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Genome-wide DNA promoter methylation and transcriptome analysis in human adipose tissue unravels novel candidate genes for obesity

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ABSTRACT

Objective/methods: DNA methylation plays an important role in obesity and related metabolic complications. We examined genome-wide DNA promoter methylation along with mRNA profiles in paired samples of human subcutaneous adipose tissue (SAT) and omental visceral adipose tissue (OVAT) from non-obese *vs.* obese individuals.
Results: We identified negatively correlated methylation and expression of several obesity-associated genes in our discovery dataset and *in silico* replicated *ETV6* in two independent cohorts. Further, we identified six adipose tissue depot-specific genes (*HAND2*, *HOXC6*, *PPARG*, *SORBS2*, *CD36*, and *CLDN1*). The effects were further supported in additional independent cohorts. Our top hits might play a role in adipogenesis and differentiation, obesity, lipid metabolism, and adipose tissue expandability. Finally, we show that *in vitro* methylation of *SORBS2* directly represses gene expression.
Conclusions: Taken together, our data show distinct tissue specific epigenetic alterations which associate with obesity.

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Keywords DNA methylation; Epigenetic mechanisms; Human adipose tissue depots; mRNA expression; Obesity-related co-morbidities

1. BACKGROUND

Abdominal omental visceral adipose tissue (OVAT) storage is more strongly associated with increased risk of obesity-related co-morbidities than subcutaneous (SAT) [1,2]. Studying adipose tissue depot specific DNA methylation and concomitant alterations in mRNA expression patterns can help to better understand the intrinsic differences between SAT and OVAT [3,4]. In addition to genetic factors ([5,6]; reviewed in [7]), epigenetic mechanisms contribute to the unexplained heritability of obesity and fat distribution. Despite recent progress [8,9], our current knowledge of adipose tissue depot specific methylation, especially in OVAT, and its impact on the development of co-morbidities is still limited. Recently, we reported significant differences in global DNA methylation levels between SAT and OVAT [10]

and at candidates genes, such as *TMEM18* [11]. Others demonstrated strong evidence for epigenetic mechanisms involved in obesity such as for *HIF3A* [12] and reported differences in DNA methylation and gene expression between human subcutaneous abdominal and gluteal adipose tissue [13] as well as between SAT and OVAT before and after gastric bypass and weight loss [14]. Furthermore, several studies reported methylation changes in skeletal muscle after bariatric surgery as well as after acute or long-term physical exercise in skeletal muscle or adipose tissue [15—18]. Recently, Barrès and colleagues demonstrated dynamic remodeling of DNA methylation in the spermatozoal epigenome after bariatric surgery [19], while others reported indications for epigenetic inheritance in mice [20].
In order to acquire deep insights into adipose tissue specific biological principles of epigenetic gene regulation and to elucidate how these

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Table 1 — Main characteristics of the Leipzig and Italian cohorts.

Subgroup	Leipzig							Italian			Italian vs. Leipzig	
	Non-obese	Obese	P-value non-obese vs. obese	Total	Methylation total	Expression total	Overlap of methylation and expression[a] total	Lean	Obese	P-value lean vs. obese	Lean vs. non-obese	Obese vs. obese
N	51	54	—	105	77	63	42	13	17	—	—	—
Male/female (N)	21/30	18/36	0.411	39/66	33/44	16/47	11/31	7/6	10/7	0.227	—	—
Lean/overweight/ obese (N)	44/7/0	0/0/54	—	44/7/54	36/5/36	23/3/37	17/1/24	13/0/0	0/0/17	—	—	—
Age (years)	64 ± 11	50 ± 15	<0.0001	57 ± 15	60 ± 10	53 ± 16	57 ± 10	42 ± 9	40 ± 10	0.576	<0.0001	0.012
BMI (kg/m^2)	23.05 ± 2.35	43.29 ± 10.90	<0.0001	33.46 ± 12.9	31.14 ± 11.5	36.07 ± 13.9	34.31 ± 13.5	24.41 ± 1.7	39.54 ± 8.4	<0.0001	0.021	0.198
Body fat (%)	22.3 ± 6.4	41.6 ± 10.4	<0.0001	32.4 ± 13.0	31.5 ± 12.7	35.44 ± 13.9	35.74 ± 14.2	—	—	—	—	—
CT-ratio (OVAT/ SAT area)	1.02 ± 0.89	0.32 ± 0.21	<0.0001	0.69 ± 0.75	0.78 ± 0.83	0.62 ± 0.51	0.69 ± 0.55	—	—	—	—	—
Waist (cm)	81.2 ± 18.4	124.9 ± 20.1	<0.0001	101.9 ± 29.1	96.9 ± 24.5	104.5 ± 30.1	99.0 ± 27.2	—	—	—	—	—
Hip (cm)	89.5 ± 11.8	124.9 ± 21.4	<0.0001	106.3 ± 24.6	100.5 ± 17.3	108.7 ± 23.9	103.1 ± 18.3	—	—	—	—	—
Waist-to-Hip ratio	0.90 ± 0.12	1.01 ± 0.16	<0.0001	0.95 ± 0.15	0.96 ± 0.15	0.95 ± 0.15	0.95 ± 0.14	—	—	—	—	—
SAT area (cm)	107.1 ± 197.2	1016.2 ± 585.3	<0.0001	533.2 ± 622.2	364.5 ± 411.1	582.6 ± 635.3	393.5 ± 400.5	—	—	—	—	—
OVAT area (cm)	69.2 ± 73.1	254.7 ± 124	<0.0001	156.2 ± 136.3	141.7 ± 125.7	164.0 ± 128.3	160.8 ± 136.0	—	—	—	—	—
Fasting plasma glucose (mmol/ l)	5.45 ± 0.88	6.40 ± 1.82	0.001	5.94 ± 1.52	5.98 ± 1.48	5.87 ± 1.31	6.00 ± 1.39	4.90 ± 0.57	5.03 ± 0.63	0.564	0.036	0.003
Fasting plasma insulin (pmol/l)	17.94 ± 47.03	134.84 ± 146.72	<0.0001	74.46 ± 121.92	75.02 ± 135.60	94.70 ± 141.10	107.59 ± 164.22	55.28 ± 35.84	111.12 ± 92.16	0.049	0.010	0.538
HbA1c (%)	5.42 ± 0.50	6.09 ± 0.76	<0.0001	5.76 ± 0.73	5.79 ± 0.80	5.71 ± 0.66	5.81 ± 0.74	—	—	—	—	—
HDL-C (mmol/l)	1.49 ± 0.48	1.15 ± 0.26	<0.0001	1.33 ± 0.43	1.34 ± 0.42	1.33 ± 0.39	1.37 ± 0.42	1.35 ± 0.29	1.14 ± 0.25	0.042	0.326	0.895
LDL-C (mmol/l)	3.06 ± 1.05	3.51 ± 1.38	0.107	3.27 ± 1.23	3.31 ± 1.23	3.28 ± 1.29	3.36 ± 1.38	2.98 ± 1.08	3.55 ± 1.07	0.161	0.812	0.916
Triglycerides (mmol/l)	1.28 ± 0.68	1.59 ± 0.94	0.150	1.41 ± 0.81	1.44 ± 0.79	1.20 ± 0.50	1.23 ± 0.51	1.11 ± 0.35	1.86 ± 0.65	0.001	0.398	0.313
Free fatty acids (mmol/l)	0.24 ± 0.25	0.48 ± 0.4	0.004	0.34 ± 0.34	0.29 ± 0.27	0.33 ± 0.36	0.23 ± 0.21	—	—	—	—	—
Urates (mg/dl)	—	—	—	—	—	—	—	5.02 ± 1.07	6.15 ± 1.07	0.008	—	—

All data are presented as mean ± SD.
[a] Expression either in SAT or OVAT.

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provoke the well-known physiological differences between SAT and OVAT, we tested the hypotheses that DNA promoter methylation levels in SAT and OVAT associate with BMI and obesity and that these profiles are adipose tissue depot specific.

2. MATERIALS AND METHODS

2.1. The Leipzig cohort

One hundred five Caucasian men ($N = 39$) and women ($N = 66$) were included in the study and paired samples of OVAT and SAT were obtained from patients who underwent open abdominal surgery for e.g. cholecystectomy or weight reduction surgery. Characteristics of the study population are summarized in Table 1. Seventy seven individuals out of 105 were included into the genome wide DNA-promoter methylation as well as in technical validation analyses. Initially, 82 individuals out of 105 were involved in mRNA expression profiling. Nineteen samples were excluded from the analysis due to insufficient RNA integrity leaving RNA expression values available from 63 individuals. Sixty three individuals out of 105 were included into mRNA expression profiling (SAT or OVAT). For a total number of 42 individuals, we were able to detect genome wide DNA promoter methylation in both tissue depots as well as genome wide expression profile in SAT or OVAT (overlap of methylation and expression in both tissues for 31 subjects, Table 1, Figure 1). Among the 105 subjects, 51 were non-obese; 44 of these were lean (mean age 65 ± 11 years, mean BMI 22.6 ± 2.2 kg/m^2), 7 were overweight (mean age 61 ± 13 years, mean BMI 25.8 ± 0.8 kg/m^2) and 54 were obese (mean age

