CDKN2C expression in adipose tissue is reduced in type II diabetes and central obesity: impact on adipocyte differentiation and lipid storage?

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CDKN2C/p18 (Cyclin-Dependent Kinase Inhibitor 2C) is a cell growth regulator that controls cell cycle progression and has previously been associated with increased risk for type II diabetes (T2D) and reduced peripheral adipose tissue (AT) storage capacity. This study explored the role of CDKN2C in AT lipid and glucose metabolism in T2D. Expression of *CDKN2C* and other genes was analyzed by transcriptomics, or real-time PCR in subcutaneous AT (SAT) samples obtained from T2D and control subjects matched for sex, age and BMI and also in paired SAT and omental AT (OAT) samples. Functional studies included adipocyte glucose uptake and lipolysis rates. CRISPR/Cas9 *CDKN2C* gene knockdown was performed in human preadipocytes to assess adipogenesis. *CDKN2C* mRNA expression in SAT and OAT was reduced in T2D and obese subjects compared to controls. *CDKN2C* expression in SAT was inversely correlated with measures of hyperglycemia, insulin resistance and visceral adiposity and positively correlated with expression of genes in several metabolic pathways, including insulin signaling and fatty acid and carbohydrate metabolism. CDKN2C protein was mainly expressed in adipocytes compared to stromal vascular cells, and its gene and protein expression was upregulated during adipocyte differentiation. Knockdown of CDKN2C did not affect the percentage of differentiating cells compared to wild type cultures. However, CDKN2C knockdown cultures had significantly lower expression of differentiation markers *CEBPA, ADIPOQ* and *FASN* and transiently reduced lipid accumulation per adipocyte during differentiation. Our findings suggest that adipose CDKN2C expression might be reduced as a consequence of insulin resistance and obesity, and this can further contribute to impairment of SAT lipid storage. (Translational Research 2022; 242:105–121)

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Abbreviations: ADIPOQ=Adiponectin; AT=Adipose tissue; CDKN2C=Cyclin-Dependent Kinase Inhibitor 2C; CEBPA=CCAAT Enhancer Binding Protein Alpha; FASN=Fatty Acid Synthase; GWAS=Genome-wide association study; MRI=Magnetic resonance imaging; OAT=Omental adipose tissue; PPARG=Peroxisome Proliferator-Activated Receptor Gamma; RNP=Ribonucleoprotein; SAT=Subcutaneous adipose tissue; sgRNA=Single-guide RNA; SVF=Stromal vascular fraction; T2D=Type II diabetes; VAT=Visceral adipose tissue; WHR=Waist-hip ratio

At A Glance Commentary

Pereira MJ, et al.

Background

CDKN2C is a cyclin-dependent kinase inhibitor that controls cell cycle progression and has been associated with impaired peripheral adipose tissue storage capacity.

Translational Significance

We found that expression of *CDKN2C* in adipose tissue is reduced in subjects with T2D and obesity, and its expression is inversely associated with markers of insulin resistance and visceral adiposity. Knockdown of CDKN2C in human preadipocytes with CRISPR/Cas9 demonstrated that CDKN2C is not essential for adipocyte proliferation or differentiation. However, loss of CDKN2C transiently reduces lipid accumulation per adipocyte during differentiation and reduces expression of genes regulating adipocyte function. This suggests that downregulation of CDKN2C in T2D and obesity might have implications for reduced lipid storage in peripheral adipose tissue.

BACKGROUND

Obesity is associated with insulin resistance and is an important risk factor for the development of type II diabetes (T2D). Adipose tissue expands by a combination of increased adipocyte number (hyperplasia) or adipocyte size (hypertrophy).[1] Although the metabolic effects of obesity are well known, there are individual differences in metabolic response to obesity. Increased adipocyte size is associated with metabolic impairments and adipose tissue dysfunction.[2] Furthermore, independent of total body fat, increased visceral fat depots with increased waist-hip-ratio (WHR) are associated with insulin resistance, T2D, and cardiovascular disease, including hypertension, and coronary heart disease.[3-5] Therefore, recruitment of precursor cells and differentiation of new adipocytes (adipogenesis) in the subcutaneous adipose tissue (SAT), rather than enlargement of existing adipocytes, would protect against obesity-associated metabolic complications.[6] For example, Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) agonists that promote adipocyte differentiation can normalize metabolic dysfunctions and reduce insulin resistance.[7]

We have previously reported that impaired SAT lipid storage, but not altered lipolysis, contributes to increased fatty acid levels in T2D.[8] Similarly, others have shown that reduced adipose tissue lipid storage capacity contributes to higher circulating lipids, which can accumulate in other organs, including the heart and liver and contribute to the development of insulin resistance and cardiovascular disease.[9,10] However, the mechanisms linking impaired peripheral adipose tissue capacity, and cardiometabolic diseases are not fully elucidated. Therefore, it is clinically relevant to find novel mechanisms that might reduce subcutaneous lipid storage capacity.

A previous genome-wide association study (GWAS) identified novel genomic regions associated with insulin resistance phenotypes and lower peripheral adipose tissue mass.[11] Cyclin-Dependent Kinase Inhibitor 2C (CDKN2C), one of the identified putative effector genes, also known as p18 and INK4C, is a member of the INK4 family of cyclin-dependent kinase inhibitors.[12] CDKN2C inhibits cyclin-dependent kinase 4 and 6, thus functioning as a cell growth regulator that controls cell cycle G1 phase progression. CDKN2C has previously been shown to inhibit cell cycle during adipocyte differentiation.[13-15] Differentiation of adipocytes is dependent on the proliferation of preadipocytes in the early stage and adipogenic differentiation in the later stage.[16] Although both are involved in adipogenesis, they oppose each other because the former requires cell cycle activity, whereas the latter requires cell cycle withdrawal. Therefore, appropriate cell cycle regulation is critical for adipogenesis, and it is reasonable to assume that molecules involved in the control of adipocyte proliferation, such as CDKN2C, play a role in the dysregulation of the adipose tissue functions found in obesity, and T2D.

This study aims to explore CDKN2C as a possible mediator for impaired peripheral adipose tissue storage capacity in obesity and T2D and functionally assess the role of CDKN2C in human adipocyte metabolism using CRISPR/Cas9 gene editing.

Table I. Clinical characteristics of the subjects

Variable	Cohort 1*			Cohort 2†	Cohort 3‡
	Control	Type II diabetes	*P*		
Men/Women (number)	10/10	10/10	-	18/50	10/17
Age, y	58 ± 11	58 ± 9	0.698	47 ± 19	49 ± 13
BMI (kg/m^2)	30.8 ± 4.6	30.7 ± 4.9	0.820	25.9 ± 2.8	37.8 ± 13.3
Waist-hip-ratio	0.96 ± 0.07	0.99 ± 0.05	0.201	0.88 ± 0.08	0.95 ± 0.11
Plasma glucose (mmol/L)	6.0 ± 0.7	8.2 ± 1.5	<0.001	5.7 ± 0.8	5.6 ± 0.7
Serum insulin (mU/L)	11.5 ± 5.2	15.5 ± 7.0	0.040	8.4 ± 3.8	16.9 ± 10.6
Serum C-Peptide (nmol/L)	0.93 ± 0.35	1.06 ± 0.33	0.157	0.68 ± 0.21	1.54 ± 0.53
HbA1C, IFCC (mmol/mol)	37.3 ± 3.7	48.8 ± 8.6	<0.001	34.6 ± 4.1	35.6 ± 3.8
Plasma total cholesterol (mmol/L)	5.69 ± 1.23	4.92 ± 0.85	0.081	5.01 ± 0.97	4.91 ± 1.07
Plasma HDL-cholesterol (mmol/L)	1.30 ± 0.26	1.15 ± 0.24	0.046	1.50 ± 0.37	1.24 ± 0.49
Plasma LDL-cholesterol (mmol/L)	3.63 ± 1.08	3.14 ± 0.78	0.221	3.02 ± 0.85	3.10 ± 0.89
Plasma triglycerides (mmol/L)	1.59 ± 0.63	1.57 ± 0.58	0.947	1.06 ± 0.45	1.47 ± 0.76
Plasma FFA (μmol/L)	293 ± 57	333 ± 80	0.114		
HOMA-IR	3.07 ± 1.58	5.27 ± 2.86	0.004	2.19 ± 1.29	4.44 ± 3.24
Matsuda index	76.6 ± 39.3	53.3 ± 28.8	0.028		
Subcutaneous adipocyte diameter (μm)	108.6 ± 9.6	106.4 ± 10.8	0.512	102.4 ± 13.7	105.2 ± 15.1
Omental adipocyte diameter (μm)					96.7 ± 18.1

Data are mean ± SD. Differences between groups were measured with the Mann-Whitney U test.
Blood chemistry is fasting.
Matsuda = 10 000/ $\sqrt{\text{fasting glucose x fasting insulin x mean glucose}_{OGTT}\text{x mean insulin}_{OGTT}}$
*Cohort 1: control and T2D subjects, SAT needle biopsy
†Cohort 2: no-diabetes, SAT needle biopsy
‡Cohort 3: paired SAT and OAT surgical biopsy

MATERIAL AND METHODS

Subjects. A *first cohort* (cohort 1) includes 20 non−diabetic controls and 20 T2D subjects' group-wise matched for sex, age, and BMI (10 men and 10 women/group, BMI: 22.4−39.9 Kg/m^2, age: 34−72 years), to study alterations in whole body and adipose tissue lipid and glucose metabolism, as previously reported.[8] The T2D subjects were on a stable dose of metformin for at least 3 months as their only anti−diabetic medication. These subjects performed an oral glucose tolerance test (OGTT) for analyses of plasma glucose, insulin, and free-fatty-acids (FFA) and a whole-body magnetic resonance imaging (MRI) for analyses of body composition, as previously reported.[8]

A *second cohort* (cohort 2) includes non−diabetic subjects (18 men and 50 women, BMI: 20.2−34.5 Kg/m^2, age: 18−72 years). SAT samples were obtained by needle aspiration of the lower part of the abdomen after local dermal anesthesia with lidocaine (Xylocaine, AstraZeneca, Södertälje, Sweden).

