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Research Article

Transgenerational inheritance of hepatic steatosis in mice: sperm methylome is largely reprogrammed and inherited but does not globally influence liver transcriptome

Sílvia Ribó[1,7], Marta Ramon-Krauel[1], Josep M. Marimon-Escude[2], Florence Busato[3], Flavio Palmieri[4], Marta Mourin-Fernandez[1,5], Ivonne Palacios-Marin[1,5], Ruben Diaz[1], Carles Lerin[1], Rafael Oliva[6], Jorg Tost[3], Josep C. Jiménez-Chillarón[1,4,*]

[1]Institut de Recerca Sant Joan de Déu (IRSJD), Endocrinology, Esplugues 08950, Spain
[2]Universitat de Barcelona-Centres Científics i Tecnològics, Barcelona 08028, Spain
[3]Laboratory for Epigenetics and Environment, Centre National de Recherche en Génomique Humaine, CEA-Institut de Biologie François Jacob, Université Paris—Saclay, Evry 91000, France
[4]Universitat de Barcelona-Facultat Medicina-Bellvitge, L'Hospitalet 08907, Spain
[5]Universitat de Barcelona-Facultat de Farmàcia, Barcelona 08028, Spain
[6]Molecular Biology of Reproduction and Development, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, and Hospital Clinic Barcelona 08036, Spain

[7]Present address: IrsiCaixa AIDS Research Institute, Campus Can Ruti, Badalona, Spain.
*Corresponding author. Department of Physiological Sciences, Biophysics Unit, School of Medicine, University of Barcelona, c/Feixa Llarga s/n, 4a planta, L'Hospitalet, Barcelona 08907, Spain. E-mail: josep.jimenez@ub.edu

Abstract

Nutritional challenges and obesity can contribute to the transmission of metabolic diseases through epigenetic mechanisms. Among them, DNA methylation stands out as a potential carrier of information because germline cytosine methylation responds to environmental factors and can be transmitted across generations. Yet, it remains unclear whether inherited DNA methylation plays an active role in the inheritance of metabolic phenotypes or solely influences expression of a few genes that cannot recapitulate the whole metabolic spectrum in the next generation offspring. Previously, we established a mouse model of childhood obesity by reducing litter size at birth. Mice raised in small litters (SL) developed obesity, insulin resistance, and hepatic steatosis. The offspring (SL-F1) and grand-offspring (SL-F2) of SL males also exhibited hepatic steatosis. Here, we aimed to investigate whether germline DNA methylation could serve as a carrier of phenotypic information, hepatic steatosis, between generations. Litter size reduction significantly altered global DNA methylation profile in the sperm of SL-F0 males. Remarkably, 8% of these methylation marks remained altered in the sperm of SL-F1 mice and in the liver of SL-F2 mice. These data suggest that germline DNA methylation is sensitive to environmental challenges and holds significant heritability, either through direct germline transmission and/or through sequential erasure and reestablishment of the marks in the following generations. Yet, DNA methylation did not strongly correlate with the hepatic transcriptome in SL-F2 mice, suggesting that it does not directly drive phenotypes in the F2. As an alternative, germline DNA methylation could potentially influence the phenotype of the next generation by modulating the expression of a reduced number of key transcription factors that, through an amplification cascade, drive phenotypic outcomes in subsequent generations.

Keywords: childhood obesity; hepatic steatosis; sperm; DNA methylation; transgenerational epigenetic inheritance; mouse model

Introduction

Heredity is defined as the tendency of offspring to resemble their parents and for biological traits (phenotypes) to run in families. It is accepted that the DNA is a major carrier of information across generations, and heredity is mediated by the transmission of alleles that remain impervious to the environment [1]. However, this view has been recently challenged: It is now recognized that several environmental cues promote phenotypic variation that can be transmitted across generations without involving genetic variation [2]. The range of reported factors inducing transgenerational/intergenerational effects comprises environmental chemicals [3, 4], nutritional cues [5–10], exercise [11, 12], obesity

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[10, 13], smoking [14–16], or stress and psychological trauma [17–21]. Transgenerational effects are mediated, at least in part, by germline epigenetic modifications that are transmitted along with the genome [2, 22]. Examples of germline epigenetic variation include DNA methylation, histone modifications, and a variety of noncoding RNAs [7, 10, 13, 23–30]. DNA methylation is the most widely studied epigenetic mark, and a few reports have shown that nutrition-obesity may alter sperm DNA methylation [31], thereby providing a means by which cytosine methylation may mediate transgenerational inheritance of metabolic traits in mammals [6, 32]. Yet, proof that germline DNA methylation is a significant carrier of metabolic phenotypes across generations remains controversial. The main reason is that germline cytosine methylation does not seem to (i) directly and (ii) globally influence the expression of target genes in metabolic tissues [24, 33, 34]. Here we explored whether sperm DNA methylation is a plausible means for the inheritance of metabolic disease in the context of childhood obesity.

We have previously developed a mouse model of childhood obesity through litter size reduction at birth [35, 36]. Briefly, we culled the progeny to four pups per female in the small litter group (SL), whereas the control females (C) nursed eight pups throughout lactation. Litter size reduction led to late-onset insulin resistance, glucose intolerance, and hepatic steatosis [37]. Strikingly, litter size reduction also influenced metabolic health in the following two generations through the paternal lineage [5, 34]. Specifically, the offspring (SL-F1) and grand-offspring (SL-F2) of the founder small litters (SL) males developed glucose intolerance and insulin resistance with aging, despite that they were not exposed to nutritional challenges throughout their life course. Therefore, we have a valuable model to study molecular mechanisms of true transgenerational inheritance of complex phenotypes in mammals [38]. In this study, we followed the paternal inheritance only, because intergenerational/transgenerational effects through the male lineage should be mediated, primarily, by epigenetic mechanisms. In contrast, maternally-mediated transgenerational effects will be due to a complex interplay between metabolic, behavioral, mitochondrial, and epigenetic modifications [2, 38].

Here we found that litter size reduction altered global DNA methylation profile in the sperm of the exposed individuals. Therefore, we confirmed that germline epigenome is extremely sensitive to environmental cues, namely early overnutrition and/or obesity. Secondly, a significant fraction of the nutritionally induced methylation marks was transmitted up to the grand offspring, suggesting that environmentally sensitive epigenetic marks can be either inherited or somehow recapitulated. Finally, DNA methylation did not influence global transcription profile in the second-generation offspring. Instead, we argue that DNA methylation may influence next generation transcriptome through modifying the expression of a small number of key upstream transcription factors (TFs) that, through an amplification process, regulate global transcription profile.

