduced by 96\% in \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice, which developed an average of 1.5 ± 0.8 adenomas per mouse, compared with 37.7 ± 4.5 in \textit{Apc\textsuperscript{Min/\textast}} mice (Fig. 1B). Average numbers of colonic tumors in the \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice were not significantly different compared with the \textit{Apc\textsuperscript{Min/\textast}} mice (0.36 ± 0.14 versus 0.50 ± 0.16 adenomas per mouse, respectively). The reduction in tumor incidence in the double heterozygous \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice compared with the \textit{Apc\textsuperscript{Min/\textast}} mice was maintained as the mice aged. At 28 weeks, \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice had an average of 0.73 ± 0.38 small intestinal adenomas per mouse (\( n = 11 \)) compared with 37.3 ± 1.8 in the \textit{Apc\textsuperscript{Min/\textast}} mice (\( n = 11 \); Fig. 1B). These results indicate that tumor formation was not merely delayed in the \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice but that haploinsufficiency of \textit{Klf5} prevents tumor initiation.

In addition to tumor numbers, tumor sizes were compared between the two genotypes. As seen in Fig. 1C, at 16 to 18 weeks, the mode for adenoma size was <1 mm for both \textit{Apc\textsuperscript{Min/\textast}} and \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice. By 28 weeks, the mode for \textit{Apc\textsuperscript{Min/\textast}} mice was increased to 1 to 2 mm, indicating growth of the tumors, yet the mode for \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice remained at <1 mm. These data suggest that \textit{Klf5} haploinsufficiency may affect tumor growth as well as tumor initiation.

\textbf{KLF5 and β-catenin levels are reduced in the \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}}} mice. To confirm that KLF5 protein was reduced in the small intestine of \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice, KLF5 levels were examined by immunofluorescence (Fig. 2A). KLF5 was restricted to the nuclei of epithelial cells in proliferating crypts of the small intestine. In \textit{Klf5\textsuperscript{+/\textast}} mice, KLF5 levels were significantly reduced to approximately half the levels in WT mice (\( P = 0.015 \)) as determined by

![Figure 2](https://www.aacrjournals.org/cancerres/69/10/4129/fig2.png)

**Figure 2.** Levels and localization of KLF5 and β-catenin in the small intestine of WT, \textit{Klf5\textsuperscript{+/\textast}}, \textit{Apc\textsuperscript{Min/\textast}}, and \textit{Apc\textsuperscript{Min/\textast}/Klf5\textsuperscript{+/\textast}} mice. A, KLF5 levels in the small intestine of 16-wk-old mice were determined by immunofluorescence staining of KLF5 in normal-appearing tissues. B, quantification of KLF5 immunofluorescence intensities. Columns, mean value of four mice per group; bars, SE. Average intensities were determined from at least 50 cells per mouse. Results are shown as KLF5 fluorescence intensity per cell relative to intensities in WT mice (*, \( P = 0.015 \); **, \( P = 0.016 \), Tukey’s test). C, immunohistochemical staining of β-catenin in small intestinal tissues from 16-wk-old mice. Bottom row, enlargements of the corresponding panels above. Arrows, nuclear staining. D, Western blot analysis of protein lysates from normal-appearing jejunum of 16-wk-old mice.
quantification of immunofluorescence intensities, correlating with haploinsufficiency of Klf5 (Fig. 2B). Similarly, KLF5 levels in Apc<sup>Min−/−</sup> mice were reduced to approximately half those in Apc<sup>Min−/−</sup> mice (P = 0.016). In the Apc<sup>Min−/−</sup> mice, KLF5 showed intense staining in the transit-amplifying cells in the crypts of normal-appearing tissue (Fig. 2A), but intensities were not significantly increased compared with WT levels.

