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Neural tube defects induced by folate deficiency in mutant *curly tail* (*Grhl3*) embryos are associated with alteration in folate one-carbon metabolism but are unlikely to result from diminished methylation

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Abstract

Background—Folate one-carbon metabolism has been implicated as a determinant of susceptibility to neural tube defects (NTDs), owing to the preventive effect of maternal folic acid supplementation and the higher risk associated with markers of diminished folate status.

Methods—Folate one-carbon metabolism was compared in *curly tail* (*ct/ct*) and genetically matched congenic (*+ct/+ct*) mouse strains using the deoxyuridine suppression test in embryonic fibroblast cells and by quantifying s-adenosylmethionine (SAM) and s-adenosylhomocysteine (SAH) in embryos using liquid chromatography tandem mass spectrometry. A possible genetic interaction between *curly tail* and a null allele of 5,10-methylenetetrahydrofolate reductase (MTHFR) was investigated by generation of compound mutant embryos.

Results—There was no deficit in thymidylate biosynthesis in *ct/ct* cells but incorporation of exogenous thymidine was lower than in *+ct/+ct* cells. In *+ct/+ct* embryos the SAM/SAH ratio was diminished by dietary folate deficiency and normalised by folic acid or *myo*-inositol treatment, in association with prevention of NTDs. In contrast, folate deficiency caused a significant increase in SAM/SAH ratio in *ct/ct* embryos. Loss of MTHFR function in *curly tail* embryos significantly reduced the SAM/SAH ratio but did not cause cranial NTDs or alter the frequency of caudal NTDs.

Conclusions—*Curly tail* fibroblasts and embryos, in which *Grhl3* expression is reduced, display alterations in one-carbon metabolism, particularly in the response to folate deficiency, compared with genetically-matched congenic controls in which *Grhl3* is unaffected. However, unlike folate deficiency, diminished methylation potential appears to be insufficient to cause cranial NTDs in the *curly tail* strain, and neither does it increase the frequency of caudal NTDs.

Keywords

Neural tube defects; folate; methylation; curly tail; inositol

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Introduction

Understanding the causes of human neural tube defects (NTDs) presents a major challenge owing to their complex multifactorial etiology, which is thought to involve multiple genetic and environmental factors (Copp and Greene 2010; Greene et al., 2009). Folate-mediated one-carbon metabolism has emerged as a key determinant of susceptibility to NTDs, and may be influenced both by environmental agents, such as supplemental folic acid, and by genetic variants, for example, in folate metabolising enzymes such as 5,10-methylenetetrahydrofolate reductase (MTHFR) (Blom et al., 2006; Molloy et al., 2009).

Mouse models have provided an opportunity to probe the relationship between folate status and NTDs (Harris 2008). For example, folic acid supplementation can prevent NTDs in some genetic mutants, including *Crooked tail*, *Spotch* (*Sp^{2H}* and *Sp*), and *Cited2* knock-outs (Carter et al., 1999; Fleming and Copp 1998; Martinez-Barbera et al., 2002; Wlodarczyk et al., 2006). Conversely, cellular folate uptake is essential for embryonic survival and neural tube closure (Piedrahita et al., 1999; Spiegelstein et al., 2004), while dietary folate deficiency has been found to exacerbate cranial NTDs in the *Spotch* (*Sp^{2H}*) mutant (Burren et al., 2008). Interestingly, spina bifida in the *curly tail* mouse is not preventable by supplemental folic acid (Seller 1994; Van Straaten and Copp 2001), whereas there is an exacerbating effect of dietary folate deficiency on cranial neurulation, such that the incidence of exencephaly is dramatically increased compared with mice raised on a standard laboratory diet (Burren et al., 2010).

Curly tail is among the most extensively characterised mouse models for NTDs and shares a number of features with the corresponding human conditions, including partial penetrance with a major influence of genetic modifiers and environmental factors (Van Straaten and Copp 2001). Homozygous mutant (*ct/ct*) embryos exhibit lumbo-sacral spina bifida (15-20% frequency) and/or tail flexion of defects (50% incidence) owing to failure or delay of closure of the posterior neuropore, respectively. Cranial NTDs (exencephaly) also occur at low frequency (approximately 5% of embryos). The major *curly tail* gene corresponds to a hypomorphic allele of *grainyhead-like-3* (*Grhl3*), and by comparison *Grhl3* null embryos develop spina bifida and exencephaly at higher frequency (Gustavsson et al., 2007; Ting et al., 2003; Yu et al., 2006). The sensitivity of cranial neurulation in *curly tail* to folate deficiency appears to result, at least in part, from modifiers in the *curly tail* genetic background. Thus, mice which are wild-type at the *Grhl3* locus (*+^{ct}*), but share the *ct* background, do not normally exhibit NTDs but develop cranial NTDs when folate-deficient, unlike several other non-mutant strains that have been tested (Burren et al., 2010).

