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Reduced PPAR γ 2 expression in adipose tissue of male rat offspring from obese dams is associated with epigenetic modifications

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Short title: epigenetic modifications and PPAR γ 2 programming

Abbreviations

AdipoQ:	adiponectin
ACOX1:	Peroxisomal acyl-coenzyme A oxidase 1
CD36:	fatty acid translocase
ChIP:	Chromatin immunoprecipitation
C/EBP α :	CCAAT/enhancer binding protein alpha
DNMT:	DNA methyltransferase
DOHaD:	Developmental Origin of Health and Disease
DGAT2:	diglyceride acyltransferase
FABP4:	fatty acid binding protein 4
FAS:	fatty acid synthase
GC:	glucocorticoid
GLUT4:	Glucose transporter type 4
HAT:	histone acetyltransferase
HDAC:	histone deacetylase
KAT2:	lysine acetyltransferase 2A
LPL:	lipoprotein lipase
MBD:	methyl-binding domain
5hmC:	5-hydroxymethylcytosine
5mC:	5-methylcytosine
MECP2:	methyl CpG binding protein 2
P300:	histone acetyltransferase P300
PGC1- α :	PPAR γ coactivator 1- α
PND:	postnatal days
PPAR:	peroxisome proliferator-activated receptor
SIRT1:	sirtuine 1
SREBP1:	sterol regulatory element-binding protein 1
TET:	Ten-eleven translocation methylcytosine dioxygenase
WAT:	white adipose tissue
iWAT:	subcutaneous inguinal
pWAT:	visceral perirenal
SVF:	stromal vascular fraction

Abstract

According to the Developmental Origin of Health and Disease (DOHaD) concept, maternal obesity and accelerated growth in neonates program obesity later in life. White adipose tissue (WAT) has been the focus of developmental programming events, although underlying mechanisms remain elusive. In rodents, WAT development primarily occurs during lactation. We previously reported that adult rat offspring from high-fat diet-fed dams (called HF) showed fat accumulation and decreased PPAR γ mRNA levels in WAT. We hypothesized that PPAR γ downregulation occurs via epigenetic malprogramming which takes place during adipogenesis. We therefore examined epigenetic modifications in the PPAR γ 1 and PPAR γ 2 promoters in perirenal (pWAT) and inguinal (iWAT) fat pads of HF offspring at weaning (postnatal days 21 (PND21) and in adulthood. PND21 is a period characterized by active epigenomic remodeling in the PPAR γ 2 promoter (DNA hypermethylation and depletion in active histone modification H3ac and H3K4me3) in pWAT, consistent with increased DNMT and DNA methylation activities. Adult HF offspring exhibited sustained hypermethylation and histone modification H3ac of the PPAR γ 2 promoter in both deposits, correlated with persistent decreased PPAR γ 2 mRNA levels. Consistent with the DOHaD hypothesis, retained epigenetic marks provide a mechanistic basis for the cellular “memory” linking maternal obesity to a predisposition for later adiposity.

Key words: perinatal programming, Developmental Origin of Health and Disease, epigenetic mechanisms, gene expression, fat expansion.

89 **Introduction**

90   Epidemiological studies demonstrated that adult-onset metabolic disorders may
91 originate from events taking place during fetal and postnatal development (1). These
92 observations led to the Developmental Origin of Health and Disease (DOHaD) hypothesis
93 also called “developmental programming” (1). In particular, maternal obesity and accelerated
94 growth in neonates program obesity later in life. Numerous studies confirmed that maternal
95 obesity is correlated with increased offspring's adiposity in rodents (2–7) and in human
96 neonates (8). Thus, white adipose tissue (WAT) has been the focus of developmental
97 programming events in a sex- and depot-specific manner (2). In rodents, adipose tissue
98 development occurs mainly during lactation (9–11), although adipose tissue remains to some
99 extent expandable throughout life (12, 13). Adipocyte progenitors are highly sensitive to
100 maternal factors. Suboptimal nutritional and hormonal milieu during the perinatal period may
101 affect adipogenesis, resulting in modified growth and persistent changes in the structure and
102 function of WAT (14–16).

