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Gene–environment interactions in the causation of neural tube defects: folate deficiency increases susceptibility conferred by loss of *Pax3* function

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Risk of neural tube defects (NTDs) is determined by genetic and environmental factors, among which folate status appears to play a key role. However, the precise nature of the link between low folate status and NTDs is poorly understood, and it remains unclear how folic acid prevents NTDs. We investigated the effect of folate level on risk of NTDs in *splotch* (*Sp*^{2H}) mice, which carry a mutation in *Pax3*. Dietary folate restriction results in reduced maternal blood folate, elevated plasma homocysteine and reduced embryonic folate content. Folate deficiency does not cause NTDs in wild-type mice, but causes a significant increase in cranial NTDs among *Sp*^{2H} embryos, demonstrating a gene–environment interaction. Control treatments, in which intermediate levels of folate are supplied, suggest that NTD risk is related to embryonic folate concentration, not maternal blood folate concentration. Notably, the effect of folate deficiency appears more deleterious in female embryos than males, since defects are not prevented by exogenous folic acid. Folate-deficient embryos exhibit developmental delay and growth retardation. However, folate content normalized to protein content is appropriate for developmental stage, suggesting that folate availability places a tight limit on growth and development. Folate-deficient embryos also exhibit a reduced ratio of s-adenosylmethionine (SAM) to s-adenosylhomocysteine (SAH). This could indicate inhibition of the methylation cycle, but we did not detect any diminution in global DNA methylation, in contrast to embryos in which the methylation cycle was specifically inhibited. Hence, folate deficiency increases the risk of NTDs in genetically predisposed *splotch* embryos, probably via embryonic growth retardation.

INTRODUCTION

Neural tube defects (NTDs), including spina bifida and anencephaly, are among the commonest human birth defects, affecting around 1 in every 1000 pregnancies. While the causes of human NTDs remain poorly understood in most cases (1–3), more than 100 single-gene defects can cause NTDs in mouse models. Nevertheless, the existence of many gene–gene and gene–environment interactions in the aetiology of mouse NTDs, and analysis of inheritance patterns in humans, argues strongly for a multi-factorial model in humans, in which susceptibility to NTDs is determined by multiple genetic and environmental factors (4–6). Among environmental factors, folate status appears to be a key determinant of NTD susceptibility since maternal supplementation with folic acid during early pregnancy reduces the risk of NTDs in the developing fetus (7–9). Conversely, reduced serum folate levels and/or elevated homocysteine levels (an inverse indicator of folate status) are observed in some mothers with NTD fetuses, and are considered risk factors for NTDs (10,11).

Despite compelling evidence that supplemental folic acid can prevent many cases of NTDs, neither the protective mechanism nor the relationship between maternal folate status and susceptibility to NTDs are well-defined. Moreover, some NTDs are not preventable by folic acid (12,13). NTDs do

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not appear to result simply from maternal folate deficiency, which is corrected by folic acid supplementation, since maternal folate levels in most affected pregnancies are within the 'normal' range (9,10). In order to investigate the role of sub-optimal folate status in causation of NTDs, studies in mice have made use of modified diets to induce severe folate deficiency in dams prior to mating. This results in reduced fetal weight, developmental delay and increased resorption rate among the offspring (14,15), although NTDs are not observed, arguing against a role for folate deficiency as a sole causative factor. Direct disruption of cellular folate uptake, by genetic ablation of folate-binding protein (Folr1), results in lethality in null embryos (16). *In vivo* supplementation with folinic acid (or 5-methyltetrahydrofolate) allows survival beyond neurulation stages, and a proportion of the embryos exhibit NTDs, suggesting that folate availability is important for neural tube closure (17).

The relatively mild reduction in folate status among human NTDs and the lack of induction of NTDs by dietary folate deficiency in animal models could be interpreted to indicate that low folate status is, at most, a minor contributory factor in determining susceptibility to NTDs. However, in some studies a dose–response relationship between maternal folate status and NTD risk has been noted (18). Thus, it has been proposed that low folate status may increase susceptibility to NTDs when present in combination with another environmental or genetic risk factor (5,13). Although this is an attractive and frequently cited hypothesis, a causative interaction between low folate status and genetic predisposition has not been demonstrated to date. Mammals cannot synthesize folates *de novo*, and intestinal absorption is therefore required to obtain this essential micronutrient from exogenous sources (19). Dietary folates are converted from polyglutamated forms to monoglutamates and preferentially absorbed in the small intestine. In addition, the bacterial microflora of the large intestine provide a second source of folates (20). In this study, we tested the hypothesis that dietary folate deficiency may increase risk of NTDs in genetically predisposed embryos, using the mutant mouse model, *splotch* (Sp^{2H}), which carries an intragenic deletion in the *Pax3* gene. Homozygous Sp^{2H} mutant embryos exhibit both cranial (exencephaly) and spinal (spina bifida) NTDs as well as neural crest and limb muscle defects (21). We found that folate deficiency results in an increased frequency of cranial NTDs in embryos carrying the Sp^{2H} mutation, and we investigated the possible mechanisms by which this effect is mediated.

RESULTS

Folate deficiency increases the incidence of NTDs in splotch mutants but not wild-type embryos

In order to investigate the relationship between folate status and genetic susceptibility to NTDs, we induced dietary folate deficiency in heterozygous female *splotch* (Sp^{2H}) mice prior to mating to generate experimental litters. Mice were maintained for a minimum of 4 weeks on a synthetic folate-free diet (designated FD), which included antibiotic to remove gut bacteria that synthesize folates. This strategy followed that of Burgoon *et al*. (15), except that mice were not

