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Obesity is associated with depot-specific alterations in adipocyte DNA methylation and gene expression

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ABSTRACT

The present study aimed to identify genes exhibiting concomitant obesity-dependent changes in DNA methylation and gene expression in adipose tissues in the mouse using diet-induced obese (DIO) C57BL/6J and genetically obese ob/ob mice as models. Mature adipocytes were isolated from epididymal and inguinal adipose tissues of ob/ob and DIO C57BL/6J mice. DNA methylation was analyzed by MeDIP-sequencing and gene expression by microarray analysis. The majority of differentially methylated regions (DMRs) were hypomethylated in obese mice. Global methylation of long interspersed elements indicated that hypomethylation did not reflect methyl donor deficiency. In both DIO and ob/ob mice, we observed more obesity-associated methylation changes in epididymal than in inguinal adipocytes. Assignment of DMRs to promoter, exon, intron and intergenic regions demonstrated that DIO-induced changes in DNA methylation in C57BL/6J mice occurred primarily in exons, whereas inguinal adipocytes of ob/ob mice exhibited a higher enrichment of DMRs in promoter regions than in other regions of the genome, suggesting an influence of leptin on DNA methylation in inguinal adipocytes. We observed altered methylation and expression of 9 genes in epididymal adipocytes, including the known obesity-associated genes, *Ehd2* and *Kctd15*, and a novel candidate gene, *Irf8*, possibly involved in immune type 1/type2 balance. The use of 2 obesity models enabled us to dissociate changes associated with high fat feeding from those associated with obesity *per se*. This information will be of value in future studies on the mechanisms governing the development of obesity and changes in adipocyte function associated with obesity.
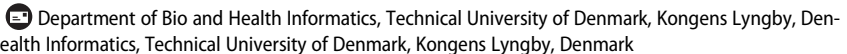
Introduction

Nutrient excess as well as deficit during fetal development may predispose to obesity and diabetes in humans and animals, and mouse studies have shown that this effect can be transmitted to subsequent generations through epigenetic changes such as histone modifications and DNA methylation in the germ line.[1,2] Obesity and accompanying insulin resistance may also affect DNA methylation in adipose tissue leading to phenotypic changes in the adipocytes.[3,4]

DNA methylation is implicated in the regulation of metabolism, and differential methylation has been identified in the promoter of several genes associated with obesity development and adipocyte function. Thus, dexamethasone treatment of mice leads to decreased DNA methylation at the *Cebpa* promoter inducing a shift in the preference of bone marrow stromal cells to favor adipocyte over osteoblast development.[5] Moreover, methylation of the cAMP response elements in the *Ucp1* promoter has been suggested to repress expression of *Ucp1* in white adipocytes.[6] Intake of high fat diet has been shown to increase methylation of the leptin promoter in retroperitoneal adipocytes in rats, and this was associated with lower circulating leptin levels.[7] A recent study also demonstrated that long-term high fat feeding induced hypermethylation of both the *Leptin* and the

Pparg promoter in gonadal, but not subcutaneous adipose tissue[8] Finally, it has been shown that the adiponectin promotor is hypermethylated in adipocytes from diet-induced obese (DIO) C57BL/6J mice and db/db mice. This was associated with decreased insulin sensitivity, which was relieved by treatment with the DNMT inhibitor RG108.[9]

The majority of studies investigating DNA methylation in mouse models of obesity has focused on specific genes.[6,7,9] Only few studies have performed genome-wide DNA methylation analysis in adipose tissue or isolated mature adipocytes from mice.[10,11]

In this study, we compared obesity-associated changes in DNA methylation of mature adipocytes from DIO C57BL/6J and genetically obese (ob/ob) mice, representing 2 widely used models for diet- and genetically induced obesity, respectively, to identify obesity associated changes. The aim was to identify genes exhibiting concomitant obesity-dependent changes in DNA methylation and gene expression in adipose tissue.

Results

The distribution of differentially methylated regions (DMRs) differs between diet-induced obese C57BL/6J and ob/ob mice

To identify changes in methylation patterns associated with genetically determined obesity and diet-induced obesity we isolated mature adipocytes from 2 obesity models. Ob/ob mice on a chow diet were chosen as a model for genetically induced obesity, whereas diet-induced obesity was induced by high fat feeding of C57BL/6J mice for 15 weeks. Age-matched chow fed C57BL/6J mice were used as lean controls in both experiments. Both DIO mice and ob/ob mice were significantly heavier than their corresponding controls, and importantly, the 2 models had similar body masses at sacrifice (Fig. S1).