103   However, the mechanisms underlying developmental programming are poorly
104 understood. Because the consequences of the events occurring in early life are maintained
105 throughout adult life, epigenetic modifications are likely to play an important role in this
106 process (17). Epigenetics is defined as heritable changes in gene expression that do not
107 involve changes to the underlying DNA sequence. Epigenetics relate to chromatin
108 modifications, including DNA methylation and histone modifications (18). DNA methylation
109 results from the transfer by DNA methyltransferase (DNMT) of a methyl group to the
110 cytosine residue within CpG dinucleotides to form 5-methylcytosine (5mC) and serves to
111 establish gene silencing (19). 5mC both positively and negatively modulates binding of
112 numerous DNA binding proteins including methyl-binding domain (MBD) proteins (MBD2,
113 MeCP2). MBD can recruit protein and chromatin remodeling complexes known to promote

the formation of a condensed chromatin structure (20). 5-hydroxymethylcytosine (5hmC) is another important cytosine modification catalyzed by the enzymes of the Ten-eleven translocation methylcytosine dioxygenase (TET) family that serves as an intermediate for demethylation. It can also promote chromatin opening at transcriptional regulatory regions (21). Chromatin structure and accessibility are also controlled by histone posttranslational modifications (18). For example, acetylation of histone H3 lysine residues (H3ac) and methylation of H3K4 (H3K4me3) are associated with active transcription while methylation of H3K9 (H3K9me3) generally indicates silenced chromatin (18). These histone posttranslational modifications are catalyzed by various enzymes including histone acetyltransferase (HAT) such as KAT2 and P300 as well as histone deacetylase (HDAC) such as SIRT1. Histone-modifying enzyme activity is dependent on intermediary metabolites and hormonal responses (22). Editing of epigenetic marks during critical developmental windows in early life may serve as a memory of exposure to potential environment insults (1). The persistence of these marks throughout life may program permanent changes in gene expression (1).

We previously showed that adult male rat offspring from high-fat diet-fed dams (called HF) displayed higher adiposity (2), hyperleptinemia and elevated leptin gene expression in WAT (25). Persistent leptin upregulation occurs via epigenetic malprogramming during lactation in a depot-specific manner (23). We also showed that adult HF offspring exhibited a decrease in PPAR γ gene expression in perirenal (pWAT) fat pad (2). PPAR γ is considered the master regulator of adipose tissue promoting all processes involved in adipocyte function and WAT expansion. PPAR γ is present in two isoforms, PPAR γ 1 and PPAR γ 2, generated by alternative promoter usage (24). PPAR γ 1 is expressed at low level in many cell types including cells from stromal vascular fraction (SVF) of adipose tissue, whereas the expression of PPAR γ 2 is mainly restricted to adipocyte (24). Although several studies reported

dysregulation of PPAR γ expression in offspring from obese dams (3–5, 7, 25, 26), scarce data is available about programming mechanisms.

Here, we hypothesized that maternal obesity programs persistent PPAR γ downregulation in HF offspring via epigenetic mechanisms malprogramming which take places during WAT development. Thus, we examined epigenetic modifications (i.e., DNA and histone modifications) focusing on both PPAR γ promoters as well as activity and expression of chromatin modifying enzymes in pWAT and inguinal (iWAT) fat pads of HF offspring at weaning and 9 months of age.

Materials and methods

Animals

Animal use accreditation by the French Ministry of Agriculture (No. 04860) has been granted to our laboratory for experimentation with rats. Experiments were conducted in accordance with the principles of laboratory animal care (European Communities Council Directive of 1986, 86/609/EEC) as previously described (2, 25). One-month-old virgin female Wistar rats were purchased from Charles River Laboratories (L'Arbresle, France) and were housed in individual cages in a humidity-controlled room with a 12:12-h light-dark cycle. Food and water were available *ad libitum*. After two weeks of acclimatization on a control (C) diet (3.85 kcal/g with 10% of total calories as fat consisting of soybean oil (5.6%) and lard (4.4%), 70% as carbohydrate and 20% as protein; D12450J, Research Diets, New Brunswick, NJ, USA), female rats were fed either a high-fat (HF) diet (5.24 kcal/g with 60% of total calories as fat consisting of soybean oil (5.6%) and lard (54.4%), 20% as carbohydrate and 20% as protein; D12492B, Research Diets, New Brunswick, NJ, USA) or a C diet for 16 weeks (n=30 per group). After mating with a male rat fed a C diet, 22-week-old pregnant

females were transferred into individual cages with free access to water and to their respective diets (C or HF diet) throughout gestation and lactation. At parturition, pups were weighed and sexed. Litter size was adjusted to 8 pups per dam (four males and four females). Body weight of pups was assessed on postnatal days 21 (PND21). Only male offspring have been used for further analysis. After weaning, male offspring from C or HF dams were housed individually with free access to water and C diet. Body weight of animals was measured weekly until 9 months of age. Male offspring were sacrificed at 2 different stages: PND21 and 9 months of age (n=10 from C or HF dams). To obviate any litter effects, animals used for each experiment were randomly chosen in different litters and only a limited number of animals (n= 1 to 2) was used from each litter.

