Defective folate metabolism causes germline epigenetic instability and distinguishes \textit{Hira} as a phenotype inheritance biomarker

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The mechanism behind transgenerational epigenetic inheritance is unclear, particularly through the maternal grandparental line. We previously showed that disruption of folate metabolism in mice by the \textit{Mtrr} hypomorphic mutation results in transgenerational epigenetic inheritance of congenital malformations. Either maternal grandparent can initiate this phenomenon, which persists for at least four wildtype generations. Here, we use genome-wide approaches to reveal genetic stability in the \textit{Mtrr} model and genome-wide differential DNA methylation in the germline of \textit{Mtrr} mutant maternal grandfathers. We observe that, while epigenetic reprogramming occurs, wildtype grandprogeny and great grandprogeny exhibit transcriptional changes that correlate with germline methylation defects. One region encompasses the \textit{Hira} gene, which is misexpressed in embryos for at least three wildtype generations in a manner that distinguishes \textit{Hira} transcript expression as a biomarker of maternal phenotypic inheritance.
Environmenal stressors can impact an individual’s health and that of their progeny\(^1\). The phenotypic risk that persists for several generations in the absence of the stressor is termed transgenerational epigenetic inheritance (TEI)\(^6\). Although the mechanism is unclear, this non-conventional inheritance likely occurs independent of DNA base-sequence mutations and involves the inheritance of an epigenetic factor(s) via the germline\(^6\). Candidate factors in mammals include DNA methylation, histone modifications, and/or non-coding RNA\(^{1,2,4,8}\). How an epigenetic message resists reprogramming and is transmitted between one or even multiple generations to cause disease remains elusive. Few mammalian models of TEI exist and most focus on paternal inheritance\(^1,2,4,11\). We previously reported the Mtrrgt mouse line, a rare model of maternal grandparental TEI in which congenital malformations are transgenerationally inherited for at least four wildtype generations\(^3\) (see below, Supplementary Fig. 1a, d–e). While the germline is implicated, the epigenetic mechanism remains unclear.

MTRR (methionine synthase reductase) is a key enzyme required for one-carbon metabolism (i.e., folate and methionine metabolism; Supplementary Fig. 2)\(^{12,14}\). Folate is a well-known vitamin important for neural tube closure, yet its function in development is complex and poorly understood. One-carbon metabolism is required for thymidine synthesis\(^{15}\) and cellular methylation. Indeed, it transmits methyl groups for the methylation of homocysteine by methionine synthase (MTR) to form methionine and tetrahydrofolate\(^{16}\). Methionine acts as a precursor for S-adenosylmethionine (SAM), which serves as the sole methyl-donor for substrates involved in epigenetic regulation (e.g., DNA, RNA, histones)\(^{17–19}\). MTRR activates MTR through the reductive methylation of its vitamin B<sub>12</sub> cofactor\(^{14}\) (Supplementary Fig. 2). Consequently, the progression of one-carbon metabolism requires MTRR to maintain genetic and epigenetic stability.

The hypomorphic Mtrrgt mutation reduces wildtype Mtrr transcript expression in mice\(^3,12\), which is sufficient to diminish MTR activity by \(\sim60\%\) (ref. \(^{12}\)). Consequently, the progression of one-carbon metabolism is inhibited by the Mtrrgt mutation. Similar to mutations in the human MTRR gene\(^{13,20–22}\) or dietary folate deficiency in humans\(^{23}\), Mtrrgt mouse display hyperhomocysteinemia\(^3,12\) and macrocytic anemia\(^24\) in adulthood, as well as altered DNA methylation patterns associated with gene misexpression\(^3\) and a broad range of incompletely penetrant developmental phenotypes at midgestation (e.g., growth defects and/or congenital malformations including heart, placenta, and neural tube closure defects)\(^3\). Therefore, Mtrrgt mice are suitable for studying defective folate metabolism.

Crucially, the Mtrrgt mouse line is a model of TEI that occurs via the maternal grandparental lineage\(^3\). Through highly controlled genetic pedigrees (Supplementary Fig. 1a, d–e), we demonstrated that an Mtrrgt heterozygous male or female mouse (i.e., the F0 generation) can initiate TEI of developmental phenotypes at embryonic day (E) 10.5 in the wildtype (Mtrrgt\(^+/-\)) descendants until the F4 generation\(^3\). This phenomenon occurs when all mice are wildtype for the Mtrr gene except for the initiating F0 Mtrrgt individual (Supplementary Fig. 1d–e). The phenotypes are similar in kind to those observed in Mtrr\(^+/-\) conceptuses (see above), though present at slightly lower frequencies\(^3\). Also, the phenotypes associate with locus-specific changes in DNA methylation that are linked to gene misexpression\(^3\) giving functional relevance to this epigenetic disruption.

Regardless of whether an F0 Mtrrgt male or female initiates TEI, the spectrum and frequency of developmental phenotypes in the F2–F4 wildtype generations are largely comparable between pedigrees when inherited via their daughters\(^3\). The exception is the F1 generation where phenotypic risk at E10.5 occurs only when F1 individuals are derived from F0 Mtrrgt\(^+/-\) females (Supplementary Fig. 1d–e)\(^3\). That is, the F1 progeny of F0 Mtrrgt\(^+/-\) males do not exhibit phenotypes at E10.5, reinforcing that these phenotypes are not transmitted through the male lineage (Supplementary Fig. 1d–e)\(^3\). However, F1 wildtype mice derived from F0 Mtrrgt\(^+/-\) males display indicators of direct epigenetic inheritance including locus-specific epigenetic dysregulation in placenta at E10.5 associated with gene misexpression in the absence of gross phenotype\(^3\), a hematopoietic phenotype later in life\(^24\), and the ability of F1 wildtype females to perpetuate epigenetically inherited phenotypes to their offspring in a manner similar to those derived from an F0 Mtrrgt\(^+/-\) female (Supplementary Fig. 1d–e)\(^3\).

Since TEI in the Mtrrgt model implicates the maternal lineage, we previously performed a blastocyst transfer experiment to show that phenotype inheritance occurred via the germline and was independent of the uterine environment. More specifically, F2 wildtype blastocysts derived from an F0 Mtrrgt\(^+/-\) maternal grandparent and F1 wildtype mother were transferred into the uteri of control females (Supplementary Fig. 1f)\(^3\). The risk for congenital malformations persisted after blastocyst transfer, indicating that phenotypic inheritance was not attributed to the uterine environment of the original mother but instead to the inheritance of an unknown epigenetic factor via the germline\(^3\). Therefore, we hypothesise that wildtype gametes from either Mtrrgt\(^+/-\) maternal grandparent can initiate phenotypic inheritance via the F1 wildtype daughters for several generations\(^3\).

However, the mechanism(s) of TEI remains unclear in the Mtrrgt model including the identity of the inherited epigenetic factor(s) and whether there are different epigenetic cues in the F0 male and female germelines that can initiate similar effects in their grandprogeny.

Here, we investigate the potential mechanism(s) of TEI in the Mtrrgt model using a genome-wide approach. First, we demonstrate that Mtrrgt\(^+/-\) mice are genetically stable and hence reassert focus on an epigenetic mechanism. Second, we show that germline DNA methylation is altered in F0 Mtrrgt\(^+/-\) males. F0 sperm were chosen for analysis because: (i) F0 Mtrrgt\(^+/-\) males initiate TEI of phenotypes in a similar manner to F0 Mtrrgt\(^+/-\) females, (ii) sperm are more experimental tractable than oocytes, and (iii) when assessing heritable effects, the uterine environment does not need to be controlled for in F0 Mtrrgt\(^+/-\) males. Even though differentially methylated regions (DMRs) in sperm of F0 Mtrrgt\(^+/-\) males are reprogrammed in somatic tissue of wildtype F1 and F2 progeny, our data shows evidence of transcriptional changes associated with germline epigenetic disruption that persists at least until the F3 generation. This proposed transcriptional memory of sperm DMRs includes misexpression of Hira, a gene important for chromatin stability\(^{25,26}\) and production of RNA\(^{27}\), which we propose as a biomarker and potential mediator of maternal phenotypic inheritance in the Mtrrgt model.

