Bioinformatic and biostatitic analysis of epigenetic data from humans and mice in the context of obesity and its complications

Sarah Voisin

UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1245

Bioinformatic and Biostatistic Analysis of Epigenetic Data from Humans and Mice in the Context of Obesity and its Complications

SARAH VOISIN

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016

ISSN 1651-6206
ISBN 978-91-554-9655-5
urn:nbn:se:uu:diva-300751

Dissertation presented at Uppsala University to be publicly examined in Uppsala, Thursday, 22 September 2016 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Associate professor Romain Barres (Section for Integrative Physiology, University of Copenhagen, Denmark).

Abstract
Voisin, S. 2016. Bioinformatic and Biostatistic Analysis of Epigenetic Data from Humans and Mice in the Context of Obesity and its Complications. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1245. 219 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9655-5.

Worldwide obesity has more than doubled since 1980 and at least 2.8 million people die each year as a result of being overweight or obese. An elevated body weight is the result of the interplay between susceptibility gene variants and an obesogenic environment, and recent evidence shows that epigenetic processes are likely involved. The growing availability of high-throughput technologies has made it possible to assess quickly the entire epigenome of large samples at a relatively low cost. As a result, vast amounts of data have been generated and researchers are now confronted to both bioinformatic and biostatistic challenges to make sense of such data in the context of obesity and its complications. In this doctoral thesis, we explored associations between the human blood methylome and obesity-associated gene variants as well as dietary fat quality and quantity. We used well described preprocessing techniques and statistical methods, along with publicly available data from consortiums and other research groups, as well as tools for pathway enrichment and chromatin state inference. We found associations between obesityassociated SNPs and methylation levels at proximal promoters and enhancers, and some of these associations were replicated in multiple tissues. We also found that contrary to dietary fat quantity, dietary fat quality associates with methylation levels in the promoter of genes involved in metabolic pathways. Then, using a gene-targeted approach, we looked at the impact of an acute environmental stress (sleep loss) on the methylation and transcription levels of circadian clock genes in skeletal muscle and adipose tissue of healthy men. We found that a single night of wakefulness can alter the epigenetic and transcriptional profile of core circadian clock genes in a tissue-specific manner. Finally, we looked at the effects of chronic maternal obesity and subsequent weight loss on the transcription of epigenetic machinery genes in the fetus and placenta of mice. We found that the transcription of epigenetic machinery genes is highly sensitive to maternal weight trajectories, and particularly those of the histone acetylation pathway. Overall, this thesis demonstrated that genetics, obesogenic environment stimuli and maternal programming impact epigenetic marks at genomic locations relevant in the pathogenesis of obesity.

Keywords: obesity, genetics, epigenetics, DNA methylation, sleep, developmental origins of health and disease, single nucleotide polymorphism, genome-wide association study

Sarah Voisin, , Department of Neuroscience, Box 593, Uppsala University, SE-75124 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-554-9655-5
urn:nbn:se:uu:diva-300751 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-300751)

An apple a day will keep anyone away if thrown hard enough.

Unknown

If your experiment needs statistics, you ought to do a better experiment.

Lord Ernest Rutherford

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Voisin S**, Almén MS, Zheleznyakova GY, Lundberg L, Zarei S, Castillo S, Eriksson FE, Nilsson EK, Blüher M, Böttcher Y, Kovacs P, Klovins J, Rask-Andersen M, Schiöth HB, Many obesity-associated SNPs strongly associate with DNA methylation changes at proximal promoters and enhancers, Genome Medicine 2015, 7:103.

II **Voisin S**, Almén MS, Moschonis G, Chrousos GP, Manios Y, Schiöth HB, Dietary fat quality impacts genome-wide DNA methylation patterns in a cross-sectional study of Greek preadolescents, Eur J Hum Genet 2014, 23(5): 654-662

III Cedernaes J, Osler ME, **Voisin S**, Broman JE, Vogel H, Dickson SL, Zierath JR, Schiöth HB, Benedict C, Acute sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes in men, J Clin Endocrinol Metab 2015, 100(9): E1255–E1261

IV Panchenko PE, **Voisin S**, Jouin M, Jouneau L, Prezelin A, Lecoutre S, Breton C, Jammes H, Junien C, Gabory A, Expression of epigenetic machinery genes is sensitive to maternal obesity and weight loss in relation to fetal growth, Clinical Epigenetics 2016, 8(22).

Additional publications

- **Voisin S**, Guilherme JPFL, Yan X, Pushkarev VP, Cieszczyk P, Massidda M, et al. ACVR1B rs2854464 Is Associated with Sprint/Power Athletic Status in a Large Cohort of Europeans but Not Brazilians. *PLoS One*. United States; 2016;11: e0156316. doi:10.1371/journal.pone.0156316
- **Voisin S**, Cieszczyk P, Pushkarev VP, Dyatlov DA, Vashlyayev BF, Shumaylov VA, Maciejewska-Karlowska A, Sawczuk M, Skuza L, Jastrzebski Z, Bishop DJ, Eynon N, EPAS1 gene variants are associated with sprint/power athletic performance in two cohorts of European athletes, *BMC Genomics*, 15(1): 382
- **Voisin S**, Eynon N, Yan X, Bishop DJ, Exercise training and DNA methylation in humans, *Acta Physiol (Oxf)* 2015, 213(1): 39–59
- Yang R, Shen X, Wang Y, **Voisin S**, Cai G, Fu Y, et al. ACTN3 R577X Gene Variant is Associated with Muscle-related Phenotypes in elite Chinese Sprint/Power Athletes. *J strength Cond Res / Natl Strength Cond Assoc*. 2016; doi:10.1519/JSC.0000000000001558
- Williams MJ, Klockars A, Eriksson A, Wiemerslage L, **Voisin S**, Dnyansagar R, Kasagiannis A, Ambrosi A, Fredriksson R , Schiöth HB, The Drosophila ETV5 homologue Ets96B: Molecular link between obesity and bipolar disorder, *PLoS Genetics* 2016
- Boström AE, Mwinyi J, Benedict C, **Voisin S**, Wu W, Schultes B, Zhang K, Schiöth HB, Longitudinal genome-wide methylation study of Roux-en-Y gastric bypass patients reveals novel CpG sites associated with essential hypertension, *BMC Medical Genomics* 2016, 9(20).
- Bandstein M, **Voisin S**, Nilsson EK, Schultes B, Ernst B, Thurnheer M, Benedict C, Mwinyi J, Schiöth HB, A genetic risk score is associated with weight loss following Roux-Y gastric bypass surgery, *Obesity Surgery* 2016
- Williams MJ, Eriksson A, Shaik M, **Voisin S**, Yamskova O, Paulsson J, Thombare K, Fredriksson R, Schiöth HB, The obesity-linked gene Nudt3 Drosophila homolog Aps is associated with insulin signalling, *Mol Endocrinol* 2015, 29(9): 1303–1319.
- Banerjee HN, Banerji A, Banerjee AN, Riddick E, Petis J, Evans S, Patel M, Parson C, Smith V, Gwebu E, **Voisin S**, Deciphering the Finger Prints of Brain Cancer Glioblastoma Multiforme from Four Different Patients by Using Near Infrared Raman Spectroscopy. *J Cancer Sci Ther* 2015, 7(2): 44–47.
- Eriksson A, Williams MJ, **Voisin S**, Hansson I, Krishnan A, Philippot G, Yamskova O, Herisson FM, Dnyansagar R, Moschonis G, Manios Y, Chrousos GP, Olszewski PK, Frediksson R, Schiöth HB, Implication of coronin 7 in body weight regulation in humans, mice and flies, *BMC Neurosci* 2015, 16(1): 13.
- Nilsson EK, Ernst B, **Voisin S**, Almén MS, Benedict C, Mwinyi J, Fredriksson R, Schultes B, Schiöth HB, Roux-en Y gastric bypass surgery induces genome-wide promoter-specific changes in DNA methylation in whole blood of obese patients, *PLoS One* 2015, 10(2): e0115186.
- Ségurel L, Austerlitz F, Toupance B, Gautier M, Kelley JL, Pasquet P, Lonjou C, Georges M, **Voisin S**, Cruaud C, Couloux A, Hegay T, Aldashev A, Vitalis R, Heyer E, Positive selection of protective variants for type 2 diabetes from the Neolithic onward: a case study in Central Asia, *Eur J Hum Genet* 2013 21(10): 1146–51.
- Zheleznyakova GY, **Voisin S**, Kiselev AV, Almén MS, Xavier MJ, Maretina MA, Tishchenko LI, Fredriksson R, Baranov VS, Schiöth HB, Genome-wide analysis shows as-

sociation of epigenetic changes in regulators of Rab and Rho GTPases with spinal muscular atrophy severity, *Eur J Hum Genet* 2013, 21(9): 988–93.

Contents

Abbreviations

5hmC	5 hydroxymethyl-cytosine
5meC	5 methyl-cytosine
ABCG1	ATP-binding cassette sub-family G member 1
ABDBR3	Adrenoceptor beta 3
APOB	Apolipoprotein B
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CGI	CpG island
CNV	Copy number variation
CREB	cAMP response element binding protein
CRISPR	Clustered regulatory interspaced short palindromic repeat
CUX1	Cut like homeobox 1
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DOHaD	Developmental origins of health and disease
EWAS	Epigenome-wide associations study
FTO	Fat mass and obesity associated
GWAS	Genome-wide association sudy
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF3A	Hypoxia inducible factor 3 alpha subunit
HMT	Histone methyltransferases
HPLC	High performance liquid chromatography
IRX3	Iroquois homeobox 3
IRX5	Iroquois homeobox 5
KAT2B	Lysine acetyltransferase 2B
KCNK9	Potassium two pore domain channel subfamily K member 9
KDM	Lysine demethylase
LBW	Low birth weight
LEP	Leptin
LGA	Large for gestational age
MBD-seq	Methyl-CpG-binding domain protein-enriched genome sequencing
MBP	Methyl-CpG binding protein
MC4R	Melanocortin 4 receptor
MeDIP	Methylated DNA immunoprecipitation
meQTL	Methylation QTL
miRNA	microRNA
ncRNA	non-coding RNA

NEGR1	Neuronal growth regulator 1
PC	Principal component
PCA	Principal component analysis
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHOSPHO1	Phosphoethanolamine/phosphocholine phosphatase
POMC	Proopiomelanocortin
PPARδ	Peroxisome proliferator-activated receptor beta
PRMT	Protein arginine methyltransferases
RRBS	Reduced representation bisulfite sequencing
SAM	S-adenosylmethionine
SH2B1	SH2B adaptor protein 1
SLC2A10	Solute carrier family 2 member 10
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
T2D	Type 2 diabetes
TALE	Transcription activator-like effector
TET	Ten-eleven translocation
TF	Transcription factor
TFAM	Transcription factor A, mitochondrial
TNF-α	Tumor necrosis factor alpha
TXNIP	Thioredoxin interacting protein
WGBS	Whole-genome bisulfite sequencing

Introduction

1. The roots of obesity and its disastrous consequences

1.1. Obesity: a huge health and economic burden

Overweight and obesity are defined as an abnormal or excessive fat accumulation that presents a risk to health. A crude population measure of obesity is the body mass index (BMI), a person's weight (in kilograms) divided by the square of his or her height (in meters). A person with a BMI of 30 or more is generally considered obese, and a person with a BMI equal to or more than 25 is considered overweight. Overweight and obesity are major risk factors for a number of chronic diseases, including type 2 diabetes (T2D) [1], cardiovascular diseases [1], certain types of cancer [1], osteoarthritis [2], nonalcoholic fatty liver disease [3]. Moreover, obesity is associated with physical conditions such as gallbladder disease [4], obstructive sleep apnoea [5] and gout [6] (Figure 1). It is also at odds with societal ideals of thinness, thus causing depression because of social stigmatization and discrimination [7], particularly in women. Despite the fact that it kills, directly or indirectly, an estimated 2.8 million people each year [8], it was only in 2013 that obesity was classified as a chronic disease by the American Medical Association [9]. Worldwide obesity has more than doubled since 1980, and what was once considered a problem only in high income countries is now hitting low- and middle-income countries, particularly urban settings. In 2014, 38% of men (Figure 2A) and 40% of women (Figure 2B) aged 18 years and over were overweight [8]. Not only does obesity shorten lifespan and lower quality of life, but it also costs billions of dollars to health care. A couple of studies have tried to give a more precise estimate of this cost, but they showed great heterogeneity in terms of scope, data sources, data quality and methodological approaches [10]. Yet, a consistent finding was that direct cost make up about half of the total cost and is attributable to treating comorbidities such as cardiovascular disease and T2D while indirect cost is attributable to work absenteeism, early retirement and disability [10]. It is therefore clear that obesity presents a huge health and economic burden, and we need to uncover the aetiology of this disease to implement the best prevention and treatment strategies.

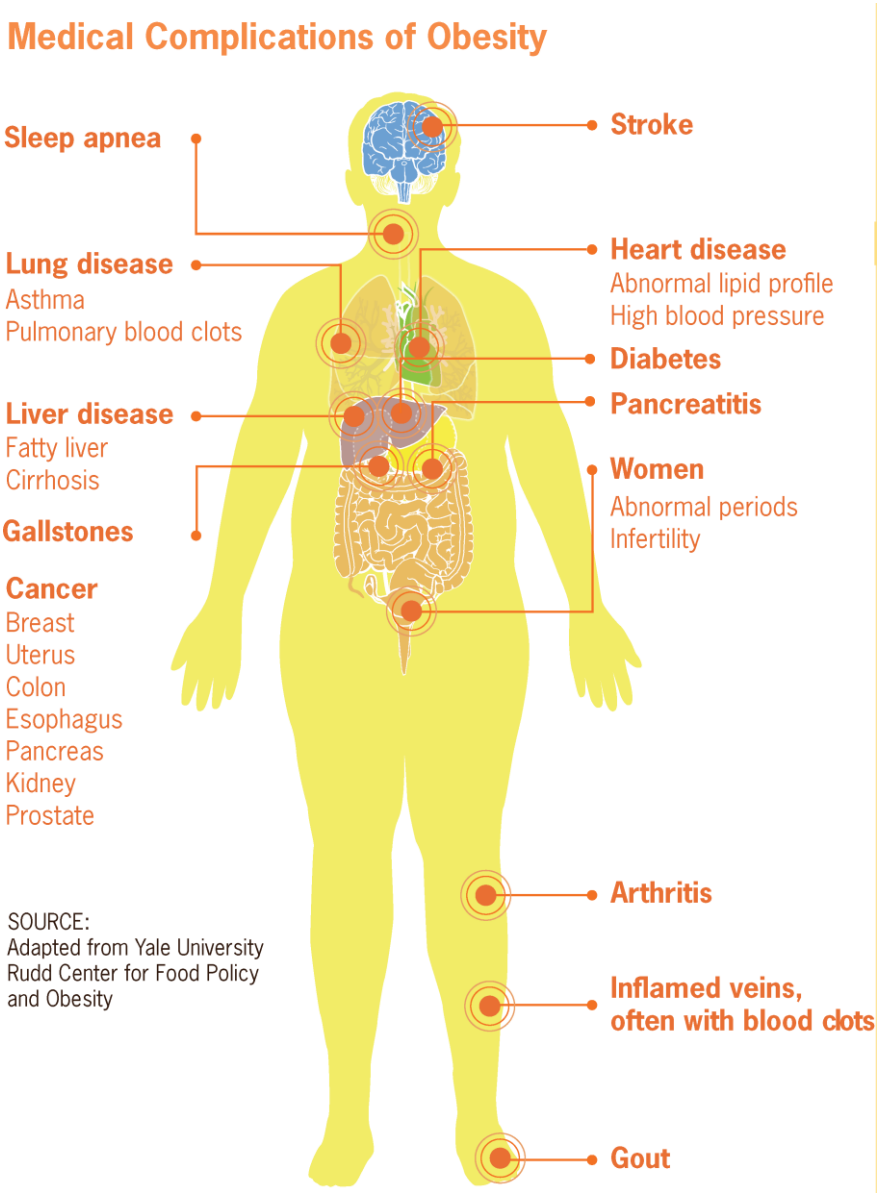


Figure 1. Obesity-associated comorbidities.

Taken from [11]

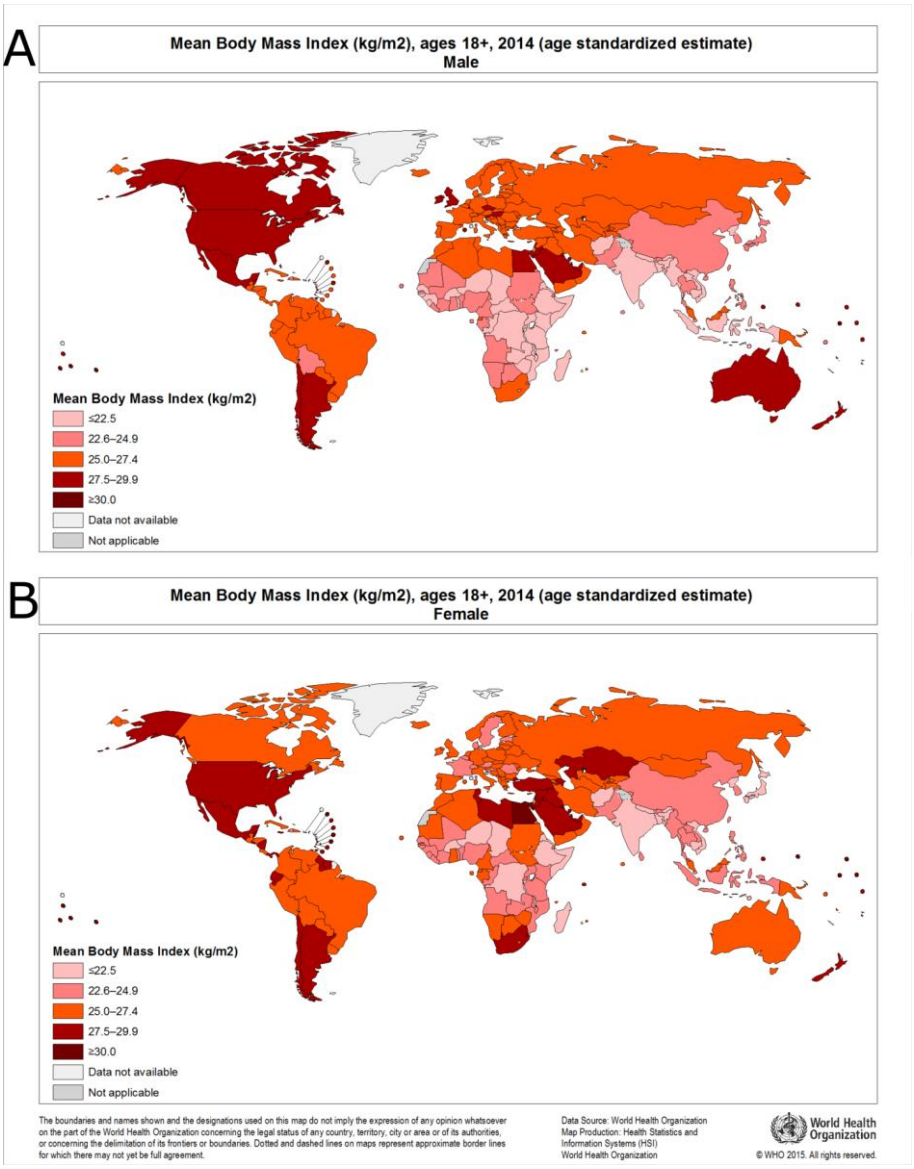


Figure 2. Mean body mass index by country, ages 18+, age standardized, 2014.

(A) Male (B) Female. Reprinted from Global Health Observatory Map Gallery, Page No.1, Copyright (2015) [12]

1.2. The relative contribution of genetics and the environment to obesity

What causes obesity? At an individual level and in most cases, a **positive energy balance** (i.e. higher energy intake than energy expenditure) is responsible for weight gain over time. Of the rare cases of early-onset, severe obesity, 7% can be explained by single point DNA mutations in genes that play a key role in appetite regulation, such as leptin (*LEP*), melanocortin 4 receptor (*MC4R*) and proopiomelanocortin (*POMC*) [13]. However, it is clear that genetic variation in humans has not changed appreciably in the past 50 years and therefore cannot account for the recent increase in obesity prevalence. It is rather the dramatic change in lifestyle in high-income and developing countries that explains the epidemic. Since the 1960s, the daily energy consumption per capita has increased by 31% worldwide, and the proportion of energy intake derived from fat by 7% [14]. Moreover, around 23% of adults and an alarming 81% of adolescents were not active enough in 2010, partly due to inaction during leisure time, sedentary behaviour on the job and at home [8]. Other obesogenic environmental exposures have been suggested to participate in the recent obesity epidemic [15] (Figure 3). (1) **Short sleep duration**, impaired sleep quality and irregular sleep-wake patterns disturb central nervous

neuroendocrine pathways controlling energy homoeostasis, food intake, and adipocyte function [16]. (2) **Endocrine disruptors** present in food, plastics, cigarettes, and fossil fuels have the ability to increase the number of adipocytes and/or the storage of fat in existing adipocytes, particularly if exposure happened early in life [17]. (3) **Increased maternal age** is associated with a higher risk of obesity in offspring, partly because older mothers produce babies that are small or large for gestational age [18,19]. (4) **Prenatal and early-postnatal nutrition**, such as under- or over-nutrition during pregnancy, can alter the projected growth pattern of various organs and systems of the body, conferring to the offspring an increased risk of metabolic disease [20]. (5) The increase in the use of certain **medications** like psychotropics [21], antihypertensives [22], and antidiabetics [23] exert a small yet significant effect on weight gain over time. Yet, it is puzzling to see that some people remain slim while others gain weight, despite being confronted to similar risk factors. Are some people genetically more susceptible to weight gain?

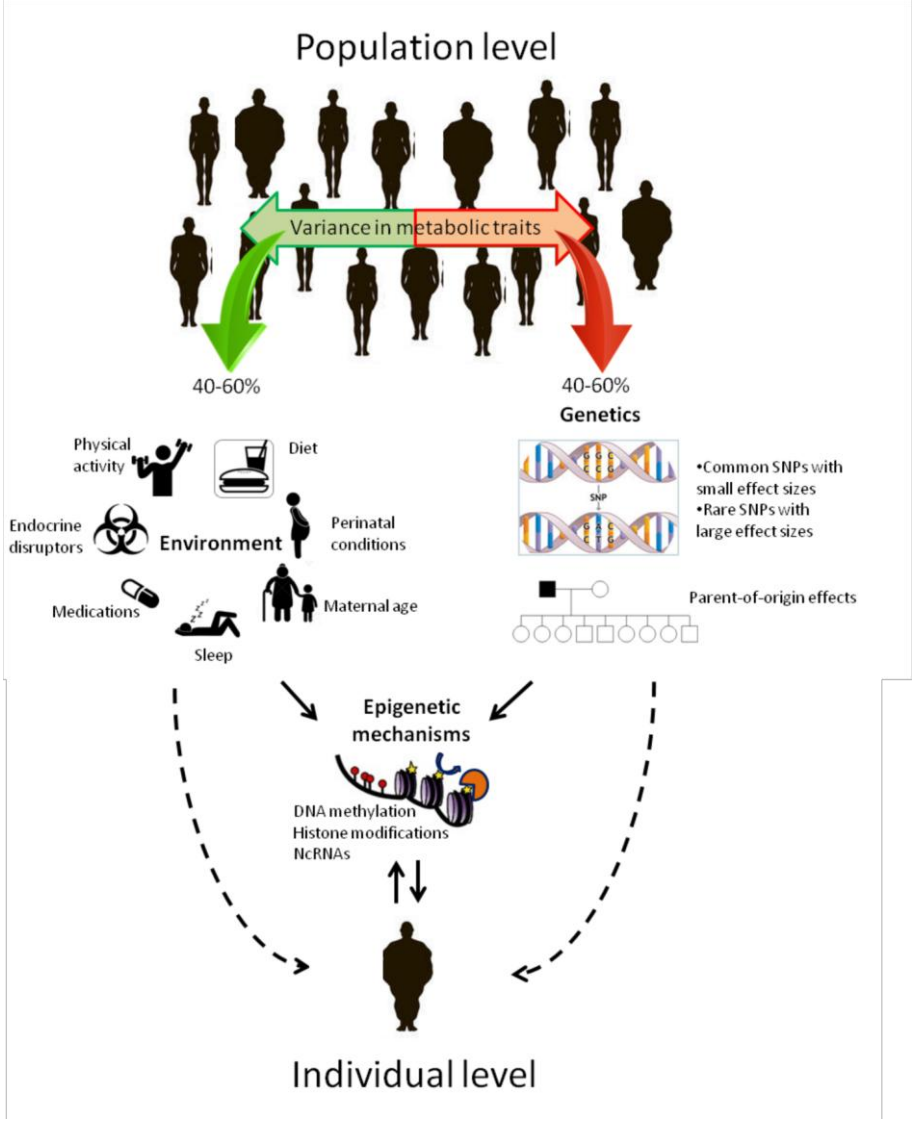


Figure 3. The relative contribution of genetics and the environment to the variance in metabolic traits and to individual metabolic parameters.

At the population level, genetics and the environment both explain ~50% of the variance in metabolic traits; at an individual level, genetics and some environmental factors (perinatal conditions, maternal age) act as predisposing agents that confer an increased or decreased risk for developing metabolic disorders. Epigenetics is the main mechanism (albeit not the only one) that has been proposed to explain how gene variants and the environment interact to impact the phenotype.

One way to answer this question is to estimate the proportion of variance in BMI or other metabolic traits (e.g. waist-to-hip ratio) in the population that is explained by genetics. This so-called "**heritability**" of metabolic traits has been estimated by family, twin and adoption studies, by comparing the observed and expected resemblance between relatives [24]. A recent meta-analysis of the heritability of human traits based on fifty years of twin studies estimated that 58% of the variance in metabolic traits, 51% of the variance in endocrine, nutritional and metabolic diseases and 48% of the variance in general metabolic functions is explained by genetic factors [25]. However, family and adoption studies usually report higher heritability estimates, because twin studies assume equal shared environments in identical and non-identical twin pairs, and because family studies cannot discriminate between genetics and shared environment [26]. Overall, these figures suggest that genetic factors account for about half of the natural variation in metabolic function in the general population, and environmental factors account for the other half.

Unfortunately, the concept of heritability is often misunderstood by the scientific community. It is important to stress that the concept of "heritability" has a meaning only at a **population level**, but does not explain a trait at the **individual level** (Figure 3). Therefore, if metabolic traits were estimated to have a heritability of ~50%, this in no way means that the metabolic traits of a given individual are due to his genetic makeup at 50% and to his environment at 50%. A heritability of 100% for a given trait does not imply that the trait is 100% genetic, but that variance in the trait in the population can be explained at 100% by genetic variation. For example, if we were to take a (hypothetical) population of elite athletes whose training regime and diet have been tightly controlled to be identical since birth (for the sake of the example, we do not consider any perinatal effects here), the observed variations in performance at a sprint test would be exclusively explained by genetic variation since their environments were strictly identical. That does not imply that the performance at the sprint test has no environmental basis: it is largely influenced by the intensity of the training regime, sleep and diet quality, etc. Conversely, a heritability of 0% does not mean that a trait has no genetic basis, but that variation in the trait in the population under study can be explained at 100% by the environment. For example, if we were to consider the population of Japan where everybody is dark-haired, observed variations in hair colour would be exclusively due to environmental factors such as sunlight exposure, use of hair dye, stress, etc. That does not mean that hair colour has no genetic basis! It is also important to note that heritability estimates change when the population under study changes. If we come back to the hair colour example and if we were to consider the world population instead of the Japanese population, heritability of hair colour would no longer be 0%, but much higher because of the added genetic diversity in genes responsible for hair colour. To further illustrate that heritability is deeply population-dependent, it is useful to note that heritability estimates can change over time because the variance in genetic values can change, the variation due to environmental factors can change, or the correlation between genes and environment can change [24]. Three questions follow on: given the high heritability of metabolic traits, which genetic variants contribute to obesity? How do they act at the molecular level? How do they interact with environmental factors?

1.3. Genome-wide association studies (GWASs) and the problem of missing heritability

The human genome is characterized by genetic variations that can happen at a small scale (1-50 bp), an intermediate scale (50-3000 bp), or at a large scale (>3000 bp) (Table 1). GWASs have been undertaken to see whether some of the ~100,000,000 single nucleotide polymorphisms (SNPs) present in the human genome [27] show allele frequencies that associate with metabolic traits and obesity risk [28–46]. Unfortunately, they have predominantly been conducted in populations of European ancestry, despite the fact that metabolic disorders are more prevalent in populations of African descent [47,48] and Hispanics [48]. Nevertheless, GWASs have identified at least 160 loci, some of which located near genes that were already discovered in monogenic forms of obesity [13], and some near genes that provide support for the central nervous system such as brain-derived neurotrophic factor (*BDNF*), *MC4R* and neuronal growth regulator 1 (*NEGR1*) [45] (Figure 4). However, the **annotation** of risk variants to specific genes is not straightforward, since most of them are located in

non-coding and regulatory regions of the genome [49], they do not necessarily regulate the nearest gene, they may regulate several genes at once, and they may regulate specific genes in specific tissues during specific developmental windows. Fat mass and obesity associated (*FTO*) is an excellent textbook case of this paradigm. SNPs in the first intron of *FTO* were found to associate with a variety of metabolic traits such as BMI, hip circumference and appetite, and they form the locus with the largest effect size on BMI [50]. Following these findings, *FTO* deletion and overexpression experiments in rodents suggested that *FTO* acts both in the brain to regulate energy balance and in other tissues to modify body composition, but links between the risk variants identified in GWASs and *FTO* expression levels have remained elusive. Recently, it has been reported that the discovered SNPs appear functionally connected not with *FTO* but with three neighbouring genes: Iroquois homeobox 3 (*IRX3*), Iroquois homeobox 5 (*IRX5*) and RPGRIP1-like (*RPGRIP1L*) [51] (Figure 5A). The first intron of *FTO* contains a binding site for the transcription factor (TF) cut like homeobox 1 (CUX1) which, through regulation of *RPGRIP1L* expression, modulates leptin receptor localization within neurons [51]. This intron also contains an enhancer sequence which directly binds to the promoter of *IRX3* and *IRX5* when the *FTO* rs1421085 risk allele is present [52], and this leads to repressed mitochondrial thermogenesis in preadipocytes [53] (Figure 5B).

Scale	Name	Type	Size	Number in the human genome
Small	Single nucleotide polymorphism (SNP)	Substitution	1 bp	~100,000,000 [27]
	Small insertion and deletion (INDEL)	Insertion	1-50 bp	~1000-200,000 [54]
		Deletion		
Intermediate (structural variation)	Copy number variation (CNV)	Deletion	50-3,000 bp	~11,000-23,000 [55]
		Duplication		~1,100-3,200 [55]
	Microsatellites	Short tandem repeats (STRs)	2-9 bp repeated 5-50 times	~3,000,000 [56]
	Minisatellites	Variable number tandem repeats (VNTRs)	10-60 bp repeated 5-50 times	~150,000 [57]
Large	Structural abnormality	Insertion	> 3,000 bp	N/A
		Deletion		
		Duplication		
		Substitution		
		Translocation		
	Numerical abnormality (aneuploidy)	Monosomy	Entire chromosome	N/A
		Trisomy		
		Tetrasomy/pentasomy		

Table 1. Types of genetic variations in the human genome.

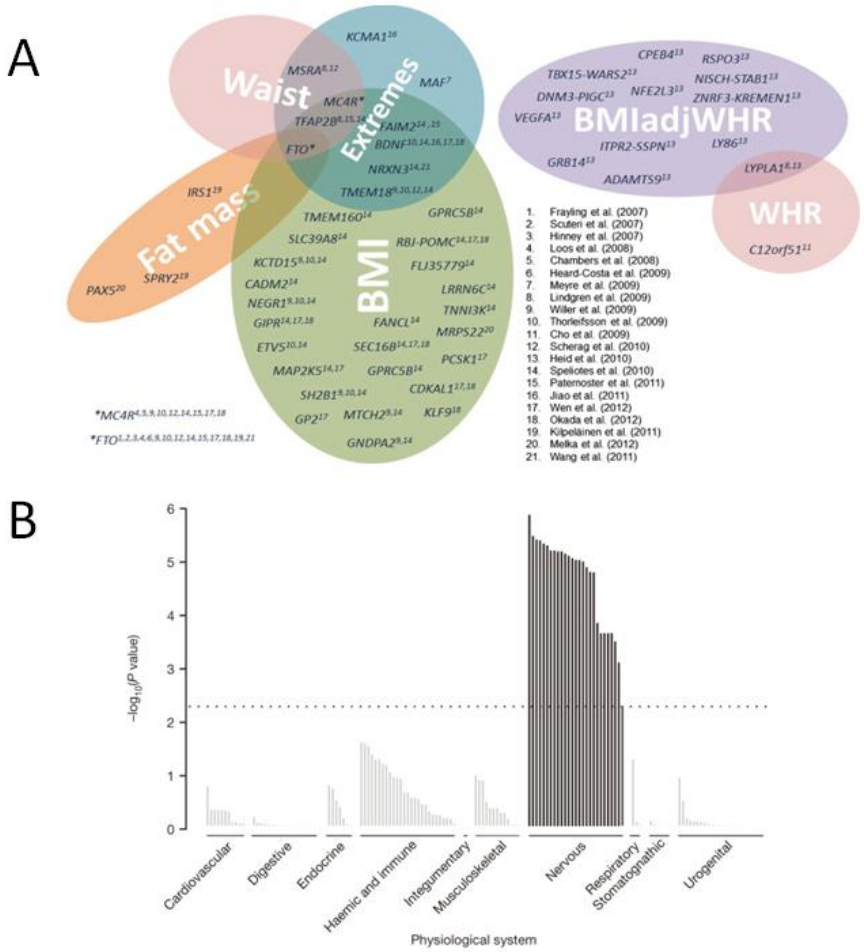


Figure 4. *Overview of the genes located in the vicinity of loci identified by GWAS*.

(A) Summary of loci found by genome-wide association studies to be associated with body mass index (BMI), waist circumference (waist), waist–hip ratio (WHR), extreme obesity phenotypes (extremes) or BMI-adjusted WHR (BMIadjWHR) with $p < 5 \times 10^{-8}$. Taken from [35] (B) Tissues and reconstituted gene sets significantly enriched for genes within BMI-associated loci. Taken from [45]

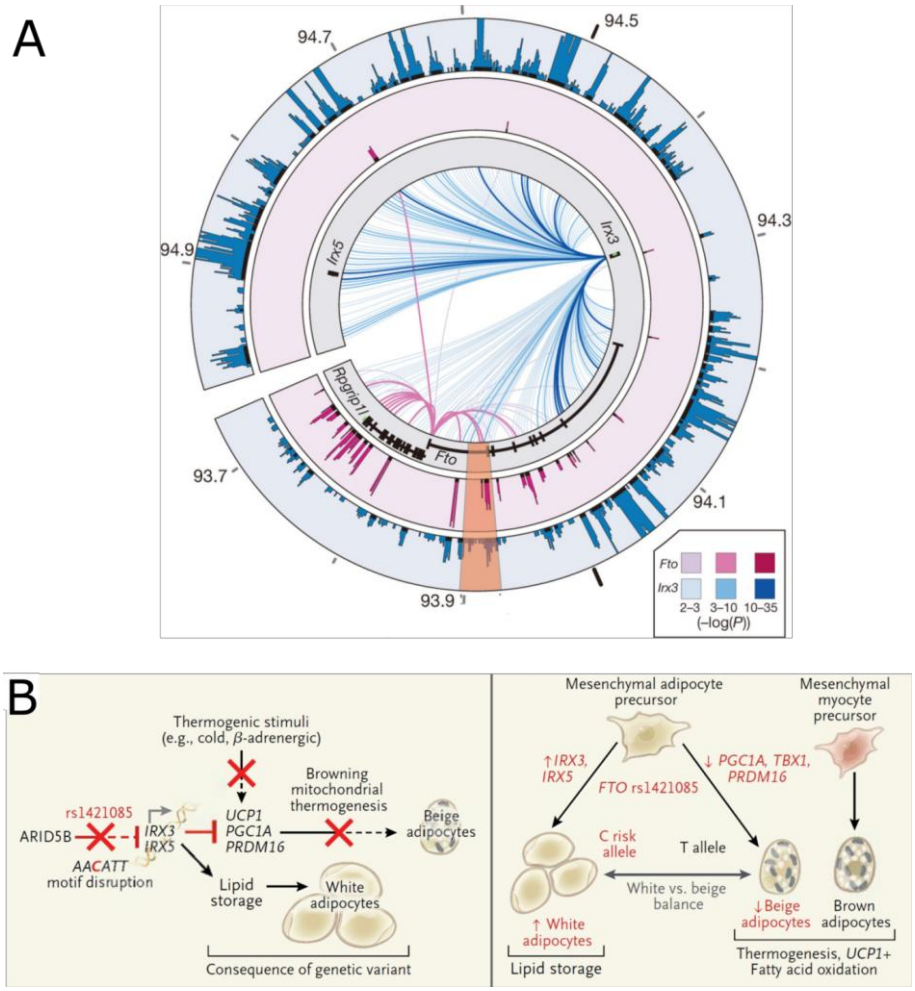


Figure 5. *The FTO locus*.

(A) Long-range interactions at the *FTO-IRX3* locus. Mouse embryo 4C-seq interactions emanating from each promoter are displayed as links across the circle (darker link implies greater significance). Outer plots show significance of interactions above background (−log(P value)). The obesity-associated interval is highlighted red. Taken from [52] (B) Summary of the mechanistic model of the *FTO* locus association with obesity, implicating a developmental shift favouring lipid-storing white adipocytes over energy-burning beige adipocytes. At its core lies a single-nucleotide T-to-C variant, rs1421085, which disrupts a conserved ARID5B repressor motif and activates a mesenchymal superenhancer and its targets (*IRX3* and *IRX5*), leading to reduced heat dissipation by mitochondrial thermogenesis (a process that is regulated by UCP1, PGC1α, and PRDM16) and to increased lipid storage in white adipocytes. Taken from [53]

Despite these well-conducted studies that show how SNPs in the first intron of *FTO* contribute to the pathogenesis of obesity, the fact remains that the effect size of these SNPs is extremely low (0.10-0.34% of explained variance in BMI) [50]. Actually, all ~160 identified loci collectively explain less than 5% of variance in BMI [58], and we still do not know what the remaining ~45% of variance stems from, a problem that has been termed "**missing heritability**" [59]. Missing heritability has been suggested to have several potential sources that are not mutually exclusive [60]:

(1) **Copy number variations** (CNVs) not investigated in most GWAS, such as deletions, insertions and duplications of segments of the genome. Genome-wide CNV analyses for obesity are scarce, partly because CNVs are notoriously difficult to measure [61]. Only one of the 84 BMI/obesity-associated CNVs discovered so far was replicated in another cohort, and it explained only 0.1% of the variance in BMI [62], suggesting that this hypothesis is unlikely.

(2) **Rare SNPs** that are not captured by the currently available commercial SNP arrays nor well tagged by the SNPs on the arrays and **with large effect sizes**. Rare variants may contribute quite sub-

stantially to the variance in BMI, and using a new analytical framework for the design of rare variant association studies will certainly help [63].

(3) **Common SNPs with** such **small effect sizes** that they cannot be identified with the current sample sizes used in GWAS. Yang *et al*. recently performed simulations based on whole-genome sequencing data, and they managed to capture ~97% and ~68% of variation at common and rare variants by imputation. They found that the added effects of ~17 million imputed variants explained 27% of the variance in BMI (25% for common variants and 2% for rare variants) [64], giving a strong case for this hypothesis.

(4) **Epistasis between SNPs** (non-additive effects). In their recent meta-analysis of twin studies, Polderman *et al*. gave the proportion of individual studies that are compatible with a parsimonious model where the variance is solely due to additive genetic variation, for a large number of traits. Interestingly, 60% of studies were compatible with this model for metabolic traits, 69% for endocrine and 72% for nutritional traits. However, only 48% of studies were compatible with this model for weight maintenance functions [25], suggesting that this hypothesis is unlikely but impossible to rule out completely.

(5) **Parent-of-origin effects** such as genomic imprinting. This hypothesis involves the ~100 genes in humans that are known to be imprinted [65], and they correspond to genes with monoallelic expression that depends upon whether it resided in a male or female the previous generation. Sequence variants located close to imprinted genes would then show consequences on the phenotype only if they are inherited from the mother of the father, but it is unclear how prevalent this phenomenon is in metabolic disorders. Three loci were suggested to have parent-of-origin effects on BMI in European American [66], and a later study on 38167 Icelanders revealed three SNPs that exhibit parental-origin specific associations for T2D [67]. More recently, potential parent-of-origin effects were reported within *FTO* [68], Apolipoprotein B (*APOB*) [69], Solute carrier family 2 member 10 (*SLC2A10*) and potassium two pore domain channel subfamily K member 9 (*KCNK9*) [70]. Animal models provide a better picture of the global contribution of parent-of-origin to the heritability, since breeding is controlled and the genotypes of both parents and offspring can be obtained. In 2014, the heterogeneous stock mouse strain descended from eight inbred progenitor strains and maintained for over 50 generations allowed the estimation of the heritability of a wide range of complex traits [71]. Strikingly, the contribution to heritability attributable to alleles shared by two animals that come from parents of the same sex (i.e., two mothers or two fathers), was *twice* that attributable to shared alleles descended from parents of the opposite sex (one from a mother and one from a father). This experiment also showed that non-imprinted genes can generate parent-of-origin effects by interaction with imprinted loci [71]. Whether and to what extent these results can be extrapolated to humans is unknown and warrants further research.

1.4. Reconciling genetics and the environment with epigenetic mechanisms

Considering the evidence discussed previously, it is now beyond doubt that both genetics and the environment contribute to the observed variance in metabolic traits, and it is also clear that the environment interacts with the genome to bring the phenotype into being. Perhaps even more fascinating is the highly debated concept of inheritance of susceptibility to metabolic disorders from one generation to the next [72–74], since the first demonstration of the inheritance of a characteristic acquired in response to an environmental stimulus by Waddington in 1956 [75]. The contribution of genetics to variance in metabolic outcomes means that we are not all equal under the same obesogenic stresses, but that does not mean that we are doomed to grow belly fat simply because we inherited a bad combination of genetic variants. It is puzzling to see that the "nature vs. nurture" debate is still very present in the scientific literature, as illustrated by the "head to head" entitled *Are the causes of obesity primarily environmental?* published by the British Medical Journal in 2012 [76,77]. Perhaps a more relevant question would be: how do genes interact with their environment to cause metabolic disorders? The main mechanism that has been proposed to mediate this interaction encompasses the addition and removal of epigenetic marks to the DNA (e.g. **DNA methylation**), **histone post-translational modifications** and **non-coding RNAs** (ncRNAs) (Figure 6). These epigenetic modifica-

tions can change chromatin structure to cause a stable modification of gene expression or gene expression potential.

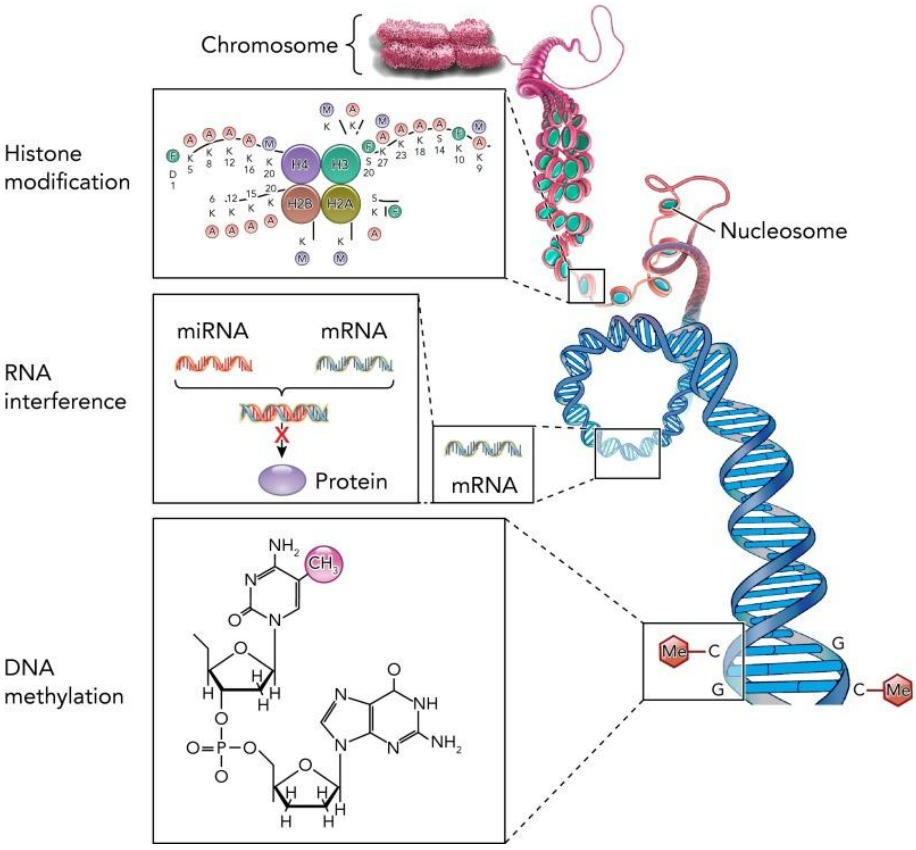


Figure 6. The three main epigenetic mechanisms involved in the control of gene expression in mammals.

Taken from [78]

2. Epigenetic modifications: focus on DNA methylation

2.1. Overview of epigenetics

2.1.1. Epigenetic modifications are diverse and determine chromatin structure

The gene expression pattern of a cell at a particular moment in time has to be adapted to the tissue it belongs and to its surrounding environment. For example, glucose must be released into the blood stream to answer the organs' glucose demand during fasting or exercise. When blood glucose is low, glucagon levels start rising while insulin levels start dropping, causing a cAMP release in hepatocytes. cAMP then triggers the phosphorylation of the TF cAMP response element binding protein (CREB), responsible for increased expression of gluconeogenic enzyme genes [79] (Figure 7A). This process requires DNA to be accessible to the transcription machinery, CREB and other TFs, so that gluconeogenic enzyme genes can be upregulated. However, DNA does not loosely float into the nucleus, but is instead wrapped around nucleosomes, forming a structure called "**chromatin**" [80]. Chromatin shows variable degrees of packaging, from the relaxed primary structure akin to beads on a string called "euchromatin", to the more tightly packaged secondary and tertiary structures called "heterochromatin" that are associated with architectural proteins (Figure 8) [81].

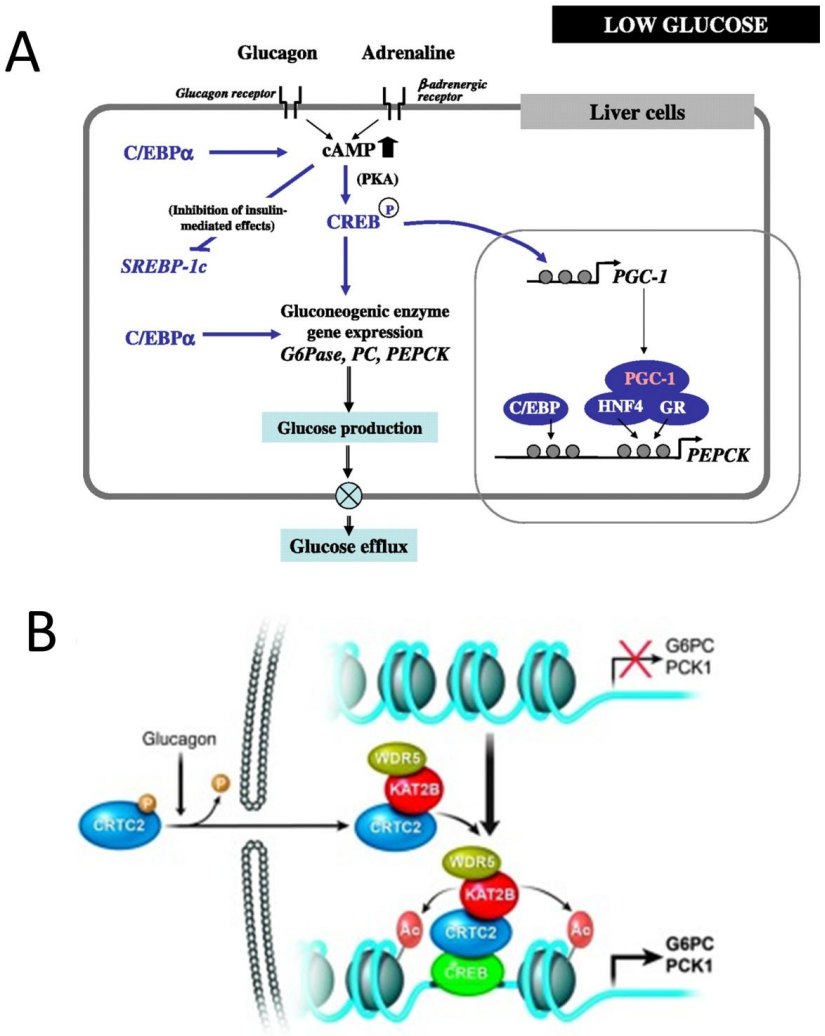


Figure 7. The three main epigenetic mechanisms involved in the control of gene expression in mammals.

(A) Transcriptional adaptation of the metabolism in liver cells upon low levels of glucose. A lack of insulin associated with increased levels of glucagon cause an increase in cAMP levels that triggers the phosphorylation of the transcription factor cAMP response element binding protein (CREB), responsible for increased expression of gluconeogenic enzyme genes. Taken from [79]. (B) Epigenetic regulation of the gluconeogenic program during fasting and in diabetes. Increases in circulating glucagon trigger the dephosphorylation and nuclear translocation of CRTC2, which associates with and mediates the recruitment of KAT2B and WDR5 to CREB binding sites on gluconeogenic promoters. In turn, KAT2B and WDR5 upregulate gluconeogenic genes through a self-reinforcing cycle in which increases in H3K9 acetylation further enhance CREB and CRTC2 occupancy**.** Taken from [82]

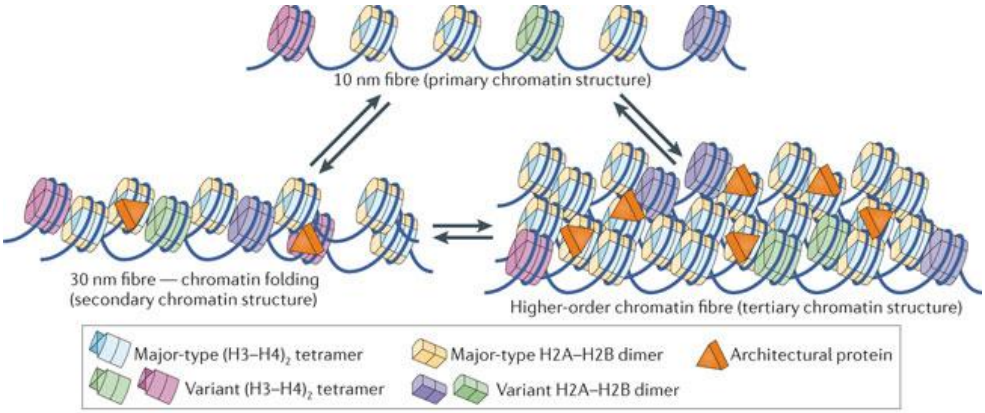


Figure 8. Primary, secondary and tertiary structure of chromatin.

The primary structure is shown as nucleosomal arrays consisting of nucleosomes with canonical histones (shown in light blue and yellow) and combinations of different histone variants (shown in green, purple and light blue). Nucleosomes with canonical or histone variants may vary in the degree of post-translational modifications, generating the possibility for nucleosomes with a large number of different 'colours'. Histone variants and PTMs may affect nucleosome structure and dynamics. The spacing between nucleosomes may vary on the basis of the underlying sequence, action of chromatin-remodelling enzymes and DNA binding by other factors (for example, transcription activators). Short-range nucleosome–nucleosome interactions result in folded chromatin fibres (secondary chromatin structure, lower left panel). Fibre–fibre interactions, which are defined by long-range interactions between individual nucleosomes, are also affected by the primary structure of chromatin fibres, including PTMs, histone variants and spacing of nucleosomes. Secondary and tertiary structures are stabilized by architectural proteins, such as linker histone H1. Transitions between the different structural states are indicated by double arrows; these may be regulated by changes in patterns of PTMs, binding or displacement of architectural proteins, exchange of histone variants and chromatin-remodelling factors. Taken from [81]

Chromatin can be seen as the "physiological form of our genetic information" [83]. Chromatin's degree of packaging and interaction with TFs depend on the combination of different covalent "epigenetic modifications" that can occur directly on DNA or on the proteins that form the nucleosomes (histones). The genome-wide pattern of epigenetic modifications (the **epigenome**) is highly **tissue-specific** because it reflects the function of the cell [84]. There are many epigenetic modifications known to date, some of which ubiquitous to all life forms while others restricted to some species, and new modifications are regularly discovered or extended to more species [85]. In mammals, amino acid residues of histone tails can undergo acetylation, mono- di- or tri-methylation, phosphorylation, ubiquitylation, and biotinylation, each of these modifications being associated with either transcriptional activation, repression or elongation as well as DNA repair, depending on which residue they target and on the genomic context [86] (Table 2). The best characterized covalent modification of DNA itself is the methylation of cytosines (5meC) in the CG context (CpG) [87], although 5meC has also been detected out of the CpG context (CpH) at substantial levels in mammals [88], notably in the mouse and human brain [89,90] as well as human skeletal muscle [91–93]. In mammals, methylation is not restricted to cytosines as adenines can become methylated, and 5meC can in turn be oxidized to form hydroxymethylcytosines (5-hmC) [94]. ncRNAs form another epigenetic mechanism; as the name suggest, ncRNAs are functional RNA molecules that are transcribed from DNA but not translated into proteins [95]. There are two major classes of ncRNAs involved in gene silencing and chromatin structure: small ncRNAs (including small interfering RNA, microRNA (miRNA), piRNA) and long ncRNAs [96]. In light of the large diversity of epigenetic modifications that coexist in the cell, it is easy to grasp why epigenetics is such a complex yet exquisite way to regulate gene expression, especially as epigenetic modifications have been demonstrated to crosstalk with one another [97–99]. We have unravelled the genetic code that determines what sequence of amino acids is translated from mRNA: could there also be an "epigenetic code" that determines what genes are allowed to be transcribed, and how cells memorize patterns of activity?

Histone	Site	Modification type	Histone-modifying enzymes	Proposed function
H2A	Lys 5	Acetylation	Tip60, p300/CBP	transcriptional activation
	Arg3	Methylation	PRMT1/6, PRMT5/7	transcriptional activation, transcriptional repression
	Ser1	Phosphorylation	MSK1	transcriptional repression
	Thr120	Phosphorylation	Bub1, VprBP	mitosis, transcriptional repression
	Lys119	Ubiquitylation	Ring2	spermatogenesis
	Lys9	Biotinylation	biotinidase	unknown
	Lys13	Biotinylation	biotinidase	unknown
H2A.X	Ser139	Phosphorylation	ATR, ATM, DNA-PK	DNA repair
	Thr142	Phosphorylation	WSTF	apoptosis, DNA repair
H2B	Lys5	Acetylation	p300, ATF2	transcriptional activation
	Lys12	Acetylation	p300/CBP, ATF2	transcriptional activation
	Lys15	Acetylation	p300/CBP, ATF2	transcriptional activation
	Lys20	Acetylation	p300	transcriptional activation
	Ser14	Phosphorylation	Mst1	apoptosis
	Ser36	Phosphorylation	AMPK	transcriptional activation
	Lys120	Ubiquitylation	UbcH6	meiosis
H3	Lys9	Acetylation	Gcn5, SRC-1	transcriptional activation
	Lys14	Acetylation	Gcn5, PCAF, SRC-1, p300	transcriptional activation
			Esa1, Tip60	DNA repair
			Elp3, Sas3	transcriptional activation (elongation)
			Hpa2	unknown
			hTFIIIC90, TAF1	RNA polymerase III transcription
			Sas2	euchromatin
	Lys18	Acetylation	Gcn5	transcriptional activation, DNA repair
			p300/CBP	DNA replication, transcriptional activation
	Lys23	Acetylation	Gcn5	transcriptional activation, DNA repair
			Sas3	transcriptional activation (elongation)
			p300/CBP	transcriptional activation
	Lys27	Acetylation	Gcn5	transcriptional activation
	Lys26	Acetylation	Gcn5	transcriptional activation
	Arg2	Methylation	PRMT5, PRMT6	transcriptional repression
	Arg8	Methylation	PRMT5, PRMT2/6	transcriptional activation, transcriptional repression
	Arg17	Methylation	CARM1	transcriptional activation
	Arg26	Methylation	CARM1	transcriptional activation
	Arg42	Methylation	CARM1	transcriptional activation
	Lys4	Tri-methylation	Set7/9	transcriptional activation
		Methylation	MLL, ALL-1	transcriptional activation

	Lys9	Tri-methylation	Suv39h, Clr4, SETDB1	transcriptional repression
		Methylation	G9a	transcriptional repression genomic imprinting
	Lys27	Methylation	Ezh2, G9a	transcriptional silencing
		Tri-methylation	Ezh2	X inactivation
	Lys36	Methylation	Set2	transcriptional activation (elongation)
	Lys79	Methylation	Dot1	euchromatin, transcriptional activation (elongation), checkpoint response
	Ser10	Phosphorylation	Aurora-B kinase	mitosis, meiosis
			MSK1, MSK2	immediate-early gene activation
			IKK-α, Snf1	transcriptional activation
	Ser28	Phosphorylation	Aurora-B kinase	mitosis
			MSK1, MSK2	immediate-early gene activation
	Thr3	Phosphorylation	Haspin/Gsg2	mitosis
	Thr11	Phosphorylation	Dlk/Zip	mitosis
	Tyr41	Phosphorylation	JAK2	transcriptional activation
	Tyr45	Phosphorylation	PKCδ	apoptosis
	Lys4	Biotinylation	biotinidase	gene expression
	Lys9	Biotinylation	biotinidase	gene expression
	Lys18	Biotinylation	biotinidase	gene expression
H4	Lys5	Acetylation	Hat1	histone deposition
			Esa1, Tip60	transcriptional activation, DNA repair
			ATF2, p300	transcriptional activation
			Hpa2	unknown
	Lys8	Acetylation	Gcn5, PCAF, ATF2, p300	transcriptional activation
			Esa1, Tip60	transcriptional activation, DNA repair
			Elp3	transcriptional activation (elongation)
	Lys12	Acetylation	Hat1	histone deposition, telomeric silencing
			Esa1, Tip60	transcriptional activation, DNA repair
			Hpa2	unknown
			p300	transcriptional activation
	Lys16	Acetylation	Gcn15, ATF2	transcriptional activation
			Esa1, Tip60	transcriptional activation, DNA repair
			Sas2	euchromatin
	Arg3	Methylation	PRMT1/6	transcriptional activation
			PRMT5/7	transcriptional repression
	Lys20	Methylation	PR-Set7	transcriptional silencing
		Tri-methylation	Suv4-20h	heterochromatin
	Ser1	Phosphorylation	CK2	DNA repair
	Lys12	Biotinylation	biotinidase	DNA damage response

Table 2. Main histone modifications known in mammals.

Adapted from [86]

2.1.2. Mitotic heritability and maintenance of epigenetic modifications

Epigenetic modifications have two fundamental properties that give cells the power to adapt to the situation while retaining a memory of their past gene expression: epigenetic modifications are **sensitive to environmental stimuli** such as diet, smoking, physical activity, and chemical pollutants [100], yet **stable in time and through cell divisions** even after the initial stimulus is gone. As Adrian Bird concisely puts it, epigenetics corresponds to "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" [101]. The pattern of epigenetic modifications at specific genomic locations reflect the developmental and functional history of the genes, and it is this pattern that will guide their present and future activity [102]. This is achieved thanks to the dynamics of a large battery of proteins that can add ("**writers**"), remove ("**erasers**") or bind ("**readers**") epigenetic modifications (Figures 9-10). DNA methyltransferases (DNMTs) are writers that add methyl groups to DNA [103], while the ten-eleven translocation (TET) family of enzymes are responsible for the oxidation of the methyl groups that can then be actively or passively removed [104] (Figure 9). Histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs) and kinases add covalent marks on histone tails, while histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphorylases remove these marks [105] (Figure 10, Table 2). There are so many known readers of epigenetic modifications that listing them exhaustively would needlessly drown this chapter [105]. Readers of histone modifications contain domains that recognize specifically acetyllysines (e.g. bromodomain), methyllysine (e.g. chromodomains) or methylarginine (e.g. Tudor domain). The reading of DNA methylation involves proteins that specifically bind methylated CpGs (MBPs), proteins that specifically bind unmethylated CpGs (CXXC domain–containing complexes), and many TFs that are sensitive to the presence of 5meC in their binding sequence [87]. However, the mitotic/meiotic heritability of epigenetic modifications has raised some debates over what truly deserves to be called "epigenetic", since many epigenetic modifications are erased at each cell division and do not generate memory [106,107], and since some epigenetic modifications quickly disappear once the initial stimulus is gone [108–110]. This is particularly true for histone marks, and Mark Ptashne has asked for the banishment of histone marks from the list of epigenetic modifications [111]. Nevertheless, previously expressed genes are frequently primed for reactivation [112] and histone modifications are involved in transcription *per se*. In the example of glycaemic control, the presence of glucagon triggers in hepatocytes the recruitment of lysine acetyltransferase 2B (KAT2B) to CREB binding sites on gluconeogenic promoters. KAT2B adds an acetyl group to the lysine 9 of histone H3, which further enhances CREB occupancy on DNA to upregulate gluconeogenic genes [113] (Figure 7B). While theoretical discussions on the definition of epigenetics are interesting, perhaps is it more important to keep in mind that the heritability of epigenetic modifications is dependent on the mark under study [114,115], and careful conclusions should be drawn whenever studying a specific mark.

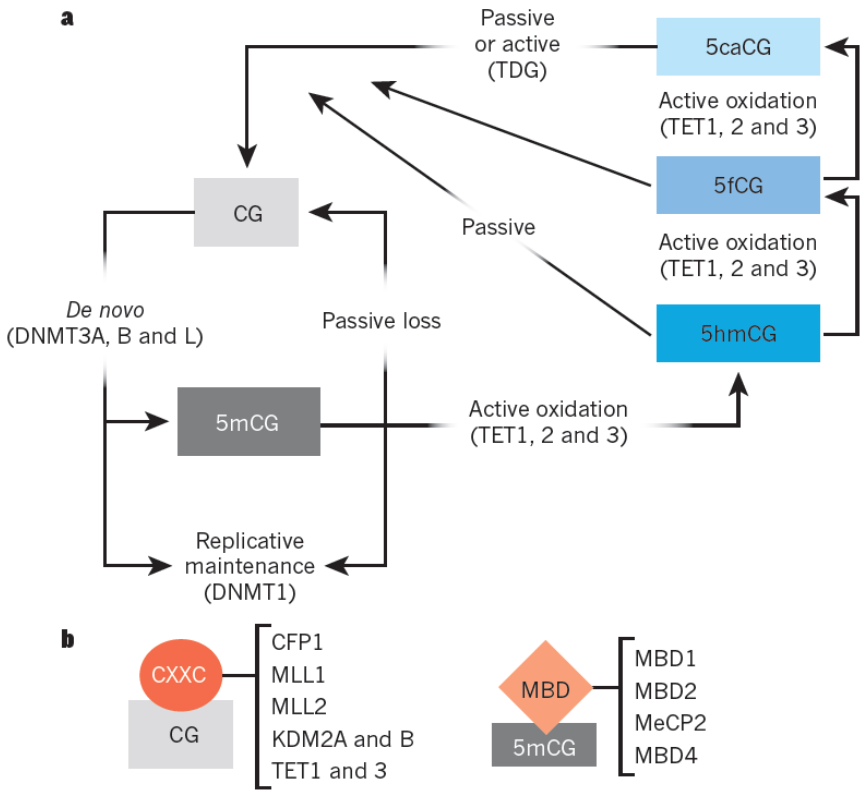


Figure 9. Setting, erasing and recognizing cytosine methylation.

a, Different methylation states of the CpG and the enzymatic pathways that set, maintain and erase the mark. The pathways leading from the oxidized forms to the unmethylated state are under debate. DNMT, DNA methyltransferase; TDG, thymine-DNA glycosylase. b, A subset of CXXC-domain-containing proteins are listed that can specifically bind to the unmethylated CpG and could potentially reinforce the unmethylated state or recruit regulatory proteins to unmethylated CpG islands. Methyl-CpG-binding domain (MBD) proteins specifically bind to the methylated CG with little or no further sequence sensitivity, potentially mediating transcriptional repression, which would be strongest in methylated CpG islands. Readers of oxidized forms are not shown owing to ongoing debate about proposed candidates. Taken from [87]

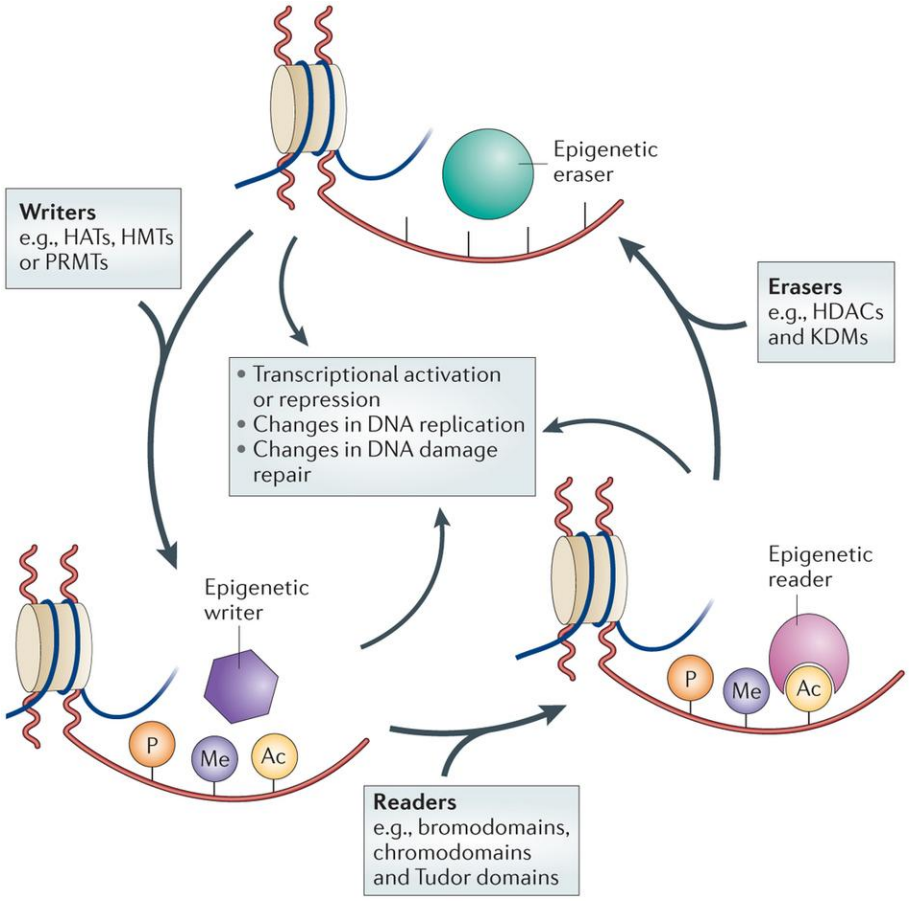


Figure 10. Setting, erasing and recognizing histone modifications.

Epigenetic regulation is a dynamic process. Epigenetic writers such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs) and kinases lay down epigenetic marks on amino acid residues on histone tails. Epigenetic readers such as proteins containing bromodomains, chromodomains and Tudor domains bind to these epigenetic marks. Epigenetic erasers such as histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphatases catalyse the removal of epigenetic marks. Addition and removal of these post-translational modifications of histone tails leads to the addition and/or removal of other marks in a highly complicated histone code. Together, histone modifications regulate various DNA-dependent processes, including transcription, DNA replication and DNA repair. Taken from [105]

2.2. Function and information content of DNA methylation in the CpG context

2.2.1. Distribution of CpG sites in the mammalian genome

The mammalian genome contains millions of CpGs, 60-90% of which are methylated [116], suggesting that methylation of CpGs is actually a default state (Figure 11A). Because methylated cytosines have a high deamination rate [117], many cytosines have mutated into thymine over the course of evolution, despite the presence of two glycosylases that are thought to repair this mismatch [118]. As a consequence, CpGs occur at only 21% of the expected frequency in the human genome [119], and the genomic regions that are typically unmethylated show an unusually high frequency of CpGs. These regions, called "**CpG islands**" (CGIs), are ~1000 base pairs long and show an elevated G+C base composition [120]. In humans, 40% of CGIs are found in annotated gene promoters [121,122] and the remaining 60% have been termed "orphan CGIs" due to the uncertainty surrounding their annotation [123] (Figure 11B). However, it seems that despite not being annotated to any specific promoter, many orphan CGIs are actual sites of transcription initiation for nearby annotated genes or ncRNAs [123,124]. Conversely, 70% of annotated gene promoters are associated with a CGI [125], especially housekeeping genes [126]. CGIs are interspaced by long stretches of highly methylated CpG-poor regions that are found both within and between genes [87]. Following the marine metaphor

of the CpG island, one can find the “CGI shore” from 0 to 2 kb on either side of a CGI, and the “CGI shelf” from 2 to 4kb on either side of a CGI (Figure 11B). Any CpG site that is not located in a promoter, a gene body, a CGI, a CGI shore or a CGI shelf is said to be located in the “**open sea**”.

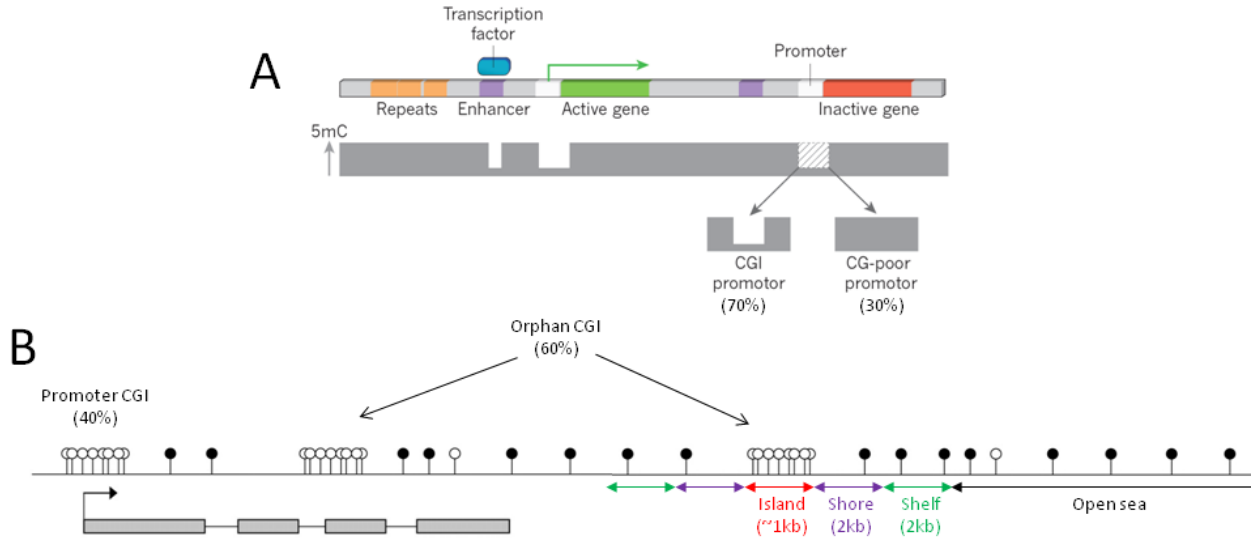


Figure 11. Distribution and organisation of 5meC in the mammalian genome.

(A) Genomic distribution of methylated cytosine in a typical mammalian genome. The representative genomic region includes an example of an active and an inactive gene with proximal (promoter) and distal (enhancer) regulatory regions. The height of the bar indicates the relative proportion of DNA methylation (5-methylcytosine, 5mC) that is observed in each region. CpG islands (CGIs), which often overlap with promoter regions, generally remain unmethylated, whereas CG-poor promoters are methylated when not active. Adapted from [87]. (B) Genomic distribution of CGIs. Many CGIs are found in gene promoters, but the majority are located in intragenic or intergenic regions and termed "orphan CGIs" due to the uncertainty surrounding their annotation. Following the metaphor of the CpG "island", the regions located up to 2kb away from the island is called "CGI shore", and the region up to 4kb termed "CGI shelf". CpG sites that are not located in a promoter, a gene body, an island, a shore or a shelf is said to be located in the "open sea". Adapted from [120].

2.2.2. DNA methylation patterns are deeply linked to the underlying chromatin state

Interestingly, DNA methylation does not have the same effects on transcription whether it occurs in promoters, enhancers or gene bodies, and whether it happens in CGIs, in the regions flanking CGIs, or in CpG-poor regions (Figure 12). What we know best is how CGIs in the promoter context work to control gene expression. Promoter CGIs are usually unmethylated, but when they become methylated, they robustly repress transcription, as notably seen in X-chromosome inactivation [127] and genomic imprinting [128]. The role of DNA methylation at CpG-poor promoters is less clear. CpG-poor promoters display tissue-specific methylation patterns [129], and there is an inverse correlation between methylation at these promoters and transcription [130]. In addition, *in vitro* removal of DNA methylation at CpG-poor promoters can result in their direct silencing [131]. This suggests that there may be a role for methylation at CpG-poor promoters in the establishment and maintenance of tissue-specific expression patterns. However, it has also been shown that CpG-poor promoters could still be expressed when they are methylated [132], and that it is the binding of TFs that shape DNA methylation profiles at CpG-poor regions [133]. Therefore, the role of methylation at CpG-poor promoters is not well understood, but potential effects might only apply to certain binding sites such as those with lower affinity, at which DNA methylation might further reduce the likelihood of binding [87]. On the contrary, intragenic methylation positively correlates with transcription [134], and it may well serve several functions at once. Like intergenic methylation [135], intragenic methylation was initially suggested to silence repetitive DNA elements that would otherwise cause genomic instability [136]. However, it was discovered that when intragenic methylation occurs at a CGI, it can suppress intragenic

promoters [124]. Also, intragenic methylation might be involved in alternative splicing events [137], but evidence for this phenomenon is scarce [138]. Recent attention has been drawn to CGI shores that lie in close proximity of CGIs [139]. In several pathologies such as cancer [140,141], intrauterine growth restriction [142], and in normal conditions that compared men and women [143], monozygotic twins [144] or simply different cell types [145], differentially methylated CpG sites were found to be enriched in CGI shores. Last but not least, CpG-poor regions span many enhancers, and methylation at enhancers is crucial for early development [146–149]. Importantly, genome-wide patterns of DNA methylation are adapted to the tissue they belong to: promoters of tissue-specific genes are often unmethylated in the tissues in which they are expressed, and methylated in the tissues where they are silenced [132,150]. In the end, all these observations show that it is not merely the presence of DNA methylation that determines its relation to transcription, but its interpretation in the context of a particular genomic region [136].

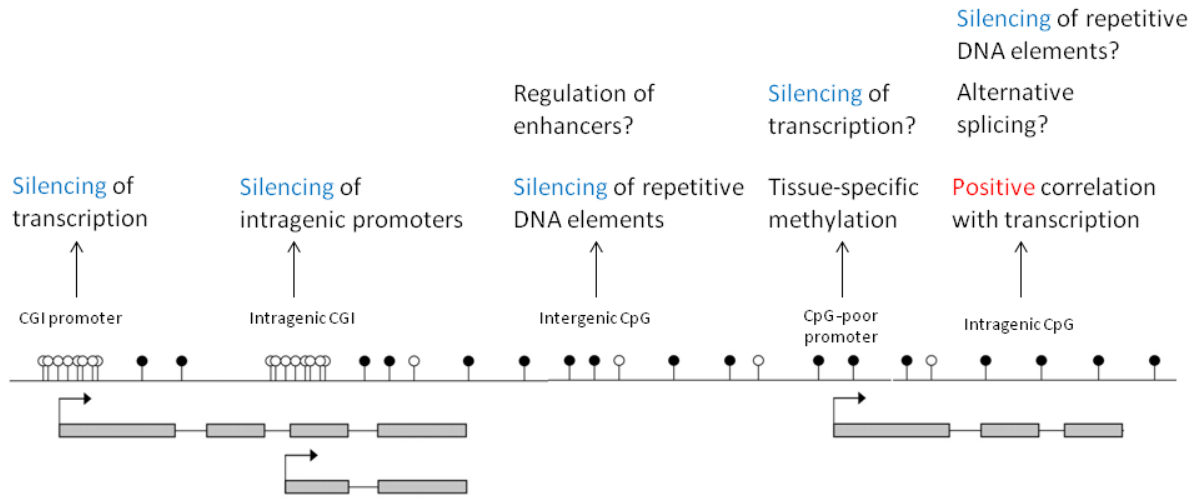


Figure 12. Proposed functions of DNA methylation in different genomic contexts.

The function of DNA methylation is well established at CGI promoters, where it stably represses transcription. Genes with CpG-poor promoters usually show tissue-specific methylation, and methylation in their promoter may repress transcription, especially at binding sites with lower affinity. Within gene bodies, CGIs are thought to silence intragenic promoters. Intragenic methylation positively correlates with transcription and could also silence repetitive elements, or participate in alternative splicing events. Intergenic methylation has a role in silencing repetitive DNA elements, and probably in regulating certain enhancers.

2.2.3. DNA methylation and transcription influence one another

Given that the roles of DNA methylation are so context-dependent, what do we know about the mechanistic and causal relationship between DNA methylation and gene expression? The emerging picture involves the complex interaction between DNA methylation, nucleosomes, MBPs and methylation-sensitive TFs [151] (Figure 13). DNA methylation can impair the binding of specific TFs to DNA [152–155], recruit transcriptionally repressive MBPs [156–159], and affect nucleosome positioning [160]. In their methylated state, promoter CGIs are bound by MBPs, which in turn prevent the binding of methyl-CpG binding TFs; in their unmethylated state, promoter CGIs are bound by CXXC domain–containing complexes that activate transcription. This scenario does not fit well transcription of genes with CpG-poor promoters though, as MBPs are sensitive to the local density in CpGs [161]. At enhancers, methylation can prevent the binding of methyl-sensitive TFs, or attract methyl-CpG binding TFs, leading to either gene activation or repression. Nevertheless, while it is now acknowledged that DNA methylation influence TF binding, it also seems that TF binding itself as well as transcription shape DNA methylation patterns, a situation very reminiscent of the feedback loops often observed in biology (Figure 14). In cancer for example, gene silencing precedes the establishment of DNA meth-

ylation at CGI promoters [130,162–164]. DNA methylation subsequently happens because inactive genes are often more susceptible to *de novo* methylation, providing added stability to the silent state [136] (Figure 15). Interestingly, while transcription of a gene harbouring a methylated CGI in its promoter is unheard of, a gene containing an unmethylated CGI in its promoter is not necessarily transcribed [132]. In this respect, methylation at promoter CGIs could be seen as a sort of genomic "rust" that accumulates when a gene is not in use [165]. Therefore, **methylation at promoter CGIs** is not the mechanism triggering silencing, but rather the mechanism allowing **gene expression to become "locked"**. At CpG-poor regions, methylation can change as a result of TF binding [166–168], and does not necessarily impair the binding of these TFs [166]. The current data suggest that **TF binding** is the central event that mediates concerted changes in other regulatory mechanisms determining chromatin states, accessibility, and conformation [169]. The issue of causality in CpG-poor regions is far from being solved, and it seems that DNA methylation is much more complex and context-dependent than previously thought.

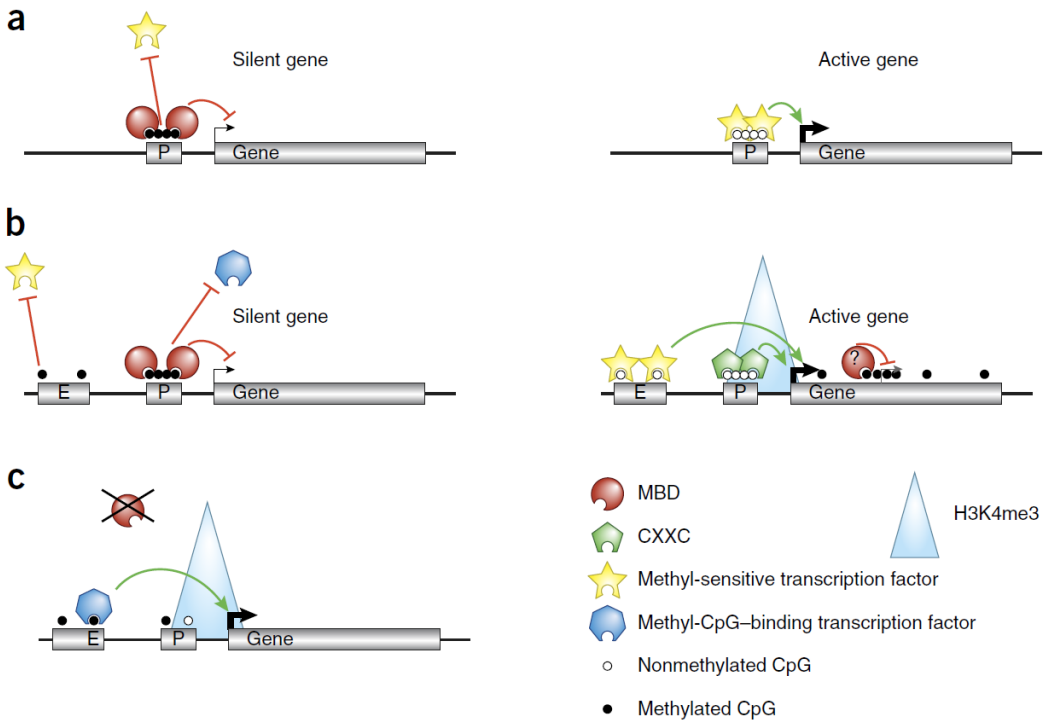


Figure 13. Models of transcriptional regulation by DNA methylation.

(a) The 'old' textbook model describing how DNA methylation regulates transcription. Left, methylated CpG-island promoters (P) recruit transcriptionally repressive MBD proteins and prevent transcription-factor binding. Right, nonmethylated CpG islands are bound by transcription factors. (b) New models describing regulation of transcription by DNA methylation. Left, genes with methylated CpG-island promoters are repressed by repressive MBD-containing complexes. In addition, methylation of an enhancer (E) can block binding of a transcription factor. Right, most active genes with nonmethylated CpG-island promoters are bound by CXXC domain–containing activator complexes. In addition, transcription factors bind to nonmethylated enhancers. Finally, gene bodies of active genes are highly methylated, and this serves to repress cryptic transcription. (c) Uncoupling between DNA methylation and repression of transcription initiation. In some cases, such as during early vertebrate development, some methylated promoters with low CpG density are actively transcribed. Transcriptionally repressive MBD proteins do not interact with these promoters, for yet-unknown reasons. Furthermore, some DNA sequences with low CpG density (including enhancers and promoters) can be bound by activating transcription factors. H3K4me3, trimethylated histone H3 Lys4, a promoter-associated histone mark associated with active transcription. Taken from [151]

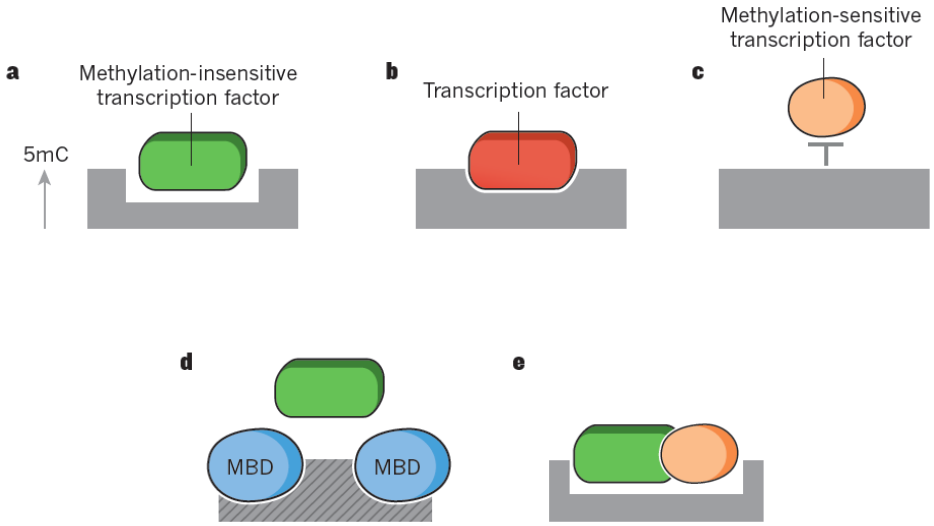


Figure 14. Potential scenarios for the interplay between cytosine methylation (shown by level of 5-methylcytosine) and transcription-factor binding.