Plasma and tissue collections

21-day-old male neonates were separated from their dams and 9-month-old (9M) male rats were fasted 16 hours before they were sacrificed by decapitation (between 9 and 10 a.m.). Trunk blood samples were collected into prechilled tubes containing EDTA (20 µl of a 5% solution) and centrifuged at 4000 g for 10 min at 4°C. Plasma was stored at -20°C. Fat pads were weighed, frozen in liquid nitrogen and stored at -80°C.

Quantification of endocrine parameters

Plasma hormone levels were evaluated in PND21 and 9M male offspring. Blood glucose was determined using a glucometer (Glucotrend 2; Roche Diagnostics, France). Plasma corticosterone levels were determined by a competitive enzyme immunoassay (Immunodiagnostic Systems Ltd, Boldon, U.K). Plasma insulin concentrations were measured by ELISA (DRG, Internatlukaional, Inc. USA). The assay sensitivity was 0.07 ng/ml (insulin), 0.55 ng/ml (corticosterone) and the intra-and inter-assay coefficients of variation

were 4% and 9.1% (insulin), 4.9% and 7.8% (corticosterone), respectively. Assay kits were applied to determine the contents of plasma triglycerides (61238 Triglyceride Enzymatique PAP100).

Western blot analysis

Immunoblotting analysis were carried out as described previously (23). Monoclonal mouse antibody against PPAR γ (catalogue no. sc-7273) was purchased from Santa Cruz Biotech (Dallas, TX, USA) and was diluted 1:200. Actin antibody (catalogue no. A2228) was purchased from Sigma Aldrich (Saint Quentin Fallavier, France) and was diluted 1:1000. Anti-mouse IgG, HRP-linked Antibody (catalogue no. 7076) secondary antibodies were purchased from Cell Signalling (Danvers, MA, USA) and were diluted 1:10,000.

Pyrosequencing analysis

Genomic DNA was extracted from frozen WAT using a DNA extraction kit (DNeasy blood and tissue kit, Qiagen, Courtaboeuf, France) and modified with sodium bisulfite using the EpiTect Fast Bisulfite Conversion kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. The percentage of cytosine methylation was determined by pyrosequencing bisulfite converted DNA using Pyro-Mark Q24 (Qiagen, Courtaboeuf, France). Pyrosequencing primers were designed to amplify PPAR γ 1 and PPAR γ 2 CpG sites (Table 1). PCR was performed with 20 μ l final reaction volumes with 1.5 ml of bisulfite DNA (10 ng), 10 μ l of QuantiTect EurobioGreen PCR Mix Hi-ROX (Eurobio, Les Ulis, France), 7.7 μ l of H₂O, and 0.4 μ l of each primer set (10 mM). Sequencing reactions used 10 μ l of PCR product and 20 μ l of 0.375 mM sequencing primer (Qiagen, Courtaboeuf, France). Pyrosequencing assays were validated with a DNA methylation scale (0%, 5%, 25%, 50%,

75% and 100%) (EpigenDX, Hopkinton, USA). Each assay included a bisulfite conversion check to verify full conversion of the DNA.

Chromatin immunoprecipitation

ChIP assays were performed as previously described (27) with antibodies against H3K4me3, H3ac and H3K9me3 (Abcam, Cambridge, UK, ab8580, ab47915 and ab1773) and IgG (Cell Signaling Technology, Danvers, USA, #2729) as a negative control. H3ac, H3K4me3 and H3K9me3 levels were analyzed using immunoprecipitated and input DNA as control.