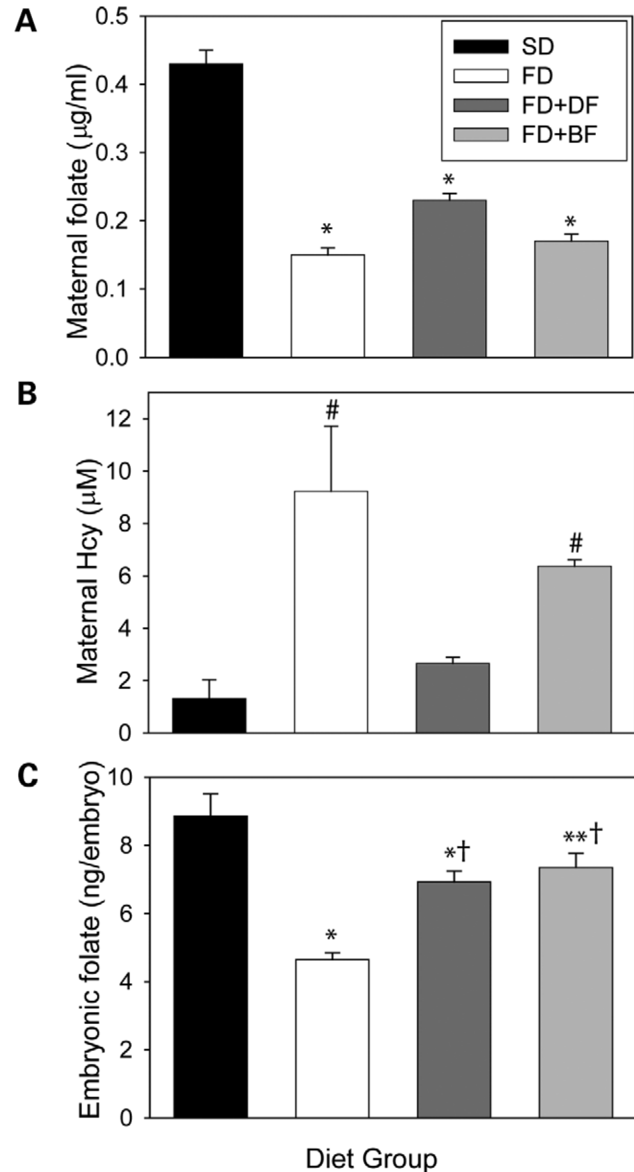

Figure 1. Embryonic folate content shows only partial correlation with maternal blood folate and homocysteine (Hcy) concentration. Whole blood folate (**A**; $n = 10–17$) and plasma homocysteine (**B**; $n = 4–6$) were quantified in $Sp^{2H/+}$ dams after maintenance for at least 6 weeks on the appropriate diet. Embryonic folate content (**C**; $n = 10–14$; Sp^{2H} genotypes pooled) was measured in stage-matched embryos at E10.5 (26–32 somite stage; mean number of somites equal for each treatment). Values are given as mean ± SEM. Maternal blood folate and embryonic folate content were significantly lower on all diets compared with the standard breeding diet ($^*P < 0.001$, $^{**}P < 0.05$, compared with SD). Embryonic folate content was significantly higher in the FD + DF and FD + BF diet groups than in the folate-deficient group ($^\dagger P < 0.001$, compared with FD). Maternal homocysteine was elevated in both the FD and FD + BF groups compared with SD and FD + DF ($^\# P < 0.001$). SD, standard breeding diet; FD, folate-deficient diet; FD + DF, folate-deficient but with dietary folate available; FD + BF, folate-deficient but with bacterial folate available.

housed on grids to prevent coprophagy. Quantification of red cell folate confirmed that, compared with the standard breeding diet (SD), there was a significant reduction in whole blood folate concentration in dams maintained on the FD diet (Fig. 1A). This correlated with an increase in plasma

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homocysteine (Fig. 1B), validating the model and confirming previous findings of an inverse correlation between folate and homocysteine concentrations in humans and animal models (9,15). To verify that maternal dietary folate deficiency can affect the embryo, folate content of individual embryos was quantified at E10.5. As expected, the total folate content was lower in embryos of the folate-deficient group compared with those on the SD (Fig. 1C).

Mice maintained under differing dietary folate regimes were mated to generate embryos, which were analysed for frequency of NTDs. Among wild-type embryos that developed under either standard or folate-deficient conditions, NTDs were never observed, in agreement with previous studies of non-mutant mouse strains (14,15). In contrast, there was a significant increase in the incidence of the cranial NTD, exencephaly, among homozygous Sp^{2H} offspring of mice maintained on the folate-deficient compared with SD (88 versus 61%; $P < 0.05$; Fig. 2A). Moreover, among heterozygous Sp^{2H} embryos, that do not develop NTDs under normal dietary conditions, 10% displayed exencephaly. Spina bifida occurs in more than 90% of Sp^{2H}/Sp^{2H} embryos developing on a normal diet, so any increased risk would be difficult to detect. However, we did not detect spina bifida among wild-type or $Sp^{2H/+}$ embryos developing under folate-deficient conditions. Sensitivity to folate level does not appear to result from diminished maternal uptake of folate by *splotch* dams, as blood folate concentrations did not differ significantly from a non-mutant strain (CD1), comparing mice either under standard or folate-deficient conditions (Supplementary Material, Fig. S1). Thus, we observe a gene–environment interaction such that folate deficiency induces exencephaly in genetically predisposed embryos carrying the Sp^{2H} allele, a proportion of which would have completed cranial neurulation under folate-replete conditions.

Embryonic folate levels correlate with NTD incidence

In order to further confirm that the observed increase in NTD incidence in Sp^{2H}/Sp^{2H} embryos was caused by folate deficiency, we tested the effect of two control diets. The first was identical to FD (including succinyl sulfathiazole antibiotic [SST]) but with the addition of dietary folic acid at 2.7 mg/kg, identical to the standard breeding diet. This diet, designated FD + DF (folate-deficient plus dietary folate) provided dietary but not bacterial folate. Both folic acid and SST antibiotic were omitted from the second diet, allowing access to bacterial folate but not to dietary folate. This diet was designated FD + BF (folate-deficient plus bacterial folate).

Inclusion of dietary folic acid (FD + DF) or removal of antibiotic (FD + BF) completely reversed the effect of the folate-deficient diet, such that the NTD frequency did not differ significantly from the standard dietary conditions (Fig. 2A). However, when we quantified maternal blood folate, mice fed on the FD + DF or FD + BF diets displayed a relatively small increase in blood folate concentration compared with FD (statistically significant only for FD + DF). Folate concentration remained significantly lower in these dams, than in mice maintained on the SD (Fig. 1A). Availability of dietary or bacterial folic acid did affect plasma homocysteine, resulting in significantly lower levels than in

