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RESEARCH ARTICLE SUMMARY

IN UTERO EFFECTS

In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism

Elizabeth J. Radford, Mitsuteru Ito, Hui Shi, Jennifer A. Corish, Kazuki Yamazawa, Elvira Isganaitis, Stefanie Seisenberger, Timothy A. Hore, Wolf Reik, Serap Erkek, Antoine H. F. M. Peters, Mary-Elizabeth Patti,* Anne C. Ferguson-Smith*

INTRODUCTION: The rapid global rise in metabolic disease suggests that nongenetic environmental factors contribute to disease risk. Early life represents a window of phenotypic plasticity important for adult metabolic health and that of future generations. Epigenetic inheritance has been implicated in the paternal transmission of environmentally induced phenotypes, but the mechanisms responsible remain unknown.

RATIONALE: We investigated the role of DNA methylation in epigenetic inheritance in an established murine model of intergenerational developmental programming. F₁ offspring of undernourished dams (UN) have low birth weight and multiple metabolic defects. Metabolic phenotypic inheritance to the F₂ generation is observed through the paternal line, even though the F₁

mice did not experience postnatal environmental perturbation. The timing of nutritional restriction coincides with methylation reacquisition in F₁ male primordial germ cells (PGCs). Therefore, we assessed F₁ sperm whole-genome methylation using immunoprecipitation of methylated DNA, combined with high-throughput sequencing, followed by independent validation. We characterized the regions susceptible to methylation change and investigated the legacy of such methylation change in the phenotypic development of the next generation.

RESULTS: In UN mice, 111 regions are hypomethylated relative to control sperm, and these changes are validated by bisulfite pyrosequencing. Methylation differences span multiple CpGs, with robust absolute changes of 10 to 30% (relative reduction ~50%). The

absolute methylation level is consistent with differentially methylated regions (DMRs) being “low-methylated regions,” known to be enriched in regulatory elements. Indeed, luciferase assays suggest a role for these DMRs in transcriptional regulation. Hypomethylated DMRs are significantly depleted from coding and repetitive regions and enriched in intergenic regions and CpG islands. They are also enriched in nucleosome-retaining regions, which suggests that, at some loci, paternal germline hypomethylation induced by in utero undernutrition is transmitted in a chromatin context. DMRs are late to regain methylation in normal male PGCs. This may render them particularly susceptible to environmental perturbations that delay or impair remethylation in late gestation.

Except for imprinted loci, gene-associated male germline methylation has generally been thought to be largely erased in the zygote, although recent studies suggest that

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resistance to reprogramming is more widespread. Indeed, 43% of hypomethylated DMRs persist and thus have the potential to affect development of the next

generation. We show that differential methylation is lost in late-gestation F₂ tissues, but considerable tissue-specific differences in expression of metabolic genes neighboring DMRs are present. Thus, it is unlikely that these expression changes are directly mediated by altered methylation; rather, the cumulative effects of dysregulated epigenetic patterns earlier in development may yield sustained alterations in chromatin architecture, transcriptional regulatory networks, differentiation, or tissue structure.

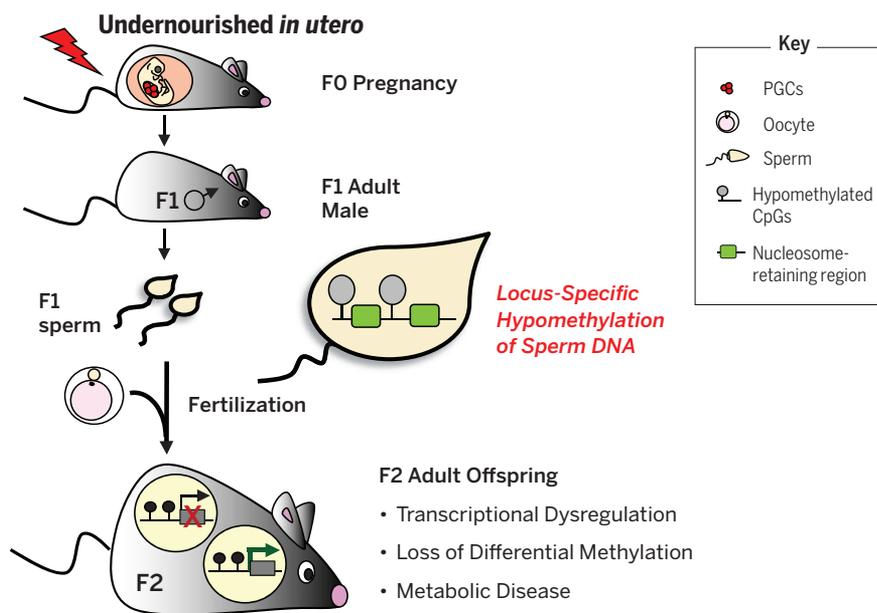
CONCLUSION: Prenatal undernutrition can compromise male germline epigenetic reprogramming and thus permanently alter DNA methylation in the sperm of adult offspring at regions resistant to zygotic reprogramming. However, persistence of altered DNA methylation into late-gestation somatic tissues of the subsequent generation is not observed. Nonetheless, alterations in gamete methylation may serve as a legacy of earlier developmental exposures and may contribute to the intergenerational transmission of environmentally induced disease. ■

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In utero undernourishment alters the adult germ cell methylome. Undernourishment during PGC reprogramming results in hypomethylation of discrete loci in adult sperm. These regions are enriched in nucleosomes and are low-methylated regions. Although partially resistant to blastocyst reprogramming, differential methylation does not persist in the next generation. However, dysregulated expression of genes neighboring DMRs is observed in F₂ offspring.

RESEARCH ARTICLE

IN UTERO EFFECTS

In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism

Elizabeth J. Radford,^{1*} Mitsuteru Ito,^{1†} Hui Shi,^{1†} Jennifer A. Corish,¹ Kazuki Yamazawa,^{1§} Elvira Isganaitis,^{2§} Stefanie Seisenberger,³ Timothy A. Hore,³ Wolf Reik,³ Serap Erkek,^{4,5,6} Antoine H. F. M. Peters,^{4,5} Mary-Elizabeth Patti,^{2||} Anne C. Ferguson-Smith^{1‡||}

Adverse prenatal environments can promote metabolic disease in offspring and subsequent generations. Animal models and epidemiological data implicate epigenetic inheritance, but the mechanisms remain unknown. In an intergenerational developmental programming model affecting F₂ mouse metabolism, we demonstrate that the in utero nutritional environment of F₁ embryos alters the germline DNA methylome of F₁ adult males in a locus-specific manner. Differentially methylated regions are hypomethylated and enriched in nucleosome-retaining regions. A substantial fraction is resistant to early embryo methylation reprogramming, which may have an impact on F₂ development. Differential methylation is not maintained in F₂ tissues, yet locus-specific expression is perturbed. Thus, in utero nutritional exposures during critical windows of germ cell development can impact the male germline methylome, associated with metabolic disease in offspring.

The rapid global rise in the incidence of diabetes, obesity, and cardiovascular disease suggests that nongenetic environmental factors are major contributors to disease risk. Epidemiological data and animal models have demonstrated that early life represents a window of phenotypic plasticity critically impor-

tant for later adult metabolic health (1). The impact of the early-life environment has been observed to extend over multiple generations in both human populations and animal models (2–8). There are at least two potential mechanisms mediating such non-Mendelian phenotypic inheritance: alterations in the parental metabolic

milieu that induce fetal developmental exposures in the second generation and epigenetic inheritance. The latter is strongly implicated when paternal transmission of environmentally induced phenotypes is observed because rodent males, present solely at breeding, contribute to the future generation only through the sperm. Although a role for histone modifications and/or RNA has been proposed (4), the epigenetic mechanism(s) responsible for intergenerational inheritance of environmentally induced phenotypes remains unknown.

