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Alkylation induced colon tumorigenesis in mice deficient in the Mgmt and Msh6 proteins

James M. Bugni, **Lisiane B. Meira**, and **Leona D. Samson**
Biological Engineering Department and Center for Environmental Health Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts

Abstract

O^6-methylguanine DNA methyltransferase (MGMT) suppresses mutations and cell death that result from alkylation damage. MGMT expression is lost by epigenetic silencing in a variety of human cancers including nearly half of sporadic colorectal cancers, suggesting that this loss maybe causal. Using mice with a targeted disruption of the *Mgmt* gene we tested whether Mgmt protects against azoxymethane (AOM) induced colonic aberrant crypt foci (ACF), against AOM and dextran sulfate sodium (DSS) induced colorectal adenomas, and against spontaneous intestinal adenomas in Apc^{Min} mice. We also examined the genetic interaction of the *Mgmt* null gene with a DNA mismatch repair null gene, namely *Msh6*. Both Mgmt and Msh6 independently suppress AOM-induced ACF, and combination of the two mutant alleles had a multiplicative effect. This synergism can be explained entirely by the suppression of alkylation-induced apoptosis when Msh6 is absent. In addition, following AOM+DSS treatment Mgmt protected against adenoma formation to the same degree as it protected against AOM-induced ACF formation. Finally, Mgmt deficiency did not affect spontaneous intestinal adenoma development in $Apc^{Min/+}$ mice, suggesting that Mgmt suppresses intestinal cancer associated with exogenous alkylating agents, and that endogenous alkylation does not contribute to the rapid tumor development seen in $Apc^{Min/+}$ mice.

Keywords

Colorectal cancer; Mgmt; Msh6; mismatch repair; azoxymethane

INTRODUCTION

O^6-methylguanine (O^6MeG) is a highly toxic and mutagenic lesion formed in DNA by S_N1 alkylating agents. This lesion is efficiently repaired by MGMT via direct transfer of the adducted methyl group to the sulfur of the active site cysteine in MGMT (Fang et al., 2005). This irreversible reaction renders MGMT inactive for further repair and induces a conformational change whereby MGMT is targeted for ubiquitin-dependent degradation (Xu-Welliver & Pegg, 2002).

O^6MeG slows but does not block DNA replication and is highly mutagenic because it preferentially mispairs with thymine, ultimately causing G:C to A:T transition mutations (Larson et al., 1985; Snow et al., 1984). The toxicity of the O^6MeG lesion is attributed to the recognition of O^6MeG:T mispairs by the DNA mismatch repair machinery (Karran & Bignami, 1992). The nascent thymine is removed during mismatch repair processing, but

Corresponding author: Leona D. Samson, 77 Massachusetts Ave., 56-235, MIT, Cambridge MA, 02139.
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another thymine is likely inserted opposite the unrepaired O^6MeG serving to reinitiate mismatch repair processing. This cycle of "futile repair" can lead to chromosomal aberrations, cell cycle arrest, or apoptosis (Hickman & Samson, 1999; Kaina, 2003; Kat et al., 1993).

