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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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 Apcmin-induced intestinal tumorigenesis called Modifier of Min 2 (Mom2). The polypt-resistant Mom2R 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.2B6 Apcmin/+ , Mom2R/+ mice. An intercross revealed that Mom2 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 in Min mice. Furthermore, we show that more adenomas progress to carcinomas in Min mice that carry the Mom2R 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 Apcmin/+ : Mom2R/+ mice is consistent with the features of human tumor initiation and progression.

[Supplemental material is available online at www.genome.org.]
altered the Mom phenotype otherwise dictated by a mutation in the Apc gene (MacPhie et al. 1995; Cormier et al. 1997).

Our laboratory identified the Mom2 locus in hybrid off-spring derived from a cross involving DBA/2J (DBA) and C57BL/6J (B6) Apc<sup>min/+</sup> 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<sup>R</sup>) arose from a spontaneous mutation in a B6 Apc<sup>min/+</sup> progenitor. On both inbred strain backgrounds, one Mom2<sup>R</sup> allele decreases small intestinal polyp number and colon polyp incidence by ∼90% (Silverman et al. 2002); thus, Mom2<sup>R</sup> has significantly greater effects on polyp multiplicity than Mom1<sup>R</sup>, 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<sup>R</sup> 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<sup>kr/+</sup> animals produced no offspring homozygous for the resistant Mom2<sup>R</sup> allele. To facilitate the genetic dissection of Mom2, we established crosses between DBA.B6 Mom2<sup>kr/+</sup> animals and Mus musculus castaneus (CAST) mice. The increase in polymorphic markers, coupled with making use of the Mom2<sup>R</sup> 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<sup>min</sup>-induced tumorigenesis and the phenotype of recessive embryonic lethality.

**Results**

Intercrosses of congenic DBA.B6 Mom2<sup>kr/+</sup> mice fail to generate homozygous Mom2<sup>R</sup> progeny

To maintain the Mom2<sup>R</sup> 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<sup>R</sup> allele on the DBA/2J (DBA) genetic background by sequential backcrosses (Silverman et al. 2003). Offspring were genotyped for the Apc<sup>min</sup> 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<sup>R</sup>), but did not carry the Apc<sup>min</sup> mutation. DBA.B6 Mom2<sup>kr/+</sup> animals from the N4–N5 backcross generations were intercrossed to generate animals homozygous for the resistant Mom2<sup>R</sup> allele. Surprisingly, our intercross resulted in 36 F<sub>2</sub> Mom2<sup>kr/+</sup> heterozygotes and 25 F<sub>2</sub> wild-type Mom2<sup>kr/+</sup> progeny, with no F<sub>2</sub> animals homozygous for the Mom2<sup>R</sup> allele. χ<sup>2</sup> analysis indicated that the expected Mendelian distribution of 1:2:1 was significantly different (P < 0.001) from the observed distribution of F<sub>2</sub> animals obtained from our Mom2<sup>R</sup> intercross (1.4:1). In addition, the observed 1.4:1 (Mom2<sup>kr/+</sup>:Mom2<sup>kr/+</sup>) ratio was not significantly different (P = 0.2) from a ratio of 2:1 (Mom2<sup>kr/+</sup>:Mom2<sup>kr/+</sup>), suggesting that the Mom2<sup>R</sup> allele is a recessive embryonic lethal mutation. Additional experiments have confirmed that homozygous Mom2<sup>R</sup> mice are never born (see below).