50 ± 15 years, mean BMI 43.3 ± 10.9 kg/m^2). Among the 77 individuals who were involved in methylation analyses, 54 did not have T2D, and 23 subjects had T2D. Eighty two individuals out of 105 were involved in mRNA expression profiling (mean age 55 ± 16 years, mean BMI 35.2 ± 13.7, non-obese $= 36$, obese $= 46$), including 61 non-diabetics and 21 subjects with T2D. Sixty three individuals out of 105 which were included into mRNA expression profiling (SAT or OVAT), included 49 non-diabetics and 14 subjects with T2D. Mature adipocytes and cells of the stromal vascular fraction were isolated from adipose tissue samples of 47 additional individuals (20 men, 27 women). Thirty three of these were obese (mean age 47 ± 11; mean BMI 55.4 ± 10.8) and 14 were lean (mean age 67 ± 9; mean BMI 23.4 ± 1.4). Paired samples of isolated adipocytes and SVF were available from 34 subjects (30 obese, mean age 47 ± 11; mean BMI 55.0 ± 11.1; 4 lean, mean age 70 ± 14, mean BMI 23.2 ± 0.4). Phenotyping was performed as previously described [21] and included anthropometric measurements, (weight, height, waist-to-hip-ratio (WHR)), body fat analysis using dual-energy X-ray absorptiometry, and laboratory parameters such as fasting plasma glucose and insulin, a 75 g oral glucose tolerance test (OGTT), and HbA1c. Insulin sensitivity was assessed with hyperinsulinemic-euglycemic clamps. Based on computed tomography scans measurement (L4–L5) of abdominal visceral and subcutaneous fat areas, obese subjects were further categorized as predominantly viscerally or subcutaneously obese as defined by a ratio of visceral/subcutaneously fat area of $>$ or <0.5. Importantly, we included only individuals with a (in part self-reported) stable body weight at least 3 months prior to surgery ($<2\%$
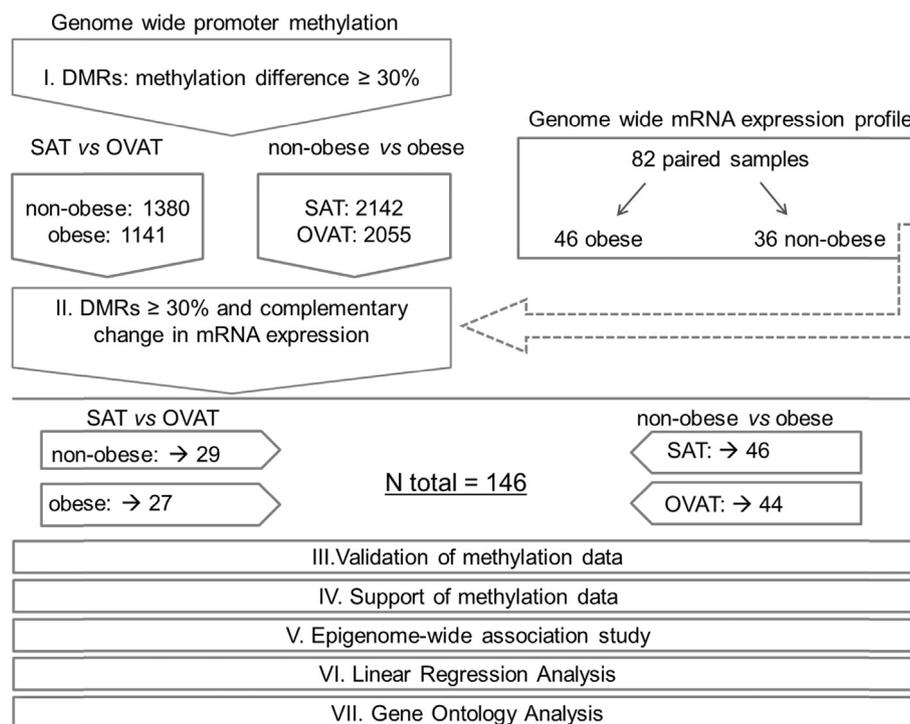
Figure 1: Experimental workflow and study design. The figure shows the experimental workflow and study design. **I**. Genome wide promoter methylation was generated and an arbitrarily chosen cut off of 30% methylation differences (=DMR; differential methylated region) in the comparisons. Identified genes are given as numbers in non-obese and obese subgroups (SAT *vs*. OVAT) and in SAT and OVAT subgroups (non-obese *vs*. obese). **II**. The identified transcripts were tested for overlapping changes in mRNA expression. The final number of genes with negatively correlation between methylation and expression is underlined. Additionally, methylation data was validated using pyrosequencing for two genes (step **III**.) and supported in 3 independent data sets (step **IV**.). Furthermore, we conducted an epigenome-wide association study (EWAS) (step **V**.). Finally most promising candidates ($N = 24$) were tested for association with anthropometric and metabolic variables using linear regression analysis (step **VI**.) and analyzed using gene ontology analyses (step **VII**.).

fluctuations of body weight). All study protocols have been approved by the ethics committee of the University of Leipzig. All participants gave written informed consent before taking part in the study.

2.2. Independent Italian cohort to support methylation effects

A total of 30 Caucasian individuals with paired samples of SAT and OVAT from an Italian cohort were included in the replication analysis. The cohort comprised 17 non-diabetic obese subjects and 13 lean (Table 1). SAT and OVAT biopsies where obtained either during bariatric surgery interventions (in obese individuals) or from abdominal surgery for non-inflammatory diseases (lean individuals). The study was approved by the Ethics Committee of Istituto Auxologico Italiano, Milan, Italy, and all patients gave written informed consent for sampling during surgical procedures. Compared to lean individuals, obese subjects were similar in terms of age and sex distribution, glucose, and total cholesterol and LDL levels. Obese subjects had a respectively significant higher mean value for BMI, triglycerides, urates levels, and white blood cells count (Table 1). HOMA-IR was ≥ 2 in 11 out of 17 obese enrolled patients and 3 had a diagnosis for hypertension. One patient was on fluoxetin treatment.

2.3. Sample preparation

Adipose tissue samples from the Leipzig cohort were taken from abdominal regions (SAT in the 5 cm periumbilical area, OVAT from the upper left part of omentum), directly frozen in liquid nitrogen after explantation, and stored at $-80\ °C$. DNA and RNA were extracted using standard approaches (SIGMA ALDRICH, Saint Louis, USA and Qiagen, Hilden, Germany). DNA from the confirmatory cohort (Italy) was extracted using DNeasy Blood and Tissue Kit (Qiagen, Milano, Italy). DNA was bisulfite converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA).

2.4. Genome wide promoter methylation

2.4.1. The Leipzig cohort

DNA promoter methylation was measured by using methylated DNA immunoprecipitation (MeDIP) combined with subsequent hybridization on tiling arrays. In brief, DNA shearing was performed by using a Bioruptor Plus, and MeDIP was performed using the MagMeDIP kit (both Diagenode, Seraing, Belgium). PCR primers for known methylated (TSH2B) and unmethylated regions (GAPDH) (MagMeDIP kit, Diagenode) were used to test MeDIP specificity by qPCR. Enriched methylated DNA was amplified using WGAII, purified using GenElute Clean-UP kit, and re-amplified using a modified protocol [22] of WGAIII (all SIGMA-ALDRICH, Saint Louis, USA). The amount of dUTP was increased to 1.8 µl to ensure length (about 66 bp) and uniformity of the double stranded DNA fragments. DNA was fragmented, labeled, and hybridized on GeneChip Human Promoter 1.0R Arrays (Affymetrix Inc., Santa Clara, USA). Arrays comprised ∼4.6 million probes covering ∼25.500 human promoter regions.

2.4.2. Italian cohort

2.4.2.1. Differential methylation (DM) analysis using Infinium HumanMethylation450 BeadChips. Infinium HumanMethylation450K BeadChips (Illumina) were used to detect methylation levels in paired SAT and OVAT samples. At gene level 450K, microarray covers 99% of RefSeq genes with multiple sites in the annotated promoter (1500 bp or 200 bp upstream of transcription start site), 5′-UTR, first exon, gene body and 3′-UTR. From the CpG context, it covers 96% of CpG islands with multiple sites in the annotated CpG islands, shores (regions flanking island) and shelves (regions flanking shores).

2.5. Genome wide expression — Leipzig cohort

Genome wide expression profiling was performed using Illumina human HT-12 expression chips. RNA integrity and concentration was examined using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RNA integrity values (RIN) of less than five were discarded from further analysis [23].

2.6. Pyrosequencing

Pyrosequencing was performed for HOXD3 and HOXD4 as described elsewhere [10,11]. PyroMark assays were designed for: NM_006898; probe number: 176736323; NM_014621, probe numbers: 176723238, 176724282. Pyrosequencing was conducted in duplicates and sequencing results were checked for consistence with MeDIP methylation array results (Supplementary Table 9).

2.7. Luciferase assays

2000 bp sized DNA fragments of the human SORBS2 and EMX2 promoter region (hg38-SORBS2: chr4:186969043-186971042; EMX2: chr10:119289946-119291945) were inserted into a CpG-free firefly luciferase reporter vector (pCpGL-basic) [24]. We performed luciferase assays as described elsewhere [18]. Briefly, DNA sequences were inserted into the vector and constructs were methylated either using SssI (methylation of double stranded dinucleotide CG sequence; results in complete promoter methylation) or HpaII (methylation of the internal cytosine residue in the CCGG sequence) methyltransferases and S-adenosylmethionine (SAM) as methyldonor (New England Biolabs, Frankfurt am Main, Germany). Unmethylated controls were treated exactly as methylated constructs including application of SAM but without using methylation enzymes. Constructs were transfected into human MCF7 cells (human breast adenocarcinoma cell line). Additionally, transfection of MCF7 cells with an empty-reporter vector serves as background control of firefly activity. The unmethylated promoter construct generates significantly more luciferase signal than the empty reporter vector (data not shown). All cells were co-transfected with pRL renilla luciferase control-reporter vector (pRL-CMV vector, Promega, Madison, USA) to control transfection efficiency. FuGene® HD transfection reagent (Promega, Madison, USA) was used for transfection of cells. Furthermore, untransfected cells were used for background correction of renilla luciferase activity. Finally, luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, USA). P-values were calculated using two sided, unpaired t-tests.

2.8. qPCR experiments in adipocytes vs. stromal vascular fractions

Adipocytes were isolated by collagenase (1 mg/ml) digestion. For expression analysis of SORBS2, HAND2, HOXC6, EMX2, PPARG, CLDN1, CD36, and ETV6 in adipocytes and stromal vascular fractions, total RNA was isolated from adipocytes and stromal vascular fractions extracted from subcutaneous and visceral adipose tissue. Relative quantification was performed with the Quantstudio 6 System (Thermo Fisher Scientific), using commercially available TaqMan Probes (Assay-on-Demand; Thermo Fisher Scientific). Relative quantities of target transcripts were calculated from samples after normalization of the data against the endogenous control, HPRT1 rRNA (Thermo Fisher Scientific).

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2.9. Data analysis and statistics

2.9.1. The Leipzig cohort

Normalized probe intensities were used. Differential methylation (two-class comparisons) was calculated using Model-based analysis of tiling-arrays for ChIP—chip (MAT) [25] as implemented in the R package 'rMAT' (http://www.bioconductor.org/packages/release/bioc/manuals/rMAT/man/rMAT.pdf). We included all probes interrogating methylation of cytosines 2000 bp upstream to 200 bp downstream the transcription start site. rMAT provides regions of differential methylation for each promoter. They were transformed into differential methylation percentage-values, ranging from 0% (no differential methylation) to 100% (all interrogated regions are affected; Tables 2 and 3). Only promoters with a minimum differential methylation of larger than 30% were considered in further analysis (Supplementary Tables 1—4). Significance was estimated by permutation tests

Table 2 — Top genes differentially methylated and expressed between non-obese and obese individuals.

Depot	Gene name	Percentage of hyper/hypomethylation		P-value		logFC
		Obese	Non-obese	Obese	Non-obese	
SAT	SETMAR	53.5	0.0	2.00E-04	1	−0.032
	HSD17B8	45.0	0.0	2.00E-04	1	−0.103
	GOLGA6L4	42.9	0.0	2.00E-04	1	−0.040
	NEDD4L	35.6	0.0	2.00E-04	1	−0.036
	THNSL2	34.7	0.0	4.00E-04	0.9996	−0.050
	RTN1	34.0	0.0	2.00E-04	1	−0.120
	NET1	33.4	0.0	2.00E-04	1	−0.158
	C8orf46	33.0	0.0	2.00E-04	1	−0.063
	TTLL7	32.1	0.0	2.00E-04	1	−0.046
	BCKDHB	31.4	0.0	2.00E-04	1	−0.085
	EPDR1	0.0	72.8	1	2.00E-04	0.169
	RUNX1	0.0	64.9	1	2.00E-04	0.051
	ATP6V1B2	0.0	61.7	1	2.00E-04	0.028
	GPR137B	0.0	50.8	1	2.00E-04	0.064
	SLC2A5	0.0	49.7	1	2.00E-04	0.141
	HCK	0.0	49.0	1	2.00E-04	0.106
	CD99L2	0.0	46.0	1	2.00E-04	0.011
	FAM46A	0.0	44.8	1	2.00E-04	0.104
	EXOSC7	0.0	44.1	1	2.00E-04	0.175
	CD9	0.0	42.8	1	2.00E-04	0.116
OVAT	SORBS2	81.9	0.0	2.00E-04	1	−0.021
	CASQ2	74.6	0.0	2.00E-04	1	−0.043
	DUSP22	59.7	12.6	2.00E-04	1	−0.059
	KCNT2	58.9	0.0	2.00E-04	1	−0.007
	SETMAR	57.1	0.0	2.00E-04	1	−0.056
	GOLGA6L4	50.4	0.0	2.00E-04	1	−0.026
	MMRN1	46.7	0.0	2.00E-04	1	−0.053
	CPE	42.2	0.0	2.00E-04	1	−0.024
	KIAA1217	40.7	0.0	2.00E-04	1	−0.098
	APMAP	40.1	0.0	2.00E-04	1	−0.107
	RGS1	0.0	64.2	1	2.00E-04	0.110
	TNFRSF21	0.0	57.0	1	2.00E-04	0.039
	DSP	0.0	47.2	1	2.00E-04	0.134
	AQP9	0.0	44.8	1	2.00E-04	0.133
	NKX3-2	0.0	43.3	1	2.00E-04	0.087
	TLE1	0.0	43.0	1	2.00E-04	0.004
	KCTD8	2.2	44.8	1	2.00E-04	0.034
	CYFIP2	14.2	56.1	1	2.00E-04	0.087
	SERPINE2	0.0	41.7	1	2.00E-04	0.091
	VAV3	0.0	39.7	1	2.00E-04	0.014

Table 2 presents the top 10 candidate genes in SAT and OVAT comparing promoter methylation levels between non-obese and obese individuals. Differences in mRNA expression are given as logarithmic fold change (logFC), consistently in comparison to non-obese subjects.

