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Original Article

ORIGINAL ARTICLE

# Testicular MTHFR deficiency may explain sperm DNA hypomethylation associated with high dose folic acid supplementation

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## Abstract

Supplementation with high doses of folic acid, an important mediator of one-carbon transfers for DNA methylation, is used clinically to improve sperm parameters in infertile men. We recently detected an unexpected loss of DNA methylation in the sperm of idiopathic infertile men after 6 months of daily supplementation with 5 mg folic acid (>10× the daily recommended intake—DRI), exacerbated in men homozygous for a common variant in the gene encoding an important enzyme in folate metabolism, methylenetetrahydrofolate reductase (*MTHFR* 677C>T). To investigate the epigenomic impact and mechanism underlying effects of folic acid on male germ cells, wild-type and heterozygote mice for a targeted inactivation of the *Mthfr* gene were fed high-dose folic acid (10× the DRI) or control diets (CDs) for 6 months. No changes were detected in general health, sperm counts or methylation of imprinted genes. Reduced representation bisulfite sequencing revealed sperm DNA hypomethylation in *Mthfr*$^{+/-}$ mice on the 10× diets. Wild-type mice demonstrated sperm hypomethylation only with a very high dose (20×) of folic acid for 12 months. Testicular MTHFR protein levels decreased significantly in wild-type mice on the 20× diet but not in those on the 10× diet, suggesting a possible role for MTHFR deficiency in sperm DNA hypomethylation. In-depth analysis of the folic acid-exposed sperm DNA methylome suggested mouse/human susceptibility of sequences with potential importance to germ cell and embryo development. Our data provide evidence for a similar cross-species response to high dose folic acid supplementation, of sperm DNA hypomethylation, and implicate MTHFR downregulation as a possible mechanism.

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## Introduction

Environmental exposures such as diet and stress may affect not only the health of individuals but may also be transmitted to their offspring (1–3). Epigenetic modifications are one of the mechanisms that can alter gene activity in response to environment, without changing the DNA sequence (4,5). Epigenetic patterning of parental gametes and *in utero* environment influence offspring susceptibility to metabolic and chronic disorders (6–9). Reports of epigenetic modifications occurring during specific developmental windows of susceptibility in the male germline suggest that sperm may transmit environmentally induced epigenetic modifications to the next generation (10). Such modifications may involve histones, coding and non-coding RNAs and DNA methylation.

DNA methylation, a well characterized epigenetic modification, occurs at the 5-position of approximately 30 million cytosine residues throughout the human genome, mainly within CpG dinucleotide sequences (11,12). DNA methylation is catalyzed by DNA methyltransferase enzymes (DNMTs) which use methyl groups provided by S-adenosylmethionine (SAM) (7,13). SAM is in turn a major product of folate-dependent one-carbon metabolism.

Several biochemical pathways, including nucleotide synthesis and production of methyl group donating enzyme cofactors, depend on one-carbon folate metabolism. The reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methylTHF via 5,10-methyleneTHF reductase (MTHFR) and the subsequent transfer of the methyl group from 5-methylTHF to homocysteine results in the production of methionine. Methionine is used to synthesize SAM which may subsequently be used in DNA methylation (14,15). Folate deficiency or its aberrant metabolism may alter SAM production and DNA methylation with possible health consequences (16–18). Furthermore, a common polymorphism in the gene for *MTHFR* (677C>T) results in lower MTHFR activity, lower folate status, higher concentrations of circulating homocysteine and reduced lymphocyte DNA methylation (19–21). Individuals with the *MTHFR* 677TT genotype are predisposed to various disorders including male infertility in some populations (22,23).

Men presenting with infertility are often supplemented with high dose folic acid (up to 5 mg daily intake; >10 times the daily recommended intake or DRI). The treatment is based on randomized studies that have shown improved sperm counts in infertile men after 5 mg daily supplementation for 6 months (24,25). In many countries, fortification of grain products with folic acid to prevent neural tube defects, in addition to other sources of supplements (e.g. other folic acid fortified products and multivitamins), have led to higher intakes of folate (26,27). Therefore, it is crucial to investigate the possible consequences of excess folic acid supplementation on the health of recipients and their offspring. We recently studied 30 normozoospermic men with idiopathic infertility, supplemented with high dose folic acid (5 mg/day) for 6 months (28). While no improvements were observed in sperm concentration, loss of methylation across the sperm DNA methylome was detected using sensitive next-generation sequencing-based approaches. Such loss of methylation was contrary to our expectations since folates mediate one-carbon transfers that supply methyl groups for SAM synthesis. In addition, individuals with the *MTHFR* 677TT genotype were more susceptible to epigenetic alterations in important disease-related genes after supplementation (28). Here, mouse studies were used to better understand the epigenetic impact of high dose folic acid supplementation on sperm and the testis. We utilized folic acid–supplemented diets and mice with mild MTHFR deficiency, a model for the human *MTHFR* 677TT genotype (29). Mouse studies enabled us to search for cross-species responses of the sperm epigenome to high dose folic acid supplements and MTHFR deficiency and to access testicular samples to study underlying mechanisms.

## Results

### High folic acid intake and *Mthfr* genotype had no effects on body and reproductive organ weights

BALB/c *Mthfr*$^{+/+}$ (a model for the human *MTHFR* 677CC genotype) and *Mthfr*$^{+/-}$ (a model for *MTHFR* 677TT individuals) male mice were randomly assigned (5/group) to control (2 mg/kg; CD) and 10× folic acid–supplemented (20 mg/kg; 10FS) amino acid–defined diets at weaning (Fig. 1A). After 6 months on the diets, mice were sacrificed, body and reproductive organ weights were recorded and sperm and testis tissues were collected. Analysis of body and reproductive organ weights showed no differences among the four groups (Fig. 1B–E). Thus, neither the *Mthfr* genotype nor folic acid supplementation showed a pronounced effect on the general health of the mice.

### Methylation of imprinted loci in sperm was unaffected by folic acid supplementation

To study the epigenetic impact of high folic acid intake on sperm, we initially investigated the methylation status of imprinted genes. Bisulfite pyrosequencing was utilized to assess the germline differentially methylated regions (DMRs) of the paternally methylated *H19* and the maternally methylated *Snrpn* imprinted loci. Assays were designed to analyze methylation status of 6 and 5 CpGs for *H19* and *Snrpn*, respectively. In somatic tissues, either the paternal or the maternal allele of imprinted genes is completely methylated while the opposite allele is not methylated. Consequently, the average methylation of imprinted genes in a somatic tissue is approximately 50%. Conversely in sperm DNA, where only one parental allele is present, methylation levels of paternally and maternally methylated loci are expected to be 100% and 0%, respectively. Analysis of sperm DNA showed that the methylation status of both imprinted loci was within the normal range in individual animals regardless of *Mthfr* genotype or diet (Fig. 2A and B). Average methylation values for the two imprinted loci were similar among different experimental groups (Fig. 2C and D). Overall, similar to our previous findings in human (28), neither *Mthfr* genotype nor high folic acid intake for 6 months affected the methylation status of imprinted genes.

### Alteration of the sperm DNA methylome depended on level of folic acid intake and *Mthfr* genotype

To analyze the global impact of high dose folic acid supplementation on the sperm DNA methylome, we used the LUminometric Methylation Assay (LUMA). The assay measured the overall proportion of methylated CpGs across the sperm epigenome and detected no significant differences among experimental groups (Fig. 3A). To further assess the sperm DNA methylome at a single nucleotide resolution level, we utilized a next-generation sequencing-based method: reduced representation bisulfite sequencing (RRBS). Analysis of methylation status of approximately 1.7 million CpGs, in 100 base pair (bp) tiles across the sperm epigenome was performed for all samples

