Zinc finger protein 277 is an intestinal transit-amplifying cell marker and colon cancer oncogene

Guofeng Xie, …, Harris Yfantis, Jean-Pierre Raufman


Graphical abstract

Find the latest version:

https://jci.me/150894/pdf
Zinc finger protein 277 (ZNF277; murine Zfp277), a transcription factor regulating cellular senescence, is overexpressed in colon cancer, but its actions in intestinal homeostasis and neoplasia are unclear. Using human and murine intestine, human colon cancer cells, and Apc<sup>Min/+</sup> mice with dysregulated β-catenin signaling and exuberant intestinal neoplasia, we explored the actions of ZNF277/Zfp277 and defined the underlying mechanisms. In normal human and murine intestine, ZNF277/Zfp277 was expressed uniquely in early stem cell progenitors, undifferentiated transit-amplifying cells (TACs). Zfp277 was overexpressed in the Apc<sup>Min/+</sup> mouse colon, implicating ZNF277/Zfp277 as a transcriptional target of β-catenin signaling. We confirmed this by showing β-catenin knockdown reduced ZNF277 expression and, using chromatin IP, identified 2 β-catenin binding sites in the ZNF277 promoter. Zfp277 deficiency attenuated intestinal epithelial cell proliferation and tumor formation, and it strikingly prolonged Apc<sup>Min/+</sup> mouse survival. RNA-Seq and PCR analyses revealed that Zfp277 modulates expression of genes in key cancer pathways, including β-catenin signaling, the HOXD family that regulates development, and p21<sup>WAF1</sup>, a cell cycle inhibitor and tumor suppressor. In both human colon cancer cells and the murine colon, ZNF277/Zfp277 deficiency induced p21<sup>WAF1</sup> expression and promoted senescence. Our findings identify ZNF277/Zfp277 as both a TAC marker and colon cancer oncogene that regulates cellular proliferation and senescence, in part by repressing p21<sup>WAF1</sup> expression.

**Conflict of interest:** Aspects of treating cancer with anticholinergic agents are the subject of a patent (“Hybrid cholinergic agents and compositions, methods of making, and methods of using to treat a cholinergic disorder,” US 6,624,155) issued on September 23, 2003, to the University of Arkansas; JPR is an inventor on this patent. JPR owns equities related to health care (Agile Therapeutics, Gilead Sciences, Merck, Proctor & Gamble).

**Copyright:** © 2022, Xie et al. This is an open access article published under the terms of the Creative Commons Attribution 4.0 International License.

**Submitted:** May 18, 2021
**Accepted:** January 5, 2022
**Published:** February 22, 2022

**Reference information:** JCI Insight. 2022;7(4):e150894.
https://doi.org/10.1172/jci.insight.150894.
found a zinc finger protein, Zfp277, was selectively downregulated in colon tumors from M_{R}R-deficient mice with reduced tumorigenesis (17). By binding to DNA, RNA, protein, or other small molecules, zinc finger protein transcription factors play key roles in regulating gene expression and thereby contribute to a variety of biological processes, including cell proliferation, differentiation, apoptosis, and metabolism (18). The human homologue of Zfp277, ZNF277, is a classic C2H2 zinc finger gene highly conserved in humans, mice, zebra fish, Drosophila, and C. elegans (19). Human ZNF277 and murine Zfp277 share 5 zinc finger domains and a 30–amino acid coiled-coil domain. Murine Zfp277 is expressed in early embryonic stem cells, suggesting it may be a critical regulator of cell proliferation, differentiation, and cell fate transitions (20). Herein, we refer to ZNF277/Zfp277 when addressing attributes relevant to both the human and murine genes and proteins.

ZNF277/Zfp277 may play important roles in senescence; Negishi et al. showed that loss of Zfp277 from mouse embryonic fibroblasts prematurely induced senescence (21). Cellular senescence is characterized by proliferative arrest and the secretion of proteins that comprise the senescence-associated secretory phenotype (22). Besides impacting aging, senescence plays an important role in cancer. Although senescent cells are initially protected against neoplastic transformation, at later stages, they may contribute to a protumorigenic microenvironment (23, 24). Although no single biomarker can identify senescent cells, cyclin-dependent kinase inhibitor 1A (CDKN1A; p21{WAF1}), β-galactosidase, CDKN2A (also known as p16{INK4A} and p19{ARF}[mouse]/p14{ARF}[human]), and CDKN2B (INK4B; p15{INK4B}) are commonly associated with senescence (22).

In the current study, we explored the role of ZNF277/Zfp277 in normal intestinal epithelial cell development, proliferation, and senescence. Additionally, we used human colon cancer cells, and animal and enteroid models, to elucidate the mechanisms whereby ZNF277/Zfp277 overexpression promotes colon neoplasia. In doing so, we identified ZNF277 as a transcriptional target of β-catenin signaling that modulates Wnt/β-catenin and polycomb protein signaling, and it represses p21{WAF1} expression, thereby regulating intestinal epithelial cell proliferation, senescence, and neoplasia. Overall, our findings uncover ZNF277/Zfp277 as a potentially novel intestinal transit-amplifying cell (TAC) marker and colon cancer oncogene.

Results
In normal intestinal epithelium, ZNF277/Zfp277 is only expressed in TACs. In normal human small intestinal and colonic mucosa, we detected ZNF277 protein expression only in early stem cell progenitors — i.e., undifferentiated proliferating TACs (Figure 1A). Rapidly cycling TACs, identified by immunostaining for the cell proliferation marker Ki67, are localized to the lower half of intestinal crypts (Figure 1A). As shown in Figure 1B, in murine small intestine and colon, Zfp277 and Ki67 colocalize in TACs. We used murine small intestinal organoids (enteroids) to confirm and extend these findings. In murine enteroids, both Zfp277 and Ki67 were expressed selectively in nuclei of basal compartment cells where stem and TA cells reside. In contrast, β-catenin was prominently expressed in the cytoplasm and membranes of all enteroid cells (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.150894DS1). These results indicate that Zfp277 is selectively expressed in murine intestinal TACs but not in differentiated enterocytes.

Murine TACS express 2 Zfp277 transcript variants. Multiple transcript isoforms with variable functions can exist in mammalian genes. In mice, 2 Zfp277 variants result from alternative splicing. Compared with Variant 1 (2454 bp; protein isoform 1; 583 aa), Variant 2 (2076 bp; protein isoform 2; 457 aa) lacks an alternate in-frame exon. Both variants share the same first 125 bp sequence (exon 1). Isoform 1 has an extra 378 bp from bp 126 to 504. We designed PCR primer pairs with the forward sequence within the first shared 125 bp sequence and the reverse primers between bp 504 and 602 (primer sequences in Methods). Variant 1 and 2 reverse transcription (RT)-PCR products resulting from the use of cDNAs prepared from WT mouse colon and enteroids were 595 and 309 bp in length, respectively (Supplemental Figure 2, A–C), and we detected both variants in colon mucosa and enteroids prepared from WT mice. In Zfp277−/− mice, Variant 1 is shorter due to removal of exon 5 in the Zfp277-KO transgenic mouse strain, and its expression is reduced compared with WT animals (Supplemental Figure 2A). Compared with WT mice, Variant 2 levels were also reduced (Supplemental Figure 2B). We detected the longer 65 kDa Zfp277 isoform 1 in murine liver (Supplemental Figure 3A) and the shorter isoform 2 (54 kDa) in mouse colon. In addition to the primary transcript (451 aa) detected in human colon cancer cells, there are 2 shorter human ZNF277 isoforms; it is unclear whether these 2 transcripts have
Figure 1. Expression and localization of ZNF277/Zfp277 in normal and neoplastic human and murine small intestine and colon. (A) In human small intestine (SI) and colonic crypts, ZNF277 is expressed selectively in the nuclei of transit amplifying cells (TACs). In the human ileum, IHC staining reveals ZNF277 and Ki67 expression in TACs. IHC reveals ZNF277 and Ki67 expression in transverse colon TACs. (B) Murine small intestinal and colonic TACs coexpress Zfp277 and Ki67. Immunofluorescence (IF) staining reveals Zfp277 and Ki67 expression in mouse ileal TACs and DAPI nuclear stains of mouse ileum. Merged Zfp277, Ki67, and DAPI confocal images reveal colocalization of Zfp277 and Ki67. IF staining reveals Zfp277 and Ki67 expression in murine colonic TACs. Merged Zfp277, Ki67, and DAPI images reveal Zfp277 and Ki67 colocalization. (C) Nuclear localization of ZNF277/Zfp277 by IHC and IF staining. IHC reveals nuclear Zfp277 expression in a colon adenoma from an ApcMin/+ mouse. IF staining reveals nuclear

