Notch Inhibits Expression of the Krüppel-Like Factor 4 Tumor Suppressor in the Intestinal Epithelium

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Abstract

The zinc finger-containing transcription factor, Krüppel-like factor 4 (KLF4), inhibits cell proliferation. An *in vivo* tumor-suppressive role for KLF4 is shown by the recent finding that *Klf4* haploinsufficiency in $Apc^{Min/+}$ mice promotes intestinal tumorigenesis. Studies also show that KLF4 is required for the terminal differentiation of goblet cells in the mouse intestine. The Notch signaling pathway suppresses goblet cell formation and is up-regulated in intestinal tumors. Here, we investigated the relationship between Notch signaling and KLF4 expression in intestinal epithelial cells. The rate of proliferation of HT29 human colon cancer cells was reduced when treated with the γ-secretase inhibitor dibenzazepine to inhibit Notch signaling or small interfering RNA directed against Notch. KLF4 levels were increased in dibenzazepine-treated or Notch small interfering RNA-treated cells. Conversely, overexpression of Notch in HT29 cells reduced KLF4 levels, suppressed *KLF4* promoter activity, and increased proliferation rate. Treatment of $Apc^{Min/+}$ mice with dibenzazepine resulted in a 50% reduction in the number of intestinal adenomas compared with the vehicle-treated group ($P < 0.001$). Both the normal-appearing intestinal mucosa and adenomas obtained from dibenzazepine-treated $Apc^{Min/+}$ mice had increased goblet cell numbers and Klf4 staining accompanied by reduced cyclin D1 and Ki-67 staining when compared with those from vehicle-treated mice. Results of these studies indicate that Notch signaling suppresses KLF4 expression in intestinal tumors and colorectal cancer cells. Inhibition of Notch signaling increases KLF4 expression and goblet cell differentiation and reduces proliferation and tumor formation. KLF4 is therefore a potential mediator for the antitumor effect of Notch inhibitors such as dibenzazepine. (Mol Cancer Res 2008;6(12):1920–7)

Introduction

Krüppel-like factor 4 (KLF4) is a zinc finger-containing transcription factor that is highly expressed in the terminally differentiated epithelial cells of the intestine (1-3). Biochemical studies indicate that KLF4 inhibits cell proliferation by blocking progression of the cell cycle at the G_1-S and G_2-M transitions (4-7). Studies also show that expression of KLF4 is reduced in colorectal neoplasia including carcinoma and adenoma relative to normal mucosa (8-11). Finally, we recently reported that haploinsufficiency of *Klf4* promotes the development of intestinal adenomas in $Apc^{Min/+}$ mice (12). Taken together, these studies are highly suggestive that KLF4 functions as a tumor suppressor in the intestinal epithelium.

Notch genes encode large, single transmembrane receptors that regulate a broad spectrum of cell fate decisions (13, 14). Notch activity is dependent on ligand binding followed by a series of proteolytic cleavage and subsequent nuclear translocation of its cytoplasmic domain, Notch intracellular domain (NICD). NICD then complexes with the DNA-binding protein RBP-J (also known as CSL or CBF1) and Mastermind-like 1 (MAML1) to exert a transcriptional effect on target gene expression (13-17). The best characterized Notch target gene is the basic helix-loop-helix protein hairy/enhancer of split 1 (HES1; ref. 18). One of the proteolytic enzymes involved in cleaving the membrane-bound Notch is a γ-secretase that includes the presenillin proteins, PS1 and PS2, which are often mutated in familial Alzheimer's disease (19). Inhibition of γ-secretase blocks activation of the Notch pathway (19, 20).

The intestinal epithelium is composed of two distinct cell lineages, one absorptive and the other secretory in nature (21). Mutational studies of the Notch downstream target HES1 in mice have revealed that the Notch pathway is involved in cell fate decision in the intestinal epithelium (22). Thus, targeted deletion of *Hes1* results in a relative increase in secretory cells at the expense of absorptive cells in the fetal intestine (22). Similarly, conditional deletion of *Rbp-j* from the intestinal epithelium leads to the conversion of proliferative crypt cells into postmitotic goblet cells (23). A recent study describing an increase in secretory cells relative to absorptive cells in the intestines of zebrafish that are mutant for a Notch ligand, *DeltaD*, also supports a role for Notch signaling in cell fate decision (24). Importantly, treatment of $Apc^{Min/+}$ mice, a model for intestinal tumorigenesis (25), with a γ-secretase inhibitor results in goblet cell differentiation in the adenomas derived

Received 5/11/08; revised 8/23/08; accepted 9/9/08.
Grant support: NIH grants DK52230, DK64399, and CA84197 (V.W. Yang) and CA130308 (A.M. Ghaleb).
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doi:10.1158/1541-7786.MCR-08-0224

from the intestine (23). Moreover, Notch signaling has been shown previously to be up-regulated in intestinal tumors (23, 26). Taken together, these studies imply that Notch inhibition by γ-secretase inhibitors may have a therapeutic potential in the treatment of intestinal neoplasm.

Previous studies also implicate a role for KLF4 in cell fate decision in the intestinal epithelium. Newborn mice with homozygous deletion of *Klf4* have a 90% reduction in the number of goblet cells in their colon and lack normal goblet cell morphology by ultrastructural analysis (27). Similarly, mice with conditional deletion of *Klf4* from the cornea show a loss of conjunctival goblet cells (28). Because both KLF4 and Notch are involved in regulating cell proliferation and cell fate decision, we sought to investigate the relationship between Notch signaling and KLF4 expression in the current study.