Results

Transgenerational inheritance of hepatic steatosis through the paternal lineage

We explored whether litter size reduction (Fig. 1a) induced metabolic imbalances in the second-generation offspring (F2) through the paternal lineage. As previously reported, males raised in small litters (SL-F0) developed adult-onset obesity, hyperinsulinemia, insulin resistance, glucose intolerance, and hepatic steatosis [5, 37] (Fig. 1b, Supplementary Table S1). In contrast, females raised in SL only developed adult-onset obesity, but not glucose intolerance and hepatic steatosis [37]. Hence, here we examined whether the parental phenotypes were transmitted to the male offspring and grand offspring. First, we found that the male offspring of SL-F0 males (SL-F1) (Fig. 1c) also developed hyperinsulinemia, insulin resistance, and hepatic steatosis, but not overweight and obesity [5, 34] (Fig. 1d, Supplementary Table S1). Finally, adult SL-F2 male mice developed moderate hyperinsulinemia, insulin resistance, and hepatic steatosis, while they retained appropriate adult body weight, adiposity, and glucose tolerance when compared to C-F2 mice (Fig. 1e, Supplementary Table S1). Together, we confirmed that litter size reduction influenced the metabolic health of the offspring (F1) and grand offspring (F2), via the paternal lineage. Paternal transmission of environmentally induced metabolic traits is likely mediated by germline epigenetic mechanisms [39].

Litter size reduction modifies DNA methylation in the founder males, the offspring, and grand offspring

To study germline epigenetic inheritance, we analyzed global DNA methylation at CpG Islands (CGIs) in the sperm of the founder males (C-F0; SL-F0) and its offspring (C-F1; SL-F1). First, we found that up to 16 000 CGIs were modified in the sperm of SL-F0 mice (Fig. 2a, Supplementary Table S2). Most changes occurred within gene bodies (72.8%) and promoter regions (25.3%) (Fig. 2b). Half of them gained methylation (log fold change > 0,2) and the other half lost it (log fold change < −0,2) (Fig. 2c). This distribution was mainly observed in intergenic regions and gene bodies, whereas promoter regions were mostly hypomethylated in SL-F0 sperm samples (Fig. 2d). Next, we found that 3057 CGIs were differentially methylated in the sperm of SL-F1 males (Fig. 2a, Supplementary Table S3). Noteworthy, the germline of SL-F1 males was not exposed to nutritional challenges nor obesity throughout life course. Therefore, differences in methylation are either transmitted or somehow re-established or maintained despite the reprogramming events that occur during spermatogenesis and early embryogenesis. Most changes occurred again within gene bodies (68%) and promoter regions (24%) (Fig. 2e), and up to 71.9% of them were hypomethylated (Fig. 2f). This trend was similar in all genomic locations, including promoters, gene bodies, intergenic regions, or divergent promoters (Fig. 2g).

As previously described, SL-F0, SL-F1, and SL-F2 mice developed hepatic steatosis (Fig. 1). Hence, we explored whether litter size reduction also influenced the methylome in the liver of the grand offspring (Fig. 2a). A total of 5018 CGIs were differentially methylated in the liver of 7.5-day-old SL-F2 mice, when compared to C-F2 mice (Fig. 2a, Supplementary Table S4). We selected this age, because at this stage SL-F2 mice have not yet developed metabolic derangements, which in turn could alter DNA methylation [33]. Like in sperm, most differences appeared in gene bodies (77%) and, to a lesser extent, in promoter regions (16%) (Fig. 2h). Again, most CGIs were hypomethylated in the livers of SL-F2 mice, as compared to the controls (Fig. 2i, j). This concordance strongly suggests transgenerational inheritance of epigenetic marks through the paternal line.

In support, 831 regions appeared differentially methylated across all three generations (Fig 3a, Supplementary Table S5). The direction and magnitude of changes were similar in all tissues: The regions that gained methylation in the sperm of SL-F0 mice also gained methylation in the sperm of SL-F1 mice (Fig 3b) and the liver of SL-F2 mice (Fig 3c). Further, the magnitude of these changes was also similar across the three generations. Most

