Human Cancer Xenografts in Outbred Nude Mice Can Be Confounded by Polymorphisms in a Modifier of Tumorigenesis

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ABSTRACT Tumorigenicity studies often employ outbred nude mice, in the absence of direct evidence that this mixed genetic background will negatively affect experimental outcome. Here we show that outbred nude mice carry two different alleles of *Pla2g2a*, a genetic modifier of intestinal tumorigenesis in mice. Here, we identify previous unreported linked polymorphisms in the promoter, noncoding and coding sequences of *Pla2g2a* and show that outbred nude mice from different commercial providers are heterogeneous for this polymorphic *Pla2g2a* allele. This heterogeneity even extends to mice obtained from a single commercial provider, which display mixed *Pla2g2a* genotypes. Notably, we demonstrated that the polymorphic *Pla2g2a* allele affects orthotopic xenograft establishment of human colon cancer cells in outbred nude mice. This finding establishes a non-cell-autonomous role for *Pla2g2a* in suppressing intestinal tumorigenesis. Using *in vitro* reporter assays and pharmacological inhibitors, we show promoter polymorphisms and nonsense-mediated RNA decay (NMD) as underlying mechanisms that lead to low *Pla2g2a* mRNA levels in tumor-sensitive mice. Together, this study provides mechanistic insight regarding *Pla2g2a* polymorphisms and demonstrates a non-cell-autonomous role for Pla2g2a in suppressing tumors. Moreover, our direct demonstration that mixed genetic backgrounds of outbred nude mice can significantly affect baseline tumorigenicity cautions against future use of outbred mice for tumor xenograft studies.

Athymic nude mice, since their first use for tumorigenicity studies in the late 1960s (Rygaard and Povlsen 1969), have served as a model in >40,000 publications. This model is used to assess the potential of tumor cells to proliferate, invade, and metastasize, as well as the efficacy of anticancer therapeutics (Singh and Ferrara 2012; Zhang *et al.* 2012). Nude mice are defective in T-cell-mediated immunity and, therefore, are less prone to reject human cancer cell xenografts (Rygaard and Povlsen 1969). Although originally maintained as an inbred BALB/c mouse strain, an outbred line of nude mice with increased fertility and vigor is now the mainstay for xenograft studies. It has been suggested that the genetic variation found in outbred mice better recapitulates the heterogeneity found in the natural population. While true, this same genetic variation also may confound experiments performed using small samples of outbred mice.

Genetic modifiers are powerful tools for elucidating protein and pathway interactions that influence a phenotype. With a C57Bl/6 (B6) genetic background, a mouse that harbors a germline mutation that truncates the tumor suppressor *Adenomatous polyposis coli* (Apc^{Min}) develops from 20 to >100 intestinal tumors (polyps) and will die at around 20 weeks of age (Moser *et al.* 1992; Zeineldin and Neufeld 2013a,b). When comparing polyp numbers in Apc^{Min} mice in different strains, an even greater variation (0.5–>300) is observed (Halberg *et al.* 2009). Some of this variation could be attributed to environmental factors. In addition, 18 genetic modifiers were found to contribute to these wide variations in intestinal tumor number (Kwong and Dove

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2009; Zeineldin and Neufeld 2013a,b). For some of these modifiers, specific sequence variants have been defined (Kwong *et al.* 2007; McCart *et al.* 2008; Oikarinen *et al.* 2009; Crist *et al.* 2011; Nnadi *et al.* 2012).

The first identified and best-characterized genetic modifier of Apc^{Min}, *Mom-1* was linked to a region of chromosome 4 containing the *Pla2g2a* gene, which encodes a secreted phospholipase A2 enzyme (Moser *et al.* 1992; Dietrich *et al.* 1993). Apc^{Min} mice develop fewer intestinal polyps if they are from strains that show high intestinal expression of *Pla2g2a* (tumor resistant) and more intestinal polyps in mouse strains that show no detectable *Pla2g2a* gene expression (tumor sensitive) (MacPhee *et al.* 1995). Overexpression of *Pla2g2a* with a transgene reduces the number of Apc^{Min}-induced polyps in the sensitive B6 reference mouse strain, indicating that *Pla2g2a* contributes to the Mom-1 phenotype (Cormier *et al.* 1997). Sequencing *Pla2g2a* cDNA from multiple mouse strains revealed an extra base in exon 3 in tumor-sensitive but not in tumor-resistant strains (Kennedy *et al.* 1995; MacPhee *et al.* 1995). This frameshift mutation results in a premature stop and was associated with an alternatively spliced RNA (Kennedy *et al.* 1995; MacPhee *et al.* 1995). Because this nonsense mutation in tumor-sensitive strains occurs before the last exon, it was predicted to lead to nonsense-mediated RNA decay (NMD) (MacPhee *et al.* 1995). NMD is a conserved eukaryotic mechanism by which to reduce the burden of mutant proteins by degrading mRNAs with premature stop codons (Palacios 2013). However, NMD of the *Pla2g2a* transcript has not been demonstrated experimentally. In addition, whether *Pla2g2a* reduces intestinal tumorigenesis in a cell autonomous or non-cell-autonomous manner is not completely understood. As nude mice are maintained as outbred colonies the question remained whether they vary at *Pla2g2a* locus and whether *Pla2g2a* genotypic variation can affect tumorigenesis in human xenograft studies using nude mice.

Here we identify new polymorphisms in the promoter, exons and introns of the *Pla2g2a* gene and confirm the previously identified exon 3 polymorphism in *Pla2g2a*. We show that these linked polymorphisms are present in tumor-resistant mouse strains ($Pla2g2a^R$) and associate with higher levels of intestinal *Pla2g2a* mRNA. Commercially available outbred nude mice are heterogeneous for the *Pla2g2a* polymorphisms, both when comparing mice from different providers and among mice from a single provider. Importantly, nude mice harboring the $Pla2g2a^R$ allele showed decreased establishment of human colon cancer cell xenografts. Finally, we also present evidence that both promoter polymorphisms and NMD participate in the mechanism underlying reduced *Pla2g2a* expression in tumor-sensitive strains.

Materials and Methods

Mice

The research using mice in this study was conducted in accordance with OLAW and AAALAC guidelines and was approved by the University of Kansas and the University of Kansas Medical Center Institutional Animal Care and Use Committees. We received two Apc^{1322T} male mice as a generous gift from Dr. Ian Tomlinson, Oxford University, United Kingdom. Apc^{Min} mice were purchased from Jackson laboratory (Bar Harbor, ME). Both Apc^{1322T} and Apc^{Min} mice were maintained in the University of Kansas Animal Care Unit by breeding males to wild-type C57Bl/6J females (Jackson laboratory). Mice were genotyped according to the published protocols (Pollard *et al.* 2009).

Screening for Pla2g2a alleles

The mouse strain that was used as the reference for the University of California, Santa Cruz (UCSC) genome sequence database came from the C57Bl/6 mouse strain (http://genome.ucsc.edu/cgi-bin/hgGateway). We refer to this reference *Pla2g2a* sequence as the "B6 allele." We refer to a *Pla2g2a* sequence that varies from that found in C57BL/6 mice as "polymorphic." Genomic DNA was purchased directly from Jackson laboratory, provided as gifts from researchers at The University of Kansas, or prepared from tail tissue or fecal pellets. One to two millimeters of the end of the mouse tail was incubated in 200 μl buffer [50 mM KCl, 50 mM Tris HCl (pH 8.0), 2.5 mM EDTA, 0.45% NP-40, and 0.45% Tween-20] containing 100 μg proteinase-K at 55° until tissue was completely digested. Proteinase-K was inactivated by heating at 95° for 5 min. For DNA isolation from fecal pellets (Figure 3B), each fecal pellet was collected in a 1.5-ml tube containing 1 ml phosphate buffered saline (PBS). The sample was vortexed for 2 min at the maximum speed followed by centrifugation at 700 × *g* for 5 min. Of the supernatant, 180 μl was taken to a new tube and 20 μl proteinase-K (20mg/ ml) was added. Proteinase-K digestion for 2 hr at 55° was followed by enzyme inactivation at 95° for 5 min. DNA was further purified using DNeasy blood and tissue kit (Qiagen) following manufacturer's instructions.