Results

Genetic stability in Mtrrgt mice. As one-carbon metabolism is directly linked to DNA synthesis\(^{15}\), we first addressed whether the Mtrrgt allele influences genetic stability. Whole-genome sequencing (WGS) was performed on phenotypically normal C57Bl/6J control embryos \((N = 2)\) and Mtrrgt\(^+/-\) embryos with congenital malformations \((N = 6)\) derived from Mtrrgt\(^+/-\) intercrosses (Supplementary Fig. 1a, c). DNA libraries were sequenced separately resulting in \(\sim30x\) coverage per embryo \((\sim3.5 \times 10^8\) paired-end reads/genome\). The sequenced genomes were compared to the C57Bl/6J reference genome to identify structural variants (SVs) and single-nucleotide polymorphisms (SNPs).
The Mtrrgt mutation was generated by a gene-trap (gt) insertion into intron nine of the Mtrr gene (Chr13) in the 129P2Ola/Hsd mouse strain before eight generations of backcrossing into the C57Bl/6J strain. As a result, the majority of variants identified in Mtrrgt/gt embryos were located on Chr13 in the genomic region surrounding the Mtrr locus (Supplementary Fig. 3a–b). These variants included the gene-trap and several SNPs that showed sequence similarity to the 129P2Ola/Hsd genome and likely persisted due to Mtrrgt genotype selection and regional crossover frequency. Variant identification in this region acted as an internal positive control of our bioinformatic method, demonstrating that it is capable of distinguishing genetic differences between experimental groups. Using these SNPs, we defined a 20 Mb region of 129P2Ola/Hsd sequence surrounding the Mtrrgt allele (Fig. 1a). When this region was bioinformatically masked, C57Bl/6 J and Mtrrgt/gt embryos contained a similar mean (±sd) frequency of SNPs (C57Bl/6 J: 4.871 ± 791 SNPs/embryo; Mtrrgt/gt: 5138 ± 398 SNPs/embryo; p = 0.781) and SVs (C57Bl/6 J: 342 SVs/embryo; Mtrrgt/gt: 301 SVs/embryo; p = 0.6886; Fig. 1b, c) implying that the de novo mutation rate was unchanged by the Mtrrgt mutation. These values were in line with expected de novo mutation rates. Only 25 (21 SNPs and 4 SVs) variants were present in all six Mtrrgt/gt embryos and absent in C57Bl/6 J embryos (Supplementary Fig. 3e, f, Supplementary Tables 1–2). When all SNPs and SVs were considered, the majority represented non-coding variants or were located in non-coding regions (Supplementary Fig. 3c, d). Moreover, genetic variation within the masked region had the minimal functional effect (beyond the gene-trap insertion) since no variant overlapped with a known enhancer, and expression of individual genes was similar among C57Bl/6 J, 129P2Ola/Hsd and Mtrrgt/gt mice (Fig. 1a, d). Genomic stability was further supported by the preserved repression of transposable elements (Supplementary Fig. 3g, 3h) in Mtrrgt/gt tissue (Fig. 1c) despite global DNA hypomethylation caused by the Mtrrgt mutation. Overall, these data support genetic integrity within the Mtrrgt model, and that phenotypic inheritance was unlikely caused by an increased frequency of de novo mutation. Therefore, the focus shifted to an epigenetic mechanism.

Germline DNA methylation is altered in the Mtrrgt model. MTRR protein has a direct role in the transmission of one-carbon methyl groups for DNA methylation. Therefore, germline DNA methylation was considered as a potential mediator of phenotype inheritance. As an Mtrrgt/gt female or male can initiate TEI (Supplementary Fig. 1d–e) and due to the experimental tractability of male gametes, we focussed our analysis on sperm. Spermato genesis and male fertility are normal in Mtrrgt/+; Mtrrgt/gt and Mtrrgt/gt males. Mature spermatozoa were collected from caudal epididymides of C57Bl/6 J, Mtrrgt/+, Mtrrgt/gt and Mtrrgt/gt mice (Supplementary Fig. 1a–c) and sperm purity was confirmed by assessing imprinted regions of known methylation status via bisulfite pyrosequencing (Supplementary Fig. 4a). Global 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) levels were consistent across all Mtrrgt genotypes relative to C57Bl/6 J control as determined by mass spectrometry (Fig. 2a).

To analyse the genome-wide distribution of sperm DNA methylation, methylated DNA Immunoprecipitation (IP) followed by sequencing (MeDIP-seq) was performed. This approach allowed the unbiased detection of locus-specific changes in DNA methylation by identifying clusters of differentially methylated cytosines, thus reducing the potential impact of single-nucleotide variants. MeDIP libraries of sperm DNA were prepared using eight males each from C57Bl/6 J, Mtrrgt/+, Mtrrgt/gt and Mtrrgt/gt genotypes (Supplementary Fig. 4b). Sequencing generated 179 million paired-end mappable reads on average per group (C57Bl/6 J: 164 million reads; Mtrrgt/+: 172 million reads; Mtrrgt/gt: 203 million reads; Mtrrgt/gt: 179 million reads). Using MEDIPS package, each Mtrr genotype was independently compared to C57Bl/6 J controls. Loci of >500 bp with a methylation change of >1.5-fold and p < 0.01 were defined as DMRs. The number of sperm DMRs identified increased with the severity of Mtrr genotype: 91 DMRs in Mtrrgt/+, 203 DMRs in Mtrrgt/gt males, and 599 DMRs in Mtrrgt/gt males (Fig. 2b, c). The presence of DMRs in sperm from Mtrrgt/+/ males indicated a parental effect of the Mtrrgt allele on offspring germline methylene since Mtrrgt/– males derive from Mtrrgt/+/ males (Supplementary Fig. 1b). Hypo- and hypermethylated regions were identified in each Mtrr genotype when compared with C57Bl/6 J controls (Fig. 2c), consistent with earlier findings in placentas. These data suggested that the Mtrrgt allele was sufficient to dysregulate sperm DNA methylation.

To ensure the robustness and reliability of the MeDIP-seq data, we randomly selected hyper- and hypomethylated DMRs to validate using bisulfite pyrosequencing. Sperm DNA from C57Bl/6 J, Mtrrgt/+, Mtrrgt/gt and Mtrrgt/gt males was assessed (N = 8 males/group; four sperm samples from MeDIP-seq experiment plus four independent samples). DMRs were validated in the Mtrr genotype in which they were identified (Figs. 2d, 3, Supplementary Fig. 5). The overall validation rate was 94.1% in hypomethylated DMRs and 58.3% in hypermethylated DMRs (Supplementary Table 3) and indicated a high degree of corroboration between techniques. The majority of DMRs that did not validate showed extensive methylation (>80% CpG methylation) in C57Bl/6 J sperm and were identified as hypermethylated in the MeDIP-seq experiment (Supplementary Fig. 5). This might reflect some false positives in line with another study.

For most DMRs assessed, methylation change was consistent across all CpG sites and the absolute change in CpG methylation ranged from 10 to 80% of control levels (Figs. 2d, 3, Supplementary Fig. 5). Within each genotypic group, a high degree of inter-individual consistency of methylation change was also observed. Therefore, we conclude that the Mtrrgt mutation, or parental exposure to it as in Mtrrgt/+, is sufficient to lead to distinct DNA methylation changes in sperm.