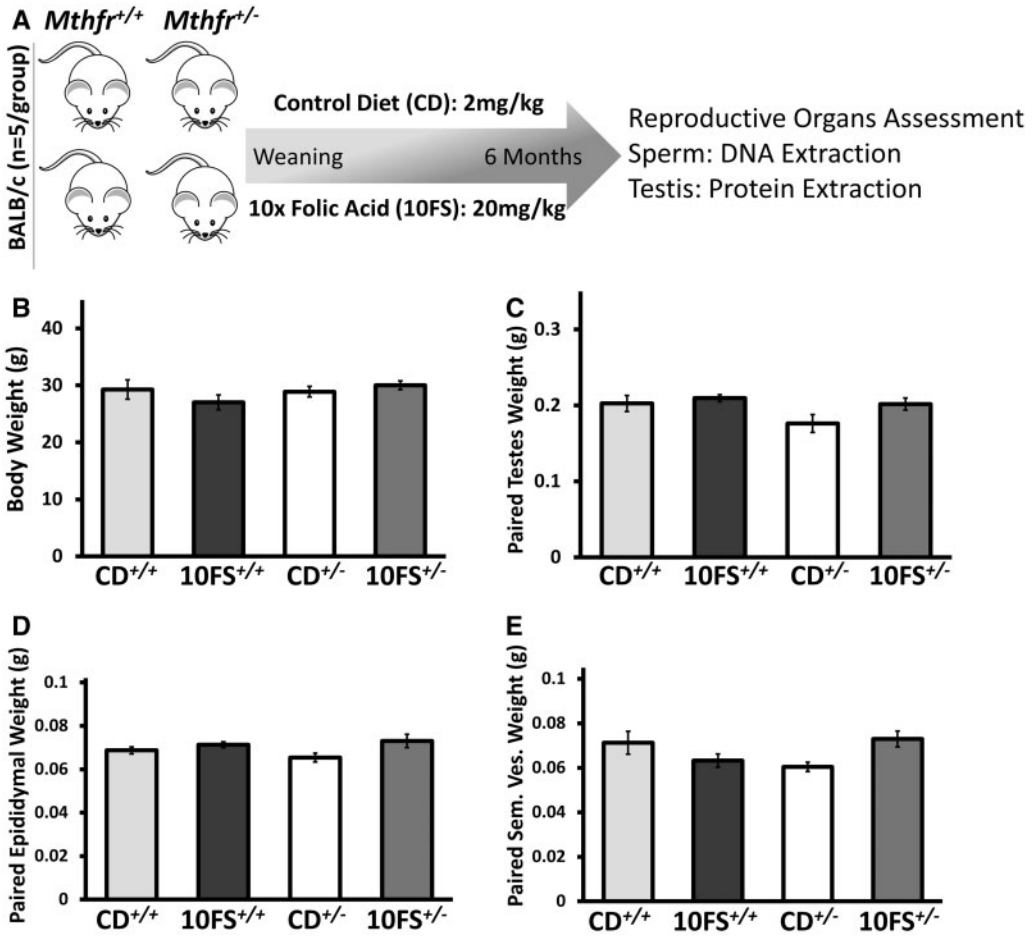


Figure 1. Experimental design and general assessment of mice. *Mthfr*$^{+/+}$ and *Mthfr* $^{+/-}$ mice were fed either high dose folic acid diet (10-times the daily recommended intake; 10FS) or CD for 6 months after weaning (**A**). At 6 months, the mice were sacrificed and body weights (**B**) and reproductive organ weights (**C–E**) were compared for the different genotype/diet groups. Error bars represent standard error of means (SEM); Sem. Ves.: seminal vesicles.

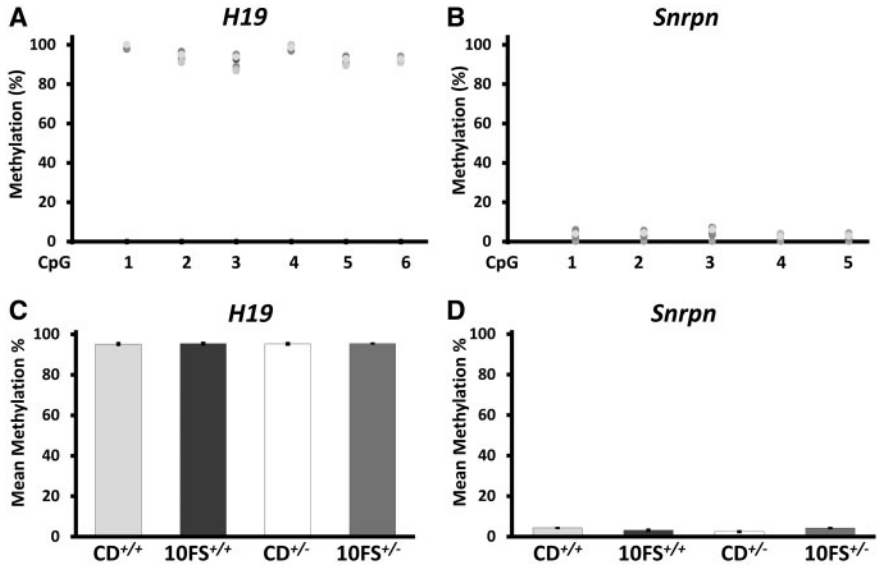


Figure 2. Methylation of germline DMRs of imprinted genes remained unchanged after high dose folic acid supplementation. Six CpGs in the paternally methylated locus *H19* and five CpGs in the maternally methylated locus *Snrpn* were examined by bisulfite pyrosequencing (**A**, **B**). Each dot ($n = 5$ dots/group or 20 dots shown per CpG site interrogated) demonstrates methylation of each individual mouse, regardless of *Mthfr* genotype or type of diet, at the corresponding CpGs. In a separate analysis, experimental groups ($n = 5$/group with average values across the CpGs averaged on a per group basis) were compared further to assess the impact of genotype/diet on mean methylation of two imprinted loci (**C, D**). Columns and error bars represent the mean methylation levels of each group and the standard error of means (SEM), respectively; CD: control diet, 10FS: 10× folic acid supplemented.

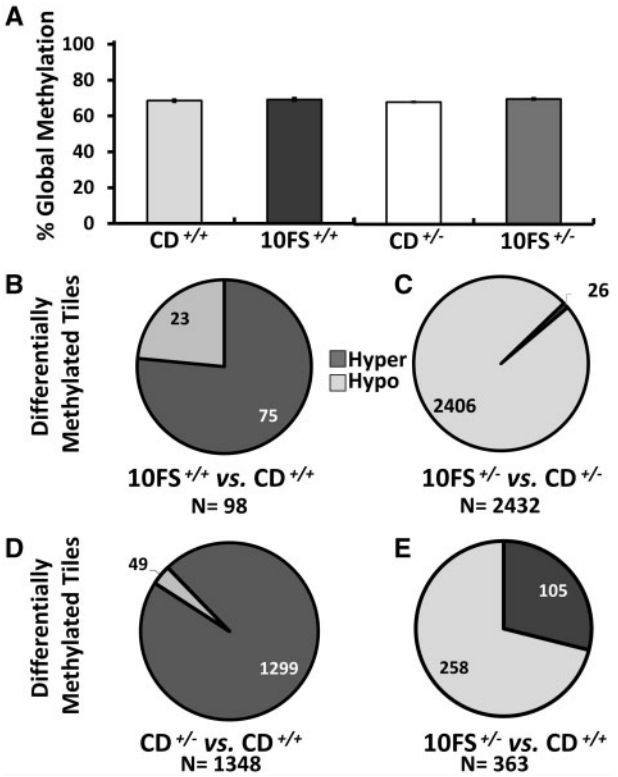


**Figure 3.** *Mthfr*$^{+/-}$ mouse sperm DNA methylome undergoes hypomethylation after 10FS diet for 6 months. Global DNA methylation levels across the mouse sperm epigenome, measured by LUMA, revealed no difference among *Mthfr* genotype/folic acid experimental groups (**A**). To assess the genome-wide DNA methylation at single nucleotide resolution, RRBS was performed followed by analysis of DMTs with significant >20% change of methylation at >10-fold coverage for *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ experimental groups (**B–E**). Pie graphs demonstrate DMT numbers (*N*) and proportion of hyper/hypo-methylated tiles (numbers on pie graphs) in pairwise comparisons of the four experimental groups. Error bars in (A) represent standard error of means (SEM); CD: control diet, 10FS: 10× folic acid supplemented.

(Supplementary Material, Table S1). In contrast to the loss of methylation previously reported in the sperm of *MTHFR* 677CC men, the sperm of their mouse homologs (*Mthfr*$^{+/+}$) were minimally affected by 10× folic acid supplementation after 6 months. Only 98 differentially methylated tiles (DMTs) with significant >20% changes in methylation at a minimum 10-fold sequencing coverage were found, most of which demonstrated hypermethylation (Fig. 3B and Supplementary Data File 1). In contrast, *Mthfr*$^{+/-}$ mice showed a similar response to *MTHFR* 677TT men and demonstrated significant hypomethylation of the sperm DNA methylome after 10× folic acid supplementation for 6 months (Fig. 3C). To study the *Mthfr* genotype effects alone, *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice on the CD were compared. While no significant difference was detected in global methylation levels (Fig. 3A), sperm DNA of *Mthfr*$^{+/-}$ mice contained 1348 DMTs, most of which were hypermethylated (Fig. 3D and Supplementary Data File 1). However, high folic acid intake appeared to counteract the genotype effect and resulted in mostly hypomethylated DMTs, as evident by comparing *Mthfr*$^{+/+}$ mice on the CD to *Mthfr*$^{+/-}$ mice on the 10FS diet (Fig. 3E and Supplementary Data File 1).

We hypothesized that providing folic acid at 10× the DRI for 6 months may be tolerated by the *Mthfr*$^{+/+}$ mouse testis, possibly due to differences between mouse and human in handling of high folate levels in the testis. Therefore, a second cohort including two separate groups of BALB/c *Mthfr*$^{+/+}$ males was randomly assigned to control (2 mg/kg CD, $n=4$) and 20× folic acid–supplemented (40 mg/kg 20FS, $n=4$) amino acid–defined diets at weaning and sacrificed after 12 months (Fig. 4A). Similar to the 10× supplemented experimental groups, no effects were observed on body weight, reproductive organ weights (Fig. 4B–E) and sperm global (LUMA) DNA methylation (Fig. 4F). However, genome-wide analysis of the sperm DNA methylome at single nucleotide resolution revealed a significant loss of methylation in the sperm of the 20FS mice marked by a high number of hypomethylated DMTs (Fig. 4G and Supplementary Data File 1). To validate the RRBS findings, we selected seven DMTs representing different genomic regions with loss/gain of methylation and analyzed their methylation status in control and FS mice by bisulfite pyrosequencing. For each DMT region, 1–2 pyrosequencing assays were designed; primers are listed in Supplementary Material, Table S2. As shown in Supplementary Material, Table S3 and Figure S1, six of seven DMTs showed significant differential methylation in the same direction as detected by RRBS. Together, our RRBS data indicated that high folic acid intakes altered the sperm DNA methylome in mice; the extent of the changes was influenced by folic acid dose, duration of exposure and *Mthfr* genotype.

