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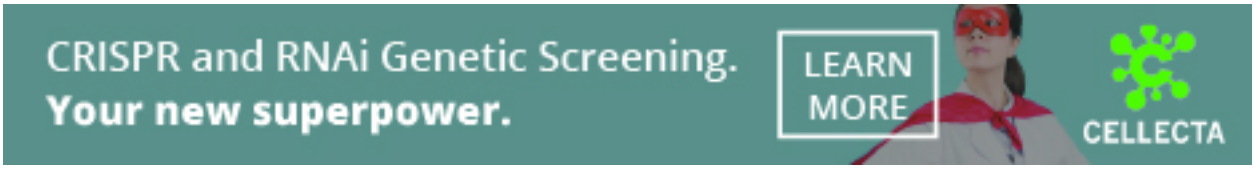

Article

The modifier of *Min* 2 (*Mom2*) locus: Embryonic lethality of a mutation in the *Atp5a1* gene suggests a novel mechanism of polyp suppression

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Inactivation of the *APC* gene is considered the initiating event in human colorectal cancer. Modifier genes that influence the penetrance of mutations in tumor-suppressor genes hold great potential for preventing the development of cancer. The mechanism by which modifier genes alter adenoma incidence can be readily studied in mice that inherit mutations in the *Apc* gene. We identified a new modifier locus of Apc^{Min}-induced intestinal tumorigenesis called Modifier of *Min* 2 (*Mom2*). The polyp-resistant $Mom2^R$ phenotype resulted from a spontaneous mutation and linkage analysis localized *Mom2* to distal chromosome 18. To obtain recombinant chromosomes for use in refining the *Mom2* interval, we generated congenic DBA.B6 $Apc^{Min/+}$, $Mom2^{R/+}$ mice. An intercross revealed that $Mom2^R$ encodes a recessive embryonic lethal mutation. We devised an exclusion strategy for mapping the *Mom2* locus using embryonic lethality as a method of selection. Expression and sequence analyses of candidate genes identified a duplication of four nucleotides within exon 3 of the α subunit of the ATP synthase (*Atp5a1*) gene. Tumor analyses revealed a novel mechanism of polyp suppression by $Mom2^R$ in *Min* mice. Furthermore, we show that more adenomas progress to carcinomas in *Min* mice that carry the $Mom2^R$ mutation. The absence of loss of heterozygosity (LOH) at the *Apc* locus, combined with the tendency of adenomas to progress to carcinomas, indicates that the sequence of events leading to tumors in $Apc^{Min/+}$ $Mom2^{R/+}$ mice is consistent with the features of human tumor initiation and progression.

[Supplemental material is available online at www.genome.org.]

Colorectal cancer is one of the leading causes of cancer deaths in the United States (www.cancer.org). The dysregulation of normal colonic epithelium leads to the formation of adenomas, which are considered a prerequisite to progression to carcinoma (Fodde et al. 2003). This multistep process involves interactions between the genome and the gut environment, leading to mutations and epigenetic changes in oncogenes and/or tumor suppressor genes (Kinzler and Vogelstein 1996; Ilyas et al. 1999; Gregorieff and Clevers 2005). However, the variation in penetrance of hereditary forms of cancer has emphasized the impact of tumor-modifier genes that can influence individual susceptibility to cancer by either enhancing or suppressing the initiation, growth, and/or progression of tumor cells. Due to environmental and genetic heterogeneity in the human population, it has been difficult to identify tumor modifier loci in humans. Therefore, complex trait analysis in experimental mouse crosses is a powerful approach to genetically dissect multigenic diseases (Moore and Nagle 2000; Threadgill et al. 2002; Siracusa et al. 2004).

The mapping, identification, and characterization of genes influencing tumor susceptibility has been facilitated by the use of mammalian models (Mao and Balmain 2003). Inbred strains of mice that differ in their susceptibility to various types of solid tumors and leukemias have been instrumental in uncovering loci that affect tumor risk (Dragani 2003; Mao and Balmain 2003). Tumor susceptibility genes may act cell autonomously within the tumor lineage, or may act in a non-cell autonomous fashion within the microenvironment leading to tumor formation (Demant 2003). Genomic differences among inbred strains of mice are being exploited to limit the regions containing the causative allelic variants and ultimately identify the genes responsible (Siracusa et al. 2004).

Modifier genes affect the phenotype caused by a mutation in another gene. A classic example of such a system with respect to tumorigenesis involves loci called the Modifiers of *Min* (*Mom*), which influence the intestinal tumor phenotype of Min mice carrying a mutation in the adenomatous polyposis coli gene (Apc^{Min}) (Dietrich et al. 1993). To date, four *Mom* loci have been identified (*Mom1*, *Mom2*, *Mom3*, and *Mom6*) (Dietrich et al. 1993; Cormier et al. 2000; Silverman et al. 2002; Haines et al. 2005), but only one gene has, as yet, been proven to be responsible for

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altering the *Mom* phenotype otherwise dictated by a mutation in the *Apc* gene (MacPhee et al. 1995; Cormier et al. 1997).

Our laboratory identified the *Mom2* locus in hybrid offspring derived from a cross involving DBA/2J (DBA) and C57BL/6J (B6) $Apc^{Min/+}$ mice (Silverman et al. 2002). We initially localized *Mom2* to a 14-cM region on distal chromosome 18, between the *D18Mit186* and *D18Mit213* markers. Inheritance patterns of the *Mom2* phenotype suggested that the resistant *Mom2* allele ($Mom2^R$) arose from a spontaneous mutation in a B6 $Apc^{Min/+}$ progenitor. On both inbred strain backgrounds, one $Mom2^R$ allele decreases small intestinal polyp number and colon polyp incidence by ~90% (Silverman et al. 2002); thus, $Mom2^R$ has significantly greater effects on polyp multiplicity than $Mom1^R$, which, when present in one copy, decreases small intestinal polyp number and size by ~50% (Cormier et al. 1997; Silverman et al. 2002).

The presence of the *Mom1* locus, in addition to other unlinked modifiers of intestinal polyp formation in the mouse genome, indicated the necessity to control genetic background for further studies of the *Mom2* locus. Therefore, we generated congenic mice carrying the $Mom2^R$ allele on both the DBA and B6 inbred strain backgrounds (Silverman et al. 2003). The *Mom2* locus was limited to <10 cM by genotyping progeny and comparing small intestinal polyp multiplicity with the distribution of recombinant breakpoints (Silverman et al. 2003).

We now report that intercrosses of DBA.B6 $Mom2^{R/+}$ animals produced no offspring homozygous for the resistant $Mom2^R$ allele. To facilitate the genetic dissection of *Mom2*, we established crosses between DBA.B6 $Mom2^{R/+}$ animals and *Mus musculus castaneus* (CAST) mice. The increase in polymorphic markers, coupled with making use of the $Mom2^R$ homozygous lethal phenotype, provided an exclusion mapping strategy that refined the *Mom2* interval to <1 Mb. Our finding of a duplication that disrupts the *Atp5a1* gene, a gene required for oxidative metabolism, provided testable hypotheses for both the modifier effect on Apc^{Min}-induced tumorigenesis and the phenotype of recessive embryonic lethality.

Results

Intercrosses of congenic DBA.B6 $Mom2^{R/+}$ mice fail to generate homozygous $Mom2^R$ progeny

To maintain the $Mom2^R$ mutation, as well as to obtain recombinant chromosomes for use in the refinement of the *Mom2* locus, we generated congenic animals carrying the C57BL/6J (B6) $Mom2^R$ allele on the DBA/2J (DBA) genetic background by sequential backcrosses (Silverman et al. 2003). Offspring were genotyped for the Apc^{Min} mutation and genotyped for the *Mom2* locus using the *Mom2* flanking markers *D18Mit80* and *D18Mit213* (Silverman et al. 2003). Progeny were selected that carried the resistant *Mom2* allele ($Mom2^R$), but did not carry the Apc^{Min} mutation. DBA.B6 $Mom2^{R/+}$ animals from the N4–N5 backcross generations were intercrossed to generate animals homozygous for the resistant $Mom2^R$ allele. Surprisingly, our intercross resulted in 36 F_2 $Mom2^{R/+}$ heterozygotes and 25 F_2 wild-type $Mom2^{+/+}$ progeny, with no F_2 animals homozygous for the $Mom2^R$ allele. χ^2 analysis indicated that the expected Mendelian distribution of 1:2:1 was significantly different ($P < 0.001$) from the observed distribution of F_2 animals obtained from our $Mom2^R$ intercross (1.4:1). In addition, the observed 1.4:1 ($Mom2^{R/+}$:$Mom2^{+/+}$) ratio was not significantly different ($P = 0.2$) from a ratio of 2:1 ($Mom2^{R/+}$:$Mom2^{+/+}$), suggesting that the $Mom2^R$ allele is a recessive embryonic lethal mutation. Additional experiments have confirmed that homozygous $Mom2^R$ mice are never born (see below).

