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Effect of maternal and post-weaning folate supply on gene-specific DNA methylation in the small intestine of weaning and adult *Apc*^{+/Min} and wild type mice

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Increasing evidence supports the developmental origins of adult health and disease hypothesis which argues for a causal relationship between adverse early life nutrition and increased disease risk in adulthood. Modulation of epigenetic marks, e.g., DNA methylation and consequential altered gene expression, has been proposed as a mechanism mediating these effects. Via its role as a methyl donor, dietary folate supply may influence DNA methylation. As aberrant methylation is an early event in colorectal cancer (CRC) pathogenesis, we hypothesized low maternal and/or post-weaning folate intake may influence methylation of genes involved in CRC development. We investigated the effects of maternal folate depletion during pregnancy and lactation on selected gene methylation in the small intestine of wild type (WT) and *Apc*^{+/Min} mice at weaning and as adults. We also investigated the effects of folate depletion post-weaning on gene methylation in adult mice. Female C57Bl6/J mice were fed low or normal folate diets from mating with *Apc*^{+/Min} males to the end of lactation. A sub-set of offspring were killed at weaning. Remaining offspring were weaned on to low or normal folate diets, resulting in four treatment groups of *Apc*^{+/Min} and WT mice. *p53* was more methylated in weaning and adult WT compared with *Apc*^{+/Min} mice ($p > 0.001$). *Igf2* and *Apc* were hypermethylated in adult *Apc*^{+/Min} compared with WT mice ($p = 0.004$ and 0.012 respectively). Low maternal folate reduced *p53* methylation in adults ($p = 0.04$). Low post-weaning folate increased *Apc* methylation in *Apc*^{+/Min} mice only ($p = 0.008$ for interaction). These observations demonstrate that folate depletion in early life can alter epigenetic marks in a gene-specific manner. Also, the differential effects of altered folate supply on DNA methylation in WT and *Apc*^{+/Min} mice suggest that genotype may modulate epigenetic responses to environmental cues and may have implications for the development of personalized nutrition.

Keywords: folate, gene-specific DNA methylation, *in utero*, *Apc*^{+/Min} mouse, CRC

INTRODUCTION

The developmental origins of health and disease (DOHaD) hypothesis proposes that exposures during early life modulate disease risk in adulthood. Indeed, there is substantial evidence for an association between lower birth weight and increased risk of type 2 diabetes, coronary heart disease, and hypertension, which has been attributed to poor nutrition *in utero* (Barker, 2004). These observations indicate the potential for a degree of plasticity during development which allows the fetal phenotype to be altered in response to environment cues (Bateson et al., 2004) in ways that may prepare it for the anticipated post-natal environment (Gluckman et al., 2005). To persist into adulthood and to affect disease risk, these environmentally orchestrated programming events must “mark” the animal at a molecular, cellular, and/or tissue level in ways that are sustained for much of the life-course and which impact on the processes which lead to the development of disease.

Epigenetic marks, including DNA methylation and covalent histone modifications, are established during embryonic and fetal development and constitute a rich information source layered on top of the DNA sequence. These marks contribute to the regulation of transcription, allowing cell specific gene expression which is essential for cell differentiation (Bird, 2002). DNA methylation, the most commonly investigated epigenetic mark, can be altered in offspring in response to maternal nutrition and these changes are associated with changes in gene expression and in the phenotype of the progeny (Waterland and Jirtle, 2003; Lillycrop et al., 2005; Dolinoy et al., 2006).

Since folate is a key dietary source of methyl groups for synthesis of S-adenosyl methionine (SAM) – the universal methyl donor – it is an attractive candidate nutrient for the modulation of DNA methylation. Furthermore, many studies highlight the effects of maternal folate status on DNA methylation in offspring

(Lillycrop et al., 2005; Sinclair et al., 2007; Kim et al., 2009; Steegers-Theunissen et al., 2009; McKay et al., 2011).

As aberrant DNA methylation is known to be an early event in the pathogenesis of colorectal cancer (CRC; Feinberg et al., 2006), we hypothesized that maternal folate supply may influence CRC development in adult offspring through manipulation of the epigenome *in utero*. Indeed, we have reported global DNA hypomethylation in the small intestine (SI) of adult mice born to folate-depleted mothers compared with those born to folate replete mothers (McKay et al., 2011). Furthermore, we established that maternal folate depletion altered DNA methylation in a gene-specific manner in the fetal gut (McKay et al., unpublished data), suggesting that some epigenetic marks in developing offspring are modifiable in response to altered maternal folate intake. Understanding these early epigenetic events may help to define appropriate standards for maternal nutrition during pregnancy to optimize adult gut health in the next generation.