Although folate metabolism was not previously found to be compromised in *curly tail* embryos at the level of nucleotide biosynthesis (Fleming and Copp 1998), alterations in abundance of homocysteine and other methylation cycle intermediates have been reported in adult mice (Tran et al., 2002). Methylation reactions are inter-linked with folate-dependent metabolism as 5-methyl tetrahydrofolate, generated by MTHFR, is required for the remethylation of homocysteine to methionine, the precursor of S-adenosylmethionine (SAM). In turn, SAM acts as the methyl donor in cellular methylation reactions, also resulting in formation of S-adenosylhomocysteine (SAH). Plasma homocysteine and liver SAH were found to be higher in *curly tail* mice than in C57Bl/6, used as control strain. The ratio of abundance of SAM/SAH was correspondingly reduced, suggestive of a possible decrease in methylation potential (Tran et al., 2002). These observations were intriguing in view of the finding that perturbation of the methylation cycle, through exposure of the embryo to inhibitors or excess methionine, is sufficient to cause cranial NTDs in mouse embryos, even in the absence of apparent genetic predisposition (Dunlevy et al., 2006a; Dunlevy et al., 2006b).

In models of dietary folate deficiency, elevated maternal plasma homocysteine has been observed along with diminished embryonic SAM/SAH ratio (Burgoon et al., 2002; Burren et al., 2008; Li et al., 2006). Similarly, elevated maternal homocysteine was also observed in folate-deficient *ct* mice (Burren et al., 2010), but the abundance of methylation cycle intermediates in the embryo was not determined. We hypothesised that *curly tail* embryos may exhibit diminished SAM/SAH ratio, as in adult liver (Tran et al., 2002), and that this may be further lowered by folate deficient conditions, which exacerbate cranial neurulation (Burren et al., 2010). Given that loss of *Mthfr* function reduces the SAM/SAH ratio we hypothesised that there may be a genetic interaction between *Mthfr* and *curly tail* leading to a further reduction in SAM/SAH ratio, and an increased frequency of cranial NTDs.

Materials and methods

Maintenance of mice, genotyping and dietary conditions

Curly tail (*ct/ct*) and genetically-matched congenic ($+^{ct}/+^{ct}$) mice were maintained as random-bred, homozygous colonies (as Burren et al., 2010). *Mthfr* knockout mice were generated previously, and were maintained on a Balb/c background and genotyped by PCR amplification as described (Chen et al., 2001). *Mthfr* heterozygous (*Mthfr*^{+/−}) mice were mated with *ct/ct* mice and the double heterozygous offspring were backcrossed to *ct/ct* to produce *ct/ct*; *Mthfr*^{+/−}; and *ct/+*; *Mthfr*^{+/−} mice, which were used to generate litters for analysis (Table 2a). Mice were maintained by intercross of *Mthfr*^{+/−}; *ct/ct* with *Mthfr*^{+/+}; *ct/ct* in order to generate *Mthfr*^{+/−}; *ct/ct* mice for further experimental matings (Table 2b). In crosses to *Mthfr* mutants, the *curly tail* allele was genotyped by PCR amplification of genomic DNA, on the basis of the putative mutation, C-21350T upstream of *Grhl3* (Gustavsson et al., 2007).

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 standard diet (SD) or to a folate-deficient diet (FD), which contained no folic acid and included the antibiotic, 1% succinyl sulfathiazole (10 g/kg). The FD diet has previously been found to result in profound folate deficiency (Burren et al., 2008; Burren et al., 2010). Females were maintained on each diet for a minimum of 3 weeks before mating to SD-fed males.