ZNF277 HT29 Xenograft  ZNF277 CRC and normal colon (10X)  ZNF277 CRC (20X)
ZNF277 expression in HT29 human colon cancer cells. DAPI staining of HT29 cell nuclei. IHC reveals nuclear ZNF277 expression of cells in an HT29 cell xenograft, nuclear ZNF277 expression in human colon cancer and adjacent normal colon (arrow). Higher-magnification image showing nuclear ZNF277 expression in human colon cancer. Size bars: 100 μM.

different intestinal functions and are expressed at different stages of development — future studies will explore these possibilities. Next, to pursue our observation that Zfp277 was selectively downregulated in colon tumors from M,R-deficient mice with attenuated tumorigenesis (17), we examined the role of ZNF277/Zfp277 in colon neoplasia.

ZNF277/Zfp277 overexpression in neoplastic colon cell nuclei. As shown in Supplemental Figure 4, in silico analysis using publicly available online databases — Oncomine (25), the Gene Expression Profiling Interactive Analysis (GEPIA) (26), and the Human Protein Atlas (27) — revealed increased ZNF277 mRNA and protein levels in colon cancer compared with normal colon. As shown in Supplemental Figure 5, in silico analysis using the UALCAN server (28) revealed that, regardless of sex, race, age, tumor stage, and nodal metastasis, ZNF277 transcript levels are 1.5- to 2.0-fold greater in tumors compared with normal tissues. Using data from the GEPIA server, we detected modest but statistically significant correlations between ZNF277 expression levels and BRAF, APC, and p21 (CDKN1A) genetic subtypes (R = 0.39 and P = 8.9 × 10 –13, R = −0.19 and P = 0.00067, and R = −0.26 and P = 2.5 × 10 –4, respectively); there was no correlation between ZNF277 and p53 levels (R = 0.016, P = 0.77). Analyzing data extracted from the Colon Adenocarcinoma (COAD) data set (GEPIA), we did not detect a relationship between ZNF277 levels and CRC survival. Because ZNF277 is expressed in many tissues, including the immune system, ZNF277 expression levels in tumor cells alone might not be sufficient to alter survival. IHC staining of murine adenomas, human colon cancer cells, colon cancer cell xenografts, and colon cancers revealed that ZNF277/Zfp277 protein overexpression was localized to cell nuclei (Figure 1C).

Zfp277 deficiency attenuates adenoma formation and progression in ApcMin/– mice. To elucidate the role of Zfp277 expression in intestinal tumorigenesis and progression, we obtained Zfp277−/− (B6.129-Zfp277<tm1>) mice wherein deletion of exon 5 of the Zfp277 gene results in complete loss of Zfp277 protein expression (21). Homozygous mutant mice backcrossed to the C57BL/6J genetic background were healthy and fertile (21). Zfp277 protein deficiency was confirmed by immunoblotting (see Methods).

To examine the effects of Zfp277 deficiency in ApcMin/+ mice with an Apc gene mutation that results in dysregulated β-catenin signaling and the development of small and large intestinal adenomas, we created ApcMin/+ mice with heterozygous and homozygous Zfp277 deficiency. Because of sex differences in human colon neoplasia, we explored the effects of Zfp277 deficiency in both male and female ApcMin/+ mice. As shown in Figure 2, A–G, the numbers of small intestine and colon tumors were substantially reduced in both male (Figure 2, A–D) and female (Figure 2, E–G) ApcMin/+ Zfp277+/– and ApcMin/+ Zfp277−/− mice compared with ApcMin/+ Zfp277+/+ littermate control mice. Male ApcMin/+ Zfp277+/– (n = 12), ApcMin/+ Zfp277−/− (n = 16), and ApcMin/+ Zfp277−/− (n = 10) mice had 46.0 ± 4.3, 16.4 ± 2.7, and 8.6 ± 2.6 (mean ± SEM) small intestinal tumors, respectively (Figure 2B). The significantly reduced small intestinal tumor burden in male ApcMin/+ mice with Zfp277 haploinsufficiency supports an important role for this gene in intestinal neoplasia.

Most tumors were localized to the distal small intestine, and in each small intestinal segment, fewer tumors were observed in ApcMin/+ Zfp277+/– compared with ApcMin/+ Zfp277−/− mice (Supplemental Figure 6A). Although — as is typical for ApcMin/+ mice — there were fewer colon tumors, Zfp277 deficiency also reduced the number of colon tumors; male ApcMin/+ Zfp277+/–, ApcMin/+ Zfp277+/–, and ApcMin/+ Zfp277+/– mice had 5.5 ± 1.4, 2.4 ± 0.7, and 0.8 ± 0.3 (mean ± SEM) colon tumors, respectively (Figure 2C). As shown in Figure 2D, in ApcMin/+ Zfp277−/− mice, colon tumors were much smaller than those in ApcMin/+ Zfp277+/– littermates; in contrast to ApcMin/+ Zfp277+/– and ApcMin/+ Zfp277+/– mice, no ApcMin/+ Zfp277−/− mice had colon tumors greater than 3 mm in largest diameter (Figure 2D).

Likewise, as shown in Figure 2, E–G, Zfp277 deficiency attenuated intestinal tumor formation in female mice. Female ApcMin/+ Zfp277+/– (n = 10), ApcMin/+ Zfp277+/– (n = 8), and ApcMin/+ Zfp277+/– mice (n = 9) had 41.1 ± 6.1, 22.5 ± 5.7, and 9.6 ± 3.9 (mean ± SEM) small intestinal tumors, respectively (Figure 2E). Again, most tumors were in the distal small intestine and, in each small intestinal segment, fewer tumors were found in ApcMin/+ Zfp277+/– compared with ApcMin/+ Zfp277+/– mice (Supplemental Figure 6B).

Zfp277 deficiency reduced the number of colon tumors 10-fold from 6.6 ± 1.6 tumors (mean ± SEM) in ApcMin/+ Zfp277+/– mice to 3.4 ± 1.4 and 0.7 ± 0.3 tumors in ApcMin/+ Zfp277+/– and Zfp277+/– ApcMin/+ littersmates, respectively (Figure 2F). Whereas ApcMin/+ Zfp277+/– mice had 3.0 ± 0.7 colon tumors that were 3 mm
in diameter or larger, \( \text{Apc}^{\text{Min}}/\text{Zfp277}^{+/–} \) and \( \text{Apc}^{\text{Min}}/\text{Zfp277}^{–/–} \) mice had only 1.1 ± 0.4 and 0.1 ± 0.1 tumors within this size range, respectively (Figure 2G). These striking changes in both tumor number and size support a role for Zfp277 in both tumor initiation and progression.