A *third cohort* (cohort 3) includes non−diabetic subjects (10 men and 17 women, BMI: 20.9−58.2 Kg/m^2, age: 19−66 years) undergone kidney donation (n = 12) at the Sahlgrenska University Hospital, or bariatric surgery (n = 15) at the Uppsala University Hospital. From these subjects, paired samples of human SAT, and omental adipose tissue (OAT) were obtained.

All subjects were phenotyped with regard to anthropometry and general medical status. Following an overnight fasting (>10h), fasting blood samples were collected to analyze HbA1c, plasma glucose and lipids, and serum insulin and C-peptide at the Department of Clinical Chemistry at the Uppsala University Hospital. The clinical characteristics of the study participants are shown in Table I.

Subjects with T1D, endocrine disorders, cancer, or other major illnesses were excluded, as were those having ongoing medication with systemic glucocorticoids, beta-blockers, and immune-modulating therapies. The study was approved by the Regional Ethics Review Board in Uppsala (Dnr2013/330, Dnr2013-183/494, Dnr2018/385) and Gothenburg (Dnr336-07, T 508-09), and the reported investigations have been carried out following principles endorsed by the Declaration of Helsinki. All participants gave their written informed consent.

One part of the adipose tissue biopsies (cohort 1-3) was snap-frozen in liquid nitrogen and used for RNA-seq and mRNA expression analyses. A segment of the SAT biopsy was used for adipocyte and stromal vascular fraction (SVF) isolation. Isolated mature adipocytes were used for adipocyte size measurements and *ex vivo* analyses of lipolysis and glucose uptake (cohort 1).[8] The SVFs were used for the differentiation of preadipocytes into adipocytes.

SAT obtained from 5 non-diabetic subjects (3 women and 2 men, BMI: 21.6−37.3 kg/m^2, age:

18−45 years) was used for CRISPR/Cas9 gene knockdown of *CDKN2C* in preadipocytes.

MRI. In cohort 1, volumes of abdominal SAT, visceral AT (VAT), and liver fat content were assessed using MRI, as previously described.[8]

Fat cell size, glucose uptake, and lipolysis in isolated adipocytes. Adipocytes and SVF were isolated from SAT as previously reported[8,17] and described in supplementary methods. Preadipocytes from SVF were differentiated into mature adipocytes (n = 8). In addition, adipocytes from cohort 1 were used for the assessment of *ex vivo* lipolysis and glucose uptake[8,18] and adipocyte size,[19] as previously reported.

SNP analyses. For the *CDKN2C* SNPs analyses, we included diabetes and obesity-related traits. The data were assessed using publicly available databases from large-scale GWAS at PhenoScanner V2.[20,21] We obtained a list with 9 traits, and therefore the *P*-values were Bonferroni-corrected with a factor of 9.

Human preadipocyte isolation and differentiation into adipocytes. The human preadipocytes isolated from SAT were differentiated up to 14 days as previously reported[17] and described in supplementary methods. The mRNA expression levels of *CDKN2C, PPARG,* and *CEBPA* (n = 8) were measured on days 0, 2, 4, 8, and 14 post-induction. CDKN2C protein levels (n = 3) were measured on days 0, 7, and 14. Preadipocytes from passage 3 were used for gene-editing experiments, and the experimental setup for these experiments is described below.

CRISPR/Cas9 gene-editing. ***CDKN2C knockdown of preadipocytes.*** CRISPR/Cas9 gene-editing of the *CDKN2C* gene (n = 5) was performed using a recently published method.[17] In short, single guide RNA (sgRNA) were designed using the Broad Institute online tool.[22] Two guides, CDKN2C G1 and CDKN2C G2 (5′- TTAACATCGAGGATAATGAAggg-3′ and 5-ACCTCTAAGTAGCAGTCTCCtgg-3′ respectively), (guide sequence in capital letters and PAM sequence in small letters), were selected targeting 2 different conserved functional domains, both important for protein-protein interactions, in exon 3, which is common for all 3 isoforms of the protein. A sgRNA targeting the safe harbor locus AAVS1 was used as a negative control (Thermo Fisher). Gene knockdown was achieved by delivering chemically modified *CDKN2C* specific sgRNA and TrueCut Cas9 protein v2 as a ribonucleoprotein (RNP) complex into cells using the Neon Transfection system (all from Thermo Fisher). Transfected cells were cultured in DMEM/Ham's F12 supplemented with 10% FBS without antibiotics for 48 hours, after which 1% PEST, 0.04 mg/mL gentamycin, and 4.125 ng/mL bFGF were added to the medium.

Assessment of CRISPR/Cas9 editing efficiency. For each transfection experiment, cells from wild type, negative control, and *CDKN2C* gene-edited cultures were collected for DNA extraction and subsequent Sanger sequencing to assess editing efficiency. Cells were collected at passages 6−7, and genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). The CRISPR/Cas9 target sequences were PCR amplified using target-specific primers 5′-ATTTGACCCCTTCAAGCCCC-3′ and 5′-TGCCTGCATCAGGCTAACAA-3′. PCR products were Sanger sequenced in both directions using the target-specific primers. Chromatograms were analyzed using online tools.[23] Knockdown efficiencies were also assessed on the protein level by immunocytochemistry and western blot, as described below.

Proliferation assay. During the preadipocyte growth period, proliferation assays were performed daily using Click-iT Plus EdU Imaging Kit (Molecular Probes) as per the manufacturer's protocol, and described in the supplementary methods.

Fluorescent staining for adipogenesis rate. Differentiation rate and lipid content were assessed on days 7 and 14 of differentiation (n = 5). Cells were fixed with 4% formaldehyde (Histolab, Gothenburg, Sweden) and stained with a combination of the fluorescent neutral lipid dye, BODIPY 493/503 (Molecular Probes, OR) and the nucleic stain Hoechst 33342. Cells were imaged using the ImageXpress Pico (Molecular Devices, CA) and the InCellis Fluorescent microscope (Bertin Instruments, Montigny-le-Bretonneux, France). Image acquisition was performed on each well using a 10x magnification, a 6 × 6 square image scan, covering ∼20% of the central area of each well, on the DAPI, FITC, and brightfield channel. Image analyses were performed on the images obtained from the ImageXpress Pico instrument using the CellReporterXpress software. Differentiation rate was calculated as the percentage of lipid-positive cells using the automated cell scoring software function "Cell differentiation" which identifies cells that either contain lipids (positive cells) or that do not contain any lipids (negative cells). Lipid content was measured as the total integrated intensity of BODIPY of lipid-positive cells per number of lipid-positive cells. Differentiation rate is a measure of how many cells in each culture became adipocytes, and lipid content is a measure of how much lipids the cells that did become adipocytes contain.

Immunocytochemistry. Immunocytochemistry was performed on day 14 of differentiation as previously described (n = 3).[17] To assess the CDKN2C protein expression in differentiated cells, formaldehyde-fixed cells were incubated with a primary antibody solution (anti−CDKN2C/p18/INK4C primary monoclonal

antibody (1:200, ab192239, Abcam), 10% normal goat serum (Sigma), and 0.1% Triton X-100 (Sigma) overnight at 4˚C followed by incubation with the secondary antibody Alexa Fluor 647 (anti−rabbit IgG [1:1000; Molecular Probes]) for 2 hours at room temperature. Imaging of the cells was done using the ImageXpress Pico automated cell imaging system, and the CellReporterXpress software was used for image analyses. The same image acquisition settings were used as described in the previous section, except the Cy5 channel for images of the Alexa Fluor 647 that was used to detect the CDKN2C antibody. Images were analysed using the software function "Cell scoring" which identifies cells that either contain CDKN2C (positive cells) or not (negative cells). CDKN2C knockdown levels were measured as the percentage of CDKN2C positive cells and compared between wild type, negative control, and gene edited cells.

