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(Epi)genetic regulation of *CRTC1* in human eating behaviour and fat distribution

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ABSTRACT

Background: In brain, CREB-regulated transcription co-activator 1 (CRTC1) is involved in metabolic dysregulation. In humans a SNP in *CRTC1* was associated to body fat percentage and two SNPs affected RNA Pol II binding and chromatin structure, implying epigenetic regulation of *CRTC1*. We sought to understand the relevance of *CRTC1* SNPs, DNA methylation and expression in human eating behaviour and its relationship to clinical variables of obesity in blood and adipose tissue.

Methods: 13 *CRTC1* SNPs were included to analyze eating behaviour. For rs7256986, follow up association analyses were applied on DNA methylation, *CRTC1* expression and clinical parameters. Linear regression was used throughout the study adjusted for age, sex and BMI. Besides data extraction from previous work, rs7256986 was de-novo genotyped and DNA methylation was evaluated by using pyrosequencing.

Findings: We found several SNPs in the *CRTC1* locus nominally associated with human eating behaviour or 2hr postprandial insulin levels and observed a correlation with alcohol and coffee intake (all $P < 0.05$). G-allele carriers of rs7256986 showed slightly increased hip circumference. We showed that rs7256986 represents a methylation quantitative trait locus (meQTL) in whole blood and adipose tissue. The presence of the SNP and/or DNA methylation correlated with *CRTC1* gene expression which in turn, related to BMI and fat distribution.

Interpretation: Our data support the known role of CRCT1 regulating energy metabolism in brain. Here, we highlight relevance of *CRTC1* regulation in blood and adipose tissue.

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1. Introduction

CRCT1 (CREB regulated transcription coactivator 1) belongs to the family of CREB (cAMP responsive element binding) transcriptional coactivators (CRTCs) which play critical roles in various biological processes including energy homeostasis [1–6]. While CRTC2 plays a role in liver gluconeogenesis during fasting [7], CRTC3 contributes to adipocyte biology and fat distribution [8]. Most studies however focused on

Abbreviations: Bdnf, brain derived neurotrophic factor; BF%, body fat percentage; BMI, body mass index; cAMP, cyclic adenosine monophosphate; CpG, Cytosine-phosphate-Guanine; CRE, cAMP responsive element; CREB, CRE binding protein; CRTC1, CREB regulated transcription co-activator 1; CT-ratio, computer tomography ratio (ratio of OVAT/SAT); DNA, deoxyribonucleic acid; FEV, Fragebogen zum Essverhalten; FEV-R18, Fragebogen zum Essverhalten-revised form 18; GWAS, genome wide association study; HbA1c, glycated hemoglobin A1c; L4-L5, lumbar vertebrae 4 and 5; LD, linkage disequilibrium; lncRNA, long non-coding RNA (ribonucleic acid); meQTL, methylation quantitative trait locus; miR, micro RNA; miRNA, micro RNA; NAFLD, non-alcoholic fatty liver diseases; OGTT, oral glucose tolerance test; OVAT, omental visceral adipose tissue; Pol2, polymerase 2; RIN, RNA integrity number; SAT, subcutaneous adipose tissue; SNP, single nucleotide polymorphism; T2D, type 2 diabetes mellitus; TFEQ, three factor eating questionnaire; WHR, waist-to-hip ratio.

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Research in context

Evidence before this study

CRTC1 plays an important role in metabolic regulation. Genetic markers in *CRTC1* were associated with body fat percentage. A role of SNPs, DNA methylation and gene expression in human eating behaviour has not been described.

Added value of this study

We show that genetic variation in *CRTC1* is related to human eating behaviour dimensions and that rs7256986 represents a meQTL in adipose tissue and blood. Furthermore, DNA methylation levels at rs7256986 correlate with *CRTC1* gene expression, which is related to BMI, fat distribution and metabolic traits.

Implications of all available evidence

Our data support the known central role of CRTC1 in regulating energy metabolism and suggest that rs7256986 is a potential epigenetic regulatory variant influencing DNA methylation levels in adipose tissue and blood.

CRTC1 which is highest expressed in the brain, specifically in prefrontal cortex, amygdala, hippocampus, and hypothalamus, hence has a central role in regulating diverse pathways [3,9,10]. CRTC1 is involved in learning and memory formation [11,12], in pathways controlling mood [3,4], regulates longevity [10], locomotor activity [13], and also energy metabolism [3,14,15], eating behaviour and obesity and diabetes development [6,2,3,14,16,17]. CRTC1 functions as co-activator of CREB by mediating its binding to cAMP responsive elements (CREs) at target gene promoters which in turn regulates gene transcription [5]. Sensing peripheral stimuli in neurons, CRTC1 affects diverse pathways through its action in the brain [3,18]. One major driver of CRTC1 function in relation to energy metabolism is leptin [6]. Leptin is secreted by white adipose tissue and mediates the translocation of CRCT1 from neuronal cytoplasm into the nucleus via affecting Ca^{2+} and cAMP-level, leading to dephosphorylation of CRTC1 [1,6]. After entering the nucleus in response to leptin, CRTC1 binds CREB and regulates transcription of a number of genes mediating hunger and satiety, but also energy expenditure [6,14]. Therefore, CRTC1 is an important regulator in energy sensing in brain. This is underlined by the Crtc1 −/− mouse model [3,6]. These mice represent a hyperphagic obesity phenotype while being resistant to the anorexigenic effects of leptin signaling [6]. In addition, these mice represent a model of depression and mood disorders [4]. There is evidence for CRTC1 in regulating *brain-derived neurothrophic factor (bdnf)* gene expression, which is linked to both depression and obesity [4]. However, despite plenty of studies focusing on CRTC1 function and gene regulation in brain, its role in adipose and other tissues is less clear. One study provides evidence for a specific role in liver, where CRCT1 represses expression of genes involved in hepatic lipid accumulation, protecting mice from the development of non-alcoholic fatty liver diseases (NAFLD) [2]. Another study showed a regulatory feedback loop between *CRTC1* and miR-212/miR-132 which impacts on glucose dependent insulin secretion from beta-cells [19]. Others linked genetic variants in *CRTC1* with BMI in patients with and without major depressive disorder underlining potentially shared pathways of obesity and psychiatric disorders [15,19]. A recent genome wide association study (GWAS) identified genetic variants within the *CRTC1* gene locus linked to overall body fat percentage (BF%) [15,16,20]. Single nucleotide polymorphisms (SNPs) in linkage disequilibrium (LD) with the body fat associating variant rs757318 affect binding of RNA polymerase II (RNA Pol II) and changes chromatin accessibility at the SNP site within the *CRTC1* gene locus. These data imply a potential relevance of epigenetic mechanisms in *CRTC1* gene regulation [16]. Taken together, *CRTC1* is an important candidate gene in metabolic dysregulation and studying (epi)genetic regulation of *CRTC1* becomes highly relevant in order to understand its contribution to obesity and fat distribution.

Here, we sought to unravel the role of *CRTC1* genetic variation in human eating behaviour, consumption of luxury goods and variables clinically relevant for obesity, and adipose tissue distribution. Further, by analyzing epigenetic alterations such as DNA methylation and its impact on gene expression in whole blood and paired samples of human omental visceral (OVAT) and subcutaneous adipose tissue (SAT), we are the first describing an epigenetic feature of rs7256986 representing a methylation quantitative trait locus (meQTL). Genotypic distribution and/or DNA methylation levels at rs7256986 correlate with *CRTC1* gene expression, which is related to BMI, fat distribution and metabolic traits. Our data suggest, that *CRTC1* genetic variation is related to dimensions of human eating behaviour, and metabolic variables, while epigenetic regulation and alterations in gene expression can either be related to such genetic variation or represent independent regulators.

2. Material and methods

2.1. Study populations

2.1.1. Sorbs cohort

The Sorbs cohort represents a German self-contained population as previously reported [21]. In the present study we included $N = 887$ non-diabetic individuals (mean BMI 26.5 ± 4.6, mean age 46 ± 16) for whom a wide range of clinical data was available. Phenotyping included measurements of anthropometric variables such as height, weight, body mass index (BMI), waist-to-hip ratio (WHR) and body fat percentage (%BF) but also clinically relevant metabolic parameters such as measures of glucose, insulin and lipid metabolism. The main study population characteristics are summarized in Table 1. Using standardized questionnaires, patient family history and history of medication were obtained as described previously [22].

2.2. Dimensions of human eating behaviour and consumption of luxury goods in the Sorbs

Of all 887 subjects, 548 (mean BMI 26.1 ± 4.4, mean age 45 ± 16) completed the German version (FEV) [23] of the 3-factor eating questionnaire (TFEQ) from Stunkard and Messick [24]. Hence, data for 3 dimensions of human eating behaviour, restraint, disinhibition and hunger feelings were available, along with data for luxury goods consumption (coffee intake, alcohol intake, smoking behaviour), which were obtained using standardized questionnaires as described elsewhere [25]. Briefly, the questionnaire included 3 scales, one for each dimension of eating behaviour (hunger/disinhibition/restraint = 21/16/14 items to be answered). Each item is to be answered either as yes/no or based on a scale ranging from a weak to a strong sense. Finally, every item scores either 1 or 0 based on a specified analyses key. Hence, a person's maximum score can reach 21-16-14. The consumption of consumer goods were recorded for the average intake of alcohol and coffee (alcohol: 1 = none; 2 = occasionally; 3 = 2–3 glasses of wine or bottle of beer per week; 4 = 1 glass of wine or bottle of beer per day; 5 = 2 glasses of wine or bottles of beer per day; 6 = more than 3 glasses of wine or bottles of beer per day; coffee: number of cups per day). Coffee consumption was further defined as high (1 = 3-4 cups per day) or low intake (0 = 1-2 cups per day). Smoking behaviour is measured as time period of smoking (former, current, never smoker) as well as in numbers of cigarettes per day. All individuals with known T2D were excluded from our analyses as diabetic individuals are trained in personal eating behaviour and food/nutrient selection, which could potentially influence the results.

2.2.1. Independent replication cohort for associations to human eating behaviour

To replicate our findings for eating behaviour, we analysed an independent German cohort comprising 314 non-diabetic, healthy individuals (mean BMI 27.0 ± 6.2, mean age 27 ± 5). Values for the dimensions of human eating behaviour of the replication cohort were obtained with the FEV (disinhibition, hunger, restraint) and FEV-R18, while the latter evaluates cognitive restraint, emotional eating and uncontrolled eating habits [26]. In addition to eating behaviour data, clinical variables were available including height weight and BMI. The main study population characteristics are summarized in Table 1.

