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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]†,||

[1]Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK. [2]Research Division, Joslin Diabetes Center and Harvard Medical School, 1 Joslin Place, Boston, MA 02215, USA. [3]The Babraham Institute, Babraham, Cambridge and Wellcome Trust Sanger Institute, Hinxton, Cambridge. [4]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland. [5]Faculty of Sciences, University of Basel, Basel, Switzerland. [6]Swiss Institute of Bioinformatics, Basel, Switzerland.

*Present address: Cambridge University Hospitals, NHS Foundation Trust, Cambridge, UK.

†Present address, Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK.

‡These authors contributed equally to this work.

§These authors contributed equally to this work.

||Corresponding author. E-mail: afsmith@mole.bio.cam.ac.uk (A.C.F.-S.); mary.elizabeth.patti@joslin.harvard.edu (M.-E.P.)

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 F_2 mouse metabolism, we demonstrate that the in utero nutritional environment of F_1 embryos alters the germline DNA methylome of F_1 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, potentially impacting F_2 development. Differential methylation is not maintained in F_2 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 non-genetic 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 important 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 which 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 nearby endogenous genes (*10*). In addition to repeat-mediated cis-acting effects, other endogenous loci that have an inherent 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*), raising 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 maximum 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, also allowing us to better model the human population. In this model, F_1 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 F_2 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 reacquisition 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 F_1 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 F_1 male mice fed standard chow, ad libitum, at 3 months of age, prior to the onset of glucose intolerance or any discernible metabolic phenotypes (*14*). These F_1 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 and C). Blood glucose and white adipose tissue mass at the time of sperm collection was not different between UN and control mice (fig. S1C). We bred F_1 control and UN males with control females prior to sperm isolation; offspring of these pregnancies were designated as CC (F_2 offspring of control males) and CU (F_2 offspring of UN males) (Fig. 1A). The F_2 offspring of F_1 sperm donors were harvested at E16.5. A contemporaneous adult cohort of F_2 CU mice demonstrates at 8 months of age similar metabolic phenotypes as 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 shows 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 F_2 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 F_1 adult sperm of males undernourished in utero