Most DMRs associate with metabolic dysregulation, not genetic effects. A proportion of the DMRs was located within the region around the gene-trap insertion site in Mtrrgt/gt and Mtrrgt/gt males (Fig. 1a, Supplementary Fig. 6b, c), consistent with Mtrrgt/gt liver and suggesting that the gene-trap or underlying 129P2Ola/Hsd sequence might epigenetically dysregulate the surrounding region. However, a comparison of the MeDIP-seq and WGS data sets revealed that genetic variation did not influence DMR calling to a great extent since only a small proportion (2.8–5.5%) of these DMRs contained one or more SNP. Eight DMRs overlapped with known enhancers (Supplementary Table 4), none of which associated with promoters containing a genetic variant. Outside of the Mtrr genomic region, 54 DMRs were common to Mtrrgt/+, Mtrrgt/gt and Mtrrgt/gt males (Fig. 2b, c, Supplementary Table 5) and were primarily located in distinct chromosomal clusters (Supplementary Fig. 6a–d). These data implicate epigenetic hotspots or underlying genetic effects. However, beyond a polymorphic duplication on Chr19 in the C57Bl/6 J strain that accounted for a minor number of DMRs (2.5–15.8% of DMRs), no DMRs overlapped with an SV or were located <1kb of an SV. Once potential genetic effects were accounted for, the majority of sperm DMRs in Mtrrgt/– and Mtrrgt/+ males (76/91 DMRs and 142/203 DMRs, respectively), and a proportion of sperm DMRs in
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**Fig. 1 The Mtrrgt mouse line is genetically stable.** a–c Whole-genome sequencing (WGS) of normal C57Bl/6 J embryos (N = 2 embryos) and severely affected Mtrrgt/gt embryos (N = 6 embryos) at embryonic day (E) 10.5 to determine the frequency of genetic variants compared to the C57Bl/6 J reference genome. a The frequency of 129P2Ola/Hsd (129P2) single-nucleotide polymorphisms (SNPs) in the region surrounding the gene-trap insertion site in the Mtrr gene (red line) on chromosome 13 (Chr13). The majority of genes within the 20 megabase (Mb) region surrounding the Mtrr gene are shown below the graph. b, c The average number of b SNPs and c structural variants (SVs) per embryo (mean ± standard deviation [sd]) in C57Bl/6 J embryos (C57, black bars) and Mtrrgt/gt embryos (gt/gt, white bars). Two-tailed unpaired t test with Welch’s correction. The 20 Mb region shown in a was masked when calculating the average number of genetic variants in b, c. d Graphs showing RT-qPCR analysis of selected genes (highlighted red in a) in embryos (E10.5; N = 6–8 embryos/group), placentas (E10.5; N = 6–8 placentas/group) and/or adult testes (N = 5–6 males/group) from C57Bl/6 J (black bars), 129P2Ola/Hsd (grey bars) and phenotypically normal Mtrrgt/gt (blue bars) mice. One-way ANOVA with Tukey’s multiple comparison test, *p = 0.022, **p = 0.007. e Graphs showing RNA expression of specific groups of transposable elements as determined by RT-qPCR in adult liver (N = 6–8 livers/group) and placentas (E10.5; N = 6–7 placenta/group) from C57Bl/6 J mice (black bars) and Mtrrgt/gt mice (blue bars; phenotypically normal; white bars: severely affected). Two-way ANOVA with Tukey’s multiple comparison test, *p = 0.011, **p = 0.005. Data from RT-qPCR analyses in d, e are shown as mean ± sd and relative to C57Bl/6 J tissue levels (normalised to 1). Source data are provided as a Source Data file.

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**Mtrrgt/gt** males (174/599 DMRs) were attributed to the long-term metabolic consequences of the Mtrrgt mutation.

**Sperm DMR genomic distribution and potential regulatory function.** DMR distribution was determined to explore the regional susceptibility of the sperm methylome to the effects of the Mtrrgt allele. First, the sperm ‘background methylome’ was established to resolve the expected genome-wide distribution of CpG methylation (see Methods). By comparing the regional distribution of sperm DMRs to the background methylome, we revealed that DMRs in all Mtrr genotypes were not significantly enriched in repetitive regions (Fig. 2e). However, sperm DMRs in Mtrr+/+ and Mtrr+/+gta males were over-represented in introns and exons, and under-represented in intergenic regions (p < 0.0003, Chi-squared test; Fig. 2f). This was not the case for Mtrrgt/gt males since DMRs were proportionately distributed among most genomic regions (Fig. 2f). Although the majority of sperm DMRs were located within CpG deserts, a proportion of DMRs from Mtrr+/gta and Mtrrgt/gta males were enriched in CpG islands (p < 0.0014, Chi-squared; Fig. 2g), which has implications for gene regulatory control. Finally, when considering only the subset of common DMRs shared by all Mtrr genotypes, a similar genomic distribution to Mtrr+/+ and Mtrr+/+gta males was observed (Supplementary Fig. 6d, e).

During sperm maturation, histones are replaced by protamines35. However, ~1% of histone-containing nucleosomes...
are retained\textsuperscript{38} providing scope for epigenetic inheritance\textsuperscript{39}. Nucleosome retention occurs primarily at promoters of developmentally regulated genes and gene-poor repeat regions, though regional distribution and frequency differ between reports\textsuperscript{40,41}. To determine whether sperm DMRs in \textit{Mtrr} males were enriched in known sites of nucleosome retention, we utilised the MNase-seq data set from C57Bl/6 J spermatozoa in Erkek et al.\textsuperscript{41}. First, by randomly selecting 10,000 regions of 500 bp as a proxy for DMRs, we determined that the expected frequency of DMR overlap with sites of nucleosome retention was 1.94%. Crucially, we observed that 14.5–34.1% of DMRs identified in sperm from \textit{Mtrr}+/+\textit{Mtrr}+/gt and \textit{Mtrrgt}/gt males were located in nucleosome retention regions (Table 1), indicating a significant enrichment (\(p < 0.0001\), binomial test). Therefore, these DMRs represented candidate regions for epigenetic inheritance.

To better understand the normal epigenetic signatures within regions identified as sperm DMRs and to predict a potential gene regulatory role, mean enrichment for histone modifications and/or chromatin accessibility in mouse spermatozoa\textsuperscript{42}, epiblast and extraembryonic ectoderm at E6.5\textsuperscript{43} was determined using published ChIP-seq and ATAC-seq data sets. All DMRs, except those surrounding the \textit{Mtrr} gene-trapped site, were analyzed (\(N = 379\) DMRs from all \textit{Mtrr} genotypes combined) alongside 379 randomly selected regions representing the ‘baseline genome’ (see Methods). Compared with the sperm baseline genome, the majority of our DMRs were likely to associate with a closed chromatin state due to collective enrichment for protamine 1 (PRM1) and repressive histone mark H3K9me3, but not active histone marks (e.g., H3K4me1, H3K27ac) or Tn5 transposase sensitive sites (THSS)\textsuperscript{42} (Supplementary Fig. 7a, b). This finding reinforces heterogeneity of DMR association with retained nucleosomes\textsuperscript{41} or protamines (Table 1). In contrast, the DMRs were more likely in an open chromatin conformation state in epiblast and extraembryonic ectoderm at E6.5 based on collective
enrichment for THSS when compared with tissue-specific baseline genomes\(^43\) (Supplementary Fig. 7c). Therefore, it is possible that the genomic regions identified as DMRs in *Mtrr* sperm have a regulatory role during development.

**Some DMRs were located in regions of reprogramming resistance.** DNA methylation is largely reprogrammed during pre-implantation development and in the developing germline\(^44,45\) to ‘reset’ the methylene between each generation. Recently, several loci were identified as ‘reprogramming resistant’\(^46–48\) and thus, are implicated in epigenetic inheritance. Using published methylome data sets\(^46,47\), we determined that 40.7–54.3% of sperm DMRs across all *Mtrr* genotypes fell within loci resistant to pre-implantation reprogramming (Table 1). Sixteen of these DMRs were common among *Mtrr\(^{+/+}\)*, *Mtrr\(^{+/-}\)* and *Mtrr\(^{gt/gt}\)* males. Fewer DMRs correlated with regions resistant to germline reprogramming (2.2–3.8% of DMRs/*Mtrr* genotype; Table 1) or both pre-implantation and germline reprogramming (2.0–2.7% of DMRs/*Mtrr* genotype; Table 1). Only one DMR located in a region resistant to germline reprogramming was common to all *Mtrr* genotypes. Furthermore, several DMRs were characterised as regions of reprogramming resistance\(^46,47\) and nucleosome retention\(^43\) (Table 1). Overall, differential methylation of these key regions in sperm of *Mtrr\(^{gt}\)* males might have important implications for epigenetic inheritance.

