Genome-wide association study identifies genetic loci associated with fat cell number and overlap with genetic risk loci for type 2 diabetes

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Tweet: Dahlman group at Karolinska Institutet reports common genetic variants in four genes of potential importance in controlling fat cell numbers, the size of body fat and diabetes risk.@RJStrawbridge and @alishaaman001

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Abstract

Interindividual differences in generation of new fat cells determine body fat and type 2 diabetes risk. We utilized the GENiAL cohort, which consists of participants who have undergone abdominal adipose biopsy, to perform a genome-wide association study (GWAS) of fat cell number (n=896). Candidate genes from the genetic study were knocked down by siRNA in human adipose derived stem cells. We report 318 SNPs and 17 genetic loci displaying suggestive ($p<1x10^{-5}$) association with fat cell number. Two loci pass threshold for GWAS-significance, on chromosome 2 (lead SNP rs149660479-G) and 7 (rs147389390-deletion). We filtered for fat cell number-associated SNPs ($p<1.00x10^{-5}$) using evidence of genotype-specific expression. Where this was observed we selected genes for follow-up investigation and hereby identified *SPATS2L* and *KCTD18* as regulators of cell proliferation consistent with the genetic data. Furthermore, 30 reported type 2 diabetes-associated SNPs displayed nominal and consistent associations with fat cell number. Functional follow up of candidate genes identified *RPL8*, *HSD17B12* and *PEPD* displaying effects on cell proliferation consistent with genetic association and gene expression findings. In conclusion findings presented herein identify *SPATS2L*, *KCTD18*, *RPL8*, *HSD17B12*, and *PEPD* of potential importance in controlling fat cell numbers (plasticity), the size of body fat and diabetes risk.

Introduction

The number and size of fat cells determine body fat and are also risk factors for type 2 diabetes as reviewed (1). Early studies demonstrated that the number of fat cells is set in adolescence (2, 3). However, more recent studies have challenged that view. There is a high rate of fat cell turnover in adult human adipose tissue and it is increased recruitment of new fat cells, rather than decreased cell death, that explains increased fat cell number in obesity (4). Furthermore, increased body weight over time, as well as weight regain after the initial drop in body weight following bariatric surgery are indeed associated with elevations in fat cell number (5-7). The plasticity of adipose cellularity has clinical consequences (8). Subjects with obesity have greater ability to increase fat cell size after overfeeding when they are insulin sensitive. Furthermore, size and number of fat cells may have impact on type 2 diabetes as discussed (1). Taken together the studies suggesting that fat cells are generated over the entire life span and that this process has clinical consequences make genetic studies of the regulation of the cell number an important issue.

A number of genes and pathways controlling recruitment of new fat cells from precursor cells cultured *in vitro* have been described but their importance for governing fat cell number and amount of body fat in humans *in vivo* is unclear (7, 9). Family history is a strong risk factor for both overweight/obesity and type 2 diabetes and recent genome wide association studies (GWAS) have identified numerous common genetic variants associated with these traits (10, 11). Interestingly, genetic predisposition to type 2 diabetes, but not obesity, is associated with impaired ability to produce adipocytes in the subcutaneous depot (12). This impairment is associated with enlarged fat cell size. Turnover studies suggest that a state of adipose hypertrophy is attributed to decreased recruitment of fat cells (13). Thus, variations in fat cell number may have impact on the development of type 2 diabetes.

In a recent study we identified a number of loci that are potentially important for fat cell volume in a GWAS (14). In the present study we have used the same unique GENetics of Adipocyte Lipolysis (GENiAL) cohort to perform a GWAS of fat cell number. We also assessed if genetic variants reported to be associated with body mass index (BMI) or type 2 diabetes displayed consistent association with fat cell number. Finally, genetic variants associated with fat cell number and displaying genotype specific gene expression in adipose tissue were taken forward for functional evaluation

Research Design and Methods

Participants

The GENiAL cohort includes 273 men and 718 women and has been described previously (15) and in Supplementary Methods. HOMA-IR as measure of insulin resistance was calculated from fasting levels of glucose and insulin as described (16). Subcutaneous adipose tissue (SAT) was obtained by a needle aspiration biopsy lateral to the umbilicus as described (15). Abdominal fat cell number was assessed in 953 subjects.

Ethics

The study was approved by the local committee on research ethics at Huddinge hospital (Sweden) and explained in detail to each participant. Informed consent was obtained from all participants.

Measurement of fat cell number in GENiAL

The method for determining fat cell number has been described in detail (17). Briefly, isolated fat cells were prepared from SAT by collagenase digestion and subjected to measures of cell

diameter, which was used to calculate the average weight of fat cells. More than ≥95% of the weight of fat cells comprises lipids. The estimated abdominal subcutaneous adipose tissue (ESAT) weight was calculated by a formula based on waist-hip ratio, sex, age, waist circumference and body fat as previously described and validated by dual X-ray absorptiometry (17). Thereafter, fat cell number in the ESAT region was calculated as the weight of ESAT divided by the mean fat cell weight.