Table 3 — Top genes differentially methylated and expressed between SAT and OVAT.

	Gene name	Percentage of hyper/hypomethylation		P-value		logFC
		SAT	OVAT	SAT	OVAT	
Obese	FAM25C	54.9	0.0	2.00E-04	1	0.509
	CKMT1B	53.2	0.0	2.00E-04	1	0.468
	SORBS2	51.8	0.0	2.00E-04	1	0.192
	TCF21	45.7	0.0	2.00E-04	1	0.521
	DFNA5	36.3	0.0	2.00E-04	1	0.270
	OLFML1	35.2	0.0	2.00E-04	1	0.142
	ANGPTL7	33.7	0.0	2.00E-04	1	0.278
	HAND2	33.5	0.0	2.00E-04	1	0.226
	PAPPA	31.0	0.0	2.00E-04	1	0.204
	CKMT1A	30.5	0.0	2.00E-04	1	0.357
	HOXC6	0.0	66.4	1	2.00E-04	−0.479
	AFF3	0.0	58.2	1	2.00E-04	−0.166
	PHLDB1	0.0	53.2	1	2.00E-04	−0.100
	EMX2	0.0	45.8	1	2.00E-04	−0.362
	COL12A12	0.0	44.5	1	2.00E-04	−0.210
	SORT1	0.0	43.5	1	2.00E-04	−0.191
	AOC3	0.0	42.5	1	2.00E-04	−0.117
	DEFB1	0.0	41.2	1	2.00E-04	−0.289
	PPARG	0.0	41.7	1	2.00E-04	−0.132
	ANXA1	0.0	39.3	1	2.00E-04	−0.138
Non-obese	TCF21	81.5	0.0	2.00E-04	1	0.399
	CKMT1A	61.0	0.0	2.00E-04	1	0.147
	SORBS2	56.8	0.0	2.00E-04	1	0.142
	CPE	53.8	0.0	2.00E-04	1	0.106
	GREM1	49.0	0.0	2.00E-04	1	0.338
	CLDN1	48.5	0.0	2.00E-04	1	0.543
	PFKM	42.8	0.0	2.00E-04	1	0.175
	PAMR1	42.4	0.0	2.00E-04	1	0.301
	PNMA2	40.2	0.0	2.00E-04	1	0.190
	DFNA5	38.3	0.0	2.00E-04	1	0.173
	HOXC6	0.00	72.2	1	2.00E-04	−0.416
	MEOX1	0.00	48.3	1	2.00E-04	−0.186
	GOS2	0.00	47.5	1	2.00E-04	−0.121
	TMEM139	0.00	46.9	1	2.00E-04	−0.063
	BHMT	0.00	46.3	1	2.00E-04	−0.085
	PPARG	0.00	46.2	1	2.00E-04	−0.035
	AOC3	0.00	44.2	1	2.00E-04	−0.097
	COL12A1	0.00	43.1	1	2.00E-04	−0.067
	ANGPT1	0.00	42.8	1	2.00E-04	−0.055
	EMX2	0.00	41.3	1	2.00E-04	−0.260

Table 3 presents the top 10 candidate genes in non-obese and obese individuals comparing promoter methylation levels between SAT and OVAT. Corresponding negatively correlated mRNA expression values are shown. Changes in mRNA expression are given as logFC, consistently standardized in relation to OVAT. Genes which are hypermethylated in SAT show increased mRNA expression in OVAT and are represented by a positive logFC value.

providing P-values, which were multitest adjusted in terms of false discovery rates (FDR) using the R package fdr-tool [26]. We applied a cut-off of FDR < 0.3.

IlluminaBeadChipsHT-12, expression data were background-corrected, log-transformed, and quantile-normalized before downstream analysis. Expression data were then matched to methylation data and ranked by using the Mann—Whitney U test. Differential expression analysis was performed using the R package oposSOM [27]. Results were listed as log fold change (logFC; Tables 2 and 3). Differential expression and methylation were then analyzed together to identify genes being either up- or down-regulated and either hyper- or hypo-methylated. For further analyses, we focused exclusively on genes showing negatively correlated DNA methylation and expression values (Figure 2). For visualization by means of circle plots, we used circos software package (version circos-0.65-pre5) [28].
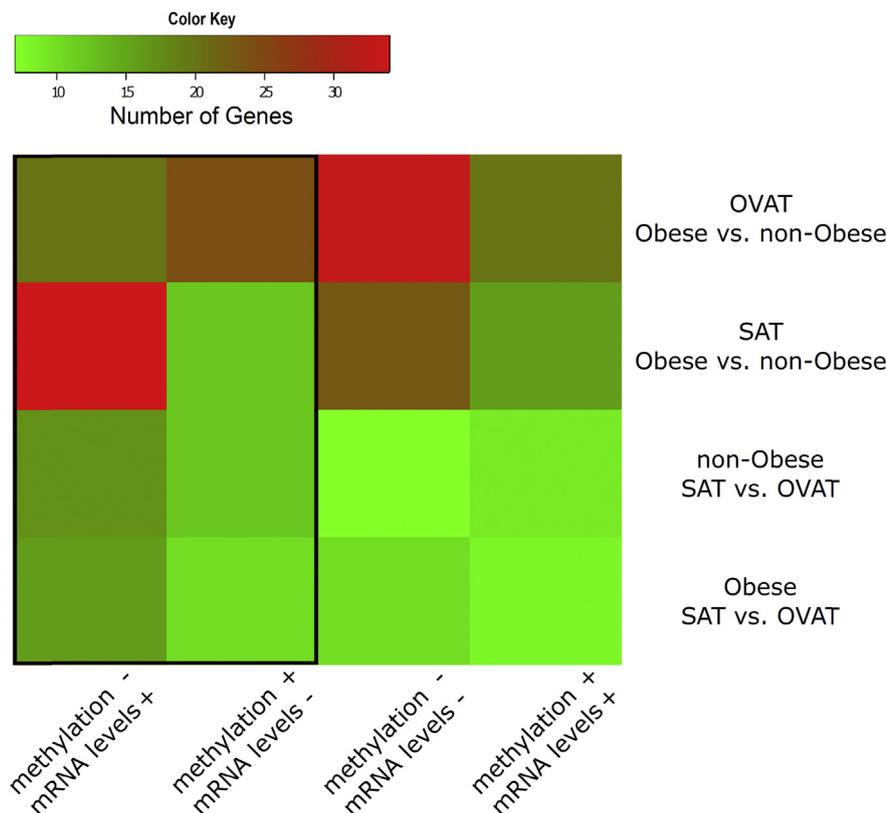
Figure 2: Numbers of identified genes showing co-regulated changes in methylation and gene expression. The heatmap [57] shows the number of genes showing significant differences in methylation and mRNA expression. The framed columns on the left side represent genes conferring negatively correlated methylation and expression levels, which were taken forward to replication analyses.

Linear regression analyses were performed using R [58] adjusted for sex, age, lnBMI (except for BMI), and type 2 diabetes. Methylation data were used as normalized probe intensities, and non-normally distributed phenotypes were log transformed to approximate normal distribution.

To analyze differences in expression levels between visceral and subcutaneous adipocytes *vs.* stromal vascular fractions as well as for group differences (non-obese *vs.* obese), unpaired two-tailed *t*-tests were applied.

2.9.2. Independent Italian cohort to support methylation effects
In order to identify differentially methylated cytosines (DMCs), we used a method based on *F*-test. We first focused on DMCs between SAT and VAT and then on those between lean and obese groups for each adipose depot. While DMCs analysis among sample group (SAT/VAT) resulted in about 100,000 DMC positions (multiple testing corrected q-value < 0.05), only about 1800 DMC positions (multiple testing corrected q-value < 0.05) could be identified between lean and obese individuals.

2.9.2.1. Data filtering.
The R package minfi was used to read differential methylation values (describing methylation level between 0 and 100%) from the .idat files. A detection *P*-value was determined for every cytosine probed in every sample. Then, the cytosine positions with *P*-value > 0.05 in more than 20% of total samples (60) were removed from the further analysis. In total, 3532 cytosines were removed out of 485512. The data were then subjected to within array normalization method SWAN which reduces technical variation within and between arrays.

3. RESULTS

3.1. General methylome and transcriptome differences
Differential methylation was estimated in the promoter range of each gene. By comparing non-obese *vs.* obese individuals in the same adipose tissue depot, we found 2142 genes which were differentially methylated in SAT, while 2055 genes were identified in OVAT (Figure 1).

In non-obese subjects, we identified 1381 differentially methylated genes when comparing SAT and OVAT. The same comparison in obese individuals yielded 1141 genes (Figure 1). All these identified genes passed through a correction for multiple testing using FDR (Supplementary Tables 1—4).

To further substantiate the results from this discovery approach, we focused on genes showing negative correlations in mRNA expression profiles along with the described methylation differences. We finally identified 29 genes differentially regulated in SAT *vs.* OVAT in the non-obese subgroup and 27 in obese individuals. Similarly, in our obesity-specific analysis, we focused only on genes fulfilling these stringent filter criteria, and, by comparing non-obese *vs.* obese subjects, we found 46 differentially regulated genes in SAT and 44 genes in OVAT (all Figures 1 and 2).