**Sperm DMRs are reprogrammed in wildtype F1 and F2 generations.** TE1 in the *Mtrr\(^{gt}\)* model occurs via the maternal grandparental lineage\(^3\) (Supplementary Fig. 1d–e). To determine the heritability of germline DMRs, bisulphite pyrosequencing was used to validate 10 sperm DMRs from F0 *Mtrr\(^{gt/gt}\)* males in the tissue of wildtype F1 and F2 progeny (i.e., the maternal grandparental pedigree). The breeding scheme was as follows: F0 *Mtrr\(^{gt/gt}\)* males were mated with C57Bl/6J control females. The resulting F1 progeny was either collected at E10.5 for analysis or allowed to litter out. In the latter case, adult F1 wildtype females were mated with C57Bl/6J control males and the F2 wildtype progeny was collected at E10.5 for analysis (Supplementary fig. 1d). The advantage of assessing inheritance of DNA methylation in F0 sperm rather than in F0 oocytes was that potential confounding effects of the F0 uterine environment could be avoided. Candidate DMRs were hyper- or hypomethylated, and localised to regions of reprogramming resistance and/or in intra- or intergenic regions (Supplementary Table 6). In general, all DMRs tested lost their differential methylation in wildtype F1 and F2 embryos and placenta at E10.5, and showed DNA methylation patterns similar to C57Bl/6J tissue (Fig. 3, Supplementary Table 7). This result occurred even when wildtype F2 conceptuses displayed congenital malformations (Fig. 3). DMRs were also assessed in *Mtrr\(^{gt/gt}\)* conceptuses at E10.5 to determine whether these regions were capable of differential methylation outside of the germline. In a manner similar to sperm from *Mtrr\(^{gt/gt}\)* males (Fig. 3), seven out of 10 DMRs were hypermethylated in *Mtrr\(^{gt/gt}\)* embryos and/or placenta compared to control conceptuses (Supplementary Fig. 8a–j). In this case, it was unclear whether DNA methylation in these regions resisted epigenetic reprogramming or was erased and abnormally re-established/maintained due to intrinsic *Mtrr\(^{gt/gt}\)* homozygosity. Overall, altered patterns of DNA methylation in sperm of *Mtrr\(^{gt/gt}\)* males were not evident in somatic tissue of wildtype progeny and grandprogeny. This result was reminiscent of mouse models of parental exposure to environmental stressors (e.g., maternal under-nutrition\(^49\), paternal folate deficiency\(^49\), or paternal cigarette smoking\(^50\)), which induced sperm DMRs associated with phenotypes in the direct offspring even though the DMRs were resolved in offspring somatic tissue. As a result, other epigenetic mechanisms (e.g., germline RNA content and/or histone modifications) with DNA methylation are implicated in phenotypic inheritance.

**Potential transcriptional memory of sperm DMRs.** A previous study in mice suggests that sperm DMRs can associate with perturbed transcription in offspring even when DNA methylation is re-established to normal levels\(^5\). To assess whether transcriptional memory associated with sperm DMRs occurred in the *Mtrr\(^{gt/gt}\)* maternal grandfather pedigree, expression of six genes located in or near sperm DMRs from F0 *Mtrr\(^{gt/gt}\)* males (Supplementary Table 6) was assessed in F1 and F2 wildtype individuals. Although all six genes displayed normal expression in F1 tissues (Fig. 4a–c), three of these genes including *Hira* (histone chaperone), *Cwc27* (spliceosome-associated protein) and *Tshz3* (transcription factor) were misexpressed in F2 wildtype embryos or adult livers compared to C57Bl/6J controls (Fig. 4d–i). This result might reflect transcriptional memory of the associated
sperm DMR or wider epigenetic dysregulation in sperm of the F0 Mtrr\+/gt males.

To further predict whether the Hira, Cwc27 and Tshz3 DMRs demarcate gene regulatory regions, their specific genetic and epigenetic characteristics beyond CpG methylation were considered. Genomically, the DMRs were located intragenically (Cwc27 and Tshz3 DMRs) or within 6 kb downstream of the gene (Hira DMR; Fig. 5a, Suppl. Figs. 9–10). Furthermore, Cwc27 DMR overlapped with a known enhancer (Supplementary Fig. 9) while Hira and Tshz3 DMRs overlapped with CpG islands\(^5\) (Fig. 5a, Supplementary Fig. 10). Next, we assessed the three DMRs for the enrichment of specific histone modifications during normal development using published ChIP-seq data sets in wildtype embryonic stem cells (ESCs) and trophoblast stem cells (TSCs). Although histone marks were largely absent at all three DMRs in TSCs, enrichment for one or more methylated histone marks (e.g., H3K4me3 and/or H3K9me3) at this DMR was apparent in ESCs (Fig. 5a, Supplementary Fig. 9–10).
Table 1 Representation of sperm DMRs in candidate regions of epigenetic inheritance with in Mtrr<sup>+/−</sup>, Mtrr<sup>/−gt</sup> and Mtrr<sup>gt</sup> males.

| Location of sperm DMRs                              | Mtrr<sup>+/−</sup> | Mtrr<sup>/−gt</sup> | Mtrr<sup>gt</sup> |
|----------------------------------------------------|-------------------|-------------------|-------------------|
| Genome-wide                                        | 91 (100%)         | 203 (100%)        | 599 (100%)        |
| Regions of nucleosome retention<sup>41</sup>        | 31 (34.1%)        | 57 (28.1%)        | 87 (14.5%)        |
| Reprogramming resistant regions in pre-implantation embryo<sup>47</sup> | 37 (40.7%)        | 96 (47.3%)        | 325 (54.3%)       |
| Reprogramming resistant regions in germline<sup>46</sup> | 2 (2.2%)          | 5 (2.5%)          | 23 (3.8%)         |
| Reprogramming resistant regions in pre-implantation embryo<sup>47</sup> & germline<sup>46</sup> | 2 (2.2%)          | 4 (2.0%)          | 16 (2.7%)         |
| Regions of nucleosome retention<sup>41</sup> & resistant to reprogramming in pre-implantation embryo<sup>47</sup> | 19 (20.9%)        | 36 (17.7%)        | 49 (8.9%)         |
| Regions of nucleosome retention<sup>41</sup> & resistant to reprogramming in germline<sup>46</sup> | 1 (1.1%)          | 2 (1.0%)          | 4 (0.67%)         |

<sup>1</sup>Number of DMRs identified compared to C57Bl/6 J controls followed by the percentage of the total number of DMRs in brackets.

Altogether, DMRs identified in sperm of Mtrr<sup>+/−gt</sup> males highlight regions that might be important for transcriptional regulation in the early conceptus by other epigenetic mechanisms. Therefore, future analyses of broader epigenetic marks at these sites are required in the Mtrr<sup>gt</sup> mouse line.

HIRA as a potential biomarker of maternal phenotypic inheritance. The importance of the Hira DMR in phenotypic inheritance was further considered based on its known resistance to germline reprogramming<sup>46</sup>, and its potential function in gene regulation (Fig. 5a) and transcriptional memory (Fig. 4d). HIRA is a histone H3.3 chaperone, which lends itself well to a role in epigenetic inheritance given its broad functionality in transcriptional regulation<sup>26</sup>, maintenance of chromatin structure in the developing oocyte<sup>25</sup>, and the male pronucleus after fertilisation<sup>27</sup>, and in rRNA transcription<sup>27</sup>. Hira<sup>−/−</sup> mice<sup>32</sup> and the Mtrr<sup>gt</sup> mouse line<sup>3</sup> display similar phenotypes including growth defects, congenital malformations, and embryonic lethality by E10.5. Furthermore, Mtrr<sup>gt</sup> genotypic severity correlated with the degree of hypermethylation in the Hira DMR in sperm (Fig. 5b, c), suggesting that the Hira DMR is responsive to alterations in folate metabolism.

