



Original / *Obesidad*

# Genome-wide screen of promoter methylation identifies novel markers in diet-induced obese mice

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## Abstract

**Objective:** To investigate the genome-wide promoter methylation and gene expression for the identification of methylation markers in obesity.

**Methods:** Using a high-fat, diet-induced obese mouse model, we performed comprehensive DNA methylation profiling of gene promoters to determine the differentially methylated genes using methylated DNA immunoprecipitation followed by hybridization to the NimbleGen MM8 CpG plus Promoter Microarray. We further integrated epigenomics data with gene expression profiling to identify promoters exhibiting an association between methylation status and the expression of downstream genes.

**Results:** A total of 24 hypermethylated promoters and 42 hypomethylated promoters in epididymal fat were selected as methylation markers, which were associated with downregulated and upregulated gene expression, respectively. The promoter methylation and differential gene expression of three markers (Mmp2, Foxj3 and Ube2q2) in the fat were validated by sequencing bisulfite-modified DNA and real-time reverse transcriptase PCR. The genes with these differentially methylated promoters and the associated transcriptional expression in the fat were primarily involved in biological activities in lipid metabolism and storage, cellular differentiation, immunity and the pathogenesis of obesity-related complications.

**Conclusions:** This study represents the first effort to determine methylation markers in obese mice that may regulate gene transcription in obesity. Our approach has potential relevance for clinical applications by identifying markers useful in elucidating the mechanisms of obesity pathogenesis and its complications.

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Key words: Obesity. Genome-wide DNA methylation. Gene expression. High-fat diet. Mouse.

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## EL CRIBADO PANGENÓMICO DE LA METILACIÓN DEL PROMOTOR IDENTIFICA MARCADORES NUEVOS EN RATONES CON OBESIDAD INDUCIDA POR LA DIETA

### Resumen

**Objetivo:** Investigar la metilación pangenómica del promotor y la expresión génica para la identificación de los marcadores de metilación en la obesidad.

**Métodos:** Empleando un modelo de ratón con obesidad inducida por la dieta con alto contenido en grasa, realizamos un perfil exhaustivo de la metilación del ADN de los genes promotores para determinar los genes metilados diferencialmente utilizando la inmunoprecipitación del ADN metilado seguida de la hibridación del NimbleGen MM8 CpG y el Promoter Microarray. Posteriormente, integramos los datos de la epigenómica con el perfil de expresión génica para identificar los promotores que mostraban una asociación entre el estado de metilación y la expresión de los genes sucesivos.

**Resultados:** Se seleccionó un total de 24 promotores hipermetilados y 42 promotores hipometilados en la grasa epididimaria como marcadores de la metilación, que se asociaron con la expresión génica regulada al alza y a la baja, respectivamente. La metilación del promotor y la expresión génica diferencial de tres marcadores (Mmp2, Foxj3 y Ube2q2) de la grasa se validaron mediante secuenciación del ADN modificado por bisulfito y por PCR de la transcriptasa reversa en tiempo real. Los genes con estos promotores metilados de forma diferencial y la expresión transcripcional asociada en la grasa estaban implicados primariamente en las actividades biológicas del metabolismo y almacenamiento de los lípidos, la diferenciación celular, la inmunidad y la patología de las complicaciones relacionadas con la obesidad.

**Conclusiones:** Este estudio representa el primer intento por determinar los marcadores de la metilación en los ratones obesos que pueden regular la transcripción génica en la obesidad. Nuestro abordaje tiene una relevancia potencial por sus aplicaciones clínicas al identificar marcadores útiles en la dilucidación de los mecanismos de la patología de la obesidad y sus complicaciones.

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Palabras clave: Obesidad. Metilación pangenómica del ADN. Expresión génica. Dieta rica en grasa. Ratón.

## Introduction

In recent years, epigenetic modifications such as DNA methylation and histone modifications have been highlighted in chronic non-communicable diseases due to their role in chromatin structure and gene expression related to physiologic and pathologic processes and their potential use as markers for disease onset, progression, diagnosis and prognosis<sup>1-4</sup>. DNA methylation is the only known modification that targets the DNA itself. Compared to histones, which undergo a variety of post-translational modifications under different conditions, DNA methylation is relatively stable over a longer period. The methylation of cytosine residues at CpG dinucleotides in gene promoters or CpG islands is well described and known to have profound effects on the regulation of gene expression<sup>1,3,5</sup>.

Emerging evidence indicates that epigenetic changes are closely associated with obesity and related complications. The candidate gene methylation studies in animal models and humans have demonstrated methylation changes in promoters of varied genes implicated in obesity, appetite control and/or metabolism, insulin signaling, immunity, growth and circadian clock regulation<sup>6-9</sup>. Genome-wide analyses in human peripheral blood cells have detected obesity-associated differentially methylated sites of genes with a wide diversity of functions, such as immune response, cell differentiation and the regulation of transcription<sup>9</sup>. Additionally, the methylation profiles of obese individuals can be modified by reductions in body weight/fat mass. For example, promoter methylation levels of the leptin and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) genes may predict the susceptibility to weight loss as well as comorbidities such as hypertension or type 2 diabetes<sup>10,11</sup>. Thus, the role of epigenetic modifications in obesity is a fruitful area for further research. Thus far, the epigenome-wide association with gene expression in obesity has never been explored.