(**a**) A methylation-insensitive transcription factor causes reduced methylation after binding. (**b**) A transcription factor binds specifically to the methylated state of its binding site. (**c**) A methylation-sensitive transcription factor is blocked by 5-methylcytosine (5mC). (**d**) Methyl-CpG-binding domain (MBD) proteins bind to the methylated state, leading to indirect repression, which probably requires high local density of CGs (shading). (**e**) A methylation-insensitive transcription factor functions as a pioneer factor and creates a site of reduced methylation that allows a methylation-sensitive factor to bind. Taken from [87]

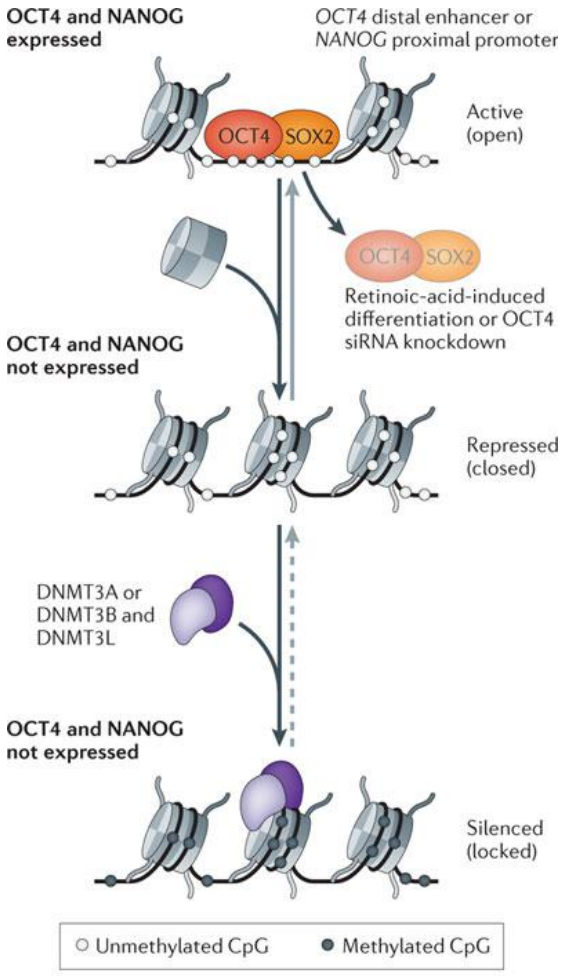


Figure 15. Silencing precedes DNA methylation.

Active promoters and enhancers have nucleosome-depleted regions (NDRs) that are often occupied by transcription factors and chromatin remodellers. Loss of factor binding — for example, during differentiation — leads to increased nucleosome occupancy of the regulatory region, providing a substrate for de novo DNA methylation. DNA methylation subsequently provides added stability to the silent state and is likely to be a mechanism for more accurate epigenetic inheritance during cell division. The example given is for the OCT4 and NANOG genes, and its generality is not yet known, but inactive genes are often more susceptible to de novo methylation than their more active counterparts. In the figure, OCT4 binding is shown and NANOG binding is not shown, although its expression is required. Recent experiments have demonstrated that the methylation must be removed by active and/or passive processes to reactivate the gene. DNMT3A, DNA methyltransferase 3A; siRNA, small interfering RNA. Taken from [136]

2.2.4. Genetic influences on DNA methylation

It is now largely documented that genetic polymorphisms located in the vicinity of TF binding sites impact TF binding [170,171], but since TF binding and DNA methylation are so tightly linked, can genetic polymorphisms impact DNA methylation patterns? Recent studies have shown that not only do genetic polymorphisms impact DNA methylation, but the vast majority of inter-individual variations in methylomes are caused by genetic polymorphisms [172,173]. In fact, methylome variations are so tightly linked to the underlying DNA sequence that Whitaker *et al*. developed a pipeline called Epigram that uses DNA motifs to predict the methylation status at tissue-specific **differentially methylated regions** (DMRs) from 18 human tissues [174]. The regions associated with differential methylation levels have been termed **methylation QTLs** (meQTLs) and have been identified in a wide variety of human tissues [175–187]. Although mostly located in *cis* (i.e. close to the CpG site they regulate), a small number of meQTLs have also been uncovered in *trans* [188,189]. Moreover, there is substantial overlap between meQTLs detected in blood and in the brain, suggesting that some meQTLs could exert ubiquitous effects on DNA methylation [182].

How do genetic variants cause differential DNA methylation? If a mutation is located directly on a CpG, a methylation site is removed or added, thus allowing or preventing methylation to happen. If a mutation is not directly located on a CpG, the proposed model is that this mutation lies within a regulatory region and affects TF binding, which in turn influences DNA methylation [87,169] (Figure 16). An alternative explanation is that the mutation happens directly in the TF, changing its ability to bind DNA, which in turn influences DNA methylation [87]. However, differences in methylation caused by genetic variants do not necessarily result in differences in gene expression [169]. Clearly, a lot more research is needed in order to assess issues of causality, the order of regulatory events, and the direction of effects.

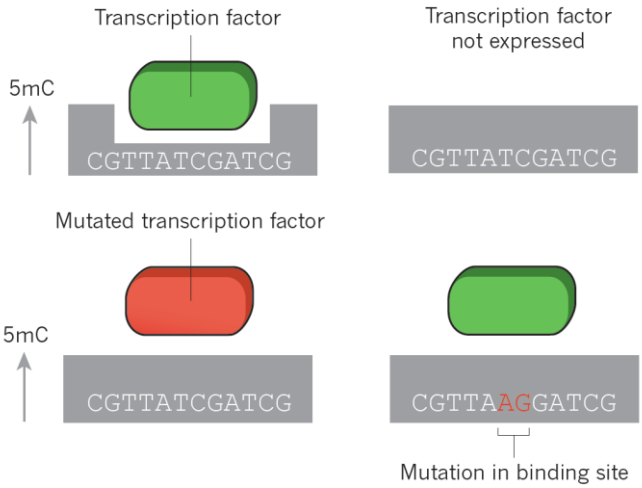


Figure 16. Potential DNA sequence determinants of cytosine methylation at CpG-poor regions.

In a simplified model, transcription-factor binding causes reduced methylation at its binding site. Loss of expression of the respective transcription factor in development or disease will cause increased methylation. Mutations to the transcription factor that affect its binding preference will influence genomic methylation patterns. Mutation in the DNA binding site will abolish binding even in a cell expressing the transcription factor, indicating how genetic variation can result in methylation differences between individuals. Taken from [87]

3. Studying DNA methylation in the context of obesity

The past few years has seen a marked increase in the number of studies investigating the relationship between DNA methylation and obesity, particularly since obesity was classified as a disease by the American Medical Association [9]. We have gained interesting insights into the importance of DNA methylation in the context of obesity, but we are now aware of the biological, bioinformatical and statistical limitations of these studies. From observational to interventional studies, from animal models to human studies, what have we learned on the implication of DNA methylation in the pathogenesis of obesity?

3.1. Measuring DNA methylation

Before reviewing the literature on what we already know about DNA methylation in the context of obesity and its complications, it is important to know that the information generated by methylation studies is highly dependent on the technique used. More than 30 different assays have been developed using PCR, endonuclease digestion, affinity enrichment or bisulfite conversion to look at **global methylation levels**, **region-specific methylation** and **genome-wide methylation** (spanning the entire genome) [190,191] (Table 3). All of these techniques have their own advantages and caveats that are important to keep in mind when interpreting results (Table 4). Techniques looking at global methylation level allow to obtain information on the % of 5meC in the sample, but do not give information on

the % of 5meC at specific locations in the genome. On the contrary, techniques looking at genome-wide methylation will give the % of 5meC at known locations in the genome. The **genome coverage** of these techniques varies greatly, from 8 to 100% for global methylation assays, and from 0.12% to 100% for site-specific methylation assays (Figure 17). For instance, high performance liquid chromatography ultraviolet (HPLC-UV) and whole-genome bisulfite sequencing (WGBS) have 100% genome coverage and represent the gold standards of global and region-specific DNA methylation assessment, respectively. Another important aspect to keep in mind is that DNA samples are usually derived from a pool of different cells which may vary in their 5meC levels [191]. Many techniques can read the methylation level of a target sequence on individual DNA strands (e.g. reduced representation bisulfite sequencing (RRBS), WGBS, methylated DNA immunoprecipitation (MeDIP)-seq) (Figures 17-18) but the more popular Infinium beadarrays read a methylation level that has been averaged over many DNA molecules (Figure 17). **Allele-specific methylation** can yield interesting information on the co-occurrence of 5meC on the same DNA strand, and potential insights into the function and regulation of DNA methylation at the target sequence (Figure 19). Finally, it should be noted that all the aforementioned techniques do not allow the discrimination of 5meC from 5hmC, but there are digestion- and antibody-based techniques available [190].

Pretreatment	Analytical step			
	Locus-specific analysis	Gel-based analysis	Array-based analysis	NGS-based analysis
Enzyme digestion	• *Hpa*II-PCR	• Southern blot • RLGS • MS-AP-PCR • AIMS	• DMH • MCAM • HELP • MethylScope • CHARM • MMASS	• Methyl–seq • MCA–seq • HELP–seq • MSCC
Affinity enrichment	• MeDIP-PCR		• MeDIP • mDIP • mCIP • MIRA	• MeDIP–seq • MIRA–seq
Sodium bisulphite	• MethyLight • EpiTYPER • Pyrosequencing	• Sanger BS • MSP • MS-SNuPE • COBRA	• BiMP • GoldenGate • Infinium	• RRBS • BC–seq • BSPP • WGSBS

Table 3. Main principles of region-specific DNA methylation analysis.

AIMS, amplification of inter-methylated sites; BC–seq, bisulphite conversion followed by capture and sequencing; BiMP, bisulphite methylation profiling; BS, bisulphite sequencing; BSPP, bisulphite padlock probes; CHARM, comprehensive high-throughput arrays for relative methylation; COBRA, combined bisulphite restriction analysis; DMH, differential methylation hybridization; HELP, HpaII tiny fragment enrichment by ligation-mediated PCR; MCA, methylated CpG island amplification; MCAM, MCA with microarray hybridization; MeDIP, mDIP and mCIP, methylated DNA immunoprecipitation; MIRA, methylated CpG island recovery assay; MMASS, microarray-based methylation assessment of single samples; MS-AP-PCR, methylation-sensitive arbitrarily primed PCR;MSCC, methylation-sensitive cut counting; MSP, methylation-specific PCR; MS-SNuPE, methylation-sensitive single nucleotide primer extension; NGS, next-generation sequencing; RLGS, restriction landmark genome scanning; RRBS, reduced representation bisulphite sequencing; –seq, followed by sequencing; WGSBS, whole-genome shotgun bisulphite sequencing. Taken from [191]

Technology	Features							Potential sources of bias							
	Unambiguous identification of CpG measured	In *cis* co-methylation information	Non-CpG methylation information	Allele-specific measurement capability	Good coverage of regions with low CpG density	Compatible with low amounts of input DNA	Full repeat-masked genome coverage	Copy-number variation bias	Fragment size bias	Incomplete bisulphite conversion bias	Bisulphite PCR bias	Cross-hybridization bias	DNA methylation status bias	GC content bias	CpG density bias
Infinium	(•)					•				•	•	•			
Enzyme–chip	(•)	(•)			(•)				•			•		•	
MeDIP–chip							•	•				•		•	•
BSPP	•	•	•	•						•	•		•		
BC–seq	•	•	•	•						•	•		•		
RRBS	•	•	•	•		•				•	•				
Enzyme–seq	•	•		•	(•)	•			•						
MeDIP–seq				•			•	•						•	•
WGSBS	•	•	•	•	•	•	•			•	•				

Table 4. Features and sources of bias for various techniques.

'•' indicates that the method has this feature or potentially has this bias; '(•)' indicates that the method has this feature to a limited extent or in some circumstances. BC–seq, bisulphite conversion followed by capture and sequencing; BSPP, bisulphite padlock probes; –chip, followed by microarray; MeDIP, methylated DNA immunoprecipitation; RRBS, reduced representation bisulphite sequencing; –seq, followed by sequencing; WGSBS, whole-genome shotgun bisulphite sequencing. Taken from [191]

Figure 17. Coverage of several global and genome-wide DNA methylation assays.

Global DNA methylation assays do not give information on methylation at specific genomic regions. LINE-1 (long interspersed nuclear elements) + pyrosequencing assesses methylation at repetitive elements; LUMA (luminometric methylation assay) assesses methylation at CCGG sites. Among the genome-wide methylation assays, only WGBS (whole genome bisulfite sequencing) and RRBS (reduced representation bisulfite sequencing) can discriminate the maternal and the paternal DNA strands and only they can detect methylation out of the CpG context (CpH). The very popular 27k, 450k and EPIC chips target 99% of Refseq gene promoters and some known enhancers but have very low whole genome coverage. MSCC (methylation sensitive cut counting) will yield information on methylation at restriction enzyme sites, such as HpaII sites.

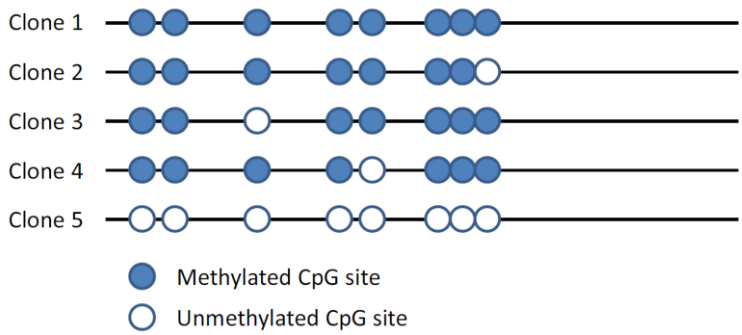


Figure 18. Typical representation of the results for DNA methylation after bisulfite sequencing.

The PCR product is cloned, and several randomly selected plasmids are sequenced. Taken from [190]

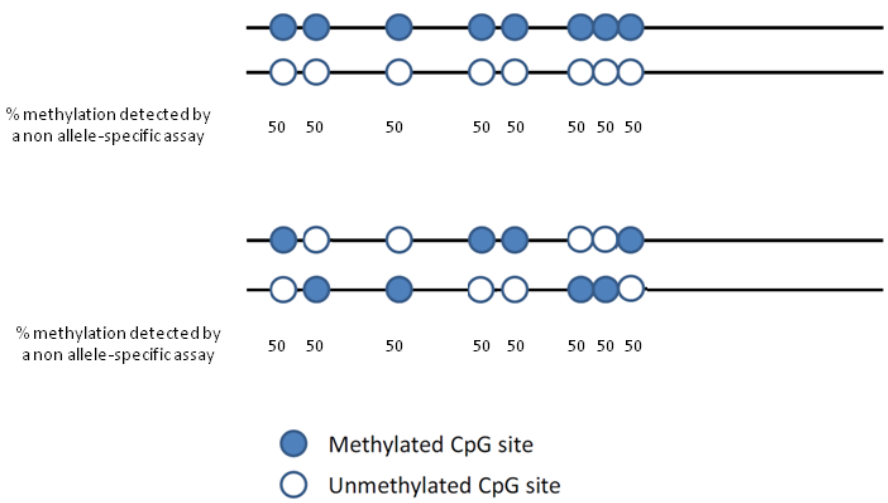


Figure 19. Illustration of the information yielded by allele-specific and non allele-specific methylation assays.

A non allele-specific methylation assay would detect a 50% methylation rate at every one of the CpG positions. However, a methylation assay that can discriminate between both DNA strands would reveal that in the top panel, one DNA strand is entirely methylated while the other is completely unmethylated, and it would reveal that in the bottom panel, methylated CpGs are not located on the same strand. This can be important to understand how DNA methylation is regulated, how genetic variants can impact DNA methylation, and how DNA methylation can act on transcription. Adapted from [190]

3.2. What we already know about DNA methylation in the context of obesity

3.2.1. The link between energy homeostasis and DNA methylation

Obesity is above all a long-term disturbance of energy homeostasis that involves major tissues such as the adipose tissue, brain, digestive tract, liver, pancreas and skeletal muscle (Figure 20). Energy homeostasis relies on the **balance between catabolism and anabolism**, two antagonist cellular processes sensitive to nutrient, endocrine and neuronal inputs. At the molecular level, the presence of amino acids, glucose, insulin, leptin, fatty acids and cholesterol (during moments of positive energy balance), or the presence of glucagon and ghrelin (during moments of negative energy balance), are sensed by ligand-dependent or cell membrane receptor-dependent TFs that translocate to the nucleus to activate or repress gene expression [192] (Figure 21). Gene expression is then finely tuned by co-regulators that include DNA methylation writers and erasers such as DNMTs and TET proteins. Yet, surprisingly few studies have investigated DNA methylation changes during the normal feeding/fasting cycle and energy homeostasis of healthy subjects. At the organismal level, the tissue-specific conditional knockout of *Dnmt3a* in the paraventricular nucleus of the hypothalamus led to hyperphagia, decreased energy expenditure, and glucose intolerance with increased serum insulin and leptin, suggesting that

Dnmt3a is required for normal energy homeostasis in mice [193]. In humans, Barrès *et al*. were the first to demonstrate that skeletal muscle contraction during a single bout exercise causes rapid DNA demethylation followed by remethylation at promoters of candidate genes important for metabolism (peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1A*), transcription factor A, mitochondrial (*TFAM*), peroxisome proliferator-activated receptor beta (*PPARδ*) and pyruvate dehydrogenase kinase 4 (*PDK4*)). Interestingly, only high-intensity (vs. low-intensity) exercise could cause these changes [93]. In a later well-designed study, Lindholm *et al*. found modest (<10%) but widespread DNA methylation changes in skeletal muscle after 3 months of endurance training in healthy men and women [194]. These methylation changes were enriched in intergenic regions and enhancers, and there were coordinated transcriptional changes corresponding to structural remodelling of the muscle and glucose metabolism, inflammatory/immunological processes and transcriptional regulation. A few *in vitro* studies also support the implication of DNA methylation in energy homeostasis to some extent. The presence of glucose and insulin leads to the enhancement of DNMT activity and global DNA methylation measured by HPLC in the human HepG2 cell line [195]. Pancreatic islets treated with palmitate [196], and THP-1 monocytes treated with arachidonic or oleic acid [197] displayed small (<3%) and widespread methylome changes that were not significant at the genome-wide level. This is in line with another study suggesting that acute changes in methylation may not be a predominant mechanism for controlling fatty acid-induced changes in mRNA in skeletal muscle, and that it may apply to only a few genes such as *PPARδ* [198]. Still, some of the top differentially methylated genes in pancreatic islets and monocytes were accompanied by corresponding changes in gene expression [196,197]. However, it should be noted that these studies used the Illumina HumanMethylation 450k beadchip that assesses only ~2% of all CpG sites in the genome.

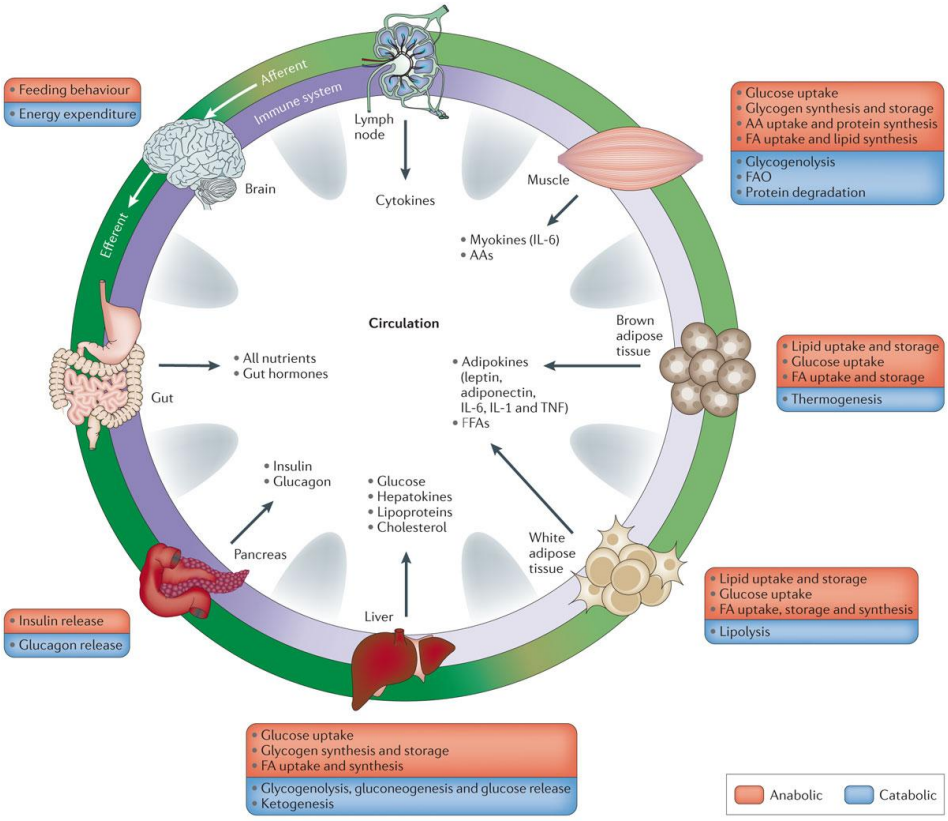


Figure 20. Whole-body energy homeostasis.

A simplified view of the major tissues involved in the regulation of whole-body energy homeostasis and their contributions to anabolic and catabolic control and hormone release is shown. AAs, amino acids; FA, fatty acid; FAO, fatty acid oxidation; FFAs, free fatty acids; IL, interleukin; TNF, tumour necrosis factor. Taken from [192]

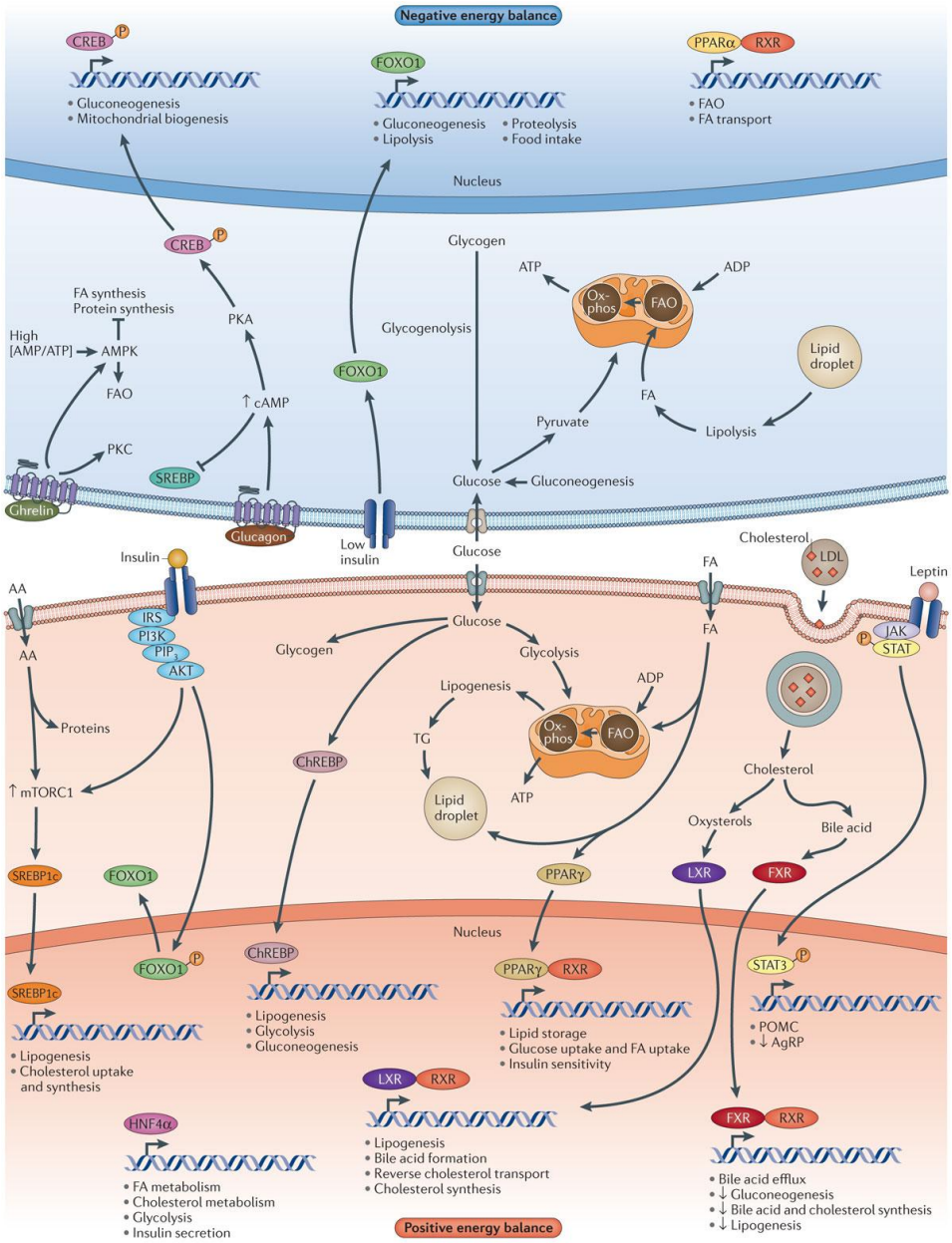


Figure 21. Transcriptional regulatory networks in positive and negative energy balance.

A schematic summarizing the transcriptional responses to energy status in the organism. The figure is a simplification, as these states and transitions between them are not always mutually exclusive. Depending on the energy state of the organism, cells have to amplify or repress specific energy pathways to guarantee appropriate energy utilization. This is accomplished in part by distinct TFs that mediate the expression of genes involved in anabolic or catabolic pathways. TFs are activated in response to metabolite signals associated with the anabolic or catabolic state, including relative concentrations of amino acids (AAs), glucose and fatty acids (FAs) present in the cytoplasm. In negative energy balance, AAs, glucose and FAs, which are generated from storage molecule breakdown (protein degradation, glycogenolysis and lipolysis, respectively), are directed primarily into energy-yielding pathways. During positive energy balance excess metabolites are directed into pathways facilitating energy storage. AgRP, Agouti-related peptide; AMPK, AMP-activated protein kinase; cAMP, cyclic AMP; ChREBP, carbohydrate-responsive element-binding protein; CREB, cAMP-responsive element-binding protein; FAO, fatty acid oxidation; FOXO1, forkhead box O1; FXR, farnesoid X receptor; HNF4α, hepatocyte nuclear factor 4α; IRS, insulin receptor substrate; JAK, Janus kinase; LDL, low-density lipoprotein; LXR, liver X receptor; mTORC1, mammalian target of rapamycin complex 1; Ox-Phos, oxidative phosphorylation; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKA, protein kinase A; PKC, protein kinase C; POMC, pro-opiomelanocortin; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein; STAT, signal transducer and activator of transcription; TG, triglyceride. Taken from [192]

It is worth stating that beside energy homeostasis, **DNA methylation and diet are intricately related** (Figure 22). For instance, curcumin [199] and green tea catechins [200] are potent DNMT inhibitors. DNA methylation requires the methyl donor S-adenosylmethionine (SAM) as a substrate which is produced from methionine and ATP in one-carbon metabolism [201]. SAM levels can be altered through diet [202], and there is now overwhelming evidence that dietary intake of methyl donors (e.g. folate) is closely linked to levels of DNA methylation [203]. Other micronutrients such as vitamins B12, B6 and B2 are involved in SAM metabolism [204] and can potentially impact DNA methylation levels, but this has not been tested. Conversely, DNA demethylation is also related to diet. The removal of methyl groups by TET proteins depends on Fe(II) and α-ketoglutarate, an intermediate of the tricarboxylic acid cycle and catabolic metabolism of glutamine [205]. Although it is not known whether TET proteins can sense α-ketoglutarate levels [201], fumarate and succinate (two other intermediates of the tricarboxylic acid cycle) inhibit TET, suggesting that the relative concentrations of these metabolites may regulate TET enzymatic activity [206]. Therefore, there is a profound link between DNA methylation and metabolism, suggesting that the long-term impairment of energy balance could lead to adverse DNA methylation changes themselves causally involved in the disease.

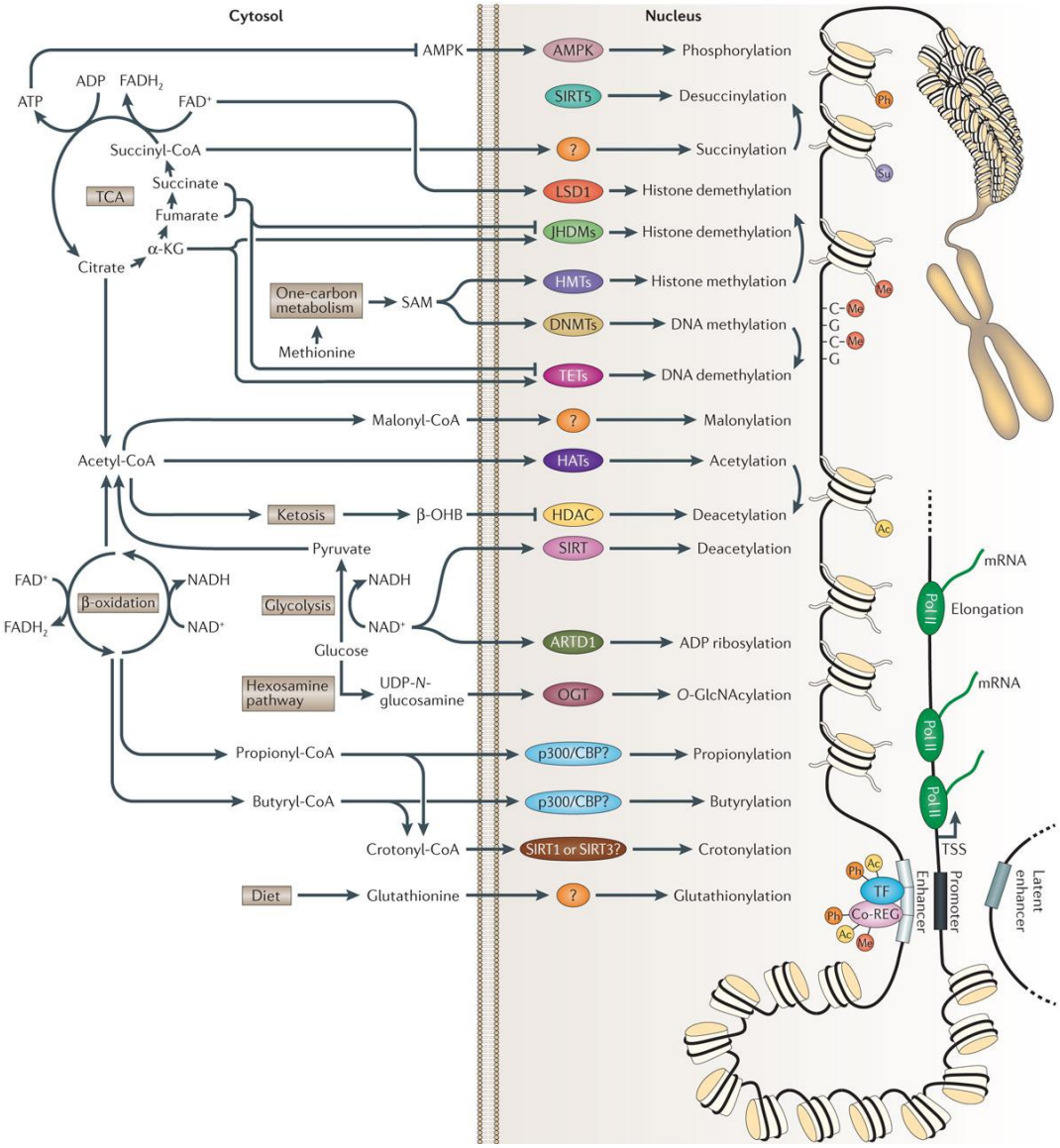


Figure 22. The interface between metabolism and epigenetic regulation of transcription.

Metabolites from major biochemical pathways participate in chromatin regulation, leading to changes in transcriptional regulation. The tricarboxylic acid (TCA) cycle and β-oxidation provide acetyl-CoA, the substrate for histone acetyltransferases (HATs) to acetylate histones. One-carbon metabolic pathways provide S-adenosylmethionine (SAM), the substrate for histone methylation. β-oxidation also leads to the production of propionyl-CoA and butyryl-CoA, which can be further processed into crotonyl-CoA; all three coenzymes are substrates for the respective histone modification. AMP/ATP levels determine the activity of AMP-activated protein kinase (AMPK), which has been shown to phosphorylate histones. α-KG, α-ketoglutarate; β-OHB, β-hydroxybutyric acid; ARTD1, ADP-ribosyltransferase diphtheria toxin-like 1; co-REG, co-regulator; DNMT, DNA methyltransferase; GlcNAc, N-acetylglucosamine; HDAC, histone deacetylase; HMT, histone methyltransferase; JHDM, JmjC domain-containing histone demethylase; LSD1, lysine-specific demethylase 1; OGT, O-linked N-acetylglucosamine transferase; p300/CBP, p300 and CREB-binding protein; Pol II, RNA polymerase II; SIRT, sirtuin; TF, transcription factor; TET, ten-eleven translocation methylcytosine dioxygenase; TSS, transcription start site. Taken from [192]

3.2.2. DNA methylation and obesity in childhood and adulthood

We have previously seen that epigenetic modifications can give cells a memory of their past gene activity and potentiate their future activity. We have also seen that DNA methylation and energy homeostasis are intricately intertwined. Is it possible then that repeated obesogenic exposures would cause shifts in DNA methylation patterns in tissues important for energy homeostasis? If so, how stable are these DNA methylation changes, and how relevant are they in the development of obesity?

If DNA methylation variations in normal metabolic conditions have received little attention, DNA methylation patterns of obese individuals have been extensively studied in both humans and mice [207–210]. In humans, studies of **global methylation** conducted in peripheral blood leukocytes did not show any consistent correlation with BMI regardless of whether methylation was assessed at repetitive elements [211–222] or as %5meC by HPLC, Elisa or flow cytometry [223–226]. This might be explained by the existence of a non-linear relationship between global methylation and BMI [222], or by methylation changes limited to specific blood cell types [226]. Conversely, a small positive association between global methylation and body fat or T2D was found in muscle [227] and adipose tissue [228–230]. A plethora of studies have chosen a **candidate-gene approach** whereby methylation levels in the promoters of genes implicated in obesity, appetite control and/or metabolism, insulin signalling, immunity, growth, circadian rhythm and imprinted genes, were tested for association with a variety of obesity markers [209]. They have notably revealed strong hypermethylation of the *PGC-1α* promoter in pancreatic islets [231], skeletal muscle [91,227,232] and liver [233] in insulin resistant patients. In blood, hypermethylation was noted at *POMC* [234,235] and hypomethylation at *TNF-α* [236,237] and *LEP* [238–241]. Finally, only two studies have looked at mitochondrial methylation in leukocytes [242] and liver [243], but they have uncovered strong hypermethylation in obesity, T2D and nonalcoholic steatohepatitis. Hypothesis-free **epigenome-wide associations studies** (EWASs) of obesity-related traits have also flourished in the past few years, to gain insight into the tissue-specific entire methylomes of metabolically sick individuals. They have revealed mostly small (<10%) but widespread methylation differences in blood [244–261], liver [262,263], adipose tissue [230,258,261,264–266], and sperm [267]. The identified DMRs are usually overrepresented in open sea and underrepresented in CGIs and promoters. Interestingly, some loci have been replicated across tissue types, ages and ethnicities, thanks to the wide use of the Illumina HumanMethylation 450k beadchip. The most promising locus lies within the first intron of hypoxia inducible factor 3 alpha subunit (*HIF3A*), where a positive association between methylation and adiposity phenotypes has been seen in neonates, children, adolescents and adults, across whole blood, adipose, peripheral blood lymphocytes, umbilical cord blood and umbilical cords, in European Caucasians, Asians and African–Americans [268]. Also, sites in ATP-binding cassette sub-family G member 1 (*ABCG1*) and phosphoethanolamine/phosphocholine phosphatase (*PHOSPHO1*) were associated not only with T2D, but also with future incidence of T2D across whole bloods, adipose tissue and skeletal muscle in European Caucasians, Mexican-Americans and Indian Asians [230,256–258,269,270]. Most of these EWAS have performed gene-enrichment analysis and have showed enrichment for pathways relevant for obesity and cancer.

Interventional studies are less common than **observational studies** for practical reasons and they often involve fewer participants, but they can give better clues about **causality** and the sequence of

events leading to obesity. Short-term high-fat overfeeding of healthy young men resulted in modest genome-wide DNA methylation changes in adipose tissue [271] and in skeletal muscle [272]. Interestingly, the methylation changes in skeletal muscle were only partly reversible after 6-8 weeks [272], suggesting that the methylome can keep track of obesogenic events with potential build-up over time. Of note, overfeeding resulted in the hypermethylation in the promoter of *PGC-1α* in skeletal muscle that was reversible [273], while 9 days of forced physical inactivity did not yield any change [274]. *In vitro*, different fatty acids were demonstrated to exert distinct effects on the methylome of monocytes [197], and severe obesity dampened the methylation changes induced by lipid exposure in skeletal muscle [198]. Weight loss interventions, either surgical or lifestyle-based, can induce important site-specific methylation changes in several tissues. In blood, there was no consistent change in methylation at the global level [221,275], but important changes at the site-specific level [276,277] after nutritional intervention. A 6 month-long exercise-based intervention lead to the remodelling of skeletal muscle [278] and adipose tissue [279] methylomes of men. Although the *LEP* promoter can be controlled by methylation, weight-loss induced changes in leptin expression do not seem to be methylation-dependent [280]. In an elegant cross-species study, DMRs identified by dietary manipulation of mice turned out to be conserved in obesity and reversed by gastric bypass in men [210]. Dramatic weight loss induced by gastric bypass was reported to cause important methylation changes in blood [281], skeletal muscle [232], adipose tissue [282] and sperm [267]. Distinct methylation patterns were observed in blood, adipose and sperm from the same individuals after surgery, suggesting that changes are tissue-specific [267]. However, methylation aging of the liver caused by obesity is not reversed after bariatric surgery [283].

Overall, these results show that DNA methylation correlates with obesity-related traits and that the methylome is sensitive to obesogenic events and reflects weight changes, in a site-specific and tissue-specific manner, although a few sites have been found across several tissues. However, it is important to keep in mind that methylation changes observed after interventions such as lipid exposure, overfeeding, weight loss programs or gastric bypass surgery might not be directly caused by these interventions. For instance, bariatric surgery is a drastic weight loss strategy that also strains the body on several other levels (e.g. nutrient deficiency [284], anaemia [285], osteoporosis [286]), so it is difficult to disentangle weight loss-induced methylation changes from methylation changes caused by other side effects.

3.2.3. DNA methylation and predisposition to obesity: effects of risk variants and developmental programming

DNA methylation is associated with obesity and is sensitive to lifestyle interventions, but can it also modulate the degree of response to obesogenic environments? Can DNA methylation levels exacerbate or dampen the effects of obesogens and weight loss interventions? Does DNA methylation explain why some people are “high responders” and others “low responders” to the same environmental exposures?

One hypothesis that has gained support recently is that parental health status and early life environment can predispose the offspring to metabolic diseases, a concept that is part of the **developmental origins of health and disease** (DOHaD) (Figure 23). One possible mechanism mediating these effects is the alteration of DNA methylation patterns. Indeed, early-life socio-economic conditions, nutrition, maternal smoking, alcohol consumption and gestational diabetes have been shown to affect DNA methylation of the offspring in a sex-specific and time-specific manner. Most of the evidence so far comes from animal models, human studies being more limited, extremely heterogeneous and somewhat inconsistent [287–290]. For instance, differential methylation at genes important for metabolism [291,292], including lower methylation at the imprinted *IGF2*/*H19* locus [293] was found in blood of adult offspring whose mothers experienced the Dutch famine of 1944. This effect was only visible for mothers who experienced famine during early gestation, and the *IGF2*/*H19* hypomethylation was more visible in men than in women [291]. While this cohort provides a unique opportunity to study the effects of undernutrition *in utero*, stress experienced by the mothers because of the war and the very harsh winter are potential confounders. It should also be noted that there was a high perinatal mortality and only the children who survived were studied. In rural Gambia where there

are strong variations of food availability depending on the time of year, Waterland *et al*. contrasted global methylation levels between children conceived during the rainy season (proxy for a period of low intake of methyl donors) and children conceived during the dry season (proxy for a period of high intake of methyl donors). They found higher methylation in the blood of 8 year-old children that had been conceived during the rainy season, which argues towards an influence of maternal diet on the offspring's methylome, but the effect was in opposite direction to what was expected (a reduction in methyl donors during the rainy season was expected to cause hypomethylation) [294]. Something may also be going on on the father's side: it was recently shown that the sperm methylome carries signs of obesity [295] and that gastric bypass remodels the sperm methylome [267], but whether these modifications are effectively transmitted to the offspring is unknown. Even if all of these studies suggest an influence of early-life experiences on the foetal methylome, we are still far from knowing to what extent it is affected, to what extent it is beneficial or detrimental and which of these effects are site-specific, tissue-specific, time-specific, sex-specific and ethnicity-specific. Heterogeneity in study designs, lack of replication and control groups as well as impossibility to assess causation make it difficult to draw definitive conclusions on the matter.

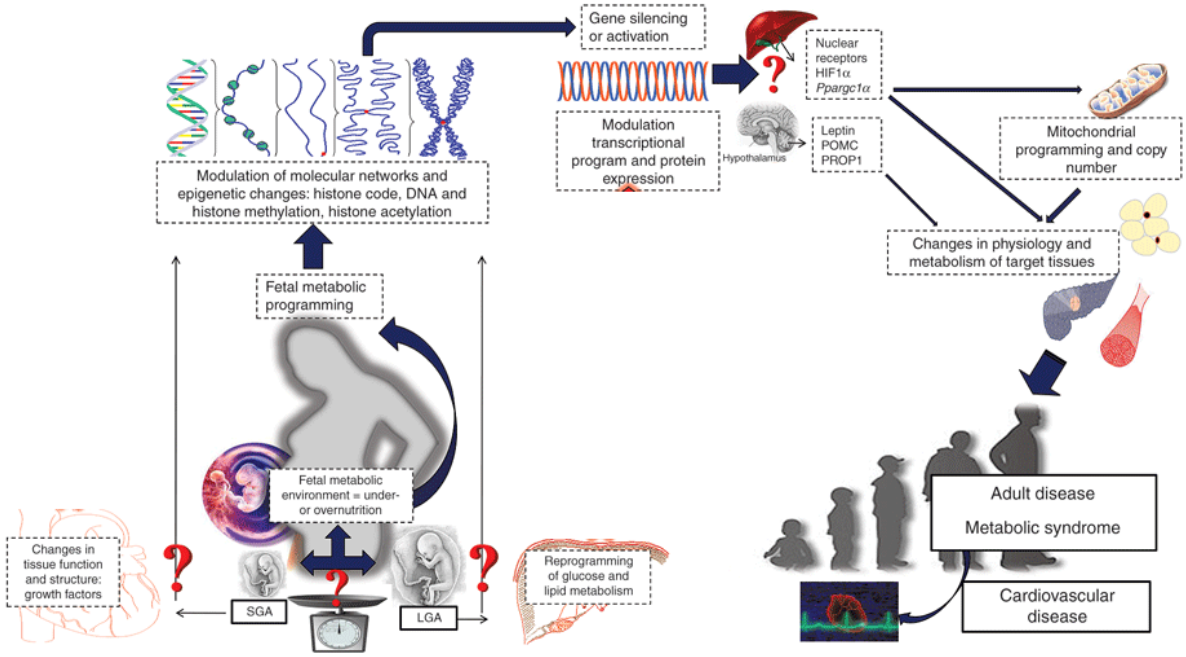


Figure 23. The complex metabolic networks that modulate fetal metabolic programming.

This picture summarizes the putative molecular mechanisms linking impaired nutrient availability during the fetal period with adult chronic diseases such as metabolic and cardiovascular disorders, including coronary heart disease, T2D, and insulin resistance. The figure illustrates the concept that fetuses adapt to an impaired supply of nutrients (under- or overnutrition) by changing their physiology and metabolism, in particular by modulating the metabolic transcriptional program of target tissues. Epigenetic modifications, such as DNA methylation and covalent posttranslational histone modifications provide a molecular explanation of how these complex metabolic networks coordinately influence fetal metabolic programming. A nutrient-restricted fetal environment may be more likely associated with the induction of changes in tissue structure and function, particularly in cardiovascular system, mainly regulated by growth factors. Conversely, a maternal "obesogenic" environment is more likely associated with metabolic reprogramming of glucose and lipid metabolism in the liver. Finally, two different hypotheses regarding whether fetal metabolic programming is controlled by metabolically active target tissues such as the liver, or is modulated by central neural pathways involved in appetite and energy balance regulation such as the hypothalamus are shown, and the concept of "mitochondrial programming" is introduced as operating on the modulation of metabolic function. IUFGR, intrauterine fetal growth restriction; LGA, large for gestational age; Mt, mitochondrial; SGA, small for gestational age. Adapted from [296]

Response to obesogenic exposures and weight loss interventions may be modulated by methylation levels at baseline [297]. Being born with a low birth weight (LBW) is associated with an increased risk

of developing metabolic disorders later in life [298] (Figure 23). LBW subjects exhibit lower methylation in the promoter of *PGC-1α* in skeletal muscle [273] and epigenetic alterations in adipose tissue that potentially influence insulin resistance and risk of T2D [271]. One study showed that when challenged with high-fat overfeeding, *PGC-1α* mRNA levels dropped only in LBW subjects [273]. However, another study found that the entire adipose methylome of LBW and control participants responded in a similar manner to high-fat overfeeding [271]. Strong genome-wide methylation differences (up to 35% [276]) between high and low responders to nutritional interventions were identified at baseline in blood and adipose tissue [297], suggesting that **DNA methylation levels can dampen or exacerbate responses to external stimuli**. Is it possible that these differences in response are caused by the presence of SNPs near the methylation sites? Some SNPs have been shown to correlate with proximal DNA methylation levels, providing a potential link between risk variants and obesity susceptibility (Figure 24). For instance, a 7.7 kb region of haplotype-specific methylation was discovered at the *FTO* locus because of the presence of CpG-creating SNPs [299]. Polymorphisms in the adrenoceptor beta 3 (*ADBR3*) candidate gene associated with *ADBR3* methylation and with metabolic disturbances in men [300], and SH2B adaptor protein 1 (*SH2B1*) CpG-SNP associated with body weight reduction in obese subjects following a dietary restriction program [301]. However, at the genome-wide level, it seems that methylation differences observed in metabolically sick individuals [253] or in response to weight loss [267] are not driven by DNA sequence differences. Actually, in one large cohort study, methylation profiles predicted BMI independently of genetic profiles in an additive manner [255].

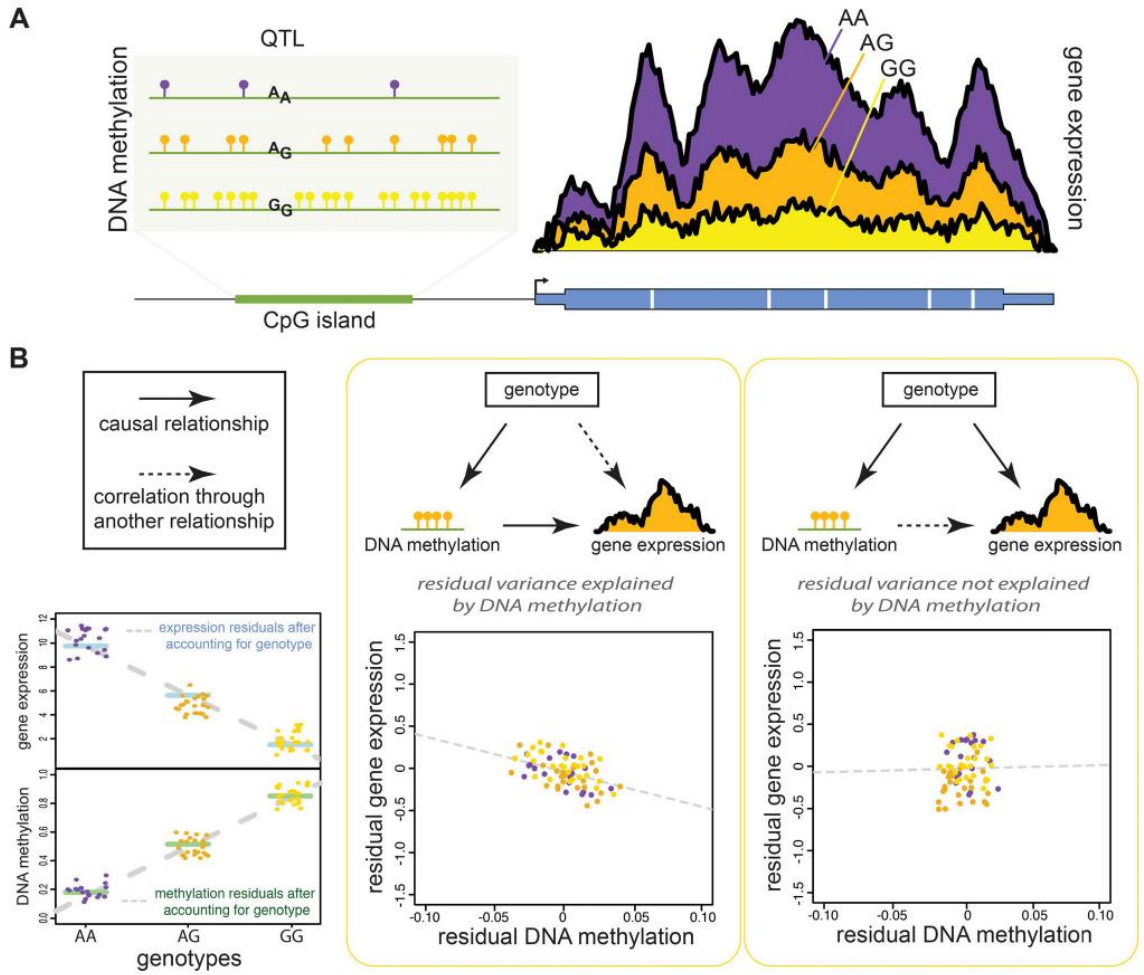


Figure 24. An approach for joint quantitative analysis of gene expression and regulatory QTLs.

(A) This example, using hypothetical data, shows a QTL that is associated with levels of both DNA methylation in an upstream CpG island (left) and gene expression (right). Though the example QTL shown here indicates higher DNA methylation due to a G allele (potentially in a CpG pair), SNPs associated with methylation do not necessarily always fall in CpG dinucleotides. (B) The observed correlation between DNA methylation and gene expression levels could be due to a few different underlying relationships, two of which we have highlighted here. The extent to which gene expression and regulatory differences are correlated through an intermediate variable is often tested using an approach called partial correlation analysis. This involves regressing out the effects of an intermediate variable—genotype in this example—from both DNA methylation and gene expression levels and then evaluating the residual correlation between the two variables (left). One possibility is that the QTL directly affects differences in DNA methylation, which then determine (cause) the gene expression level. Thus, gene expression is regulated by the genotype through the DNA methylation effects (middle), and the residual variance in gene expression levels will still be correlated to residual DNA methylation levels. Alternatively, genotype is independently associated with both DNA methylation and gene expression levels—for instance, by directly influencing changes in an upstream mechanism (such as transcription factor binding) that affects DNA methylation and gene expression levels. This would make DNA methylation and gene expression appear to be correlated, but not causally related (right), and the residual values no longer show any significant correlation). Taken from [169]

Collectively, there is accumulating evidence that **predisposition to obesity** and **responsiveness to lifestyle interventions** is partly embedded in the methylome. The presence of SNPs at certain loci may contribute to this susceptibility through DNA methylation changes, but more research is needed to give a full picture of how the genome and the methylome interact in the pathogenesis of obesity.

3.3. Challenges to face

By nature, epigenomic studies, especially the ones performed at the genome-wide level, combine many of the difficulties of genetic studies, with difficulties inherent to epigenomics [302–305]. We will now review some of the most important biological and statistical issues that have not yet been fully addressed.

3.3.1. Biological challenges

In **observational studies**, it is usually impossible to determine whether the methylated regions that correlate with obesity-related traits are a **cause** or simply a **consequence** of the metabolic disturbance. In light of what we know about the sensitivity of DNA methylation to various environments and the establishment of obesity, it is likely that some methylation changes are slow, progressive and result from accumulated repetitions of metabolic stress while others, established early by risk variants or adverse early-life conditions, give an increased susceptibility to develop obesity. It is also conceivable that some of the DNA methylation changes caused by obesity would in turn confer an increased susceptibility to further develop obesity, thus fuelling a vicious cycle. However, we currently have very little idea of whether this is true or not and what specific genomic regions or tissues are involved. **Interventional studies**, **longitudinal studies** of monozygotic twins and **randomized controlled trials** are good designs to answer questions of causality, but they are extremely expensive and would need to be conducted over extended periods of time to truly mimic the pathogenesis of obesity and to accurately reproduce human weight trajectories. A technique called **Mendelian randomization** that uses meQTL has been recently developed to help answer causality issues [306] (Figure 25), and it was successfully used to show that *HIF3A* methylation is likely a consequence of obesity [249,307], and that maternal hyperglycemia is part of causal pathways influencing offspring *LEP* epigenetic regulation in newborns [308].

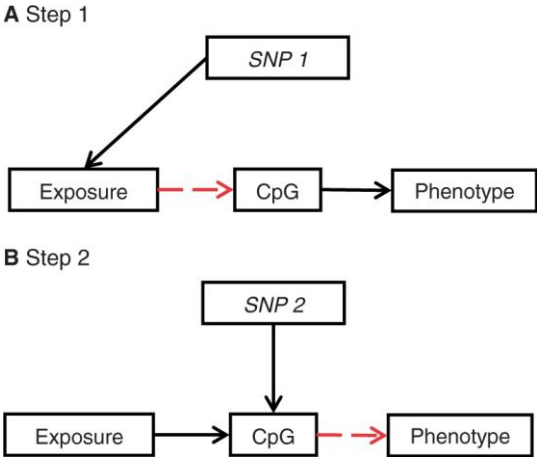


Figure 25. Two-step epigenetic Mendelian randomization: applying the principle of Mendelian randomization to DNA methylation as an intermediate phenotype.

Genetic variants can be used as instrumental variables in a two-step framework to establish whether DNA methylation is on the causal pathway between exposure and disease. An overview of the two-step framework of this approach is shown. (A) First, an SNP is used to proxy for the environmentally modifiable exposure of interest and (B) secondly, a different SNP is used to proxy for DNA methylation levels. Taken from [306]

This causality issue brings about another problem: how do we appropriately define individuals at risk? Obesity and BMI are widely considered to be appropriate proxies for metabolic risk, but as much as a third of individuals deemed obese are in fact metabolically healthy [309,310] (Figure 26)! And even if these variables may be useful at a large-scale population level, the sample sizes often used in the aforementioned studies are probably too small to justify the use of BMI or weight category. Using better proxies for metabolic risk such as waist circumference and central adiposity will likely give better estimates and statistical inferences. The methylomes of obese individuals are often tested at a single point in time, but their **weight trajectories** (i.e. weight gain, loss and regain) may be more important for disease susceptibility. A recent study revealed important differences in mortality between those who lost weight, those who remained obese and those who were never obese; strikingly, mortality was similar in individuals who remained obese and formerly obese individuals who lost weight [311] (Figure 27). This could be explained by the fact that obesity at a particular age may predispose to illness, regardless of weight at higher ages, and the fact that weight loss is often associated with illness such as diabetes and cardiovascular disease. Studies considering weight trajectories and weight maintenance will therefore yield results that are more useful from a clinical perspective.

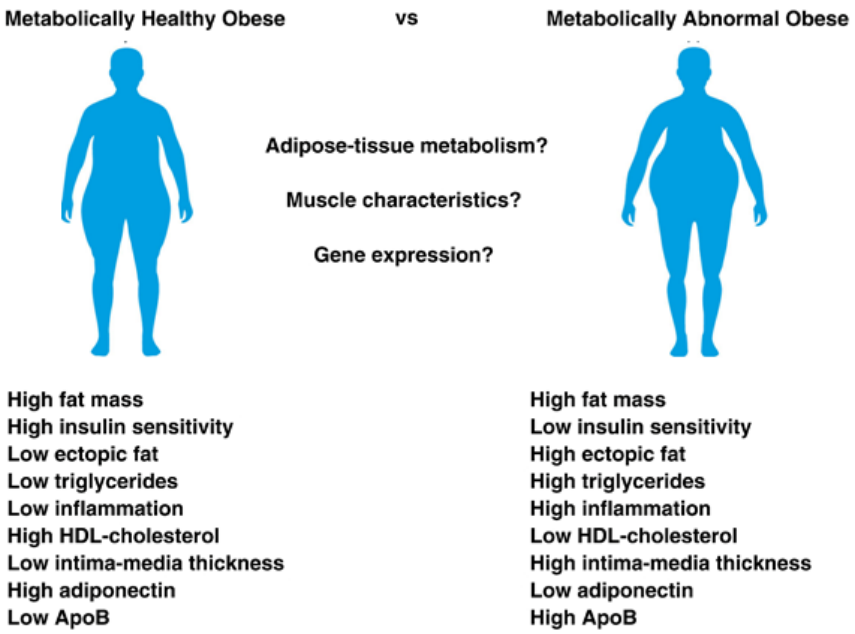


Figure 26. Factors that might distinguish metabolically healthy but obese individuals from metabolically abnormal obese subjects despite a similar fat mass.

Adapted from [310]

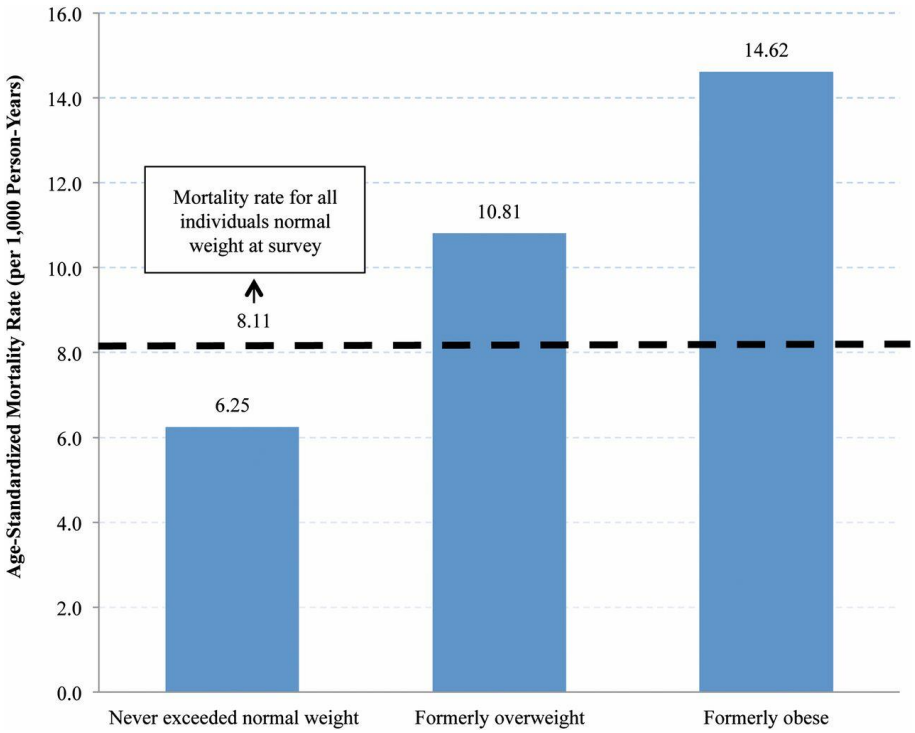


Figure 27. Age-standardized mortality rates for individuals who were normal weight at survey, stratified by maximum BMI.

Normal weight, 18.5–24.9 kg/m^2; overweight, 25.0–29.9 kg/m^2; obese, 30 kg/m^2 and above. Mortality rates were age-standardized to the US 2000 Census, using 5-y age groups between 50 and 84 y. Estimates are weighted and account for complex survey design. Taken from [311]

The **temporal** aspect is really a central issue in epigenomic studies. Contrary to the genome (with the exception of rare mutations), the methylome is flexible and sensitive to many environmental in-

sults throughout life. And precisely because the epigenome can be modulated by so many factors (e.g. SNPs, early-life environment, diet, physical activity, age, smoking, sex, etc.), **confounders** are omnipresent and difficult to fully account for. It would help to have a better picture of what a "normal" epigenome looks like in different tissues, but the natural and stochastic variations of the methylome are poorly characterized. However, what is perhaps more important than trying to account for all potential confounders is to know the effect sizes of these confounders, and to what extent they can bias the results of a given study. In particular, given the high **sexual dimorphism** often seen in animal models of early-life exposures, differences between sexes as well as ethnicities need to be better considered.

Perhaps the thorniest and most recurrent problem in epigenomics is **tissue-specificity**. DNA methylation patterns are highly tissue-specific [84], to the point where intra-individual between-tissue variation in DNA methylation greatly exceeds inter-individual differences [176]. For instance, the association between BMI and *HIF3A* methylation was seen in blood but not in skin [249]. However, there is often limited availability of metabolically relevant tissues in humans so many studies are performed in blood. Some methylation changes associated with metabolic traits may be found both in blood and other tissues [176], but as methylation changes reflect tissue-specific responses to environmental stimuli, this will likely concern only a limited number of methylation sites. Depending on the goal of the study, it is important to replicate changes seen in blood in the relevant tissue: studies that seek to better understand the **mechanistic link** between the epigenome and the pathogenesis of obesity need to replicate their findings in their tissue of interest; however, studies that only aim at finding **biomarkers** for specific outcomes (e.g. obesity risk, weight loss response to T2D susceptibility) do not need to replicate their findings in other tissues, as it is blood that will be used to assess risk in patients [312]. Moreover, **cell-type heterogeneity** within a given tissue poses an additional problem, as observed methylation differences may actually be only due to differences in cell type composition. This is particularly true in blood, a tissue made of widely heterogeneous cells such as monocytes, granulocytes, T-cells and B-cells [313]. This is a huge concern in obesity, since obesity itself results in an inflammatory state in metabolic tissue and a change of blood cell type composition [314] (Figure 28). When flow cytometry is possible, cell counts can be used to adjust for cell type composition [315], but recent bioinformatical techniques have been developed to infer this cell type composition by relying only on DNA methylation patterns [313,316]. However, these adjustments may not be sufficient if cell type compositions are too heterogeneous, so studying the methylomes of specific cell types may be necessary yet difficult to achieve.

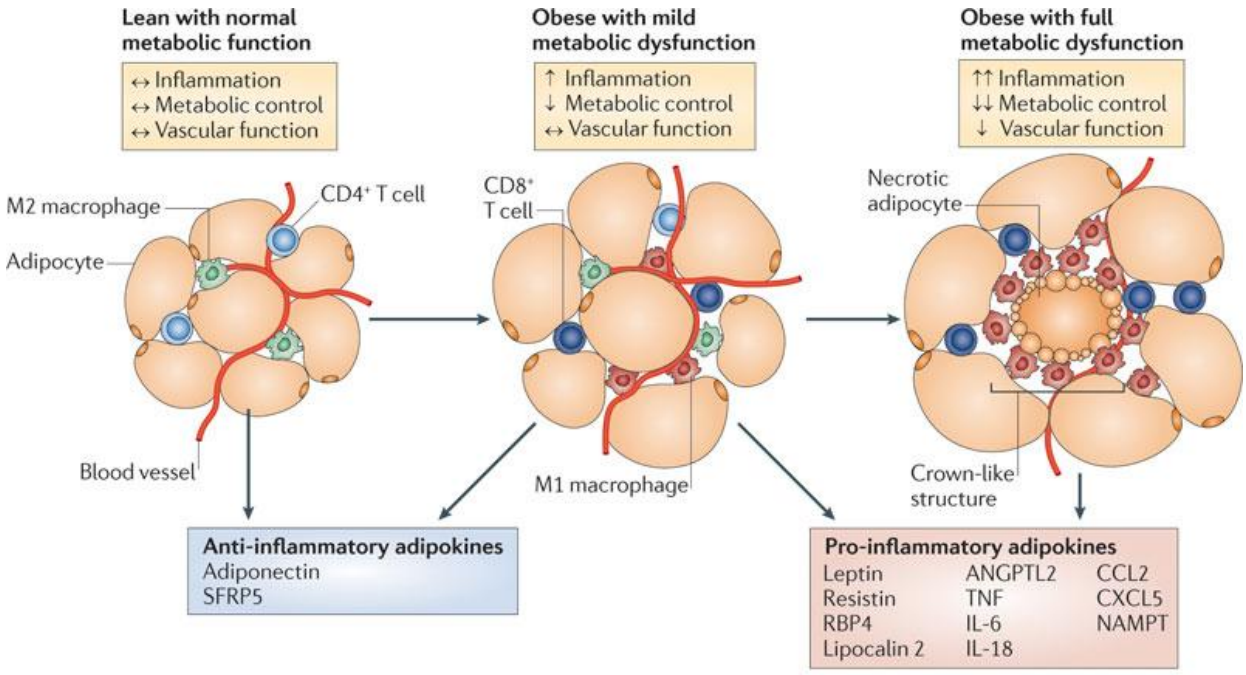


Figure 28. Phenotypic modulation of adipose tissue.

Adipose tissue can be described by at least three structural and functional classifications: lean with normal metabolic function, obese with mild metabolic dysfunction and obese with full metabolic dysfunction. As obesity develops, adipocytes undergo hypertrophy owing to increased triglyceride storage. With limited obesity, it is likely that the tissue retains relatively normal metabolic function and has low levels of immune cell activation and sufficient vascular function. However, qualitative changes in the expanding adipose tissue can promote the transition to a metabolically dysfunctional phenotype. Macrophages in lean adipose tissue express markers of an M2 or alternatively activated state, whereas obesity leads to the recruitment and accumulation of M1 or classically activated macrophages, as well as T cells, in adipose tissue. Anti-inflammatory adipokines, including adiponectin and secreted frizzled-related protein 5 (SFRP5), are preferentially produced by lean adipose tissue. In states of obesity, adipose tissue generates large amounts of pro-inflammatory factors, including leptin, resistin, retinol-binding protein 4 (RBP4), lipocalin 2, angiopoietin-like protein 2 (ANGPTL2), tumour necrosis factor (TNF), interleukin-6 (IL-6), IL-18, CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 5 (CXCL5) and nicotinamide phosphoribosyltransferase (NAMPT). Obese individuals with adipose tissue in a metabolically intermediate state have improved metabolic parameters, diminished inflammatory marker expression and better vascular function compared with individuals that have metabolically dysfunctional adipose tissue. Metabolically dysfunctional adipose tissue can be associated with higher levels of adipocyte necrosis, and M1 macrophages are arranged around these dead cells in crown-like structures. Taken from [317]

3.3.2. Statistical and bioinformatical challenges

The bioinformatic and biostatistic treatment of DNA methylation data is a field in rapid expansion, especially for DNA methylation data generated with no gold standard defined so far.

Where in the genome should we look to find relevant DMRs in relation to obesity? Given our limited knowledge on the function of DNA methylation outside of promoters, most candidate-gene studies have focused on **gene promoters**, and the first genome-wide DNA methylation chip developed by Illumina (HumanMethylation 27k beadchip) also targeted gene promoters. While these regions are known to be involved in the regulation of developmentally expressed housekeeping genes and have an important role in the pathogenesis of cancer [303], we do not know whether they are prominent genomic loci in the pathogenesis of obesity. The development of the **HumanMethylation 450k beadchip** by Illumina has extended the interrogation of methylation sites within gene bodies, CpG-poor promoters and between genes. Interestingly, most of the EWASs previously mentioned found an enrichment of DMRs in the **open sea** and **enhancers**, suggesting that regulatory regions and not promoters may be the prominent targets in the pathogenesis of obesity. However, is the **annotation of CpGs** to certain genes and regulatory regions as straightforward as often mentioned in papers? A CpG that falls into the promoter of a gene or within a gene body is often automatically annotated to the gene

in question, but we can very well imagine that a region would serve as an enhancer in a tissue and as a promoter in another tissue. We need more information on the underlying **chromatin state** of specific tissues to appropriately assign CpGs to certain genes, and some international **consortia** (ENCODE, Roadmap Epigenomics, FANTOM5) have generated data that should help with that. Moreover, non-CpG methylation deserves more attention since it was associated with T2D [91], markers of obesity [318], and it was shown to be sensitive to acute exercise [93] and gastric bypass surgery [318]. Finally, many studies have used gene enrichment tools to find significant enrichment in certain pathways. However, the gene enrichment tools that were initially developed for transcription microarray data may not be appropriate for methylation data. For instance, using gene set enrichment analysis on methylation data yields biased results because of differences in the numbers of CpG sites associated with different classes of genes and gene promoters [319].

Even if it covers 99% of RefSeq genes, the HumanMethylation 450k beadchip interrogates less than 2% of all CpG sites in the human genome, so human methylome changes with obesity remain largely unknown. And what if obesity actually causes methylome changes that are very small but that target a very high number of genes and pathways, leading to big effects? Now that techniques like WGBS have been developed and become increasingly cheaper, we have the ability to assess the entire methylome. The question is: do we have the appropriate statistical tools to analyse it (Figure 29)? Studies have already had to deal with the **multiple testing problem** and often report only a couple of significant DMRs to keep the false positive rate at a certain level. To circumvent this issue, it is possible to restrict the analysis to certain regions, but then the global picture of the methylome is lost. It is also possible to average the methylation levels of proximal CpG sites since they are often correlated, but if only a single CpG site is important for the regulation of gene transcription, its variations with obesity would be diluted and go unnoticed. A recently developed R package called *DMRcate* makes use of methylation differences at single sites to find DMRs without loss of information, and it should be very useful in future EWASs [320]. Moreover, the reported **effect sizes** of most studies are extremely small and often close to the technical variability of the Illumina HumanMethylation beadchip (<5% methylation difference). Can a sample of only a few hundred individuals suffice to find robust methylation differences between groups that are that small? More importantly, what do these methylation differences actually mean? Are they biologically active?

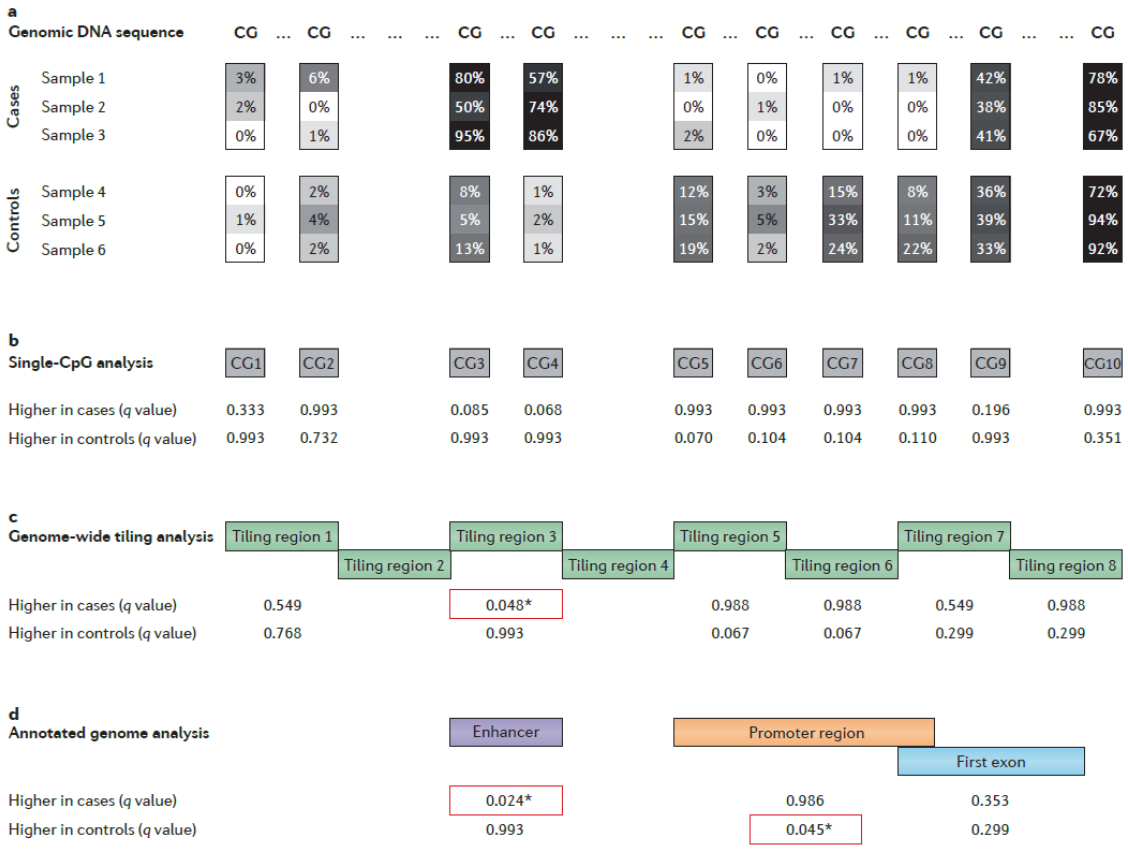


Figure 29. Effective identification of differentially methylated regions in a highly annotated genome.

a | An illustrative example of differences in DNA methylation within the promoter region of a gene and at an upstream enhancer. For easier visualization, DNA methylation data are shown for only three cases and three controls (a realistic number would be hundreds of samples) and for ten CpGs in total (dozens to hundreds of CpGs are realistic numbers for a typical promoter region). **b** | When DNA methylation levels between cases and controls are compared at the resolution of single CpGs, all multiple-testing-corrected q values > 0.05 and are therefore considered to be insignificant. **c** | When combining statistical evidence from neighbouring CpGs over a fixed distance (tiling regions highlighted in green), one region is identified as being significantly more methylated in cases than in controls (q value = 0.048). **d** | When combining statistical evidence across all CpGs that can be assigned to the same functional element on the basis of external genome annotation data, two DMRs are identified: the upstream enhancer (highlighted in purple) is significantly more methylated in cases (q value = 0.024), and the promoter region (in orange) is significantly more methylated in controls (q value = 0.045). The figure is based on the following statistical methods. Differences in DNA methylation at single CpGs (in **b**) are identified by unpaired, one-sided t tests, which assess whether or not the DNA methylation levels at the specific CpG are significantly higher in cases than in controls, and vice versa. The reason for using two separate one-sided tests lies in the ability to combine their results as described below; nevertheless, one two-sided test works equally well if no combination of P values is intended. For the tiling region analysis (in **c**), the locus is segmented into equally spaced regions, and the statistical significance for each of these regions is assessed using a generalization of Fisher's method. This method combines the P values of all single CpGs that fall into the region while accounting for linear correlations between neighbouring CpGs (which are estimated to be ≤ 0.8 on the basis of empirical observations for genome-wide bisulphite-sequencing data). The annotated genome analysis (in **d**) uses external genome annotation data to focus the statistical analysis on those combinations of CpGs that are likely to work together as an epigenetic switch: for example, by deactivating a known promoter or enhancer element. In all three cases, q values are calculated as estimates of the multiple testing- corrected false discovery rate, and a q value of 0.05 is used as the significance threshold for each direction of the comparison. Note that in this example the analysis of tiling regions increases statistical power because neighbouring CpGs exhibit correlated changes in DNA methylation, and the incorporation of genome annotation data leads to further improvements, because the CpGs in the enhancer as well as those in the promoter exhibit a coordinated switch of their DNA methylation levels. Adapted from [321]

Clearly, many biological and bioinformatical issues need to be solved if we want to understand how the methylome is modulated in the pathogenesis of obesity. How do obesity-associated gene variants act at the molecular level to cause an increased susceptibility to metabolic disorders? Are the obesogenic effects of environmental factors such as sleep deprivation and diet mediated by changes in the epigenome? Do weight trajectories of mothers impact their offspring's phenotype, and if so, is the epigenome involved? The following four papers are the result of three years of intense research, with the constant wish to keep updated with the latest development in the field and they give some new interesting insights into the interplay between our methylome and obesity.

Aims

The overall aim of this thesis was to investigate epigenetic changes associated with genetic and environmental obesogenic exposures. We particularly focused on DNA methylation in relation to genetic risk variants, nutrition, sleep and developmental programming.

More specifically, the aim of each paper was as follows:

- In paper I, the aim was to see whether SNPs discovered in GWASs for obesity-related traits associated with proximal DNA methylation in blood. Furthermore, we wanted to functionally characterize the methylated CpGs associated with SNPs.
- In paper II, the aim was to see whether dietary measures of fat intake associated with DNA methylation levels in blood. We specifically wanted to compare results obtained for measures of dietary fat quantity and dietary fat quality.
- In paper III, the aim was to see whether one night of complete sleep deprivation could impact the transcription and DNA methylation of four core circadian genes (*CLOCK*, *BMAL1*, *PER1*, *CRY1*) in adipose tissue and skeletal muscle of young healthy men.
- In paper IV, the aim was to see whether chronic maternal obesity impacts foeto-placental growth and the expression of 60 epigenetic machinery genes and 32 metabolic genes in foetal liver and placenta, using a mouse model.

Results

Paper I

In this first study, we tested associations between 52 SNPs that were previously identified in GWASs or meta-analyses to be associated with obesity traits, and proximal DNA methylation in whole blood of 355 healthy young individuals. The rationale for only testing methylation sites that were within 500kb of each SNP was based on previous studies in various tissues that showed the presence of meQTLs essentially within 500kb of CpGs. Using a linear model adjusted for age, sex, weight category and proxy for blood cell counts, we found that alleles at 28 of the 52 obesity-associated SNPs associate with methylation levels at 107 proximal CpG sites. This suggests that carriers of obesity-associated risk alleles display complex alterations of the gene regulatory landscape.

To link these methylation differences to the pathogenesis of obesity, we then annotated these CpG sites to genes and chromatin states. Out of 107 CpG sites, 38 are located in gene promoters, including genes strongly implicated in obesity (*MIR148A*, *BDNF*, *PTPMT1*, *NR1H3*, *POMC*, *LGR4*, *TUFM*, *SULT1A1*, *SULT1A2*, *APOBR*, *CLN3*, *SH2B1*). We applied the chromHMM software to seven genome-wide patterns of histone modifications shared by the Roadmap Epigenomics project, in order to infer chromatin states in eleven metabolically relevant tissues. Interestingly, the 107 CpG sites were enriched in enhancers in PBMCs, and we sought to identify genes putatively regulated in *cis* or in *trans* by these CpGs. Using data from the FANTOM5 project and ChIA-PET libraries, we also found that the CpG sites were located in regions showing putative long-range interactions with nearby promoters, including genes for which the corresponding SNPs are known eQTLs (*C1QTNF4*, *CELF1*, *NUP160* and *ADCY3*). These results suggest that carriers of obesity-associated risk alleles display complex alterations of the gene regulatory landscape. We finally tested some of our initial findings in other tissues (skin fibroblasts, brain and adipose tissue), and some SNP-CpG associations were indeed replicated in skin fibroblasts.

Our results strongly suggest that many obesity-associated SNPs are associated with proximal gene regulation, which was reflected by association of obesity risk allele genotypes with differential DNA methylation. This study highlights the importance of DNA methylation and other chromatin marks as a way to understand the molecular basis of genetic variants associated with human diseases and traits.

Paper II

In this second study, we tested associations between genome-wide blood methylation levels at gene promoters and dietary fat intake variables in a cohort of 69 Greek preadolescents. Not only did we investigate quantitative measures of dietary fat intake (proportion of energy intake derived from fat, cholesterol intake), but also qualitative measures of dietary fat intake (ratios between different fatty acids: MUFA/SFA, PUFA/SFA, (MUFA+PUFA)/SFA). Using a robust linear model adjusted for sex, weight category, Tanner stage and white blood cell count, we found that quantitative measures of fat intake associate with very few DMRs while qualitative measures of fat intake associate with many DMRs. This suggests that specific dietary fat profiles might cause physiological responses underpinned by DNA methylation changes.

To link these DNA methylation associations to metabolic pathways, we looked more closely at the genes containing the DMRs. One of the five DMRs identified for the proportion of energy intake derived from fat lied in the promoter of *TXNIP*, a gene known to play a role in inflammation, insulin secretion and sensitivity, glucose uptake and gluconeogenesis, and a gene that was found to be

upregulated by a high-fat diet in rodents. We performed gene enrichment analysis on the DMRs found for qualitative measures of fat intake, using two different online-based tools (CPDB and g:profiler): while no enrichment was found for MUFA/SFA, (MUFA+PUFA)/SFA showed enrichment in 5 pathways and PUFA/SFA showed enrichment in 34 pathways, including mechanism of gene regulation by peroxisome proliferators via PPARα, adipogenesis, the leptin pathway and IL6.

Despite some limitations (small sample size, replication not tested in other tissues, impossibility to determine causality), this work was the first of its kind conducted in children and it led to the conclusion that fat quality may influence DNA methylation on a large genomic scale. DNA methylation could be one of the molecular underpinnings of the different cell and physiological responses associated with different types of dietary fatty acids.

Paper III

In this third study, we tested whether one night of complete sleep deprivation could impact the transcription and DNA methylation of four core circadian genes (*CLOCK, BMAL1, PER1, CRY1*) in adipose tissue (AT) and skeletal muscle (SM) of young healthy men. We set a randomized controlled trial in which subjects were put under two conditions in random order: one full night of sleep and one full night of wakefulness. Tissue samples were taken in the morning at a set time, and transcription was assessed by qPCR while DNA methylation was assessed with the Illumina HumanMethylation 450k beadchip. This methylation array contains CpGs that are spread over the entire genome, and we needed to select CpG sites that were putatively involved in the regulation of the four investigated genes. First, we selected all CpG sites located within 1500bp of the TSS of each gene and likely directly involved in the regulation of transcription. Then, we made use of publicly available epigenetic information from the Roadmap Epigenomics Project to infer chromatin states in AT and SM, and open-access ChIA-PET long-range interactions to identify putative enhancers for the four genes under investigation. We also made use of the presence of technical replicates to make sure that the observed methylation changes were not purely due to the technical variability of the chip.

We identified transcription changes in SM, and methylation changes in AT. In SM, *BMAL1* and *CRY1* were downregulated by ~20%; in AT, one CpG site in the promoter of *CRY1* and two CpG sites located in a *PER1* enhancer were hypermethylated by ~0.5%. The methylation effect size may seem extremely small, but we had four pairs of technical replicates at our disposal and we ensured that at least half of our samples showed a methylation difference between conditions greater than the mean methylation difference in technical replicates. These results show that a single night of wakefulness can alter the epigenetic and transcriptional profile of core circadian clock genes in key metabolic tissues. Tissue-specific clock alterations could explain why shift work may disrupt metabolic integrity.

Paper IV

In this fourth study, we used a mouse model to test the effects of chronic maternal obesity on foeto-placental growth and on the expression of 60 epigenetic machinery genes and 32 metabolic genes in foetal liver and placenta. This is of fundamental importance to understand the molecular basis of the developmental origins of health and disease. We also tested whether preconceptional weight loss could alleviate the effects seen in the offspring of the obese mothers, to explore whether weight loss prior to pregnancy is actually beneficial for the offspring.