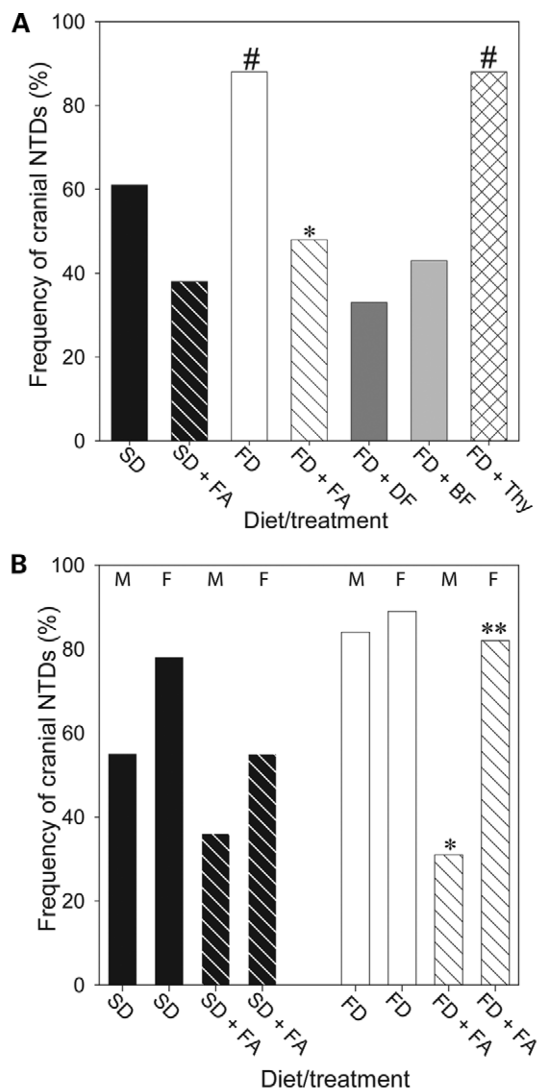

Figure 2. Frequency of cranial NTDs among Sp^{2H}/Sp^{2H} embryos varies with folate status. (**A**) Among Sp^{2H}/Sp^{2H} embryos that developed under folate-deficient (FD, $n = 33$) conditions, a significantly higher incidence of exencephaly (at E10.5–11.5) was observed than among embryos developing under conditions of standard diet (SD, $n = 34$); # indicates significant difference compared with SD ($P < 0.05$, Fisher exact test). In contrast, incidence of exencephaly under conditions of folate deficiency but with dietary folate available (FD + DF, $n = 12$) or folate deficiency but with bacterial folate available (FD + BF, $n = 14$) did not differ from the SD ($P > 0.05$). Maternal supplementation with folic acid reduced the incidence of exencephaly among both SD embryos (SD + FA, $n = 24$) and FD embryos (FD + FA, $n = 23$), although this effect reached statistical significance only for the FD + FA group (*indicates significant difference compared with FD, $P < 0.01$, Fisher exact test). Thymidine treatment (FD + Thy, $n = 7$) did not reduce the frequency of exencephaly. (**B**) The sex of a subset of embryos was determined (indicated as M or F above bar). On the SD the frequency of exencephaly was higher among females (F, $n = 7$) than males (M, $n = 11$), and remained higher after folic acid treatment (SD + FA; $n = 14$ males, 11 females), although these sex differences were not statistically significant. Under folate-deficient (FD) conditions both sexes exhibited a high frequency of exencephaly ($n = 19$ males, $n = 9$ females). However, while folic acid significantly reduced the frequency of exencephaly among males ($n = 17$, *indicates significant difference compared with FD, $P < 0.01$, Fisher exact test), females were almost completely resistant to folic acid ($n = 14$) such that the frequency of exencephaly was significantly higher among folic acid-treated FD females than males (**indicates significant difference compared with FD + FA males, $P < 0.02$).

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severe folate deficiency (Fig. 1B). Most strikingly, despite the minimal effect on maternal blood folate concentration, embryonic folate content was significantly higher in embryos developing under conditions where either dietary (FD + DF) or bacterial folate (FD + BF) were available, compared with neither (FD) (Fig. 1C). Thus, available folate may be preferentially taken up by the embryo, allowing the embryo to 'amplify' a small increase in maternal folate into a larger increase in embryonic folate. These data demonstrate that removal of both dietary folic acid and gut bacterial-derived folate is necessary to generate significant folate deficiency, and to enhance a genetic predisposition to NTDs.

We next tested the ability of exogenous folic acid to prevent folate deficiency-induced NTDs, by administering folic acid to pregnant dams prior to and during the stage of cranial neurulation. This *in vivo* folic acid treatment reduced the frequency of exencephaly in Sp^{2H}/Sp^{2H} embryos developing under normal dietary conditions, as previously observed (22,23), and also in folate-deficient embryos (Fig. 2A). The magnitude of decrease in NTD frequency was greater in the FD than the SD group, despite the difference in maternal 'baseline' folate status. Perhaps the folate deficiency-induced NTDs are more amenable to prevention than NTDs which occur under folate-replete conditions. Moreover, around one-third of cranial NTDs are not prevented by folic acid in either treatment group. The lower rate of NTDs in folate-treated groups is unlikely to result from folic acid-induced lethality of affected embryos as the number of implantation and resorption rates did not differ between diets or treatment groups (Supplementary Material, Table S1).

Folic acid treatment of folate-deficient dams (FD + FA) elevates maternal blood folate to 0.28 ± 0.03 µg/ml ($n = 4$), a level which is intermediate between normal and folate-deficient conditions (0.43 ± 0.02 and 0.15 ± 0.01 µg/ml, respectively, as was shown in Fig. 1A). In the offspring of these FD + FA mice, embryonic folate content was restored to apparently normal levels, 5.41 ± 0.5 ng/embryo in FD + FA group ($n = 9$) compared with 5.14 ± 0.3 ng/embryo ($n = 6$) in SD group at the 22–26 somite stage (note that for SD embryos at this stage the values are lower than for SD embryos at the later, 26–32, somite stage shown in Fig. 1C). Hence, the effect of exogenous folic acid treatment in increasing embryonic folate concentration correlates well with its ability to prevent a significant proportion of the cranial NTDs seen in folate-deficient dams.

Female embryos are thought to be more susceptible than males to cranial NTDs since there is a preponderance of female embryos among anencephalic human fetuses (24) and exencephaly is more frequent among female than male embryos in several mouse strains (25,26). Therefore, we determined the sex of a subset of Sp^{2H}/Sp^{2H} embryos that developed under SD and FD conditions, both with and without folic acid treatment. Under standard dietary conditions, the incidence of exencephaly was higher among female than male embryos (Fig. 2B), although this difference did not reach statistical significance. Folic acid reduced the incidence of NTDs by a similar magnitude in both sexes. Under folate-deficient conditions exencephaly occurred at similarly high frequency in male and female embryos (Fig. 2B). However, there was a remarkable difference between sexes in the efficacy of folic acid treatment, such that folic acid produced a significant reduction in the frequency of exencephaly among male embryos, but had almost no effect on females (Fig. 2B).

