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Paternally Induced Transgenerational Environmental Reprogramming of Metabolic Gene Expression in Mammals

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SUMMARY

Epigenetic information can be inherited through the mammalian germline and represents a plausible transgenerational carrier of environmental information. To test whether transgenerational inheritance of environmental information occurs in mammals, we carried out an expression profiling screen for genes in mice that responded to *paternal* diet. Offspring of males fed a low-protein diet exhibited elevated hepatic expression of many genes involved in lipid and cholesterol biosynthesis and decreased levels of cholesterol esters, relative to the offspring of males fed a control diet. Epigenomic profiling of offspring livers revealed numerous modest (~20%) changes in cytosine methylation depending on paternal diet, including reproducible changes in methylation over a likely enhancer for the key lipid regulator *Ppara*. These results, in conjunction with recent human epidemiological data, indicate that parental diet can affect cholesterol and lipid metabolism in offspring and define a model system to study environmental reprogramming of the heritable epigenome.

INTRODUCTION

The past few decades have seen an important expansion of our understanding of inheritance, as a wide variety of epigenetically inherited traits have been described (Jablonka and Lamb, 1995, 2005; Rando and Verstrepen, 2007). One implication of epigenetic inheritance systems is that they provide a potential mechanism by which parents could transfer information to their offspring about the environment they experienced. In other words, mechanisms exist that could allow organisms to "inform" their progeny about prevailing environmental conditions. Under certain historical circumstances—for example, repeated exposure over evolutionary time to a moderately toxic environment that persists for tens of generations—such non-Mendelian information transfer could be adaptive (reviewed in Jablonka and Lamb, 1995; Rando and Verstrepen, 2007). Whether or not organisms can inherit characters induced by ancestral environments has far-reaching implications, and this type of inheritance has come to be called "Lamarckian" inheritance after the early evolutionary theorist J.B. Lamarck, although it is worth noting that both Darwin and Lamarck believed in the inheritance of acquired characters.

Despite these theoretical considerations, at present there is scant evidence for transgenerational effects of the environment, particularly in mammals. The majority of examples of transgenerational environmental effects described have been maternal effects (see Harris and Seckl, 2010; Whitelaw and Whitelaw, 2008; Youngson and Whitelaw, 2008 for review), including in utero passage of photoperiod information in various rodents (Horton, 2005), cultural inheritance of stress reactivity and maternal grooming behavior in rats (Meaney et al., 2007; Weaver et al., 2004), and metabolic and psychiatric sequelae of fetal malnutrition in humans and rodents (Hales and Barker, 2001; Harris and Seckl, 2010; Symonds et al., 2009). However, maternal effects are difficult to separate from direct effects of in utero environmental exposure on offspring.

A small number of studies have identified *heritable* epigenetic effects of environmental perturbations on offspring. Treatment of pregnant rat mothers with the endocrine disruptor vinclozolin results in decreased fertility and behavorial changes in several generations of offspring (Anway et al., 2005; Crews et al., 2007). In another study, withholding methyl donors from pregnant female mice resulted in decreased cytosine methylation across the agouti viable yellow A^{vy} reporter locus (Waterland and Jirtle, 2003), and the altered cytosine methylation profile persisted well beyond the first generation (Cropley et al., 2006).

Whereas demonstration of multigenerational changes (e.g., an F2 effect) is important when using maternal treatment protocols to rule out simple plastic responses of offspring to the in utero environment, paternal effects avoid this issue as fathers often contribute little more than sperm to offspring. A handful of paternal effects have been documented in the literature—pre-mating fasting of male mice has been reported to affect serum glucose levels in offspring (Anderson et al., 2006), and chronic exposure of male rats to high-fat diet affects pancreatic islet biology in offspring (Ng et al., 2010). Furthermore, epidemiological data from human populations link experience of famine in paternal grandfathers to obesity and cardiovascular disease two generations later (Kaati et al., 2002; Pembrey et al., 2006). These results motivate a deeper exploration of the mechanisms of pre-mating paternal diet on offspring phenotype.

It is therefore of great interest to determine what environmental conditions have transgenerational effects in mammals, and to characterize the mechanisms that mediate these effects. Here, we describe a genomic screen for transgenerational effects of paternal diet on gene expression in offspring in mice. Expression of hundreds of genes changes in the offspring of males fed a low-protein diet, with coherent upregulation of lipid and cholesterol biosynthetic pathways. Epigenomic profiling in offspring livers identified changes in cytosine methylation at a putative enhancer for the key lipid transcription factor *Ppara*, and these changes correlated with the downregulation of this gene in offspring. Interestingly, we did not find effects of paternal diet on methylation of this locus in sperm, and overall sperm cytosine methylation patterns were largely conserved under various dietary regimes. These results establish an inbred, genetically tractable model system for the study of transgenerational effects of diet and may have implications for the epidemiology of several major human diseases.

RESULTS

Experimental Paradigm

Male mice were fed control or low-protein diets (11% rather than 20% protein, with the remaining mass made up with sucrose) from weaning until sexual maturity. Note that although the relevant dietary change in this experiment could be protein content, sucrose content, fat/protein ratio, etc., for simplicity we refer to the diet as low protein throughout the text. Mice on either diet were then mated to females reared on control diet (Figure 1A and Figure S1A available online). Fathers were removed after 1 or 2 days of mating, limiting their influence on their progeny to the mating itself. All mothers were maintained on control diet throughout the course of the experiment. After birth, the offspring were reared with their mothers until 3 weeks old, at which point their livers were harvested for RNA isolation. DNA microarrays were used to profile global gene expression differences in the livers of the offspring from the two types of crosses (Table S1).

Testing for differences between 26 matched pairs of mice from the two F1 groups, we found a significant overabundance of differentially expressed genes, relative to the null hypothesis that the parental treatment does not affect offspring (1595 genes at a false discovery rate—FDR—of 0.001, Figures S1B and S1C). We also identified a more robust (t test with null hypothesis of mean change 0.2, FDR of 0.01) group of 445 genes whose expression strongly depended on the diet consumed by their fathers (Figure 1B). In our analysis we focus on this more robust group of genes; however, all the phenomena described below are true for the larger group as well. These gene expression changes were observed in 13 (7 low-protein, 6 control) litters in experiments spanning several years, carried out in three different animal facilities (Figures S2A and S2B). In principle, random factors should be distributed equally between our two groups given the numbers of offspring examined, but we directly address a number of potential artifacts nonetheless, including changes in cell populations, circadian cycle, litter size, order of sacrifice, and cage location (Figure S2, see Experimental Procedures).

We confirmed our results by q-RT-PCR (Figures 1C, Figure S1A). Squalene epoxidase (*Sqle*), which catalyzes the first oxygenation step in sterol biosynthesis, exhibited an ~3-fold increase in the low-protein cohort in our microarray data, and q-RT-PCR showed a similar average expression difference across over 25 animals, gathered in crosses carried out several years apart (Figure 1C). The differences we observe occur in both male and female progeny (Figure 1C, Figure S2C), though these dietary history-dependent differences are superimposed on a baseline of differential expression between the sexes.