Female mice were fed either a control diet (CTRL group), a high-fat diet (obese (OB) group), or a high-fat diet switched to a control diet two months before conception (weight loss (WL) group). Foetuses from OB mothers showed foetal growth restriction, and 28% of the foetuses were small for gestational age, but foetuses from WL mothers were comparable to foetuses from CTRL mothers. Foetal liver and placental labyrinth were more responsive to maternal obesity than junctional zone in terms of transcriptional response. No less than 30% of the 60 epigenetic machinery genes and 16% of the 32 metabolic genes showed transcription difference between at least two groups, in at least one of the

three investigated tissues. Genes involved in the histone acetylation pathway were particularly impacted by maternal weight trajectories. In foetuses from OB mothers, lysine acetyltransferases and Bromodomain-containing protein 2 were upregulated, while most histone deacetylases were downregulated. Importantly, the expression of only a subset of these genes was normalized in foetuses from WL mothers.

This study showed the high sensitivity of the epigenetic machinery genes to maternal weight trajectories, especially in foetal liver and placental labyrinth. A difference in expression of these genes may lead to epigenetic alteration of these tissues, leading to foetal growth restriction. Preconceptional weight loss seemed to have beneficial effects on foetal growth restriction, but normalization of gene expression did not happen for all genes, showing a putative "memory" of weight trajectories at the level of gene transcription.

Discussion

1. What this thesis has made possible and improved

1.1. An increased knowledge on the relationship between obesity and the epigenome

Before this thesis started, some important issues in the field had not been addressed or were only started to be investigated: how do obesity-associated gene variants act at the molecular level to cause an increased susceptibility to metabolic disorders? Are the obesogenic effects of environmental factors such as sleep deprivation and diet mediated by changes in the epigenome? Do weight trajectories of mothers impact their offspring's phenotype, and if so, is the epigenome involved?

While we are far from having addressed these issues exhaustively, we have slightly lifted the veil on these important questions. We have shown that obesity-associated gene variants are associated with differential DNA methylation in *cis*, and the impacted methylation sites are located in promoters as well as functional regions of the genome relevant for the pathogenesis of obesity. We observed extremely diverse effect sizes, ranging from 0.5 to 57% of variance in methylation explained by genotypes, which is consistent with results obtained in other genome-wide meQTLs explorations [173] (Table 5). We did not test whether these DNA methylation differences between genotypes are actively and causatively implicated in modifications of chromatin activity at genes implicated in metabolism, or whether they simply reflect complex alterations of the gene regulatory landscape. Nevertheless, these results resonate strongly with the recently discovered mechanism that explains the association between *FTO* risk variants and metabolic traits and that involves disruption of long-range interactions between a super enhancer and its targets [52,53] (Figure 5). Interestingly, a genome-wide study integrating genetic, epigenetic, transcriptomic and phenotypic data in 119 men demonstrated that some genetic variants mediate their effects on metabolic traits via altered DNA methylation in human adipose tissue [322]. In our study, no less than half of the 52 obesity-associated SNPs we tested showed associations with proximal DNA methylation levels, indicating that a disturbed chromatin activity and long-range interactions may indeed be common in the presence of risk alleles. It is however important to stress that these associations were essentially found in blood, and we still do not know whether the very same associations exist in other tissues that are more relevant for metabolism (e.g. brain, adipose tissue, pancreas, liver).

Organism	Population	Tissue	*N* ind.	*N* marks	Method	FDR control	*cis* (kb)	% assoc.	% *cis*	% *trans*	% var. *cis*	% var. *trans*
Human	Natural	CRBLM, FCTX, TCTX and pons	150	1	GWA	NR	10^3	4–5.1	30–40	60–70	18–88[§§]	18–88[§§]
Human	Natural	CRBLM	153	1	GWA	0.05	10^3	8.71[‖‖]	98	2	17–73	NR
Human	Natural	LCL	77	1	GWA	0.1	50	0.17	73	27	22–63	NR
Human	Natural	LCL	180[§]	1	GWA-cis	0.3–0.4	100	0.12–0.38	100	NT	36–92[¶¶]	NT
Human	Natural	WB	201	1	GWA-cis	NR	500	11.93[‖‖]	100	NT	NR	NT
Human	Natural	LCL	133[§]	1	GWA-cis	0.05	100	5.1–5.8	100	NT	30[¶¶,##]	NT
Human	Natural	FIB, T cells and LC	66–111	1	GWA-cis	0.1	5	3.4–7.8	100	NT	10–90	NT
Human	Natural	HPI	89	1	GWA	0.05	500	2.57	96.8	3.2	NR	NR
Human	Natural	FIB	62	1	GWA-cis	0.05	250	2[‖‖]	100	NT	NR	NT
Human	Natural	LCL	133[§]	1	GWA	0.05	10^3	1.8–2.6	100	NT	23–97[¶¶]	NT
Human	Natural	LCL	34[§]	1	GWA-cis	NR	200	NR	100	NT	26[‖‖]	NT
Human	Natural	WB	697	1	GWA	0.01	10^3	15	99.4	0.6	NR	NR

Table 5. Overview of genetics studies of population DNA methylation variation in humans.

Studies were selected if they included more than 10 individuals, used genome-wide methods for measuring epigenomic variation and applied mapping approaches to identify cis- and/or trans-acting genetic variant. Assoc., association; CRBLM, cerebellum; FCTX, frontal cortex; FDR, false discovery rate; FIB, fibroblasts; GWA, genome-wide association mapping; GWA-cis, genome-wide association mapping that tests only for associations in cis; HPI, human pancreatic islets; ind., individuals; LC, lymphoblastoid cells; LCL, lymphoblastoid cell lines; NA, not available; NR, not reported; NT, not tested; TCTX, temporal cortex; WB, whole blood; var., variation.

[§]Data contains individuals from diverse populations.

[§§]Percent of variance explained by *cis* and *trans* loci combined.

[‖‖]Reported numbers refer to a conditional analysis in which variable probes were pre-selected.

[¶¶]Numbers are based on within-population analysis.

[##]Average estimate.

Taken from [173]

We have also revealed DNA methylation differences with measures of dietary fat quality and after sleep deprivation. Contrary to dietary fat quantity, the ratios of different fatty acids were associated with many DMRs at gene promoters. The DMRs were in promoters of genes showing enrichment for a group of pathways related to adipogenesis and mechanism of gene regulation by peroxisome proliferators via PPARa and another group of pathways related to leptin and IL6. This is particularly interesting given that a recent review on the effect of diets on morbid tissues concluded: "the type of the lipid provided in diets appears to be more important than its quantity, especially when considering body fat accumulation and distribution, and metabolic influences" [323]. Our study was the first to use a single measure that includes all three fatty acids as a proxy for the overall quality of fat intake. Unfortunately, our data did not allow to discriminate PUFA from *trans* fatty acids that show the strongest association with all-cause mortality [324] (Figure 30). Nevertheless, MUFA and PUFA induce greater diet-induced thermogenesis, energy expenditure, and fat oxidation than SFA [325]. We do not know whether the observed associations are indeed caused by the fat intake profile, whether the observed DNA methylation differences play a role in metabolic risk, or whether these associations are also

found in other tissues. Only a couple of studies have tested associations between blood DNA methylation and intake of PUFA [326,327] or MUFA [327], and although they found suggestive associations, they did not show any causal relationship. Using an interventional design, we were able to show that one night of total sleep deprivation impacts the methylation and transcription of four key circadian genes in a tissue-specific manner. The observed methylation differences were extremely small (~0.5% methylation difference after sleep deprivation), but above the technical variability observed at these probes. Could there be a "build-up" of DNA methylation over time if sleep deprivation is repeated? And if so, would these DNA methylation differences be functionally important for metabolic risk?

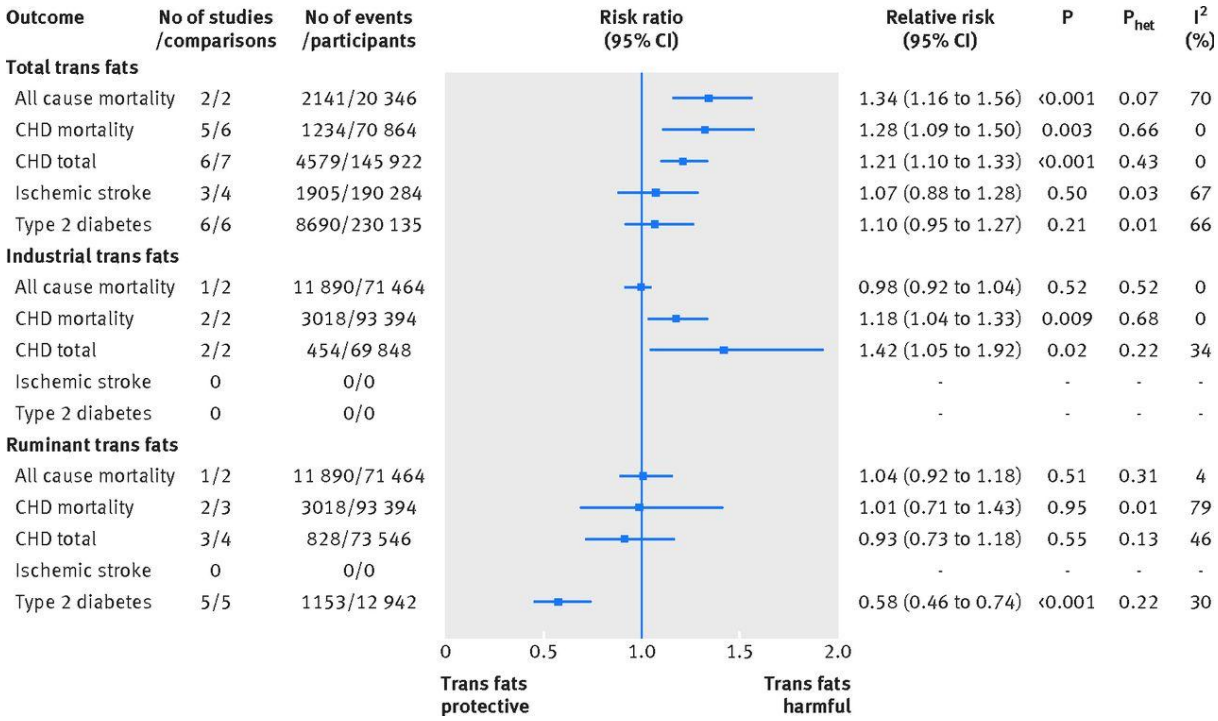


Figure 30. Summary most adjusted relative risks of total trans *fat, industrial* trans *fat, and ruminant* trans *fat and all cause mortality, CHD mortality, total CHD, ischemic stroke, and type 2 diabetes.*

For total *trans* fats effect estimate for is fixed effect analysis; all others random effects analyses. P value is for Z test of no overall association between exposure and outcome; P_{het} is for test of no differences in association measure among studies; I^2 is proportion of total variation in study estimates from heterogeneity rather than sampling error. Taken from [324]

Last but not least, we have used a mouse model to circumvent the ethical and technical difficulties of studying the influence of parental health on the offspring susceptibility to metabolic diseases in humans. We have shown that transcription of epigenetic machinery genes in the offspring is sensitive to maternal weight trajectories. Enzymes of the histone acetylation pathway were particularly impacted by maternal obesity, and preconceptional weight loss restored the expression of only a subset of these genes in liver and placenta. This study strengthens the probable involvement of epigenetics in the DOHaD paradigm, but there is still some way to go before we prove that the same mechanisms exist in humans.

1.2. A variety of models and study designs

This thesis has looked at the involvement of epigenetics in obesity from different angles, which has shed light on the strengths, difficulties and weaknesses of each approach. We have directly looked at the expression of epigenetic machinery genes in our mouse model (paper IV), but we have mostly studied DNA methylation in the CpG context using Illumina HumanMethylation beadchips (paper I, II

and III). The HumanMethylation beadchips come with inherent preprocessing difficulties for which there is no gold standard, so we chose widely-used and well-described methods (e.g. adjustment for type I and type II probes, batch correction, quantile normalization). We have performed one genome-wide, hypothesis-free investigation (paper II), as well as targeted approaches (paper I, III and IV). For practical reasons, human methylation profiles were derived from blood (paper I and II) but as this thesis aimed at understanding molecular mechanisms taking place in metabolically relevant tissues, we looked for extra data in brain and adipose tissue (paper I), and we directly investigated skeletal muscle and adipose tissue (paper III), as well as liver and placenta (paper IV). The observational designs of paper I and II allowed us to test more subjects (n=69 and n=355), but there are numerous potential confounders and uncertainty surrounding dietary reports that are inherent to such designs. In the interventional designs of paper III and IV the same individuals were assessed at several time points, thus greatly reducing uncontrolled, external influences that could bias our results. Finally we worked on preadolescents (paper II), adolescents (paper I), adults (paper I and III) and mice (paper IV) as this thesis aimed for a general understanding of the link between epigenetics and obesity in the general population.

1.3. Enriching the annotation of DNA methylation using open-access data

One major challenge in this thesis was the interpretation of differences in DNA methylation profiles [321]. As we highlighted in the introduction, the function of DNA methylation is highly context-dependent: when 5meC has a role in gene regulation, it does not necessarily regulate the closest gene, and the regulated gene(s) can differ depending on the tissue and the developmental window. Once we find differences in methylation between genotypes (paper I), how do we identify the putatively impacted gene(s)? How can we identify CpG sites that are indeed involved in the regulation of the four core circadian genes we are interested in (paper III)? This can be inferred if we know the underlying chromatin state, transcription factor binding and long-range chromatin interactions at the precise location of our CpGs of interest (Figure 29). Performing the actual experiments to obtain that information (e.g. ChIP-seq of histone modifications, chromosome conformation capture or chromatin interaction analysis by paired-end tag sequencing) would have definitely been preferred but extremely time-consuming and costly. Instead, we chose to make use of recent data generated by large consortia such as the GTEx consortium (2013), the FANTOM5 Project (2014) and the Roadmap Epigenomics Project (2015), as well as older data generated by the Genome Institute of Singapore (2011). By cross-checking chromatin states, active enhancers, eQTLs in important tissues and long-range interaction information, we found that the CpGs showing differential methylation between genotypes at obesity-associated SNPs were enriched in enhancers in blood, and we could pinpoint the putatively impacted genes. We could also identify which CpGs are located in probable enhancers for the four core circadian genes (paper III), and it proved useful since two of these CpGs showed consistent hypermethylation after sleep deprivation. Finally, we obtained replication datasets in other tissues thanks to the shared data that is regularly uploaded on open-access platforms such as Gene Expression Omnibus, ArrayExpress and dbGAP.

While the use of data generated by big consortia definitely helped to make sense of the differential methylation signal, there are limitations that are important to consider. It is now acknowledged that the epigenome is under strong genetic control [173]. Moreover, males and females show marked differences in their epigenome and they respond differently to environmental stimuli such as social behaviour, nutrition and chemical compounds [328–331]. The reference epigenomes generated by the Roadmap Epigenomics Project [332] and the active enhancers detected by the FANTOM5 Project [333] are often derived from pools of individuals or single individuals with various ethnicities and sexes, and there is no available information on their lifelong environmental exposures. It is therefore possible that the reference chromatin states and active enhancers we used in paper I and III did not accurately reflect the chromatin states and active enhancers present in our samples. Nevertheless, the inter-individual variability in DNA methylation for a given tissue is typically much smaller than the inter-tissue variability for a given individual [303,312]. Moreover, differential DNA methylation at enhancer elements, with concurrent changes in histone modifications and transcription factor binding,

is common at the cell, tissue, and individual levels, whereas promoter methylation is more prominent in reinforcing fundamental tissue identities [334]. Therefore, using reference epigenomes and reference chromatin activities in different tissues is likely useful to have a general idea of chromatin activity in a tissue of interest, especially at promoters.

2. The difficulties of studying the epigenome in the context of human obesity

2.1. Time: a crucial and underestimated factor

2.1.1. The power of longitudinal and prospective epigenetic studies

Metabolic disorders are chronic diseases that develop progressively over several months to several years, and they are not permanent as individuals can lose weight and show improvements in their metabolic parameters. However, the body remembers the hardships it goes through. Indeed, individual **weight trajectories** are important when considering mortality risk, as mortality was similar in individuals who remained obese and formerly obese individuals who lost weight [311] (Figure 27). That is because obesity at a particular age may predispose to illness, regardless of weight at higher ages, and weight loss is often associated with illness such as diabetes and cardiovascular disease. Therefore, **longitudinal studies**, particularly in **twins** (Figure 31), have the ability to capture the **dynamics of the epigenome** during individual weight trajectories, to disentangle **causality** issues between the epigenome and metabolic disorders or mortality risk, and understand whether and how the body can memorize past metabolic disturbances [49]. To our knowledge, no study has taken such an approach yet. From a more clinical standpoint, **prospective studies** can identify **epigenetic markers** of individuals at higher risk of developing metabolic diseases. A recent prospective EWAS performed in ~25,000 Indian Asians and Europeans identified several CpG sites whose methylation levels in blood associated with future incidence of T2D [257]. Two of these sites were recently confirmed in the blood of another independent prospective cohort of T2D [269], showing the potential for such approaches and their therapeutic applications.

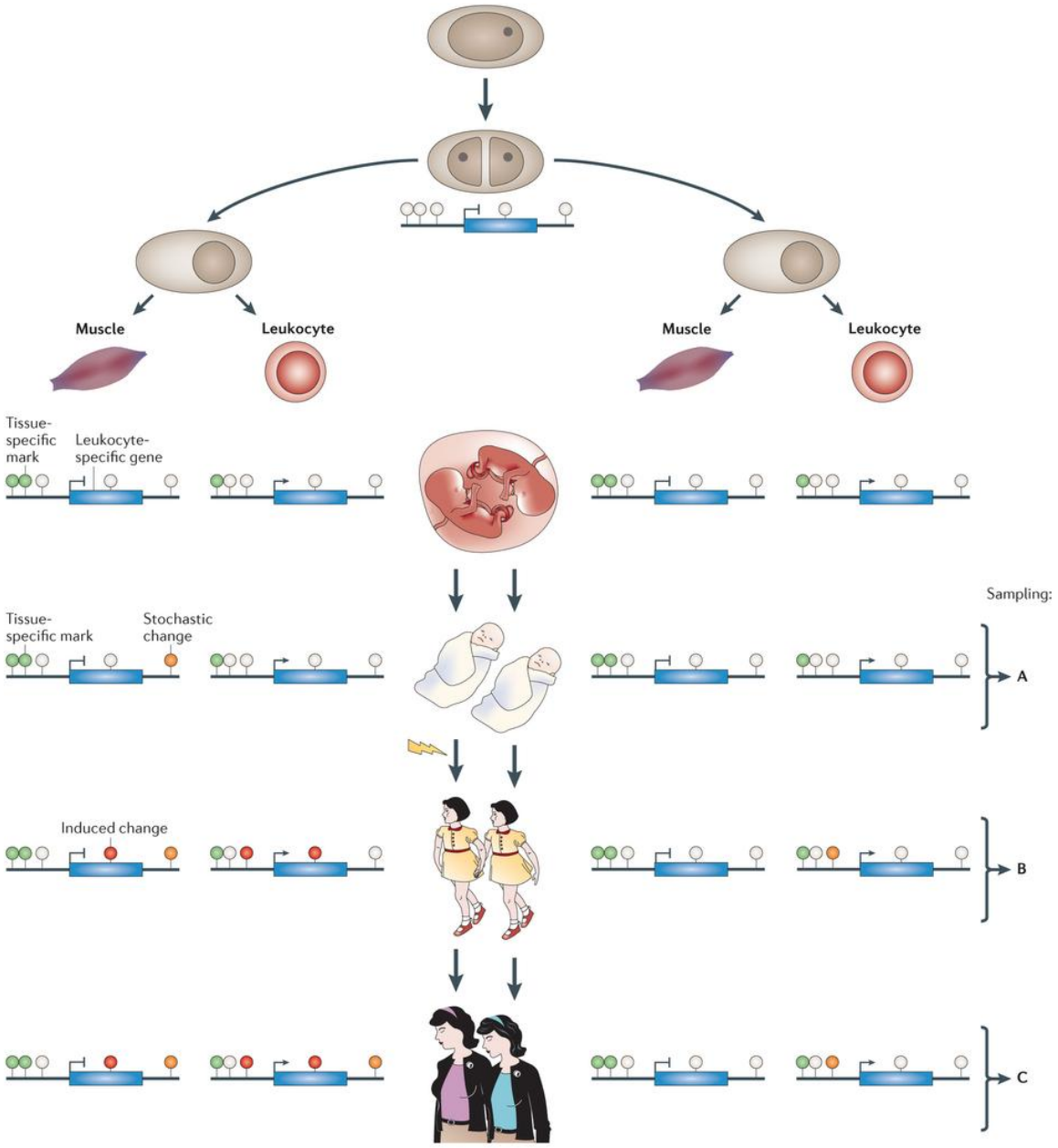


Figure 31. Longitudinal analysis of epigenetic changes in a population cohort of monozygotic twins.

The longitudinal analysis of epigenetic changes in a population cohort of monozygotic (MZ) twins is a strategy that can be particularly informative for understanding epigenetic variation and its links to disease. MZ twins share their DNA sequence, parents, birth date and sex, and are likely to have experienced a very similar prenatal environment. The figure highlights tissue-specific epigenetic marks (green) being established during gestation, which are stably maintained through life in both twins. Some stochastic epigenetic drift (orange) occurs during development, resulting in some epigenetic discordance between the two twins, but this is not necessarily related to the phenotype of interest. Non-shared environmental risk factors (lightning symbol) can induce exposure-specific alterations (red), which may be present across cell types but induce pathogenic changes only in tissues in which the disease-associated gene (blue bar) is expressed. The longitudinal sampling from these twins would highlight high phenotypic and epigenetic concordance at time A, with some stochastic epigenetic drift. After exposure (time B), the twins might become discordant for disease and show epigenetic differences at the disease-associated locus. Further sampling at time C might identify changes caused by the disease itself or perhaps a tissue-specific effect of medication. Taken from [303]

Interventional studies that look at the epigenetic basis of individual **sensitivity to weight gain or weight loss** can help identify individuals at highest risk of developing metabolic diseases and individuals most likely to benefit from weight loss interventions [297]. To date, few markers have been

consistently found across studies, but *TNF-α* is a promising candidate since it was found hypomethylated in both blood and adipose tissue of the high responders to weight loss interventions [335,336]. Nevertheless, considering the health risks associated with past obesity, more studies investigating personal sensitivity to weight gain would be welcome. Such interventional studies can also help answer questions such as: how **plastic** and **resilient** is the epigenome after exposure to an obesogenic stress? Is there a "**build-up**" of epigenetic changes over time, and if so, how long does it take to reach a build-up with significant effects on the phenotype? Are there environmentally-induced epigenetic changes that are **irreversible**? Short-term experiments have demonstrated the potential of high-fat overfeeding to induce genome-wide DNA methylation changes in adipose tissue [271] and skeletal muscle [272]. Interestingly, the methylation changes in skeletal muscle were only partly reversible after 6-8 weeks [272], suggesting that the methylome can keep track of obesogenic events with potential build-up over time. Paper III in this thesis also showed that acute sleep deprivation can induce methylation changes in core circadian genes in adipose tissue, and it would be extremely interesting to investigate a potential "build-up" of methylation changes with chronic sleep deprivation.

2.1.2. Discrepancy between DNA methylation and gene transcription: a time lag problem, an annotation problem, or not a problem at all?

A recurrent problem in the field is the apparent lack of correlation between DNA methylation and transcription. Most observational and interventional studies investigating the link between metabolic disorders and DNA methylation have looked for DNA methylation changes that are concomitant with transcriptional changes [91,194,278–280,337]. While this is a perfectly valid approach that can give information on the function of DNA methylation at the detected sites, it must be interpreted with caution. As we mentioned in the introduction, the relationship between transcription and DNA methylation is **complex and context-dependent**; transcription relies heavily on the presence or absence of other factors such as the transcription machinery and activating TFs. Therefore, while DNA methylation changes concomitant with transcriptional changes are indicative of a probable causal relationship between DNA methylation and transcription, a lack of significant correlation between DNA methylation and transcription cannot be interpreted as a lack of causal relationship. Indeed, if DNA methylation reflects past chromatin activity and can "prime" genes to be activated or repressed, in many cases there would be the need for an environmental stress or stimulus to reveal the effects that DNA methylation has on transcription (Figure 32). The relevant question to understand the role of DNA methylation in metabolic disorders then becomes: **can baseline methylation differences induce a difference in transcriptional response to a specific environmental stimulus**, and if so, is this response related to metabolic disorders? One study has taken such an approach by comparing the transcriptional response of lean and obese women after exposure of their skeletal muscle to lipids [198]. They found that obese women had an impaired ability to upregulate global transcriptional regulators of fatty acid oxidation in response to lipid exposure, but these differences were not mediated by a difference in baseline methylation at *PPARδ* [198]. In addition, the **speed** at which DNA methylation and gene transcription change may explain why methylation and transcription often do not correlate. In a study looking at the influence of acute exercise on DNA methylation and transcription, promoters of *TFAM* and *PPARδ* were hypomethylated and transcribed at the same time, while promoters of *PGC-1α* and *PDK4* were hypomethylated immediately after exercise but transcribed only 3h after [93] (Figure 33). Therefore, there seems to be variability in the timing of DNA hypo- or hyper-methylation induced by environmental stimuli and the corresponding gene transcription [338].

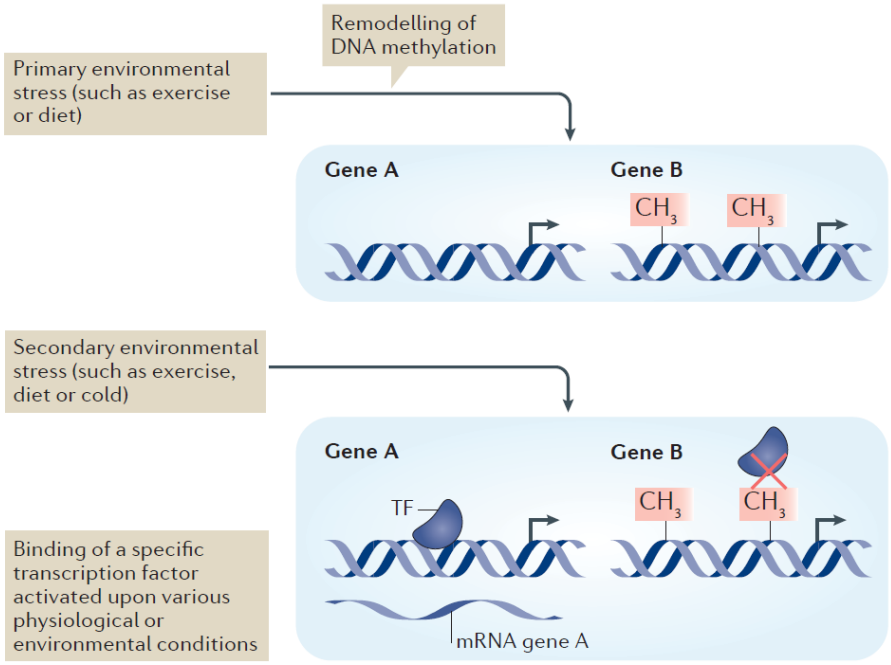


Figure 32. Potential effect of environmentally induced epigenetic changes on gene expression.

In this hypothetical model, gene A is hypomethylated, while gene B is hypermethylated in response to environmental cues. Gene expression changes might not occur until a secondary environmental stress or specific physiological state is altered, thereby enabling specific transcription factors to bind to the hypomethylated gene A, but not to hypermethylated gene B, which results in transcription of gene A. This model also explains the often observed discrepancy between DNA methylation and transcriptional changes. In this model, the same epigenetic modification in multiple tissues might only be functionally relevant in a given tissue if a specific transcription factor is also activated. Taken from [339]

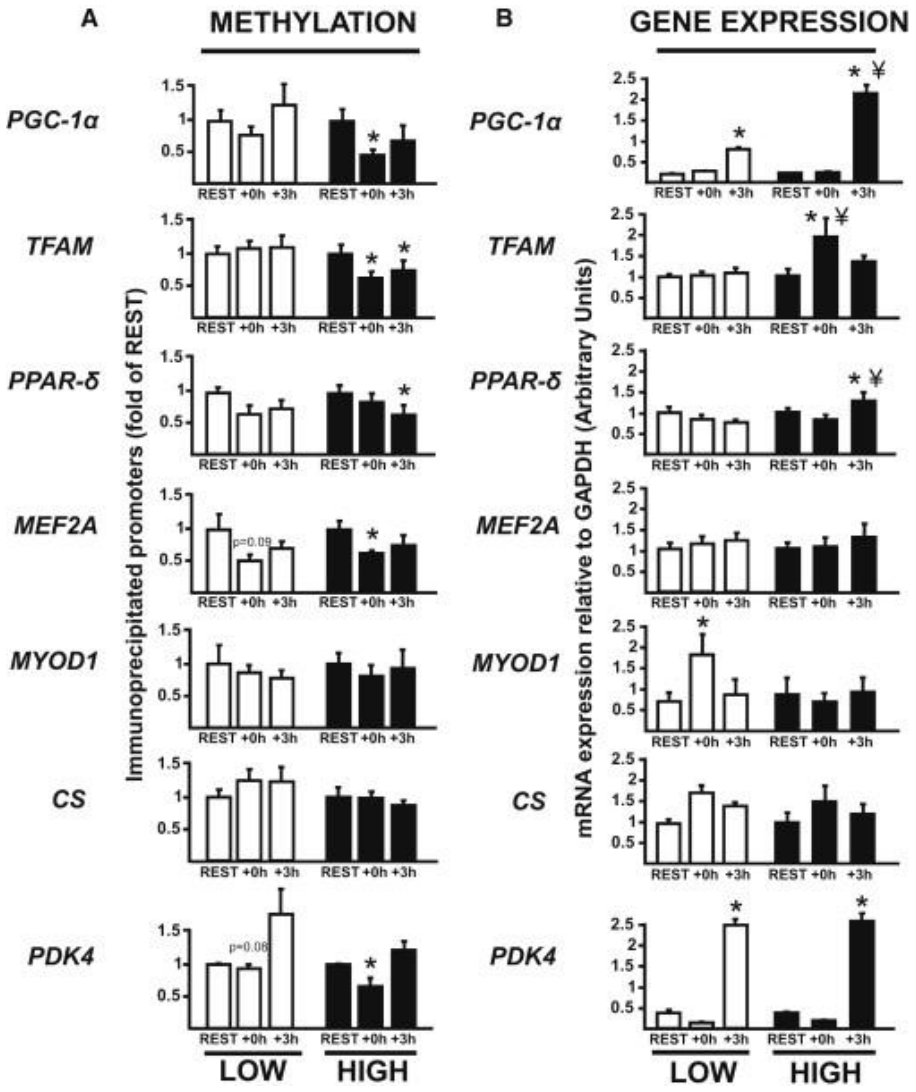


Figure 33. Exercise-induced promoter hypomethylation is intensity-dependent (A and B)

Promoter methylation (A) and respective mRNA level of genes (B) involved in fuel utilization and mitochondrial biogenesis as measured by MeDIP-qPCR at REST, 0 hr, or 3 hr after low- or high-intensity acute exercise (light or dark bars, respectively); n = 8 subjects. Results are mean ± SEM. ∗p < 0.05 versus REST, ¥p < 0.05 versus low-intensity exercise. Taken from [93].

Another hypothesis that can explain the apparent lack of correlation between DNA methylation and transcription has to do with the **annotation of 5meC**. As mentioned in the introduction, chromatin states at given positions vary between tissues, so 5meC may regulate different genes depending on the tissue. For instance, elements called *cis*-regulatory elements with dynamic signatures show a strong promoter signature in one tissue but an enhancer signature other tissues [332,340,341]. Therefore, a given 5meC can be located in the promoter of gene G_1 and regulate gene G_1 in tissue T_1, but if that genomic location also corresponds to an enhancer for gene G_2, the 5meC would then regulate gene G_2 in tissue T_2. Unfortunately, little effort has been made to annotate 5meC with accuracy: when 5meC falls inside a gene promoter or a gene body, it is often annotated to that gene, without consideration for the underlying chromatin state. 5meC located in open sea is either annotated to the closest gene or left without annotation. We suggest that this annotation problem is actually quite important. We tried to annotate the ~450,000 CpGs of the widely-used Illumina HumanMethylation 450k beadchip to specific genes in female skeletal muscle. The 450k beadchip mostly targets CpG islands and gene promoters, but also gene bodies and open seas. We downloaded the extended chromatin state annotation

of female skeletal muscle built with 6 histone marks (H3K4me3, H3K4me1, H3K36me3, H3K27me3, H3K9me3 and H3K27ac) and released by the Roadmap Epigenomics Project [342]. Then, we calculated the proportion of these ~450,000 CpGs that are located in genomic regions matching the underlying chromatin state in female skeletal muscle (e.g. if a CpG falls inside a gene body and corresponds to an "active transcription" state, the genomic location (gene body) matches the chromatin state (active transcription). However, if this CpG falls inside a gene body and corresponds to an "active enhancer" state, the genomic location does not match the chromatin state). Only 44% of the CpGs from the chip were located in a genomic region matching the underlying chromatin state in female skeletal muscle. This problem is not limited to the HumanMethylation 450k beadchip, but can also arise with any other methylation assessment technique (e.g. MeDIP-seq, RRBS, WGBS).

2.1.3. Are natural circadian (and circannual) variations in the epigenome confounding studies?

Circadian variations of the epigenome were discovered in the human brain [343–346] and mouse liver [347–349] (Figure 34), meaning that **some of our epigenome is highly flexible and can change within just a few hours as part of our natural daily cycle**. However, no study has established the exhaustive natural circadian variations of the epigenome in tissues important for metabolism, or in tissues often used in human studies (e.g. blood). One study claimed to have detected sine-like oscillations in DNA methylation over a whole day in blood of healthy subjects, but they did not adjust their analysis for possible changes in blood cell-type composition during this 24h period [350]. However, changes in blood cell-type composition was recently found to occur after a the ingestion of a single meal, and explained >99% of all the methylation differences detected 160 min after the meal [351] (Figure 35). Besides, individuals do not necessarily have circadian rhythms that have similar phases or periods, which is reflected by their **chronotype**, also called morningness–eveningness (i.e., the tendency to be an early “lark” or a late “owl”) [352]. Chronotype can change with age: older people tend to be more skewed towards morningness than younger individuals, and it is still debated whether chronotypes differ between sexes and ethnicities [352,353]. This issue is particularly relevant when considering metabolic disorders, since the evening chronotype is associated with metabolic traits such as obesity, diabetes and sarcopenia [354–356]. Finally, it was recently discovered that 4,000 protein-coding mRNAs in white blood cells and subcutaneous adipose tissue have **seasonal expression profiles in humans**, with inverted patterns observed between Europe and Oceania [357] (Figure 36). Importantly, in adipose tissue, as in blood, metabolic pathways were among the most associated seasonal pathways [357]. The authors observed seasonal variations in blood cell-type composition that probably explain part of the seasonal variations in mRNA, but it is also possible that the epigenome shows seasonal variations and contributes to this seasonality. Therefore, studies comparing individuals at a single time point, or comparing the same individuals over time need to ensure that the epigenetic differences they observe are not due to natural daily or yearly oscillations. Nevertheless, even if epigenetic differences are observed at CpG sites known to follow circadian or circannual oscillations, these oscillations would not confound the results as long as the magnitude of the epigenetic differences exceeds by far the effect sizes of the oscillations.

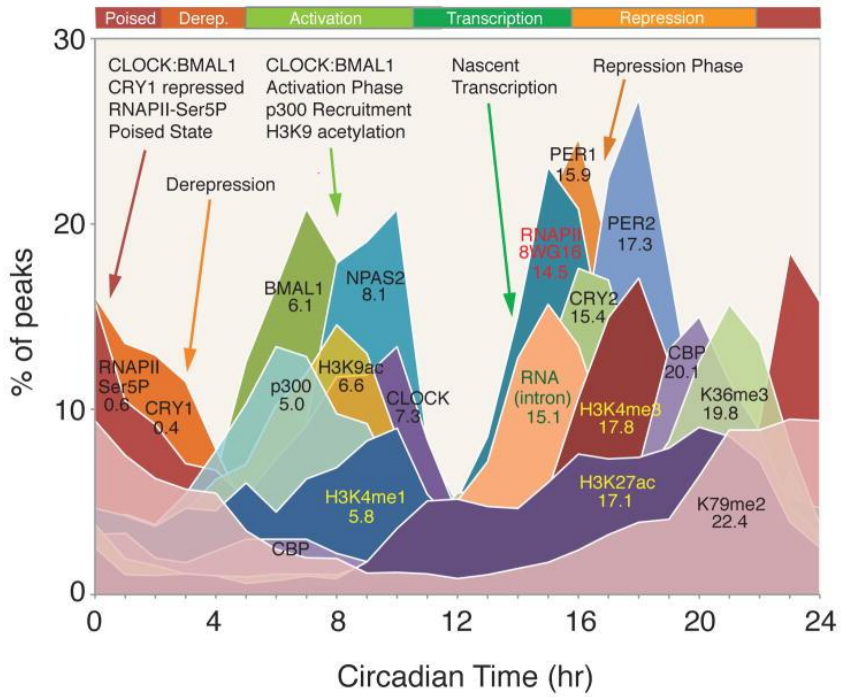


Figure 34. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals.

Circadian landscape of the cistrome and epigenome of the murine liver. Taken from [349]

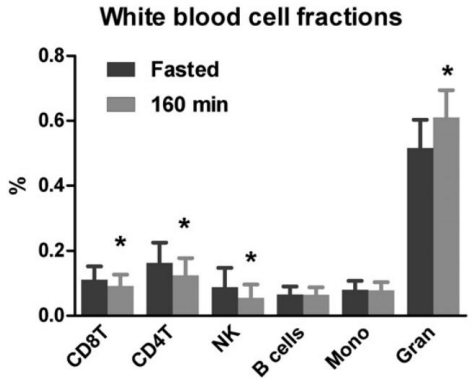


Figure 35. Changes in fractions of WBC after food intake.

WBC fractions were inferred from methylation data. Paired t-tests were used to test for differences in WBC fractions in participants (n = 25) between the fasted and postprandial states. *P , 0.001. Gran, granulocyte; Mono, monocyte; NK, natural killer; WBC, white blood cell. Taken from [351].

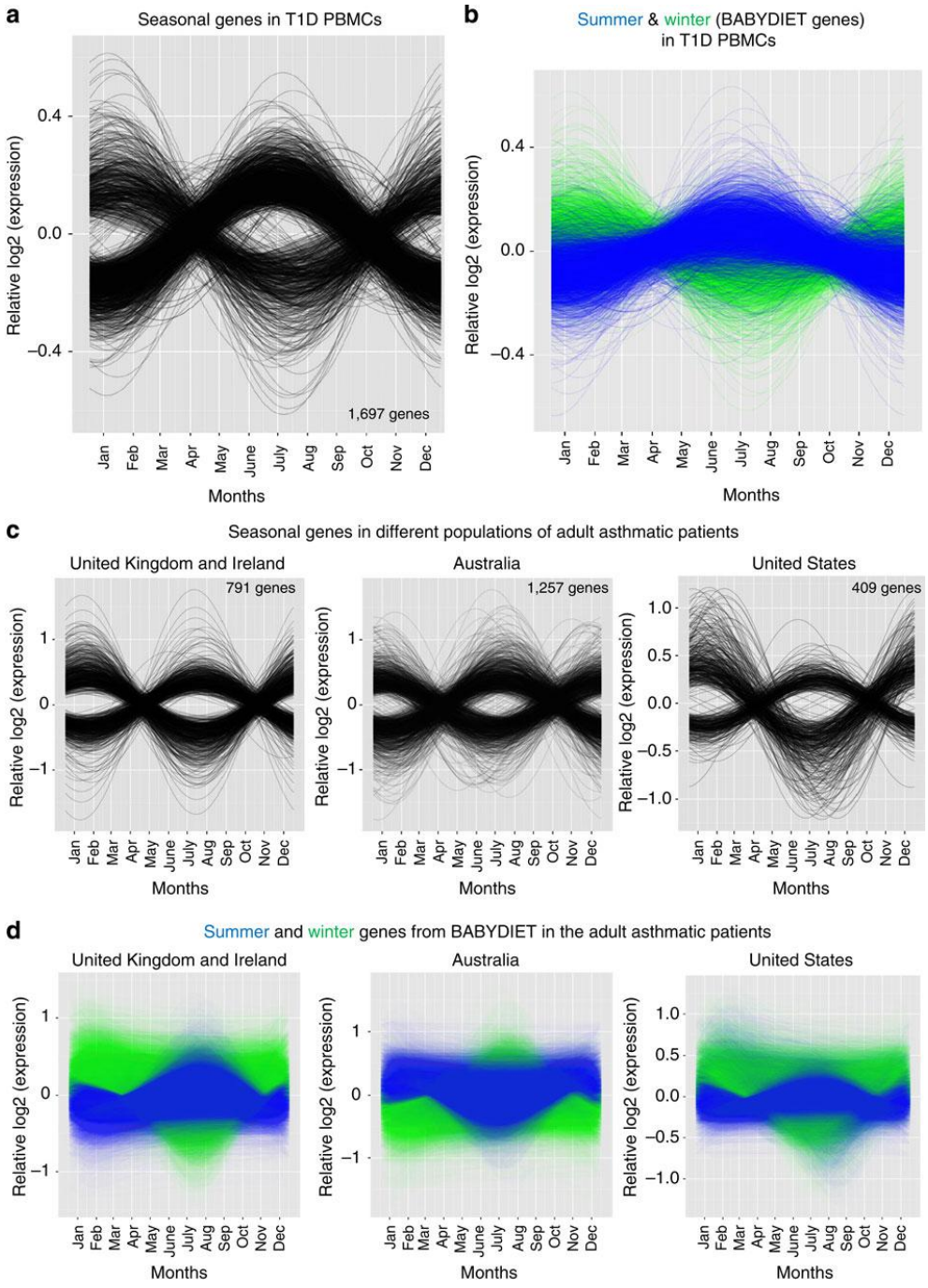


Figure 36. Seasonal gene expression in geographically distinct cohorts.

(a) Seasonality was also observed in PBMCs collected from T1D patients in the United Kingdom (n=236 individuals). A total of 1,697 genes were seasonal in this data set. (b) The previously defined summer and winter genes from the BABYDIET data set maintained their seasonal expression patterns in the T1D samples. (c) PBMCs from asthmatic patients collected from different countries also showed seasonal gene expression. In the United Kingdom/Ireland (n=26 asthmatic individuals; 85 PBMC samples), 791 genes were seasonal, while 1,257 and 409 genes were seasonal in Australia (n=26 individuals; 85 samples) and United States (n=37 individuals; 123 samples), respectively. (d) Summer and winter BABYDIET genes maintained their seasonal expression patterns in the asthmatic PBMC samples, with their patterns inverted in Australia. Taken from [357].

2.2. Tissue-specificity of DNA methylation

2.2.1. Choosing the right tissue to study

The methylome is notoriously **tissue-specific**, and this tissue specificity is believed to be responsible for establishing and maintaining cell-type identity [358,359]. Even different blood cell types (T-cells, B-cells, NK-cells, granulocytes and monocytes) show marked methylation differences [360,361]. Therefore, it seems only natural to investigate DNA methylation in tissues implicated in metabolism, but these tissues are unfortunately difficult to collect in humans. The hardest to access is the brain since it can only be sampled post-mortem. The digestive tract, liver, visceral fat and pancreas may be sampled during surgery, but diseased individuals are typically overrepresented and participants remain scarce. Subcutaneous adipose tissue and skeletal muscle biopsies are feasible without resorting to surgery, but they can only be performed a limited number of times per day because of the discomfort they cause. Saliva and blood are undeniably the easiest tissues to sample in a non-invasive manner and that is the reason why most methylation studies to date were performed in blood. But what is the point of studying a tissue that is not directly related to metabolism? It all depends on the aim of the study. First of all, **obesity is characterized by low-grade, chronic inflammation** [314,362] whereby white blood cells are more abundant in the circulation [363–365] and they infiltrate the adipose tissue [366] (Figure 28). As such, studying epigenetic changes with obesity in blood cells can yield interesting insights into this inflammatory process. Second, studies whose aim is to identify **biomarkers** that can be useful in a clinical setting do not need to worry about the possible function of their biomarker at the molecular level: as long as the biomarker shows good sensitivity and specificity for patients at risk, it has fulfilled its purpose. However, when the focus of a study is the brain, does it make any sense to investigate blood? When methylation changes associated with obesity are observed in blood, how should we interpret them and what information on other organs do they truly give?

M. Szyf proposed three hypotheses that could explain the association between DNA methylation in blood and behaviour or brain-related phenotypes [367], and these hypotheses can also apply to the case when DNA methylation changes in blood associate with metabolic disorders. First of all, it is possible that the DNA methylation changes observed in blood do not reflect what is happening in other tissues because these changes are the consequence of the **blood-specific response to the environmental insult**, and other tissues would show DNA methylation changes of their own. Second, if DNA methylation changes are studied in the DOHaD context, we can very well imagine that **an environmental insult that took place early in life** impacted common precursor cells to blood and other tissues, and has therefore caused the same methylation changes in blood and other tissues. Third, **circulating molecules** such as hormones, leptin, adipokines, insulin, and perhaps even miRNAs that are released in response to an experience or an exposure **will cause similar methylation changes in blood and other tissues**. For instance, the leptin receptor is abundant on the membrane of hypothalamus cells, but it is also present on the membrane of blood cells [368], so circulating leptin might cause similar methylation changes in both the hypothalamus and the blood. Interestingly, in abdominal skeletal muscle of women with T2D (manuscript under preparation), we have detected DNA methylation changes at one CpG in thioredoxin interacting protein (*TXNIP*) and at one CpG in Suppressor of cytokine signaling 3 (*SOCS3*) that are similar in direction and magnitude to DNA methylation changes observed in blood in other independent T2D cohorts [253,256,257,369,370]. To summarise, it is likely that the DNA methylation changes observed in blood are a combination of at least the three aforementioned hypotheses to varying degrees, but these changes cannot be extrapolated to other tissues without investigating them directly.

2.2.2. Adjusting for cell-type composition: a major issue

The human body is made of highly specialized tissues, and these tissues are themselves made of a variety of highly specialized cells. Importantly, environmental stimuli can change **the relative proportion of these different cell types** at various rates depending on the tissue. For instance, skeletal muscle is made of ~90-100% muscle cells and ~1-10% satellite cells that have a role in muscle repair [371] (Figure 37). Satellite cell content increases with long-term endurance and strength training [371,372] and declines after two weeks of bed rest [373]. Another tissue that undergoes drastic

changes in cell type proportions is blood. It was recently shown that the estimated leukocyte population in whole blood changes just 160 min after a meal (Figure 35), and these changes in blood cell type proportion explained >99% of the detected methylation changes [351]. Moreover, as we mentioned earlier, obesity is characterized by low-grade, chronic inflammation [314,362] whereby white blood cells are more abundant in the circulation [363–365] and they infiltrate the adipose tissue [366] (Figure 28). The issue of cell-type composition of tissues is particularly worrying given the fact that reported **DNA methylation changes with obesity are often <10% methylation difference**, in blood [244–261], liver [262,263], adipose tissue [230,258,261,264–266], and sperm [267]. How can we unsure that these methylation changes are not an artefact and do not reflect a simple change in cell type composition?

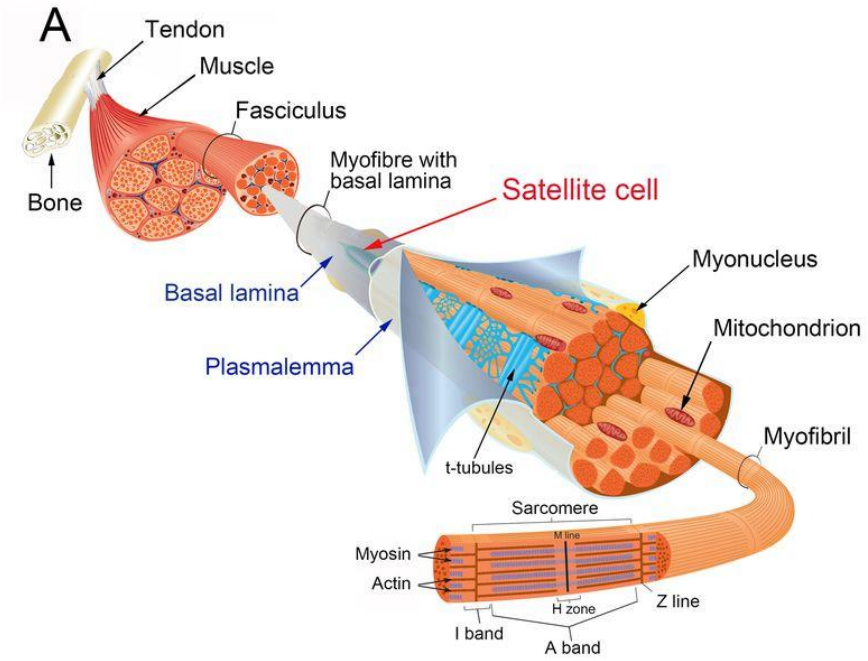


Figure 37. Muscle structure and the satellite cell niche.

(A) The structure and ultra-structure of skeletal muscle. The satellite cell niche is on the surface of the myofibre, beneath the surrounding basal lamina, as indicated. Taken from [374]

Depending on the tissue under study, it may be directly possible to estimate the relative cell-type proportions with **histological techniques** or **flow cytometry**. For instance, the relative proportions of pancreatic α and β cells can be estimated with histologic analysis [375] and the relative proportions of blood cell types with flow cytometry [376]. However, flow cytometry requires a large amount of fresh blood and laborious antibody tagging [313]. Several **bioinformatical techniques** have been developed to capture and account for differences in cell type composition without having to resort to these laborious techniques: a method implemented in the *minfi* R package [377], a method similar to regression calibration developed by Houseman et al. [313], FaST-LMM-EWASher [378], RefFreeEWAS [316] and one method called surrogate variable analysis (SVA) [379]. Some of these techniques rely on reference methylomes of purified cells while others are reference-free, and they offer the scientific community a fast and easy way to detect true methylation differences with high sensitivity and specificity [380]. In particular, SVA outperforms all other reference-free techniques [380] (Table 6) and can account for other sources of heterogeneity between groups, such as genetic, environmental, demographic, and technical factors [379]. A last method consists in using a set of CpGs that are known to vary considerably between cell types and to perform a principal component analysis (PCA) on them; the top principal components (PCs) can then be included as covariates in the analysis to account for the cell type profile of individual samples [188,381]. Therefore, we believe that given the immense amount of possible confounders in human EWAS, one of the aforementioned techniques should be

routinely used regardless of the study design and investigated tissue, in order to increase the biological accuracy and reproducibility of analyses.

	Sensitivity: Median (95% CI)	Specificity: Median (95% CI)
FaST-LMM-EWASher	0.00 (0.00, 0.52)	1.00 (0.99, 1.00)
RefFreeEWAS	0.98 (0.00, 1.00)	0.94 (0.93, 1.00)
SVA	1.00 (0.98, 1.00)	0.94 (0.93, 0.94)

Table 6. Sensitivity and specificity with respect to truly identified variables using 100 simulated data

CI: confidence interval. Taken from [380]

2.3. Genetic influences on the methylome

2.3.1. Heritability of the methylome

The strong associations between obesity-associated SNPs and proximal DNA methylation we detected in paper I are not restricted to disease variants (Figure 38). Twin studies have found a relatively low mean heritability across tissues (12-19%), but heritability was extremely variable at different sites (ranging 0-100%) [178,180,382]. In what is probably the largest and least biased human meQTL study to date, McClay *et al*. used methyl-CpG-binding domain protein-enriched genome sequencing (MBD-seq) to uncover meQTLs genome-wide [189]. They found that 15% of all the ~3.2 million CpGs they assessed were associated with meQTLs in *cis*. Array-based meQTL studies typically reported a smaller fraction of the surveyed CpG to be associated with meQTLs in *cis* (0.12-15%) [172,177,178,181,184,187,383–388]. MeQTLs are enriched in heterochromatin and quiescent regions of the genome, and 75% of them are caused by the presence of SNPs inside CpGs that impair their methylation potential [189]. Thus, although the majority of the meQTLs may not be functional, a substantial proportion of them are present in active chromatin states such as active/weak enhancers and active TSSs [189]. Interestingly, disease variants detected in GWASs are enriched within 200 bp of these meQTLs [189], and it is likely that they are also associated with other epigenetic marks such as histone modifications [383] (Figure 39). Only two studies have had the statistical power to test the presence of meQTLs in *trans* using two different techniques (one next-generation sequencing method and one array-based method), and they both found the same proportion of the tested CpGs to be associated with *trans*-meQTLs (0.5% and 0.6%) [188,189]. In summary, **the overall proportion of CpGs that are under genetic control may seem low, but there is a high heterogeneity in heritability at different CpGs**. For further reading, we recommend an excellent review on the topic that was published in May 2016 [173].

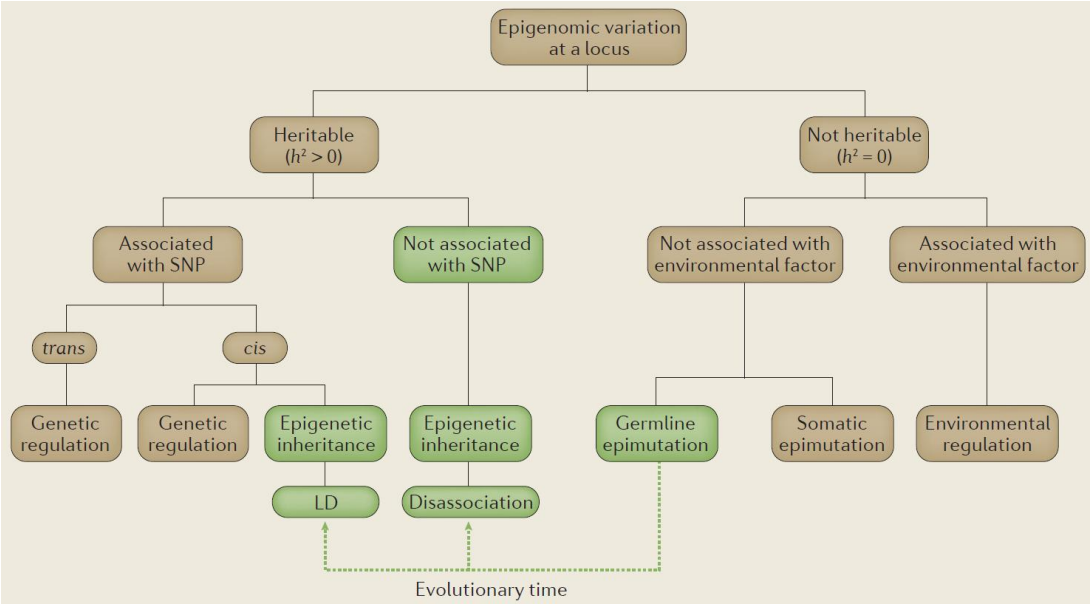


Figure 38. Sources of population epigenomic variation.

Epigenomic variation at a locus can be treated as a quantitative trait. Heritability estimates can be obtained using classical variance components analysis using pedigree data (for example, parent–offspring, twins, and so on). In the absence of epigenetic inheritance, a non-zero heritability estimate ($h^2 > 0$) implies that epigenomic variation at the locus is under genetic control by *cis*- or *trans*-acting sequence variants. When epigenomic variation is not heritable ($h^2 = 0$), variation could be the result of differential exposures to past or current environmental factors. Systematic identification of such environmental factors should be possible and is one goal of epigenome-wide association studies. In the absence of causative environmental factors, epigenomic variation may be the outcome of stochastic somatic epimutations that lead to intra-individual tissue heterogeneity and inter-individual 'epigenetic drift'. Detection of such somatic epimutations will require advances in single-cell epigenomic sequencing technologies. The often stated conclusion that epigenomic variation is under genetic control whenever *cis*-SNP associations are detected, or non-zero heritability estimates are found, is strictly only valid if epigenetic inheritance can be assumed absent. This assumption should always be checked against emerging experimental data. Adapted from [173]

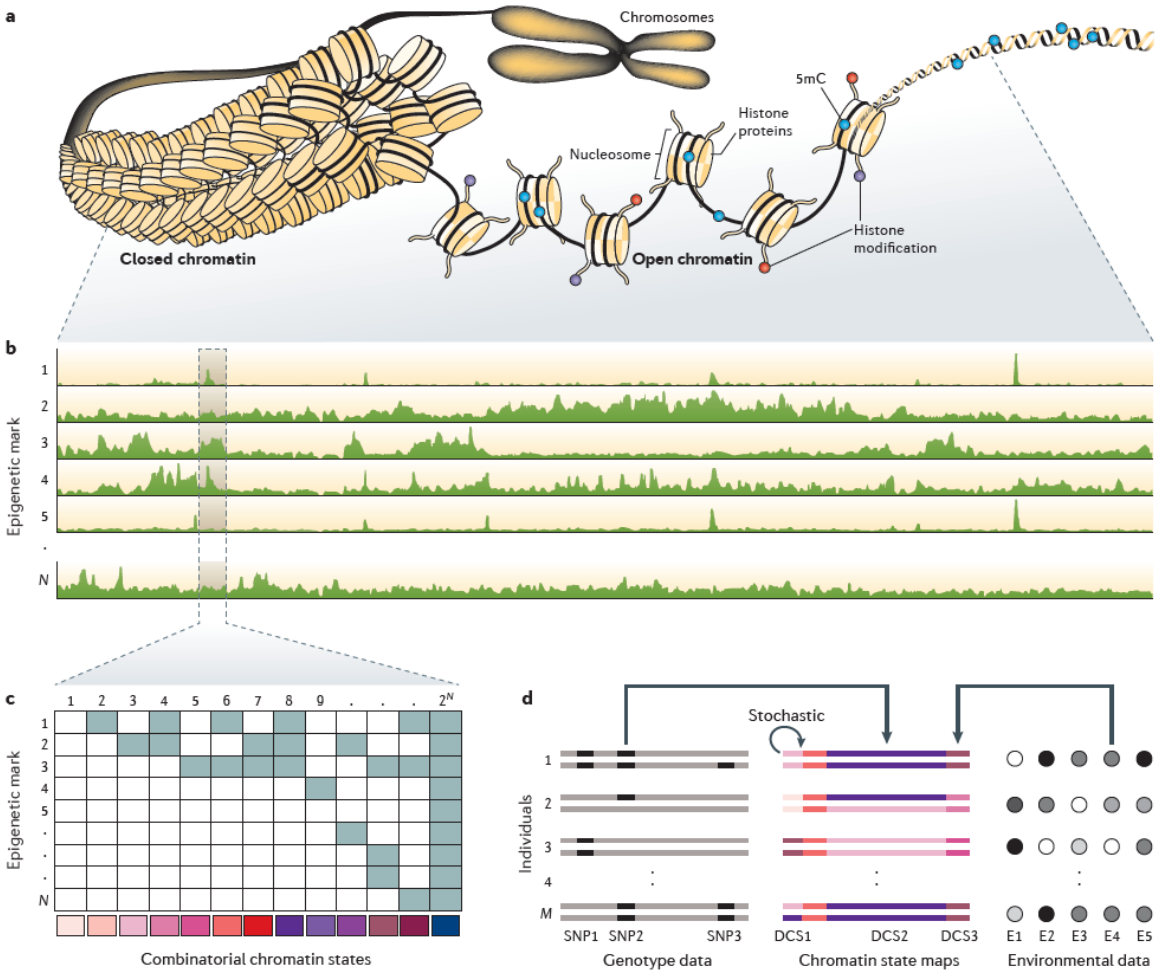


Figure 39. Main steps in population epigenomic analysis.

a | DNA is tightly packaged in cells and is functionally modified by a variety of epigenetic marks, such as cytosine methylation (5mC) or post-translational changes in histone proteins. The co-occurrence of specific epigenetic marks in a genomic region defines its functional state. Of note, histones in closed chromatin also contain repressive marks (not shown). b | The genome-wide distribution of different epigenetic marks can be measured using next-generation sequencing (NGS) technologies. Shown are the read-tracks from NGS measurements of N different epigenetic marks along the genome. c | The computational challenge is to infer distinct chromatin states for each genomic position. These chromatin states are defined by the joint presence and absence patterns of the different epigenetic marks. With N marks there can be 2N possible combinatorial states. The colour code on the bottom denotes each unique state. This analysis leads to the construction of chromatin state maps. d | Shown are the chromatin state maps of M diploid individuals. Individuals differ in their chromatin states in three genomic regions. These differential chromatin states (DCSs) can originate from DNA sequence polymorphisms, environmental factors or from stochastic changes. DCS2 is caused by a single-nucleotide polymorphism (SNP2), DCS3 is caused by exposure to environmental factor E4 and DCS1 is the result of stochastic processes in the mitotic maintenance of the chromatin state at that locus. The statistical challenge is to try to identify these causal factors from millions of measured SNPs and a large number of environmental factors. Taken from [173]

2.3.2. Accounting for population stratification in epigenetic studies

Why does this all matter when studying epigenetics in the context of obesity and its complications? If not properly accounted for, **genetic differences can be a major confounder in epigenetic studies, especially when sample sizes are low**. If the allele frequency at a meQTL is unbalanced between the groups being compared, a DMR will be detected between the groups, despite the fact that it is only due to the presence of the SNP and not the condition of interest. There are several ways to unsure that SNPs are not confounding the DNA methylation analysis: the first method consists in sequencing the region surrounding the DMR and see if any SNP in the sequence correlates with the DMR. However, depending on the population under study, the effects of SNPs can span several thousand bp [389,390]

and it is often not possible to sequence such a large region for all detected DMRs. Another method has been used in GWAS and consists in performing a PCA on genome-wide genetic data for all individuals. The top PCs of the PCA can then be used in the association tests as a proxy for ancestry [391]. However, genome-wide genetic data is often not available in EWAS, so it has been proposed to use genome-wide DNA methylation data as a proxy for ancestry, given that some 5meC are under strong genetic control [173]. Unfortunately, this technique poses problem, as DNA methylation levels are associated with many more factors than genetic variants: age, sex, and plethora of environmental factors. Therefore, the use of PCs based on genome-wide methylation data may remove important sources of variability, a problem that Barfield *et al*. addressed by computing the PCs of sets of CpG sites near SNPs to compute PCs that are better proxies for ancestry [392]. They have made available their lists of CpG sites from the Illumina 450K that are located within 0-100bp of 1000 Genomes Project variants with minor allele frequency >.01, along with R code to compute the PCs (http://genetics.emory.edu/research/conneely/) [392].

3. Unanswered questions and future research directions

3.1. The biological meaning of DNA methylation changes: (effect) size matters

The **direction** of an effect is just as important as the **magnitude** of this effect, but this magnitude cannot be inferred simply by looking at the most common statistic reported in papers: **the p-value**. Typical DNA methylation changes detected in cross-sectional or prospective studies of obesity and related comorbidities have consistently found widespread but small DNA methylation changes (< 10% methylation change) between groups. This means that only a tiny proportion of cells have a methylation status that differs between groups at a given location (Figure 40). What does this **biologically** mean? It may mean that obesity and related comorbidities impact **plethora of genes at a low level**, which results in **large disturbances** of pathways important for metabolism. But can a difference of methylation in only a few cells cause a significant change in transcription and eventually, in phenotype? Surprisingly few papers have addressed that question, but it is an important one [393]. Murphy *et al*. examined the correlation between *IGF2* transcription and the methylation level of the *IGF2* DMR, and they suggested that a change in methylation as little as 1% at this DMR can lead to either a doubling or halving of transcription, depending on the direction of methylation [394]. However, these estimations were based on pyrosequencing whose technical variability can go up to 5% methylation difference between technical replicates [395], and using a very small sample size (n=41) [394]. Not only do small effect sizes pose biological questions, but they also raise an important **statistical problem**. If effect sizes are that small, wouldn't we need very large sample sizes to detect methylation differences that are significant at the genome-wide level? Tsai & Bell performed simulations to estimate power under the case-control and discordant MZ twin EWAS study designs, under a range of epigenetic risk effect sizes and conditions [396]. They found that to detect a mean methylation difference of 7% at genome-wide significance with 80% power, 178 pairs of twins or 211 cases and 211 controls would be needed [396] (Figure 41). In addition, a high variance in methylation in cases or controls, as well as genetic and environmental variables influencing DNA methylation levels are likely to inflate these numbers [396]. The rapid development of targeted **epigenome editing**, via an adaptation of the clustered regulatory interspaced short palindromic repeat (CRISPR)-Cas system [397–399] (Figure 42) or the transcription activator-like effector (TALE) protein [400] will allow researchers to cause site-specific DNA methylation and answer both questions of causality and effect sizes. Effect size may also be important on the **clinical side**, since we need biomarkers that can identify individuals at risk with high sensitivity and specificity. If DNA methylation differences between diseased individuals and controls are extremely small, it will be difficult to find a reliable biomarker, and we may need to use a combination of several biomarkers to increase sensitivity and specificity. Zeevi *et al*. took a brilliant machine-learning approach to identify individuals at high risk of showing high glucose peaks after certain foods and paved the way to personalized nutrition [401]. It would be extremely interesting to see re-

search teams take a similar approach to test the reliability of the few epigenetic biomarkers identified to date (e.g. CpGs in *PHOSPHO1* and *ABCG1* as predictors of T2D).

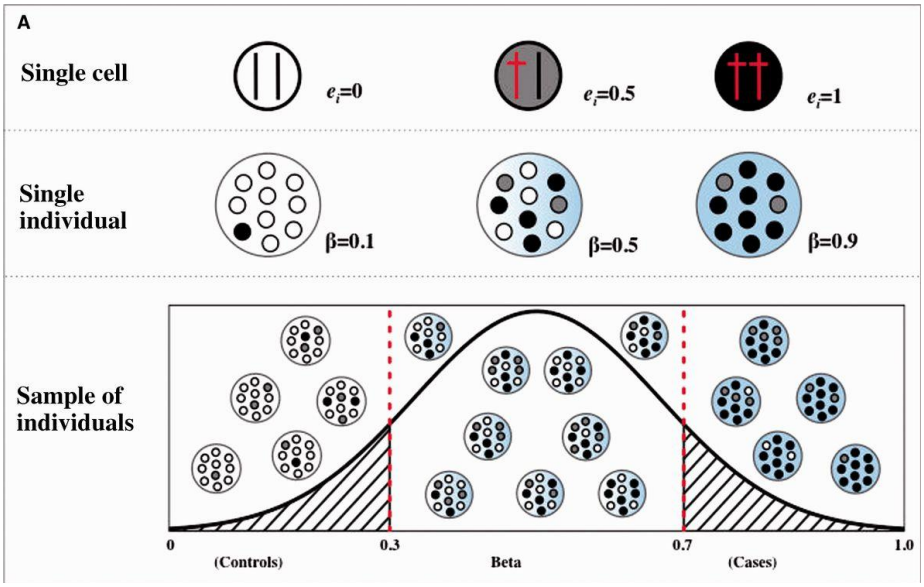


Figure 40. DNA methylation patterns at the cellular and individual levels.

We assume that a cell can have two methylated alleles ($e_i = 1$), one methylated allele ($e_i = 0.5$) or two unmethylated alleles ($e_i = 0$), and one sample from an individual contains different frequencies of these cells (upper panel). The methylated allele is shown as a dagger symbol, and the colour of each cell represents its methylation status: un-methylated (white), hemi-methylated (grey) and methylated (black) (upper panel). The methylation in each sample is represented as the summary of the methylated epi-allele, denoted here as beta (middle panel) which can range from 0 to 1 (lower panel). We assume that cases have greater mean methylation levels compared with controls, and we propose one control and eight case distributions. Taken from [396]

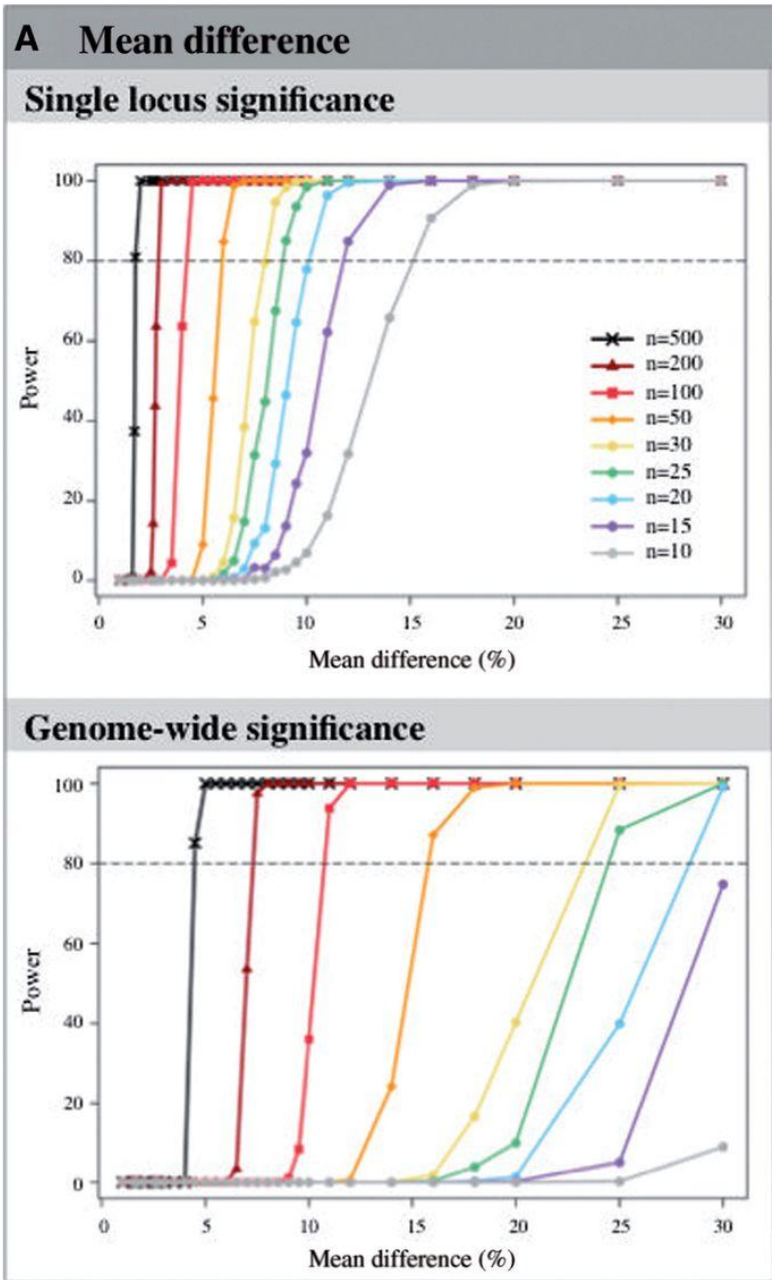


Figure 41. Power of case-control EWAS.

Estimates are obtained for a range of sample sizes, using (A) mean difference and (B) methOR effects, at nominal (upper panel) and genome-wide (lower panel) significance thresholds. Each line represents the power curve under different case-control sample sizes from 10 to 500 pairs of cases and controls. Taken from [402]

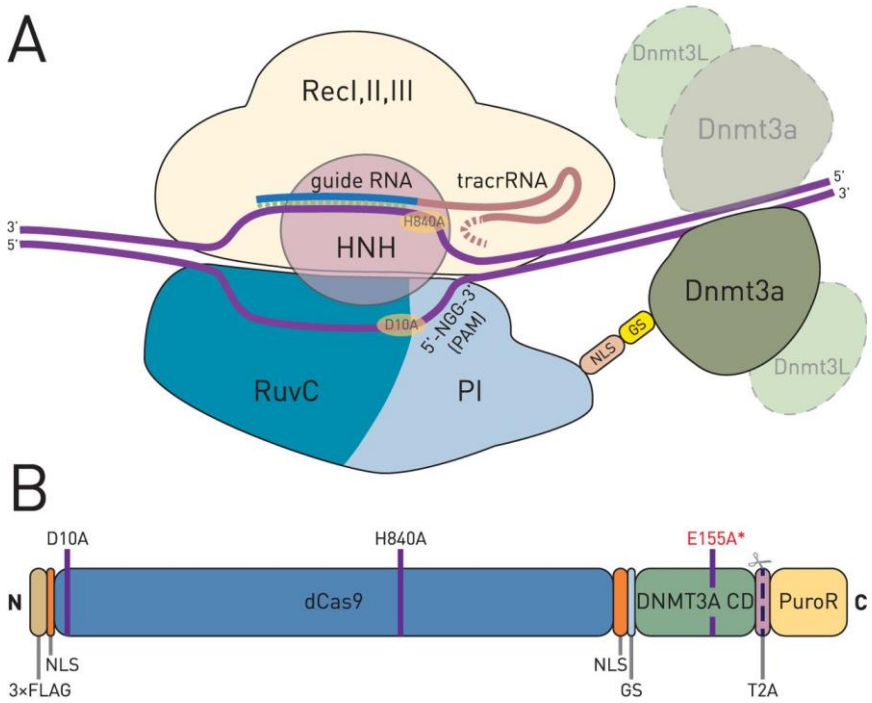


Figure 42. Schematic representation of a dCas9-DNMT3A fusion protein in complex with sgRNA and its target DNA sequence.

The sgRNA is bound in a cleft between the recognition lobe (RecI, II and III domains) and the nuclease lobe (HNH, RuvC and PI domains) of Cas9 protein. The C–terminus of Cas9 is located on the PAM–interacting (PI) domain and faces the side where the bound genomic DNA protrudes with its 3′ end relative to the sgRNA sequence. The sgRNA is a synthetic fusion between bacterial crRNA and tracrRNA, with guide sequence and tracrRNA part shown in different colours. The catalytic domain of DNMT3A recruits its partner for dimerization and DNMT3L proteins in vivo (dashed lightened symbols). NLS, nuclear localization signal; GS, Gly4Ser peptide linker. (B) Domain structure of the dCas9–DNMT3A fusion protein. The nuclease-inactivating mutations D10A and H840A of Streptococcus pyogenes Cas9 are indicated. Deactivated Cas9 was fused to the catalytic domain of the human de novo DNA methyltransferase 3A (DNMT3A CD) using a short Gly4Ser peptide (GS). The dCas9–DNMT3A is expressed as a bicistronic mRNA, along with puromycin resistance gene (PuroR, shown) or EGFP gene, thus enabling selection of transfected cells. The PuroR (or EGFP) moiety is separated during translation by action of the T2A self-cleaving peptide. The inactive fusion methyltransferase (dCas9-DNMT3A-ANV) for use as a negative control contains an additional substitution (E155A*) in the active site of DNMT3A. 3x FLAG, epitope tag; NLS, nuclear localization signal. Adapted from [397]

3.2. The developmental origins of leanness and obesity

As we highlighted in the introduction, the obesity epidemic has happened over just a few decades, which means that it is mainly driven by environmental factors such as excess caloric intake, reduced physical activity, poor sleep and endocrine disruptors. Interestingly, Daalgard *et al*. observed that in contrast to the popularized notion that the population as a whole is significantly gaining weight, the calculated mean BMI of the major (lean) population increased by only +0.4% from 1963–1994 to 1999–2012, while the heavy sub-population, with a mean BMI 4–5 points above ‘‘normal,’’ more than tripled, from 12% to 38% of all individuals [403] (Figure 43). Does that mean that **obesity is fuelling itself** in a vicious cycle, whereby obese parents can transmit to their offspring a susceptibility to metabolic disorders (Figure 44)? And if so, does this transmission occur via a **biological programming of organs**, or through **cultural and behavioural transmission**? Do moms and dads play an equally large role in this transmission? **How many generations** does it last, and can it be **reversed**? These questions are among the most heavily debated at the moment, since we have little data in humans so far [404,405]. Indeed, the obesity epidemic is relatively recent, we need extensive epidemiological and longitudinal data to appreciate this phenomenon, and human studies are limited by ethical issues and

many confounders. However, a starting point is the observation that overnutrition *in utero* gives the offspring a higher risk of being born large for gestational age (LGA) [406,407]. Or, being born LGA is associated with an increased risk of obesity and diabetes in later life [408]. During the developmental period, rapidly growing foetuses and neonates are vulnerable to perturbations of the maternal nutritional and non-nutritional milieu, resulting in programmed changes in organ structure, cellular responses and gene expression that impact metabolism and physiology of the offspring [290]. Does this programming involve the epigenome? While this hasn't directly been tested in humans, work in primates suggest that this is likely the case: maternal high-fat feeding causes site-specific alterations in foetal hepatic H3 acetylation [409], and this is likely due to a downregulation of lysine deacetylase SIRT1 [410]. Although there were some indications of DNA methylation changes associated with maternal obesity in humans, investigated tissues were limited to cord blood [411–413] and placenta [412], which does not necessarily reflect the soma. However, a longitudinal study has found that methylation at a specific site in the retinoid X receptor alpha gene was both inversely correlated with maternal carbohydrate intake in early pregnancy and positively correlated with the children's adiposity at age 9, and results were replicated in an independent cohort [414]. The emphasis has undeniably and understandably been put on the mother as she carries the baby during its most vulnerable developmental windows, but **what about the father**? Recent epigenetic analyses of human sperm have shown that obese men carry a distinct epigenetic signature compared to lean men, in particular at genes controlling brain development and function, concomitant with an altered expression of small ncRNAs [267]. Whether these alterations are effectively transmitted to the offspring, whether they are persistent and whether they pose a risk for the offspring remain unknown.

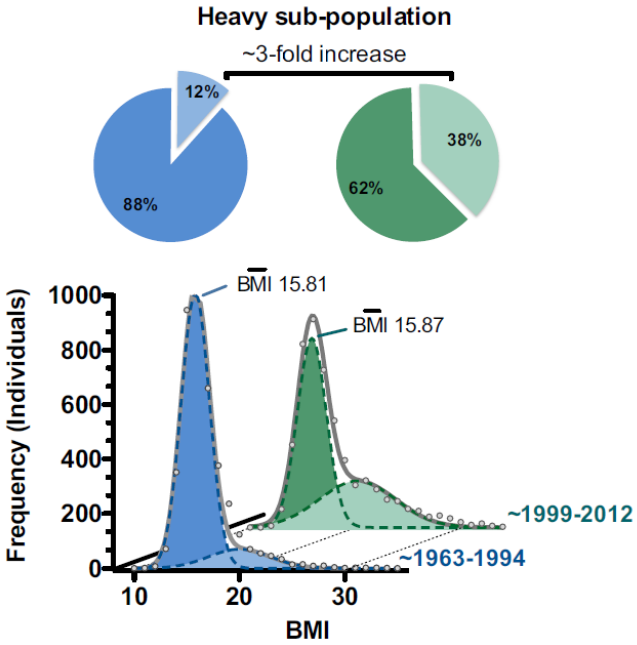


Figure 43. Comparison of similar fitting of childhood data from continuous NHANES 1999–2012 (CDC, 2012) and prior NHANES/NHES surveys (1963–1994) (CDC, 1994).

This shows a marked shift in recent decades where the heavy sub-population triples in size (pie charts). Adapted from [403]

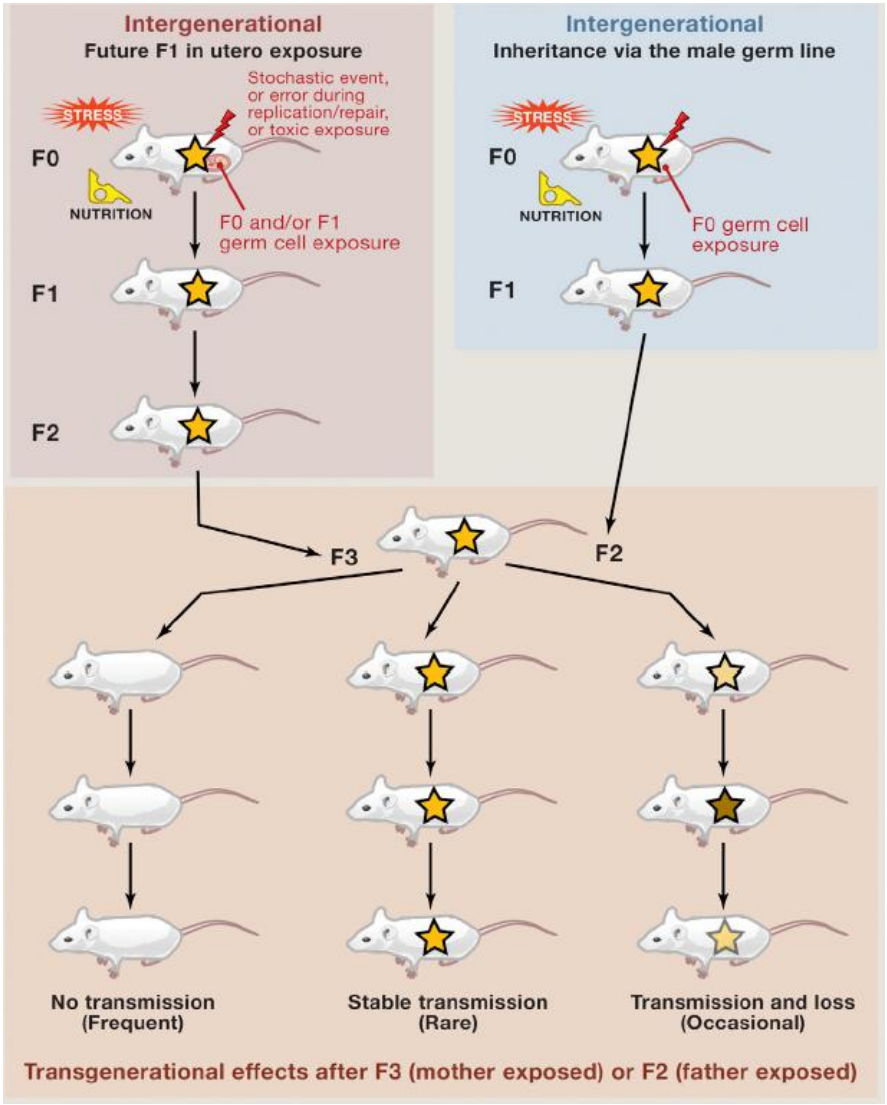


Figure 44. Transgenerational and intergenerational epigenetic effects.

Epigenetic changes in mammals can arise sporadically or can be induced by the environment (toxins, nutrition, and stress). In the case of an exposed female mouse, if she is pregnant, the foetus can be affected in utero (F1), as can the germline of the fetus (the future F2). These are considered to be parental effects, leading to intergenerational epigenetic inheritance. Only F3 individuals can be considered as true transgenerational inheritance in the absence of exposure. In the case of males in which an epigenetic change is induced, the individual (F0) and his germline (future F1) are exposed; the F1 is thus considered as intergenerational. Only F2 and subsequent generations can be considered for evidence of transgenerational inheritance. Taken from [405]

Until we have more data in humans, hypotheses on the mechanisms mediating a possible transmission of metabolic risk from parents to offspring via the epigenome have to face a thorny problem: the efficient **epigenetic reprogramming** that takes place both in the germline and in the early embryo and that erases nearly all epigenetic marks acquired during development or imposed by the environment [405] (Figure 45). Could there be regions important for metabolism that escape this reprogramming in humans, as was discovered in mice [415]?