Results

Suppression of Notch Signaling in HT29 Cells by Dibenzazepine Reduces Proliferation and Increases KLF4 Levels

We first determined the effect of Notch inhibition on the rate of proliferation and KLF4 expression in the HT29 human colon cancer cells. HT29 cells were treated for progressively longer periods with increasing amounts of the γ-secretase inhibitor, dibenzazepine, to inhibit Notch signaling. As seen in Fig. 1A, there was a gradual reduction in the rate of proliferation of HT29 cells with increasing concentrations of dibenzazepine when compared with control. To determine whether the decreased rate of proliferation was due to cell death from drug toxicity, we measured viable cell counts using trypan blue staining and fluorescence-activated cell sorting analysis as well as Western blotting for cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase. As seen in Supplementary Fig. S1A, there were no significant differences in cell viability between treated and untreated cells at all time points as measured by trypan blue exclusion. There were also no significant differences in the percentages of cells in the sub-G_1 population between treated and untreated cells as measured by flow cytometry (Supplementary Fig. S1B). Moreover, there were no detectable levels of either cleaved caspase-3 or cleaved poly(ADP-ribose) polymerase at any given drug concentration and time point (Supplementary Fig. S1C). These results indicate that the effect of dibenzazepine on HT29 cells is mainly due to inhibition of cell proliferation rather than induction of apoptosis.

Western blot analysis of proteins extracted from HT29 cells treated with various concentrations of dibenzazepine for various periods showed a dose-dependent reduction in the levels of the activated form of Notch, NICD, along with a dose-dependent increase in the levels of KLF4 (Fig. 1B). The increase in KLF4 levels was accompanied by an increase in the levels of p21 (Fig. 1B), a downstream mediator of the cell cycle effect of KLF4 (4). In contrast, there was only a modest increase in the levels of p27 but no change in the levels of p57 on treatment of dibenzazepine (Supplementary Fig. S2).

Notch Signaling Inhibits Expression of KLF4 in HT29 Cells

To independently confirm the observation that suppression of Notch signaling with dibenzazepine results in the increase in KLF4, we inhibited Notch using small interfering RNA (siRNA) and determined the effect of such suppression on KLF4 expression. As with dibenzazepine, suppression of Notch by siRNA targeted against full-length Notch resulted in elevated levels of KLF4 mRNA and protein (Fig. 2A and B, respectively). Conversely, the level of KLF4 was reduced on overexpression of either NICD or full-length Notch in HT29 cells (Fig. 2C).

We then correlated Notch activity with the rate of cell proliferation following genetic manipulations in HT29 cells. As seen in Supplementary Fig. S3A, overexpression of NICD increased the rate of proliferation of HT29 cells. Conversely, inhibition of Notch by siRNA reduced the rate of proliferation (Supplementary Fig. S3B) in a manner that is similar to inhibition of Notch by dibenzazepine (Fig. 1A). These experiments further show that Notch promotes proliferation of HT29 cells and inhibits KLF4 expression.

In addition to inhibiting KLF4 expression at the levels of mRNA and protein as shown in Fig. 2A to C, activation of Notch by overexpression of NICD in HT29 cells led to a decrease in the luciferase activity directed by the mouse *Klf4* promoter (Fig. 2D, compare *lanes 1* and *2*). Moreover, cotransfection of the dominant-negative MAML1 abrogated the ability of NICD to suppress *Klf4* promoter activity in a dose-dependent manner (Fig. 2D, *lanes 4-7*). These results indicate that Notch inhibits KLF4 expression by suppressing *KLF4* promoter.

HES1, a transcription repressor, is known to mediate Notch signaling. Sequence analysis of 1.0 kb of the 5′-flanking sequence and 550 bp of the 5′-untranslated region of the mouse *Klf4* gene (29) revealed five potential HES1-binding sites (refs. 18, 30; Supplementary Fig. S4). Consequently, cotransfection experiments showed that HES1 significantly reduced luciferase activity driven by the *Klf4* promoter (Fig. 2E, compare *lanes 1* and *2*). Of note is that overexpression of dominant-negative MAML1 had no effect on the ability of HES1 to suppress *Klf4* promoter activity (Fig. 2E, *lanes 4-7*). This is consistent with previous findings that MAML1 exerts its effect on NICD but not on HES1 (13-18). Deletion analysis of the *Klf4* promoter showed that the minimal region required for HES1 to suppress *Klf4* promoter activity is between nucleotides -168 and +144 (29), a region that contains a single reverse class C HES1-binding sequence (Supplementary Fig. S4; data not shown). These results show that HES1 is the mediator for the suppressive effect of Notch on the KLF4 promoter.

Dibenzazepine Treatment Results in a Reduction of the Number of Intestinal Adenomas in $Apc^{Min/+}$ *Mice*

To determine whether inhibition of Notch signaling has an effect on intestinal tumor formation *in vivo*, we treated $Apc^{Min/+}$ mice at ages 10, 14, and18 weeks with 10 μmol/kg dibenzazepine or vehicle alone every other day for a total of 10 days. The number of adenomas in the intestine was assessed 2 days following the last injection. As seen in Fig. 3A, there was on average a 50% reduction in the number of adenomas in the small intestine of dibenzazepine-treated $Apc^{Min/+}$ mice compared with vehicle-treated $Apc^{Min/+}$ mice ($P < 0.001$, paired two-tailed t test) combining all three age groups. There was no statistical difference in the number of adenomas in the

colons of dibenzazepine- and vehicle-treated $Apc^{Min/+}$ mice, although the average number of colonic adenomas per mouse was <2 (data not shown). When the tumor burdens at the three different ages were separately analyzed (Fig. 3B), there was a statistically significant reduction in the number of small intestinal adenomas per mouse at weeks 12 and 16 ($P < 0.005$ and $P < 0.01$, respectively) in dibenzazepine-treated mice when compared with control. At 20 weeks, there was a trend toward reduction in the number of small intestinal adenomas due to dibenzazepine treatment, although the difference did not reach statistical significance ($P = 0.07$).