The methylation microarrays and high-throughput sequencing technologies allow for large-scale epigenome-wide association studies (EWASs) and the determination of epigenomic events in the development of metabolic diseases<sup>12</sup>. In the present study, we used the methylated DNA immunoprecipitation (MeDIP) approach, which generates an enrichment of methylated genomic fragments by means of an anti-5-methylcytosine antibody combined with the hybridization of the fragments to a whole-genome promoter array. We then performed the MEDME (modeling experimental data with MeDIP enrichment) post-processing routine to determine the methylation levels in diet-induced obese (DIO) mice. Finally, an analysis of the potential methylation markers, along with additional gene expression experiments, revealed promoter features that appeared to be relevant for transcriptional regulation. The identification of methylation changes in specific genes will provide important targets for

further study into the underlying mechanisms and the therapeutic potential for obesity and its comorbidities.

## Materials and methods

### *Diets and animals*

Based on the high-fat diet formula (D12492) for DIO mice from Research Diets Inc. (New Brunswick, NJ), a high-fat diet (HFD) (34.9% fat by wt., 60% kcal) with soy oil and lard oil as the main sources of fat was designed with a standard diet (SD) (4.3% fat by wt; 10% kcal) as a control. Forty male C57BL/6J mice at 3 to 4 weeks of age were obtained from the Laboratory Animal Center, Hospital 304 affiliated with the General Hospital of the People's Liberation Army in China. All mice were housed at the animal facilities in a temperature-, light- and humidity-controlled room with a 12-h (h) light/12-h dark cycle. After one week of recovery from transportation, mice were classified into two groups (20 mice in each group) and fed with one of the two types of diets for 4 months. The body weight was measured weekly to confirm that the HFD-fed mice gained more weight than the SD-fed mice. At the end of the experiment, fasted mice were anesthetized by an intraperitoneal injection of Avertin (2,2,2-tribromoethanol, T-4840-2; Sigma-Aldrich Chemie GmbH, Steinheim, Germany; 125 mg/kg) to obtain blood samples by heart puncture. An evaluation of blood triglyceride levels and leptin concentration was performed at the end of the experiment to confirm that the HFD-fed mice developed an obese phenotype. The mice were then immediately euthanized by decapitation. The epididymal fat and the brain were dissected free of the surrounding tissue, immediately frozen in liquid N<sub>2</sub> and stored at -80°C until analysis. All of the animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Hospital 304 affiliated with the General Hospital of the People's Liberation Army in China.

### *Gene-expression microarrays and data analysis*

Total RNA from the adipose tissue or the brain hypothalamus was quantified using a NanoDrop ND-1000 spectrophotometer, and the RNA integrity was assessed using standard denaturing agarose gel electrophoresis. For microarray analysis, an Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Briefly, 1  $\mu$ g of total RNA from each sample was amplified and transcribed into fluorescent cRNA using Agilent's Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The labeled cRNAs were hybridized onto the Whole Genome Oligo Array (4 $\times$ 44K, Agilent Technologies) with over 39,000 mouse genes and trans-

cripts represented. After the slides were washed, the arrays were scanned with an Agilent Scanner G2505B.

Agilent Feature Extraction software (version 10.7.3.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, differentially expressed genes with statistical significance were identified through Volcano Plot filtering between the two groups, with a threshold of fold change at  $\geq 1.5$  and  $P \leq 0.05$ . Pathway analysis and GO analysis were applied to determine the roles of these differentially expressed genes in these biological pathways or GO terms. Finally, hierarchical clustering was performed to determine the distinguishable gene expression pattern among samples.

#### *DNA methylation microarrays and data analysis*

##### Genomic DNA extraction

Genomic DNA (gDNA) was extracted from samples using a DNeasy Blood & Tissue Kit (Qiagen, Fremont, CA). A total of 10  $\mu\text{g}$  gDNA of each sample was sonicated to approximately 200 – 1000 bp with a Bioruptor sonicator (Diagenode) on “Low” mode for 10 cycles of 30 seconds “ON” and 30 seconds “OFF.” The gDNA and each sheared DNA were agarose analyzed.

##### Immunoprecipitation

Then, 1  $\mu\text{g}$  of sonicated gDNA was used for immunoprecipitation using a mouse monoclonal anti-5-methylcytosine antibody (Diagenode). For this procedure, DNA was heat-denatured at 94°C for 10 min, rapidly cooled on ice and immunoprecipitated with 1  $\mu\text{L}$  primary antibody overnight at 4°C with rocking agitation in 400  $\mu\text{L}$  immunoprecipitation buffer (0.5% BSA in PBS). To recover the immunoprecipitated DNA fragments, 200  $\mu\text{L}$  of anti-mouse IgG magnetic beads was added and incubated for an additional 2 h at 4°C with agitation. After immunoprecipitation, a total of five immunoprecipitation washes were performed with ice-cold immunoprecipitation buffer. The washed beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg/mL proteinase K for 2 h at 65°C and then allowed to cool to room temperature. MeDIP DNA was purified using Qiagen MinElute columns (Qiagen).

##### Whole-genome amplification

The MeDIP-enriched DNA was amplified using a GenomePlex® Complete Whole Genome Amplification (WGA2) kit from Sigma-Aldrich. The amplified DNA samples were then purified with a QIAquick PCR purification kit (Qiagen).

#### DNA labeling and array hybridization

The NimbleGen Dual-Color DNA Labeling Kit (NimbleGen Systems, Inc., Madison, WI, USA) was used for labeling according to the manufacturer’s guidelines. Briefly, 1  $\mu\text{g}$  DNA of each sample was incubated for 10 min at 98°C with 1 OD of Cy3-9mer primer (IP sample) or Cy5-9mer primer (Input sample). Then, 100 pmol of deoxynucleoside triphosphates and 100 U of the Klenow fragment (New England Biolabs, USA) were added, and the mix was incubated at 37°C for 2 h. The reaction was halted by adding 0.1 volume of 0.5 M EDTA, and the labeled DNA was purified by isopropanol/ethanol precipitation and hybridized to genomic promoter arrays. For array hybridization, a NimbleGen MM8 CpG plus Promoter Microarray was used, which is a single array design containing all known CpG islands annotated by UCSC and all well-characterized RefSeq promoter regions (from approximately -1300 bp to +500 bp of the TSSs) entirely covered by approximately 385,000 probes. Scanning was performed with the Axon GenePix 4000B microarray scanner.