To confirm the purity of F_1 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), hence minimizing outcomes that might be associated with inter-individual genetic differences. Mass spectrometry analysis of F_1 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, offering near-unbiased genome-wide coverage with under-representation of low density mC/mCG (*22*), thus minimizing the possible influence of single nucleotide variants and allowing identification of clusters of differentially methylated cytosines. Optimization of antibody specificity was carried out to ensure no cross sampling of hydroxymethylated or unmethylated cytosine (fig. S2B, 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. Two independent comparisons between the control and UN pools were conducted 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 employed bisulfite pyrosequencing assays on 32 regions using an expanded panel of sperm samples: 12 control males from 5 litters, and 11 UN males from 4 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, suggesting 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 non-repetitive, unique hypomethylated regions was 90%. Differences in methylation at these loci span multiple CpGs, with robust absolute changes of 10–30%, a relative reduction of up to 50% (Fig. 2). Moreover, these differences are remarkably consistent among individual animals from multiple independent litters, indicating 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 germline 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 (χ^2 $P < 0.0001$) with under-representation of LINEs (χ^2 $P = 0.001$) and SINEs (χ^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 re-acquisition of methylation in developing F_1 male 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 13.5, with evidence of remethylation by E16.5 (Fig. 4A, grey 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 (χ^2 $P < 0.0001$; Fig. 4A), suggesting that these regions are late to re-methylate and may be susceptible to environmental perturbations which 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 (χ^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 nucleosomes. In mature sperm, 23/111 (21%) hypomethylated DMRs retain nucleosomes (Fig. 4B). Bootstrap re-sampling 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 germline 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*), suggesting that differential methylation in the paternal germline may persist into the early embryo and 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 compromised germline. 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 datasets (*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, 5/6 regions significantly suppressed reporter activity when inserted in both the forward and reverse orientation in neural stem cells, and 3/6 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 seventeen germ-line DMRs, using quantitative polymerase chain reaction (qPCR) in liver and brain of F_2 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 twelve genes not associated with DMRs (fig. S5). Because methylation differences are not observed in E16.5 tissues of these same F_2 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 F_2 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 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 (F_2 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 through measuring the response to treatment with agents which 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 prior to diazoxide challenge was significantly reduced (Fig. 6F) (*3*). Consistent with this, CU individuals secrete significantly less insulin during glucose tolerance testing (Fig. 6G) (*3*). 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 relationship between compromised F_1 germline reprogramming at these loci and F_2 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 following dietary protein restriction in adult males (*4*). Disparities may be due to the use of caloric rather than protein restriction, 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 as 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 primordial germ cells (PGCs) in the developing embryo are undergoing re-establishment 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 has suggested that a high fat diet during adult life might alter sperm DNA methylation, indicating 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 following in utero undernourishment at regions 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 F_2 offspring at regions of F_1 germline differential methylation. Such differences in gene expression could reflect the impact of altered methylation during early development, with subsequent transcriptional patterns which persist despite DNA remethylation in later gestation. Alternatively, altered F_2 expression may be the cumulative result of multiple locus-specific defects in germline chromatin 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 which lacks DNA methylation, and such mechanistic processes may also be involved in mammals. Indeed, there is evidence that sperm borne miRNAs 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 miRNAs (*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, permanently altering DNA methylation in the germline 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.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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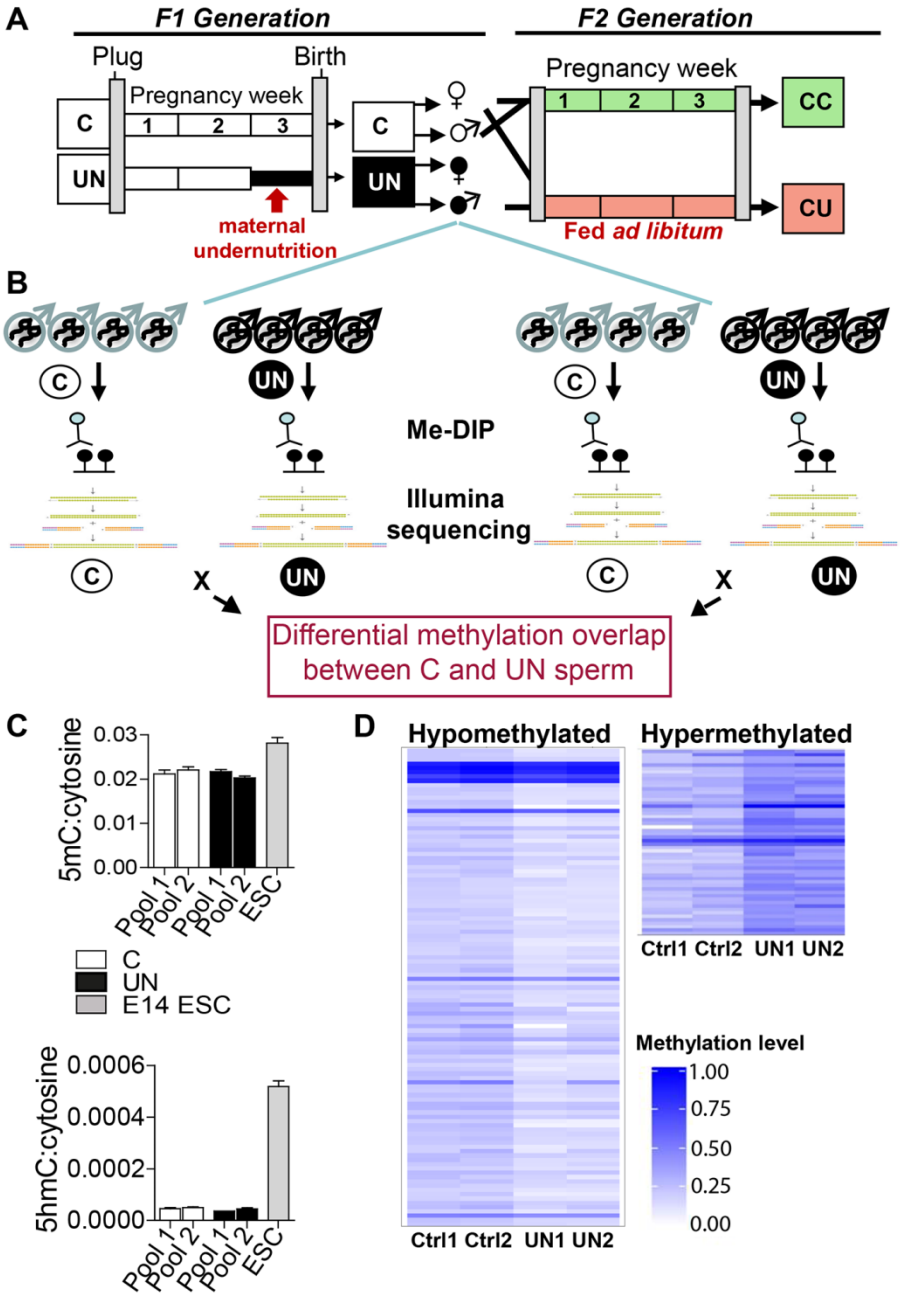

