Secretory phospholipase Pla2g2a confers resistance to intestinal tumorigenesis

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Individuals inheriting the same mutation predisposing to cancer may show very different outcomes, ranging from early aggressive cancer to disease-free survival. Experimental mouse models can provide a powerful tool to identify factors in the environment and genetic background that account for such modifications. The Min mouse strain[1], in which the *Apc*^{Min} mutation disrupts the mouse homologue of the human familial polyposis gene[2], develops intestinal neoplasms whose multiplicity is strongly affected by genetic background[3]. We previously mapped[4,5] a strong modifier locus, *Mom1* (modifier of Min-1), to a 4-cM region on mouse chromosome 4 containing a candidate gene *Pla2g2a* encoding a secretory phospholipase[6]. Here, we report that a cosmid transgene overexpressing *Pla2g2a* caused a reduction in tumour multiplicity and size, comparable to that conferred by a single copy of the resistance allele of *Mom1*. These results offer strong evidence that this secretory phospholipase can provide active tumour resistance. The association of *Pla2g2a* with *Mom1* thus withstands a strong functional test and is likely to represent the successful identification of a polymorphic quantitative trait locus in mammals.

Intestinal tumour number in *Apc*^{Min}/+ mice varies widely with genetic background[3]. We previously mapped a major modifier locus, *Mom1*, to a 15-cM region on distal chromosome 4, and showed that it explained about 50% of the genetic variance in tumour number in AKR×B6 backcrosses[4]. To examine the action of *Mom1* in the absence of other modifier genes, we created a line (designated B6.*Mom1*$^{AKR/B6}$) by breeding a 35-cM region surrounding *Mom1* from the AKR strain onto an otherwise B6 genetic background[7]. By using this strain to construct heterozygotes (*Mom1*$^{AKR/B6}$), we found that a single copy of the *Mom1*^{AKR} allele reduces tumour multiplicity twofold and tumour diameter by 20%. *Mom1* action is localized to an intestinal crypt lineage[8,9], and the resistant allele of *Mom1* is retained within the tumour lineage when *Min*-induced tumours form in *Mom1* heterozygotes[5].

MacPhee and colleagues[6] recently proposed the calcium-dependent non-pancreatic secretory phospholipase gene *Pla2g2a* (ref. 10) as a candidate gene for *Mom1*, on the basis of their finding that the gene mapped to the interval containing *Mom1* and carried a frameshift mutation eliminating expression in B6 but not in AKR, MA or CAST. As McPhee *et al.* noted, this evidence is intriguing but hardly compelling. Indeed, the initial 15-cM *Mom1* region[4] is large enough to contain about 1,000 average-sized genes, many of which are likely to have mutations in B6. We subjected the hypothesis to preliminary tests[5] by localizing the *Mom1* locus more precisely to a 4-cM interval and by determining the presence of the *Mom1* allele carried in five additional inbred strains. The results were consistent with, but, of course, do not prove the hypothesis that *Pla2g2a* is *Mom1*.

To test this hypothesis more directly, we constructed a transgenic mouse carrying the functional *Pla2g2a*^{AKR} allele on a B6 background. We isolated cosmid KH1 containing *Pla2g2a* from a library prepared from AKR genomic DNA and characterized the clone by determining its complete 41,125-bp DNA sequence. The *Pla2g2a* gene is intact and is surrounded by 26.9 kb of 5′ flanking sequence and 10.9 kb of 3′ flanking sequence (Fig. 1). DNA sequence analysis revealed that fragments of two other genes are carried on the cosmid: the first two of four exons of another phospholipase gene *Pla2g5* (previously reported to map to the region[10]) and an expressed sequence tag (EST) reported in a mouse heart library. Whether the EST represents a *bona fide* transcript is unclear: it is represented in the public repositories by only a single clone, whose sequence lacks introns or a poly-A tract and contains several repeat elements. Hybridization of a probe from the clone gives a weak signal on northern blots of intestinal mRNA, with no differences seen between B6 and AKR or between transgenic and non-transgenic mice.