The effect of folate deficiency is not mediated through excess homocysteine

Elevated homocysteine concentration in maternal blood is a risk factor for human NTDs (5) and was found to occur in association with reduced maternal and embryonic folate in our dietary models (Fig. 1). A prevalent idea is that homocysteine itself may cause NTDs (13,27). Arguing against this hypothesis, we and others found that direct exposure of mouse or rat embryos to excess homocysteine is toxic but does not result in isolated NTDs (28,29).

The previous experimental studies were performed in non-mutant strains and since we also find no effect of folate deficiency in wild-type embryos, we reasoned that elevated homocysteine might cause an increased rate of NTDs only among 'at risk' Sp^{2H}/Sp^{2H} and $Sp^{2H/+}$ embryos, analogous to the effect of folate deficiency. In order to test this idea directly, we exposed cultured *splotch* embryos to exogenous homocysteine throughout a period of 40 h from E8.5, which encompasses the entire period of cranial neurulation. As previously reported in CD1 mice (28), homocysteine concentrations of 500 µM and above resulted in significant embryotoxicity (data not shown), whereas 250 µM homocysteine was well-tolerated by cultured embryos. Thus, in comparison to vehicle-treated controls, there was no apparent effect of 250 µM homocysteine on embryo viability, growth or developmental progression as indicated by yolk sac circulation score, crown-rump length or somite number after culture, respectively (Table 1). Moreover, exposure to homocysteine had no apparent effect on cranial neurulation, as the rate of exencephaly among homozygous mutant embryos was not increased compared with vehicle-treated controls. No neurulation defects were observed in heterozygous or wild-type embryos (Table 1), in agreement with reported *in vivo* studies (30). Similarly, spinal neural tube closure was not delayed by homocysteine exposure, as indicated by comparable posterior neuropore lengths in treated and control embryos of equivalent genotype (Table 1).

Folate concentration limits embryonic growth and developmental progression

In addition to variation in the frequency of NTDs, maternal diet had a striking effect on embryonic growth and developmental progression, as indicated by crown-rump length and somite number, respectively. For example, embryos developing in folate-deficient dams had a significantly smaller crown-rump length and fewer somites than embryos developing under other dietary conditions (Fig. 3), an effect that was irrespective of embryonic genotype (Supplementary Material, Table S2). Developmental delay appeared to become progressively worse as development proceeded, such that folate-deficient embryos had on average 5.3 fewer somites than normal at E10.5 and 6.6 fewer somites at E11.5 (Fig. 3A). Thus, assuming that embryos typically gain one somite approximately every 2 h during neurulation stages, a

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Table 1. Development, growth and incidence of NTDs among *splotch* embryos cultured in the presence of 250 μM homocysteine (Hcy)

Treatment/genotype	No.	Cranial NTDs	PNP length (mm)	Somites	Crown-rump length (mm)	Yolk sac score
Control						
+/+	2	0	0.49 ± 0.09	25.0 ± 0.0	3.33 ± 0.17	2.5 ± 0.5
$Sp^{2H/+}$	10	0	0.42 ± 0.12	25.7 ± 0.78	3.37 ± 0.13	2.4 ± 0.3
Sp^{2H}/Sp^{2H}	5	2 (40%)	1.54 ± 0.15*	24.6 ± 0.51	3.47 ± 0.14	1.6 ± 0.4
Hcy						
+/+	4	0	0.18 ± 0.09	23.0 ± 1.9	3.19 ± 0.30	1.3 ± 0.3
$Sp^{2H/+}$	12	0	0.19 ± 0.05	25.4 ± 0.6	3.44 ± 0.10	2.1 ± 0.2
Sp^{2H}/Sp^{2H}	8	2 (25%)	1.32 ± 0.19*	25.0 ± 0.7	3.6 ± 0.20	2.0 ± 0.3

Embryos were cultured for 40 h from E8.5, and no difference was observed between treatment groups in number of somites, crown-rump length (indicators of developmental progression and growth respectively) or yolk sac circulation score (indicator of viability). As expected the PNP is significantly enlarged in Sp^{2H}/Sp^{2H} embryos compared with other genotypes (*$P < 0.05$; one-way ANOVA), but no difference was observed between treatments for any genotype. The frequency of cranial NTDs among Sp^{2H}/Sp^{2H} embryos was not affected by homocysteine treatment ($P > 0.05$; Fisher exact test).

folate-deficient embryo would take 10–13 h longer to reach a specific somite stage, than controls.

As another measure of embryonic growth, we compared protein content with somite number for individual embryos developing under standard or folate-deficient conditions. At a given somite number (used as a measure of developmental stage) there was no apparent difference in protein content between dietary groups, although the folate-deficient embryos were older in gestational age (Fig. 4). Thus, folate deficiency appears to perturb the relationship between growth/developmental progression and gestational age, but does not dissociate embryonic growth from developmental progression.

We investigated in further detail the changing folate status of embryos as they progressed through development, under either standard or folate-deficient conditions. The total folate content of embryos on the SD increased linearly with somite stage, whereas folate-deficient embryos exhibited an unchanging folate content from 15 to 40 somites (i.e. over a 50-h developmental period; Fig. 5A). However, after normalization to total embryonic protein content, folate concentration was found to decrease with increasing somite number in both control and folate-deficient embryos. Prior to the 20 somite stage, folate concentrations for SD and FD embryos were non-overlapping (Fig. 5B). Strikingly, at later stages, the data overlapped and there was no significant difference in the rate of decline of folate concentration in embryos on the standard and folate-deficient diets (Fig. 5B). We conclude, therefore, that folate concentration is maintained with strict limits in the embryo, suggesting a controlling effect of folate content on embryonic growth.

Normalization of NTD incidence in the FD + DF and FD + BF control diets correlated with enhanced embryonic growth and development compared with the FD diet (Fig. 3). The protective effect of folic acid cannot, however, be entirely attributed to correction of overall growth retardation. Intraperitoneal injection of folic acid into folate-deficient dams significantly reduced the frequency of cranial NTDs (Fig. 2A) and yet had no detectable effect on overall embryonic growth and development at E11.5, despite the significant reduction in NTD rate (Fig. 3). To further analyse the possible role of overall embryonic growth in rescue of NTDs, a further set of folate-deficient mice were treated with thymidine. This treatment led to a significant improvement of growth parameters (Fig. 3), but did not diminish the number of Sp^{2H}/Sp^{2H} embryos with cranial NTDs (Fig. 2A). However, only very small open regions of cranial neural tube were present in the thymidine-treated embryos (compare Fig. 6C and D), suggesting that the closure process was enhanced, although ultimately failed to be completed.