Upregulation of Proliferation and Lipid Biosynthesis Genes in Low-Protein Offspring

To help define the physiological differences between our cohorts, we calculated enrichments of various Gene Ontology (GO) processes in the differentially expressed genes. Genes upregulated in our treatment group's offspring were enriched for a number of categories of genes involved in fat and cholesterol biosynthesis, including lipid biosynthesis ($p < 9 \times 10^{-26}$), steroid biosynthesis ($p < 3 \times 10^{-19}$), cholesterol biosynthesis ($p < 2 \times 10^{-12}$), and oxidation-reduction ($p < 4 \times 10^{-10}$). Another major group of upregulated genes are annotated to be involved in S phase, such as DNA replication ($p < 2 \times 10^{-9}$) and related annotations. Downregulated genes were enriched for GO annotations such as sequence-specific DNA binding ($p < 6 \times 10^{-6}$) and ligand-dependent nuclear receptor activity ($p < 6 \times 10^{-5}$), although the number of genes matching these annotations was small (14 and 5, respectively).

The increase in S phase genes likely indicates a hyperproliferative state, whereas the metabolic expression differences suggest that lipid metabolism is altered in these animals. To explore the mechanisms responsible for these altered gene expression programs, we asked whether the observed gene expression

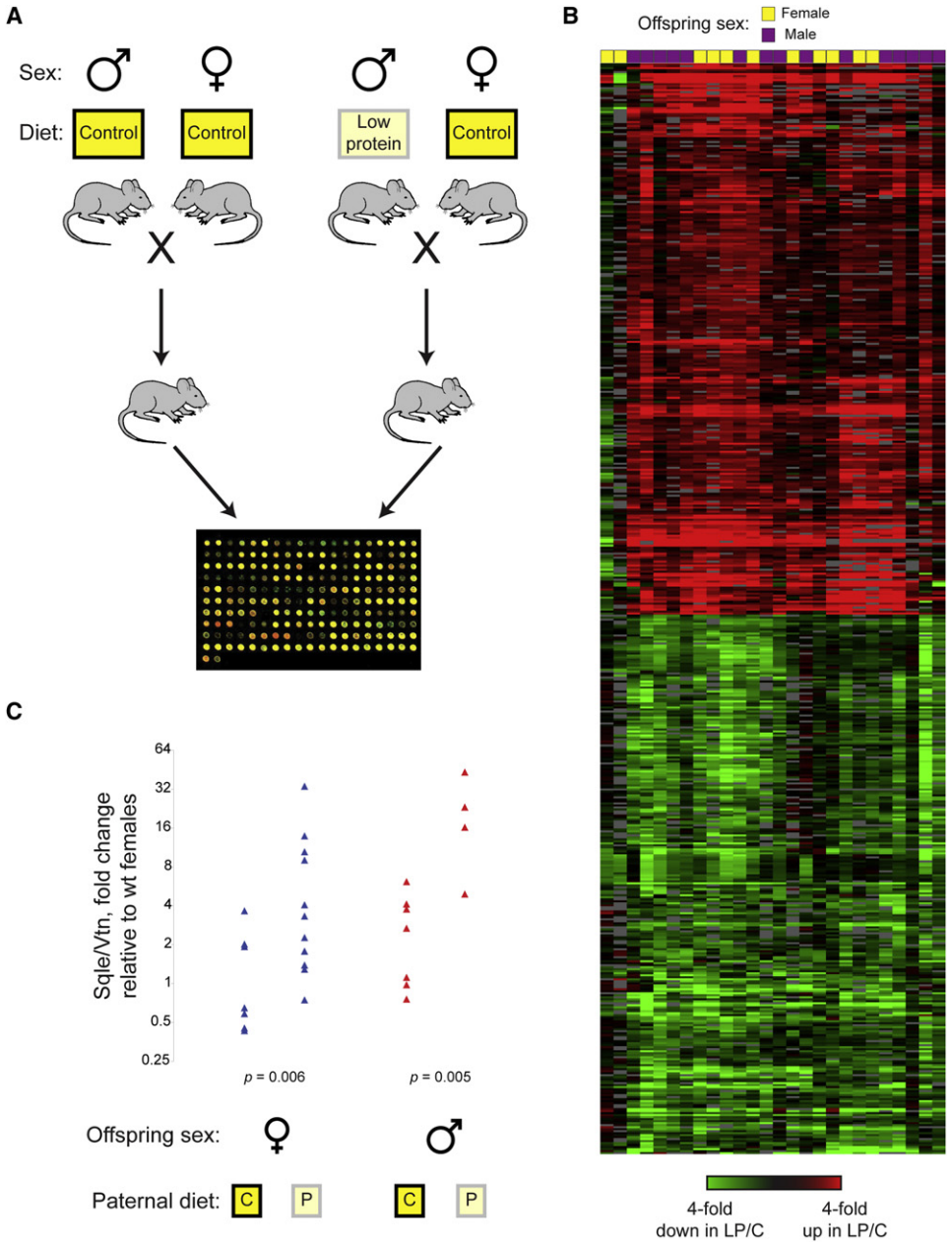

Figure 1. A Screen for Genes Regulated by Paternal Diet

(A) Experimental design. Male mice were fed control or low (11%) protein diet from weaning until sexual maturity, then were mated to females that were raised on control diet. Males were removed after 1 or 2 days of mating. Livers were harvested from offspring at 3 weeks, and RNA was prepared, labeled, and hybridized to oligonucleotide microarrays.

(B) Overview of microarray data, comparing offspring of sibling males fed different diets—red boxes indicate higher RNA levels in low-protein than control offspring, green indicates higher expression in controls. Boxes at the top indicate comparisons between two male (purple) or two female (yellow) offspring. Each column shows results from a comparison of a pair of offspring. Only genes passing the stringent threshold for significant change (Figure S1B) are shown. Data are clustered by experiment (columns) and by genes (rows).

(C) Validation of microarray data. Quantitative RT-PCR was used to determine levels of Squalene epoxidase (*Sqle*) relative to the control gene Vitronectin (*Vtn*), which showed no change in the microarray dataset. Animals are grouped by paternal diet and by sex, and data are expressed as ΔC_T between *Sqle* and *Vtn*, normalized relative to the average of control females.

Additional validation is shown in Figure S1A. p values were calculated using t test. See also Table S1, Figure S1, and Figure S2.

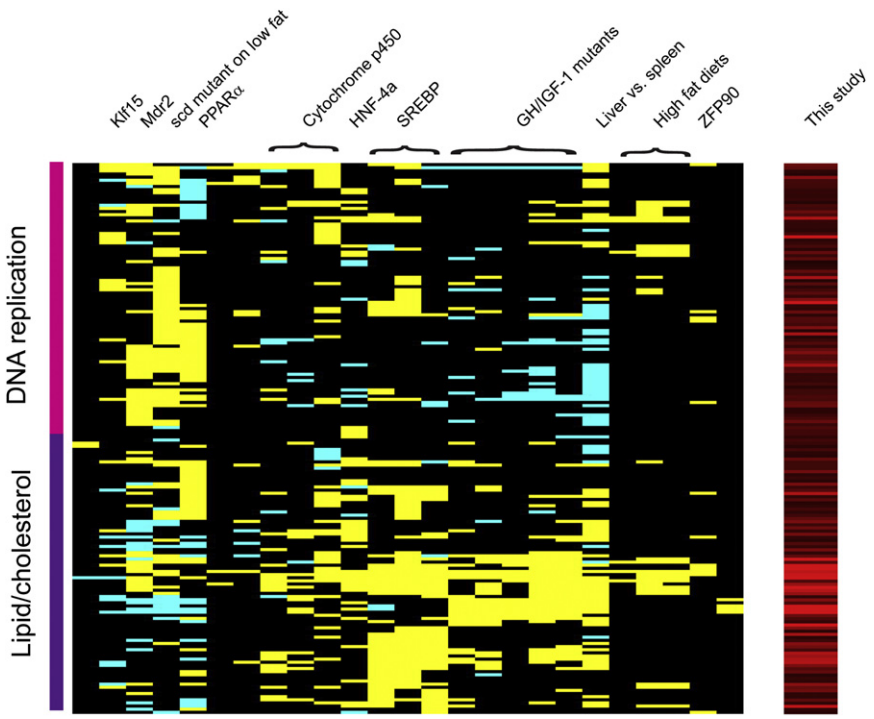

Figure 2. Multiple Pathways Are Affected by Paternal Diet

Comparison of upregulated gene expression profile with a compendium of public datasets of hepatic gene expression. A clustering of our upregulated genes according to their notation in the 28 significant ($p < 0.00025$) overlapping signatures from an assembled compendium of 120 publicly available murine liver signatures under various conditions and genetic perturbations (GEO; Horton et al., 2003; Yang et al., 2009). For each significant profile, the majority of overlapping genes are shown as yellow, whereas genes with opposite regulation (i.e., down rather than up in the dataset in question) are blue. The genes divide into two distinct clusters, one enriched in DNA replication and the other in various categories of fat and cholesterol biosynthesis. See also Table S2 and Figure S3.