Pronuclear injection into B6 eggs yielded a single initial transgenic founder, which was subsequently bred to B6 mice to produce a B6 transgenic line (designated here as TgKH1(+)*Mom1*$^{B6/B6}$). DNA analysis showed that the transgenic line carries approximately nine copies of the cosmid, which segregate as a single autosomal mendelian locus (not linked to either *Mom1* or *Apc*) and are likely to be arranged as a tandem array. The transgenic mice express the *Pla2g2a*^{AKR} allele: northern blots showed the expected 769-bp mRNA (not shown) and western blots showed the expected 13-kD polypeptide (Fig. 2), both of which are absent from the B6 parental strain. The level of polypeptide was substantially elevated throughout the intestinal tracts of transgenic animals relative to non-transgenic animals heterozygous for the *Mom1*^{AKR} allele (Fig. 2). The spatial distribution of Pla2g2a in the intestine was characterized by immunohistochemistry (Fig. 3). Non-transgenic *Mom1*$^{AKR/B6}$ animals showed staining in the vast majority of intestinal crypts (Fig. 3*b*), with positive crypts showing an evenly distributed staining pattern. Pla2g2a is not restricted to Paneth cells in the lower regions of intestinal crypts—in contrast to the localization of lysozyme (Fig. 3*d*), which is found only in Paneth cells[11]. Pla2g2a is also found in the colon (R.B.H., data not shown), which lacks Paneth cells[12]. Transgenic animals showed a similar distribution

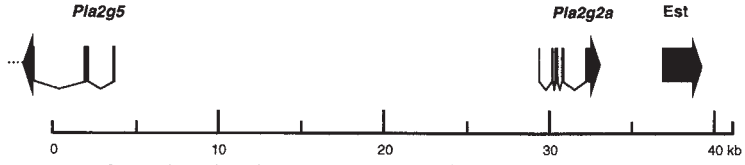

Fig. 1 Map of cosmid KH1 based on its complete 41,125-bp DNA sequence, showing the position of two identified genes, *Pla2g2a* (GenBank U28244) and *Pla2g5*, and one EST. *Pla2g5* is only partly contained on the cosmid. Intron–exon boundaries are based on comparison with published cDNA sequences; exon positions are drawn approximately to scale.

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Table 1 • Effect of *Pla2g2a*^{AKR} transgene on tumour multiplicity and size in Min mice

Class	Genotype		N	Tumour Number			Tumour Diameter (mm)
	Transgene	*Mom1*		Small Intestine	Large Intestine	Total mean ± s.e.m.	mean ± s.e.m.
1	–	B6/B6	16	104	2.0	106 ± 8	1.35 ± 0.05
2	–	AKR/B6	12	42	1.3	43 ± 5	1.04 ± 0.04
3	+	B6/B6	10	44	0.5	45 ± 6	1.14 ± 0.08
4	+	AKR/B6	11	59	1.2	60 ± 8	1.03 ± 0.01

of Pla2g2a (Fig. 3*c*), but the proportion of positive crypts in the small intestine was notably lower, and the level of Pla2g2a in the colon was much higher (R.B.H., data not shown).

We tested whether the transgene conferred resistance to the Min phenotype. The B6.*Mom1*^{AKR} congenic line was used to construct *Mom1*$^{AKR/B6}$ *Apc*^{Min}/+ mice, which were then crossed to the transgenic line TgKH1(+)*Mom1*$^{B6/B6}$ (Table 1). Progeny mice carrying either the *Mom1*^{AKR} allele or the transgene displayed a twofold reduction in tumour multiplicity when compared with control littermates carrying neither factor ($P<0.0002$ for each comparison). Each class also showed a significant decrease in tumour diameter compared with the control ($P<0.009$ for each comparison). The transgene thus has a similar qualitative and quantitative effect as *Mom1*^{AKR}. Notably, mice carrying both the transgene and the *Mom1*^{AKR} allele do not differ in tumour multiplicity and diameter from animals carrying either factor alone ($P>0.12$).

The transgene derived from within the *Mom1* region thus clearly acts as a resistance modifier of the Min intestinal phenotype. Moreover, the transgene closely reproduces the quantitative reduction in tumour multiplicity seen in *Mom1*$^{AKR/B6}$ heterozygotes. The most straightforward interpretation is that the *Mom1* locus resides on the cosmid and is, in fact, *Pla2g2a*. We cannot formally exclude the possibility that the transgene insertion has disrupted an unrelated locus that is a modifier of Min or that the modification is due to another gene on the cosmid (for example, the above-mentioned EST), but such explanations seem unlikely.