2.2.2. Adiposity cohort

In this study we included a total of 168 non-diabetic individuals (mean BMI 35.3 ± 12.7, mean age 55 ± 17.5) from the Leipzig Adiposity cohort [27–29]. Paired adipose tissue samples from omental visceral and subcutaneous depots were obtained from individuals who underwent open abdominal surgeries including, for example, weight reduction surgery, explorative laparotomy or cholecystectomy as described previously [27,29]. A wide range of clinical variables were available as earlier reported [27,29]. Phenotyping 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 75g oral glucose tolerance test (OGTT) and HbA1c. Insulin sensitivity was assessed with hyper-insulinemic-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% 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. Main characteristics of the study population are summarized in Table 1.

2.3. Extraction of SNP data and individual genotyping

Genotype data of 13 *CRTC1* SNP markers were extracted from a genome wide SNP data set available for 887 non-diabetic individuals from the German Sorbs population [22]. The SNP variant rs7256986 was de novo genotyped in all 3 study cohorts. Genomic DNA was extracted from whole blood tissue samples in the Sorbs ($N = 887$) and replication cohort ($N = 314$) using the QIAmp DNA Blood Midi Kit (Qiagen Inc., Valencia, CA, USA) or QuickGene DNA Whole Blood Kit (Kurabo, Japan). Genomic DNA was extracted from adipose tissue in the Adiposity cohort ($N = 168$) using the GenEluteTM Mammalian Genomic DNA Miniprep Kit (SIGMA-ALDRICH, USA) according to the manufacturer's protocols. SNP data were obtained by using Allelic Discrimination TaqMan SNP Genotyping System (Applied Biosystems by Life-Technologies Carlsbad, CA, USA). Fluorescence signals were detected by the ABI 7500 Real-Time PCR system. Genotyping errors were excluded by random re-genotyping (5% of all samples) while all genotypes matched with initially obtained results. Further we used water as non-template controls ($N = 6$ per run). All SNPs were in Hardy–Weinberg equilibrium (all $P < 0.05$).

2.4. Pyrosequencing at SNP-CpG and CpG2

Pyrosequencing analyses were performed in 45 individuals from the Sorbs cohort and 30 subjects from the Adiposity cohort, respectively. Main characteristics of the subgroups are presented in Supplementary Table S4. 500ng genomic DNA was bisulfite converted (EpiTect Fast DNA Bisulfite Kit (Qiagen, Hilden, Germany)) according to the manufacturer's protocol. Using PyroMark Assay Design Software 2.0 (Qiagen, Hilden, Germany), we custom-designed a PyroMark Assay (Fig. 1, Table 2). Pyrosequencing was performed with the PyroMark Q24 system (Qiagen, Hilden, Germany). All samples were analyzed in duplicates and the mean of duplicates were taken forward for analyses. Variation coefficient between duplicates was <0.1 for the SNP-CpG and CpG2 in both cohorts. Water was used as non-template control. Only data that reached "passed quality" in the pyrosequencing run were taken forward for final statistical analyses.

2.5. Extraction of gene expression and DNA promoter methylation data

Mean promoter wide *CRTC1* methylation data were available for $N = 54$ subjects and were extracted from a genome wide data set that had previously been generated for the Adiposity cohort by using MeDIP (methylated DNA immunoprecipitation) on a Chip procedure (GeneChip Human Promoter 1.0R Arrays (Affymetrix Inc., Santa Clara, USA), Fig. 1) [27]. *CRTC1* mRNA expression data were available for the Adiposity cohort from the same study [27] for $N = 44$ (SAT) and $N = 36$ (OVAT). *CRTC1* mRNA expression data were available for the Sorbs

Table 1
Main characteristics of the study cohorts.

Cohort	Sorbs	Replication cohort	Adiposity cohort
Characteristics	Mean ± SD	Mean ± SD	Mean ± SD
(Total *N*)	(887)	(314)	(168)
Gender (m/f)	354/533	173/141	62/106
Age (years)	46 ± 16	27 ± 5	55 ± 17.5
BMI (kg/m^2)	26.5 ± 4.6	27 ± 6.2	35.3 ± 12.7
WHR	0.87 ± 0.096	n.a.	0.92 ± 0.12
FPG (mMol)	5.27 ± 0.51	n.a.	5.38 ± 0.92
FPI (pMol)	38.53 ± 23.26	n.a.	61.39 ± 65.31
Total Chol (mMol)	5.32 ± 1.05	n.a.	5.03 ± 0.92

Cohort	Sorbs	Replication cohort	Adiposity cohort
Dimensions of human eating behaviour	Mean ± SD	Mean ± SD	Mean ± SD
(Total *N*)	(548)	(314)	(0)
Restraint	7.89 ± 4.88	6.14 ± 4.5	n.a.
Disinhibition	4.34 ± 2.98	6.20 ± 3.16	n.a.
Hunger	3.92 ± 2.82	5.54 ± 3.41	n.a.

Data are represented as mean ± SD (standard deviation); only non-diabetic subjects are represented. Restraint, Disinhibition and Hunger values were obtained using the German version of the Three Factor Eating Questionnaire. *N* = number; m = male; f = female; BMI = body mass index; WHR = waist to hip ratio; FPG = fasting plasma glucose; FPI = fasting plasma insulin; Chol = plasma total cholesterol level. n.a. = not available.

cohort and were extracted from a genome wide data set for $N = 886$ non-diabetic subjects [30].

Briefly, mRNA expression data for both studies was generated using Illumina human HT-12 expression chips. RNA integrity and concentration were examined using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RNA integrity values (RIN) of less than 5 were discarded from further analysis [27].

2.6. Statistics

All statistical analyses were performed using SPSS statistics software 24 (SPSS, Inc. Chicago, IL) and GraphPad Prism 7 (GraphPad, San Diego, Ca, USA). Prior to analyses, data were tested for normal distribution using one-sample Kolmogorov-Smirnov test and were logarithmically transformed to approximate normal distribution. Data are presented as mean ± standard deviation if not otherwise stated. Independent group statistics were performed to test for differences of DNA methylation and gene expression between genotype groups of rs7256986 and between SAT and OVAT using paired Student's t-tests. All association analyses with the dimensions of human eating behaviour, anthropometric and clinically relevant metabolic parameters with either the SNP variants, DNA methylation or gene expression were performed using linear regression analyses adjusted for age, gender and BMI (except for BMI in analyses where BMI was used as an independent variable). All analyses regarding *CRTC1* SNPs were performed for 3 modes of inheritance (m = minor allele; M = major allele; additive (mm vs Mm vs MM), dominant (mm + Mm vs MM) and recessive (mm vs Mm + MM)). Results were standardized for the minor-alleles of the SNPs. Sample-size weighted meta-analyses for each dimension of human eating behaviour were performed using METAL [31] in order to evaluate a standardized effect size over both, the Sorbs and replication cohort. Bonferroni correction was used to take into account multiple testing (0.05/44 (number of tests)). We lowered the study-specific significance threshold to $P = 0.0011$. All *P*-values >0.0011 but <0.05 were considered to be of nominal statistical significance. All *P*-values given are uncorrected for multiple testing.

3. Results

3.1. Role of CRTC1 in whole blood

3.1.1. CRTC1 genetic variants associate with eating behaviour, intake of luxury goods and insulin metabolism in the Sorbs

A growing body of evidence describes an important role of CRTC1 in brain in energy sensing, influencing hunger and satiety. For example, *Crtc1*$^{-/-}$ knock out mice are hyperphagic and resist towards anorexic leptin effects [6]. Male mice show alterations in hypothalamic orexigenic and anorexigenic gene expression levels [3]. Data in our initial study cohort, the human Sorbs population support *CRTC1*s role on human eating behaviour (measured via the German version of the three factor eating questionnaire (FEV)) [23] while providing evidence for genetic variants affecting the eating behaviour dimensions disinhibition and hunger, but also luxury goods consumption (Table 3). We tested 13 SNP variants within the *CRCT1* gene locus for association with dimensions of eating behaviour, coffee and alcohol intake, tobacco smoking and metabolic variables. Of the 13 SNPs, 10 variants were in either complete (1.0) or incomplete but high (0.8-0.9) LD with rs757318, a SNP previously identified as a risk variant for body fat percentage [16]. Here, we found all 10 variants of this LD block nominally associated with alcohol intake and 2h postprandial insulin levels (Table 3 and Supplementary Table S1 (summarizing variants in complete LD)). rs10404726 ($0.8 < LD < 1.0$) was in addition related to insulin levels 30 min postprandial. Two variants (rs11881199 and rs11878507, both $0.8 < LD < 1.0$) were additionally related to restraint eating behaviour and to the amount of coffee intake (Table 3). Moreover, three independent genetic variants (rs7256986, rs2023878, rs6510997) nominally associated with either restraint, or hunger and disinhibition (Table 3). We further observed a relationship to 2h postprandial insulin levels and/or coffee intake for those SNPs. All associations were performed in non-diabetic individuals and were statistically nominally significant at a level of $P < 0.05$ and adjusted for age, sex and ln BMI. However, none of these associations would withstand correction for multiple testing.

3.2. Follow up of rs7256986 in the Sorbs and in an replication cohort for eating behaviour

Of all tested variants, we selected rs7256986 for additional replication analyses based on the following criteria: First, this SNP represents an independent tagging variant for a smaller LD block containing three *CRCT1* SNPs (rs11085244; rs11085242; rs2051816; SNAPproxy search 09/2017: http://archive.broadinstitute.org). Second, in the initial Sorbs cohort the variant related to increased disinhibition and hunger feelings, with decreased coffee intake (all Table 3) and is the only genetic variant that is related to anthropometric measures (hip circumference) as shown in Table 4. This SNP variant introduces a CpG site, hence represents a potential epigenetic regulatory variant.

3.2.1. Replication of the associations to eating behaviour for rs7256986

For this particular variant, we first sought to replicate our findings on eating behaviour in an independent replication cohort including healthy, non-diabetic subjects (Table 1) for which eating behaviour data were available. Although non-significant, we observed the same effect directions for increased restraint and disinhibition in G-allele carriers while we found inconsistent results for hunger (Supplementary Table S2). A sample-size weighted meta-analysis resulted in no statistical significant associations (Supplementary Table S3).