The $Mom2^R$ locus encodes a recessive embryonic lethal mutation

We tested the hypothesis that the mutant $Mom2^R$ allele may impair the normal function of either oocytes or sperm by determining whether $Mom2^R$ could be equally inherited from a heterozygous $Mom2^{R/+}$ mother or a heterozygous $Mom2^{R/+}$ father. Table 1 shows the crosses, number of mating cages, and genotypes of the resulting progeny. In two of the four backcrosses, offspring could inherit a $Mom2^R$ allele from their heterozygous mother, whereas in the remaining two backcrosses, offspring could inherit a $Mom2^R$ allele from their heterozygous father. Analysis of the distribution of $Mom2^{R/+}$ versus $Mom2^{+/+}$ offspring by the χ^2 test revealed no significant differences in inheritance patterns between the mutant $Mom2^R$ and wild-type $Mom2^+$ loci (Table 1). Therefore, the function of oocytes and sperm do not appear to be impaired by the presence of the $Mom2^R$ mutation. The data suggest that the death of $Mom2^{R/R}$ embryos occurs sometime after fertilization, but before birth.

To investigate the developmental stage of embryonic lethality, timed pregnancies from intercrosses of DBA.B6 $Mom2^{R/+}$ mice were established. A total of 41 embryos, which includes embryonic tissue from six resorption sites, were obtained from six females at 12.5 d post coitus (dpc). Genomic DNA from each embryo was genotyped for the *Mom2* flanking markers, *D18Mit80* and *D18Mit213*, to distinguish the presence of the B6 $Mom2^R$ locus from the wild-type DBA $Mom2^+$ locus (Silverman et al. 2003). Ten embryos were homozygous for the wild-type DBA allele at both loci, 24 embryos were heterozygous for the B6 and DBA alleles at both loci, and seven embryos were single recombinants (three embryos were D/B at the *D18Mit80* locus and D/D at the *D18Mit213* locus; two embryos were D/D at the *D18Mit80* locus and D/B at the *D18Mit213* locus; and two embryos were D/B at the *D18Mit80* locus and B/B at the *D18Mit213* locus). None of the 41 embryos were homozygous for B6 alleles at both the *D18Mit80* and *D18Mit213* loci, demonstrating that $Mom2^{R/R}$ embryos die before 12.5 dpc.

Refining the localization of *Mom2* by an exclusion mapping strategy

The proof of embryonic lethality coupled with the ability to detect recombinants in the *Mom2* region provided a strategy to

Table 1. The $Mom2^R$ allele can be transmitted to offspring from both female and male parents

Cross[a]	No. of mating cages	$Mom2^{+/+}$	$Mom2^{R/+}$	χ^2	*P* value
DBA.B6 $Mom2^{R/+}$ × B6 $Apc^{Min/+}$	6	41	31	1.4	$P > 0.05$
DBA.B6 $Apc^{Min/+}$, $Mom2^{R/+}$ × DBA	6	31	27	0.3	$P > 0.05$
DBA × DBA.B6 $Mom2^{R/+}$	5	50	53	0.1	$P > 0.05$
DBA × DBA.B6 $Apc^{Min/+}$, $Mom2^{R/+}$	10	88	93	0.1	$P > 0.05$

The *D18Mit80* and *D18Mit213* markers were used to distinguish the presence of the B6 $Mom2^R$ locus from the wild-type DBA $Mom2^+$ locus (Silverman et al. 2003).
[a]For each cross, the female is listed first.

further define the boundaries of the *Mom2*^{R} locus. To facilitate the genetic dissection and further refinement of the *Mom2* interval, we initiated crosses with CAST/Ei (CAST) mice (Silver 1995). The CAST subspecies diverged from common laboratory strains by one to two million years, thus providing genetic diversity to increase the number of polymorphisms (Silver 1995). Six mating cages of CAST × DBA.B6 *Mom2*$^{R/+}$ animals from the N7–N8 backcross generations (Fig. 1A) produced 94 F_1 offspring that were genotyped for the *Mom2* region. While all F_1 offspring inherited a CAST allele at each marker, either a DBA allele (*Mom2*$^+$) or the B6 allele (*Mom2*^{R}) could be inherited from the heterozygous DBA.B6 *Mom2*$^{R/+}$ congenic parent. Since the *Mom2*^{R} mutation arose on a B6 background, F_1 progeny were selected that carried the B6 allele at both the proximal *D18Mit186* and distal *D18Mit213* markers. These [CAST × DBA.B6 *Mom2*$^{R/+}$]F_1 hybrid mice were intercrossed to generate F_2 progeny that were genotyped for *D18Mit186* and *D18Mit213*. Only F_2 animals recombinant within the *Mom2* interval were subjected to further screening (Fig. 1B).

We used an exclusion mapping technique for the *Mom2* locus based on the lack of *Mom2*$^{R/R}$ homozygous liveborn progeny from the DBA.B6 *Mom2*$^{R/+}$ intercross. Since *Mom2*^{R} is a recessive embryonic lethal mutation, no animals homozygous for the B6 *Mom2*^{R} allele should survive. Therefore, any marker within the *Mom2* region that was homozygous for the B6 allele would be excluded as being part of the *Mom2* locus (Fig. 1B,C). Progeny from the [CAST × DBA.B6 *Mom2*$^{R/+}$]F_1 intercross were identified that were B/B at either the proximal (*D18Mit186*) or distal (*D18Mit213*) markers, and were B/C or C/C at the other, indicating that a recombination occurred within the *Mom2* region (Fig. 1B). Of 536 F_2 offspring, 22 were informative recombinants. No F_2 progeny were identified that were B/B at both the *D18Mit186* and *D18Mit213* markers; 452 nonrecombinant F_2 animals showed a distribution of 159: 293: 0 (C/C: B/C: B/B), with a 1: 1.8 ratio instead of a Mendelian 1:2:1 ratio ($\chi^2 = 129.4$, $P < 0.0001$), confirming the recessive embryonic lethality of the *Mom2*^{R} mutation.

To further dissect the *Mom2* locus, the 22 recombinant F_2 animals were genotyped for additional markers (Fig. 1C). The spontaneous mutation that created the *Mom2*^{R} allele did not appear to cause a gross deletion, inversion, or large alteration that affected genes in the region. All markers, with the exception of *D18Mit47* and *D18Mit106*, were homozygous for B/B alleles in at least one mouse, thus excluding them from consideration (Fig. 1C). Analysis of recombination breakpoints indicates that the *Mom2* locus must reside between the *D18Kcc2* (within the *St8sia5* [formerly known as *Siat8e*] gene) and *D18Kcc10* (within the *Slc14a2* gene) markers (Supplemental Table 1).

To further narrow the location of the *Mom2* gene, F_2 progeny that were heterozygous for the *Mom2* region were sequentially intercrossed. F_3 offspring were identified that carried crossovers between the *St8sia5* and *Slc14a2* genes. Intercrosses of these recombinant F_3 progeny produced 64 F_4 mice that were either pure CAST or mice that were heterozygous throughout the *Mom2* region. No mice that were homozygous for B6 alleles in the *Mom2* region were born, consistent with the recessive embryonic lethality of the *Mom2*^{R} mutation. These data demonstrated that the region distal to *St8sia5* and proximal to *D18Kcc7* (within the *5430411K18Rik* gene) contained a recessive embryonic lethal mutation and excluded both the *Slc14a1* and *Slc14a2* genes as candidates (Supplemental Table 1). Based on Build 34 from the Ensembl database, the *Mom2* interval is ~800 kb in length and encodes six known genes, one novel gene, one pseudogene, and one predicted gene (www.ensembl.org/Mus musculus/October 2005).