We also examined whether there were any differences between the size of adenomas formed in the small and large intestines of dibenzazepine-treated $Apc^{Min/+}$ mice and control. As shown in Fig. 3C, the adenomas that developed in the small intestines of $Apc^{Min/+}$ mice at 12 weeks were mostly small (<1 and 1-2 mm). By week 16, there was a shift in the size of adenomas to larger ones and this trend continued at 20 weeks. Dibenzazepine-treated $Apc^{Min/+}$ mice had a similar pattern of size distribution of adenomas as the vehicle-treated $Apc^{Min/+}$ mice at any given age, although there was a significant reduction in the number of adenomas in some of the size range per age group in the treated $Apc^{Min/+}$ mice compared with control mice (*double-headed arrows*). There was no significant difference in the size of adenomas formed in the large intestines of untreated and treated $Apc^{Min/+}$ mice (data not shown).

Dibenzazepine Treatment Leads to Increased Numbers of Goblet Cells and Klf4 Levels in the Intestinal Mucosa of Wild-type and $Apc^{Min/+}$ *Mice*

Previous studies indicate that dibenzazepine treatment in mice leads to intestinal goblet cell metaplasia (23, 31). To confirm these findings and to investigate whether dibenzazepine treatment results in increased Klf4 expression *in vivo*, we examined intestinal tissues obtained from dibenzazepine- or vehicle-treated wild-type and $Apc^{Min/+}$ mice using Alcian blue/periodic acid-Schiff (AB/PAS) staining to identify goblet cells and immunostaining to identify Klf4. Results in Fig. 4 show that both the number of goblet cells and the intensity of Klf4 immunostain (Fig. 4C and D, respectively) were increased in the normal-appearing small intestinal mucosa from $Apc^{Min/+}$ mice treated with dibenzazepine when compared with vehicle-treated mice (Fig. 4A and B). A similar increase in both is noted in the wild-type mice treated with dibenzazepine (Supplementary Fig. S5). Importantly, the number of goblet cells and Klf4 staining (Fig. 5C and D, respectively) were also increased in adenomas derived from the small intestines of dibenzazepine-treated $Apc^{Min/+}$ mice compared with control (Fig. 5A and B). In agreement with a previous report (23), the efficiency of the dibenzazepine treatment in converting tumor cells into nonproliferating goblet cells was relatively low. In the large intestine, dibenzazepine treatment led to only a modest increase in goblet cells and Klf4 staining in both wild-type and $Apc^{Min/+}$ mice when compared with control mice (Supplementary Figs. S6 and S7). This may explain the finding that dibenzazepine treatment did not lead to a significant reduction in the number of large intestinal adenomas in $Apc^{Min/+}$ mice.

KLF4 has been shown to negatively regulate cell cycle progression (4, 32). In addition to activating p21 expression

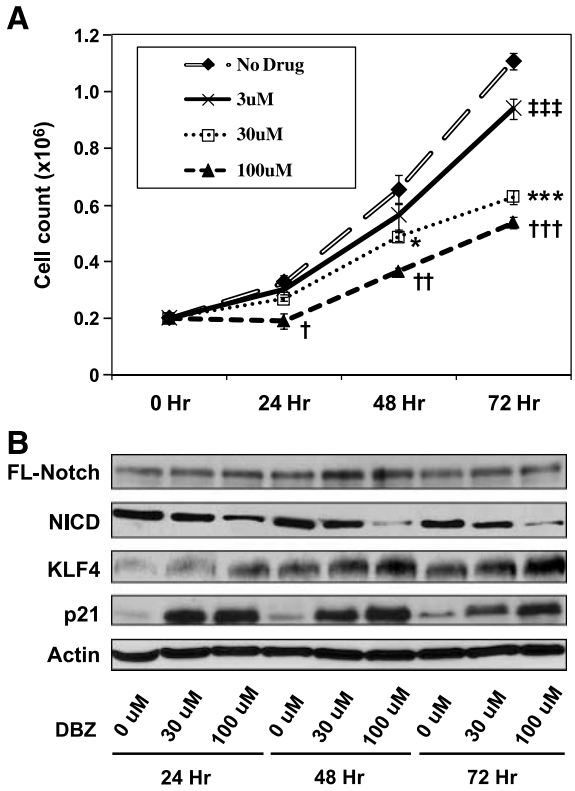

FIGURE 1. Suppression of Notch signaling by dibenzazepine in HT29 cells results in decreased proliferation and increased KLF4 levels. Human colon cancer cell line HT29 was treated with increasing concentrations of the γ-secretase inhibitor, dibenzazepine, for up to 72 h. **A.** Rates of proliferation were assessed by counting the number of cells daily. * and †, $P < 0.05$; ††, $P < 0.01$; ‡‡‡, ***, and †††, $P < 0.001$, compared with control (paired two-tailed *t* test; $n = 4$). **B.** Western blot analysis for the indicated proteins. FL, full-length.