DNA methylation in mature adipocytes from inguinal and epididymal fat pads was assessed using MeDIP-seq. On average, 170 million paired-end reads per sample were obtained from sequencing. After filtering PCR duplicates, mapped reads without proper pairing, and poor quality alignments (Mapping quality <30), approx. 35% of reads from each sample were used for further analyses (Fig. S2). Assignment of DMRs to promoter, exon, intron and intergenic regions demonstrated that changes in DNA methylation associated with DIO in C57BL/6J mice were primarily enriched in exons (Fig. S3). This tendency was less pronounced in ob/ob mice. Compared to epididymal adipocytes in ob/ob and both epididymal and inguinal adipocytes in DIO mice,

inguinal adipocytes of ob/ob mice exhibited a higher enrichment of DMRs in promoter regions than in other regions of the genome, suggesting a specific influence of leptin on DNA methylation in the more plastic inguinal adipocytes.

Obesity is associated with hypomethylation

Differential methylation as represented in volcano plots (Fig. 1) revealed that hypomethylation was dominating in epididymal and inguinal adipose tissue from both obesity models (details are summarized in Table 1, and DMR region details for individual comparisons are listed in Supplementary Tables ST1a-d, available at http://www.cbs.dtu.dk/suppl/Sonne2016/). Nutritional factors influencing the supply of methyl donors are known to affect DNA methylation.[12] Therefore, amino acid and homocysteine levels were measured in plasma samples from the DIO mice. Amino acid profiling showed that the plasma levels of 3 amino acids, 1-methyl-L-histidine, L-methionine and arginine were significantly lower (p-value < 0.01) in obese than in lean mice (Table ST2). By contrast, we detected no significant difference in the level of total homocysteine in the plasma samples from obese and lean mice. To further investigate whether the observed obesity related hypomethylation reflected a general decrease in methylation, global DNA methylation was assessed based on methylation of LINE repeats. We observed no changes in global methylation in obese mice indicating that the observed hypomethylation was not a result of methyl donor insufficiency (Fig. S4).

DMRs are predominantly shared between inguinal and epididymal tissues in the DIO mice

The mechanism by which DNA methylation modulates transcription is not completely understood, but it may affect distant regions as well as regions proximal to the promoter.[13] DMRs were assigned to genes using a distance-based strategy (+/− 10kb of gene boundaries) as described in the methods. Comparison of DMR-associated genes revealed that the highest number of shared genes (2004) were found between inguinal and epididymal adipocytes in the DIO mice, whereas only a few of the identified DMR-associated genes were shared between adipocytes from the 2 models (Fig. 2A).

Among the DMRs found in DIO and ob/ob mice, 306 genes in epididymal adipocytes and 16 genes in inguinal adipocytes contained DMRs in both models. We term such sites obesity associated (Fig. 2B, Table ST3a and ST3b, available at http://www.cbs.dtu.
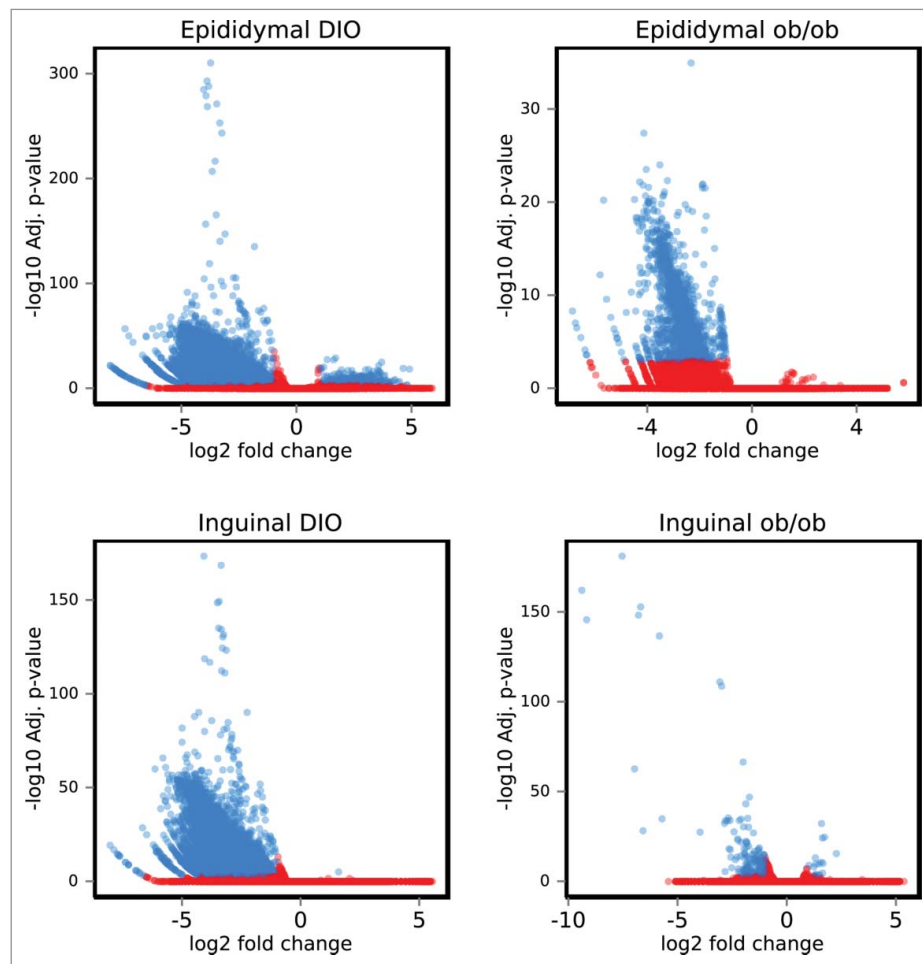
Figure 1. DNA methylation in diet-induced obese and genetically obese ob/ob mice. Volcano plots showing mean methylation differences between mature adipocytes from inguinal and epididymal adipose tissue from obese vs lean mice. Ob/ob (n = 4) or DIO (n = 3). Significantly different methylation sites are shown as blue spots, non-significant are shown as red spots (adjusted p-value <0.001). DIO: Diet-induced obese.