Paternal transgenerational epigenetic inheritance of altered DNA methylation has been demonstrated previously: for example, in rodents exposed to the endocrine disruptor vinclozolin (9) and in mice with variable methylation at the *Agouti viable yellow*, *A^{vy}*, and *Axin-Fused*, *Axin^{Fu}*, alleles formed by insertion of IAPs (intracisternal A particles) into or near endogenous genes (10). In addition to repeat-mediated cis-acting effects, other endogenous loci that have an inherent

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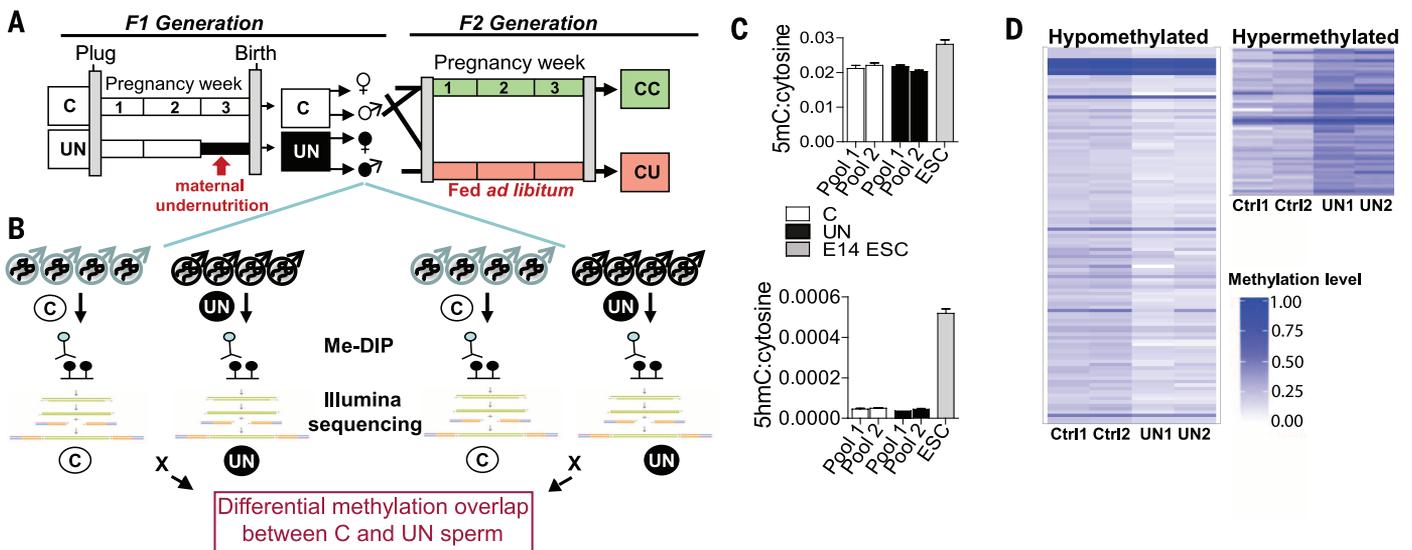


Fig. 1. Total methylation is stable in UN sperm, with significant locus-specific changes. (A) Experimental design. F₁ generation: Dams were randomized on pregnancy day 12.5 to control (C) or undernutrition (UN) groups, and UN food intake was restricted to 50%. Postnatal litters were equalized to eight pups, and animals were fed ad libitum. F₂ generation: Control F₁ females mated at age 2 months with nonsibling control or UN males and fed ad libitum to produce: CC, both parents controls; CU, control dam, UN sire. (B) Independent sperm DNA samples were quantified and pooled in equimolar ratios to generate

two pools per condition. Control pools: $n = 8$, five litters. UN pools: $n = 8$, four litters. After MeDIP-seq, two independent C versus UN comparisons identified DMRs where methylation fold change $>1.5\times$ and binomial $P < 0.0001$ in both independent biological replicates. (C) Mass spectrometry quantification of control and UN sperm 5-methyl-cytosine (above) and 5-hydroxymethyl-cytosine (below). E14 embryonic stem cells (ESCs) are shown for comparison. (D) Heat map of 111 hypomethylated DMRs (left) and 55 hypermethylated DMRs (right). Hypomethylated DMRs did not validate.

epigenetic vulnerability to environmental conditions may contribute to intergenerational phenotypes and play an important role in the developmental origins of health and disease. Furthermore, recent studies have suggested that resistance to zygotic DNA methylation reprogramming extends beyond imprinted domains (11–13), which raises the possibility that gametic methylation may play a larger role than previously recognized in early development. A key unanswered question is whether an altered in utero environment or nutritional insult might affect the DNA methylation profile of adult germ cells.

Our aim was to investigate the role of DNA methylation in epigenetic inheritance in an established in utero murine model of intergenerational developmental programming (3). To produce the most robust phenotype, the maxi-

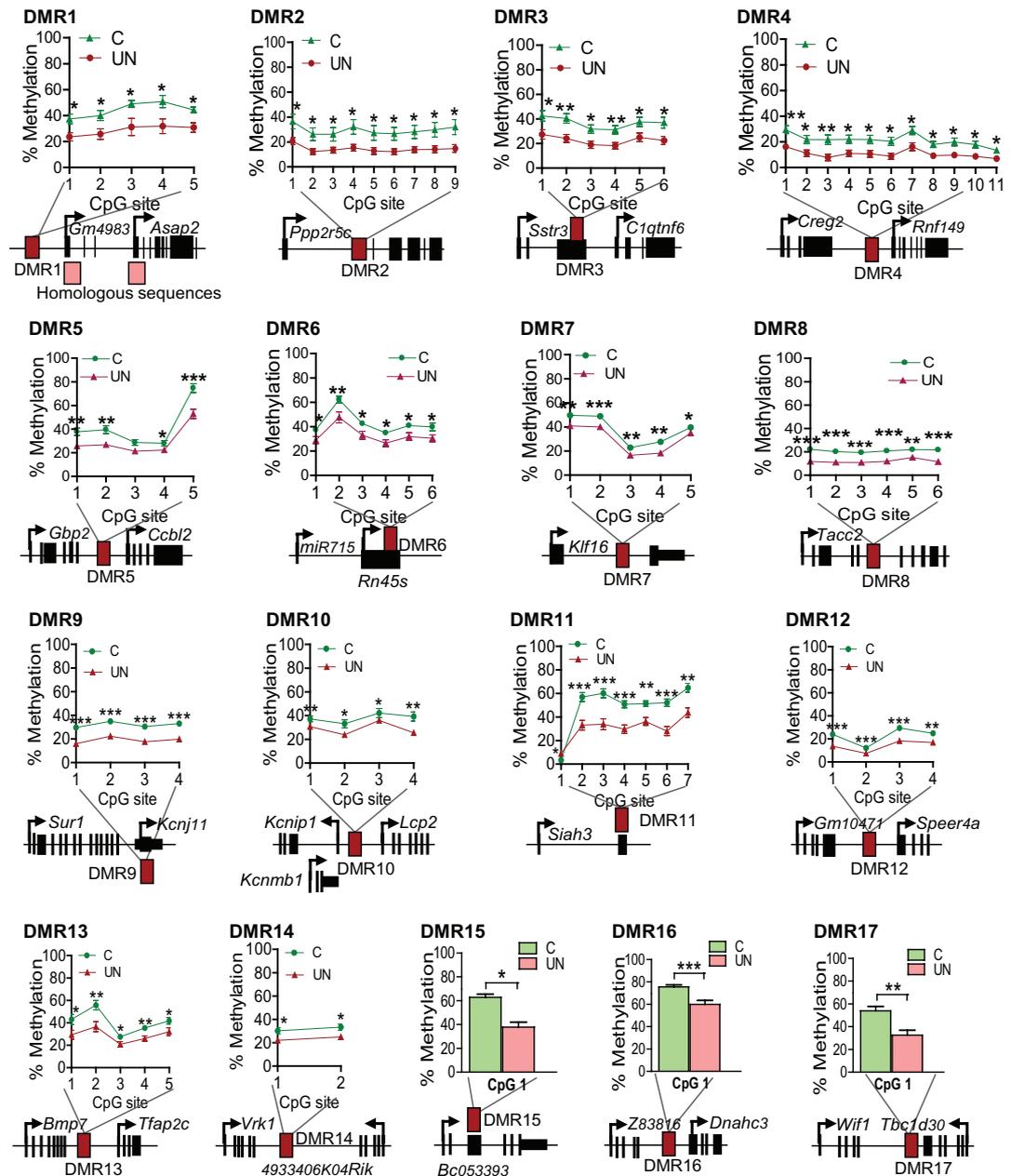
imum caloric restriction that does not cause significant fetal loss was chosen (Fig. 1A). This regime is largely incompatible with successful pregnancy in inbred mouse strains. Consequently, we used the outbred ICR strain, which also allowed us to better model the human population. In this model, F₁ offspring of undernourished dams have low birth weight as well as early-life adiposity, reduced muscle stem cell number and function, impaired pancreatic function, and progressive glucose intolerance (14–16). Inheritance of significantly reduced birth weight and glucose intolerance to the F₂ generation is observed through the paternal line in the absence of any further environmental perturbation (fig. S1, D to H) (3). The period of experimentally induced nutritional restriction in this model (day 12.5 to 18.5 of pregnancy) coincides with the reacquisi-

tion of methylation in male primordial germ cells as they are epigenetically reprogrammed (17). The dynamics of such methylation changes have been best studied at imprinting control regions (ICRs). However, we have already excluded a substantial perturbation of methylation at ICRs in this model (18). Thus, we now assess the whole-genome distribution of methylation in F₁ sperm, using immunoprecipitation of methylated DNA combined with high-throughput sequencing (MeDIP-seq) (19–21), followed by independent validation by bisulfite sequencing.