Genetic analysis

Genotyping in GENiAL has been described previously (15). After quality control, 896 samples and 9,714,326 SNPs were available for phenotypic analysis. Fat cell number is normally distributed according to visual inspection of data in the cohort. A GWAS of abdominal fat cell number was conducted in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) (15) using linear regression, assuming an additive genetic model, and adjusting for population structure, age, and sex. Genome-wide significance was set at $p < 5.00 \times 10^{-8}$ and suggestive significance was set at $p < 1.00 \times 10^{-5}$. Results were visualized using FUMA (18) and LocusZoom (19). Conditional analyses, where the lead SNP was included as a covariate, was used to assess the number of signals in loci with multiple GWAS-significant SNPs. Sensitivity analysis was conducted, whereby BMI was also included as a covariate.

Mendelian Randomization analysis

Genetic variants associated with fat cell number were used in a Mendelian Randomization (MR) analysis, to examine the possible causal effect of fat cell number on BMI, type 2 diabetes, and waist-hip ratio adjusted for BMI (WHRadjBMI) (see also Online Supplemental Materials and Supplementary Table 1). R package TwoSampleMR (20) was used to perform a

bidirectional MR analysis. For the first analysis, fat cell number was used as an exposure to obtain the causal estimate of its association with outcomes BMI, type 2 diabetes, and WHRadjBMI. For the second analysis BMI, type 2 diabetes, and WHRadjBMI were used as exposures while fat cell number was used as the outcome. Summary statistics were obtained using the IEU GWAS R package (21). MR analyses was conducted using the inverse-variance weighted (IVW) method to obtain the causal estimate of the fat cell number on the respective outcomes (for sensitivity analyses, see online Supplemental Materials).

Data mining

Data from GWAS Catalogue (https://www.ebi.ac.uk/gwas/) were retrieved on March 31, 2020. Evidence of genotype specific gene expression was retrieved from GTEx database (www.gtexportal.org) (22) and were retrieved August 2020. The purpose of the analysis was to determine whether there was evidence that the SNPs have effects on gene expression levels in relevant tissues. All SNP-gene pairs listed in GTEx and reported herein reach FDR <5%. There was no threshold for physical distance. VEP was used to assess predicted functional consequences of SNPs (https://www.ensembl.org/info/docs/tools/vep/index.html, accessed 2021-08-02) (23). The FANTOM 5 dataset (24) was used to assess gene expression in human adipose-derived stem cells (hASCs) undergoing *in vitro* differentiation to adipocytes. Transcripts whose expression were >10 tags per million (TPM) at some point during the time course were defined as detected in hASCs.

Cell culture and transfection with small interfering RNA

hADSCs were isolated from subcutaneous adipose tissue of a male donor (16 years old, BMI 24 kg/m^2) and have been described in detail previously (25). One million proliferating hASCs suspended in 90 µl of R buffer were transfected using 200 pmol ON-TARGETplus

SMARTpool small interfering RNAs (siRNAs) targeting candidate genes or control siRNA #1 (Dharmacon, Lafayette, US) using NEON transfection system (Invitrogen, Carlsbad, CA, US) according to the manufacturer's protocol and previously described (14). Subsequently, cells were plated in antibiotic free medium. This was annotated as day -4. Medium was replaced 24 hours post-transfection and differentiation to adipocytes was started by medium replacement 96 hours post-transfection (day 0) following medium replacement every third day. The RNA and medium were collected at proliferation stage 2 days post-transfection (day -2), at differentiation start (day 0), day 3 and 7 after the induction of differentiation. Product identification numbers for siRNA are summarized in Table 1.

Measurement of cell proliferation rate

hASCs were electroporated at proliferation stage (day -4 before initiation of differentiation). Two days post-transfection (day -2), the cells were incubated with media containing 5 µM 5-ethynyl-20-deoxyuridine (EdU) for 24 h. EdU-positive cells and total cell number (nuclear staining) were assessed using the Click-iT®Plus EdU Alexa Fluor555 kit (C10352, Invitrogen, Carlsbad, CA) according to manufacturer protocols. Rate of proliferation (EdU positive cells) was normalized to the number of nuclei representing number of cells in each well. Quantification of cells were performed using CellInsight™CX5 High Content Screening (HCS) Platform (Thermo Fischer Scientific, Waltham, MA, US) with integrated "Object detection" protocol.

Measurement lipid accumulation

Lipids were stained and quantified at differentiation day 7, e.g. 11 days post-transfection using Bodipy 493/503 (0.2 µg/mL; Molecular Probes, Thermo Fisher Scientific, Waltham, MA, US) as previously described (15).

RNA isolation and analysis of gene expression

NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) and the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, US) were used to prepare RNA and synthesize cDNA. RNA concentration and quality were measured using Varioskan™ LUX multimode microplate reader and µDrop plate (ThermoFisher Scientific). Quantitative RT-PCR was performed using 1 ng of cDNA and SybrGreen primer pairs (Sigma Aldrich) in 10 µL reaction on the QuantStudio Real-Time PCR maschine (Thermo Fisher Scientific). Relative gene expression was calculated using the $2^{-\Delta Ct}$ method (26). Primer pairs are listed in Table 1.

Data and resource availability

Summary statistics from the GWAS analysis and experimental data are available upon request from I.D.

Statistical analysis

Clinical data were analyzed by multiple regression in JMP v 14. Data from *in vitro* experiments were analyzed by t-test. All p-values are two-tailed. When specific hypotheses are tested, a nominal $p<0.05$ is used as the significance threshold. Se = standard error.