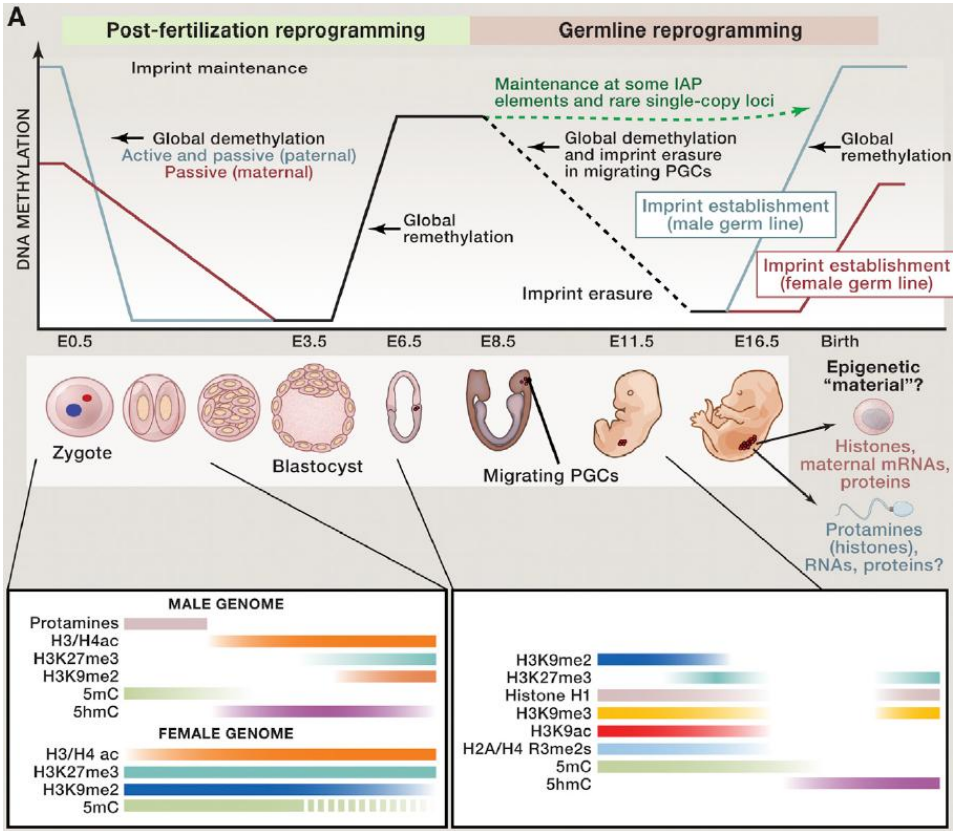


Figure 45. Germline Reprogramming of DNA methylation in mice.

In mice, there are at least two rounds of genome-wide DNA methylation reprogramming. The first occurs just after fertilization, in the zygote and early cleavage stages, to erase gametic (sperm and oocyte) epigenomic marks. During this phase of reprogramming, genomic imprints are maintained. The other major reprogramming process occurs in the germline, where the paternal and maternal somatic programs are erased, together with imprints, and the inactive X is reactivated. Subsequent to this, parent-specific imprints are laid down in the germline. In each reprogramming window, a specific set of mechanisms regulates erasure and re-establishment of DNA methylation. Recent studies have uncovered roles for the TET3 hydroxylase and passive demethylation, together with base excision repair (BER) and the elongator complex, in methylation erasure from the zygote. In the germline, deamination by AID, BER, and passive demethylation has been implicated in reprogramming, but the processes are still poorly understood. Taken from [405]

4. Conclusion and future perspectives

Through the use of large datasets and complex bioinformatical analyses, this thesis has contributed to extend our knowledge on epigenetics in the context of obesity and its complications. Genetic variants associated with metabolic risk are likely causing a local deregulation of chromatin states that could translate into large and complex modulation of gene transcription. Dietary fat quality was associated with blood DNA methylation changes at genes involved in metabolic pathways, and sleep restriction caused tissue-specific methylation and transcriptional changes at core circadian genes. In mice, maternal obesity was associated with an alteration of histone deacetylases in foetal liver and placenta, thus reinforcing the potential involvement of the epigenome in the DOHaD paradigm.

As I went through this thesis, I got increasingly convinced that the bioinformatical and biostatistical tools to analyse the gargantuan amount of data being generated in the field of epigenomics are painfully lagging behind. "Acquiring data is the easy part. What is direly needed are innovative approaches for mining multiple levels of "omics" and other data to discern patterns of data-disease relationships that may be used for decision-making in clinical treatment. Although the statistical approaches lag behind the technology and our ability to gather data, the potential is great to make substantial progress in this area." [49]

I would like to end by saying that as I was writing this thesis, I stumbled upon a Nature news article entitled “What’s the point of the PhD thesis?” [416]. I read the article apprehensively, although I do not really know why (maybe I was afraid to read something like “There is no point. You wrote your thesis for nothing.”). But before I finished reading the article, I realized that this thesis had taught me numerous things that are both science- and human-related. I realized that like John Snow, I know nothing (or very little); I am definitely no longer impressed when I get p-value<0.05; finally, imagination and ability to teach are on my top 10 skills that make a good scientist. The Nature news article ended with the following: “In the end, the only way you can assess [the PhD thesis] is whether the graduates of the programme become successful scientists. If they do, you've done a good job. If they haven't, you haven't.” [416]. Only the future will tell if that is true for me.

Abstract en français

Analyse biostatistique et bioinformatique de données épigénétiques chez l'homme et la souris en lien avec l'obésité et ses complications

L'obésité dans le monde a plus que doublé depuis 1980 et au moins 2,8 millions de personnes meurent chaque année des conséquences directes du surpoids ou de l'obésité. Un poids corporel élevé est la résultante de l'interaction entre des variants génétiques à risque et un environnement obésogène, et des données récentes montrent que des processus épigénétiques sont probablement impliqués. La disponibilité croissante des technologies à haut débit a permis d'évaluer rapidement l'épigénome de nombreux échantillons à un coût relativement faible. En conséquence, de vastes quantités de données ont été générées et les chercheurs sont maintenant confrontés à des défis bioinformatiques et biostatistiques pour extraire de ces données de l'information en lien avec l'obésité et ses complications.

Dans cette thèse de doctorat, nous avons exploré des associations entre le méthylome sanguin humain et des polymorphismes nucléotidiques (SNPs) liés à l'obésité, ainsi que des mesures qualitatives et quantitatives de l'apport journalier en acides gras. Nous avons utilisé des techniques de prétraitement des données et des méthodes statistiques bien décrites, une large batterie de données en libre accès générées par des consortia et d'autres groupes de recherche, ainsi que des outils pour l'enrichissement de pathways et l'inférence d'états de la chromatine. Nous avons trouvé des associations entre des SNPs liés à l'obésité et la méthylation de promoteurs et enhancers proximaux, et certaines de ces associations ont été répliquées dans plusieurs autres tissus. Nous avons également constaté que, contrairement à la quantité d'apport journalier en acides gras, la qualité de l'apport journalier en acides gras est associée à une méthylation différentielle de promoteurs de gènes impliqués dans des voies métaboliques. Puis, en utilisant une approche ciblée, nous avons examiné l'impact d'un stress environnemental aigu (une nuit blanche) sur la méthylation et la transcription de gènes du rythme circadien dans le muscle squelettique et le tissu adipeux d'hommes sains. Nous avons constaté qu'une seule nuit blanche peut modifier le profil épigénétique et transcriptionnel de gènes clés du rythme circadien, et ce de manière tissu-spécifique. Enfin, nous avons examiné les effets de l'obésité maternelle chronique et de la perte de poids ultérieure sur la transcription de gènes de la machinerie épigénétique dans le fœtus et le placenta de souris. Nous avons constaté que la transcription de ces gènes est très sensible aux trajectoires pondérales maternelles, et en particulier ceux de la voie d'acétylation des histones. Dans l'ensemble, cette thèse a démontré que la génétique, des stimuli environnementaux obésogènes et l'environnement maternel agissent sur les marques épigénétiques à des endroits génomiques pertinents dans la pathogenèse de l'obésité.

Mots clés : obésité, génétique, épigénétique, méthylation de l'ADN, sommeil, origines développementales de la santé et des maladies, polymorphisme nucléotidique, étude d'association pangénomique

Acknowledgements

As I am deeply convinced that contrary to a second language, the mother tongue stops by the heart before reaching the brain, I will try to squeeze in one page the gratitude I feel towards all the people who have helped me during these three years, in their native languages.

Först och främst vill jag varmt tacka kommittéemedlemmarna för sin insats. Jag är djupt övertygad om att deras insatser och kommentarer kommer att kasta nytt ljus på avhandlingen och berika dess innehåll. Merci Romain de m'avoir fait l'honneur d'être rapporteur et "opponent" pour ma thèse, et un grand merci à Alain de m'avoir sauvée d'un désistement inopiné.

Jag vill tacka Helgi för att ge mig den hjälpen och samtidigt vetenskapligt självständighet som tillät mig att växa som forskare. Jag njöt av min tid på ditt lab och i Sverige. Tack för att du var en tolerant, dynamisk och entusiastisk handledare som alltid värdesätter sina studenters arbete. Jag vill också tacka mina collegor som alltid fick mig att skratta och som alltid ställde upp för vetenskapliga diskussioner. Ni främjade min kärlek för vetenskap. Tack Markus, Robert, Linda, Anders, Marcus, Mathias, Frida, Nathalie, Hanna, Jonathan; thank you Mike, Lyle and Rohit; gracias Ernesto; спасибо Olga; danke Christian, Thomas, Nina und Nadine. Mitt särskilda tack gäller dig, Markus: Du var alltid den biostatistikgenin som visste vart jag skulle gräva för att hitta en skatt.

Un grand merci à l'unité de Biologie du Développement et Reproduction, dont les chercheurs, gestionnaires et techniciens m'ont permis de faire ma thèse dans les meilleures conditions. Je voudrais chaleureusement remercier toute l'ER5 pour son accueil et son soutien. Merci à Hélène, Sara, Polina, Mélanie et Sofiane pour vos réflexions et la bonne ambiance que vous insufflez à l'équipe. Merci à vous Claudine d'avoir partagé avec moi vos idées et votre savoir, merci Luc de t'être lancé à corps perdu dans des prises de tête bioinformatiques avec moi, et merci en particulier à Anne de m'avoir donné à la fois toute l'attention et l'indépendance dont j'ai besoin. Anne, si tu es aussi bonne mère que directrice de thèse, la petite Marion a de merveilleux jours devant elle...

Tusen tack Karin och Bernt, de bästa hyresvärdar i världen. Er värme, generositet, nyfikenhet och öppenhet gjorde att Mat och jag trivdes och kännde oss välkomna i Sverige. Om det var möjligt att anta morföräldrar på samma sätt föräldrar kan adoptera barn, skulle vi inte tveka att gå igenom all nödvändig pappersarbete för att kunna ha er som vår tredje par farföräldrar.

Merci à mes chers amis d'enfance, d'adolescence et autres périodes plus (ou moins) sérieuses de ma vie de m'avoir tenu compagnie, supportée, sermonnée, écoutée, appréciée et fait grandir. Ma grande sœur Carine, mes amis Tom, William, Jill-Jênn, Victor, Anael, Jérémie et mon cher Tangue. Le généreux, mystérieux et profondément gentil roi Arthur qui est toujours là quand on a besoin de lui (et même quand on a pas besoin de lui); Samantha, la femme de ma vie qui, par ses sourires et ses silences bienveillants a redonné de la consistance à mon corps et de la couleur à mon âme quand elle était diaphane. Ne t'inquiète pas, je ne te lâcherai jamais et je ne t'oublierai pas, où que je sois.

Merci à mes très chers parents de m'avoir offert tout leur amour et leur aide, ainsi qu'à mes deux grands frères d'avoir toujours été là pour moi; si je peux aller de l'avant aussi vite et sereinement, c'est que je sais que vous êtes tous là pour me soutenir. Mat, my dear Moomba, I want to thank you for entering my life, for marrying me, for offering me to defend my PhD with a sword and a shield, and simply for being awesome on a daily basis. I love you.

References

1. O'Neill S, O'Driscoll L. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obes Rev. 2015;16: 1–12. doi:10.1111/obr.12229
2. Vincent HK, Heywood K, Connelly J, Hurley RW. Obesity and weight loss in the treatment and prevention of osteoarthritis. PM R. United States; 2012;4: S59–67. doi:10.1016/j.pmrj.2012.01.005
3. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology. United States; 2010;51: 679–689. doi:10.1002/hep.23280
4. Aune D, Norat T, Vatten LJ. Body mass index, abdominal fatness and the risk of gallbladder disease. Eur J Epidemiol. Netherlands; 2015;30: 1009–1019. doi:10.1007/s10654-015-0081-y
5. Kuna ST, Reboussin DM, Borradaile KE, Sanders MH, Millman RP, Zammit G, et al. Long-term effect of weight loss on obstructive sleep apnea severity in obese patients with type 2 diabetes. Sleep. United States; 2013;36: 641–649A. doi:10.5665/sleep.2618
6. Aune D, Norat T, Vatten LJ. Body mass index and the risk of gout: a systematic review and dose-response meta-analysis of prospective studies. Eur J Nutr. Germany; 2014;53: 1591–1601. doi:10.1007/s00394-014-0766-0
7. Sikorski C, Spahlholz J, Hartlev M, Riedel-Heller SG. Weight-based discrimination: an ubiquitary phenomenon? Int J Obes (Lond). 2015; doi:10.1038/ijo.2015.165
8. World Health Organization. Obesity and overweight. In: Media Center [Internet]. 2015 p. Fact sheet N°311. Available: http://www.who.int/mediacentre/factsheets/fs311/en/
9. Pollack A. A.M.A. Recognizes Obesity as a Disease. New York Times. 2013.
10. Dee A, Kearns K, O'Neill C, Sharp L, Staines A, O'Dwyer V, et al. The direct and indirect costs of both overweight and obesity: a systematic review. BMC Res Notes. England; 2014;7: 242. doi:10.1186/1756-0500-7-242
11. Doheny K. Obesity, Cancer: Evidence Grows Supporting the Link. In: Highlight from Obesity Week 2015 [Internet]. 2015. Available: http://www.endocrineweb.com/professional/obesity/obesity-cancer-evidence-grows-supporting-link
12. Global Health Observatory Map Gallery [Internet]. 2015 p. 1. Available: http://gamapserver.who.int/mapLibrary/app/searchResults.aspx
13. O'Rahilly S, Farooqi IS. The Genetics of Obesity in Humans. In: De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM, et al., editors. South Dartmouth (MA); 2000.
14. FAO statistics division. Food balance [Internet]. 2015. Available: http://faostat3.fao.org/browse/FB/*/E
15. McAllister EJ, Dhurandhar N V, Keith SW, Aronne LJ, Barger J, Baskin M, et al. Ten putative contributors to the obesity epidemic. Crit Rev Food Sci Nutr. England; 2009;49: 868–913. doi:10.1080/10408390903372599
16. Schmid SM, Hallschmid M, Schultes B. The metabolic burden of sleep loss. lancet Diabetes Endocrinol. England; 2015;3: 52–62. doi:10.1016/S2213-8587(14)70012-9
17. Heindel JJ, Newbold R, Schug TT. Endocrine disruptors and obesity. Nat Rev Endocrinol. 2015; doi:10.1038/nrendo.2015.163
18. Surkan PJ, Hsieh C-C, Johansson AL V, Dickman PW, Cnattingius S. Reasons for increasing trends in large for gestational age births. Obstet Gynecol. United States; 2004;104: 720–726. doi:10.1097/01.AOG.0000141442.59573.cd
19. Stettler N, Zemel BS, Kumanyika S, Stallings VA. Infant weight gain and childhood overweight status in a multicenter, cohort study. Pediatrics. United States; 2002;109: 194–199.
20. Burgio E, Lopomo A, Migliore L. Obesity and diabetes: from genetics to epigenetics. Mol Biol Rep. Netherlands; 2015;42: 799–818. doi:10.1007/s11033-014-3751-z
21. Nihalani N, Schwartz TL, Siddiqui UA, Megna JL. Obesity and psychotropics. CNS Neurosci Ther. England; 2012;18: 57–63. doi:10.1111/j.1755-5949.2011.00232.x
22. Mosenkis A, Townsend RR. Antihypertensive medications and weight gain. J Clin Hypertens (Greenwich). United States; 2004;6: 90.
23. McFarlane SI. Antidiabetic medications and weight gain: implications for the practicing physician. Curr

Diab Rep. United States; 2009;9: 249–254.
24. Wray N, Visscher P. Estimating trait heritability. Nat Educ. 2008;1: 29.
25. Polderman TJC, Benyamin B, de Leeuw CA, Sullivan PF, van Bochoven A, Visscher PM, et al. Meta-analysis of the heritability of human traits based on fifty years of twin studies. Nat Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;47: 702–709. Available: http://dx.doi.org/10.1038/ng.3285
26. Elks CE, den Hoed M, Zhao JH, Sharp SJ, Wareham NJ, Loos RJF, et al. Variability in the Heritability of Body Mass Index: A Systematic Review and Meta-Regression. Front Endocrinol (Lausanne). Frontiers Research Foundation; 2012;3: 29. doi:10.3389/fendo.2012.00029
27. NCBI dbSNP Build 147 [Internet]. 2016 [cited 19 Jun 2016]. Available: http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&build_id=147
28. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. United States; 2007;316: 889–894. doi:10.1126/science.1141634
29. Scuteri A, Sanna S, Chen W-M, Uda M, Albai G, Strait J, et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet. United States; 2007;3: e115. doi:10.1371/journal.pgen.0030115
30. Loos RJF, Lindgren CM, Li S, Wheeler E, Zhao JH, Prokopenko I, et al. Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nat Genet. 2008;40: 768–775.
31. Chambers JC, Elliott P, Zabaneh D, Zhang W, Li Y, Froguel P, et al. Common genetic variation near MC4R is associated with waist circumference and insulin resistance. Nat Genet. Nature Publishing Group; 2008;40: 716–718. Available: http://dx.doi.org/10.1038/ng.156
32. Willer CJ, Speliotes EK, Loos RJF, Li S, Lindgren CM, Heid IM, et al. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. Nat Genet. 2009;41: 25–34.
33. Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. Nat Genet. 2009;41: 18–24.
34. Meyre D, Delplanque J, Chèvre J-C, Lecoeur C, Lobbens S, Gallina S, et al. Genome-wide association study for early-onset and morbid adult obesity identifies three new risk loci in European populations. Nat Genet. 2009;41: 157–159.
35. Lindgren CM, Heid IM, Randall JC, Lamina C, Steinthorsdottir V, Qi L, et al. Genome-wide association scan meta-analysis identifies three Loci influencing adiposity and fat distribution. PLoS Genet. United States; 2009;5: e1000508. doi:10.1371/journal.pgen.1000508
36. Heard-Costa NL, Zillikens MC, Monda KL, Johansson A, Harris TB, Fu M, et al. NRXN3 is a novel locus for waist circumference: a genome-wide association study from the CHARGE Consortium. PLoS Genet. 2009;5: e1000539.
37. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet. United States; 2010;42: 937–948. doi:10.1038/ng.686
38. Heid IIM, Jackson AUA, Randall J, Winkler T. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. Nat Genet. 2010;42: 950. doi:10.1038/ng.685
39. Scherag A, Dina C, Hinney A, Vatin V, Scherag S, Vogel CIG, et al. Two new Loci for body-weight regulation identified in a joint analysis of genome-wide association studies for early-onset extreme obesity in French and german study groups. PLoS Genet. United States; 2010;6: e1000916. doi:10.1371/journal.pgen.1000916
40. Kilpeläinen TO, Zillikens MC, Stančákova A, Finucane FM, Ried JS, Langenberg C, et al. Genetic variation near IRS1 associates with reduced adiposity and an impaired metabolic profile. Nat Genet. 2011;43: 753–760.
41. Wen W, Cho Y-S, Zheng W, Dorajoo R, Kato N, Qi L, et al. Meta-analysis identifies common variants associated with body mass index in east Asians. Nature Genetics. 2012. pp. 307–311. doi:10.1038/ng.1087
42. Okada Y, Kubo M, Ohmiya H, Takahashi A, Kumasaka N, Hosono N, et al. Common variants at CDKAL1 and KLF9 are associated with body mass index in east Asian populations. Nature Genetics. 2012. pp. 302–306.
43. Bradfield JP, Taal HR, Timpson NJ, Scherag A, Lecoeur C, Warrington NM, et al. A genome-wide

association meta-analysis identifies new childhood obesity loci. Nat Genet. United States; 2012;44: 526–531. doi:10.1038/ng.2247
44. Monda KL, Chen GK, Taylor KC, Palmer C, Edwards TL, Lange LA, et al. A meta-analysis identifies new loci associated with body mass index in individuals of African ancestry. Nat Genet. 2013;45: 690–6. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3694490&tool=pmcentrez&rendertype=abstract
45. Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;518: 197–206. Available: http://dx.doi.org/10.1038/nature14177
46. Shungin D, Winkler TW, Croteau-Chonka DC, Ferreira T, Locke AE, Magi R, et al. New genetic loci link adipose and insulin biology to body fat distribution. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;518: 187–196. Available: http://dx.doi.org/10.1038/nature14132
47. Schmiegelow MD, Hedlin H, Mackey RH, Martin LW, Vitolins MZ, Stefanick ML, et al. Race and Ethnicity, Obesity, Metabolic Health, and Risk of Cardiovascular Disease in Postmenopausal Women. J Am Hear Assoc . 2015;4 . doi:10.1161/JAHA.114.001695
48. DeBoer MD. Ethnicity, obesity and the metabolic syndrome: implications on assessing risk and targeting intervention. Expert Rev Endocrinol Metab. 2011;6: 279–289. doi:10.1586/eem.11.17
49. Bray MS, Loos RJF, McCaffery JM, Ling C, Franks PW, Weinstock GM, et al. NIH working group report-using genomic information to guide weight management: From universal to precision treatment. Obesity (Silver Spring). United States; 2016;24: 14–22. doi:10.1002/oby.21381
50. Loos RJF, Yeo GSH. The bigger picture of FTO: the first GWAS-identified obesity gene. Nat Rev Endocrinol. England; 2014;10: 51–61. doi:10.1038/nrendo.2013.227
51. Tung YCL, Yeo GSH, O'Rahilly S, Coll AP. Obesity and FTO: Changing Focus at a Complex Locus. Cell Metab. United States; 2014;20: 710–718. doi:10.1016/j.cmet.2014.09.010
52. Smemo S, Tena JJ, Kim KH, Gamazon ER, Sakabe NJ, Gomez-Marin C, et al. Obesity-associated variants within FTO form long-range functional connections with IRX3. Nature. 2014;507: 371–375. Available: http://www.ncbi.nlm.nih.gov/pubmed/24646999
53. Claussnitzer M, Dankel SN, Kim K-H, Quon G, Meuleman W, Haugen C, et al. FTO Obesity Variant Circuitry and Adipocyte Browning in Humans. N Engl J Med. Massachusetts Medical Society; 2015;373: 895–907. doi:10.1056/NEJMoa1502214
54. Mullaney JM, Mills RE, Pittard WS, Devine SE. Small insertions and deletions (INDELs) in human genomes. Hum Mol Genet . 2010;19 : R131–R136. doi:10.1093/hmg/ddq400
55. Zarrei M, MacDonald JR, Merico D, Scherer SW. A copy number variation map of the human genome. Nat Rev Genet. England; 2015;16: 172–183. doi:10.1038/nrg3871
56. Payseur BA, Jing P, Haasl RJ. A Genomic Portrait of Human Microsatellite Variation. Mol Biol Evol. Oxford University Press; 2011;28: 303–312. doi:10.1093/molbev/msq198
57. Naslund K, Saetre P, von Salome J, Bergstrom TF, Jareborg N, Jazin E. Genome-wide prediction of human VNTRs. Genomics. United States; 2005;85: 24–35. doi:10.1016/j.ygeno.2004.10.009
58. Waalen J. The genetics of human obesity. Transl Res. United States; 2014;164: 293–301. doi:10.1016/j.trsl.2014.05.010
59. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, et al. Finding the missing heritability of complex diseases. Nature. Macmillan Publishers Limited. All rights reserved; 2009;461: 747–753. Available: http://dx.doi.org/10.1038/nature08494
60. Eichler EE, Flint J, Gibson G, Kong A, Leal SM, Moore JH, et al. Missing heritability and strategies for finding the underlying causes of complex disease. Nat Rev Genet. England; 2010;11: 446–450. doi:10.1038/nrg2809
61. Cantsilieris S, White SJ. Correlating multiallelic copy number polymorphisms with disease susceptibility. Hum Mutat. United States; 2013;34: 1–13. doi:10.1002/humu.22172
62. Peterson RE, Maes HH, Lin P, Kramer JR, Hesselbrock VM, Bauer LO, et al. On the association of common and rare genetic variation influencing body mass index: a combined SNP and CNV analysis. BMC Genomics. England; 2014;15: 368. doi:10.1186/1471-2164-15-368
63. Zuk O, Schaffner SF, Samocha K, Do R, Hechter E, Kathiresan S, et al. Searching for missing heritability: designing rare variant association studies. Proc Natl Acad Sci U S A. United States; 2014;111: E455–64. doi:10.1073/pnas.1322563111

64. Yang J, Bakshi A, Zhu Z, Hemani G, Vinkhuyzen AAE, Lee SH, et al. Genetic variance estimation with imputed variants finds negligible missing heritability for human height and body mass index. Nat Genet. United States; 2015;47: 1114–1120. doi:10.1038/ng.3390
65. Geneimprint database: human imprinted genes [Internet]. 2012.
66. Dong C, Li W-D, Geller F, Lei L, Li D, Gorlova OY, et al. Possible Genomic Imprinting of Three Human Obesity-Related Genetic Loci. Am J Hum Genet. Elsevier; 2016;76: 427–437. doi:10.1086/428438
67. Kong A, Steinthorsdottir V, Masson G, Thorleifsson G, Sulem P, Besenbacher S, et al. Parental origin of sequence variants associated with complex diseases. Nature. 2009;462: 868–874.
68. Liu X, Hinney A, Scholz M, Scherag A, Tonjes A, Stumvoll M, et al. Indications for Potential Parent-of-Origin Effects within the FTO Gene. PLoS One. Public Library of Science; 2015;10: e0119206.
69. Hochner H, Allard C, Granot-Hershkovitz E, Chen J, Sitlani CM, Sazdovska S, et al. Parent-of-Origin Effects of the APOB Gene on Adiposity in Young Adults. PLoS Genet. United States; 2015;11: e1005573. doi:10.1371/journal.pgen.1005573
70. Hoggart CJ, Venturini G, Mangino M, Gomez F, Ascari G, Zhao JH, et al. Novel Approach Identifies SNPs in <italic>SLC2A10</italic> and <italic>KCNK9</italic> with Evidence for Parent-of-Origin Effect on Body Mass Index. PLoS Genet. Public Library of Science; 2014;10: e1004508.
71. Mott R, Yuan W, Kaisaki P, Gan X, Cleak J, Edwards A, et al. The architecture of parent-of-origin effects in mice. Cell. United States; 2014;156: 332–342. doi:10.1016/j.cell.2013.11.043
72. Huypens P, Sass S, Wu M, Dyckhoff D, Tschop M, Theis F, et al. Epigenetic germline inheritance of diet-induced obesity and insulin resistance. Nat Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2016;48: 497–499. Available: http://dx.doi.org/10.1038/ng.3527
73. Wei Y, Yang C-R, Wei Y-P, Zhao Z-A, Hou Y, Schatten H, et al. Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. Proc Natl Acad Sci U S A. National Academy of Sciences; 2014;111: 1873–1878. doi:10.1073/pnas.1321195111
74. Heard E, Martienssen RA. Transgenerational Epigenetic Inheritance: Myths and Mechanisms. Cell. Elsevier; 2016;157: 95–109. doi:10.1016/j.cell.2014.02.045
75. Waddington CH. Genetic Assimilation of the Bithorax Phenotype. Evolution (N Y). [Society for the Study of Evolution, Wiley]; 1956;10: 1–13. doi:10.2307/2406091
76. Frayling TM. Are the causes of obesity primarily environmental? No. BMJ. England; 2012;345: e5844.
77. Wilding J. Are the causes of obesity primarily environmental? Yes. BMJ. England; 2012;345: e5843.
78. Hagood JS. Beyond the genome: epigenetic mechanisms in lung remodeling. Physiology (Bethesda). United States; 2014;29: 177–185. doi:10.1152/physiol.00048.2013
79. Desvergne B, Michalik L, Wahli W. Transcriptional Regulation of Metabolism. Physiol Rev. 2006;86: 465–514. Available: http://physrev.physiology.org/content/86/2/465.abstract
80. Wolffe AP. Chromatin: Structure and Function [Internet]. Elsevier Science; 2012. Available: https://books.google.fr/books?id=cY2WLTwo0EIC
81. Luger K, Dechassa ML, Tremethick DJ. New insights into nucleosome and chromatin structure: an ordered state or a disordered affair? Nat Rev Mol Cell Biol. England; 2012;13: 436–447. doi:10.1038/nrm3382
82. Ravnskjaer K, Hogan MF, Lackey D, Tora L, Dent SYR, Olefsky J, et al. Glucagon regulates gluconeogenesis through KAT2B- and WDR5-mediated epigenetic effects. J Clin Invest. American Society for Clinical Investigation; 2013;123: 4318–4328. doi:10.1172/JCI69035
83. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nat Rev Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2016;advance on. Available: http://dx.doi.org/10.1038/nrg.2016.59
84. Roadmap Epigenomics C, Kundaje A, Meuleman W, Ernst J, Bilenky M, Yen A. Integrative analysis of 111 reference human epigenomes. Nature. 2015;518. doi:10.1038/nature14248
85. Pfeifer GP. Epigenetics: An elusive DNA base in mammals. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2016;advance on. Available: http://dx.doi.org/10.1038/nature17315
86. Histone Modification Table. In: Cell Signalling Technology [Internet]. 2014 [cited 20 Apr 2016]. Available: www.cellsignal.com/common/content/content.jsp?id=science-tables-histone#references
87. Schubeler D. Function and information content of DNA methylation. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;517: 321–326. Available: http://dx.doi.org/10.1038/nature14192

88. Yan J, Zierath JR, Barres R. Evidence for non-CpG methylation in mammals. Exp Cell Res. United States; 2011;317: 2555–2561. doi:10.1016/j.yexcr.2011.08.019
89. Xie W, Barr CL, Kim A, Yue F, Lee AY, Eubanks J. Base-resolution analyses of sequence and parent-of-origin dependent DNA methylation in the mouse genome. Cell. 2012;148. doi:10.1016/j.cell.2011.12.035
90. Guo JU, Su Y, Shin JH, Shin J, Li H, Xie B. Distribution, recognition and regulation of non-CpG methylation in the adult mammalian brain. Nat Neurosci. 2014;17. doi:10.1038/nn.3607
91. Barres R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. Cell Metab. United States; 2009;10: 189–198. doi:10.1016/j.cmet.2009.07.011
92. Barres R, Kirchner H, Rasmussen M, Yan J, FR K, Krook A. Weight loss after gastric bypass surgery in human obesity remodels promoter methylation. Cell Rep. 2013;3: 1020–1027. Available: http://dx.doi.org/10.1016/j.celrep.2013.03.018
93. Barrès R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, et al. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. Cell Metab. Cell Press,; 2012;15: 405–411. doi:10.1016/j.cmet.2012.01.001
94. Breiling A, Lyko F. Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. Epigenetics Chromatin. 2015;8: 1–9. doi:10.1186/s13072-015-0016-6
95. Holoch D, Moazed D. RNA-mediated epigenetic regulation of gene expression. Nat Rev Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;16: 71–84.
96. Holoch D, Moazed D. RNA-mediated epigenetic regulation of gene expression. Nat Rev Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;16: 71–84. Available: http://dx.doi.org/10.1038/nrg3863
97. Grummt I. Different epigenetic layers engage in complex crosstalk to define the epigenetic state of mammalian rRNA genes. Hum Mol Genet . 2007;16 : R21–R27. doi:10.1093/hmg/ddm020
98. Molina-Serrano D, Schiza V, Kirmizis A. Cross-talk among epigenetic modifications: lessons from histone arginine methylation. Biochem Soc Trans. England; 2013;41: 751–759. doi:10.1042/BST20130003
99. Ben-Porath I, Cedar H. Epigenetic Crosstalk. Mol Cell. 2001;8: 933–935. doi:10.1016/S1097-2765(01)00399-9
100. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2012;13: 97–109. Available: http://dx.doi.org/10.1038/nrg3142
101. Bird A. Perceptions of epigenetics. Nature. 2007;447. doi:10.1038/nature05913
102. Jablonka E, Lamb MJ. The inheritance of acquired epigenetic variations. Int J Epidemiol . 2015;44 : 1094–1103. doi:10.1093/ije/dyv020
103. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. Annu Rev Biochem. 2005;74. doi:10.1146/annurev.biochem.74.010904.153721
104. Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;502: 472–479. Available: http://dx.doi.org/10.1038/nature12750
105. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014;13: 673–691. Available: http://dx.doi.org/10.1038/nrd4360
106. Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, et al. Molecular Biology of the Cell, Sixth Edition [Internet]. Taylor & Francis Group; 2014. Available: https://books.google.fr/books?id=jK6UBQAAQBAJ
107. Ptashne M. Epigenetics: Core misconcept. Proc Natl Acad Sci U S A. National Academy of Sciences; 2013;110: 7101–7103. doi:10.1073/pnas.1305399110
108. Katan-Khaykovich Y, Struhl K. Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. Genes Dev . 2002;16 : 743–752. doi:10.1101/gad.967302
109. Radman-Livaja M, Liu CL, Friedman N, Schreiber SL, Rando OJ. Replication and Active Demethylation Represent Partially Overlapping Mechanisms for Erasure of H3K4me3 in Budding Yeast. PLoS Genet. Public Library of Science; 2010;6: e1000837. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1000837

110. Ringrose L, Paro R. Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development. England; 2007;134: 223–232. doi:10.1242/dev.02723
111. Ptashne M. Epigenetics: Core misconcept. Proc Natl Acad Sci U S A. National Academy of Sciences; 2013;110: 7101–7103. doi:10.1073/pnas.1305399110
112. D'Urso A, Brickner JH. Mechanisms of epigenetic memory. Trends Genet. 2014;30: 230–6. doi:10.1016/j.tig.2014.04.004
113. Ravnskjaer K, Hogan MF, Lackey D, Tora L, Dent SYR, Olefsky J, et al. Glucagon regulates gluconeogenesis through KAT2B- and WDR5-mediated epigenetic effects. J Clin Invest. American Society for Clinical Investigation; 2013;123: 4318–4328. doi:10.1172/JCI69035
114. Byun H-M, Nordio F, Coull BA, Tarantini L, Hou L, Bonzini M, et al. Temporal Stability of Epigenetic Markers: Sequence Characteristics and Predictors of Short-Term DNA Methylation Variations. PLoS One. Public Library of Science; 2012;7: e39220. Available: http://dx.doi.org/10.1371%2Fjournal.pone.0039220
115. Talens RP, Boomsma DI, Tobi EW, Kremer D, Jukema JW, Willemsen G, et al. Variation, patterns, and temporal stability of DNA methylation: considerations for epigenetic epidemiology. FASEB J Off Publ Fed Am Soc Exp Biol. United States; 2010;24: 3135–3144. doi:10.1096/fj.09-150490
116. Tucker KL. Methylated Cytosine and the Brain. Neuron. 2001;30: 649–652. doi:10.1016/S0896-6273(01)00325-7
117. Shen JC, Rideout WM, Jones PA. The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA. Nucleic Acids Res. 1994;22: 972–976. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC307917/
118. Cortazar D, Kunz C, Selfridge J, Lettieri T, Saito Y, MacDougall E, et al. Embryonic lethal phenotype reveals a function of TDG in maintaining epigenetic stability. Nature. England; 2011;470: 419–423. doi:10.1038/nature09672
119. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature. England; 2001;409: 860–921. doi:10.1038/35057062
120. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev . 2011;25 : 1010–1022. doi:10.1101/gad.2037511
121. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A. United States; 2002;99: 3740–3745. doi:10.1073/pnas.052410099
122. Zhao Z, Han L. CpG islands: algorithms and applications in methylation studies. Biochem Biophys Res Commun. United States; 2009;382: 643–645. doi:10.1016/j.bbrc.2009.03.076
123. Illingworth RS, Gruenewald-Schneider U, Webb S, Kerr ARW, James KD, Turner DJ, et al. Orphan CpG Islands Identify Numerous Conserved Promoters in the Mammalian Genome. PLoS Genet. Public Library of Science; 2010;6: e1001134. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1001134
124. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D/'Souza C, Fouse SD, et al. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2010;466: 253–257. Available: http://dx.doi.org/10.1038/nature09165
125. Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A. United States; 2006;103: 1412–1417. doi:10.1073/pnas.0510310103
126. Zhu J, He F, Hu S, Yu J. On the nature of human housekeeping genes. Trends Genet. England; 2008;24: 481–484. doi:10.1016/j.tig.2008.08.004
127. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. United States; 2003;33 Suppl: 245–254. doi:10.1038/ng1089
128. Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. Nature. 1993;366. doi:10.1038/366362a0
129. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, et al. DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet. 2006;38: 1378–1385.
130. Gal-Yam EN, Egger G, Iniguez L, Holster H, Einarsson S, Zhang X, et al. Frequent switching of Polycomb repressive marks and DNA hypermethylation in the PC3 prostate cancer cell line. Proc Natl Acad Sci U S A. United States; 2008;105: 12979–12984. doi:10.1073/pnas.0806437105
131. Han H, Cortez CC, Yang X, Nichols PW, Jones PA, Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. Hum Mol Genet . 2011;20 : 4299–4310. doi:10.1093/hmg/ddr356
132. Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, et al. Distribution, silencing potential

and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet. 2007;39: 457–466. doi:10.1038/ng1990
133. Li R, Mav D, Grimm SA, Jothi R, Shah R, Wade PA. Fine-tuning of epigenetic regulation with respect to promoter CpG content in a cell type-specific manner. Epigenetics. Taylor & Francis; 2014;9: 747–759. doi:10.4161/epi.28075
134. Wolf SF, Jolly DJ, Lunnen KD, Friedmann T, Migeon BR. Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation. Proc Natl Acad Sci U S A. UNITED STATES; 1984;81: 2806–2810.
135. Lippman Z, Gendrel A-V, Black M, Vaughn MW, Dedhia N, McCombie WR, et al. Role of transposable elements in heterochromatin and epigenetic control. Nature. England; 2004;430: 471–476. doi:10.1038/nature02651
136. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet. Nature Publishing Group; 2012;13: 484–492. doi:10.1038/nrg3230
137. Naftelberg S, Schor IE, Ast G, Kornblihtt AR. Regulation of Alternative Splicing Through Coupling with Transcription and Chromatin Structure. Annu Rev Biochem. Annual Reviews; 2015;84: 165–198. doi:10.1146/annurev-biochem-060614-034242
138. Shukla S, Kavak E, Gregory M, Imashimizu M, Shutinoski B, Kashlev M, et al. CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011;479: 74–79. Available: http://dx.doi.org/10.1038/nature10442
139. Portela A, Esteller M. Epigenetic modifications and human disease. Nat Biotech. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2010;28: 1057–1068. Available: http://dx.doi.org/10.1038/nbt.1685
140. Rechache NS, Wang Y, Stevenson HS, Killian JK, Edelman DC, Merino M, et al. DNA Methylation Profiling Identifies Global Methylation Differences and Markers of Adrenocortical Tumors. J Clin Endocrinol Metab. Chevy Chase, MD: Endocrine Society; 2012;97: E1004–E1013. doi:10.1210/jc.2011-3298
141. Qu X, Davison J, Du L, Storer B, Stirewalt DL, Heimfeld S, et al. Identification of differentially methylated markers among cytogenetic risk groups of acute myeloid leukemia. Epigenetics. United States; 2015;10: 526–535. doi:10.1080/15592294.2015.1048060
142. He Z, Lu H, Luo H, Gao F, Wang T, Gao Y, et al. The promoter methylomes of monochorionic twin placentas reveal intrauterine growth restriction-specific variations in the methylation patterns. Sci Rep. England; 2016;6: 20181. doi:10.1038/srep20181
143. Singmann P, Shem-Tov D, Wahl S, Grallert H, Fiorito G, Shin S-Y, et al. Characterization of whole-genome autosomal differences of DNA methylation between men and women. Epigenetics Chromatin. England; 2015;8: 43. doi:10.1186/s13072-015-0035-3
144. Zhang N, Zhao S, Zhang S-H, Chen J, Lu D, Shen M, et al. Intra-Monozygotic Twin Pair Discordance and Longitudinal Variation of Whole-Genome Scale DNA Methylation in Adults. PLoS One. United States; 2015;10: e0135022. doi:10.1371/journal.pone.0135022
145. Doi A, Park I-H, Wen B, Murakami P, Aryee MJ, Irizarry R, et al. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet. Nature Publishing Group; 2009;41: 1350–1353. Available: http://dx.doi.org/10.1038/ng.471
146. Hon GC, Rajagopal N, Shen Y, McCleary DF, Yue F, Dang MD, et al. Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. Nat Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;45: 1198–1206. Available: http://dx.doi.org/10.1038/ng.2746
147. Hogart A, Lichtenberg J, Ajay SS, Anderson S, Margulies EH, Bodine DM. Genome-wide DNA methylation profiles in hematopoietic stem and progenitor cells reveal overrepresentation of ETS transcription factor binding sites. Genome Res. United States; 2012;22: 1407–1418. doi:10.1101/gr.132878.111
148. Ziller MJ, Gu H, Muller F, Donaghey J, Tsai LT-Y, Kohlbacher O, et al. Charting a dynamic DNA methylation landscape of the human genome. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;500: 477–481. Available: http://dx.doi.org/10.1038/nature12433
149. Xie W, Schultz MD, Lister R, Hou Z, Rajagopal N, Ray P. Epigenomic analysis of multilineage differentiation of human embryonic stem cells. Cell. 2013;153. doi:10.1016/j.cell.2013.04.022

150. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol. UNITED STATES; 1999;19: 7327–7335.
151. Spruijt CG, Vermeulen M. DNA methylation: old dog, new tricks? Nat Struct Mol Biol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014;21: 949–954. Available: http://dx.doi.org/10.1038/nsmb.2910
152. Iguchi-Ariga SM, Schaffner W. CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev. UNITED STATES; 1989;3: 612–619.
153. Campanero MR, Armstrong MI, Flemington EK. CpG methylation as a mechanism for the regulation of E2F activity. Proc Natl Acad Sci . 2000;97 : 6481–6486. doi:10.1073/pnas.100340697
154. Prendergast GC, Ziff EB. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science. UNITED STATES; 1991;251: 186–189.
155. Blattler A, Farnham PJ. Cross-talk between site-specific transcription factors and DNA methylation states. J Biol Chem. United States; 2013;288: 34287–34294. doi:10.1074/jbc.R113.512517
156. Bakker J, Lin X, Nelson WG. Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. J Biol Chem. United States; 2002;277: 22573–22580. doi:10.1074/jbc.M203009200
157. Jiang CL, Jin SG, Lee DH, Lan ZJ, Xu X, O'Connor TR, et al. MBD3L1 and MBD3L2, two new proteins homologous to the methyl-CpG-binding proteins MBD2 and MBD3: characterization of MBD3L1 as a testis-specific transcriptional repressor. Genomics. United States; 2002;80: 621–629.
158. Bogdanović O, Veenstra GJC. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosoma. Berlin/Heidelberg: Springer-Verlag; 2009;118: 549–565. doi:10.1007/s00412-009-0221-9
159. Curradi M, Izzo A, Badaracco G, Landsberger N. Molecular mechanisms of gene silencing mediated by DNA methylation. Mol Cell Biol. United States; 2002;22: 3157–3173.
160. Huff JT, Zilberman D. Dnmt1-Independent CG Methylation Contributes to Nucleosome Positioning in Diverse Eukaryotes. Cell. Elsevier; 2016;156: 1286–1297. doi:10.1016/j.cell.2014.01.029
161. Baubec T, Ivanek R, Lienert F, Schubeler D. Methylation-dependent and -independent genomic targeting principles of the MBD protein family. Cell. United States; 2013;153: 480–492. doi:10.1016/j.cell.2013.03.011
162. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet. United States; 2007;39: 237–242. doi:10.1038/ng1972
163. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet. United States; 2007;39: 232–236. doi:10.1038/ng1950
164. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, et al. Epigenetic stem cell signature in cancer. Nat Genet. 2007;39: 157–158. Available: http://dx.doi.org/10.1038/ng1941
165. Medvedeva YA, Khamis AM, Kulakovskiy I V, Ba-Alawi W, Bhuyan MSI, Kawaji H, et al. Effects of cytosine methylation on transcription factor binding sites. BMC Genomics. 2014;15: 1–12. doi:10.1186/1471-2164-15-119
166. Stadler MB, Murr R, Burger L, Ivanek R, Lienert F, Scholer A, et al. DNA-binding factors shape the mouse methylome at distal regulatory regions. Nature. 2011;480.
167. Han L, Lin IG, Hsieh C-L. Protein Binding Protects Sites on Stable Episomes and in the Chromosome from De Novo Methylation. Mol Cell Biol. American Society for Microbiology; 2001;21: 3416–3424. doi:10.1128/MCB.21.10.3416-3424.2001
168. Kress C, Thomassin H, Grange T. Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. Proc Natl Acad Sci U S A. United States; 2006;103: 11112–11117. doi:10.1073/pnas.0601793103
169. Pai AA, Pritchard JK, Gilad Y. The Genetic and Mechanistic Basis for Variation in Gene Regulation. PLoS Genet. Public Library of Science; 2015;11: e1004857. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1004857
170. Kasowski M, Grubert F, Heffelfinger C, Hariharan M, Asabere A, Waszak SM, et al. Variation in transcription factor binding among humans. Science (80-). 2010;328: 232–235.
171. McDaniell R, Lee BK, Song L, Liu Z, Boyle AP, Erdos MR, et al. Heritable individual-specific and allele-specific chromatin signatures in humans. Science (80-). 2010;328: 235–239.

172. Gutierrez-Arcelus M, Lappalainen T, Montgomery SB, Buil A, Ongen H, Yurovsky A, et al. Passive and active DNA methylation and the interplay with genetic variation in gene regulation. Ponting CP, editor. Elife. eLife Sciences Publications, Ltd; 2013;2: e00523. doi:10.7554/eLife.00523
173. Taudt A, Colome-Tatche M, Johannes F. Genetic sources of population epigenomic variation. Nat Rev Genet. England; 2016;17: 319–332. doi:10.1038/nrg.2016.45
174. Whitaker JW, Chen Z, Wang W. Predicting the human epigenome from DNA motifs. Nat Meth. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;12: 265–272. Available: http://dx.doi.org/10.1038/nmeth.3065
175. Quon G, Lippert C, Heckerman D, Listgarten J. Patterns of methylation heritability in a genome-wide analysis of four brain regions. Nucleic Acids Res. 2013;41: 2095–2104.
176. Davies MN, Volta M, Pidsley R, Lunnon K, Dixit A, Lovestone S, et al. Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. Genome Biol. 2012;13: R43. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3446315&tool=pmcentrez&rendertype=abstract
177. Gibbs JR, van der Brug MP, Hernandez DG, Traynor BJ, Nalls MA, Lai SL, et al. Abundant quantitative trait loci exist for DNA methylation and gene expression in Human Brain. PLoS Genet. 2010;6: 29.
178. Zhang D, Cheng L, Badner JA, Chen C, Chen Q, Luo W, et al. Genetic Control of Individual Differences in Gene-Specific Methylation in Human Brain. Am J Hum Genet. 2010;86: 411–419.
179. Drong AW, Nicholson G, Hedman ??sa K., Meduri E, Grundberg E, Small KS, et al. The Presence of Methylation Quantitative Trait Loci Indicates a Direct Genetic Influence on the Level of DNA Methylation in Adipose Tissue. PLoS One. 2013;8: e55923.
180. Grundberg E, Meduri E, Sandling JK, Hedman ??sa K., Keildson S, Buil A, et al. Global analysis of dna methylation variation in adipose tissue from twins reveals links to disease-associated variants in distal regulatory elements. Am J Hum Genet. 2013;93: 876–890.
181. van Eijk K, de Jong S, Boks M, Langeveld T, Colas F, Veldink J, et al. Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects. BMC Genomics. 2012;13: 636. doi:doi: 10.1186/1471-2164-13-636
182. Smith AK, Kilaru V, Kocak M, Almli LM, Mercer KB, Ressler KJ, et al. Methylation quantitative trait loci (meQTLs) are consistently detected across ancestry, developmental stage, and tissue type. BMC Genomics. 2014;15: 145. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4028873&tool=pmcentrez&rendertype=abstract
183. Shi J, Marconett CN, Duan J, Hyland PL, Li P, Wang Z, et al. Characterizing the genetic basis of methylome diversity in histologically normal human lung tissue. Nat Commun. Nature Publishing Group; 2014;5: 3365. doi:10.1038/ncomms4365
184. Wagner JR, Busche S, Ge B, Kwan T, Pastinen T, Blanchette M. The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. Genome Biol. 2014;15: R37. doi:10.1186/gb-2014-15-2-r37
185. Gutierrez-Arcelus M, Lappalainen T, Montgomery SB, Buil A, Ongen H, Yurovsky A, et al. Passive and active DNA methylation and the interplay with genetic variation in gene regulation. Elife. 2013;2: e00523. doi:10.7554/eLife.00523
186. Gertz J, Varley KE, Reddy TE, Bowling KM, Pauli F, Parker SL, et al. Analysis of DNA Methylation in a Three-Generation Family Reveals Widespread Genetic Influence on Epigenetic Regulation. PLoS Genet. Public Library of Science; 2011;7: e1002228. Available: http://dx.doi.org/10.1371/journal.pgen.1002228
187. Bell J, Pai A, Pickrell J, Gaffney D, Pique-Regi R, Degner J, et al. DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol. 2011;12: R10. Available: http://genomebiology.com/2011/12/1/R10
188. Lemire M, Zaidi SHE, Ban M, Ge B, Aïssi D, Germain M, et al. Long-range epigenetic regulation is conferred by genetic variation located at thousands of independent loci. Nat Commun. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;6. Available: http://dx.doi.org/10.1038/ncomms7326
189. McClay JL, Shabalin AA, Dozmorov MG, Adkins DE, Kumar G, Nerella S, et al. High density methylation QTL analysis in human blood via next-generation sequencing of the methylated genomic DNA fraction. Genome Biol. England; 2015;16: 291. doi:10.1186/s13059-015-0842-7
190. Kurdyukov S, Bullock M. DNA Methylation Analysis: Choosing the Right Method. Biology (Basel).

Switzerland; 2016;5. doi:10.3390/biology5010003
191. Laird PW. Principles and challenges of genomewide DNA methylation analysis. Nat Rev Genet. 2010;11. doi:10.1038/nrg2732
192. Lempradl A, Pospisilik JA, Penninger JM. Exploring the emerging complexity in transcriptional regulation of energy homeostasis. Nat Rev Genet. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;16: 665–681. Available: http://dx.doi.org/10.1038/nrg3941
193. Kohno D, Lee S, Harper MJ, Kim KW, Sone H, Sasaki T, et al. Dnmt3a in Sim1 Neurons Is Necessary for Normal Energy Homeostasis. J Neurosci . 2014;34 : 15288–15296. doi:10.1523/JNEUROSCI.1316-14.2014
194. Lindholm ME, Marabita F, Gomez-Cabrero D, Rundqvist H, Ekstrom TJ, Tegner J, et al. An integrative analysis reveals coordinated reprogramming of the epigenome and the transcriptome in human skeletal muscle after training. Epigenetics. United States; 2014;9: 1557–1569. doi:10.4161/15592294.2014.982445
195. Chiang E-PI, Wang Y-C, Chen W-W, Tang F-Y. Effects of insulin and glucose on cellular metabolic fluxes in homocysteine transsulfuration, remethylation, S-adenosylmethionine synthesis, and global deoxyribonucleic acid methylation. J Clin Endocrinol Metab. United States; 2009;94: 1017–1025. doi:10.1210/jc.2008-2038
196. Hall E, Volkov P, Dayeh T, Bacos K, Rönn T, Nitert MD, et al. Effects of palmitate on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. BMC Med. 2014;12: 1–15. doi:10.1186/1741-7015-12-103
197. Silva-Martinez GA, Rodriguez-Rios D, Alvarado-Caudillo Y, Vaquero A, Esteller M, Carmona FJ, et al. Arachidonic and oleic acid exert distinct effects on the DNA methylome. Epigenetics. 2016; 1–14. doi:10.1080/15592294.2016.1161873
198. Maples JM, Brault JJ, Shewchuk BM, Witczak CA, Zou K, Rowland N, et al. Lipid exposure elicits differential responses in gene expression and DNA methylation in primary human skeletal muscle cells from severely obese women. Physiol Genomics. United States; 2015;47: 139–146. doi:10.1152/physiolgenomics.00065.2014
199. Boyanapalli SSS, Kong A-NT. "Curcumin, the King of Spices": Epigenetic Regulatory Mechanisms in the Prevention of Cancer, Neurological, and Inflammatory Diseases. Curr Pharmacol Reports. 2015;1: 129–139. doi:10.1007/s40495-015-0018-x
200. Yiannakopoulou EC. Targeting DNA methylation with green tea catechins. Pharmacology. Switzerland; 2015;95: 111–116. doi:10.1159/000375503
201. Gut P, Verdin E. The nexus of chromatin regulation and intermediary metabolism. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;502: 489–498. Available: http://dx.doi.org/10.1038/nature12752
202. Poirier LA, Wise CK, Delongchamp RR, Sinha R. Blood determinations of S-adenosylmethionine, S-adenosylhomocysteine, and homocysteine: correlations with diet. Cancer Epidemiol Biomarkers Prev. United States; 2001;10: 649–655.
203. Lu C, Thompson CB. Metabolic Regulation of Epigenetics. Cell Metab. Elsevier; 2016;16: 9–17. doi:10.1016/j.cmet.2012.06.001
204. Kaelin Jr. WG, McKnight SL. Influence of Metabolism on Epigenetics and Disease. Cell. Elsevier; 2016;153: 56–69. doi:10.1016/j.cell.2013.03.004
205. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324: 930–935.
206. Xiao M, Yang H, Xu W, Ma S, Lin H, Zhu H, et al. Inhibition of alpha-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes Dev. United States; 2012;26: 1326–1338. doi:10.1101/gad.191056.112
207. Ronn T, Ling C. DNA methylation as a diagnostic and therapeutic target in the battle against Type 2 diabetes. Epigenomics. England; 2015;7: 451–460. doi:10.2217/epi.15.7
208. Martinez-Jimenez CP, Sandoval J. Epigenetic crosstalk: a molecular language in human metabolic disorders. Front Biosci (Schol Ed). United States; 2015;7: 46–57.
209. van Dijk SJ, Molloy PL, Varinli H, Morrison JL, Muhlhausler BS. Epigenetics and human obesity. Int J Obes (Lond). England; 2015;39: 85–97. doi:10.1038/ijo.2014.34
210. Multhaup ML, Seldin MM, Jaffe AE, Lei X, Kirchner H, Mondal P, et al. Mouse-human experimental epigenetic analysis unmasks dietary targets and genetic liability for diabetic phenotypes. Cell Metab.

United States; 2015;21: 138–149. doi:10.1016/j.cmet.2014.12.014
211. Zhang FF, Cardarelli R, Carroll J, Fulda KG, Kaur M, Gonzalez K, et al. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics. 2011;6: 623–629. Available: http://www.ncbi.nlm.nih.gov/pubmed/21739720
212. Piyathilake CJ, Badiga S, Alvarez RD, Partridge EE, Johanning GL. A Lower Degree of PBMC L1 Methylation Is Associated with Excess Body Weight and Higher HOMA-IR in the Presence of Lower Concentrations of Plasma Folate. PLoS One. Public Library of Science; 2013;8: e54544. Available: http://dx.doi.org/10.1371%2Fjournal.pone.0054544
213. Cash HL, McGarvey ST, Houseman EA, Marsit CJ, Hawley NL, Lambert-Messerlian GM, et al. Cardiovascular disease risk factors and DNA methylation at the LINE-1 repeat region in peripheral blood from Samoan islanders. Epigenetics. Landes Bioscience; 2011;6: 1257–1264. doi:10.4161/epi.6.10.17728
214. Zhu Z-Z, Hou L, Bollati V, Tarantini L, Marinelli B, Cantone L, et al. Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. Int J Epidemiol. England; 2012;41: 126–139. doi:10.1093/ije/dyq154
215. Pearce MS, McConnell JC, Potter C, Barrett LM, Parker L, Mathers JC, et al. Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. Int J Epidemiol. England; 2012;41: 210–217. doi:10.1093/ije/dys020
216. Zhang FF, Santella RM, Wolff M, Kappil MA, Markowitz SB, Morabia A. White blood cell global methylation and IL-6 promoter methylation in association with diet and lifestyle risk factors in a cancer-free population. Epigenetics. Landes Bioscience Inc.; 2012;7: 606–614. Available: http://www.landesbioscience.com/journals/epigenetics/article/20236/
217. Figueiredo JC, Grau M V, Wallace K, Levine AJ, Shen L, Hamdan R, et al. Global DNA hypomethylation (LINE-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. Cancer Epidemiol Biomarkers Prev. United States; 2009;18: 1041–1049. doi:10.1158/1055-9965.EPI-08-0926
218. Nomura Y, Lambertini L, Rialdi A, Lee M, Mystal EY, Grabie M, et al. Global methylation in the placenta and umbilical cord blood from pregnancies with maternal gestational diabetes, preeclampsia, and obesity. Reprod Sci. United States; 2014;21: 131–137. doi:10.1177/1933719113492206
219. Perng W, Mora-Plazas M, Marin C, Rozek LS, Baylin A, Villamor E. A Prospective Study of LINE-1DNA Methylation and Development of Adiposity in School-Age Children. PLoS One. Public Library of Science; 2013;8: e62587. Available: http://dx.doi.org/10.1371%2Fjournal.pone.0062587
220. Jintaridth P, Tungtrongchitr R, Preutthipan S, Mutirangura A. Hypomethylation of Alu Elements in Post-Menopausal Women with Osteoporosis. PLoS One. Public Library of Science; 2013;8: e70386. Available: http://dx.doi.org/10.1371%2Fjournal.pone.0070386
221. Duggan C, Xiao L, Terry MB, McTiernan A. No effect of weight loss on LINE-1 methylation levels in peripheral blood leukocytes from postmenopausal overweight women. Obesity. 2014;22: 2091–2096. doi:10.1002/oby.20806
222. Na YK, Hong HS, Lee DH, Lee WK, Kim DS. Effect of body mass index on global DNA methylation in healthy Korean women. Mol Cells. Korea (South); 2014;37: 467–472. doi:10.14348/molcells.2014.0073
223. Maghbooli Z, Hossein-Nezhad A, Larijani B, Pasalar P, Keshtkar AA. Association between alterations in global DNA methylation and predisposing factors in diabetes: a high pressure liquid chromatography based study. Minerva Med. Italy; 2015;106: 221–231.
224. Maghbooli Z, Larijani B, Emamgholipour S, Amini M, Keshtkar A, Pasalar P. Aberrant DNA methylation patterns in diabetic nephropathy. J Diabetes Metab Disord. England; 2014;13: 69. doi:10.1186/2251-6581-13-69
225. Gomes MVM, Toffoli L V, Arruda DW, Soldera LM, Pelosi GG, Neves-Souza RD, et al. Age-Related Changes in the Global DNA Methylation Profile of Leukocytes Are Linked to Nutrition but Are Not Associated with the MTHFR C677T Genotype or to Functional Capacities. PLoS One. 2012;7: e52570. doi:10.1371/journal.pone.0052570
226. Simar D, Versteyhe S, Donkin I, Liu J, Hesson L, Nylander V, et al. DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human. Metabolism. United States; 2014;63: 1188–1197. doi:10.1016/j.metabol.2014.05.014
227. Ribel-Madsen R, Fraga MF, Jacobsen S, Bork-Jensen J, Lara E, Calvanese V, et al. Genome-wide analysis of DNA methylation differences in muscle and fat from monozygotic twins discordant for type 2 diabetes. PLoS One. United States; 2012;7: e51302. doi:10.1371/journal.pone.0051302
228. Keller M, Kralisch S, Rohde K, Schleinitz D, Dietrich A, Schon MR, et al. Global DNA methylation

levels in human adipose tissue are related to fat distribution and glucose homeostasis. Diabetologia. Germany; 2014;57: 2374–2383. doi:10.1007/s00125-014-3356-z
229. Nicoletti CF, Nonino CB, de Oliveira BAP, Pinhel MA de S, Mansego ML, Milagro FI, et al. DNA Methylation and Hydroxymethylation Levels in Relation to Two Weight Loss Strategies: Energy-Restricted Diet or Bariatric Surgery. Obes Surg. United States; 2016;26: 603–611. doi:10.1007/s11695-015-1802-8
230. Nilsson E, Jansson PA, Perfilyev A, Volkov P, Pedersen M, Svensson MK, et al. Altered DNA methylation and differential expression of genes influencing metabolism and inflammation in adipose tissue from subjects with type 2 diabetes. Diabetes. United States; 2014;63: 2962–2976. doi:10.2337/db13-1459
231. Ling C, Del Guerra S, Lupi R, Rönn T, Granhall C, Luthman H, et al. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. Diabetologia. Berlin/Heidelberg: Springer-Verlag; 2008;51: 615–622. doi:10.1007/s00125-007-0916-5
232. Barres R, Kirchner H, Rasmussen M, Yan J, Kantor FR, Krook A, et al. Weight Loss after Gastric Bypass Surgery in Human Obesity Remodels Promoter Methylation. Cell Rep. Elsevier; 2016;3: 1020–1027. doi:10.1016/j.celrep.2013.03.018
233. Sookoian S, Rosselli MS, Gemma C, Burgueno AL, Fernandez Gianotti T, Castano GO, et al. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1alpha promoter. Hepatology. United States; 2010;52: 1992–2000. doi:10.1002/hep.23927
234. Kuehnen P, Mischke M, Wiegand S, Sers C, Horsthemke B, Lau S, et al. An Alu Element?Associated Hypermethylation Variant of the POMC Gene Is Associated with Childhood Obesity. PLoS Genet. Public Library of Science; 2012;8: e1002543. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1002543
235. Crujeiras AB, Campion J, Diaz-Lagares A, Milagro FI, Goyenechea E, Abete I, et al. Association of weight regain with specific methylation levels in the NPY and POMC promoters in leukocytes of obese men: a translational study. Regul Pept. Netherlands; 2013;186: 1–6. doi:10.1016/j.regpep.2013.06.012
236. Gomez-Uriz AM, Goyenechea E, Campion J, de Arce A, Martinez MT, Puchau B, et al. Epigenetic patterns of two gene promoters (TNF-alpha and PON) in stroke considering obesity condition and dietary intake. J Physiol Biochem. Spain; 2014;70: 603–614. doi:10.1007/s13105-014-0316-5
237. Hermsdorff HH, Mansego ML, Campion J, Milagro FI, Zulet MA, Martinez JA. TNF-alpha promoter methylation in peripheral white blood cells: relationship with circulating TNFalpha, truncal fat and n-6 PUFA intake in young women. Cytokine. United States; 2013;64: 265–271. doi:10.1016/j.cyto.2013.05.028
238. Obermann-Borst SA, Eilers PHC, Tobi EW, de Jong FH, Slagboom PE, Heijmans BT, et al. Duration of breastfeeding and gender are associated with methylation of the LEPTIN gene in very young children. Pediatr Res. United States; 2013;74: 344–349. doi:10.1038/pr.2013.95
239. Lesseur C, Armstrong DA, Paquette AG, Koestler DC, Padbury JF, Marsit CJ. Tissue-specific Leptin promoter DNA methylation is associated with maternal and infant perinatal factors. Mol Cell Endocrinol. Ireland; 2013;381: 160–167. doi:10.1016/j.mce.2013.07.024
240. Garcia-Cardona MC, Huang F, Garcia-Vivas JM, Lopez-Camarillo C, Del Rio Navarro BE, Navarro Olivos E, et al. DNA methylation of leptin and adiponectin promoters in children is reduced by the combined presence of obesity and insulin resistance. Int J Obes (Lond). England; 2014;38: 1457–1465. doi:10.1038/ijo.2014.30
241. Houde A-A, Legare C, Biron S, Lescelleur O, Biertho L, Marceau S, et al. Leptin and adiponectin DNA methylation levels in adipose tissues and blood cells are associated with BMI, waist girth and LDL-cholesterol levels in severely obese men and women. BMC Med Genet. England; 2015;16: 29. doi:10.1186/s12881-015-0174-1
242. Zheng LD, Linarelli LE, Liu L, Wall SS, Greenawald MH, Seidel RW, et al. Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. Clin Epigenetics. Germany; 2015;7: 60. doi:10.1186/s13148-015-0093-1
243. Pirola CJ, Fernández Gianotti T, Burgueño AL, Rey-Funes M, Loidl CF, Mallardi P, et al. Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. Gut. 2012;62: 1356–1363. doi:10.1136/gutjnl-2012-302962
244. Carless M a, Kulkarni H, Kos MZ, Charlesworth J, Peralta JM, Göring HHH, et al. Genetic effects on DNA methylation and its potential relevance for obesity in mexican americans. PLoS One. 2013;8: e73950. doi:10.1371/journal.pone.0073950

245. Xu X, Su S, Barnes V a, De Miguel C, Pollock J, Ownby D, et al. A genome-wide methylation study on obesity: differential variability and differential methylation. Epigenetics. 2013;8: 522–33. doi:10.4161/epi.24506
246. Almén MS, Nilsson EK, Jacobsson JA, Kalnina I, Klovins J, Fredriksson R, et al. Genome-wide analysis reveals DNA methylation markers that vary with both age and obesity. Gene. 2014;548: 61–67.
247. Wang X, Zhu H, Snieder H, Su S, Munn D, Harshfield G, et al. Obesity related methylation changes in DNA of peripheral blood leukocytes. BMC Med. 2010;8: 87.
248. Feinberg AP, Irizarry RA, Fradin D, Aryee MJ, Murakami P, Aspelund T, et al. Personalized epigenomic signatures that are stable over time and covary with body mass index. Sci Transl Med. 2010;2: 49ra67.
249. Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aïssi D, Wahl S, et al. DNA methylation and body-mass index: a genome-wide analysis. Lancet. 7AD;383: 1990–1998. doi:http://dx.doi.org/10.1016/S0140-6736(13)62674-4
250. Irvin MR, Zhi D, Joehanes R, Mendelson M, Aslibekyan S, Claas SA, et al. Epigenome-wide association study of fasting blood lipids in the Genetics of Lipid-lowering Drugs and Diet Network study. Circulation. United States; 2014;130: 565–572. doi:10.1161/CIRCULATIONAHA.114.009158
251. Almén MS, Schiöth HB, Fredriksson R, Moschonis G, Chrousos GP, Jacobsson JA, et al. Genome wide analysis reveals association of a FTO gene variant with epigenetic changes. Genomics. 2012. pp. 132–137. doi:10.1016/j.ygeno.2011.12.007
252. Al Muftah WA, Al-Shafai M, Zaghlool SB, Visconti A, Tsai P-C, Kumar P, et al. Epigenetic associations of type 2 diabetes and BMI in an Arab population. Clin Epigenetics. Germany; 2016;8: 13. doi:10.1186/s13148-016-0177-6
253. Mamtani M, Kulkarni H, Dyer TD, Goring HHH, Neary JL, Cole SA, et al. Genome- and epigenome-wide association study of hypertriglyceridemic waist in Mexican American families. Clin Epigenetics. Germany; 2016;8: 6. doi:10.1186/s13148-016-0173-x
254. Mansego ML, Milagro FI, Zulet MA, Moreno-Aliaga MJ, Martinez JA. Differential DNA Methylation in Relation to Age and Health Risks of Obesity. Int J Mol Sci. Switzerland; 2015;16: 16816–16832. doi:10.3390/ijms160816816
255. Shah S, Bonder MJ, Marioni RE, Zhu Z, McRae AF, Zhernakova A, et al. Improving Phenotypic Prediction by Combining Genetic and Epigenetic Associations. Am J Hum Genet. United States; 2015;97: 75–85. doi:10.1016/j.ajhg.2015.05.014
256. Kulkarni H, Kos MZ, Neary J, Dyer TD, Kent JWJ, Goring HHH, et al. Novel epigenetic determinants of type 2 diabetes in Mexican-American families. Hum Mol Genet. England; 2015;24: 5330–5344. doi:10.1093/hmg/ddv232
257. Chambers JC, Loh M, Lehne B, Drong A, Kriebel J, Motta V, et al. Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study. lancet Diabetes Endocrinol. England; 2015;3: 526–534. doi:10.1016/S2213-8587(15)00127-8
258. Demerath EW, Guan W, Grove ML, Aslibekyan S, Mendelson M, Zhou Y-H, et al. Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. Hum Mol Genet. England; 2015;24: 4464–4479. doi:10.1093/hmg/ddv161
259. Ding X, Zheng D, Fan C, Liu Z, Dong H, Lu Y, et al. Genome-wide screen of DNA methylation identifies novel markers in childhood obesity. Gene. Netherlands; 2015;566: 74–83. doi:10.1016/j.gene.2015.04.032
260. Ollikainen M, Ismail K, Gervin K, Kyllonen A, Hakkarainen A, Lundbom J, et al. Genome-wide blood DNA methylation alterations at regulatory elements and heterochromatic regions in monozygotic twins discordant for obesity and liver fat. Clin Epigenetics. 2015;7: 39. Available: http://www.clinicalepigeneticsjournal.com/content/7/1/39
261. Rönn T, Volkov P, Gillberg L, Kokosar M, Perfilyev A, Jacobsen AL, et al. Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. Hum Mol Genet . 2015;24 : 3792–3813. doi:10.1093/hmg/ddv124
262. Kirchner H, Sinha I, Gao H, Ruby MA, Schonke M, Lindvall JM, et al. Altered DNA methylation of glycolytic and lipogenic genes in liver from obese and type 2 diabetic patients. Mol Metab. Germany; 2016;5: 171–183. doi:10.1016/j.molmet.2015.12.004
263. Nilsson E, Matte A, Perfilyev A, de Mello VD, Kakela P, Pihlajamaki J, et al. Epigenetic Alterations in Human Liver From Subjects With Type 2 Diabetes in Parallel With Reduced Folate Levels. J Clin

Endocrinol Metab. United States; 2015;100: E1491–501. doi:10.1210/jc.2015-3204
264. Guénard F, Tchernof A, Deshaies Y, Pérusse L, Biron S, Lescelleur O, et al. Differential methylation in visceral adipose tissue of obese men discordant for metabolic disturbances. Physiol Genomics. 2014;46: 216–222. Available: http://physiolgenomics.physiology.org/content/46/6/216.abstract
265. Pietilainen KH, Ismail K, Jarvinen E, Heinonen S, Tummers M, Bollepalli S, et al. DNA methylation and gene expression patterns in adipose tissue differ significantly within young adult monozygotic BMI-discordant twin pairs. Int J Obes (Lond). England; 2016;40: 654–661. doi:10.1038/ijo.2015.221
266. Dahlman I, Sinha I, Gao H, Brodin D, Thorell A, Ryden M, et al. The fat cell epigenetic signature in post-obese women is characterized by global hypomethylation and differential DNA methylation of adipogenesis genes. Int J Obes (Lond). England; 2015;39: 910–919. doi:10.1038/ijo.2015.31
267. Donkin I, Versteyhe S, Ingerslev LR, Qian K, Mechta M, Nordkap L, et al. Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans. Cell Metab. Elsevier; 2016;23: 369–378. doi:10.1016/j.cmet.2015.11.004
268. Holbrook JD. Does diet interact with genotype to cause epigenetic modification of angiogenesis genes, on the trajectory to obesity? Epigenomics. 2016; doi:10.2217/epi-2016-0023
269. Dayeh T, Tuomi T, Almgren P, Perfilyev A, Jansson P-A, de Mello VD, et al. DNA methylation of loci within ABCG1 and PHOSPHO1 in blood DNA is associated with future type 2 diabetes risk. Epigenetics. 2016; 0. doi:10.1080/15592294.2016.1178418
270. Ding J, Reynolds LM, Zeller T, Müller C, Lohman K, Nicklas BJ, et al. Alterations of a Cellular Cholesterol Metabolism Network Are a Molecular Feature of Obesity-Related Type 2 Diabetes and Cardiovascular Disease. Diabetes . 2015;64 : 3464–3474. doi:10.2337/db14-1314
271. Gillberg L, Perfilyev A, Brons C, Thomasen M, Grunnet LG, Volkov P, et al. Adipose tissue transcriptomics and epigenomics in low birthweight men and controls: role of high-fat overfeeding. Diabetologia. Germany; 2016;59: 799–812. doi:10.1007/s00125-015-3852-9
272. Jacobsen SC, Brons C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, et al. Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. Diabetologia. Germany; 2012;55: 3341–3349. doi:10.1007/s00125-012-2717-8
273. Brøns C, Jacobsen S, Nilsson E, Rönn T, Jensen CB, Storgaard H, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab. 2010;95: 3048–3056. Available: http://www.ncbi.nlm.nih.gov/pubmed/20410232
274. Alibegovic AC, Sonne MP, Højbjerre L, Bork-Jensen J, Jacobsen S, Nilsson E, et al. Insulin resistance induced by physical inactivity is associated with multiple transcriptional changes in skeletal muscle in young men. Am J Physiol - Endocrinol Metab. 2010;299: E752–E763. Available: http://www.ncbi.nlm.nih.gov/pubmed/20739510
275. Delgado-Cruzata L, Zhang W, McDonald JA, Tsai WY, Valdovinos C, Falci L, et al. Dietary modifications, weight loss, and changes in metabolic markers affect global DNA methylation in Hispanic, African American, and Afro-Caribbean breast cancer survivors. J Nutr. United States; 2015;145: 783–790. doi:10.3945/jn.114.202853
276. Milagro FI, Campion J, Cordero P, Goyenechea E, Gomez-Uriz AM, Abete I, et al. A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. FASEB J. 2011;25.
277. do Amaral CL, Milagro FI, Curi R, Martinez JA. DNA methylation pattern in overweight women under an energy-restricted diet supplemented with fish oil. Biomed Res Int. United States; 2014;2014: 675021. doi:10.1155/2014/675021
278. Nitert MD, Dayeh T, Volkov P, Elgzyri T, Hall E, Nilsson E, et al. Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes. Diabetes. 2012;61: 3322–3332.
279. Rönn T, Volkov P, Davegårdh C, Dayeh T, Hall E, Olsson AH, et al. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. PLoS Genet. 2013;9. doi:10.1371/journal.pgen.1003572
280. Marchi M, Lisi S, Curcio M, Barbuti S, Piaggi P, Ceccarini G, et al. Human leptin tissue distribution, but not weight loss-dependent change in expression, is associated with methylation of its promoter. Epigenetics. United States; 2011;6: 1198–1206. doi:10.4161/epi.6.10.16600
281. Nilsson EK, Ernst B, Voisin S, Almén MS, Benedict C, Mwinyi J, et al. Roux-En Y Gastric Bypass Surgery Induces Genome-Wide Promoter-Specific Changes in DNA Methylation in Whole Blood of Obese Patients. PLoS One. Public Library of Science; 2015;10: e0115186. Available:

http://dx.doi.org/10.1371%2Fjournal.pone.0115186
282. Benton MC, Johnstone A, Eccles D, Harmon B, Hayes MT, Lea RA, et al. An analysis of DNA methylation in human adipose tissue reveals differential modification of obesity genes before and after gastric bypass and weight loss. Genome Biol. 2015;16: 1–21. doi:10.1186/s13059-014-0569-x
283. Horvath S, Erhart W, Brosch M, Ammerpohl O, von Schönfels W, Ahrens M, et al. Obesity accelerates epigenetic aging of human liver. Proc Natl Acad Sci . 2014;111 : 15538–15543. doi:10.1073/pnas.1412759111
284. Xanthakos SA. Nutritional deficiencies in obesity and after bariatric surgery. Pediatr Clin North Am. United States; 2009;56: 1105–1121. doi:10.1016/j.pcl.2009.07.002
285. von Drygalski A, Andris DA. Anemia after bariatric surgery: more than just iron deficiency. Nutr Clin Pract. United States; 2009;24: 217–226. doi:10.1177/0884533609332174
286. Scibora LM, Ikramuddin S, Buchwald H, Petit MA. Examining the link between bariatric surgery, bone loss, and osteoporosis: a review of bone density studies. Obes Surg. United States; 2012;22: 654–667. doi:10.1007/s11695-012-0596-1
287. Navarro E, Funtikova AN, Fito M, Schroder H. Prenatal nutrition and the risk of adult obesity: Long-term effects of nutrition on epigenetic mechanisms regulating Gene expression. J Nutr Biochem. 2016;39: 1–14. doi:10.1016/j.jnutbio.2016.03.012
288. Barua S, Junaid MA. Lifestyle, pregnancy and epigenetic effects. Epigenomics. Future Medicine; 2015;7: 85–102. doi:10.2217/epi.14.71
289. Demetriou CA, van Veldhoven K, Relton C, Stringhini S, Kyriacou K, Vineis P. Biological embedding of early-life exposures and disease risk in humans: a role for DNA methylation. Eur J Clin Invest. England; 2015;45: 303–332. doi:10.1111/eci.12406
290. Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. Int J Obes (Lond). England; 2015;39: 633–641. doi:10.1038/ijo.2015.13
291. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet. England; 2009;18: 4046–4053. doi:10.1093/hmg/ddp353
292. Tobi EW, Goeman JJ, Monajemi R, Gu H, Putter H, Zhang Y, et al. DNA methylation signatures link prenatal famine exposure to growth and metabolism. Nat Commun. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014;5. Available: http://dx.doi.org/10.1038/ncomms6592
293. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A. United States; 2008;105: 17046–17049. doi:10.1073/pnas.0806560105
294. Waterland RA, Kellermayer R, Laritsky E, Rayco-Solon P, Harris RA, Travisano M, et al. Season of Conception in Rural Gambia Affects DNA Methylation at Putative Human Metastable Epialleles. PLoS Genet. Public Library of Science; 2010;6: e1001252. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1001252
295. Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, et al. Obesity-related DNA methylation at imprinted genes in human sperm: Results from the TIEGER study. Clin Epigenetics. Germany; 2016;8: 51. doi:10.1186/s13148-016-0217-2
296. Sookoian S, Gianotti TF, Burgueno AL, Pirola CJ. Fetal metabolic programming and epigenetic modifications: a systems biology approach. Pediatr Res. International Pediatric Research Foundation, Inc.; 2013;73: 531–542. Available: http://dx.doi.org/10.1038/pr.2013.2
297. Goni L, Milagro FI, Cuervo M, Martinez JA. Single-nucleotide polymorphisms and DNA methylation markers associated with central obesity and regulation of body weight. Nutr Rev. United States; 2014;72: 673–690. doi:10.1111/nure.12143
298. Harder T, Rodekamp E, Schellong K, Dudenhausen JW, Plagemann A. Birth weight and subsequent risk of type 2 diabetes: a meta-analysis. Am J Epidemiol. United States; 2007;165: 849–857. doi:10.1093/aje/kwk071
299. Bell CG, Finer S, Lindgren CM, Wilson GA, Rakyan VK, Teschendorff AE, et al. Integrated genetic and epigenetic analysis identifies haplotype-specific methylation in the FTO type 2 diabetes and obesity susceptibility locus. PLoS One. United States; 2010;5: e14040. doi:10.1371/journal.pone.0014040
300. Guay S-P, Brisson D, Lamarche B, Biron S, Lescelleur O, Biertho L, et al. ADRB3 gene promoter DNA methylation in blood and visceral adipose tissue is associated with metabolic disturbances in men. Epigenomics. Future Medicine; 2014;6: 33–43. doi:10.2217/epi.13.82
301. Mansego ML, Milagro FI, Zulet MA, Martinez JA. SH2B1 CpG-SNP Is Associated with Body Weight

Reduction in Obese Subjects Following a Dietary Restriction Program. Ann Nutr Metab. 2014;66: 1–9. Available: http://www.karger.com/DOI/10.1159/000368425
302. Heijmans BT, Mill J. Commentary: The seven plagues of epigenetic epidemiology. Int J Epidemiol. Oxford University Press; 2012;41: 74–78. doi:10.1093/ije/dyr225
303. Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. Nat Rev Genet. England; 2013;14: 585–594. doi:10.1038/nrg3405
304. Murphy TM, Mill J. Epigenetics in health and disease: heralding the EWAS era. Lancet (London, England). England; 2014;383: 1952–1954. doi:10.1016/S0140-6736(14)60269-5
305. Callaway E. Epigenomics starts to make its mark. Nature. 2014;508: 22. doi:10.1038/508022a
306. Relton CL, Davey Smith G. Two-step epigenetic Mendelian randomization: a strategy for establishing the causal role of epigenetic processes in pathways to disease. Int J Epidemiol. England; 2012;41: 161–176. doi:10.1093/ije/dyr233
307. Richmond RC, Sharp GC, Ward ME, Fraser A, Lyttleton O, McArdle WL, et al. DNA Methylation and BMI: Investigating Identified Methylation Sites at HIF3A in a Causal Framework. Diabetes. United States; 2016;65: 1231–1244. doi:10.2337/db15-0996
308. Allard C, Desgagne V, Patenaude J, Lacroix M, Guillemette L, Battista MC, et al. Mendelian randomization supports causality between maternal hyperglycemia and epigenetic regulation of leptin gene in newborns. Epigenetics. United States; 2015;10: 342–351. doi:10.1080/15592294.2015.1029700
309. Stefan N, Häring H-U, Hu FB, Schulze MB. Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. Lancet Diabetes Endocrinol. Elsevier; 2016;1: 152–162. doi:10.1016/S2213-8587(13)70062-7
310. Primeau V, Coderre L, Karelis AD, Brochu M, Lavoie M-E, Messier V, et al. Characterizing the profile of obese patients who are metabolically healthy. Int J Obes. Macmillan Publishers Limited; 2011;35: 971–981. Available: http://dx.doi.org/10.1038/ijo.2010.216
311. Stokes A, Preston SH. Revealing the burden of obesity using weight histories. Proc Natl Acad Sci U S A. United States; 2016;113: 572–577. doi:10.1073/pnas.1515472113
312. Hannon E, Lunnon K, Schalkwyk L, Mill J. Interindividual methylomic variation across blood, cortex, and cerebellum: implications for epigenetic studies of neurological and neuropsychiatric phenotypes. Epigenetics. Taylor & Francis; 2015;10: 1024–1032. doi:10.1080/15592294.2015.1100786
313. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13.
314. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annu Rev Immunol. United States; 2011;29: 415–445. doi:10.1146/annurev-immunol-031210-101322
315. Heijmans BT, Mill J. Commentary: The seven plagues of epigenetic epidemiology. Int J Epidemiol. Oxford University Press; 2012;41: 74–78. doi:10.1093/ije/dyr225
316. Houseman EA, Molitor J, Marsit CJ. Reference-Free Cell Mixture Adjustments in Analysis of DNA Methylation Data. Bioinforma . 2014; doi:10.1093/bioinformatics/btu029
317. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011;11: 85–97. Available: http://dx.doi.org/10.1038/nri2921
318. Barres R, Kirchner H, Rasmussen M, Yan J, Kantor FR, Krook A, et al. Weight Loss after Gastric Bypass Surgery in Human Obesity Remodels Promoter Methylation. Cell Rep. Elsevier; 2016;3: 1020–1027. doi:10.1016/j.celrep.2013.03.018
319. Geeleher P, Hartnett L, Egan LJ, Golden A, Raja Ali RA, Seoighe C. Gene-set analysis is severely biased when applied to genome-wide methylation data. Bioinforma . 2013; doi:10.1093/bioinformatics/btt311
320. Peters TJ, Buckley MJ, Statham AL, Pidsley R, Samaras K, V Lord R, et al. De novo identification of differentially methylated regions in the human genome. Epigenetics {&} Chromatin. 2015;8: 1–16. doi:10.1186/1756-8935-8-6
321. Bock C. Analysing and interpreting DNA methylation data. Nat Rev Genet. England; 2012;13: 705–719. doi:10.1038/nrg3273
322. Volkov P, Olsson AH, Gillberg L, Jorgensen SW, Brons C, Eriksson K-F, et al. A Genome-Wide mQTL Analysis in Human Adipose Tissue Identifies Genetic Variants Associated with DNA Methylation, Gene Expression and Metabolic Traits. PLoS One. United States; 2016;11: e0157776. doi:10.1371/journal.pone.0157776
323. Henkin Y, Kovsan J, Gepner Y, Shai I. Diets and morbid tissues--history counts, present counts. Br J Nutr. England; 2015;113 Suppl : S11–8. doi:10.1017/S0007114514003730