Experimental design and metabolic phenotype

Mature sperm was isolated from F₁ male mice fed standard chow, ad libitum, at 3 months of age, before the onset of glucose intolerance or

Fig. 2. Bisulfite mutagenesis validation of hypomethylated DMRs in an expanded panel of F₁ males' sperm. Seventeen genomic regions validated (Table 1). Data plotted: means ± SEM. C: n = 12, five litters; UN: n = 11, four litters. *P < 0.05, **P < 0.01, ***P < 0.001; unpaired two-tailed t test.



any discernible metabolic phenotypes (14). These F₁ males, previously exposed to experimental undernutrition in utero (UN), were smaller at birth (UN 1.34 ± 0.025 g, controls 1.65 ± 0.028 g, $P < 0.0001$) and at 3 months of age (UN 41.4 ± 0.82 g, controls 44.2 ± 0.94 g, $P = 0.04$) (fig. S1, B

Table 1. Validation of hypomethylated DMRs by bisulfite pyrosequencing. Absolute methylation level calculated by bisulfite mutagenesis combined with pyrosequencing in C and UN F₁ sperm ($n \geq 11$, ≥ 4 litters), F₂ E16.5 brain and liver ($n \geq 12$, ≥ 3 litters) at hypomethylated DMRs. DMRs at nonrepetitive, distinct loci are indicated by an asterisk (*). Blastocyst methylation level was extracted from (12) and (26).

DMR coordinates	Sperm methylation (%)	Blastocyst methylation (%)	F ₂ E16.5 methylation (%)	
			Liver	Brain
DMR1: MMU12:19181482-19182200	Ctrl = 50	4 (12)	CC = 13	CC = 10
	UN = 24	8 (26)	CU = 14	CU = 10
	$P < 0.0001$			
DMR2: MMU12:111666100-111666400	Ctrl = 29	0 (12)	CC = 85	CC = 82
	UN = 14	7 (26)	CU = 88	CU = 83
	$P < 0.0001$			
DMR3*: MMU15:78370350-78370800	Ctrl = 37	0 (12)	CC = 81	CC = 76
	UN = 23		CU = 81	CU = 73
	$P < 0.0001$			
DMR4*: MMU1:39654450-39655100	Ctrl = 20	5 (12)	CC = 88	CC = 88
	UN = 12		CU = 88	CU = 87
	$P < 0.0001$			
DMR5: MMU3:142351001-142351500	Ctrl = 41	13 (12)	CC = 64	CC = 62
	UN = 29	25 (26)	CU = 61	CU = 56
	$P = 0.0004$			
DMR6*: MMU17:39984601-39985700	Ctrl = 43	7 (26)	CC = 30	CC = 34
	UN = 33	26 (12)	CU = 26	CU = 33
	$P < 0.0001$			
DMR7*: MMU10:80033801-80034300	Ctrl = 38	22 (26)	CC = 8	CC = 8
	UN = 30	28 (12)	CU = 8	CU = 9
	$P = 0.001$			
DMR8*: MMU7:137835001-137836500	Ctrl = 21	22 (26)	CC = 18	CC = 20
	UN = 12	25 (12)	CU = 19	CU = 20
	$P < 0.0001$			
DMR9*: MMU7:53354201-53354900	Ctrl = 18	0 (26)	CC = 78	CC = 95
	UN = 11	9 (12)	CU = 80	CU = 94
	$P = 0.0003$			
DMR10: MMU11:33922001-33922500	Ctrl = 39	16 (26)	CC = 38	CC = 28
	UN = 29		CU = 38	CU = 32
	$P < 0.0001$			
DMR11*: MMU14:75925601-75926100	Ctrl = 48	0 (26)	CC = 64	CC = 78
	UN = 30	3 (26)	CU = 66	CU = 83
	$P < 0.0001$			
DMR12*: MMU5:26397201-26397700	Ctrl = 23	24 (12, 26)	CC = 80	CC = 85
	UN = 14		CU = 79	CU = 85
	$P < 0.0001$			
DMR13*: MMU2:172688001-172688500	Ctrl = 41	6 (26)	CC = 74	CC = 73
	UN = 29		CU = 72	CU = 69
	$P < 0.0001$			
DMR14: MMU12:107752401-107752500	Ctrl = 32	27.6 (26)	CC = 54	CC = 58
	UN = 24		CU = 55	CU = 55
	$P = 0.001$			
DMR15*: MMU11:46390601-46391100	Ctrl = 63	4.2 (26)	CC = 59	CC = 84
	UN = 38		CU = 57	CU = 82
	$P < 0.0001$			
DMR16: MMU7:10836601-10837100	Ctrl = 75	56 (26)	CC = 70	CC = 82
	UN = 54		CU = 71	CU = 82
	$P < 0.0001$			
DMR17: MMU10:120699201-120699700	Ctrl = 54	12.3 (26)	CC = 70	CC = 82
	UN = 33		CU = 73	CU = 82
	$P = 0.001$			

and C). Blood glucose and white adipose tissue mass at the time of sperm collection were not different between UN and control mice (fig. S1C). We bred F₁ control and UN males with control females before sperm isolation; offspring of these pregnancies were designated as CC (F₂ offspring of control males) and CU (F₂ offspring of UN males) (Fig. 1A). The F₂ offspring of F₁ sperm donors were harvested at embryonic day 16.5 (E16.5). A contemporaneous adult cohort of F₂ CU mice demonstrated, at 8 months of age, metabolic phenotypes similar to those previously observed (3), including reduced muscle mass and increased adiposity, with no difference in overall body or brain weight (fig. S1D). Furthermore, this CU cohort also showed glucose intolerance, particularly in the first-phase response to a glucose challenge (fig. S1E), as was previously observed. Pyruvate tolerance tests suggest that increased gluconeogenesis may contribute to this glucose intolerance (fig. S1F).

To assess whether a metabolic phenotype is discernible at E16.5 in the F₂ generation, we examined lipid metabolism. There is an overall trend toward increased lipid abundance, particularly for saturated fatty acid-conjugated triglycerides (fig. S1G). This is associated with a significant increase in expression of genes involved in lipid oxidation in E16.5 CU liver, such as *PPARα*, *Pgc1α*, and *Pgc1β*, and a trend toward down-regulation of genes involved in lipid synthesis, including *Scd1*, *Srebp1*, and *Dgat1* (fig. S1H), likely secondary to the increased hepatic lipid abundance at E16.5. Together, these data suggest that CU individuals have altered metabolism even in utero.

Hypomethylation of discrete loci in F₁ adult sperm of males undernourished in utero

To confirm the purity of F₁ sperm samples, bisulfite sequencing of imprinting control regions was carried out (fig. S2A). Independent sperm DNA samples were pooled in equimolar ratios to make two pools for each condition, each pool comprising four individuals from four independent litters (Fig. 1B), which minimized outcomes that might be associated with interindividual genetic differences. Mass spectrometry analysis of F₁ sperm DNA demonstrates that in utero nutrition does not affect the total level of DNA methylation or hydroxymethylation (Fig. 1C). It is also notable that the level of hydroxymethylation in sperm is only 2.1% of that observed in embryonic stem cells (Fig. 1C). Consequently, only the genomic distribution of DNA methylation was analyzed further.