Results

Characteristics of the GENiAL cohort has been reported (15). Obesity was positively associated with abdominal subcutaneous fat cell number in the cohort (R^2 0.40, P <0.001), whereas insulin resistance as assessed by HOMA-IR, was inversely correlated with fat cell number in multiple regression adjusting for BMI of study participants (partial R^2 0.057, P <0.001).

GWAS of abdominal cell number

In a GWAS we identified 44 SNPs in nine genetic loci (as defined by FUMA (18)) which passed threshold for GWAS-significance for association with fat cell number ($p < 5.00 \times 10^{-8}$, Figure 1A-B, Supplementary Figure 1, Supplementary Table 2). Conditional analyses, using the lead or a proxy where FUMA lacks information on the lead SNP as a covariate, provided no evidence for additional signals in any loci (Supplementary Table 2). A further 337 SNPs demonstrated suggestive associations ($p < 1 \times 10^{-5}$) with fat cell number (Supplementary Table 3). Very few genetic signals for complex traits contain only one or two SNPs, instead most contain a large number of SNPs albeit at different levels of association due to being co-inherited to varying degrees with the lead SNP. Therefore, to prioritize loci for functional follow-up, we focused on loci with >3 SNPs associated with fat cell number according to visual inspection in Locus Zoom and, for SNPs displaying suggestive association with fat cell number, MAF >2%. The analysis resulted in 17 genetic loci associated with fat cell number, of which two loci pass threshold for GWAS-significance, on chromosomes 2 (lead SNP rs149660479-G, Beta 0.183, Se 0.029, $P = 8.71 \times 10^{-10}$, Figure 1C, Supplementary Table 4) and 7 (rs147389390-deletion, Beta 0.113, Se 0.018, $P = 1.49 \times 10^{-9}$, Figure 1D-E, Supplementary Table 4). The locus on chromosome 2 overlaps *SPTBN1,* and the locus on chromosome 7 *C1GALT1* and *LOC101927354*. Fat cell number associated SNPs in *SPTBN1* and *C1GALT1* are located within introns or 3'UTR regions of these genes. No SNP in Supplementary Table 4 has been reported to be associated with clinical traits according to GWAS catalogue.

Sensitivity analyses including BMI as an additional covariate provided similar results (Supplementary Figure 2A), however the QQ-plot suggests that adding BMI as a covariate introduces inflation of test statistics (Supplementary Figure 2B). We believe this inflation is

due to the skewed distribution of BMI in the cohort (Supplementary Figure 2C). The SNPs with suggestive evidence of association in the original analyses demonstrated similar effect sizes in both analyses (Spearman's rank correlation coefficient=0.93, Supplementary Figure 2D).

MR analysis

Bi-directional MR analysis was conducted to assess direction of possible causal relationships between fat cell number and BMI, WHRadjBMI, or type 2 diabetes (Online Supplemental Materials). A total of 9 instrumental variables (Supplementary Table 5) were used for fat cell number. Fat cell number showed no significant causal association with BMI using the IVW method (OR 0.98, CI 95% 0.93-1.03, p=0.54). Similar estimates were obtained while accounting for uncorrelated horizontal pleiotropy with MR Egger (OR 0.92, CI 95% 0.73-1.151, p=0.52) and accounting for correlated horizontal pleiotropy using MR weighted median (OR 0.97, CI 95% 0.92-1.03, p=0.47) or weighted mode (OR 0.97, CI 95% 0.89-1.05, p=0.49). To check if BMI instead leads to a difference in fat cell number, MR was conducted using BMI as the exposure and fat cell number as the outcome. There was no significant association of the effect of BMI on fat cell number (Supplementary Table 6). Similarly, the was no significant casual association between fat cell number, WHRadjBMI and type 2 diabetes (Online Supplemental Materials, Supplementary Table 5 and 6). MR Steiger estimated the direction of causality to be fat cell number leading to a difference in BMI (Steiger $p=7.23x10^{-74}$), WHRadjBMI (Steiger $P=1.21x10^{-8}$) and type 2 diabetes (Steiger $p=1.4x10^{-8}$).

Genotype-specific gene expression patterns

We also explored the fat cell number-associated SNPs ($p<1.00x10^{-5}$) for evidence of genotype-specific gene expression patterns and, where this was observed, we selected the protein-encoding genes for follow-up investigation (Table 2). Selected genes were in the same linkage disequilibrium block as the SNP according to Locus Zoom. Of note, of the SNPs in Table 2, inclusion of BMI as a covariate had limited impact on the association effect size; maximum change was for rs141481897: $Beta_{original}$=0.119, $Beta_{BMI}$=0.095. The protein-encoding genes also had to be expressed in our hASCs. We identified five genes that were taken forward, i.e. *SPATS2L, KCTD18, MAST4, FAH* and *HEATR3.* These genes were knocked down by siRNA in proliferating hASCs. For three genes knock-down efficiency varied between 90-70 % whereas for *HEATR3* knock-down efficiency of 50 to 70 % was observed (Figure 2a). We could not achieve satisfactory knockdown of *MAST4* and this gene was therefore not taken forward for further functional studies (data not shown). Knock-down of two genes, *SPATS2L* and *KCTD18*, decreased number of proliferating cells. The other investigated genes had no impact on proliferation (Figure 2b). The impact of *SPATS2L* and *KCTD18* on proliferation was not associated with altered accumulation of lipids as measure of adipogenesis (Supplementary Figure 3). Interestingly, among fat cell number associated SNPs, rs565245989 in the *KCTD18* locus is the only one which comprises a frameshift mutation and is predicted by VEP to have a deleterious effect on gene function (Supplementary table 3).