Raw data were extracted as pair files using NimbleScan software. We performed median-centering, quantile normalization and linear smoothing using the Bioconductor software packages Ringo, limma and MEDME, respectively. After normalization, normalized log<sub>2</sub>-ratio data were created for each sample. From the normalized log<sub>2</sub>-ratio data, a sliding-window peak-finding algorithm provided by NimbleScan v2.5 aodrqEW (Roche-NimbleGen) was applied to determine the enriched peaks with specified parameters (sliding window width: 750 bp; mini-probes per peak: 2; P value minimum cutoff: 2; maximum spacing between nearby probes within peak: 500 bp). The identified peaks were mapped to the following genomic features: transcripts and CpG Islands.

Promoters or regions were further classified into three categories to distinguish strong CpG islands, weak CpG islands and sequences with a low abundance of CpG sites<sup>13</sup>. High CpG promoters/regions (HCP) contain a 500-bp sequence with GC content above 55%, a CpG ratio above 0.75 and a CpG observed to expected ratio greater than 0.6. Intermediate CpG promoters/regions (ICP) contain a region below 500 bp and have moderate CpG richness (GC content less than 55%) with a CpG ratio between 0.48 and 0.75 and a CpG observed to expected ratio between 0.4 and 0.6. Low CpG promoters/regions (LCP) do not have a region of 500 bp with a CpG ratio above 0.48 or a CpG observed to expected ratio above 0.4<sup>14</sup>.

##### Identification of biomarkers

Overlapping between gene expression and DNA methylation was conducted using the R gplots package

(cran.r-project.org) to screen the possible biomarkers involved in the pathogenesis of obesity. Only promoters whose downstream gene was differentially expressed at least 1.5-fold ( $P < 0.05$ ) through Volcano Plot filtering were considered. For the last filter, methylation markers in adipose tissue of DIO mice were required to display an inverse relationship to gene expression, i.e., hyper- and hypomethylated states being down- and upregulated, respectively.

#### Sequencing of bisulfite-modified DNA

The methylation levels of the selected site-specific CpG sites from the three genes (Ube2q2, Foxj3 and Mmp2) were determined by bisulfite sequencing. The target regions of the relevant genes and the primers used for amplification are listed in table I. Briefly, DNA from the epididymal fat was isolated and purified with the DNA Purification Kit (cat. no. DN 1008, Biofuture Group Inc., Beijing, China) and modified with bisulfate using the Methylamp™ DNA Modification Kit (cat. no. P-1001, Epigentek Group Inc., Brooklyn, NY). The methyl-modified DNA was amplified by nested PCR, and products were sequenced directly. DNA methylation was calculated from the amplitude of cytosine and thymine within each CpG dinucleotide,  $C/(C+T)$ , as described by Lewin et al.<sup>15</sup>.

#### Gene expression by quantitative RT-PCR

Total RNA was extracted from mouse epididymal fat using TRIzol Reagent (cat. no. 15596-026, Invitrogen, Carlsbad, CA, USA), and cDNA was prepared from the total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (cat. no. 18080-051, Invitrogen, Carlsbad, CA, USA) according to the procedures provided by the manufacturer. The genes' mRNA levels in the fat were measured using real-time quantitative RT-PCR with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA).

The oligonucleotide primers were designed with the assistance of PrimerExpress software: Ube2q2 (NM\_180600) (Forward: GACCAAACCTGCTT-CCTG; Reverse: GTGCCAACCATTCATAA), Foxj3 (NM\_172699) (Forward: AGGTCCTCAG-TAGTTTGC; Reverse: AGACTGCGATACTC-CAAC), Mmp2 (NM\_008610) (Forward: CCCC-GATGCTGATACTGA; Reverse: CTGTCCGCCA-AATAAACC), beta-actin (NM\_007393) (forward: ACCAGTTCGCCATGGATGAC; reverse: TGCCG-GAGCCGTTGTC). The co-amplification of mouse beta-actin mRNA, an invariant internal control, was performed in all the samples. The assays were performed in triplicate, and the results were normalized to the beta-actin mRNA levels using the  $2^{-\Delta CT}$  method.

#### Statistical analysis

All statistical analyses for gene expression microarray and DNA methylation microarray were conducted with R statistical packages v2.15.0 (www.r-project.org) and SPSS (Statistical Package for the Social Sciences) version 11.5 for Windows. For site-specific CpG sites from the three genes, Student's *t*-test was used to investigate whether their methylation or expression levels differ between the obese and the lean group. The two-sided level of significance was set at  $P < 0.05$ .

