DNA Hypomethylation Contributes to Genomic Instability and Intestinal Cancer Initiation
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Abstract
Intestinal cancer is a heterogeneous disease driven by genetic mutations and epigenetic changes. Approximately 80% of sporadic colorectal cancers are initiated by mutation and inactivation of the adenomatous polyposis coli (APC) gene, which results in unrestrained intestinal epithelial growth and formation of adenomas. Aberrant DNA methylation promotes cancer progression by the inactivation of tumor suppressor genes via promoter methylation. In addition, global DNA hypomethylation is often seen before the formation of adenomas, suggesting that it contributes to neoplastic transformation. Previous studies employed mice with a hypomorphic mutation in DNA methyltransferase 1 (Dnmt1), which exhibited constitutive global DNA hypomethylation and decreased tumorigenesis in the Apc<sup>Min</sup> mouse model of intestinal cancer. However, the consequences of intestinal epithelial-specific acute hypomethylation during Apc<sup>Min</sup> tumor initiation have not been reported. Using temporally controlled intestinal epithelial-specific gene ablation, we show that total loss of Dnmt1 in the Apc<sup>Min</sup> mouse model of intestinal cancer causes accelerated adenoma initiation. Deletion of Dnmt1 precipitates an acute response characterized by hypomethylation of repetitive elements and genomic instability, which surprisingly is followed by remethylation with time. Two months post-Dnmt1 ablation, mice display increased macroadenoma load, consistent with a role for Dnmt1 and DNA methylation in maintaining genomic stability. These data suggest that DNA hypomethylation plays a previously unappreciated role in intestinal adenoma initiation.

Introduction
Intestinal and colorectal cancers are multifactorial diseases with various inputs including diet, environment, genetic mutations, and epigenetic abnormalities. The disease first manifests as an overproliferation defect in the form of polyps that, if not removed, can progress to precancerous adenomas. Further transition to invasive and metastatic cancer is due to the accumulation of multiple genetic mutations and epigenetic changes that alter gene expression patterns, driving neoplastic transformation and growth (1). However, the extent to which epigenetic modifications, and specifically DNA methylation, contribute to the initiation and progression of intestinal cancer is unclear.

Neoplastic tissues frequently display global DNA hypomethylation, which can occur passively through loss of the maintenance methyltransferase, DNMT1, or actively by TET enzyme-mediated oxidation of methyl cytosine, followed by base excision repair (2–4). Alternatively, some tumors exhibit increased DNA methylation on specific tumor suppressor gene promoters, which requires the de novo DNA methyltransferases, DNMT3A and DNMT3B. Hypomethylation of tumor suppressor gene promoters causes decreased activity of the associated genes and promotes cancer cell growth in vitro (5). Comparison of human colon adenomas to control tissue revealed that although proximal promoter CpG islands are often hypermethylated in tumors, DNA hypomethylation is found genome-wide, including neighboring CpG island shores and repetitive elements (6, 7). Hypomethylated intergenic and intronic regions appear early in the transition from normal to neoplastic (8, 9), suggesting a more important role for DNA hypomethylation in cancer initiation than previously appreciated.

Previously, the role of Dnmt1 in intestinal tumorigenesis was studied in the Apc<sup>Min</sup> mouse model. This paradigm mimics the hereditary human colon cancer syndrome Familial Adenomatous Polyposis (FAP), which is caused by germline mutations of the APC gene (10). LOH at the APC locus causes β-catenin stabilization and unrestricted Wnt activity, resulting in the formation of macroscopic adenomas by 3 months of age (11–13). LOH is also a common triggering mechanism for sporadic intestinal and colorectal tumor development (1), rendering the Apc<sup>Min</sup> mouse a useful model for the study of intestinal cancer initiation.

Multiple hypomorphic Dnmt1 paradigms show complete block of macroscopic tumor formation (14–18). The authors concluded that Dnmt1 and DNA methylation are required for adenoma development in the Apc<sup>Min</sup> model. These studies employed hypomorphic Dnmt1 mice, which express constitutively reduced Dnmt1 levels from earliest development onward in all tissues (15), including nonepithelial cells, such as myofibroblasts. Mesenchymally expressed genes have been shown to be strong modifiers of the Apc<sup>Min</sup> tumor load (19) and could thus contribute to the observed phenotype in the Dnmt1-hypomorphic...
Apc\textsuperscript{Min+/+} intestine. Furthermore, continuous DNA hypomethylation may mask acute affects during the neoplastic transformation, which occurs prior to reported observations of macroadenomas at 6 months of age. Because of these limitations, the precise mechanism of how DNA methylation acts within the intestinal epithelium during adenoma initiation remains to be determined.

To address this important knowledge gap, we employed a temporally controlled, intestinal epithelial-specific gene ablation model to delete Dnmt1 and decrease maintenance DNA methylation. Ablation of Dnmt1 causes an acute phenotype characterized by global DNA hypomethylation, genome instability, and apoptosis. The severe effects of Dnmt1 ablation result in a dramatic increase in adenoma initiation by accelerated LOH at the Apc locus. These results strongly support a role for DNA hypomethylation in chromosomal instability and tumor initiation.