RNA isolation and real-time PCR

PPAR γ isoforms (PPAR γ 1, PPAR γ 2), PPAR γ targets (C/EBP α , SREBP1, FAS, DGAT2, LPL, CD36, FABP4, GLUT4, ACOX1, AdipoQ, Resistin), enzymes of the epigenetic machinery (DNMT1, MBD2, MECP2, TET2, TET3, KAT2, P300, HDAC1, SIRT1, LSD1, SUV39) mRNA levels were determined by RT-qPCR using SyberGreen-based chemistry as previously described (2). Cyclophilin A (PpiA) and Ribosomal Protein Lateral Stalk Subunit P1 (RPLP1) were used as reference genes for RT-qPCR. Primer sequences are listed in Table 1.

Global DNA methylation and hydroxymethylation

Relative levels of global DNA methylation were assessed by me-CpG ELISA using the MethylFlash^{TM} Methylated DNA Quantification Kit (Cat # P-1034; Epigentek, Mundolsheim, France). Global DNA hydroxymethylation were measured using the Quest 5-hmC^{TM} DNA ELISA Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's

instructions. The amount of DNA methylation and DNA hydroxymethylation was calculated based on a standard curve generated using the kit controls.

Enzyme activity

DNMT, TET, HAT and HDAC activities were studied using the EpiQuick DNMT Assay kit (Cat # P-3001-2; Epigentek, Mundolsheim, France), TET Hydroxylase Activity Quantification kit (Cat # ab156912; Abcam, Cambridge, UK), EpiQuick HAT Assay kit (Cat # P-4003-96; Epigentek, Mundolsheim, France) and EpiQuick HDAT Assay kit (Cat # P-4002-96; Epigentek, Mundolsheim, France). These assays were performed according to manufacturer's instructions.

Statistical analysis

All data are expressed as means $\pm$ standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism6. A direct comparison between a pair of groups was made using an unpaired Student's t test. *P* values < 0.05 was considered statistically significant.

Results

Effects of maternal obesity on HF offspring metabolic parameters

Given that metabolic signals including hormonal signaling pathways play critical roles in determining chromatin structure (28), we assessed the plasma metabolic profile in HF offspring. We previously reported that weaned and adult HF offspring exhibited hyperleptinemia (2, 23). Here, we show that serum triacylglycerol and insulin levels were elevated in HF male offspring at PND21 (Table 2). At 9 months of age, HF male offspring

still displayed hyperinsulinemia and had hypercorticosteronemia (2). No difference in plasma glucose concentration was observed at both stages (Table 2, (2)).

Offspring from obese dams show persistent decreased PPAR γ expression levels in adipose tissue

At PND21, HF offspring showed a decrease in PPAR γ mRNA and protein expression in pWAT and iWAT, consistent with global reduction in PPAR γ 1 and PPAR γ 2 expression (Fig 1A-D). At 9M, among PPAR γ 1 and PPAR γ 2, only the latter showed a persistent downregulation in both fat pads. The impact of maternal obesity on HF offspring's PPAR γ target genes was more pronounced in pWAT at both stages, suggesting a depot-specific programming of adipogenesis and lipogenesis (Fig 1E, F).

Persistent lower PPAR γ expression is associated with CpG hypermethylation in the PPAR γ 2 promoter of HF offspring

We first assessed whether maternal obesity affects CpG methylation status of the PPAR γ 1 and PPAR γ 2 proximal promoter regions (Fig 2A) in offspring. To do this, we analyzed single CpG sites by bisulfite pyrosequencing. For the PPAR γ 1 promoter, at PND21, higher 5mC levels with hypermethylation at two out of seven CpG sites analyzed (-364/-346) were observed in pWAT of HF offspring (Fig 2B). Only hypermethylation of CpG -346 persisted in HF offspring at 9 months of age (Fig 2B). No variation in DNA methylation levels were detected in iWAT (Fig 2C). For the PPAR γ 2 promoter, higher 5mC levels were measured in both fat pads and stages in HF offspring. Two out of four CpG dinucleotides (-215/-199) were similarly affected by maternal obesity. Both CpG were hypermethylated at PND21 whereas only the CpG -199 remained hypermethylated at 9M (Fig 2D and E).

Consistently, CpG hypermethylation were correlated with PPAR γ mRNA levels at PND21 and 9M in both deposits.

Weaned offspring from obese dams display higher DNMT activity and global DNA methylation in pWAT

We then assessed whether maternal obesity affects the epigenetic machinery activity in offspring. At PND21, DNMT activity (Fig 2F) and global DNA methylation (Fig 2G) were higher in pWAT, consistent with the CpG hypermethylation observed in the PPAR γ promoters of HF offspring. No variations in TET activity (Fig 2J) and global hydroxymethylation (Fig 2K) were observed. No changes of DNMT1, MBD2, MECP2, TET2 and TET3 mRNA levels were detected (Fig S1). No gross modifications were observed in iWAT at PND21 and in both fat pads at 9M for all parameters (Fig S1).