3.2. Obesity associated differences in DNA methylation and mRNA expression
The top ten candidate genes showing the biggest differences in the ratio of hyper/hypomethylation between non-obese and obese individuals in SAT and OVAT are presented in Table 2. Effect directions of methylation and expression of differentially regulated genes and their

distribution over the genome are visualized in circle plots (Figure 3C,D). We observed several novel candidates such as the *empty spiracles homeobox 2* (*EMX2*), *EPDR1* (*ependymin related 1*), *RUNX1* (*runt-related transcription factor 1*), *DUSP22* (*dual specificity phosphatase 22*), and *RGS1* (*regulator of G-protein signaling 1*). Complete lists of differentially methylated and expressed transcripts are presented in Supplementary Tables 4 and 5

3.3. Adipose tissue depot specific differences

The top ten candidate genes showing the largest differences in the ratio of hyper/hypomethylation between SAT and OVAT in both non-obese and obese subjects along with corresponding changes in gene expression are presented in Table 3. Further, effect directions and their distribution over the genome are visualized in circle plots (Figure 3A,B). We identified novel candidates that may play a role in adipose tissue development and differentiation such as the *transcription factor 21* (*TCF21*), also known as an epigenetically regulated white adipocyte marker [29], which was strongly hypermethylated and less expressed in SAT among non-obese subjects. Another identified candidate was *claudin 1* (*CLDN1*) encoding an integral membrane protein and a component of tight junction strands. We further observed the *heart and neural crest derivatives expressed 2* (*HAND2*) which may play a role in adipogenic differentiation via *NOTCH* signaling [30,31] and the *cluster of differentiation 36* (*CD36*), which is involved in lipid metabolism [32,33]. Further, we also confirmed known candidate genes such as the *homeobox C6* (*HOXC6*) and the *peroxisome proliferator-activated receptor gamma* (*PPARG*). Complete lists of differentially methylated and expressed genes are presented in Supplementary Tables 7 and 8

3.4. Gene expression analysis in isolated adipocytes and SVF

To further substantiate our findings and to shed more light on potential functional effects of these methylation events, we analyzed gene expression of the top genes (*SORBS2, HAND2, HOXC6, EMX2, PPARG, CLDN1, CD36,* and *ETV6*) separately in mature adipocytes and stromal vascular fractions (Supplementary Figure 1). Our results largely confirm the initially observed effects performed in adipose tissue biopsies. However, although non-significant we discovered different effects in adipocytes compared to SVF for two genes (*PPARG* and *CD36*), which might have influenced our initial analyses.

3.5. Technical validation of methylation data

Pyrosequencing was used to validate methylation data. Two genes demonstrating high differences of DNA methylation between SAT and OVAT (Supplementary Tables 1 and 2) were selected for validation. The results revealed similar directions in DNA methylation changes of the analyzed transcripts of *homeobox D3* (*HOXD3*); and *homeobox D4* (*HOXD4*) compared to the genome wide array derived data (Supplementary Table 9).

3.6. Support of methylation effects in independent cohorts

We further sought to support our methylation data in three independent data sets from analyses of DNA methylation pattern in SAT *vs.* OVAT and/or in non-obese *vs.* obese subjects. We used a data set comprising six subjects with data available for SAT and OVAT (obtained post mortem) [34] publicly available via MARMAL-Aid [35]. We observed nine genes showing similar effect directions of differential methylation in SAT *vs.* OVAT. Further, we used another independent cohort from Italy comprising 30 individuals, which supported methylation effects of 44 genes compared to our initial methylation data. Among these three cohorts, we finally observed 6 genes, which were consistently

supported: *HAND2, HOXC6, PPARG, sorbin and SH3 domain containing 2* (*SORBS2*), *CD36,* and *CLDN1* (all Table 4).

Moreover, to add further weight to our finding of methylation differences between non-obese *vs.* obese subjects, we used publicly available data of 15 individuals as reported by Benton et al. (E-MTAB-3052) [14]. By considering individuals as obese before bariatric surgery while categorizing subjects after the operation as lean(er), we used this dataset to look for genes showing similar effect directions as compared to our initial methylation data in SAT and OVAT. Our analysis revealed two genes exhibiting the same effect direction. Again, we also used the Italian cohort and supported methylation directions at 42 genes. Among all three cohorts, *ets variant 6 (ETV6)* (Table 4) showed consistent effects.

3.7. In-vitro methylation decreases gene expression

Next, we tested whether induced changes in promoter DNA methylation of two identified top genes *SORBS2* and *EMX2* truly affect the transcriptional activity *in vitro* using a firefly luciferase assay as described elsewhere [18]. The results clearly show that methylation by *Sss*I methyltransferase significantly reduced the luciferase activity for both constructs as shown in Figure 4.

### 3.8. Epigenome wide analysis (EWAS) for BMI

EWAS was conducted in the total cohort for which methylation data were available ($N = 77$). We tested for association of mean DNA methylation levels per promoter (for 22.625 transcripts) with BMI as a continuous variable in SAT and OVAT, separately. In SAT, we identified the *sarcospan* gene (*SSPN*) significantly and negatively associated to BMI (Figure 5, Table 5). In OVAT, we observed the *coiled-coil domain containing protein 125* (*CCDC125*) showing the strongest positive effect on BMI (Figure 5, Table 5). Both top hits reached genome wide statistical significance according to multiple testing (Bonferroni corrected 0.05/22.625 transcripts = $P < 2.20 \times 10^{-6}$).

3.9. Gene ontology analysis

Next, we used the successfully replicated genes from i) non-obese *vs.* obese and ii) SAT *vs.* OVAT (Tables 2 and 3; total $N = 7$), the top three candidates selected from our top ten hits ($N = 17$) and the top candidates from the EWAS ($N = 2$) to generate a final list of the most promising candidate genes ($N = 24$), which were taken forward to gene ontology analyses and extended association studies with multiple variables of anthropometric and metabolic phenotypes. We performed gene ontology analyses (https://david.ncifcrf.gov/) and found that identified genes are most likely involved in transcription factor activity (enrichment score ~ 1.87), regulation of transcription (enrichment score ~ 1.65), transcriptional regulation (enrichment score ~ 1.56), and DNA-binding (enrichment score ~ 1.19) (Figure 6, Supplementary Table 10).

3.10. Association with phenotypic traits

Linear regression analyses were performed in the total cohort for which methylation data were available ($N = 77$) (Supplementary Tables 11—14). We found several genes associated to BMI in OVAT (nine out of 24) while only four genes show similar effects in SAT. The strongest associations with BMI in OVAT were observed for *SORBS2* and *CASQ2* (Supplementary Table 12), although these genes did not show any additional associations to anthropometric traits. Recently, it was reported that DNA methylation at *SORBS2* and *CASQ2* is related to BMI in SAT [36]. We observed methylation of *RUNX1* in OVAT associated significantly with parameters of fat distribution such as CT-ratio (OVAT/SAT area), waist, waist-to-hip ratio (WHR), and visceral fat area
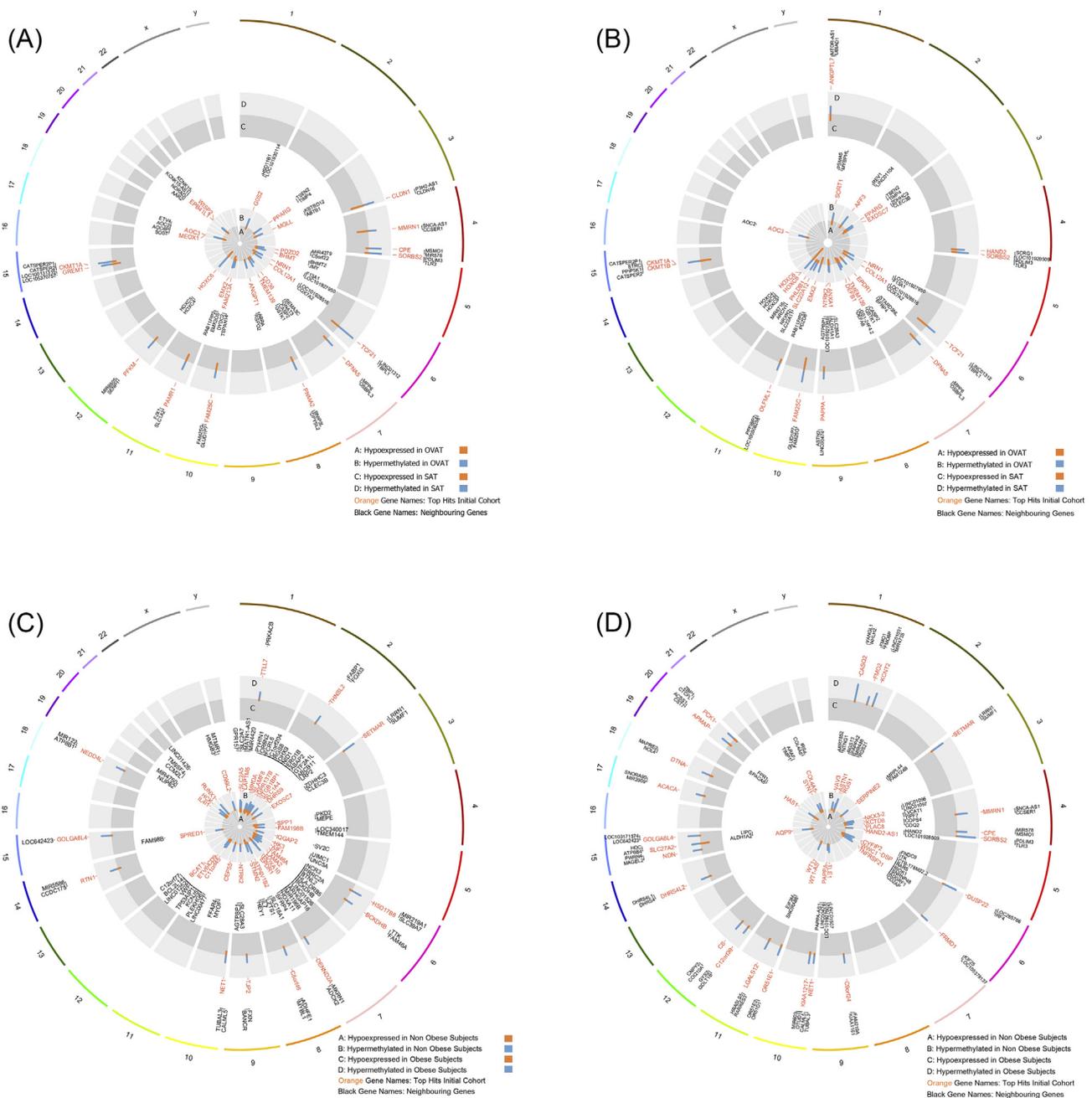
Figure 3: Circle plots showing the genome wide distribution of differentially regulated genes. The figure shows all genes conferring significant changes in DNA methylation and negatively correlated gene expression levels. Blue bars represent methylation intensities; orange bars represent expression levels (logFC values). Outside circles represent chromosomes, the light grey circles represent methylation levels, and the grey circles represent expression values. **A)** non-obese subjects hypermethylated/lower expressed in SAT (middle circle) and hypermethylated/lower expressed in OVAT (inner circle); **B)** obese subjects hypermethylated/lower expressed in SAT (middle circle) and hypermethylated/lower expressed in OVAT (inner circle); **C)** SAT: genes hypermethylated/lower expressed in obese (middle circle) and hypermethylated/lower expressed in non-obese individuals (inner circle); **D)** OVAT: genes hypermethylated/lower expressed in obese (middle circle) and hypermethylated/lower expressed in non-obese individuals (inner circle).

(Supplementary Table 12). Interestingly, *RUNX1* methylation in SAT was also related to CT-ratio but showed an effect opposite in direction on subcutaneous fat area.