The potential regulatory legacy of the Hira DMR in sperm was further explored in the Mtrr<sup>+/−gt</sup> maternal grandfather pedigree. The Hira DMR, which was 6 kb downstream of the Hira gene and overlaps with a CpG island and CTCF binding site in ESCs and TSCs (Fig. 5a), was substantially hypermethylated in sperm of F0 Mtrr<sup>+/−gt</sup> males compared with controls (39.0±4.1% more methylated CpGs per CpG site assessed; Fig. 5b, c). As with the other sperm DMRs assessed (Fig. 3), the Hira DMR was reprogrammed in F1–F3 wildtype embryos and placentas at E10.5 (Fig. 3a, Supplementary Fig. 11a). Although we originally assessed Hira mRNA in the F1–F2 generations in the maternal grandfather pedigree (Fig. 4), further analysis revealed that Hira isoforms (mRNA and long non-coding RNA (lincRNA 209); Fig. 5a) were differentially regulated at E10.5 (Fig. 6a–c). Hira lincRNA function is unknown, though lincRNA-based mechanisms often control cell fates during development by influencing the nuclear organisation and transcriptional regulation<sup>35</sup>. Notably, this pattern of RNA expression was associated with generational patterns of phenotypic inheritance. For instance, we observed down-regulation of Hira mRNA in F2–F3 wildtype embryos and not F1 wildtype embryos at E10.5 (Fig. 6a–c). Conversely, significant upregulation of Hira lincRNA expression was apparent only in F1 wildtype embryos at E10.5 (Fig. 6a–c). This expression pattern was embryo-specific since the corresponding placentas showed normal Hira transcript levels in each generation assessed compared to controls (Fig. 6a–b). Since phenotypes at E10.5 were apparent in F2 generation onwards, yet were absent in the F1 generation of the Mtrr<sup>+/−gt</sup> maternal grandfather pedigree<sup>3</sup>, embryo-specific Hira RNA misexpression reflected the pattern of phenotypic inheritance.

To further investigate a potential link between Hira expression and phenotypic inheritance, we analysed wildtype F1–F3 conceptuses at E10.5 derived from F0 Mtrr<sup>+/−gt</sup> females (i.e., the maternal grandmother pedigree; Supplementary Fig. 1e). The breeding scheme was as follows: F0 Mtrr<sup>+/−gt</sup> females were mated with C57Bl/6 J control males. The F1 progeny was collected at E10.5 for genotype and phenotype analysis or allowed to litter out. The resulting adult F1 wildtype females were mated with C57Bl/6 J males. The F2 wildtype progeny was similarly collected at E10.5 for analysis or adult F2 wildtype females were mated with C57Bl/6 J control males to generate F3 wildtype conceptuses for analysis at E10.5 (Supplementary Fig. 1e). In this pedigree, all generations, including the F1 generation, display a broad spectrum of developmental phenotypes<sup>3</sup>. Supporting our hypothesis, Hira mRNA expression was downregulated in F1–F3 wildtype embryos derived from an F0 Mtrr<sup>+/−gt</sup> female compared to C57Bl/6 J controls (Fig. 6d), thus correlating with maternal
phenotypic inheritance. As expected, Hira lncRNA transcripts were unchanged in F1 and F3 wildtype embryos (Fig. 6d). However, Hira lncRNA was downregulated in F2 wildtype embryos (Fig. 6d), which display the highest frequency of phenotypes among the three generations. Regardless, these data suggested that altered Hira mRNA transcripts might be a potential biomarker of maternal phenotypic inheritance since dysregulation of Hira mRNA occurred only in wildtype embryos with high phenotypic risk as a result of their derivation from an oocyte with Mtrr<sup>gt</sup> ancestry rather than sperm (Fig. 7).

How the Hira gene is regulated is unknown. Mtrr<sup>gt</sup> embryos at E10.5 (derived from Mtrr<sup>gt</sup>/gt intercrosses), which demonstrate a greater phenotypic risk than the Mtrr<sup>+/+</sup> maternal grandparental pedigrees, also displayed dysregulation of Hira mRNA and lncRNA expression (Supplementary Figs. 8k–m, 11d). This finding was in association with normal DNA methylation at the Hira DMR in Mtrr<sup>gt</sup>/gt embryos at E10.5 (Supplementary Fig. 8b), implicating additional mechanisms of epigenetic regulation. Histone methylation profiles at the Hira locus in normal ESCs and TSCs indicate a potential role for the Hira promoter and Hira DMR (Fig. 5a) in gene regulation that will require future investigation.

HIRA protein levels were also dysregulated in Mtrr<sup>gt</sup>/gt embryos and F2 wildtype embryos and placentas (Fig. 6e–h, Supplementary Fig. 11e, f). The pattern of dysregulation did not always occur in a manner predicted by the direction of Hira mRNA expression. For example, there was an increase in HIRA protein levels in F2 wildtype embryos when Hira mRNA was downregulated (Fig. 6b, d–h). This discrepancy might result from defective HIRA protein degradation, drastic translational upregulation of HIRA protein to compensate for low mRNA levels, or alternatively, negative feedback to down-regulate HIRA mRNA owing to high levels of HIRA protein in the embryo. Further work will be required to delineate whether the HIRA chaperone mediates maternal phenotypic inheritance in the Mtrr<sup>gt</sup> model and other models of TEI.

Discussion
We investigated potential mechanisms contributing to epigenetic inheritance in Mtrr<sup>gt</sup> mice, a unique model of mammalian TEI<sup>3</sup>. In the Mtrr<sup>gt</sup> model, inheritance of developmental phenotypes and epigenetic instability occurs via the maternal grandparental lineage with an F0 Mtrr<sup>gt/−</sup> male or female initiating the TEI effect<sup>3</sup>. Here, we assessed DNA methylation in spermatozoa to understand how the germline epigenome was affected by the Mtrr<sup>gt</sup> allele. We chose sperm due to its experimental tractability and to avoid the confounding factors of the F0 uterine environment when assessing epigenetic inheritance via the germline. We identified several distinct DMRs in regions of predicted importance in transcriptional regulation and epigenetic inheritance including regions of nucleosome retention and reprogramming resistance. This result illustrates widespread epigenetic instability in the male germline of the Mtrr<sup>gt</sup> model, particularly in the F0 Mtrr<sup>gt/−</sup> males of the maternal grandfather pedigree. While largely resolved in somatic tissue of subsequent wildtype generations, some germline DMRs were correlated with transcriptional changes at associated loci in the F1–F3 progeny. This proposed transcriptional memory of a germline DMR persisted for at least three generations, longer than previously reported in another model<sup>1</sup>. This observation indicates additional epigenetic factors beyond DNA methylation in the mechanism of TEI. Furthermore, the histone chaperone gene Hira emerged as a transcriptional biomarker and potential mediator of maternal phenotypic inheritance.
The extent to which genetic and epigenetic factors interact in this and other TEI models is unclear. One-carbon metabolism is involved in thymidine synthesis, and DNA breaks triggered by folate deficiency-induced uracil misincorporation were demonstrated in erythrocytes of splenectomised patients and prostate adenoma cells. However, WGS of Mtrrgt/gt embryos excluded genetic instability in the Mtrr mouse line because de novo mutations occurred at an expected frequency and comparable frequency to control embryos. The WGS data also discounted alternative phenotype-causing mutations outside of the Mtrr locus and, when compared with our sperm methylome data set, showed that differential CpG methylation was unlikely due to underlying genetic variation in the Mtrrgt mouse line. Therefore, the epigenetic consequences of the Mtrrgt allele rather than genetic

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**Fig. 5 Epigenetic characteristics of Hira DMR in mice.**

**a** Enrichment of DNA binding proteins (CCTC-binding factor, CTCF) and histone modifications (H3K27ac, H3K4me3, H3K4me1, H3K9me3) in the Hira locus on mouse chromosome (Chr) 16 using published data sets of chromatin immunoprecipitation followed by sequencing (ChiP-seq) analyses in cultured wildtype embryonic stem cells (ESCs) and trophoblast stem cells (TSCs). Dark grey rectangles indicate enrichment peak calling for each histone modification. Pink rectangle and light grey shading indicate the Hira differentially methylated region (DMR) identified in sperm of Mtrr+/gt and Mtrrgt/gt males. Blue rectangles indicate CpG islands. Green rectangles indicate enhancers. Schematics of protein-encoding (brown) and long non-coding RNA (lncRNA) encoding (purple) Hira isoforms are shown. Region of qPCR primer sets 1 and 2 are also indicated (black boxes).