We assessed the genome-wide distribution of sperm methylation by MeDIP-seq (Fig. 1B). This approach is most suited to the detection of robust regional changes in DNA methylation, as it offers near-unbiased genome-wide coverage with underrepresentation of low-density mC/mCG (22), which minimizes the possible influence of single-nucleotide variants and allows identification of clusters of differentially methylated cytosines. Optimization of antibody specificity was carried out

to ensure that there was no cross-sampling of hydroxymethylated or unmethylated cytosine (fig. S2B) (see materials and methods). Sequencing of antibody-enriched samples generated a total of 322.6 million mappable reads for control and 301.8 million for UN libraries. We conducted two independent comparisons between the control and UN pools, using the MEDIPS package (23) (see materials and methods for more details). Loci with a methylation change >1.5 -fold and a binomial $P < 0.0001$ in both of the independent comparisons were selected for further study and clustered into 166 differentially methylated regions (DMRs), of which 111 were hypomethylated and 55 hypermethylated in UN relative to control sperm (Fig. 1D).

Bisulfite pyrosequencing validation of MeDIP-seq DMRs

To independently validate regions of altered methylation using a different technology, we used bisulfite pyrosequencing assays on 32 regions, using an expanded panel of sperm samples: 12 control males from five litters and 11 UN males from four litters. Twenty-four hypomethylated regions and eight hypermethylated regions were randomly chosen for validation, distributed throughout the range of fold change and P values. No significant difference in methylation was found at any hypermethylated DMR, which suggests that these regions may be false-positives (table S1). In contrast, significant loss of methylation was confirmed at 17 of the hypomethylated regions in the expanded panel of F_1 UN sperm samples (Fig. 2 and Table 1). The validation rate of the nonrepetitive, unique hypomethylated regions was 90%. Differences in methylation at these loci span multiple CpGs, with robust absolute changes of 10 to 30%, a relative reduction of up to 50% (Fig. 2). Moreover, these differences are remarkably consistent among individual animals from multiple independent litters, which indicates that they are unlikely to be caused by genetic variation (fig. S3). The bisulfite sequencing data show identical absolute levels of methylation in the two replicate pools assessed by MeDIP-seq (fig. S3). Furthermore, the absolute methylation level (generally, under 50% in both groups) is consistent with these DMRs being “low-methylated regions,” previously shown to be enriched in regulatory elements (24). Together, these data demonstrate that discrete loci in the adult male germ line are susceptible to changes in methylation as a result of nutritional stress in utero.

DMRs are not distributed randomly through the genome

We examined the distribution of unique and repetitive elements among DMRs. Hypomethylated DMRs are significantly depleted from coding regions but enriched in intergenic regions and CpG islands (Fig. 3). Repetitive elements are significantly depleted from hypomethylated DMRs ($\chi^2 P < 0.0001$) with underrepresentation of long interspersed nuclear elements (LINEs) ($\chi^2 P = 0.001$) and short interspersed nuclear elements

(SINEs) ($\chi^2 P < 0.0001$) and no significant enrichment of IAPs (Fig. 3).

The predominance of hypomethylated DMRs is striking. This is consistent with in utero undernutrition during the final third of gestation impairing the reacquisition of methylation in developing F_1 male primordial germ cells (PGCs). The nutritional insult experienced by the fetus worsens with increasing gestation as maternal energy reserves are depleted. Therefore, we hypothesize that the likelihood of remethylation being disrupted by in utero undernutrition increases toward term. Analysis of the temporal dynamics of methylation reprogramming in normal PGCs (25) suggests that this is indeed the case. In normal male PGCs, whole-genome methylation is progressively reduced from E6.5 to E13.5, with evidence of remethylation by E16.5 (Fig. 4A, gray bars). In contrast, those DMRs found to be hypomethylated in adult UN sperm (green bars) exhibit a distinct temporal pattern of reprogramming. These DMRs have significantly lower methylation levels at E16.5 in normal male PGCs ($\chi^2 P < 0.0001$) (Fig. 4A), which suggests that these regions are late to remethylate and may be susceptible to environmental perturbations that delay or impair remethylation at this stage. In normal adult sperm, methylation has largely been regained, but a minority of regions retain low methylation levels (26). UN-associated hypomethylated DMRs are enriched in these low methylated regions ($\chi^2 P < 0.0001$) (Fig. 4A).

During spermiogenesis, 99% of histones are exchanged for protamines, but nucleosomes are particularly retained in regions of high CpG density and low DNA methylation (27). Given the low methylation level of our DMRs, we assessed whether these regions are also enriched in nu-

cleosomes. In mature sperm, 23 out of 111 (21%) hypomethylated DMRs retain nucleosomes (Fig. 4B). Bootstrap resampling of randomly selected regions from the background methylome demonstrates that this is a significant enrichment, $P < 0.0001$, and a feature of low methylated regions (see fig. S4 for details). This suggests that, at some loci, paternal germline hypomethylation induced by in utero undernutrition is transmitted in a chromatin context.

The developmental legacy of germline DMRs in late gestation of the F_2 generation

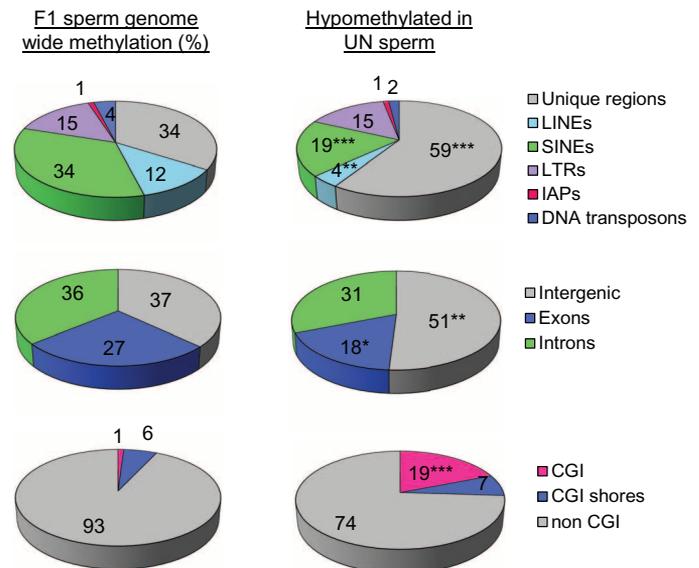
With the exception of imprints, it has been thought that gene-associated methylation in the male germ line is largely reprogrammed in the zygote by active DNA demethylation (17). However, recent studies suggest that resistance to DNA methylation reprogramming extends beyond imprinted domains (11–13). Indeed, 43% of our hypomethylated DMRs are resistant to zygotic reprogramming (26), which suggests that differential methylation in the paternal germ line may persist into the early embryo and may affect the development of the next generation (Table 2).

To determine whether altered F_1 sperm methylation persisted as a “memory” of sperm compromise in F_2 offspring, we bred F_1 control and UN males with control females. Offspring were designated as CC (F_2 offspring of control males) and CU (F_2 offspring of UN males) as noted above (Fig. 1A). Using liver and brain samples from late-gestation (E16.5) CC and CU embryos, we analyzed DNA methylation at validated germline DMRs. Differential methylation has been lost in F_2 E16.5 brain and liver (Table 1 and Fig. 5) and is therefore not a long-term heritable memory of a

Fig. 3. DMRs are enriched in intergenic nonrepetitive regions and CpG islands. (Top)

Relative distribution (%) of F_1 sperm methylated regions among distinct sequence and repetitive elements genome-wide (left) and among the F_1 UN sperm hypomethylated DMRs (right). Distinct regions are significantly enriched ($\chi^2 P < 0.0001$), whereas LINEs and SINEs are significantly depleted from hypomethylated DMRs ($\chi^2 P = 0.001$; $\chi^2 P < 0.0001$, respectively), relative to all

methylated regions detected in F_1 sperm. (Middle) Relative distribution (%) of methylated regions among coding and noncoding sequences. Exons are significantly depleted ($\chi^2 P = 0.036$), and intergenic regions are significantly enriched ($\chi^2 P = 0.0012$) among hypomethylated DMRs. (Bottom) Relative distribution (%) of methylated regions detected by MeDIP-seq among CpG islands (CGI) and CGI shores. CGIs are significantly enriched among hypomethylated DMRs ($\chi^2 P < 0.0001$).



compromised germ line. These data indicate that any functional consequences of germline DMRs are likely to be established early in development and/or linked to associated, but currently unknown, regulatory effects that may persist despite DNA remethylation in later development.