Apc^{+/*Min*} mice, which carry a STOP mutation at codon 850 in the *Apc* gene and develop multiple intestinal neoplasms spontaneously, have been used widely as a model of CRC (Su et al., 1992). Following depleted or normal maternal folate supply from mating until the end of lactation, we investigated the methylation status of candidate genes in *Apc*^{+/*Min*} and in wild type (WT) littermates to explore the potential effects of a nutritional insult (reduced folate supply) during early life on DNA methylation in the context of risk of CRC. For this purpose, we selected candidate genes implicated in the development of CRC and which have exhibited methylation changes in CRC (*Igf2*, *p53*, *p16*, and *Apc*; Burri et al., 2001; Feinberg et al., 2006; Segditsas et al., 2008; Baba et al., 2010). Furthermore, to investigate the potentially cumulative effects of both maternal and post-weaning folate depletion (a double nutritional insult), we also examined DNA methylation in adult *Apc*^{+/*Min*} and WT mice fed the low folate diet from weaning.

MATERIALS AND METHODS

ANIMAL HOUSING AND HUSBANDRY

All animal procedures were approved by the Newcastle University Ethics Review Committee and the Home Office. Animals were housed in the Comparative Biology Centre (Newcastle University) at 20–22°C with 12 h light/dark cycles. Fresh water was available *ad libitum*. Experimental diets were based on the AIN-93G rodent diets (Reeves et al., 1993) and have been described previously (McKay et al., 2008). Mating trios [two C57BL/6J females (8 weeks old), one *Apc*^{+/*Min*} male] were offered 6 g/day/mouse of a normal folate (2 mg folic acid/kg diet) or low folate (0.4 mg folic acid/kg diet) diet. Pregnant females were re-caged and offered 10 g/day/mouse of their allocated diet throughout pregnancy. At 2 weeks post-partum, diet quantity was increased (20 g/day/mouse).

At weaning (mean 32 days post-partum), offspring were genotyped (as described in McKay et al., 2008) and a randomly chosen sub-set of offspring, and all dams were killed for sample collection. The remaining offspring were re-caged (one to four per cage) and randomly assigned to a post-weaning diet of either normal folate (2 mg folic acid/kg diet) or low folate (0.4 mg folic acid/kg diet) diet (6 g/day/mouse). At an average of 96 days old, mice were killed for sample collection.

The effects of a low folate diet during pregnancy and/or post-weaning on the outcome of pregnancy, offspring growth, folate status, and tumor number and size (in *Apc*^{+/*Min*} mice), have been reported previously (McKay et al., 2008).

SAMPLE COLLECTION

Animals were anesthetized using gaseous isoflurane followed by cervical dislocation. The entire SI from the stomach to the cecum was removed and cut into two sections of equal length, the proximal and the terminal SI. These sections were then opened longitudinally, washed with PBS, and examined for tumors before being wrapped in foil and snap frozen in liquid nitrogen. Samples were stored at –80°C until DNA was extracted.

DNA EXTRACTION AND GENE-SPECIFIC DNA METHYLATION ASSESSMENT

DNA was extracted (including RNase treatment) using a Qiagen DNA mini kit (Qiagen-51306) following the manufacturer's protocol from normal appearing (i.e., an area of the gut where no macroscopic tumors were present) proximal SI tissue of juvenile and adult *Apc*^{+/*Min*} and WT mice.