**b** Partial schematic drawing of Hira transcripts and Hira DMR (pink rectangle) in relation to MeDIP-seq reads in sperm from C57Bl/6 J (black), wildtype (Mtrr+/+; purple), Mtrr+/gt (green) and Mtrrgt/gt (blue) males. N = 8 males/group.

**c** The average percentage methylation at individual CpG sites (mean ± standard deviation) in the Hira DMR in sperm from C57Bl/6 J males (black circles; N = 4 males), Mtrr+/gt males (purple circles; N = 4 males), Mtrrgt/gt males (blue circles; N = 8 males), and Mtrrgt/gt males (green circles; N = 8 males). Two-way ANOVA with Sidak’s multiple comparisons test performed on mean methylation per CpG site. *p* values are indicated for each male genotype compared to C57Bl/6 J and were similar for all CpG sites assessed within one genotype as indicated: C57Bl/6 J versus Mtrr+/+; not significant (n.s.); C57Bl/6 J versus Mtrr+/gt males or Mtrrgt/gt males, *p* < 0.0001. Source data are provided as a Source Data file.
Iinstability are more likely to instigate TEI in this model. Generating alternative Mtrr mutations and/or backcrossing the Mtrrgt allele into a different mouse strain will further assess whether genetics has a role in TEI.

Our data show that inheritance of sperm DMRs by offspring somatic tissue was unlikely, as have other studies4,49,50. Instead, somatic cell lineages might inherit germline epigenetic instability in a broader sense. For instance, despite normal DNA methylation in F1–F3 wildtype embryos and placentas (E10.5) at the genomic locations highlighted by sperm DMRs, epigenetic instability associated with gene misexpression is still evident in F1 and F2 wildtype placentas at several loci3. It is possible that abnormalities in the sperm epigenome of F0 Mtrr+/gt males might be reprogrammed and then stochastically and abnormally re-established/maintained in other genomic regions in wildtype offspring. This hypothesis might explain inter-individual phenotypic variability in the F2–F3 generations. However, we showed that sperm of Mtrr+/+ males exhibited several DMRs that overlapped with sperm DMRs in Mtrr+/gt males (representing their fathers). Therefore, reconstruction of specific atypical F0 germline methylation patterns in the F1 wildtype germline is possible in the Mtrrgt model. Vinclozolin toxicant exposure model of TEI shows dissimilar DMRs in spermatozoa of F1 and F3 offspring8, which suggests that epigenetic patterns might shift as the generational distance from the F0 individual increases.

Dietary folate deficiency causes differential methylation in sperm and craniofacial defects in the immediate offspring 49, though whether it leads to TEI is unknown. There was no overlap of DMRs when sperm methylomes were compared between the diet model and Mtrr+/gt males. This result disputes the existence of folate-sensitive epigenomic hotspots in sperm. However, the severity of insult or technical differences (e.g., MeDIP-array49 versus MeDIP-seq) might explain the discrepancy. Why only one Mtrrgt allele sufficiently initiates TEI is unknown since a direct
Fig. 6 Dysregulation of Hira RNA expression in embryos aligns with a pattern of maternal phenotypic inheritance. a–d RT-qPCR analysis of Hira mRNA (solid bars, primer set 1) and Hira IncRNA (striped bars, primer set 2) expression in embryos and placentas at E10.5. The tissue and pedigrees were assessed (see also Supplementary Fig. 1a, d–e for the breeding scheme): a F1 wildtype (Mtrr+/+) conceptuses from F0 Mtrr+/gt males (orange bars; N = 4–5 embryos, N = 8 placentas), b F2 wildtype conceptuses from F0 Mtrr+/gt males (purple bars, N = 5 embryos and N = 7–8 placentas from phenotypically normal (n) conceptuses; pink bars, N = 5 severely affected (a) embryos), c F3 wildtype conceptuses from F0 Mtrr+/gt males (teal bars, N = 3–4 phenotypically normal embryos; turquoise bars, N = 3 severely affected embryos), and d F1 wildtype embryos from F0 Mtrr+/gt females (orange bars, N = 7 phenotypically normal embryos; green bars, N = 3 severely affected embryos), F2 wildtype embryos from F0 Mtrr+/gt females (purple bars, N = 7 phenotypically normal embryos; pink bars, N = 7 severely affected embryos), and F3 wildtype embryos from F0 Mtrr+/gt females (teal bars, N = 5–7 phenotypically normal embryos; turquoise bars, N = 3 severely affected embryos). C57Bl/6 J conceptuses were controls (black bars, N = 4–8 embryos or placenta/experiment). e–h Western blot analysis showing HIRA protein expression in F1 wildtype (orange bars) and F2 wildtype (purple bars) conceptuses derived from e to f F0 Mtrr+/gt males or g–h F0 Mtrr+/gt females. C57Bl/6 J (C57) conceptuses (black bars) were assessed as controls. e, f C57Bl6/J: N = 4–5 embryos, N = 4–5 placentas; F1: N = 4 embryos, N = 3–8 placentas; F2: N = 7–9 embryos, N = 7–8 placentas. Images of western blot gels showing HIRA protein with β-actin as a loading control in f and h were quantified in e and g, respectively, using the background subtraction method. HIRA protein levels were normalised to β-actin. All RNA and protein data were plotted as mean ± standard deviation and relative to C57Bl/6 J (normalised to 1). Experiments were conducted in technical duplicates (protein) or triplicates (RNA). Statistical analyses: a–d Two-tailed independent t test or Kruskal–Wallis test with Dunn’s multiple comparison. a ***p = 0.0034; b **p = 0.0049, ***p = 0.0002; c *p = 0.0004, ***p < 0.0001; d F1: **p = 0.0056, F2 (primer set 1): *p = 0.06, *p = 0.0203, F2 (primer set 2): *p = 0.0457, F3: *p = 0.016. e One-way ANOVA. p values are shown on graphs. Pedigree legend: circle, female; square, male; blue outline, C57Bl/6 J line; black outline, Mtrr+/+ mouse line; white fill, Mtrr+/+; half-white/half-black fill, Mtrr+/gt, black fill, Mtrrgt/gt, F0, parental generation; F1, first filial generation; F2, second filial generation; F3, third filial generation. Source data are provided as a Source Data file.

Fig. 7 Proposed model of maternal grandparental phenotype inheritance in Mtrrgt model that implicates epigenetic instability in the germline. A model proposing how epigenetic instability generated by the Mtrrgt mutation might be differently inherited over multiple generations depending upon whether TEI is initiated by a an oocyte or b sperm of an F0 Mtrr+/gt individual. Trends of Hira mRNA expression are shown. Pedigree legend: circle, female; square, male; blue outline, C57Bl/6 J control; black outline, Mtrr+/+ mouse line; white filled, Mtrr+/+; half-white/half-black filled, Mtrr+/gt. See also Supplementary Fig. 1 for breeding schemes. Scale bar: 500 μm. F0, parental generation; F1, first filial generation; F2, second filial generation; F3, third filial generation.
paramutation effect was not evident and mice do not display similar metabolic derangement to Mtrrgt/+ females. Whether specific DNA methylation patterns observed in F0 sperm of the maternal grandfather pedigree are reconstructed in F1 oocytes is yet-to-be determined. Extensive differences between sperm and oocytes methylomes will make this difficult to resolve and yet also emphasizes that additional epigenetic mechanisms are likely involved, such as histone modifications, DNA methylation and emphasise the complexity of epigenetic mechanisms involved in TEI. Ultimately, our data indicate the importance of normal folate metabolism in both women and men of reproductive age for healthy pregnancies in their daughters and granddaughters.