Production of mouse embryonic fibroblasts and deoxyuridine (dU) suppression test

Embryonic fibroblast primary cell lines were prepared by standard methodology and cultured in DMEM medium containing 10% fetal bovine serum and 100 units penicillin/100 µg streptomycin. Confluent cells were trypsinised, washed in PBS and divided between two tubes for resuspension in either RPMI 1640 medium containing 10% fetal bovine serum and 100 units penicillin/100 µg streptomycin or RPMI 1640 without folic acid containing 10% charcoal-stripped fetal bovine serum and 100 units penicillin/100 µg streptomycin. Cells (0.5×10^6 cells /well) were seeded in triplicate into a 12-well plate and cultured overnight. Deoxyuridine (dU; 10 µM) or PBS were added followed, after 2 hours culture, by [³H]thymidine (0.5 µCi/ml). After overnight culture, cell pellets were collected for DNA extraction and measurement of [³H]thymidine incorporation (Dunlevy et al., 2007). DNA concentration was determined by NanoDrop. The assay was performed in triplicate for each cell type and repeated once.

Quantification of S-adenosylmethionine and S-adenosylhomocysteine

Samples consisting of individual embryos were analysed in duplicate by liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) as described previously (Burren et al., 2006).

RT-PCR and Quantitative Real Time RT-PCR

Total mRNA was extracted from mouse embryonic fibroblasts by the Trizol method and first strand cDNA was synthesized by reverse transcription using Superscript II (Invitrogen) with random hexamers. Gene expression was determined by RT-PCR and by quantitative real time RT-PCR using MESA Blue pPCR Mastermix (Eurogentec) according to the manufacturer's instructions on a 7500 Fast Real Time PCR system (Applied Biosystems), with each sample analysed in triplicate. Specific primers used to amplify *Grhl3* were 5'-CTCGGAGAAGGAAGATGTCG and 5'-AGTATCCGCTTCTCCTTGG and results were normalized with respect to *Gapdh* (as previously; Gustavsson et al., 2007).

Results

Thymidylate biosynthesis is not deficient in curly tail embryos

Owing to the sensitivity of cranial neural tube closure in the *curly tail* strain to folate deficiency, we tested for an inherent defect of folate metabolism. Primary fibroblastic cell lines were generated from *ct/ct* and *+^{ct}/+^{ct}* embryos and RT-PCR analysis confirmed that *Grhl3* is expressed in primary embryonic fibroblasts (data not shown). Quantitative real time RT-PCR showed that the level of *Grhl3* expression in *ct/ct* primary fibroblasts is only around half of that in *+^{ct}/+^{ct}* cells (44-49% relative expression).

Cell lines were subjected to the deoxyuridine suppression test to provide a measure of the efficiency of pyrimidine biosynthesis, as used previously in primary fibroblast cell lines (Dunlevy et al., 2007). Deoxythymidine monophosphate (dTMP), the precursor of pyrimidines, can be generated from dU monophosphate (dUMP) by the folate-dependent action of thymidylate synthase (*de novo* synthesis) or from phosphorylation of thymidine (the salvage pathway). Provided that 5,10-methylenetetrahydrofolate is available as co-factor, addition of dU to the culture medium stimulates *de novo* synthesis and thereby suppresses incorporation of exogenous thymidine into DNA by the salvage pathway. In both standard and low-folate media, addition of dU suppressed [³H] thymidine incorporation as expected, in *ct/ct* as well as *+^{ct}/+^{ct}* cells (Fig. 1A). In standard medium, the magnitude (%) of suppression was significantly greater in *curly tail* than in *+^{ct}/+^{ct}* fibroblasts (Fig. 1B), but in neither case was the finding suggestive of a major deficit in folate metabolism. This result is in accordance with previous use of the dU suppression test in cultured *curly tail* embryos, but for which a genetically matched wild-type control strain was not available (Fleming and Copp 1998).

In low-folate medium, thymidine incorporation was increased in all experimental groups (Fig. 1A), presumably reflecting increased demand for exogenous thymidine as folate availability is limited, and thymidylate biosynthesis is thereby reduced. The magnitude of suppression by dU was comparable in low folate and standard conditions (Fig. 1B). Although the percentage suppression of [³H] thymidine uptake did not differ between *ct/ct* and *+^{ct}/+^{ct}* cells under low-folate conditions, we noted a significantly lower uptake of [³H] thymidine into DNA in *curly tail* cells than in *+^{ct}/+^{ct}* cells under low-folate conditions, both in the presence and absence of dU (Fig. 1A).