## Results

#### *Differences in promoter methylation between obese mice and lean mice*

DNA methylation was determined using MeDIP followed by hybridization to the NimbleGen MM8 CpG plus Promoter Microarray, which probes 385,000 RefSeq mouse promoters. In adipose tissues between the obese mice and the control mice, 626 differentially methylated promoters were found, with 277 gene promoters hypermethylated and 349 hypomethylated

**Table I**  
*Bisulfite sequencing primers used in this study*

<i>Gene Name</i>	<i>Forward (F) and Reverse (R) Primers</i>
Ube2q2	Outer F: (-499) TATAAGTTTGGAGAGAAGGATTTTT (-475) Inner F: (-472) GGAGTTATATTTAGAAAAGTGGTG (-449) R: (-212) AAAATAAACCTAAACTCCCCTAC (-234)
Foxj3	Outer F: (-566) GTTTTATTTGTTGATAGTGAGGTTA (-542) Inner F: (-535) GGTTAGTTAATTTGAGATGGAG (-514) R: (-293) AAATTCCAACTCCTAAAAAATAC (-315)
Mmp2	Outer R: (+473) AAACCAACATAACAACCTTACCAC (+451) Inner R: (+453) CACTACCAACTCTTATCTATTTTA (+429) F: (+255) ATAGTTAGAGATTTTAGGGTGATA (+278)

**Table II**  
The number of differentially methylated gene promoters in the DIO mice compared with the lean mice

	Adipose tissue			Hypothalamu		
	Hypermethyl	Hypomethyl	Total	Hypermethyl	Hypomethyl	Total
HCP	185	207	392	157	189	346
ICP	53	93	146	72	62	134
LCP	39	49	88	103	63	166

in obese mice. In the brain hypothalamus of obese mice, 646 differentially methylated promoters were found, with 332 hypermethylated and 314 hypomethylated compared to that of control mice (table II).

#### Identification of gene methylation markers in obesity

Gene expression was evaluated for each methylation profile to assess the promoter methylation relevant to transcriptional repression. An analysis of the gene expression microarray showed that compared to the lean mice, there were 3284 genes transcriptionally upregulated and 3390 genes downregulated in the adipose tissue; in the brain, 275 genes were transcriptionally upregulated and 120 genes were downregulated. An overlapping analysis of gene expression and promoter methylation provided a list of differently methylated genes whose transcriptional expressions were associated with their promoter methylation profiles in the adipose tissue of DIO mice compared to the control mice (table III and table IV). A total of 24 hypermethylated promoters and 42 hypomethylated promoters in adipose tissue were selected as methylation markers. In the brain hypothalamus, only the promoter of gene *tmem204* was hypermethylated and that of gene *olfr485* was hypomethylated, which were associated with downregulated and upregulated gene expression, respectively.

Then, gene expression was evaluated for each methylation profile to assess promoter CpG content relevant to transcriptional repression. The results indicated that the expression levels of genes under the control of HCPs is negatively related to the promoter methylation levels (trend *P* value 0.009), whereas no significant correlation was found between the methylation levels of ICPs or LCPs and gene transcriptional expression (*P* = 0.364 and 0.677, respectively) (fig. 1). These filters increased the likelihood of identifying promoters with a dysregulated methylation pattern causally related to the differential expression of downstream genes.

GO and Pathway analysis indicated the specific roles of these differentially methylated and expressed genes in the biological pathways or GO terms. The genes with hypermethylated promoters and under-expression were potentially relevant to metabolic processes (GO: 0008152) including lipid metabolism

and storage (Abhd5, 4921517L17Rik); the insulin-like growth receptor signaling pathway (Atxn7); obesity-linked diseases such as diabetes mellitus (Foxj3, Hmg20a) and thrombosis (Zfp161); cellular processes (GO: 0009987) including protein modification, such as lipidation and ubiquitination (Atxn7, Hecw2, Ube2q2, Dpm1); nuclear import/export pathways (Xpo4, Npm1) and transcription activator/repressors (Cebpd, Mamstr); cellular component organization (GO: 0071840), including nucleosome and cell junction assembly (Nap111, Pard3), spliceosome (Clk4), telomere organization and maintenance (Tnks2) and microtubule cytoskeleton organization (Npm1); and growth (GO: 0040007), including cell differentiation of the epidermis, lung alveolus, neural crest cell and bone (Lamas, Npm1, Errfil, Co19a1); and gland morphogenesis (Lama5). The genes that were hypomethylated in promoters with transcriptional overexpression were associated with metabolic processes (GO: 0008152), including lipid metabolism (Asah1, Acaa2); biological regulation (GO: 0065007), including the positive regulation of fat cell differentiation (Sh3pxd2b) and the negative regulation of T and B cell proliferation and immunity (Pawr, Trpm4, Usp25); signaling (GO: 0023052), including the insulin-like growth receptor signaling pathway (wdr11) and the TLR4-dependent innate immune response (Usp25); response to stimulus (GO: 0050896), including the response to estradiol stimulus (Mmp2), macrophage colony stimulating factor (csflr), tumor from hypoxia (Car9) and ischemic stroke (Arl6ip6); and localization (GO: 0051179), including activating delayed rectifier K<sup>+</sup> current (Kcnh2) and the G2/M transition checkpoint (Fzr1).

#### Further validation of promoter methylation and gene expression

Three genes (Ube2q2, Foxj3 and Mmp2) were chosen for further validation of promoter methylation and gene expression in the adipose tissue. The methylation status of the promoters of these three genes was evaluated by sequencing the bisulfite-converted DNA. An increased total averaged methylation level of CG sites was found in promoters of the Ube2q2 and Foxj3 genes in DIO mice compared to the control mice. The total averaged CG site methy-