The Mom2<sup>R</sup> locus encodes a recessive embryonic lethal mutation

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

To investigate the developmental stage of embryonic lethal-ity, timed pregnancies from intercrosses of DBA.B6 Mom2<sup>kr/+</sup> 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<sup>R</sup> flanking markers, D18Mit80 and D18Mit213, to distinguish the presence of the B6 Mom2<sup>R</sup> locus from the wild-type DBA Mom2<sup>R</sup> loci (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<sup>kr</sup> 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<sup>R</sup> allele can be transmitted to offspring from both female and male parents**

<table>
<thead>
<tr>
<th>Cross†</th>
<th>No. of mating cages</th>
<th>Mom2&lt;sup&gt;kr/+&lt;/sup&gt;</th>
<th>Mom2&lt;sup&gt;kr/+&lt;/sup&gt;</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA.B6 Mom2&lt;sup&gt;kr/+&lt;/sup&gt; × B6 Apc&lt;sup&gt;min&lt;/sup&gt;</td>
<td>6</td>
<td>41</td>
<td>31</td>
<td>1.4</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>DBA.B6 Apc&lt;sup&gt;min&lt;/sup&gt; × DBA Mom2&lt;sup&gt;kr/+&lt;/sup&gt;</td>
<td>6</td>
<td>31</td>
<td>27</td>
<td>0.3</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>DBA × DBA.B6 Mom2&lt;sup&gt;kr/+&lt;/sup&gt;</td>
<td>5</td>
<td>50</td>
<td>53</td>
<td>0.1</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>DBA × DBA.B6 Apc&lt;sup&gt;min&lt;/sup&gt; × Mom2&lt;sup&gt;kr/+&lt;/sup&gt;</td>
<td>10</td>
<td>88</td>
<td>93</td>
<td>0.1</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

The D18Mit80 and D18Mit213 markers were used to distinguish the presence of the B6 Mom2<sup>R</sup> locus from the wild-type DBA Mom2<sup>R</sup> locus (Silverman et al. 2003).

†For each cross, the female is listed first.
further define the boundaries of the Mom2 region. 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 Mom2R/− animals from the N7–N8 backcross generations (Fig. 1A) produced 94 F1 offspring that were genotyped for the Mom2 region. While all F1 offspring inherited a CAST allele at each marker, either a DBA allele (Mom2+) or the B6 allele (Mom2−) could be inherited from the heterozygous DBA.B6 Mom2+/- congenic parent. Since the Mom2R mutation arose on a B6 background, F1 progeny were selected that carried the B6 allele at both the proximal D18Mit186 and distal D18Mit213 markers. These [CAST × DBA.B6 Mom2+/-]F1 hybrid mice were intercrossed to generate F2 progeny that were genotyped for D18Mit186 and D18Mit213. Only F2 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 Mom2R+/− homozygous liveborn progeny from the DBA.B6 Mom2+/- intercross. Since Mom2− is a recessive embryonic lethal mutation, no animals homozygous for the B6 Mom2+ 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+/-]F1 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 F2 offspring, 22 were informative recombinants. No F2 progeny were identified that were B/B at both the D18Mit186 and D18Mit213 markers; 452 nonrecombinant F2 animals showed a distribution of 159:293:0 (C/C: B/C: B/B), with a 1:1:1 ratio instead of a Mendelian 1:2:1 ratio ($\chi^2 = 129.4$, $P < 0.0001$), confirming the recessive embryonic lethality of the Mom2R mutation.

To further dissect the Mom2 locus, the 22 recombinant F2 animals were genotyped for additional markers (Fig. 1C). The spontaneous mutation that created the Mom2− 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 Slt8sia5 [formerly known as Siat8e] gene) and D18Kcc10 (within the Slc14a2 gene) markers (Supplemental Table 1).