**Materials and Methods**

**Mice**

Dnmt1\textsuperscript{loxP/loxP} mice were provided by Rudolf Jaenisch (20). Villin-Cre-ERT2 mice were received from Sylvia Robine (21), and Apc\textsuperscript{Min+/+} mice from the Jackson Laboratories (10, 12). For Dnmt1 deletion experiments, Cre recombination activity was induced by 3 daily intraperitoneal injections of 1.6 mg tamoxifen (Sigma) in accordance with approved Institutional Animal Care and Use Committee protocols.

**Histology**

Tissues were isolated and fixed using 4% paraformaldehyde in PBS and then embedded in paraffin. Antigen retrieval was performed using the 2100 Antigen-Retriever in Buffer A (Electron Microscopy Sciences) and standard immunostaining procedures were performed for Dnmt1 (Santa Cruz), E-cadherin (BD Transduction Laboratories), Sox9 (Millipore), Ki67 (BD Pharmingen), β-catenin (BD Transduction Laboratories), Cyclin D1 (Biocare Medical), E-cad (Abcam), and pH2AX (Cell Signaling Technology). TUNEL staining was performed using TUNEL Label and Enzyme (Roche) and AlexaFluor 555-aha-dUTP (Life Technologies). Percentages of TUNEL+ nuclei were determined by counting the number of nuclei positive for TUNEL staining and dividing by the total number of nuclei in the respective tissue. All microcopy was performed on a Nikon Eclipse 80i.

**Laser capture microdissection**

Tumor and nontumor epithelial cell DNA was collected using a Leica LMD7000 Laser Microdissection microscope and the Arcturus PicoPure DNA Isolation Kit (Applied Biosystems).

**Targeted bisulfite sequencing**

Mouse gDNA (100 ng) was bisulfite converted using the Epitect Bisulfite Kit (Qiagen). Template DNA was amplified using KAPA HIF1 Uracl+ (KAPA) with primers directed to the LINE1 elements (22), the H19 imprinting control region, Olfm4 and Hes1 (23), Vdr, Hic1, Sftp5, Mgmt, Dusp6, e-Fos, Msln, and S100a4. Primer sets and regions subsequently sequenced can be found in Supplementary Table S1. Sequencing libraries were prepared and analyzed using the BiSPCR\textsuperscript{2} strategy (LINE1, H19) or the standard protocol (Vdr, Hic1, Sftp5, Mgmt, Dusp6, e-Fos, Msln, S100a4), described previously (23, 24).

**Phosphorylated H3 cell-cycle quantification**

Apc\textsuperscript{Min+/+};Dnmt1\textsuperscript{loxP/loxP} and Apc\textsuperscript{Min+/+};Dnmt1\textsuperscript{loxP/loxP} Villin-Cre-ERT2 mutants and sibling Apc\textsuperscript{Min+/+};Dnmt1\textsuperscript{loxP/loxP} controls were injected with tamoxifen at 1 month of age. Small intestines were collected 1 week, 1 month, and 2 months following tamoxifen administration, in separate cohorts of mice. Following fixation, tissue sections were stained for phosphorylated histone H3 (PH3) to identify cells in M phase and β-catenin to identify regions of hyperproliferative neoplastic epithelium. We counted the number of PH3+ nuclei and divided it by the total number of nuclei in the respective region. This calculation was also performed for neoplastic intestinal epithelium at both 1 and 2 months post-tamoxifen time points, for controls and mutants.

**qRT-PCR**

Intestines isolated 1 week post-tamoxifen were gently scraped to remove villi and treated with EDTA to isolate crypt cells. Macroscopic tumors and adjacent normal tissue, from intestines isolated 2 months post-tamoxifen, were visualized and dissected using a stereoscope. RNA was extracted using the TRIzol RNA isolation protocol (Invitrogen), followed by RNA cleanup using the RNeasy Mini Kit (Qiagen). mRNA expression was measured using quantitative RT-PCR, as described previously (27). The SYBR green qPCR master mix (Agilent) was used in all qPCR reactions, and the fold change was calculated relative to the geometric mean of Tbp and β-actin, using the ΔΔC\textsubscript{T} method. The method of normalizing to the geometric mean of a set of reference genes has been described previously (28). Primer sequences can be found in Supplementary Table S1.

