

Epigenetic Repression of DNA Mismatch Repair by Inflammation and Hypoxia in Inflammatory Bowel Disease–Associated Colorectal Cancer

Robert A. Edwards,¹ Mavee Witherspoon,² Kehui Wang,¹ Kambiz Afrasiabi,² Trang Pham,² Lutz Birnbaumer,³ and Steven M. Lipkin²

Departments of ¹Pathology and ²Medicine, University of California-Irvine, Irvine, California; and ³Laboratory of Neurobiology/Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

Abstract

Sporadic human mismatch repair (MMR)–deficient colorectal cancers account for ~12.5% of all cases of colorectal cancer. MMR-deficient colorectal cancers are classically characterized by right-sided location, multifocality, mucinous histology, and lymphocytic infiltration. However, tumors in germ-line MMR-deficient mouse models lack these histopathologic features. Mice lacking the heterotrimeric G protein α subunit $G\alpha 2$ develop chronic colitis and multifocal, right-sided cancers with mucinous histopathology, similar to human MMR-deficient colorectal cancer. Young $G\alpha 2^{-/-}$ colonic epithelium has normal MMR expression but selectively loses MLH1 and consequently PMS2 expression following inflammation. $G\alpha 2^{-/-}$ cancers have microsatellite instability. *Mlh1* is epigenetically silenced not by promoter hypermethylation but by decreased histone acetylation. Chronically inflamed $G\alpha 2^{-/-}$ colonic mucosa contains patchy hypoxia, with increased crypt expression of the hypoxia markers DEC-1 and BNIP3. Chromatin immunoprecipitation identified increased binding of the transcriptional repressor DEC-1 to the proximal *Mlh1* promoter in hypoxic YAMC cells and colitic $G\alpha 2^{-/-}$ crypts. Treating $G\alpha 2^{-/-}$ mice with the histone deacetylase inhibitor suberoylanilide hydroxamic acid significantly decreased colitis activity and rescued MLH1 expression in crypt epithelial cells, which was associated with increased acetyl histone H3 levels and decreased DEC-1 binding at the proximal *Mlh1* promoter, consistent with a histone deacetylase–dependent mechanism. These data link chronic hypoxic inflammation, epigenetic MMR protein down-regulation, development of MMR-deficient colorectal cancer, and the first mouse model of somatically acquired MMR-deficient colorectal cancer. [Cancer Res 2009;69(16):6423–9]

Introduction

DNA mismatch repair (MMR)–deficient colorectal cancers have distinct features that distinguish them from other colorectal cancer types (1). These include increased frameshift mutation rates [commonly called microsatellite instability (MSI)], multifocality,

increased likelihood of right-sided colon cancer location, non-polyposis pathology, mucinous histology, and presence of a Crohn's-like lymphocytic infiltrate in tumors (2, 3).

MMR-deficient colorectal cancers arise through three distinct genetic mechanisms. First, monoallelic germ-line MMR mutation and somatic loss of the second MMR allele causes hereditary nonpolyposis colon cancer, also known as Lynch syndrome. Lynch syndrome carriers body tissues are MMR-proficient because of the other allele. As predicted by the Knudson hypothesis, the second MMR gene allele is somatically lost in some cells, which then become MMR-deficient and transform into colorectal cancer and other cancers. The second mechanism is biallelic germ-line constitutive MMR gene mutation, where all body tissues are MMR-deficient. These patients have a different clinical phenotype, with very early-onset hematologic, brain, and small intestinal cancers (4–6). Although colorectal cancers can develop, penetrance is low and arise more commonly in the left colon (4, 5).

The third genetic mechanism, *de novo* sporadic MMR-deficient colorectal cancers, accounts for ~12.5% of colorectal cancer (6). Both MMR alleles are somatically mutated or epigenetically inactivated in some cells, which transform into cancer. *Mlh1* promoter hypermethylation is the most common cause of sporadic MMR-deficient colorectal cancer (7–9). *In vitro* studies have shown that epigenetic *Mlh1* inactivation can also occur via hypoxia-induced transcriptional repression of the proximal *Mlh1* promoter by histone deacetylases (HDAC; ref. 9). However, whether this mechanism is relevant to *in vivo* colorectal cancer tumorigenesis is unknown.

Multiple biallelic MMR germ-line knockout mouse models have been created (Supplementary Table S1). These mutations primarily cause lymphomas and small intestine adenomas (10–16) Only rarely do these models develop colorectal cancer, and mucinous, nonpolyposis, or proximal colon colorectal cancers essentially never arise. These models are therefore more similar to human biallelic germ-line constitutive MMR deficiency than somatically acquired MMR deficiency syndromes.

$G\alpha 2$ is a heterotrimeric G protein α subunit, the deletion of which in mice leads to spontaneous colitis and nonpolyposis, right-sided, multifocal colorectal cancers with mucinous histology and a Crohn's-like inflammatory infiltrate (17–19). The similarities between $G\alpha 2^{-/-}$ mouse colorectal cancer and somatically acquired human MMR-deficient colorectal cancer (Supplementary Table S1) led us to investigate whether acquired MMR silencing might contribute as a mechanism of colorectal cancer tumorigenesis in $G\alpha 2^{-/-}$ mice. We found that MLH1 and PMS2 protein levels in crypts are initially normal but are suppressed following the onset of hypoxic colitis. Hypoxia induces colonic expression of the bHLH transcriptional repressor DEC-1 and enhances its binding to the proximal *Mlh1* promoter, thereby repressing MLH1 expression and

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

Requests for reprints: Robert A. Edwards, Department of Pathology, University of California-Irvine, D449 Med Sci I, Irvine, CA 92697-4800. Phone: 949-824-8576; Fax: 949-824-2160; E-mail: redwards@uci.edu and Steven M. Lipkin, Department of Genetic Medicine, Weill Cornell Medical College, Room 718 HSS, New York, NY 10021. Phone: 212-774-7160; E-mail: stl2012@med.cornell.edu.

©2009 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-09-1285

leading to MSI. Treatment of $\text{G}\alpha 2^{-/-}$ mice with HDAC inhibitor (HDACi) decreases colitis and relieves epigenetic repression of MLH1 expression. In summary, we describe the first mouse model of somatically acquired MMR deficiency and show that hypoxia-induced *Mlh1* silencing may be another important epigenetic mechanism contributing to the development of MMR-deficient colorectal cancer.

Materials and Methods

Mice. Wild-type (WT) and $\text{G}\alpha 2^{-/-}$ mice on a cross-bred 129Sv/C57BL6 background were genotyped as described previously (19). All experiments were approved by the Institutional Animal Care and Use Committee at University of California-Irvine.

In vivo assessment of apoptosis induction. Age-matched WT and $\text{G}\alpha 2^{-/-}$ mice were injected i.p. with 20 mg/kg 1,2-dimethylhydrazine for 24 h. Sections (5 μm) of fixed control and 1,2-dimethylhydrazine-treated colons were stained for apoptotic cells using an Apoptag staining kit (Chemicon). The data are expressed as the mean \pm SE of the normalized ratio of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive/propidium iodide-positive cells.