Several of the here reported associations were still significant after correction for multiple testing according to Bonferroni (0.05/16 traits = $P < 3.1 \times 10^{-3}$). Notably, we found SAT derived *SSPN* methylation levels significantly related to WHR, while methylation

levels in OVAT correlated with serum levels of free fatty acids (FFA). Further, methylation at four genes (*HAND2*, *SORBS2, CASQ2*, and *CCDC125*) in OVAT was significantly associated with BMI (Supplementary Tables 11 and 12).

Next, we sought to investigate the association of methylation levels with variables related to glucose and lipid metabolism (Supplementary Tables 13 and 14). We observed the strongest relationship between

Table 4 — In-silico replication of methylation data.

SAT vs. OVAT (combined non-obese and obese)			Obese vs non-obese (combined SAT and OVAT)		
Own initial data	Replication Italy	Replication Slieker et al. (33)	Own initial data	Replication Italy	Replication Benton et al. (27) (SAT)
TMEM139	TCF21	DMRT2	SETMAR	BCKDHB	AIF1
EPDR1	CKMT1B	DMRT3	**ETV6**	ETV6	ETV6
HAND2	HAND2	HAND2	HSD17B8	HSD17B8	
HOXC6	HOXC6	HOXC6	NEDD4L	NEDD4L	
PPARG	PPARG	PPARG	THNSL2	NET1	
SORBS2	SORBS2	SORBS2	TTLL7	SETMAR	
CD36	CD36	CD36	C8orf46	TJP2	
CLDN1	CLDN1	CLDN1	RTN1	C11orf45	
DEFB1	TMEM139	TBX15	NET1	DHRS9	
AOC3	ANGPTL7		TJP2	C8orf46	
COL12A1	EPDR1		DENND2A	FAM46A	
EMX2	NTRK2		BCKDHB	GPR137B	
AFF3	EXOSC7		SPP1	HOXA10	
NTRK2	ANXA1		STMN2	IL4I1	
CKMT1A	HOXC8		LHCGR	IQGAP2	
PAPPA	AOC3		FAM198B	LAPTM5	
HOXC8	EMX2		NPL	LHCGR	
ANGPTL7	OLFML1		LAPTM5	RUNX1	
OLFML1	DEFB1		SLAMF8	SLAMF8	
DFNA5	NRN1		EXOSC7	ACACA	
TCF21	COL12A1		HK3	C9orf24	
NRN1	AFF3		EPDR1	CPE	
CKMT1B	GREM1		MNDA	DHRS4L2	
FAM25C	CKMT1A		C11orf45	DTNA	
TPGS2	CPE		GOLGA6L4	DUSP22	
CDH13	MMRN1		BCAT1	FMO2	
EXOSC7	PFKM		SLC2A5	FRMD1	
PDZD2	PAPPA		DHRS9	KIAA1217	
EPB41L1	PAMR1		ITGB1BP1	LGALS12	
WISP2	FAM25C		HLA-DRA	NDN	
PHLDB1	PNMA2		CD9	OR51E1	
ANGPT1	CDH13		IL4I1	SORBS2	
FAM213A	DFNA5		IQGAP2	AQP9	
LHX6	PDZD2		CEP55	ASTN1	
G0S2	EPB41L1		AIF1	CYFIP2	
BHMT	WISP2		HCK	HAS1	
TMEM139	PHLDB1		LAMA2	KCTD8	
MGLL	FAM213A		FAM46A	NKX3-2	
MEOX1	G0S2		SLC1A4	SERPINE2	
ANXA1	BHMT		NTRK2	WT1	
GREM1	ANGPT1		HOXA10	WT1-AS	
PAMR1	LHX6		SPRED1	WWC1	
CPE	MGLL		GPR137B		
MMRN1	MEOX1		RUNX1		
PFKM			CD99L2		
			ATP6V1B2		
			SORBS2		
			CASQ2		
			KCNT2		
			DUSP22		
			CPE		
			MMRN1		
			KIAA1217		
			CS		
			APMAP		
			NDN		
			OR51E1		
			FMO2		
			C9orf24		
			LGALS12		
			FRMD1		
			DTNA		
			SLC27A2		
			DHRS4L2		
			PCK1		
			C12orf39		

Table 4 — (continued)

ACACA
HAS1
WWC1
DSP
WT1-AS
AQP9
WT1
SYN1
PLAC8
RGS1
CYFIP2
SERPINE2
COL4A5
NKX3-2
PAPPA
ASTN1
KCTD8
HAND2-AS1
VAV3
TNFRSF21
TLE1

Replication analyses are summarized. Input data are genes from the initial sample set (Leipzig cohort) conferring negatively correlated DNA methylation and expression levels. Genes marked in bold were successfully replicated in two independent cohorts.

RGS1 methylation and *HbA1c* levels in OVAT. Similar associations were found for *HOXC6* and *HAND2* with *HbA1c* in OVAT. However, the observed relationships do not withstand correction for multiple testing.

3.11. Differential methylation of GWAS loci, imprinted genes, and epigenetic regulators

We further compared our methylation data with known GWAS genes/ loci for BMI [5] and fat distribution (WHR) [6]. In 16 BMI loci, we found differential methylation levels between SAT *vs.* OVAT and in 24 genes when comparing non-obese *vs.* obese in either depot (Supplementary Table 15). In 22 genes of the WHR loci, we found differential methylation in each comparison (Supplementary Table 16). Further, we mapped our differentially methylated genes against a catalogue of known imprinted loci (http://www.geneimprint.org/). As demonstrated in Supplementary Table 17, most of the imprinted genes were differentially methylated between non-obese *vs.* obese subjects (SAT $N = 37$, OVAT $N = 37$). To test whether genes encoding enzymes regulating epigenetic mechanisms or adapter proteins may be regulated by DNA methylation themselves, we extracted genes from the literature and searched for potential overlaps with our methylation data (Supplementary Table 18).

4. DISCUSSION

4.1. Adipose tissue depot specific candidate genes

We discovered fat distribution candidate genes and, for 6 of these genes, methylation effects were further supported in additional cohorts; *HAND2, HOXC6, PPARG, SORBS2, CD36,* and *CLDN1.* Among them, the best studied candidate is *PPARG,* which is widely known as a key regulator of adipogenesis and differentiation (reviewed in [37]). Our data demonstrate that the *PPARG* promoter region is significantly hypermethylated in OVAT along with decreased gene expression in OVAT compared to SAT. This may indicate reduced adipogenesis in OVAT leading to impaired triglyceride uptake and insulin sensitivity as previously reported [38]. However, although non-significant, we observed different expression effects in isolated adipocytes. As we were unable to analyze adipocytes and SVF from the same individuals
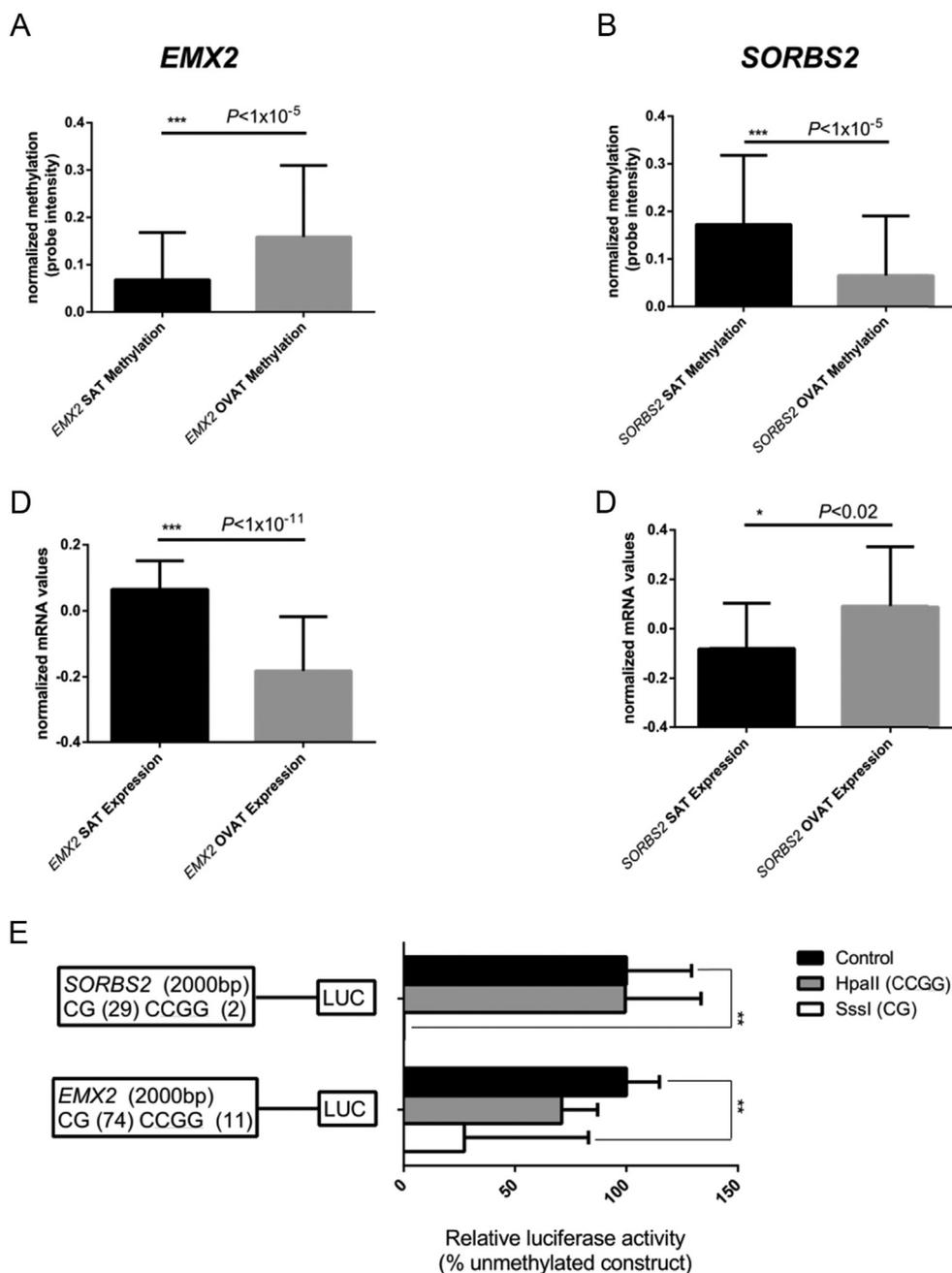
Figure 4: Luciferase assays for *SORBS2* and *EMX2*. The figure shows effect directions of methylation and expression in SAT and OVAT, respectively. Data shown represent results from the two luciferase reporter plasmids which were used to test effects of DNA methylation in *SORBS2* and *EMX2* promoter regions on transcriptional activity. Both plasmids comprised 2000 bp of either *SORBS2* or *EMX2* promoter regions inserted into a pCpGl-basic vector which was either methylated by HpaII (which methylates the internal Cs of the CCGG sequence) or *SssI* (which methylates all CpG sites) and then transfected into *MCF7* cells. Data were normalized using a co-transfected renilla luciferase vector and presented as methylated promoter constructs (grey and white bars) relative to un-methylated constructs (black bars). Experiments were performed six times (*SORBS*) or eight times (*EMX2*) including four replicates in each experiment. Cells transfected with an empty pCpGL-basic vector and untransfected cells activities were used as background correction for firefly and renilla luciferase activity, respectively. *$P < 0.05$ **$P < 0.005$. Data are shown as mean $\pm$ SEM.

as in our discovery cohort, this may have contributed to the observed inconsistencies.