Methods

Ethics statement. This research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body.

Animal model. Mtrr+/+Gt(GX)343TG (Mgl:35261359) mouse line, referred to as the Mtrr model, was generated when a β-geo gene trap (gt) vector was inserted into intron 9 of the Mtrr gene in 129P2Ola/Hsd ESCs. Mtrr ESCs were injected into C57Bl/6j blastocysts. Upon germline transmission, the Mtrr allele was backcrossed into the C57Bl/6j genetic background for at least eight generations. The F1 generation Mtrr+/+ males were produced from Mtrr+/+ intercrosses (Supplementary Fig. 1b). Mtrr+/- mice were produced from Mtrr+/+ intercrosses (Supplementary Fig. 1c). C57Bl/6f1 mice from The Jackson Laboratories (www.jaxmice.jax.org) and 129P2Oa/Hsd from Envigo (previously Harlan Laboratories [www.envigo.com]) were used as controls and were bred in house and separately from the Mtrr mouse line. Mice were housed in a temperature- and humidity-controlled environment with a 12 h light-dark cycle. All mice were fed a normal chow diet (Rodent No. 3 chow, Special Diet Services) ad libitum from weaning, which included (per kg of diet): 1.6 g choline, 2.73 mg folate acid, 26.8 μg vitamin B12, 3.4 g methionine, 51.3 mg zinc. Mice were killed via cervical dislocation. Genotyping for the Mtrr+/- and Mtrrgt+/- alleles and for sex (Rbm31) was performed using PCR on DNA extracted from ear tissue or yolk sac using primer sequences in Supplementary Table 8.

To determine the multigenerational effects of the Mtrr+/- allele in the maternal grandfather, the following mouse pedigrees was established (Supplementary Fig. 2). For the F1 generation, F0 Mtrr+/+ females were mated with C57Bl/6j males (Supplementary Fig. 1) and the resulting Mtrr+/- progeny were analysed. For the F2 generation, F1 Mtrr+/- females were mated with C57Bl/6j males and the resulting Mtrr+/- progeny were analysed. For the F3 generation, F2 Mtrr+/- females were mated with C57Bl/6j males and the resulting Mtrr+/- progeny were analysed. A similar pedigree was established to assess the effects of the Mtrr+/- allele in the maternal grandmother with the exception of the F0 generation, which involved the mating of an Mtrr+/- female with a C57Bl/6j male (Supplementary Fig. 1e).

Tissue dissection and phenotyping. For embryo and placenta collection, timed matings were established and noon on the day that the vaginal plug was detected was considered embryonic day (E) 0.5. Embryos and placentas were dissected at E10.5. Embryos and placentas were dissected at E10.5 in cold 1X phosphate-buffered saline and were scored for phenotypes (see below), photographed, weighed, and snap frozen in liquid nitrogen for storage at −80°C. All conceptuses were dissected using a Zeiss SteREO Discovery V8 microscope with an AxioCam MRC5 camera (Carl Zeiss). Livers were collected from pregnant female mice (gestational day 10.5), weighed and snap frozen in liquid nitrogen for storage at −80°C. Both male and female conceptuses at E10.5 were assessed, as no sexual dimorphism is apparent at this stage. While many individual mice were assessed over the course of this study, some of the individual tissue samples were assessed for the expression of multiple genes or for methylation at multiple DMRs. A rigorous phenotyping regime was performed at E10.5 as previously described. In brief, all conceptuses were scored for one or more congenital malformation (as appropriate for the developmental stage) including failure of the neural tube to close in the cranial or spinal cord region, malformed branchial arches, pericardial edema, reversed heart looping, enlarged heart, and/or eccentric chorioallantoic attachment. Twining or haemorrhaging was also scored as a severe abnormality. Conceptuses with severe abnormalities were categorised separately from resorptions, the latter of which consisted of maternal decidua surrounding an indistinguishable mass of fetal derived tissue. Resorptions were not assessed in this study as they represented dead conceptuses. Embryos with <30 somite pairs were considered developmentally delayed. Embryos with 30–39 somite pairs but a crown-rump length more than two standard deviations (sd) from the mean crown-rump length (Mtrr+/+) were considered growth restricted or growth enhanced. Conceptuses were considered phenotypically normal if they were absent of congenital malformations, had 30–39 somite pairs, and had crown-rump lengths within two sd of controls. AxioVision 4.7.2 imaging software was used to measure crown-rump lengths (Carl Zeiss).

Conceptus size at E10.5 was unaffected by litter size in all Mtrr+/- pedigrees assessed.

Spermatozoa collection. Spermatozoa from cauda epididymides and vas deferens were collected from 16 to 20 week-old fertile mice using a swim-up procedure as previously described with the following amendments. Spermatozoa were released from 20 min at 37°C in Donners Medium (25 mM NaHCO3, 20 mg/mL bovine serum albumin, 1 mM sodium pyruvate and 0.53% (vol/vol) sodium d-lactate in Donners stock (135 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, and 30 mM HEPES)). Samples were centrifuged at 500 × g (21°C) for 10 min. The supernatant was transferred and centrifuged at 1300 × g (14°C) for 15 min. After the majority of
supernatant was discarded, the samples were centrifuged at 13,000 x g (4°C) for 5 min. Further supernatant was discarded and the remaining spermatozoa were centrifuged at 12,000 x g for 1 min and stored at −80°C.

**Nucleic acid extraction.** For embryo, trophoblast and liver tissue, genomic DNA (gDNA) was extracted using DNeasy Blood and Tissue kit (Qagen) according to the manufacturer’s instructions. RNA was extracted from tissues using the AllPrep DNA/RNA Mini Kit (Qagen). For sperm, Solution A (75 mM NaCl pH 8; 25 mM EDTA) and Solution B (10 mM Tris-HCl pH 8; 10 mM EDTA; 1% SDS; 80 mM DTT) were added to the samples followed by RNase A incubation (37°C, 1 h) and Proteinase K incubation (55°C or overnight) respectively. Non-degraded gDNA was extracted using phenol/chloroform/isomyl alcohol mix (25:24:1) (Sigma–Aldrich) as per the manufacturer’s instructions and pelleted using standard methods in TE buffer. DNA quality and quantity were confirmed using gel electrophoresis and QuantFlour dsDNA Sample kit (Promega) as per the manufacturer’s instructions.

**Whole-genome sequencing.** Non-degraded gDNA from two whole C57Bl/6 J embryos at E10.5 (one male, one female) and six whole Mtrr<sup>−/−</sup> embryos with congenital malformations (four male and two females) were extracted using phenol/chloroform/isomyl alcohol mix (25:24:1) (Sigma–Aldrich) as per the manufacturer’s instructions and pelleted using standard methods in TE buffer. DNA quality and quantity were confirmed using gel electrophoresis and QuantFlour dsDNA Sample kit (Promega) as per the manufacturer’s instructions.