Colonic crypt isolation, SDS-PAGE, and Western blotting. Rinsed small intestine and colon segments were reverted on a Labconco vibratory stirrer and shaken in 30 mmol/L EDTA in HBSS. Crypts liberated between 20 and 40 min (>98% pure by cytospin analysis) were used for Western blotting and quantitative real-time PCR. Antibodies used include MLH1 (BD Pharmingen; clone G168-728), PMS2 (BD Pharmingen; clone A16-4), MSH2 (Calbiochem; clone FE11), MSH6 (Abcam; clone 44), DEC-1 (Santa Cruz Biotechnology; clone S-8), BNIP-3 (Santa Cruz Biotechnology; clone Ana40), and β -actin (Sigma; clone 20-33).

Immunohistochemistry. WT ($n = 24$) and $\text{G}\alpha 2^{-/-}$ ($n = 22$) colon sections were microwaved (20 min) in citrate buffer. After peroxidase, biotin, and endogenous IgG blocking, anti-MMR antibodies (described above) were added overnight at 4°C and then developed using peroxidase-based detection. Eight mucinous and 17 nonmucinous carcinomas located throughout the colon were stained.

Laser capture microdissection of mouse crypt epithelium and colon cancer cells. Formalin-fixed, deparaffinized WT and $\text{G}\alpha 2^{-/-}$ colon sections on PENfoil slides (Leica) were microdissected using a Leica AS-LMD. Genomic DNA from regions of WT or inflamed $\text{G}\alpha 2^{-/-}$ crypts and $\text{G}\alpha 2^{-/-}$ cancers was purified using a PureGene DNA isolation kit (Qiagen) and used for MSI and promoter methylation studies.

MSI analysis. Individual microsatellite templates were analyzed for insertion/deletion mutations by single-molecule PCR using standard protocols (20, 21). Briefly, DNA isolated from laser capture-microdissected $\text{G}\alpha 2^{-/-}$ and WT colons was diluted to 0.5 to 1.5 single-genome equivalents followed by PCR amplification by mononucleotide microsatellite markers A33 and L24375.

DNA methylation analysis. Genomic DNA (650 ng) from WT and colitic $\text{G}\alpha 2^{-/-}$ crypts ($n = 7$ each) was modified using an Imprint DNA Modification Kit (Sigma). PCR primers, complementary to the modified or unmodified sequence, were designed for the proximal *Mlh1* promoter region. For the analysis of the proximal area of the *Mlh1* promoter, a region expanding from -324 to -288 bp of the ***PENDING****Mlh1* promoter was amplified by hemineated PCR using the following conditions: unmethylated primers 5'-ACTACAATCTACGCCGTCT-3' (sense), 5'-TTCGACCGACTACAATCCTA-3' (antisense for first PCR), and 5'-TTTAGTTTGGTGTCCGGTC-3' (antisense for second PCR); methylated primers: 5'-AACTACAATCCTACCATCTTC-3' (sense), 5'-ACTTCAACCAACTACAATCCTA-3' (antisense for first PCR), and 5'-TTTTAGTTTGGTGTGGGTT-3' (antisense for second PCR). Bisulfite-treated DNA was subjected to nested PCR and subsequent sequence verification. One microliter of the first PCR product was used as template for the second PCR.

Semiquantitative real-time PCR. Real-time PCR was done using an Applied Biosystems Step One Plus instrument using SYBR Green detection reagents. Oligonucleotide sequences were as follows: murine MLH1

forward 5'-GTTTACTCCATTCGGAAGCAGT-3', murine MLH1 reverse 5'-TGTGAGCGGAAGGCTTTATAGAT-3', murine PMS2 forward 5'-ACCTCACGAGTTGACTT-3', murine PMS2 reverse 5'-AGTCGAGTCCCAACGCTTG-3', murine BNIP3 forward 5'-CGAAGCGCACAGCTACTCTC-3', murine BNIP3 reverse 5'-GCCTTCCAATGTAGATCCCCAAG-3', murine DEC-1 forward 5'-TACAAGCTGGTGTATTGTCCG-3', and murine DEC-1 reverse 5'-CTGGGAAGATTTCAGGTCCCG-3'. Normalization was done using murine RPA2 (forward 5'-ATACCAGACAACAGAGGG-3' and reverse 5'-GCCAGTCCGCTCAATCACC-3').

In vitro hypoxia treatment. Subconfluent cultures of YAMC cells (a kind gift of Dr. Robert Whitehead) were grown in RPMI with 5% fetal bovine serum, 100 ng/ μL IFN- γ , and 1 \times insulin/transferrin/selenium and incubated in a Biospherix hypoxia chamber at 37°C in 1% O₂ for 24 h and total RNA was analyzed by quantitative real-time PCR.

Suberoylanilide hydroxamic acid treatment of $\text{G}\alpha 2^{-/-}$ mice. Groups of 32- to 36-week-old $\text{G}\alpha 2^{-/-}$ mice ($n = 4$) were treated with daily gavage feedings with a 5 mg/mL solution of suberoylanilide hydroxamic acid (SAHA; Cayman Chemical) solubilized in PBS containing a 5 molar excess of β -hydroxypropyl cyclodextrin (Sigma) for 7 days (final dose 50 mg/kg). Control animals received only cyclodextrin solution. Colons were harvested and crypts isolated as described above and analyzed for MLH1 expression by Western blot analysis.

Chromatin immunoprecipitation. Cells and crypts were cross-linked in 1% formaldehyde for 10 min and then lysed in SDS lysis buffer and sonicated to a fragment length of 300 to 1,000 bp. The sonicate was precleared and the supernatant was incubated overnight at 4°C with the following antibodies: anti-DEC-1 (Santa Cruz Biotechnology; clone S-8) and anti-acetyl histone H3 (Millipore). Rabbit IgG was used as a negative control antibody. Following immunoprecipitation, washing, crosslink reversal, and DNA cleanup, the proximal *Mlh1* promoter region was amplified for an optimized number of cycles using the following primers: forward 5'-GTTTAGAGCGGGACAGAGAT-3' and reverse 5'-CCTGCTACAAACGCCATATT-3'.

Histopathologic assessment of $\text{G}\alpha 2^{-/-}$ and SAHA-treated mice. The severity of colitis and assessment of cancer histology for $\text{G}\alpha 2^{-/-}$ mice was done as described previously (19).

Statistical analyses. For MSI analysis, statistically significant mutation frequency differences between genomic DNA from different genotypes were tested by Fisher's exact test. Statistical significance was assessed using Student's paired *t* test for linear data and the Mann-Whitney *U* test for nonlinear data.