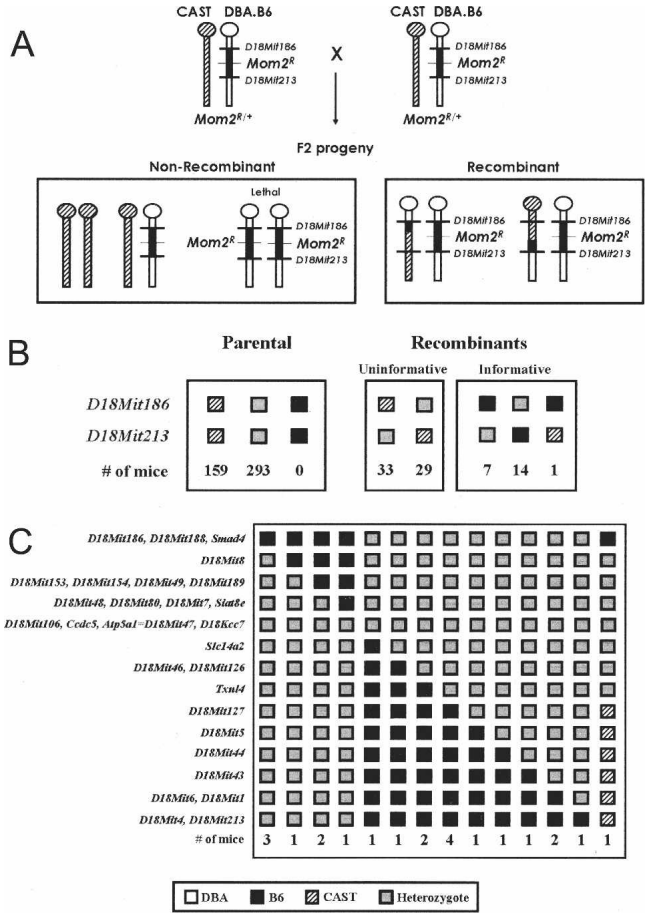

Figure 1. Exclusion mapping strategy to refine the *Mom2* locus. (*A*) Based on the observation that the *Mom2*^{R} locus encodes a recessive embryonic lethal mutation, we devised a strategic intercross of (CAST × DBA.B6 *Mom2*$^{R/+}$)F_1 mice to limit the boundaries of the *Mom2* locus. Since mice homozygous for the B6 *Mom2*^{R} allele (B/B) should not survive, markers within the *Mom2* region that were homozygous for B6 alleles would be excluded. DBA.B6 *Mom2*$^{R/+}$ animals from the N7–N8 generations were mated to CAST mice. DBA chromosomes are shown in white; the B6 *Mom2*^{R} region is shown in black; CAST chromosomes are represented by diagonal stripes and heterozygotes are shown in gray. The possible F_2 progeny are shown in black boxes; all offspring were genotyped for proximal (*D18Mit186*) and distal (*D18Mit213*) markers flanking *Mom2* (Silverman et al. 2002, 2003). (*B*) The number of resulting F_2 progeny are shown below the boxes. Nonrecombinant F_2 progeny are in the black box on the *left;* representative recombinant progeny are in the boxes to the *right*. F_2 offspring that were B/B at either the proximal (*D18Mit186*) or distal (*D18Mit213*) marker and were B/C or C/C at the other, indicated the occurrence of a recombination within the *Mom2* region. Nonrecombinant CAST DBA.B6 *Mom2*^{R} animals were uninformative for linkage analysis. (*C*) Haplotype analysis of recombinant F_2 animals from the [CAST × DBA.B6 *Mom2*$^{R/+}$]F_1 intercross. Mapped loci are listed on the *left*. Numbers below each column of boxes represent the number of F_2 progeny that fall into each haplotype. F_2 progeny were identified that were B/B at either the proximal or distal marker, and were B/C or C/C at the other, indicating an informative recombinant within the *Mom2* region; these 22 mice were genotyped by PCR for polymorphic markers within the *Mom2* region. This analysis positioned *Mom2* between the *D18Mit7* locus and the *Slc14a2* gene.

Expression analysis and sequencing of genes within the *Mom2* region reveals a mutation in the *Atp5a1* gene

Based on an early-acting recessive embryonic lethal mutation at the *Mom2* locus, we hypothesized that the mutated gene would most likely represent a null allele. Therefore, we examined the expression levels of genes within the *Mom2* interval by real-time PCR. Assays-on-Demand (ABI) provided tests for *Atp5a1, Ccdc5, Loxhd1, Pstpip2, 8030462N17Rik,* and *St8sia5*. Total RNA was isolated from the proximal and distal small intestine and colon of congenic DBA.B6 $Mom2^{R/+}$ mice along with control DBA and B6 mice. Of all the genes tested, only the *Atp5a1* gene showed a significant decrease in mRNA levels compared with that predicted for a DBA × B6 F_1 hybrid (Fig. 2A).

Results of real-time PCR were consistent with the *Atp5a1* gene being expressed solely from the DBA allele in the congenic DBA.B6 $Mom2^{R/+}$ mice. Therefore, we sequenced each exon of the *Atp5a1* gene from genomic DNA of congenic DBA.B6 $Mom2^{R/+}$ and B6 $Mom2^{R/+}$ mice, along with control DBA and B6 mice (Supplemental Table 2). The results revealed a 4-bp duplication in the coding region (exon 3), which was present in mice carrying the $Mom2^{R}$ allele and absent in $Mom2^{+/+}$ controls (Fig. 2B). This duplication is predicted to cause a frameshift mutation, which would result in aberrant incorporation of 42 amino acids followed by a stop codon in exon 4, downstream of the mutation site (Fig. 2C). If the transcript from the $Mom2^{R}$ allele was equally stable as from the $Mom2^{+}$ allele, the predicted mutant mRNA would be the same size as the wild-type mRNA, and was therefore not detectable by Northern blot analyses (data not shown). However, sequencing cDNA generated from $Mom2^{R/+}$ intestines failed to identify any mutant *Atp5a1* transcripts, suggesting that this allele does not generate a stable transcript (data not shown). In addition, sequencing of the flanking *Ccdc5* and *Pstpip2* genes showed no differences between $Mom2^{R/+}$ mice and wild-type mice (Supplemental Tables 3, 4).

To further cement the relationship between the 4-bp duplication in the *Atp5a1* gene and the $Mom2^{R}$ phenotype, we sequenced the original B6 $Apc^{Min/+}$ father who first transmitted the $Mom2^{R}$ allele to his progeny (Silverman et al. 2002); results showed that he indeed carried the mutation. Sequencing of five $Mom2^{R/+}$ mice, three from the real-time PCR studies, and the two F_3 parents (see above), further confirmed the presence of the mutation in mice carrying the $Mom2^{R}$ allele. No polymorphisms between the DBA and B6 strains in coding regions, untranslated regions, or splice junctions were found.

We designed a positive/negative PCR-based assay to detect the presence of the mutated *Atp5a1* allele using a forward primer that incorporated the 4-bp duplication in its sequence (see Methods). The specificity of the primer ensured that it would only bind a genomic fragment that contains the mutant *Atp5a1* allele. A PCR fragment of 147 bp was detected if the *Atp5a1* duplication was present, whereas no fragment was produced from the wild-type allele. Further studies compared the number of polyps in $Apc^{Min/+}$ mice carrying the mutated $Atp5a1^{Mom2R}$ allele with those carrying the wild-type $Atp5a1^{+}$ allele. Twenty DBA.B6 $Apc^{Min/+}$ mice were randomly selected from the N7–N16 backcross generations and genotyped to determine whether they carried mutant or wild-type *Atp5a1* alleles. The average polyp number (± SD) for the 10 mice carrying the mutant $Atp5a1^{Mom2R}$ allele was 9.6 ± 4.8 polyps, whereas the average polyp number (± SD) for the 10 mice carrying the wild-type $Atp5a1^{+}$ allele was 78.3 ± 32.9 polyps. Additional offspring have shown an identical relationship (data not shown). Thus, there is a 100% concordance between the presence of the mutant *Atp5a1* allele and resistance to Apc^{Min}-induced intestinal polyposis.