We also identified *HOXC6* among the top ranked candidate genes with highest methylation levels in OVAT along with decreased mRNA expression, which was further supported in pure adipocytes. Homeobox genes are involved in developmental processes [39]. *HOXC6* was also demonstrated by others to be differentially expressed in human SAT before and after bariatric surgery [40], between SAT and

gluteofemoral adipose tissue [13] and skeletal muscle [17]. Our findings largely support these data.

Furthermore, *HAND2* was higher methylated in SAT from obese subjects along with decreased expression levels in both adipose tissue biopsies and isolated adipocytes. *Hand2* is involved in *Notch* signaling in heart development of mice [41]. Considering that *NOTCH* signaling also plays a major role in adipogenic differentiation [30,31], mainly through inhibiting ASC (Adipose tissue-Derived-Stem Cells)
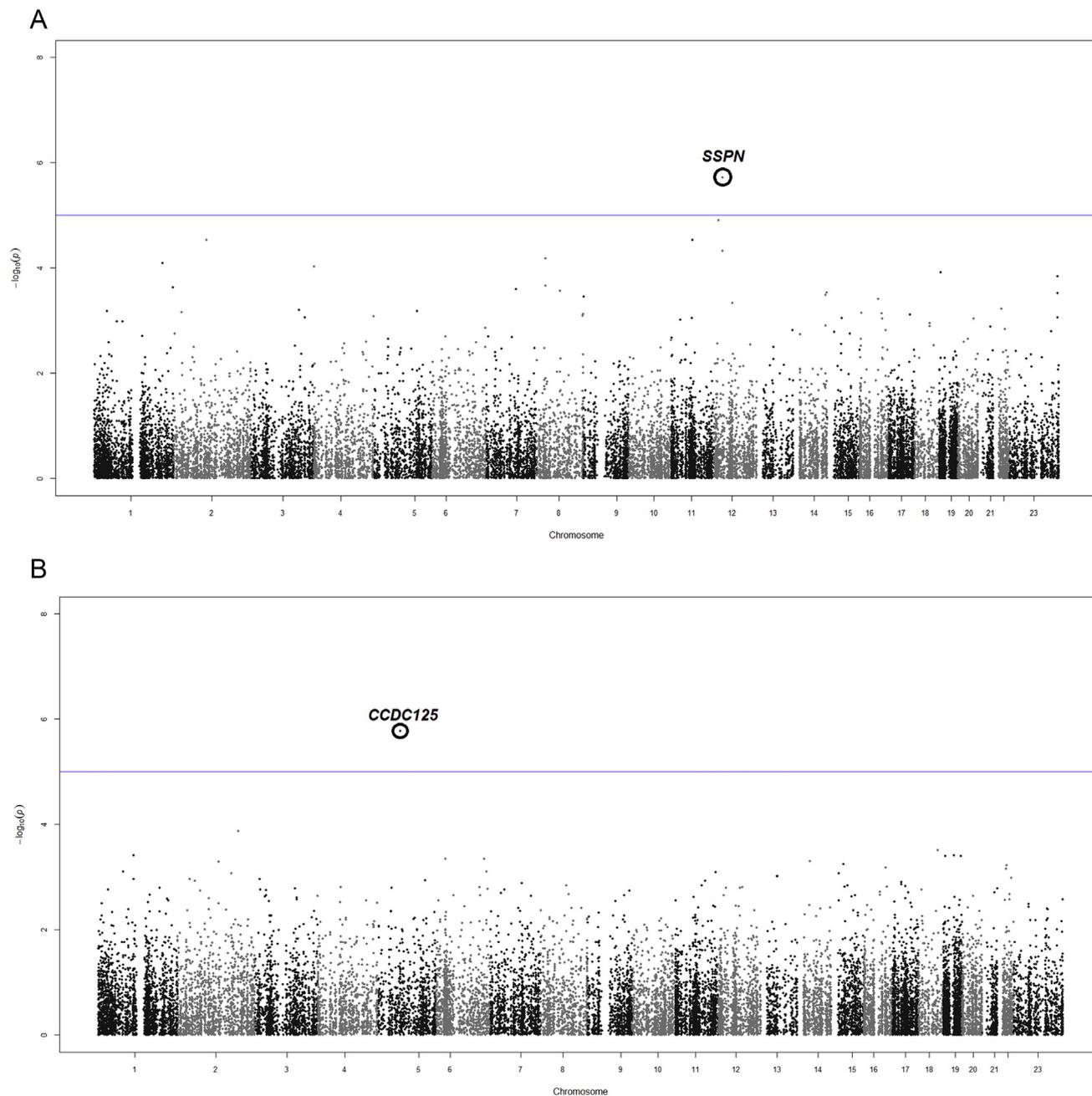
Figure 5: Epigenome wide association study (EWAS) between DNA promoter methylation and BMI. EWAS was applied to test for a relationship (linear regression) of DNA promoter methylation per transcript with BMI. *P*-values for epigenome wide association analysis with BMI were calculated using an R (version 3.0.2) package called CpGassoc [58]. All analyses were adjusted for age, gender, and type 2 diabetes, separately in a) SAT and b) OVAT in the samples from the Leipzig cohort, for which methylation data were available (*N* = 77). Different transcripts per gene showing exactly the same association results are summarized in the same dot. Bonferroni correction was used to correct for multiple testing.

differentiation to adipocytes and thereby affecting the adipose tissue expansion capacity [30], this might indicate reduced adipocyte differentiation in OVAT. In line with this, increased *Notch* signaling in mice blocked the expansion of white adipose tissue, ectopic fat accumulation and insulin resistance [42], which further supports the potential dysfunctional role of OVAT [1,2].

Among the successfully replicated hits was *SORBS2*, which was more methylated and less expressed in SAT versus OVAT in both non-obese and obese subjects. We substantiated these results in isolated adipocytes. Linear regression analyses revealed a strong association of

methylation with BMI for *SORBS2* in OVAT. *SORBS2* might be involved in insulin mediated translocation of *GLUT4* [43] and *t* thereby might affect energy storage. Furthermore, we clearly show that *in vitro* promoter methylation of *SORBS2* directly represses the transcriptional activity of the gene-reporter constructs. We observed similar effects for *EMX2*, a developmental gene which was already shown by others to be upregulated after weight loss in SAT [40]. Our data provide functional evidence that promoter methylation in these genes directly influences gene activity and thereby contributes to the well-known biological distinctions between SAT and OVAT.

Depot	Gene ID	Transcript	T-statistic	P-value	Function	Literature
SAT	*SSPN*	NM_005086	−5.19	1.9×10^{-06}	Sarcospan	Obesity, WHR PMID: 20935629 PMID: 26449484
OVAT	*CCDC125*	NM_176816	5.22	1.7×10^{-06}	Coiled-coil domain containing 125	Isaac's syndrome PMID: 19787194

Table 5 — Top EWAS hits in SAT and OVAT.

Top candidate transcripts from EWAS analyses in SAT and OVAT are shown approaching genome-wide significance ($P < 2.2 \times 10^{-6}$). T-statistic and P-values were generated by applying linear regression analysis.
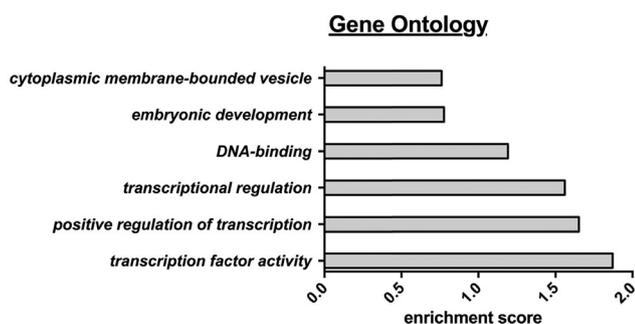
Gene Ontology

Figure 6: Gene ontology analysis. Gene ontology analysis was performed using the DAVID program (http://david.abcc.ncifcrf.gov/summary.jsp) with default settings, high classification stringency and 0.5 as cut off for the enrichment score. Enriched pathways include the following genes: transcription factor activity: *HAND2, PPARG, RUNX1, HSD17B8, HOXC6, TCF21*; positive regulation of transcription: *TCF21, MEOX1, HAND2, PPARG, RUNX1*; transcriptional regulation: *HOXC6, TCF21, MEOX1, HAND2, PPARG, ETV6, RUNX1, HSD17B8, AFF3*; DNA-binding: *HOXC6, TCF21, MEOX1, HAND2, PPARG, AFF3, ETV6, RUNX1*.

The *CD36* gene was more methylated and less expressed in OVAT among non-obese subjects and affects metabolism through several mechanisms. First, *CD36* increases attraction to fatty foods in rodents while it is also expressed in human taste receptor cells, which, in turn, may also affect human eating behavior [44]. Secondly, it is involved in lipid metabolism by taking up long chain fatty acids and oxidized low-density lipoproteins [32,33]. Finally, *CD36* silencing prevents lipid accumulation and reduces proliferation in vascular smooth muscle cells induced by adipocyte-conditioned medium or oleic acid [45]. These data support our finding of reduced gene expression in OVAT among non-obese individuals, which, however, we could not confirm in isolated adipocytes.

We identified increased methylation of the tight junction protein *CLDN1* together with lower expression levels in SAT compared to OVAT from non-obese individuals. *CLDN1* is expressed in the intestinal membrane, and the expression can be reduced by fat emulsion, which, in turn, results in increased membrane permeability as demonstrated in rats [46]. Considering the fact that OVAT is located close to the intestine and recent reports about the leaky gut hypothesis [47], the increased expression of *CLDN1* in OVAT compared to SAT suggest a functional role of *CLDN1* in visceral fat. Similar effects were seen in isolated adipocytes, which adds further evidence to these findings.

4.2. Candidate genes differentially regulated between non-obese and obese subjects

We found several candidate genes and further supported *ETV6* in two additional independent cohorts. *ETV6* functions as a transcriptional repressor and is involved in acute lymphoblastic leukemia [48]. Moreover, *ETV6* was reported in several GWAS influencing human height [49,50]. In our dataset, *ETV6* is hypermethylated in SAT and pure adipocytes from non-obese subjects compared to obese individuals. A potential role for *ETV6* in obesity or related phenotypes has not been reported so far but seems plausible in light of its association with other anthropometric measures such as height. Consistently, *RUNX1* is also significantly hypermethylated in SAT from non-obese subjects compared to obese individuals. Others have shown that *RUNX1* DNA methylation is significantly decreased in response to exercise training in skeletal muscle [17]. Interestingly, in our data, *RUNX1* showed a wide range of associations to parameters of fat distribution in SAT and OVAT, further strengthening its potential role in obesity. Although we confirmed several genes in additional cohorts, we consider data from our initial analysis as the most important original results. Therefore, we also consider genes as true signals that have not shown similar effect directions in other cohorts. Among these newly discovered obesity candidate genes is *DUSP22*, which is hypermethylated in OVAT from obese subjects and was previously shown to be differentially methylated between high and low responders to a weight loss intervention [51]. It was suggested, that reduced methylation along with increased expression of *DUSP22* might be indirectly involved in obesity by inhibiting the IL6/LIF/STAT3 pathway [52]. However, we observed no correlation between methylation levels of *DUSP22* and IL6-serum levels in our subjects.