Results

Previously, we detailed the natural history and histopathology of $\text{G}\alpha 2^{-/-}$ colorectal cancer (19). We have now extended our colorectal cancer analysis in a cohort ($n = 47$) of $\text{G}\alpha 2^{-/-}$ mice (Table 1). Among $\text{G}\alpha 2^{-/-}$ mice sacrificed after age 20 weeks, 89.8% had at least one colorectal cancer present, with a mean tumor incidence of 1.89 colorectal cancers per mouse. In contrast, germline *Mlh1* and *Msh2* MMR-deficient mice rarely develop colonic adenomas or colorectal cancer; <50% develop small intestine adenomas by 12 months and only rarely develop colorectal cancer (22). $\text{G}\alpha 2^{-/-}$ mice colorectal cancers were predominantly right-sided (75%). Like human MMR-deficient colorectal cancers, all $\text{G}\alpha 2^{-/-}$ colorectal cancers were nonpolypoid and had a Crohn's like lymphocytic infiltrate. (See Supplementary Fig. S3 for a histopathologic comparison of a Lynch syndrome colorectal cancer versus $\text{G}\alpha 2^{-/-}$ colorectal cancer.) Mucinous colorectal cancers are more common in MMR-deficient colorectal cancers (15-25%; refs. 23-25) than sporadic colorectal cancer patients (5-10%). $\text{G}\alpha 2^{-/-}$ tumors develop a higher rate of mucinous colorectal cancers (29%) than has previously been reported in any mouse model of colorectal cancer. In summary, $\text{G}\alpha 2^{-/-}$ colorectal cancer are more similar to Lynch syndrome and sporadic MMR-deficient

Table 1. Histopathology of colorectal cancers in $\text{G}\alpha 2^{-/-}$ mice

Location/histology	Nonmucinous	Mucinous	Total no.	% of Total
Cecum	39	7	46	51.7
Proximal	15	7	22	24.7
Mid	9	5	14	15.7
Distal	0	7	7	7.9
Totals	63	26	89	100
% of Total	70.7	29.3		

NOTE: Colons from 47 mice with cancer between ages 12 and 53 were evaluated for the number, location, and histologic differentiation of colorectal cancers.

colorectal cancer (26) than the existing MMR-deficient mouse models (Supplementary Table S1) or MMR-proficient colorectal cancer.

Acquired suppression of MLH1 and PMS2 protein in $\text{G}\alpha 2^{-/-}$ knockout colon epithelium. Epigenetic silencing of MLH1 causes the majority of sporadic MMR-defective colorectal cancer (27, 28). Because $\text{G}\alpha 2^{-/-}$ colorectal cancer phenocopy Lynch syndrome and sporadic MMR-deficient colorectal cancer, we stained colonic epithelium from young versus old WT and $\text{G}\alpha 2^{-/-}$ mice for MMR expression. $\text{G}\alpha 2^{-/-}$ colons from young (<6 weeks) mice have no inflammation or colorectal cancer (19), and immunohistochemistry performed on 8-week-old $\text{G}\alpha 2^{-/-}$ mice showed WT levels of MMR proteins MLH1, PMS2, MSH2, and MSH6 (Supplementary Fig. S1). By age 40 weeks, immunohistochemistry of $\text{G}\alpha 2^{-/-}$ colitis and colorectal cancer showed a consistent, marked reduction in MLH1 and PMS2 protein levels in inflamed and cancerous $\text{G}\alpha 2^{-/-}$ mucosa (Supplementary Fig. S2) in both epithelium and fixed stromal cells (E, J, G, and K). MLH1 expression was preserved in infiltrating lymphocytes in the $\text{G}\alpha 2^{-/-}$ tissues (white boxed region, F). PMS2 is an obligate heterodimeric partner of MLH1. With MLH1 silencing, undimerized PMS2 protein becomes unstable and is degraded (20). Expression of the MSH2 and MSH6 MMR proteins is not affected by MLH1 silencing.

To confirm the immunohistochemical results, we quantified MMR protein expression in purified epithelia from WT and inflamed $\text{G}\alpha 2^{-/-}$ colons. In young mice, MMR protein expression was unchanged (Fig. 1A, top). However, after 20 weeks, total crypt MLH1 and PMS2 were markedly decreased in $\text{G}\alpha 2^{-/-}$ colonic epithelial cells (Fig. 1A, bottom). Consistent with the immunohistochemical result (Fig. 1S-H), MSH2 expression was unchanged.

To corroborate the acquired loss of MLH1 expression between ages 8 and 20 weeks, quantitative PCR analysis of MLH1 expression in 8- and 20-week-old $\text{G}\alpha 2^{-/-}$ crypt epithelium revealed normal MLH1 expression at the early time point but a ~75% drop in MLH1 expression in inflamed 20-week-old $\text{G}\alpha 2^{-/-}$ crypts (Fig. 1B). $\text{G}\alpha 2^{-/-}$ crypt PMS2 mRNA levels were not significantly different from WT at either time point.

In summary, both immunohistochemistry and Western analysis show that MLH1 and PMS2 protein levels are markedly reduced in inflamed $\text{G}\alpha 2^{-/-}$ colon. This decrease is somatically acquired, as it follows the onset of inflammation. MSH2 and MSH6 levels are not affected. *Mlh1* is transcriptionally repressed, whereas PMS2

reduction is post-translational likely in response to decreased MLH1 protein levels.

MSI in inflamed $\text{G}\alpha 2^{-/-}$ mucosa. MMR-deficient colorectal cancers include increased MSI and impaired DNA damage response. Previously, we have shown hypomorphic MSI in *Mlh3*^{-/-} and *Pms2*^{-/-} mice (because of redundancy between *Mlh3* and *Pms2*) and full-blown MSI in *Mlh3*^{-/-};*Pms2*^{-/-} mice, indistinguishable from *Mlh1*^{-/-} mice (29). Using mouse microsatellite markers previously studied in other MMR-deficient mouse models (16, 30), we performed MSI testing on laser capture-microdissected $\text{G}\alpha 2^{-/-}$ tumor DNA and WT colon epithelium DNA. Consistent with previous studies of both mouse and human, almost all MSI was caused by mononucleotide repeat deletions that shortened microsatellites (31). For each marker tested, $\text{G}\alpha 2^{-/-}$ colorectal cancers had significantly increased MSI versus WT (both $P < 0.0001$; Table 2). At the same time, young precolitic $\text{G}\alpha 2^{-/-}$ colon showed no MSI (data not shown).

MLH1 promoter methylation is not increased in $\text{G}\alpha 2^{-/-}$ mice. Epigenetic silencing of the proximal *Mlh1* promoter by CpG methylation is a common mechanism for sporadic MMR-deficient colorectal cancer tumorigenesis (32). We therefore studied the orthologous CpG island in the mouse *Mlh1* promoter in $\text{G}\alpha 2^{-/-}$ inflamed colon. Using bisulfite-treated WT and colitic $\text{G}\alpha 2^{-/-}$ crypt genomic DNA, we observed no significant differences in the intensity of methylated versus unmethylated PCR products between WT colon and $\text{G}\alpha 2^{-/-}$ inflamed colon samples (Supplementary Fig. S4). Methylation of the proximal *Mlh1* promoter does not appear to contribute to epigenetic silencing of *Mlh1* in $\text{G}\alpha 2^{-/-}$ inflamed colons and colorectal cancer.