Because β-catenin is the key effector of APC mutations in intestinal cancers, we examined whether β-catenin levels and/or localization were influenced by Klf5 haploinsufficiency. Immuno-histochemical staining of small intestinal tissues showed strong nuclear and cytoplasmic staining of β-catenin at the base of the crypts in the Apc<sup>Min−/−</sup> mice, whereas nuclear staining was less pronounced in the WT, Klf5<sup>−/−</sup>, and Apc<sup>Min−/−</sup>/Klf5<sup>−/−</sup> mice (Fig. 2C). By Western blot analysis, β-catenin levels were higher in intestinal tissues from Apc<sup>Min−/−</sup> mice compared with WT or Klf5<sup>−/−</sup> mice (Fig. 2D), confirming previous findings that mutations in the Apc gene increase the stability and accumulation of β-catenin (7). Importantly, this effect was abrogated in Apc<sup>Min−/−</sup>/Klf5<sup>−/−</sup> mice, where β-catenin levels were comparable with those in the WT and Klf5<sup>−/−</sup> mice (Fig. 2C and D). Similarly, when comparing levels of β-catenin in nuclear extracts from intestinal tissues by Western blot analysis, β-catenin was reduced in Apc<sup>Min−/−</sup>/Klf5<sup>−/−</sup> mice compared with Apc<sup>Min−/−</sup> mice (Supplementary Fig. S1), agreeing with results in Fig. 2C indicating reduced nuclear localization of β-catenin in the crypts of Apc<sup>Min−/−</sup>/Klf5<sup>−/−</sup> mice. Thus, haploinsufficiency of klf5 may "offset" the effect of the Apc<sup>Min</sup> mutation on the β-catenin protein.

**KLF5 interacts with β-catenin.** To investigate the mechanisms by which KLF5 affects β-catenin protein levels and localization, KLF5 and β-catenin were coexpressed in COS-1 cells. Protein levels of β-catenin were higher in cells expressing both ectopic β-catenin and KLF5 compared with cells expressing β-catenin alone (Supplementary Fig. S2A). This affect was not due to higher transcript levels, as both endogenous and exogenous β-catenin mRNA levels were unchanged in the presence of ectopic KLF5 (Supplementary Fig. S2B). Another potential mechanism by which KLF5 could affect β-catenin protein levels is through physical interaction and stabilization. Therefore, immunoprecipitation experiments were conducted in cells expressing KLF5-HA and β-catenin-FLAG to determine whether the two proteins interact. β-Catenin-FLAG coprecipitated with KLF5-HA, and likewise, KLF5-HA immunoprecipitated with β-catenin-FLAG (Fig. 3A). Neither protein was precipitated with the IgG antibody control. To verify interaction of endogenous β-catenin and KLF5 proteins, KLF5 was immunoprecipitated from DLD-1 human colorectal cancer cells.
colon cancer cell lysates. β-catenin coprecipitated with KLF5 but was not precipitated in the preimmune serum negative control (Fig. 3B).

To further evaluate the interaction between β-catenin and KLF5, the two proteins were examined for colocalization by immunofluorescence staining. Expression of KLF5-HA alone showed intense, diffuse staining of KLF5 throughout the nucleus, whereas β-catenin-FLAG-alone transfected cells showed both cytoplasmic and nuclear staining of β-catenin (Fig. 3C). When KLF5 and β-catenin were coexpressed, KLF5 was redistributed within the nucleus in a pattern that corresponded to β-catenin localization. In addition, there was an increase in nuclear staining for β-catenin in the cotransfected cell (Fig. 3C). Redistribution of KLF5 was also seen for endogenous KLF5 in cells transfected with β-catenin-FLAG alone. Although KLF5 exhibited diffuse nuclear staining in cells lacking ectopic β-catenin (Fig. 3D, orange arrow), KLF5 was redirected to distinct intranuclear structures in cells expressing β-catenin–FLAG (Fig. 3D, white arrow). This altered localization of KLF5 correlated with the expression pattern of ectopic β-catenin. KLF5 facilitates nuclear localization of β-catenin. To investigate in-depth the ability of KLF5 to promote nuclear localization of β-catenin, cells transfected with either β-catenin alone or β-catenin and KLF5 were analyzed for subcellular distribution of β-catenin by immunofluorescence and protein fractionation. In examining a field of cells stained for β-catenin and KLF5, β-catenin exhibited more intense nuclear localization in the presence of ectopic KLF5 than when expressed alone (Fig. 4A). β-catenin localization was also analyzed by subcellular fractionation, with protein lysates separated into nuclear, cytoplasmic, and membrane fractions (Fig. 4B). Amounts of β-catenin localized to the membrane fraction were approximately equal in the two sets of transfections. However, a significant proportion of β-catenin was redistributed from the cytoplasm to the nucleus upon coexpression with KLF5. Thus, cells with ectopic expression of both β-catenin and KLF5 had higher levels of nuclear β-catenin.