Zfp277 deficiency substantially prolongs \( \text{Apc}^{\text{Min}}/\text{Apc}^{\text{Min}} \) mouse survival. As shown in Supplemental Figure 6C, by 15 weeks of age, whereas female \( \text{Apc}^{\text{Min}}/\text{Zfp277}^{+/–} \) mice weighed the same as \( \text{Apc}^{+/+}/\text{Zfp277}^{+/+} \) mice, they weighed significantly more than \( \text{Apc}^{\text{Min}}/\text{Zfp277}^{–/–} \) mice. As shown in Supplemental Figure 6D, at 15 weeks,
whereas $Apc^{+/+}$ Zfp277+/+, Zfp277−/−, and $Apc^{Min/+}$ Zfp277−/− mice had normal hematocrits, both male and female $Apc^{Min/+}$ littermate mice had become severely anemic.

As shown in Figure 2H, both male and female $Apc^{Min/+}$ Zfp277−/− mice lived much longer than $Apc^{Min/+}$ Zfp277+/+ littermates; mean survival for male $Apc^{Min/+}$ Zfp277+/+ mice ($n = 11$) was 207 days versus 299 days for male $Apc^{Min/+}$ Zfp277−/− mice ($n = 10$, $P < 0.001$). Notably, this survival advantage was even more striking for female Zfp277-deficient mice; mean survival for female $Apc^{Min/+}$ Zfp277−/− littermate controls ($n = 12$) was 168 days versus 325 days for female $Apc^{Min/+}$ Zfp277+/+ mice ($n = 9$) — an almost 2-fold difference ($P < 0.001$). In contrast, the mean survival of male $Apc^{Min/+}$ and female $Apc^{Min/+}$ mice ($P = 0.692$) and the survival of female versus male $Apc^{Min/+}$ Zfp277−/− mice were not significantly different ($P = 0.21$). Likewise, we did not observe differences in the survival of male and female WT or Zfp277-KO mice (data not shown).

Zfp277 promotes intestinal epithelial cell proliferation. To determine if changes in cell proliferation or apoptosis played roles in attenuating tumorigenesis and progression in Zfp277-deficient mice, we measured (a) Ki67 and BrdU staining and (b) caspase-3 activation, respectively. We found markedly reduced indices of cell proliferation in both the small intestine (Figure 3, A and E) and colon (Figure 3C) of Zfp277-deficient mice. TAC proliferation, measured by BrdU staining, was reduced in both the small intestine (Figure 3B) and colon (Figure 3D) of Zfp277-deficient mice possessing either WT or mutated $Apc$. Zfp277 deficiency did not alter apoptosis; we detected similar numbers of apoptotic cells in adenomas and normal intestinal tissues from $Apc^{Min/+}$ and $Apc^{Min/+}$ Zfp277−/− mice (Supplemental Figure 7).

ZNF277 promotes cell proliferation in vitro and in vivo. To gain additional functional and mechanistic insights into the role of ZNF277 in Zfp277+, we examined the effects of modulating ZNF277 expression in human colon cancer cells. As shown in Figure 4, A and B, in HT29, H508, and SNUC4 cells, small interfering RNA (siRNA) ZNF277 knockdown inhibited in vitro cell proliferation. As anticipated from these findings, overexpressing ZNF277 in HT29 cells increased cell proliferation (Figure 4, C and D). To determine whether ZNF277 regulates cell proliferation in nonintestinal epithelial cells, we examined the effect of ZNF277 knockdown on HEK293 cells, a commonly used kidney epithelial cell line. As shown in Figure 4, E and F, CRISPR KO of ZNF277 using guide RNAs (gRNAs) significantly attenuated cell proliferation in HEK293 epithelial cells, indicating that ZNF277 can regulate the proliferation of multiple epithelial cell types.

To determine whether ZNF277 deficiency affects cell proliferation in vivo, we examined the effect of CRISPR KO of ZNF277 on the growth of human colon cancer cell xenografts. First, we generated a pooled ZNF277-KO cell line using ZNF277 CRISPR gRNA constructs in HT29 cells (see Methods). As shown in Figure 5, A and B, respectively, CRISPR KO of ZNF277 in pooled HT29 cells resulted in negligible ZNF277 protein expression and attenuated cell proliferation. Over the 3 weeks after cells were injected into the flanks of nude mice, compared with xenografts created using control cells, we observed greatly diminished ZNF277 CRISPR xenograft volumes and weights (Figure 5, C–E). In addition — as shown in Figure 5, F and G, respectively — IHC and immunoblots revealed robustly increased p21 expression levels in xenografts generated from CRISPR ZNF277-KO cells. Interestingly, p53 protein expression was undetectable in xenografts with low ZNF277 expression (Figure 5, F and G). Collectively, these results suggest that ZNF277 promotes intestinal epithelial cell proliferation, in part by inhibiting p21 expression.

ZNF277 is a transcriptional target of β-catenin. The Wnt signaling pathway plays an instrumental role in regulating intestinal epithelial homeostasis; β-catenin is the most prominent downstream effector of Wnt signaling (29–32). To determine whether β-catenin regulates ZNF277 expression, we examined the effect of β-catenin knockdown. As shown in Figure 6A, in 3 human colon cancer cell lines, siRNA knockdown of β-catenin (CTNNB1) dose-dependently reduced ZNF277 protein expression. In control experiments, ZNF277 knockdown did not alter β-catenin expression in either HT29 or H508 cells (Figure 6B). As anticipated from these findings, Zfp277 protein levels were increased in the colons of $Apc^{Min/+}$ mice with activated β-catenin signaling (Figure 6C). Collectively, these results indicate that ZNF277 expression is regulated by Wnt/β-catenin signaling.

Canonical actions of β-catenin require it to form a transcriptional complex with T cell factor (TCF) and lymphoid enhancing factor (LEF) (29). We used ChIP assays to determine if such a complex could regulate ZNF277 transcription by binding to its promoter. Consensus TCF/LEF binding sites in human colon cancer cells contain the CTTTG(A/T) (A/T) sequence (33). Four consensus TCF/LEF binding sites are located within 5.0 kb of the 5′ ZNF277 promoter from the transcriptional start site: 5′GCTTTGTAAAA at -265, 5′CCTTTGTTG at -1390, 5′ACTTTGAAAG at -2012 and 5′ACTTTGACACCTTGATC at -4762. To identify the binding site(s) for β-catenin/TCF/LEF in the ZNF277 promoter in HT29 human colon cancer cells with robust β-catenin and ZNF277 expression, we performed ChIP assays using quantitative PCR (qPCR)
primers for each potential site (Supplemental Table 2) with ChIP-grade anti–β-catenin antibodies. As shown in Figure 6D, ChIP qPCR results indicated 6.5- and 4.5-fold enhanced binding by β-catenin at the –265 and –2012 sites, respectively, but we failed to detect enhanced binding at the other 2 sites. These results support the conclusion that, in human colon cancer cells, β-catenin regulates ZNF277 transcription. Potential cotranscriptional factors of ZNF277 in TACs remain to be identified.