Fig. 1. Total methylation is stable in UN sperm, with significant locus specific changes. (**A**) Experimental design: F_1 generation: Dams were randomized on pregnancy day 12.5 to control (C) or undernutrition (UN) groups and UN food intake restricted to 50%. Postnatal litters were equalized to eight pups and animals fed ad libitum. F_2 generation: control F_1 females mated at age 2 months with non-sibling 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, 5 litters. UN pools: *n* = 8, 4 litters. Following MeDIP-seq two independent C vs UN comparisons identified DMRs where methylation FC >1.5× and binomial *P* value <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 ESCs are shown for comparison. (**D**) Heatmap of 111 hypomethylated DMRs (left) and 55 hypermethylated DMRs (right). Hypermethylated DMRs did not validate.

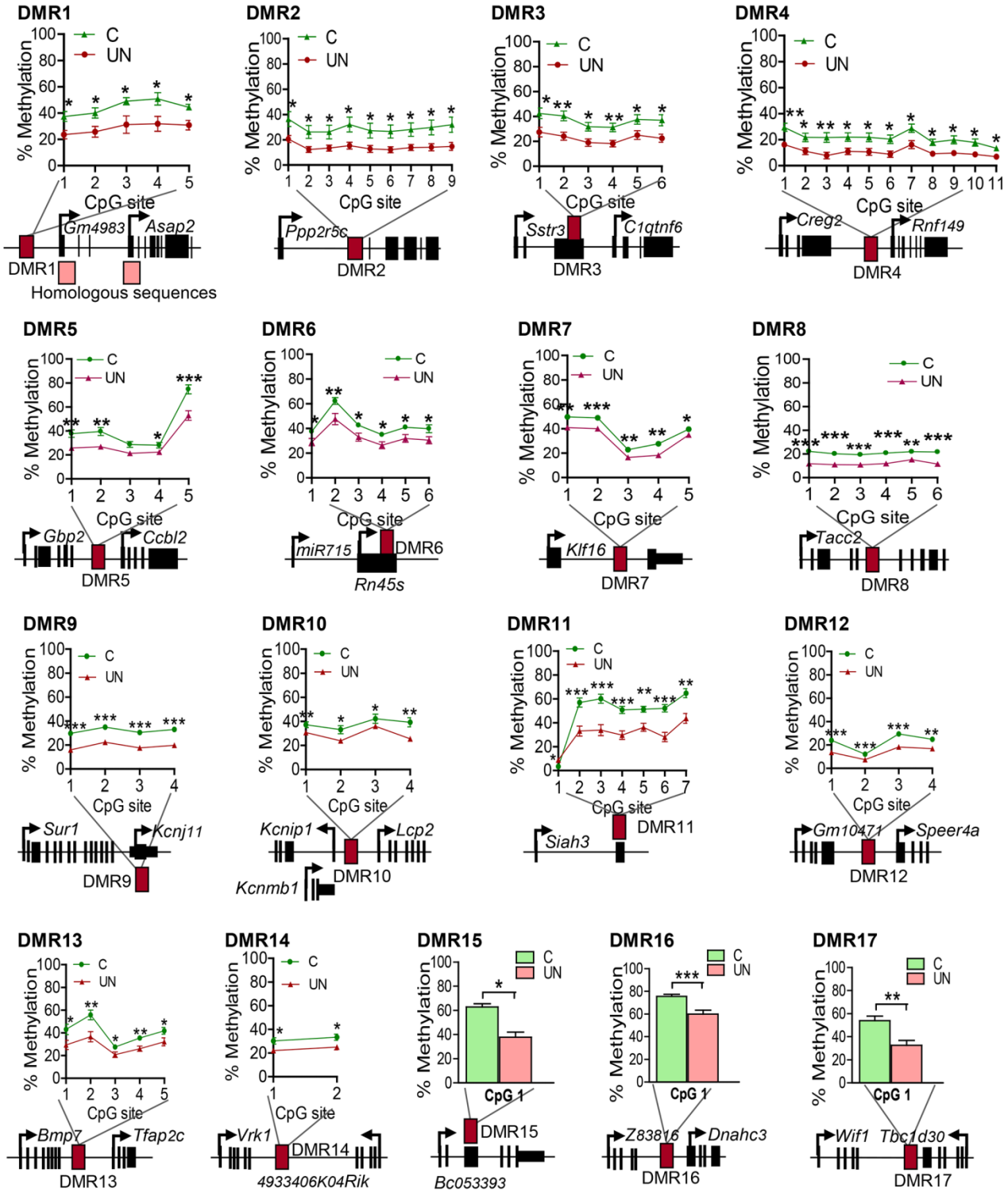