MGMT is highly conserved phylogenetically, and yeast cells mutated for the *MGMT* homolog, *Mgt1*, have higher spontaneous mutation rates than wild type cells (Xiao & Samson, 1993). While MGMT-deficiency has not been shown to increase spontaneous mutation rate in cultured mammalian cells, wild type cells have a smaller fraction of spontaneous G to A transition mutations compared to mutant cells (Aquilina et al., 1992). Similarly, *Mgmt* null mice show little increase in spontaneous mutation or spontaneous tumor susceptibility but are extremely sensitive to the cytotoxic and carcinogenic effects of alkylating agents (Glassner et al., 1999; Sakumi et al., 1997; Sandercock et al., 2004). Sekiguchi and coworkers demonstrated that methylnitrosourea-induced, O^6MeG dependent, lethality in mice is relieved by the addition of a DNA mismatch repair deficiency, but at the expense of increased thymic lymphoma development (Sakumi et al., 1997). However, this model of alkylation tolerance/tumor susceptibility has not has not been demonstrated at other anatomic sites. Here, we used mice with a targeted deletion of the *Mgmt* active site region to monitor the effect of unrepaired O^6MeG lesions on intestinal cancer susceptibility and to examine the interaction with DNA mismatch repair (Glassner et al., 1999). MGMT may be important in protecting against sporadic human colorectal cancer as its expression is lost through epigenetic silencing of the *MGMT* gene in nearly 50% of these cancers (Lind et al., 2004). Strengthening the argument for a causal association, tumors with silenced *MGMT* tend to have higher incidences of G:C to A:T transitions in *KRAS2* and *TP53* (Esteller et al., 2001; Nagasaka et al., 2008). Inhibition of MGMT with O^6-benzylguanine (O^6BG) in rats sensitizes to AOM induced colon tumors, and transgenic expression of *Mgmt* in mice protects against AOM induced ACF (Wali et al., 1999; Zaidi et al., 1995). In this study, we examined the interaction of *Mgmt* with the mismatch repair gene *Msh6* in different models of intestinal tumorigenesis. Both genes are ubiquitously expressed, and Msh6 deficient mice develop spontaneous intestinal tumors at approximately one year of age (Acharya et al., 1996; Edelmann et al., 1997; Harris et al., 1991; Myrnes et al., 1984). We found that $Mgmt^{-/-}$ mice are sensitive to AOM induced ACF and that this sensitivity is synergistically enhanced in $Msh6^{-/-}$ mice. Furthermore, the effect of Msh6 on this carcinogen-induced endpoint could be explained entirely by its supression of apoptosis. However, while Msh6-mediated apoptosis was sufficient to account for the suppression of O^6MeG-induced ACF formation, it did not result in suppression of adenoma formation in a chronic inflammation dependent model of tumorigenesis.

MATERIALS AND METHODS

Experimental Animals

Targeted deletion within *Mgmt* was previously reported (Glassner et al., 1999). *Msh6* knockout mice ($Msh6^{tmRak}$, hereafter called $Msh6^{-/-}$) mice were obtained from Winfried Edelmann via Frank Jirik (Edelmann et al., 1997). These mutations have been backcrossed to the C57BL6/J strain for over 12 generations. C57BL6/J-Apc^{Min} animals were purchased from the Jackson Laboratory.

Chemical Carcinogenesis

Six to 8 week-old mice were injected with AOM at doses from 1 to 12.5 mg/kg. For ACF studies, animals were euthanized 15 or 25 weeks after treatment by CO_2 asphyxiation. Alternatively, 5 days after AOM treatment, mice were administered 5 cycles of 2.5% DSS in the drinking water; each cycle consisted of 5 days of DSS followed by 16 days of tap water.

The protocol included the following exceptions: the last cycle was for 4 days and contained 2.0% DSS; also, the 3rd cycle was stopped erroneously after two days, and five days later the full 5 day treatment was administered. All animals receiving DSS were treated contemporaneously and were euthanized 4 or 5 days after the last cycle of DSS. Colons were removed from cecum to anus, flushed with cold PBS, splayed open and fixed overnight in formalin. ACF and tumors were scored in whole mounts under a stereomicroscope after briefly staining in 1% methylene blue to provide contrast. Subsequently, colons were embedded as Swiss rolls for general pathology.

Spontaneous tumorigenesis in $Apc^{Min/+}$ mice

Apc$^{Min/+}$ mice were crossed with *Mgmt*$^{-/-}$ mice, and F_2 or F_3 generation littermates were used in tumor studies. The study was performed in two independent sets of crosses at different times with different *Apc*$^{Min/+}$ animals received from the Jackson Laboratory; the results from these independent experiments were analyzed separately. Animals were euthanized at 130 days of age. Small intestines and colons were splayed open and fixed overnight in formalin. Tumors greater than 0.5 mm in diameter were enumerated under a stereoscope and measured with an ocular reticle. In addition, the position of each tumor along the length of the intestine was recorded. The non-parametric Mann-Whitney test was used to determine statistical differences in ACF or tumor multiplicities.