There is, however, one respect in which the data conflict with expectations. We previously showed that the 35-cM segment around *Mom1*^{AKR} acts as a semi-dominant modifier, with one copy causing a twofold reduction and two copies causing a fourfold reduction[7]. However, overexpression of *Pla2g2a* from the transgene fails to confer more than a 2-fold reduction—either alone or in combination with *Mom1*^{AKR}. This may be because of improper expression or regulation of the transgene, as suggested by the fact that Pla2g2a is found in only a minority of crypts (Fig. 3*c*). Alternatively, *Pla2g2a* may act as a simple dominant resistance factor, and the semi-dominance of the *Mom1*^{AKR} region may be due to a second modifier locus in the large congenic segment. Indeed, we have reported a recombinant in this region[5] that seems to subdivide the dominant resistance of the AKR haplotype and expresses *Pla2g2a* (R.B.H., data not shown). Possible candidates for such an additional modifier locus could include other phospholipase genes known to lie in the region[10].

Although such caveats remain, the results provide clear functional evidence that *Pla2g2a* provides resistance to tumour development. Additional transgenic lines carrying a smaller transgene would provide useful confirmation, but definitive proof would require a knock-in experiment, such as restoring a functional *Pla2g2a* gene in the B6 strain.

The mechanism by which the secretory phospholipase reduces the net growth rate and multiplicity of Min-induced adenomas remains as mysterious as when *Pla2g2a* was first proposed[6] as a candidate for *Mom1*. Several hypotheses have been proposed, but none is satisfactory. One hypothesis concerns prostaglandin biosynthesis. In light of the observations that inhibition of the prostaglandin-producing cyclo-oxygenase Cox2 diminishes adenoma size and multiplicity in mice with *Apc* mutations[13] and that Pla2g2a produces a lipid substrate of Cox2 (refs 6,14), it has been suggested that a *Pla2g2a* mutation might act by affecting prostaglandin production. However, this explanation would predict that a nonfunctional *Pla2g2a* allele in B6 would confer resistance rather than susceptibility. Another hypothesis for the action of a secretory phospholipase in reducing tumorigenesis involves bactericidal activity[15] resulting in decreased bacterial production of mutagens or clastogens. This hypothesis is rendered unlikely, however, by recent experiments demonstrating that the Min phenotype is relatively unaffected when B6 *Min*/+ animals are raised under germ-free conditions[16]. Another hypothesis is that lipids released by Pla2g2a stimulate surveillance by lymphoid cells[17] or apoptosis, thereby attenuating the adenoma stem-cell population. A key consideration in elucidating the mechanism of action is the fact that the resistance allele of *Mom1* is not lost in the tumour lineage in heterozygotes[5], in contrast to the situation for classic cell-autonomous tumour suppressor genes.

Understanding the mechanism by which *Pla2g2a* affects tumours in Min mice may also elucidate the biology of intestinal tumours in humans. Regardless of whether germline sequence variation in the human *PLA2G2A* gene accounts for variability in tumour number in human familial polyposis patients[18,19], knowledge of the biological mechanism for *Pla2g2a*-mediated resistance in mice may lead to important therapeutic approaches in humans.

Recent years have seen great progress in genetic mapping of QTLs[20] in rodent models of many diseases, including cancer. The challenge now is to identify the genes at the molecular level. In the case of *Mom1*, the process has benefited from the fortunate proposal of a candidate gene. In general, it is likely to require fine-structure genetic mapping, using congenic strains to separate the locus from other modifier genes and progeny testing to measure quantitative phenotypes, as done for *Mom1* (refs 5,7), and large-insert transgenesis (as in the recent cloning of the *vibrator* and *Clock* genes[21,22]) to narrow the search to a region small enough to allow systematic analysis to discover the reponsible gene and muta-