Glucose uptake on differentiated adipocytes. Glucose uptake was performed on days 13−14 of the differentiation (n = 4) in wild type, negative control and edited cells using the luminescence Glucose Uptake-GLO kit (Promega, Madison, WI) according to the manufacturer's instructions. In brief, cells were washed 2 times with warmed PBS and then incubated for 2 hours in Krebs Ringer HEPES (KRH) buffer containing 0.01% BSA (Sigma), with 5 mM glucose (Sigma), 200 nM adenosine (Sigma), and pH 7.4. Afterwards, cells were washed with KRH without glucose 2 times. To assess basal and insulin-stimulated glucose uptake, cells were incubated at 37˚C for a total of 30 minutes in KRH no-glucose medium without or with insulin (25 and 1000 μU/mL). The glucose analog 2-deoxy-2-glucose was added for the last 10 minutes of the incubation. The quantification was done as per manufacture's instruction and described in supplementary methods.

Gene expression. ***Transcriptomics.*** SAT from cohort 1 was used for RNAseq at Exiqon A/S (Vedbaek, Denmark), as previously reported.[8]

The data used to determine the tissue distribution of *CDKN2C* were obtained from the GTEx Portal (dbGaP Accession phs000424.v8.p2).[24] The GTEx project include tissue samples from nearly 1000 individuals (67% men), age: 20−70 years, BMI: 18.5-35 Kg/m^2 and with different ethnicities. CDKN2C was determined using ENSG00000123080.10 gene code ID.

Real-time quantitative PCR. Total RNA was extracted from SAT and OAT (cohort 2 and 3, n = 95), differentiating preadipocytes (days 0, 7 and 14 post-induction) (n = 8) and from knockdown cell cultures on days 0, 7, and 14 of differentiation (n = 5) using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using a High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. The concentration and purity of total RNA were measured with the Nanodrop (Thermo Scientific). mRNA expression was determined using TaqMan gene expression assays (Thermo Fisher) of *CDKN2C* (Hs00176227), and differentiation markers *CEBPA* (Hs00269972), *PPARG* (Hs01115513), *ADIPOQ* (Hs00605917), *FASN* (Hs01005622), *LEP* (Hs00174877). The gene expression was detected using the QuantStudio 3 sequence detection system (Applied Biosystem) and calculated using a $2^{-\text{deltaCT}}$. The gene expression levels were normalized to the housekeeping genes *18S* or *GUSB,* and all samples were run in duplicates.

Western-blot. Total protein lysates were prepared from differentiating preadipocytes (days 0, 7, and 14 post-induction, n = 3); knockdown cell cultures on day 14 of differentiation (n = 3); mature adipocytes and SVF fraction (n = 5); and SAT and OAT, as previously reported[17] and described in supplementary methods. Samples with extreme values in *CDKN2C* mRNA expression in lean and obese groups were used for AT protein expression analyses. The primary antibodies anti−CDKN2C/p18 INK4C (1:1000, #2896, Cell Signaling, Beverly, MA), anti−PPARG (1:1000, #2443, Cell Signaling Technologies), and anti−glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a protein loading control (1:2000, Millipore, Temecula, CA) were used for experiments.

Pathway analysis. Transcriptomics expression levels of all identified genes were correlated with *CDKN2C* expression in T2D and control groups of cohort 1 SAT samples. Gene lists of all significant correlations ($P < 0.05$) from the 2 groups were used for functional annotation analysis with DAVID Bioinformatics Resources v6.8 (the Database for Annotation, Visualization, and Integrated Discovery).[25] The list of functional annotations with enrichment *P*-value (Benjamini correction, $P < 0.05$) was used for visual presentation.

Statistical analysis. Descriptive data are presented as mean ± SEM unless stated otherwise. All data were first checked for normality using the Shapiro-Wilk test and by analyzing histograms. Spearman's correlation test and multiple linear regressions were performed between *CDKN2C* mRNA expression and markers of insulin resistance and obesity. Mann-Whitney U test was used for analyses between 2 independent groups. A comparison of the mean of more than 2 groups was made using repeated measures ANOVA or Fridman test for normally distributed or not normally distributed data, respectively. Multiple comparisons were corrected for the false discovery rate using the original Benjamini and Hochberg method. All statistical analyses were performed using IBM SPSS Statistics 26 and GraphPad Prism 9 software, and a *P*-value <0.05 was considered statistically significant.

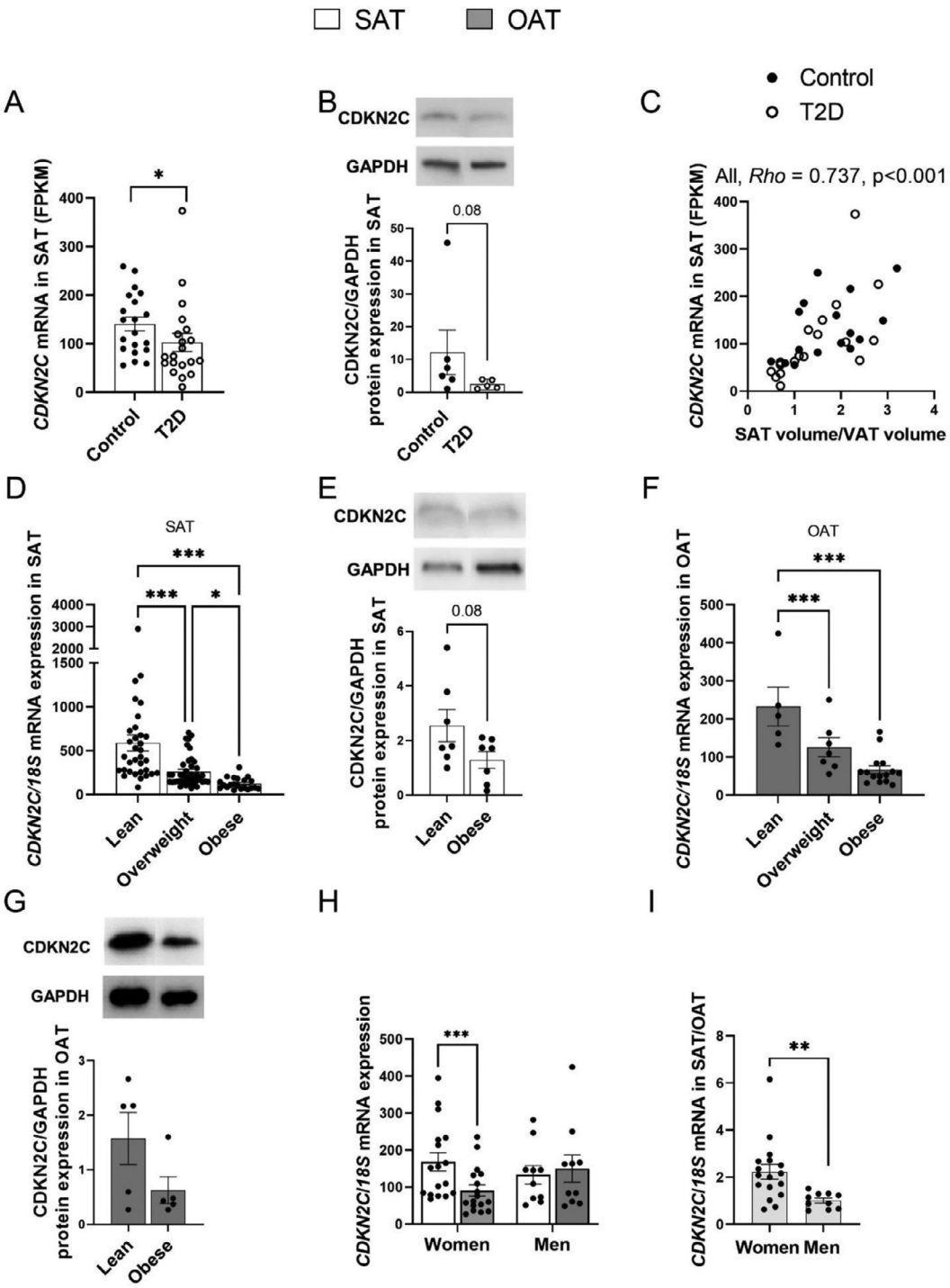

Fig 1. C*DKN2C* mRNA and protein expression in human adipose tissue. CDKN2C mRNA expression in adipose tissue from (A) control and T2D subjects (n = 20/group); (B) CDKN2C protein expression in adipose tissue from control (n = 6) and T2D (n = 5) subjects. (C) Correlation of *CDKN2C* mRNA levels with SAT volume/VAT volume. (D, E) *CDKN2C* mRNA and protein expression in SAT of lean (n = 33 and 7, respectively), overweight (n = 41) and obese (n = 21 and 6, respectively) subjects without T2D. (F, G) *CDKN2C* mRNA and protein expression in OAT of lean (n = 5), overweight (n = 7) and obese (n = 17 and 5, respectively) subjects without T2D. (H) Paired SAT and OAT (n = 23) *CDKN2C* mRNA expression in woman and men without T2D. (I) *CDKN2C* mRNA expression in SAT /OAT ratio in women and men without T2D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data is shown as mean ± SEM.