Two micrograms of genomic DNA were bisulfite treated, purified, desulfonated, and ethanol precipitated as detailed in Belshaw et al. (2004). A one-twentieth volume was used as a template in PCR reactions to amplify CpG rich regions of *p53*, *Igf2*, *Apc*, and *p16* genes using primers detailed in Table 1. Nested PCR reactions were required to amplify *p53* and *Igf2*, therefore from the initial PCR, using F1 and R1 primers, 2 µl was transferred to a subsequent PCR reaction using F2 and R2 primers (see Table 1). All PCR reactions were carried out in 20 µl volume and contained 1 Unit HotStarTaq (Qiagen) and 0.75 pmol of each forward and reverse primer. Other reagents used for specific PCR reaction are detailed in Table 1. Cycling conditions were 95°C for 15 min × 1, 35 cycles of 95°C for 30 s, annealing temperature (see Table 1) for 30 s, 72°C for 30 s or 1 min (Table 1) followed by a final 10 min extension at 72°C, and then held at 4°C. CpG methylation status of the four genes was then determined by a combined bisulfite restriction analysis (COBRA) assay (Xiong and Laird, 1997). PCR products were digested overnight with the appropriate restriction enzyme (Table 1). Digested DNA was separated by gel electrophoresis (3% agarose) and gels stained with 0.01% SYBR green I in 1× TBE for 4 h. DNA SYBR green fluorescence was captured using a UV camera (UVItech Limited) and band intensities were quantified using UViband software (UVItech Limited). The percentage DNA methylation was then calculated from the relative intensities of the digested and undigested PCR products.

STATISTICAL ANALYSIS

Data distributions from this 2 × 2 factorially designed study (two levels of maternal folate supply and two levels of folate supply to the offspring post-weaning) were examined by the Kolmogorov–Smirnov test. All data sets were normally distributed. Analysis of variance was used to examine the effects of sex, genotype, and maternal and post-weaning diets, and interactions between maternal and post-weaning folate supply, on methylation of selected genes in the SI of weaning aged and adult mice.

Table 1 | PCR and enzyme digestion details for COBRA assays to detect gene-specific DNA methylation.

Gene	GenBank number	Primers (100 pmol/ μ l)	Annealing temperature (°C)	Extension time (s)	MgCl ₂ (mM)	Q solution (μ l)	Product size (bp)	Restriction enzyme	Incubation temperature (°C)	Fragment size (bp)
<i>p53</i>	AF287146	F1 ttttttaagtgatatttagttaagag	56.5	30	3	4	500	-	-	-
		R1 ataacaataaataaacaacaaatcctcc								
		F2 ttggaagggttaagtaggaaga								
		R2 ctataaatattaccctcaacaaaa								
<i>Igf2</i>	U71085	F1 atttatttttgattg999tatta	58	30	3	4	437	-	-	-
		R1 aaatcactaataataactaaacatctcc								
		F2 tgaatttggttagtgatatttttt								
		R2 ttaacctctaaactctttaaactcttta								
<i>p16</i>	AF332190	R taaacctctaaactctttaaactcttta	58	30	1.5	0	436	<i>Hpy</i> CH4IV	37	355/81
		F tgaatttgatattttttgtagtt								
		F gtttaggttggaatttggaggttag								
		R acactccattctatctccaataa								
<i>Apc</i>	AF209032		61	60	1.5	0	563	<i>FauI</i>	56	332/231

RESULTS

EFFECTS OF SEX AND GENOTYPE ON GENE-SPECIFIC DNA METHYLATION IN SI

There were no differences between male and female mice in methylation at any of the loci investigated in offspring killed at either weaning or in adulthood (aged 96 days).

At weaning there was no difference in percentage methylation at the *Igf2*, *p16*, or *Apc* loci in *Apc*^{+/*Min*} compared with WT mice but the *p53* locus was significantly ($p < 0.001$) more methylated in WT mice (Figure 1).

In adult mice, genotype did not affect DNA methylation at the *p16* locus. As we observed at weaning, methylation at the *p53* locus was significantly ($p < 0.001$) higher in WT mice (Figure 2) in adulthood. Furthermore, both the *Igf2* and *Apc* loci were more highly methylated in *Apc*^{+/*Min*} mice compared with WT mice ($p = 0.004$ and 0.012 respectively).

EFFECTS OF LOW MATERNAL DIETARY FOLATE ON GENE-SPECIFIC DNA METHYLATION

No significant effects of low maternal dietary folate during pregnancy and lactation were observed on DNA methylation at the loci investigated in weaning mice (data not shown).