To determine whether loss of KLF5 would have a reciprocal effect on β-catenin localization, HCT116 human colon cancer cells were transfected with KLF5-specific siRNA and examined for subcellular localization of β-catenin by immunofluorescence (Fig. 4C). In cells transfected with nonspecific siRNA, β-catenin exhibited visible nuclear staining. However, this localization was in large part disrupted in cells transfected with two different KLF5-specific siRNAs, where β-catenin was localized predominantly to the cell membranes and cytoplasm. A similar effect was seen in another colon cancer cell line, DLD-1 (Fig. 4D). Taken together, these data indicate that KLF5 facilitates localization of β-catenin to the nucleus. KLF5 modulates transcriptional activity of β-catenin. Because increased expression of KLF5 enhances nuclear localization of β-catenin, KLF5 may also promote the transcriptional

![Figure 4](image-url)

Figure 4. Alterations in KLF5 expression affect β-catenin nuclear localization. A, COS-1 cells were transfected as indicated and examined by immunofluorescence for nuclear β-catenin (red); KLF5 (green). Cells were counterstained with Hoechst 33,352 (blue) to visualize nuclei. B, COS-1 cells were transfected with either β-catenin or β-catenin and KLF5 and fractionated into nuclear, cytosolic, and membranous components. Lysates were blotted for histone H1 (nuclear), β-actin (cytosolic), and Na+/K+ ATPase (membranous) to confirm fractionation of compartments. C, HCT116 colon cancer cells were transfected with nonspecific siRNA or two distinct KLF5 siRNAs (1126 or 1127). Seventy-two hours after transfection, cells were stained by immunofluorescence for expression and localization of KLF5 (green) and β-catenin (red). D, repeat of experiment in C using DLD-1 colon cancer cells.
Expression constructs as indicated and measured for activity of TOPFLASH and Ccnd1. Coexpression of β-catenin and KLF5 resulted in a 7.6-fold increase in luciferase activity compared with cells transfected with empty vector. In cells transfected with KLF5 alone, luciferase activity was increased by 3.2-fold over vector control (P < 0.001). Coexpression of β-catenin and KLF5 resulted in a 7.6-fold increase in activity, which was significantly greater than with β-catenin alone (P < 0.001) or KLF5 alone (P < 0.001). Moreover, when β-catenin and KLF5 were coexpressed, they interacted to produce a synergistic effect on Ccnd1 promoter luciferase activity (P < 0.001). Thus, coexpression of KLF5 with β-catenin enhances the transcription of β-catenin targets.

To determine if loss of KLF5 would have a corresponding negative effect on TCF/β-catenin transcriptional activity, HCT 116 and DLD-1 cells were transfected with a nonspecific siRNA or two different siRNAs specific for KLF5 and the TOPFLASH luciferase reporter (Fig. 5B). Transfection with either KLF5-specific siRNA dramatically reduced TOPFLASH activity in both cell lines compared with transfection with nonspecific siRNA (P < 0.001), correlating with loss of β-catenin from the nucleus in Fig. 4C and D.