Analysis of validated DMRs in publicly available data sets (28) indicates that these loci have cell type-specific enrichment of histone modifications and transcription factor binding, characteristic of a role in cis regulation of transcription (table S2). To assess the function of a randomly selected subset of six DMRs, we conducted luciferase reporter assays in neural stem cells (29) and NIH3T3 cells in culture, using methylation-stable regions (non-DMRs) validated by pyrosequencing as additional negative controls. No significant enhancer function could be attributed to any of the regions tested in either cell type. In contrast, in vectors designed to assess a negative influence on transcription, such as an enhancer blocking or silencer function, when inserted in both the forward and reverse orientation, five out of six regions showed significantly suppressed reporter activity in neural stem cells as did three out of six regions in NIH3T3 cells (Fig. 6A). Taken together, the data suggest that these germline DMRs may play cell-specific regulatory roles in the modulation of transcription.

To assess this possibility, we examined expression of genes neighboring the 17 germline DMRs, using quantitative polymerase chain reaction (qPCR) in liver and brain of F₂ CC and CU fetuses at E16.5. Genes associated with DMRs 15 and 16 were not expressed in these tissues. Eight DMRs showed significant tissue-specific differences in expression of neighboring genes (Fig. 6, B and C). In contrast, no change in expression was found at 12 genes not associated with DMRs (fig. S5). Because methylation differences are not observed in E16.5 tissues of these same F₂ offspring (Fig. 5), it is unlikely that these expression changes are directly mediated by alterations in methylation. Rather, the cumulative effects of dysregulated epigenetic patterns earlier in development may yield sustained alterations in chromatin architecture, transcriptional regulatory networks, cell type, or tissue structure.

Several affected genes, including *Sstr3*, *C1qntf6*, *Tbc1d30*, *Kcnj11*, and *Sur1*, are candidate contributors to the F₂ phenotypes, given their known roles in glucose tolerance and metabolism (30–36). For example, the DMR9 lies within the *Kcnj11* gene, immediately downstream of *Sur1*. These genes encode the two subunits of the pancreatic β -cell adenosine triphosphate (ATP)-dependent K⁺ channel, which are necessary for the physiological control of insulin secretion (34, 36). Furthermore, polymorphisms at these loci are associated with type 2 diabetes (35, 37). In pancreatic islets isolated from 4-month-old CU mice (F₂ generation), expression of *Sur1* is reduced by 33% ($P < 0.05$) (Fig. 6D) (3). The function of β -cell ATP-dependent K⁺ channels in controlling insulin secretion can be assessed by measuring the response to treatment with agents that inhibit and activate these channels, such as sulfonylureas and

diazoxide, respectively, or through the insulin secretory response to a glucose challenge. Freshly isolated 4-month-old CU pancreatic islets demonstrate impaired insulin secretion in response to the sulfonylurea tolbutamide (Fig. 6E) and absence of suppression of insulin secretion to diazoxide (Fig. 6F) (3). Furthermore, basal insulin secretion before diazoxide challenge was significantly reduced (3) (Fig. 6F). Consistent with this, CU individuals secrete significantly less insulin during glucose tolerance testing (3) (Fig. 6G). These data strongly suggest impaired function of ATP-dependent K⁺ channels in the adult CU pancreas and implicate this in the altered glucose tolerance observed in CU individuals (fig. S1E). Further work will be required to delineate

the precise relation between compromised F₁ germline reprogramming at these loci and F₂ phenotypes.

Discussion

Our data indicate that nutritional perturbations during in utero development can alter male germline methylation at discrete loci. In turn, some of these DMRs are associated with differential transcript expression during offspring embryonic life. Our findings contrast with the largely negative data of Carone *et al.* in which no significant changes were observed in sperm DNA methylation after dietary protein restriction in adult males (4). Disparities may be due to the use of caloric rather than protein restriction,

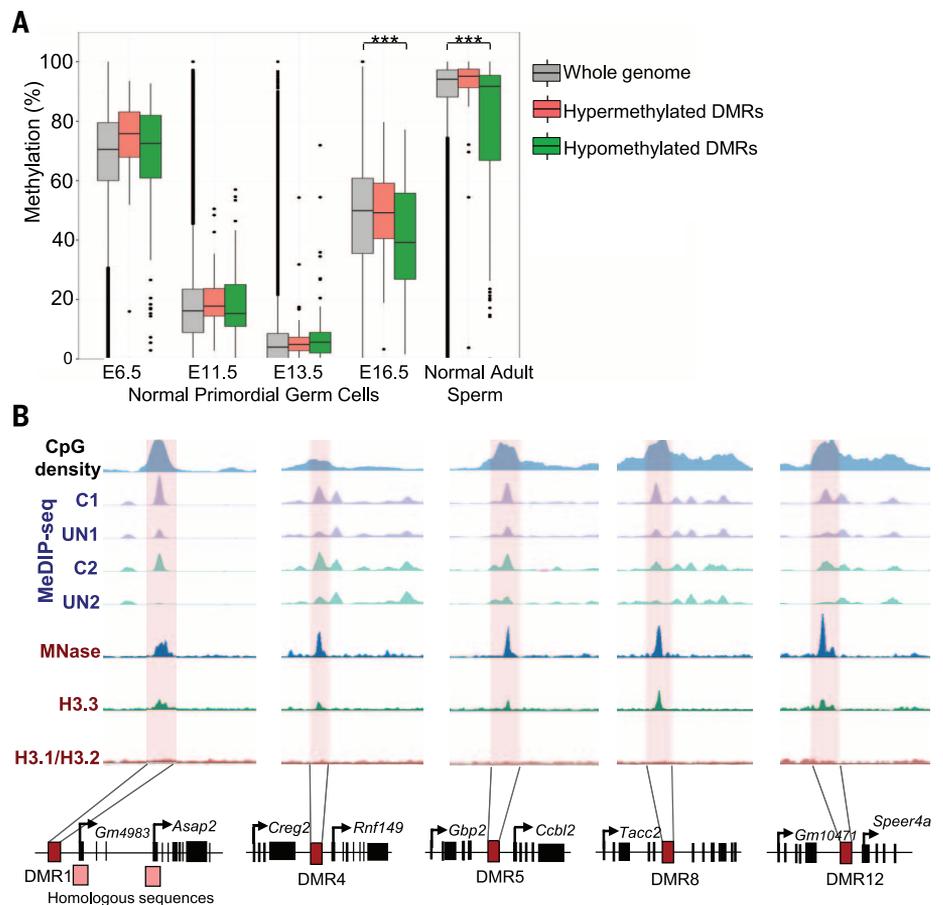


Fig. 4. DMRs regain methylation late during PGC reprogramming and retain nucleosomes in mature sperm. (A) Methylation level of hypomethylated (green) and hypermethylated (red) DMRs in our data set versus the whole genome (gray) in normal PGCs (25) and mature sperm from adult males (26). Hypermethylated DMRs act as an additional negative control because they did not validate. E13.5 and E16.5 are male PGCs. E6.5 and E11.5 are mixed-sex PGCs (25). (B) Nucleosome enrichment (27) at five representative hypomethylated DMRs.

Table 2. Of hypomethylated DMRs, 43% are resistant to zygotic demethylation. Hypomethylated DMRs susceptible (<20% methylation) or partially resistant (>20% methylation) to blastocyst reprogramming (28).

	Number (%) of hypomethylated DMRs
Blastocyst methylation < 20% (26)	63 (57)
Blastocyst methylation > 20% (26)	48 (43)

strain differences, or the greater number of individuals assessed in our analysis. Alternatively it may be due to differences in the timing of the nutritional insult, because of Carone and colleagues' imposed protein restriction during adult life. By contrast, the nutritional perturbation in our model occurs exclusively during late prenatal life, precisely when male PGCs in the developing embryo are undergoing reestablishment of their epigenetic profile. At this time, PGCs may be particularly vulnerable to epigenetic perturbation. It is notable that intergenerational phenotypic inheritance caused by endocrine disruptors

associated with altered sperm DNA methylation also involves prenatal exposure (9, 38). However, recent data have suggested that a high-fat diet during adult life might alter sperm DNA methylation, which indicates that the adult germline methylome may be more susceptible to environmental conditions than previously thought (8).