Persistent lower PPAR γ expression is associated with depletion in H3ac/H3K4me3 in the PPAR γ 2 promoter of HF offspring

Finally, we assessed whether maternal obesity affects histone modifications in both promoters of offspring. For the PPAR γ 1 promoter, at PND21, ChIP-qPCR revealed less enrichment of H3ac and H3K4me3 (active marks) in both fat pads of HF offspring (Fig 3A-F). These differences were no longer observable at 9 months of age (Fig 3A-F). For the PPAR γ 2 promoter, at PND21, a reduction of active marks H3ac and H3K4me3 and a tendency towards enrichment of inactive mark H3K9me3 were evidenced only in pWAT of HF offspring (Fig 3A-F). At 9 months of age, persistent reduction of active mark H3ac was measured in both fat pads of HF offspring (Fig 3 A-F). No changes in global histone acetyl transferase and histone deacetylase activities were measured (Fig G and J). No variations of KAT2, P300, HDAC1, SIRT1, LSD1 and SUV39 mRNA levels were evidenced (Fig S1).

Discussion

Here, we show, for the first time, that persistent decreased mRNA PPAR γ 2 levels in adult HF offspring's WAT may occur via epigenomic remodeling in the PPAR γ 2 promoter which take place during lactation.

We extended our previous work (2) reporting that obesity-prone 9-month-old HF offspring had lower PPAR γ 2 mRNA expression levels in both pWAT and iWAT fat pads. Given that PPAR γ 2 promotes adipocyte differentiation and triglyceride storage in WAT (24), it may appear paradoxical that maternal obesity reduces PPAR γ 2 expression in WAT of HF offspring. However, others have also shown that obesity is associated with a decline in the PPAR γ 2 activity and expression (29–32). These modifications appear to be strongly associated with the pathogenesis of metabolic syndrome (30–32). The decrease in PPAR γ 2 gene expression might be seen as an adaptive mechanism to prevent further fat accumulation in WAT (33). It may, in turn, durably change the expression of PPAR γ target genes. Consistent with this hypothesis, recent findings have demonstrated that PPAR γ downregulation in obese mice is associated with a decrease in genome-wide association occupancy of PPAR γ (31).

We previously reported that HF neonates exhibited a rapid weight gain during lactation. At PND21, HF male offspring displayed higher iWAT and pWAT deposit weights with increased leptin mRNA levels, marked adipocyte hypertrophy and hyperplasia. HF 9-month-old male rats showed persistent increased pWAT mass with elevated leptin mRNA levels whereas this "expandable" phenotype was no longer observed in iWAT (2, 23). By contrast, both fat pads had persistent lower PPAR γ expression. These varying outcomes may reflect difference in postnatal and depot-specific programming. Depot-specific adipogenesis is regulated by the anatomic location, the unique signature of developmental gene expression and the hormonal microenvironment of WAT (35). PPAR γ governs biological processes by

modifying the expression of a large number of target genes. As already reported (2, 25), gene expression profiling revealed differences in adipogenic and lipogenic activities between iWAT and pWAT of adult HF offspring, suggesting a depot-specific programming. It may appear paradoxical that persistent reduced PPAR γ 2 expression was correlated with an increase in expression of genes associated with fat accumulation (i.e., FAS, DGAT2 and LPL) in pWAT. One possible explanation is that regulation of lipogenic genes in adult HF offspring's WAT may occur, at least in part, via additional PPAR γ -independent signaling pathways. Consistent with this hypothesis, HF adult male offspring exhibited hyperinsulinemia with elevated SREBP-1c mRNA levels within pWAT (2) that might be due for activation of insulin signaling. Indeed, offspring from obese dams showed greater AKT phosphorylation in response to insulin challenge associated with up-regulation of lipogenic pathways in WAT (5).