To distinguish the polymorphic from the B6 *Pla2g2a* allele, we designed a primer that is complementary to the polymorphic sequence and is different from the B6 sequence in its last two 3′ nucleotides (Figure 1B). This primer also has a mismatch mutation at the invariable nucleotide at position −4 from the 3′ end. Introducing this mismatch reduces primer annealing to the B6 *Pla2g2a* DNA at its 3′ region, preventing nonspecific amplification without interfering with annealing of the same primer to the polymorphic allele DNA. We used the same strategy to design a primer specific for the B6 allele that does not amplify the polymorphic allele (Table 1 shows primers sequences). PCR was performed in a 25-μl reaction containing 2 μl genomic DNA (5 μl for DNA isolated from fecal pellets), 2 mM $MgCl_2$, 0.4 mM dNTPs (NEB), 15 pmol of the common primer (Pla2ga2F), 3 pmol of the Pla2g2a^{S}R primer, 10 pmol of the Pla2g2a^{R}R primer (Table 1 shows primers sequences), and 1 unit of Crimson *Taq* DNA polymerase (NEB). The reaction conditions were 94° for 5 min and then 30 cycles of denaturation at 94° for 30 sec, annealing at 52° for 30 sec and extension at 68° for 30 sec followed

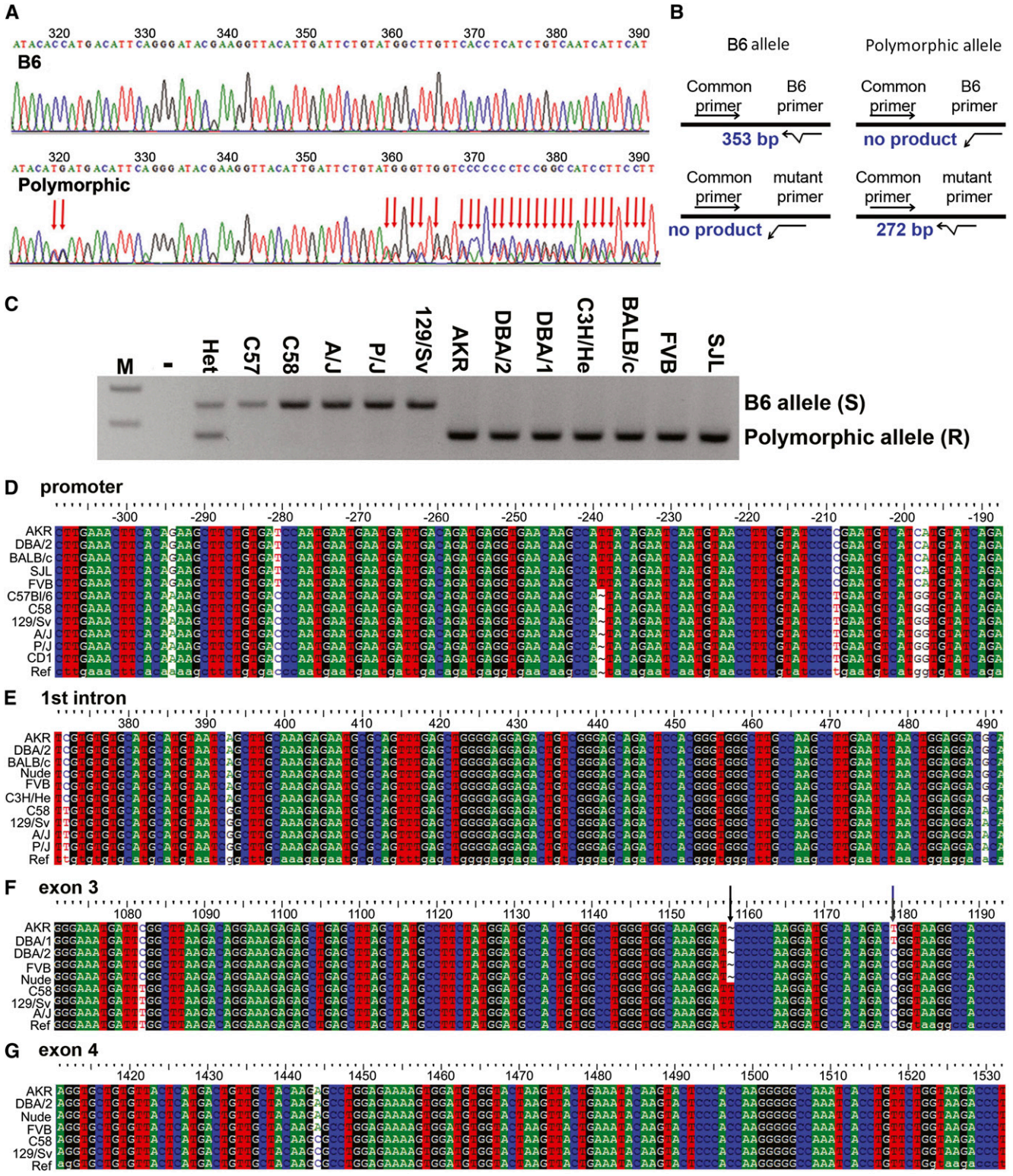

Figure 1 *Pla2g2a* polymorphisms identified in *Apc*^{Min} mice. (A) Chromatograph showing *Pla2g2a* promoter sequence for a short-lived (B6) and a long-lived (Polymorphic) *Apc*-mutant mouse. Polymorphisms designated by red arrows. (B) PCR-based assay for *Pla2g2a* polymorphisms identified in promoter sequence of long-lived (polymorphic) *Apc*-mutant mouse. (C) Screening different mouse strains for the *Pla2g2a* promoter polymorphisms using the PCR-based test. Strains AKR, DBA/2, DBA/1, C3H/He, BALB/c, FVB, and SJL carry the polymorphic promoter while strains C57, C58, A/J, P/J, and 129/Sv carry the B6 promoter. Het, heterozygous control sample from our mouse colony; −, PCR product without template; M, 100-bp DNA ladder. (D–G) Sequence results illustrate *Pla2g2a* polymorphisms found in different strains in the promoter area (D), first intron (E), exon 3 (F), and exon 4 (G). For exon 3 polymorphisms (E), sequence results illustrate the previously reported exon 3 nucleotide polymorphism marked by a black arrow; a newly identified R63W polymorphism in AKR and DBA/1 strains is marked by a blue arrow. Numbers represent nucleotide location, with +1 as the transcription start site.

by a final extension at 68° for 3 min. PCR products were separated by electrophoresis in 2% agarose gels and detected by ethidium bromide staining and UV visualization.

Sequencing the Pla2g2a gene and regions of Mom-2, Mom-5, and Mom-7

The expected life spans of Apc^{Min} and Apc^{1322T} mice are 20 and 16 weeks, respectively (Moser *et al.* 1990; Pollard *et al.* 2009). Initial analysis of long-lived mice (all C57Bl/6 strain) included one Apc^{Min} mouse that lived for 57 weeks and one Apc^{1322T} mouse that lived for 37 weeks. These mice were compared to an Apc^{Min} mouse that lived for 18 weeks and another Apc^{1322T} mouse that became sick and was killed at 18 weeks as short-lived controls. We also included two wild-type C57Bl/6J mice purchased directly from the commercial provider (Jackson Laboratories).