(33), KLF4 is known to repress cyclin D1 expression (34). To determine whether dibenzazepine-induced elevation in Klf4 expression in both normal intestinal mucosa and adenomas of $Apc^{Min/+}$ mice is correlated with changes in cyclin D1 levels and cellular proliferation, we performed immunostaining of intestinal tissues from dibenzazepine- or vehicle-treated $Apc^{Min/+}$ mice for cyclin D1 and Ki-67. As seen in Fig. 6, cyclin D1 staining was significantly reduced in the crypts of the normal-appearing small intestinal mucosa in dibenzazepine-treated $Apc^{Min/+}$ mice (Fig. 6C) compared with control (Fig. 6A). Similarly, cyclin D1 staining was reduced in intestinal adenomas obtained from dibenzazepine-treated $Apc^{Min/+}$ mice (Fig. 6D) compared with control (Fig. 6B). A similar effect was observed for the Ki-67 staining (Supplementary Fig. S8). The reduced cyclin D1 and Ki-67 may therefore explain the suppressive effect of dibenzazepine on adenoma formation in $Apc^{Min/+}$ mice.

Discussion

Previous studies indicate that KLF4 inhibits cell proliferation by activating crucial checkpoints in the cell cycle on overexpression of exogenous KLF4 or following DNA damage (4-7, 32, 35). A critical transcriptional target of KLF4 in these

conditions is the cyclin-dependent inhibitor, p21 (4-7, 32). These findings led to the suggestion that KLF4 may function as a tumor suppressor in colorectal cancer (8-11). Our recent study showing that haploinsufficiency of *Klf4* promotes the development of intestinal adenomas in $Apc^{Min/+}$ mice supports this notion (12). *In vivo*, KLF4 has been shown to be required for the terminal differentiation of goblet cells in the colon (27) and conjunctiva (28) of newborn mice. Like KLF4, Notch pathways are known to be involved in cell fate decision (13, 14). In the intestinal epithelium, Notch is active in the proliferative crypt compartment (22, 36, 37), in contrast to the preferential expression of KLF4 in the postmitotic differentiated cell population (1, 2). Deletion of key component of the Notch pathways such as Rbp-j and Hes1 from the intestine results in a shift from absorptive cells to secretory cells, including goblet cells (22, 23). The opposing effects of KLF4 and Notch on cellular proliferation and goblet cell formation provide the rationale for our current study that attempts to establish a regulatory relationship between the two molecules.

Several lines of evidence from our studies indicate that KLF4 is a downstream target of Notch signaling. In HT29 cells, overexpression of either NICD or full-length Notch inhibits KLF4 expression and promoter activity (Fig. 2C and D), which is accompanied by an increase in the rate of proliferation (Supplementary Fig. S3A). Conversely, inhibition of Notch signaling by the γ-secretase inhibitor dibenzazepine or siRNA against Notch results in an increase in KLF4 expression (Figs. 1B and 2A and B) and a decrease in cellular proliferation (Fig. 1A;