The underlying molecular mechanisms regulating PPAR γ gene expression remain unclear. However, decreased PPAR γ expression via modifications of epigenetic mechanisms may play a pivotal role in the development of obesity and diabetes (29, 30, 32, 35–37). Few studies have shown that DNA methylation and histone modifications regulate the expression of PPAR γ in WAT (32, 35, 36, 38–40). First, during adipogenesis, DNA demethylation (33) as well as enrichment of active marks H3ac (41) and H3K4me3 (42, 43) were observed in the PPAR γ promoter region that positively regulates gene expression. Second, luciferase reporter assays demonstrated that PPAR γ promoter methylation may repress reporter gene expression (33). Third, hypermethylation of the PPAR γ 2 promoter, which was negatively associated with PPAR γ 2 expression, was evidenced in visceral WAT of obese animals (32, 36, 38, 40). However, to our knowledge, no data are available regarding PPAR γ programming mechanisms in offspring from obese dams.

We showed that PND21 is a period characterized by active epigenomic remodeling (i.e., higher DNA methylation and lower active histone modification H3ac and H3K4me3) of the PPAR γ 2 promoter in pWAT, consistent with decreased PPAR γ 2 mRNA expression levels. Higher DNMT activity parallels the increase in global DNA methylation and CpGs methylation in the PPAR γ 2 promoter. During lactation, adipocyte stem cells are plastic and highly sensitive to maternal factors (44). Thus, maternal obesity and modified milk composition may influence offspring's nutritional and hormonal status (45, 46). Indeed, epigenetic enzyme activities are particularly sensitive to intracellular fluctuation in energy as well as metabolic intermediates (22). In line with this notion, weaned HF offspring exhibited higher plasma leptin levels (2) as well as insulin and triglycerides concentrations. Consistent with epigenomic remodeling, global enzyme activity modifications were observed at PND21. However, the increase in DNMT and DNA methylation activities were restricted to pWAT in weaned HF offspring. It may rely on spatiotemporal developmental differences that occur between depots. The DNMT activity is maximal during adipogenesis (47). In rodents, iWAT depots begin to develop during embryogenesis and the progenitor compartment is established before the first few days of life whereas pWAT depots mainly develop postnatally during lactation which coincides with the period of maximum adipogenesis (11, 48). As already suggested (2, 25), offspring's pWAT seems to be much more sensitive to maternal obesity.

Some of these epigenetic modifications were still observable in the PPAR γ 2 promoter in adult HF offspring. Sustained epigenetic modifications (i.e., higher DNA methylation and lower active histone modification H3ac) were observed in both fat pads of adult HF offspring, consistent with persistent decreased PPAR γ gene expression. 5mC is the only known modification that targets the DNA itself. In most cases, the extent of 5mC of a promoter is inversely correlated with the activity of the respective gene (19). We also showed that the global 5mC level of the PPAR γ 2 promoter in adult HF offspring was lower than weaned HF

pups, suggesting that a demethylation process occurs during development of WAT. The enrichment of H3ac is characteristic of promoter activation (43). A limitation of our work lies in the fact that DNA analysis was performed on entire tissue without distinguishing adipocyte and SVF fractions. Extracts may contain different cell type depending on the stage of development, tissue expansion and physiological states. However, gene expression profiling using several cell markers suggests that the composition of HF offspring's WAT is not modified regardless of the stage of development.

Our results provide compelling evidence that downregulation of PPAR γ expression may have developmental origins through epigenetic mechanisms. Thus, editing of early-life epigenetic markings that occurs during development of WAT may persist throughout life. Consistent with the DOHaD hypothesis, retained marks may provide a mechanistic basis for the cellular “memory” linking maternal obesity to a predisposition for later adiposity.

Author contributions: S.L., F.O., D.E., A.G., J.E., C.B. designed the experiments, analyzed data and contributed to discussion and manuscript. C.P., L.B., L.M. performed the experiments. C.L., C.G., J.L., D.V. contributed to discussion.

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Conflicts of interest: The author declares that he has no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1

Primers used for RT-qPCR and pyrosequencing. Primer sequences are indicated (see text for definitions).