ZNF277 is associated with B lymphoma Mo-MLV insertion region 1 homolog (BMI1). In mouse embryonic fibroblasts, Negishi et al. showed physical association of Bmi1 with the N-terminal domain (1–58 aa) of Zfp277 (21). BMI1 is an important component of polycomb group complex 1 (PRC1). To determine whether ZNF277 interacts physically with BMI1 in humans, we performed co-IP using protein extracts from

![Figure 3: Zfp277 deficiency attenuates epithelial cell proliferation in murine small intestine and colon.](image)

(A) Zfp277 deficiency reduces small intestinal Ki67 expression. Brackets indicate staining at the base of the crypts. Representative Ki67 IHC staining of ileal segments from mice with the indicated genotypes. (B) Numbers of Ki67+ cells per ileal crypt in mice of the indicated genotype. (C) Representative Ki67 IHC staining of distal colons from mice with the indicated genotype. (D) Numbers of Ki67+ cells per colon crypt. All mice were 15-week-old males. Scale bar: 100 μM. Values represent mean ± SEM from 3 mice for each genotype. (E) Representative confocal microscopy images of BrdU (green; 2-hour labeling) and Ki67 (red) IF staining and merged images of BrdU, Ki67, and DAPI of ileal segments from 15-week-old male mice with the indicated genotype. Arrows indicate fluorescence signals at the base of the crypts. Scale bar: 100 μM.
SNUC4 human colon cancer cells; these experiments showed robust ZNF277 and BMI1 protein expression. As shown in Figure 6E, we detected abundant ZNF277 in BMI1 immunoprecipitates, indicating that ZNF277 forms a protein complex with BMI1. This finding suggests that ZNF277 is a component of the PRC1 protein complex in human colon cancer cells. In addition, this physical ZNF277-BMI1 association provides a potential target for small molecule inhibitors to disrupt this protein-protein interaction.

ZNF277 inhibits cellular senescence in vitro by repressing p21WAF1 expression. Zfp277 regulates mouse embryonic fibroblast senescence (21). To gain additional insights into the role of ZNF277 in modulating cellular senescence, we examined the effect of ZNF277 CRISPR KO in colon cancer cells on markers for the senescence-associated secretory phenotype. As shown in Figure 7A, using ZNF277 CRISPR gRNA
Figure 5. ZNF277 deficiency attenuates xenograft growth. (A) Immunoblots of HT29 cell extracts without (control) or with CRISPR knockdown of ZNF277 expression. β-Actin was used as a loading control. (B) ZNF277 deficiency attenuates HT29 cell proliferation in vitro from 7 separate experiments. (C) ZNF277 deficiency attenuates xenograft growth. Time-course reveals reduced volume of ZNF277 CRISPR HT29 cell–derived xenografts (n = 8) compared with control xenografts (n = 7). (D) Representative images of s.c. and excised xenografts from HT29 ZNF277 CRISPR versus control cells. Arrows and arrowheads indicate control and HT29 ZNF277 CRISPR xenografts, respectively. (E) Reduced weights of xenografts with ZNF277 deficiency (n = 8). *P < 0.01 versus controls (n = 7); 2-tailed Student’s t test. (F) Representative microscopic images of control and ZNF277-deficient xenografts stained for H&E, p21WAF1, and p53. (G) ZNF277, p21WAF1, p53, and β-catenin immunoblots of proteins extracted from ZNF277 CRISPR cell– and control cell–derived tumors (2 separate tumors from each group). Values represent mean ± SD. Scale bar: 100 μM.

constructs, we generated several ZNF277 CRISPR KO cell lines in HT29 (pools) and HEK293 cells (clones). Compared with control cell pools derived from nonspecific (scrambled) gRNA, this greatly reduced or completely abolished ZNF277 protein expression.

In human HT29 and H508 colon cancer cells and HEK293 cells with siRNA- or CRISPR-induced reduction of ZNF277 expression, we detected strikingly increased p21WAF1 levels (Figure 7A). Compared with WT mice, as shown in Figure 7B, we also detected robustly increased murine p21WAF1 expression in the colon of both ApcMin/+ and Zfp277-deficient mice, whereas p27 levels were not affected. In HT29 cells with reduced ZNF277 expression, we detected no changes in the expression of p27 or p57, other members of the p21WAF1 family (Figure 7A); we also detected no changes in p16INK4A or p14ARF expression (not shown). In HT29 cells, ZNF277 knockdown induced more than a 2-fold increased p21WAF1 mRNA levels (Figure 7D). In addition, as shown in Figure 7C, p53 knockdown in HT29 cells increased p21WAF1 expression and did not alter the upregulation of p21WAF1 observed after ZNF277 knockdown. These results suggest that, in colon cancer, ZNF277 selectively regulates p21WAF1 expression independently of p53.

As anticipated from the above findings, CRISPR KO of ZNF277 in HT29 cells increased β-galactosidase staining, a marker of augmented senescence (Figure 7E). As shown in Figure 7F, compared with the small intestine of Apc+/+ mice and adenomas from ApcMin/+ mice, we detected increased p21WAF1 expression in...
the small intestine of Zfp277−/− mice and in adenomas from ApcMin/+ Zfp277−/− mice. Collectively, these results suggest that ZNF277/Zfp277 represses p21WAF1 expression, at least partially via transcriptional regulation, to inhibit senescence. Our findings are consistent with observations by el-Deiry et al. that human intestines express p21WAF1 only in nonproliferating epithelial cells (i.e., differentiated enterocytes with absent ZNF277 expression) but not in TACs that express ZNF277 (34).

RNA-Seq reveals ZNF277/Zfp277 transcriptional targets. To identify murine Zfp277 transcriptional target genes, we performed RNA-Seq using RNA isolated from normal colonic mucosa from 8-week-old WT and Zfp277−/− male littermate mice (n = 3 for both). As shown by the heatmap in Figure 8A, based on a FDR cutoff of 0.05 and a log2 fold change (LFC) ≥ ± 1, in Zfp277-deficient versus WT mice, 758 genes were upregulated and 263 downregulated. Several homeobox genes belonging to the posterior Hoxd gene cluster (Figure 8, B and C) were dramatically increased, including Hoxd13, EVX2, Hoxd12,
Hoxd11, and Hoxd10 (all greater than 6-fold). Of the top 16 upregulated nonimmunoglobulin genes, 4 belonged to the posterior Hoxd gene cluster. Because read counts showed that the Hoxd13 transcript was more abundant than the other 3 Hoxd cluster genes, we chose Hoxd13 for more detailed characterization.

As shown in Figure 8D, using qPCR, we observed more than a 30-fold increase in Hoxd13 mRNA levels in colonic mucosa from Zfp277-deficient mice, confirming the RNA-Seq results. Consistent with these results, we detected increased HOXD13 protein expression in ZNF277-deficient xenografts (Figure 8E). Collectively, these results identify posterior Hoxd genes as ZNF277/Zfp277 transcriptional targets that are normally repressed in colon epithelium.

As shown in Supplemental Table 3, gene pathway analysis using DAVID 6.8 identified several enriched functionally related gene groups, including anterior-posterior pattern specification, pathway in cancer, regulation of MAPK, ERK1/2 cascades, canonical Wnt signaling, and cytokine-cytokine receptor interaction involved in immune system response/process. These findings suggest that ZNF277/Zfp277 has a broad range of transcriptional target genes and may play important roles at different stages of development and cancer progression. The observation that murine Zfp277-deficient mice are healthy and fertile (21) suggests that other transcription factors may replace Zfp277 function when it is absent.