RGS1 methylation is higher in OVAT from non-obese subjects compared to obese individuals and negatively associated with HbA1c (Supplementary Table 14). Our results are in line with animal data showing an increased expression of *Rgs1* in epididymal white adipose tissue due to high-fat diet (HFD)-induced obesity in mice [53].

4.3. EWAS

A EWAS for BMI in paired samples of SAT and OVAT has never been performed. We identified the two candidate genes *SSPN* and *CCDC125* as significantly associated to BMI in SAT and OVAT, respectively. The *SSPN* gene has been previously described as one of 13 WHR loci [54]. *SSPN* encodes for a protein which is part of the dystrophin-glycoprotein complex and is predominantly expressed in muscle, adipose tissue, thyroid, and retina. In muscle dystrophy, reduced muscle mass is compensated for by increased fat tissue and connective tissue. Another sub-complex of the dystrophin-glycoprotein complex is the sarcoglycan-complex which is also present in adipose tissue. β/δ-sarcoglycan null mice show reduced sarcoglycan-complexes along with reduced protein levels of SSPN in white adipocytes [55]. While no established role for *CCDC125* in obesity is known so far, it is involved in Isaac's syndrome [56], a movement disorder caused by increased sensitivity of peripheral motor nerves. *CCDC125* is mainly expressed in immune associated tissues such as thymus, spleen and bone marrow [56].

No association was found for *HIF3A*, which was recently reported to be a significant correlate to BMI, most likely due to the fact that we did not measure the same CpG sites but measured mean DNA promoter methylation levels per transcript [12]. Therefore, we cannot rule out that *HIF3A* may also be related to BMI in our samples.

4.4. Limitations

Albeit greater compared to other studies, our sample size may still limit our ability to identify small effects and may have led to false positive and false negative results. Moreover, we included 77 paired samples of SAT and OVAT in the methylation profiling and 63 in the mRNA profiling. However, overlapping methylation and expression data (either SAT or OVAT) are only available for 42 subjects (overlap

with expression from both SAT and OVAT, 31 subjects), which may have prevented us from identifying causal relationships. Further, in our discovery cohort, we used MeDIP to enrich methylated regions followed by array hybridization, which naturally has lower coverage than NGS based methods. Therefore, we may have missed important, physiologically meaningful, differentially methylated regions in our analysis. However, we further substantiated the results from our initial dataset by comparing those with effect directions derived from 450K arrays in several independent cohorts. The here presented results from functional analyses such as luciferase assays originate from MCF7 cells and need to be established in adipocytes in the future. We have only limited knowledge of the individual environmental factors, which may have theoretically influenced our results. Importantly, adipose tissue is a heterogeneous sample *per se* containing multiple cell types, and we cannot rule out that methylation levels originating from other cell types such as macrophages may have an impact on our results. However, for most of our top genes, we provide similar effect directions when analyzing gene expression in isolated adipocytes. As we were unable to perform these experiments in the same individuals included in our initial analyses, this may be one reason causing the observed differences.

5. CONCLUSIONS

Using a combined genome wide epigenetic and transcriptomic analysis, we confirmed obesity and fat distribution candidate genes and identified genes which have been previously unrecognized in the pathophysiology of obesity. Our data suggest that DNA promoter methylation of specific genes is directly associated with BMI and obesity while we clearly demonstrate adipose tissue depot specific differences. Confirming known candidate genes such as *PPARG* underlines the credibility of the here identified genes.

AUTHOR CONTRIBUTIONS

Y.B. and M.K. conceptualized, designed, and conceived the study. M.B. and Y.B. supervised the study. M.K. performed sample preparation for the arrays and gene ontology- and validation analyses, designed tables and figures, and wrote the first draft of the manuscript. L.H. performed bioinformatics analyses of methylation and expression array data. X.L. performed EWAS analyses, designed Manhattan plots and circle plots, and performed methylation *in-silico* replications by using publicly available data sets. T.W. performed linear regression analyses. R.C. performed methylation *in-silico* replications in the Italian cohort. K.R. performed luciferase experiments, edited the manuscript, and contributed to the discussion and data interpretation. M.Klös. assisted in sample preparation for expression analyses. F.E. assisted in validation analyses by using pyrosequencing. A.D., M.R.S., D.G., T.L., and M.D. contributed samples. M.S. and P.K. edited the manuscript. A.M.D. supervised in-silico replication analyses (PI of the Italian cohort). C. L. and K. B. supervised *in-silico* methylation analyses and luciferase assays, and contributed to data interpretation. M. Kern performed gene expression analyses in isolated adipocytes and SVF (supervised by M.B.) H.B. supervised bioinformatics analyses and contributed to discussion and data interpretation. M.B. edited the manuscript, contributed to the data analysis and discussion, and is PI of the Adipose Tissue Biobank (Leipzig Cohort). Y.B. conceived and designed experiments, edited the manuscript, and contributed to discussion and data interpretation. All authors contributed to the final version of the manuscript, guarantee for the data and analyses, and agreed to publish the data.

CONFLICT OF INTEREST

The authors have no conflict of interests.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.11.003.

REFERENCES

[1] St-Pierre, J., Lemieux, I., Perron, P., Brisson, D., Santuré, M., Vohl, M.-C., et al., 2007. Relation of the "Hypertriglyceridemic Waist" phenotype to earlier manifestations of coronary artery disease in patients with glucose intolerance and type 2 diabetes mellitus. The American Journal of Cardiology 99(3):369–373. http://dx.doi.org/10.1016/j.amjcard.2006.08.041.

[2] Vega, G.L., Adams-Huet, B., Peshock, R., Willett, D., Shah, B., Grundy, S.M., 2006. Influence of body fat content and distribution on variation in metabolic risk. The Journal of Clinical Endocrinology & Metabolism 91(11):4459–4466. http://dx.doi.org/10.1210/jc.2006-0814.

[3] Gerhard, G.S., Styer, A.M., Strodel, W.E., Roesch, S.L., Yavorek, A., Carey, D.J., et al., 2014 Mar. Gene expression profiling in subcutaneous, visceral and epigastric adipose tissues of patients with extreme obesity. International Journal of Obesity (Lond) 38(3):371–378. http://dx.doi.org/10.1038/ijo.2013.152 [Epub 2013 Aug 16].

[4] Trujillo, M.E., Scherer, P.E., 2006. Adipose tissue-derived factors: impact on health and disease. Endocrine Reviews 27(7):762–778. http://dx.doi.org/10.1210/er.2006-0033.

[5] Locke, A.E., Kahali, B., Berndt, S.I., Justice, A.E., Pers, T.H., Day, F.R., et al., 2015. Genetic studies of body mass index yield new insights for obesity biology. Nature 518(7538):197—206. http://dx.doi.org/10.1038/nature14177.

[6] Shungin, D., Winkler, T.W., Croteau-Chonka, D.C., Ferreira, T., Locke, A.E., Mägi, R., et al., 2015. New genetic loci link adipose and insulin biology to body fat distribution. Nature 518(7538):187—196. http://dx.doi.org/10.1038/nature14132.

[7] Schleinitz, D., Böttcher, Y., Blüher, M., Kovacs, P., 2014. The genetics of fat distribution. Diabetologia 57(7):1276—1286. http://dx.doi.org/10.1007/s00125-014-3214-z.

[8] Dahlman, I., Sinha, I., Gao, H., Brodin, D., Thorell, A., Rydén, M., et al., 2015 Jun. The fat cell epigenetic signature in post-obese women is characterized by global hypomethylation and differential DNA methylation of adipogenesis genes. International Journal of Obesity (Lond) 39(6):910—919. http://dx.doi.org/10.1038/ijo.2015.31 [Epub 2015 Mar 18].

[9] Nilsson, E., Jansson, P.A., Perfilyev, A., Volkov, P., Pedersen, M., Svensson, M.K., et al., 2014. Altered DNA methylation and differential expression of genes influencing metabolism and inflammation in adipose tissue from subjects with type 2 diabetes. Diabetes 63(9):2962—2976. http://dx.doi.org/10.2337/db13-1459.

[10] Keller, M., Kralisch, S., Rohde, K., Schleinitz, D., Dietrich, A., Schön, M.R., et al., 2014. Global DNA methylation levels in human adipose tissue are related to fat distribution and glucose homeostasis. Diabetologia 57(11): 2374—2383. http://dx.doi.org/10.1007/s00125-014-3356-z. PubMed Central PMCID: PMC25145546.

[11] Rohde, K., Keller, M., Klös, M., Schleinitz, D., Dietrich, A., Schön, M.R., et al., 2014. Adipose tissue depot specific promoter methylation of TMEM18. Journal of Molecular Medicine 92(8):881—888. http://dx.doi.org/10.1007/s00109-014-1154-1.

[12] Dick, K.J., Nelson, C.P., Tsaprouni, L., Sandling, J.K., Aïssi, D., Wahl, S., et al., 2014. DNA methylation and body-mass index: a genome-wide analysis. The Lancet 383(9933):1990—1998. http://dx.doi.org/10.1016/s0140-6736(13)62674-4.

[13] Gehrke, S., Brueckner, B., Schepky, A., Klein, J., Iwen, A., Bosch, T.C.G., et al., 2013. Epigenetic regulation of depot-specific gene expression in adipose tissue. PLoS One 8(12):e82516. http://dx.doi.org/10.1371/journal.pone.0082516.

[14] Benton, M.C., Johnstone, A., Eccles, D., Harmon, B., Hayes, M.T., Lea, R.A., et al., 2015. An analysis of DNA methylation in human adipose tissue reveals differential modification of obesity genes before and after gastric bypass and weight loss. Genome Biology 16:8. http://dx.doi.org/10.1186/s13059-014-0569-x.

[15] Barres, R., Kirchner, H., Rasmussen, M., Yan, J., Kantor, F.R., Krook, A., et al., 2013. Weight loss after gastric bypass surgery in human obesity remodels promoter methylation. Cell Reports 3(4):1020—1027. http://dx.doi.org/10.1016/j.celrep.2013.03.018.

[16] Barrès, R., Yan, J., Egan, B., Treebak, J.T., Rasmussen, M., Fritz, T., et al., 2012. Acute exercise remodels promoter methylation in human skeletal muscle. Cell Metabolism 15(3):405—411. http://dx.doi.org/10.1016/j.cmet.2012.01.001.

[17] Nitert, M.D., Dayeh, T., Volkov, P., Elgzyri, T., Hall, E., Nilsson, E., et al., 2012. Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes. Diabetes 61(12):3322—3332. http://dx.doi.org/10.2337/db11-1653.

[18] Rönn, T., Volkov, P., Davegårdh, C., Dayeh, T., Hall, E., Olsson, A.H., et al., 2013. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. PLoS Genetics 9(6):e1003572. http://dx.doi.org/10.1371/journal.pgen.1003572.

[19] Donkin, I., Versteyhe, S., Ingerslev, L.R., Qian, K., Mechta, M., Nordkap, L., et al., 2015. Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans. Cell Metabolism 23(2):369—378. http://dx.doi.org/10.1016/j.cmet.2015.11.004. PubMed Central PMCID: PMC26669700.

[20] Huypens, P., Sass, S., Wu, M., Dyckhoff, D., Tschop, M., Theis, F., et al., 2016. Epigenetic germline inheritance of diet-induced obesity and insulin resistance. Nature Genetics 48(5):497—499. http://dx.doi.org/10.1038/ng.3527. Epub 2016/03/15. PubMed PMID: 26974008.