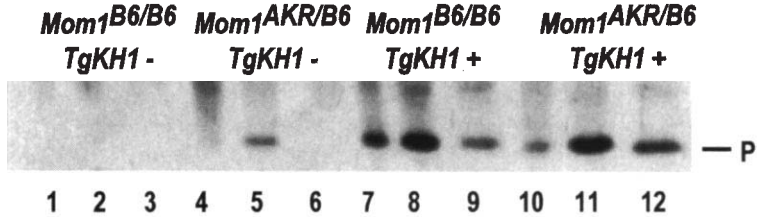

Fig. 2 Level of Pla2g2a in the intestine. Acid-soluble protein extracts (5 μg) were prepared from the proximal half of the small intestine (lanes 1,4,7,10), distal half of the small intestine (lanes 2,5,8,11) and colon (lanes 3,6,9,12) of non-transgenic *Mom1*$^{B6/B6}$ homozygotes (lanes 1–3), non-transgenic *Mom1*$^{B6/AKR}$ heterozygotes (lanes 4–6), transgenic *Mom1*$^{B6/B6}$ homozygotes (lanes 7–9) and transgenic *Mom1*$^{B6/AKR}$ heterozygotes (lanes 10–12). These extracts were subjected to western-blot analysis with Pla2g2a antiserum (see Methods). Note that *Mom1*$^{B6/AKR}$ heterozygotes show detectable levels only in the distal small intestine (lane 5) under the conditions used here; positive signals are detected in the proximal small intestine and colon when a western blot is overloaded with 20 μg. These observations are consistent with previous studies[29] on the localization of Pla2g2a.