**Results and Discussion**

**Deletion of Dnmt1 in the intestinal epithelium of adult Apc\textsuperscript{Min+/+} mice induces acute global hypomethylation**

To determine the role of Dnmt1 in intestinal adenoma initiation, we employed Apc\textsuperscript{Min+/+};Dnmt1\textsuperscript{loxP/loxP};Villin-Cre-ERT2 mice to inducibly delete Dnmt1 throughout the intestinal epithelia. Apc\textsuperscript{Min+/+};Dnmt1\textsuperscript{loxP/loxP};Villin-Cre-ERT2 mice and their Apc\textsuperscript{Min+/+};Dnmt1\textsuperscript{loxP/loxP} siblings (referred to as ‘mutants’ and ‘controls’, respectively) were injected with tamoxifen at 4 weeks of age to induce Dnmt1 deletion (Supplementary Fig. S1). On the basis of our previous analysis of Dnmt1 ablation in the intestinal epithelium, we isolated the small intestine 1 week after tamoxifen treatment (23). To address the role of Dnmt1 in adenoma development, we analyzed a second cohort of mice in which we harvested the intestine and colon 2 months post-tamoxifen treatment, at 3 months of age. By 3 months of age, Apc\textsuperscript{Min+/+} mice...
display macroscopic lesions (11, 12). Thus, 2 months post-tamoxifen allowed us to contrast adenoma development in Dnmt1 mutants with Apc\(^{Min/}\);Dnmt1\(^{loxP/loxP}\) controls. At 1 week and 2 months following tamoxifen treatment, Dnmt1 deletion was effective throughout the mutant epithelia of small intestine (Fig. 1A–D and C). Dnmt1 deletion was also maintained in mutant adenomas (Fig. 1E, F, and H). As Apc\(^{Min/}\) mice exhibit tumors predominantly in the small intestine (12, 29), we focused our studies on adenoma development in the small intestinal epithelium. First, we analyzed the effects of Dnmt1 deletion in the non-neoplastic epithelium of Apc\(^{Min/}\) mice.

To estimate global DNA methylation levels, we performed targeted bisulfite sequencing of the H19 imprinting control region (ICR) and the Long Interspersed Nucleotide Element 1 (LINE1). The process of imprinting is dependent on DNA methylation and refers to the methylation of alleles in a parental-specific manner to restrict gene expression to one allele (30). The H19 imprinting control region is methylated on the paternally inherited allele, thereby ensuring maternal allele expression (31). LINE1 retrotransposon loci comprise approximately 20% of the mouse and human genome, are maintained in a highly methylated state to inhibit transposase activity, and are a representative of genome-wide methylation levels (32–34). We employed laser capture microdissection (LCM) to collect crypts from mutant and control intestines 1 week following tamoxifen treatment. H19 methylation was maintained despite loss of Dnmt1 (Fig. 2A), implicating a role for Dnmt3a and Dnmt3b in maintaining genomic imprints, as suggested previously (35, 36). Strikingly, within 1 week following Dnmt1 deletion, we observed a 35% reduction in DNA methylation at the LINE1 loci in mutant crypts compared with the control crypt epithelium (Fig. 2B). Thus, Dnmt1 is required acutely to maintain DNA methylation levels on LINE1 elements but is not required for maintenance of imprinted loci in the rapidly proliferating intestinal epithelium.

To determine whether global hypomethylation of repetitive elements was sustained over time, we isolated crypt epithelium from mutants and controls 1 and 2 months following tamoxifen treatment. Surprisingly, we found no differences in methylation levels in all conditions tested, demonstrating that DNA methylation had been fully restored after temporary loss following Dnmt1 ablation (Fig. 2C–F). These results were unexpected, as Dnmt1 deletion was maintained at 2 months after tamoxifen administration (Fig. 1C–F). Recovery of DNA methylation was likely driven by the de novo DNA methyltransferases Dnmt3a and 3b, which are also expressed in the intestinal epithelium (23).

Dnmt1 mutants display increased tumor initiation

One and 2 months following tamoxifen injection, the small intestine was examined for neoplastic transformation. Surprisingly, H&E staining revealed a dramatic increase in the number of neoplastic lesions throughout the mutant small intestine at 2 months, but not 1 month, post-tamoxifen (Fig. 3A–D). Indeed, mutants displayed more than 6-fold more macroscopic adenomas than controls at 2 months following tamoxifen injection (Fig. 3E; \(n = 17–20\) per group). In addition, mutant small intestinal tumors were on average twice as large as those of controls at 2 months post-tamoxifen injection (Fig. 3F; \(n = 5–8\) per group). Furthermore, we observed 58% incidence of neoplastic transformation in colons of mutant mice (\(n = 12\)) compared with 14% in controls (\(n = 7\); Supplementary Fig. S2).

Previously, it had been reported that partial loss of Dnmt1 produces a block in the progression of adenomas in the Apc\(^{Min/}\) mouse paradigm (14–18), which is in sharp contrast to our findings of an increased number of macroscopic lesions in the Apc\(^{Min/}\);Dnmt1\(^{loxP/loxP}\);VillinCreERT2 deletion model. To confirm that these lesions are bona fide adenomas, we consulted with a pathologist to perform histopathologic assessment on the intestines of mutant and control mice, 1 and 2 months following.