Mechanism of protection from polyp development by the *Mom2* locus

The finding of a mutation in the *Atp5a1* gene suggested a potential mechanism, whereby the formation of polyps is inhibited in the presence of the mutation. Since the *Atp5a1* gene is essential for aerobic respiration (Comelli et al. 1998, 2003; Schnaufer et al. 2005), its absence would result in cell death. This action would be cell autonomous. B6 $Apc^{Min/+}$ mice develop polyps along the intestinal tract; nearly 100% of polyps that develop in the small intestine occur by somatic recombination, which results in loss of heterozygosity (LOH) for the Apc^{Min} mutation (Shoemaker et al. 1998; Haigis et al. 2002, 2004; Haigis and Dove 2003). Therefore, if the $Mom2^{R}$ mutation acted in a dominant fashion, its action would be independent of whether it was located on the same chromosome as the Apc^{Min} allele. However, if the mechanism of action were by LOH, then the mutant *Atp5a1* allele would only prevent polyps when it was on the same chromosome as the Apc^{Min} mutation (in *cis*); elimination of both the wild-type *Apc* and *Atp5a1* genes on the wild-type homolog would lead to cell death, since there would no longer be a functional *Atp5a1* gene.

One method to test this hypothesis is to determine polyp number in mice where the $Mom2^{R}$ mutation resides on the same chromosome as the Apc^{Min} mutation (in *cis*) as opposed to the $Mom2^{R}$ mutation and the Apc^{Min} mutation residing on different chromosome 18 homologs (in *trans*). Crosses were performed to generate offspring with the $Mom2^{R}$ mutation in both the *cis* and *trans* configurations with respect to the Apc^{Min} mutation (Fig. 3). Quantitation of the number of polyps was performed on progeny aged for 150 d. Control mice that carried only the Apc^{Min} mutation developed an average of 88 polyps in the small intestine, whereas those mice that carried Apc^{Min} and $Mom2^{R}$ on the same chromosome (in *cis*) developed a significantly lower average of only six polyps (Student's *t*-test, $P < 0.0001$); these data are consistent with our previous findings for the $Mom2^{R}$ phenotype (Silverman et al. 2002, 2003). However, mice that carried the Apc^{Min} and $Mom2^{R}$ mutations on different homologs (in *trans*) developed an average of 99 polyps, which is not significantly different from the Apc^{Min}-only control group (Student's *t*-test, $P > 0.5$). The results shown in Figure 3 clearly demonstrate that on a DBA background, $Mom2^{R}$ functions as a potent suppressor of polyp formation only when in *cis* with respect to the Apc^{Min} mutation.

Previous studies have shown that on a B6 background, polyps that develop have not only lost their Apc^{+} allele, but have lost the entire chromosome 18 (Shoemaker et al. 1998; Haigis and Dove 2003; Haigis et al. 2004). We determined whether LOH for Apc^{+} is occurring in adenomas from $Apc^{Min/+}$ mice compared with adenomas from $Apc^{Min/+}$ $Mom2^{R/+}$ mice (Fig. 4). The data clearly show that the majority of adenomas (12/16) from $Apc^{Min/+}$ mice have lost their Apc^{+} allele, whereas only one adenoma (1/21) from $Apc^{Min/+}$ $Mom2^{R/+}$ mice has lost its Apc^{+} allele ($P < 10^{-7}$). Histopathological analysis of the adenomas used in the LOH analysis revealed that three of 21 tumors in $Apc^{Min/+}$ $Mom2^{R/+}$ mice progressed to carcinomas, whereas none of the adenomas in $Apc^{Min/+}$ mice progressed (Figs. 4, 5).

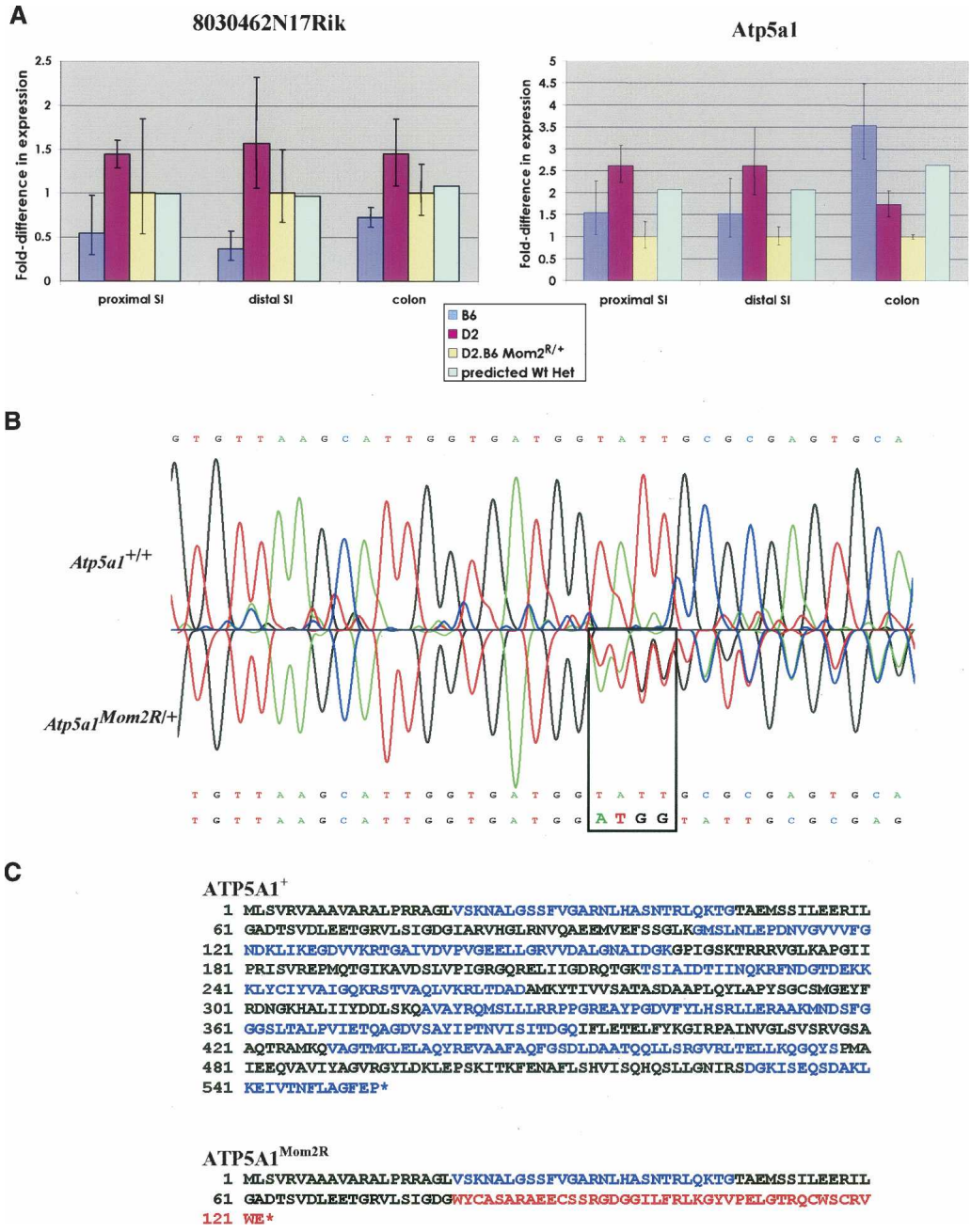

Figure 2. The coding region of the *Atp5a1* gene contains a 4-bp insertion. (*A*) Results of the real-time PCR analysis of intestinal expression of *Atp5a1* and *8030462N17Rik* expression in B6, DBA, and DBA.*Mom2*$^{R/+}$ congenic mice. A graphical representation of the relative expression levels of *Atp5a1* and *8030462N17Rik* in the proximal small intestine, distal small intestine, and colon are shown. Experimentally determined expression levels in wild-type B6, wild-type DBA, and the *Mom2*$^{R/+}$ congenic are shown. The predicted WT bar indicates the expected expression level in the *Mom2*$^{R/+}$ congenic animal, assuming 50% contribution from both parental alleles. *Atp5a1* reproducibly demonstrated an expression of 50% of the DBA/2J allele, with no contribution from the B6 allele. *8030462N17Rik* was representative of the other genes that mapped to the congenic interval, demonstrating an expression level equivalent to the predicted level (Supplemental Methods 1). (*B*) The sequence of exon 3 of the *Atp5a1* gene in a *Mom2*$^{R/+}$ mouse reveals a divergence beginning after the 100th nucleotide of exon 3. The mutation is a 4-bp duplication of ATGG (shown in box) within exon 3. (*C*) The amino acid sequence of the wild-type *Atp5a1* gene is shown in comparison to the amino acid sequence of the mutant *Atp5a1*^{Mom2R} allele. The 4-bp insertion results in a frameshift that is predicted to generate a severely truncated protein.