## Testicular MTHFR protein levels decreased in response to 20FS diets

The fact that sperm from *Mthfr*$^{+/-}$ mice and *MTHFR* 677TT men were both hypomethylated in response to 10× folic acid supplementation suggested possible mechanistic involvement of the key enzyme in folate metabolism, MTHFR. Since we had observed that MTHFR protein expression was downregulated in liver following 10× folic acid supplementation in mice (30), we hypothesized that a similar phenomenon might be occurring in testis. MTHFR bands on immunoblots were identified using testis samples from *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ C57BL/6 mice (Supplementary Material, Fig. S2). As depicted in Figure 5A and B, testicular MTHFR protein levels in BALB/c mice were decreased as expected in *Mthfr*$^{+/-}$; however, there was no significant effect of 10FS diets on MTHFR levels in mice of either genotype. In contrast, testicular MTHFR expression decreased significantly in *Mthfr*$^{+/+}$ mice fed 20FS diets for 12 months as compared with CDs (Fig. 5C and D). The 20FS diet (Fig. 5D) resulted in a similar decrease in testicular MTHFR levels as the *Mthfr*$^{+/-}$ genotype (Fig. 5B). Our data demonstrate significant downregulation of testicular MTHFR levels as a possible mechanism of sperm DNA hypomethylation in wild-type mice with very high folic acid intakes.

## Intergenic and testis enhancer regions were susceptible to DNA methylation changes after high dose folic acid supplementation

To further investigate the sperm hypomethylating effects observed in the 20FS *Mthfr*$^{+/+}$ and the 10FS *Mthfr*$^{+/-}$ mice due to high dose folic acid supplementation, we next analyzed the distribution of DMTs among different genomic regions. Distributions of all sequenced tiles, regardless of their methylation levels, were compared with DMTs. Specific genomic regions were considered potentially more susceptible to excess folic acid when larger fractions of DMTs were observed within these regions. Distributions of sequenced tiles were first

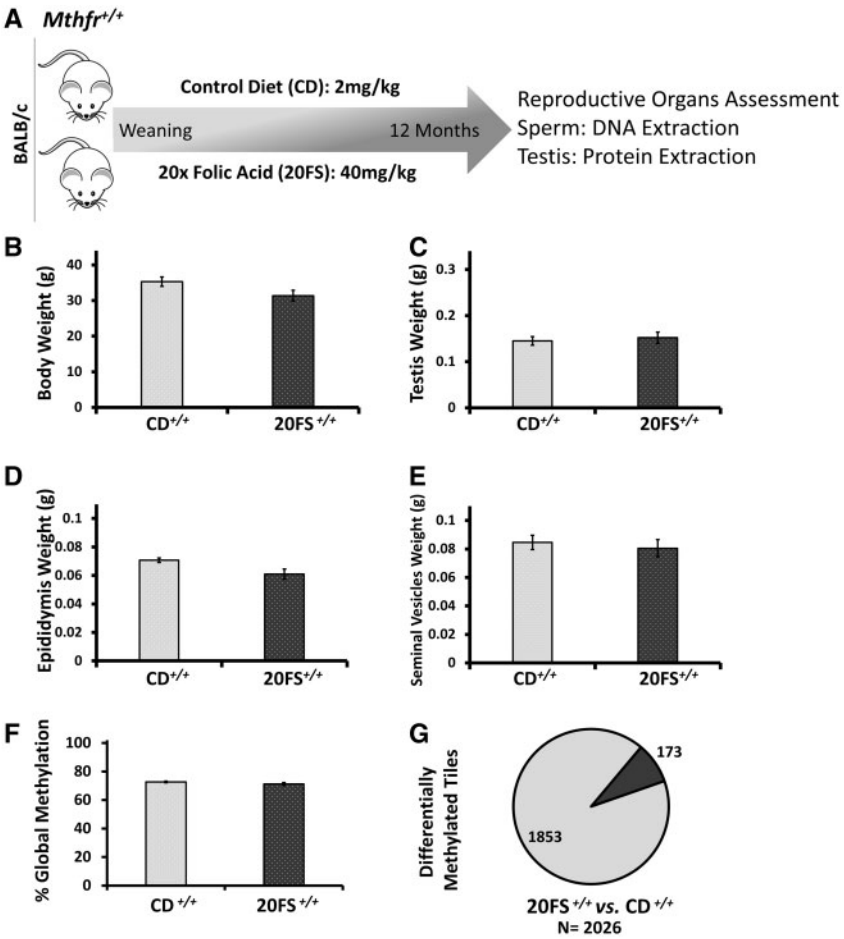


**Figure 4.** *Mthfr*$^{+/+}$ mouse sperm DNA methylome is hypomethylated after 20FS diet for 12 months. **(A)** Experimental design and general assessment of the second cohort of mice. *Mthfr*$^{+/+}$ mice were supplemented with either high dose folic acid including 20-times daily recommended intake (20FS) or CD for 12 months after weaning. At 12 months, the mice were sacrificed and further assessment was performed to compare the body weight (B) and reproductive organ weights **(C–E)** in different genotype/diet groups. Global DNA methylation levels across the mouse sperm epigenome, measured by LUMA, revealed no difference among the folic acid experimental groups **(F)**. To assess the genome-wide DNA methylation at single nucleotide resolution, RRBS was performed followed by analysis of DMTs with significant >20% change of methylation at >10-fold coverage **(G)**. Pie graph demonstrate DMT numbers (N) and proportion of hyper (dark gray)/hypo (light gray)-methylated tiles (numbers on pie graphs). Error bars represent standard error of mean (SEM); CD: control diet, 20FS: 20× folic acid supplemented.

analyzed among different intergenic and genic (exons, introns, promoters, 3′ and 5′ untranslated regions and transcription termination sites) regions and revealed higher proportions of DMTs in intergenic regions, similar to our previous findings in human sperm. Moreover, the proportion of DMTs located within promoters in the sperm DNA of folic acid–supplemented mice was lower than the overall proportion of tiles localized to promoters (Fig. 6A). Alterations of DNA methylation in intergenic regions may affect important regulatory elements such as enhancers that control gene expression. Utilizing available data of the recently identified enhancer–promoter units (31), we investigated possible enrichment for mouse testis enhancer regions in DMTs affected by high or very high dose folic acid. Indeed, the proportion of tiles corresponding to enhancer regions was significantly higher in the DMTs of the *Mthfr*$^{+/-}$ mice (FS vs. CD) when compared with the overall proportion of tiles localized to enhancers (Fig. 6B) suggesting a higher susceptibility of the sperm epigenome in regions potentially important for germ cell development, when *Mthfr* haploinsufficiency is involved. Interestingly, a subset of 163 DMTs was common between the 20FS *Mthfr*$^{+/+}$ and 10FS *Mthfr*$^{+/-}$ mice (Fig. 6C). The latter finding suggests there may be sperm epigenomic regions particularly susceptible to high folic acid intakes and MTHFR deficiency.

## High folic acid intakes affected sperm genomic regions known to escape DNA demethylation during the preimplantation and germ cell reprogramming stages

Since most DNA across the genome undergoes demethylation during the preimplantation reprogramming period, it is possible that any dietary or environmentally altered sperm DNA methylation patterns would be corrected after fertilization. However, recent data suggest that methylation at a subset of sites in sperm DNA escape demethylation and thus transmit male gamete–derived methylation patterns with possible functional consequences for the embryo (32). To assess the possibility of intergenerational transmission of DNA methylation alterations from sperm to embryo, we reanalyzed available mouse RRBS data (33) and searched for genomic regions (1000 bp tiles) that are hypermethylated (>75% methylation) in sperm and hypomethylated (<35% methylation) in oocytes (see Materials and Methods). During preimplantation reprogramming, these regions are normally expected to undergo demethylation and

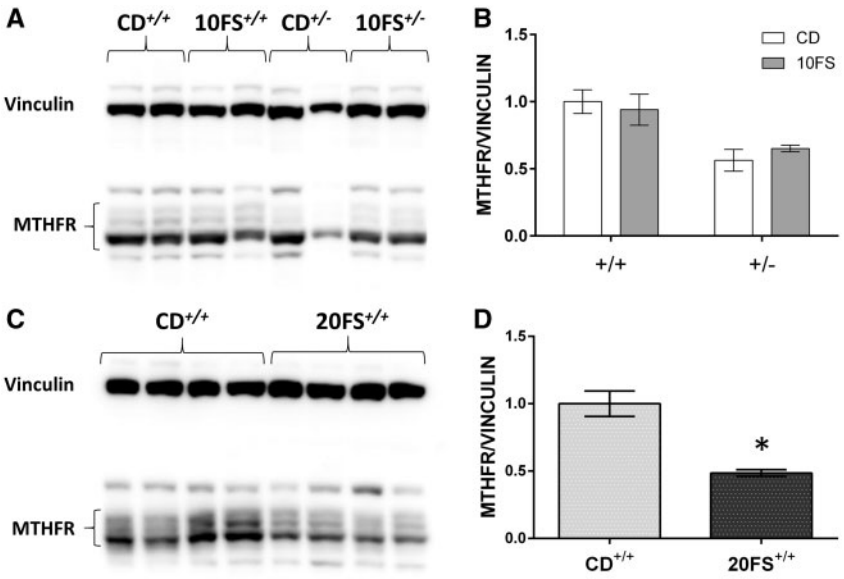


**Figure 5.** MTHFR protein levels in mouse testis decreased after 20× folic acid supplementation. Immunoblots of testis samples from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice supplemented with 10× folic acid ($n = 5$ per group) for 6 months showed that MTHFR protein levels did not change due to diet [representative blot (**A**); quantification (**B**)]. Western blot of testis samples from *Mthfr*$^{+/+}$ mice after 12 months of 20× folic acid supplementation (**C**) demonstrated significant MTHFR downregulation in 20FS mice (**D**). Error bars represent standard error of means (SEM). Asterisk shows the significant difference between control and supplemented groups (*t*-Test, $P = 0.0019$, $n = 4$ per group); CD: control diet, 10FS: 10× folic acid supplemented, 20FS: 20× folic acid supplemented.