Curly tail embryos exhibit abnormalities in the methylation cycle under normal and folate-deficient dietary conditions

We tested whether the methylation cycle disturbance reported in adult *ct/ct* mice (Tran et al., 2002) is also present in the developing embryo and in primary embryonic fibroblasts. The abundance of s-adenosylmethionine (SAM) and s-adenosylhomocysteine (SAH) was determined in both embryos and cell lines from *ct/ct* and *+^{ct}/+^{ct}* mice. SAM/SAH ratio did not differ between *ct/ct* and *+^{ct}/+^{ct}* cell lines (73.0 ± 1.8 compared with 72.6 ± 0.7). In *+^{ct}/*

$+^{ct}$ embryos, the SAM/SAH ratio declined with gestational age from E10.5 to 11.5 (Table 1), as we found previously in CD1 and *splotch* (Sp^{2H}) embryos (Burren et al., 2006; Burren et al., 2008). In contrast, ct/ct embryos did not show this decline, but instead there was a significant increase in SAM/SAH ratio from E10.5 to 11.5 under normal dietary conditions (Table 1).

Comparison of stage-matched embryos of the differing strains showed that the SAM/SAH ratio in ct/ct embryos was significantly lower than in $+^{ct}/+^{ct}$ at E10.5, coincident with a greater abundance of SAH (Table 1). In contrast, one day later, at E11.5, the SAM/SAH ratio did not significantly differ between ct/ct and $+^{ct}/+^{ct}$ embryos. Since dietary folate-deficiency has been found to exacerbate cranial NTDs in ct/ct embryos and to induce exencephaly in $+^{ct}/+^{ct}$ embryos, we investigated the effect of a folate-deficient (FD) diet on SAM and SAH abundance. The effect of maternal dietary folate deficiency on maternal blood levels of folate and homocysteine, and on embryonic growth, developmental progression and folate status have been described previously (Burren et al., 2010). Owing to the developmental delay imposed by the FD diet (Burren et al., 2010), we compared E11.5 embryos developing under FD conditions with embryos raised on the standard diet (SD) matched for either gestational age (E11.5) or somite stage (E10.5). In $+^{ct}/+^{ct}$ embryos, FD conditions resulted in a significant elevation in concentration of both SAM and SAH compared with both age- and stage-matched SD embryos (Table 1). The greater magnitude of change in SAH concentration resulted in a reduction of the SAM/SAH ratio, as we previously observed in folate-deficient *splotch* embryos (Burren et al., 2008), which is indicative of reduced methylation potential. In contrast, folate deficiency had no apparent effect on SAH abundance in E11.5 ct/ct embryos compared with E10.5 (stage-matched by somite number) embryos on the normal diet (Table 1). However, there was a dramatic increase in abundance of SAM, resulting in a significant increase in the SAM/SAH ratio. Hence, methylation potential appears to be paradoxically increased in ct/ct embryos by dietary folate deficiency.

We found previously that maternal supplementation with folic acid results in a small, although statistically insignificant, reduction in frequency of cranial NTDs in folate-deficient $+^{ct}/+^{ct}$ and, to a lesser extent, in ct/ct embryos (Burren et al., 2010). Here, we found that folic acid treatment reduced abundance of both SAM and SAH, and increased the SAM/SAH ratio, in $+^{ct}/+^{ct}$ embryos compared with untreated folate-deficient embryos (Table 1). In fact, the SAM and SAH values reverted to levels observed in embryos that developed to the same stage under standard dietary conditions. In *curly tail* mutant embryos, SAM abundance also decreased after exogenous folic acid treatment (as did SAH abundance although this was not statistically significant). However, there was no significant effect of folic acid treatment on SAM/SAH ratio. Maternal supplementation with *myo*-inositol was found to prevent folate-related NTDs in $+^{ct}/+^{ct}$ embryos (Burren et al., 2010), and the levels of methylation cycle intermediates appeared to be normalised in inositol-treated embryos (Table 1).

Deletion of 5,10-methylenetetrahydrofolate reductase does not exacerbate curly tail NTDs

Owing to our finding of perturbed SAM and SAH levels in ct/ct embryos we reasoned that genetic manipulation of the methylation cycle might alter the penetrance of NTDs. To test this hypothesis we intercrossed *curly tail* mice with mice carrying a null allele of *Mthfr*. MTHFR generates 5-methylTHF, a key component in the remethylation of homocysteine to methionine. Loss of MTHFR function has been found to diminish SAM/SAH ratio and decrease methylation activity (Chen et al., 2001). Although *Mthfr* and *Grhl3* are only 12.3 Mb apart on Chromosome 4, we were able to generate ct/ct ; *Mthfr* $^{+/-}$ mice through successive rounds of intercrossing, and these mice were both viable and fertile. Two sets of

experimental matings were then performed to investigate the effect of *Mthfr* genotype on the *ct* phenotype (Table 2).