Discussion

The observation that the *Mom2*^{R} locus carried a recessive embryonic lethal mutation initially enabled the use of an exclusion mapping technique to identify the *Mom2* gene as well as to provide a testable hypothesis for *Mom2*^{R} function. This strategy excludes any marker homozygous for the B6 allele within the *Mom2* region as being a part of the *Mom2* locus (Fig. 1). This exclusion mapping technique should be applicable to any mutation that results in embryonic lethality (Williams 1999). Our mapping re-

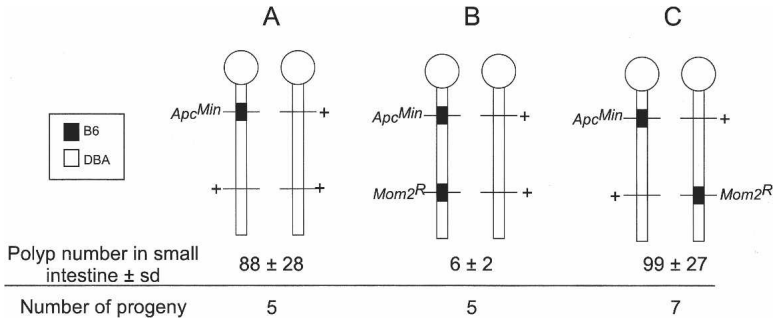

Figure 3. The *cis–trans* test reveals that the *Mom2*^{R} mutation functions only when in *cis* with the *Apc*^{Min} mutation. Two crosses were established to test whether *Mom2*^{R} could function in *trans* as well as in *cis*. Three mating cages of DBA.B6 *Mom2*$^{R/+}$ × DBA.B6 *Apc*$^{Min/+}$ mice produced progeny that carried only the *Apc*^{Min} mutation on chromosome 18 (*A*) or progeny that carried the *Apc*^{Min} mutation on one chromosome 18 homolog and the *Mom2*^{R} mutation on the other chromosome 18 homolog (*C*, in *trans*). Three mating cages of DBA/2J × DBA.B6 *Apc*$^{Min/+}$, *Mom2*$^{R/+}$ mice produced progeny that carried both the *Apc*^{Min} and *Mom2*^{R} mutations on the same chromosome 18 homolog (*B*, in *cis*). All parents of these progeny were at the N9–N12 backcross generations. The chromosomes show the positions of the *Apc* and *Mom2* alleles on chromosome 18 homologs. Below the chromosomes is shown the average number of polyps ± standard deviation. Statistical analyses showed that there is no significant difference in polyp numbers between the control group carrying only the *Apc*^{Min} allele and the group that has *Apc*^{Min} and *Mom2*^{R} in *trans*. (*A* vs. *C*, $P > 0.5$). In contrast, the only progeny that show highly significant differences with decreased polyp numbers is the *Apc*^{Min} group that has *Mom2*^{R} in *cis* (*B* vs. *A*, $P < 0.0001$; *B* vs. *C*, $P < 0.0001$).

sults from the intersubspecific intercross, which utilized the phenotype of lethality, are consistent with the mapping results from the B6 and DBA backcrosses, which utilized the phenotype of polyp multiplicity (Silverman et al. 2003). While more than one gene in the region could be affected, the simplest hypothesis is that a spontaneous mutation in the *Atp5a1* gene has a dominant phenotype of polyp suppression and a recessive phenotype of embryonic lethality. Understanding the biological function of the *Atp5a1* gene will provide important insights concerning its potential role as a modifier of human cancer.

Embryonic lethality of the *Mom2*^{R} mutation

The *Mom2*^{R} allele is an early embryonic recessive lethal mutation. In timed pregnancies, we were unable to recover homozygous *Mom2*^{R} embryos by 12.5 d of gestation. Since we propose that *Mom2*^{R} is a null allele for the *Atp5a1* gene, leading to a nonfunctional ATP synthase α subunit, it is not surprising that it is a recessive lethal mutation. Silencing of the *Atp5a1* gene in *Trypanosoma brucei* and *Trypanosoma evansi* results in lethality (Schnaufer et al. 2005). Mitochondrial synthesis dramatically increases during early to late blastocyst stage, comprising up to 7% of total protein synthesis in the embryo (Taylor and Piko 1995). The energy need of developing embryos dramatically increases during the formation of the blastocoel. Anoxic respiration is the primary form of ATP production until implantation and the formation of the placenta (Houghton et al. 1996). Aerobic respiration increases during late blastocyst stage (Ginsberg and Hillman 1975). With the increasing demands for aerobic respiration and energy needs during blastocoel formation and implantation, it is likely that the homozygous *Mom2*^{R} embryos die around this stage of development due to their inability to produce sufficient energy for their needs.

The *Atp5a1* gene and aerobic respiration

The *Atp5a1* gene encodes the α subunit of the ATP synthase complex, which is composed of 16 protein subunits (Boyer 1997). Two of these subunits are encoded by mitochondrial genes; the remainder are encoded by nuclear genes. The assembled complex, composed of the F_0 and F_1 subunits, is localized to the inner mitochondrial membrane. The F_0 complex spans the membrane and is a hydrogen ion (H^+) pump, while the F_1 complex is a molecular motor harnessing the H^+ gradient to produce ATP by oxidative phosphorylation (Boyer 1997). Atp5a1 is one of the five subunits (α, β γ, δ, and ε) comprising the F_1 complex of ATP synthase. The human ortholog (*ATP5A1*) has been mapped to chromosome 18q12-q21 (Godbout et al. 1997). *Atp5a1* is encoded by 12 exons and at least two splice variants have been detected that differ in the 3′ UTR (Yotov and St-Arnaud 1993). Inherited mutations affecting ATP synthase function have mainly been identified in the mitochondrial-encoded *ATP6* gene, resulting in NARP (neuropathy, ataxia, and retinitis pigmentosa) and a form of Leigh syndrome (Nijtmans et al. 2001). A hypomorphic mutation in a nuclear-encoded ATP synthase chaperone, *ATPAF2* (formerly known as *ATP12*), has been identified (De Meirleir et al. 2004). We report here the first mutation identified in the *Atp5a1* gene of mice or humans.

The *ATP5A1* gene and cancer

The *ATP5A1* gene may provide a novel target to aid in diagnostics, prevention, and/or treatment of cancer. Several studies have identified an important link between the respiratory state of cancer cells and the tendency of those cells to be malignant. The Warburg hypothesis originally proposed that cancer cells have impaired mitochondrial function leading to an elevated rate of glycolysis in tumor cells (Warburg 1956). While the identification of the genetic basis of cancer has "ruled out" the causal relationship between elevated levels of glycolysis and transformation, the basic tenet of the Warburg hypothesis has been demonstrated in many tumor types (Cuezva et al. 2002, 2004). Studies have demonstrated a significant decrease in oxidative phosphorylation with an increase in glycolysis in cancer tissues. In lung, liver, kidney, and colon carcinomas, a decrease in the expression of *ATP5B* has been observed (Cuezva et al. 2002, 2004). This decrease in ATP synthase production is also associated with resistance to chemo- and radiotherapies in several cancers. Decreased expression of *ATP5A1* has been detected in colon cancer cells that exhibit resistance to 5-fluorouracil in culture; however, the decrease in ATP5A1 levels may precede and contribute to the ability of these colon cancer cells to attain resistance to 5-fluorouracil (Shin et al. 2005).