3.2.2. Rs7256986 represents a meQTL in whole blood from the Sorbs

Since genetic variation at rs7256986 (A > G) introduces a CpG site, we tested for differential DNA methylation levels at this particular site (so-called SNP-CpG) and for a potential co-methylation profile at a neighboring CpG site (so-called CpG2, according to ENSEMBL search 08/2017). We applied pyrosequencing to genomic DNA extracted

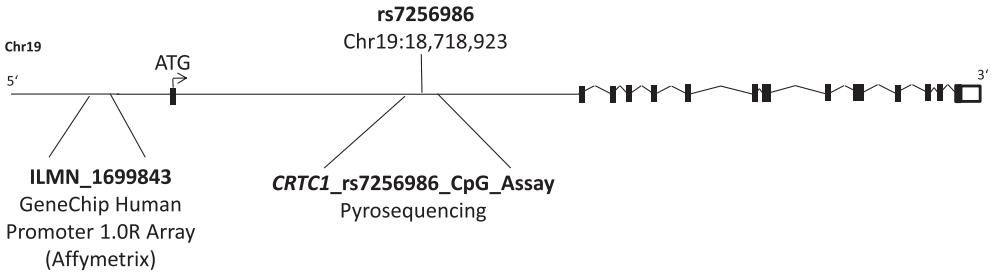

Fig. 1. Schematic representation of the analysed *CRTC1* locus Location of promoter methylation locus (ILMN_1699843 = analyed ilumina probe ID) and CpG Assay for pyrosequencing of rs7256986 (SNP-CpG) and CpG2 is shown. Figure not scaled. Details of the CpG Assay are shown in Table 2. Chr = Chromosome, ATG = translation start site, ILMN = Ilumina

Table 2
Details of *CRTC1*_rs7256986_CpG_Assay.

	Sequences
Forward primer	GGGGGTTTATTTTAAATAGGATGG
Reverse primer (Biotin)	ATAACTACCCAAACTCCTTCATTTTCTTCT
Sequencing primer	ATGGAAGTTTATTGGTGT
Sequence to analyze	GTTTAAAA**Y**[1]GGGAGTATAATA**Y**[2]GATTTGAATTG AATGAATAAAGAGAA GAAAAT
Sequence to analyze before bisulfite treatment	GTT/CTAAAA**C**A[1]/**G**GG/TAGCATAACA**C**A[2]/**G**ATTT GAACTGAAT/AGAATA A/CAGAGA/GAGAA/CAAT/C

Assay was designed using PyroMark Assay Design Software 2.0 (Qiagen, Hilden, Germany). The Reverse Primer was biotinylated. Analyzed CpG sites are indicated as Y or CG in bold while rs7256986 is underlined or presented as A/G (minor allele frequency G = 0.1360/681 (1000 Genomes)). Further SNP variants are indicated as allele 1/allele 2. Superscript numbers indicate SNP-CpG site ([1]) or CpG2 ([2]). Of note: assay design was performed according to ensemble release form 08/2017 where CpG2 was not announced to be a SNP site. An updated release from 07/2018, showed indeed CpG2 being a second CpG-SNP (rs571408084 A/G). However, no additional genotyping of this variant was performed due to a minor allele frequency of A = 0.0002/1 (1000 Genomes) proofing this SNP to be an extremely rare variant.

from whole blood in a subset of 45 individuals from the Sorbs population (primer sequences Table 2).

In homozygous G-allele carriers ($N = 13$) we observed 88.4% higher methylation at the SNP-CpG site compared to non-carriers (Fig. 2a). Heterozygous individuals ($N = 12$) show an intermediate methylation pattern of about 50% at the SNP-CpG site. CpG2, which is 12bp away from the SNP-CpG, is co-methylated showing a 10.8% higher methylation in G allele carriers compared to non-carriers (Fig. 2a, 87.8% vs. 77.0%). Similarly, we observed that heterozygous GA carriers ($N = 12$) have significantly increased methylation levels of 3.7% at CpG2 compared to AA-allele carriers ($N = 20$). All *P*-values are statistically nominally significant, while all but the latter withstand correction for multiple testing ($P < 0.0011$ and $P < 0.05$, respectively, Fig. 2a). Hence, we observed a mean per-allele increase in DNA methylation per G allele of 44.2% at SNP-CpG and 5.4% at CpG2 (Fig. 2a).

Next, we tested whether gene expression of *CRTC1* is regulated by DNA methylation and whether this is influenced by genetic variation. For these analyses overlapping gene expression and genotype data were available for 866 individuals from the Sorbs population. While we genotyped rs7256986 *de novo* in the Sorbs, we extracted mRNA expression values from our previously published data set [27]. When *CRTC1* gene expression was stratified for genotype groups, we found no significant association (Fig. 2b). Further, higher DNA methylation levels at SNP-CpG and CpG2 are negatively correlated with gene expression (Fig. 2c–d).

3.2.3. Association of genotype, DNA methylation and gene expression with clinical variables in the Sorbs

We first tested for a relationship of genetic variation, gene expression and DNA methylation with clinical variables involved in glucose and lipid metabolism, fat distribution and eating behaviour. We found a genetic association of nominal significance between the G-allele at rs7256986 with increased hip circumference ($P = 0.046$, Table 4). Further, *CRTC1* gene expression levels associate negatively with hip circumference ($P = 0.032$, Table 5), which remains nominally significant at the level of $P < 0.05$ when applying conditional analysis adjusting for rs7258696 genotypes ($P = 0.049$). These data indicate an independent negative relationship between *CTRC1* expression and hip circumference. Further, another clinical trait related to adipose tissue distribution, WHR, is positively correlated to gene expression ($P = 0.006$, Table 5), withstanding conditional analysis $P = 0.012$.

Table 3
Association analyses of *CRTC1* SNPs with the dimensions of human eating behaviour, luxury food consumption and metabolic parameters in the Sorbs.

CRTC1 SNP ID	Allele frequencies (*N*)	Restraint	Disinhibition	Hunger	Coffee intake^	Alcohol intake^	Smoking^	Ins 30min oGTT	Ins 120min oGTT
SNPs with incomplete LD with rs757318									
rs1166897	TT (195)	8.18 ± 4.94	4.64 ± 2.86	4.31 ± 3.15	0.79 ± 0.41	2.73 ± 1.18	2.42 ± 0.82	323.4 ± 214.1	186.7 ± 153.9
	TC (368)	7.48 ± 4.7	4.33 ± 3.17	3.81 ± 2.74	0.83 ± 0.37	2.88 ± 1.25	2.51 ± 0.73	278.2 ± 170	169.7 ± 156.6
	CC (193)	8.01 ± 5.08	3.81 ± 2.63	3.73 ± 2.6	0.85 ± 0.36	2.94 ± 1.17	2.51 ± 0.72	302.1 ± 168.4	166.4 ± 154.3
	P-value	n.s.	n.s.	n.s.	n.s.	**°0.007 $0.042 "0.017**	n.s.	n.s.	**°0.021 "0.044**
rs10404726	CC (206)	8.01 ± 4.87	4.53 ± 2.86	4.27 ± 3.1	0.8 ± 0.4	2.71 ± 1.15	2.43 ± 0.81	324.9 ± 212	190.4 ± 160.6
	CT (380)	7.44 ± 4.76	4.32 ± 3.17	3.79 ± 2.74	0.83 ± 0.38	2.88 ± 1.27	2.51 ± 0.73	276.9 ± 167.8	173.3 ± 158
	TT (188)	8.18 ± 5	3.87 ± 2.67	3.78 ± 2.59	0.85 ± 0.35	2.95 ± 1.18	2.52 ± 0.71	303.7 ± 172.3	165.2 ± 157.6
	P-value	n.s.	n.s.	n.s.	n.s.	**°0.002 $0.018 "0.006**	n.s.	**"0.026**	**°0.006 $0.018 "0.031**
rs11881199	TT (166)	8.71 ± 4.84	4.59 ± 3	4.15 ± 3.05	0.77 ± 0.42	2.75 ± 1.16	2.42 ± 0.82	318.6 ± 217.5	180.3 ± 147.3
	TC (369)	7.48 ± 4.69	4.46 ± 3.16	3.96 ± 2.83	0.83 ± 0.37	2.84 ± 1.27	2.50 ± 0.74	284.1 ± 171.6	173.5 ± 160.5
	CC (255)	7.87 ± 5.14	3.85 ± 2.59	3.63 ± 2.47	0.87 ± 0.33	2.94 ± 1.16	2.50 ± 0.72	297.2 ± 170.3	167.8 ± 168.1
	P-value	**"0.038**	n.s.	n.s.	**°0.015 "0.025**	**°0.028 $0.044**	n.s.	n.s.	**°0.044 $0.046**
rs11878507	GG (171)	8.75 ± 4.84	4.56 ± 3.01	4.13 ± 3.03	0.78 ± 0.42	2.75 ± 1.15	2.44 ± 0.81	316.3 ± 215.8	178.7 ± 146.6
	GA (369)	7.48 ± 4.68	4.48 ± 3.18	3.96 ± 2.83	0.83 ± 0.37	2.84 ± 1.26	2.49 ± 0.75	283.4 ± 170.4	172.1 ± 159.2
	AA (221)	7.92 ± 5.12	3.85 ± 2.6	3.61 ± 2.47	0.88 ± 0.33	2.92 ± 1.13	2.50 ± 0.73	293.8 ± 165.3	162.5 ± 156.7
	P-value	**"0.040**	n.s.	n.s.	**°0.015 "0.032**	**°0.042**	n.s.	n.s.	**°0.037$0.030**
Independent *CRTC1* SNPs									
rs7256986	GG (37)	9.14 ± 4.88	4.66 ± 3.01	3.84 ± 3.28	0.76 ± 0.43	2.76 ± 1.14	2.51 ± 0.77	297.3 ± 145.5	231.4 ± 165.1
	GA (309)	7.85 ± 4.95	4.75 ± 3.25	4.52 ± 3.08	0.81 ± 0.4	2.77 ± 1.15	2.46 ± 0.77	305.3 ± 200.2	162.6 ± 140.9
	AA (541)	7.84 ± 4.84	4.07 ± 2.79	3.56 ± 2.57	0.86 ± 0.34	2.92 ± 1.27	2.49 ± 0.76	287.8 ± 172.1	176.8 ± 169
	P-value	n.s.	**"0.044**	**°0.011; "0.002**	**°0.020; "0.040**	n.s.	n.s.	n.s.	**"0.052**
rs2023878	TT (29)	8.74 ± 5.67	3.34 ± 2	3.53 ± 2.55	0.86 ± 0.35	3.14 ± 1.38	2.59 ± 0.68	287.8 ± 150.7	195.5 ± 144.2
	TC (234)	8.18 ± 4.95	3.89 ± 2.66	3.48 ± 2.29	0.83 ± 0.38	2.89 ± 1.22	2.54 ± 0.73	278.4 ± 166.1	159.1 ± 151.6
	CC (567)	7.79 ± 4.85	4.58 ± 3.1	4.13 ± 2.96	0.83 ± 0.37	2.83 ± 1.22	2.46 ± 0.77	302.8 ± 190.5	178.5 ± 163.1
	P-value	n.s.	**°0.009; "0.015**	**°0.026; "0.018**	n.s.	n.s.	n.s.	n.s.	**"0.033**
rs6510997	TT (35)	8.47 ± 4.92	4.21 ± 2.2	3.71 ± 2.57	0.91 ± 0.29	3.2 ± 1.08	2.49 ± 0.7	299.6 ± 192.2	145.7 ± 117.8
	TC (286)	7.05 ± 4.91	4.37 ± 2.95	3.85 ± 2.67	0.88 ± 0.32	2.81 ± 1.23	2.45 ± 0.74	289.4 ± 167.9	174.8 ± 169.1
	CC (516)	8.33 ± 4.84	4.28 ± 3.02	3.93 ± 2.87	0.8 ± 0.4	2.87 ± 1.23	2.51 ± 0.76	298.8 ± 189.6	176.8 ± 159.2
	P-value	**°0.024; "0.008**	n.s.	n.s.	**°0.002; "0.002**	n.s.	n.s.	n.s.	n.s.