[21] Kloting, N., Fasshauer, M., Dietrich, A., Kovacs, P., Schon, M.R., Kern, M., et al., 2010. Insulin-sensitive obesity. AJP: Endocrinology and Metabolism 299(3):E506—E515. http://dx.doi.org/10.1152/ajpendo.00586.2009.

[22] O'Geen, H., Nicolet, C., Blahnik, K., Green, R., Farnham, P., 2006. Comparison of sample preparation methods for ChIP-chip assays. Biotechniques 41(5): 577—580. http://dx.doi.org/10.2144/000112268.

[23] Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., et al., 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Molecular Biology 7:3. http://dx.doi.org/10.1186/1471-2199-7-3.

[24] Klug, M., Rehli, M., 2014. Functional analysis of promoter CPG-methylation using a CpG-free luciferase reporter vector. Epigenetics 1(3):127—130. http://dx.doi.org/10.4161/epi.1.3.3327.

[25] Johnson, W.E., Li, W., Meyer, C.A., Gottardo, R., Carroll, J.S., Brown, M., et al., 2006. Model-based analysis of tiling-arrays for ChIP-chip. Proceedings of National Academy of Sciences United States of America 103(33):12457—12462. http://dx.doi.org/10.1073/pnas.0601180103.

[26] Strimmer, K., 2008. A unified approach to false discovery rate estimation. BMC Bioinformatics 9:303. http://dx.doi.org/10.1186/1471-2105-9-303.

[27] Löffler-Wirth, H., Kalcher, M., Binder, H., 2015. oposSOM: R-package for high-dimensional portraying of genome-wide expression landscapes on bio-conductor. Bioinformatics (Oxford, England) 31(19):3225—3227. http://dx.doi.org/10.1093/bioinformatics/btv342.

[28] Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., et al., 2009. Circos: an information aesthetic for comparative genomics. Genome Research 19(9):1639—1645. http://dx.doi.org/10.1101/gr.092759.109.

[29] Elsen, M., Raschke, S., Tennagels, N., Schwahn, U., Jelenik, T., Roden, M., et al., 2014. BMP4 and BMP7 induce the white-to-brown transition of primary human adipose stem cells. AJP: Cell Physiology 306(5):C431—C440. http://dx.doi.org/10.1152/ajpcell.00290.2013.

[30] Ferrer-Lorente, R., Bejar, M.T., Badimon, L., 2014. Notch signaling pathway activation in normal and hyperglycemic rats differs in the stem cells of visceral and subcutaneous adipose tissue. Stem Cells and Development 23(24):3034—3048. http://dx.doi.org/10.1089/scd.2014.0070.

[31] Song, B.-Q., Chi, Y., Li, X., Du, W.-J., Han, Z.-B., Tian, J.-J., et al., 2015. Inhibition of notch signaling promotes the adipogenic differentiation of mesenchymal stem cells through autophagy activation and PTEN-PI3K/AKT/mTOR pathway. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 36(5): 1991—2002. http://dx.doi.org/10.1159/000430167.

[32] Kennedy, D.J., Kuchibhotla, S., Westfall, K.M., Silverstein, R.L., Morton, R.E., Febbraio, M., 2011. A CD36-dependent pathway enhances macrophage and adipose tissue inflammation and impairs insulin signalling. Cardiovascular Research 89(3):604—613. http://dx.doi.org/10.1093/cvr/cvq360.

[33] Love-Gregory, L., Abumrad, N.A., 2011. CD36 genetics and the metabolic complications of obesity. Current Opinion in Clinical Nutrition and Metabolic Care 14(6):527—534. http://dx.doi.org/10.1097/MCO.0b013e32834bbac9. PubMed Central PMCID: PMC21912245.

[34] Slieker, R.C., Bos, S.D., Goeman, J.J., Bovée, J.V., Talens, R.P., van der Breggen, R., et al., 2013. Identification and systematic annotation of tissue-specific differentially methylated regions using the Illumina 450k array. Epigenetics & Chromatin 6(1):26. http://dx.doi.org/10.1186/1756-8935-6-26. PubMed Central PMCID: PMC23919675.

[35] Lowe, R., Rakyan, V.K., 2013. Marmal-aid—a database for Infinium Human-Methylation450. BMC Bioinformatics 14:359. http://dx.doi.org/10.1186/1471-2105-14-359.

[36] Ronn, T., Volkov, P., Gillberg, L., Kokosar, M., Perfilyev, A., Jacobsen, A.L., et al., 2015. 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. Human Molecular Genetics 24(13):3792—3813. http://dx.doi.org/10.1093/hmg/ddv124. PubMed Central PMCID: PMC25861810.

[37] Kawai, M., Rosen, C.J., 2010. PPARγ: a circadian transcription factor in adipogenesis and osteogenesis. Nature Reviews Endocrinology 6(11):629—636. http://dx.doi.org/10.1038/nrendo.2010.155.

[38] Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., et al., 1999. PPARγ is required for the differentiation of adipose tissue in vivo and in vitro. Molecular Cell 4(4):611—617. http://dx.doi.org/10.1016/s1097-2765(00)80211-7.

[39] Yamamoto, Y., Gesta, S., Lee, K.Y., Tran, T.T., Saadatirad, P., Ronald Kahn, C., 2010. Adipose depots possess unique developmental gene signatures. Obesity 18(5):872—878. http://dx.doi.org/10.1038/oby.2009.512.

[40] Dankel, S.N., Fadnes, D.J., Stavrum, A.-K., Stansberg, C., Holdhus, R., Hoang, T., et al., 2010. Switch from stress response to homeobox transcription factors in adipose tissue after profound fat loss. PLoS One 5(6):e11033. http://dx.doi.org/10.1371/journal.pone.0011033.

[41] VanDusen, N.J., Casanovas, J., Vincentz, J.W., Firulli, B.A., Osterwalder, M., Lopez-Rios, J., et al., 2014. Hand2 is an essential regulator for two notch-dependent functions within the embryonic endocardium. Cell Reports 9(6):2071—2083. http://dx.doi.org/10.1016/j.celrep.2014.11.021.

[42] Chartoumpekis, D.V., Palliyaguru, D.L., Wakabayashi, N., Khoo, N.K.H., Schoiswohl, G., O'Doherty, R.M., et al., 2015. Notch intracellular domain overexpression in adipocytes confers lipodystrophy in mice. Molecular Metabolism 4(7):543—550. http://dx.doi.org/10.1016/j.molmet.2015.04.004.

[43] Kimura, A., Baumann, C.A., Chiang, S.H., Saltiel, A.R., 2001. The sorbin homology domain: a motif for the targeting of proteins to lipid rafts. Proceedings of National Academy of Sciences United States of America 98(16):9098—9103. http://dx.doi.org/10.1073/pnas.151252898.

[44] Degrace-Passilly, P., Besnard, P., 2012. CD36 and taste of fat. Current Opinion in Clinical Nutrition and Metabolic Care 15(2):107—111. http://dx.doi.org/10.1097/MCO.0b013e32834ff19c.

[45] Schlich, R., Lamers, D., Eckel, J., Sell, H., 2015. Adipokines enhance oleic acid-induced proliferation of vascular smooth muscle cells by inducing CD36 expression. Archives of Physiology and Biochemistry 121(3):81—87. http://dx.doi.org/10.3109/13813455.2015.1045520.

[46] Suzuki, T., Hara, H., 2010. Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. Nutrition & Metabolism 7:19. http://dx.doi.org/10.1186/1743-7075-7-19.

[47] Geurts, L., Neyrinck, A.M., Delzenne, N.M., Knauf, C., Cani, P.D., 2014. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism: novel insights into molecular targets and interventions using prebiotics. Beneficial Microbes 5(1):3—17. http://dx.doi.org/10.3920/bm2012.0065.

[48] Ellinghaus, E., Stanulla, M., Richter, G., Ellinghaus, D., te Kronnie, G., Cario, G., et al., 2012. Identification of germline susceptibility loci in ETV6-RUNX1-rearranged childhood acute lymphoblastic leukemia. Leukemia 26(5):902—909. http://dx.doi.org/10.1038/leu.2011.302.

[49] Berndt, S.I., Gustafsson, S., Mägi, R., Ganna, A., Wheeler, E., Feitosa, M.F., et al., 2013. Genome-wide meta-analysis identifies 11 new loci for anthropometric traits and provides insights into genetic architecture. Nature Genetics 45(5):501—512. http://dx.doi.org/10.1038/ng.2606.

[50] Lango Allen, H., Estrada, K., Lettre, G., Berndt, S.I., Weedon, M.N., Rivadeneira, F., et al., 2010. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature 467(7317):832—838. http://dx.doi.org/10.1038/nature09410.

[51] Moleres, A., Campión, J., Milagro, F.I., Marcos, A., Campoy, C., Garagorri, J.M., et al., 2013. Differential DNA methylation patterns between high and low responders to a weight loss intervention in overweight or obese adolescents: the EVASYON study. FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 27(6):2504—2512. http://dx.doi.org/10.1096/fj.12-215566.

[52] Sekine, Y., Tsuji, S., Ikeda, O., Sato, N., Aoki, N., Aoyama, K., et al., 2006. Regulation of STAT3-mediated signaling by LMW-DSP2. Oncogene 25(42):5801—5806. http://dx.doi.org/10.1038/sj.onc.1209578. PubMed Central PMCID: PMC16636663.

[53] Choi, M.-S., Kim, Y.-J., Kwon, E.-Y., Ryoo, J.Y., Kim, S.R., Jung, U.J., 2015. High-fat diet decreases energy expenditure and expression of genes controlling lipid metabolism, mitochondrial function and skeletal system development in the adipose tissue, along with increased expression of extracellular matrix remodelling- and inflammation-related genes. The British journal of nutrition 113(6):867—877. http://dx.doi.org/10.1017/s0007114515000100. PubMed Central PMCID: PMC25744306.

[54] Heid, I.M., Jackson, A.U., Randall, J.C., Winkler, T.W., Qi, L., Steinthorsdottir, V., et al., 2010. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. Nature Genetics 42(11):949—960. http://dx.doi.org/10.1038/ng.685.

[55] Groh, S., Zong, H., Goddeeris, M.M., Lebakken, C.S., Venzke, D., Pessin, J.E., et al., 2009. Sarcoglycan complex: implications for metabolic defects in muscular dystrophies. The Journal of Biological Chemistry 284(29):19178—19182. http://dx.doi.org/10.1074/jbc.C109.010728.

[56] Araya, N., Arimura, H., Kawahara, K.-I., Yagishita, N., Ishida, J., Fujii, R., et al., 2009. Role of Kenae/CCDC125 in cell motility through the deregulation of RhoGTPase. International Journal of Molecular Medicine 24(05):605—611. http://dx.doi.org/10.3892/ijmm_00000271.

[57] Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A., et al., 2015. gplots: Various R programming tools for plotting data. R package version 2.17.0 ed: CRAN.R-project.org.

[58] Barfield, R.T., Kilaru, V., Smith, A.K., Conneely, K.N., 2012. CpGassoc: an R function for analysis of DNA methylation microarray data. Bioinformatics (Oxford, England) 28(9):1280—1281. http://dx.doi.org/10.1093/bioinformatics/bts124. PubMed Central PMCID: PMC22451269.