RT-qPCR primer sequences		Targeted mRNA	Pyrosequencing primer sequences		Targeted regions
Forward	TTGGCCATATTATAGCTGTCAATTATT	PPAR γ	Forward	ATGTTGGTTAAATGAGTTTATTTAGTG	PPAR γ 2 promoter #1
Reverse	TGTCTGGATGGGCTTCA		Reverse	<i>Biotin</i> -ACACTATCCTAATTAAAAACCACTA	
Forward	GCACTGCCTATGAGCACTTC	PPAR γ 1	Sequence	ATTGAATGTGTGGG	PPAR γ 2 promoter #2
Reverse	GTGGCCTGTTGTAGAGTTGG		Forward	TTGTTTAGTTTTGTGGAATGTGGAGAGTAG	
Forward	GCTGCAGCGCTAAATTCATC	PPAR γ 2	Reverse	<i>Biotin</i> -ACTATCTCCCCAATAACCCACACATTCA	PPAR γ 2 promoter #3
Reverse	GCTGATTCCGAAGTTGGTGG		Sequence	TGTTGAATA-AATTATTTTAGAAGT	
Forward	CCAATCACGCAATAGTTCTGG	Acox	Forward	GGGGGATTATTGTTATGTGATATGTT	PPAR γ 2 promoter #3
Reverse	CGCTGTATCGTATGGCGAT		Reverse	<i>Biotin</i> -ATAAAAAAAATTAAAAACCTCCCTAAAAAT	
Forward	CAGGAGATGCTGGAATGACA	AdipoQ	Sequence	ATTAGGTTATTATGTGATAAGG	PPAR γ 1 promoter #1
Reverse	CTACGCTGAATGCTGAGTGAT		Forward	AACTATCACCAAATCCACACAAT	
Forward	TGCATGAATTAGTTGAACCAAGGCCA	CD36	Reverse	<i>Biotin</i> -CCCAAAACAATCTAACACCAACCATAA	
Reverse	CCACAGTTCGATCGCAGCCCC		Sequence 1	GGTTTTTTTAGAAGGTGTT	
Forward	AGTTGACCAGTGACAATGACCG	C/EBP α	Sequence 2	GTGAGAGTTAGGAGTAGGGTA	
Reverse	TCAGGCAGCTGGCGGAAG		Sequence 3	TTTGATAGGAAAGTTAGGT	
Forward	AGGCCTTGATGGTTTCTATCCA	DGAT2	ChIP-qPCR primer sequences		Targeted regions
Reverse	GCTGCCCTTCCCAATTAAC		Forward	ACAGTTTTTCAGAGAGGGGCT	PPAR γ 1 promoter
Forward	TAGTTCGGTGGCTACGAGGA	DNMT1	Reverse	CGACAGTGACCCCATTTTCC	PPAR γ 2 promoter
Reverse	CGTTTAGCGGGACCCCTTGAA		Forward	ACAGTTCACACCCCTCACAA	
Forward	GACCTGGAAACTCGTCTCCA	FABP4	Reverse	TGGCACTGTCCTGATTGAGA	Control A
Reverse	CATACCGGCCACTTTCCTGG		Forward	AGAGCTGAAAGAGGGCAAGA	
Forward	GGACATGGTCACAGACGATGAC	FAS	Reverse	GTCTCTCCTGCCCTCAAAC	Control B
Reverse	GTCGAACTTGGACAGATCCTTCA		Forward	CAGGCTGTTGAGTGGGTAGA	
Forward	AGGCACCCTCACTACCCTTT	GLUT4	Reverse	GGGCCACTGTTGCCATTTTA	
Reverse	ATAGCCCTTTTCTCTCCCAA				
Forward	CCAGAAGCCAAAGGGGTCAA	HDAC1			
Reverse	TGCGCTGGTCCCTATCTAGT				
Forward	ACTCCCATCTTCAGTCCTTC	KAT2A			
Reverse	CTTCTCTCTCTCTCCTGGC				
Forward	AAGGTCATCTTCTGTGCCA	LPL			
Reverse	CAGCCGACTTCTTCAGAGA				
Forward	GGCAAGAGCGATGTCTACTA	MBD2			
Reverse	CTGGACCGACTCCTTGAAGA				
Forward	CAGCTCCAACAGGATTCATGGT	MeCP2			
Reverse	TGATGTCTCTGCTTTGCCTGCCT				
Forward	AGATTAGAGGGCAGCAGAGAC	P300			
Reverse	GCCATAGGAGGTGGGTTCATAC				
Forward	AGACTTCAGTCCCTACTG	Resistin			
Reverse	CTAGTGACGGTTGTGCCTT				
Forward	CTGTTTCTGTGGGATACCTGACT	Sirt1			
Reverse	ATCGAACATGGCTTGAGGATC T				
Forward	CATCGACTACATCCGCTTCTTACA	SREBP1			
Reverse	GTCTTTCAGTGATTTGCTTTTGTGA				
Forward	AATCGCCTTCGGATTACAGACTC	TET2			
Reverse	CTTGACCTCCGATACACCCAT T				
Forward	TCAGCAACACCTTCATCACAA	TET3			
Reverse	TTTTCTTGGGTGGTTTGTA				
Forward	ATTCATGTGCCAGGGTGGT	Ppia			
Reverse	GATGCCAGGACCTGTATGCT				
Forward	GACGGTCACGGAGGATAAGATC	Rplp1			
Reverse	GCAAACAAGCCAGGCCAGAAA				