Data are presented as mean ± SD (Standard Deviation). *P*-values were calculated using linear regression analyses adjusted for age, sex and ln_BMI for three modes of inheritance indicated as; additive = °; dominant = $; recessive = "; nominal significant *P*-values are highlighted in bold. Results are standardized for the minor allele of the SNPs. SNP = single nucleotide polymorphism; N = number; ID = identity; LD = linkage disequilibrium; Ins = Insulin in pmol/l; oGTT = oral glucose tolerance test; n.s. = not significant.

^ Coffee intake is defined as: drinking 1-2cups coffee/day (low intake = 0) or drinking 3-4cups coffee/day (high intake = 1), alcohol consumption was measured as glasses/week, smoking was defined as number of cigarettes/day.

Next, we tested whether DNA methylation, which we have shown to be associated with genetic variation at rs7256986 (Fig. 2a), correlates with eating behaviour dimensions and clinical variables. Indeed, higher DNA methylation is related to increased restraint eating behaviour ($P = 0.001$ and $P = 0.008$ for SNP-CpG site and CpG2, respectively, Table 5). Further, methylation levels at CpG2 are positively correlated to increased glucose levels 2 h post prandial ($P = 0.008$, Table 5). The described relationship of DNA methylation with these traits does not withstand conditional analyses for rs7256986, hence might be dependent on the SNP variant (data not shown). From these data we conclude that rs7256986 is a methylation quantitative trait locus (meQTL) in the Sorbs. Further, although the relationship is moderate, anthropometric variables such as hip circumference and WHR are independently associated on a nominal level with both rs7256986 genotypes and *CRTC1* gene expression in whole blood in the Sorbs population. All associations are adjusted for age, gender and ln BMI.

3.3. Role of CRTC1 in human adipose tissue

Although predominantly expressed in brain, CRTC1 expression in liver was recently described to affect hepatic lipid accumulation and the etiology of NAFLD [2]. Given the fact that CRTC1 function in brain strongly depends on leptin [14], we next wanted to elucidate a potential role of *CRTC1* regulation in human adipose tissue, the primary source of leptin. We evaluated gene expression data in two different human adipose tissue depots (SAT vs. OVAT) in non-diabetic individuals and tested for fat depot-specific *CRTC1* mRNA expression. We next analyzed the role of rs7256986 genotypes in establishing DNA methylation patterns at SNP-CpG and CpG2 in adipose tissue. Further, we re-visited the interrelationship of genotypes, DNA methylation and gene expression in adipose tissue and its correlation to clinical variables.

Table 4
Linear regression analysis for rs7256986 genotypes and clinically relevant variables in the Sorbs.

Genotype (*N*)	*CRTC1* rs7256986		
	GG $N = 37$	GA $N = 309$	AA $N = 541$
Association analysis with anthropometric traits			
BMI (kg/m^2)	27.4 ± 4.5	26.1 ± 4.8	26.6 ± 4.5
P-value	n.s.		
Waist (cm)	91.1 ± 14.3	88.9 ± 13.6	89.8 ± 13.1
P-value	n.s.		
Hip (cm)	104.8 ± 9	103.1 ± 9.4	103.3 ± 8.2
P-value	**$0.046** ($R^2 = 0.797$; $F = 866.34$; $P(F) = 1.80x10^{-302}$)		
WHR	0.87 ± 0.11	0.86 ± 0.09	0.87 ± 0.1
P-value	n.s.		
Body fat (%)	22.5 ± 9.2	20.3 ± 9.3	20.7 ± 8.4
P-value	n.s.		
Association analysis with glucose/insulin metabolism			
FPG (mMol)	5.28 ± 0.55	5.26 ± 0.49	5.28 ± 0.53
P-value	n.s.		
Glucose_2hOGTT (mMol)	5.98 ± 1.51	5.21 ± 1.65	5.48 ± 1.8
P-value	n.s.		
FPI (pMol)	37.42 ± 20.78	38.54 ± 22.67	38.61 ± 23.78
P-value	n.s.		
Insulin_2hOGTT(pMol)	231.38 ± 165.08	162.61 ± 140.94	176.79 ± 169.04
P-value	"0.052		
Association analysis with fat metabolism			
Total C (mMol)	5.47 ± 0.87	5.23 ± 1.06	5.36 ± 1.04
P-value	n.s.		
HDL (mMol)	1.66 ± 0.39	1.68 ± 0.41	1.64 ± 0.39
P-value	n.s.		
LDL (mMol)	3.51 ± 0.9	3.27 ± 0.97	3.42 ± 0.97
P-value	n.s.		
TG (mMol)	1.25 ± 0.57	1.19 ± 0.7	1.28 ± 0.88
P-value	n.s.		

Data are presented as mean ± SD (standard deviation); *P*-values were calculated using linear regression analyses adjusted for age, sex and ln_BMI (except for BMI) using three modes of inheritance indicated as additive = °; dominant = $; recessive = "; nominal significant *P*-values are highlighted in bold; variables of the full model for the association with Hip(cm) are given as R^2 = corrected R^2; F = changes in F; P(F) = significant changes of F; *N* = number; BMI = body mass index; WHR = waist to hip ratio; FPG = fasting plasma glucose; FPI = fasting plasma insulin; 2hr oGTT = values after 2 h oral glucose tolerance test; Total Chol = plasma total cholesterol level; HDL = high density lipoprotein; LDL = low density lipoprotein; TG = Triglycerides; n.s. = not significant.

3.3.1. Rs7256986 genotypes associate with anthropometric variables in the adiposity cohort

In the Adiposity cohort our genetic data for rs7256986 show association on nominal significance level with variables related to obesity (BMI, Table 6) and adipose tissue distribution (waist, Table 6), which is, in line with our data originating from the Sorbs (Table 4). Overall, GG-carriers represent an unfavorable anthropometric profile as compared to homozygous carriers of the A-allele. All data are adjusted for age, sex and ln BMI (except for BMI). No eating behaviour data were available in this cohort.

3.3.2. Rs7256986 represents a meQTL in adipose tissue from the adiposity cohort

Similarly to our analyses in whole blood, we performed pyrosequencing on genomic DNA extracted from SAT and OVAT in a subset of the adipose tissue cohort ($N = 30$). We tested for differential DNA methylation levels at SNP-CpG and for a potential co-methylation profile at CpG2. We found in SAT and OVAT a similar methylation pattern as in whole blood with higher methylation in G allele carriers (Fig. 3a–b).

Further, to test whether gene expression is correlated with this DNA methylation profile and whether a potential relationship relates to genetic variation at SNP-CpG we analyzed gene expression categorized for genotype data in the adiposity cohort. We extracted gene expression values from our previously published genome wide data set [27]. Gene expression data from 44 and 36 individuals (SAT and OVAT respectively) were available, all with corresponding genotype data. When stratifying *CRTC1* gene expression levels per genotype group, we observed no differences in SAT (Fig. 3c) while increased expression in OVAT among G-allele carriers were present (Fig. 3d). From these data we conclude that indeed genetic variation correlates with the degree of DNA methylation in adipose tissue and may directly influence *CRTC1* expression in OVAT, hence rs7256986 represents a meQTL in both adipose tissue depots.

3.3.3. CRTC1 gene expression and DNA promoter methylation in SAT and OVAT

To further strengthen our results, we extracted from our recently published data set DNA promoter methylation data for SAT and OVAT [27], from the Adiposity cohort ($N = 54$). We tested whether DNA promoter methylation correlates with gene expression. *CRTC1* expression level in OVAT (0.012 ± 0.065) was slightly higher than in SAT (-0.0152 ± 0.068) ($P = 0.066$, Fig. 4a). Further, DNA promoter methylation profiles were comparable in both depots (Fig. 4b). Increased *CRTC1* promoter methylation positively correlates with its gene expression in OVAT ($P = 0.050$, Fig. 5a) and promoter methylation of both fat depots is correlated ($P = 0.050$, Fig. 5b).