324. de Souza RJ, Mente A, Maroleanu A, Cozma AI, Ha V, Kishibe T, et al. Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies. BMJ. 2015;351. Available: http://www.bmj.com/content/351/bmj.h3978.abstract
325. Krishnan S, Cooper J a. Effect of dietary fatty acid composition on substrate utilization and body weight maintenance in humans. Eur J Nutr. 2014;53: 691–710. doi:10.1007/s00394-013-0638-z
326. Aslibekyan S, Wiener HW, Havel PJ, Stanhope KL, O'Brien DM, Hopkins SE, et al. DNA methylation patterns are associated with n-3 fatty acid intake in Yup'ik people. J Nutr. United States; 2014;144: 425–430. doi:10.3945/jn.113.187203
327. Milagro FI, Gómez-Abellán P, Campión J, Martínez JA, Ordovás JM, Garaulet M. CLOCK, PER2 and BMAL1 DNA Methylation: Association with Obesity and Metabolic Syndrome Characteristics and Monounsaturated Fat Intake. Chronobiol Int. 2012; 1–15. doi:10.3109/07420528.2012.719967
328. Gabory A, Attig L, Junien C. Sexual dimorphism in environmental epigenetic programming. Mol Cell Endocrinol. Ireland; 2009;304: 8–18. doi:10.1016/j.mce.2009.02.015
329. Mamrut S, Avidan N, Staun-Ram E, Ginzburg E, Truffault F, Berrih-Aknin S, et al. Integrative analysis of methylome and transcriptome in human blood identifies extensive sex- and immune cell-specific differentially methylated regions. Epigenetics. United States; 2015;10: 943–957. doi:10.1080/15592294.2015.1084462
330. Gabory A, Attig L, Junien C. Developmental programming and epigenetics. Am J Clin Nutr . 2011;94 : 1943S–1952S. Available: http://ajcn.nutrition.org/content/94/6_Suppl/1943S.abstract
331. Nugent BM, McCarthy MM. Epigenetic Underpinnings of Developmental Sex Differences in the Brain. Neuroendocrinology. 2011;93: 150–158. Available: http://www.karger.com/DOI/10.1159/000325264
332. Kundaje A, Meuleman W, Ernst J, Bilenky M, Yen A, Heravi-Moussavi A, et al. Integrative analysis of 111 reference human epigenomes. Nature. England; 2015;518: 317–330. doi:10.1038/nature14248
333. Andersson R, Gebhard C, Miguel-Escalada I, Hoof I, Bornholdt J, Boyd M, et al. An atlas of active enhancers across human cell types and tissues. Nature. 2014;507: 455–61. Available: http://www.ncbi.nlm.nih.gov/pubmed/24670763
334. Zhang B, Zhou Y, Lin N, Lowdon RF, Hong C, Nagarajan RP, et al. Functional DNA methylation differences between tissues, cell types, and across individuals discovered using the M&M algorithm. Genome Res. 2013;23: 1522–1540. doi:10.1101/gr.156539.113
335. Campion J, Milagro FI, Goyenechea E, Martinez JA. TNF-alpha promoter methylation as a predictive biomarker for weight-loss response. Obesity (Silver Spring). United States; 2009;17: 1293–1297. doi:10.1038/oby.2008.679
336. Cordero P, Campion J, FI M, Goyenechea E, Steemburgo T, BM J. Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a low-calorie diet. J Physiol Biochem. 2011;67: 463–470. Available: http://dx.doi.org/10.1007/s13105-011-0084-4
337. Brons C, Jacobsen S, Nilsson E, Ronn T, Jensen CB, Storgaard H, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab. United States; 2010;95: 3048–3056. doi:10.1210/jc.2009-2413
338. Voisin S, Eynon N, Yan X, Bishop DJ. Exercise training and DNA methylation in humans. Acta Physiol. 2015;213: 39–59. doi:10.1111/apha.12414
339. Barres R, Zierath JR. The role of diet and exercise in the transgenerational epigenetic landscape of T2DM. Nat Rev Endocrinol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2016;advance on. Available: http://dx.doi.org/10.1038/nrendo.2016.87
340. Leung D, Jung I, Rajagopal N, Schmitt A, Selvaraj S, Lee AY, et al. Integrative analysis of haplotype-resolved epigenomes across human tissues. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;518: 350–354. Available: http://dx.doi.org/10.1038/nature14217
341. Andersson R. Promoter or enhancer, what's the difference? Deconstruction of established distinctions and presentation of a unifying model. Bioessays. United States; 2015;37: 314–323. doi:10.1002/bies.201400162
342. Chromatin state learning [Internet]. [cited 5 Jul 2016]. Available: http://egg2.wustl.edu/roadmap/web_portal/chr_state_learning.html#exp_18state
343. Azzi A, Dallmann R, Casserly A, Rehrauer H, Patrignani A, Maier B, et al. Circadian behavior is light-reprogrammed by plastic DNA methylation. Nat Neurosci. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014;17: 377–382. Available:

http://dx.doi.org/10.1038/nn.3651
344. Lim ASP, Srivastava GP, Yu L, Chibnik LB, Xu J, Buchman AS, et al. 24-Hour Rhythms of DNA Methylation and Their Relation with Rhythms of RNA Expression in the Human Dorsolateral Prefrontal Cortex. PLoS Genet. Public Library of Science; 2014;10: e1004792. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1004792
345. Sahar S, Sassone-Corsi P. The epigenetic language of circadian clocks. Handb Exp Pharmacol. Germany; 2013; 29–44. doi:10.1007/978-3-642-25950-0_2
346. Masri S, Sassone-Corsi P. The circadian clock: a framework linking metabolism, epigenetics and neuronal function. Nat Rev Neurosci. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;14: 69–75. Available: http://dx.doi.org/10.1038/nrn3393
347. Aguilar-Arnal L, Sassone-Corsi P. Chromatin landscape and circadian dynamics: Spatial and temporal organization of clock transcription. Proc Natl Acad Sci U S A. National Academy of Sciences; 2015;112: 6863–6870. doi:10.1073/pnas.1411264111
348. Xia L, Ma S, Zhang Y, Wang T, Zhou M, Wang Z, et al. Daily Variation in Global and Local DNA Methylation in Mouse Livers. Oster H, editor. PLoS One. San Francisco, CA USA: Public Library of Science; 2015;10: e0118101. doi:10.1371/journal.pone.0118101
349. Koike N, Yoo S-H, Huang H-C, Kumar V, Lee C, Kim T-K, et al. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science. United States; 2012;338: 349–354. doi:10.1126/science.1226339
350. Bonsch D, Hothorn T, Krieglstein C, Koch M, Nehmer C, Lenz B, et al. Daily variations of homocysteine concentration may influence methylation of DNA in normal healthy individuals. Chronobiol Int. United States; 2007;24: 315–326. doi:10.1080/07420520701290565
351. Rask-Andersen M, Bringeland N, Nilsson EK, Bandstein M, Olaya Bucaro M, Vogel H, et al. Postprandial alterations in whole-blood DNA methylation are mediated by changes in white blood cell composition. Am J Clin Nutr. 2016; doi:10.3945/ajcn.115.122366
352. Goel N, Basner M, Rao H, Dinges DF. Circadian Rhythms, Sleep Deprivation, and Human Performance. Prog Mol Biol Transl Sci. 2013;119: 155–190. doi:10.1016/B978-0-12-396971-2.00007-5
353. Paine S-J, Gander PH, Travier N. The epidemiology of morningness/eveningness: influence of age, gender, ethnicity, and socioeconomic factors in adults (30-49 years). J Biol Rhythms. United States; 2006;21: 68–76. doi:10.1177/0748730405283154
354. Ribas-Latre A, Eckel-Mahan K. Interdependence of nutrient metabolism and the circadian clock system: Importance for metabolic health. Mol Metab. Germany; 2016;5: 133–152. doi:10.1016/j.molmet.2015.12.006
355. Ruiz-Lozano T, Vidal J, de Hollanda A, Canteras M, Garaulet M, Izquierdo-Pulido M. Evening-chronotype associates with obesity in severe obese subjects: interaction with CLOCK 3111T/C. Int J Obes (Lond). 2016; doi:10.1038/ijo.2016.116
356. Yu JH, Yun C-H, Ahn JH, Suh S, Cho HJ, Lee SK, et al. Evening chronotype is associated with metabolic disorders and body composition in middle-aged adults. J Clin Endocrinol Metab. United States; 2015;100: 1494–1502. doi:10.1210/jc.2014-3754
357. Dopico XC, Evangelou M, Ferreira RC, Guo H, Pekalski ML, Smyth DJ, et al. Widespread seasonal gene expression reveals annual differences in human immunity and physiology. Nat Commun. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2015;6. Available: http://dx.doi.org/10.1038/ncomms8000
358. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. 2009;462: 315–322.
359. Kitamura E, Igarashi J, Morohashi A, Hida N, Oinuma T, Nemoto N, et al. Analysis of tissue-specific differentially methylated regions (TDMs) in humans. Genomics. 2007;89: 326–337. doi:10.1016/j.ygeno.2006.11.006
360. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012. p. 86. doi:10.1186/1471-2105-13-86
361. Koestler DC, Marsit CJ, Christensen BC, Accomando W, Langevin SM, Houseman EA, et al. Peripheral blood immune cell methylation profiles are associated with nonhematopoietic cancers. Cancer Epidemiol Biomarkers Prev. 2012;21: 1293–1302.
362. Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res Clin Pract. 2014;105: 141–150. doi:http://dx.doi.org/10.1016/j.diabres.2014.04.006

363. Fisch IR, Freedman SH. Smoking, oral contraceptives, and obesity. Effects on white blood cell count. JAMA. UNITED STATES; 1975;234: 500–506.
364. Dixon JB, O'Brien PE. Obesity and the white blood cell count: changes with sustained weight loss. Obes Surg. England; 2006;16: 251–257. doi:10.1381/096089206776116453
365. Xu X, Su S, Wang X, Barnes V, De Miguel C, Ownby D, et al. Obesity is associated with more activated neutrophils in African American male youth. Int J Obes. Macmillan Publishers Limited; 2015;39: 26–32. Available: http://dx.doi.org/10.1038/ijo.2014.194
366. Mraz M, Haluzik M. The role of adipose tissue immune cells in obesity and low-grade inflammation. J Endocrinol . 2014;222 : R113–R127. doi:10.1530/JOE-14-0283
367. Szyf M. Examining peripheral DNA methylation in behavioral epigenetic and epigenetic psychiatry: opportunities and challenges. Epigenomics. England; 2014;6: 581–584. doi:10.2217/epi.14.57
368. Tsiotra PC, Pappa V, Raptis SA, Tsigos C. Expression of the long and short leptin receptor isoforms in peripheral blood mononuclear cells: implications for leptin's actions. Metabolism. United States; 2000;49: 1537–1541. doi:10.1053/meta.2000.18519
369. Soriano-Tarraga C, Jimenez-Conde J, Giralt-Steinhauer E, Mola-Caminal M, Vivanco-Hidalgo RM, Ois A, et al. Epigenome-wide association study identifies TXNIP gene associated with type 2 diabetes mellitus and sustained hyperglycemia. Hum Mol Genet. England; 2016;25: 609–619. doi:10.1093/hmg/ddv493
370. Florath I, Butterbach K, Heiss J, Bewerunge-Hudler M, Zhang Y, Schottker B, et al. Type 2 diabetes and leucocyte DNA methylation: an epigenome-wide association study in over 1,500 older adults. Diabetologia. Germany; 2016;59: 130–138. doi:10.1007/s00125-015-3773-7
371. Kadi F, Charifi N, Denis C, Lexell J, Andersen JL, Schjerling P, et al. The behaviour of satellite cells in response to exercise: what have we learned from human studies? Pflügers Arch. 2005;451: 319–327. doi:10.1007/s00424-005-1406-6
372. Hoedt A, Christensen B, Nellemann B, Mikkelsen UR, Hansen M, Schjerling P, et al. Satellite cell response to erythropoietin treatment and endurance training in healthy young men. J Physiol. 2016;594: 727–743. doi:10.1113/JP271333
373. Arentson-Lantz EJ, English KL, Paddon-Jones D, Fry CS. Fourteen days of bed rest induces a decline in satellite cell content and robust atrophy of skeletal muscle fibers in middle-aged adults. J Appl Physiol. 2016;120: 965–975. Available: http://jap.physiology.org/content/120/8/965.abstract
374. Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development. 2012;139: 2845–2856. Available: http://dev.biologists.org/content/139/16/2845.abstract
375. Linnemann AK, Baan M, Davis DB. Pancreatic β-Cell Proliferation in Obesity. Adv Nutr An Int Rev J . 2014;5 : 278–288. doi:10.3945/an.113.005488
376. Brown M, Wittwer C. Flow Cytometry: Principles and Clinical Applications in Hematology. Clin Chem . 2000;46 : 1221–1229. Available: http://www.clinchem.org/content/46/8/1221.abstract
377. Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. Genome Biol. 2014;15.
378. Zou J, Lippert C, Heckerman D, Aryee M, Listgarten J. Epigenome-wide association studies without the need for cell-type composition. Nat Meth. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014;11: 309–311. Available: http://dx.doi.org/10.1038/nmeth.2815
379. Leek JT, Storey JD. Capturing heterogeneity in gene expression studies by surrogate variable analysis. PLoS Genet. 2007;3: 1724–1735.
380. Kaushal A, Zhang H, Karmaus WJJ, Wang JSL. Which methods to choose to correct cell types in genome-scale blood-derived DNA methylation data? BMC Bioinformatics. BioMed Central; 2015;16: P7–P7. doi:10.1186/1471-2105-16-S15-P7
381. Voisin S, Almen MS, Zheleznyakova GY, Lundberg L, Zarei S, Castillo S, et al. Many obesity-associated SNPs strongly associate with DNA methylation changes at proximal promoters and enhancers. Genome Med. England; 2015;7: 103. doi:10.1186/s13073-015-0225-4
382. Gordon L, Joo JE, Powell JE, Ollikainen M, Novakovic B, Li X, et al. Neonatal DNA methylation profile in human twins is specified by a complex interplay between intrauterine environmental and genetic factors, subject to tissue-specific influence. Genome Res. United States; 2012;22: 1395–1406. doi:10.1101/gr.136598.111
383. Banovich NE, Lan X, McVicker G, van de Geijn B, Degner JF, Blischak JD, et al. Methylation QTLs Are Associated with Coordinated Changes in Transcription Factor Binding, Histone Modifications, and Gene Expression Levels. PLoS Genet. Public Library of Science; 2014;10: e1004663. Available:

http://dx.doi.org/10.1371%2Fjournal.pgen.1004663
384. Carja O, MacIsaac JL, Mah SM, Henn BM, Kobor MS, Feldman MW, et al. Worldwide patterns of human epigenetic variation. bioRxiv. 2015; Available: http://biorxiv.org/content/early/2015/07/05/021931.abstract
385. Fraser HB, Lam LL, Neumann SM, Kobor MS. Population-specificity of human DNA methylation. Genome Biol. 2012;13: 1–12. doi:10.1186/gb-2012-13-2-r8
386. Moen EL, Zhang X, Mu W, Delaney SM, Wing C, McQuade J, et al. Genome-wide variation of cytosine modifications between European and African populations and the implications for complex traits. Genetics. United States; 2013;194: 987–996. doi:10.1534/genetics.113.151381
387. Olsson AH, Volkov P, Bacos K, Dayeh T, Hall E, Nilsson EA, et al. Genome-Wide Associations between Genetic and Epigenetic Variation Influence mRNA Expression and Insulin Secretion in Human Pancreatic Islets. PLoS Genet. Public Library of Science; 2014;10: e1004735. Available: http://dx.doi.org/10.1371%2Fjournal.pgen.1004735
388. Zhang X, Moen EL, Liu C, Mu W, Gamazon ER, Delaney SM, et al. Linking the genetic architecture of cytosine modifications with human complex traits. Hum Mol Genet. England; 2014;23: 5893–5905. doi:10.1093/hmg/ddu313
389. Kilpinen H, Waszak SM, Gschwind AR, Raghav SK, Witwicki RM, Orioli A, et al. Coordinated Effects of Sequence Variation on DNA Binding, Chromatin Structure, and Transcription. Science (80-). 2013;342: 744–747. Available: http://science.sciencemag.org/content/342/6159/744.abstract
390. McVicker G, van de Geijn B, Degner JF, Cain CE, Banovich NE, Raj A, et al. Identification of Genetic Variants That Affect Histone Modifications in Human Cells. Science (80-). 2013;342: 747–749. Available: http://science.sciencemag.org/content/342/6159/747.abstract
391. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006;38: 904–909. Available: http://dx.doi.org/10.1038/ng1847
392. Barfield RT, Almli LM, Kilaru V, Smith AK, Mercer KB, Duncan R, et al. Accounting for Population Stratification in DNA Methylation Studies. Genet Epidemiol. 2014;38: 231–241. doi:10.1002/gepi.21789
393. Ek WE, Rask-Andersen M, Johansson A. The role of DNA methylation in the pathogenesis of disease: what can epigenome-wide association studies tell? Epigenomics. England; 2016. pp. 5–7. doi:10.2217/epi.15.99
394. Murphy SK, Adigun A, Huang Z, Overcash F, Wang F, Jirtle RL, et al. Gender-Specific Methylation Differences in Relation to Prenatal Exposure to Cigarette Smoke. Gene. 2012;494: 36–43. doi:10.1016/j.gene.2011.11.062
395. Vilahur N, Baccarelli AA, Bustamante M, Agramunt S, Byun H-M, Fernandez MF, et al. Storage conditions and stability of global DNA methylation in placental tissue. Epigenomics. 2013;5: 10.2217/epi.13.29. doi:10.2217/epi.13.29
396. Tsai P-C, Bell JT. Power and sample size estimation for epigenome-wide association scans to detect differential DNA methylation. Int J Epidemiol . 2015; doi:10.1093/ije/dyv041
397. Vojta A, Dobrinić P, Tadić V, Bočkor L, Korać P, Julg B, et al. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucleic Acids Res . 2016; doi:10.1093/nar/gkw159
398. McDonald JI, Celik H, Rois LE, Fishberger G, Fowler T, Rees R, et al. Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. Biol Open. 2016; Available: http://bio.biologists.org/content/early/2016/05/03/bio.019067.abstract
399. Xu X, Tao Y, Gao X, Zhang L, Li X, Zou W, et al. A CRISPR-based approach for targeted DNA demethylation. Cell Discov. SIBS, CAS; 2016;2: 16009. Available: http://dx.doi.org/10.1038/celldisc.2016.9
400. Maeder ML, Angstman JF, Richardson ME, Linder SJ, Cascio VM, Tsai SQ, et al. Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. Nat Biotech. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;31: 1137–1142. Available: http://dx.doi.org/10.1038/nbt.2726
401. Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, et al. Personalized Nutrition by Prediction of Glycemic Responses. Cell. Elsevier; 2016;163: 1079–1094. doi:10.1016/j.cell.2015.11.001
402. Tsai P-C, Bell JT. Power and sample size estimation for epigenome-wide association scans to detect differential DNA methylation. Int J Epidemiol . 2015; doi:10.1093/ije/dyv041
403. Dalgaard K, Landgraf K, Heyne S, Lempradl A, Longinotto J, Gossens K, et al. Trim28 Haploinsufficiency Triggers Bi-stable Epigenetic Obesity. Cell. Elsevier; 2016;164: 353–364. doi:10.1016/j.cell.2015.12.025

404. Waterland RA. Epigenetic Mechanisms Affecting Regulation of Energy Balance: Many Questions, Few Answers. Annu Rev Nutr. Annual Reviews; 2014;34: 337–355. doi:10.1146/annurev-nutr-071813-105315
405. Heard E, Martienssen RA. Transgenerational Epigenetic Inheritance: Myths and Mechanisms. Cell. Elsevier; 2016;157: 95–109. doi:10.1016/j.cell.2014.02.045
406. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics. United States; 2005;115: e290–6. doi:10.1542/peds.2004-1808
407. Oken E, Rifas-Shiman SL, Field AE, Frazier AL, Gillman MW. Maternal gestational weight gain and offspring weight in adolescence. Obstet Gynecol. United States; 2008;112: 999–1006. doi:10.1097/AOG.0b013e31818a5d50
408. Armitage JA, Poston L, Taylor PD. Developmental origins of obesity and the metabolic syndrome: the role of maternal obesity. Front Horm Res. Switzerland; 2008;36: 73–84. doi:10.1159/0000115355
409. Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, et al. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J Mol Endocrinol. England; 2008;41: 91–102. doi:10.1677/JME-08-0025
410. Suter MA, Chen A, Burdine MS, Choudhury M, Harris RA, Lane RH, et al. A maternal high-fat diet modulates fetal SIRT1 histone and protein deacetylase activity in nonhuman primates. FASEB J Off Publ Fed Am Soc Exp Biol. United States; 2012;26: 5106–5114. doi:10.1096/fj.12-212878
411. Gemma C, Sookoian S, Alvarinas J, Garcia SI, Quintana L, Kanevsky D, et al. Maternal pregestational BMI is associated with methylation of the PPARGC1A promoter in newborns. Obesity (Silver Spring). United States; 2009;17: 1032–1039. doi:10.1038/oby.2008.605
412. Michels KB, Harris HR, Barault L. Birthweight, Maternal Weight Trajectories and Global DNA Methylation of LINE-1 Repetitive Elements. PLoS One. Public Library of Science; 2011;6: e25254. Available: http://dx.doi.org/10.1371%2Fjournal.pone.0025254
413. El Hajj N, Pliushch G, Schneider E, Dittrich M, Müller T, Korenkov M, et al. Metabolic programming of MEST DNA methylation by intrauterine exposure to gestational diabetes mellitus. Diabetes. 2013;62: 1320–8. doi:10.2337/db12-0289
414. Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, et al. Epigenetic gene promoter methylation at birth is associated with child's later adiposity. Diabetes. 2011;60.
415. Borgel J, Guibert S, Li Y, Chiba H, Schubeler D, Sasaki H, et al. Targets and dynamics of promoter DNA methylation during early mouse development. Nat Genet. 2010;42. doi:10.1038/ng.708
416. Gould J. What's the point of the PhD thesis? [Internet]. 2016 [cited 10 Jul 2016]. Available: http://www.nature.com/news/what-s-the-point-of-the-phd-thesis-1.20203

Appendix

Poster presented at the Epigenetics, Obesity and Metabolism conference, Oxford, UK, 11-14 October 2015

Obesity-associated SNPs associate with DNA methylation changes at proximal promoters and enhancers

Sarah Voisin, Markus Sällman Almén, Galina Y Zheleznyakova, Lina Lundberg, Sanaz Zarei, Sandra Castillo, Fia E. Eriksson, Emil K. Nilsson, Matthias Blüher, Yvonne Böttcher, Peter Kovacs, Janis Klovins, Mathias Rask-Andersen, Helgi B. Schiöth

Genome Medicine, in press

Background

The mechanisms of how genetic variants (SNPs) identified in GWAS act to influence body mass remain unknown for most of these SNPs. Recent evidence points to epigenetic and chromatin state of the genome to have an important role. For instance, obesity-associated SNPs could influence DNA methylation, leading to gene deregulation.

Scientific questions

- Do obesity-associated SNPs associate with proximal DNA methylation (within 500 kb)?
- If so, what are the characteristics of the methylated regions controlled by obesity-associated SNPs?
- If so, are these SNP-CpG associations tissue-specific?

Results

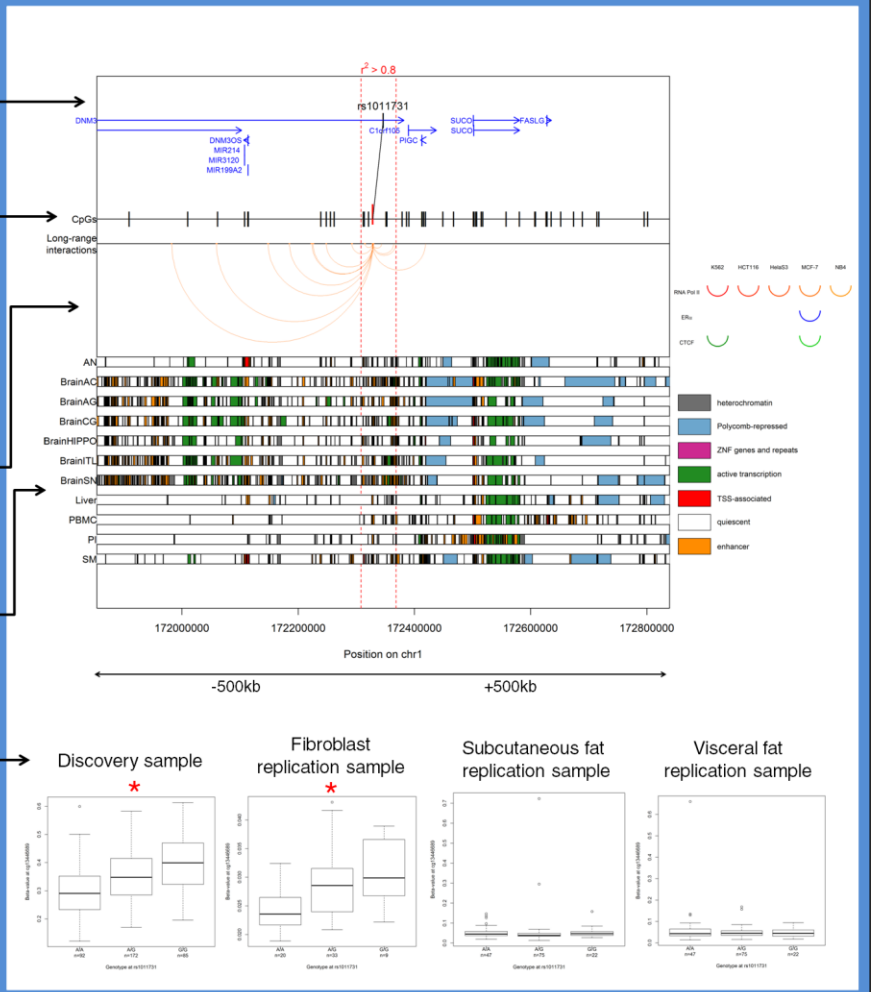
- Alleles at **28** of the 52 obesity-associated SNPs associate with methylation levels at **107 proximal CpG sites** in blood of 355 healthy young individuals.
- Out of these 107 CpG sites, **38 are located in gene promoters**, including **genes strongly implicated in obesity** (*MIR148A*, *BDNF*, *PTPMT1*, *NR1H3*, *HOXC12*, *ITIH4*, *POMC*, *ADCY3*, *LGR4*, *TUFM*, *SULT1A1*, *SULT1A2*, *APOBR*, *CLN3*, *SH2B1* and *IL27*). Interestingly, the associated SNPs are in known eQTLs for some of these genes. We also found that the 107 CpGs are **enriched in enhancers** in peripheral blood mononuclear cells.
- Finally, our results indicate **that some of these associations are not blood-specific** as we successfully replicated four associations in skin fibroblasts.

Material and Methods

- **355 healthy young individuals** were genotyped for **52 SNPs** previously associated with obesity-related traits (BMI, waist-to-hip ratio, waist circumference, etc.).

	Discovery sample
n	355
n_{males}	214 (60%)
Age (years)	20.6±1.2
Weight (kg)	71.6±13.7
Height (m)	1.76±0.092
Weight category	76% lean, 20% overweight, 4% obese

- We also obtained genome-wide **blood DNA methylation** in these individuals, using the 450k HumanMethylation beadchip. However, we only analysed CpGs located **within 500 kb of the 52 SNPs**.
- To see whether any of the significant CpG was in putative **long-range interactions**, we used open-access long-range interactions as defined by ChIA-PET libraries from five cell lines and three transcription factors
- To see whether the significant CpGs were over- or under-represented in particular **chromatin states**, we used seven open-access histone modifications to infer putative chromatin states in **eleven tissues relevant in obesity**
- For replication in other tissues, we used two open-access datasets (**skin fibroblasts**, n=62; **four brain regions**, n=121-133) and one additional dataset of **subcutaneous and visceral fat** (n=149)



Conclusion and perspectives

- Our results strongly suggest that **many obesity-associated SNPs** are associated with **proximal gene regulation**, which was reflected by association of obesity risk allele genotypes with differential DNA methylation.
- This study highlights **the importance of DNA methylation** and **other chromatin marks** as a way to understand the molecular basis of genetic variants associated to human diseases and traits.

Functional pharmacology lab
Neurosciences department

Helgi B Schiöth
Marcus Sällman Almén
Sarah Voisin
Anne Gabory
Claudine Junien

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1245

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-300751

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016

Paper I [redacted]

Voisin et al. Genome Medicine (2015) 7:103
DOI 10.1186/s13073-015-0225-4

Genome Medicine

RESEARCH Open Access

Many obesity-associated SNPs strongly associate with DNA methylation changes at proximal promoters and enhancers

Sarah Voisin[1*], Markus Sällman Almén[1,2], Galina Y. Zheleznyakova[1], Lina Lundberg[1], Sanaz Zarei[1], Sandra Castillo[1], Fia Ence Eriksson[1], Emil K. Nilsson[1], Matthias Blüher[3], Yvonne Böttcher[3], Peter Kovacs[3], Janis Klovins[4], Mathias Rask-Andersen[1] and Helgi B. Schiöth[1]

Abstract

Background: The mechanisms by which genetic variants, such as single nucleotide polymorphisms (SNPs), identified in genome-wide association studies act to influence body mass remain unknown for most of these SNPs, which continue to puzzle the scientific community. Recent evidence points to the epigenetic and chromatin states of the genome as having important roles.

Methods: We genotyped 355 healthy young individuals for 52 known obesity-associated SNPs and obtained DNA methylation levels in their blood using the Illumina 450 K BeadChip. Associations between alleles and methylation at proximal cytosine residues were tested using a linear model adjusted for age, sex, weight category, and a proxy for blood cell type counts. For replication in other tissues, we used two open-access datasets (skin fibroblasts, $n = 62$; four brain regions, $n = 121–133$) and an additional dataset in subcutaneous and visceral fat ($n = 149$).

Results: We found that alleles at 28 of these obesity-associated SNPs associate with methylation levels at 107 proximal CpG sites. Out of 107 CpG sites, 38 are located in gene promoters, including genes strongly implicated in obesity (*MIR148A*, *BDNF*, *PTPMT1*, *NR1H3*, *MGAT1*, *SCGB3A1*, *HOXC12*, *PMAIP1*, *PSIP1*, *RPS10-NUDT3*, *RPS10*, *SKOR1*, *MAP2K5*, *SIX5*, *AGRN*, *IMMP1L*, *ELP4*, *ITIH4*, *SEMA3G*, *POMC*, *ADCY3*, *SSPN*, *LGR4*, *TUFM*, *MIR4721*, *SULT1A1*, *SULT1A2*, *APOBR*, *CLN3*, *SPNS1*, *SH2B1*, *ATXN2L*, and *IL27*). Interestingly, the associated SNPs are in known eQTLs for some of these genes. We also found that the 107 CpGs are enriched in enhancers in peripheral blood mononuclear cells. Finally, our results indicate that some of these associations are not blood-specific as we successfully replicated four associations in skin fibroblasts.

Conclusions: Our results strongly suggest that many obesity-associated SNPs are associated with proximal gene regulation, which was reflected by association of obesity risk allele genotypes with differential DNA methylation. This study highlights the importance of DNA methylation and other chromatin marks as a way to understand the molecular basis of genetic variants associated with human diseases and traits.

Background

Genome-wide association studies (GWASs) have identified a plethora of common genetic variants that are associated with obesity-associated traits (e.g., body mass index (BMI) [1–11], fat mass [12, 13], low lean body mass [14], blood lipid levels [15], waist circumference [13, 16], BMI-adjusted waist-to-hip ratio [17, 18]). Some of these single nucleotide polymorphisms (SNPs) are located near genes whose role in obesity is well established, such as *MC4R* [19]. However, most of these SNPs are located near genes whose role in obesity is still unclear, and the mechanisms through which they act remain unknown. Part of this lack of understanding may be due to a focus on the genes in closest proximity to these SNPs. Actually, these SNPs may regulate genes that are located quite far away, as recently demonstrated for genetic variants within *FTO*. In human brains, obesity-associated SNPs in *FTO* were found to be associated with

* Correspondence: sarah.voisin.aeris@gmail.com
[1]Department of Neuroscience, Functional Pharmacology, Uppsala University, Uppsala, Sweden
Full list of author information is available at the end of the article

expression of *IRX3*, a gene located more than half a million base pairs downstream of the body mass-associated genetic locus [20]. Another instance is the rs4537545 SNP previously associated with coronary heart disease [21] and located within *IL6R*: this SNP was recently found to be associated with blood mRNA levels of *ATP8B2*, a gene located 115 kb away [22]. Thus, obesity-associated SNPs might act through long-range interactions (for example, by disrupting enhancers) and potentially through epigenetic mechanisms.

The epigenome represents the pattern of chemical and structural modifications to DNA that are heritable through mitosis and/or meiosis, but that do not entail changes in DNA sequence. Epigenetic mechanisms encompass DNA methylation, histone modifications, and non-coding RNAs, and have the potential to modify gene expression. Recent attention has been drawn to the possible role of epigenetics in the pathogenesis of obesity [23, 24]. Moreover, while the epigenome is known to be modulated by the environment, this modulation can also be affected by genetic variants. Studies in brain [25–28], adipose tissue [29, 30], blood [26, 31, 32], lung [33], fibroblasts [34, 35], T cells [35], leukocytes [36], and lymphoblastoid cells [35, 37] have shown that the genome contains quantitative trait loci (QTLs) for DNA methylation, also called methylation QTLs (meQTLs). DNA methylation levels correlate with the presence of specific alleles at nearby SNPs, and meQTLs tend to locate outside of promoters, especially in intergenic regions. In a study conducted in adipose tissue [29], meQTLs overlapping metabolic disease loci were enriched in histone marks predictive of genetic enhancers. Interestingly, top associations from a GWAS of bipolar disorder were enriched in meQTLs [38], suggesting that this could be a powerful approach to better understand the molecular basis of candidate SNPs from GWASs.

In the present study, we tested associations between 52 SNPs that were previously identified in GWASs or meta-analyses to be associated with obesity traits, and proximal DNA methylation in whole blood of 355 healthy young individuals. We then tested the tissue specificity of the majority of these associations in four brain regions (n = 121–133), visceral adipose tissue (VAT; n = 149), subcutaneous adipose tissue (SAT; n = 149) and fibroblasts (n = 62). Finally, the genomic context of associated CpG sites was explored, using chromatin segmentation on publicly available histone marks from 11 tissues and long-range interactions from five cell lines.

Methods

Discovery study group

Ethics, consent and permissions

The discovery study group comprised two sub-groups of healthy young Caucasians from two different age ranges (Table 1). All participants and their guardians gave informed written consent and the study was approved by the local ethics committee in Uppsala, EPN, diary number 2011/446; this study was conducted in accordance with the principles of the Declaration of Helsinki. The first sub-group comprised 130 individuals aged 14–16 years who were recruited by visiting schools in Uppsala county and by post. Two 6-ml blood samples were drawn for genotyping and DNA methylation measurement, at any time during the day. The other sub-group comprised 225 individuals of white European descent aged 18–34 years, also recruited in Uppsala. Subjects were fasting (at least 10 h) when blood samples were taken for genotyping and DNA methylation measurement. For individuals aged under 18 years, we used Cole et al.'s definition to determine weight category [39]. For individuals aged 18 years and older, the following cutoffs were used: lean, BMI < 25; overweight, 25 ≤ BMI < 30; obese, BMI ≥ 30. We chose to use weight category instead of BMI since our cohort includes individuals aged under 18 years whose BMI scales differ from the BMI scales of individuals aged over 18 years.

Genotyping

We selected 52 SNPs that have been associated by GWASs or meta-analyses of GWASs with obesity-associated traits (BMI [1–10], BMI-adjusted waist-to-hip ratio [18], fat mass [12], low lean body mass [14], blood lipid levels [15] and waist circumference [16]) and the discovery study group was genotyped for these SNPs

Table 1 Description of the discovery samples

	Sub-group 1	Sub-group 2	Total
n	130	225	355
n_{males}	37 (29 %)	177 (79 %)	214 (60 %)
Age (years)[a]	15.3 ± 0.64	23.6 ± 3.3	20.6 ± 1.2
Weight (kg)[a]	72.9 ± 11.4	76.6 ± 12.4	71.6 ± 13.7
Height (m)[a]	1.70 ± 0.081	1.79 ± 0.078	1.76 ± 0.092
Weight category[b]	77 % lean, 18 % overweight, 5 % obese	74 % lean, 21 % overweight, 5 % obese	76 % lean, 20 % overweight, 4 % obese

[a]Mean ± standard deviation. [b]For individuals aged under 18 years, we used Cole et al.'s definition to determine weight category [39]. For individuals aged 18 years and older, the following cutoffs were used: lean, BMI < 25; overweight, 25 ≤ BMI < 30; obese, BMI ≥ 30

(Additional file 1). Genotyping of the 52 SNPs was carried out at the SNP technology platform at Uppsala University [40] using an Illumina Golden Gate Assay (Illumina Inc., San Diego, CA, USA). There were missing genotypes for 8 of the 52 tested SNPs, ranging from one individual to 52 individuals with missing genotypes (Additional file 1). Individuals with missing genotypes were removed from the analysis.

DNA methylation profiling

The genome-wide Illumina Infinium HumanMethylation450 BeadChip (Illumina), which allows interrogation of 485,512 CpG dinucleotides covering 25,953 genes, was applied to determine the methylation profile of genomic DNA isolated and purified from the peripheral whole blood. This chip has been shown to give a reliable and reproducible estimation of the methylation profile on a genomic scale [15]. First, bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research) according to the manufacturer's protocol. Briefly, 500 ng of DNA was sodium bisulfite-treated, denatured at 98 °C for 10 min, and bisulfite converted at 64 °C for 2.5 h. After conversion, samples were desulfonated and purified using column preparation. Approximately 200 ng of bisulfate-converted DNA was processed according to the Illumina Infinium Methylation Assay protocol. This assay is based on the conversion of unmethylated cytosine (C) nucleotides into uracil/thymine (T) nucleotides by the bisulfite treatment. The DNA was whole-genome amplified, enzymatically fragmented, precipitated, resuspended, and hybridized overnight at 48 °C to locus-specific oligonucleotide primers on the BeadChip. After hybridization, the C or T nucleotides were detected by single-base primer extension. The fluorescence signals corresponding to the C or T nucleotides were measured from the BeadChips using the Illumina iScan scanner. Phenotypes, genotypes, raw data, and processed DNA methylation data are available through the Gene Expression Omnibus (GEO) database [41] with accession number [GEO:GSE73103].

DNA methylation processing

All downstream data processing and statistical analyses were performed with the statistical software R [42] together with the *minfi* [43], *ChAMP* [44], *sva* [45], and *MethylAid* [46] packages of the Bioconductor project.

Background correction and adjustment of type I and type II probes

Fluorescence data were preprocessed using the GenomeStudio 2009.2 (Illumina) software. First, we background corrected the data using NOOB [47]. In the Illumina Infinium HumanMethylation450 BeadChip array, the probes come in two different designs, characterized by widely different DNA methylation distributions and dynamic range, which may bias downstream analyses. Therefore, we applied the BMIQ algorithm to adjust for the two different probe designs [48].

Removal of batch effects

The plates on which samples are run introduce a known batch effect that is important to correct for. We used the ComBat function to adjust directly for this batch effect [45].

Principal component analysis

We performed a principal component analysis (PCA) using the PCA function of the *FactoMineR* package [49], first calculating the covariance matrix between all samples using only the most variable autosomal CpG sites, measured in terms of their 95 % reference range: the range of methylation values observed in the central 95 % of the samples or, more precisely, the difference between the 97.5 and 2.5 % percentiles. Using a 95 % reference range of at least 0.20, 103,408 CpG sites were used in the covariance matrix calculation. Together, the two first principal components explain over 39 % of the total variance. Each subsequent vector does not add substantially to the variance explained: 285 vectors would be necessary to explain 95 % of the total variance.

Sample exclusion

We excluded from association analyses: (1) samples that were outliers in any one of the quality control plots generated by MethylAid [46] (rotated M versus U plot, overall sample-dependent control plot, bisulfite conversion control plot, overall sample-independent control plot and detection p value plot) using the default thresholds (0 samples); (2) samples that were outliers with respect to any one of the first eight principal components (corresponding to the approximate location of the elbow of the eigenvalue scree plot; six samples). After exclusion of samples, we were left with 349 samples: 128 from the first sub-group (29 % males; mean age ± standard deviation 15.3 ± 0.64 years) and 221 from the second sub-group (78 % males; mean age ± standard deviation 23.6 ± 3.3 years).

Probe exclusion

We removed probes with missing β values, probes having less than 75 % of samples with detection p value < 0.01, and probes located on the sex chromosomes. Using the annotation generated by Chen et al. [50], we also removed cross-reactive probes and probes containing SNPs with minor allele frequency > 1 % in European populations. In total, 397,615 probes were included in the analysis.

Choice of investigated CpGs

We selected the probes within 500 kb of each SNP. A total of 8485 probes were analyzed, with an average of 163 CpGs per SNP (Additional file 1).

Cell-type proportions

Because differences in cell-type proportions between DNA samples can confound association results [51], we adjusted our analyses using a surrogate for cell-type proportions derived from 43 differentially methylated CpG sites present on the HumanMethylation450 array that have the ability to discriminate between blood cell types [52]. As a surrogate for cell-type proportions, and to reduce the number of variables, we used the first two principal components associated with these 43 sites that together explain over 70 % of the total variance in methylation at these 43 CpG sites.

To verify that the first two principal components that we derived from the list of 43 differentially methylated CpG sites [52] can indeed serve as a surrogate for blood cell proportions, we tested for associations between the principal components and the methylation levels at all of our sites, adjusting our analyses for sex, age, weight category, and batch. We selected the top 10 % of the sites that showed the strongest associations (49,035 sites, all associated at levels $p < 10^{-8}$) and extracted these sites in data sets of purified human leukocyte subtypes [53] [GEO:GSE39981]; 2564 sites were overlapping. A dendrogram representation of our top sites in this data set [53] reveals clear clustering of samples according to cell type, indicating a good ability for principal components to discriminate between samples with different cell compositions (Additional file 2).

Validation of methylation with bisulfite sequencing

The methylation levels of two of the associated CpG sites (cg15576492 and cg2204028, at position chr1:1015257–1015540) were validated using bisulfite sequencing. The sequences including target CpG sites were obtained from the University of California, Santa Cruz (UCSC) Genome Browser database. The sequences (bisulfite-converted DNA template) for the primers were forward (biotin labeled)-5′-ATGGATGTTGGTGTGAGTATT-3′ and reverse 5′-CCCTCTACACATCTAAACCCT-3′. Bisulfite sequencing primers were designed with Methyl Primer Express® v.1.0 (Applied Biosystems) so that the amplicons covered target CpG sites. These regions were PCR amplified in duplicate from bisulfite-treated DNA. Similar efficiency in PCR amplification for unmethylated and methylated fragments was controlled for using Human Methylated & Non-methylated DNA Set (Zymo Research). PCR reactions were performed in a final volume of 25 μl and contained 2.5 μl of bisulfite-treated DNA (10–15 ng/μl), 0.05 μl of each primer (100 pmol/μl), 1 μl DMSO, 0.5 μl of SYBR Green I (1:50,000; Invitrogen, Sweden) in TE buffer (pH 7.8), 0.25 μl of 25 mM dNTP mix (Fermentas), 2.5 μl 10× buffer, 4 μl of 25 mM $MgCl_2$, 1 U of Hot Start Taq DNA polymerase (Thermo Scientific). Cycling conditions were as follows: 10-min initial denaturation step at 95 °C, followed by 45 cycles of 95 °C for 20 s, 30 s at optimal annealing temperature of primers, 20–45 s at 72 °C, 5 min of final elongation at 72 °C. Fluorescence was measured after the elongation phase. Melting curve analysis consisted of 81 cycles of 10 s at 55 °C with increasing increments of 0.5 °C per cycle. Bio-Rad iQ5 version 2.0 software (Bio-Rad Laboratories) was used to process real-time PCR data.

Amplicons were purified using GeneJET PCR Purification Kit (Thermo Scientific).

DNA sequencing was performed using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI3730XL DNA Analyzer (Applied Biosystems) at Uppsala Genome Center. Cycle sequencing was as follows: 30 s initial denaturation step at 94 °C, followed by 35 cycles of 94 °C for 25 s, 50 °C for 15 s, 60 °C for 120 s. Each sample was sequenced twice and the two methylation levels were averaged. Amplification primers were used for sequencing. All samples were analyzed in duplicates on different plates and the mean methylation levels in percentage per sample were used for further analyses. Methylation levels of CpG sites for all amplicons were quantified using Epigenetic Sequencing Methylation analysis software [54]. The software was repeatedly used to determine the methylation profile of several genes [55, 56]. The software algorithm analyzes the methylation percentage of each CpG site in an amplicon without cloning stage.

Replication study groups

VAT and SAT

VAT and SAT samples were used to test specifically the association between alleles at rs1011731 and methylation at cg13446689. Paired samples of VAT and SAT from 149 Caucasian subjects (35 % male) who underwent open abdominal surgery were included in the study. This subset is part of a study group that had already been genotyped for rs1011731, described in detail elsewhere [57]. Thirty-two individuals were lean (aged 63 ± 11 years, BMI 22.1 ± 2.5 kg/m^2), 22 were overweight (67 ± 12 years, BMI 27.1 ± 1.4 kg/m^2) and 94 were obese (age 47 ± 13 years, BMI 48.1 ± 9.7 kg/m^2); BMI was missing for one individual and 46 subjects had diabetes type 2. Patients with severe conditions, including generalized inflammation or end-stage malignant diseases, were excluded from the study. Samples of VAT and SAT were immediately frozen in liquid nitrogen after explantation. The study was approved by the ethics committee of the

University of Leipzig and all subjects gave written informed consent.

Genomic DNA was extracted from frozen adipose tissue samples using GenElute™ Mammalian Genomic DNA Miniprep Kit (SIGMA-ALDRICH, USA). All samples were bisulfite converted using Qiagen Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and applied to whole bisulfitome amplification (EpiTect Whole Bisulfitome Kit, Qiagen, Hilden, Germany). Finally, all samples were purified using GenElute PCR Clean-up Kit (Sigma-Aldrich, USA). Methylation levels of cg13446689 were determined using a custom designed PyroMark CpG assay (Qiagen, Hilden, Germany). The sequences (bisulfite-converted DNA template) for the primers were forward (biotin labeled)-5′-AAGTGATGG GAGTTGTTGG-3′ and reverse 5′- ACCCCAAAACAAT TCAAACAAACCATA-′3. Using the sequencing primer 5′-ACAATTCAAACAAACCATACTTA-3′ the following sequence was analyzed (5′- CACAAC[R]ACTAACTAA TCTATAC[R]ACCTCAAACCAA AAACAACA ACCAAC AACTCC-3′). The pyrosequencing was run on a PyroMark Q24 (Qiagen, Hilden, Germany). All samples were analyzed in duplicates on different plates and the mean methylation levels in percentage per sample were used for further analyses. Water was used as a non-template control using the same PCR conditions.

Fibroblasts

Methylation, SNP genotyping, and gene expression data from primary skin fibroblasts from Caucasian individuals ($n = 62$) [34] were obtained from GEO (accession number [GEO:GSE53261]).

Brain regions (cerebellum, frontal cortex, caudal pons, and temporal cortex)

SNP genotyping data from four different brain regions ($n = 121–133$) [58] were obtained from dbGAP (accession number phs000249.v1.p1). All individuals were of Caucasian descent, but two individuals from the cerebellum study samples were of African and Asian descent, respectively. We removed these two individuals from our analysis. Methylation data were obtained from GEO (accession number [GEO:GSE15745]).

Annotation

Genes

The genomic positions of RefSeq genes were downloaded from the UCSC genome browser, and the location of each CpG site was determined as promoter (within 1500 bp of the transcription start site (TSS)), gene body, intergenic, or ambiguous (overlapping a promoter and a gene body).

Linkage disequilibrium

Linkage disequilibrium (LD) data were obtained from SNAP Proxy, using CEU as the "population panel" and the 1000 Genomes Pilot 1 as "SNP dataset" [59].

Chromatin states

ChromHMM [60] was applied for seven publicly available histone modifications (H3K4me1, H3K4me3, H3K9ac, H3K9me3, H3K27ac, H3K27me3, and H3K36me3) from 11 tissues: adipose nuclei (AN), pancreatic islets (PI), peripheral blood mononuclear primary cells (PBMC), skeletal muscle (SM), liver, brain angular gyrus (BrainAG), brain anterior caudate (BrainAC), brain cingulate gyrus (BrainCG), brain hippocampus (BrainHIPPO), brain inferior temporal lobe (BrainITL), and brain substantia nigra (BrainSN). Data were downloaded from NIH Roadmap Epigenomics Project Data Listings. An 18-state model was learned from all binarized data and was used to produce segmentations based on the most likely state assignment of the model. Then, each state was assigned to one of the following seven categories: enhancer, active TSS/poised TSS/flanking TSS, active transcription, quiescent, heterochromatin, Polycomb-repressed, ZNF genes/repeats.

Ubiquitous, tissue-specific, and cell-specific in vivo transcribed enhancers

Ubiquitous, tissue-specific (adipose tissue, blood, brain, liver, pancreas, and skeletal muscle) and cell type-specific (preadipocytes, fat cells, hepatocytes, and skeletal muscle cells) enhancers, as well as TSS–enhancer associations, as defined by CAGE tags in the FANTOM5 project, were downloaded from the Transcribed Enhancer Atlas website [61, 62].

Long-range interactions

We used publicly available chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) libraries to map long-range interactions in five different cell lines, with three different transcription factors [63] (Additional file 3). Data were downloaded from the WashU Epigenome Browser.

Expression QTLs

We used the following publicly available expression QTL (eQTL) browsers to see whether any of the associated SNPs or SNPs in strong linkage with them ($r^2 > 0.8$) were eQTLs for our genes of interest: the eQTL browser of the Genotype-Tissue Expression (GTEx) project [64], the eQTL Browser of the National Center for Biotechnology Information, the eQTL resources from the Gilad/Pritchard group [65], and the blood eQTL browser developed by Westra *et al.* [66].

Statistics

For statistical analysis, we used the $\log_2$ ratio of the intensities of methylated probe and unmethylated probe, also called M value, which is more statistically valid for the differential analysis of methylation levels [67].

Linear model

We developed the following linear model for each CpG site k:

$$M_k = a_k + b_{kS}S + b_{kA}A + b_{kW}W + b_{kG}G + b_{kPC1}PC1 + b_{kPC2}PC2 + \varepsilon_k$$

where M_k is the M value of CpG site k, S is the dichotomized sex (female = 1 and male = 0), A is the age, W is the weight category (normal weight = 0, overweight = 1, obese = 2), G is the genotype at the investigated SNP (homozygotes for non-risk allele = 0, heterozygotes = 1, homozygotes for risk allele = 2), PC1 and PC2 are the first two principal components derived from the list of 43 differentially methylated CpG sites in blood cell types, and ε_k is the unexplained variability. We chose to use weight category instead of BMI since our study samples include individuals aged under 18 years whose BMI scales differ from the BMI scales of individuals aged over 18 years. Rare homozygous genotypes (count of less than 10) were combined with heterozygotes.

The coefficients b_{kx} summarize the association between methylation levels and the variables of interest. The p value for the SNP was determined using a likelihood ratio test, using the lrtest function of the *lmtest* package [68], and we report the effect size as the proportion R^2 of the CpG methylation variance that is explained by the SNP, among the variance not already explained by the covariates. To control the proportion of false positives, q values were calculated using the qvalue function of the *qvalue* package [69]. A SNP was considered significant if its q value was < 0.05.

Enrichment of associated CpGs in genomic regions, in vivo transcribed enhancers, and chromatin states

To test whether associated CpGs were enriched or underrepresented in different genomic regions (promoter, gene body, etc.), chromatin states (enhancer, TSS, heterochromatin, etc.) and in vivo transcribed enhancers, we used Fisher's exact test. To control the proportion of false positives, q values were calculated using the qvalue function of the *qvalue* package [69]. Significance was considered at a q value < 0.05.

Number of long-range interactions

The distributions of the numbers of long-range interactions per CpG were skewed. Thus, to see whether associated CpGs had a higher or lower number of long-range interactions, we used Mann–Whitney U-test.

Power calculations

We used the pwr.f2.test function of the *pwr* package in R to determine the statistical power in the replication datasets (fibroblasts, brain and SAT/VAT).

Results

Obesity-associated SNPs associate with methylation at proximal CpGs in whole blood samples from healthy individuals

We tested associations between 52 obesity-associated SNPs and M values of all CpG sites 500 kb upstream and 500 kb downstream of each SNP in the blood of 355 individuals (Table 1), using a linear regression model adjusted for age, sex, blood cell type surrogate, and weight category (i.e., lean, overweight, or obese) instead of BMI since our study samples include individuals aged under 18 years whose BMI scales differ from the BMI scales of individuals aged over 18 years. In total, 8485 probes were tested, with an average of 163 probes per SNP (Additional file 1). Methylation levels at 107 CpGs associated with genotypes at 28 SNPs (likelihood ratio test, q value < 0.05; Additional file 4) and most of the associations were between SNPs and CpGs that are close to each other (50 % of the associations are between SNPs and CpGs that are within 40 kb of each other; Fig. 1). Also, the closer the SNP and CpG are, the stronger the statistical significance is (Fig. 1). One example of these SNP–CpG associations is depicted in Fig. 2. The rs713586 SNP explains 53.8 % of the total variance in methylation at cg01884057, with carriers of the risk allele (*C*) at rs713586 having higher methylation.

The two sub-groups that were pooled for the discovery analysis were of two different age ranges (see "Methods"), but they did not significantly differ in terms of global DNA methylation patterns, as shown by PCA (Additional file 5). To make sure that the two sub-groups were comparable and could effectively be combined for the discovery analysis, we tested the significance of the 107 CpGs separately in each. SNP effects were in the same directions for all 107 CpGs in the two separate sub-groups; 105 of the 107 CpGs were significant (raw p value < 0.05) in the first, while 86 of the 107 CpGs were significant (raw p value < 0.05) in the second. This suggests that our results are not driven by a specificity of one of the two sub-groups and that it was reasonable to pool them for the discovery analysis.

Genomic context of CpGs associated with obesity-associated SNPs

To understand the functional significance of the CpGs associated with alleles at obesity-associated SNPs, we

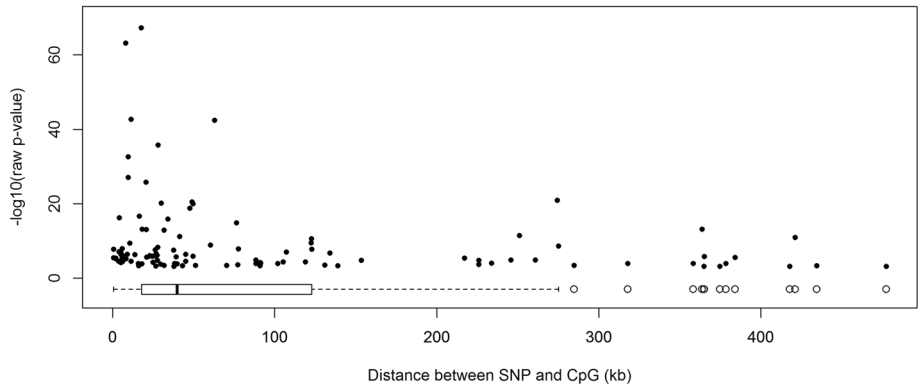


Fig. 1 Raw *p* value as a function of distance between SNP and CpG. Each point represents an associated SNP–CpG pair (107 pairs). Most associated SNP–CpG pairs are close to each other, as illustrated by the box plot of the distance between SNP and CpG (bottom of the plot). The closer the associated SNP–CpG pairs are, the lower the *p* value

analyzed their genomic location in relation to genes, chromatin states in 11 tissues, ubiquitous, tissue-specific, or cell-specific in vivo transcribed enhancers, and long-range interactions in five cell lines.

CpGs associated with obesity-associated SNPs are depleted in promoters and enriched in intergenic regions

Thirty-eight of the associated CpGs were located in gene promoters (*MIR148A*, *BDNF*, *PTPMT1*, *NR1H3*, *MGAT1*, *SCGB3A1*, *HOXC12*, *PMAIP1*, *PSIP1*, *RPS10-NUDT3*, *RPS10*, *SKOR1*, *MAP2K5*, *SIX5*, *AGRN*, *IMMP1L*, *ELP4*, *ITIH4*, *SEMA3G*, *POMC*, *ADCY3*, *SSPN*, *LGR4*, *TUFM*, *MIR4721*, *SULT1A1*, *SULT1A2*, *APOBR*, *CLN3*, *SPNS1*, *SH2B1*, *ATXN2L*, and *IL27*), including eight also located in a gene body (Additional file 4). Thus, associated CpGs were underrepresented in promoters (28 % of CpGs, Fisher's exact test p value = 0.0097). In contrast, 31 associated CpGs were located in intergenic regions, which is more than expected by chance (30 % of CpGs, Fisher's exact test p value = 0.0087; Fig. 3). This is consistent with previous studies on meQTLs [27, 30].

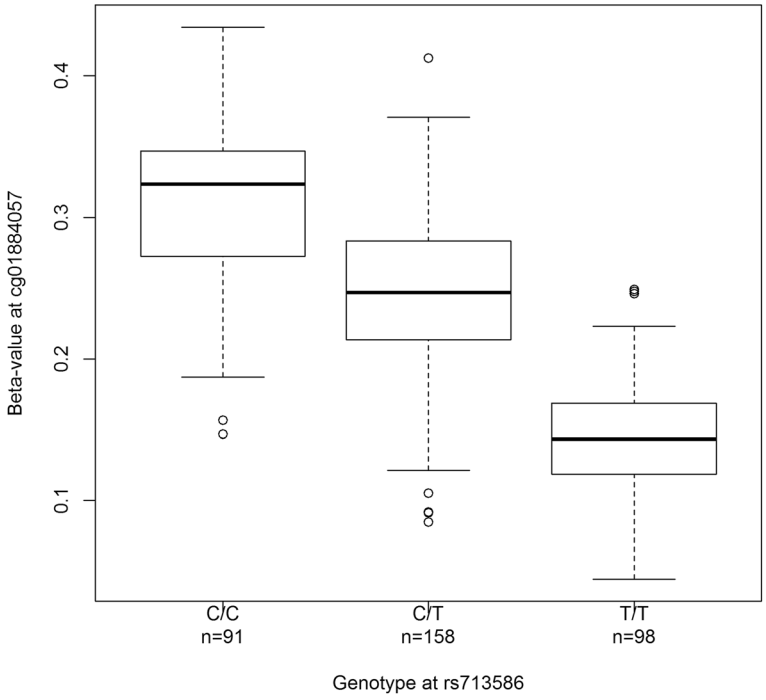


Fig. 2 Associations between genotypes at rs713586 and methylation at cg01884057. Distribution of methylation levels at cg01884057 is displayed for individuals carrying zero (C/C), one (T/C), or two (T/T) risk alleles at rs713586

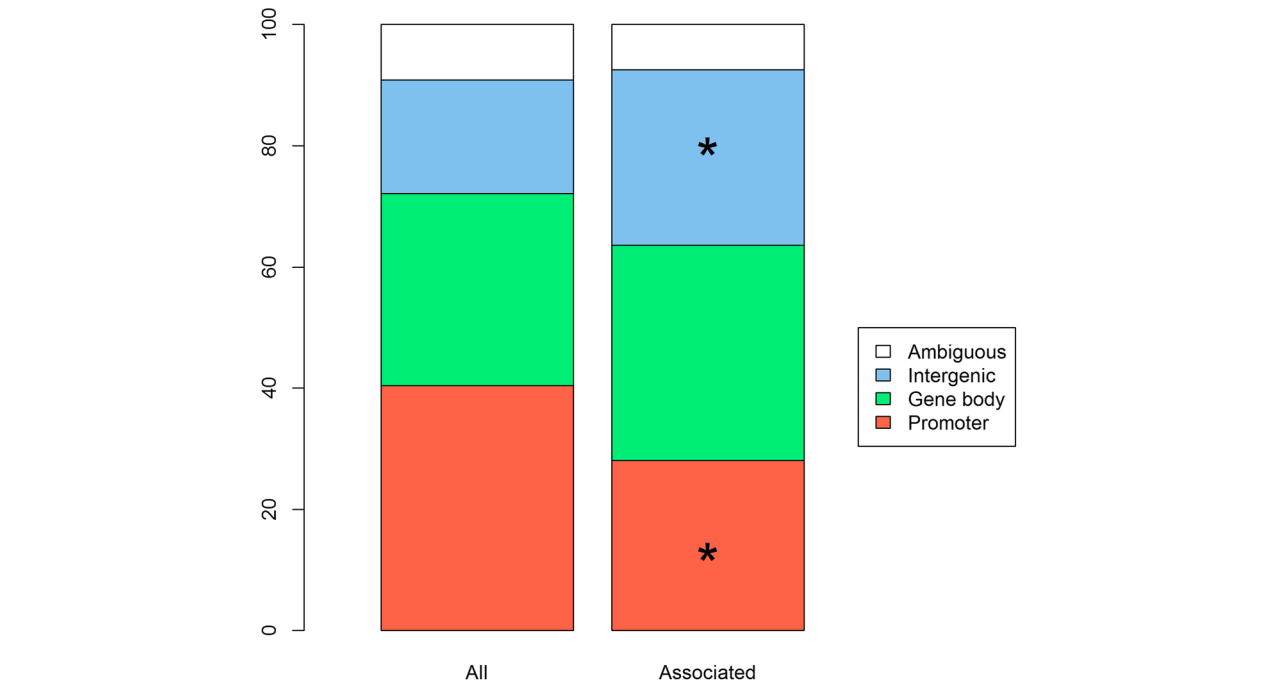


Fig. 3 Distribution of associated versus all tested CpGs in promoters, gene bodies, and intergenic regions. A CpG was classified as "promoter" if located within 1500 bp of the TSS of a gene, and as "ambiguous" if it was both in a promoter and within a gene body. Associated CpGs (*top*) were underrepresented in promoters, and overrepresented in intergenic regions (Fisher's exact test). *q value < 0.05

CpGs associated with obesity-associated SNPs are enriched in enhancers in PBMCs

The activity of functional genomic elements is associated with the state of the chromatin at these sites, such as histone modification patterns and access of transcription factors to DNA. The recently developed chromHMM tool allows interpreting chromatin states in a particular tissue or cell type by integrating histone marks and transcription factor binding data [60]. Using seven publicly available histone marks in 11 tissues relevant in the pathogenesis of obesity (AN, six brain regions, liver, PBMCs, PIs, and SM), we interpreted the chromatin states of all regions containing the tested CpGs (Additional file 6). Consistent with the enrichment of associated CpGs in intergenic regions (Fig. 3), associated CpGs were enriched in enhancers in PBMCs (Fisher's exact test, q value = 0.0019) (Fig. 4).

Only one CpG associated with obesity-associated SNPs is located in in vivo transcribed enhancers

The enrichment of associated CpGs in enhancers is a prediction by chromHMM that relies on histone marks, but we wanted to test whether associated CpGs were also found in active enhancers, as defined by cap-analysis of gene expression (CAGE) in the FANTOM5 project [61]. cg04588972, whose methylation was lower in carriers of the risk allele at rs1878047, was in a ubiquitous enhancer showing long-range interactions with the TSS of *KLK14*, *IGLON5*, *LRRC4B*, and *SYT3*. However, associated CpGs were not underrepresented in ubiquitous, tissue-specific, or cell-specific enhancers (Fisher's exact tests, all p values > 0.05). FANTOM5 uses very stringent criteria to detect active enhancers using whole transcriptome sequencing [61], thus possibly explaining why none of the associated CpGs were in active enhancers as defined by CAGE in the FANTOM5 project. Indeed, chromHMM predicted 33–266 times more active enhancers from the FANTOM5 project depending on the tissue, and there was little overlap between the two.

CpGs associated with obesity-associated SNPs show long-range interactions with promoters and other genomic regions

Following the enrichment of associated CpGs at enhancers, we mapped all tested CpGs to long-range interactions as defined by ChIA-PET libraries from five cell lines and three transcription factors (Additional file 3) [63]. Of the 107 associated CpGs, 103 (96 %) were located in regions with at least one long-range interaction with another genomic region, and 73 of the 107 associated CpGs (68 %) were located in regions with at least one long-range interaction with a gene promoter (Additional file 4). For instance, five CpGs negatively associated with rs2444217 and located in enhancers in brain, PBMCs, liver, PIs, and SM showed long-range interactions with the same five

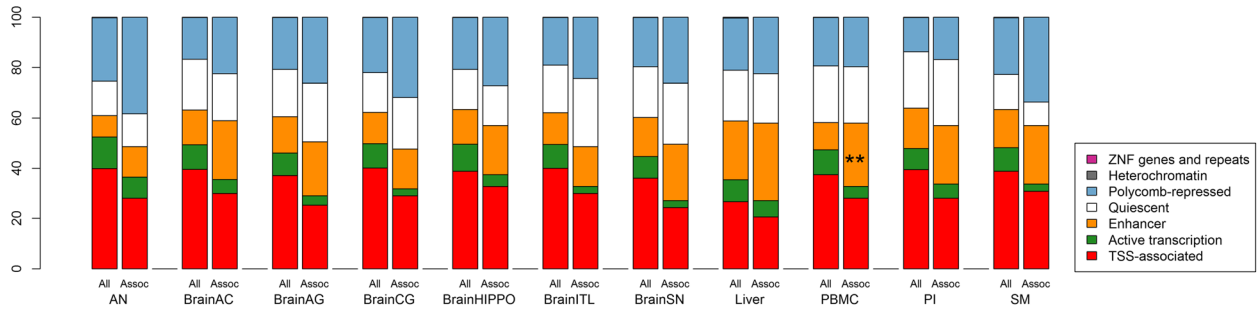


Fig. 4 Distribution of associated versus all tested CpGs in seven chromatin states in 11 tissues. Associated CpGs were overrepresented in enhancers in PBMCs (Fisher's exact test). **q value < 0.01

gene promoters (Additional file 4; Fig. 5). Also, 6 of the 15 CpGs associated with alleles at rs3934834 were found to interact with no less than 11 promoters, and were in enhancers in PIs (Additional files 4 and 6; Fig. 6). The graphic results for all significant SNPs can be found in Additional file 7. Associated CpGs were not enriched in long-range interactions across all five cell lines and three target transcription factors (Mann–Whitney U-test p value > 0.05; Fig. 7).

Associated CpGs are located in or show long-range interactions with the promoters of genes for which the corresponding SNPs are known eQTLs

We showed that some of the associated CpGs are located in gene promoters, and some are in regions

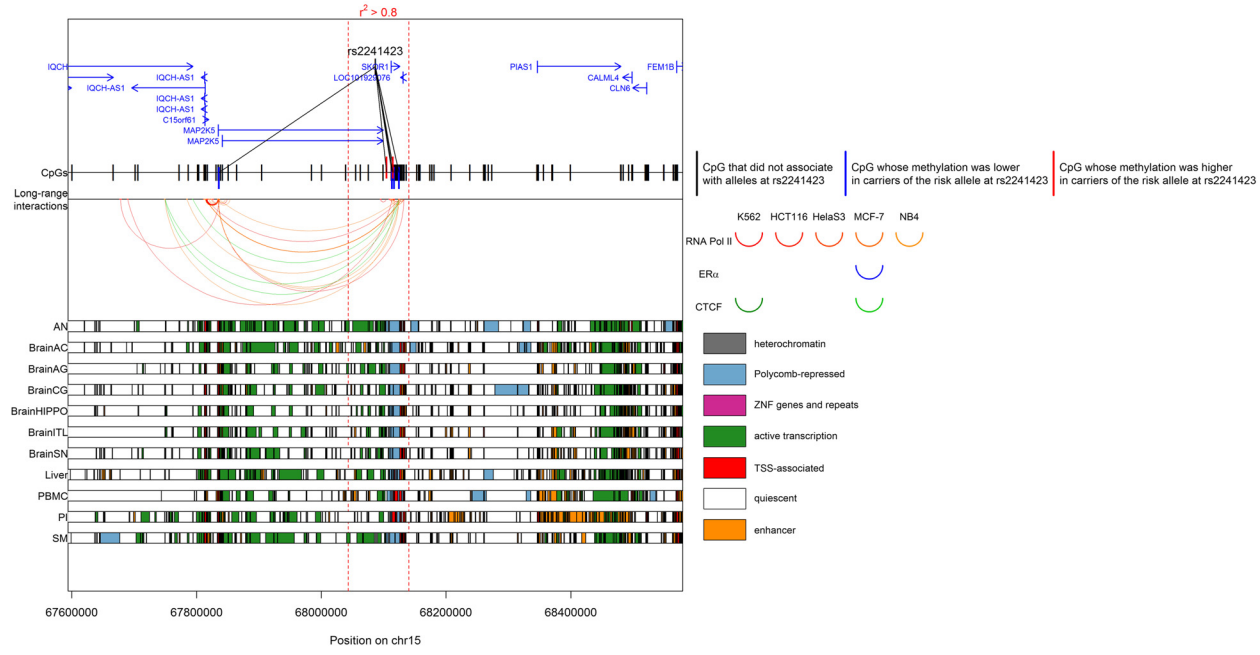


Fig. 5 Genomic context of the CpGs associated with rs2444217. Genomic positions of RefSeq genes and rs2444217 are displayed in the *top panel*. Within the two *vertical red dotted lines*, the LD $r^2 > 0.8$. The positions of the tested CpGs are displayed. Long-range interactions as defined by ChIA-PET libraries from five cell lines using chromatin immunoprecipitation with antibodies targeting three transcription factors (Additional file 5) are displayed as arcs. For clarity of visualization, we chose to display only the long-range interactions of genomic regions containing associated CpGs. Two interacting genomic regions are represented by an arc that links them, and the thickness of the arc line is proportional to the strength of this interaction. The color of the arc corresponds to the target transcription factor and the shade of the color corresponds to the cell line: *red* for RNA polymerase II, *blue* for ERα, and *green* for CTCF. In the *bottom panel*, chromatin states in 11 tissues are displayed. Chromatin states were obtained using chromHMM prediction using data on seven histone marks (see "Methods"). The color of each band corresponds to a particular state. *AN* adipose nuclei, *BrainAC* brain anterior caudate, *BrainAG* brain angular gyrus, *BrainCG* brain cingulate gyrus, *BrainHIPPO* brain hippocampus, *BrainITL* brain inferior temporal lobe, *BrainSN* brain substantia nigra, *PBMC* peripheral blood mononuclear primary cells, *PI* pancreatic islets, *SM* skeletal muscle

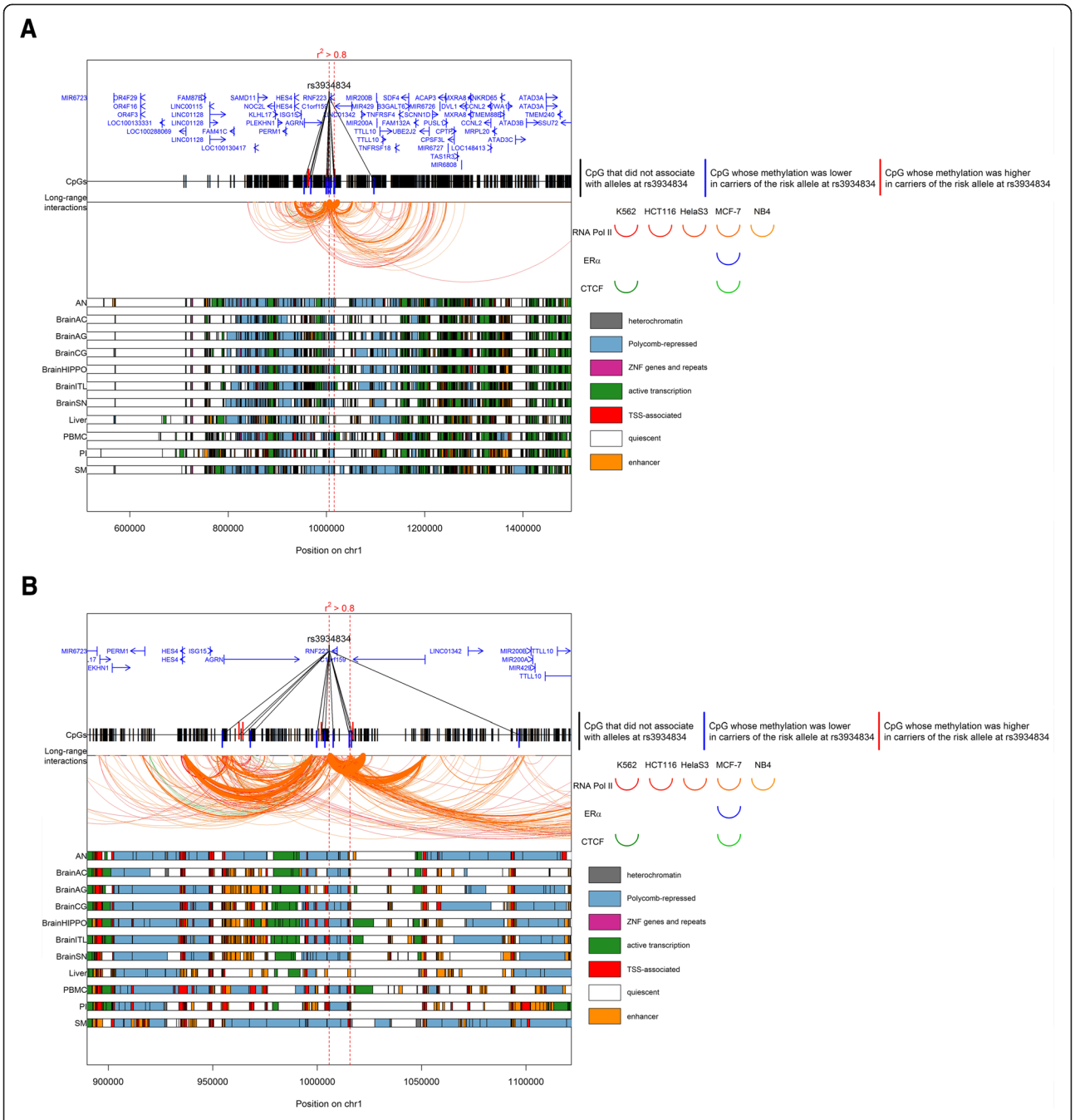


Fig. 6 Genomic context of the CpGs associated with rs3934834. **a** The entire investigated region. **b** Zoom on the region surrounding rs3934834. Genomic positions of RefSeq genes and rs3934834 are displayed in the *top panel*. Within the two *vertical red dotted lines*, the LD $r^2 > 0.8$. The positions of the tested CpGs are displayed. Long-range interactions as defined by ChIA-PET libraries from five cell lines using chromatin immunoprecipitation with antibodies targeting three transcription factors (Additional file 5) are displayed as arcs. For clarity of visualization, we chose to display only the long-range interactions of genomic regions containing associated CpGs. Two interacting genomic regions are represented by an arc that links them, and the thickness of the arc line is proportional to the strength of this interaction. The color of the arc corresponds to the target transcription factor and the shade of the color corresponds to the cell line: *red* for RNA polymerase II, *blue* for ERα, and *green* for CTCF. In the *bottom panel*, chromatin states in 11 tissues are displayed. Chromatin states were obtained using chromHMM prediction using data on seven histone marks (see "Methods"). The color of each band corresponds to a particular state. *AN* adipose nuclei, *BrainAC* brain anterior caudate, *BrainAG* brain angular gyrus, *BrainCG* brain cingulate gyrus, *BrainHIPPO* brain hippocampus, *BrainITL* brain inferior temporal lobe, *BrainSN* brain substantia nigra, *PBMC* peripheral blood mononuclear primary cells, *PI* pancreatic islets, *SM* skeletal muscle

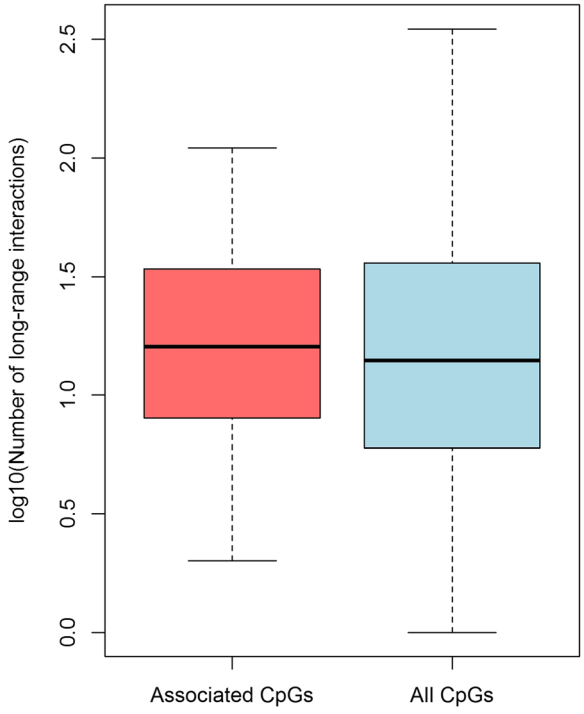


Fig. 7 Distribution of the number of long-range interactions for associated versus all tested CpGs. For each associated and tested CpG, we counted the number of genomic regions containing the CpG that interacted with another genomic region. For clarity and because the number of interactions was skewed, we chose to display the $\log_{10}$(number of interactions)

showing putative long-range interactions with gene promoters. In order to make the link between SNP, methylation, and mRNA expression, we searched four eQTL databases (see "Methods"); we browsed all associated SNPs and SNPs in strong LD with them (with $r^2 > 0.8$), and retrieved the genes for which they were eQTLs (Additional file 8). We found that associated CpGs are located in or show long-range interactions with the promoters of genes for which the corresponding SNPs are known eQTLs. For instance, rs10838738 is a known eQTL for several genes in blood, including *C1QTNF4*, *CELF1*, and *NUP160*. Interestingly, rs10838738 associated with three CpGs showing long-range interactions with *C1QTNF4* (Additional file 4), four CpGs showing long-range interactions with *CELF1* (Additional file 4), including three in enhancers in PBMCs (Additional file 6), and one CpG showing long-range interactions with *NUP160* (Additional file 4) that was in an enhancer in PBMCs (Additional file 4). Another example is rs713586, a known eQTL for *ADCY3* in blood and monocytes. rs713586 associated with a CpG located in the promoter of *ADCY3* (Additional file 4) that was also promoter-associated in PBMCs (Additional file 6).

Genome-scale measurements are validated by bisulfite sequencing

We validated one of the tested CpGs (cg15576492) by bisulfite sequencing, using DNA from 17 individuals from the discovery study group who were homozygous for rs3934834 (six *A*/*A* and 11 *G*/*G*). Our criteria for choosing this site were the following: 1) significant association with risk alleles; 2) strongest association with risk alleles; 3) located in a gene promoter and/or having long-range interactions with a gene promoter. The correlation between methylation assessed by Illumina and bisulfite sequencing was good (Pearson's correlation coefficient r = 0.68, p value = 0.0025; Additional file 9). Methylation in *A*/*A* was higher than methylation in *G*/*G*, but the methylation difference did not reach statistical significance (p value > 0.05), which could be explained by reduced statistical power (17 individuals).

SNP–CpG associations might not be blood-specific

Four of the initial SNP–CpG associations in blood are replicated in skin fibroblasts

The open-access dataset of skin fibroblasts consists of DNA methylation data assessed with the Illumina HumanMethylation450 BeadChip and genotype data assessed with the Illumina Human1M-Duov3 DNA Analysis BeadChip. Thus, we had data to test 65 of the 107 significant SNP–CpG associations in skin fibroblasts ($n = 62$). Fourteen SNP–CpG associations had a raw p value < 0.05, and seven had a q value < 0.05, including four having a concordant effect sign with results obtained in blood (Additional file 10). Notably, genotypes at rs1011731 associated with methylation at cg13446689 (regression coefficient = 0.254, q value = 0.012).

The single SNP–methylation association tested in SAT and VAT was not significant

The SAT and VAT study group of 149 individuals (mostly overweight/obese) was used to test specifically the association between genotypes at rs1011731 and methylation at cg13446689, which was assessed by bisulfite sequencing. We chose to test this SNP–CpG pair because there was an association between cg13446689 and rs1011731 in both blood and fibroblasts, and because this study group had already been genotyped for rs1011731. There was no association between methylation at cg13446689 in VAT or SAT and genotypes at rs1011731 (p value > 0.05; Additional file 10).

The two SNP–methylation associations tested in cerebellum, frontal cortex, caudal pons, and temporal cortex were not significant

The open-access dataset of four brain regions consists of DNA methylation assayed using the Illumina

HumanMethylation27 BeadChip, and genotype data assessed with the Illumina Human1M-Duov3 DNA Analysis BeadChip. Thus, we had data to test two of the 107 associated CpGs (cg05585544 and cg11385473). There was no association between genotypes at rs10838738 and methylation at cg05585544 in any of the four brain regions; there was no association between genotypes at rs652722 and methylation at cg11385473 in any of the four brain regions (p values > 0.05; Additional file 10).

Discussion

Our findings suggest that carriers of obesity-associated risk alleles display complex alterations of the gene regulatory landscape. We find that obesity-associated SNPs can be linked to DNA methylation levels in several proximal locations, which implies that they may affect multiple genes. These SNPs associated with proximal DNA methylation levels in whole blood of healthy individuals, but these associations might not be blood-specific. Interestingly, several obesity-associated SNPs associated with CpGs that were in the promoters of genes known to participate in the pathogenesis of obesity, or were located in regions that interact with such genes. In addition, associated CpGs were enriched in enhancers in blood, which highlights their potential in gene regulation.

It is well established that DNA methylation levels correlate with the presence of specific alleles at nearby SNPs [25–37], such as Grundberg et al. [29], who found that 28 % of CpGs were associated with SNPs within 100 kb in adipose tissue. If we restrict our analysis to 100 kb, we find 103 SNP–CpG associations at a q value < 0.05, corresponding to 27 unique SNPs (52 % of tested SNPs). It would be difficult to assess whether this percentage is particularly high, but the present study shows that obesity-associated SNPs discovered in GWASs may mediate their effect through alterations of the regulation of transcription. Indeed, the global results for each significant SNP display fascinating patterns. Several obesity-associated SNPs may affect "transcription factories", clusters of gene promoters and their enhancers that interact in three-dimensional space and are brought together by DNA-binding proteins such as CTCF [70]. The most striking example is rs7498665 since the 12 CpGs associated with this SNP are located in ten distinct gene promoters. rs3888190, one of the top loci of the most recent BMI GWAS [11], is in perfect LD with rs7498665 ($r^2 = 1$) and is known to be an eQTL for five of these ten gene promoters (*APOBR* [71], *SH2B1* [71], *SULT1A2* [72], *ATXN2L* [11], and *TUFM* [11]). Another interesting example is rs10838738, which associated with three CpGs showing long-range interactions with *C1QTNF4*, four CpGs showing long-range interactions with *CELF1*, and one CpG showing long-range interactions with *NUP160*. rs10838738 is a known eQTL for these three genes in blood [64, 66]. Thus, our results suggest that the effect of obesity-associated SNPs may be mediated by multiple and quite distant genes, as illustrated by three of our investigated SNPs (rs3934834, rs2287019, and rs7498665) that associated with CpGs interacting with no less than 15 promoters (Additional files 4 and 7). This underlines the importance for a rational and inclusive selection process for candidate genes for GWAS hits rather than the common practice of only focusing on the closest gene.