Genomic DNA was amplified with overlapping primers covering the entire *Pla2g2a* gene, including promoter, 5′-UTR, exons, introns, and 3′-UTR (Table 1). For analysis of *Mom2*, *Mom5*, and *Mom7*, sequence variation-specific primers spanning published sequences were used to amplify DNA by PCR (Table 1) (Baran *et al.* 2007; Kwong *et al.* 2007; Oikarinen *et al.* 2009). This examination did not lead to identification of a candidate modifier. Gel-purified PCR products were sent for Sanger sequencing (ACGT Inc. or Genewiz). Sequences were compared to the C57Bl/6 mouse reference sequences at the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). The complete *Pla2g2a* sequence from different strains has been uploaded in GenBank (accession nos. KF564039, KF564040, KF564041, KF564042, KF564043, KF564044, KF564045, KF564046, KF564047, KF564048, KF564049, and KF564050).

Analysis of mRNA by real-time reverse transcription– PCR

Intestinal epithelial cells were isolated from three mice with B6 *Pla2g2a* and three mice with polymorphic *Pla2g2a* alleles as described (Zeineldin *et al.* 2012). RNA isolation and cDNA formation from intestinal epithelial cells, HCT116 cells, or cecum of the nude mice was performed as previously described (Zeineldin *et al.* 2012). Briefly, 1 ml of Trizol (Invitrogen) was added to 200 μl of suspended intestinal epithelial cells. For cultured HCT116 cells, cell media was removed and cells were then scraped in 1 ml of Trizol (Invitrogen) and moved into 1.5-ml tubes. Samples were stored at −80° until use. RNA isolation and purification using Trizol (Invitrogen) was performed according to manufacturer's instructions. For nude mouse cecal tissue, the cecum was opened and washed in PBS and a small piece (~3 mm) was placed into a 1.5-ml tube, snap frozen in dry ice, and kept at −80° until use. Samples were homogenized in 1 ml Trizol (Invitrogen) using a hand-held homogenizer. RNA was isolated from 350 μl of the homogenized lysate using Direct-Zol RNA miniprep (Zymo reseach) following manufacturer's protocol. The quality of the isolated RNA was assessed using 1% agarose gel electrophoresis and quantity was determined by measuring the OD_{260}. For preparing cDNA, 1 μg total RNA was incubated for 1 hr at 42° in 1× M-MLuV enzyme buffer (NEB, Ipswich, MA) containing 1 mM dNTPs, 1 μg random hexamer primers (NEB), and 200 units of M-MLuV reverse transcriptase enzyme (NEB). The reverse transcriptase enzyme was then inactivated by heating at 95° for 5 min. Quantitative PCR was performed for mouse *Pla2g2a*, mouse *Hprt*, bacterial *Amp*, and Human *HPRT* using specific primers listed in Table 1 and using a DNA engine Opticon 2 instrument (MJ Research, Waltham, MA). The total reaction volume was 25 μl, containing 1× DyNAmo HS SYBR Green qPCR kit (Thermo), 15 pmol of each primer, and 2 μl of cDNA at a 1:5 dilution. Each reaction was performed in duplicate or triplicate. The reaction steps were initial denaturation at 95° for 15 min, followed by 40 cycles of denaturation at 94° for 20 sec, annealing at 54° for 30 sec, and extension at 72° for 30 sec. Fluorescence was measured at the end of every cycle and a melting curve was analyzed between 40° and 95° with 0.2° increments. Samples were included in the analysis only if the melting curve was a single peak at the expected temperature. For quantifying *Pla2g2a* mRNA in intestinal epithelial cells and ceca of nude mice, the average $\Delta C(t)$ was calculated for every sample relative to the housekeeping gene transcript *Hprt*. In calculating fold change of *Pla2g2a* mRNA from intestinal epithelial cells, $\Delta\Delta C(t)$ method was used relative to the C57Bl/6 mice with B6 *Pla2g2a* sequence. For quantification of *Pla2g2a* transcript in the ceca from nude mice, the relative abundance for every sample was normalized to the median value for resistant nude mice that did not establish tumors. In quantifying mouse *Pla2g2a* transcript in HCT116 cells, $\Delta C(t)$ was calculated relative to *Amp*-resistant sequence within the transfected plasmid. To correct for any variation in transfection efficiency between samples, *Amp* abundance was normalized to human *HPRT* expression and *GAPDH* DNA sequences. *P*-values were calculated using the two-tailed Mann–Whitney nonparametric test and GraphPad Prism software.

Cloning and plasmids

To test the effect of promoter polymorphisms on *Pla2g2a* expression, *Pla2g2a-Prom(AKR):pGL3* and *Pla2g2a-Prom (C58):pGL3* reporter plasmids were made by inserting *Pla2g2a* promoter (a 1.5-kb DNA piece upstream to *Pla2g2a* first codon) and 5′-UTR DNA from AKR or C58 mouse strains upstream of the Luciferase reporter gene in the pGL3 promoter vector using Pla2g2a Prom and Pla2g2a 5′-UTR primers (listed in Table 1). For testing *Pla2g2a* mRNA stability, all *Pla2g2a* coding exons and intervening introns from C58 and AKR mouse strains were used to replace the *Luciferase* gene in the pGL3 promoter vector using Pla2g2a whole primers (listed in Table 1). All the cloned plasmids were sequenced at ATCG and only those with no cloning-introduced mutations were included in the study.

Luciferase reporter assay

Using GeneExpresso transfection reagent following manufacturer's instructions, 2.25 μg of either *Pla2g2a-Prom*

Table 1 Primers used in this study

Primer name	Forward primer	Backward primer	Notes
Mom1-1	5′-gtaaggtggctccgtggtaa-3′	5′-catacccaatgccctttttg-3′	Promoter region
Mom1-5′-UTR-1	5′-cccagtcaggagaggtttca-3′	5′-ggactcattccccagaattg-3′	5′-UTR region
Mom1-5′-UTR-2	5′-atcggcttgcaaagagaatg-3′	5′-ctggaaaccactgggacact-3′	5′-UTR region
Mom1-2	5′-ggcagttggaattcaggaaa-3′	5′-ttgagcctgaaaggaaatgg-3′	
Mom1-3	5′-tcaccacccctttaccaggtc-3′	5′-acctcggctggctggaaac-3′	
Mom1-4	5′-gtaaggccaccccagttctc-3′	5′-atcttttggccacactctgc-3′	
Mom1-5	5′-cctaaaacagggcacacaca-3′	5′-agtggctgaggatgaccttg-3′	
Mom1-6	5′-gccctctgcagtgtatgaaa-3′	5′tccaagttatgagaacacacacg-3′	
Mom1-7	5′-aggccctcacaagtaaagca-3′	5′-cctggtttttgatggctctc-3′	3′-UTR region
Mom-2*	5′-accatctctccagcaccaag-3′	5′-ggcaaaatgagacttaaatgctt-3′	Flanks exon 3 of *Atp5a*
Mom-5*	5′-tgttggaacgtgtttttgga-3′	5′-aatcgcatagatacactgtctgag-3′	
Mom-7*	5′-aacccaggactgcttccttt-3′	5′-ttagaaggcaggagcagagg-3′	
Primers for screening for *Pla2g2a* promoter polymorphisms			
Pla2g2aF	5′-tgattttgaaacctctcctga-3′		Common primer
Pla2g2a^{S}R	5′-acttcacaaaagcttctgtaac -3′		Wild-type- specific primer
Pla2g2a^{R}R	5′-tcgtatccctgaatgtcttca		Polymorphism- specific primer
Primers for cloning *Pla2g2a* promoter and 5′-UTR regions			
Pla2g2a Prom	5′-ggccgcggtaccgtgttctgcctcagctccat- 3′	5′-gcggcgcctaggtttcctgaattccaactgccc tt-3′	
Pla2g2a 5′-UTR	5′-ggccgccctaggaacaagacaaggccttgaacaa-3	5′-gcggcgccatgggctgtcagctctgtgaaggaca-3′	
Primers for cloning Pla2g2a coding exons and intervening introns			
Pla2 W	5′-ggccgcccatggatgaaggtcctcctgctgctag-3′	5′-gcggcgtctagattgtctgatgaattgctttacttg-3′	
Primers for QRT-PCR for *Pla2g2a* mRNA			
Pla2g2a	5′-tacaagcgcctggagaaaag-3′	5′-ggccttatcgcactgacaca-3′	
Mouse *Hprt*	5′-tgctcgagatgtcatgaagg-3′	5′-tatgtcccccgttgactgat-3′	Mouse *Hprt*
Amp	5′-ggtcctccgatcgttgtcag-3′	5′-cggtcgccgcatacactatt-3′	Vector *Amp*
Human *HPRT*	5′-tgacactggcaaaacaatgca-3′	5′-ggtccttttcaccagcaagct-3′	
Human *GAPDH*	5′-ccatcactgccacccagaag-3′	5′-agcttcccgttcagctcagg-3′	DNA sequences