In several studies, it has been demonstrated that resveratrol inhibits adenoma development in *Apc*$^{Min/+}$ mice (Schneider et al. 2001; Ziegler et al. 2004; Sale et al. 2005). Resveratrol is a phytoalexin found in grape skins that has diverse effects on cell cycle control and is a cancer chemopreventative agent (Ulrich et al. 2005). One of its many biological effects is the binding and inhibition of the F_1 subunit of ATP synthase, resulting in the inhibition of ATP production (Zheng and Ramirez 2000). Alteration

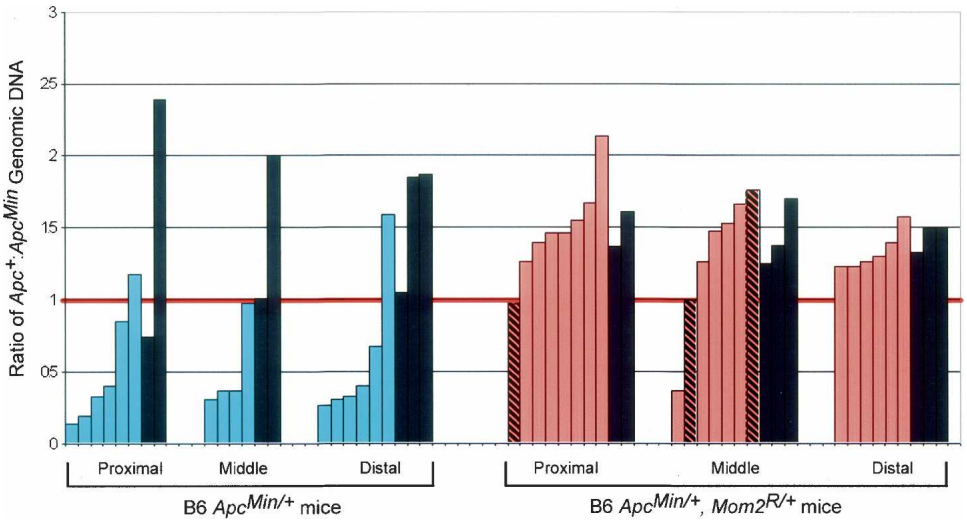

Figure 4. Quantitation of the Apc^{+}:Apc^{Min} ratio in normal and adenoma tissues in $Mom2^{R/+}$ mice. Paraffin-embedded polyp and normal tissues were microdissected from slides. DNA was isolated using Qiagen DNeasy Tissue Kit. Genomic DNA was genotyped for the Apc^{Min} mutation, PCR products run on a 4% agarose gel, and scanned using an EtBr filter on a Typhoon scanner. Ratios were quantified using ImageQuant 5.2. Values reflect the average of duplicate trials. Black bars represent normal tissue; turquoise bars represent polyps from B6 $Apc^{Min/+}$, $Mom2^{+/+}$ mice (aged ~4–5 mo); salmon bars represent polyps from B6 $Apc^{Min/+}$, $Mom2^{R/+}$ mice (aged ~15–17 mo). Hatched bars represent tumors that progressed to carcinomas. A ratio of 1.0 (red line) represents the ideal Mendelian 1:1 ratio of Apc^{+}:Apc^{Min} alleles expected in genomic DNA from a normal heterozygote. Ratios below 0.5 suggest loss of the Apc^{+} allele, whereas ratios above 1.5 suggest loss of the Apc^{Min} allele. The ratios of normal tissue from $Apc^{Min/+}$, $Mom2^{+/+}$ mice (1.56 ± 0.62) do not appear to vary from the ratios of normal tissue from $Apc^{Min/+}$, $Mom2^{R/+}$ mice (1.46 ± 0.15) ($P > 0.6$, *t*-test). Interestingly, comparison of the ratios of tumor (1.38 ± 0.35) to normal (1.46 ± 0.15) tissues from $Apc^{Min/+}$, $Mom2^{R/+}$ mice indicate that they are quite similar ($P > 0.5$, *t*-test), suggesting that most tumors from $Apc^{Min/+}$, $Mom2^{R/+}$ mice retain both *Apc* alleles. In contrast, comparison of the ratios of tumor (0.54 ± 0.41) to normal (1.56 ± 0.62) tissues from $Apc^{Min/+}$, $Mom2^{+/+}$ mice show a striking difference ($P < 2 \times 10^{-4}$, *t*-test), suggesting that most polyps lose the Apc^{+} allele. Furthermore, the ratios of tumor tissue from $Apc^{Min/+}$, $Mom2^{+/+}$ mice (0.54 ± 0.41) differ significantly from the ratios of tumor tissue from $Apc^{Min/+}$, $Mom2^{R/+}$ mice (1.38 ± 0.35) ($P < 3 \times 10^{-7}$, *t*-test). These data indicate that most tumors in $Apc^{Min/+}$, $Mom2^{R/+}$ mice arise by a mechanism different from that occurring in $Apc^{Min/+}$, $Mom2^{+/+}$ mice.

of the mitochondrial membrane potential is one of the primary responses to treatment by resveratrol (Sareen et al. 2006). It is tempting to speculate that the mode of action of resveratrol is to inhibit Apc^{Min} adenoma formation through the inhibition of ATP synthase in the intestinal stem cells. Therefore, in the presence of resveratrol and after loss of the chromosome containing the Apc^{+} allele (along with an $Atp5a1^{+}$ allele), there is insufficient ATP synthesis, that results in a metabolic stress on the initiating tumor cell resulting in cell death.

The decrease in ATP synthase activity in later stages of cancer is at odds with the finding that a mutation in *Atp5a1* results in a decrease of polyp multiplicity. However, this discrepancy most likely reflects the differing energy requirements for early-stage tumor initiation in contrast to later stages of cancer progression.

Mechanism of polyp suppression by the $Mom2^{R}$ mutation

The mechanism of polyp formation on a B6 genetic background was originally proposed to result from loss of the entire chromosome containing the wild-type *Apc* allele (Levy et al. 1994; Luongo et al. 1994; Shoemaker et al. 1998; Haigis et al. 2004). However, current studies indicate that the loss of heterozygosity of the Apc^{+} allele results primarily from somatic recombination, with a small fraction of polyps exhibiting chromosomal homozygosis (Haigis et al. 2002; Haigis and Dove 2003). These mechanisms provide a working hypothesis for the $Mom2^{R}$ allele in polyp protection. Inhibition of ATP synthase activity using oligomycin has demonstrated that cells experiencing a 65%–85% decrease in ATP synthase activity undergo apoptosis in vitro (Comelli et al. 1998, 2003). Therefore, any mechanism resulting in loss of both wild-type *Atp5a1* alleles should cause cell death, due to the absence of ATP synthase activity. Since the chromosome carrying the Apc^{Min} allele also carries the mutant *Atp5a1* allele, loss of the Apc^{+} $Mom2^{+}$ chromosome 18 would result in loss of the only functional *Atp5a1* allele (Fig. 6A). Furthermore, any stem cell in intestinal crypts that undergoes somatic recombination and becomes homozygous for the Apc^{Min} mutation (which would normally lead to polyposis), would undergo cell death in $Mom2^{R/+}$ mice, due to the fact that the cell is now homozygous for the *Atp5a1* mutation (Fig. 6B). Similarly, chromosomal homozygosis of the chromosome carrying both the Apc^{Min} and *Atp5a1* mutations (*cis* configuration) would also result in cell death (Fig. 6C). Thus, when the $Mom2^{R}$ allele is in *cis* with the Apc^{Min} mutation, LOH of the wild-type chromosome results in a dramatic ~90% reduction in polyp formation. It is interesting to note that this degree of reduction in polyp numbers is comparable and consistent with the decrease in polyp numbers seen in Apc^{Min} mice carrying the Robertsonian translocation Rb(7.18)9Lub, which has been shown to suppress somatic recombination in Apc^{Min} mice (Haigis and Dove 2003).

How do the few polyps arise in $Apc^{Min/+}$, $Mom2^{R/+}$ mice?

The finding of a few polyps in some mice that carry Apc^{Min} and $Mom2^{R}$ in *cis* suggested several mechanisms by which intestinal cells can escape polyp suppression (Fig. 6). The data indicate that the few polyps that form in $Mom2^{R/+}$ animals maintain heterozygosity for both *Apc* alleles (Fig. 4). Therefore, since the wild-type chromosome 18 is retained, the Apc^{+} allele must be inactivated. This event could occur by either epigenetic silencing (Esteller et al. 2000; Chen et al. 2005) or somatic mutation (Miyaki