differences might reflect altered regulation of a small number of pathways. We checked for significant overlaps of the gene expression profile observed in our low-protein offspring with a compendium of 120 publicly available murine liver gene expression datasets (Experimental Procedures). Our low-protein offspring gene expression profile significantly ($p < .05$ after Bonferroni correction) overlapped gene expression changes from 28 published profiles (Figure 2, Table S2), including gene expression profiles associated with perturbation of transcription factors that regulate cholesterol and lipid metabolism (SREBP [Horton et al., 2003], KLF15 [Gray et al., 2007], PPARα [Rakhshandehroo et al., 2007], and ZFP90 [Yang et al., 2009]). Our gene expression dataset also significantly matched hepatic gene expression in a variety of mice with mutations affecting growth hormone (GH) and insulin-like growth factor 1 (IGF-1) levels (Boylston et al., 2004; Madsen et al., 2004; Tsuchiya et al., 2004). Hierarchical clustering according to the enriched public profiles revealed two types of prominent gene functions in our data: DNA replication ($p < 6 \times 10^{-14}$) and lipid or cholesterol biosynthesis ($p < 2 \times 10^{-27}$) (Figure 2). The partial overlap observed with each of many different transcription factor and growth factor profiles suggests that the altered gene expression profile observed in low-protein offspring is likely related to reprogramming of multiple distinct pathways.

To assess whether the reprogrammed state in offspring reproduces the paternal response to low-protein diet, we measured global gene expression changes in the livers of pairs of animals weaned to control or low-protein diet as in Figure 1A. Genes that change in offspring are not the same as the genes induced in the parental generation by these protocols (Figure S3). Instead, males fed the low-protein diet upregulate immune response and apoptosis-related genes and downregulate genes involved in carboxylic acid metabolism (analysis not shown).

Transgenerational Effects on Lipid Metabolism

We further focused on cholesterol biosynthesis genes. Coherent upregulation of genes involved in cholesterol metabolism is observed in the offspring of low-protein fathers (Figure 3A). Figure 3B shows a more detailed comparison between our upregulated dataset and published data (Horton et al., 2003) for genes activated by a major transcriptional regulator of cholesterol metabolism, SREBP. Many of the genes upregulated in low-protein offspring have previously been shown to be upregulated by overexpression of SREBP-1a or SREBP-2 or downregulated by loss of the SREBP-activating gene *Scap*.

To explore the correspondence between hepatic gene expression and physiology, we measured lipid levels in three pairs of control and treatment livers to determine whether increased levels of lipid biosynthesis genes were associated with changes in lipid levels (Figure 3C, Experimental Procedures). Livers in the cohort with low-protein diet fathers were depleted of cholesterol and cholesterol esters (whose levels were reduced more than 2-fold). Additional differences were found in specific lipid classes, such as substantial increases in relative levels of saturated cardiolipins, saturated free fatty acids, and saturated and monounsaturated triacylglycerides in low-protein offspring (Table S3). Together, these results demonstrate that paternal diet affects metabolites of key biomedical importance in offspring.

MicroRNAs in Offspring

Small (19–35 bp) RNAs such as microRNAs (miRNAs) have recently been implicated in epigenetic inheritance in mice (Wagner et al., 2008). To determine whether altered small RNA populations might drive our reprogramming effect, we characterized by high-throughput sequencing the small (19–35 bp) RNA population from control and low-protein offspring livers (Ghildiyal et al., 2008) and mapped reads to known miRNAs (Table S4).

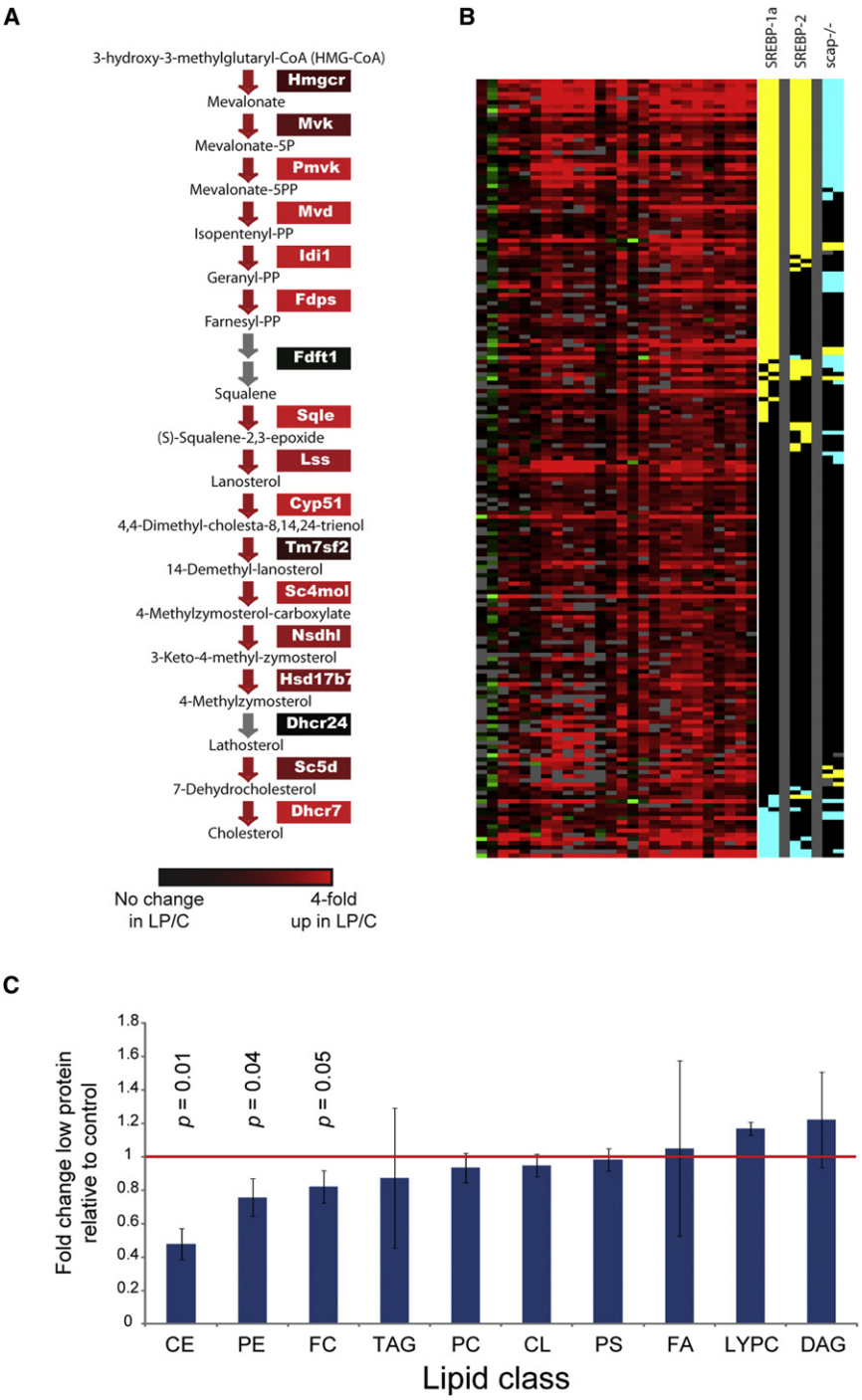

Figure 3. Altered Cholesterol Metabolism in the Low-Protein Cohort

(A) Cholesterol biosynthesis. Genes annotated as cholesterol biosynthesis genes are shown, with colors indicating average difference in expression in low-protein versus control comparisons.