(AKR):pGL3 promoter or *Pla2g2a-Prom(C58):pGL3* promoter was cotransfected with 0.25 μg of Renilla luciferase (transfection control) into HCT116 cells. After 48 hr, cells were harvested and luciferase activity was assessed using a Dual Luciferase reporter assay system (Promega, Madison, WI) and a LMAXII 384 microplate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase data were normalized to Renilla luciferase. Fold change of the normalized firefly luciferase from the *Pla2g2a-Prom(AKR):pGL3* promoter was calculated relative to the normalized firefly luciferase from the *Pla2g2a-Prom(C58):pGL3* promoter. Data were collected from five independent experiments. *P*-values were calculated using the Mann–Whitney nonparametric test and GraphPad Prism software.

Testing Pla2g2a mRNA stability and NMD

To test *Pla2g2 amRNA* stability, HCT116 cells were transfected with 2.5 μg of *Pla2g2a(AKR):pGL3* or *Pla2g2a(C58):pGL3* plasmid using GeneExpresso transfection reagent following manufacturer's instructions. After 24 hr, RNA was isolated and mouse *Pla2g2a* transcripts were quantified by RT–PCR using mouse-specific *Pla2g2a* primers. Data were collected from four independent experiments including 11 samples transfected with *Pla2g2a(AKR):pGL3* and 10 samples transfected with *Pla2g2a(C58):pGL3* DNA. To test NMD, transfected cells were treated with DMSO vehicle or 20 μM wortmannin for 2 hr, 100 nM cyclohexamide for 6 hr, or both before quantifying mouse *Pla2g2a* mRNA levels. Data were collected from three independent experiments.

Nude mice xenograft experiment

Thirty-five 6- to 8-week-old female Nu/Nu mice (provider B) were orthotopically inoculated with HCT116 cells expressing m-cherry/Luciferase. Implantation of HCT116 cells and imaging of mice were performed by investigators blind to the mouse genotypes. Briefly, mice were anesthetized with pentobarbital (50 mg/kg) and the cecum was accessed via a small incision into the peritoneal wall. With the aid of a dissecting microscope, 1×10^6 cells were injected in a volume of 60 μl into the submucosal layer of the cecum. Following survival surgery and while animals were recovering, a tail snip was collected from each animal to allow for genotyping as described above. The mice were allowed to recover and then returned to housing. The study continued for 28 days from the date of cell implantation and animals were imaged once each week using bioluminescence imaging (described below) to quantify tumor burden.

Bioluminescence imaging

Animals were injected with potassium salt of D-luciferin (150 mg/kg body weight). Following isoflurane-induced anesthesia, animals were imaged at 15 min after D-luciferin injection using a Xenogen IVIS system coupled to Living Image acquisition and analysis software version 4.0 (Perkin Elmer, Waltham, MA). Region-of-interest (ROI) boxes were drawn around the entire body (excluding tail) of the animals or around the tissue specimen for *ex vivo* imaging. Measurements were expressed as total flux, *i.e.*, photons per second.

Results

Novel Pla2g2a polymorphisms identified

Genetic modifiers can alter tumorigenicity in mouse cancer models. One such modifier, *Pla2g2a*, has long been known to affect tumor formation in mice genetically predisposed to intestinal tumorigenesis, Apc^{Min}. Sequence analysis of the entire *Pla2g2a* gene including promoter, 5′-UTR, exons, and introns revealed previously unknown sequence differences in a heterozygous state in a long-lived Apc^{Min} mouse from our colony, but not in Apc^{Min} mice with more typical short life spans or in wild-type C57Bl/6J mice obtained directly from a commercial provider (Figure 1A). We refer to this sequence variation as "polymorphic" when compared to the C57BL/6 reference sequence (UCSC genomic sequence database), which is referred to as B6. Many of these polymorphisms were found in the promoter region of *Pla2g2a*; however, some were also found in other coding and noncoding regions of the gene. The long-lived Apc^{Min} mouse did not have alterations in other described genetic "modifiers of Min" (*Mom2*, *Mom5*, and *Mom7*) as determined by direct sequencing of PCR amplified genomic DNA (Baran *et al.* 2007; Kwong *et al.* 2007; Oikarinen *et al.* 2009).

To screen for mice with *Pla2g2a* polymorphisms, we developed a simple test that could accurately differentiate between the B6 allele and the polymorphic allele in a single PCR reaction. Using a common forward oligomer/primer that anneals to *Pla2g2a* promoter DNA in both the B6 and polymorphic alleles and two specific reverse primers that recognize either the B6 or polymorphic allele (Figure 1B), two PCR products (272 and 353 bp) are amplified.

Polymorphisms found throughout the Pla2g2a gene in different mouse strains

To unambiguously determine the sequences of the polymorphic loci, we amplified the promoter regions from each *Pla2g2a* allele obtained from a heterozygous mouse, introduced this DNA into a plasmid, and sequenced these plasmid DNAs. We found 26 bases that differed between the B6 and the polymorphic alleles in the *Pla2g2a* promoter, 5′-UTR region, and first intron. The sites of these polymorphisms relative to the transcriptional start site are; −882, −879, −874, −869, −865, −857, −851, −832, −804, −801, −692, −689, −529, −517, −479, −359, −348, −347, +48, +319, +361, +382, +479, +599, +648, and +711. As these *Pla2g2a* polymorphisms were not previously reported, we screened additional mouse strains using our PCR-based screening protocol and found that 7 of the 11 congenic strains also contained the polymorphisms (Figure 1C). Sequencing the *Pla2g2a* promoter region and 5′-UTR DNA from these strains confirmed the PCR results (Figure 1, D and E). Of note, we found that the polymorphic *Pla2g2a* allele was present in strains that were previously shown to be tumor resistant; AKR, DBA/1, DBA/2, BALB/c, and C3H/He (Dietrich *et al.* 1993; MacPhee *et al.* 1995; Halberg *et al.* 2009).

We tested whether mice with *Pla2g2a* promoter polymorphisms identified in our colony would also carry a previously identified *Pla2g2a* exon 3 alteration (absence of the T insertion found in the reference sequence from B6 mice) (Kennedy *et al.* 1995). Sequencing exon 3 in five mice that were heterozygous for *Pla2g2a* promoter polymorphisms as shown by PCR screening and promoter sequencing (Figure 1, C and D) revealed that all were heterozygous for the exon 3 T nucleotide alteration as well (not shown). In contrast, five mice that were homozygous for the B6 *Pla2g2a* promoter sequence were also homozygous for the corresponding B6 sequence alteration in exon 3 (a T nucleotide insertion). We conclude that the *Pla2g2a* allele detected in our colony and referred to as polymorphic compared to the reference B6, also has the T alteration in exon 3 that was previously reported. In addition to this alteration, we found many other "linked" polymorphisms in coding and noncoding sequences that associated with tumor-sensitive or tumor-resistant strains. These polymorphisms include a missense mutation 76 bp upstream from the original exon 3 insertion that is predicted to result in a tryptophan to arginine substitution in all tumor-sensitive strains (Figure 1F) and a similar missense mutation 21 bp downstream only in AKR and DBA/1 strains. Polymorphisms were also detected in other intron and coding exons. Figure 1G shows a polymorphic locus in exon 4. The complete *Pla2g2a* sequence from different strains has been uploaded in GenBank. These linked polymorphisms indicate extensive *Pla2g2a* sequence variation between sensitive and resistant mouse strains.