Our experiment was designed to minimize detection of single CpG methylation differences, which we a priori hypothesize to be more likely to be due to genetic differences. Our results indicate that robust germ cell methylation changes do occur after in utero undernourishment at re-

gions partially resistant to zygotic reprogramming. However, persistence of altered DNA methylation into late-gestation somatic tissues of the subsequent generation was not observed. Nonetheless, gene expression is altered in these F₂ offspring at regions of F₁ germline differential methylation. Such differences in gene expression could reflect the impact of altered methylation during early development, with subsequent transcriptional patterns that persist despite DNA remethylation in later gestation. Alternatively, altered F₂ expression may be the cumulative result of multiple locus-specific defects in germline chromatin

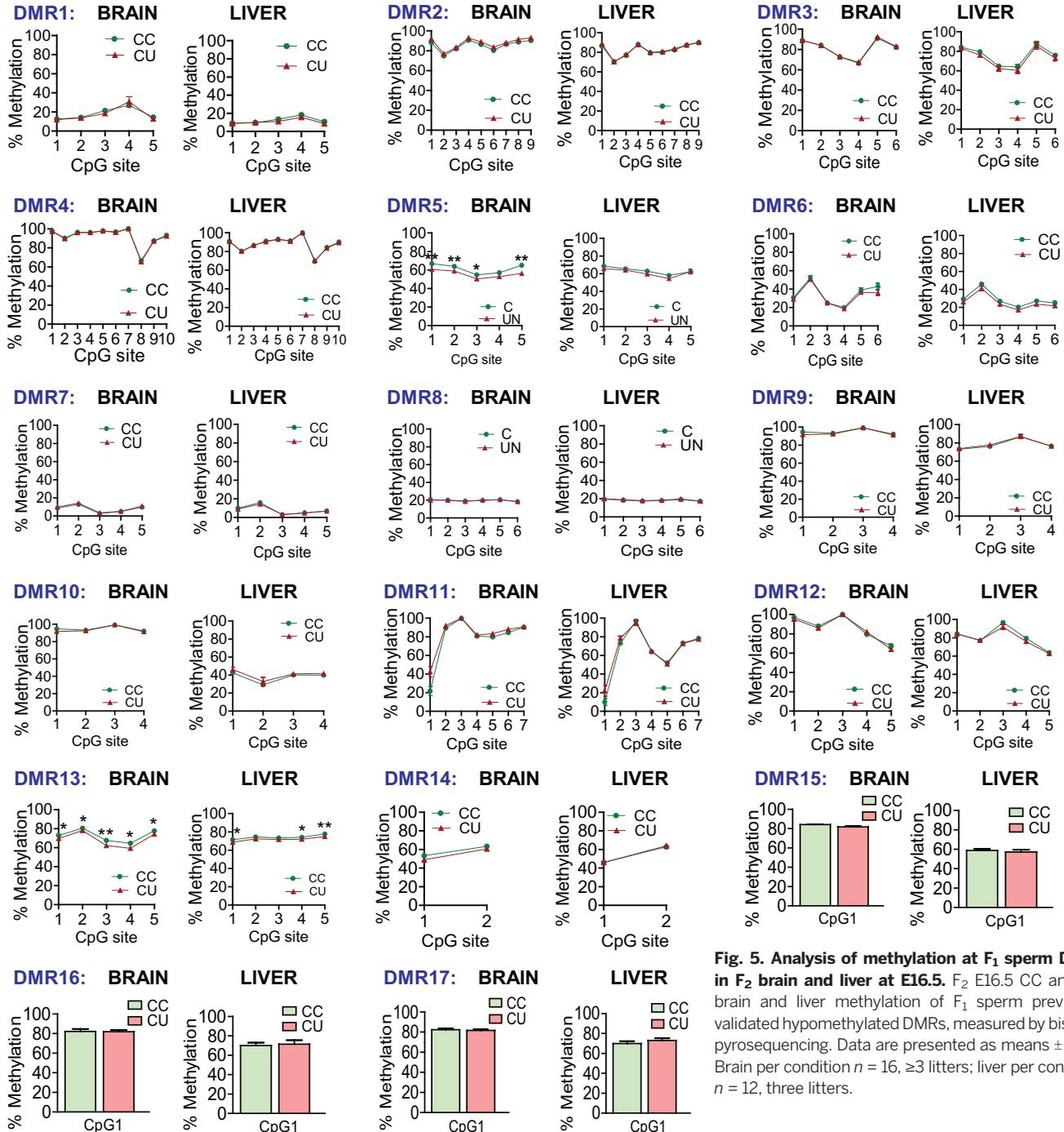


Fig. 5. Analysis of methylation at F₁ sperm DMRs in F₂ brain and liver at E16.5. F₂ E16.5 CC and CU brain and liver methylation of F₁ sperm previously validated hypomethylated DMRs, measured by bisulfite pyrosequencing. Data are presented as means ± SEM. Brain per condition *n* = 16, ≥3 litters; liver per condition *n* = 12, three litters.