To further narrow the location of the Mom2 gene, F2 progeny that were heterozygous for the Mom2 region were sequentially intercrossed. F3 offspring were identified that carried crosses between the Slt8sia5 and Slc14a2 genes. Intercrosses of these recombinant F2 progeny produced 64 F3 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 mutation. These data demonstrated that the region distal to Slt8sia5 and proximal to D18Kcc7 (within the Slat8sia5 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).
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, Cdc5, Lox1d, Pstpip2, 8030462N17Rik, and S8isaz5. Total RNA was isolated from the proximal and distal small intestine and colon of congenic DBA.B6 Mom2Mom2 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 F1 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 Mom2Mom2 mice. Therefore, we sequenced each exon of the Atp5a1 gene from genomic DNA of congenic DBA.B6 Mom2Mom2 and B6 Mom2Mom2 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 Mom2Mom2 allele and absent in Mom2Mom2 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 Mom2Mom2 allele was equally stable as from the Mom2Mom2 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 Mom2Mom2 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 Cdc5 and Pstpip2 genes showed no differences between Mom2Mom2 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 Mom2Mom2 phenotype, we sequenced the original B6 ApcMinMom2R father who first transmitted the Mom2Mom2 allele to his progeny (Silverman et al. 2002); results showed that he indeed carried the mutation. Sequencing of five Mom2Mom2 mice, three from the real-time PCR studies, and the two F2 parents (see above), further confirmed the presence of the mutation in mice carrying the Mom2Mom2 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 ApcMin Mom2Mom2 mice carrying the mutated Atp5a1Mom2R allele with those carrying the wild-type Atp5a1 allele. Twenty DBA.B6 ApcMinMom2R 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 Atp5a1Mom2R 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 ApcMin-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 ApcMinMom2R mice develop polypos 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 ApcMin mutation (Shoemaker et al. 1998; Haigis et al. 2002, 2004; Haigis and Dove 2003). Therefore, if the Mom2Mom2 mutation acted in a dominant fashion, its action would be independent of whether it was located on the same chromosome as the ApcMin 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 ApcMin allele (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 Mom2Mom2 mutation resides on the same chromosome as the ApcMin mutation (in cis) as opposed to the Mom2Mom2 mutation and the ApcMin mutation residing on different chromosome 18 homologs (in trans). Crosses were performed to generate offspring with the Mom2Mom2 mutation in both the cis and trans configurations with respect to the ApcMin mutation (Fig. 3). Quantitation of the number of polyps was performed on progeny aged for 150 d. Control mice that carried only the ApcMin mutation developed an average of 88 polyps in the small intestine, whereas those mice that carried ApcMin and Mom2Mom2 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 Mom2Mom2 phenotype (Silverman et al. 2002, 2003). However, mice that carried the ApcMin and Mom2Mom2 mutations on different homologs (in trans) developed an average of 99 polyps, which is not significantly different from the ApcMin-only control group (Student’s t-test, P > 0.5). The results shown in Figure 3 clearly demonstrate that on a DBA background, Mom2Mom2 functions as a potent suppressor of polyp formation only when in cis with respect to the ApcMin 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 ApcMinMom2R mice compared with adenomas from ApcMin Mom2Mom2 mice (Fig. 4). The data clearly show that the majority of adenomas (12/16) from ApcMinMom2R mice have lost their Apc+ allele, whereas only one adenoma (1/21) from ApcMinMom2Mom2 mice has lost its Apc+ allele (P < 0.001). Histopathological analysis of the adenomas used in the LOH analysis revealed that three of 21 tumors in ApcMinMom2R Mom2Mom2 mice progressed to carcinomas, whereas none of the adenomas in ApcMinMom2R mice progressed (Figs. 4, 5).
Discussion

The observation that the Mom2R 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 function. This strategy excludes any marker homozygous for the B6 allele within the Mom2 region as being a part of the Mom2R locus (Fig. 1). This exclusion mapping technique should be applicable to any mutation that results in embryonic lethality (Williams 1999). Our mapping re-
Effects of Mom2 on intestinal tumorigenesis