AOM-induced apoptosis

Mice were treated intraperitoneally with AOM at doses of 1, 5, 10, or 50 mg/kg and were sacrificed 24 or 48 hours later. Colons were removed and flushed with ice cold PBS, and the distended colons were fixed overnight in formalin. Cylinders were cut from the mid to distal colon and were used to score for apoptosis. Apoptoses were scored in hematoxylin and eosin stained sections by morphological criteria (pyknotic nuclei, nuclear fragmentation, cytoplasmic blebbing). Regions from 3 or 4 cylindrical cross sections per animal were scored where high quality crypt architecture was observed, and data are represented as apoptoses/crypt. Three to 5 animals were used per experimental group and data from male and female mice are combined as there were no gender differences. We scored a minimum of 250 crypts or 100 apoptotic events per animal.

RESULTS

Mgmt and Msh6 act synergistically to suppress ACF formation in response to AOM

To determine whether Mgmt protects against alkylation induced cancer or precancerous ACF we treated mice with a single dose of the carcinogen AOM. The dose of 10 mg/kg was chosen because it yields a modest number of ACF in wild type C57Bl/6J mice (Sohn et al., 2001). On this resistant genetic background numerous ACF will form after 5 consecutive weekly treatments with AOM, but only a small fraction of these will progress to adenomas. We attempted successive weekly doses to induce tumors, but all the *Mgmt*$^{-/-}$ died during the course of treatment (data not shown). AOM is an S_N1 alkylating agent whose mutagenicity is dependent on activation by mixed function oxidases of the cytochrome p450 superfamily (primarily Cyp2e1) causing breakdown to the ultimate carcinogen, the methyl diazonium ion (Sohn et al., 2001). Following treatment with 10 mg/kg AOM and euthanization 15 weeks after treatment, *Mgmt*$^{-/-}$ mice developed 9-fold more ACF than wild type mice ($p < 0.0001$), and *Msh6*$^{-/-}$ mice developed 4-fold more ACF than wild type mice ($p = 0.003$) (Fig 1B). The effect of combining both mutant alleles on ACF formation was nearly perfectly multiplicative with *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ animals developing 38-fold more ACF than wild type mice (Fig 1B). Also, the number of ACF did not change from 15 to 25 weeks, as wild type and *Mgmt*$^{-/-}$ animals that were sacrificed at 25 weeks after treatment had nearly identical average ACF numbers compared to the respective groups at the 15 week

time point (Fig 1B). Importantly, *Mgmt* heterozygosity also caused a significant 3.3-fold increase in ACF multiplicity relative to wild type ($p = 0.03$) (Fig 1C). *Msh6* heterozygosity on the other hand did not significantly affect ACF development, as $Msh6^{+/-}$ mice had similar ACF multiplicities as $Msh6^{+/+}$ mice on the $Mgmt^{+/+}$, $^{+/-}$, and $^{-/-}$ backgrounds (Fig 1C).

When mice were treated with a single dose of AOM (10 mg/kg) there was no significant effect of Mgmt or Msh6 deficiency on tumor development at either the 15 or 25 week time points. One of 16 $Mgmt^{-/-}$ mice and 1 of 20 $Mgmt^{-/-}Msh6^{+/-}$ mice euthanized 15 weeks after treatment each had a single adenoma, and no tumors were seen in either the $Mgmt^{+/+}$ or $Mgmt^{-/-}$ mice euthanized 25 weeks after AOM treatment. This result is consistent with the resistance of C57BL6/J mice to tumor development in response to AOM, and demonstrates that Mgmt deficiency does not significantly affect progression to adenomas in this experimental timeframe.