Figure 1.
Conditional ablation of Dnmt1 in the Apc\(^{Min/}\) intestinal epithelium. A–F, Dnmt1 deletion is maintained 1 week (B) and 2 months (D) after tamoxifen treatment in the adult mouse small intestinal epithelium. Immunohistochemical staining of Dnmt1 protein is evident in the crypt epithelia located just above the submucosa (outlined in black) of control mice at both time points (A, C) but is absent in mutant mice (B, D). Neoplastic epithelia (outlined in red) display high levels of Dnmt1 protein in control animals (E). Deletion of Dnmt1 protein is maintained in the neoplastic epithelium (outlined in red) in mutant animals 2 months after tamoxifen treatment (F). G and H, qRT-PCR analysis comparing the relative gene expression levels of Dnmt1 in controls and mutants 1 week (G) and 2 months (H) after tamoxifen treatment (\(n = 3–5\) per genotypes). Compared with controls, Dnmt1 mutants express significantly lower levels of Dnmt1, and deletion is maintained in macroscopic tumors isolated 2 months post-tamoxifen. Gene expression is calculated relative to the geometric mean of TBP and β-actin. All scale bars are 50 μm. \(^*\), \(P < 0.05\); \(^*\), \(P < 0.01\); Student’s t test.
tamoxifen treatment at 4 weeks of age. Using criteria described by Biovin and colleagues (25), we found 15-fold more lesions that had progressed to adenomas in mutant mice compared with ApcMin/þ controls (Supplementary Fig. S3B) at 2 months post-tamoxifen injection. At 1 month post-tamoxifen, all lesions observed in both controls and Dnmt1 mutants displayed similar histopathology and were characterized as gastrointestinal intraepithelial neoplasias (Supplementary Fig. S3A).

DNA hypomethylation is observed at specific genes in tumors

DNA methylation is important for maintaining appropriate intestinal stem cell gene expression, and loss of Dnmt1 in the intestinal epithelium results in increased crypt cell proliferation (23). Previously, we observed that 1 week following deletion of Dnmt1, DNA methylation was decreased at enhancers associated with genes critical for intestinal stem cell proliferation, Hes1 and Olfm4 (23). Thus, altered DNA methylation could be responsible for the dramatic increase in lesions observed in our mutant animals. We employed LCM to collect crypt epithelia and neoplastic tumors from mutant and control intestines 2 months following tamoxifen treatment. We employed targeted bisulfite sequencing of the H19 imprinting control region (Chr7: 149,766,621–149,766,690). At 1 week (A), 1 month (C), and 2 months (E) following tamoxifen administration, H19 methylation levels are comparable between Dnmt1 mutants and controls. B, D, F, targeted bisulfite sequencing of 9 CpGs in the LINE1 repetitive elements (NCBI Accession #D84391: 976–1,072). LINE1 methylation levels 1 week after tamoxifen injection are significantly reduced in crypts of mutant mice compared with controls (B). One month (D) and 2 months (F) after tamoxifen-induced ablation of Dnmt1, global DNA methylation levels have been restored to baseline. For all graphs, data are presented as average ± SEM. **, P < 0.01 by 2-tailed Student t test.
significantly hypomethylated at the Hes1 enhancer compared with control tumors (Fig. 4C). Furthermore, mutant tumors are significantly hypomethylated at both Hes1 and Olfm4 loci compared with adjacent non-neoplastic epithelia (4C, dashed lines). These data are intriguing because Hes1 expression is increased in stem cell–like cells in colon cancer (37), and our results suggest that DNA methylation may play a role in activating Hes1 expression in tumors.

Aberrant DNA methylation drives intestinal cancer cell growth in multiple paradigms (14–18) and could be responsible for the dramatic increase in lesion growth observed in our mutant animals. Hypermethylation of tumor suppressor genes allows unrestrained growth and is a hallmark of the colorectal cancer CpG island methylator phenotype (CIMP; ref. 38). We tested promoter methylation of four genes that have been previously shown to be hypermethylated in adenomas, Vdr, Hic1, Sfrp5, and Mgmt (39–43). To our surprise, only Vdr and Mgmt showed increased methylation in adenomas, relative to nontumor epithelia (Fig. 4D). Dnmt1 deficiency reduced methylation in mutant tumors compared with control tumors in an inconsistent manner (Fig. 4D), suggesting that hypermethylation at these loci is not required for tumor growth. DNA hypomethylation has also

Figure 3.
Deletion of Dnmt1 in adult Apc<sup>Min/+</sup> mice accelerates adenoma initiation. A–D, H&E staining of control and mutant intestinal epithelium 1 month (A and B) and 2 months (C and D) after tamoxifen injection. H&E staining shows disruption of normal intestinal epithelial morphology in the small intestine of tamoxifen-treated mutants compared with control mice (B vs. A, D vs. C). Scale bars are 50 μm. C, intestinal epithelial-specific Dnmt1 deletion causes increased incidence of macroscopic tumors. Total number of macroscopic tumors was counted throughout the entire small intestine of mutant and control mice, 1 month (n = 5–7 per group) and 2 months (n = 17–20 per group) following tamoxifen treatment. E, Dnmt1 deletion results in increased size of neoplastic lesions in the small intestine 2 months after tamoxifen treatment. Neoplastic lesions were measured in control and mutant mice at 1 month (n = 2 for controls, n = 3 for mutants) and 2 months (n = 5 for controls, n = 8 for mutants) following tamoxifen injection. For all graphs, data are presented as average ± SEM. ***, P < 0.001 by one-way ANOVA.
Dnmt1 in Genomic Stability during Intestinal Tumorigenesis

Figure 4.