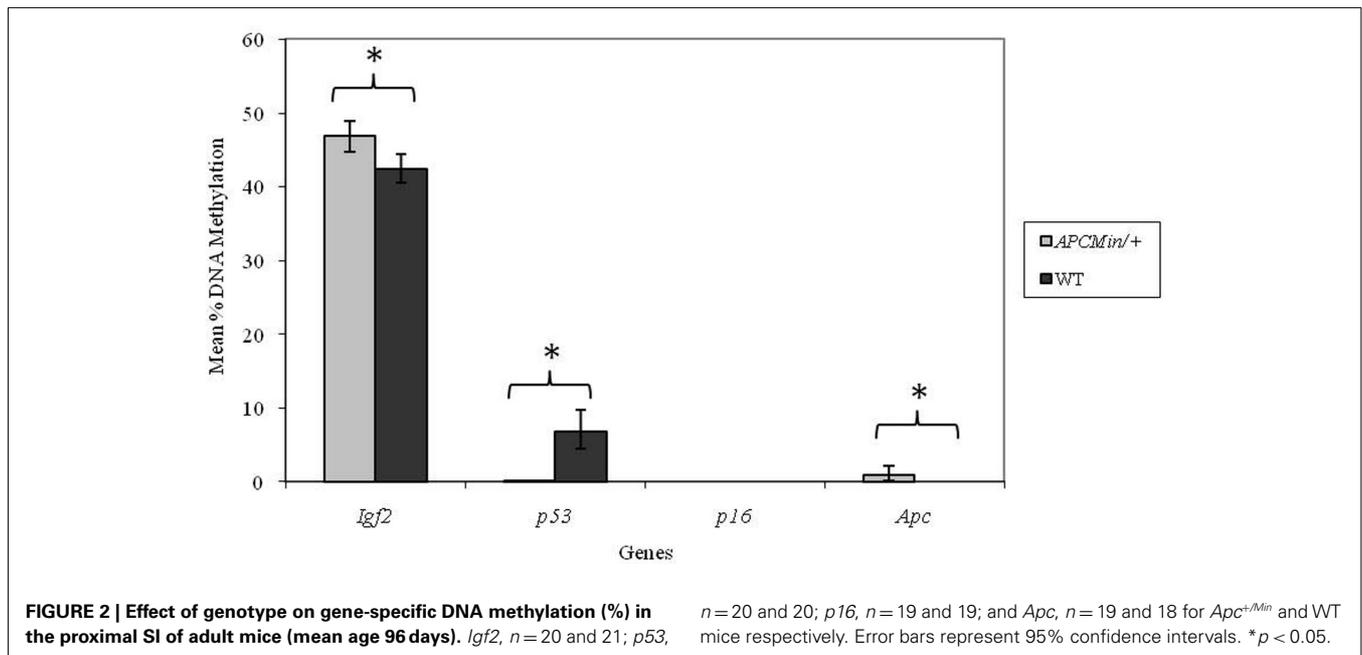
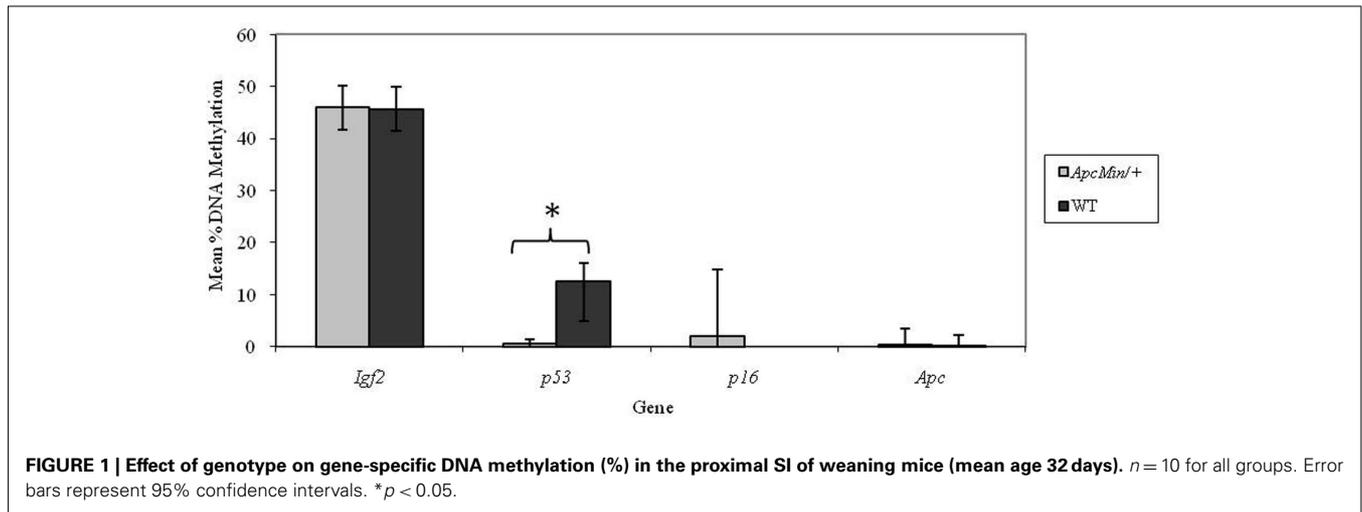
However, adult mice born to low folate fed mothers had a lower percentage methylation at the *p53* locus compared with mice born to normal folate fed mothers ($p = 0.04$; Figure 3). There were no detectable effects ($p > 0.05$) of low maternal folate intake at other loci in adult mice (Figure 3).