dk/suppl/Sonne2016/). Interestingly, a subset of these revealed opposite methylation patterns, underlining the difference between the 2 obesity models. Of the 306 identified DMRs in epididymal adipocytes, 302 were hypomethylated. A motif search of the 16 and 302 hypomethylated regions identified 3 (Fig. 2C) and 7

significant motifs, respectively (Table ST4). Of the 7 significant motifs in the epididymal adipocytes motif 3, 4 and 6 harbor binding sites for transcription factors Zic1, a reported marker for classical brown adipocytes,[14] Myf6, a myocyte marker,[15] and the cell cycle regulators E2f2/E2f3,[16] respectively (Fig. 2D).

Table 1. Differentially methylated regions in epididymal and inguinal adipocytes from diet-induced and ob/ob mice. Proportion of hyper- and hypo-methylated regions and number of genes mapped within the $+/-10$kb region of the DMRs. DMRs: Differentially methylated regions. DIO: Diet-induced obese.

| Origin of isolated mature adipocytes	Number of DMRs (p-value <0.001) as compared with lean mice	Number of Hypermethylated regions (obese > lean)	Number of Hypomethylated regions (lean > obese)	Number of genes mapped by DMRs
Epididymal tissue from DIO mice (n = 3)	5393	512	4881	3013
Epididymal tissue from ob/ob mice (n = 4)	773	0	773	318
Inguinal tissue from DIO mice (n = 3)	4606	1	4605	2603
Inguinal tissue from ob/ob mice (n = 4)	146	15	131	57
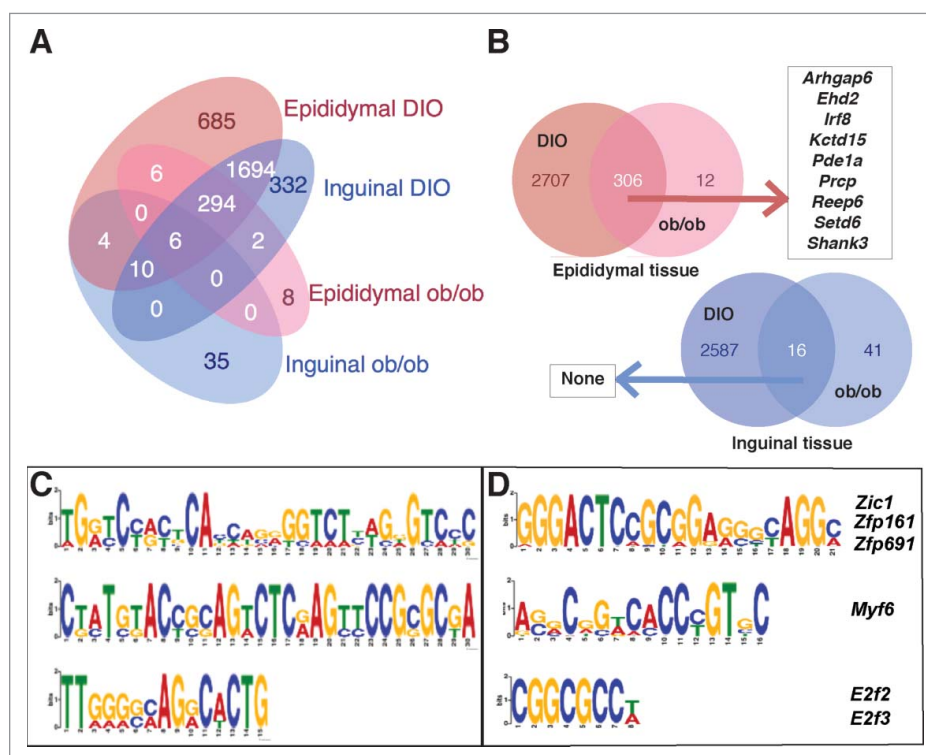
Figure 2. Differentially methylated regions in diet-induced obese and genetically obese ob/ob mice. [A] Genes harboring differentially methylated regions in adipocytes in inguinal and epididymal adipose tissue of diet-induced obese C57BL/6J mice (n = 3) and genetically obese ob/ob mice (n = 4). The highest overlap of 2004 genes was found between inguinal and epididymal adipose tissue from DIO mice (adjusted p-value <0.001). DIO: Diet-induced obese. [B] Venn diagram showing the number of overlapping genes harboring differentially methylated regions in diet-induced obese male C57BL/6J mice (n = 3) and genetically obese male ob/ob mice (n = 4). [A] epididymal adipose tissue and [B] inguinal adipose tissue. Of the 55 overlapping genes with obesity-associated methylation sites as well as altered expression in epididymal adipocytes (adjusted p-value <0.1), 9 genes with abs. log2FC in gene expression in epididymal tissue >1 are enlisted in the figure. DIO: Diet-induced obese. [C] Top 3 motifs predicted in the DMRs based on 16 