3.3.4. CRTC1 gene expression and DNA promoter methylation in SAT and OVAT associate with clinical variables

CRTC1 expression in OVAT negatively correlates with BMI ($P = 0.007$, Table 7) and this correlation withstands conditional analyses for rs7256986 genotypes ($P = 0.014$) hinting at a genotype independent relationship Further, WHR is negatively related to DNA promoter methylation in OVAT ($P = 0.012$), also independently from genotype distribution at rs7256986 ($P = 0.013$). Total cholesterol levels ($P = 0.022$), triglycerides ($P = 0.005$) plasma cytokine IL6 levels ($P = 0.021$) are positively correlated with OVAT DNA promoter methylation, and again these relationships were independent from rs7256986

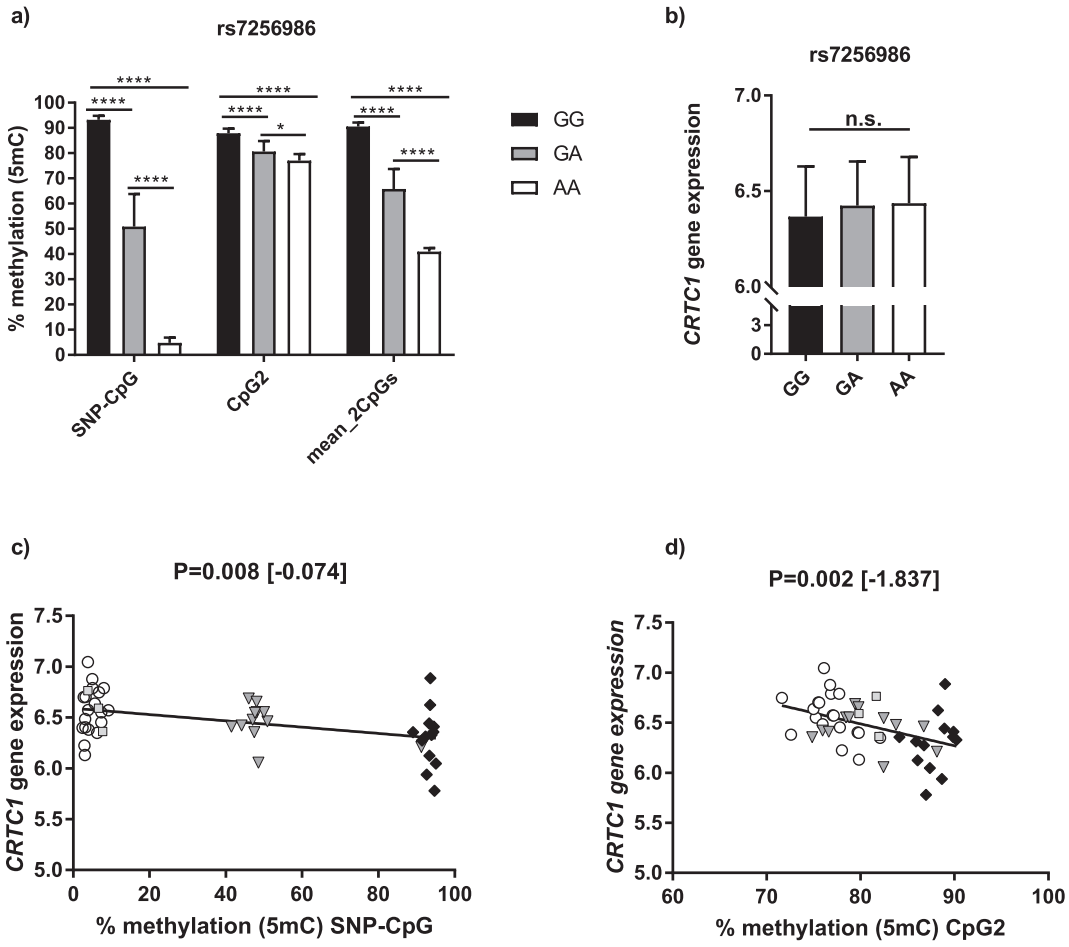

Fig. 2. DNA methylation at SNP-CpG and CpG2 and *CRTC1* expression categorized per rs7256986 genotypes in the Sorbs a) DNA methylation at SNP-CpG and CpG2 categorized for genotypes at rs7256986. Data are presented as mean ± SD. *P*-values were calculated using independent students t-tests. *P*-values<0.0001 = **** and <0.005 = *. 5mC = 5-Methylcytosine; Numbers per genotype: GG = 13; GA = 12; AA = 20. b) *CRTC1* gene expression stratified per genotypes at rs7256986. Data are presented as mean ± SD. *P*-values were calculated using independent students t-tests. Numbers per genotype: GG = 37; GA = 298; AA = 531 c-d) Correlation of *CRTC1* expression with DNA methylation at SNP-CpG and CpG2. Data were obtained using linear regression analysis adjusted for age, sex and ln_BMI; beta coefficient is given in brackets; white dots = AA = 20; grey triangles = GA = 12; black diamonds = GG = 13; light grey quarters = no genotype data.

genotypes ($P = 0.026$, $P = 0.005$ and $P = 0.019$ respectively). A nominal significant association was detected for SAT DNA methylation with decreased waist circumference ($P = 0.046$). All data are summarized in Table 7.

3.3.5. DNA methylation at SNP-CpG and CpG2 is related to anthropometric parameters in the adiposity cohort

In addition to *CRTC1* DNA promoter methylation we sought to understand whether DNA methylation at SNP-CpG and CpG2 is related to metabolic and anthropometric variables of obesity. In both fat depots, DNA methylation at SNP-CpG and CpG2 shows a negative association profile (nominal level) to several variables related to fat distribution (Table 8). Especially, visceral fat area is negatively correlated to SNP-CpG methylation levels in SAT ($P = 0.039$) and OVAT ($P = 0.016$). We found similar relationships of OVAT SNP-CpG methylation to CT-ratio ($P = 0.027$) and adipocyte size (maximum diameter) in SAT ($P = 0.016$) and OVAT ($P = 0.009$). Further data are summarized in Table 8. Overall, with increased methylation we observed a favorable outcome for anthropometric and metabolic variables. All data are adjusted for sex, age and ln_BMI, while several associations seem to be independent from genetic variation withstanding conditional analyses for rs2756986 (Table 8).

4. Discussion

Our study focuses on *CRTC1* as a candidate gene for fat distribution as recently described in a GWAS [16]. Here, we sought to better understand (i) the role of genetic variation in human eating behaviour, consumption of luxury goods and variables clinically relevant for obesity, and adipose tissue distribution. We used three independent cohorts to perform these analyses. (ii) We tested for a potential epigenetic regulation of *CRTC1* and an interrelationship of genetic variation with DNA methylation in whole blood and two intra-individually paired human adipose tissue depots (OVAT and SAT). (iii) We next used *CRTC1* expression data from whole blood and adipose tissue to further understand whether (epi)genetic alterations correlate with *CRTC1* gene activity.

4.1. CRTC1 genetic variants are related to dimensions of human eating behaviour and metabolic variables

In our initial data set, the Sorbs, we first focused on genetic variants within a large LD block of the *CRTC1* gene locus including 10 variants in LD with the previously published body fat associating variant rs757318 [16]. All variants are related to 120min postprandial insulin levels and alcohol intake. Most interestingly, two variants (rs11881199 and rs11878507) are also related to increased restraint eating behaviour.

Table 5
Association of rs7256986 SNP methylation and *CRTC1* gene expression with clinical variables in the Sorbs.

	N	rs7256986 methylation			*CRTC1* gene expression	
		SNP-CpG	CpG2	mean_2CpGs	*N*	*P*-value [B]
		P-value [B]				
Association analysis with dimensions of human eating behaviour						
Restraint	41	**0.001 [0.504]**	**0.008 [0.419]**	**0.001 [0.533]**	536	0.972 [0.029]
Disinhibtion	41	0.731 [−0.059]	0.382 [−0.146]	0.720 [−0.063]	536	0.789 [0.126]
Hunger	41	0.255 [−0.192]	0.133 [−0.247]	0.202 [−0.219]	535	0.484 [0.330]
Association analysis with luxury food consumption						
Smoking behaviour^	46	0.590 [−0.084]	0.676 [−0.063]	0.758 [−0.047]	867	0.470 [−0.073]
Cigaretts per day	17	0.677 [0.104]	0.837 [−0.055]	0.862 [0.045]	296	0.508 [−1.123]
Coffee consumption^	46	0.233 [−0.186]	0.131 [−0.234]	0.200 [−0.201]	859	0.780 [−0.015]
Coffee cups per day	38	0.359 [−0.147]	0.587 [−0.088]	0.391 [−0.138]	713	0.416 [−0.095]
Alcohol consumption^	46	0.569 [0.076]	0.635 [0.063]	0.567 [0.077]	873	0.694 [−0.059]
Association analysis with anthropometric traits						
BMI (kg/m^2)	47	0.599 [0.009]	0.798 [−0.098]	0.708 [0.027]	877	0.225 [0.066]
Waist (cm)	47	0.613 [−0.028]	0.618 [−0.028]	0.609 [−0.029]	877	0.096 [0.014]
Hip (cm)	46	0.579 [0.039]	0.992 [0.001]	0.709 [0.027]	875	**0.032 [−0.011]***
WHR	46	0.318 [−0.089]	0.537 [−0.056]	0.388 [−0.078]	875	**0.006 [0.025]***
Body fat (%)	47	0.101 [−0.107]	0.236 [−0.078]	0.105 [−0.107]	877	0.167 [0.039]
Association analysis with glucose/insulin metabolism						
FPG (mMol)	47	0.070 [0.205]	0.772 [0.033]	0.145 [0.168]	876	0.563 [0.007]
Glucose_2hOGTT (mMol)	47	0.066 [0.239]	**0.008 [0.335]**	**0.039 [0.270]**	864	0.151 [−0.059]
FPI (pmol)	47	0.233 [−0.165]	0.381 [−0.122]	0.289 [−0.149]	876	0.781 [0.020]
Insulin_2hOGTT (pmol)	47	0.359 [0.136]	0.110 [0.234]	0.251 [0.172]	863	0.265 [−0.133]
Association analysis with fat metabolism						
Total Chol (mMol)	47	0.055 [−0.272]	0.476 [−0.103]	0.092 [−0.243]	877	0.362 [0.023]
HDL Chol (mMol)	47	0.833 [−0.027]	0.995 [−0.001]	0.725 [−0.046]	877	0.466 [−0.021]
LDL Chol (mMol)	47	0.040 [−0.272]	0.235 [−0.160]	0.059 [−0.254]	877	0.456 [0.028]
TG (mMol)	47	0.362 [−0.115]	0.860 [0.022]	0.478 [−0.091]	877	0.950 [−0.004]

P-values were calculated using linear regression analyses adjusted for age, sex and ln_BMI; B = regression coefficient beta, shown in square brackets; nominal significant *P*-values are highlighted in bold; coffee intake is defined as: drinking 1-2cups coffee/day (low intake = 0) or drinking 3-4cups coffee/day (high intake = 1);, alcohol consumption was measured as glasses/week. *N* = number; BMI = body mass index; WHR = waist to hip ratio; FPG = fasting plasma glucose; FPI = fasting plasma insulin; 2hr oGTT = values after 2 h oral glucose tolerance test; Total Chol = plasma total cholesterol level; HDL = high density lipoprotein; LDL = low density lipoprotein; TG = Triglycerides.

* Indicates associations withstanding conditional analysis for rs7256986 genotype.

^ Smoking behaviour is defined as time period of smoking (former, current, never smoker) as well as numbers of cigarettes per day.