Dnmt1-mutant tumors show increased hypomethylation at potential oncogenes. Targeted bisulfite sequencing was performed to estimate maintenance methylation of Dnmt1-deficient tumors, relative to controls tumors and to adjacent non-neoplastic crypt epithelium. Tumors and crypt epithelium were isolated by LCM from controls and mutants 2 months following tamoxifen treatment. A and B, analysis of 9 CpGs in the LINE1 repetitive elements (NCBI Accession #D84391: 976–1,072) revealed no alterations in methylation of the repetitive transposable elements (A). Analysis of 6 CpGs in the H19 imprinting control region (Chr7: 149,766,621-149,766,690) showed that H19 methylation levels are comparable between Dnmt1 mutants and controls, in both tumor and nontumor epithelium. C, potential enhancer regions of genes involved in intestinal stem cell identity and proliferation, Olfm4 and Hes1 were analyzed for methylation changes in all conditions. Regional averages of 7 CpGs in the Olfm4 enhancer (chr14: 80,399,983–80,400,271) and 6 CpGs in the Hes1 enhancer (chr16: 30,055,572–30,055,738) are shown. The Hes1 enhancer shows increased methylation in control tumors compared with adjacent tissue, which is abolished by Dnmt1 deletion. D, genes with cancer-specific promoter hypermethylation, Vdr, Hic1, Sfrp5, Mgmt were analyzed for methylation changes in all conditions. Regional averages of 8 CpGs in the Vdr promoter (chr15: 97,731,208–97,731,590), 16 CpGs in Hic1 promoter (chr1: 74,963,743–74,963,918), 16 CpGs in the Sfrp5 promoter (chr19: 42,276,328–42,276,453), and 19 CpGs in Mgmt promoter (chr7: 144,086,076–144,086,345) are shown. Only Vdr and Mgmt displayed significant hypermethylation in control tumors compared with adjacent tissue. Loss of Dnmt1 caused significant loss of hypermethylation at both loci, suggesting that hypermethylation of these genes is not required for tumor progression. E, genes with cancer-specific promoter hypomethylation, Dusp6, c-Fos, Msln, St100a4, were analyzed for methylation changes in all conditions. Regional averages of 8 CpGs in the Dusp6 promoter (chr10: 98,728,738–98,729,028), 14 CpGs in the c-Fos promoter (chr12: 86,817,243–86,817,533), 6 CpGs in the Msln promoter (chr17: 25,891,502–25,891,643), and 3 CpGs in the St100a4 promoter (chr3: 90,407,319–90,407,489) are shown. Only Dusp6 and c-Fos displayed significant hypomethylation in control tumors compared with adjacent tissue. Dnmt1 deletion caused even more drastic loss of methylation at both promoters, suggesting that loss of Dnmt1 may be driving activation of potential oncogenes during tumor growth.
been reported at several potential oncogenes including Dusp6, c-Fos, Msln, and S100a4 (43–46). Although none of these gene promoters show significant loss of methylation in tumors compared with the respective normal epithelia, we observed significantly decreased methylation at Dusp6 and c-Fos in Dnmt1 mutant relative to control adenomas (Fig. 4E). c-Fos is a potent driver of colon cancer cell growth (47), and its expression is regulated by DNA hypomethylation in several cancer types (48, 49). These data suggest that hypomethylation at oncogenes may be driving tumor growth in Dnmt1-deficient ApcMin/+ mutant animals.

**Dnmt1-null tumors are initiated by LOH and Wnt activation**

We further investigated tumor initiation to determine whether the observed changes in DNA methylation were a causative factor of neoplastic transformation. Adenoma formation in the ApcMin/+ mouse model is driven by LOH at the Apc locus, which causes nuclear accumulation of β-catenin and activation of Wnt signaling (13). LOH is observed in virtually all ApcMin/+ intestinal tumors (26, 50). To characterize adenoma initiation by LOH in detail, we employed LCM to isolate neoplastic and normal intestinal epithelial cells from mutant mice and their control siblings, 2 months following tamoxifen treatment. Since the number of macroscopic lesions found in controls at 1-month post-tamoxifen is very small (Fig. 3E, Supplementary Fig. S3), we focused on the 2-month time point for LOH analysis. DNA was isolated from these samples (~1,000 cells per sample), and the status of the Apc alleles was examined as described previously (26). Mutant tumors displayed LOH at a rate similar to that of control tumors (Fig. 5A), demonstrating that Dnmt1-mutant ApcMin/+ mice initiate tumors by the same mechanism.