hypomethylated genes shared by inguinal adipocytes in genetically obese male ob/ob mice (n = 3) and diet-induced obese male C57BL/6J mice (n = 3). [D] Three of the top 7 motifs predicted in the DMRs (see Table S4 for a full list), based on 302 hypomethylated genes shared by epididymal adipocytes in genetically obese male ob/ob mice (n = 3) and diet-induced obese male C57BL/6J mice (n = 3). DIO: Diet-induced obese. Letter size reflects the prevalence of the nucleotide at the position in the motif.

DNA methylation changes in epididymal fat from both models are associated with changes in gene expression of known obesity-related genes

To examine if the obesity-associated changes in DNA methylation were accompanied by changes in gene expression, a second experiment was performed to isolate RNA from mature adipocytes from inguinal and epididymal fat pads from DIO mice and corresponding lean controls. As in the first experiments, DIO mice gained significantly more weight than their RD littermates and exhibited significantly higher masses of inguinal and epididymal white, as well as interscapular brown adipose tissue, and liver (Fig. S5). In this experiment, we found 1135 genes differentially expressed in mature adipocytes from epididymal tissue of DIO vs control mice, with a fold change >2 and adjusted *p*-value <0.1. In line with the observed hypomethylation patterns, expression of the majority, 757 genes, was upregulated while expression of 378 genes was downregulated in DIO mice (Figure S6a, Table ST5, available at http://www.cbs.dtu.dk/suppl/Sonne2016/). Comparing obese mice with the lean control mice, 411 genes were differentially expressed in mature adipocytes from inguinal adipose tissue (Figure S6b, Table ST6, available at http://www.cbs.dtu.dk/suppl/Sonne2016/). Of these, expression of 304 genes was upregulated and expression of 107 genes downregulated in the DIO mice.

Of the obesity-associated methylation sites shared between genetically obese and DIO mice, 55 genes in epididymal adipocytes and one gene in inguinal adipocytes also showed altered expression (adjusted p-value < 0.1). Of the 55 genes in epididymal adipocytes, 9 showed a fold change of more than 2. These were all hypomethylated and expression of 5 genes (*Arhgap6, Irf8, Prcp, Setd6, Shank3*) was upregulated

whereas expression of 4 genes (*Ehd2, Kctd15, Pde1a, Reep6*) was downregulated (Table ST7, available at http://www.cbs.dtu.dk/suppl/Sonne2016/).