Genetics of abdominal fat cell number in susceptibility to type 2 diabetes

We next assessed the importance of impaired fat cell number for genetic predisposition to type 2 diabetes by overlapping GWAS results for the two traits. SNPs associated with type 2 diabetes or related traits were retrieved from GWAS catalogue (n=1,569) and the more recent paper by Vujkovic et al (n=174) (27); 1382 of these SNPs were assayed in our analysis of fat

cell number. 48 of the type 2 diabetes SNPs displayed nominal association with fat cell number of which 30 SNPs showed consistent association i.e. the type 2 diabetes risk allele was associated with lower fat cell number (Table 3). Eleven risk alleles for type 2 diabetes were associated with larger fat cell number whereas for seven SNPs risk allele could not be determined in GWAS catalogue. 16 of the 30 consistent type 2 diabetes – fat cell number SNPs demonstrated expression quantitative loci (eQTLs) in adipose tissue according to GTEx (Table 3); 13 eQTL genes were expressed in our hASCs and were therefore taken forward for functional evaluation by siRNA-mediated knock down in proliferating hASCs (*SETD2, RPL8, ZNF34, ZNF251, ZNF7, COMMD5, HSD17B12, CYB5D2, ZZEF1, PEPD, MAP1LC3A, STOML1* and *THOC5*). The knockdown efficiency was satisfactory for most studied genes and varied between 90 and 50% (Figure 3a). Knockdown of *ZNF34, ZNF7* and *ZZEF1* were at about 50% at day -2 or at differentiation start (day 0) but less efficient at the end of differentiation. We could not achieve satisfactory knockdown for *ZNF251* and this gene was therefore not taken forward for further functional studies (data not shown). Knock-down of seven genes (*RPL8, ZNF7, HSD17B12, ZZEF1, PEPD, MAP1LC3A* and *THOC5*) decreased number of proliferating cells (Figure 3b). Results for *RPL8, HSD17B12* and *PEPD* were consistent with genetic data e.g. rs2294120-G is associated with higher *RPL8* expression, more fat cells, and protection against type 2 diabetes, and *RPL8* knockdown inhibited fat cell proliferation. The impact of *HSD17B12* and *PEPD* on proliferation was not associated with altered accumulation of lipids as measure of adipogenesis (Supplementary Figure 3). The few cells that survived knockdown of *RPL8* and continue to differentiate developed very aberrant adipocyte and lipid droplets morphology. This precluded quantification of lipids (data is not shown).

Genetics of abdominal fat cell number in susceptibility to obesity

For comparison we also retrieved SNPs associated with BMI (n=3,254) from the GWAS catalogue. 2,987 of these SNPs were assayed in our analysis of fat cell number. 136 of the BMI SNPs displayed nominal association with fat cell number of which 38 SNPs displayed consistent association, i.e. the BMI increasing allele was associated with higher fat cell number (Supplementary table 7).

Effect of candidate genes on markers of cell cycle progression and apoptosis

For the five genes displaying consistent effects according to genetic analysis and knockdown experiments (*SPATS2L*, *KCT18*, *RPL8*, *HSD17B12*, and *PEPD*) we next evaluated potential mechanisms by which these genes influenced fat cell number. We explored the impact of gene knockdown on genes encoding central stimulators of cell cycle progression in hASCs, that is cyclins *CCND1*, *CCND3* and *CCNG2* (28). We also measured *TP53*, which has ability to induce cell cycle arrest and apoptosis; to differentiate between the two cell fates we measured the proapoptotic marker *BAX* in parallel. Knock down of *RPL8* upregulated expression of *CCND1*. In contrast, expression of *CCND3* was first downregulated (day -2) and then upregulated at day 0 (Figure 4a). Knock down *of PEPD* temporally downregulated all three cell cycle markers (Figure 4b). Knockdown of *RPL8* upregulated expression of *TP53* and *BAX* (Figure 4c-d). *PEPD* knockdown reduced *BAX* at day -2 but otherwise had no impact on cell cycle arrest and apoptosis markers. Knockdown of *SPATS2L*, *KCT18*, and *HSD17B12* did not show any effect on the expression of studied cell cycle markers (data not shown). Thus, available data does not support that impact on expression of cycle progression and apoptosis makers is a major mechanism of action of identified candidate genes for fat cell number.

Discussion

The genetic factors that may be in control of fat cell size number and function have predominantly been investigated previously using a candidate gene approach (14). Only two studies using GWAS have been published (14, 15) dealing with fat cell size and lipolysis. In this present study we have, by a large scale GWAS, demonstrated two genome wide significant loci (*SPTBN1, C1GALT1*) associated with abdominal subcutaneous fat cell number. These loci have no such association with fat cell size in the presently investigated cohort (14). We also describe in the present study a region on chromosome 2 reaching the suggestive threshold for which eQTL together with siRNA knockdown experiments support to the notion that *SPATS2L* and *KCTD18* control adipose cell number. Furthermore, observed enrichment for SNP alleles with opposing effects on fat cell number and type 2 diabetes risk is consistent with the notion that an inherited impaired ability to expand adipose cell number predisposes to diabetes. By further eQTL analysis together with knock down experiments we identified shared genetic loci for type 2 diabetes and fat cell number, highlight potential underlaying candidate genes (*RPL8, HSD17B12* and *PEPD*), and demonstrated their involvement in promoting proliferation of adipose precursor cells.