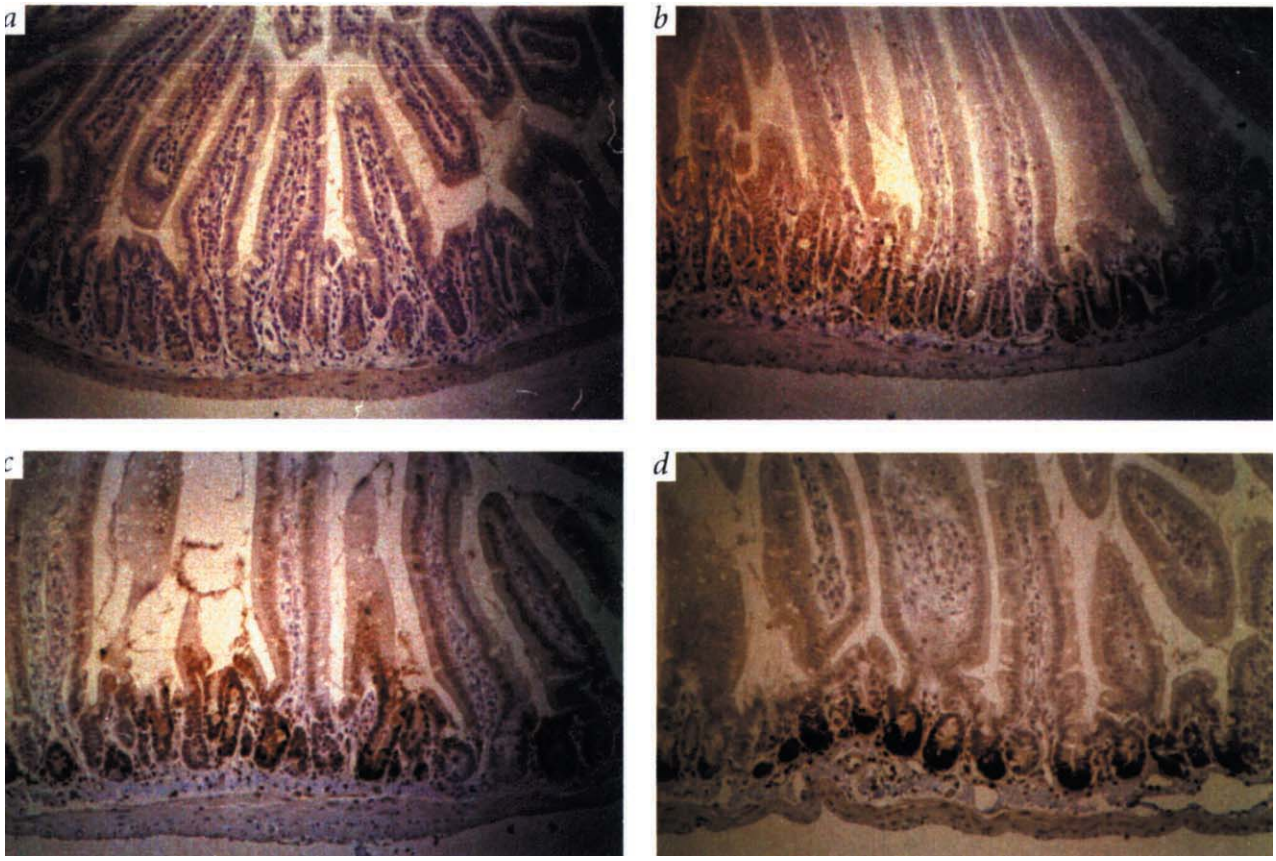

Fig. 3 Spatial distribution of Pla2g2a in the intestine. Sections were prepared from the medial region of the small intestines of the following classes of mice: the negative control, TgKH1 (–) *Mom1*$^{B6/B6}$ (**a**); positive control, TgKH1 (–) *Mom1*$^{AKR/B6}$ (**b**); and a *Mom1*$^{B6/B6}$ animal carrying the *Pla2g2a* transgene TgKH1 (**c**,**d**). Sections were subjected to peroxidase-based immunohistochemistry with Pla2g2a antiserum (*a*–*c*) and lysozyme antiserum (*d*) (see Methods). The scale bar in *d* corresponds to 500 μm and is representative of all panels.

tion. The problem is especially challenging in the case of quantitative trait loci defined by polymorphic strain variation (such as *Mom1*), as there may be many sequence differences in the critical region[22], and functional assays will be required for proof. However, the resulting genes are likely to shed much light on physiological mechanisms important in health and disease.

Methods

Animal care and breeding. Experimental mice were bred at the McArdle Laboratory for Cancer Research. Mice were fed and handled as previously described[1] and were given bottled tap water *ad libitum*. To avoid nurturing defects commonly observed in C57BL/6 Min/+ females, neonates were transferred to the care of ICR foster mothers (Sprague Dawley) until weaning.

Strain designations. Strains are abbreviated throughout as B6, C57BL/6J; AKR, AKR/J; MA, MA/J; CAST, CAST/EiJ.

Construction of transgenic line. Cosmid KH1 was isolated by screening an AKR genomic cosmid library, cloned in the vector SuperCos1 (Stratagene) with a 193-bp probe derived from exon 5 of *Pla2g2a*. The cosmid was digested with *Not*1 to liberate the insert, the DNA was electrophoresed in a 0.8% agarose gel and the 41-kb insert was electro-eluted in a pulsed-field gel apparatus (200 volts, 20 s switch time, 4 °C, overnight). Injection of the purified fragment into pronuclei of fertilized B6 mouse eggs was performed by DNX Transgenics, and transgenic mice were screened by PCR with DNA prepared from tail biopsies and primers T7 (5′–TAATACGACTCACTATAGGG–3′, located in the vector arm) and Pla47b (5′–AGAGACTTTTTTCTCTGATT–3′, located in the insert). A single male transgenic founder was produced and mated with B6 females to produce a transgenic line. Presence of the transgene was detected by PCR.

Experimental classes of mice. These were generated by a cross between B6.*Apc*$^{Min/+}$*Mom1*$^{AKR/B6}$ females[7] and B6 males carrying the *Pla2g2a*^{AKR} transgene, *TgKH1*. These littermate classes provide internal controls for comparing tumour numbers and diameters. Progeny bearing the *Apc*^{Min} mutation were identified by DNA analysis. Mice were allowed to mature to 90 days, and were then killed. Tumours were counted along the entire length of the small intestine and colon.

DNA analysis. The copy number of the transgene was determined by comparison of the *Pla2g2a*^{AKR} allele carried on the transgene to the endogenous *Pla2g2a*^{B6} allele. These alleles differ by a *BamH*I site that is present in the AKR allele but absent from the B6 allele. The comparison was performed by two independent methods: Southern hybridization and PCR. In the first method, Southern blots containing 10 μg of spleen DNA digested with *Bam*HI were prepared from the line B6.*Mom1*$^{AKR/B6}$ (which has one copy of each allele) and from the transgenic line TgKH1 (which contains two copies of the B6 allele and an unknown number of copies of the AKR allele). The Southern blots were hybridized with a probe derived from a PCR product spanning most of the *Pla2g2a* genomic locus (from primer 5′–GAGAGAAACCATACCACCATCC–3′ in exon 1 to 5′–TGCTTTACTTGTGAGGGCCT–3′ in exon 5). The AKR allele yields two fragments of 2.5 and 6.5 kb, while the B6 allele yields a single fragment of 9.0 kb. A Fuji X-BAS 2000 Phosphor-Imager was used to compare the intensities of the two AKR bands with the B6 band in the two strains. In the second method, we amplified a 476-bp PCR product spanning the polymorphic *Bam*HI site. The resulting product was digested with *Bam*HI, electrophoresed in a 2% agarose gel and visualized with ethidium-bromide staining. We compared the transgenic line and the congenic strain with a control, mixing series of varying amounts of AKR and B6 DNA. Both methods indicated that the transgenic line carried 9 ± 1 copies of the AKR allele.