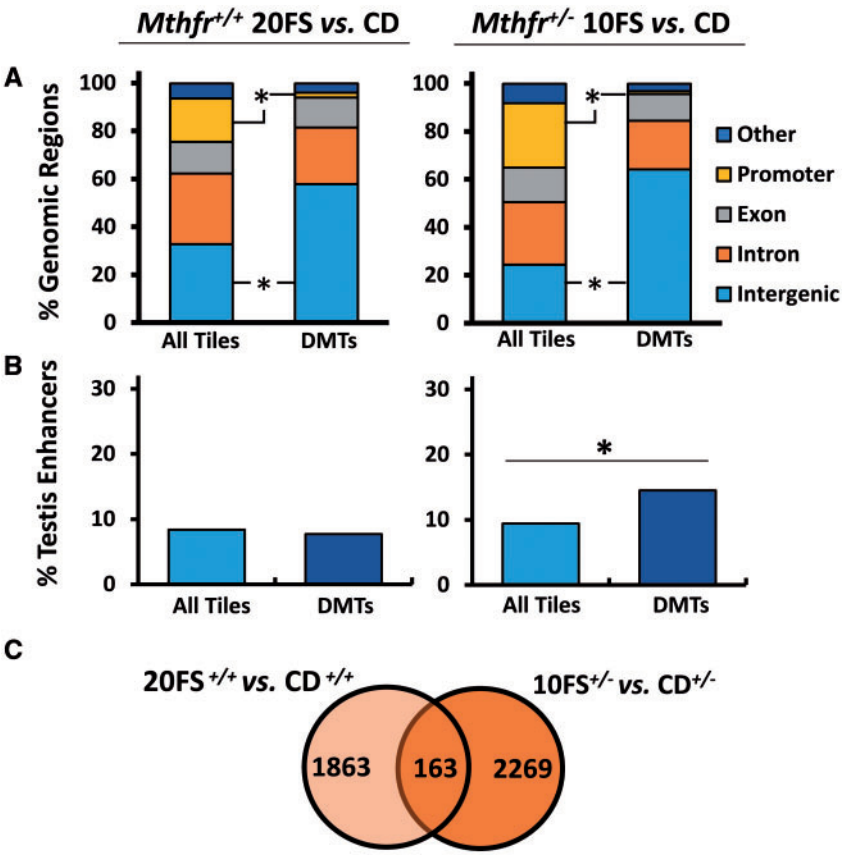


**Figure 6.** High folic acid intakes affected intergenic and enhancer regions across the mouse sperm DNA methylome. Distributions of all sequenced tiles, regardless of their methylation changes, and proportion of DMTs (DMTs with >20% change of methylation) among the two experimental groups affected by high dose folic acid are presented for different genomic regions (**A**). Enrichments for testis enhancer regions were compared between all sequenced tiles and DMTs (**B**). Common DMTs were found to be affected in both the 20FS *Mthfr*$^{+/+}$ and 10FS *Mthfr*$^{+/-}$ mice (**C**). Venn diagram demonstrates the number of DMTs specific to both groups and common between two groups. Asterisks show the significant differences between all tiles and DMTs (chi-square, $P < 0.05$); DMTs: differentially methylated tiles, CD: control diet, 10FS: 10× folic acid supplemented, 20FS: 20× folic acid supplemented.

**Table 1.** Gene ontology (GO) terms associated with mouse and human sperm genomic regions affected by high dose folic acid

| | GO terms | Count | Enrichment score[a] |
|---|---|---|---|
| **Mouse** | ***Mthfr*$^{+/-}$ 20 FS vs. CD** | | |
| | Cell signaling | 228 | 6.25 |
| | Ion binding/transport | 271 | 2.17 |
| | Nervous system development | 65 | 1.49 |
| | Reproductive structure development | 17 | 1.22 |
| | ***Mthfr*$^{+/-}$ 10FS vs. CD** | | |
| | Nervous system development | 114 | 9.7 |
| | Embryonic morphogenesis | 45 | 4.5 |
| | Intracellular signaling cascade | 90 | 2.94 |
| | Cell adhesion | 55 | 2.8 |
| | Transcription factor activity | 80 | 2 |
| | Reproductive structure development | 22 | 1.99 |
| | Neural tube development | 14 | 1.56 |
| **Human** | ***MTHFR 677 CC after 10× folic acid*** | | |
| | None | — | — |
| | ***MTHFR 677 CT after 10× folic acid*** | | |
| | Cell migration | 12 | 2.02 |
| | Nervous system development | 16 | 1.72 |
| | ***MTHFR 677 TT after 10× folic acid*** | | |
| | Cell migration | 14 | 1.74 |
| | Ion binding/transport | 27 | 1.25 |

[a]Enrichment score calculated as the log of the modified Fisher exact P-value, see Materials and Methods.

present very low methylation levels in the inner cell mass (ICM). We followed the fate of sperm-specific hypermethylated regions and found potential preimplantation 'escapees' that demonstrated medium methylation (35–65%) in the ICM. Such medium methylation levels would presumably be due to the average methylation of a paternally hypermethylated, and not reprogrammed, allele and a maternally hypomethylated allele. We could identify 4340 preimplantation 'escapee' regions by this approach; 90 DMTs in 10FS *Mthfr*$^{+/-}$ mice and 20FS *Mthfr*$^{+/+}$ mice were in such regions (Supplementary Material, Table S4). The second wave of embryonic DNA demethylation occurs during germline methylation reprogramming in primordial germ cells (PGCs). Recent data suggest that not all DNA regions in PGCs undergo demethylation (34). We asked whether any of the germ cell 'escapee' regions may be among the tiles affected by high dose folic acid in mouse sperm. To do this, available mouse RRBS data (35) were reanalyzed and revealed 2435 sperm-specific regions which remained highly methylated after germline demethylation in the mouse. Overall, 31 DMTs affected in 10FS *Mthfr*$^{+/-}$ mice and 20FS *Mthfr*$^{+/+}$ mice were from regions that may escape the germline reprogramming phase (Supplementary Material, Table S4).

Next, we reanalyzed our previous human RRBS data, which reported loss of methylation in infertile men after high dose folic acid supplementation, to search for enrichment of human preimplantation or germ cell escapee regions. A preimplantation escapee list including 3349 regions was prepared by reanalysis of available human RRBS data (36). The list of human germ cell escapees (7070 regions) was obtained from Dr. Azim Surani (37). A small subset (20 of 847) of DMTs were in either the preimplantation or germ cell escapee regions (Supplementary Material, Table S4). With few human DMTs found to be overlapping with escapees, motif enrichment was not examined. However, in the mouse, lists were combined to examine sequence features found in DMTs overlapping with preimplantation or germline escapees. The overlapping mouse DMTs were found mainly in intergenic regions (80% in preimplantation and 61% in germline escapees, respectively). Using HOMER for analysis, several known motifs were found to be enriched in the sequences of both escapee lists (Supplementary Material, Tables S5 and S6). Motifs related to SMAD protein members were enriched in both lists of escapees. SMADs are a family of signal transduction proteins that are activated through TGF-beta signaling. These proteins help to mediate multiple signaling pathways including possible roles in the regulation of carcinogenesis (38). Another common enriched motif was that of the transcriptional factor Eomes, which is crucial for embryonic development and the CNS in vertebrates (39). Together, our human and mouse data suggest that high dose folic acid and *MTHFR* haploinsufficiency may affect the sperm DNA methylome in regions that may not be reprogrammed during developmental methylation resetting stages. This could result in a potential loss of important paternal DNA methylation information between generations.

### Common cellular pathways were affected in both mouse and human with high folic acid intakes

Our genome-wide approach to investigate the impact of high folic acid intakes on the sperm DNA methylome in mouse and human revealed loss of methylation in potentially important genomic regions. We postulated that such affected regions may represent important cellular pathways and utilized the DAVID online tool to test this hypothesis. As shown in Table 1, several cellular pathways were affected by high folic acid in *Mthfr*$^{+/+}$ mice on the 20FS diet and in *Mthfr*$^{+/-}$ mice on the 10FS diet. In human, fewer cellular pathways survived the statistical tests for significance while more pathways were affected in *MTHFR* 677CT and TT individuals (Table 1). Genes related to 'nervous system development' and 'ion binding and transport' pathways were commonly affected in both mouse and human with high folic acid intakes. We also carried out a direct comparison of orthologous regions for DMTs found after supplementation in our human and mouse studies. In these comparisons, we found little evidence of overlap of orthologous affected regions. Thus, we cannot conclude there is sequence conservation of induced hypomethylation.