The GWAS significant locus on chromosome 2 harbors the *SPTBN1* gene. *SPTBN1* encodes beta II spectrin, which is important for maintaining the cell structure and act as scaffold for proteins within cells. *SPTBN1* has been implicated in cancer development and cardiovascular disease (29). Furthermore, GWAS have implicated *SPTBN1* in osteoporosis development, possibly by facilitating cell cycle progression (30). Interestingly, adipocytes and bone cells have the same stem cell origin (31). The GWAS significant locus on chromosome 7 harbors *C1GALT1*, which encodes T-synthase and regulates *O*-glycosylation of proteins. *C1GALT1* and glycosylation are essential for normal development and have been implicated in inflammatory disease and oncogenesis (32). However, although *SPTBN1* and *C1GALT1* are

candidate genes in the chromosome 2 and 7 loci, we could not functionally link the fat cell number associated SNPs directly to the function of these genes and we did therefore not take these genes forward for functional follow up.

Among SNPs displaying suggestive association with fat cell number, their role as eQTL together with siRNA knockdown results give support to the notion that *SPATS2L* and *KCTD18* on chromosome 2 control adipose cell number. Consistent with our findings in hASCs, knockdown of *SPATS2L* in myoblast has been reported to induce growth arrest (33). Furthermore, one SNP in *SPATS2L* was associated with fasting plasma glucose in a Chinese GWAS (34). The function of *KCTD18* has to our knowledge not been described (35).

A possible causal link between adipose morphology, i.e. the relationship between fat cell size and number, and type 2 diabetes has so far only been examined epidemiologically and for fat cell volume (36, 37). By overlapping established risk alleles for type 2 diabetes with our GWAS of fat cell number, we revealed an overrepresentation of type 2 diabetes risk alleles that were associated with a lower number of fat cells. In these analyses we used nominal association with fat cell number since we analysed predetermined hypotheses, i.e. SNP with established role in type 2 diabetes development. Bidirectional MR analyses to investigate potential causal relationships suggested that, whilst no significant associations were identified, fat cell number contributes to BMI, WHR and type 2 diabetes, but there is no evidence for the opposite direction of effect.

Our analyses highlighted three genes controlling hASCs proliferation and comprising candidate genes for type 2 diabetes, *RPL8*, *HSD17B12*, and *PEPD*. *RPL8* encodes a ribosomal protein; however, a peptide of RPL8 has previously been reported to stimulate T

cell proliferation (38) suggesting that the gene has functions beyond translation. mRNA measurements of cyclins suggested that *RPL8* could control proliferation by controlling progression through the cell cycle, but possible also by influencing apoptosis. The enzyme encoded by *HSD17B12* control elongation of long chain fatty acids. Knockdown of *HSD17B12* has been reported to either stimulate or inhibit cell proliferation (39). *PEPD* encodes Pepdidase C, which cleaves di- and tripeptides containing carboxyl-terminal proline and is involved in protein degradation. However, as a ligand, *PEPD* can bind directly to the epidermal growth factor receptor and regulate cellular metabolism and cell proliferation through stimulation of PI3K/Akt (40). Our analysis or cell cycle markers suggested that *PEPD* knockdown inhibited cell cycle progression. Thus, all three herein described candidate GWAS candidate genes for fat cell number and type 2 diabetes have been linked to fat cell number by diverse mechanisms. The analysis presented herein complement previous -omics analysis to identify type 2 diabetes GWAS signals with impact in adipose tissue (41, 42).

There are some caveats with the present investigation. Adipocyte number is higher in obesity, which is strongly determined by CNS pathways although peripheral pathways are also important (4, 43, 44). One limitation of the present study is that we focused on local subcutaneous adipose regulation of fat number and ignored CNS effects. The GTEx database used for analysis of eQTLs has poor coverage of specific types of neurons and the functional studies were focused on one adipose region. Therefore, we cannot say if detected GWAS signals have impact on CNS pathways or if similar results are true for other adipose depots such as the visceral. Also GTEx is lacking data on as adipose precursor cells. In the type 2 diabetes analyses we focused functional follow up studies on SNPs displaying opposing effect on at cell number and diabetes risk; this does not exclude that alleles associated with more fat cells and increased diabetes risk could be important. We used nominal association as

threshold is analysis of type 2 diabetes SNPs which increases the risk of false positive findings. Another limitation is that siRNA experiments assess the function of genes and not the role of specific genetic variants. Although a large number of subjects were investigated there is no independent confirmation of the findings.

To conclude the findings presented herein identify *SPATS2L, KCTD18, RPL8, HSD17B12,* and *PEPD* of potential importance in controlling fat cell numbers (plasticity), the size of body fat and diabetes risk.

Acknowledgments

Author contribution: I.D. designed the study, analyzed data, and wrote the manuscript. A.K. designed and performed experiments, analyzed data, and wrote the manuscript together with I.D.. A.A., R.S. and P.A. analyzed data and reviewed/edited the manuscript. I.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest No potential conflicts of interest relevant to this article were reported.