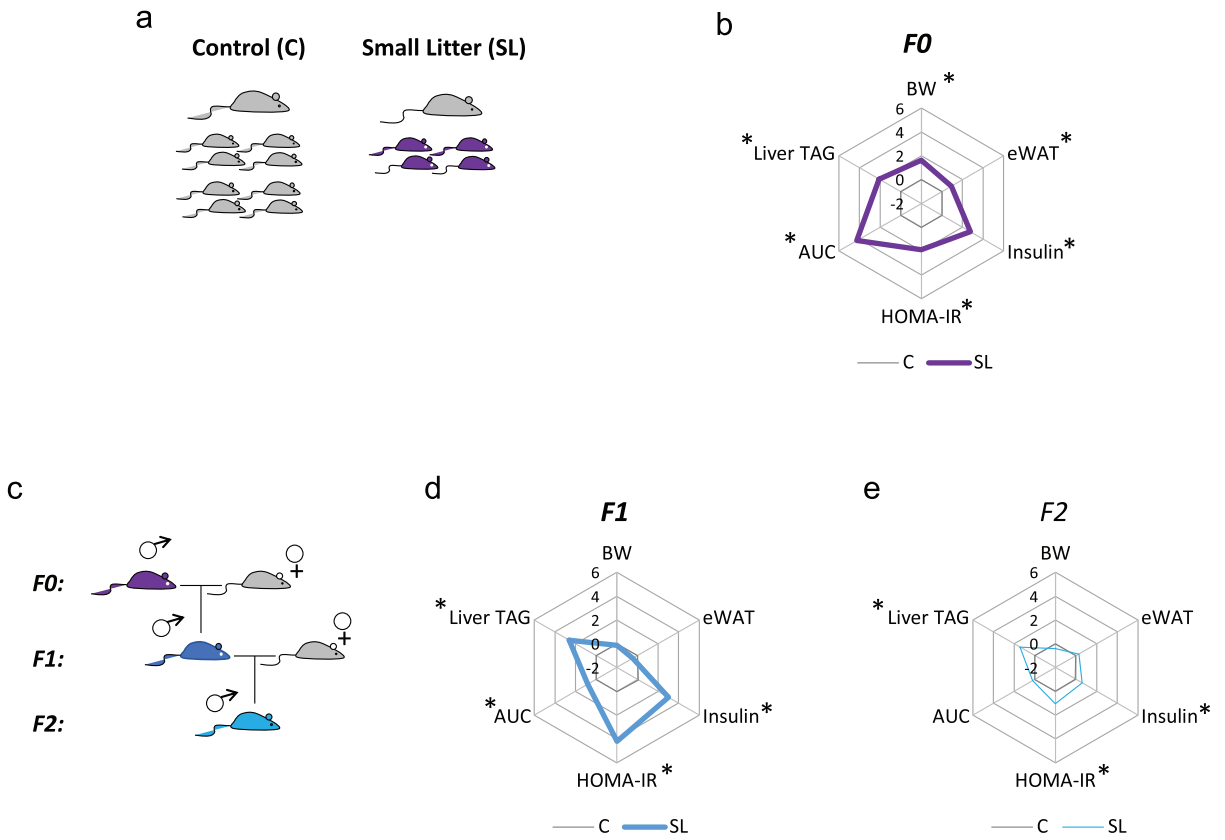

Figure 1. Litter size reduction leads to metabolic deregulation in the offspring and grand offspring, through the paternal lineage. (a) Mouse model of childhood obesity. Control females nursed eight pups through the lactation period (21 days), whereas the females in the small liter group (SL) nursed four pups. (b) Spider plot that summarizes the physiology of the parental generation (C-F0; Sl-F0 males). All physiological data (linked to Supplementary Table S1) was converted into Z-scores (Supplementary Table S17) to facilitate comparison between different measurements and across generations. The average Z-score for the control group was near 0.00 for all measured parameters. The legend in the figure is as follows: BW= Body weight; eWAT= epididymal white adipose tissue; HOMA-IR= Homeostatic model assessment of insulin resistance; AUC= Area Under the Curve for a glucose tolerance test. All determinations that were statistically significant between groups are designated with an asterisk. (c) Scheme representing the breeding strategy. Control and SL males of the parental generation were crossed with external control females to generate the first-generation offspring (C-F1; SL-F1). Likewise, C-F1 and SL-F1 males were crossed with another group of external control females to generate the second-generation offspring, F2. (d and e) Spider plots detailing physiological information for the offspring and grand offspring of the parental groups, respectively. *P value <.05, student's t-test.

changes occurred again within the gene bodies (80.2%) and promoter regions (14%) (Fig 3d) and appeared largely demethylated in SL samples as compared to the controls (Fig 3e).

Together, these data support that early nutrition/obesity largely influences cytosine methylation in the germline (sperm) of the exposed SL-F0 mice. A significant fraction of these CGIs (8%) was recapitulated in the following generations (SL-F1, SL-F2). Hence, this "line of continuity" across multiple generations strongly suggests the potential of DNA methylation for being inherited, or somehow recapitulated in the following generations [2, 40].

Transcription profile in the liver of the grand offspring during early development

We addressed whether the methylation marks that are potentially transmitted from the F0 to the F2 influence phenotypic outcomes through tackling the grand offspring transcriptome. Therefore, we first measured global transcription profile (Affymetrix) in the liver of PD7.5 neonates (Fig. 4a). In all, 1090 genes were differentially expressed in the liver of SL-F2 mice, when compared to C-F2 mice (Fig. 4a, Supplementary Tables S6). Significantly, these changes arose despite the SL-F2 mice not being exposed to nutritional challenges through their life course. Therefore, these changes should be attributed, at least in part, to the (inherited) methylation marks. We next quantified the correlation between DNA methylation and transcription in the liver of SL-F2 mice. Interestingly, of the 1090 dysregulated genes only 124 genes, associated to 290 differentially methylated regions, seemed to be likely directly regulated by DNA methylation (Fig. 4b, Supplementary Table S7). This correlation was even lower when we considered the methylation marks that were altered across the three generations (i.e. inherited) (Fig. 4c, Supplementary Table S8). Only 40 CGIs, corresponding to 24 genes, appeared commonly deregulated. These data suggest that germline-transmitted cytosine methylation does not seem to exert a direct impact on phenotypic variation. In support, the ontologies associated with the methylation marks in sperm-F0-F1 and liver-F2 corresponded to "Cell adhesion, Regulation of Transcription, Glucuronidation and Development" (Fig. 4d), whereas the ontology associated with the transcription was "Lipid Metabolic Process" (Fig. 4e). Furthermore, mRNA expression and DNA methylation in either tissue did not significantly correlate at all (Fig. 4f).

Given this "disconnection," we speculated that that sperm-worn methylation marks might influence F2 phenotypes through (i) impacting early embryonic development, and/or (ii) regulating