Pla2g2a polymorphisms in outbred nude mice

Unlike congenic inbred mouse strains that are homozygous for virtually all chromosomal loci, outbred mice maintain genetic variation in individual mice. Using our PCR-based test, we found two of the five outbred CD1 mice analyzed were heterozygous for the *Pla2g2a* polymorphic allele ($Pla2g2a^R$) while the other three mice were homozygous for the B6 allele ($Pla2g2a^S$), consistent with a previous report of the *Pla2g2a* exon 3 base insertion in CD1 mice (Figure 2A) (Kennedy *et al.* 1995). We confirmed these results by sequencing *Pla2g2a* from one mouse of each genotype and show the promoter sequence from a CD1 mouse in Figure 1D.

Because most nude mice are maintained as outbred colonies, genetic variation is preserved among these mice. Using our PCR protocol to screen genomic DNA isolated from 103 nude mice (Nu/Nu) from two different providers, we found all 57 mice from provider A were homozygous for the *Pla2g2a* allele carried by resistant strains ($Pla2g2a^R$, Figure 2B). In contrast, nude mice from provider B displayed various combinations of both alleles ($Pla2g2a^S$ and $Pla2g2a^R$). Out of 46 mice from provider B, 10 (21.7%) were $Pla2g2a^{S/S}$, 28 (60.9%) were $Pla2g2a^{S/R}$, and 8 (17.4%) were $Pla2g2a^{R/R}$ (Figure 2C). Therefore, the frequency of the $Pla2g2a^S$ and $Pla2g2a^R$ allele in these mice was 0.543 and 0.457, respectively. DNA sequencing of the *Pla2g2a* promoter region and exon 3 from four $Pla2g2a^{S/S}$, four $Pla2g2a^{R/R}$, and three $Pla2g2a^{R/S}$ nude

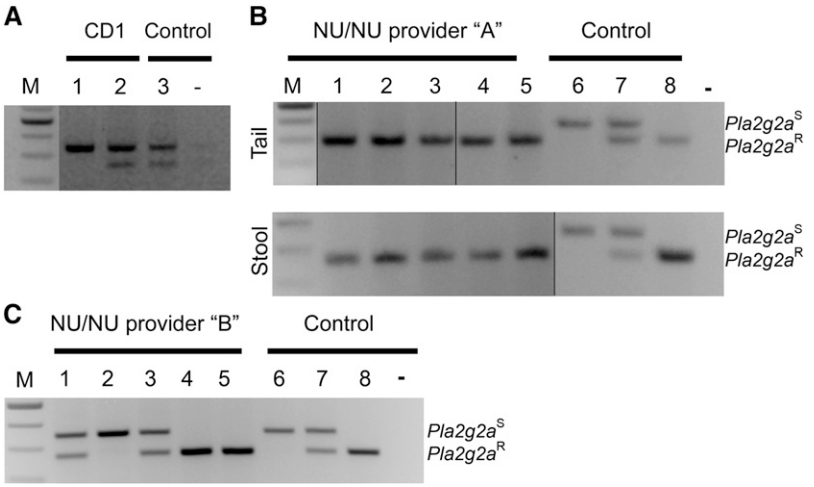

Figure 2 *Pla2g2a* alleles in outbred mouse colonies. (A) PCR screening results from two CD1 mice are shown next to a control sample from a *Pla2g2a*$^{R/S}$ mouse (3). Mouse 1 is homozygous for the *Pla2g2a*^{S} allele while mouse 2 is heterozygous (*Pla2g2a*$^{R/S}$). (B) Screening nude mice from provider A using DNA from tail (top) or stool (bottom) revealed all mice to be homozygous for *Pla2g2a*^{R}. (C) Screening nude mice from provider B revealed heterogeneity at the *Pla2g2a* locus. Mice 1 and 3 are *Pla2g2a*$^{R/S}$, mouse 2 is *Pla2g2a*$^{S/S}$, and mice 4 and 5 are *Pla2g2a*$^{R/R}$. As controls for B and C, mice 6, 7, and 8 are *Pla2g2a*$^{S/S}$, *Pla2g2a*$^{R/S}$, and *Pla2g2a*$^{R/R}$. −, PCR product without template. M, 100-bp DNA ladder.

mice confirmed the presence of the B6 (*Pla2g2a*^{S}) or polymorphic (*Pla2g2a*^{R}) alleles, including the previously reported exon 3 insertion of T nucleotide, as predicted by PCR screening results, again indicating that these polymorphisms are linked (data not shown).

Pla2g2a affects orthotopic tumor establishment of colon cancer cells in nude mice, functioning in a non-cell-autonomous manner

Whether *Pla2g2a* suppression of tumorigenesis is cell autonomous or non-cell autonomous is not settled. We reasoned that, as nude mice were heterogeneous for the polymorphic *Pla2g2a* allele, orthotopic injection of colon cancer cells in the cecum would allow us to directly test a cell-autonomous *vs.* a non-cell-autonomous role for *Pla2g2a* in intestinal tumor suppression. In addition, this system would also allow us to test our hypothesis that *Pla2g2a* heterogeneity can affect the outcome of xenograft studies where human cancer cells are injected into nude mice and then tumor development and growth is monitored. To test these hypotheses, we purchased 35 female nude mice from provider B and injected luciferase-labeled HCT116 human colon cancer cells into the cecal wall of these mice. Xenograft establishment, growth, and local and distant metastasis were monitored (Figure 3A). Because the *Pla2g2a*^{R} allele appears to be dominant (Dietrich *et al.* 1993; Cormier *et al.* 1997), we combined 5 *Pla2g2a*$^{R/R}$ and 22 *Pla2g2a*$^{S/R}$ mice into a single resistant group (R) with the remaining 8 *Pla2g2a*$^{S/S}$ mice forming the sensitive group (S). HCT116 cells established cecal tumors in 75% of the sensitive group mice but only 34.6% of the resistant group mice ($\chi^2 = 4.047$, two-tailed $p = 0.0443$, Figure 3C). Over the 4-week duration of the experiment, four of the six tumors (67%) that developed in the sensitive mice grew bigger while only 44.4% of the tumors established in resistant mice expanded (Table 2). At the end of the experiment, two tumors (33%) from mice in the sensitive group demonstrated invasion, one local, and one more distant metastasis to the liver (Figure 3B and Table 2). In the resistant group, only one tumor had local metastasis (11.1%). These results demonstrate a non-cell-autonomous role for Pla2g2a in suppression of colon tumors. Of potentially broader impact, this analysis also provides a concrete example of how a genetic modifier can significantly alter the outcome of a xenograft study performed using outbred nude mice.

Mice with Pla2g2a polymorphisms have more Pla2g2a mRNA in intestinal cells

The *Pla2g2a* allele in C57Bl/6 mice (*Pla2g2a*^{S}) has been described as virtually null (MacPhee *et al.* 1995). However, the reduction in *Pla2g2a* expression was attributed to the exon 3 T insertion. Since we found previously unreported polymorphisms in *Pla2g2a*, with many of them in the promoter region, we were interested to measure the effects of these polymorphisms on *Pla2g2a* expression. We first compared *Pla2g2a* expression in intestinal epithelial cells from our C57BL6 mice with known *Pla2g2a* genotypes. We found that mice with a polymorphic allele (*Pla2g2a*^{R}) had higher *Pla2g2a* mRNA levels in epithelial cells from jejunum, ileum, and colon (177-, 31- and 77-fold by qRT–PCR, respectively, data not shown) than mice with only the B6 allele (*Pla2g2a*^{S}). Figure 3E shows RT–PCR products from jejunal RNA isolated from mice with different *Pla2g2a* alleles amplified with *Pla2g2a*- or *Hprt*-specific primers. Therefore, the *Pla2g2a* polymorphisms we initially identified in long-lived mice associate with increased expression of the gene.