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The data presented herein have not been submitted or presented elsewhere.

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Table 1. siRNA and primers used in the study

Gene	siRNA *	Primer (sequence 5'-3') †	
		Forward	Reverse
COMMD5	L-015390-02	CCCTGTACATTTGTCTTTGG	AAGGCAGAAGAGTGAAAATG
CYB5D2	L-004706-02	ATATAGATGTTGAGGCCGAG	AATTGTGAAGTGTCAGCATC
FAH	L-009635-00	AGTCATCATAACAGGGTACTG	CCAGAAGAGCAGAGAAAATC
HEATR3	L-020982-02	GGCTGAGGAAATATTAGAGAAC	GGCGGAAGTAAATCTGAAAC
HSD17B12	L-008474-02	TGCAACCAAGACTTTTGTAG	CGACTGTTTTAATTGCAGAC
KCTD18	L-016962-02	AGAAGAGGTGCTAGATGTTC	CCTGACTCATCTGTTTTTAGAG
MAP1LC3A	L-013579-00	AGAAAGGATTTTGAGGAGGG	TTCATCTGCAAAACTGAGAC
MAST4	L-031608-00	AAGAGTGGGAATAAGGTGT	CTTATAGCTGTTTCTCCTGG
PEPD	L-005990-02	CAGTACGTAGATGAGATTGC	CCATATCCGTCTTAAACACTC
RPL8	L-013721-02	AGAAGGTTATCTCCTCAGC	AAGATGGGTTTGTCAATTCG
SETD2	L-012448-00	AGAACAGCCAGATAAAACAG	CTCCTTTAGGTCTTTCCAAC
SPATS2L	L-020248-01	CAGAAGAACTAAAGAGACTCAC	CAAAGTGCTTAATTTCTGCC
STOML1	L-009360-01	CTGCTACCAGTTCAATGTC	CTTCCTCGTCCTGTAGTG
THOC5	L-015317-01	GGCAATAGAAATAGAAGAACGG	TTGGTGATCTCCTTCTGTAG
ZNF251	L-025858-01	AAGTAGACACCAGAGAAGTC	AACAAGATTTGAGCTGTGAG
ZNF34	L018566-02	AGTCTCACTGGGAGTAGG	CAAATGTCTCCTGTGAAGTC
ZNF7	L-019776-00	GACAGATTCTACGATTAGGAC	GAATCAGAAACGTCTCCAAAG
ZZEF1	L-031841-02	AGAGGTAGAACTGACTCTTC	GGTTTACCATATTTGAGGAGC
Non-targeting siRNA			
#1	D-001810-01	not relevant	not relevant
CCND1	not used	GCCTCTAAGATGAAGGAGAC	CCATTTGCAGCAGCTC
CCND3	not used	CTGTGATTGCACATGATTTC	GGCAAAGGTATAATCTGTAGC
CCNG2	not used	GATGAAAGTGAAAGTGAGGAC	TTCTAAGATGGAAAGCACAG
TP53	not used	TTCCCTGGATTGGCAG	TCAAATCATCCATTGCTTGG
BAX	not used	AACTGGACAGTAACATGGAG	TTGCTGGCAAAGTAGAAAAG

* On-Target Plus Human Smart pool (Horizon)
† Pre-designed oligos (Sigma Aldrich)

Table 2. Genetic loci associated abdominal fat cell number and harboring eQTL*

CHROM	POS	ID	REF	ALT	A1	A1_FREQ	BETA	L95	U95	P	eQTL
1	47080679	rs6671527	A	G	G	0.491	-0.028	-0.040	-0.016	7.66E-06	*LURAP* in SAT
2	54911968	rs75654812	C	T	C	0.059	0.071	0.046	0.097	8.06E-08	*PRORSD1P* in other organs
2	201020141	rs141481897	C	T	C	0.016	0.119	0.071	0.167	1.15E-06	*SATB2-AS1* in ileum
2	201119367	rs115947566	A	G	A	0.031	0.077	0.044	0.111	6.97E-06	*SPATS2L* in skin
2	201354937	rs565245989	D	I	D	0.009	0.154	0.088	0.219	4.61E-06	*KCTD18* SAT
5	66306930	rs537382	A	G	G	0.124	0.042	0.024	0.060	6.70E-06	*MAST-AS1* SAT, *MAST4* muscle
5	145491112	rs151111196	C	T	C	0.018	0.119	0.067	0.171	7.09E-06	*LARS* SAT
6	109636621	rs76809124	G	A	G	0.027	0.085	0.048	0.121	6.13E-06	*CCDC162P* SAT
7	136866494	rs147782262	A	G	A	0.042	0.070	0.039	0.101	9.94E-06	*PTN* esofagus
15	80519402	rs764503	G	A	G	0.302	0.031	0.017	0.044	7.17E-06	*FAH* SAT , *LINC01314* other organs
16	50203171	rs9302747	A	G	G	0.022	0.096	0.057	0.135	1.99E-06	*HEATR3* lung

* SNPs associated with fat cell number with $p<10^{-5}$ after filtering for genetic loci with >3 SNPs associated with fat cell number according to visual inspection in Locus Zoom and, for SNPs displaying suggestive but not GWAS significant association with fat cell number, MAF >2%. Beta is calculated for allelele A1. SAT = subcutaneous adipose tissue; eQTL = expression quantitative trait locus