Among the genes downregulated in epididymal adipocytes, a few have earlier been described in the context of obesity. The *Ehd2* gene is involved in trafficking of SLC2A4 to the plasma membrane, a process that is impaired in hypertrophic adipocytes independent of inflammation.[17] In the *Kctd15* risk gene, SNPs were found to be associated with BMI (P = 2.6E–07, β = 0.06)[18] and with obesity and obesity-related traits (P < 0.005, odds ratio = 1.54).[19] The upregulated genes include *Prcp*, previously associated with obesity. PRCP-deficient mice have decreased fat mass and improved glucose tolerance and insulin sensitivity.[20] In line with this, elevated levels of serum PRCP have been described in obese subjects.[21]

DNA methylation changes in diet-induced obesity associate with expression of genes involved in adipogenesis

As the overlap in obesity-associated DMRs between epididymal and inguinal adipocytes was higher in DIO mice than in ob/ob mice (Fig. 2), results obtained from DIO mice were used for further analyses integrating DNA methylation and gene expression data. The analyses identified subsets of genes exhibiting the canonical negative correlation between methylation and gene expression, where hypomethylation of gene was associated with increased expression (Fig. 3A, green box). On the other hand, a subset of genes exhibited unexpected correlations between methylation and gene expression, where hypomethylation in various gene regions was associated with decreased expression (Fig. 3A, red box), a phenomenon described previously in transformed cells.[22]

In total, we identified 103 genes in epididymal adipocytes and 39 genes in inguinal adipocytes with concomitant differential methylation (adjusted p-value < 0.001) and expression (adjusted p-value < 0.1) (Fig. 3B). Of these, 24 genes were shared between the 2 adipose tissues (Table ST8, available at http://www.cbs.dtu.dk/suppl/Sonne2016/). Among the shared downregulated genes was *Lipe*, encoding one of the major enzymes involved in lipolysis in adipocytes. Further, expression of *Aacs*, encoding a ketone-using enzyme, providing acetyl-CoA used for synthesis of fatty acids and cholesterol, was downregulated, and knockout of *Aacs* in mice leads to suppressed expression of adipocyte markers like *Pparg* and *Cebpa*.[23] Furthermore, *Nrp2*, a lymphatic vessel developmental gene was hypomethylated and
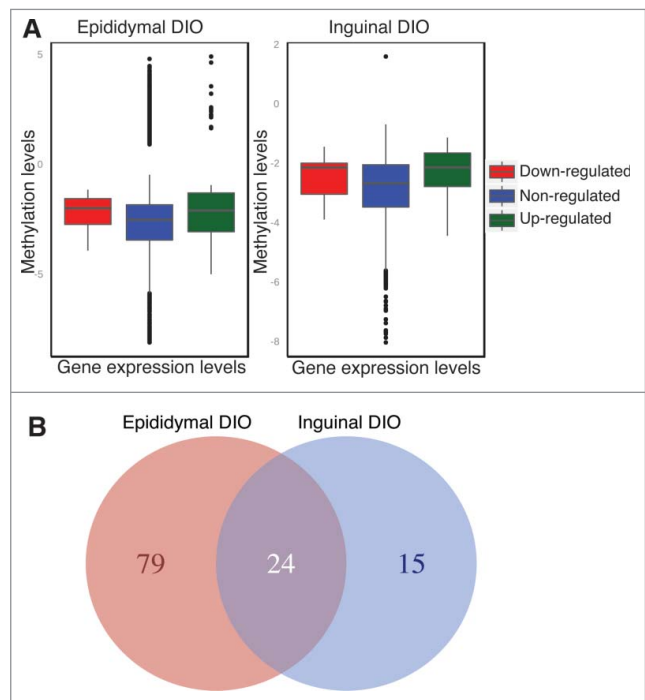

Figure 3. Correlation between DNA methylation and gene expression changes. [A] Box plot showing the median methylation fold changes in the 3 classes of gene expression, upregulated (log2(FC)>1, green), downregulated (log2(FC)<−1, red) and genes with no significant difference in gene expression (gray). The methylation levels on the y-axis were quantified as hypermethylated in diet-induced obesity if methylation level was greater than zero where as hypomethylated if methylation level was less than zero. The box represents the distribution of methylation states in each gene expression class, where the horizontal line denotes median methylation status of the group and the box represents the first and third quantiles. The dots represent above third quantile and below first quantile. [B] Number of overlapping genes exhibiting differences in gene expression (n = 5) and DNA methylation (n = 3) in epididymal and inguinal adipocytes from diet-induced obese C57BL/6J mice. Venn diagram represents the overlap between genes with differential gene expression along with differential methylation between inguinal and epididymal adipocytes from DIO C57BL/6J mice. DIO: Diet-induced obese.