EFFECTS OF LOW POST-WEANING DIETARY FOLATE ON GENE-SPECIFIC DNA METHYLATION

Altering dietary folate supply from weaning had no significant effect on DNA methylation at the *Igf2*, *p53*, or *p16* loci in adult mice (Figure 4). However, the *Apc* locus was found to be unmethylated in adult mice fed normal folate post-weaning diets, but methylated in mice fed folate-depleted diets. This difference in mean *Apc* methylation was statistically significant ($p = 0.009$; Figure 4). Furthermore, there was a significant interaction between genotype and post-weaning diet for methylation at the *Apc* locus ($p = 0.008$). Mean methylation of the *Apc* gene in *Apc*^{+/*Min*} mice fed the folate-depleted diet post-weaning was 4.2% whereas methylation was undetectable in *Apc*^{+/*Min*} and WT mice fed the normal folate diet. There were no significant interactions between maternal and post-weaning folate supply for methylation at any of the loci investigated.

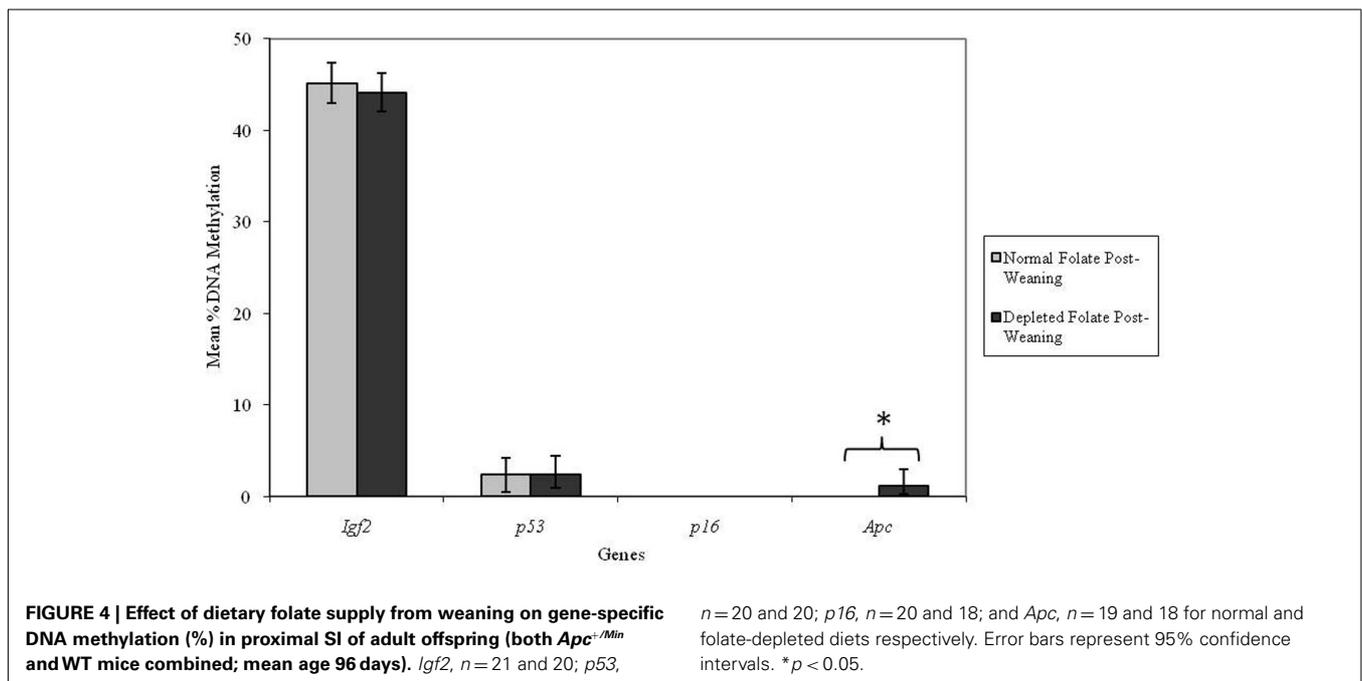
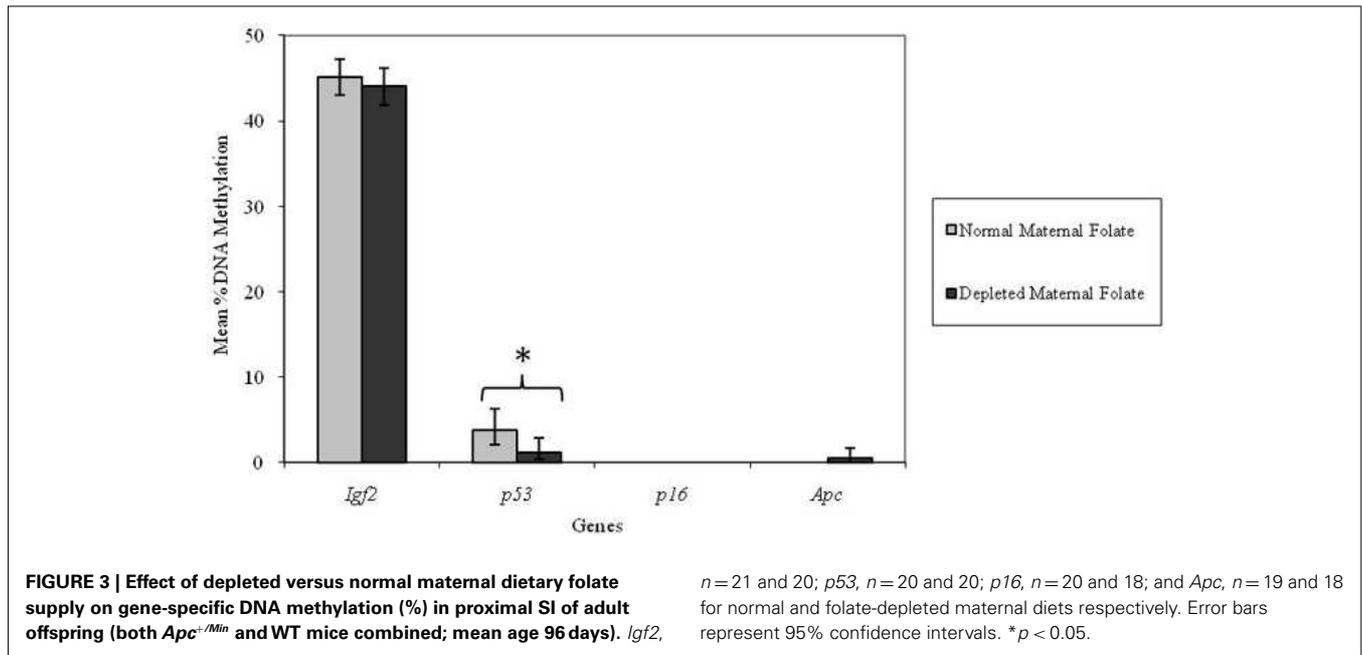
DISCUSSION