Table II. Correlation coefficient between *CDKN2C* mRNA and markers of hyperglycemia, insulin resistance and obesity

	Cohort 1						*Cohort 2+3*			
	***CDKN2C* mRNA in SAT**						***CDKN2C* mRNA in SAT**		***CDKN2C* mRNA in OAT**	
	All (n = 40)		**Control (n = 20)**		**T2D (n = 20)**		**(n = 95)**		**(n = 27)**	
	Rho	***P-value***	***Rho***	***P-value***	***Rho***	***P-value***	***Rho***	***P-value***	***Rho***	***P-value***
Hyperglycemia and insulin resistance markers										
HbA1c	**-0.376**	**0.017**	0.117	0.625	**-0.565**	**0.009**	-0.089	0.397	-0.200	0.339
HOMA-IR	**-0.434**	**0.005**	-0.197	0.405	**-0.457**	**0.043**	**-0.373**	**<0.001**	**-0.726**	**<0.001**
Fasting glucose	**-0.466**	**0.002**	-0.014	0.952	**-0.731**	**<0.001**	-0.138	0.182	**-0.565**	**0.002**
Fasting insulin	-0.278	0.082	-0.280	0.232	-0.021	0.930	**-0.416**	**<0.001**	**-0.727**	**<0.001**
Fasting C-peptide	**-0.332**	**0.037**	-0.413	0.070	-0.065	0.786	**-0.513**	**<0.001**	-0.300	0.277
HDL-cholesterol	**0.564**	**<0.001**	**0.637**	**0.003**	0.257	0.274	**0.333**	**0.001**	**0.504**	**0.007**
Fasting FFA	-0.011	0.947	-0.024	0.920	0.192	0.416	na		na	
OGTT AUC glucose	**-0.474**	**0.002**	-0.108	0.650	**-0.689**	**0.001**	na		na	
OGTT AUC insulin	-0.012	0.942	-0.394	0.086	0.208	0.380	na		na	
OGTT AUC FFA	**-0.513**	**0.001**	-0.362	0.116	-0.442	0.051	na		na	
OGTT AUC Glycerol	-0.158	0.331	-0.269	0.251	-0.096	0.686	na		na	
Matsuda	**0.421**	**0.007**	0.287	0.220	0.295	0.207	na		na	
Insulinogenic index	**0.329**	**0.038**	-0.045	0.850	0.441	0.052	na		na	
Adipocyte glucose uptake, ex vivo[a]										
Subcutaneous basal	0.259	0.112	0.011	0.965	0.342	0.152	0.133	0.364	-0.538	0.071
Subcutaneous insulin-stimulated[b]	**0.358**	**0.025**	-0.025	0.910	0.435	0.063	0.262	0.069	-0.427	0.167
Omental basal	na		na		na		0.336	0.312	-0.391	0.235
Omental insulin-stimulated[b]	na		na		na		0.600	0.051	-0.155	0.650
Obesity markers										
BMI	-0.060	0.711	-0.263	0.262	0.156	0.510	**-0.659**	**<0.001**	**-0.693**	**<0.001**
Waist-hip-ratio	**-0.622**	**<0.001**	**-0.487**	**0.029**	**-0.683**	**0.001**	**-0.361**	**<0.001**	0.203	0.309
SAT volume	0.230	0.184	-0.194	0.471	**0.456**	**0.050**	na		na	
VAT volume	**-0.770**	**<0.001**	**-0.729**	**0.001**	**-0.686**	**0.001**	na		na	
SAT/VAT volume	**0.737**	**<0.001**	**0.559**	**0.024**	**0.812**	**<0.001**	na		na	
Liver Fat %	**-0.527**	**0.001**	-0.288	0.279	**-0.684**	**0.001**	na		na	
Subcutaneous adipocyte size	0.092	0.572	-0.035	0.885	0.182	0.443	**-0.247**	**0.028**	**-0.549**	**0.007**
Omental adipocyte size	na		na		na		**-0.496**	**0.016**	**-0.451**	**0.031**

Table presents Spearman's rho correlation coefficient.
Significant Spearman rank correlation values are shown in bold: $P < 0.05$.
T2D, type 2 diabetes; SAT, subcutaneous adipose tissue; OAT, omental adipose tissue; VAT, visceral adipose tissue. na=not available
[a]For Cohort 2+3, SAT, n = 49 and OAT, n = 11.
[b]Glucose uptake with 1000 μU/mL of insulin.

RESULTS

CDKN2C mRNA expression in adipose tissue. In subjects with T2D *CDKN2C* expression was down-regulated by 45% on mRNA level ($P < 0.05$), and by 80% on protein level ($P = 0.08$), compared to controls (Fig 1A–B). In both groups, a positive correlation was found between *CDKN2C* mRNA and SAT/VAT volume (Fig 1C and Table II).

CDKN2C mRNA and protein levels in SAT and OAT were assessed in subjects without T2D and with different degrees of obesity (Fig 1D–G). *CDKN2C* mRNA expression was reduced in both SAT and OAT by 50% in overweight and by 75% in obese subjects, as compared to lean subjects (Fig 1D, F). CDKN2C protein expression followed a similar reduction in obese, compared to lean subjects, as observed at mRNA levels (Fig 1E and G).

CDKN2C tissue distribution was determined in different human tissues, including SAT from beneath the leg's skin, and OAT. *CDKN2C* mRNA is widely expressed, with the highest gene expression in adipose tissue (Supplementary Fig 1).

Associations of SNPs in *CDKN2C* regions and T2D and obesity. Associations of polymorphisms in the *CDKN2C* gene were associated with diabetes and obesity-related phenotypes by performing analyses in publically available databases from genome-wide

Table III. Top hits for the association of single nucleotide polymorphism in the CDKN2C region with diabetes and obesity and body fat

Trait	SNP	Effect allele	Non−effect allele	Beta	Direction	*P*-value	Adjusted P-value	N
Whole body fat-free mass	rs78181360	A	G	-0.02121	-	2.69E−09	2.42E−08	331291
Whole body fat-free mass	rs3176447	A	T	0.02364	+	1.05E−17	9.43E−17	331291
Whole body fat-free mass	rs3176459	A	G	0.00895	+	4.16E−08	3.74E−07	331291
Whole body fat-free mass	rs3176466	T	C	0.02366	+	1.60E−18	1.44E−17	331291
Diabetes	rs3176466	T	C	-0.0041	-	7.17E−06	6.45E−05	336473
Whole body fat-free mass	rs3176468	T	C	-0.02348	-	3.24E−18	2.92E−17	331291
Diabetes	rs3176468	T	C	0.00409	+	7.73E−06	6.96E−05	336473
Whole body fat-free mass	rs1043141	T	C	0.02355	+	3.16E−18	2.84E−17	331291
Whole body fat-free mass	rs12855	T	C	0.0235	+	3.00E−18	2.70E−17	331291

N, number of subjects in analysis

association studies. We found 2 SNPs associated with T2D and 7 with whole-body fat-free mass (Table III).

***CDKN2C* mRNA expression in SAT and OAT is sex-dependent.** In women without T2D, *CDKN2C* expression was 50% lower ($P < 0.001$) in OAT compared to SAT, while in men it did not differ (Fig 1H). Accordingly, the SAT/OAT ratio of *CDKN2C* mRNA expression was higher in women than men ($P < 0.01$, Fig 1I). The sex-dependent difference in OAT vs SAT *CDKN2C* gene expression was observed across BMI (data not shown). Multiple linear regression was used to investigate whether sex and BMI could predict *CDKN2C* expression in SAT vs OAT. In regression analyses, sex (standardized beta for male sex = -0.570, $P = 0.005$), but not BMI, (NS) explained 36% of the variability in the SAT/OAT *CDKN2C* mRNA expression ($r^2 = 0.36$). This suggests that the SAT vs OAT differences between women and men are independent of BMI.

Association between *CDKN2C* mRNA in SAT and markers of hyperglycemia, insulin resistance, obesity, and cardiovascular disease. Associations between CDKN2C mRNA levels and markers of hyperglycemia, insulin resistance and obesity in T2D and control subjects were explorative, and are shown in Table II. In the T2D subjects, *CDKN2C* mRNA expression in SAT was negatively correlated with hyperglycemia (HbA1C, fasting glucose and glucose AUC during OGTT) and with the insulin resistance markers HOMA-IR, ($P < 0.05$ for all) and FFA during OGTT ($P = 0.051$), but positively with subcutaneous adipocyte insulin-stimulated glucose uptake *ex vivo* ($P = 0.063$). In addition, there were negative associations with WHR, VAT volume, and liver fat percentage ($P < 0.01$, all). In contrast, *CDKN2C* mRNA expression correlated positively with SAT volume ($P = 0.05$) and with SAT/VAT volume ($P < 0.001$), but no association with BMI was found. No significant associations were found with adipocyte lipolysis *ex vivo* (data not shown) and glycerol AUC during OGTT.