At a more detailed level, patterns of DNA methylation at specific CpGs between carriers and non-carriers of risk alleles were consistent with previous studies. Alleles at rs713586 explained 54 % of the variance in methylation at cg01884057, with an increase of almost 10 % methylation for each risk allele. The very same pattern was also found in adipose tissue in another study [29]. More interestingly, some patterns of DNA methylation between carriers and non-carriers of risk alleles was consistent with what is known about these genes and obesity. For instance, *MIR148A* is upregulated during normal adipogenesis but downregulated in obese adipocytes [73], and its expression is regulated by DNA methylation at its CpG island [74]. Consistently, carriers of the risk allele at rs1055144 had higher methylation levels in the promoter of *MIR148A*. Also, carriers of the risk allele at rs10838738 had lower methylation in the promoter of *PTPMT1*, a gene that codes for a mitochondrial phosphatase whose inhibition lowers glucose concentration [75] and a suggested drug target for treatment of type II diabetes [76]. Last but not least, three of the associated CpGs were located within two of the numerous promoters of *BDNF*, which encodes a neurotrophin that plays several roles in regulating energy homeostasis [77]. It is suggested that *BDNF* is finely regulated by DNA methylation and histone modifications [78, 79], and differential *BDNF* transcripts are expressed at different time points and in different cellular compartments [79]. Carriers of the risk allele at rs10767664 had higher methylation in the pII promoter of *BDNF*, and lower methylation in the pVI promoter of *BDNF*. However, the roles of specific *BDNF* promoters in obesity remain unexplored. Also, the SNPs may affect other genes linked to obesity: NR1H3, a member of the liver X receptors that regulate cholesterol catabolism [80] and expressed during adipose tissue remodeling [81]; PACSIN3, a kinase that induces glucose uptake by adipocytes [82]; LGR4, a G protein-coupled receptor whose ablation potentiates the white-to-brown fat transition [83]; POMC, a peptide that decreases food intake and increases energy expenditure [84]; CLN3 and ITH4, two proteins positively associated with obesity [85, 86]; and the developmental genes *HOTAIR* and *HOXC11*, responsible for differential fat accumulation between upper and lower body, and under epigenetic control [85, 87].

Our study aimed at unraveling the molecular effects of body mass-associated genetic variants on chromatin structure, with a special focus on DNA methylation. We benefited from a large sample size for the discovery analysis ($n = 355$) and from the use of a large battery of open access datasets to map associated CpGs to meaningful genomic annotations, such as promoters, predicted and in vivo transcribed enhancers, and long-range interactions. In addition, we tested the tissue-specificity of 65 of the initial associations in skin fibroblasts, two of the initial associations in four brain regions, and one in SAT and VAT. It is possible that some of the associations discovered in blood are limited to this tissue, or that unmeasured environmental factors such as smoking, diet, physical activity, and tissue-specific molecular factors impacted DNA methylation at the measured CpG sites and confounded our results. It should be noted, however, that we could not test all of the 107 initial associations in the replication samples, and the sample sizes of the replication samples were smaller than the discovery study group. Analysis of statistical power (probability of detecting a "true" effect when it exists) suggests that we have a high probability of replicating our results in the VAT and SAT replication samples, where power was 95 %. In contrast, power was only 23–25 % for cg11385473 and 42–47 % for cg05585544 for the brain replication samples and 39 % on average for the skin fibroblast replication samples, which implies that we are likely unable to replicate our results due to too small sample groups for these conditions. Besides, pan-tissue SNP–CpG associations are consistent with a genome-wide study where genotype-dependent methylation differences between blood and brain were associated, making genetic influence on DNA methylation in blood relevant for other tissues [26]. Finally, it should be kept in mind that the probes of the methylation array used in this study (Illumina 450 k) are enriched in CpG islands, gene promoters, and gene regions; it is thus possible that we missed important CpGs linked to obesity-associated SNPs.

In the paradigm of genetics–epigenetics–environment relationships, it is still unknown whether obesity-associated SNPs directly cause differential DNA methylation at genes and enhancers that contribute to the pathogenesis of obesity, or if the observed differential methylation levels are merely a consequence of a modified gene regulation caused by the presence of risk alleles at obesity-associated SNPs. In a recent review on the function and information content of DNA methylation, DNA methylation is thought to have both an active and passive role in gene regulation, and it seems to be highly contextual [88]. In particular, it has been proposed that mutations within regulatory regions affect binding of transcription factors, which in turn influence DNA methylation [88]. If DNA methylation does not necessarily actively impact on gene regulation, it is at least an informative marker of the underlying regulatory activity. Therefore, the differential methylation observed in carriers of risk alleles at obesity-associated SNPs in our study likely reflects allele-specific effects on gene regulatory mechanisms.

Conclusions

In this study we report strong associations between obesity-associated SNPs discovered in GWASs and methylation levels at proximal CpG sites. The methylation sites associated with alleles at obesity-associated SNPs were enriched in enhancers in PBMCs, and some of these sites were located in the promoters of genes, or were located in regions showing long-range interactions with established roles in appetite regulation as well as regulation of body mass. We also found indications that some of these genotype–methylation associations exist in different tissues. This study has implications for understanding how obesity-associated variants mediate their effects. Further studies are needed to unravel the mechanisms that govern the interplay between genetic variants and the activity of functional DNA elements.

Additional files

The following additional data are available with the online version of this paper. Additional file 1 is a is a table describing the 52 investigated SNPs. Additional file 2 is a heatmap showing the top CpG sites associated with blood cell type surrogates (principal components), evaluated in purified human leukocyte subtype methylation data sets. Additional file 3 is a table listing the cell lines and target transcription factors of the ChIA-PET libraries. Additional file 4 is a table describing the 107 associated CpGs found in blood and their annotation. Additional file 5 is a PCA showing the two discovery study samples on the first three principal components, using only the most variable autosomal CpG sites. Additional file 6 is a table showing the chromatin state at the genomic position of the 107 associated CpG, in 11 tissues. Additional file 7 is a figure showing the genomic context of the CpGs associated with the 28 significant SNPs. Additional file 8 is a table showing the eQTLs found in four eQTL databases for each of the 28 significant SNPs. Additional file 9 is a figure showing the correlations between Illumina 450 K and pyrosequence analysis of cg15576492. Additional file 10 is a table summarizing the replication of the 107 SNP-CpG associations found in blood.

Additional file 1: Description of the 52 investigated SNPs. SNPs in bold are SNPs for which significant associations with DNA methylation were found. *Number in parenthesis = number of individuals with missing genotypes. (DOCX 108 kb)

Additional file 2: Top CpG sites associated with blood cell type surrogates (principal components), evaluated in purified human leukocyte subtype methylation data sets. (TIFF 26367 kb)

Additional file 3: Cell lines and target transcription factors of the ChIA-PET libraries. #Cell types are described in ENCODE project [89]. *Target transcription factors and their corresponding antibodies are described in the ENCODE project [90]. (DOCX 11 kb)

Additional file 4: Description of the 107 associated CpGs found in blood and their annotation. *Coefficient of the linear model associated with the obesity-associated SNP: positive for increased methylation with presence of risk allele; coefficients are calculated using M values. (DOCX 38 kb)

Additional file 5: Principal component analysis of the two study groups ($n = 355$) on the first three principal components, using only the most variable autosomal CpG sites. *Red dots* are individuals from study sub-group 1 ($n = 130$), *black dots* are individuals from study sub-group 2 ($n = 225$). (TIFF 26367 kb)

Additional file 6: Chromatin states at the genomic position of the 107 CpGs, in the 11 investigated tissues. *AN* adipose nuclei, *BrainAC* brain anterior caudate, *BrainAG* brain angular gyrus, *BrainCG* brain cingulate gyrus, *BrainHIPPO* brain hippocampus, *BrainITL* brain inferior temporal lobe, *BrainSN* brain substantia nigra, *PBMC* peripheral blood mononuclear primary cells, *PI* pancreatic islets, *SM* skeletal muscle. (DOCX 36 kb)

Additional file 7: Genomic context of the CpGs associated with the significant SNPs. Each plot corresponds to a SNP for which associations with DNA methylation were found (28 plots in total). Genomic positions of RefSeq genes and the obesity-related SNP are displayed in the *top panel*. Within the two *vertical red dotted lines*, the linkage disequilibrium $r^2 > 0.8$. The positions of the tested CpGs are displayed. Long-range interactions as defined by ChIA-PET libraries from five cell lines using chromatin immunoprecipitation with antibodies targeting three transcription factors (Additional file 4) are displayed as arcs. For clarity of visualization, we chose to display only the long-range interactions of genomic regions containing associated CpGs. Two interacting genomic regions are represented by an arc that links them, and the thickness of the arc line is proportional to the strength of this interaction. The color of the arc corresponds to the target transcription factor and the shade of the color corresponds to the cell line: *red* for RNA polymerase II, *blue* for ERα, and *green* for CTCF. In the *bottom panel*, chromatin states in 11 tissues are displayed. Chromatin states were obtained using chromHMM prediction using data on seven histone marks (see "Methods"). The color of each band corresponds to a particular state. *AN* adipose nuclei, *BrainAC* brain anterior caudate, *BrainAG* brain angular gyrus, *BrainCG* brain cingulate gyrus, *BrainHIPPO* brain hippocampus, *BrainITL* brain inferior temporal lobe, *BrainSN* brain substantia nigra, *PBMC* peripheral blood mononuclear primary cells, *PI* pancreatic islets, *SM* skeletal muscle. (TIFF 9613 kb)

Additional file 8: eQTLs found in four eQTL databases for each of the significant 28 SNPs. [1]Investigated tissues: subcutaneous adipose tissue, aorta artery, tibial artery, esophagus mucosa, esophagus muscularis, heart left ventricle, lung, skeletal muscle, tibial nerve, sun-exposed skin, lower leg, stomach, thyroid, whole blood. [2]Investigated tissues/cell lines: lymphoblastoid cell line (LCL), liver, monocytes, fibroblasts, T cells, brain cortex. [3]Investigated tissues/cell lines: lymphoblastoid cell line (LCL), liver, brain cerebellum, brain frontal cortex, brain temporal cortex, brain pons. (DOCX 25 kb)

Additional file 9: Correlations between Illumina 450 K and pyrosequence analysis of cg15576492. Methylation at cg15576492, as determined by the Illumina 450 k Chip and expressed as β value, is plotted against methylation at cg15576492, as determined by pyrosequencing and expressed as β value ($n = 17$). (TIFF 12920 kb)

Additional file 10: Replication of the 107 SNP-CpG associations found in blood. *Coefficient of the linear model associated with the obesity-associated SNP: positive for increased methylation with presence of risk allele; coefficients are calculated using M values. Coefficients with a hash symbol correspond to associations with raw p value < 0.05 and coefficients in bold correspond to associations with q value < 0.05. (DOCX 31 kb)

Abbreviations

AN: adipose nuclei; BMI: body mass index; BrainAC: brain anterior caudate; BrainAG: brain angular gyrus; BrainCG: brain cingulate gyrus; BrainHIPPO: brain hippocampus; BrainITL: brain inferior temporal lobe; BrainSN: brain substantia nigra; CAGE: cap-analysis of gene expression; ChIA-PET: chromatin interaction analysis by paired-end tag sequencing; eQTL: expression quantitative trait locus; GEO: Gene Expression Omnibus; GWAS: genome-wide association study; LD: linkage disequilibrium; meQTL: methylation quantitative trait locus; PBMC: peripheral blood mononuclear primary cell; PCA: principal component analysis; PCR: polymerase chain reaction; PI: pancreatic islet; QTL: quantitative trait locus; SAT: subcutaneous adipose tissue; SM: skeletal muscle; SNP: single nucleotide polymorphism; TSS: transcription start site; UCSC: University of California, Santa Cruz; VAT: visceral adipose tissue.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SV participated in the design of the study, performed the statistical analysis and drafted the manuscript. MSA participated in the design of the study and helped to draft the manuscript. GYZ carried out the confirmative bisulfite sequencing analysis in blood. LL, SZ, SC, and FE recruited participants and collected blood samples. EN and JK carried out DNA preparation and contributed to genotyping. MRA participated in the design of the study. PK, MB, and YB carried out the genotyping and pyrosequencing in adipose tissue. HBS conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The methylation array was performed at the Genotyping Technology Platform, (http://www.genotyping.se), the sequencing was performed at the Uppsala Genome Center (http://www.igp.uu.se/facilities/genome_center), Uppsala, Sweden with support from Uppsala University and the Knut and Alice Wallenberg foundation and at the Uppsala Genome Centre. The studies were supported by the Swedish Research Council (VR, medicine) and the Novo Nordisk Foundation. M.R.A. is supported by the Swedish Brain Research foundation, the Fredrik O. Ingrid Thuring foundation and the Lars Hierta Memorial Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This project was supported by grants from the Boehringer Ingelheim Foundation to P.K., from the IFB AdiposityDiseases (ADI-K50D; ADI-K7-39 and ADI-K7-45 to Y.B. and ADI-K60E to P.K.). IFB AdiposityDiseases is supported by the Federal Ministry of Education and Research (BMBF), Germany, FKZ: 01EO1001. This work was further supported by grants from the DDG and DDS to Y.B. and from the Collaborative Research Center funded by the German Research Foundation (CRC 1052; B01 and B03 to M.B. and P.K., respectively) and by the Kompetenznetz Adipositas (Competence network for Obesity) funded by the German Federal Ministry of Education and Research (German Obesity Biomaterial Bank; FKZ 01GI1128).

Author details

[1]Department of Neuroscience, Functional Pharmacology, Uppsala University, Uppsala, Sweden. [2]Department of Medical Biochemistry and Microbiology, Uppsala University, SE-751 23 Uppsala, Sweden. [3]Medical Faculty, IFB Adiposity Diseases, University of Leipzig, Liebigstrasse 21, 04103 Leipzig, Germany. [4]Latvian Biomedical Research and Study Center, Ratsupites 1, Riga LV-1067, Latvia.

Received: 22 July 2015 Accepted: 21 September 2015
Published online: 08 October 2015

References

1. Willer CJ, Speliotes EK, Loos RJF, Li S, Lindgren CM, Heid IM, et al. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. Nat Genet. 2009;41:25–34.
2. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal eighteen new loci associated with body mass index. Nat Genet. 2011;42:937–48.

3. Okada Y, Kubo M, Ohmiya H, Takahashi A, Kumasaka N, Hosono N, et al. Common variants at CDKAL1 and KLF9 are associated with body mass index in east Asian populations. Nat Genet. 2012;44:302–6.
4. Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. Nat Genet. 2009;41:18–24.
5. Liu JZ, Medland SE, Wright MJ, Henders AK, Heath AC, Madden PAF, et al. Genome-wide association study of height and body mass index in Australian twin families. Twin Res Hum Genet. 2010;13:179–93.
6. Johansson A, Marroni F, Hayward C, Franklin CS, Kirichenko AV, Jonasson I, et al. Linkage and genome-wide association analysis of obesity-related phenotypes: association of weight with the MGAT1 gene. Obesity (Silver Spring). 2010;18:803–8.
7. Cotsapas C, Speliotes EK, Hatoum IJ, Greenawalt DM, Dobrin R, Lum PY, et al. Common body mass index-associated variants confer risk of extreme obesity. Hum Mol Genet. 2009;18:3502–7.
8. Wen W, Cho Y-S, Zheng W, Dorajoo R, Kato N, Qi L, et al. Meta-analysis identifies common variants associated with body mass index in east Asians. Nat Genet. 2012;44:307–11.
9. Ng MCY, Hester JM, Wing MR, Li J, Xu J, Hicks PJ, et al. Genome-wide association of BMI in African Americans. Obesity. 2012;20:622–7.
10. Field SF, Howson JMM, Walker NM, Dunger DB, Todd JA. Analysis of the obesity gene FTO in 14,803 type 1 diabetes cases and controls. Diabetologia. 2007;50:2218–20.
11. Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. 2015;518:197–206.
12. Loos RJF, Lindgren CM, Li S, Wheeler E, Zhao JH, Prokopenko I, et al. Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nat Genet. 2008;40:768–75.
13. Jamshidi Y, Snieder H, Ge D, Spector TD, O'Dell SD. The SH2B gene is associated with serum leptin and body fat in normal female twins. Obesity (Silver Spring). 2007;15:5–9.
14. Liu XG, Tan LJ, Lei SF, Liu YJ, Shen H, Wang L, et al. Genome-wide association and replication studies identified TRHR as an important gene for lean body mass. Am J Hum Genet. 2009;84:418–23.
15. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466:707–13.
16. Heard-Costa NL, Zillikens MC, Monda KL, Johansson A, Harris TB, Fu M, et al. NRXN3 is a novel locus for waist circumference: a genome-wide association study from the CHARGE Consortium. PLoS Genet. 2009;5, e1000539.
17. Shungin D, Winkler TW, Croteau-Chonka DC, Ferreira T, Locke AE, Magi R, et al. New genetic loci link adipose and insulin biology to body fat distribution. Nature. 2015;518:187–96.
18. Heid IIM, Jackson AUA, Randall J, Winkler T. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. Nat Genet. 2010;42:950.
19. Hinney A, Vogel CIG, Hebebrand J. From monogenic to polygenic obesity: Recent advances. Eur Child Adolesc Psychiatry. 2010;19:297–310.
20. Smemo S, Tena JJ, Kim KH, Gamazon ER, Sakabe NJ, Gomez-Marin C, et al. Obesity-associated variants within FTO form long-range functional connections with IRX3. Nature. 2014;507:371–5.
21. Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, et al. Genetic Loci associated with C-reactive protein levels and risk of coronary heart disease. JAMA. 2009;302:37–48.
22. Mansego ML, Milagro FI, Zulet MA, Martinez JA. SH2B1 CpG-SNP is associated with body weight reduction in obese subjects following a dietary restriction program. Ann Nutr Metab. 2014;66:1–9.
23. Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aïssi D, Wahl S, et al. DNA methylation and body-mass index: a genome-wide analysis. Lancet. 2014;6736:1–9.
24. Almén MS, Nilsson EK, Jacobsson JA, Kalnina I, Klovins J, Fredriksson R, et al. Genome-wide analysis reveals DNA methylation markers that vary with both age and obesity. Gene. 2014;548:61–7.
25. Quon G, Lippert C, Heckerman D, Listgarten J. Patterns of methylation heritability in a genome-wide analysis of four brain regions. Nucleic Acids Res. 2013;41:2095–104.
26. Davies MN, Volta M, Pidsley R, Lunnon K, Dixit A, Lovestone S, et al. Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. Genome Biol. 2012;13:R43.
27. Gibbs JR, van der Brug MP, Hernandez DG, Traynor BJ, Nalls MA, Lai SL, et al. Abundant quantitative trait loci exist for DNA methylation and gene expression in Human Brain. PLoS Genet. 2010;6:29.
28. Zhang D, Cheng L, Badner JA, Chen C, Chen Q, Luo W, et al. Genetic control of individual differences in gene-specific methylation in human brain. Am J Hum Genet. 2010;86:411–9.
29. Grundberg E, Meduri E, Sandling JK, Hedman AK, Keildson S, Buil A, et al. Global analysis of dna methylation variation in adipose tissue from twins reveals links to disease-associated variants in distal regulatory elements. Am J Hum Genet. 2013;93:876–90.
30. Drong AW, Nicholson G, Hedman AK, Meduri E, Grundberg E, Small KS, et al. The presence of methylation quantitative trait loci indicates a direct genetic influence on the level of DNA methylation in adipose tissue. PLoS One. 2013;8, e55923.
31. Smith AK, Kilaru V, Kocak M, Almli LM, Mercer KB, Ressler KJ, et al. Methylation quantitative trait loci (meQTLs) are consistently detected across ancestry, developmental stage, and tissue type. BMC Genomics. 2014;15:145.
32. Van Eijk K, de Jong S, Boks M, Langeveld T, Colas F, Veldink J, et al. Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects. BMC Genomics. 2012;13:636.
33. Shi J, Marconett CN, Duan J, Hyland PL, Li P, Wang Z, et al. Characterizing the genetic basis of methylome diversity in histologically normal human lung tissue. Nat Commun. 2014;5:3365.
34. Wagner JR, Busche S, Ge B, Kwan T, Pastinen T, Blanchette M. The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts. Genome Biol. 2014;15:R37.
35. Gutierrez-Arcelus M, Lappalainen T, Montgomery SB, Buil A, Ongen H, Yurovsky A, et al. Passive and active DNA methylation and the interplay with genetic variation in gene regulation. Elife. 2013;2, e00523.
36. Gertz J, Varley KE, Reddy TE, Bowling KM, Pauli F, Parker SL, et al. Analysis of DNA methylation in a three-generation family reveals widespread genetic influence on epigenetic regulation. PLoS Genet. 2011;7:e1002228.
37. Bell J, Pai A, Pickrell J, Gaffney D, Pique-Regi R, Degner J, et al. DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol. 2011;12:R10.
38. Gamazon ER, Badner JA, Cheng L, Zhang C, Zhang D, Cox NJ, et al. Enrichment of cis-regulatory gene expression SNPs and methylation quantitative trait loci among bipolar disorder susceptibility variants. Mol Psychiatry. 2012;18:340–6.
39. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000;320:1240–3.
40. SNP&SEQ Technology Platform. http://molmed.medsci.uu.se/SNP+SEQ+Technology+Platform/.
41. Gene Expression Omnibus. http://www.ncbi.nlm.nih.gov/geo/.
42. The R Project for Statistical Computing. https://www.r-project.org/.
43. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA Methylation microarrays. Bioinformatics. 2014;10:1363–9.
44. Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, et al. ChAMP: 450 k chip analysis methylation pipeline. Bioinformatics. 2014;30:428–30.
45. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The SVA package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics. 2012;28:882–3.
46. Van Iterson M, Tobi EW, Slieker RC, den Hollander W, Luijk R, Slagboom PE, et al. MethylAid: Visual and interactive quality control of large Illumina 450 k data sets. Bioinformatics. 2014;30:3435–7.
47. Triche TJ, Weisenberger DJ, Van Den Berg D, Laird PW, Siegmund KD. Low-level processing of Illumina Infinium DNA Methylation BeadArrays. Nucleic Acids Res. 2013;41:e90.
48. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics. 2013;29:189–96.
49. Lê S, Josse J, Husson F. FactoMineR: An R package for multivariate analysis. J Stat Softw. 2008;25:1–18.

50. Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, et al. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics. 2013;8:203–9.
51. Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol. 2013;31:142–7.
52. Koestler DC, Marsit CJ, Christensen BC, Accomando W, Langevin SM, Houseman EA, et al. Peripheral blood immune cell methylation profiles are associated with nonhematopoietic cancers. Cancer Epidemiol Biomarkers Prev. 2012;21:1293–302.
53. Accomando WP, Wiencke JK, Houseman EA, Butler RA, Zheng S, Nelson HH, et al. Decreased NK cells in patients with head and neck cancer determined in archival DNA. Clin Cancer Res. 2012;18:6147–54.
54. Lewin J, Schmitt AO, Adorján P, Hildmann T, Piepenbrock C. Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. Bioinformatics. 2004;20:3005–12.
55. Heberlein A, Muschler M, Frieling H, Behr M, Eberlein C, Wilhelm J, et al. Epigenetic down regulation of nerve growth factor during alcohol withdrawal. Addict Biol. 2013;18:508–10.
56. Domschke K, Tidow N, Schrempf M, Schwarte K, Klauke B, Reif A, et al. Epigenetic signature of panic disorder: A role of glutamate decarboxylase 1 (GAD1) DNA hypomethylation? Prog Neuropsychopharmacol Biol Psychiatry. 2013;46:189–96.
57. Schleinitz D, Klöting N, Lindgren CM, Breitfeld J, Dietrich A, Schön MR, et al. Fat depot-specific mRNA expression of novel loci associated with waist-hip ratio. Int J Obes (Lond). 2014;1:120–5.
58. Gibbs JR, van der Brug MP, Hernandez DG, Traynor BJ, Nalls MA, Lai SL, et al. Abundant quantitative trait Loci exist for DNA methylation and gene expression in human brain. PLoS Genet. 2010;6:e1000952.
59. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, De Bakker PIW. SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics. 2008;24:2938–9.
60. Ernst J, Kellis M. ChromHMM: automating chromatin-state discovery and characterization. Nat Methods. 2012;9:215–6.
61. Andersson R, Gebhard C, Miguel-Escalada I, Hoof I, Bornholdt J, Boyd M, et al. An atlas of active enhancers across human cell types and tissues. Nature. 2014;507:455–61.
62. Forrest ARR, Kawaji H, Rehli M, Baillie JK, de Hoon MJL, Lassmann T, et al. A promoter-level mammalian expression atlas. Nature. 2014;507:462–70.
63. Hillmer AM, Yao F, Inaki K, Lee WH, Ariyaratne PN, Teo ASM, et al. Comprehensive long-span paired-end-tag mapping reveals characteristic patterns of structural variations in epithelial cancer genomes. Genome Res. 2011;21:665–75.
64. Consortium TG. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013;45:580–5.
65. Veyrieras JB, Kudaravalli S, Kim SY, Dermitzakis ET, Gilad Y, Stephens M, et al. High-resolution mapping of expression-QTLs yields insight into human gene regulation. PLoS Genet. 2008;4:e1000214.
66. Westra HJ, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J, et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. Nat Genet. 2013;45:1238–43.
67. Du P, Zhang X, Huang C-C, Jafari N, Kibbe WA, Hou L, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics. 2010;11:587.
68. Zeileis A, Hothorn T. Diagnostic checking in regression relationships. R news. 2002;2:7–10.
69. storey j: qvalue: q-value estimation for false discovery rate control. 2015:r package version 2.0.0.https://www.bioconductor.org/packages/release/bioc/html/qvalue.html.
70. Ong C-T, Corces VG. CTCF: an architectural protein bridging genome topology and function. Nat Rev Genet. 2014;15:234–46.
71. Emilsson V, Thorleifsson G, Zhang B, Leonardson AS, Zink F, Zhu J, et al. Genetics of gene expression and its effect on disease. Nature. 2008;452:423–8.
72. Zhong H, Yang X, Kaplan LM, Molony C, Schadt EE. Integrating pathway analysis and genetics of gene expression for genome-wide association studies. Am J Hum Genet. 2010;86:581–91.
73. Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes. 2009;58:1050–7.
74. Long XR, He Y, Huang C, Li J. MicroRNA-148a is silenced by hypermethylation and interacts with DNA methyltransferase 1 in hepatocellular carcinogenesis. Int J Oncol. 2014;45:1915–22.
75. Nath AK, Ryu JH, Jin YN, Roberts LD, Dejam A, Gerszten RE, et al. PTPMT1 inhibition lowers glucose through succinate dehydrogenase phosphorylation. Cell Rep. 2015;10:694–701.
76. Boisclair Y, Tremblay ML. Firing up mitochondrial activities with PTPMT1. Mol Cell. 2005;19:291–2.
77. Marosi K, Mattson MP. BDNF mediates adaptive brain and body responses to energetic challenges. Trends Endocrinol Metab. 2014;25:89–98.
78. Martínez-Levy G, Cruz-Fuentes CS. Genetic and epigenetic regulation of the brain-derived neurotrophic factor in the central nervous system. Yale J Biol Med. 2014;87:173–86.
79. Karpova NN. Role of BDNF epigenetics in activity-dependent neuronal plasticity. Neuropharmacology. 2014;76:709–18.
80. Cruz-Garcia L, Schlegel A. Lxr-driven enterocyte lipid droplet formation delays transport of ingested lipids. J Lipid Res. 2014;55:1944–58.
81. Laurencikiene J, Rydén M. Liver X receptors and fat cell metabolism. Int J Obes (Lond). 2012;36:1494–502.
82. Roach W, Plomann M. PACSIN3 overexpression increases adipocyte glucose transport through GLUT1. Biochem Biophys Res Commun. 2007;355:745–50.
83. Wang J, Liu R, Wang F, Hong J, Li X, Chen M, et al. Ablation of LGR4 promotes energy expenditure by driving white-to-brown fat switch. Nat Cell Biol. 2013;15:1455–63.
84. Kim JD, Leyva S, Diano S. Hormonal regulation of the hypothalamic melanocortin system. Front Physiol. 2014;5:480.
85. Pinnick KE, Nicholson G, Manolopoulos KN, McQuaid SE, Valet P, Frayn KN, et al. Distinct developmental profile of lower-body adipose tissue defines resistance against obesity-associated metabolic complications. Diabetes. 2014;11:3785–97.
86. Choi J-W, Liu H, Choi DK, Oh TS, Mukherjee R, Yun JW. Profiling of gender-specific rat plasma proteins associated with susceptibility or resistance to diet-induced obesity. J Proteomics. 2012;4:1386–400.
87. Karpe F, Pinnick KE. Biology of upper-body and lower-body adipose tissue[mdash]link to whole-body phenotypes. Nat Rev Endocrinol. 2015;11:90–100.
88. Schubeler D. Function and information content of DNA methylation. Nature. 2015;517:321–6.
89. ENCODE target transcription factors and their corresponding antibodies.http://genome.ucsc.edu/ENCODE/antibodies.html.
90. http://genome.ucsc.edu/ENCODE/antibodies.html.

Paper II [redacted]

European Journal of Human Genetics (2015) 23, 654–662

www.nature.com/ejhg

EJHG Open

ARTICLE

Dietary fat quality impacts genome-wide DNA methylation patterns in a cross-sectional study of Greek preadolescents

Sarah Voisin*[,1,4], Markus S Almén[1,4], George Moschonis[2], George P Chrousos[3], Yannis Manios[2] and Helgi B Schiöth[1]

The type and the amount of dietary fat have a significant influence on the metabolic pathways involved in the development of obesity, metabolic syndrome, diabetes type 2 and cardiovascular diseases. However, it is unknown to what extent this modulation is achieved through DNA methylation. We assessed the effects of cholesterol intake, the proportion of energy intake derived from fat, the ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA), the ratio of monounsaturated fatty acids (MUFA) to SFA, and the ratio of MUFA + PUFA to SFA on genome-wide DNA methylation patterns in normal-weight and obese children. We determined the genome-wide methylation profile in the blood of 69 Greek preadolescents (~10 years old) as well as their dietary intake for two consecutive weekdays and one weekend day. The methylation levels of one CpG island shore and four sites were significantly correlated with total fat intake. The methylation levels of 2 islands, 11 island shores and 16 sites were significantly correlated with PUFA/SFA; of 9 islands, 26 island shores and 158 sites with MUFA/SFA; and of 10 islands, 40 island shores and 130 sites with (MUFA + PUFA)/SFA. We found significant gene enrichment in 34 pathways for PUFA/SFA, including the leptin pathway, and a significant enrichment in 5 pathways for (MUFA + PUFA)/SFA. Our results suggest that specific changes in DNA methylation may have an important role in the mechanisms involved in the physiological responses to different types of dietary fat.
European Journal of Human Genetics (2015) **23,** 654–662; doi:10.1038/ejhg.2014.139; published online 30 July 2014

INTRODUCTION

According to the World Health Organization[1], worldwide obesity has nearly doubled since 1980, resulting in an increase in cardiovascular diseases and diabetes type 2. One of the possible causes to this negative development is the increase of consumption of energy-dense foods that are high in fat. Dietary guidelines do not only recommend to eat a moderate amount of fat, but they also recommend to consume the right type of fat.[2] Fatty acids include saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA), and their structural differences explain why they have different biological effects.[3] Consuming PUFA or MUFA instead of SFA is known to improve the blood lipid profile.[4] Moreover, consumption of SFA in place of MUFA may worsen glucose-insulin homeostasis.[5] Finally, replacing SFA with PUFA has been reported to lower coronary heart disease risk.[6]

Some of the effects of the qualitative and quantitative aspects of fat intake have been imputed to a modification of the transcription of key genes involved in pathways related to lipid and glucose metabolism, and/or inflammation.[7] The regulation of gene expression can be achieved by mechanisms other than changes in the nucleotide sequence, namely epigenetic processes. Such processes are responsible for the establishment, maintenance, and reversal of metastable transcriptional states.[8] One major example of such processes is the methylation of cytosine, usually at CpG dinucleotides, called DNA methylation. Regions rich in CpGs are called 'CpG islands' and are mostly unmethylated when located in the promoter of active genes. Conversely, methylated promoters are associated with reduced gene expression.[9]

Five studies have investigated the link between DNA methylation and fat intake in humans, but the methylation assays in those studies were limited to only few key genes. One study found a significantly higher methylation in the peroxisome proliferator-activated receptor coactivator-1 gene (*PPARGC1A*) in high-fat overfed men.[10] Another study found that the clock circadian regulator gene (*CLOCK*) methylation was negatively associated with MUFA intake, but positively associated with PUFA intake.[11] A third study showed that higher n-6 PUFA intake was associated with lower methylation in the promoter of tumor necrosis factor-α (*TNFα*).[12] A fourth study found no significant correlation between a diet rich in fat and sucrose, and methylation of hydroxyacyl-coenzyme A dehydrogenase (*HADH*) and glucokinase (*GCK*) genes.[13] The fifth paper reported a lack of correlation between four diets enriched in different types of fat and the methylation levels of leptin (*LEP*), leptin receptor (*LEPR*), and pro-opiomelanocortin (*POMC*) genes.[14]

Here we explore the genome-wide DNA methylation profiles of Greek preadolescents with respect to parameters related to dietary fat quantity, and dietary fat quality. To our knowledge, this is the first time that parameters related to both quantitative and qualitative

[1]Department of Neuroscience, Functional Pharmacology, Uppsala University, Uppsala, Sweden; [2]Department of Nutrition and Dietetics, Harokopio University, Athens, Greece; [3]First Department of Pediatrics, Athens University Medical School, Aghia Sophia Children's Hospital, Athens, Greece
*Correspondence: S Voisin, Department of Neuroscience, Functional Pharmacology, Uppsala University, BMC, Box 593, Uppsala 751 24, Sweden. Tel: +46762122936; Fax: +4618511540; E-mail: sarah.voisin.aeris@gmail.com
[4]These authors contributed equally to this work.
Received 9 January 2014; revised 16 June 2014; accepted 20 June 2014; published online 30 July 2014

aspects of fat intake with respect to DNA methylation are investigated at a genome-wide scale. Moreover, no such studies have been performed in children.

MATERIALS AND METHODS

Genome-wide changes of DNA methylation pattern associated with parameters related to fat intake were assessed. Two variables related to dietary fat quantity (proportion of energy intake derived from fat, cholesterol intake) and three related to dietary fat quality (MUFA/SFA, PUFA/SFA and (MUFA + PUFA)/SFA) were analyzed. A linear model that explains the methylation level for each CpG site/island corrected for gender, weight category, Tanner stage (an estimation of physical development), and white blood cell count was utilized. The ratios between the unsaturated and saturated fatty acid intakes were preferred to their individual values, as they have been reported to have antagonistic effects. A higher fatty acids ratio would account for a 'healthier' fatty acid intake profile, while a lower ratio would account for an 'unhealthier' fatty acid intake profile.

Ethics

All participants and their guardians gave informed written consent and the study was approved by the Greek Ministry of National Education (7055/C7-Athens, 19-01-2007) and the Ethical Committee of Harokopio University (16/ Athens, 19-12-2006).

Subjects

The 'Healthy Growth Study' was a cross-sectional epidemiological study initiated in May 2007. Approval to conduct the study was granted by the Greek Ministry of National Education (7055/C7-Athens, 19-01-2007) and the Ethics Committee of Harokopio University of Athens (16/Athens, 19-12-2006). The study population comprised school children attending the fifth and sixth grades of primary schools located in the regions of Attica, Etoloakarnania, Thessaloniki and Heraklion. The sampling procedure is fully described elsewhere.[15] For the purpose of the current analysis, a subsample of 24 obese and 23 normal-weight preadolescent girls, as well as 11 obese and 11 normal-weight preadolescent boys (Table 1) was selected. This subsample was initially used to investigate the effect of polymorphism in the *FTO* gene on genome-wide DNA methylation patterns.[16]

Dietary assessment

Dietary intake data was obtained for two consecutive weekdays and one weekend day, via morning interviews with the children at the school site using the 24-h recall technique. More specifically, all study participants were asked to describe the type and amount of foods and beverages consumed during the previous day, provided that it was a usual day according to the participant's perception. To improve the accuracy of food descriptions, standard household measures (cups, tablespoons, etc) and food models were used to define amounts. At the end of each interview, the interviewers, who were dietitians rigorously trained to minimize interviewer's effect, reviewed the collected food intake data with the respondent to clarify entries, servings and possible forgotten foods. Food intake data were analyzed using the Nutritionist V diet analysis software (version 2.1, 1999, First Databank, San Bruno, CA, USA), which was extensively amended to include traditional Greek recipes, as described in Food Composition Tables of Greek Cooked Foods and Dishes. Furthermore, the database was updated with nutritional information of processed foods provided by independent research institutes, food companies and fast-food chains.

Table 1 Demographic data stratified for weight category and gender

Gender	*Normal-weight*	*Obese*	P-*value*[a]
Male			
N	11	11	
Age (years)	10.34 ± 0.25	10.82 ± 0.56	0.03
Height (*z*-score)[b]	−1.0 ± 0.20	0.44 ± 0.21	<0.001
Weight (*z*-score)[b]	−0.94 ± 0.093	1.5 ± 0.15	<0.001
BMI (*z*-score)[b]	−0.71 ± 0.11	1.6 ± 0.19	<0.001
White blood cell count (10^3/mm^3)	8.56 ± 3.85	6.93 ± 1.27	n.s.
Tanner stage[c] (*z*-score)[b]	−1.5 ± 0.27	−1.5 ± 0.32	n.s.
Total fat intake (% of total energy intake)	39.92 ± 8.96	45.82 ± 9.54	n.s.
MUFA intake (% of total energy intake)	19.00 ± 5.97	21.74 ± 5.91	n.s.
PUFA intake (% of total energy intake)	7.83 ± 7.99	4.77 ± 0.92	n.s.
SFA intake (% of total energy intake)	13.32 ± 3.62	16.29 ± 3.54	n.s.
Cholesterol (g/day)	188.90 ± 101.84	304.49 ± 137.54	n.s.
Female			
N	23	24	
Age (years)	10.54 ± 0.46	10.94 ± 0.71	0.05
Height (*z*-score)[b]	−0.76 ± 0.16	0.30 ± 0.24	0.001
Weight (*z*-score)[b]	−0.86 ± 0.088	1.5 ± 0.19	<0.001
BMI (*z*-score)[b]	−0.72 ± 0.079	1.8 ± 0.13	<0.001
White blood cell count (10^3/mm^3)	7.26 ± 2.07	7.28 ± 1.70	n.s.
Tanner stage[c] (*z*-score)[b]	−1.45 ± 0.14	−0.70 ± 0.21	n.s.
Total fat intake (% of total energy intake)	42.22 ± 6.59	38.42 ± 7.66	n.s.
MUFA intake (% of total energy intake)	18.83 ± 4.85	17.96 ± ± 5.15	n.s.
PUFA intake (% of total energy intake)	7.56 ± 11.42	4.30 ± 2.13	0.04
SFA intake (% of total energy intake)	15.55 ± 2.98	13.67 ± 4.28	0.04
Cholesterol (g/day)	216.25 ± 89.86	211.04 ± 125.58	n.s.

Abbreviations: BMI, body mass index; MUFA, monounsaturated fatty acid; n.s., nonsignificant; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.
[a]Indicates *P*-value for significant or n.s. differences between obese and normal-weight individuals. All values are means ± SEs.
[b]*z*-Scores were calculated using all samples from the Healthy Growth Study as a reference population.
[c]Describes pubertal development.

DNA methylation profiling

The genome-wide Illumina Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA, USA), which allows interrogation of 27 578 CpG dinucleotides covering 14 495 genes was applied to determine the methylation profile of genomic DNA isolated and purified from the peripheral whole blood. This chip has been shown to give a reliable and reproducible estimation of the methylation profile on a genomic scale.[17] First, bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Briefly, 500 ng of DNA was sodium bisulfite-treated, denatured at 98 °C for 10 min, and bisulfite converted at 64 °C for 2.5 h. After conversion, samples were desulfonated and purified using column preparation. Approximately 200 ng of bisulfate-converted DNA was processed according to the Illumina Infinium Methylation Assay protocol. This assay is based on the conversion of unmethylated cytosine (C) nucleotides into uracil/thymine (T) nucleotides by the bisulfite treatment. The DNA was whole-genome amplified, enzymatically fragmented, precipitated, resuspended, and hybridized overnight at 48 °C to locus-specific oligonucleotide primers on the BeadChip. After hybridization, the C or T nucleotides were detected by single-base primer extension. The fluorescence signals corresponding to the C or T nucleotides were measured from the BeadChips using the Illumina iScan scanner. Phenotypes, raw data and background-corrected normalized DNA methylation data are available through the GEO database (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE27860 for the girls and GSE57484 for the boys.

Data processing

All downstream data processing and statistical analyses were performed with the statistical software R (www.r-project.org) together with the *lumi*,[18] *limma*[19] and *IMA*[20] packages of the Bioconductor project.

Data preprocessing. The fluorescence data were preprocessed using the GenomeStudio 2009.2 (Illumina) software. We used the $\log_2$ ratio of the intensities of methylated probe *versus* unmethylated probe, also called *M*-value, which is more statistically valid for the differential analysis of methylation levels.[21]

Quality control. The data were imported and submitted to quality control using a modified version of the *IMA.methy450PP* function of the *IMA* package. The following CpG sites and samples were removed: the sites with missing β-values, the sites with detection *P*-value > 0.05, the sites having $< 75\%$ of samples with detection *P*-value $< 10^{-5}$, the samples with missing β-values, the samples with detection *P*-value $> 10^{-5}$ and the samples having $< 75\%$ of sites with detection *P*-value $< 10^{-5}$. A total of 26 168 probes were included in the analysis, after discarding 328 probes that did not reach the quality control together with 1082 probes from the sex chromosomes.

Normalization. Quantile normalization was performed on the *M*-values of all the 26 168 CpG sites using the *lumiMethyN* function of the *lumi* package.

Annotation. For better interpretation of the genome-wide methylation patterns, we chose to use the expanded annotation table for the Illumina Infinium HumanMethylation450 BeadChip array generated by Price *et al.*[22] There are a total of 27 578 loci for 27k array, and 1600 of them are not mapped to 450k array. For those unmapped loci, we kept their original annotation from the 27k array. The expanded annotation file was used to determine the average methylation value of CpG sites belonging to the same island or island shores (all sites with the same name in the 'HIL_CpG_Island_Name' column of the annotation file were averaged). We obtained the average methylation value of 5980 islands/island shores, which reduced the number of interrogated locations to 19 437 sites/islands. The CpG island classification developed by Price *et al*[22] provides good enrichment discrimination of CpG islands. This classification is a combination of Weber *et al*'s classification[23] where CpG islands are defined according to the GC content, the Obs/Exp CpG ratio and the island length, and Illumina's classification, where CpG islands are defined according to their physical position (islands, island shores, and shelves). The location within a CpG island or shore are suggested to be relevant,[24] and Price *et al*'s definition of CpG islands allowed to distinguish different methylation distribution between probes, which remained undetectable with the Illumina CpG island classification.[22] Besides, their classification demonstrated a more extreme DNA methylation profile and a larger proportion of differentially methylated regions between different tissues.

The expanded annotation file was also used to determine which gene each interrogated CpG site/island may be associated with ('Closest_TSS_gene_name' column of the annotation file), the distance of each interrogated CpG site/island to the closest TSS (transcription start site) ('Distance_closest_TSS' column of the annotation file) and the CpG density surrounding each interrogated CpG site/island ('HIL_CpG_class' column of the annotation file). Each site can either be located in a high-density CpG island, an intermediate-density CpG island, a region of intermediate-density CpG island that borders HCs, or a non-island. Indeed, the local CpG density has been shown to influence the role of methylated cytosines, with methylation having more transcriptional effect in high-density CpG island and less at non-islands.[25]

The Illumina-provided MAPINFO GenomeStudio column was used to determine the genomic location of each interrogated CpG site. For CpG islands, the name of the island was used to determine its genomic location (eg the island 'chr19_IC:17905037-17906698' would be a CpG island of intermediate density located on chromosome 19, between 17 905 037 and 17 906 698).

Statistics

Linear model. We developed the following linear model for each CpG site k, using *limma*'s robust regression method, with a maximum number of iteration equal to 10 000:

$$M_k = a_k + b_{kG}G + b_{kT}T + b_{kW}W + b_{kB}B + b_{kV}V + \varepsilon_k$$

where M_k is the *M*-value of CpG site/island *k*, *G* is the dichotomized gender (female = 1 and male = 0), *T* is the Tanner stage, *B* is the white blood cell count, *W* is the dichotomized weight category (normal-weight = 0 and obese = 1), ε_k is the unexplained variability, and *V* is one of the following variables: proportion of energy intake derived from total fat intake, cholesterol intake (g/day), MUFA/SFA, PUFA/SFA, or (MUFA + PUFA)/SFA.

The coefficients b_{kx} summarize the correlation between the methylation level and the variables of interest. Moderated *t*-statistics for each contrast and CpG site/island were created using an empirical Bayes model, to rank genes in order of evidence for differential methylation.[19] To control the proportion of false positives, *P*-values were adjusted for multiple comparisons as proposed by Benjamini and Hochberg (BH).[26] An adjusted *P*-value > 0.05 was considered nonsignificant.

Three children from the cohort had a MUFA/SFA, a PUFA/SFA, and a (MUFA + PUFA)/SFA higher than the mean $\pm 3 \times$ SD. Thus, they were excluded from the linear models developed for MUFA/SFA, PUFA/SFA, and (MUFA + PUFA)/SFA.

Functional enrichment analysis. The unique Entrez Gene ID associated with each significant gene-based site/island was identified. Three gene lists were generated for MUFA/SFA, PUFA/SFA, and (MUFA + PUFA)/SFA, respectively.

We used the web-based ConsensusPathDB-human (CPDB)[27,28] to determine the significant pathways each gene list may be involved in. On the basis of the reference gene set (all Entrez Gene IDs from the 27k BeadChip annotation file were used as a background), the expected number of genes in each pathway of the CPDB database is compared with the actual number of genes found for this pathway. For each pathway, a *P*-value and a *q*-value are calculated according to the hypergeometric test. The pathways with a raw *P*-value < 0.05 together with a *q*-value < 0.05 were selected. As CPDB includes information from 30 databases, the pathways often overlap with each other to some extent. Thus, to show the relationships between the different pathways, we constructed a heatmap of the proportion of shared input genes between the significant pathways. For instance, if P1 is a given pathway containing genes *A*, *B*, and *C* from the input gene list, and P2 is a given pathway containing genes *B*, *C*, *D*, and *E* from the input gene list, the

proportion of shared genes between P1 and P2 is:

$$\frac{|P1 \cap P2|}{|P1 \cup P2|} = \frac{2}{5} = 40\%$$

We also used the web-based g:Profiler[29,30] as an alternative method for pathway analysis, to confirm the significant results obtained with CPDB. The g:GOSt tool was used for enrichment analysis, with the same background gene list, and the g:GOSt native method g:SCS for multiple testing correction. The pathways with an adjusted *P*-value <0.05 were selected. It is important to note that g:Profiler only includes pathways from two databases: KEGG and Reactome.

RESULTS

Four CpG sites and one CpG island were found to be significantly associated with the proportion of overall fat intake (Figure 1a), while no significance was found for cholesterol intake. The methylation levels of 2 islands, 11 island shores, and 16 sites were significantly correlated with PUFA/SFA; 9 islands, 26 island shores, and 158 sites for MUFA/SFA; 10 islands, 40 island shores, and 130 sites for (MUFA + PUFA)/SFA (Figure 1b and Supplementary Tables 1–3).

What genes are associated with the significant CpG sites/islands?

To determine which gene may be regulated by each CpG site and island, we identified the gene whose TSS is closest to each CpG site and island. Each significant site, island or island shore can show either

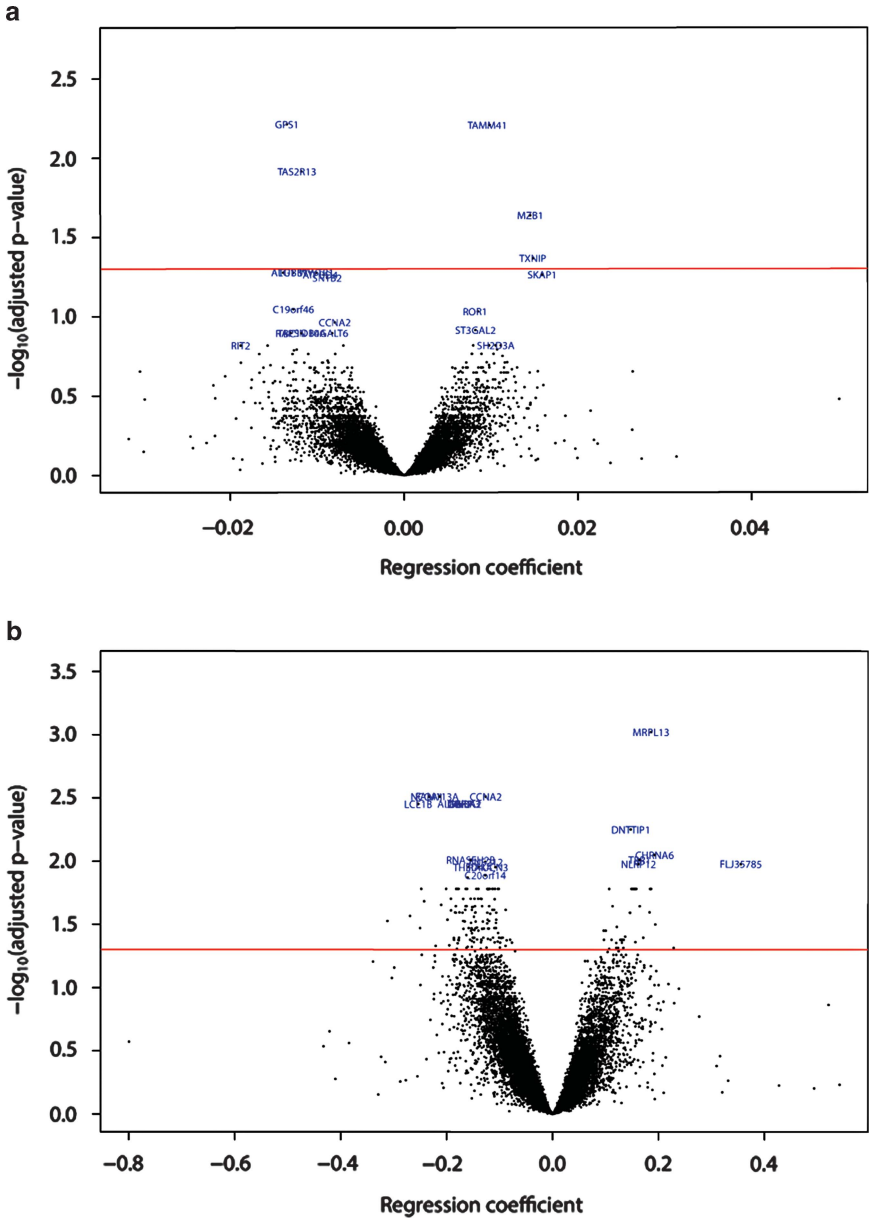


Figure 1 Volcano plots for proportion of total energy intake derived from fat (**a**) and (MUFA + PUFA)/SFA (**b**). The regression coefficient refers to the coefficient of the linear model and each point represents a CpG site or a CpG island. The red horizontal line is the significance threshold (*P*-value = 0.05) and all points above this line are significant. (**a**) proportion of total energy intake derived from fat (positive coefficients refer to an increased methylation in children for whom fat represents a higher proportion of total energy intake). (**b**) (MUFA + PUFA)/SFA (positive coefficients refer to an increased methylation in children having a higher (MUFA + PUFA)/SFA).

Table 2 Information on the significant CpG sites/island found for proportion of energy intake derived from fat and the top 10 most significant CpG sites/islands found for MUFA/SFA, PUFA/SFA, and (MUFA + PUFA)/SFA

Gene	Entrez Gene ID	Genomic location of the probe/island (hg19)	HIL class[a]	Genomic location of the closest TSS (hg19)	Coefficient[b]	Adjusted P-value[c]
Proportion of energy intake derived from fat						
GPS1	2873	chr17:80009807	HC	80 009 762	−0.0135	0.00612
TAMM41	132001	chr3_HCshore:11887600_11888782; chr3_ICshore:11887684_11888691	HC	11 888 351	0.00987	0.00621
TAS2R13	50838	chr12:11061985	LC	11 062 160	−0.0118	0.0121
MZB1	51237	chr5:138725350	LC	138 725 604	0.0145	0.023
TXNIP	10628	chr1:145438031	IC	145 438 461	0.0148	0.043
MUFA/SFA						
ALDH3A2	224	chr17:19552343	HC	19 552 063	−0.289	0.00097
MYLK3	91807	chr16:46782176	LC	46 782 220	−0.238	0.00363
LOC642852	257103	chr21:46716835	LC	46 707 966	−0.317	0.00364
TPPP2	122664	chr14:21498837	IC	21 498 344	−0.309	0.00364
RXFP2	122042	chr13:32313824	NA	32 313 679	−0.262	0.00364
TMEM80	283232	chr11_HCshore:694282_696564; chr11_ICshore:694282_697179	HC	695 615	−0.245	0.00364
SEMA3G	56920	chr3:52478874	HC	52 479 042	0.28	0.00388
VCAM1	7412	chr1:101185020	LC	101 185 195	−0.259	0.00482
KRT73	319101	chr12:53013281	LC	53 012 342	−0.245	0.00496
KRTCAP2	200185	chr1:155145737	HC	155 145 803	−0.301	0.0051
PUFA/SFA						
CBR1	873	chr21_HCshore:37441920_37443032; chr21_ICshore:37442016_37442892	HC	37 442 284	1.28	4.02e–06
RBCK1	10616	chr20:388351	HC	388 708	0.687	2.3e–05
ABHD16A	7920	chr6_HCshore:31670422_31671462; chr6_ICshore:31670279_31671902	HC	31 671 136	−0.302	7.18e–05
KRT23	25984	chr17:39095141	LC	39 093 835	−0.326	0.00536
PDE3A	5139	chr12_HCshore:20521268_20523183; chr12_ICshore:20520944_20523341	HC	20 522 178	−0.274	0.0066
NCOA1	8648	chr2:24806720	LC	24 807 344	−0.42	0.00722
PCED1A	64773	chr20:2822804	LC	2 821 796	−0.412	0.00914
MRPL13	27085	chr8:121457500	HC	121 457 646	0.308	0.0193
AKR7A2	54896	chr1_HCshore:19638013_19639253; chr1_ICshore:19637904_19639606;	HC	19 638 639	0.237	0.0193
FAM154A	158297	chr9_IC:19032509_19033364	IC	19 033 255	−0.357	0.0193
(MUFA + PUFA)/SFA						
MRPL13	27085	chr8:121457500	HC	121 457 646	0.186	0.000952
NCOA1	8648	chr2:24806720	LC	24 807 344	−0.233	0.00308
PCED1A	64773	chr20:2822804	LC	2821796	−0.213	0.00308
CCNA2	890	chr4_HCshore:122744257_122745486; chr4_ICshore:122744093_122745437	HC	122 745 087	−0.126	0.00308
LCE1B	353132	chr1:152783674	LC	152 784 446	−0.254	0.00352
ALDH3A2	224	chr17:19552343	HC	19 552 063	−0.176	0.00352
MYLK3	91807	chr16:46782176	LC	46 782 220	−0.166	0.00352
GBP7	388646	chr1:89641121	LC	89 641 722	−0.175	0.00352
DGKI	9162	chr7_HCshore:137530917_137532628; chr7_ICshore:137530976_137532560	HC	137 531 608	−0.178	0.00352
DNTTIP1	140686	chr20:44421526	LC	44 420 575	0.148	0.00561

Abbreviations: HC, high-density CpG island; IC, intermediate-density CpG island; LC, non-island; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.
[a]CpG density surrounding each interrogated CpG site/island.
[b]Value of the coefficient of the linear model associated with (MUFA + PUFA)/SFA.
[c]*P*-value calculated by moderated t-statistics and adjusted for multiple comparisons according to Benjamini and Hochberg.

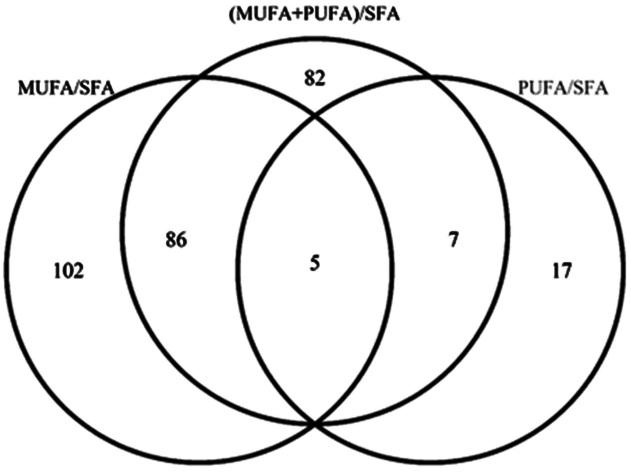


Figure 2 Venn diagram of the significant CpG sites and islands found for MUFA/SFA, PUFA/SFA, and (MUFA + PUFA)/SFA.

a positive fold change if its methylation is higher in children having an elevated dietary variable (eg, a higher cholesterol intake), or a negative fold change if its methylation is lower in children having an elevated dietary variable.

Regarding the proportion of fat intake, one CpG site associated with taste receptor, type 2, member 13 (*TAS2R13)* that may have a role in the perception of bitterness, while another site associated with thioredoxin interacting protein (*TXNIP)*, a regulator of cellular oxidative stress downregulated by SFA uptake[31] (Table 2).

The 10 most significant sites/islands/island shores found for MUFA/SFA included one CpG site associated with aldehyde dehydrogenase 3 family, member A2 (*ALDH3A2)* ($P = 0.00097$), whose expression is reduced in insulin-resistant murine models.[32] It also included a CpG site associated with sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G (*SEMA3G*) ($P = 0.0039$), whose expression increases during adipogenesis.[33] Among the top 10 found for PUFA/SFA, there was 1 CpG site associated with nuclear receptor coactivator 1 (*NCOA1)* ($P = 0.0072$) and another 1 associated with PC-esterase domain containing 1A (*PCED1A*) ($P = 0.0091$), as well as an island shore associated with phosphodiesterase 3A, cGMP-inhibited (*PDE3A*) ($P = 0.0066$; Table 2).

There were only 4 sites and 1 island shore found significant for all fatty acid ratios, but 86 sites/islands/island shores in common between MUFA/SFA and (MUFA + PUFA)/SFA, and 7 in common between PUFA/SFA and (MUFA + PUFA)/SFA (Figure 2). Notably, the four sites found significant for all fatty acid ratios contained some of previously mentioned sites (Table 2), for example, the ones associated with *NCOA1* ($P = 0.0031$) (Figure 3a) and *PCED1A* ($P = 0.0031$) (Figure 3b). It also included an island shore associated with *CCNA2* (Figure 3c), a gene recently shown to be associated with serum phosphatidylcholine concentration in mice.[34]

In which pathways are the significant genes involved?

Instead of going through all the genes associated with the significant sites found for MUFA/SFA, PUFA/SFA, and (MUFA + PUFA)/SFA, it was preferred to perform a gene enrichment analysis. Using CPDP,[27] we identified the significant pathways for each of the fatty acid ratios. We considered a pathway significant if the significant CpG sites/island/island shores were associated with a high proportion of genes involved in this particular pathway.

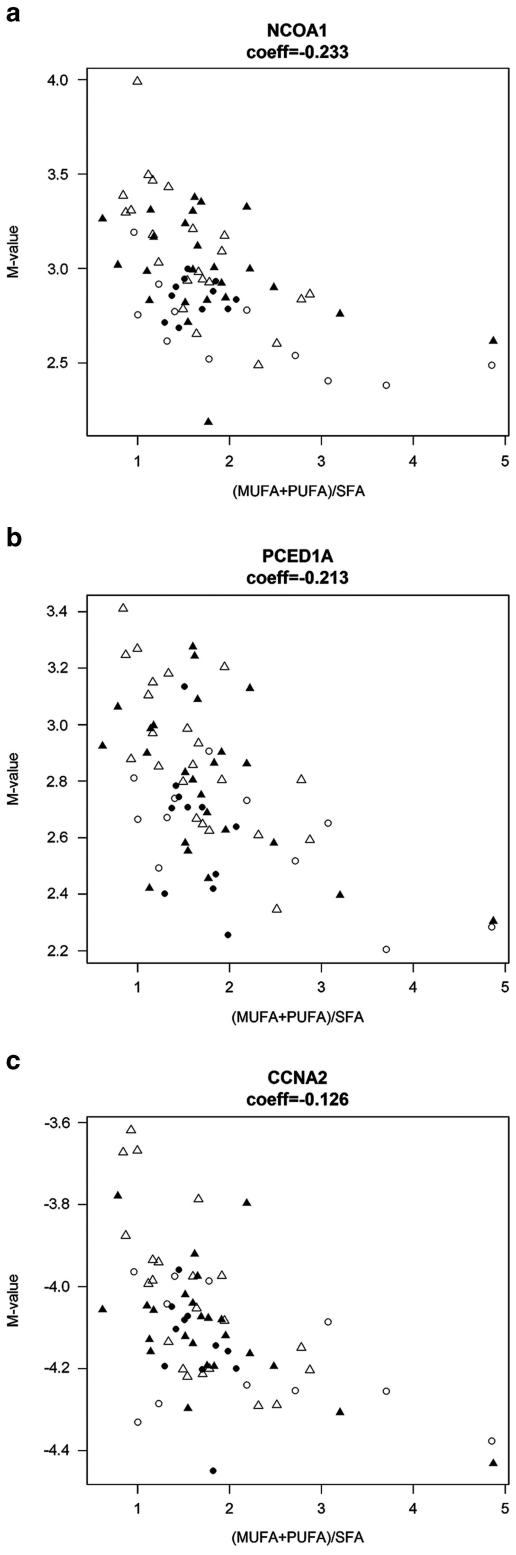


Figure 3 Correlation between methylation of three sites associated with NCOA1 (**a**), PCED1A (**b**), *CCNA2* (**c**), and (MUFA + PUFA)/SFA. Coeff (coefficient) of the linear model associated with (MUFA + PUFA)/SFA; full triangles, obese girls ($n = 23$); full circles, obese boys ($n = 11$); empty triangles, normal-weight girls ($n = 22$); empty circles, normal-weight boys ($n = 10$).

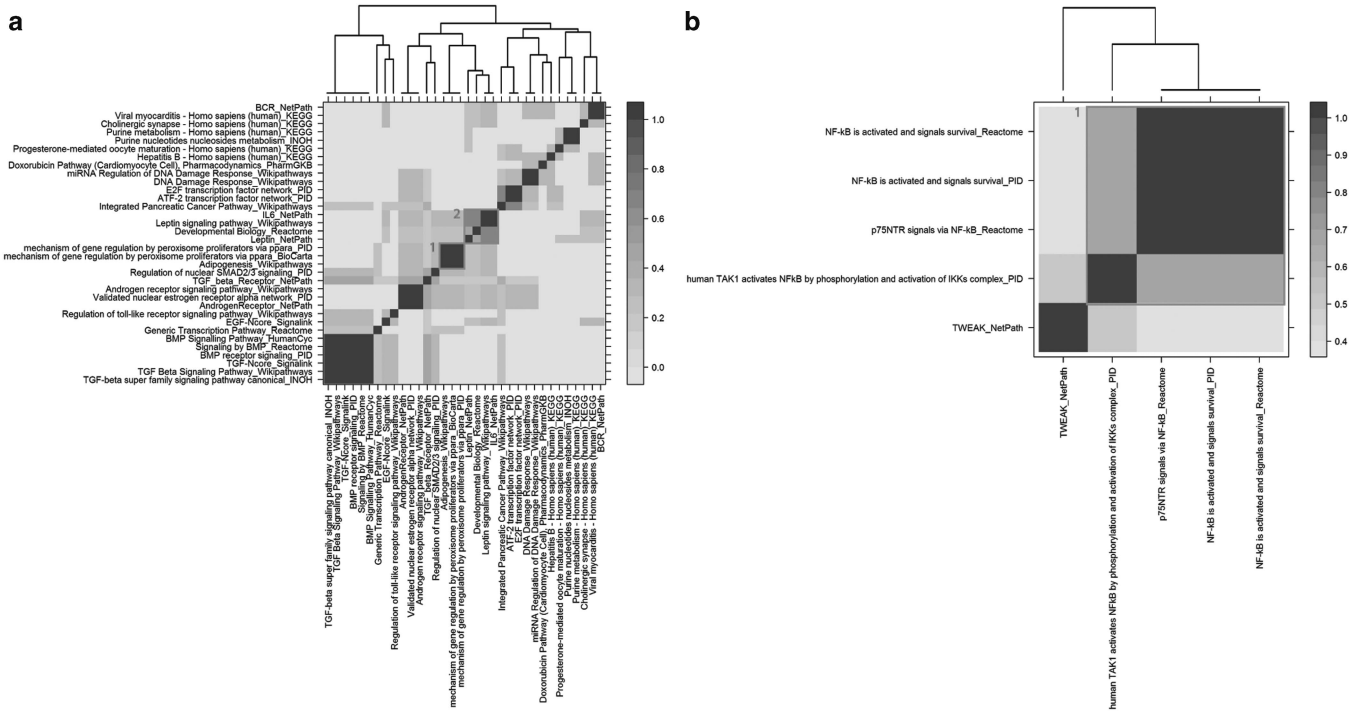


Figure 4 Heatmap representation of the proportion of shared genes between the significant pathways found for PUFA/SFA (**a**) and (MUFA + PUFA)/SFA (**b**). Each significant pathway retrieved from CPDB (P-value < 0.05 and q-value < 0.05) is represented on the graph, along with the database it comes from. A stronger color indicates a higher proportion of shared genes between two pathways. (**a**) Red rectangle 1: group of pathways related to adipogenesis and mechanism of gene regulation by peroxisome proliferators *via* PPARα; red rectangle 2: group of pathways related to leptin and IL6. (**b**) Red rectangle 1: group of pathways related to NF-κB. The full colour version of this figure is available at *European Journal of Human Genetics* online.

Neither CPDB nor g:Profiler identified significant pathways for MUFA/SFA, but CPDB found 34 significant pathways for PUFA/SFA (Supplementary Table 4), including 1 group of pathways related to adipogenesis and mechanism of gene regulation by peroxisome proliferators *via* PPARα (Group 1, Figure 4a), and another group of pathways related to leptin and IL6 (Group 2, Figure 4a). Five significant pathways were identified for (MUFA + PUFA)/SFA using CPDB (Supplementary Table 4), including one group of pathways linked to NF-κB (Group 1, Figure 4b). g:Profiler identified only one significant pathway for (MUFA + PUFA)/SFA, also linked to NF-κB (IKKβ phosphorylates IkB causing NF-κB to dissociate, P-value = 0.041).

DISCUSSION

In the present study of Greek preadolescents, we found a large number of CpG sites and regions significantly associated with variables related to the quality of fat intake and few sites significantly associated with variables related to the quantity of fat intake.

Our findings suggest that fat quality is likely to influence DNA methylation on a large genomic scale. *NCOA1*, one of the most significant gene found for all fatty acids ratios, is involved in the mechanism of gene regulation by peroxisome proliferators *via* PPARα, a master gene whose regulation is altered in obesity.[35] NCOA1 is a transcriptional coactivator whose ablation confers susceptibility to diet-induced obesity.[36] Interestingly, various fatty acids, but especially PUFAs, act as ligands for PPARα. Moreover, along with *PDE3A,* the fifth most significant gene found for PUFA/SFA, *NCOA1*, is part of the leptin pathway. Leptin is an adipokine that has a key role in regulating energy intake by inhibiting the sensation of hunger.[37] Fish oil has been reported to increase plasma leptin concentrations,[38] and leptin induces the expression of *NCOA1* in human cells.[39] Besides, *PDE3A*'s expression is enhanced in cows fed with a diet enriched in fish oil or in SFA.[40] Interestingly, an island shore located near the TSS of *PDE3A* was less methylated in children with a higher PUFA/SFA. All this information is consistent with the negative fold change observed for *NCOA1* in our cohort.

There was substantial overlap between the significant sites/islands/island shores found for the different fatty acid ratios, but little overlap between all fatty acid ratios. This may reflect how MUFA and PUFA affect DNA methylation in a different way. Interestingly, the site associated with *NCOA1* was more significant for (MUFA + PUFA)/SFA than for PUFA/SFA or MUFA/SFA, suggesting that PUFA and MUFA affect the methylation of this gene in an identical way. A similar observation can be made for *PCED1A* and *CCNA2* that were more significant for (MUFA + PUFA)/SFA than for PUFA/SFA or MUFA/SFA. However, this may also be due to differences in power to detect significant correlations, as the fatty acids ratios distributions were quite different (Supplementary Figure 1).

It should be noted that two of the four individual CpG sites found to be significantly associated with the proportion of energy intake derived from fat might be relevant to obesity. It has been hypothesized that individuals with increased bitter taste sensitivity avoid antioxidant-rich vegetables because of their perceived bitterness, consuming instead sweet, fatty foods.[41] The site associated with *TAS2R13* was more methylated in children for whom fat represents a higher proportion of the total energy intake. In addition, children with a higher proportion of energy intake derived from fat had a higher methylation at a site located in an island shore near *TXNIP*, which is consistent with the observed downregulation of *TXNIP* by SFA uptake.[31] None of these genes were previously reported to be

differentially methylated depending on fat intake, probably because the methylation assays of previous studies were limited in scope only addressing key genes.

The present work was not devoid of limitations. First of all, our sample size is limited ($n = 69$) and therefore replication is needed to confirm our findings and to allow generalization to larger populations. Second, the fatty acid ratios investigated herein are among the most interesting to compare with respect to health, as their roles are heavily debated and researched. However, other fatty acids not examined in this study may reflect other aspects of the quality of fat intake. For example, unsaturated fatty acids includes *trans* unsaturated fatty acids, which have been demonstrated to have adverse effects on health.[42] In addition, we did not separate n-3 and n-6 PUFA in our study, but these two fatty acids do not have the same effects; while both n-3 and n-6 PUFA have beneficial effects, an excess of n-6 PUFA can cause health disorders.[43] DNA methylation was assessed in whole peripheral blood, which is the case for most epigenetic studies focused on nutrition, as peripheral changes may occur in relation to overall energy balance.[44] However, the methylation pattern observed in blood may not always reflect the pattern in other tissues.[45] The other weakness of this approach is that DNA methylation can vary by blood cell type, and thus the methylation changes associated with the variables investigated in this study may represent an alteration in blood cell composition, rather than a change in methylation. However, no correlation was found between any of the investigated variables and the relative proportions of granulocytes, lymphocytes, or mid cells (P-value > 0.05 on Spearman's correlation test). Finally, an increasing number of human studies suggest that parental BMI impacts DNA methylation in the offspring, especially at imprinted genes.[46–48] However, evidences in humans are still scarce and limited to two available tissues at birth: umbilical cord and/or placenta; thus, we did not take parental BMI into account in our analysis.

In conclusion, this study is the first to demonstrate the roles of fat quantity and quality in DNA methylation patterns at a genome-wide scale. Our results suggest that specific changes in DNA methylation may have an important role in the mechanisms involved in the physiological responses to different types of dietary fat. Future studies could reveal other potential impacts of dietary fat quality on DNA methylation in controlled, randomized designs, and perhaps investigate further the downstream effects of this process.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The methylation array was performed at the Genotyping Technology Platform, Uppsala, Sweden (http://www.genotyping.se), with support from Uppsala University and the Knut and Alice Wallenberg foundation, and at the Uppsala Genome Centre. The studies were supported by the Swedish Research Council (VR, medicine), Ecole Normale Superieure de Lyon and the Novo Nordisk Foundation. We thank Miguel Xavier for his guidance, advice and support.

1 World Health Organization. Obesity and overweight. *Media Cent.* Fact sheet N 311 (2013). Available at: http://www.who.int/mediacentre/factsheets/fs311/en/.
2 UN Food and Agriculture Organization. *Fats and Fatty Acids in Human Nutrition.* Rome, Italy: Publishing Policy and Support Branch, Office of Knowledge Exchange, Research and Extension, FAO, 2010.
3 Tvrzicka E, Kremmyda L-S, Stankova B, Zak A: Fatty acids as biocompounds: their role in human metabolism, health and disease–a review. Part 1: classification, dietary sources and biological functions. *Biomed Pap Med Fac Univ Palacky Olomouc Czechoslov* 2011; **155**: 117–130.
4 Micha R, Mozaffarian D: Trans fatty acids: effects on metabolic syndrome, heart disease and diabetes. *Nat Rev Endocrinol* 2009; **5**: 335–344.
5 Vessby B, Uusitupa M, Hermansen K *et al*: Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia* 2001; **44**: 312–319.
6 Jakobsen MU, Reilly EJO, Heitmann BL *et al*: Major types of dietary fat and risk of coronary heart disease: a pooled analysis of 11 cohort studies 1–3. *Am J Clin Nutr* 2009; **89**: 1425–1432.
7 Kremmyda L-S, Tvrzicka E, Stankova B, Zak A: Fatty acids as biocompounds: their role in human metabolism, health and disease - a review. Part 2: Fatty acid physiological roles and applications in human health and disease. *Biomed Pap* 2011; **155**: 195–218.
8 Bonasio R, Tu S, Reinberg D: Molecular signals of epigenetic states. *Science (80-)* 2010; **330**: 612–616.
9 Hashimshony T, Zhang J, Keshet I, Bustin M, Cedar H: The role of DNA methylation in setting up chromatin structure during development. *Nat Genet* 2003; **34**: 187–192.
10 Brøns C, Jacobsen S, Nilsson E *et al*: Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. *J Clin Endocrinol Metab* 2010; **95**: 3048–3056.
11 Milagro FI, Gómez-Abellán P, Campión J *et al*: CLOCK, PER2 and BMAL1 DNA methylation: association with obesity and metabolic syndrome characteristics and monounsaturated fat intake. *Chronobiol Int* 2012; **29**: 1–15.
12 Hermsdorff HH, Mansego ML, Campión J *et al*: TNF-alpha promoter methylation in peripheral white blood cells: Relationship with circulating TNFα, truncal fat and n-6 PUFA intake in young women. *Cytokine* 2013; **64**: 265–271.
13 Lomba A, Milagro FI, García-Díaz DF *et al*: Obesity induced by a pair-fed high fat sucrose diet: methylation and expression pattern of genes related to energy homeostasis. *Lipids Health Dis* 2010; **9**: 60.
14 Fan C, Liu X, Shen W, Deckelbaum RJ, Qi K: The regulation of leptin, leptin receptor and pro-opiomelanocortin expression by n-3 PUFAs in diet-induced obese mice is not related to the methylation of their promoters. *Nutr Metab (Lond)* 2011; **8**: 31.
15 Moschonis G, Tanagra S, Vandorou A *et al*: Social, economic and demographic correlates of overweight and obesity in primary-school children: preliminary data from the Healthy Growth Study. *Public Health Nutr* 2010; **13**: 1693–1700.
16 Almén MS, Schiöth HB, Fredriksson R *et al*: Genome wide analysis reveals association of a FTO gene variant with epigenetic changes. *Genomics* 2012; **99**: 132–137.
17 Bibikova M, Le J, Barnes B *et al*: Genome-wide DNA methylation profiling using Infinium®® assay. *Epigenomics* 2009; **1**: 24.
18 Du P, Kibbe WA, Lin SM: lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 2008; **24**: 1547–1548.
19 Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article 3.
20 Wang D, Yan L, Hu Q *et al*: IMA: an R package for high-throughput analysis of Illumina's 450K Infinium methylation data. *Bioinformatics* 2012; **28**: 729–730.
21 Du P, Zhang X, Huang C *et al*: Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics* 2010; **11**: 587.
22 Price ME, Cotton AM, Lam LL *et al*: Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. *Epigenetics Chromatin* 2013; **6**: 4.
23 Weber M, Hellmann I, Stadler MB *et al*: Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 2007; **39**: 457–466.
24 Michels KB, Binder AM, Dedeurwaerder S *et al*: Recommendations for the design and analysis of epigenome-wide association studies. *Nat Methods* 2013; **10**: 949–955.
25 Hackett JA, Surani MA: DNA methylation dynamics during the mammalian life cycle. *Philos Trans R Soc B Biol Sci* 2013; **368**: 20110328.
26 Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B Methodol* 1995; **57**: 289–300.
27 Kamburov A, Wierling C, Lehrach H, Herwig R: ConsensusPathDB—a database for integrating human functional interaction networks. *Nucleic Acids Res* 2009; **37**: D623–D628.
28 Kamburov A, Stelzl U, Lehrach H, Herwig R: The ConsensusPathDB interaction database: 2013 update. *Nucleic Acids Res* 2013; **41**: D793–D800.
29 Reimand J, Arak T, Vilo J: g:Profiler–a web server for functional interpretation of gene lists (2011 update). *Nucleic Acids Res* 2011; **39**: W307–W315.
30 Reimand J, Kull M, Peterson H, Hansen J, Vilo J: g:Profiler–a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res* 2007; **35**: W193–W200.
31 Shaked M, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G: AMP-activated protein kinase (AMPK) mediates nutrient regulation of thioredoxin-interacting protein (TXNIP) in pancreatic beta-cells. *PLoS One* 2011; **6**: e28804.
32 Demozay D, Rocchi S, Mas J *et al*: Fatty aldehyde dehydrogenase: potential role in oxidative stress protection and regulation of its gene expression by insulin. *J Biol Chem* 2004; **279**: 6261–6270.
33 Ullah M, Stich S, Häupl T *et al*: Reverse differentiation as a gene filtering tool in genome expression profiling of adipogenesis for fat marker gene selection and their analysis. *PLoS One* 2013; **8**: e69754.

34 Schäfer N, Yu Z, Wagener A *et al*: Changes in metabolite profiles caused by genetically determined obesity in mice. *Metabolomics* 2014; **10**: 461–472.
35 Holloway GP, Bonen A, Spriet LL: Regulation of skeletal muscle mitochondrial fatty acid metabolism in lean and obese individuals. *Am J Clin Nutr* 2009; **89**: 455S–462S.
36 Picard F, Gehin M, Anicotte J *et al*: SRC-1 and TIF2 control energy balance between white and brown adipose tissues. *Cell* 2002; **111**: 931–941.
37 Brennan AM, Mantzoros CS: Drug Insight: the role of leptin in human physiology and pathophysiology[mdash]emerging clinical applications. *Nat Clin Pr End Met* 2006; **2**: 318–327.
38 Puglisi MJ, Hasty AH, Saraswathi V: The role of adipose tissue in mediating the beneficial effects of dietary fish oil. *J Nutr Biochem* 2011; **22**: 101–108.
39 Yin N, Wang D, Zhang H *et al*: Molecular mechanisms involved in the growth stimulation of breast cancer cells by leptin. *Cancer Res* 2004; **64**: 5870–5875.
40 Schmitt E, Ballou MA, Correa MN *et al*: Dietary lipid during the transition period to manipulate subcutaneous adipose tissue peroxisome proliferator-activated receptor-gamma co-regulator and target gene expression. *J Dairy Sci* 2011; **94**: 5913–5925.
41 Garcia-Bailo B, Toguri C, Eny KM, El-Sohemy A: Genetic variation in taste and its influence on food selection. *Omi J Integr Biol* 2009; **13**: 69–80.
42 White B: Dietary fatty acids. *Am Fam Phys* 2009; **80**: 345–350.
43 Lands B: Consequences of essential fatty acids. *Nutrients* 2012; **4**: 1338–1357.
44 Pjetri E, Schmidt U, Kas MJ, Campbell IC: Epigenetics and eating disorders. *Curr Opin Clin Nutr Metab Care* 2012; **15**: 330–335.
45 McKay JA, Xie L, Harris S *et al*: Blood as a surrogate marker for tissue-specific DNA methylation and changes due to folate depletion in post-partum female mice. *Mol Nutr Food Res* 2011; **55**: 1026–1035.
46 Soubry A, Murphy SK, Wang F *et al*: Newborns of obese parents have altered DNA methylation patterns at imprinted genes. *Int J Obes (Lond)* 2013; e-pub ahead of print 25 October 2013; doi:10.1038/ijo.2013.193.
47 Soubry A, Schildkraut JM, Murtha A *et al*: Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort. *BMC Med* 2013; **11**: 29.
48 Liu X, Chen Q, Tsai H, Wang G: Maternal preconception body mass index and offspring cord blood DNA methylation: exploration of early life origins of disease. *Environ Mol Mutagen* 2013; **55**: 223–230.

Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)

Paper III [redacted]

Acute Sleep Loss Induces Tissue-Specific Epigenetic and Transcriptional Alterations to Circadian Clock Genes in Men

Jonathan Cedernaes, Megan E. Osler, Sarah Voisin, Jan-Erik Broman, Heike Vogel, Suzanne L. Dickson, Juleen R. Zierath, Helgi B. Schiöth, and Christian Benedict

Department of Neuroscience (J.C., S.V., J.E.B., H.B.S., C.B.), Uppsala University, 751 24 Uppsala, Sweden; Department of Molecular Medicine and Surgery (M.E.O., J.R.Z.), Karolinska Institutet, 171 77 Stockholm, Sweden; Department of Experimental Diabetology (H.V.), German Institute of Human Nutrition Potsdam-Rehbruecke, 14558 Nuthetal, Germany; and Department of Physiology, Institute of Neuroscience and Physiology (H.V., S.L.D.), The Sahlgrenska Academy at the University of Gothenburg, 411 37 Gothenburg, Sweden

Context: Shift workers are at increased risk of metabolic morbidities. Clock genes are known to regulate metabolic processes in peripheral tissues, eg, glucose oxidation.

Objective: This study aimed to investigate how clock genes are affected at the epigenetic and transcriptional level in peripheral human tissues following acute total sleep deprivation (TSD), mimicking shift work with extended wakefulness.

Intervention: In a randomized, two-period, two-condition, crossover clinical study, 15 healthy men underwent two experimental sessions: x sleep (2230–0700 h) and overnight wakefulness. On the subsequent morning, serum cortisol was measured, followed by skeletal muscle and subcutaneous adipose tissue biopsies for DNA methylation and gene expression analyses of core clock genes (*BMAL1*, *CLOCK*, *CRY1*, *PER1*). Finally, baseline and 2-h post-oral glucose load plasma glucose concentrations were determined.

Main Outcome Measures: In adipose tissue, acute sleep deprivation vs sleep increased methylation in the promoter of *CRY1* (+4%; $P = .026$) and in two promoter-interacting enhancer regions of *PER1* (+15%; $P = .036$; +9%; $P = .026$). In skeletal muscle, TSD vs sleep decreased gene expression of *BMAL1* (−18%; $P = .033$) and *CRY1* (−22%; $P = .047$). Concentrations of serum cortisol, which can reset peripheral tissue clocks, were decreased (2449 ± 932 vs 3178 ± 723 nmol/L; $P = .039$), whereas postprandial plasma glucose concentrations were elevated after TSD (7.77 ± 1.63 vs 6.59 ± 1.32 mmol/L; $P = .011$).

Conclusions: Our findings demonstrate that a single night of wakefulness can alter the epigenetic and transcriptional profile of core circadian clock genes in key metabolic tissues. Tissue-specific clock alterations could explain why shift work may disrupt metabolic integrity as observed herein.
(*J Clin Endocrinol Metab* 100: E1255–E1261, 2015)

Animals studies have convincingly demonstrated that the circadian clock allows gene expression to coincide with anticipated metabolic requirements throughout day/night variations via *CLOCK* and *BMAL1* as positive transcriptional regulators and *PERIOD* and *CRYPTOCHROME* as negative transcriptional regulators (1). The

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received May 13, 2015. Accepted July 7, 2015.
First Published Online July 13, 2015

Abbreviations: OGTT, oral glucose tolerance test; TSD, total sleep deprivation.

lack of clock genes, even when ablated only in skeletal muscle or adipose tissue (2, 3), results in systemic metabolic perturbations in animal models (4). These metabolic responses include hyperglycemia and insulin resistance, and can also result in obesity and type 2 diabetes in animals (3–5). As reviewed in Cedernaes et al (6) and Schmid et al (7), similar metabolic phenotypes have been observed in humans subjected to experimental paradigms mimicking night shift work, comprising reduced energy expenditure, impaired systemic glucose disposal, and increased food intake. Over time, these conditions may thus result in metabolic dysregulation and weight gain (6, 7). Although shortened sleep leads to genome-wide changes in the leukocyte transcriptome comprising clock genes (8), the influence of overnight wakefulness, as occurs in night shift work, on the circadian machinery in tissues critically involved in whole-body energy homeostasis is, however, unknown. The importance of this research is highlighted by the fact that today, at least 15% of the workforce—numbering 15 million in the United States alone— carry out shift work, with job activities scheduled during the biological night.