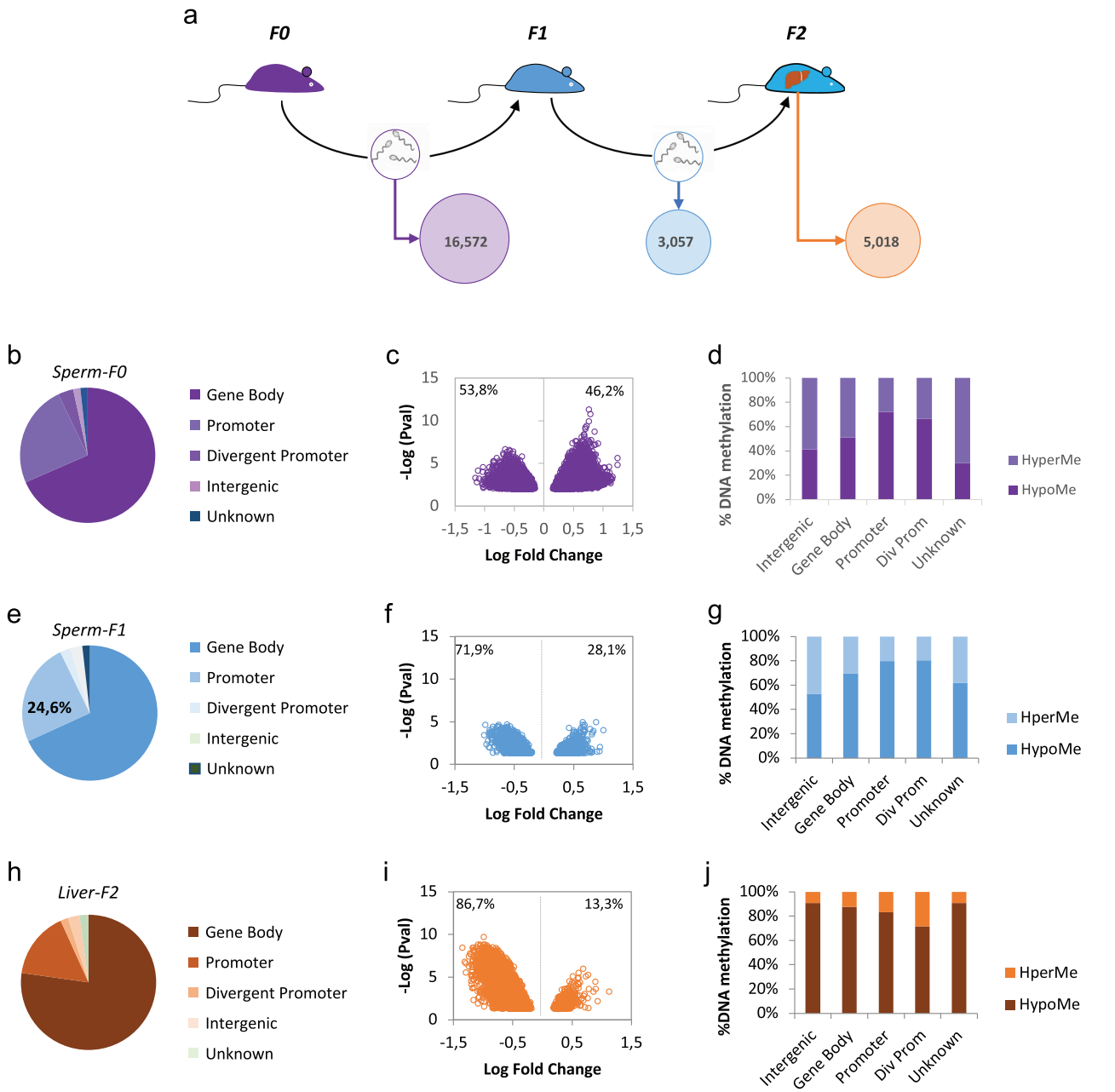

Figure 2. Litter size reduction leads to altered patterns of cytosine methylation in the sperm of the exposed individuals (SL-F0) and their offspring (SL-F1), and the liver of the grand offspring. (a) Number of CGIs that are differentially methylated in sperm of SL-F0 mice, sperm of SL-F1 mice, and liver of SL-F2 mice as compared to their matched controls. (b) Genomic distribution of the differentially methylated regions (F0). (c) Volcano plot. Distribution of the CGIs that are differentially methylated in the sperm of SL-F0 males. (d) Distribution of hypermethylated (Hyperme) and hypomethylated (Hypome) CGIs based on their genomic localization (F0). (e) Number of CGIs that are differentially methylated in sperm of SL-F1 mice, as compared to the C-F1. (f) Genomic distribution of the differentially methylated regions (F1). (g) Volcano plot. Distribution of the CGIs that are differentially methylated between hypermethylated and hypermethylated in the sperm of SL-F1 males. (h) Number of CGIs that are differentially methylated in the liver of SL-F2 mice, as compared to the controls. (i) Volcano plot. Distribution of the CGIs that are differentially methylated between hypermethylated and hypermethylated in the liver of SL-F2 males. (j) Genomic distribution of the differentially methylated regions (F2).

the expression of key upstream TFs that, secondarily, regulate the hepatic transcriptome. Hence, we analyzed the global gene expression profile in whole ED7.5 embryos and the liver of ED14.5 embryos of the second-generation offspring (Fig. 5a). In all, 452 and 612 genes were differentially expressed in ED7.5 and ED14.5 samples, respectively (Fig. 5a, Supplementary Tables S9, S10). Interestingly, the ontologies most significantly altered were, again, "Lipid Metabolic Processes" (Fig. 5b). Yet, despite this concordance, the genes contributing to these pathways were different at each stage (Fig. 5c). Furthermore, the correlation between DNA methylation and expression of these genes was zero (data not shown). Together, these data suggest that DNA methylation does not influence next generation offspring through global modulation of developmental pathways.

Since sperm DNA methylation does not globally regulate offspring transcriptome, we next explored whether a reduced number of specific TFs may play this role (Fig. 6a). Essentially, a small set of TFs may coordinately regulate a larger group of downstream

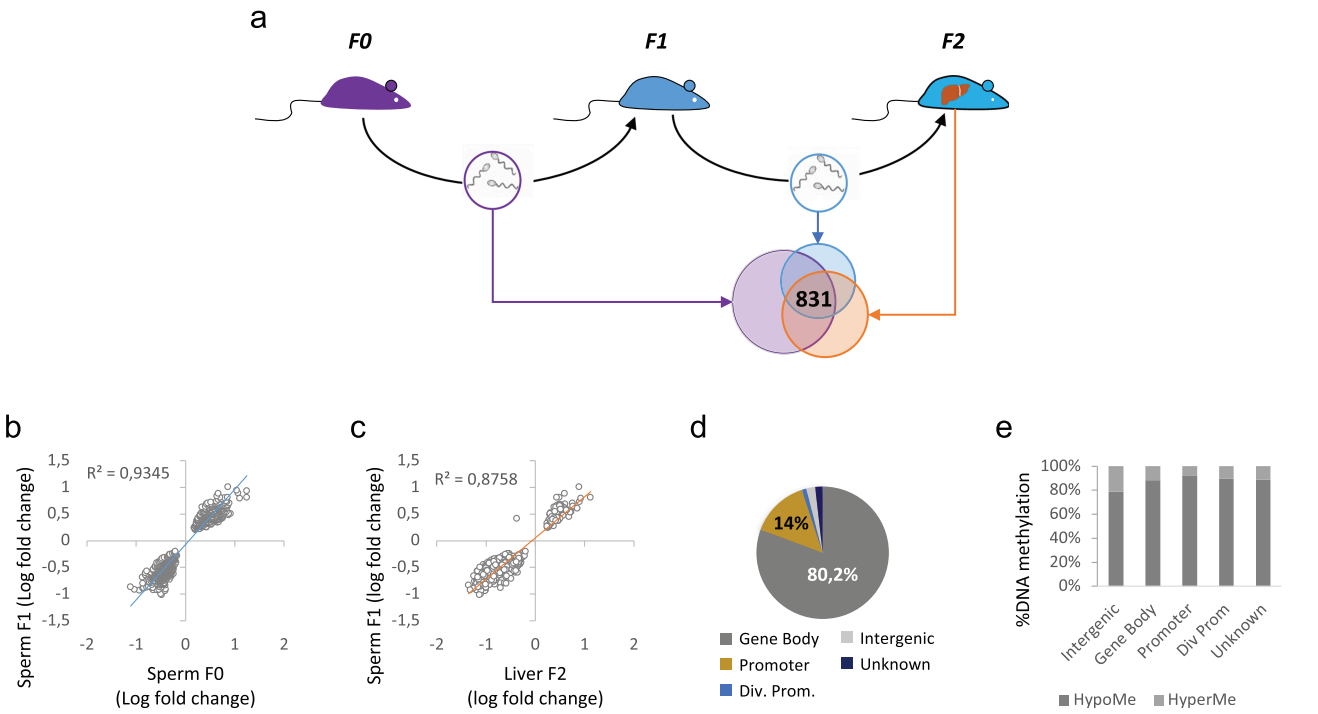

Figure 3. A total of 831 nutritionally induced methylation marks are transmitted (i.e. inherited) from the exposed mice to the F2. (a) Schematic representation that details the number of CGIs that appeared differentially methylated across the three generations (Venn diagram). (b) Correlation of cytosine methylation between sperm samples of F0 males and sperm samples of F1 mice. The axes represent the fold change (Log scale) between Control and SL mice. Positive values specify that SL samples gained methylation, compared to the controls, whereas negative values designate that SL samples are hypomethylated when compared to controls. (c) Correlation of cytosine methylation between liver samples of F2 males and sperm samples of F1 mice. (d) Distribution of the differentially methylated CGIs. (e) Distribution of hypermethylated and hypomethylated CGIs based on their genomic localization.