In an initial cross, we generated litters containing embryos of *ct/ct* and *ct/+* genotypes together with differing combinations of *Mthfr* alleles (Table 2a). This experiment produced a small number of *ct/+*; *Mthfr*^{+/+} and *ct/ct*; *Mthfr*^{-/-} embryos compared with other genotypes, owing to the requirement for recombination between the *Grhl3* and *Mthfr* loci to generate these genotypes in this cross (Table 2). As expected, there was a low frequency of tail flexion defects, and no spina bifida, among *ct/+* embryos. Interestingly, an apparent inverse relationship occurred between the frequency of tail flexion defects and the number of mutant *Mthfr* alleles but this was difficult to evaluate owing to the small number of embryos with *ct/+*; *Mthfr*^{+/+} genotype. Among *ct/ct* embryos the frequency of tail flexion defects (+ spina bifida) was 40-50%, as in the *curly tail* strain, and did not differ with *Mthfr* genotype.

A second set of experimental matings was performed in which all mice were homozygous for the *ct* mutant allele (Table 2b). The frequency of tail flexion defects among offspring of this cross was also 40-50%, as in the *curly tail* strain, and did not differ with *Mthfr* genotype. No spina bifida was detected among *ct/ct*; *Mthfr*^{-/-} embryos but, owing to the low incidence of spina bifida in all groups, the sample size in this study was too small to determine whether there is truly a protective effect of nullizygosity for *Mthfr* on the *ct/ct* NTD phenotype. Nevertheless, it is clear that loss of *Mthfr* function does not alleviate or exacerbate *curly tail* spinal NTDs. Moreover, cranial NTDs were not observed in any embryos from these matings.

In order to verify a functional effect of the *Mthfr* null allele, we quantified SAM and SAH in compound mutant embryos. As expected, the abundance of SAH was significantly elevated, and SAM/SAH ratio was reduced, in *ct/ct*; *Mthfr*^{-/-} embryos compared with *ct/ct*; *Mthfr*^{+/+} embryos (Table 3).

Discussion

The link between folate status and NTD risk is not fully understood. Sub-optimal folate status and associated markers such as elevated homocysteine are associated with increased risk of an affected pregnancy, but NTDs are not simply secondary to folate deficiency as maternal serum folate levels in most cases are within the 'normal' range (Blom et al., 2006; Daly et al., 1995; Shaw et al., 1995). Moreover, experimental studies in mice suggest that folate uptake by the developing embryo can respond to diminished maternal circulating folate, such that severe folate deficiency is required to significantly affect embryonic folate content (Burren et al., 2008). When experimental dietary folate deficiency has been generated, using synthetic diet combined with antibiotics, embryos exhibit developmental delay and growth retardation (Burgoon et al., 2002; Burren et al., 2008). However, NTDs have not been found to arise in these circumstances unless additional genetic risk factors are present. Thus, in folate deficient conditions neural tube closure is able to proceed to completion in the Swiss-Webster, ICR and CD1 strains as well as in wild-type embryos on a mixed C3H/101/CBA background (Burgoon et al., 2002; Heid et al., 1992). In contrast, the frequency of cranial NTDs was significantly increased in homozygous *spotch* (*Sp*^{2H}) and *curly tail* mutants when maintained on a folate-deficient diet (Burren et al., 2008; Burren et al., 2010). Folate deficiency also caused cranial NTDs in congenic *ct/+* embryos, that carry modifier genes of the *ct* genetic background (Burren et al. 2010). Such a model may be analogous to the situation in some human NTDs, in which it is envisaged that folate status interacts with multiple genetic risk factors which are individually insufficient to cause NTDs.