Several lines of evidence confirm that the numerous adenomas that develop in the Dnmt1-deficient ApcMin/+ mice are the result of Wnt pathway activation. Mutant adenomas at both 1 and 2 months following tamoxifen treatment exhibited increased nuclear β-catenin protein (Fig. 5B–D) relative to 2-month controls. Multiple Wnt signaling targets, such as CyclinD1 (Fig. 5E–G) and Sox9 (Fig. 5H–J), were also strongly activated. These data demonstrated that total loss of Dnmt1 in the mature small intestinal epithelium promotes neoplastic progression, most likely through a mechanism that enhances LOH at the ApcMin/+ locus.

**Dnmt1-mutant mice display acute increases in proliferation and apoptosis**

Because of the dramatic difference in lesion size and hypomethylation at potential oncogenes observed in the inducible Dnmt1-mutant intestine, we investigated whether Dnmt1 deficiency had a direct impact on cell proliferation in ApcMin/+ adenomas or the non-neoplastic crypt epithelium. We determined percentages of control and mutant epithelial cells in M phase by staining for pH3, at 1 week, 1 month, and 2 months post-tamoxifen treatment. One week post-tamoxifen (Fig. 6A–C), the percentage of pH3+ nuclei was significantly increased in the Dnmt1-deficient epithelium relative to control (n = 4 per group). Interestingly, at 1 month (Fig. 6D–F) and 2 months (Fig. 6G–I) following tamoxifen injection, the proliferation rate was equivalent in Dnmt1-positive or -negative tumors and in the adjacent crypt epithelium.

We also examined the small intestine at all three time points for signs of cell death using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL), which detects DNA fragmentation in apoptotic cells. We quantified the percent of epithelial cells that were TUNEL+, similar to the pH3 staining quantification. Mutants 1 week after tamoxifen treatment displayed a significantly higher proportion of TUNEL+ cells compared with controls (Fig. 7A–C). Both control and mutant adenomas exhibited only rare epithelial cells positive for TUNEL, and we found no differences in cell death between mutant and control neoplastic and non-neoplastic epithelia, at 1 month (Fig. 7D–F) or 2 months (Fig. 7G–I) following tamoxifen administration. However, many subepithelial cells were TUNEL-positive (Fig. 7D, E, G, and H). These data demonstrate that loss of Dnmt1 in the adult intestinal epithelium in vivo has acute temporary effects on intestinal proliferation and apoptosis but no effect on ApcMin/+ adenoma proliferation or cell death. Overall, our results suggest that the increase in adenoma number and size found in the Dnmt1-mutant mice is driven by accelerated tumor initiation through LOH.

**Dnmt1-deficient ApcMin/+ intestinal epithelia exhibit increased genomic instability**

Next, we considered the molecular mechanisms behind the accelerated LOH we observed in the Dnmt1-deficient intestinal epithelium. We hypothesized that the established role of Dnmt1 in the preservation of genomic stability might contribute to the phenotype. DNA methylation has been linked to mismatch repair (MMR) deficiency and genomic instability in multiple contexts, in both cell lines and disease. In the HCT116 colorectal cancer cell line, ablation of catalytically active DNMT1 causes cell-cycle arrest and apoptosis due to increased chromosomal instability (51, 52). In mouse embryonic stem cells, loss of Dnmt1 also causes global hypomethylation and increased mutation rates (53).

To determine levels of DNA damage in mutant and control mice, we performed γH2AX staining, which visualizes DNA double-strand breaks as a marker of chromosomal instability. One week following tamoxifen administration, we discovered a dramatic increase in the fraction of γH2AX+ epithelial cells in Dnmt1-mutant versus controls, which contained no γH2AX+ epithelial cells (Fig. 8A–C). One month post-tamoxifen injection, mutants and controls displayed a similar number of γH2AX+ nuclei (Fig. 8D–F). There was a significant difference between neoplastic and non-neoplastic epithelium in control mice, and a similar statistically relevant change was observed in mutants (Fig. 8F). Two months following tamoxifen treatment, nonmutant epithelia in both the control and mutant mice showed very little γH2AX (Fig. 8G–I). Although we found many more epithelial cells within Dnmt1-deficient tumors to be γH2AX+ compared with control tumors (Fig. 8I), these data were not statistically significant. These results demonstrate that increased genomic instability occurs as a result of Dnmt1 deletion in the ApcMin/+ model and that genomic instability occurs prior to tumor development. We propose that this temporary decrease in genomic stability, combined with elevated proliferation and apoptosis levels, contributes to accelerated LOH and enhanced tumorigenesis in the inducible ApcMin/+;Dnmt1loxP/loxP;Villin-Cre-ERT2

**Discussion**

The results presented above have major implications for the fields of DNA methylation and intestinal cancer biology. We show that loss of the DNA methyltransferase Dnmt1 in the ApcMin/+ cancer model results in acute hypomethylation and DNA damage...
Figure 5.