Fig. 2. Bisulfite mutagenesis validation of hypomethylated DMRs in an expanded panel of F_1 males' sperm. Seventeen genomic regions validated (Table 1). Data plotted: mean ± SEM. (C) n = 12, 5 litters; UN: n = 11, 4 litters. * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$ unpaired two-tailed t test.

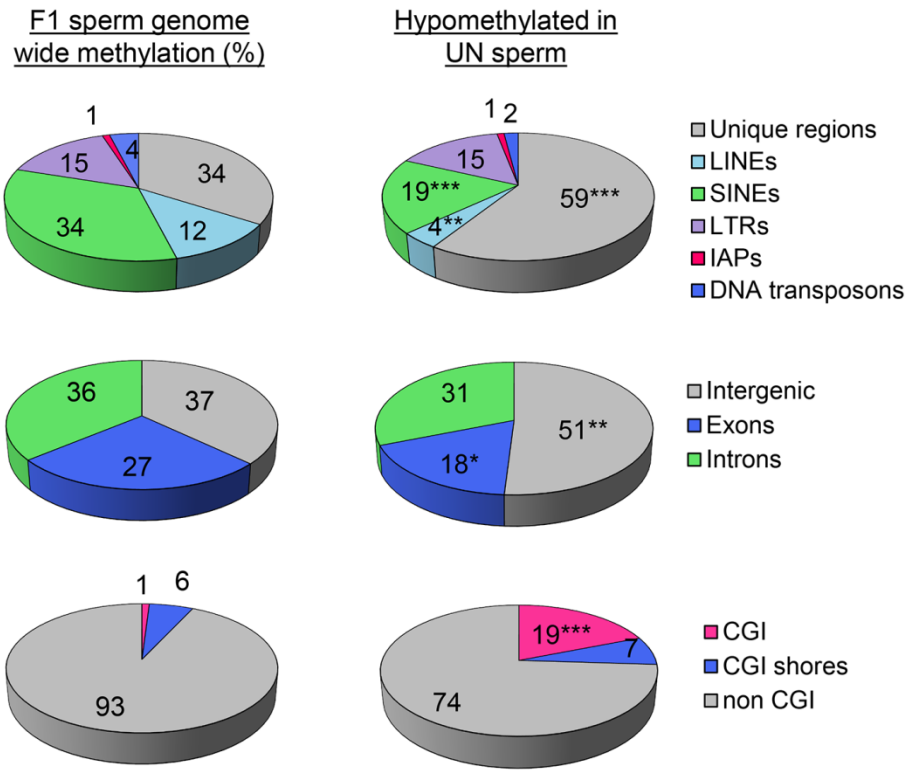

Fig. 3. DMRs are enriched in intergenic non-repetitive regions and CpG islands. (**Top**) Relative distribution (%) of F_1 sperm methylated regions among unique sequence and repetitive elements genome wide (left) and among the F_1 UN sperm hypomethylated DMRs (right). Unique regions are significantly 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 non-coding sequence. Exons are significantly depleted ($\chi 2$ $P = 0.036$), and intergenic regions 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 ($\chi 2$ $P < 0.0001$).