We hypothesised that sensitivity of neural tube closure to folate deficiency, in embryos with a *ct* genetic background, could be associated with an underlying defect in folate metabolism resulting in diminished thymidylate biosynthesis, as has been proposed in the *splotch* mutant (Fleming and Copp 1998). Our findings do not support this hypothesis, however, as results with the dU suppression test on fibroblastic cell lines from $+^{ct}/+^{ct}$ and *ct/ct* embryos appeared normal and did not differ between normal and low folate conditions. The observed increase in exogenous thymidine incorporation under low folate conditions presumably reflects reduced thymidylate biosynthesis in conditions of folate scarcity, together with a 'preferred' use of the salvage pathway. However, under low folate conditions the magnitude of suppression by dU was comparable to that in standard medium, suggesting that there is no diminution of the capacity for up-regulation of thymidylate synthase, whose activity depends on availability of 5,10-methylenetetrahydrofolate.

Interestingly, we observed differences in both dU suppression (standard conditions) and [^3H]thymidine incorporation (low-folate conditions) in comparisons of *ct/ct* and $+^{ct}/+^{ct}$ cell lines. These differences are likely to relate to the reduced level of *Grhl3* expression, which is known to persist in cultured *ct/ct* fibroblasts, as in neurulation-stage embryos (Gustavsson et al., 2007). The small increase in % dU suppression in *ct/ct* cells is suggestive of improved efficacy of thymidylate biosynthesis compared with $+^{ct}/+^{ct}$ cells, although this difference was not observed under low folate conditions. The reduced incorporation of exogenous thymidine into DNA in *ct/ct* compared with $+^{ct}/+^{ct}$ cells, in the absence of any proliferation deficit (data not shown), also suggests that the requirement for one-carbon units for thymidylate synthesis can be readily met from folate metabolism.

We next focussed on the methylation cycle owing to the previous observation of altered homocysteine and SAM/SAH levels in adult *curly tail* mice (Tran et al., 2002) and the fact that inhibition of the methylation cycle inhibits neural tube closure in mouse and chick embryos (Afman et al., 2005; Dunlevy et al., 2006b). A possible role for diminished function of methylation reactions in some human NTDs is also suggested by the increased risk of NTDs associated with polymorphisms in *MTHFR* (Amorim et al., 2007; Molloy et al., 2009), and combinations of methionine synthase (*MTR*) and methionine synthase reductase (*MTRR*) polymorphisms (Gueant-Rodriguez et al., 2003; Zhu et al., 2003) (see Greene et al., 2009 for list of studies). It is thus possible that cranial NTDs induced by folate deficiency in $+^{ct}/+^{ct}$ embryos result in part from the observed reduction in SAM/SAH ratio, which is hypothesised to suppress methylation reactions. However, this is unlikely to be the sole factor as folate deficiency does not induce NTDs in other wild-type strains (which lack the *ct* background modifiers). Nevertheless, the protective effect of folic acid supplements in folate-deficient $+^{ct}/+^{ct}$ embryos (Burren et al., 2010) was associated with normalisation of the SAM/SAH ratio. *Myo*-inositol has a greater preventive effect than folic acid in this model (Burren et al., 2010), and remarkably this treatment also normalised SAM/SAH ratio.

The diminished SAM/SAH ratio in *ct/ct* compared with $+^{ct}/+^{ct}$ embryos at E10.5, could be suggested to contribute to the cranial NTDs that are observed in around 5-10% of *ct/ct* embryos on a standard diet. However, arguing against this idea, the significant further reduction in SAM/SAH ratio in *ct/ct*, *Mthfr*^{-/-} compound mutant embryos was not sufficient to cause cranial NTDs under standard dietary conditions. This finding also supports the idea that folate-related cranial NTDs in $+^{ct}/+^{ct}$ embryos do not result primarily from diminished methylation (see above). It also appears unlikely that diminished (E10.5) or elevated (E11.5) SAM/SAH ratio contributes to the development of spinal NTDs as the frequency of tail flexion defects was unaffected by embryonic *Mthfr* genotype and hence was neither exacerbated nor rescued by a significant reduction in SAM/SAH ratio. Whilst we did not observe spina bifida in *curly tail* embryos that were null for MTHFR, suggesting possible rescue, the frequency did not exceed 10% in any genotype, making a significant reduction in

spina bifida occurrence difficult to detect. While embryonic SAM/SAH was clearly reduced in compound mutant embryos, the experimental design did not allow evaluation of a possible effect of maternal *Mthfr* genotype (Li et al., 2005; Pickell et al., 2009), which could potentially influence embryonic development perhaps through a different mechanism.