(B) Many genes upregulated in the low-protein cohort are SREBP targets. Upregulated cluster from Figure 1B is shown, along with data from Horton et al. (2003). Genes scored as up in both replicates from Horton et al. (2003) are shown as yellow, genes scored as down are blue. Columns show data from transgenic mice overexpressing SREBP-1a or SREBP-2 or from *Scap* knockout mice.

(C) Cholesterol levels are decreased in livers of low-protein offspring. Data from lipidomic profiling of liver tissue from three control and three low-protein animals are shown as mean ± standard deviation. Red line indicates no change. p values were calculated using a paired t test on log-transformed lipid abundance data. Cholesterol esters, CE; phosphatidylethanolamine, PE; free cholesterol, FC; triacylglycerol, TAG; phopshatidylcholine, PC; cardiolipin, CL; phosphatidylserine, PS ; free fatty acid, FA; lysophosphatidylcholine, LYPC; and diacylglycerol, DAG.

See also Table S3.

A number of miRNAs changed expression in the offspring from low-protein diet fathers (Figure 4). Changes were often subtle in magnitude (~50%), but were reproduced in four control versus low-protein comparisons (paired t test), and given the number of sequencing reads obtained for these RNAs this magnitude of difference is well outside of counting error (Table S4). Offspring of low-protein cohort upregulated miR-21, let-7, miR-199, and miR-98 and downregulated miR-210. Many of these upregulated miRNAs are associated with proliferation in liver, with miR-21 and miR-199 both associated with hepatocellular carcinoma (Jiang et al., 2008), whereas let-7 is well-known as a tumor suppressor (Jerome et al., 2007). The increase in growth-associated miRNAs is consistent with the hyperproliferative gene expression profile observed in the offspring of low-protein diet fathers.

We found no statistically significant overlap ($p > 0.05$) between the predicted targets of the miRNAs here and the gene expression changes we observe, though the subtle (~50%) changes in miRNA abundance we observe might be expected to have little effect on mRNA—even when specific miRNAs are artificially introduced in cells, downregulation of target mRNAs is less than 2-fold for the majority of predicted targets (Hendrickson et al., 2008). Our results therefore suggest that miRNAs are likely to be additional targets of the reprogramming pathway yet are likely not the direct upstream regulators of the entire response (but see Wagner et al., 2008).

Cytosine Methylation in Offspring

How are offspring reprogrammed by paternal diet? Cytosine methylation is a widespread DNA modification that is

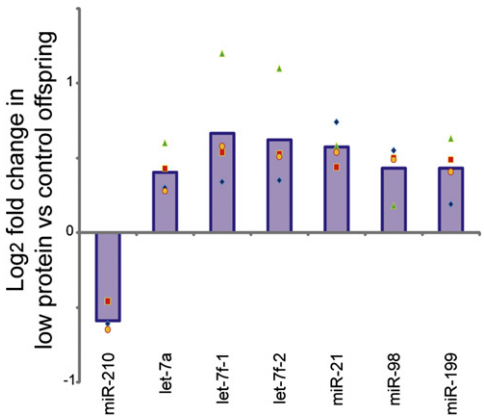

Figure 4. Proliferation-Related MicroRNAs Respond to Paternal Diet
Small (<35 nt) RNAs from the livers of eight offspring (four control, four low-protein) were isolated and subjected to high-throughput sequencing. MicroRNAs that exhibited consistent changes in all four pairs of animals are shown, with average change shown as a bar and individual comparisons shown as points. See also Table S4.

environmentally responsive and carries at least some heritable information between generations (Bartolomei et al., 1993; Cropley et al., 2006; Holliday, 1987; Rakyan et al., 2003; Waterland and Jirtle, 2003). As imprinted loci are often involved in growth control (Moore and Haig, 1991), we first asked whether a subset of candidate imprinted loci exhibited altered cytosine methylation in low-protein offspring (Figure S4A). As these loci did not exhibit significant changes in methylation, we therefore turned to genome-scale mapping studies to search for differentially methylated loci between control and low-protein offspring.

We performed reduced representation bisulfite sequencing (RRBS) (Meissner et al., 2008) to characterize cytosine methylation at single-nucleotide resolution across ~1% of the mouse genome (Table S5). RRBS was performed for livers from a pair of control and low-protein offspring, and fraction of methylated CpGs was calculated for a variety of features such as promoters, enhancers, and other nongenic CpG islands. In general, we found that cytosine methylation was well-correlated between control and low-protein offspring (Figures 5A and 5B). However, we did observe widespread modest (~10%–20%) changes in CpG methylation between the two samples (red and green dots in Figures 5A and 5B), consistent with many observations indicating that environmental changes tend to have small quantitative effects on cytosine methylation in the next generation (Blewitt et al., 2006; Heijmans et al., 2008; Ng et al., 2010; Weaver et al., 2004). Importantly, changes in promoter methylation did not globally correlate with changes in gene expression in offspring, indicating that the gene expression program in offspring is unlikely to be epigenetically specified at each individual gene (Figure 5C). Of course, widespread gene expression differences can be caused by changes to a small number of upstream regulators, and a number of differentially methylated regions are associated with cholesterol- or lipid-related genes (Table S5).

Most interestingly, we found a substantial (~30%) increase in methylation at an intergenic CpG island ~50 kb upstream of *Ppara* (Figure 6A). This locus is likely an enhancer for *Ppara*, as it is associated with the enhancer chromatin mark H3K4me1 (Heintzman et al., 2007) in murine liver (F. Yue and B. Ren, personal communication). *Ppara* is downregulated in the majority (but not all) of offspring livers (Table S1, Figure 6B), and the overall gene expression profile in our offspring livers significantly matches the gene expression changes observed in *Ppara* knockout mice (Figure 2), suggesting that epigenetic regulation of this single locus could drive a substantial fraction of the observed gene expression changes in offspring. Indeed, variance of *Ppara* mRNA levels alone can be used to explain ~13.7% of the variance in the entire gene expression dataset (although this of course does not determine causality).

We therefore assayed the methylation status of this locus by bisulfite sequencing in an additional 17 offspring livers (8 control and 9 low-protein), finding average differences of up to 8% methylation between low-protein and control livers at several CpGs in this locus (Figure 6C). Importantly, these pooled data underestimate the potential role of this locus in reprogramming as they include animals exhibiting a range of changes in *Ppara* gene expression—individual animal pairs with large differences in *Ppara* mRNA levels exhibit methylation differences of up to 30% at various cytosines across this locus. Figure 6D shows individual bisulfite clones for three pairs of animals with varying extents of *Ppara* downregulation (not all animals used for methylation analysis were analyzed by microarray). Taken together, these results identify a differentially methylated locus that is a strong candidate to be one of the upstream controllers of the hepatic gene expression response.