Figure 7. ZNF277 deficiency augments p21WAF1 expression. (A) siRNA and CRISPR knockdown of ZNF277 expression augments p21WAF1 levels in HT29, H508, and HEK293 cells. β-Actin was used as a loading control. (B) Levels of murine p21WAF1 expression are augmented in colon tissue extracts from ApcMin/+ and Zfp277 –/– mice. Experiments were performed using tissues from 2 separate 8-week-old mice of each genotype. (C) Upregulated p21WAF1 expression after p53 and ZNF277 knockdown in HT29 cells. All siRNAs were 25 nM, except lane 4 (50 nM). (D) ZNF277 knockdown augments p21WAF1 mRNA levels in HT29 cells. Data are shown as mean ± SEM from 3 separate experiments. *P < 0.01 (2-tailed Student’s t test). (E) Zfp277 deficiency stimulates cellular senescence. β-Galactosidase staining in control HT29 cells (A) and HT29 cells with CRISPR knockdown of ZNF277 (B). Scale bar: 50 μM. (F) Zfp277 deficiency increases p21WAF1 expression. IHC of p21WAF1 in the normal small intestine of WT (A) and Zfp277 –/– (B) mice, as well as in small intestine adenomas from Zfp277 –/– ApcMin/+ (C) and Zfp277 –/– ApcMin/+ (D) mice. Arrows indicate adenomas. Scale bar: 100 μM.
To identify human ZNF277 target genes in cancer cells, we performed RNA-Seq using RNA isolated from HT29 cells with and without ZNF277 CRISPR KO (n = 3 for both). As shown in Supplemental Table 4, based on a FDR cutoff of 0.05 and a LFC ≥ ± 1, in ZNF277-deficient compared with control HT29 cells, 657 genes were upregulated and 93 were downregulated. The top 4 altered KEGG signaling pathways in cancer included increased expression of p21 WAF1, pathways regulating pluripotency in stem cells, proteoglycans, and PI3K/AKT signaling. These findings provide additional evidence that ZNF277 plays a prominent role in human CRC by transcriptional regulation of many genes involved in cancer progression, including p21WAF1 and Wnt signaling. Consistent with our Zfp277 RNA-Seq findings, many HOX genes were identified as ZNF277 targets, revealing Hox genes as transcriptional targets of Zfp277, most likely playing a role in early development.

**Discussion**

Intestinal epithelial homeostasis is maintained by intestinal stem and early progenitor TACs (35, 36). Stem cells can divide asymmetrically and generate 2 daughter cells, a stem cell and pluripotent TAC. TACs proliferate rapidly to generate differentiated cell types, including goblet cells and functional enterocytes, thereby stimulating cell proliferation and attenuating cell senescence, as well as enhancing tumorigenesis and progressive neoplasia.
these mechanisms is the expression by TACs of a unique set of genes, including the cell proliferation marker Ki67 and protooncogene MYC. MYC, a prominent oncogene and transcription factor, is also expressed primarily in TACs, plays an essential role in early embryonic development, and acts as a protooncogene in tumor cells (38). Myc, the mouse homologue of human MYC, is expressed only in intestinal TACs and is required for intestinal crypt formation but dispensable for epithelial homeostasis (39). A Myc-null mutation results in embryonic lethality, whereas Apc\textsuperscript{Min/−} mice in which Myc is haploinsufficient survive longer than control littermates due to delayed intestinal adenoma formation (40).

Our findings support a key role for ZNF277, an evolutionarily conserved zinc finger transcription factor, in cell senescence and oncogenesis. We identified ZNF277/Zfp277 as a potentially novel TAC-specific transcription factor that promotes TAC proliferation and intestinal tumorigenesis, thereby shedding new light on the regulation of TACs, a crucial link between intestinal stem cells and differentiated enterocytes. Indeed, the expression pattern and functions of ZNF277 appear to closely mimic those of MYC. The mouse ENCODE transcriptome database (NCBI Gene; https://www.ncbi.nlm.nih.gov/) and Human Protein Atlas (27) reveal ZNF277/Zfp277 mRNA and protein expression in a wide variety of normal murine and human tissues, including the intestines, with strong nuclear staining in 22 of 24 human colon tumors. Previously, using 12 archived human colon cancer tissues, we detected ZNF277 mRNA overexpression in cancer compared with adjacent normal colon mucosa (17). Likewise, in an archived set of 23 formalin-fixed paraffin-embedded human colon cancer tissues, we used IHC to detect ZNF277 protein overexpression in tumors compared with adjacent normal colon from the same person; ZNF277 was primarily localized to tumor cell nuclei (17). These findings are supported by in silico analysis of the NCBI Gene Expression Omnibus (GEO) profile database that revealed upregulated ZNF277 expression in 33 of 34 colon tumors (97%) compared with adjacent normal colon (17, 41).

In the present study, analysis of ZNF277 expression using 4 online cancer databases revealed that both ZNF277 transcript and protein levels were increased in CRC specimens from men and women of all ages and races, with a variety of cancer stages and lymph node metastasis. Per cBioPortal, ZNF277 gene mutations causing mostly missense changes were identified in approximately 2.5% of more than 3000 colon cancer specimens. Because we identified ZNF277 as a potential colon cancer oncogene, it is not surprising that its rates of mutation in colon cancer (mostly loss of function) are not as high as reported for tumor suppressors like APC (42) and p53 (43). Indeed, our studies in a murine model of genetic colon cancer, Apc\textsuperscript{Min/−} mice, supports an important role for ZNF277/Zfp277 in intestinal neoplasia. Zfp277 deficiency profoundly reduced tumor formation in both the small intestine and colon of Apc\textsuperscript{Min/−} mice. Reduced tumor formation was associated with strikingly prolonged survival in both male and female mice, suggesting that ZNF277 may be an important CRC oncogene.

The polycomb proteins, polycomb repressive complex 1 and 2 (PRC1 and PRC2), play pivotal roles in stem cell fate determination and development, primarily by maintaining the repressed state of target genes via histone modifications (44–47). PRCs are important gatekeepers that establish and maintain cell identity. In addition to pluripotent embryonic stem cells, PRCs and associated proteins — such as BMI1, a polycomb ring finger protein — also function in tissue-specific stem cells. PRC1 preserves intestinal stem cell identity by suppressing non-lineage-specific transcription factors, thereby sustaining Wnt/\(\beta\)-catenin transcriptional activity (48). In mouse embryonic fibroblasts, Negishi et al. showed that Zfp277 mediates transcriptional repression of p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} via interaction with Bmi1 in the PRC1 complex (21). Loss of Zfp277 in mouse embryonic fibroblasts caused dissociation of PRC1 proteins from the Ink4A/ARF locus, resulting in premature senescence associated with derepressed p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} expression (21). Liu et al. showed that mouse embryonic fibroblasts with strong p16\textsuperscript{INK4A} promoter activation in vivo display features of senescence (49). The INK/ARF locus, which generates tumor suppressors p16 INK4A and p15 INK4B, is a pivotal node between senescence and cancer (23, 24). p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} bind to CDK4/6 to induce cell cycle arrest via retinoblastoma protein, whereas p14\textsuperscript{ARF}/19ARF arrests the cell cycle by stabilizing p53, trapping MDM2, and increasing expression of p21\textsuperscript{WAF1}, an important cell cycle inhibitor and tumor suppressor for many cancers including those of the colon. Here, we showed that ZNF277 also interacts with BMI1 in human colon cancer cells, although the functional significance of this interaction and whether ZNF277 is a component of the PRC1 complex in intestinal epithelial cells remains to be determined.