**Table III**  
The screened genes transcriptionally upregulated by their promoters' hypomethylation

Gene Name	Classification	Accession No.
G protein-coupled receptor 137B, pseudogene (Gpr137b-ps)	HCP	NR_003568
RIKEN cDNA 2610524H06 gene (2610524H06Rik)	HCP	NM_181075
transmembrane protein 189 (Tmem189)	HCP	NM_145538
<b>collagen, type IV, alpha 1 (Col4a1)</b>	<b>HCP</b>	<b>NM_009931</b>
MKL (megakaryoblastic leukemia)/myocardin-like 1 (Mkl1)	HCP	NM_001082536
<b>potassium voltage-gated channel, subfamily H (eag-related), member 2 (Kcnh2)</b>	<b>HCP</b>	<b>NM_013569</b>
membrane bound O-acyltransferase domain containing 7 (Mboat7)	HCP	NM_029934
<b>PRKC, apoptosis, WT1, regulator (Pawr)</b>	HCP	NM_054056
fizzy/cell division cycle 20 related 1 (Drosophila) (Fzr1)	HCP	NM_019757
Williams-Beuren syndrome chromosome region 17 homolog (human) (Wbscr17)	HCP	NM_145218
ring finger protein 26 (Rnf26)	HCP	NM_153762
protein phosphatase 1, regulatory (inhibitor) subunit 16A (Ppp1r16a)	HCP	NM_033371
SH3 and PX domains 2B (Sh3pxd2b)	HCP	NM_177364
asparagine-linked glycosylation 13 homolog (S. cerevisiae) (Alg13)	HCP	NM_026247
<b>growth factor receptor bound protein 2 (Grb2)</b>	<b>HCP</b>	<b>NM_008163</b>
potassium channel tetramerisation domain containing 5 (Kctd5)	HCP	NM_027008
<b>RIKEN cDNA 1810058I24 gene (1810058I24Rik)</b>	<b>HCP</b>	<b>NR_027875</b>
ubiquitin specific peptidase 25 (Usp25)	HCP	NM_0113918
ADP-ribosylation factor-like 6 interacting protein 6 (Arl6ip6)	HCP	NM_022989
AFG3(ATPase family gene 3)-like 1 (yeast) (Afg3l1)	HCP	NM_054070
adaptor-related protein complex AP-1, mu subunit 1 (Ap1m1)	HCP	NM_007456
round spermatid basic protein 1-like (Rsb11)	HCP	NM_001080977
transient receptor potential cation channel, subfamily M, member 4 (Trpm4)	ICP	NM_175130
N-acylsphingosine amidohydrolase 1 (Asah1)	ICP	NM_019734
colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage) (Csf2ra)	ICP	NM_009970
UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4 (B3galt4)	ICP	NM_019420
zinc finger protein 7 (Zfp7)	ICP	NM_145916
<b>RIKEN cDNA 5730508B09 gene (5730508B09Rik)</b>	<b>ICP</b>	<b>NM_027482</b>
signal sequence receptor, beta (Ssr2)	ICP	NM_025448
histocompatibility 2, Q region locus 10 (H2-Q10)	ICP	NM_010391
Rab acceptor 1 (prenylated) (Rabac1)	ICP	NM_010261
<b>Sjogren's syndrome/scleroderma autoantigen 1 homolog (human) (Sssca1)</b>	<b>ICP</b>	<b>NM_020491</b>
WD repeat domain 11 (Wdr11)	ICP	NM_172255
aldo-keto reductase family 1, member A4 (aldehyde reductase) (Akr1a4)	ICP	NM_021473
ubiquitin-conjugating enzyme E2E 1, UBC4/5 homolog (yeast) (Ube2e1)	ICP	NM_009455
acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) (Acaa2)	ICP	NM_177470
<b>ryanodine receptor 2, cardiac (Ryr2)</b>	<b>ICP</b>	<b>NM_023868</b>
<b>colony stimulating factor 1 receptor (Csf1r)</b>	<b>LCP</b>	<b>NM_001037859</b>
carbonic anhydrase 9 (Car9)	LCP	NM_139305
solute carrier family 6 (neurotransmitter transporter, GABA), member 1 (Slc6a1)	LCP	NM_178703
<b>spondin 2, extracellular matrix protein (Spon2)</b>	<b>LCP</b>	<b>NM_133903</b>
<b>matrix metalloproteinase 2 (Mmp2) *</b>	<b>LCP</b>	<b>NM_008610</b>

Bold font indicates genes whose promoter methylation has been studied in cancers.

\* chosen for further validation of promoter methylation and gene expression.

lation level in the Mmp2 promoter was significantly lower in DIO obese mice than in the control mice (fig. 2). Quantitative real-time RT-PCR validated the inverse correlation between gene expression (fig. 3) and promoter methylation.

## Discussion

Recent studies have demonstrated that DNA methylation in promoters of varied genes is closely associated with obesity and its complications<sup>6-9</sup>. In the present

study, we performed comprehensive DNA methylation profiling of gene promoters in DIO mice and control lean mice to determine the differentially methylated genes using MeDIP followed by hybridization to the NimbleGen MM8 CpG plus Promoter Microarray. We further integrated epigenomic data with gene expression profiling to identify promoters that exhibited an association between methylation status and the expression of downstream genes. We identified 66 methylation markers in the adipose tissue of DIO mice, of which 24 were hypermethylated and 42 were hypomethylated.

Table IV

The screened genes transcriptionally downregulated by their promoters' hypermethylation