Aberrant DNA methylation is a cardinal feature of many cancers (Watanabe and Maekawa, 2010). Although abnormal DNA methylation patterns are an early event in CRC development, it is currently unclear whether the observed changes in DNA methylation are causal for cancer development or a consequence of it. In the study described here, we report hypomethylation at the *p53* locus in the SI of *Apc*^{+/*Min*} mice compared with WT mice at weaning. At this age (mean 32 days), no macroscopic lesions were apparent in the SI so the observed difference in *p53* methylation appears to precede tumor development in this model. Furthermore, the difference in *p53* methylation between *Apc*^{+/*Min*} and WT



mice was also observed in adults (mean 96 days of age), indicating that these epigenetic changes are sustained during the period of development of macroscopic tumors (see McKay et al., 2008 for details of tumor size and multiplicity). Previously, hypomethylation within exons 5–8 of the *p53* gene has been associated with colon tumorigenesis in the DMH rat model (Kim et al., 1996) and *p53* hypomethylation has been associated with increased mutation rates within this gene (Tornaletti and Pfeifer, 1995). In addition, loss of function of the *p53* tumor suppressor gene is common in the progression of late adenoma to carcinoma in humans (Fearon, 2011). In our study, mice with genetically inherited loss of function of one *Apc* allele had lower methylation at the *p53* locus than their WT littermates. To our knowledge, this is the first report of a collateral epigenetic effect on a tumor suppressor gene (*p53*) resulting from a germ-line mutation in another tumor suppressor

gene (*Apc*). Such effects might be anticipated in tumors *per se* but the present discovery is all the more remarkable because it was observed in macroscopically normal tissue. It will be important to seek confirmation of this finding in independent studies and to investigate the mechanism responsible. Since reduced *p53* methylation is expected to be accompanied by increased expression of the corresponding tumor suppressor gene and hence greater resistance to tumorigenesis, the sustained lower *p53* methylation in the tumor-prone *ApC^{+/Min}* mice is surprising. It is possible that the altered *p53* methylation is unconnected mechanistically with tumor development. This would be expected if the methylation change at the specific CpG site probed by the present COBRA assay does not signal a change in *p53* transcription. Further investigation of the functional consequences of this apparently novel genotype:epigenetic interaction is warranted.



In contrast to our findings of genotype-related *p53* hypomethylation, we observed that the *Igf2* and *Apc* genes were more highly methylated in adult *Apc*^{+/*Min*} mice compared with WT mice. Hypermethylation at the *Apc* locus is widely documented in human CRC (Venkatchalam et al., 2010) and may contribute to the loss of function through silencing of the second (non-mutated) allele of this tumor suppressor gene described as the gate-keeper of the colorectum (Kwong and Dove, 2009). Both hypo- and hypermethylation at the *Igf2* locus have been reported in human cases of CRC (Issa et al., 1996; Nakagawa et al., 2001;

Cui et al., 2002; Baba et al., 2010) with loss of imprinting at this locus believed to contribute to tumor growth (Cui et al., 2002).

Although dietary and other exposures during fetal and early post-natal life might be expected to influence risk of many complex diseases in adulthood, to date there is limited and conflicting evidence of associations between early life exposures and adult cancer incidence (Johnson et al., 2009). It is plausible that environmental factors that alter DNA methylation patterns during development may be early events influencing cancer development in later life, particularly as aberrant DNA methylation patterns are associated

with most cancers (Watanabe and Maekawa, 2010) and is an early event in CRC (Feinberg et al., 2006). We therefore hypothesized that reducing maternal folate supply during pregnancy and lactation would stress the developing mouse's capacity to methylate cellular macromolecules. This may therefore alter patterns of DNA methylation during fetal and early post-natal development. If these putative epigenetic derangements occurred in genes critical for cancer development, e.g., tumor suppressor genes then the maternal dietary insult might influence CRC risk in adult offspring. Indeed it was reported recently that early life exposure to famine was associated with the decreased risk of developing a CpG island methylator phenotype (CIMP) CRC (Hughes et al., 2009). Since the presence of a CIMP tumor is indicative of epigenetic instability coupled with transcriptional silencing of gene expression and microsatellite instability (Ferguson et al., 2004; Slattery et al., 2009), these findings suggest that early life exposures may indeed result in persistent epigenetic alterations that may influence risk of CRC development in later life. In the present study, we did not observe any differences in response to maternal dietary folate supply in methylation of our panel of cancer-related genes when examined in mice at weaning (mean 32 days of age). However, in adult offspring (mean age 96 days) we observed reduced methylation at the *p53* locus (but no changes in the other three genes investigated) in offspring born to mothers fed the low folate diet. Previously we reported reduced genomic DNA methylation in the SI of adult offspring in response to low maternal folate intake (McKay et al., 2011) and we have reported elsewhere that the response to maternal folate supply was gene, and CpG site, specific in the fetal gut (McKay et al., unpublished data). Our observations in the present study support these earlier reports since we found that methylation changes in the offspring in response to reduced maternal folate supply are gene-specific. Taken together, these data strengthen the hypothesis that maternal folate intake modulates epigenomic patterns in the offspring. Furthermore, the changes reported here in respect of *p53* methylation may indicate that such exposures have the potential to influence gut health in adulthood via epigenetic programming of some cancer related genes.