Inflamed $\text{G}\alpha 2^{-/-}$ colons and colorectal cancers are hypoxic. The lack of promoter hypermethylation in the setting of suppressed MLH1 expression led us to test other potential mechanisms shown to silence *Mlh1*. *In vitro* studies have shown that hypoxia can repress

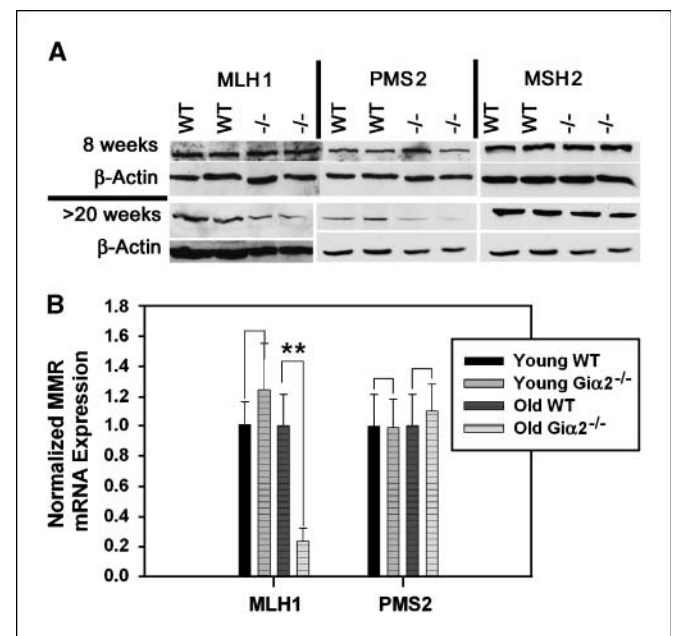


Figure 1. A, acquired suppression of MLH1 and PMS in $\text{G}\alpha 2^{-/-}$ crypts. Purified 8-wk-old (top) and >20-wk-old (bottom) WT and $\text{G}\alpha 2^{-/-}$ crypt epithelial cells were immunoblotted for MLH1, PMS2, and MSH2. B, quantitative PCR for MLH1 and PMS2 in young and old crypts ($n = 3$ each) reveals a significant down-regulation of MLH1 in older $\text{G}\alpha 2^{-/-}$ crypts. Representative of three separate experiments. **, $P < 0.01$.

Table 2. Microsatellite analysis

MSI marker	Genotype	n	No. variant alleles	Total mutation frequency	P vs WT
A33	WT	235	8	0.03	<0.0001
	$Gi\alpha 2^{-/-}$	229	62	0.27	
	$Mlh3^{-/-};Pms2^{-/-}$	235	107	0.46	
L24375 (A23)	WT	248	4	0.02	<0.0001
	$Gi\alpha 2^{-/-}$	275	91	0.33	
	$Mlh3^{-/-};Pms2^{-/-}$	258	104	0.4	

NOTE: Total mutation frequencies were determined using unique mouse mononucleotide microsatellite markers A33 and L24375 (A23). The total number of mutant size variant alleles divided by the total number of alleles is shown for each microsatellite marker. We compared the mutation frequency in colonic tumor DNA from $Gi\alpha 2^{-/-}$, $Mlh3^{-/-};Pms2^{-/-}$, or normal WT tissue among pairs of genotypes. A Fisher's exact test determined statistically significant differences. *, $P < 0.0001$, significant when compared with WT controls.

MLH1 mRNA expression in a HDAC-dependent manner (33). Because other mouse models of colitis have been shown to have mucosal hypoxia (34), we evaluated whether the $Gi\alpha 2^{-/-}$ inflamed colon might be hypoxic and whether hypoxic repression of MLH1 might be occurring *in vivo*. DEC-1 and BNIP3 are well-characterized markers of hypoxia (35, 36). First, we validated DEC-1 (37) and BNIP3 (38) as hypoxic markers in colonocytes by incubating YAMC cells *in vitro* under hypoxia (1% O_2) or normoxia for 24 h. Consistent with previous studies in human colon cancer cell lines and other cells types, exposure to hypoxic conditions resulted in a significant up-regulation of BNIP3 (9.00 ± 1.07 -fold; $P = 0.009$) and DEC-1 (2.11 ± 0.27 -fold; $P = 0.033$) mRNA expression. Western analysis confirmed proportional increases in DEC-1 and BNIP3 protein in hypoxic YAMC cells (Fig. 2A), confirming that DEC-1 and BNIP3 are inducible markers of hypoxia in mouse colonocytes. Next, we determined that these hypoxia markers were up-regulated *in vivo* in colonocytes in crypts from colitic $Gi\alpha 2^{-/-}$ mice compared with WT mice.

To show directly that $Gi\alpha 2^{-/-}$ inflamed colons are hypoxic, we used EF5 (kindly provided by Dr. Cameron Koch), a well-characterized direct probe of tissue hypoxia (39). We injected WT and colitic $Gi\alpha 2^{-/-}$ mice with EF5. Four hours after i.v. injection, in WT colon tissues, the surface epithelium contains a

faint diffuse staining, difficult to capture in unenhanced immunofluorescence images (Fig. 2B, a). In contrast, in $Gi\alpha 2^{-/-}$ inflamed colon, hypoxia is greatly increased in the surface epithelium and in the deeper crypts in $Gi\alpha 2^{-/-}$ mice. Notably, hypoxia is particularly prominent in areas with cancer and adjacent underlying stroma (Fig. 2B, b and c). Treatment of the same cancer sample with precompeted anti-EF5 abrogated staining (Fig. 2B, d). The corresponding areas following hematoxylin counterstaining are shown in Supplementary Fig. S6. The pattern of EF5 staining seen in inflamed $Gi\alpha 2^{-/-}$ colons is similar to that seen in the mouse model of TNBS colitis (34). In summary, DEC-1 and BNIP3 are biomarkers of hypoxia in mouse colonocytes and in $Gi\alpha 2^{-/-}$ inflamed colon epithelium. Furthermore, EF5 dye studies confirm that, similar to TNBS-induced colitis, EF5 trapping in hypoxic tissue is increased in $Gi\alpha 2^{-/-}$ inflamed colon.