target genes that collectively are involved in specific pathways (Fig. 6b). We first identified a set of TFs that could potentially explain global changes in the transcriptome of ED7.5, ED14.5, and PD7.5 (Ingenuity Pathway Analysis, IPA; Qiagen). Specifically, 562, 204, and 258 putative upstream regulators appeared significantly enriched in ED7.5, ED14.5, and PD7.5 samples, respectively (Fig. 6c, Supplementary Tables S11, S12, S13). We selected TFs that, in addition, appeared differentially methylated in the sperm of F0-F1 males. We identified 15, 6, and 3 upstream TFs whose methylation was altered in the sperm of SL-F0/SL-F1 males (Fig. 6d, Supplementary Tables S14, S15, S16). Remarkably, many of them (*Runx3, Nfatc2, Gata3, Gata1, Bhlh40, Zfp36, Zfp36l1*) are known to be involved on hepatic steatosis [41–45]. However, their targets, that were differentially expressed in SL-F2 embryos, were not directly related with lipid metabolic pathways (Fig. 6e). Instead, they were associated with proliferation and differentiation, which are involved on developmental processes. Together, these data suggest that a small group of upstream TFs might be sufficient to ignite an amplification cascade that, indirectly, modifies the global patterns of hepatic-lipid gene expression.

Discussion

It is now widely recognized that environmentally induced phenotypes may be inherited through nongenetic mechanisms [22]. Forms of nongenetic inheritance include maternal physiology, parental behavior, cultural processes, or germline epigenetic modifications [2]. In turn, epigenetic mechanisms include DNA methylation, histone modifications, and a plethora of regulatory noncoding RNAs [46]. We have developed a mouse model of early adiposity (i.e. childhood obesity) through litter size reduction [35]. Mice reared in SL developed obesity, glucose intolerance, insulin resistance, and hepatic steatosis with ageing [37, 47]. Interestingly, insulin resistance and hepatic steatosis were transmitted into the first- and second-generation offspring via the paternal lineage [5], which strongly supports epigenetic inheritance. In our experimental setting, we can rule out other forms of nongenomic inheritance, including paternal/maternal behavior, or maternal physiology. Therefore, in our model, males can only contribute to the following generations through the information contained in the sperm, namely the epigenome [2].

Many studies [6, 7] have focused on paternal transmission from father to offspring, that is, from the F0 to F1. This is referred to as intergenerational inheritance [39]. Under this paradigm, when founders (F0) are exposed to an environmental challenge, the germline, which will give rise to the next generation offspring (F1), is also directly exposed. Therefore, transmission of phenotypes from F0 to F1 might be attributed not only to the epigenome but also to gamete dysfunction and/or the induction of novel mutations. However, transmission of phenotypes to the second-generation (F2) requires the involvement of the germline from F1 individuals, which have not been exposed to the environmental insult. Hence, transmission of phenotypes up to the F2 implies epigenetic reprogramming of the germline, which is referred to as transgenerational inheritance [39].

Here we explored the role of DNA methylation at CGIs in mediating the inheritance of the metabolic phenotypes. The rationale is that nutritional imbalances, including high-fat feeding, neonatal overnutrition, or fetal undernutrition, may alter sperm

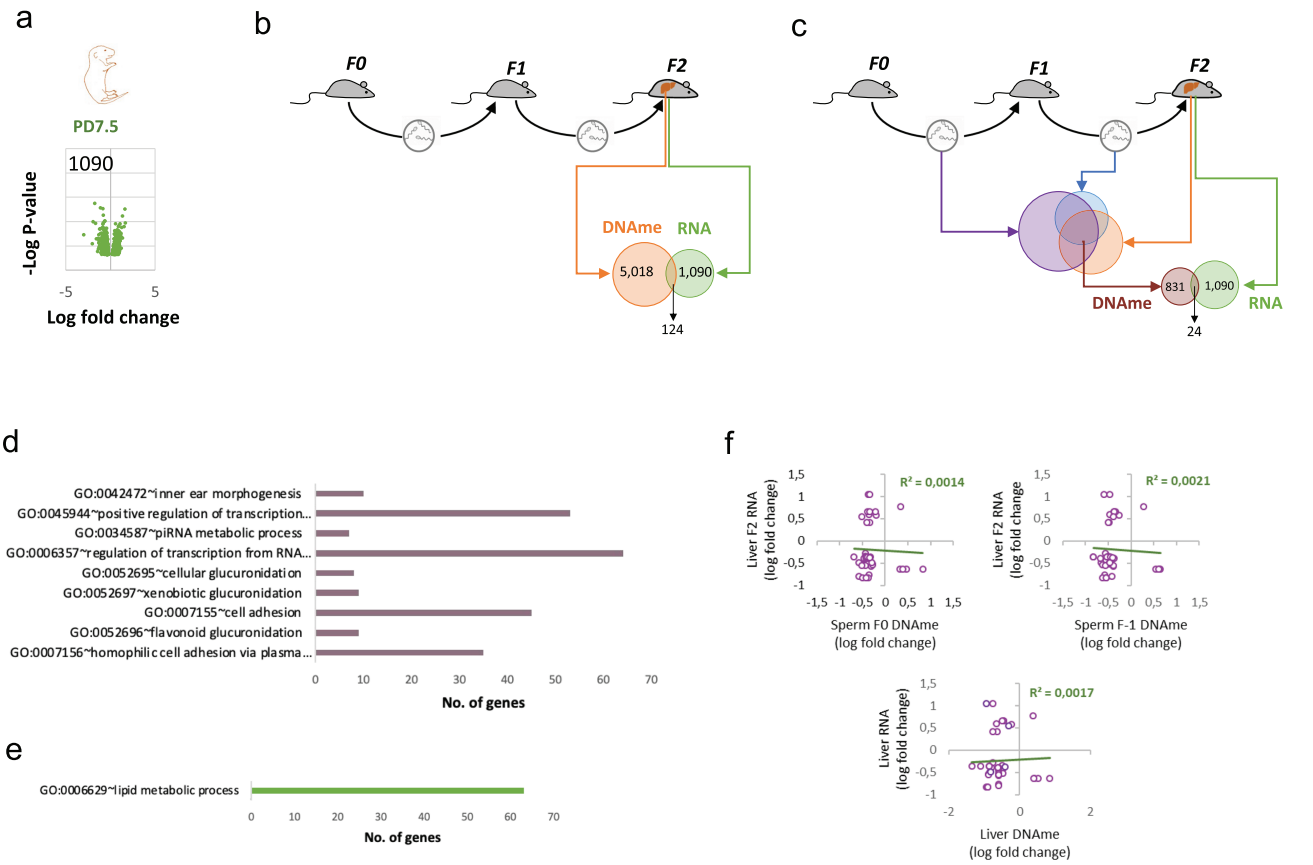

Figure 4. Global gene expression is altered in the liver of the grand offspring. (a) volcano plot. Gene expression profile in the liver of SL-F2 neonates (PD7.5) compared to C-F2 neonates. (b) Venn diagram: intersection between regions differentially methylated and differentially expressed in the liver of 7.5 mice. (c) Venn diagram: Intersection between differentially methylated CGIs that are potentially inherited across three generations and liver transcriptome (F2). (d) Gene ontologies associated with DNA methylation changes. (e) Gene ontologies associated with transcriptomic changes. (f) Correlation between gene expression (liver-F2) and DNA methylation at sperm-F0, sperm-F1, and liver-F2.