We found that ZNF277 inhibits cellular senescence by repressing p21\textsuperscript{WAF1} expression in human colon cancer cells. p21\textsuperscript{WAF1} is a potent cell cycle inhibitor and tumor suppressor (50, 51) whose expression is lost in ~80% of colon cancers (52); p21\textsuperscript{WAF1} loss correlates with a poor prognosis (50). p21\textsuperscript{WAF1}-deficient mice develop spontaneous intestinal tumors (53), and in human fibrosarcoma cells, p21\textsuperscript{WAF1} overexpression
induces growth arrest and senescence by inhibiting cell cycle progression and DNA repair (54). p21WAF1 is also a critical determinant of intestinal cell responses to the nonsteroidal antiinflammatory drug sulindac; in ApcMin/+ mice, inactivating p21WAF1 eliminates the ability of sulindac to inhibit intestinal tumor formation (55). Many FDA-approved anticancer drugs, including histone deacetylase inhibitors, function at least in part by inducing p21WAF1 expression. Campaner et al. showed in various cell types, including MEFs and a p53-null human cancer cell line, that p21WAF1 suppresses cellular senescence induced by MYC activation (56). Here, we found that ZNF277 inhibits p21WAF1 expression in human CRC cells by a p53-independent mechanism. These findings are also consistent with our finding that increased ZNF277 expression also is a critical determinant of intestinal cell responses to the nonsteroidal antiinflammatory drug sulindac; part by inducing p21WAF1 expression. Campaner et al. showed in various cell types, including MEFs and a p53-null human cancer cell line, that p21WAF1 suppresses cellular senescence induced by MYC activation (56). Here, we found that ZNF277 inhibits p21WAF1 expression in human CRC cells by a p53-independent mechanism. These findings are also consistent with our finding that increased ZNF277 levels in CRC correlate significantly with reduced p21WAF1 (CDKN1A) expression. Whether ZNF277-mediated suppression of p21WAF1 expression depends on PRC1 remains to be determined.

To define how silencing ZNF277 affects transcriptomes related to proliferation in human CRC cells and to identify potential ZNF277 target genes in CRC, we performed RNA-Seq in HT29 cells with and without ZNF277 deficiency. The leading pathways impacted by ZNF277 deficiency included increased p21WAF1 expression in cancer stem cell signaling, proteoglycans in cancer, and PI3K/AKT signaling. These findings reveal that ZNF277 may regulate CRC progression via a variety of molecular mechanisms involving p21WAF1, intestinal stem cells, and Wnt/β-catenin and other cancer signaling pathways. Notably, HOX genes were again identified and confirmed the key role of ZNF/Zfp277 in development and cancer progression. A limitation of an RNA-Seq–based approach is that genes identified by RNA-Seq differential gene expression may not be direct transcriptional targets of Zfp277. This limitation will be overcome when ChIP quality anti-ZNF/Zfp277 antibodies become available for direct ChIP-Seq.

Wnt/β-catenin signaling is essential for normal intestinal homeostasis. Hence, the major Wnt signaling components and key downstream targets such as APC, CTNNB1, and MYC are challenging therapeutic targets. In contrast, several observations suggest that ZNF277 may be a more promising and druggable target. First, Zfp277-deficient mice are healthy (21), suggesting a limited risk of off-target toxicity. Second, the interaction between ZNF277 and BMI1 in the PRC1 complex provides a useful screening tool to test potential small molecule inhibitors of ZNF277. Lastly, inhibiting ZNF277 may increase p21WAF1 expression and promote cell cycle arrest in cancer cells. As summarized by the illustration in Figure 8F, the present work newly identifies ZNF277/Zfp277 as an intestinal TAC marker and colon cancer oncogene. ZNF277 modulates intestinal β-catenin signaling and tumorigenesis by acting as a key transcription factor and component of the PRC1 complex that regulates cell proliferation and senescence. It would be of interest to determine...
whether the oncogenic effects of ZNF277/Zfp277 depend solely on its expression in intestinal epithelial cells; this question will be addressed in future studies by examining the effects on intestinal tumorigenesis in mice with conditional intestinal epithelial cell–selective Zfp277 deficiency.

**Methods**

**Chemicals**

Chemicals were purchased from Sigma-Aldrich and cell culture media were purchased from Thermo Fisher Scientific.

**Online cancer databases**

*Oncomine database and platform.* Oncomine was an online cancer microarray database and an integrated data-mining platform. ZNF277 mRNA levels in colon cancer tumor specimens were compared with normal surrounding tissues. The threshold and threshold P value used were 1.5-fold and 0.001, respectively.

*GEPIA server.* GEPIA (gepia.cancer-pku.cn) is an interactive web server for analyzing RNA-Seq expression data using tumor and normal samples from the National Cancer Institute Cancer Genome Atlas Program (TCGA) database and the Genotype-Tissue Expression (GTEx) project (https://www.genome.gov/Funded-Programs-Projects/Genotype-Tissue-Expression-Project). It provides customizable functions according to cancer types, including differential gene expression. The Colon Adenocarcinoma (COAD) data sets were used in our analysis of ZNF277 transcript.

*The Human Protein Atlas program.* This online database (https://www.proteinatlas.org/) provides information on human proteins in cells, tissues, and organs using integration of various omics technologies, including antibody-based imaging, mass spectrometry–based proteomics, transcriptomics, and systems biology.

*UALCAN server.* UALCAN (ualcan.path.uab.edu) is an interactive web resource for analyzing cancer OMICS data, and it provides easy access to OMICS data (TCGA and MET500). The TCGA COAD data set was used in our analysis of ZNF277 transcript.

*Experimental animals.* Six- to 8-week-old WT C57BL/6J and Apc<sup>Min</sup>/+ (C57BL/6J-Apc<sup>Min</sup>/J) mice (The Jackson Laboratory, stock no. 002020) with female Apc<sup>Min/+</sup>. Six- to 8-week-old WT C57BL/6J and Apc<sup>Min</sup>/+ Zfp277+/+ male mice were purchased from the Riken BioResource Research Center. We generated WT, Apc<sup>Min</sup>/+ Zfp277<sup>−/−</sup>, and Apc<sup>Min</sup>/+ Zfp277<sup>+/−</sup> mice by breeding Apc<sup>Min</sup>/+ Zfp277<sup>−/−</sup> male mice with Zfp277<sup>−/−</sup> female mice. Briefly, we first crossed male Apc<sup>Min</sup>/+ (C57BL/6J-Apc<sup>Min</sup>/J) mice (The Jackson Laboratory, stock no. 002020) with female Zfp277<sup>−/−</sup> mice and then crossed Apc<sup>Min</sup>/+ Zfp277<sup>−/−</sup> male mice with Zfp277<sup>−/−</sup> female mice to generate Apc<sup>Min</sup>/+ Zfp277<sup>−/−</sup>, Apc<sup>Min</sup>/+ Zfp277<sup>+/−</sup>, and Apc<sup>Min</sup>/+ Zfp277<sup>+/+</sup> mice. Genotyping for Zfp277<sup>−/−</sup> and Apc<sup>Min</sup> status was performed using tail genomic DNA per instructions from the Riken BioResource Research Center and The Jackson Laboratory, respectively (15–17, 21), and Zfp277 protein deficiency was confirmed by immunoblotting. Using different anti-Zfp277 antibodies directed against various portions of the Zfp277 protein, including the N-terminus, we failed to detect Zfp277 protein in selected tissues from Zfp277<sup>−/−</sup> mice including liver (Supplemental Figure 3A) and colon (Figure 6C). At 15 weeks of age, mice were euthanized, the intestines were harvested, and tumors were counted as previously described (15–17, 69). After counting tumors, tissues were fixed in formalin and stained for H&E in Swiss Roll configurations (Supplemental Figure 3, B–D). For experiments involving immunoblotting and qPCR, we used 6- to 8-week-old mice. To characterize intestinal tumors, we used 15-week-old mice. We housed mice under identical conditions in the same pathogen-free room with free access to commercial rodent chow and water; we allowed mice to acclimatize in the vivarium for 2 weeks prior to breeding. For survival studies, mice were observed daily and euthanized when moribund or achieving predetermined endpoints with at least 4 of the following signs of severe distress: dehydration, rectal prolapse or bleeding, anorexia, weight loss >20% initial body weight, hunched posture, lethargy, persistent recumbency, dyspnea, ruffled fur, and inability to rise or ambulate.