Further, for another three independent variants, we observed relationships to restraint, disinhibition and/or hunger, coffee consumption, 120min postprandial insulin levels and hip circumference. These data support the role of *CRTC1* in eating behaviour. To further strengthen our results we selected one of the top genetic variants, rs7256986, to replicate the relationship to eating behaviour dimensions in another independent replication cohort (whole blood) and the effects on metabolic and anthropometric variables in the Adiposity cohort (adipose tissue). Rs7256986 represents a tagging SNP for a smaller LD block that is independent from the previously published lead SNP rs757318 [16]. Although the effects for eating behaviour in the replication cohort are non-significant we observed the same trends for restraint and disinhibition adding plausibility to our initial results. In the Adiposity cohort we found that rs7256986 correlates with BMI and waist circumference, clinically relevant variables for obesity and fat distribution. Together with the correlations to postprandial insulin and hip circumference in the Sorbs these data corroborate studies reporting a relationship of *CRTC1* SNPs with obesity-related and cardio-metabolic traits [15,27,20]. Others have reported that in mice, *Crtc1* expression in liver is related to hepatic lipid accumulation [2], while *Crtc1* deficiency leads to development of hepatic steatosis in young animals. Interestingly, the studied *Crtc1* knock out mouse model is obese with more pronounced visceral fat accumulation [2]. Hence, it is worth focusing on *CRTC1* genetic variants and gene function in other tissues than brain to better understand how CRTC1 contributes to obesity and metabolism. Moreover, we are the first providing a detailed analysis for a role of *CRTC1* genetic variants in human eating behaviour, which is in line with the known central role of CRTC1 in brain in regulating energy metabolism and thereby also obesity and diabetes development [2,3,6,14,16,17]. Larger studies are needed to confirm the results found in this study.

4.2. Epigenetic circuitry – rs7256986 is a meQTL in whole blood and adipose tissue

Next, as rs7256986 introduces a CpG site we sought to test for a potential epigenetic regulation of *CRTC1* and if so, whether this correlates with genetic variation. We measured DNA methylation at SNP-CpG and a nearby CpG site in two cohorts by using genomic DNA extracted from whole blood from the Sorbs, and SAT and OVAT depots from the Adiposity cohort. We observed that DNA methylation was significantly higher in individuals carrying the G allele, hence rs7256986 represents a meQTL in both cohorts in all three tissues. In whole blood from Sorbs DNA methylation levels at the two analyzed CpG sites were positively related with eating behaviour and most likely dependent on genotype distribution. Further, while DNA methylation levels impact on glucose metabolism in the Sorbs, we show in the Adiposity cohort a relationship with variables related to adipose tissue distribution. Some of these associations in adipose tissue are, in contrast to the results from whole blood, independent from rs7256986, indicating at potential tissue-specific epigenetic mechanism.

4.3. CRTC1 gene expression correlates with clinical variables

Further, to better understand whether DNA methylation relates to *CRTC1* expression we found that, although non-significant, in whole blood from the Sorbs, G allele carries show lower gene expression and a negative relationship between methylation and expression. In line

Table 6
Linear regression analysis of rs7256986 in the Adiposity cohort.

Genotype (*N*)	*CRTC1* rs7256986		
	GG *N* = 16	GA *N* = 67	AA *N* = 85
Association analysis with anthropometric traits			
Height (m)	1.68 ± 0.11	1.68 ± 0.08	1.7 ± 0.1
P-value	n.s.		
Weight (kg)	114.7 ± 38.6	91.6 ± 36.8	106.7 ± 39
P-value	n.s.		
BMI (kg/m^2)	40.2 ± 12.1	32.4 ± 12.3	36.7 ± 12.8
P-value	**$0.016**		
Waist (cm)	115.13 ± 28.528	98.42 ± 29.197	112.86 ± 27.568
P-value	**°0.045 $0.035**		
Hip (cm)	124.38 ± 30.201	110.57 ± 31.223	120.96 ± 29.849
P-value	n.s.		
WHR	0.93 ± 0.12	0.89 ± 0.11	0.94 ± 0.12
P-value	n.s.		
Body fat (%)	34.82 ± 15.147	26.26 ± 8.154	32.7 ± 12.318
P-value	n.s.		
Subcutaneous fat area (cm^2)	1071 ± 791.866	547.6 ± 703.179	841.2 ± 758.246
P-value	n.s.		
Visceral fat area (cm^2)	217.27 ± 138.532	123.4 ± 122.386	178.9 ± 132.809
P-value	n.s.		
Vis/sc ratio	0.3 ± 0.2	0.6 ± 0.5	0.5 ± 0.5
P-value	n.s.		
Mean adipocyte size SAT (um)	135.2 ± 30.228	115.48 ± 22.416	123.01 ± 24.109
P-value	n.s.		
Mean adipocyte size OVAT (um)	134.97 ± 17.654	113.79 ± 19.452	121.81 ± 23.574
P-value	n.s.		
Max adipocyte size SAT (um)	423.13 ± 192.29	233.26 ± 96.481	265.53 ± 122.115
P-value	n.s.		
Max adipocyte size OVAT (um)	382.87 ± 185.959	215.93 ± 68.348	251.08 ± 109.649
P-value	n.s.		
Association analysis with glucose/insulin metabolism			
FPG (mMol)	5.6 ± 0.523	5.24 ± 0.532	5.44 ± 0.519
P-value	"0.051		
Glucose_2hOGTT (mMol)	6.34 ± 0.923	6.22 ± 1.29	6.77 ± 1.142
P-value	$0.054		
FPI (pMol)	55.98 ± 45.347	54.32 ± 73.087	67.21 ± 64.076
P-value	n.s.		
HbA1c	5.63 ± 0.296	5.38 ± 0.36	5.55 ± 0.345
P-value	n.s.		
Association analysis with fat metabolism			
Total C (mMol)	4.97 ± 0.807	4.98 ± 0.929	5.09 ± 0.954
P-value	n.s.		
HDL (mMol)	1.35 ± 0.259	1.45 ± 0.434	1.35 ± 0.358
P-value	n.s.		
LDL (mMol)	3.31 ± 0.798	3 ± 1.093	3.4 ± 1.049
P-value	n.s.		
TG (mMol)	1.39 ± 1.191	1.29 ± 0.78	1.14 ± 0.387
P-value	n.s.		
Association analysis with (Adipo)cytokines			
Adiponectin (mMol)	6.64 ± 2.754	10.44 ± 6.066	8.54 ± 4.566
P-value	n.s.		
Leptin (mMol)	34.77 ± 18.204	24.7 ± 24.751	27.88 ± 20.505
P-value	n.s.		
IL6 (pg/ml)	5.88 ± 5.34	3.4 ± 4.078	4.52 ± 4.877
P-value	n.s.		

Data are presented as mean ± SD (standard deviation); *P*-values were calculated using linear regression analyses adjusted for age, sex and ln_BMI (except for BMI) using three modes of inheritance indicated as additive = °; dominant = $; recessive = "; nominal significant *P*-values are highlighted in bold; *N* = number; BMI = body mass index; WHR = waist to hip ratio; FPG = fasting plasma glucose; FPI = fasting plasma insulin; 2hr oGTT = values after 2 h oral glucose tolerance test; HbA1c = glycosylated hemoglobin; Total Chol = plasma total cholesterol level; HDL = high density lipoprotein; LDL = low density lipoprotein; TG = Triglycerides; IL6 = Interleukin 6; n.s. = not significant.

with this, the levels of mRNA expression correlate with fat distribution (hip circumference). In adipose tissue we find a higher gene expression in G allele carriers in OVAT along with a positive correlation between DNA methylation and *CRTC1* expression. However, the relationship of mRNA expression with clinical variables such as hip circumference in the Adiposity cohort seems to be, at least partly, driven by an independent regulatory mechanism as several associations withstand conditional analyses for rs7256986. Taken together, we conclude that rs7256986 is a potential epigenetic regulatory variant and functions in general as a meQTL influencing DNA methylation levels. Hence, this SNP may affect gene expression, which can translate into alterations in clinical traits. However, due to the small size of the cohort, we were not able to perform Mendelian randomization analyses to draw causative conclusions. We clearly find tissue-specific differences as the relationship between gene expression and clinical traits is partly independent from genotype distribution in adipose tissue. Therefore, although our results are novel and imply an important regulatory role of *CRTC1* in blood and adipose tissue in addition to its described effects in brain, it is noteworthy to mention that larger studies are warranted to confirm or reject our results. Studies show that Crtc1 −/− mice are hyperphagic and obese [3,6]. While the low expression in brain might mainly affect eating behaviour with subsequent development of obesity, an upregulation in other tissues such as adipose tissue could have additional negative influence on metabolic variables. As we found a slightly higher gene expression in the visceral adipose compartment and among GG carries, one can speculate that this potentially translates into higher metabolic activity of this fat depot and/or among GG carriers. However, this remains speculative as our data are non-significant. CRTC3 was shown to be secreted from adipocytes and likely to be a regulating factor for adipokine production and insulin resistance [8]. Hence, it would be of high interest to understand whether CRCT1 plays an additional or complementary role in addition to CRTC3 in adipose biology and fat distribution.