Expression of β-catenin targets is reduced in ApcMin+/+/Klf5−/− mice. To address whether reduced KLF5 expression affects transcriptional activity of β-catenin in vivo, RNA was isolated from the jejunum of WT, Klf5−/−, ApcMin+/+−, and ApcMin+/+−/Klf5−/− mice, and expression of two transcriptional targets of β-catenin that promote proliferation were examined by reverse transcriptase-quantitative PCR. Transcript levels of Ccnd1, the gene encoding cyclin D1, were increased 1.9-fold in the ApcMin+/+− Compared with WT (P = 0.009; Fig. 5C). In contrast, levels of Ccnd1 in the ApcMin+/+−/Klf5−/− mice were similar to WT controls (Fig. 5C). As a transcriptional target of KLF5 (24, 25), Ccnd1 transcript levels were significantly reduced in the Klf5−/− mice compared with WT (0.41-fold; P < 0.001), suggesting that this target gene is strongly influenced by KLF5 levels. In examining c-Myc transcript levels, c-Myc was increased 1.4-fold (P = 0.002) in ApcMin+/+− mice compared with WT mice. This change was offset in the ApcMin+/+−/Klf5−/− mice, with levels comparable with WT (P < 0.001). No significant change in c-Myc levels was observed in the Klf5−/− mice. Target gene expression was further confirmed by immunohistochemical staining of the protein products (Fig. 5D). In concordance with mRNA levels, cyclin D1 staining was more intense in small intestinal tissues of ApcMin+/+− mice compared with WT mice (Fig. 5D), whereas no induction of cyclin D1 was observed in the ApcMin+/+−/Klf5−/− mice. Likewise, c-Myc expression was increased in the ApcMin+/+− mice, but this effect was abrogated in the ApcMin+/+−/Klf5−/− animals, which exhibited c-Myc levels similar to WT (Fig. 5D). Therefore, KLF5 is necessary for the downstream activation of β-catenin target genes in the ApcMin+/+−/Klf5−/− mouse.

KL5 and β-catenin colocalize in intestinal adenomas. Because KLF5 seems to contribute to the tumorigenic effects of β-catenin in the ApcMin+/+− setting, immunohistochemical analyses were performed to determine the distribution of KLF5 and β-catenin in intestinal tumors derived from ApcMin+/+− mice. As seen in Fig. 6A, there was intense nuclear staining of KLF5 in epithelial cells in the dysplastic epithelium adjacent to the adenomas as well as cells throughout the adenomas. β-catenin activity of β-catenin. To test this possibility, KL5 and β-catenin were coexpressed in COS-1 cells with pTOPFLASH, a luciferase reporter construct that measures TCF/β-catenin activity (8). Compared with empty vector, cells transfected with β-catenin alone showed a 3.6-fold increase in TOPFLASH luciferase activity (P = 0.003), whereas ectopic expression of KL5 alone had no effect (Fig. 5A). Cells transfected with both β-catenin and KL5 showed a 6.2-fold increase in luciferase activity, an effect that was significantly greater than with β-catenin alone (P = 0.006). As a transcriptional target for both KL5 and β-catenin (24, 25), Ccnd1 promoter activity was also measured (Fig. 5A). Transfection with β-catenin alone did not significantly increase Ccnd1 luciferase activity compared with cells transfected with empty vector. In cells transfected with KL5 alone, luciferase activity was increased by 3.2-fold over vector control (P < 0.001). Coexpression of β-catenin and KL5 resulted in a 7.6-fold increase in activity, which was significantly greater than with β-catenin alone (P < 0.001) or KL5 alone (P < 0.001). Moreover, when β-catenin and KL5 were coexpressed, they interacted to produce a synergistic effect on Ccnd1 promoter luciferase activity (P < 0.001). Thus, coexpression of KL5 with β-catenin enhances the transcription of β-catenin targets.