Mgmt deficiency enhances AOM-induced apoptosis

To examine the short term cytotoxic effect of the carcinogen AOM, apoptosis was scored in the colon 24 and 48 hours after a single injection; Figure 2 shows one representative photomicrograph of colonic apoptosis after AOM treatment in a $Mgmt^{-/-}$ animal (see arrows). Over 90% of the total apoptoses in any group occurred within 5 cells from the base of the crypt (data not shown). $Mgmt^{-/-}$ mice were dramatically more sensitive to AOM induced apoptosis than wild type mice. The sensitivity of the $Mgmt^{-/-}$ strain is apparent at 24h post AOM for the 10 mg/kg dose (Fig 2B) and by 48h after the same dose $Mgmt^{-/-}$ mice had an apoptotic index 20-fold greater than that of wild type mice, with $Mgmt^{-/-}$ mice showing 1.2 ± 0.2 apoptoses per crypt, and wild type mice showing 0.05 ± 0.02 apoptoses per crypt (Fig 2C). In fact, even $Mgmt^{-/-}$ animals treated with 1 mg/kg AOM still had over a 10-fold higher apoptotic index than that of wild type mice treated with 10 mg/kg AOM. The sensitivity of $Mgmt^{-/-}$ mice to AOM induced apoptosis was entirely rescued by Msh6 deficiency (Figures 2B and 2C). 48h after exposure to 10 mg/kg AOM, the double mutant $Mgmt^{-/-}Msh6^{-/-}$ mice had an apoptotic index indistinguishable from either wild type or $Msh6^{-/-}$ mice, and 20-fold lower than $Mgmt^{-/-}$ mice (Fig 2C).

Differences in apoptotic index among genotypes were consistent between the 24 and 48 hr time points (compare Figs 2B and C). The apoptotic response of $Mgmt^{-/-}$ mice was lower and was more variable at the 24h versus the 48h time point, for all genotypes. This variability may be explained by the fact that O^6MeG dependent apoptosis occurs in the second cell cycle after adduct formation, and cells at the base of the crypt in the colon divide approximately once a day (Kaina et al., 1997; Kellett et al., 1992); thus at the 48 hour time point, the majority of dividing cells would have completed two cell cycles. 48h after a dose of 50 mg/kg, $Mgmt^{-/-}$ mice were again scored as being more sensitive to apoptosis than wild type mice, and again the sensitivity was relieved by Msh6 deficiency. At this dose, however, the magnitude of the difference between $Mgmt^{-/-}$ and other genotypes was not as great as that at 10 mg/kg, possibly because of the induction of apoptosis by lesions other than O^6MeG. This is consistent with the observed rise in apoptotic index only in $Mgmt^{-/-}$ mice from 24 to 48 hours after treatment in contrast to a leveling or decline in the other genotypes (Fig 2B and C).

Mgmt-deficiency sensitizes to tumorigenesis when tumors are promoted with DSS

To determine whether Mgmt deficiency can sensitize animals to colon tumor development, mice were treated with a classic two-stage colon carcinogenesis protocol: namely a single injection of AOM followed by repeated exposure to DSS in the drinking water. Oral delivery of DSS to mice injures the colonic epithelial barrier resulting in inflammation, and

tumor promotion associated with this inflammation occurs almost exclusively in the mid to distal colon. Following treatment with 1 mg/kg AOM + DSS, *Mgmt*$^{-/-}$ mice developed 9-fold more tumors than *Mgmt*$^{+/+}$ mice ($p = 0.003$) (Fig 3A). Histopathologic analysis of tumors showed polypoid or sessile adenoma with moderate to severe dysplasia and frequent mitoses. No differences in histopathology were observed among different genotypes (data not shown). The combination of 5 mg/kg AOM followed by DSS treatment was highly toxic to *Mgmt*$^{-/-}$ mice, and 12 days after termination of the first cycle of DSS, only 2 of 15 *Mgmt*$^{-/-}$ mice survived (Fig 3B) (these mice survived to the end of the study). *Mgmt*$^{-/-}$ mice had severe weight loss and rectal bleeding during the first cycle of DSS demonstrating that the epithelial damage caused by AOM when combined with the epithelial damage caused by DSS was lethal in this genotype. In contrast, wild type, *Msh6*$^{-/-}$, and *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ mice showed full survival during this period. Paralleling the apoptosis results, the addition of Msh6 deficiency to the *Mgmt*$^{-/-}$ background completely eliminated whole animal lethality induced by 5 mg/kg AOM followed by DSS administration. The two surviving *Mgmt*$^{-/-}$ animals had more tumors than *Mgmt*$^{+/+}$ mice at the end of the study (Fig 3C). The double mutant *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ mice had 27-fold more tumors than wild type mice ($p = 0.02$). Thus, the induction of tumors did not parallel the induction of ACF (Fig 1C), as the tumor response of *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ mice did not significantly differ from that of *Msh6*$^{-/-}$ mice (Fig 3C).