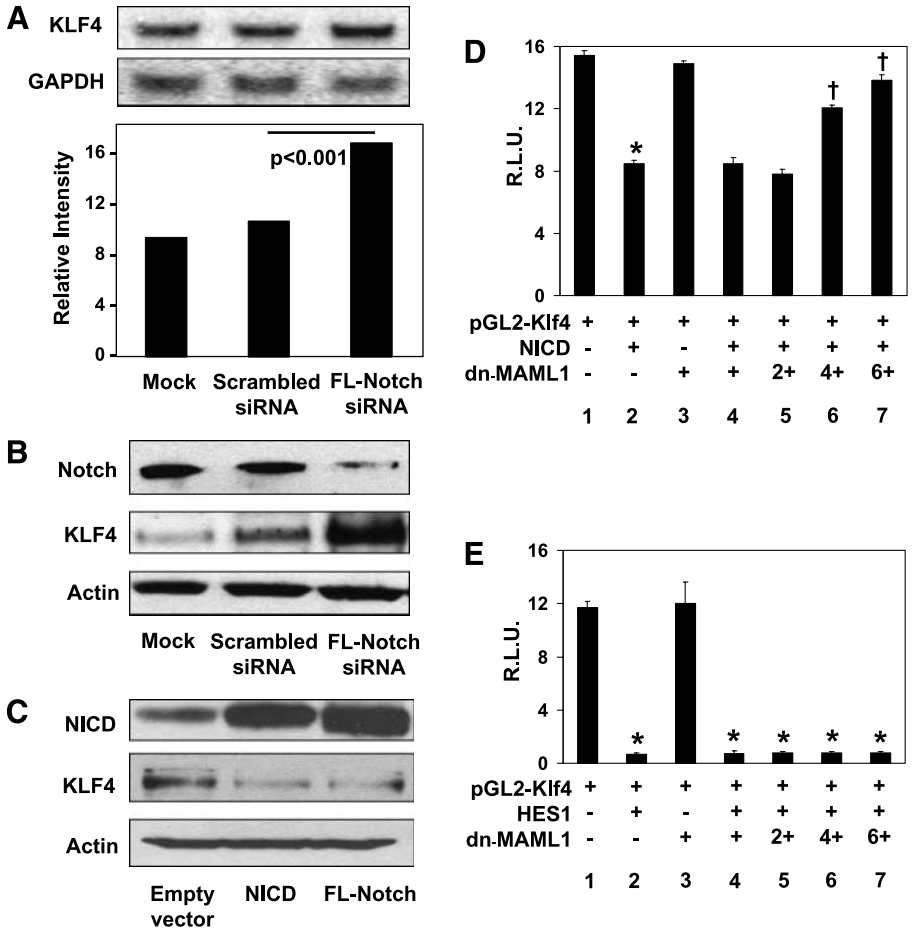

FIGURE 2. Notch suppresses KLF4 expression in HT29 cells. **A.** HT29 cells were mock-transfected or transfected with scrambled siRNA or siRNA directed against full-length Notch. Twenty-four hours following transfection, RNA was prepared and analyzed by Northern blot analysis for KLF4 and GAPDH. Bottom, densitometric tracing of KLF4 band intensities after normalizing to those of GAPDH ($n = 3$). **B.** Western blot analysis of Notch, KLF4, and actin in mock-transfected HT29 cells or cells transfected with scrambled siRNA or siRNA directed against full-length Notch. **C.** HT29 cells were transfected with empty vector or an expression construct for NICD or full-length Notch. Western blot analysis for NICD, KLF4, and actin was done 24 h following transfection. **D.** HT29 cells were cotransfected with the pGL2-Klf4 luciferase reporter (*lanes 1-7*), an expression vector containing NICD (MIGR1-ICNX; *lanes 2* and *4-7*), and an expression vector containing the dominant-negative MAML1 (MIGR1-MAML1) at increasing concentrations (*lanes 3-7*) along with an internal *Renilla* luciferase control. Twenty-four hours following transfection, luciferase activities were determined and normalized to the internal standard *Renilla* luciferase activities. Normalized mean relative luciferase activities (*R.L.U.*) of four independent experiments. *, $P < 0.001$, compared with *lane 1*; †, $P < 0.001$, compared with *lane 2* (paired two-tailed *t* test). **E.** HT29 cells were cotransfected with the pGL2-Klf4 luciferase reporter (*lanes 1-7*), an expression vector containing HES1 (pCMV-HES1; *lanes 2* and *4-7*), and an expression vector containing the dominant-negative MAML1 (MIGR1-MAML1) at increasing concentrations (*lanes 3-7*) along with an internal *Renilla* luciferase control. Twenty-four hours following transfection, luciferase activities were determined and normalized to the internal standard *Renilla* luciferase activities. Normalized mean relative luciferase activities of four independent experiments. *, $P < 0.001$, compared with lane 1 (paired two-tailed *t* test).

Supplementary Fig. S3B). In the normal intestinal mucosa of wild-type or $Apc^{Min/+}$ mice, dibenzazepine treatment leads to an increase in Klf4 staining (Fig. 4; Supplementary Fig. S5), which is accompanied by an increase in goblet cell numbers (Fig. 4; Supplementary Fig. S5). A similar finding is noted in intestinal adenomas derived from $Apc^{Min/+}$ mice (Fig. 5). These findings strongly suggest that KLF4 is negatively regulated by Notch signaling. On inhibition of Notch, KLF4 expression is increased with an associated conversion of proliferative cells to goblet cells. Importantly, the increase in KLF4 is correlated with the antitumor activity of dibenzazepine in $Apc^{Min/+}$ mice (Fig. 3) and the antiproliferative effect of dibenzazepine on the normal mucosa and adenomas from $Apc^{Min/+}$ mice (Fig. 6; Supplementary Fig. S8) and on HT29 cells (Fig. 1A). In view of the established inhibitory effect of KLF4 on cell proliferation (1, 4, 7, 32), it is likely that KLF4 is responsible at least in part for the antitumor activity of dibenzazepine.

Results of our studies indicate that Notch signaling negatively regulates the activity of the mouse *Klf4* promoter including 1.0 kb of the 5′-flanking region and 550 bp of the 5′-untranslated region (Fig. 2D). Transcriptional regulation by Notch is dependent on the nuclear translocation and binding of NICD to the DNA-binding protein RBP-J (also known as CSL or CBF1) and MAML1 (13-17), where MAML1 is required to stabilize the interaction between NICD and RBP-J (13, 14). HES1 is one of the best studied downstream targets and mediator of Notch signaling (18). Within the mouse *Klf4* promoter examined in this study, there are five potential binding sites for HES1 (Supplementary Fig. S4). Indeed, cotransfection experiments show that both NICD and HES1 suppress *Klf4* promoter activity (Fig. 2D and E). These experiments also show that a dominant-negative MAML1 construct is capable of competing with Notch but not HES1 in their ability to inhibit the *Klf4* promoter (Fig. 2D and E). This is consistent with the fact that MAML1 regulates Notch signaling at the level of NICD and RBP-J but not at the level of HES1 (13-18).

Previous studies show that KLF4 level is reduced in intestinal adenomas from $Apc^{Min/+}$ mice (12, 38, 39) and colonic adenomas from patients with familial adenomatous polyposis (39) when compared with their respectively matched normal-appearing mucosa. Studies also indicate that KLF4 level is reduced in colorectal adenomas and carcinomas and many colon cancer cell lines (8-11). Moreover, haploinsufficiency of *Klf4* results in an increase in intestinal tumor burden in $Apc^{Min/+}$ mice (12). Furthermore, KLF4 has been shown to regulate normal intestinal homeostasis and tumor repression by interacting with β-catenin and repressing β-catenin-mediated gene expression (40). Taken together, these studies corroborate the tumor-suppressive effect of KLF4 in the intestinal epithelium. Importantly, ectopic expression of KLF4 in colorectal cancer cells that lack endogenous KLF4 expression results in reduced tumorigenecity (41). Results of the current study showing a correlation between Klf4 induction and cyclin D1 repression in the intestinal mucosa and adenomas of $Apc^{Min/+}$ mice as a consequence of dibenzazepine treatment further highlight the significance of KLF4 in intestinal tumor development. The reduced cyclin D1 levels could be due to direct suppression of KLF4 on the cyclin D1 promoter (34) or indirect suppression of cyclin D1 expression as a consequence