Figure 3. The cis–trans test reveals that the Mom2<sup>+</sup> mutation functions only when in cis with the Apc<sup>Min</sup> mutation. Two crosses were established to test whether Mom2<sup>+</sup> could function in trans as well as in cis. Three mating cages of DBA.B6 Mom2<sup>Min+/+</sup> × DBA.B6 Apc<sup>Min+/+</sup> mice produced progeny that carried only the Apc<sup>Min</sup> mutation on chromosome 18 (A) or progeny that carried the Apc<sup>Min</sup> mutation on one chromosome 18 homolog and the Mom2<sup>+</sup> mutation on the other chromosome 18 homolog (C, in trans). Three mating cages of DBA/2J × DBA.B6 Apc<sup>Min+/+</sup>, Mom2<sup>Min+/+</sup> mice produced progeny that carried both the Apc<sup>Min</sup> and Mom2<sup>+</sup> 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<sup>Min</sup> allele and the group that has Apc<sup>Min</sup> and Mom2<sup>+</sup> 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<sup>Min</sup> group that has Mom2<sup>+</sup> 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<sup>+</sup> mutation

The Mom2<sup>+</sup> allele is an early embryonic recessive lethal mutation. In timed pregnancies, we were unable to recover homozygous Mom2<sup>+</sup> embryos by 12.5 d of gestation. Since we propose that Mom2<sup>+</sup> is a null allele for the Atp5a1 gene, leading to a nonfunctional ATP synthase 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 (Schnauber 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 blastocoele. 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 blastocoele formation and implantation, it is likely that the homozygous Mom2<sup>+</sup> 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<sub>0</sub> and F<sub>1</sub> subunits, is localized to the inner mitochondrial membrane. The F<sub>0</sub> complex spans the membrane and is a hydrogen ion (H<sup>+</sup>) pump, while the F<sub>1</sub> complex is a molecular motor harnessing the H<sup>+</sup> 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<sub>1</sub> complex of ATP synthase. The human ortholog (ATPS1) 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<sup>Min+/+</sup> 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<sub>1</sub> subunit of ATP synthase, resulting in the inhibition of ATP production (Zheng and Ramirez 2000). Alteration
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 Atp5α1 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<sup>−</sup> allele (along with an Atp5α1<sup>−</sup> allele), there is insufficient ATP synthase 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 Atp5α1 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<sup>R</sup> 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; Haigs et al. 2004). However, current studies indicate that the loss of heterozygosity of the Apc<sup>−</sup> allele results primarily from somatic recombination, with a small fraction of polyps exhibiting chromosomal homozygosis (Haigs et al. 2002; Haigs and Dove 2003). These mechanisms provide a working hypothesis for the Mom2<sup>R</sup> 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 Atp5α1 alleles should cause cell death, due to the absence of ATP synthase activity. Since the chromosome carrying the Apc<sup>Min</sup> allele also carries the mutant Atp5α1 allele, loss of the Apc<sup>−</sup> chromosome 18 would result in loss of the only functional Atp5α1 allele (Fig. 6A). Furthermore, any stem cell in intestinal crypts that undergoes somatic recombination and becomes homozygous for the Apc<sup>Min</sup> mutation (which would normally lead to polyposis), would undergo cell death in Mom2<sup>R</sup>/ mice, due to the fact that the cell is now homozygous for the Atp5α1 mutation (Fig. 6B). Similarly, chromosomal homozygosis of the chromosome carrying both the Apc<sup>Min</sup> and Atp5α1 mutations (cis configuration) would also result in cell death (Fig. 6C). Thus, when the Mom2<sup>R</sup> allele is in cis with the Apc<sup>Min</sup> 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<sup>Min</sup> mice carrying the Robertsonian translocation Rb(7.18)9Lub, which has been shown to suppress somatic recombination in Apc<sup>Min</sup> mice (Haigs and Dove 2003).

How do the few polyps arise in Apc<sup>Min</sup><sup>/+</sup>, Mom2<sup>R</sup> mice?