Folate deficiency influences abundance of methylation cycle intermediates

Folate metabolism is inter-linked with the methylation cycle by the remethylation of homocysteine to methionine. We showed previously that suppression of the methylation cycle leads to cranial NTDs in mouse embryos (31,32). We hypothesized that a less severe effect of folate deficiency on the methylation cycle might also contribute to development of NTDs in genetically susceptible embryos. Therefore, the key methylation cycle intermediates s-adenosylmethionine (SAM) and s-adenosylhomocysteine (SAH) were quantified in folate-deficient embryos, and in somite-matched and gestational age-matched controls. In embryos developing under control conditions, we observed a reduction in SAM/SAH ratio with development from E10.5 to E11.5 (Table 2), as found in previous studies (33).

Comparison of folate-deficient and control dietary groups showed that, for gestationally matched embryos at E11.5, there were small but non-significant differences in both SAM and SAH concentrations, and in SAM/SAH ratio (Table 2). In contrast, comparison of folate-deficient embryos at E11.5 with somite-matched E10.5 controls showed a significant elevation of SAH levels, resulting in a significantly lower SAM/SAH ratio (Table 2). Hence, SAM/SAH ratio in folate-deficient embryos is consistent with gestational age but abnormally low for developmental stage (as indicated by somite number). Comparison of *splotch* genotypes did not reveal any significant difference in SAM or SAH levels between genotypes (Supplementary Material, Fig. S2).

Supplementation of folate-deficient embryos with exogenous folic acid did not significantly alter SAM or SAH abundance, or SAM/SAH ratio, compared with untreated FD

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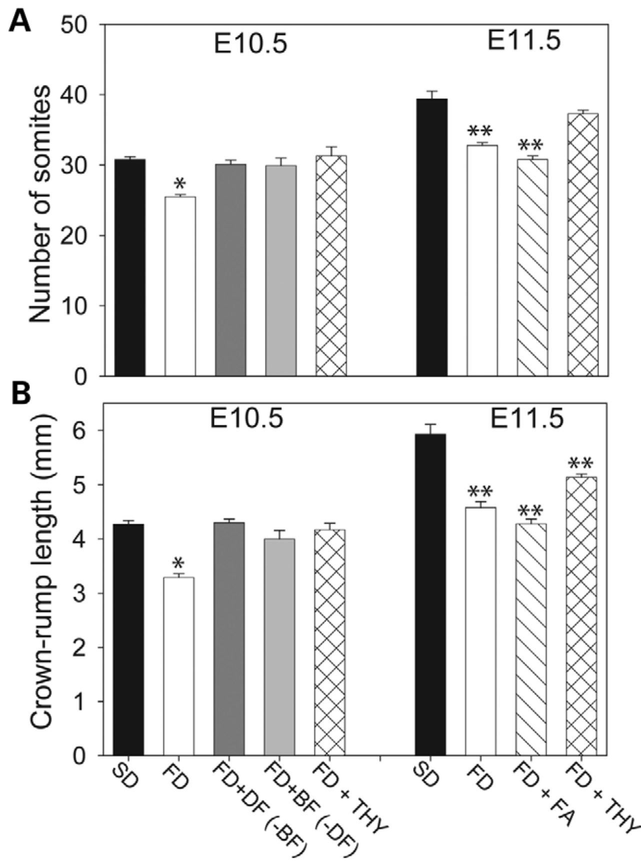

Figure 3. Developmental stage and size of embryos after development under varying levels of folate. *Splotch* embryos (genotypes pooled) developing under folate-deficient (FD) conditions had fewer somites (**A**) and smaller crown-rump length (**B**) at E10.5 than embryos in all other dietary groups (*significant difference compared with other groups, $P < 0.001$, One-way ANOVA). At E11.5, FD embryos still had significantly fewer somites and smaller crown-rump length than embryos from the SD (**$P < 0.001$). This difference was not rescued by folic acid treatment (FD + FA; **$P < 0.001$ compared with SD). In contrast, thymidine treatment (FD+THY) resulted in normalization of somite number in folate-deficient embryos ($P > 0.05$ compared with SD), although FD + THY embryos still had reduced crown-rump length (**$P < 0.001$ compared with SD). Values are given as mean ± SEM. Numbers of embryos at E10.5: 46 SD; 93 FD; 34 FD + DF; 21 FD + BF; 24 FD+THY. At E11.5: 19 SD; 49 FD; 28 FD + FA; 21 FD+THY. See Figure 1 for dietary group abbreviations.

embryos. In contrast, thymidine treatment resulted in a significant reduction in SAM/SAH ratio primarily owing to increased SAH abundance (Table 2).

DNA methylation

The observed reduction of SAM/SAH ratio in folate-deficient embryos could indicate reduced methylation potential. In order to detect an effect of folate deficiency on overall DNA methylation, we measured the ratio of methylated to unmethylated cytosine in genomic DNA extracted from stage-matched (28–29 somite) SD and FD embryos. Interestingly, despite the difference in SAM/SAH ratio at this stage (Table 2), there was no apparent effect of folate deficiency on methylation of total genomic DNA (Table 3). As a positive control, non-mutant CD1 embryos were cultured in the presence of the methylation

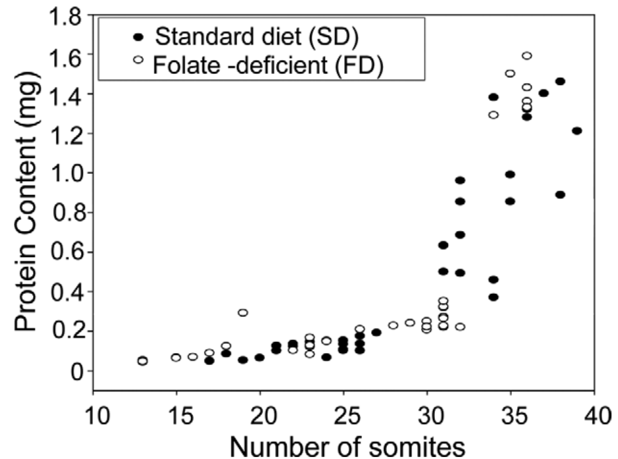

Figure 4. Relationship between embryonic growth (protein content per embryo) and developmental stage (somite number) in *splotch* embryos developing under differing dietary folate conditions. Black circles indicate standard diet (SD), white circles indicate folate-deficient diet (FD). All *splotch* genotypes were pooled for analysis. Note the exponential increase in protein content with somite stage, with no differences between SD and FD dietary groups. Hence, embryonic growth and developmental progression are not dissociated in folate deficiency.

cycle inhibitor, ethionine, at a concentration known to cause cranial NTDs (31). Following 24-h culture, from E8.5 to E9.5, genomic DNA methylation was significantly reduced in embryos exposed to ethionine, compared with control cultured embryos (Table 3). Hence, there is no evidence for a detrimental effect of folate deficiency on methylation capacity in neurulation-stage mouse embryos.