## Discussion

In countries where food is fortified with folic acid, most individuals now have adequate folate intakes. However, with the current ready access to prescription of folic acid supplements, concerns have arisen about the potential adverse effects of excess folic acid intakes (40,41). Little is known about the effects of folic acid supplements on the sperm epigenome; such studies are needed because animal studies indicate that abnormal sperm methylation patterns can be transmitted to the offspring. Evidence that normal levels of folate are important for male germ cells includes a study connecting lifetime folate deficiency with epigenetic defects in sperm and birth defects in the offspring of exposed males (42). While our recent study in infertile men showed that administration of high dose folic acid resulted in sperm hypomethylation, the findings were counter-intuitive as folic acid might have been expected to increase DNA methylation levels (28). Here, we report that, similar to our results in human, the mouse sperm DNA methylome also became

hypomethylated in response to high dose folic acid supplementation. Such hypomethylation affected regions and genes of potential functional importance for the offspring. We identified downregulation of testicular MTHFR as a possible mechanism contributing to the observed effects on the sperm epigenome.

Based on our previous studies, as compared with the CD, the 10FS and 20FS diets result in approximately two-fold increases in blood folate levels (43,44). The high folic acid diets did not have major effects on general or reproductive health of males, as evidenced by similar body, testes, epididymal and seminal vesicle weights across the groups. Seminal vesicle weights provide a sensitive measure of testosterone output while testis and epididymal weights reflect sperm numbers. Similarly, sperm concentrations did not change after folic acid supplementation in our human study (28).

In studies of male infertility, global and imprinted gene methylation are frequently measured to assess the sperm epigenome. The global CpG assay, LUMA, provided a preliminary indication that sperm methylation at the ∼20 million CpG sites in the mouse genome was not affected in a major way by the diets. At the single gene level, we initially chose to examine the methylation of imprinted genes, abnormalities of which have been reported in the sperm of infertile men (45,46). The results indicated that neither high dose folic acid nor the *Mthfr*$^{+/-}$ genotype resulted in hyper- or hypomethylation of imprinted loci. Similarly, we previously assessed several imprinted genes in human including *H19* and *SNRPN* and did not detect significant alterations because of high dose folic acid supplementation or *MTHFR* 677 genotype (28). In the current study, with *H19* methylation close to 100% in all samples and *Snrpn* methylation close to 0%, we were confident of the purity of our sperm samples and their lack of contamination with somatic cell DNA, prior to moving to the more sensitive assay of DNA methylation, RRBS.

RRBS was used to examine genome-wide single nucleotide effects of folic acid supplementation on the sperm epigenome. For studies of the DNA methylome, RRBS enriches for approximately 10% of the total CpGs mostly located in GC-rich genomic loci, avoids repeat regions and provides a cost-effective approach to analyze multiple samples individually as compared with whole genome bisulfite sequencing. Contrary to sperm DNA hypomethylation in *MTHFR* 677CC infertile men following 10× folic acid consumption, little effect on the mouse sperm DNA methylome was observed in the 10FS *Mthfr*$^{+/+}$ group. We hypothesized that mouse testes may be less sensitive (or have a higher threshold) to folic acid intakes above the DRI than human. Thus, to test the response to a higher dose of folic acid, we treated *Mthfr*$^{+/+}$ mice with either CD or 20FS for 12 months and detected sperm hypomethylation in the 20FS group demonstrating that DNA hypomethylation in response to high folic acid intake occurs in both mouse and human testes, albeit at a higher threshold intake for mice. In contrast, *Mthfr*$^{+/-}$ mice, known to have lower MTHFR levels in testis (29), responded in a similar manner to *MTHFR* 677TT men showing sperm DNA hypomethylation after exposure to the 10FS diet.

Our results suggested that DNA hypomethylation associated with high dose folic acid supplements might be related to effects on the folate metabolic pathway and possibly MTHFR. The availability of testis tissue in mice enabled us to investigate MTHFR protein expression levels. In human, the *MTHFR* 677C>T polymorphism results in the substitution of valine for alanine in the MTHFR protein and a subsequent 50–60% reduction in enzyme activity (20). *Mthfr*$^{+/-}$ mice express 40–50% less MTHFR protein as compared with wild-type animals, thus resembling the changes in humans with the *MTHFR* 677C>T polymorphism (29). With an antibody previously used to examine MTHFR localization in mouse testis (47), we initially used testis extracts from C57BL/6 *Mthfr* mice to identify MTHFR isoforms by immunoblotting. C57BL/6 strain mice were used to characterize testicular MTHFR since adult BALB/c *Mthfr*$^{-/-}$ mice present complete absence of germ cells in the testis (48). In contrast, C57BL/6 *Mthfr*$^{-/-}$ mice have decreased testis weights, the full complement of germ cells and about a 50% decrease in sperm counts compared with their *Mthfr*$^{+/+}$ littermates. Thus, testes from the C57BL/6 *Mthfr*$^{-/-}$ mice provide an appropriate control for the specificity of the MTHFR antibody on Western. As expected, testicular MTHFR protein levels were ∼50% lower in *Mthfr*$^{+/-}$ mice; however, there were no significant effects of the 10FS diet, regardless of *Mthfr* genotype. Our results in testis contrast with those we recently reported in the liver where the same 10FS diets resulted in a significant decrease of MTHFR protein expression (30). A different response to the 10FS diet in testis could be due to metabolic differences between the two tissues, other regulatory mechanisms or to the fact that MTHFR protein level and activity in testis are much higher than liver (29).

Notably, there was a significant ∼50% decrease in testicular MTHFR levels detected in the *Mthfr*$^{+/+}$ mice on the 20FS diet. We propose that sperm DNA hypomethylation occurred in the 20FS mice due to downregulation of MTHFR enzyme and reduced availability of methyl groups for DNA methylation, producing a state equivalent to folate deficiency despite the high folate levels. Testicular downregulation of MTHFR may possibly explain the sperm DNA hypomethylation in high dose folic acid–treated men (28). In *Mthfr*$^{+/-}$ mice, MTHFR levels were resistant to downregulation by the 10FS diet, however, the combination of the ∼50% decrease in MTHFR and excess folic acid consumption appears to compromise DNA methylation resulting in the hypomethylation of sperm DNA observed in both the *Mthfr*$^{+/-}$ 10FS and the *Mthfr*$^{+/+}$ 20FS mice. In contrast, reduced MTHFR expression alone did not result in sperm DNA hypomethylation in mice fed the DRI for folic acid, which also suggested that an interaction between decreased MTHFR levels and excess folic acid intake is driving these effects. In liver, the 10FS diet favoured the phosphorylation of MTHFR, which lowers the activity of the enzyme (30). A comprehensive evaluation of one-carbon metabolites would have been ideal. Unfortunately, there was not enough testes tissue available for that analysis. However, we have analyzed metabolites in liver in mice fed 10FS that show similar reductions in MTHFR protein. Decreased MTHFR expression in the liver of 10FS mice resulted in lower concentrations of hepatic methylTHF and SAM, and increased SAH (30). Similarly, in pregnant mice fed 10FS, MTHFR expression in liver decreased relative to controls, leading to decreased hepatic SAM, and increased SAH (49). These results suggest that SAM, and therefore methylation, may also be affected by decreased MTHFR expression in the testes. It should be noted that MTHFR expression is much higher in testes than in liver, which may explain why liver is more sensitive to high folate than testes. Further studies would be required to determine whether less active versions of MTHFR are also produced in the human testis in response to high dose folic acid.

Interestingly, sperm DNA hypermethylation was seen in the *Mthfr*$^{+/-}$ mice on CDs. Since both histone and DNA methylation patterns are inter-related and acquired in developing male germ cells starting prenatally (reviewed in (10)), we would speculate that either one or both epigenetic modifications could be affected by *Mthfr* haploinsufficiency. For instance, decreases in H3K4me3, a mark anti-correlated with DNA methylation, could result in aberrant DNA methylation at sites normally marked by

histone modification. Thus, it is possible that the DNA methylation disturbance caused by *Mthfr* haploinsufficiency (affecting prenatal and postnatal germ cells) is distinct from the disturbance caused by postnatal folic acid supplementation, such that combining the *Mthfr* haploinsufficiency and 10FS diets, results in modest sperm hypomethylation when compared with wild-type mice on CDs (Fig. 3E).

The amino acid sequences of mouse and human MTHFR are ~90% identical (50). An important difference between mouse and human is the relative activity of dihydrofolate reductase (DHFR), the enzyme responsible for the conversion of folic acid to THF. The activity of DHFR is much higher in murine tissues than in human—reviewed in (51). Therefore, mice may have a greater capacity to convert folic acid to a usable form than humans. This would reduce exposure to folic acid in mice as compared with humans. This underlying difference in folic acid metabolism may explain why a higher dose of folic acid is required in mouse to elicit a response similar to humans.