**MultiQC package (version 1.4, http://multiqc.info).** FastQ files were merged using seqkit (version 0.8.0).<sup>72</sup> Reads were locally realigned and SNPs and short indels identified using BWA (version 0.7.12–r1039).<sup>71</sup> FastQ files were merged using seqkit (version 0.8.0).<sup>72</sup> Sequencing reads were aligned to the C57Bl/6 J reference genome (mm10) using Bowtie2 (version 2.3.4).<sup>73</sup> Reads were locally realigned and SNPs and short indels identified using GenomeAnalysisTK (version 3.7).<sup>77</sup> Homozygous variants were called when >90% of reads at the locus supported the variant call, whereas variants with at least 30% of reads supporting the variant calls were classified as heterozygous. Two rounds of filtering of variants were performed as follows. First, variants with low mapping quality (<40), overlapping annotated repeats or segmental duplications, and (vi) >3 heterozygous variants fell within a 10 kb region were removed using vcftools (version 0.1.15).<sup>75</sup> In order to identify SNPs, the data were remapped using the mouse genome (https://fantom.gsc.riken.jp/5/mm10.enhancers.bed.gz) was used. All.bed files were converted to bigwig using UCSC tools. The distance in base pairs between DMRs and genes or between enhancers and genes was calculated using the closest coordinates (the start/end of DMR or enhancer) to the transcriptional start site (TSS) minus the TSS and then plus 1. To determine further interactions between enhancers and promoters, we analysed public Hi-C data sets for ESC and TSC (E-MTAB-6585; ESC_promoter-other_interactions_GOTHiC.txt) and random hexamer primers (Thermo Scientific) and random hexamer primers (Thermo Scientific) using 1–2 μg of RNA in a 20-μl reaction according to manufacturer’s instructions. PCR amplification was conducted using MESA Green qPCR MasterMix Plus for SYBR Assay (Eurogentec Ltd.) on a DNA Engine Opticon2 thermocycler (BioRad). The following cycling conditions were used: 95°C for 10 min, 40 cycles: 95°C for 30 sec, 60°C for 1 min, followed by melt curve analysis. Transcript levels were normalised to Hprt and/or Gapdh RNA levels. Relative CDNA expression levels were analysed using the 2(−ΔΔCt) method.<sup>87</sup> Experiments were conducted in technical triplicate with biological replicates indicated in the figure legends. Transcript levels in C57Bl/6 J tissue were normalised to 1. For primer sequences and concentrations, refer to Supplementary Table 8.

**Enhancer analysis.** To determine whether genetic variants or DMRs overlapped with known enhancer regions, FANTOM5 enhancer database<sup>85</sup> for GRCm38 mouse genome (https://fantom.gsc.riken.jp/5; F5.mm10.enhancers.bed.gz) was used. All.bed files were applied to UCSC Genome Browser<sup>86</sup> to check for region-specific features. The distance in base pairs between DMRs and genes or between enhancers and genes was calculated using the closest coordinates (the start/end of DMR or enhancer) to the transcriptional start site (TSS) minus the TSS and then plus 1. To determine further interactions between enhancers and promoters, we analysed public Hi-C data sets for ESC and TSC (E-MTAB-6585; ESC_promoter-other_interactions_GOTHiC.txt and TSC_promoter-other_interactions_GOTHiC.txt) using WashU Epigenome Browser<sup>86</sup>.

**Quantitative reverse transcription PCR (RT-qPCR).** For RNA expression analysis, cDNA was synthesised using RevertAid H Minus reverse transcriptase (Thermo Scientific) and random hexamer primers (Thermo Scientific) using 1–2 μg of RNA in a 20-μl reaction according to manufacturer’s instructions. PCR amplification was conducted using MESA Green qPCR MasterMix Plus for SYBR Assay (Eurogentec Ltd.) on a DNA Engine Opticon2 thermocycler (BioRad). The following cycling conditions were used: 95°C for 10 min, 40 cycles: 95°C for 30 sec, 60°C for 1 min, followed by melt curve analysis. Transcript levels were normalised to Hprt and/or Gapdh RNA levels. Relative CDNA expression levels were analysed using the 2(−ΔΔCt) method.<sup>87</sup> Experiments were conducted in technical triplicate with biological replicates indicated in the figure legends. Transcript levels in C57Bl/6 J tissue were normalised to 1. For primer sequences and concentrations, refer to Supplementary Table 8.

**Bisulfitte mutation and pyrosequencing.** Between 250 ng and 2 μg of gDNA extracted from each tissue sample was bisulfitte treated with an ImPrima DNA Modification Kit (Sigma). Control samples lacking DNA template were run to ensure that there was no contamination during the bisulfitte conversion of DNA. To quantify DMR CpG methylation, pyrosequencing was performed. In all, 30 ng of bisulfitte-converted DNA was used as a template for PCR, together with 0.2 μM of each bisulfitte primer and 0.25 μl of HotStarTag PlusDNA Polymerase (Qiagen). Refer to Supplementary Table 11 for primer sequences, which were designed using PyroMark Assay Design Software 2.0 (Qiagen). PCR was performed in triplicate.
using the following conditions: 95°C for 5 min, 40 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 55 sec and then 72°C for 5 min. PCR products were purified using Sepharose 6 Fast Flow Performance beads (GE Healthcare). The beads bound to DNA were washed in 70% ethanol, 0.1 M NaOH and 10 mM Tris-acetated (pH 7.6) and then hybridized to the sequencing primer in PyroMark annealing buffer according to the manufacturer’s instructions. Pyrosequencing was conducted using PyroMark Gold reagents kit (Qagen) on a PyroMark MD pyrosequencer (Biotage). The mean CpG methylation was calculated using three to eight biological replicates and at least two technical replicates. Analysis of methylation status was performed using Pyro Q-CpG software (version 1.0.9, Biotage).

**Mass spectrometry.** Sperm gDNA was digested into individual nucleoside components using the DNA Degradase Plus kit (Zymo Research) according to the manufacturer’s instructions. In all, 100 ng of degraded DNA per individual was sent to the Babraham Institute Mass Spectrometry Facility (Cambridge, UK), where global cytosine, 5mC and 5hmC was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described.28 The sperm of nine males were assessed per genotype and analysed in pools each containing three unique individuals. All pooled samples were analysed in triplicate. Global 5mC and 5hmC levels are reported as percentiles relative to C.

**Western blotting.** Embryos and placenats at E10.5 were homogenised in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4 and complete mini EDTA-protease inhibitor cocktail [Roche Diagnostics]) with Lysing Matrix D ceramic beads (MP Biomedical) using a MagNA Lyser (Roche Diagnostics) at 5500 rpm for 20 sec. Samples were incubated on ice for 5 min and then homogenised again at 5500 rpm for 20 sec. Homogenates were then incubated on ice for 20 min with brief intervening vortexing steps occurring every 5 min. Samples were then centrifuged at 10,000 x g for 5 min. Supernatant from each sample was transferred to a new tube and centrifuged again at 10,000 x g for 5 min to ensure that all residual tissue was removed. The protein concentration of tissue lysates was determined using bicinchoninic acid (Sigma-Aldrich). Proteins were denatured with gel loading buffer (50 mM Tris [pH 6.8], 100 mM DTT, 2% SDS, 10% glycerol and a trace amount of bromophenol blue) at 70°C for 10 min. Equivalent amounts of protein were resolved by 8–10% SDS–PAGE and blotted onto nitrocellulose (0.2 µm, Amersham Protran) with a semi-dry blotter (GE Healthcare). The membrane was stained with Ponceau S to ensure equal loading. Band intensities were determined using the following conditions: 95°C for 5 min, 40 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 55 sec and then 72°C for 5 min. PCR products were purified using Sephadex 6 Fast Flow Performance beads (GE Healthcare). The beads bound to DNA were washed in 70% ethanol, 0.1 M NaOH and 10 mM Tris-acetated (pH 7.6) and then hybridized to the sequencing primer in PyroMark annealing buffer (Qagen) according to the manufacturer’s instructions. Pyrosequencing was conducted using PyroMark Gold reagents kit (Qagen) on a PyroMark MD pyrosequencer (Biotage). The mean CpG methylation was calculated using three to eight biological replicates and at least two technical replicates. Analysis of methylation status was performed using Pyro Q-CpG software (version 1.0.9, Biotage).

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E.D.W. conceived the project. G.E.T.B. collected sperm, performed DNA/RNA extractions, generated WGS and MeDIP libraries, and performed RT-qPCR and bisulphite pyrosequencing analyses. G.E.T.B. and E.D.W. dissected tissue and phenotyped conceptuses. G.E.T.B. and E.D.W. collected and analysed the data. X.Z., R.S.H. and G.E.T.B. designed and performed bioinformatics analyses. H.W.Y. performed the western blotting analysis. G.E.T.B., A.C.F.-S., G.J.B. and E.D.W. designed the experiments and interpreted the results. E.D.W. and G.E.T.B. wrote the manuscript. All authors read and revised the manuscript.

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The authors declare no competing interests.

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