demonstrated increased gene expression in both inguinal and epididymal adipocytes possibly reflecting neovascularization associated with adipose tissue growth.[24]

Genes exhibiting tissue-specific regulation included Zinc-finger nuclear protein (*Zfp521*), which acts as a repressor of adipogenesis.[25] Surprisingly, we found *Zfp521* expression upregulated in epididymal adipose tissue in obese mice. In the inguinal tissue, we observed upregulation of *Pkig* expression, possibly favoring adipocyte differentiation over osteogenesis[26] and increased expression of *Cpt1a*, reported to promote adipocyte differentiation by preserving insulin sensitivity.[27]

Discussion

MeDIP sequencing of DNA from mature adipocytes demonstrated profound changes in patterns and levels of DNA methylation associated with both diet-induced and genetically determined obesity. Interestingly, DMRs were predominantly hypomethylated in the obese state. Global hypomethylation has in previous studies been associated with methyl donor insufficiency due to lack of essential nutrients such as folate, vitamin B6 and B12, riboflavin or choline in the diet.[28] In the current study, lower serum levels of 1-methyl-L-histidine, L-methionine and arginine were observed in DIO mice compared with lean controls, but homocysteine levels were unchanged. Furthermore, no differences in methylation of LINE repeat regions indicated that the observed hypomethylation was not global, and probably not a result of methyl donor insufficiency. Hence, it is likely that obesity leads to specific changes in DNA methylation, and the majority of differentially methylated regions are demethylated leading to increased DNA accessibility. This is in line with a previous study reporting global hypomethylation in adipocytes of post-obese vs never-obese women.[29]

Comparison of the 2 obesity models identified a subset of 306/16 genes that were differentially methylated in mature adipocytes from epididymal/inguinal adipose tissue, irrespective of whether the excess fat mass was due to intake of a high fat diet (diet-induced obesity) or chow overeating (ob/ob). Interestingly, we found more differentially methylated genes in the diet-induced obesity model than the ob/ob model. This seemed inversely correlated with tissue expansion, as a higher proportion of the weight gain in ob/ob mice is due to increased fat mass.[30] However, it has been shown that adipose tissues from DIO mice are more prone to inflammation than ob/ob mice,[31] and inflammation is more pronounced in visceral compared with subcutaneous adipose tissue.[31] Since we observed more changes in epididymal than inguinal adipose tissue, we suggest that the differential methylation may reflect the inflammatory state of the tissue. In line with this, a large proportion of the DMRs is associated with pro-inflammatory genes expressed in the mature adipocytes themselves, including *Ccl2* which is expressed in mature adipocytes, or residual immune cells remaining in the fraction containing the mature adipocytes (Table ST9).

Previous studies of obesity-related methylation changes in mice are not directly comparable. Fan *et al.* used whole epididymal fat pads[10] while Multhaup *et al.* did not specify the adipose tissue for collection of mature adipocytes.[11] Similar to our findings, Fan *et al.* found more hypo- than hyper-methylated regions in epididymal tissue after high fat feeding. The diet-associated genes identified by Fan et al.,[10] *Kcnh2, Mboat7, Fzr1, Kctd5, Trpm4 and Ssr2* were also hypomethylated in mature adipocytes from both epididymal and inguinal adipose tissue in our study and *Hmg20a* was hypermethylated in epididymal adipose tissue in both studies.

Multhaup *et al.* used a more complicated cross-species setup, comparing DNA methylation changes in mature adipocytes from DIO vs lean mice to subcutaneous adipocytes from obese and lean humans.[11] In line with the observed hypermethylation of *Tmcc3* in epididymal adipose tissue of DIO mice in our study, Multhaup *et al.* observed hypermethylation of *Tmcc3* in high fat fed mice and obese humans. However, this was not accompanied by significant changes in gene expression. Comparing the findings by Multhaup *et al.* to our results, 16 DMRs displayed the same direction of methylation changes in epididymal tissue in DIO.[11]