Mgmt does not affect tumor formation in *Apc*^{Min} mice

To determine whether Mgmt influences spontaneous intestinal tumor development we crossed *Mgmt*$^{-/-}$ mice with *Apc*$^{Min/+}$ mice. We found that Mgmt deficiency did not significantly affect intestinal tumor development on an *Apc*$^{Min/+}$ background. (Fig 4A). Mean tumor numbers in *Mgmt*$^{-/-}$ *Apc*$^{Min/+}$and *Mgmt*$^{+/-}$ *Apc*$^{Min/+}$ mice were 1.8 and 1.9-fold higher, respectively, compared to *Mgmt*$^{+/+}$ *Apc*$^{Min/+}$ mice, but neither difference was statistically significant ($p = 0.08$ and 0.13, respectively). Furthermore, the results in Fig 4 represent a combination of data from two independent experiments, and the small and statistically insignificant fold-differences in tumor multiplicity could be attributed to a modest, between-experiment difference in tumor response (data not shown). Moreover, there was no effect of Mgmt on tumor size or tumor distribution throughout the length of the intestine (Fig 4B and C).

DISCUSSION

Our studies show that Mgmt deficiency sensitizes animals to the development of colonic ACF after treatment with an exogenous alkylating agent, and also sensitizes to the development of colorectal adenomas when this alkylation is followed by inflammation associated tumor promotion. In fact, the magnitude of the difference between *Mgmt*$^{-/-}$ and wild type mice for ACF following AOM treatment was exactly the same as the difference in tumor multiplicity following AOM+DSS. Almost all tumors that develop after AOM+DSS treatment have GC to AT transition mutations in the β-catenin oncogene, *Ctnnb1*. This type of mutation is caused by AOM and prevented by Mgmt, and thus it is possible that Mgmt deficiency is increasing the AOM-induced mutation frequency in *Ctnnb1*. In addition, Mgmt and Msh6 had a synergistic effect on suppressing AOM induced ACF, a synergy that can be explained entirely by the Msh6 deficiency relieving the sensitivity to AOM induced apoptosis on an *Mgmt*$^{-/-}$ background. Mismatch repair has been shown to trigger apoptosis in response to several types of DNA damage (Jiricny, 2006; Stojic et al., 2004). Loss of this function allows cells to survive in the presence of damage, increasing the chances of accumulating oncogenic mutations. The results of our study demonstrate that for the endpoint of alkylation induced ACF formation, this mechanism is sufficient to explain the observed increase of ACF in *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ mice relative to *Mgmt*$^{-/-}$ mice. Our results

also demonstrate the enormous sensitivity of Mgmt deficient animals to alkylation induced carcinogenesis. Mgmt deficient mice had a 20-fold higher apoptotic index at the dose of AOM used in the carcinogenesis experiments. Potentially, 20-fold more would-be-initiated cells are removed by apoptosis from the colonic crypts, and still *Mgmt*$^{-/-}$ mice developed 9-fold more ACF.