DISCUSSION

Dietary factors have long been thought to play a role in determining susceptibility to NTDs, and attention has particularly focused on folic acid-related molecules. Previous studies in mice showed that dietary folate deficiency is insufficient to induce NTDs in non-mutant strains (14,15). Similarly, we found that folate deficiency did not cause NTDs in wild-type embryos from *splotch* litters, despite depletion of maternal blood and embryonic folate and an elevated circulating homocysteine concentration. In contrast, induction of folate deficiency in *splotch* (Sp^{2H}) mutant embryos resulted in a significant increase in cranial NTD frequency, indicating a gene–environment interaction between loss of *Pax3* function and folate status. Notably, the effect of folate deficiency appears more deleterious in female embryos than males, since defects were not prevented by treatment with exogenous folic acid. Increased female susceptibility to cranial NTDs is also observed among human fetuses and other mouse strains (24–26), but the mechanism remains unresolved. We are not aware of published data suggesting an increased proportion of females among apparently folic acid-resistant NTDs (i.e. those cases where NTDs occurred despite folic acid supplementation), but this area deserves further attention.

Previously, induction of folate deficiency in a different *splotch* allele, *Sp*, was reported to have no effect on the incidence of NTDs (34), although, in another study of *Sp*, NTDs

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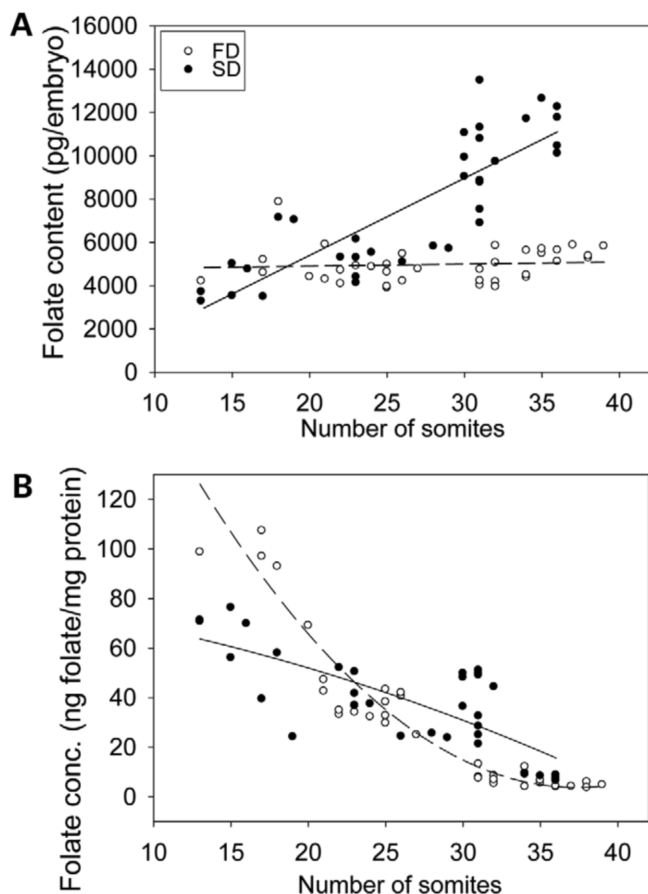

Figure 5. Developmental change in embryonic folate status. Folate and protein content were measured for a series of *splotch* embryos (genotypes pooled for analysis) at different developmental stages (as indicated by somite number). Each data point represents an individual embryo. Embryonic folate content (**A**) increases with developmental stage for embryos developing under standard dietary conditions (SD, solid line), whereas there is no discernible increase for embryos developing under folate-deficient (FD, dashed line) conditions. Linear regression lines were fitted (FD, solid line, $r^2 = 0.700$; SD, dashed line, $r^2 = 0.024$) and show significant difference in gradient between dietary groups ($P < 0.001$, *t*-test). In contrast, folate concentration (**B**), normalized to protein content, declines with development. Prior to 20 somites the rate of decline is greater in FD than SD embryos, but after the 20 somite stage there is no apparent difference between SD and FD groups. Thus, at a given somite stage the protein concentration of FD embryos is the same as for SD embryos, although the embryo is gestationally older.

could be prevented by folic acid and 5-methyltetrahydrofolate supplementation (23). In the present work, both folate deficiency and folate supplementation were found to affect the frequency of NTDs in *splotch* mice carrying the Sp^{2H} allele. Although both *Sp* and Sp^{2H} are thought to be effectively null alleles (35), the frequency of cranial NTDs differs markedly. For example, Li *et al*. (34) found exencephaly in only around 8% of *Sp/Sp* embryos, whereas we observed this NTD in ~60% of Sp^{2H}/Sp^{2H} embryos. In contrast, spina bifida is almost fully penetrant in both *Sp* (96%) and Sp^{2H} (90%) alleles. The frequency of exencephaly, but not spina bifida, can be predictably altered by variation in the location of Closure 2, the site of onset of cranial neural tube closure (36). Although this closure event occurs most commonly at the forebrain–midbrain boundary, some genetic backgrounds exhibit Closure 2 more caudally, in the midbrain, and these strains are resistant to exencephaly. Conversely, strains with Closure 2 at a more rostral location, within the forebrain, are predisposed to exencephaly. It remains to be determined whether this genetic background difference can explain the differing propensities of existing mouse colonies carrying the *Sp* and Sp^{2H} alleles to develop cranial NTDs.

Another factor that may explain the apparent variation in response to folate deficiency of *Sp* and Sp^{2H} mice is the differing methodology used to deplete folate in the two studies. Our analysis of maternal blood folate demonstrated that both dietary and bacterial sources of folate must be removed to achieve profound folate deficiency and an elevated homocysteine concentration. In fact, some previous studies have shown further reduction in folate status by maintaining dams on grids to prevent coprophagy, another source of folate (15). The study of *Sp* mice (34) used diets containing reduced amounts, but not complete absence, of folic acid. In the present study, the FD + BF or FD + DF diets, in which bacterial or dietary folate was still available, resulted in a smaller reduction in embryonic folate content than when both dietary and bacterial folate were removed, and did not increase the frequency of cranial NTDs, similar to the observations of Li *et al*. (34).