Cosmid sequencing and analysis. The nucleotide sequence of cosmid KH1 was determined by standard shotgun sequencing of M13 clones, with fluorescent dye-primer sequencing reactions electrophoresed on an ABI377 DNA sequencer. The shotgun sequencing provided approximately 15-fold coverage, permitting assembly into a single-sequence contig. The sequence was analysed by use of the BLAST computer program[24].

Genotyping. The genotype of the *Apc* locus was determined as previously described[4]. The genotype at the *Mom1* locus was assigned on the basis of genotypes at the closely linked flanking markers *D4Mit54* and *D4Mit13*, as previously described[6].

RNA analysis. Total RNA was isolated from frozen small-intestinal and colonic tissue with Trizol reagent (GIBCO BRL) as described[25]. For northern blots, total RNA (10 μg) was treated with glyoxal, electrophoresed through 1% agarose gels and transferred to nylon membranes; the blots were hybridized in a formamide-based hybridization solution with radioactively labelled probes for *Pla2g2a* and, as a control, *GAPDH*. Procedures have been described[25]. The intensity of the bands was measured with a Fuji X-BAS 2000 Phosphor-Imager. *Pla2g2a* mRNA levels were normalized to *GAPDH* mRNA levels.

Western-blot analysis. The intestine was removed, opened, washed in phosphate-buffered saline (PBS), divided into segments and minced with a razor blade. The tissue was suspended in 1 M acetic acid and incubated overnight at 4 °C. The sample was centrifuged at 4,000 r.p.m. in a swinging bucket rotor for 30 min. The supernatant was transferred to a clean tube and stored at −20 °C. Western-blot analysis was performed as described[26], except that 0.1 volume of 10 N NaOH was added to samples before electrophoresis and blots were probed with a 1:2,000 dilution of rabbit polyclonal antiserum against 'enhancing factor', which was an early name[27] for what was subsequently shown[28] to be phospholipase 2g2a. Immunodetection with goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) and ECL western blotting kit (Amersham) was performed according to the manufacturer's instructions. Signals were quantitated with a Molecular Dynamics densitometer and software. All signals were within the linear response range, as determined by a titration curve that was generated by loading increasing amounts of extract prepared from the distal half of the small intestine of transgenic animals.

Immunohistochemistry. The *Pla2g2a* antiserum at a 1:20,000 dilution and the lysozyme antiserum (DAKO) at a 1:1,000 dilution were used for standard peroxidase-based immunohistochemistry. Tissue was fixed in phospholipase for 1 h at room temperature, embedded in paraffin and cut into 5-mm sections. The sections were then dewaxed and rehydrated through a graded ethanol series. Antigen retrieval was performed by heating the slides in citrate buffer (pH 6) for 25 min on full power in a 650-W Kenmore microwave oven. Endogenous peroxidase activity was quenched by incubation of the slides in 0.03% hydrogen peroxide solution for 15 min at room temperature. Immunohistochemistry was performed with the ABC elite peroxidase system (Vector) according to the manufacturer's instructions. Sections were developed in diaminobenzidine substrate (Sigma) and counterstained with hematoxylin for 5 s.

Tumour scoring. All mice were killed by CO_2 asphyxiation at 90 days of age. The entire intestinal tract was removed, prepared and fixed as previously described[1]. Tumours (0.4 mm) were scored from post-fixed tissues with a Nikon SMZ-U dissecting microscope at ×10 magnification. All tumours were scored by a single observer (R.T.C.), and a subset of tumours was verified by a second observer (R.B.H.).

Statistics. *P* values for tumour numbers and sizes were determined by comparison of each modified class with $Apc^{Min/+}Mom1^{B6/B6}$ TgKH1(−) control mice by use of the non-parametric Wilcoxon Rank Sum test.

GenBank accession numbers. AC002108, whole cosmid sequence; U28244, *Pla2g2a*; U66873, *Pla2g5*; AA114569, EST next to *Pla2g2a*.

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