Table 2

Plasma parameters of weaned male offspring from obese dams fed either a control (C) or a high-fat (HF) diet

Variable	Male Offspring PND21		
	C	HF	<i>Maternal diet effect p value</i>
Glucose (dg/ml)	168.22 ± 5.49	171.14 ± 2.54	ns
Triacylglycerol(mg/dl)	210.9 ± 11.28	281.9 ± 13.10	*
Insulin (μU/ml)	34.35 ± 5.94	60.99 ± 5.49	*
Corticosterone (ng/ml)	221.21 ± 16.87	216.63 ± 20.84	ns

All data are presented as means +/- SEM. Data were analyzed using Student's *t* test. Effects of maternal obesity at PND21 (*P < 0.05). n = 20 per group.

Figure legends

Figure 1: Maternal obesity induces persistent lower PPAR γ expression in adipose tissue of offspring

PPAR γ expression was assessed in male offspring from dams fed a control diet (called C) or high-fat diet-fed dams (called HF) on postnatal days 21 (PND21) and 9 months of age (9M). PPAR γ , PPAR γ 1 and PPAR γ 2 mRNA levels were determined in pWAT (**A**) and iWAT (**B**) by RT-qPCR. Representative Western blot of PPAR γ (55 KDa) and β -Actin (43 KDa) were indicated in pWAT (**C**) and iWAT (**D**). mRNA levels of PPAR γ target genes were determined in pWAT (**E**) and iWAT (**F**) by RT-qPCR. Relative gene expression in the C group at PND21 was set to 1. Ppia and Rplp1 were used as standard genes. All data are presented as means $\pm$ SEM. Data were analyzed using Student's *t* test. Effects of maternal obesity at PND21 (*P < 0.05) and at 9M (#*P* < 0.05). *n* = 5-10 per group.

Figure 2: Persistent lower PPAR γ expression is associated with CpG hypermethylation in the PPAR γ 2 promoter of HF offspring

Epigenetic modifications were assessed in male C and HF offspring in two fat pads (pWAT and iWAT) at PND21 and 9M. Schematic positions and sequences of rat PPAR γ 1 and PPAR γ 2 promoters were shown (**A**). CpG methylation in PPAR γ 1 promoter (**B**, **C**) and PPAR γ 2 promoter (**D**, **E**) were assessed by bisulfite pyrosequencing in pWAT (**B**, **D**) and iWAT (**C**, **E**). DNMT activity from nuclear protein (**F**) and global DNA methylation (**G**) were assessed in pWAT and iWAT. TET activity from nuclear protein (**H**) and global DNA hydroxymethylation (**I**) were assessed in pWAT and iWAT. All data are presented as means

+/- SEM. Data were analyzed using Student's *t* test. Effects of maternal obesity at PND21 (*P < 0.05) and at 9M (#*P* < 0.05). *n* = 5 per group.

Figure 3: Persistent lower PPAR γ expression is associated with depletion in H3ac/H3K4me3 in the PPAR γ 2 promoter of HF offspring

Epigenetic modifications were assessed in male C and HF offspring in two fat pads (pWAT and iWAT) at PND21 and 9M. Histone modifications were measured by ChIP assays with antibodies against H3ac (**A, B**), H3K4me3 (active mark) (**C, D**) and H3K9me3 (inactive mark) (**E, F**) in pWAT (**A, C, E**) and iWAT (**B, D, F**). HAT (**G**) and HDAC (**H**) activities were assessed from nuclear protein of pWAT and iWAT. All data are presented as means +/- SEM. Data were analyzed using Student's *t* test. Effects of maternal obesity at PND21 (*P < 0.05) and at 9M (#*P* < 0.05). *n* = 5 per group.

Figure 1

Figure 2

Figure 3