In controls, the *CDKN2C* expression was negatively correlated with the obesity markers WHR and VAT volume and positively with the SAT/VAT volume ($P < 0.05$), but no significant associations were found with hyperglycemia and insulin resistance markers. In addition, there was a positive association with HDL-cholesterol ($P < 0.01$). Similar and stronger associations were found when pooling T2D subjects and controls together (Table II). The possible associations for *CDKN2C* expression were repeated in cohorts 2 and 3, including more SAT and OAT samples and wide variation in BMI (20.2−58.2 kg/m^2) and HOMA-IR (0.58−14.08) (Table II). In both SAT and OAT, there were negative associations with HOMA-IR, fasting insulin, BMI, and subcutaneous and omental adipocyte size. *CDKN2C* expression in SAT was positively correlated with subcutaneous glucose uptake, while in OAT, the correlation was negative (did not reach significance).

In multiple regression analysis, SAT/VAT volume (stand beta coefficient = 0.455, $P = 0.003$), BMI (stand beta coefficient = -0.442, $P = 0.001$) and sex (women vs men stand beta coefficient = -0.300, $P = 0.035$) were significantly associated with *CDKN2C* mRNA expression ($P < 0.001$, adjusted $R^2 = 0.68$ for model), while HOMA-IR and adipocyte glucose uptake were not, when pooling T2D subjects and controls together.

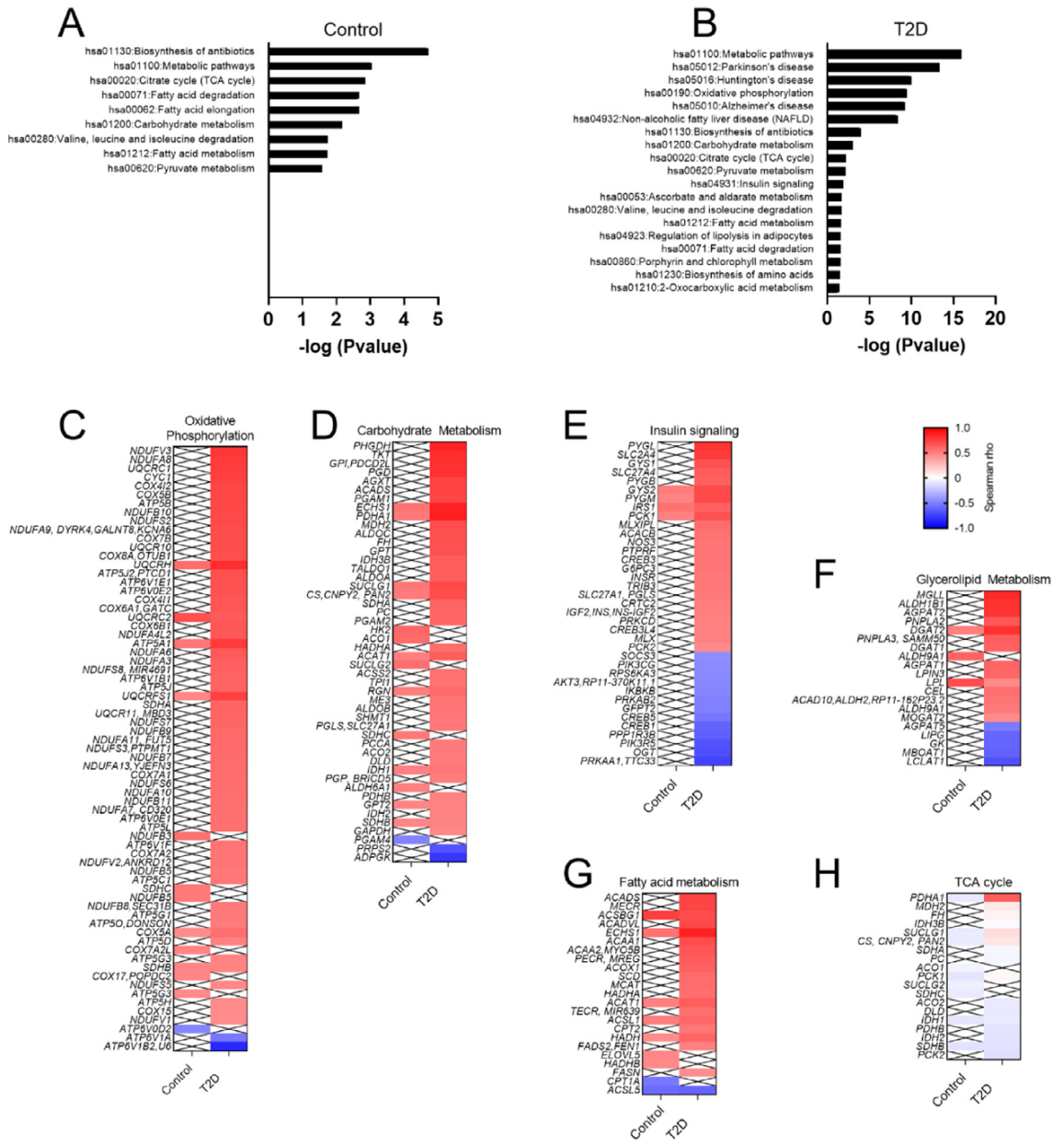

Fig 2. Correlation analysis of *CDKN2C* mRNA using transcriptomic data in adipose tissue of control and T2D subjects. Pathway analysis of all genes significantly correlated with *CDKN2C* in SAT of (A) control (n = 20) and (B) T2D subjects (n = 20). The bar graph shows −log (*P*-value) of significant pathways. (C−H) Spearman rank correlations of the genes significantly associated with *CDKN2C* in selected pathways.

Genes associated with *CDKN2C* in adipose tissue. To gain insights into the potential function of *CDKN2C* in adipose tissue, the list of genes identified with transcriptomic profiling of adipose tissue and whose expression levels were significantly associated with *CDKN2C* expression in control and T2D subjects (n = 750 and 3601 genes, respectively) were used for enrichment analysis. Terms that were enriched in this analysis were dominated by links to adipocyte metabolism (Fig 2A-B and Supplementary Table I and II). Specifically, enriched terms included largely positive associations with oxidative phosphorylation, carbohydrate, glycerolipid and fatty acid metabolism, and insulin signaling (Fig 2C−G). In particular, several genes involved in lipogenesis (eg *FASN, ACACB, PDHA1, AGPAT1* and *2, DGAT1* and *2*) were significantly positively associated with the expression of *CDKN2C* in SAT of T2D subjects. In contrast,