Reduced *Pla2g2a* mRNA level also correlates with tumor sensitivity in *Apc*^{Min} mice (MacPhee *et al.* 1995). To examine the link between establishment of orthotopic xenograft tumors and *Pla2g2a* expression, we performed quantitative RT–PCR using mouse-specific *Pla2g2a* primers and RNA isolated from ceca of nude mice. We observed wide variation in *Pla2g2a* mRNA levels in the cecum from nude mice, especially among the resistant group. This variation may represent heterogeneous genetic elements controlling *Pla2g2a* expression in these mice with mixed genetic background. Nonetheless, mice carrying the *Pla2g2a*^{R} allele showed higher levels of *Pla2g2a* mRNA than the sensitive group

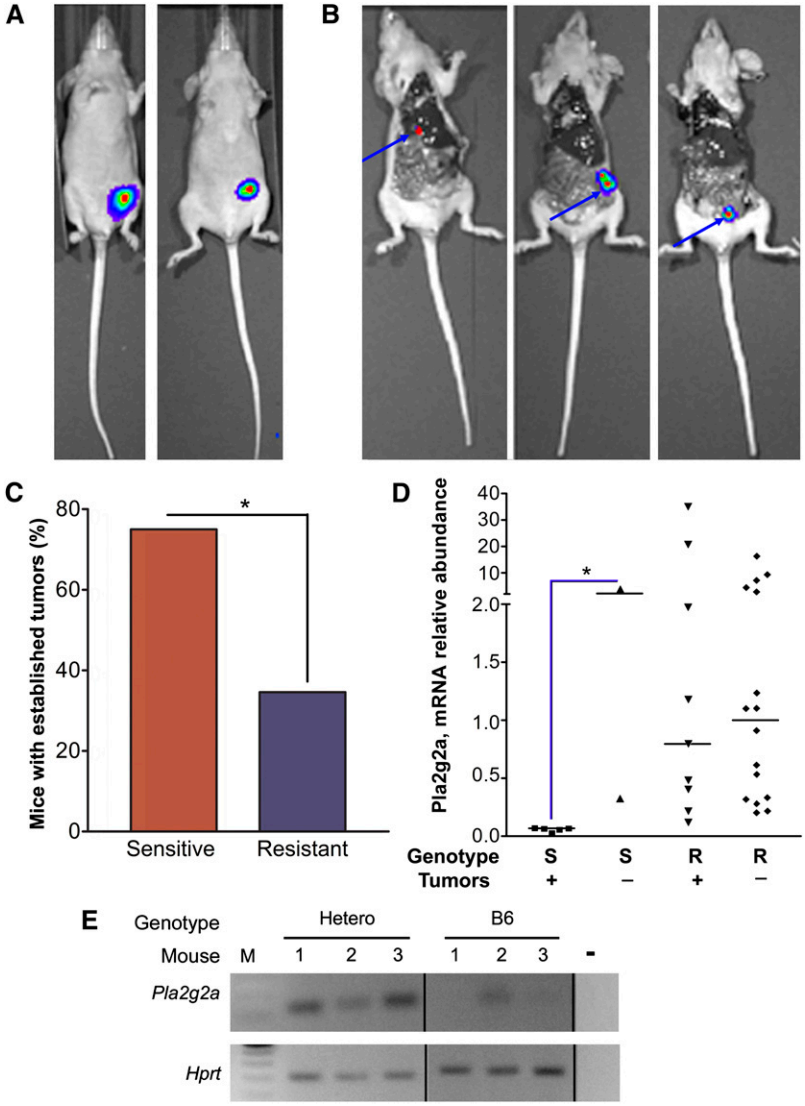

Figure 3 The *Pla2g2a*^{R} allele decreases colon cancer cell establishment in the cecum of nude mice. (A) *In vivo* image of luciferase-labeled HCT116 grafted in cecal wall of a sensitive (left) or resistant (right) nude mouse. (B) *Ex vivo* image of HCT116 luciferase-labeled cells showing hepatic metastasis (left), and local invasion (middle), both in sensitive nude mice, and local invasion (right) in a resistant nude mouse. (C) Percentage of sensitive and resistant nude mice in which HCT116 cells established tumors in the cecal wall. (*) $P < 0.05$ (χ^2 test). (D) Quantitative RT–PCR revealed relative expression of *Pla2g2a* RNA in the cecum of sensitive and resistant nude mice that established or did not establish tumors in colon cancer cell orthotopic xenografts. Line represents median. (E) RT–PCR of *Pla2g2a* mRNA isolated from jejunal intestinal epithelial cells of B6–*Pla2g2a*$^{S/S}$ mice or mice heterozygous for the polymorphic allele (hetero–*Pla2g2a*$^{R/S}$). The housekeeping *Hprt* was used as an internal control.

mice (Figure 3D, $P = 0.003$). Within the sensitive group, the mice that did not establish tumors had significantly more *Pla2g2a* mRNA than those that established tumors (Figure 3D). The same trend of higher *Pla2g2a* mRNA level in mice that did not establish tumors was also seen in the resistant group, although this did not reach statistical significance.

Pla2g2a polymorphisms associate with prolonged survival of Apc^{1322T} mice

It was critical to identify all mice in our colony that had *Pla2g2a* polymorphisms so that they could be excluded from future studies. Using the PCR-based test to screen our mice, we found the *Pla2g2a* polymorphic allele in only two *Apc^{Min}* mice, but in many mice with another germline *Apc* mutation, *Apc^{1322T}* (Figure 4A). As most *Apc^{1322T}* mice with the polymorphic allele were killed at different time points and many of these mice underwent different experimental conditions, compiling their intestinal tumor data were not informative. However, we found that five *Apc^{1322T}* mice with the B6 *Pla2g2a* allele and seven *Apc^{1322T}* mice heterozygous for the polymorphic allele died or were killed when they were moribund, thus providing useful survival data. While *Apc^{1322T}* with the B6 *Pla2g2a* gene lived for 16.4 ± 1.9 weeks, which is comparable to the reported survival of *Apc^{1322T}* mice (Pollard *et al.* 2009; Lewis *et al.* 2010), *Apc^{1322T}* mice heterozygous for the polymorphic allele lived significantly longer, until 26.3 ± 2.3 weeks ($P = 0.0075$) (Figure 4B). Together, these data indicate that the *Pla2g2a* polymorphic allele associates with prolonged survival in *Apc^{1322T}* mice.

To trace the source of the *Pla2g2a* polymorphic allele in our *Apc^{Min}* and *Apc^{1322T}* colonies, we screened the available DNA from many generations of mice. We conclude that the polymorphic *Pla2g2a* allele *was* introduced into our *Apc^{Min}* and *Apc^{1322T}* mice by a breeding female carrying the polymorphic allele obtained from another provider.

Promoter polymorphisms affect Pla2g2a expression

To understand the mechanisms underlying the low expression of the B6 *Pla2g2a* allele, we initially examined the

Table 2 Orthotopic tumor establishment of human colon cancer cells in nude mice

	Pla2g2a = sensitive	Pla2g2a = resistant
Number	8	27
Mice with established tumors	6 (75%)	9 (34.6%)
Tumors growing	4 (67%)	4 (44.4%)
Liver metastasis	1 (16.7%)	0
Local invasion	1 (16.7%)	1 (11.1%)

newly identified *Pla2g2a* promoter polymorphisms for their contribution to gene expression. Using several programs to predict differences in transcription factor binding between the B6 and polymorphic *Pla2g2a* alleles, we did not identify a consistent set of transcription factors (data not shown). Nonetheless, to look for global effects of the promoter polymorphisms, we generated firefly luciferase reporter constructs driven by the *Pla2g2a* promoter and 5′-UTR regions, including the first intron, from the AKR strain ($Pla2g2a^R$) or C58 strain ($Pla2g2a^S$ allele identical to that of C57Bl/6 mice) (Figure 5A). We found significantly more firefly luciferase activity in cells transfected with the AKR-derived *Pla2g2a* promoter and 5′-UTR reporter than in cells transfected with the C58-derived *Pla2g2a* promoter and 5′-UTR (Figure 5A). We conclude that the *Pla2g2a* promoter polymorphisms affect *Pla2g2a* expression, resulting in *Pla2g2a* RNA levels that are higher in tumor-resistant strains than in tumor-sensitive strains.