The mechanism for Msh6 suppressing adenoma formation following AOM+DSS treatment is somewhat different than for AOM induced ACF: *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ mice had a greater tumor response than *Mgmt*$^{+/+}$ mice and the two surviving *Mgmt*$^{-/-}$ animals, but the response was similar to mice deficient for Msh6 alone. Our data do not rule out the possibility that initiated cells that go on to form ACF are qualitatively different from those that form adenomas. Another possible explanation for our results is that replication errors that occur during IBD-associated cell proliferation are driving mutagenesis and tumor development in Msh6-deficient mice to a point that mutations resulting from O^6MeG do not further enhance tumor development. Our results are consistent with previous reports demonstrating the extreme sensitivity of mismatch repair deficient mice (*Msh2*$^{-/-}$ and *Mlh1*$^{-/-}$) to IBD-associated tumor development causes by DSS administration (Kohonen-Corish et al., 2002; Taniguchi et al., 2006).

Our data on alkylation induced ACF and apoptosis are consistent with the paradigm established by Sekiguchi and coworkers for thymic lymphoma formation in response to MNU (Kawate et al., 1998). That is, mismatch repair deficiency relieves the short-term cytotoxic effects of O^6MeG at the expense of grossly increased sensitivity to a carcinogenic endpoint. Our study contrasts somewhat with previous reports showing that inhibition of Mgmt through the delivery of the inhibitor O^6-benzylguanine had no effect apoptosis induced by the S_N1 alkylating agents MNU and temozolamide in the mouse small intestine (Toft et al., 2000; Toft et al., 1999). This discrepancy could result from the different alkylating agents used, the small intestine versus the colon, the use of a pharmacologic versus a genetic method for eliminating Mgmt activity, or a combination of these factors.

Mgmt status did not affect progression to adenomas following AOM treatment alone, nor did it affect intestinal tumor development on the *Apc*$^{Min/+}$ background. Other DNA repair deficiencies including Msh2, Msh6, Mlh1, Pms2, Myh, and Mbd4 all result in more tumors on an *Apc* mutant background (either *Min* or *1638N*) (Baker et al., 1998; Edelmann et al., 1999; Kuraguchi et al., 2001; Millar et al., 2002; Reitmair et al., 1996; Sieber et al., 2004). They also cause a shift towards mutational loss of the wild type allele of *Apc* away from the predominant mechanism for loss via mitotic recombination that normally occurs in C57BL6/J mice (Haigis et al., 2002). Most of these mutants also show an increase in spontaneous mutation frequencies in the intestine in transgenic reporter mice such as Big Blue. In contrast, Mgmt deficiency causes no such increase in spontaneous mutation frequency (Sandercock et al., 2004). We conclude that any endogenous formation of O^6MeG that would be repaired by Mgmt is not sufficient to enhance tumor development in the experimental timeframes that we examined.

Consistent with other studies, our results show that Mgmt deficiency strongly sensitizes to cancer development that is induced upon exposure to an alkylating carcinogen (Wali et al., 1999; Zaidi et al., 1995). Human exposure to S_N1 type alkylating carcinogens can come in the diet, cigarette smoke, fuel combustion products, or the enzymatic nitrosation of amides and amines mediated by enteric bacteria (Hecht, 1999; Sedgwick, 1997). Enterocytes that lose expression of MGMT would be significantly more sensitive to these exposures. Interestingly, promoter hypermethylation dependent loss of Mgmt expression is more frequent in colorectal carcinomas than in adenomatous polyps or in normal mucosa (Nagasaka et al., 2008). This silencing is associated with higher fractions of tumors with

G:C -> A:T transitions in *KRAS2* and *TP53*, mutations thought to drive malignant progression in the colon (Vogelstein & Kinzler, 1993). Therefore, it is possible that loss of *MGMT* expression during early tumor development sensitizes to malignant progression.