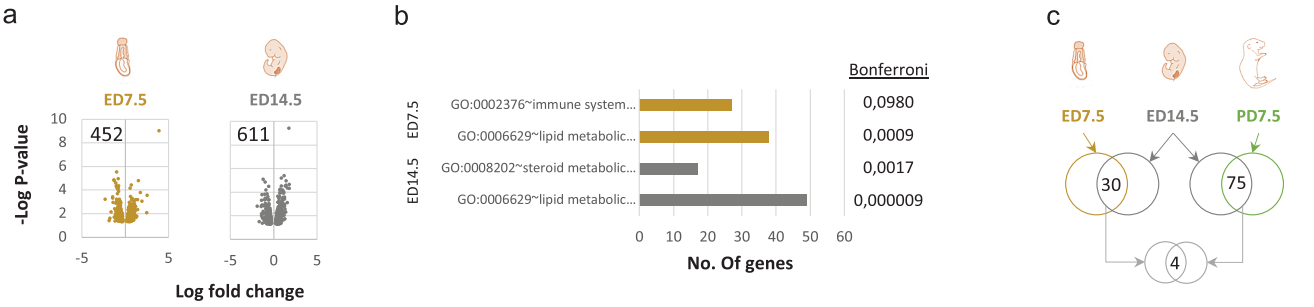

Figure 5. DNA methylation profile does globally not correlate with gene expression changes. (a) Volcano plot. Gene expression profile in ED7.5 whole embryos and the liver of ED14.5 embryos (F2). (b) Gene ontologies associated with differential gene expression between C-F2 and SL-F2 embryos at ED7.5 and Ed14.5. (c) Venn diagram showing the number of genes that are differentially expressed across three developmental stages, including ED7.5, ED14.5, and PD7.5.

DNA methylation at CGIs [48]. However, the involvement of these methylation marks in the inheritance of metabolic diseases remains unclear. The main concern is that several studies failed to demonstrate that sperm-DNA methylation profoundly influences the expression of their target genes in the offspring and/or grand offspring [24, 33]. Here we aimed to address this issue through careful analysis of (I) cytosine methylation profile in the sperm of carriers (F0 and F1) and target tissues of the grand offspring (liver), and (II) gene expression profile in the liver of the grand offspring (F2).

First, we confirmed that litter size reduction massively reprogrammed sperm DNA methylation in SL-F0 mice. In agreement, several studies have reported that paternal obesity, diabetes, exercise, and nutritional challenges (high fat diet, protein/folate deficiencies) modify the sperm epigenome of the exposed individuals [23, 25, 38, 49]. These data confirm that the germline epigenome is extremely sensitive to environmental cues, including nutritional challenges. Second, we found that DNA methylation profile in the sperm of SL-F0 mice was largely recapitulated in the sperm of the offspring (SL-F1) and, more importantly, the liver of the grand