Cytosine Methylation, RNA, and Chromatin in Sperm

The link between paternal diet and offspring methylation patterns lead us to consider the hypothesis that paternal diet affects cytosine methylation patterns in sperm. We therefore isolated highly pure (>99%) sperm from the caudal epididymis of males consuming control or low-protein diet. We assayed the *Ppara* enhancer for methylation by bisulfite sequencing but found no significant changes between males consuming control or low-protein diet (Figure S4B). These results indicate either that cytosine methylation in sperm is not the relevant paternally transmitted dietary information at this locus (but changes at some point during development; Blewitt et al., 2006), or that we captured animals whose offspring would not manifest significant changes in expression of the associated genes—as seen in Figure 1B or Figure 6B, *Ppara* downregulation is variably penetrant in low-protein offspring.

To globally investigate effects of paternal diet on sperm cytosine methylation, we isolated sperm from four males—two consuming control diet, one consuming low-protein diet, and one subjected to a caloric restriction regimen. We then surveyed cytosine methylation patterns across the entire genome via MeDIP-Seq (immunoprecipitation using antibodies against 5me-C followed by deep sequencing; Jacinto et al., 2008; Weber et al., 2005) (Figure 7A, Figure S5A, and Figure S6). Notably, global cytosine methylation profiles were highly correlated between any pair of samples, indicating that the sperm "epigenome" is largely unresponsive to these differences in diet (Figures 7B–7D, Figures S5B–S5E). Indeed, littermates on different diets (Figures 7B and 7C) were better-correlated for promoter methylation than were the pairs of control animals

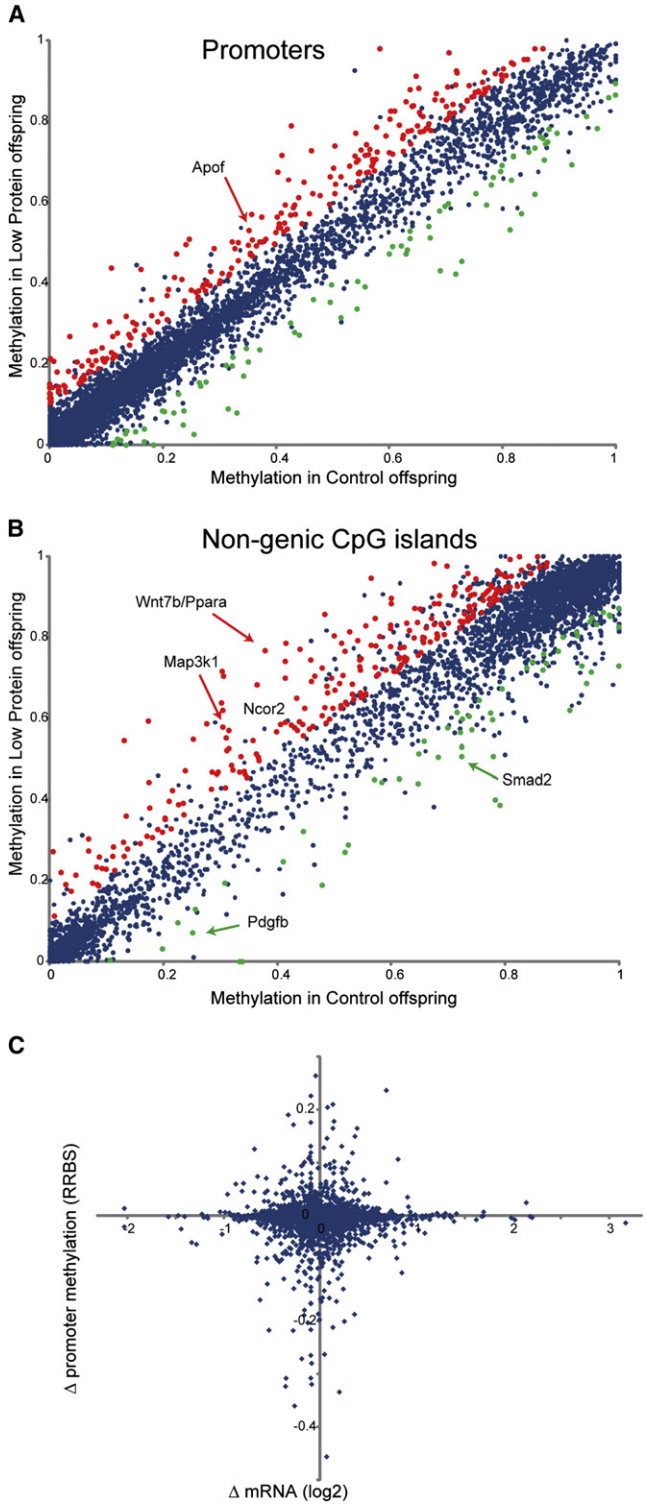

Figure 5. Transgenerational Effects of Paternal Diet on Hepatic Cytosine Methylation

(A) Genomic DNA from control and low-protein offspring livers was subjected to reduced representation bisulfite sequencing (RRBS). For all annotated promoters, average fraction of CpGs that were methylated is shown for the control sample (x axis) compared to the low-protein sample (y axis). Red and green dots indicate promoters with significant ($p < 0.05$) methylation changes of over 10%.

(B) As in (A), for nongenic CpG islands.

(C) Promoter cytosine methylation changes are uncorrelated with gene expression changes. For each promoter, the average change in cytosine methylation is compared to the change in mRNA abundance from Figure 1B. See also Table S5 and Figure S4.

from different litters (Figure 7D). Although these results do not rule out cytosine methylation in sperm as the relevant carrier of epigenetic information about paternal diet, the high correlation between samples, coupled with the absence of cytosine methylation changes at the *Ppara* enhancer in sperm, leads us to consider alternative epigenetic information carriers including RNA (Rassoulzadegan et al., 2006; Wagner et al., 2008) and chromatin (Arpanahi et al., 2009; Brykczynska et al., 2010; Hammoud et al., 2009; Ooi and Henikoff, 2007).

We used Affymetrix microarrays to analyze RNA levels for three pairs of males and for two matched epididymis samples (Figure S6, Figure S7A, Table S6). Curiously, low-protein and caloric restriction samples consistently exhibited more "sperm-like" RNA populations (as opposed to epididymis RNA) than did control samples (Figures S7B and S7C). Whether this reflects systematic contamination issues or biological differences in sperm maturity or quality is presently unknown, although we note that we confirmed consistently higher levels of the sperm-specific *Dnahc3* by q-RT-PCR in an additional 7/8 low-protein sperm samples (Figure S7E). We note that control sperm samples were routinely >99.5% sperm as assayed by microscopy (Figure S6), but nonetheless we cannot completely rule out systematic contamination issues. With this possibility in mind, we identified genes that were differentially packaged in control versus low-protein sperm by correcting for potential epididymal contamination (Figures S7B–S7F). Interestingly, we observed downregulation of a number of transcription factors and chromatin regulators such as *Smarcd3* and *Ppard*, although q-RT-PCR validation was not statistically significant due to high inter-animal variability (Figure S7F).