How does folate deficiency predispose to NTDs? Folate one-carbon metabolism plays several key cellular roles including provision of nucleotides for DNA synthesis and generation of SAM, the methyl donor in cellular methylation reactions. We found that lack of folates caused a profound retardation of embryonic growth and developmental progression, suggesting that cell proliferation is compromised. Thus, at a given gestational age, folate-deficient embryos contained less protein, and had smaller crown-rump length and fewer somites, than embryos developing under folate-replete conditions. Strikingly, analysis of individual embryos showed that folate concentration at a specific developmental stage (as defined by somite number) does not differ between conditions where folate is deficient or replete. Neither is there a dissociation of embryonic growth and developmental progression. These findings suggest that embryonic folate availability places an absolute limit on the rate of growth and developmental progression. Cranial neural tube closure appears particularly susceptible to factors that suppress proliferation (26), and we speculate that the developmental delay imposed by folate deficiency may exacerbate the underlying defect in *splotch* mutant embryos, thereby increasing risk of NTDs. In particular, the observed developmental delay implies that the period of neural tube closure is longer in folate-deficient embryos, perhaps increasing vulnerability to factors, such as loss of *Pax3* function, that inhibit closure. Although treatment of folate-deficient litters with exogenous folic acid did not ameliorate overall developmental delay or growth retardation, there was a significant preventive effect on cranial NTDs. We speculate that exogenous folic acid may cause a localized stimulation of proliferation in the cranial neural folds that opposes the cellular defects imposed by loss of *Pax3* function, thereby enhancing closure and diminishing NTD risk.

In addition to growth retardation, folate deficiency also caused a significant decrease in SAM/SAH ratio, compared

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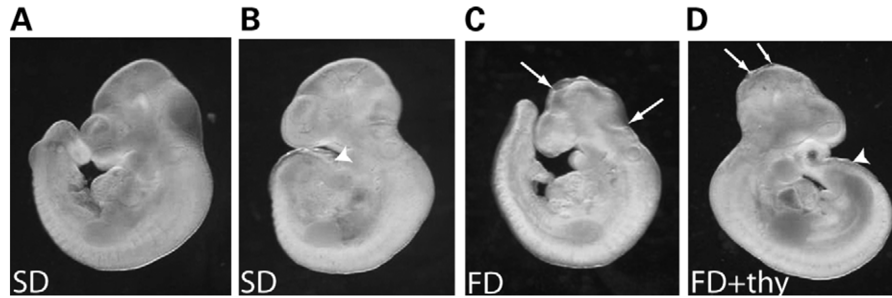

Figure 6. NTDs in *splotch* embryos Neural tube closure is completed among all wild-type embryos (SD embryo shown in **A**), whereas Sp^{2H}/Sp^{2H} embryos (**B**–**D**) exhibit NTDs, comprising spina bifida (arrowhead in B and D) and/or exencephaly (C and D, region of open neural folds is indicated by arrows). In FD conditions, the region of open neural folds encompasses the midbrain or mid- and hindbrain (C), whereas thymidine-treated embryos exhibit a small region of open neural folds (D).

Table 2. Abundance of methylation cycle intermediates is affected by folate-deficient diet

Diet/treatment	Gest. age	No.	No. somites	SAM (nmol/mg protein)	SAH (nmol/mg protein)	Ratio SAM/SAH
SD	E10.5	40	29.8 ± 0.3	3.35 ± 0.26	0.024 ± 0.002	147 ± 7
SD	E11.5	14	40.1 ± 0.6	3.28 ± 0.29	0.033 ± 0.004*	111 ± 18*
FD	E11.5	18	29.6 ± 0.9	2.94 ± 0.23	0.050 ± 0.011**	96 ± 12**
FD + FA	E11.5	29	28.7 ± 0.7	3.29 ± 0.35	0.041 ± 0.005	97 ± 14
FD + THY	E11.5	13	35.5 ± 0.3	3.58 ± 0.20	0.063 ± 0.004	59 ± 4#

SAM and SAH were quantified in individual *splotch* embryos from different dietary groups (genotypes pooled). Owing to the developmental retardation of folate-deficient (FD) embryos, both somite-matched (E10.5) and gestational age-matched (E11.5) control embryos (SD) were analysed. SAH abundance increases and SAM/SAH ratio decreases with stage, under normal dietary conditions (* significant difference compared with E10.5 SD, $P < 0.015$). In folate-deficient embryos, SAH abundance is elevated and SAM/SAH ratio decreased compared with somite-matched controls (** compared with E10.5 SD, $P < 0.002$), whereas SAM, SAH and SAM/SAH ratio do not differ significantly from gestation age-matched controls (compared with E11.5 SD, $P > 0.05$). Folic acid treatment does not significantly alter SAM and SAH abundance compared with FD alone. Thymidine treatment of FD embryos results in a significant decrease in SAM/SAH ratio (# indicates significant difference from FD, $P < 0.002$).

Table 3. Genomic DNA methylation in the embryo is apparently unaffected by folate-deficient diet

Diet/treatment	Gest. age	No	No. somites	Methyl Cyt/total Cyt (%)
SD	E10.5 (early)	5	28.2 ± 0.4	2.76 ± 0.24
FD	E10.5 (late)	9	28.4 ± 0.2	2.75 ± 0.15
Cultured embryos				
Control	E9.5	2 pools	19.5 ± 0.3	3.26 ± 0.13
Ethionine	E9.5	2 pools	19.2 ± 0.7	1.80 ± 0.02

The ratio of 5-methylcytosine to total cytosine was measured in genomic DNA from somite-matched *splotch* embryos that developed *in vivo* under differing dietary conditions (no significant difference). No differences were apparent between genotypes and data has been pooled in the table. As a control experiment, non-mutant CD1 embryos were cultured for 24 h, from E8.5 to 9.5, with 5 mM ethionine (an inhibitor of the methylation cycle). As predicted, DNA methylation was reduced in ethionine-treated embryos ($n = 5$) compared with controls ($n = 4$), and three out of five ethionine-treated CD1 embryos exhibited cranial NTDs.

with somite-matched controls, suggesting that the methylation cycle might be suppressed. Indeed, inhibition of the methylation cycle is known to induce a high frequency of cranial NTDs in cultured mouse embryos and in chick embryos (31,32,37). On the other hand, global DNA methylation was not affected in our folate-deficient embryos, in contrast to embryos exposed to the methylation cycle inhibitor, ethionine, which showed significant inhibition of global DNA methylation. Currently, we cannot rule out the possibility that folate deficiency specifically suppresses methylation of certain critical DNA sequences. For example, adult mice or rats maintained on folate-deficient diets exhibit hypomethylation at the p53 locus (38,39), although effects on the developing embryo have not been reported. Cell culture models also suggest that low folate status may affect expression of other genes involved in cell cycle regulation (40). It is also possible that methylation of other macromolecules, such as proteins or RNA, may be compromised. Nevertheless, taken together, we currently find no evidence to indicate that folate deficiency exacerbates NTDs in predisposed *splotch* embryos via inhibition of cellular methylation.