Figure 5. Changes in KLF5 expression affect activation of β-catenin transcriptional targets. A, luciferase assays in COS-1 cells transfected with expression constructs as indicated and measured for activity of TOPFLASH and Ccnd1 promoters. Rel. luciferase activity: relative luciferase activity. Coexpression of β-catenin and KL5 produced significant synergistic effects for Ccnd1 (F [1,20] = 74.0; P < 0.001). Comparison of means (Tukey’s procedure): for TOPFLASH: 1*, P = 0.003; 1**, P = 0.006; n = 3 per group; for Ccnd1: 1*, P < 0.001; n = 6 per group. B, luciferase assays in HCT116 and DLD-1 colon cancer cells transfected with indicated siRNAs. 1*, P < 0.001; n = 3 per group. C, quantitative RT-PCR analysis of Ccnd1 and c-Myc mRNA levels using RNA from distal jejunum. Results were normalized to β-actin levels. For Ccnd1: 1*, P = 0.009; 1**, P < 0.001; n = 3; for c-Myc: 1*, P = 0.002; 1**, P = 0.009; n = 3. D, immunohistochemical staining of cyclin D1 and c-Myc in jejunum from mice at age 16 wk.
exhibited strong cytoplasmic staining throughout the adenomas, and showed a high degree of nuclear staining, particularly in cells at the leading edges of the adenomas. To examine colocalization of KLF5 and β-catenin in Apc<sup>Min/+</sup> adenomas, tissues were stained by immunofluorescence (Fig. 6B). Both KLF5 and β-catenin showed nuclear staining in a majority of the cells in the adenomas, with considerable colocalization of the two proteins in individual cells. Thus, KLF5 and β-catenin are coexpressed in Apc<sup>Min/+</sup> adenomas, and can work together to drive growth-promoting transcriptional activity in those tissues.

When comparing β-catenin levels in the adenomas from Apc<sup>Min/+</sup> and Apc<sup>Min/+Klf5<sup>+/−</sup></sup> mice by immunofluorescence, no significant differences were observed in β-catenin intensities in small-size adenomas (<1 mm) from the two genotypes (Supplementary Fig. S3). Both sets of adenomas exhibited nuclear localization of β-catenin. The only subjective difference seemed to be a more diffuse cytoplasmic localization of β-catenin in the Apc<sup>Min/+</sup> mice compared with a more intense localization to cell junctions in the Apc<sup>Min/+Klf5<sup>+/−</sup></sup> mice. These results reinforce the notion that haploinsufficiency of Klf5 affects adenoma formation at the earliest stages; and that these effects can be bypassed at low frequency to result in high β-catenin levels and subsequent establishment of adenomas.

**Discussion**

Mutational inactivation of the APC tumor suppressor gene and subsequent activation of the Wnt cascade is the rate-limiting event in CRC (26). In cases of CRC in which APC is not mutated, other mutations disrupting the normal regulation of β-catenin occur in a mutually exclusive manner (27, 28), highlighting the importance of dysregulation of β-catenin in the transformation of epithelial cells. Here, we show that KLF5 is a critical component for maintaining increased levels and activities of β-catenin in the Apc<sup>Min/+</sup> mouse model of intestinal tumorigenesis. With heterozygous deletion of Klf5, levels of β-catenin in the Apc<sup>Min/+</sup> setting are reduced to WT levels, resulting in reduced β-catenin activity as reflected in reduced expression of transcriptional targets including Ccdn1 and c-Myc. Other genetic crosses with Apc<sup>Min/+</sup> mice have been reported to have significant effects on intestinal tumorigenesis, including Apc<sup>Min/+MyD88<sup>−/−</sup></sup> (80% reduction in number of adenomas; ref. 29) and Apc<sup>Min/+cyclin D1<sup>−/−</sup></sup> (84% reduction; ref. 30). However, the Apc<sup>Min/+Klf5<sup>+/−</sup></sup> mouse is the first example of haploinsufficiency of a gene rescuing the tumorigenic effects of the Apc<sup>Min</sup> mutation.