Although the downregulation of *Smarcd3* was not significantly confirmed by q-RT-PCR, this could reflect the variable penetrance of paternal diet on offspring described above. Given that heterozygous mutants in chromatin remodelers can affect offspring phenotype even when the mutant allele segregates away (Chong et al., 2007), we used an initial genome-wide mapping (not shown) of overall histone retention (pan-H3 ChIP) abundance and the key epigenetic histone modification H3K27me3 in sperm to identify targets for single locus analysis. We observed a consistent decrease in H3K27me3 in low-protein sperm at the promoter of *Maoa* (Monoamine oxidase) in 5/5 pairs of sperm samples and a decrease in H3K27me3 at *Eftud1* in 4/5 paired samples (Figures S7G and S7H). These results demonstrate proof of principle that the sperm epigenome is regulated by dietary conditions, although the biological implications of these observations are not yet clear.

DISCUSSION

Taken together, our results demonstrate that paternal diet affects lipid- and proliferation-related gene expression in the

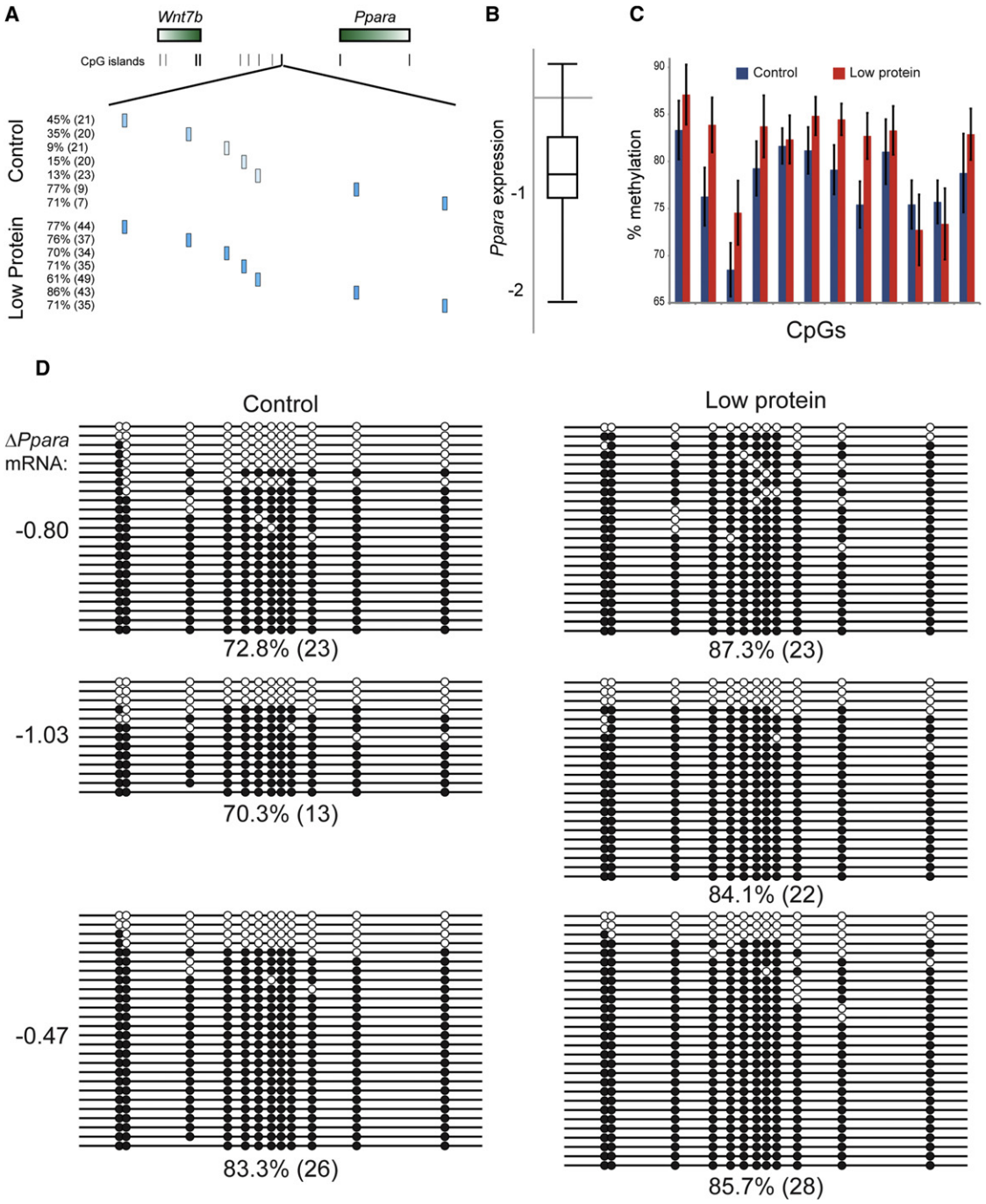

Figure 6. Effects of Paternal Diet on Methylation of a Putative *Ppara* Enhancer

(A) Differential methylation of a putative *Ppara* enhancer. Top panel shows a schematic of chromosome 15: 85,360,000–85,640,000. Zoomed in region represents chr15: 85,514,715-85,514,920. RRBS data for one control and one low-protein offspring pair are shown below, with assayed CpGs represented as boxes colored to indicate % of clones methylated. Numbers to the left indicate % methylation, with number of sequence reads covering the CpG in parentheses.

(B) *Ppara* is downregulated in most low-protein offspring livers. Box plot shows mean, quartiles, and highest and lowest values from Table S1.

(C) Putative enhancer methylation correlates with *Ppara* downregulation. DNA from eight control and nine low-protein pairs of offspring livers was bisulfite treated, and at least 13 clones were analyzed for each animal. Percent methylation at each of the 12 CpGs in this region plotted on the y axis; data are shown as mean ± standard error of the mean (SEM).

(D) Individual bisulfite clones are shown for three control and three low-protein offspring. White circles indicate unmethylated CpGs, black circles indicate methylated CpGs. Microarray data for change in *Ppara* RNA levels between the paired animals are shown to the left, in $\log_2$. Values under each bisulfite grouping indicate overall % methylation, with number of clones analyzed in parentheses.