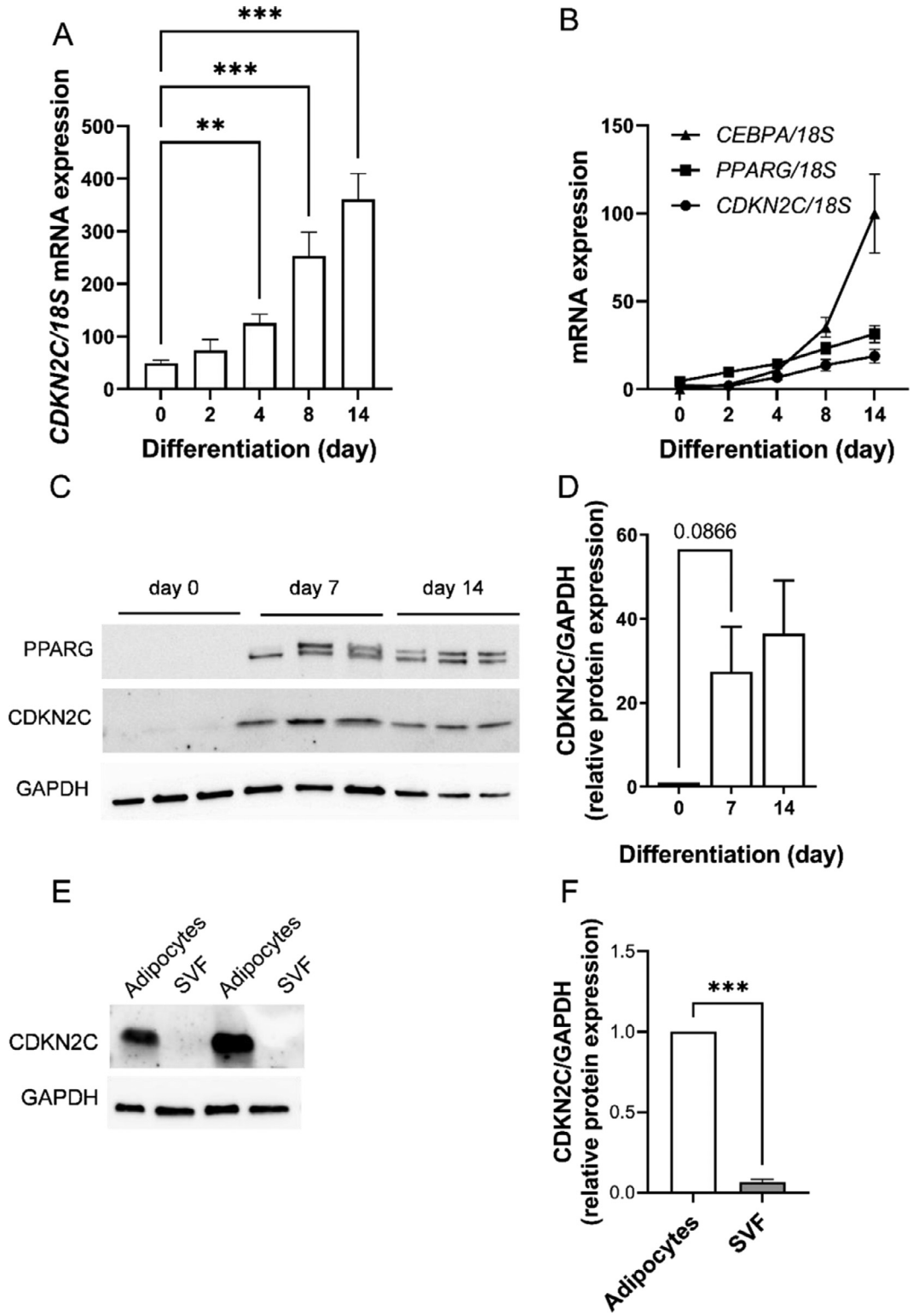

Fig 3. CDKN2C expression in adipocytes and stromal vascular cells. Human preadipocytes (n = 8) were differentiated into adipocytes, and on days 0, 2, 4, 8, and 14 of differentiation samples were analyzed for (A−B) *CDKN2C* mRNA expression and (B) *CEBPA* and *PPARG* mRNA expression during preadipocyte differentiation. (C) CDKN2C and PPARG protein at days 0 and 7 and 14 after induction of adipocyte differentiation and (D) quantification of protein levels (n = 3). (E) CDKN2C protein in isolated adipocytes and SVF cells and (F) quantification of protein levels (n = 5). ** $P < 0.01$, *** $P < 0.01$. Data is shown as means ± SEM.

CDKN2C negatively correlated to several genes in the TCA cycle (eg *PCK1, PDHB*) (Fig 2H).

Expression of CDKN2C during human preadipocyte differentiation. CDKN2C mRNA and protein expression were up-regulated during human preadipocyte differentiation into adipocytes (Fig 3A-D). At the mRNA level, *CDKN2C* expression was significantly up-regulated from day 4 ($P < 0.01$, Fig 3A), and its expression showed a pattern similar to *PPARG* (Fig 3B). *CDKN2C* expression highly correlated with *PPARG* gene expression (rho = 0.87, $P < 0.001$). In addition, CDKN2C protein was measured in adipocytes and SVF. The SVF contains all non−adipocyte cells from adipose tissue (eg, immune cells, fibroblasts, endothelial cells, adipose-derived stem cells, preadipocytes), allowing for determination of the relative contribution of adipocytes vs SVF to CDKN2C levels in adipose tissue. Most of the CDKN2C protein found in adipose tissue could be attributed to the adipocytes (>95%, $P < 0.001$), while the SVF only contributed to a minor fraction (Fig 3E−F).

Assessment of *CDKN2C* gene knockdown. *CDKN2C* gene knockdown was performed efficiently using the delivery method of sgRNA and Cas9 protein complex by electroporation. Knockdown efficiency was determined at the DNA, mRNA, and protein levels (Fig 4). Sanger sequencing knockdown efficiency scores indicate that CDKN2C G1 achieved 94% while CDKN2C G2 achieved 92% efficiency (Fig 4A−D). When assessed at the mRNA levels, *CDKN2C* expression was significantly lower in CDKN2C G1 cultures on days 7 and 14 ($P < 0.05$), compared to wild type and negative cultures. CDKN2C G2 cultures had modestly decreased *CDKN2C* mRNA levels on both time points compared to wild type, and significantly decreased compared to negative control on day 14 ($P < 0.05$) (Fig 4E). Immunocytochemistry data revealed that, compared to wild type and negative control, CDKN2C protein expression in the knockdown cultures was downregulated by 90% for CDKN2C G1 and 94% for G2 (both $P < 0.001$) (Fig 4F−G). This was further confirmed by western blot data where CDKN2C protein levels for both guides were undetectable (Fig 4H). The negative control sgRNA did not have any effect on the expression of *CDKN2C* (Fig 4E−H).

Impact of *CDKN2C* knockdown on adipocyte metabolism. The loss of CDKN2C did not affect preadipocyte proliferation for CDKN2C G1 and G2, compared to wild type (data not shown). CDKN2C was dispensable for preadipocyte differentiation since no difference in the percentage of differentiating cells was observed between edited and wild type or negative cultures (Fig 5A−B). However, relative lipid content accumulated per cell was reduced transiently in CDKN2C G1 cultures by 13 and 16% compared to wild type and negative cultures, respectively ($P < 0.05$ both) on day 7 of differentiation with a similar trend for CDKN2C G2 (Fig 5C and Supplementary Fig 2). CDKN2C knockdown, on day 14 of differentiation, significantly reduced expression of the differentiation marker *CEBPA* ($P < 0.01$ for CDKN2C G1 and $P < 0.05$ for CDKN2C G2, vs wild type) and *ADIPOQ* ($P < 0.05$ for both guides vs wild type and CDKN2C G1 vs negative control) (Fig 5E−F). *FASN* was significantly down-regulated in CDKN2C G1 cultures ($P < 0.05$) and nominally decreased in CDKN2C G2 cultures (Fig 5G, $P = 0.07$), compared to wild type. *PPARG* expression (Fig 5D) and *LEP* levels (data not shown) were not affected by CDKN2C knockdown. Down-regulation of CDKN2C did not affect mature adipocyte glucose uptake (Fig 5H). The negative control sgRNA did not have any effect on the adipocyte differentiation (Fig 5A−G).

To test whether *CDKN2C* expression was dependent on PPARG or adipocyte differentiation, *PPARG* knockouts (PPARG KO) were performed in human preadipocytes, as previously described.[17] *CDKN2C* expression was reduced in PPARG KO, on days 7 and 14 post-induction of preadipocyte differentiation (Fig 5I, $P < 0.05$), compared to wild type and negative cultures. mRNA expression of differentiation markers in PPARG KO cultures, such as *CD36, ADIPOQ,* and *CEBPB* have previously been published.[17]

DISCUSSION

This study shows that CDKN2C mRNA expression in adipose tissue is down-regulated in T2D and obese subjects, and it is negatively correlated with hyperglycemia, insulin resistance and central obesity phenotypes. We hypothesized that CDKN2C could have a role in adipocyte metabolism. CDKN2C knockdown by CRISPR/Cas9 in subcutaneous human preadipocytes caused a minor and transient decrease in relative lipid content during differentiation and it reduced expression of genes regulating adipocyte function at the end of differentiation. These findings could suggest a potential role of CDKN2C in lipid storage dysregulation in obesity and T2D.

Our association analyses for CDKN2C vs markers of hyperglycemia, insulin resistance and obesity were explorative, but provided corroborative evidence for a negative association between *CDKN2C* and several markers of insulin resistance and central obesity (eg HOMA-IR, OGTT AUC for FFA, WHR, liver fat %) and positive with the ratio of SAT to VAT volume. This suggests a link between lower CDKN2C