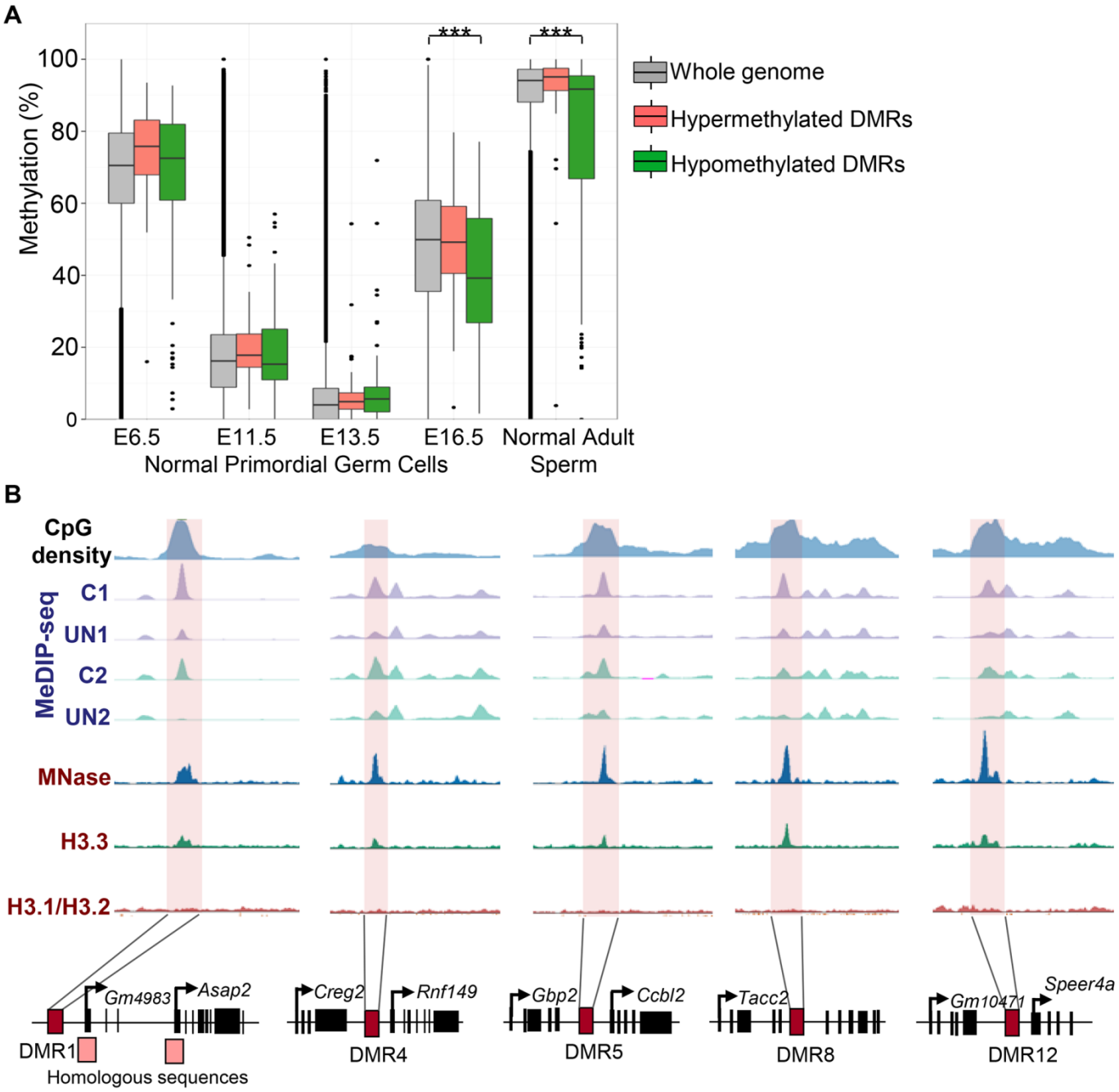

Fig. 4. DMRs regain methylation late during PGC reprogramming and retain nucleosomes in mature sperm. (**A**) Methylation level of hypomethylated (green) and hypermethylated (red) DMRs in our data set versus the whole genome (grey) in normal PGCs (*25*) and mature sperm from adult males (*26*). Hypermethylated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