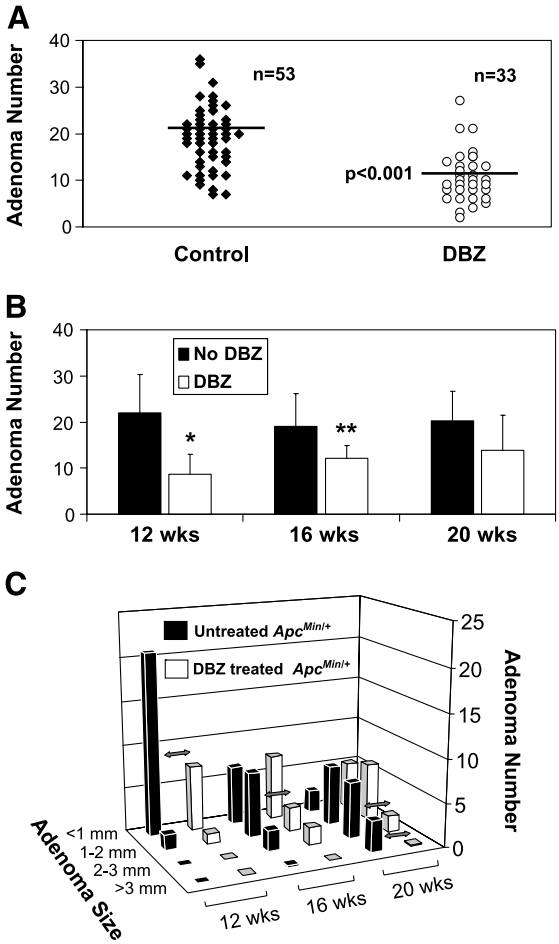

FIGURE 3. Effects of dibenzazepine treatment on intestinal adenoma formation in $Apc^{Min/+}$ mice. $Apc^{Min/+}$ mice at age 10, 14, or 14 wk received an intraperitoneal injection of vehicle or 10 μmol/kg dibenzazepine every other day for 10 d. Two days following the last injection, mice were euthanized to determine the intestinal tumor burden. **A.** Comparison of the number of adenomas per mouse in the small intestine between control and dibenzazepine-treated mice in the three age groups combined. Columns, mean in each group. $P < 0.001$ (paired two-tailed *t* test). **B.** Comparison of the number of adenomas developed in the small intestine of control and dibenzazepine-treated mice at 12, 16, and 20 wk. *, $P < 0.005$; **, $P < 0.01$, compared with control (two-tailed *t* test). **C.** Size distribution of adenomas developed in the small intestine at 12, 16, and 20 wk, respectively, in control and dibenzazepine-treated $Apc^{Min/+}$ mice. Double-headed arrows, significant difference between the two groups ($P < 0.01$).

of the inhibitory effect of KLF4 on β-catenin (40). Be that as it may, the collective studies suggest that KLF4 is a potential therapeutic target for antitumor drugs.

A recent study shows that conditional ablation of Notch1 and Notch2 or Rbp-j from the intestine of transgenic mice results in the derepression of p27 and p57 but not p21 (42). In comparison, our study shows that dibenzazepine-mediated Notch inhibition in HT29 cells results in a significant induction in p21 (Fig. 1B) but only a modest effect on p27 and no effect on p57 (Supplementary Fig. S2). The differences seen between the two studies may be due to the difference in species (mouse versus human) or methods of manipulation (genetic versus pharmacologic). It should be noted that KLF4 has recently been shown to activate expression of p27 in human pancreatic cells

(43), suggesting that both p21 and p27 may play a role in the antiproliferative effect of Notch inhibition.

Notch activity has been shown to be required for the maintenance of the proliferative progenitor crypt cells in the intestine (37). Overactivity of Notch in the intestine inhibits differentiation of the secretory lineage, including goblet cells (37). Conversely, suppression of Notch pathways by genetic (22, 23) or pharmacologic (23, 31, 44) means leads to goblet cell metaplasia. In view of the findings that KLF4 is required for terminal differentiation of goblet cells in the intestine (27) and that KLF4 induction is accompanied by increased goblet cell formation following dibenzazepine treatment (this study), it is possible that KLF4 is the mediator for the goblet cell metaplasia secondary to treatment with γ-secretase inhibitors. Although goblet cell metaplasia following Notch suppression has been considered an undesirable toxic side effect (31, 44, 45), there is conceivably a therapeutic window between the antitumor and goblet cell metaplastic effect on KLF4 induction. The exact mechanism by which up-regulation of KLF4 in the intestine after γ-secretase treatment leads to goblet cell metaplasia and reduction in adenoma formation is under investigation.

In conclusion, we show for the first time that the Notch cascade directly regulates KLF4 expression and that increased *Klf4* expression *in vivo* plays a role in the reduction of intestinal adenoma formation in $Apc^{Min/+}$ mice following Notch inhibition. Our findings suggest that events that inactivate KLF4 may promote tumorigenesis in human colorectal cancers and that the reversal of the KLF4 inactivation might lead to a reduction in the proliferation rate and ultimately a reduction in tumor burden.