With this background, we characterized the effects of one night of sleep deprivation on gene expression and DNA methylation of core circadian clock genes in peripheral tissues. DNA methylation of gene promoters and promoter-interacting enhancers is one epigenetic mechanism involved in the control of gene expression (9) and is a malleable process following acute lifestyle interventions (10). We obtained subcutaneous adipose tissue and skeletal muscle biopsies from fasted healthy young men following both acute sleep deprivation and normal sleep. In addition, fasting serum cortisol and plasma glucose were measured, the latter before and 120 minutes after an oral glucose tolerance test (OGTT).

Materials and Methods

Study design

This randomized crossover within-subject trial was conducted from March through September 2013 at Uppsala Biomedical Centre, Uppsala University, Sweden. The sessions were relatively evenly distributed across the study period. Study procedures and written consent forms were approved by the Regional Ethical Review Board in Uppsala (EPN 2012/477). The study was conducted in accordance with the Helsinki Declaration. Each enrolled participant voluntarily signed the consent form.

Participants

Sixteen of 17 enrolled subjects participated in two sessions of this study. Participants were of self-reported good health, free from chronic medical conditions or chronic medication, nonsmokers, and had normal sleeping habits (7–9 h of sleep/night; Pittsburgh Sleep Quality Index score ≤ 5) (extended screening protocol in Supplement Part 1).

Study protocol and interventions

All 16 participants engaged in two conditions (acute sleep deprivation vs sleep), in which each condition was separated by at least 4 weeks. Participants came in a semifasted state (fasted since 1500 h) to the laboratory two evenings before each session's final experimental morning, and remained in the laboratory under constant supervision until the end of the experimental session (ie, approximately a 42-h laboratory stay).

Participants were provided with breakfast, lunch, and dinner during their 24-hour baseline period (each meal providing one third of the participants' individually calculated energy requirements; based on the Harris-Benedict equation factored 1.2 for light physical activity), and had an 8.5-hour sleep opportunity during the first night (2230–0700 h). During the first baseline day, participants were provided with two standardized and supervised 15-minute walks. During nonexperimental time periods, participants were confined to their rooms but were free to engage in sedentary-level activities.

Randomization to the first experimental condition (sleep or acute sleep deprivation) was generated by drawing lots, with a fixed block size of 2 and allocation ratio of 1:1. Participants were randomly assigned after having been screened by J.C. as eligible, and were scheduled in pairs for the next available session slot. The allocation sequence was only known by one of the researchers (C.B.) but was concealed from the participants, with the experimenters only notified 2 weeks in advance of each new session for experimental preparation. Participants were blinded to the experimental condition (sleep or acute sleep deprivation) until 90 minutes in advance of onset of the nighttime intervention, which took place during the second night (2230–0700 h). During this period in the sleep condition, room lights were kept off and sleep was monitored. In contrast, in the sleep deprivation condition, participants were under constant supervision 2230–0700 h to ensure wakefulness, remaining bed-restricted and fasted.

Blood sampling, biopsy collection, and OGTT

After fasting, blood samples were obtained at 0730 h. Tissue biopsies were also obtained in the fasted state, 2–3 hours after subject wake-up time, with the collection of the adipose tissue preceding that of the skeletal muscle. Following the biopsy collection, participants completed a 75g OGTT (further details provided in the Supplement).

DNA extraction and epigenetic analyses

DNA extraction and epigenetic analysis with Illumina's HumanMethylation450 BeadChip are further described in Supplement Part 1.

DNA methylation preprocessing consisted of probe filtering (removal of probes with missing β-values; probes with less than 75% of samples with detection $P < .01$; or nonspecific or single nucleotide polymorphism–coinciding probes), followed by adjustment of type I and type II probes using BMIQ (11), and removal of batch effects using ComBat (12). We ran four pairs of technical replicates, including at least one from each experimental condition (sleep and acute sleep deprivation), to estimate the inner variability of each probe. We only considered for further analysis the probes for which at least half of the subjects showed

a methylation difference between conditions greater than the mean difference in technical replicates.

CpG sites within 1500 bp of the transcription start site of *CLOCK*, *ARNTL*, *CRY1*, and *PER1* were analyzed (15 CpG sites for adipose tissue and 9 nine for skeletal muscle). The promoter is a key part of a gene, but enhancers also prominently contribute to the regulation of gene expression (13). To identify putative enhancers of *CLOCK*, *ARNTL*, *CRY1*, and *PER1*, we inferred chromatin states in adipose nuclei and skeletal muscle, and mapped long-range interactions in five different cells lines, with three different transcription factors (14). CpG sites located in chromatin states indicative of enhancers in adipose nuclei and skeletal muscle and in regions having long-range interactions with the promoters of *CLOCK*, *ARNTL*, *CRY1*, and *PER1*, were also analyzed (six CpG sites for adipose tissue and four for skeletal muscle).

Methylation levels are presented as β-values (ranging from zero to one, corresponding to zero and 100% methylation, respectively). *P*-values were adjusted for multiple testing according to the Benjamini-Hochberg method within each tissue (15).

RNA extraction and qPCR analysis of gene expression

Methods used for RNA extraction and qPCR analysis of gene expression are described in further detail in **Supplement Part 1.** The gene expression of *CLOCK*, *ARNTL*, *CRY1*, and *PER1* was analyzed with qPCR in adipose tissue and skeletal muscle. All analyses were run in duplicates (primer information in **Supplement Part 2**). The ΔCt method was used to normalize data (16).

Statistics

Normal-distribution criteria of analyzed data were assessed with Kolmogorov-Smirnov's test of normality. Normally distributed data was analyzed with paired Student *t* tests, whereas nonnormally distributed variables were analyzed with Wilcoxon signed-rank test. Methylation data was analyzed using the software package R (version 3.1); we used the $\log_2$ ratio of the intensities of methylated probe vs unmethylated probe, also called M-value, which is more statistically valid for the differential analysis of methylation levels (17). All other data was analyzed using the software SPSS (version 21; SPSS Inc.) and are presented as means $\pm$ SD. Two-sided $P < .05$ were considered significant. For the adipose tissue, one individual was excluded for all gene expression analyses (expression values greater than mean + 2 SD for several genes). The significance values were, however, not changed when the analysis was run with or without this subject (data not shown). For *PER1* in skeletal muscle, an outlier was excluded from both conditions (expression values in the sleep deprivation condition greater than mean + 2 SD), but significance values were not altered when the analysis was run with or without this subject (data not shown).

Results

Of 17 enrolled subjects, 16 completed participation in both sessions (sleep and acute sleep deprivation). One participant was excluded from later analysis due to insufficient sleep (< 7 h) in the sleep condition. Fifteen participants were therefore included in the final analysis (age, 22.3 $\pm$ 1.9 y; body mass index, 22.6 $\pm$ 1.8 kg/m^2). Sleep data are presented in Supplement Part 3.

Effect of acute sleep deprivation on methylation and expression of circadian genes in adipose tissue and skeletal muscle

Methylation levels at cg04674060 (+15%; adjusted $P = .036$) and cg19308989 (+9%; adjusted $P = .026$; both CpG sites located in enhancers interacting with the promoter of *PER1*), and at cg20193872 (located in the promoter of *CRY1*; +4%; adjusted $P = .026$), increased after acute sleep deprivation, compared with the sleep condition, in adipose tissue (shown in Figure 1). In skeletal muscle, the investigated CpG sites were not altered (detailed probe results in Supplement Part 4).

In skeletal muscle, mRNA expression of *BMAL1* and *CRY1* was decreased following acute sleep deprivation (−18 and −22% compared with expression levels found after sleep; $P = .033$ and $P = .047$, respectively; see Figure 2 and Supplement Part 4). Skeletal muscle *CLOCK* or *PER1* gene expression was unaltered. Moreover, the adipose tissue genes were unaltered following acute sleep deprivation.

Effect of acute sleep deprivation on fasting cortisol and glucose tolerance

Following acute sleep deprivation, fasting serum cortisol concentrations were decreased at 0730 h (2449 $\pm$ 932 vs 3178 $\pm$ 723 nmol/L; $P = .039$), compared with after sleep. Plasma glucose concentrations at 120 minutes post-OGTT were higher following acute sleep deprivation, compared with the values obtained after sleep (pre-OGTT: 5.36 $\pm$ 0.30 vs 5.38 $\pm$ 0.36 mmol/l; $P = .705$; post-OGTT: 7.77 $\pm$ 1.63 vs 6.59 $\pm$ 1.32 mmol/l; $P = .011$).

Discussion

We determined the effect of one night of wakefulness, as occurs during night shift work, on DNA methylation and mRNA expression of key circadian genes (ie, *BMAL1*, *CLOCK*, *CRY1*, and *PER1*) in human skeletal muscle and adipose tissue. We provide evidence that acute sleep deprivation increases promoter methylation and reduces transcription of circadian genes in a tissue-specific manner. Our analysis reveals increased methylation of transcription-regulating regions of *PER1* and *CRY1* in adipose tissue and reduced gene expression of *CRY1* and *BMAL1* in skeletal muscle. We also observed an impaired glucose response following an OGTT after acute sleep deprivation. Our results suggest that acute sleep loss alters clock gene regulation, concomitant with deleterious met-

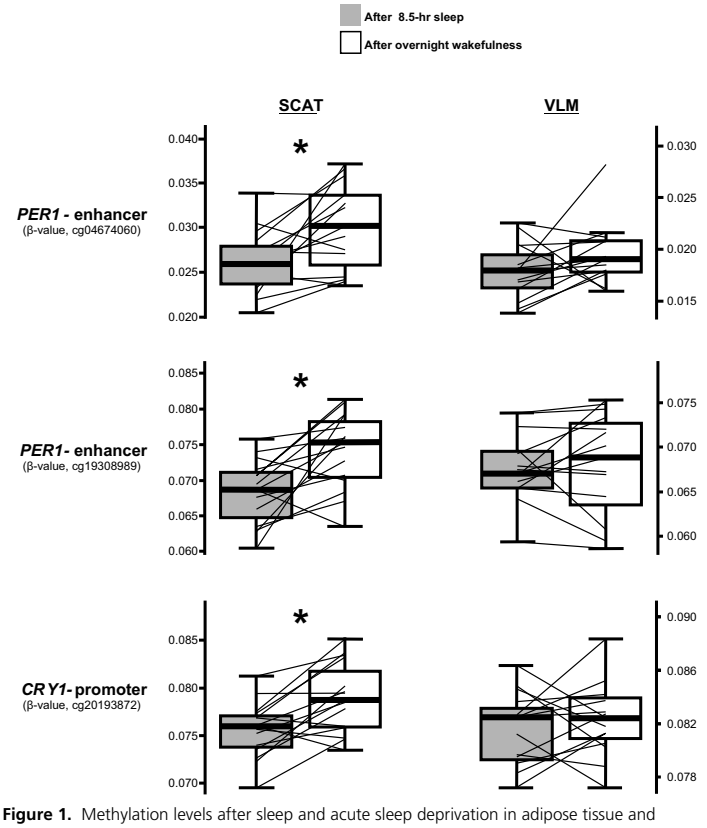


Figure 1. Methylation levels after sleep and acute sleep deprivation in adipose tissue and skeletal muscle. Methylation levels in two putative enhancers interacting with the promoter of *PER1* (probes cg04674060 and cg19308989) were increased following overnight wakefulness (ie, acute sleep deprivation), in adipose tissue (SCAT) compared with after sleep. Methylation levels in the promoter of *CRY1* (probe cg20193872) were also increased following overnight wakefulness (acute sleep deprivation) compared with after sleep, in adipose tissue (SCAT). No differences were seen, however, between the two conditions in skeletal muscle (VLM). Methylation levels are shown as beta values (β-value; ranging from zero to one, corresponding to zero and 100% methylation, respectively). Horizontal line represents median, box interquartile range, whiskers represent spread of remaining values. Two points that are linked by a line show the difference in methylation levels in overnight wakefulness vs sleep conditions for each individual. *, $P < .05$; n = 15 for all analyses. Abbreviations: SCAT, sc adipose tissue; VLM, vastus lateralis muscle.

abolic effects, which are differential, rather than uniform across key peripheral metabolic tissues in healthy humans.

Our results of altered DNA methylation for promoter and promoter-interacting enhancer regions of core clock genes in adipose tissue suggest that acute sleep deprivation can cause acute epigenetic remodeling of the circadian clock. Similar acute epigenetic changes occur following other types of physiological or metabolic interventions, including acute high-intensity exercise (10). We provide additional evidence that challenges the conventional view that epigenetic regulation is largely a mitotically stable process resistant to the effect of environmental factors. Hypermethylation of core clock genes in humans is linked to insulin resistance in humans (18), and this has also partially been observed in blood of people who chronically work shifts (19). Given that the circadian clock affects key metabolic processes (1), our results suggest that sleep loss–induced hypermethylation of *PER1* and *CRY1* in adipose tissue may contribute to glucose intolerance as measured by the 120-minute post-OGTT glucose value.

We found that mRNA expression of the core clock genes *BMAL1* and *CRY1* was decreased in skeletal muscle following acute sleep deprivation. Similar changes occur in circulating leukocytes following longer periods of shortened sleep in humans (8). Skeletal muscle–specific deletion of *Bmal1*, or global deficiency of *Cry1*, impairs insulin sensitivity and glucose metabolism in mouse models (2, 20). Moreover, clock gene expression is altered in peripheral blood cells from type 2 diabetic vs nondiabetic patients (21), with an inverse correlation between clock gene expression (*BMAL1*, *PER1*, and *PER3*) and glycosylated protein (HbA1c) level noted. Thus, our observed transcriptional changes in circadian clock genes in skeletal muscle in response to acute sleep deprivation may impair glucose tolerance.

Although the design of our study did not allow us to ascertain the molecular cause of the observed epigenetic and transcriptional changes in skeletal muscle and adipose tissue following sleep loss, several putative candidate mechanisms can be implicated. For instance, whereas glucocorticoid levels may be slightly elevated during nocturnal wakefulness (22), glucocorticoids—as also shown in our study—are reduced during typical awakening hours (eg, between 0700 and 0800 h) (23). Glucocorticoids reset circadian rhythms of peripheral circadian clocks (24). Thus, resetting of peripheral circadian clocks may be hampered by a blunted cortisol awakening response after acute sleep deprivation.

At both the epigenetic and transcriptional level, we demonstrate tissue-specific alterations in core clock genes

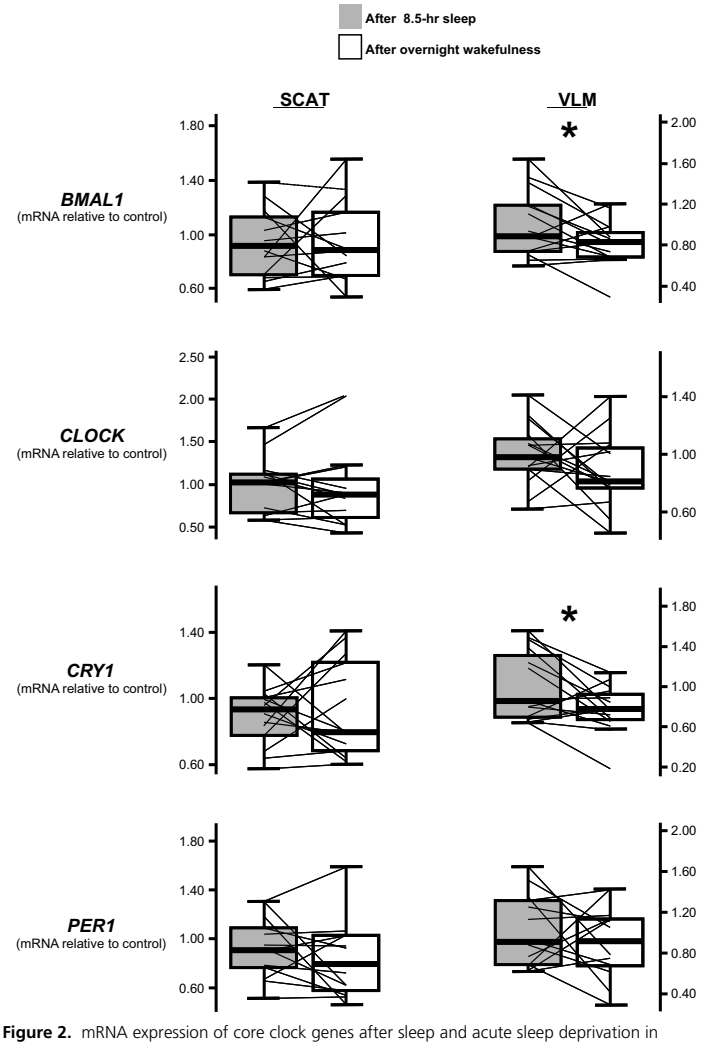


Figure 2. mRNA expression of core clock genes after sleep and acute sleep deprivation in adipose tissue and skeletal muscle. mRNA expression of *BMAL1* and *CRY1* was down-regulated in skeletal muscle (VLM) from humans following overnight wakefulness (ie, acute sleep deprivation) compared with after sleep. No differences between the two conditions were found for the other genes or in the adipose tissue (SCAT). Values are shown as expression levels relative to the control condition (sleep). Horizontal line represents median, box interquartile range, whiskers represent spread of remaining values. Two points that are linked by a line show the difference in mRNA expression in overnight wakefulness vs sleep conditions for each individual. *, $P < .05$; n = 14 for all analyses in adipose tissue; n = 15 for all analyses in skeletal muscle except for *PER1* (see main text for descriptions of excluded values). Abbreviations: SCAT, sc adipose tissue; VLM, vastus lateralis muscle.

under conditions of acute sleep deprivation, consistent with animal studies, in which the circadian machinery exhibits tissue-specific changes in rhythm following shift-work-mimicking sleep-wake paradigms (25). Such internal desynchrony has been hypothesized to underlie metabolic effects of shift work (26–28). The physiological relevance of tissue-specific circadian clocks is further supported by aforementioned and other animal studies in which core clock genes have been ablated or rescued in a tissue-specific manner (3, 29), (eg, an adipose tissue–targeting *Bmal1* deletion in mice resulting in an obese phenotype) (3). Furthermore, insulin-dependent peripheral tissues, ie, adipose tissue and skeletal muscle, shift toward a diabetes-like phenotype following sleep loss (30, 31). Our tissue-differential effects further reinforce the notion that acute circadian misalignment can produce desynchrony of peripheral circadian clocks, with possibly tissue-specific downstream metabolic effects.

Limitations

Several limitations should be kept in mind when interpreting our results. Lights were on in the TSD condition but not in the sleep condition (~300 lux vs darkness). Given that light can entrain the human circadian clock (32), our experimental design does not allow us to disentangle if the observed effects of overnight wakefulness on core circadian genes were either driven by loss of sleep, light exposure, or both. However, it is important to note that our experiment aimed at mimicking night shift work, which is typically performed under ambient light exposure. Another limitation of our study is that expression and methylation of clock genes was measured only at a single time point, ie, under fasting conditions in the morning following each sleep intervention. Thus, our study does not allow firm conclusions on how the circadian pattern of expression and methylation pattern of clock genes is influenced by overnight wakefulness. This would, however, have required repeated tissue sampling, which is much more feasible using animal models; models that can also be maintained longer in a fasted state to avoid the entraining effect of meals on clock genes. Finally, hypermethylation of transcription-regulatory regions of core circadian genes in the

adipose tissue were not paralleled by concomitant reduced expression of these genes. Given that we only sampled biopsies at one time point, a possible explanation might be that acute promoter hypermethylation altered circadian gene mRNA expression at subsequent points in the sleep deprivation condition, (ie, following biopsy collection). Supporting this assumption are results from a separate study examined the effects of an acute bout of exercise on skeletal muscle promoter methylation and corresponding gene expression (10). There, remodeling of promoter methylation of *PGC-1α*, a gene involved in the circadian machinery of the skeletal muscle (33), was accompanied by a delayed (ie, 3 h later), but not concomitant change in gene expression (10).

Conclusions

One night of sleep loss results in hypermethylation of regulatory regions of key clock genes. These effects are tissue specific, and occur in adipose tissue, but not in skeletal muscle. Gene expression differences were observed for the investigated clock genes in skeletal muscle, but not in adipose tissue. Shift work is associated with many of the same phenotypes observed in transgenic animal models in which the circadian clock is disrupted, (eg, glucose intolerance) (34–36). This suggests that our findings of altered peripheral clocks at the epigenetic and transcriptional level, with ensuing glucose intolerance, following acute sleep loss may contribute to metabolic disruptions typically observed in humans with activities regularly scheduled during times that produce chronic desynchrony between tissue-specific clocks.

Perspectives

Given that recurrent partial sleep deprivation decreases insulin sensitivity at the systemic and adipose tissue level in humans (30, 37), future studies to examine whether similar changes occur under conditions of recurrent partial sleep deprivation are of interest. Using repeated biopsy collection, eg, also under insulin-stimulated conditions, may decipher the time-dependent dynamics of peripheral circadian misalignment and how this might relate to metabolic perturbations, including impaired glucose tolerance. Whether our findings can be extrapolated to females or older participants is currently unknown and warrants investigation. Large interindividual differences were observed in our data for how sleep deprivation altered peripheral tissues' clock-gene methylation and gene expression. Contributing factors may be subjects' chronotype—linked to differential responses' to sleep deprivation— or seasonality; with recent studies demonstrating seasonal circadian clock gene variability in animals as well as humans (38), supported by summer-winter variation in human adipose tissue. Ambient light can influence circadian rhythms (39). Thus, light can also resynchronize peripheral circadian rhythms in the absence of a functioning central pacemaker (40); and enhance the cortisol awakening response (41). The mechanism by which different ambient light exposures influences the peripheral clock under conditions of extended wakefulness remains to be investigated. Finally, although the absence of nighttime meals in our sleep deprivation condition precludes the synchronizing influence from such a zeitgeber on peripheral clocks (42), nighttime meal intake is common in shift workers and may thereby modulate effects on tissue-specific circadian clocks.

Acknowledgments

Methylation profiling was performed by the SNP&SEQ Technology Platform in Uppsala. The platform is part of Science for Life Laboratory at Uppsala University and supported as a national infrastructure by the Swedish Research Council. We thank Dr Brendan Egan and Dr Alexander Chibalin for valuable discussions regarding experimental procedures, and Olof Ros and Jon Brandell for their help in conducting the experiments.

Address all correspondence and requests for reprints to: Jonathan Cedernaes, Department of Neuroscience, Uppsala University, Husargatan 3, Box 593, 751 24 Uppsala, Sweden. E-mail: jonathan.cedernaes@neuro.uu.se.

Author Contributions: J.C. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. J.C. and C.B. designed the study; J.C. and C.B. wrote the protocol; J.C. and J.E.B. collected the data; J.C., S.V., J.E.B., H.V., and C.B. conducted the analyses; J.C., M.E.O., S.V., J.E.B., H.V., S.D., J.Z., H.S., and C.B. interpreted the data; J.C., M.E.O., S.V., J.E.B., H.V., S.L.D., J.R.Z., H.B.S., and C.B. contributed to writing; J.C., M.E.O., S.V., J.E.B., H.V., S.L.D., J.R.Z., H.B.S., and C.B. approved the final manuscript.

This study was registered in ClinicalTrials.gov as trial number NCT01800253.

This work was supported by the Swedish Brain Research Foundation (J.C., C.B.), AFA Försäkring (C.B.), the Novo Nordisk Foundation (C.B., J.Z.), the Swedish Society of Medicine (J.C.), Magnus Bergvall's Foundation (J.C.), Thuring's Foundation (J.C.), Tore Nilsson's Foundation (J.C.), and the Swedish Research Council (H.B.S., J.R.Z.). The funding sources had no input in the design and conduct of this study, in the collection, analysis, and interpretation of the data, or in the preparation, review, or approval of the manuscript.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Bass J.** Circadian topology of metabolism. *Nature*. 2012;491:348–356.

2. **Dyar KA, Ciciliot S, Wright LE, et al.** Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. *Mol Metab*. 2014;3:29–41.
3. **Paschos GK, Ibrahim S, Song WL, et al.** Obesity in mice with adipocyte-specific deletion of clock component Arntl. *Nat Med*. 2012; 18:1768–1777.
4. **Marcheva B, Ramsey KM, Buhr ED, et al.** Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature*. 2010;466:627–631.
5. **Turek FW, Joshu C, Kohsaka A, et al.** Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*. 2005;308:1043–1045.
6. **Cedernaes J, Schiöth HB, Benedict C.** Determinants of shortened, disrupted, and mistimed sleep and associated metabolic health consequences in healthy humans. *Diabetes*. 2015;64:1073–1080.
7. **Schmid SM, Hallschmid M, Schultes B.** The metabolic burden of sleep loss. *Lancet Diabetes Endocrinol*. 2015;3(1):52–62.
8. **Möller -Levet CS, Archer SN, Bucca G, et al.** Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc Natl Acad Sci U S A*. 2013;110: E1132–E1141.
9. **Kundaje A, Meuleman W, Ernst J, et al.** Integrative analysis of 111 reference human epigenomes. *Nature*. 2015;518:317–330.
10. **Barres R, Yan J, Egan B, et al.** Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metab*. 2012;15:405–411.
11. **Teschendorff AE, Marabita F, Lechner M, et al.** A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics*. 2013;29:189–196.
12. **Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods.** *Biostatistics*. 2007;8:118–127.
13. **Andersson R, Gebhard C, Miguel-Escalada I, et al.** An atlas of active enhancers across human cell types and tissues. *Nature*. 2014;507: 455–461.
14. **Hillmer AM, Yao F, Inaki K, et al.** Comprehensive long-span paired-end-tag mapping reveals characteristic patterns of structural variations in epithelial cancer genomes. *Genome Res*. 2011;21:665–675.
15. **Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I.** Controlling the false discovery rate in behavior genetics research. *Behav Brain Res*. 2001;125:279–284.
16. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
17. **Du P, Zhang X, Huang CC, et al.** Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics*. 2010;11:587.
18. **Milagro FI, Gómez-Abellán P, Campión J, Martínez JA, Ordovás JM, Garaulet M.** CLOCK, PER2 and BMAL1 DNA methylation: Association with obesity and metabolic syndrome characteristics and monounsaturated fat intake. *Chronobiol Int*. 2012;29:1180–1194.
19. **Zhu Y, Stevens RG, Hoffman AE, et al.** Epigenetic impact of long-term shiftwork: Pilot evidence from circadian genes and whole-genome methylation analysis. *Chronobiol Int*. 2011;28:852–861.
20. **Lamia KA, Papp SJ, Yu RT, et al.** Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*. 2011;480:552–556.
21. **Ando H, Takamura T, Matsuzawa-Nagata N, et al.** Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. *Diabetologia*. 2009;52:329–335.
22. **Benedict C, Hallschmid M, Lassen A, et al.** Acute sleep deprivation reduces energy expenditure in healthy men. *m J Clin Nutr*. 2011; 93:1229–1236.
23. **Backhaus J, Junghanns K, Hohagen F.** Sleep disturbances are correlated with decreased morning awakening salivary cortisol. *Psychoneuroendocrinology*. 2004;29(9):1184–91.
24. **Balsalobre A, Brown SA, Marcacci L, et al.** Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*. 2000; 289:2344–2347.
25. **Yamazaki S, Numano R, Abe M, et al.** Resetting central and peripheral circadian oscillators in transgenic rats. *Science*. 2000;288: 682–685.
26. **Arendt J.** Shift work: Coping with the biological clock. *Occup Med*. 2010;60:10–20.
27. **Bass J, Takahashi JS.** Circadian integration of metabolism and energetics. *Science*. 2010;330:1349–1354.
28. **Eckel-Mahan K, Sassone-Corsi P.** Metabolism and the circadian clock converge. *Physiol Rev*. 2013;93:107–135.
29. **McDearmon EL, Patel KN, Ko CH, et al.** Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. *Science*. 2006;314:1304–1308.
30. **Broussard JL, Ehrmann DA, Van Cauter E, Tasali E, Brady MJ.** Impaired insulin signaling in human adipocytes after experimental sleep restriction: A randomized, crossover study. *Ann Intern Med*. 2012;157:549–557.
31. **Vondra K, Brodan V, Bass A, et al.** Effects of sleep deprivation on the activity of selected metabolic enzymes in skeletal muscle. *Eur J Appl Physiol Occup Physiol*. 1981;47:41–46.
32. **Wright KP Jr, McHill AW, Birks BR, Griffin BR, Rusterholz T, Chinoy ED.** Entrainment of the human circadian clock to the natural light-dark cycle. *Curr Biol*. 2013;23(16):1554–1558.
33. **Liu C, Li S, Liu T, Borjigin J, Lin JD.** Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature*. 2007;447:477–481.
34. **Nagaya T, Yoshida H, Takahashi H, Kawai M.** Markers of insulin resistance in day and shift workers aged 30–59 years. *Int Arch Occup Environ Health*. 2002;75:562–568.
35. **Buxton OM, Cain SW, O'Connor SP, et al.** Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. *Sci Transl Med*. 2012;4:129ra143.
36. **Leproult R, Holmbäck U, Van Cauter E.** Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes*. 2014;63:1860–1869.
37. **Rao MN, Neylan TC, Grunfeld C, Mulligan K, Schambelan M, Schwarz JM.** Subchronic sleep restriction causes tissue-specific insulin resistance. *J Clin Endocrinol Metab*. 2015;100(4):1664–1671.
38. **Dopico XC, Evangelou M, Ferreira RC, et al.** Widespread seasonal gene expression reveals annual differences in human immunity and physiology. *Nat Commun*. 2015;6:7000.
39. **Chang AM, Aeschbach D, Duffy JF, Czeisler CA.** Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness. *Proc Natl Acad Sci U S A*. 2015;112(4): 1232–1237.
40. **Izumo M, Pejchal M, Schook AC, et al.** Differential effects of light and feeding on circadian organization of peripheral clocks in a forebrain Bmal1 mutant. *Elife*. 2014;3.
41. **Scheer FA, Buijs RM.** Light affects morning salivary cortisol in humans. *J Clin Endocrinol Metab*. 1999;84:3395–3398.
42. **Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M.** Entrainment of the circadian clock in the liver by feeding. *Science*. 2001; 291:490–493.

Paper IV [redacted]

Acute Sleep Loss Induces Tissue-Specific Epigenetic and Transcriptional Alterations to Circadian Clock Genes in Men

Jonathan Cedernaes, Megan E. Osler, Sarah Voisin, Jan-Erik Broman, Heike Vogel, Suzanne L. Dickson, Juleen R. Zierath, Helgi B. Schiöth, and Christian Benedict

Department of Neuroscience (J.C., S.V., J.E.B., H.B.S., C.B.), Uppsala University, 751 24 Uppsala, Sweden; Department of Molecular Medicine and Surgery (M.E.O., J.R.Z.), Karolinska Institutet, 171 77 Stockholm, Sweden; Department of Experimental Diabetology (H.V.), German Institute of Human Nutrition Potsdam-Rehbruecke, 14558 Nuthetal, Germany; and Department of Physiology, Institute of Neuroscience and Physiology (H.V., S.L.D.), The Sahlgrenska Academy at the University of Gothenburg, 411 37 Gothenburg, Sweden

Context: Shift workers are at increased risk of metabolic morbidities. Clock genes are known to regulate metabolic processes in peripheral tissues, eg, glucose oxidation.

Objective: This study aimed to investigate how clock genes are affected at the epigenetic and transcriptional level in peripheral human tissues following acute total sleep deprivation (TSD), mimicking shift work with extended wakefulness.

Intervention: In a randomized, two-period, two-condition, crossover clinical study, 15 healthy men underwent two experimental sessions: x sleep (2230–0700 h) and overnight wakefulness. On the subsequent morning, serum cortisol was measured, followed by skeletal muscle and subcutaneous adipose tissue biopsies for DNA methylation and gene expression analyses of core clock genes (*BMAL1*, *CLOCK*, *CRY1*, *PER1*). Finally, baseline and 2-h post-oral glucose load plasma glucose concentrations were determined.

Main Outcome Measures: In adipose tissue, acute sleep deprivation vs sleep increased methylation in the promoter of *CRY1* (+4%; $P = .026$) and in two promoter-interacting enhancer regions of *PER1* (+15%; $P = .036$; +9%; $P = .026$). In skeletal muscle, TSD vs sleep decreased gene expression of *BMAL1* (−18%; $P = .033$) and *CRY1* (−22%; $P = .047$). Concentrations of serum cortisol, which can reset peripheral tissue clocks, were decreased (2449 ± 932 vs 3178 ± 723 nmol/L; $P = .039$), whereas postprandial plasma glucose concentrations were elevated after TSD (7.77 ± 1.63 vs 6.59 ± 1.32 mmol/L; $P = .011$).

Conclusions: Our findings demonstrate that a single night of wakefulness can alter the epigenetic and transcriptional profile of core circadian clock genes in key metabolic tissues. Tissue-specific clock alterations could explain why shift work may disrupt metabolic integrity as observed herein.
(*J Clin Endocrinol Metab* 100: E1255–E1261, 2015)

Animals studies have convincingly demonstrated that the circadian clock allows gene expression to coincide with anticipated metabolic requirements throughout day/night variations via *CLOCK* and *BMAL1* as positive transcriptional regulators and *PERIOD* and *CRYPTOCHROME* as negative transcriptional regulators (1). The

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received May 13, 2015. Accepted July 7, 2015.
First Published Online July 13, 2015

Abbreviations: OGTT, oral glucose tolerance test; TSD, total sleep deprivation.

lack of clock genes, even when ablated only in skeletal muscle or adipose tissue (2, 3), results in systemic metabolic perturbations in animal models (4). These metabolic responses include hyperglycemia and insulin resistance, and can also result in obesity and type 2 diabetes in animals (3–5). As reviewed in Cedernaes et al (6) and Schmid et al (7), similar metabolic phenotypes have been observed in humans subjected to experimental paradigms mimicking night shift work, comprising reduced energy expenditure, impaired systemic glucose disposal, and increased food intake. Over time, these conditions may thus result in metabolic dysregulation and weight gain (6, 7). Although shortened sleep leads to genome-wide changes in the leukocyte transcriptome comprising clock genes (8), the influence of overnight wakefulness, as occurs in night shift work, on the circadian machinery in tissues critically involved in whole-body energy homeostasis is, however, unknown. The importance of this research is highlighted by the fact that today, at least 15% of the workforce—numbering 15 million in the United States alone—carry out shift work, with job activities scheduled during the biological night.

With this background, we characterized the effects of one night of sleep deprivation on gene expression and DNA methylation of core circadian clock genes in peripheral tissues. DNA methylation of gene promoters and promoter-interacting enhancers is one epigenetic mechanism involved in the control of gene expression (9) and is a malleable process following acute lifestyle interventions (10). We obtained subcutaneous adipose tissue and skeletal muscle biopsies from fasted healthy young men following both acute sleep deprivation and normal sleep. In addition, fasting serum cortisol and plasma glucose were measured, the latter before and 120 minutes after an oral glucose tolerance test (OGTT).

Materials and Methods

Study design

This randomized crossover within-subject trial was conducted from March through September 2013 at Uppsala Biomedical Centre, Uppsala University, Sweden. The sessions were relatively evenly distributed across the study period. Study procedures and written consent forms were approved by the Regional Ethical Review Board in Uppsala (EPN 2012/477). The study was conducted in accordance with the Helsinki Declaration. Each enrolled participant voluntarily signed the consent form.

Participants

Sixteen of 17 enrolled subjects participated in two sessions of this study. Participants were of self-reported good health, free from chronic medical conditions or chronic medication, nonsmokers, and had normal sleeping habits (7–9 h of sleep/night; Pittsburgh Sleep Quality Index score ≤ 5) (extended screening protocol in Supplement Part 1).

Study protocol and interventions

All 16 participants engaged in two conditions (acute sleep deprivation vs sleep), in which each condition was separated by at least 4 weeks. Participants came in a semifasted state (fasted since 1500 h) to the laboratory two evenings before each session's final experimental morning, and remained in the laboratory under constant supervision until the end of the experimental session (ie, approximately a 42-h laboratory stay).

Participants were provided with breakfast, lunch, and dinner during their 24-hour baseline period (each meal providing one third of the participants' individually calculated energy requirements; based on the Harris-Benedict equation factored 1.2 for light physical activity), and had an 8.5-hour sleep opportunity during the first night (2230–0700 h). During the first baseline day, participants were provided with two standardized and supervised 15-minute walks. During nonexperimental time periods, participants were confined to their rooms but were free to engage in sedentary-level activities.

Randomization to the first experimental condition (sleep or acute sleep deprivation) was generated by drawing lots, with a fixed block size of 2 and allocation ratio of 1:1. Participants were randomly assigned after having been screened by J.C. as eligible, and were scheduled in pairs for the next available session slot. The allocation sequence was only known by one of the researchers (C.B.) but was concealed from the participants, with the experimenters only notified 2 weeks in advance of each new session for experimental preparation. Participants were blinded to the experimental condition (sleep or acute sleep deprivation) until 90 minutes in advance of onset of the nighttime intervention, which took place during the second night (2230–0700 h). During this period in the sleep condition, room lights were kept off and sleep was monitored. In contrast, in the sleep deprivation condition, participants were under constant supervision 2230–0700 h to ensure wakefulness, remaining bed-restricted and fasted.

Blood sampling, biopsy collection, and OGTT

After fasting, blood samples were obtained at 0730 h. Tissue biopsies were also obtained in the fasted state, 2–3 hours after subject wake-up time, with the collection of the adipose tissue preceding that of the skeletal muscle. Following the biopsy collection, participants completed a 75g OGTT (further details provided in the Supplement).

DNA extraction and epigenetic analyses

DNA extraction and epigenetic analysis with Illumina's HumanMethylation450 BeadChip are further described in Supplement Part 1.

DNA methylation preprocessing consisted of probe filtering (removal of probes with missing β-values; probes with less than 75% of samples with detection $P < .01$; or nonspecific or single nucleotide polymorphism–coinciding probes), followed by adjustment of type I and type II probes using BMIQ (11), and removal of batch effects using ComBat (12). We ran four pairs of technical replicates, including at least one from each experimental condition (sleep and acute sleep deprivation), to estimate the inner variability of each probe. We only considered for further analysis the probes for which at least half of the subjects showed

a methylation difference between conditions greater than the mean difference in technical replicates.

CpG sites within 1500 bp of the transcription start site of *CLOCK*, *ARNTL*, *CRY1*, and *PER1* were analyzed (15 CpG sites for adipose tissue and 9 nine for skeletal muscle). The promoter is a key part of a gene, but enhancers also prominently contribute to the regulation of gene expression (13). To identify putative enhancers of *CLOCK*, *ARNTL*, *CRY1*, and *PER1*, we inferred chromatin states in adipose nuclei and skeletal muscle, and mapped long-range interactions in five different cells lines, with three different transcription factors (14). CpG sites located in chromatin states indicative of enhancers in adipose nuclei and skeletal muscle and in regions having long-range interactions with the promoters of *CLOCK*, *ARNTL*, *CRY1*, and *PER1*, were also analyzed (six CpG sites for adipose tissue and four for skeletal muscle).

Methylation levels are presented as β-values (ranging from zero to one, corresponding to zero and 100% methylation, respectively). *P*-values were adjusted for multiple testing according to the Benjamini-Hochberg method within each tissue (15).

RNA extraction and qPCR analysis of gene expression

Methods used for RNA extraction and qPCR analysis of gene expression are described in further detail in **Supplement Part 1.** The gene expression of *CLOCK*, *ARNTL*, *CRY1*, and *PER1* was analyzed with qPCR in adipose tissue and skeletal muscle. All analyses were run in duplicates (primer information in **Supplement Part 2**). The ΔCt method was used to normalize data (16).

Statistics

Normal-distribution criteria of analyzed data were assessed with Kolmogorov-Smirnov's test of normality. Normally distributed data was analyzed with paired Student *t* tests, whereas nonnormally distributed variables were analyzed with Wilcoxon signed-rank test. Methylation data was analyzed using the software package R (version 3.1); we used the $\log_2$ ratio of the intensities of methylated probe vs unmethylated probe, also called M-value, which is more statistically valid for the differential analysis of methylation levels (17). All other data was analyzed using the software SPSS (version 21; SPSS Inc.) and are presented as means ± SD. Two-sided $P < .05$ were considered significant. For the adipose tissue, one individual was excluded for all gene expression analyses (expression values greater than mean + 2 SD for several genes). The significance values were, however, not changed when the analysis was run with or without this subject (data not shown). For *PER1* in skeletal muscle, an outlier was excluded from both conditions (expression values in the sleep deprivation condition greater than mean + 2 SD), but significance values were not altered when the analysis was run with or without this subject (data not shown).

Results

Of 17 enrolled subjects, 16 completed participation in both sessions (sleep and acute sleep deprivation). One participant was excluded from later analysis due to insufficient sleep (< 7 h) in the sleep condition. Fifteen participants were therefore included in the final analysis (age, 22.3 ± 1.9 y; body mass index, 22.6 ± 1.8 kg/m^2). Sleep data are presented in Supplement Part 3.

Effect of acute sleep deprivation on methylation and expression of circadian genes in adipose tissue and skeletal muscle

Methylation levels at cg04674060 (+15%; adjusted $P = .036$) and cg19308989 (+9%; adjusted $P = .026$; both CpG sites located in enhancers interacting with the promoter of *PER1*), and at cg20193872 (located in the promoter of *CRY1*; +4%; adjusted $P = .026$), increased after acute sleep deprivation, compared with the sleep condition, in adipose tissue (shown in Figure 1). In skeletal muscle, the investigated CpG sites were not altered (detailed probe results in Supplement Part 4).

In skeletal muscle, mRNA expression of *BMAL1* and *CRY1* was decreased following acute sleep deprivation (−18 and −22% compared with expression levels found after sleep; $P = .033$ and $P = .047$, respectively; see Figure 2 and Supplement Part 4). Skeletal muscle *CLOCK* or *PER1* gene expression was unaltered. Moreover, the adipose tissue genes were unaltered following acute sleep deprivation.

Effect of acute sleep deprivation on fasting cortisol and glucose tolerance

Following acute sleep deprivation, fasting serum cortisol concentrations were decreased at 0730 h (2449 ± 932 vs 3178 ± 723 nmol/L; $P = .039$), compared with after sleep. Plasma glucose concentrations at 120 minutes post-OGTT were higher following acute sleep deprivation, compared with the values obtained after sleep (pre-OGTT: 5.36 ± 0.30 vs 5.38 ± 0.36 mmol/l; $P = .705$; post-OGTT: 7.77 ± 1.63 vs 6.59 ± 1.32 mmol/l; $P = .011$).

Discussion

We determined the effect of one night of wakefulness, as occurs during night shift work, on DNA methylation and mRNA expression of key circadian genes (ie, *BMAL1*, *CLOCK*, *CRY1*, and *PER1*) in human skeletal muscle and adipose tissue. We provide evidence that acute sleep deprivation increases promoter methylation and reduces transcription of circadian genes in a tissue-specific manner. Our analysis reveals increased methylation of transcription-regulating regions of *PER1* and *CRY1* in adipose tissue and reduced gene expression of *CRY1* and *BMAL1* in skeletal muscle. We also observed an impaired glucose response following an OGTT after acute sleep deprivation. Our results suggest that acute sleep loss alters clock gene regulation, concomitant with deleterious met-

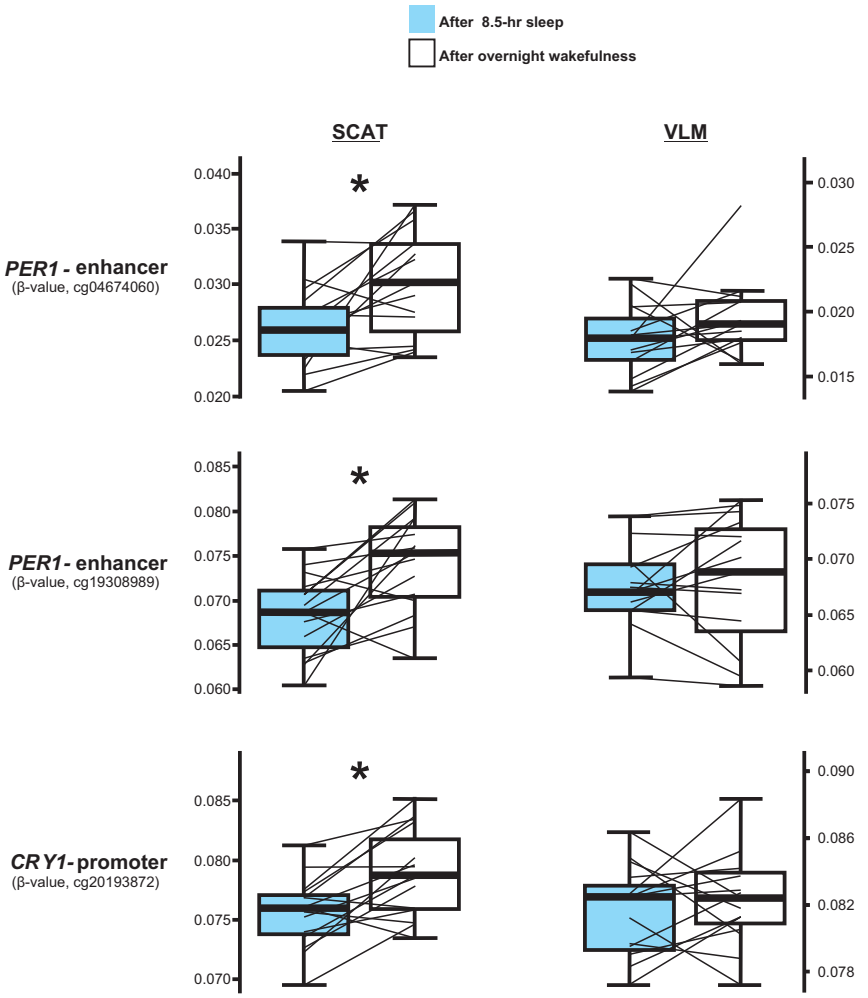


Figure 1. Methylation levels after sleep and acute sleep deprivation in adipose tissue and skeletal muscle. Methylation levels in two putative enhancers interacting with the promoter of *PER1* (probes cg04674060 and cg19308989) were increased following overnight wakefulness (ie, acute sleep deprivation), in adipose tissue (SCAT) compared with after sleep. Methylation levels in the promoter of *CRY1* (probe cg20193872) were also increased following overnight wakefulness (acute sleep deprivation) compared with after sleep, in adipose tissue (SCAT). No differences were seen, however, between the two conditions in skeletal muscle (VLM). Methylation levels are shown as beta values (β-value; ranging from zero to one, corresponding to zero and 100% methylation, respectively). Horizontal line represents median, box interquartile range, whiskers represent spread of remaining values. Two points that are linked by a line show the difference in methylation levels in overnight wakefulness vs sleep conditions for each individual. *, $P < .05$; n = 15 for all analyses. Abbreviations: SCAT, sc adipose tissue; VLM, vastus lateralis muscle.

abolic effects, which are differential, rather than uniform across key peripheral metabolic tissues in healthy humans.

Our results of altered DNA methylation for promoter and promoter-interacting enhancer regions of core clock genes in adipose tissue suggest that acute sleep deprivation can cause acute epigenetic remodeling of the circadian clock. Similar acute epigenetic changes occur following other types of physiological or metabolic interventions, including acute high-intensity exercise (10). We provide additional evidence that challenges the conventional view that epigenetic regulation is largely a mitotically stable process resistant to the effect of environmental factors. Hypermethylation of core clock genes in humans is linked to insulin resistance in humans (18), and this has also partially been observed in blood of people who chronically work shifts (19). Given that the circadian clock affects key metabolic processes (1), our results suggest that sleep loss–induced hypermethylation of *PER1* and *CRY1* in adipose tissue may contribute to glucose intolerance as measured by the 120-minute post-OGTT glucose value.

We found that mRNA expression of the core clock genes *BMAL1* and *CRY1* was decreased in skeletal muscle following acute sleep deprivation. Similar changes occur in circulating leukocytes following longer periods of shortened sleep in humans (8). Skeletal muscle–specific deletion of *Bmal1*, or global deficiency of *Cry1*, impairs insulin sensitivity and glucose metabolism in mouse models (2, 20). Moreover, clock gene expression is altered in peripheral blood cells from type 2 diabetic vs nondiabetic patients (21), with an inverse correlation between clock gene expression (*BMAL1*, *PER1*, and *PER3*) and glycosylated protein (HbA1c) level noted. Thus, our observed transcriptional changes in circadian clock genes in skeletal muscle in response to acute sleep deprivation may impair glucose tolerance.

Although the design of our study did not allow us to ascertain the molecular cause of the observed epigenetic and transcriptional changes in skeletal muscle and adipose tissue following sleep loss, several putative candidate mechanisms can be implicated. For instance, whereas glucocorticoid levels may be slightly elevated during nocturnal wakefulness (22), glucocorticoids—as also shown in our study—are reduced during typical awakening hours (eg, between 0700 and 0800 h) (23). Glucocorticoids reset circadian rhythms of peripheral circadian clocks (24). Thus, resetting of peripheral circadian clocks may be hampered by a blunted cortisol awakening response after acute sleep deprivation.

At both the epigenetic and transcriptional level, we demonstrate tissue-specific alterations in core clock genes

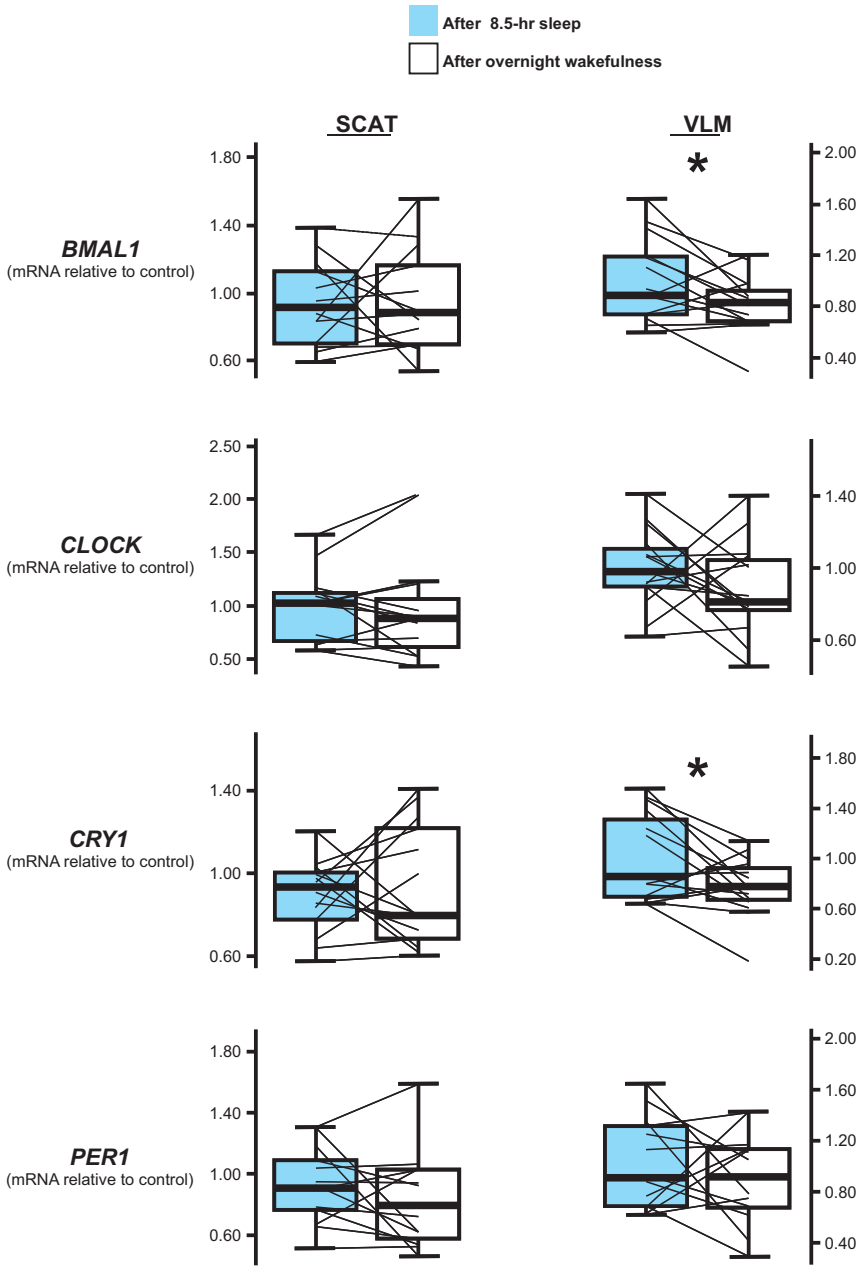


Figure 2. mRNA expression of core clock genes after sleep and acute sleep deprivation in adipose tissue and skeletal muscle. mRNA expression of *BMAL1* and *CRY1* was down-regulated in skeletal muscle (VLM) from humans following overnight wakefulness (ie, acute sleep deprivation) compared with after sleep. No differences between the two conditions were found for the other genes or in the adipose tissue (SCAT). Values are shown as expression levels relative to the control condition (sleep). Horizontal line represents median, box interquartile range, whiskers represent spread of remaining values. Two points that are linked by a line show the difference in mRNA expression in overnight wakefulness vs sleep conditions for each individual. *, $P < .05$; n = 14 for all analyses in adipose tissue; n = 15 for all analyses in skeletal muscle except for *PER1* (see main text for descriptions of excluded values). Abbreviations: SCAT, sc adipose tissue; VLM, vastus lateralis muscle.

under conditions of acute sleep deprivation, consistent with animal studies, in which the circadian machinery exhibits tissue-specific changes in rhythm following shift-work-mimicking sleep-wake paradigms (25). Such internal desynchrony has been hypothesized to underlie metabolic effects of shift work (26–28). The physiological relevance of tissue-specific circadian clocks is further supported by aforementioned and other animal studies in which core clock genes have been ablated or rescued in a tissue-specific manner (3, 29), (eg, an adipose tissue–targeting *Bmal1* deletion in mice resulting in an obese phenotype) (3). Furthermore, insulin-dependent peripheral tissues, ie, adipose tissue and skeletal muscle, shift toward a diabetes-like phenotype following sleep loss (30, 31). Our tissue-differential effects further reinforce the notion that acute circadian misalignment can produce desynchrony of peripheral circadian clocks, with possibly tissue-specific downstream metabolic effects.

Limitations

Several limitations should be kept in mind when interpreting our results. Lights were on in the TSD condition but not in the sleep condition (~300 lux vs darkness). Given that light can entrain the human circadian clock (32), our experimental design does not allow us to disentangle if the observed effects of overnight wakefulness on core circadian genes were either driven by loss of sleep, light exposure, or both. However, it is important to note that our experiment aimed at mimicking night shift work, which is typically performed under ambient light exposure. Another limitation of our study is that expression and methylation of clock genes was measured only at a single time point, ie, under fasting conditions in the morning following each sleep intervention. Thus, our study does not allow firm conclusions on how the circadian pattern of expression and methylation pattern of clock genes is influenced by overnight wakefulness. This would, however, have required repeated tissue sampling, which is much more feasible using animal models; models that can also be maintained longer in a fasted state to avoid the entraining effect of meals on clock genes. Finally, hypermethylation of transcription-regulatory regions of core circadian genes in the

adipose tissue were not paralleled by concomitant reduced expression of these genes. Given that we only sampled biopsies at one time point, a possible explanation might be that acute promoter hypermethylation altered circadian gene mRNA expression at subsequent points in the sleep deprivation condition, (ie, following biopsy collection). Supporting this assumption are results from a separate study examined the effects of an acute bout of exercise on skeletal muscle promoter methylation and corresponding gene expression (10). There, remodeling of promoter methylation of *PGC-1α*, a gene involved in the circadian machinery of the skeletal muscle (33), was accompanied by a delayed (ie, 3 h later), but not concomitant change in gene expression (10).

Conclusions

One night of sleep loss results in hypermethylation of regulatory regions of key clock genes. These effects are tissue specific, and occur in adipose tissue, but not in skeletal muscle. Gene expression differences were observed for the investigated clock genes in skeletal muscle, but not in adipose tissue. Shift work is associated with many of the same phenotypes observed in transgenic animal models in which the circadian clock is disrupted, (eg, glucose intolerance) (34–36). This suggests that our findings of altered peripheral clocks at the epigenetic and transcriptional level, with ensuing glucose intolerance, following acute sleep loss may contribute to metabolic disruptions typically observed in humans with activities regularly scheduled during times that produce chronic desynchrony between tissue-specific clocks.

Perspectives

Given that recurrent partial sleep deprivation decreases insulin sensitivity at the systemic and adipose tissue level in humans (30, 37), future studies to examine whether similar changes occur under conditions of recurrent partial sleep deprivation are of interest. Using repeated biopsy collection, eg, also under insulin-stimulated conditions, may decipher the time-dependent dynamics of peripheral circadian misalignment and how this might relate to metabolic perturbations, including impaired glucose tolerance. Whether our findings can be extrapolated to females or older participants is currently unknown and warrants investigation. Large interindividual differences were observed in our data for how sleep deprivation altered peripheral tissues' clock-gene methylation and gene expression. Contributing factors may be subjects' chronotype—linked to differential responses' to sleep deprivation— or seasonality; with recent studies demonstrating seasonal circadian clock gene variability in animals as well as humans (38), supported by summer-winter variation in human adipose tissue. Ambient light can influence circadian rhythms (39). Thus, light can also resynchronize peripheral circadian rhythms in the absence of a functioning central pacemaker (40); and enhance the cortisol awakening response (41). The mechanism by which different ambient light exposures influences the peripheral clock under conditions of extended wakefulness remains to be investigated. Finally, although the absence of nighttime meals in our sleep deprivation condition precludes the synchronizing influence from such a zeitgeber on peripheral clocks (42), nighttime meal intake is common in shift workers and may thereby modulate effects on tissue-specific circadian clocks.

Acknowledgments

Methylation profiling was performed by the SNP&SEQ Technology Platform in Uppsala. The platform is part of Science for Life Laboratory at Uppsala University and supported as a national infrastructure by the Swedish Research Council. We thank Dr Brendan Egan and Dr Alexander Chibalin for valuable discussions regarding experimental procedures, and Olof Ros and Jon Brandell for their help in conducting the experiments.

Address all correspondence and requests for reprints to: Jonathan Cedernaes, Department of Neuroscience, Uppsala University, Husargatan 3, Box 593, 751 24 Uppsala, Sweden. E-mail: jonathan.cedernaes@neuro.uu.se.

Author Contributions: J.C. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. J.C. and C.B. designed the study; J.C. and C.B. wrote the protocol; J.C. and J.E.B. collected the data; J.C., S.V., J.E.B., H.V., and C.B. conducted the analyses; J.C., M.E.O., S.V., J.E.B., H.V., S.D., J.Z., H.S., and C.B. interpreted the data; J.C., M.E.O., S.V., J.E.B., H.V., S.L.D., J.R.Z., H.B.S., and C.B. contributed to writing; J.C., M.E.O., S.V., J.E.B., H.V., S.L.D., J.R.Z., H.B.S., and C.B. approved the final manuscript.

This study was registered in ClinicalTrials.gov as trial number NCT01800253.

This work was supported by the Swedish Brain Research Foundation (J.C., C.B.), AFA Försäkring (C.B.), the Novo Nordisk Foundation (C.B., J.Z.), the Swedish Society of Medicine (J.C.), Magnus Bergvall's Foundation (J.C.), Thuring's Foundation (J.C.), Tore Nilsson's Foundation (J.C.), and the Swedish Research Council (H.B.S., J.R.Z.). The funding sources had no input in the design and conduct of this study, in the collection, analysis, and interpretation of the data, or in the preparation, review, or approval of the manuscript.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Bass J.** Circadian topology of metabolism. *Nature*. 2012;491:348–356.

2. **Dyar KA, Ciciliot S, Wright LE, et al.** Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. *Mol Metab*. 2014;3:29–41.
3. **Paschos GK, Ibrahim S, Song WL, et al.** Obesity in mice with adipocyte-specific deletion of clock component Arntl. *Nat Med*. 2012; 18:1768–1777.
4. **Marcheva B, Ramsey KM, Buhr ED, et al.** Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature*. 2010;466:627–631.
5. **Turek FW, Joshu C, Kohsaka A, et al.** Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*. 2005;308:1043–1045.
6. **Cedernaes J, Schiöth HB, Benedict C.** Determinants of shortened, disrupted, and mistimed sleep and associated metabolic health consequences in healthy humans. *Diabetes*. 2015;64:1073–1080.
7. **Schmid SM, Hallschmid M, Schultes B.** The metabolic burden of sleep loss. *Lancet Diabetes Endocrinol*. 2015;3(1):52–62.
8. **Möller -Levet CS, Archer SN, Bucca G, et al.** Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc Natl Acad Sci U S A*. 2013;110: E1132–E1141.
9. **Kundaje A, Meuleman W, Ernst J, et al.** Integrative analysis of 111 reference human epigenomes. *Nature*. 2015;518:317–330.
10. **Barres R, Yan J, Egan B, et al.** Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metab*. 2012;15:405–411.
11. **Teschendorff AE, Marabita F, Lechner M, et al.** A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics*. 2013;29:189–196.
12. **Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods.** *Biostatistics*. 2007;8:118–127.
13. **Andersson R, Gebhard C, Miguel-Escalada I, et al.** An atlas of active enhancers across human cell types and tissues. *Nature*. 2014;507: 455–461.
14. **Hillmer AM, Yao F, Inaki K, et al.** Comprehensive long-span paired-end-tag mapping reveals characteristic patterns of structural variations in epithelial cancer genomes. *Genome Res*. 2011;21:665–675.
15. **Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I.** Controlling the false discovery rate in behavior genetics research. *Behav Brain Res*. 2001;125:279–284.
16. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
17. **Du P, Zhang X, Huang CC, et al.** Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics*. 2010;11:587.
18. **Milagro FI, Gómez-Abellán P, Campión J, Martínez JA, Ordovás JM, Garaulet M.** CLOCK, PER2 and BMAL1 DNA methylation: Association with obesity and metabolic syndrome characteristics and monounsaturated fat intake. *Chronobiol Int*. 2012;29:1180–1194.
19. **Zhu Y, Stevens RG, Hoffman AE, et al.** Epigenetic impact of long-term shiftwork: Pilot evidence from circadian genes and whole-genome methylation analysis. *Chronobiol Int*. 2011;28:852–861.
20. **Lamia KA, Papp SJ, Yu RT, et al.** Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*. 2011;480:552–556.
21. **Ando H, Takamura T, Matsuzawa-Nagata N, et al.** Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. *Diabetologia*. 2009;52:329–335.
22. **Benedict C, Hallschmid M, Lassen A, et al.** Acute sleep deprivation reduces energy expenditure in healthy men. *m J Clin Nutr*. 2011; 93:1229–1236.
23. **Backhaus J, Junghanns K, Hohagen F.** Sleep disturbances are correlated with decreased morning awakening salivary cortisol. *Psychoneuroendocrinology*. 2004;29(9):1184–91.
24. **Balsalobre A, Brown SA, Marcacci L, et al.** Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*. 2000; 289:2344–2347.
25. **Yamazaki S, Numano R, Abe M, et al.** Resetting central and peripheral circadian oscillators in transgenic rats. *Science*. 2000;288: 682–685.
26. **Arendt J.** Shift work: Coping with the biological clock. *Occup Med*. 2010;60:10–20.
27. **Bass J, Takahashi JS.** Circadian integration of metabolism and energetics. *Science*. 2010;330:1349–1354.
28. **Eckel-Mahan K, Sassone-Corsi P.** Metabolism and the circadian clock converge. *Physiol Rev*. 2013;93:107–135.
29. **McDearmon EL, Patel KN, Ko CH, et al.** Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. *Science*. 2006;314:1304–1308.
30. **Broussard JL, Ehrmann DA, Van Cauter E, Tasali E, Brady MJ.** Impaired insulin signaling in human adipocytes after experimental sleep restriction: A randomized, crossover study. *Ann Intern Med*. 2012;157:549–557.
31. **Vondra K, Brodan V, Bass A, et al.** Effects of sleep deprivation on the activity of selected metabolic enzymes in skeletal muscle. *Eur J Appl Physiol Occup Physiol*. 1981;47:41–46.
32. **Wright KP Jr, McHill AW, Birks BR, Griffin BR, Rusterholz T, Chinoy ED.** Entrainment of the human circadian clock to the natural light-dark cycle. *Curr Biol*. 2013;23(16):1554–1558.
33. **Liu C, Li S, Liu T, Borjigin J, Lin JD.** Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature*. 2007;447:477–481.
34. **Nagaya T, Yoshida H, Takahashi H, Kawai M.** Markers of insulin resistance in day and shift workers aged 30–59 years. *Int Arch Occup Environ Health*. 2002;75:562–568.
35. **Buxton OM, Cain SW, O'Connor SP, et al.** Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. *Sci Transl Med*. 2012;4:129ra143.
36. **Leproult R, Holmbäck U, Van Cauter E.** Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes*. 2014;63:1860–1869.
37. **Rao MN, Neylan TC, Grunfeld C, Mulligan K, Schambelan M, Schwarz JM.** Subchronic sleep restriction causes tissue-specific insulin resistance. *J Clin Endocrinol Metab*. 2015;100(4):1664–1671.
38. **Dopico XC, Evangelou M, Ferreira RC, et al.** Widespread seasonal gene expression reveals annual differences in human immunity and physiology. *Nat Commun*. 2015;6:7000.
39. **Chang AM, Aeschbach D, Duffy JF, Czeisler CA.** Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness. *Proc Natl Acad Sci U S A*. 2015;112(4): 1232–1237.
40. **Izumo M, Pejchal M, Schook AC, et al.** Differential effects of light and feeding on circadian organization of peripheral clocks in a forebrain Bmal1 mutant. *Elife*. 2014;3.
41. **Scheer FA, Buijs RM.** Light affects morning salivary cortisol in humans. *J Clin Endocrinol Metab*. 1999;84:3395–3398.
42. **Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M.** Entrainment of the circadian clock in the liver by feeding. *Science*. 2001; 291:490–493.

Paper IV [redacted]

Panchenko et al. Clinical Epigenetics (2016) 8:22
DOI 10.1186/s13148-016-0188-3

Clinical Epigenetics

RESEARCH Open Access

Expression of epigenetic machinery genes is sensitive to maternal obesity and weight loss in relation to fetal growth in mice

Polina E. Panchenko[1,2], Sarah Voisin[1,2], Mélanie Jouin[1], Luc Jouneau[1], Audrey Prézelin[1], Simon Lecoutre[3], Christophe Breton[3], Hélène Jammes[1], Claudine Junien[1,4] and Anne Gabory[1*]

Abstract

Background: Maternal obesity impacts fetal growth and pregnancy outcomes. To counteract the deleterious effects of obesity on fertility and pregnancy issue, preconceptional weight loss is recommended to obese women. Whether this weight loss is beneficial/detrimental for offspring remains poorly explored. Epigenetic mechanisms could be affected by maternal weight changes, perturbing expression of key developmental genes in the placenta or fetus. Our aim was to investigate the effects of chronic maternal obesity on feto-placental growth along with the underlying epigenetic mechanisms. We also tested whether preconceptional weight loss could alleviate these effects.

Results: Female mice were fed either a control diet (CTRL group), a high-fat diet (obese (OB) group), or a high-fat diet switched to a control diet 2 months before conception (weight loss (WL) group). At mating, OB females presented an obese phenotype while WL females normalized metabolic parameters. At embryonic day 18.5 (E18.5), fetuses from OB females presented fetal growth restriction (FGR; −13 %) and 28 % of the fetuses were small for gestational age (SGA). Fetuses from WL females normalized this phenotype. The expression of 60 epigenetic machinery genes and 32 metabolic genes was measured in the fetal liver, placental labyrinth, and junctional zone. We revealed 23 genes altered by maternal weight trajectories in at least one of three tissues. The fetal liver and placental labyrinth were more responsive to maternal obesity than junctional zone. One third (18/60) of the epigenetic machinery genes were differentially expressed between at least two maternal groups. Interestingly, genes involved in the histone acetylation pathway were particularly altered (13/18). In OB group, *lysine acetyltransferases* and *Bromodomain-containing protein 2* were upregulated, while most histone deacetylases were downregulated. In WL group, the expression of only a subset of these genes was normalized.

Conclusions: This study highlights the high sensitivity of the epigenetic machinery gene expression, and particularly the histone acetylation pathway, to maternal obesity. These obesity-induced transcriptional changes could alter the placental and the hepatic epigenome, leading to FGR. Preconceptional weight loss appears beneficial to fetal growth, but some effects of previous obesity were retained in offspring phenotype.

Keywords: Maternal obesity, Preconceptional weight loss, Fetal growth restriction, Epigenetic machinery, Histone deacetylases (HDACs), Lysine acetyltransferases (KATs), Placenta, Liver

* Correspondence: anne.gabory@jouy.inra.fr
[1]UMR BDR, INRA, ENVA, Université Paris Saclay, 78350 Jouy en Josas, France
Full list of author information is available at the end of the article

Background

The worldwide prevalence of obesity in women was 38 % in 2013 [1]. Obesity during pregnancy comprises increased risks for metabolic and obstetrical complications (e.g., gestational hypertension and diabetes, and preeclampsia) but also stillbirth, prematurity, and congenital malformations [2]. Fetal growth could be particularly impacted by maternal obesity. Maternal obesity is associated with macrosomia or, on the contrary, with fetal growth restriction (FGR) [3–6]. FGR is associated with a high incidence of metabolic diseases in adulthood [7, 8], which is consistent with the *developmental origins of health and disease* (DOHaD) concept. This concept, also named "developmental programming" or "conditioning," states that environmental factors during early development could predispose an individual to chronic diseases [9].

Despite the high incidence of FGR in obese women, little is known about the underlying mechanisms. FGR could result from insufficient oxygen supply due to disturbed vascularization, increased lipid accumulation, and macronutrients transport in the placenta, crucial organ regulating appropriate fetal development [10–12]. Importantly, different placental parts have different functions and cellular populations [13]. The labyrinth is a zone of active exchange between maternal and fetal blood, while the junctional zone provides hormone production and storage of nutrients that are necessary for fetal development [13]. The structure and the function of several organs could be affected in the offspring of obese mothers. In utero alterations of hepatic development and function by maternal obesity could disturb metabolic homeostasis [14, 15]. The effect of obesity on organogenesis and gene expression in growth-restricted fetuses needs further investigation; the current efforts are indeed focused on FGR induced by poor maternal nutrition [16].

Modulation of offspring phenotype in response to maternal environment could be mediated by epigenetic mechanisms. Epigenetic marks (e.g., DNA methylation, histone posttranslational modifications) are stable but reversible covalent modifications that are regulated by a complex epigenetic machinery. Its actors "write," "erase" or "read" epigenetic marks, establishing the epigenome of the cell in conjunction with environmental factors. This epigenetic landscape is dynamic during development and controls gene expression patterns in a tissue-specific manner. Alterations of DNA methylation, histone modifications and, to a lesser extent, of their regulators were observed in relation to FGR caused by different factors [17–22]. Epigenetic disturbances in growth-restricted fetuses in the context of maternal diet-induced obesity need further investigation. Maternal unbalanced nutrition and metabolic state could impact certain epigenetic enzymes in the developing organs of the offspring, affecting the epigenome [23, 24]. As epigenetic marks can be transmitted through generations of cell divisions, epigenetics has emerged as a plausible mechanism for long-term memory of environmental insults [23, 25].

To counteract the negative effects of obesity on pregnancy outcomes, preconceptional weight loss is currently recommended to women with high body mass index [26]. Nevertheless, very few studies have assessed the consequences of maternal weight loss on fetal growth [27]. In humans, weight loss between two pregnancies reduces the risk of macrosomia [28]. However, in another cohort, weight loss between the age of 20 years and conception had a negative impact on birth weight [29]. Thus, the impact of maternal preconceptional weight changes on fetal growth and underlying epigenetic processes needs to be clarified. Currently, there is no relevant mouse model to study this important issue for public health.

Based on the observations that maternal obesity impairs feto-placental development, our aim was to examine the impact of maternal weight trajectories (obesity or weight loss) on the expression of epigenetic and metabolic genes in the fetal liver and in the placental labyrinth and junctional zone. We showed that maternal obesity induced FGR, which was associated with an altered expression of histone acetylation modifiers in the fetal liver and labyrinth, but not in the junctional zone. In contrast, correction of obesity during the preconceptional period by nutritional intervention normalized fetal weight and induced an adaptation at the transcriptional level. This study provides a novel mouse model for investigating the molecular mechanisms of obesity-induced FGR and highlights the sensitivity of the epigenetic machinery to maternal nutrition and metabolism.

Results

High-fat diet (HFD) induced severe obesity in female mice; switching to a control diet (CD) induced weight loss and normalization of metabolic parameters

Female mice were fed either a CD (control females (CTRL)) or a HFD (obese females (OB)) for 4 months during the preconceptional period (Fig. 1). From the start of the diet and up to mating, OB females put on weight faster ($\beta = 0.52$, $P < 0.001$) and were heavier than CTRL females on a CD ($P < 0.001$, Fig. 2a). OB females weighed 27 and 36 % more than CTRL females after 2 and 4 months of diet, respectively. After 2 months on a HFD, we replaced HFD by CD for a subset of the OB females to induce weight loss (WL females) (Fig. 1). The weight of WL females was lower than the weight of OB females as early as 3 days after CD initiation and afterwards ($P < 0.05$ at all time points, Fig. 2a). WL females tended to normalize their weight ($P = 0.105$, week 19.5,

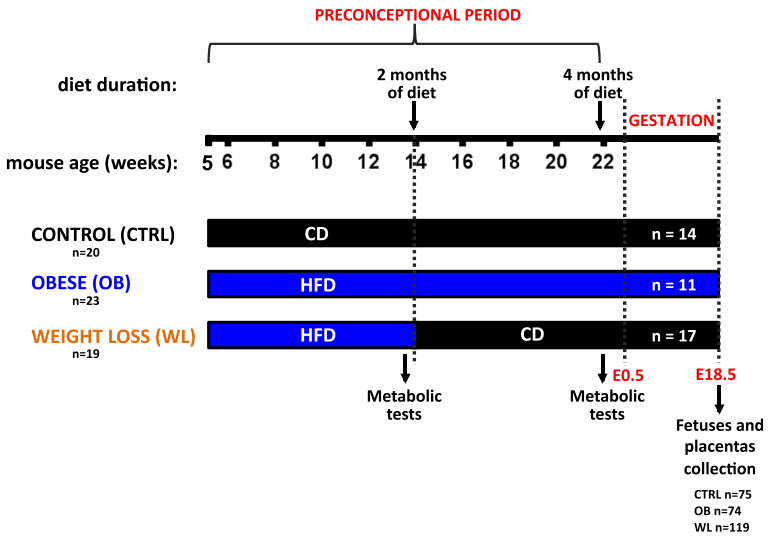


Fig. 1 Experimental design. C57BL/6j females were fed a control diet (*CD*) or a high-fat diet (*HFD*) throughout the study. After 2 months of diet, HFD was switched to CD for the weight loss group. After 2 and 4 months of diet, females' metabolic parameters (cholesterol, fasting glucose levels, and glucose tolerance) were measured. Females were mated with males on a standard diet and sacrificed at E18.5, then fetuses and placentas were weighed and collected

$P = 0.062$, week 21, $P = 0.051$, week 21.5, $P = 0.031$, week 22, WL vs. CTRL). However, they remained 5 % heavier than CTRL females before mating.

The caloric intake of OB females was higher than the caloric intake of CTRL females during 4 months of preconceptional diet ($P < 0.001$, Fig. 2b) but OB and CTRL females had a similar food intake to body weight ratio (kcal/kg/day) (Additional file 1: Figure S1). WL females drastically decreased their caloric intake right after the nutritional intervention ($P < 0.001$, WL vs. CTRL/OB; Fig. 2b) and normalized this parameter 1 week later ($P = 0.15$, week 15, WL vs. CTRL; $P < 0.05$, WL vs. OB) and thereafter. Metabolic parameters were assessed after 2 and 4 months of diet (Fig. 1). OB females were hypercholesterolemic, hyperglycemic, and glucose intolerant compared to CTRL females ($P < 0.001$, $P < 0.05$, and $P < 0.001$ at each time point, respectively, Fig. 2c–f). The nutritional intervention normalized these parameters in WL females.

In summary, OB females were obese and had an impaired glucose metabolism and hypercholesterolemia. Switching to a CD allowed complete restoration of all these parameters although WL females remained 5 % heavier than CTRL females before mating.

Obese dams on a HFD gained less weight at term of pregnancy

To determine the potential effects of maternal obesity and preconceptional weight loss on fetal outcomes, we mated CTRL, OB, and WL females after 4 months of preconceptional diet with males on a standard laboratory diet (Fig. 1). Preconceptional diet was maintained during pregnancy. OB dams had reduced total pregnant body weight gain and carcass weight compared with CTRL and WL dams at embryonic day 18.5 (E18.5) ($P < 0.001$, Fig. 3a, b). There were no differences between WL and CTRL dams ($P = 0.83$ for total weight gain; $P = 0.56$ for carcass weight). The maternal group explained 29 % of the variance in total body weight gain and 37 % of the variance in carcass weight at E18.5. OB and WL dams had increased litter size vs. CTRL dams at E18.5 ($P < 0.001$; CTRL 5.5 ± 2.18, $n = 14$ litters; OB 6.8 ± 1.99, $n = 11$ litters; WL 7.0 ± 2.26, $n = 17$ litters). There was no difference in litter size between OB and WL dams ($P = 0.13$).

Maternal obesity induced fetal growth restriction, while preconceptional weight loss allowed restoration of fetal weight

As several studies identified sex-specific effects of maternal obesity on fetuses and placentas, we tested the effect of sex on fetal and placental weights [12, 30–32]. Sex did not affect fetal weight ($P = 0.17$) but affected placental weight and fetal-weight-to-placental-weight ratio index (FPI). Male placentas were heavier than female placentas ($P < 0.001$; difference in CTRL 11 %, OB 10 %, and WL 7 %), and FPI was lower in males than in females for all maternal groups ($P < 0.001$). We therefore adjusted for sex in the placental weight and FPI analysis only. Moreover, litter size affected fetal and placental weights ($P < 0.001$), and even if it explained only 4.6 and 7.7 % of the variance, respectively (Additional file 1: Figure S2 and S3), we adjusted all analyses for this parameter. There were no differences in the mother's age at mating between the three investigated groups (CTRL 26.1 ± 1.94 weeks; OB 25.6 ± 1.83 weeks; WL 25.6 ± 1.71 weeks). Maternal age/

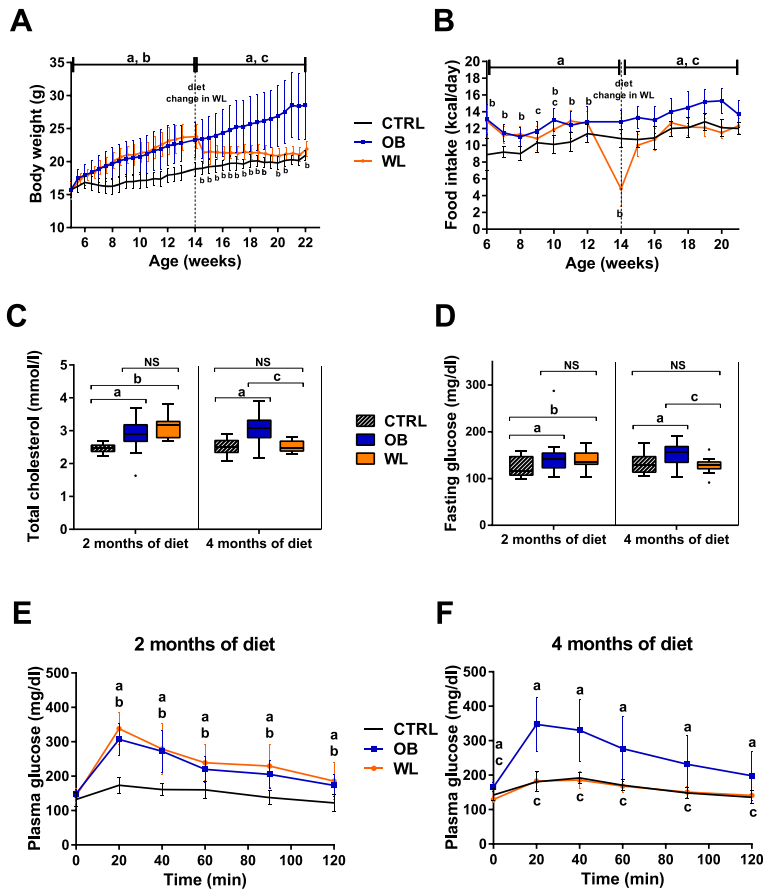


Fig. 2 Body weight and metabolic parameters of OB and WL females during the preconceptional period. **a** Body weight. (*a*) $P < 0.05$ OB vs. CTRL, (*b*) $P < 0.05$ WL vs. CTRL, (*c*) $P < 0.05$ WL vs. OB. $n = 20$ CTRL, 23 OB, 19 WL. **b** Caloric intake. (*a*) $P < 0.001$ OB vs. CTRL, (*b*) $P < 0.001$ WL vs. CTRL, (*c*) $P < 0.05$ WL vs. OB. $n = 18$–20 CTRL, 23 OB, 17–19 WL. **c** Plasma cholesterol levels after 2 and 4 months of preconceptional diet. *NS* nonsignificant. (*a*) $P < 0.001$ OB vs. CTRL, (*b*) $P < 0.001$ WL vs. CTRL, (*c*) $P < 0.001$ WL vs. OB. $n = 17$–19 CTRL, 22–23 OB, 19 WL. **d** Fasting glucose level after 2 and 4 months of preconceptional diet. *NS* nonsignificant. (*a*) $P < 0.05$ OB vs. CTRL, (*b*) $P < 0.05$ WL vs. CTRL, (*c*) $P < 0.05$ WL vs. OB. $n = 17$–19 CTRL, 23 OB, 19 WL. **e** Plasma glucose levels during oral glucose tolerance test (OGTT) after 2 months of diet. (*a*) $P < 0.001$ OB vs. CTRL, (*b*) $P < 0.001$ WL vs. CTRL, 11–12 females per group. **f** Plasma glucose levels during OGTT after 4 months of diet. (*a*) $P < 0.001$ OB vs. CTRL, (*c*) $P < 0.001$ WL vs. OB, 12 females per group. Data are expressed as mean ± St. Dev (*a*, *b*, *e*, *f*) or as Tukey's boxplot (*c*, *d*)

diet duration correlated with fetal weight and FPI but not with placental weight (Pearson's correlation test, $r = 0.39$, $P < 0.001$, $r = 0.40$, $P < 0.001$, $r = -0.08$, $P = 0.17$, respectively, Additional file 1: Figure S4). All studies were therefore adjusted for maternal age, which was also equivalent to diet duration in our experimental protocol.

We then examined the effect of maternal metabolism on fetal and placental weights at E18.5. We observed a 13 % reduction of the weight in fetuses of OB dams compared with CTRL dams ($P < 0.001$, Fig. 3c). Fetuses of WL dams had similar weight to fetuses of CTRL dams ($P = 0.17$, Fig. 3c) but heavier than fetuses of OB dams ($P < 0.001$). Overall, maternal group and age explained 15 and 14 % of the variance in fetal weight, respectively. We determined the proportion of small for gestational age (SGA) fetuses, defined as fetal weight <10th percentile of CTRL population. There were 28.4 % of SGA fetuses in OB dams and 11.8 % in the WL dams (Fig. 3d). The odds of being SGA was increased in OB group by a factor of 3.2 (logistic regression, 95 % CI 1.19–9.76, $P = 0.028$). In WL group, the odds of being SGA was not altered compared to CTRL group ($P = 0.48$). Increase in maternal age/diet duration for 1 week decreased the odds by a factor of 0.61 ($P < 0.001$). Gaussian distributions of fetal weight in the three maternal groups are available in Additional file 1: Figure S5.