Hypoxia modulates acetyl histone H3 levels and DEC-1 association with the proximal MLH1 promoter *in vitro* and *in vivo*. Low levels of acetyl histone H3 are associated with transcriptional silencing of many genes (40). Using chromatin immunoprecipitation, we tested whether hypoxia decreases acetyl histone H3 content at the proximal *Mlh1* promoter in YAMC cells and whether treatment with HDACi can increase acetyl histone H3

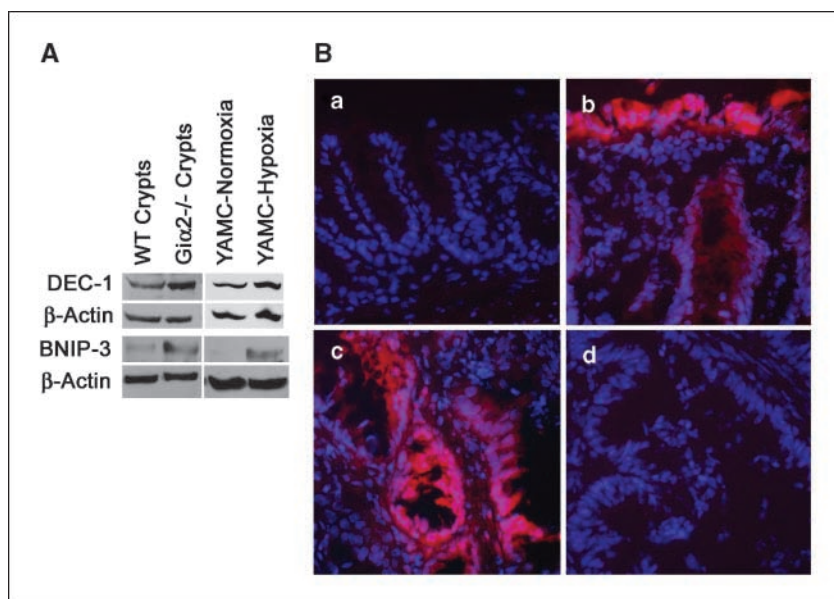
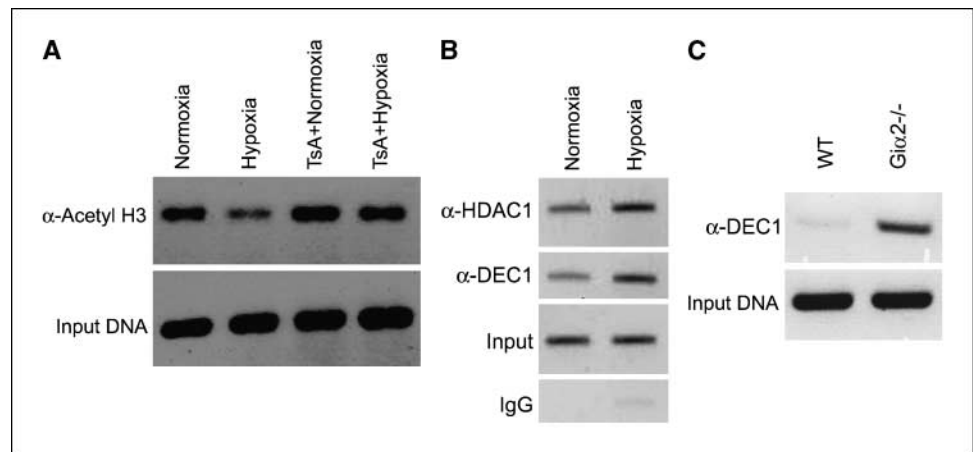


Figure 2. Inflamed $Gi\alpha 2^{-/-}$ crypts are hypoxic. A, YAMC cells grown in 1% O_2 (right) up-regulate the hypoxia markers DEC-1 and BNIP3, which are also up-regulated in purified crypt lysates from colitic $Gi\alpha 2^{-/-}$ mice (left). Representative of three separate YAMC and crypt isolation experiments. B, frozen sections from EF5-treated WT and $Gi\alpha 2^{-/-}$ mice (age 20-27 wk) were stained with Cy3-conjugated anti EF5. WT colon (a) had minimal EF5 staining, whereas colitic $Gi\alpha 2^{-/-}$ mucosa (b) was profoundly hypoxic in surface epithelium and in some deeper crypt cells. EF5 staining of $Gi\alpha 2^{-/-}$ cancers (c) was patchy and involved primarily malignant epithelial cells and the adjacent stroma. As a negative control, preadsorbed anti EF-5 (d) revealed no staining.

Figure 3. Hypoxia modulates histone H3 acetylation and DEC-1 and HDAC1 binding to the proximal MLH1 promoter. **A**, hypoxia induces histone H3 deacetylation at the MLH1 promoter in a HDAC-dependent manner. **B**, hypoxia enhances DEC-1 and HDAC1 binding to the proximal MLH1 promoter in YAMC cells. **C**, in colitic $\text{Gi}\alpha 2^{-/-}$ crypts, DEC-1 binding to the MLH1 promoter is also enhanced. TSA, trichostatin A.



levels. Figure 3A shows that hypoxia suppressed histone H3 acetylation at the proximal *Mlh1* promoter, whereas pretreatment with trichostatin A enhanced *Mlh1* promoter histone H3 acetylation in hypoxic (as well as normoxic) culture conditions. This indicates that hypoxia decreases acetyl histone H3 content at the *Mlh1* promoter *in vitro*, which can be rescued by HDACi treatment. Consistent with other cell lines rendered hypoxic *in vitro*, hypoxia suppresses MLH1 expression at the mRNA and protein level by quantitative PCR and Western blot, respectively (data not shown).

Hypoxia is known to enhance DEC-1 binding to the proximal *Mlh1* promoter in cell culture, suppressing *Mlh1* expression (41). We performed chromatin immunoprecipitation on genomic DNA from hypoxic YAMC cells and from colonic crypt epithelium from 28-week-old WT and colitic $\text{Gi}\alpha 2^{-/-}$ mice and analyzed the proximal murine *Mlh1* promoter using oligos spanning this region. We identified a marked increase in DEC-1 at the proximal *Mlh1* promoter in hypoxic YAMC cells as well as $\text{Gi}\alpha 2^{-/-}$ crypts mice *in vivo* (Fig. 3 B and C). Consistent with previous reports indicating the ability of DEC-1 to bind HDAC1 (42), we also found an increase in HDAC1 bound to the *Mlh1* promoter in hypoxic YAMC cells. In hypoxic YAMC cells and $\text{Gi}\alpha 2^{-/-}$ inflamed crypts, DEC-1 occupancy at the proximal *Mlh1* promoter is increased, as is HDAC1 binding that may be responsible for decreased acetyl histone H3 levels.