NMD contributes to instability of Pla2g2a transcript in tumor-resistant strains

It was previously proposed that low levels of *Pla2g2a* mRNA in tumor-sensitive mice were the result of NMD (MacPhee *et al.* 1995). This assumption, based on the known polymorphism in *Pla2g2a* positioned in exon 3 and thus before the last exon, has not been directly tested. To examine the potential involvement of NMD in controlling *Pla2g2a* levels, a second set of constructs was generated by cloning the *Pla2g2a* coding exons and the intervening introns from tumor-sensitive or tumor-resistant (AKR) strains after the SV40 promoter. We reasoned that as the *Pla2g2a* gene from each strain was controlled by the same strong promoter, any changes in transcript level would likely result from inherent RNA stability differences rather than transcription rates. Using qRT–PCR and primers specific for mouse *Pla2g2a* mRNA, we observed that *Pla2g2a* mRNA levels are significantly lower in human cells transfected with the construct containing the C58-derived *Pla2g2a* sequence than in those transfected with the AKR-derived *Pla2g2a* sequence (Figure 5B).

To further confirm a role for NMD in reduction of *Pla2g2a* mRNA levels in tumor-sensitive strains, we treated cultured cells with pharmacologic NMD inhibitors. Wortmannin is a PI3K inhibitor that can also inhibit the PI3K-related protein hSMG1, a component of the NMD surveillance machinery (Pal *et al.* 2001). Cyclohexamide inhibits eukaryotic

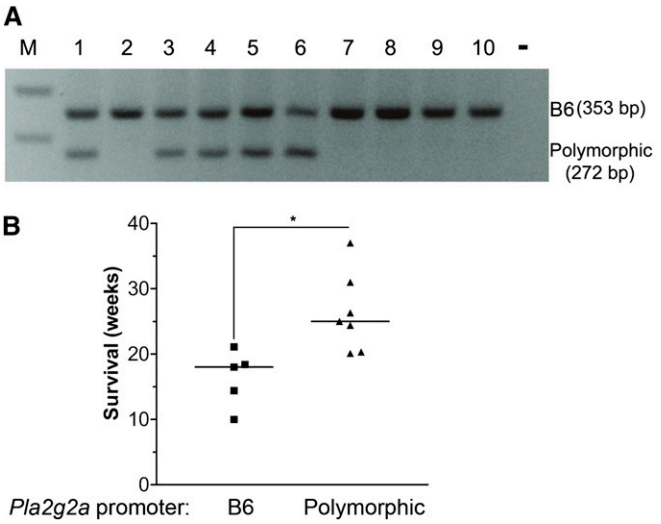

Figure 4 *Pla2g2a* polymorphisms associate with prolonged survival of Apc^{1322T} mice. (A) Analysis of genomic DNA from 10 Apc^{1322T} mice. Samples 1, 3, 4, 5, and 6 are from mice heterozygous for the *Pla2g2a* polymorphic allele, samples 2, 7, 8, 9, and 10 are from B6 mice. —, PCR product without template; M, 100-bp DNA ladder. (B) the survival of Apc^{1322T} mice with B6 *Pla2g2a* status (squares) or heterozygous for the polymorphic *Pla2g2a* allele (triangles). Lines indicate median survival for each group. (*) $P < 0.05$ (two-tailed *t*-test).

protein synthesis and thereby also affects NMD, which requires protein synthesis for completion (Palacios 2013). When human cells transfected with the SV40-driven C58-derived *Pla2g2a* gene construct were treated with wortmannin alone or in combination with cyclohexamide, the mouse *Pla2g2a* mRNA level significantly increased relative to vehicle-treated cells (Figure 5C). In contrast, a parallel control experiment performed using the SV40-driven AKR-derived *Pla2g2a* gene construct revealed no change in mouse *Pla2g2a* transcript level in the cells treated with wortmannin, cyclohexamide, or both drugs (Figure 5C). We conclude that *Pla2g2a* promoter polymorphisms and NMD each contribute to *Pla2g2a* mRNA levels in tumor-sensitive strains.

Discussion

Genetic and environmental factors modify tumorigenicity in humans and in model organisms such as mice. Identification and characterization of genetic modifiers leads to greater appreciation for the different pathways contributing to a given phenotype (Kwong and Dove 2009). In this study, we showed that there are two distinct *Pla2g2a* alleles in different mouse strains: one allele is similar to that described in the reference sequence (B6 allele, $Pla2g2a^S$) and the other is polymorphic ($Pla2g2a^R$). The $Pla2g2a^R$ allele is different from the B6 ($Pla2g2a^S$) allele in the promoter, coding, and noncoding sequences. We first identified this *Pla2g2a* polymorphic allele in long-lived Apc^{Min} mice in our colony. Most of these polymorphisms are conserved in tumor-resistant mouse strains. We showed that *Pla2g2a*

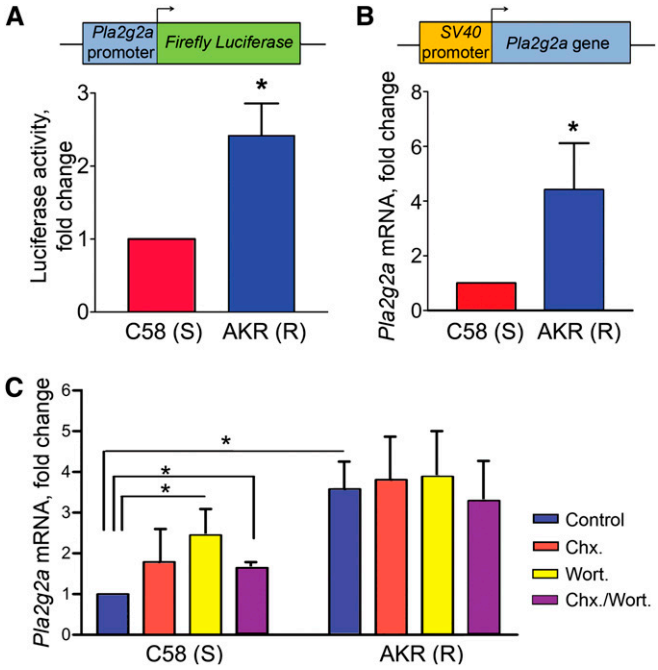

Figure 5 Promoter polymorphisms and NMD alter *Pla2g2a* RNA level (A) A luciferase reporter was expressed in HCT116 cells under the control of *Pla2g2a* promoter DNA from the tumor-sensitive (S) C58 or the tumor-resistant (R) AKR strain. The *Pla2g2a* promoter from the tumor-resistant strain resulted in more luciferase than that from the sensitive strain. (*) $P < 0.05$ (Mann–Whitney nonparametric test). (B) qRT–PCR analysis of mouse *Pla2g2a* mRNA levels from the tumor-sensitive strain (C58) and the tumor-resistant strain (AKR) *Pla2g2a* genes each transcribed from the SV40 promoter in HCT116 cells. (*) $P < 0.05$ (Mann–Whitney nonparametric test). (C) NMD was inhibited using 20 μM wortmannin (Wort.) and/or 100 nM cyclohexamide (Chx.). RNA levels were quantified by qRT–PCR. Note that NMD inhibitors significantly increased C58 *Pla2g2a* mRNA level. (*) $P < 0.05$ (Mann–Whitney nonparametric test).

polymorphisms in outbred nude mice affect the orthotopic establishment of human cancer cells. In addition, we provide evidence that promoter polymorphisms affect *Pla2g2a* transcription and that *Pla2g2a* transcripts from tumor-sensitive mice undergo NMD.