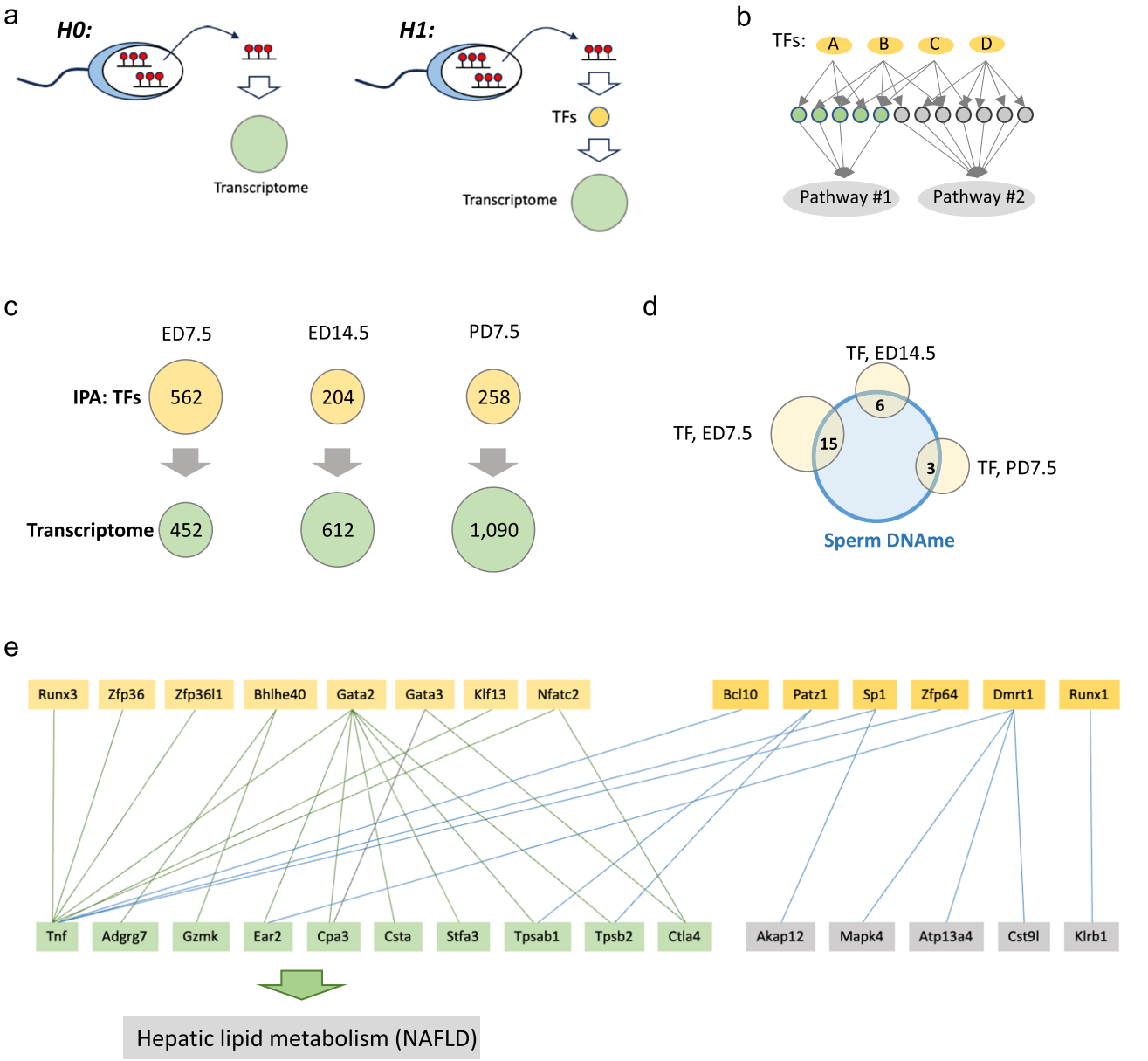

Figure 6. A few TFs may modify the global pattern of gene expression. (a) Alternative hypotheses addressing how sperm DNA methylation mya regulate offspring gene expression. Null hypothesis (H0) proposes that sperm DNA methylation may influence global gene expression profile. Our previous data does not support H0. The alternative hypothesis (H1) proposes that sperm DNA methylation may influence a small number of TFs that, in turn, regulate offspring transcriptome. (b) Theoretical scheme representing how a small set of TFs (yellow dots) may regulate a larger dataset (green and grey dots) that are involved in specific pathways (grey). (c) Following the previous scheme, we identified a large dataset of TFs (IPA; yellow circles) that may potentially regulate transcription in ED7.5 embryos, the liver of ED14.5 embryos, and the liver of PD7.5 neonates (blue circles). (d) Number of TFs (identified via IPA) that, in addition, were differentially methylated in the sperm of SL-F1 males. (e) Connectome. The 14 TFs that appeared differentially methylated at ED7.5 and their differentially expressed downstream targets are presented. The targets colored in green have been related to hepatic steatosis.

offspring (SL-F2). This transgenerational line of continuity, from F0-to-F1-to-F2, strongly suggests that DNA methylation may be inherited across generations. Therefore, we addressed whether these (potentially) inherited methyl-marks were also carriers of phenotypic information across generations. To this end, we identified the methylation marks that were differentially methylated in all three generations and correlated them with the hepatic transcriptome in 7.5-day-old mice. We selected this developmental stage because 7.5-day-old mice do not show metabolic derangements [33, 46]. Studying this issue in adults is challenging because, instead of being inherited, modifications in cytosine methylation and gene expression may arise secondary to the metabolic deregulation that SL-F2 mice acquire with ageing.

We found that the overlapping between the inherited methylation marks and the transcriptome was rather poor. Indeed, the ontologies associated with cytosine methylation corresponded to "Development and Regulation of Transcription," whereas the ontologies associated with the transcriptome were associated with "Lipid Metabolism." We can conclude that inherited global changes in DNA methylation are not associated with the global

transcriptome. Others have also found that changes in sperm DNA methylation are inherited, but do not carry functional consequences because the methylation marks do not directly influence the expression of target genes in metabolic tissues [24, 33, 49]. Collectively, these data put into question whether DNA methylation plays a true essential role in mediating transgenerational inheritance of phenotypes, at least in the context of obesity and nutritionally-induced phenotypes [24, 33, 46, 50].

As an alternative possibility, we explored whether DNA methylation might influence offspring's phenotypes through indirect mechanisms. We considered two possibilities. Firstly, the methyl marks being transmitted via the sperm might influence the expression of developmental genes during early embryonic/fetal growth. Indeed, it has been postulated that sperm-borne small noncoding RNAs influence offspring physiology through modifying embryonic development [20, 26–28]. Hence, we analyzed the transcriptome in ED7.5 embryos, and the liver of ED14.5 fetuses. At these two stages, no ontology terms were associated with developmental processes. Instead, the ontologies associated with lipid metabolic process were already significantly deregulated during embryogenesis. This data led us to conclude that sperm methyl-marks do not seem to mediate the transmission of metabolic phenotypes through global modulation of embryonic and/or fetal development. Instead, hepatic lipid metabolism is already primed in F2 embryos, suggesting that it constitutes a driver of the disease rather than a consequence of other ageing factors.

Secondly, we explored whether the sperm-derived methyl marks might influence the expression of specific TFs that, through an amplification process, influence large transcriptional datasets related to hepatic lipid metabolism. Here we do provide evidence that a reduced set of TFs may participate in regulating genes associated with lipid metabolism, from embryonic Day 7.5 (ED7.5) to postnatal Day 7.5 (PD7.5). We identified 14 TFs in which cytosine methylation was altered in sperm and were able to regulate the expression of target genes in ED7.5 and ED14.5 embryos. Some of them are known to be involved in the development of non-alcoholic fatty liver disease. For example, *Bhlhe40, Zfp36l1*, and *Zfp36* have been involved in the development of alcoholic fatty liver disease in rodents [41–44]. *Klf3* plays an important role in triglyceride (TAG) accumulation through regulating lipoprotein assembly, secretion, and fatty acid oxidation, at least in *Caenorhabditis elegans* [45]. Others, including *Runx3*, *Nfatc2*, or *Gata3*, are potentially good biomarkers for the diagnosis of NAFLD (Non-Alcoholic Fatty Liver Disease) and Non-Alcoholic SteatoHepatitis [51–53]. While the involvement of these TFs in the development of NAFLD in our model is plausible, this possibility deserves further investigation.

Taking together the previous results, we conclude that only a small number of upstream TFs, which exhibit altered DNA methylation profiles, may possibly influence a vast array of genes secondarily, as development proceeds, through an amplification cascade. In support, we had previously described an example in which the methylation of the TF *Lxra* might drive, in part, hepatic lipid metabolism in the offspring of low-birth-weight male mice [6]. Yet, we recognize that our study has some limitations. An alternative possibility that we have not tested here would be that DNA methylation might impact on noncoding RNAs (i.e. miRNAs or similar) that secondarily regulate the transcriptome. Unfortunately, we cannot test this possibility with our current approach. Here we aimed at detecting changes in DNA methylation in regions that are associated with the coding transcriptome, including promoter and intragenic regions. Elucidating the potential involvement of sperm DNA methylation on noncoding regions would require bisulfite conversion followed by whole genome sequencing in parallel with the sequencing of the noncoding RNA. The interrelation between DNA methylation and noncoding regulation is a possibility that deserves future studies.

In conclusion, here we provide evidence that sperm DNA methylation is a plausible carrier of environmentally induced information across generations. Specifically, we found that early overfeeding and/or childhood obesity-related dysfunction dramatically alters global DNA methylation profile in the male germline. A significant fraction of these methylation marks (8%) is still present in the sperm of the offspring and, strikingly, the somatic tissue of the grand offspring. This line of continuity strongly supports true epigenetic inheritance. Finally, most differentially methylated CGIs do not seem to influence the expression of the associated genes in somatic tissues of the offspring. Instead, cytosine methylation of a few key TFs might be inherited and influence, through an amplification cascade, a set of genes involved in lipid metabolism in the liver of SL-F2 mice.