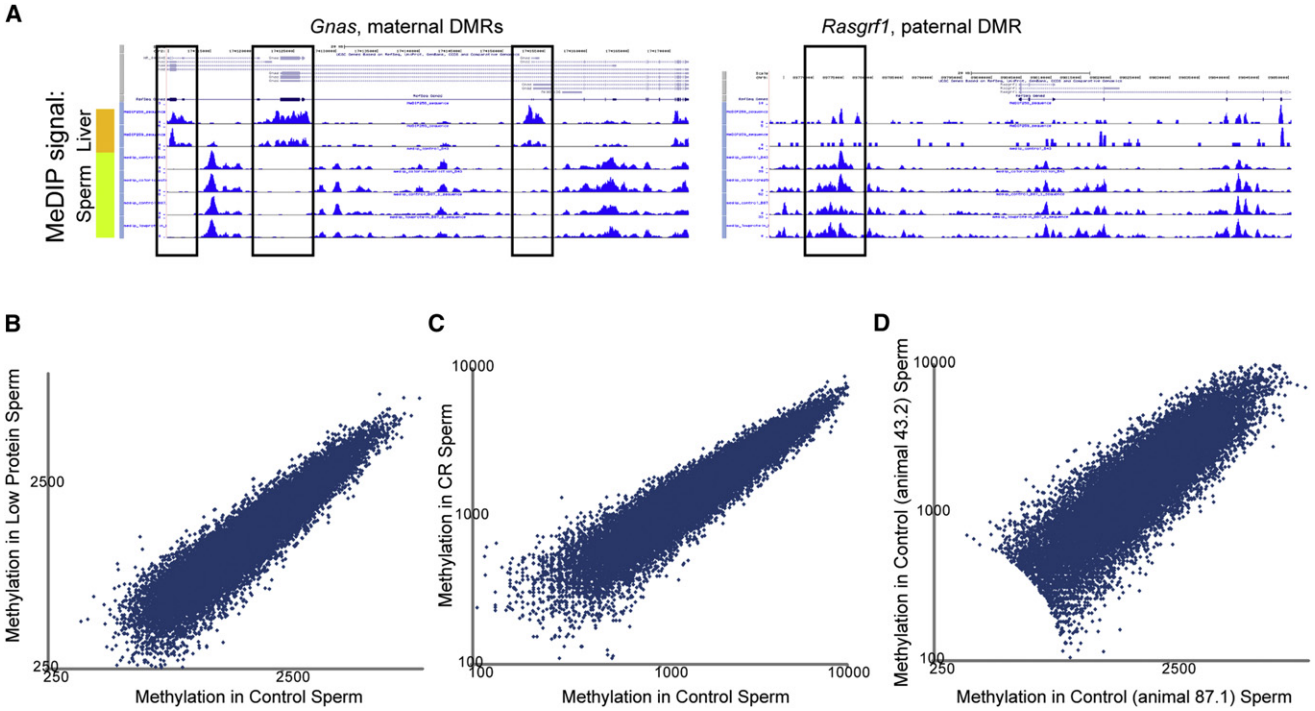

Figure 7. Modest Effects of Diet on the Sperm Epigenome

(A) MeDIP sequencing data are shown for two liver samples (top two tracks) and four sperm samples (bottom four) at a maternally methylated region (*Gnas*, left) and a paternally methylated region (*Rasgrf1*, right).

(B) Comparison of control and low-protein methylation. For each promoter, methylation levels were averaged for 8 kb surrounding the TSS, and values are scatterplotted for control sperm (x axis) versus low-protein sperm (y axis). x and y axes are plotted on logarithmic scales.

(C) As in (B), but for control versus caloric restriction.

(D) As in (B), but for the pair of control samples.

Similar results for (B)–(D) are found when focusing on the 1 kb surrounding the TSS (not shown). See Figure S7 for analyses of consistent RNA and chromatin differences between low-protein and control sperm.

offspring of inbred mice, and that epigenetic information carriers in sperm respond to environmental conditions. These results have potential implications for human health and raise numerous mechanistic questions, discussed below.

Paternal Diet Affects Metabolism in Offspring

Our results clearly identify a set of physiological pathways whose expression is sensitive to paternal diet. Specifically, we find that hepatic expression of genes involved in proliferation and cholesterol biosynthesis can be regulated by paternal diet, and these changes are reflected in levels of several lipid metabolites. Combined with data showing that offspring glucose levels are affected by paternal fasting in mice (Anderson et al., 2006), these results demonstrate that paternal diet has wide-ranging effects on the metabolism of offspring in rodents. Interestingly, a very recent study from Ng et al. (Ng et al., 2010) reported that chronic exposure of male rats to high-fat diet was associated with pancreatic beta cell dysfunction in female offspring. It will naturally be of great interest in the near future to compare the transgenerational effects of high-fat and low-protein diets, although one clear difference is that in our system a transgenerational effect is observed in both sex offspring.

Whether the effects we observe on cholesterol metabolism prove advantageous in low-protein conditions remains to be tested, but it will be important to investigate ecologically relevant diets in order to speculate more firmly about adaptive significance of any observed transgenerational effects. For example, at present we cannot say with certainty what aspect of the low-protein regimen is sensed by males—it is possible that offspring metabolism is affected by overall protein consumption, or high sucrose, or fat/protein ratio, or even levels of micronutrients, as our males consumed diets ad libitum and thus might have overconsumed the low-protein diet.

The Reprogrammed State: Liver

What is the mechanistic basis for the reprogrammed gene expression state? Genome-scale analyses of cytosine methylation in offspring livers identified several lipid-related genes that were differentially methylated depending on paternal diet. Most notably, a putative enhancer for a major lipid regulator, *Ppara*, exhibited generally higher methylation in low-protein offspring than in control offspring. Methylation at this locus was variable between animals, consistent with the partial penetrance of *Ppara* downregulation in our dataset. The overall gene expression profile observed in low-protein offspring significantly overlaps

gene expression changes observed in *Ppara* knockout mice (Rakhshandehroo et al., 2007), leading to the hypothesis that epigenetic *Ppara* downregulation via enhancer methylation is an upstream event that affects an entire downstream regulon in reprogrammed animals. Note that although the hepatic downregulation of *Ppara* suggests a liver-autonomous epigenetic change, we cannot rule out that hepatic gene expression changes result from global physiological changes resulting from downregulation of *Ppara* in some other tissue.

Interestingly, *Ppara* expression in liver is also regulated by *maternal* diet—offspring of female mice consuming a high-fat diet exhibit altered hepatic *Ppara* expression, with increased expression at birth but decreased expression at weaning (Yamaguchi et al., 2010). Together with our data, these results suggest that *Ppara* is a key nexus that integrates ancestral dietary information to control offspring metabolism.

Mechanistic Basis for Transgenerational Paternal Effects

Paternal diet could potentially affect offspring phenotype via a number of different mechanisms. Although we focus here on epigenetic inheritance systems, it is important to note that parental information can also be passed to offspring via social or cultural inheritance systems (Avital and Jablonka, 2000; Champagne and Meaney, 2001; Jablonka and Lamb, 1995; Meaney et al., 2007; Weaver et al., 2004). Although such maternally provided social inheritance is unlikely in our paternal effect system—males were typically only in females' cages for one day—it is known that in some animals females can judge mate quality and allocate resources accordingly (Pryke and Griffith, 2009), and that seminal fluid can influence female postcopulatory behavior in *Drosophila* (Fricke et al., 2008; Wolfner, 2002). These and other plausible transgenerational information carriers cannot be excluded at present—ongoing artificial insemination and in vitro fertilization experiments will determine whether sperm carry the relevant metabolic information in our system.

Here we focused on the hypothesis that paternal dietary information does indeed reside in sperm epigenetic information carriers. First, a subset of cytosine methylation patterns in sperm are known to be heritable (Chong et al., 2007; Cropley et al., 2006; Rakyan et al., 2003; Waterland and Jirtle, 2003). Second, several reports suggest that RNA molecules packaged in sperm can affect offspring phenotype (Rassoulzadegan et al., 2006; Wagner et al., 2008). Third, chromatin structure has been proposed to carry epigenetic information, as sperm are largely devoid of histone proteins but retain them at a subset of developmentally important loci (Arpanahi et al., 2009; Brykczynska et al., 2010; Chong et al., 2007; Hammoud et al., 2009). Finally, it is conceivable that additional or novel epigenetic regulators (such as prions) are packaged into sperm, or that sperm quality is affected by diet, or that genetic changes are directed by the environment (although it is important to emphasize that inbred mouse strains were used in this study).

Here, we report whole-genome characterization of cytosine methylation patterns and RNA content in sperm obtained from mice maintained on control, low-protein, and caloric restriction diets. Globally, cytosine methylation patterns are similar in all three conditions, indicating that the sperm epigenome is largely unaffected by these diets. Nonetheless, changes in relatively few loci can have profound effects in the developing animal, and our data do not rule out the possibility of inheritance through sperm cytosine methylation, especially given that MeDIP is unlikely to identify ~10%–20% of differences in methylation at a small number of cytosines. Importantly, the putative enhancer of *Ppara* (Figure 6) was not differentially methylated in sperm. It will therefore be of great interest in the future to determine when during development the differential methylation observed in liver is established and to identify the upstream events leading to differential methylation (Blewitt et al., 2006).