The most significant effects of high folic acid diets were visible in DMTs identified by RRBS. Interestingly, DMTs were mostly located in the intergenic regions of the mouse sperm genome and not promoter regions, similar to our findings in human (28). In addition to repeat sequences, intergenic regions contain less-characterized gene regulatory regions such as enhancers (52). We found enrichment in testis enhancer regions for DMTs affected by high folic acid intakes in 10FS *Mthfr*$^{+/-}$ mice. The results suggest that intergenic regions involved in controlling gene expression in testis may be susceptible to *Mthfr* haploinsufficiency and high dose folic acid supplements. To date, human testis enhancers have not been characterized. More research is required to characterize the change of methylation in these regions and their possible impact on gene expression. Our cross-species findings in mouse and human on susceptibility of intergenic regions to high folic acid intakes implicate the importance of assessing such regions in environment/epigenome interaction studies.

Alterations in sperm DNA methylation in response to diet or environment could be functionally important due to the possibility of transmission of epigenetic changes to offspring. This is supported by several studies showing an impact of paternal exposures on the sperm DNA methylome and the health of the offspring of the exposed males (6,16,42). In considering intergenerational effects, there is a caveat for studies of dietary components such as folate; since one-carbon metabolism is crucial for DNA synthesis, it is difficult to rule out the possibility that some of the inherited effects result from genetic rather than epigenetic changes. Most sperm DNA methylation perturbations are likely corrected between generations at times of epigenetic reprogramming, either in the embryo during preimplantation development or in the germline in primordial germ cells (53,54). However, recent studies utilizing sensitive genome-wide approaches identified specific genomic regions that are not reprogrammed and escape the two demethylation stages in mouse and human (33,35–37). It is proposed that such retained methylation between generations may be of functional relevance for embryo or germline development. We reanalyzed published datasets and identified the sperm-specific genomic regions that escape (termed 'escapee' regions) the preimplantation and germ cell demethylation phases. A small subset of tiles with significant changes of methylation in the sperm of the 20FS *Mthfr*$^{+/+}$ and 10FS *Mthfr*$^{+/-}$ mice were in such 'escapee' regions. DMT sequences overlapping these regions demonstrated enrichment for several motifs with roles in transcription factor binding and signal transduction. Alterations in DNA methylation at these 'escapee' regions may contribute to altered gene expression in the next generation and possible negative embryonic outcomes. The relatively low percentage of tiles located in such regions may be explained by a limitation of our RRBS approach which provides information on ~10% of the total CpGs in the genome. It would be interesting to specifically target all the known 'escapee' regions in future studies of the effects of folic acid on the sperm epigenome. A further indication that functional sites in the genome might be impacted by high dose folic acid supplements and MTHFR deficiency came from the submission of mouse and human DMTs to pathway analysis. We detected genomic regions affected by high folic acid intake which are related to important cellular and molecular pathways such as nervous system development and ion transport, with overlaps between the mouse and human studies. Together, finding DMTs amongst testis enhancers, 'escapee' regions, and genomic regions related to common physiological processes and disease in mouse and man, provide indications that the impact of high folic acid intake and MTHFR deficiency on sperm DNA may be occurring at regions of functional relevance for the embryo.

## Materials and Methods

### Mice, diets and sample collection

All experiments were performed according to the Canadian Council on Animal Care guidelines and approved by the Animal Care Committee of the Research Institute of the McGill University Health Centre. BALB/c *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ males (29) were placed on control (2 mg/kg CD) and 10× folic acid–supplemented (20 mg/kg 10FS) amino acid–defined diets (Envigo) at weaning ($n = 5$/group), as described (30). A second cohort of *Mthfr*$^{+/+}$ males was assigned to CD and 20× folic acid–supplemented (40 mg/kg 20FS) amino acid–defined diets (Envigo) at weaning ($n = 4$/group). Mice were housed under specific pathogen-free conditions in a controlled environment (12 h/12 h light/dark cycle, 18–24°C) with *ad libitum* access to food and water. After 6 months on CD/10FS diets and 12 months on CD/20FS diets, mice were sacrificed in random order by $CO_2$ asphyxiation and body weights recorded. Reproductive organs were collected, weighed and rinsed with cold phosphate-buffered saline. Mature sperm samples were collected from the cauda-epididymis. All tissues and sperm samples were snap frozen on dry ice and stored at −80°C.

### Sperm DNA extraction

Sperm DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) per the manufacturer's protocols, with minor modifications as described previously (28). Prior to proceeding with DNA extraction, sperm samples were incubated overnight at 37°C in lysis buffer containing EDTA, tris and dithiothreitol (DTT). Genotyping for the *MTHFR* 677C>T polymorphism was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as described previously (29).

### Bisulfite pyrosequencing

Quantitative measurement of DNA methylation levels was performed by bisulfite pyrosequencing on CpG dinucleotides located in the germline DMRs of imprinted loci as well as in the DMTs selected for RRBS validation. Isolated sperm genomic DNA was subjected to bisulfite conversion using the EpiTect

Bisulfite kit (Qiagen #59104). Pyrosequencing was performed as described previously (55). Amplicons were sequenced using the PyroMark Q24 kit and the PyroMarkR Q24 Vacuum Workstation (QIAGEN) using the manufacturer's protocol. Biotinylated and sequencing primers for assessment of *H19* and *Snrpn* were used as described before (55). Primers for validation of RRBS DMTs are listed in Supplementary Material, Table S3. Analysis of germline DMRs of imprinted loci was also used to determine possible contamination of sperm DNA with somatic cell DNA, as marked by aberrant methylation detected in both imprinted regions.

### LUminometric methylation assay

LUMA was performed to assess genomic DNA methylation at a global level using restriction enzymes and pyrosequencing analysis. It was done as described before (56) with the following modifications. For each sample, duplicate digestion was carried out for both restriction enzymes *Hpa*II and *Msp*I. For each digestion, 500 ng of DNA diluted in 10 µl of DNase-free water was digested in 10µl of enzyme restriction master mix [7µl DNase-free water, 2µl 10× Tango Buffer, 0.5µl *Eco*RI (10 U/µl), 0.5 µl restriction enzyme (*Hpa*II or *Msp*I) (10 U/µl)]. Following the 4 h digestion at 37°C, samples were analyzed on a Pyrosequencer Q24 from Qiagen under the 'AQ' mode. To do so, 20µl of pyrosequencing annealing buffer was added to each digested DNA sample for a total of 40µl, and 30µl (375ng of DNA) was loaded onto a pyrosequencing plate. The nucleotides were not diluted and the pyrosequencing reagents were added to the cartridge according to the volumes determined from the run preparation using the PyroMark Q24 software.

### Reduced representation bisulfite sequencing (RRBS)

RRBS libraries were generated for individual sperm samples ($n = 5$–6 per group) using previously published protocols and the gel-free technique (55,57,58). Briefly, 1 µg of sperm DNA was digested using the methylation-sensitive restriction enzyme, *Msp*I, followed by end repair and A-tailing. Small fragments of DNA were then removed utilizing AMPure XP beads (Beckman Coulter), followed by ligation to methylated adapters (Illumina), two rounds of bisulfite conversion and clean up. RRBS libraries were prepared by large-scale PCR and assessed for quality prior to sequencing. Ten samples of sperm DNA libraries were then multiplexed for paired end sequencing in one lane of a HiSeq 2000 sequencer (Illumina). The RRBS analysis pipeline has been established and is maintained at the McGill University Epigenomics Mapping Centre; analysis of data was performed as described previously (28). Briefly, analysis steps included alignment of reads (UCSC mm10 genome version) by the software pipeline bsmap version 2.6 (59) and determination of DMTs by the MethylKit software (version 0.5.3) which implemented the Benjamini-Hochberg false discovery (FDR)-based method for *P*-value correction. Only DMTs passing the *q*-value threshold ($q = 0.01$) were considered (60). Analysis was based on 100 bp step-wise tiling windows, ≥2 CpG per tile, ≥1 CpG in each DNA strand, as well as ≥10× CpG coverage of each tile per sample. Methylation level of each 100 bp tile was the average of all CpGs within the tile. If significant absolute differences in DNA methylation between two experimental groups exceeded 20%, the tile was designated as a DMT; further annotation of DMTs was performed by HOMER software version 3.51. The list of 63 742 testis-enhancer regions was obtained from (31), after converting the sequences to UCSC mm10 genome version. Intersection of extended DMTs (±500 bp) and additional tiles to these tracks were performed with bedtools (v2.23.0) utilities. RRBS data have been submitted to the NCBI Gene Expression Omnibus under the accession number GSE100849.

### Immunoblotting

Testis extracts for immunoblotting were prepared from ∼100 mg frozen tissue under denaturing conditions, as described previously (30). Tissue lysis was performed in RIPA buffer containing complete-mini protease inhibitor (Roche) and Halt phosphatase inhibitor (ThermoFisher Scientific) using a bead mill; lysates were cleared by centrifugation. Protein concentration was determined by Qubit Protein Assay Kit (ThermoFisher Scientific). Protein samples were boiled in reducing sample buffer containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol. Western blotting was performed using ∼15 µg of protein, as described (30,61). Briefly, proteins were resolved on an 8% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with anti-MTHFR (20) and anti-vinculin (Cell Signalling Technology) primary antibodies diluted in 4% milk TBST. Immunoreactive protein was detected using horseradish peroxidase-coupled anti-rabbit IgG (GE Healthcare Life Sciences) and visualized with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences). Blots were documented and quantified using the Amersham Imager 600 (GE Healthcare Life Sciences). MTHFR densitometry results were normalized to the vinculin loading control and reported relative to the mean value for CD-fed *Mthfr*$^{+/+}$ mice, which was standardized to a reference value of 1.