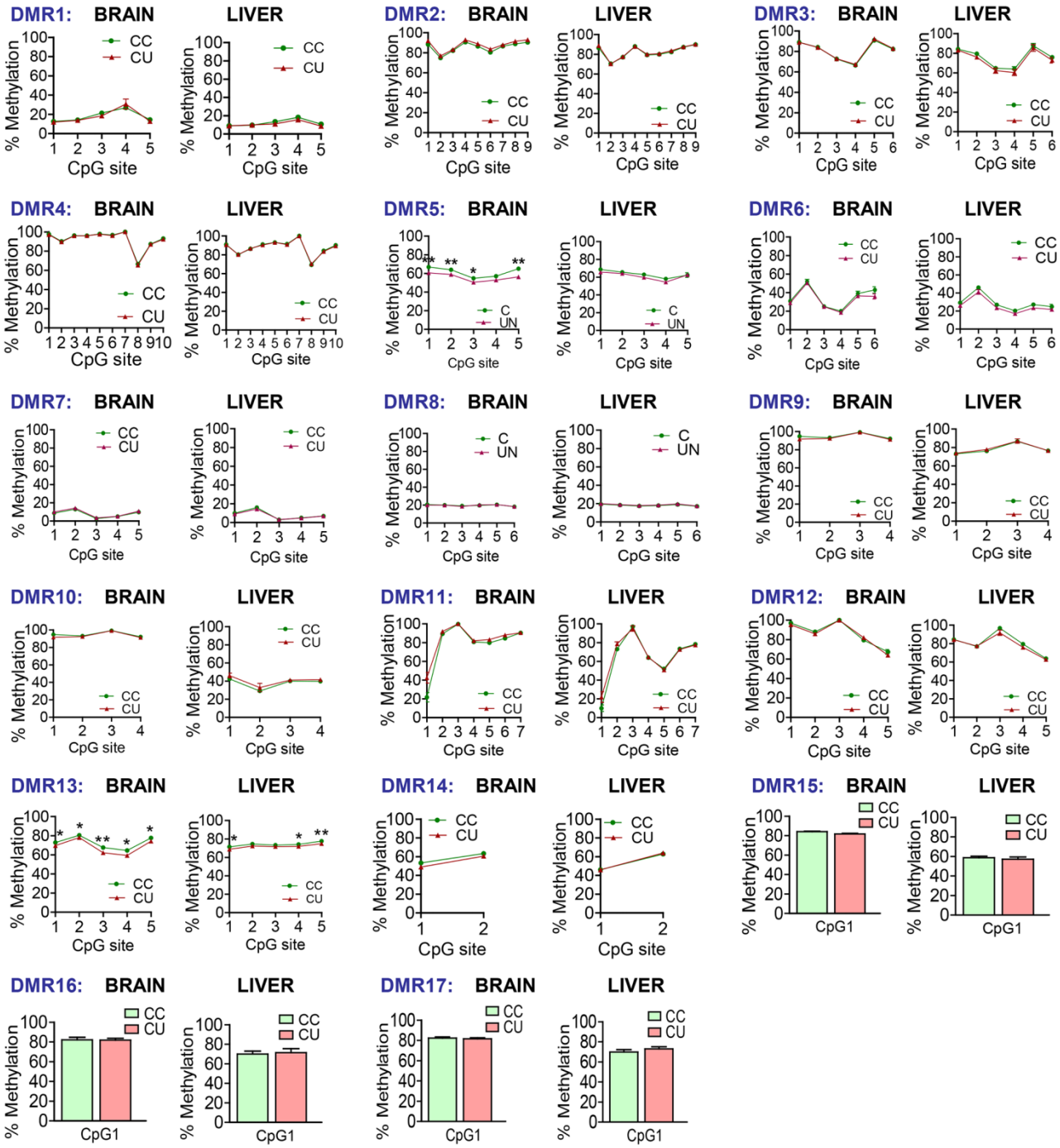

Fig. 5. Analysis of methylation at F_1 sperm DMRs in F_2 brain and liver at E16.5. F_2 E16.5 CC and CU brain and liver methylation of F_1 sperm previously validated hypomethylated DMRs, measured by bisulfite pyrosequencing. Data presented as mean ± SEM. Brain per condition *n* = 16, ≥3 litters; Liver per condition *n* = 12, 3 litters.