Interestingly, we did identify effects of diet on RNA content and chromatin packaging of sperm. For example, sperm from control animals were consistently depleted of the highly sperm-specific *Dnahc3* gene (Figure S7) relative to sperm from low-protein animals. We cannot presently determine whether this represents reproducible differences in contamination, differences in sperm maturity, or something else. Finally, based on our observation that low-protein sperm tended to be depleted of genes encoding a number of chromatin regulators, we have begun to search for dietary effects on sperm chromatin structure. Interestingly we found that the *Maoa* promoter was consistently depleted of the key Polycomb-related chromatin mark H3K27me3 (Figure S7G), demonstrating as a proof of concept that chromatin packaging of the sperm genome is responsive to the environment and motivating genome-wide investigation into dietary effects on sperm chromatin. Given the common behavorial changes observed in many transgenerational inheritance paradigms, the possibility that H3K27me3 at *Maoa* affects offspring behavior (potentially via altered offspring responses to maternal stress; Harris and Seckl, 2010) will be of great future interest.

Relevance to Human Disease

These results are likely to be relevant for human disease because not only is *maternal* starvation in humans correlated with obesity and diabetes in children (Lumey et al., 2007), but also, remarkably, limited food in paternal grandfathers has been associated with changed risk of diabetes and cardiovascular disease in grandchildren (Kaati et al., 2002; Pembrey et al., 2006). Interestingly, in these studies ancestral access to food and disease risk were not associated with disease risk in the next generation but were only associated with F2 disease risk. However, it is important to note that the transgenerational effects of food availability for paternal grandfathers depend on the exact period during childhood of exposure to rich or poor diets (Pembrey et al., 2006), whereas our experimental protocol involved continuous low-protein diet from weaning until mating. Thus, future studies are required to more precisely define when and how ancestral exposure to a low-protein diet affects epigenetic programming of offspring metabolism.

Together, these results suggest rethinking basic practices in epidemiological studies of complex diseases such as diabetes, heart disease, or alcoholism. We believe that future environmental exposure histories will need to include parental exposure histories as well as those of the patients to disentangle induced epigenetic effects from the currently sought genetic and environmental factors underlying complex diseases. Our observations

provide an inbred mammalian model for transgenerational reprogramming of metabolic phenotype that will enable dissection of the exposure history necessary for reprogramming and genetic analysis of the machinery involved in reprogramming, and they suggest a number of specific pathways likely to be the direct targets of epigenetic reprogramming.

EXPERIMENTAL PROCEDURES

Mouse Husbandry

All animal care and use procedures were in accordance with guidelines of the Institutional Animal Care and Use Committee. C57/Bl6 mice were obtained from Jackson Labs and from Charles River Laboratories (for different iterations of this experiment). All experiments were performed with mice that had been raised for at least two generations on control diet to attempt to minimize any transgenerational effects of transitioning to control diet from chow provided by animal provider. For all comparisons shown, male mice were weaned from mothers at 21 days of age, and sibling males were put into cages with low-protein or control diet (moistened with water to allow mice to break the hard pellets). Females were weaned to control diet. Males were raised on diet until 9–12 weeks of age, at which point they were placed with females for 1 or 2 days. Control and low-protein mating cages were always interspersed with one another. Note that we always used virgin females to avoid confounding effects of the female's litter number, although this results in many lost litters as first litters were often consumed by their mothers. After 1 to 2 days, males were removed, and pregnant females were left alone with control diet and a shepherd shack until their litters were 3 weeks of age. At 3 weeks of age offspring were sacrificed by isoflurane and cervical dislocation, and median lobe of liver was rapidly dissected out and flash-frozen in liquid N_2.

Diets

Diets were obtained from Bio-serv, and compositions are listed in Table S7. For most experiments only low-protein diet was sterilized per standard protocol at Bio-serv. For later experiments, both diets were sterilized.

RNA Extraction

Liver samples were ground with a liquid N2-cooled mortar and pestle. Total RNA for microarray analysis was extracted from liver powder using Trizol.

Microarray Hybridization

Thirty micrograms of total RNA was labeled for 2 hr at 42°C with Superscript II reverse transcriptase using 4 μg of random hexamer and 4 μg of oligo dT. Cy3- and Cy5-labeled samples were hybridized to home-printed "MEEBO" microarrays. MEEBO information is available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL6352. Microarrays were hybridized at 65°C for 16 hr, washed as previously described (Diehn et al., 2002), and scanned using an Axon Genepix 4000B microarray scanner.

Comparison to Public Murine Liver Microarray Data

We built a compendium of public microarray data consisting of 120 gene expression profiles in the murine liver under various conditions and genetic perturbations. Signatures of differentially expressed genes were determined using a combination of two one-tailed t tests, with FDR correction of 0.1. Profiles significantly enriched with up- or downregulated genes in low-protein offspring were defined by a hypergeometric p value ≤ 0.05 after correction for multiple hypotheses ($p < 0.00025$).

Lipid Measurements

~50–100 mg of ground liver tissue from six animals (three paired sets) was sent to Lipomics for "Truemass" mass spectrometry characterization of 450 lipid levels (Table S4). Note that samples 73-1 and 76-1 come from PBS-perfused livers, whereas the other four samples were dissected without perfusion.

Small RNA Cloning and Sequencing

Total RNA was isolated from ground liver tissue using mirVana (Ambion). 18–35 nt small RNA was purified from 100 μg of total RNA, ligated to adaptors, amplified, gel-purified, and sequenced using a Solexa Genome Analyzer (Illumina) (Ghildiyal et al., 2008).

RRBS

Reduced representation bisulfite sequencing was carried out as previously described (Meissner et al., 2008). Data are available at http://thrifty-epigenome.computational-epigenetics.org.

Sperm Isolation

Caudal epididymis was dissected from sacrificed animals, punctured, and incubated for 30 min in M2 media (Sigma) at 37°C. Supernatant was removed, pelleted (3000 g for 5 min), washed 2× with PBS and 1× in water, and incubated in Somatic Cell Lysis buffer. Sperm preparations were used only if they were >99.5% pure as assessed by microscopy, and q-RT-PCR was also used to reject any sperm samples based on the ratio between epididymis-specific genes *Actb* or *Myh11* and sperm-specific genes *Smcp* or *Odf1* (Figure S6).

MeDIP

Methyl-DNA immunoprecipitation was carried out essentially as described (Weber et al., 2005, 2007). Four micrograms of purified genomic DNA was fragmented to a mean size of 300 bp using a Covaris machine, denatured, and immunoprecipitated with 5mC antibody (Eurogentec). ChIP material was Solexa sequenced, with ~21 million uniquely mappable reads per library.

ACCESSION NUMBERS

All microarray data and deep sequencing data used in this study have been deposited to GEO (http://www.ncbi.nlm.nih.gov/projects/geo/), accession # GSE25899.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and seven tables and can be found with this article online at doi:10.1016/j.cell.2010.12.008.

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O.J.R. and H.A.H. designed the original expression experiment, and a pilot was carried out by C.E.H., O.J.R., and H.A.H. O.J.R., B.C, L.F., and J.S. carried out animal husbandry and gene expression experiments, and L.F. and C.L. carried out miRNA profiling. C.B. and A.M. carried out RRBS experiments, L.F. carried out sperm RNA profiling, and J.S. carried out MeDIP experiments. J.S. and B.C. carried out bisulfite sequencing, and B.C. carried out chromatin ChIPs. N.H., O.J.R., and N.F. analyzed the gene expression and microRNA data, R.L., Z.W., and O.J.R. analyzed the MeDIP data. O.J.R., P.D.Z., and N.F. wrote the paper.

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