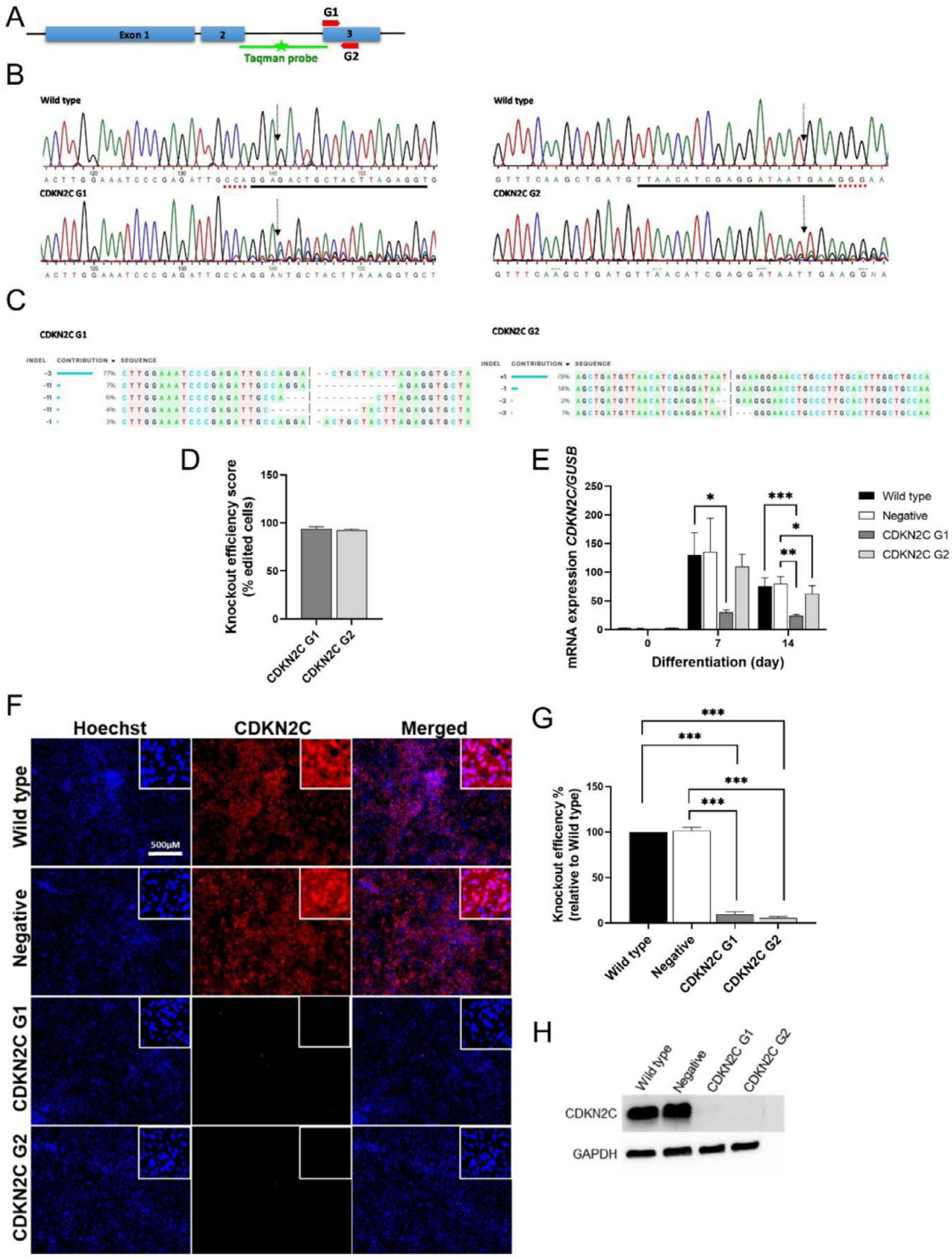

Fig 4. CDKN2C knockdown efficiency in primary human preadipocytes. (A) Gene model of the *CDKN2C* gene showing exon numbers and introns. The binding site for the TaqMan probe used for qPCR detection is indicated as a green line with a star, spanning the exon junction 2−3. Also indicated are the target sites and directions of the CRISPR/Cas9 *CDKN2C* specific sgRNAs, G1, and G2. The schematic organization of the gene was adapted from the GTEx Portal (www.gtexportal.org). (B) Representative Sanger sequencing chromatograms of the Cas9 cut sites for CDKN2C G1 (right) and G2 (left) with the wild type reference chromatogram above (n = 3). SgRNA sequences are shown as black lines and PAM sequences as dotted red lines. The cut site is indicated by a vertical dotted black arrow shown for both the edited and the reference sequences. (C) Relative indel

expression in SAT and a preferential fat deposition in visceral rather than SAT depots and increased insulin resistance. These findings are in line with a previous microarray study, in which CDKN2C was shown to be down-regulated in SAT in morbidly obese individuals.[26] Furthermore, the negative association of CDKN2C expression with FFA AUC during OGTT, but not with glycerol AUC and adipocyte lipolysis, supports that adipose tissue lipid storage is impaired when CDKN2C expression is low. This is in agreement with our previous study indicating that dysregulated lipid storage, rather than lipolysis, contributes to increased FFA levels in T2D.[8]

Adipose tissue plays a central role in regulating whole-body energy and glucose homeostasis through its hormonal and metabolic functions both within AT and via communication with other tissues. Fat accumulation in visceral depots is associated with insulin resistance and an increased risk for developing T2D and cardiovascular diseases, whereas a relative increase of subcutaneous fat has been shown to be protective against these metabolic abnormalities.[27] At the cellular level, this difference appears to be related to depot-specific differences in the expression of multiple genes involved in embryonic development, adipocyte differentiation, and growth factors.[28-31] Therefore, our findings that CDKN2C was reduced with obesity and insulin resistance phenotypes, and its expression in adipose tissue was associated with several metabolic pathways, including insulin signaling and carbohydrate metabolism, suggest a potential role of CDKN2C in regulating energy metabolism. However, we also need to consider that the low expression of *CDKN2C* in adipose tissue in T2D, and obesity could be largely a secondary phenomenon to the metabolic dysregulation in these conditions.

Interestingly, we found sex differences in *CDKN2C* expression in SAT and OAT; *CDKN2C* expression was higher in SAT compared to OAT in women only (>2-fold). Generally, there are sex differences in adipose tissue distribution, with women storing fat predominantly in SAT depot, while men store mainly in VAT.[32] We show *CDKN2C* associations with central adiposity and altered lipid storage (eg lipogenic genes, FFA levels during OGTT), which together with depot-specific gene expression could contribute to different fat accumulation patterns in women and men. Additional studies are needed to investigate whether the sex and depot differences in *CDKN2C* gene expression are a cause or a consequence of sexual dimorphism in lipid storage and mobilization.

To gain additional evidence of an association between CDKN2C with obesity and diabetes, we identified CDKN2C SNPs that could affect specific metabolic phenotypes in publicly available databases. Significant associations were found for CDKN2C SNPs related to fat mass and the risk for developing T2D. A recent GWAS has also supported that CDKN2C plays a role in obesity and T2D pathogenesis.[33] However, whether these polymorphisms lead to changes in CDKN2C protein function is unknown, and requires further studies.

The differentiation of preadipocytes into adipocytes is coordinated by the expression of genes involved in the cell cycle and proliferation, such as cyclins, and cyclin-dependent kinases.[34] Results from our human preadipocyte differentiation experiments showing an increase in CDKN2C expression during preadipocyte differentiation into adipocytes are consistent with previous findings in pig and 3T3-L1 adipocytes.[13,35] Furthermore, we demonstrated that the expression of CDKN2C is low in proliferating preadipocytes or SVF cells compared to mature adipocytes. Thus, CDKN2C may have a regulatory role only after the adipogenic program is initiated.[15] We also demonstrated strong similarities in the expression of CDKN2C and PPARG during adipocyte differentiation. PPARG is a master regulation of adipogenesis, and PPARG KO completely blocks preadipocyte differentiation.[17] We found that expression of *CDKN2C* was undetectable in PPARG KO cultures, and this suggests that expression of CDKN2C requires adipocyte differentiation. In addition, we cannot exclude the possibility that PPARG can directly regulate *CDKN2C* expression.

To explore a potential role of CDKN2C in regulating preadipocyte proliferation or adipogenesis, we

contribution estimations for each sequence for CDKN2C G1 (left) and CDKN2C G2(right), analyzed and quantified by ICE v2 (Inference of CRISPR edits). Predominant editing events for the CDKN2C G1 is a 2-base pair deletion and for G2 a 1 base pair insertion. 1 representative experiment out of 3 is shown. (D) Total knockdown efficiency score based on gene-editing events on the DNA level of CDKN2C G2 and G2 indicating an almost complete knockdown of the gene for both guide RNAs (n = 3). (E) mRNA levels of *CDKN2C* during differentiation (days 0, 7, and 14) in wild type, negative control and *CDKN2C* gene edited cultures normalized to *GUSB* as a reference gene. (F) Representative immunofluorescent images of wild type, negative control, CDKN2C G1 and G2 cultures showing Hoechst staining (blue) to visualize nuclei, Alexa Fluor 647 (red) to visualize CDKN2C protein, and both combined shown as Merged. Scale bar in 500 μM. (G) Quantification of the images as the percentage of CDKN2C positive cell. (H) Representative western blot showing CDKN2C protein levels of wild type, negative control, and CDKN2C G1 and G2 cultures on day 14 of differentiation (n = 5). *$P <$ 0.05, ***$P < 0.001$. Data is shown as means ± SEM (Color version of the figure is available online.)