Dnmt1-deficient Apc<sup>Min</sup> intestinal tumors display increased Wnt signaling. A, control and mutant mice were tamoxifen-treated at 4 weeks of age, and intestines were harvested 2 months later. Intestinal tumors from control and mutant mice were isolated by LCM for PCR testing of LOH at the Apc locus. Results of PCR testing demonstrate that mutant tumors display LOH at a rate similar to that of controls. B–J, control and mutant mice were tamoxifen-treated at 4 weeks of age, and intestines were harvested 1 or 2 months later for immunostaining analysis of Wnt signaling targets. B–D, nuclear β-catenin, as visualized by immunohistochemical staining, is increased in Dnmt1-mutant intestine 2 months after tamoxifen treatment (D), compared with tamoxifen-treated, age-matched control small intestine (B). One month following Dnmt1 ablation, mutant small intestine displays nuclear β-catenin levels similar to that observed in the 2-month controls (C compared with B, respectively). E–G, expression of the Wnt signaling target cyclin D1 (CyclinD1) is increased in Dnmt1-deficient tumors (G) relative to age-matched control tumors (E). At 1-month post-Dnmt1 ablation, CyclinD1 staining appears slightly increased compared with the 2-month controls (F vs. E, respectively). CyclinD1 (red), DAPI (blue). H–J, Sox9 immunofluorescent staining shows increased Sox9 expression in Dnmt1-deficient tumors (J) compared with control tumors (H) 2 months following tamoxifen treatment. At 1 month after Dnmt1 ablation, increased Sox9 expression is visible by immunostaining (I compared with control, H). Sox9 (red), DAPI (blue). All scale bars are 50 μm.
but does not affect tumor cell proliferation or apoptosis. We posit that decreased genomic stability accelerates LOH at the Apc locus, resulting in increased tumor initiation and larger tumors over time. Our work adds to the body of evidence that implicates a crucial role for Dnmt1 and DNA methylation in maintaining genome stability.

Figure 6.
Loss of Dnmt1 in the ApcMin+/+ intestine causes an acute increase in crypt cell proliferation. Percentage of cells in mitosis was determined by immunostaining and counting of pH3+ nuclei in the small intestine 1 week, 1 month, and 2 months following tamoxifen treatment. The percentage of pH3+ cells was calculated as the number of pH3+ nuclei divided by the total number of epithelial nuclei. A–C, one week following tamoxifen injection, mutants displayed a significantly increased percentage of pH3+ cells (C, n = 4 per genotype). Representative images of pH3 immunostaining are shown for controls (A) and Dnmt1 mutants (B). D–F, one month after tamoxifen treatment, mutants (E) and controls (D) displayed comparable percentages of pH3+ cells, in both tumors and noncancerous epithelia (F). Twenty-four tumors across 5 mice were counted for Dnmt1 mutants; 4 tumors across 4 mice were counted for controls. Approximately 1,500 noncancerous cells were counted from the same biologic replicates to calculate pH3 frequency in nontumor tissue. G–I, two months after tamoxifen treatment, mutants (H) and controls (G) displayed comparable percentages of pH3+ cells, in both tumors and noncancerous epithelia (I). Twenty-four tumors across 7 mice were counted for Dnmt1 mutants; 8 tumors across 3 mice were counted for controls. Nontumor epithelia quantified as in (D–F). E-cadherin (green) outlines the intestinal epithelium. All scale bars are 50 μm. For all graphs, data are presented as average ± SEM. **, P < 0.01 by Student t test (C). One-way ANOVA was performed for (F, I), and the results were not significant.
Importantly, our study demonstrates increased intestinal adenoma formation after deletion of Dnmt1. Our experimental paradigm results in intestine-specific, temporal loss of Dnmt1 and acute hypomethylation, followed by recovery of global genome DNA methylation, in contrast to prior work, which had employed germline hypomorphic Dnmt1 alleles (14–18). These previous studies thus differed both in the timing of Dnmt1 deletion and in the lack of tissue specificity and described a