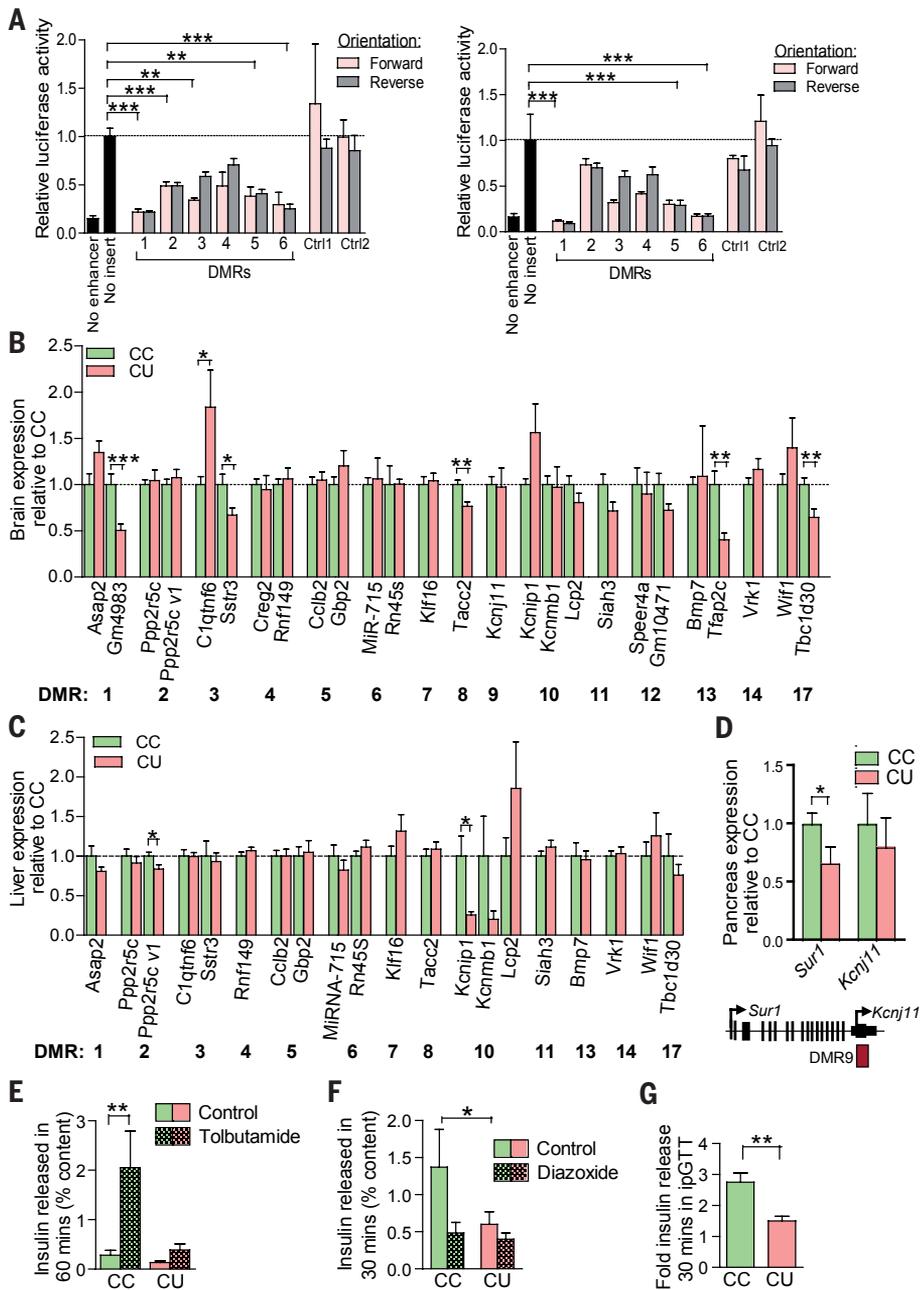


Fig. 6. Developmental legacy of altered UN sperm methylation in the F₂ generation. (A) Luciferase assay for a negative effect on transcription in 46C neural stem cells (29) (left) and NIH3T3 cells (right). Sequences were inserted between the promoter and enhancer of the control pGL3 vector. The pGL3 promoter vector (lacking an enhancer) was used as a positive control. Two regions validated by pyrosequencing as having unaffected F₁ sperm methylation were used as negative controls. Control 1: MMU2:77723600–77723900, control 2: MMU17:87639700–87640000. Data are plotted as means ± SEM, normalized to activity of the control pGL3 vector with no insert. One-way analysis of variance (ANOVA), Dunnett's post hoc test ***P < 0.001, **P < 0.0001. **(B)** F₂ E16.5 brain expression of genes neighboring F₁ sperm DMRs. Data are plotted as means ± SEM. *MIR-715* expression normalized to *SnoRNA 202*; all other expression normalized to *Hprt*. *Hprt* and *SnoRNA202* were unaffected. Unpaired two-tailed *t* test: *Gm4983*, *P* = 0.0004; *C1qtnf6*, *P* = 0.049; *Sstr3*, *P* = 0.02; *Tacc2*, *P* = 0.0018; *Tfap2c*, *P* = 0.015; and *Tbc1d30*, *P* = 0.006. Per condition *n* = 16, ≥3 litters. **(C)** F₂ E16.5 liver expression of genes neighboring F₁ sperm DMRs. Data are plotted as means ± SEM. Normalized as for (B). Unpaired two-tailed *t* test: *Ppp2r5c variant1*, *P* = 0.03; *Kcnip1*, *P* = 0.011. Per condition *n* = 12, three litters. **(D)** F₂ pancreatic expression at 4 months. Per condition *n* ≥ 5, **P* < 0.05, unpaired two-tailed *t* test (3). **(E)** Tolbutamide (200 μM)–stimulated insulin secretion, freshly isolated 4-month-old islets; *n* ≥ 4, ≥2 isolations. ***P* < 0.01, unpaired two-tailed *t* test (3). **(F)** Diazoxide (250 μM) inhibition of insulin secretion, freshly isolated 4-month-old islets; *n* = 4 per group, ≥2 isolations. **P* < 0.05, unpaired two-tailed *t* test (3). **(G)** Fold change in serum insulin 30 min after intraperitoneal glucose bolus (1 mg/kg). ***P* < 0.01, *n* ≥ 8, unpaired two-tailed *t* test (3).

state. Further work will be required to explore these possibilities.

Recent work in cultured cells demonstrates that regional methylation levels can be a secondary consequence of changes in DNA binding factors (24). Thus, it is possible that the germline DMRs identified in our study are secondary to other chromatin perturbations. Consistent with this, we observed enrichment of nucleosome occupancy at DMRs. Further studies are required to examine whether these represent regions of vulnerability in the sperm genome. Histone modifications and small RNA molecules are known to be required for multigenerational gene-silencing effects in *Caenorhabditis elegans* (39, 40), an animal that lacks DNA methylation, and such mechanistic processes may also be involved in mammals. Indeed, there is evidence that sperm-borne microRNAs play an important role in early mammalian development (41), and the early-life environment may have the potential to alter the abundance of some sperm microRNAs (42).

Conclusion

Data presented here serve as a proof of principle that undernutrition during prenatal life, even when followed by normal postnatal nutrition, can compromise male germline development and epigenetic reprogramming and so permanently alter DNA methylation in the germ line of the adult offspring. Alterations in adult gamete methylation may serve as a legacy of earlier developmental exposures that may contribute to the intergenerational transmission of environmentally induced disease.

REFERENCES AND NOTES

- P. D. Gluckman, M. A. Hanson, C. Cooper, K. L. Thornburg, Effect of in utero and early-life conditions on adult health and disease. *N. Engl. J. Med.* **359**, 61–73 (2008). doi: [10.1056/NEJMr0708473](https://doi.org/10.1056/NEJMr0708473); pmid: 18596274
- A. J. Drake, B. R. Walker, The intergenerational effects of fetal programming: Non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk. *J. Endocrinol.* **180**, 1–16 (2004). doi: [10.1677/joe.0.1800001](https://doi.org/10.1677/joe.0.1800001); pmid: 14709139
- J. C. Jimenez-Chillaron et al., Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. *Diabetes* **58**, 460–468 (2009). doi: [10.2337/db08-0490](https://doi.org/10.2337/db08-0490); pmid: 19017762
- B. R. Carone et al., Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* **143**, 1084–1096 (2010). doi: [10.1016/j.cell.2010.12.008](https://doi.org/10.1016/j.cell.2010.12.008); pmid: 21183072
- S. F. Ng et al., Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* **467**, 963–966 (2010). doi: [10.1038/nature09491](https://doi.org/10.1038/nature09491); pmid: 20962845
- M. E. Pembrey et al., ALSPAC Study Team, Sex-specific, male-line transgenerational responses in humans. *Eur. J. Hum. Genet.* **14**, 159–166 (2006). doi: [10.1038/sj.ejhg.5201538](https://doi.org/10.1038/sj.ejhg.5201538); pmid: 16391557
- I. C. Weaver et al., Epigenetic programming by maternal behavior. *Nat. Neurosci.* **7**, 847–854 (2004). doi: [10.1038/nrn1276](https://doi.org/10.1038/nrn1276); pmid: 15220929
- Y. Wei et al., Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 1873–1878 (2014). doi: [10.1073/pnas.1321195111](https://doi.org/10.1073/pnas.1321195111); pmid: 24449870
- M. D. Anway, A. S. Cupp, M. Uzumcu, M. K. Skinner, Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308**, 1466–1469 (2005). doi: [10.1126/science.1108190](https://doi.org/10.1126/science.1108190); pmid: 15933200
- V. K. Rakyatan et al., Transgenerational inheritance of epigenetic states at the murine *Axin(Fu)* allele occurs after maternal and paternal transmission. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2538–2543 (2003). doi: [10.1073/pnas.0436776100](https://doi.org/10.1073/pnas.0436776100); pmid: 12601169