Material and methods

Animal procedures and experimental design

All procedures have been approved by the Committee of Animal Experimentation from the University of Barcelona and the Conselleria de Ramaderia i Pesca de la Generalitat de Catalunya. Mouse strain ICR (ICR-CD1; Envigo, Spain) was chosen for this study. Eight-week-old virgin females were mated with 8-week-old males. Mice were housed on a 12-h light-dark cycle with free access to food and water. At delivery day, cohorts were adjusted to eight male pups for the control group (C), and to four male pups for the over nutrition or small litter size group (SL). Pups from both groups were breastfed freely by the dam. This constituted the parental generation (F0). In the successive generations, 8-week-old males from F0 generation were mated with 8-week-old virgin females for both groups to form the F1 offspring generation. To obtain the F2 offspring generation, we mated 8-week-old F1 males with 8- week-old virgin females. All cohorts (control and SL) were adjusted to eight male pups, to constitute the F1 and F2 offspring generations. Weaning of the pups was done at 3 weeks of age. They were housed in cages, with six animals/group, maintaining separation between control and SL groups. They had free access to water and food ad libitum 2014 Teklad diet (Envigo, Spain).

In vivo metabolic testing

The intraperitoneal glucose tolerance test (ipGTT, 1.5 g/kg) was performed on unrestrained conscious mice after an overnight-fast. Blood glucose was monitored with a Glucometer Elite (Menarini, Barcelona, Spain) at 0 min, 15 min, 30 min, 90 min, and 120 min after glucose injection. Glucose tolerance was calculated as the area under the glucose curve (AUC).

Insulin sensitivity was determined by HOMA-IR, as previously described [5]. HOMA was calculated by using fasting glucose and fasting insulin as follows: HOMA-IR = Glucose * Insulin/405, where glucose is given in mg/dl and insulin is given in μU/ml. Insulin was measured by ELISA in 5 μl of plasma (Crystal Chem-Europe, the Netherlands).

Tissue sample collection

Adult male mice were euthanized (CO_2 overdose) at 20 weeks of age after being mated with control dams, and sperm, liver, epididymal white adipose tissue (eWAT) were retrieved. Tissues were snap frozen in liquid nitrogen and stored at –80°C until analyzed. Epididymal fat mass content was calculated as a percentage of wet tissue per whole body weight.

Triglyceride liver quantification

Triglyceride content was assessed by following the Bligh and Dyer method [54], as previously described [47]. Briefly, 50 mg of frozen liver were homogenized, and lipids were extracted through an overnight incubation in a methanol-chloroform (1:2, v/v) solution. TAG content was then quantified through colorimetric method by using a Triglyceride Quantification kit (BioSystems, Barcelona, Spain) following the manufacturer's instructions.

Sperm isolation

Cauda epididymis and ductus deferens were dissected, cut into several small pieces and incubated in Hepes buffered Ham's F10 medium (Gibco), supplemented with sodium bicarbonate, sodium pyruvate, and sodium lactate. Tissues were incubated at 37°C for 15 min with gentle agitation (80 rpm) to assist with sperm extraction. Epididymal tissue was filtered out using a 30-μm disposable filter (Cell Trics).

Finally, the homogenate was layered on top of a Percoll density gradient (40%:80%; GE Healthcare, reference 17-0897-02) and centrifuged at 400× *g* for 30 min at room temperature. The resulting sperm pellets were washed and resuspended in fresh medium, and the final concentration was determined prior to freezing.

DNA and RNA extraction

Genomic DNA from tissues was extracted using the Wizard® Genomic DNA Purification Systems Kit (Promega Biotech Ibérica S.L., Madrid, Spain). Sperm DNA was isolated by using the DNeasy Blood & Tissue Kit (Izasa-Qiagen, Barcelona, Spain). Total RNA was isolated by using TriReagent (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's protocol.

Affymetrix microarrays

Microarray hybridization and analysis have been performed as previously described, using Affymetrix Clariom S Mouse Arrays® (Thermo Fisher Scientific, Barcelona, Spain). Briefly, we hybridized four samples for each group (C, SL) on each developmental stage (ED7.5, ED14.5, PD7.5). Expression values were summarized after background correction and normalization steps using the RMA methodology [55]. Differential expression analysis was performed by the nonparametric approach Rank Prod [56]. Oligonucleotides presenting changes between groups with q-values lower than 0.05 were considered significant. The software tool David [57] was used for the calculation of the functional clustering enrichment statistical analysis of the Gene Ontology Terms and Kegg Pathways databases considering the list of significant genes. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [58] and are accessible through GEO Series accession number GSE286077 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE286077).

Global DNA methylation profile

Global DNA methylation profile was assessed using the Agilent Mouse DNA Methylation Microarray (Agilent Technologies, Santa Clara, CA, USA). Microarrays were scanned, using Agilent Microarray Scanner G2505 with a resolution of 2-μm. The resulting .tif images were processed with the Agilent Feature Extraction 11.0.1.1 and Agilent Workbench 6.5.0.18 software, according to the manufacturer's instructions. These software products gave the position of the CpG island in the gene structure: promoter, intragenic, downstream, divergent promoter.

DNA methylation was determined via MedIP. Briefly, 500 ng genomic DNA was divided into two fractions. One of them (250 ng) was subjected to immunoprecipitation with anticytosine antibodies. The DNA libraries of immunoprecipitated and nonprecipitated samples were labeled (Cy3 and Cy5, respectively) and hybridized onto Agilent 105 K Mouse CpG Island microarrays (ID 015279). Before microarray data analysis, outliers and low signal intensity within 2.6 standard deviations of background were removed (Feature Extraction software v.10.7, Agilent Technologies, Santa Clara, CA, USA). After normalization, sample DNA methylation and detection were performed by using the Agilent Genomic Workbench, which provides the algorithms for methylation detection. Here, for measuring the degree of enrichment (or de-enrichment), we used the the Bayesian tool for methylation analysis [59], which enables the estimation of absolute methylation levels from immunoprecipitation-based DNA methylation profiles. This parameter can have the following values: −1 (hypomethylation), 1 (hypermethylation), or 0 (uninterpretable) [59]. The methylation status is based on the percentage of methylated probes in the island. Genes with uninterpretable results were excluded from the analysis.

The datasets included here have been deposited in NCBI's Gene Expression Omnibus [58] and are accessible through GEO Series accession number GSE286036 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE286036).

Ingenuity pathway analysis

Ingenuity pathway analysis (IPA, Qiagen) was used to explore and compare the gene expression differences between the SL and Control liver samples at each developmental embryo stage. When uploading the dataset of each list of genes, the "Expr log Ratio" option was chosen. We used the comparison analysis function in IPA to find the upstream regulators. Networks were generated to visualize the canonical pathways.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using a two-tailed *t* test or a one-way analysis of variance (ANOVA) as indicated (IBM SPSS Statistics 19, Madrid, Spain). A *P* value <.05 was considered significant.

Supplementary data

Supplementary data is available at *EnvEpig* online.

Conflict of interest: None declared.

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Data availability

The datasets included in this project have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE286036 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE286036) and GSE286077 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE286077).

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