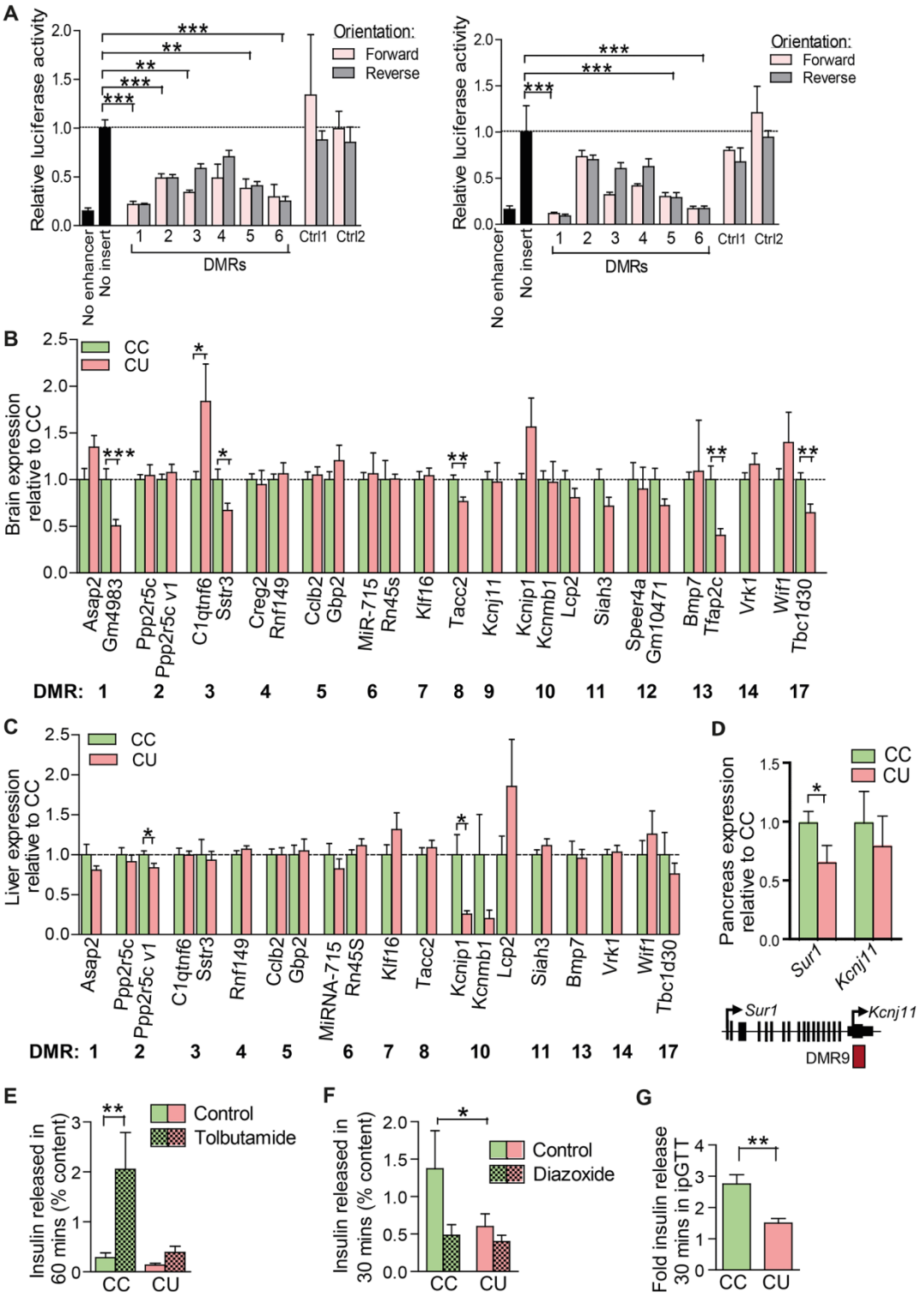
A
Relative luciferase activity
Orientation:
Forward
Reverse
No enhancer
No insert
DMRs
Ctrl1
Ctrl2
B
Brain expression relative to CC
CC
CU
DMR: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 17
C
Liver expression relative to CC
D
Pancreas expression relative to CC
Sur1
Kcnj11
DMR9
E
Insulin released in 60 mins (% content)
Control
Tolbutamide
F
Insulin released in 30 mins (% content)
Diazoxide
G
Fold insulin release 30 mins in ipGTT

Fig. 6. Developmental legacy of altered UN sperm methylation in the F_2 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 F_1 sperm methylation were used as negative controls. Control 1: MMU2:77723600–77723900, Control 2: MMU17:87639700-87640000. Data are plotted as mean ± SEM, normalized to activity of the Control pGL3 vector with no insert. One way ANOVA, Dunnett's post-test $^{**}P < 0.001$, $^{***}P < 0.0001$. (**B**) F_2 E16.5 brain expression of genes neighboring F_1 sperm DMRs. Data plotted as mean ± SEM. *MiR-715* expression normalised to *SnoRNA 202*, all other expression normalised to *Hprt. Hprt* and *SnoRNA202* were unaffected. Unpaired two-tailed *t* test: *Gmf4983* $P = 0.0004$, *C1qtnf6* $P = 0.049$, *Sstr3* $P = 0.02$, *Tacc2* $P = 0.0018$, *Tfap2c* $P = 0.015$, *Tbc1d30* $P = 0.006$. Per condition $n = 16$, ≥3 litters. (**C**) F_2 E16.5 liver expression of genes neighboring F_1 sperm DMRs. Data plotted as mean ± SEM. Normalized as for (B). Unpaired two-tailed *t* test: *Ppp2r5c variant1* $P = 0.03$, *Kcnip1* $P = 0.011$. Per condition $n = 12$, 3 litters. (**D**) F_2 Pancreatic expression at 4 months. Per condition $n \geq 5$ * $P < 0.05$, unpaired two-tailed *t* test (*3*). (**E**) Tolbutamide (200 μM) stimulated insulin secretion, freshly isolated 4 month old islets*;* $n \geq 4$, ≥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 following intraperitoneal glucose bolus (1 mg/kg). $^{**}P < 0.01$, $n \geq 8$, unpaired two-tailed *t* test (*3*).