Gene Name	Classification	Accession No.
ataxin-7 (Atxn7)	HCP	NM_139227
<b>CCAAT/enhancer-binding protein delta (Cebpd)</b>	<b>HCP</b>	<b>NM_007679</b>
<b>RIKEN cDNA 4921517L17 gene (4921517L17Rik)</b>	<b>HCP</b>	<b>NM_027585</b>
splicing factor, arginine/serine-rich 4 (Srsf4)	HCP	NM_020587
<b>abhydrolase domain containing 5 (Abhd5)</b>	<b>HCP</b>	<b>NM_026179</b>
HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 (Hecw2)	HCP	NM_172655
<b>exportin-4 (Xpo4)</b>	<b>HCP</b>	<b>NM_020506</b>
ERBB receptor feedback inhibitor 1 (Errfi1)	HCP	NM_133753
<b>RIKEN cDNA 1700096K18 gene (1700096K18Rik)</b>	<b>HCP</b>	<b>NR_027388</b>
forkhead box J3 (Foxj3) *	HCP	NM_172699
<b>cDNA sequence BC049762 (BC049762)</b>	<b>HCP</b>	<b>NM_177567</b>
<b>zinc finger protein 161 Gene (Zfp161)</b>	<b>HCP</b>	<b>NM_009547</b>
tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2 (Tnks2)	HCP	NM_001163635
<b>nucleosome assembly protein 1-like 1 (Nap1l1)</b>	<b>HCP</b>	<b>NM_015781</b>
<b>Rap1 interacting factor 1 homolog (yeast) (Rif1)</b>	<b>HCP</b>	<b>NM_175238</b>
ubiquitin-conjugating enzyme E2Q (putative) 2 (Ube2q2) *	HCP	NM_180600
<b>par-3 (partitioning defective 3) homolog (C. elegans) (Pard3)</b>	<b>HCP</b>	<b>NM_001122850</b>
CDC like kinase 4 (Clk4)	HCP	NM_007714
CDC like kinase 4 (Clk4)	HCP	NM_007714
<b>nucleophosmin 1 (Npm1)</b>	<b>HCP</b>	<b>NM_008722</b>
dolichol-phosphate (beta-D) mannosyltransferase 1 (Dpm1)	HCP	NM_010072
<b>MEF2 activating motif and SAP domain containing transcriptional regulator (Mamstr)</b>	<b>ICP</b>	<b>NM_172418</b>
high mobility group 20A (Hmg20a)	ICP	NM_025812
laminin, alpha 5 (Lama5)	ICP	NM_001081171
<b>collagen, type IX, alpha 1 (Col9a1)</b>	<b>LCP</b>	<b>NM_007740</b>

Bold font indicates genes whose promoter methylation has been studied in cancers.

\* chosen for further validation of promoter methylation and gene expression.

Previous experiments have indicated that the relationship between promoter methylation and gene expression is related to the CpG density of the promoter<sup>14</sup>. To examine this relationship, we divided promoters into three types according to CpG content: high CpG promoters (HCPs), low CpG promoters (LCPs) and intermediate CpG promoters (ICPs). A stronger correlation was shown between DNA methylation levels and transcriptional repression in HCPs compared with ICPs and LCPs. Weber et al. reported that LCPs show no significant correlation between gene activity and the abundance of methylated cytosines, implying that low concentrations of methylated cytosines do not preclude gene activity, whereas the activity of ICPs and HCPs was negatively correlated with their DNA methylation status<sup>14</sup>.

The GO and Pathway analyses suggested that the genes with differentiated methylation and transcriptional expression were associated with biological activities in lipid metabolism and storage, adipocyte differentiation, immunity and the pathogenesis of obesity-related complications. However, the epigenetic control and involvement in obesity have not yet been established for any of the methylation markers found in this study. Interestingly, among these markers, there were 11 hypomethylated and 13 hypermethylated promoters that have been previously

studied, almost all of which are involved in the pathogenesis of cancers such as follicular lymphoma and Burkitt's lymphoma, breast cancer, neuroblastoma, myeloid leukemia and others<sup>16-18</sup>. This finding implies that these differentially methylated genes most likely play important roles in the abnormal adipocyte proliferation and differentiation in obese individuals, which should be further studied.

We assessed the methylation status of Mmp2, Foxj3 and Ube2q2 in the adipose tissue of DIO mice and found a significant methylation change between the obese and control groups. The protein encoded by the Mmp2 gene is a matrix metalloproteinase, which degrades type IV collagen, the component of the basement cellular membrane, and is essential for proper extracellular matrix remodeling in adipose tissue<sup>19</sup>. It has been reported that Mmp2 may facilitate the hypertrophic development of adipocytes, the formation of adipose clusters<sup>20</sup>, and the early phase of murine preadipocyte differentiation<sup>21</sup>. Moreover, preadipocyte treatment with MMP inhibitors or antibodies markedly decreases adipocyte differentiation by inhibiting the lipogenesis of triglycerides and its hydrolysis<sup>22</sup>. In addition, Mmp2 is strongly upregulated by macrophage-secreted factors, inducing a major inflammatory response in human adipocytes<sup>23</sup>. In the current study, the methylation fraction of the Mmp2 promoter