Outbred mice have genetic variation that is lacking in inbred congenic mouse lines. But this same feature that provides more natural population characteristics may also confound experimental results, particularly in studies using only a small sample size (Chia *et al.* 2005). One of the most unexpected results from the current study was the finding that commercially available outbred nude mice are heterogeneous for *Pla2g2a* alleles and this heterogeneity leads to variation in the capacity of each outbred mouse to support growth of a human cancer cell xenograft (Figure 2 and Figure 3). Nude mice from two different commercial providers were not consistent in *Pla2g2a* genotype. While all nude mice from one provider were homozygous for the $Pla2g2a^R$ allele, both the $Pla2g2a^R$ and $Pla2g2a^S$ alleles were present in the mice from a second provider. These findings highlight the potential danger of comparing the results from studies performed using nude mice from different providers and even results from a heterogeneous mix of outbred nude mice from a single provider. For instance, nude mice in our study were shipped in seven cages with five mice per cage. As expected, there was variable distribution of *Pla2g2a* alleles between cages, with the frequency of $Pla2g2a^{S/S}$ mice in each cage ranging from 0/5 (0%) to 3/5 (60%). Results from this study unambiguously demonstrate the necessity of a large sample size in studies using outbred nude mice.

The heterogeneity of outbred nude mice is further illustrated by the varied *Pla2g2a* transcript levels, even in nude mice with the same *Pla2g2a* genotype (Figure 3D). This variation presumably results from additional differences in genetic elements that regulate gene expression. It seems likely, but remains to be tested, whether the related *Pla2g2a* transcript level will affect orthotopic xenografts of other colon cancer cells and other tumorigenesis studies with different cancer cells and injection sites. Nonetheless, the inability to completely monitor or control for varied expression of tumorigenesis modifiers such as *Pla2g2a* makes tumorigenicity studies using outbred mice problematic.

This is the first report of a genetic modifier of Apc^{Min} that also affects the phenotype of Apc^{1322T} mice. The Apc^{1322T} model was developed to more closely recapitulate the longer truncated APC proteins (approximately half full length) found in human colorectal cancers (Nieuwenhuis and Vasen 2007; Pollard *et al.* 2009). In this study we showed that $Pla2g2a^R$ prolongs the survival of Apc^{1322T} mice. Mom-1 was originally described as a quantitative trait locus that affects polyp multiplicity, size, and distribution in Apc^{Min} mice. Although survival is a good indicator for polyp multiplicity, we looked retrograde at collected polyp data from our Apc^{1322T} mice. Unfortunately, most of these mice carrying the $Pla2g2a^R$ allele were killed at different time points and were under different experimental conditions, making this analysis uninformative. A future study of Apc^{1322T} mice with different *Pla2g2a* genotypes and killed at the same age would be necessary to detect any effect of Pla2g2a on polyp multiplicity, size, and distribution.

Another Apc model, the $Apc^{\Delta 242}$ mouse, with a much shorter truncated Apc protein, showed fewer polyps in the first generation of C3H/He × C57Bl/6 mice (Crist *et al.* 2010). Notably, the C3H/He mouse strain has the tumor-resistant *Pla2g2a* allele (Figure 1C) (Markova *et al.* 2005). Taken together, our results in Apc^{1322T} mice and reported data in Apc^{Min} and $Apc^{\Delta 242}$ mice (Dietrich *et al.* 1993; Crist *et al.* 2010) indicate that *Pla2g2a* polymorphisms can affect intestinal tumorigenesis in *Apc*-mutant mice, independent of the nature of *Apc* mutation.

Although *Pla2g2a* is responsible for most of the Mom-1 phenotype in Apc^{Min} mice, the *Pla2g2a* gene is only one part of the *Mom-1* locus. Another locus distal to *Pla2g2a*, but still within the *Mom-1* locus, has a modest effect on polyp multiplicity in small intestines of Apc^{Min} mice (Cormier *et al.* 2000). In this study, we examined only *Pla2g2a* polymorphisms in Apc^{1322T} and nude mice and not the distal *Mom-1*

locus. Perhaps the distal *Mom-1* locus accounts for some of the prolonged survival phenotype in Apc^{1322T} mice. The distal *Mom-1* locus might also contribute to the variation in xenograft establishment in nude mice ceca. However, the previous finding that *Pla2g2a* and not the distal *Mom-1* locus is responsible for all the resistance phenotype in the colon (Cormier *et al.* 2000) argues against this possibility.

Pla2g2a does not appear to function as a universal tumor suppressor or oncogene. *PLA2G2A mRNA* is upregulated in lung and prostate cancer cells (Oleksowicz *et al.* 2012; Yu *et al.* 2012a). Furthermore, reducing expression of *PLA2G2A* by hairpin RNA reduces the proliferation of lung cancer cells, consistent with an oncogenic role for PLA2G2A (Yu *et al.* 2012b). On the other hand, low *PLA2G2A* expression in gastric tumors is associated with increased invasiveness and metastasis, with poor prognostic outcome (Ganesan *et al.* 2008). Additionally, mouse strains with little or no expression of *Pla2g2a* have increased susceptibility to intestinal tumorigenicity driven by germline *Apc* mutations (Dietrich *et al.* 1993; Crist *et al.* 2010), *Muc2* mutations (Fijneman *et al.* 2008), or inflammation (Fijneman *et al.* 2009). Here we show that orthotopic establishment of colon cancer cells is also reduced with higher *Pla2g2a* expression in nude mice. These data clearly establish a protective effect for Pla2g2a against intestinal tumorigenesis in mice.

The mechanism by which the phospholipase 2A enzyme Pla2g2a decreases intestinal tumor burden in resistant strains is not completely understood. *Pla2g2a* is expressed in Paneth cells in the small intestine and goblet cells in the large intestine. Secreted Pla2g2a may function in a non-cell-autonomous manner by controlling bacterial flora and, in so doing, reduce inflammatory mediators that would otherwise promote tumor growth (Fijneman and Cormier 2008; Fijneman *et al.* 2008). Pla2g2a can also modulate signaling pathways, including Wnt signaling, potentially through regulating production of fatty acids such as arachidonic acid, a precursor for a variety of prostaglandin signaling mediators (Fijneman *et al.* 2009). Our orthotopic xenograft study clearly demonstrates a non-cell-autonomous role for Pla2g2a. HCT116 human colon cancer cells express little endogenous *Pla2g2a* (Belinsky *et al.* 2007), yet we found that their growth in the cecal wall of nude mice was affected in a manner that correlated with the level of cecal *Pla2g2a* transcript (Figure 3D). The number of polyps that develop when fetal small intestinal tissues from Apc^{Min} mice were grafted subcutaneously was previously shown to correlate with the *Mom-1* allele status (sensitive or resistant) of the isograft's donor rather than of the host mouse (Gould and Dove 1996). This result does not necessarily conflict with our cecal xenograft data, as *Mom-1* could still affect intestinal tumorigencity in a non-cell-autonomous manner within isograft's intestinal tissue microenvironment in addition to a systemic effect in the host mouse.

It was previously reported that overexpression of mouse *Pla2g2a* in HCT116 cells increased xenograft tumor size when these colon cancer cells were implanted subcutaneously in nude mice (Belinsky *et al.* 2007). This seemingly contradictory result might be explained by differences in the cell expressing *Pla2g2a* (implanted human cancer cells *vs.* surrounding mouse tissues) or the site of the xenograft implantation (subcutaneous *vs.* cecal wall). Moreover, in the previous study, the *Pla2g2a* genotype of the nude mice was not reported and this later variable would be expected to affect the experimental outcome.

In conclusion, we showed that the *Pla2g2a* sequence varies between tumor-resistant and tumor-sensitive mouse strains. Some of these variations in the promoter region affect *Pla2g2a* expression and we provide direct evidence that NMD also contributes to reducing *Pla2g2a* mRNA level in tumor-sensitive strains. The *Pla2g2a* genotype affects not only the Apc^{Min} phenotype, but also that of Apc^{1322T} mice. Remarkably, the *Pla2g2a* genotype varies in outbred nude mice from different providers and this variation affects orthotopic establishment of colon cancer cell xenografts in these mice.

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