Table 1. Validation of hypomethylated DMRs by bisulfite pyrosequencing. Absolute methylation level calculated by bisulfite mutagenesis combined with pyrosequencing in C and UN F_1 sperm ($n \geq 11$, ≥4 litters), F_2 E16.5 brain and liver ($n \geq 12$, ≥3 litters) at hypomethylated DMRs. DMRs at non-repetitive, unique loci are indicated by an asterisk (*). Blastocyst methylation level extracted from (*12*) and (*26*).

DMR coordinates	Sperm methylation (%)	Blastocyst methylation (%)	F_2 E16.5 methylation (%)	
			Liver	Brain
DMR1: MMU12:19181482-19182200	Ctrl = 50 UN = 24 $P < 0.0001$	4 (*12*) 8 (*26*)	CC = 13 CU = 14	CC = 10 CU = 10
DMR2: MMU12:111666100-111666400	Ctrl = 29 UN = 14 $P < 0.0001$	0 (*12*) 7 (*26*)	CC = 85 CU = 88	CC = 82 CU = 83
DMR3*: MMU15:78370350-78370800	Ctrl = 37 UN = 23 $P < 0.0001$	0 (*12*)	CC = 81 CU = 81	CC = 76 CU = 73
DMR4*: MMU1:39654450-39655100	Ctrl = 20 UN = 12 $P < 0.0001$	5 (*12*)	CC = 88 CU = 88	CC = 88 CU = 87
DMR5: MMU3:142351001-142351500	Ctrl = 41 UN = 29 $P = 0.0004$	13 (*12*) 25 (*26*)	CC = 64 CU = 61	CC = 62 CU = 56
DMR6*: MMU17:39984601-39985700	Ctrl = 43 UN = 33 $P < 0.0001$	7 (*26*) 26 (*12*)	CC = 30 CU = 26	CC = 34 CU = 33
DMR7*: MMU10:80033801-80034300	Ctrl = 38 UN = 30 $P = 0.001$	22 (*26*) 28 (*12*)	CC = 8 CU = 8	CC = 8 CU = 9
DMR8*: MMU7:137835001-137836500	Ctrl = 21 UN = 12 $P < 0.0001$	22 (*26*) 25 (*12*)	CC = 18 CU = 19	CC = 20 CU = 20
DMR9*: MMU7:53354201-53354900	Ctrl = 18 UN = 11 $P = 0.0003$	0 (*26*) 9 (*12*)	CC = 78 CU = 80	CC = 95 CU = 94
DMR10: MMU11:33922001-33922500	Ctrl = 39 UN = 29 $P < 0.0001$	16 (*26*)	CC = 38 CU = 38	CC = 28 CU = 32
DMR11*: MMU14:75925601-75926100	Ctrl = 48 UN = 30 $P < 0.0001$	0 (*26*) 3 (*26*)	CC = 64 CU = 66	CC = 78 CU = 83
DMR12*: MMU5:26397201-26397700	Ctrl = 23 UN = 14 $P < 0.0001$	24 (*12*, *26*)	CC = 80 CU = 79	CC = 85 CU = 85
DMR13*: MMU2:172688001-172688500	Ctrl = 41 UN = 29 $P < 0.0001$	6 (*26*)	CC = 74 CU = 72	CC = 73 CU = 69
DMR14:MMU12:107752401-107752500	Ctrl = 32 UN = 24 $P = 0.001$	27.6 (*26*)	CC = 54 CU = 55	CC = 58 CU = 55
DMR15*: MMU11:46390601-46391100	Ctrl = 63 UN = 38 $P < 0.0001$	4.2 (*26*)	CC = 59 CU = 57	CC = 84 CU = 82
DMR16: MMU7:10836601-10837100	Ctrl = 75 UN = 54 $P < 0.0001$	56 (*26*)	CC = 70 CU = 71	CC = 82 CU = 82
DMR17: MMU10:120699201-120699700	Ctrl = 54 UN = 33 $P = 0.001$	12.3 (*26*)	CC = 70 CU = 73	CC = 82 CU = 82

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)