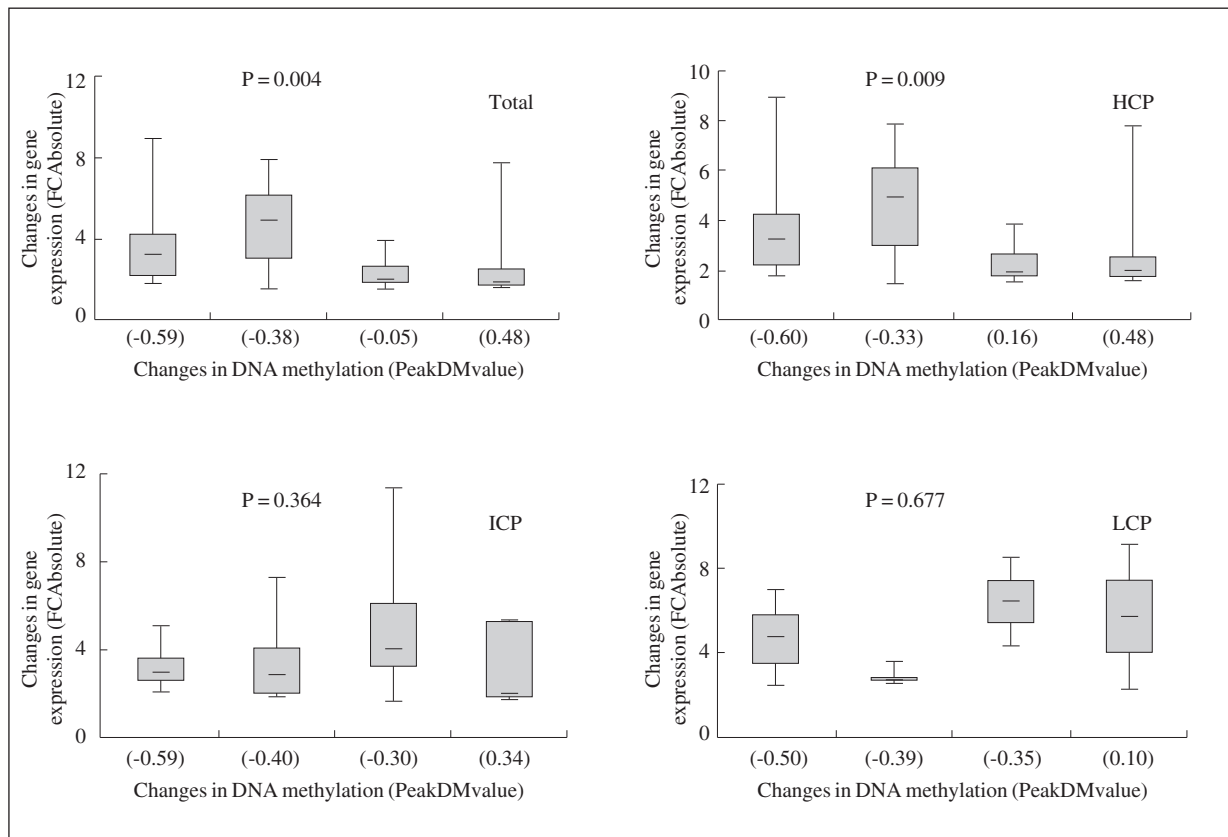


Fig. 1.—Association of transcriptional changes with DNA methylation. Promoters or regions were further classified into three categories: HCPs, ICPs and LCPs. P values were determined by the linear regression to analyze the correlation between promoter methylation levels and gene transcriptional changes.

was reduced with the correlated upregulation of transcription in DIO mice, which is consistent with the findings that the increased expression of *Mmp2* is associated with overweight/obese subjects<sup>24,25</sup>.

The product of the *Foxj3* gene is a forkhead/winged helix transcription factor that functions to regulate cellular fate determination, proliferation and differentiation<sup>26</sup>. *Foxj3* may regulate a network of zinc finger proteins (ZFPs)<sup>27</sup>, which is a key transcriptional regulator involved in adipocyte proliferation and differentiation<sup>28</sup>. Additionally, *Foxj3* activates the transcriptional expression of the *Mef2c* (myocyte enhancer factor) gene involved in adipogenesis<sup>29</sup>. Grant et al. reported that the knockdown of *Foxj3* and *Foxk1* eliminated cell cycle-dependent oscillations and resulted in decreased cell proliferation rates<sup>30</sup>. In response to feeding with a high-calorie diet, *Foxj3* expression is downregulated, with a concomitant decrease in *Mef2c* expression<sup>31</sup>. In keeping with this finding, we found that high-fat feeding to mice downregulated the expression of *Foxj3*, most likely by enhancing the methylation level of the *Foxj3* promoter.

The *Ube2q2* protein, one of the functional ubiquitin-conjugating enzymes, is responsible for the intermediate phase of the ubiquitination pathway, which is

fundamental in the regulation of protein stability and turnover. In adipocytes, the ubiquitin-dependent proteasomal pathway controls several highly regulated proteins, such as fat-specific protein 27 (*Fsp27*), peroxisome proliferator-activated receptor- $\gamma$  (*Ppar- $\gamma$* ) and pigment epithelium-derived factor (*Pedf*), which participate in lipid storage, adipocyte development and insulin sensitivity, respectively<sup>32-34</sup>. Transcriptome analysis has demonstrated that the expression of several genes in the hypothalamus, including *Ube2q2*, is modulated by high fat intake<sup>35</sup>. In the present study, our findings showed that the expression of *Ube2q2* in the adipose tissue of DIO mice was decreased, which may be attributable to the higher methylation of its promoter.

In summary, we have shown that the integration of high-throughput data from the genome-wide integrative analysis of promoter methylation and gene expression in DIO mice, compared to the lean controls, can identify new DNA methylation markers related to obesity. The aberrant methylated promoters reported here have potential relevance in clinical applications as markers for elucidating the mechanisms of obesity pathogenesis (abnormal lipid metabolism, storage and adipocyte proliferation and differentiation, etc.) and its complications.