### Bioinformatics reanalysis

The two windows of DNA methylation reprogramming in both mouse and humans were compared with the all sequenced tiles and DMTs found in our study. Publically available data, as described below, were examined and reanalyzed. Human data on the germline reprogramming stage determined genomic regions (7070) with greater than 30% methylation by the end of germline reprogramming with <10% overlap with repetitive elements (37). Similarly, for mouse germ cell reprogramming data on E10.5 male embryos (35), 1000 bp tiles with a minimum of 5 CpGs (each having ≥5× coverage) were constructed and the average methylation of each tile determined. Similar to human data, tiles with >30% methylation and <10% overlap with repetitive elements were retained for analysis (2435 tiles). To examine preimplantation DNA methylation reprogramming, data from oocytes, sperm and ICM samples for human (36) and mouse (33) were used. Average CpG methylation levels for each 100 bp tile were determined in each tissue type. Sperm specific escapees of preimplantation reprogramming were defined as tiles having ≤35% methylation in the oocyte, ≥75% methylation in sperm, and between 35–65% methylation in the ICM (3349 human tiles 'escapees' and 4340 mouse tiles 'escapees'). The DMTs found to overlap with both the germline and preimplantation escapees were analyzed for known motif enrichment using HOMER software version 3.51 (findMotifsGenome.pl) with default parameters. Briefly, a differential motif discovery algorithm was used to compare the genomic sequences found in the DMTs versus random background sequence regions that are selected to match the GC content of our sequences of interest.

HOMER then screened its library of reliable motifs against the regions of interest (i.e. overlapping DMTs) and the background regions, returning a list of enriched motifs.

### Pathway analysis

To investigate potential cellular and molecular pathways associated with identified DMTs, DAVID Bioinformatics Resources version 6.7 was used (62,63). Each group of genes corresponding to the DMT list was compared with the background list based on all tiles sequenced. A modified, and more conservative, Fisher Exact test was performed to determine the significance of enrichment which was then presented as the enrichment score ($-\log P$-value).

### Statistical analysis

Statistical Package for Social Sciences (SPSS) version 16 and Microsoft Excel 365 ProPlus spreadsheet were used for data entry and analysis. Significant changes after high dose folic acid supplementation were detected by *t*-test. Differences among experimental groups as well as RRBS genomic regions were assessed by analysis of variance (ANOVA) and chi square/Fisher exact tests. $P < 0.05$ was considered statistically significant.

## Supplementary Material

Supplementary Material is available at *HMG* online.

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## References

1. Rodgers, A.B. and Bale, T.L. (2015) Germ cell origins of post-traumatic stress disorder risk: the transgenerational impact of parental stress experience. *Biol. Psychiatry*, **78**, 307–314.
2. Simpkin, A.J., Hemani, G., Suderman, M., Gaunt, T.R., Lyttleton, O., McArdle, W.L., Ring, S.M., Sharp, G.C., Tilling, K., Horvath, S. *et al.* (2016) Prenatal and early life influences on epigenetic age in children: a study of mother-offspring pairs from two cohort studies. *Hum. Mol. Genet.*, **25**, 191–201.
3. Eclarinal, J.D., Zhu, S., Baker, M.S., Piyarathna, D.B., Coarfa, C., Fiorotto, M.L. and Waterland, R.A. (2016) Maternal exercise during pregnancy promotes physical activity in adult offspring. *FASEB J.*, **30**, 2541–2548.
4. Joubert, B.R., Felix, J.F., Yousefi, P., Bakulski, K.M., Just, A.C., Breton, C., Reese, S.E., Markunas, C.A., Richmond, R.C., Xu, C.J. *et al.* (2016) DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am. J. Hum. Genet.*, **98**, 680–696.
5. de Waal, E., Vrooman, L.A., Fischer, E., Ord, T., Mainigi, M.A., Coutifaris, C., Schultz, R.M. and Bartolomei, M.S. (2015) The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model. *Hum. Mol. Genet.*, **24**, 6975–6985.
6. Radford, E.J., Ito, M., Shi, H., Corish, J.A., Yamazawa, K., Isganaitis, E., Seisenberger, S., Hore, T.A., Reik, W., Erkek, S. *et al.* (2014) In utero effects. In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. *Science*, **345**, 1255903.
7. Hara, S., Takano, T., Fujikawa, T., Yamada, M., Wakai, T., Kono, T. and Obata, Y. (2014) Forced expression of DNA methyltransferases during oocyte growth accelerates the establishment of methylation imprints but not functional genomic imprinting. *Hum. Mol. Genet.*, **23**, 3853–3864.
8. Wei, Y., Yang, C.R., Wei, Y.P., Zhao, Z.A., Hou, Y., Schatten, H. and Sun, Q.Y. (2014) Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. *Proc. Natl. Acad. Sci. U. S. A.*, **111**, 1873–1878.
9. Dominguez-Salas, P., Moore, S.E., Baker, M.S., Bergen, A.W., Cox, S.E., Dyer, R.A., Fulford, A.J., Guan, Y., Laritsky, E., Silver, M.J. *et al.* (2014) Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. *Nat. Commun.*, **5**, 3746.
10. Ly, L., Chan, D. and Trasler, J.M. (2015) Developmental windows of susceptibility for epigenetic inheritance through the male germline. *Semin. Cell Dev. Biol.*, **43**, 96–105.
11. Weichenhan, D. and Plass, C. (2013) The evolving epigenome. *Hum. Mol. Genet.*, **22**, R1–R6.
12. Powell, W.T. and LaSalle, J.M. (2015) Epigenetic mechanisms in diurnal cycles of metabolism and neurodevelopment. *Hum. Mol. Genet.*, **24**, R1–R9.
13. Goll, M.G. and Bestor, T.H. (2005) Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.*, **74**, 481–514.
14. Hazra, A., Kraft, P., Lazarus, R., Chen, C., Chanock, S.J., Jacques, P., Selhub, J. and Hunter, D.J. (2009) Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. *Hum. Mol. Genet.*, **18**, 4677–4687.
15. Crider, K.S., Yang, T.P., Berry, R.J. and Bailey, L.B. (2012) Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Adv. Nutr.*, **3**, 21–38.
16. Padmanabhan, N., Jia, D., Geary-Joo, C., Wu, X., Ferguson-Smith, A.C., Fung, E., Bieda, M.C., Snyder, F.F., Gravel, R.A., Cross, J.C. *et al.* (2013) Mutation in folate metabolism causes epigenetic instability and transgenerational effects on development. *Cell*, **155**, 81–93.
17. Jadavji, N.M., Deng, L., Malysheva, O., Caudill, M.A. and Rozen, R. (2015) MTHFR deficiency or reduced intake of folate or choline in pregnant mice results in impaired short-term memory and increased apoptosis in the hippocampus of wild-type offspring. *Neuroscience*, **300**, 1–9.
18. Christensen, K.E., Deng, L., Leung, K.Y., Arning, E., Bottiglieri, T., Malysheva, O.V., Caudill, M.A., Krupenko, N.I., Greene,