11. J. Borgel *et al.*, Targets and dynamics of promoter DNA methylation during early mouse development. *Nat. Genet.* **42**, 1093–1100 (2010). doi: [10.1038/ng.708](https://doi.org/10.1038/ng.708); pmid: [21057502](https://pubmed.ncbi.nlm.nih.gov/21057502/)
12. S. A. Smallwood *et al.*, Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat. Genet.* **43**, 811–814 (2011). doi: [10.1038/ng.864](https://doi.org/10.1038/ng.864); pmid: [21706000](https://pubmed.ncbi.nlm.nih.gov/21706000/)
13. Z. D. Smith *et al.*, A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature* **484**, 339–344 (2012). doi: [10.1038/nature10960](https://doi.org/10.1038/nature10960); pmid: [22456710](https://pubmed.ncbi.nlm.nih.gov/22456710/)
14. J. C. Jimenez-Chillaron *et al.*, Beta-cell secretory dysfunction in the pathogenesis of low birth weight-associated diabetes: A murine model. *Diabetes* **54**, 702–711 (2005). doi: [10.2337/diabetes.54.3.702](https://doi.org/10.2337/diabetes.54.3.702); pmid: [15734846](https://pubmed.ncbi.nlm.nih.gov/15734846/)
15. E. Isganaitis *et al.*, Accelerated postnatal growth increases lipogenic gene expression and adipocyte size in low-birth weight mice. *Diabetes* **58**, 1192–1200 (2009). doi: [10.2337/db08-1266](https://doi.org/10.2337/db08-1266); pmid: [19208909](https://pubmed.ncbi.nlm.nih.gov/19208909/)
16. M. Woo *et al.*, Early life nutrition modulates muscle stem cell number: Implications for muscle mass and repair. *Stem Cells Dev.* **20**, 1763–1769 (2011). doi: [10.1089/scd.2010.0349](https://doi.org/10.1089/scd.2010.0349); pmid: [21247245](https://pubmed.ncbi.nlm.nih.gov/21247245/)
17. H. Sasaki, Y. Matsui, Epigenetic events in mammalian germ-cell development: Reprogramming and beyond. *Nat. Rev. Genet.* **9**, 129–140 (2008). doi: [10.1038/nrg2295](https://doi.org/10.1038/nrg2295); pmid: [18197165](https://pubmed.ncbi.nlm.nih.gov/18197165/)
18. E. J. Radford *et al.*, An unbiased assessment of the role of imprinted genes in an intergenerational model of developmental programming. *PLoS Genet.* **8**, e1002605 (2012). doi: [10.1371/journal.pgen.1002605](https://doi.org/10.1371/journal.pgen.1002605); pmid: [22511876](https://pubmed.ncbi.nlm.nih.gov/22511876/)
19. M. Weber *et al.*, Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat. Genet.* **37**, 853–862 (2005). doi: [10.1038/ng1598](https://doi.org/10.1038/ng1598); pmid: [16007088](https://pubmed.ncbi.nlm.nih.gov/16007088/)
20. M. Weber *et al.*, Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat. Genet.* **39**, 457–466 (2007). doi: [10.1038/ng1990](https://doi.org/10.1038/ng1990); pmid: [17334365](https://pubmed.ncbi.nlm.nih.gov/17334365/)
21. T. A. Down *et al.*, A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. *Nat. Biotechnol.* **26**, 779–785 (2008). doi: [10.1038/nbt1414](https://doi.org/10.1038/nbt1414); pmid: [18612301](https://pubmed.ncbi.nlm.nih.gov/18612301/)
22. O. Taiwo *et al.*, Methylome analysis using MeDIP-seq with low DNA concentrations. *Nat. Protoc.* **7**, 617–636 (2012). doi: [10.1038/nprot.2012.012](https://doi.org/10.1038/nprot.2012.012); pmid: [22402632](https://pubmed.ncbi.nlm.nih.gov/22402632/)
23. L. Chavez *et al.*, Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage. *Genome Res.* **20**, 1441–1450 (2010). doi: [10.1101/gr.110114.110](https://doi.org/10.1101/gr.110114.110); pmid: [20802089](https://pubmed.ncbi.nlm.nih.gov/20802089/)
24. M. B. Stadler *et al.*, DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature* **480**, 490–495 (2011). pmid: [22170606](https://pubmed.ncbi.nlm.nih.gov/22170606/)
25. S. Seisenberger *et al.*, The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Mol. Cell* **48**, 849–862 (2012). doi: [10.1016/j.molcel.2012.11.001](https://doi.org/10.1016/j.molcel.2012.11.001); pmid: [23219530](https://pubmed.ncbi.nlm.nih.gov/23219530/)
26. H. Kobayashi *et al.*, Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish oocyte-specific heritable marks. *PLoS Genet.* **8**, e1002440 (2012). doi: [10.1371/journal.pgen.1002440](https://doi.org/10.1371/journal.pgen.1002440); pmid: [22242016](https://pubmed.ncbi.nlm.nih.gov/22242016/)
27. S. Erkek *et al.*, Molecular determinants of nucleosome retention at CpG-rich sequences in mouse spermatozoa. *Nat. Struct. Mol. Biol.* **20**, 868–875 (2013). doi: [10.1038/nsmb.2599](https://doi.org/10.1038/nsmb.2599); pmid: [23770822](https://pubmed.ncbi.nlm.nih.gov/23770822/)
28. K. R. Rosenbloom *et al.*, ENCODE whole-genome data in the UCSC Genome Browser: Update 2012. *Nucleic Acids Res.* **40** (D1), D912–D917 (2012). doi: [10.1093/nar/gkr1012](https://doi.org/10.1093/nar/gkr1012); pmid: [22075998](https://pubmed.ncbi.nlm.nih.gov/22075998/)
29. Q. L. Ying, M. Stavridis, D. Griffiths, M. Li, A. Smith, Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* **21**, 183–186 (2003). doi: [10.1038/nbt780](https://doi.org/10.1038/nbt780); pmid: [12524553](https://pubmed.ncbi.nlm.nih.gov/12524553/)
30. T. Iwanaga, T. Miki, H. Takahashi-Iwanaga, Restricted expression of somatostatin receptor 3 to primary cilia in the pancreatic islets and adenohypophysitis of mice. *Biomed. Res.* **32**, 73–81 (2011). doi: [10.2220/biomedres.32.73](https://doi.org/10.2220/biomedres.32.73); pmid: [21383513](https://pubmed.ncbi.nlm.nih.gov/21383513/)
31. J. F. Bruno, Y. Xu, J. Song, M. Berelowitz, Pituitary and hypothalamic somatostatin receptor subtype messenger ribonucleic acid expression in the food-deprived and diabetic rat. *Endocrinology* **135**, 1787–1792 (1994). pmid: [7956902](https://pubmed.ncbi.nlm.nih.gov/7956902/)
32. T. Takeuchi, Y. Adachi, T. Nagayama, Expression of a secretory protein C1qTNF6, a C1qTNF family member, in hepatocellular carcinoma. *Anal. Cell. Pathol. (Amst.)* **34**, 113–121 (2011). pmid: [21508531](https://pubmed.ncbi.nlm.nih.gov/21508531/)
33. J. R. Huyghe *et al.*, Exome array analysis identifies new loci and low-frequency variants influencing insulin processing and secretion. *Nat. Genet.* **45**, 197–201 (2013). doi: [10.1038/ng.2507](https://doi.org/10.1038/ng.2507); pmid: [23263489](https://pubmed.ncbi.nlm.nih.gov/23263489/)
34. A. P. Babenko *et al.*, Activating mutations in the *ABCC8* gene in neonatal diabetes mellitus. *N. Engl. J. Med.* **355**, 456–466 (2006). doi: [10.1056/NEJMoa055068](https://doi.org/10.1056/NEJMoa055068); pmid: [16885549](https://pubmed.ncbi.nlm.nih.gov/16885549/)
35. O. Laukkanen *et al.*, Polymorphisms of the *SUR1* (*ABCC8*) and *Kir6.2* (*KCNJ11*) genes predict the conversion from impaired glucose tolerance to type 2 diabetes. The Finnish Diabetes Prevention Study. *J. Clin. Endocrinol. Metab.* **89**, 6286–6290 (2004). doi: [10.1210/jc.2004-1204](https://doi.org/10.1210/jc.2004-1204); pmid: [15579791](https://pubmed.ncbi.nlm.nih.gov/15579791/)
36. A. L. Gloyn *et al.*, Activating mutations in the gene encoding the ATP-sensitive potassium-channel subunit *Kir6.2* and permanent neonatal diabetes. *N. Engl. J. Med.* **350**, 1838–1849 (2004). doi: [10.1056/NEJMoa032922](https://doi.org/10.1056/NEJMoa032922); pmid: [15115830](https://pubmed.ncbi.nlm.nih.gov/15115830/)
37. K. E. Lohmueller, C. L. Pearce, M. Pike, E. S. Lander, J. N. Hirschhorn, Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat. Genet.* **33**, 177–182 (2003). doi: [10.1038/ng1071](https://doi.org/10.1038/ng1071); pmid: [12524541](https://pubmed.ncbi.nlm.nih.gov/12524541/)
38. C. Guerrero-Bosagna, M. Settles, B. Lucker, M. K. Skinner, Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. *PLoS ONE* **5**, e13100 (2010). doi: [10.1371/journal.pone.0013100](https://doi.org/10.1371/journal.pone.0013100); pmid: [20927350](https://pubmed.ncbi.nlm.nih.gov/20927350/)
39. A. Ashe *et al.*, piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99 (2012). doi: [10.1016/j.cell.2012.06.018](https://doi.org/10.1016/j.cell.2012.06.018); pmid: [22738725](https://pubmed.ncbi.nlm.nih.gov/22738725/)
40. M. Shirayama *et al.*, piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* **150**, 65–77 (2012). doi: [10.1016/j.cell.2012.06.015](https://doi.org/10.1016/j.cell.2012.06.015); pmid: [22738726](https://pubmed.ncbi.nlm.nih.gov/22738726/)
41. W. M. Liu *et al.*, Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 490–494 (2012). doi: [10.1073/pnas.1110368109](https://doi.org/10.1073/pnas.1110368109); pmid: [22203953](https://pubmed.ncbi.nlm.nih.gov/22203953/)
42. K. Gapp *et al.*, Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat. Neurosci.* **17**, 667–669 (2014). doi: [10.1038/nn.3695](https://doi.org/10.1038/nn.3695); pmid: [24728267](https://pubmed.ncbi.nlm.nih.gov/24728267/)

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SUPPLEMENTARY MATERIALS

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