**Hematocrit measurements.** At the age 15 weeks, blood was obtained from 3 male and 3 female mice by cardiac puncture after mice were anesthetized preceding euthanasia as described (70). After centrifuging heparin-coated microhematocrit tubes at 12,000g for 30 seconds at room temperature, the ratio of the volume occupied by RBCs to the volume of whole blood was measured and expressed as a fraction in percentage.

**Tumor measurement, histological, and IHC analyses.** At age 15 weeks, experimental mice were weighed and euthanized. The small intestine and colon were harvested, and segments were opened longitudinally and placed flat with the luminal surface facing up on transparent films. Tumors were identified by visual inspection and counted using a Nikon SMZ1500 dissecting microscope by investigators masked to genotypes and
experimental groups. Colon tumor sizes were measured using calipers. For histological analysis, tissues were fixed in 4% paraformaldehyde and paraffin-embedded in Swiss Roll configurations (Supplemental Figure 3D). Sections (5 μm) were stained with H&E (Supplemental Figure 3B). For IHC and immunofluorescent analysis, we used primary and secondary antibodies listed in Supplemental Table 1, including names of manufacturers, antibody catalog numbers, and lot numbers. Immunoglobulins and secondary antibodies were used as negative controls.

In vivo and in vitro measurement of cell proliferation and apoptosis. Active proliferating intestinal epithelial cells were labeled with BrdU 2 hours before euthanasia (15). Briefly, to label S-phase cells, 2 hours before euthanasia, mice were administered an i.p. injection of 50 mg/kg BrdU (Sigma-Aldrich), a marker of cell proliferation. The percentage of BrdU+ cells was determined after immunostaining with anti-BrdU antibody (Supplemental Table 1). We also performed Ki67 IHC staining to measure actively proliferating cells; we counted Ki67+ nuclei in intestinal crypts (data expressed as percentage of total cells that were Ki67+ in each crypt). A total of 10 random crypts per mouse were examined in tissue sections from 3 mice of each genotype. To identify apoptotic cells, we immunostained tissue sections with anti–activated caspase-3 antibody (Supplemental Table 1). Only complete crypts were evaluated, and investigators were masked to genotype and experimental groups.

Murine enteroid cultures. Small intestinal enteroids were generated from primary tissues using the mouse Intesticult Organoid Growth Medium (Stem Cell Technologies [SCT]; catalog 06005) and Corning Matrigel Matrix (Corning Inc.; catalog 356231) per SCT protocol. Enteroids were maintained with media changes every 2–3 days and passaging when growth reached appropriate densities. ROCK 1/2 inhibitor (Y-27632) at 10 μM was added for the first 2 days after generation from primary tissue.

Cell lines and cell culture. HT29, H508, and SNUC4 human colon cancer cells and HEK293 human kidney epithelial cells were purchased from the American Type Culture Collection (ATCC) and maintained in growth media supplemented with 10% FBS. HT29 and SNUC4 cells were grown in McCoy’s 5A Media (Thermo Fisher Scientific). H508 and HEK293 cells were grown in RPMI-1640 and Leibovitz’s L-15 media, respectively. We passaged adherent cells weekly at subconfluence after trypsinization and maintained cultures in incubators at 37°C in an atmosphere of 5% CO2 and 95% air. All cell lines were authenticated a minimum of every 6 months by short tandem repeat sequencing in the UMB Genomic Core.

Plasmids and transient transfection. Transient ZNF277 overexpression in human HT29 colon cancer cells was achieved by transfecting 1 μg of plasmid pcDNA3.1-ZNF277 (clone OHu03772; GenScript) for 48 hours. Nuclear lysates were then analyzed for ZNF277 levels using immunoblotting and compared with HT29 cells transfected with pcDNA3.1.

Measurement of in vitro cell proliferation. Cells were seeded in 96-well plates at approximately 10% confluence and allowed to attach for 24 hours. After an additional 24 hours, cell proliferation was determined by adding 20 μL CellTiter 96 AQueous One solution (Promega) to each well. After a 1- to 2-hour incubation at 37°C, absorbance was measured at 490 nm using a 96-well microtiter plate reader (SpectraMax384).

siRNA transfection. We purchased the following siRNAs from Invitrogen: Silencer Select Negative Control No. 1 siRNA, ZNF277 SILEE SELECT SIRNA ASSAY ID S22065, and CTNNB1 SILEER SIRNA ASSAY ID 146154. For siRNA transfection experiments, human colon cancer cells and HEK293 cells were seeded in 6- and 96-well plates at approximately 10% confluence and incubated at 37°C for 24 hours. siRNA duplex oligos targeting ZNF277 or nontargeting control oligos were transfected into cells with Lipofectamine Transfection Agent (Thermo Fisher Scientific) according to the manufacturer’s instructions. One to 2 days following transfections, cells in 96-well plates were used for cell proliferation assay, and cells in the 6-well plates were harvested for immunoblotting to confirm and quantify siRNA knockdown.

Generation of CRISPR KO cell lines. We purchased 2 ZNF277 CRISPR gRNA plasmids (project name U4370DK190-1; clone C93266, gRNA TTGCAAGTTTACCATGTGTC; project name U4370DK190-2; clone C93269, gRNA AGACAGTAAGCATTGTATCC) from GenScript. The cloning vector for both gRNAs is the pS.pCas9 BB-2A-Puro vector (PX459) v2.0 plasmid. After verifying these gRNA plasmid sequences by DNA-Seq, we used SuperFect transfection reagent (QIAGEN, catalog 30130S) to transfect HT29 and HEK 293 cells and used HT29 and HEK 293 cells transfected with the pS.pCas9 BB-2A-Puro vector as controls. Stably transfected cells were selected using 10 μg/mL puromycin based on killing curves. Finally, we used immunoblotting to determine ZNF277 protein levels. Using the protocol above, we generated several pools of mixed ZNF277 CRISPR cells with low or absent ZNF277 expression in HT29 and HEK 293 cells.
**Generation of human colon cancer xenografts.** To generate xenografts, 1 million pooled CRISPR ZNF277-KO HT29 cells (pool no. 1) or nonspecific CRISPR HT29 cells were injected s.c. into both flanks of 6-week-old female nude mice (stock no. 002019; Homozygous for Foxn1<sup>n</sup>; The Jackson Laboratory) in 100 μL mixtures (50% Matrigel) as previously described (71). ZNF277 CRISPR HT29 cells were injected into the left flanks of nude mice, whereas the nonspecific CRISPR HT29 cells were injected into the right flanks of the same nude mouse. Tumor size was measured biweekly with calipers and tumor volumes calculated using the formula: tumor volume = (length × width<sup>2</sup>) / 2. Mouse body weight was measured twice per week. At the end of the study, xenografts were excised, photographed, and weighed. Tumors were bisected and half stored in liquid nitrogen for molecular studies and half fixed for IHC studies.