How might our findings relate to the causation of human NTDs? Clinical trials have shown a clear protective effect of folic acid supplementation, but the link between maternal dietary folate and risk of human NTDs is not well-understood (13). One of the difficulties of assessing risk factors for human

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NTDs is the inaccessibility of the neurulation-stage human embryo for biochemical analysis. In most cases, the folate status of the embryo has been inferred from maternal folate concentrations, sometimes measured at much later stages of pregnancy. In the present study, measurement of embryonic folate concentrations showed that removal of either dietary or gut bacterial folate sources had only minimal effects on embryonic folate, and did not increase NTD frequency, whereas maternal blood folate was markedly depleted under such dietary conditions. Thus, embryonic folate content, but not maternal blood folate, correlates with NTD risk in Sp^{2H} mice. These findings do not support a model based purely on maternal folate status, and emphasize the need for caution in interpreting human data based on relatively small changes in maternal blood folate concentrations. On the other hand, our data provide considerable support for the idea that genetic factors, in addition to folate status, are critical in determining risk of NTDs. The complex aetiology of human NTDs certainly provides scope for the majority of cases to involve multiple interacting factors, both genetic and environmental. For example, the pattern of recurrence of human NTDs favours a polygenic or oligogenic inheritance model, rather than dominant or recessive single-gene inheritance (6). Moreover, mouse studies suggest that many different genes have the potential to play a role, perhaps in numerous combinations (6).

Recently, using fibroblastic cell lines derived from human fetuses with NTDs, we found that a subset of NTDs are associated with abnormal folate metabolism (41). It could be envisaged that reduced folate availability would further enhance such a defect, increasing NTD risk. In addition, the apparent effects of folate deficiency on embryonic growth and development suggest that depletion of embryonic folate could also exacerbate more general cellular risk factors for NTDs. A goal for the future, therefore, is to identify the genetic variants that are responsible for abnormal cellular folate metabolism in a proportion of human fetuses with NTDs, and to determine the mechanisms by which such genetic predisposition can be exacerbated by embryonic folate deficiency, and ameliorated by folic acid supplementation.

MATERIALS AND METHODS

Maintenance of mice, dietary conditions and collection of embryos

Sp^{2H} mice were maintained as a random-bred colony, and genotyped by PCR using primers to *Pax3* (21). Mice were routinely fed a standard breeding diet (Harlan Teklad) containing 2.7 mg/kg folic acid. For dietary studies, female mice were randomly allocated at 6 weeks of age to the SD or to one of three synthetic protein-based diets (TD.04083, TD.04297 and TD.04296) that were matched to the SD for micronutrient levels, with the exception of folic acid. No folic acid was present in diets FD and FD + BF, while 1% succinyl sulfathiazole (10 g/kg) was included in diets FD and FD + DF. Females were maintained on each diet for a minimum of 4 weeks before mating to males which had been fed the standard breeding diet.

Experimental litters were generated by overnight matings between heterozygous mice, the day of finding a copulation plug being designated embryonic day 0.5 (E0.5). *In vivo* supplementation was by intraperitoneal injection, daily from E7.5 to 10.5, using 1 mg/ml folic acid (Sigma) in sterile phosphate buffered saline (PBS), to a dose of 10 mg/kg, or 0.1 mg/ml thymidine (Sigma) in PBS, to a dose of 1 mg/kg. Mice were killed by cervical dislocation and embryos were dissected from the uterus in Dulbecco's Modified Eagle's medium (Invitrogen) containing 10% fetal calf serum. Embryos were rinsed in PBS and immediately frozen on dry ice for biochemical analysis or fixed in 4% paraformaldehyde for other studies.

Genotyping of embryos

Genotyping was performed by PCR using DNA extracted from yolk sacs. *Splotch* genotype was determined using primers to *Pax3* (21) and embryonic sex was determined using sex-specific primers to amplify the *Smcx* and *Smcy* genes (42).

Quantification of folate and homocysteine in maternal blood

Mice were anaesthetized with halothane and blood was collected by cardiac puncture and immediately transferred to lithium–heparin-coated tubes (Microtainer^{TM}, BD). Whole blood folate was measured using the *L. Casei* microbiological method (43). A second aliquot was centrifuged and plasma was stored at −70°C prior to quantification of homocysteine by standard methods (44).

Quantification of folate in embryo samples

Embryonic folate was measured using an adapted version of the *L. Casei* microbiological method (43). Briefly, each embryo was sonicated in water, on ice, to produce a homogeneous solution. An aliquot was taken for determination of protein concentration using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). To include measurement of both mono- and polyglutamylfolates (total folate), the sample was incubated with conjugase extract (partially purified rat serum) in a 1% ascorbic acid buffer for 60 min at 37°C to convert polyglutamylfolates to assayable monoglutamylfolates and then diluted within the range of the standard curve with 0.5% sodium ascorbate.

Quantification of SAM and SAH

Samples consisting of a pool of 2–4 embryos from a specific treatment group were analysed in duplicate by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) as described previously (33).

DNA methylation assay

Splotch embryos developing under folate-deficient ($n = 9$) or standard conditions ($n = 5$) were collected at the 28–29 somite stage. CD1 mouse embryos were cultured for 24 h from E8.5 to E9.5 in the presence of 5 mM ethionine or PBS (control group), as described previously (31). Genomic DNA

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was prepared from individual *splotch* embryos and from pools of three CD1 embryos following culture ($n = 6$ embryos for each treatment), using a QIAamp DNA mini kit (Qiagen). DNA was hydrolyzed by formic acid and analysed by LC-ESI-MS/MS, as described previously (45).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

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Conflict of Interest statement. None declared.

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