Table 3. Type 2 diabetes SNPs from GWAS associated with abdominal subcutaneous fat cell number*

							Published GWAS of type 2 diabetes				GTEx¶	
Assoc †	**Chrom**	**ID**	**A1**	**A1 freq**	**beta A1**	**P**	**Pubmed‡**	**Risk allele§**		**OR or beta**		**Gene#**	**High**
No	2	rs12463719	A	0.273	0.016	3.E-02	Vujkovic	G	0.72	-0.03	D		
Yes	3	rs2688419	C	0.46	0.016	7.E-03	26818947	T	0.65	1.07			
Yes	3	rs11926707	T	0.36	0.015	2.E-02	30054458	T	0.37	0.05	D	*PTH1R*	T
												PRSS45	T
												SETD2	C
No	3	rs831571	T	0.18	-0.025	2.E-03	22158537	C	0.61	1.09			
Yes	3	rs844215	T	0.374	0.013	4.E-02	Vujkovic	C	0.59	0.03	I		
Yes	3	rs2063640	A	0.12	-0.022	3.E-02	21490949	A	0.17	1.23			
	4	rs17447640	G	0.11	0.021	3.E-02	28060188		NR				
Yes	6	rs9379084	A	0.10	0.032	4.E-03	29632382	G	0.89	1.09			
	7	rs11298745	I	0.07	0.026	4.E-02	28060188		NR				
Yes	7	rs10231619	T	0.16	-0.023	9.E-03	25102180	T	0.74	1.13			
Yes	7	rs2299383	T	0.46	0.014	3.E-02	30054458	T	0.42	0.04	I		
Yes	7	rs10229583	A	0.28	0.013	5.E-02	23532257	G	0.83	1.14			
Yes	8	rs1561927	C	0.26	-0.018	1.E-02	24509480	C		1.06			
Yes	8	rs2294120	G	0.46	0.012	4.E-02	30054458	G	0.46	0.04	D	*RPL8*	G
												ZNF34	A
												ZNF251	A
												ZNF517	G

												ZNF7	A
												COMMD5	G
	9	rs10973627	G	0.12	-0.023	2.E-02	28060188		NR				
No	9	rs17791483	G	0.08	-0.024	4.E-02	30054458	G	0.06	0.10	D		
No	9	rs13292262	C	0.08	-0.023	4.E-02	26818947	T	NR	1.17			
No	9	rs13292136	T	0.08	-0.026	2.E-02	20581827	C		1.11			
Yes	10	rs1955163	A	0.10	0.031	5.E-03	30718926	G	0.51	1.05			
No	10	rs2812533	T	0.16	-0.026	3.E-03	24509480	C		1.07			
Yes	11	rs8181588	C	0.06	0.039	3.E-03	24101674	A	0.48	1.30			
Yes	11	rs163177	T	0.46	0.013	4.E-02	28406950	C	0.43	1.21			
Yes	11	rs2237892	T	0.07	0.042	5.E-04	30054458	T	0.06	0.10	D	*AC108448.2*	C
Yes	11	rs2283228	C	0.09	0.037	1.E-03	25102180	A	0.89	1.19		*AC108448.2*	A
Yes	11	rs10769936	T	0.254	0.017	2.E-02	Vujkovic	C	0.71	0.04	I	TRIM66	C
												SCUBE2	T
Yes	11	rs1061810	A	0.28	-0.014	4.E-02	28566273	A	0.28	1.07		*HSD17B12*	C
												RP11-613D13.5	C
Yes	11	rs7113297	T	0.35	-0.015	3.E-02	29358691	T	NR	1.11			
Yes	11	rs10830963	G	0.30	-0.014	4.E-02	24509480	G	0.27	1.11			
Yes	12	rs11063069	G	0.19	-0.020	2.E-02	22885922	G	0.21	1.08			
Yes	12	rs7132908	A	0.42	-0.01	4.E-02	Vujkovic	G	0.61	-0.03	D		
	13	rs7988007	C	0.19	-0.018	2.E-02	30595370		NR				
No	14	rs730570	G	0.18	0.018	3.E-02	21573907	G	NR	1.14			
	15	rs34715063	C	0.12	-0.027	5.E-03	30595370		NR				
Yes	15	rs12917449	C	0.205	-0.02	3.E-02	Vujkovic	C	0.2	0.04	I	*STOML1*	A
No	17	rs623323	T	0.14	0.027	2.E-02	23300278	T	0.15	1.28			
Yes	17	rs781831	C	0.38	-0.019	3.E-03	29632382	C	0.42	1.04		*CYB5D2*	C