**Antibodies and immunoblotting.** Immunoblotting were performed as described previously (72). To ensure equal loading of protein samples, protein concentrations of cell lysates were determined using the BCA Protein Assay kit (catalog 23227; Thermo Fisher Scientific). Protein (10–20 μg) was loaded into each lane. Antibodies used in immunoblotting, including manufacturers, catalogs, and lot numbers, are listed in Supplemental Table 1. After probing with primary antibodies, immunoblots were incubated with horseradish peroxide-conjugated secondary antibodies and visualized by chemiluminescence (Pierce) using the ChemiDoc Touch Imaging System (Bio-Rad). To avoid saturation areas of the bands and apply the linear ranges only, we performed semiquantifications using the Quantity One software (Bio-Rad).

**IP.** IP was performed following New England Biolab protocols with minor modifications. Briefly, cells were lysed in RIPA buffer with proteinase and phosphatase inhibitors. Lysates were precleared with magnetic protein G agarose beads (New England Biolabs), incubated with 2 μg anti-BMI1 antibody (Supplemental Table 1) overnight. Normal mouse IgG (2 μg) was used as a negative control. Anti-ZNF277 and anti-BMI1 antibodies were used to detect ZNF277 and BMI1, respectively. IP was performed using actively growing cells with 10% FBS.

**qPCR and RT-PCR.** We performed qPCR and quantification of mRNA levels as described previously (73). We confirmed the specificity of amplifications by melting-curve analysis and calculated relative levels of mRNA according to the standard ΔΔCt method. We normalized expression values by comparison with GAPDH. qPCR and RT-PCR primer sequences are listed in Supplemental Table 2.

**ChIP assays.** We performed ChIP assays using the Pierce Magnetic ChIP Kit (catalog 26157; Thermo Fisher Scientific) per manufacturer’s instructions as previously described (74). Briefly, in vivo crosslinking was performed using 4 million cultured cells using 1% formaldehyde. Cell lysis was performed using buffers containing proteinase inhibitor cocktails. Lysates were then digested with Micrococcal Nuclease (MNase; Pierce Kit, Thermo Fisher Scientific) to generate random DNA fragments from 160 to 320 bp. Chromatin was obtained after brief sonication to rupture nuclei. Aliquots (5 μL) were removed for agarose gel analysis. IP of crosslinked protein/DNA were performed overnight at 4°C using antibodies against mouse β-catenin (Supplemental Table 1), RNA Polymerase II (positive control) and rabbit or goat IgG (negative controls). Elution and reverse crosslinks of protein/DNA complexes to free DNA were performed using the ChIP Elution Buffer without protein kinase K, using a magnet. Eluted DNA was purified using DNA spin columns. qPCR was performed with primers listed in Supplemental Table 2 using eluted DNA and 1% input. Antibodies used in this ChIP assay included mouse monoclonal anti–human β-catenin antibody, rabbit anti–RNA Polymerase II, and mouse IgG (Supplemental Table 1). GAPDH was used as a negative control.

**RNA-Seq.** RNA-Seq was performed by the Genomic Resource Center (GRC) at the Institute for Genome Sciences at the University of Maryland Baltimore. Briefly, we provided high-quality total RNA and submitted the samples to GRC, which then conducted quality control tests, prepared Illumina RNA-Seq libraries, and performed sequencing using the Illumina HiSeq4000 System. Differentially expressed transcripts were identified based on a FDR cutoff of 0.05 and a LFC ≥ ±1. Gene pathway analysis was performed using DAVID Bioinformatics Resources 6.8 (David.ncifcrf.gov). For Zfp277 RNA-Seq, we used RNA isolated from normal colonic mucosa from 8-week-old WT and Zfp277–/– male littermate mice (n = 3 for both). Comparisons were made using WT (control no. 3). For ZNF277 RNA-Seq, we used RNA isolated from control HT29 cells and 3 HT29 cell lines with CRISPR KO (n = 3 for both; Figure 7A). Comparisons were made using control no. 1. The GEO accession for this work is GSE192559.

**Statistics.** Data were expressed as mean ± SEM from a minimum of 3 independent experiments. We performed Student’s 2-tailed t test, Mann-Whitney U test, 1-way ANOVA, with either Tukey’s HSD post hoc or Dunn’s tests using SigmaPlot 13.0 (Systat Software Inc.) and considered P < 0.05 to be statistically significant.
Study approval. For human samples, deidentified preexisting formalin-fixed paraffin-embedded sections from surgically resected human colon cancers and adjacent normal colon or small intestine tissues from the same individuals were obtained from the Department of Pathology at the University of Maryland Baltimore (an exemption for these studies was obtained from the IRB at the University of Maryland Baltimore). This study abides by the Declaration of Helsinki principles. The animal study was approved by the Office of Animal Welfare Assurance at the University of Maryland School of Medicine and by the Research and Development Committee at the VA Maryland Health Care System.

Author contributions
ZP, JL, GX, and SML performed experiments. CBD and HY provided human tissues and expertise in pathology. GX performed online cancer database analysis. GX and JPR designed and supervised the study and wrote the manuscript. All authors reviewed, edited, and approved the submitted manuscript.

Acknowledgments
We thank Lisa Sadzewicz, Heather Huot Creasy, and Carrie McCracken from the GRC at the University of Maryland Institute for Genome Sciences for technical assistance on RNA-Seq and data analysis. We also thank Cindy Xie for assistance with medical illustration (Figure 8F). This work was supported by VA Merit Awards BX002777 (to GX), BX002129 (to JPR), and BX004890 (to JPR) from the US Department of Veterans Affairs Biomedical Laboratory Research and Development Program and T32 DK067872 (to JPR and supporting SML) from the NIH. The contents do not represent the views of the US Department of Veterans Affairs or the United States Government.

Address correspondence to: Jean-Pierre Raufman, Department of Medicine, Division of Gastroenterology and Hepatology, University of Maryland School of Medicine, 22 S. Greene Street, N3W62, Baltimore, Maryland 21201, USA. Phone: 410.328.8728; Email: jraufman@som.umaryland.edu.

41. Khamas A, et al. Screening for epigenetically masked genes in colorectal cancer Using 5-Aza-2'-deoxycytidine, microarray and
40. Ciznadija D, et al. Intestinal adenoma formation and MYC activation are regulated by cooperation between MYB and Wnt sig-
54. Chang BD, et al. Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and
52. Ogino S, et al. Screening for epigenetically masked genes in colorectal cancer Using 5-Aza-2'-deoxycytidine, microarray and
51. Al Bitar S, Gali-Muhtashib H. The role of the cyclin dependent kinase inhibitor p21^{CDK4}\textsuperscript{INKi} in targettig cancer: molecular mecha-
44. Ciznadija D, et al. Intestinal adenoma formation and MYC activation are regulated by cooperation between MYB and Wnt sig-
30. Fevr T, et al. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol Cell Biol.