Figure 7.
Loss of Dnmt1 results in a short-term increase in intestinal epithelial apoptosis but is not altered 1 or 2 months following Dnmt1 deletion. TUNEL staining (red) was performed to detect apoptotic cells, with E-cadherin (green) to outline the epithelium. The percentage of apoptotic cells was determined by counting of the TUNEL-positive (TUNEL⁺) nuclei in the small intestine 1 week, 1 month, and 2 months following tamoxifen treatment. The percentage of TUNEL⁺ cells was calculated as the number of TUNEL⁺ nuclei divided by the total number of epithelial nuclei. A–C, one week following tamoxifen treatment, Dnmt1 mutants have significantly higher rates of apoptosis than controls (B vs. A; quantitation in C). N = 4 per group. *** P < 0.001 by Student t-test. D–F, one month after tamoxifen injection, both tumor and nontumor epithelia contain TUNEL-positive cells, which is unchanged in mutant mice (D vs. E; quantitation in F). n = 3 biologic replicates per group, with 8 tumors counted from controls and 14 tumors counted from mutants. G–I, two months after tamoxifen injection, both tumor and nontumor epithelia contain TUNEL-positive cells at low frequency in control mice, which is unchanged in mutant mice (H vs. G; quantitation in I). n = 3–5 biological replicates per group; two tumors were counted from controls, and 10 tumors were counted from mutants. All scale bars are 50 μm. For all graphs, data are presented as average ± SEM. One-way ANOVA test was performed in (F, I), and results were not significant.
constitutive hypomethylation phenotype which may be a contributing factor to our disparate results. Our Dnmt1-deficient tumors 2 months after tamoxifen administration exhibit significant hypomethylation of several potential oncogenes, yet display minimal DNA methylation changes genome-wide. We cannot rule out that the genome remethylation occurring from 1 week to 1 month post-tamoxifen contributes to accelerated tumorigenesis in our conditional Dnmt1 ablation intestinal cancer model. However, the significantly enhanced genomic instability, proliferation, and apoptosis observed immediately following Dnmt1 ablation,

Figure 8.
The Dnmt1-deficient Apc<sup>Min</sup>/+ intestine displays increased chromosomal instability. Mice were tamoxifen-treated at 4 weeks of age, and intestines were harvested 1 week, 1 month, or 2 months later for immunofluorescent staining analysis. γH2AX (red) was used to visualize DNA double-strand breaks as a marker of genome instability, and E-cadherin (green) was used to outline the epithelium. The percentage of γH2AX-positive (γH2AX<sup>+</sup>) cells was calculated as the number of γH2AX<sup>+</sup> nuclei divided by the total number of epithelial nuclei. A–C, One week following tamoxifen treatment, the number of γH2AX<sup>+</sup> nuclei is increased in mutant compared with control small intestines (B compared with A, quantification in C). n = 4 biological replicates per group. *** P < 0.001 by Student t test. D–F, one month post-tamoxifen injection, both mutant and control non-neoplastic epithelia display low levels of DNA damage, which is significantly increased in tumors of both groups (F). n = 3 biological replicates per group; 2 tumors were counted for controls, 8 tumors were counted for mutants. G–I, two months following tamoxifen treatment, increased levels of γH2AX<sup>+</sup> foci continue to be seen in mutant tumors compared with mutant non-neoplastic epithelium (I). There is no significant difference between control and mutant γH2AX<sup>+</sup> levels (G vs. H, quantification in I). n = 3–4 biological replicates per group; 3 tumors were counted from controls, and 12 tumors were counted from mutants. All scale bars are 50 μm. For F and I, *, P < 0.05; **, P < 0.01; ***, P < 0.001, one-way ANOVA.
combined with the increased adenoma formation at 1 and 2 months post-tamoxifen, strongly suggest a role for Dnmt1 in maintaining genomic stability and preventing intestinal cancer initiation.

Interestingly, our model results in acute global hypomethylation at LINE1 repetitive elements. DNA methylation is associated with the silencing of LINE1 transcription (54), and LINE1 hypomethylation is highly variable in colorectal carcinomas (55). It has been hypothesized that LINE1 retrotransposition events can interrupt critical genes, such as APC, and drive tumorigenesis (54). In particular contexts, such as APC heterozygosity, changes in genome stability are critical drivers of cancer initiation. Our data support the general theory that DNA hypomethylation causes increased DNA damage, and LINE1 hypomethylation may also be contributing to our observed phenotype.

The DNA demethylating agents azacitidine and decitabine have been tested as anticancer therapeutics in colorectal cancer to abrogate DNA hypermethylation and the silencing of tumor suppressor genes (56, 57). Decitabine and azacitidine are most commonly used to treat acute myeloid leukemia (AML) and myelodysplastic syndromes, with variable success rates (58–60). Our results indicate that DNA demethylation actually contributes to cancer formation, and warrant caution in treating patients with gastrointestinal cancer with a potential tumor-enhancing drug.

In conclusion, we show that deletion of Dnmt1 in the adult intestinal epithelium of Apcmin/+ mice causes accelerated formation of adenomas. Loss of Dnmt1 results in acute hypomethylation and genomic instability, accompanied by increased proliferation and apoptosis. Although Dnmt1-deficient adenomas eventually recover global DNA methylation, they continue to display hypomethylation at several potential oncogenes, highlighting an unappreciated role for DNA hypomethylation in intestinal tumor development. These results support a fundamental role for DNA methylation in preserving genomic integrity during intestinal tumor formation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: K.L. Sheaffer, K.H. Kaestner
Development of methodology: K.L. Sheaffer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Sheaffer, E.N. Elliott
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.L. Sheaffer, E.N. Elliott
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