HDACi relieve hypoxia, reduce inflammation, and normalize MLH1 expression in $\text{Gi}\alpha 2^{-/-}$ colorectal cancers. Recent studies have shown both anti-inflammatory and antineoplastic effects of HDACi in mouse models of chronic colitis and colitis-associated colorectal cancer (43, 44). These results suggest that chronic inflammation alters histone acetylation in the inflamed colon, and this epigenetic change may contribute to tumorigenesis. Because HDACi have been shown to block hypoxia-induced MLH1 silencing *in vitro* (33), we tested whether HDACi treatment could enhance MLH1 expression *in vivo*. We gavaged groups of 33- to 36-week-old $\text{Gi}\alpha 2^{-/-}$ mice with 50 mg/kg SAHA daily for 8 days and evaluated MLH1 expression in crypt epithelium by Western blot and quantitative PCR. SAHA caused a significant reduction in colitis severity (Fig. 4A) using a previously published histologic activity index (45). We found significant increases in MLH1 mRNA and protein expression (Fig. 4B and C; Supplementary Fig. S5). Overall acetyl histone H3 levels in SAHA-treated crypts were increased (data not shown). By chromatin immunoprecipitation analysis, SAHA treatment enhanced acetyl histone H3 levels and decreased DEC-1 occupancy at the *Mlh1* promoter (Fig. 4D). These experiments indicate that HDACi relieve *Mlh1* transcriptional repression

in chronically inflamed $\text{Gi}\alpha 2^{-/-}$ crypts via epigenetic alterations of the *Mlh1* promoter involving histone acetylation and DEC-1.

Discussion

Human MMR-deficient colorectal cancers have histopathologic features that distinguish them from MMR-proficient sporadic colorectal cancer (1), including right-sidedness, nonpolyposis and mucinous histology, earlier age of onset, multifocality, a Crohn's-like lymphocytic infiltrate, and MSI (2, 3). Although multiple germ-line MMR-deficient mouse models have been generated, a

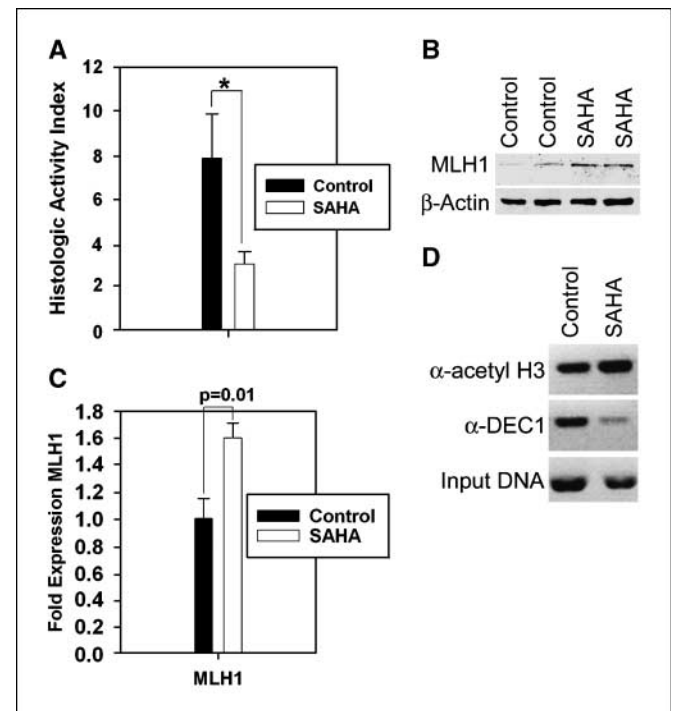


Figure 4. HDACi treatment improves colitis and rescues MLH1 expression by decreasing DEC-1 occupancy at the proximal MLH1 promoter. **A**, age-matched 33- to 36-wk-old $\text{Gi}\alpha 2^{-/-}$ mice were treated with vehicle or 50 mg/kg SAHA ($n = 4$ each) for 8 d and then assessed for disease severity using a histologic activity scoring index. *, $P < 0.05$. Representative of three separate experiments. Purified crypt lysates were evaluated for MLH1 protein (**B**) and mRNA (**C**) by Western blotting and quantitative PCR, respectively. **D**, chromatin immunoprecipitation reveals an increase in acetyl histone H3 levels and decreased DEC-1 binding in SAHA-treated crypts.

persistent mystery is their very different histologic phenotype versus that seen in human Lynch syndrome and MMR-deficient sporadic colorectal cancer.

The histopathology of $\text{G}\alpha 2^{-/-}$ colorectal cancer is very similar to Lynch syndrome and MMR-deficient sporadic colorectal cancer. We speculate that the mechanism of inheritance of MMR deficiency may explain these observations. When all mouse/human body tissues are MMR-deficient, intestinal cells transform into cancers in a MMR-deficient microenvironment in the absence of hypoxic inflammation. This may enhance tumor growth in the tissues seen in human and mouse biallelic germ-line MMR deficiency. When hypoxic inflammation-induced MMR-deficient cells transform into cancers surrounded by a partially MMR-proficient microniche (given the hypomorphic MSI that we observe), tumor growth is enhanced with the spectrum seen in Lynch syndrome, sporadic MMR-deficient colorectal cancer, and $\text{G}\alpha 2^{-/-}$ mice. Determining the relative importance of hypoxia-induced signaling versus inflammation-derived mediators in MMR silencing is under way. The MMR status of tumor microniche may also explain why $\text{Mlh}3^{-/-}$ mice (which are MMR-deficient hypomorphs) develop a higher proportion of colorectal cancers versus small intestinal adenomas than completely MMR-deficient mouse models (16), albeit with overall lower intestinal cancer penetrance.

$\text{G}\alpha 2^{-/-}$ mice have somatically acquired MMR deficiency that occurs in both inflamed and cancerous colon mucosa. This repression of MMR activity is specific to the MutL homologues *Mlh1* and *Pms2*, as the MutS homologues *Msh2* and *Msh6* are not affected.

Consistent with this reduction in MLH1/PMS2 protein levels, inflamed $\text{G}\alpha 2^{-/-}$ colorectal cancers have MSI. However, some residual MLH1 protein remains. Therefore, $\text{G}\alpha 2^{-/-}$ mice have a hypomorphic MSI phenotype. Hypomorphic MSI likely reflects two factors: (a) decreased but not absent MLH1/PMS2 protein and (b) MSI arises only in older mice after colon inflammation, so mouse tissue is MMR-deficient for a shorter period. Together, these factors would be expected to cause lower MSI frequencies compared with germ-line MMR-deficient knockout mice. The same issue may also explain why the impaired DNA damage response in $\text{G}\alpha 2^{-/-}$ inflamed colon (19) is also not as dramatic as that seen in germ-line MMR-deficient models (46). Studies using inducible *Mlh1* knockouts showed that low-level MLH1 expression has ~20% of the MSI mutation rate compared with complete loss of MLH1 expression and near-proficiency in cell extract-based single-base substitution repair assays (47). Another example of hypomorphic MMR and MSI/frameshift capacity was seen in studies with *Pms2*^{-/-} and *Mlh3*^{-/-} mice. There is redundancy between *Pms2* and *Mlh3* in MSI/frameshift repair. Loss of each of these genes individually causes hypomorphic MSI, but together, *Mlh3*^{-/-};*Pms2*^{-/-} mice have “full” MSI that is indistinguishable from *Mlh1*^{-/-} mice. Because both *Mlh3*^{-/-} and *Pms2*^{-/-} mice develop tumors, these data are consistent with a mechanism whereby partial MMR loss causes cancer susceptibility. The precise relationship between the degree of MMR capacity and MSI rates is likely to depend on the cell lineage, residual MMR capacity, and the specific repeat sequences analyzed to quantify MSI mutation rates. Future studies that use genome-wide assays of MSI mutation rates in MMR-proficient, hypomorphic, and deficient colon will be helpful to resolve the relationship between MMR capacity, hypomorphic MMR, and MSI mutations in colorectal cancer “driver” CAN genes and noncoding sequences important for colorectal cancer tumorigenesis.