Comparison of the regions that were differentially methylated in epididymal and inguinal adipose depots in the 2 mouse models of obesity allowed us to dissociate changes in methylation patterns associated with HF feeding from those associated with obesity. In epididymal adipose tissue, we identified a subset of 9 genes, which were differentially methylated and associated with changes in gene expression in both obesity models. Of these *Ehd2, Kctd15* and *Prcp* have previously been associated with obesity.[17,18,21] The gene encoding the transcription factor, IRF8, which is necessary for production of IgG2a and mounting a type 1 immune response[32] was hypomethylated and exhibited upregulated expression in obesity. This is in line with the finding that visceral adipose tissue inflammation and insulin resistance is dominated by a type 1 response.[33] Interestingly, a type 1 rather than a type 2 response dominates in C57BL/6J mice,[34] which may explain why C57BL/6J mice are more prone to inflammation, obesity and insulin resistance. Furthermore, IRFs are suggested to play a role in adipocyte differentiation and *Irf8* has been shown to have a higher expression in mature adipocytes as compared with the stromal-vascular fraction and F4/80+ macrophages.[35]

One problem associated with the present study and previous studies relates to the presence of cells from the stromal vascular fraction, including immune cells, in the isolated mature adipocytes. While elaborate FACS-based protocols minimizing the presence of cells from the stromal vascular fraction in the fraction representing the mature adipocytes have been developed,[36] they still do not guarantee the complete absence of cells from the stromal vascular fraction. To completely rule out such contamination, analyses based on single isolated adipocytes would seem to be required. We used available published data and BioGPS[37] (http://biogps.org) to examine

to what extent the genes exhibiting concurrent changes in DNA methylation and expression represented genes expressed in mature adipocyte. This survey showed that all these genes with the exception of *Arhgap6* are reported to be expressed in mature adipocytes, but also showed that *Irf8, Prcp, Setd6, Shank3, Nrp2 Zfp521, Pkig,* and *Cpt1a* are expressed in cell of the stromal vascular fraction that may contaminate the mature adipocytes. However, the use of 2 distinct models of obesity in this study would point to the mature adipocytes as a prominent contributor to the observed changes in DNA methylation and gene expression reported in the present study.

Thus, in summary we provide information on the correlation between changes in DNA methylation patterns and gene expression in a preparation of mature adipocytes from epididymal and inguinal adipose tissues in relation to obesity. By comparing DIO and genetically obese mice, we were able to dissociate changes associated with high fat feeding from those associated with obesity *per se*. This information will be of value in future studies on the mechanisms governing the development of obesity and changes in adipocyte function associated with obesity. In general, obesity was associated with pronounced global DNA hypomethylation, with epididymal fat exhibiting more obesity-associated DMRs than inguinal fat. Among genes exhibiting concurrent changes in methylation and gene expression we identified several known obesity-associated genes, and in addition, our analysis pointed to the transcription factor *Irf8* as a new candidate gene possibly involved in the function of adipose tissue in obese mice through hypomethylation driven expression changes.

Methods

Experimental design (animal model and DNA isolation)

Diet-induced obesity model: Four-week old male C57BL/6J (wt) mice obtained from the Jackson Laboratory were fed a regular diet (RD: ~20 E% fat Mouse diet 20, 5058, Picolab) or a high fat diet (HFD: 60 E% fat, D12492, Research Diets Inc.) for 15 weeks (n = 3). Genetically obese model: Nine-week old male ob/ob and wild type (wt) C57BL/6J mice (n = 4) were obtained from the Jackson Laboratory and fed a chow diet corresponding to the regular diet in the diet-induced obesity model.

Mature adipocytes were isolated from epididymal and inguinal adipose tissues from lean and obese mice by digesting with collagenase D (1.5U/mL) and dispase II (2.4U/mL) in 4% BSA in PBS supplemented with 10mM CaCl$_2$ at 37°C with constant agitation for 15 or 25 minutes for eWAT and iWAT, respectively, the completely digested sample was washed twice with 4% BSA in PBS and the floating layer of mature adipocytes collected. DNA was isolated using the DNeasy kit (Qiagen) and used for MeDIP sequencing.

A separate experiment was performed to investigate global gene expression differences in mature adipocytes corresponding to methylation changes. Twenty 4-week old male C57BL/6J mice were obtained from the Jackson Laboratory and fed either RD (n = 10) or a HFD (n = 10) as described above. After 15 weeks of feeding, plasma was collected for quantification of amino acids and metabolites. Mature adipocytes were collected from epididymal and inguinal adipose tissue as described above. Total RNA was extracted using Trizol LS (Invitrogen), DNAse treated (Qiagen) and LiCl precipitated.