												ZZEF1	C
Yes	17	rs781852	G	0.36	-0.018	6.E-03	29632382	G	0.39	1.05		*CYB5D2*	G
												ATP2A3	G
												ZZEF1	G
Yes	17	rs8068804	A	0.30	-0.015	3.E-02	30054458	A	0.33	0.06	I	*CYB5D2*	A
												ZZEF1	A
No	17	rs1656794	A	0.285	-0.02	3.E-02	Vujkovic	G	0.72	0.03	I		
	19	rs10406327	G	0.50	-0.014	3.E-02	30718926	C	0.53	1.04		*PEPD*	C**
No	20	rs6515236	C	0.25	-0.016	3.E-02	30054458	C	0.25	0.05	D		
	20	rs7274168	C	0.41	0.022	5.E-04	30595370		NR			*CHMP4B*	C
Yes	20	rs6059662	A	0.29	0.016	2.E-02	30054458	A	0.34	0.04	D	*RP5-1125A11.7*	A
												MAP1LC3A	G
Yes	20	rs6017317	G	0.23	-0.019	7.E-03	22158537	G	0.48	1.09			
Yes	20	rs4812829	A	0.20	-0.018	2.E-02	24509480	A	0.16	1.07		*OSER1*	A
Yes	20	rs16988991	A	0.20	-0.018	1.E-02	30718926	A	0.45	1.05		*OSER1*	A
No	20	rs1800961	T	0.06	0.039	4.E-03	29632382	T	0.04	1.09			
Yes	22	rs75401573	T	0.062	0.028	3.E-02	Vujkovic	C	0.92	0.05	I	*THOC5*	T

* SNPs associated with type 2 diabetes in GWAS catalogue were overlapped with SNPs nominally associated with abdominal fat cell number. Consistent association Yes = allele associated with increased risk of type 2 diabetes is associated with lower number of abdominal fat cells.
† Consistent association between fat cell number and type 2 diabetes. Empty cell = excluded due to unclear type 2 diabetes-risk allele.
‡ If more than one study has reported a specific SNP we have filtered for publications with alleles reported and for studies with large sample size.
§ The cell is left empty if no risk allele is reported in GWAS catalogue.
¶ Only SNPs with consistent results for fat cell number and type 2 diabetes risk have been chosen for eQTL analysis.
# ZZEF1 comprises an eQTL in VAT, all other listed genes comprise eQTL in SAT.

Figure Legends

Figure 1. QQ and Manhattan plots for adipose fat cell number.

a. Manhattan plot: SNPs are aligned along the X axis by their position on the chromosome, and on the Y axis by their association with fat cell number. The horizontal red line represents the threshold for GWAS significance ($p < 5x10^{-8}$). b. The QQ plot demonstrates the SNPs (grey dots) aligned by their observed P value (Y axis) in comparison to their expected P values (X axis). The red dotted (diagonal) line indicates the null distribution. c-e. Regional plots show a zoomed-in version of the Manhattan plot for the GWAS-significant loci on chromosome 2, and on chromosome 7. Linkage disequilibrium information is not available in LocusZoom for rs147389390, whereas this is available for another SNP in the chromosome 7 locus with very similar p-value.

Figure 2. Effects of siRNA-mediated knock-down of candidate genes for fat cell number on human adipose-derived stem cell proliferation. hASCs were transfected with control siRNA oligonucleotide (siNegC) or siRNAs targeting *SPATS2L, KCTD18, FAH* and *HEATR3* in proliferating cells 4 days prior induction of differentiation. a. RNA samples for the evaluation of knockdown were collected at day -2, day 0, day 3 and 7 and gene expression was monitored by quantitative RT-PCR. Relative gene expression was normalized to the reference gene *18s*. Results are based on three biological/independent experiments and were analyzed using t-test and presented as fold change ± SD relative to control siRNA (NegC) of corresponding time point. b. Two days post transfection the cells were treated with EdU for 24 h. Three days post transfection number of proliferating cells were evaluated. Results are based on three biological/independent experiments and were analyzed using t-test and presented in fold change ± SD relative to negative control NegC.

***P < 0.005, **P < 0.01, *P < 0.05.

Figure 3. Effects of siRNA-mediated knock-down of shared candidate genes for type 2 diabetes and fat cell number on human adipose-derived stem cell proliferation. hASCs were transfected with control siRNA oligonucleotide (siNegC) or siRNAs targeting *SETD2, RPL8, ZNF34, ZNF7, COMMD5, HSD17B12, CYB5D2, ZZEF1, PEPD, MAP1LC3A, STOML1* and *THOC5* in proliferating cells 4 days prior induction of differentiation. a. RNA samples for evaluation of knockdown were collected at day -2, day 0, day 3 and 7 and the gene expression was monitored by quantitative RT-PCR. Relative gene expression was normalized to the reference gene *18s*. Results are based on three biological/independent experiments and were analyzed using t-test and presented as average ± SD of fold change relative to control siRNA (NegC) of corresponding time point. b. Two days post transfection the cells were treated with EdU for 24 h. Three days post transfection number of proliferating cells were evaluated. Results are based on three biological/independent experiments and were analyzed using t-test and presented in fold change ± SD relative to NegC. ***P < 0.005, **P < 0.01, *P < 0.05.

Figure 4. Effects of RNA interference-mediated knock-down of candidate genes on proliferation and apoptosis markers in human adipose-derived stem cells. hASCs were transfected with control (siNegC) or target gene siRNA. Expression of *RPL8* and *PEPD* were knocked down in proliferating cells 4 days prior induction of differentiation. Samples were collected at day -2, day 0, day 3 and 7 when the expression of *CCND1*, *CCN3*, *CCNG2* (a-b), *TP53* and *BAX* (c-d) was monitored. Relative gene expression was normalized to the reference gene *18s*. Results are based on three biological/independent experiments and were analyzed using t-test and presented as fold change ± SD relative to NegC of corresponding time point. ***P < 0.005, **P < 0.01, *P < 0.05.