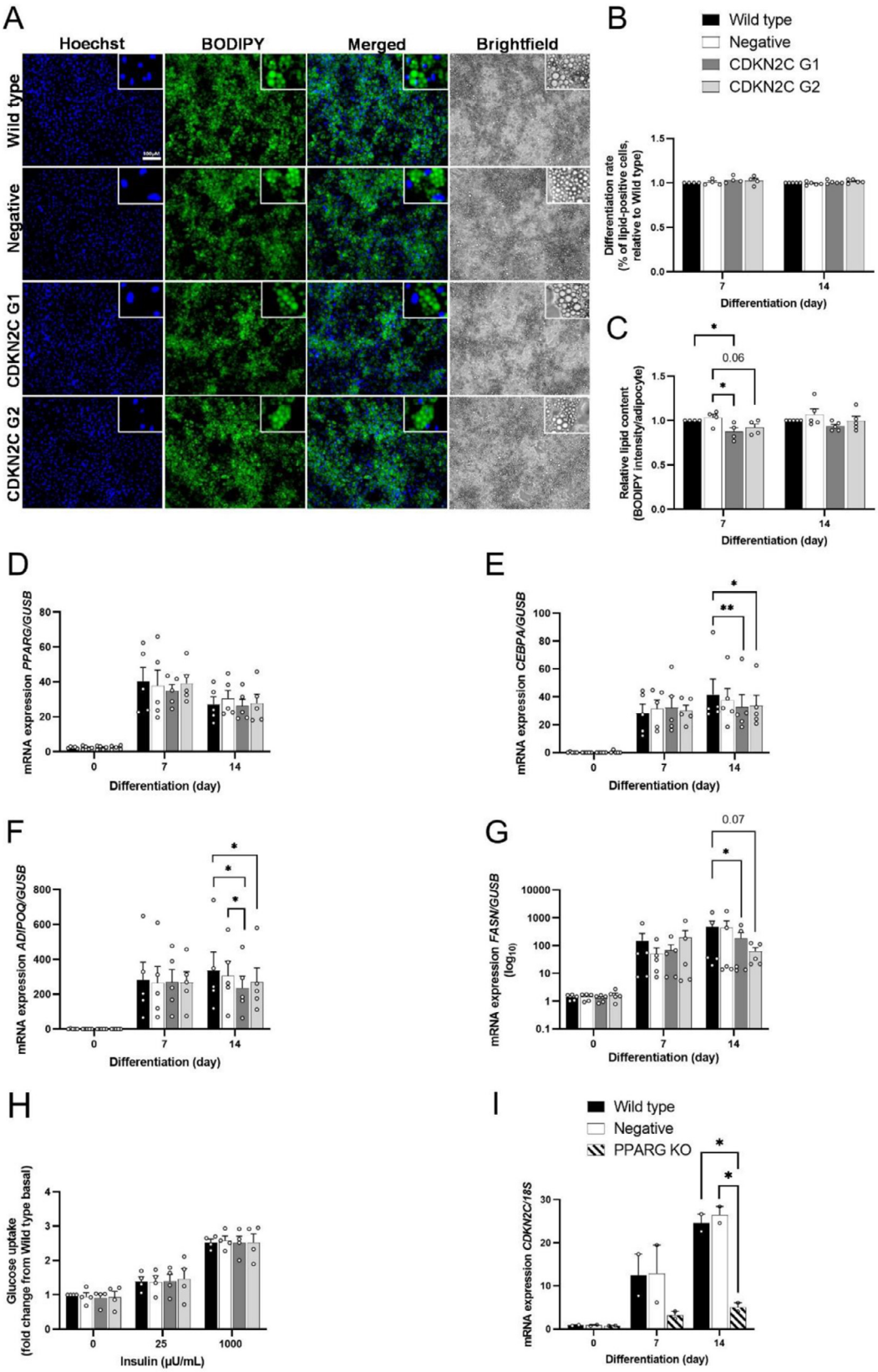

Fig 5. CDKN2C knockdown effects on adipocyte metabolism. (A) Representative images of adipocyte cultures on day 14 of differentiation stained with Hoechst nuclear stain, BODIPY lipid stain, both combined shown as

performed CDKN2C knockdown in human primary preadipocytes. The knockdown efficiency achieved was over 90% for both sgRNAs used, as confirmed by Sanger sequencing, and protein expression levels. We observed that CDKN2C knockdown in human preadipocytes did not significantly affect preadipocyte proliferation rates. It has been shown that CDKN2C deficient mice have increased body size and widespread organomegaly, including adipose tissue, caused by the increase in cell number.[36] The divergent effects of CDKN2C knockdown in human and rodent cells may be explained by the differences in experimental designs, primarily short-term *in vitro* vs long-term *in vivo* setup, or attributed to species-specific differences in cell cycle regulation.

Furthermore, we showed that CDKN2C knockdown in human preadipocytes did not directly regulate adipogenesis. Although we only see a minor and transient effect on preadipocyte lipid content, the down-regulation of *CEBPA, FASN* and *ADIPOQ* in already differentiated adipocytes could indicate altered adipocyte function. These data align with our association study, showing that *CDKN2C* expression in adipose tissue is positively correlated with several markers of lipogenesis, including *FASN, PPARG, CEBPA, DGAT1,* and *2*. Our transcriptomics data also suggest that *CDKN2C* gene expression is positively associated with several metabolic pathways, including oxidative phosphorylation, carbohydrate, and fatty acid metabolism. Potential alterations in these metabolic pathways in subjects with low levels of CDKN2C could impact energy metabolism and lipid storage. However, these associations need to be clarified, and their impact should be studied further in *in vitro* as well as *in vivo* models of genetically or pharmacologically modified CDKN2C. Different CDK inhibitors (eg CDKN2A, CDKN1A, and CDKN1B) have been suggested to play critical roles to maintain cell cycle arrest at various stages during preadipocyte differentiation, ie exponential growth phase, initiation of differentiation, and terminal phase of differentiation.[13,37,38] Our data suggest that CDKN2C might be relevant only after the adipogenesis program has been initiated, as suggested before.[15] This would be consistent with the fact that *CDKN2C* expression in preadipocytes is low compared to mature adipocytes.

We observed a 30% reduction in adiponectin expression in the CDKN2C knockdown cultures. The clinical significance of this reduction is unknown, but adiponectin is a well-known adipokine with insulin-sensitizing characteristics and on average individuals with obesity and T2D have 30%−40% reduction in plasma adiponectin levels compared to control subjects.[39,40] Even though our data suggest no direct effect of CDKN2C knockdown on adipocyte glucose metabolism per se, we cannot exclude effects on whole-body glucose turnover, and insulin sensitivity via secreted adiponectin or other adipose-derived mediators.

This study has some limitations. The number of paired SAT and OAT samples is limited, and it does not allow for analyses of depot differences based on sex and BMI. The associations obtained in the observational part do not allow any causal inference. For that reason, we performed functional analyses with CRISPR/Cas9 gene-editing. However, *in vitro* knockdown of CDKN2C was performed in a limited number of experiments, and with adipose progenitor cells only from subcutaneous depot. This does not necessarily reflect the *in vivo* setting. Therefore, further functional characterisation of adipose tissue CDKN2C should be performed *in vivo*, for example, by using tissue-specific gene knockdown in animals.

In summary, *CDKN2C* expression in SAT and OAT is reduced in obesity and T2D and it is inversely associated with measures of visceral adiposity, hyperglycemia, and insulin resistance. CDKN2C is dispensable for human preadipocyte differentiation, but knockdown of CDKN2C led to reduced expression of several genes that are of importance to maintain normal adipocyte metabolism. Our findings suggest that *CDKN2C* expression might be reduced as a consequence of insulin resistance and obesity, and this can further contribute to impairment of SAT lipid storage.

FUNDING SOURCE

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Merged, as well as corresponding brightfield image for wild type, negative control, CDKN2C G1, and G2 cultures (with 20x magnification segments compared to images). (B) Differentiation rate, measured as the percentage of lipid-positive cells during differentiation (days 7 and 14), shows how many cells in total became adipocytes (n=5). (C) Relative lipid content of edited and non−edited cells quantified during differentiation (days 7 and 14), measured as total integrated intensity of BODIPY signal per number of lipid-positive cells (n = 5), shows the amount of lipids accumulated per adipocyte. (D−G) Differentiation rate determined by measuring gene expression of differentiation markers in preadipocytes and adipocytes on days 7 and 14 post-induction (n = 5). (H) Basal and insulin-stimulated glucose uptake assessed in differentiated adipocytes and shown as relative to basal wild type (n = 4). (I) mRNA expression of *CDKN2C* quantified in PPARG knockout cultures on days 0, 7, and 14 of differentiation (n = 2). $*P < 0.05$, $**P < 0.01$. *FASN* values have been log transformed. Data is shown as means ± SEM.

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CONTRIBUTION STATEMENT

MJP, MV, and SH contributed to study design, acquisition, and interpretation of data and wrote the manuscript; PK, HJ, RK, EH, JK contributed to data acquisition; SS and MKS contributed to study design and acquisition of data; JWE contributed to study design and data interpretation. All co-authors revised the manuscript critically and approved a final version.

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CONFLICTS OF INTEREST

All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest. Stanko Skrtic was an employee of AstraZeneca R&D at the time of the study, and he is currently an employee of Novo Nordisk. The other authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.12.003.

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