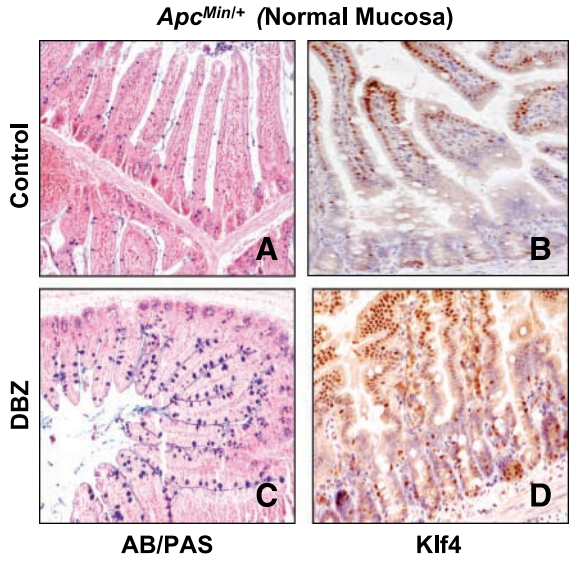

FIGURE 4. Histochemical staining for goblet cells and immunostaining for Klf4 in the normal-appearing small intestinal mucosa of control and dibenzazepine-treated $Apc^{Min/+}$ mice. The normal-appearing small intestinal tissues obtained from age-matched control (**A** and **B**) and dibenzazepine-treated (**C** and **D**) $Apc^{Min/+}$ mice were stained for goblet cells (*blue*) using AB/PAS stain (**A** and **C**) or immunostained for Klf4 (**B** and **D**).

Materials and Methods

Cell Lines, Reagents, and Plasmid Constructs

The human colon cancer cell line HT29 was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum. The γ-secretase inhibitor, dibenzazepine, was purchased from Sigma-Aldrich and dissolved in DMSO (EMD Chemicals). For *in vitro* experiments, solubilized dibenzazepine was suspended in growth medium containing 0.01% (v/v) Tween 80 to the final concentrations as indicated. Antibodies used for Western blot analyses were obtained from the following sources: rabbit anti-KLF4 (H180), rabbit anti-p57 (C20), and rabbit anti-p27 (Santa Cruz Biotechnology); mouse monoclonal anti-full-length Notch (Abcam); rabbit anti-NICD (Millipore); mouse monoclonal anti-p21 (BD Biosciences); mouse monoclonal anti-β-actin (BD Biosciences); and rabbit anti-cleaved caspase-3 and rabbit anti-cleaved poly(ADP-ribose) polymerase (Cell Signaling Technology).

The expression construct containing full-length Notch1 (pCS2-MT-Notch1; ref. 46) was kindly provided by Dr. Raphael Kopan (Washington University), and those containing NICD (MIGR1-ICNX; ref. 47) and dominant-negative MAML1 peptide [MIGR1-MAML1 (13-74); ref. 48] were kindly provided by Dr. Warren Pear (University of Pennsylvania). The expression vector expressing human HES1 (pCMV-HES1) was purchased from Origene. A construct linking 1.0 kb of the 5′-flanking region and 550 bp of the untranslated region of the mouse *Klf4* gene to the pGL2-basic luciferase reporter (Promega), pGL2-Klf4, was generated previously in our laboratory (29). siRNA directed against human Notch was obtained from Invitrogen.

Treatment of Animals with the γ-Secretase Inhibitor Dibenzazepine and Assessment of Tumor Burden

$Apc^{Min/+}$ mice on a B6 background were crossbred. Wild-type B6 mice served as controls. Dibenzazepine solubilized in DMSO was suspended in PBS containing 0.5% (w/v) hydroxypropylmethylcellulose (Methocel E4M; Dow Chemicals) and 0.01% (v/v) Tween 80, as vehicle (23), to the final concentrations indicated below. At 10, 14, and 18 weeks, mice were given intraperitoneal injections of 10 μmol/kg dibenzazepine or carrier alone every other day for a total of 10 days. Two days following the last injection (on weeks 12, 16, and 20), mice were euthanized by CO_2 asphyxiation and the small and large intestines were longitudinally dissected in their entirety. After washing in PBS, the intestines were examined under a dissection microscope for the presence of adenomas. The number and size of adenomas in both 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).

AB/PAS Staining for Goblet Cells

Goblet cell staining was carried out as described with slight modifications. In brief, intestinal tissues were fixed in 10% formalin in PBS and subsequently embedded in paraffin. Paraffin sections (5 μm thick) were cut and applied to Superfrost Plus slides (VWR). Sections were deparaffinized in xylene, rehydrated in ethanol, and then brought to distilled

water for 5 min. Alcian blue [1% (w/v) Alcian blue 8GX (Sigma-Aldrich) in 3% acetic acid] was applied to the sections for 15 min at room temperature followed by 2 min wash in running tap water. Periodic acid (Biocare Medical) was then applied for 5 min at room temperature, and slides were washed in distilled water and then stained with Schiff's reagent (Biocare Medical) for 15 min at room temperature followed by 5 min wash in running tap water. The sections were then counterstained for nuclei with Nuclear Fast Red (Biocare Medical) for 15 min and then washed in running tap water for 2 min followed by dehydration (twice in 95% ethanol then twice in 100% ethanol) and coverslipping.