Hypermethylation of the proximal *Mlh1* promoter is a well-established mechanism for MLH1 silencing in sporadic MMR-

deficient colorectal cancer (reviewed in ref. 48). In inflamed $\text{G}\alpha 2^{-/-}$ crypt gDNA, we did not find increased methylation of the orthologous sequence in the mouse proximal *Mlh1* promoter (Supplementary Fig. S4). We cannot, however, rule out that the *Mlh1* promoter is methylated in $\text{G}\alpha 2^{-/-}$ cancers. Future studies treating $\text{G}\alpha 2^{-/-}$ mice with hypomethylating agents and assessing the effect on tumorigenesis and *Mlh1* silencing may be helpful to address this issue.

Recently, tissue hypoxia has emerged as a mechanism of *Mlh1* silencing and MMR deficiency *in vitro* but has not yet been shown to occur *in vivo*. Using hypoxia biomarkers BNIP3, DEC-1, and EF5 (49), we show that $\text{G}\alpha 2^{-/-}$ inflamed colon has regional hypoxia, likely resulting from the dense infiltrate of chronic inflammatory cells, and increased metabolic activity in damaged/regenerating epithelium. Hypoxia-induced epigenetic MLH1 silencing involves up-regulation of the bHLH transcription factor DEC-1/Sharp2/Stra13 via a hypoxia-inducible factor-1 α response element in its promoter (42). DEC-1 contains a NH₂-terminal DNA-binding domain that recognizes a CANNTG- E-box consensus sequence in the proximal *Mlh1* promoter (41) and a COOH-terminal transactivation domain that binds the class 1 HDAC (42). *In vitro*, DEC-1 expression inhibits MLH1 expression in a HDAC-dependent manner (33, 41, 50), as HDACi prevent hypoxic DEC-1-mediated MLH1 repression. Consistent with predictions based on *in vitro* studies, we showed increased DEC-1 expression in inflamed $\text{G}\alpha 2^{-/-}$ crypts (Fig. 2A) and increased DEC-1 occupancy at the proximal *Mlh1* promoter *in vivo* (Fig. 3C). Because colitis (and hypoxia) is patchy, the DEC-1 Western and chromatin immunoprecipitation data likely underestimate the degree of hypoxia-induced DEC-1 expression and its binding to the *Mlh1* promoter, as both techniques assay all crypts isolated from a segment of colon, not all of which is necessarily hypoxic. A model of epigenetic *Mlh1* silencing by hypoxia is illustrated in Supplementary Fig. S7.

Recent reports indicate that treatment with HDACi have anti-inflammatory and antineoplastic effects in animal models of inflammatory bowel disease and inflammatory bowel disease-associated colorectal cancer. In dextran sulfate sodium and TNBS colitis models, the HDACi VPA and SAHA dose-dependently increase acetyl histone H3 in lamina propria mononuclear cells, inhibit proinflammatory cytokines, and reduce colitis severity (43). Another HDACi, ITF2357, can suppress inflammatory bowel disease-associated colorectal cancer development in interleukin-10^{-/-} and azoxymethane/dextran sulfate sodium-treated mice apparently by lowering nuclear factor- κ B activity and enhanced apoptotic activity in lamina propria mononuclear cells (44). Interestingly, the antineoplastic effect was only seen in the right colon, suggesting differential sensitivity of right- and left-sided colitis-associated colorectal cancers to HDACi. We found that daily oral gavage with 50 mg/kg SAHA caused a significant decrease in colitis and up-regulated MLH1 expression, suggesting that decreased hypoxic inflammation results in increased reversal of *Mlh1* silencing via increased tissue oxygenation.

Chronic inflammation is a risk factor for colorectal cancer. Although the suppressive effects of HDACi on nuclear factor- κ B activity and inflammation undoubtedly contribute to their chemopreventive effect in azoxymethane/dextran sulfate sodium and interleukin-10 knockout mice, our studies indicate that the hypoxic tissue microenvironment may also contribute to the development of MSI via *Mlh1* suppression and that HDACi treatment can reverse *Mlh1* silencing. Most importantly, in concert with previous work, our studies provide additional preclinical mechanistic support that

colorectal cancer prevention trials in inflammatory bowel disease patients with HDACi should be explored in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 2003;349:247–57.
2. Greenson JK, Bonner JD, Ben-Yzhak O, et al. Phenotype of microsatellite unstable colorectal carcinomas: well-differentiated and focally mucinous tumors and the absence of dirty necrosis correlate with microsatellite instability. *Am J Surg Pathol* 2003;27:563–70.
3. Jenkins MA, Hayashi S, O'Shea AM, et al. Pathology features in Bethesda guidelines predict colorectal cancer microsatellite instability: a population-based study. *Gastroenterology* 2007;133:48–56.
4. Felton KE, Gilchrist DM, Andrew SE. Constitutive deficiency in DNA mismatch repair: is it time for Lynch III? *Clin Genet* 2007;71:499–500.
5. Lindor NM, Rabe K, Petersen GM, et al. Lower cancer incidence in Amsterdam-I criteria families without mismatch repair deficiency: familial colorectal cancer type X. *JAMA* 2005;293:1979–85.
6. Barnetson RA, Tenesa A, Farrington SM, et al. Identification and survival of carriers of mutations in DNA mismatch-repair genes in colon cancer. *N Engl J Med* 2006;354:2751–63.
7. Samowitz WS, Albertsen H, Sweeney C, et al. Association of smoking, CpG island methylator phenotype, and V600E BRAF mutations in colon cancer. *J Natl Cancer Inst* 2006;98:1731–8.
8. Shen L, Issa JP. Epigenetics in colorectal cancer. *Curr Opin Gastroenterol* 2002;18:68–73.
9. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;38:787–93.
10. Baker SM, Bronner CE, Zhang L, et al. Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell* 1995;82:309–19.
11. de Wind N, Dekker M, Berns A, Radman M, te Riele H. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* 1995;82:321–30.
12. Edelmann W, Umar A, Yang K, et al. The DNA mismatch repair genes Msh3 and Msh6 cooperate in intestinal tumor suppression. *Cancer Res* 2000;60:803–7.
13. Edelmann W, Yang K, Kuraguchi M, et al. Tumorigenesis in Mlh1 and Mlh1/Apc1638N mutant mice. *Cancer Res* 1999;59:1301–7.
14. Edelmann W, Yang K, Umar A, et al. Mutation in the mismatch repair gene Msh6 causes cancer susceptibility. *Cell* 1997;91:467–77.
15. Lipkin SM, Moens PB, Wang V, et al. Meiotic arrest and aneuploidy in MLH3-deficient mice. *Nat Genet* 2002;31:385–90.
16. Chen PC, Dudley S, Hagen W, et al. Contributions by MutL homologues Mlh3 and Pms2 to DNA mismatch repair and tumor suppression in the mouse. *Cancer Res* 2005;65:8662–70.