We showed that loss of mismatch repair and its ability to signal apoptosis dramatically sensitizes animals to the development of O^6MeG-induced colon cancer precursor lesions, namely ACF. Interestingly, the mismatch repair gene *MLH1* is also epigenetically silenced in almost half of sporadic colorectal cancers (Esteller et al., 2001), but silencing of *MLH1* and *MGMT* do not appear to occur in the same tumors (Fox et al., 2006). Also, the ability of the O^6MeG lesion to signal apoptosis involves p53-independent pathways (Hickman & Samson, 1999). Our results clearly demonstrate that loss of the ability to signal apoptosis in response to O^6MeG lesions dramatically sensitizes enterocytes to tumor formation. Thus, inactivation of O^6MeG-initiated apoptosis may be an important step in human colorectal cancer development, particularly in those cancers that have lost expression of MGMT.

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Abbreviations

ACF	Aberrant crypt foci
AOM	Azoxymethane
DSS	Dextran sulfate sodium
Mgmt	O^6-methylguanine-DNA methyltransferase
O^6MeG	O^6-methylguanine

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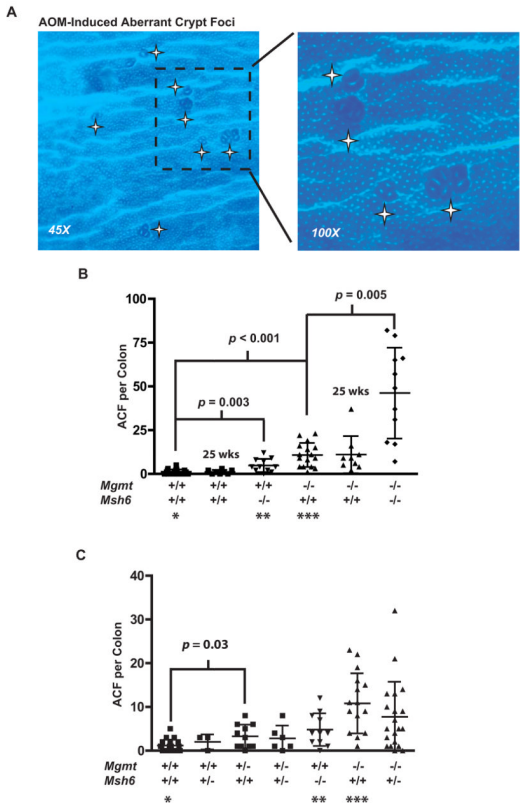

Figure 1. AOM-induced aberrant crypt foci

Mice were treated with 10 mg/kg AOM and sacrificed 15 or 25 weeks after treatment. (A) Fixed colons were stained briefly with methylene blue and destained in PBS. Whole mount of a colon from an AOM-treated *Mgmt*$^{-/-}$*Msh6*$^{-/-}$ mouse. Several ACF are apparent in this field (marked by the white stars, 45X original magnification). (B) Numbers of ACF for all homozygous mice are shown. Mice were sacrificed at 15 weeks after treatment, unless otherwise noted as being sacrificed at 25 weeks. (C) Numbers of ACF for heterozygous mice sacrificed at 15 weeks compared to selected homozygous mice; *, **, and *** indicate the same data in (B) and (C).