There was no effect of maternal group on placental weight at E18.5 ($P = 0.42$, Fig. 3e). FPI, which represents placental efficiency, was reduced in the fetuses of OB dams compared with those of CTRL and WL dams ($P < 0.001$, Fig. 3f). However, WL and CTRL dams had similar FPI ($P = 0.16$). Maternal group explained 12 % of the variance in FPI. In female fetuses, there was a correlation between fetal and placental weight in all maternal groups

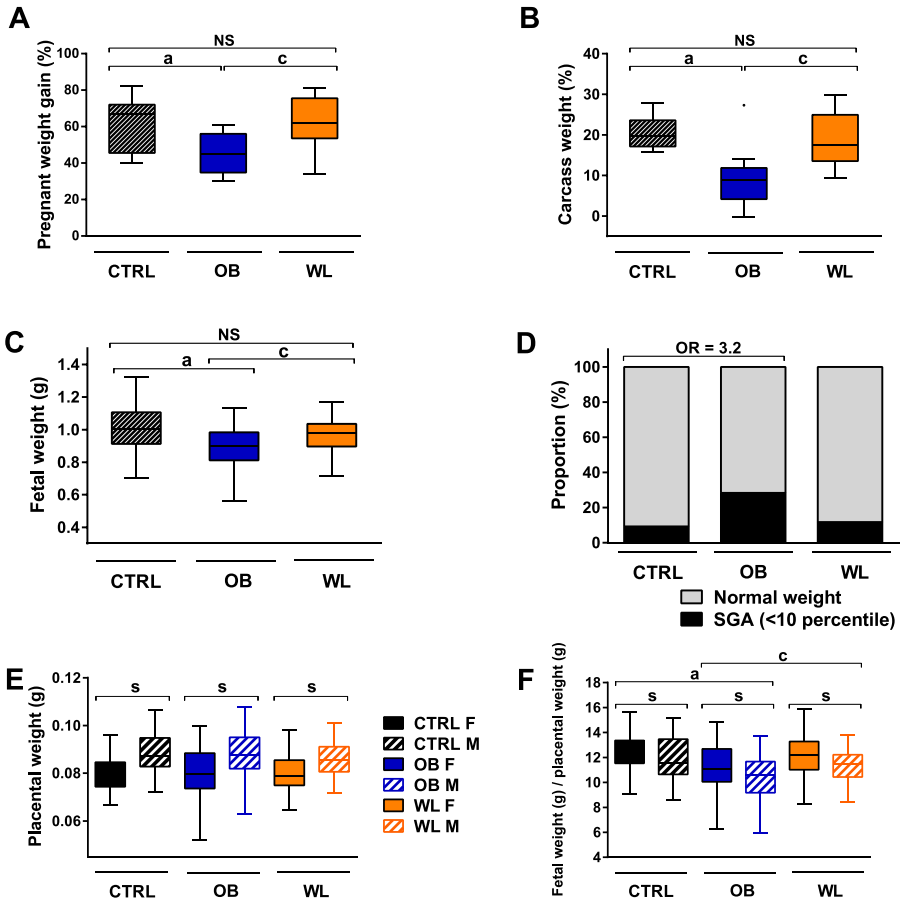


Fig. 3 Body weight and fetal and placental weights in OB and WL dams at E18.5. **a** Dams pregnant body weight gain (percentage of initial weight). *NS* nonsignificant. (*a*) $P < 0.001$ OB vs. CTRL. (*c*) $P < 0.001$ WL vs. OB. $n = 13$ CTRL, 11 OB, 17 WL. **b** Dams carcass weight (percentage of initial weight) at sacrifice. *NS* nonsignificant. (*a*) $P < 0.001$ OB vs. CTRL, (*c*) $P < 0.001$ WL vs. OB. $n = 13$ CTRL, 10 OB, 16 WL. **c** Fetal weight. *NS* nonsignificant. (*a*) $P < 0.001$ OB vs. CTRL. (*c*) $P < 0.001$ WL vs. OB. Data from males and females were combined as there was no effect of sex on fetal weight. $n = 75$ CTRL, 74 OB, 119 WL. **d** Proportion of small for gestational age (SGA) fetuses. Data from males and females were combined as there was no effect of sex on fetal weight. CTRL ($n = 7$ SGA/75 fetuses), OB ($n = 21/74$), WL ($n = 14/119$). Maternal obesity: odds ratio (OR) of being SGA = 3.2 (95 % CI 1.19–9.76, $P = 0.028$). **e** Placental weight. (*s*) $P < 0.001$ males vs. females. $n = 36$ CTRL F, 39 CTRL M, 35 OB F, 39 OB M, 61 WL F, 58 WL M. **f** Fetal-weight-to-placental weight ratio index. (*a*) $P = 0.001$ OB vs. CTRL. (*c*) $P < 0.001$ WL vs. OB. (*s*) $P = 0.001$ males vs. females. $n = 36$ CTRL F, 39 CTRL M, 35 OB F, 39 OB M, 61 WL F, 58 WL M

(Pearson's correlation test, adjusting for factor "mother": CTRL $r = 0.50$, $P_{adj} = 0.006$; OB $r = 0.49$; $P_{adj} = 0.006$; WL $r = 0.36$, $P_{adj} = 0.0063$; Additional file 1: Figure S5). In male fetuses, we observed a correlation in WL ($r = 0.45$, $P_{adj} = 0.002$), but not in CTRL and OB groups ($r = 0.28$, $P_{adj} = 0.097$ and $r = 0.1$, $P_{adj} = 0.54$, respectively).

Thus, maternal chronic obesity caused FGR in both sexes and impaired placental efficiency. Preconceptional weight loss induced by nutritional intervention abolished this FGR and restored placental efficiency.

Maternal obesity altered gene expression in the fetal liver and placental labyrinth, but not in junctional zone

To unravel the molecular mechanisms of the impact of maternal obesity and preconceptional weight loss in growth-restricted offspring, we assessed the gene expression at E18.5 using custom TaqMan low-density arrays (TLDAs). We tested the expression of 60 epigenetic machinery genes and 32 genes involved in metabolism or in development (Additional file 2: Table S1). Based on the literature and our previous studies, these epigenetic genes were selected because of their implication in metabolic processes and obesity or type 2 diabetes [30, 33]. Some of the metabolic genes assessed in our study are known targets of developmental conditioning, and for a subset of these genes, the epigenetic alterations are documented in this context. A description of the selection criteria of genes for the custom TLDA design is available in Additional file 3.

The vast majority of expression studies are performed in whole placentas, but epigenetic and metabolic processes

may not be the same in different placental layers since they have different functions and cell populations [30, 34, 35]. The liver is a major organ regulating the metabolic processes and it is particularly affected by obesity [14, 15]. Thus, the expression study was performed in placental labyrinth and junctional zone separately, as well as in the fetal liver, as we also aimed to evaluate the impact of maternal nutrition on fetal tissues.

Hierarchical clustering based on mean gene expression revealed that gene expression was affected by maternal diet in the liver and labyrinth: the OB group clustered away from the CTRL and WL groups (Fig. 4a, b). The effect of obesity was weaker in the junctional zone as OB males and females clustered with WL and CTRL males (Fig. 4c). In the liver, groups clustered according to maternal diet while in placental layers, CTRL and WL groups clustered according to fetal sex. Thus, maternal obesity affected the mean expression of all tested genes in the fetal liver and labyrinth, while maternal weight loss restored it.

We showed that 23 genes were significantly altered by maternal weight trajectories in at least one of three tissues (Table 1). The full list of mean expression level per gene and per group and adjusted *p* values are presented in Additional file 4: Table S2.

The expression of *Kdm5d* (*Jarid1d*) and *Uty* genes, which are localized on the Y chromosome, was restricted to male samples. Their paralogs located on the X chromosome (*Kdm5c/Jarid1c* and *Kdm6a/Utx*), and which partially escape X inactivation, were not differentially expressed between males and females, except for *Kdm6a* in the junctional zone ($p = 0.003$). Two other genes showed sex differences in this tissue independent of maternal dietary group: *Bdnf* and *Lpl* were also more expressed in female ($P = 0.039$ and 0.033, respectively). We then pooled male and female data from the same maternal group to assess the effect of maternal diet on gene expression. Maternal age did not correlate with gene expression in any of the three investigated tissues (Pearson's correlation test; $P > 0.05$ for all genes).

Maternal obesity and weight loss altered the expression of epigenetic machinery genes in the fetal liver

Maternal weight trajectories affected the transcription of nine epigenetic genes in the liver (Fig. 5, Table 1). Expression of the histone deacetylase *Hdac2* was reduced in OB fetuses compared to CTRL fetuses (Table 1, Fig. 5e). On the contrary, expression of the lysine acetyltransferases *Kat2a* (*Gcn5*), *Kat3a* (*Creb binding protein*), *Kat6b* (*Myst4*) and *Kat13d* (*Clock*), the arginine methyltransferases *Prmt1* and *Prmt7*, the histone deacetylase *Hdac6*, and the bromodomain protein *Brd2* was increased in fetuses from OB dams. Maternal weight loss

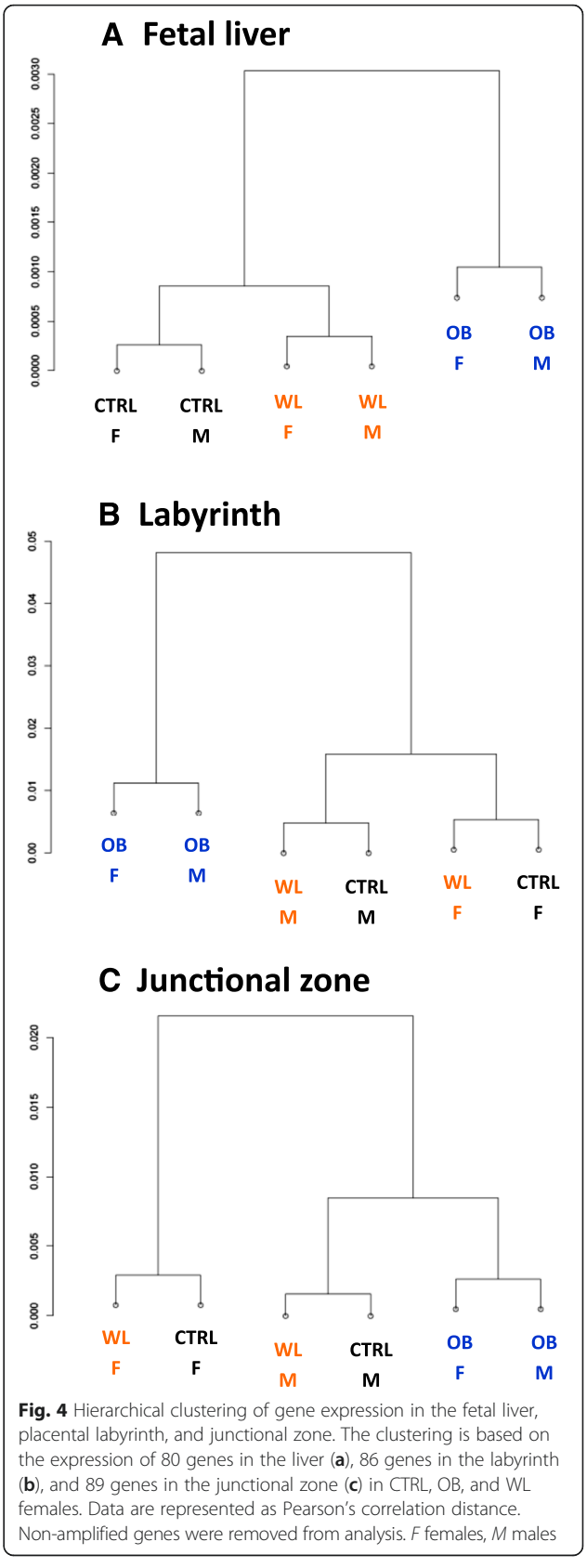


Fig. 4 Hierarchical clustering of gene expression in the fetal liver, placental labyrinth, and junctional zone. The clustering is based on the expression of 80 genes in the liver (**a**), 86 genes in the labyrinth (**b**), and 89 genes in the junctional zone (**c**) in CTRL, OB, and WL females. Data are represented as Pearson's correlation distance. Non-amplified genes were removed from analysis. *F* females, *M* males

Table 1 Differentially expressed genes in the fetal liver, placental labyrinth, and junctional zone at E18.5

Tissue	Gene	CTRL group	OB group	WL group	*P* value		
					OB vs. CTRL	WL vs. CTRL	WL vs. OB
Fetal liver	*Kat2a* (*Gcn5*)	2.92 ± 0.90	4.77 ± 1.48	4.14 ± 1.51	↗ 0.004	0.118	0.622
	Kat3a (*Crebbp*)	0.71 ± 0.19	1.14 ± 0.34	1.04 ± 0.37	↗ 0.008	0.055	0.745
	Kat6b (*Myst4*)	0.97 ± 0.29	1.65 ± 0.48	1.26 ± 0.41	↗ 0.004	0.209	0.209
	Kat13d (*Clock*)	2.19 ± 0.59	3.67 ± 0.89	3.16 ± 1.23	↗ <0.001	0.307	0.445
	Hdac2	0.43 ± 0.17	0.20 ± 0.07	0.26 ± 0.13	↘ 0.004	0.065	0.496
	Hdac6	1.44 ± 0.43	2.29 ± 0.71	1.78 ± 0.74	↗ 0.012	0.445	0.339
	Brd2	0.25 ± 0.07	0.34 ± 0.07	0.37 ± 0.10	↗ 0.039	0.024	0.731
	Prmt1	0.50 ± 0.15	0.78 ± 0.21	0.61 ± 0.23	↗ 0.008	0.431	0.279
	Prmt7	7.1 ± 2.6	11.6 ± 3.9	8.5 ± 3.0	↗ 0.016	0.496	0.166
	Gck	0.81 ± 0.56	0.26 ± 0.24	0.76 ± 0.61	↘ 0.031	0.940	0.088
	Lepr	0.22 ± 0.09	0.25 ± 0.07	0.17 ± 0.05	0.637	0.377	↘ 0.039
Labyrinth	*Kat1* (*Hat1*)	0.024 ± 0.011	0.041 ± 0.013	0.037 ± 0.010	↗ 0.012	↗ 0.029	0.463
	Kat3a (*Crebbp*)	1.22 ± 0.42	1.49 ± 0.43	1.84 ± 0.60	0.215	↗ 0.029	0.192
	Kat3b (*Ep300*)	0.012 ± 0.006	0.030 ± 0.014	0.029 ± 0.014	↗ 0.003	↗ 0.006	0.907
	Kat13b (*Ncoa3*)	0.045 ± 0.022	0.084 ± 0.033	0.087 ± 0.028	↗ 0.017	↗ 0.003	0.871
	Hdac2	0.35 ± 0.15	0.23 ± 0.15	0.18 ± 0.08	0.124	↘ 0.014	0.448
	Hdac3	4.03 ± 1.41	2.60 ± 0.62	3.81 ± 1.43	↘ 0.021	0.797	0.053
	Hdac10	0.22 ± 0.08	0.10 ± 0.05	0.22 ± 0.10	↘ 0.003	0.978	↗ 0.003
	Sirt4	0.32 ± 0.14	0.23 ± 0.07	0.37 ± 0.16	0.124	0.478	↗ 0.046
	Brd2	0.73 ± 0.26	0.91 ± 0.18	1.06 ± 0.31	0.124	↗ 0.036	0.266
	Kmt1d (*Ehmt1*)	2.09 ± 0.74	1.34 ± 0.37	2.50 ± 1.15	↘ 0.029	0.402	↗ 0.017
	Mbd5	0.031 ± 0.010	0.022 ± 0.007	0.035 ± 0.013	0.053	0.480	↗ 0.021
	Mecp2	0.035 ± 0.012	0.029 ± 0.010	0.045 ± 0.012	0.247	0.112	↗ 0.007
	Hsd11β1	49.7 ± 25.5	26.1 ± 13.8	52.1 ± 32.0	↘ 0.046	0.892	0.065
	Irs1	0.71 ± 0.36	0.38 ± 0.19	0.65 ± 0.41	↘ 0.043	0.777	0.124
	Tph1	0.021 ± 0.010	0.012 ± 0.006	0.021 ± 0.009	↘ 0.033	0.978	↗ 0.04951
Junctional zone	*Kat3b* (*Ep300*)	0.029 ± 0.008	0.042 ± 0.012	0.045 ± 0.012	0.101	↗ 0.040	0.743

Data are represented as mean expression levels ± St.Dev. When the *p* value was significant, an arrow showing the sense of variation was added (↘ downregulation, ↗ upregulation)

induced various transcriptional responses of epigenetic machinery genes. On the one hand, *Brd2* expression remained increased in WL compared to CTRL fetuses, showing no normalization of its expression (Fig. 5g). Expression of *Hdac2* and *Kat3a* tended to remain altered in the liver of WL fetuses (P_{adj} = 0.065 and P_{adj} = 0.055, respectively, WL vs. CTRL). On the other hand, expression of *Kat2a*, *Kat6b*, *Kat13d*, *Hdac6*, *Prmt1*, and *Prmt7* was similar in WL and CTRL fetuses. Therefore, maternal weight loss restored the expression of some of the genes that were altered by maternal obesity, but not of all.

Among the investigated metabolic genes, only glucokinase (*Gck*) was affected. Its expression was reduced in the liver of OB fetuses compared to that of CTRL and normalized in WL fetuses (Table 1, Fig. 6a).

Maternal obesity and weight loss altered the expression of epigenetic machinery and metabolic genes in the placenta

Maternal obesity and weight loss altered the expression of 12 epigenetic genes in the placental labyrinth and one gene in junctional zone (Fig. 7, Table 1). Expression of the lysine acetyltransferases *Kat1* (*Hat1*), *Kat3b* (*Ep300*), and *Kat13b* (*Ncoa3*) was higher in the labyrinth of OB dams compared to that of CTRL dams (Fig. 7a, c, e). Expression of the lysine methyltransferase *Kmt1d* (*Ehmt1*) and the histone deacetylases *Hdac3* and *Hdac10* was reduced in the labyrinth of OB dams (Table 1, Fig. 7g, h). In WL dams, responses in the placenta highly differed between the genes. There was no restoration of expression for *Kat1*, *Kat3b*, and *Kat13b*. On the contrary, *Kmt1d*, *Hdac3*, and *Hdac10* expression was restored.

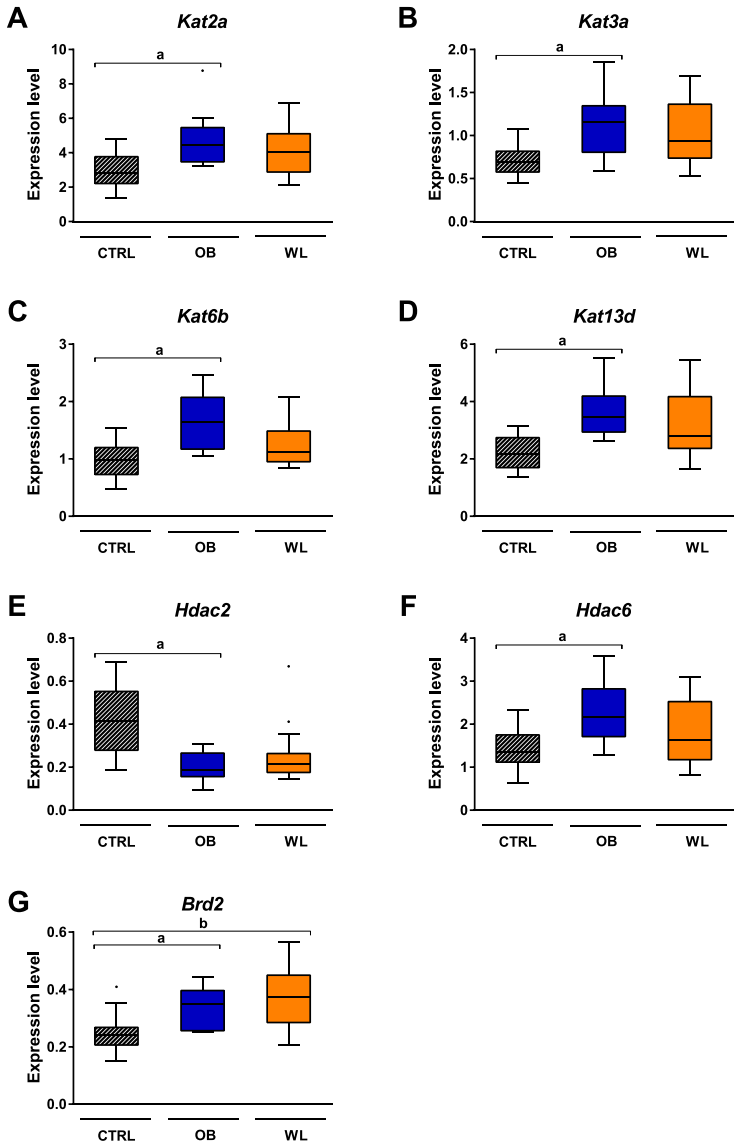


Fig. 5 Genes implicated in histone acetylation are differentially expressed in the fetal liver at E18.5. We assessed the expression level of 60 genes of the epigenetic machinery using TaqMan low-density arrays. The expression of *Kat2a* (**a**), *Kat3a* (**b**), *Kat6b* (**c**), *Kat13d* (**d**), *Hdac2* (**e**), *Hdac6* (**f**), and *Brd2* (**g**) was affected by maternal obesity. (*a*) $P_{adj} < 0.05$ OB vs. CTRL, (*b*) $P_{adj} < 0.05$ WL vs. CTRL. CTRL ($n = 16$), OB ($n = 14$), WL ($n = 16$)

Expression of *Sirtuin 4*, *Mecp2*, and *Mbd5* was higher in WL than in OB labyrinth, but similar to CTRL (Table 1, Fig. 7i). Finally, some genes were not altered by maternal obesity but by maternal weight loss: *Kat3a* and *Brd2* were upregulated and *Hdac2* was downregulated in the labyrinth; *Kat3b* was upregulated in the junctional zone (Fig. 7b, d, f, j). For these four genes, the medians and the distributions in OB group were intermediate between CTRL and WL groups.

We showed that the expression of hydroxysteroid 11-beta dehydrogenase 1 (*Hsd11β1*), insulin receptor substrate 1 (*Irs1*), and tryptophan hydroxylase 1 (*Tph1*) was downregulated in the placental labyrinth of OB dams (Table 1, Fig. 6b–d). There was no difference between WL and CTRL for these metabolic genes. None of the investigated metabolic or developmental genes were affected in the junctional zone.

Thus, the expression of 30 % of epigenetic machinery genes (18 out of 60 studied) was altered by maternal weight trajectories in three tissues. Fifteen percent of genes (9/60) was differentially expressed in the fetal liver, 20 % (12/60) in the labyrinth, and 1.7 % (1/60) in the junctional zone. The histone acetylation pathway (KATs, HDACs, and BRDs) was particularly altered: 78 % of differentially expressed genes in the liver (7/9), 75 % in the labyrinth (9/12), and the only gene in the junctional zone.

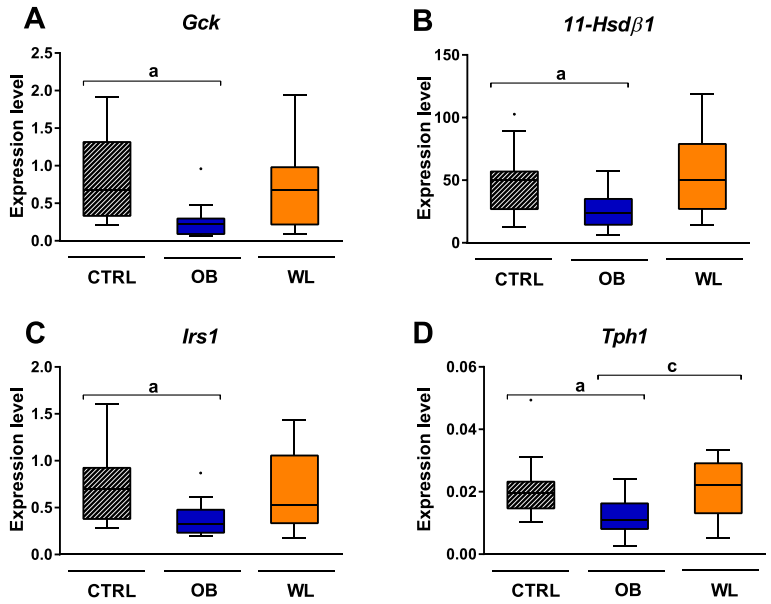


Fig. 6 Maternal weight trajectories affect the expression of genes implicated in metabolism. We assessed the expression level of 32 genes implicated in metabolism or development using TaqMan low-density arrays. The expression of *glucokinase* (**a**) in the fetal liver and *hydroxysteroid 11-β dehydrogenase 1* (**b**), *insulin receptor substrate 1* (**c**), and *tryptophan hydroxylase 1* (**d**) in the placental labyrinth was affected by maternal obesity or weight loss at E18.5. (*a*) $P_{adj} < 0.05$ OB vs. CTRL, (*c*) $P_{adj} < 0.05$ WL vs. OB. CTRL ($n = 16$), OB ($n = 14$), WL ($n = 16$)

Maternal obesity had an important effect on the expression of metabolic and epigenetic genes in the fetal liver and placental labyrinth, but not in the junctional zone. Maternal preconceptional weight loss allowed a global restoration of transcription to CTRL levels. However, the expression of certain genes (for example, *Kat1*, *Kat3b*, and *Kat13b* in the labyrinth and *Brd2* in the liver) was not restored.

Discussion

In the present study, we showed that maternal chronic obesity lead to FGR at E18.5, while the weight loss induced by a nutritional intervention performed in preconceptional period allowed a partial fetal weight restoration. These phenotypic changes were associated with a transcriptional response in the fetal liver and placental labyrinth: we showed that 23 genes were significantly altered by maternal weight trajectories in at least one of three tissues between two maternal groups (Fig. 8). Our results identified that epigenetic machinery gene expression is clearly sensitive to maternal weight trajectories, especially for genes involved in histone acetylation.

Maternal obesity-induced FGR is associated with altered expression of epigenetic modifiers in the fetal liver and placental labyrinth

In our model, OB mice were severely obese and presented the characteristics of metabolic syndrome after 4 months of HFD. This resulted in FGR at E18.5, which is consistent with the previous studies in mice [11, 15, 36, 37]. However, in some mouse models, fetuses from OB dams displayed an overgrowth, and it associated with altered placental transport [34, 38]. These discrepancies could be explained by differences in diet composition, especially by high-sugar content. In humans and rats, maternal obesity also associates with an elevated fetal weight [3, 4, 39]. As maternal obesity associates also with FGR in humans, our study provides a mouse model for investigation of the molecular mechanisms of this pathology, which remain unknown [6].

Maternal HFD alters hepatic function and structure in the fetus [14, 15]. In OB dams, we report reduced transcription of *Gck*, the enzyme controlling the synthesis of hepatic glycogen and implicated in type 2 diabetes. *Gck* expression could remain reduced after birth and lead to impaired glucose metabolism [40, 41]. Placental function is also affected by obesity or HFD [11, 12, 34, 38, 42, 43]. In our study, *Hsd11β1*, *Irs1*, and *Tph1* were downregulated in the labyrinth of OB dams, suggesting that alterations of placental function could contribute to FGR. Hydroxysteroid dehydrogenases 11β control the passage of glucocorticoids from the mother to the fetus. In humans, placental expression of *HSD11β1* is associated with birth weight [44]; placental *HSD11βs* expression is reduced in SGA neonates [45]. Impaired placental insulin signaling is associated with obesity, gestational diabetes mellitus, or intrauterine growth restriction (IUGR) [46, 47]. Insulin regulates placental growth, vascularization, glycogen, and lipid storage [48]. Reduced expression of *Irs1* in OB dams could lead to insulin

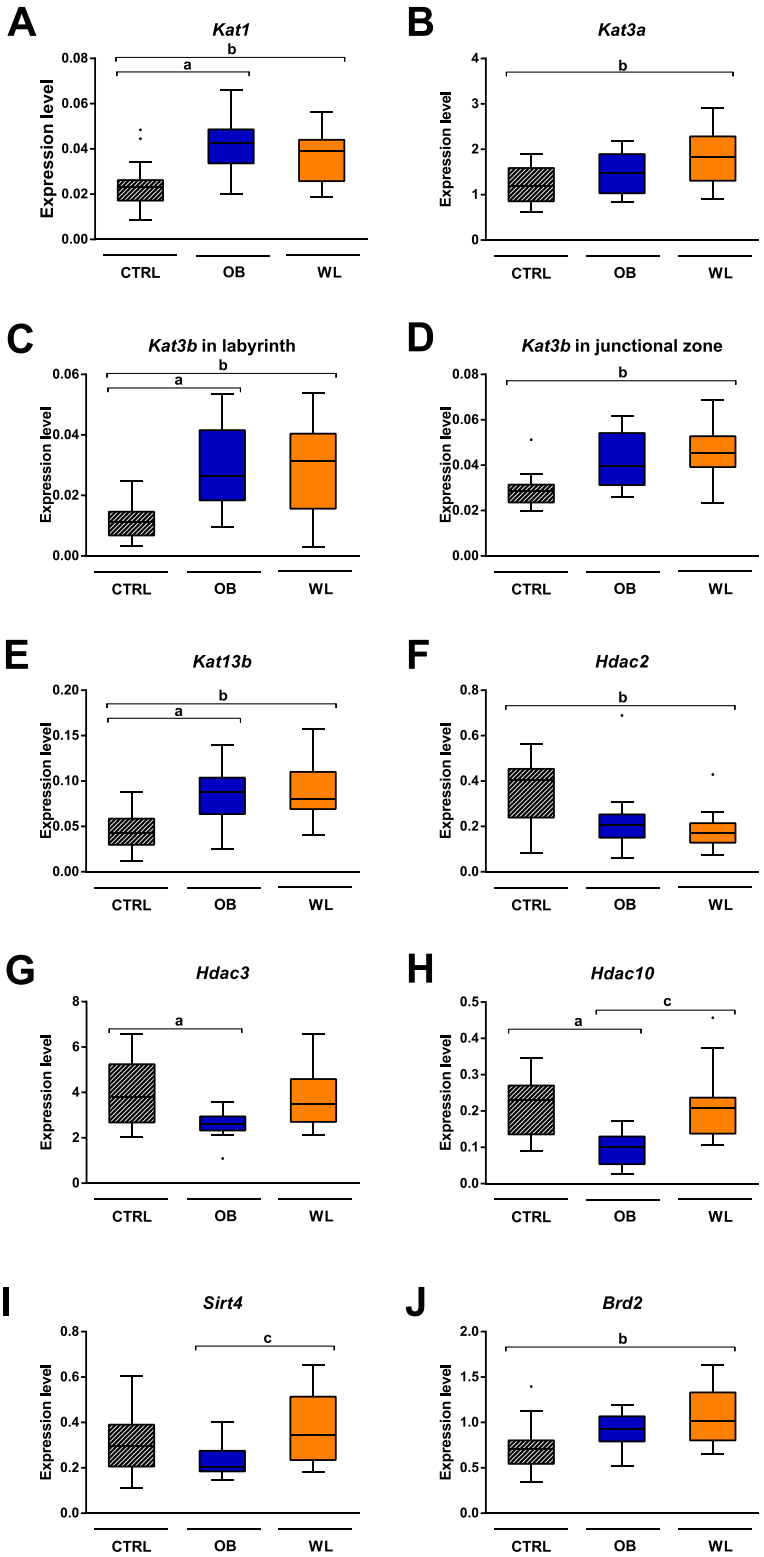


Fig. 7 Genes implicated in histone acetylation are differentially expressed in the placenta at E18.5. We assessed the expression level of 60 epigenetic machinery genes using TaqMan low-density arrays. The expression of *Kat1* (**a**), *Kat3a* (**b**), *Kat3b* (**c**), *Kat13b* (**e**), *Hdac2* (**f**), *Hdac3* (**g**), *Hdac10* (**h**), *Sirt4* (**i**), and *Brd2* (**j**) was affected by maternal obesity and weight loss in the labyrinth. Only *Kat3b* (**d**) was differentially expressed in the junctional zone. (*a*) $P_{adj} < 0.05$ OB vs. CTRL, (*b*) $P_{adj} < 0.05$ WL vs. CTRL, (*c*) $P_{adj} < 0.05$ WL vs. OB. CTRL ($n = 16$), OB ($n = 14$), WL ($n = 16$)

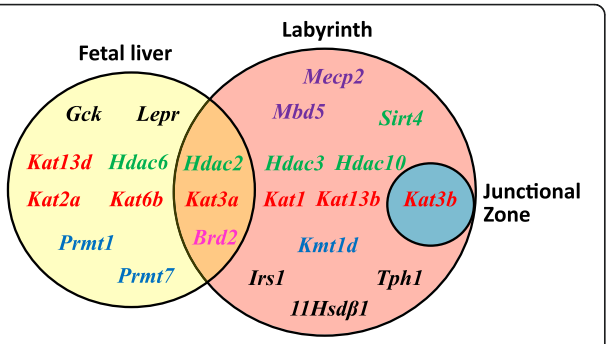


Fig. 8 Differentially expressed genes in the fetal liver, placental labyrinth, and junctional zone. *Venn diagram* shows the names of the genes altered by maternal weight trajectories in the three tissues. Metabolic genes are represented in *black*, methyl CpG-binding proteins in *violet*, arginine methyltransferases and lysine methyltransferases in *blue*, histone deacetylases in *green*, lysine acetyltransferases in *red*, and bromodomain-containing proteins in *pink*

resistance in placental labyrinth and affect these processes. Finally, downregulation of *Tph1* could impair the transformation of maternal tryptophan to serotonin, a process that is necessary for proper fetal brain development [49, 50].

Metabolic diseases are associated with alteration of epigenetic marks [51]. A key finding in this study is that maternal obesity has a major impact on the expression of epigenetic regulators in the fetal liver and placental labyrinth at term. The actors of the histone acetylation pathway were particularly affected. Some families of epigenetic modifiers were not affected by obesity in our study: DNA methyltransferases, TET methylcytosine dioxygenase (TET) proteins, methyl-binding proteins, and lysine demethylases. But this does not preclude changes at previous developmental stages.

Transcript levels of arginine methyltransferases *Prmt1* and *Prmt7* were upregulated in the liver of OB fetuses. Prmts, which catalyze the methylation of arginine histone residues, are implicated in hepatic gluconeogenesis [52]. Prmt1 dimethylates the arginines on forkhead box protein O1 (FOXO1), inducing the translocation of this transcription factor into the nucleus and activation of its target metabolic genes [53]. No implication of Prmt7 in hepatic metabolism or development is known. Implications of the histone arginine methyltransferase activity in FGR or in response to maternal nutritional environment remain to be determined. Only one of the seven studied lysine methyltransferases was differentially expressed in our study: *Kmt1d* was downregulated in the labyrinth of OB dams. No implication of this lysine methyltransferase in placental biology has yet been reported.

Preconceptional weight loss is beneficial to fetal growth and induces an adaptation at the transcriptional level

To our knowledge, it is the first mouse model to study the effects of preconceptional weight loss induced by nutritional intervention on fetal growth and on the transcriptional response in fetal and placental tissues. Despite the normalization of maternal phenotype at conception (Fig. 2), fetuses of WL dams presented transcription differences compared with fetuses of CTRL dams.

The nutritional intervention and preconceptional weight loss restored the FGR observed in OB fetuses. Interestingly, the proportion of SGA fetuses was comparable to the proportion of SGA fetuses from CTRL dams. The first human cohort studies point at rather beneficial effects of maternal weight loss on fetal outcomes, namely a lower risk of large for gestational age (LGA) infants compared to obese patients [27, 28, 54]. In sheep, maternal obesity induced macrosomia and elevated heart, liver, and perirenal adipose tissue weights. A nutritional intervention normalized the fetal and organ weights [55]. In rat studies, there was no effect of maternal obesity or weight loss on fetal weight [56, 57]. Taking into account our results, preconceptional weight loss appears beneficial for fetal growth, counteracting the adverse effects of maternal obesity.

In our expression study, we observed different profiles of transcriptional response to maternal weight loss: mRNA levels of a subset of genes were completely or partially normalized, while other genes remained altered. Overall, the placenta and fetal liver in WL group presented an adaptation in gene expression with high individual variability for some genes.

Finally, some genes were differentially expressed only in WL group: the methyl-DNA binding proteins *Mecp2* and *Mbd5* were upregulated in the labyrinth of WL compared with CTRL and OB. These DNA methylation "readers" are implicated in obesity (MeCP2) and glucose homeostasis regulation (MBD5) [58–60]. In the placenta of calorie-restricted mice, MeCP2 binding to hypermethylated CpG island of *Glut3* was enhanced [61].

We observed a restoration of *Gck* mRNA levels in the fetal liver of WL offspring, and a restoration of *Hsd11β1*, *Irs1*, and *Tph1* levels in the placental labyrinth. Similar results were obtained for *Hsd11β1* in a sheep model of maternal obesity and nutritional preconceptional intervention [62]. The normalization of expression of these genes involved in glucose, glucocorticoid and serotonin metabolism, and insulin signaling by maternal weight loss could abolish the negative effects induced by maternal obesity.

In this study, maternal obesity, despite the nutritional intervention prior to conception, was retained in the gene expression pattern. WL females were severely obese throughout their puberty, when HFD consumption and high adiposity could have affected their gonads. Obesity causes lipid accumulation, mitochondrial dysfunction, and chromosomal abnormalities in oocytes, as well as a modification of follicular liquid content [63, 64]. This can

induce FGR and developmental defects [65]. The negative consequences of maternal obesity could be transmitted, at least in part, via alteration of oocyte function and epigenome. Methylation levels of genes involved in the lipid metabolism could be transmitted to the blastocyst and to adult progeny in mice [66]. In another study, maternal HFD and obesity induces hypermethylation of *Lep* and hypomethylation of *Pparα* in the oocytes and in the liver of adult offspring [67]. The oocyte epigenetic machinery and acetylation levels are sensitive to maternal diabetes [68]. Therefore, in our study, despite the normalization of metabolism by nutritional intervention, some epigenetic marks affected by obesity could be retained in the WL group. Weight loss could also trigger the addition of novel epigenetic marks in oocytes. These epigenetic alterations could provide a mechanism that explains the "memorization" of an obesogenic environment.

The balance between "writers" and "erasers" of histone acetylation could play a role in obesity-induced FGR

In our expression profiling, we studied the transcription of 29 genes involved in histone acetylation (KATs, HDACs, and BRDs), out of 60 epigenetic genes (48 %). Remarkably, 78 % of differentially expressed epigenetic genes in the fetal liver (7 out of 9) and 75 % in the labyrinth (9 out of 12) are involved in histone acetylation. The only differentially expressed gene in the junctional zone (*Kat3b*) is also involved in acetylation pathway. The three genes in common between the liver and labyrinth and the gene differentially expressed in the two placental layers are involved in histone acetylation. Seven members of KAT family, "writers" of lysine acetylation, were upregulated in the fetal liver and placental labyrinth of OB dams. Interestingly, their expression was restored in the fetal liver (*Kat2a*, *Kat3a*, *Kat6b*, and *Kat13d*) of WL dams, but there was clearly no restoration in the labyrinth (*Kat1*, *Kat3a*, *Kat3b*, and *Kat13b*). Moreover, the expression of the majority of HDACs (*Hdac2*, *Hdac3*, and *Hdac10*) that mediate the opposite reaction was reduced in the fetal liver and placental labyrinth of OB dams.

Therefore, we observed a disruption in the balance between the expression of "writers" and "erasers" of lysine acetylation in offspring from obese mother at term of gestation (Table 2). This could lead to an increased level of histone acetylation in FGR offspring of OB dams. In primates, global levels of histone 3 (H3)K14ac were upregulated in the liver at term and after birth in the case of chronic maternal HFD consumption [69]. This hyperacetylation is consistent with reduced expression and enzymatic activity of Hdac1 and Sirt1 [70]. In mice, maternal HFD increased the level of H3K14ac in the fetal liver at E18.5, and this effect persisted after birth [71]. Interestingly, expression of *Hdac1*, *Hdac3*, and *Sirt1* was unaltered at the same stage. In rats, the *Pepck* gene implicated in gluconeogenesis is enriched in H4ac in the liver of fetuses from dams on a HFD, which is consistent with its high expression and fetal hyperglycaemia [39]. In diabetic mice, the expression of *KATs* was upregulated in oocytes, while the expression of *HDACs* was downregulated, as in our study, and the dynamics of acetylation at different lysine residues was disturbed during oocyte maturation [68].

These studies mainly tested the expression of acetylation-related genes. One purpose of our study was to analyze the expression of different families of epigenetic modifiers. The only other extensive report focused on 67 epigenetic modifiers in transcriptomic data from fetal lung, liver, kidney, heart, and placenta of IUGR fetuses, induced by low-protein maternal diet in rats [72]. Histone acetylation modifiers were unchanged while DNA methyltransferases (DNMTs) were differentially expressed in the fetal liver and HDACs in the lung. Therefore, maternal undernutrition or overnutrition could affect different biological processes yet both resulting in FGR.

Some of the differentially expressed KATs or HDACs revealed in our study are implicated in metabolic processes and associated with metabolic diseases, according to different studies in mice and humans. *Hdac2* and *Hdac6* are involved in adipogenesis [73]. HDAC3 is an important regulator of hepatic lipid metabolism in a circadian manner and invalidation of its gene induces hepatic steatosis [74]. Single nucleotide polymorphisms in *KAT13D* (*CLOCK*) gene are associated with obesity and weight loss success in humans [75, 76]. CLOCK is a major circadian regulator: in mice, its mutation disrupts the circadian rhythms and induces a metabolic syndrome [77]. *Kat13b* is involved in energy expenditure and therefore obesity [78]. *Kat3a* is implicated in diabetes by acting on

Table 2 Maternal weight trajectories affect the expression of genes involved in histone acetylation

	Writers: KATs		Erasers: HDACs		Readers: Brd2	
	OB	WL	OB	WL	OB	WL
Fetal liver	↑	Restoration	↑ or ↓	No restoration (trend)/restoration	↑	No restoration
Labyrinth	↑	No restoration	↓	No restoration/restoration	=	↑
Junctional zone	=	↑	-	-	-	-

↑ upregulation, ↓ downregulation, = no significant difference, - no differential expression in this tissue, *KAT* lysine acetyltransferases, *HDAC* histone deacetylases, *BRD* bromodomain-containing proteins

the transcription of gluconeogenetic genes [79]. Kat3b regulates the expression of genes involved in lipogenesis and gluconeogenesis in the liver [80]. Thus, KATs and HDACs have important roles in metabolism and their alteration in obese offspring could indicate major changes in placental and hepatic function.

Bromodomain proteins recognize acetylated lysines and recruit other enzymes to form a multiprotein complex that further enhances transcriptional activity [81]. Brd2 is a negative regulator of adipogenesis via transcriptional repression of *Ppary*. Heterozygous mice invalidated for *Brd2* are obese and have surprisingly better glucose tolerance [82]. The meaning for the increased *Brd2* expression in OB group observed in our study (Table 2, Fig. 5) remains to be determined.

Overall, our results show that epigenetic machinery genes are sensitive to maternal environment and that histone acetylation pathway is particularly affected. Epigenetic modifiers should be considered when studying the offspring's response to maternal metabolic disturbances.

Limitations and strength of the study

In this study, we used a mouse model of obesity-induced FGR, relevant for the investigation of epigenetic mechanisms underlying this pathology, in relation to feto-placental development. This is also the first report of the effects of preconceptional maternal weight loss induced by nutritional intervention on feto-placental growth and gene expression at term. This important contribution highlights the consequences of maternal body weight changes on fetal outcomes and molecular processes, providing evidence to the elaboration the preconceptional counseling for obese women. In our nutritional protocol, we used well-controlled purified HFD containing 59.9 % calories from lipids (mainly saturated and monounsaturated fatty acids). This diet is largely used in metabolic studies in rodents and is different from the "Western diet" characterized by a high-lipid and high-sucrose content [36, 38]. Dietary lipids can have a direct impact on gene transcription by regulation of transcription factors and epigenetic enzymes [24, 83]. However, it is not possible to distinguish the proper effects of maternal obesity (hormonal and inflammatory factors, insulin resistance, and hyperglycemia) and that of dietary lipids in our study. Others tried to address this issue showing differential effects of maternal adiposity and HFD on offspring phenotype, but the effects of lipids on epigenetic processes during early development need further investigation [84, 85]. Another important point to notice is that in our model, 7 weeks of mating were necessary to obtain a large enough sample size. We therefore included maternal age/diet duration (indistinguishable according to our protocol) in our statistical model. Interestingly, the proportion of variance in fetal weight and FPI explained by the maternal age are comparable to the proportion of variance explained by group. This highlights the relevance of maternal age/diet duration for fetal weight and the importance to include it as a covariate.

In our expressional screening, we used a high-output reverse transcription (RT)-real-time polymerase chain reaction (qPCR) technique that simultaneously assessed 96 transcripts. We have studied 60 epigenetic machinery genes, which gives a large overview of the maternal impact on epigenetic regulation processes. These genes were chosen based on their implication in metabolic processes or on our previous study where the effect of maternal HFD during pregnancy showed an altered expression of seven epigenetic genes [30]. Our goal was to study different families of epigenetic modifiers (DNMTs, TETs, lysine methyltransferases (KMTs), lysine-specific demethylases (KDMs), KATs, HDACs, BRDs) because the literature was particularly focused on histone acetylation and DNA methylation. However, some interesting epigenetic families were not taken into account, like the gene-encoding proteins from the polycomb or tritorax families, or nucleosome remodelers, such as switch/sucrose non-fermentable (SWI/SNF). The expression of these genes has never been studied in the context of obesity. Moreover, the expression changes reported here are transcript levels. There may be differences between mRNA levels and proteins level or enzymatic activity. In other studies, Hdac1 and Sirt1 mRNA and protein levels were both affected in the same direction in the context of maternal obesity or HFD [69, 70].

The originality of our study was to compare different parts of the placenta—labyrinth and junctional zone—-which have different structures, cellular content, and function. Indeed, we observed a striking difference in terms of gene expression in these two placental layers, highlighting that obesity could have plausible different impacts on them. We observed a smaller transcriptional response in junctional zone in comparison with labyrinth. A plausible explanation could be that the labyrinth, due to its intimate contact with maternal blood, is more reactive at the transcriptional level, in order to adapt to a dynamic environment. Interestingly, the only affected gene in the junctional zone (*Kat3b*) is differentially expressed in a similar pattern in the labyrinth. KAT3b is implicated in preeclampsia, a disease associated with placental insufficient vascularization, and with the regulation of placental *Hsd11β2* expression via elevated H3K9ac and H3K27ac in humans [86, 87]. Our study highlights a potential important role of KAT3b in the placenta in response to maternal metabolism. Its epigenetic targets need further investigation.

Conclusions

In our mouse model, maternal obesity induced FGR and reduced placental efficiency at term. These phenotypic

changes were associated with alterations of the expression of genes involved in epigenetic processes in the fetal liver and placenta. Nutritional intervention during the preconceptional period allowed maternal weight loss and the normalization of metabolic parameters at mating. In the offspring of WL mothers, fetal growth was partially restored and the transcription was normalized only for a subset of genes affected by maternal obesity. Thus, the history of maternal obesity has an impact on fetal growth and transcriptional activity. The epigenetic machinery is highly sensitive to maternal weight trajectories, which could lead to an altered epigenome in the offspring. Histone acetylation modifiers represented a major part of the differentially expressed genes in OB and WL groups that could account for reminiscence of the obese status.

This study highlights the importance of investigating the mechanisms of regulation of histone marks in response to environmental insults. The link between histone modifiers, histone acetylation levels, and placental and hepatic function should be established. Alteration of the epigenome early during ontogenesis, could be a mechanism of "memorization" of the environment in utero, contributing to particular gene expression patterns and thus to adult phenotype establishment. It could be an underlying mechanism explaining the conditioning of the offspring health later in life [9, 88]. Advances in this direction should help to unravel the molecular mechanisms of developmental conditioning induced by maternal weight trajectories.

Methods

Animal experiments

The COMETHEA ethical committee (Comité d'éthique pour l'expérimentation animale), registered with the national Comité National de Réflexion Ethique sur l'Expérimentation Animale under the no. 45 approved this protocol (visa 12/062). Four-week-old female and 7-week-old male C57Bl/6J OlaHsd mice were received from Harlan Laboratory (Venray, Netherlands) and housed in Unité d'Infectologie Expérimentale des Rongeurs et Poissons (IERP; INRA, Jouy-en-Josas, France). After 1 week of adaptation, the mice were placed in individual cages at controlled temperature (22 ± 2 °C) with a 12-h-light/12-h-dark cycle. Mice had ad libitum access to water and food, and paper towel was provided for nest building.

Five-week-old control females (CTRL) were fed a control diet (CD, 10 % from fat, 70 % from carbohydrates, 20 % from protein; #D12450K) and obese females (OB) were fed a high-fat diet (HFD, 59.9 % from fat, 20.1 % from carbohydrates, 20 % from protein; #D12492) during 4 months before conception and throughout gestation (Fig. 1). Diets were purchased in pellet form from Research Diets (New Brunswick, NJ, USA). After 2 months of HFD (at 14 weeks of age), a subset of the OB females was placed on a CD in order to induce a weight loss (WL group) for the remaining 2 months before conception. Overall, 20 CTRL, 23 OB, and 19 WL females followed nutritional protocol. Females and food on the grid were weighed twice a week. Food intake (FI) was calculated as (food day n (g) – food day 0 (g))/n days, with 3.85 kcal/g for CD and 5.24 kcal/g for HFD. FI-to-body-weight ratio (kcal/kg of body weight/day) was calculated for each mouse. Measurement of fasting cholesterolemia, glycaemia, and oral glucose tolerance test (OGTT) were performed at age 13 and 22 weeks (following 2 and 4 months of diet).

Females were mated individually with randomly assigned, chow-fed (Special Diets Services, Witham, Essex, England; #801030 RM3A) 8–9-week-old males ($n = 12$). The presence of vaginal plug represented embryonic day 0.5 (E0.5). If no plug was observed, females were mated with another male according to their oestrus cycle. All females were mated between 23 and 30 weeks of age and respective diets were maintained throughout mating period and gestation. Body weight of pregnant females was recorded at E0.5 and E18.5. Gestational weight gain was calculated as (body weight at E18.5 (g) – body weight at E0.5 (g))/body weight at E0.5 (g). Pregnant CTRL ($n = 14$), OB ($n = 11$), and WL ($n = 17$) females were weighed and sacrificed by cervical dislocation at E18.5. Tissue sampling was performed on a table maintained at 4 °C. Fetuses and placentas were removed from the uterine horn and placed in a solution of PBS 1×. Maternal carcasses (bodies without the uterus, fetuses, and placentas) were weighed, and proper maternal body weight gain was calculated as (carcass weight at sacrifice (g) – body weight at E0.5 (g))/body weight at E0.5 (g). Fetal development stage was in accordance with the "Theiler Staging Criteria for Mouse Embryo Development" (TS 26). One CTRL fetus and one OB fetus were removed from analysis and tissue collection because of placental necrosis associated with weak fetal weight. Fetal sex was determined by visual examination of the gonads. Fetuses and placentas were weighed (36 CTRL females (F) and 39 CTRL males (M); 35 OB F and 39 OB M; 61 WL F and 58 WL M). Maternal group did not alter the sex ratios of the litters (proportion test: $p = 0.81$; CTRL 48 % F, 52 % M; OB 47 % F, 53 % M; WL 51 % F, 49 % M). The placental labyrinth and junctional zone were separated and collected, along with the fetal liver. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C.

Experimental procedures

Fasting blood glucose levels were measured in all females (20 CTRL, 23 OB, 19 WL). OGTTs were performed in a subset of females on a HFD, which showed

a maximal body weight and weight gain ($n = 11–12$ per group) and in CTRL females within an average range of weight. After 6 h of fasting (8:00 a.m. to 2:00 p.m.), a bolus of glucose (2 g/kg of body weight) was delivered into the stomach of conscious mice with a gavage needle. Tail vein blood glycaemia was measured in duplicates using an Accu-Chek Performa blood glucose meter (Roche diagnostics GmbH, Germany) at time 0, 20, 40, 60, 90, and 120 min. This protocol was described as the best discriminating glucose tolerance between HFD vs. CD mice [89].

Submandibular vein blood (300 μl) from conscious mice was collected in heparinized tubes (Choay heparin, Sanofi-aventis, Paris, France) after 6 h of fasting. Blood samples were centrifuged for 10 min at 1500*g* at 20 °C. Plasma was collected and stored at −20 °C. Total cholesterol plasma levels were measured by colorimetric dosage on Vitros system in Ambroise Paré Hospital (Boulogne-Billancourt, France).

Statistical analysis of physiological data

All analyses were performed with the R statistical software. For the entire preconceptional period, analyses were performed on all mice (20 CTRL, 23 OB, 19 WL). After mating, analyses were restricted to pregnant females (14 CTRL, 11 OB, 17 WL) and their litters. Linear mixed models were used to model the evolution of parameters such as weight, food intake, and food efficiency with time, using the lmer function of the lme4 package in R [90]. Estimates of the slopes for each parameter were reported as "β." Reported p values were obtained with a likelihood ratio test, thanks to the lrtest function of the lmtest package [91]. ANOVA was used to test differences at each time point, thanks to the aov function. Reported p values were obtained with a likelihood ratio test, thanks to the lrtest function of the lmtest package [91]. We performed complete case analyses (i.e., we removed all missing data for each individual, none were imputed). All p values were adjusted for multiple comparisons using the p.adjust function with BH correction [92]. If significance was found, Tukey's post hoc test was used to determine which groups differ from one another. Effect size was reported as the proportion R^2 of the variance in the variable of interest (e.g., body weight, cholesterol, fetal weight) that is explained by maternal group, among the variance not already explained by the covariates. For most variables of interest, groups showed unequal variances. Therefore, investigated parameters were Box-Cox transformed, using the powerTransform and bcPower functions of the car package [93]. This function estimates a transformation for the variable z from the family of transformations indexed by the parameter lambda that makes the residuals from the regression of the transformed z on the predictors as close to normally distributed as possible. We used Pearson's product moment correlation coefficient to test a correlation between fetal weight and placental weight. To adjust for factor "mother," we conducted a regression of fetal weight on mother and a regression of placental weight on mother. The correlation test was performed on the residuals of both models. We used a proportion test to determine differences in sex ratios in the offspring. We calculated a proportion of SGA fetuses as described previously, using Z-score of 1.28 [94]. Logistic regression was used to see the impact of maternal group on the odds of being SGA, using the SGA status of the fetus as the response variable (0 = not SGA and 1 = SGA) and age, sex, and litter size as explanatory variables. Data are represented as Tukey's boxplots indicating the median, 25th, and 75th percentiles. Whiskers indicate the 5th and 95th percentiles. Outliers are shown as dots.

Expression analysis

RNA extraction and DNAse treatment

Only litters with 6–9 fetuses were included in the expression analysis. For each fetus, total RNA was extracted from 20 to 50 mg of the placental labyrinth, junctional zone, or fetal liver. Tissue was reduced to a powder in liquid nitrogen and homogenized in 500 μl of TRIZOL reagent in Mixer Mill MM300 (Qiagen) with one tungsten ball for 2 min at 20 Hz twice. Then, RNA was extracted according to the manufacturer's instructions for RNA isolation (Life Technologies). The aqueous phase containing RNA was collected using Phase Lock Gel Heavy tubes (5 Prime, Hamburg, Germany). The extracted RNA was resuspended in 100 μl of RNase-free water and stored at −20 °C. RNA concentration and purity (A260/A280) was measured using NanoDrop spectrophotometer (NanoDrop Technologies), and a DNase treatment was performed (DNA-free kit, AM1906, Ambion, Life Technologies). The quality of RNA samples was assessed using Bio-Analyzer Agilent 2100 (Agilent Technologies). The RNA integrity number (RIN) of all samples was between the range of 8.8 and 10. For each tissue, 2.5 μg of RNA per fetal sample was pooled according to litter, sex, and maternal diet ($n = 8$ CTRL F, 8 CTRL M, 7 OB F, 7 OB M, 8 WL F, 8 WL M).

Reverse transcription

Six hundred nanograms of RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) according to the manufacturer's recommendations. Reverse transcription were performed in duplicates that were pooled and stored at +4 °C.

Quantitative real-time PCR

Gene expression was quantified using custom TaqMan low-density arrays (TLDAs) (Applied Biosystems). Each array consisted of a 384-well microfluidic card preloaded

with primer sets and 6-FAM-labeled TaqMan probes. The format we chose (96a; Cat. No. 4342261) contained four samples per card; for each sample, the expression of 96 genes was measured, including four control assays. The first set of 60 target genes included genes implicated in epigenetic processes and the second set of 32 target genes included genes implicated in placental/hepatic development or energetic metabolism (Additional file 3). We studied the expression of five DNA methyltransferases (DNMTs), 18 histone deacetylases (HDACs), seven KMTs, seven KDMs, nine lysine acetyltransferases (KATs), five methyl-binding domain proteins (MBDs), two bromodomain proteins (BRDs), four protein arginine N-methyltransferases (PRMTs), and three enzymes of DNA hydroxymethylation (TETs). All assays and their assay ID numbers are listed in Additional file 2: Table S1. All probes spanned a gene, an exon-exon junction, except for the following assays: Actb (Mm00607939_s1), Mrpl32 (Mm00777741_sH), Bdnf (Mm04230607_s1), Cebpα (Mm00514283_s1), and Cebpβ (Mm00843434_s1), where primers and probes mapped within a single exon. In addition, the following assays may detect genomic DNA: 18S (Hs99999901_s1), Hdac1 (Mm02391771_g1), Hdac10 (Mm01308118_g1), and Mecp2 (Mm01193537_g1). Thus, all samples were treated with DNase. The experiment was performed on the BRIDGE-ICE platform (INRA, Jouy-en-Josas, France) according to the manufacturer's instructions. Four samples were run on each TLDA card in simplicates. Each sample reservoir on the card was loaded with 100 μl of the reaction mix: cDNA template (600 ng) mixed with TaqMan Gene Expression Master Mix (Applied Biosystems). After centrifugation (twice 1 min at 1200 rpm, Heraeus Multifuge 3S Centrifuge), the wells were sealed with a TLDA Sealer (Applied Biosystems). Real-time PCR amplification was performed on the 7900HT Real-Time PCR System (Applied Biosystems) using SDS 2.4 software with standard conditions: 2 min 50 °C, 10 min 94.5 °C, 30 s 97 °C (40 cycles), 1 min 59.7 °C.

Normalization of expression level

Six potential reference genes were tested on the fetal liver and placental tissue samples: *Eif4a2* (eukaryotic translation initiation factor 4A2), *ActB* (Actin beta), *Tbp* (TATA box-binding protein), *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *Mrpl32* (mitochondrial ribosomal protein L32), and *Sdha* (succinate dehydrogenase complex, subunit A, flavoprotein). Using the GeNorm software, *ActB*, *Sdha*, and *Mrpl32* were chosen for normalization of expression levels [95]. Threshold cycle (Ct) values were calculated with the ExpressionSuite v1.0.3 software (Applied Biosystems). The detection threshold was set manually for all genes and was the same for each assay in all tissues. Ct = 39 was used as the cutoff: above this value, expression level was set to 0. Genes were assigned as non-amplified (NA) if more than 15 % of samples were NA. All NA genes (Additional file 4: Table S2; 12 in the fetal liver, 6 in the placental labyrinth, and 3 in the junctional zone) were removed from the analysis. Normalization was performed independently for same-sex samples within each maternal group (CTRL F, CTRL M, OB F, OB M, WL F, WL M). For each of these groups, $Ct_{[ref]}$ was the mean of the three Ct values of the reference genes. Then, expression level of target genes was calculated as $2^{-(Ct[target\ gene] - Ct[ref])}$, as previously described [96].

Hierarchical clustering

For each tissue, transcription values of each target gene were averaged across same-sex samples within each maternal group (CTRL F, CTRL M, OB F, OB M, WL F, WL M). Only genes with expression >0 were taken into account (80 in the liver, 86 in the labyrinth, and 89 in the junctional zone). Missing Ct values were imputed (7 in the liver, 7 in the labyrinth, and 4 in the junctional zone out of 4416 values per tissue), assigning the mean Ct value of the samples with similar sex and maternal diet. Then, hierarchical clustering was performed using Pearson's correlation coefficient as distance function and Ward as linkage method.

Statistical analysis of TLDA expression study

Analysis was performed with the R statistical software. For each gene, we compared the expression values between males and females within each maternal group. As we did not detect an effect of sex on the expression for any of the studied genes (except for *Kdm5d* and *Uty*, both located on Y chromosome), we pooled males and females within each maternal group. Pairwise comparisons of maternal groups were conducted using a permutation test, as implemented in the oneway_test function of the *coin* package in R [97]. For each set of tests (i.e., all tested genes for a given pair of maternal groups), p values were adjusted for multiple testing as proposed by Benjamini and Hochberg [92]. Differences were considered significant when $P_{adj} < 0.05$. Data are shown as means ± St. Dev or as Tukey's boxplots.

Additional files

Additional file 1: Supplementary Figures S1 to S6. Food intake (FI) to body weight (BW) ratio in females during the preconceptional period. (a) $P < 0.05$ OB vs. CTRL, (b) $P < 0.05$ WL vs. CTRL, (c) $P < 0.05$ WL vs. OB. $n = 18$–20 CTRL, 23 OB, 17–19 WL. **Figure S2.** Fetal weight as a function of litter size in dams at E18.5. Both sexes were combined as there was no effect of sex on fetal weight. **Figure S3.** Placental weight as a function of litter size and sex at E18.5. **Figure S4.** Effect of maternal age on fetal parameters. (A) Relationship between maternal age and fetal weight. (B) Relationship between maternal age and fetal-weight-to-placental-weight

ratio index (FPI). For statistical analysis, see text. **Figure S5.** Distribution of fetal weight in CTRL, OB, and WL dams at E18.5. CTRL dams are represented in black, WL dams in brown, and OB dams in blue. The red line represents the 10th percentile of CTRL population. **Figure S6.** Relationship between fetal and placental weight in female and male offspring at E18.5. For statistical analysis, see text and "Methods" section. M: males, F: females.

Additional file 2: List of the gene symbol and name, and the design strand and TaqMan® Gene Expression Assay IDentification reference for the 96 genes studied on the Custom TLDA.

Additional file 3: Description of the selection criteria of genes for the custom TLDA design and related bibliographic references.

Additional file 4: Expression level and adjusted *P* values of 92 genes in the fetal liver, placental labyrinth, and junctional zone. We assessed the expression level of 60 epigenetic machinery genes and 32 genes implicated in metabolism or development in CTRL, OB, and WL females at E18.5 using TaqMan low-density arrays. Data are represented as mean expression levels ± St.Dev. *—differentially expressed genes. NA—non-amplified. Significant differences ($P_{adj} < 0.05$) are indicated in red.

Abbreviations

BRDs: bromodomain proteins; CD: control diet; CTRL: control group; DNMTs: DNA methyltransferases; DOHaD: developmental origins of health and disease; E18.5: embryonic day 18.5; FGR: fetal growth restriction; FPI: fetal-weight-to-placental-weight ratio index; H3: histone 3; HDACs: histone deacetylases; HFD: high-fat diet; KATs: lysine acetyltransferases; IUGR: intrauterine growth restriction; KDMs: lysine-specific demethylases; KMTs: lysine methyltransferases; LGA: large for gestational age; MBDs: methyl-binding domain proteins; NA: non-amplified; OB: obese group; OGTT: oral glucose tolerance test; PRMTs: protein arginine N-methyltransferases; P_{adj}: adjusted *p* value; qPCR: real-time polymerase chain reaction; RT: reverse transcription; SGA: small for gestational age; TETs: TET methylcytosine dioxygenases; TLDA: TaqMan low-density array; WL: weight loss group.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AG and CJ conceived, designed, and supervised the project. PEP, MJ, AP, and AG performed the experiments. PEP, SV, LJ, and AG performed the analysis of the data. PEP, AG, HJ, SL, CB, CJ, and AG analyzed and discussed the data. PEP and AG wrote the draft manuscript. HJ, CB, and CJ critically reviewed the manuscript for important intellectual content. All authors read and approved the final manuscript.

Acknowledgements

The help of Marion Lemaire with the expression study is greatly acknowledged. We are grateful to Dr. Amanda Sferruzzi-Perri for sharing with us her expertise in placental layers sampling. We thank the members of the Infectiologie Expérimentale des Rongeurs et Poissons Unit (UE 0907) of the INRA (Jouy-en-Josas) for their professionalism and help with animal experiments. We thank Bénédicte Langelier (Micalis), Nicolas Crapart, and Claudia Bevilacqua (BRIDGE-ICE Platform) of the INRA (Jouy-en-Josas) for technical assistance with TLDA expression study. We wish to thank the members of Neurobiologie de l'Olfaction Unity (UR1197) of INRA for help with tissue sampling. This study was supported by the Fondation Cœur et Artères (FCA 13T1) and the Institut Benjamin Delessert. PEP and SV were supported by the Ministère de la Recherche.
The data sets supporting the results of this article are included within the article and its additional files, available at the Clin Epigenetic's website.

Author details

[1]UMR BDR, INRA, ENVA, Université Paris Saclay, 78350 Jouy en Josas, France. [2]Ecole Doctorale 394 "Physiologie, physiopathologie et thérapeutique", Université Pierre et Marie Curie, 75252 Paris, France. [3]Univ. Lille, EA4489, Équipe Malnutrition Maternelle et Programmation des Maladies Métaboliques, F59000 Lille, France. [4]Université Versailles Saint-Quentin en Yvelines (UVSQ), Guyancourt, France.

Received: 24 November 2015 Accepted: 12 February 2016
Published online: 27 February 2016

References

1. Ng M. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 384: ed. 2014 May 29;766–81.
2. Lee CYW, Koren G. Maternal obesity: effects on pregnancy and the role of pre-conception counselling. J Obstet Gynaecol. 2010;30:101–6.
3. Acosta O, Ramirez VI, Lager S, Gaccioli F, Dudley DJ, Powell TL, et al. Increased glucose and placental GLUT-1 in large infants of obese nondiabetic mothers. Am J Obstet Gynecol. 2015;212:227. e1–227.e7.
4. Gaudet L, Ferraro ZM, Wen SW, Walker M. Maternal obesity and occurrence of fetal macrosomia: a systematic review and meta-analysis. BioMed Res Int. 2014;2014:1–22.
5. Liu L, Hong Z, Zhang L. Associations of prepregnancy body mass index and gestational weight gain with pregnancy outcomes in nulliparous women delivering single live babies. Sci Rep. 2015;5:12863.
6. Rajasingam D, Seed PT, Briley AL, Shennan AH, Poston L. A prospective study of pregnancy outcome and biomarkers of oxidative stress in nulliparous obese women. Am J Obstet Gynecol. 2009;200:395. e1–395.e9.
7. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, et al. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ. 1991;303: 1019–22.
8. Fernandez-Twinn DS, Ozanne SE. Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome. Physiol Behav. 2006;88:234–43.
9. Hanson MA, Gluckman PD. Early developmental conditioning of later health and disease: physiology or pathophysiology? Physiol Rev. 2014;94:1027–76.
10. Hayes EK, Lechowicz A, Petrik JJ, Storozhuk Y, Paez-Parent S, Dai Q, et al. Adverse fetal and neonatal outcomes associated with a life-long high fat diet: role of altered development of the placental casculature. Reiss I, editor. PLoS ONE. 2012;7:e33370.
11. Sferruzzi-Perri AN, Vaughan OR, Haro M, Cooper WN, Musial B, Charalambous M, et al. An obesogenic diet during mouse pregnancy modifies maternal nutrient partitioning and the fetal growth trajectory. FASEB J. 2013;27:3928–37.
12. Tarrade A, Rousseau-Ralliard D, Aubrière M-C, Peynot N, Dahirel M, Bertrand-Michel J, et al. Sexual dimorphism of the feto-placental phenotype in response to a high fat and control maternal diets in a rabbit model. Zenclussen AC, editor. PLoS ONE. 2013;8:e83458.
13. Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. Placenta. 2002;23:3–19.
14. McCurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. J Clin Invest. 2009;119(2):323–35.
15. Plata M del M, Williams L, Seki Y, Hartil K, Kaur H, Lin C-L, et al. Critical periods of increased fetal vulnerability to a maternal high fat diet. Reprod Biol Endocrinol RBE. 2014;12:80.
16. Swanson AM, David AL. Animal models of fetal growth restriction: considerations for translational medicine. Placenta. 2015;36:623–30.
17. Attig L, Gabory A, Junien C. Early nutrition and epigenetic programming: chasing shadows. Curr Opin Clin Nutr Metab Care. 2010;13:284–93.
18. Delahaye F, Wijetunga NA, Heo HJ, Tozour JN, Zhao YM, Greally JM, et al. Sexual dimorphism in epigenomic responses of stem cells to extreme fetal growth. Nat Commun. 2014;5:5187.
19. Fu Q. Uteroplacental insufficiency induces site-specific changes in histone H3 covalent modifications and affects DNA-histone H3 positioning in day 0 IUGR rat liver. Physiol Genomics. 2004;20:108–16.
20. MacLennan NK. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. Physiol Genomics. 2004;18:43–50.
21. Park JH, Stoffers DA, Nicholls RD, Simmons RA. Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. J Clin Invest. 2008;118:2316–24.
22. Raychaudhuri N, Raychaudhuri S, Thamotharan M, Devaskar SU. Histone code modifications repress glucose transporter 4 expression in the intrauterine growth-restricted offspring. J Biol Chem. 2008;283:13611–26.
23. Gabory A, Attig L, Junien C. Developmental programming and epigenetics. Am J Clin Nutr. 2011;94:1943S–52S.
24. Donohoe DR, Bultman SJ. Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. J Cell Physiol. 2012;227:3169–77.

25. Waterland RA, Michels KB. Epigenetic epidemiology of the developmental origins hypothesis. Annu Rev Nutr. 2007;27:363–88.
26. Paden MM, Avery DM. Preconception counseling to prevent the complications of obesity during pregnancy. Am J Clin Med. 2012;9:30–5.
27. Forsum E, Brantsæter AL, Olafsdottir A-S, Olsen SF, Thorsdottir I. Weight loss before conception: a systematic literature review. Food Nutr Res. 2013;57.
28. Villamor E, Cnattingius S. Interpregnancy weight change and risk of adverse pregnancy outcomes: a population-based study. The Lancet. 2006;368:1164–70.
29. Diouf I, Charles MA, Thiebaugeorges O, Forhan A, Kaminski M, Heude B, et al. Maternal weight change before pregnancy in relation to birthweight and risks of adverse pregnancy outcomes. Eur J Epidemiol. 2011;26:789–96.
30. Gabory A, Ferry L, Fajardy I, Jouneau L, Gothié J-D, Vigé A, et al. Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta. Aguila MB, editor. PLoS ONE. 2012;7:e47986.
31. Mao J, Zhang X, Sieli PT, Falduto MT, Torres KE, Rosenfeld CS. Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. Proc Natl Acad Sci. 2010;107:5557–62.
32. Clifton VL. Review: sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta. 2010;31:S33–9.
33. Attig L, Vigé A, Gabory A, Karimi M, Beauger A, Gross M-S, et al. Dietary alleviation of maternal obesity and diabetes: increased resistance to diet-induced obesity transcriptional and epigenetic signatures. PloS One. 2013;8, e66816.
34. Rosario FJ, Kanai Y, Powell TL, Jansson T. Increased placental nutrient transport in a novel mouse model of maternal obesity with fetal overgrowth: maternal obesity and placental nutrient transport. Obesity. 2015;23:1663–70.
35. Gheorghe CP, Goyal R, Mittal A, Longo LD. Gene expression in the placenta: maternal stress and epigenetic responses. Int J Dev Biol. 2010;54:507–23.
36. King V, Hibbert N, Seckl JR, Norman JE, Drake AJ. The effects of an obesogenic diet during pregnancy on fetal growth and placental gene expression are gestation dependent. Placenta. 2013;34:1087–90.
37. Sasson IE, Vitins AP, Mainigi MA, Moley KH, Simmons RA. Pre-gestational vs gestational exposure to maternal obesity differentially programs the offspring in mice. Diabetologia. 2015;58:615–24.
38. Jones HN, Woollett LA, Barbour N, Prasad PD, Powell TL, Jansson T. High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. FASEB J. 2009;23:271–8.
39. Strakovsky RS, Zhang X, Zhou D, Pan Y-X. Gestational high fat diet programs hepatic phosphoenolpyruvate carboxykinase gene expression and histone modification in neonatal offspring rats: programming gluconeogenesis by gestational high fat diet. J Physiol. 2011;589:2707–17.
40. Lane RH, MacLennan NK, Hsu JL, Janke SM, Pham TD. Increased hepatic peroxisome proliferator-activated receptor- coactivator-1 gene expression in a rat model of intrauterine growth retardation and subsequent insulin resistance. Endocrinology. 2002;143:2486–90.
41. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic β cell-specific gene knock-outs using Cre recombinase. J Biol Chem. 1999;274:305–15.
42. Mele J, Muralimanoharan S, Maloyan A, Myatt L. Impaired mitochondrial function in human placenta with increased maternal adiposity. AJP Endocrinol Metab. 2014;307:E419–25.
43. Dube E, Gravel A, Martin C, Desparois G, Moussa I, Ethier-Chiasson M, et al. Modulation of fatty acid transport and metabolism by maternal obesity in the human full-term placenta. Biol Reprod. 2012;87:14–4.
44. Muramatsu-Kato K, Itoh H, Kobayashi-Kohmura Y, Murakami H, Uchida T, Suzuki K, et al. Comparison between placental gene expression of 11β-hydroxysteroid dehydrogenases and infantile growth at 10 months of age: placental 11βHSD and infantile growth. J Obstet Gynaecol Res. 2014;40:465–72.
45. Struwe E, Berzl GM, Schild RL, Beckmann MW, Dörr HG, Rascher W, et al. Simultaneously reduced gene expression of cortisol-activating and cortisol-inactivating enzymes in placentas of small-for-gestational-age neonates. Am J Obstet Gynecol. 2007;197:43. e1–43.e6.
46. Street ME, Viani I, Ziveri MA, Volta C, Smerieri A, Bernasconi S. Impairment of insulin receptor signal transduction in placentas of intra-uterine growth-restricted newborns and its relationship with fetal growth. Eur J Endocrinol Eur Fed Endocr Soc. 2011;164:45–52.
47. Colomiere M, Permezel M, Riley C, Desoye G, Lappas M. Defective insulin signaling in placenta from pregnancies complicated by gestational diabetes mellitus. Eur J Endocrinol. 2009;160:567–78.
48. Hiden U, Glitzner E, Hartmann M, Desoye G. Insulin and the IGF system in the human placenta of normal and diabetic pregnancies. J Anat. 2009;215:60–8.
49. Bonnin A, Goeden N, Chen K, Wilson ML, King J, Shih JC, et al. A transient placental source of serotonin for the fetal forebrain. Nature. 2011;472:347–50.
50. Goeden N, Velasquez JC, Bonnin A. Placental tryptophan metabolism as a potential novel pathway for the developmental origins of mental diseases. Transl Dev Psychiatry. 2013;1:20593.
51. Martinez JA, Milagro FI, Claycombe KJ, Schalinske KL. Epigenetics in adipose tissue, obesity, weight loss, and diabetes. Adv Nutr Int Rev J. 2014;5:71–81.
52. Han H-S, Choi D, Choi S, Koo S-H. Roles of protein arginine methyltransferases in the control of glucose metabolism. Endocrinol Metab. 2014;29:435.
53. Yamagata K, Daitoku H, Takahashi Y, Namiki K, Hisatake K, Kako K, et al. Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. Mol Cell. 2008;32:221–31.
54. Bogaerts A, Ameye L, Martens E, Devlieger R. Weight loss in obese pregnant women and risk for adverse perinatal outcomes. Obstet Gynecol. 2015;125:566–75.
55. Tuersunjiang N, Odhiambo JF, Long NM, Shasa DR, Nathanielsz PW, Ford SP. Diet reduction to requirements in obese/overfed ewes from early gestation prevents glucose/insulin dysregulation and returns fetal adiposity and organ development to control levels. Am J Physiol Endocrinol Metab. 2013;305:E868–78.
56. Srinivasan M. Maternal hyperinsulinemia predisposes rat fetuses for hyperinsulinemia, and adult-onset obesity and maternal mild food restriction reverses this phenotype. AJP Endocrinol Metab. 2005;290:E129–34.
57. Zambrano E, Martínez-Samayoa PM, Rodríguez-González GL, Nathanielsz PW. RAPID REPORT: dietary intervention prior to pregnancy reverses metabolic programming in male offspring of obese rats: dietary intervention to reverse metabolic programming outcomes. J. Physiol. 2010;588:1791–9.
58. Du Y, Liu B, Guo F, Xu G, Ding Y, Liu Y, et al. The essential role of Mbd5 in the regulation of somatic growth and glucose homeostasis in mice. Chowen JA, editor. PLoS ONE. 2012;7:e47358.
59. Wang X, Lacza Z, Sun YE, Han W. Leptin resistance and obesity in mice with deletion of methyl-CpG-binding protein 2 (MeCP2) in hypothalamic pro-opiomelanocortin (POMC) neurons. Diabetologia. 2014;57:236–45.
60. Fyffe SL, Neul JL, Samaco RC, Chao H-T, Ben-Shachar S, Moretti P, et al. Deletion of Mecp2 in Sim1-expressing neurons reveals a critical role for MeCP2 in feeding behavior, aggression, and the response to stress. Neuron. 2008;59:947–58.
61. Ganguly A, Chen Y, Shin B-C, Devaskar SU. Prenatal caloric restriction enhances DNA methylation and MeCP2 recruitment with reduced murine placental glucose transporter isoform 3 expression. J Nutr Biochem. 2014;25:259–66.
62. Nicholas LM, Rattanatray L, MacLaughlin SM, Ozanne SE, Kleemann DO, Walker SK, et al. Differential effects of maternal obesity and weight loss in the periconceptional period on the epigenetic regulation of hepatic insulin-signaling pathways in the offspring. FASEB J. 2013;27:3786–96.
63. Wu LL-Y, Dunning KR, Yang X, Russell DL, Lane M, Norman RJ, et al. High-fat diet causes lipotoxicity responses in cumulus–oocyte complexes and decreased fertilization rates. Endocrinology. 2010;151:5438–45.
64. Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, et al. Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. J Clin Endocrinol Metab. 2009;94:1533–40.
65. Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, et al. High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. Clarke H, editor. PLoS ONE. 2012;7:e49217.
66. Wei Y, Yang C-R, Wei Y-P, Ge Z-J, Zhao Z-A, Zhang B, et al. Enriched environment-induced maternal weight loss reprograms metabolic gene expression in mouse offspring. J Biol Chem. 2015;290:4604–19.
67. Ge Z-J, Luo S-M, Lin F, Liang Q-X, Huang L, Wei Y-C, et al. DNA methylation in oocytes and liver of female mice and their offspring: effects of high-fat-diet-induced obesity. Environ Health Perspect. 2014;122:159–64.
68. Ding L, Pan R, Huang X, Wang J-X, Shen Y-T, Xu L, et al. Changes in histone acetylation during oocyte meiotic maturation in the diabetic mouse. Theriogenology. 2012;78:784–92.
69. Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, et al. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J Mol Endocrinol. 2008;41:91–102.
70. Suter MA, Chen A, Burdine MS, Choudhury M, Harris RA, Lane RH, et al. A maternal high-fat diet modulates fetal SIRT1 histone and protein deacetylase activity in nonhuman primates. FASEB J. 2012;26:5106–14.

71. Suter MA, Ma J, Vuguin PM, Hartil K, Fiallo A, Harris RA, et al. In utero exposure to a maternal high-fat diet alters the epigenetic histone code in a murine model. Am J Obstet Gynecol. 2014;210:463. e1–463.e11.
72. Vaiman D, Gascoin-Lachambre G, Boubred F, Mondon F, Feuerstein J-M, Ligi I, et al. The intensity of IUGR-induced transcriptome deregulations is inversely correlated with the onset of organ function in a rat model. Baud O, editor. PLoS ONE. 2011;6:e21222.
73. Yoo EJ, Chung J-J, Choe SS, Kim KH, Kim JB. Down-regulation of histone deacetylases stimulates adipocyte differentiation. J Biol Chem. 2006;281: 6608–15.
74. Feng D, Liu T, Sun Z, Bugge A, Mullican SE, Alenghat T, et al. A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. Science. 2011;331:1315–9.
75. Sookoian S, Gemma C, Gianotti TF, Burgueño A, Castaño G, Pirola CJ. Genetic variants of Clock transcription factor are associated with individual susceptibility to obesity. Am J Clin Nutr. 2008;87:1606–15.
76. Garaulet M, Corbalan MD, Madrid JA, Morales E, Baraza JC, Lee Y-C, et al. CLOCK gene is implicated in weight reduction in obese patients participating in a dietary programme based on the Mediterranean diet. Int J Obes. 2010;34:516–23.
77. Turek FW. Obesity and metabolic syndrome in circadian Clock mutant mice. Science. 2005;308:1043–5.
78. Coste A, Louet J-F, Lagouge M, Lerin C, Antal MC, Meziane H, et al. The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1α. Proc Natl Acad Sci. 2008;105:17187–92.
79. Zhou XY, Shibusawa N, Naik K, Porras D, Temple K, Ou H, et al. Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB-binding protein. Nat Med. 2004;10:633–7.
80. Bricambert J, Miranda J, Benhamed F, Girard J, Postic C, Dentin R. Salt-inducible kinase 2 links transcriptional coactivator p300 phosphorylation to the prevention of ChREBP-dependent hepatic steatosis in mice. J Clin Invest. 2010;120:4316–31.
81. Marmorstein R, Zhou M-M. Writers and readers of histone acetylation: structure, mechanism, and inhibition. Cold Spring Harb Perspect Biol. 2014;6:a018762–2.
82. Wang F, Liu H, Blanton WP, Belkina A, Lebrasseur NK, Denis GV. *Brd2* disruption in mice causes severe obesity without type 2 diabetes. Biochem J. 2010;425:71–83.
83. Jump DB, Tripathy S, Depner CM. Fatty acid-regulated transcription factors in the liver. Annu Rev Nutr. 2013;33:249–69.
84. White CL, Purpera MN, Morrison CD. Maternal obesity is necessary for programming effect of high-fat diet on offspring. AJP Regul Integr Comp Physiol. 2009;296:R1464–72.
85. Howie GJ, Sloboda DM, Kamal T, Vickers MH. Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet: maternal high fat nutrition and obesity in offspring. J Physiol. 2009;587:905–15.
86. Li J, Wang W, Liu C, Wang W, Li W, Shu Q, et al. Critical role of histone acetylation by p300 in human placental 11β-HSD2 expression. J Clin Endocrinol Metab. 2013;98:E1189–97.
87. Vaiman D, Calicchio R, Miralles F. Landscape of transcriptional deregulations in the preeclamptic placenta. PLoS ONE. 2013;8(6), e65498.
88. Portha B, Fournier A, Ah Kioon MD, Mezger V, Movassat J. Early environmental factors, alteration of epigenetic marks and metabolic disease susceptibility. Biochimie. 2014;97:1–15.
89. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. AJP Endocrinol Metab. 2008;295:E1323–32.
90. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015;67.
91. Zeileis A, Hothorn T. Diagnostic checking in regression relationships. R News. 2002;2:7–10.
92. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol. 1995;57:289–300.
93. Fox J, Sanford W. An {R} companion to applied regression. second. Thousand Oaks {CA}: Sage; 2011.
94. Dilworth MR, Kusinski LC, Baker BC, Renshall LJ, Greenwood SL, Sibley CP, et al. Defining fetal growth restriction in mice: a standardized and clinically relevant approach. Placenta. 2011;32:914–6.
95. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:research0034.
96. Gabory A, Ripoche M-A, Le Digarcher A, Watrin F, Ziyyat A, Forné T, et al. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. Dev Camb Engl. 2009;136:3413–21.
97. Zeileis A, Wiel MA, Hornik K, Hothorn T. Implementing a class of permutation tests: the coin package. J Stat Softw. 2008;28:1–23.