Acknowledgments

Received 4/6/09; revised 6/15/09; accepted 6/16/09; published OnlineFirst 7/28/09.
Grant support: NIH grants K08 DK59816 and R21 DK071591 (R.A. Edwards) and CA98626 (S.M. Lipkin) and Intramural Research Program of the NIH Project Z01-ES101643.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

17. Rudolph U, Finegold MJ, Rich SS, et al. Ulcerative colitis and adenocarcinoma of the colon in Gai2-deficient mice. *Nat Genet* 1995;10:143–50.
18. Hornquist CE, Lu X, Rogers-Fani PM, et al. G(α)2-deficient mice with colitis exhibit a local increase in memory CD4⁺ T cells and proinflammatory Th1-type cytokines. *J Immunol* 1997;158:1068–77.
19. Edwards RA, Wang K, Davis JS, Birnbaumer L. Role for epithelial dysregulation in early-onset colitis-associated colon cancer in Gi2-α^{-/-} mice. *Inflamm Bowel Dis* 2008;14:898–907.
20. Yao X, Buermeier AB, Narayanan L, et al. Different mutator phenotypes in Mlh1- versus Pms2-deficient mice. *Proc Natl Acad Sci U S A* 1999;96:6850–5.
21. Yang G, Scherer SJ, Shell SS, et al. Dominant effects of an Msh6 missense mutation on DNA repair and cancer susceptibility. *Cancer Cell* 2004;6:139–50.
22. Taketo MM, Edelmann W. Mouse models of colon cancer. *Gastroenterology* 2009;136:780–98.
23. Alexander J, Watanabe T, Wu TT, Rashid A, Li S, Hamilton SR. Histopathological identification of colon cancer with microsatellite instability. *Am J Pathol* 2001;158:527–35.
24. Jass JR. HNPCC and sporadic MSI-H colorectal cancer: a review of the morphological similarities and differences. *Fam Cancer* 2004;3:93–100.
25. Yearsley M, Hampel H, Lehman A, Nakagawa H, de la Chapelle A, Frankel WL. Histologic features distinguish microsatellite-high from microsatellite-low and microsatellite-stable colorectal carcinomas, but do not differentiate germline mutations from methylation of the MLH1 promoter. *Hum Pathol* 2006;37:831–8.
26. Raut CP, Pawlik TM, Rodriguez-Bigas MA. Clinicopathologic features in colorectal cancer patients with microsatellite instability. *Mutat Res* 2004;568:275–82.
27. Whitelaw NC, Whitelaw E. Transgenerational epigenetic inheritance in health and disease. *Curr Opin Genet Dev* 2008;18:273–9.
28. Ligtenberg MJ, Kuiper RP, Chan TL, et al. Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3' exons of TACSTD1. *Nat Genet* 2009;41:112–7.
29. Chen PC, Kuraguchi M, Velasquez J, et al. Novel roles for MLH3 deficiency and TLE6-like amplification in DNA mismatch repair-deficient gastrointestinal tumorigenesis and progression. *PLoS Genet* 2008;4:e1000092.
30. Kabbarah O, Mallon MA, Pfeifer JD, Edelmann W, Kucherlapati R, Goodfellow PJ. A panel of repeat markers for detection of microsatellite instability in murine tumors. *Mol Carcinog* 2003;38:155–9.
31. Umar A, Boland CR, Terdiman JP, et al. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* 2004;96:261–8.
32. Deng G, Chen A, Hong J, Chae HS, Kim YS. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res* 1999;59:2029–33.
33. Mihaylova VT, Bindra RS, Yuan J, et al. Decreased expression of the DNA mismatch repair gene Mlh1

- under hypoxic stress in mammalian cells. *Mol Cell Biol* 2003;23:3265–73.
34. Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J Clin Invest* 2004;114:1098–106.
35. Koukourakis MI, Giatromanolaki A, Polychronidis A, et al. Endogenous markers of hypoxia/anaerobic metabolism and anemia in primary colorectal cancer. *Cancer Sci* 2006;97:582–8.
36. Turley H, Wykoff CC, Troup S, Watson PH, Gatter KC, Harris AL. The hypoxia-regulated transcription factor DEC1 (Stra13, SHARP-2) and its expression in human tissues and tumours. *J Pathol* 2004;203:808–13.
37. Wykoff CC, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling. *Oncogene* 2000;19:6297–305.
38. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* 2000;97:9082–7.
39. Koch CJ. Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. *Methods Enzymol* 2002;352:3–31.
40. Vaissiere T, Sawan C, Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutat Res* 2008;659:40–8.
41. Nakamura H, Tanimoto K, Hiyama K, et al. Human mismatch repair gene, MLH1, is transcriptionally repressed by the hypoxia-inducible transcription factors, DEC1 and DEC2. *Oncogene* 2008;27:4200–9.
42. Ivanov SV, Salnikow K, Ivanova AV, Bai L, Lerman MI. Hypoxic repression of STAT1 and its downstream genes by a pVHL/HIF-1 target DEC1/STRA13. *Oncogene* 2007;26:802–12.
43. Glauben R, Batra A, Fedke I, et al. Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *J Immunol* 2006;176:5015–22.
44. Glauben R, Batra A, Stroh T, et al. Histone deacetylases: novel targets for prevention of colitis-associated cancer in mice. *Gut* 2008;57:613–22.
45. Edwards RA, Smock AZ. Defective arachidonate release and PGE2 production in Giα2-deficient intestinal and colonic subepithelial myofibroblasts. *Inflamm Bowel Dis* 2006;12:153–65.
46. Edelmann W, Cohen PE, Kane M, et al. Meiotic pachytene arrest in MLH1-deficient mice. *Cell* 1996;85:1125–34.
47. Cejka P, Stojic L, Mojas N, et al. Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1. *EMBO J* 2003;22:2245–54.
48. Grady WM. Genomic instability and colon cancer. *Cancer Metastasis Rev* 2004;23:11–27.
49. Taylor CT, Colgan SP. Hypoxia and gastrointestinal disease. *J Mol Med* 2007;85:1295–300.
50. Rodriguez-Jimenez FJ, Moreno-Manzano V, Lucas-Dominguez R, Sanchez-Puelles JM. Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells. *Stem Cells* 2008;26:2052–62.