The response of *ct/ct* embryos to folate deficiency was notable in that the SAM/SAH ratio increased rather than being reduced, as is observed in *+^{ct}/+^{ct}* embryos (this study), other mouse strains (Burren et al., 2008) or predicted by computational models (Nijhout et al., 2006). This is despite the fact that maternal plasma homocysteine is significantly elevated by folate deficiency in both *ct/ct* and *+^{ct}/+^{ct}* embryos (Burren et al., 2010). It appears unlikely, therefore, that folate deficiency-induced cranial NTDs in *ct/ct* embryos result from a global loss of methylation potential, particularly as cranial NTDs were not induced by loss of MTHFR (which resembles folate deficiency in causing elevated homocysteine) (Chen et al., 2001). Together these data suggest that the deleterious effect of folate deficiency on cranial neural tube closure involves factors other than suppression of methylation. We speculate that such a mechanism could involve diminished nucleotide biosynthesis or depletion of choline metabolites (as utilisation of choline for remethylation of homocysteine is enhanced by low folate/high homocysteine), which has been associated with NTDs in mice and humans (Fisher et al., 2002; Schwahn et al., 2003; Shaw et al., 2009).

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Figure 1. Analysis of folate-dependent thymidylate biosynthesis using the deoxyuridine (dU) suppression test in curly tail (ct/ct) and congenic ($+^{ct}/+^{ct}$) cells

(A) Incorporation of [3 H]thymidine into dTMP (and thence to DNA), via the thymidine kinase-mediated salvage pathway (white bars), is suppressed by addition of dU which stimulates *de novo* synthesis of dTMP by thymidylate synthase (grey bars). Reduction of folate availability (culture in low folate medium) increases the incorporation of [3 H]thymidine into cells of both genotypes (# indicates significant difference compared with equivalent genotype/condition for standard medium, $p < 0.001$). Under 'low folate' conditions the incorporation is diminished in ct/ct cells compared with $+^{ct}/+^{ct}$ (* indicates significant difference compared with $+^{ct}/+^{ct}$ cells under the same conditions, $p < 0.02$). (B)

The % suppression of thymidine incorporation by dU is higher in *ct/ct* cells than in *+^{ct}/+^{ct}* cells under standard culture conditions (* indicates significant difference compared with *+^{ct}/+^{ct}*, $p < 0.05$), but not under 'low folate' conditions.

Table 1
Quantification of methylation cycle intermediates in $+^{ct}/+^{ct}$ and curly tail embryos

Measurement of s-adenosylmethionine (SAM) and s-adenosylhomocysteine (SAH) was by LC-MS/MS, with normalisation to protein content. Values are given as mean $\pm$ SEM. Whereas SAM/SAH ratio declined with developmental age in $+^{ct}/+^{ct}$ embryos, the SAM/SAH ratio was higher at E11.5 compared with E10.5 in ct/ct embryos (** indicates significant difference compared with E10.5 ct/ct embryos on the standard (SD) diet, $p < 0.01$). At E10.5, the SAM/SAH ratio was significantly lower in ct/ct than in $+^{ct}/+^{ct}$ embryos, coincident with increased SAH abundance (* indicates significant difference compared with age-matched $+^{ct}/+^{ct}$ embryos on the same diet, $P < 0.025$). The folate deficient (FD) diet caused significant changes in levels of SAM, SAH and the SAM/SAH ratio (# shows significant difference compared with all other groups within the same strain, $p < 0.05$, ANOVA). Notably, FD caused a reduction of the SAM/SAH ratio in $+^{ct}/+^{ct}$ embryos, but its elevation in ct/ct embryos (## shows significant difference compared with somite-matched (E10.5) ct/ct embryos on SD, $p < 0.05$, ANOVA).