N.D., Jerome-Majewska, L. *et al.* (2013) A novel mouse model for genetic variation in 10-formyltetrahydrofolate synthetase exhibits disturbed purine synthesis with impacts on pregnancy and embryonic development. *Hum. Mol. Genet.*, **22**, 3705–3719.
19. Crider, K.S., Zhu, J.H., Hao, L., Yang, Q.H., Yang, T.P., Gindler, J., Maneval, D.R., Quinlivan, E.P., Li, Z., Bailey, L.B. *et al.* (2011) MTHFR 677C->T genotype is associated with folate and homocysteine concentrations in a large, population-based, double-blind trial of folic acid supplementation. *Am. J. Clin. Nutr.*, **93**, 1365–1372.
20. Frosst, P., Blom, H.J., Milos, R., Goyette, P., Sheppard, C.A., Matthews, R.G., Boers, G.J., den Heijer, M., Kluijtmans, L.A., van den Heuvel, L.P. *et al.* (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.*, **10**, 111–113.
21. Friso, S., Choi, S.W., Girelli, D., Mason, J.B., Dolnikowski, G.G., Bagley, P.J., Olivieri, O., Jacques, P.F., Rosenberg, I.H., Corrocher, R. *et al.* (2002) A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc. Natl. Acad. Sci. U. S. A.*, **99**, 5606–5611.
22. Gong, M., Dong, W., He, T., Shi, Z., Huang, G., Ren, R., Huang, S., Qiu, S. and Yuan, R. (2015) MTHFR 677C>T polymorphism increases the male infertility risk: a meta-analysis involving 26 studies. *PLoS One*, **10**, e0121147.
23. Liew, S.C. and Gupta, E.D. (2015) Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: epidemiology, metabolism and the associated diseases. *Eur. J. Med. Genet.*, **58**, 1–10.
24. Wong, W.Y., Merkus, H.M., Thomas, C.M., Menkveld, R., Zielhuis, G.A. and Steegers-Theunissen, R.P. (2002) Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. *Fertil. Steril*, **77**, 491–498.
25. Ebisch, I.M., Thomas, C.M., Peters, W.H., Braat, D.D. and Steegers-Theunissen, R.P. (2007) The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Hum. Reprod. Update*, **13**, 163–174.
26. Sweeney, M.R., McPartlin, J. and Scott, J. (2007) Folic acid fortification and public health: report on threshold doses above which unmetabolised folic acid appear in serum. *BMC Public Health*, **7**, 41.
27. De Wals, P., Tairou, F., Van Allen, M.I., Uh, S.H., Lowry, R.B., Sibbald, B., Evans, J.A., Van den Hof, M.C., Zimmer, P., Crowley, M. *et al.* (2007) Reduction in neural-tube defects after folic acid fortification in Canada. *N. Engl. J. Med*, **357**, 135–142.
28. Aarabi, M., San Gabriel, M.C., Chan, D., Behan, N.A., Caron, M., Pastinen, T., Bourque, G., MacFarlane, A.J., Zini, A. and Trasler, J. (2015) High-dose folic acid supplementation alters the human sperm methylome and is influenced by the MTHFR C677T polymorphism. *Hum. Mol. Genet.*, **24**, 6301–6313.
29. Chen, Z., Karaplis, A.C., Ackerman, S.L., Pogribny, I.P., Melnyk, S., Lussier-Cacan, S., Chen, M.F., Pai, A., John, S.W., Smith, R.S. *et al.* (2001) Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum. Mol. Genet.*, **10**, 433–443.
30. Christensen, K.E., Mikael, L.G., Leung, K.Y., Levesque, N., Deng, L., Wu, Q., Malysheva, O.V., Best, A., Caudill, M.A., Greene, N.D. *et al.* (2015) High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice. *Am. J. Clin. Nutr*, **101**, 646–658.
31. Shen, Y., Yue, F., McCleary, D.F., Ye, Z., Edsall, L., Kuan, S., Wagner, U., Dixon, J., Lee, L., Lobanenkov, V.V. *et al.* (2012) A map of the cis-regulatory sequences in the mouse genome. *Nature*, **488**, 116–120.
32. Burgess, D.J. (2014) Stem cells: epigenome reprogramming–of mice and men. *Nat. Rev. Genet.*, **15**, 571.
33. Smith, Z.D., Chan, M.M., Mikkelsen, T.S., Gu, H., Gnirke, A., Regev, A. and Meissner, A. (2012) A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature*, **484**, 339–344.
34. Cowley, M. and Oakey, R.J. (2012) Resetting for the next generation. *Mol. Cell.*, **48**, 819–821.
35. Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W. and Reik, W. (2012) The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Mol. Cell*, **48**, 849–862.
36. Guo, H., Zhu, P., Yan, L., Li, R., Hu, B., Lian, Y., Yan, J., Ren, X., Lin, S., Li, J. *et al.* (2014) The DNA methylation landscape of human early embryos. *Nature*, **511**, 606–610.
37. Tang, W.W., Dietmann, S., Irie, N., Leitch, H.G., Floros, V.I., Bradshaw, C.R., Hackett, J.A., Chinnery, P.F. and Surani, M.A. (2015) A unique gene regulatory network resets the human germline epigenome for development. *Cell*, **161**, 1453–1467.
38. Samanta, D. and Datta, P.K. (2012) Alterations in the Smad pathway in human cancers. *Front. Biosci. (Landmark Ed)*, **17**, 1281–1293.
39. Simon, C.S., Downes, D.J., Gosden, M.E., Telenius, J., Higgs, D.R., Hughes, J.R., Costello, I., Bikoff, E.K. and Robertson, E.J. (2017) Functional characterisation of cis-regulatory elements governing dynamic Eomes expression in the early mouse embryo. *Development*, **144**, 1249–1260.
40. Smith, A.D., Kim, Y.I. and Refsum, H. (2008) Is folic acid good for everyone? *Am. J. Clin. Nutr*, **87**, 517–533.
41. Reynolds, E.H. (2016) What is the safe upper intake level of folic acid for the nervous system? Implications for folic acid fortification policies. *Eur. J. Clin. Nutr.*, **70**, 537–540.
42. Lambrot, R., Xu, C., Saint-Phar, S., Chountalos, G., Cohen, T., Paquet, M., Suderman, M., Hallett, M. and Kimmins, S. (2013) Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nat. Commun.*, **4**, 2889.
43. Pickell, L., Brown, K., Li, D., Wang, X.L., Deng, L., Wu, Q., Selhub, J., Luo, L., Jerome-Majewska, L. and Rozen, R. (2011) High intake of folic acid disrupts embryonic development in mice. *Birth Defects Res. A. Clin. Mol. Teratol.*, **91**, 8–19.
44. Mikael, L.G., Deng, L., Paul, L., Selhub, J. and Rozen, R. (2013) Moderately high intake of folic acid has a negative impact on mouse embryonic development. *Birth Defects Res. A. Clin. Mol. Teratol.*, **97**, 47–52.
45. Poplinski, A., Tuttelmann, F., Kanber, D., Horsthemke, B. and Gromoll, J. (2010) Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *Int. J. Androl.*, **33**, 642–649.
46. Li, B., Li, J-b., Xiao, X-f., Ma, Y-f., Wang, J., Liang, X-x., Zhao, H-x., Jiang, F., Yao, Y-q., Wang, X-h. and Sobol, R.W. (2013) Altered DNA methylation patterns of the H19 differentially methylated region and the DAZL gene promoter are associated with defective human sperm. *PLoS One*, **8**, e71215.
47. Garner, J.L., Niles, K.M., McGraw, S., Yeh, J.R., Cushnie, D.W., Hermo, L., Nagano, M.C. and Trasler, J.M. (2013) Stability of DNA methylation patterns in mouse spermatogonia under

conditions of MTHFR deficiency and methionine supplementation. *Biol. Reprod.*, **89**, 125.
48. Chan, D., Cushnie, D.W., Neaga, O.R., Lawrance, A.K., Rozen, R. and Trasler, J.M. (2010) Strain-specific defects in testicular development and sperm epigenetic patterns in 5,10-methylenetetrahydrofolate reductase-deficient mice. *Endocrinology*, **151**, 3363–3373.
49. Christensen, K.E., Hou, W., Bahous, R.H., Deng, L., Malysheva, O.V., Arning, E., Bottiglieri, T., Caudill, M.A., Jerome-Majewska, L.A. and Rozen, R. (2016) Moderate folic acid supplementation and MTHFD1-synthetase deficiency in mice, a model for the R653Q variant, result in embryonic defects and abnormal placental development. *Am. J. Clin. Nutr.*, **104**, 1459–1469.
50. Goyette, P., Pai, A., Milos, R., Frosst, P., Tran, P., Chen, Z., Chan, M. and Rozen, R. (1998) Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). *Mamm. Genome*, **9**, 652–656.
51. Wright, A.J., Dainty, J.R. and Finglas, P.M. (2007) Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. *Br. J. Nutr.*, **98**, 667–675.
52. Chandra, S., Terragni, J., Zhang, G., Pradhan, S., Haushka, S., Johnston, D., Baribault, C., Lacey, M. and Ehrlich, M. (2015) Tissue-specific epigenetics in gene neighborhoods: myogenic transcription factor genes. *Hum. Mol. Genet.*, **24**, 4660–4673.
53. Molaro, A., Falciatori, I., Hodges, E., Aravin, A.A., Marran, K., Rafii, S., McCombie, W.R., Smith, A.D. and Hannon, G.J. (2014) Two waves of de novo methylation during mouse germ cell development. *Genes Dev.*, **28**, 1544–1549.
54. Smith, Z.D., Chan, M.M., Humm, K.C., Karnik, R., Mekhoubad, S., Regev, A., Eggan, K. and Meissner, A. (2014) DNA methylation dynamics of the human preimplantation embryo. *Nature*, **511**, 611–615.
55. McGraw, S., Zhang, J.X., Farag, M., Chan, D., Caron, M., Konermann, C., Oakes, C.C., Mohan, K.N., Plass, C., Pastinen, T. *et al.* (2015) Transient DNMT1 suppression reveals hidden heritable marks in the genome. *Nucleic Acids Res.*, **43**, 1485–1497.
56. Luttropp, K., Sjoholm, L.K. and Ekstrom, T.J. (2015) Global analysis of DNA 5-methylcytosine using the luminometric methylation assay, LUMA. *Methods Mol. Biol.*, **1315**, 209–219.
57. Gu, H., Smith, Z.D., Bock, C., Boyle, P., Gnirke, A. and Meissner, A. (2011) Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. *Nat. Protoc.*, **6**, 468–481.
58. Boyle, P., Clement, K., Gu, H., Smith, Z.D., Ziller, M., Fostel, J.L., Holmes, L., Meldrim, J., Kelley, F., Gnirke, A. *et al.* (2012) Gel-free multiplexed reduced representation bisulfite sequencing for large-scale DNA methylation profiling. *Genome Biol.*, **13**, R92.
59. Xi, Y. and Li, W. (2009) BSMAP: whole genome bisulfite sequence MAPping program. *BMC Bioinformatics*, **10**, 232.
60. Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F.E., Figueroa, M.E., Melnick, A. and Mason, C.E. (2012) methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol.*, **13**, R87.
61. Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S.I., Sutovsky, P., Librach, C.L. and Oko, R. (2014) Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil. Steril.*, **102**, 440–447.
62. Huang da, W., Sherman, B.T. and Lempicki, R.A. (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.*, **37**, 1–13.
63. Huang da, W., Sherman, B.T. and Lempicki, R.A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.*, **4**, 44–57.