MeDIP-seq library preparation

A library was prepared from 10 μg of DNA as described previously.[38] Briefly, DNA was fragmented. End repair, <A> base addition and adaptor ligation were performed using Illumina's Paired-End DNA Sample Prep kit following the manufacturer's instructions. Adaptor-ligated DNA was immunoprecipitated by anti-5mC, and MeDIP products were validated by qPCR using SYBR green mastermix (Applied Biosystems) and primers for positive and negative control regions supplied in the MeDIP kit (Diagenode). MeDIP DNA was purified with ZYMO DNA Clean & Concentrator-5 column following the manufacturer's instructions and amplified by adaptor-mediated PCR in a final reaction volume of 50 μL. Amplification quality and quantity were evaluated using Agilent 2100 bioanalyzer and DNA 1000 chips (Agilent Technology). Paired-end libraries were constructed and subjected to Illumina HiSeq2000 sequencing.

MeDIP-Seq data analysis

The paired-end reads from the MeDIP sequencing were checked for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The quality checked reads were mapped to the mouse reference genome build mm9 using Bowtie2[39] for each sample independently. Mapped reads were filtered for mapping quality 30 and sorted using Picard (http://picard.sourceforge.net) and SAMtools,[40] whereas duplicates were removed using Picard MarkDuplicates (http://picard.sourceforge.net). Aligned reads were filtered for missing mates in the alignments. Using the mapped reads, the correlation between replicates was checked using Spearman correlation coefficient. Mapped data in BAM format

were further analyzed to find differentially methylated regions (DMRs) between the ob/ob vs wt and HFD vs RD in epididymal and inguinal adipose tissue. In the MEDIPS[41] package of R, reads mapped to the genome were extended to 300 nucleotides to account for all CpGs in the region. The genome was divided into non-overlapping bins of 250 nucleotides during this analysis and reads mapped per region were counted. Relative methylation scores (rms) were calculated for each bin of the genome by counting the number of mapped reads. Normalization was applied on this count data to convert it to reads per million. MEDIPS internally uses the edgeR package to identify differentially methylated regions between the samples. EdgeR uses a negative binomial distribution (especially useful for discrete count data) to identify the DMRs between the 2 states under comparison, and thus, calculates mean methylation values (rpm, rms, ams), log fold changes, variances and p-values by comparing 2 sample sets. The DMRs were mapped and assigned to a gene if they were placed within 10kB +/− of the gene boundary.

Microarray analysis

The quality and quantity of RNA were determined using a Bioanalyzer nano kit (Agilent Technologies) and Qubit RNA BR Assay (Life Technologies), respectively. RNA from the 5 mice with the best RNA quality (RIN > 6.7) was chosen for subsequent analysis. Gene expression profiles were determined using the Mouse Agilent 4 × 44 v2 gene expression arrays (Agilent Technologies).

The single color microarray data was analyzed using the limma package.[42] Background correction and normalization of the data was done based on the negative controls. After normalization, we used the Bayesian method in the limma package to identify genes that exhibited differential expression between RD and HFD mice. Gene expression levels were determined as the median expression value of the probes corresponding to a given gene. Differential expression was filtered based on an FDR corrected p-value of less than 0.1 (corresponding to an FDR of 10%).

Integrative methods

The regions exhibiting differential methylation (DMRs) between RD and HFD were mapped to genes with differential gene expression (DGE). If the DMR fell within the gene boundary or +/− 10 KB of the gene boundary, the DMR was considered likely to affect gene expression. The genes with DMRs and DGE were only considered for further analysis if the fold change in expression between the 2 conditions was greater than 2.

Functional analysis

Using the MEME suite,[43] we performed de novo motif analysis of genomic regions, which were shared between DIO and ob/ob mice in epididymal and inguinal adipose tissues.

Determination of free amino acids and homocysteine in plasma

Plasma samples were collected from RD and HFD fed mice at termination and profiled for levels of amino acids. Plasma samples were first deproteinized by adding 10% sulfosalicylic acid (1:1 v/v) and centrifuged. The supernatants were filtered and the concentration of free amino acids were determined by using a Biochrom 30+ Amino Acid Analyzer as described previously.[44] For the analysis of total homocysteine, DL-dithiothreitol (DTT) was added to a final concentration of 1.2%. Samples were deproteinized and further analyzed as described for free amino acids.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

SK, KK, JW, RG and LM designed and supervised the study. SK, SBS, MDD and LSM performed the experiments, RY and YG did the data analyses and SBS, RY, SK, LM and KK wrote the manuscript.

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