The effect of KLF5 on β-catenin seems to be through physical interaction. We showed here that the two proteins are communoprecipitated and that they colocalize within the cell. In addition, we showed that overexpression of KLF5 enhances nuclear localization of β-catenin, and that reducing KFL5 expression, both in vitro and in vivo, significantly reduces concentrations of β-catenin in the nucleus. Because KLF5 contains a putative nuclear localization signal with homology to the nuclear localization signal confirmed in the related protein KLF4 (31), KLF5 may bind and assist β-catenin in localizing to the nucleus. Enhanced levels of nuclear β-catenin would therefore increase its availability to bind and activate transcriptional partners, such as TCF-4. This premise is supported by increased nuclear

![Figure 6](https://www.aacrjournals.org/4131 figure)
localization of β-catenin and increased TOPFLASH luciferase activity in the presence of ectopically expressed KLF5 (Figs. 4A, B and 5A).

KLF5 has been reported to promote proliferation of intestinal epithelial cells, both in vitro and in vivo (13, 14). KLF5 is a target of the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase pathway (32, 33), a signaling pathway critical for the G1-S phase cell cycle transition (34). As a downstream target of ERK1/2 signaling, KLF5 is activated in response to a number of mitogenic and stress stimuli (14, 35, 36) and thus may play a role in enhancing proliferation in intestinal tissues in situations of damage or stress. In support of this point, our laboratory has shown that KLF5 contributes to colonic hyperplasia in mice infected with the bacterial pathogen, *Citrobacter rodentium* (15).

In human cancer, KLF5 has been reported as a downstream target of oncogenic HRas in vitro and shown to mediate pro-proliferative and transforming activities of HRas (24). In addition, KLF5 is highly expressed in human primary colorectal cancers exhibiting Kras mutations (19). Thus, in addition to its potential involvement with the APC/β-catenin pathway, KLF5 may contribute to colorectal carcinogenesis through mutational activation of KRas. In a study of 247 gastric carcinoma patients, high KLF5 expression is found in 63% of early-stage gastric cancer patients (18). Furthermore, KLF5 expression has been reported as a prognostic factor for overall survival in patients with sporadic breast cancer, with higher KLF5 expression correlating with shorter disease-free survival and poorer overall survival (20). In contrast to these studies, a number of reports examining KLF5 status in various types of tumors show that KLF5 is down-regulated by several mechanisms, implicating KLF5 as a tumor suppressor (37–39). This apparent discrepancy may be attributable to the timing of changes in KLF5 expression, where increased expression early in tumor formation is growth-promoting, and loss of expression in later stages of cancer aids tumor cell survival. Studies that examine KLF5 expression by stage support this possibility, reflecting high KLF5 expression in early stages of cancer progression and lower KLF5 expression in later stages (18). What is clear from the results presented here is that reduced levels of KLF5 in the *Ap* min/+ context inhibit tumor initiation, implying that the activity of KLF5 contributes to early events in adenoma formation.

It has been argued that there may be an “optimal” level of Wnt signaling for colorectal tumorigenesis to occur, given that different functional mutations in the APC gene have varying abilities to drive formation of colorectal polyps (40). Thus, modulation of β-catenin activity, especially in patients with hereditary APC mutations, may be therapeutically beneficial. Given that partial reduction of KLF5 has a significant effect on β-catenin activity and adenoma formation in the *Ap* min/+ intestinal tumorigenesis model, KLF5 may present an attractive target for the development of novel therapeutic agents for early stage CRC treatment or prevention.

**Disclosure of Potential Conflicts of Interest**

The authors have no potential conflicts of interest.

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**References**


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Haploinsufficiency of Krüppel-Like Factor 5 Rescues the Tumor-Initiating Effect of the $Apc^{Min}$ Mutation in the Intestine

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