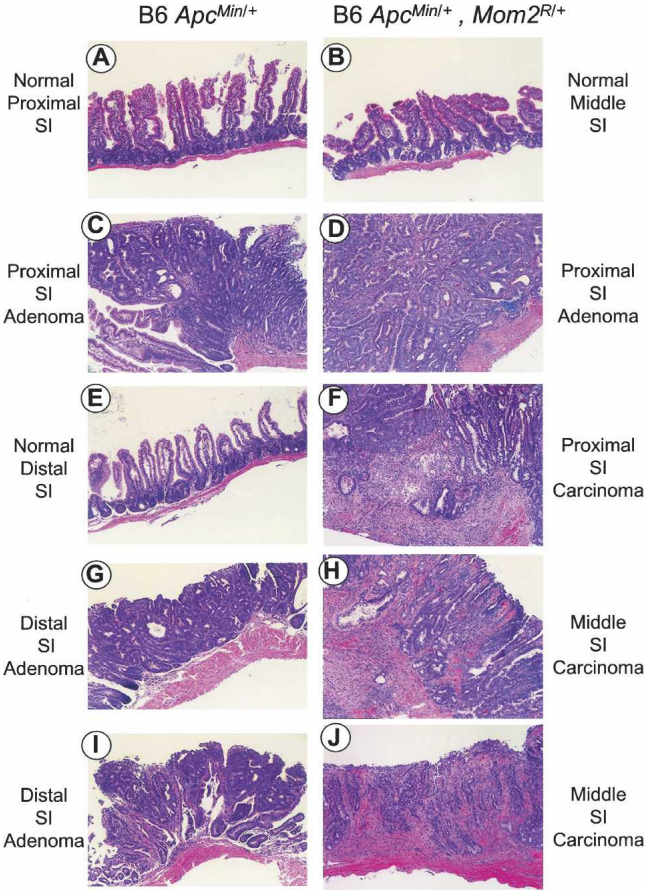

Figure 5. Polyps from B6 $Apc^{Min/+}$, $Mom2^{R/+}$ mice tend to be more advanced than polyps from B6 $Apc^{Min/+}$ mice. Microdissected polyps were stained with hematoxylin and eosin and photographed on a Nikon Eclipse E600 microscope at 100× magnification. (*A*,*C*,*E*,*G*,*I*) Isolated from B6 $Apc^{Min/+}$ $Mom2^{+/+}$ mice as indicated. (*B*,*D*,*F*,*H*,*J*) Isolated from B6 $Apc^{Min/+}$ $Mom2^{R/+}$ mice as indicated. Size measurements revealed that adenomas in $Apc^{Min/+}$ $Mom2^{R/+}$ mice were threefold larger than the adenomas in $Apc^{Min/+}$ $Mom2^{+/+}$mice ($P = 0.03$) (data not shown).

et al. 1995; Crabtree et al. 2003) of the Apc^{+} allele. Alternatively, somatic recombination could occur between the Apc^{Min} and $Mom2^{R}$ mutations, resulting in a chromosome that has only the Apc^{Min} mutation and a wild-type *Atp5a1* allele. Chromosomal homozygosis (Haigis et al. 2002, 2004) and/or loss of the reciprocal recombinant would result in a cell that carries only the Apc^{Min} mutation and the $Atp5a1^{+}$ gene, thus resulting in a polyp (Fig. 6D–F).

Several features of the adenomas that develop in $Apc^{Min/+}$ $Mom2^{R/+}$ mice are reminiscent of human tumorigenesis. The most common mechanisms of LOH for the *APC* gene involve: (1) epigenetic silencing, (2) base substitutions within the coding region, or (3) small intragenic deletions (Fearnhead et al. 2001). However, human colon tumors have not been shown to lose the entire chromosome carrying the *APC* gene, as has been demonstrated for chromosome 18 in B6 $Apc^{Min/+}$ mice (Levy et al. 1994; Luongo et al. 1994). Therefore, retention of the Apc^{+} allele in polyps from $Apc^{Min/+}$ $Mom2^{R/+}$ mice is one feature that accurately recapitulates human tumorigenesis. In addition, the heavy polyp load in B6 $Apc^{Min/+}$ mice usually leads to death by 5–6 mo of age, and adenomas in these mice rarely progress to carcinomas (Halberg et al. 2000; Cooper et al. 2005). In contrast, the light polyp load in $Apc^{Min/+}$ $Mom2^{R/+}$ mice allows for a significantly longer lifespan (>2 yr) and ~15% of adenomas progress to carcinomas by 15–17 mo of age (Figs. 4, 5). Whether progression is related to the longer lifespan or, alternatively, to the retention of the Apc^{+} chromosome, remains to be determined. Regardless of the mechanism, $Apc^{Min/+}$ $Mom2^{R/+}$ mice provide informative vehicles to test therapeutic agents designed to prevent tumor progression.

Generation of congenic mice and the chance for any mutation on mouse chromosome 18 to result in a similar phenotype for *Min*

Our identification of a recessive lethal mutation that is physically linked to the Apc^{Min} mutation suggests that a recessive cell-lethal mutation in any gene on mouse chromosome 18 would result in a reduction in the number of intestinal polyps (if it resided in *cis* with the Apc^{Min} mutation). This statement would be true in any mouse strains (such as B6) in which the major mechanism of Apc^{+} inactivation involved loss of the chromosome carrying the Apc^{+} allele. In addition, continued selection for a specific mutation or chromosomal region (such as occurs during the derivation of congenic lines) may increase the chances of such mutations becoming fixed within a colony. The $Mom2^{R}$ mutation may have been perpetuated within the Apc^{Min} colony at The Jackson Laboratory, because the mice that carried $Mom2^{R}$ would have appeared more fit, had higher reproductive capacity, and lived longer than their susceptible $Mom2^{+}$ littermates. Such mutations, if providing a selective advantage, could eventually become the predominant allele within a colony, thus altering the characteristics of the inbred strain over time.

Potential examples of other such mutations occurring on the Apc^{Min} chromosome are suggested in the literature. For example, Wilson et al. (1997) reported that B6 $Apc^{Min/+}$ mice developed an average of 25.4 polyps (range 20–38 polyps) when aged for 120 d (Wilson et al. 1997); a later publication using the same Min colony showed an average tumor number of only 18 polyps (Wagenaar-Miller et al. 2003). Similarly, Javid et al. (2005) reported an average of 18.9 ± 6.8 (SD) polyps in C57BL/6J Apc^{Min} mice obtained directly from The Jackson Laboratory. These numbers are substantially smaller and have a much lower variance than the number of polyps commonly reported for B6 $Apc^{Min/+}$ mice (Siracusa et al. 2004). These almost uniform types of polyp distributions suggest that other loci tightly linked to the Apc^{Min} mutation are responsible for resistance to polyp formation. In contrast, a recent report has identified a novel modifier closely linked to Apc^{Min} that results in a dramatic increase in polyp multiplicity that arose during selective inbreeding of a hybrid strain (Haines et al. 2005).

Several publications that describe polyp multiplicity in Apc^{Min} mice on a C57BL/6J (or congenic) background demonstrate that there exists much variation in polyp multiplicity (Song et al. 2000; Tucker et al. 2002; Mai et al. 2003; Haines et al. 2005; Moran et al. 2005). While some of this variation might be explained by environmental differences, it is clear from our studies that heterogeneity exists within inbred strains of mice, and could be due to the presence of spontaneous mutations that alter the genetic "homogeneity" of a model system.