### Ube2q2

-499

tacaagtctggagagaaggaccttctgggagtcacacttagaaaagtgggtgggtggttcagc**cgcgaggcgagagcaa**  
1 2 3  
cata**cg**tgaaggtgctagg**cg**cacacctctcatctccccatcc**cggcgcgc**ccaggccaggtgagtggggtggcc**cggt**  
4 5 6 7 8 9  
ggacaacca**cgcgaggag**caacctccaggggccc**cgcgcg**tttctacc**cgagcgcag**tgg**cgcgcg**gataggc  
10 11 12 13 14 15 16 17 18 19 20  
**cgcg**cag**cg**tctctggccagaggccc**cggt**gcag**cg**cagagg**cgcg**caggggagctcaggttcac**cg**t  
21 22 23 24 25 26 27 -212

### Foxj3

-566

gctccacctgttgatagtgaggccacgacctggccagctaaacttgagatggagaggatgac**cgcg**gaggggtaatgac  
1 2  
aagatgacatgctgggtagggaggggcct**cg**aacc**cg**ggttcttgacattc**cg**aaggac**cg**g**cg**tgtcccaagcaagg  
3 4 5 6 7  
tcaaag**cg**ccc**cg**tgg**cg**gc**cg**gaaaatc**cg**g**cg**tgggtgcttgac**cg**ggg**cg**cgagaggcagaggctgga  
8 9 10 11 12 13 14 15 16  
agg**cg**gggaggtcac**cg**agg**cg**cattcctcaggagttggaatcc  
17 18 19 -293

### Mmp2

+255

acagccagagacctcaggggtgacac**cgcg**gagcc**cg**gggag**cg**caacgatggaggca**cg**agtggtggggag**cg**  
1 2 3 4 5 6 7  
tg**cg**ccgagacctct**cg**gggttctct**cg**tctctgctgctgctggtgg**cg**cgccat**cg**ctgcaccat**cg**cccatcaatcaa  
8 9 10 11 12 13 14  
gttccc**cg**cgatg**cg**cccctaaacagacaaagagttggcagtggttaagttgctatgctggctt  
15 16 17 +473

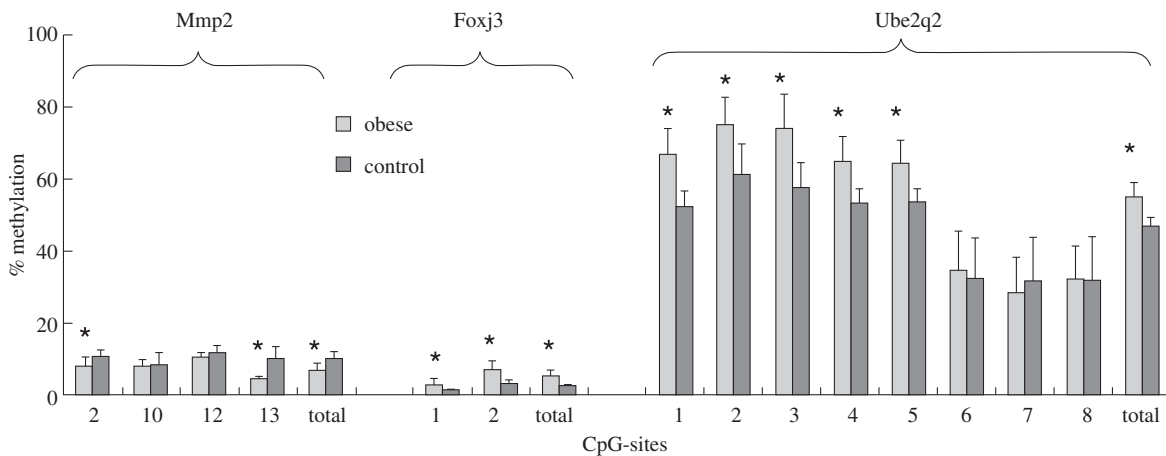


Fig. 2.—Validation of promoter methylation for the selected markers. Genomic DNA isolated from the epididymal fat was analyzed for the methylation levels of CpG sites at the indicated positions of the gene promoters (the upper panel). Except for the methylated CpG sites shown in the lower panel, the rests were all completely demethylated. The total indicated the averaged methylation levels from the CpG sites shown. Data represent means±SD determined from 15 mice in each group. \* Compared to the control group,  $P < 0.05$ .

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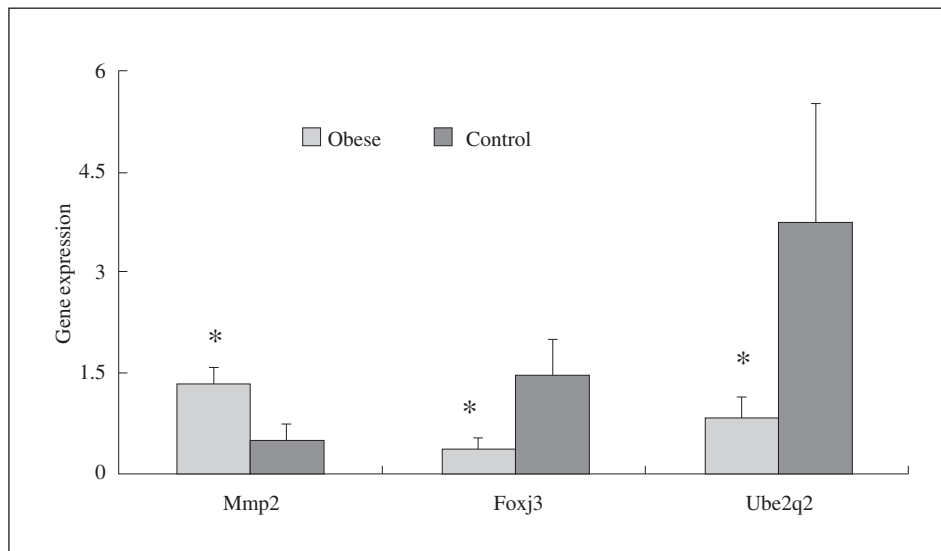


Fig. 3.—The mRNA expression of the selected gene markers. Gene transcripts in the epididymal fat were measured by real-time RT-PCR, and the expression levels were normalized to that of beta-actin using 2<sup>-ΔCT</sup> method. Data represent the mRNA levels determined from 15 mice in each group. \*Compared to the control group,  $P < 0.05$ .

### Conflict of Interest

There are no competing financial interests related to the work described.

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