The finding of a few polyps in some mice that carry Apc<sup>Min</sup> and Mom2<sup>R</sup> 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<sup>R</sup> animals maintain heterozygosity for both Apc alleles (Fig. 4). Therefore, since the wild-type chromosome 18 is retained, the Apc<sup>−</sup> 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 4.** Quantitation of the Apc<sup>+</sup>:Apc<sup>Min</sup> ratio in normal and adenoma tissues in Mom2<sup>R</sup>/ 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<sup>Min</sup> 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 occurring in Apc<sup>Min</sup>/+ mice. (A) The mechanism of polyp suppression by the Mom2<sup>R</sup> allele results primarily from somatic recombination, with a small fraction of polyps exhibiting chromosomal homozygosis (Haigs et al. 2002; Haigs and Dove 2003). These mechanisms provide a working hypothesis for the Mom2<sup>R</sup> 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 Atp5α1 alleles should cause cell death, due to the absence of ATP synthase activity. Since the chromosome carrying the Apc<sup>Min</sup> allele also carries the mutant Atp5α1 allele, loss of the Apc<sup>−</sup> chromosome 18 would result in loss of the only functional Atp5α1 allele (Fig. 6A). Furthermore, any stem cell in intestinal crypts that undergoes somatic recombination and becomes homozygous for the Apc<sup>Min</sup> mutation (which would normally lead to polyposis), would undergo cell death in Mom2<sup>R</sup> mice, due to the fact that the cell is now homozygous for the Atp5α1 mutation (Fig. 6B). Similarly, chromosomal homozygosis of the chromosome carrying both the Apc<sup>Min</sup> and Atp5α1 mutations (cis configuration) would also result in cell death (Fig. 6C). Thus, when the Mom2<sup>R</sup> allele is in cis with the Apc<sup>Min</sup> 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<sup>Min</sup> mice carrying the Robertsonian translocation Rb(7.18)9Lub, which has been shown to suppress somatic recombination in Apc<sup>Min</sup> mice (Haigs and Dove 2003).
Effects of Mom2 on intestinal tumorigenesis

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 x magnification. (A,C,E,G,I) 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^R/+ 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 mutation and Mom2^R mutations, resulting in a chromosome that has only the Apc^Min mutation and a wild-type Atp5a1 allele. Chromosomal homozygosis (Hagis 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 C3BL/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.
Methods

Mice

Mice were bred at the AAALAC-accredited TJU Animal Facility, except for the original C57BL/6J (B6) ApcMin/+ 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, CcS, 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 Mom2R/+ intercross

Congenic mice were generated through selection and backcrossing of DBA.B6 ApcMin/+; Mom2R/N# mice with DBA animals; offspring were genotyped by PCR for the ApcMin 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 (Mom2R/+), but did not carry the ApcMin mutation. DBA.B6 Mom2R/+ animals from the N4–N5 backcross generations were intercrossed to generate Mom2R homozygotes. Each F2 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 Mom2R/R, Mom2R/+ or Mom2R/+. 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 ApcMin 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 MgCl2, 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-32P]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/mussssr.html). Several primer pairs (D18Kcc1 loci) in the Mom2 region detected polymorphisms between B6, DBA, and CAST mice (supplemental Table 1).

Genotyping by the mutation in the Atpsa1 gene
The mutation in the Atpsa1 gene was detected by designing primers that incorporated the 4-bp insertion into the sequence. The forward primer (5’-GTGAACGGTTGGATGATGTC-3’) lies at the site of the insertion (middle of exon 3) and will only bind the mutant Atpsa1 allele. The reverse primer (5’T-TGAGA GAGACACGTACAGACCC-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, dATP, and dTTP), in a buffer (final concentration 50 mM Tris-HCl at pH 8.0, 1.5 mM MgCl2, 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 Atpsa1 allele was present, whereas no fragment was detected in mice homozygous for the wild-type Atpsa1 gene.

Assessment of intestinal polyp number in Atpsa1 mice
Atpsa1<sup>Min</sup> mice generated from crosses of DBA × DBA.B6 Atpsa1<sup>Min</sup> mice were aged and then euthanized by CO<sub>2</sub> asphyxiation. The small intestine and colon were dissected and cleared of residual debris as described (Koratkar et al. 2002, 2004). Polyps were assessed by decline of mitochondrial ATP synthesis in erythroleukemia cells. Free Radic. Biol. Med. 24: 924–932.

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