4.4. DNA methylation correlates with gene expression and clinical variables

We show that DNA promoter methylation and gene expression in OVAT are positively, but weakly correlated. Traditionally, CpG hypermethylation at gene promoters leads to transcriptional repression and vice versa. In general, the correlation of promoter-DNA methylation on gene expression is dependent on the density of CpG sites [32]. Therefore, methylation at promoters with a high density of CpG sites may not be strongly correlated with gene expression and other factors such as histone modifications may be taken into account. It is worth mentioning that the meQTL identified here is located in the first intron of *CRTC1* and therefore a site within the gene body. In line with our data and although not well understood, methylation within the gene body is related to increased gene expression [33,34]. Further, the meQTL may have additional functional effects such as on splicing of *CRTC1*, transcription factor binding and three-dimensional chromatin looping if the region acts as a distal enhancer for other genes. Overall, studying the gene-wide methylation pattern of *CRTC1* would shed light on how exactly transcription might be regulated by its DNA methylation. As we found OVAT gene expression negatively related to BMI and several other clinical traits such as WHR and furthermore lipid species positively related to DNA promoter methylation in OVAT, one can speculate that *CRTC1* upregulation in OVAT may result in negative consequences for metabolic health. This might be mediated through increased inflammatory potential in OVAT, as our data suggest by increased plasma cytokine IL6 levels, which positively associate with OVAT DNA promoter methylation. Additionally, promoter methylation in both fat depots is correlated. These data hint on a cross tissue regulatory mechanism. It might be speculated that dysregulation of one fat depot impacts on inter-depot gene regulation probably via secreted molecules such as miRNAs or lncRNAs. That miRNAs are a source for *CRTC1* gene regulation was recently shown in liver and pancreas [2]. While in pancreatic

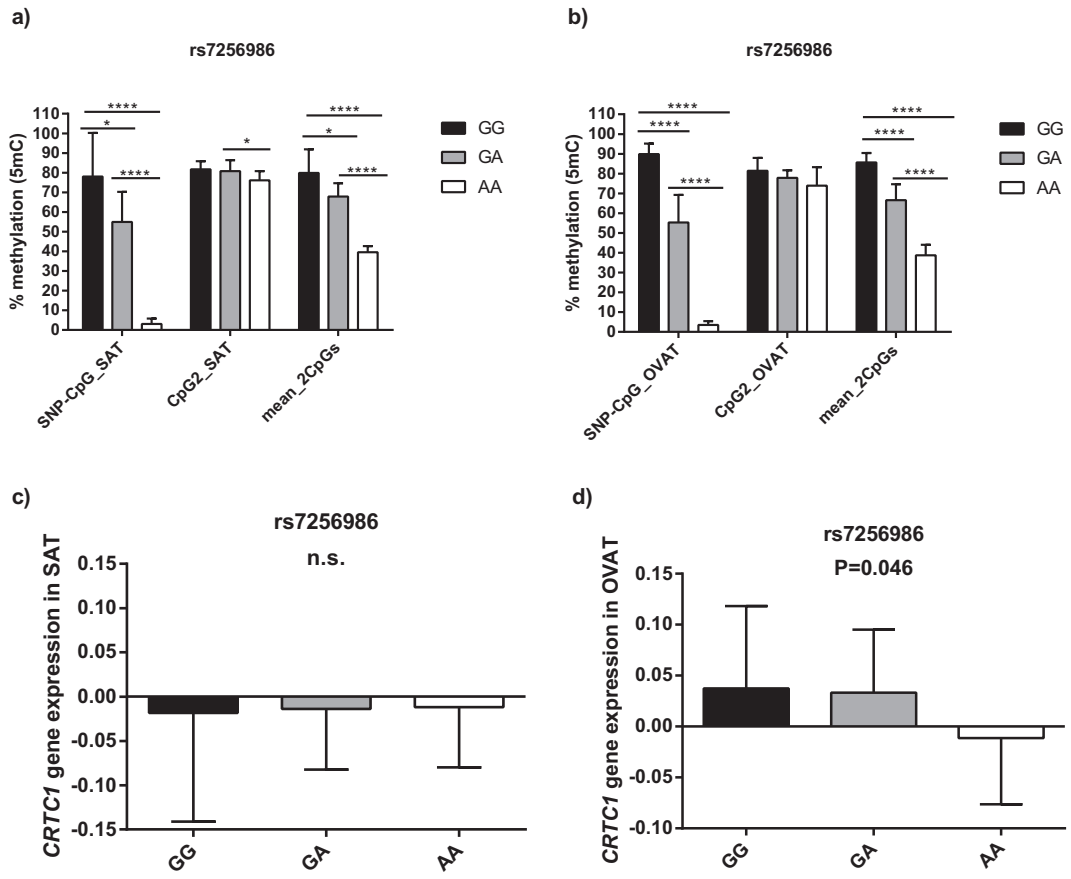

Fig. 3. DNA methylation at SNP-CpG and CpG2, and *CRTC1* expression categorized per rs7256986 genotypes in the adiposity cohort a-b) DNA methylation at the SNP-CpG site and CpG2 categorized for genotypes at rs7256986 in a) SAT and b) OVAT. Data are presented as mean ± SD. *P*-values were calculated using independent students t-tests. *P*-values<0.0001 = **** and <0.005 = *. 5mC = 5-Methylcytosine; n.s. = not significant; Numbers per genotype a) GG = 10; GA = 10; AA = 10 and b) GG = 10; GA = 10; AA = 5. c) and d) *CRTC1* gene expression in SAT and OVAT. Data are presented as mean ± SD. *P*-value was calculated using independent group comparison for GG + GA (*N* = 22) vs AA (*N* = 14) of rs7256986. All data are shown for non-diabetics only.

beta-cells *CRTC1* expression is related to the microRNAs miR-212/miR-132 in a regulatory loop impacting insulin signaling [19] hepatic miR-34a was shown to directly target *CRTC1* in the liver, regulating its mRNA expression and being protective against hepatic metabolic disturbances [2].

In light of the worldwide efforts in anticipating precision medicine tools, our results might be of clinical relevance in the future. Genetic variance at *CRTC1* might be taken into account as individual susceptibility variants related to dimensions of human eating behaviour to design individualized treatment strategies.

4.5. Limitations

Although we included three different cohorts in our analyses and several types of tissues such as whole blood and paired human OVAT/SAT samples, we are aware that the samples size is still limiting the

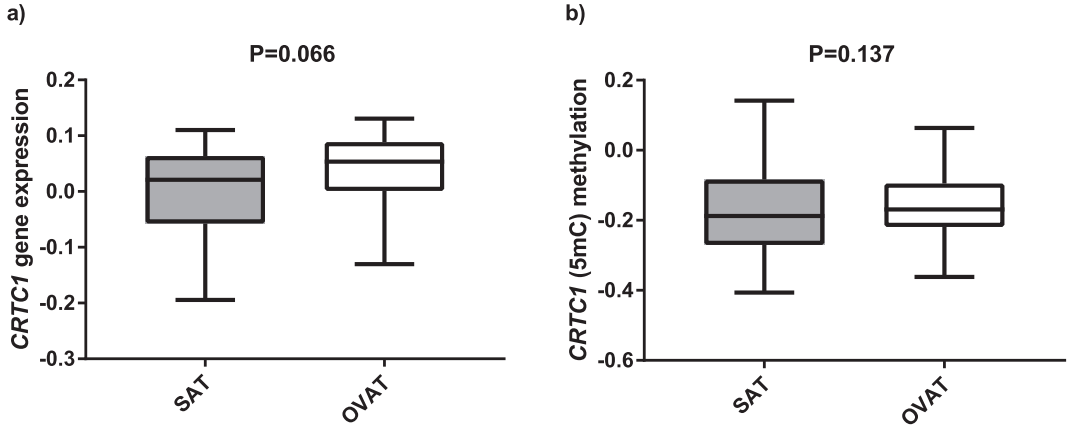

Fig. 4. *CRTC1* gene expression and promoter DNA methylation stratified for SAT and OVAT in non-diabetic subjects from the adipose tissue cohort Data are presented as mean ± SD. *P*-values were calculated using paired samples t-tests. a) gene expression in SAT and OVAT (*N* = 31). b) promoter DNA methylation in SAT and OVAT (*N* = 54).

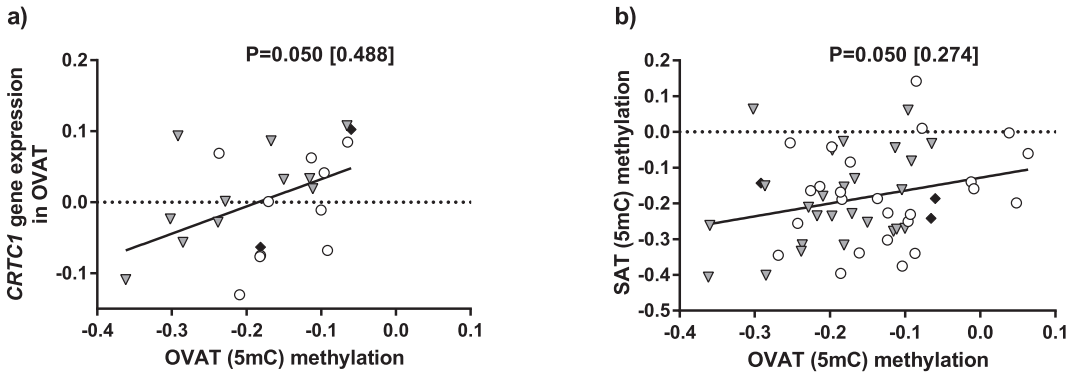

Fig. 5. Correlations of *CRTC1* promoter DNA methylation *P*-values and beta values (in square brackets) were calculated using linear regression models adjusted for age, sex and ln_BMI. OVAT = omental visceral adipose tissue; SAT = subcutaneous adipose tissue; white dots = AA; gray triangles = GA; black diamonds = GG; light gray quarters = no genotype data; *N* numbers = *N* a) Correlation of expression with promoter DNA methylation in OVAT (*N* = 23) b) Correlation of promoter DNA methylation between SAT and OVAT (*N* = 23).

power of the here presented study to reach statistically significant results. This may limit our ability to identify small effects and may have led to false positive and false negative results. Especially the numbers of homozygous G allele carriers is small and may have influenced our results for association analyses. Similarly, most of the observed nominal significant associations would not withstand correction for multiple testing. Therefore, our results should be cautiously interpreted avoiding overestimation of the observed effects. Further, we have only limited knowledge of the individuals´ environmental factors which may have theoretically influenced DNA methylation levels. Importantly, adipose tissue is a heterogeneous sample per se containing multiple cell types, and we cannot rule out that methylation/expression levels originating from other cell types such as macrophages may influence the results. Bioinformatic estimation of cell composition in adipose tissue and cell counts of blood samples is not applicable due to missing information and is further limiting the here presented work.

5. Conclusion

We provide evidence that genetic variation in *CRTC1* is related to dimensions of human eating behaviour. Further, we are the first describing an epigenetic feature of rs7256986 representing a meQTL in whole blood and adipose tissue. Genetic variation and DNA methylation levels at rs7256986 correlate with *CRTC1* gene expression, which is related to BMI, fat distribution and metabolic traits. In addition, gene expression in adipose tissue is correlated to clinically relevant variables independently from rs7256986.

Taken together, our data suggest a role of *CRTC1* genetic variants in dimensions of human eating behaviour and metabolic variables, whilst

Table 7
Association of *CRTC1* gene expression and DNA promoter methylation with phenotypical variables in adipose tissue.