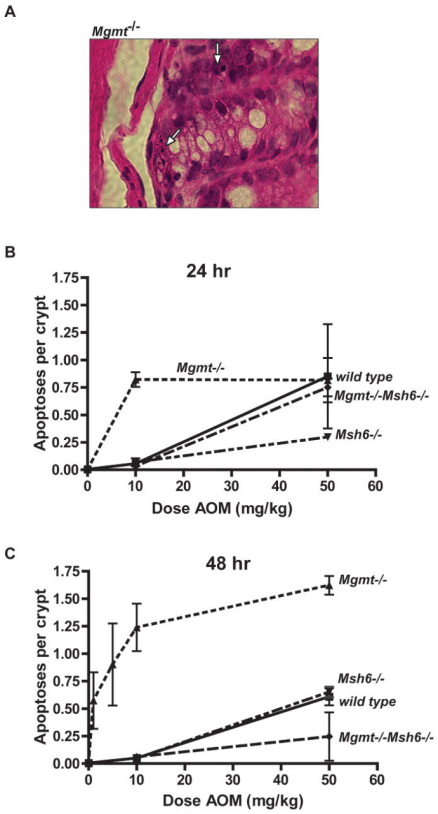

Figure 2. AOM-induced apoptosis in the colon

Mice were treated with AOM and sacrificed 24 or 48 hours after treatment. H&E stained sections from the distal colon were used to score apoptosis by morphological assessment (pyknotic nuclei, nuclear fragmentation, cytoplasmic blebbing) (A). Representative field from an *Mgmt*$^{-/-}$ mouse (10 mg/kg AOM, 48 hr). Apoptoses are marked with arrows (600X original magnification). B. Apoptotic indices scored 24 (B) or 48 (C) hours after AOM treatment and represented as number of apoptoses per crypt.

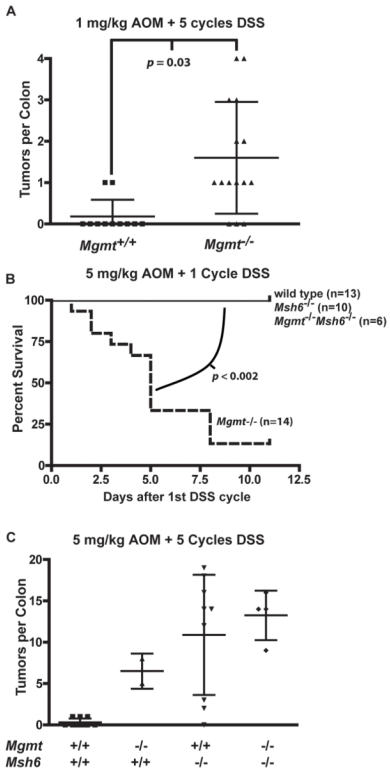

Figure 3. AOM + DSS

Mice were treated with 1 or 5 mg/kg AOM and subsequently administered 5 cycles of 2.5% DSS in the drinking water. (A) Tumors per animal when treated with 1 mg/kg AOM. (B) Survival following the first cycle of DSS in the drinking water. (C) Tumors per animal at the end of the experiment from the animals treated in (B).

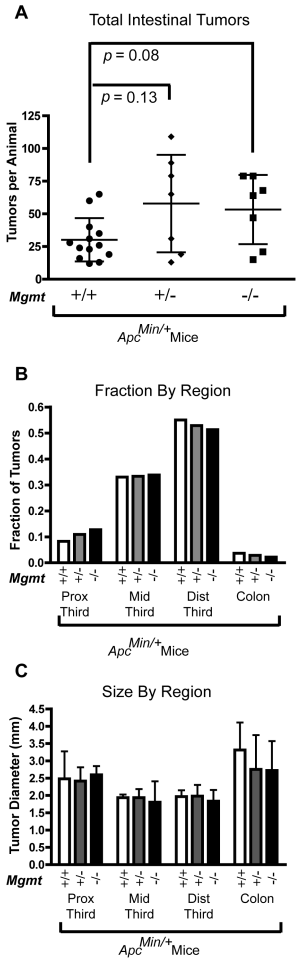

Figure 4. Tumor development in *Apc*^{Min} mice

Apc$^{Min/+}$ mice that were either *Mgmt*$^{+/+}$ or *Mgmt*$^{-/-}$ were sacrificed at 130 days of age. The number, size and positions of tumors in the small intestine or colon were recorded. (A) Scatter plot showing tumor multiplicities for total small intestinal tumors from two experiments. Each point represents the tumor count for one animal. *Mgmt*$^{-/-}$ lines were highly unproductive in breeding pairs with *Apc*$^{Min/+}$mice, and thus the experiments had to be conducted twice with separate *Apc*$^{Min/+}$ mice obtained from the Jackson Laboratory. (B) Bar graphs showing the fractional distribution of tumors in the small intestine and colon. The small intestine was divided into proximal, middle, and distal thirds. (C) Tumor sizes by intestinal region; diameter in mm.