Immunohistochemistry

Intestinal tissues for immunohistochemistry were fixed in 10% formalin in PBS and subsequently embedded in paraffin. Paraffin sections (5 μm thick) were cut and applied to Superfrost Plus slides. Sections were deparaffinized in xylene, rehydrated in ethanol, and then treated with 10 mmol/L sodium citrate (pH 6.0) at 120°C for 10 min in a pressure cooker. The histologic sections were incubated with a blocking buffer (2% nonfat dry milk, 0.01% Tween 20 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 [rabbit anti-GKLF (H-180); Santa Cruz Biotechnology], cyclin D1 (rabbit monoclonal anti-cyclin D1; Biocare Medical), and Ki-67 (rabbit anti-Ki-67; Abcam) at a dilution of 1:200, 1:100, and 1:1,000, respectively, in the blocking buffer overnight at 4°C. Detection of primary antibodies was carried out using appropriate biotinylated secondary antibodies at 1:500 dilution for 20 min at room temperature, and color development was done using the Vectastain ABC kit (Vector Laboratories). Sections were then counterstained with hematoxylin, dehydrated, and coverslipped. Sections were also stained separately for goblet cells using AB/PAS stain. Images were acquired using an Axioskop 2 plus microscope (Zeiss) equipped with an AxioCam MRC5 CCD camera (Zeiss).

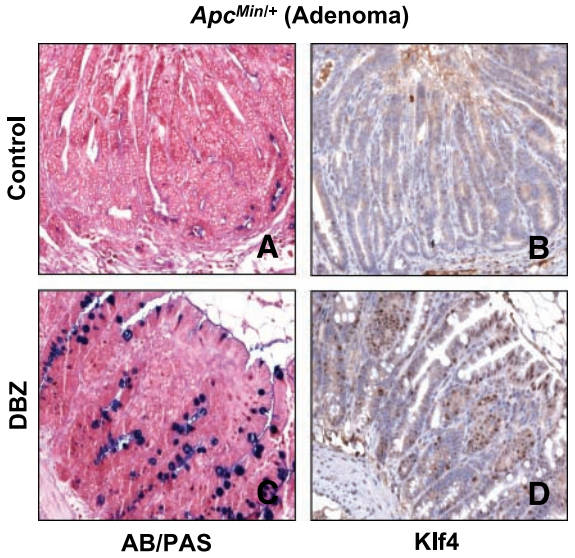

FIGURE 5. AB/PAS staining and Klf4 immunostaining in intestinal adenomas from control and dibenzazepine-treated $Apc^{Min/+}$ mice. Small intestinal adenomas obtained from age-matched control (**A** and **B**) and dibenzazepine-treated (**C** and **D**) $Apc^{Min/+}$ mice were stained for goblet cells (*blue*) using AB/PAS stain (**A** and **C**) or immunostained for Klf4 (**B** and **D**).

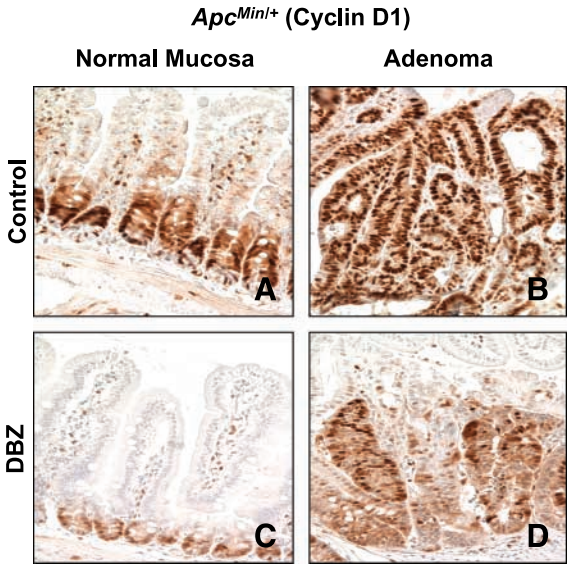

FIGURE 6. Cyclin D1 immunostaining in the normal-appearing small intestinal mucosa and adenomas from control and dibenzazepine-treated $Apc^{Min/+}$ mice. Normal-appearing small intestinal tissues (**A** and **C**) and small intestinal adenomas (**B** and **D**) obtained from age-matched control $Apc^{Min/+}$ mice (**A** and **B**) and dibenzazepine-treated $Apc^{Min/+}$ mice (**C** and **D**) were immunostained with antibodies against cyclin D1.

Trypan Blue Stain for Viable Cell Count

Following trypsinization, cells were collected by low-speed centrifugation, washed once in PBS, and resuspended in 200 μL PBS. An equal volume of 0.4% trypan blue stain (Mediatech) was added to the cell suspension, mixed gently, and allowed to stand for 5 min at room temperature and the percent of viable (unstained) and dead (stained) cells was counted using a hemocytometer.

Flow Cytometry

Cell cycle analysis by fluorescence-activated cell sorting was done as described previously (5). Cells were rinsed twice in PBS, treated with trypsin, and resuspended in their corresponding medium containing 10% fetal bovine serum. Cells were then collected by centrifugation, washed with PBS, collected again by centrifugation, resuspended in 70% ethanol, and fixed at −20°C overnight. Cells were pelleted once again by centrifugation and resuspended in a solution containing 50 mg/mL propidium iodide, 50 mg/mL RNase A, 0.1% Triton X-100, and 0.1 mmol/L EDTA at room temperature for 30 min. Flow cytometry was done on a FACSCalibur cytometer (Becton Dickinson).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mol Cancer Res 2008;6:1920-1927.