	N	*CRTC1*_meth_SAT	*CRTC1*_meth_OVAT	*CRTC1*_expr_SAT	*CRTC1*_expr_OVAT
Association analysis with anthropometric traits					
BMI (kg/m^2)	44/36/54/54	0.839 [0.022]	0.472 [−0.105]	0.408 [0.179]	**0.007 [−0.687]***
Waist (cm)	42/34/53/53	**0.046 [−0.172]**	0.213 [−0.080]	0.646 [0.029]	0.660 [−0.036]
Hip (cm)	42/34/53/53	0.334 [−0.069]	0.194 [0.093]	0.556 [0.041]	0.345 [0.089]
WHR	42/34/53/53	0.186 [−0.149]	**0.012 [−0.276]***	0.992 [−0.001]	0.182 [−0.210]
Body fat (%)	26/21/33/33	0.993 [0.001]	0.673 [−0.042]	0.733 [0.034]	0.170 [0.187]
Subcutaneous fat area (cm^2)	43/35/54/54	0.692 [0.028]	0.623 [0.035]	0.273 [−0.082]	0.664 [0.044]
Visceral fat area (cm^2)	43/35/54/54	0.451 [−0.070]	0.954 [−0.005]	0.235 [−0.125]	0.424 [0.108]
vis/sc ratio	43/35/54/54	0.341 [−0.117]	0.617 [−0.062]	0.992 [−0.001]	0.739 [0.053]
Mean adipocyte size SAT (um)	20/18/17/17	0.785 [0.069]	0.259 [−0.287]	0.146 [−0.275]	0.516 [0.142]
Mean adipocyte size OVAT (um)	20/18/17/17	0.215 [0.320]	0.291 [0.284]	0.716 [−0.075]	0.653 [0.075]
Max adipocyte size SAT (um)	20/18/17/17	0.604 [−0.093]	0.525 [−0.117]	0.715 [−0.062]	0.588 [−0.121]
Max adipocyte size SAT (um)	20/18/17/17	0.698 [−0.073]	0.946 [0.013]	0.553 [0.103]	0.977 [−0.007]
Association analysis with glucose/insulin metabolism					
FPG (mMol)	44/36/54/54	0.474 [−0.095]	0.496 [−0.091]	0.737 [0.050]	0.418 [−0.160]
Glucose_2hOGTT (mMol)	35/27/40/40	0.341 [−0.150]	0.080 [−0.263]	0.762 [0.049]	0.687 [−0.088]
FPI (pmol)	21/18/24/24	0.933 [0.014]	0.595 [−0.091]	0.401 [0.154]	0.490 [−0.206]
HbA1c	43/35/53/53	0.272 [−0.172]	0.888 [−0.016]	0.443 [−0.099]	0.877 [−0.026]
Association analysis with fat metabolism					
Total C (mMol)	19/15/28/28	0.856 [−0.038]	**0.022 [0.449]***	0.514 [−0.170]	0.455 [0.273]
HDL (mMol)	32/27/38/38	0.687 [0.059]	0.095 [−0.238]	0.811 [0.038]	0.719 [−0.070]
LDL (mMol)	32/27/38/38	0.222 [−0.192]	0.967 [0.006]	0.847 [−0.036]	0.897 [−0.028]
TG (mMol)	20/16/31/31	0.489 [0.117]	**0.005 [0.444]***	0.888 [−0.032]	0.475 [0.236]
Association analysis with (adipo)cytokines					
Adiponectin (mMol)	35/29/40/40	0.800 [−0.034]	0.937 [−0.011]	0.122 [0.172]	0.183 [0.206]
Leptin (mMol)	36/29/42/42	0.201 [−0.116]	0.458 [0.070]	0.947 [0.007]	0.238 [0.175]
IL6 (pg/ml)	38/32/45/45	0.098 [0.229]	**0.021 [0.322]***	0.767 [−0.041]	0.298 [0.182]

P-values were calculated using linear regression analyses adjusted for age, sex and ln_BMI; B = regression coefficient beta, shown in square brackets; nominal significant *P*-values are highlighted in bold; *N* = number meth_SAT/OVAT/exp_SAT/OVAT; BMI = body mass index; WHR = waist to hip ratio; FPG = fasting plasma glucose; FPI = fasting plasma insulin; 2hoGTT = values after 2 h oral glucose tolerance test; Total Chol = plasma total cholesterol level; HDL = high density lipoprotein; LDL = low density lipoprotein; TG = Triglyceride.
* Indicates associations withstanding conditional analysis for rs7256986 genotype.

Table 8
Association of rs7256986 SNP methylation with clinical variables in the Adiposity cohort.*

	N	*CRTC1* rs7256986					
		SNP-CpG_SAT	CpG2_SAT	mean_2CpGs_SAT	SNP-CpG_OVAT	CpG2_OVAT	mean_2CpGs_OVAT
Association analysis with anthropometric traits							
BMI (kg/m^2)	30/25	0.802 [0.057]	0.921 [0.051]	0.826 [0.055]	0.175 [0.268]	0.140 [0.310]	0.135 [0.317]
Waist (cm)	29/25	0.161 [−0.105]	0.302 [−0.078]	0.144 [−0.109]	0.231 [−0.104]	0.737 [−0.029]	0.252 [−0.100]
Hip (cm)	29/25	0.466 [−0.075]	0.939 [−0.008]	0.490 [−0.071]	0.332 [−0.117]	0.166 [−0.163]	0.257 [−0.237]
WHR	29/25	0.483 [−0.110]	0.320 [−0.153]	0.424 [−0.124]	0.845 [−0.036]	0.299 [0.187]	0.993 [0.002]
Body fat (%)	19/14	0.118 [−0.180]	0.055 [−0.225]	0.089 [−0.195]	0.220 [−0.138]	0.695 [0.045]	0.308 [−0.117]
Subcutaneous_fat_cm^2	29/24	0.598 [−0.057]	0.440 [0.082]	0.700 [−0.042]	0.836 [0.019]	0.34 [−0.090]	0.970 [0.004]
Visceral_fat_cm^2	29/24	**0.039 [−0.271]**	0.644 [−0.062]	**0.045 [−0.263]**	**0.016 [−0.272]***	0.093 [−0.212]	**0.014 [−0.282]***
vis/sc ratio	29/24	0.215 [−0.200]	0.239 [−0.188]	0.182 [−0.215]	**0.027 [−0.342]**	0.624 [−0.087]	**0.039 [−0.326]**
sc_mean	12/8	0.052 [−0.264]	0.443 [−0.137]	0.059 [−0.255]	0.128 [−0.340]	0.915 [−0.035]	0.178 [−0.322]
vis_mean	12/8	0.076 [−0.334]	**0.048 [−0.425]**	0.057 [−0.349]	0.090 [−0.506]	0.698 [−0.174]	0.121 [−0.492]
sc_max	12/8	0.343 [−0.175]	0.344 [−0.204]	0.321 [−0.181]	**0.016 [−0.380]**	0.185 [−0.333]	**0.013 [−0.392]**
vis_max	12/8	0.322 [−0.185]	0.563 [−0.130]	0.324 [−0.182]	**0.009 [−0.467]**	0.432 [−0.264]	**0.018 [−0.465]**
Association analysis with glucose/insulin metabolism							
FPG (mMol)	30/25	0.409 [0.146]	0.211 [−0.217]	0.554 [0.105]	0.635 [0.100]	0.166 [0.282]	0.497 [0.144]
Glucose_2hOGTT (mMol)	24/19	0.414 [−0.177]	0.262 [0.241]	0.523 [−0.139]	0.338 [−0.246]	0.648 [0.119]	0.418 [−0.210]
FPI (pmol)	18/15	**0.017 [−0.444]**	**0.003 [−0.533]***	**0.010 [−0.472]***	0.059 [−0.442]	0.411 [0.208]	0.111 [−0.383]
HbA1c	30/25	0.324 [−0.153]	0.516 [−0.100]	0.311 [−0.157]	0.301 [−0.188]	0.362 [0.164]	0.443 [−0.141]
Association analysis with fat metabolism							
Total C (mMol)	17/16	0.232 [0.338]	0.612 [−0.168]	0.293 [0.297]	0.756 [0.102]	0.190 [0.382]	0.593 [0.171]
HDL (mMol)	24/21	0.566 [0.106]	0.410 [−0.149]	0.691 [0.073]	0.613 [0.109]	0.156 [−0.283]	0.791 [0.057]
LDL (mMol)	24/21	0.415 [−0.157]	0.750 [−0.061]	0.429 [−0.151]	0.576 [−0.126]	0.630 [0.103]	0.653 [−0.101]
TG (mMol)	18/17	0.992 [0.003]	0.167 [0.388]	0.820 [0.061]	0.630 [−0.136]	**0.039 [−0.584]***	0.462 [−0.204]
Association analysis with (adipo)cytokines							
Adiponectin	23/18	0.795 [−0.050]	0.760 [−0.059]	0.774 [−0.057]	0.664 [0.108]	**0.050 [0.381]***	0.399 [0.212]
Leptin	24/19	0.131 [−0.214]	0.465 [−0.103]	0.126 [−0.221]	**0.037 [−0.323]**	0.590 [0.076]	0.079 [−0.282]
IL6	26/21	**0.018 [−0.432]***	0.769 [0.056]	**0.030 [−0.405]**	0.146 [−0.333]	0.388 [−0.179]	0.129 [−0.348]

P-values were calculated using linear regression analyses adjusted for age, sex and ln_BMI; B = regression coefficient beta, shown in square brackets; nominal significant *P*-values are highlighted in bold; *N* = number SAT/OVAT; BMI = body mass index; WHR = waist to hip ratio; FPG = fasting plasma glucose; FPI = fasting plasma insulin; 2hoGTT = values after 2 h oral glucose tolerance test; Total Chol = plasma total cholesterol level; HDL = high density lipoprotein; LDL = low density lipoprotein; TG = Triglyceride.
* Indicates associations withstanding conditional analysis for rs7256986 genotype.

epigenetic regulation and alterations in gene expression can either be related to such genetic variation or represent independent regulators.

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Declaration of competing interests

None.

Ethics approval and consent to participate

The Ethics Committee of the University of Leipzig approved this study, while it was performed in accordance to the declaration of Helsinki. All subjects gave written informed consent before taking part in this study.

Availability of data and material

The dataset supporting the conclusions of this article is included within the article and its additional files.

Author contributions

KR performed all laboratory work, data analysis, statistical work and wrote the manuscript draft. MK contributed to sample preparation. LlCP, TR and PK supported with critical data discussion. MS and AT are PIs of the Sorbs cohort. AH and AV are PIs for independent replication cohort. MB is PI for the Adiposity cohort and the German Obesity Biobank. YB initiated, conceived and designed the study, contributed to critical data discussion and wrote the final version of the manuscript. All authors contributed to the final manuscript by proof reading, editing and critical discussing the obtained results.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.050.

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