Diet/treatment	Gest. age	No.	No. somites	SAM (nmol/mg protein)	SAH (nmol/mg protein)	Ratio SAM/SAH
$+^{ct}/+^{ct}$						
SD	E10.5	15	29.2 $\pm$ 0.5	2.81 $\pm$ 0.18	0.031 $\pm$ 0.002	93 $\pm$ 5
SD	E11.5	8	38.6 $\pm$ 0.9	1.98 $\pm$ 0.08	0.025 $\pm$ 0.001	79 $\pm$ 3
FD	E11.5	7	32.6 $\pm$ 1.2	4.01 $\pm$ 0.47 [#]	0.116 $\pm$ 0.030 [#]	48 $\pm$ 11 [#]
FD+FA	E11.5	5	31.4 $\pm$ 0.9	2.44 $\pm$ 0.16	0.029 $\pm$ 0.003	87 $\pm$ 6
FD+MI	E11.5	5	33.2 $\pm$ 1.9	2.38 $\pm$ 0.16	0.03 $\pm$ 0.008	104 $\pm$ 26
ct/ct						
SD	E10.5	15	29.5 $\pm$ 0.3	3.27 $\pm$ 0.25	0.044 $\pm$ 0.004*	76 $\pm$ 3 *
SD	E11.5	9	39.8 $\pm$ 0.8	2.84 $\pm$ 0.27*	0.027 $\pm$ 0.003	119 $\pm$ 19**
FD	E11.5	16	29.8 $\pm$ 1.4	5.18 $\pm$ 0.75 [#]	0.045 $\pm$ 0.012	195 $\pm$ 43 ^{##}
FD+FA	E11.5	9	27.6 $\pm$ 0.8	2.98 $\pm$ 0.24	0.025 $\pm$ 0.004	152 $\pm$ 38 ^{##}

Table 2
NTDs among curly tail; *Mthfr* compound mutants

Sixteen litters were initially examined (a) and a further twelve litters were examined from matings in which all mice were homozygous for the *ct* mutation (b). Embryos were examined at E11.5 for the presence of NTDs and/or tail flexion defects and no statistically significant differences were noted. Note that in mating (a), the *ct* and *Mthfr*⁻ alleles were in repulsion in the double heterozygote parent. Hence, the offspring genotypes *ct*/+; *Mthfr*^{+/+} and *ct*/*ct*; *Mthfr*^{-/-} could only be generated following a recombination event between the two loci. These recombinant genotypes occurred at low frequency compared with the other, non-recombinant, genotypes for this reason.

Mating & embryonic genotypes	No. embryos	Tail defect/NTD phenotype		
		No defects	Curly/bent tail	Spina bifida & curly tail
a. <i>ct/ct</i> ; <i>Mthfr</i>^{+/-} X <i>ct/+</i> ; <i>Mthfr</i>^{+/-}				
<i>ct/+</i> ; <i>Mthfr</i> ^{+/+}	4	2 (50%)	2 (50%)	0
<i>ct/+</i> ; <i>Mthfr</i> ^{+/-}	27	23 (85%)	4 (15%)	0
<i>ct/+</i> ; <i>Mthfr</i> ^{-/-}	16	16 (100%)	0	0
<i>ct/ct</i> ; <i>Mthfr</i> ^{+/+}	16	8 (50%)	8 (50%)	0
<i>ct/ct</i> ; <i>Mthfr</i> ^{+/-}	17	7 (41%)	8 (47%)	2 (12%)
<i>ct/ct</i> ; <i>Mthfr</i> ^{-/-}	2	1 (50%)	1 (50%)	0
b. <i>ct/ct</i> ; <i>Mthfr</i>^{+/-} X <i>ct/ct</i> ; <i>Mthfr</i>^{+/-}				
<i>ct/ct</i> ; <i>Mthfr</i> ^{+/+}	31	16 (52%)	13 (42%)	2 (6%)
<i>ct/ct</i> ; <i>Mthfr</i> ^{+/-}	37	20 (54%)	16 (43%)	1 (3%)
<i>ct/ct</i> ; <i>Mthfr</i> ^{-/-}	23	13 (57%)	10 (43%)	0

Table 3
Quantification of methylation cycle intermediates in *curly tail/Mthfr* compound mutant embryos

Stage-matched embryos were analysed at E10.5 and values are given as mean $\pm$ SEM. Homozygosity for the null *Mthfr* allele resulted in a significant elevation of SAH abundance and reduction in SAM/SAH ratio (* indicates significant difference compared with *ct/ct; Mthfr*^{+/+}, $p < 0.001$).

Embryo genotype	No. embryos	SAM (nmol/mg protein)	SAH (nmol/mg protein)	Ratio SAM/SAH
<i>ct/ct; Mthfr</i> ^{+/+}	3	2.11 ($\pm$ 0.56)	0.045 ($\pm$ 0.009)	44.6 ($\pm$ 4.5)
<i>ct/ct; Mthfr</i> ^{-/-}	4	3.34 ($\pm$ 0.32)	0.776 ($\pm$ 0.065)*	4.4 ($\pm$ 0.4)*