We report here that low post-weaning dietary folate intake caused hypermethylation at the *Apc* locus in *Apc^{+/-Min}* mice only. Although increased methylation in response to decreased folate intake may seem paradoxical, hypermethylation at the *Apc* locus has been reported in association with consumers of low folate/high alcohol in human cases of sporadic CRC (van Engeland et al., 2003). That this effect on *Apc* methylation occurred in *Apc^{+/-Min}* mice only (not in *WT* mice), suggests that there may be aberrations in the methylation machinery within SI of these tumor-predisposed mice. Since folate depletion is predicted to reduce cellular SAM supply, this will have implications for resource (methyl group) allocation to competing pathways within the cell and it may be that such competition, rather than derangements in the DNA methylation machinery *per se*, is responsible for the altered *Apc* methylation that we have observed. Indeed, in human CRC, hypermethylation of the *APC* promoter by folate depletion has been hypothesized to reduce expression of the gene (Esteller et al., 2000), which may be sufficient to initiate tumor development.

Finally, our observation of gene-specific responses to altered folate supply indicate that some genomic loci appear to be more labile than others in response to environmental cues such as altered nutrition. For example, it is well documented that metastable epialleles are epigenetically labile ("epilabile") in response to folate and other dietary factors (Waterland and Jirtle, 2003; Dolinoy et al., 2006; Waterland et al., 2006). Currently, relatively little is known about the characteristics of DNA domains which make them more or less epilabile. In this study we measured DNA methylation of a panel of genes known to be involved in the development of CRC and sensitive to methylation changes in CRC. Of the four genes we studied, methylation of *p53* was altered by maternal folate intake in both *Apc^{+/-Min}* and *WT* mice whereas *Apc* methylation was altered in *Apc^{+/-Min}* mice only, in response to low folate intake after weaning. The COBRA assay probes a single CpG site and it remains to be discovered whether the CpG site investigated here is characteristic of all the cytosine residues in the corresponding CpG island. It would be useful to extend this analysis using additional methodologies such as bisulfite sequencing or pyrosequencing which can measure several CpG sites within a given DNA domain. The only gene for which we did not observe any variation in DNA methylation was *p16*. Although aberrant DNA methylation of this gene has been observed in several human cancers (Auerkari, 2006), *p16* appears to be largely unmethylated in non-neoplastic tissue (Belshaw et al., 2008). In agreement with these findings, in the present study we analyzed DNA from macroscopically normal mucosa and found that *p16* methylation was undetectable in most samples – only very low levels of *p16* methylation were detected in *Apc^{+/-Min}* mice at weaning. The present study adopted a candidate gene approach and it is also possible that other genes may also have altered DNA methylation in response to maternal and/or post-weaning dietary folate depletion. Now that we have established proof of principle that reduced folate supply in early life can modulate gene methylation, further studies using genome-wide approaches such as methylation arrays or next generation sequencing are warranted to identify "epilabile" loci and to investigate both the molecular and overall health consequences of such epigenetic events.

In summary, the results presented here provide proof of principle that reduced maternal folate supply during pregnancy and lactation and/or reduced folate supply from weaning can alter the methylation of genes involved in CRC development. In particular, we have shown that reduced maternal folate intake during pregnancy and lactation alters *p53* methylation in the adult offspring. In addition, we have reported the apparently novel observation that genetically inherited loss of function of one *Apc* allele resulted in lower methylation at the *p53* locus. To our knowledge this is the first reported observation of a collateral epigenetic effect on a tumor suppressor gene (*p53*) resulting from a germ-line mutation in another tumor suppressor gene (*Apc*). It will be important to replicate these novel findings in independent studies.

CONTRIBUTION

The study was designed by John C. Mathers and Elizabeth A. Williams and laboratory work was undertaken by Jill A. McKay. All authors contributed to data analysis. Jill A. McKay and John C. Mathers wrote the manuscript with contributions from

Elizabeth A. Williams. All authors read and approved the final manuscript.

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