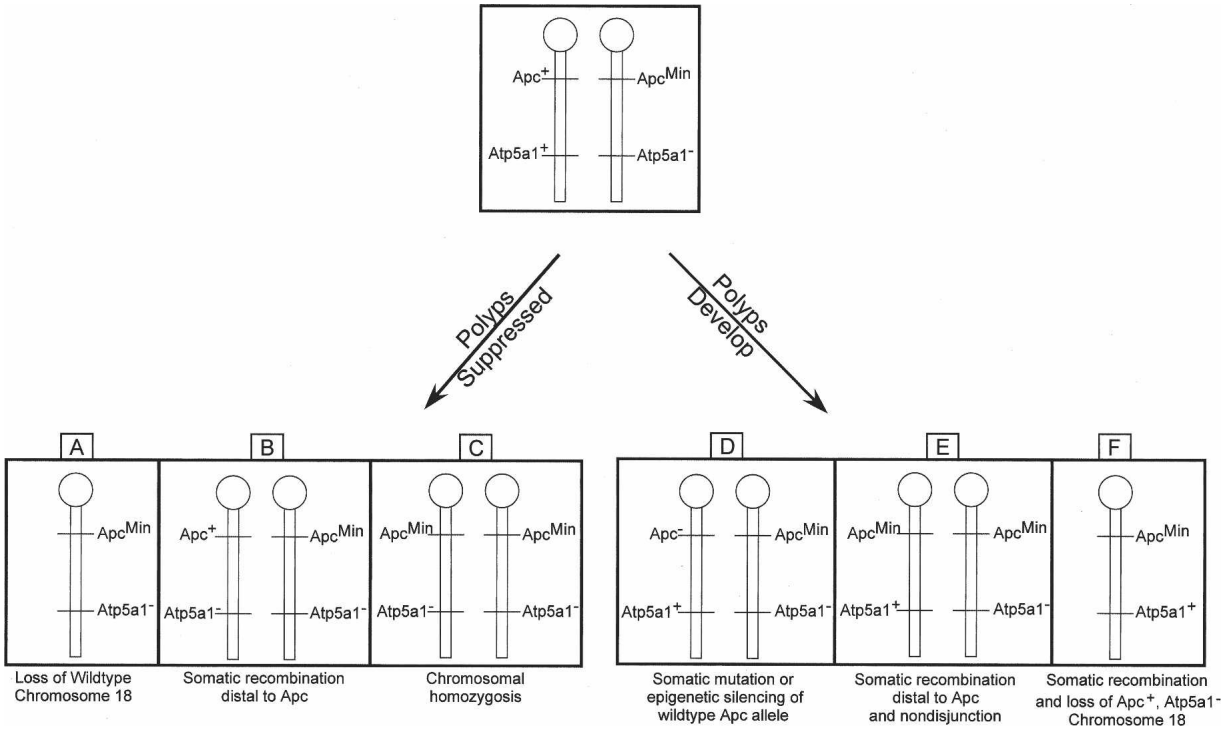

Figure 6. Mechanism of *Mom2*^{R} suppression of *Apc*^{Min}-induced polyposis. The chromosome 18 homologs present in an *Apc*$^{Min/+}$ *Atp5a1*$^{+/-}$ mouse are shown. Previous work has shown that in the B6 mouse, the major mechanism of polyp formation involves loss of the entire wild-type chromosome 18 (*A*). If this occurs, a cell would lose its wild-type *Atp5a1*$^{+}$ allele and die. Similarly, if somatic recombination distal to *Apc* (*B*) or chromosomal homozygosis (*C*) of the *Apc*^{Min} *Atp5a1*$^{-}$ chromosome occurs, the cell would have no wild-type *Atp5a1*$^{+}$ allele and die. Therefore, these mechanisms would prevent stem cells from developing into polyps. However, if both chromosome 18 homologs remain (*D*), polyps could develop in cells that have their wild-type *Apc* allele silenced or inactivated. In this case, a cell would retain its wild-type *Atp5a1*$^{+}$ allele. Similarly, recombination between the *Apc* and *Atp5a1* loci could occur, followed by loss of the chromosome containing the *Apc*$^{+}$ and *Atp5a1*$^{-}$ alleles (*F*) or nondisjunction (*E*). In these cases, cells retaining the mutant *Apc*^{Min} allele and the wild-type *Atp5a1*$^{+}$ allele could form polyps.

Methods

Mice

Mice were bred at the AAALAC-accredited TJU Animal Facility, except for the original C57BL/6J (B6) *Apc*$^{Min/+}$ males, DBA/2J (DBA) mice, and CAST/Ei (CAST) mice, which were purchased from The Jackson Laboratory. Animals were fed laboratory autoclavable rodent diet 5010 (PMI Nutrition International, Inc.). Special husbandry protocols were followed to ensure both reproductive fecundity and viability of CAST mice; these mice were housed in a designated "quiet room" in large microisolator cages with specially designed lids to prevent escape. Cages were prepared with autoclaved aspen shavings, 2–3 nestlets, food, and water bottles.

Genomic DNA isolation and sequencing

Genomic DNA was isolated from tail biopsies as described (Silverman et al. 2002). Genomic DNA from organs was isolated using a standard phenol-chloroform method and resuspended in water (Sambrook and Russell 2001). Genomic DNA was subjected to PCR using primer pairs derived from intronic sequences designed to amplify exons of *Atp5a1*, *Ccdc5*, and *Pstpip2* (Supplemental Tables 2, 3, 4). Resulting fragments were purified and sequenced with a 377-DNA Sequencer from ABI Prism by the KCC Nucleic Acids Facility. Sequencing was performed using both forward and reverse primers and the resulting sequences were aligned using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

DBA.B6 *Mom2*$^{R/+}$ intercross

Congenic mice were generated through selection and backcrossing of DBA.B6 *Apc*$^{Min/+}$, *Mom2*$^{R/+}$ N# mice with DBA animals; offspring were genotyped by PCR for the *Apc*^{Min} mutation as well as for the *Mom2* locus using the flanking markers *D18Mit186* and *D18Mit213* (Silverman et al. 2003). Progeny were selected that carried the *Mom2*-resistant region (*Mom2*$^{R/+}$), but did not carry the *Apc*^{Min} mutation. DBA.B6 *Mom2*$^{R/+}$ animals from the N4–N5 backcross generations were intercrossed to generate *Mom2*$^{R/R}$ homozygotes. Each F_2 offspring was genotyped for the *Mom2* region by PCR with *D18Mit80* and *D18Mit213* as described (Silverman et al. 2003), and to determine whether they were *Mom2*$^{R/R}$, *Mom2*$^{R/+}$, or *Mom2*$^{+/+}$. Four offspring that showed recombination within the *Mom2* region were not informative and were therefore excluded from the analyses (data not shown).

Genotyping for molecular markers

The *Apc*^{Min} mutation was genotyped by modification of a previously published protocol (Luongo et al. 1994) as described (Silverman et al. 2003). SSLP markers were selected based on polymorphisms that were identified between the B6 and CAST strains. Primer pairs were purchased from Invitrogen. A total of 50–100 ng of genomic tail DNA was amplified along with 50 ng each of the oligomers, 0.5 mM of each nucleotide (dCTP, dGTP, dTTP, and dATP), in a buffer (final concentration 50 mM KCl, 10 mM Tris-HCl at pH 8.0, 1.5 mM $MgCl_2$, and 0.1 mg/mL gelatin), along with 5 U/μL of Taq DNA polymerase (Roche Molecular

Biochemicals). Samples were amplified under the following conditions: one cycle at 94°C for 4 min, followed by 35 cycles at 94°C for 30 sec, 58°C or 60°C for 45 sec, 72°C for 30 sec, and ending with one cycle at 72°C for 7 min. Polymorphisms of 10 bp or higher were detected on 3% TBE agarose gels and visualized by staining with EtBr (Sambrook and Russell 2001). If the polymorphism was <10 bp, the forward primer was end-labeled with [γ^{32}P]dATP using polynucleotide kinase prior to PCR (Sambrook and Russell 2001); 6% denaturing polyacrylamide urea gels were used for resolution of the radiolabeled products, followed by autoradiography (Sambrook and Russell 2001).

Identification of new SSLP markers

Candidate SSR marker primer pairs were generated using the Mouse Genome SSR search website, Massachusetts General Hospital (Charlestown, MA; http://danio.mgh.harvard.edu/mouseMarkers/musssr.html). Several primer pairs (D18Kcc# loci) in the *Mom2* region detected polymorphisms between B6, DBA, and CAST mice (Supplemental Table 1).

Genotyping for the mutation in the *Atp5a1* gene

The mutation in the *Atp5a1* gene was detected by designing primers that incorporated the 4-bp insertion into the sequence. The forward primer (5′-GTTAAGCATTGGTGATGGATGG-3′) lies at the site of the insertion (middle of exon 3) and will only bind the mutant *Atp5a1* allele. The reverse primer (5′-TGAGA GAGCAAAGCTACAGATCC-3′) lies within intron 3. PCR reactions contained 100 ng of genomic DNA, 50 ng each of the forward and reverse oligomers, 0.5 mM of each nucleotide (dCTP, dGTP, dTTP, and dATP), in a buffer (final concentration 50 mM KCl, 10 mM Tris-HCl at pH 8.0, 1.5 mM $MgCl_2$, and 0.1 mg/mL gelatin), along with 5 U/µL of Taq DNA polymerase (Roche Molecular Biochemicals). Samples were amplified under the following conditions: one cycle at 94°C for 4 min, followed by 35 cycles at 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, ending with one cycle at 72°C for 7 min. A 147-bp fragment was detected when the mutant *Atp5a1* allele was present, whereas no fragment was detected in mice homozygous for the wild-type *Atp5a1* gene.

Assessment of intestinal polyp number in *Apc^{Min}* mice

Apc$^{Min/+}$ mice generated from crosses of DBA × DBA.B6 *Apc$^{Min/+}$* mice were aged and then euthanized by CO_2 asphyxiation. The small intestine and colon were dissected and cleared of residual debris as described (Koratkar et al. 2002, 2004). Polyps were counted using a Nikon SMZ-U dissection scope at 15× magnification.

Acknowledgments

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